Characterisation of glycosidase enzymes of wine Lactic Acid Bacteria

By

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THE UNIVERSITY
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To my family,
who supported and encouraged me
in this adventure.

Abstract

Many compounds important for wine quality are in the glycosidic form, that is a sugar moiety is attached, through a glycosidic linkage, to the alcoholic group of the compound. Such glycosidic compounds comprise several groups, all having great relevance to wine quality and can be broadly grouped as the aromatic compounds and compounds that contribute to colour. The former group, when in the glycosidic form are, unfortunately, odourless and do not contribute to the aroma of wine. However, once the sugar moiety is cleaved, they regain their aromatic characteristics. This is the reason why glycosyl-terpenols, for example, are considered a potential source of aroma in wine. On the other hand, anthocyanins need to be in the glycosidic form to contribute to colour in wine, especially red wines. Once de-glycosylated, these compounds tend to lose a substantial colour capacity (at least in wine conditions, particularly at low pH) and become more chemically reactive. In red wines this outcome may be undesirable but, conversely, for rose or 'blanc de noir' wines, decolourisation might be beneficial.

Of the many methods to increase the amount of aroma or manipulate colour, enzymatic hydrolysis seems to be the most appropriate for wine since it has lesser drawbacks compared to methods such as acidic hydrolysis or heating. Given the particular nature of glycosides in wine, attention has to focus on five glycosidase enzymes: β-D-glucopyranosidase, α-D-glucopyranosidase, α-L-rhamnopyranosidase, α-L-arabinofuranosidase and β-D-xylopyranosidase. This project has investigated the presence and distribution of glycosidases amongst 40 isolates of Lactic Acid Bacteria (LAB) (22 of which being *Oenococcus oeni* and the remaining 18 equally represented by *Lactobacillus* and *Pediococcus*), as a start to investigating their potential application in winemaking. Three lines of research were followed in this study: a) a biochemical investigation of glycosidase activities using artificial (*p*-nitrophenol-linked) substrates, b) an investigation of the enzymatic effect of LAB on wine anthocyanins, and c) identification and cloning of a putative β-glucosidase gene from *Oenococcus oeni*.

From this work it was clear that all *O. oeni* strains studied showed activity against most of the glycosides tested, both under optimized and wine-like conditions. Most importantly, some strains showed an increase or no effect by the presence of glucose and a few cases glycosidases, such as α-arabinosidase and α-rhamnosidase,

were in fact highly stimulated when fructose was present in the assay medium. By comparison, *Lactobacillus* and *Pediococcus* isolates showed activity only against selected glycosidase substrates. Highest enzymatic activities were observed for all tested strains at pHs nearer neutral with the nature and magnitude of such activities being highly strain-dependent.

In order to increase understanding of the interaction of LAB glycosidases with natural substrates, the ability of arbutin, salicin and the anthocyanin, malvidin-3-glucose, to induce these activities were studied. In subsequent experiments, the ability for anthocyanins to be decolourised when LAB isolates were present in the incubation media was examined, with the resulting reduction in colour intensity be obvious to the naked eye. While loss of malvidin-3-glucoside was monitored by HPLC analysis, the appearance of the expected breakdown product(s) could not be confirmed.

β-D-glucopyranosidase is a well characterised enzyme in many organisms, including several LAB. With this information it was possible to locate three β-D-glucopyranosidases in the *Oenococcus oeni* genome, which has recently been sequenced and published on GenBank. These enzymes were inserted in what, most likely, seemed operons of the phosphotransferase system (PTS) of the carbohydrate catabolism. Two of these enzymes were in the same operon, which showed a higher PTS structure than the other. In fact there was a gene codifying for putative a transcriptional regulator, most probably of the PTS domain EII, representing a cellobiose permease. Genomic DNA was extracted from strain Oen2, being the bacterium that showed the highest glycosidase activity. One of the b-D-glucopyranosidase enzyme was PCR amplified and sequenced, showing 25 nucleotide modifications that produced 6 amino acid substitutions.

Wine LAB therefore may represent a valid alternative as a source of enzymes for use in winemaking and other food industry processes. They clearly possess and range of glycosidase activities, which in some cases appear to overcome many of the drawbacks (e.g. inhibition by pH, ethanol and/or sugar) found with enzymes derived from other wine organisms or fungi commonly exploited in food technology. Wine LAB also influence the persistence of malvidin-3-glucoside in incubation assays, which may have important repercussions for wine quality.

Declaration of Authorship

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

Components of Chapter 1 and essentially all of the work detailed in Chapters 3 and 4 has been published in peer-reviewed scientific journals:

Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P.R. and Jiranek, V. (2004) Lactic acid bacteria as a source of enzymes for use in vinification. *Applied and Environmental Microbiology.* **70**, 5715-5731.

Grimaldi A., Bartowsky, E. and Jiranek, V. (2005) A survey of glycosidase activities of commercial strains of *Oenococcus oeni. International Journal of Food Microbiology.* **105**, 233-244.

Grimaldi, A., Bartowsky, E. and Jiranek, V. (2005) Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. *Journal of Applied Microbiology.* **99**, 1061-1069.

Copies of these papers are included in Appendix 11.

I give consent to this copy of my thesis being made available in the University Library. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

Antonio Grimaldi

15 June 2006

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TABLE OF CONTENTS

7 GENERAL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

1. LITERATURE REVIEW

1.1. Introduction

Aroma along with flavour (aroma detected retronasally), taste, colour and clarity is an important factor for wine quality and represents one of the main characteristics defining a wine. Aroma is the term for all the odorous and perfumed compounds that are derived from grape. In addition, bouquet is normally used to define the smell originating from the processing of wine, including both alcoholic and malolactic fermentations and aging in wood barrels and bottles (Margalit, 1997). Many compounds are involved in the formation of aroma in a wine, and from a chemical point of view they can be very different, but with one characteristic in common: volatility. In fact, these compounds must be volatile to be perceived by human senses.

In several wines, such as Muscat, Gewürztraminer and Riesling, terpenols are the main compounds responsible for the aromatic profile. These compounds often have a very low aroma detection threshold, thus they are perceived even when present at a few parts per million (ppm) in wine. Their typical aroma is floral and fruity. Unfortunately, terpenols exist in wine mostly in a non-volatile and odourless form, bound with sugars to form compounds called glycosides. In very aromatic wines it has been found that the ratio between bound and free terpenols can range between 1:1 and 5:1 (Muscat) and even up to 15:1 for Gewürztraminer (Günata et al., 1988). For this reason, these compounds are considered a *potential* source of aroma in such wines and are defined as aroma precursors. As such, it is important to find a method to liberate terpenols from glycosides to increase aroma intensity and/or complexity and ideally the quality of wines.

Enzymes of the glycosidase family hydrolyse glycosides, and in this context are able to liberate terpenols without negatively affecting other quality characteristics of wines. These enzymes have been studied in a great number of different organisms, including human beings (Coutinho and Henrissat, 1999). However, wine, with its low pH (typically between 2.8 and 4), high ethanol and sugar content, is a harsh environment for such enzymes. Many of these factors have proven highly inhibitory, rending some of the enzymatic preparations useless in winemaking. Glycosidase

preparations that show a low inhibition, such as those of fungal origin, are often too impure for use in wine, and can possess some negative enzymatic characteristics. Esterases, which hydrolyse esters that contribute to fruit flavour, or polyphenol-oxidases, which oxide polyphenols and thereby change or degrade the colour of wines, are frequently found as contaminants in these glycosidase preparations. For all these reasons, it is important to find glycosidases which have a high specificity for wine glycosides, have no secondary enzymatic action and above all are not inhibited by wine components.

While many studies of glycosidases of wine yeast have been conducted, there is an almost complete lack of information about the enzymes from wine bacteria, and in particular the lactic acid bacterium *Oenococcus oeni*. Our preliminary work on *O. oeni* glycosidases, and β-glucosidases in particular, confirms that these activities exist and that they seem to bypass the limitations encountered by glycosidases from other sources (Grimaldi et al., 2000).

The aim of this project is to investigate the occurrence of glycosidases in wine Lactic Acid Bacteria (LAB) and evaluate their possible use in winemaking to increase the aroma and therefore the quality of wines. The next sections will describe the presence of glycosidases in plants, with a special regard to grapevines, and the studies carried out in wine on glycosidases derived from several organisms.

1.2. Glycosides as precursors of aroma

Glycosides are formed by one or more carbohydrates (sugars) and a compound which has at least one alcoholic (hydroxyl) group; the latter is called an aglycone in glycosides and its nature can range from a simple to a complicated alcohol, such as methanol or a sterol, respectively. Sugars and alcohols are bound together by a glycosidic linkage, which can involve any of the hydroxyl groups in the sugar moiety. Glycosides have different physical and chemical characteristics to their single components. They are normally soluble in water and non-volatile, whereas many of the

aglycones are hydrophobic (Marais, 1983; Margalit, 1997; Ribéreau-Gayon et al., 2004b; Strauss et al., 1986)

In grape and wine, glycosides are in two forms; di- or mono-saccharides, depending on the number of sugars that are attached to the aglycone. In the former, two sugars are present, one of which is always D-glucopyranose and the other either α-L-rhamnopyranose (also known as rutinose), α-L-arabinofuranose or β-D-apiofuranose. In the case of the monosaccharide form, where D-glucopyranose is the only sugar, they are generally called glucosides (Fig. 1.1). Typically, glycosides are the most abundant in grapes, whereas glucosides are in much lower concentrations (Bayonove et al., 1992; Stahl-Biskup et al., 1993; Winterhalter and Skouroumounis, 1997). It was thought that D-glucopyranose was only in the beta form in grape/wine but recent work revealed the alpha form in Riesling wine (Winterhalter et al., 1998). More studies are needed, however, to determine if such compounds are also present in other grape varieties.
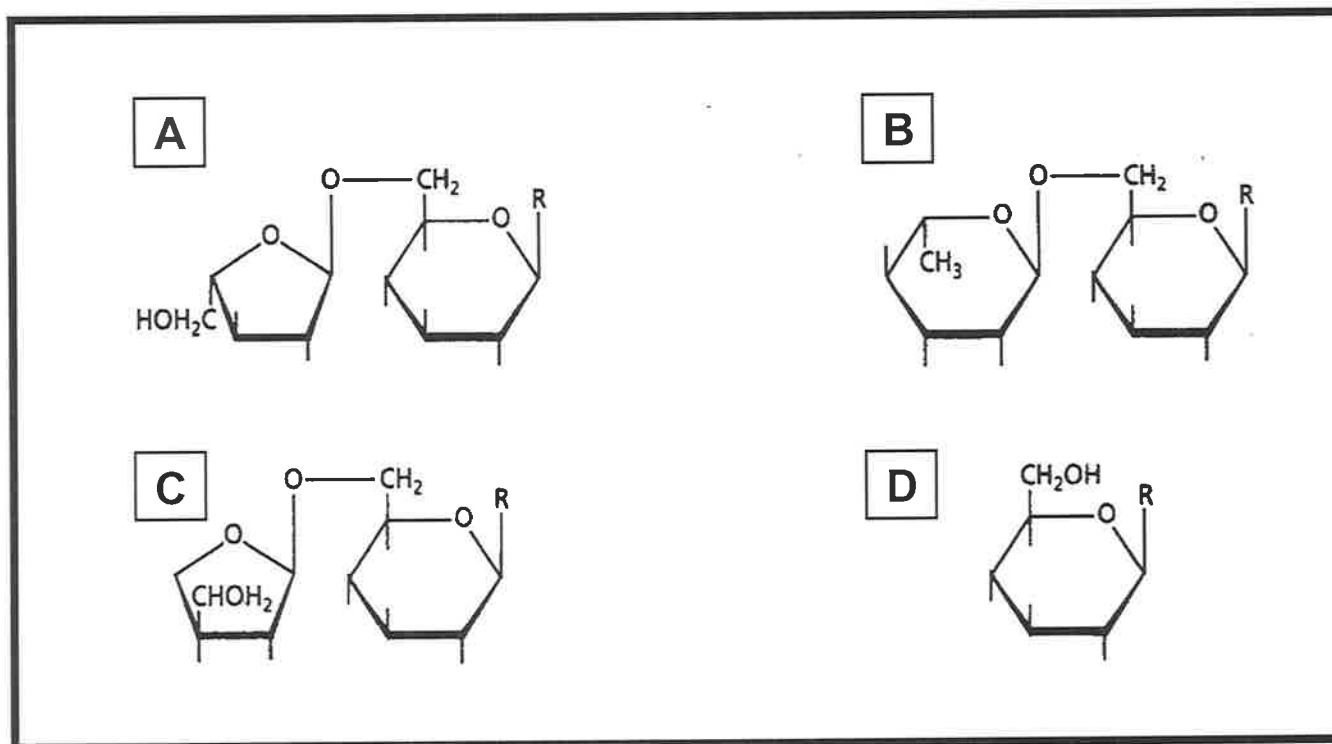
Figure 1.1. Most abundant glycosides in wine (Ribéreau-Gayon et al., 2004b). R, aglycone, which may be represented by either monoterpenes, C_{13} norisoprenoids, benzene derivatives or aliphatic compounds (Winterhalter and Skouroumounis, 1997).

A, 6-O-α-L-arabinofuranosyl-β-D-glucopyranoside; B, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside;

C, 6-O-β-D-apiofuranosyl-β-D-glucopyranoside; D, β-D-glucopyranoside.

1.2.1. Types of aglycones and sugars

In grapes the nature of aglycones is more diverse than the linked sugars. They can be norisoprenoids, benzene derivatives, aliphatic compounds and monoterpenes (terpenols), and their presence and concentration in grapes varies enormously. Of all of these, terpenols are the most important constituents of aroma in several wines, mainly those classified as aromatic such as Muscat, Gewürztraminer, Riesling and Malvasia (Mateo and Jimenez, 2000).

Terpenols are alcohols of terpenoids, polymers of the compound isoprene. Typically, terpenols found in grape/wine are dimers of isoprene and have one alcoholic group. The most common are linalol, geraniol and nerol, but α-terpineol and citronellol are also present, though in much lower concentrations (details in section 3.2.2). As their names suggest, they are responsible for floral and fruit aromas and have low aroma detection thresholds by human senses: from 18 to 400 µg/L being required in wine (Marais, 1983; Ribéreau-Gayon et al., 2004b). When a terpenol or any other odorous aglycone is linked to a sugar forming a glycoside, it loses its volatility and thus its aromaticity. This is why glycosides are considered aroma precursors or a potential source of aroma in grape and wine.

1.3. Presence of glycosides and their aglycones in plants

1.3.1. The plant kingdom

Glycosides are widespread in nature and a great number of biologically important molecules contain glycosidic linkages (McMurry, 1992), both in the animal and plant kingdoms. In plants, the number of compounds linked to sugars by a glycosidic linkage is so high that even today the physiological and chemical role of many is not clear. It has been recognised that plants tend to accumulate and store many secondary products of their metabolism as glycosides. As stated, from a chemical point

5

of view, the glycosides differ very much from their single components. In fact glycosides are normally soluble in water and have a lower chemical reactivity. Presumably, this simplifies the storage of toxic molecules (such as some polyphenols) in plant cell vacuoles. Normally, a large range of enzymes hydrolyse glycosides when plant cells require the particular aglycones. This is the case during defensive action against fungal and bacterial infections. Some aglycones such as phloridzin and arbutin in apple and leaves, respectively, are believed to have anti-microbial activity (Goodman et al., 1986). When these plants are infected by certain bacterial pathogens, endogenous plant β-glucosidases hydrolyse the glycosides that contain arbutin. During such infections, it has been demonstrated that plants produce a high concentration of glycosidase enzymes, and glycosides are removed from the storage compartments to be transferred to the infected areas.

1.3.2. Grapevine and cultivated grape varieties

Grape glycosides and their aglycone varieties have been quantified in several studies, but the use of different quantitation methods makes a comparison between these studies difficult. Furthermore, the studied grape varieties were harvested from different regions of the world. Of all the grape varieties, particular attention has been paid to Muscat and Gewürztraminer, given their great aromaticity. Some of these studies will be discussed in detail in the next sections and have been chosen for the fact that they represent the most exhaustive study of all the glycosides in several grape varieties.

1.3.2.1. Sugar moieties of grape glycosides

As described in Section 1.2, four different forms of glycosides can be identified in grapes and wine according to their sugar moiety: 6-O-α-rhamnopyranosyl-β-D-glucopyranosides (Rha-Glu), 6-O-α-arabinofuranosyl-β-D-glucopyranosides (Ara-Glu),

6-O-β-apiofuranosyl-β-D-glucopyranosides (Apio-Glu) and β-D-glucopyranosides (Glu).

α-rhamnopyranosides (Rha), α-arabinifuranosides (Ara), β-apiofuranosides (Apio) and β-xylopyranosides (Xylo) are also present, but usually in very low concentrations (Razungles et al., 1993; Winterhalter and Skouroumounis, 1997). The composition of single glycosides differs greatly by grape variety, growing area and maturity of the grapes (Bureau ct al., 2000; Günata et al., 1985; Marais and van Wyk, 1986). A selection of findings from some of these studies is reported in Table 1.1.

Considering only the glycosides containing the most aromatic aglycones (i.e. having the same oxidation state as linalol), Apio-Glu and Ara-Glu are the most abundant (30% and up to 60%, respectively) with Rha-Glu and Glu occurring in much lower concentrations, around 15 and 10%, respectively (Bayonove et al., 1992).

Table 1.1. Nature of the sugar moiety of aromatic glycosides in various grape varieties

Grape variety	Relative occurrence of sugar moieties (%)						Reference
	Rha-Glu[1]	Apio-Glu[2]	Ara-Glu[3]	Glu[4]	Rha[5]	Ara[6]	
Muscat Ottonel	5	20	25	50	nd	nd	(Bayonove et al., 1992)[a]
Muscat Frontignan	1	33	33	33	nd	nd	
Muscat of Alexandria	10	30	20	40	nd	nd	
Muscat Hambourg	6	36	41	17	nd	nd	
Gewürztraminer	7	30	50	12	nd	nd	
Muscat of Alexandria	24	nd	37	19	10	10	(Razungles et al., 1993)[b]
Muscat of Alexandria	26	10	25	39	nd	nd	(Bureau et al., 1996)[b]
Parellada	51	nd	25	24	nd	nd	(Lopez-Tamames et al., 1997)
Xarel.lo	53	nd	18	29	nd	nd	
Listan	21	nd	27	52	nd	nd	

[1] 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides

[2] 6-O-β-D-apiofuranosyl-β-D-glucopyranosides

[3] 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides

[4] β-D-glucopyranosides

[5] α-L-rhamnopyranosides

[6] α-L-arabinofuranosdies

[a] glycoside extraction method and analysis not mentioned

[b] glycosides hydrolysed by glycosidases and aglycones measured by GC

nd: not determined.

1.3.2.2. Aromatic aglycones of grape varieties

Several compounds are found in the glycosidic form in grapes and wine and can be grouped into 4 different categories: monoterpenes and C_{13} norisoprenoids (Fig. 1.2 and 1.3, respectively), benzene derivatives and aliphatic compounds (Sefton et al., 1993; Winterhalter and Skouroumounis, 1997). Of all these compounds, monoterpenes and their alcoholic forms, terpenols, are the most common in grapes and play an important role in determining the aroma of Muscat and Gewürztraminer (Ribéreau-Gayon et al., 1975).

Vitis vinifera is a plant rich in terpenols, with up to 44 identified compounds, although all are not always present in all grape varieties nor at the same concentrations. In fact a classification of many grape varieties has been proposed based on their content of terpenols (Strauss et al., 1986):

a) Aromatic: all Muscat varieties (total concentration more than 6 mg/L);
b) Non-Muscat aromatic varieties: Gewürztraminer, Müller-Thurgau, Riesling and Sylvaner (1 to 4 mg/L);
c) Neutral varieties: all red varieties, Chardonnay, Sauvignon Blanc, Trebbiano, Semillon, Verdelho and Chenin Blanc (lower than 1 mg/L).

Several studies have been dedicated to quantifying terpenols in different grape varieties, both in the free and glycosidic forms. A selection of findings from such studies is summarised in Table 1.2. Both enzymatic and acidic hydrolysis were used to obtain free terpenols and enable detection of the aglycone by gas chromatography (GC). Most of the authors of these works found that terpenols were largely present in the glycosidic form, although the nature of the sugars present was often not specified.
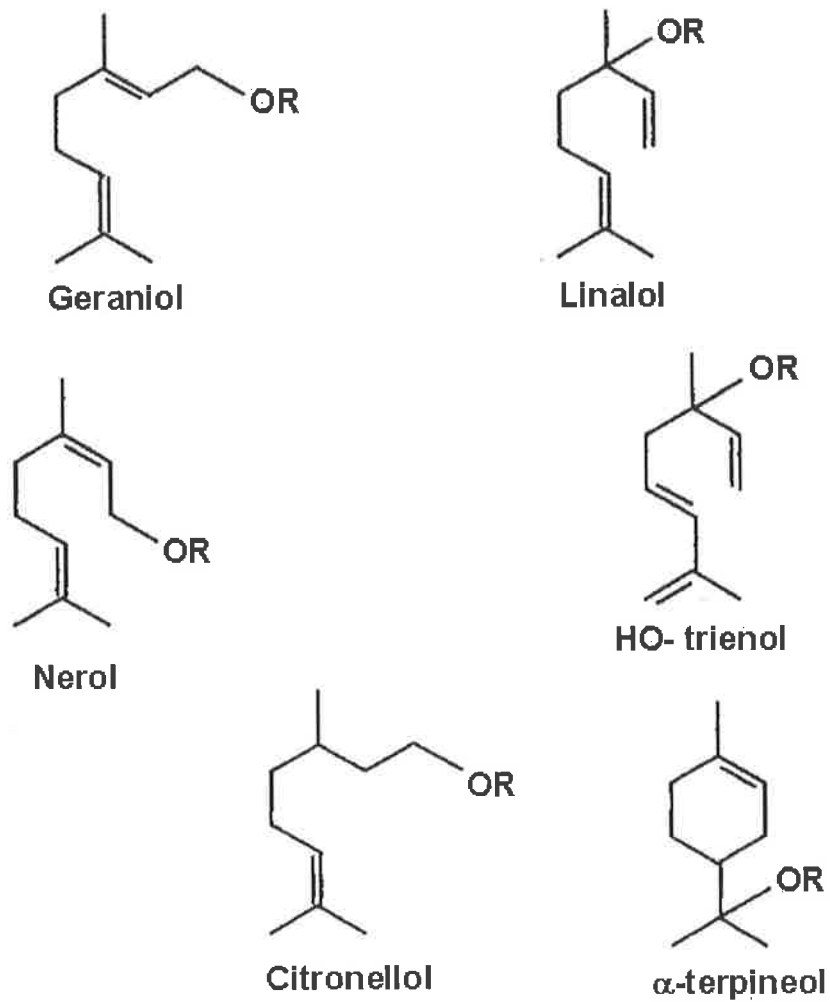
Figure 1.2. Most abundant terpenols (monoterpene alcohols) found in wine. R, either -H or a sugar moiety, forming a glycoside (see Section 1.3.2.1) (adapted from (Ribéreau-Gayon et al., 2004b).
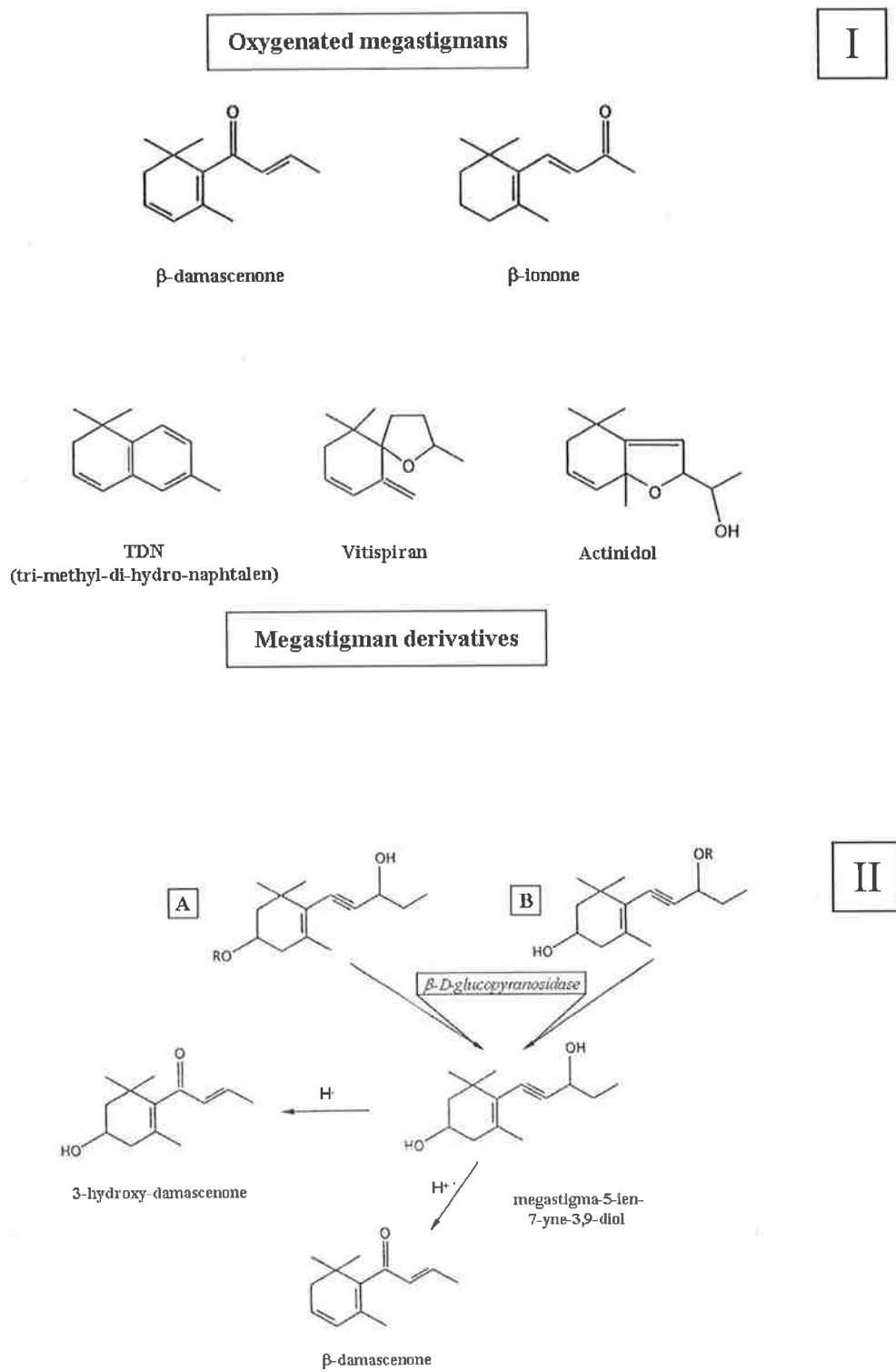
Figure 1.3. Most common C_{13} norisoprenoids found in wine (I) and formation of damascenone (II) from the precursor megastima-5-ien-7-yne-3,9diol-β-D-glucopyranoside, having a glucose in either position 3 (A) or 7 (B) (adapted from (Ribéreau-Gayon et al., 2004b).

Table 1.2. Terpenol content of various grape varieties

Grape variety	Predominant terpenols	Reference
Muscat (5 varieties)	Linalol, nerol, geraniol, α-terpineol	(Ribéreau-Gayon et al., 1975)
Muscat of Alexandria	Linalol, geraniol, nerol, dimethyl-2,6-octadiene-3,7-dio-2,6	(Razungles et al., 1993)
Cabernet Sauvignon	E dimethyl-2,6-octadiene-2,7-dio-1,6	
Chardonnay	Dimethyl-2,6-octadiene-1,7-dio-3,6	
Riesling	Linalol, unknown monoterpene, Z dimethyl-2,6-octadiene-2,7-dio-1,6	
Sauvignon Blanc	α-terpineol, geraniol, Z dimethyl-2,6-octadiene-2,7-dio-1,6	
Semillon	Z dimethyl-2,6-octadiene-2,7-dio-1,6	
Syrah	Dimethyl-2,6-octadiene-1,7-dio-3,6	
Muscat of Alexandria	Linalol, α-terpineol, citronellol, nerol, geraniol	(Gunata et al., 1986)
Merlot	E dimethyl-2,6-octadiene-3,7-dio-1,6	(Sefton, 1998)
Muscat of Alexandria	Nerol, geraniol, geramic acid	(Wirth et al., 2001)
Shiraz (Syrah)	Geraniol hydrate + Z 8-hydroxylinalool	
Chardonnay	E dimethyl-2,6-octadiene-2,7-dio-1,6	(Sefton et al., 1993)
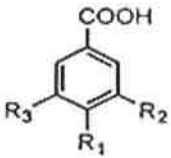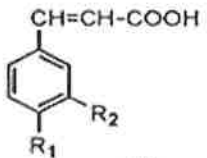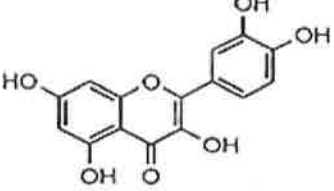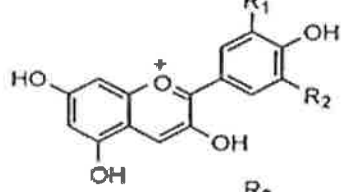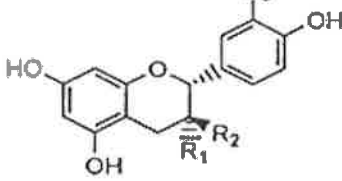
As was the case for the sugar moieties, the concentration of single terpenols is so different, depending on methods used for extraction of glycosides, viticultural region, grape variety and maturity, that it is difficult to make comparisons. Nevertheless, from these works it is evident that the hydrolysis of glycosides increases the free concentration of terpenols and other aglycones enormously, even by several hundred-fold. The implications for wine aroma are clearly shown by several authors (Gunata et al., 1986; Razungles et al., 1993; Ribéreau-Gayon et al., 1975).

1.3.2.3. Grape glycosides important for wine colour

A further group of molecules found in grapes and wine in the glycosidic form is that represented by the benzene derivatives. These compounds are important in wine because of their contribution, either singly or in a polymerised form, to the colour and structure of red and, in minimal part, white wines. Some of these benzene compounds, when deglycosylated, become volatile with the potential for substantial contributions, both positive and negative, to wine aroma. A further subset, which includes compounds such as resveratrol has recently been established as having a positive effect on human health through the prevention or reduction of cardio-vascular diseases and colon cancer (Birt et al., 2004; La Torre et al., 2004; Vrhovsek et al., 1997).

As mentioned, benzene derivatives are present both in a monomeric and a polymeric form. Though the latter plays a major role in determining the structure of wines, the former is of greater interest in the context of this project, since this category is often found in the glycosidic form. In grapes, depending on the chemical structure, three groups are found (Fig. 1.4), these are:

a) phenolic acids and their derivatives (single benzene ring);

b) flavonoids (two benzene rings linked by an O-heterocycle);

c) anthocyanins (two benzene rings linked by an unsaturated O-heterocycle)

Compound	Structure
Phenolic acids	
Hydroxycinnamic acids	
Flavonols	
Anthocyanins	
Flavonols	

Figure 1.4. Monomeric structure of wine phenols (Ontario Wild Blueberry Phytochemical Research Centre). R_1, R_2 and R_3, may be represented by either –H, –OH or –OCH_3.

Most often glucose is the sugar in these glycosides, but galactose, rhamnose and arabinose can also be present. These glycosides may sometimes be further acylated (with p-coumaric, caffeic and acetic acid), with the acids being linked to the sugar. Once again, the glycosidic form of these species is more stable than the corresponding aglycone and the tastes of each may differ. As an example, esculin has a bitter taste, whereas its aglycone (esculetin) has a sour taste (Ribéreau-Gayon et al., 2004b).

Anthocyanins are key contributors to the red colour of wines and occur in both mono- or di-glucosylated forms (Fig. 1.5). In varieties of *Vitis vinifera*, anthocyanins occur almost entirely in the former form, while the phylloxera resistant American varieties, *V. riparia* and *V. rupestris*, have mostly the di-glycosylated forms. This fact has been used to recognise and differentiate wines made from the different grape varieties, after they were introduced to overcome phylloxera (Usseglio-Tomasset, 1995).

Normally five anthocyanins are present in wine: cyanidin, peonidin, delfinidin, petunidin and malvidin. Though all are typically present, the most abundant in all varieties is malvidin, representing around 50% of all anthocyanins in Sangiovese and up to 90% in Grenache (Ribéreau-Gayon et al., 2004b). Total anthocyanin content largely varies across grape varieties and with ripening. Pinot noir, for example, has around 100 mg/L while Syrah or Cabernet Sauvignon can reach a content of 1,500 mg/L (Ribéreau-Gayon et al., 2004b). The highest concentrations of anthocyanins are usually found in the ripest grapes, a fact commonly used, amongst other parameters, as a maturation index (Peynaud, 1985).

The hydrolysis of anthocyanins (either enzymatic or acidic) produces aglycones called anthocyanidins (Fig. 1.6). In wine these aglycones have a lower colour intensity and tend to quickly precipitate if not polymerised with other phenolic compounds (Blom, 1983; Eskin, 1979).
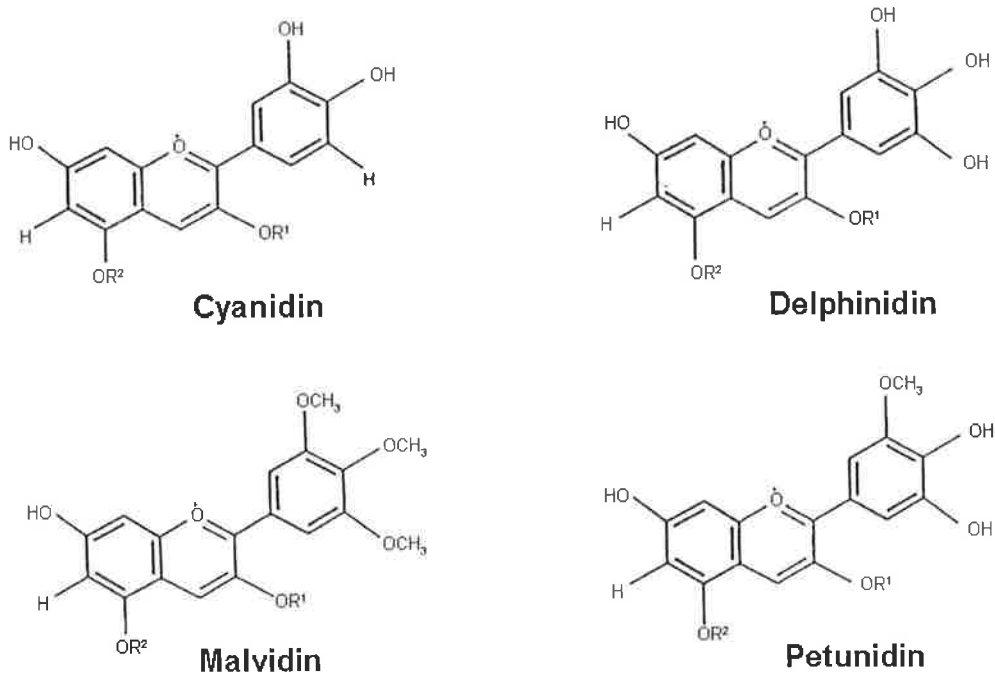
Figure 1.5. Most abundant anthocyanins found in grape and wine (adapted from Ontario Wild Blueberry Phytochemical Research Centre). Sugar moieties, either glucose, rhamnose or galactose, are attached on the R^1 only (*Vitis vinifera*), or R^1 and R^2 (non *Vitis vinifera* grape varieties) (Ribéreau-Gayon et al., 2004b).

Malvidin-3-β-D-glucoside

β-D-glucopyranosidase

Malvidin
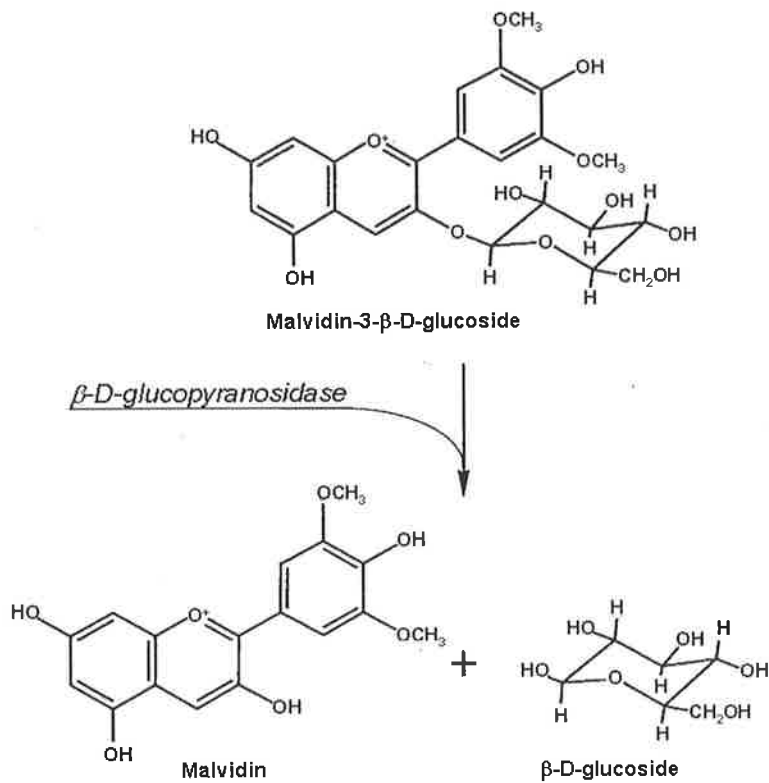
β-D-glucoside

Figure 1.6. Scheme of either the enzymatic hydrolysis malvidin-3-β-D-glucose through a β-D-glucopyranosidase (i.e. in this case an anthocyanase), or its acidic hydrolysis. Adapted from Wrolstad et al., 1994.

1.4. Methods used to liberate aromatic compounds from glycosides in wine

Across nature and the research laboratory, several mechanisms can be used to hydrolyse glycosides to liberate aglycones. In a wine context, the impact of these treatments on other quality and sensory aspects of wine is of paramount importance.

1.4.1. Acidic hydrolysis and heating

Acid hydrolysis has often been used to prepare wine samples for quantification of the free aglycones, usually by gas-chromatographic techniques (Francis et al., 1992; Sefton, 1998; Sefton et al., 1993; Skouroumounis and Sefton, 2000). The pH values used are generally those found in wine (about 2.8 – 4.0), but studies at pH values approaching 1.0, have also been performed. In all cases, the samples were also held at temperatures ranging from 45°C to 100°C, for periods of a few days and up to 4 weeks. Both model wine solutions and actual wine samples were used in an effort to understand the influence of ethanol on glycoside hydrolysis.

Of all these studies, only Francis (1992) analysed the hydrolysates to define the sensorial effect of enzymatic hydrolysis. He showed that in all cases acidic hydrolysis enhanced aromatic characteristics. However further research is needed to understand whether such results can be reproduced in varieties other than the those investigated, that is, Semillon, Chardonnay and Sauvignon blanc. Nevertheless, considering the fact that winemakers prefer to use temperatures typically below 30°C in order to preserve aroma volatiles, the application of induced acidic hydrolysis at higher temperatures is likely to be met with strong objections in the industry. The exception may be the case where thermovinification is applied (Boulton et al., 1996a). For this reason, other approaches are must be found to effect the liberation of aglycones.

1.4.2. Enzymatic hydrolysis

The use of enzymes has long been common in food processes because of their high substrate specificity, low cost and few secondary effects (Berger, 1995; Mittal, 1992). In terms of aglycone liberation, further information is still needed to understand which enzymes are most suitable for a particular reaction and most importantly, how they function. Because of the particular structure of grape and wine glycosides, it was clear from initial studies that usually more than a single enzyme is involved in the hydrolysis. The exact mechanism and sequence of enzymatic action of glycosidases on wine glycosides was elucidated by Günata et al (Günata et al., 1988) (Fig. 1.7). Using several solutions containing either di- or mono-saccharide glycosides or mixtures of both, it was possible to demonstrate that hydrolysis occurs in two separate and sequential steps, each carried out by different enzymes. In the first step, one of the following three glycosidases breaks the linkage between the terminal sugar and the β-D-glucose: α-L-arabinofuranosidase, α-L-rhamnopyranosidase and β-D-apiofuranosidase, depending on which sugar is present in the terminal position. In the next step, β-D-glucopyranosidase breaks the β-glucosidic linkage to liberate the aglycone. It is important to note that without the first hydrolysis, it is not possible for the second to occur.
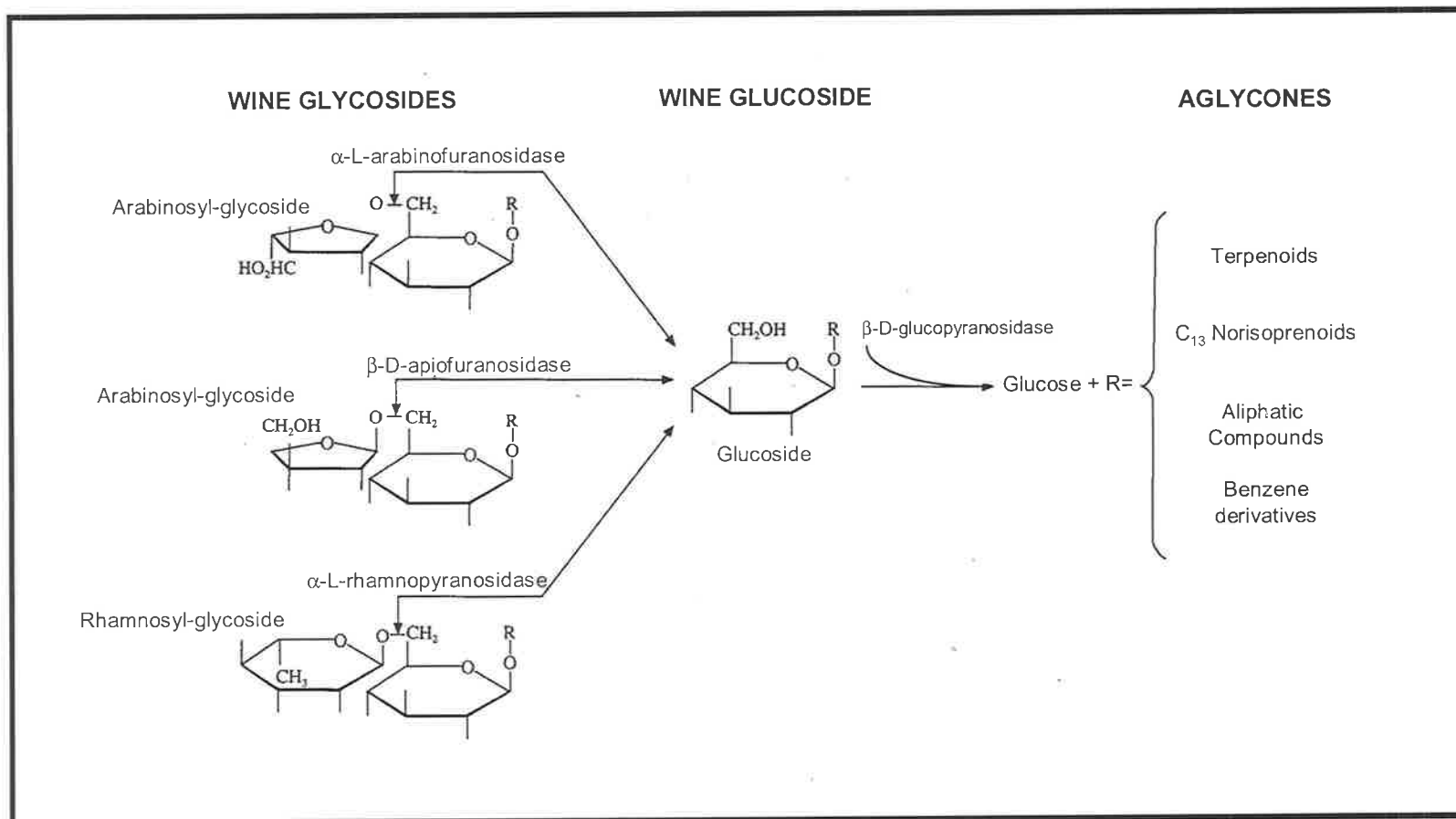
Figure 1.7. Sequential enzymatic hydrolysis (Günata et al., 1988) of wine glycosides (adapted from (Vincenzini et al., 2005), with modifications).

1.5. Studies of glycosidases in wine and grape juice

Since the discovery of terpenyl-glycosides as precursors of aroma in wines such as Muscat and Gewürztraminer, many researchers have sought methods to liberate terpenols from these precursors to increase the aroma and ideally the quality of wine. As mentioned in Section 1.4, there are several ways to achieve this outcome, however, enzymatic hydrolysis appears to be the only one that avoids deterioration of wine quality. Enzymes can have a high substrate specificity, are typically easy to apply and can be economical to produce with clear benefits for quality and processing efficiency. For all these reasons, enzymes have been widely used in food technology. However the very specificity which can be a key benefit of some enzymes can also be a limitation to their use: it may be difficult to find the appropriate enzyme to suit the desired reaction. As a result, much research energy has been spent, and is still being spent, on finding glycosidases that are able to hydrolyse wine glycosides and do so effectively in the inhibitory environment of wine.

The outcomes of studies of glycosidase enzymes from several organisms and as candidates for use in wine will be reviewed below, with special reference to microorganisms associated with wine.

1.5.1. Grapevine glycosidases

It has been known for a long time that plants and grapevines in particular possess glycosidases. In comparison with those of yeasts and fungi, grapevine enzymes have not been studied extensively in terms of their usefulness for the enhancement of aroma in wine. The main reason is that results with these enzymes show their inefficiency in hydrolysing grape glycosides. As several authors indicated, glycosidases from both berries and leaves were strongly inhibited by sugar and the low pH of grape juice. In addition, they show no specificity for glycosides containing tertiary alcohol aglycones, such as linalol, one of the most abundant terpenols in wine (Aryan et al., 1987; Günata et al., 1990a). The potential benefits of the application of these glycosidases to flavour liberation in wine therefore seems extremely limited.

21

1.5.2. Fungal glycosidases

Fungi are an abundant source of enzymes, though their nature is highly varied (Prell and Day, 2001). Of all the fungal genera, those consisting of plant pathogens seem to have the highest production of glycosidases, presumably due to their importance during the first stages of plant infection, when these enzymes are needed to hydrolyse the cellulosic plant cell wall (Lalaoui et al., 2000). Consequently, many studies have been dedicated to *Aspergillus* and *Botrytis* glycosidases. As shown in Table 1.3, individual *Aspergillus* enzymes show very different characteristics in terms of optimum pH, temperature and substrate specificity. All four glycosidases important for wine have been found in this fungus, namely, β-D-glucopyranosidase (β-glu), α-L-arabinofuranosidase (α-ara), α-L-rhamnopyranosidase (α-rha) and β-D-apiofuranosidase (β-apio). Unfortunately, commercial fungal preparations are impure as indicated by the requirement for further purification prior to their characterisation in the laboratory (Spagna et al., 2000; Spagna et al., 1998).

Optimum pHs were normally found to be around 4.0, but for β-apio the optimum was higher at around 5.0 – 6.0. In terms of temperature optima, the majority of enzymes function best in the range 60 – 65°C, whereas for β-apio the optimum operational temperature was 50°C. The most important finding for all enzymes is that glucose and low pH (approx. 3.0 – 4.0) were not inhibitory. This fact, added to the high specificity of hydrolysis for the most common glycosides in wine, suggests that fungal glycosidases may be useful in winemaking to enhance the aroma of wines. The main drawback of these commercial fungal glycosidases was their poor stability in wine, combined with the often high enzymatic impurity of such preparations (Wightman et al., 1997). More studies are clearly needed before these enzymes can be used fully and successfully in winemaking.

Table 1.3. Selected pertinent fungal glycosidases investigated for the enhancement of aroma in wine.

Enzyme[1]	Organism	Reference
β-glu	*Aspergillus niger*	(Le Traon-Masson and Pellerin, 1998)
α-ara, β-glu	*Aspergillus niger*	(Spagna et al., 1998)
α-rha	*Aspergillus nidulans*	(Manzanares et al., 2000a)
β-glu	*Aspergillus niger*	(Günata et al., 1990b)
β-apio	*Aspergillus niger*	(Guo et al., 1999)
α-rha	*Aspergillus niger*	(Spagna et al., 2000)
β-apio	*Aspergillus niger*	(Günata et al., 1997)
β-apio	*Aspergillus niger*	(Dupin et al., 1992)
β-glu	*Botrytis cinerea*	(Sasaki and Nagayama, 1997)
β-glu	*Botrytis cinerea*	(Gueguen et al., 1995b)

[1] β-glu, β-D-glucopyranosidase; α-ara, α-L-arabinofuranosidase; α-rha, α-L-rhamnopyranosidase; β-apio, β-D-apiofuranosidase.

1.5.2.1. Immobilised enzymes extracted from fungi

Where it is not possible to use a whole organism with a particular enzymatic activity, extraction and at times purification of the desired enzyme is necessary. As a result such purified enzymes may be costly, thus making their recovery from the substrate matrix and reuse highly desirable. Alternatively, removal of the enzyme from the substrate matrix and hence the finished product may be necessary or in fact may be the only way to stop the reaction. In these cases, immobilisation of the purified enzymes on a mineral support such as hydroxyapatite or a resin represents an effective solution (Dupin et al., 1992; Gueguen et al., 1996; Gueguen et al., 1997; Riccio et al., 1999; Shoseyov et al., 1990). Other advantages of immobilisation are that the amount of enzyme that is in contact with the substrate is more easily regulated thereby allowing the efficiency of the hydrolysis to be maximised over that seen for a simple addition of free enzyme. A comparison of results obtained with free and immobilised enzymes shows that, although the total glycosidase activity may be lower, immobilisation increases the stability of the enzymes and decreases the inhibitory effect of wine and fruit juice components (Gueguen et al., 1996; Riccio et al., 1999). Therefore, while enzyme immobilisation has proven useful in food and other industries, in the wine industry it remains a largely untested but highly promising approach to the application of enzymes.

1.5.2.2. Commercial enzymatic preparations having glycosidase activity

Fungal pectolytic enzymes (pectinases), purified mainly from *Aspergillus spp.*, have been widely used in several food processes since the beginning of the modern food industry. Pectins are important constituents of plant cells but, being water insoluble, they can greatly hamper filtration processes. In winemaking, pectinases are used to aid grape maceration so as to increase juice yield and extraction of colour from the grape skins (Canal-Llauberes, 1993; Van Rensburg and Pretorius, 2000).

In recent years it has been established that these commercial pectinases have a secondary glycosidic activity (Villettaz, 1996; Wightman et al., 1997; Wightman and Wrolstad, 1996; Wrolstad et al., 1994). This effect, combined with the fact that at high

concentrations pectinases reduce the colour intensity of wine, has raised questions regarding the action of these enzymes on polyphenols, and in particular, the anthocyanins. In the fruit juice industry the loss of colour is considered a desirable attribute of enzymes but in oenology it may be a serious limitation, particularly in red wine production. Most studies regarding this aspect have centred on the former industry, meanwhile very little research has been dedicated to exploring this issue in winemaking. One of the most complete works on this topic has been undertaken by Wrolstad and coworkers. Initiating out of a study of several types of fruit juices (Wightman and Wrolstad, 1995; Wightman and Wrolstad, 1996; Wrolstad et al., 1994) this group undertook an exhaustive study on wine anthocyanins and phenolics in Pinot noir and Cabernet Sauvignon wines (Wightman et al., 1997). Several pectolytic preparations, having known glycosidic activities, were tested to define the degree to which the polyphenol profile was affected. The results clearly show that there was a hydrolysis of large amounts of anthocyanidin-glycosides (up to 60%) and quercetin-glucoside by those enzymes in wine conditions (Table 1.4). In addition, the authors noted that treated wines possessed a greater amount of polymeric anthocyanins, following the inverse trend of their monomeric form.

The complete implications of such hydrolysis on total polyphenols and consequently the colour of wines is, to date, still unknown. After malolactic fermentation, for example, wines undergo a decrease in colour intensity that is certainly caused by an increase of pH, but it may also be attributed to the hydrolysis of some of the anthocyanins ((Ducruet, 1998; Piffaut et al., 1994); Professor Rocco Di Stefano, personal communication).

Table 1.4. Concentrations of anthocyanin and phenolic glycosides after pectolytic treatment in Cabernet Sauvignon wine (Wightman et al., 1997).[*]

Phenolic glycosides	Pinot Noir (mg/L)					Cabernet Sauvignon (mg/L)				
	Control	Rapidase[1]	Rohapect[2]	AR 2000[3]	Cytolase[4]	Control	Rapidase[1]	Rohapect[2]	AR 2000[3]	Cytolase[4]
Total monomeric anthocyanin-glucoside	170	160	150	90	110	360	350	340	180	280
Malvidin-3G[a]	130	135	125	65	75	150	140	140	60	100
Malvidin-3GA[b]	nd	nd	nd	nd	nd	50	50	50	40	45
Malvidin-3GC[c]	nd	nd	nd	nd	nd	15	14	12	6	8
Polymeric anthocyanin (%)	36	36	40	54	45	7	8	9	19	12
Quercetin aglycone	360	400	340	510	460	250	265	270	410	290
trans-resveratrol (HPLC peak areas)	60	120	300	730	660	200	260	240	410	275

[a] malvidin-3-glucoside; [b] malvidin-3-glucosylacetate; [c] malvidin-3-glucosylcoumarate;
[1] Rapidase EX Color, [3] AR 2000 and [4] Cytolase PLC5, Gist Brocades/DSM-Enology, Delft, The Netherlands; [2] Rohapect VR Super L, Rohm, Darmstadt, Germany;
[*] data taken after 160 days from beginning of fermentation.

1.5.3. Enzymes from yeasts

Wine yeasts have been widely studied for their impact on wine flavour and aroma. Thus it is not surprising that many works dedicated to yeast glycosidases have been carried out using *Saccharomyces cerevisiae*, the most important microorganism in wine. The findings from such studies as well as those performed on non-*Saccharomyces* yeasts are summarised in Table 1.5. Unfortunately, regardless of which world wine region it has been isolated from, *S. cerevisiae* has shown very limited production of glycosidases. These enzymes are not normally inhibited by glucose, but actually are slightly induced by small amounts of glucose, approximately 0.1% w/v. Even so, they are never produced in sufficient amounts to obtain detectable hydrolysis of any substrate (Grimaldi, 2000).

Studies performed on non-*Saccharomyces* wine yeasts have yielded more promising results. Many apiculate yeasts (*Hanseniaspora/Kloeckera*) show high enzymatic activity, but one that is completely inhibited by even very low concentrations of sugars, 0.1% w/v. Speculations have been made about the mechanism of glucose inhibition on the glycosidases. Direct binding of glucose with the active site of the enzyme, thereby impeding its proper functioning has been proposed (Iolanda Rosi, personal communication). Other yeasts (*Pichia*, *Candida* and *Brettanomyces*) showed high activity but were inhibited by the increasing formation of ethanol in the grape juice/wine. In addition, these yeasts are largely considered spoilage microorganisms and so their presence in wine is typically considered undesiderable.

Generally it was found that the glycosidic activity of wine-related yeasts was localised intracellularly. The few exceptions included *Debaryomyces hansenii* and *Candida molischiana*, in which the enyzme is almost completely localised extracellularly (Rosi et al., 1994; Strauss et al., 2001; Yanai and Sato, 1999). Another important aspect of the findings for yeasts is that the main glycosidase enzyme observed was β-D-glucopyranosidase, with little or no production of the other three enzymes.

Table 1.5. Yeast glycosidases studied for enhancing wine aroma

Enzyme[1]	Organism	Reference
β-glu, α-ara, α-rha	*Saccharomyces cerevisiae*	(Delcroix et al., 1994)
β-glu	*Zygosaccharomyces bailii*	(Gueguen et al., 1995a)
β-glu	*Dekkera intermedia*	(Blondin et al., 1983)
β-glu	*Candida molischiana*	(Gondé et al., 1985)
β-glu	*Saccharomyces cerevisiae*	(Dubourdieu et al., 1988)
β-glu	*Hanseniaspora vinae*	(Vasserot et al., 1989)
β-glu	non-*Saccharomyces*[b]	(Charoenchai et al., 1997)
β-glu	*Debaryomyces hansenii*	(Yanai and Sato, 1999)
β-glu	wine yeasts[c]	(Rosi et al., 1994)
β-glu	*Saccharomyces spp.*	(Mateo and di Stefano, 1997)
glycosidases[a]	non-*Saccharomyces*[b]	(Mendes Ferreira et al., 2001)
β-glu	non-*Saccharomyces*[b]	(Strauss et al., 2001)
β-glu	wine yeasts[c]	(Ubeda Iranzo et al., 1998)
β-glu	*Candida molischiana*	(Gueguen et al., 1996)
β-glu	*Debaryomyces hansenii*	(Riccio et al., 1999)
α-ara	*Pichia capsulata*	(Yanai and Sato, 2000)
glycosidases[a]	wine yeasts[c]	(McMahon et al., 1999)

[1] β-glu, β-D-glucopyranosidase; α-ara, α-L-arabinofuranosidase ; α-rha, α-L-rhamnopyranosidase.

[a] enzymes not fully characterised/described. [b] wine yeasts other than *Saccharomyces cerevisiae*. [c] mixed cultures used in the experimental assays.

In summary, it is possible to state that the most common yeasts found in wine show either very low glycosidase activity or else enzymes whose activity is greatly inhibited by wine components, such as sugars (glucose) and ethanol. Despite the fact that some yeasts are potentially good enzyme producers, the properties of these enzymes are far from ideal and so other enzymatic sources should be investigated to obtain a better enhancement of aroma of wine.

1.5.4. Glycosidases of wine bacteria

1.5.4.1. Lactic Acid Bacteria in wine

Without considering spoilage bacteria *sensu strictu*, such as acetic acid producing bacteria, the only group that has a relevant importance in winemaking is the Lactic Acid Bacteria (LAB) (Fugelsang, 1997; Vincenzini et al., 2005). These bacteria form a large group of gram positive bacteria, which are able to ferment several carbon sources to produce lactic acid. In wine their importance comes from the fact that they carry out the malolactic fermentation, which decreases the acidity and the herbaceous taste of wine. This is achieved by the conversion of L-malic acid in L-lactic acid, together with CO_2. Only three genera of LAB are normally found in wine, *Lactobacillus*, *Pediococcus* and *Oenococcus*, however, the last, comprising the sole species *O. oeni*, is recognised as the main malolactic bacterium (Davis et al., 1985; Davis et al., 1988; Van Vuuren and Dicks, 1993). Deacidification is not the only important contribution of LAB to wine, both having positive and negative effects, depending on their intensity or combination of the produced compounds (Vincenzini et al., 2005). Their citric acid metabolism produces compounds such as di-acetyl which are responsible for the "buttery" or "nutty" aroma (reviewed by (Bartowsky and Henschke, 2004)). When produced at above 0.57 g/L, 2,3-butan-diol tends to increase the viscosity of wine with important effects on its structure (Sponholz et al., 1993).

1.5.4.2. Glycosidases of wine Lactic Acid Bacteria

Very little information is available about enzymatic activities of LAB other than the malic acid dehydrogenase enzyme (reviewed by (Matthews et al., 2004; Van Vuuren and Dicks, 1993)). Regarding glycosidase enzymes, some β-D-glucosipyranodases of *Lactobacillus plantarum* (Marasco et al., 1998; Marasco et al., 2000; Muscariello et al., 2001) and α-D-glucosipyranodase of *Pediococcus pentosaceus* (genetic sequence of amylase operon submitted to GenBank by (Leenhouts et al., 1994)) have been characterised. Data regarding this group of enzymes of *Oenococcus oeni*, instead, is minimal. In a survey of wine yeasts and bacteria for glycosidase activity, no action against the test substrate, the artificial glucoside arbutin, was detected from the LAB included (McMahon et al., 1999). However, as pointed out by the authors, the fastidious nature of *O. oeni* and difficulties in cultivating this organism are likely to have contributed to the lack of enzyme production.

Using other media and growth conditions, it has been possible to demonstrate that *O. oeni* in fact has a high glycosidase activity against artificial substrates under aerobic conditions (Grimaldi et al., 2000). Not only was this one of the first studies to report data regarding this enzyme for *O. oeni*, the detected activity was not completely inhibited by low pH or sugars. Further characterisation of this activity is needed, particularly, to define the extent of hydrolysis of natural substrates under anaerobic conditions. Recently, Boido and co-workers (Boido et al., 2002) quantified glycosides during malolactic fermentations carried out by commercial strains of *O. oeni*. While some increases of free aroma compounds were observed, the authors suggest the possibility that the majority of aglycones liberated during the MLF could be absorbed by bacterial polysaccharides and peptidoglycans. However, no direct evidence was given to show that these modifications could be attributed to the action of bacterial enzymes.

Data relating to glycosidase activity of wine LAB are therefore very limited. While the previous studies show great promise, it is not yet possible to state the best conditions needed for *O. oeni* to produce glycosidase enzymes. Furthermore, the precise specificity of these enzymes for grape glycosides remains unclear. In fact, it may be that

the best conditions for growth of LAB and conduct of the malolactic fermentation are incompatible with those for optimal glycosidase production and action.

1.6. Conclusions

Despite the importance to winemaking of wine Lactic Acid Bacteria, particularly *Oenococcus oeni* through their role in the malolactic fermentation, research regarding the aroma and flavour impact of wine microorganisms has been almost exclusively focused on *Saccharomyces cerevisiae*. No doubt this is due to the pivotal importance of this yeast to the alcoholic fermentation. Unfortunately, after almost a decade of intensive research effort, all indications are that *S. cerevisiae* is a poor producer of relevant extracellular enzymes in general, but particularly under oenological conditions.

Enzymes extracted from organisms such as fungi of the genus *Aspergillus* have demonstrated a greater efficiency, combined with a high specificity for wine glycosides. However, commercial preparations derived from these fungi often contain several other undesirable enzymes such as esterases and polyphenol-oxidases.

Colour modifications and reductions occurring during and after bacterial malolactic fermentation have usually been attributed to the pH increase occurring during this process. The possibility that such effects are in fact due to an enzymic hydrolysis of anthocyanins by LAB glycosidase enzymes needs to be considered.

Thus, a glycosidase enzyme preparation that does not have any secondary enzymatic actions and it is able to work under wine conditions without being inhibited by wine components would be an extremely useful tool for winemaking. Preliminary research suggests that wine LAB glycosidases and *O. oeni* β-glucosidase in particular, may satisfy all these prerequisites. However, very little information is available in the literature about the characterisation of these enzymes.

The aim of this project is therefore to characterise wine LAB glycosidases and to determine their impact on wine aroma and other glycosidic compounds in wine.

2. RESEARCH PLAN

2.1. Significance of the project

Glycosides are considered a potential aromatic source in wine because they contain terpenols as aglycones. Terpenols are aromatic only when they are free, while their glycosidic form is odourless and non-volatile. Any method to liberate terpenols from glycosides will increase the aromatic characteristics of a wine and potentially its quality. Important colour compounds in wine, anthocyanins, are also in glycosidic form. An eventual hydrolysis would change quite remarkably their chemistry with important repercussions, both positive and negative, on wine colour.

Of all the methods known to liberate terpenols, enzymatic hydrolysis is the only one which does not have any negative effect on the structure and quality of wines. The enzymes involved in glycoside hydrolysis are called glycosidases, and a lot of information is available on many glycosidases from several organisms. Unfortunately, the presence of many inhibiting agents in winemaking makes most of the studied glycosidases unsuitable to this process; it is therefore highly desiderable to find glycosidases which may overcome the above mentioned problems.

Wine lactic acid bacteria appear to have glycosidases which are less inhibited than enzymes from other sources by wine parameters. In addition, these microorganisms play an important role in winemaking because they carry out the malolactic fermentation. They also represent the only group of wine related microorganisms with virtually no dedicated studies regarding glycosidases and their impact on wine glycosides.

The combination of low inhibition and lack of available information makes wine lactic acid bacteria a very interesting source for the study of glycosidases for use in winemaking to improve the aromatic profile of wines and consequently their quality.

2.2. Objectives of the project

The main purpose of this project was to characterise glycosidase enzymes of wine lactic acid bacteria in order to identify suited enzymes:

a) to increase the aromatic compounds on wine, through the liberation of terpenols from glycosides;

b) to lessen the reduction of colour in red wines or, alternatively, to help decolourisation of white wines obtained by "white fermentation" of red grapes (e.g.: sparkling wines from Pinot Noir).

This characterisation was obtained working on two separate research levels: at a biochemical and a genetic one.

To understand the biochemistry, LAB strains in our collection were screened using artificial substrates, having chosen broad parameters that are important when working with wine: pH, temperature, acidity ethanol/sugar content and inducers. After preliminary screenings, the attention focused on a reduced number of strains, to identify the highest enzyme producers.

The recently published *Oenococcus* oeni genome generated the basic knowledge to identify and localise glycosidase genes. This information was used to initiate the isolation process of the enzymatic proteins.

3. BIOCHEMICAL CHARACTERISATION OF *OENOCOCCUS OENI* GLYCOSIDASE ENZYMES[1]

3.1. Introduction

Present knowledge about the glycosidases of *Oenococcus oeni* is modest compared to that of other industrially important LAB, particularly those applied in the manufacture of dairy products (Antuna and Martinez-Anaya, 1993; Bianchi-Salvadori et al., 1995; De Vos and Gasson, 1989; Marasco et al., 1998; Marasco et al., 2000; Tzanetakis and Litopoulou-Tzanetaki, 1989). Most previous studies of the topic have examined the activity of these enzymes during the malolactic fermentation (MLF) of wine (Barbagallo et al., 2004; Boido et al., 2002; D'Incecco et al., 2004; Ugliano et al., 2003). This study has sought to define the nature and extent of glycosidase activity of 22 strains of *O. oeni* under optimised and reproducible conditions, initiating with the indications found with a previous work (Grimaldi et al., 2000). As such, these organisms were grown in defined media, and presented with *p*-nitrophenyl-linked glycosides of relevance to wine. The influence of critical wine parameters, such as the concentrations of residual sugars and ethanol, pH and temperature were investigated both individually and in combination to identify the most promising candidates for application to wine.

3.2. Materials and methods

3.2.1. Bacterial strains and cultivation

The strains of *O. oeni* used in this study are listed in Table 3.1. Most were single colony isolates from commercial preparations (some formulated as mixtures of strains) of freeze-dried starter cultures used for initiation of MLF in winemaking. Long term storage of the bacteria was achieved in vials with treated beads in a cryopreservative fluid at -80°C (Protect®, Technical Service Consultants Ltd.). Pre-cultures of each

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[1] This chapter is largely based on: *Grimaldi. A., Bartowsky, E., and Jiranek, V. (2005) A survey of glycosidase activities of commercial strains of* Oenococcus oeni. *Internat. J. Food Microbiol. 105:233-44,* and has essentially only been reformatted to conform with the rest of this thesis.

strain were prepared on an ongoing basis (each 2 – 4 days) by adding one bead into de Mann/Rogosa/Sharp broth medium supplemented with preservative-free apple juice (20%), adjusted to pH 5.0 (MRSA) (Kelly et al., 1989) and incubated at 25°C.

Cultures used for the study of glycosidase activities were prepared by inoculating precultures into 5 mL of MRSA to an optical density measured at a wavelength of 600 nm (OD_{600}) of approximately 1 and incubating at 25°C for 42 h (66 h for *O. oeni* strains Oen3 and Oen5). Cells were harvested and washed twice by centrifugation (20,000 x g for 5 min) with 0.85% w/v NaCl in distilled RO water.

3.2.2. Determination of glycosidase activity of *O. oeni*.

Glycosidase activity was determined according to a method described previously (Grimaldi et al., 2000), with modifications to allow the use of a micro-plate spectrophotometer. Assays were reduced to a final volume of 80 µl and performed in standard 96-wells plates. For each reaction, 40 µl of 0.2 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO_4) was used (Dawson et al., 1986). When required McIlvane buffer was prepared to pH 4.0 and included the following at the final assay concentrations indicated: ethanol (0, 4, 8 and 12 v/v), glucose or fructose (0, 0.01, 0.1, 0.75 and 2% w/v). Each well was then dosed with 20 µl of a suspension of the appropriate *O. oeni* strain prepared in 0.85% NaCl and standardised to yield a final OD_{600} in the assay of 0.5. Substrate solutions (20 µl) were added to give the following final concentrations: *p*-nitro-phenyl β-D-glucopyranoside (10 mM), *p*-nitro-phenyl α-D-glucopyranoside (10 mM), *p*-nitro-phenyl xylopyranoside (7.5 mM), *p*-nitro-phenyl α-L-rhamnopyranoside (7.5 mM), and *p*-nitro-phenyl α-L-arabinofuranoside (7.5 mM) (Sigma). Assays were incubated at 37°C and enzymatic activity was stopped after 1 h by the addition of 160 µl of 0.5 M Na_2CO_3 and the 96-well plate centrifuged (2,500 x g for 18 min) to remove the cells from the reaction. Supernatants (200 µl) were transferred into corresponding wells in a fresh 96-well plate and the absorbance of each at 400_{nm} determined with a multi-plate spectrophotometer (µQuant®, BIO-TEK Instruments Inc.) set to automatic path-length correction. Blanks were prepared without

Table 3.1. Strains and source of *Oenococcus oeni* used in this study[a].

Isolate	Origin
Oen1	Lallemand O.S.U. (VI 77)
Oen2	Lalvin 4X (VL 92)
Oen3	Inobacter
Oen4	Bitec Vino
Oen5	Lalvin-Inobacter
Oen6	Lalvin MT01 Standard
Oen7	Lallemand 3X (E218)
Oen8	CHR Hansen Viniflora
Oen9	CHR Hansen Viniflora
Oen10	Lalvin EQ54 MBR
Oen11	Lalvin MCW
Oen12	Lalvin 3X 1Step
Oen13	Lalvin IB Standard
Oen14	Enoferm Alpha
Oen16	Lalvin 3X Standard
Oen17	Lalvin OSU MBR
Oen20	Lallemand No 3
Oen21	Lallemand No 4
Oen22	Lallemand No 5
Oen23	Lallemand No 8
Oen28	Lallemand No 2
Oen29	Lallemand No 9

[a] Strains 20 – 29 were derived from pre-commercial trails samples kindly provided by Lallemand.

bacterial cells but otherwise treated in the same manner. All reactions were performed in at least duplicate with replicate values typically being within 5% of one another. Non-enzymic hydrolysis of p-nitro-phenyl α-L-arabinofuranoside under alkaline conditions occurred after the addition of the 0.5 M Na_2CO_3 solution, and resulted in a doubling of sample absorbance approximately every 20 min. A similar increase was observed, in both samples and blanks (data not shown) and thus samples were processed as quickly as possible. Accordingly, centrifugation was applied for only 12 min after which supernatants were immediately transferred and analysed in the multi-plate spectrophotometer. In all cases, one unit of activity was defined as mmols of p-nitro-phenol liberated per min per milligram of cell dry weight. Culture dry cell weight was determined from 10 mL cultures which had been grown for 42 h.

3.2.3. Temperature dependence of glycosidase activity

Where the influence of temperature on glycosidase activity was investigated the same method as described above was used with the exception that all volumes were increased by 25% to give a final assay volume of 100 μl. Assays were performed in 200 μl disposable PCR tubes thereby allowing the use of a PCR thermocycler (Mastercycler Gradient®, Eppendorf) for accurate temperature control. Tubes were incubated for 1 h at temperatures between 14.9°C and 57.7°C. At the end of the incubation period, 80 μl from each assay were transferred to a well of a 96-well plate to which had previously been added 160 μl of 0.5 M Na_2CO_3. The 96-well plate was then centrifuged as above to pellet cells and 200 μl of supernatant transferred to a fresh 96-well plate for quantification of sample absorbance at 400_{nm} as described.

3.3. Results

With few exceptions, previous studies of glycosidases of wine LAB have been limited to investigations of β-glucosidase (β-D-glucopyranosidase) activity. Our earlier study (Grimaldi et al., 2000) appears to be the first to use other substrates to specifically identify enzymatic activities, albeit limited, beyond β-glucosidase. Regardless of the

source organism or the enzyme activities under investigation, an influence by parameters including pH, temperature and the presence of inhibitors such as sugars and ethanol has been a common observation (Aryan et al., 1987; Barbagallo et al., 2004; Grimaldi et al., 2000; Spagna et al., 2002; Winterhalter and Skouroumounis, 1997). In this study we sought to expand our earlier preliminary findings by more fully defining the nature and functional limitations of the glycosidases associated with up to 22 different *O. oeni* strains.

3.3.1. pH and substrate interactions

Strains of *O. oeni* were cultured in MRSA and evaluated for their ability to liberate nitrophenol from each of five glycosylated *p*-nitrophenyl-substrates over a range of pH values (2.6 and 7.0). General observations from this survey (see Figs. 3.1 – 3.4) include that *O. oeni* glycosidase activities were widely distributed, with most strains acting on several of the substrates tested. Also, in confirmation of previous findings, assay pH greatly altered the glycosidase activity for the majority of *O. oeni* strain and substrate combinations. This finding reinforces the necessity of examining these properties over a broad range of pH values in order to develop a fuller view of the potential of individual *O. oeni* strains.

3.3.1.1. β-D-Glucopyranosidase

All strains tested possessed a detectable β-glucosidase activity against *p*-nitro-phenyl β-D-glucopyranoside (*p*NP-βGlu). The magnitude of this activity ranged by up to 16-fold in a strain and pH dependent manner with strains Oen2, Oen5, Oen16, Oen21 and Oen22 typically showing highest activities, whilst strains Oen6A, Oen13, Oen14 and Oen28 were representative of the lower extreme (Fig. 3.1). Across the 17 strains surveyed for this activity, it is evident that pH of the assay buffer markedly influenced β-glucosidase activity. Some strains in fact displayed two peaks of activity: one at approximately pH 3.4 and a second at a pH >5.8, while in other strains maximal activity was centred at a single pH of 3.8.

3.3.1.2. α-D-Glucopyranosidase

As observed for β-D-glucopyranosidase activity, all isolates possessed an activity against *p*-nitro-phenyl α-D-glucopyranoside (*p*NP-αGlu) (Fig. 3.2). Values ranged by approximately 10-fold across the strains and pH values studied.

The optimal pH for α-L-glucopyranosidase activity varied such that it occurred at approximately pH 3.8 for strain Oen22, pH 4.6 for strain Oen2 and pH 6.6 for strains Oen1, Oen5, Oen12, Oen16 and Oen17. Dual pH optima of the sort observed for β-D-glucopyranosidase activity in many strains, were atypical for α-D-glucopyranosidase activity, and were most obviously seen with strain Oen17 at pH 3.8 and 6.6.

3.3.1.3. β-D-Xylopyranosidase

Liberation of nitrophenol from *p*-nitro-phenyl β-D-xylopyranoside (*p*NP-βXyl) was most effective at lower pH values, with a peak in activity typically occurring at pH 3.0 – 3.4 (Fig. 3.3). Maximum activities of approximately 0.9 units were seen for strain Oen21 and Oen22 at pH 3.0. Strain Oen16 demonstrated a duality of pH optima, being at 3.0 and 5.4.

3.3.1.4. α-L-Rhamnopyranosidase and α-L-Arabinofuranosidase

The response of individual strains to these two substrates were similar and thus only selected data are shown in Figure 3.4. For the remaining data, see Appendix 1 (α-L-Rhamnopyranosidase) and Appendix 2 (α-L-arabinofuranosidase) Despite an oenological importance being placed on α-L-rhamnopyranosidase because of the frequent occurrence of rhamnose-linked aroma compounds in grapes (Bayonove et al., 1992; Bureau et al., 1996; Razungles et al., 1993), poor hydrolysis of the corresponding *p*-nitrophenyl substrate (*p*NP-αRha), with a maximum of only 0.2 units was observed by *O. oeni* strains (Fig. 3.4A). Again, lower pH values gave optimal hydrolysis of *p*NP
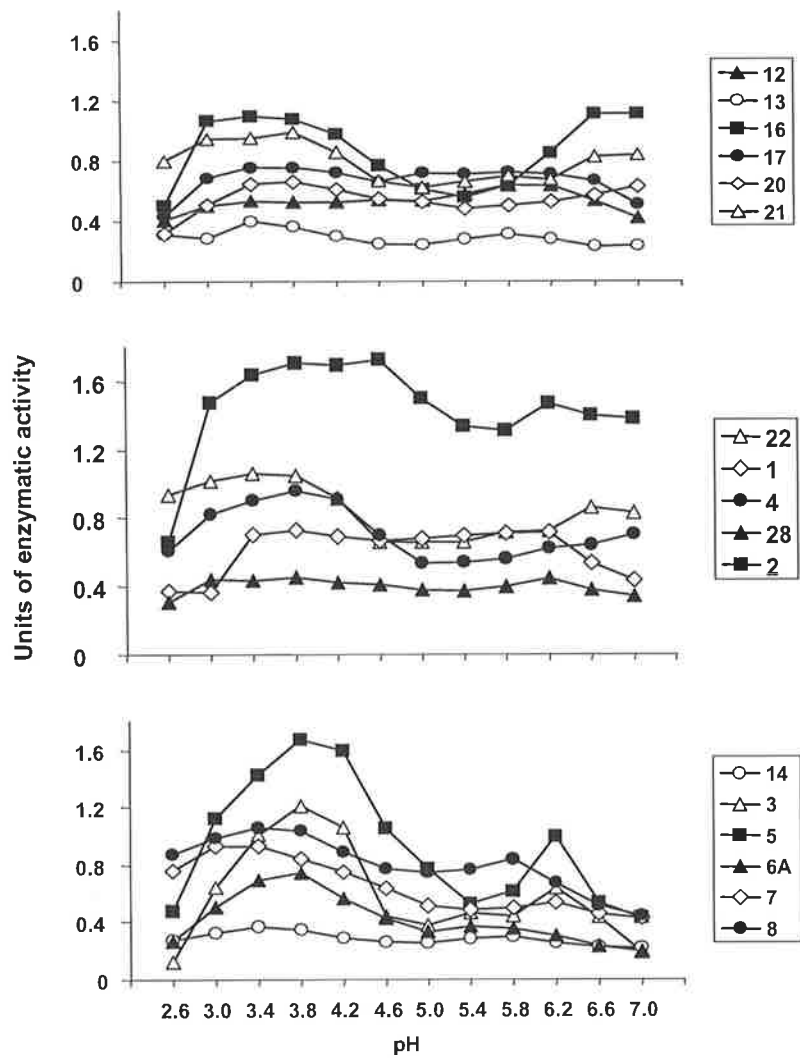
Figure 3.1. Influence of pH on the glycosidase activity of *O. oeni* strains against *p*-nitro-phenyl β-D-glucopyranoside. Values are the mean of duplicate determinations.
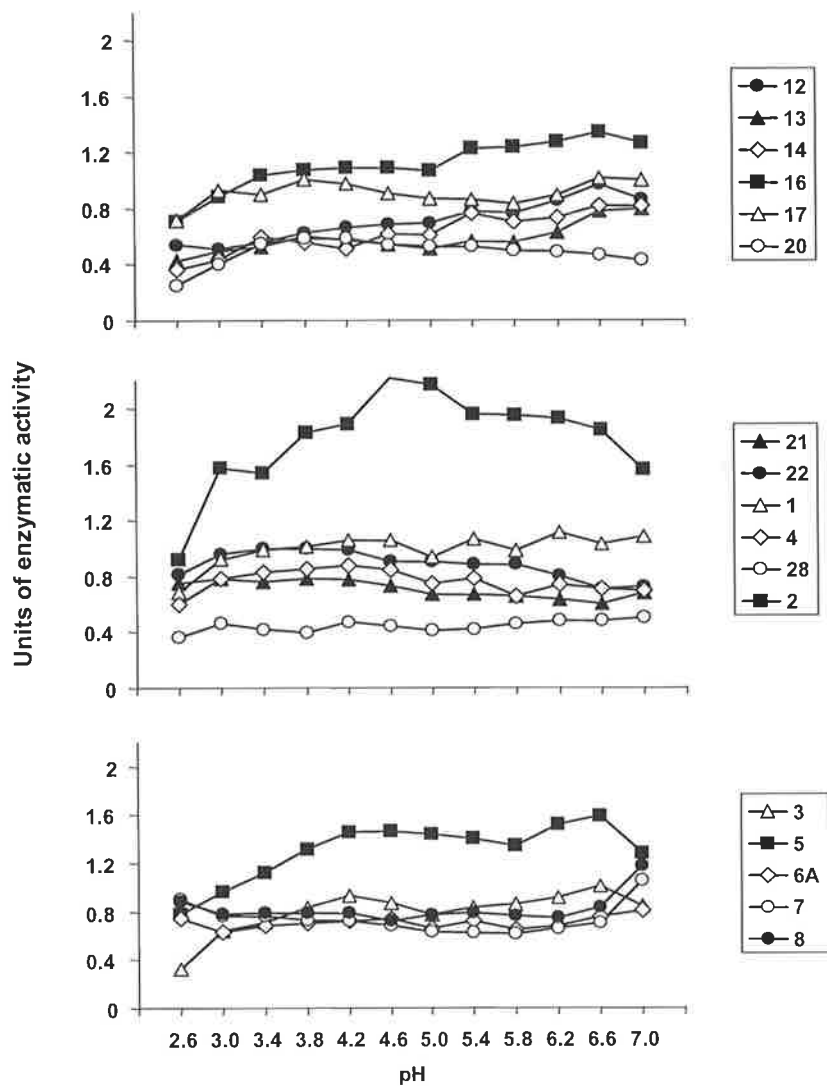
Figure 3.2. Influence of pH on the glycosidase activity of *O. oeni* strains against *p*-nitro-phenyl α-D-glucopyranoside. Values are the mean of duplicate determinations.
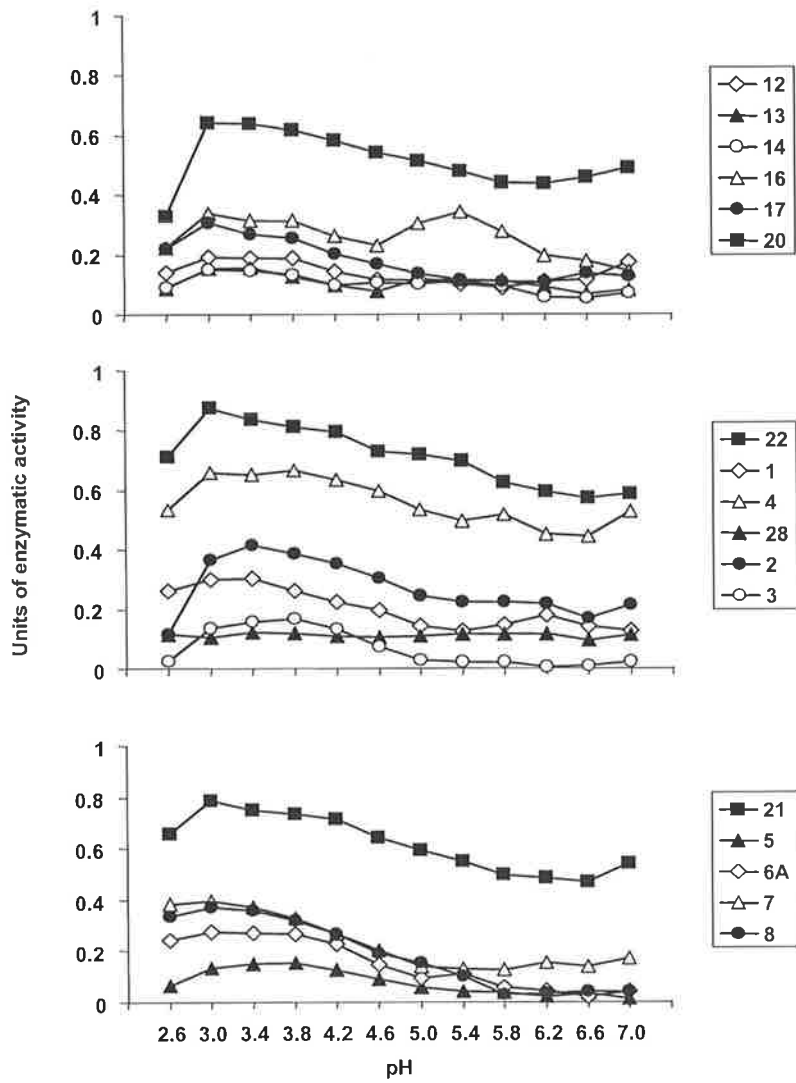
Figure 3.3. Influence of pH on the glycosidase activity of *O. oeni* strains against *p*-nitro-phenyl β-D-xylopyranoside. Values are the mean of duplicate determinations.
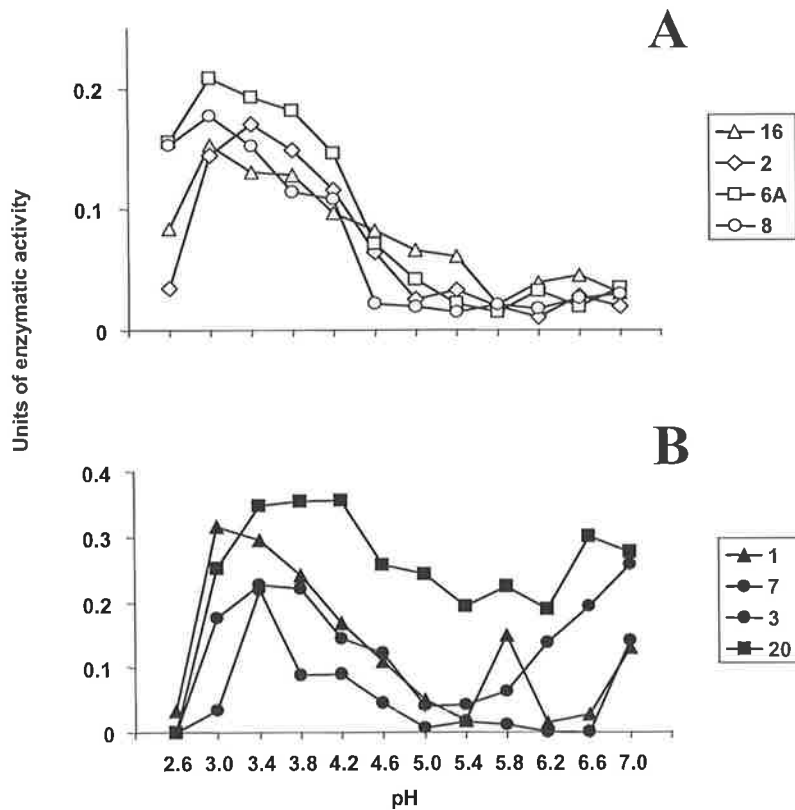
Figure 3.4. Influence of pH on the glycosidase activity of *O. oeni* strains against *p*-nitro-phenyl-α-L-rhamnopyranoside (A) and *p*-nitro-phenyl-α-L-arabinofuranoside (B). Values are the mean of duplicate determinations.

-αRha, whereas trace or no activity was seen above pH values of 4.6 – 5.0. Given the scarcity of this activity any further discussion of it is limited. Hydrolysis of *p*-nitrophenyl α-L-arabinopyranoside (*p*NP-αAra) was readily evidenced by all but two of the 21 strains studied (Fig. 3.4B). In several strains two pH optima seemed apparent: one at around 3.4 – 3.8 and a second above pH 6.6. Highest activities of the order of 0.4 units, as typified by strain Oen20, occurred at approximately pH 3.4.

A ranking of strains according to their ability to hydrolyse a given substrate was made difficult by virtue of the fact that such rankings changed according to the pH at which activity was quantified. For this reason, 'total' glycosidase activity was determined for each strain by summing activity measurements made across all pH values for a given substrate (Table 3.2). In this way, the strains most active against a particular substrate could be identified while also revealing trends across the five substrates tested. Thus strains Oen16, Oen22, Oen2, Oen5 and Oen8 were amongst the most highly active group for both *p*NP-βGlu and *p*NP-αGlu, whereas strains Oen21, Oen22 and Oen4 were prominent amongst the remaining substrates. For practical reasons, all substrates were not applied at the same concentration and thus a summation of total activities for each strain across all substrates and pH values will not equate to an absolute measure of total glycosidic activity for that strain. Nevertheless, such summation provides a useful means of achieving an overall ranking of the strains studied. This cumulative value highlights *O. oeni* strains Oen2 and then Oen22, Oen5, Oen16 and Oen21 as possessing the greatest overall glycosidic activity (Table 3.2)

3.3.2. Temperature optima of glycosidase activities

The ability of observed glycosidase activities to operate over a broad range of temperatures was determined. A selection of strains showing the highest activities were chosen to perform this test (Fig. 3.5). In general terms, maximal hydrolysis of any of the substrates occurred at temperatures close to 40°C. Nitrophenol release was rapidly reduced as the temperature increased to 60°C, while a more gradual decline was observed at temperatures below the optimum. Only low activities were observed at temperatures of 20°C and 23°C, which might be considered more relevant to winemaking conditions.

Table 3.2. Total glycosidic activities for *O. oeni* strains to each of five substrates[a].

Strain	Substrate*					TOTAL[b]
	*p*NP-βGlu	*p*NP-αGlu	*p*NP-βXyl	*p*NP-αAra	*p*NP-αRha	
Oen28	4.76	5.31	1.34	0.32	0.04	**11.77**
Oen13	3.47	6.99	1.28	0.74	1.34	**12.48**
Oen14	3.39	7.51	1.21	0.69	1.35	**14.15**
Oen6	4.96	8.48	1.78	**1.12**	0.80	**17.14**
Oen12	6.34	8.50	1.66	0.46	1.16	**18.12**
Oen3	7.00	9.54	0.79	0.57	0.65	**18.55**
Oen7	7.74	8.84	2.80	0.91	1.62	**21.91**
Oen20	6.51	5.84	**6.15**	0.80	**2.99**	**21.96**
Oen1	7.28	**11.92**	2.40	0.77	1.55	**23.22**
Oen17	8.04	**10.89**	2.16	0.86	**1.64**	**23.59**
Oen8	**9.57**	9.84	2.23	0.85	1.28	**23.77**
Oen4	8.51	9.12	**6.70**	**1.05**	**1.67**	**27.05**
Oen21	9.52	8.48	**7.41**	**1.10**	**1.66**	**28.17**
Oen16	**10.35**	**13.27**	3.11	**0.94**	0.63	**28.30**
Oen5	**11.19**	**15.66**	0.91	0.57	ND[c]	**28.33**
Oen22	**10.06**	10.57	**8.55**	**1.15**	**1.65**	**31.98**
Oen2	**17.29**	**21.37**	**3.23**	0.81	0.96	**43.66**

[a] Values are a summation of activities determined at each of the examined pH values between 2.6 and 7.0 (see Figure 3.1);

[b] Measures of total glycosidic activity are derived from the totals determined for each substrate;

[c] ND, Not determined.

* Top five (red) and highest (blue) glycosidase enzyme producers in each category.
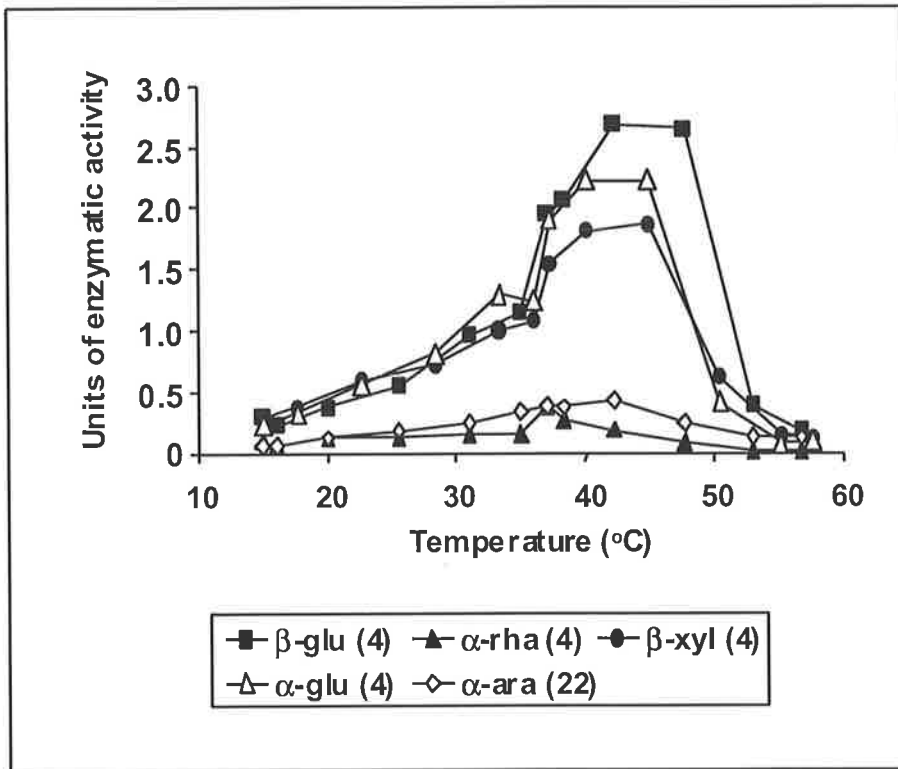
Figure 3.5. Influence of temperature on glycosidic activity for a selection of *O. oeni* strains (shown in brackets) and substrates. Values are the mean of duplicate determinations.

3.3.3. Influence of ethanol, glucose or fructose on glycosidase activity

Ethanol and sugars (glucose and fructose) have often been reported as the cause of inhibition of glycosidase enzymes (Aryan et al., 1987; Barbagallo et al., 2004; Grimaldi et al., 2000; Sanchez-Torres et al., 1998; Spagna et al., 2002; Winterhalter and Skouroumounis, 1997). The influence of these compounds was therefore investigated in this study. Examination of all 22 strains revealed a limited number of patterns of responses to the inclusion of either ethanol, glucose or fructose in the assay buffer.

Ethanol at 4% v/v resulted in a marked increase in β-D-glucopyranosidase activity by strains Oen2 and Oen4 (Fig. 3.6A), a trend repeated for 70% of all strains studied. A reduced or no enhancement was seen for the remaining strains or at higher ethanol concentrations. In strains with low initial activities, as typified by Oen6A and Oen13, higher ethanol concentrations were typically inhibitory to β-glucopyranosidase. Inhibition of β-glucopyranosidase activity was also observed for glucose and fructose, even when present at only 0.01% w/v (Fig. 3.7A). Such reductions, typically of the order of 40%, were seen for approximately 80% of the 15 strains studied. Interestingly, a 200-fold increase in the concentration of glucose or fructose did not greatly increase enzyme inhibition. Strain Oen13 stood out from others by displaying a marked enhancement of activity, particularly in the presence of fructose.

Ethanol enhancement was also observed for α-D-glucopyranosidase, again being most apparent at 4% v/v (Fig. 3.6B), albeit to a lesser degree and only occurring in 60% of all strains examined. Glucose, still at only 0.01% w/v, was highly inhibitory to α-D-glucopyranosidase. Across all strains, glucose produced an average decline in activity of approximately 45% (Fig. 3.7B). The response to fructose was lower (Fig. 3.8B).
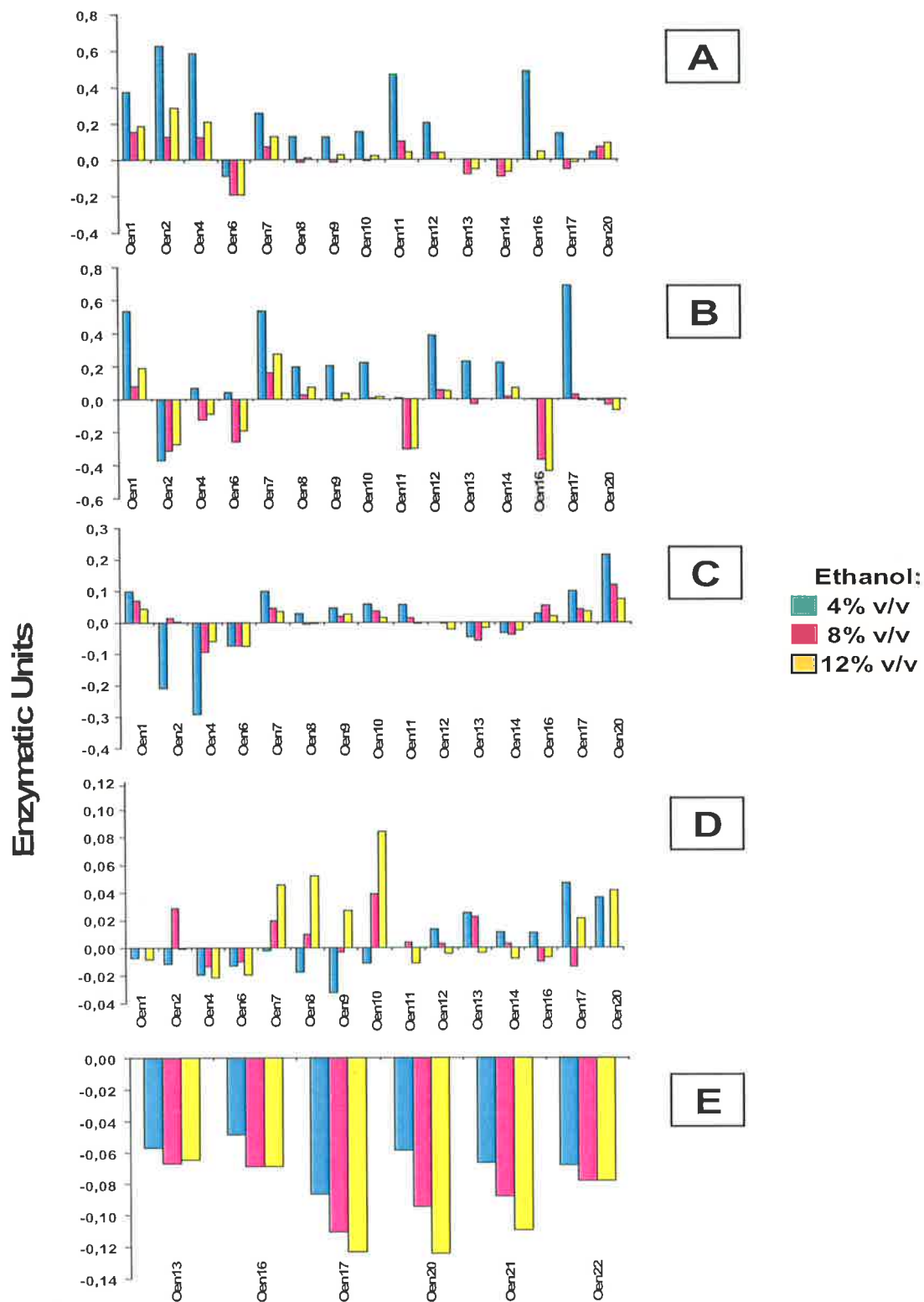
Figure 3.6. Glycosidic activity of *O. oeni* strains observed in the presence of ethanol against *p*-nitro-phenyl β-D-glucopyranoside (A), *p*-nitro-phenyl-α-D-glucopyranoside (B), *p*-nitro-phenyl-β-D-xylopyranoside (C), *p*-nitro-phenyl-α-L-rhamnopyranoside (D) and *p*-nitro-phenyl-α-L-arabinofuranoside (E). Values are relative (EU) to the activity observed for the control performed under the same conditions in the absence of either ethanol or added sugars. Values are the mean of duplicate determinations.
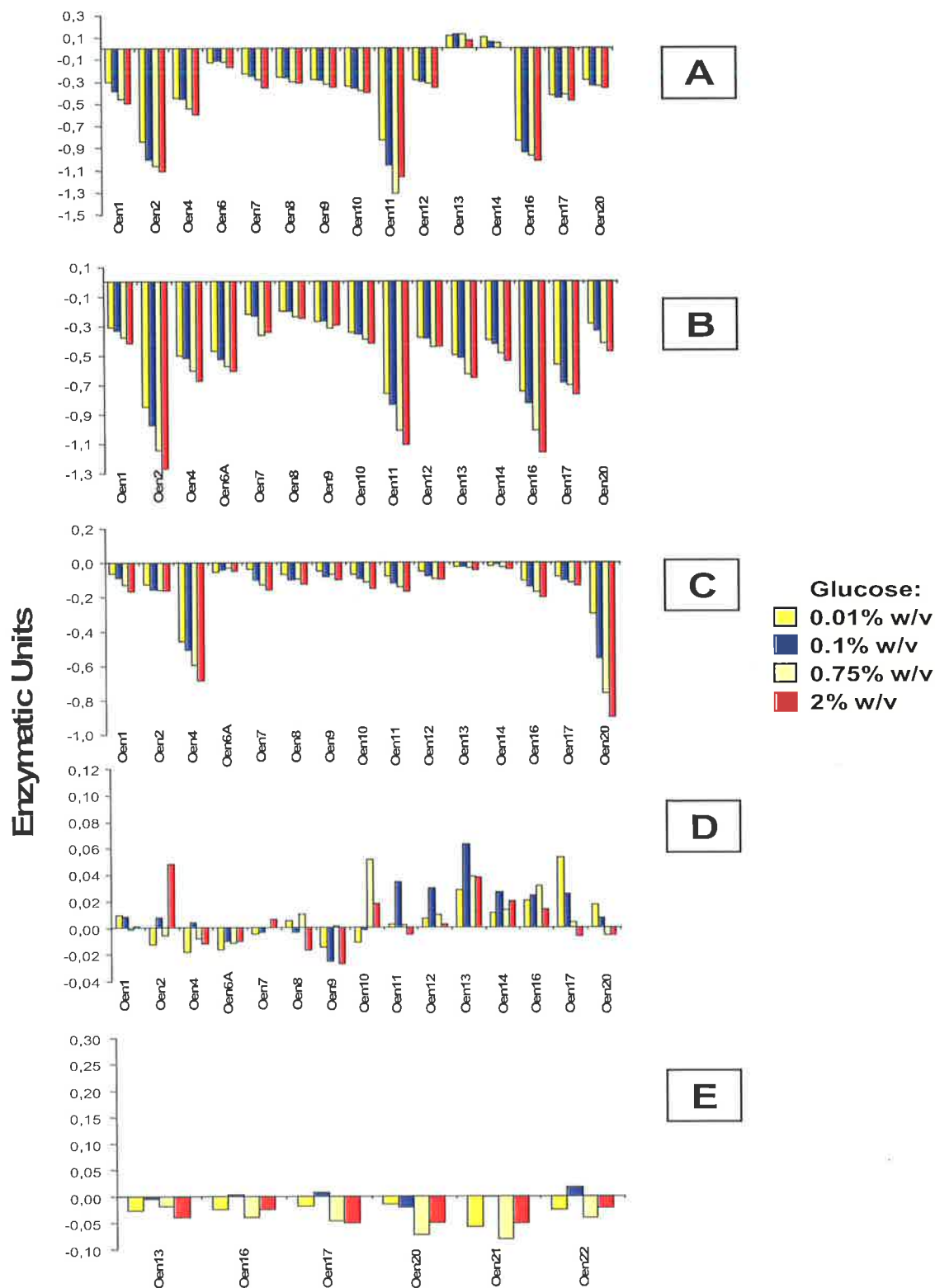
Figure 3.7. Glycosidic activity of *O. oeni* strains observed in the presence of glucose against *p*-nitro-phenyl β-D-glucopyranoside (A), *p*-nitro-phenyl-α-D-glucopyranoside (B), *p*-nitro-phenyl-β-D-xylopyranoside (C), *p*-nitro-phenyl-α-L-rhamnopyranoside (D) and *p*-nitro-phenyl-α-L-arabinofuranoside (E). Values are relative (E.U.) to the activity observed for the control performed under the same conditions in the absence of either ethanol or added sugars. Values are the mean of duplicate determinations.

49

When considering β-D-xylopyranosidase activity, the impact of ethanol was modest and variable (Fig. 3.6C). At lower concentrations, glucose more so than fructose, produced a modest drop in nitrophenol liberation from pNP-Xyl (Figures 3.6C, 3.7C and 3.8C, respectively). Determinations of the influence of ethanol, glucose and fructose on α-L-rhamnopyranosidase activity were performed, however, the minimal nature of this activity made some responses difficult to discern clearly (Figures 3.6D, 3.7D and 3.8D, respectively). It is noteworthy however that several strains, that is Oen2, Oen7, Oen8, Oen9, Oen10 and Oen20, tended towards enhanced activity in the presence of concentrations of ethanol greater than 0.01%, as well as in the presence of sugars.

Results for the α-L-arabinofuranoside substrate showed a strong inhibition by ethanol, so much so that at higher ethanol concentrations (8 and 12%) there was a total lack of enzymatic activity (Fig. 3.6E). Glucose was also inhibitory, though not as completely, particularly at lower concentrations (Fig. 3.7E). The most interesting result was obtained with the inclusion of fructose in the assay medium. Most strains exhibited a large increase in activity (> 200% for strain Oen13) over that of the control when incubated with fructose (Fig. 3.8E). Only at the highest concentration (2%) was fructose inhibitory for some strains.
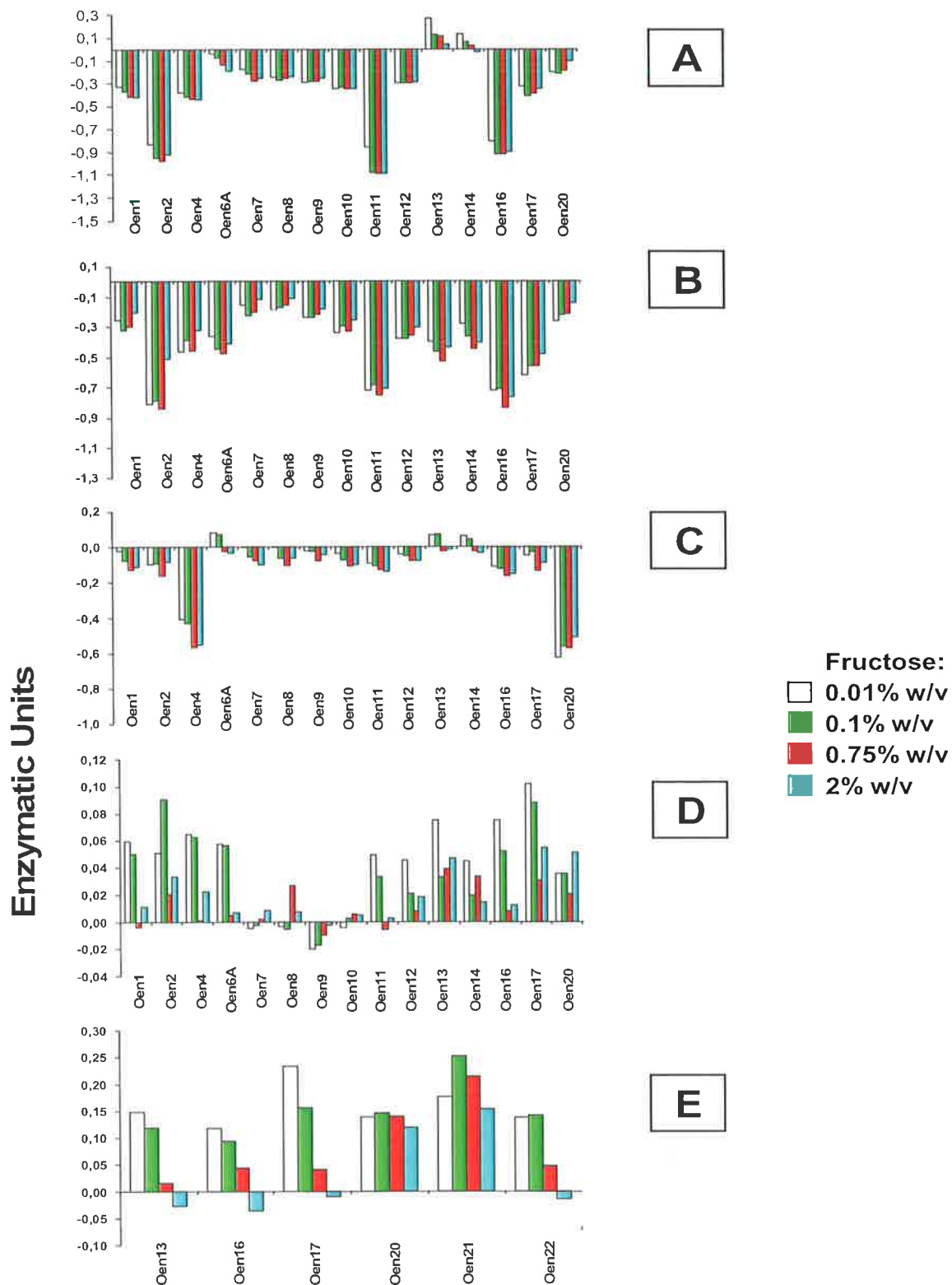
Figure 3.8. Glycosidic activity of *O. oeni* strains observed in the presence of fructose against *p*-nitro-phenyl β-D-glucopyranoside (A), *p*-nitro-phenyl-α-D-glucopyranoside (B), *p*-nitro-phenyl-β-D-xylopyranoside (C), *p*-nitro-phenyl-α-L-rhamnopyranoside (D) and *p*-nitro-phenyl-α-L-arabinofuranoside (E). Values are relative (EU) to the activity observed for the control performed under the same conditions in the absence of either ethanol or added sugars. Values are the mean of duplicate determinations.

3.3.4. Influence of multiple parameters on glycosidase activity

In most settings outside the laboratory, bacteria are likely to face a combination of the chemical and physical factors investigated above. This is certainly true for winemaking. To study the interplay of these factors, glycosidase activity in representative strains was assayed at pH 3.5 with or without 10% v/v ethanol, 0.2% w/v glucose and/or 0.1% w/v fructose, for four substrates. pNP-αRha was omitted from this survey due to the low activity previously seen for this substrate. For most of the strains examined, the inclusion of potential inhibitors, ethanol and glucose had little affect on glycosidase activities against pNP-βGlu, pNP-αGlu and pNP-βXyl substrates (Fig. 3.9). Inhibition was most apparent for the action of strain Oen4 against pNP-βXyl. For several other strain and substrate combinations, an enhancement of activity was observed, particularly for strains Oen21 and Oen22 and pNP-βGlu. Given the stimulatory affect of fructose on α-arabinofuranosidase activity (Fig. 3.8), an additional assay condition incorporating fructose instead of glucose was tested. For all five strains studied, the inclusion of ethanol and glucose or fructose was highly inhibitory on α-L-arabinofuranosidase activity (Fig. 3.9). Only in the case of strain Oen22 incubated with ethanol and fructose was there any clear suggestion that fructose was at least less inhibitory than glucose.
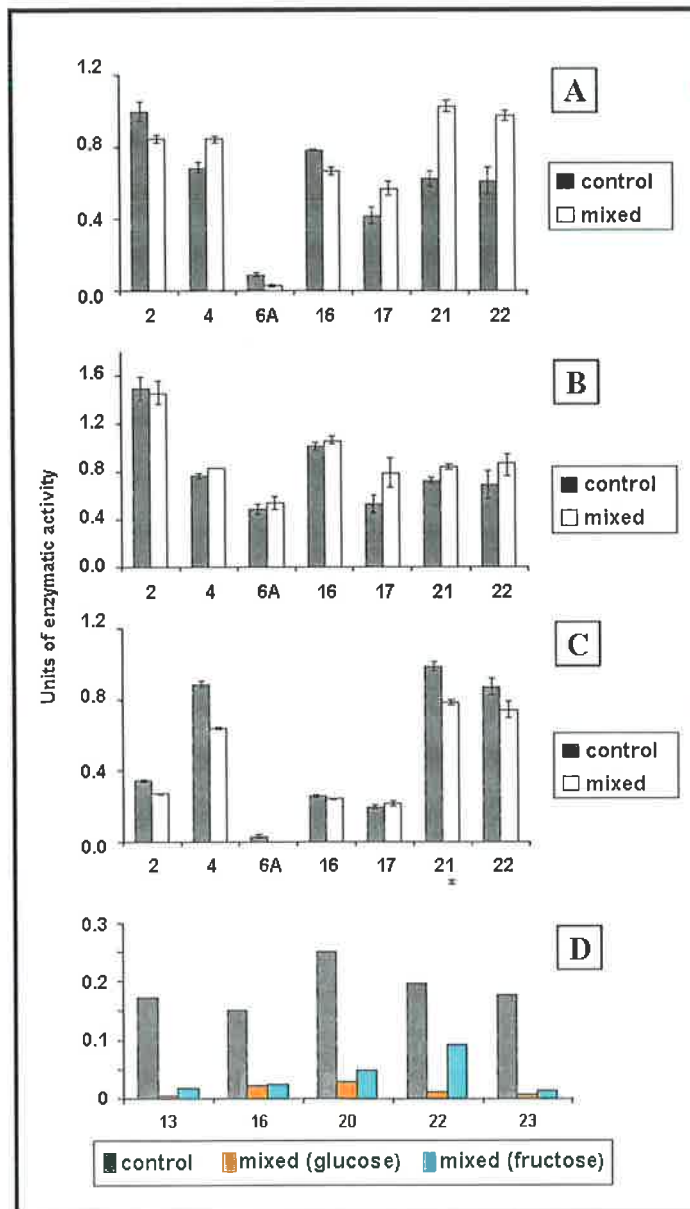
Figure 3.9. Influence of ethanol and glucose (A-C) or ethanol and fructose (D) on the glycosidic activities of selected *O. oeni* strains against (A) *p*-nitro-phenyl β-D-glucopyranoside, (B) *p*-nitro-phenyl α-D-glucopyranoside, (C) *p*-nitro-phenyl β-D-xylopyranoside and (D) *p*-nitro-phenyl α-L-arabinofuranoside. Values are the mean of triplicate determinations.

3.4 Discussion

Previous studies have either indirectly or specifically sought to demonstrate the presence of glycosidic, typically β-glucosidase (β-glucopyranosidase), activities amongst *O. oeni* associated with wine (Grimaldi et al., 2000; Mansfield et al., 2002; McMahon et al., 1999). To the best of our knowledge, the present work represents the most comprehensive investigation of this type in terms of numbers of strains and particularly, substrates and impacting parameters. This work has succeeded in demonstrating that the possession of glycosidic activities is both widespread amongst strains of *O. oeni* and is not limited to only β-glucosidase. We have also examined the influence of potentially inhibitory parameters such as pH, temperature and selected wine components (ethanol, glucose and fructose), both singularly and in combination on the activity of five glycosidases.

At the commencement of this study, it was decided to modify the methodology used in our preliminary study (Grimaldi et al., 2000) and made several improvements in order to accommodate the range of compositional parameters used and the variability in growth seen for the expanded strain collection under investigation. Specifically, a 0.5 M Na_2CO_3 solution was used prior to spectrophotometric quantification of *p*-nitrophenol because of its greater ability to alkalise assay samples of low pH. Also, the addition of bacterial biomass was standardised to a final OD_{600} of 0.5 in the assay. With these modifications it has been possible to more precisely assess the influence of pH on glycosidic activities. In keeping with their anticipated appropriateness for use in the liberation of glycosides under wine conditions, most of the *O. oeni* strains studied had relatively high glycosidase activity at pH values between 3.0 and 4.0 (i.e. wine pH). The fact that this pH optimum does not correspond with the optimal pH for the growth of these organisms (~ pH 4.2 – 4.8; (Van Vuuren and Dicks, 1993)) argues against the influence of pH merely being a consequence of differential growth of the biomass during the assay period.

For the three glycosidases, β-D-glucopyranosidase, α-D-glucopyranosidase and β-D-xylopyranosidase, marked hydrolytic activity was retained above wine pH values, up to neutral, suggesting the possible use of these enzymes at such pH values. In contrast, hydrolysis of *p*NP-αRha and *p*NP-αAra quickly diminished with pH values

54

above 4.0. This latter finding also explains our previous inability to detect significant α-L-rhamnopyranosidase activity in 13 *O. oeni* isolates, where measurements were conducted at pH 5.0 (Grimaldi et al., 2000). Also of note is the existence of two peaks of β-D-glucopyranosidase activity at quite distinct pH values (Fig. 3.1). This result might be indicative of the involvement of multiple enzymes, each with their own pH optimum. Alternatively, it is possible this study has actually revealed another example of that uncommon group of enzymes that have dual pH optima (Gee et al., 1988; Levin and Bodansky, 1966; Nagashima et al., 1999). Opposing the latter notion are reports of multiple β-D-glucopyranosidases in *Lactobacillus plantarum* ((Marasco et al., 1998; Marasco et al., 2000) and other lactic acid bacteria. A preliminary search of the *Oenococcus* genome sequence (Coutinho and Henrissat, 1999) also reveals the presence of multiple putative open reading frames with high homology to a consensus sequence derived from more fully characterised β-D-glucopyranosidases from other organisms (data not shown). Detailed characterisation of purified forms of the enzyme(s) in question will resolve this issue.

The finding that few *O. oeni* strains possessed consistently high activities for all substrates used (Table 3.2), reiterates the importance of strain selection when considering the application of *O. oeni* in the modification of the glycoside profile of wine. Such variability might also be considered indicative of the involvement of multiple enzymes with limited substrate specificity, rather than a single enzyme able to act on all the substrates tested. The presence of minimal α-L-rhamnopyranosidase and α-L-arabinofuranosidase activities in particular agrees with recent findings that *O. oeni* has low specificity for this important group of disaccharide aroma-related compounds in wine (D'Incecco et al., 2004). The industrial significance of these activities, which is nonetheless detectable in these strains, does still warrant further investigation.

In investigating the influence of specific physiochemical parameters, some light has been shed on the scope of the applicability of the enzymes examined in this study. Retention of some glycosidic activity at temperatures below 20°C (Fig. 3.2) is of importance for the wine industry, given that wines are usually stored in this range during the MLF. The greatest activity was observed above 35°C. Whilst this is outside the typical operating range for winemaking, it becomes more important for alternate

processing of small batches of juice or wine or else other industrial applications (Girard et al., 1997; Mourgues and Bénard, 1982).

The enhancing effect of ethanol, occurring most often at lower concentrations (e.g. 4% v/v; Figure 3.3), mirrors previous findings for *O. oeni* (Grimaldi et al., 2000) and yeast biomass (Blondin et al., 1983; Gondé et al., 1985), though expression of a β-glucosidase gene from *Lactobacillus plantarum* has been shown to be repressed by 12% (v/v) ethanol (Spano et al., 2005). Given the duration of the assay used in this study it is more likely that observed influences of ethanol related to existing enzyme rather than *de novo* synthesis. Here, ethanol partially inhibited glycosidase activities, with complete inhibition being most often seen for α-arabinofuranosidase activity. The retention of glycosidase activity in the presence of up to 10% ethanol supports the potential use of such enzymes in the processing of alcoholic beverages, such as wine and beer, with ethanol concentrations within this range. A characterisation of purified enzymes is necessary to determine the precise manner in which ethanol influences their activity.

Non-distilled alcoholic beverages often contain residual sugars, which in the case of wine are mainly glucose and fructose (Boulton et al., 1996a). In keeping with numerous reports for glycosidase enzymes from various sources (Aryan et al., 1987; Cordonnier et al., 1989), the presence of such sugars was found to reduce activity (Fig. 3.3). The key exception being a marked enhancement of some activities by fructose even when present at concentrations as high as 0.75 % v/v, the specified maximum residual sugar content for dry table wines (Iland and Gago, 2002). As a general trend, although the threshold concentration for appearance of fructose inhibition was as at least as low as 0.01%, a 75-fold increase in fructose concentration did not produce a proportional increase in inhibition. This represents an important distinction between *O. oeni* and other sources of glycosidases wherein the degree of inhibition is much more closely linked to the concentration of sugar present (Aryan et al., 1987; Cordonnier et al., 1989).

In summary, in the single parameter experiments, ethanol, sugars (glucose and fructose) and pH, are able to impact upon the glycosidic ability of *O. oeni* cells in a manner that ranges between highly inhibitory to highly stimulatory. By additionally examining the influence of these parameters when applied in a multifactorial fashion, it

has been possible to gain an insight into the potential of these activities under conditions more analogous to an industrial setting. Most notably, combination of these parameters generally resulted in a more moderate influence on detectable glycosidase activity, especially that against pNP-βGlu, pNP-αGlu and pNP-βXyl (Fig. 3.4). The ability to hydrolyse pNP-αAra was again found to be weak and, in this multifactorial experiment, one sensitive to sugars. Thus, whereas fructose on its own was at times seen to be stimulatory, when supplied with ethanol at pH 3.5 a marked inhibition was apparent. This observation is a likely explanation for the inability of D'Incecco and coworkers (D'Incecco et al., 2004) to detect any glycosidase activity in wine (containing ethanol) other than β-glucosidase. There are no obvious links between the findings for the multifactorial experiments compared to those in which single parameters were studied. These observations serve only to highlight the complexity of the interactions involved and reiterates the importance of further work with purified glycoside enzymes. Nevertheless, several strains stand out as possessing high levels of activity against the various substrates studied. Consideration of such findings along with the relative sensitivity of each activity to the wine-related inhibitors studied allows for the selection of strains for further evaluation: either during MLF of wine or else upon incubation with purified grape glycosides.

4. BIOCHEMICAL CHARACTERISATION OF *LACTOBACILLUS* AND *PEDIOCOCCUS* GLYCOSIDASES[2]

4.1. Introduction

Three genera of lactic acid bacteria (LAB) are commonly associated with the winemaking process: *Oenococcus*, *Pediococcus* and *Lactobacillus* (Fugelsang, 1997; Wibowo et al., 1985). Of these, often only *Oenococcus* is able to survive in the later stages of the process when ethanol concentrations are high (Liu, 2002; Lonvaud-Funel, 1999; Wibowo et al., 1985), up to 15% w/v (Iland and Gago, 2002). This makes *Oenococcus oeni* the main bacterium of the malolactic fermentation (MLF) in wine. While some species of *Lactobacillus* are thought to strongly contribute to MLF (Sieiro et al., 1990), the main influence on wine by *Pediococcus* and *Lactobacillus* is likely to originate from their presence outside of alcoholic fermentation. That is, in the earlier stages of a normal or delayed fermentation (prior to accumulation of high concentrations of ethanol) or in a spoilage context in wines with low sulfur dioxide and/or higher pH values (> 3.5) (Costello et al., 1983; Fugelsang, 1997). Given their natural association with the winemaking process and the fact that *O. oeni* growth performance in wines is at times poor, *Pediococcus* and particularly *Lactobacillus* are attracting greater interest as MLF starters (Pilatte and Prahl, 1997).

Whereas *Lactobacillus* and *Pediococcus* have been extensively studied in the dairy industry where they play a pivotal role in determining the flavour and textural properties of several dairy products (e.g. see reviews by (Matthews et al., 2004; Torriani et al., 1994), comparable data obtained under oenological conditions or for strains from wine are distinctly lacking. One mechanism by which these organisms may influence wine sensory properties is as a consequence of the production of glycosidase activities, which can act on compounds with potential sensory significance.

Numerous studies have investigated the glycosidase activity of *O. oeni* strains demonstrating a range of capabilities (Boido et al., 2002; D'Incecco et al., 2004;

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[2] This chapter is largely based on: *Grimaldi, A., Bartowsky, E., and Jiranek, V. (2005) Screening of Lactobacillus spp. and Pediococcus spp. for glycosidase activities that are important in oenology. J. Appl. Microbiol. 99:1061-9*, and has essentially only been reformatted to conform with the rest of this thesis.

58

Grimaldi et al., 2005; Grimaldi et al., 2000; Mansfield et al., 2002; McMahon et al., 1999; Ugliano et al., 2003), however, there has been minimal characterisation of glycosidases of *Lactobacillus* and *Pediococcus*. These findings have implications for the selection and, ultimately, the application of these organisms in winemaking to effect predictable alterations in the aroma properties of wine.

4.2. Materials and methods

4.2.1. Bacterial strains and cultivation

The strains of *Lactobacillus* and *Pediococcus* used in this study are listed in Table 4.1. Most were single colony isolates from commercial preparations of freeze-dried starter cultures (containing *Oenococcus oeni* strains) used in initiation of the MLF in winemaking. *Pediococcus* strain 18X and *Lactobacillus* strain 19Z were isolated from commercial olive products and identified to the genus level (as were all other unidentified isolates) using physiological test strips (API 50 CHL, bioMérieux).

Cultivation conditions were identical to *Oenococcus oeni* strains (see section 3.2.1).

4.2.2 .Determination of glycosidase activity

Enzymatic activity was measured as described previously for *Oenococcus oeni* (see sections 3.2.2 and 3.2.3).

Table 4.1. Bacterial strains used in this study.

Strain	Genus/species	Origin
Lac6A	*Lactobacillus*	Lalvin MT01 Standard
Lac12	*Lactobacillus*	Lalvin 3X 1 Step
Lac13	*Lactobacillus*	Lalvin IB Standard
Lac15	*Lactobacillus*	Lavin 31 MBR
Lac16	*Lactobacillus*	Lalvin 3X Standard
Lac24	*Lactobacillus*	Experimental starter culture[a]
Lac25	*Lactobacillus*	Experimental starter culture[a]
Lac19	*Lactobacillus*	Commercial olive product
Lac26	*Lactobacillus plantarum*	Experimental starter culture[a]
Ped3	*Pediococcus*	Inobacter
Ped10	*Pediococcus*	Lalvin EQ54 MBR
Ped11	*Pediococcus*	Lavin MCW
Ped15	*Pediococcus*	Lalvin 31 MBR
Ped16	*Pediococcus*	Lalvin 3X Standard
Ped17	*Pediococcus*	Lalvin OSU MBR
Ped24	*Pediococcus*	Experimental starter culture[a]
Ped27	*Pediococcus*	Experimental starter culture[a]
Ped18	*Pediococcus*	Commercial olive product

[a]Strains derived from pre-commercial trails samples kindly provided by Lallemand, Montpellier, France.

4.3. Results

Lactobacillus and *Pediococcus* strains were tested with all the *p*-nitrophenyl forms of the key glycosides of importance in winemaking. Unlike earlier work which showed detectable and variable activity by *Oenococcus oeni* against five substrates (see Chapter 3), preliminary work showed *Lactobacillus* spp. and *Pediococcus* spp. only acted against β- and α-D-glucopyranosides (see Appendix 3 to 8 for these activities). Accordingly, only these substrates were included in the more detailed investigation described herein. The following sections present the results concerning β- and α-D-glucopyranosidase activities and the manner in which they are influenced by various wine parameters.

4.3.1 Effect of pH

The effect of pH on the hydrolysis of β-D-glucopyranoside is shown in Figure 4.1. For both *Lactobacillus* spp. (Fig. 4.1A and 4.1B) and *Pediococcus* spp. (Fig. 4.1C and 4.1D) maximum activities ranged between 0.1 and 0.4 units, with only strain Ped18 showing a higher activity (1.2 units). Several strains (Ped17, Ped3, Ped11, Ped24 and Lac25) possessed either low activity or else appeared unable to hydrolyse *p*NP-βGlu under the conditions used. *Pediococcus* strains with marked activity showed a conventional bell curve response to pH whereas, for most *Lactobacillus* strains, activities were less influenced by pH. Importantly, at wine pH values (~ pH 3.0 – 4.0) *Lactobacillus* spp. retained most of their activity, whereas *Pediococcus* spp. most typically did not affect hydrolysis until above pH 3.4 - 3.8. Again, the activity of strain Ped18 stood out, in this case being able to act below this pH range. All *Lactobacillus* strains acted against *p*NP-αGlu as substrate (Fig. 4.2A and 4.2B), but strain Ped18 was the only *Pediococcus* spp. able to do so (Fig. 2C). The range of maximum activities was similar to that seen for *p*NP-βGlu with strong activity being retained at wine pH values.
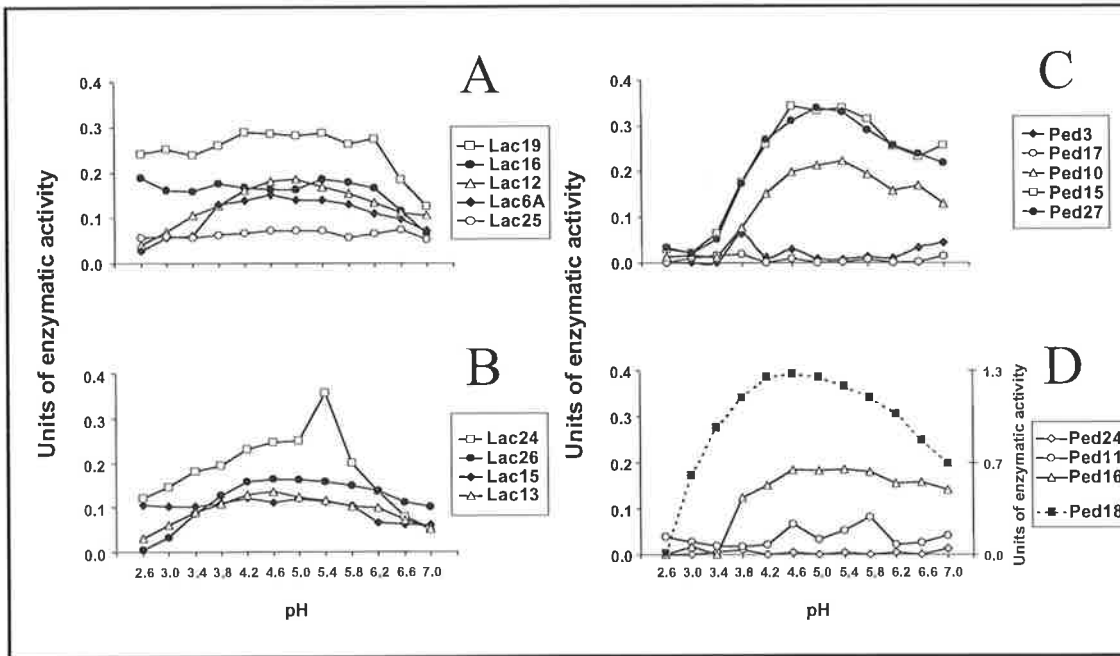
Figure 4.1. Influence of pH on the enzymatic activities of *Lactobacillus* spp. (A and B) and *Pediococcus* spp. (C and D) against *p*-nitrophenyl β-D-glucopyranoside. Values are the means of duplicate determinations. NB: Values for Ped18 are plotted against the right hand axis.
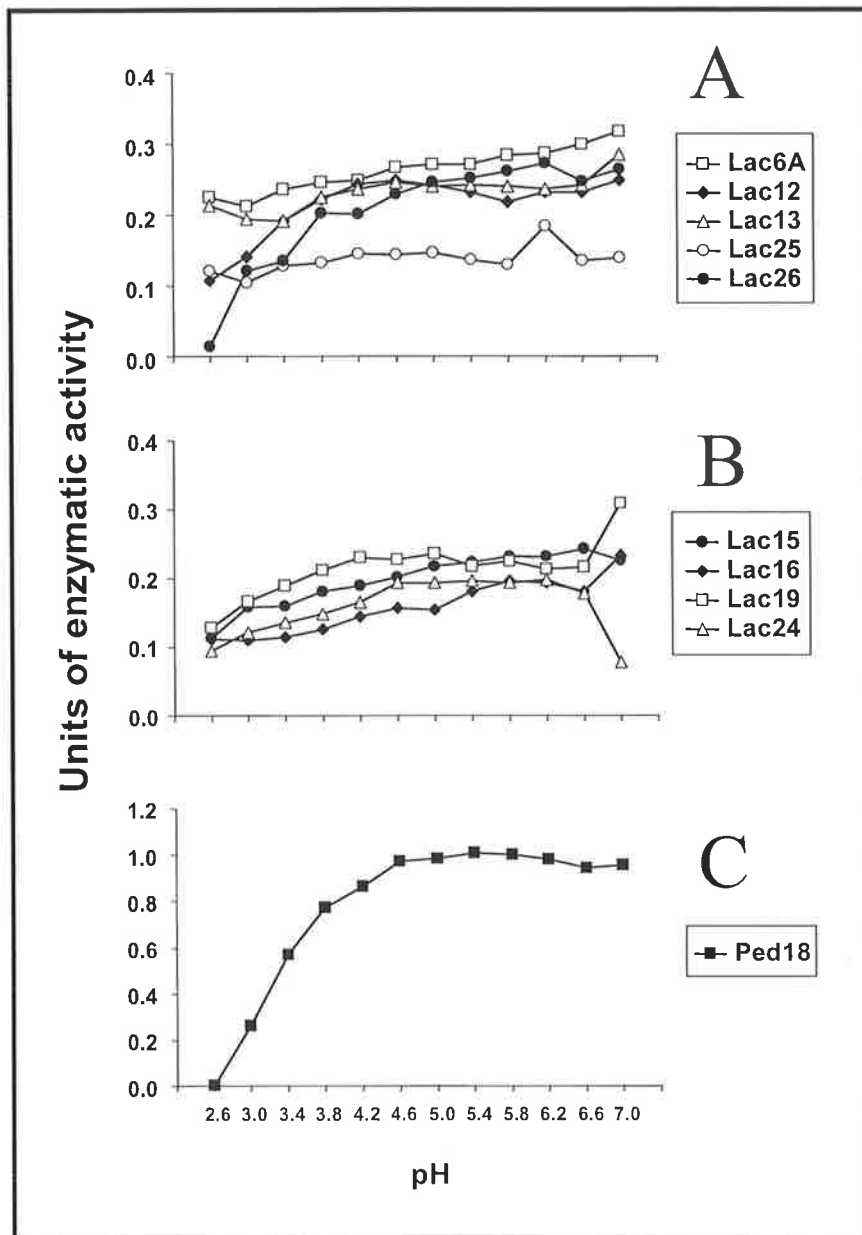
Figure 4.2. Influence of pH on the enzymatic activities of *Lactobacillus* spp. (A and B) and *Pediococcus* spp. (C) against *p*-nitrophenyl α-D-glucopyranoside. Values are the means of duplicate determinations. NB: a different scale is used in panel C.

4.3.2. Effect of ethanol

Ethanol can occur in wine at concentrations up to and above 15% (v/v) and has been repeatedly shown to influence glycosidase activities both positively and negatively in a highly strain- and concentration-dependent manner ((Grimaldi et al., 2000; Williams, 1993); Chapter 3). Spano et al. (Spano et al., 2005) also showed a β-glucosidase gene from *Lactobacillus plantarum* to be repressed by 12% (v/v) ethanol. When ethanol was present in the assay medium used in this study, responses were again highly strain-dependent (Fig. 4.3). Nevertheless, β-D-glucopyranosidase activities were typically enhanced amongst *Lactobacillus* spp. (Fig. 4.3A), whereas most often for *Pediococcus* spp., little change was seen (Fig. 3C). The greatest enhancement of β-D-glucopyranosidase activity occurred at 4 or 8% (v/v) ethanol. Strains Lac15 and Lac16 showed increases of 50% or more at 4% ethanol. For all *Lactobacillus* strains except Lac12, lowest activities were seen at 12% ethanol (v/v). Hydrolysis of *p*NP-βGlu was strongly inhibited for *Pediococcus* strain Ped3 at all ethanol concentrations. When considering α-D-glucopyranosidase in *Lactobacillus* spp., the response tended toward an enhanced activity with increasing amounts of ethanol (Fig. 4.3B), and only strain Lac12 showing marked inhibition at all ethanol concentrations. The response of *Pediococcus* strains was opposite: all but strain Ped18 being strongly inhibited (Fig. 4.3D).
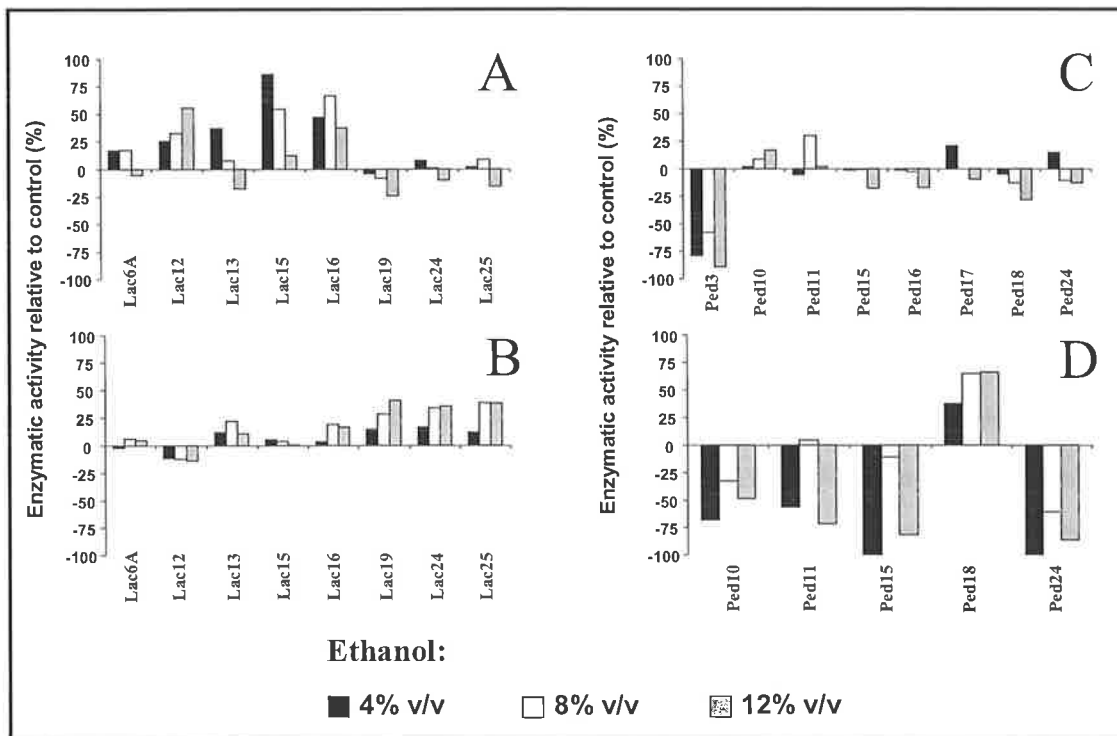
Figure 4.3. Influence of ethanol on the enzymatic activities of *Lactobacillus* spp. against *p*-nitrophenyl β-D-glucopyranoside (A) or *p*-nitrophenyl α-D-glucopyranoside (B) and *Pediococcus* spp. against *p*-nitrophenyl β-D-glucopyranoside (C) or *p*-nitrophenyl α-D-glucopyranoside (D). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the means of duplicate determinations.

4.3.3. Effect of glucose and fructose

The natural grape sugars glucose and fructose have been shown to be inhibitory to glycosidase enzymes even at the residual concentrations found in wines (Grimaldi et al., 2005; Grimaldi et al., 2000; Williams, 1993; Winterhalter and Skouroumounis, 1997). Inclusion of glucose at one of the four concentrations examined in this study (0.1 – 20 g l^{-1}) produced a similar pattern of inhibition for both β-D-glucopyranosidase and α-D-glucopyranosidase activities in *Lactobacillus* spp. (Fig. 4.4A and 4.4B). While glucose inhibition increased with sugar concentration, the response was not linear and strong inhibition was apparent even at 0.01% glucose. In *Pediococcus* strains Ped10 and Ped15, β-D-glucopyranosidase was completely inhibited at all glucose concentrations (Fig. 4.4C). In strain Ped24 inhibition was minimal while in strain Ped17 the response was one of enhancement with 1 g l^{-1} glucose mediating a 125% increase of activity against *p*NP-βGlu compared to the glucose-free control (Fig. 4.4C).

The typical response to fructose (Fig. 4.5) was also an inhibition and in fact was similar to that seen in the presence of glucose. Again *Pediococcus* strain Ped17 defied the trend by possessing a β-D-glucopyranosidase that was enhanced in the presence of fructose.
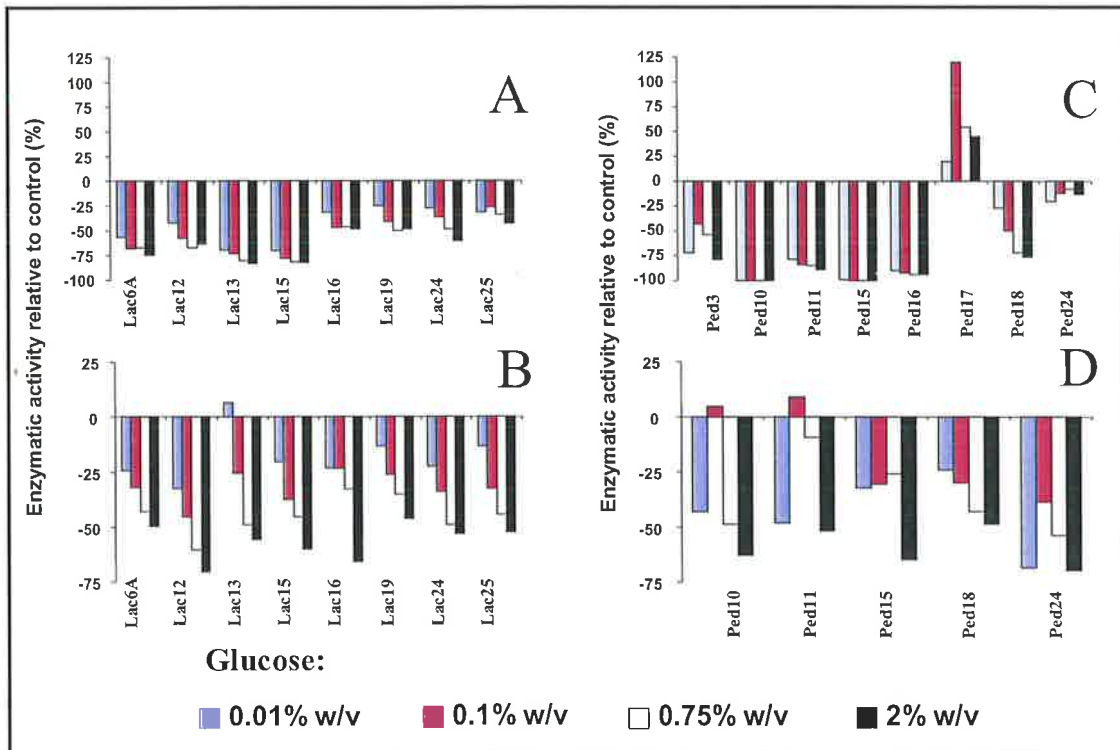
Figure 4.4. Influence of glucose on the enzymatic activities of *Lactobacillus* spp. against *p*-nitrophenyl β-D-glucopyranoside (A) or *p*-nitrophenyl α-D-glucopyranoside (B) and *Pediococcus* spp. against *p*-nitrophenyl β-D-glucopyranoside (C) or *p*-nitrophenyl α-D-glucopyranoside (D). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the means of duplicate determinations.
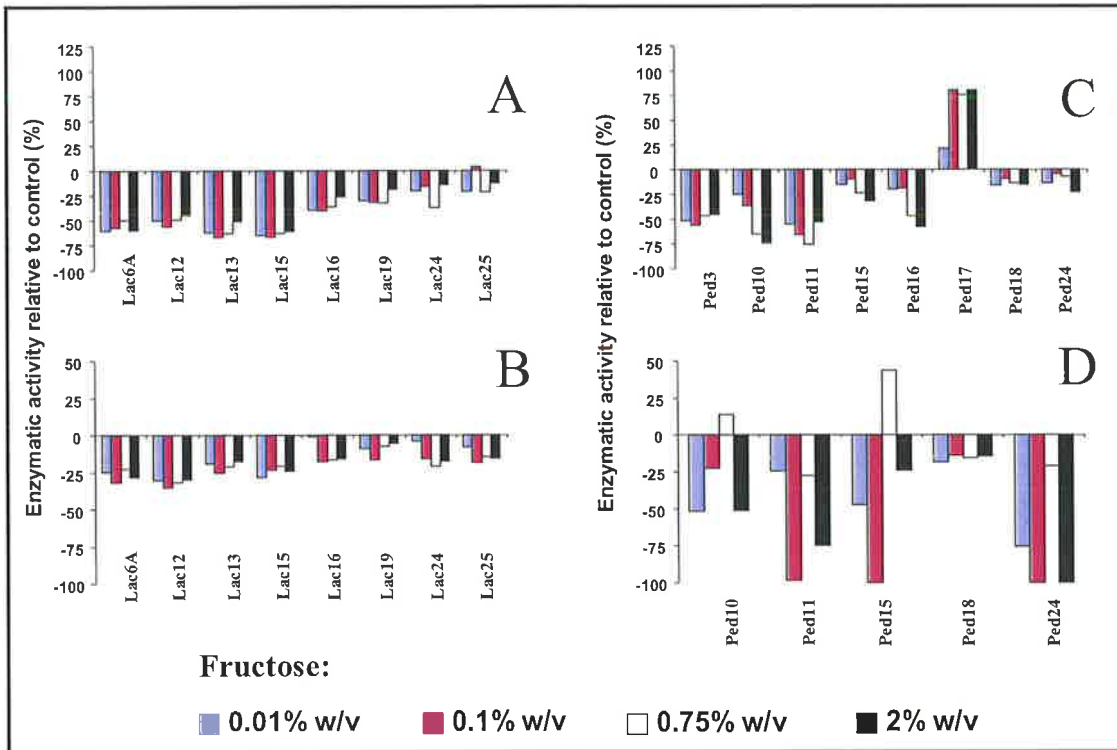
Figure 4.5. Influence of fructose on the enzymatic activities of *Lactobacillus* spp. against *p*-nitrophenyl β-D-glucopyranoside (A) or *p*-nitrophenyl α-D-glucopyranoside (B) and *Pediococcus* spp. against *p*-nitrophenyl β-D-glucopyranoside (C) or *p*-nitrophenyl α-D-glucopyranoside (D). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the means of duplicate determinations.

4.3.4. Effect of temperature

Given that much of the winemaking process occurs at temperatures between approximately 10 and 30°C, the impact of incubation temperature on the glycosidic activities of a selection of the *Lactobacillus* and *Pediococcus* strains under investigation was examined. In all cases temperature optima occurred at or above 35°C for both enzymatic activities (Fig. 4.6). For *Lactobacillus* strains Lac16 and Lac19, maximal hydrolysis of pNP-αGlu occurred around 45°C. In all cases, as temperatures approached 50°C both activities were rapidly reduced to zero at 60°C. At temperatures used in winemaking, significant activities were retained.

4.3.5. Effect of combined parameters

Evaluation of strains for hydrolysis of pNP-βGlu and pNP-αGlu in the presence of a combination of the parameters tested above revealed similar response patterns for many strains. Therefore, a selection of the most representative strains was made and these isolates were chosen for this test (Fig. 4.7). Both activities displayed by *Lactobacillus* strain Lac12 and Lac19 showed a small increase when assayed in the presence of 10% ethanol and 2 g l^{-1} glucose (Fig. 4.7A and 4.7B). Meanwhile, β-D-glucopyranosidase activity of *Pediococcus* strain Ped18 was about 30% of that observed for the control (Fig. 4.7A) whereas α-D-glucopyranosidase activity was increased by approximately 100% relative to the control (Fig. 7B). Strain Ped15 displayed minimal activity.
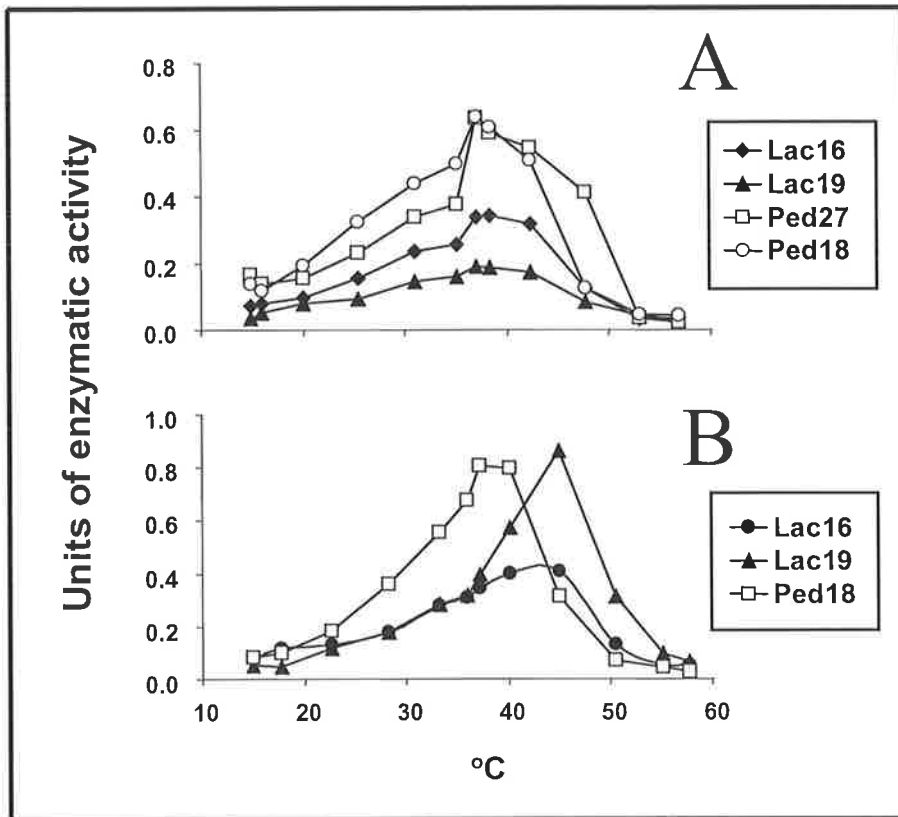
Figure 4.6. Influence of temperature on enzymatic activities of *Lactobacillus spp.* and *Pediococcus spp.* against *p*-nitrophenyl β-D-glucopyranoside (A) and *p*-nitro-phenyl α-D-glucopyranoside (B). Values are the mean of duplicate determinations.
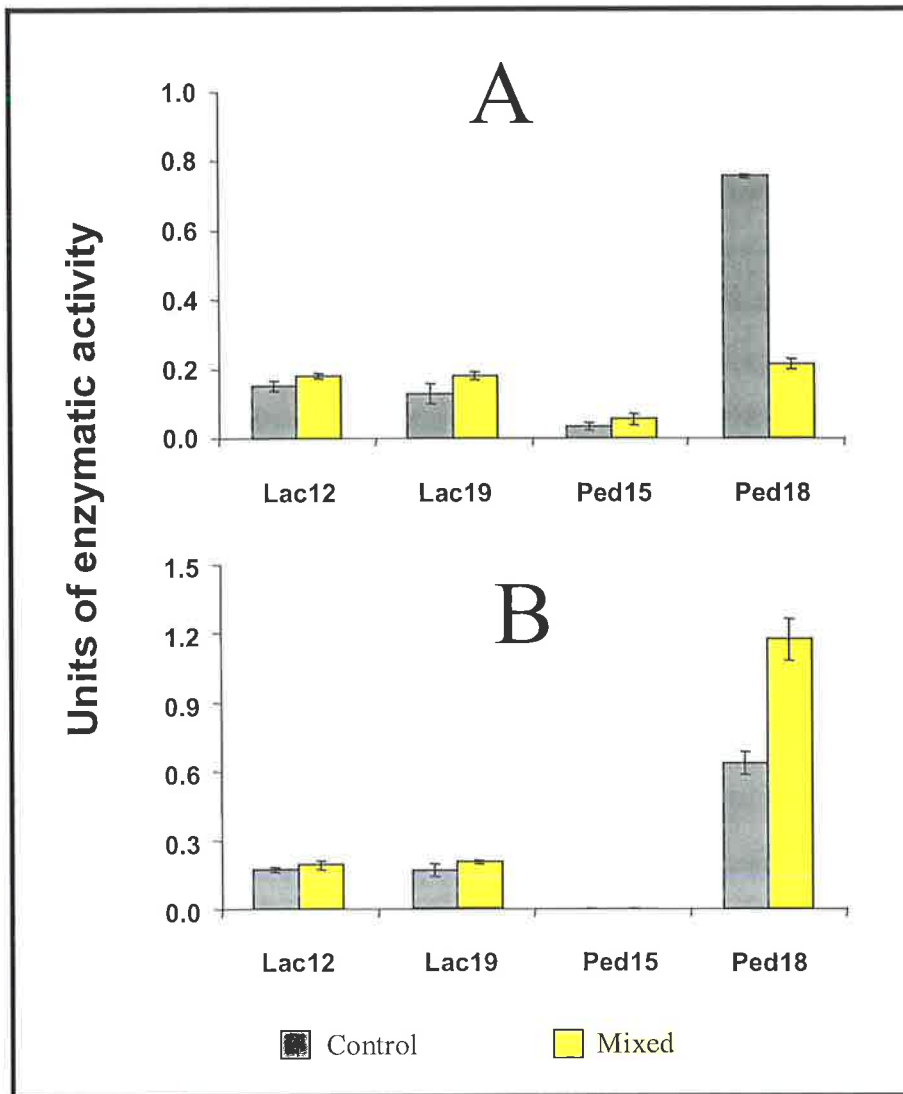
Figure 4.7. Influence of ethanol and glucose on *Lactobacillus spp.* and *Pediococcus spp.* against *p*-nitrophenyl β-D-glucopyranoside (A) and *p*-nitrophenyl α-D-glucopyranoside (B). Assays were conducted at pH 3.5, in the presence or absence of ethanol (10%, v/v) and glucose (2 g l^{-1}). Values are the mean of triplicate determinations ± standard deviation.

4.4. Discussion

Many studies have been carried out to investigate the ability of *Lactobacillus* and *Pediococcus* species to produce glycosidase enzymes (Antuna and Martinez-Anaya, 1993; Bianchi-Salvadori et al., 1995; De Vos and Gasson, 1989; Marasco et al., 1998; Marasco et al., 2000; Tzanetakis and Litopoulou-Tzanetaki, 1989). The majority of such works, however, have concentrated on dairy isolates. Little attention, if any, has been dedicated to the characterization of the glycosidases of wine *Lactobacillus* and *Pediococcus* species. Nevertheless, given that these organisms form part of the natural microflora of the winemaking process (Boulton et al., 1996a; Costello et al., 1983; Fugelsang, 1997) and have become commercially available as alternate malolactic fermentation starters (Pilatte and Prahl, 1997), a more detailed characterization is justified.

This work has shown that the *Lactobacillus* and *Pediococcus* strains studied here produce little beyond α- and β-D-glucopyranosidase activities. The former activity may be of importance in the liberation of nutrients from yeast-derived macromolecules which can enhance the growth of lactic acid bacteria (Guilloux-Benatier et al., 1993). While β-D-glucopyranosidases can play a role in liberation of sensorially detectable grape aglycones from their glycosylated forms, this process may require the removal of multiple glycoside species, potentially in a sequential manner (Günata et al., 1988). Accordingly, the limited number of additional glycosidase activities from these organisms reduces the potential impact of *Lactobacillus* and *Pediococcus* strains on the typically mixed population of flavour precursors found in grapes. Furthermore these activities were approximately one order of magnitude less than those seen for *O. oeni* in previous work ((Grimaldi et al., 2000); Chapter 3). Even so, the activities studied here might still have marked impact on the flavour profile of wine. To begin to determine their relative importance in a wine-like environment, several key parameters were examined individually and in combination.

Mirroring recent findings for *O. oeni* ((Barbagallo et al., 2004; Grimaldi et al., 2000; Ugliano et al., 2003); Chapter 3), both β- and α-D-glucopyranosidase activities were influenced by pH, though to varying degrees (Fig. 4.1). The relative stability of *Lactobacillus* β- and α-D-glucopyranosidase activities to pH changes and low pH

values suggests that these isolates rather than the wine-related *Pediococcus* would be of greater importance in wines with typical pH values between 3.0 and 4.0. The sole exception, *Pediococcus* strain Ped18, which was not of wine origin, might enjoy oenological applications given its high overall activity which afforded it residual activities at wine pH that were comparable to the *Lactobacillus* strains.

The variability of the response of the studied glycosidase activities to ethanol, glucose and fructose is not without precedent in other organisms, or *O. oeni* ((Grimaldi et al., 2000; Williams, 1993; Winterhalter and Skouroumounis, 1997); Chapter 3). While still showing strong strain dependence, our findings reveal ethanol to induce a trend toward enhancement of hydrolysis of both substrates amongst *Lactobacillus* strains compared with a reduction or unaltered hydrolysis by *Pediococcus* strains (Fig. 4.3). While this data offers no further clues as to the basis for these responses, the availability of considerable strain variation introduces the possibility of selecting and applying isolates according the ethanol concentration of the fermenting juice or wine. Contrasting the variability of the response to ethanol, glucose (a possible end-product of glycosidase activity) was seen to be inhibitory to hydrolysis of both pNP-βGlu and pNP-αGlu with few exceptions (Figures 4.4 and 4.5). Again the existence of an isolate (Ped17) whose β-D-glucopyranosidase activity is strongly enhanced by both glucose and fructose, offers a potentially valuable source from which a superior enzyme might be purified. Such a preparation or strain is an excellent candidate for overcoming the marked sensitivity seen amongst existing enzyme preparations to these sugars (reviewed by (Winterhalter and Skouroumounis, 1997)).

The final parameter considered in this study was included out of recognition of the fact that it varies widely during winemaking. Optimal temperatures for pNP-βGlu and pNP-αGlu hydrolysis (35 - 45°C) were higher than the typically working range seen during fruit processing, fermentation and maturation (Boulton et al., 1996a). Compared to *O. oeni* (Chapter 3), *Lactobacillus* and *Pediococcus* strains showed a reduced sensitivity to temperatures outside the optima. Again this fact suggests that these genera may be a better source of enzymes for application to low temperature juices or wines.

An analysis of the impact of the simultaneous exposure of bacterial biomass to multiple parameters proved informative. Both *Lactobacillus* strains Lac12 and Lac19

tended toward enhanced activities, suggesting that the response to ethanol was the dominant effect. The overriding influence on activity by *Pediococcus* strain Ped15 was the low pH of the medium (i.e. 3.5) resulting in minimal activity upon which the influences of ethanol and glucose were minor. The nature of the response to ethanol and glucose was more clearly seen against the background of the higher activity of strain Ped18. In this case, β-D-glucopyranosidase activity was reduced, replicating the pattern seen in the presence of ethanol alone (Fig. 4.3C). Similarly, α-D-glucopyranosidase activity was increased, again as seen previously (Fig. 4.3D) when this strains was exposed to ethanol only.

In summary, this study completes the survey performed on wine LAB strains on glycosidase enzymes, initiated with *Oenococcus oeni* (Chapter 3). Having demonstrated that all tested strains have at least one clear detectable glycosidic activity, some points can be made regarding differences between the genera, *Lactobacillus*, *Pediococcus* and *Oenococcus*. While the latter have a very wide range of activities, the former effect hydrolysis of only a limited number of substrates, specifically the β- and α-D-glucopyranosides. The ability of *Lactobacillus* and *Pediococcus* to hydrolyse the *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-α-D-glucopyranoside substrates was also lower than *Oenococcus*. Importantly, the presence of sugars in the assay medium was highly inhibitory to both enzymatic activities. At face value this might imply that use of such organisms for flavour liberation is limited to the latter stages of fermentation when juice sugar contents are low. Interestingly, sugar concentrations as low as 0.1 g l^{-1} were inhibitory to glycosidic activities, and concentrations up to 20 g l^{-1} were only marginally more inhibitory. In addition, enhancement of hydrolysis by ethanol appeared to override inhibition by sugar. Thus the window of sugar concentrations within which such strains might be applied during the fermentation may well be wider than that examined here. It is also worth reiterating that glycosidic activities by one strain in this survey, Ped17, were in fact enhanced by sugars.

The findings reported here form the basis for the selection of isolates for the purification and further characterization of potentially useful glycosidase enzymes. In addition, this study is being extended to consider the ability of selected isolates to act on natural grape glycosides under wine-like conditions and during winemaking, with the ultimate aim of tailoring wine composition.

5. INFLUENCE OF LACTIC ACID BACTERIA ON WINE PIGMENTS.

5.1. Introduction

Colour is of critical importance for red wine quality. As a result research aimed at understanding how colour is formed and develops has been extensive and continues to add to the knowledge in this area. It is well established that wine colour is the result of a very large interaction among several compounds of the phenol family, together with their polymerised forms (Boulton, 2001; Brouillard et al., 2003; Ribéreau-Gayon et al., 2004b). But wine colour is rarely inert. It is clear from comparison of young and old wines that there are changes that occur during aging. However, changes in colour intensity and/or hue do not only occur after the wine is bottled. The winemaking process features many influential parameters.

The major source of colour in red wines are the flavonoid molecules, the anthocyanins, which are derived largely from the skin of grapes (Ribéreau-Gayon et al., 2004b). In grape juice and at the early stages of alcoholic fermentation most anthocyanins are in the glycosidic form (Ribéreau-Gayon et al., 2004b). These compounds are chemically stable, water soluble and dark red in colour (Boulton et al., 1996b). When hydrolysed, the resulting anthocyanidins become unstable and, above all, are easily converted to brown or colourless compounds (Fig. 5.1) (Blom, 1983; Eskin, 1979). Their high reactivity accelerates the polymerisation process, resulting in more stable and larger molecules with time (Wightman et al., 1997). However, the full implications for wine colour of this process are still unknown.

Winemaking practices have important implications for colour, both in increasing and decreasing its intensity. The attention of this project was focused on the latter only. Throughout the winemaking process yeasts and bacteria have the opportunity to decrease wine colour.
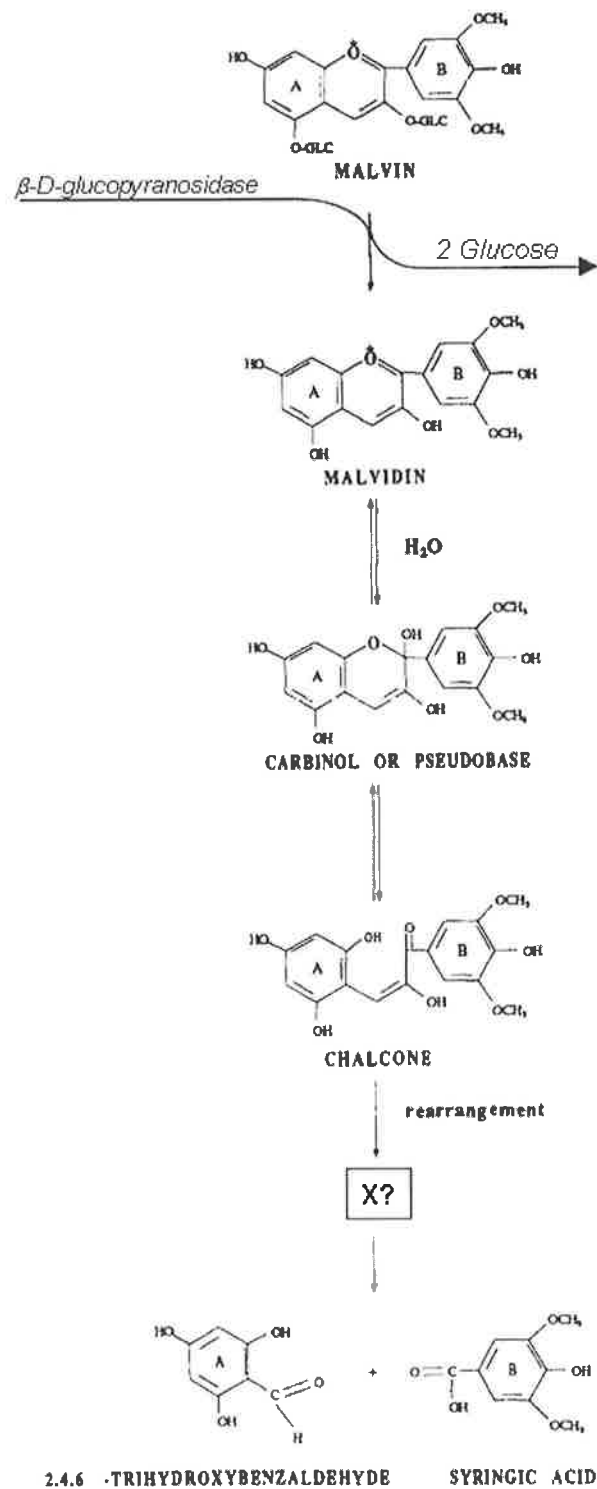
Figure 5.1. Hydrolysis and subsequent degradation of an anthocyanin, malvidin-3,5-diglucoside (adapted from (Piffaut et al., 1994)). X, according to Ducruet (Ducruet, 1998), it might be represented by the complex 2,4,6-trihydroxybenzaldehyde-glucose.

Visual examination of the yeast lees from a red wine fermentation makes it clear that these cells adsorb colour compounds on their cell wall: the quantitative importance of this and the extent to which bacterial cells are able to do the same has not been defined. Anecdotal evidence from winemakers suggests that colour changes occur during the malolactic fermentation (MLF), carried out by lactic acid bacteria. During this fermentation, malic acid is converted to lactic acid, which imparts a less sour taste to wines. Wine acidity is also reduced and pH is increased. An increase in pH of even a few points is enough to induce chemical variations in many phenolic compounds, especially anthocyanins (Ribéreau-Gayon et al., 2004b; Usseglio-Tomasset, 1995). These molecules tend to be in a colourless form at higher pHs (Fig. 5.2). Therefore the decrease in colour which occurs after this fermentation (Davis et al., 1985) may be quite substantial, both from a chemical and sensorial point of view.

However, it is noted that despite the lower colour intensity (OD_{420} + OD_{520} + OD_{620} measurements), colour stability increases after MLF, and it is greater when MLF is carried out in stainless steel rather than in oak barrels (Henick-Kling and Acree, 1998). This fact represents an important step in considering that other modifications may occur to anthocyanins, in addition to the modification of their structure due to the increase of pH. Henick-Kling (1998) points out that the possible explanations may reside in the increased polymerisation and condensation phenomena between tannins and anthocyanins. This is also confirmed by a study performed on German wines, where after MLF these regained colour intensity due to the higher condensation and polymerisation rates of anthocyanins with tannins (Rauhut et al., 1995). This may be due to a possible hydrolysis of anthocyanins, in which corresponding aglycones becomes highly reactive and further polymerised (Ducruet, 1998; Piffaut et al., 1994; Wightman et al., 1997), or completely degraded (Ducruet, 1998). In addition, the observation that in many cases MLF only marginally increases the pH of wines, raises the possibility that LAB glycosidase enzymes might in fact play a more important role in the colour loss process.

OCH₃ ... OH ... OCH₃
O ... O ... O-Glucose ... OH
Quinone form (violet)

pK = 4.25 ... H⁺ ... -H⁺

OCH₃ ... OH ... OCH₃
HO ... Ö ... O-Glucose ... OH
Malvidin-3-Glucoside Flavylium ion (red)

pK = 2.60 ... -H⁺ +H₂0 ... H⁺

OCH₃ ... OH ... OCH₃
HO ... OH ... O ... O-Glucose ... OH
Pseudobase form (colourless)
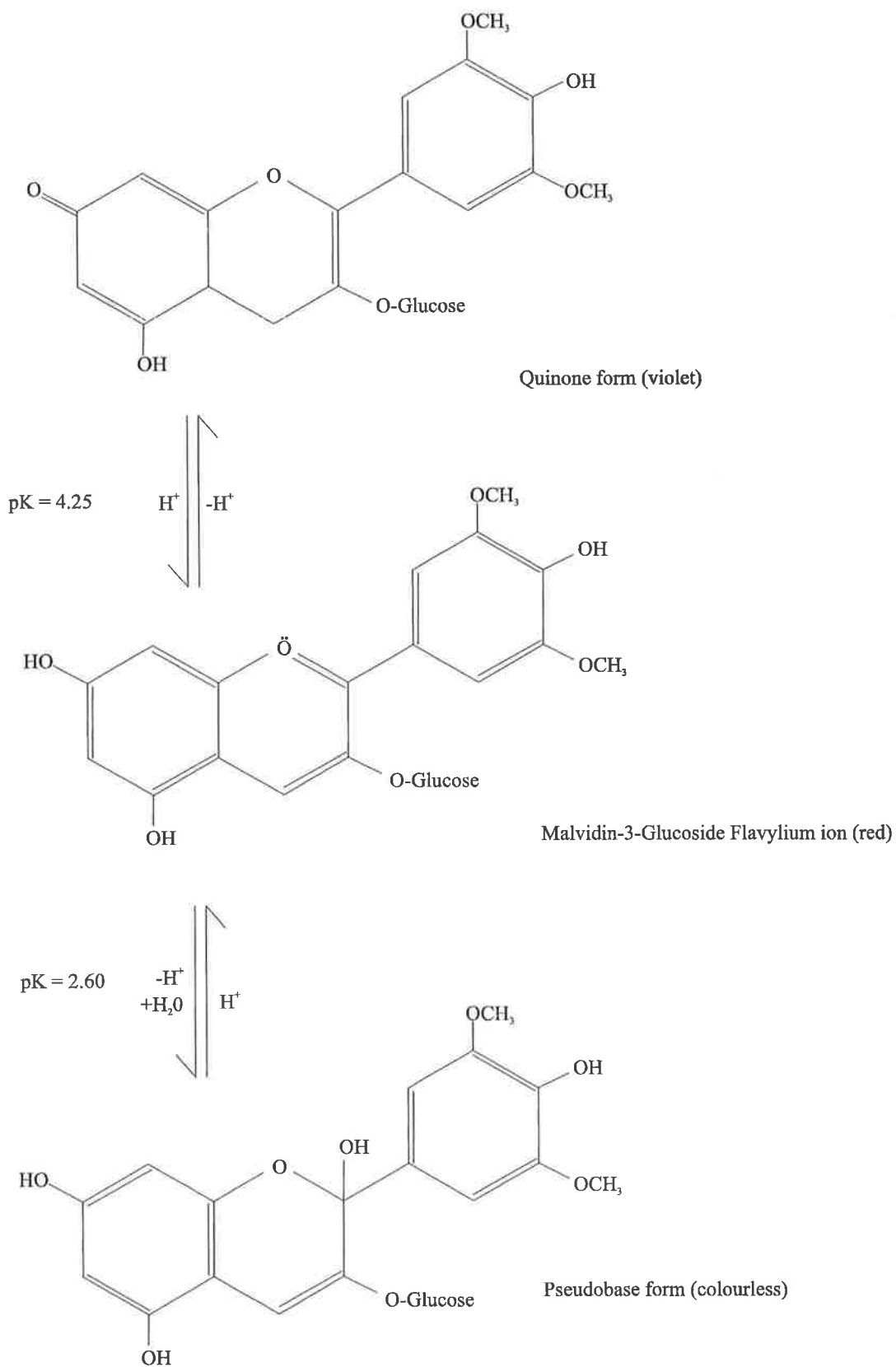
Figure 5.2. pH dependency of malvidin-3-glucoside pigment cation (School of Agriculture, Food and Wine, The University of Adelaide).

Anthocyanin hydrolysing enzymes (anthocyanases) have generally been considered negative in the wine industry because of their action in reducing wine colour intensity, particularly for red table wines. In fact their influence on colour is often a parameter used to determine the quality of commercial pectolytic preparations (eg.: DSM-Enology, Delft, The Netherlands). Despite this obvious drawback, these enzymes may represent an important application in the production of some specific wines:

a) White champagne-style wines produced from red varieties, such as Pinot noir, where red colouration is largely undesirable may benefit from decolourisation.

b) Still, white table wines produced from red varieties would benefit in terms of a reduced reliance on chemical and physical finings to ameliorate their colour.

c) Rose wines may often require some adjustment, specifically a reduction, of their colour intensity.

d) Red table wines produced from Syrah (Shiraz) and Cabernets grown in hot and dry regions, can contain high concentrations of anthocyanins and anthocyanin-tannin complex, and thus may require treatment with anthocyanases to reduce the strong sensorial impact that those molecules can have.

Given the potential application and impact of LAB glycosidases, particularly β-D-glucopyranosidases, on wine colour a more detailed characterisation of these enzymes will assist in their fuller exploitation as tools with a wide spectrum of applications in winemaking. A better understanding of their contribution to wine colour will also provide a better foundation for when or where they might be best applied or alternatively the situations under which they may be detrimental to wine quality.

5.2. Materials and methods

5.2.1. Anthocyanin extraction

All anthocyanin preparations were kindly provided by Dr Ewald Swinny of the School of Agriculture and Wine, The University of Adelaide. The preparation was prepared from unspecified red grape varieties and mainly consisted of malvidin-3-glucoside, representing 95% of total anthocyanins.

5.2.2. Induction of β-glucopyranosidase activity by natural glycosides

5.2.2.1. LAB growth

LAB strains were inoculated into MRS supplemented with 20% apple juice (pH 5.0) and grown at 26°C for 42h (66 h for *O. oeni* strains Oen3 and Oen5). The cells were harvested and twice washed with 0.85% w/v NaCl solution.

5.2.2.2 Induction assay conditions

Cells were resuspended in 10 ml of modified Chemically Defined Wine Medium (modCDWM, Table 5.1, with no carbon sources and adjusted to pH 4.5), to a final OD_{600} of 0.6 in 10 ml of the appropriate modCDWM in 50 ml flasks and incubated for 6 h at 26°C with shaking.

To detect the induction of LAB β-D-glucopyranosidase(s), modCDWM was supplemented with several inducers, as shown in Table 5.2. At the end of the 6 h induction, the cells were twice washed with 0.85% w/v NaCl solution, resuspended in duplicate aliquots of 0.5 ml of 0.2 M citrate/phosphate buffer containing 10 mM *p*-nitrophenyl-β-D-glucopyranoside. Vials were incubated for 1 h at 37°C. The reaction was stopped with 2 volumes of 0.5 M Na_2CO_3. The extent of *p*-nitrophenol liberation was measured spectrophotometrically at 400 nm, against cell-free blanks which had been treated in the same manner as the samples. Results are given as units of enzymatic activity where one unit equates to mmol of liberated *p*-nitrophenol per minute per mg of dry cells and is the average of duplicates.

Table 5.1. Modified Chemically Defined Wine Medium (Patynowski et al., 2002)*

Component	Concentration (per litre)	Component	Concentration (per litre)
L-tartaric acid	3 g	Adenine	10 mg
L-malic acid	3 g	Guanine	10 mg
Citric acid	0.5 g	Uracil	10 mg
		Xanthine	5 mg
K_2HPO_4	1 g	Thymine	10 mg
KH_2PO_4	1 g		
$MgSO_4 \bullet 7H_2O$	200 mg	Alanine	75 mg
$MnSO_4 \bullet 4H_2O$	50 mg	Arginine	200 mg
$FeSO_4 \bullet 7H_2O$	50 mg	Aspartic acid	50 mg
		Cystine	50 mg
Myo-Inositol	20 mg	Cysteine	500 mg
Pyridixine•HCl	1.5 mg	Glutamic acid	200 mg
Nicotinic acid	2 mg	Glycine	20 mg
Calcium pantothenate	5 mg	Histidine	20 mg
Thiamin•HCl	1 mg	Isoleucine	30 mg
ρ-aminobenzoic acid	0.05 mg	Leucine	30 mg
Riboflavin	1 mg	Lysine	40 mg
Biotin	0.05 mg	Methionine	25 mg
Folic acid	0.05 mg	Phenylalanine	20 mg
Cobalamine B12	0.07 mg	Proline	100 mg
		Serine	40 mg
Tween 80	1 mL	Threonine	100 mg
		Tryptophan	10 mg
		Tyrosine	20 mg
		Valine	20 mg

* ethanol absent and pH adjusted to 4.5.

Table 5.2. Nature and concentration of additions to modified CDWM.

Designation	Addition	Concentration
modCDWM (control)	-	-
modCDWM-Arb	Arbutin	0.2 w/v (g/L)
modCDWM-Sal	Salicin	0.2 w/v (g/L)
modCDWM-Anth	Anthocyanin solution	100 mg/L

5.2.3. Action of LAB β-D-glucopyranosidase on wine anthocyanins

5.2.3.1. Anthocyanin medium and incubation conditions

The medium used was modCDWM-Anth, with an anthocyanin concentration of 80 mg/L instead of 100 mg/L, and a pH of 4.0. Cells were grown in 20% apple juice MRS, at 26°C for 42 h. They were twice washed with 0.85% NaCl solution and resuspended in 10 ml modCDWM-Anth in 30ml flasks. Cultures were incubated at 26°C and stirred on a shaking platform. Samples were taken at regular intervals and prepared for HPLC analysis: 0.5 ml of methanol was added to 0.5 ml of sample and the mixture filtered through a 0.22 μm cellulose filter to eliminate particulates. An aliquot of this solution (20 μL) was then analysed by HPLC.

5.2.3.2. HPLC analysis of hydrolysed anthocyanins

Long HPLC analysis: Acidic hydrolysis was performed on a solution containing malvidin-3-glucoside to obtain the corresponding anthocyanidin malvidin and glucose. This solution was used to detect the retention times of both derivative compounds. HPLC conditions were as follows: A 20 μL sample was injected directly onto a 250 mm x 4.6 mm I.D. Alltima C18 5 micron end-capped column (Alltech) and eluted with a gradient solvent system comprising 2% formic acid (solvent A) and acetonitrile (solvent B). The linear gradient commenced with 10% solvent B for 4 min, increasing to 20% at 15 min, 50% at 35 min, 80% at 38 min and 100% at 40 min, before re-equilibration of the column. The detector was set at 520 nm and the peaks were identified on the basis of the on-line spectrum recorded for each identifiable peak.

Rapid HPLC analysis:. The reaction solutions were monitored with a shorter HPLC method involving chromatography on a Platinum* "EPS C18 100Å, 1.5μm, 33mm x 7mm Rocket" column and a 2% acetonitrile gradient solvent system flowing at 1.5 ml/min, starting with 10% CH₃CN for 2 minutes and proceeding to 58% (7 minutes) and 100% (10-12 minutes) CH₃CN, followed by re-equilibration of the column. Detection was performed at 520nm.

5.2.3.3. Colour measurement at 520 nm

Samples were taken at the end of the incubation of the various media and analysed spectrophotometrically. In each case 0.25 ml samples were added to 1 ml of 10% HCl in distilled water and centrifuged (20,000 g x 5 min) before measurement in a spectrophotometer at 520 nm against blanks without cells, treated as sample.

5.3. Results

5.3.1. Induction of LAB β-D-glucopyranosidase activity

Effect of Arbutin and Salicin on β-glucosidase Activity: Both arbutin and salicin are natural glycosides present in many organisms of the Plant Kingdom and have been used extensively as glucosidase substrates (Caridi et al., 2005; De Angelis et al., 2005; Gueguen et al., 1997; Mendes Ferreira et al., 2001; Rosi et al., 1994; Sanchez-Torres et al., 1998). The presence in the incubation medium of these compounds had a varied impact on the β-D-glucopyranosidase activity amongst the LAB isolates assessed (Figs. 5.3 – 5.5). For most (12/22) of the *Oenococcus* isolates examined, little change in activity was noted (Fig. 5.3). However, where induction was observed for these strains, it was not necessarily seen for both glycosides but rather was most apparent following exposure to arbutin. For example strain Oen2 displayed a ~70% increase in detected β-glucosidase activity following exposure to arbutin, with little response to salicin. Alternatively activity by strain Oen3 was increased by ~25% by arbutin but reduced by approximately 20% by salicin. Finally, both glycosides reduced the detectable activity in strain Oen7, the only LAB isolate to have such result.

For all *Lactobacillus* and most *Pediococcus* isolates, detected β-glucosidase activity was increased with both glycosides producing a similar response (Figs. 5.4 and 5.5). Strains Lac15 and Lac16 displayed strong induction with activities being up to 16- and 7-fold over that of the control, respectively. Where induction was observed in *Pediococcus* it did not occur to the same extent. Thus for strains Ped16 and Ped18, post-induction levels were of the order of 50% greater than the control. Unlike isolates of *Oenococcus*, no reductions in activity were seen for *Pediococcus*.

84
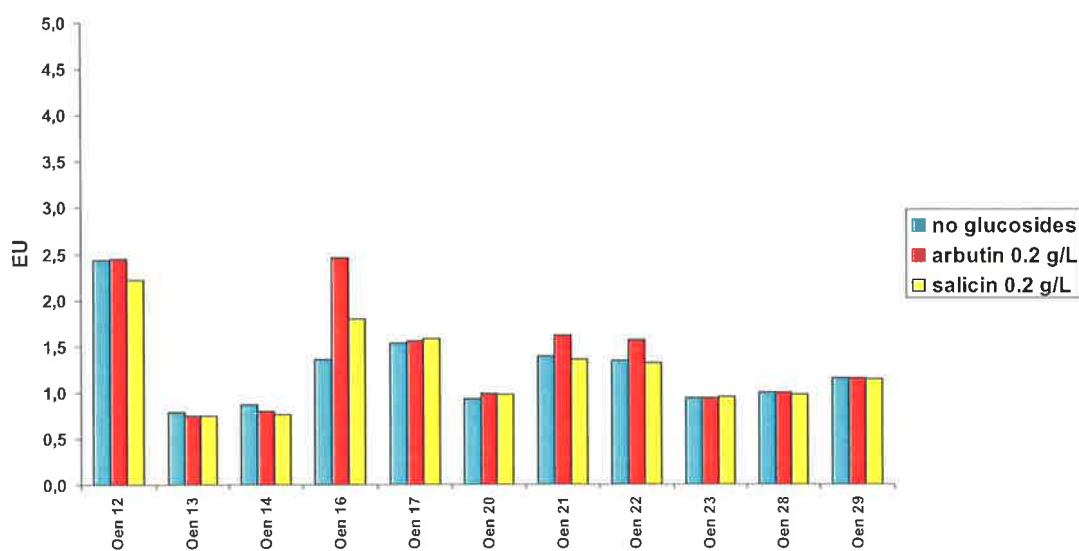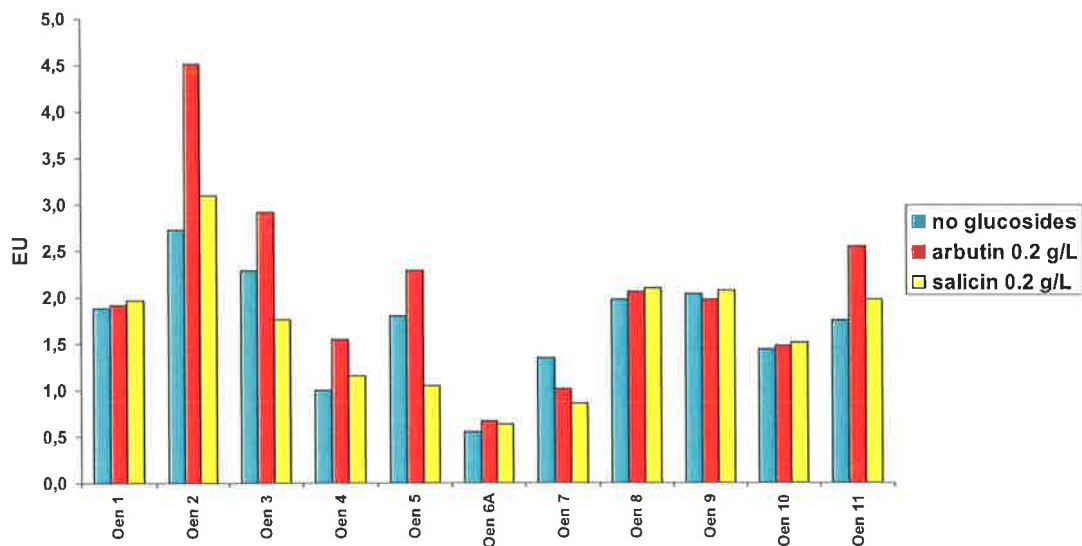
Figure 5.3. Effect of 6 h pre-exposure to glycosides on β-D-glucopyranosidase activity of *O. oeni* strains. EU, enzymatic units. Values are the mean of duplicate determinations, which in each case were within no more than 1.3% of one another.
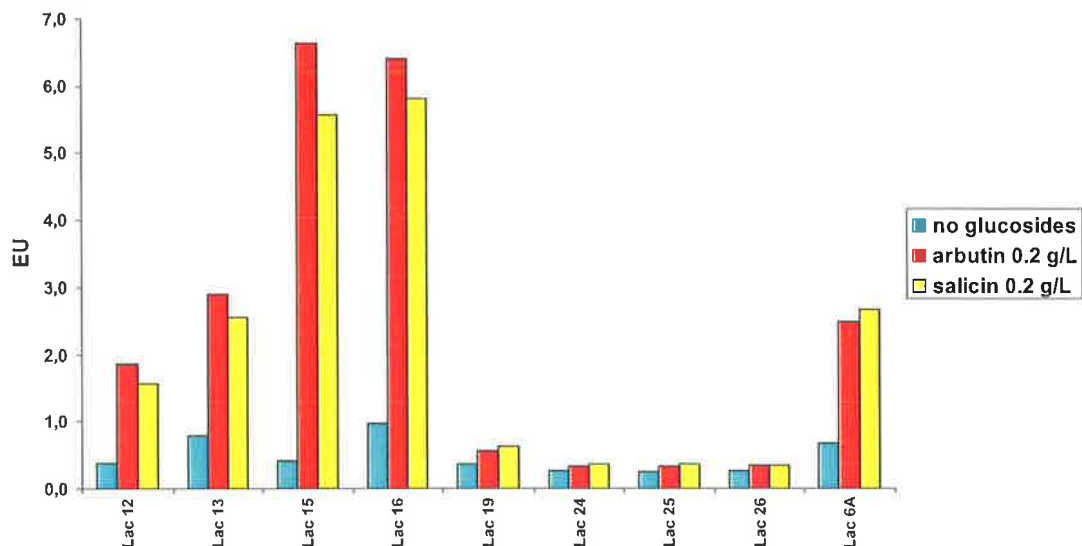
Figure 5.4. Effect of 6 h pre-exposure to glycosides on β-D-glucopyranosidase activity of *Lactobacillus* isolates. EU, enzymatic units. Values are the mean of duplicate determinations, which in each case were within no more than 4.3% of one another.
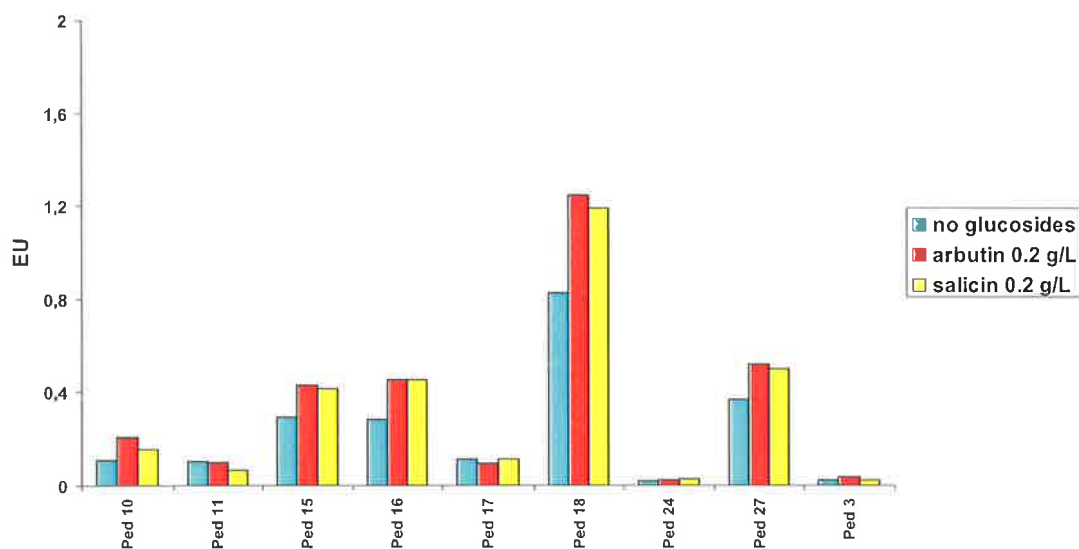
Figure 5.5. Effect of 6 h pre-exposure to glycosides on β-D-glucopyranosidase activity of *Pediococcus* isolates. EU, enzymatic units. Control, no glycosides; arbutin and salicin, 0.2 g/L. Values are the mean of duplicate determinations, which in each case were within no more than 6.3% of one another.

86

Effect of Anthocyanin on β-glucosidase Activity: Contrary to some reports about glycosidases being inhibited by anthocyanins (Adisakwattana et al., 2004; Matsui et al., 2001a; Matsui et al., 2001b; McDougall et al., 2005), in this study LAB β-glucopyranosidases were stimulated by prior exposure to anthocyanins. Compared to *Oenococcus* (Table 5.3) and *Pediococcus* (Table 5.4) isolates, the influence of anthocyanins was greatest for *Lactobacillus*, both in terms of percentage change and absolute values (Table 5.5). Despite the fact that almost half of the tested strains did not show significant increases, several isolates were highly induced by the presence of anthocyanins in the incubation medium. Thus the average increase across each set of isolates was 34.7% for *Lactobacillus*, 11.4% for *Oenococcus* and 10.6% for *Pediococcus*.

The above experiment highlighted the possible interactions occurring between wine phenolic glycosides and the β-D-glucopyranosidase enzyme of LAB. Further experiments were undertaken to investigate this possibility in greater detail.

5.3.2. Action of LAB β-glucopyranosidase on wine anthocyanins

Given that wine anthocyanins were able to induce greater β-D-glucopyranosidase activity amongst some strains of LAB, a more detailed study of the impact of LAB cells on a malvidin-3 glucoside preparation was undertaken. It is clear from Figure 5.6 that pigmentation of an anthocyanin-containing growth medium was greatly reduced when LAB cells were included. HPLC analysis was used in an attempt to determine the basis for this observation. A solution containing malvidin-3-glucoside was hydrolysed with HCl for 15 minutes in order to obtain the malvidin-aglycone. This solution was resolved by HPLC to determine the exact retention times for both molecules (Fig. 5.7).

During a time-course study of the effect of incubating LAB strains in modCDWM containing anthocyanins, malvidin-3-glucoside progressively decreased in concentration in the medium, but without being counterbalanced by the appearance of

Table 5.3. Difference in β-D-glucopyranosidase activity for *Oenococcus* isolates following incubation with or without the presence of anthocyanins.

LAB	ModCDWM Units	ModCDWM-Anth Units	Variation Units	%
Oen 11	1.75	2.15	**0.40**	22.9
Oen 16	1.36	1.66	**0.30**	22.4
Oen 7	1.35	1.63	**0.28**	20.7
Oen 4	1.01	1.26	**0.26**	25.5
Oen 12	2.43	2.68	**0.25**	10.2
Oen 23	0.94	1.18	**0.24**	25.1
Oen 13	0.78	1.02	**0.24**	30.1
Oen 6A	0.55	0.77	**0.22**	41.2
Oen 14	0.86	1.08	**0.22**	25.6
Oen 8	1.98	2.19	**0.21**	10.5
Oen 20	0.93	1.10	**0.17**	18.1
Oen 3	2.29	2.44	**0.16**	6.8
Oen 9	2.03	2.18	**0.15**	7.5
Oen 10	1.44	1.59	**0.15**	10.3
Oen 1	1.89	2.03	**0.14**	7.5
Oen 2	2.72	2.82	**0.10**	3.5
Oen 22	1.35	1.41	**0.06**	4.5
Oen 29	1.15	1.19	**0.04**	3.3
Oen 21	1.39	1.42	**0.03**	2.4
Oen 28	0.99	1.02	**0.02**	2.3
Oen 17	1.53	1.53	**0.00**	0.0
Oen 5	1.79	1.71	**-0.08**	-4.5

Values are the mean of duplicates, which in each case were within no more than 1.3% of one another.

Table 5.4. Increase in β-glucosidase activity for *Lactobacillus* isolates following incubation with or without the presence of anthocyanins.

LAB	ModCDWM Units	ModCDWM-Anth Units	Variation Units	%
Lac 16	0.98	1.53	**0.55**	56.3
Lac 15	0.42	0.87	**0.45**	108.7
Lac 6	0.68	0.88	**0.21**	30.9
Lac 12	0.38	0.52	**0.14**	37.2
Lac 13	0.79	0.92	**0.13**	16.6
Lac 25	0.25	0.28	**0.03**	12.6
Lac 26	0.26	0.28	**0.01**	4.7
Lac 24	0.26	0.26	**0.01**	2.3
Lac 19	0.36	0.34	**-0.02**	-4.7

Values are the mean of duplicates, which in each case were within no more than 4.3% of one another.

Table 5.5. Difference in β-glucosidase activity for *Pediococcus* isolates following incubation with or without the presence of anthocyanins.

LAB	ModCDWM Units	ModCDWM-Anth Units	Variation Units	%
Ped 16	0.28	0.36	**0.08**	26.4
Ped 15	0.29	0.34	**0.05**	17.7
Ped 27	0.37	0.42	**0.05**	13.7
Ped 10	0.11	0.16	**0.05**	41.4
Ped 17	0.11	0.14	**0.03**	22.0
Ped 24	0.02	0.04	**0.02**	77.2
Ped 3	0.02	0.02	**0.00**	9.6
Ped 18	0.83	0.82	**0.00**	-0.5
Ped 11	0.10	0.07	**-0.04**	-35.2

Values are the mean of duplicates, which in each case were within no more than 1.3% of one another.
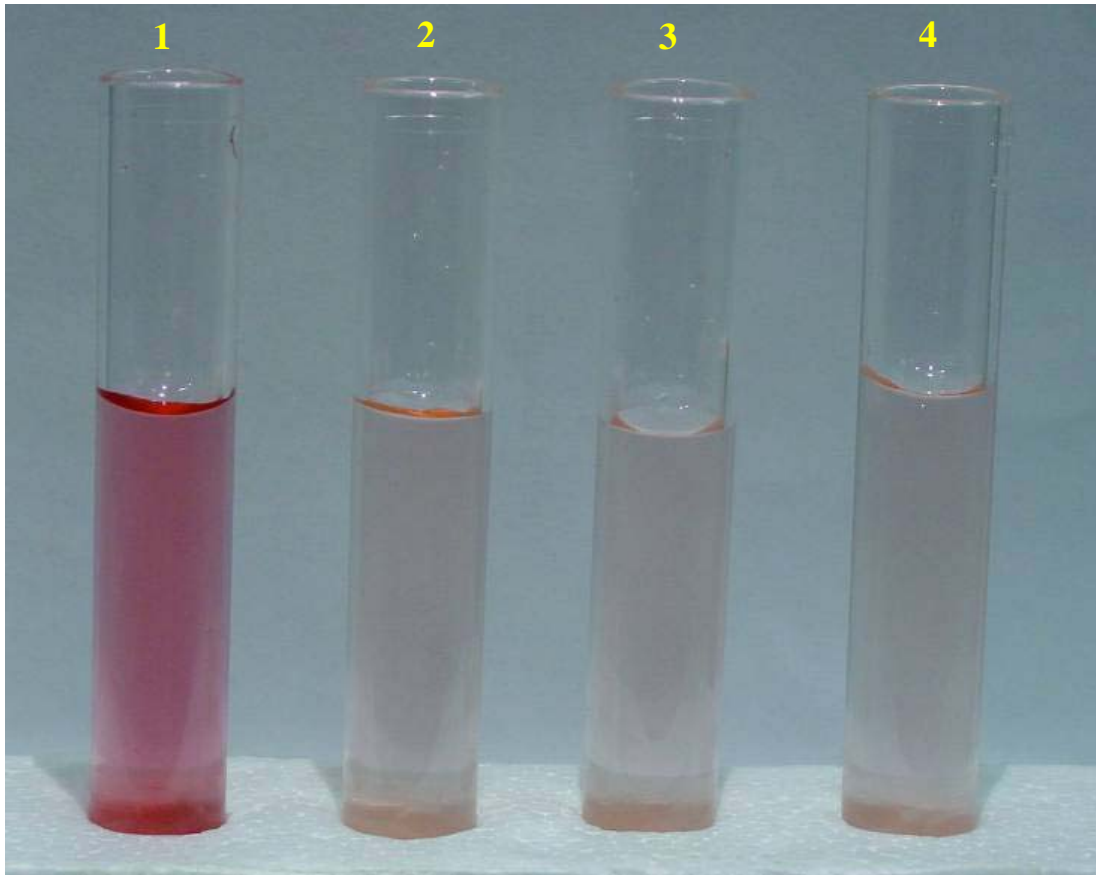
Figure 5.6. Variation in pigmentation of a malvidin-3-glucoside containing medium following growth of *O. oeni* strains for 72 h (modCDWM-Anth with 80 mg/L malvidin-3-glucoside, 5% v/v ethanol, 0.2% w/v glucose at 26ºC in shaking mode). Cultures were centrifuged (20,000 x *g* 5 min) and the supernatant collected. Anthocyanin solution containing no cells (1, control). or cells of Oen7 (2), Oen11 (3) and Oen16 (4).
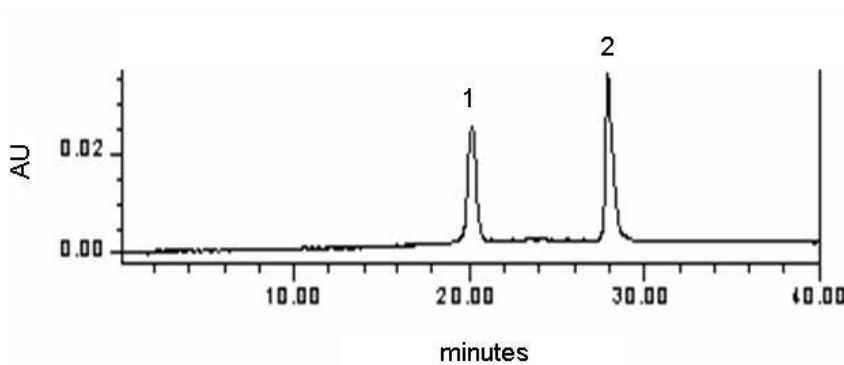
Figure 5.7. HPLC chromatogram showing retention times for malvidin-3-glucoside (peak 1) and its aglycone (peak 2) after acidic hydrolysis with 1 M HCl.

the aglycone, malvidin (Fig. 5.8). This was observed with all three LAB genera used (data not shown). During such incubations, colour intensity decreased quite noticeably compared to a cell-free control (Fig. 5.9). The high chemical reactivity of malvidin, combined with its tendency to quickly precipitate (Blom, 1983; Eskin, 1979; Ribéreau-Gayon et al., 2004b), suggested that any delay in analysis by HPLC might reduce the possibility of this product being detected. Other researchers have found that it may be difficult to detect some anthocyanidins due to their rapid chemical and possible microbial degradation (Aura et al., 2005). Consequently, it became apparent that it would be fundamentally important to hasten the analysis of the anthocyanin hydrolysates, so as to avoid substantial modification/degradation of the products. To this end a more rapid processing regime and HPLC analysis was used. Thus, LAB cells were incubated at 26°C in 0.2 M citrate/phosphate buffer containing 100 mg/L of malvidin-3-glucoside. The pH was reduced to 3.4, in order to better reproduce wine conditions. The rapid HPLC analysis (see section 5.2.3.2) was used as it was able to resolve both malvidin-3-glucoside and its aglycone with retention times of 5'40" and 7'50", respectively and within a total cycle duration of only 15 min (data not shown).

At the point of the first sample being taken at 30 minutes of incubation, malvidin-3-glucoside was the only compound detected (Fig. 5.10). Again no malvidin-aglycone peak appeared, even after 240 minutes by which time significant degradation of malvidin-3-glucoside had occurred. Interestingly, while analysis at 520 nm did not show any change in the medium composition, at 280 nm another peak appeared, with a retention time of about 9.5 min. This peak started to appear from 130 minute incubation and became larger in subsequent samples. To ensure that it was not an impurity, a further measurement was taken after 24 h incubation. Again, malvidin-aglycone was not detected at 520 nm, but analysis at 280 nm showed a clear and large peak at 9.5 min (Fig. 5.11). Due to time constraints and difficulties with further and alternate analyses, it was not possible to identify the compound(s) responsible for this peak at 280 nm. Also, given the small volume of this initial trial, β-glucosidase activity within this incubation assay was not specifically assayed. Further characterisation carried out outside of this PhD project would ideally consider relative β-glucosidase activity as well as examine the influence of inactivated cells so as to determine the extent of absorption of malvidin-3-glucoside to the bacterial biomass.
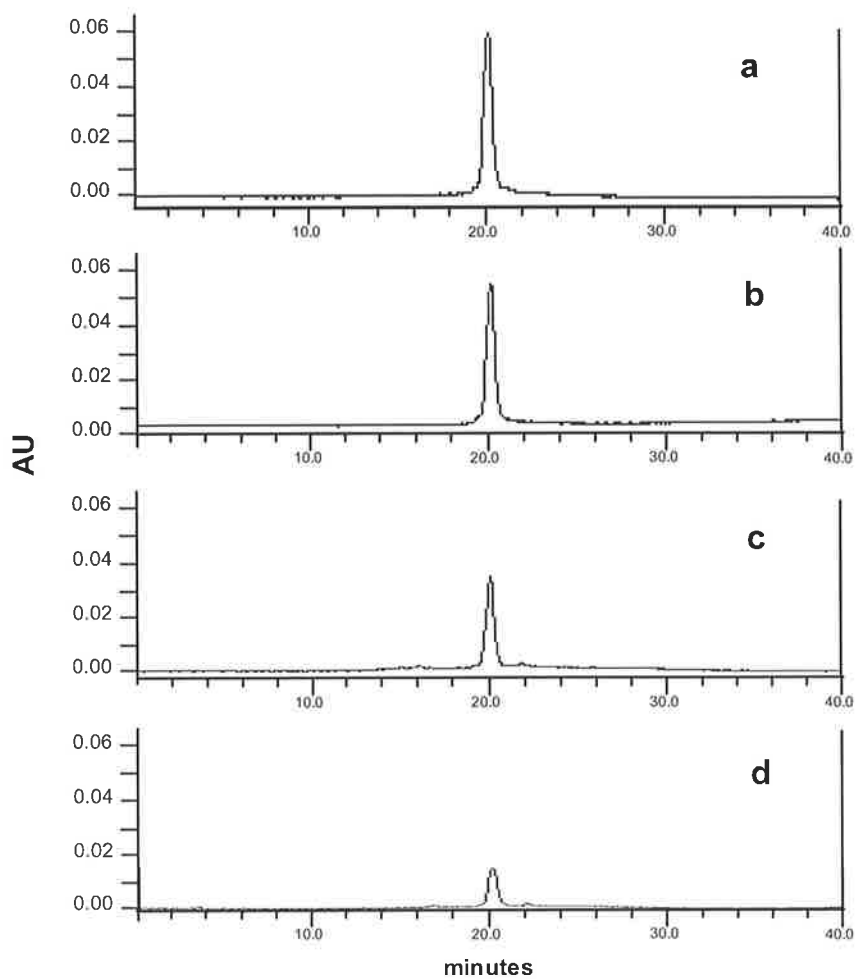
Figure 5.8. HPLC analysis (520 nm) of culture samples from *O. oeni* (strain Oen11) incubated for 4 h (a), 24 h (b), 48 h (c) or 72 h (d) in modCDWM-Anth. Peaks correspond to malvidin-3-glucoside.
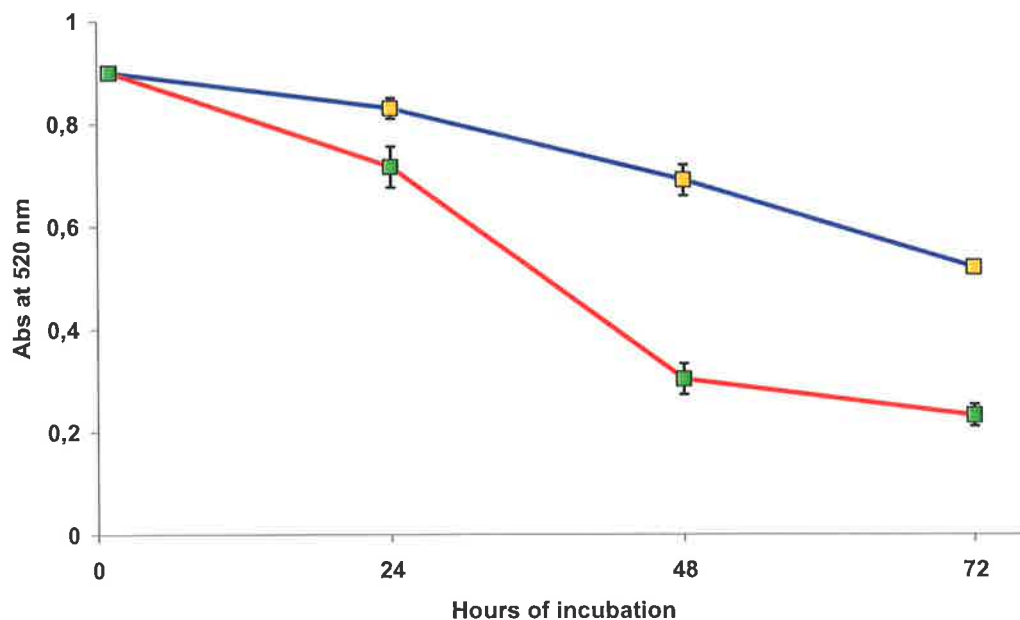
Figure 5.9. Absorbance at 520 nm of the supernatant of a malvidin-3-glucoside preparation as modCDWM-Anth incubated with *O oeni* strain Oen16 (red curve) compared to a control without cells (blue curve). Values are the mean of duplicate determinations.
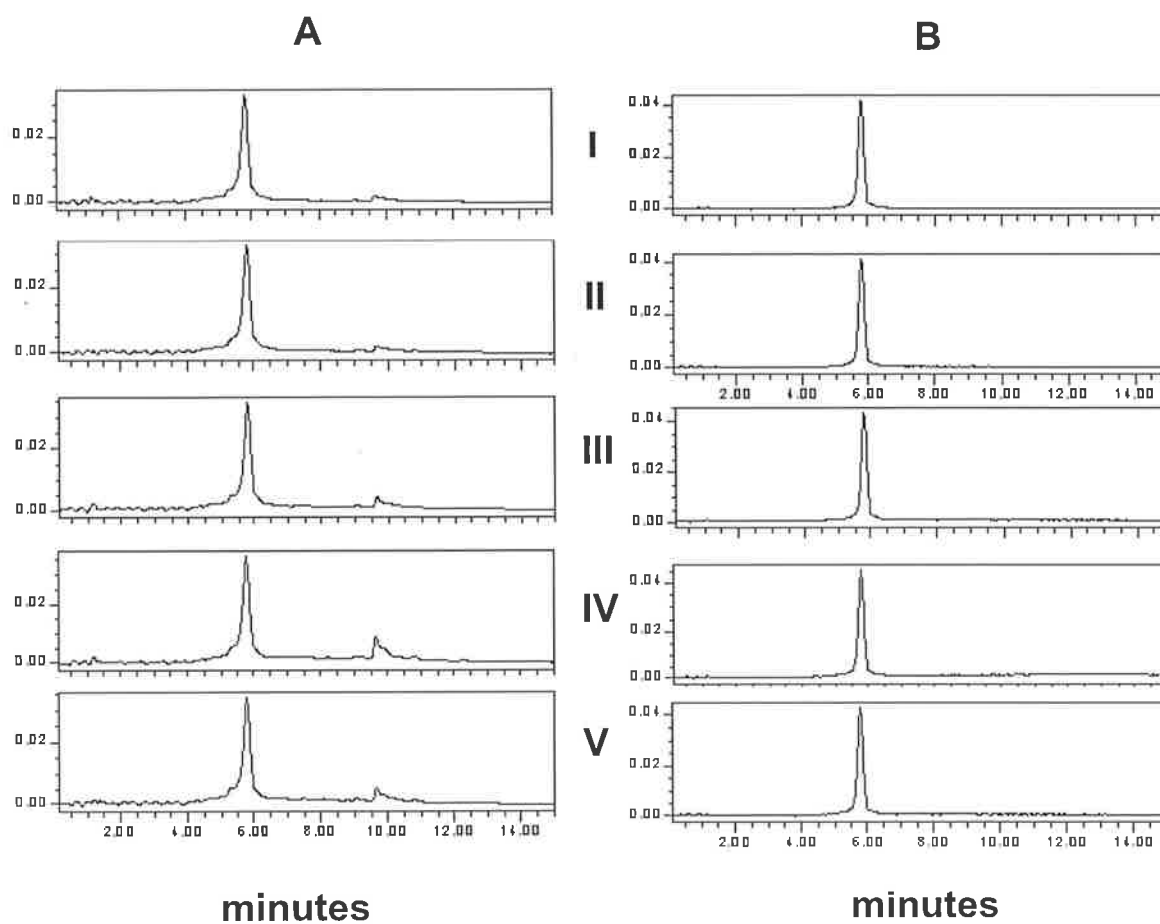
Figure 5.10. HPLC analysis of culture samples from *Lactobacillus* (Lac16) incubated for 30 min (I), 80 min (II), 130 min (III), 190 min (IV) or 240 min (V) in modCDWM-Anth containing malvidin-3-glucoside. Peaks detected at 280 nm (A) and at 520 nm (B) are shown.
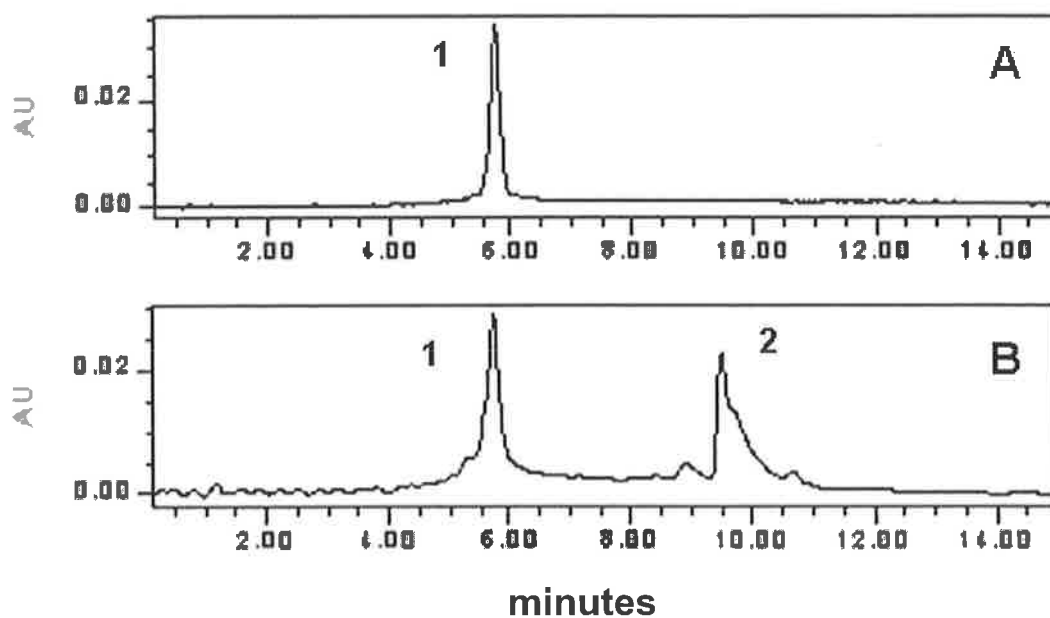
Figure 5.11. HPLC analysis of *Lactobacillus* (Lac16) incubated with malvidin-3-glucoside for 72 h (modCDWM-Anth with 5% ethanol, pH 3.4, 0.2% w/v glucose at 26°C). Chromatograms produced at 520 nm (A) and 280 nm (B). Peaks: 1, malvidin-3-glucoside; 2, unknown compound.

5.4 Conclusions

Several glycosides were used in this project to evaluate the ability of wine LAB to hydrolysis natural glycosidic substrates. As found by others (Marasco et al., 1998; Marasco et al., 2000), arbutin and salicin had a stimulating effect on β-D-glucopyranosidase activity, the former having a higher stimulus than the latter when supplied at the same concentration. The higher levels of enzymatic activity after only a few hours *in Lactobacillus* isolates are likely a reflection of the faster growth of this isolates compared to *Oenococcus* and *Pediococcus* strains.

While malvidin-3-glucoside is the most abundant anthocyanin in wine, it also is the most susceptible to precipitation and physical, chemical and microbiological transformations (Blom, 1983; Eskin, 1979; Ribéreau-Gayon et al., 2004b). Some bacteria, in fact, are able to break down anthocyanins to yield derivatives for their metabolism (Aura et al., 2005). Unquestionably, in all experiments reported here malvidin-3-glucoside decreased in concentration over time upon exposure to certain LAB. This decrease could be seen with the naked eye (Fig. 5.6) as well as through the measurement of malvidin-3-glucoside by HPLC (Figures 5.8 and 5.9). The peak detected by HPLC and corresponding to malvidin-3-glucoside was easily observed and seen to decline, but the breakdown products were not readily apparent. Thus it is possible that such products are either metabolised by the bacteria, precipitated or absorbed to bacterial cells or a combination of some or all of these. The cell wall is a very effective interface between extracellular and intracellular domains and can adsorb molecules and often trap larger ones in its structure (Stanier et al., 1986). Without further investigation it is impossible to evaluate whether the aglycone was adsorbed by or into the cells. As already stated further should consider i) quantitation of β-glucosidase activity during this experiment, ii) the evaluation of inactivated biomass for its ability to decrease malvidin-3-glucoside content and iii) a more extensive search for degradation products. The use of glycosides whose degradation products are more stable than malvidin would assist such an investigation. In fact Aura and coworkers (Aura et al., 2005) focused on quercetin-rutinoside and quercetin-glucoside, because their aglycones were more stable and easily detectable than malvidin.

In conclusion, it is possible to say that the amount of β-D-glucopyranosidase activity of LAB could be altered by prior exposure to the glycosides arbutin, salicin or anthocyanins. Also LAB biomass was seen to reduce malvidin-3-glucoside content to yet to be determined derivatives. This latter finding has clear implications for wine colour. Although not definitively proven in this project, bacteria appear capable of reducing anthocyanin content, perhaps by hydrolysis, to an extent which is yet to be fully quantified.

6. GENETIC CHARACTERISATION OF *OENOCOCCUS OENI* GLUCOSIDASES

6.1. Introduction

Lactic Acid Bacteria (LAB) are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. These bacteria are heterotrophic and because they lack many biosynthetic capabilities, generally have complex nutritional requirements. LAB belong to the *Clostridium* phylum, are gram positive and their G+C content is lesser than 50% (Gasser et al., 1994). Table 6.1 summarises phenotypic and genetic characteristics of LAB that are most commonly isolated from wine. These characteristics are often used as markers for genera identification. Lactic acid formation and carbohydrate catabolism are often the only common characteristics within this group, therefore their taxonomy has frequently been modified due to the vast diversity of the bacteria included (Salminen et al., 2004).

When analysing bacterial carbohydrate catabolic systems, we have to consider the complete set of proteins involved in this process, in which enzymes, and glycosidases in particular, play a fundamental role. One of these systems is represented by the phospho-enol-pyruvate (PEP) dependant carbohydrate phosphotransferase system (PTS), common in both gram negative and positive bacteria (Postma et al., 1993). This system is involved in both the transport and phosphorylation of a large number of carbohydrates, in movement toward these carbon sources (chemotaxis), and in regulation of a number of other metabolic pathways. Typically, proteins involved are phosphorylated at histidine residues (Postma and Lengeler, 1985; Postma et al., 1993). Regardless of the microorganism or carbohydrate, all PTSs that have been characterised catalyse the overall process shown below (Fig. 6.1).

$$\text{PTS}$$
$$\text{P-enol-pyruvate}_{(in)} + \text{carbohydrate}_{(out)} \longrightarrow \text{pyruvate}_{(in)} + \text{carbohydrate}_{(in)}$$
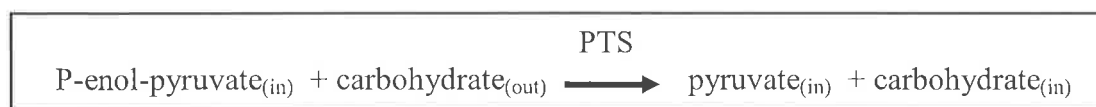
Figure 6.1. Phospho-transferase system (PTS) general mechanisms in bacteria. In, cytoplasm; Out, periplasm (Postma et al., 1993).

Table 6.1. Key phenotypic and genetic characteristics of Lactic Acid Bacteria found in wine (Ribéreau-Gayon et al., 2004a).

Cell morphology	Type of glucose Fermentation	Wine species	Genome Size (Mb)	G+C content	Cell size (μm)
Rods (singly, pairs, short chains)	Facultative heterolactic (group II)	*Lactobacillus casei*	2.8	36-47%	0.5-1.2 1.0-10.0
		Lactobacillus plantarum	3.3		
	Obligate heterolactic (group III)	*Lactobacillus brevis*	1.8		
		Lactobacillus hilgardii	~2		
Cocci (tetrads, pairs)	Homolactic	*Pediococcus damnosus*	~2	34-42%	1.0/2.0
		Pediococcus pentosaceus	1.8		
Cocci/Rods (pairs, short and long chains)	Heterolactic	*Oenococcus oeni*	1.8	38-44%	0.5-0.7 0.7-1.2
		Leuconostoc mesenteroides	2.0		

The organisation of the PTS systems involves both extracellular and intracellular elements, as depicted in the general scheme shown in Figure 6.2. EI and HPr are non-sugar specific and general proteins for all PTSs. They capture and transport a phosphate group from PEP to the EII domains, which consist of three elements (A, B and C, as represented in Figure 6.2). Hydrophobic domains EIIC (and EIID when present) are sugar specific, membrane bound and work as permeases, introducing the sugar moieties into the cytoplasm. Domains EIIA and EIIB, instead, are hydrophilic and located in the cytoplasm. These function as phosphate group carriers to the sugar moiety, EIIA contains the first phosphorylation site (P-His) while EIIB contains a second one (either a P-Cys or a P-His residue). This phosphorylation process is fundamental to "activate" molecules to be used in the catabolic pathways (Postma et al., 1993).

All studied PTS genes are grouped into specific operons, often coupled with glycosidase enzymes, in accordance with the EIIC domains. Several other genes may be present, that encode for proteins (such as antiterminators) that regulate the transcription of the other genes within the operon, and in many cases they have signals to regulate the amount of protein produced. Also LAB glycosidases are often inserted into the PTS operon. (Knezevic et al., 2000; Kruger and Hecker, 1995; Rutberg, 1997).

β-D-glucopyranosidase of *O. oeni* was chosen for further study given that a large number of such enzymes have been studied in many other organisms. In particular, the β-D-glucopyranosidases (both phospho- and non-) of *Lactobacillus plantarum* have been sequenced (Marasco et al., 1998; Marasco et al., 2000; Muscariello et al., 2001), thereby providing information which could be used to guide identification of these enzymes in *O. oeni*. We therefore sought to clone and sequence β-D-glucopyranosidase(s) from *O. oeni*.

The genome of *O. oeni* (together with many other LAB bacteria) has been recently sequenced by a consortium of laboratories around the world (http://genome.jgi-psf.org/draft_microbes/oenoe/oenoe.home.html), and despite the fact that it is still at a provisional stage, almost the entire genome has been published on Gene Bank (www.ncbi.nlm.nih.gov), albeit with some gaps in certain regions. The data utilised in this project were part of the first and second draft of the Gene Bank publication.
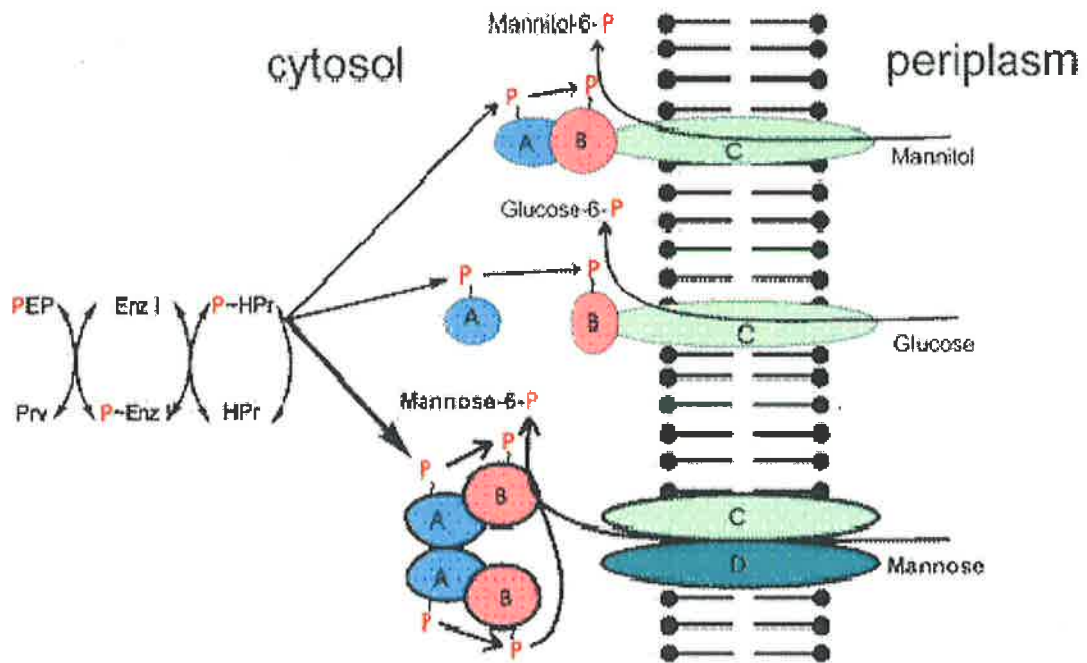
Figure 6.2. General structure of the most common bacterial PTS systems. Enz I represents domain EI. A, B and C represent domains EII of the respective carbohydrates. Some EIIC may be split into 2 domains, EIIC and EIID (e.g.: mannose). P~ indicates the phosphorylated form of the various proteins (adapted from (Gschwind et al., 1997) and (Postma et al., 1993)).

6.2 Materials and methods

6.2.1. DNA extraction and purification from *Oenococcus oeni*

The procedure used to extract and purify *O. oeni* DNA was as described in Zavaleta *et al.* (Zavaleta et al., 1997), with some modifications. The strain utilised for this genetic analysis was Oen2, the isolate that showed the highest and most varied activity.

Cells were harvested after 48 h growth (MRS + 20% apple juice, 26°C at pH 5.0), washed and resuspended (0.7 ml) in modified TE buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA). The cell wall was digested with 10 mg/ml of lysozyme at 37°C for 1 h. To the resulting homogenate, 80 µl of 10% w/v SDS and 5 µl of 500 µl/ml RNase cocktail were added with incubation for 1 h at 37°C. Proteins were digested with 6 µl of 20 mg/ml proteinase K at 50°C for 5 h. Digested proteins were eliminated by washing twice with 1 volume of phenol-chloroform (5:1) and the genomic DNA was precipitated with a mixture of 0.1 volumes of 3M NaCl and 1.5 volumes of isopropanol. DNA was finally washed with 0.5 ml of 75% v/v ethanol and was resuspended in 200 µl of sterile MQ water and stored at –70°C.

6.2.2. PCR conditions

PCR was carried out with 30 amplification cycles (hot start for 3 min at 95°C) of 30 seconds at 95°C, 1 min at 50°C and 2 min at 68°C. $MgCl_2$ was used at 3.5 mM. Taq polymerase was Dynazyme EXT (Finnzymes) at a concentration of 0.01U/µL.

6.2.3. Design of primers

The exact sequence of the region flanking the putative glucosidase open reading frames (ORFs) was available due to the publication of the entire *O. oeni* genome (JGI, http://genome.jgi-psf.org/draft_microbes/oenoe/oenoe.home.html). This enabled the design of primers that could include the entire ORFs. Both operons were large, more

102

than 2 kb, therefore it was decided to concentrate on the sequences encoding the enzymes only. Given that the two β-glucosidases in operon 2 were contiguous, the amplified region included both of them. Thus, two sets of primers were designed: BG3 fwd/BG4 rev and BG5 fwd/BG6 rev for operon 1 and 2, respectively. Table 6.2 lists their sequences and location in the *O. oeni* genome. The PCR amplified regions were of 2718 (operon 1) and 4186 bp (operon 2).

6.2.4. Sequencing of *O. oeni* β-D-glucopyranosidase genes

6.2.4.1. Nucleotide analysis

Sequencing of the PCR amplified regions was obtained using a 'primer walking' approach. Three amplified PCR bands were cleaned with the Qiaquick gel extraction kit (Qiagen) and used as templates for sequencing, utilising the ABI Big dye sequencing protocol. PCR primers used for sequencing are listed in Table 6.3. PCR cycles were as follows: 1 minute at 94°C, followed by 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C. Products were precipitated with 75% isopropanol at room temperature for 15 minutes, centrifuged (20,000 x g, 15 min) and pellets washed with 75% isopropanol before analysis of products by a commercial sequencing facility (Institute of Medical and Veterinary Science, Adelaide). Alignments and production of a consensus sequence from sequencing runs were performed using a trial version of Sequencher and then compared to the published *O. oeni* sequence of JGI using Clustal (WebAngis 1997).

Table 6.2. Primers sequence and location for *Oenococcus oeni* putative β-D-glucopyranosidase Operons 1 and 2.

Code	Location (scaffold 3)	Size (bp)	Sequence 5' – 3'	Amplified region (bp)
BG3 fwd[a]	9289-9253	37	GGCGCGATCCATATTCTGCCAATATTCCTGAAGTTCC	2718
BG4 rev[a]	6571-6605	35	CAGCTCAACTATCTCACCGGCCAGATCACGAAAGG	
BG5 fwd	125811-125847	37	CTGAAATCTTCGCTACTGCCGCTGCTGATGCTGATGC	4186
BG6 rev	129997-129963	35	GAGAACGGTTCCGGTTGGAACGGATCAATGATCGG	

[a] complementary sequence.

Table 6.3. Primers used to sequence *O. oeni* β-D-glucopyranosidase gene Operon 1.

Homology	Code	Sequence
pGemT	T7-	TAATACGACTCACTATAGGG
	SP6-	TATTTAGGTGACACTATAG
β-D-glucopyranosidase	BG3Fb.seq-	CGGGAGATTACCGATGGAATTG
	BG4Rb.seq-	ATCGACATAGATGAAACCGTACC
	BG3Rc.seq-	CAATTCCATCGGTAATCTCCCG
	BG4Fc.seq-	GGTACGGTTTCATCTATGTCGAT
	BG5Fc.seq-	GGCTGCATGATCAACATGAC
	BG5Rc.seq-	GTCATGTTGATCATGCAGCC

6.3. Results

6.3.1. Localisation of the putative genes for β-D-glucopyranosidase in *Oenococcus* and *Pediococcus*

Several LAB as key representatives of the food industry were part of the genome sequencing project coordinated by the Joint Genome Institute, JGI (http://genome.jgi-psf.org/mic_cur1.html). The most common *Lactobacillus* in wine is often *L. plantarum*, whose numerous glycosidases have been studied quite extensively (listed in (Coutinho and Henrissat, 1999)). In particular, a glucosidase (phospo-β-glucopyranosidase) has been well characterised to date ((Marasco et al., 2000), Lac 2 in this project). Using this *L. plantarum* Lac 2 sequence as the basis for a genome sequence search, it was possible to identify the following regions based on high nucleotide sequence similarity from sequence data bases (Table 6.4):

a) three putative β-glucosidase ORFs in *Oenococcus oeni*;

b) five ORFs in *Pediococcus pentosaceus*;

c) a further two ORFs in *Lactobacillus plantarum*.

When aligning the putative amino acid sequences of the different LAB strains, the homology was even higher. In particular in two very distinct regions, approximately between amino acids 70 and 180 and between amino acids 320 and near the end of the genes gave homologies of the order of 80-85% (Figures 6.3A and 6.3B).

These putative ORFs appear to be inserted in what are, most likely, PTS system operons, with an organisation (such as presence of genes for the typical PTS carbohydrate domains EII) that is similar to many other bacterial glucosidase operons (Cote et al., 2000; Cote and Honeyman, 2002; Kruger and Hecker, 1995; Le Coq et al., 1995; Postma et al., 1993; Schnetz et al., 1987).

Table 6.4. Nucleotide homology of *Lactobacillus plantarum* Lac2 β-D-glucopyranosidase gene with other wine associated LAB.

LAB species	Gene Designation	Nucleotide homology (%)
Oenococcus oeni	Oen 3	73
Oenococcus oeni	Oen 1	72
Oenococcus oeni	Oen 2	<60
Pediococcus pentosaceus	Ped 1	73
Pediococcus pentosaceus	Ped 2	~70
Pediococcus pentosaceus	Ped 3	<50*
Pediococcus pentosaceus	Ped 4	<50*
Pediococcus pentosaceus	Ped 5	<50*
Lactobacillus plantarum	Lac 1	73
Lactobacillus plantarum	Lac 3	70

* localised using Oen 3 sequence (performed with Blast program at http://www.ncbi.nlm.nih.gov/BLAST).
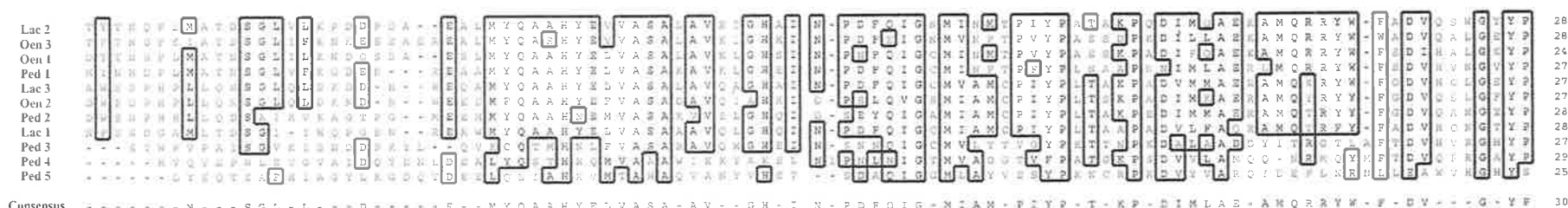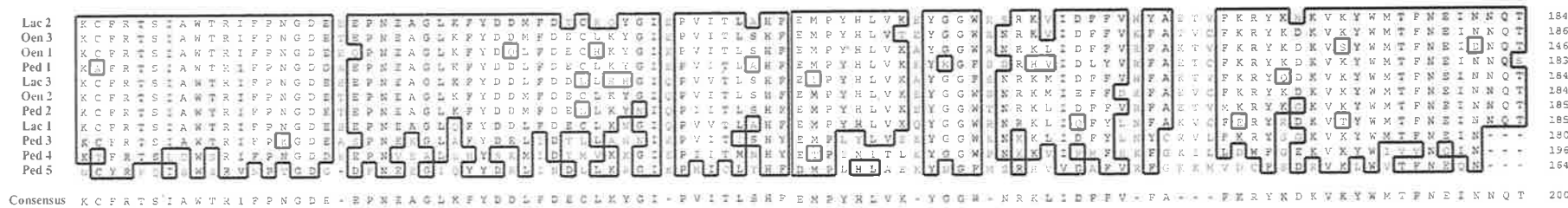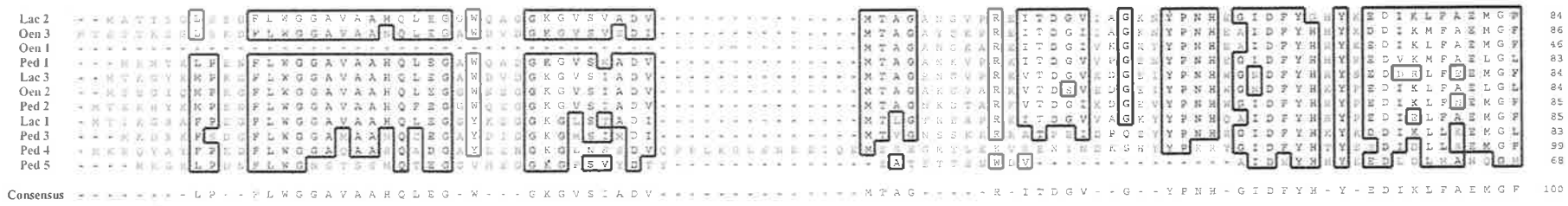
Figure 6.3A. Putative LAB β-D-glucopyranosidase genes. Amino acid alignment obtained with WebAngis 1997 (Sydney, Australia). Oen, *Oenococcus oeni*; Lac, *Lactobacillus plantarum*; Ped, *Pediococcus pentosaceus*.

108

Figure 6.3B. Putative LAB β-D-glucopyranosidase genes. Amino acid alignment obtained with WebAngis 1997 (Sydney, Australia). Oen, *Oenococcus oeni*; Lac, *Lactobacillus plantarum*; Ped, *Pediococcus pentosaceus*.

Putative *O. oeni* glucosidases are located in two distinct operons in a region of the genome that the JGI has provisionally classified in scaffold 3 (GeneBank accession number NZ_AABJ02000003). The first operon (Operon 1) is smaller than the second (Operon 2), having a size of 3509 bp compared to 6043 bp, respectively (Fig. 6.4). The main features of the key putative genes in each operon is shown in Table 6.5.

Operon 1 is comprised of 4 ORFs: a putative phospho-β-glucopyranosidase and three genes that encode proteins of the PTS system class II A, B and C of the cellobiose group. Operon 2 has a higher number of ORFs: 2 adjacent putative β-glucopyranosidases, three genes of the PTS systems class II A, B and C (cellobiose group) and a transcriptional regulator. PTS genes of both operons display a great similarity with analogous predicted proteins (Table 6.6), demonstrating the high genetic conservation through the bacterial world of proteins with the same function (Postma et al., 1993).

6.3.1.1 Operon 1

The four ORFs of this operon are situated on the complementary strand. Based on nucleotide sequences, the core enzyme is most probably a phospho-β-glucopyranosidase. In fact it is 110 bp shorter than the other typical operon β-glucosidases (1332 bp; (Coutinho and Henrissat, 1999)). PTS genes of this operon are part of the cellobiose group, which recognise and hydrolyse either simple disaccharides or other large glucosides that have a β-glucosidic linkage. This operon does not show any specific regulatory components. This may be confirmed by the absence of any transcriptional regulatory gene or antiterminator structure in this operon.

6.3.1.2 Operon 2

As mentioned above, this operon is larger both in size and ORF number than Operon 1. In fact, it has two putative glucosidases genes that have high similarities with other bacterial β-glucopyranosidases. PTS genes were similar to those of Operon 1, being of the cellobiose group as well. In addition, among the two enzymes there is an
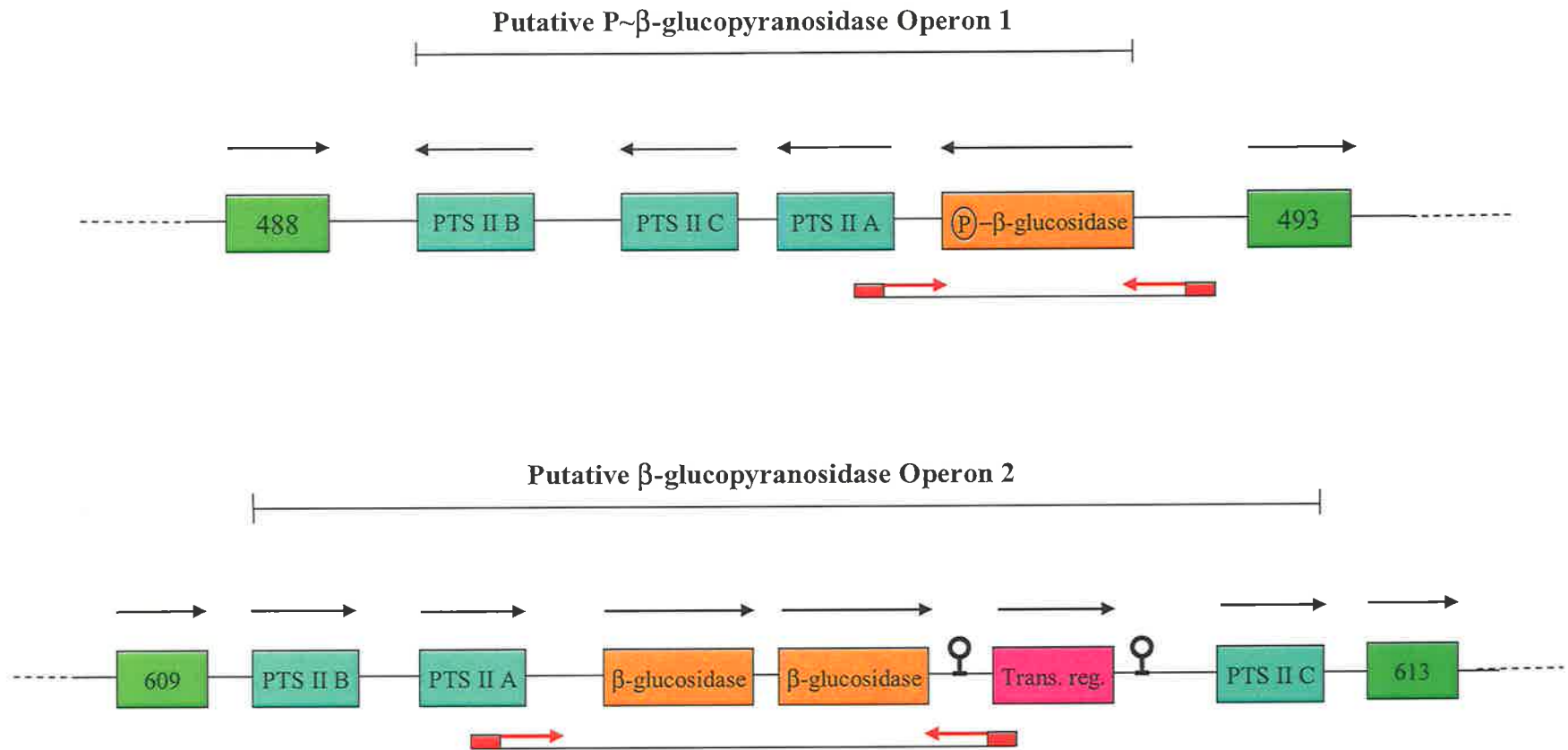
Figure 6.4. Scheme of the putative operons and β-D-glucopyranosidase ORFs in *O. oeni*. Putative stem-loop structures are indicated in Operon 2 by ⚲. Black arrows indicate the direction of transcription. Red boxes and arrows indicate primers and direction of amplification, respectively. Drawings are not to scale (from www.ncbi.nlm.nih.gov).

Table 6.5. Features of selected genes of *Oenococcus oeni* β-D-glucopyranosidase operons

Gene N°	Position		Strand	Amino acids	Similar to
Oen489	5020	6372	-	451	EII C (cellobiose family)
Oen490	6547	6870	-	108	EII A (cellobiose family)
Oen491	6872	7195	-	108	EII B (cellobiose family)
Oen492	7198	8529	-	444	P~β-D-glucopyranosidase*
Oen606	125711	126019	+	103	EII B (cellobiose family)
Oen607	126135	126470	+	112	EII A (cellobiose family)
Oen608	126477	127922	+	482	β-D-glucopyranosidase
Oen609	127939	129396	+	486	β-D-glucopyranosidase
Oen610	129474	129860	+	129	Transcriptional Regulator
Oen612	130453	131754	+	434	EII C (cellobiose family)

* phospho-β-D-glucopyranosidase.

Table 6.6. Amino acid similarity of *Oenococcus oeni* EIIC genes with analogous genes from other wine LAB*.

LAB species	Operon 1		Operon 2	
	Identity %	Similarity %	Identity %	Similarity %
Lactobacillus plantarum	35	54	75	89
Lactobacillus acidophilus	81	93	43	61
Lactobacillus gasseri	65	81	38	58
Lactobacillus johnsonii	69	84	41	68
Lactobacillus casei	50	68	-	-
Lactobacillus sakei	-	-	37	57
Pediococcus pentosaceus	31	51	54	76
Leuconostoc mesenteroides	46	66	-	-
Lactococcus lactis	34	51	37	51

* performed with Blast program at http://www.ncbi.nlm.nih.gov/BLAST. Identity, based on same amino acid encoded; Similarity, same amino acids and conservative amino acid substitutions.

ORF (*Oen610*) that bears a high resemblance, up to 74% (see Appendix 9), to the transcriptional regulator for PTSs: a domain containing a helix-turn-helix motif, of which the best characterised member of this family is RpiR, a regulator of the expression of the rpiB gene (Yamamoto et al., 2001). The encoded protein may be combined with sections functioning as an antiterminator, but unfortunately despite the presence of stem-loop structures, none have the typical sequence common in many LAB and other bacteria (Rutberg, 1997). The two glucosidase genes have similar length and present high homology, particularly when compared with *L. plantarum* β-glucosidases that have already been well characterised (Marasco et al., 1998; Marasco et al., 2000).

This operon appears to contain two stem-loop structures inserted around the putative transcriptional regulator of *Oen0610* (Fig. 6.5). Stem-loop A is located between this gene and the putative β-D-glucopyranosidase ORF Oen609, 9 bp downstream of the termination site of *Oen610*. It has a length of 44 bp and is very AT rich (64%). While stem-loop B is located 153 bp downstream of Oen610, with a length of 52 bp, and is also AT rich (~74%).

These structures most probably form part of the termination signals for the transcription of the *Oen609* and *Ooen610* ORFs, encoding for the putative β-glucopyranosidase and the transcriptional regulator, respectively. The encoded protein of the latter would probably bind to a ribonucleic antitermination (RAT) site that precedes the gene encoding for the PTS domain EIIC (*Oen612*) (Rutberg, 1997). This protein would overlap the stem-loop, allowing its transcription and therefore functioning as an operon regulator.

```
                                            T
                                          T   T
                                        T       T
        A                               C       T       B
                                        T   C
            AA                            AT
            TA                          T     G
          C   T                         G     G
        A       C                         AT
        G       A                       T     G
          C   C                           TA
            AT                          T     G
            AT                            TA
            CG                            GC
            AT                            AT
            GC                          T     G
            AT                            TA
            GC                            AT
          T   G                         T     G
            CG                            TA
            GC                            TA
            AT                            CG
            AT                            AT
            AT                          G     A
            AT                            AT
            AT                            AT
            AT                            CG
```
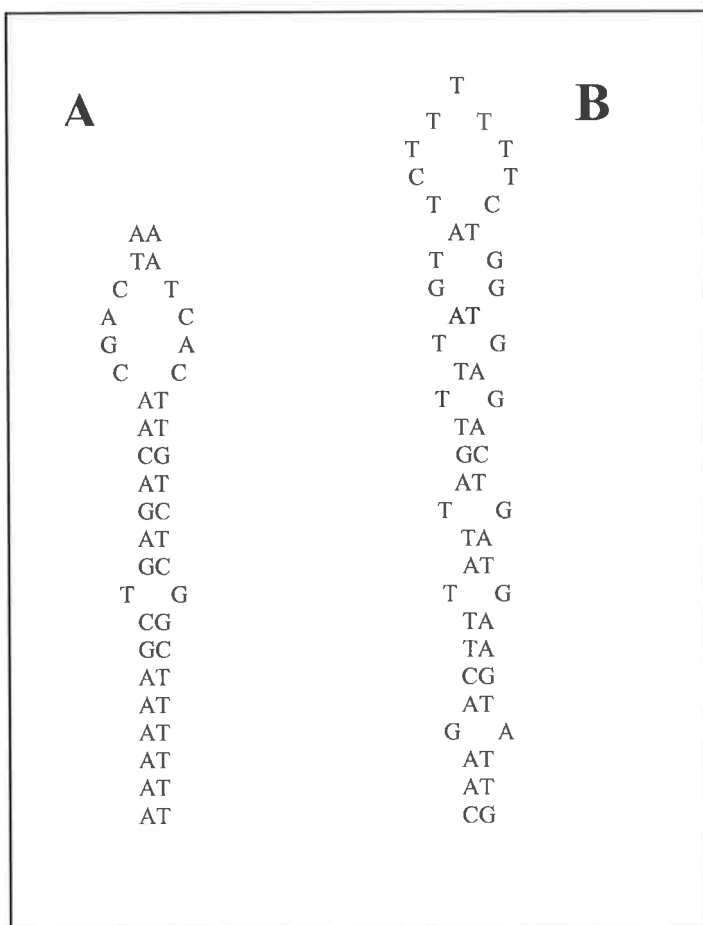
Figure 6.5. Putative stem-loop structures in *Oenococcus oeni* (Oen 2) Operon 2. A, located between *Oen619* and *Oen610* ORFs; B, located downstream *Oen610*.

6.3.2. Sequencing of the putative *O. oeni* β-D-glucopyranosidase genes

Although the theoretical PCR conditions for the two sets of primers were almost identical, repeated attempts to amplify Operon 2 did not succeed. Changes to the PCR conditions (e.g. annealing temperatures, $MgCl_2$ concentrations, etc) were not met with success. It has therefore only been possible to amplify Operon 1. The nucleotide analysis showed some modifications, both in the entire amplified region and inside the putative β-glucopyranosidase gene. In total there were 55 and 25 base modifications, respectively (Fig. 6.6). Regarding the putative β-D-glucopyranosidase gene, the identified differences produced six amino acid substitutions, as shown in Figure 6.7.

A recent report by Spano and co-workers (2005), in which an *O. oeni* β-D-glucopyranosidase was sequenced and purified, revealed an enzyme of 463 amino acids. Based on amino acid alignment (ClustalW) with the other three β-D-glucopyaranosidases studied in this project, little homology was seen (Fig. 6.8), that is homology was only 23% with Oen492, 21% with Oen608 and 20% with Oen609.

Given the great number of β-D-glucopyranosidases present in bacteria, it is possible that there might be a specific enzyme for each glucoside substrate. In addition, the subcellular localisation of each enzyme may require produce different biochemical characteristics in the enzyme.

Identities: 1306/1332 (98%)

```
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Query: 61     tattaccccaatcaagaggccatcgactttatcatcgctataaagaagatatcaagtta 120
              |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
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Query: 121    ttcgccgagatgggtttttaaatgttttcgaacctcgattgcctggacgaggatctttccc 180
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Sbjct: 4551   ttcgccgagatgggtttttaaatgttttcgaacctcgattgcctggacgaggatctttccc 4492


Query: 181    aatggagacgaagagcaacccaacgaagccggcttgaagttttacgaccagctctttgat 240
              |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
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Query: 241    gaatgccacaagtatggtatcgaaccggtcattaccctctcgcattttgaaatgccctat 300
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Query: 301    cacttggttaaagtctacggcggctggcgtaaccgaaaactaatcgatttctttgttcac 360
              || ||||||||| ||||||||||||||||||||||||||||||||||||||||||||| |
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Query: 361    tttgccaagacggtcttcaaacgttataaagacaaagttagctactggatgacctttaat 420
              |||||||||||||||||| |||||||||||||||||||||||||||||||||||| |||||
Sbjct: 4311   tttgccaagacggtctttaaacgttataaagacaaagttagctactggatgactttttaat 4252


Query: 421    gagatcgacaaccaaaccgattatacaaatcgcttcttaatggctactaattccggtttg 480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 481    atattaaaaaatgatcaaagtgatgcagaaagcttaatgtatcaagcggctcattacgaa 540
              |||||||||||||||||||||||||| |||||||||||||||||| ||||||||||||||
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Query: 541    ctggttgccagtgctctagccgtcaagcttggccatagtattaatcctgattttcagatc 600
              |||||||||||||||||||||||||||||||||||| ||||||||||||||| ||||||||
Sbjct: 4131   ttggttgccagtgctctagccgtcaagcttggccacagtattaatcctaattttcagatc 4072


Query: 601    ggctgcatgatcaacatgacgcctgtttacccggcttcttcaaaaccagctgatatcttt 660
              |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct: 4071   ggctgcatgatcaacatgacgcctgtttacccggcttcctcaaaaccagctgatatcttt 4012


Query: 661    caagcagaaaaagcaatgcaaaggcgctattggttttccgacattcacgctctgggcaaa 720
              ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||| ||
Sbjct: 4011   caagcagaaaaagcaatgcaaaggcgctactggttttccgacattcacgctctgggaaaa 3952


Query: 721    tatccagaaaacatggaagtattttttgaaacaaaacaattttcgcccggatattacgagc 780
              ||||| |||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct: 3951   tatccggaaaacatggaagtattttttgaaacaaaacaattttcgcccggatatcacgagc 3892
```

Figure 6.6. Nucleotide alignment between amplified *Oenococcus oeni* (strain Oen2) β-D-glucopyranosidase gene (Query) and data published on JGI (Sbjct). Base differences are highlighted (*). Figure continues over page.

117

```
Query: 781   gaggaccgaatagtattaaaagaaggaactgtcgactatattggattgagttattacaat 840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3891  gaggaccgaatagtattaaaagaaggaactgtcgactatattggattgagttattacaat 3832


Query: 841   tcaatgaccgttcaatcaaaagaaagcaacccgggttttcatttcattggtcccgaactg 900
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||| || || |||||||
Sbjct: 3831  tcaatgaccgttcaatcaaaagaaagcaacccgggctttcatttcatcggtccggaactg 3772


Query: 901   accgttgataatccaaatgttgaaaaaagcgattggggatggccgatcgatccgttggga 960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 961   cttaggtattctttaaactggctggccgaccactatcacaagcccttgttcattgttgaa 1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3711  cttaggtattctttaaactggctggccgaccactatcacaagcccttgttcattgttgaa 3652


Query: 1021  aacggtctgggagcctatgacaaagtcgaaaataaccaacagatccatgacccttatcga 1080
             ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 3651  aacggtctgggagcctatgacaaagtcgaaaatagccaacagatccatgacccttatcga 3592


Query: 1081  atcgcttatctaaaagctcatatccaggcaatgatcgatgcagttcaagaagacggggtt 1140
             |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct: 3591  atcgcttatctaaaagctcatatccaggcaatgatcgatgcggttcaagaagacggggtt 3532


Query: 1141  aaggtcattggttatacgccctggggttgtatcgatctggtttccgccggaaccggacag 1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 1201  atgtccaaaaggtacggtttttatctatgtcgataaagacgaccagggcaaaggtagctta 1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct: 3471  atgtccaaaaggtacggtttttatctatgtcgataaagacgaccagggcaaaggcagctta 3412


Query: 1261  aaaagactgaaaaaggattcctttttctggtatcaacaggttattaagtcaaacggcagt 1320
             ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
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Figure 6.6. (cont).

Identities: 437/443 (98.9%)
Positives: 440/443 (99.3%)

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Query:   61    NGDEEQPNEAGLKFYDQLFDECHKYGIEPVITLSHFEMPYHLVK YGGWRNRKLIDFFV  120
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Query:  121    FAKTVFKRYKDKVSYWMTFNEIDNQTDYTNRFLMATNSGLILKNDQSDAESLMYQAAHYE 180
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Sbjct: 4311    FAKTVFKRYKDKVSYWMTFNEIDNQTDYTNRFLMATNSGLILKNDQSDAESLMYQAAHYE 4132

Query:  181    LVASALAVKLGHSINP FQIGCMINMTPVYPASSKPADIFQAEKAMQRRYWFSDIHALGK 240
               LVASALAVKLGHSINP+FQIGCMINMTPVYPASSKPADIFQAEKAMQRRYWFSDIHALGK
Sbjct: 4131    LVASALAVKLGHSINPNFQIGCMINMTPVYPASSKPADIFQAEKAMQRRYWFSDIHALGK 3952

Query:  241    YPENMEVFLKQNNFRPDITSEDRIVLKEGTVDYIGLSYYNSMTVQSKESNPGFHFIGPEL 300
               YPENMEVFLKQNNFRPDITSEDRIVLKEGTVDYIGLSYYNSMTVQSKESNPGFHFIGPEL
Sbjct: 3951    YPENMEVFLKQNNFRPDITSEDRIVLKEGTVDYIGLSYYNSMTVQSKESNPGFHFIGPEL 3772

Query:  301    TVDNPNVEKSDWGWPIDPLGLRYSLNWLADHYHKPLFIVENGLGAYDKVEN QQIHDPYR 360
               TVDNPNVEKSDWGWPIDPLGLRYSLNWLADHYHKPLFIVENGLGAYDKVEN+QQIHDPYR
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Sbjct: 3591    IAYLKAHIQAMIDAVQEDGVKVIGYTPWGCIDLVSAGTGQMSKRYGFIYVDKDDQGKGSL 3412

Query:  421    KRLKKDSFFWYQQVI SNGSQLD 443
               KRLKKDSFFWYQQVI+SNGSQLD
Sbjct: 3411    KRLKKDSFFWYQQVIQSNGSQLD 3343
```

Figure 6.7. Amino acid alignment between amplified *Oenococcus oeni* putative β-glucosidase gene (see Figure 6.6) (Query) and data published on JGI (Sbjct). Amino acid substitutions marked with colours: blue for conservative amino acid substitution, green for complete substitution. The central row reiterates regions of identity with mismatches left blank and conservative changes indicated with a "+".

```
Oen492    ------------------------------------MTAGANGKAREITDGIVKGK  20
Oen609    MTETTKSGLRKDFLWGGAVAANQLEGAWDVDGKGVSVSDIMTAGAYQKPREITDGIIAGK  60
Oen608    MSEG--IQMPKGFLWGGAVAAHQLEGGWNEGGKGVSIADVMTAGAKGVARRVTDSVEDGE  58
Spano     ---MTMVEFPEGFVWGAATSGPQTEGNFHKQHQN-----VFDYWFATEPEQFDAGVG---  49
                         :    ::                        ::        .. ..   . :

Oen492    YYPNHEAIDFYHRYKEDIKLFAEMGFKCFRTSIAWTRIFPNGDEEQPNEAGLKFYDQLFD  80
Oen609    NYPNHEAIDFYHHYKDDIKMFAEMGFKCFRTSIAWTRIFPNGDETEPNEAGLKFYDDMFD  120
Oen608    IYPNHWGNDFYHKYPEDIKLFAELGLKCFRTSIAWTRIFPNGDETEPNEAGLKFYDDMFD  118
Spano     ---PDTASNFYNDYDHDLALMAQAGVQGLRTSIQWTRLIDDFETASLNADGVAFYNHVID  106
           .  .  :**: *  .*: ::*: *.: :**** ***:: : :  .  *  *: **:..:*

Oen492    ECHKYGIEPVITLSHFEMPYHLVKAYGGWRNRKLIDFFVRFAKTVFKRYKDKVSYWMTFN  140
Oen609    ECLKYGIEPVITLSHFEMPYHLVTEYGGWRNRKVIDFFVKFATVCFKRYKDKVKYWMTFN  180
Oen608    ECLKYGIQPVITLSHFEMPYHLVKEYGGWSNRKMIEFFDRFAEVCFKRYKDKVKYWMTFN  178
Spano     SMLAHHITPYINLHHFDLPVALYDKYHGWESKHVVELFVKFAEQCFKLFGDRVDHWYTFN  166
           .   :  * * *.*  **::*   *    * **  .:::::::*  :**    **  : *:*.:* ***

Oen492    EIDNQTDYTNRFLMATNSGLILKNDQSDAE--SLMYQAAHYELVASALAVKLGHS--INP  196
Oen609    EINNQTTFTNDFSIATDSGLIFRNKESEAEREALMYQASHYEVVASALAVKIGHK--INP  238
Oen608    EINNQTDWPDPHPLLQNSGLQLDKNDNWEE---EMFQAAHYEFVASADAVQIAHR--IDP  233
Spano     EPK----------VVVDGQYLYGWHYPQVINGPKAVQVAYNMNLASAKTVARFHELCVRP  216
           *  .            :  :.      .           *.::   :*** :*   *  :  :  *

Oen492    NFQIGCMINMTPVYPASSKPADIFQAEKAMQRR-YWFSDIHALGKYPENMEVFLKQNNFR  255
Oen609    DFEIGNMVNFTPVYPASSDPKDILLAEKAMQRR-YWWADVQALGEYPVGMEAYFKNHDLR  297
Oen608    SLQVGSMIAMCPIYPLTSKPADIMKAERAMQYR-YYFGDVQSLGFYPEWIQKYWARKGYN  292
Spano     EQQIGIILNLTPAYAASDDPADLAAAEFAELWSNNLFLDPAVLGHFPEKLVERLTMDGVL  276
           .  ::* ::  :  *  *.  :..*  *:  ** *     :  *   ** :*   :      ..

Oen492    PDITSEDRIVLKEGTVDYIGLSYYNSMTVQSKESNPG-----FHFIGPELTVDNPNVEKS  310
Oen609    PDITAEDRVVLREGTVDYVGFSYYNSMTVKYSDDNPE-----FKFVGDREAVKNPNLKYN  352
Oen608    LDISASDLATIKAGTVDYVGFSYYMSFATKAHEGETH-----FDYDEHDDLVSNPYVEKS  347
Spano     WDATPTELAIIAANPVDCLGVNYYHPFRVQRPDISPKSLQPWMPDIYFKEYDMPGRMMNV  336
           *  :.  :     :    ..** :*..**  .:  .:  :  ..   :    :          :

Oen492    DWGWPIDPLGLRYSLNWLADHY-HKPLFIVENGLGAYDK---VENSQQIHDPYRIAYLKA  366
Oen609    DWGWPVDPVGLRYSMNWLTEHY-HKPVMIVENGFGAYDK---VESDGSIHDDYRVDYLRA  408
Oen608    DWGWQIDPVGLRYAMNWMTTRW-HKPLFIVENGFGAYDK---VEDDGSIHDPYRIQYFHD  403
Spano     DRGWEIYPQAMTDIARNIQKNYGNIPWMISENGMGVAGEERFLDKQGVVQDDYRIDFMKE  396
           * **  : *  .:    .  :    .:  :  *  :* ***:*.  .:    :::..    ::* **: : :::

Oen492    HIQAMIDAVQEDGVKVIGYTPWGCIDLVSAGTGQMSKRYGFIYVDKDDQGKGSLKRLKKD  426
Oen609    HVKQMITAVNEDGVDLMGYTPWGCIDLVSAGTGQMSKRYGFIYVDKDDEGNGTLERSKKD  468
Oen608    HILQMEKAVKEDGVQLLGYTPWGHIDLVSASTGEMKKRYGMIYVDEDDEGHGSLKRSKKD  463
Spano     HLTALAKGIAAG-SNCQGYFVWSGIDCWSWNH-AYHNRYGLIRNDIHTQT-----KTLKK  449
           *:   :   .:  .    .   **  *. **  *  .     :.***:*  *  .:  :     :  *.

Oen492    SFFWYQQVIQSNGSQLD-  443
Oen609    SFYWYQKVIRSNGLDLD-  485
Oen608    SFYWYKKVIESNGKDLDI  481
Spano     SAKWFAELGERNGF----  463
          *   *:  ::  .  **
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Figure 6.8. Amino acid alignment between a published *Oenococcus oeni* β-D-glucopyranosidase gene (Spano et al., 2005) and the three putative translated glucosidases studied in this project. Alignment was performed with ClustalW (European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw). * consensus

6.4. Conclusions

The organisation of the putative *O. oeni* β-D-glucopyranosidase operon, localised in the studied isolate (Oen2), was found to resemble closely the known Phospho Transferase System (PTS) operons found in many other LAB bacteria. Despite the fact it was not possible to amplify and sequence the other glucosidases of Operon 2, the information regarding its ORFs found on Gene Bank revealed that the similarity can be extended to this operon too. This is confirmed by the presence, in proximity of the glucosidase gene, of several genes that encode for characteristic proteins of the PTS class. The high homology of these structures with other LAB, both at the operon and gene level, suggests a conserved mechanism in carbohydrate catabolism in the group.

While the three β-D-glucopyranosidase genes identified in *O. oeni* showed great similarity, they differed from the glucosidase characterised by Spano and co-workers (Spano et al., 2005). There might be several reasons for the observed differences. Only a detailed analysis of the structure of the isolated enzyme will answer these questions.

At the conclusion of this project, it was only possible to PCR amplify and sequence one β-D-glucopyranosidase gene. Results of the sequencing showed few modifications at nucleotide levels, causing six amino acid substitutions. Despite the presence of a transcriptional regulator in Operon 2, no structure involved in the transcription of the operon genes has been found. Obviously, the composition of the operon as described in this project is speculative, being based on preliminary genomic data, and as such it is possible that such signals (specific terminators and antiterminators) if present might be found outside of the proposed region. A more extensive sequencing of this operon will be required in order to more fully characterise the putative *O .oeni* β-D-glucopyranosidase enzymes.

7. GENERAL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

This project has investigated the occurrence of glycosidase enzymes amongst Lactic Acid Bacteria (LAB) and their potential to hydrolyse wine glycosides. The nature of glycosides in wine is quite varied and ranges from aromatic compounds, such as C_{13} norisoprenoids, aliphatic compounds and monoterpenes (terpenols), to colour compounds, such as benzene derivatives (anthocyanins)(Winterhalter and Skouroumounis, 1997). The hydrolysis of these would have several repercussions on wine quality, both positive and negative. In the former category, aromatic compounds could be liberated thereby enhancing the flavour and aroma of wines, otherwise trapped in a flavourless, glycosylated form. On the other hand, colour compounds may be degraded, resulting in losses of wine colour. There is also the possibility that glycosidases may liberate phenolic off-flavours, which are otherwise neutral or odourless in the glycosidic form, such as p-vinyl-guaiacol by the liberation of its precursor, ferulic acid (Wrolstad et al., 1994). For all these reasons, it is of importance to understand how widely distributed LAB glycosidases are and how well they may function under wine-like conditions to either improve or else spoil wine quality.

To begin with, it was sought to create a collection of wine LAB strains, comprised of or derived mostly from commercial starter cultures for malolactic fermentation (MLF), with the sole exception being two isolates from a commercial olive product. The intention was to utilise bacteria that had already been tested and isolated for winemaking, and therefore to better define their potential impact on winemaking by understanding their ability to produce glycosidase enzymes. In fact, the collection comprised a wide range of biotypes of *Oenococcus oeni*, the most important bacterium for MLF in wine (Costello et al., 1983; Davis et al., 1985; Fugelsang, 1997; Van Vuuren and Dicks, 1993).

To investigate LAB glycosidases, a multi-step research approach has been used. Initially, a conventional biochemical characterisation was performed on up to 22 *O. oeni* isolates, 10 lactobacilli and 9 pediococci in order to gain a clear idea of the most appropriate operational parameters of these enzymes. Since this project had a winemaking context, comparisons were constantly made between the optimal

conditions and those of winemaking. In a second stage of the study, attention was concentrated on the effect of LAB glycosidases on wine colour, and their potential to destroy, or limit, an important component of wine quality. While such an outcome would be generally undesirable, it is nonetheless recognised that in some specific instances of wine production, e.g. rose and white wines made from red grapes, colour reduction may in fact be beneficial. Finally, an investigation into the genetic bases of the synthesis and production of the glycosidase protein was carried out. Of the many glycosidases considered in this project, the enzyme which had the greatest number of related studies was undoubtedly β-D-glucopyranosidase. Thus this enzyme was chosen as the topic for this latter phase of the study. Published sequence information from a large number of bacteria and LAB, some being wine related, was consulted during this work.

In the following sections the general conclusions and significance of findings from this study, as well as key targets for future investigations, are discussed under the three headings discussed above.

Biochemical characterisation

Given the difficulties in obtaining purified natural substrates from grape or wine, it was decided to opt for synthetic substrates that have been used widely in the literature (Günata et al., 1988; Manzanares et al., 2000b; Mateo and di Stefano, 1997; Mendes Ferreira et al., 2001) as alternative substrates, particularly for early stage screening studies such as that undertaken here. A range of substrates were used in the first screening of LAB. Considering that many of them were not hydrolysed to any significant degree (e.g. α- and β-D-galactopyranoside and β-D-mannopyranoside), attention was focused on those that were wine related. They were the nitro-phenol linked β- and α-D-glucopyranosides, β-D-xylopyranoside, α-L-arabinofuranoside and α-L-rhamnopyranoside. Unfortunately, it was not possible to locate commercial source of one of the most abundant glycoside in wine, β-D-apiofuranoside, therefore the substrate had to be excluded from this project.

The use of microplate technology enabled the achievement of the largest biochemical screening for glycosidase enzymes of wine LAB to date. The number of conditions and substrates analysed provide a strong indication that wine LAB have several glycosidase enzymes. Another important finding was that most enzymatic activities were highly strain-dependent. This was particularly true for the *O. oeni* isolates in regard to β-D-glucopyranosidase (Table 3.2) for which it was found that differences in activity of about 6-fold existed between the highest (Oen2) and lowest (Oen14) activity strains. Significantly, these differences were not always maintained with other glycosidase activities. In fact, in the same experiment, strain Oen20, despite being generally a medium glucosidase producer, presented very high levels of β-D-xylopyranosidase activity, almost twice the activity of strain Oen2. Even higher β-D-xylopyranosidase activities were seen for strains Oen 4, 21 and 22 and yet all exhibited only moderate β-D-glucopyranosidase activity. These sorts of findings support the notion that there exist in *Oenococcus oeni* different and distinct glycosidase enzymes. In fact, the five glycosidase enzymes studied in this project, have a high substrate specificity, with only β-D-glucopyranosidase being able to hydrolyse, in particular conditions, β-galactosides, β-arabinosides and β-xylosides also (enzyme nomenclature site, www.expasy.org/enzyme/) The *Pediococcus* and *Lactobacillus* isolates in comparison do not show the same comprehensive range of activities. Instead greatest activities were seen against the β- and α-D-glucopyranosides (Fig. 4.1 and 4.2, respectively), thereby suggesting that perhaps at most only two types of enzymes are present.

In addition to indications that multiple enzymes exist in *O. oeni*, the pH-dependent behaviour suggests a multiplicity of enzymes with the same substrate specificity. As discussed in Chapter 3, an unusual finding was observed in some *O. oeni* isolates when their activity was tested along the pH spectrum: two distinct peaks of activity were seen for the β-D-glucopyranoside hydrolysing activity. The number of replicates and strains for which this property was seen eliminates the possibility of experimental error. Also the fact that dual pH optima were not seen for all strains argues against this finding being an artefact of the experimental system. Initially it was thought that some other glycosidases, might have β-D-glucopyranosidase activity as a secondary activity apparent only as pH approached neutrality. However, results obtained with the great number of glycosidase substrates do not support this notion as hydrolysis of

124

substrates other than the β-D-glucopyranoside was typically poor above pH 5.8 (e.g. Fig. 3.3 and 3.4). Clarification of this issue will best be achieved through study of the purified enzymes, hence efforts within this project (Chapter 6) towards cloning and ultimately heterologously expressing β-D-glucopyranosidase structural genes to produce a source of a pure single enzyme.

The fact that the highest levels of activity for all the tested glycosidases were measured at the lower spectrum of pH, very close to the values found in wine is highly significant. This clearly demonstrates that wine LAB glycosidases may represent a good source of enzymes and in fact an alternative to the commercial fungal preparations currently used in winemaking. The presence of glycosidase activity may also represent a good selection criterion for isolation of new malolactic strains for use in winemaking, with the specific aim of modifying the aroma profile of the wine in addition to conducting the MLF.

Colour modifications

It is recognised that yeasts, in particular situations, can modify wine colour, though the mechanisms are not well defined (Bartowsky et al., 2004). Proposed routes include the prevention of precipitation of tannins and anthocyanins by releasing a greater amount of cell wall derived polysaccharides into the must (Escot et al., 2001), or by the adsorption to or absorption into intact cell walls (Caridi et al., 2004; Escot et al., 2001; Morata et al., 2003). Studies in both model solutions and must based fermentations have shown that the presence of yeast metabolites pyruvate and acetaldehyde increases the rate of formation of larger, more stable molecules from anthocyanins and other phenolics (Drinkine et al., 2005; Fulcrand et al., 1998; Romero and J., 2000). This in turn can slow the browning of aging wines.

By comparison to the actions of yeast, very little is known of the action of LAB on the same compounds, apart from their indirect effect resulting from the increase of pH, which drives anthocyanins away from their red coloured, flavylium ion form (Ribereau-Gayon et al., 2004b). This fact might at times not be enough to explain all the modifications of wine colour seen during and after malolactic fermentation. It is

possible to hypothesize that LAB may in fact hydrolyse these compounds, producing unstable and colourless molecules that inevitably reduce the colour intensity of wine. However, despite this initial loss of colour intensity, the compounds that contribute to colour actually increase in stability. This has been observed in several studies (Amati et al., 1998; Rauhut et al., 1995; Wightman et al., 1997), where the loss in anthocyanins was counterbalanced by an increased polymerisation rate, thereby leading to a more complex and stable pigments, which impart a brighter colour to wines for a longer time.

There is very little data showing the hydrolysis of anthocyanins in wine. Other fields have expended more research effort on this topic than has been seen in oenology. In fact, the importance of understanding how to reduce (and often completely remove), or else increase, the colour intensity plays a major role in the fruit juice industry. Wrolstad and his team have thoroughly investigated the action of several pectolytic enzymes of commercial preparations on the colour of berry fruit juices (Wightman and Wrolstad, 1995; Wightman and Wrolstad, 1996; Wrolstad et al., 1994). These preparations contained high levels of β-D-glucopyranosidase as a secondary enzymatic activity. It was found that they had strong activity towards the monomeric anthocyanins of the juices, resulting in their hydrolysis and subsequent destruction. This fact may explain the loss of colour intensity when pectolytic enzyme preparations are used during long macerations in grape and raspberry wines (Montedoro and Bertuccioli, 1976; Withy et al., 1993), despite a better phenolic extraction from the fruit skin. These anthocyanin transformations produce several modifications to wine colour; a reduction in intensity but with gains in stability due to the higher polymerisation rates, which tend to preserve the coloured forms of phenols in wine ((Rauhut et al., 1995; Wightman et al., 1997); Rocco Di Stefano, personal communication). Enzymatic reactions, again using pectolytic enzyme preparations, were performed on malvidin-glucosides to understand what product(s) this reaction might yield (Ducruet, 1998; Piffaut et al., 1994). The studies highlighted the subsequent destruction of the malvidin-glucoside once it has been hydrolysed, however, the process was still to be demonstrated to occur in wine.

In the present project the ability of LAB to affect wine colour, and anthocyanins particularly, was investigated. The initial work investigated what effect malvidin-3-glucoside might have on the β-D-glucopyranosidase activity. After 6 h of contact, an

increase of up to 0.4 and 0.55 units of β-D-glucopyranosidase activity was observed for *Oenococcus* and *Lactobacillus* isolates, respectively (Fig. 5.3 and 5.4, respectively). A much lower effect was seen in *Pediococcus* strains. These results follow the same pattern seen for other glucosides known to induce glucosidase activity, such as the two used in this project, arbutin and salicin. This finding therefore strongly suggests that anthocyanins might be recognised as substrate for this group of enzymes.

Subsequent work was directed at determining the fate of anthocyanins in a wine-like medium containing growing LAB. During these tests a dramatic reduction in colour, quite obvious by eye, was evident in all samples containing bacterial biomass. Unfortunately, the expected product of the eventual hydrolysis of malvidin-3-glucoside, malvidin-aglycone, while detectable by the HPLC analysis used was not seen to appear in the samples. The biomass did however develop a strong reddish coloration (data not shown), suggesting that part of the loss might have been due to an absorbance to the bacterial cell wall. Nevertheless, some products were appearing on the HPLC chromatograms after few hours of incubation and with the decline of malvidin-3-glucoside. As it was not possible to identify these compounds, it was postulated that they might be some degradation products of the anthocyanin, but which has been further degraded or modified from the expected end products of anthocyanin hydrolysis. This may be in accordance with what has been found with the aforementioned studies of Piffaut (1994) and Ducruet (1998). The high instability of malvidin-glucoside necessitated the use of alternative anthocyanins or wine phenolic-glucosides as substrates, such as quercetin-rutinoside, for similar studies of glucoside utilisation (Aura et al., 2005).

Genetic characterisation

With the recent publication of genome sequences of both *Oenococcus oeni* and *Pediococcus pentosaceus*, it was possible to localise several putative open reading frames (ORFs) that have high homology to β-D-glucopyranosidases of the related LAB, *Lactobacillus plantarum*. The organisation of these ORFs followed the general scheme for the carbohydrate catabolism phospho-transferase system (PTS), which is very common through the bacterial world. The three putative glucosidases were grouped into

127

two distinct operons, comprising 4 and 6 ORFs, respectively. Both operons had ORFs with a high resemblance to domain II of the cellobiose family, which includes a cellobiose hydrolysing enzyme (Postma et al., 1993). In addition, the second and larger operon included a transcriptional regulator gene, which might have a role in regulation the glucosidase genes. Given the size of both operons, cloning attempts were limited to the enzyme ORF and immediate flanking regions. To date, regions from the smaller operon were successfully amplified and sequenced to reveal few amino acid substitutions when compared to the predicted sequence. Further characterisation is now underway to determine the implications of these modifications as well as the function of the gene product.

Future Research Directions

This project has established that Lactic Acid Bacteria (LAB) possess a wide range of glycosidase enzymes, especially those that may enhance wine quality. Their ability to function in a harsh environment similar to that of wine is of great interest and warrants continuation of the characterization of these enzymes. Activities in *Oenococcus oeni*, rather than in lactobacilli or pediococci, are of particular interest because of their magnitude, the multiplicity or apparent dual pH optima of the enzymes as well as the importance of this organism to winemaking. Thus it would be highly desirable to complete the isolation and purification of these enzymes, particularly the β-D-glucopyranosidases. Further work to characterize these enzymes might include:

a) extraction, purification and biochemical characterization of the enzyme(s) of interest directly from *Oenococcus oeni*;

b) cloning and expression of the putative structural genes into a host organism, such as *Escherichia coli*, to allow its production in isolation from other glycosidases as is otherwise the case in the native organism;

c) application of over-expression vectors to allow increased yields of the protein in the *E. coli* host, thereby facilitating purification.

Given the difficulties associated with the disruption of *Oenococcus* cells (Stanier et al., 1986) and the potentially large number of interfering enzymes, heterologous

production and purification of the gene might prove most successful. Protocols for this have been already published and *E. coli* strains that have their native glucosidase genes deleted are available as hosts (Marasco et al., 1998; Marasco et al., 2000). An added benefit of over-production of the enzyme is that it may allow for more precise characterization of the enzymes under oenological conditions without necessarily requiring growth of the source organism in the wine.

The effect of LAB glycosidases on wine colour compounds has also been investigated. The complexity of anthocyanin chemistry has made this analysis difficult. However, colour intensity was clearly found to decrease markedly after incubation of anthocyanins with LAB cells. Therefore further characterization of the basis for this phenomenon is of great interest. It may be useful to use alternate substrates or analytical methodologies for such a study, since the wine anthocyanins are highly reactive, unstable or metabolised by the bacteria, thereby making their quantitative study difficult. Once again application of purified enzymes will allow separation of the de-glycosylation capability from the bacterial biomass which may be binding or metabolizing the aglycones.

Other important objectives remain. Having proven that LAB have the ability to hydrolyse synthetic glycosides, it would be crucial to define this ability on wine glycosides and/or in actual wines. The defined conditions used here, while highly beneficial in the execution of the initial screening and characterization of activities in many LAB, they nonetheless represent an environment of greatly simplified chemical composition. Also, this project has concentrated on the sugar fraction of glycosides, therefore the obvious next step is to determine the abilities and specificities of these enzymes for the aglycone component of the potential substrates.

The data produced from this project will help to better select LAB strains for use in winemaking. The microplate procedure developed here is quick and simple to use, with the additional advantage of saving a considerable amount of reagents when compared to similar methods. The range of abilities for hydrolysis of wine glycosides could make LAB strains a powerful tool in the hands of the informed winemaker, in that it could be possible to enhance specific subsets of aromas, that are linked to specific sugars, instead of others. Even without this degree of sophistication, it is possible to say

that LAB have the potential to contribute to the complexity of wine, enhancing its varietal characteristics, no doubt with fewer harmful side effects than chemical or thermal treatments. The action of these enzymes on wine colour compounds may even be considered an improvement for some wine styles.

APPENDIX 1. Influence of pH on the α-L-rhamnopyranosidase activity (expressed as units of activity) of *Oenococcus oeni**.

LAB strain	pH											
	2.6	3.0	3.4	3.8	4.2	4.6	5.0	5.4	5.8	6.2	6.6	7.0
Oen12	0.04	0.07	0.06	0.06	0.05	0.05	0.02	0.03	0.02	0.02	0.03	0.03
Oen13	0.04	0.11	0.11	0.11	0.08	0.08	0.04	0.03	0.03	0.04	0.05	0.03
Oen14	0.06	0.12	0.12	0.10	0.07	0.06	0.03	0.04	0.03	0.03	0.03	0.03
Oen17	0.09	0.13	0.11	0.10	0.07	0.07	0.06	0.05	0.05	0.07	0.05	0.05
Oen20	0.07	0.09	0.14	0.13	0.06	0.05	0.03	0.05	0.05	0.06	0.04	0.06
Oen21	0.18	0.15	0.12	0.14	0.11	0.08	0.04	0.07	0.06	0.06	0.06	0.04
Oen22	0.17	0.13	0.16	0.15	0.14	0.08	0.05	0.05	0.04	0.07	0.06	0.06
Oen1	0.05	0.13	0.10	0.12	0.09	0.05	0.07	0.03	0.04	0.05	0.03	0.03
Oen4	0.06	0.10	0.13	0.12	0.12	0.13	0.12	0.07	0.07	0.06	0.04	0.04
Oen28	0.02	0.02	0.04	0.04	0.03	0.03	0.04	0.02	0.04	0.02	0.03	0.02
Oen3	0.03	0.11	0.11	0.11	0.10	0.05	0.02	0.02	0.01	0.01	0.01	0.01
Oen5	0.05	0.08	0.12	0.08	0.08	0.05	0.05	0.02	0.01	0.01	0.01	0.02
Oen7	0.19	0.19	0.17	0.11	0.08	0.04	0.03	0.01	0.02	0.03	0.02	0.03

* these data represent the enzymatic activity of the *O. oeni* not shown in Figure 3.4,. Chapter 3.

APPENDIX 2a.   Influence of pH on the α-L-arabinofuranosidase activity (expressed as units of activity) of *Oenococcus oeni**.

LAB strain	pH											
	2.6	**3.0**	**3.4**	**3.8**	**4.2**	**4.6**	**5.0**	**5.4**	**5.8**	**6.2**	**6.6**	**7.0**
Oen2	0,07	0,12	0,14	0,16	0,12	0,07	0,04	0,02	0,07	0,00	0,03	0,10
Oen4	0,09	0,15	0,17	0,18	0,17	0,15	0,09	0,05	0,16	0,01	0,13	0,30
Oen6A	0,00	0,11	0,14	0,12	0,06	0,04	0,03	0,01	0,01	0,05	0,06	0,12
Oen8	0,02	0,09	0,10	0,09	0,05	0,05	0,02	0,10	0,05	0,12	0,26	0,31
Oen9	0,03	0,15	0,20	0,14	0,12	0,11	0,11	0,06	0,08	0,16	0,06	0,06
Oen10	0,05	0,11	0,16	0,10	0,09	0,08	0,14	0,08	0,10	0,19	0,09	0,09
Oen11	0,04	0,13	0,14	0,15	0,14	0,10	0,11	0,07	0,12	0,13	0,13	0,12
Oen12	0,05	0,16	0,17	0,13	0,14	0,06	0,08	0,04	0,06	0,05	0,13	0,07
Oen13	0,06	0,18	0,21	0,21	0,14	0,05	0,05	0,02	0,05	0,05	0,15	0,14

* these data represent the enzymatic activity of the *O. oeni* isolates not shown in Figure 3.4, Chapter 3.

APPENDIX 2b. Influence of pH on the α-L-arabinofuranosidase activity (expressed as units of activity) of *Oenococcus oeni**.

LAB strain	pH											
	2.6	3.0	3.4	3.8	4.2	4.6	5.0	5.4	5.8	6.2	6.6	7.0
Oen14	0,07	0,18	0,19	0,15	0,12	0,06	0,06	0,04	0,10	0,06	0,13	0,16
Oen16	0,02	0,08	0,10	0,11	0,10	0,05	0,01	0,04	0,00	0,02	0,00	0,08
Oen17	0,02	0,32	0,30	0,32	0,22	0,14	0,01	0,01	0,08	0,00	0,00	0,19
Oen21	0,05	0,15	0,17	0,18	0,18	0,11	0,11	0,05	0,03	0,07	0,24	0,29
Oen22	0,05	0,16	0,16	0,18	0,18	0,11	0,10	0,03	0,04	0,07	0,25	0,30
Oen23	0,00	0,10	0,15	0,16	0,11	0,03	0,00	0,00	0,00	0,00	0,07	0,00
Oen28	0,00	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Oen29	0,00	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,01	0,11	0,01	0,12

* these data represent the enzymatic activity of the *O. oeni* isolates not shown in Figure 3.4, Chapter 3.

Appendix 3. Influence of pH on the β-D-xylopyranosidase activity (expressed as units of activity) of *Pediococcus spp.*

LAB strain	pH											
	2.6	3.0	3.4	3.8	4.2	4.6	5.0	5.4	5.8	6.2	6.6	7.0
Ped3	0.01	0.01	0.00	0.00	0.00	0.05	0.01	0.02	0.02	0.01	0.03	0.02
Ped16	0.00	0.01	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.01	0.00
Ped17	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.01	0.00
Ped24	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ped10	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.03
Ped11	0.03	0.03	0.02	0.02	0.01	0.00	0.02	0.01	0.02	0.01	0.02	0.03
Ped15	0.03	0.02	0.01	0.01	0.01	0.02	0.00	0.01	0.01	0.01	0.01	0.02
Ped27	0.03	0.02	0.01	0.01	0.02	0.01	0.02	0.02	0.01	0.01	0.02	0.02
Ped18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 4. Influence of pH on the β-D-xylopyranosidase activity (expressed as units of activity) of *Lactobacillus spp.*

LAB strain	pH											
	2,6	3,0	3,4	3,8	4,2	4,6	5,0	5,4	5,8	6,2	6,6	7,0
Lac6A	0,03	0,01	0,02	0,03	0,01	0,01	0,01	0,01	0,02	0,02	0,03	0,01
Lac12	0,03	0,01	0,01	0,00	0,01	0,01	0,00	0,00	0,02	0,01	0,02	0,00
Lac13	0,05	0,02	0,02	0,02	0,01	0,01	0,00	0,01	0,02	0,01	0,02	0,01
Lac25	0,04	0,03	0,04	0,03	0,02	0,01	0,02	0,01	0,02	0,03	0,03	0,02
Lac26	0,00	0,02	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01
Lac15	0,01	0,02	0,02	0,03	0,03	0,03	0,02	0,03	0,01	0,04	0,03	0,03
Lac16	0,02	0,01	0,01	0,01	0,02	0,01	0,01	0,02	0,01	0,03	0,02	0,02
Lac19	0,03	0,01	0,00	0,01	0,02	0,01	0,01	0,02	0,02	0,03	0,02	0,02
Lac24	0,08	0,06	0,05	0,06	0,06	0,06	0,06	0,05	0,02	0,03	0,03	0,03

Appendix 5. Influence of pH on α-L-rhamnopyranosidase activity (expressed as units of activity) of *Pediococcus spp.*

LAB strain	pH											
	2,6	3,0	3,4	3,8	4,2	4,6	5,0	5,4	5,8	6,2	6,6	7,0
Ped3	0,03	0,00	0,01	0,03	0,03	0,01	0,03	0,01	0,04	0,04	0,03	0,04
Ped16	0,03	0,00	0,01	0,02	0,01	0,00	0,02	0,00	0,04	0,01	0,01	0,02
Ped17	0,01	0,00	0,01	0,03	0,00	0,01	0,02	0,00	0,03	0,01	0,01	0,02
Ped24	0,01	0,00	0,00	0,02	0,00	0,10	0,02	0,00	0,03	0,00	0,09	0,02
Ped10	0,07	0,03	0,03	0,04	0,07	0,03	0,03	0,03	0,04	0,03	0,03	0,03
Ped11	0,03	0,02	0,02	0,03	0,03	0,02	0,02	0,03	0,03	0,02	0,02	0,03
Ped15	0,03	0,03	0,02	0,03	0,03	0,02	0,03	0,02	0,03	0,03	0,03	0,04
Ped27	0,05	0,03	0,05	0,04	0,03	0,03	0,02	0,03	0,04	0,03	0,04	0,03
Ped18	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,01

Appendix 6. Influence of pH on the α-L-rhamopyranosidase activity (expressed as units of activity) of *Lactobacillus spp.*

LAB strain	pH											
	2,6	3,0	3,4	3,8	4,2	4,6	5,0	5,4	5,8	6,2	6,6	7,0
Lac6A	0,01	0,02	0,02	0,01	0,03	0,02	0,02	0,02	0,03	0,03	0,02	0,04
Lac12	0,02	0,01	0,01	0,01	0,02	0,01	0,02	0,02	0,02	0,02	0,05	0,02
Lac13	0,02	0,01	0,01	0,02	0,01	0,02	0,01	0,01	0,02	0,02	0,02	0,02
Lac25	0,03	0,03	0,01	0,02	0,03	0,01	0,03	0,02	0,04	0,02	0,03	0,03
Lac26	0,01	0,01	0,01	0,00	0,03	0,01	0,02	0,03	0,02	0,01	0,01	0,02
Lac15	0,03	0,04	0,02	0,01	0,01	0,03	0,03	0,02	0,04	0,02	0,03	0,03
Lac16	0,02	0,03	0,02	0,01	0,00	0,02	0,01	0,00	0,01	0,00	0,02	0,07
Lac19	0,04	0,06	0,02	0,01	0,02	0,02	0,03	0,02	0,02	0,01	0,06	0,04
Lac24	0,03	0,07	0,02	0,03	0,02	0,02	0,04	0,02	0,01	0,02	0,05	0,10

Appendix 7. Influence of pH on the α-L-arabinofuranosidase activity (expressed as units of activity) of *Pediococcus spp.*

LAB strain	pH											
	2,6	3,0	3,4	3,8	4,2	4,6	5,0	5,4	5,8	6,2	6,6	7,0
Ped10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Ped3	0,03	0,00	0,03	0,01	0,00	0,00	0,00	0,00	0,06	0,01	0,01	0,01
Ped11	0,01	0,04	0,01	0,00	0,00	0,00	0,00	0,00	0,04	0,02	0,02	0,02
Ped16	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,07	0,00	0,01
Ped17	0,01	0,00	0,01	0,00	0,01	0,00	0,00	0,00	0,02	0,02	0,01	0,01
Ped18	0,01	0,06	0,00	0,00	0,00	0,01	0,00	0,00	0,01	0,03	0,00	0,00
Ped24	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,03	0,07	0,02	0,00

Appendix 8. Influence of pH on α-L-arabinofuranosidase activity (expressed as units of activity) of *Lactobacillus spp.*

LAB strain	pH											
	2,6	3,0	3,4	3,8	4,2	4,6	5,0	5,4	5,8	6,2	6,6	7,0
Lac19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Lac6A	0,00	0,01	0,00	0,02	0,05	0,01	0,01	0,00	0,02	0,01	0,01	0,02
Lac12	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Lac13	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,02
Lac15	0,00	0,00	0,00	0,03	0,00	0,00	0,01	0,00	0,00	0,01	0,01	0,00
Lac16	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,00	0,01	0,01
Lac24	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,02	0,01	0,01	0,00

Appendix 9. Homology between *Oenococcus oeni* transcriptional regulator *Oen610* with similar bacterial genes*.

Species	Gene regulated	Identity[a] (%)	Positive[b] (%)
Lactobacillus plantarum	PTS EIIC cellobiose family	53	74
Yersinia frederiksenii	PTS EIIC glucose/ maltose/N-acetylglucosamine family	46	66
Yersinia mollarti	Phospho-β-glucosidase	46	66
Pediococcus pentosaceus	Phospho-β-glucosidase/β-galactosidase	45	65
Clostridium beijerincki	PTS EIIB cellobiose family	43	63
Enterococcus faecalis	Glycosyl hydrolase	40	59
Lactococcus lactis	PTS EIIB cellobiose family	34	58
Listeria monocytogenes	PTS EIIA cellobiose family	33	59

* performed with Blast program at http://www.ncbi.nlm.nih.gov/BLAST.

[a] based on same amino acid encoded.

[b] same amino acids and conservative amino acid substitutions.

Appendix 10. Classes of terpenols found in wine.

3,7-Dimethyl-2,7-octadien-1,6-diol (E)

3,7-Dimethyl-2,5-octadien-1,7-diol (E,E)

3,7-Dimethyl-1-octen-3,6,7 triol

3,7-Dimethyl-1,7-octadien-3,6-diol (diendiol 2)

3,7-Dimethyl-1-octen-6-one-7-triol

3,7-Dimethyl-2,7-octadien-1,7-diol (E)

8-Hydroxy geraniol

8-Hydroxy linalol

3,7-Dimethyl-1,5-octadien-3,7-diol (diendiol 1)

3,7-Dimethyl-1-octen-3,7-diol

3,7-Dimethyl-1,6-octadien-3,5-diol

Geranic acid

Geraniol

Linalol

Linalol oxides

Pyranic form

Furanic form

Main monoterpenols

Nerol

Ho-trienol

3,7-Dimethyl-2-octen-1,7-diol (Z)

3,7-Dimethyl-2,7-octadien-1,6 diol (Z)

Nerol oxide

Rose oxide

Citronellol

3,7-Dimethyl-1,7-octadienol

3,7-Dimethyl-7-octen-1,6 diol

Alpha-terpineol

Terpinen-4-ol

p-Menthane-1,8-diol

p-Menth-2-ene-1,8-diol

p-Menth-1-ene-6,8-diol (menthenediol-1)

p-Menth-1-ene-7,8-diol (menthenediol-2)

141

Appendix 11. Publications arising from this study.

Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P.R. and Jiranek, V. (2004) Lactic acid bacteria as a source of enzymes for use in vinification. *Applied and Environmental Microbiology.* **70**, 5715-5731.

Grimaldi A., Bartowsky, E. and Jiranek, V. (2005) A survey of glycosidase activities of commercial strains of *Oenococcus oeni. International Journal of Food Microbiology.* **105**, 233-344.

Grimaldi, A., Bartowsky, E. and Jiranek, V. (2005) Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. *Journal of Applied Microbiology.* **99**, 1061-1069.

MINIREVIEW

Lactic Acid Bacteria as a Potential Source of Enzymes for Use in Vinification

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Two key groups of organisms are involved in the production of red, white, and sparkling wine. The yeasts, typically strains of *Saccharomyces cerevisiae*, carry out the primary or alcoholic fermentation, in which sugars are converted to ethanol and CO_2. Lactic acid bacteria (LAB), especially *Oenococcus oeni* (formerly *Leuconostoc oenos* [51]), conduct the secondary or malolactic fermentation (MLF) of wine by decarboxylating L-malic acid to L-lactic acid and CO_2 (292). Apart from these two crucial reactions in grape vinification, a myriad of other changes occur to complete the transformation of grape juice to wine. Compounds that stimulate our visual, olfactory, gustatory, and tactile senses are either released from the various ingredients or are synthesized, degraded, or modified during vinification. Many of these processes involve the action of enzymes. Such enzymes can be free or cell associated and originate from sources that include enzyme addition, the grapes themselves, the grape microflora (fungi, yeast, or bacteria), the inoculated microbes, or microbes associated with winery equipment and storage vessels to which the wine is exposed during production.

Current viticultural practices and vinification processes are essentially protocols for favoring the activities of certain enzymes while discouraging the activities of others. Thus, winemakers can broadly achieve desirable outcomes during fermentation by using a selected wine yeast strain characterized by desirable physiological and hence enzymatic properties (149, 225). Conversely, adverse reactions, such as the browning associated with polyphenoloxidases, can be minimized by excluding oxygen from the grape juice or through addition of sulfur dioxide (SO_2) to inhibit enzyme activity (25).

A more recent strategy in the history of winemaking is the addition to juice or wine of a microbial culture or enzyme preparation that confers a specific or select group of enzymatic activities. These activities can either amplify the effect of indigenous enzymes or be novel (40, 285). Initially, such additives addressed issues of juice-processing efficiency and wine recovery. Thus, the gelling seen in many fruit juices as a result of pectins has for many decades been reduced or eliminated with pectinase enzymes, most often derived from *Aspergillus* fungi (283), which increase juice extraction or minimize filter blockage. Enzyme-based solutions that provide a broader range of benefits, such as flavor enhancement or manipulation of color, have now become available.

In the development of new enzyme treatments, efforts have often been centered on desirable activities identified in the microorganisms used or encountered during vinification, especially the yeast (35, 83, 267, 274). In part, this approach has been taken because of legal restrictions on the nature of additives that can be added to wine. It is unlikely that the use in winemaking of wine yeast with a novel enzymatic capability would require regulatory approval, whereas the addition of an enzyme extract or purified enzyme preparation may require such approval. Despite the appeal of this approach, extensive efforts have yielded only a small number of technologically important enzymes, and even fewer of these enzymes perform satisfactorily under winemaking conditions, which include a high sugar (glucose and fructose) content, a low pH (pH 3.0 to 4.0), low temperatures (<15°C), and the presence of ethanol (up to 15% [vol/vol] or more) or SO_2.

Interestingly, the LAB that grow and thrive in grape juice or wine under conditions that interfere with the production and activity of desirable enzymes in yeast or fungi have been poorly studied as a source of enzymes with potential usefulness in vinification. Young wine can be a nutritionally deficient environment that could be expected to lead to the elaboration by LAB of numerous enzymatic activities for nutrient scavenging. Emerging findings detailed throughout this review are confirming this notion. In this review we examine the potential of LAB as a source of enzymes that could improve wine quality and complexity. Also discussed are the malolactic enzyme, proteases and peptidases, glycosidases, polysaccharide-degrading enzymes, esterases, ureases, phenoloxidases, and lipases. We emphasize findings from investigations in which wine LAB were used or which were performed under wine-like conditions. Where there are no oenological data, we refer to studies of LAB from other processes, such dairy processes, but the analysis is limited to those species also found in wine (based on a consensus derived from references 25, 62, 95, and 231). The activities of greatest interest are those conferred by a single enzyme, ideally one with an extracellular localization. Such

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TABLE 1. Proteolytic and peptidolytic activities of LAB[a]

Organism	Enzyme location	Proteinase and endopeptidase	Other peptidase	Carboxy-peptidase	Aminopeptidase	Dipeptidase	Dipeptidyl peptidase	Tripeptidase	Proline-specific peptidase
Lactobacillus brevis	CFE	Casein (74, 107, 128)[b]	Succinyl-Phe-p-NA and Gln-Phe-p-NA (74)	CBZ derivatives (128)	p-NA derivatives (74, 107, 128, 248, 294) AMC derivatives (295)	Dipeptides (74, 107, 128)	p-NA derivatives (294, 295) AMC derivatives (295)	Tripeptides (107)	p-NA derivatives (107, 248, 294) Pro-AMC (295)
		Gluten (107)							
		BZ-amino-p-NA (294) BZ-peptide-p-NA (294) CBZ-amino-p-NA (294) α-Lactalbumin whey protein (74) β-Lactoglobulin whey protein (74) ABZ-peptide-p-NA (294)			NAm derivatives (74) Dipeptide (74) Casein (294)				
	CD	Casein (107) Gluten (107)			p-NA derivatives (107)	Dipeptides (107)		Tripeptides (107)	
	WC	Casein (52, 107)	Digest of albumin and globulin polypeptides (50)		p-NA derivatives (107)	Dipeptides (107)		Tripeptides (107)	p-NA (107)
		Gluten (107) Gelatine (52) Milk protein (128)			ArAm derivatives (128)				
Lactobacillus buchneri	CFE	Casein (74)			p-NA derivatives (74, 248, 294) AMC derivatives (295)	Dipeptides (74, 294)	p-NA derivatives (294) AMC derivatives (295)	Tripeptides (294)	Dipeptides (294) Pro-AMC (295)
		Resorutin-labeled casein (248)							
		MeOsuc-Arg-Pro-Tyr-p-NA (248) α-Lactalbumin whey protein (74)			NAm derivatives (74) Dipeptides (74)				p-NA (248) N-terminal pentapeptide of bradykinin (294)
		β-Lactoglobulin whey protein (74)							
Lactobacillus casei	CFE	Casein (12, 26, 67, 123, 128, 272)	Water-soluble cheddar cheese peptides (216)	CBZ derivatives (2, 67, 69, 128)	p-NA derivatives (2, 4, 46, 67, 69, 73, 84, 85, 87, 94, 123, 128, 216, 228, 243, 248, 272, 294)	Dipeptides (2, 27, 67, 69, 87, 88, 94, 128, 228, 248, 294)	p-NA derivatives (46, 294, 295)	Tripeptide (2, 27, 248, 294)	p-NA derivatives (85, 123, 228, 248, 272, 294)
		N,N-Dimethyl casein (94)	Succinyl-Phe-p-NA and Gln-Phe-p-NA (aryl-peptidyl amidase) (68)		AMC derivatives (243, 247, 295)		AMC derivatives (295)		Dipeptides (84, 89, 248, 294)
		Resorutin-labeled casein (248)			Unspecified aminopeptidase substrates (216) NAm derivatives (2, 5, 248)		NAm derivatives (248)		Tripeptides (84)
		Milk protein (122)							AMC derivatives (117, 216, 295)
		ABZ-peptide-p-NA (294)			Dipeptides (4, 69, 85, 87)				N-terminal pentapeptide of bradykinin (294, 295)
		BZ-peptide-p-NA (294) CBZ-peptide-p-NA (67, 272, 294)			Tripeptides (69, 85, 87) Goat's milk curd peptides (217)				NAm derivatives (117, 248)
		MeOsuc-tripeptide-p-NA (248) Peptide-p-NA derivatives (123) N-Gln-Phe-2-NAm (5, 216) Methionine enkephalin (272) Casein (70, 86, 146, 198, 272)		CBZ-tripeptide-p-NA (272)	p-NA derivatives (272)				p-NA derivatives (272)
	CD	Hemoglobin (198)			Goat's milk curd peptides (217) NAm derivatives (5)				
	WC	CBZ-tripeptide-p-NA (272) MeOsuc-tripeptide-p-NA (85) Casein (122, 129, 268)	Cheddar cheese slurry peptides (197)		p-NA derivatives (227)				
	WC	Casein-FITC (243) Milk protein (8, 26, 58, 128, 227) N-benzoyl-DL-Phe-2-NAm (227, 268)			ArAm derivatives (128) NAm derivatives (227, 268) Goat's milk curd peptides (217) p-NA derivatives (227)				
	CD + CFE								
Lactobacillus curvatus	CFE	ABZ-peptide-p-NA (294)			p-NA derivatives (78, 168, 170, 221, 242, 294) Dipeptides (168, 170)	Dipeptides (169, 294)	p-NA derivatives (221, 294) AMC derivatives (295)	Tripeptides (294)	Dipeptides (294) Pro-AMC (295)
		BZ-amide-p-NA (294)							

Species	Type	Substrate	Substrate						
		BZ-peptide-p-NA (294); CBZ-amide-p-NA (294)			Tripeptides (168); Tetrapeptides (168); Pentapeptides (168); AMC derivatives (78, 242); ArAm derivatives (213, 270)				N-terminal pentapeptide of bradykinin (294, 295); X-Pro-p-NA derivatives (171); X-Pro-X tripeptides (171)
	WC	Casein-FITC (78, 242)	Cheddar cheese slurry peptides (197)		AMC derivatives (242, 295)				
	CS	Milk protein (221); Casein (213); Azocasein (220); Azoalbumin (220)							
Lactobacillus fermentum	CFE	Casein (74, 107); Resorutin-labeled casein (248); Gluten (107); β-Lactoglobulin whey protein (74); BZ-tripeptide-pNA (248)	Succinyl-Phe-p-NA and Gln-Phe-p-NA (74)		p-NA derivatives (74, 107, 248, 294); AMC derivatives (295); NAm derivatives (74); Dipeptides (74)	Dipeptides (74, 107, 294)	p-NA derivatives (294); AMC derivatives (295)	Tripeptides (107, 294)	p-NA (107, 248); Dipeptides (294); Pro-AMC (295)
	CD / WC	Casein (107); Gluten (107)	Digest of albumin and globulin polypeptides (50)		ArAm derivatives (204, 270)	Dipeptides (107); Dipeptides (107)		Tripeptides (107); Tripeptides (107)	p-NA derivatives (107)
Lactobacillus fructivorans	CFE	Casein (107); Gluten (107)		CBZ-Leu (107)	p-NA derivatives (107)	Dipeptides (107)		Tripeptides (107)	Pro-p-NA (107)
	CD / WC	Casein (107); Gluten (107)	Digest of albumin and globulin polypeptides (50)	CBZ-Leu (107)	p-NA derivatives (107)	Dipeptides (107)		Tripeptides (107)	
Lactobacillus hilgardii	WC	Casein (52); Gelatin (52)	Digest of albumin and globulin polypeptides (50)						
Lactobacillus homohiochii	CS	Azocasein (220); Azoalbumin (220)							
Lactobacillus paracasei	CFE	Casein (272); Resorutin-labeled casein (248); Ovine casein (22); Benzoyl-amino-p-NA (22); ABZ-peptide-pNA (294); BZ-peptide-p-NA (248, 294); CBZ-peptide-p-NA (272, 294); MeOsuc-tripeptide-p-NA (248); Acetyl-amino-p-NA (22); N-Succinyl-amino-p-NA (22, 294); Methionine eukephalin (272)	8 to 13-residue oligopeptides (271)	N-CBZ-linked dipeptides (22, 108); Hippuryl-Arg (22)	p-NA derivatives (22, 108, 221, 248, 269, 272); AMC derivatives (295)	Dipeptides (22, 108, 294); p-NA derivatives (294)	p-NA derivatives (22, 108, 221, 294); AMC derivatives (295)	Tripeptides (108); p-NA derivatives (294)	X-Pro-p-NA (269, 272); Dipeptides (294); Pro-AMC (295); N-terminal pentapeptide of bradykinin (294, 295); p-NA derivatives (248)
	CD	Casein (272); Methionine eukephalin (272)			p-NA derivatives (272)				X-Pro-p-NA (272)
	WC	Casein (221, 257); Milk protein (8, 58, 64, 221); Caprine and ovine curdled milk protein (93)	Cheddar cheese slurry peptides (197); Caprine and ovine curdled milk peptides (93)		ArAm derivatives (270); Caprine and ovine curdled milk substrates (93)				
Lactobacillus pentosus	CFE / WC	Milk protein (58)			AMC derivatives (295)		AMC derivatives (295)		
Lactobacillus plantarum	CFE	Casein (107, 123, 128, 167, 303)	β-Casein hydrolysate (143)	CBZ derivatives (2, 107, 108, 128)	p-NA derivatives (46, 77, 107, 108, 123, 128, 221, 228, 248, 294, 303)	Dipeptides (107, 108, 128, 228)	p-NA derivatives (46, 108, 221, 294, 295)	Tripeptides (107, 108, 294)	p-NA derivatives (107, 123, 228, 248, 294)

TABLE 1—*Continued*

Organism	Enzyme location	Proteinase and endopeptidase	Other peptidase	Carboxy-peptidase	Aminopeptidase	Dipeptidase	Dipeptidyl peptidase	Tripeptidase	Proline-specific peptidase
		Resorutin-labeled casein (248)		Dipeptides (2)	AMC derivatives (77, 295)		AMC derivatives (295)		Dipeptides (294)
		Gluten (107) ABZ-peptide-*p*-NA (294)		Tripeptides (2)					Pro-AMC (295) N-terminal pentapeptide of bradykinin (294, 295)
	CD	BZ-peptide-*p*-NA (294) Peptide-*p*-NA derivatives (123) Casein (70, 107) Gluten (107)			*p*-NA derivatives (107) Goat's milk curd peptides (217)	Dipeptides (107)		Tripeptides (107)	
	WC	Casein (93, 107, 129, 213, 221, 268)	Digest of albumin and globulin polypeptides (50) Caprine and ovine curdled milk peptides (93)	CBZ derivatives (107)	*p*-NA derivatives (107, 227)	Dipeptides (107)		Tripeptides (107)	*p*-NA derivatives (107)
		Casein-FITC (77)			ArAm derivatives (128, 184, 213, 270)				
	WC	Milk protein (8, 64, 122, 128, 221, 227, 298, 303) Caprine and ovine curdled milk (93) Gluten (107, 219) *N*-benzoyl-DL-Phe-2-NAm (268)			NAm derivatives (227, 268) Caprine and ovine curdled milk substrates (93)				
	CD + CFE				*p*-NA derivatives (227)				
Lactobacillus sakei	CFE				*p*-NA derivatives (78, 242, 245, 247) AMC derivatives (78, 242, 245, 247) Dipeptides (245, 247) Oligopeptides (247)	Dipeptides (193)		Tripeptides (244)	X-Pro-*p*-NA derivatives (246) X-Pro-AMC derivatives (246) Tripeptides (246) Pentapeptides (246)
	WC	Casein (213) Casein-FITC (78, 242) Gluten (219)			ArAm derivatives (213)				
Pediococcus acidilactici	CFE	Casein (20)			*p*-NA derivatives (47) NAm derivatives (20)	Dipeptides (20)	*p*-NA derivatives (47) NAm derivative (20)		
Pediococcus pentosaceus	CFE	Casein (20, 281) BZ-Arg-*p*-NA (281) *N*-Acetyl-Ala-*p*-NA (281) *N*-Succinyl-Phe-*p*-NA (281)	β-Casein hydrolysate (143)	CBZ-linked dipeptides (281) Hippuryl-Arg (281)	*p*-NA derivatives (281) NAm derivatives (20)	Dipeptides (20, 281)	*p*-NA derivatives (281) NAm derivative (20)	Tripeptides (254)	
	WC				ArAm derivatives (189, 204)				
Leuconostoc mesenteroides	CFE	¹⁴C-methylated casein (74)		CBZ-Leu (128)	*p*-NA derivatives (66, 76, 128, 221, 252) NAm derivatives (66, 252)	Dipeptides (66, 76, 128)	*p*-NA derivatives (221)		X-Pro-*p*-NA (252)
	CD WC	Casein (128) ¹⁴C-methylated casein (76) Casein (221) Milk protein (128, 221, 252)			*p*-NA derivatives (76) ArAm derivative (128)	Dipeptides (76)			

a Activities are listed on the basis of their cellular location and the substrates used to detect and quantify their presence. Abbreviations: CFE, cell extract of disrupted cells; CD, cell debris; WC, whole cells in liquid or solid culture; CS, culture supernatant; ABZ, aminobenzoyl; AMC, 7-amino-4-methyl coumarin; Arg, arginine; ArAm, arylamide; BZ, benzyl; CBZ, benzyloxycarbonyl; FITC, fluorescein isothiocyanate; Gln, glutamine; Leu, leucine; MeOsuc, methyoxysuccinyl; NAm, naphthylamide; *p*-NA, *p*-nitroanilide; Phe, phenylalanine; Pro, proline; Tyr, tyrosine; X, amino acid (various). The data are limited to data derived from species which can be found in association with grape juice or wine.

b The numbers in parentheses are references.

enzymes are most amenable to separation from the cell biomass or preparation as an enzyme-enriched extract, which may be desirable when the originating organism is difficult to grow or is not wanted in grape juice or wine. Some topics that fall outside the scope of this review are covered elsewhere, including in broader reviews of the role of enzymes in winemaking (283) or of the implications of the growth and metabolic activity of LAB in grape juice and wine (158).

THE MALOLACTIC ENZYME

The LAB most commonly associated with wine belong to *O. oeni* and select *Lactobacillus* and *Pediococcus* spp. The major function of LAB is the conversion of L-malic acid to L-lactic acid during the MLF. This conversion may be achieved by one of three pathways (reviewed in references 124 and 284). Most wine-borne LAB decarboxylate L-malic acid to L-lactic acid and carbon dioxide in a reaction catalyzed by the malolactic enzyme without the release of intermediates. One exception to this is observed in *Lactobacillus casei* and *Lactobacillus faecalis*, which use a malic enzyme (malate dehydrogenase) to metabolize L-malic acid to pyruvate. L-Lactate dehydrogenase then acts on pyruvate to produce L-lactic acid. A second exception is evident in *Lactobacillus fermentum*, in which metabolism of L-malic acid yields D-lactic acid, L-lactic acid, acetate, succinate, and carbon dioxide.

Despite the importance of the MLF, its occurrence is both highly unpredictable and difficult to control or manipulate (125). Consequently, techniques that facilitate the efficient and complete conversion of L-malic acid to L-lactic acid in grape juice and wine have been sought. Such techniques aim to separate this central enzyme-driven conversion from the often problematic growth of the source LAB in the wine. Examples from the beverage and food industries include bioreactor systems comprising LAB cells immobilized alone (44, 45, 54, 182, 200, 256, 262), LAB cells coimmobilized with yeast (201), or free *O. oeni* cells (99) or enzymes and cofactors (91, 182, 282). The ability of the malolactic enzyme, as a single enzyme, to conduct the conversion of L-malic acid to L-lactic acid has made it the activity of choice for such bioreactor systems, as well as heterologous expression studies. A bioreactor containing NAD, manganese ions, and the malolactic enzyme from *L. oenos* strain 84.06 achieved a 62 to 75% conversion rate for L-malic acid to L-lactic acid in wine (91). Incomplete conversion was attributed to enzyme inactivation and instability of the cofactor NAD at the wine pH (40, 102). The expression of the malolactic enzyme encoded by the *mleS* gene from *Lactococcus lactis* in an *S. cerevisiae* wine yeast enabled it to effect the MLF and alcoholic fermentation simultaneously (289). Whether achieved via such recombinant methods or via bioconversions with cells or enzyme preparations, the potential benefits of enhanced application of malolactic enzyme warrant further research. Identification of a malolactic enzyme that is more resilient under wine conditions and improved delivery systems is of foremost interest.

PROTEOLYTIC AND PEPTIDOLYTIC ENZYMES

Grape juice nitrogen compounds include compounds that are variously essential or detrimental to successful fermenta-

tion and wine quality. The bulk of the nitrogenous fraction is comprised of the alpha amino acids and ammonium (25, 126), which along with peptides containing up to five amino acid residues (16, 199) represent the assimilable nitrogen that is vital for yeast growth and fermentative activity (30, 139, 191, 238) and suppression of hydrogen sulfide (100, 112, 138, 263). Conversely, the proteins of grapes are considered a nuisance as they become unstable in the finished wine and can precipitate to produce a haze (15, 133, 290).

Bentonite fining remains the most common and effective method for removal of haze-forming proteins from wine despite the unwanted effects of removing some assimilable nitrogen, modifying the flavor, and changing the kinetics of fermentation (9, 32, 121, 203, 211, 265). Proteases have been sought from a variety of sources, and they have been evaluated as an alternative to bentonite treatment to remove unwanted proteins while possibly also liberating assimilable nitrogen for exploitation by yeast (9, 188, 297). Commercial proteolytic preparations, such as trypsin and pepsin, do not function optimally at the low temperatures and pH used during winemaking (40). Proteases from *Aspergillus niger* have similarly been unsuccessful under winemaking conditions (9, 121). Modra et al. (188) studied five commercial peptidase preparations in wine, but none was found to significantly reduce the bentonite concentration required to achieve heat stability. Researchers have investigated wine and beer yeasts as alternate sources of such enzymes, reasoning that these organisms would be more suited to the conditions of the corresponding fermentations, but generally the results have been disappointing (21, 55, 148, 202, 236, 267). Alternate enzymes or alternate sources are clearly called for, and thus the proteolytic and peptidolytic activities of LAB are receiving greater attention.

LAB are fastidious in their amino acid requirements (95, 101), and there is clear evidence that some LAB produce the activities needed to procure peptides and amino acids to meet these requirements (81, 190). Comprehensive reviews covering proteolytic and peptidolytic activities across all genera of LAB are available elsewhere (38, 111, 154, 155). Most work in this field has been performed by workers in the dairy industry, in which these enzymes are directly involved in flavor and texture development (65, 287, 294) and are indirectly involved in the maximization of microbial cell growth by provision of essential amino acids (111). Here, only findings from studies of wine-related species of LAB are summarized. From Table 1 it is evident that activities designated proteinases and several types of peptidases are widely distributed across the three genera and 15 wine-related species included. The potential importance of these activities for winemaking is in part linked to the nature of the enzyme, its cellular location, and how it is applied to the wine.

Activities that are lost to the culture supernatant or are associated with whole cells have been reported for most species (Table 1). As a result, such activities could be evident in intact cells of a LAB when it is grown in grape juice or wine. Organisms whose growth mirrors that of *O. oeni* and is most apparent after or toward the end of the primary fermentation are unlikely to have an impact on yeast growth. At this time yeast needs minimal assimilable nitrogen; therefore, any proteolytic or peptidolytic activity of LAB is beneficial mainly for haze reduction. Conversely, the sensitivity of *Lactobacillus* and

147

Pediococcus to ethanol (95, 284, 292) relegates their growth in mixed cultures with yeast to the early stages of the primary fermentation. Degradation of proteins and peptides at this early stage might not only affect protein haze formation in the finished wine but also release assimilable nitrogen to benefit yeast growth.

The application of a cell-free enzyme extract is one way to dissociate a desired enzymatic activity from the need to grow a particular LAB in grape juice or wine. This approach also introduces the possibility of exploiting the considerable cohort of intracellular enzymes identified to date, but it might be necessary to consider the stability of these enzymes under wine conditions. In considering the importance of individual enzyme types, proline-specific peptidases might be less important in providing assimilable nitrogen since the liberation of proline has little nutritional value to yeast cells because of their inability to exploit this amino acid under oenological conditions (135, 239).

In the absence of extensive studies of wine LAB, the nature and frequency of proteolytic and peptidolytic activities identified by dairy researchers strongly suggest that similar activities also exist in wine LAB. What data have been reported for wine show promise. It is recognized that the levels of individual peptides and amino acids can increase or decrease during LAB growth in wine, and the only general point of agreement is that the arginine concentration decreases while the ornithine concentration increases during MLF (124, 292). More detailed information comes from a series of studies conducted by Manca de Nadra and coworkers. These workers described two enzymes, proteases I and II, which are produced by several strains of *O. oeni* during the early and final phases of growth, respectively (233). Protease I displayed optimal activity at pH 4.0 and 30°C, while the optimal protease II activity occurred at pH 5.5 and 40°C (232). Both proteases were apparently repressed by ammonium, tryptone, and casein hydrolysate, were induced by nutrient starvation, and were able to liberate detectable concentrations of amino acids from protein and polypeptide extracts from red and white wines (81, 174, 175, 234). When applied to sterile grape juice, a concentrated, purified exoprotease is thought to degrade proteins at a high rate (80). As encouraging as these findings are, there are some questions that remain to be answered. For example, it is not known whether the observed degradation of grape proteins releases peptides and amino acids in amounts that provide a nutritional benefit to the yeast or bacteria involved in the winemaking process and whether these activities are able to reduce the potential for haze formation in wine in which protein is unstable.

GLYCOSIDASES

The sensory properties of wine are the result of a multitude of individual compounds. Four groups of these compounds, the monoterpenes, C_{13}-norisoprenoids, benzene derivatives, and aliphatic compounds, all can occur linked to sugars to form glycosides (250, 296). Monoterpenes and some benzene derivatives and C_{13}-norisoprenoids play an important role in determining wine aroma, particularly for varieties such as Muscat, Gewürztraminer, and Riesling. Aliphatic compounds, which include the aliphatic alcohols, carboxylic acids, lactones, and

ethyl esters, are more related to the flavor of a wine. The remaining benzene derivatives include the anthocyanins, which contribute to wine color. Importantly, the characteristics of the glycosides differ from those of the corresponding aglycones. Generally, the glycosides are water soluble and less reactive and volatile than the aglycones, possibly explaining why plants store a great number of compounds in the glycosidic form (130). In wine, volatile, aromatic compounds that are otherwise detectable by human senses are nonvolatile and undetectable when they are in the glycosidic form. Accordingly, because as much as ~95% or more of such aromatic compounds is present in the glycosidic form, most of the aromatic potential of these compounds is not realized (116). Conversely, monoglucoside anthocyanins represent the principal form in which the anthocyanins that contribute to color in red wines are found (230). When these color compounds are deglycosylated, the corresponding anthocyanidin is less stable and is readily converted to a brown or colorless compound (23, 134). While this outcome may be undesirable in a red wine, these enzymes have been proposed as a means to reduce the color intensity in white or rose wines produced from red grapes (241).

The glycosidase enzymes that cleave the sugar moiety from glycosides can therefore have a major impact on the sensory profile of a wine. The occurrence of many types of such enzymes is a reflection of the complexity of their glycoside substrates, which can contain either mono- or disaccharides. The terminal sugar can be either β-D-glucopyranoside, α-L-rhamnopyranoside, α-L-arabinofuranoside, β-D-apiofuranoside, or β-D-xylopyranoside, and the additional central sugar in disaccharides is always β-D-glucopyranoside (296). Removal of these sugars requires a glycosidase specific for the terminal sugar, followed by, in the case of a disaccharide, a β-D-glucopyranosidase (116). The latter enzyme is essential for liberation of aglycones from all diglycosides and β-D-glucopyranosides; hence, research efforts are concentrated on this enzyme to the almost complete exclusion of other enzymes.

With the aim of increasing the aromaticity of wines, glycosidases have been widely studied in several organisms, including both wine-related and non-wine-related organisms. Grapevines produce glycosidases, although these enzymes have little activity against wine glycosides (6). Given its importance in winemaking, much attention has been paid to *S. cerevisiae*, but this yeast shows very limited production of glycosidases, much of which is intracellular (49, 179). Studies of other wine yeasts, including the apiculates and the spoilage yeasts, have yielded wide-ranging levels of activities, primarily β-D-glucosidase (β-D-glucopyranosidase) activities (35, 176, 237). Sanchez-Torres and coworkers (241) have also heterologously expressed a β-D-glucosidase from *Candida molischiana* in an *S. cerevisiae* wine strain and have demonstrated readily observable anthocyanase (decolorizing) activity in microvinification experiments. Several grapevine fungal pathogens, such as *Aspergillus* and *Botrytis*, produce large amounts of glycosidase activities that also have high levels of specificity for purified wine glycosides (177). Accordingly, *Aspergillus* is a common source of commercial enzyme preparations that have glycosidic activities; however, these preparations are often impure, requiring resolution before characterization in the laboratory (258, 259, 261), and they have undesirable effects on the wine (1, 115, 296). More importantly, the enzymes of fungi are frequently ineffective in

wine (6, 43, 114). The same is true for many of the glycosidic activities from the various source organisms examined to date, which can be limited by sensitivity to one or more of the following key wine parameters: low pH (pH 3.0 to 4.0), ethanol content (9 to 16%, vol/vol), or residual sugar content (<10 g/liter) (reviewed in reference 296). Interestingly, the LAB, which can thrive under these conditions, have received little attention as a potential source of glycosidic enzymes.

While the glycosidases of some LAB have been studied, wine isolates have only recently been included. Limited data have been reported for *O. oeni*, and no data are available for wine *Lactobacillus* and *Pediococcus* spp. McMahon and coworkers (183) observed no enzymatic activity by *O. oeni* against arbutin, an artificial glycosidic substrate. In another study (24), changes in the glycoside content of Tannat wines during MLF indirectly supported the existence of such activities in the commercial *O. oeni* strains used. More specific data have come from examinations of commercial wine *O. oeni* isolates (11, 110, 176), which were shown to have the potential for high glycosidase activity against nitrophenyl glycosides. β-D-Glucosidase was the predominant activity, and some β-D-xylopyranosidase and α-L-arabinopyranosidase activities were also detected (110). Notably, these activities were only partially inhibited under wine-like conditions. At pH 3.5 and in the presence of glucose (20 g/liter) and ethanol (12%, vol/vol), one isolate retained ~50% of the activity seen under optimized conditions (110). Mansfield and coworkers (176) could not detect activity against glycosides extracted from the Viognier variety; however, more recent work demonstrated that some *O. oeni* strains are able to act on glycosides extracted from the highly aromatic Muscat variety (275) or the nonaromatic Chardonnay variety (53). In agreement with results obtained with synthetic substrates, the pattern of hydrolysis of selected glycosides from the Chardonnay variety showed that strain EQ 54 had little activity other than a β-D-glucosidase activity, and greater hydrolysis of the mixture occurred only after addition of commercial α-L-rhamnopyranosidase and α-L-arabinofuranosidase preparations (53).

While the use of enzymes and/or selected cultures to liberate aroma compounds from natural grape aroma glycosides is still in the early stages of development, the findings to date for LAB and synthetic or natural glycosides are very encouraging and justify further investigation. LAB appear to possess the full array of glycosidases needed to hydrolyze many of the glycosides found in grapes and wine, although some enzymes have limited activity. Determining the precise sensory significance of glycosidic activities, as well as the longevity of any positive effects, is an important objective of future studies.

POLYSACCHARIDE-DEGRADING ENZYMES

The polysaccharides of higher plant cell walls and middle lamellae that affect wine production include cellulose (primarily β-glucans), hemicellulose (primarily xylans), and pectic substances (291). Such compounds are present in grape juice as a result of berry disruption or release through the action of degradative enzymes from the grapes. In fruit infected with the mold *Botrytis cinerea*, β-glucans are excreted by this pathogen directly into the berry (32, 59), and fungal enzymes release grape polysaccharides, particularly type II arabinogalactan and rhamnogalacturonan II (92). While fungal enzymes appeared

not to enhance the release of polysaccharides (mannoproteins) from yeast (92), these compounds can be released during yeast cell growth, through exposure to shear (e.g., during pumping and centrifugation) (157, 251), and particularly upon autolysis (56, 90).

Collectively, polysaccharides reduce juice extraction and are primarily responsible for fouling of filters during clarification steps. Wine quality also can be affected through changes in clarity (60), while an effect on viscosity may influence mouth feel (240) and the perception of tastes and aromas (61, 119, 120, 151, 173). Enzymes capable of degrading polysaccharides therefore have the potential to improve juice yields (18, 118, 208, 210) and wine processability through the removal of problem colloids, to increase wine quality via breakdown of grape cell walls to yield better extraction of color and aroma precursors (10, 98, 215, 229, 293), and to alter the perception of wine components (61, 151). These complex macromolecules are hydrolyzed by a number of distinct enzymes, including pectinases (protopectinase, pectin methylesterase, polygalacturonase, and pectin and pectate lyase activities), cellulases (endoglucanase, exoglucanase, and cellobiase activities), and hemicellulases (β-D-galactanase, β-D-mannase, and β-D-xylanase activities) (reviewed in reference 283). There have been few reports of attempts to specifically identify polysaccharide-degrading enzymes in LAB, despite the importance of these enzymes to winemaking.

The pectinolytic activities of LAB have largely been addressed in studies of fermentation processes other than winemaking, in which their significance remains unclear. For example, early work on silage microflora suggested that cellulases and hemicellulases are produced (195), whereas more recent work indicated that combinations of *Lactobacillus plantarum* and *Pediococcus cerevisiae* (*Pediococcus damnosus*) had negligible ability to degrade plant cell walls (147). Pectin methylesterase and polygalacturonate lyase activities have been detected in the spontaneous fermentation of cassava roots, but the study neither confirmed nor discounted the involvement of the LAB present in the fermentation (28). At the very least, *L. plantarum* is able to liberate reducing sugars from polymeric carbohydrates during corn straw ensiling (299).

An extracellular glucanase that is produced early in the stationary phase of cell growth has been demonstrated in *O. oeni* (113). This enzyme was determined to be β-1,3 in nature and to be capable of hydrolyzing yeast cell wall macromolecules; thus, it was proposed that the enzyme plays a role in yeast cell autolysis following alcoholic fermentation. Further work is required to confirm the significance of this activity along with its efficacy at temperatures below 10°C, at which currently available glucanases are insufficiently active (32, 286). Similarly, the absence of additional polysaccharide-degrading enzymes cannot be assumed until a comprehensive and specific search for such activities, such as the search conducted for wine yeasts and fungi, has been completed for LAB.

ESTERASES

Esters are a large group of volatile compounds that are usually present in wine at concentrations above the sensory threshold. Most wine esters are produced by yeast as secondary products of sugar metabolism during alcoholic fermentation

TABLE 2. Esterases of LAB[a]

Organism	Enzyme location	Substrates hydrolyzed			
		Acetate	Propionate	Butyrate	Valerate
Lactobacillus brevis	CFE	α-N- (71, 280), β-N- (71), o-NP- (71), p-NP- (71, 294)[b]	α-/β-N- (71, 280), p-NP- (71)	α-N- (71), β-N- (70, 103), p-NP- (71, 294), o-NP- (71)	α-/β-N- (71), p-NP- (71)
	CD			β-N- (103)	
Lactobacillus buchneri	CFE	α-N- (280), p-NP- (294)	α-/β-N- (280)	α-/β-N- (280), p-NP- (71, 294)	
Lactobacillus casei	CFE	α-N- (71, 141), β-N- (71, 141, 223), o-NP- (70), p-NP- (37, 71, 294)	α-N- (71, 141), β-N- (71), p-NP- (37, 71)	α-N- (71, 141), β-N- (33, 71, 103), p-NP- (33, 37, 71, 73, 156, 294), o-NP- (71, 73)	α-/β-N- (71), p-NP- (71)
	CD	β-N- (223)		β-N- (103)	
	WC	α-N- (194)		α-N- (227)	
Lactobacillus curvatus	CFE	p-NP- (294)		β-N- (103), p-NP- (294)	
	WC			β-N- (213)	
Lactobacillus fermentum	CFE	α-/β-N- (71), p-NP- (71, 294), o-NP- (71)	α-/β-N- (71), p-NP- (71)	α-N- (71), β-N- (71, 103), p-NP- (71, 294), o-NP- (71)	α-/β-N- (71), p-NP- (71)
	CD	β-N- (106)		β-N- (103, 106)	
	WC	α-N- (194)			
Lactobacillus hilgardii	CFE		α-N- (280)		
Lactobacillus paracasei	CFE	α-/β-N- (22), p-NP- (294)	α-/β-N- (22)	α-/β-N- (22), p-NP- (294)	
	WC				
Lactobacillus plantarum	CFE	α-N- (71, 141), β-N- (71, 104, 141), p-NP- (71, 294, 303), o-NP- (71)	α-/β-N- (71, 141), p-NP- (71)	α-N- (71, 141), β-N- (71, 103, 104, 141), p-NP- (71, 294, 303), o-NP- (71)	α-/β-N- (71), o-NP- (71)
	CD			β-N- (103)	
	WC				
Lactobacillus sakei	WC			β-N- (213)	
Pediococcus pentosaceus	CFE	α-/β-N- (19)	α-/β-N- (19)	β-N- (19), p-NP- (281)	
Leuconostoc mesenteroides	CFE WC	α-/β-N- (141, 280, 281)	α-N- (141, 280)	α-N- (280), β-N- (141, 280)	

[a] Activities are listed on the basis of their cellular location, the ester substrate and, where appropriate, the form of the chromogenic linker. Abbreviations: CFE, cell extract of disrupted cells; CD, cell debris; WC, whole cells in liquid or solid culture; α-N-, α-naphthyl; β-N-, β-naphthyl-; o-NP-, o-nitrophenyl; p-NP-, p-nitrophenyl-; FAX, 2-O-[5-O-(trans-feruroyl)-β-L-arabinofuranosyl]-D-xylopyranose. Data are limited to data derived from species which can be found in association with grape juice or wine.

[b] The numbers in parentheses are references.

(149, 300). Esters can also be derived from the grape (226) and from the chemical esterification of alcohols and acids during wine aging (75). The importance of esters in winemaking lies in their prominent role in determining the aroma and, by extension, the quality of wine. Esters are responsible for the desirable, fruity aroma of young wines (149, 178), although they can also have a detrimental effect on wine aroma when they are present at excessive concentrations (264).

Quantitatively, the most important wine esters are mainly yeast derived and include (i) ethyl esters of organic acids, (ii) ethyl esters of fatty acids, and (iii) acetate esters (75). Ethyl acetate is usually the predominant ester in wine, and with a low sensory threshold, it is often an important contributor to wine aroma (75). At low concentrations, ethyl acetate aroma is desirable and described as fruity, but at higher concentrations it imparts an undesirable nail polish remover character to wine (13). Other important wine esters and their aromas include isoamyl acetate (banana), ethyl hexanoate (fruity, violets), ethyl octanoate (pineapple, pear), and ethyl decanoate (floral) (149, 178). Esterolytic activity during wine production could result in either an increase or a decrease in wine quality, depending on the ester involved (47). In addition, the compounds liberated by the esterases (for example fatty acids and higher alcohols) could contribute to wine aroma (75, 149).

While the esterases of yeast have been extensively researched (for example, see references 180, 218, and 235), there

TABLE 2—*Continued*

		Substrates hydrolyzed				
Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Others
α-N- (280), β-N- (103, 280), p-NP- (71, 294) β-N- (103)	α-N- (280), β-N- (103), p-/o-NP- (71, 73) β-N- (103)	β-N- (103, 280)	β-N- (103)	β-N- (103)		Triacetin (206)
α-/β-N- (280), p-NP- (294)	α-N- (280)	β-N- (280)		p-NP- (294)		
β-N- (33, 103), p-NP- (33, 71, 73, 156, 294), o-NP- (71, 73) β-N- (103)	β-N- (33, 103), p-NP- (37, 71, 73, 156), o-NP- (71, 73) β-N- (103)	p-NP- (37), β-N- (103)	β-N- (33, 103)	β-N- (33, 103), p-NP- (294)	p-NP- (294)	Triacetin (206), Tributyrin (33), unspecified C4 and C8 (216)
β-N- (103), p-NP- (294)	β-N- (103)	β-N- (103)	β-N- (103)	β-N- (103), p-NP- (294)		Unspecified C4 and C8 (270)
β-N- (103), p-NP- (71, 294), o-NP- (71) β-N- (103, 106)	β-N- (103), p-/o-NP- (71) β-N- (103, 106)	β-N- (103) β-N- (103, 106)	β-N- (103) β-N- (103, 106)	β-N- (103), p-NP- (294)		FAX, methyl ferulate, methyl coumarate (57) Ethyl/methyl ferulate (57), unspecified C4 and C8 (204, 270)
α-N- (280)	α-N- (280)	β-N- (280)				
α-/β-N- (22), p-NP- (294)	α-/β-N- (22)	α-/β-N- (22)		p-NP- (294)	p-NP- (294)	Unspecified C4 and C8 (270)
α-N- (141), β-N- (103, 104), p-NP- (294)	β-N- (103, 104), o-/p-NP- (166)	β-N- (103, 104)	β-N- (103, 104)	p-NP- (294), β-N- (103)	β-N- (104), p-NP- (294)	β-N-Stearate, β-N-oleate, milk fat, tributyrin, tricaprylin, trilaurin (104), triacetin (206)
β-N- (103)	β-N- (103)					Tributyrin, oleuropein (153), unspecified C4 and C8 (270)
	β-N- (213)					
α-N- (280)	α-N- (280)					Triacetin (206) Tributyrin, oleuropein (153)

has been little work focusing on the esterases of wine LAB. Our current knowledge of LAB esterases is based primarily on work carried out in the dairy industry, in which such enzymes contribute to the characteristic flavors and defects of cheeses (132). Most of this work has focused on the metabolism of esters by LAB, and it is now suspected that these enzymes have the ability to both synthesize and hydrolyze esters (158). Thus, dairy LAB synthesize esters, including ethyl butanoate and ethyl hexanoate, while ester hydrolysis is also supported by abundant experimental evidence (82, 131, 159). A summary of the literature describing hydrolytic esterases from dairy *Lactobacillus* and *Pediococcus* species is presented in Table 2. Similar results have been obtained for LAB genera not endogenous to wine, including *Streptococcus* (160), *Leuconostoc* (141, 280), *Lactococcus* (41), and *Enterococcus* (141). The appearance of esterase activity in association with whole cells or in culture supernatants of some LAB shown in Table 2 implies

that growth in grape juice or wine of these species may modify the ester profile of the beverage. Where intracellular esterase activities are reported, cell disruption would presumably be required in order for these activities to have an impact on wine.

In a screening of the enzymatic activities of wine LAB, Davis et al. (47) found 23 strains that were able to hydrolyze an ester substrate, but no steps were taken to characterize the enzymes further or to determine their ability to synthesize esters. In some wine flavor studies workers have reported changes in the concentration of individual esters during MLF. For example, increases in ethyl acetate (48, 172), isoamyl acetate (152, 172), and ethyl lactate (48, 97) levels have been observed, while Zeeman et al. (301) reported a decrease in the levels of some esters following MLF. These results suggest that like the esterases of dairy isolates, esterases of wine LAB are involved in both the synthesis and hydrolysis of esters. No further investigation has been reported. Therefore, further research into the

esterase systems of wine LAB should help determine the precise nature of these enzymes and their effects on the sensory properties of wine.

UREASES

Ethyl carbamate is a known carcinogen and is formed in wine via the spontaneous acid ethanolysis of certain carbamyl precursors, including urea and citrulline (145, 192, 207, 209, 266). Due to the health risks associated with elevated levels of ethyl carbamate in wine, the sources of the precursor compounds have been studied extensively. The pathways by which LAB can contribute to the ethyl carbamate precursor pool in wine have also been investigated (161).

Ureases produced by microorganisms are substrate-specific enzymes that catalyze the hydrolysis of urea (249). Urea-degrading enzymes are a potential tool for reducing the concentration of urea in wine in order to avoid dangerous and illegal concentrations of ethyl carbamate. Most commercial urease products are derived from nonwine sources (e.g., beans) and are unsuitable for use in wine, which has a pH that typically is well below the neutral pH optima (144). Ureases derived from LAB have been investigated as alternate enzymes for the removal of excess urea in wine. The acid ureases produced by *L. fermentum* (144, 212, 273) and *Lactobacillus reuteri* (140) were very effective over the pH range from 2.0 to 4.0, which included typical wine pH values. Wine components, such as phenolic compounds (e.g., grape seed tannins), sulfur dioxide, ethanol, and organic acids (e.g., malic acid, lactic acid, and pyruvic acid), did, however, inhibit the activity of the urease from *L. fermentum* (79, 273). Whether such inhibition limits the usefulness of ureases, at least in wine of a certain type or composition, remains to be determined through further study.

PHENOLOXIDASES

Laccases (*p*-benzendiol:oxygen oxidoreductase) and tyrosinases (monophenol monooxygenase) are two groups of phenoloxidases which are widely distributed in nature. They have been found in bacteria (39), filamentous fungi (181, 196), insects (7, 253), and higher plants (109, 181). Both groups of enzymes catalyze the transformation of a large number of (poly)phenolic and nonphenolic aromatic compounds and thus have potential uses in bioremediation processes in the paper and pulp, tanning (63, 196), and food industries (olive mill and brewery wastewater) (187). One of the main applications of laccases in the food industry is product stabilization in fruit juice, beer, and wine processing (187).

A myriad of phenolic compounds are found in musts and wine; these compounds range from simple hydroxybenzoic acid and cinnamic acid derivatives to more complex molecules, such as catechins, anthocyanins, flavonols, flavanones, and tannins (178). Such compounds are responsible for the desirable attributes of color, astringency, flavor, and aroma of wine, as well as unwanted attributes, including browning, flavor and aroma alterations, and some forms of haze, which are a consequence of enzymatic and chemical oxidoreduction in white musts and wines. In vinification two main approaches have been taken to combat oxidative decolorization and flavor alteration (madei-

rization). One of these approaches is inhibition of enzymes in the must by using sulfur dioxide as a reductant and inhibitor; alternatively, the polyphenol substrate content of white wine is reduced by limiting maceration of the must. Where introduction has not been avoided or reduced, polyphenols are removed from the must or wine with fining agents, such as polyvinylpolypyrrolidone, gelatin, casein, and egg albumin, in addition to bentonite (187). More recently, chitosan, a polymeric adjuvant, was used as a fining agent and was found to be comparable to potassium caseinate in terms of wine stabilization (260).

Treatment of the must with enzymes such as laccases, tyrosinases, tannases, and peroxidases has been considered an alternative to treatment with physical-chemical adsorbents (302). Laccases are thought to be the most promising enzymes, because they have broader specificity for phenolic compounds, are more stable at the pH of must and wine, and are less affected by sulfur dioxide (32). With these enzymes, wine stabilization is achieved through preferentative treatment to bring about oxidation of polyphenol substrates normally involved in the madeirization process. The oxidized products polymerize and precipitate, and they are subsequently removed by conventional clarifiers and filtration. To date, laccases from lignin-degrading fungi, such as *Trametes versicolor*, *Coriolus versicolor*, and *Agaricus bisporus*, and tyrosinases from mushrooms have been used in wine processing with promising results (32). When the enzymes were applied either singularly or in combination and immobilized on supports ranging from metal chelate affinity matrices to molecular sieves, good results were observed in terms of polyphenol removal, retention of enzyme activity, and reuse of the carrier support (see reviews in references 63 and 187).

The occurrence of (poly)phenoloxidases in LAB, particularly those commonly associated with winemaking, is also of interest. The work of Benz and coworkers (17) and Lamia and Moktar (150) suggests that these enzymes are present in fermentative LAB. Specifically, *L. lactis* is able to reduce humic acids (17), a constituent of soil humus containing substituted phenols and polyphenols (137), which implies that a polyphenoloxidase is involved. Lamia and Moktar (150) demonstrated that *L. plantarum* grown on diluted olive mill wastewater induced the depolymerization of polyphenols (presumably by the action of tannases) and inhibited the polymerization of simple phenolic compounds which occurs at lower postfermentation pH values.

Studies of wine LAB, particularly *O. oeni*, have revealed that the growth and rate of MLF of these organisms are influenced by phenolic compounds. Gallic acid and anthocyanins, metabolized by growing cells, have a positive effect on growth and malolactic activity, whereas tannins are inhibitory (288). Other workers have observed that growth of *O. oeni* is inhibited by phenolic acids, including *p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, protocatechuic, gallic, vanillic, and syringic acids, while growth of *Lactobacillus hilgardii* was stimulated (31). These findings are indicative of the evolution of polyphenoloxidases (and possibly tannases) from some of these organisms. If such activities do exist, their contribution to browning or decolorization is yet to be determined, but it might be expected to be most relevant to species that grow in grape juice early in

TABLE 3. Lipase activities of species of LAB that can be found in association with grape juice or wine[a]

Organism	Enzyme location	Substrates hydrolyzed[b]
Lactobacillus brevis	CFE	Butter oil (34), coconut oil (34), glyceromonobutyrate (72), milk fat (72, 103), tributyrin (34, 72, 103, 206), tricaproin (34), tricaprylin (34, 72, 103), trilaurin (72, 103), triolein (34, 103), tripropionin (34), tripalmitin (72)
	WC	Tributyrin (206), tricaproin (206)
Lactobacillus casei	CFE	Butterfat (163, 222, 278), milk fat (103), composite butter (278), glyceromonobutyrate (72), β-naphthyl laurate (223), olive oil (162, 278), tributyrin (72, 103, 156, 162, 206, 255, 278), tricaprylin (72, 103), trilaurin (103), tripalmitin (103), triolein (103), Tween 80 (156)
	CD	β-Naphthyl laurate (223)
	WC	Butter fat (277), butter oil (185), milk fat (29), α-naphthyl caprylate (227), olive oil (276), tributyrin (206), tricaproin (206), triolein (185)
Lactobacillus curvatus	CFE	Tributyrin (103, 214), tricaprylin (103, 214), tricaprin (214), trilaurin (103, 214), tripalmitin (214), triolein (214), milk fat (103)
	WC	Tributyrin (214)
	Not specified	Tributyrin (213)
Lactobacillus fermentum	CFE	Glyceromonobutyrate (72), milk fat (72, 103), tributyrin (72, 103), triolein (103), tricaprylin (72, 103), trilaurin (72, 103), tripalmitin (72, 103)
Lactobacillus paracasei	WC	Tributyrin (93)
Lactobacillus plantarum	CFE	Butterfat (278), composite butter (278), glyceromonobutyrate (72), lard (3), milk fat (72, 103, 105, 141), olive oil (278), tributyrin (3, 72, 103, 105, 141, 206, 255, 278), tricaproin (206), tricaprylin (72, 103, 106), trilaurin (72, 103, 105), triolein (103, 255), tripalmitin (72, 103, 105, 255)
	WC	Milk fat (29), olive oil (278), tributyrin (206), tricaproin (206)
	CS	Milk fat (141), olive oil (164, 165), tributyrin (141)
Lactobacillus sakei	WC	Tributyrin (214)
	Not specified	Tributyrin (213)
Pediococcus damnosus	CFE	Tributyrin (206), tricaproin (206)
Pediococcus pentosaceus	CFE	Tributyrin (205), tricaprylin (205)
	WC	Unspecified C_{14} substrate (204)
Leuconostoc mesenteroides	CFE	Tributyrin (141)
	CS	Milk fat (141), tributyrin (141)

[a] Abbreviations: CFE, cell extract of disrupted cells; CD, cell debris; WC, whole cells in liquid or solid culture; CS, culture supernatant.
[b] The numbers in parentheses are references.

fermentation. This could include LAB which are inoculated early, such as *Lactobacillus*, or contaminating LAB. Spectrophotometric assays analogous to those described for plant polyphenoloxidases, in which substrates such as catechol, catechin, and *p*-courmaric acid are used (109), should facilitate extended surveys of wine LAB for such activities.

LIPASES

Wine lipids can be derived from the grape berry (96, 186) or can be released from yeast during autolysis (224). Grape lipids can originate from a number of sources within the berry, including the skin, seeds, and berry pulp. The lipid profiles of each of these sources have been shown to be different, due to variation in both the concentration and the fatty acid composition of neutral lipids, glycolipids, and phospholipids (186). The grape lipid profile also varies with grape maturation (14), climate (136), and variety; red varieties tend to have greater total lipid concentrations than white varieties (96). Yeast autolysis following fermentation releases many different types of lipids, including tri-, di-, and monoacylglycerols and sterols, in amounts and proportions which vary with the yeast strain (224). Such lipids are known to influence not only the sen-

sory profile of sparkling wine but also foam characteristics (224).

The action of lipases on wine lipids could yield a range of volatile compounds, including fatty acids. The low aroma thresholds of fatty acids allows them to contribute to wine aroma, but since their odors are described as vinegar, cheesy, and sweaty, their impact might not be desirable (75). A more positive contribution to the aroma profile of wine can develop when volatile compounds such as esters, ketones, and aldehydes are derived from these fatty acids (36).

The lipolytic activity of wine LAB has not been thoroughly investigated, but preliminary work with nonwine substrates suggests that some LAB may produce lipases. In a study of LAB isolated from wines, Davis et al. (47) observed lipase activity in several strains of *L. oenos* (*O. oeni*) and one species of *Lactobacillus*. By contrast, a more recent study failed to find lipolytic activity in wine isolates comprising 32 *Lactobacillus* strains, two *Leuconostoc* strains, and three *Lactococcus* strains (127). The lipolytic activity of LAB has been more extensively researched in other areas of food production. In dairy foods, lipases can contribute to flavor and processability (279). On the basis of this work, LAB are now generally acknowledged to be weakly lipolytic (93, 142), and their lipases display substrate

specificity which is both strain and species dependent (141). By utilizing numerous substrates and various degrees of cell fractionation, several such studies have provided information about activities in genera that are of interest in winemaking, namely, *Lactobacillus* and *Pediococcus* (Table 3). A broader review of the lipolytic activity of all dairy LAB was produced by Collins et al. (42). Because lipases are located extracellularly or are associated with the whole cells, LAB (Table 3) have the potential to influence the wine lipid content when they are grown in grape juice or wine. The ability of any of these enzymes to attack membranes of yeast and grape cells and to influence wine aroma remains to be determined.

CONCLUSIONS

A considerable amount of research has been conducted to determine the enzymatic properties of LAB specifically isolated from wine or, more commonly, the enzymatic properties of analogous species isolated from other foods or beverages. From this work it is clear that these organisms possess an extensive collection of enzymatic activities, many of which have the potential to influence wine composition and therefore the processing, organoleptic properties, and quality of wine. In many cases the precise nature and extent of this influence has yet to be delineated for the LAB that are associated with winemaking and grow under the conditions encountered during grape juice fermentation and wine maturation. Ideally, the potential for these organisms that has been highlighted in this review will stimulate fuller characterization of wine LAB so that at the very least these strains will be able to be applied by the winemaker in a more informed manner. In other cases, these organisms may serve as a source for the preparation of enzyme extracts that are better able to function under the harsh and changing environmental conditions of wine fermentation.

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A survey of glycosidase activities of commercial wine strains of *Oenococcus oeni*

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Abstract

Lactic acid bacteria play an important role in winemaking by undertaking the malolactic fermentation, yet little information is available on other aspects of their physiology, such as their profile of external enzymatic activities. In this study we sought evidence for the existence and action of glycosidase enzymes in wine isolates of *Oenococcus oeni*. This group of enzymes is of interest because of their potential for liberation of grape-derived aroma compounds from their natural glycosylated state. This comprehensive study reveals that these bacteria produce glycosidases that might be important in winemaking. Strains did not necessarily hydrolyse all substrates tested, but rather were grouped according to substrate specificity. Thus a subset comprising strains 2, 5 and 16 possessed high cumulative activities against β-D- and α-D-glucopyranoside substrates, while a group comprising strains 4, 21 and 22 was noted for superior hydrolysis of β-D-xylopyranoside, α-L-rhamnopyranoside and α-L-arabinofuranoside substrates. Key physico-chemical inhibitors of analogous systems from other microorganisms were seen to produce variable responses across the strains investigated here. Accordingly, several strains retained significant hydrolytic activity at typical wine pH values (~3.0–4.0), residual glucose and fructose contents (up to 20 g/L), and ethanol contents (up to 12%). These findings highlight the potential of *O. oeni* as a useful alternative source of glycosidase enzymes for use in winemaking.
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1. Introduction

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Many studies have been carried out to determine the impact of wine microorganisms on wine aroma and flavour (Antonelli et al., 1999; Kotseridis and Baumes, 2000; Lambrechts and Pretorius, 2000; Reynolds et al., 2001). Few of these works, however, have focused on Lactic Acid Bacteria (LAB) and in parti-

160

cular, *Oenococcus oeni*, the main bacterial species that conducts the malolactic fermentation (MLF) in wine. It is only recently that interest in this organism has shifted from the MLF to consider its impact on wine aroma, especially through elaboration of glycosidase enzymes. Many chemical compounds with the potential to contribute significantly to wine aroma, including monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols, can occur in grapes in free form or as odourless non-volatile glycosides (Günata et al., 1988; Williams et al., 1982; Winterhalter and Skouroumounis, 1997). Included are aroma compounds occurring in either the α-L-arabinofuranoside, α-L-rhamnopyranoside, β-D-apiofuranoside, β-D-glucopyranoside, or β-D-xylopyranoside forms (Williams et al., 1982; Günata et al., 1985; Strauss et al., 1986; Voirin et al., 1990). These glycoconjugates can be either monoglucosides or disaccharide glycosides where the glucose bound to the aglycon is further substituted with an α-L-arabinofuranoside, α-L-rhamnopyranoside, β-D-xylopyranoside or β-apiofuranoside. The release of the aglycon from the disaccharide involves the sequential release of the sugar moieties (Günata et al., 1988; D'Incecco et al., 2004). Specific glycosidases are now recognised as effective tools for the liberation of these aroma compounds (van Rensburg and Pretorius, 2000), with fewer of the negative impacts on wine structure and quality found with other treatments such as heating, acid hydrolysis or application of crude enzyme preparations (Francis et al., 1992; Spagna et al., 1998; Schneider et al., 2001).

Present knowledge about the glycosidases of *O. oeni* is modest compared to that of other industrially important LAB, particularly those applied in the manufacture of dairy products (De Vos and Gasson, 1989; Tzanetakis and Litopoulou-Tzanetakis, 1989; Antuna and Martinez-Anaya, 1993; Bianchi-Salvadori et al., 1995; Marasco et al., 1998, 2000). Most previous studies of the topic have examined the activity of these enzymes during the MLF of wine (Boido et al., 2002; Ugliano et al., 2003; Barbagallo et al., 2004; D'Incecco et al., 2004). In this study we have sought to define the nature and extent of glycosidase activity of 22 strains of *O. oeni* under optimised and reproducible conditions. As such, these organisms were grown in defined media, and presented with *p*-nitro-phenyl-linked glycosides of relevance to wine.

The influence of critical wine parameters, such as the concentrations of residual sugars and ethanol, pH and temperature were investigated both individually and in combination to identify the most promising candidates for application to wine.

2. Materials and methods

2.1. Bacterial strains and cultivation

The strains of *O. oeni* used in this study are listed in Table 1. Most were single colony isolates from commercial preparations (some formulated as mixtures of strains) of freeze-dried starter cultures used for the initiation of MLF during winemaking. Long term storage of the bacteria was achieved using vials containing treated beads in a cryopreservative fluid at -80 °C (Protect®, Technical Service Consultants Ltd.). Pre-cultures of each strain were prepared on an ongoing basis (each 2–4 days) by adding one bead into de Mann/Rogosa/Sharp broth medium supplemented with preservative free apple juice (20%),

Table 1
Strains and source of *Oenococcus oeni* used in this study[a]

Isolate	Origin
1	Lallemand O.S.U. (VI 77)
2	Lalvin 4× (VL 92)
3	Inobacter
4	Bitec Vino
5	Lalvin-Inobacter
6A	Lalvin MT01 Standard
7	Lallemand 3× (E218)
8	CHR Hansen Viniflora
9	CHR Hansen Viniflora
10	Lalvin EQ54 MBR
11	Lalvin MCW
12	Lalvin 3× 1Step
13	Lalvin IB Standard
14	Enoferm Alpha
16	Lalvin 3× Standard
17	Lalvin OSU MBR
20	Lallemand No. 3
21	Lallemand No. 4
22	Lallemand No. 5
23	Lallemand No. 8
28	Lallemand No. 2
29	Lallemand No. 9

[a] Strains 20–29 were derived from pre-commercial trails samples kindly provided by Lallemand.

adjusted to pH 5.0 (MRSA) (Kelly et al., 1989) and incubated at 25 °C.

Cultures used for the study of glycosidase activities were prepared by inoculating precultures into 5 mL of MRSA to an optical density measured at a wavelength of 600 nm (OD_{600}) of approximately 1 and incubating at 25 °C for 42 h (66 h for *O. oeni* strains 3 and 5). Cells were harvested and washed twice by centrifugation (20,000 × g for 5 min) with 0.85% w/v NaCl in distilled water. All strains were not necessarily analysed in a single experiment since occasionally one or more strains grow insufficiently.

2.2. Determination of glycosidase activity of culture biomass

Glycosidase activity was determined according to a method described previously (Grimaldi et al., 2000), with modifications to allow the use of a micro-plate spectrophotometer. Assays were reduced to a final volume of 80 μl and performed in standard 96-wells plates. For each reaction, 40 μl of 0.2 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO_4) was used (Dawson et al., 1986). When required McIlvane buffer was prepared to pH 4.0 and included the following at the final assay concentrations indicated: ethanol (0, 4, 8 and 12 v/v), glucose or fructose (0, 0.01, 0.1, 0.75 and 2% w/v). Each well was then dosed with 20 μl of a suspension of the appropriate *O. oeni* strain prepared in 0.85% NaCl and standardised to yield a final OD_{600} in the assay of 0.5. Substrate solutions (20 μl) were added to give the following final concentrations: *p*-nitro-phenyl β-D-glucopyranoside (10 mM), *p*-nitro-phenyl α-D-glucopyranoside (10 mM), *p*-nitro-phenyl xylopyranoside (7.5 mM), *p*-nitro-phenyl α-L-rhamnopyranoside (7.5 mM), and *p*-nitro-phenyl α-L-arabinofuranoside (7.5 mM) (Sigma). Assays were incubated at 37 °C and enzymatic activity was stopped after 1 h by the addition of 160 μl of 0.5 M Na_2CO_3 and the 96-well plate centrifuged (2500 × g for 18 min) to remove the cells from the reaction. Supernatants (200 μl) were transferred into corresponding wells in a fresh 96-well plate and the absorbance of each at 400 nm determined with a multi-plate spectrophotometer (μQuant®, BIO-TEK Instruments Inc.) set to automatic path-length correction. Blanks were prepared without bacterial cells but otherwise treated in the same manner. All reactions were performed in at least duplicate with replicate values typically being within 5% of one another. Non-enzymic hydrolysis of *p*-nitro-phenyl α-L-arabinofuranoside under alkaline conditions occurred after the addition of the 0.5 M Na_2CO_3 solution, and resulted in a doubling of sample absorbance approximately every 20 min. A similar increase was observed, in both samples and blanks (data not shown) and thus samples were processed as quickly as possible. Accordingly, centrifugation was applied for only 12 min after which supernatants were immediately transferred and analysed in the multi-plate spectrophotometer. In all cases, one unit of activity was defined as mmols of *p*-nitro-phenol liberated per min per milligram of cell dry weight. Culture dry cell weight was determined from 10 mL cultures which had been grown for 42 h.

2.3. Temperature dependence of glycosidase activity

Where the influence of temperature on glycosidase activity was investigated the same method as described above was used with the exception that all volumes were increased by 25% to give a final assay volume of 100 μl. Assays were performed in 200 μl disposable PCR tubes thereby allowing the use of a PCR thermocycler (Mastercycler Gradient®, Eppendorf) for accurate temperature control. Tubes were incubated for 1 h at temperatures between 14.9 °C and 57.7 °C. At the end of the incubation period, 80 μl from each assay were transferred to a well of a 96-well plate to which had previously been added 160 μl of 0.5 M Na_2CO_3. The 96-well plate was then centrifuged as above to pellet cells and 200 μl of supernatant transferred to a fresh 96-well plate for quantitation of sample absorbance at 400 nm as described.

3. Results

With few exceptions, previous studies of glycosidases of wine LAB have been limited to investigations of β-glucosidase (β-D-glucopyranosidase) activity. Our earlier study (Grimaldi et al., 2000) appears to be the first to use other substrates to specifically identify enzymatic activities, albeit limited, beyond β-glucosidase. Regardless of the source

organism or the enzyme activities under investigation, an influence by parameters including pH, temperature and the presence of inhibitors such as sugars and ethanol has been a common observation (e.g. Aryan et al., 1987; Winterhalter and Skouroumounis, 1997; Grimaldi et al., 2000; Spagna et al., 2002; Barbagallo et al., 2004). In this study we sought to expand our earlier preliminary findings by more fully defining the nature and functional limitations of the glycosidases associated with up to 22 different *O. oeni* strains.

3.1. pH and substrate interactions

Strains of *O. oeni* were cultured in MRSA and evaluated for their ability to liberate nitrophenol from each of five glycosylated *p*-nitro-phenyl-substrates over a range of pH values (2.6 and 7.0). General observations from this survey (Fig. 1) include that *O. oeni* glycosidase activities were widely distributed, with most strains acting on several of the substrates tested. Also, in confirmation of previous findings, assay pH greatly altered the glycosidase activity for the majority of *O. oeni* strain and substrate combinations. This finding reinforces the necessity of examining these properties under a broad range of pH values in order to develop a fuller view of the potential of individual *O. oeni* strains.

3.1.1. β-D-Glucopyranosidase

All strains tested possessed a detectable β-glucosidase activity against *p*NP-βGlu. The magnitude of this activity ranged by up to 16-fold in a strain and pH dependent manner with strains 2, 5, 16, 21 and 22 typically showing highest activities, whilst strains 6A, 13, 14 and 28 were representative of the lower extreme (Fig. 1A). Across the 17 strains surveyed for this activity, it is evident that pH of the assay buffer markedly influenced β-glucosidase activity. Some strains in fact displayed two peaks of activity: one near pH 3.4 and a second at a pH > 5.8, while in other strains maximal activity was centred around a single pH of 3.8.

3.1.2. α-D-Glucopyranosidase

As observed for β-D-glucopyranosidase activity, all isolates possessed an activity against *p*NP-αGlu (Fig. 1B). Values ranged by approximately 10-fold across the strains and pH values studied. The optimal

pH for α-L-glucopyranosidase activity varied such that it occurred at around pH 3.8 for strain 22, pH 4.6 for strain 2 and pH 6.6 for strains 1, 5, 12, 16 and 17. Dual pH optima of the sort observed for β-D-glucopyranosidase activity in many strains, were atypical for α-D-glucopyranosidase activity, and were most obviously seen with strain 17 at pH 3.8 and 6.6.

3.1.3. β-D-Xylopyranosidase

Liberation of nitrophenol from *p*-nitro-phenyl β-D-xylopyranoside was most effective at lower pH values, with a peak in activity typically occurring at pH 3.0–3.4 (Fig. 1C). Maximum activities of approximately 0.9 units were seen for strain 21 and 22 at pH 3.0. Strain 16 demonstrated a duality of pH optima, being at 3.0 and 5.4.

3.1.4. α-L-Rhamnopyranosidase and α-L-arabinofuranosidase

The response of individual strains to the final pair of substrates studied were similar and thus only selected data are shown in Fig. 1D. Despite an oenological importance being placed on α-L-rhamnopyranosidase because of the frequent occurrence of rhamnose-linked aroma compounds in grapes (Bayonove et al., 1992; Razungles et al., 1993; Bureau et al., 1996), poor hydrolysis of the corresponding *p*-nitrophenyl substrate (*p*NP-αRha), with a maximum of only 0.2 units was observed. Again, lower pH values gave optimal hydrolysis of *p*NP-αRha, whereas trace or no activity was seen above pH values of 4.6–5.0. Given the scarcity of this activity any further discussion of it is limited. Hydrolysis of *p*-nitro-phenyl α-L-arabinopyranoside was readily evidenced by all but two of the 21 strains studied. Again, only the findings for a selection of strains is reported here (Fig. 1D). In several strains two pH optima seemed apparent: one at around 3.4–3.8 and a second above pH 6.6. Highest activities of the order of 0.4 units, as typified by strain 20, occurred near pH 3.4.

A ranking of strains according to their ability to hydrolyse a given substrate was made difficult by virtue of the fact that such rankings changed according to the pH at which activity was quantified. For this reason, 'total' glycosidase activity was determined for each strain by summing activity measurements made across all pH values for a given substrate (Table 2). In this way, the strains most active against a particular
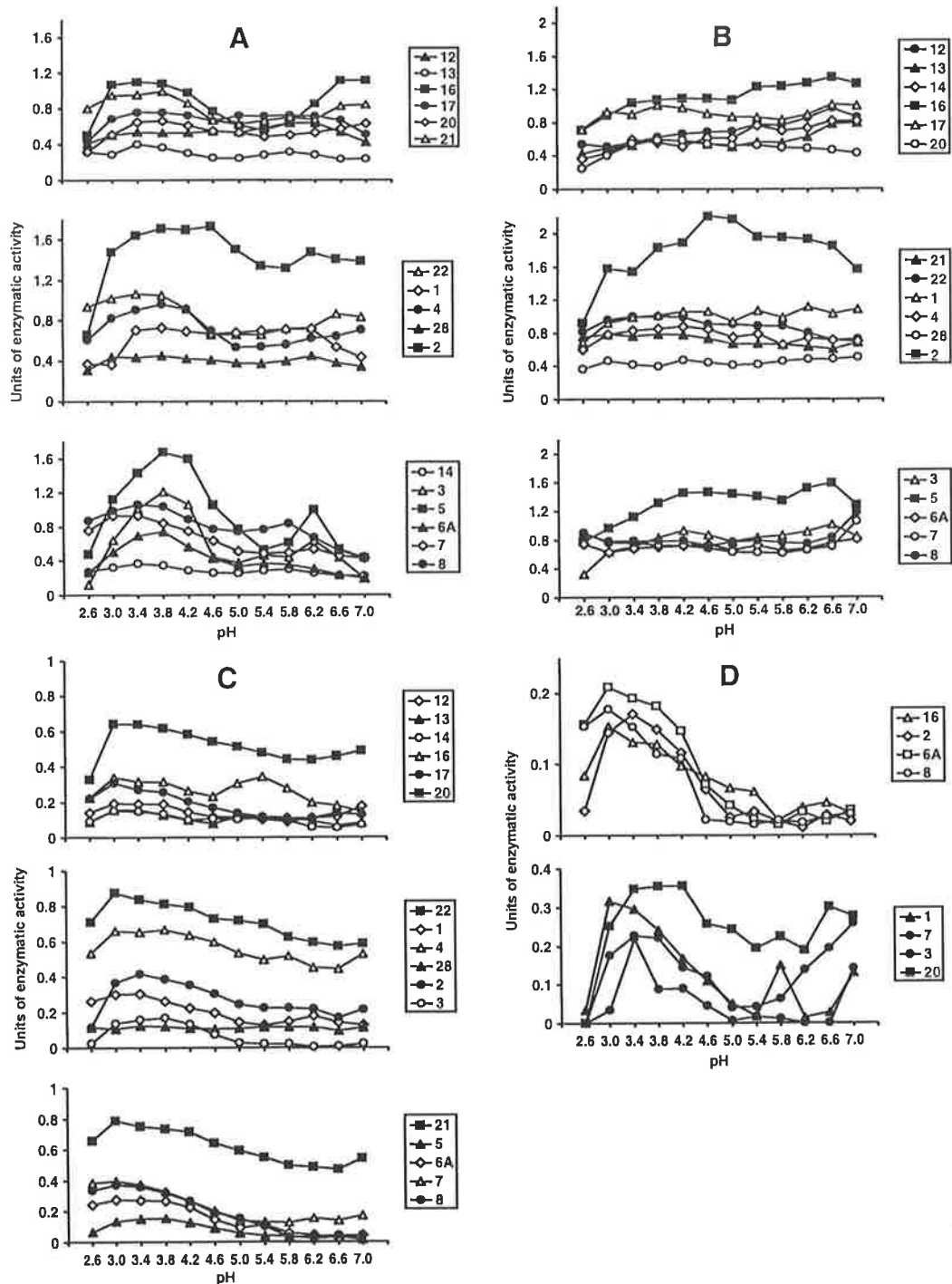
Fig. 1. Influence of pH on the glycosidase activity of *Oenococcus oeni* strains against *p*-nitro-phenyl β-D-glucopyranoside (A), *p*-nitro-phenyl α-D-glucopyranoside (B), *p*-nitro-phenyl β-D-xylopyranoside (C), *p*-nitro-phenyl α-L-rhamnopyranoside (D, open symbols) and *p*-nitro-phenyl α-L-arabinofuranoside (D, close symbols). Values are the mean of duplicate determinations.

Table 2
Total glycosidic activities for *Oenococcus oeni* strains against each of five substrates[a]

Strain	Substrate					Total[b]
	pNP-βGlu	pNP-αGlu	pNP-βXyl	pNP-αAra	pNP-αRha	
28	4.76	5.31	1.34	0.32	0.04	11.77
13	3.47	6.99	1.28	0.74	1.34	12.48
14	3.39	7.51	1.21	0.69	1.35	14.15
6A	4.96	8.48	1.78	1.12	0.80	17.14
12	6.34	8.50	1.66	0.46	1.16	18.12
3	7.00	9.54	0.79	0.57	0.65	18.55
7	7.74	8.84	2.80	0.91	1.62	21.91
20	6.51	5.84	6.15	0.80	2.99	21.96
1	7.28	11.92	2.40	0.77	1.55	23.22
17	8.04	10.89	2.16	0.86	1.64	23.59
8	9.57	9.84	2.23	0.85	1.28	23.77
4	8.51	9.12	6.70	1.05	1.67	27.05
21	9.52	8.48	7.41	1.10	1.66	28.17
16	10.35	13.27	3.11	0.94	0.63	28.30
5	11.19	15.66	0.91	0.57	ND[c]	28.33
22	10.06	10.57	8.55	1.15	1.65	31.98
2	17.29	21.37	3.23	0.81	0.96	43.66

[a] Values are a summation of activities determined at each of the examined pH values between 2.6 and 7.0 (see Fig. 1).

[b] Measures of total glycosidic activity are derived from the totals determined for each substrate.

[c] ND, not determined.

substrate could be identified while also revealing trends across the five substrates tested. Thus strains 16, 22, 2, 5, and 8 were amongst the most highly active group for both pNP-βGlu and pNP-αGlu, whereas strains 21, 22, and 4 were prominent amongst the remaining substrates. For practical reasons, all substrates were not applied at the same concentration in this survey and thus a summation of total activities for each strain across all substrates and pH values will not equate to an absolute measure of total glycosidic activity for that strain. Nevertheless, such summations provide a useful means of achieving an overall ranking of the strains studied. This cumulative value highlights *O. oeni* strains 2 and then 22, 5, 16 and 21 as possessing the greatest overall glycosidic activity (Table 2).

3.2. Temperature optima of glycosidase activities

The ability of observed glycosidase activities to operate over a broad range of temperatures was determined. While absolute values differed, general temperature trends were largely consistent for all strains.

As such, only a selection of strains are reported here, and for clarity, typically those with highest activities (Fig. 2). In general terms, maximal hydrolysis of any of the substrates occurred at temperatures near 40 °C. Nitrophenol release was rapidly reduced as the temperature increased to 60 °C, while a more gradual decline was observed at temperatures below the optimum. Only low activities were observed at temperatures of 20 and 23 °C, which might be considered more relevant to winemaking conditions.

3.3. Influence of ethanol, glucose or fructose on glycosidase activity

Ethanol and sugars (glucose and fructose) have often been reported as the cause of inhibition of glycosidase enzymes (Aryan et al., 1987; Winterhalter and Skouroumounis, 1997; Sanchez-Torres et al., 1998; Grimaldi et al., 2000; Spagna et al., 2002; Barbagallo et al., 2004). The influence of these compounds was therefore investigated in this study. Examination of all 22 strains revealed a limited number of patterns of responses to the inclusion of either ethanol, glucose or fructose in the assay buffer. Accordingly, a representative selection only of the studied strains is reported.

Ethanol at 4% v/v resulted in a marked increase in β-D-glucopyranosidase activity by strains 2 and 4 (Fig. 3, A1), a trend repeated for 70% of all strains studied (data not shown). A reduced or no enhancement was seen for the remaining strains or at higher ethanol contents. In strains with low initial activities,
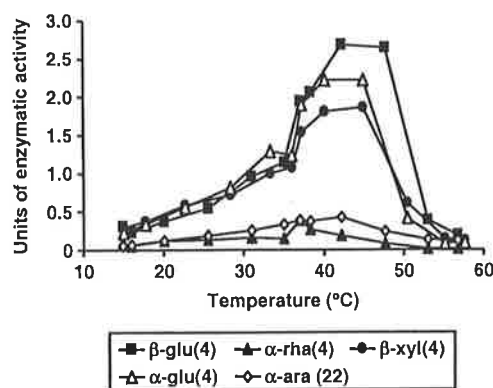

Fig. 2. Influence of temperature on glycosidic activity for a selection of *Oenococcus oeni* strains (in parentheses) and substrates. Values are the mean of duplicate determinations.
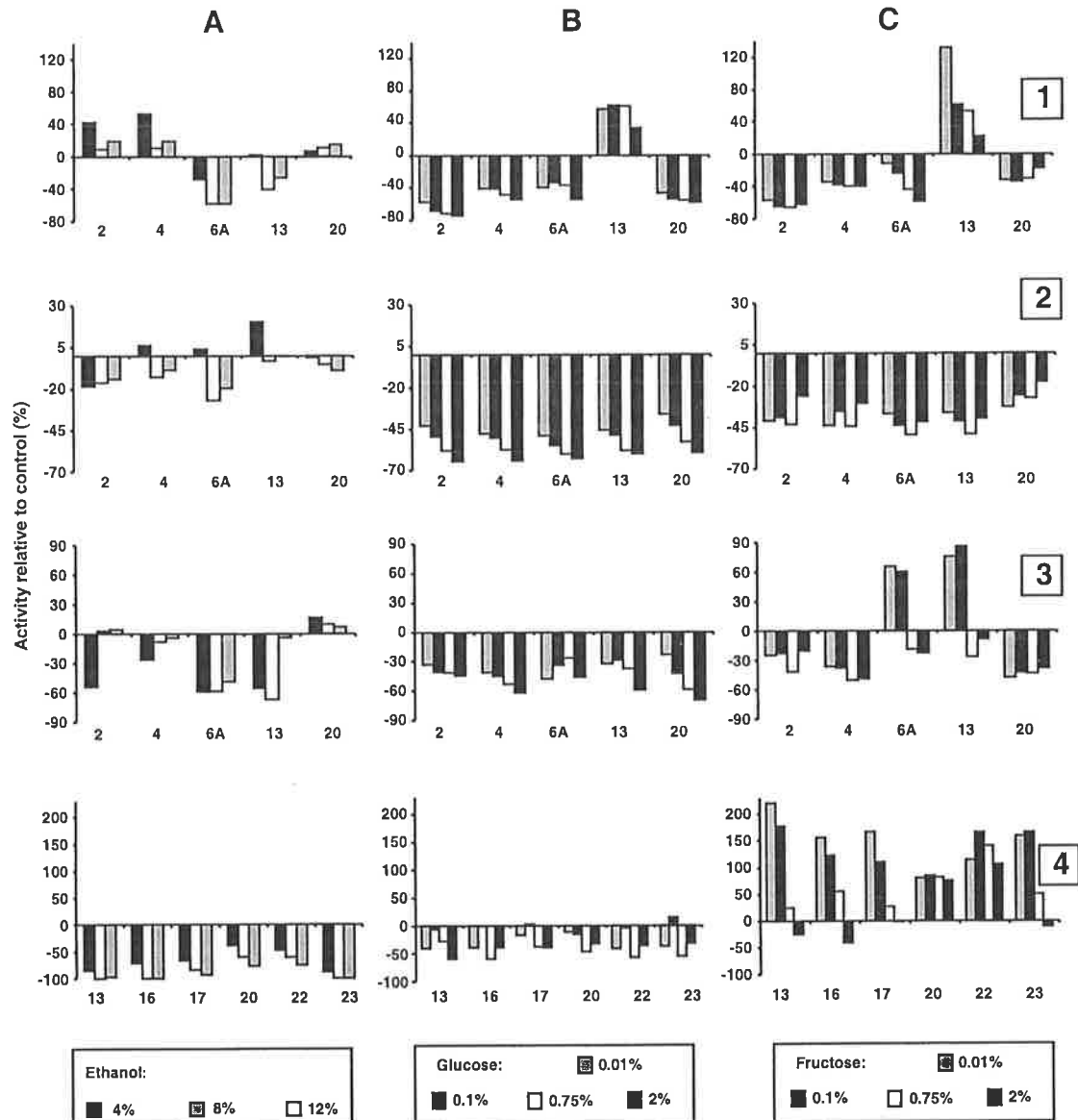
Fig. 3. Glycosidic activity of *Oenococcus oeni* strains observed in the presence of ethanol (column A), glucose (column B) or fructose (column C) against *p*-nitro-phenyl β-D-glucopyranoside (row 1), *p*-nitro-phenyl α-D-glucopyranoside (row 2), *p*-nitro-phenyl β-D-xylopyranoside (row 3) and *p*-nitro-phenyl α-L-arabinofuranoside (row 4). Values are relative (%) to the activity observed for the control performed under the same conditions in the absence of either ethanol or added sugars. Values are the mean of duplicate determinations.

as typified by 6A and 13, higher ethanol contents were typically inhibitory to β-glucopyranosidase. Inhibition of β-glucopyranosidase activity was also observed for glucose and fructose, even when present at only 0.01% w/v (Fig. 3, B1 and C1). Such reductions, typically of the order of 40%, were seen for

approximately 80% of the 15 strains studied (data not shown). Interestingly, a 200-fold increase in the concentration of glucose or fructose did not greatly increase inhibition. Strain 13 stood out from others by displaying a marked enhancement of activity, particularly in the presence of fructose.

Ethanol enhancement was also observed for α-D-glucopyranosidase, again being most apparent at 4% v/v (Fig. 3, A2), albeit to a lesser degree and only occurring in 60% of all strains examined (data not shown). Glucose, still at only 0.01% w/v, was highly inhibitory to α-D-glucopyranosidase. Across all strains, glucose produced an average decline in activity of approximately 45% (Fig 3, B2). The response to fructose was lower (Fig 3, C2).

When considering β-D-xylopyranosidase activity, the impact of ethanol was modest and variable (Fig. 3, A3). At lower concentrations, glucose more so than fructose, produced a modest drop in nitrophenol liberation from pNP-Xyl (Fig. 3, B3 and C3). Determinations of the influence of ethanol, glucose and fructose on α-L-rhamnopyranosidase activity were performed, however, the minimal nature of this activity made some responses difficult to discern clearly. It is noteworthy however that several strains, that is 2, 7, 8, 9, 10 and 20, tended towards enhanced activity in the presence of concentrations of ethanol greater than 0.01%, as well as in the presence of sugars (data not shown).

Results for the α-L-arabinofuranoside substrate showed a strong inhibition by ethanol, so much so that at higher ethanol levels (8% and 12%) there was a total lack of enzymatic activity (Fig. 3, A4). Glucose was also inhibitory, though not as completely, particularly at lower concentrations. The most interesting result was obtained with the inclusion of fructose in the assay medium. Most strains exhibited a large increase in activity (>200% for strain 13) over that of the control when incubated with fructose (Fig. 3, C4). Only at the highest concentration (2%) was fructose inhibitory for some strains.

3.4. Influence of multiple parameters on glycosidase activity

In most settings outside the laboratory, bacteria are likely to face a combination of the factors investigated above. This is certainly true for winemaking. To study the interplay of these factors, glycosidase activity in representative strains was assayed at pH 3.5 with or without 10% v/v ethanol, 0.2% w/v glucose and/or 0.1% w/v fructose, for four substrates. Once again, pNP-αRha was omitted from this survey due to the low activity previously seen for this substrate. For

most of the strains examined, the inclusion of potential inhibitors, ethanol and glucose had little affect on glycosidase activities against pNP-βGlu, pNP-αGlu and pNP-βXyl substrates (Fig. 4). Inhibition was most apparent for the action of strain 4 against pNP-βXyl. For several other strain and substrate combinations, an enhancement of activity was observed, particularly for strains 21 and 22 and pNP-βGlu. Given the stimula-
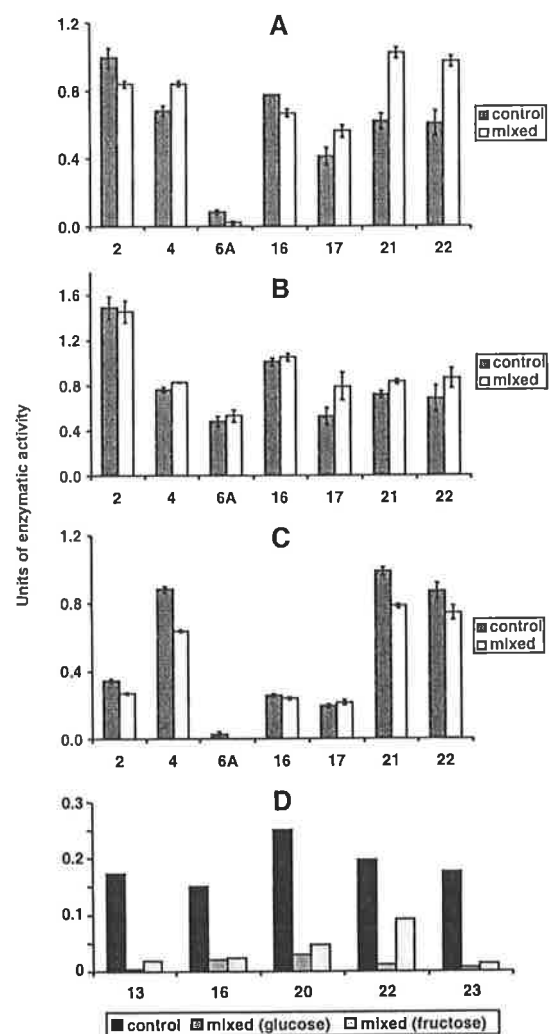

Fig. 4. Influence of ethanol and glucose (A–C) or ethanol and fructose (D) on the glycosidic activities of selected *Oenococcus oeni* strains against (A) p-nitro-phenyl β-D-glucopyranoside, (B) p-nitro-phenyl α-D-glucopyranoside, (C) p-nitro-phenyl β-D-xylopyranoside and (D) p-nitro-phenyl α-L-arabinofuranoside. Values are the mean of triplicate determinations.

tory affect of fructose on α-arabinofuranosidase activity (Fig. 3), an additional assay condition incorporating fructose instead of glucose was tested. For all five strains studied, the inclusion of ethanol and glucose or fructose was highly inhibitory on α-arabinofuranosidase activity (Fig. 4). Only in the case of strain 22 incubated with ethanol and fructose was there any clear suggestion that fructose was at least less inhibitory than glucose.

4. Discussion

Previous studies have either indirectly or specifically sought to demonstrate the presence of glycosidic, typically β-glucosidase (β-glucopyranosidase), activities amongst *O. oeni* associated with wine (McMahon et al., 1999; Grimaldi et al., 2000; Mansfield et al., 2002). To the best of our knowledge, the present work represents the most comprehensive investigation of this type in terms of numbers of strains and particularly, substrates and impacting parameters. This work has succeeded in demonstrating that the possession of glycosidic activities is both widespread amongst strains of *O. oeni* and is not limited to only β-glucosidase. We have also examined the influence of potentially inhibitory parameters such as pH, temperature and selected wine components (ethanol, glucose and fructose), both singularly and in combination on the activity of five glycosidases.

At the commencement of this study, we modified the methodology used in our preliminary report (Grimaldi et al., 2000) and made several improvements in order to accommodate the range of compositional parameters used and the variability in growth seen for the expanded strain collection under investigation. Specifically, a 0.5 M Na_2CO_3 solution was used prior to spectrophotometric quantitation of p-nitrophenol because of its greater ability to alkalise assay samples of low pH. Also, the addition of bacterial biomass was standardised to a final OD_{600} of 0.5 in the assay. With these modifications it has been possible to more precisely assess the influence of pH on glycosidic activities. In keeping with their anticipated appropriateness for use in the liberation of glycosides under wine conditions, most of the *O. oeni* strains studied had relatively high glycosidase activity at pH values between 3.0 and 4.0 (i.e. wine pH). The fact that

this pH optimum does not correspond with the optimal pH for the growth of these organisms (~pH 4.2–4.8; van Vuuren and Dicks, 1993) argues against the influence of pH merely being a consequence of differential growth of the biomass during the assay period.

For the three glycosidases, β-D-glucopyranosidase, α-D-glucopyranosidase and β-D-xylopyranosidase, marked hydrolytic activity was retained above wine pH values, up to neutral, suggesting the possible use of these enzymes at such pH values. In contrast, hydrolysis of pNP-αRha and pNP-αAra quickly diminished with pH values above 4.0. This latter finding also explains our previous inability to detect significant α-L-rhamnopyranosidase activity in 13 *O. oeni* isolates, where measurements were conducted at pH 5.0 (Grimaldi et al., 2000). Also of note is the existence of two peaks of β-D-glucopyranosidase activity at quite distinct pH values (Fig. 1). This result might be indicative of the involvement of multiple enzymes, each with their own pH optimum. Alternatively, it is possible this study has actually revealed another example of that uncommon group of enzymes that have dual pH optima (e.g. Levin and Bodansky, 1966; Gee et al., 1988; Nagashima et al., 1999). Opposing the latter notion are reports of multiple β-D-glucopyranosidases in *Lactobacillus plantarum* (Marasco et al., 1998, 2000) and other lactic acid bacteria. A preliminary search of the *Oenococcus* genome sequence (Coutinho and Henrissat, 1999) also reveals the presence of multiple putative open reading frames with high homology to a consensus sequence derived from more fully characterised β-D-glucopyranosidases from other organisms (data not shown). Detailed characterisation of purified forms of the enzyme(s) in question will resolve this issue.

The finding that few *O. oeni* strains possessed consistently high activities for all substrates used (Table 2), reiterates the importance of strain selection when considering the application of *O. oeni* in the modification of the glycoside profile of wine. Such variability might also be considered indicative of the involvement of multiple enzymes with limited substrate specificity, rather than a single enzyme able to act on all the substrates tested. The presence of minimal α-L-rhamnopyranosidase and α-L-arabinofuranosidase activities in particular agrees with recent findings that *O. oeni* has low specificity for this important group of disaccharide aroma-related com-

pounds in wine (D'Incecco et al., 2004). The industrial significance of these activities, which is nonetheless detectable in these strains, does still warrant further investigation.

In investigating the influence of specific physiochemical parameters, some light has been shed on the scope of the applicability of the enzymes examined in this study. Retention of some glycosidic activity at temperatures below 20 °C (Fig. 2) is of importance for the wine industry, given that wines are usually stored in this range during the MLF. The greatest activity was observed above 35 °C. Whilst this is outside the typical operating range for winemaking, it becomes more important for alternate processing of small batches of juice or wine or else other industrial applications (e.g. Mourgues and Bénard, 1982; Girard et al., 1997).

The enhancing effect of ethanol, occurring most often at lower concentrations (e.g. 4% v/v; Fig 3), mirrors previous findings for *O. oeni* (Grimaldi et al., 2000) and yeast biomass (Blondin et al., 1983; Gondé et al., 1985), though expression of a β-glucosidase gene from *L. plantarum* has been shown to be repressed by 12% (v/v) ethanol (Spano et al., 2005). Given the duration of the assay used in this study it is more likely that observed influences of ethanol related to existing enzyme rather than *de novo* synthesis. Here, ethanol partially inhibited glycosidase activities, with complete inhibition being most often seen for α-arabinofuranosidase activity. The retention of glycosidase activity in the presence of up to 10% ethanol supports the potential use of such enzymes in the processing of alcoholic beverages, such as wine and beer, with ethanol concentrations within this range. A characterisation of purified enzymes is necessary to determine the precise manner in which ethanol influences their activity.

Non-distilled alcoholic beverages often contain residual sugars, which in the case of wine are mainly glucose and fructose (Boulton et al., 1996). In keeping with numerous reports for glycosidase enzymes from various sources (Aryan et al., 1987; Cordonnier et al., 1989), the presence of such sugars was found to reduce activity (Fig 3). The key exception being a marked enhancement of some activities by fructose even when present at concentrations as high as 0.75% v/v, the specified maximum residual sugar content for dry table wines (Iland and Gago, 2002). As a general

trend, although the threshold concentration for appearance of fructose inhibition was as at least as low as 0.01%, a 75-fold increase in fructose concentration did not produce a proportional increase in inhibition. This represents an important distinction between *O. oeni* and other sources of glycosidases wherein the degree of inhibition is much more closely linked to the concentration of sugar present (Aryan et al., 1987; Cordonnier et al., 1989).

In summary, in the single parameter experiments, ethanol, sugars (glucose and fructose) and pH, are able to impact upon the glycosidic ability of *O. oeni* cells in a manner that ranges between highly inhibitory to highly stimulatory. By additionally examining the influence of these parameters when applied in a multifactorial fashion, it has been possible to gain an insight into the potential of these activities under conditions more analogous to an industrial setting. Most notably, combination of these parameters generally resulted in a more moderate influence on detectable glycosidase activity, especially that against pNP-βGlu, pNP-αGlu and pNP-βXyl (Fig. 4). The ability to hydrolyse pNP-αAra (and pNP-αRha; data not shown) was again found to be weak and, in this multifactorial experiment, one sensitive to sugars. Thus, whereas fructose on its own was at times seen to be stimulatory, when supplied with ethanol at pH 3.5 a marked inhibition was apparent. This observation is a likely explanation for the inability of D'Incecco et al. (2004) to detect any glycosidase activity in wine (containing ethanol) other than β-glucosidase. There are no obvious links between the findings for the multifactorial experiments compared to those in which single parameters were studied. These observations serve only to highlight the complexity of the interactions involved and reiterates the importance of further work with purified glycoside enzymes. Nevertheless, several strains stand out as possessing high levels of activity against the various substrates studied. Consideration of such findings along with the relative sensitivity of each activity to the wine-related inhibitors studied allows for the selection of strains for further evaluation: either during MLF of wine or else upon incubation with purified grape glycosides. In addition, work is currently underway in this laboratory to purify the probably numerous glycosidase enzymes found in these bacteria, ahead of their more precise characterisation.

243

Acknowledgments

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