Co-expression of aroma liberating enzymes in a wine yeast strain

by

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Summary

Monoterpenes are important aroma compounds in certain grape varieties such as Muscat, Gewürztraminer and Riesling and are present as either odourless, glycosidically bound complexes or as free aromatic monoterpenes. These complexes occur as monoglucosides or, when present as diglycosides, most commonly as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides of mainly linalool, geraniol, nerol and citronellol.

The release of monoterpenes from non-volatile glycosidically bound precursors occurs either by acid hydrolysis or enzymatic hydrolysis. High temperature acid hydrolysis causes a rearrangement of the monoterpene aglycones and a decrease in the aroma and changes in the aromatic characteristics of monoterpenes and is therefore not suitable. Enzymatic hydrolysis does not modify the monoterpene aglycones and can be an efficent method to release potentially volatile monoterpenes.

α-L-arabinofuranosidase and β-glucosidase are important enzymes responsible for the liberation of monoterpene alcohols from their glycosides. Glycosidases from *Vitis vinifera* and *Saccharomyces cerevisiae* are severely inhibited by winemaking conditions and this leads to unutilized aroma potential, while commercial preparations of aroma liberating enzymes are crude extracts that often have unwanted and unpredictable side effects. It is therefore of interest to investigate alternative measures to release glycosidically bound monoterpenes to increase the floral aroma of wine without side activities that impact negatively on wine.

Heterologous α-L-arabinofuranosidases and β-glucosidases have previously been expressed in *S. cerevisiae* and these studies have evaluated and found increased glycosidic activities against both natural and synthetic substrates.

In this study, we expressed the *Aspergillus awamori* α-L-arabinofuranosidase (*AwAbfB*) in combination with either the β-glucosidases *Bgl2 f*rom *Saccharomycopsis fibuligera* or the *BglA* from *Aspergillus kawachii* in the industrial yeast strain *S. cerevisiae* VIN13 to facilitate the sequential enzymatic hydrolysis of monoterpene diglycosides. Enzyme assays and GC-FID (Gas Chromatography with a Flame Ionization Detector) results show a significant increase in the amount of free monoterpene concentrations under winemaking conditions in the strain co-expressing the *AwAbfB* and the *Bgl2*. The increases in free monoterpene levels obtained were similar to those obtained with a commercial enzyme preparation, LAFAZYM AROM. Sensorial evaluation confirmed the improvement in the wine aroma profile, particularly the floral character. This yeast strain permits a single culture fermentation which improves the sensorial quality and complexity of wine. Further investigations on the factors influencing the stability and reactivity of monoterpenes during alcoholic fermentation are needed.

Opsomming

Monoterpene speel ‘n belangrike rol in die aroma van sekere wyndruifkultivars soos Muskaat, Gewürztraminer en Riesling en kom voor as reuklose, glikosidies gebonde verbindings of as vry aromatiese monoterpene. Hierdie verbindings bestaan in die vorm van monoglukosiede of as diglikosiedies gebonde monoterpene. Die meeste diglikosiedies gebonde monoterpene kom voor as 6-*O*-α-L-arabinofuranosiel-β-D-glukopiranosiede van hoofsaaklik linalool, geraniol, nerol en sitronellol.

Die vrystelling van monoterpene vanaf nie-vlugtige glikosiedies gebonde voorlopers geskied deur middel van ‘n suurhidroliese of ‘n ensiematiese hidroliese. Die vrystelling van monoterpene deur middel van suurhidroliese veroorsaak veranderinge in die monoterpene asook veranderinge in die aromatiese eienskappe van die monoterpene en dit lei tot ‘n afname in die aroma van monoterpene en is as gevolg hiervan nie geskik vir die vrystelling van glikosidies gebonde monoterpene nie. Die ensiematiese hidroliese lei nie tot veranderinge in die monoterpene nie en kan ‘n effektiewe metode wees om potensieël vlugtige monoterpene vry te stel.

α-L-arabinofuranosidase en β-glukosidase is belangrike ensieme wat monoterpeen alkohole vrystel van hul glikosiedies gebonde voorloper molekules. Die glikosidases van *Vitis vinifera* en *Saccharomyces cerevisiae* word sterk geïnhibeer onder wynmaaktoestande en dit lei tot onbenutte aroma potensiaal, terwyl kommersieële aromavrystellings-ensiembereidings uit kru ekstrakte bestaan wat oor ongewenste en onvoorspelbare newe-effekte beskik. Dit is om hierdie rede van belang om alternatiewe maniere te vind om glikosidies gebonde monoterpene vry te stel ten einde die blom en vrugtige aroma te vermeerder sonder newe-effekte wat die wyn negatief kan beïnvloed.

Heterologiese α-L-arabinofuranosidases and β-glukosidases is al van tevore in *S. cerevisiae* gekloneer en in hierdie studies is verhoogde glikosidiese aktiwiteit aangeteken teenoor beide natuurlike en sintetiese substrate.

In die huidige studie is die *Aspergillus awamori* α-L-arabinofuranosidase (*AwAbfB*) in kombinasie met een van die β-glukosidases *Bgl2* vanaf *Saccharomycopsis fibuligera* òf die *BglA* vanaf *Aspergillus kawachii* in die industriële gisras *S. cerevisiae* VIN13 uitgedruk om die sekwensiële ensiematiese hidroliese van diglikosidies gebonde monoterpene te fasiliteer. Ensiemtoetse en GC-VIO (Gas Chromatografie met ‘n Vlam Ionisasie Opnemer) resultate dui merkwaardige verhogings aan in die vlakke van vry monoterpeen konsentrasies onder wynmaaktoestande in ‘n gisras wat die *AwAbfB* tesame met die *Bgl2* uitdruk. Die verhoging in vry monoterpeenvlakke is gelykstaande aan wat verkry is met ‘n kommersiële ensiempreparaat, LAFAZYM AROM. Sensoriese evaluering het die verbetering in die aromatiese profiel, veral van die blomkarakter van die wyn bevestig. Hierdie gisras stel enkelkultuurfermentasies in staat wat die sensoriese kwaliteit en die kompleksiteit van wyn kan verbeter. Verdere navorsing is nodig om die stabiliteit en reaktiwiteit van monoterpene tydens alkoholiese fermentasie beter te verstaan.

This thesis is dedicated to my parents Daniël and Helena de Klerk, Dirk, Lana, Dian, Frits, Charné, Sarah and the love of my life, Meaghan Kok.

Biographical sketch

Daniël de Klerk was born in Pretoria, South Africa on 25 April 1982. He matriculated at Die Hoërskool Menlopark in 2000. Daniël enrolled at the University of Pretoria in 2001 and obtained a BSc degree in Biotechnology in 2004. In 2005 he enrolled at the University of Stellenbosch and obtained a BScHons degree in Wine Biotechnology in 2005.

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Preface

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

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Liberation of monoterpenes in wine by α-L-arabinofuranosidase and β-glucosidase.

Chapter 3 **Research results**
Co-expression of α-L-arabinofuranosidase and β-glucosidase in *Saccharomyces cerevisiae*

Chapter 4 **General discussion and conclusions**

Contents

Chapter 1

Introduction and project aims

1.1 INTRODUCTION

Monoterpenes play a fundamental role in the aroma of especially white wine varieties and a supplemental role in the aroma of red wines. However, many wines lack sufficient varietal aroma because a large proportion of monoterpenes are glycosidically conjugated in young wines; a condition in which monoterpenes do not contribute to the aroma of a wine. The result of this glycoconjugation is unutilized aroma and flavour potential (Esteve-Zarzoso *et al.*, 1998; Manzanares *et al.*, 2003). This implies that an increase in the content of monoterpenes with pleasant floral notes might enhance the characteristic aroma of certain white grape varieties.

Glycoconjugated monoterpenes can occur as monosaccharide glycosides, wherein the sugar moiety consists of a β-D-glucose unit, and as disaccharide glycosides, wherein the monosaccharide glycoside is further supplemented with a second sugar unit, usually an α-L-arabinofuranoside (Günata *et al.*, 1988). Monoterpenes at the oxidation state of linalool are mainly present in the form of disaccharide conjugates in Muscat grape juice (Reynolds and Wardle, 1989). The level of glycoconjugation also varies greatly between vintages, classes of volatiles, and between individual metabolites (Reynolds and Wardle, 1989; Sefton *et al.*, 1994).

Monoterpenes can be liberated from their glycosides by the action of glycoside hydrolases α-L-arabinofuranosidase and β-D-glucosidase. β-glucosidases that are capable of monoterpene release from monoglucosides do not have endoglucanase activity; therefore disaccharides can not be released with the action of only β-glucosidase. Hydrolysis of grape monoterpenyl disaccharide-glycosides takes place sequentially (Günata *et al.*, 1988). First, the (1→6) linkage is cleaved by either α-L-arabinofuranosidase or α-L-rhamnosidase, resulting in the release of arabinose or rhamnose, respectively, and the corresponding monoterpenyl β-D-glucoside. Subsequently, the action of a β-D-glucosidase liberates a monoterpenol and β-D-glucose.

The β-D-glucosidase produced by commercial yeast strains has poor activity towards these monoterpene glycosides, and yeasts do not produce α-L-arabinofuranosidase. The α-L-arabinofuranosidases produced by some fungi, and β-D-glucosidases produced by some fungi and non-*Saccharomyces* yeasts have strong activity towards monoterpene glycosides. Introduction of these heterologous genes for α-L-arabinofuranosidase and β-D-glucosidase into *Saccharomyces cerevisiae* could consequently enhance the liberation of monoterpenes from their glycosidic precursors to increase the aroma of wine.

The main aim of this study is the development of a wine yeast strain capable of expression and secretion of heterologous α-L-arabinofuranosidase and β-D-glucosidase during fermentation of grape must to release monoterpenes from their glycosylated forms. Wines produced with this yeast should contain increased levels of free monoterpenes and a pronounced floral and fruit aroma character.

1.2 AIMS

The specific aims of this study were:

- construction of single-copy yeast integration vectors containing expression cassettes of the α-L-arabinofuranosidase and β-glucosidases under control of constitutive promoters and terminators.
- transformation of all constructs into an industrial wine yeast strain: *Saccharomyces cerevisiae* VIN13
- confirmation of integration and expression of heterologous genes
- evaluation of α-L-arabinofuranosidase and β-glucosidase activity
- evaluation of the effect of α-L-arabinofuranosidase and β-glucosidase activity on the release of glycosidically bound precursors during and after fermentation

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Chapter 2

Literature review

Liberation of monoterpenes in wine by α-L-arabinofuranosidase and β-glucosidase.

2. Liberation of monoterpenes in wine by α-L-arabinofuranosidase and β-glucosidase

2.1 Introduction

Wine is a complex beverage containing numerous compounds responsible for the wide range of different flavours and aromas. Although yeast is credited to contribute the most to the overall wine aroma (Nykänen, 1986), the typical varietal flavor of wines is mainly due to volatile aromatic compounds that come from the grape (Cabaroglu *et al.*, *2003*). In aromatic white grape varieties, for example Muscat, Gewürztraminer and Riesling, monoterpenes are key aromatic compounds influencing the typical varietal character of these fruit and their respective wines. Aromatic descriptors for monoterpenes include rose, violet, geranium and fruit.

In grapes, musts and wines, these aromatic compounds essentially exist in two forms: volatile and odorous, a case in which they contribute to the aroma of the wine, or glycosidically bonded, a situation where they do not contribute to the aroma of the wine, but are considered to be aroma precursors that, once liberated, can contribute to the aroma (Günata *et al.*, 1986; Williams *et al.*, 1982). These complexes most often occur as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides of mainly linalool, geraniol, nerol, α-terpineol, citronellol and hotrienol (Marais, 1983; Mateo and Jiménez, 2000; Williams *et al.*, 1982). In the production of wine, glycosidases are involved in the release of monoterpenes from their conjugated forms. α-L-arabinofuranosidase and β-glucosidase are the two foremost glycosidases concerned with the liberation of grape aroma precursors.

2.2 The flavour and aroma of wine

The flavour of wines is due to the presence of a diverse amount of grape derived as well as yeast metabolism products. Some of the grape derived volatile precursors include monoterpenes, norisoprenoids and thiols. Sensorially significant volatiles derived from yeast metabolism are the esters, alcohols and acetates.

Wine quality is determined by a complex balance of all the wine aroma components (Marais, 1983). Although the aroma of wine is formed primarily by fermentation, formation of varietal aroma compounds requires the presence of certain non-floral precursors that are converted to aroma-active compounds (Hernández-Orte *et al.*, 2008). The relative combinations of these compounds define the unique varietal characteristics of a wine, rather than a single odourant, although some compounds have more impact than others. These aroma compounds can complement one another because perception threshold of a compound within this mixture is often lower than it might be as an individual component (Ribéreau-Gayon *et al.*, 1998).

Many wines lack sufficient varietal aroma because a large proportion of glycosides are still present in young wines. The result is that a large fraction of the monoterpene alcohols in wine

remain bound to glycosides resulting in unutilized aroma and flavour potential (Esteve-Zarzoso *et al.*, 1998; Manzanares, *et al.*, 2003).

A deduction from these observations is that an increase in the content of monoterpenes with pleasant floral notes might enhance the characteristic aroma of certain white grape varieties, and as a consequence, processes which might enhance the amount of the bound fraction in the must or favour the liberation of volatile components from this bound fraction are of major interest.

Mainly the effects of enzymatic release of glycosidically bound monoterpenes that influence wine aroma, are considered in this review. Therefore, other aromatic compounds that occur in wine such as esters and fusel alcohols that are formed by yeast metabolism are not discussed further.

2.3 Glycosidically bound aroma precursors

Glycosylated precursors can occur as monosaccharide glycosides, wherein the sugar moiety consists of a β-D-glucose unit, and as disaccharide glycosides, wherein the monosaccharide glycoside is further supplemented with a second sugar entity, usually an α-L-arabinofuranoside, α-L-rhamnopyranoside, or β-D-apiofuranoside (Günata *et al.*, 1988). Günata and colleagues have shown that grapes contain arabinofuranosyl- and rhamnopyranosyl-glucosides and that monoterpenyl β-D-glucosides are mostly bound to arabinose by a ratio of 3 to 1 compared to rhamnose. Monoterpenes at the oxidation state of linalool are mainly present in the form of disaccharide conjugates in Muscat grape juice while monoterpenes at the higher oxidation state are primarily present as monoglucosides (Reynolds and Wardle, 1989). This observation explains why the extent of hydrolysis of bound terpenols varies with the nature of the aglycone (Günata *et al.*, 1986). The level of glycoconjugation also varies greatly between vintages, classes of volatiles, and between individual metabolites (Reynolds and Wardle, 1989; Sefton *et al.*, 1994).

Monoterpenes are essentially present as either free or glycosidically conjugated precursors. The free fraction can be divided further into two groups: free monoterpenes and oxides or polyhydroxylated forms of free monoterpenes, or free odourless polyols (Mateo and Jiménez, 2000). The monoterpene polyols are highly reactive intermediate forms of free monoterpenols formed by hydrolysis of their conjugated precursors (Williams *et al.*, 1981). The free odourless polyols do not contribute directly to the aroma of grapes and wine, but they can be broken down to form volatile compounds such as hotrienol and nerol oxide (Williams *et al.*, 1980).

The amount of conjugated volatile secondary metabolites can be more than 95% (Sefton *et al.*, 1996). Monoterpenes account for about 80% of the total amount of aglycons in highly aromatic white grape varieties, compared to only 10% in red grape varieties where shikimate derivatives are dominant (Wirth *et al.*, 2001). Although there are many other aglycones that are volatile when free, like aliphatic alcohols, alkyl phenols, norisoprenoids, resveratrol and sesquiterpenoids (Arévalo Villena *et al.*, 2007; Bloem *et al.*, 2008; Whiton and Zoecklein, 2002),

this review focuses on the monoterpene alcohols and glycosides, which play a fundamental role in the aroma of especially white wine varieties. Based on the aforementioned, the collection of glycoconjugates partially represents the potential aroma of most white grape varieties (Zoecklein *et al.*, 1997a).

The majority of the free monoterpenes in wine are formed by hydrolysis of the conjugated monoterpene precursors (Williams *et al.*, 1981). A common shortcoming in many vinifications is that the low levels of activity exhibited by glycosidases from *Vitis vinifera* and *Saccharomyces cerevisiae* under winemaking conditions result in a considerable proportion of glycosides still being present in young wines, and these glycosides have the capability to greatly enhance wine aroma (Palmeri and Spagna, 2007).

It is well known that the odor-active aroma molecule is not always formed by hydrolysis, but by chemical rearrangement of different precursors, some of which require previous hydrolysis, as observed for C_{13}-norisoprenoidic aglycons (Baumes *et al.*, 2002). C_{13}-norisoprenoids are also influential in the aroma of wine. C_{13}-norisoprenoids are secondary metabolites that are formed in the grape berry and a large proportion accumulates as non-volatile glycosides (Swiegers *et al.*, 2005). β-Damascenone, β-ionone and α-ionone are some examples that are present in most grape varieties.

A variety of glycosyltransferases catalyze the formation of glycosylated compounds. The multiple glycosyltransferases show high specificities for the sugar-acceptor substrate and the position of glycosyl addition (Ford and Høj, 1998). Glycosylation of compounds results in enhanced water solubility and lower chemical reactivity (Sarry and Günata, 2004). This is demonstrated by the fact that free monoterpenes are soluble in organic solvents whereas glycosidically bound precursors are water-soluble (Williams *et al.*, 1981). Glycosylation might lead to distribution of glycosides throughout the berry. After synthesis in the hypodermal cells of fruit (Williams *et al.*, 1985), geraniol is either stored in the berries or transported in glycosylated form to the leaves, where the total concentration of monoterpene glycosides can be between 2 to 5 times higher than in the berries (Wirth *et al.*, 2001). Glycosylated compounds are therefore regarded as transportable storage compounds or detoxification products that have little or no physiological activity (Sarry and Günata, 2004). The prevalence of glycosylated secondary metabolites, including flavonols, anthocyanins, monoterpenes, norisoprenoidic compounds and plant hormones illustrates that both glycoside hydrolases and glycoside transferases that are responsible for their metabolism play a central role in a large number of major biological processes.

2.4 Monoterpenes

Monoterpenes are widespread aroma compounds that are responsible for the characteristic fragrance of many fruits, flowers, leaves and wood. Unlike many other wine aroma compounds, monoterpenes are primarily derived from the grapes. Some monoterpenes, particularly geraniol, nerol and linalool are responsible for the most significant influence on the overall aroma and

flavour of the berry due to their low perception threshold and relatively significant concentrations (see table 2.1) (Wilson *et al.*, 1986).

Monoterpenes can be classified according to one of three oxidation levels:

1. the lowest oxidation state in which citronellol is the sole member.
2. the linalool oxidation state which includes the largest group of grape monoterpenes (linalool, α-terpineol, geraniol, nerol).
3. the hotrienol oxidation state which includes the linalool oxides, nerol oxide, hotrienol and the anhydrofuran linalool oxides (Williams *et al.*, 1981).

Citronellol levels are not enhanced by hydrolysis (Williams *et al.*, 1981). This suggests that citronellol is probably a conversion product of other monoterpenes. Monoterpenes at the oxidation state of linalool are generally direct products of their monoterpene precursors, and in some cases they are descendent from an enediol. Monoterpenes at the oxidation state of hotrienol, excluding pyran linalool oxides, are products from monoterpene polyols. Significant changes in the types and proportions of monoterpenes in wine occur during processing and ageing. Examples include linalool which is readily oxidised into linalool oxide and geraniol and nerol which can be transformed into α-terpineol (Ribéreau-Gayon *et al.*, 1975). α-terpineol and its glycosides are only minor components of all grape varieties and levels of this monoterpene increase during and after fermentation (Wilson *et al.*, 1986).

These chemical changes that individual monoterpenes undergo during processing or storage of wine are responsible for the decrease in aroma of some wines (Ribéreau-Gayon *et al.*, 1975). Indeed, monoterpenes have varying characteristics with regards to their stability, water solubility, volatility and odour threshold (Williams *et al.*, 1981).

2.5 Role of monoterpenes in wine aroma

Although monoterpenes are responsible for the characteristic aroma of aromatic wines like Muscat, Weisser Riesling, Scheurebe, Gewürztraminer and Bukettraube, they also play a supplemental role in the aroma of non-aromatic red and white wines, albeit to a lesser extent mainly due to their low concentration in these wines (Boido *et al.*, 2003; Marais, 1983; Sefton *et al.*, 1994).

The most prominent terpene compounds occuring generally and in high concentrations in Muscat and aroma related grapes and wines are the highly aromatic, acyclic monoterpenes linalool, geraniol, nerol and citronellol (Marais, 1983). Except for α-terpineol, monocyclic monoterpenes have limited sensory impact (Sefton *et al.*, 1994).

Despite the fact that monoterpenes are present in high concentrations in Muscat grapes and wine, they do not produce a Muscat character but they contribute a floral character to the wine and have a synergistic effect on the aroma of white wine varieties. Ethyl-cinnamate and

β-ionone are compounds that is largely responsible for the typical aroma of Muscat wine (Etievant *et al.*, 1983). Due to the strong influence that monoterpenes have on the varietal aroma of wine, it is a determining factor in the perceived quality of many wines.

2.5.1 Perception threshold of monoterpenes.

Of the various volatile compounds present in wine, only a subset contributes to the wine flavour (Francis and Newton, 2005). Determination of the perception thresholds of compounds provides insight on the significance of their contribution towards the wine flavour. However, as remarked by Francis and Newton, (2005), the establishment of aroma thresholds is subject to a degree of uncertainty, and threshold values in the published literature have been determined using different methods with different degrees of exactitude and in diverse matrices. It is nonetheless useful to compare the different matrices and threshold values in order to obtain approximate thresholds for individual compounds, to evaluate their relative effects on aroma and to gain information regarding the stability and overall activity of monoterpenes in different matrices.

The wine matrix can have a significant influence on the perception of some compounds to such an extent that compounds that are present at levels above the critical value are not distinguished sensorially (Cabaroglu *et al.*, 2003). Conversely, flavour compounds that are present below their perception threshold value can sometimes become detectible if they are present in a mixture due to interactions between these volatile components (Francis and Newton, 2005). When terpenes are present in a mixture, they can react with each other which can lead to an increase in aroma. In such cases, the mixture is more aromatic than the most aromatic compound in that mixture (Ribéreau-Gayon *et al.*, 1975). An additional effect of these synergistic interactions is the masking of other aromas (Francis and Newton, 2005). An increase in one aroma often leads to a decrease in the perception of another, and since monoterpenes contribute positively to the aroma of wine, it often masks negative odours.

Geraniol and linalool are considered to be the most important monoterpenes, both due to their high concentrations and low perception thresholds (Ribéreau-Gayon *et al.*, 1975; Sánchez Palomo *et al.*, 2007). Geraniol and linalool have similar aromatic strengths and are present at similar levels in Muscat grapes whereas nerol and terpineol have perception thresholds that are four times higher. The terpene oxides have perception thresholds that are 30 to 60 times higher than that of linalool (Ribéreau-Gayon *et al.*, 1975).

Monoterpene glycosides contribute almost nothing to the sensory character of a wine and are basically odourless. In terms of taste, the monoterpene glycosides are almost tasteless at normal thresholds in wine, presenting only a faint floral and fruity character. At ten times the concentration normally found in wine, a faint bitterness can be observed (Noble *et al.*, 1988). The concentrations in grape juice and wine, as well as perception thresholds of the sensorially most significant monoterpenes are summarized in Table 2.1.

Table 2.1 Concentrations and perception thresholds of monoterpenes

Monoterpene	Concentration in grape juice (µg/l)	Concentration in wine (µg/l)	Perception threshold in water/sugar (µg/l)	Perception threshold in 10% ethanol (µg/l)
Linalool	0.6[e]-1056[a]	2.0[e]-219[e]	100[a] 50[b]	15[d]
Geraniol	2.5[e]-1059[a]	13.2[e]-51.1[e]	132[a]	30[d]
Citronellol	1.1[e]-9.3[a]	7.7[e]-42.2[e]	18[b]	100[d]
Nerol	0[e]-447[a]	7.8[e]-46.3[e]	400[a]	
α-terpineol	0.2[e]-145[a]	8.2[e]-23.2[e]	500[a]	
Total	5[e]-500[c]-600[c]			

[a]Ribéreau-Gayon *et al.*, 1975. Muscat grape juice.
[b]Ribéreau-Gayon *et al.*, 2000.
[c]Reynolds and Wardle, 1989. Sauvignon blanc juice.
[d]Guth, 1997.
[e]Castro-Vázquez *et al.*, 2002. Various wines.

2.5.2 Aroma properties of monoterpenes.

For the most part, monoterpenes have a floral aroma and contribute to the floral and fruity aroma of wine to different degrees. Some monoterpenes are dominant in certain grape varieties, for example geraniol is an important monoterpene in Gewürztraminer aroma and is present at high levels in both glycosidically bound and free forms, while linalool is less abundant in Gewürztraminer (Ong and Acree, 1999; Vaudano *et al.*, 2004; Wilson *et al.*, 1986). Geraniol has a geranium or rose-like aroma, α-terpineol a sweet, lilac type scent, and linalool a muscaty, iris-like aroma (Baek *et al.*, 1997). Other monoterpenes, for instance citronellol with its citrus aroma are sometimes only present in low concentrations, yet they still have an influence on the aroma of the wine due to their supporting role and synergystic action with other monoterpenes (Reynolds and Wardle, 1989).

These variations in monoterpene levels are characteristic of the different grape varieties that rely on monoterpenes as their major aroma contributors, for example Gewürztraminer and Muscat grapes are dependant on the monoterpenes for their aroma (Aryan *et al.*, 1987). The linalool oxides and monoterpene polyols have high threshold values and are almost odourless (Wilson *et al.*, 1986).

2.6 Monoterpene synthesis and function

Monoterpenoids are produced by higher plants, algae and fungi, from the common precursor geranyl pyrophosphate (GPP) (King and Dickinson, 2000). Synthesis of monoterpenes, particularly linalool, proceeds via the action of monoterpene synthases and continues past veraison until the berries are mature (Baumes *et al.*, 2002). *V. vinifera* undoubtedly contributes the majority of monoterpenes found in wine. The contribution of monoterpenes synthesized by some *S. cerevisiae* strains to the final monoterpene concentration is small when compared to acid hydrolysis of monoterpene precursors present in the must (Ugliano *et al.*, 2006).

2.6.1 Function of monoterpenes.

Monoterpenes have a wide range of functions not yet completely studied and understood. Some functions might simply be based on the aroma properties of monoterpenes, for example the scent of flowers, in order to attract pollinating insects. Certain bark beetle genera are capable of biosynthesizing monoterpenes that function in intraspecific chemical communication as aggregation and dispersion pheromones. The release of a monoterpenic aggregation pheromone facilitates host colonization and mating (Gilg *et al.*, 2005). Monoterpenes from plants might play a similar role in affecting insect behaviour.

Monoterpenes can also function as a defence mechanism. They have been reported to be cytotoxic compounds and thus been used as antifungal drugs since ancient times (Oswald *et al.*, 2007). Geraniol and linalool have been shown to have antimicrobial activity against bacteria and fungi (Pattnaik *et al.*, 1997). More specifically, geraniol was found to inhibit growth of *Candida* and *Saccharomyces* strains by enhancement of the rate of potassium leakage out of whole cells and was also shown to increase membrane fluidity (Bard *et al.*, 1988; Chambon *et al.*, 1990). The minimum inhibitory concentration of geraniol towards yeast is 2-3 mg/l, far in excess of concentrations normally found in wine (Bard *et al.*, 1988). In another study, King and Dickinson, (2000) demonstrated that terpenoids do not affect the growth of yeast at a concentration of 25 mg/ml. A possible mechanism for yeast resistance to monoterpenes is the amplification of extrusion proteins belonging to the ATP binding cassette (ABC) superfamily (Oswald *et al.*, 2007). The presence of geraniol does not appear to inhibit ergosterol biosynthesis (Bard *et al*., 1988). An interesting property of geraniol with potential medical application is antitumor activity against murine Leukemia cells (Crowell, 1999).

2.6.2 Synthesis and location of monoterpenes

2.6.2.1 *V. vinifera*

The more odourant monoterpenes of *V. vinifera* (linalool, nerol, and geraniol) are present at higher levels in berries than in leaves (Wirth *et al.*, 2001). Monoterpene concentrations are also higher in the skins of the berries than in the juice (Castro Vázquez *et al.*, 2002). The distributions of monoterpenes throughout the berry are different for the different types of monoterpenes. For instance, in *V. vinifera*, both free geraniol and nerol, as well as their glycosylated forms are associated with the skins of the berries, whereas free and glycosylated linalool is uniformly distributed between the skins and juice (Williams *et al.*, 1985, Wilson *et al.*, 1986). Free and glycosidically bound α-terpineol is usually only present at low concentrations in the berries (Wilson *et al.*, 1986). Free geraniol occurs at high levels in the skins of *V. vinifera* berries, suggesting that hypodermal cells of the fruit are sites of biosynthesis and/or storage of this compound. Although free monoterpenes are compartmentalized in different parts of the berry, glycosylation leads to a widespread distribution of monoterpenes throughout the berry.

As mentioned above, free monoterpenes are present at higher concentrations in the skins of the berries, with the exception of linalool. Although this might suggest that increased skin

contact will improve the extraction of monoterpenes from the skins, a collateral effect will be the excessive extraction of undesirable compounds such as tannins and other compounds that are sensitive to autooxidative breakdown that have a detrimental effect on the flavour and aroma of the juice and wine (Wilson *et al.*, 1986). The presence of monoterpene glycosides away from the epidermis highlights the importance to investigate and pursue processes other than skin contact for aroma enhancement.

The mevalonate-independent 1-deoxy-D-xylulose 5-phosphate/2C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway is the dominant metabolic route for monoterpene biosynthesis in the grape berry exocarp and mesocarp and in grape leaves (Luan and Wüst, 2002). The MEP pathway (Figure 2.1), located in the chloroplast, is responsible for the formation of volatile monoterpenes in plants by provision of isopentenyl pyrophosphate (IPP) precursors for plastidial monoterpene and cytosolic sesquiterpene biosynthesis.

The trafficking of IPP occurs unidirectionally from the plastids to the cytosol in *V. vinifera* (Dudareva *et al.*, 2005). High enzyme activity of certain terpene synthases in aromatic plants could therefore create a shift in part of the metabolic flux toward monoterpenoid production (see Figure 2.1) (Oswald *et al.*, 2007).

The MEP pathway operates in a rhythmic manner controlled by the circadian clock, which in turn influences the production of monoterpenes (Dudareva *et al*., 2005). Monoterpenes are formed directly from Geranyl Pyrophosphate (GPP) by monoterpene synthases produced by *V. vinifera* while monoterpene diols originate from the monoterpene alcohols by hydrolysis or via photo-oxidation (Martin and Bohlmann, 2004, Rapp, 1987).

2.6.2.2 *S. cerevisiae*

In *S. cerevisiae*, GPP occurs exclusively as an intermediate of farnesyl pyrophosphate (FPP) synthesis and there is no monoterpene synthase gene present in the genome of *S. cerevisiae* (Oswald *et al*., 2007). Any monoterpenes that are possibly produced in yeast are therefore products of GPP and FPP phosphatase activities.

Most yeasts do not form monoterpenes under normal fermentation conditions (King and Dickinson, 2000). However, when sterol synthesis is inhibited, some strains are capable of producing small amounts of monoterpenes at concentrations well below threshold. *S. cerevisiae* strains produce only trace amounts of monoterpenes. The use of *S. cerevisiae* mutants blocked in FPP synthetase lead to increased levels of geraniol and farnesol due to synthesis of the phosphorylated forms of these molecules as intermediates of the ergosterol pathway (Chambon *et al.*, 1990). Dephosphorylation of FPP and GPP might involve phosphatase activities, whereafter excretion of geraniol and farnesol could occur via single diffusion due to their hydrophobic character (Chambon *et al.*, 1990; Oswald *et al.*, 2007).

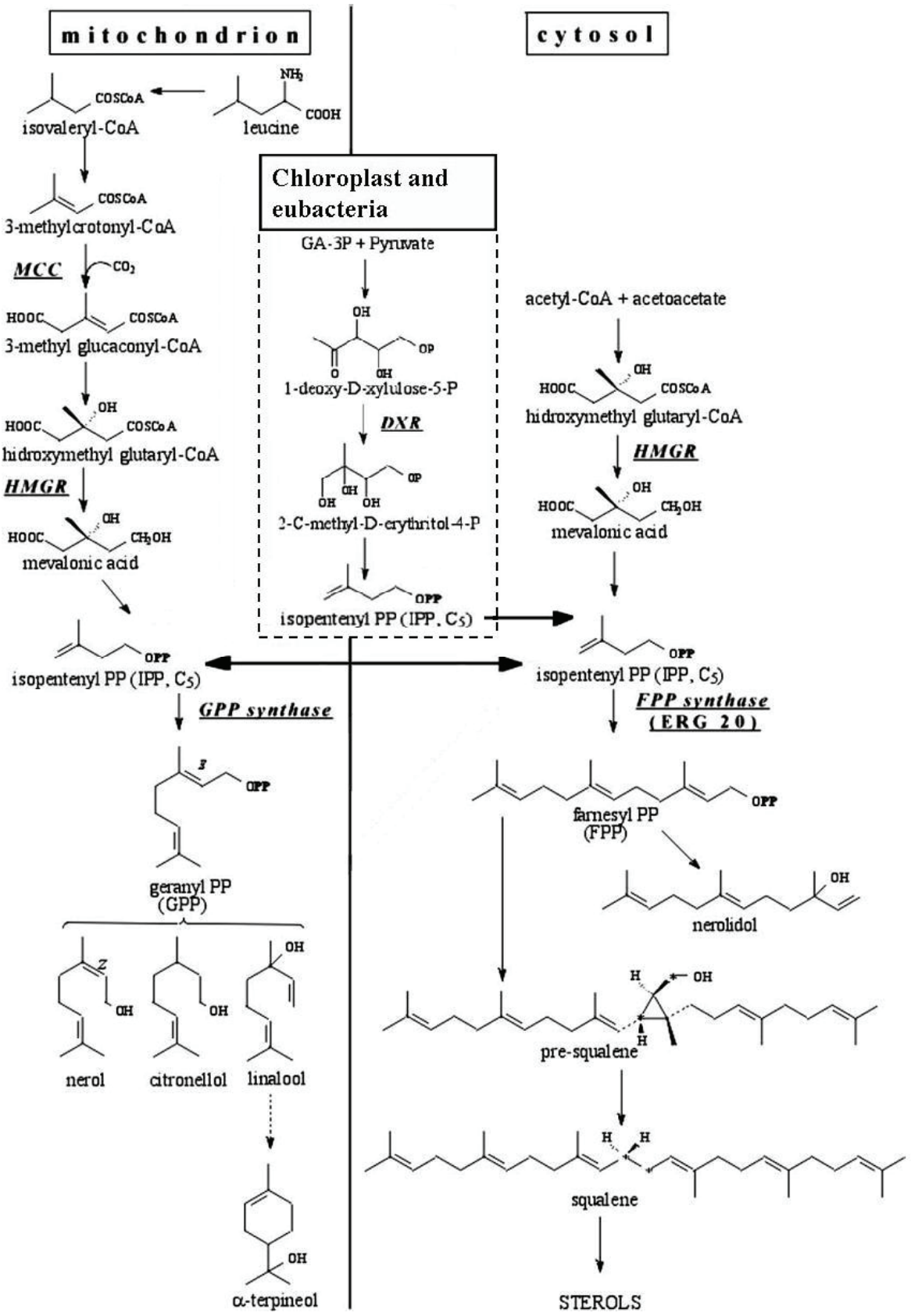

Figure 2.1 Sterol biosynthetic pathways and possible relationships with monoterpene formation in *S. cerevisiae* and *V. vinifera*. (Carrau *et al.*, 2005).

Another study has shown that *S. cerevisiae* is capable of *de novo* synthesis of monoterpenes in the absence of grape derived precursors by an alternative pathway which does not involve the sterol pathway from which sesquiterpenes are derived (Carrau *et al.*, 2005). In this alternative pathway (Figure 2.1), which is located in the mitochondrion, leucine is converted to mevalonic acid. Higher concentrations of free assimilable nitrogen increase the accumulation of linalool and citronellol. This is probably due to increased leucine production which requires the availability of nitrogen and is stimulated by microaerobic conditions. Linalool and α-terpineol are the terpenes produced in greatest abundance by *S. cerevisiae* at levels of up to 5 μg/l. The low amounts of citronellol produced despite the relatively high amount of geraniol could be explained by the inability of some strains to reduce geraniol to citronellol. The trafficking of IPP occurs bidirectionally between the mitochodrion and the cytosol in *S. cerevisiae*.

A few non-*Saccharomyces* yeasts seem to have some ability to synthesize monoterpenes, for example *Kluyveromyces lactis* has been shown to produce the monoterpenes citronellol, linalool and geraniol (Drawert and Barton, 1978). The levels of production are however low, with citronellol and linalool accumulating at about 50 μg/l in culture broth while geraniol is only detected in traces. When geraniol is added to cultures of *K. lactis*, it is quantitatively reduced to citronellol. The addition of asparagine stimulates the increased production of citronellol.

During alcoholic fermentation of must by *S. cerevisiae*, the monoterpenes geraniol, linalool, α-terpineol, and nerolidol have been shown to accumulate intracellularly (Zea *et al.*, 1995). This might be due to inhibited sterol biosynthesis from squalene by anaerobic conditions, leading to intracellular accumulation of intermediate terpenic precursors for squalene that are presumed to undergo cyclizations, isomerizations, and enzymatic conversions to form terpenes that are excreted into the wine (Figure 2.2). Since this study was done in must, it is possible that free monoterpenes might have entered the cell by diffusion.

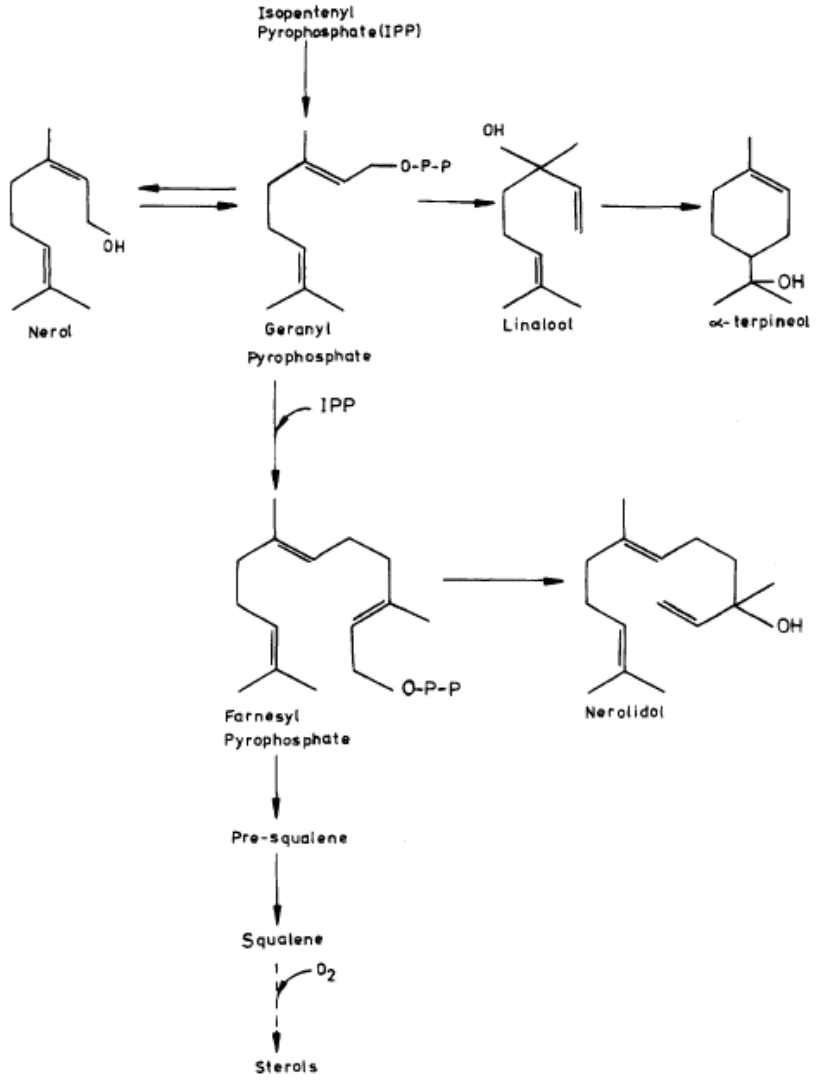

Figure 2.2 Sterol biosynthesis pathway and terpene formation in yeasts (Zea *et al.*, 1995).

Attempts have been made to clone and express monoterpene synthases in wine yeast, for instance the *Clarkia breweri* S-linalool synthase gene has been expressed in *S. cerevisiae* and linalool was efficiently excreted at levels above the perception threshold (Herrero *et al.*, 2008). Although this experiment has shown that a single monoterpene synthase expression does not influence growth rates or result in unforeseen changes in metabolite profiles, the possibility exists that overexpression of monoterpene synthases in *S. cerevisiae* might lead to a redirection of the isoprenoid precursors dimethylallyl diphosphate and isopenthenyl diphosphate towards GPP. This pathway will compete with FPP formation, which is required to produce sterols involved in membrane function. In addition, the accumulation of monoterpenes inside the yeast cell might be toxic to the yeast. Expression of a single monoterpene synthase will increase the levels of only one of the monoterpenes, and as a consequence, the relative balance of monoterpenes that is potentially and naturally present in must and wine might be disturbed. Metabolic engineering of *S. cerevisiae* to produce monoterpenes might be useful for industrial production of monoterpenes, but application in winemaking will require the expression of

several genes to produce different monoterpenes. This might put an excessive metabolic burden on the cell and it will also be difficult to control the production of monoterpenes, leading to a change in the intrinsic character of the wine.

Maximum levels of linalool were already reached within two days of fermentation in the study by Herrero *et al.*, (2008), and levels declined after this, indicating that early production or liberation of monoterpenes could have the disadvantage that monoterpenes are converted, broken down or simply evaporate in early or later stages of fermentation.

In another study, geraniol synthase from *Ocimum basilicum* has been expressed in *S. cerevisiae* which facilitated the excretion of geraniol whereas mutants defective in farnesyl diphosphate synthase excreted similar quantities of geraniol and linalool (Oswald *et al.*, 2007). The expression of geraniol synthase has an impact on the general ergosterol pathway. Geraniol synthesis is increased even more by the use of yeast mutants that are defective in FPP synthase.

Any approach which decreases the production of FPP will have a negative influence on the cell membrane because FPP is the precursor of vital products such as sterols, dolichols and geranylgeranyl pyrophosphate and therefore it contributes to membrane structure, cell-wall synthesis, protein prenylation or ubiquinone synthesis (Oswald *et al.*, 2007).

2.7 Influence of viticultural practices on monoterpenes

Viticultural practices inevitably have an effect on the absolute and relative concentrations of monoterpenes in grapes. Comparisons between studies are complicated due to differences in cultivar, temperatures during ripening, and time interval between onset of veraison and maturity. General observations do provide some insight into certain factors since monoterpene levels can differ drastically between vintages (Masa and Vilanova, 2008).

Free volatile terpenes (FVT) and potentially volatile terpenes (PVT) accumulates along with the maturation of the berry and the simultaneous increase in sugar concentration, but they are not necessarily mutually dependant processes (Reynolds and Wardle 1989; Sánchez Palomo *et al.*, 2007). Although the levels of monoterpenes increases with maturation of the berries, postponing of the harvest date can lead to decreases in the aromas of white wines that are described as fresh (Gómez-Míguez *et al.*, 2007).

Wines made from exposed fruit contain greater amounts of FVT and PVT than wines from shaded fruit, and these differences remained in the wines after ageing (Macaulay and Morris, 1993; Reynolds and Wardle, 1989). Monoterpene accumulation seems to be temperature related because the highest FVT concentrations tended to be in partially shaded fruit, wherein temperatures for biosynthesis of monoterpenes were adequate, but not so high as to cause excessive volatilization (Reynolds and Wardle 1989). This is consistent with the observation that white cultivar wines are often deficient in characteristic aromas in warm wine-producing countries (Marais, 1988).

Terroir also seem to have a major influence in view of the fact that overall terpene production as well as the concentration of free monoterpenes vary greatly between different vineyards and soil types (Gómez-Míguez *et al.*, 2007; Vilanova *et al.*, 2007). Monoterpene glycoconjugate accumulation in berries can be increased by draining of water in superficial soil, harvesting at a more advanced maturity, or by increasing the exposure of the berry to sunlight (Schneider *et al*., 2002). Terpene content may decrease once optimal sugar levels are attained, although this may be influenced by temperature and water availability during ripening (Sánchez Palomo *et al.*, 2007).

To produce higher aromatic potential wines, viticultural and oenological processes and practices which increase glycoconjugate levels in berries, improve their extraction and also favour their hydrolysis in the wine with minimal transformation into less aromatic compounds should be carefully considered (Schneider *et al.*, 2002).

2.8 Influence of oenological practices on monoterpenes

Different Oenological practices can also have a great deal of influence on the monoterpene composition of wine. Judicious use of pressing and skin contact times can potentially contribute to the monoterpene levels in juice since extended skin contact times and vigorous pressing may result in unwanted and increased phenol concentrations in wine. Macaulay and Morris, (1993) have shown that skin contact does not increase PVT levels, while Reynolds *et al.*, (1993) as well as Sánchez Palomo *et al.*, (2006) have shown that the highest FVT and PVT levels are obtained in juices from grapes subjected to skin contact compared with grapes crushed and immediately pressed. In the study by Reynolds *et al.*, (1993) it was reported that substantial amounts of FVT and PVT were lost between the berry and juice stages. These two studies have inconsistent results possibly due to the distribution of glycosylated monoterpenes throughout the berry.

Some other factors lead to a decrease in monoterpene content, for example the cell debris and seeds of the pulp fractions can absorb the various monoterpenes present in the juices (Williams *et al.*, 1985). During alcoholic fermentation, some of the aromatic compounds are released by volatilization (Günata *et al.*, 1986). This loss of monoterpenes due to volatilization can be reduced by fermenting at lower temperatures which allows better retention of volatile aroma compounds.

Hydrolytic breakdown of glycoconjugates can be induced by post-fermentation thermal storage (Zoecklein *et al.*, 1997a). Thermal treatment can also have a negative effect on the aroma due to the oxidation and isomerization of monoterpenes into less flavoursome products. Increased terpene concentrations in wine and juice as a result of heat treatment and skin contact are not always evident in the sensorial properties of these wines and juices due to the concomitant extraction of tannins and other compounds that are responsible for coarse tastes (Marais, 1988). Another post-fermentation process, lees contact, can lower the content of

glycosyl-glucose by about 50% without an increase in the amount of free monoterpenes and should consequently also be avoided (Zoecklein *et al.*, 1997a).

2.9 Liberation of monoterpenes from glycosidically bound precursors

During and after alcoholic fermentation, some of the glycosidically bound aromatic compounds are released (Günata *et al.*, 1986). This increase in the levels of free monoterpenes and other aromatic compounds can be explained by the efficient hydrolysis of corresponding glycosides (Martino *et al.*, 2000). This process occurs via two mechanisms: Acid hydrolysis, which is a normal part of the juice and must processing, or enzymatic hydrolysis (Williams *et al.*, 1981). Enzymatic hydrolysis is the preferred mechanism for the release of monoterpenes because it is rapid, efficient and does not result in modification of the intrinsic aromatic character of the wine as opposed to acid hydrolysis (Swiegers *et al.*, 2005). High temperature acidic hydrolysis of monoterpene glycosides cause a molecular rearrangement and transformation of the monoterpene aglycones into other, unwanted compounds and is therefore not suitable (Mateo and Jiménez, 2000; Williams *et al.*, 1982).

Significant sensorial differences exist between volatiles released from a precursor fraction by hydrolysis with a glycosidase enzyme, and those released by acid hydrolysis (Abbott *et al.*, 1991). Water solubility and the presence of an allylic glycosidic linkage have a large effect on the reactivity of these monoterpenes. The presence of an allylic glycosidic linkage facilitates the formation of a carbocation which is an important step in the hydrolytic process (Williams *et al.*, 1982).

2.9.1 Acid hydrolysis

During vinification and wine ageing, the mild acidic conditions (pH 3.0-3.8) cause aromatic precursors to undergo a natural process of slow chemical hydrolysis of the glycosidic bond resulting in the release of fragrant monoterpenes (Sefton *et al.*, 1994; Spagna *et al.*, 1998a). Under acidic conditions (as are found in wine, pH 2.5-3.8) these monoterpenes can be converted into less fragrant compounds such as α-terpineol, monoterpene diols and monoterpene oxides which can have a significant effect on the composition of free monoterpenes and the aroma of the wine. An additional disadvantage is that acid hydrolysis proceeds slowly under winemaking conditions (Palmeri and Spagna, 2007). Glycosidases do not present these disadvantages and are therefore the accepted mechanism of monoterpene release (Martino *et al.*, 2000).

Under very acidic and high temperature conditions, the primary allylic glucosides are cleaved at the ether linkage and not at the glycosidic bond (Skouroumounis and Sefton, 2000). These conditions tend to favour the formation of myrcenol and isomeric ocimenols at the expense of linalool, geraniol and nerol which result in a decrease in fruity and floral aromas and an increase in unwanted butter, eucalyptus, rubber and tobacco aromas (De La Presa-Owens and Noble, 1997; Fariña *et al.*, 2005; Williams *et al.*, 1981). Very low pH levels (pH 1.0) give rise

to α-terpineol, 1,4- and 1,8 cineoles, the isomeric 2,2-dimethyl-5-(1-methylprop-1-enyl)-tetrahydrofurans, the hydrated forms of these two oxides, *p*-cymene, 1-terpineol, myrcenol, the isomeric ocimenols, 4-terpineol and γ-terpineol (Williams *et al.*, 1982).

Phenolic-free glycosides can be present at about 80% of the total glycosides present in aged wines, indicating that the acid hydrolysis is incomplete and not very efficient (Zoecklein *et al.*, 1998). The rate of acid hydrolysis is closely dependent on the pH and temperature of the medium and on the structure of the aglycone moiety, since it has been observed that glycosides of tertiary alcohols are more readily hydrolysed than those of primary alcohols, such as geraniol and nerol (Williams *et al.*, 1981).

2.9.2 Enzyme hydrolysis

A common characteristic of β-glucosidases that are capable of monoterpene release from monoglucosides is that they do not have endoglucanase activity; therefore disaccharides can not be released with the action of only β-glucosidase. Günata *et al.*, (1988) have studied and elegantly displayed the sequential mode of action whereby hydrolysis of grape monoterpenyl disaccharide-glycosides takes place. First, the (1→6) linkage is cleaved by either α-L-arabinofuranosidase or α-L-rhamnosidase, resulting in the release of arabinose or rhamnose, respectively, and the corresponding monoterpenyl β-D-glucoside. Subsequently, the action of a β-D-glucosidase liberates a monoterpenol and β-D-glucose (Figure 2.3).

The simultaneous addition of the purified enzymes β-D-glucosidase, α-L-arabinofuranosidase and α-L-rhamnopyranosidase lead to the greatest release of glycosidically bound precursors when compared to the individual addition of these enzymes (Bloem *et al.*, 2008). This serves as a practical example of glycosidases working in concert to liberate diglycosidically bound monoterpenes.

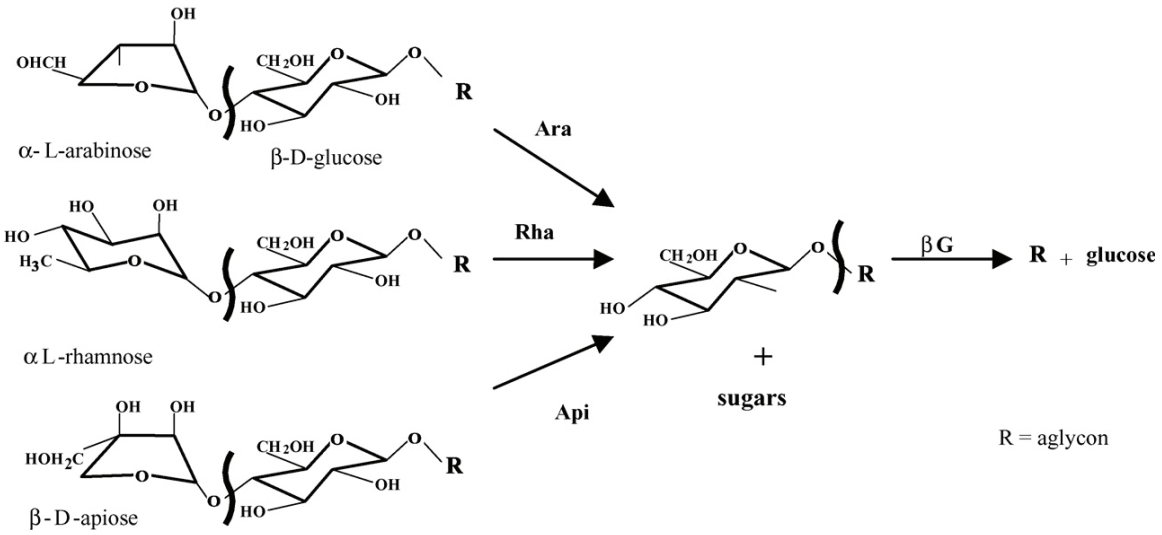

Figure 2.3 Sequential hydrolytic mechanism of glycosidases. Ara: α-L-arabinofuranosidase; Rha: α-L-rhamnopyranosidase; Api: β-D-apiofuranosidase; βG: β-D-glucopyranosidase. R = monoterpenes, sesquiterpenes, norisoprenoids, benzene derivatives, aliphatic alcohols. (Palmeri and Spagna, 2007).

Enzymatic hydrolysis is more efficient than natural acid hydrolysis, since the amount of free monoterpenes is higher in fermented samples compared to non-fermented samples (Hernández-Orte *et al.*, 2008). However, during the handling of the juice and under winemaking conditions, endogenous grape glycosidases of *V. vinifera* and *S. cerevisiae* show very low activity towards the monoterpene glycosides (Delcroix *et al.*, 1994; Aryan *et al.*, 1987). The total concentration of compounds of the oxidation level of the linalool oxides is greater in the enzyme-liberated aglycon plus free fractions than in the corresponding acid hydrolysates (Reynolds and Wardle, 1989). In addition, certain glycosides are known to hydrolyse either slowly or not at all under mild acid conditions resembling that of wine.

There are a few qualities that are required from these enzymes to be used under winemaking conditions. Firstly, they should have a high affinity for grape-derived terpenoid aglycones. Secondly, they should possess decent activity at wine pH (pH 2.5-3.8). The third requirement is resistance to glucose inhibition, the fourth is that the enzyme should be tolerant to ethanol, and the fifth is that it should be active at winemaking temperature (10-30°C).

Monoterpene concentrations can increase significantly in enzyme-treated wines (Cabaroglu *et al.*, 2003). This enzymatic increase is largely dependant on the amount of glycosidic precursors that are present in the juice, and since precursors are present in varying concentrations relative to each other, some monoterpenes might increase more than others. Glycosidically bound C_{13}-norisoprenoids can also be released by enzymatic treatment. Different genera of organisms also present a diverse array of enzymatic activities that inevitably have an influence on the sensorial properties of a wine (Hernández-Orte *et al*., 2008). Therefore, the aromatic profile obtained will depend both on the varietal glycoside composition and the origin of the enzyme employed.

2.10 Immobilization of glycosidases

There has been substantial effort made in the development of methods for immobilization of arabinofuranosidase and β-glucosidase onto various substrates in an attempt to stabilize these enzymes, to enhance their activity and to develop practical commercial products. Enzymes are either immobilized on a chemical substrate, or anchored to the cell wall of the yeast. Immobilization can have a stabilizing effect on the enzyme, as seen for the *Candida molischiana* 35M5N β-glucosidase when immobilized to Duolite A-568 resin (Gueguen *et al.*, 1996). Although immobilization increases the stability of the enzyme, it might decrease the activity in some instances (Spagna *et al.*, 1998b).

Anchor sequences might aid in more effective secretion or less miss-folding and degradation of active β-glucosidase. Conversely, overexpression of proteins bound to the cell-wall might have an obstructing effect on the diffusion of hydrolysis products (van Rooyen *et al*., 2005).

If an enzyme is shown to be stable and active under winemaking conditions, these qualities might obliviate the need for immobilization.

2.11 Stability of liberated monoterpenes

It has been demonstrated that the levels of free monoterpenes can decrease in juice, during fermentation, and during processing and storage of the wine due to transformation reactions (Ribéreau-Gayon *et al.*, 1975; Rocha *et al.*, 2005). These changes in the level of free monoterpenes observed in an aqueous acidic system can be explained mainly by acid-catalyzed terpene reactions and to a minor degree by glycosidic release. These reactions are part of a complex system where compounds are formed and degraded simultaneously (Varming *et al.*, 2006). In general, all allylic alcohols are unstable in acidic solutions (Luan *et al.*, 2004).

Modifications of the monoterpenes may not only alter the types of aroma, but also the intensity. A number of the more fragrant precursors (linalool, nerol, geraniol) turn into less fragrant compounds under acidic conditions (α-terpineol, diols, triols, oxides) (Spagna *et al.*, 1998a). This decline in free and aromatic monoterpenes is the result of oxidation reactions that lead to the production of monoterpene oxides and monoterpene diols with sensory thresholds that are approximately ten times higher than that of their precursors (Strauss *et al.*, 1988). In addition, these changes also impact negatively on the aroma quality of monoterpenes. For example, the iris-like odour of linalool is substituted by the musty, pine-like scent of α-terpineol, its main conversion product (Varming *et al.*, 2006).

2.11.1 Stability in juice

The stability of free monoterpenes in grape juice has not been studied in detail and monoterpenes are believed to slowly undergo some of the mild acid transformation reactions. Due to the fact that these reactions proceed very slowly, and that juice is generally not stored for extended periods of time before fermentation the effect on the final product should be relatively small when compared to the effects of fermentation and ageing. Certain treatments like heat treatment of juice produces *cis*-terpin, an end product of monoterpene glycoside hydrolysis and also leads to the formation of 1,8 cineole (Williams *et al.*, 1982).

2.11.2 Stability in fermenting juice

The average concentration of free monoterpenes can decrease even during normal fermentations with *S. cerevisiae* (Zoecklein *et al.*, 1997a). Possible explanations for this loss of terpenes include degradation due to transformation and isomerization, volatilization, or adsorption/metabolism by the yeast cell walls (Günata *et al.*, 1986). The liberation of glycosidically bound terpenes during fermentation might therefore in some instances lead to a decrease in the monoterpene concentrations of the finished wine. Consequently, decreases in the amount of monoterpene glycosides do not necessarily result in a corresponding increase in the amount of free monoterpenes (Martino *et al.*, 2000).

Although α-terpineol is present in grape juice at low concentrations, it can also appear during wine production from rearrangement of acyclic monoterpenes such as geraniol, nerol, and linalool under the acid conditions encountered in wine (Martin and Bohlmann, 2004).

2.11.3 Stability in wine

Free monoterpenes are not stable in wine in comparison to bound monoterpenes, since monoterpenols are known to rapidly rearrange under acidic conditions to form transformation products, while their corresponding glycosides are more stable (Skouroumounis and Sefton, 2000; Voirin *et al.*, 1990). The result of this instability is often a reduction in the amount of more aromatic monoterpenes (Numan and Bhosle, 2006). Although these transformation reactions are known to occur under acidic conditions as are found in wine, the effect of additional factors and their affects are not known and consequently it is currently impossible to make predictions concerned with the rate at, and extent to which these reactions will occur.

Levels of some free monoterpenes, such as linalool, vary little after fermentation; for others, such as geraniol, the decrease can be considerable. Free linalool and α-terpineol levels sometimes increase while levels of geraniol and nerol usually decrease in older wines (Günata *et al.*, 1986; Zoecklein *et al.*, 1997b). This is due to nerol being converted mainly to α-terpineol, while geraniol forms mainly linalool under acidic conditions (Pedersen *et al.*, 2003).

During bottle maturation of wine, some monoterpenes undergo a complex pattern of transformations. For example, linalool is transformed to other terpene compounds. The main reaction occurs via α-terpineol and 1,8-terpin. 1,8-Terpin is not present in young white wine (Rapp, 1987). Other transformation products of geraniol, nerol and linalool include the monoterpene polyols and oxides. These diols and oxides are thermodynamically stable end-products of mild acid hydrolysis of the major grape and wine monoterpenes at the oxidation level of linalool (Reynolds and Wardle, 1989).

Derivatives of monoterpenes are formed through biochemical oxidation as well as hydration/dehydration (Reynolds and Wardle, 1989). Cyclization reactions cause some monoterpenes to form lactones, for example, 2-vinyl-2 methyltetrahydrofuran-5-one is formed from linalool oxides. As mentioned earlier, these cyclic monoterpenes have a lower sensory impact than acyclic monoterpenes. Under acidic conditions, linalool and linalyl acetate are converted mainly to α-terpineol and 3,7-dimethyloct-1-en-3,7-diol, while geraniol, geraniol acetate, nerol and nerol acetate are converted to α-terpineol, linalool and the isomeric 3,7-dimethyloct-2-en-1,7-diols (Baxter *et al.*, 1978; Skouroumounis and Sefton, 2000; Williams *et al.*, 1982). This partially explains why linalool does not decrease as much as geraniol and nerol, since some linalool is produced from these two monoterpenes. Citronellol levels usually increase in wine while geraniol and nerol are the monoterpenes that decrease the most as wine ages (Pedersen *et al.*, 2003; Sánchez Palomo *et al.*, 2007).

2.12 Commercial glycosidase preparations

The release of glycosidically bound monoterpenes is enhanced by addition of commercial enzyme preparations that are derived from fungi, mainly *Aspergillus* spp. which have GRAS status (Querol and Rámon, 1996; Spagna *et al.*, 1998a). The composition of these preparations is not always constant, and they are an undefined, crude mixture of non-specific glucanases which make it difficult to control their effect on the aroma of the wine since they give inconsistent results and can cause the level of free monoterpenes to decrease (Macaulay and Morris, 1993; Rocha *et al.*, 2005).

In a commercial preparation isolated from *Aspergillus niger* and described by Günata *et al.*, (1997), multiple forms of β-apiosidase, β-glucosidase, α-rhamnosidase, and α-arabinofuranosidase are present. Many of these preparations are intended to be used to improve juice yield and clarification. They do however possess non-specific side activities that can be useful in liberating glycosidically bound compounds.

Liberation of aglycones by using exogenous glycosidases can enhance the floral and fruity aroma of wines, but they can also generate new compounds that influence wine aroma negatively such as vinyl phenols and vinyl guaiacol (Sánchez Palomo *et al.*, 2005). Increased levels of vinyl guaiacol and vinyl phenol following enzyme treatment in wines may be due either to the hydrolysis of glycosylated forms in the wine, or to the additional cinnamate esterase activity of the enzyme preparation used (Cabaroglu *et al.*, 2003; Sánchez Palomo *et al.*, 2005). The cinnamate esterase activity from added enzyme preparations lead to an increase in the concentration of cinnamic acids liberated from their corresponding tartaric acid esters, and the cinnamic acids are in turn decarboxylated to vinyl phenols and vinyl guaiacol by decarboxylase produced by *S. cerevisiae* during fermentation (Dugelay *et al.*, 1993).

Vinyl guaiacol and vinyl phenol are cinnamic acid derivatives that may also be formed by fermentation yeasts and are responsible for unpleasant phenolic off-flavors if they are present in high concentrations (Sánchez Palomo *et al.*, 2005). The majority of enological yeast strains possess decarboxylase activity (Dugelay *et al.*, 1993). Other detrimental side activities of these preparations can include esterase, anthocyanase and polyphenoloxidase activities (Palmeri and Spagna, 2007). Pure enzymes should therefore be used to avoid loss of aroma, colour, and antioxidants and to avoid the formation of unpleasant phenolic off-flavors during winemaking.

Substantial efforts have been made to obtain new preparations that do not have collateral or unpredictable effects on the wine. These endeavours have focused on the purification and characterization of new enzymes with specific activities from fungal preparations, but these purification procedures are generally expensive. Additional disadvantages of some purified enzymes is that they are strongly inhibited by glucose, and that they possess poor stability and therefore would have to have their stability increased, for example, by adopting immobilization techniques or by chemical modification (Aryan *et al.*, 1987; Spagna *et al.*, 1998a).

The use of yeast that produces a functional glycosidases capable of releasing monoterpenes from their glycosidically bound precursors should be more reliable and less troublesome than added enzyme preparations (Spagna *et al.*, 1998a).

2.13 Release of anthocyanin

β-glucosidases used for the release of monoterpenes should have low anthocyanase activity to prevent loss of colour in wine. Anthocyanins are responsible for the pigment of red wine and are formed by the addition of a mono- or disaccharide to anthocyanidins. Some glycosidases can decompose anthocyanins in juice by hydrolyzing the glycosidic linkage which results in the release of the unstable pigment aglycon which degrades spontaneously into colourless compounds (Wightman and Wrolstad, 1995). Since glucose is the most common sugar moiety of anthocyanins, β-glucosidases with anthocyanase activity could contribute significantly to the loss of colour in red wine (Palmeri and Spagna, 2007; Sánchez-Torres *et al.*, 1998).

A problem with commercial fungal (*A. niger*) enzyme preparations is that they exert a significant decolourizing effect on extracts of pigments derived from berry fruits due to the enzymatic hydrolysis of the anthocyanin glucoside and the natural transformation of the liberated anthocyanin into a colourless derivative (Huang, 1952). Two enzyme preparations, AR 2000 (aroma-releasing) and Cytolase PCL5 (pectolytic), have been shown to have pronounced effects on wine colour (Wightman *et al.*, 1997). Monomeric anthocyanin, malvidin-3-glucoside, 3-glucosylacetate, and 3-glucosylcoumarate concentrations decrease when commercial enzyme preparations are used.

2.14 Biotransformation of monoterpenes

Different yeasts have the capability to transform monoterpenes during fermentation using a variety of reactions. The reactions catalyzed by the yeasts are summarized in Figure 2.4. These reactions 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) (King and Dickinson, 2000; Vaudano *et al.*, 2004). King and Dickinson, (2000) have shown that monoterpenoids do not undergo spontaneous transformation and that *S. cerevisiae* does not synthesize these compounds.

In general, the reactions catalyzed by the yeasts lead mainly to the formation of linalool and α-terpineol. Although only single strains from each species were used in the study by King and Dickinson, (2000), the different levels of transformation of monoterpenes observed were quite dramatic.

Some yeast can produce acetate esters of geraniol and citronellol. For example, although geraniol is mainly converted to citronellol and nerol, traces of geranyl acetate and citronellyl acetate can be found (King and Dickinson, 2003).

Sporulated surface cultures of *A. niger* are able to convert citronellol into *cis*- and *trans*-rose oxides and nerol oxide (Demyttenaere *et al.*, 2004). Other bioconversion products were

6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, limonene, terpinolene, linalool and α-terpineol. Limonene has been shown to be transformed to α-terpineol by *Fusarium oxysporum* (Maróstica and Pastore, 2007).

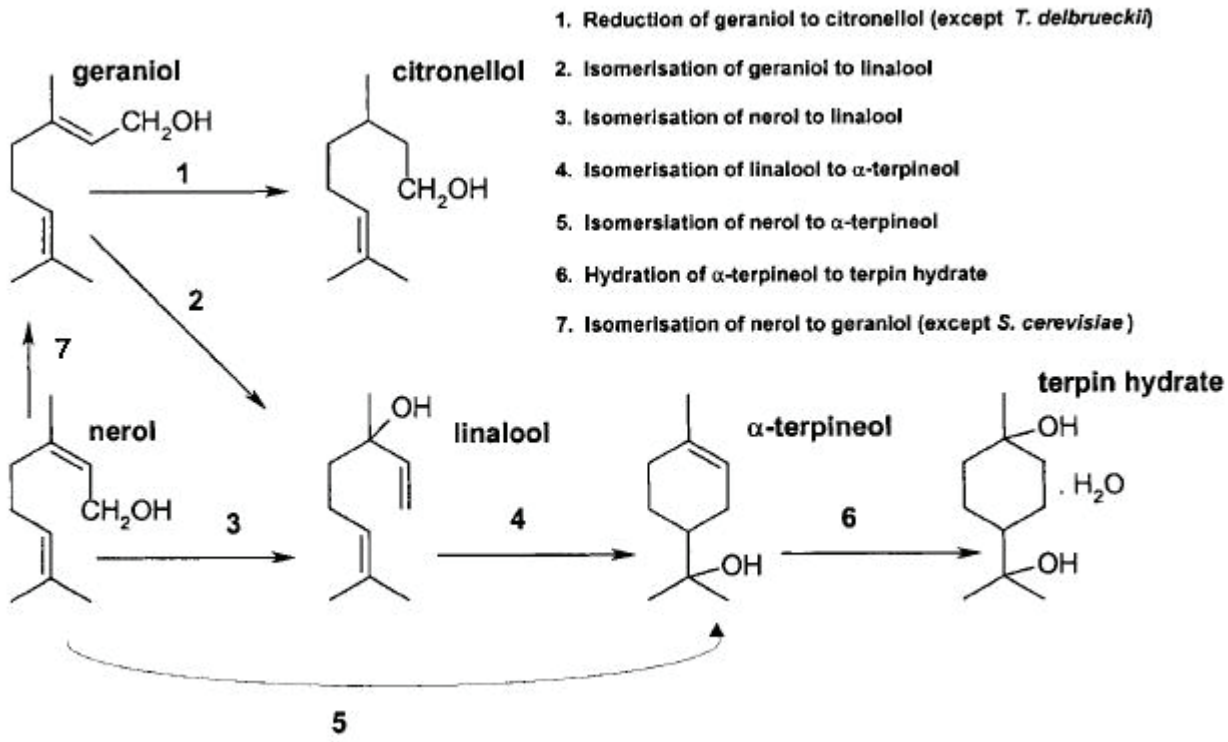

Figure 2.4 Monoterpenoid biotransformation reactions catalyzed by *S. cerevisiae*, *T. delbrueckii*, and *K. lactis*. (King and Dickinson, 2000).

2.15 Monoterpene metabolism in yeast

Free monoterpenes undergo an acid-catalyzed rearrangement and might be used in yeast metabolism (Martino *et al.*, 2000). For instance, the reduction of geraniol at the beginning of fermentation, without the production of a corresponding amount of monoterpenols led Vaudano *et al.*, (2004) to the hypothesis that geraniol is used in other yeast metabolism pathways that are involved in the synthesis of molecules which are required during the rapid growth phase. Monoterpenes might evaporate during and after fermentation due to their volatile character. However, it has been shown that free monoterpenes undergo metabolic turnover rather than evaporative losses (Paisarnrat and Ambid, 1985).

Terpenic compounds occur as intermediates in the production of sterols, for example geraniol is present in an active form as geranyl pyrophosphate, and geraniol can consequently become depleted due to the biosynthesis of steroids that are vital for eukaryotic cellular growth (Vaudano *et al.*, 2004). Geraniol is metabolized in the first 48 hours of growth, both aerobically

and anaerobically. In the first 24h, the synthesis rate of citronellol is high, and lower as fermentation progresses. Linalool is also consumed during fermentation.

Citronellol production is higher in the presence of ergosterol because geraniol is primarily reduced to citronellol under high oxygen and high ergosterol conditions and it is hypothesized that geraniol is primarily a biosynthetic intermediate of sterol metabolism under low oxygen and low ergosterol conditions (Vaudano *et al.*, 2004). At the beginning of the growth phase, when steroid requirements are higher, presence or lack of oxygen can have a significant influence on the degradation of geraniol. After the first few hours of fermentation, the yeast needs fewer sterols and consumes lower quantities of geraniol.

The introduction of geraniol into the sterol biosynthetic pathway probably entails transforming geraniol into the metabolic form of geranyl pyrophosphate with an enzyme reaction similar to that used for introducing farnesol into eukaryotic cells (Vaudano *et al.*, 2004).

2.16 Specificity of glycosidases

Different β-glucosidases have different affinities for substrates (van Rooyen *et al.*, 2005). The species and strain of the organism from which the enzyme is derived has a great influence on the specificity and activity of the enzyme.

The use of mixtures of glycosidases rather than homogenous enzyme preparations is one reason for the inaccurate observations and erroneous conclusions that glycosidases lack aglycone specificity. Utilization of artificial substrates such as benzyl-, nitrophenyl-, or 4-methylumbelliferyl-glycosides (4-MUG) during characterization of glycosidases can also be blamed (Hösel and Conn, 1982). α-L-arabinofuranosidase and β-glucosidase show less activity against natural substrates than towards synthetic substrates (Margolles-Clark *et al.*, 1996). Enzymes should also be evaluated for their activity against target or native precursors, because many yeast strains, including *Debaryomyces hansenii* and *Metschnikowia pulcherrima* exhibit high glycosidase activity against synthetic substrates, but show low activity towards native grape precursors (Fernández-González *et al.*, 2003). The use of nitrophenyl-glycosides does not provide information about the substrate specificity of the enzyme towards the native glycosidically bound monoterpenes that are present in grape must. It is therefore important to test enzyme activity towards target substrates when conducting screening assays.

Another problem with *para*-nitrophenyl (pNP) substrates is that some enzymes show side activity against synthetic model substrates (Margolles-Clark *et al.*, 1996). It has been shown that *S. cerevisiae* has some constitutive β-glucosidase activity on *para*-nitrophenyl-β-D-glucopyranoside (pNPG), while an assay on terpene glycosides has shown that this activity is induced (Mateo and Di Stefano, 1997). The constitutive activity of non-induced yeasts is probably due to another *S. cerevisiae* exoglucanase able to hydrolyse pNPG. This is an example of one of the problems faced when using pNPG as a substrate.

Sometimes, different strains show different levels of activity towards synthetic substrates as well, for example, Fia *et al.*, (2005) showed that some strains showed higher activity against

4-MUG and esculin than arbutin. Although *D. hansenii* exhibit high β-glucosidase activity against synthetic substrates, it showed especially low activity towards isolated linalool and geraniol precursors (Fernández-González *et al.*, 2003). These are the most abundant and aromatically important monoterpenes and monoterpene precursors in wine. Due to the fact that sugar moieties of pNP substrates are identical to those of natural glycosides, substrate specificity must be influenced by the aglycon if there are differences in activity towards native and synthetic substrates under identical conditions (Günata *et al.*, 1988). Non-specific activity against natural and synthetic model substrates has been reported for α-L-arabinofuranosidases and β-glucosidases (Koseki *et al.*, 2006; Margolles-Clark *et al.*, 1996).

The activity of β-glucosidases towards monoterpene glycosides is influenced by the aglycone moiety (Günata *et al.*, 1988). This is demonstrated by the fact that during fermentation, linalyl glucoside is the monoterpene glucoside that is released the most at up to 70%, while geraniol, nerol and α-terpineol is not released by more than 40% by yeast enzymatic activity (Ugliano *et al.*, 2006). On the contrary, β-glucosidase form *A. niger* hydrolyze the glucosides of primary terpene alcohols, such as geraniol and nerol, more readily than the glucosides of tertiary terpene alcohols (linalool and α-terpineol), due to lower steric hindrance (Günata *et al.*, 1990). This seems to indicate that *S. cerevisiae* β-glucosidases has a propensity to hydrolyze tertiary monoterpene glucosides while *A. niger* β-glucosidases act more specific towards primary monoterpene glucosides.

Plant *β*-glycosidases often display a pronounced specificity for specific aglycones. The electron attracting power of the aglycone might play a role in determining this specificity (Hösel and Conn, 1982). Some plant *β*-glucosidases display a low rate of hydrolysis for aromatic glucosides and for artificial substrates while others have a very pronounced specific activity for the aglycone moiety of their substrate. This aglycone specificity of glycosidases is due to the liberation of certain aglycones to obtain a specific physiological goal.

Only some of the aglycons are present as monoglucosides, therefore, use of substrates such as pNPG, arbutin or 4-MUG might indicate the presence of a β-glucosidase, but not guarantee activity against natural wine glycosides.

Activity of the exoglycosidase arabinofuranoside is not affected by the aglycone moiety of diglycosides, but rather by the chemical structures of the sugar moieties of these glycosides (Ugliano *et al.*, 2006). A possible explanation for this observation is that the terminal sugars have the same orientation in the interglycosidic bonds in related glycosides, regardless of the structure of the aglycon (Voirin *et al.*, 1990).

Specificity of glycosidases towards certain substrates can have a marked influence on the observed activity and efficiency of hydrolysis. The efficiency of the hydrolysis of monoterpenyl β-D-glucosides by β-glucosidases is therefore determined by both the origin of the enzyme and the structure of the aglycon while the efficiency of hydrolysis of arabinofuranosides by α-L-arabinofuranosidases is determined by the origin of the enzyme and the structure of the sugar moieties.

2.17 Glycosidase activity under winemaking conditions

There is numerous potentiallly inhibiting or stimulating factors present in grape must and wine with regards to glycosidase enzyme activity. Furthermore, it is usually a combination of these factors that can have a severe inhibitory effect on the activity of an enzyme. For example, enzymes which are not inhibited by ethanol, low pH, or sugar when these factors are present separately, and are even sometimes stimulated by some of these factors, may become inactive when these factors are applied in combination (Grimaldi *et al.*, 2000). Grimaldi and co-workers showed that some β-glucosidases are stimulated by ethanol at moderate pH values (pH 4.5 to 6.0), but at low pH conditions ethanol can inhibit activity.

The goal of studies focussing on enzymatic release of monoterpenes should be to find an enzyme with large aglycon specificity and good stability at low pH, as well as tolerance to glucose, ethanol and other factors present in wine (Colagrande *et al.*, 1994). Even if an enzyme can maintain only partial activity under winemaking conditions, it can have a significant effect on the wine aroma.

2.18 α-L-Arabinofuranosidase

The possible effect of α-L-arabinofuranosidase on wine parameters was previously investigated with satisfactory results (Sánchez-Torres *et al.*, 1996; Spagna *et al.*, 1998b; Crous *et al.*, 1996). For a comprehensive review on the applications of arabinofuranosidase, see Numan and Bhosle (2006).

Arabinofuranosidases have not enjoyed as much attention as the β-glucosidases in terms of application in the release of monoterpenes in wine. α-L-arabinofuranosidase is considered to be part of the hemicellulase complex, working in synergy with xylanase and β-xylosidase. Some of the α-L-arabinofuranosidases have activity against diglycosidically bound monoterpenes and are potentially useful in the aromatization of wine.

2.18.1 Classification

The α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55, ABF), are exohydrolases able to hydrolyze terminal non-reducing α-L-1,2-, α-L-1,3- and α-L-1,5-α-L-arabinofuranosyl linkages from oligo- or polysaccharides which contain α-linked L-arabinofuranosyl residues at non-reducing ends (Chacón-Martínez *et al.*, 2004; Flipphi *et al.*, 1993).

2.18.2 Sources and characteristics

The enzyme α-L-arabinofuranosidase has been isolated from a number of bacteria, fungi and plants (Le Clinche *et al.*, 1997). 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. Grapevine glycosidases for example have poor stability at wine pH and is inhibited by glucose and ethanol.

Although numerous sources have been investigated for the production and isolation of effective α-L-arabinofuranosidases, the most promising enzymes came from fungi from the genus *Aspergillus*. This activity proceeds mostly extracellular and production of this enzyme is induced in a medium containing L-arabinose or L-arabinan, and not by glucose (Flipphi *et al.*, 1993; Tagawa and Kaji, 1988).

The malolactic bacterium *Oenococcus oeni* does have α-L-arabinofuranosidase activity against synthetic substrates as well as purified grape glycosides in a chemically defined medium (D'Incecco *et al.*, 2004; Grimaldi *et al.*, 2005). These enzymes do not release monoterpenes in wines, suggesting that these enzymes are simply not active under actual winemaking conditions.

S. cerevisiae does not have a gene encoding an α-L-arabinofuranosidase, yet it has been reported that some *Saccharomyces* strains have enzymatic activities capable of hydrolyzing the diglycosidic conjugates (McMahon *et al.*, 1999; Ugliano *et al.*, 2006). These activities may have been caused by the broad activity of a glycoside hydrolase and this particular investigation was carried out in synthetic medium at pH 5.0 to favour enzymatic hydrolysis above acid hydrolysis. Under winemaking conditions, these activities are probably severely inhibited.

α-L-arabinofuranosidase from *Aspergillus oryzae* and *Aspergillus awamori* can function in presence of high salt concentration and have a wide range of substrates (Hashimoto and Nakata, 2003). This arabinofuranosidase has also been shown to have different activities towards different *para*-nitrophenyl and native substrates, necessitating the need to investigate the activity of this enzyme towards native grape monoterpene precursors under winemaking conditions (Koseki *et al.*, 2006).

Expression of these arabinofuranosidases are stimulated by low pH conditions and induced by the presence of arabitol but is inhibited by glucose (Le Clinche *et al.*, 1997; Gielkens *et al.*, 1999). It is known that the DNA-binding protein CreA mediates carbon catabolite repression (van Peij *et al.*, 1998). The consensus CreA recognition sequences were found at several sites in the promoter regions of the *AwabfA* gene (Koseki *et al.*, 2003). A consensus sequence for the binding of a transcriptional activator of the xylanolytic enzymes is present 390 and 400 bases upstream of the translation start codon and might be responsible for activation of transcription.

The activity of α-L-arabinofuranosidase in commercial enzyme preparations are highly variable, since these enzymes are crude preparations from *A. niger* made up of mainly β-glucosidase. In addition, these glycosidases from *A. niger* are inhibited by glucose and gluconolactone (Le Traon-Masson and Pellerin, 1998). It would therefore be interesting to investigate the influence of α-L-arabinofuranosidase from *A. awamori* towards monoterpene glycosides.

The nucleotide and protein sequences of the different α-L-arabinofuranosidase-B or α-L-arabinofuranosidase-2 genes from *Aspergillus* spp. show very strong homology. The single

amino acid difference between *A. niger* and *A. awamori* α-L-arabinofuranosidases might have a positive effect on the stability and activity of the latter's enzyme

In terms of activity under wine-like conditions, the *A. awamori* α-L-arabinofuranosidase-B (AwabfB) appears to be a suitable enzyme with favourable characteristics for the release of diglycosidically bound monoterpenes. This enzyme contributes to the formation of aroma compounds in the production of Awamori, an alcoholic beverage indigenous to Japan (Koseki *et al.*, 2003). The *AwabfB* gene from *A. awamori* has been described and the enzyme has also been purified, characterised and shown to be active at low pH and low temperature conditions similar to that of wine (Kaneko *et al.*, 1998; Koseki *et al.*, 2003). The AwabfB has an optimum activity at pH 4 and 55°C and is stable between pH 3 to pH 7 at temperatures of up to 60°C. Heavy metal ions, such as Hg^{2+} and Cu^{2+} decrease activity of arabinofuranosidases while ethanol can also inhibit activity, although activity is still sufficient at ethanol concentrations of 12% (Le Clinche *et al.*, 1997).

2.18.3 Cloning and expression in *S. cerevisiae*

Genes encoding α-L-arabinofuranosidases have been cloned from various organisms, including bacteria, fungi and yeasts (Margolles-Clark *et al.*, 1996).

α-L-arabinofuranosidases from strains of *A. niger* have enjoyed the most attention in terms of purification and cloning towards application in winemaking. The α-L-arabinofuranosidase B gene (*abfB*) from *A. niger* was successfully cloned and expressed in *S. cerevisiae* and functional α-L-arabinofuranosidase was secreted from the yeast cells (Crous *et al.*, 1996). The *abfB* from *A. niger* was also successfully cloned into a wine yeast strain under control of the yeast actin gene promoter (Sánchez-Torres *et al.*, 1996). This heterologous abfB is not degraded in wine and displays similar physicochemical properties to the native enzyme and seems to be resistant to inhibition by glucose (0.5M) and SO_2 (150ppm). Ethanol at a concentration of 12.5% reduces activity of this enzyme by 40%.

Enzyme activity towards synthetic substrates was evaluated, but the monoterpene levels were not measured in these studies. Nonetheless, these studies have shown the feasibility of using recombinant yeast strains expressing arabinofuranosidase.

2.19 β-glucosidase

The β-glucosidases catalyze the hydrolysis of aryl- and alkyl-β-D-glucosides as well as glucosides with only a carbohydrate moiety, e.g. cellobiose (Bhatia *et al.*, 2002). Enzymatic hydrolysis of glycosides proceeds similarly to that of acid hydrolysis of glycosides, therefore both involve a carbocation intermediate. Although β-glucosidases have many functions based on hydrolytic and synthetic activity, only hydrolysis of monoterpenyl precursors are discussed here. For a review on applications of β-glucosidases, see Bhatia *et al.*, (2002).

2.19.1 Classification

The β-glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21, BGL), constitute a group of well characterized, biologically important enzymes that catalyze the transfer of a glycosyl group between oxygen nucleophiles. Under physiological conditions, such a transfer reaction generally results in hydrolysis of a bond linking carbohydrate residues in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glucosides, short chain oligosaccharides and disaccharides (Bhatia *et al.*, 2002). β-glucosidase hydrolyzes the bond between glucose and the substrate bound at the anomeric carbon.

On the basis of substrate specificity, β-glucosidases can be grouped into three classes:

1. aryl-β-glucosidases
2. cellobiases
3. broad substrate specificity enzymes.

The exo-acting aryl-β-glucosidases are of interest in the release of monoterpenes from their glycosidic precursors due to their specific exoglucosidase activity. It is not necessary to hydrolyze cellobiose and broad substrate specificity enzymes might lead to the release of unwanted compounds.

2.19.2 Sources and characterisitics

β-glucosidases are present in most organisms, including plants, fungi bacteria, insects and mammals where they catalyze diverse but specific hydrolysis reactions. In fungi and bacteria, β-glucosidase is involved in cellulose and cellobiose catabolism as part of the cellulase complex. In insects and plants, β-glucosidase is involved in the release of cyanides from cyano-glucoside precursors. This is part of a defense mechanism (Bhatia *et al.*, 2002). Additionally, its functions in plants include the hydrolysis of phytohormone precursors, pigment metabolism, seed development, and biomass conversion. In humans, membrane-bound lysosomal acid β-glucosidase is implicated in Gaucher's disease as the cells deficient in this enzyme are unable to hydrolyze glycosylceramides. Depending on the origin, each β-glucosidase has different physicochemical characteristics, and only those pertaining to wine production will be discussed (Barbagallo *et al.*, 2004b).

Glucose and especially glucono-δ-lactone are powerful inhibitors of most β-glucosidases (Riou *et al.*, 1998). Synthesis of β-glucosidase is induced by the substrate carbon source and can be found either intracellularly or extracellularly, depending on the source organism and the presence of secretion signal sequences (Arévalo Villena *et al.*, 2005; Manzanares *et al.*, 2000).

2.19.2.1 β-glucosidases in *V. vinifera*

β-glucosidase activities are widely distributed in *V. vinifera* grapes, and some of these enzymes evidently have functions unrelated to terpene glycoside hydrolysis. Enzymes in the berry have lower activity than enzymes in the leaves and are strongly inhibited by glucose, low pH, and ethanol (Aryan *et al.*, 1987).

Grape β-glucosidase has high specific activity towards the aglycon part of the substrate (Aryan *et al.*, 1987). Some β-glucosidases have low activity towards, or are incapable of releasing tertiary monoterpenes such as linalool and α-terpineol, but they are capable of hydrolyzing monoglucosides of terpenes with primary alcohol groups such as geraniol, nerol and citronellol. This means that one of the most important monoterpenes in terms of aroma, linalool, can not be released by grape-derived β-glucosidase. This aglycone specificity of these plant-derived β-glucosidases limits the usefulness of these enzymes to release glycosidically bound monoterpenes in wine. In addition, grapevine β-glucosidases have poor stability at wine pH and as a consequence they have limited capability to hydrolyze monoterpene glycosides.

The native β-glucosidase activity of *V. vinifera* is not useful for the purpose of releasing bound aroma compounds during winemaking.

2.19.2.2 β-glucosidases in commercial yeast strains

Although β-glucosidase is produced by commercial and wild *S. cerevisiae* strains, activity levels demonstrated under winemaking conditions are much lower than those observed in some non-*Saccharomyces* yeast and fungi and is therefore unsuitable for the release of monoterpenes (Barbagallo *et al.*, 2004b; Dubourdieu *et al.*, 2002; Fia *et al.*, 2005; Zoecklein *et al.*, 1998). In commercial yeast strains, β-glucosidase activity appear to be located intracellularly due to the fact that extracellular β-glucosidase activity is weak and intracellular activity much more noticable (Arévalo Villena *et al.*, 2005). This might be explained by the observation that the enzyme is stabilized by the intracellular pH of the cell which is at pH 5.0-6.0 (Zoecklein *et al.*, 1997b).

Activity of yeast β-glucosidase is strongly inhibited at wine pH values due to the instability of this enzyme (Fia *et al.*, 2005). Some studies have reported optimal β-glucosidase activity in strains of *S. cerevisiae* and *Saccharomyces bayanus* at pH 5 and 50 °C (Delcroix *et al.*, 1994; Ugliano *et al.*, 2006). The *S. cerevisiae* β-glucosidase is not very sensitive to glucose, ethanol or SO_2, but the enzyme that is excreted or released in the juice is strongly inhibited by glucono-δ-lactone at fairly low levels of 10 mM and is highly unstable below pH 3.5 (Delcroix *et al.*, 1994). Mateo and Di Stefano, (1997), however reported severe inhibition of *S. cerevisiae* β-glucosidase activity by ethanol.

2.19.2.3 β-glucosidases in fungi and non-*Saccharomyces* yeasts

The fungi and other non-*Saccharomyces* yeasts like *Candida molischiana* and *Hanseniaspora uvarum* produce β-glucosidases that are more suitable for application in winemaking than *Saccharomyces yeasts* (Fernández-González *et al.*, 2003). The major drawback when using these yeast in winemaking is that they are poor fermenters and produce unwanted compounds such as acetic acid (Mendes Ferreira *et al.*, 2001; Zoecklein *et al.*, 1997b).

It appears that β-glucosidase synthesis in non-*Saccharomyces* is closely correlated to the active growth phase. Increase in enzymatic activity upon addition of glucose may therefore be the result of higher levels of biomass production rather than a stimulatory effect by glucose (Fia

et al., 2005). In most cases however, glucose is inhibitory. Most of these organisms also only possess intracellular β-glucosidase activity (Arévalo Villena *et al.*, 2005; Mendes Ferreira *et al.*, 2001).

Although the majority of β-glucosidases are inhibited by ethanol, most β-glucosidases of fungal and yeast origin show increased activity under mild (10%) ethanol conditions (Grimaldi *et al.*, 2000; Manzanares *et al.*, 2000; McMahon *et al.*, 1999). There are three ways in which ethanol might increase activity:

1. Ethanol increases reaction rates by acting as a preferential acceptor (rather than water) of a key glycosyl intermediate (Riou *et al.*, 1998.)
2. Higher ethanol concentrations may alter membrane permeability thereby allowing easier access between the intracellular enzyme and the substrate.
3. Ethanol can also influence the polarity of the medium which in turn can have an influence on the enzyme conformation and its activity (Barbagallo *et al.*, 2004b).

Pichia and *Hanseniaspora* yeasts show high activity at a pH range of 4.0 to 5.5, but these enzymes are cell wall bound, and are not excreted into the medium (Arévalo Villena *et al.*, 2005; Barbagallo *et al.*, 2004b; Manzanares *et al.*, 2000).

Yeasts from the genus *Debaryomyces* have in recent years shown promising β-glucosidase activity. *Debaryomyces pseudopolymorphus* produces an extracellular β-glucosidase when grown on cellobiose. This enzyme has optimum activity at pH 4.0 and 40°C with a strong affinity for cellobiose and maltose and wide substrate specificity. The *D. pseudopolymorphus* β-glucosidase is inhibited by heavy metals and acetic acid, but not by glucose and its activity is enhanced by low concentrations of ethanol (Arévalo Villena *et al.*, 2006). The presence of β-glucosidases from non-*Saccharomyces* organisms at the beginning of alcoholic fermentation can have a significant effect on the aroma of wine (Mendes Ferreira *et al.*, 2001). Cofermentation with *S. cerevisiae* VIN13 and *D. pseudopolymorphus* increases the liberation of linalool, nerol and citronellol (Cordero-Otero *et al.*, 2003). In contrast, Arévalo Villena *et al.*, (2007) showed that although the β-glucosidases from *D. pseudopolymorphus* show significant *in vitro* activity, it does not release much of the glycosidically bound precursors, and a large proportion of the potential aroma is unutilized. Levels of extracellular β-glucosidase production and activity of *D. vanrijiae* is similar to *D. pseudopolymorphus* and its enzyme is tolerant to glucose, ethanol and has wide substrate specificity (Belancic *et al.*, 2003). *D. vanrijiae* β-glucosidase however has low activity towards tertiary alcohols which can result in unutilized aroma potential.

Aspergillus spp. are the source of β-glucosidase for the commercial enzyme preparations and their β-glucosidase are considered to have very suitable characteristics for the release of monoterpenes in wine. β-Glucosidase from *A. niger* has optimal activity at pH 4.5-5.0 and optimum temperature of 62-70°C with 40-50% relative activity at 20-25°C, is slightly activated by ethanol and is severely inhibited by glucose (Barbagallo *et al.*, 2004b). When glucose is present

at 1g/l, the enzyme retains 64% activity and at 100 g/l the activity decreases to only 2% (Martino *et al.*, 2000).

A. kawachii produces both extracellular and intracellular β-glucosidases that are derived from the same gene (Iwashita *et al.*, 2001). Fungi are known to synthesize and/or secrete many forms of the same enzyme depending on the strain and environmental conditions (Riou *et al.*, 1998). An example is when complex flavonoidic compounds like quercetin and rutin induce the production of a specific β-glucosidase in *Aspergillus oryzae* which might play a role during plant attack by the fungus. The β-glucosidase-A (BGLA) from *A. kawachii* degrades cellobiose, but is also capable of releasing glycosidically bound monoterpenes and is therefore considered to be a wide substrate specificity enzyme. *A. kawachii* produces an extracellular soluble polysaccharide under solid culture conditions that seems to stabilize and localize the extracellular β-glucosidase (Iwashita *et al.*, 2001). The BGLA enzyme has good stability between pH 3.0 and pH 7.0 and has optimal activity at pH 4.0.

The yeast *Saccharomycopsis fibuligera* has two genes coding for two different β-glucosidases. These BGL1 and BGL2 enzymes from *S. fibuligera* have different substrate specificities. For example, the *S. cerevisiae* transformant expressing the *BGL1* ferments cellobiose to ethanol but the *BGL2* transformant does not (Machida *et al.*, 1988). The BGL1 has a high specific activity towards, and affinity for cellobiose (van Rooyen *et al.*, 2005), while the BGL2 has specific activity towards aryl β-D-glucosides (Machida *et al.*, 1988). The BGL2 protein from *Saccharomycopsis fibuligera* has optimum pH 5.0, and a temperature optimum of 40-50°C. Lower phenolic contents were reported in wines fermented with *S. cerevisiae* expressing *BGL2*, indicating that this enzyme might have low activity towards glycosides of vinyl guaiacol and vinyl phenol (van Rensburg *et al.*, 2005).

2.19.2.4 β-glucosidases in malolactic bacteria

O. oeni has the ability to hydrolyse glycosylated compounds in oak wood (Bloem *et al.*, 2008). This explains the dramatic increase of aroma, especially vanillin, when malolactic fermentation (MLF) is conducted in oak wood barrels. This increase can be due to the activity of the β-glucosidase produced by this bacteria. The *O. oeni* β-glucosidase is activated by ethanol and does not show anthocyanase activity (Barbagallo *et al.*, 2004a). This activity is present in both the supernatant and cellular fractions (Grimaldi *et al.*, 2000).

According to Mansfield *et al.*, (2002), the *O. oeni* β-glucosidase is incapable of hydrolyzing native grape glycosides. Both *Brettanomyces bruxellensis* and *O. oeni* show β-glucosidase activity towards synthetic substrate pNPG, but they have no activity against native grape glycosides. In contrast, D'Incecco *et al.*, (2004) showed that *O. oeni* does have β-glucosidase activity towards synthetic substrates as well as purified grape glycosides in a chemically defined medium, suggesting that the production and hydrolytic activity of these enzymes may be inhibited in wine. Barbagallo *et al.*, (2004b) have shown that β-glucosidase of *O. oeni* is inhibited by high glucose and fructose concentrations as are found in wine.

Another study has shown that *O. oeni* β-glucosidase is in fact active against native grape precursors, but these experiments have also been conducted in synthetic medium (Bartowsky *et al.*, 2004). This study shows a decrease in the amount of glycosyl-glucose precursors, not specifically monoterpenyl glycosides, therefore one can not conclude from this study that these enzymes have specific activity towards native grape monoterpenyl glycoside precursors. Release of linalool, nerol and geraniol was not detected.

MLF can decrease the amount of glycosides, without a concomitant increase in aglycone concentration (Boido *et al.*, 2002). This can be due to binding of the aglycones with bacterial polysaccharides.

2.19.3 Cloning and expression in *S. cerevisiae*

β-Glucosidases from various sources including bacteria, moulds, yeasts and plants have been cloned in *S. cerevisiae* (Bhatia *et al.*, 2002; Raynal *et al.*, 1987). The aims of most of these studies were concerned with the fermentation of cellobiose (Adam *et al.*, 1995). The capability to ferment cellobiose is not important in the production of wine. The release of glycosidically bound aroma precursors is the main aim in projects concerned with the heterologous expression of β-glucosidases in wine yeast strains.

The *BGL2* gene from *S. fibuligera* has previously been expressed in *S. cerevisiae* under its native promoter and secretion signal (van Rensburg *et al.*, 1998). Wines produced with VIN13 expressing *BGL2* have shown slightly increased levels of monoterpenes (van Rensburg *et al.*, 2005). The *BGLA* gene from *A. kawachii* has also been expressed in *S. cerevisiae* with the objective to enable utilization of cellobiose as carbon source (van Rooyen *et al.*, 2005). This BGLA has wide substrate specificity and can hydrolyze various glucosidic bonds, releasing monoterpenes from their glycosidic precursors.

2.20 Importance of this study

The glycoside composition of different grape varieties as well as the origin of the enzyme can have an immense influence on the aroma of the final product. Often, a large proportion (70% to 90% of the initial amount in juice) of the monoterpene glycosides is not hydrolyzed during fermentation. Many of these monoterpenes are diglycosidically bonded and can therefore only be efficiently released by the action of both an α-L-arabinofuranosidase and a β-glucosidase. Use of arabinofuranosidase and β-glucosidase in combination has been shown to be more effective in releasing monoterpenes from their glycosides than only β-glucosidase (Genovés *et al.*, 2005).

S. cerevisiae does not have arabinofuranosidase activity, and the β-glucosidases of most commercial wine yeast strains are either not expressed, has low affinity for monoterpene glycosides or the enzyme is inhibited under winemaking conditions.

Since cofermentation or the addition of exogenous enzymes are difficult to control with regards to unwanted reactions, and are also sometimes expensive, it is necessary to investigate other avenues for the efficient liberation of monoterpenes from their glycosyl precursors.

Although glycosidases have previously been expressed in *S. cerevisiae*, they were not expressed simultaneously in a commercial wine yeast strain. The co-expression of α-L-arabinofuranosidase and a β-glucosidase is therefore a logical avenue to investigate for the liberation of monoterpenes during fermentation. A genetically engineered wine yeast strain of *S. cerevisiae* that secretes the *A. awamori* AwabfB in conjunction with a β-glucosidase into the must during fermentation, could be useful in improving the flavour and aroma of wines by releasing the glycosidically bound monoterpenols.

β-glucosidase activity might also have health benefits due to the hydrolysis of glycosylated resveratrol isomers (*trans*- and *cis*-piceid) to free resveratrol isomers (*trans*- and *cis*-resveratrol) (González-Candelas *et al.*, 2000).

The use of enzymes with specific activity towards monoterpene glycosides can restrict the release of unwanted compounds like vinyl phenols, and can limit the decolorizing effect that many commercial enzyme preparations confer. Control and predictability of the final monoterpene levels in wine will be beneficial to the winemaker.

2.21 Conclusion

The significant role of monoterpenes in wine aroma has been established. These compounds are sometimes glycosylated to a large extent, serving as a potential source of free, aromatic monoterpenes.

Although numerous studies have examined widely varying techniques for the improvement of wine aroma, the general consensus among researchers seems to be that enzymatic release is the most efficient method. Glycosidases with specific activity towards certain grape precursors do not modify the monoterpenes as is the case with acid hydrolysis. Some α-L-arabinofuranosidases and β-glucosidases possess suitable properties for the liberation of monoterpenes under winemaking conditions. Co-expression of these enzymes in a wine yeast strain could facilitate aroma-enhancing single-strain fermentations. A subtle increase in the content of free terpenols with pleasant floral notes might enhance the characteristic aroma of certain white grape varieties.

Monoterpenes are known to be unstable and rearrange under the acidic conditions of wine. A better understanding is still needed of the dynamics of monoterpene conversion and factors that influence interconversion of monoterpenes.

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Chapter 3

RESEARCH RESULTS

Co-expression of α-L-arabinofuranosidase and β-glucosidase in *Saccharomyces cerevisiae*

Co-expression of α-L-arabinofuranosidase and β-glucosidase in *Saccharomyces cerevisiae*

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Abstract:

Monoterpenes are important aroma compounds in certain grape varieties such as Muscat, Gewürztraminer and Riesling and are present as either odourless, glycosidically bound complexes or as free aromatic monoterpenes. These complexes occur as monoglucosides or, when present as diglycosides, most commonly as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides of mainly linalool, geraniol, nerol and citronellol. α-L-arabinofuranosidase and β-glucosidase are important enzymes responsible for the liberation of monoterpene alcohols from their glycosides. Grape and yeast glycosidases are severely inhibited by winemaking conditions and this leads to unutilized aroma potential. Commercial enzyme preparations can be used to liberate the monoterpenes but they commonly consist of crude extracts that often have unwanted and unpredictable side effects on wine aroma. This project aims to address these problems by expression and secretion of the *Aspergillus awamori* α-L-arabinofuranosidase in combination with either the β-glucosidases *BGL2 f*rom *Saccharomycopsis fibuligera* or the *BGLA* from *Aspergillus kawachii* in the industrial yeast strain *Saccharomyces cerevisiae* VIN13. Enzyme assays and GC-FID results show a significant increase in the amount of free monoterpenes produced in wine fermented with the recombinant *S. cerevisiae* strains. Sensorial evaluation confirms the improvement in the wine aroma profile, particularly the floral character. Recombinant yeast strains constructed during this study have the potential to produce wines with an enhanced aroma profile eliminating the need for the addition of commercial enzyme preparations.

3.1 INTRODUCTION

Selected strains of *Saccharomyces cerevisiae* are widely used as wine yeast starter cultures whose primary role is to convert the grape sugar into alcohol. In addition, the wine yeast's metabolic activities result in the production of higher alcohols, fatty acids and esters, which are important flavour and aroma compounds that influence the sensory profile of the wine.

Monoterpene alcohols also contribute to the flavour and aroma of grapes and wines (Günata *et al*., 1986). This is especially applicable to wines of Gewürztraminer and Muscat varieties, but these flavour compounds are also present in other grape varieties where they supplement other varietal flavours and aromas (Marais, 1983; Wenzel and de Vries, 1968). Geraniol and linalool are considered to be the most important of the monoterpene alcohols since they are present in greater concentrations and have lower perception thresholds than other major wine monoterpenes (Manzanares *et al*., 2003).

Monoterpenes are essentially present in two states: volatile and odourous, or non-volatile and flavourless glycosidically bound complexes (aroma precursors) (Günata *et al*., 1985; Mateo and Jiménez, 2000; Williams *et al*., 1982a). These complexes most often occur as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides, 6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides, 6-*O*-β-D-glucopyranosyl-β-D-glucopyranosides, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides, or 6-*O*-β-D apiofuranosyl-β-D-glucopyranosides of mainly linalool, geraniol, nerol, α-terpineol and hotrienol (Marais, 1983; Mateo and Jiménez, 2000; Williams *et al*., 1982a).

Monoterpenes can be released from their glycosides either by acid or enzymatic hydrolysis. Enzymatic hydrolysis of disaccharide glycosides takes place in two successive steps: α-L-arabinofuranosidase cleaves a glycosidic linkage to yield an arabinose and a monoterpenyl glucoside, and β-glucosidase then liberates the monoterpene alcohol (De Vries, 2000; Flipphi *et al*., 1993; Le Clinche *et al*., 1997; Miyanaga *et al*., 2004). In the case of monoglucosides, the glucosidase acts directly. β-Glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21, BGL) that are capable of monoterpene release from monoglucosides do not have endoglucanase activity; therefore disaccharides can not be released by the action of only β-glucosidase (Günata *et al.*, 1988). The α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55, ABF), are able to hydrolyze the terminal non-reducing α-L-arabinofuranosyl linkages from α-L-arabinofuranosides (Chacón-Martínez *et al.*, 2004).

During the handling of the juice and under winemaking conditions, endogenous grape glycosidases of *Vitis vinifera* and *S. cerevisiae* show very low activity towards the monoterpene glycosides (Aryan *et al.*, 1987; Delcroix *et al.*, 1994). The result is that a large fraction of the monoterpene alcohols in wine remain bound to glycosides resulting in unutilized aroma and flavour potential (Esteve-Zarzoso *et al*., 1998; Manzanares *et al.*, 2003).

High temperature acidic hydrolysis of monoterpene glycosides cause a molecular rearrangement and transformation of the monoterpene aglycones into other, unwanted compounds

and is for this reason not suitable (Mateo and Jiménez, 2000; Williams *et al.*, 1982b). The result of high temperature acid hydrolysis is a decrease in fruity and floral aromas and an increase in butter, rubber and tobacco aromas (De La Presa-Owens and Noble, 1997). Significant sensorial differences exist between volatiles released from a precursor fraction by hydrolysis with a glycosidase enzyme, and those released by acid hydrolysis. The enzymatic hydrolysis is the preferred method (Abbott *et al.*, 1991).

Focus has increasingly fallen on the introduction of exogenous β-glucosidases to wines and juices. These enzymes are mainly of a fungal origin (Colombatto *et al.*, 2003; Mateo and Jiménez, 2000), and are currently produced on an industrial scale from cultures of *Aspergillus niger* (Spagna *et al*., 1998a; Querol and Ramón, 1996). The drawback of commercial enzyme preparations is that they are complex, undefined mixtures of enzymes with non-specific side activities which make it difficult to control their effect on the aroma of the wine (Rocha *et al.*, 2005). Substantial efforts have been made to obtain new preparations without negative or unpredictable effects on the wine, mostly focusing on the purification and characterization of new enzymes with specific activities from fungal preparations. Some purified enzymes have poor stability and therefore would have to have their stability increased, for example, by adopting immobilization techniques or by chemical modification (Spagna *et al.*, 1998a).

Genes encoding α-L-arabinofuranosidases and β-glucosidases, which are responsible for the two-step enzymatic hydrolysis (Günata *et al.*, 1988) of the monoterpenes from their sugar moieties, have been cloned from various organisms, including bacteria, fungi and yeasts. 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 (Le Clinche *et al*., 1997).

The α-L-arabinofuranosidase gene (*AwAbfB*) from *Aspergillus awamori* has been described and the α-L-arabinofuranosidase protein has also been purified, characterised and shown to be active at low pH and low temperature conditions as are found in wine (Kaneko *et al.*, 1998; Koseki *et al.*, 2003). The *A. awamori* α-L-arabinofuranosidase has an optimum activity at pH 4 and 55°C and has been shown to be stable between pH 3 to pH 7 at temperatures of up to 60°C. The possible effect of α-L-arabinofuranosidase on wine parameters was previously investigated with satisfactory results (Crous *et al.*, 1996; Sánchez-Torres *et al.*, 1996; Spagna *et al*., 1998b).

The β-glucosidase A from *A. kawachii* (BGLA) degrades cellobiose, but is also capable of releasing glycosidically bound monoterpenes and is therefore considered to be a wide substrate specificity enzyme. The BGLA enzyme has moderate tolerance towards ethanol, is highly glucose tolerant, shows good stability between pH 3.0 and pH 7.0 and has optimal activity at pH 4.0 (Iwashita *et al.*, 2001). The *BGLA* gene from *A. kawachii* has also been expressed in *S. cerevisiae* with the objective to enable utilization of cellobiose as sole carbon source (van Rooyen *et al.*, 2005).

The *BGL2* gene from the yeast *Saccharomycopsis fibuligera* has specific activity towards aryl β-D-glucosides and has optimum activity at pH 5.0 and a temperature optimum of 40-50°C (Machida *et al.*, 1988). The *BGL2* gene from *S. fibuligera* has previously been expressed in *S. cerevisiae* under its native promoter and secretion signal (van Rensburg *et al.*, 1998). Wines produced with VIN13 (a commercial wine yeast strain) expressing *BGL2* have shown slightly increased levels of monoterpenes (van Rensburg *et al.*, 2005). All of the enzymes used in this study have good stability in wine-like conditions which obliviates the need for immobilization.

S. cerevisiae VIN13 has been chosen for this study due to the fact that it is a widely used industrial wine yeast strain with strong fermentation capability and aroma enhancing characteristics.

S. cerevisiae does not have ABF activity, and the BGL of most commercial wine yeast strains are either not expressed, has low affinity for monoterpene glycosides or this enzyme is inhibited under winemaking conditions.

Since cofermentation (the simultaneous fermentation with yeast and an enzyme producing microbe) or the addition of exogenous enzymes are difficult to control with regards to unwanted reactions, and are also sometimes expensive, it is necessary to investigate other avenues for the efficient liberation of monoterpenes from their glycosyl precursors.

Although glycosides have previously been expressed in *S. cerevisiae*, they were not expressed simultaneously in a commercial wine yeast strain. The co-expression of α-L-arabinofuranosidase and a β-glucosidase is therefore a logical avenue to investigate for the liberation of monoterpenes during fermentation. A genetically engineered wine yeast strain of *S. cerevisiae* that secretes the *A. awamori* AwAbfB in conjunction with a β-glucosidase into the must during fermentation, could be useful in improving the flavour and aroma of wines by releasing the glycosidically bound monoterpenols.

The use of enzymes with specific activity towards monoterpene glycosides can restrict the release of unwanted compounds like vinylphenols (Sánchez Palomo *et al.*, 2005), and can limit the decolorizing (Huang, 1952) effect that many commercial enzyme preparations confer. Control and predictability of the final monoterpene levels in wine will be an additional benefit to the winemaker.

The aim of this study is to develop and evaluate a wine yeast strain that produces enzymes capable of releasing monoterpenes from their glycosylated precursor forms. A subtle increase in the content of free terpenols with pleasant floral notes might enhance the characteristic aroma of certain white grape varieties. The possible impact of these changes on the overall aroma of the wine is also discussed.

3.2 MATERIALS AND METHODS

3.2.1 MICROBIAL STRAINS AND CULTURE CONDITIONS

The sources and relevant genotypes of bacterial and yeast strains, as well as plasmids used in this study, are listed in Table 1. *Escherichia coli* transformants were grown in Luria-Bertani (LB) broth (Biolab, Midrand, South Africa) containing 12 g/l tryptone, 12 g/l NaCl and 6 g/l yeast extract supplemented with 100 μg/ml ampicillin (Roche, Mannheim, Germany) for plasmid selection.

Table 3.1 Microbial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype	Source or reference
Bacterial strain		
E. coli DH5α	F'ϕ80Δ*lacZ*Δ*M15*Δ(*lacZYA-argF*)*U169 deoR reA1 hsdR17* (r_κ^- m_κ^+) *supE44*λ^- *thi1 gyr A96 relA1/F'proABlacI*^{q}*Z*Δ*M15,zzf::Tn5*[*Km*^{r}]	[a]GIBCO-BRL/Life Technologies
Yeast strains		
S. cerevisiae VIN13	Commercial diploid strain	Anchor yeast technologies.
VpSPs	VIN13 *ILV2::* pSPs	This study
VA	VIN13 *ILV2::* pSa	This study
VBa	VIN13 *ILV2::* pSBa	This study
VB2	VIN13 *ILV2::* pSB2	This study
VABa	VIN13 *ILV2::* pSaBa	This study
VAB2	VIN13 *ILV2::* pSaB2	This study
Plasmids		
pGEM®-T Easy	Ap^R Δ*lacZ*	Promega Corporation, Madison, WI, USA
pCEL15AwAbfB	Ap^R ura3 *PGK1*$_P$-*MF*α1$_S$-*AwAbf*B-*PGK1*$_T$	This laboratory
pSPs	pSP73 (Ap^R) *PGK1*$_P$-*MF*α*1*$_S$-*PGK1*$_T$ *SMR1*	This laboratory
pSa	Ap^R *PGK1*$_P$-*MF*α*1*$_S$-*AwAbf*B-*PGK1*$_T$ *SMR1*	This study
pSBa	Ap^R *ENO1*$_P$-*XYN2*$_S$-*BGLA-ENO1*$_T$ *SMR1*	This study
pSB2	Ap^R *ENO1*$_P$-*XYN2*$_S$-*BGL2-ENO1*$_T$ *SMR1*	This study
pSaBa	Ap^R *PGK1*$_P$-*MF*α*1*$_S$-*AwAbf*B-*PGK1*$_T$ *ENO1*$_P$-*XYN2*$_S$-*BGLA-ENO1*$_T$ *SMR1*	This study
pSaB2	Ap^R *PGK1*$_P$-*MF*α*1*$_S$-*AwAbf*B-*PGK1*$_T$ *ENO1*$_P$-*XYN2*$_S$-*BGL2-ENO1*$_T$ *SMR1*	This study
pSFB2	Ap^R *BGL2*	This study
pDLG98	Ap^R *ENO1*$_P$-*XYN2*$_S$-*BGLA-AG*α*1-ENO1*$_T$	Kindly provided by D.C. la Grange, Stellenbosch University
pAZ21	Ap^R *ENO1*$_P$-*XYN2*$_S$-*CBH2-ENO1*$_T$	Den Haan *et al*., 2007
pSFB2	Ap^R *BGL2*	This laboratory
YSF1	Ap^R *PGK1*$_P$-*XYN2*$_S$-*BGL1-AG*α*1-PGK1*$_T$	Van Rooyen *et al.*, 2005

[a]GIBCO/Bethesda Research Laboratories, Life Technologies Ltd., 3 Fountain Drive, Ichinnan Business Park, Paisley, PA4 9RF.

Selection of *E. coli* transformants were carried out with the blue/white selection method on LB agar (Biolab) plates containing 100 μg/ml ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Roche) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) (Sigma, St. Louis, MO, USA).

Yeast peptone dextrose (YPD) broth (Biolab) containing 1% yeast extract, 2% peptone and 2% glucose were used for the general culturing of yeast cells. *S. cerevisiae* transformants were cultured in synthetic complete (SC) medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, U.S.A.), 2% glucose and all the required growth factors except leucine (SC^{-Leu}). Solid media contained 1.5% agar (Difco Laboratories). Selection of *S. cerevisiae* transformants was carried out on SC^{-Leu} media containing 18% D-sorbitol and with the pH adjusted to 5.6. Bacteria and yeasts were routinely cultured at 37°C and 30°C, respectively. Yeast liquid culture growth was measured spectrophotometrically at 600nm and expressed as viable cell number using a calibration curve relating the two parameters.

3.2.2 DNA MANIPULATIONS AND PLASMID CONSTRUCTION

Standard methods were used for plasmid DNA isolation, restriction enzyme digestion, ligation reactions and transformation of *E. coli* DH5α. (Sambrook *et al*., 1989). TaKaRa ExTaq™ DNA polymerase (TAKARA BIO INC; Shiga, Japan) was used in polymerase chain reactions (PCR) required for the manipulation of DNA. *Taq* DNA polymerase (Bioline U.S.A. Inc.) was used for screening and confirmation PCR reactions.

Ligation reactions were carried out with 1 unit of T4 DNA ligase (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Restriction enzyme digests of DNA were carried out with DNA restriction endonucleases from Fermentas and Roche. All gel isolations of DNA fragments were performed with the Zymoclean DNA recovery kit (Zymo Research Corp., U.S.A.).

The primers used in this study are listed in Table 3.2 and the PCR programmes are listed in Table 3.3. PCR amplification was carried out in a final volume of 50 ml of reaction mixture using a thermocycler (Hybaid PCR express, UK). PCR product fragments from each reaction were isolated from an agarose gel, ligated into pGEM®-T Easy and transformed into *E. coli* DH5α. Plasmids were isolated from *E. coli* transformants and analyzed with restriction endonuclease digestions, agarose gel electrophoresis and sequencing.

Table 3.2 Primers used in this study. Specific properties of primers are underlined.

Primer name	Sequence (5'→3')	Properties
Integ 5'F	GCT AAG AGG AGA TAA ATA CAA CAG AAT C	
Integ 3'R	GAC TAC CGA TTT GGT AGA ATG TCA TAT A	
AbFB 3'R	**CTT AAG** GGA TGT GCT GCA AGG CGA TT	*Afl*II
XYNSECF	GAT C**CT CGA G**AT GGT CTC CTT CAC CTC CCT	*Xho*I
XYNSECR	GAT C**TC GCG A**GC GCT TCT CCA CAG C	*Nru*I
BGL2 5'	**TCG CGA** CTT CCT GTT CAA ACT CAT AAT CTG ACT G	*Nru*I
BGL2 3'	**CTC GAG** TGC TCA AAA ATC AAA TAG TAA ACA GGA	*Xho*I
Enot F	GAT CGT TCC TGT AAG GAG AT**C TCG AG**	*Xho*I
Enot R	GAT C**GA GCT C**AA AGA GGT TTA GAC ATT G	*Sac*I
Enop F	GAT C**CT GCA G**TC TTC TAG GCG GGT TAT CTA	*Pst*I
Enop R	GAT C**CT CGA G**TG ATT TAG TGT TTG TGT GTT GAT A	*Xho*I
pDlg98 BGLA 5'	GAT C**CA TAT G**CT AGT CTT CTA GGC GGG TTA TCT	*Nde*I
pDlg98 BGLA 3'	GAT CTT AGT GAA CAG TAG GCA GAG ACG C	Termination codon
pAZ21ENOt5'	GAT C**AC TAG T**AG ATC TCG AGA GCT TTT GAT	*Spe*I
pAZ21ENOt3'	GAT C**CA TAT G**CC GCA AAG AGG TTT AGA CAT	*Nde*I
ACTf	TAA AGC CGG TTT TGC CGG TGA C	
ACTr	CGA TAG ATG GAC CAC TTT CGT CG	
Abf 5'seq	CTG CTA AAG AAG AAG GGG TAT CT	
Abf 3'seq	AGT TAC GAA GCA AAC GCC GT	

*Restriction enzyme sites are indicated in bold

Table 3.3 PCR programmes used in this study.

	Initial denaturation		Amplification		Final elongation	
Program number	Temperature (°C)	Time (min)	Temperature (°C)	Time	Temperature (°C)	Time (min)
1	94	5	94	20s	72	5
			50	35s		
			72	4 min		
2	94	3	94	10s	72	4
			47	30s		
			70	40s		
3	94	3	94	15s	72	5
			49	30s		
			71	3 min		
4	94	3	94	10s	72	3
			44	10s		
			70	25s		

Three expression cassettes were constructed for constitutive expression and secretion of glycoside hydrolases in *S. cerevisiae* VIN13:

1. *AwAbfB* from *A. awamori* under control of phosphoglycerate kinase promoter and terminator, with mating factor α secretion signal.

2. *BGL2 f*rom *S. fibuligera* under control of enolase promoter and terminator, with *Trichoderma reesei* xylanase 2 secretion signal.

3. *BGLA* from *A. kawachii* under control of enolase promoter and terminator, with *Trichoderma reesei* xylanase 2 secretion signal.

3.2.2.1 Construction of plasmid pSa

Plasmid pCel15AwAbfB was used as source of the *A. awamori AwAbf*B expression cassette *PGK1*$_P$-*MF*α*1*$_S$-*AwAbf*B-*PGK1*$_T$ in which the *A. awamori AwAbf*B gene is under control of the constitutive *S. cerevisiae* phosphoglycerate kinase gene promoter (*PGK1*$_P$). The mating pheromone α-factor secretion signal is fused in-frame to the arabinofuranosidase protein to facilitate excretion to the outside of the cell. Plasmids pCel15AwAbfB and pSPs were digested with *Sac*I and *Xho*I. The 2465 bp *PGK1*$_P$-*MF*α*1*$_S$-*AwAbf*B fragment obtained from pCel15AwAbfB were ligated to the 5241 bp fragment of pSPs to obtain plasmid pSa (7716 bp) (Figure 3.1).

3.2.2.2 Construction of plasmid pSaBa

The *ENO1*$_P$-*XYN2*$_S$-*BGLA* fragment was amplified from plasmid pDLG98 with primers pDlg98 BGLA 5' and pDlg98 BGLA 3' using PCR program 1 (Table 3.3) and the product was ligated into the pGEMTEasy vector to create plasmid pGa1. The orientation was verified by restriction enzyme digests to ensure that the *Spe*I site of the pGEMTEasy vector was on the 3' end of the *ENO1*$_P$- *XYN2*$_S$-*BGLA* fragment. The *ENO1*$_T$ terminator sequence was amplified from plasmid pAZ21 with primers pAZ21ENOt5' and pAZ21ENOt3' using PCR program 2 and ligated into pGEMTEasy to create plasmid pGa2. The orientation of this fragment was also verified by restriction enzyme digests to ensure that the *Spe*I site of the pGEMTEasy vector was on the 3' end of the *ENO1*$_T$ fragment. The *ENO1*$_T$ fragment had the *Spe*I site introduced on the 5' end by PCR. Consequently, plasmids pGa1 and pGa2 were restricted with *Spe*I and the *ENO1*$_T$ fragment was introduced after the *ENO1*$_P$-*XYN2*$_S$-*BGLA* fragment in pGa1. This plasmid was designated pBac. Primers pDLG98 BGLA 5' and pAZ21ENOt3' introduced the *Nde*I restriction sites on both sides of the *ENO1*$_P$-*XYN2*$_S$-*BGLA*-*ENO1*$_T$ expression cassette. Plasmids pBac and pSa were digested with *Nde*I and the *ENO1*$_P$-*XYN2*$_S$-*BGLA*-*ENO1*$_T$ cassette was ligated into plasmid pSa to obtain plasmid pSaBa (11421bp) (Figure 3.1).

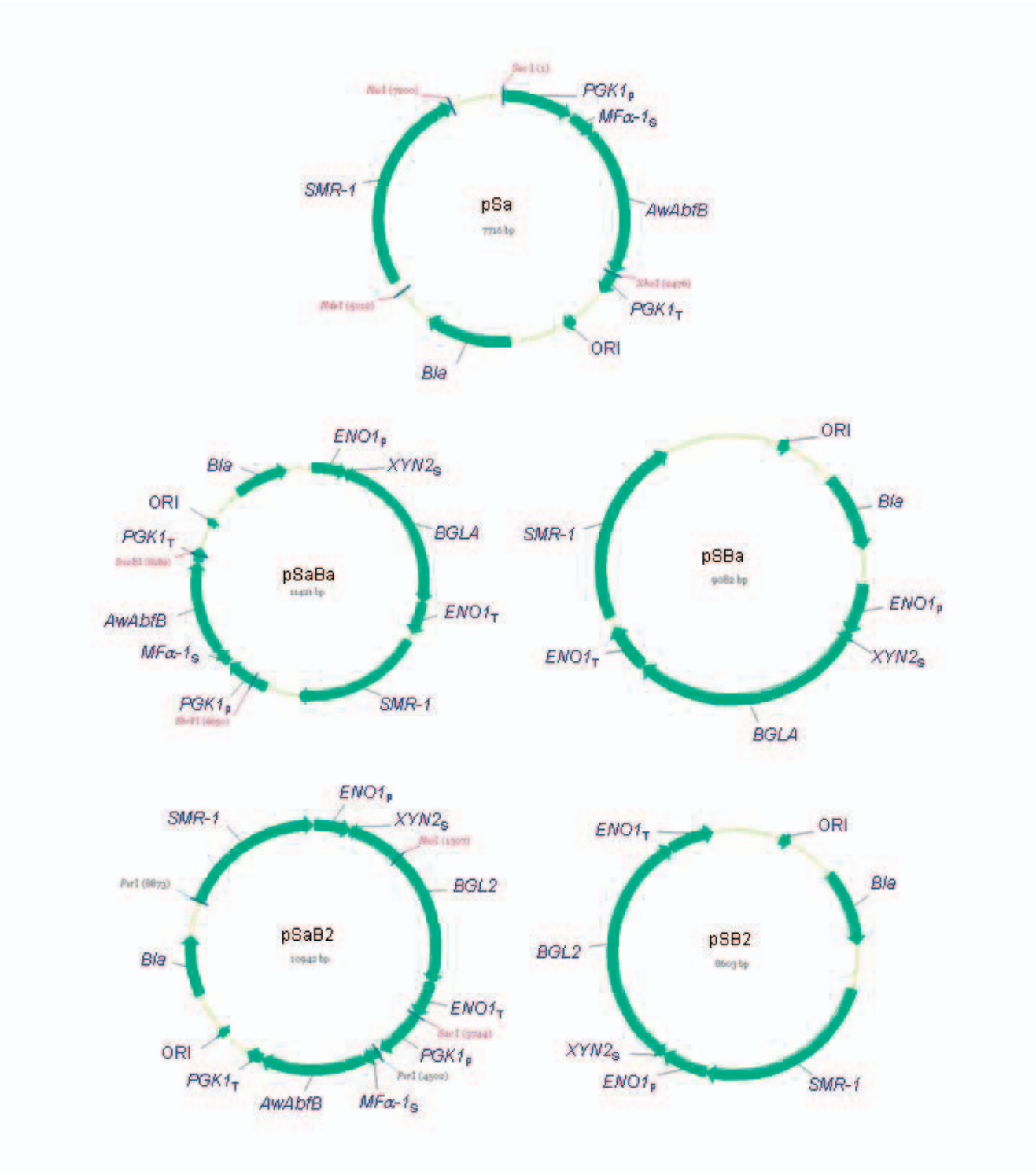

Figure 3.1 Plasmids constructed for integration into *S. cerevisiae* VIN13.

3.2.2.3 Construction of plasmid pSBa

Plasmid pSBa was created by removing the 2339 bp fragment (*PGK1*$_P$-*MF*α*1*$_S$-*AwAbfB-PGK1*$_T$) with restriction enzymes *Bbr*PI and *Sna*BI from plasmid pSaBa. The resulting 9082 bp fragment was isolated and circulized with ligase to retain the *ENO1*$_P$-*XYN2*$_S$-*BGLA-ENO1*$_T$ expression cassette and to remove the arabinofuranosidase expression cassette (Figure 3.1).

3.2.2.4 Construction of plasmid pSaB2

The *BGL2* gene was amplified from plasmid pSFB2 with primers BGL2 5' and BGL2 3' with PCR program 3 and subsequently ligated into the pGemTEasy vector to create pGemB2. The Xylanase 2 secretion signal sequence was amplified from plasmid YSF1 with primers XYNSECF and XYNSECR using PCR program 4 and subsequently ligated into pGemTEasy to create plasmid pGXs. Plasmids pGemB2 and pGXs were digested with *Nru*I and *Spe*I and the 2640bp *BGL2* and 3100bp pGxs fragments were ligated to produce plasmid pGXsB2. The *ENO1* promoter sequence was amplified from plasmid pAZ21 with primers Enop F and Enop R using PCR program 2 and ligated into pGemTeasy to create plasmid pGEp. The *ENO1* terminator sequence was amplified from plasmid pAZ21 using PCR program 2 with primers Enot F and Enot R and ligated into pGemTeasy to create plasmid pGEt. Plasmids pGEp and pGEt were digested with *Sph*I and *Xho*I restriction endonucleases and the 557bp *ENO1* promoter fragment was ligated upstream of the *ENO1* terminator sequence in pGEt to create plasmid pGEpt.

Plasmids pGXsB2 and pGEpt were digested with restriction endonuclease *Xho*I and the 2713 bp *XYN2*$_S$-*BGL2* fragment was ligated between the *ENO1* promoter and terminator sequences in plasmid pGEpt to create plasmid pGB2c. Plasmid pGB2c was digested with *Pst*I and *Sac*I and the 3743 bp *ENO1*$_P$-*XYN2*$_S$-*BGL2-ENO1*$_T$ expression cassette was isolated and ligated into plasmid pSa previously digested with *Nsi*I and *Sac*I to obtain plasmid pSaB2 (10942bp) (Figure 3.1).

3.2.2.5 Construction of plasmid pSB2

Plasmid pSB2 was created by removing the 2339 bp fragment (*PGK1*$_P$-*MF*α*1*$_S$-*AwAbfB-PGK1*$_T$) with restriction enzymes *Bbr*PI and *Sna*BI from plasmid pSaB2. The resulting 8603 bp fragment was isolated and circulized with ligase to retain the *ENO1*$_P$-*XYN2*$_S$-*BGL2-ENO1*$_T$ expression cassette and to remove the arabinofuranosidase expression cassette (Figure 3.1).

3.2.3 YEAST TRANSFORMATION

Plasmids pSa, pSBa, pSaBa, pSb2, pSab2 and pSPS were linearized at the *Eco*NI restriction site located in the *SMR* marker and transformed into *S. cerevisiae* strain VIN13 by electroporation at 1.5kV, 25µF and 200Ω. The transformed strains are listed in Table 3.1.

The integration occurs at the *ILV2* gene on Chromosome XIII of the host genome. The *ILV2* gene codes for the amino acid biosynthetic enzyme acetolactate synthase (ALS) (Falco *et al*., 1985). Putative *S. cerevisiae* tansformants were selected on SC^{-Leu} media supplemented with sulfometuron methyl (SMM) at 50 μg/μl (Casey *et al*., 1988). *The SMR1* gene confers resistance to the herbicide sulfometuron methyl. Both untransformed VIN13 yeast and empty vector-transformed (VIN13 *ILV2::*pSPs) were used as negative controls.

The stability of transformants was tested by culturing the yeast non-selectively in YPD medium over 20 generations (three successive inoculations from OD_{600} 0.1 to OD_{600} 7.0 after the initial inoculation from OD_{600} 0.1 to OD_{600} 1.0). The individual colonies were replicated onto SD plates containing SMM media. All colonies showed SM^R phenotypes and presented the expected enzyme activities against *p*-nitrophenyl substrates.

3.2.4 INTEGRATION PCR ANALYSIS

Yeast genomic DNA was prepared according to standard methods (Sambrook *et al*., 1989).

Positive transformants were isolated and a genomic DNA PCR screen was done to confirm integration of the expression cassettes with primers that bind on genomic DNA and another primer that bind to the inserted fragment at either the start or end of the expression cassette.

Primers that were used for integration and stability studies are Integ 5'F and Enot R for the *ENO1*$_P$-*XYN2*$_S$-*BGL2*-*ENO1*$_T$ cassette, Integ 3'R and Enop F for the *ENO1*$_P$-*XYN2*$_S$-*BGLA*-*ENO1*$_T$ cassette, and Integ 5'F and *AbF*B 3'R for the *PGK1*$_P$-*MF*a*1*$_S$-*AwAbf*B-*PGK1*$_T$ cassette.

3.2.5 REVERSE TRANSCRIPTION PCR ANALYSIS

Transcription of integrated genes was verified by reverse-transcription PCR analysis using the Superscript™ III First-Strand Synthesis System from Invitrogen life technologies, Carlsbad, California, U.S.A.). Total RNA was isolated from yeast cells in the exponential growth phase (at OD_{600} 2.0) according to the acid phenol and chloroform extraction protocol devised by Schmitt *et al*., (1990). RNA samples were subjected to PCR and gel electrophoresis to test for DNA contamination. The manufacturer's protocol for reverse transcription was followed using an oligo $(dT)_{20}$ primer and 5 μg of total cellular RNA.

After first strand DNA synthesis of total RNA, the RNA was digested by addition of RNase H. Gene-specific primers were used for the PCR amplification of reverse transcribed genes using 1μl of the RT-PCR products. The BGL2 3' and BGL2 5' primer pair was used for the *XYN2*$_S$-*BGL2* sequence, pDLG BGLA 3' and Bacpg1 primer pair for the *XYN2*$_S$-*BGLA* sequence, and the Abf 5'seq and Abf 3'seq primer pair for the *MF*a*1*$_S$-*AwAbf*B sequence. Primers for yeast actin ACTf and ACTr were used for positive control reactions to test for specimen adequacy, RNA extraction, and the presence of PCR inhibitors.

3.2.6 ENZYME ASSAYS

3.2.6.1 Liquid culture conditions for production of heterologous glycosidases

Yeast cells were cultured in 5 ml of non-selective YPD medium, inoculated at 10^6 cells per ml from overnight cultures and incubated at 30°C on an orbital shaker at 180 rpm for a total of 96 hours. Prior to inoculation, cells from overnight cultures were centrifuged for 2 min at 3000 rpm at 4°C and washed with distilled water to prevent carryover of excreted enzyme from the preculture. At different intervals, 100 µl of culture was collected under aseptic conditions and assayed for enzymatic activities present in the supernatant. For each assay, cells were plated out in a series of dilutions to determine live cell numbers. Cell growth was measured as absorbance at 600 nm and viable cell numbers were calculated by linear regression to the logarithmic curve of the OD_{600} versus time during exponential growth.

3.2.6.2 Enzyme activity assays

Enzyme assays were done according to the method of Machida *et al*., (1988). Samples for assay were grown to OD_{600} 1.0 and then clarified by centrifugation (5000 x g, 10 min, 4°C). The supernatant was used to measure extracellular activity, hence 39 µl of the supernatant was transferred to a 1.5 ml tube containing 10 µl of McIlvaine buffer pH 5.0 (0.2 M Na_2HPO_4 and 0.1 M citric acid), 1 µl of 200 mM pNP substrate (*p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, Sigma-Aldrich) was added, and the mixture was incubated for 80 min at 42°C. The reaction was then stopped and the pH raised by the addition of 500 µl of 0.25 M Na_2CO_3, and the absorbance was measured at 405 nm against a blank containing YPD in place of the sample. Enzyme stability was evaluated in McIlvaine buffer at pH 5.0 to calculate and confirm applicable incubation times with the *p*-nitrophenol substrate. A substrate blank (buffer and substrate) and sample blanks (cell preparation and buffer) were prepared and subtracted from experimental absorbance readings. All assays were performed in triplicate. A series of standard solutions of *p*-nitrophenol containing 0 to 200 nM pNP were prepared to make a calibration curve and calculate the molar extinction coefficient of 16600 $M^{-1}cm^{-1}$ for pNP-arabinofuranoside and pNP-β-D-glucopyranoside. Results are given as micromoles of pNP released per milliliter per hour.

The activity of the enzymes was determined over the pH range of 2-7 at 42°C. The influence of temperature was determined at the optimum pH of each enzyme over the temperature range of 5 to 65°C for a period of 80 minutes.

To determine enzyme stability at different temperatures, the supernatant was incubated for 30 min at temperature values in multiples of 5°C between 5 and 65°C and the remaining activity was measured by incubating for another hour with McIlvaine buffer and the appropriate pNP substrate at 42°C. Similarly, enzyme stability at different pH levels was determined by incubating

the supernatant adjusted with NaOH or HCl in the pH range 2-7 for 30 min, and then incubating for another hour with McIlvaine buffer and the appropriate pNP substrate at 42°C.

To determine the effect of glucose and ethanol at concentrations ranging from 0 to 1 M and from 0 to 16% (v/v), respectively, on the activity of arabinofuranosidase and β-glucosidase, activity was measured and calculated as a percentage of activity for a solution without these factors in McIlvaine buffer pH 5.0.

3.2.6.3 Enzyme activity location

The supernatant was used for determination of extracellular activity, whereas the cell-bound activity was determined after isolation of cells. Cells were harvested from 5 ml of culture by centrifugation of 1 ml at 5000 rpm for 2 minutes at 4°C and washed twice with cold distilled water. The pellet was resuspended in 0.2 ml McIlvaine buffer pH 5.0 and assayed for activity.

3.2.7 MICROVINIFICATION

Common fermentation parameters for the production of white wines were used. 200 kg of Gewürztraminer grapes were destemmed and crushed and allowed to undergo skin contact for 24 h at 15°C after addition of 40 mg/l SO_2. The juice and skins were then pressed and the juice was kept at 15°C for 24 h for clarification. Juice was siphoned from sediment, analyzed and kept at 4°C. Analysis of juice revealed a titratable acid level of 6.7 g/l, a pH of 3.7, and total reducing sugar of 210 g/l. The FOSS Grapescan 2000 (Foss Electric, Denmark) were used to measure initial sugar content of must. The final point of fermentation was determined using glucose as standard. At the end of fermentation (sugar content below 2 g/l), 1.5 l of grape juice was dispensed in triplicate for each fermentation into sterile 2 l glass bottles.

Yeast were grown in YPD up to an OD_{600} of 4.0 at 30°C, and then centrifuged at 500 x g for 2 min to collect the cells. Yeasts were inoculated into the must at a concentration of 2 x 10^6 cells/ml at 15°C. Fermentation was conducted in an incubator regulated at 15°C and was monitored by CO_2 release: the amount of CO_2 released was determined by measuring weight loss at least every 24 h. Once weight loss stabilised for 48 h, the fermentation was considered complete. 10 mg/l SO_2 was added, the wines were racked from the lees and kept in 750 ml screw cap bottles at 15°C. All wine samples collected during and after fermentation was centrifuged to remove yeast cells and debris and kept at 4°C until analysis.

To compare the aroma liberating capacity of the recombinant yeast strains produced in this study with that of a commercial glycosidase preparation, we added LAFAZYM AROM (Laffort Oenologie, Bordeaux, France) to wine prepared by the untransformed VIN13 yeast. LAFAZYM AROM is described as a pectolytic enzyme preparation for revealing terpenic varietal aromas from their precursors and is specified to contain high activity of β-glucosidase which enables hydrolysis of sugars at the terminal extremities of terpenic precursors. This preparation is obtained from

purified and concentrated extracts of selected strains of *A. niger*. Because the activity of LAFAZYM AROM is inhibited by sugar, it is used at the end of fermentation at a dosage of 5 g/hl by dissolving 5 g in 50 ml water and using it immediately with a contact time of three weeks.

3.2.8 WINE ANALYSIS

Fourier Transform Infrared Spectroscopy (FTIR) utilizing the GrapeScan 2000 was used during and after fermentation to determine ethanol, reducing sugar, pH, volatile acidity, total acidity, malic acid and lactic acid levels.

3.2.9 GLYCOSIDICALLY BOUND MONOTERPENE ANALYSIS

Solid phase extraction was used for the isolation of glycosidically bound monoterpenes whereafter the glycosidically bound monoterpenes were liberated by acid hydrolysis.

Glycosidically bound aroma precursors were quantified in must and wines fermented with *S. cerevisiae* VIN13 and VAB2 using an adaptation of the method by Williams *et al*., (1995) as modified by Arévalo Villena *et al.*, (2006) to determine the potential hydrolysis of monoterpenes.

An equimolar proportion of free aglycons and glucose is obtained after acid hydrolysis. Glucose is then measured with an enzyme kit and the aroma precursors quantified. This gives an estimation of the total concentration of glycosylated secondary metabolites. Evaluation of the method with regards to retention and recovery of glycosidically bound precursors was performed with a range of concentrations of the synthetic glycoside *N*-octyl β-D-glucoside solutions (Sigma) from 0 to 50 μM. Actual *vs*. expected recovery was almost equivalent, yielding a correlation coefficient (r^2) of 0.996, identical to that obtained by Arévalo Villena *et al*., (2006).

3.2.9.1 Isolation of glycosidically bound monoterpenes

Must and wine samples were centrifuged at 4000 x g for 5 minutes. The 500 mg C_{18} HF Bond Elut LRC-C18 OH cartridges (Varian, Palo Alto, CA, U.S.A.) were pretreated with methanol, washed with water and then with wine simulant. Samples were then passed (15 ml must, 20 ml wine) through the column, observing similar flow rates. The column was then washed three times with 3 ml water and was run dry under vacuum for 5 minutes. Glycosides were eluted with 1.5 ml ethanol and then followed with 3 ml dH_2O at a flow rate of approximately 1 ml/min. The samples were adjusted to a final volume of 5 ml with dH_2O.

3.2.9.2 Acid hydrolysis

To 0.5 ml aliquots of the above glycoside eluate was added H_2SO_4 (1.0 ml, 2.25M). A control solution was similarly prepared for each eluate, with 1 ml water added in place of the H_2SO_4 solution, for determination of the free glucose concentraion of the eluate. A reagent blank was

made with 30% ethanol in place of the glycoside eluate. Samples and the reagent blank were brought to boiling temperature (107°C) for 1 h, while controls were held at room temperature.

3.2.9.3 Analysis of D-Glucose

The glucose assay kit from Megazyme International, Wicklow, Ireland was used according to the manufacturer's instructions. A 262 μl aliquot of solutions after hydrolysis was transferred to plate wells to which 3 M NaOH solution (260 μl) was added. NaOH was substituted for water in control solutions. Fifty μl of these samples were assayed for D-glucose according to the manufacturer's instructions and the potential amounts of glycosidically bound precursors were calculated from the D-glucose values obtained.

3.2.10 FREE MONOTERPENE ANALYSIS

Free monoterpenes were isolated by solid phase extraction and quantified by GC-FID.

3.2.10.1 Extraction of monoterpenes

The wine samples were analyzed every third day of the fermentation. Each sample of wine was passed through a 500 mg C_{18} Varian HF Bond Elut LRC-C18 OH cartridge, previously conditioned with 4 ml dichloromethane, solvated with 4 ml methanol and rinsed with 4 ml of wine simulant (12% ethanol, 2.5 g/l tartaric acid pH 3.5).

The wine samples (50 ml) were spiked with 50 μl internal standard (2.5 μg/l of 2-6-dimethyl-6-hepten-2-ol in ethanol) and passed through the cartridge. The cartridge was washed with 4 ml water and then dried under vacuum for 15 minutes. The free monoterpenes were eluted with 2 ml of dichloromethane and dried with anhydrous Na_2SO_4. This dichloromethane extract was analyzed by GC-FID.

3.2.10.2 Gas chromatography – flame ionization detector (GC – FID) analysis

One microliter of extract was injected into a DB-FFAP capillary column (60 m × 0.32 mm × 0.25 μm) (Agilent, Little Falls, Wilmington, USA) with a splitless system for 2 min. GC-FID analysis was carried out using a 6890 Plus Gas chromatograph (Hewlett-Packard, Palo Alto, CA) interfaced with an FID detector. The carrier gas was Helium with a flow rate of 30 ml/min. The injection volume was 1 μl, the injector temperature was 220°C and the detector temperature was 250°C. Oven temperature was programmed at 40°C for 12 min, from 40 to 190°C at a rate of 12°C/min, from 190 to 250°C at a rate of 15°C/min, and then held at 250°C for 2 min.

3.2.10.3 Data analysis

Each wine was extracted once, but injected into the instrument three times from the same extract. The average of the three injections for each compound present in the wine is then calculated and the reported monoterpene concentrations represent the means and standard deviations of three

independent vinifications. Quantitative data were obtained by comparison of relative peak areas of each compound in the graph to that of the internal standard. The statistical significance of the results has been calculated through one-way analysis of variance (ANOVA). Tukey's test was used to determine significant differences between group means. A significance level of 5% was used in all cases.

3.2.11 SENSORIAL ANALYSIS

Sensory analysis of wines was suitably conducted by a panel of 14 trained wine-testers, who had previous experience in wine sensory analysis. Samples were coded and randomly presented to minimize positional bias and expectation error. Triangle tests were performed to determine if the controls and enzyme-treated wines were significantly different and the significance of the test was established from statistical tables.

Wines were only sniffed and only aroma attributes were considered. Wines were presented in coded standard wine-testing glasses and covered with a petri dish to minimize the escape of volatile components. The testing temperature was 10°C. Preference testing was conducted using attributes previously agreed upon as best for describing sensorial characteristics and capable of distinguishing one from another.

3.3 RESULTS AND DISCUSSION

S. cerevisiae VIN13 was transformed with heterologous genes encoding α-L-arabinofuranosidase and β-glucosidase singly or in combination. It has been shown in previous studies that yeast can produce functional heterologous glycosidases. In the current study, we co-expressed the two most notable genes coding for enzymes responsible for the release of glycosidically bound precursors and analyzed the effect on the monoterpene concentrations of the wine.

3.3.1 INTEGRATION AND EXPRESSION OF α-L-ARABINOFURANOSIDASE AND β-GLUCOSIDASE IN *SACCHAROMYCES CEREVISIAE*

The *N*-terminal residue of the *AwAbfB* and *BGL2/BGLA* mature protein-coding DNA sequences are preceded by the yeast mating pheromone α-factor secretion signal sequence and the yeast xylanase secretion signal sequences, respectively. *S. cerevisiae* strain VIN13 was transformed with the linearized plasmids by electroporation. After 72 h of growth, between 1 and 20 sulfometuron methyl resistant transformants developed on SMM-containing SC^{-LEU} selection plates. A yeast strain was constructed containing only the empty pSPs plasmid and was used as the negative control. The growth rate of transformants and controls are similar to that of *S. cerevisiae* VIN13.

PCR, restriction enzyme digests and gel electrophoresis of genomic DNA was used to confirm integration of the *AwAbfB* and *BGL2/BGLA* expression cassettes in the expected combinations at

the ILV2 locus in *S. cerevisiae* VIN13. Although the production of acetolactate synthase (encoded by ILV2) is important for aroma compound formation (higher alcohols), the gene remains active, therefore no influence is expected on higher alcohol levels after fermentation with these transformed yeasts. On the basis of band sizes of PCR products of genomic DNA of transformants, the genes appear to be integrated in single copy (Figures 3.2 – 3.4). In Figure 3.4 primers were used that bind to the genomic DNA on either side of the ILV2 locus. This PCR reaction yielded a 2205 bp product, indicating that one of the ILV2 loci were not changed by integration of the plasmids in all the transformed strains.

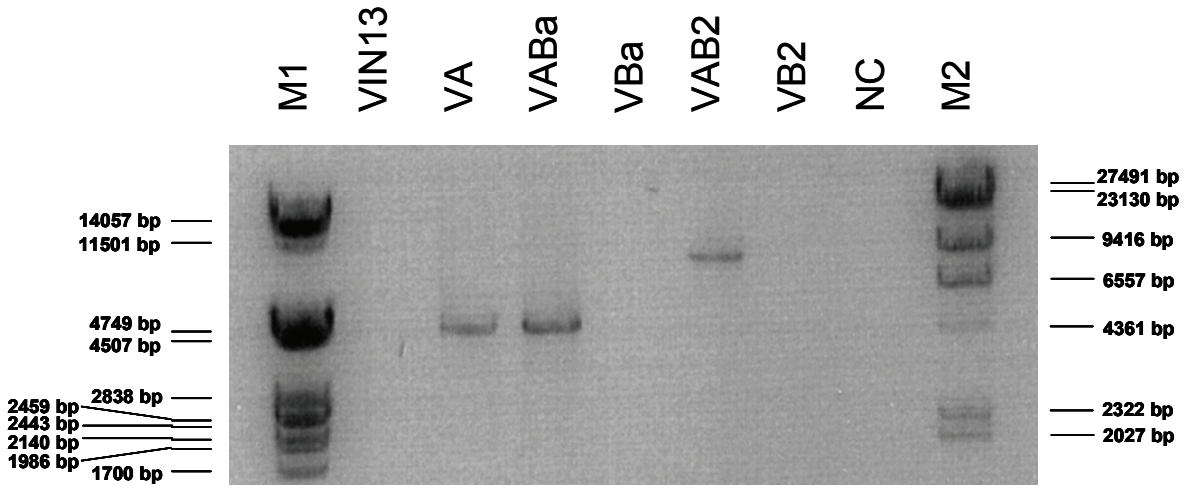

Figure 3.2 Detection of integrated arabinofuranosidase by PCR. NC, Negative control; M1, Marker (λ DNA digested with *Pst*I), M2, Marker (λ DNA digested with *Hind*III).

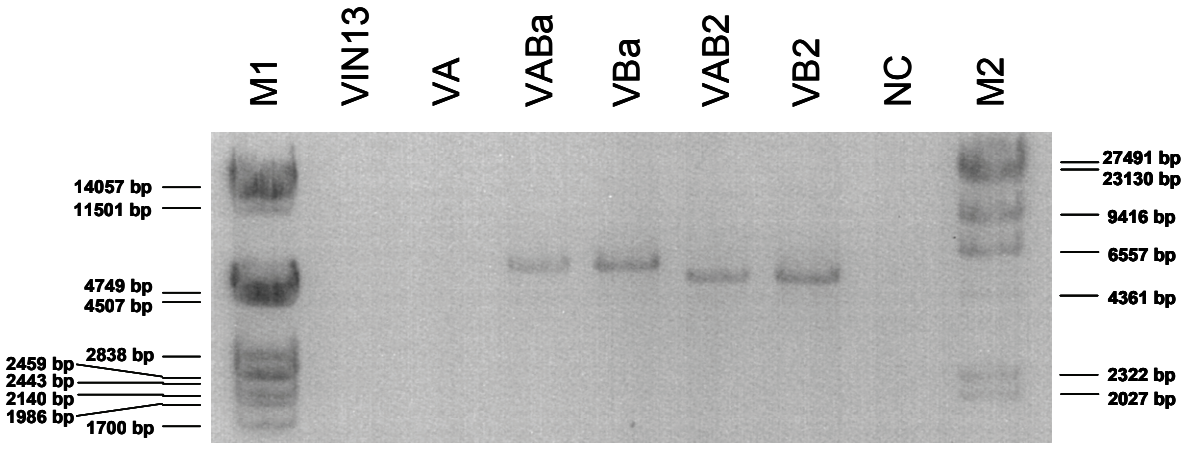

Figure 3.3 Detection of integrated Beta-glucosidases by PCR. NC, Negative control; M1, Marker (λ DNA digested with *Pst*I), M2, Marker (λ DNA digested with *Hind*III).

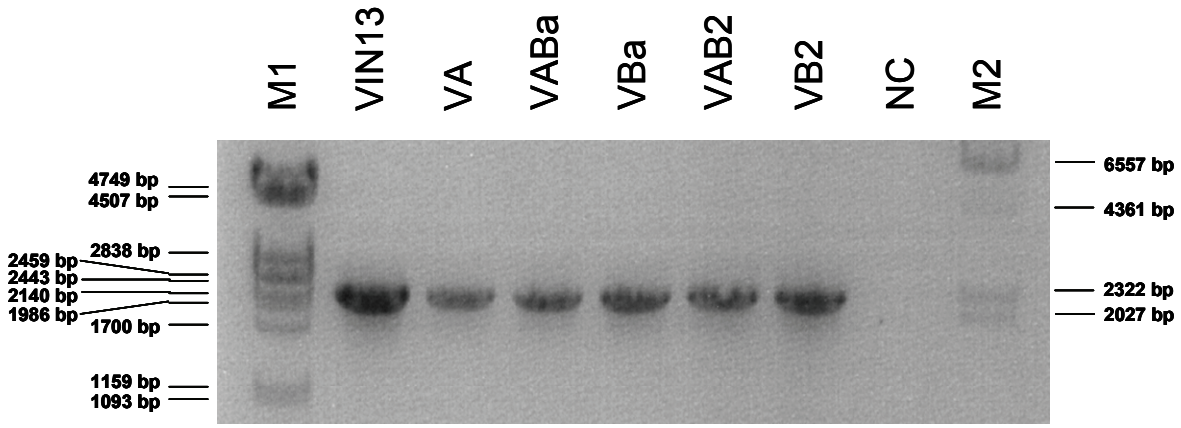

Figure 3.4 Control PCR showing single copy integration of expression cassettes. NC, Negative control; M1, Marker (λ DNA digested with *Pst*I), M2, Marker (λ DNA digested with *Hind*III).

Reverse-Transcription-PCR was used to confirm expression of integrated genes in VIN13 (Figures 3.5 – 3.6). Fragments obtained on agarose gels after electrophoresis correspond to the expected size of the gene coding sequences of *AwAbfB* and *BGL2/BGLA.* Restriction enzyme digests of the reverse-transcribed genes also confirm the identity of these genes (data not shown).

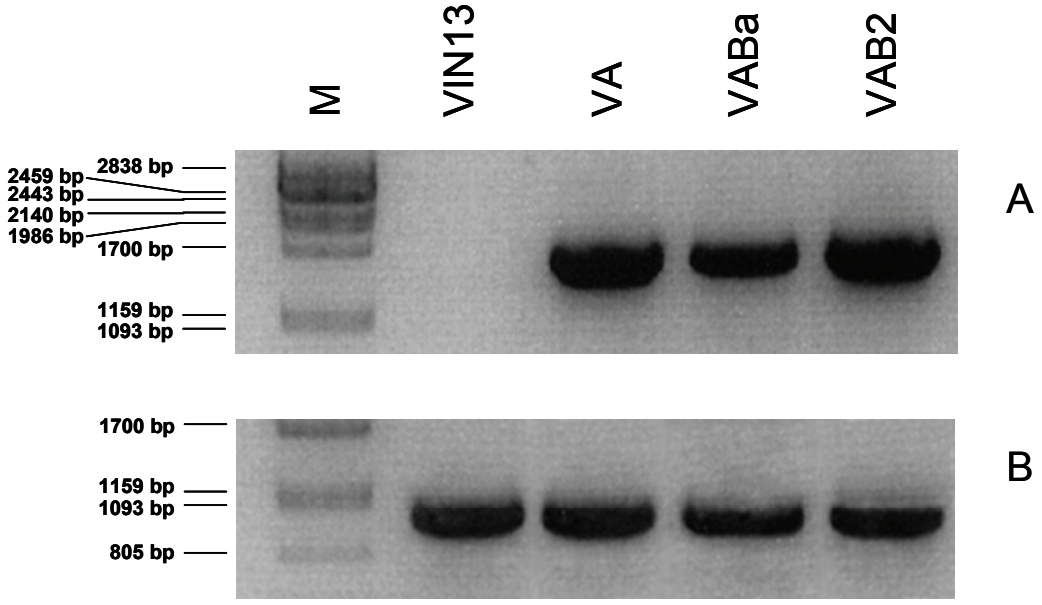

Figure 3.5 Reverse-transcription PCR of the 1.5 kbp α-L-arabinofuranosidase confirms expression of heterologous genes in *S. cerevisiae*. M= Marker (λ DNA digested with *Pst*I), NC= negative control (**A**). The yeast actin gene was used as internal control (**B**).

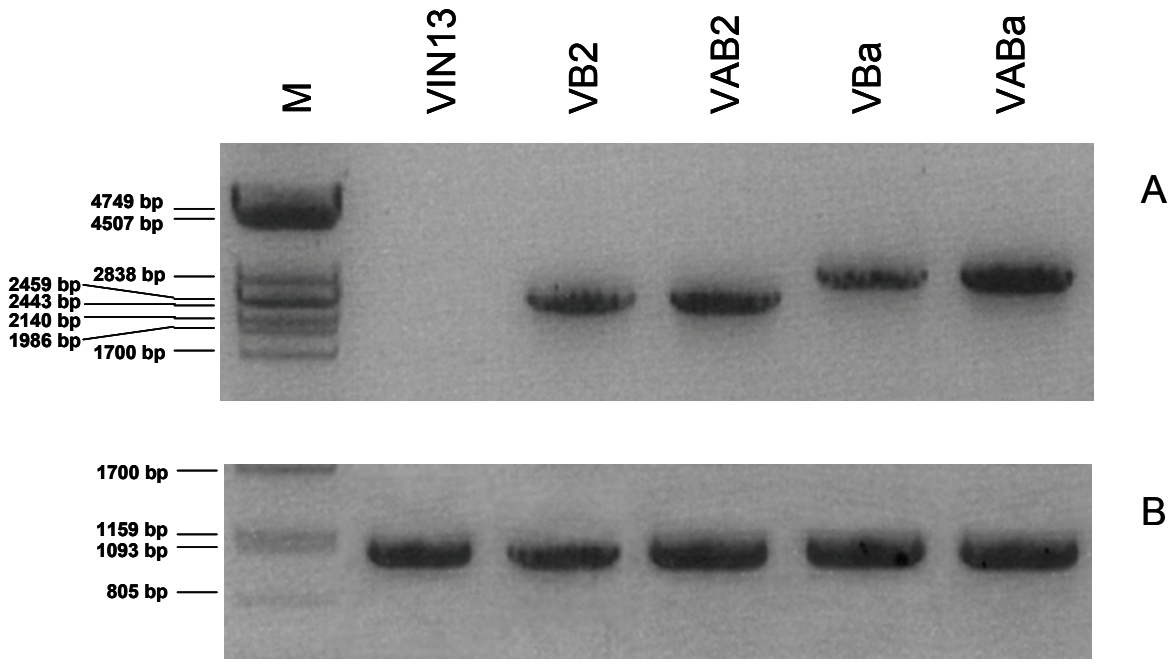

Figure 3.6 Reverse-transcription PCR of β-glucosidase (BGL2: 2.6 kbp, BGLA: 2.7 kbp) confirms expression of heterologous genes in *S. cerevisiae*. M= Marker (λ DNA digested with *Pst*I), NC= negative control (**A**). The yeast actin gene was used as positive control (**B**).

3.3.2 CO-EXPRESSION OF α-L-ARABINOFURANOSIDASE AND β-GLUCOSIDASE IN *S. CEREVISIAE*

ABF activity towards pNP substrate was only detected in transformants VA, VABa and VAB2, indicating that the *AwAbfB* gene was functionally expressed. Although *S. cerevisiae* VIN13 has some BGL activity towards the synthetic pNP substrate, it is clear that transformants harbouring the *BGLA* and *BGL2* genes (VBa, VABa, VB2, VAB2) have greatly increased levels of activity in YPD media that follows a similar pattern of expression to *AwAbfB*. The greatest increase in enzyme activity took place during the first two days of growth, in agreement with the constitutive expression of the *AwAbfB* gene by the *PGK* promoter and the *BGLA* and *BGL2* genes each under control of the *ENO1* promoter (Figure 3.7). Microscope examination of the culture broth confirmed the presence of yeast and the absence of bacteria.

The activity of α-L-arabinofuranosidase and β-glucosidases increased sharply up to 18 h, increased slowly from 18 up to 72 h, and then declined slowly. Secretion of the α-L-arabinofuranosidase and the β-glucosidase into the culture fluid by the recombinant yeast was demonstrated by doing the enzyme assay with the supernatant of the growth medium after centrifuging the culture broth. Enzyme activity, based on hydrolytic activity on pNP, was mainly located in the extracellular fraction. Trace levels of activity were obtained with the cell-bound fraction. The growth rates of the recombinant strains were identical to each other and to that of the untransformed *S. cerevisiae* VIN13 (data not shown).

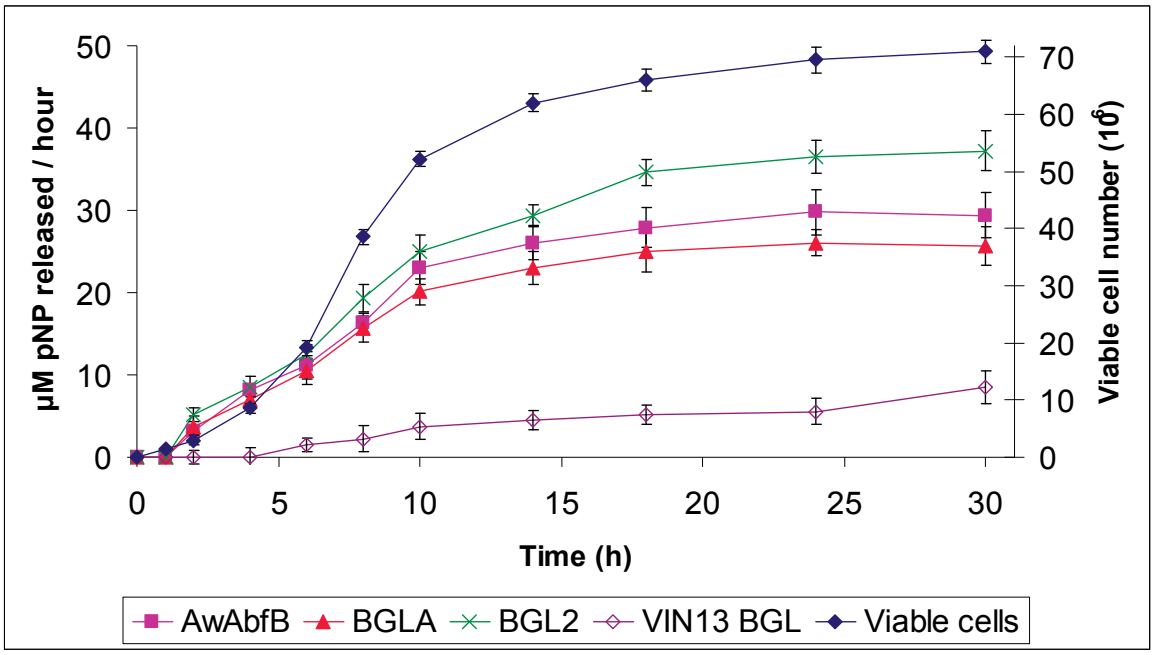

Figure 3.7 The α-L-arabinofuranosidase and β-glucosidase activities in *S. cerevisiae* VIN13 control strain and strains transformed with glycosidase genes *AwAbfB* from *A. awamori*, *BGL2* from *S. fibuligera* and *BGLA* from *A. kawachii*. The mean growth curve of all strains is denoted by (◆). The backround β-glucosidase activity in *S. cerevisiae* VIN13 control strain is denoted by (◇). No arabinofuranosidase activity was detected in *S. cerevisiae* VIN13. Enzyme activity is expressed as the amount of *para*-nitrophenol (pNP) released from *para*-nitrophenyl substrates per hour. Error bars indicate 1.96 standard deviations of two independent experiments performed in triplicate. The average enzyme activity between different strains harbouring the same heterologous gene did not vary by more than 4% and are therefore combined to avoid complicating the graph.

It has been suggested that the native yeast β-glucosidase might be located in the periplasmic space of the yeast cell and is released upon cell death and autolysis (McMahon *et al*., 1999). The low levels of β-glucosidase activity observed in *S. cerevisiae* is probably due to exoglucanases (Gil *et al*., 2005). Assays on cell-bound activity done on the cellular fraction of cultures have revealed that very little activity is associated with the cellular fraction of the liquid culture (data not shown).

3.3.3 pH DEPENDANCE AND STABILITY OF HETEROLOGOUSLY EXPRESSED α-L-ARABINOFURANOSIDASE AND β-GLUCOSIDASE.

The activity and stability of the enzymes were determined at a range of pH values. The AwAbfB retained 18% activity after 30 min at pH 3.5 and also had optimum activity at pH 3.5 (Figure 3.8). BGLA is very unstable below pH 3.5 with a 95% loss in activity after 30 min at pH 3.5 (Figure 3.9). BGL2 is more stable at low pH values than BGLA and retains 17% of its activity after 30 min at pH 3.5 (Figure 3.10). Both BGLA and BGL2 possess optimum activity at pH 4.0. All three of the enzymes expressed in *S.cerevisiae* have characteristics similar to what they have when expressed in their native host (Iwashita *et al*., 2001; Kaneko *et al*., 1998; Machida *et al*., 1988).

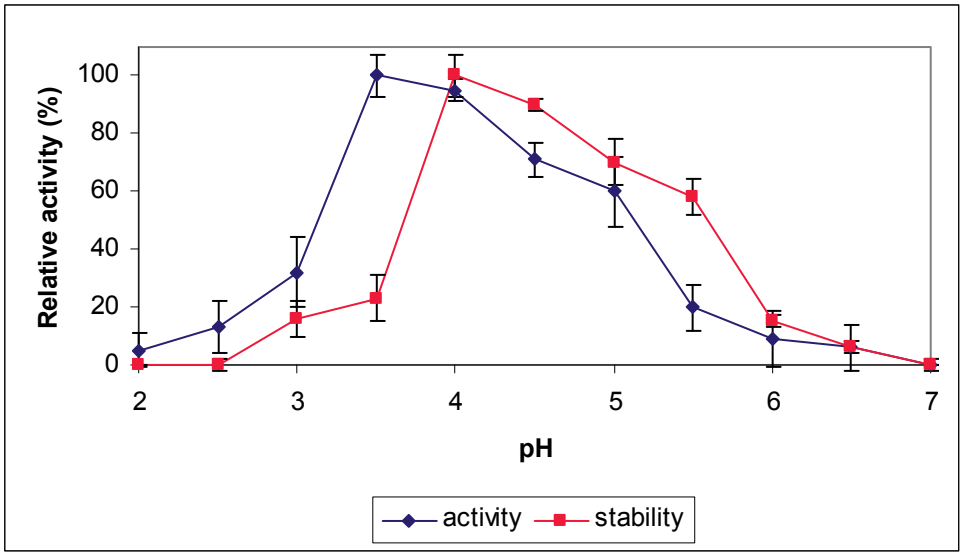

Figure 3.8 Relative pH activity and stability of AwAbfB expressed in VIN13. Values are averages of two independent experiments performed in triplicate. Error bars indicate 1.96 standard deviations.

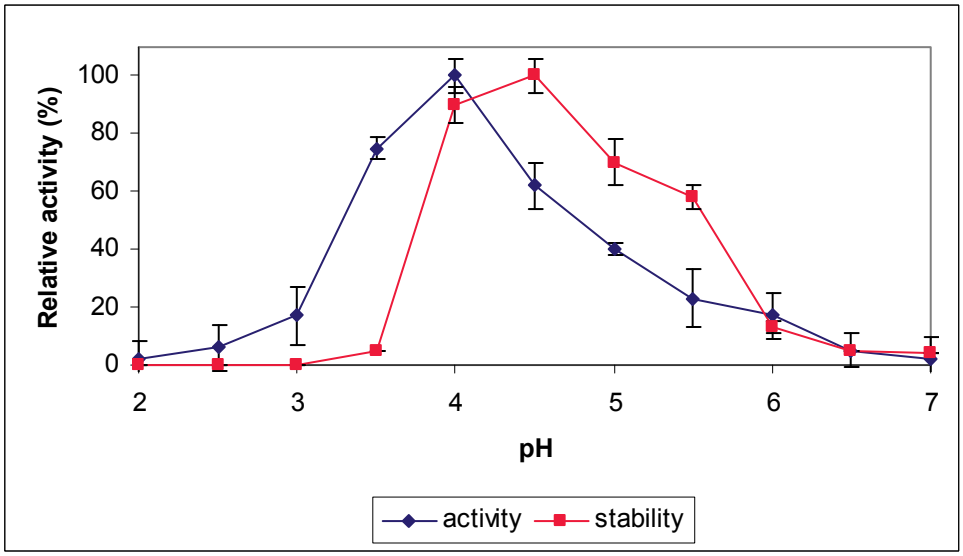

Figure 3.9 Relative pH activity and stability of BGLA expressed in VIN13. Values are averages of two independent experiments performed in triplicate. Error bars indicate 1.96 standard deviations.

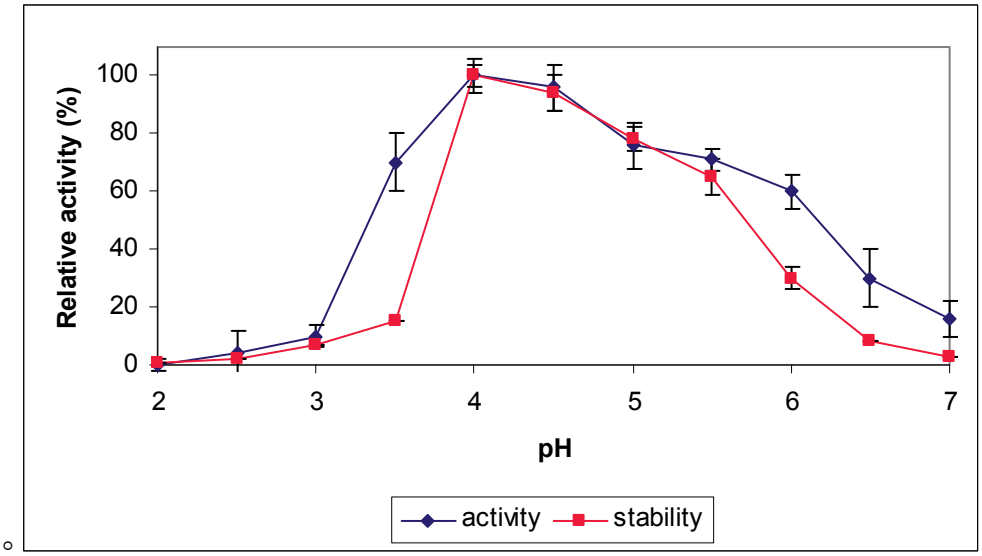

Figure 3.10 Relative pH activity and stability of BGL2 expressed in VIN13. Values are averages of two independent experiments performed in triplicate. Error bars indicate 1.96 standard deviations.

3.3.4 TEMPERATURE DEPENDANCE AND STABILITY OF HETEROLOGOUSLY EXPRESSED α-L-ARABINOFURANOSIDASE AND β-GLUCOSIDASE.

The activity and stability of AwAbfB, BGLA, and BGL2 expressed in *S. cerevisiae* VIN13 was determined at the pH levels at which optimum levels of activity were obtained. The optimum temperature for AwAbfB activity is 55°C and stability of the enzyme declined sharply at temperatures above 60°C (Figure 3.11). Maximum enzyme activity obtained with AwAbfB-harbouring VIN13 strains was 2.58 µM pNP/hour/10^6. BGLA was also optimally active at 55°C, releasing 1.29 µM pNP/hour/10^6 cells (Figure 3.12). Stability and activity of this enzyme declined sharply at temperatures above 60°C. BGL2 was more sensitive to high temperatures and had optimal activity between 45°C and 50°C (Figure 3.13). The BGL2 was highly unstable at temperatures above 55°C. Maximum activity for this enzyme, when expressed in VIN13 was 2.23 µM pNP/hour/10^6 cells. To put these numbers into perspective, the native *S. cerevisiae* VIN13 had no ABF activity, and released only 0.18 µM pNP/hour/10^6 cells from pNP-β-D-glucopyranoside.

Although these enzymes have only 8-10% relative activity at the general white wine fermentation temperature of 15°C, this level of activity (0.26, 0.10, and 0.20 µM pNP/hour/10^6 cells for AwAbfB, BGLA and BGL2 respectively) is sufficient to potentially bring about a marked change in the level of free monoterpenols.

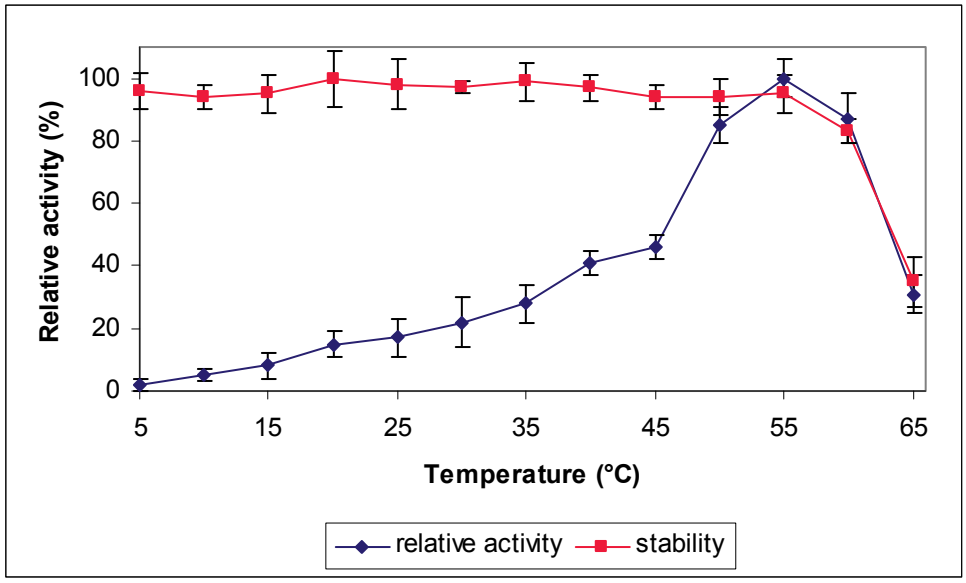

Figure 3.11 Relative temperature activity and thermostability of AwAbfB expressed in VIN13. Values are averages of two independent experiments performed in triplicate. Error bars indicate 1.96 standard deviations.

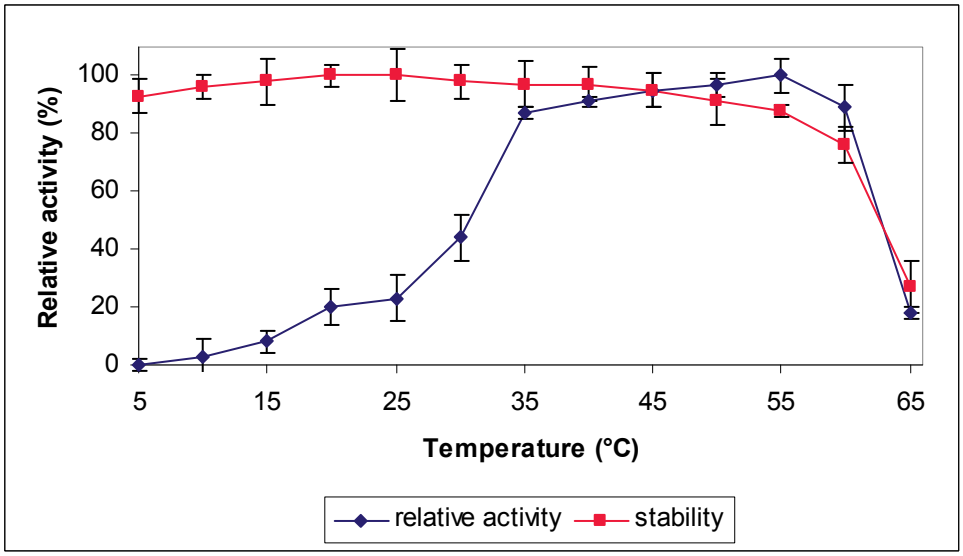

Figure 3.12 Relative temperature activity and thermostability of BGLA expressed in VIN13. Values are averages of two independent experiments performed in triplicate. Error bars indicate 1.96 standard deviations.

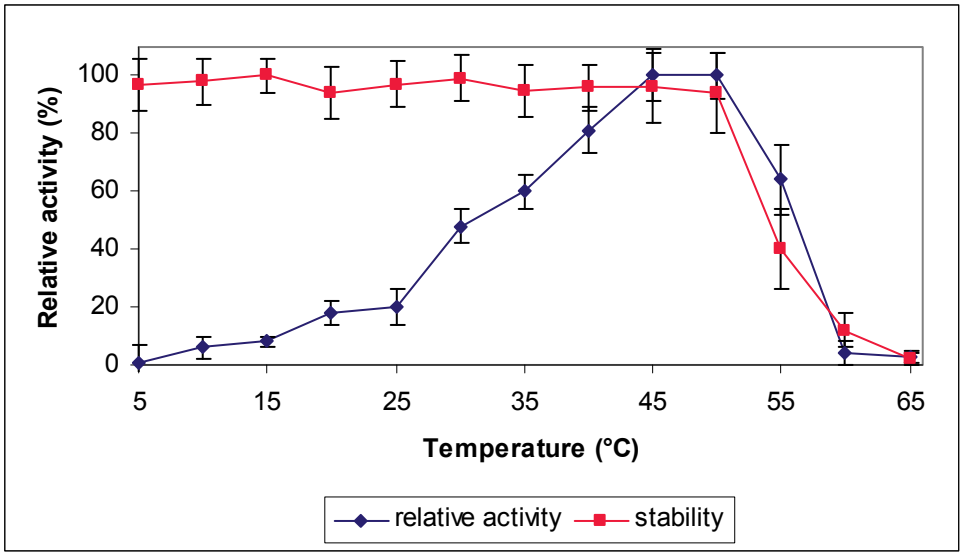

Figure 3.13 Relative temperature activity and thermostability of BGL2 expressed in VIN13. Values are averages of two independent experiments performed in triplicate. Error bars indicate 1.96 standard deviations.

3.3.5 INFLUENCE OF INHIBITORY FACTORS ON HETEROLOGOUSLY EXPRESSED α-L-ARABINOFURANOSIDASE AND β-GLUCOSIDASE.

Glucose has an inhibitory effect on all three enzymes, although BGL2 is inhibited the most, retaining 34% activity at a concentration of 1 M glucose (Figure 3.14). BGLA and AwAbfB retains 58% and 50% activity, respectively at 1 M glucose.

An experiment was done to determine whether the glucose inhibition displayed, could be reversed with a decrease in glucose concentration. Samples containing 0.5 M glucose and ten times the amount of enzyme normally used for enzyme assays from the supernatant were left at room temperature for 1 hour. These samples were then diluted to contain 0.1 M glucose and activity was measured against a second set of samples that had 0.1 M and 0.5 M glucose added just before addition of the pNP substrates. Both sample sets containing 0.1 M glucose had similar enzyme activities that were higher than the freshly incubated 0.5 M glucose-containing sample (data not shown). This implies that inhibition by glucose is reversible for these enzymes. There might thus be an increase in enzymatic activity as fermentation progresses due to the progressive disappearance of glucose.

Ethanol does not inhibit these glycosidases much, since they all retain more than 60% relative activity at an ethanol concentration of 16% (Figure 3.15). At an ethanol concentration of 4%, BGL2 activity is slightly enhanced. Inhibition at higher ethanol concentrations might be due to denaturation of the enzymes.

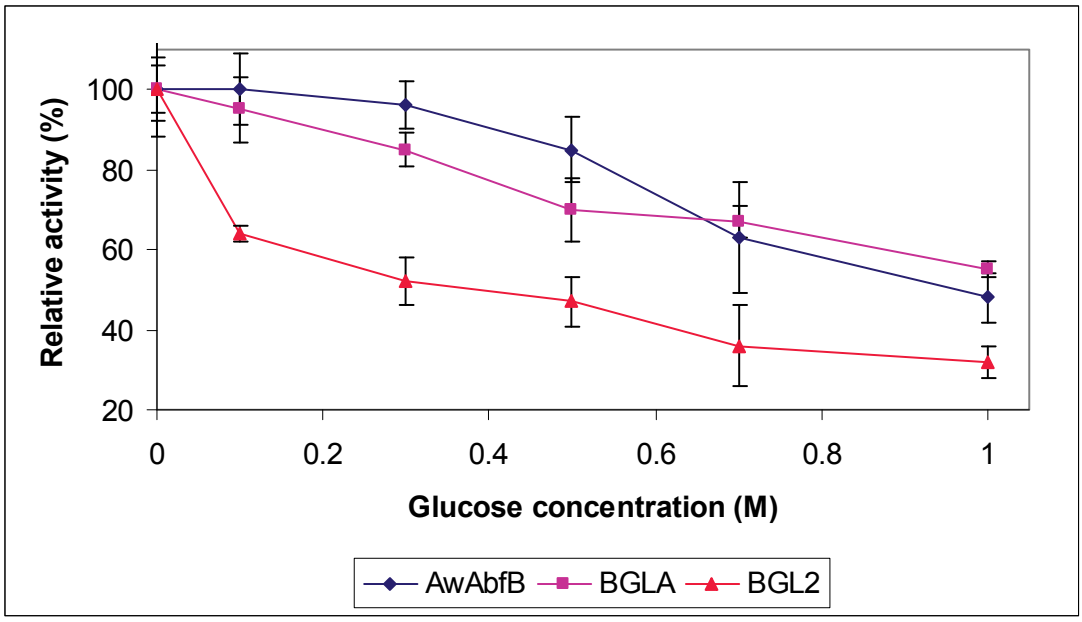

Figure 3.14 Effect of glucose on glycosidase activities. Values are the mean of three determinations and are expressed as a percentage relative to the activity without glucose. Error bars indicate 1.96 standard deviations.

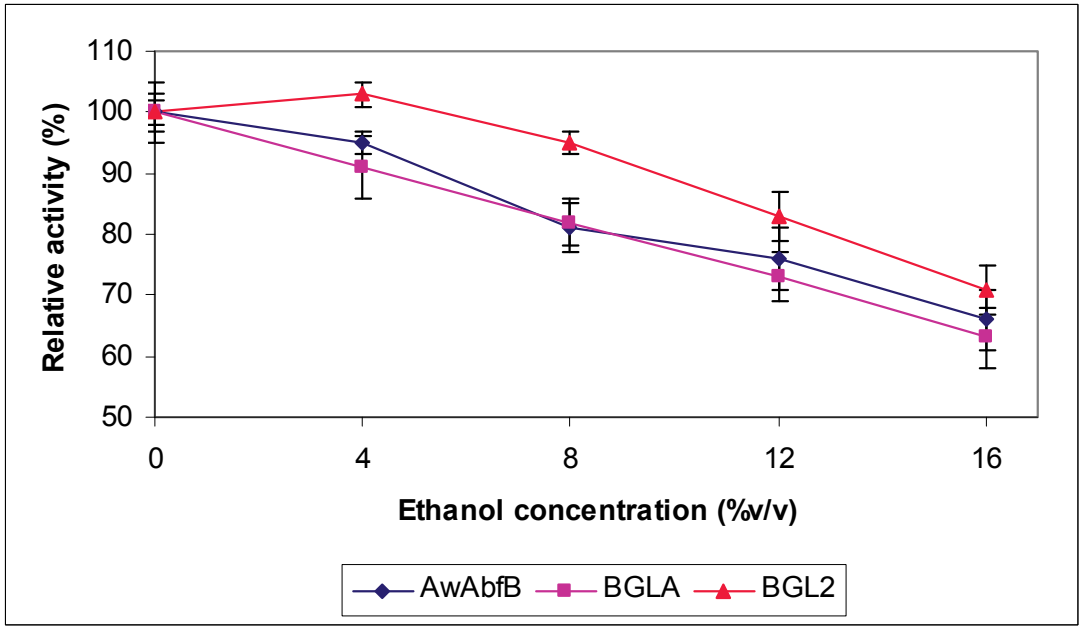

Figure 3.15 Effect of ethanol on glycosidase activities. Values are the mean of three determinations and are expressed as a percentage relative to the activity without ethanol. Error bars indicate 1.96 standard deviations.

3.3.6 VINIFICATION

All the transformants and control samples had the same rate of fermentation. Fermentation was essentially completed after nine days, but the weight only properly stabilized after ten to twelve days (Figure 3.16). After 12 days, wine samples were racked and samples of the yeast lees were taken and inoculated into YPD broth. Yeast genomic DNA was isolated from these cultures and screened by PCR for the presence of integrated genes to confirm that the fermentation was conducted with the recombinant yeast strains.

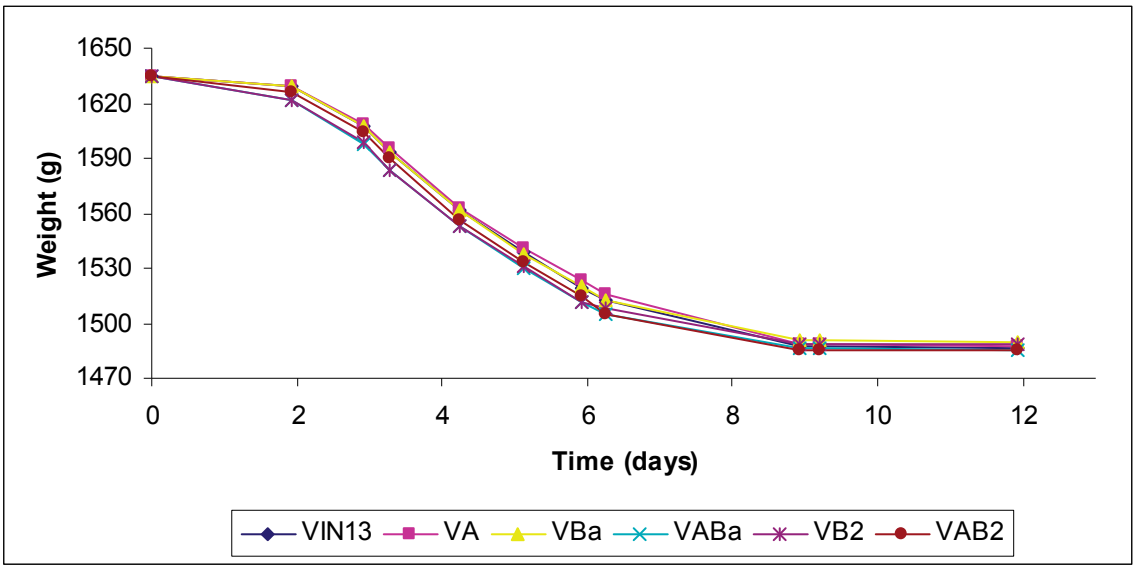

Figure 3.16 Fermentation weight loss corrected to 1.5L. Standard deviation among sample treatments <1%.

No significant differences were observed in the physico-chemical characteristics of the different treatments of wines over the entire course of fermentation (Table 3.4). The expression of AwAbfB singly or in combination with BGLA or BGL2 does not seem to put a significant metabolic burden on the yeast.

Table 3.4 Effect of different yeast strains on physico-chemical characteristics of the Gewürztraminer microvinifications after 12 days of fermentation. Values are the mean ± standard deviation of three replicates.

Component	Yeast strain					
	VIN13	VA	VBa	VABa	VB2	VAB2
Ethanol (%)	12.91±0.0608	12.92±0.0896	13.01±.0265	12.82±0.035	13.02±0.0635	13.03±0.0321
pH	3.83±0.012	3.81±0.006	3.84±0.015	3.87±.014	3.87±0.01	3.86±0.015
Total acid (g/l)	4.32±0.0361	4.37±0.0115	4.41±.0436	4.31±.0424	4.35±0.0208	4.39±0.0513
Malic acid (g/l)	1.69±0.0289	1.74±0.02	1.72±0.01	1.73±0.007	1.78±0.0321	1.78±0.0603
Lactic acid (g/l)	0.3±0.0153	0.28±0.01	0.34±0.02	0.35±0.014	0.32±0.01	0.33±0.0208
Volatile acid (g/l)	0.24±0.0058	0.23±0.0058	0.26±0.0060	0.25±0	0.25±0.0058	0.25±0.0058
Residual sugar (g/l)	0.89±0.1466	0.96±0.1296	0.88±.0841	0.87±0.07	0.97±0.2003	0.8±0.1151

3.3.7 LIBERATION OF MONOTERPENES DURING FERMENTATION

It is well known that monoterpenes have varying characteristics with regards to their stability and volatility and that significant change in the type and proportion of individual monoterpenes can occur in wine during processing and ageing (Ribéreau-Gayon *et al.*, 1975; Williams *et al.*, 1981). These chemical changes that individual monoterpenes undergo during processing or storage of wine can lead to a decrease or an increase in the aroma of some wines (Ribéreau-Gayon *et al.*, 1975). It is for this reason that the concentrations of the aromatically most significant monoterpenes were determined at various stages, before, during and after fermentation. The evolution profiles during fermentation of the free fractions was highly variable for the different monoterpenes

There was an increase going from juice to wine in the total as well as the individual concentration of all monoterpenes in all wines fermented with VIN13 as well as the recombinant strains. The exception was nerol, which decreased in all cases, except in the wine fermented with VAB2 (Table 3.5). This decrease in the overall concentration of nerol might be due to volatilization due to CO_2 release, or the transformation of nerol to diols, triols, or oxides (Spagna *et al.*, 1998a). The yeast might also have the capability to transform nerol by a variety of mechanisms e.g. translocations (nerol to linalool) and *cis* to *trans* isomerizations (nerol to geraniol) (King and Dickinson, 2000; Vaudano *et al.*, 2004). The most probable reason that nerol concentrations

increased only in the wine fermented with VAB2 is that the α-L-arabinofuranosidase (AwAbfB) releases the arabinofuranosyl residue; whereafter the BGL2 is capable of releasing the monoterpene from the resulting monoglucoside. A gradual increase after 6 days of fermentation in the concentration of nerol might be due to increased activity of the BGL2 due to the decrease in glucose concentration (Figure 3.17). Wine fermented with strain VABa had lower nerol concentrations possibly due to the inability of BGLA to hydrolyse nerol glucosides. Although there was a significant increase in the concentration of nerol in wine produced with VAB2, its level at 33.18 µg/l is still well below the perception threshold of 400 µg/l in sugar (Ribéreau-Gayon *et al.*, 1975). Nonetheless, this increase in nerol level might have an influence on the aroma of the wine due to a supporting role in wine aroma and synergystic action with other monoterpenes (Reynolds and Wardle, 1989).

Table 3.5 Concentrations of a selected subset of monoterpenes in µg/l before fermentation and at the end of fermentation as determined by GC-FID. * Indicates values that are significantly different from VIN13 according to Tukey's test ($p<0.05$). n.d. Indicates not detected.

		Yeast strain					
		VIN13	**VA**	**VBa**	**VABa**	**VB2**	**VAB2**
Linalool	Before fermentation	1.63±0.034	1.63±0.034	1.63±0.034	1.63±0.034	1.63±0.034	1.63±0.034
	End of fermentation	2.52±0.122	2.5±0.191	2.54±0.25	2.44±0.023	2.67±0.101	3.05*±0.131
Citronellol	Before fermentation	4.21±0.11	4.21±0.11	4.21±0.11	4.21±0.11	4.21±0.11	4.21±0.11
	End of fermentation	13.01±0.429	11.62±1.041	18.34±1.385	19.18±1.41	19.04±1.19	38.31*±2.788
Geraniol	Before fermentation	74.95±3.73	74.95±3.73	74.95±3.73	74.95±3.73	74.95±3.73	74.95±3.73
	End of fermentation	619.62±31.72	578.57*±40.4	516*±28.89	602.25±45.35	524.59*±35.67	555.39*±28.29
Nerol	Before fermentation	16.36±0.795	16.36±0.795	16.36±0.795	16.36±0.795	16.36±0.795	16.36±0.795
	End of fermentation	12.21±0.535	10.74±0.728	11.74±0.687	11.17±0.945	11.78±0.242	33.18*±1.874
α-Terpineol	Before fermentation	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	End of fermentation	n.d.	n.d.	n.d.	n.d.	4.52*±2.979	4.58*±1.642
Total	Before fermentation	97.15±4.313	97.15±4.313	97.15±4.313	97.15±4.313	97.15±4.313	97.15±4.313
	End of fermentation	647.36±21.44	603.43±28.63	548.62±28.04	635.04±43.79	562.6±22.78	634.51±27.53

α-terpineol was only detected at the end of fermentation and only in wines fermented with VB2 and VAB2 at concentrations of 4.52 µg/l and 4.58 µg/l, respectively. This might be due to BGL2 having the ability to hydrolyse tertiary monoterpene alcohols from their glucosides, and BGLA not being capable of hydrolysing tertiary monoterpene alcohol glucosides like linalool and α-terpineol.

Conversions of other monoterpenes are excluded as the reason for this increase in α-terpineol due to the fact that the physico-chemical characteristics of all the wines were very similar. Steric hindrance has been implicated as a factor contributing to the inability of some glucosidases to liberate tertiary monoterpene alcohols from their glucosides (Günata *et al.*, 1990).

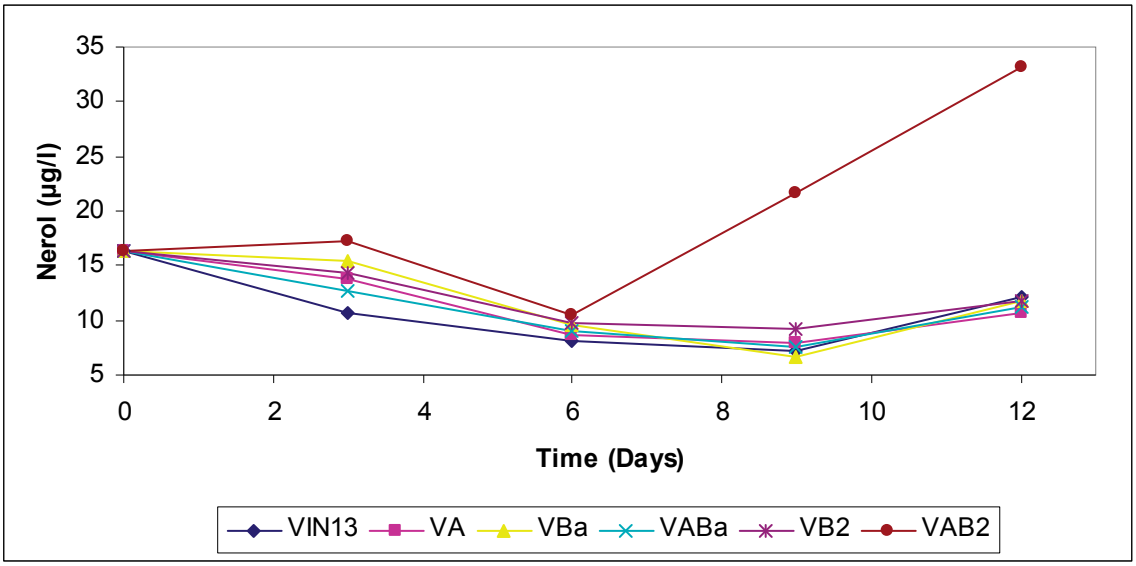

Figure 3.17 Evolution profiles of nerol during fermentation of Gewürztraminer must with recombinant *S. cerevisiae* VIN13 strains expressing AwAbfB from *A. awamori*, BGL2 from *S. fibuligera* and BGLA from *A. kawachii* (SD<5%).

Linalool levels increased in all samples fermented with both native VIN13 and recombinant yeasts (Figure 3.18). Linalool concentrations were significantly higher only in wines produced with VAB2 ($p<0.05$). At 3.05 µg/l, the concentration of linalool in VAB2 wines was still below the perception threshold of 15 µg/l in 10% ethanol (Guth, 1997). A possible explanation for this slight increase is that some of the liberated linalool might have been oxidised to form linalool oxide or polyhydroxylated forms of linalool (Ribéreau-Gayon *et al.*, 1975; Spagna *et al.*, 1998a). Linalool might also be consumed by the yeast during fermentation for the production of sterols (Vaudano *et al.*, 2004).

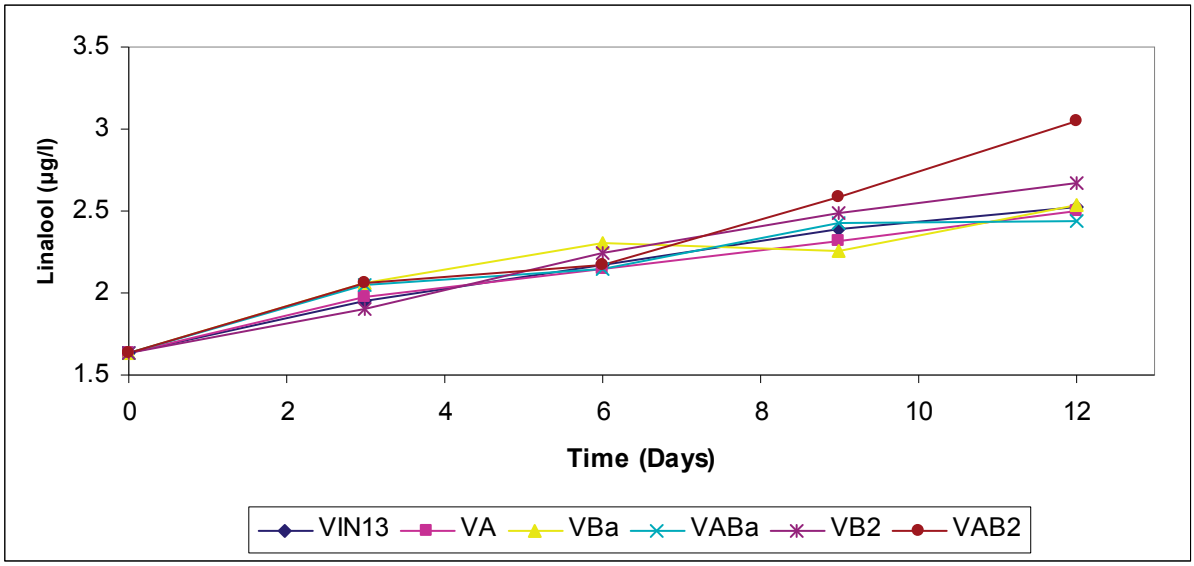

Figure 3.18 Evolution profiles of linalool during fermentation of Gewürztraminer must with recombinant *S. cerevisiae* VIN13 strains expressing AwAbfB from *A. awamori*, BGL2 from *S. fibuligera* and BGLA from *A. kawachii* (SD<5%).

Terpenic compounds occur as intermediates in the production of sterols, for example geraniol is present in an active form as geranyl pyrophosphate, and geraniol can consequently be used in the biosynthesis of steroids that are vital for eukaryotic cellular growth (Vaudano *et al.*, 2004). Geraniol is metabolized in the exponential phase of rapid growth, both aerobically and anaerobically. After the first few hours of fermentation, the yeast needs fewer sterols and consumes lower quantities of geraniol. This observation, together with the fact that ergosterol has a regulating effect on the metabolism of monoterpenes by regulating transcription of the sterol biosynthetic pathway at various biosynthesis points partially explains the interesting occurrence that geraniol concentrations are significantly lower in fermentations conducted with strains VA, VBA, VABa, VB2 and VAB2 than the control strains at the end of fermentation (Figure 3.19). The large amount of geraniol released by the AwAbfB, BGLA and BGL2 during the first three days of fermentation is sufficient to cause a downregulation of the sterol biosynthetic pathway due to the introduction of geraniol as an intermediate in this pathway. Levels of geraniol are higher at the end of fermentation in wine produced with *S. cerevisiae* VIN13 due to the sterol biosynthesis pathway not being inhibited to the same extent as that of the recombinant strains. The native VIN13 does therefore not release as much geraniol early on in fermentation and needs to maintain production of geranyl pyrophosphate. Geraniol that is liberated during the middle to later stages of fermentation (days 4 to 12) is therefore free in the must and is not taken up by the yeast for sterol biosynthesis. The BGL2 appears to have a slightly better activity towards geranyl glucosides than the BGLA. Geraniol levels increased in all wines to values between 17 and 20-fold the perception threshold of 30 µg/l in 10% ethanol (Guth, 1997). The increases in geraniol between day 3 and 6 is probably due to increased enzyme

synthesis and activity as a result of decreasing sugar concentrations and an increase in pH from 3.4 to 3.7.

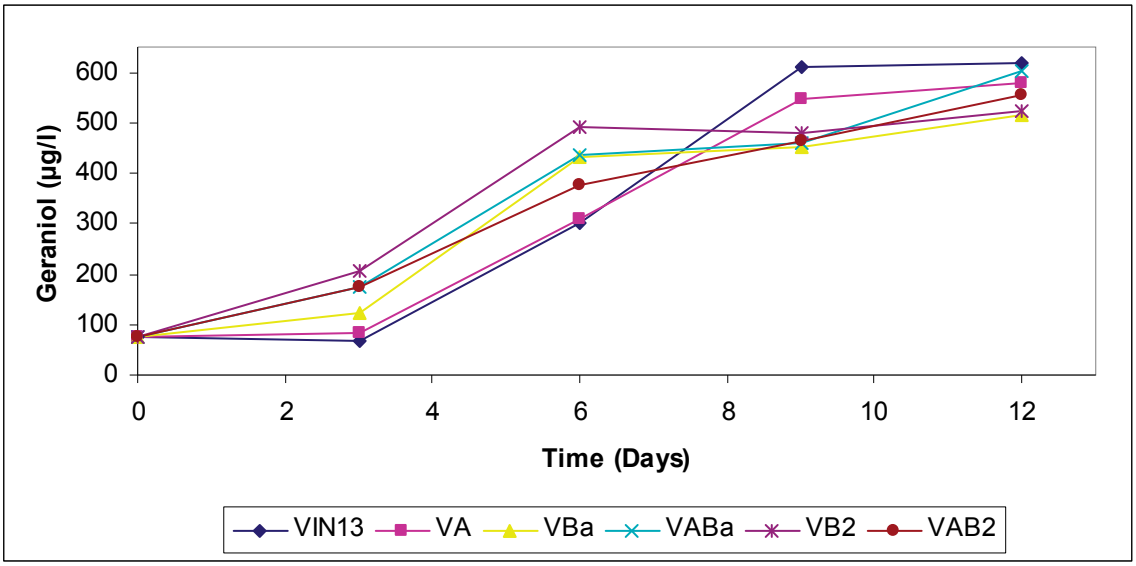

Figure 3.19 Evolution profiles of geraniol during fermentation of Gewürztraminer must with recombinant *S. cerevisiae* VIN13 strains expressing AwAbfB from *A. awamori*, BGL2 from *S. fibuligera* and BGLA from *A. kawachii* (SD<5%).

Citronellol concentrations increased significantly only in wines fermented with VAB2 from 4.21 µg/l to 38.31 µg/l, however it remained below the perception threshold of 100 µg/l in 10% ethanol (Guth, 1997) (Figure 3.20). Although an increase is observed up to day 3, there is a decrease in citronellol during the period from day 3 to day 6 possibly due to volatilization. The significant increase in citronellol in wines produced with VAB2 compared to wines produced with VB2, VABa and VBa indicates that BGL2 is more active towards citronellol glucosides than BGLA, and that a significant proportion of the citronellol was diglycosidically bonded and could only be released by the action of both AwAbfB and BGL2.

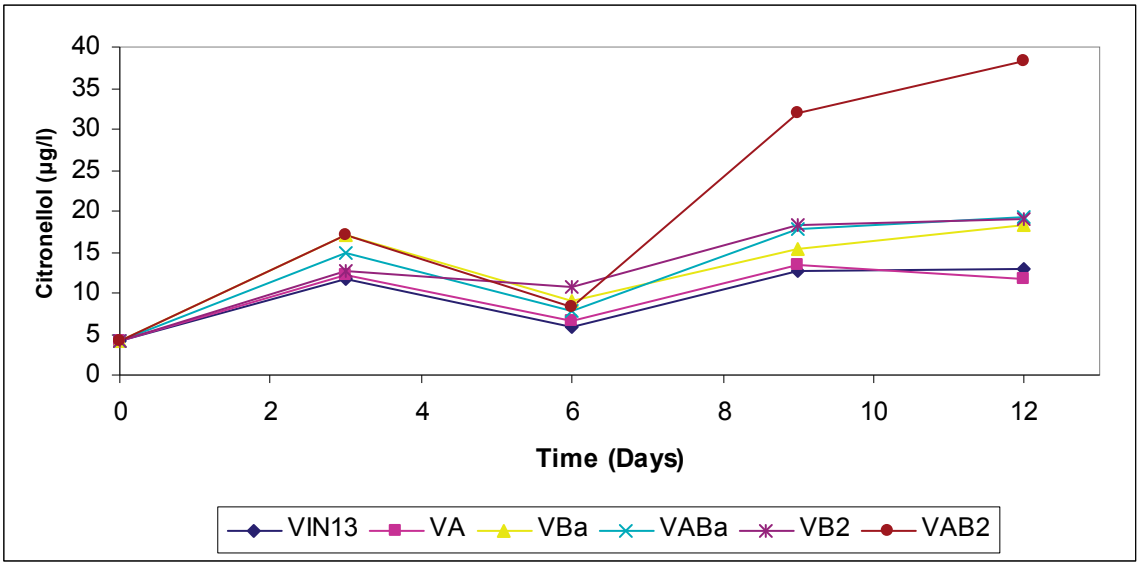

Figure 3.20 Evolution profiles of citronellol during fermentation of Gewürztraminer must with recombinant *S. cerevisiae* VIN13 strains expressing AwAbfB from *A. awamori*, BGL2 from *S. fibuligera* and BGLA from *A. kawachii* (SD<5%).

The changes in monoterpene concentrations are more significant in wines fermented with strain VAB2 both due to the synergistic action of both the AwAbfB and BGL2 towards diglycosidically bonded monoterpenes, and also due to the fact that the BGL2 has activity and specificity towards the native grape glycosides, whereas the BGLA has wide substrate activity and is not as dedicated towards the release of grape monoterpenyl glycosides. This decreased specificity of BGLA is aggravated by instability at low pH values and inhibition by low temperatures, as well as high glucose and ethanol concentrations.

The slight increase in concentrations of some monoterpenes, for example linalool does not seem to be due to limited precursor concentrations since the concentration of linalool increased after fermentation as will be demonstrated in the section 3.3.9.

3.3.8 PRECURSOR ANALYSIS

Glycosyl glucose concentrations were analysed in must and wines fermented with VIN13 and VAB2 to provide an estimation of the total pool of glycosides, of which a significant proportion is bound to monoterpenes (Zoecklein *et al*., 1997). As can be seen in Figure 3.21, there is a significant decrease in the concentration of glycosidically bonded precursors between the must and wine stages. It is also notable that wine fermented with VAB2 has 110 µM or 29% less glycosyl glucose precursor molecules than the control sample fermented with VIN13. This difference is much greater than the monoterpene levels measured by GC-FID at the end of fermentation. It can be explained by the fact that although monoterpene glycosides make up the bulk of the glycosides, they are not the only glycosides and some monoterpenes are consumed by the yeast, degraded, evaporate or are converted to other forms that are not detected (oxides, diols and triols). Nonetheless it is clear

that AwAbfB and BGL2 have the ability to hydrolyse glycosyl glucose molecules under winemaking conditions.

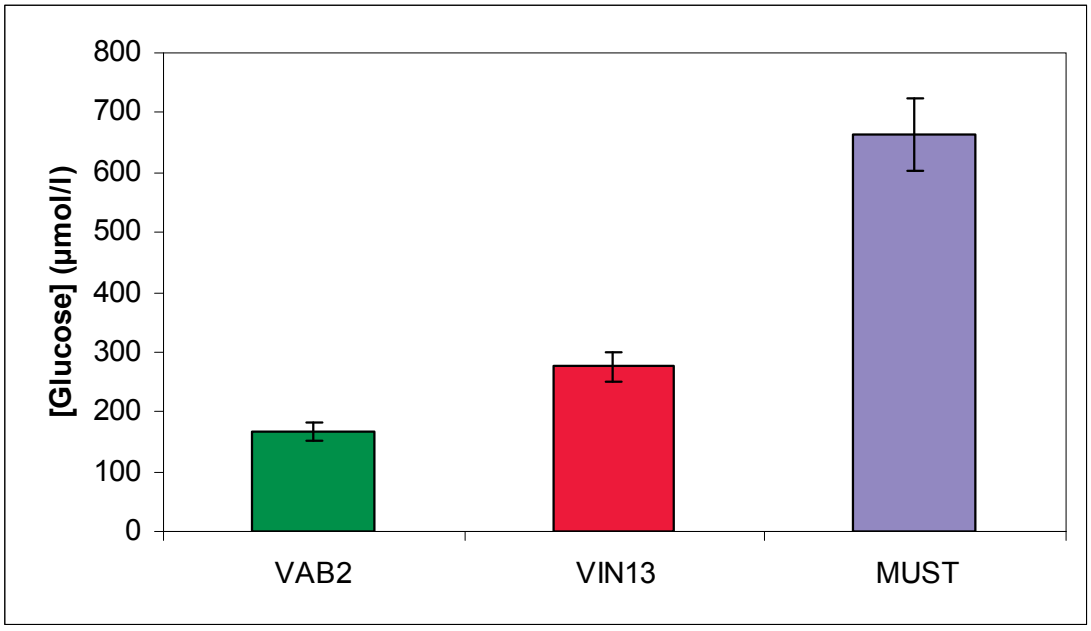

Figure 3.21 Aroma precursor concentrations (expressed as µmol glucose/l) in must and wine. Error bars indicate standard deviation.

3.3.9 MONOTERPENE ANALYSIS AFTER FERMENTATION

Monoterpene levels were also analysed for linalool, geraniol, nerol, citronellol and α-terpineol at two additional stages at 9 and 38 days after fermentation and racking of the wine. The effect of the recombinant yeast on monoterpene concentrations was also compared to that of commercial enzyme preparation LAFAZYM AROM. It is interesting to note that the total levels of all the monoterpenes analysed had decreased from the end of fermentation to nine days after fermentation, but increased between 9 and 38 days after fermentation (Figures 3.22 to 3.26).

Volatilization during the racking process might have caused evaporation of monoterpenes. Another explanation for the decrease might be oxidation of monoterpenes or formation of polyols from monoterpenes. The increase in free monoterpenes can be explained by the hydrolysis of monoterpene glycosides after the equilibrium for the formation of polyols and oxides has been reached. These hydrolysis reactions can only be partially ascribed to the slow acid hydrolysis reactions normally found in wine. The majority of the increase in monoterpene concentrations can be credited to residual enzymatic activity. Strain VAB2 had the most significant effect on the concentration of free monoterpenes and released more monoterpenes than LAFAZYM AROM.

Only strain VAB2 had the ability to release significant ($p<0.05$) amounts of monoterpenes in the bottle after fermentation when compared to VIN13. This is once again due to the stability, relative activity and temperature advantage that BGL2 has compared to BGLA. In addition, these increases are not observed in wines fermented with strain VB2 due to the inability of BGL2 to liberate monoterpenes from diglycosides. The BGLA is not active towards grape glucosides under these high ethanol concentration conditions.

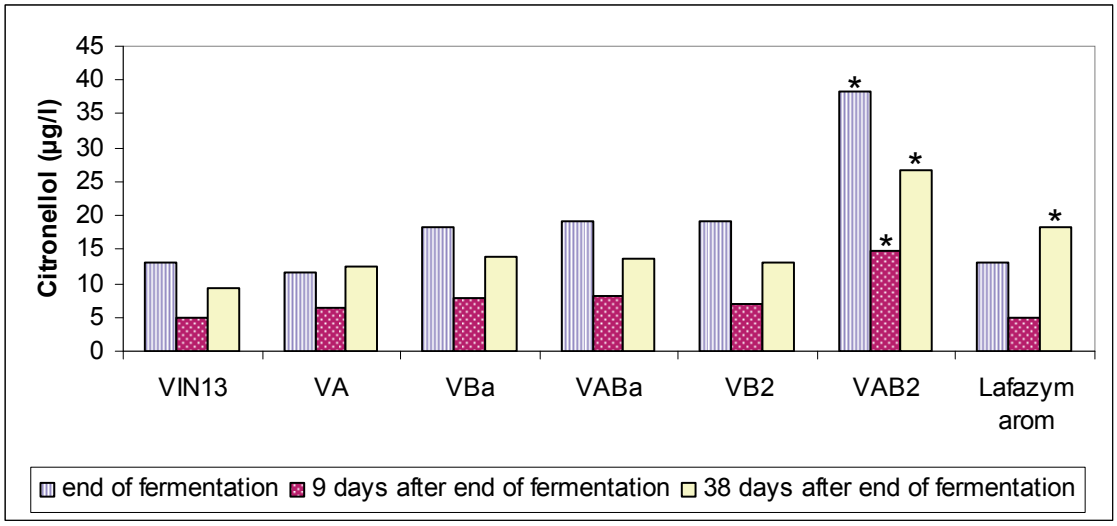

Figure 3.22 Citronellol concentrations in wine after fermentation (SD<5%). * Indicates values that are significantly different from VIN13 according to Tukey's test ($p<0.05$).

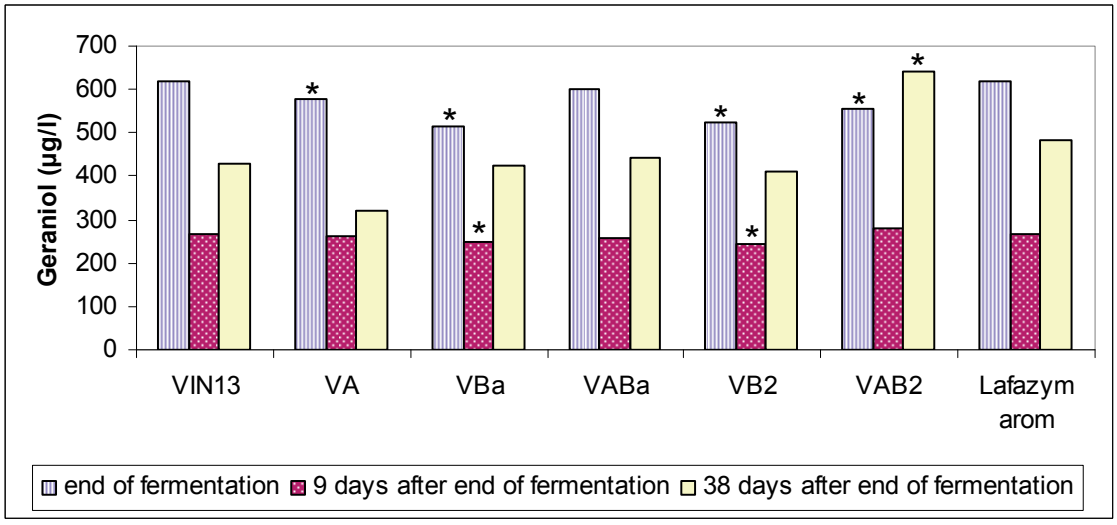

Figure 3.23 Geraniol concentrations in wine after fermentation (SD<5%). * indicates values that are significantly different from VIN13 according to Tukey's test ($p<0.05$).

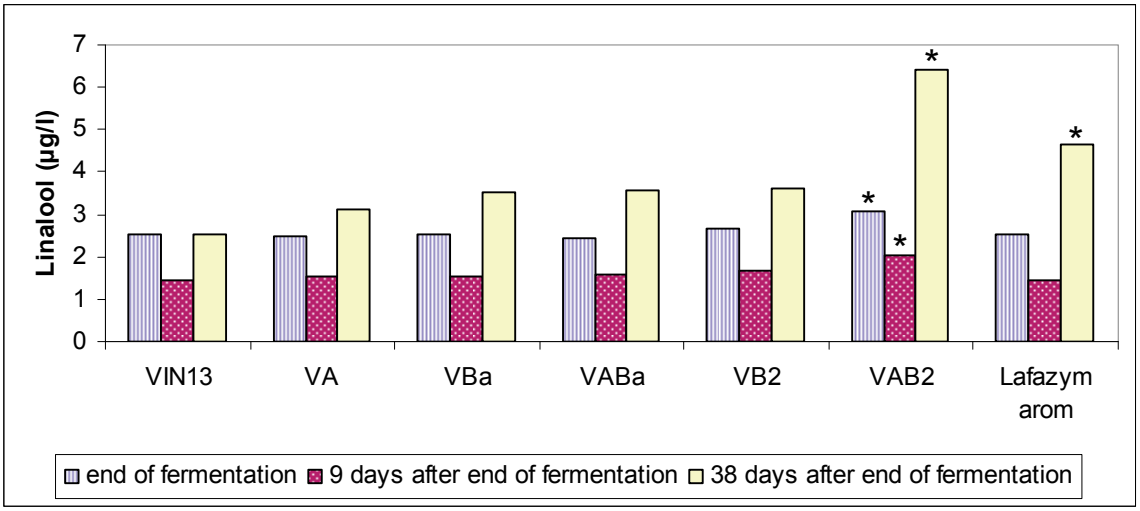

Figure 3.24 Linalool concentrations in wine after fermentation (SD<5%). * Indicates values that are significantly different from VIN13 according to Tukey's test ($p<0.05$).

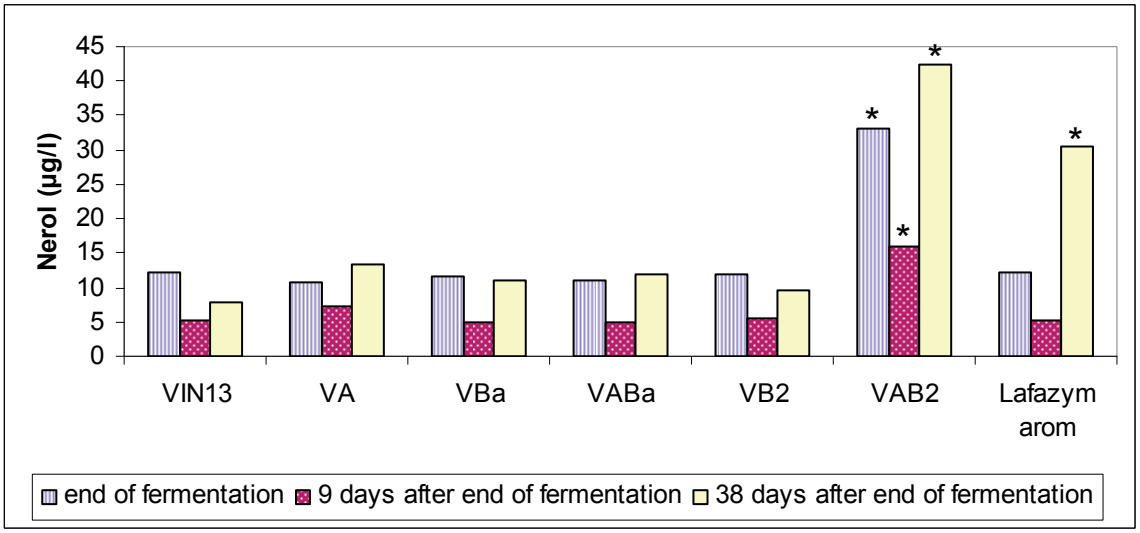

Figure 3.25 Nerol concentrations in wine after fermentation (SD<5%). * Indicates values that are significantly different from VIN13 according to Tukey's test ($p<0.05$).

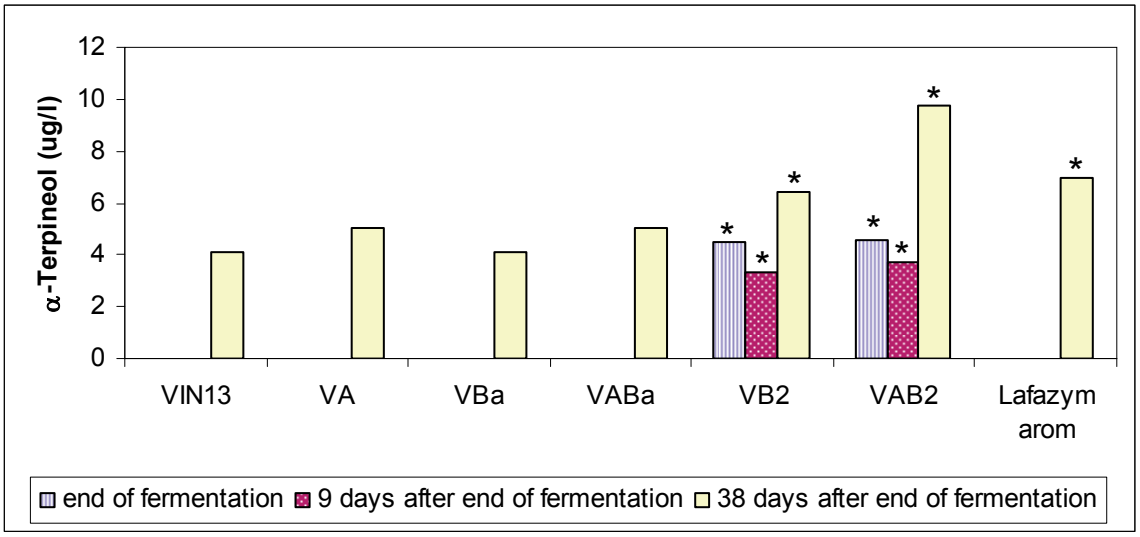

Figure 3.26 α-Terpineol concentrations in wine after fermentation (SD<5%). * Indicates values that are significantly different from VIN13 according to Tukey's test ($p<0.05$).

3.3.10 SENSORIAL ANALYSIS

Although variation was high in evaluation of the wines, trends were observed. Wines produced with strains VBa, VABa, VB2 and VAB2 as well as the sample treated with LAFAZYM AROM showed different sensorial attributes from control wines and were judged as wines with more floral and fruity aroma with some lychee notes (Table 3.6). High monoterpene concentrations seem to mask vegetable aromas (Figure 3.27). Wines fermented by strain VAB2 had the most intense perceived fruity and floral aroma.

Table 3.6 Intensity scores of aroma descriptors of Gewürztraminer wine produced with recombinant yeast strains. Values are expressed as the average of all trials (n=14) ± standard deviation.

Aroma attribute	Yeast strain utilized/Fermentation treatment						
	VIN13	VA	VBa	VABa	VB2	VAB2	VIN13+Lafazym arom
Fruit	24±19.9	30±26.7	54±28.4	47±28.0	49±28.2	62±19.2	61±21.8
Floral	33±21.8	30±26.1	53±23.5	53±28.4	53±29.3	69±21.2	49±25.8
Vegetable	56±26.1	47±33.5	25±29.5	17±29.4	13±14.9	6±19.6	12±13.5

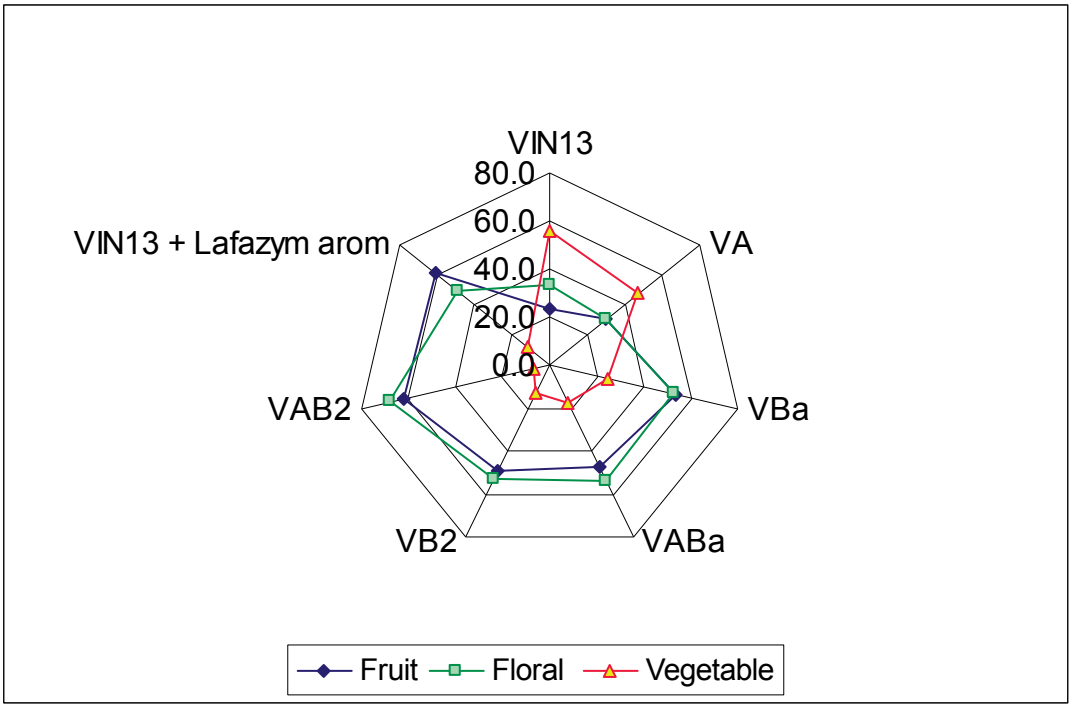

Figure 3.27 Sensorial attributes of Gewürztraminer wine fermented with recombinant *S. cerevisiae* VIN13 strains expressing AwAbfB from *A. awamori*, BGL2 from *S. fibuligera* and BGLA from *A. kawachii.* Wine samples fermented with yeast strains co-expressing arabinofuranosidase and β-glucosidase were judged as having more floral and fruity aroma.

The triangular test data in table 3.7 confirm that the panel could distinguish between wines fermented with strains VIN13 and recombinant strains expressing heterologous glycosidases. All panellists preferred wines produced with strain VAB2 to VIN13.

Table 3.7 Triangle test data of sensory analysis of wines by 14 panellists

Sample Comparison	Correct response	Preference of V13	Significance (P) of one-tailed test
VIN13 and VA	7/14	2/9	n.s.
VIN13 and VBa	8/14	0/9	n.s.
VIN13 and VABa	8/14	1/8	n.s.
VIN13 and VB2	9/14	0/10	0.05
VIN13 and VAB2	13/14	0/13	0.001
VIN13 and Lafazym arom	12/14	1/12	0.001

n.s. – not significant

3.4 CONCLUSION

Recombinant *S. cerevisiae* VIN13 yeast strains have been constructed to express and secrete the *A. awamori* gene encoding a B-type α-L-arabinofuranosidase (*AwAbfB*) in combination with either the β-glucosidases *BGL2 f*rom *S. fibuligera* or the *BGLA* from *A. kawachii*. Co-expression of AwAbfB and BGL2 in VIN13 leads to a noticable increase in free monoterpenes in wines due to the α-L-arabinofuranosidase and β-glucosidase activities. This increase in free monoterpene levels are similar to those obtained with a commercial enzyme preparation, LAFAZYM AROM. It is of great interest to note that the liberation of monoterpenes early in fermentation may lead to decreased levels of final monoterpene concentrations especially that of geraniol, when compared to the wild type VIN13 strain. Sensorial evaluation confirmed the improvement in the wine aroma profile, particularly the floral character.

Recombinant yeast strains constructed during this study have the potential to produce wines with an enhanced aroma profile eliminating the need for the addition of commercial enzyme preparations. The AwAbfB and BGL2 show activity towards the glycosidically bound monoterpenes and are stable and active under the harsh conditions found in must and wine while the BGLA is less stable and active than BGL2. Appearance of the wine was also unaffected, but this observation was not supported by colorimetric data.

It is difficult to correlate changes in monoterpene concentrations exclusively to the action of glycosidases hydrolysing a corresponding glycoside because monoterpenes undergo a variety of chemical changes due to their instability under acidic conditions, and furthermore the yeast has the capability to consume and transform monoterpenes. The significant increase in levels of some monoterpenes compared in wines produced by certain recombinant strains to wines fermented with the control strain are attributable to enzymatic hydrolysis of monoterpene glycosides. These results indicate the need for further detailed investigations on the factors influencing the behaviour of monoterpenes during alcoholic fermentation.

3.5 ACKNOWLEDGEMENTS

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Chapter 4

General discussion and conclusions

4.1 GENERAL DISCUSSION AND CONCLUSIONS

It is well established that monoterpenes are important aroma compounds in different grape varieties and wines. Monoterpenes are present as either odourless, glycosidically bound complexes or as free aromatic monoterpenes (Abbott *et al*., 1991). These complexes occur as monoglucosides or, when present as diglycosides, most commonly as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides of mainly linalool, geraniol, nerol and citronellol. The α-L-arabinofuranosidases and β-glucosidases are important enzymes responsible for the liberation of monoterpene alcohols from their glycosides. Grape and yeast glycosidases are severely inhibited by winemaking conditions and this leads to unutilized aroma potential, while commercial aroma liberating enzyme preparations are crude extracts that often have unwanted and unpredictable side effects on wine aroma.

In this study the expression and secretion of the *Aspergillus awamori* α-L-arabinofuranosidase B in combination with either the β-glucosidases *BGL2* from *Saccharomycopsis fibuligera* or the *BGLA* from *Aspergillus kawachii* in the industrial yeast strain *Saccharomyces cerevisiae* VIN13 facilitated the release of monoterpenes during and after fermentation. Enzyme assays demonstrated the production and efficient secretion of the functional AwAbfB, BGLA and BGL2 from early in the exponential growth phase through to the stationary phase. GC-FID results show a significant increase in the amount of free monoterpenes produced in wine fermented with the recombinant *S. cerevisiae* strains.

The most significant increase in free monoterpenes was observed in wines produced with the VIN13 strain co-expressing the *A. awamori AwAbfB* and the *BGL2* from *S. fibuligera*. Sensorial evaluation confirmed the improvement in the wine aroma profile, particularly the floral character.

The possible posttranslational modifications that the host organism, *S. cerevisiae,* might have performed on the heterologously expressed AwAbfB, BGLA and BGL2 did not appear to influence the activities of the enzymes; they displayed hydrolytic properties similar to those of enzymes expressed in their native host (Iwashita *et al.*, 2001; Kaneko *et al*., 1998; Machida *et al.*, 1988).

The lower level of enzymatic activity exhibited by the BGLA compared to the BGL2 is probably due to instability and low activity of the BGLA under the combination of a low pH and high ethanol concentration of wine. In addition, the superior activity of BGL2 might be due to its ability to hydrolyse a wider range of substrates, while BGLA is unable to hydrolyse tertiary monoterpene alcohol glucosides like linalool and α-terpineol. Steric hindrance has been implicated as a factor contributing to the inability of some glucosidases to liberate tertiary monoterpene alcohols from their glucosides (Günata *et al.*, 1990). In agreement with the data describing the influence of glucose and ethanol on the activity of BGL2, there seems to be a significant increase in the activity

of this enzyme towards the end of fermentation as the levels of glucose decrease and the levels of ethanol increase.

There was an increase in the total as well as the individual concentration of all monoterpenes in all wines fermented with VIN13 as well as the recombinant strains, except for nerol. Nerol decreased in all cases, except in the wine fermented with VAB2. The increased levels of free monoterpenes in the wine fermented with VAB2 are due to the α-L-arabinofuranosidase (AwAbfB) releasing the arabinofuranosyl residue; whereafter the BGL2 is capable of releasing the monoterpene from the resulting monoglucoside.

Less significant increases in free monoterpene levels were observed in cases where the BGLA and BGL2 were singularly produced in *S.cerevisiae* VIN13. This is due to a proportion of the grape glycosides being present as further substituted disaccharides. In this case, the β-glucosidases could not liberate the aglycones until the terminal sugars were removed by the arabinofuranosidase.

Geraniol is used in yeast metabolism pathways that are involved in the synthesis of steroid molecules required in the rapid growth phase (Vaudano *et al*., 2004). This observation, together with the fact that ergosterol has a regulating effect on the metabolism of monoterpenes by regulating transcription of the sterol biosynthetic pathway at various biosynthesis points partially explains the interesting occurrence that geraniol concentrations are significantly lower in fermentations conducted with strains VA, VBA, VABa, VB2 and VAB2 than the control strains at the end of fermentation. The large amount of geraniol released by the AwAbfB, BGLA and BGL2 during the first three days of fermentation is sufficient to cause a down regulation of the sterol biosynthetic pathway due to the introduction of geraniol as an intermediate in this pathway. Levels of geraniol are higher at the end of fermentation in wine produced with *S. cerevisiae* VIN13 due to the sterol biosynthesis pathway not being inhibited to the same extent as that of the recombinant strains. The native VIN13 does therefore not release as much geraniol early on in fermentation and needs to maintain production of geranyl pyrophosphate. Geraniol that is liberated during the middle to later stages of fermentation (days 4 to 12) is therefore free in the must and is not taken up by the yeast for sterol biosynthesis. The BGL2 appears to have a slightly better activity towards geranyl glucosides than the BGLA. Geraniol levels increased in all wines to values between 17 and 20-fold the perception threshold of 30 µg/l in 10% ethanol (Guth, 1997).

The total levels of all the monoterpenes analysed had decreased from the end of fermentation to nine days after fermentation, but increased between 9 and 38 days after fermentation. Volatilization during the racking process might have caused evaporation of monoterpenes. Another explanation for the initial decrease in monoterpenes might be oxidation of monoterpenes or formation of polyols (Strauss *et al.*, 1988).

The subsequent increase in free monoterpenes can be explained by the hydrolysis of monoterpene glycosides after the equilibrium for the formation of polyols and oxides has been

reached. These hydrolysis reactions can only be partially ascribed to the slow acid hydrolysis reactions normally found in wine. The majority of the increase in monoterpene concentrations can be credited to residual enzymatic activity. This can be verified by treating a sample of wine fermented by strain VAB2 with proteinase; if the increaese in monoterpene levels are dependent on residual enzymatic activity, there would not be as significant an increase in the amount of free monoterpenes. Only strain VAB2 had the ability to release significant ($p<0.05$) amounts of monoterpenes in the bottle after fermentation when compared to VIN13. VAB2 also released more monoterpenes than the commercial enzyme preparation LAFAZYM AROM. This is once again due to the specificity, stability, relative activity and low temperature activity advantage that BGL2 has when compared to BGLA. In addition, these increases are not observed in wines fermented with strain VB2 due to the inability of BGL2 to liberate monoterpenes from diglycosides. The BGLA is not active towards grape glucosides under these high ethanol concentration conditions.

The recombinant yeast strains constructed during this study have the potential to produce wines with an enhanced aroma profile eliminating the need for the addition of commercial enzyme preparations. Expression of the *A. awamori* α-L-arabinofuranosidase in combination with the β-glucosidase *BGL2* from *S. fibuligera* in the industrial yeast strain *S. cerevisiae* VIN13 allows efficient hydrolysis of monoterpenes from their glycosylated precursors.

However, a better understanding of the dynamics of free monoterpenes is necessary in order to predict the effect of releasing additional monoterpenes on the final aroma composition of wine. It has become apparent that studies on the enzymatic release of monoterpenes should not be considered in isolation, but should also take into account the transformations that monoterpenes undergo under acidic conditions.

Due to the fact that monoterpenes undergo a variety of chemical changes owing to their instability under acidic conditions, and also that yeast has the capability to consume and transform monoterpenes, it is difficult to correlate changes in monoterpene concentrations exclusively to the action of glycosidases hydrolysing a corresponding glycoside (Martino *et al.*, 2000). These reactions are part of a complex system where compounds are formed and degraded simultaneously (Varming *et al.*, 2006). Thus, further detailed investigations on the factors influencing the behaviour of monoterpenes during alcoholic fermentation are needed.

In addition, the long-term effects of enzymatic release of monoterpenes still need to be explored. It would be interesting to see if the early release of glycosidically bound monoterpenes leads to a shift in the balance of end-product monoterpenes or if they chemically react to form non-aromatic compounds.

Late fermentation expression under a different promoter might prevent excessive volatilization of free monoterpenes and downregulation of the sterol biosynthesis pathway by decreasing the amount of monoterpenes available during the early growth stage. Fewer monoterpenes will then be

incorporated into the synthesis of ergosterol. The effect of glycosidase enzyme activity on vinyl phenol and anthocyanins should also be investigated.

When the chemistry and biochemistry of monoterpenes and monoterpene glycosides are fully understood, it will be possible for winemakers to produce wines with improved floral aroma by employing a recombinant *S. cerevisiae* strain co-expressing monoterpene-liberating enzymes.

4.2 LITERATURE CITED

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