The use of enzymes for increased aroma formation in wine

by

Tanya Gwendryth Stidwell

Thesis presented in partial fulfilment of the requirements for the degree of Master of Sciences at the University of Stellenbosch.

December 2001

Supervisor:
Prof. I.S. Pretorius

Co-supervisors:
Dr. P. Van Rensburg
Dr. M.G. Lambrechts

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

T. G. Stidwell   **Date**

SUMMARY

Monoterpene alcohols (monoterpenols) play an important role in the flavour and aroma of grapes and wine. This is especially applicable to wines of a muscat variety, but these flavour compounds are also present in other non-muscat grape varieties, where they supplement other varietal flavours and aromas. These monoterpenols can be found in grapes and wine as free, volatile and odorous molecules, as well as in flavourless, non-volatile glycosidic complexes. These complexes most often occur as 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-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides), or 6-O-β-D-apiofuranosyl-β-D-glucopyranosides of mainly linalool, geraniol, nerol, α-terpineol and hotrienol. These precursors are, however, hydrolyzed only to a limted extent by endogenous glycosidases during the fermentation process, as they exhibit very low activity in wine conditions.

The monoterpenols can be released from their sugar moieties by one of two methods: either an acid or an enzymatic hydrolysis. The enzymatic hydrolysis mechanism is fully understood, and the process functions in two successive steps: firstly, depending on the precursor, the glycosidic linkage is cleaved by an α-L-arabinofuranosidase, an α-L-rhamnosidase, a β-D-xylosidase, or a β-D-apiosidase. The second step involves the liberation of the monoterpene alcohol by a β-glucosidase. This enzymatic hydrolysis does not influence the intrinsic aromatic characteristics of the wine, as opposed to acid hydrolysis.

As the endogenous grape glycosides of *Vitis vinifera* and the yeast *Saccharomyces cerevisiae* show very low activity towards these aromatic precursors during the handling of the juice and winemaking processes, the focus has increasingly fallen on introducing exogenous β-glucosidases to wines and juices. Genes encoding β-glucosidases and α-L-arabinofuranosidases have been cloned from various organisms, including bacteria, fungi and yeasts. However, the activities and properties of these enzymes are not always suitable for exploitation under winemaking conditions, where a low pH, low temperatures, and high ethanol and glucose concentrations prevail. A genetically engineered wine yeast strain of *S. cerevisiae* that expresses glycosidases that are active in these conditions would be useful in improving the flavour and aroma of wines, thereby adding to the complexity and value of the wine.

Two β-glucosidase genes, *BGL1* and *BGL2* from *Saccharomycopsis fibuligera*, were subcloned into two *Escherichia coli*-yeast shuttle vectors. A dominant selectable marker gene (*SMR1*) was also inserted onto these plasmids. These plasmids were designated

pBGL1 (containing the *BGL1* gene) and pBGL2 (containing the *BGL2* gene) respectively. Introduction of the two plasmids into two strains of *S. cerevisiae* then followed. A laboratory strain, Σ1278, was transformed to confirm the effective secretion of the expressed protein. An industrial yeast strain, VIN13, was subsequently transformed by making use of the selectable marker (resistance against sulfometuron). Enzyme assays with the synthetic substrate ρ-nitrophenol-β-D-glucopyranoside (ρNPG) were performed to determine the activity of the β-glucosidases over a period of days, as well as at certain temperatures and pH values. The stability of the enzymes was also investigated.

These recombinant yeasts were able to degrade the ρNPG efficiently. They showed promising results concerning pH optima, with a substantial amount of activity found at the pH levels as found in the wine environment. There was also a slight increase in specific activity at lower temperatures. The recombinant yeast strains were also tested in small-scale fermentations. Three wines were made, of which two were from white cultivars (Chenin blanc and Gewürtztraminer) and one from red (Pinotage). Results obtained from micro-extraction from the finished wines showed that the terpenol content did increase, although this was not the only wine component influenced. Other flavour compounds also showed increases, especially the esters. This also played a role in the flavour increase in the wine.

Future work would include optimizing the available results. This would entail the addition of another glycosidic enzyme, such as α-L-arabinofuranosidase, to the genome of the wine yeast to aid the further breakdown of glycosidic bonds. The cloning or engineering of a β-glucosidase enzyme that is more active at low temperatures would also yield better results and release even more of the aroma of the wine.

OPSOMMING

Monoterpeenalkohole (monoterpenole) speel 'n belangrike rol in die geur en aroma van druiwe en wyn. Dit is veral van toepassing op wyne van Muskaat-variëteite, maar hierdie geurkomponente is ook teenwoordig in ander nie-Muskaat druifsoorte, waar dit bydra tot die variëteitsgeur en aroma. Hierdie monoterpenole kom voor in druiwe as vry, vlugtige en aromatiese molekules, of as geurlose, nie-vlugtige glikosidies-gebonde komplekse. Hierdie komplekse is meestal in die vorm van 6-O-α-L-arabinofuranosiel-β-D-glukopiranosiede, 6-O-β-D-xilopiranosiel-β-D-glukopiranosiede (primverosiede), 6-O-β-D-glukopiranosiel-β-D-glukopiranosiede (gentiobiosiede), 6-O-α-L-ramno-pyranosiel-β-D-glukopiranosiede (rutinosiede), of 6-O-β-D-apiofuranosiel-β-D-glukopirano-siede van hoofsaaklik linalool, geraniol, nerol, α-terpineol en hotrienol. Hierdie geurvoorlopers word egter slegs tot 'n beperkte mate tydens die proses van fermentasie deur die endogene glikosidase ensieme gehidroliseer, aangesien hulle baie min aktiwiteit toon onder wynbereidingstoestande.

Die monoterpenole kan op een van twee wyses van hul suikermolekules vrygestel word: 'n suurhidrolise, of ensiematiese hidrolise. Die ensiematiese hidroliseproses word baie goed begryp en behels twee opeenvolgende stappe: eerstens, afhangende van die aard van die voorloper, word die glikosidiese verbinding deur 'n α-L-arabinofuranosidase, 'n α-L-ramnosidase, 'n β-D-xilosidase, of 'n β-D-apiosidase gebreek. In die tweede stap word die monoterpeenalkohol deur 'n β-glukosidase vrygestel. Hierdie ensiematiese afbraakproses verander nie die intrinsieke aromatiese kenmerke van die wyn, soos wat met suurhidrolise die geval is nie.

Omdat die endogene glikosidases van *Vitis vinifera* en dié van die gis *Saccharomyces cerevisiae* baie lae aktiwiteit ten opsigte van die aromatiese voorlopers gedurende die hantering van die druiwesap en wynmaakprosesse toon, val die fokus al hoe meer op die inkorporering van eksogene β-glukosidases in wyn en sappe. Gene wat vir β-glukosidases en α-L-arabinofuranosidases kodeer, is al vanuit verskeie organismes gekloneer, insluitende bakterieë, fungi en giste. Die aktiwiteite en kenmerke van hierdie ensieme is egter nie altyd wenslik vir hul gebruik in wyn nie, aangesien dit 'n omgewing is met 'n lae pH, lae temperatuur, en hoë etanolvlakke en glukose-konsentrasies. 'n Geneties veranderde wyngis van *S. cerevisiae* wat in staat is om glikosidases uit te druk wat onder hierdie kondisies aktief is, sal baie handig te pas kom in die verbetering van die geur en aroma van wyne, om daardeur die kompleksiteit en die waarde van die wyn te verhoog.

Twee β-glukosidasegene, *BGL1* en *BGL2* vanaf die gis *Saccharomycopsis fibuligera*, is in twee afsonderlike *Esccherichia coli*-gis-pendelplasmiede gesubkloneer. 'n Dominante selekteerbare merkergeen (*SMR1*) is ook in hierdie plasmiede gekloneer. Hierdie plasmiede word onderskeidelik pBGL1 (met die *BGL1*-geen) en pBGL2 (bevattende

die *BGL2*-geen) genoem. Hierdie twee plasmiede is hierna apart na twee rasse van *S. cerevisiae* getransformeer. Eerstens is 'n laboratoriumras, $\Sigma1278$, getransformeer om te bevestig dat effektiewe sekresie en uitdrukking van die proteïen plaasvind. Hierna is 'n industriële gisras, VIN13, getransformeer deur gebruik te maak van die selektiewe merker (bestandheid teen sulfometuron). Ensiem-bepalings met behulp van die sintetiese substraat ρ-nitrofeniel-β-D-glukopiranosied (ρNPG) is gedoen om die aktiwiteit van die β-glukosidases oor 'n aantal dae te bepaal, asook om die aktiwiteit by sekere temperature en pH-vlakke te meet. Die stabiliteit van die ensieme is ook bepaal.

Hierdie rekombinante giste was in staat om ρNPG effektief af te breek. Hulle het belowende resultate betreffende die pH-optima getoon, met 'n aansienlike hoeveelheid aktiwiteit by die pH-vlakke soos dit in die wynomgewing voorkom. Daar was ook 'n effense verhoging in die ensieme se aktiwiteite by laer temperature. Die rekombinante gisrasse is ook in kleinskaalse wynfermentasies gebruik. Drie verskillende wyne is gemaak, waarvan twee wit kulitvars was (Chenin blanc en Gewürtztraminer) en een 'n rooi kulitvar (Pinotage). Resultate wat deur die mikro-ekstraksie van die voltooide wyne verkry is, het getoon dat die terpenolinhoud wel verhoog het, alhoewel dit nie die enigste wynkomponente was wat beïnvloed is nie. Ander geurkomponente het ook 'n verhoging in konsentrasie getoon, veral die esters. Hierdie verbindings het ook 'n rol in die verhoging van geur in die wyne gespeel.

Toekomstige werk sal die beskikbare resultate verder optimaliseer. Dit sal insluit die byvoeging van nog 'n glikosidiese ensiem, soos α-L-arabinofuranosidase, tot die genoom van die wyngis, om verdere afbraak van glikosidiese verbindings teweeg te bring. Die klonering of verandering van 'n β-glukosidase-ensiem met verhoogde aktiwiteit by laer temperature sal ook beter resultate toon en meer geur in die wyn kan vrystel.

This thesis is dedicated to my family.
Hierdie tesis is opgedra aan my familie.

BIOGRAPHICAL SKETCH

Tanya Gwendryth Stidwell was born in Beaufort West, South Africa, on 6 December 1976. She attended Saffier Primary School, and matriculated at Tygerberg High School in 1994.

Tanya began her studies at the University of Stellenbosch in 1995 and obtained a BSc degree in Genetics and Microbiology in 1997. In 1998 she completed a BscHons degree in Wine Biotechnology at the same university. In 1999 she enrolled for an MSc in Wine Biotechnology.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

Prof IS Pretorius, Director of the Institute for Wine Biotechnology, University of Stellenbosch, for accepting me as a student, for invaluable council, and for his enthusiasm throughout this project;

Dr P Van Rensburg, Institute for Wine Biotechnology, University of Stellenbosch, who acted as my project leader and co-supervisor, for his encouragement and thought-inspiring discussions;

Dr MG Lambrechts, Distell, Stellenbosch, who acted as my co-supervisor, for the critical evaluation of this thesis;

The students and staff of the Institute for Wine Biotechnology, for their assistance and discussions;

The National Research Foundation (NRF) and Winetech, for financial assistance;

My family and friend Wessel, for their encouragement and patience;

The Almighty, for giving me this opportunity.

PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal *Food Microbiology*, to which Chapter 3 will be submitted for publication.

Chapter 1   **General Introduction and Project Aims**

Chapter 2   **Literature Review**
The effect of glycosidases on the aroma of wine
– a review.

Chapter 3   **Research Results**
The expression of two *Saccharomycopsis fibuligera* β-glucosidase genes (*BGL1* and *BGL2*) in an industrial wine yeast strain of *Saccharomyces cerevisiae*.

Chapter 4   **General Discussion and Conclusions**

CONTENTS

CHAPTER 3. THE EXPRESSION OF TWO *SACCHAROMYCOPSIS FIBULIGERA* β-GLUCOSIDASE GENES (*BGL1* AND *BGL2*) IN AN INDUSTRIAL WINE YEAST STRAIN OF *SACCHAROMYCES CEREVISIAE*

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION

1.1. The flavour of wine

The flavour of wine is a very complex interaction of the flavour compounds and how the consumer perceives it through taste and smell. How the wine tastes and smells is the combination of a multitude of different components, each contributing to the final flavour and aroma of the wine. The aroma and flavour of a wine can basically be divided into two groups, volatile and non-volatile components. The sensation of taste can be atttributed to the non-volatile components, whereas the volatile components are responsible for the odour of the wine. The volatile compounds can usually be perceived at very low concentrations, whereas the non-volatiles have to be present in larger quantities to make a difference in the taste, i.e. higher than levels of 1% or even more. Three major distinctions concerning the origin of the aroma of wine are traditionally made. The first is the aroma that is contributed by the grapes themselves, and this also includes the aroma changes due to modifications during the processing of the grapes. The second is the aroma that is formed during the fermentation process, and the third is the bouquet of the wine, which is the result of the transformation of the aroma during aging, whether it is in the bottle or in wooden casks.

1.2 The importance of terpenic compounds

All grapes possess a generic grape aroma that forms the basis of its varietal aroma. Most varieties also possess a further distinctive aroma that distinguishes these varieties from others. The generic grape aroma consists mainly of a combination of esters, aldehydes, ketones, alcohols, phenols, organic sulphur compounds and acetates. Other compounds then add to this generic grape aroma, lending it a distinctive aroma unique to that specific cultivar. Examples of these compounds are the methoxypyrazines present in Sauvignon blanc juice, which gives it its distinctive grassy and vegetative aroma. Another group of compounds that contribute in a very large way to typical varietal character is the monoterpene alcohols in the Muscat varieties. These compounds, as well as their derivatives, have been studied extensively and to date more than 50 of these compounds are known in grapes and wines. The most common monoterpene alcohols found are linalool, geraniol, nerol, citronellol, α-terpineol and hotrienol. Linalool and geraniol are the most aromatic, imparting flavour at the lowest thresholds. Oxides of these monoterpene alcohols are also very common, especially those of linalool and nerol, linalool-oxide and nerol-oxide. Although their perception thresholds are much higher than that of the

monoterpene alcohols, they play a synergistic role in the overall mixture of aromatic constituents (Ribéreau-Gayon et al., 1975).

The aromas imparted by these monoterpene alcohols (monoterpenols) are not limited to the muscat-like smell, but can range from spicy, peppery and smoky to grassy. They therefore can play supporting roles in the aroma of other cultivars as well, such as Chardonnay, Cape Riesling and Sauvignon blanc (Rapp and Mandery, 1986).

However, the terpenols in grapes are not always free and therefore volatile. Actually, a large fraction of these monoterpene alcohols are bound to glycosides, which renders them non-volatile and odourless. Conjugates of linalool, geraniol, nerol and α-terpineol are detected most often. Mulkens (1987) also observed that glycosylesters of monoterpenoid aglycones exist. The glycosidically bound fraction is often found exceeding the amount of free aroma compounds in a ratio ranging from 2:1 to 5:1. The aglycones are bound to include basically all types of saccharides found in grapes, and include α-L-rhamnopyranose, α-L-arabinofuranose, β-D-xylopyranose and β-D-apiofuranose, among others. A general feature of the glycosidically bound volatiles is that the sugar that is bound directly to the aglycone is a β-D-glucose. Other sugar units as mentioned are then added onto this molecule (Williams, 1993).

During fermentation the monoterpene alcohol profile does not change significantly, although it may undergo small changes (Rapp and Mandery, 1986). This is mainly due to the fact that wine yeasts cannot synthesize monoterpenes and are generally unable to convert the glycosidically bound fraction of these monoterpenols to free volatile compounds. The small changes they are reported to undergo include basic conformation shifts and oxidation. During post-fermentation processes and aging, however, the terpenic profile of a wine can change in a more significant manner. According to Rapp et al. (1985a, b) various additional chemical reactions occur during bottle maturation, and some of the monoterpenes in wine undergo drastic changes. Linalool in particular is transformed to other terpenes, decreasing the levels of this highly perceptible compound and increasing the levels of other terpenes that may have higher perception thresholds. An increase in these other compounds may either have no or little influence (e.g. linalool oxide), where the heightened levels of others, for example α-terpineol, may have a negative influence (Güntert, 1984; Rapp et al., 1985c). Since the olfactory perception threshold for the monoterpenes in wine varies, ranging from 100 µg/l to 700 µg/ml, it has been found that additive effects of these compounds may lower the perception threshold. It is thus clear that it remains very difficult to predict the aroma changes during aging (Ribéreau-Gayon, 1978).

2. THE INCREASE OF VOLATILE FLAVOUR IN WINE

Since it has become apparent that the release of the monoterpene alcohols from their glycones could increase the aroma of wine considerably, this subject has become the focus point of various studies. It was concluded early on that these terpenols could be liberated by two mechanisms. The first is an acid hydrolysis (Williams et al., 1982), which, due to its nature, can produce modifications in the aromatic character of the wine. The second method is by enzymatic hydrolysis (Günata et al., 1988; 1990a). This mechanism has been studied extensively (Aryan et al., 1987; Günata et al., 1990b). It has been concluded that glycosidic enzymes are able to split the β-glucosidic bond between the terpenes and sugars without modifying the aromatic characteristics, as observed with acid hydrolysis.

This enzymatic liberation of the monoterpene alcohols occurs in two steps. The first step requires the action of an α-arabinofuranosidase, α-rhamnosidase, β-xylosidase, or a β-apiosidase for the enzymatic cleavage of the (1→6)-intersugar-linkage. This results in a monoterpenyl glucoside, since the sugar moiety bound to the terpenol is always a β-D-glucose. The second step then involves a β-glucosidase to liberate the terpene alcohol (Günata et al., 1988). When the disaccharide moiety connected to the monoterpenol consists of two glucose units, both the hydrolytic steps can be performed by a β-glucosidase in a two-step mechanism (Haisman and Knight, 1967).

2.1 Properties of endogenous and exogenous glycosidases

Endogenous glycosidases, as present in the wine, are mainly from *Vitis vinifera*, the grape plant itself. However, not all of these enzymes are able to function in winemaking conditions. This environment is particularly harsh, as low pH levels, an increasing ethanol content and high glucose concentrations prevail. In fact, plant glycosidases are poor performers in wine conditions, with a very low tolerance for low pH levels. They are also easily inhibited by the high glucose and ethanol levels present in wine (Aryan et al., 1987). Since these enzymes cannot reveal the whole aromatic potential, the focus is falling increasingly on the use of exogenous glycosidases.

Glycosidase enzymes, and specifically β-glucosidases, are present in a variety of organisms where they perform a multitude of functions. Bacterial glycosidases showed better activity in the limiting conditions offered by wine, but high temperature optima and inhibition by glucose levels as found in wine constrained the heterologous use of these enzymes. Most fungal β-glucosidases are also inhibited by glucose, even at levels as low as 90 g/L, or 9% v/v (Riou et al., 1998).

Yeasts show promise as sources for exogenous glycosidases. Over the years, however, the data on *Saccharomyces cerevisiae* β-glucosidases have been contradictory. It has been reported that a structural gene for this enzyme exists in the yeast genome, but it is very poorly expressed (Duercksen and Halvorsen, 1958, 1959). More recent results obtained by Günata et al. (1990a) concluded that *S. cerevisiae* exhibits very low β-glucosidase activity, but Delcroix et al. (1994) isolated three yeast strains that showed high enzymatic activity in trials, but low activity in wine itself. On the other hand, Darriet et al. (1988) postulated that it was rather the activity of certain oxidases located in the periplasmic space of a strain of *S. cerevisiae* that was able to hydrolyze monoterpene glycosides. Later work done by Mateo and Di Stefano (1997) revealed that wine yeasts have some constitutive β-glucosidase activity, but that this enzyme is still inhibited by winemaking conditions. Other yeasts have shown to have glycosidases that are more active in the wine environment. Examples of sources of glycosidases that have been studied in depth include *Candida molischiana* and *C. wickerhamii* (Günata et al., 1990b; Gueguen et al., 1996; Skory et al., 1996); *Debaryomyces hansenii* (Rosi et al., 1994; Yanai and Sato, 1999); *Saccharomycopsis fibuligera* (Machida et al., 1988); *Kluyveromyces fragilis* (Raynal and Guerineau, 1984); *Zygosaccharomyces bailii* (Gueguen et al., 1995); *Candida entomophila* (Gueguen et al., 1994); *Candida peltata* (Saha and Bothast, 1996); *Aspergillus niger* (Yan and Lin, 1997); and *Aspergillus oryzae* (Riou et al., 1998). These glycosidases show more promise as endogenous enzymes and could be used as heterologous enzymes for the release of more aroma in wine.

3. OTHER GLYCOSIDICALLY BOUND COMPOUNDS

It is not only monoterpene alcohols that are found as glycosidically bound structures in wine. In fact, approximately 200 aglycones within 150 plant species are known. Some of these more commonly found in wine include aliphatic alcohols, alkylphenols, sesquiterpenoids (C_{15}-compounds), norisoprenoids (C_{13}-compounds, derived from carotenoid aroma compounds) (Winterhalter and Skouroumounis, 1997), anthocyanins (Wightman and Wrolstad, 1995), as well as resveratrol (Vrhovsek et al., 1997). This information holds the promise that glycosidase enzymes could not only increase the aroma of wine by releasing monoterpene alcohols, but also that of the other aroma compounds. It could also increase the intensity of the colour, as well as of resveratrol levels in red wine, an added health benefit.

4.   AIMS OF THIS STUDY

S. cerevisiae is a model organism that presents the simplest eukaryotic model of gene expression. It has been used widely to study the molecular details of gene expression and product secretion, and has gained much significance over past years as a host of heterologous proteins of biotechnological interest. As a wine yeast it also acts as a vehicle for many biotechnological changes that one would like to introduce to wine. The primary aim of this study was to introduce two exogenous β-glucosidase genes from the yeast *S. fibuligera* (Machida et al., 1988) to the genome of a laboratory and a wine yeast strain of *S. cerevisiae*. Further aims and approaches that flowed from this were:

(i)   the monitoring of the expression, secretion, activity and stability of these β-glucosidase enzymes in *S. cerevisiae*, as expressed under their native promoters;

(ii)   small-scale fermentations with yeast transformants containing these enzymes to study the expression and activity of these enzymes under the limiting conditions of wine.

5.   REFERENCES

Aryan, A.P., Wilson, B., Strauss, C.R., and Williams, P.J. (1987) The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible application in enology. *Am J. Enol. Vitic.* **38**, 182-188.

Darriet, P., Boidron, J.N., and Dubordieu, D. (1988) L'hydrolyse des hétérosides terpéniques du Muscat a Petit Grains par les enzymes périplasmiques de *Saccharomyces cerevisiae*. *Conn. Vigne Vin.* **22**, 189-195.

Delcroix, A., Günata, Z., Sapis, J., Salmon. J., and Bayonove, C. (1994) Glycosidase activities of three enological yeast strains during winemaking: Effect on the terpenol content of Muscat wine. *Am J. Enol. Vitic.* **45**, 291-296.

Duerksen, J.D., and Halvorson, H. (1958) Purification and properties of an inducible β-glucosidase of yeast. *J. Biol. Chem.* **233**, 1113-1120.

Duerksen, J.D., and Halvorson, H. (1959) The specificity of induction of β-glucosidase in *Saccharomyces cerevisiae*. *Biochem. Biophys. Acta.* **36**, 47-55.

Gueguen, Y., Chemardin, P., Arnaud, A., and Galzy, P. (1994) Purification and characterization of the endocellular β-glucosidase of a new strain of *Candida entomophila* isolated from fermenting agave (*Agave* sp.) juice. *Biotechnol. Appl. Biochem.* **20**, 185-198.

Gueguen, Y., Chemardin, P., Arnaud, A., and Galzy, P. (1995) Comparative study of extracellular and intracellular β-glucosidases of a new strain of *Zygosaccharomyces bailii* isolated from fermenting agave juice. *J. Appl. Bact.* **78**, 270-280.

Gueguen, Y., Chemardin, P., Janbon, G., Arnaud, A., and Galzy, P. (1996) A very efficient β-glucosidase catalyst for the hydrolysis of flavour precursors of wines and fruit juices. *J. Agric. Food Chem.* **44**, 2336-2340.

Günata, Z., Bitteur, S., Brillouet, J.M., Bayonove, C., and Cordonnier, R. (1988) Sequential enzymic hydrolysis of potentially aromatic glycosides from grape. *Carbohydr. Res.* **184**, 139-149.

Günata, Y.Z., Bayonove, C.L., Tapiero, C., and Cordonnier, R.E. (1990a) Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. *J. Agric. Food. Chem.* **38**, 1232-1236.

Günata, Y.Z., Bayonove, C.L., Arnaud, A., and Galzy, P. (1990b) Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamii* β-glucosidases. *J. Sci. Food. Agric.* **50**, 499-506.

Güntert, M. (1984) Gaschromatographisch-massenspektrometrische Untersuchungen flüchtiger Inhaltstoffe des Weinaromas: Beitrag zur Sortencharakterisierung der Rebsorte Weisser Riesling. Thesis, Universität Karlsruhe, Karlsruhe 1984.

Haisman, D.R. and Knight, D.J. (1967) The enzymatic hydrolysis of amygdalin. *Biochem J.* **103**, 528.

Machida, M., Ohtsuki, I., Fukui, S., and Yamashita, I. (1988) Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular β-glucosidases as expressed in *Saccharomyces cerevisiae.* *Appl. Environ. Microbiol.* **54**, 3147-3155.

Mateo, J.J. and Di Stefano, R. (1997) Description of the β-glucosidase activity of wine yeasts. *Food Microbiol.* **14**, 583-591.

Mulkens, A. (1987) Flavonoids of the leaves of *Melissa officinalis* L. *Pharm Acta Helv.* **62**, 229.

Rapp, A., Almy, J., and Güntert, M. (1985a) Identification of several sulphur-containing compounds in wine. *Am. J. Enol. Vitic.* **36**, 219–221.

Rapp, A., Güntert, M., and Rieth, W. (1985b) Einfluss der Maischestandzeit auf die Aromazusammensetzung des Traubenmostes und Weines. *Dt. Lebensm. Rdsch.* **81**, 69-72.

Rapp, A., Güntert, M., and Ullemeyer, H. (1985c) Über Veränderungen der Aromastoffe während der Flaschenlagerung von Weissweinen der Rebsorte Riesling. *Z. Lebensmittelnunters. – Frorsch.* **180**, 109–116.

Rapp, A., and Mandery, H. (1986) Wine Aroma. *Experimentia.* **42**, 873-884.

Raynal, A. and Guerineau, M. (1984) Cloning and expression of the structural gene for β-glucosidase of *Kluyveromyces fragilis* in *Escherichia coli* and *Saccharomyces cerevisiae. Mol. Gen. Genet.* **195**, 108-115.

Ribéreau-Gayon, P., Boidron, J. H., and Terrier, A. (1975) Aroma of muscat grape varieties. *J. Agric. Food Chem.* **23**, 1042–1047.

Ribéreau-Gayon, P. (1978) "Wine-Flavor". In: *Flavor of foods and beverages.* (Eds. G. Charalambous, G. E. Inglet) Academic Press, New York / San Francisco / London.

Riou, C., Salmon, J.C., Valiier, M.J., Günata, Z., and Barre, P. (1998) Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae. Appl. Environ. Microbiol.* **64**, 3607-3614.

Rosi, I., Vinella, M., and Domizio, P. (1994) Characterization of β-glucosidase activity in yeasts of oenological origin. *J. Appl. Bacteriol.* **77**, 519-527.

Saha, B.C., Freer, S.N., and Bothast, R.J. (1995) Thermostable β-glucosidases. Enzymatic degradation of insoluble carbohydrates. (Eds. J. N. Saddler, N. H. Penner) American Chemical Society, Washington, D.C.

Skory, C.D., Freer, S.N., and Bothast, R.J. (1996) Properties of an intracellular β-glucosidase purified from the cellobiose-fermenting yeast *Candida wickerhamii. Appl. Microbiol. Biotechnol.* **46**, 353-359.

Vrhovsek, U., Wendelin, S., and Eder, R. (1997) Effects of various vinification techniques on the concentration of *cis*-and *trans*-resveratrol and resveratrol glucoside isomers in wine. *Am. J. Enol. Vitic.* **48**, 214-219.

Wightman, J.D. and Wrolstad, R.E. (1995) Anthocyanin analysis as a measure of glycosidase activity in enzymes for juice processing. *J. Food. Sci.* **60**, 862-867.

Williams, P.J., Strauss, C.R., Wilson, B., and Massy-Westropp, R.A. (1982) Studies on the hydrolysis of *Vitis vinifera* monoterpene precursor compounds and model monoterpene β-D-glucosides rationalizing the monoterpene composition of grapes. *J. Agric. Food Chem.* **30**, 1219-1223.

Williams, P.J. (1993) Hydrolytic flavour release in fruit and wines through hydrolysis of non-volatile precursors. *Flavor Science – Sensible Principles and Techniques.* p 287. (Eds Acree, T.E., Teranishi, R.) American Chemical Society, Washington DC.

Winterhalter, P. and Skouroumounis, G. K. (1997) Glycoconjugated aroma compounds: occurrence, role and biotechnological transformation. *Adv. Biochem. Eng. Biotech.* **55**, 73-105.

Yan, T.-R, and C.-L. Lin. (1997) Purification and characterization of a glucose tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. *Biosci. Biotechnol. Biochem.* **61**, 965-970.

Yanai, T., and Sato, M. (1999) Isolation and properties of β-glucosidase produced by *Debaryomyces hansenii* and its application in winemaking. *Am. J. Enol. Vitic.* **50**, 231-235.

CHAPTER 2

LITERATURE REVIEW

The effect of heterologous glycosidases on the aroma of wine – a review

1.   THE AROMA OF WINE

Wine is a beverage that is greatly enjoyed and admired by people the world over.   Various factors contribute to the popularity and enjoyment of wine, of which its flavour is probably the most important.   The flavour of wine, however, is a very complex interaction between the flavour components in the wine itself, and the sense of smell and taste of the consumer. The flavour of wine is in essence the culmination of a multitude of components, each contributing in its special way to the final aroma and flavour of the wine.

The concept of "flavour" in a wine is basically a composition of volatile and non-volatile components.   Volatile components are mainly responsible for the odour of the wine, and non-volatiles cause the sensation of taste.   These sensations could be that of sweetness,   sourness,   bitterness   or   saltiness,   or   a   combination   of   any   of   the aforementioned.   These are, however, limited taste sensations, and are usually formed by the presence of sugars, organic acids, polymeric phenols and mineral substances.   To influence taste, these compounds mostly have to be present at levels of 1% or even more (Rapp and Mandery, 1986).

The volatile components in wine, however, can usually be perceived when present in much lower concentrations.   This is because our sense of smell is developed to a far greater extent than our sense of taste, being extremely sensitive to certain aroma substances.   Some compounds can be smelled by individuals at thresholds as low as 10^{-4} and 10^{-12} g/l (Guadigni et al., 1963). Several hundred different compounds are simultaneously responsible for the odour released by the wine, and since there is no real character impact compound, the aroma of the wine can be said to be a delicate balance of all these components.

The aroma of wine was first studied by Hennig and Villforth in 1942. They identified a few basic compounds using classical chemical methods, but in the late fifties the process of gas chromatography was first applied to identify more complex compounds, for instance higher alcohols and esters (Bayer and Bässler, 1961; Bayer et al., 1958).   Gas chromatography extended the field for research in wine aroma enormously, giving scientists the tool for extracting aroma substances from grapes and wine, and then following the change in these substances in the evolution of grape juice to wine.

There are a few factors influencing the amount of aroma compounds present in the final wine product. These include environmental factors, e.g. the climate and soil conditions in which the grapes were grown, the cultivar of the grapes, the fermentation conditions (pH, temperature, juice nutrients and microflora) as well as the various post-fermentation treatments that the wine is sometimes subjected to, for instance blending, clarification and filtration.

Three major distinctions are made in connection with the aroma of wine. The first is the aroma that originates from the grapes itself, including the aroma changes due to modifications during the processing of the grapes. The second is the aroma produced during the fermentation process, and the third is the bouquet which results from the transformation of the aroma during aging, whether it be in wooden casks, or in the bottle (Rapp and Mandery, 1986).

1.1   The aroma of grapes

Different varieties of grapes have different tastes. There is, however, a generic grape taste that all grapes possess, and which distinguishes them from other types of fruit. This neutral aroma will be discussed first, followed by a discussion of the distinctive aroma of some grape varieties.

There are a few compounds in grapes that are either present in such low quantities that they cannot be detected in grape must, or their poor water solubility prevents them from effectively making an impact on the aroma of the must. These include the aliphatic n-alkanes and some aromatic hydrocarbons like toluene, xilene and alkylbenzenes (Schreier et al., 1976; Stevens et al., 1957, 1967, 1969). These compounds may precipitate with the must slurry during wine making, rendering them even more insignificant.

Very few esters are present in grapes of *Vitis vinifera* species. In grapes they are mainly acetate esters of short chain alcohols, and the acetates of some monoterpene alcohols (Rapp and Knipser, 1980) and (*E*)-methyl geranoate are found in muscat grape varieties (Schreier et al., 1976).

Aldehydes play a large role in the aroma of wine, as enzymatic processes that form C_6-aldehydes and alcohols take over at the moment of grape cell destruction (Rapp and Mandery, 1986). These compounds are quantitatively dominant, so the aroma of the grape must would be highly dependent on which of these compounds were present. *Trans*-2-hexanal and n-hexanal occur in large amounts In Sultana and Grenache varieties (Ramshaw and Hardy, 1985; Stevens et al., 1967). Due to enzyme inhibition, the aldehydes can be either saturated or unsaturated (Schreier et al., 1976), which makes it very difficult to determine exactly to what extent the aldehydes contribute to the original grape aroma.

Small fractions of ketones are also present, with 2- and 3-n-alkanones occuring in the highest concentrations.   n-Alcohols with a chain length of four to 11 carbons compromise the alcohol fraction (Schreier, 1979). Generally, these alcohols do not contribute very much to the aroma imparted by the alcohol fraction in the final wine product, but according to Welch et al. (1982), they could play a role in the varietal aroma of

Muscadine grapes. This can be due to the presence of isoamyl alcohol, hexanol, benzaldehyde and 2-phenylethanol and its derivatives.

Monoterpene alcohols and their derivatives play an important role in the aroma of wine, especially in the muscat cultivars. This is a group of aroma compounds that has been studied extensively, and to date more than 50 monoterpene compounds in grapes and wines are known. The most common monoterpene alcohols found are linalool, geraniol, nerol, citronellol, α-terpineol and hotrienol. Ribéreau-Gayon et al. (1975) found that linalool and geraniol are the most aromatic within the terpene fraction. The other monoterpene alcohols generally have much higher perception thresholds than that of linalool, which is quite low at 100 μg/l.
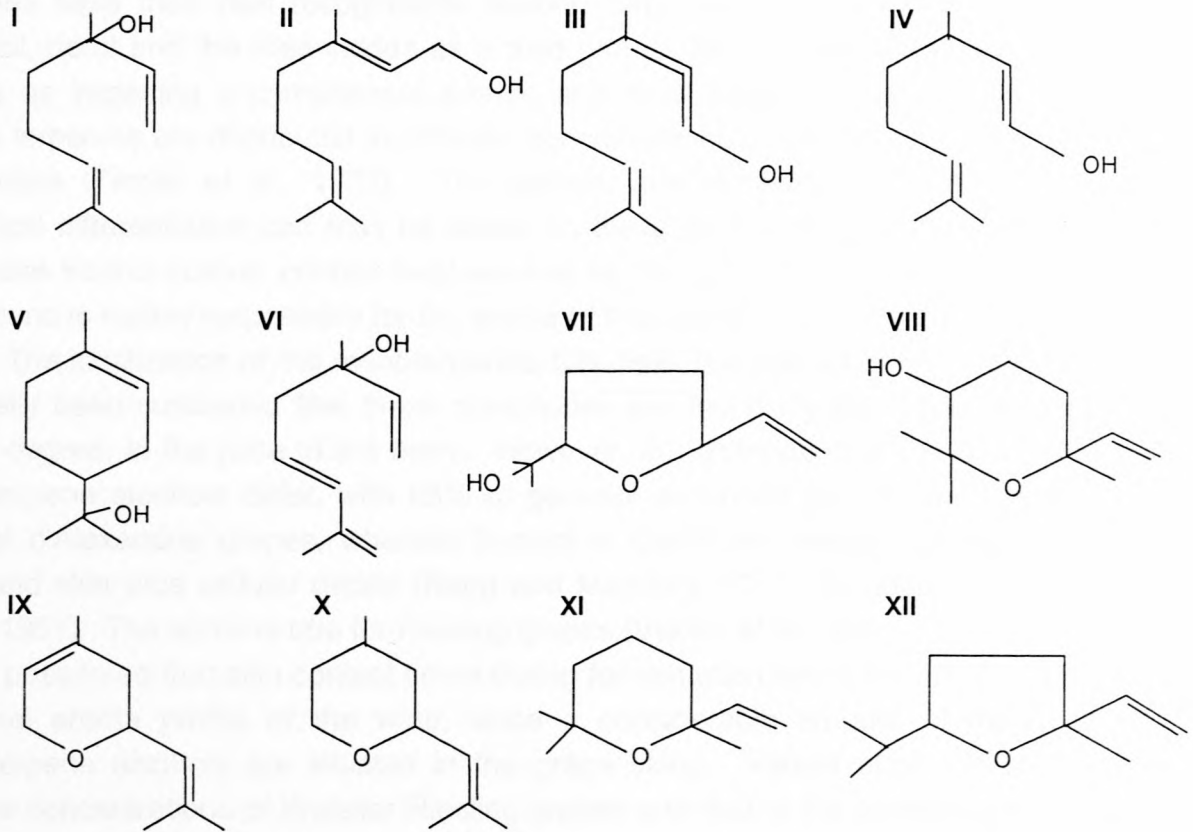
Figure 1. Volatile monoterpenes found in wine. **I** – linalool, **II** – geraniol, **III** – nerol, **IV** – citronellol, **V**-α-terpineol, **VI** – hotrienol, **VII & VIII** – linalool oxides, **IX** – nerol oxide, **X** – rose oxide, **XI & XII** – ethers (Rapp and Mandery, 1986).

Oxides of these monoterpene alcohols are also common, especially those of linalool and nerol. Their perception thresholds are high (3000-5000 µg/l). However, although they would not be detected on their own, the perception threshold is lower than the most aromatic single component as a result of the synergistic effect of the mixture of the grape aroma constituents (Ribéreau-Gayon et al., 1975).

It is possible to distinguish between different cultivars of grapes and wine made from these cultivars according to their unique terpene profiles. As mentioned before, the monoterpene alcohol fraction contributes largely to the varietal aroma of muscat cultivars, such as Muscat d'Alexandrie, Morio Muscat and Muscat de Frontignan. They also play roles in other varieties, existing as subtle supporting flavour components in cultivars such as Chardonnay, Sauvignon blanc, Kerner and Cape Riesling. The aroma imparted by the terpenes is therefore not only limited to a muscat-like smell, but includes various other aromas, such as spicy, peppery, smoky and grassy. It has been observed that most of the terpenes have their own recognizable flavour. Simpson (1979) described the aroma of geraniol, nerol and the rose oxides as a rose aroma, that of linalool as coriander, linalool oxides as imparting a campherous aroma, and nerol oxide as smelling like vegetation. These terpenols are distributed in different concentrations in the different white varieties of *V. vinifera* (Terrier et al., 1972). The patterns are constant for each variety and an analytical differentiation can thus be based on these profiles. For instance, grapes of the Japanese Koshu cultivar contain terpinen-4-ol as the main volatile monoterpene and this compound is mainly responsible for the aroma of this specific cultivar.

The localization of the monoterpenols has been the subject of many studies. It has generally been concluded that these compounds are found mostly in the skins and, to a lesser degree, in the juice of the berry. However, the distribution of the different types of monoterpene alcohols differ, with 95% of geraniol and nerol concentrated in the skin of Muscat d'Alexandrie grapes, whereas linalool is distributed almost equally between the juice and skin plus cellular debris (Rapp and Mandery, 1986; Cordonnier and Bayonove, 1978, 1981). The same is true for Riesling grapes (Versini et al., 1981).

It was postulated that skin contact times during fermentation could thus play a large role in the final aroma profile of the wine, since a considerable amount of these important monoterpene alcohols are situated in the grape skins. Versini et al. (1981) compared terpene concentrations of Weisser Riesling grapes with that of the corresponding must and wine. Pressing of the grapes showed a clear increase in terpene concentrations from grapes to must, for instance seven to eight times for geraniol, nerol and citronellol. This was applicable for the wines as well, with a five to tenfold increase in the levels of linalool, nerol, hotrienol, α-terpineol and the linalool oxides. These authors also noted the differences that certain treatments and conditions have on the extraction of these terpenes. These included different skin contact periods, a carbon dioxide atmosphere, the use of

pectolytic enzymes, as well as heating of the press juice. A definite increase in the terpene concentration was observed. Rapp et al. (1985c), however, showed that increased skin contact times rather correlated with an increase in acetamide concentration, whereas the amounts of the terpenes stayed constant.

Table 1. The fractions of the three most common terpenols as they occur in the grape berry (Marais, 1985, and references therein).

	mg/100g berries			% of each alcohol		
	Linalool	Nerol	Geraniol	Linalool	Nerol	Geraniol
Skins	14.2	15.2	100	26%	95.6%	94.6%
Flesh	13.5	0.45	3.5	24%	2.7%	3.3%
Juice	27.5	0.3	2.5	50%	1.7%	2.5%

There are external factors that also influence the terpenol content in grapes. One of these is the mould, *Botrytis cinerea*. This fungus causes the rotting of grapes, but under special climatic conditions it is responsible for noble rot. This rot is a prerequisite for the production of botrytised wines, which have a very distinct aroma and are highly sought after. *B cinerea* is incapable of producing terpenoids in grape varieties in which they are not present, but the mould can transform linalool, which has been added to grape must, into other monoterpenes (Shimuzu et al., 1982). Small amounts of geraniol could also be formed from nerol, which is about 100 times higher in concentration (Rapp, 1987). Two of the compounds formed from the monoterpenes are mainly responsible for the distinct aroma imparted to the wine by *B. cinerea*. They are ethyl 9-hydroxynonanoate and 4,5-dimethyl-3-hydroxy-2(5H)-furanone (sotolone). The sotolone imparts a sweet, sugar- and caramel-like aroma, with a threshold value of 2.5 ppb. The content of this compound in botrytised wines is usually about 5-20 ppb, but in normal wines made from uninfected grapes, the value is below 1 ppb. Sotolone also plays a role in the flavour of sugar molasses, aged saké and flor sherry (Masuda et al., 1984).

However, the terpenols present in grapes are not always free and therefore volatile. In fact, in most cases they are bound to glycosides, which makes them non-volatile and therefore odourless. Conjugates of geraniol, nerol, linalool and α-terpineol are detected most often (Voirin et al., 1992). Mulkens (1987) also mentioned glycosylesters of monoterpenoid aglycones. As mentioned before, glycosidically bound flavour components

are very common, and they are often found exceeding the amount of free aroma compounds in a ratio range of 2:1 to 5:1. One can thus easily see the importance of this component of the grape aroma.

1.1.1 Chemical composition of aglycone structures

The aglycone structures found most commonly are medium-chain alkanols and alkenols, shikimic acid metabolites, as well as mevalonate-derived compounds with 10 (monoterpenoids), 13 (C_{13}-norisoprenoids) and 15 carbon atoms (sesquiterpenoids). The aglycones of plant glycosides are structurally complex and highly diverse. The disaccharide moieties to which they are bound are also a very diverse group, as can be seen in Figure 2.

I - α-L-Arabinofuranosyl-

II - α-L-Rhamnopyranosyl-

III - β-D-Xylopyranosyl-

IV - β-D-Apiofuranosyl-

V - β-D-Glucopyranosyl-

VI - -β-D-Glucopyranoside
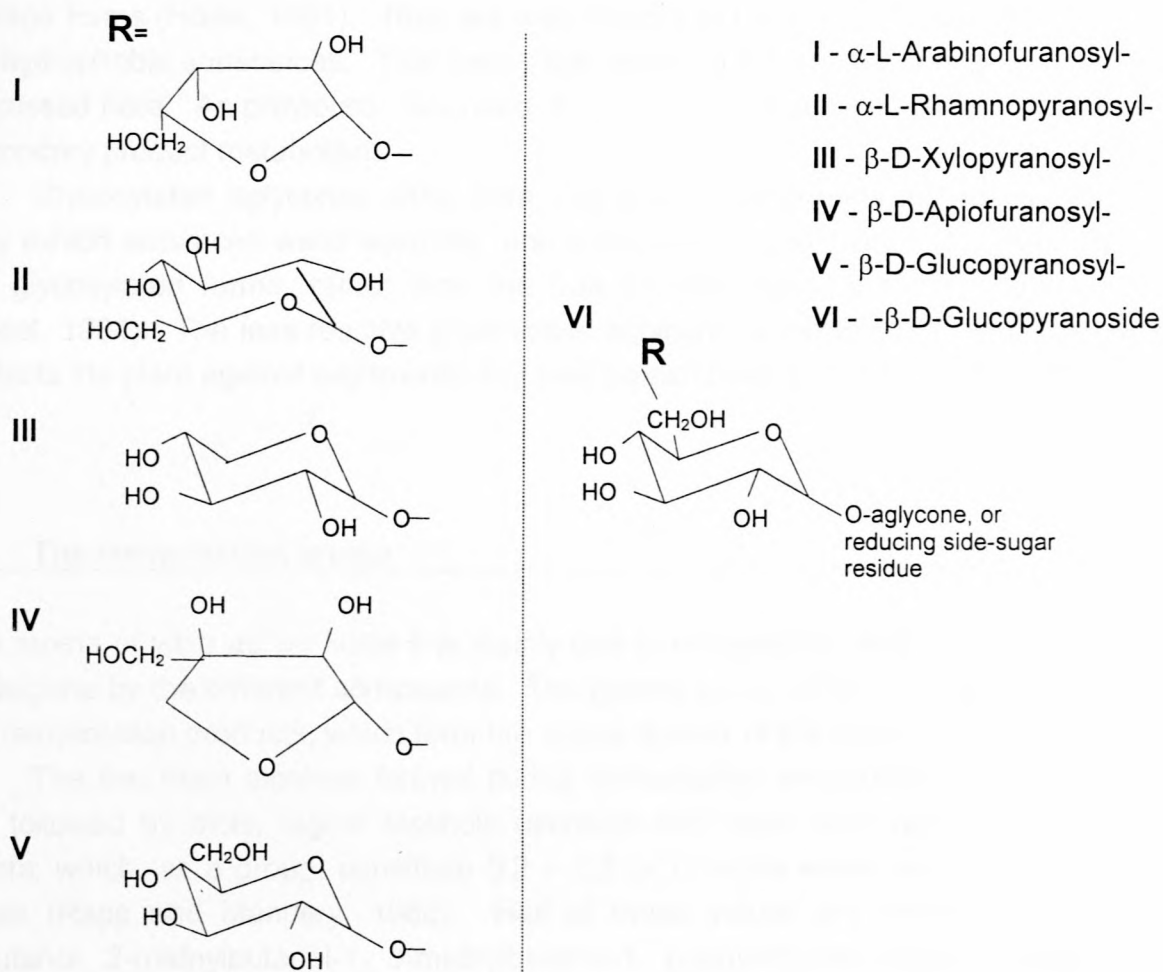
Figure 2. Disaccharide sugar moieties that have been identified in flavour precursors (adapted from Williams, 1993.)

A general feature of glycosidically bound volatiles is that the sugar that is bound directly to the aglycone is always a β-D-glucose. This glucose molecule may have other sugar units added to it. These types of sugars have been identified and consist of α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, β-D-apiofuranose and β-D-glucose (Williams, 1993).

1.1.2 The importance of glycoconjugated volatiles in the grapevine

Plant glycosides, and in this case grapevine glycosides, are obviously involved in different biochemical processes. It is thought that they play important roles as accumulation and storage forms (Hösel, 1981). They are also thought to have importance as transport forms for hydrophobic substances. This theory still needs to be studied, though, and will not be discussed here. As previously described, they also play a general role as intermediates of secondary product metabolism.

Glycosylated aglycones differ from free aroma compounds in two main properties: they exhibit enhanced water solubility, and a decrease in reactivity. This may explain why the glycosylated forms, rather than the free volatile forms, are accumulated in plants (Hösel, 1981). The less reactive glycosilated aglycone is easily stored in the vacuole and protects the plant against any toxicity that may be exhibited by the free aroma compound.

1.2   The fermentation aroma

The aroma of wine as we know it is mainly due to fermentation and the chemical changes undergone by the different compounds. The grapey odour of the must is superimposed by the fermentation products, which form the vinous flavour of the wine product (Rapp, 1987).

The two main alcohols formed during fermentation are ethanol and glycerol. They are followed by diols, higher alcohols (alcohols with more than two carbon atoms) and esters, which, as a group, constitute 0.2 – 1.2 g/l of white wines and 0.4 – 1.4 g/l of red wines (Rapp and Mandery, 1986). Half of these values are made up of n-propanol, n-butanol, 2-methylbutanol-1, 3-methylbutanol-1, phenylethanol, ethyl acetate and ethyl lactate. The formation of some compounds, for instance, the acetates, takes place in conjunction with and in parallel to the formation of ethanol by the yeast, which performs the fermentation. Other compounds, such as methanol, which arise from the degradation of

pectins, remain uninfluenced by the activity of most yeasts during fermentation, although some wine yeasts are known to form methanol to a small degree.

The role played by ethanol in wine is a very important one. It acts mainly as a solvent for a large number of the constituents in the wine and balances the taste sensations. It has a role as a fixer for odours and ultimately determines the viscosity (body) of the fermented product (Rapp, 1987).

The higher alcohols (fusel alcohols) can occur in varying concentrations, mostly in concentrations above their perception thresholds. When they are present at concentrations below 300 mg/l, they add to the complexity of the wine and make a pleasant contribution. When they exceed concentrations above 400 mg/l, however, they are generally regarded as a negative factor (Rapp, 1987).

The terpene alcohols and their oxides are also present in the wine. Some of these monoterpenols undergo small changes during alcoholic fermentation (Rapp and Mandery, 1986), but this has a very small influence on their concentrations in the final wine product. Since yeasts are unable to synthesize monoterpenes, these compounds are of specific importance as varietal characteristic substances (Rapp, 1987).

Monoterpene profiles of the varieties Riesling (WR), Paarl Riesling (PR) and Chenin blanc (CB)

Legend:
- 1 - trans-linox(f)
- 2 - cis-linox (f)
- 3 - Neroloxide
- 4 - Linalool
- 5 - Hotrienol
- 6 - alpha-Terpineol
- 7 - trans-linox (p)
- 8 - cis-Linox (p)
- 9 - Terpindiol-I
- 10 - Hydroxylinalool

Figure 3. A "terpene profile" of the Riesling varieties: Weisser Riesling, Paarl Riesling and Chenin blanc cultivated in South Africa (adapted from Rapp, 1987).

pectins, remain uninfluenced by the activity of most yeasts during fermentation, although some wine yeasts are known to form methanol to a small degree.

The role played by ethanol in wine is a very important one. It acts mainly as a solvent for a large number of the constituents in the wine and balances the taste sensations. It has a role as a fixer for odours and ultimately determines the viscosity (body) of the fermented product (Rapp, 1987).

The higher alcohols (fusel alcohols) can occur in varying concentrations, mostly in concentrations above their perception thresholds. When they are present at concentrations below 300 mg/l, they add to the complexity of the wine and make a pleasant contribution. When they exceed concentrations above 400 mg/l, however, they are generally regarded as a negative factor (Rapp, 1987).

The terpene alcohols and their oxides are also present in the wine. Some of these monoterpenols undergo small changes during alcoholic fermentation (Rapp and Mandery, 1986), but this has a very small influence on their concentrations in the final wine product. Since yeasts are unable to synthesize monoterpenes, these compounds are of specific importance as varietal characteristic substances (Rapp, 1987).
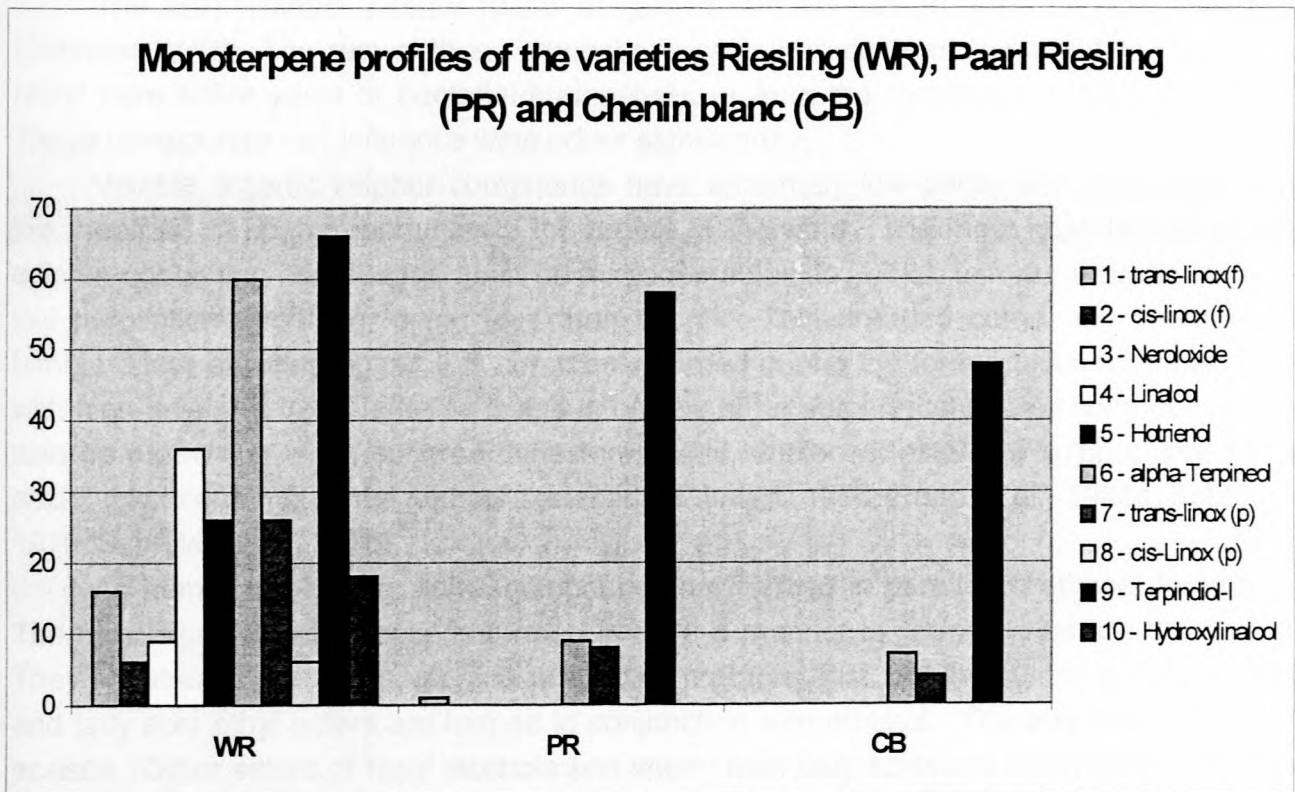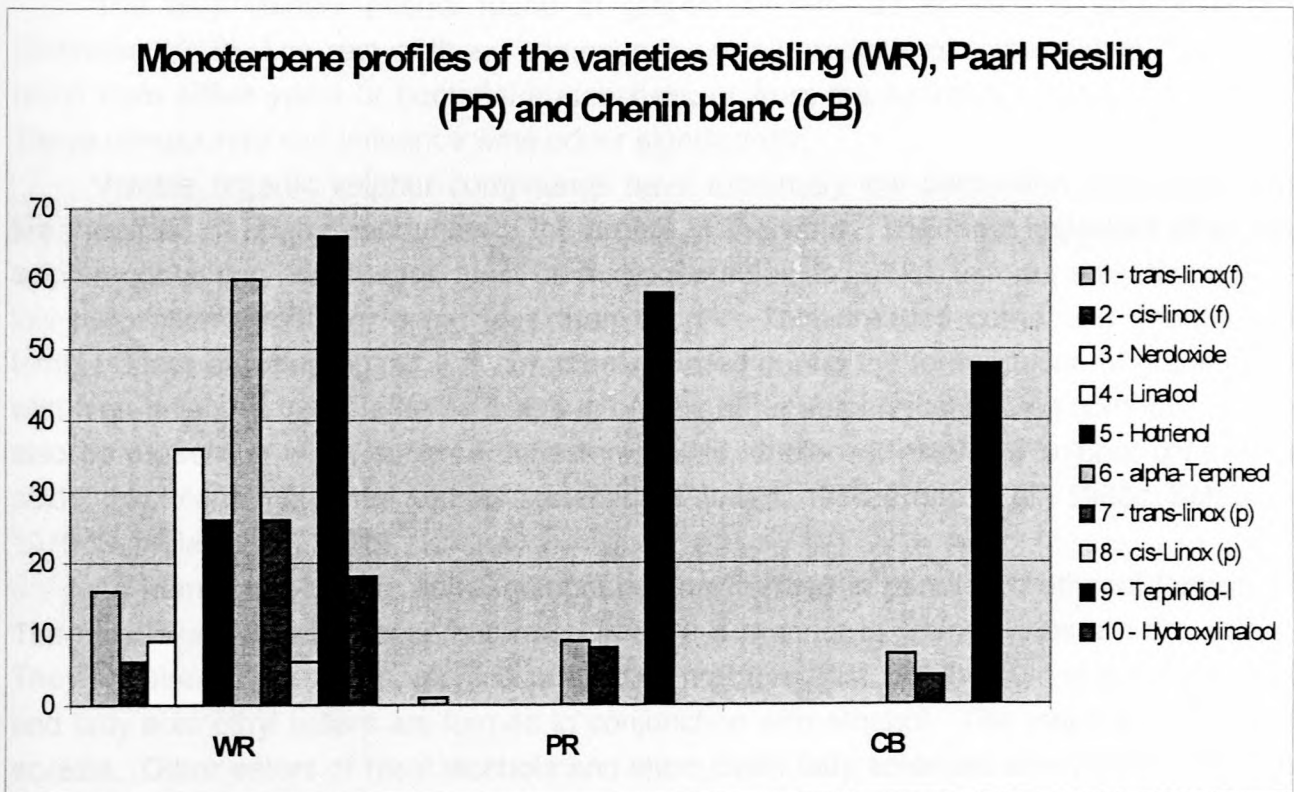

Figure 3. A "terpene profile" of the Riesling varieties: Weisser Riesling, Paarl Riesling and Chenin blanc cultivated in South Africa (adapted from Rapp, 1987).

As can be seen in Figure 3, it is possible to do an analytical characterization of different wines based on their terpene profile. Only a few compounds are necessary to perform this task. With this method it was possible for Rapp (1987) to distinguish between wines from different varieties that are named "Riesling" but which were not originally produced from the Riesling (Weisser Riesling) variety. The difference between Weisser Riesling, Paarl Riesling and Chenin blanc is clearly visible in Figure 3.

Most of the aldehydes present in the grape must are only detected in the initial phases of fermentation (Rapp et al., 1985c) and are most probably reduced to alcohols by the yeast. The aldehydes in wine, except for acetaldehyde, arise from carbohydrate degradation, originate from lignins, or are formed during the aging process. Ketones are found in small quantities and the sensory impact imparted by them seems to be very low., They usually possess negative flavour sensations when they do occur in higher concentrations, for instance diacetyl, which has a buttery odour.

Only a few organic acids contribute to the volatile odour of wine. The acids that possess odour include acetic acid (vinegary), propanoic acid (goaty), butanoic acid (spoiled butter) and lactic acid. These acids are usually present below the perception threshold, with the exception of acetic acid (Rapp and Mandery, 1986).

The only volatile phenol found in grapes as well as in wine is acetovanillone (Schreier, 1979). The rest of these compounds are all products of fermentation. They can result from either yeast or bacterial metabolism, or from the hydrolysis of higher phenols. These compounds can influence wine odour significantly.

Volatile organic sulphur compounds have extremely low perception thresholds and are therefore of huge importance to the aroma of the wine. The most important of all the sulphur-containing compounds must be hydrogen sulphide, which unfortunately has a very low perception threshold, being less than 1 µg/l. This dreaded compound imparts the terrible odour of rotten eggs. It is commonly formed during the fermentation of grape must with high levels of free elemental sulphur. A few other sulphur-containing compounds can also be present in wine, such as thioesters, thiols, thiolanes, esters of sulphur-containing acids, thiazols, a mercaptal and an acetamide (Güntert, 1984; Rapp et al., 1985c; Schreier, 1979; Schreier et al., 1976).

As mentioned before, some compounds are formed in parallel to ethanol formation. The most significant compounds formed in such a manner in young wines are the esters. They are already present in very low quantities in grape must, but the bulk of acetate esters and fatty acid ethyl esters are formed in conjunction with ethanol. The major ester is ethyl acetate. Other esters of fusel alcohols and short chain fatty acids are also present and are generally named "fruit esters" because of the pleasant aroma that they impart. They have low perception thresholds and are especially important in white wines. The esters that play a role here are ethyl butanoate, caproate, caprate and laurate, which are present at

concentrations below 10 mg/l, but this value is still approximately ten times that of their perception threshold. Howver, the odour of esters is not always pleasant. As the acid carbon number increases, the odour of the esters tends to become soft, then soapy, and finally stearic (Rapp and Mandery, 1986).

The fermentation conditions and the type of yeast used to perform the fermentation play a large role in the final constitution of the ester content of the wine, as well as. It is concluded that the basic odour of wines is caused by the four esters (ethyl acetate, isoamyl acetate, ethyl caproate and caprylate), two alcohols (isobutyl and isoamyl) and acetaldehyde. The other components are considered to only modify the basic odour (Avakyants et al., 1981).

1.2.1 Post-fermentation processes

It is known that the technological processes performed at the completion of fermentation can change the composition of some compounds in the wine. One of these groups of compounds is the monoterpenes (Rapp, 1987). They are not affected during bentonite treatment of juice and wine, but carbon fining has a major influence on their concentration. This is ironic, as carbon fining is most commonly used to eliminate undesirable aroma notes. Geraniol, nerol and citronellol are absorbed more strongly than linalool.

1.3   Wine bouquet

Several chemical reactions influence the constitution of the volatile compounds in wine, and this leads to the transformation of the aroma into the bouquet. These transformations depend largely on the storage conditions. There are two types of bouquet: the bouquet of oxidation, due to the presence of aldehydes and acetals, and the bouquet of reduction, which is formed after bottle aging (Ribéreau-Gayon, 1978).

Fine red wines in particular benefit from storage in wooden casks. During this aging process, a large number of aromatic elements are extracted from the wood, adding to the complexity of the wine without diminishment of the wine character afterwards. The main compounds that are derived from the wood are phenolic compounds and lactones (Masuda and Nishimura, 1971; Kepner et al., 1972).

Reazin et al. (1976) conducted experiments that showed that, in aging whiskey distillates, acetaldehyde, acetic acid and ethyl acetate are produced from ethanol by oxidation and esterification reactions. It is thought that similar reactions may occur in wine during aging in wooden barrels, and to this end Ribéreau-Gayon (1971) had shown that

there are variable increases in the levels of volatile acidity and ethyl acetate. The major acid contributing to the volatile acidity is acetic acid (Onishi et al., 1977). Some of this increased acetic acid comes from direct hydrolysis and extraction from the wood, but a much larger portion, up to 4% of the wood, can be released by alkaline hydrolysis and somewhat less by strong acid hydrolysis, evidently from the hemicelluloses (Nishimura et al., 1983).

Acetates are produced enzymatically in excess of their equilibrium concentrations and contribute to the pleasant, fruity aroma of young wines. This equilibrium is eventually restored during storage, when they gradually hydrolyze until their levels are equal to that of their corresponding acids and alcohols (Simpson, 1978a; Marais and Pool, 1980). In contrast to the decrease in acetate levels, the ethyl esters of diprotic acids show a constant increase, caused by the chemical esterification during the course of aging.

The amount of vitispiranes, a compound resulting from carotinoid degradation, also increases. These vitispiranes have a camphoraceous eucalyptus-like odour, which increases with storage (Simpson, 1978b; Simpson et al., 1977) and can result in a wine with an off-flavour (Simpson, 1978b).

According to Rapp et al. (1985a, 1985b), various additional chemical reactions occur during bottle maturation, and these play a large role in the change of the aroma of the wine. The reactions can typically be divided into four groups of reactions:
- changes in the concentration of esters (increase of ethyl esters, decrease of acetates);
- formation of compounds from carbohydrate degradation;
- formation of compounds from carotinoid degradation;
- changes in the terpenoid constitution.

As with aging in wooden barrels, the decrease in the levels of acetates during bottle aging severely depletes the wine fruitiness. However, the levels of some other compounds may increase, and not always with pleasant results. For instance, a hydrocarbon, 1,1,6-trimethyldihydronaphtalene (TDN) – a compound arising from carotinoid degradation – causes the well-known petrol-like character in older wines, especially Riesling. Damascenone is also a product of carotene degradation, but shows a decline in concentration during storage (Güntert, 1984). The furane derivatives are an example of carbohydrate degradation. Furfural and ethyl furoate are formed readily in young wines, but unlike other compounds, the amounts of furfural actually increase during wine storage Rapp et al., 1985c).

Some of the monoterpenes in wine undergo rather drastic changes. For example, linalool is transformed to other terpene compounds, as shown in Figure 4. The main reaction occurs via α-terpineol to 1,8-terpin, a compound only formed during the aging of wine. The reactions that take place can be summarized as follows:
- monoterpene alcohols, like linalool, geraniol and citronellol, decrease;

- the linalool oxides, as well as nerol oxide, hotrienol, hydroxylinalool and hydroxycitronellol, increase;
- 2,6,6-trimethyl-2-vinyl-tetrahydropyran, the anhydrolinalool oxides, 2,2-dimethyl-5-(1-methylpropyl)-tetrahydrofuran and cis-1,8-menthandiol are formed (Hennig and Villforth, 1942; Buttery et al., 1971).
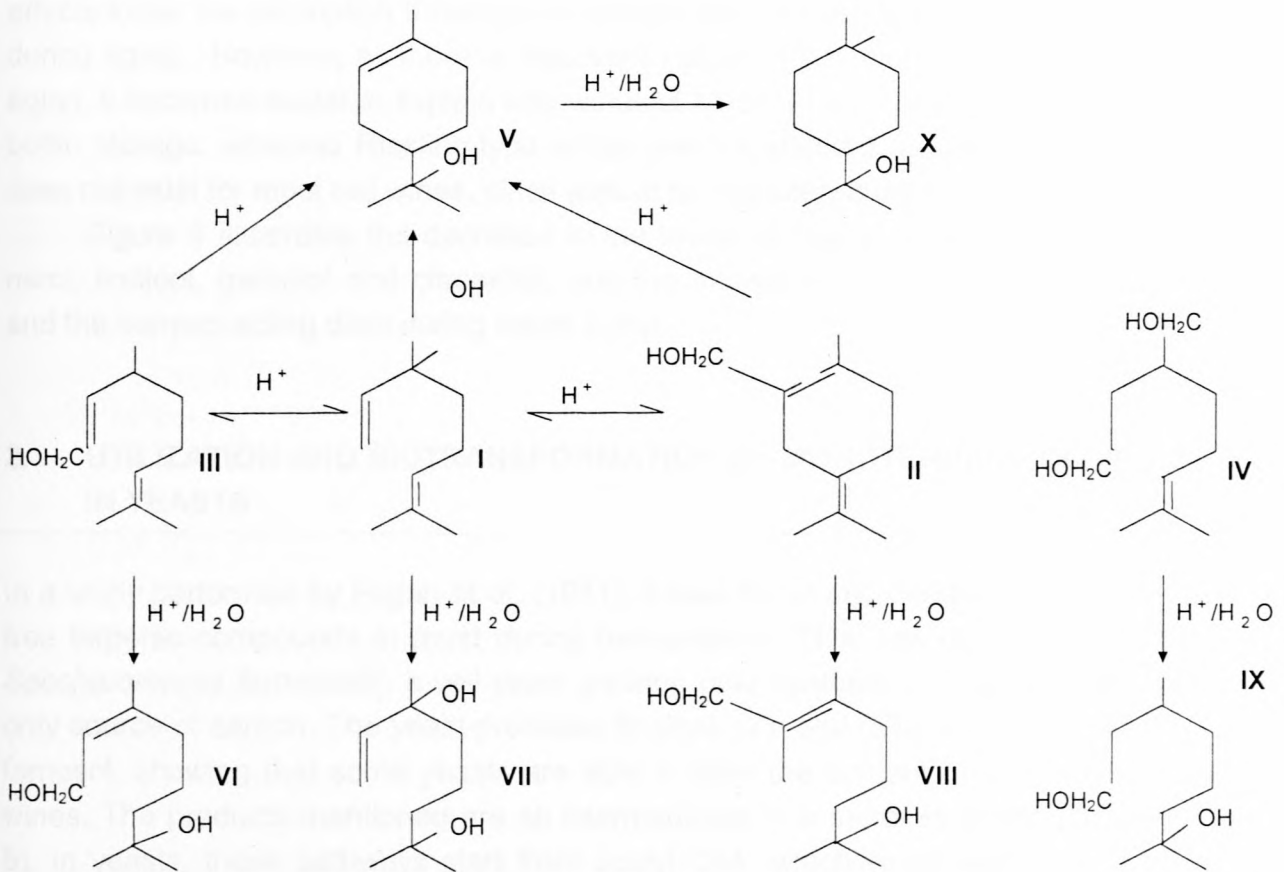
Figure 4. The proposed reaction scheme of monoterpene alcohols during bottle aging in wine. I – linalool, II – geraniol, III – nerol, IV – citronellol, V - α-terpineol, VI – hydroxynerol, VII – hydroxyinalool, VIII hydroxygeraniol, IX – hydroxycitronellol, X – cis-1,8-terpine (adapted from Güntert, 1984; Rapp et al., 1985c).

It can be expected that the changes in the terpene composition should have an enormous influence on the character of the wine. For instance, the linalool content decreases to about 10 % of its original amount after ten years of storage. If one can estimate the

average concentration of the linalool content to be about 400 µg/l in the unaged wine, then it is clear that the remaining linalool content would be below 100 µg/l after a few years. This value is below the perception threshold for this compound (Güntert, 1984; Rapp et al., 1985b). The increase in linalool oxide does not significantly affect the bouquet of the wine. An increase in α-terpineol, however, could probably affect the wine flavour in a negative sense. Since the olfactory perception threshold varies for the monoterpenes in wine, ranging from 100 to 700 µg/l, and it has been found by Ribéreau-Gayon (1978) that additive effects lower the perception threshold, it remains very difficult to predict the aroma changes during aging. However, as more is discovered about the monoterpene composition during aging, it becomes easier to explain why wines of Muscat-type cultivars seldom improve with bottle storage, whereas Riesling-type wines are not affected as negatively. This problem does not exist for most red wines, since almost no monoterpenes contribute to the flavour.

Figure 4 illustrates the decrease in the levels of four monoterpene alcohols, namely nerol, linalool, geraniol and citronellol, and the increase of α-terpineol, cis-1,8-mentandiol and the corresponding diols during bottle aging.

2. UTILIZATION AND BIOTRANSFORMATION OF MONOTERPENE COMPOUNDS IN YEASTS

In a study performed by Fagan et al. (1981), it was found that certain yeasts may increase free terpenic compounds in must during fermentation. This was observed with the yeast *Saccharomyces fermentatii*, a veil yeast growing over synthetic media, with ethanol as the only source of carbon. The yeast produced linalool, (Z)- and (E)-nerolidol, as well as (E,E)-farnesol, showing that some yeasts are able to alter the content of aroma compounds in wines. The products mentioned are all intermediates in a sterol synthetic pathway (Figure 5). In yeasts, these pathways start from acetyl CoA, which yields squalene, a triterpene, and continue via mevalonic acid and several terpenic compound intermediates, such as geranylpyrophosphate and farnesyl pyrophosphate (Parks, 1978). The pathway proceeds anaerobically up until the epoxidation of squalene and the demethylation and dehydrogenation of lanosterol – two essential steps in the formation of ergosterol. Both processes require the presence of molecular oxygen. Therefore it can be said that oxygen availability decisively influences the synthesis of sterols and other terpenic compounds, as well the formation of intermediary products in yeast (Ratledge and Evans, 1989).

Zea et al. (1995) studied the relationship between the contents of some free monoterpenes in yeast cells and musts subjected to vinification for a few days. Three *S. cerevisiae* strains were used, namely the *cerevisiae*, *bayanus* and *capensis* strains. α-Terpineol was found to occur at the highest concentrations of all the terpenic alcohols

studied in the three yeast strains, and was particularly high in the *cerevisiae* strain. Linalool was the major alcohol produced and detected in the wine by the *cerevisiae* strain during the first two days of fermentation, but the levels of this monoterpenol then dropped, possibly as a result of its cyclization to α-terpineol. Geraniol, on the other hand, occurred in undetectable quantities in the *cerevisiae* strain. However, the geraniol content in the *bayanus* strain increased during the first three days of fermentation and peaked after 134 days. Linalool found in the cell body of the *capensis* strain was detected only after alcoholic fermentation (10 days), and then remained constant. The geraniol content also stayed constant. During fermentation, (*E*)-nerolidol was found to be the terpene that accumulated to the highest concentration. Its *Z* isomer was found to accumulate only after fermentation was completed, and then only in small amounts. As a rule, linalool and α-terpineol accumulated throughout the period of the study (134 days). Linalool levels decreased during the first 24 hours, but this was found to be concomitant with an increase in α-terpineol content, except in the must fermented by the *capensis* strain. Geraniol accumulated in must during the fermentation by all three strains (Zea et al., 1995). The overall terpenes measured revealed that the highest increase in concentrations occurred during the third and tenth day of fermentation, when the fermentation virtually ceased. After this point, their concentrations in the wines containing dead cells of the *cerevisiae* strain fell, while those in the wines produced by the veil-forming strains increased significantly.

It is thought that terpenes undergo intracellular isomerizations, mutual enzymatic conversions and cyclizations. King and Dickinson (2000) confirmed this when they examined the biotransformations of monoterpene alcohols by *S. cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. *S. cerevisiae* and *T. delbrueckii* were chosen for this study, as they play a role in vinification, while *K. lactis* was chosen to determine whether similar reactions occurred in a yeast not associated with brewing or wine production. Previously it was found that *K. lactis* produced geraniol, linalool and citronellol at low concentrations (50 µg/l), with added geraniol being converted to citronellol (Drawert and Barton, 1978).

T. delbrueckii (formerly *S. fermentatii*) produced linalool, (*E*)-nerolidol, (*Z*)-nerolidol, and *trans, trans*-farnesol (Fagan et al., 1981). The studies showed that the terpenoids were accumulating intracellularly in the yeast cells, and there was speculation that this may be due to accumulation of sterol pathway metabolites as a result of anaerobiosis. Also, *S. cerevisiae* strains with an altered farnesyl phosphatase synthase (*FPPS*) gene have been shown to secrete the monoterpenoids geraniol, citronellol and linalool, due to the intracellular accumulation of GPP (Chambon et al., 1990; Javelot et al., 1990).

Isopentyl Pyrophosphate (IPP)

Nerol

Geranyl Pyrophosphate

Linalool

α-terpineol

-O-P-P'

OH

OH

IPP

Farnesyl Pyrophosphate

Nerolidol

OH

O-P-P

Pre-squalene

Squalene

O₂
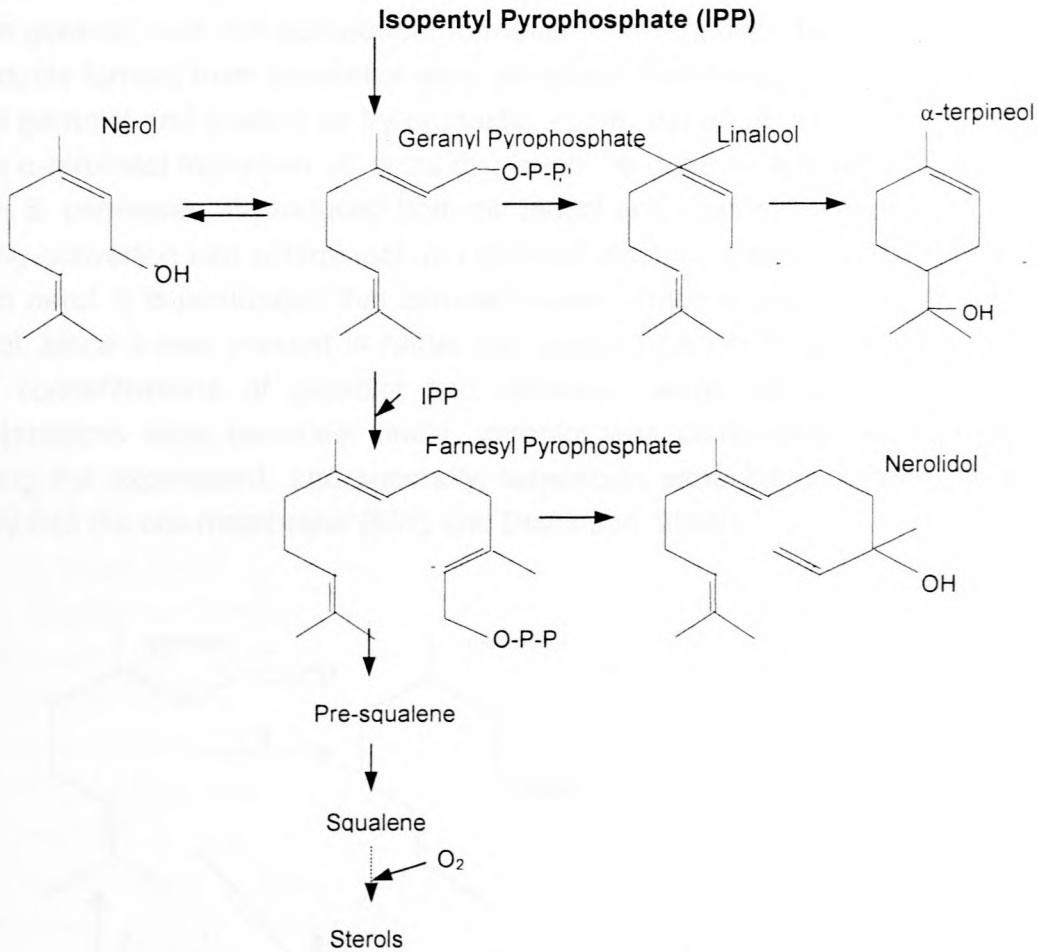
Sterols

Figure 5. Sterol biosynthesis and terpene formation in yeast (Zea et al., 1995).

Overall, however, these compounds consist only of alcohols in yeast strains that produce monoterpenoids (be it naturally or due to altered enzymes). As no terpenoid cyclase genes like those found in plants have yet been identified in yeast, it seems likely that they are capable of biotransformation reactions. To determine whether and how these reactions take place, King and Dickinson (2000) added monoterpenoid alcohols to base cultures of the three yeast strains mentioned earlier, namely *K. lactis*, *T. delbrueckii* and *S. cerevisiae*. The products were then detected. *S. cerevisiae* converted geraniol into citronellol and linalool, with α-terpineol also being detected. Supplemented linalool was converted into α-terpineol. Nerol also underwent cyclization to the form of α-terpineol. No products were detected with citronellol. Traces of *cis*-terpin hydrate were also found in all cases where α-terpineol was present.

In their study, King and Dickinson (2000) also found that *T. delbrueckii* produced linalool from geraniol, with the subsequent formation of α-terpineol. Neither citronellol formation nor products formed from citronellol were observed. Nerol was also converted into α-terpineol, with geraniol and linalool as by-products. Again, traces of *cis*-terpin hydrate were detected with α-terpineol formation. *K. lactis* displayed the greatest amount of reactions in this study. Like *S. cerevisiae*, it produced both citronellol and linalool from geraniol, with linalool then being converted into α-terpineol. α-Terpineol, linalool, geraniol and citronellol were formed from nerol. It is postulated that citronellol was formed via geraniol rather than directly from nerol, since it was present in rather low concentrations (King and Dickinson, 2000). Also, the concentrations of geraniol and citronellol were not mutually compensatory. Two explanations were provided: firstly, geraniol was continually being produced from nerol during the experiment, and secondly, terpenoids were being lost due to evaporation and entry into the cell membrane (King and Dickinson, 2000).
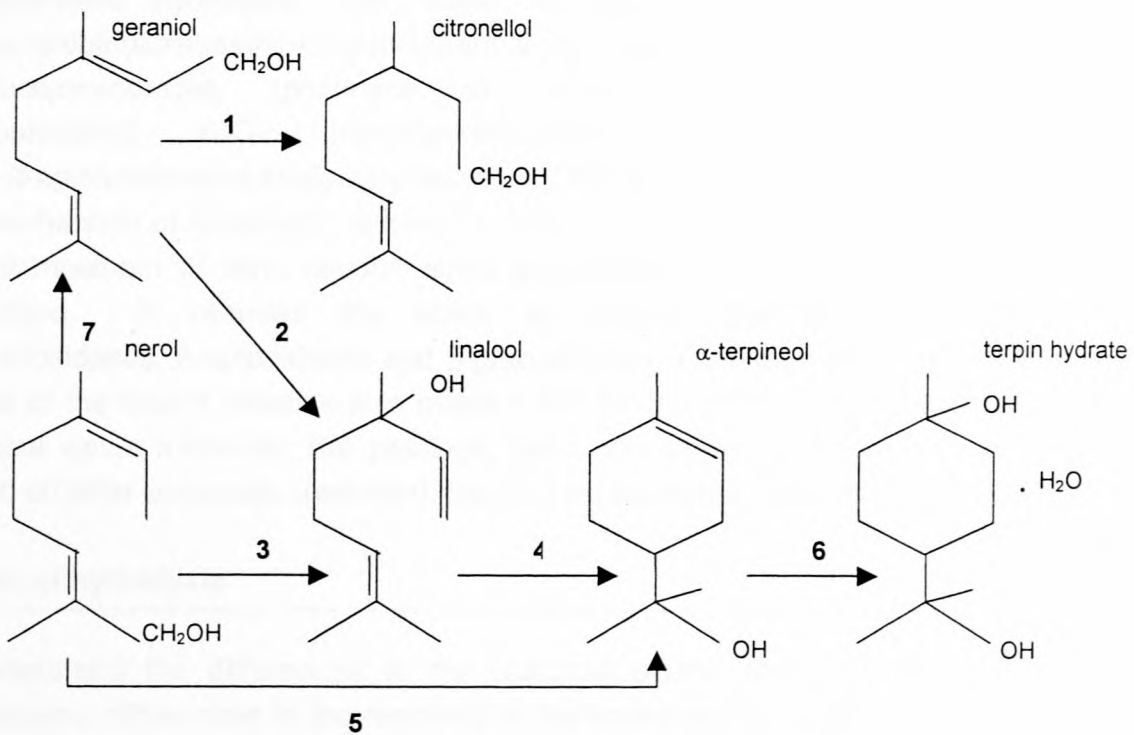
Figure 6. A scheme showing the monoterpenol biotransformation reactions catalyzed by *S. cerevisiae*, *T. delbrueckii* and *K. lactis*: **1** – reduction of geraniol to citronellol (except *T. delbrueckii*); **2** – isomerization of geraniol to nerol; **3** – isomerization of nerol to linalool; **4** – isomerization of linalool to α-terpineol; **5** – isomerization of nerol to α-terpineol; **6** – hydration of α-terpineol to terpin hydrate; **7** – isomerization of nerol to geraniol (except *S. cerevisiae*) (King and Dickinson, 2000).

These reactions catalyzed by the yeasts are summarized in Figure 6. They consist of reductions (geraniol to citronellol), translocations (geraniol and nerol to linalool), *cis* to *trans* isomerizations (nerol to geraniol), and cyclizations (nerol and linalool to α-terpineol). The formation of terpin hydrate probably involves the hydroxylation of α-terpineol to form terpin. This terpin may then be hydrated via an enzymatic reaction, although this reaction probably occurs spontaneously, as no terpin was detected (King and Dickinson, 2000).

3.   THE INCREASE OF VOLATILE FLAVOUR IN WINE

Monoterpenes play an important role in the aroma of some wine grape cultivars, as mentioned before. Major fractions of these terpenols exist as glycosidically bound forms and, their release from their glycones is subsequently of major interest. These terpenols can be liberated by one of two mechanisms: the first is an acid hydrolysis, and the second an enzymatic hydrolysis. The sugar moieties of these compounds are either 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides (vicianosides), 6-O-β-D-xylopyranosyl-β-D-glucopyranosides (primverosides), 6-O-β-D-glucopyranosyl-β-D-glucopyranosides (gentiobiosides), 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides), or 6-O-β-D-apiofuranosyl-β-D-glucopyranosides (Williams et al., 1982a; Günata et al., 1985). The mechanism of enzymatic release in particular attracts great interest when it comes to the enhancement of wine flavour, since this mode of liberation is well established and understood.   It requires the action of specific glycosidases: α-arabinosidases, α-rhamnosidases, β-apiosidases and β-glucosidases (Günata et al., 1988). The enzymatic release of the bound volatiles also offers a tool for the production of natural flavours from otherwise waste materials, like peelings, skins and stems. The liberated volatiles can be distilled off after enzymatic treatment, before the residues are discarded.

3.1   Acid hydrolysis

To understand the differences in the outcome of the acid and enzymatic hydrolysis mechanisms, differences in the reactivity of the bound aroma compounds have to be taken into account. Not considering the obvious conversion of flavourless polyol structures into odour-active forms, it has been confirmed that the glycosidic linkage has a significant influence on the chemical reactivity of the bound aglycone.

Acid hydrolysis is definitely promoted by heating (Williams et al., 1980). However, this does not provide the ideal scenario, since most volatile constituents would become so volatile as to escape the solution. It also has the disadvantage of artifact formation as a result of chemical rearrangement of the monoterpene aglycones (Williams et al., 1982b),

and it can thus be said that acidic hydrolysis changes the intrinsic varietal aroma of the wine.

3.2   The action of hydrolytic enzymes

The glycosidases are mostly exogenous enzymes. The liberation of the disaccharidic conjugates depends on the sequential action of these hydrolytic enzymes. The first step requires the action of an α-arabinofuranosidase, α-rhamnosidase, β-xylosidase, or β-apiosidase for the enzymatic cleavage of the intersugar-linkage, a (1→6)-link. This results in the production of monoterpenyl glucosides. The second hydrolytic step involves β-glucosidase activity to liberate the aglycone moiety (Günata et al., 1988). Only in the case of the gentiobiosides (β-D-glucopyranosyl-(1-6)-β-D-glucopyranosides), where the disaccharide moiety consists of two glucose units, the β-glucosidase activity is able to liberate the aglycone in a two-step mechanism (Haisman and Knight, 1967). Unlike acidic hydrolysis, the enzymatic mechanism allows only for minimal changes in the natural monoterpenol distribution (Günata et al., 1988).
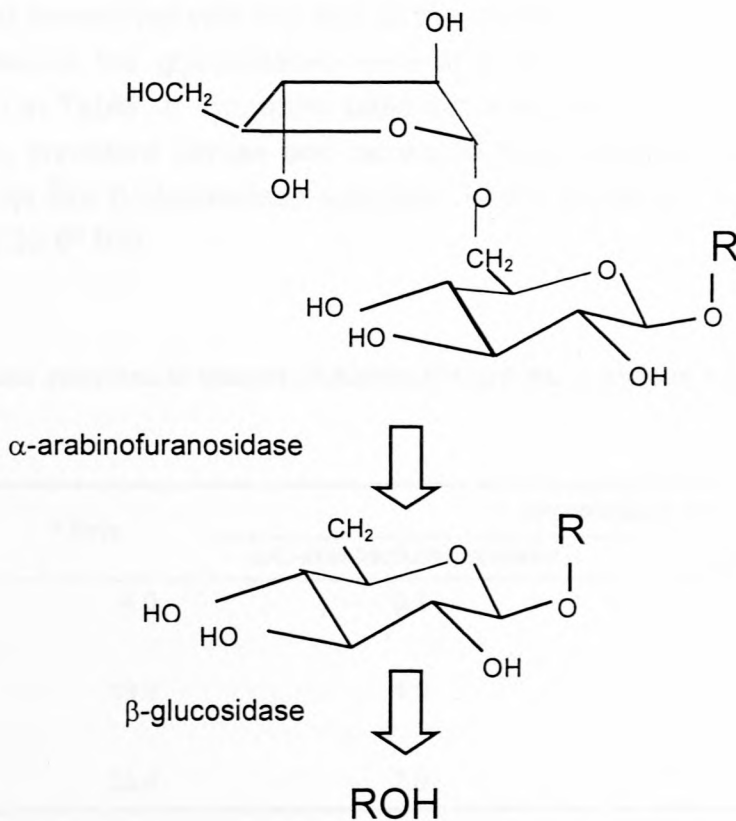
Figure 7.   A schematic representation of the enzymatic action of glycosidases, in this case α-L-arabinofuranosidase and β-glucosidase (Spagna et al., 1998).

3.3   Properties of endogenous glycosidases

A number of different glycosidases, from both endogenous and exogenous sources, have been tested with regard to their activities during the practical applications concerning flavour release. It became apparent very early on in these studies that there are a lot of problems relating to indigenous plant glycosidases.   The main limiting factors were the most important ones, namely the wine conditions. The enzymes originating from *V. vinifera* showed a pH optimum at 5.0, but importantly exhibited a strong inhibition in the presence of glucose and ethanol. These enzymes would therefore be virtually inactive during winemaking procedures.

The endogenous β-glucosidases of *V. vinifera* were examined intensively by Aryan et al. (1987).   In their study they searched for the localization of these enzymes, and the data obtained indicated that the enzymes were concentrated in the grape pulp and juice. Enzyme activity was measured by making crude extractions of these enzymes from the parts of the plants where they were localized. The enzymes were then partially purified and their activities were determined using the appropriate glycosides of ρ-nitrophenol as substrates. The effect of glucose inhibition could be curtailed in this manner. Only residual activity was found associated with the skin of the berries. The data collected also showed when the activities of the glycosidases were at a peak during the development of the berries, as shown in Table 2. From this table it can be seen that the glycosidase activities were weak in the immature berries and increased with fruit ripening. This increase was most significant for the β-glucosidase activities, which increased almost eight-fold in the fruit, from 13.8 to 23.6° Brix.

Table 2.   Glycosidase activities in Muscat of Alexandria grapes at various stages of maturity (Aryan et al., 1987).

Harvest date	° Brix	Glycosidase activity	
		α-L-arabinofuranosidase	β-glucosidase
7 January	4.0	0.1	12.0
5 February	13.8	1.0	32.6
10 April	23.6	7.0	252.7

It is clear from the study of Aryan et al. (1987) that β-glucosidase activities are widely distributed in *V.vinifera* grapes, and in some cultivars the enzymes obviously function for purposes unrelated to terpene glycoside hydrolysis, as will be explained later. For example, in Chardonnay and Thompson Seedless, two varieties known for their low monoterpene concentration (Dimitriadis and Williams; 1984), the β-glucosidase activity was higher than that found in juices of cultivars which are monoterpene-dependant for varietal character, i.e. Muscat Hamburg and Gewürztraminer.

As mentioned before, plant glycosidases are inhibited by low pH levels, as well as by high levels of glucose and ethanol in the wine. A further constraint to the application of these enzymes stems from their aglycone specificities. These enzymes were all found to be incapable of hydrolyzing the sugar conjugates of tertiary alcohols. Thus, glycosides of some of the most flavour-important monoterpenes, for example linalool, are unaffected by the enzymes even under ideal conditions. The authors of this study conclude that there is no practical advantage in seeking to exploit the grape's native β-glucosidase activity for the purpose of releasing glycosidically bound flavourants during winemaking (Aryan et al., 1987).

Similar profiles of these enzymes were exhibited by other endogenous plant and yeast glycosidases, showing strong inactivity at low pH levels and grape juice sugar levels. The only exception to this rule, however, was observed for the wine yeast, *S. cerevisiae* itself, of which the periplasmic glucosidase showed strong activity even at a glucose concentration of 5% (280 mmol/l). Further limiting conditions in the use of endogenous glycosidases in winemaking are due to their known aglycone specificity and low ethanol tolerance levels (Günata et al., 1993; Williams, 1993).

Over the past few years, data on β-glucosidase in *Saccharomyces* have been contradictory. It has been reported that the structural gene for β-glucosidase is present in *S. cerevisiae*, but it is very poorly expressed (Duerksen and Halvorson, 1958; 1959). This could explain why *S. cerevisiae* is unable to grow on a medium containing cellobiose as sole carbon source. The function of this β-glucosidase would probably be to enable the yeast to utilize β-D-glycosides as a source of carbon and energy, specifically if other sources of carbon and energy were to be depleted. 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 is glucose independent. On the other hand, results obtained by Günata et al. (1990a) concluded that these yeasts do have very low β-glucosidase activity, but Delcroix et al. (1994) found three oenological strains showing high β-glucosidase activity. Later work done by Mateo and Stefano (1997) revealed that wine yeasts have some constitutive β-glucosidase activity, but this enzyme activity is still inhibited under winemaking conditions. The pH optimum of these enzymes originating from

S. cerevisiae ranges between pH 5.7 and 6.8, and they are unstable at temperatures exceeding 50°C (Woodward and Wiseman, 1982).

This endogenous yeast β-glucosidase was tested under wine conditions by various authors and the results were not very promising. Delcroix et al., (1994) showed that the pH level for maximal activity was at 5.0, which is similar to that of the β-glucosidases originating from grapes and fungi (Aryan et al., 1987). Stability was found to be maximal at pH 6 and fell rapidly when the pH decreased. Under the authors' test conditions (20°C, 90 min), the loss of activity was 95 % at pH 2.8. They postulated that the low β-glucosidase activity in fermenting juice is probably due to the instability of the enzyme at low pH levels.

S. cerevisiae β-glucosidase activity was the highest at 50°C, again similar to that of grape and fungal orientated enzymes (Aryan et al., 1987). At the usual temperatures at which fermentation takes place (15 to 20°C), β-glucosidase activity from yeasts was only 10 to 15% of the maximum.

Aryan et al. (1987) found that, in contrast to grape and fungal β-glucosidases, yeast β-glucosidase was only weakly sensitive to the presence of glucose. At 100 g/l of glucose, the activity was reduced by only about 20%. The enzyme is not affected greatly by ethanol either, as the activity decreased by only 15%. SO_2 had no influence whatsoever at a concentration of 50 mg/l, the average concentration used in winemaking (Aryan et al., 1987).

These results that were found by Aryan et al. (1987) have important consequences. They indicate that the glycosidases present in the fruit or grape juice do not hydrolyze the abundance of glycosides found in them. During the fermentation process the activity of the yeast glycosidases also will just barely affect the glycoside content of the must, before being inhibited by the ethanol concentration, or any of the other limiting factors, such as the pH. Thus a huge amount of glycoconjugated flavour compounds, a vast source of potential aroma remains in the wine. Consequently, numerous authors have proposed that the use of exogenous glycosidases should be considered for the aroma enrichment of wine and fruit juices (Aryan et al., 1987).

3.4 Properties of exogenous glycosidases

Glycosidases are a group of enzymes that are exceptionally widespread in occurrence. For example, the sources of β-glucosidases are shown in Table 3. β-Glucosidases have been detected in a large number of organisms, ranging from prokaryotic bacteria to eukaryotic fungi, yeasts, plants, insects and even humans. A number of these sources have been

tested for glycosidic activity that may be of use under the limiting conditions offered by wine. These sources include mainly plants, fungi, bacteria and yeasts.

Table 3. Sources of β-glucosidases.

Source of β-glucosidase	Gene name	Reference	Cloned
Acetovibrio cellulolyticus		Saddler and Khan (1980)	
Aspergillus kawachii		Iwashita et al. (1998, 1999)	Yes
Aspergillus niger CCRC 31494		Yan and Lin (1997)	Yes
Aspergillus oryzae		Mega and Matsushima (1979), Riou et al. (1998)	
Aspergillus phoenicis		Sternberg et al. (1977)	
Aspergillus wentii		Woodward and Wiseman (1982)	
Bacteroides succinogenes		Groleau and Forsberg (1981)	
Bifidobacterium breve clb		Nunoura et al. (1996)	
Botryodiplodia theobromae		Woodward and Wiseman (1982)	
Candida entomophila		Gueguen et al. (1994)	
Candida guilliermondii		Woodward and Wiseman (1982)	
Candida molischiana		Günata et al. (1990c); Gueguen et al. (1996)	
Candida peltata		Saha and Bothast (1996)	
Candida wickerhamii		Günata et al. (1990c), Skory et al. (1996)	
Clostridium thermocellum		Ait et al. (1979)	
Debaryomyces castellii		Rosi et al. (1994)	
Debaryomyces hansenii		Rosi et al. (1994); Yanai and Sato (1999)	
Debaryomyces polymorphus		Rosi et al. (1994)	
Dekkera intermedia		Blondin et al. (1983)	
Erwinia herbicola		Garibaldi and Gibbins (1975)	
Hansenula anomala		Rosi et al. (1994)	
Humicola grisea var. thermoidea		Peralta et al. (1997)	
Kloeckera apiculata		Rosi et al. (1994)	
Klueveromyces fragilis		Raynal and Guerineau (1984)	Yes
Mucor miehei 7H-10		Woodward and Wiseman (1982)	
Mucor racemosus		Woodward and Wiseman (1982)	
Neurospora crassa		Eberhart and Beck (1973)	
Saccharomycopsis fibuligera	BGL1 & BGL2	Machida et al. (1988); Van Rensburg et al. (1998)	Yes
Sporotrichum (Chrysosporium) thermophile		Woodward and Wiseman (1982)	
Thermoascus aurantiacus		Woodward and Wiseman (1982)	
Trichoderma reesei		Woodward and Wiseman (1982)	
Trichoderma viride		Herr (1979)	
Zygosaccharomyces bailii		Gueguen et al. (1995)	
Bacillus polymyxa	bglA & bglB	Adam et al. (1995) ; Günata et al. (1996)	Yes
Saccharomyces cerevisiae		Mateo and Di Stefano (1997) ; Iranzo et al. (1998)	
Vitis vinifera		Aryan et al. (1987)	

As these glycosidases are to be exploited to release potential aroma, they must satisfy a few prerequisites, including specificity, pH optimum and tolerance to glucose and ethanol.

Another property exhibited by these enzymes should be a temperature optimum that corresponds with the temperatures at which wine fermentation takes place. The general activity displayed by these enzymes can also be taken into consideration. Two of these glycosidases, β-glucosidase and α-arabinofuranosidase, will be discussed in greater detail in the following sections.

3.4.1 β-Glucosidases

Almost all β-glucosidases have subunit molecular weights of 55-65 kDa, acid pH optima of pH 4-6, and an absolute requirement for a β-glycoside (i.e. glucoside, and to a much lesser extent fucoside and galactoside) as substrate. Even though they differ in source they show remarkable similarity in substrate specificity for glycone (glucose), as well as for some non-physiological aglycones, for example nitrophenols. However, they may have widely different physiological glucosidic substrates with different aglycone moieties (Esen, 1993).

β-Glucosidases from different orders and kingdoms seem to differ in their specificities for the aglycone (an aryl or alkyl group) linked to the glucosyl group by a β-glucosidic bond. β-Glucosidases of fungi, bacteria, humans and dicotylodonous plants (for example *V. vinifera*) have been shown to be glycosilated, while those of monocotylodonous plants, such as maize and sorghum, are not. In dicots, β-glucosidases are localized in the cell wall (Kakes, 1985; Frehner and Conn, 1987) or protein bodies (Swain et al., 1992) whereas in monocots, the enzymes are found mainly in the plastids (Thayer and Conn, 1981; Nisius and Ruppel, 1987; Esen, 1992).

In plants, β-glucosidases are thought to perform a wide variety of metabolic roles, for example in relation to productivity, as well as food and feed toxicity-related reactions (Jones 1988, Poulton, 1990). They have also been implicated in growth-related responses. However, most of the functions have not been fully documented and elucidated.

One of the functions of plant β-glucosidases is thought to be cyanogenesis (Jones, 1988; Poulton, 1990). This is the process in which the release of hydrogencyanide, or HCN, takes place as the result of the hydrolysis of cyanogenic glucosides. It is a very common process in plants, as it has been shown to occur in over 3000 plant species belonging to 110 different families. As the enzyme is present in cellular compartments, the enclosures have to be disrupted, as in fact happens when herbivores and pathogens cause injury to cells and tissue. This would imply that cyanogenesis is a chemical defence response to organisms feeding on intact parts of the plant, or attacking the plant through a site of previous injury.

Apart from cyanogenesis, plant β-glucosidases have also been associated with the hydrolysis of glucosides of phytohormones, and thus play an important role in activating these hormones. This has been shown in maize β-glucosidase (Campos et al., 1993).

Another physiological function found in maize β-glucosidase is the hydrolysis of the hydroxamic acid glucoside (DIMBOA-glc), as suggested by Cuevas et al. (1992). Hydroxamic acids play a major role in the defence of the plant against insects. They are present in the plant body as 2-O-β-D-glucopyranosides and, upon disruption of plant tissue, aglycones are liberated that are chemically labile and of higher toxicity than the parent glucosides. Studies by Babcock and Esen (as quoted by Esen, 1993) demonstrated this, and furthermore determined that there was a direct correlation between the hydroxamic acid glucoside content of maize organs and parts of the plant and their β-glucosidase activity.

Little is known about insect β-glucosidases and their substrates, but a β-glucosidase from the larvae of the moth *Zyaena trifolii* has been described by Nahrstedt and Mueller (1993). The system is very similar to that of cyanogenic plants belonging to the family Fabaceae, which may imply that the same function of protection against predators is performed.

3.4.1.1 Specificity of β-glucosidases

It has been observed that, when it comes to substrate specificity, β-glucosidases can be divided into three subgroups (Rojas et al., 1995). This group of enzymes can catalyze the hydrolysis of i) alkyl- and aryl- β-D-glucosides (e.g. methyl-β-D-glucoside and ρ-nitrophenyl-β-D-glucoside), ii) glycosides containing only carbohydrate residues (e.g. cellobiose), and iii) a broad spectrum of substrate types. The last group represents most of the β-glucosidases that have been cloned.

The third group of β-glucosidases is the one that facilitates the liberation of most of the glycosidic precursors from glycosidically bound structures in wine, such as the monoterpene alcohols, resveratrol and colour components. It has been found that some of these β-glucosidases have wide specificity ranges, for instance the β-glucosidase isolated from the yeast *Candida molischiana* strain 35M5N. This enzyme possesses a wide spectrum of activity, including α-L-arabinfuranosidase and α-L-rhamnosidase activities (Guegen et al., 1996). Another example is the β-glucosidase isolated from *Bifidobacterium breve* clb., which has β-D-fucosidase and β-D-galactosidase activity besides the expected β-glucosidase activity (Nunoura et al., 1996). Some of the β-glucosidases show preference towards certain monoterpene glucosides. Geranyl-β-D-glucoside seems to be a preferred substrate of the β-glucosidase encoded by the *bglA* gene cloned from *Bacillus polymyxa*

(Günata et al., 1996). This is representative of the tendency of the β-D-glucosides of primary alcohols, such as geraniol, nerol and citronellol, to be hydrolyzed more easily by most β-glucosidases than that of tertiary alcohols (linalool and α-terpineol). This β-glucosidase encoded by the *bglA* gene is a well-characterized, multimeric enzyme, able to hydrolyze not only β-glucosides as already mentioned, but also β-galactosides (Gonzáles-Candelas et al., 1989; Painbeni et al., 1992; López-Camacho and Polaina, 1993; Sanz-Aparicio et al., 1994). More examples of a broad-specificity type β-glucosidase derive from *Aspergillus oryzae* (Riou et al., 1998) and *Zygosaccharomyces bailii* (Gueguen et al., 1995). The enzyme from *A. oryzae* can hydrolyze a range of (1→3)-, (1→4)-, and (1→6)- β-digly-cosides, as well as aryl-and alkyl-β-glycosides. The β-glucosidase from *Z. bailii* was active against (1→4)- β (cellobiose, aryl-β-glucosides), (1→6)- β (gentiobiose), (1→4)- α-(α-ρNPG) and (1→6)- α (ρ-nitrophenyl-α-L-arabinofurano-side for intracellular β-glucosidase and ρ-nitrophenyl-α-rhamnopyranoside for extracellular β-glucosidase) configurations.

The enzymes usually are defined clearly in the aforementioned groups, although some activity regarding substrates in the other group may be observed. An example of this can be seen in a study done by Peralta et al. (1997), in which a highly thermostable β-glucosidase was isolated from the thermophilic fungi, *Humicola grisea*, var. *thermoidea*. This enzyme hydrolyzes mainly aryl β-D-glucosidases (100%), but cellobiose was hydrolyzed as well, at a percentage of 20%.

Cellobiose is a byproduct of cellulose degradation performed by some microorganisms. This product usually accumulates because of the weak β-glucosidase, or cellobiase, activity of most cellulolytic microorganisms (Woodward and Wiseman, 1982). Two β-glucosidase genes were cloned from the cellobiose-assimilating yeast *S. fibuligera*, named *BGL1* and *BGL2* (Machida et al., 1988; Van Rensburg et al., 1998). It was discovered that these two genes are structural genes for two β-glucosidases of different substrate specificities. They were called *BGL1*, encoding for an enzyme hydrolyzing cellobiose and cellooligosaccharides efficiently, and *BGL2*, encoding an enzyme whose substrate of preference is alkyl- or aryl-β-D-glucosides. The same is true of the *bglB* gene, an anamorph of the *bglA* gene, both of which were isolated from *B. polymyxa*. The *bglB* gene shows sequence homology to the *bglA* gene, but the encoded enzymes have different substrate specificities. Comparative studies reveal that amino acid sequences in yeast β-glucosidases are conserved relatively well, which may be essential to enzyme function (Machida et al., 1988).

3.4.1.2   pH optima of β-glucosidases

Rosi et al. (1994) did a study on the activity of β-glucosidase activity in yeasts of oenological origin. This study focused mainly on wild yeasts found on the grapes, and various properties of the β-glucosidases produced by them were examined. The wild yeast *Debaryomyces hansenii* was grown over a pH range of 3 to 7 and it was concluded that the different localizations of the β-glucosidase favoured different pH optima. The extracellular and parietal enzymatic activity had an optimum pH of 4 to 5, while the intracellular enzyme pH optimum was close to that of the endocellular environment (pH 6). This is the trend followed by most β-glucosidases, with the pH optimum for the extracellular enzyme being slightly lower than that of the intracellular enzyme. For instance, this is also true for the β-glucosidase cloned from *Z. bailli* (Gueguen et al., 1995), as well as for the *Candida wickerhamii* β-glucosidase, which has a pH optimum of 6.0 to 6.5 (Skory et al., 1996). The enzyme from *A. oryzae* has a pH optimum of 5, which was measured under optimal temperature conditions (Riou et al., 1998). At pH 3.5, the activity is 50% of the maximal activity, and only 25% of the maximal activity remains at pH 3.0. The activity of the β-glucosidase isolated from *Aspergillus niger* is also at a maximum at pH 6, but there is still an activity level of about 40% at pH 3.3 (Spagna et al., 1998).

One exception with regard to these high optimum pH levels is displayed by the cloned β-glucosidase of *C. molischiana.* In a study done by Gueguen et al. (1996), free and immobilized cells were immersed in wine preparations, after which the activity of the enzymes were evaluated. It was found that the pH optimum of the β-glucosidase of both the free and immobilized cells was 3.5. Another source of an enzyme with a lower pH optimum is *S. fibuligera.* Both β-glucosidases isolated from this organism has a pH optimum of 4.5 (Machida et al., 1988). The desired pH levels where there should be maximal activity of the exogenous β-glucosidases are in the range of 3.0 to 3.6. However, any enzyme that shows a good amount of activity, even if not the absolute maximum, would be suitable. Therefore it is a worthwhile exercise to continue the search for β-glucosidases for the enhancement of wine flavour and aroma.

3.4.1.3   Glucose and ethanol tolerance of β-glucosidases

It has been found that there is a certain amount of glucose inhibition in wild yeast strains (Rosi et al., 1994). This was shown, for example, with the yeast *D. hansenii*. With increasing levels of glucose present in the medium, the activity of the exocellular β-glucosidase decreased. Thus, at a level of 9% glucose in the growth medium, the enzymatic activity was only at about 30% of the maximum value. Ethanol had a limited

inhibitory effect on the enzyme, and even showed a stimulatory effect on the enzymatic activity of whole yeast cells.

The same type of glucose inhibition was observed for both the β-glucosidases (intracellular and extracellular) isolated from *Z. bailii*. Moreover, the enzymes were completely inhibited by D-gluconic-acid-lactone. As with the yeast *Debaryomyces*, ethanol had a stimulatory effect with concentrations up to 15% v/v (Rosi et al., 1994). This phenomenon has been described before by Pemberton et al. (1980) as the probable result of glycosyl transferase activity. However, at very high concentrations of alcohol, e.g. those higher than found in wine (above 15% v/v) these enzymes are inhibited strongly by ethanol, probably because of protein denaturation. The same trends were observed for the extracellular and intracellular β-glucosidases of *C. molischiana* 35M5N. Ethanol acted as an activator up to 1 M concentration and no inhibition was observed at a concentration of 2 M ethanol, the average value found in wine. Glucose was again shown to be a competitive inhibitor. Another species of this genus, *C. wickerhamii*, however, showed different kinetic properties for its β-glucosidase (Skory et al., 1996). This enzyme is fairly resistant to end-product inhibition by glucose, retaining 58% of its activity at 100 mM glucose. In this case alcohol has an adverse effect on the activity of the β-glucosidase. As the concentration of the ethanol increases to levels as low as 0.5 M, the activity decreases rapidly. It seems that this effect is more pronounced as the length of the alcohol chain increases. The *n*-alcohols have just the opposite effect and result in a greater than 1.6-fold increase in β-glucosidase activity for 1-butanol and 1-pentanol. The increase in the observed activity is thought to occur because alcohols are generally stronger nucleophiles than water in reactions involving nucleophilic substitutions (Sinnot, 1990; Withers and Street, 1989).

This same phenomenon is observed with some fungal β-glucosidases, for instance the enzyme isolated from *A. niger*, and used in a commercial enzyme preparation (Caldini et al., 1993). Ethanol once again was a strong activator of the β-glucosidase activity, with an increase in ethanol concentration corresponding to a linear increase in activity of the enzyme, even up to a concentration of 16% (v/v). However, as with β-glucosidases isolated from other sources, glucose was a competitive inhibitor of the enzyme, with an inhibition constant (K_i) of 1.4 mM. Most microbial enzymes show inhibition constants ranging from as low as 0.5 mM to no more than 100 mM for glucose (Saha et al., 1996; Woodward and Wiseman, 1982). K_i values for enzymes from *Aspergillus* species have been reported to range from 3 to 14 mM (Kwon et al., 1992; Yan and Lin, 1997). A β-glucosidase from another strain of this genus, *A. niger* CCRC 31494, has a K_i of 543 mM, which, according to the authors of the study performed, indicates that the enzyme is highly glucose tolerant (Yan and Lin, 1997). An even higher tolerance for glucose was seen to be exhibited by another species in this genus, *A. oryzae*, with a K_i value of 1.36 M. The high level of glucose tolerance also implies that these enzymes are relatively insensitive to end-

product inhibition. These β-glucosidases also exhibit a heightened activity as the ethanol concentration increases.

3.4.1.4   Temperature optima of β-glucosidase

Most β-glucosidases tend to have high temperature optima. The basic trend that is observed most commonly is the linear increase in activity with an increase in temperature, and then a sharp decrease in activity as soon as that temperature is exceeded.

An example of a wild yeast in which this trend is followed is *D. hansenii* (Yanai and Sato, 1999). The enzymatic activity of the β-glucosidase increased when the temperature was increased up to 40°C, and then decreased. At 60°C, the activity was only between 15 and 20% of the maximum activity. In the temperature interval between 20°C and 30°C, the activity was between 40 and 70% of the maximum reached (Rosi et al., 1994). In *S. fibuligera* the maximum activity was displayed at 50°C. The activity then decreases sharply so that it is less than 15% at 60°C. At lower temperatures (20 - 30°C) the activity was about 30 to 60% of the maximum (Machida et al., 1988). Another yeast, *Z. bailii*, shows low activity at low temperatures, then a gradual increase as the temperature rises, and then, after a maximal activity peak is reached between 55 and 70°C, the activity rapidly falls to 0% at 80°C (Gueguen et al., 1995). However, all yeasts do not display this high optimum temperature. The β-glucosidase isolated from *C. wickerhamii* had a comparatively low optimum temperature of "only" 35°C, but thermal-stability analysis of the β-glucosidase revealed that it is quite labile at this temperature. The temperature where this enzyme is stable is at 24°C, but the enzyme is rapidly inactivated at temperatures of 28°C and above (Skory et al., 1996).

Fungal β-glucosidases also have very high optimum temperatures. The enzyme isolated from *A. niger* CCRC 31494 had optimum activity at 55°C, but was stable below that temperature (Yan and Lin, 1997). The enzyme originating from *A. oryzae* showed a slightly lower optimum temperature at 50°C.

3.4.1.5.1.1   Stability of β-glucosidases

When enzymes are studied under conditions that denature and inactivate typical proteins, it can provide considerable information about their structures. For example, when maize β-glucosidase was observed in the presence of anionic detergents such as SDS and

deoxycholate, it was shown that the enzyme was very stable and active. Two fungal β-glucosidases derived from *Trichoderma* and *Penicillium* were also found to be active in the presence of denaturants, with the *Trichoderma* enzyme even more active than other β-glucosidases. The stability and activity data obtained suggest that β-glucosidases have a compact and rigid structure similar to that of thermophilic enzymes. In fact, when tested, maize β-glucosidase retained its structure and functional integrity after exposure to trypsin, chemotrypsin and proteinase K under both denaturing and non-denaturing conditions (Esen, 1993).

3.4.2 α-L-Arabinofuranosidases

3.4.2.1 Specificity of α-L-arabinofuranosidases

α-Arabinofuranosidase was purified for the first time by Kaji et al. (1967). Since then, numerous studies on this enzyme have been performed by various investigators to further determine its properties and applications. A study by Kaneko et al. (1998) shifted the attention more towards the specific substrates preferred by this enzyme.

In their study, Kaneko et al. (1998) investigated the substrate specifities of two α-L-arabinofuranosidases. One was isolated from *A. niger* 5-16. The other enzyme also originated from *A. niger*, but was part of a commercial enzyme preparation produced by Megazyme. Both these enzymes hydrolyzed arabinan and debranched-arabinan at almost the same rate, but the enzyme from *A. niger* (Megazyme) preferentially released arabinosyl side-chains of arabinan. The latter enzyme also hydrolyzes methyl 2-*O*-, methyl 3-*O*- and methyl 5-*O*-α-L-arabinofuranosyl-α-L-arabinofuranosides to arabinose and methyl α-L-arabinofuranoside in the order of (1→5)- >(1→2)- >(1→3)-linkages. On the other hand, the α-L-arabino-furanosidase from *A niger* 5-16 successively releases the arabinose of arabinan from non-reducing terminals. This enzyme performs the action of hydrolysis in the order of (1→2)- >(1→3)- >(1→5)-linkages. Both of the enzymes hydrolyze the (1→3)-linkage more efficiently than the (1→5)-linkage of methyl 3,5-di-*O*-α-L-arabinofuranosyl-α-L-arabinofuranoside.

Günata et al. (1990b) performed a study on an α-L-arabinofuranosidase from *A. niger*, probably the most common source of this enzyme. This fungus is known to produce three different types of L-araban-degrading activities: an endo-1,5-α-L-arabinase (EC 3.2.1.99) and two distinct α-L-arabinofuranosidases, ABFA and ABFB (EC 3.2.1.55) Kaji (1984). It was found that these α-L-arabinofuranosidases liberated arabinose and the corresponding monoterpenyl β-D-glucosides. These arabinosidases were able to cleave quantitatively the (1→6)-linkage between the terminal arabinofuranosyl unit and the intermediate glucose unit attached to the monoterpenyl, regardless of the structure of the

liberated monoterpenyl β-D-glucosides. These results were confirmed in a study done by Crous et al. (1996). It should be noted that some plant and fungal β-D-glucosidases are unable to hydrolyze some of the monoterpene glycosides (Günata et al., 1986) to yield the aromatic volatile terpenols. This apparent difference in specificity could be related to the structure of substrates, the α-L-arabinofuranosidic linkage being farther from the aglycon moiety than the β-D-glycopyranosidic linkage. The enzyme is devoid of β-D-glucosidase activity since glucose is not released at all. Moreover, the α-L-arabinofuranosidase is inactive toward monoterpenyl rhamnosylglucosides.

A. niger α-L-arabinofuranosidase A (ABFA) is active on small 1,5-linked α-L-arabinofuranosides, such as ρ-nitrophenyl-α-L-arabinofuranoside and unbranched 1,5-α-L-arabinofuranose oligosaccharides (Flipphi et al., 1993a). According to Komae et al. (1982), an α-L-arabinofuranosidase produced by Streptomyces purperascens IF 03389 shows a similar substrate specificity. There are also indications that such an enzyme could be produced by Bacillus subtilis F-11 (Weinstein and Albersheim, 1979). A gene encoding another A. niger α-arabinofuranosidase, ABFB, was cloned and characterized by Flipphi et al. (1993b). It was determined that this enzyme differed from the one encoded by the abfA gene in respect of their natural substrate specificities. Though both of these enzymes are active on the non-natural substrate ρ-nitrophenyl-α-L-arabinofuranoside, ABFA acts only on small linear 1,5-α-linked L-arabinofuranosyl oligosaccharides while ABFB is less specific. ABFB is able to hydrolyze 1,5-, 1,3-, and 1,2-α-linkages not only in L-arabinofuranosyl oligosaccharides, but also in polysaccharides containing terminal non-reducing L-arabinofuranoses in side chains, like L-arabinan, arabinogalactan and arabinoxylan. In contrast to the different forms of β-glucosidases found in the same organism, these two forms of α-arabinofuranosidases do not show significant homology with regard to their sequences.

Spagna et al. (1998) conducted a study on A. niger α-L-arabinofuranosidase (EC 3.2.1.55) and it was determined that, in wine, the substrates preferred by this enzyme included a considerable amount of glycosidically bound terpenic alcohols. As predicted, the primary ones (citronellol, nerol and geraniol) were hydrolyzed more effectively than the tertiary alcohols (linalool, α-terpineol). These authors postulated that this behaviour may be the result of the fact that nerol and geraniol are present essentially in a bound form in the grapes on which they conducted the study.

3.4.2.2 pH Optima of α-L-arabinofuranosidases

Limited information is available on the properties of the different α-L-arabinofuranosidases isolated from different sources. About the only data that could be found were of enzymes

isolated from a commercial enzyme preparation from *A. niger* (Spagna et al., 1998). The optimum pH value for the α-L-arabinofuranosidase was found to be 4.0. A relatively small amount of activity of 40% was detected at pH 3.3, which is close to that of wine.

In another study performed on the enzyme from *A. niger*, it was found that the arabinosidase exhibited maximum activity in the pH range of 3.7 - 4.0 (Günata et al., 1990b). The activity was stable between pH 3.8 and 4.9, but its activity decreased rapidly at pH levels lower than 3.5 and higher than 5.5. According to Kaji (1984), this instability in the neutral and alkaline range is unusual for arabinosidases of fungal origin.

3.4.2.3 Temperature optima of α-L-arabinofuranosidases

In the study by Spagna et al. (1998), the optimum temperature for the α-L-arabinofuranosidase was found to be 65°C. At 20 - 25°C, which is considered by the authors to be the temperature at which this enzyme will potentially be used in oenology, the relative activity was found to be only 10 – 30%.

The arabinofuranosidase studied by Günata et al. (1990b) exhibited an optimum temperature of 60°C, which is slightly lower than that of the enzyme studied by Spagna et al. (1998). However, the same low activity was observed at the lower temperatures at which vinification would take place.

4. COMMERCIAL ENZYME PREPARATIONS

The use of commercially available enzyme preparations in the food and wine industry is well established. Many enzymes, such as pectinases, proteases, cellulases, as well as glycosidases, are produced on a commercial scale. Although the use of these enzymes is more widespread in the food industry, only a few enzymes have application in the wine industry. Examples of these include the pectinases, used for clarification and extraction in wine, as well as the glycosidases, used to increase the aroma of some wines. Most of the enzymes originate from *Aspergillus* species. These preparations are sold as single enzyme preparations, where only one enzyme is present, or as mixed enzymes, for example composite mixtures of pectinases, cellulases, hemicellulases and glycosidases.

As can be seen throughout this study, β-glucosidase and α-L-arabinofuranosidase are enzymes of considerable importance in food technology (Aryan et al., 1987). These enzymes are currently produced on an industrial scale from a variety of cultures, including

A. niger. In addition to producing optimum activity, this organism has GRAS status, which makes it acceptable when it comes to producing foodstuffs or related products fit for human consumption.

In a study by Park (1996), a commercially available enzyme preparation containing β-glucosidase was added to wines made from Muscat of Alexandria grapes. These grapes are known for their high glycosidically bound monoterpene content. The wine was made without the addition of enzymes, as the enzymes were only available after the wines were aged for 7.8 months. The enzyme-treated wines were then stored immediately in an aging cellar for 14 months at 9.5°C. During the study it became clear that the time when the enzymes are added is crucial. As can be seen in this chapter, both the β-glucosidase and α-L-arabinofuranosidase have specific pH optima and are influenced significantly by the glucose and ethanol concentrations of the must. Generally, low pH and high ethanol content are not favourable for enzymatic reactions. Aryan et al. (1987) reported on Rohapect C, another enzyme preparation of which the enzymes showed optimal activity at pH 6.0. However, Shoseyov et al. (1988) described different optimal conditions for an enzyme isolated from *A. niger*, with a wide active pH range and an optimal pH of 3.4. However, glucose remains a strong inhibitor of both enzymes, and it is therefore better to add the enzyme toward the end of the fermentation, or even to the finished wine (Großmann et al., 1987; Shoseyov et al., 1988).

The results of the study indicated that the enzyme Rohapect 7104 can effectively be used to hydrolyze glycosidically bound monoterpenes. However, according to the author there a few factors that should be considered if the preparation is to be used. In normal wines, the glycosidically bound monoterpenes are relatively stable and are hydrolyzed very slowly due the acidic conditions in the wine. The release of the floral aroma therefore occurs over a long period of aging. Acidic hydrolysis, however, is also responsible for the interconversion of the released monoterpenes to more stable forms, although less desirable compounds, such as α-terpineol. When enzymes are added, the floral aroma is released all at once, so there are very little or no bound monoterpenes left to maintain a constant level of terpenic-floral aroma in the wine over a long aging period. Therefore it could be considered beneficial if some of the bound monoterpenes were left to provide a constant aroma throughout the "naturally controlled-release" of monoterpenes (Großmann et al., 1987; Shoseyov et al., 1988).

5. EXPRESSION OF HETEROLOGOUS β-GLUCOSIDASE AND α-L-ARABINOFURANOSIDASE GENES IN *SACCHAROMYCES CEREVISIAE*

5.1 β-Glucosidase

As mentioned before, the β-glucosidase enzyme has a wide host of substrates. So far, the exploitation of this enzyme in the industry has focused mainly on an increase in cellobiose degradation. Cellobiose is a byproduct of cellulose degradation performed by some microorganisms. This product usually accumulates because of the weak β-glucosidase, or cellobiase, activity of most cellulolytic microorganisms (Woodward and Wiseman, 1982). The yeast *S. cerevisiae* is able to ferment maltose and sucrose, but cannot ferment either cellobiose or lactose. These four sugars are the most important disaccharides in nature. Maltose and cellobiose owe their importance mainly to being products of the enzymatic hydrolysis of starch and cellulose respectively. Cellobiose and lactose are β-glycosides, but since *Saccharomyces* lacks β-glycosidases, consequently no β-glycosides are fermented naturally by this yeast (Barnett, 1976; 1981).

In a study performed by Adam et al. (1995), the authors aimed to express bacterial β-glucosidase activity in *S. cerevisiae*. This would enable the yeast to utilize cellobiose as a fermentable carbon source. Three factors concerning the heterologous expression were considered to be of great importance: the genotypic stability, gene copy number and strain ploidy. The common procedure to solve the instability of phenotypes conveyed by genes cloned in plasmids is to integrate them at specific genomic locations by means of homologous recombination. Usually, integration of a DNA fragment into the genome occurs in single copy, although sometimes several tandem repeats can be found at the site of integration (Orr-Weaver et al., 1981; Orr-Weaver and Szostak, 1983; Rothstein, 1991). The stabilization of a gene by means of its integration has the effect that its level of expression is reduced. The use of strains with different ploidy, the third factor under consideration, may add another variable to control heterologous gene expression.

In the study by Adam et al. (1995) several *S. cerevisiae* strains were constructed that expressed the *bglA* gene derived from *B. polymyxa* (discussed earlier). The authors then examined the β-glucosidase activity produced by these yeast strains. The strains differed in i) the location of the gene (either in a plasmid or integrated in the genome); ii) the number of *bglA* copies (single or multiple); and iii) ploidy.

From the results of the experimental work done by Adam et al. (1995), it was noted that increased activity of β-glucosidase was observed in the strains that contained an integrated *bglA* gene. This activity was more than in the case of the strain that contained an autonomously replicating plasmid carrying the *bglA* gene. Both of these strains showed much higher activity than the control strain, which exhibited some activity towards

ρ-nitrophenyl-β-D-glucopyranoside (ρNPG), the universal substrate to test β-glucosidase activity. The authors postulated that this may be due to another enzyme encoded by *S cerevisiae* and able to hydrolyze ρNPG. This postulation is supported by the fact that a gene from *S. cerevisiae* encoding an exo-glucanase cloned in a multi-copy plasmid was able to hydrolyze ρNPG (Nebrada et al., 1986).

The stability of multiple integrations at a highly expressed site in the yeast genome (repeated ribosomal-DNA sequences) was also examined. Five out of seven strains showed very high stability, retaining the altered phenotype after 60 generations. (Nebrada et al. (1986) constructed different strains of different ploidy to examine what effect the ploidy may have on the expression of the *bglA* gene. The results are summarized in Table 4.

Table 4. β-Glucosidase activity in cell extracts prepared from cultures of diploid, triploid and tetraploid strains, grown in minimal medium, at different times (Adam et al., 1995).

Strain (ploidy)	Specific activity (mU/mg)				
	0h	6h	12h	18h	24h
XAA14 (2n)	13	121	449	538	533
XAA15 (2n)	20	441	816	891	888
ADAA1 (2n)	6	142	597	1295	1695
ADAA2 (2n)	4	340	795	1441	1741
XAA10-6D (2n)	4	176	400	684	985
TRPAA1 (3n)	5	295	828	1188	1088
TRPAA7 (3n)	12	396	882	1133	1536
TTPAA3 (4n)	38	379	1144	1449	1740
TTPAA20 (4n)	5	245	682	981	1357

All these strains contained multiple integrated copies of the *bglA* gene. In general, while per-cell activity increased with ploidy, the values of specific activity were similar for strains of equivalent genotype, independently of their ploidy. In another report, Takagi et al. (1985) measured the expression of the β-galactosidase gene encoded by the *lacZ* gene of *E. coli*, cloned under control of a yeast promoter in different plasmids, in strains of different ploidy. It was observed that slightly lower enzyme activities occur in diploids and tetroids than in haploids.

One important observation in this report is that of the high susceptibility of triploids and tetraploids to cell lysis, most likely due to the construction of the promoter sequences, and perhaps not linked to the expression of the β-glucosidase gene itself.

5.2   α-L-Arabinofuranosidase

In a study by Crous et al. (1996), the α-L-arabinofuranosidase gene (*ABF2*) of *A. niger* was expressed in *S. cerevisiae*. *S. cerevisiae* was transformed with a multi-copy episomal plasmid containing the *ABF2* gene sandwiched between yeast promoter and terminator sequences. This gene displays a 94% correspondence with the α-L-arabinofuranosidase B gene cloned from *A. niger* N400 (Flipphi et al., 1993b). The recombinant α-L-arabinofuranosidase was secreted into the surrounding medium despite the absence of a typical –Lys-Arg- or Arg-Arg- dipeptide sequence at the NH_2-proximal region of the Abf2 protein, which can be recognized by the *KEX2* protease of *S. cerevisiae*. Secretion could possibly be attributed to the high degree of hydrophobic residues in the first 18 amino-acid residues of the Abf2 protein.

The highest Abf2 activity of 0.002 U/ml was determined after 48 h for *S. cerevisiae* strain Y294, a laboratory strain, grown in synthetic (SC) medium. This compared well with the values obtained by Van der Veen et al. (1991) for the native AbfB protein of *A. niger* with glucose as sole carbon source. However, it was observed that a substantially higher level of α-L-arabinofuranosidase activity was detected in the supernatant of the recombinant *S. cerevisiae* strain when grown on complex YPD media.

6.   CONCLUDING REMARKS

As can be seen from this literature review, it is clear that:

- there are a multitude of flavour and fragrance compounds that play a role in the sensorial quality of wine, of which terpenols and their alcohols are only a part;
- these terpenols and their alcohols have been studied intensively, as have the enzymes that may increase their concentrations in wine;
- β-glucosidases and α-arabinofuranosidases from diverse sources have already been cloned and expressed in other organisms in order to elucidate their functions in that specific organism, or to understand their activity better.

This is all done to better our knowledge about glycosidases and their substrates, so that when the time is ripe, a biotechnological increase of flavour in wine will not be such a

strange concept. In fact, because the sensorial quality of a product like wine plays such an important role in the preference of the public, the concept of genetically enhanced wine may be one of the more exciting applications of gene technology that exists today.

7. REFERENCES

Adam, A. C., Rubio-Texeira, M., and Polaina, J. (1995) Induced expression of bacterial β-glucosidase activity in *Saccharomyces. Yeast* **11**, 395-406.

Ait, N., Cruezet, N., and Cattanéo, J. (1979) Characterization and purification of thermostable β-glucosidases from *Clostridium thermocellum. Biochem. Biophys. Res. Comm.* **90**, 537-546.

Aryan, A.P., Wilson, B., Strauss, C.R., and Williams, P.J. (1987) The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible application in enology. *Am J. Enol. Vitic.* **38**, 182-188.

Avakyants, S. P., Rastyannikov, E.G., Chernyaga, B.S., and Navrotskii, V.J. (1981) Khromato-mass-spektrometrischeskoe issledovarie letuchikh vesnchestv vina. *Vinodel. Vinograd. SSSR* **41**, 50-53.

Barnett, J. A. (1976) The utilization of sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* **32**, 125-234.

Barnett, J. A. (1981) The utilization of disaccharides and some other sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* **39**, 347-405.

Bayer, E., and Bässler, E. (1961) Systematische Identifizierung von Estern im Weinaroma. II. Mitteilung zur systematischen Identifizierung verdampfbarer organischer Substanzen. *Z. analyt. Chem.* **181**, 418-424.

Bayer, E., Kupfer, G., and Reuther, K.H. (1958) Anwendung der Gaschromatographie zur Analyse künstlicher und natürlicher Aromastoffe. *Z. analyt. Chem.* **164**, 1-10.

Blondin, B., Ratomahenina, R., Arnaud, A., and Galzy, P. (1983) Purification and properties of the β-glucosidase of a yeast capable of fermenting cellobiose to ethanol: *Dekkera intermediata* Van der Walt. *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 1-6.

Buttery, R.G., Seifert, R.M., Gaudigni, D.G., and Luig, L.C. (1971) Characterization of additional volatile components of tomato. *J. Agric. Food Chem.* **19**, 524–529.

Caldini, C., Bonomi, F., Pifferi, P. G., Lanzarini, G., and Galante, Y. (1993) Kinetic and immobilization studies on fungal glycosidases for aroma enhancement in wine. *Enzyme Microb. Technol.* **16**, 286-291.

Campos, N., Bako, L., Brzobohaty, B., Feldwisch, J., Zettl, R., Boland, W., and Palme, K. (1993) Identification and characterization of a novel phytohormone conjugate specific β-glucosidase activity from maize. *β-glucosidases: Biochemistry and molecular biology.* (Ed. A. Esen) ACS Symposium Series 533. Washington DC. pp. 205-213.

Chambon, C., Ladeveze, V., Oulmouden, A., Servouse, M., and Karst, F. (1990) Isolation and properties of yeast mutants affected in farnesyl diphosphate synthetase. *Curr. Genet.* **18**, 41-46.

Cordonnier, R., and Bayonove, C. (1978) Les composantes varietales et prefermentaires de l'arôme de vins. *Extrait Parfum Cosmet. Arômes.* **24**, 67-77.

Cordonnier, R., and Bayonove, C. (1981) Etude de la phase prefermentaire de la vinification: Extraction et formation de certain composé de l'arôme: cas des terpenol, des aldehydes et des alcools en C_6. *Conn. Vigne Vin.* **15**, 267-286.

Crous, J.M., Pretorius, I.S., and Van Zyl, W.H. (1996) Cloning and expression of the α-L-arabinofuranosidase gene (*ABF2*) of *Aspergillus niger* in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **46**, 256-260.

Cuevas, L., Niemeyer, H.M., and Jonsson, L.M. (1992) Partial purification and characterization of a hydroxamic acid glucoside from β-D-glucosidase from maize. *Phytochem.* **31**, 2609-2612.

Darriet, P., Boidron, J.N., and Dubordieu, D. (1988) L'hydrolyse des hétérosides terpéniques du Muscat a Petit Grains par les enzymes périplasmiques de *Saccharomyces cerevisiae*. *Conn. Vigne Vin.* **22**, 189-195.

Delcroix, A., Günata, Z., Sapis, J., Salmon. J., and Bayonove, C. (1994) Glycosidase activities of three enological yeast strains during winemaking: Effect on the terpenol content of Muscat wine. *Am J. Enol. Vitic.* **45**, 291-296.

Dimitriadis, E., and Williams, P. J. (1984) The development and use of a rapid analytical technique for estimation of free and potentially volatile monoterpene flavorants of grapes. *Am. J. Enol. Vitic.* **35**, 66-71.

Drawert, F., and Barton, H. (1978) Biosynthesis of flavor compounds by micro-organisms. 3. Production of monoterpenes by the yeast *Kluyveromyces lactis*. *J. Agric. Food. Chem.* **26**, 765-767.

Duerksen, J.D., and Halvorson, H. (1958) Purification and properties of an inductible β-glucosidase of yeast. *J. Biol. Chem.* **233**, 1113-1120.

Duerksen, J.D., and Halvorson, H. (1959) The specificity of induction of β-glucosidase in *Saccharomyces cerevisiae*. *Biochem. Biophys. Acta.* **36**, 47-55.

Eberhart, B.M., and Beck, R.S. (1973) Induction of β-glucosidases in *Neurospora crassa*. *J. Bact.* **116**, 295-303.

Esen, A. (1992) Purification and partial characterization of maize (*Zea mays* L.) β-glucosidase. *Plant Physiol.* **98**, 174-182.

Esen, A. (1993) β-glucosidases: Biochemistry and Molecular Biology. *ACS Symposium Series*. (Eds. M. J. Comstock, A. Esen) Washington D.C. No 533, pp. 11-12.

Fagan, G.L., Kepner, R.E., and Webb, A.D. (1981) Production of linalool, *cis*-nerolidol and *trans*-nerolidol, and *trans, trans*-farnesol by *Saccharomyces fermentati* growing as a film on simulated wine. *Vitis* **20**, 36-42.

Flipphi, M.J.A., Van Heuvel, M., Van der Veen, P., Visser, J., and De Graaff, L.H. (1993b) Cloning and characterization of the *abfB* gene coding for the major α-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. *Curr Genet.* **24**, 525-532.

Flipphi, M.J.A., Visser, J., Van der Veen, P., and De Graaff, L.H. (1993a) Cloning of the *Aspergillus niger* gene encoding α-L-arabinofuranosidase A. *Appl. Microbiol. Biotechnol.* **39**, 335-340.

Frehner, M., and Conn, E.E. (1987) The linamarin β-glucosidase in Costa Rican wild lima beans (*Phaseolus lunatis* L.) is apoplastic. *Plant Physiol.* **84**, 1296-1300.

Garibaldi, A., and Gibbins, L.N. (1975) Partial purification and properties of a β-glucosidase from *Erwinia herbicola* Y46. *Can. J. Microbiol.* **21**, 513-520.

Gaudigni, D.G., Buttery, R.G., and Okano, S. (1963) Odour threshold of some organic compounds associated with food flavours. *J. Sci. Food Agric.* **14**, 366–368.

Gonzáles-Candelas, L., Aristoy, M.C., Polaina, J., and Flors, A. (1989) Cloning and characterization of two genes from *Bacillus polymyxa* expressing β-glucosidase activity in *Escherichia coli. Appl. Environ. Microbiol.* **55**, 3173-3177.

Großmann, M., Rapp, A., and Reith, W. (1987) Enzymatische freisetzung gebundener Aromastoffe in Wein. *Deutsche Lebensm-rundshau*, **83**, 7.

Groleau, D., and Forsberg, C.W. (1981) Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes. Can. J. Microbiol.* **27**, 517-530.

Gueguen, Y., Chemardin, P., Arnaud, A., and Galzy, P. (1994) Purification and characterization of the endocellular β-glucosidase of a new strain of *Candida entomophila* isolated from fermenting agave (*Agave* sp.) juice. *Biotechnol. Appl. Biochem.* **20**, 185-198.

Gueguen, Y., Chemardin, P., Arnaud, A., and Galzy, P. (1995) Comparative study of extracellular and intracellular β-glucosidases of a new strain of *Zygosaccharomyces bailii* isolated from fermenting agave juice. *J. Appl. Bacteriol.* **78**, 270-280.

Gueguen, Y., Chemardin, P., Janbon, G., Arnaud, A., and Galzy, P. (1996) A very efficient β-glucosidase catalyst for the hydrolysis of flavour precursors of wines and fruit juices. *J. Agric. Food Chem.* **44**, 2336-2340.

Günata, Z., Bayonove, C., Baumes, R., and Cordonnier, R. (1985) The aroma of grapes. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr.* **331**, 83-90.

Günata, Z., Bayonove, C., Baumes, R.L., and Cordonnier, R.E. (1986) Specificity of free and bound fractions of some aroma components of grapes cv. Muscat during wine processing. Preliminary results. *Am. J. Enol. Vitic.* **37**, 112-114.

Günata, Y.Z., Bayonove, C.L., Tapiero, C., and Cordonnier, R.E. (1990a) Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. *J. Agric. Food. Chem.* **38**, 1232-1236.

Günata, Z., Brillouet, J.-M., Voirin, S., Baumes, R., and Cordonnier, R. (1990b) Purification and some properties of an α-L-arabinofuranosidase from *Aspergillus niger*. Action on grape monoterpenyl arabinofuranosylglucosides. *J. Agric. Food Chem.* **38**, 772-776.

Günata, Y.Z., Bayonove, C.L., Arnaud, A., and Galzy, P. (1990c) Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamii* β-glucosidases. *J. Sci. Food. Agric.* **50**, 499-506.

Günata, Z., Bitteur, S., Brillouet, J.M., Bayonove, C., and Cordonnier, R. (1988) Sequential enzymic hydrolysis of potentially aromatic glycosides from grape. *Carbohydr. Res.* **184**, 139-149.

Günata, Z., Dugelay, I., Sapis, J.C., Baumes, R., and Bayonove, C. (1993) Role of enzymes in the use of the flavour potential from grape glycosides in winemaking. In: *Progress in Flavour Precursor Studies – Analysis, Generation, Biotechnology.* p 219. (Eds. P. Schreier, P. Winterhalter) p219. Allured Publ. Carol Stream.

Günata, Z., Vallier, M.J., Sapis, J.C., Bayonove, C., Arnaudon, V., Madarro, A., and Polaina, J. (1996) Hydrolysis of monoterpenyl-β-D-glucosides by cloned β-glucosidases from *Bacillus polymyxa. Enzyme Microb. Tech.* **18**, 286-290.

Güntert, M. (1984) Gaschromatographisch-massenspektrometrische Untersuchungen flüchtiger Inhaltstoffe des Weinaromas: Beitrag zur Sortencharakterisierung der Rebsorte Weisser Riesling. Thesis, Universität Karlsruhe, Karlsruhe 1984.

Haisman, D.R., and Knight, D.J. (1967) The enzymatic hydrolysis of amygdalin. *Biochem J.* **103**, 528.

Hennig, K. and Villforth, F. (1942) Die Aromastoffe der Weine. *Vorratspfl. Lebensmittelforsch.* **5**, 181–200, 313–333.

Herr, D. (1979) Secretion of cellulase and β-glucosidase by *Trichoderma viride* ITCC-1433 in submerged cultures of different substrates. *Biotech. Bioeng.* **21**, 1361-1371.

Hösel, W. (1981) Glycosylation and glycosidases. *The Biochemistry of Plants.* (Eds. P. K Stumpf, E. E. Conn) Vol 7, p 725. Academic Press, New York.

Iranzo, J.F.U., Pérez, A.I.B., and Cañas, P.M.I. (1998) Study of the oenological characteristics and enzymatic activities of wine yeasts. *Food Microbiol.* **15**, 399-406.

Iwashita, K., Kouichi, T., Hitoshi, K., Hitoshi, S., and Kiyoshi, I. (1998) Purification and characterization of extracellular and cell wall-bound β-glucosidases from *Aspergillus kawachii. Biosci. Biotech. Biochem.* **62**, 1938-1946.

Iwashita, K., Tatsuya, N., Hitoshi, K., Makoto, T., Hitoshi, S., and Kioshi, I. (1999) The *bglA* gene of *Aspergillus kawachii* encodes both extracellular and cell wall-bound β-glucosidases. *Appl. Environ. Mcrobiol.* **65**, 5546-5553.

Javelot, C., Karst, F., Ladeveze, V., Chambon, C., and Vladescu, B. (1990) Production of monoterpenes by yeast mutants defective in sterol biosynthesis. In: *Microbiology Applications in Food Technology.* (Eds. B.H. Nga, Y. K. Lee) Vol 1, pp. 101-122. Elsevier. New York.

Jones, D. A. (1988) In *Cyanide compounds in Biology: CIBA Foundation Symposium.* (Eds. D. Evered, S. Hamett) John Wiley and Sons, Chichester, UK. No 140. pp151-170.

Kaji, A. (1984) L-Arabinosidases. *Adv. Carbohydr. Chem. Biochem.* **42**, 383-394.

Kaji, A., Tagawa, K., and Matsubara, K. (1967) Studies on the enzymes acting on arabinase. Part VIII: Purification and properties of arabinase produced by *Aspergillus niger. Agric. Biol. Chem.* **31**, 1023-1028.

Kakes, P. (1985) Linamarase and other β-glucosidases are present in the cell walls of *Trifolium repens* L. leaves. *Planta* **166**, 156-160.

Kaneko, S., Ishii, T., Kobayashi, H., and Kusakabe, I. (1998) Substrate specificities of α-L-arabinofuranosidases produced by two species of *Aspergillus niger*. *Biosci. Biotechnol. Biochem.* **62**, 695-699.

Kepner, R.E., Webb, A.D., and Muller, C.J. (1972) Identification of 4-hydroxy-3-methyloctonoic acid gamma-lactone (5-butyl-4-methyl-dihydro-2-(3H)-furanone) as a volatile component of oak-wood aged wines of *Vitis vinifera* var. "Cabernet-Sauvignon". *Am. J. Enol. Vitic.* **23**, 103-105.

King, A., and Dickinson, R. (2000) Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae, Torulaspora delbrueckii* and *Kluyveromyces lactis. Yeast* **16**, 499-506.

Komae, K., Kaji, A., and Sato, M. (1982) An α-L-arabinofuranosidase from *Streptomyces purperascens* IF 03389. *Agric. Biol. Chem.* **46**, 1899-1905.

Kwon, K.-S., Kang, H.G., and Hah, Y.C. (1992) Purification and characterization of two extracellular β-glucosidases from *Aspergillus nidulans. FEMS Micobiol. Lett.* **97**, 149-154.

López-Camacho, C., and Polaina, J. (1993) Random mutagenesis of a plasmid-borne glycosidase gene and phenotypic selection of mutants in *Escherichia coli. Mutat. Res.* **301**, 73-77.

Machida, M., Ohtsuki, I., Fukui, S., and Yamashita, I. (1988) Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular β-glucosidases as expressed in *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **54**, 3147-3155.

Marais, J. (1985) Die effek van druifkultivar, rypheid en mosbehandelings op terpeenkonsentrasies en wynkwaliteit. M Sc-thesis, University of Stellenbosch, Stellenbosch, Republic of South Africa.

Marais, J., and Pool, H.J. (1980) Effect of storage time and temperature on the on the volatile composition and quality of dry white table wines. *Vitis* **19**, 151-164.

Masuda, M., and Nishimura, K. (1971) Branched nonalactones from some *Quercus* species. *Phytochem.* **10**, 1401-1402.

Masuda, M., Okawa, E., Nishimura, K., and Yunome, H. (1984) Identification of 4,5-dimethyl-3-hydroxy-2(5H)-furanone (Sotolone) and ethyl-9-hydroxy-nonanoate in botrytised wine and evaluation of the roles of compounds characteristic of it. *Agric. biol. Chem.* **48**, 2707–2710.

Mateo, J.J., and Di Stefano, R. (1997) Description of the β-glucosidase activity of wine yeasts. *Food Microbiol.* **14**, 583-591.

Mega, T., and Matsushima, Y. (1979) Comparative studies of three exo-β-glycosidases of *Aspergillus oryzae. J. Biochem.* **85**, 335-341.

Mulkens, A. (1987) Flavonoids of the leaves of *Melissa officinalis* L. *Pharm Acta Helv.* **62**, 229.

Narhrstedt, A., and Mueller, E. (1993) β-glucosidase *Zygaena trifolii* and its inhibition by some alkaline earth metal ions. *β-glucosidases: Biochemistry and molecular biology.* (Ed. A. Esin) ACS Symposium Series 533. Washington DC. pp 132-144.

Nebrada, A.R., Villa, T.G., Villanueva, J.R., and Del Rey, F. (1986) Cloning of genes related to exo-β-glucanase production in *Saccharomyces cerevisiae*: characterization of an exo-β-glucanase structural gene. *Gene.* **47**, 245-259.

Nishimura, K., Ohnishi, M., Masuda, M., Koga. K., and Matsuama, R. (1983) Reactions of wood components during maturation. *Flavour of Distilled Beverages.* pp. 241-255. (Ed. J.R. Piggott) Ellis Horwood, Chichester.

Nisius, A., and Ruppel, H.G. (1987) Immunocytological and chemical studies on the stroma-centre-forming protein from *Avena* plastids. *Planta* **171**, 443-452.

Nunoura, N., Ohdan, K., Tanaka, K., Tamaki, H., Yano, T., Inui, M., Yukawa, H., Yamamoto, K., and Kumagai, H. (1996) Cloning and nucleotide sequence of the β-D-glucosidase gene from *Bifidobacterium breve* clb, and expression of β-D-glucosidase activity in *Escherichia coli. Biosci. Biotech. Biochem.* **60**, 2011-2018.

Onishi, M., Guymon, J.F., and Crowell, E.A. (1977) Changes in some volatile constituents of brandy during aging. *Am. J. Enol. Vitic.* **28**, 192-198.

Orr-Weaver, T.L., and Szostak, J.W. (1983) Multiple tandem plasmid integration in *S. cerevisiae. Mol. Cell. Biol.* **3**, 747-749.

Orr-Weaver, T.L., Szostak, J.W., and Rothstein, R.J. (1981) Yeast transformation: A model study for the study of recombination. *Proc. Natl. Acad. Sci. USA.* **78**, 6354-6358.

Painbeni, E., Vallés, S., Polaina, J., and Flors, A. (1992) Purification and characterization of a *Bacillus polymyxa* β-glucosidase expressed in *Escherichia coli. J. Bacteriol.* **174**, 3087-3091.

Park, S. K. (1996) Changes in free and glycosidically bound monoterpenes as a function of fermentation, wine aging and enzyme treatment. *Foods and Biotechnol.* **5**, 280-286.

Parks, L.W. (1978) Metabolism of sterols in yeast. *CRC Crit. Rev. Microbiol.* **6**, 301-341.

Pemberton, M.S., Brown, R.D., and Emert, G.H. (1980) The role of β-glucosidase in the bioconversion of cellulose to ethanol. *Can. J. Chem. Eng.* **58**, 723-729.

Peralta, R.M., Kadowaki, M.K., Terenzi, H.F., and Jorge, J.A. (1997) A highly thermostable β-glucosidase activity from the thermophilic fungus *Humicola grisea* var. *thermoidea*: purification and biochemical characterization. *FEMS Microbiol. Lett.* **146**, 291-295.

Poulton, J.E. (1990) Cyanogenesis in plants. *Plant Physiol.* **94**, 401-405.

Ramshaw, E.H., and Hardy, P.J. (1985) Volatile compounds in dried grapes. *J. Sci. Food. Agric.* **20**, 619–621.

Rapp, A., and Knipser, W. (1980) Eine neue Methode zur Einreicherung von Dampfraumkomponenten. Dargestellt am Beispiel des Weines. *Chromatographia.* **13**, 698–702.

Rapp, A., and Mandery, H. (1986) Wine Aroma. *Experimentia.* **42**, 873-884.

Rapp, A. (1987) Studies on terpene compounds in wines. In: *Frontiers of Flavour*, Proceedings of the 5th International Flavour Conference, Greece, 1987. (Ed. G. Charalambous). Elsevier Science Publishers, Amsterdam.

Rapp, A., Almy, J., and Güntert, M. (1985a) Identification of several sulphur-containing compounds in wine. *Am. J. Enol. Vitic.* **36**, 219–221.

Rapp, A., Güntert, M., and Rieth, W. (1985b) Einfluss der Maischestandzeit auf die Aromazusammensetzung des Traubenmostes und Weines. *Dt. Lebensm. Rdsch.* **81**, 69-72.

Rapp, A., Güntert, M., and Ullemeyer, H. (1985c) Über Veränderungen der Aromastoffe während der Flaschenlagerung von Weissweinen der Rebsorte Riesling. *Z. Lebensmittelunters. – Forsch.* **180**, 109–116.

Ratledge, C., and Evans, C.T. (1989) Lipids and their metabolism. In: *The Yeasts.* Vol 3, pp. 367-455. Academic Press, London.

Raynal, A., and Guerineau, M. (1984) Cloning and expression of the structural gene for β-glucosidase of *Kluyveromyces fragilis* in *Escherichia coli* and *Saccharomyces cerevisiae. Mol. Gen. Genet.* **195**, 108-115.

Reazin, G. H., Baldwoin, S., Scales, H.S., Washington, H.W., and Andreasen, A. (1976) Determination of the congeners produced from ethanol during whiskey maturation. *J. Ass. Off. Analyt. Chem.* **59**, 770-776.

Ribéreau-Gayon, P. (1971) Recherches technologiques sur les composés phénoliques des vins rouges. III. Influence du mode de logement sur les caractères chimiques et organoleptiques des vins rouges, plus particulièrement sur leur couleur. *Conn. Vigne Vin.* **5**, 87-97.

Ribéreau-Gayon, P. (1978) "Wine-Flavor" In: *Flavor of foods and beverages.* (Eds. G. Charalambous, G.E. Inglett) Academic Press, New York / San Francisco / London.

Ribéreau-Gayon, P., Boidron, J.H., and Terrier, A. (1975) Aroma of muscat grape varieties. *J. Agric. Food Chem.* **23**, 1042–1047

Riou, C., Salmon, J.C., Valiier, M.J., Günata, Z., and Barre, P. (1998) Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae. Appl. Environ. Microbiol.* **64**, 3607-3614.

Rojas, A., Arola, L., and Romeu, A. (1995) β-Glucosidase families revealed by computer analysis of protein sequences. *Biochem. Mol. Biol. Int.* **35**, 1223-1231.

Rosi, I., Vinella, M., and Domizio, P. (1994) Characterization of β-glucosidase activity in yeasts of oenological origin. *J. Appl. Bacteriol.* **77**, 519-527.

Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast. *Meth. Enzymol.* **194**, 281-301.

Saddler, J.N., and Khan, A.W. (1980) Cellulolytic enzyme system of *Acetovibrio cellulolyticus. Can. J. Microbiol.* **27**, 288-294.

Saha, B.C., and Bothast, R.J. (1996) Production, purification and characterization of a highly glucose-tolerant novel β-glucosidase from *Candida peltata. Appl. Environ. Microbiol.* **62**, 3165-3170.

Saha, B.C., Freer, S.N., and Bothast, R.J. (1996) Thermostable β-glucosidases. In: *Enzymatic degradation of insoluble carbohydrates.* (Eds. J. N. Saddler, N. H. Penner) American Chemical Society, Washington, D.C.

Sanz-Aparicio, J., Romero, A., Martinez-Ripoll, M., Madarro, A., Flors, A., and Polaina, J. (1994) Crystallization and preliminary X-ray diffraction analysis of a type I β-glucosidase encoded by the *bglA* gene of *Bacillus polymyxa. J. Mol. Biol.* **240**, 267-270.

Schreier, P. (1979) Flavor composition of wines: a review. *CRC. Food Sci. Nutr.* **12**, 59-111.

Schreier, P., Drawert, F., and Junker, A. (1976) Identification of volatile constituents from grapes. *J. Agric. Food Chem.* **24**, 331-336.

Shimuzu, J., Nokara, M., and Watanabe, M. (1982) Transformation of terpenoids in grape must by *Botrytis cinerea. Agric. Biol. Chem.* **46**, 1339–1344.

Shoseyov, O., Bravdo, B.-A., Ikan, R., and Chet, I. (1988) Endo-β-glucosidase from *Aspergillus niger* grown on a monoterpene glycosidase-containing medium. *Phytochem.* **27**:1973.

Simpson, R.F. (1978a) Aroma and compositional changes in wine with oxidation, storage and aging. *Vitis* **17**, 274-287.

Simpson, R.F. (1978b) 1,1,6-Trimethyl-1,2-dihydronaphtalene: an important contributor to the bottle aged bouquet of wine. *Chemy Ind.* 37.

Simpson, R.F. (1979) Volatile aroma components of Australian port wines. *J. Sci. Food Agric.* **31**, 214–222.

Simpson, R.F., Strauss, C.R., and Williams, P.J. (1977) Vitispirane: a C_{13} spiro-ether in the aroma volatiles of grape juice, wine and distilled grape-spirits. *Chemy Ind.* 663–664.

Sinnot, M.L. (1990) Catalytic mechanisms of enzymatic glycosyl transfer. *Chem. Rev.* **90**, 1171-1202.

Skory, C.D., Freer, S.N., and Bothast, R.J. (1996) Properties of an intracellular β-glucosidase purified from the cellobiose-fermenting yeast *Candida wickerhamii. Appl. Microbiol. Biotechnol.* **46**, 353-359.

Spagna, G., Romagnoli, D., Angela, M., Bianchi, G., and Pifferi, P.G. (1998) A simple method for purifying glycosidases: α-L-arabinofuranosidase and β-D-glucopyranosidase from *Aspergillus niger* to increase the aroma of wine. Part I. *Enzyme Microb. Tech.* **22**, 298-304.

Sternberg, D., Vijayakumar, P., and Reese, E.T. (1977) β-glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. *Can. J. Microbiol.* **23**, 139-147.

Stevens, K.L., Bomben, J.L., and McFadden, W.H. (1967) Volatiles from grapes. *Vitis vinifera* (Linn.) cultivar Grenache. *J. Agric. Food Chem.* **15,** 378-380.

Stevens, K.L., Bomben, J.L., Lee, A., and McFadden, W.H. (1957) Volatiles from grapes. Muscat of Alexandria. *Food Res.* **22**, 384–95.

Stevens, K.L., Flath, A., Lee, A., and Stern, D.J. (1969) Volatiles from grapes. Comparison of Grenache juice and Grenache rosé wine. *J. Agric. Food Chem.* **17**, 1102-1106.

Swain, E., Li, C.P., and Poulton, J.E. (1992) Tissue and subcellular localization of enzymes catabolizing (*R*)-amygdalin in mature *Prunus serotina* seeds. *Plant Phys.* **100**,291-300.

Takagi, A., Chua, E.N., Bonnchird, C., Harashima, S., and Oshima, Y. (1985). Constant copy numbers of plasmids in *Saccharomyces cerevisiae* hosts with different ploidies *Appl. Microbiol. Biotehnol.* **23**, 123-129.

Terrier, A., Boidron, J. N., and Ribéreau-Gayon, P. (1972) Teneurs en composés terpéniques des raisins de *V. vinifera. C. r. Acad. Sci. Ser. D.* **275**, 941-944.

Thayer, S.S., and Conn, E.E. (1981) Subcellular localization of dhurrin β-glucosidase and hydroxynitrile lyase in the mesophyll cells of Sorghum leaf blades. *Plant Physiol.* **67**, 617-622.

Van der Veen, P., Flipphi, M.J.A., Voragen, A.G.J., and Visser, J. (1991) Induction, purification and characterization of arabinases produced by *Aspergillus niger. Arch. Microbiol.* **15**, 723-728.

Van Rensburg, P., Van Zyl, W.H., and Pretorius, I.S. (1998) Engineering yeast for efficient cellulose degradation. *Yeast* **14**: 67-76.

Voirin, S.G., Baumes, R.L., Sapis, J.C., and Bayonove, C.L. (1992) Analytical methods for monoterpene glycosides in grape and wine. II. Qualitative and quantitative determination of monoterpene glycosides in grape. *J. Chromatogr.* **595**: 269-281.

Versini, G., Inama, S., and Sartori, G. (1981) A capillary column gaschromatographic research into the terpene constituents of "Riesling Renano" (Rhine Riesling) wine from Trentino Alto Adige: their distribution within berries, their passage into must and their presence in the wine according to different wine making procedures, organoleptic considerations. *Vini Ital.* **23**, 189-211.

Weinstein, L., and Albersheim, P. (1979) Structure of plant cell walls IX. Purification and partial characterization of a wall-degrading endo-arabinase and an arabinosidase from *Bacillus subtilis*. *Plant Physiol.* **63**, 425-432.

Welch, R.C., Johnston, J.C., and Hunter, G.L.K. (1982) Volatile constituents of the Muscadine grape (*V. rotundifolia*). *J. Agric. Food. Chem.* **30**, 681-684.

Williams, P.J., Strauss, C.R., and Wilson, B. (1980) Hydroxylized linalool derivatives as precursors of volatile monoterpenes of Muscat grapes. *J. Agric. Food. Chem.* **28**, 766-771.

Williams, P.J., Strauss, C.R., Wilson, B., and Massy-Westropp, R.A. (1982a) Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochem.* **21**, 2013-2010.

Williams, P.J., Strauss, C.R., Wilson, B., and Massy-Westropp, R.A. (1982b) Studies on the hydrolysis of *Vitis vinifera* monoterpene precursor compounds and model monoterpene β-D-glucosides rationalizing the monoterpene composition of grapes. *J. Agric. Food Chem.* **30**, 1219-1223.

Williams, P.J. (1993) Hydrolytic flavour release in fruit and wines through hydrolysis of non-volatile precursors. In: *Flavor Science – Sensible Principles and Techniques.* (Eds. T. E. Acree, R. Teranishi) p 287. American Chemical Society, Washington DC.

Withers, S.G., and Street, I.P. (1989) β-Glucosidase: mechanisms and inhibition. In: *Plant cell wall polymers: biogenesis and biodegradation.* (Ed. N.G. Lewis) pp. 597-607. American Chemical Society, Washington D.C.

Woodward, J., and Wiseman, A. (1982) Fungal and other β-glucosidases – their properties and applications. *Enzyme Microb. Technol.* **4**, 73-79.

Yan, T.-R., and Lin, C.-L. (1997) Purification and characterization of a glucose tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. *Biosci. Biotechnol. Biochem.* **61**, 965-970.

Yanai, T., and Sato, M. (1999) Isolation and properties of β-glucosidase produced by *Debaryomyces hansenii* and its application in winemaking. *Am. J. Enol. Vitic.* **50**, 231-235.

Zea, L., Moreno, J., Ortega, J.M., and Medina, M. (1995) Content of free terpenic compounds in cells and musts during vinification with three *Saccharomyces cerevisiae* races. *J. Agric. Food Chem.* **43**, 1110-1114.

CHAPTER 3

:

RESEARCH RESULTS

The expression of two *Saccharomycopsis fibuligera* β-glucosidase-encoding genes (*BGL1* and *BGL2*) in an industrial wine yeast strain

This manuscript is to be submitted for publication in
Food Microbiology

The expression of two *Saccharomycopsis fibuligera* β-glucosidase-encoding genes (*BGL1* and *BGL2*) in an industrial wine yeast strain

Tanya Stidwell, Pierre van Rensburg, Marius G. Lambrechts, Ricardo Cordero Otero, Isak S. Pretorius

Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Stellenbosch 7600, South Africa

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 α-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 are able to hydrolyze these glycosidic bonds, thereby releasing the aromatic terpenols as well as other aglycones bound to saccharides. However, grape-derived β-glucosidases are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine. By contrast, some yeast β-glucosidases are not inhibited by these limiting factors and have been shown to liberate terpenols from terpenyl-glycoside precursors. Since these β-glucosidases are absent in most *Saccharomyces cerevisiae* starter culture strains, two β-glucosidase genes (*BGL1* and *BGL2*) from the yeast *Saccharomycopsis fibuligera* were sub-cloned with a dominant selectable marker (*SMR1*) into an integrating *Escherichia coli-S. cerevisiae* shuttle plasmid and expressed in a commercial wine yeast strain (VIN13). Using ρ-nitrophenyl-β-D-glucopyranoside (ρNPG) 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. This study therefore paves the way for the development of wine yeast starter culture strains that could liberate monoterpenols from grape-derived non-volatile *O*-glycosides, thereby intensifying the varietal flavour of wine.

INTRODUCTION

Monoterpenes and monoterpene alcohols play a very important role in the grape and wine flavour of most Muscat varieties. These compounds also occur in other cultivars, existing as subtle supporting flavours, although they are less pronounced and other flavour compounds may play a larger role in the varietal flavour of the wine. The 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. These monoterpene alcohols include geraniol, nerol, citronellol, linalool, α-terpineol and hotrienol among others. The major fractions of these compounds occur in the grape as glycosidically bound forms (Günata et al., 1985; Voirin et al., 1992; Williams et al., 1982), which renders them non-volatile and flavourless. The sugar moieties towhich 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). These glycosidic bonds can 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 a lot of attention as a means of improving the varietal aroma and flavour of wine. The mechanism by which this hydrolysis works is now well established (Günata et al., 1985; 1988) and entails specific glycosidases active in two successive steps. In the first step, the action of an α-arabinofuranosidase, α-rhamnosidase, β-xylosidase or β-apiosidase is necessary to cleave the intersugar-linkage (Günata et al., 1988), and this is followed by the second step, in which a β-glucosidase liberates the aglycone. In the case where the disaccharide moiety consists of two glucose units, only the action of a β-glucosidase is needed to facilitate the complete reaction (Haisman et al., 1967). The aglycone does not neccesarily consist of a terrpenol; 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 flavour, as well as having other beneficial properties, especially concerning the release of resveratrol (Vrhovsek et al., 1997).

The use of heterologous enzymes for increased flavour production has attracted more interest in recent times, as enzyme systems in native organisms sometimes are not efficient enough for flavour release under the conditions that apply, for instance the low pH and high glucose environment of wine and juices. Glycosidases occur in nearly every organism. They are present in plants, fungi, bacteria, yeasts and even humans, where they perform a variety of functions. However, not all of these enzymes are suitable for heterologous expression in other organisms, for example those from plants exhibit high pH

optima (pH 5 for *Vitis vinifera*), 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 9% (Riou et al., 1998). These enzymes are also more active at high pH values (Woodward and Wiseman, 1982). Bacterial glycosidases generally have the disadvantage of being active at high temperatures (50°C and higher) (Woodward and Wiseman, 1982). Yeast glycosidases are now becoming the focus point for the characterization and use of heterologous expression in other strains, as they mostly exhibit more favourable characteristics. These β-glucosidases are among those that have been most intensively studied for 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).

In this study we have subcloned and expressed two β-glucosidase genes from *Saccharomycopsis fibuligera*, *BGL1* and *BGL2* (Machida et al., 1988), in a commercial starter culture strain of the yeast *Saccharomyces cerevisiae*, VIN13. The aim of the study was to ferment muscat wine, as well as another cultivar with little varietal flavour, to determine whether the expression of these genes and the extracellular production of their encoded β-glucosidases would modify the flavour of the wine by increasing the released terpenol fraction. Here we present the results of the expression of these enzymes in wine yeast in the laboratory as well as during winemaking.

MATERIALS AND METHODS

Microbial strains and plasmids. The bacterial and yeast strains used, as well as the plasmids created and used in this study, are listed with their sources and relevant genotypes in Table 1. The β-glucosidase genes (*BGL1* and *BGL2*) were cloned previously (Van Rensburg et al., 1998). Bacterial strains were grown at 37°C and yeast strains at 30°C.

Media and screening procedures. Transformants of *Escherichia coli* were grown and maintained on Luria Bertani broth and agar (Sambrook et al., 1989), supplemented with 100 µg/ml ampicillin (Sigma) to support selective pressure. Yeast cells (both Σ1278 and VIN13) were grown in rich YPD media (1% yeast extract, 2% glucose and 2% peptone). Laboratory yeast strain Σ1278 was transformed using the lithium acetate method (Gietz and Schiestl, 1991) and transformants were plated on SC^{-Ura} / SC^{-Leu} media (containing 1%

yeast nitrogen base without amino acids, 2% glucose, 2% agar and all the required growth factors lacking uracil or leucin). The industrial yeast strain, VIN13, was transformed via electroporation 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 sulfometuron methyl (SMM) (Dupont, USA), from 10 µg/ml to 100 µg/ml in 10 µg/ml increments]. The yeast transformants were then restreaked on fresh SD-plates containing 100 µg/ml SMM, and then inoculated in test tubes containing 10 ml YPD cultures. Extracellular enzyme assays were performed on these cultures.

Table 1. Microbial strains and plasmids used in this study.

Strain / Plasmid	Relative genotype	Source /Reference
Escherichia coli strain:		
DH5α	supE44 ΔlacU169 (∅80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Sambrook et al., 1989
Saccharomyces cerevisiae strains:		
Σ1278	MATα/MATα ura3/ura3	Radcliffe et al., 1997
Y294	α 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., 2001
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

Construction of the genomic library. Total genomic DNA from *S. fibuligera* carrying the β-glucosidase gene was isolated and prepared as described by Yamashita et al. (1985). The genomic DNA was partially digested with the restriction endonuclease *Sau*3A. DNA fragments larger than 5 kb were recovered and inserted in the *Bam*HI site of the *E. coli-S. cerevisiae* shuttle plasmid (YEp13) by ligation. The resulting recombinant DNA

molecules were used to transform *E. coli* (Ausubel et al., 1987) to ampicillin resistance (Ap^R). Approximately 20 000 Ap^R clones were obtained 90% of which were tetracycline sensitive (Tc^S). Selection for plasmids containing the *BGL2* gene was carried out in *S. cerevisiae* Y294. The plasmid carrying the *BGL2* gene (pBGL) was characterized and its DNA insert sequenced by the dideoxy chain-terminating method (Sanger et al., 1977) for double-stranded plasmid templates using a sequence kit (United States Biochemical Corporation, Cleveland, OH, U.S.A.). PSFB219 was originally constructed by combining the pUC19 vector with the cloned *BGL2* gene, under the control of its native promoter, from *S. fibuligera*.

Recombinant DNA and genetic techniques. DNA fragments were manipulated and subcloned by standard molecular cloning techniques. Standard methods, namely agarose gel electrophoresis and Southern hybridizations (Ausubel et al., 1987; Sambrook et al., 1989) were also applied for the transformation of *E. coli*. The restriction endonucleases and T$_4$ DNA ligase were obtained from Boehringer Mannheim, and the shrimp alkaline phosphatase from Amersham Life Science. These enzymes were used according to the suppliers' specifications and recommendations.

Yeast transformations. Transformation of the *S. cerevisiae* strain VIN13 was done by electroporation using the EasyjecT Electroporator at 1300 V, 25µF and 321 Ω. Strain Σ1278 was transformed using the lithium acetate method, as mentioned previously. The presence of the constructed plasmids was verified by restreaking recombinant colonies on SD-plates containing 100 µg/ml SMM. Strong growth (large colonies after overnight incubation at 30°C) was indicative of a positive transformation.

Assay for β-glucosidase activity. β-Glucosidase activity of the yeast strains was measured as follows: VIN13 and Σ1278 transformants were grown in 10 ml of non-selective YPD media on a rotator machine at 30°C for a total of 96 h. Assays were performed for four consecutive days at 24-h intervals. Activity was determined by mixing samples of the culture supernatant (195 µl) with 20 µl of 0.05 M sodium acetate buffer, as well as 2 µl of 0.2 M ρ-nitrophenyl-β-D-glucopyranoside (ρNPG) (supplied by Sigma, St. Louis, MO, USA). Incubation took place at 42°C for 3 h, and then the reaction was stopped by the addition of 500 µl 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°C to 60°C, and then incubated for another hour with the sodium acetate buffer (pH

4.5) and $pNPG$ 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°C) for a period of 1 h. The activity at certain pH values (pH 2 to 8) at 50°C was determined in the same way with the sodium acetate buffer adjusted. Plate assays were also performed. Colonies were spotted on SD-plates supplemented with $pNPG$ and grown overnight at 30°C. 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.

Winemaking. Wine was made from juice derived from three grape cultivars, namely Chenin blanc, Gewürtztraminer and Pinotage. A total of 60 kg of grapes of each of the two white varieties was destemmed and crushed and then pressed. Overnight settling with the addition of 30 mg/L 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 culture flasks of YPD 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 diammonium phosphate (DAP) was added to the must to adjust the nitrogen concentration. The yeast was also inoculated into the must to a final concentration of 1 x 10^6 cells/ml 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 stabilized. Upon completion of fermentation, the wines were racked off their lees and then left at -4°C to settle and undergo tartrate stabilization, after which the wines were filtered and bottled. Of the red variety, namely 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 stabilization. After this period the wines were filtered and bottled. SO_2 was added for all the wines as prescribed, before tartrate stabilization and again at bottling.

Chemical composition. The levels of ethanol (% v/v), reducing sugar, pH, titratable acidity, volatile acidity, malic acid and lactic acid in the finished wines were determined using the methods described by Iland et al. (2000). The values obtained were confirmed using the Foss Wine Scan (Wine Scan Technologies, Denmark).

Colour/browning. The colour of the red wine was measured by absorbance at 420 nm and 520 nm (Zoecklein et al., 1990). Samples were membrane filtered (0.22 μm) 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.

Phenolics. The phenol content of both the red and white wines was measured by absorbance at 280 nm (Zoecklein et al., 1990). Samples were prepared as for the colour measurements.

Gas Chromatography

Apparatus: 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 μm film thickness. Chromatographic conditions: carrier gas, He; head pressure, 140 kPa; flow, 12.5 ml/min; purge flow, 7.0 ml/min; 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, then to 160°C at 5°C/min and then to 220°C at 10°C/min and held for 10 min; make-up gas N_2 at 30 ml/min; detector FID, H_2 at 40 ml/min; air, 450 ml/min; injected volume, 2 μl.

Method: Introduce 10 ml of wine into the extraction tube. Add 200 μl of Freon 113 (1,1,2-Trichloro-1,2,2-trifluoroethane, obtained from Aldrich) as extracting agent and 2 μl of a solution of 2,6-dimethylheptenol (400 mg/l in ethanol as internal standard), as well as 1.2 g of NaCl. Cap the tubes and shake for 30 min in an automatic shaker at maximum speed. Centrifuge tubes (5 min @ 3000 rpm) and recover the organic phase with a pasteur pipette, transfer it over 50 mg of Na_2SO4 into a HP 2 ml vial with a 200 μl glass insert, and analyze 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; pH 3.2) with an alcohol content similar to that of the analyzed wine.

RESULTS

Construction of recombinant E. coli-yeast shuttle plasmids

The plasmid pBGL1 was constructed to create a plasmid containing both a β-glucosidase gene and a selectable marker gene, *SMR1-410*. This was done by linearizing plasmid pSFB15 with *Hind*III. pDLG31 was also digested with *Hind*III, resulting in two linear fragments of DNA, one containing the *SMR1-410* gene. This fragment of DNA was subsequently ligated to the linearized pSFB15, resulting in plasmid pBGL1. Plasmid pSFB15 is a recombinant YIp5 shuttle vector containing the *BGL1* gene under the control of its native promoter, originally cloned from *S. fibuligera* in this laboratory. Plasmid pBGL2 was constructed by using a previously constructed plasmid, pSFB219, to subclone the *SMR1-410* gene into it. The pSFB219 plasmid was linearized with *Bam*HI. The *SMR1-410* gene was isolated from pDLG31 by digestion with the same enzyme and then ligated to the linearized pSFB219. This resulted in the shuttle plasmid designated pBGL2. These shuttle plasmids are illustrated in Figure 1. The native secretion signal was also used for both cloned genes.
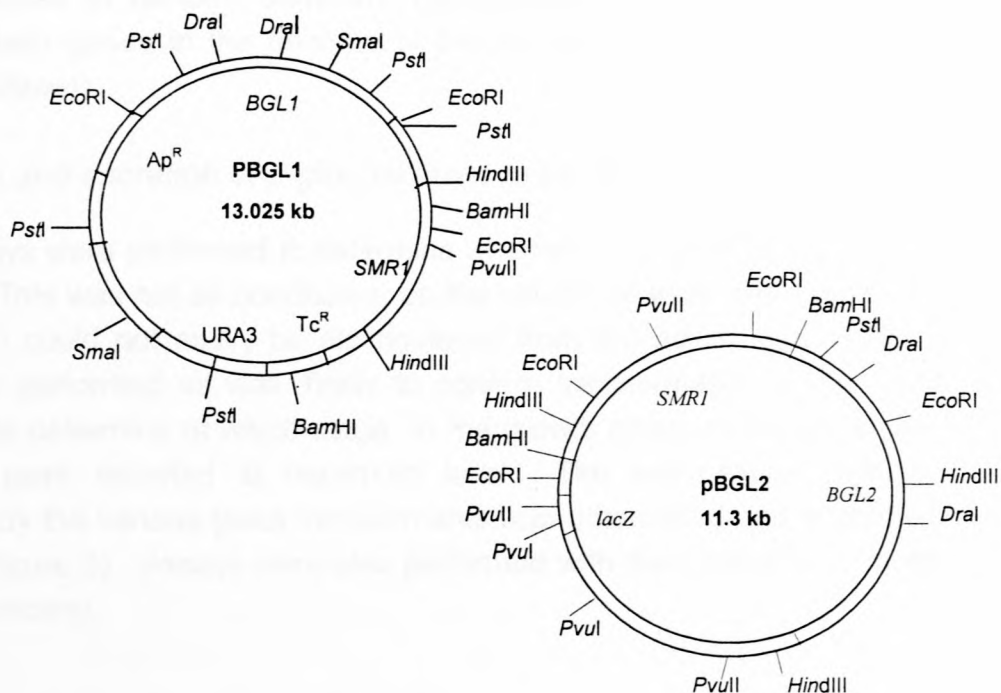

Figure 1. Schematic representation of the two shuttle plasmids containing the *BGL1* and *BGL2* genes.

The *SMR1* gene is used as a "natural" selectable marker, as it originated from a natural 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 Σ1278

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 utilizing the subcloned *SMR1-410* gene. As this gene confers resistance to the herbicide sulfometuron methyl (SMM), SD-plates containing SMM were used for selection. Transformants were restreaked on SD plates containing 100 μg/ml SMM, and if these showed growth, preliminary enzyme assays were performed to confirm that the enzyme had been expressed effectively.

Laboratory strain Σ1278 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 hybridizations. This revealed the presence of the β-glucosidase genes in the genome of the transformed yeast strains VIN13 and Σ1278 (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 (Figure 2). Assays were also performed with the intracellular fraction of the yeast (data not shown).

Properties of the expressed β-glucosidases

When strain Σ1278 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 thinner cell wall than the 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 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 enzyme was taking place and in what quantities. This could be accomplished, as the amount of ρ-nitrophenol released could be determined per million cells per time unit, in this case 180 min.
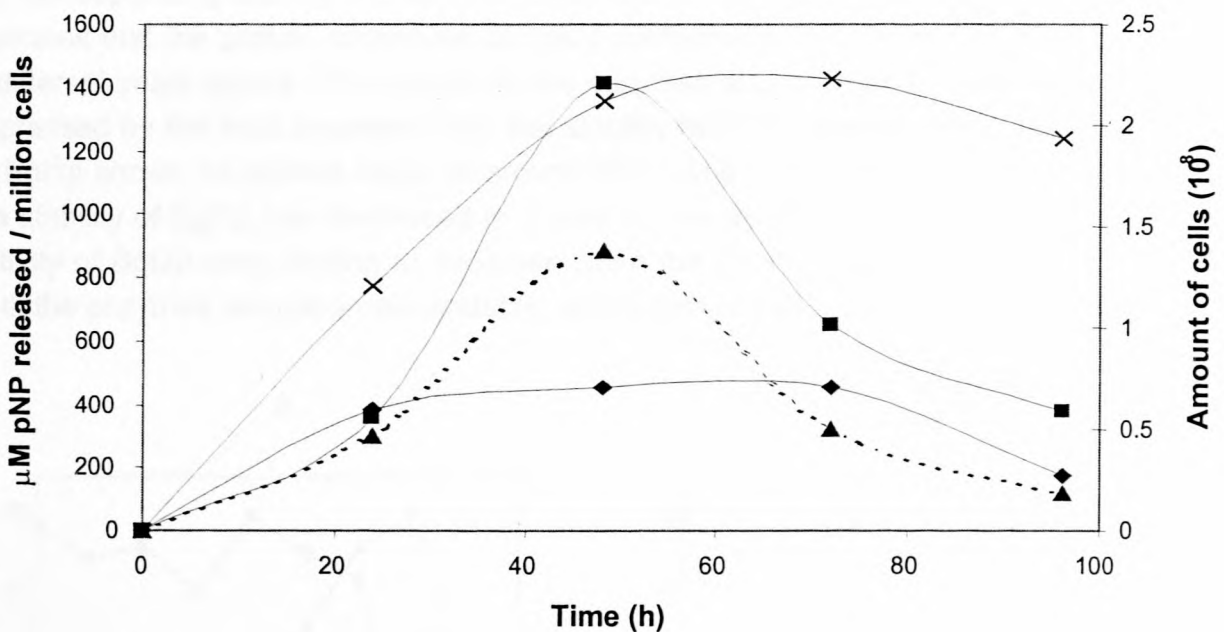

Figure 2.   This graph illustrates the β-glucosidase activity produced by the *S. cerevisiae* transformants. The Bgl1p values are indicated by (■) and the Bgl2p values by (▲). (♦) Represents the control, non-transformed VIN13. The amount of ρ-nitrophenol released per million cells during a 96-h period is shown. A mean curve (X), representing the growth of all the strains, is also shown.

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 Figure 2 that both the yeast-derived Bgl1 and Bgl2 enzymes exhibited the highest activity after 48 h of growth at 30°C. This is important, as this is when the cell growth 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 Figure 3. Both the enzymes showed a gradual increase in activity up to a temperature of 50°C, and the activity then decreased sharply to levels under 15% at higher temperatures. This trend is consistent with the corresponding activity in their host organism, *S. fibuligera* (Machida et al., 1988). This indicates that the protein conserves its basic conformation, even though it is expressed by a different yeast specie. The stability of the enzymes also showed the same trend as when expressed by the host organism, with the stability high at lower temperatures. The stability of Bgl1p shows an optimal range at around 45°C, while that of Bgl2p is at 50°C. At 50°C, the stability of Bgl1p has decreased to a level as low as 20% of the relative value, with the activity of Bgl2p only starting to decrease when the temperature exceeds 50°C. At 60°C, both the enzymes are extremely unstable, with activity evels under 10% being observed.
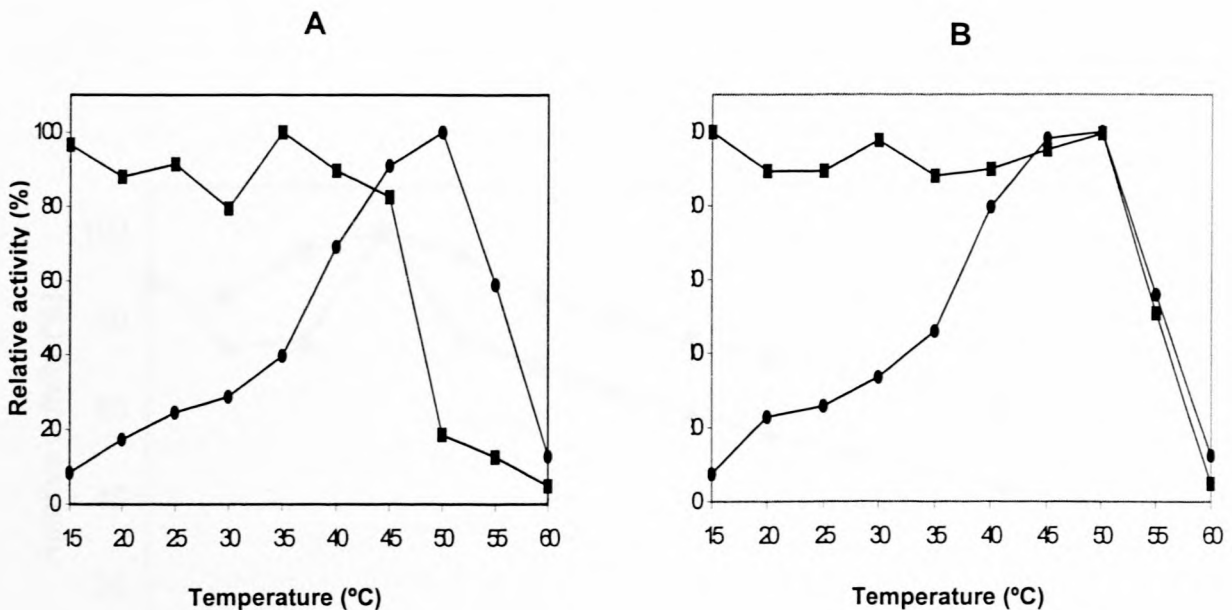

Figure 3. The activity (•) and stability (■) of Bgl1p (Panel A) and Bgl2p (Panel B) at certain temperatures.

The activity of the enzymes at certain pH values is presented in Figure 4. Both *S. cerevisiae*-derived enzymes seem to be relatively stable at low pH values, and this is contradictory to what was observed by Machida et al. (1988) when they characterized 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 pNP 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 pNP would be released at a lower pH value. The same is true for the temperature assays, which were done at 42°C where the activity was only 70% of the maximum. The assays were therefore repeated, this time at a higher temperature (45°C), the stability at 50°C taken into account, but no substantially higher values were recorded.
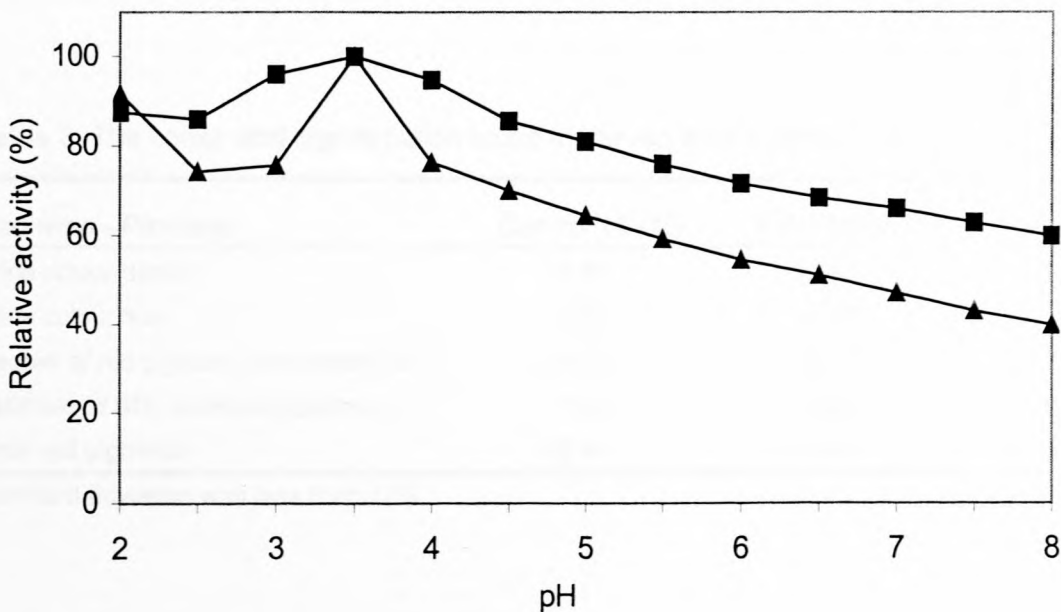

Figure 4. The activity of Bgl1p (■) and Bgl2p ( ) at certain pH values.

Expression of the β-glucosidase enzymes in wine

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

Table 2. Analysis of wines. All values are given as g/l, except ethanol (%v/v).

Sample	Ethanol (%v/v)	Residual sugar	pH	Volatile acidity	Titratable acidity	Malic acid	Lactic acid
Pinotage C	16.56	0	3.94	0.37	5.21	3.62	0
Pinotage B1	16.05	0.24	3.91	0.37	5.29	2.86	0.14
Pinotage B2	16.24	0	4.05	0.37	5.23	3.46	0
Gewürtz C	15.02	0.11	3.61	0.31	4.23	1.98	0.25
Gewürtz B1	15.28	0.51	3.63	0.23	4.01	2.08	0.18
Gewürtz B2	15.26	0.52	3.63	0.23	4.01	2.02	0.18
Chenin bl C	14.51	0.57	3.18	0.22	5.52	2.49	0.22
Chenin bl B1	14.50	0.52	3.17	0.24	5.56	2.57	0.16
Chenin bl B2	14.60	0.52	3.17	0.23	5.59	2.60	0.17

C denotes the control, B1 the VIN13[*BGL1*] strain, and B2 the VIN13[*BGL2*] strain.

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
Gewürztraminer	2.12	2.10	2.09
Estimate of brown pigments			
Chenin blanc	0.05	0.05	0.05
Gewürztraminer	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.

Total 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
- Gewürztraminer	1.16	0.96	0.85

Standard deviation was less than 12 %.

Table 6. The volatile components as found by microextration of Pinotage wines. All values are in mg/l, unless otherwise specified.

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. nd – not detectable.

† The ratio of the values obtained for the control VIN13 and the respective transformant strains, VIN13[*BGL1*] and VIN13[*BGL2*].

Table 7. The volatile components as found by microextration of Gewürtztraminer wines. All values are in mg/l, unless otherwise specified.

Volatile components-Gewürtztraminer	VIN13	VIN13[BGL1]	† [BGL1]/VIN13	VIN13[BGL2]	† [BGL2]/VIN13
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 µg/l.

† The ratio of the values obtained for the control VIN13 and the respective transformant strains, VIN13[BGL1] and VIN13[BGL2].

DISCUSSION

There is much contradictory data available in the literature on the occurrence and activity of β-glucosidase genes in *S. cerevisiae*. It was previously determined 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 Stefano (1997) revealed that wine yeasts had some constitutive β-glucosidase activity, but that this enzyme activity was still inhibited under winemaking conditions. There also is another group of enzymes, called exo-glucanases, that are produced by *S. cerevisiae* and show activity against the assay substrate, ρ-nitrophenyl-β-D-glucopyranoside (Kuranda and Robbins, 1987). These exo-glucanases are also glycosides, but the main difference between these enzymes and the exogenous enzymes that we introduced into *S. cerevisiae* is substrate specificity. The exo-(1→3)-β-glucanases act at non-reducing ends of the cell wall polysaccharide, (1→3)-β-D-glucan,

releasing glucose. This group of enzymes thus plays a nutritional role in fungi and yeasts. In *S. cerevisiae*, this group of enzymes 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 β-1,6-linkages. However, it 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 that both the transformants show far greater activity than the control, as the values presented consist of both the exo-glucanase and β-glucosidase 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 ρNP 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 waySuch influence may 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 yeast 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 enzymes, which took place during the exponential growth phase of the yeast cells. Activity thereafter started to decline as cells entered a stationary phase. Activity was very low when the dying phase was reached after 96 h. This indicates that residual activity and stability of the enzymes in the growth medium are not very high. The production of the enzymes did not affect the growth of the cells and showed no detrimental effect towards normal cell propagation.

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°C). The pH profiles of the two enzymes also differed from those observed in the original study. We found that the enzymes were surprisingly active at low pH values, which is in complete 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 enzymes expressed 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 influence the activity of the enzymes.

According to Machida et al. (1988) the two β-glucosidases encoded by *BGL1* and *BGL2* respectively have different substrate preferences. They stated that the Bgl1 enzyme hydrolyzed cellobiose and cellooligosaccharides more efficiently, whereas the *BGL2-*

encoded enzyme showed an affinity for aryl- and alkyl-β-D-glucosides. The *BGL1*-encoded enzyme was less active on the synthetic substrate ρNPG (43.3 U/mg of protein released) than the *BGL2*-encoded β-glucosidase (168 U/mg of protein released). We found that the *BGL1*-encoded β-glucosidase released just as much, if not more, ρNP than the *BGL2*-encoded β-glucosidase.

The next step was to test these transformed wine yeasts in small-scale wine fermentations. The results are shown in Tables 2 to 7. The fermentations were performed in duplicate for the white wines. 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 show no significant differences between the wines made with the genetically altered yeast and the wine made with the control yeast. This leads us to assume that the enzyme expressed by the yeast shows puts so little metabolic burden on the yeast as to be of little or no effect. This is because the fermentations, as well as 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. The wines do, however, differ in their respective densities, with the control having the most intense colour. This may 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 severed, 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 usualy 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 modified wine yeasts. In general, however, the browning seems 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 and the experimental wines, 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. One prime example of this is the increase in the concentration of some esters found in the wines made with the VIN13[*BGL1*] and VIN13[*BGL2*] yeasts. Each of these esters impart a different aroma to the wine. For example, hexanoic, octanoic and decanoic acid ethyleseters 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 thus be postulated that the two β-glucosidase enzymes may play a role during ester synthesis in the yeast. It may 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 may also be possible that the substrate specificity of the enzyme as expressed in its new host may have been altered and could have become more suitable for substrate hydrolysis in the ester formation biosynthetic pathways.

Conclusion

From the results it is possible to deduce that, in an indirect manner, the two β-glucosidase enzymes exhibit activity under wine conditions. They are thus functionally expressed. For future applications, further studies could be undertaken to optimize the results already available. These would include the addition of another enzyme involved in the first step of enzymatic hydrolysis of the glycosidic compounds, such as α-arabinofuranosidase. Engineering a β-glucosidase that is more active at low temperatures would also yield far better results. Studies are underway in our laboratory to continuously select for new β-glucosidase enzymes by screening different microorganisms found in nature.

Acknowledgements

References

Aryan, A.P., Wilson, B., Strauss, C.R., and Williams, P.J. (1987) The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible application in enology. *Am. J. Enol. Vitic.* **38**, 182-188.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) Current protocols in Molecular Biology. John Wiley and Sons, New York.

Casey, G.P., Kiao, W., and Rank, G.H. (1988) A convenient dominant selectable marker for gene transfer in industrial strains of *Saccharomyces* yeast: *SMR1* encoded resistance to the herbicide sulfometuron methyl. *J. Inst. Brew.* **94**, 93-97.

Darriet, P., Boidron, J.N., and Dubordieu, D. (1988) L'hydrolyse des hétérosides terpéniques du Muscat a Petit Grains par les enzymes périplasmiques de *Saccharomyces cerevisiae*. *Conn. Vigne Vin.* **22**,189-195.

Delcroix, A., Günata, Z., Sapis, J., Salmon, J., and Bayonove, C. (1994) Glycosidase activities of three enological yeast strains during winemaking: Effect on the terpenol content of Muscat wine. *Am J. Enol. Vitic.* **45**, 291-296.

Duerksen, J.D., and Halvorson, H. (1958) Purification and properties of an inducible β-glucosidase of yeast. *J. Biol. Chem.* **233**, 1113-1120.

Duerksen, J.D., and Halvorson, H. (1959) The specificity of induction of β-glucosidase in *Saccharomyces cerevisiae*. *Biochem. Biophys. Acta.* **36**, 47-55.

Gietz, R.H., and Schiestl, R.D. (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acid as carrier. *Yeast.* **7**, 253-263.

Gueguen, Y., Chemardin, P., Arnaud, A., and Galzy, P. (1994) Purification and characterization of the endocellular β-glucosidase of a new strain of *Candida entomophila* isolated from fermenting agave (*Agave* sp.) juice. *Biotechnol. Appl. Biochem.* **20**, 185-198.

Gueguen, Y., Chemardin, P., Arnaud, A., and Galzy, P. (1995) Comparative study of extracellular and intracellular β-glucosidases of a new strain of *Zygosaccharomyces bailii* isolated from fermenting agave juice. *J. Appl. Bacteriol.* **78**, 270-280.

Günata, Z., Bayonove, C., Baumes, R., and Cordonnier, R. (1985) The aroma of grapes. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr.* **331**, 83-90.

Günata, Z., Bitteur, S., Brillouet, J.M., Bayonove, C., and Cordonnier, R. (1988) Sequential enzymic hydrolysis of potentially aromatic glycosides from grapes. *Carbohydr. Res.* **184**, 139-149.

Günata, Y. Z., Bayonove, C.L., Cordonnier, R.E., Arnaud, A., and Galzy, P. (1990a) Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamii* β-glucosidases. *J. Sci. Food. Agric.* **50**, 499-506.

Günata, Y.Z., Bayonove, C.L., Tapiero, C., and Cordonnier, R.E. (1990b) Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. *J. Agric. Food. Chem.* **38**, 1232-1236.

Gundllapalli Moses, S.B., Cordero-Otero, R.R., La Grange, D.C., Pretorius, I.S., and Van Rensburg, P. (2001) The influence of different genetic backgrounds on amylase production. *Biotechnol. Lett.* (submitted).

Haisman, D.R., and Knight, D.J. (1967) The enzymatic hydrolysis of amygdalin. *Biochem J.* **103**, 528.

Iland, P., Ewart, A., Sitters, J., Markides, A., and Bruer, N. (2000) Techniques for chemical and quality monitoring during winemaking. Patrick Iland Wine Promotions, Compbeltown.

Kuranda, M.J., and Robbins, P.W. (1987) Cloning and heterologous expression of glycosidase genes from *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA.* **84**, 2585-2589.

Machida, M., Ohtsuki, I., Fukui, S., and Yamashita, I. (1988) Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular β-glucosidases as expressed in *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **54**, 3147-3155.

Manners, D.J., Masson, A.J., and Patterson, J.C. (1973) The structure of a β-(1-3)-D-glucan from yeast cell walls. *Biochem. J.*, **135**, 19-30.

Mateo, J.J., and Di Stefano, R. (1997) Description of the β-glucosidase activity of wine yeasts. *Food Microbiol.* **14**, 583-591.

Radcliffe, P.A., Binley, K.M., Trevethick, J., Hall, M., and Sudbery, P.E. (1997) Filamentous growth of the budding yeast *Saccharomyces cerevisiae* induced by overexpression of the *WHI2* gene. *Microbiol.* **143**, 1867-1876.

Raynal, A., and Guerinaeu, M. (1984) Cloning and expression of the structural gene for β-glucosidase of *Kluyveromyces fragilis* in *Escherichia coli* and *Saccharomyces cerevisiae. Mol. Gen. Genet.* **195**, 108-115.

Riou, C., Salmon, J-M., Vallier, M-J., Gunata, Z., and Barre, P. (1998) Purification, characterization, and substrate specificity of a novel highly glucose tolerant β-glucosidase from *Aspergillus oryzae. Appl. Environ. Microbiol.* **64**, 3607-3614.

Rosi, I., Vinella, M., and Domizio, P. (1994) Characterization of β-glucosidase activity in yeasts of oenological origin. *J. Appl. Bacteriol.* **77**, 519-527.

Saha, B.C., and Bothast, R.J. (1996) Production, purification, and characterization of a highly glucose-tolerant novel β-glucosidase from *Candida peltata. Appl. Environ. Microbiol.* **62**, 3165-3170.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular cloning. A laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Skory, C.D., Freer, S.N., and Bothast, R.J. (1996) Properties of an intracellular β-glucosidase from the cellobiose-fermenting yeast *Candida wickerhamii. Appl. Microbiol. Biotechnol.* **46**, 353-359.

Van Rensburg, P., Van Zyl, W.H., and Pretorius, I.S. (1998) Engineering yeast for efficient cellulose degradation. *Yeast* **14**: 67-76.

Voirin, S.G., Baumes, R.L., Sapis, J.C., and Bayonove, C.L. (1992) Analytical methods for monoterpene glycosides in grape and wine. II. Qualitative and quantitative determination of monoterpene glycosides in grape. *J. Chromatogr.* **595**, 269-281.

Vrhovsek, U., Wendelin, S., and Eder, R. (1997) Effects of various vinification techniques on the concentration of *cis*- and *trans*-resveratrol and resveratrol isomers in wine. *Am. J. Enol. Vitic.* **48**, 214-219.

Williams, P.J., Strauss, C.R., Wilson, B., and Massy-Westropp, R.A. (1982) Novel monoterpene disaccharide glycoside of *Vitis vinifera* grapes and wines. *Phytochem.* **21**, 2013-2020.

Woodward, J., and Wiseman, A. (1982) Fungal and other β-glucosidases – their properties and applications. *Enzyme Microb. Technol.* **4**, 73-79.

Yan, T-R., and Lin, C-L. (1997) Purification and characterization of a glucose tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. *Biosci. Biotechnol. Biochem.* **61**, 965-970.

Yamashita, I., Itoh, T., and Fukui, S. (1985) Cloning and expression of *Saccharomycopsis fibuligera* glycoamylase gene in *Saccharomyces cerevisiae*. *Appl. Microb. Biotech.* **23**, 130-133.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., and Nury, F.S. (1994) *Wine analysis and production*. Chapman & Hall, New York.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

1.   Perspectives

Wine is probably one of the most complex beverages known to man. Nowhere else is there such a wide spectrum of flavours in one defined substance. All these flavours stem from a complex, completely non-linear system of interactions between hundreds of compounds originating from the grapes, the fermentation aroma and the aging bouquet. Aroma is generally the collective term for what is perceived as the smell, or *bouquet* of the wine. In wine this cannot be attributed only to a single, or even a few aromatic compounds. Indeed, the aroma of wine is a cumulative result of absolute amounts and specific ratios of many interactive compounds, rather than being attributable to a single "impact" compound (Noble, 1994; Cole and Noble, 1995).

It is claimed that yeast and fermentation conditions are the most important factors influencing the flavours in wine. This is because the principal part of the flavour of wine is derived from the secondary metabolites of the grapes. These compounds undergo various changes in biosynthetic pathways. Fermentation increases the chemical and flavour complexity of wine by assisting in the extraction of compounds from solids present in grape must, modifying some grape derived compounds, and producing a substantial amount of yeast metabolites (Schreier, 1979; Rapp and Versini, 1991).

However, a high percentage of these metabolites occur as their respective, non-volatile *O*-glycosides. Several studies have shown that increased enzymatic hydrolysis of aroma precursors present in grape juice can liberate the aglycone to intensify the varietal character of wines (Canal-Llaubères, 1993). An example of this is the grape monoterpene alcohols that naturally occur as glycosidically bound structures in grapes. There are enzymes, such as β-glucosidases, that are able to cleave these glycosidically bound aroma precursors. Some of these enzymes are not active in the wine environment, as they are inhibited by the low pH and high glucose and ethanol levels. An example of these are the indigenous grape glycosidases (Canal-Llaubères, 1993). In contrast, the β-glucosidases of *Aspergillus* and other fungal species are mostly tolerant of the pH, glucose and ethanol levels that prevail in wine. These enzymes have been developed and incorporated as components of commercially available enzyme preparations, which are added to fermented juice as soon as the glucose is depleted. They can also be added to young wines (Canal-Llaubères, 1993). The use of these enzyme preparations, however, can be a very costly practice, especially in large cellars. The addition of the preparations also has the disadvantage of being viewed by purists as an "unnatural" or "artificial" intervention by winemakers. This has led to renewed interest in wine yeast starter cultures that are able to produce and secrete "robust" β-glucosidases during wine fermentations,

thereby liberating aroma compounds without the addition of exogenous enzyme preparations.

Most yeast β-glucosidases are active under wine conditions, i.e. low pH and high glucose and ethanol levels. Examples of sources of these enzymes include *Candida, Debaryomyces, Dekkera* and *Hanseniaspora* species. Since most starter cultures of *Saccharomyces cerevisiae* do not express β-glucosidases, the functional expression of a foreign β-glucosidase by *S. cerevisiae* would provide an alternative to enzyme preparations. To this end we have expressed the two β-glucosidase genes (*BGL1* and *BGL2*) from *Saccharomycopsis fibuligera* (Van Rensburg et al., 1998) in the VIN13 starter culture strain.

Chapter 1 of this thesis elaborates in more detail on wine aroma and the important role played by monoterpene alcohols in the varietal flavour of some wines. A small fraction of monoterpene alcohols found in wine and juices is free, and therefore volatile. As mentioned before, however, a larger fraction is bound to saccharide moieties in the wine, rendering them non-volatile and odourless (Günata et al., 1985; Voirin et al., 1992; Williams et al., 1982). This chapter explores the possibility of releasing these bound monoterpene alcohols and thus increasing the volatile flavour of the wine through enzymatic hydrolysis, versus the practices of acidic hydrolysis and enzyme preparations (Günata et al., 1988). It is also mentioned that other glycosidically bound compounds, such as other aroma compounds (Winterhalter and Skouroumounis, 1997), colour pigments (Wightman and Wrolstad, 1995) and resveratrol (Vrhovsek et al., 1997), could also be liberated by enzymatic hydrolysis. This would increase the aroma even more, as well as increasing the colour intensity and health benefits of red wines.

The first section of Chapter 2 focuses in even greater depth on the aroma of wine, distinguishing between the precursor compound from which it originates (Rapp and Mandery, 1986). The first is the aroma of the grapes itself, and also includes the aroma changes due to modifications during the processing of the grapes. The second is the aroma produced during the fermentation process, and the third is the bouquet that results from the transformation of the aroma compounds during aging, whether it is in the bottle or in wooden casks. Again, emphasis is placed on the importance of monoterpene compounds and the role they play in the aroma of wine. It includes discussion on how volatile flavour in the wine could be increased, whether it is through acid or enzymatic hydrolysis. The advantages and disadvantages of both methods are also discussed.

The second part of Chapter 2 introduces properties and characteristics of the glycosidic enzymes. Contradictory data on *S. cerevisiae* β-glucosidases are discussed, as well as the possibility of introducing exogenous glycosidases to the genome of this organism to release the full aroma potential of the wine. Two glycosidases, β-glucosidase and α-L-arabinofuranosidase, are discussed in depth. Not all β-glucosidases and

α-L-arabinofuranosidases are suitable for releasing aroma compounds from their glycones in the wine environment (Aryan et al., 1987; Woodward and Wiseman, 1982; Günata et al., 1990). As mentioned before, inhibiting factors such as low pH and low temperatures, as well as an increasing ethanol content and high glucose concentrations, prevail in wine, and these factors play an enormous role in the selection of glycosidases that have to display sufficient activity under these circumstances. Various β-glucosidases and α-L-arabinofuranosidases from a variety of sources are discussed and evaluated according to these criteria. Commercial enzyme preparations are also described and assessed.

The last section of this chapter discusses results obtained from the heterologous expression of heterologous glycosidases in *S. cerevisiae*. In this section, it was determined that three major factors had an influence on the heterologous expression of β-glucosidase genes: the genotypic stability, the gene copy number, and strain ploidy (Adam et al., 1995). The instability of phenotypes conveyed by genes cloned into plasmids is solved by homologous recombination at specific genomic sites. Usually, integration of a DNA fragment into the yeast genome occurs in single copy, although it has been observed that several tandem repeats can be found at the location of integration (Orr-Weaver et al., 1981; Orr-Weaver and Szostak, 1983; Rothstein, 1991). Increasing the stability of a gene by means of its integration inevitably means that the level of its expression is reduced. Good results were obtained, with *S. cerevisiae* efficiently expressing a β-glucosidase from *Bacillus polymyxa*. Crous et al. (1996) also expressed the α-L-arabino-furanosidase gene (*ABF2*) of *Aspergillus niger* in *S. cerevisiae*. The recombinant enzyme was secreted into the surrounding medium, even though a dipeptide sequence was absent. It was postulated that the enzyme was probably still being secreted due to the high degree of hydrophobic residues on the first section of the Abf2 protein. This again emphasizes the ability of *S. cerevisiae* to act as vehicle for heterologous gene expression for biotechnological applications.

Chapter 3 describes the subcloning and characterization of two β-glucosidase genes from *Saccharomycopsis fibuligera*, *BGL1* and *BGL2* (Machida et al., 1988), into yeast integration vectors, with the dominant selectable marker *SMR1* (Casey et al., 1988). These plasmids were designated pBGL1 (containing the *BGL1* gene) and pBGL2 (with the *BGL2* gene) respectively. The two genes differ in respect of their substrate specificity, with *BGL1* preferring cellobiose as substrate, and *BGL2* rather acting on alkyl-and aryl-β-D-glucosides.

These plasmids were then transformed into a laboratory strain of *S. cerevisiae* (Σ1278), as well as into an industrial wine strain, VIN13. The genes were first expressed in the laboratory strain to confirm expression of *S. fubiligera BGL1* and *BGL2* genes, the synthesis of the encoded proteins and the secretion of the corresponding biologically active β-glucosidase activities. Expression of the enzyme in VIN13 was also tested and enzyme assays were performed with the synthetic substrate ρ-nitrophenyl-β-D-glucopyranoside

(ρNPG). The results obtained showed that the two β-glucosidase enzymes were correctly synthesized and expressed. Small-scale fermentations were also set up with the transformed wine yeast strains, and the wines analysed. As could be seen, the β-glucosidase enzymes did not have a significant influence on the monoterpene content of the wines, but they did increase the ester content to a large extent. In addition, the wine colour density of the red wines was influenced, with the wines made with the genetically altered yeast showing less dense colouring. It was postulated that the enzymes might be able to cleave the glucose units bonded to the anthocyanins, making them less stable and more susceptible to decolouration by SO_2. The phenolics show the same pattern among the wines.

From the data presented in this chapter it therefore can be stated that two transformant strains of the wine yeast strain of *S. cerevisiae*, VIN13, now exists and are able to secrete biologically active β-glucosidase enzymes. In view of increasing the aroma potential of wine, this can be seen as an important step towards development of a wine yeast strain that can be used in normal fermentations to increase the aroma of wine. This would also be advantageous in view of cutting the costs of supplementing wines and juices with costly non-specific enzyme preparations.

Future work would entail the addition of another glycosidic enzyme, such as α-L-arabinofuranosidase, to the genome of the wine yeast to aid the further breakdown of glycosidic bonds. The cloning or engineering of a β-glucosidase enzyme that is more active at low temperatures would also yield better results and release even more of the aroma of the wine.

2.   REFERENCES

Adam, A. C., Rubio-Texeira, M., and Polaina, J. (1995) Induced expression of bacterial β-glucosidase activity in *Saccharomyces*. *Yeast* **11**, 395-406.

Aryan, A. P., Wilson, B., Strauss, C.R., and Williams, P.J. (1987) The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible application in enology. *Am J. Enol. Vatic.* **38**, 182-188.

Canal-Laubères, R-M. (1993) Enzymes in Winemaking. In: *Wine Microbiology and Biotechnology*. (Ed G.H. Fleet) pp 447-506. Harwood Academic Publishers, Switserland.

Casey, G.P., Kiao, W., and Rank, G.H. (1988) A convenient dominant selectable marker for gene transfer in industrial strains of *Saccharomyces* yeast: *SMR1* encoded resistance to the herbicide sulfometuron methyl. *J. Inst. Brew.* **94**, 93-97.

Cole, V.C. and Noble, A.C. (1995). Flavor chemistry and assessment. In: *Fermented Beverage Production*. (Eds. A.G.H. Lea, J.R. Piggot) pp 361-385. Blackie Academic and Proffesional: London.

Crous, J.M., Pretorius, I.S., and Van Zyl, W.H. (1996) Cloning and expression of the α-L-arabinofuranosidase gene (*ABF2*) of *Aspergillus niger* in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **46**, 256-260.

Günata, Z., Bayonove, C., Baumes, R., and Cordonnier, R. (1985) The aroma of grapes. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr.* **331**, 83-90.

Günata, Z., Bitteur, S., Brillouet, J.M., Bayonove, C., and Cordonnier, R. (1988) Sequential enzymic hydrolysis of potentially aromatic glycosides from grape. *Carbohydr. Res.* **184**, 139-149.

Günata, Y.Z., Bayonove, C.L., Tapiero, C., and Cordonnier, R.E. (1990) Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. *J. Agric. Food. Chem.* **38**, 1232-1236.

Machida, M., Ohtsuki, I., Fukui, S., and Amashita, I. (1988) Nucleotide sequences of *Saccharomycopsis fibuligera* genes for extracellular β-glucosidases as expressed in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **54**, 3147-3155.

Noble, A.C. (1994) Wine flavour. In: *Understanding Natural Flavours*. (Eds. J.R. Piggott, A. Patterson) pp 228-242. Blackie Academic and Proffessional: Glasgow.

Orr-Weaver, T.L., and Szostak, J.W. (1983) Multiple tandem plasmid integration in *S. cerevisiae*. *Mol. Cell. Biol.* **3**, 747-749.

Orr-Weaver, T.L., Szostak, J.W., and Rothstein, R.J. (1981) Yeast transformation: A model study for the study of recombination. *Proc. Natl. Acad. Sci. USA*. **78**, 6354-6358.

Rapp, A. and Mandery, H. (1986) Wine Aroma. *Experimentia*. **42**, 873-884.

Rapp, A. and Versini, G. (1991) Influence of nitrogen compounds in grapes on aroma compounds of wine. In: *Proceedings of the International Symposium on nitrogen in grapes and wines*. (Ed. Rantz) pp 156-164. American Society for Enology and Viticulture.

Romanos, M.A., Scorer, C.A., and Clare, J.J. (1992) Foreign gene expression in yeast: a review. *Yeast*. **8**, 423-488.

Rothstein, R. (1991) Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast. *Meth. Enzymol.* **194**, 281-301.

Schreier, P. (1979) Flavour composition of wines: a review. *CRC. Food Sci. Nutr.* **12**, 59-111.

Van Rensburg, P., Van Zyl, W.H. and Pretorius, I.S. (1998) Engineering yeast for efficient cellulose degradation. *Yeast* **14**: 67-76.

Voirin, S.G., Baumes, R.L., Sapis, J.C., and Bayonove, C.L. (1992) Analytical methods for monoterpene glycosidases in grape and wine. II. Qualitative and quantitative determination of monoterpene glycosides in grape. *J. Chromatogr.* **595**, 269-281.

Vrhovsek, U., Wendelin, S., and Eder, R. (1997) Effects of various vinification techniques on the concentration of *cis*-and *trans*-resveratrol and resveratrol glucoside isomers in wine. *Am. J. Enol. Vatic.* **48**, 214-219.

Wightman, J.D., and Wrolstad, R.E. (1995) Anthocyanin analysis as a measure of glycosidase activity in enzymes for juice processing. *J. Food. Sci.* **60**, 862-867.

Williams, P.J., Strauss, C.R., Wilson, B., and Massy-Westropp, R.A. (1982a) Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochem.* **21**, 2013-2010.

Winterhalter, P., and Skouroumounis, G.K. (1997) Glycoconjugated aroma compounds: occurrence, role and biotechnological transformation. *Adv. Biochem. Eng. Biotech.* **55**, 73-105.

Woodward, J., and Wiseman, A. (1982) Fungal and other β-glucosidases – their properties and applications. *Enzyme Microb. Technol.* **4**, 73-79.