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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Keywords: Glycosidase; Lactic acid bacteria; Wine; MLF; Aroma; Flavour

1. Introduction

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-
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

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strains and source of Oenococcus oeni used in this study^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lallemand O.S.U. (VI 77)</td>
</tr>
<tr>
<td>2</td>
<td>Lalvin 4× (VL 92)</td>
</tr>
<tr>
<td>3</td>
<td>Inobacter</td>
</tr>
<tr>
<td>4</td>
<td>Bitec Vino</td>
</tr>
<tr>
<td>5</td>
<td>Lalvin-Inobacter</td>
</tr>
<tr>
<td>6A</td>
<td>Lalvin MT01 Standard</td>
</tr>
<tr>
<td>7</td>
<td>Lallemand 3× (E218)</td>
</tr>
<tr>
<td>8</td>
<td>CHR Hansen Viniflora</td>
</tr>
<tr>
<td>9</td>
<td>CHR Hansen Viniflora</td>
</tr>
<tr>
<td>10</td>
<td>Lalvin EQ54 MBR</td>
</tr>
<tr>
<td>11</td>
<td>Lalvin MCW</td>
</tr>
<tr>
<td>12</td>
<td>Lalvin 3× 1Step</td>
</tr>
<tr>
<td>13</td>
<td>Lalvin IB Standard</td>
</tr>
<tr>
<td>14</td>
<td>Enoferm Alpha</td>
</tr>
<tr>
<td>15</td>
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<td>16</td>
<td>Lalvin OSU MBR</td>
</tr>
<tr>
<td>17</td>
<td>Lallemand No. 3</td>
</tr>
<tr>
<td>18</td>
<td>Lallemand No. 4</td>
</tr>
<tr>
<td>19</td>
<td>Lallemand No. 5</td>
</tr>
<tr>
<td>20</td>
<td>Lallemand No. 8</td>
</tr>
<tr>
<td>21</td>
<td>Lallemand No. 2</td>
</tr>
<tr>
<td>22</td>
<td>Lallemand No. 9</td>
</tr>
</tbody>
</table>

^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<sub>600</sub>) 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<sub>2</sub>HPO<sub>4</sub>) 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<sub>600</sub> in the assay of 0.5. Substrate solutions (20 μl) were added to give the following final concentrations: p-nitrophenyl 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<sub>2</sub>CO<sub>3</sub> 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 (μQuantr®, 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<sub>2</sub>CO<sub>3</sub> 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<sub>2</sub>CO<sub>3</sub>. 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 pNP-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 pH>5.8, while in other strains maximal activity was centred around a single pH of 3.8.

3.1.2. α-L-Glucopyranosidase

As observed for β-D-glucopyranosidase activity, all isolates possessed an activity against pNP-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 (pNP-αRha), with a maximum of only 0.2 units was observed. Again, lower pH values gave optimal hydrolysis of pNP-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.
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 \( p\)-NP-\( \beta\)-Glu and \( p\)-NP-\( \alpha\)-Glu, 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 \( \beta\)-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, 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 \( p\)-NP-\( \beta\)-Glu and \( p\)-NP-\( \alpha\)-Glu, 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).

### Table 2

Total glycosidic activities for \( Oenococcus\) oeni strains against each of five substrates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Total(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( p)-NP-( \beta)-Glu</td>
<td>( p)-NP-( \alpha)-Glu</td>
</tr>
<tr>
<td>28</td>
<td>4.76</td>
<td>5.31</td>
</tr>
<tr>
<td>13</td>
<td>3.47</td>
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<tr>
<td>14</td>
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<tr>
<td>6A</td>
<td>4.96</td>
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<tr>
<td>12</td>
<td>6.34</td>
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<tr>
<td>2</td>
<td>17.29</td>
<td>21.37</td>
</tr>
</tbody>
</table>

\(^a\) 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.
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-
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 Na₂CO₃ 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 OD₆₀₀ 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. Mourguès 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 glycosidas 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 glycosidases 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.
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