Chasing wine aroma —

Does *Oenococcus oeni* have the potential to release aroma compounds from authentic grape precursors?

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The essence of wine lies predominantly in its colour, aroma and flavour. It is the seemingly endless diversity of these attributes that keeps the consumer fascinated with wine. Winemakers are therefore continually seeking new and rediscovering old techniques to enhance varietal character and to modulate and increase the sensorially diverse qualities of wine. Biological processes feature heavily among the many options available to the winemaker.

Wine colour, aroma and flavour are defined by a suite of compounds that are largely derived from naturally occurring constituents of the grape berry (including sugars, acids, nutrients and glycosylated aglycons), oak wood when used, and micro-organisms during fermentation. Yeasts, with the ability to ferment a large amount of sugar, are able to perform the primary fermentation. This concomitantly leads to a dramatic increase in volatile and non-volatile constituents that largely characterise the wine. Thus, fermentation produces these various components overlaid with the varietal character of wine.

On the other hand, the secondary malolactic fermentation (MLF), as its name suggests, is principally a decacidification step, which is used to manicure the acidity of certain wine types and confer added microbial stability to the product. In terms of aroma impact, the MLF is not a passive process as is seen with diacetyl production. Indeed, recent research suggest that malolactic (ML) bacteria not only interact with the yeast secondary metabolites (fatty acids, carboxyls, esters, acids and alcohols), but also with several of the important non-volatile glycoconjugates, the grape derived flavour precursor compounds that distinguish wine varietal character (Figure 1).

The latent pool of aroma compounds

Grape-derived glycoconjugates constitute a latent pool of volatile aglycons that can be an additional source of wine aroma and flavour compounds (Williams et al. 1989). Broadly, these aglycons are grouped together according to their structure; norisoprenoids (e.g. damascenone), volatile phenols and other benzene derivatives (e.g. raspberry ketone), monoterpenes (e.g. linalool, nerol and geraniol), and aliphatics (e.g. hexanol). In the non-floral variety, Chardonnay, which is extensively grown throughout Australia, almost 200 different aglycons have been identified in grape juice, prior to fermentation (Sefton et al. 1993).

The flavourless glycoconjugates are either glucosides or disaccharide or trisaccharide glycosides. They all contain a glucosyl moiety, but for the disaccharide glycosides, the glucose is further substituted with α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-xylopyranosyl or β-apiofuranosyl sugars (Figure 2). In the grape, the disaccharide glycosides are
the dominant storage form of aroma substances (Williams et al. 1982). Many of the wine volatile compounds can be released from their flavourless glycoconjugate precursors by either acid or enzymatic hydrolysis.

The glycosidases, which are involved with the enzymatic cleavage of the disaccharide glycosides, include α-L-arabinofuranosidase, α-L-rhamnopyranosidase, β-D-xylopyranosyl, β-apiofuranosyl and β-D-glucopyranosidase (also referred to as β-D-glucosidase). The liberation of the volatile aglycon from the disaccharide by yeast involves the sequential release of the sugar moieties (Günata et al. 1988), where the first step is the hydrolysis of the inter-sugar link and the second step, a β-glucosidase activity, for the cleavage of the remaining β-glucosidic moiety.

**IN SEARCH OF SOURCES OF GLYCOSIDASES FOR AGLYCON LIBERATION IN WINE**

The yeast most commonly used to initiate alcoholic fermentation, *Saccharomyces cerevisiae*, has only limited ability to unleash the aroma compounds bound in the glycoconjugate form. This leaves a substantial pool of glycosides for other micro-organisms present during grape vinification to act upon.

In contrast, several non-*Saccharomyces* genera, including strains of *Brettanomyces/Dekkera* sp., *Candida stellata*, *Debaryomyces* sp., *Kloeckera apiculata*, *Metschnikowia pulcherrima* and *Pichia anomala*, all of which are often isolated from grape juice, have been shown to exhibit varying degrees of β-glucosidase activity (Charoenchai et al. 1997). Most of these studies either used *p*-nitrophenol-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-glucopyranoside or arbutin as a substrate to demonstrate the presence of β-glucosidase activity. However, this assay technique does not necessarily predict activity against natural wine glycosides. This is because only a limited number of aglycons are present as mono-glucosides in grape juice. Few studies have investigated other glycosidase activities with these non-*Saccharomyces* species. The release of numerous volatile compounds by a strain of *K. apiculata* from a glycosidic extract of Muscat grape juice infers that this species does possess other glycosidases in addition to β-glucosidase enabling the yeast to liberate aglycons from the glycoconjugates (Mendes Ferreira et al. 2001). The co-fermentation by a *Debaryomyces pseudopolymorphus* strain with commercial wine yeast *S. cerevisiae* VIN13 of a Chardonnay grape must increased the liberation of citronellol, geraniol and nerol (Cordero Otero et al. 2003). Thus, the presence of non-*Saccharomyces* species at the onset of alcoholic fermentation may have a greater potential to contribute to the liberation of some aglycons from the flavourless precursor glycoside during fermentation.

It is intriguing that yeast have a large array of various extracellular hydrolytic enzymes (i.e. pectinases, proteases, glucanases, lichenases, cellulases, xylanases and amylases). However, the glycosidases do not feature prominently and, when present, they appear not to be produced in abundant quantities.
Grapevine glycosidases, even though present in grape juice, may have minimal effect on enhancing varietal aroma during vinification principally because of poor stability at wine pH and high glucose concentration affects enzyme activity. Several grapevine fungal pathogens (such as *Aspergillus* and *Botrytis*) produce numerous glycosidases that have high activity for purified wine glycosides. Commercial preparations of fungal glycosidases, usually sourced from *Aspergillus sp.*, can be used to liberate more flavour aglycons into the wine, but may not function well under key wine conditions of low pH (3.0-4.0), ethanol content (9-16% v/v) or residual sugar content (< 10 g/L) (Winterhalter and Skouroumounis 1997). These commercial enzyme preparations often contain other activities that may have undesirable effects on the wine.

An alternative approach to preparing a crude or pure extract of glycosidases for addition to grape juice, is to express the appropriate enzyme gene in *S. cerevisiae*. Several studies have demonstrated the heterologous expression of fungal or non-*Saccharomyces* β-D-glycosidases, α-L-rhamnosidases or α-L-arabinofuranosidases in industrial wine yeast strains as successful tools to increase the free monoterpenic content of wine. For example, the *rhaA* gene (α-L-rhamnosidase) from *Aspergillus aculeatus* has been successfully expressed in conjunction with the *Candida molischiana* β-D-glucosidase in an industrial wine yeast strain to increase the pool of isoalol, nerol and α-terpineol in Muscat wine (Manzanares et al. 2003). This type of approach using genetically modified wine yeast to increase wine volatile content might be an alternative option for the future, subject to market acceptance of this approach.

Bacteria represent another group of micro-organisms that are commonly associated with grapes and winemaking. Those belonging to the family of lactic acid bacteria (LAB) are extensively used in the fermentation of various foodstuffs. The other most commonly encountered group of bacteria are from the acetic acid bacteria (AAB) family. The AAB are considered as spoilage organisms in wine as they convert the ethanol to acetic acid, the basis of vinegar production. Species of *Acetobacter* are commonly isolated from a large variety of fruits, including grape berries. There is limited knowledge on the glycosidases of AAB; however, a β-glucosidase has recently been described from *Acetobacter xylinium* (Tajima et al. 2001), and it is postulated that the β-glucosidase may play a role in the cellulytic activity of this species. The *Acetobacter* species found in wine may also be capable of liberating some of the latent aroma compounds of wine, however, their contribution to this aroma pool is insignificant, as the wine will also be spoiled.

Glycosidase activities in LAB have been observed in *Lactobacillus* sp. isolated from beer (α-glucosidase) or cassava (β-glucosidase), *Leuconostoc mesenteroides* isolated from cassava (β-glucosidase) and *Oenococcus oeni* from wine (several glycosidase activities, in particular β-glucosidase). The β-glucosidase characterised from *Lactobacillus plantarum* (isolated from fermented cassava) can degrade cyanogenic glycosides and this has been proposed as a mechanism to detoxify food plants (Lei et al. 1999). β-Glucosidases or other glycosidase activities have not been studied in wine lactobacilli and pediococci.

**MLF and Aroma/Flavour Aglycone Liberation by *Oenococcus oeni***

*Oenococcus oeni* is the preferred LAB for initiating MLF in Australian wine. However, the first studies on the presence and type of glycosidic activities in *O. oeni* were equivocal. Using *p*-nitrophenol conjugates as substrates, McMahon et al. (1999) were unable to demonstrate any significant glycosidase activities. Vivas et al. (1997) inferred glycosidase activity in *O. oeni*, as this bacterium was able to release the anthocyanin malvidin from its glycosylated form (malvidin-3-glucoside). Studies at the AWRI also obtained evidence for β-glucosidase activity in commercial strains of *O. oeni* by cleavage of the chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside) on solid media. In a comprehensive study using synthetic *p*-nitrophenol substrates, Grimaldi et al. (2006) showed that several commercial *O. oeni* strains possess disaccharidic as well as glucosidic activities, but the activity of these enzymes was strongly dependent upon wine conditions, such as pH, ethanol and residual sugar content. Only recently had the glycosidase activities of *O. oeni* been tested with authentic wine glycosidic substrates, again with equivocal results (McMahon et al. 1999). Whereas Boido et al. (2002) quantified the release of glycosylated volatile precursors in a Tannat wine, Mansfield et al. (2002) surprisingly observed no glycosidic activity in Viognier glycosidic extracts.

As part of a project conducted by the AWRI Wine Microbiology Team by a visiting Italian PhD student, Nadia D’Incecco, we investigated the ability of *O. oeni* to liberate Chardonnay volatile aroma compounds from their glycoconjugates during MLF. To ensure that the wine glycosides used in this work had not been subjected to glycosidic activities of bacterial origin, we prepared the wine in the Hickinbotham Roseworthy Wine Science Laboratory from hand-picked, disease-free Chardonnay grapes (Barossa Valley 2000) as the source of wine glycosides. A glycosidic extract was prepared from the Chardonnay wine and introduced into a chemically defined wine medium (CDW). Various combinations of MLF and enzymatic treatments were used to specifically characterise the glycosidic activities of *O. oeni* strain Lalvin EQ54, a popular MLF strain. The enzymatic treatments involved the addition of commercial preparations of β-glucosidase, α-L-rhamnopyranosidase and α-L-arabinofuranosidase; however, α-L-rhamnopyranosidase is known to contain 0.006% of β-glucosidase (Cordonnier et al. 1989). Earlier work had suggested that *O. oeni* strains might only possess limited activity of the exo-glycosidases, including α-L-rhamnopyranosidase and α-L-arabinofuranosidase (Grimaldi et al. 2000). Using various combinations of bacteria and the exogenous enzymes, we were able to demonstrate sequential release of the sugar moieties from disaccharide glycosides; a fact that had not
been previously verified (D’Incecco et al. 2004).

Residual sugar in wine, in particular glucose, has been previously shown to reduce bacterial β-glucosidase activity, and the inhibitory effect of glucose on yeast and fungal glycosidases, in particular β-glucosidase, has been well documented. Despite the reduction in β-glucosidase activity by the residual sugar of wine, prolonged exposure of wine glycosides to glycosidic enzymes may lead to significant release of aroma aglycons. It may be the case, therefore, that the recent trend to shortening the exposure of white wines to MLF by using high cell density inocula will reduce the effectiveness of MLF associated hydrolytic activities. For the purpose of experimentation, we wanted to minimise any effect of sugar inhibition and therefore optimised the sugar concentration to sustain bacterial growth while reducing the effect on the enzyme activities. From preliminary experiments that examined the ability of O. oeni to grow in CDW with different concentrations and proportions of glucose and fructose, it was found that 1 g/L glucose and 10 g/L fructose in the CDW allowed satisfactory growth while glucose and fructose, it was found that 1 g/L glucose and 10 g/L fructose in the CDW allowed satisfactory growth while minimising the potential inhibition by glucose of the various bacterial glycosidic activities examined in this study.

The O. oeni strain Lalvin EQ54 was inoculated into CDW in the presence and absence of the glycosidic extract, which was added to a concentration equivalent to that found in the Chardonnay wine from which they were extracted. As the bacterial culture had been preadapted to the CDW, there was no initial lag period or decline in bacterial number, thus metabolism of the L-malic acid commenced almost immediately (Figure 3). Bacterial growth in CDW was not affected by the presence of the wine glycosides, but the rate of malic acid metabolism was greatly enhanced (Figure 3). The stimulation of bacterial growth, but not of MLF, by wine components, including glycosylated components, has, however, been noted by other researchers (Guilloux-Benatier et al. 1995).

Glycosidase activity by the O. oeni strain Lalvin EQ54 was monitored in the various treatments following MLF using the glycosyl-glucose assay developed at the AWRI in collaboration with The University of Adelaide (Iland et al. 1996). The Chardonnay glycosidic extract, which was added to the synthetic wine at a concentration of 65 µM, and is in the lower range expected for a Chardonnay wine (Selton et al. 1999), would be expected to contain both monosaccharide and disaccharide glycosides (Williams et al. 1989). Both the commercially available β-glucosidase preparation and O. oeni-derived β-glucosidase enzyme, reduced the glycosyl-glucose concentration by 17-20%, inferring that the Chardonnay glycoside extract contained at least this proportion of monosaccharide glycosides. The possibility that the enzyme has restricted specificity for some glucosides cannot be discounted (Figure 4). The presence of the α-L-rhamnopyranosidase and α-L-arabinofuranosidase preparations in combination with O. oeni Lalvin EQ54 derived β-glucosidase, greatly increased the hydrolysis of sugar moieties from the wine glycosides (approximately 45% released), which is similar to that formed when all three enzyme preparations were added together to the synthetic wine with glycoside extract. A 100% recovery of glycosyl-glucose would have been expected with these two enzymes alone; however, the commercially available α-L-rhamnopyranosidase preparation contains some β-glucosidase activity (Cordonnier et al. 1989). The release of glycosyl-glucose by the O. oeni Lalvin EQ54 ferment β-glucosidase plus commercial α-L-rhamnopyranosidase and α-L-arabinofuranosidase preparations was equivalent to the treatment with all three enzyme preparations.

O. oeni strain Lalvin EQ54 enzyme activity for β-glucosidase, α-L-rhamnopyranosidase and α-L-arabinofuranosidase was determined at the end of MLF using p-nitrophenol substrates. Cell associated activity for all three enzymes was detected at the end of MLF.

Identity of the Chardonnay wine aglycons released by the various MLF or enzymatic treatments was investigated by
of the three commercial enzyme preparations; however, not with the bacterial β-glucosidase. Similar low concentrations of α-terpineol released from a Muscat wine glycosidic extract have been observed (Ugliano et al. 2003), and from glycosylated Tannat wine components (Boido et al. 2002). Again the bacterial metabolism of α-terpineol cannot be discounted. The monoterpenes, linalool, nerol and geraniol, were not detected in this Chardonnay extract. However, release of these volatile compounds from Tannat wine and Muscat wine have been observed.

There was some release of the volatile phenol vanillin and benzene derivative 4-hydroxybenzoic acid by the O. oeni Lalvin EQ54 β-glucosidase. Methyl vanillate, a volatile phenol, was released in the presence of the three commercial enzyme preparations and to a small degree by the two enzymes, α-L-rhamnopyranosidase and α-L-arabinofuranosidase. Tyrosol, a benzenoid compound was also identified in some of the treatments. O. oeni Lalvin EQ54 was unable to liberate tyrosol; however, when α-L-rhamnopyranosidase and α-L-arabinofuranosidase were added, there was a consequent release of tyrosol from the glycosidic extract.

The greater release of aglycons by treatment with the three glycosidic enzyme preparations treatment compared to the treatment with bacteria and purified α-L-rhamnopyranosidase and α-L-arabinofuranosidase preparations could be due to limiting β-glucosidase activity associated with O. oeni Lalvin EQ54. However, the partial metabolism of aglycons by the bacterial culture cannot be discounted in these experiments.

CONCLUSIONS
The results from this study and those of others indicate that O. oeni strains do possess limited glycosidase activities that will result in the liberation of volatile compounds from their glycoconjugate precursors during MLF as a result of the metabolic activity of O. oeni. Many of these aglycons liberated by O. oeni are volatile compounds that, in appropriate concentration, would contribute to the aroma or bouquet of wine. In conclusion, this work demonstrates that an O. oeni strain has the potential to release aroma compounds from authentic grape precursors. The extent to which this capacity is of practical significance in commercial wine production is at present unclear but presents a proposition that clearly can be tested.

ACKNOWLEDGMENTS
Nadia D’Incecco undertook her PhD studies at the University of Padova, Italy, with Drs Anna Lante and Paolo Spettoli, and as part of a collaboration, a portion of her studies were conducted at the AWRI. The authors thank Dr Mark Sefton for assisting with the interpretation of the GC/MS data and expert advice. This project is supported by Australia’s grapegrowers and winemakers through their investment body the Grape and Wine Research Development Corporation, with matching funds from the Australian Government.
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