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PROOF

Development and assessment of a recombinant *Saccharomyces cerevisiae* wine yeast producing two aroma-enhancing β-glucosidases encoded by the *Saccharomycopsis fibuligera* BGL1 and BGL2 genes

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Abstract - The distinctive varietal flavour of grapes and wine is affected by the absolute and relative concentrations of many compounds, including monoterpene alcohols such as citronellol, geraniol, hotrienol, linalool, nerol and a-terpineol. Monoterpenols in grapes and wine can occur either as free volatile and odorous molecules, or as glycosidically bound, odourless, non-volatile complexes. β-Glucosidases constitute one group of glycosidases that can help to unleash this latent pool of grape-derived volatile aglycons and provide an additional source of wine aroma and flavour compounds. However, yeast (*Saccharomyces cerevisiae*) and bacteria (*Oenococcus oeni*) most commonly used to initiate alcoholic and malolactic fermentation during winemaking have only limited ability to liberate the aromatic terpenols as well as other aglycones bound to saccharides. Therefore, the purpose of this study was to clone, integrate and express two β-glucosidase genes (*BGL1* and *BGL2*) from the dimorphic yeast, *Saccharomycopsis fibuligera*, in a commercially available wine yeast strain, VIN13. Using *p*-nitrophenyl-β-D-glucopyranoside as a synthetic substrate, enzyme assays and kinetic studies indicated that both these two extracellularly produced β-glucosidases were able to cleave glycosidic bonds efficiently. Subsequently, wine from Chenin blanc, Gewürtztraminer and Pinotage grapes was made with the transformed and untransformed *S. cerevisiae* VIN13 strains, and compared. A series of analyses indicated that wines produced by the β-glucosidase-producing VIN13 strain contained slightly higher levels of terpenols in comparison with the wines produced by the untransformed control VIN13 strain. Surprisingly, the wines produced by the transformed strain also contained increased ester concentrations. Many of these fragrant compounds, when produced in appropriate concentrations, would contribute to the fermentation bouquet of wine. The extent to which this acquired capacity of the transformed wine yeast is of practical significance in large-scale wine production is at present unclear but presents a worthwhile proposition for further investigation.

Key words: β-glucosidases; *Saccharomycopsis fibuligera*; *Saccharomyces cerevisiae*; wine yeast; wine aroma.

INTRODUCTION

The essence of wine lies primarily in its colour, aroma and flavour. The seemingly endless and fascinating diversity of these attributes is defined by a set 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 microorganisms during fermentation (Lambrechts and Pretorius, 2000; Pretorius, 2000, 2003). Winemakers are continually seeking techniques to unleash the latent pool of volatile aglycons masked in the flavourless grape-derived glycoconjugates in order to enhance varietal character and to modulate and increase the sensorially diverse qualities of wine. These aglycons include norisoprenoids (e.g. damascenone), volatile phenols and other benzene derivatives (e.g. raspberry ketone), aliphatics (e.g. hexanol) and monoterpenes (e.g. citronellol, geraniol, hotrienol, linalool, nerol and a-terpineol) (Bartowsky *et al*., 2004).

Monoterpenes and monoterpene alcohols play an important role in the aroma and flavour of Muscat grape varieties and wine. However, these compounds also occur in other cultivars, such as the non-floral variety, Chardonnay, in which 200 different aglycons have been identified (Sefton *et al*., 1993). In the latter, these compounds exist as subtle supporting aromas and flavours, although they are less pronounced, and other odorous flavour compounds might play a larger role in the varietal aroma and flavour of the wine. The fragrances and flavours they impart are therefore not limited to the perfume-like aroma of muscat, but also include other aromas, such as spicy, peppery, smoky and grassy odours. The major fractions of the monoterpenes and monoterpene alcohols occur in the grape as glycosidically bound forms (Williams *et al.*, 1982; Günata *et al*., 1985; Voirin *et al*., 1992), which render them non-volatile and flavourless. The flavourless glycoconjugates are either glucosides or disaccharide or trisaccharide glycosides (Bartowsky *et al*., 2004). The sugar moieties to which these monoterpenols are bound include 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides (vicianosides), 6-*O*-β-D-xylopyranosyl-β-D-gluco-pyranosides (primverosides), 6-*O*-β-D-glucopyranosyl-β-D-glucopyranosides (gentio-biosides), 6-*O*-β-D-rhamnopyranosyl-β-D-glucopyranosides (rutino-sides), and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides (Williams *et al*., 1982; Günata *et al*., 1988).

The glycosidic bonds present in the glycoconjugates can

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be liberated by two methods: the first, acid hydrolysis, has the disadvantage of possibly changing the intrinsic varietal aroma of the wine. The second method is by way of enzymatic hydrolysis, which has attracted attention as a means of improving the varietal aroma and flavour of wine. The mechanism by which this hydrolysis works is now well established and entails specific glycosidases active in two successive steps (Günata *et al*., 1985, 1988). In the first step, the action of an a-arabinofuranosidase, a-rhamnosidase, b-xylosidase or b-apiosidase is necessary to cleave the inter-sugar-linkages and this is followed by the second step, during which β-glucosidases liberate the aglycones (Günata *et al*., 1988). In the case where the disaccharide moiety consists of two glucose units, only the action of a β-glucosidases is needed to facilitate the complete reaction (Haisman and Knight, 1967). The aglycone does not necessarily consist of a terpenol; other aglycones include aliphatic alcohols, alkylphenols, sesquiterpenoids, norisoprenoids and resveratrol. The release of these molecules can also be to the advantage of the improvement of wine aroma and flavour, as well as having other beneficial properties, especially concerning the release of the antioxidant, resveratrol (Vrhovsek *et al.,* 1997*;* Becker *et al.,* 2003).

Glycosidases occur in nearly every organism where they perform a variety of functions. However, not all of these enzymes are suitable for use in winemaking; for example, those from plants exhibit high pH optima (pH 5 for *Vitis vinifera* grapevine), and the enzymes are virtually inactive at pH 3–4, the pH of wines and grape juices (Aryan *et al*., 1987). Also, the glycosidases from fungi are notoriously inhibited by glucose concentrations even as low as 1 to 1.5%. These enzymes are also more active at high pH values (Woodward and Wiseman, 1982). Several fungal β-glucosidasess have been studied with the aim of future applications (Raynal and Guerineau, 1984; Kuranda and Robbins, 1987; Machida *et al*., 1988; Günata *et al*., 1990a; Gueguen *et al*., 1994; Rosi *et al*., 1994; Gueguen *et al*., 1995; Saha and Bothast, 1996; Skory *et al*., 1996; Yan and Lin, 1997; Riou *et al*., 1998). Bacterial glycosidases generally have the disadvantage of being active at high temperatures (50 ºC and higher) (Woodward and Wiseman, 1982). Commercial strains of the yeast (*Saccharomyces cerevisiae*) and bacterium (*Oenococcus oeni*) most commonly used to initiate alcoholic fermentation and malolactic fermentation, respectively, have only limited aroma-enhancing enzyme activities and this leaves a substantial pool of grape-derived glycosides untapped (Cordero Otero *et al*., 2003; D'Incecco *et al*., 2004).

An alternative approach to preparing a crude or pure extract of glycosidases for addition to grape juice, is to use mixed yeast cultures to initiate wine fermentations. Recently, it has been reported that 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 might have a greater potential to contribute to the liberation of some aglycons from the flavourless precursor glycoside during fermentation. However, the presence of certain non-*Saccharomyces* yeasts during wine fermentations might also cause off-flavours. Therefore, the risk associated with mixed culture fermentations has prompted wine scientists to investigate the expression of heterologous genes encoding aroma-enhancing enzymes in wine yeast strains of *S. cerevisiae* as an alternative strategy.

Several recent studies have demonstrated the potential of heterologous expression of fungal and non-*Saccharomyces* β-glucosidasess, a-arabinofuranosidases and a-rhamnosidases in industrial wine yeast strains as a viable option to increase the free monoterpene content of wine. For example, the α-rhamnosidase gene (*rhaA*) from *Aspergillus aculeatus* has been successfully expressed in conjunction with the *Candida molischiana* β-glucosidases gene in an industrial wine yeast strain to increase the pool of linalool, nerol and a-terpineol in Muscat wine (Manzanares *et al*., 2003). The use of such genetically modified wine yeast to increase wine volatile content might be an alternative option for the future, subject to market acceptance of this approach.

The present study forms an integral part of an extensive research program aimed at the liberation of authentic grape-derived volatile compounds in appropriate concentrations that would improve the aroma or bouquet of wine. More specifically, the purpose of this study was to express two β-glucosidases genes (*BGL1* and *BGL2*) from the dimorphic yeast, *Saccharomycopsis fibuligera* (Machida *et al*., 1988; Van Rensburg *et al*., 1998), in a widely used commercial starter culture wine strain (VIN13) of *Saccharomyces cerevisiae*. The transformed and untransformed VIN13 strains were used to ferment juice from muscat and non-muscat grapes. The resulting wines were analysed to determine whether the expression of the *BGL1* and *BGL2* genes and the extracellular production of their encoded β-glucosidasess would modify the aroma and flavour of the wine by increasing the released terpenol fraction.

MATERIALS AND METHODS

Microbial strains, plasmids, media and screening procedures. The microbial strains and plasmids used in this study are listed in Table 1.

Transformants of *Escherichia coli* were grown in Luria Bertani (LB) broth and maintained on LB agar (Sambrook *et al*., 1989) plates, supplemented with 100 μg ml^{-1} ampicillin (Sigma) to support selective pressure. Laboratory (Σ1278) and industrial (VIN13) strains of *S. cerevisiae* were grown in a rich YPD medium (1% yeast extract, 2% glucose and 2% peptone). Strain Σ1278 was transformed using the lithium acetate method (Gietz and Schiestl, 1991) and Σ1278 transformants were plated on SC^{-Ura} and SC^{-Leu} media (containing 1% yeast nitrogen base without amino acids, 2% glucose, 2% agar and all the required growth factors lacking uracil or leucine). Ura^{+} and Leu^{+} colonies were picked as positive transformants. Electroporation (utilising an EasyjecT Electroporator set at 1300 V, 25 μF and 321 W) was used in combination with the lithium acetate method to transform strain VIN13, and transformants were selected on SD-agar plates [0.67% nitrogen base without amino acids, 0.05% glucose, 2% agar, containing a range of concentrations of the herbicide sulfometuron methyl (SMM; Dupont, USA), from 10 to 100 μg ml^{-1} in 10 μg ml^{-1} increments]. These transformants were then re-streaked on fresh SD-plates containing 100 μg ml^{-1} SMM, and strong growth (large colonies after overnight incubation at 30 ºC) was indicative of SMM resistance (Smm^{R}) and a positive transformation. The Smm^{R} strains were then inoculated in test tubes containing 10 ml YPD cultures. Extracellular β-glucosidase assays were per-

TABLE 1 – Microbial strains and plasmids used in this study

Strains and Plasmids	Relative genotype	Source /Reference
Escherichia coli strain:		
DH5a	*sup*E44 D*lac*U169 (Δ80*lacZ*DM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Sambrook *et al.,* 1989
Saccharomyces cerevisiae strains:		
Σ1278	*MATα/MATa ura3/ura3*	Radcliffe *et al*., 1997
Σ294	*a leu2-3 112 ura3-52 his3 trp1-2a*	This laboratory
VIN13	Commercial diploid strain	Anchor Yeast Technologies
Plasmids:		
pSFB15	Ap^R Tc^R *URA3 BGL1*	This laboratory
pSFB219	Ap^R *lacZ lacI BGL2*	This study
pDLG31	Ap^R $PGK1_p$ *LKA1* $PGK1_T$ *SMR1*	Gundllapalli Moses *et al*., 2002
pBGL1	Ap^R *lacZ lacI BGL1 SMR1*	This study
pBGL2	Ap^R *lacZ lacI BGL2 SMR1*	This study
pBGL	Ap^R *LEU2 BGL2*	This study

formed on these cultures. Bacterial strains were grown at 37 ºC and yeast strains at 30 ºC.

Cloning of the *BGL1* and *BGL2* β-glucosidase genes. A genomic library of *S. fibuligera* that was previously constructed in our laboratory (Van Rensburg *et al*., 1998) was used to clone the *BGL1* and *BGL2* genes according to the selection procedure described by Yamashita *et al*. (1985). Standard methods were used for the manipulation, subcloning, sequencing and Southern hybridisation of DNA fragments containing the *BGL1* and *BGL2* β-glucosidase genes and the *SMR1* marker gene (Sanger *et al*., 1977; Ausubel *et al*., 1987; Sambrook *et al*., 1989). The *SMR1* gene, conferring Smm^R, originates from the *S. cerevisiae ILV2* gene carrying a single base pair transition (Casey *et al*., 1988). Yeast integration plasmids (YIp), pBGL1 (containing *BGL1* and *SMR1*) and pBGL2 (containing *BGL2* and *SMR1*) were used to transform *S. cerevisiae* strains and VIN13 (Fig. 1).

DISEGNARE?

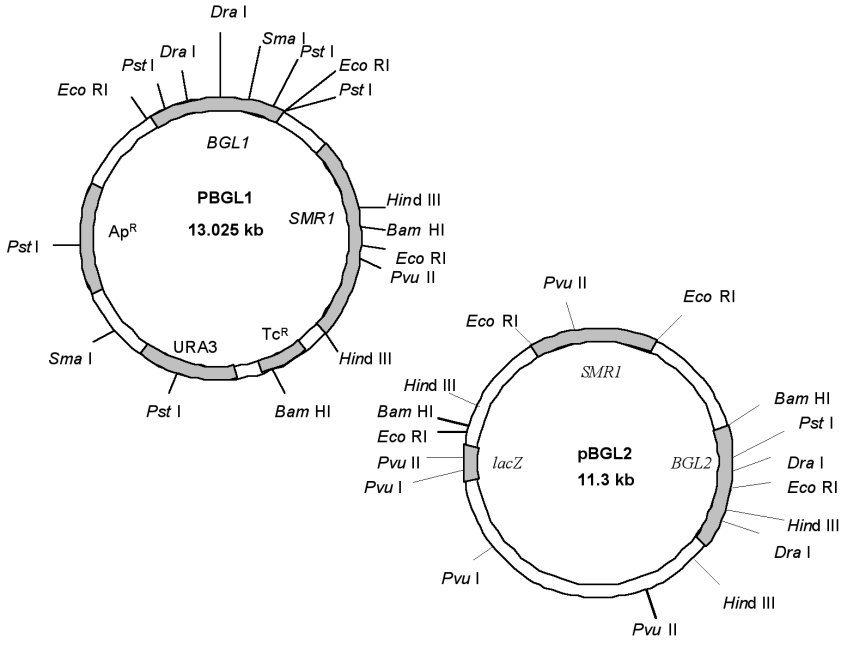

FIG. 1 – A schematic representation of the two *Eschericia coli-Saccharomyces cerevisiae* shuttle plasmids containing the *Saccharomycopsis fibuligera* β-glucosidase genes, *BGL1* and *BGL2*.

Assay for β-glucosidase activity. β-Glucosidase activity of the yeast strains, transformed with plasmids pBGL1 and pBGL2, was measured as follows: *S. cerevisiae* VIN13 and transformants were grown in 10 ml of non-selective YPD medium on a rotator machine at 30 ∞C for a total of 96 h. Assays were performed for four consecutive days at 24 h intervals. β-Glucosidase activity was determined by mixing samples of the culture supernatant (195 µL) with 20 µL of 50 mM sodium acetate buffer (pH 4.5), as well as 2 µL of 0.2 M *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; Sigma, St. Louis, MO, USA). Incubation took place at 42 ºC for 3 h, and then the reaction was stopped by the addition of 500 µL of 1 M sodium carbonate. These assays were performed in duplicate. Plate cell counts were done simultaneously for each assay in the applicable dilutions to determine live cell numbers. Assays were also performed to determine the temperature stability and activity of the enzymes, as well as the activity of the enzymes throughout a pH range. One colony of each recombinant yeast strain was inoculated in 10 ml YPD and grown for 48 h. For temperature stability, the supernatant was incubated for 30 min at temperatures ranging from 15 to 60 ºC, and then incubated for another hour with the sodium acetate buffer and *p*NPG as the substrate. The residual activity of the enzyme was then assayed. To determine the activity at certain temperatures, the culture supernatant with added buffer and substrate was incubated at a range of temperatures (15 to 60 ºC) for a period of 1 h. The activity at certain pH values (pH 2 to 8) at 50 ºC was incubated for 1 h with the pH of the sodium acetate buffer adjusted. Plate assays were also performed. Colonies were spotted on SD-plates supplemented with *p*NPG and grown overnight at 30 ºC. After the incubation period a volume of 1 M sodium acetate was poured over the agar, and a positive reaction was observed as a yellow halo developing around the colony.

Vinification. Wine was made in duplicate from juice derived from three grape cultivars, namely Chenin blanc, Gewürztraminer and Pinotage. A total of 60 kg of grapes of each of the two white varieties (Chenin blanc and Gewürztraminer) was destemmed and crushed and then pressed. Overnight settling with the addition of 30 mg L^{-1} SO_2 then took place. Standard analyses (titratable acids, pH, sugar content) were done on the unfermented must. The control yeast, VIN13, and the two recombinant strains, VIN13[*BGL1*] and VIN13[*BGL2*], were grown in 500 ml YPD-containing culture flasks for 2 days at 30 ∞C, and then centrifuged to collect the biomass. After settling without the addition of commercial enzyme preparations, 0.75 g L^{-1} diammonium phosphate (DAP) was added to the must to adjust the nitrogen concentration. The yeast was also inoculated into the grape must to a final concentration of 1 x 10^6 cells ml^{-1} and fermented in 4.5-liter fermentation flasks at 15 ∞C. The fermentation process was followed by measuring the decrease in the weight of the bottles, and alcoholic fermentation was considered complete when the weight of the bottles stabilised. Upon completion of fermentation, the wines were racked off their lees and then left at −4 ∞C to settle and undergo tartrate stabilisation, after which the wines were filtered and bottled. Of the red variety (Pinotage), 80 kg of grapes were destemmed and crushed. Cold maceration was done for 3 days at 15 ∞C, while the three yeast strains were propagated in 500 ml flasks of YPD at 30 ∞C. The grape must on the skins was then separated into three fermentation containers and inoculated with the three different yeast strains, respectively. Fermentation took place at 25 ∞C and was monitored by taking readings of the sugar content as it decreased. After fermentation was completed, the wine on the grape skins was pressed and drawn over into flasks. One week of settling was allowed, after which the wines were placed at −4 ∞C for tartrate stabilisation. After this period the wines were filtered and bottled. SO_2 was added for all the wines as prescribed, before tartrate stabilisation and again at bottling.

Standard wine analysis. Fourier Transform Infrared Spectroscopy (FTIR), using the GrapeScan 2000 instrument (FOSS Electric, Denmark), was used to determine the chemical characteristics of the fermented wines. The commercial calibrations for the analysis of the wine were provided by FOSS Electric, and all calibrations were validated.

Measurement of colour and phenolic content of wine. The colour/browning of the red wine was measured by absorbance at 420 nm and 520 nm (Zoecklein *et al*., 1997). Samples were membrane filtered (0.22 mm) and measured in duplicate using 2 mm quartz cells against a water reference. The white wine samples were prepared in the same way as the red wine samples and absorbance readings were taken at 320 nm and 420 nm using 2 mm cells. The phenol content of both the red and white wines was measured by absorbance at 280 nm (Zoecklein *et al*., 1997). Samples were prepared as for the colour measurements.

GC measurement of volatile compounds.
Volumetric material: HP 6890 series gas chromatograph, fitted with a FID, fitted with split-splitless injector, and automatic sampler 7683 were used. Column: Supelco SPB5, 60 m x 0.32 mm i.d., 0.25 mm film thickness. Chromatographic conditions: carrier gas, He; head pressure, 140 kPa; flow, 12.5 ml min^{-1}; purge flow, 7.0 ml min^{-1}; injector and detector temperature, 250 ∞C; initial column temperature, 50 ∞C, held for 2 min and then raised to 150 ∞C at 10 ∞C min^{-1}, then to 160 ∞C at 5 ∞C min^{-1} and then to 220 ∞C at 10 ∞C min^{-1} and held for 10 min; make-up gas N_2 at 30 ml min^{-1}; detector FID, H_2 at 40 ml min^{-1}; air, 450 ml min^{-1}; injected volume, 2 ml.

Method: A volume of 10 ml of wine was decanted into an extraction tube. An aliquote of 200 mL of Freon 113 (1,1,2-Trichloro-1,2,2-trifluoroethane, obtained from Aldrich) was added as an extracting agent, and 2 mL of a solution of 2,6-dimethylheptenol (400 mg L^{-1} in ethanol as internal standard) and 1.2 g of NaCl was also added to the extraction tube. The tubes were capped and shaken for 30 min in an automatic shaker at maximum speed. The tubes were centrifuged (5 min at 3000 rpm). The organic phase was recovered with a pasteur pipette, transferred over 50 mg of Na_2SO4 into a 2 mL vial with a 200 µL glass insert, and analysed under the chromatographic conditions as described above. After the chromatographic analysis, the relative areas or heights of the calibrated peaks are interpolated from calibration graphs created using synthetic wine solutions (ethanol 12% for white wine, 16% for red wine v/v; tartaric acid 6 g L^{-1}; pH 3.2) with an alcohol content similar to that of the analysed wine.

RESULTS

Construction of recombinant *Escherichia coli*-yeast shuttle plasmids

The plasmid pBGL1 was constructed to create a plasmid containing the *S. fibuligera BGL1* β-glucosidase gene and a selectable marker gene, *SMR1*. This was done by linearising plasmid pSFB15 with *Hin*dIII. Plasmid pDLG31 was also digested with *Hin*dIII, resulting in two linear fragments of DNA, one containing the *SMR1* gene. This fragment of DNA was subsequently ligated to the linearised pSFB15, resulting in plasmid pBGL1. Plasmid pSFB15 is a recombinant YIp5-based shuttle integration vector containing the *BGL1* gene under the control of its authentic *S. fibuligera* promoter. Plasmid pBGL2 was constructed by using a previously constructed plasmid, pSFB219, to subclone the *SMR1* gene into it. The pSFB219 plasmid was linearised with *Bam*HI. The *SMR1* gene was isolated from pDLG31 by digestion with the same enzyme and then ligated to the linearised pSFB219. This resulted in the shuttle plasmid designated pBGL2. These shuttle plasmids are illustrated in Fig. 1. The native secretion signal was also used for both cloned genes.

The *SMR1* gene is used as a 'natural' selectable marker, as it originated from a spontaneous mutation of the *ILV2* gene from *S. cerevisiae* due to a single base pair transition (Casey *et al*., 1988). This gene also presents the added advantage of not only acting as a selectable marker gene, but also directing the integration of the recombinant DNA to a known locus on chromosome XIII.

Expression of the *BGL1* and *BGL2* genes in the industrial yeast strain VIN13 and laboratory Strain

Both plasmids (pBGL1 and pBGL2) were individually transformed into the industrial yeast strain VIN13 using the technique of electroporation. Transformants that effectively took up and integrated the recombinant DNA were selected by utilising the subcloned *SMR1* gene. As this gene confers resistance to the herbicide sulfometuron methyl (SMM), SD-plates containing SMM were used for selection. Transformants were re-streaked on SD plates containing 100 mg ml^{-1} SMM, and if these showed growth, preliminary enzyme assays were performed to confirm that the relevant β-glucosidase gene had been expressed effectively.

Laboratory strain was transformed to Ura^+ with pBGL1 and pBGL2. Yeast genomic DNA was prepared from both strains of the transformants and digested with endonucleases to perform Southern hybridisations. This revealed the presence of the β-glucosidase genes in the genome of the transformed yeast strains VIN13 and (data not shown).

Synthesis and secretion of β-glucosidases in yeast

Plate assays were performed to determine whether the β-glucosidase enzymes were being secreted. This was not as conclusive, as the halos that were observed were light yellow in colour and could not easily be distinguished from the actual agar colour. Liquid assays were then performed as well, firstly to confirm transformation of the yeast strains, and secondly to determine at which stage in the growth phase of the yeast the β-glucosidase enzymes were secreted at maximum levels. The extracellular β-glucosidase activity produced by the various yeast transformants was determined and quantified over a period of 96 h (Fig. 2). Assays were also performed with the intracellular fraction of the yeast (data not shown).

Properties of the expressed β-glucosidases

When strain was transformed with the two β-glucosidase genes, it was shown in the assays that the proteins were being processed and secreted correctly and efficiently. The activity measured was indeed higher, and this could be attributed to the assumption that the laboratory strain has a less rigid cell wall than the more robust wine yeast strain, which has been adapted to the high ethanol and high SO_2 concentrations in the wine environment. The activity of the β-glucosidase activity as secreted by the VIN13 transformants was thereafter measured in intervals of 24 h for a total period of 96 h. This was done in conjunction with live cell counts from plates to determine in which growth phase secretion of the β-glucosidase was taking place and in what quantities. This could be accomplished, as the amount of *p*NP released could be determined per million cells per time unit, in this case 180 min.

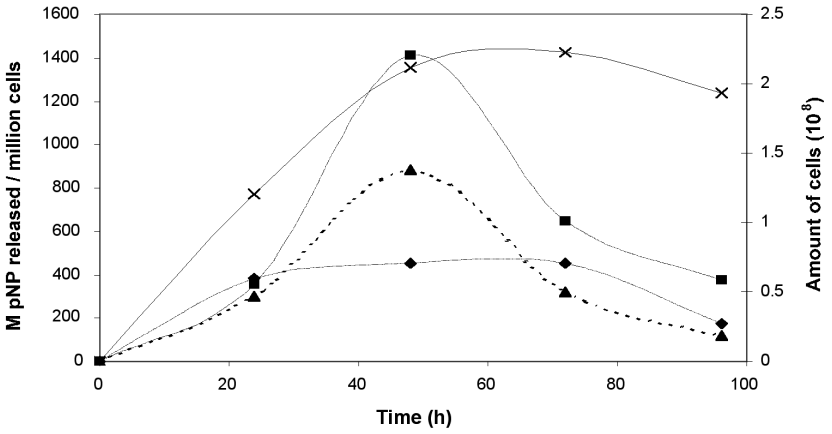

FIG. 2 – The β-glucosidase activities produced by a commercially available *Saccharomyces cerevisiae* wine yeast strain (VIN13) and a VIN13 strain transformed with the *Saccharomycopsis fibuligera* β-glucosidase genes, *BGL1* and *BGL2*. The Bgl1p values are indicated by (■) and the Bgl2p values by (▲). (◆) Represents the control, non-transformed VIN13 host strain. The amount of *p*-nitrophenol released per million cells during a 96-h period is shown. A mean curve (X), representing the growth of all the strains, is also shown.

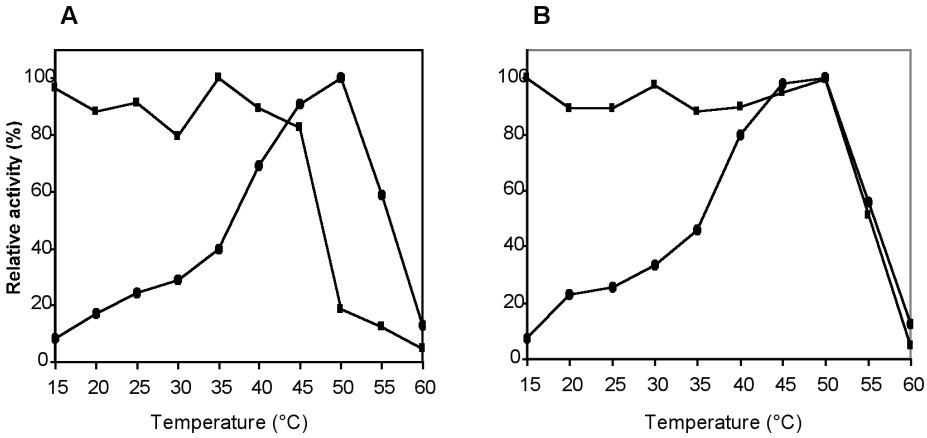

FIG. 3 – The activity (●) and stability (■) of two *Saccharomycopsis fibuligera* β-glucosidases, Bgl1p (Panel A) and Bgl2p (Panel B), at certain temperatures. Both β-glucosidases were produced by *Saccharomyces cerevisiae* transformed with the *S. fibuligera BGL1* and *BGL2* genes.

It was shown that the VIN13 strain containing the *BGL1* gene (VIN13[*BGL1*]) exhibited nearly three and a half times the borderline activity of the control (non-transformed VIN13), and the VIN13 strain containing the *BGL2* gene (VIN13[*BGL2*]) exhibited more than double the activity. It can be seen from Fig. 2 that both the yeast-derived Bgl1 and Bgl2 enzymes exhibited the highest activity after 48 h of growth at 30 ºC. This is important, as this is when the yeast culture enters the stationary phase and metabolic activity is at its highest. During winemaking this will also be when the highest fermentation rates are observed. It is therefore significant to note that the enzyme is active and produced efficiently during this period. The production of the enzyme also has no adverse or detrimental effect on the growth rate of the yeast itself, as proliferation continues at a predicted rate.

Temperature and pH dependence of the enzymes

The temperature activity and stability curves are shown in Fig. 3. Both the enzymes showed a gradual increase in activity up to a temperature of 50 ºC, and the activity then decreased sharply to levels under 15% at higher temperatures. This trend is consistent with the corresponding activity in their authentic host organism, *S. fibuligera* (Machida *et al*., 1988). This indicates that the protein conserved its basic conformation, even though it is expressed by a different yeast species. The stability of the β-glucosidases also showed the same trend as when expressed by the authentic host organism, with the stability high at lower temperatures. The stability of Bgl1p showed an optimal range at around 45 ºC, while that of Bgl2p was at 50 ºC. At 50 ºC, the stability of Bgl1p decreased to a level as low as 20% of the relative value, with the activity of Bgl2p only starting to decrease when the temperature exceeded 50 ºC. At 60 ºC, both the enzymes were extremely unstable, with activity levels under 10% being observed.

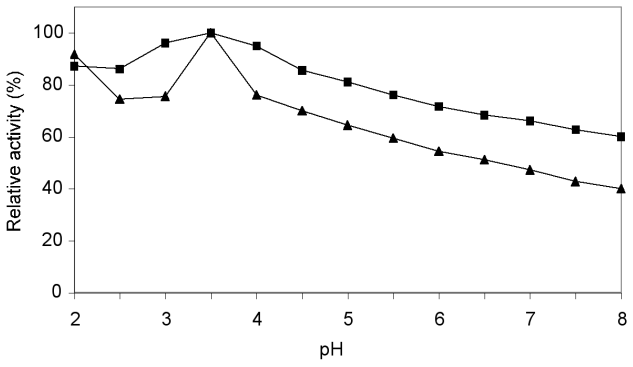

FIG. 4 – The activity of two *Saccharomycopsis fibuligera* β-glucosidases, Bgl1p (■) and at certain pH values. Both β-glucosidases were produced by *Saccharomyces cerevisiae* transformed with the *S. fibuligera BGL1* and *BGL2* genes.

The activity of the enzymes at certain pH values is presented in Fig. 4. Both *S. cerevisiae*-derived enzymes (Bgl1p and Bgl2p) seemed to be relatively stable at low pH values, and this is contradictory to what was observed by Machida *et al*. (1988) when they characterised the enzyme in *S. fibuligera*. They noted that the activities of Bgl1p and Bgl2p were basically non-existent at pH 2, and even at a pH of 3 the relative activity of both enzymes was less than 10%. An increase in activity was seen only when the pH of the buffer neared 4, and the optimal pH value was noted at 4.5. This was also the pH value at which we initially performed the assays, and the amount of *p*NP released was measured as such. However, when we measured the pH activity, it became apparent that a pH optimum for both enzymes could be found at around pH 3.5, which is very encouraging, as this is the approximate pH of most grape juices and wines. Activity was seen to decrease with a gentle slope as the pH was increased, but even at pH 8 the activity of Bgl1p was 40% and that of Bgl2p 60% of the maximum activity. This presented the question whether a greater amount of rNP would be released at a lower pH value. The same was true for the temperature assays, which were done at 42 ºC where the activity was only 70% of the maximum. The assays were therefore repeated, this time at a higher temperature (45 ºC), the stability at 50 ºC taken into account, but no substantially higher values were recorded.

Expression of the β-glucosidase enzymes in wine

The wines were analysed with the Foss Grape Scan on completion of fermentation. The results are shown in Table 2. Other analyses are shown in Tables 3-7.

TABLE 2 –Analysis of wines as determined by FOSS Grape Scan

Sample	Ethanol (%v/v)	Residual Sugar	pH	VA	TA	Malic acid	Lactic acid
Pinotage Control	16.56	0	4.15	0.37	5.21	3.62	0
Pinotage BGL1	16.05	0.24	3.91	0.37	5.29	2.86	0.14
Pinotage BGL2	16.24	0	4.05	0.36	5.23	3.46	0
Gewürtz Control	15.02±0.071	0.11±0.106	3.61±0.007	0.31±0.064	4.23±0.127	1.98±0.049	0.25±0.035
Gewürtz BGL1	15.28±0.064	0.505±0.219	3.63±0	0.23±0.014	4.01±0	2.08±0.007	0.18±0
Gewürtz BGL2	15.26±0.021	0.52±0.099	3.63±0	0.23±0.007	4.01±0.042	2.03±0.064	0.18±0
Chenin blanc Control	14.51±0.007	0.56±0.049	3.18±0.021	0.22±0.0354	5.52±0.1131	2.49±0.0212	0.22±0.0212
Chenin blanc BGL1	14.50±0.035	0.52±0.035	3.17±0	0.24±0.014	5.56±0.014	2.57±0.007	0.16±0.007
Chenin blanc BGL2	14.6±0.028	0.52±0	3.18±0.007	0.23±0.006	5.59±0.007	2.55±0.071	0.17±0

Residual sugar, VA, T.A., malic acid and lactic acid is measured in g l^{-1}.

TABLE 3 – The colour and pigmentation found in the red wine expressed as absorbance units

Red wine - Pinotage	Control VIN13	VIN13 [*BGL1*]	VIN13 [*BGL2*]
Wine colour density	17.81	13.91	14.84
Wine colour hue	0.62	0.61	0.62
Degree of red pigment colouration (%)	25.80	24.12	25.70
Estimate of SO_2 resistant pigments	9.58	8.32	9.36
Total red pigments	42.42	35.80	35.56

Standard deviation was less than 12%.

TABLE 4 – The colour and browning found in the white wines expressed as absorbance units

White wine	Control VIN13	VIN13 [*BGL1*]	VIN13 [*BGL2*]
Total hydroxycinnamates			
Chenin blanc	2.65	2.48	2.40
Gewürtztraminer	2.12	2.10	2.09
Estimate of brown pigments			
Chenin blanc	0.05	0.05	0.05
Gewürtztraminer	0.08	0.07	0.07

Standard deviation was less than 12%.

TABLE 5 – Total phenolics expressed as absorbance units as found in the wine

White phenolics	Control VIN13	VIN13 [*BGL1*]	VIN13 [*BGL2*]
Red wine			
Pinotage	69.33	61.55	54.58
White wine			
Chenin blanc	0.71	0.40	0.48
Gewürtztraminer	1.16	0.96	0.85

Standard deviation was less than 12%.

DISCUSSION

There is much contradictory data available in the literature on the occurrence of β-glucosidase genes and their encoded activities in *S. cerevisiae*. It was previously reported that *S. cerevisiae* contains a structural gene for β-glucosidase, though it is very poorly expressed (Duerksen and Halvorson, 1958, 1959). Results obtained by Günata *et al*. (1990b) concluded that these yeasts have very low β-glucosidase activity, but Delcroix *et al*. (1994) found three oenological strains showing high β-glucosidase activity. On the other hand, Darriet *et al*. (1988) reported that it was rather certain oxidases located in the periplasmic space of a strain of *S. cerevisiae* that were responsible for the hydrolysis of monoterpene glycosides of Muscat grapes, and that the activity of yeast β-glucosidases was glucose independent. Later work done by Mateo and Di Stefano (1997) revealed that wine yeasts had some constitutive β-glucosidase activity, but that this enzyme activity was inhibited under wine-making conditions.

There also is another group of enzymes, called exo-glucanases, that are produced by *S. cerevisiae* and that show activity against the assay substrate, *p*-nitrophenyl-β-D-glucopyranoside (Kuranda and Robbins, 1987). The main difference between these exo-glucanases and the heterologous β-glucosidases that we introduced into *S. cerevisiae* is substrate specificity. The exo-1,3-b-glucanases act at non-reducing ends of the cell wall polysaccharide, 1,3-b-D-glucan, releasing glucose. This group of enzymes plays a nutritional role in fungi and yeasts. In *S. cerevisiae*, this group of exo-glucanases is only slightly (3%) able to release glucose and other sugar moieties from other compounds, such as terpenic alcohols (Manners *et al*., 1973), as these bonds are usually b-1,6-linkages. However, these activities will influence the results of laboratory enzyme assays, as the control in all the experiments will exhibit a baseline activity that can be attributed to the endogenous exo-glucanase activity. It is clear from the graphs in Fig. 2 that both the transformants show far greater activity than the control, as the values presented consist of both the exo-glucanase and β-glucosidase

TABLE 6 –The volatile components as found by microextration of Pinotage wines

Volatile components - Pinotage	Control VIN13*	VIN13 [BGL1]*	[BGL1]/ VIN13**	VIN13 [BGL2]*	[BGL2]/ VIN13**
Butanol-3-methyl	147.99	222.97	1.50	182.98	1.2
Butanol-2-methyl	37.54	222.97	5.94	nd	_
Propanol	1.65	4.29	2.59	1.12	0.68
Hexanoic acid ethylester	0.26	0.30	1.15	0.23	0.90
Heptanoic acid ethylester	0.59	0.08	0.13	nd	_
Diethylsuccinate	0.62	0.90	1.47	0.51	0.82
Octanoic acid ethylester	0.25	0.26	1.07	0.20	0.82
Decanoic acid ethylester	0.09	0.10	1.02	0.0	0.82
Citronellol*	17.60	21.06	1.20	14.85	0.84
Nerol*	14.23	14.88	1.05	14.89	1.05
Geraniol*	8.46	nd	_	7.04	0.83

* Values are given as μg l^{-1}.
** The ratio of the values obtained for the control VIN13 yeast strain and the respective transformed strains, VIN13[*BGL1*] and VIN13[*BGL2*].
nd and – : not detectable.

TABLE 7 – The volatile components as found by microextration of Gewürtztraminer wines

Volatile components	VIN13*	VIN13[*BGL1*]*	[BGL1]**	VIN13[*BGL2*]*	[BGL2]**
Butanol-3-methyl	234	126.50	0.54	144.50	0.62
Butanol-2-methyl	42	33.50	0.80	39.50	0.94
Hexanoic acid ethylester	0.66	0.96	1.46	1.06	1.61
Octanoic acid ethylester	0.75	1.05	1.40	1.30	1.74
Decanoic acid ethylester	0.19	0.33	1.72	0.46	2.42
Acetic acid-2-phenylester	0.43	0.85	1.99	0.94	2.20
Nerol*	15.59	17.55	1.13	17.39	1.12
Geraniol*	15.34	20.03	1.31	21.03	1.37
Terpineol*	10.18	10.31	1.01	nd	_

* Values are given as mgl^{-1}.
** The ratio of the values obtained for the control VIN13 and the respective transformant strains, VIN13[*BGL1*] and VIN13[*BGL2*].
nd and – : not detectable.

activity. However, merely subtracting the values of the control from the values of the transformants would give an inadequate view of the true amount of rNP released and thus of the activity of the enzymes. This is because it is unknown whether the production of the β-glucosidase influences the production and/or activity of the exo-glucanase in any way. Such influence might be possible, as lower activity values for the β-glucosidase were recorded at 24 h for both of the transformants, and after 96 h for VIN13[*BGL2*].

The production of β-glucosidase enzymes by the VIN13 transformants was found to be dependent on the metabolic state of the cells, i.e. the growth phase. Maximum activity could be connected with maximum production of the heterologous β-glucosidases, which took place during the beginning of the stationary phase of the VIN13 transformants. Activity thereafter started to decline as the VIN13 cultures entered the stationary phase. Activity was very low when the dying phase was reached after 96 h. This indicated that residual activity and stability of the enzymes in the growth medium were not very high. The production of the heterologous β-glucosidases did not affect the growth of the transformed VIN13 cells and showed no detrimental effect towards normal cell propagation.

In this study, the optimum temperature for activity of the β-glucosidases was found to be the same as reported by Machida *et al*. (1988). However, the β-glucosidase encoded by *BGL2* was found to be more stable at the higher temperatures than what was found originally (up to 50 ºC). The pH profiles of the *S. cerevisiae*-derived Bgl1p and Bgl2p also differed from those observed by Machida *et al*. (1988). We found that the both Bgl1p and Bgl2p were surprisingly active at low pH values, which is in contradiction to previous reports. Enzymes are usually most stable and active at the pH levels of the surrounding environment; for instance, intracellular enzymes would be most stable at pH levels of 6-7. However, the two *S. fibuligera* β-glucosidases expressed in *S. cerevisiae* were secreted into the growth medium, which makes it more difficult to predict at what pH levels they would be most active. It could be that the expression by another yeast species changed the conformation of the protein in such a fashion that it developed higher resistance against the denaturing effect of acidic conditions. However, this supposed protective change did not seem to influence the activity of Bgl1p and Bgl2p.

According to Machida *et al*. (1988) the two β-glucosidases encoded by *BGL1* and *BGL2* have different substrate preferences. They stated that the *BGL1*-encoded enzyme hydrolysed cellobiose and cellooligosaccharides more efficiently, whereas the *BGL2*-encoded enzyme showed an affinity for aryl- and alkyl-b-D-glucosides. Our results confirmed the data of Machida *et al*. (1988) and we found that Bgl1p was less active on the synthetic substrate *p*NPG (43.3 U mg^{-1} of protein released) than Bgl2p (168 U $mg^{-1}$of protein released).

The next step was to test these transformed wine yeasts in small-scale wine fermentations. The results from duplicate wine fermentations are shown in Tables 2 to 7. The general analyses of all the wines are generally very much the same as those of the control wines. The pH values, alcohol values, volatile acidity, titratable acidity and other acids showed no major differences between the wines made with the genetically altered yeast and the wine made with the control yeast. This has lead us to assume that the *S. fibuligera* β-glucosidases expressed by *S. cerevisiae* transfomants have not put a detectable metabolic burden on the transformed cells. This assumption is based on the observation that the end-products of the fermentations of all the yeast strains were similar to that of the control yeast.

Generally, wine colour density values relate to the visual description of wines; wines with density values of 0 to 6 are described as lightly coloured, from about 6 to about 10 as medium red and more than 10 as deep red. As all the values in Table 3 are above 10, it is clear that the wines are very dark red in colour. However, the wines differ in their respective densities, with the control having the most intense colour. This might be due to the fact that, in a normal wine, the red colour pigments, or anthocyanins, are generally conjugated to glucose moieties. When this bond is cleaved, the anthocyanins become less stable and are more susceptable to bleaching by SO_2. In this unstable form they can also transform spontaneously to a more stable form. These forms are usually colourless. This also explains the lower values obtained for the estimate of SO_2 resistant pigments, as well as the values for the total red pigments in the wines made with the genetically modified yeasts.

As can be seen in Table 4, the colour and browning were not influenced much by fermentation with the transformed wine yeasts. In general, however, the browning seemed slightly less in the Gewürtztraminer wine made by VIN13[*BGL1*] and VIN13[*BGL2*] than that of the control. The values are not as significant though, as the differences are very small.

In red wines, phenols and phenolic substances are quite prevalent, and the values are very high compared to that of the white wines. In Table 5, however, it can be seen that the values differ in the control wines and the experimental wines fermented by the transformed strains, with the phenols in the wine made with the genetically altered yeasts having lower phenolic contents. This is also true for the white wines. We assume that as the anthocyanins and phenols are so closely related, the same processes and factors influence both in the same manner.

The microextraction done during the GC-analyses showed not only that differing terpenol amounts were present, but also that concentrations of other substances showed variation in the different wines (Tables 6 and 7). For example, an increase in the concentration of some esters was found in the wines made with the VIN13[*BGL1*] and VIN13[*BGL2*] yeasts. It is known that each of these esters impart a different aroma to the wine. For instance, hexanoic, octanoic and decanoic acid ethyl esters impart apple-like, fruity aromas, while acetic acid-2-phenylester adds a honey, fruity and flowery fragrance to the wine. There were also variations between higher alcohols, such as propanol.

It can be postulated that the two *S. fibuligera* β-glucosidases expressed in VIN13 might play a role during ester synthesis in the transfomed cells. It might be that more esters are formed, since more pyruvate and acetyl-CoA are formed in direct relation to more glucose being available. On the other hand, it might also be possible that the substrate specificity of the enzyme as expressed in its new host might have been altered and could have become more suitable for substrate hydrolysis in the ester formation biosynthetic pathways.

In conclusion, the results from this study indicate that the VIN13 wine strain of *S cerevisiae* transformed with the *BGL1* and *BGL2* genes of *S. fibuligera* secreted biologically active β-glucosidases under winemaking conditions. The transformed wine yeasts appear to posses limited glycosidase activities that will result in the liberation of volatile compounds from their glyconjugate precursors during the fermentation of Chenin blanc, Gewürtztraminer and Pinotage grape juice. The β-glucosidase-producing VIN13 wine yeast contained slightly higher levels of terpenols in comparison with the wines produced by the untransformed control VIN13 host strain. Surprisingly, the wines produced by the transformed strain also contained increased ester concentrations. Many of these fragrant compounds, when produced in appropriate concentrations, would contribute to the fermentation bouquet of wine. The extent to which this acquired capacity of the transformed wine yeast is of practical significance in large-scale wine production is at present unclear but presents a proposition that clearly can be assessed.

In light of the results obtained in this study, we believe that it is reasonable to further pursue expression in wine yeast strains of novel combinations of heterologous β-glucosidase and other glycosidase-encoding genes at sufficient levels to enable the liberation aroma-enhancing compounds from authentic grape-derived non-volatile *O*-glycosides, thereby intensifying the varietal flavour of wine.

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