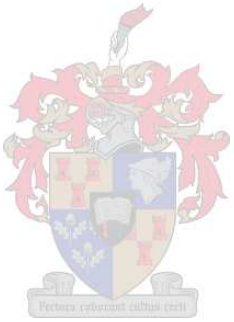
Characterization and Evaluation of Glucose oxidase Activity in Recombinant *Saccharomyces cerevisiae* Strains

by

Daniël Francois Malherbe



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Declaration

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Date: 18th February 2010

Summary

Characterization and Evaluation of Glucose oxidase Activity in Recombinant *Saccharomyces cerevisiae* Strains

DF Malherbe

at

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Popular wine styles prepared from fully-ripened, more mature grapes are characterized by intense fruitiness and varietal flavors. However, lengthy maturation of grapes in the vineyard does not only translate into higher flavor intensity but also into higher sugar levels, which, in turn, leads to wines with higher concentrations of alcohol. Excessive alcohol levels can compromise wine flavor and render wine unbalanced. This, along with health issues and anti-social behavior linked to high-risk alcohol consumption patterns, stricter legislation and increased tax rates associated with high-alcohol wines, have increased demand for wines with reduced alcohol concentrations, without loss of the intense fruity aromas. Although low-alcohol wines can be made using physical post-fermentation processes, such approaches are often expensive and can impact adversely on wine flavor. As an alternative strategy, yeast strains are being developed by several research groups to convert some of the grape sugars into metabolites other than ethanol.

Based on promising results from previous preliminary work, this study focused on the development of an industrial *Saccharomyces cerevisiae* wine strain producing glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4). GOX oxidizes β-D-glucose to D-glucono-δ-lactone and gluconic acid (GA) extracellularly, thus preventing its entry into glycolysis, thereby diverting a portion of

the sugar carbon away from ethanol. The GOX-encoding gene from the food-grade fungus, *Aspergillus niger* was used to construct three cassettes (*GOX1*, *GOX2* and *GOX2LOX*). In these gene cassettes, the *A. niger* GOX gene was placed under the regulation of the *S. cerevisiae* phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). To facilitate secretion, in *GOX1* the yeast mating pheromone-factor α secretion signal ($MF\alpha1_S$) was fused to the *GOX* gene, and in *GOX2* the native *A. niger* secretion signal of GOX was used. These gene cassettes were each integrated into the genome of two laboratory yeast strains (BY4742 and Σ1278b) and one industrial wine yeast strain (VIN13). An additional integration cassette, designated *GOX2LOX*, was constructed to knock out the *IME1* gene in *S. cerevisiae*. In *GOX2LOX*, *GOX2* was fused to a loxP cassette. VIN13-Δ1 was obtained by integrating a single copy of *GOX2LOX* into the *IME1* locus. To generate an asporogenic, GOX-producing wine yeast, VIN13-Δ2 was created by sporulation, micromanipulation and re-diploidisation of VIN13-Δ1. Comparative analysis indicated that (i) *GOX2* resulted in higher levels of extracellular glucose oxidase activity than *GOX1*; and that (ii) the levels of secreted glucose oxidase activity in the wine yeast transformants were sufficiently high to conduct follow-up small-scale wine fermentation trials.

The wine yeast transformant, VIN13-Δ1 was evaluated under red and white experimental winemaking conditions. Results from this work indicated that glucose oxidase was produced and secreted by VIN13-Δ1 that dominated the fermentation to the end, but also that the enzyme was not highly active under the evaluated winemaking conditions. Consequently, no significant decrease in ethanol concentrations was observed in the wine made from VIN13-Δ1 when compared to that from VIN13. Wine samples were analyzed by Fourier transform-middle infrared spectrometry (FT-MIR) to determine the chemical composition and Gas chromatography with a flame ionization detector (GC-FID) to evaluate the concentrations of aroma compounds. The levels of gluconic acid were determined by enzymatic assays. Multivariate data analysis (PCA and PLS1-discrim) was applied to highlight significant differences between the wines made by VIN13 (wild-type) and VIN13-Δ1. Chemometric projections of the score plots for all results allowed insight into all significant variation up to three principal components (PCA) or PLS components, which showed very clearly that GA is a key factor in evaluating the effect of GOX in VIN13-Δ1 fermentation with regard to VIN13 fermentations. The VIN13-Δ1 effect manifestations were best shown on PLS1-discrim score plots that revealed

that, of the restricted variable subsets the FT-MIR-compounds and GC-compounds yielded better results, with the GC-compounds displaying greater discriminability between cultivars and VIN13 / VIN13-Δ1. It can be concluded from these results that the greatest influence of VIN13-Δ1 produced wines could be observed in the aroma components, but, because there were also discriminability effects discernable in the FT-MIR-compounds, thus the flavor components were also affected.

The activity of GOX in grape juice was further investigated in controlled small scale fermentations performed in a bio-reactor. It was confirmed that GOX is active under aerobic conditions, inactive under anaerobic conditions, and can be activated instantly when an anaerobic culture is switched to aerobic conditions (simulated micro-oxygenation). These fermentations showed that glucose oxidase is active in grape juice, and that oxygen play a key-role in the enzyme's activation. Finally, it was shown with the help of a simplified model, that under ideal conditions, GOX secreted from VIN13-Δ1, can be employed to reduce the ethanol by a predefined concentration for the production of low alcohol wines.

This work gives more insight into how to employ a GOX-producing wine yeast during winemaking and strongly suggests the use of micro-oxygenation to activate the enzyme in order to reduce available glucose, thereby diverting a portion of the sugar carbon away from ethanol production.

Opsomming

Karakterisering en Evaluering van Glukose-oksidase Aktiwiteit in Rekombinante *Saccharomyces cerevisiae* Rasse

("Characterization and Evaluation of Glucose oxidase Activity in Recombinant *Saccharomyces cerevisiae* Strains")

DF Malherbe

by

Universiteit van Stellenbosch

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Gewilde wynstyle word dikwels gemaak van volryp, goed ontwikkelde druiwe, gekarakteriseer deur intense aromas en smaakkomponente wat direk met spesifike kultivars geassosieer word. 'n Nadelige gevolg om druiwe te lank aan die wingerdstok te laat bly hang sodat meer intense geurkomponente kan ontwikkel, is die toename in suikerinhoud. Hierdie addisionele suiker lei tot wyne met hoër alkoholvlakke. Te hoë alkoholvlakke kan wyn ongebalanseerd laat voorkom en die smaak nadelig beïnvloed. Dit, tesame met gesondheidsredes en anti-sosiale gedrag wat gekoppel kan word aan die inname van te veel alkohol, strenger wetgewing aangaande dronkbestuur en die toename in belasting op wyne met 'n hoër alkoholinhoud, het aanleiding gegee tot 'n aanvraag vir wyn met 'n verlaagte alkoholinhoud, sonder dat aroma- en geurkomponente ingeboet word. Alhoewel daar sekere fisiese/gemeganiseerde prosesse beskikbaar is om die alkohol in wyn te verwyder of te verminder, is 'n nadeel dat hierdie prosesse baie duur en arbeidsintensief is, en dat dit deur sommige wynpuriste as te ingrypend in die 'natuurlike' proses van wynmaak beskou word. Sommige van hierdie alkoholverwyderingsprosesse kan ook

die wyn se geur- en aromakomponente nadelig beïnvloed. As alternatief tot hierdie fisies-chemiese prosesse word wyngiste reg oor die wêreld deur verskillende navorsingsgroepe ontwikkel sodat van die druifsuikers nie na alkohol omgeskakel word nie, maar eerder ander metaboliete.

Belowende navorsingsresultate in 'n voorafgaande studie het aanleiding gegee tot hierdie navorsingsprojek. In hierdie studie word daar klem gelê op die ontwikkeling, deur middel van genetiese manipulering, van 'n industriële wynras van *Saccharomyces cerevisiae* sodat dit in staat sal wees om glukose-oksidase (GOX; β-D-glukose:suurstof oksidoreduktase, EC 1.1.3.4) te produseer. GOX kan reeds β-D-glukose in die medium oksideer na glukoonsuur (GA), wat sodoende verhoed dat dit verder gemetaboliseer word via glukolise, en dit het tot gevolg dat 'n gedeelte van die beskikbare suiker nie omgeskakel word na alkohol nie. Die strukturele glukose-oksidase-geen (*GOX*) van die voedsel-gegradiëerde fungus, *Aspergillus niger* is gebruik tydens die konstruksie van drie kassette (*GOX1*, *GOX2* en *GOX2LOX*). Binne hiedie geenkassette is *A. niger* se *GOX*-geen se transkripsie-inisiëring en -terminering onafhanklik deur die fosfogliseraat-kinase-1-promotor ($PGK1_P$) en termineerder ($PGK1_T$) bewerkstellig. Om uitskeiding van GOX uit die gis te bewerkstellig, is daar van die α-spesifieke gisferomoon-α-faktor ($MF\alpha1_S$) in *GOX1* gebruik gemaak, en in *GOX2*, van *A. niger* se eie natuurlike sekresiesein. Hierdie geenkassette is binne-in die genoom van twee labaratoriumgisrasse van *S. cerevisiae* (BY4742 en Σ1278b) asook een industriële wyngisras (VIN13) geintegreer. 'n Addisionele integreringskasset (die sogenaamde *GOX2LOX*-kasset) is gemaak om die *IME1*-geen van *S. cerevisiae* te elimineer. Binne die *GOX2LOX*-kasset is *GOX2* aan 'n loxP-kasset gekoppel. Die nuwe wyngis VIN13-Δ1 is verkry deur 'n genomiese integrasie van *GOX2LOX* binne-in die *IME1*-lokus. Om die niesporulerende GOX-produserende wyngis VIN13-Δ2 te verkry, is VIN13-Δ1 gesporuleer, onderwerp aan mikromanipulasie en toegelaat om te herdiploidiseer. Ontledings het aangedui dat (i) *GOX2* aanleiding gegee het tot hoër vlakke van ekstrasellulêre glukose-oksidase aktiwiteit in vergelyking met *GOX1*; en (ii) dat die vlakke van uitgeskeide biologies-aktiewe glukose-oksidase vir die wyngisrasse aansienlik hoër was. Dit het verdere kleinskaalse wynfermentasies geregverdig.

Die getransformeerde wyngis VIN13-Δ1 is op eksperimentele skaal in die maak van rooi- en witwyn geëvalueer. Ontledings van hierdie eksperimentele wyne het daarop gedui dat glukose-oksidase deur die VIN13-Δ1-gisselle geproduseer en suksesvol uitgeskei tydens die wynmaakproses is, en dat VIN13-Δ1 die fermentasie

gedomineer het en die alkoholiese gisting voltooi het. Resultate het egter ook aangedui dat die geproduseerde glukose-oksidase nie baie aktief was onder die wynmaaktoestande wat in hierdie eksperimentele wynmaakproses gegeld het nie, en gevolglik was daar nie 'n drastiese verlaging in die alkoholvlakke sigbaar toe VIN13-Δ1 se wyne met VIN13 se wyne vergelyk is nie. Wynmonsters is deur middel van Fourier-transformasie-mid-infrarooispektroskopie (FT-MIR) ontleed ten einde die chemiese samestelling te bepaal, en gaschromatografie-massaspektrometrie (GC-MS) is aangewend om die wynaromakomponente te bepaal. Die vlakke van glukoonsuur is deur middel van ensiematiese reaksies bepaal. Multiveranderlike data-analise [hoofkomponentanalise (PCA) en parsiële kleinte kwadrate (PLS1) diskriminantanalise] is op die data van die verskeie analitiese tegnieke toegepas om onderliggende veskille tussen die wyne van VIN13 (wilde-tipe) en VIN13-Δ1 uit te wys. Chemometriese projeksies het aangetoon dat daar wel beduidende variasies sigbaar was tot en met drie hoofkomponente en/of PLS-komponente wat duidelik aandui dat glukoonsuur 'n sleutelfaktor was ten opsigte van die uitwerking wat GOX op VIN13-Δ1-fermentasies in vergelyking met VIN13-fermentasies. VIN13-Δ1 effek manifestasies is die beste waargeneem op grafieke wat PLS1-diskriminantanalise-data bevat. Verder het PLS1-diskriminantanalise ook aangetoon dat van die 'groepe' wat gebruik was tydens die analise, die FT-MIR-komponente en die GC-komponente beter resultate gelewer het. Die GC-komponente het hulle verder daartoe geleen om tussen die verskillende kultivars en VIN13/VIN13-Δ1-fermentasies te diskrimineer. Daar kan dus met sekerheid gesê word dat die grootste invloed in VIN13-Δ1 wyne sigbaar is in die aromakomponent, maar omdat daar wel ook variasies sigbaar was in die MIR-komponente, dat die smaakkomponente ook beïnvloed was.

Die aktiwiteit van GOX in druiwesap is verder ondersoek deur gebruik te maak van kleinskaalse fermentasies in bioreaktors. Daar is bevestig dat die VIN13-Δ1-geproduseerde GOX biologies-aktief was tydens aerobiese kondisies, onaktief was tydens anaerobiese kondisies, en onmiddelik geaktiveer kon word wanneer 'n anaerobiese fermentasie aerobies gemaak word (gesimuleerde mikro-oksigenasie). Hierdie verskillende fermentasies dui daarop dat glukose-oksidase inderdaat aktief is in druiwesap, en dat suurstof 'n sleutelfaktor is tydens die aktivering van die ensiem. Met behulp van 'n vereenvoudigde model kon aangetoon word dat tydens ideale toestande dit wel moontlik is om die alkoholvlakke te verlaag na 'n voorafbepaalde konsentrasie vir die bereiding van lae-alkohol wyne.

Hierdie studie verskaf verdere insig hoe om 'n GOX-produserende wyngis ge-

durende die wynmaakproses vir die verlaging van die alkoholvlakke te benut. Verder is dit duidelik dat suurstof van kardinale belang is vir die aktivering van die glukose-oksidase-ensiem en dat 'n tegniek soos mikro-oksigenasie 'n belangrike rol in hierdie verband tydens die wynmaakproses sou kon speel.

To Professor IS (Sakkie) Pretorius who introduced me to the wonderful world of molecular yeast microbiology. His human kindness, passion for the sciences, as well as his continuous encouragement and support throughout my studies, is contagious and an inspiration.

Biographical Sketch

Daniël Francois Malherbe was born in Bellville, South Africa on 1 February 1975. He attended Eikestad Primary School and matriculated at the Paul Roos Gymnasium, Stellenbosch in 1993.

He entered the University of Stellenbosch (US, Stellenbosch) and obtained a BSc degree in Microbiology and Genetics in 1998. In 1999 he completed a HonsBSc degree in Wine Biotechnology at the Institute for Wine Biotechnology (IWBT, US). After the graduation of his MSc degree in 2003, Danie enrolled for an PhD degree in Wine Biotechnology.

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Preface

This dissertation is presented as a compilation of six chapters, consisting of three research chapters with each of the chapters being introduced separately. All chapters are written closely in accordance to the style of the *American Journal of Enology and Viticulture*.

Chapter 1	**General Introduction and Project Aims**
Chapter 2	**Literature Review** Influence of Alcohol in Wine – From Yeast to Consumer
Chapter 3	**Research Results (Will be submitted for publication)** Evaluating the production and secretion of glucose oxidase by *Saccharomyces cerevisiae* yeast strains
Chapter 4	**Research Results (Will be submitted for publication)** Evaluation of a glucose-oxidase producing wine yeast in small-scale winemaking conditions imploring chemometrics
Chapter 5	**Research Results (Will be submitted for publication)** Impact of oxygen on glucose oxidase activity evaluated using model fermentations
Chapter 6	**Overview, Final Discussion, Concluding Remarks and Strategies for Future Research**

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Chapter 1

General Introduction and Project Aims

1.1 General Introduction

Serving that 'Perfect bottle' at a table starts with balancing correct viticultural techniques, harvesting fully matured fruit at the right time, and using up to date enological practices along with the right microbe interaction once fermentation starts. This is not an easy task and is precisely the reason why viticulture and enology of the twenty-first century are both finely tuned scientific fields which rely on continuous research and the use of cutting edge technologies to improve current and outdated practices. Once these practices are blended to promote one another the 'perfect bottle' of wine is not hovering on the horizon any more, but are within the winemakers grasp.

As consumers embrace a lifestyle of relaxing entertainment and wine-oriented social gatherings it is necessary to determine what wine consumers, connoisseurs and markets demand nationally as well as internationally. Questions such as these will assist the wine industry in helping to determine which area should be addressed first and which could be facilitated, for local consumption, export to foreign markets or for commercial benefits (Pickering *et al.*, 1998; Gladstones and Tomlinson, 1999; Gladstones, 2000; Høj and Pretorius, 2004).

In an effort to 'bottle sunshine', grape must is typically prepared from fully

matured grapes with intense varietal flavors. It is, therefore, becoming common practice to harvest fully ripened grapes. However, sunshine can be a very sharp double-edged sword and it is the application of wine biotechnology that permits its benefits to be harnessed, while at the same time negating its detrimental influences. This approach not only gives the high flavor intensity required, but also results in a more than adequate concentration of sugar.

This high concentration of sugar invariably leads to the production of wines that contain high levels of alcohol, with some wines reaching ethanol concentrations above 15% (v/v) (Godden, 2000). This has several implications: first, a high ethanol concentration can affect the sensory properties of the wine (Guth and Sies, 2002).

Depending on wine style, alcohol can be perceived as a 'hotness' on the palate, making the wines appear unbalanced. Furthermore, the higher alcohol content can mask the overall aroma and flavor of the wine. Second, health consciousness and increasingly strict road traffic laws pertaining to drinking and driving seem to be the main reasons for a worldwide decline in the consumption of alcohol (Pickering, 2000). This has increased the demand for reduced alcohol, low alcohol and even de-alcoholized wines (Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998), putting a great deal of pressure on wine producers, particularly those in warm climate regions where grape sugar levels can become high. Third, there can be increased tax rates associated with high alcohol wines (Pickering *et al.*, 1998; Gladstones and Tomlinson, 1999; Gladstones, 2000).

Several physical processes are used for the removal or reduction of alcohol in wine and some of them are sometimes used in combination. These processes tend to involve expensive equipment and can be intensive from a processing point of view (Bui *et al.*, 1986; Pickering *et al.*, 1999; Mermelstein, 2000).

An alternative approach was introduced with the concept of treating grape must with glucose oxidase (GOX) to reduce the glucose content of the must (the enzyme convert glucose to gluconic acid before the yeast cells are able to metabolize the glucose to ethanol), and therefore produce a wine with a reduced alcohol content after fermentation. This method was met with success, but is still labour intensive, as the winemaker has to add the enzyme to the must at a specific time. Concerns have also been raised as to the sensory quality of the finished product (wine) and,

in addition, the partial or incomplete fermentation of immature grapes with a low sugar content can have the inherent problem of excess residual sugar and a lack of flavor development in the resulting wine (Pickering *et al.*, 1998). When must is treated with GOX, the enzyme converts glucose into gluconic acid (which also has GRAS status), which is not metabolized by wine yeasts. Wines produced in this way should have reduced levels of ethanol and higher acidity. Furthermore, this technology could also be employed to produce a reserve of acidic musts or wines for blending purposes (Canal-Llaubères, 1993).

The winemaking process constitutes a unique ecological niche that involves the interaction of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). *Saccharomyces cerevisiae* has established its importance as wine yeast and also proven itself as a reliable starter culture organism. Its primary role is to convert the grape sugar into alcohol and, secondly, its metabolic activities result in the production of higher alcohols, fatty acids and esters, which are important flavor and aroma compounds that are essential for consistent and predictable wine quality (Fleet, 1993).

Enzymes play a crucial role in the winemaking process, and many of these enzymes originate from the grape itself, from the indigenous microflora on the grapes and from the microorganisms present during winemaking. In addition to enzymes that occur in pre- and post-fermentation practices, there are at least ten different enzymes driving the fermentation kinetics that convert grape juice into wine. The endogenous enzymes of grapes, yeasts and other microorganisms present in must and wine often are neither efficient nor sufficient under winemaking conditions to effectively catalyze the various biotransformation reactions. Commercial enzyme preparations therefore are used widely to enhance wine fermentations. Consequently, it is of key importance to understand the nature and behavior of these enzymes and to create the optimal conditions to exploit those enzymes that are valuable, while inhibiting those that may be harmful to the quality of the wine. Research in this field is very active and continually expanding. Recently, it also was suggested to use aerobic yeasts for the production of low-alcohol wines (Erten and Campbell, 2001), but the prospect of developing wine yeast strains expressing heterologous enzymes is also available (Whittington *et al.*, 1990; Park *et al.*, 2000; Van Rensburg and Pretorius, 2000; Kapat *et al.*, 2001).

Due to the demanding nature of modern winemaking practices and sophisticated wine markets, there is an ever-growing quest for specialized wine yeast strains possessing a wide range of optimized, improved or novel enological properties. Thus it only makes sense to perform additional research and finding a way to develop the ultimate wine yeast that are able to reduce the amount alcohol during fermentation without effecting the overall chemical and sensory properties of the finished product (Malherbe *et al.*, 2003).

1.2 Project Aims

Based on market research the world over, there is growing consumer demand, both in the domestic and export markets, for low-alcohol, 'reduced-alcohol' and de-alcoholized wines. It is therefore important to create a fast, reliable and inexpensive method and strategies to reduce the concentration of alcohol in wine. This study forms part of a comprehensive research program on reduced alcohol wines within the Institute for Wine Biotechnology, which is funded by Winetech (Wine Industry Network of Expertise and Technology), THRIP (The Technology and Human Resources for Industry Programme) and the NRF (National Research Foundation). The focus of the present study was to further explore the potential use of yeast-derived enzymes and the development of *Saccharomyces cerevisiae* wine strains yielding lower levels of alcohol during the winemaking process. More specifically, with this project it was attempted to genetically alter an industrial wine strain to produce less ethanol with minimal changes to wine composition.

The specific aims of this study were the following:

(i) to design a cloning system that will simplify integration of DNA into yeast chromosomal DNA, as well as selection of transformants, in diploid and polyploid host strains;

(ii) to construct all relevant vectors necessary to introduce the glucose oxidase gene cassettes, (*GOX1* and *GOX2*) from *Aspergillus niger* into laboratory and industrial strains of *S. cerevisiae*, targeting the *IME1* gene location thereby minimizing the chances for horizontal DNA transfer from genetically modified strains to ambient yeasts;

(iii) to verify the successful integration of *GOX1* and *GOX2* knock-out cassettes in yeast transformants;

(iv) to verify the deletion of the *IME1* gene;

(v) to screen laboratory and industrial yeast transformants for the production and secretion of biological active glucose oxidase;

(vi) to quantitatively determine the levels of glucose oxidase produced by GOX-producing laboratory and industrial yeast, both intracellularly and extracellularly;

(vii) to quantitatively determine the levels of gluconic acid produced by glucose oxidase-producing laboratory and industrial yeast, both intracellularly and extracellularly;

(viii) to conduct fermentation trials to evaluate the ability of newly constructed yeasts to transcribe the GOX gene and secrete glucose oxidase under wine-making condition;

(ix) to evaluate glucose oxidase's ability to convert glucose into gluconic acid under winemaking conditions;

(x) to demonstrate that the integrated GOX gene cassettes were stable and that the alcoholic fermentation was completed by the genetically engineered yeasts;

(xi) to evaluate the experimental wines chemically using Fourier transform infrared analysis in the mid-infrared area (FT-MIR) and gas-chromatography with a flame ionization detector (GC-FID);

(xii) to use chemometrics and multivariate data analysis to build a model for the prediction of gluconic acid levels and decreased levels of ethanol;

(xiii) to assess the effect of micro-oxygenation on the efficiency of GOX-producing yeasts in synthetic media in bioreactor trials;

(xiv) to assess the effect of micro-oxygenation on the efficiency of GOX-producing yeasts in grape must in bioreactor trials;

(xv) to determine if it is possible to predict a specific level of alcohol reduction, using an enzymatic approach; and

(xvi) to show that the genetically engineered wine strains are unable to sporulate and thus limiting unintended exchange of genetic material with ambient sporulating yeasts.

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Chapter 2

Influence of Alcohol in Wine – From Yeast to Consumer

2.1 Introduction

Alcoholic fermentation, the conversion of the principal grape sugars, glucose and fructose, to ethanol and carbon dioxide (CO_2) is conducted by yeasts of the genus *Saccharomyces*, generally *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (Boulton *et al.*, 1996). This complex microbial process probably represents the oldest form of a biotechnological application of a microorganism and has been used by humans for several thousand years (Griffith, 2004; McGovern *et al.*, 2004, 2009). This momentous innovation of "viniculturies" (encompassing both viticulture, vine cultivation, and winemaking) came together for the first time in human prehistory when the necessary preconditions were met during the Neolithic period, from about 8500 to 4000 B.C. (McGovern, 2007). Wine, as we know it, was available in the Shiraz area of present Iran over 6,000 years ago; but if we expand the concept of "wine" to fruits other than *Vitis*, the Chinese made wine, and drank it, some 9,000 years ago (McGovern, 2007; Jiang *et al.*, 2009).

Fermented products are healthier, safer and can be kept or stored for much longer periods than the raw material they originate from; this proved most important in warm or hot climates (Halpern, 2008). This is the reason why fermentation technology is such an important biotechnological tool, even in our modern world today.

Winemaking starts in the vineyard. The grapevines must be tended to year-round to ensure that they are adequately watered, receive enough nutrients, are protected from animals, which might trample them, graze on the leaves, or eat the fruit. Pests, such as mites, lice, fungi, and bacteria also have to be controlled (McGovern, 2007). These are some of the factors that contribute to healthy fully matured fruit that can be harvested at optimal ripeness.

Once the grapes arrive at the winery the viticulturist hands over to the oenologist. The winemaker has to apply different techniques and philosophies to produce a range of wines, white or red, for a variety of different styles. He has to understand the grapes he works with, as well as the style of wine that he wishes to produce. In the end, what consumers taste in wine is the culmination of management practices, talents and passion from the vineyard to the glass.

The supply of wine to the consumer has become intriguingly complicated as the wine marketplace has become more competitive than ever with improved wine quality and marketing sophistication. Furthermore, local markets are also influenced by global competitor countries' wine markets as trends indicate that the health benefits of wine lays in the balance of a good diet supplemented with moderate levels of good quality wine (Peregrin, 2005; Paganini-Hill *et al.*, 2007; Walzem, 2008). In addition, wines containing less alcohol provide all the benefits without the toxicity, and is much more affordable because of reduced taxation of these products (Halpern, 2008).

Therefore it comes as no surprise that over the past decade, a significant drop in the consumption of high alcohol beverages (10–13%) was observed. At the same time, the consumption of beverages with a lower alcohol content has shown an increase. This might be partly because of stricter drink-driving legislation, the awareness of health risks arising from excessive alcohol intake (Shults *et al.*, 2001; Erten and Campbell, 2001; Room *et al.*, 2005), or new fashion trends. These observations contribute to the development of new consumer markets which could benefit the wine industry as it increases the demand for wines with alcohol concentrations lowered in the final product. Such wines can be categorized as reduced alcohol (1.2% to 5.5–6.5% v/v), low alcohol (0.5–1.2% v/v) and even de-alcoholized (not above 0.5% v/v) wines (Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998*a*; Gladstones and Tomlinson, 1999; Gladstones, 2000; Pickering, 2000*a*).

In warmer wine regions, wine fermentation usually starts with about 23–25% sugar (22–25 Brix), leading to finished wines containing between 13 and 14% alcohol (v/v). This alcohol content increases even more when grapes are left longer on vines to obtain optimum ripeness and allow for enhanced flavor and aroma compound synthesis. In these circumstances, very high sugar concentrations may cause problem fermentations, either in prefermented grape juice with an inhibitory effect of sugar concentration, or towards the end of alcoholic fermentation with high ethanol concentration.

These types of problems and consumer demands call for research in methods to reduce or remove excess alcohol. There are a number of physicochemical and biological de-alcoholization methods available, of which some are rather expensive, labour intensive or result in wines with impaired organoleptic properties.

In this review, we will focus on what makes ethanol toxic to yeast cells and how it might induce suboptimal fermentation performance during winemaking. We will discuss the implications of high alcohol wines on human health as well as consumer preference, and give an overview of the financial implications from a commercial point of view. Finally, we will suggest possible solutions to address these high ethanol levels and means to reduce them to more acceptable levels employing physicochemical or biological methods for de-alcoholization of wine.

2.2 Sugar metabolism and ethanol production – Basics

Yeast metabolism can be either aerobic (respiratory) or anaerobic (fermentative) depending on the availability of molecular oxygen (see Figure 2.1). During winemaking respiration is very limited, even at the beginning of fermentation because of the high concentration of sugar which triggers fermentative metabolism. This effect where fermentation is triggered even with the availability of oxygen is called the Crabtree effect. *Saccharomyces* displays typical exponential growth kinetics in grape juice. Approximately 50% of the total available glucose and fructose may be metabolized during the growth phase (five to seven generations) to pyruvate via the glycolytic pathway. The remainder of the sugar will be fermented during the stationary phase after the exponential growth phase has ended and a final cell den-

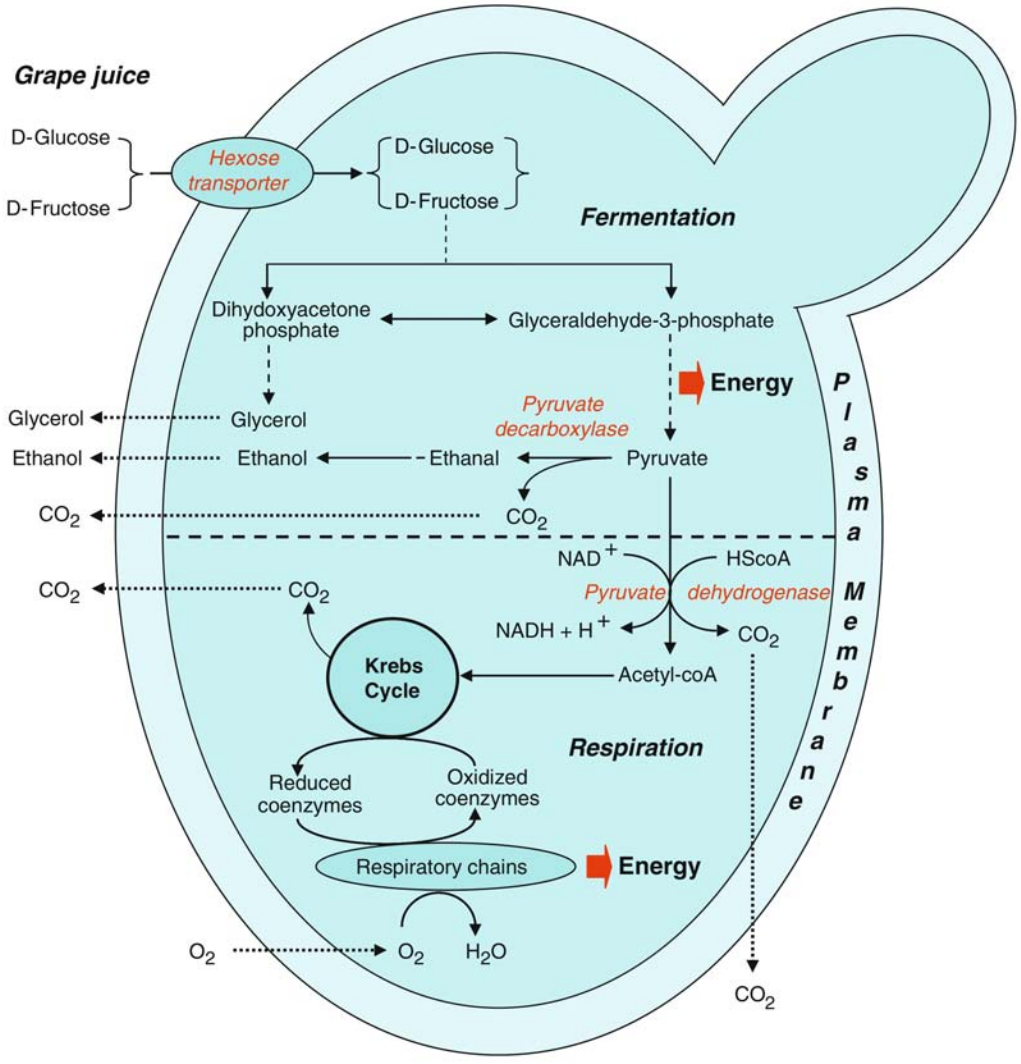


Figure 2.1: Fermentation and respiration in yeast cells during alcoholic fermentation of grape must. The source of the figure is Zamora (2009).

sity of 1–2 $\times$ 10^8 cells/mL has been reached. Figure 2.2 presents typical yeast and fermentation profile of grape must and Figure 2.3 presents the basic metabolic pathway in the yeast during the fermentation of grape must.

During glycolysis (see Figure 2.3 and Figure 2.4), one molecule of glucose is phosphorylated in two steps using 2 ATP to produce fructose 1,6-bisphosphate which is split by aldolase to form two 3-carbon triose phosphates. Inorganic phosphate is assimilated to form two triose diphosphates from which four protons (H^+ atoms) are accepted by two molecules of oxidized NAD^+. In the next four steps,

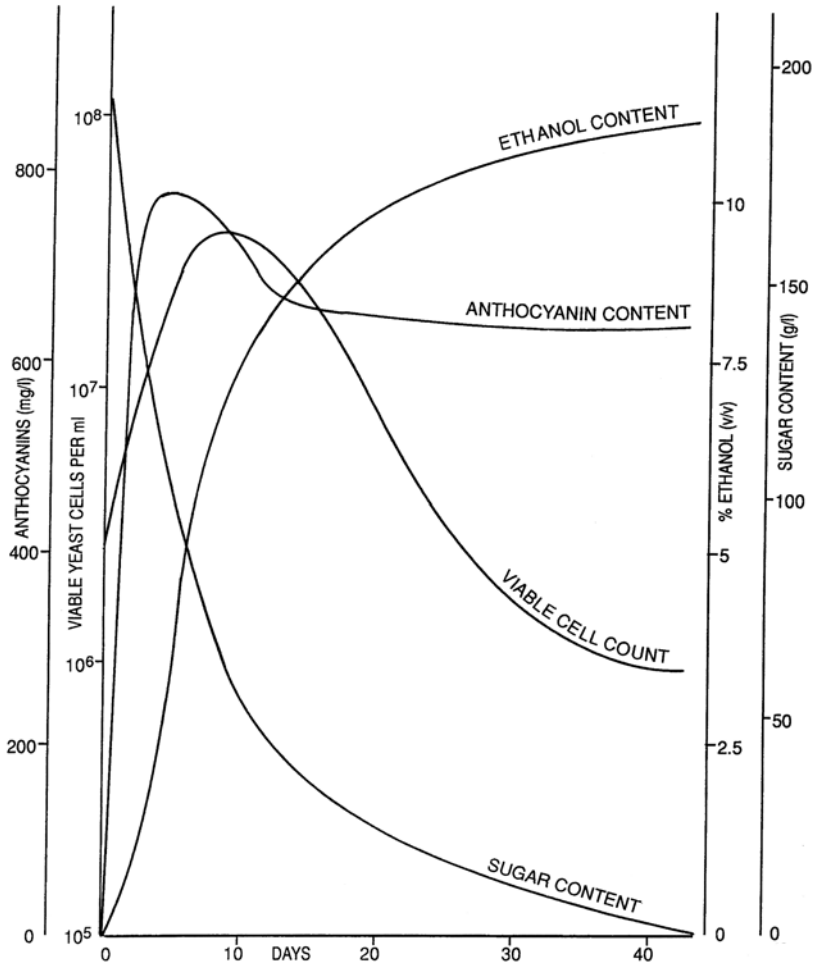


Figure 2.2: Yeast and fermentation profiles of grape must. The source of the figure is Jackson (2000*b*).

4 ATPs are formed by transfer of phosphate from the triose diphosphate to ADP resulting in the formation of two molecules pyruvate (Walker, 1998*b*). These reactions can be summarized:

$$\text{Glucose} + 2\,\text{ADP} + 2\,\text{P}_i \longrightarrow 2\,\text{Pyruvate} + 2\,\text{ATP} + 2\,\text{NADH} + \text{H}^+$$

The yeast uses the extra 2 ATP to increase in biomass and sustain growth. Each molecule of pyruvate is decarboxylated to acetaldehyde (ethanal), which is then reduced to ethanol. The reduction of acetaldehyde is important in order to recycle co-factors (NAD^+), thereby maintaining the redox balance and prevent the stalling

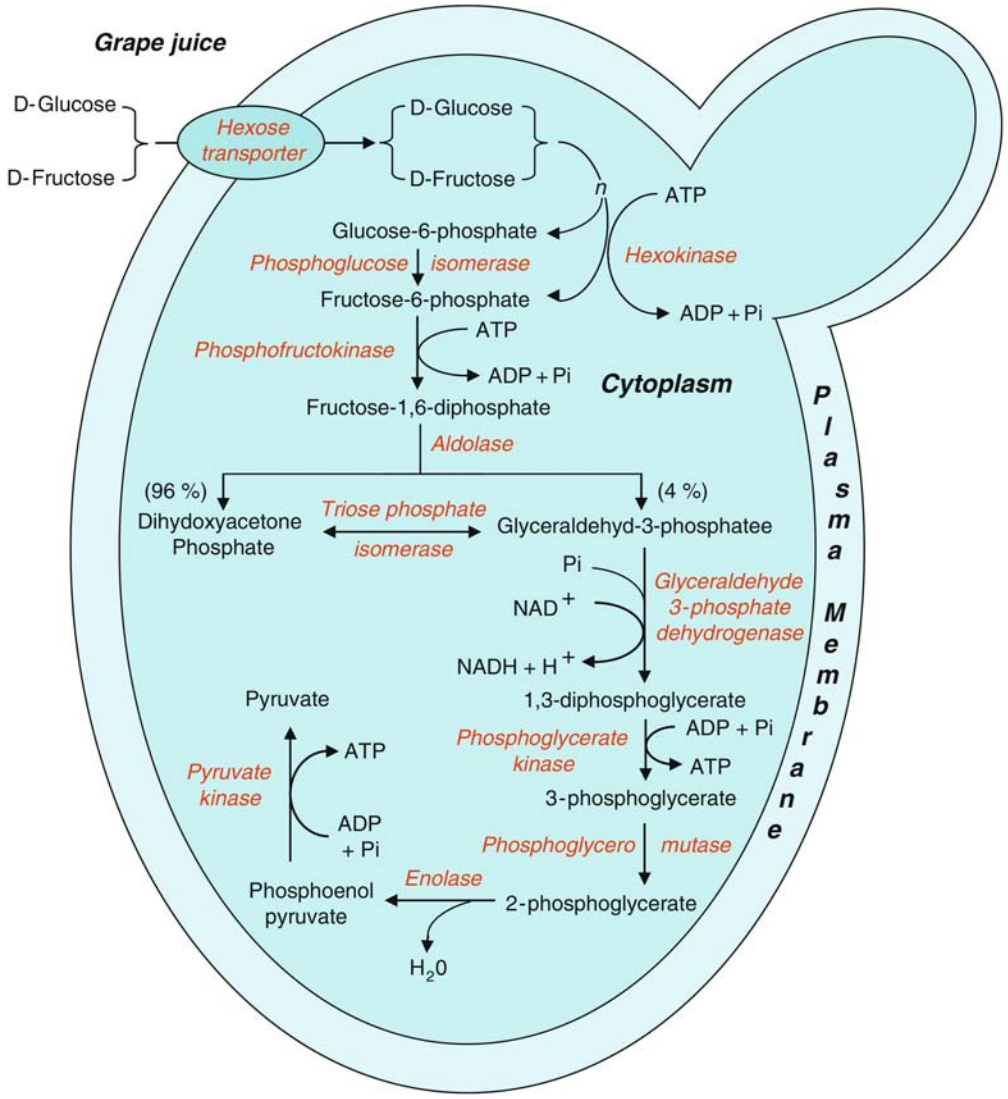


Figure 2.3: Glycolysis in yeast during alcoholic fermentation in grape must. The source of the figure is Zamora (2009).

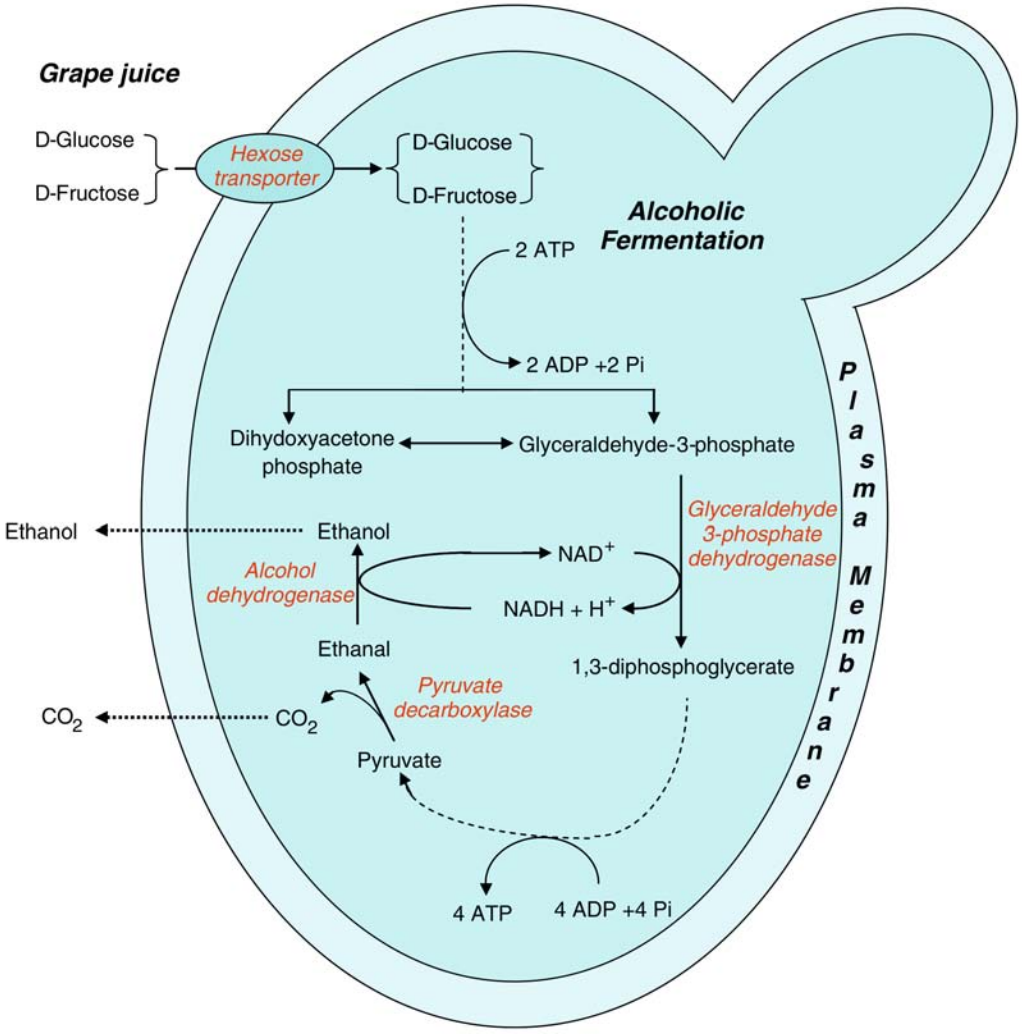


Figure 2.4: Alcoholic fermentation in grape must. The source of the figure is Zamora (2009).

of glycolysis. Thus, one molecule of glucose/fructose yields two molecules each of ethanol and carbon dioxide (CO_2).

$$\text{Glucose} \longrightarrow 2\,\text{Ethanol} + 2\,CO_2$$

The amount of ethanol produced per unit of sugar during wine fermentation is of considerable commercial importance. The theoretical conversion of 180 g of sugar into 88 g of CO_2 and 92 g of ethanol (51.1% on a weight basis) could only be expected in the absence of any yeast growth and loss of ethanol as vapor (Boulton *et al.*, 1996). This however is unrealistic and over the years a number of studies have tried to determine empirical conversion factors that can convert sugar directly to the final ethanol content on a volume basis:

$$\text{Ethanol (\%v/v)} = a + (b \times \text{Brix})$$

The value of the factor 'b' range from 0.55 to 0.63 depending on the yeast strain and its ability, or rather its efficiency to produce ethanol. Factor 'a' would then constitute the balance: glycerol, succinate and other products including biomass (Boulton *et al.*, 1996).

In warmer wine regions (e.g. South Africa) where fermentation starts with approximately 23–25% sugar (22–25 Brix), a quick calculation makes it clear that the finished wine will have a high alcohol content of between 12–14% (v/v). An ever greater problem arises when grapes are left longer on vines to obtain optimal ripeness and allow for enhanced flavor and aroma development. In these circumstances very high sugar concentrations cause problem fermentations, either in prefermented grape juice with an inhibitory effect of to high a sugar concentration, or towards the end of alcoholic fermentation with a to high ethanol concentration.

2.3 The effect of alcohol on yeast fermentation performance

During winemaking the yeast may be challenged by a variety of chemical stress factors. These could derive from the grape must (e.g. osmotic stress/high sugar (200 g/L), or low levels of assimilable nitrogen (150 g/L)), or as a result of the

winemaking process (e.g. temperature or presence of SO_2), or due to fermentation byproducts (Walker, 1998*a*; Carrasco *et al.*, 2001). Examples of the latter would be ethanol and acetaldehyde (Walker, 1998*a*).

Ethanol, or alcohol as it is commonly referred to is a major metabolic product of wine yeast fermentation and the winemaking process. Ethanol can be a two edged sword during winemaking.

It can act as a 'guardian', that governs the growth and influence of non- *Saccharomyces* species during fermentation. The quantity of alcohol necessary to block a fermentation and the growth of non-*Saccharomyces* species depends on many factors, including specific strain, temperature and aeration to name a few (Ribéreau-Gayon *et al.*, 2006*b*), and only becomes visible near the stationary phase of growth. Generally, the species of *Hanseniaspora*, *Pichia*, *Kluyveromyces*, *Metschnikowia*, *Candida* and *Issatchenkia* found in grape juice are not tolerant to ethanol concentrations exceeding 5–7%, and this explains their decline and death as the fermentation progresses beyond the mid-stage (Gao and Fleet, 1988; Heard and Fleet, 1988). There are exceptions where e.g. *Candida* can be present at the end of alcoholic fermentation and contribute to a higher wine quality (Jolly *et al.*, 2003), but in general the higher ethanol concentrations ensures that *Saccharomyces* dominates the fermentation, and limits the chances of wine spoilage by off-flavor/aroma production by other microorganisms.

On the other hand, ethanol may also act as a potent chemical stress factor for the yeast cells and cause serious problems for winemakers. As ethanol accumulates during the fermentation process, and its concentration increases, it will first have an inhibitory effect on growth, but as the process continues it will become lethal to the yeast and would result ultimately in cell death (Fleet, 2003).

For the purpose of this review, we will only discuss the effect of ethanol on the yeast cell and the physiological responses to ethanol toxicity.

2.3.1 Alcohol stress and alcohol tolerance in wine yeast

The mechanisms of ethanol toxicity have been studied extensively, as it is of considerable commercial importance to alcohol producers due to the fact that the fermentation is impaired by the main product (Walker, 1998*a*). Ethanol-induced tox-

Table 2.1: Important effects of ethanol on yeast cell physiology.

Physiological function	Ethanol influence
Cell viability	• General inhibition of growth, cell division and cell viability • Decrease in cell volume • Induction of morphological transitions • Enhancement of thermal death
Intermediary metabolism and macromolecular biosynthesis	• Denaturation of intracellular proteins and glycolytic enzymes • Lowered rate of RNA and protein accumulation • Reduction of V_{max} of main glycolytic enzymes • Enhancement of petite mutation • Induction of heat shock-like stress proteins • Increase in oxygen free radicals • Induced synthesis of cytochrome P450
Membrane structure and function	• Alteration of fatty acid and sterol composition • Induced lipolysis of cellular phospholipids • Increased ionic permeability • Inhibition of nutrient uptake • Inhibition of H^+-ATPase and dissipation of proton-motive force • Uncoupling of electrogenic processes by promoting passive re-entry of protons and consequential lowering of cytoplasmic pH • Hyperpolarization of plasma membrane

Table adapted from Walker (1998*a*).

icity and ethanol tolerance in the yeast have been reviewed by Ingram and Buttke (1985); Casey and Ingledew (1986); D'Amore *et al.* (1989); Carrasco *et al.* (2001); You *et al.* (2003); Hu *et al.* (2007); Nevoigt (2008).

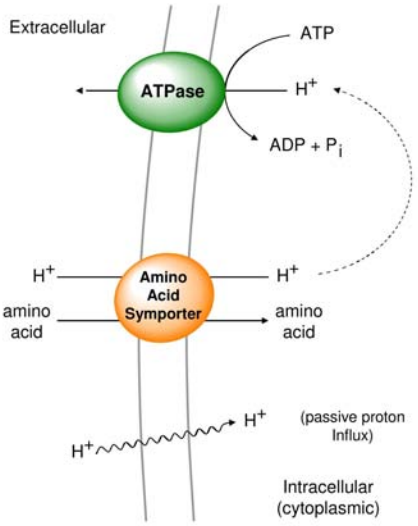
Ethanol acts as a non-competitive inhibitor of yeast growth rate at relatively low concentration. The glycolytic metabolism of the yeast seems to be comparatively resistant to the inhibitory effects of ethanol, and little effect on enzyme denaturation is detected below around 13% (w/v) ethanol (Walker, 1998*a*). Furthermore, growth inhibition (Jones and Greenfield, 1985) by ethanol is not due to enzyme inhibition as higher intracellular concentrations would be required for toxicity and these would not be achieved because of the passive diffusion of ethanol from the yeast cells to the wine environment (Guijarro and Lagunas, 1984). The principal inhibitory effects of ethanol toxicity on a yeast physiology is summarized in Table 2.1.

Despite the large range of influences that ethanol toxicity has on the physiology

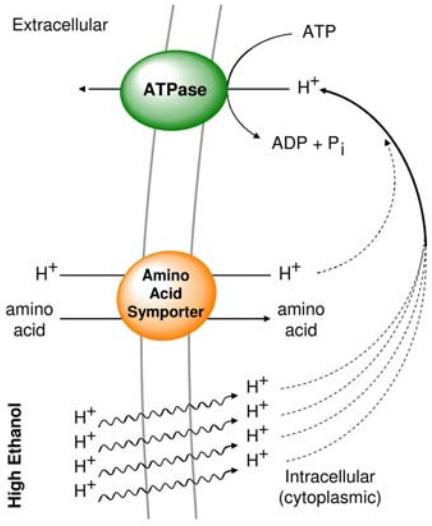
of the yeast cell, it would appear that the predominant target of ethanol is the yeast plasma membrane and specifically the membrane's permeability (Jones and Greenfield, 1985; Boulton *et al.*, 1996; Learmonth and Gratton, 2002). Several authors have suggested a relationship between the fatty acid composition of phospholipid membranes and ethanol stress tolerance. There is, in fact, a well-documented correlation between ethanol tolerance and the increased degree of fatty acid unsaturation of membrane lipids in *S. cerevisiae* (Mishra and Prasad, 1989; Mishra and Kaur, 1991; Alexandre *et al.*, 1994; You *et al.*, 2003). Thus, ethanol affects the yeast's transport of essential nutrients by impairing the hydrophobicity of the cell wall, which in turn results in increased membrane fluidity and decreased membrane structural integrity (Walker, 1998*a*; Mishra and Kaur, 1991). This makes the cells more permeable to hydrogen ions from the medium (Leão and van Uden, 1984*a*; Cartwright *et al.*, 1986) and increases the rate of passive proton influx into the yeast cells. Figure 2.5 presents graphically how passive proton influxin 2.5 (a) helps facilitate amino acid uptake and 2.5 (b) decreased membrane structural integrity when high ethanol levels are present. This increase of the passive proton influx, causes acidification of the cytoplasm and leads eventually to cell death as metabolism can not be maintained.

Other alcohols also contribute to the toxicity of the yeast and a correlation exists between the lipid solubility of the alcohols and their toxic effects. Furthermore, as the concentration decreases of any particular alcohol necessary to cause cell death, it is noticeable that at the same time the alcohol's carbon chain length increases that correlates to an increase in the hydrophobicity (Leão and Uden, 1982). In addition, Curran and Khalawan (1994) demonstrated that the alcohol concentration necessary to induce the heat-shock response also decreases as the carbon chain length increases. This would suggest that alcohol and heat both affect membrane lipids which are important components of the cell membrane and will have a great influence in the stress physiology of yeasts during wine fermentations (Walker, 1998*a*; Coleman *et al.*, 2007).

The toxicity by ethanol is further enhanced by the combined effect of a number of additional factors. These include metabolic by-products such as other alcohols, aldehydes, esters, organic acids (especially octanoic and decanoic acids), fatty acids, carbonyl and phenolic compounds, high temperatures and nutrient lim-



(a) Facilitated amino acid uptake during a normal fermentation with no stress on the yeast.



(b) High ethanol stress environment results in increased proton influx.

Figure 2.5: Passive proton influx (a) helps facilitate amino acid uptake and (b) cause decreased membrane structural entegrity when high ethanol levels are present which can acidify the cytoplasm of the yeast cell in grape must.

itations, specifically Mg^{2+} ions (Walker, 1998*a*).

2.3.2 Response of wine yeast to alcohol stress

It is important for a yeast cell during alcoholic fermentation is to maintain viability and stay metabolically active in the presence of ever increasing concentrations of ethanol. There is a number of physiological adaptions the yeast undergo which are thought to give some kind of protection against ethanol toxicity, these are outlined in Table 2.2 (Walker, 1998*a*).

These adaptions by the yeast ranges from alterations in its membrane fluidity to synthesis of detoxification enzymes. These alterations were confirmed by Mizoguchi and Hara (1997), where ethanol-induced alterations in the lipid composition of *S. cerevisiae*, grown in the presence of exogenous fatty acids, were studied. With the addition of both palmitic acid and ethanol (4–8%) to the medium, there was a striking increase in the palmitic acid content of the plasmamembrane and de-

Table 2.2: Adaptive physiological responses of yeasts to ethanol.

• Decrease in membrane saturated fatty acids (e.g. palmitic acid)
• Increase in membrane unsaturated fatty acids (e.g. oleic acid)
• Acceleration of squalene and ergosterol biosynthesis
• Increase in the phospholipid:protein ratio (e.g. enhanced phosphatidylinositol biosynthesis)
• Enhanced mitochondrial superoxide dismutase activity
• Elevated levels of cellular trehalose
• Stimulation of stress protein biosynthesis and acquisition of thermotolerance
• Increased synthesis of cytochrome P450 and increased ethanol metabolism

Table adapted from Walker (1998*a*).

crease in the content of myristoleic, palmitoleic, and oleic acids in the phospholipid fatty acid composition, as compared to their contents in the absence of ethanol. The palmitic acid-enriched cells had additional ethanol-endurability and a higher cell-membrane integrity than linoleic acid-enriched cells (Mizoguchi and Hara, 1996, 1997). New research by You *et al.* (2003) demonstrated that oleic acid is the most effective unsaturated fatty acid (UFA) in overcoming the toxic effects of ethanol in growing yeast cells. Furthermore, the only other UFA tested that conferred a nominal degree of ethanol tolerance was cis-vaccenic acid (Delta11Z-C18:1), whereas neither Delta11Z-C16:1 nor palmitoleic acid (Delta9Z-C16:1) conferred any ethanol tolerance. These findings of You *et al.* (2003) are consistent with the hypothesis that ethanol tolerance in yeast results from incorporation of oleic acid into lipid membranes, effecting a compensatory decrease in membrane fluidity that counteracts the fluidizing effects of ethanol.

A common response of yeasts confronted by ethanol toxicity is an increase in the fatty acyl chain length and an increase in the proportion of USF and sterols within the plasma membrane. This suggests that fermentations supplemented with membrane lipids (fatty acids, assimable nitrogen and metal ions) would enhance cell tolerance to ethanol toxicity (Casey and Ingledew, 1986; D'Amore *et al.*, 1989).

Metal ions have also been implicated in providing tolerance to ethanol and one of the major ions to provide this tolerance is Mg^{2+}. It has been found that Mg^{2+}-supplemented yeast cultures retain a higher viability rate in otherwise lethal heat shock and ethanol treatments. Elevated levels of Mg^{2+} have also shown that it could prevent stress protein biosynthesis (Birch and Walker, 1996; Ciesarová *et al.*, 1996), indicating the need for damage repair mechanisms to be activated has been averted

Table 2.3: Important effects of Mg^{2+} on yeast cell physiology resulting from ethanol stress.

Experimental observation	Reference
Mg^{2+} partially prevents the increase in proton and anion permeability caused by ethanol	Petrov and Okorokov (1990)
Mg^{2+} supplementations reduce the decline in yeast fermentative activity	Dombek and Ingram (1986)
Mg^{2+} increases ethanol production during fermentation of high-sugar media	D'Amore *et al.* (1988); Walker and Maynard (1996)
Mg^{2+} maintains high cell viability and ethanol production in rapid fermentations	Dasari *et al.* (1990)
Mg^{2+} is responsible for the differences in toxicity between produced and added ethanol	Dasari *et al.* (1990)
Mg^{2+} protects cells from ethanol toxicity and prevents ethanol-stress protein synthesis	Birch and Walker (1996); Ciesarová *et al.* (1996)

Table adapted from Walker (1998*a*).

by Mg^{2+}. Table 2.3 summarizes the role of Mg^{2+} in ethanol stress and tolerance.

Other responses to ethanol relate to the biosynthesis of heat-shock like proteins, or so called "ethanol stress proteins". Heat and ethanol stress cause similar changes to plasma membrane protein composition, i.e. reducing the levels of plasma membrane H^{+}-ATPase protein and inducing the plasma membrane-associated Hsp30 protein. Both stresses also stimulate the activity of the fraction of H^{+}-ATPase remaining in the plasma membrane. The resulting enhancement to catalyze proton efflux from the cell represents a considerable energy demand, yet may help to counteract the adverse effects for homeostasis of the increased membrane permeability that results from heat and ethanol stress (Piper, 1995; Swan and Watson, 1999).

Furthermore, an increase in activity of mitochondrial superoxide dismutases have been observed in yeast cells when increased levels of ethanol are detected. This observation suggests that ethanol induced oxygen free radical synthesis may be associated with ethanol toxicity and that antioxidant enzymes play a crucial role

in the defense mechanism in order to protect the cells from the damaging effects of ethanol (Costa *et al.*, 1997).

These physiological changes occurring in response to increased concentrations of ethanol help us understand how a yeast cell can tolerate higher than usual levels of ethanol and stay viable within a wine fermentation. It would seem that there is no single response but rather a unified response, or cascade of responses to ethanol toxicity that give a particular yeast strain the ability, or added advantage, to tolerate ethanol without any deleterious effects on its metabolic activities. This makes research in this field difficult but will undoubtedly be ongoing.

2.3.3 Nutrient limitations resulting from ethanol toxicity

The plasma membrane provides the semi-permeable barrier that allows all cells to exist. When yeasts encounter changes in the environment the plasma membrane adapt to these stimuli in order to remain viable. The presence of ethanol also affects the plasma membrane and the damage caused results in altered membrane organization and permeability. Ethanol affects the plasma membrane, possibly by entering the hydrophobic interior and thereby increasing the polarity of this region, thus permitting the free exchange of polar molecules and weakening the hydrophobic interactions (Alexandre and Charpentier, 1998). As the concentration of ethanol increases in the medium, so does the rate of passive proton influx into the yeast cells. As the cells become more permeable, the increased passive proton influx results in the pooling hydrogen ions intra cellularly (Leão and van Uden, 1984*a*; Cartwright *et al.*, 1986). The accumulation of hydrogen ions results in the acidification of the yeast cells and death.

Many amino acid transport systems are proton symporters, and are typically coupled to the movement of hydrogen ions (Boulton *et al.*, 1996; Goossens *et al.*, 2000). Thus when an amino acid molecule enters the cell, a proton (H^+) also enters the cell. The use of proton symporters can be regarded as a metabolic strategy considering that the difference in the grape juice pH relative to that of the yeast cytoplasm is typically a least three pH units less at the start of fermentation. In this way a component running along a strong gradient (pH) is energetically linked to uptake of amino acids as compared to one that is running against its gradient (Boulton *et al.*, 1996).

The passive proton influx that help facilitate the uptake of the metabolic components accumulate protons in the cell and can cause acidification of the cytoplasm and eventually cell death. Therefore, they have to be excreted from the cell. The cells can eliminate the excess hydrogen ions, making use of active transport (energy coupled transport), via the plasma membrane ATPase. The ATPase function as a hydrogen ion pump, which uses energy from the hydrolysis of one ATP molecule for each hydrogen ion pumped out of the cell (Serrano, 1978; Eraso and Portillo, 1994; Goossens *et al.*, 2000; Souza *et al.*, 2001). This ability of the yeast to excrete the access protons is an important regulatory factor to prevent acidification of the cytoplasm and to maintain amino acid uptake (Roon *et al.*, 1975*a*,*b*, 1977*a*,*b*). When the levels of excess cytoplasmic protons become too high the yeast may have to shut down hydrogen ion-coupled transporters to avoid overloading the capacity of the ATPase. This results also in the shut down of facilitated amino acid uptake during grape must fermentation (Boulton *et al.*, 1996). Additionally, mutations of the plasma membrane ATPase activity decreases permeability of amino acids and ammonium ions (Serrano, 1978), and render the cells sensitive to growth inhibition at low pH. The mechanism of amino acid transport is shown in Figures 2.5 (a) and 2.5 (b). Therefore, amino acid transport is strongly inhibited by ethanol and could result in problem fermentations (Leão and van Uden, 1984*a*,*b*, 1985; Ferreras *et al.*, 1989; Lglesias *et al.*, 1990) as a result of nutrient limitations (Learmonth and Gratton, 2002).

The yeast typically consume most of the available amino acids early during grape juice fermentation before the appearance of significant amounts of ethanol in the medium to ensure that their transport will not be compromised. The yeast is able to store amino acids in the vacuole to use at leisure when needed for biosynthesis and thus keep cytoplasmic pools of amino acids low for metabolic regulatory purposes while total cellular levels are in great excess over what is needed to produce a new cell (Boulton *et al.*, 1996). In addition, more energy must be consumed to deal with the increase in hydrogen ions due to increased passive flux into the cells as a consequence of ethanol production, the nitrogen has long since been depleted from the medium and the proton-coupled transport systems are not necessary. This also explains why the late addition of nitrogen to correct for nitrogen deficiency during alcoholic fermentation may have little to no impact on yeast metabolism simply because the cells are unable to transport the added compounds.

2.3.4 Development of alcohol tolerant wine yeast

The process of alcoholic fermentation can microbiologically be simplified by inoculating with a pure wine yeast strain/culture as it has been shown that it will rapidly dominate the fermentation (Boulton *et al.*, 1996), guaranteeing the outcome of a fermentation to a certain extend. This has led to the generation of large collections of wine yeasts that can be used by winemakers to ensure reliability of performance and quality (Schmidt *et al.*, 2006).

However, as fermentation conditions have been manipulated to meet a variety of challenges (Rosenfeld *et al.*, 2003), the emphasis has once again fallen on ethanol toxicity and the need for strains that are ethanol tolerant.

Much effort has been devoted to explore biochemical/physiological determinants of ethanol tolerance in yeast (Hu *et al.*, 2007). Thus, when considering a wine fermentation and the accumulation of ethanol during alcoholic fermentation, adaptation and survival of yeasts to increasing concentrations of ethanol are important criteria in selecting the correct wine yeast strain.

Many factors are responsible for variation in ethanol tolerance of budding yeasts. Some include: lipid composition of the plasma membrane (You *et al.*, 2003; Takagi *et al.*, 2005), accumulation of trehalose (Mansure *et al.*, 1994; Sharma, 1997) or heat-shock protein Hsp104 (Piper, 1995), activity of plasma membrane H^+-ATPase (Aguilera *et al.*, 2006), and mitochondrial stability (Ibeas and Jimenez, 1997; Castrejon *et al.*, 2002).

In addition, wineries nowadays use sophisticated temperature control systems (Carrasco *et al.*, 2001), thus temperature is an excellent example of a physical factor that can influence winemaking (Phisalaphong *et al.*, 2006). Ethanol tolerance of some yeast species depends on the temperature (Casey and Ingledew, 1986; D'Amore *et al.*, 1988; Gao and Fleet, 1988; D'Amore *et al.*, 1989). Torija *et al.* (2003) evaluated how temperature (15–35°C) affects the dynamics of a known population of *Saccharomyces* during alcoholic fermentation. Their work concluded that alcoholic fermentations at lower temperatures yield less ethanol compared to fermentations at higher temperatures. This could possibly be explained by a reduction in the use of substrates (carbon source) and an increase in secondary product formation such as glycerol and succinic acid. Higher temperatures correlate with higher yeast mortality and may induce a slower final rate of fermentation that can result in stuck fermentations with higher sugar contents (Torija *et al.*, 2003).

Additionally, as yeast cells have to be produced industrially prior to inoculation, it is worth noting that there are other conditions during the production processes (e.g. biomass production and drying) to which yeast cells are subjected (e.g. oxidative and desiccation stresses) that may have adverse effects that should also be taken into consideration (Carrasco *et al.*, 2001).

It is clear that a wine fermentation is a dynamic and complex process in which the yeast cell is subjected to multiple stress conditions. Successful adaptation to these stresses involves changes in gene expression profiles where a large number of genes are either up or down regulated (Varela *et al.*, 2005). Uncovering the genetic determinants for variation in ethanol tolerance in *Saccharomyces* wine yeasts is essential for understanding the evolution of fermentation, how certain species gain dominance during a fermentation, and ultimately improving the efficiency of how we select a new wine yeast strain that has better adapted to ethanol toxicity (Hu *et al.*, 2007).

2.4 The effect of alcohol on wine sensory characteristics

Wine consists of two primary ingredients, water and ethanol. However, how we sense wine depends on an additional 20 or more basic flavor compounds. The subtle differences distinguishing one varietal wine from another depend on an even larger number of compounds. More than 500 compounds have been isolated and identified from different types of wines. Alone, over 160 esters have been distinguished. Most of these occur at concentrations between 10^{-4} to 10^{-9} g/L. At these levels, most esters are below the threshold of human sensory perception. Therefore, most of the compounds that contribute to the characteristics of a wine, have no sensorial impact individually. In combination though, they may be very significant (Jackson, 2000*a*). Because of the bewildering array of compounds found in grapes and wine, the main focus of this section will be on the effect of alcohol on wine sensory characteristics.

The reason why ethanol has such a large impact on wine composition is that it is the most abundant compound in wine, second to water. Therefore, alcohol

defines a wine's strength, and does so in terms of alcohol content, or percentage of alcohol by volume. Because ethanol has a density of 0.789 g/mL, a wine with an alcohol content of 10% vol (v/v) contains 78.9 g/L of ethanol by weight. The alcoholic strength of wine is on average 100 g/L (12.5% v/v), although it could be higher (13–14.5%) in warmer climate countries (103–115 g/L). This increase in the alcohol content in temperate climates, depends directly on grape ripeness at the time of harvest.

During alcoholic fermentation, approximately 18 g/L of sugar will be converted to 1% (v/v) of ethanol (Ribéreau-Gayon *et al.*, 2006*a*). Thus, winemakers can use this value to estimate the amount of alcohol in a finished wine.

Due to the low density of ethanol, dry wines, containing negligible amounts of sugar, have densities below that of water (1.00), ranging from 0.91 to 0.94. This value decreases as the alcohol content increases. It would seem that the amount of ethanol in wine affect the overall aroma and flavor, and that there is a relationship between the vinous character ("wine" aroma without any elements that really stand out for description) and alcohol content, on the one hand, and a soft, full-bodied flavor, on the other hand (Ribéreau-Gayon *et al.*, 2006*a*).

2.4.1 Chemical interactions between alcohol and other wine components

Alcohols are organic compounds containing one or more hydroxyl groups (–OH). Simple alcohols contain only a single hydroxyl group, whereas diols and polyols contain two or more hydroxyl groups, respectively (Jackson, 2000*a*).

Ethanol's affinity for water and its solubility, by forming hydrogen bonds, makes it a powerful dehydrant. This property is useful in flocculating hydrophilic colloids, proteins and polysaccharides. It also gives anti-microbial properties that are particularly valuable in wines left to age. Ethanol can act as a solvent in pigment and tannin extraction from grapes. Furthermore, ethanol's unique properties are also useful for dissolving phenols from pomace during fermentation and is therefore involved in solubilizing certain odoriferous molecules and certainly contributes to the expression of aromas in wine (Jackson, 2000*a*; Ribéreau-Gayon *et al.*, 2006*a*).

Looking at the specific chemical interactions, the substitution of the hydrogen in a hydroxyl group by a carbon yields the 'alkoxy' group that characterizes ethers.

Another common oxygen-based functional group is the carbonyl group. Depending on its location in a group of atoms, it characterizes aldehydes (terminal position) or ketones (internal position). Combinations with an hydroxyl group on the same carbon generates the carboxyl group, giving organic molecules the properties of an acid. When an organic alcohol (hydroxyl) group reacts with an acid (carboxyl) group, it produces the ester linkage. It can therefore esterify with acetic, tartaric, malic and lactic acids in wine. In addition, alcohol can also be involved in the formation of ring structures (cyclic esters), called lactones. Furthermore, interaction of the hydroxyl group of an alcohol with the carbonyl group of aldehydes (e.g. acetaldehyde) or ketones, generates the a hemi-acetal and/or acetal compound (Jackson, 2000*a*; Ribéreau-Gayon *et al.*, 2006*a*).

Reactions between alcohols and hydrogen sulfide, resulting from the residues remaining of some vineyard treatments or produced by fermenting yeasts, form thiols which have most often a very unpleasant smell that is difficult to eliminate (Ribéreau-Gayon *et al.*, 2006*a*).

2.4.1.1 Balance between alcohol, glycerol and polysaccharides

When sweetness is detected in dry wines, it usually comes from the presence of aromatic compounds, combined with the mild sweet tastes of ethanol and glycerol (Jackson, 2002). Several alcohols are present in wine, but only ethanol occurs in sufficient concentrations to evoke taste sensations. It directly contributes to wine aroma and overall flavor since it is substantially above its perception threshold (from 0.1 to 100 ppm) (Pozo-Bayón and Reineccius, 2009).

Although ethanol possesses a sweet aspect, the acidity of wine diminishes its sensory significance. However, ethanol does enhance the sweetness of sugars that may be residing in finished wines (Zamora, 2009). Ethanol also reduces the perception of acidity, making acidic wines appear less sour and more balanced. At high concentrations (> 14%), alcohol increasingly causes a burning sensation (perceived as hotness) that may also contribute to the body (feeling of weight or fullness), especially in dry wines. In a study by Pickering *et al.* (1998*b*) an increase in ethanol concentration series, of added ethanol (0, 3%, 7%, 10%, 12%, and 14% v/v), could be correlated with perceived intensity (‘hotness’) in wines ranging from 0–14% v/v ethanol and contributed to the perception of ‘fullness’ in white table wines. Ethanol also can increase the perceived intensity of bitter phenolic compounds, while decreasing the sensation of tannin-induced astringency (Jackson, 2002; Gawel *et al.*,

2007).

Because ethanol can influence viscosity of a beverages, it could modify aroma release and thus aroma perception (Nurgel and Pickering, 2005). Nevertheless, the most studied ethanol effect is related to its capacity to modify solution polarity, thus altering the gas-liquid partition coefficient. An increase in ethanol content has been shown to decrease the activity coefficients of many volatile compounds in wine because of an increase in solubility (Voilley *et al.*, 1990).

Glycerol is the most prominent wine polyol. In dry wines its concentration is only exceeded by water and ethanol and is therefore an important by-product of alcoholic fermentation (Lubbers *et al.*, 2001). Glycerol production takes place in order to maintain the redox balance within the yeast. Growth of yeast on amino acids, as the main nitrogen source, limits the need for amino acid biosynthesis, and hence little associated NADH is generated. This limits the need for NADH reoxidation, which is coupled to glycerol production. On the other hand, growth on ammonium salts requires *de novo* amino acid synthesis, which results in significant glycerol production in order to maintain redox balance (Ugliano and Henschke, 2009). Figure 2.6 demonstrate production of glycerol and the associated redox reactions with flavor implications. Generally, dry red wines contain more glycerol than dry white wines (Ough *et al.*, 1972; Ribéreau-Gayon *et al.*, 2006*a*) and concentrations can range from 4–10 g/L in dry table wines but may reach values as high as 15–20 g/L in dry red wines, depending on factors, such as grape variety, degree of ripeness, fermentation temperature, SO_2 concentration, pH of grape must, nitrogen composition, aeration, yeast strain and inoculation level (Ough *et al.*, 1972; Sehović *et al.*, 2004; Ribéreau-Gayon *et al.*, 2006*a*).

Consequently, glycerol has often been assumed to be responsible in generating a smooth mouth-feel and the perception of viscosity. Thus it would appear that glycerol plays an important role in the organoleptic properties of wine (Lubbers *et al.*, 2001). The threshold taste level of glycerol is observed at 5.2 g/L in wine. Glycerol is a non-volatile compound but contributes significantly to the sweetness, body and fullness of wines, although a mass concentration of 25.8 g/L has been proposed as a level at which an increase in viscosity can be perceived (Noble and Bursick, 1984). Glycerol has a sweet taste that reinforces the sweetness of ethanol in dry wines, but it is not responsible for any of the sweetness in sweet wines (Ribéreau-Gayon *et al.*,

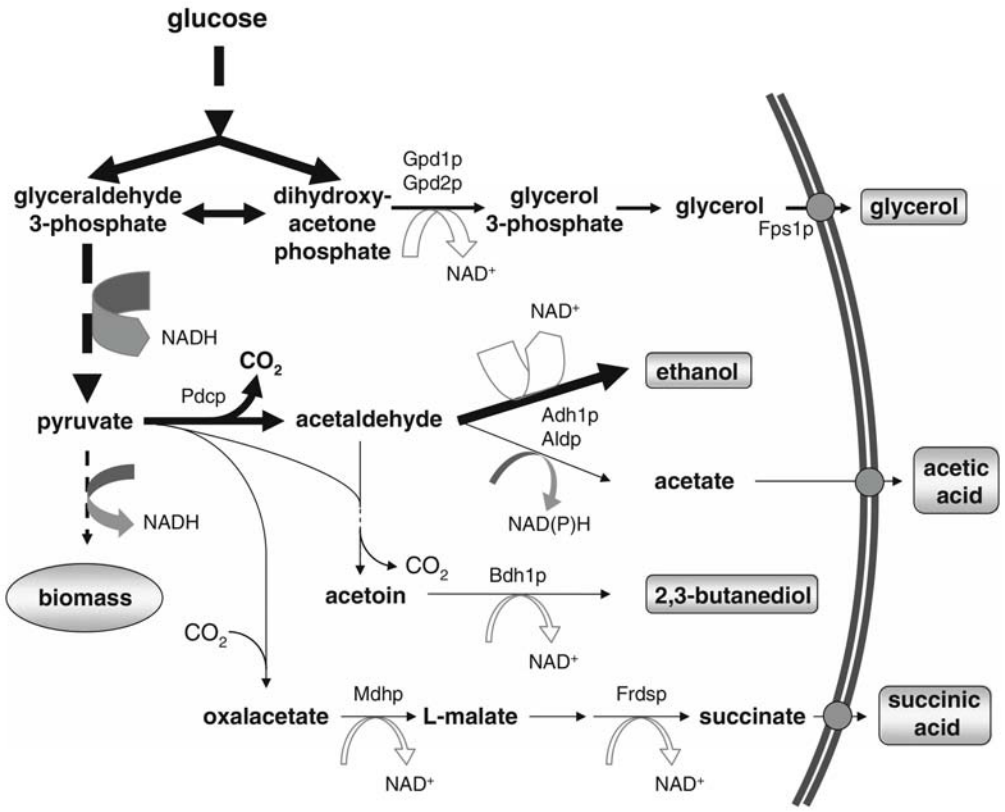


Figure 2.6: Production of glycerol and associated redox reactions with flavor implications. Ethanol production is essentially redox neutral; however metabolism associated with biomass production generates nett NADH, which is oxidized largely by glycerol production. Other important NADH oxidizing reactions with flavor implications are the production of 2,3-butanediol, L-malic acid and succinic acid. When glycerol production is stimulated by non-growth associated reactions (i.e. osmotic stress) the reduction of NAD^+ occurs by other reactions including the oxidation of acetaldehyde to acetic acid. The source of the figure is Ugliano and Henschke (2009).

2006*a*; Zamora, 2009).

Viscosity is known to contribute to the oral sensations when drinking wine. These include saltiness, sweetness, bitterness, flavor and astringency (Christensen, 1979; Christensen and Casper, 1987; Smith *et al.*, 1996; Hollowood *et al.*, 2002; Nurgel and Pickering, 2005). However, glycerol rarely reaches a concentration that perceptibly affects viscosity ($\geq$26 g/L) (Noble and Bursick, 1984). Nevertheless, it may still be sufficient to play a minor role in suppressing the perception of acidity, bitterness and astringency. The slightly sweet taste of glycerol may also play a minor role in dry wines, in which the concentration of glycerol often surpasses its sensory threshold for sweetness ($\geq$5 g/l) (Nieuwoudt *et al.*, 2004). However, it is unlikely to contribute detectably to the sweetness of dessert wines that contain more sugar compared to a standard table wine (Jackson, 2002).

Therefore, glycerol production is for the above mentioned reasons, one of the desirable features during grape must fermentation (Rankine and Bridson, 1971; Scanes *et al.*, 1998; Sehović *et al.*, 2004).

Polysaccharides are carbohydrates ("polyhydroxy-aldehydes/ketones") with a degree of polymerization higher than 20 saccharite units. Their presence in wines is due to the contribution of the cell walls of either microorganisms during alcoholic fermentation or grape berries after degradation by pectic enzymes during grape ripening or winemaking. Polysaccharides constitute one of the main groups of macromolecules in wine and contribute by increasing its viscosity and stability (Sanz and Martínez-Castro, 2009).

The levels of polysaccharides in finished wine is generally low and the significance of polysaccharides to the sensory properties of wine has not been adequately studied (Jackson, 2000*a*). Sanz and Martínez-Castro (2009) reported that polysaccharides have been linked to the organoleptic quality of wines (Vidal *et al.*, 2003) because their interactions with other constituents such as polyphenols (Riou *et al.*, 2002) and other aromatic compounds (Chalier *et al.*, 2007), etc. An increase in ethanol would caused a decrease in the solubility of the polysaccharides (Bouchard *et al.*, 2007), resulting in less interaction with other aromatic compounds and a decrease in the organoleptic quality of the wine.

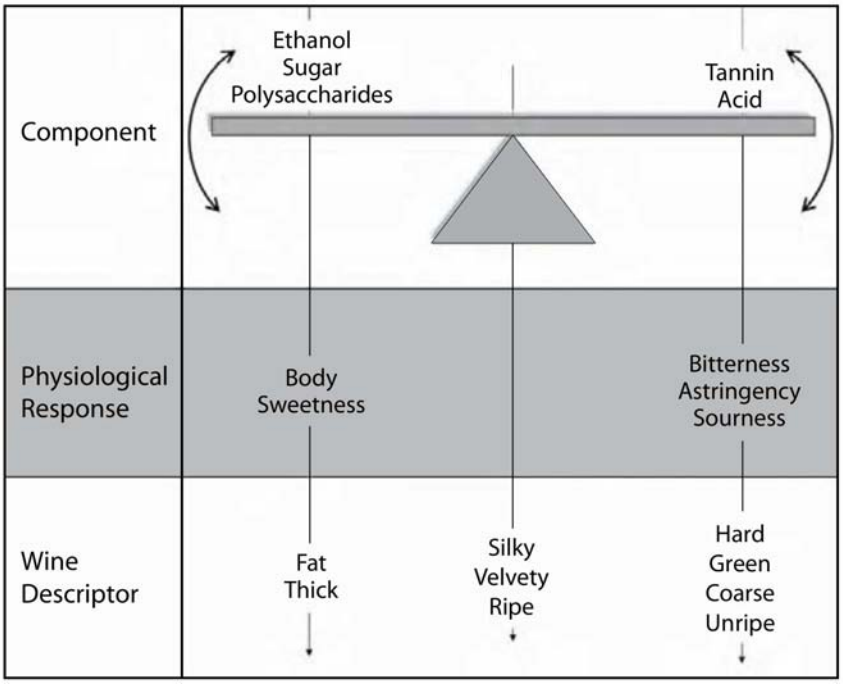


Figure 2.7: Interaction of wine components: Tannins and acid are balanced with ethanol sugar and polysaccharides. The source of the figure is Kennedy (2008).

Ultimately, the goal is to balance all the chemical components in a wine. This can be difficult for a winemaker if initially, the grapes were picked early, as a wine would have a tendency to have excess tannins and acidity with a deficiency in polysaccharides, sugar and ethanol. If the fruits were more mature, the composition becomes more balanced and the descriptors become more positive (Kennedy, 2008). See Figure 2.7 to conceptualize the relationship between the different grape components and how they influence each other during winemaking.

2.4.1.2 Influence of alcohol on volatile chemical compounds

Ethanol also has the ability to help dissolve volatile compounds that are produced during fermentation and those that are formed during maturation in wooden barrels. The dissolving action of the alcohol probably reduces the loss of aromatic compounds with carbon dioxide during alcoholic fermentation. Conversely, at low concentrations (0.50–0.75%), alcohol enhances the release of certain aromatic compounds (Williams and Rosser, 1981; Jackson, 2000*a*).

The effect of ethanol on the volatility of aroma compounds is understood and it clearly appears that ethanol leads to modification in macromolecule conformation

(e.g. protein structure), which changes the binding capacity of the macromolecule (Voilley and Lubbers, 1998).

2.5 The link between wine alcohol content, human health and consumer preferences

2.5.1 Alcohol consumption and implications on human health

The contrasting social and antisocial effects of moderate versus excessive alcohol consumption must have become evident almost as soon as wine was discovered (Griffith, 2004; McGovern *et al.*, 2004, 2009). The use of grape wine as a medicine or as a carrier solution to dispensed natural products, specifically herbs and tree resins as drugs has a long history, going back at least to the ancient Egyptians (McGovern *et al.*, 2009). The ancient Greek and Roman societies also used wine extensively for medicinal purposes. This practice continued until the beginning of the twentieth century (Jackson, 2000*d*).

Modern consumer trends associated with regular and moderate consumption of wine should be placed in context with the constituents and characteristics of a healthy diet and lifestyle (Stockley and Høj, 2005). The benefits of wine for health are mostly associated with polyphenols, which are absorbed better from wine but poorly from unfermented grape juice. Dealcoholised wine provides all the benefits without the toxicity, and is more affordable in some contries as a result of how wines are taxed (Halpern, 2008). These findings benefit the wine industry as ever changing alcohol taxation policies in the beverage industry and stricter drinking driving legislation also increased the demand for methods to produce wines containing less alcohol and include reduced alcohol (1.2% to 5.5–6.5% v/v), low alcohol (0.5–1.2% v/v) and even de-alcoholized (not above 0.5% v/v) wines (Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998*a*; Gladstones and Tomlinson, 1999; Gladstones, 2000; Pickering, 2000*a*).

2.5.1.1 Advantages of alcohol for human well-being

Over the years, studies consistently demonstrated an inverse relationship between alcohol consumption and the occurrence of myocardial infarction and cardiac death, with a J-shaped curve relating alcohol intake to mortality (Connor, 2006), favor-

ing moderate alcohol drinkers compared with non-drinkers or heavy drinkers who overindulge themselves (Goldfinger, 2003). The 'French paradox' and the benefits of drinking wine presented by Renaud and de Lorgeril (1992); Gall (2001) enhanced an interest in wine world wide (Goldfinger, 2003). In addition, the reduction in risk for wine consumers is similar to that of consumers of fruits, wholegrain and vegetables. This diet in conjunction with wine, presents the core components of a 'Mediterranean-style diet' (Stockley and Høj, 2005; Walzem, 2008).

The chemical components in wine considered primarily responsible for its health benefit are ethanol (75–85% of cardioprotective properties), the phenolic compounds and the polyphenolic forms of the phenolic compounds (Stockley and Høj, 2005). Furthermore, the variety of wines available to consumers can be expected to effect health differently in accordance with a particular wine's total polyphenolic content and spectrum of individual polyphenols (Walzem, 2008). Hence the reason red wine is supposedly healthier as compared to white wine (Stockley and Høj, 2005).

The beneficiary effects of individual components in wine for moderate wine consumption may be summarized as follow (Bujanda, 2000; Estruch, 2000; Goldfinger, 2003; Peregrin, 2005; Stockley and Høj, 2005; Crozier, 2006; Walzem, 2008; Covas *et al.*, 2009):

Ethanol

- increases high density lipoprotein (HDL)-cholesterol (anti-antiatherogenic effect),
- inhibits platelet aggregation (thrombus formation – acute chardiacischaemia),
- reduces systemic inflammation, and
- oxidative damage

Polyphenols

- increase the anti-oxidant protection,
- decrease platelet aggregation,
- decrease systemic inflammation,
- increase vasodilatation

Interestingly, a study indicated that wine buyers made more purchases of healthy food items compared to people who buy beer (Johansen *et al.*, 2006). Wine consumers generally have fewer risk factors for cardiovascular disease compared with beer and spirits consumers, which is reflected in an approximately 25% to 35% lower risk of cardiovascular disease for wine consumers compared to consumers of beer and spirits (Stockley and Høj, 2005). This trend indicates that the health benefits of type of alcohol consumed (Paganini-Hill *et al.*, 2007), lay in the balance of a good diet supplemented with moderate levels of wine (Peregrin, 2005; Crozier, 2006; Walzem, 2008).

2.5.1.2 Disadvantages of alcohol for human well-being

For humans, ethanol is toxic and over consumption often has devastating consequences. The lethal dose (LD_{50}) by oral consumption is 1400 mg/Kg body weight (Ribéreau-Gayon *et al.*, 2006*a*). When one thinks of the disadvantages alcohol poses to human well-being that what comes to mind first and foremost is alcohol abuse. This is governed by an individual's tolerance to alcohol (level of intoxication) and/or if that person is addicted to it. Alcohol abuse can ultimately lead to alcoholism (Mayer, 1983).

Because the problems associated with alcoholism have been well-documented elsewhere (Buckland, 2001; Room *et al.*, 2005; Edenberg *et al.*, 2006; Babor, 2008), they will not be discussed here.

It is however worth mentioning that excessive ethanol consumption or abuse (Jackson, 2000*d*) may cause:

1. cirrhosis of the liver (Cederbaum *et al.*, 2009),
2. increase the likelihood of hypertension and stroke (Barden *et al.*, 2007),
3. the development of cancers of the mouth, pharynx, larynx, esophagus, and liver (Longnecker, 1995; McPherson, 2007; Seitz and Meier, 2007), and
4. increase the risk for fetal alcohol syndrome (Abel and Hannigan, 1995; May *et al.*, 2007)

Although various factors contribute to alcohol-associated cancer development, research has shown that acetaldehyde rather than alcohol itself is carcinogenic. Acetaldehyde is highly toxic, mutagenic, and carcinogenic. Furthermore, it interferes at many sites with DNA synthesis and repair and, can consequently result in tumor development (Seitz and Meier, 2007). Acetaldehyde is produced from ethanol by alcohol dehydrognase (ADH) or cytochrome P4502E1 (CYP 2E1). These enzymes rapidly metabolize alcohol leading to increased concentrations of acetaldehyde and 'alcohol-associated' cancer development (Seitz and Meier, 2007; Edenberg *et al.*, 2006).

In addition, it has also been shown that alcohol can influence highly active antiretroviral treatment (HAART) and result in poorer viro-immune outcomes, thereby increasing the susceptibility to AIDS in HIV infected individuals. Although there were discrepancies in the findings, they could be related to confounding variables, including gender, patterns of alcohol abuse and type of alcohol beverage beyond the amount consumed (Míguez-Burbano *et al.*, 2009).

Figure 2.8 presents the possible long-term effects of excess and moderate alcohol consumption.

2.5.2 Health regulations and consumer preferences

2.5.2.1 Food safety regulations pertaining to alcoholic beverages

Recent years have brought substantial advances in our understanding of the risk relations of alcohol consumption and specific disorders (Room *et al.*, 2005; Jernigan, 2006). Advertising campaigns by companies selling alcoholic beverages is well received by the public and increase the sale of these beverages. Unfortunately, public information campaigns regarding alcohol consumption and abuse is not well received and the general experience is negative (Babor *et al.*, 2005). Unless governments are willing to proceed with intensive counter-advertising campaigns, which the alcohol industry will probably interpret as an attack, the most promising path forward for public information campaigns in the alcohol field is rather in terms of building support for implementing proven prevention strategies. These could be: support for control policies on advertising, availability and price of alcoholic beverages, availability restrictions, and the enforcement of conditions on licensed

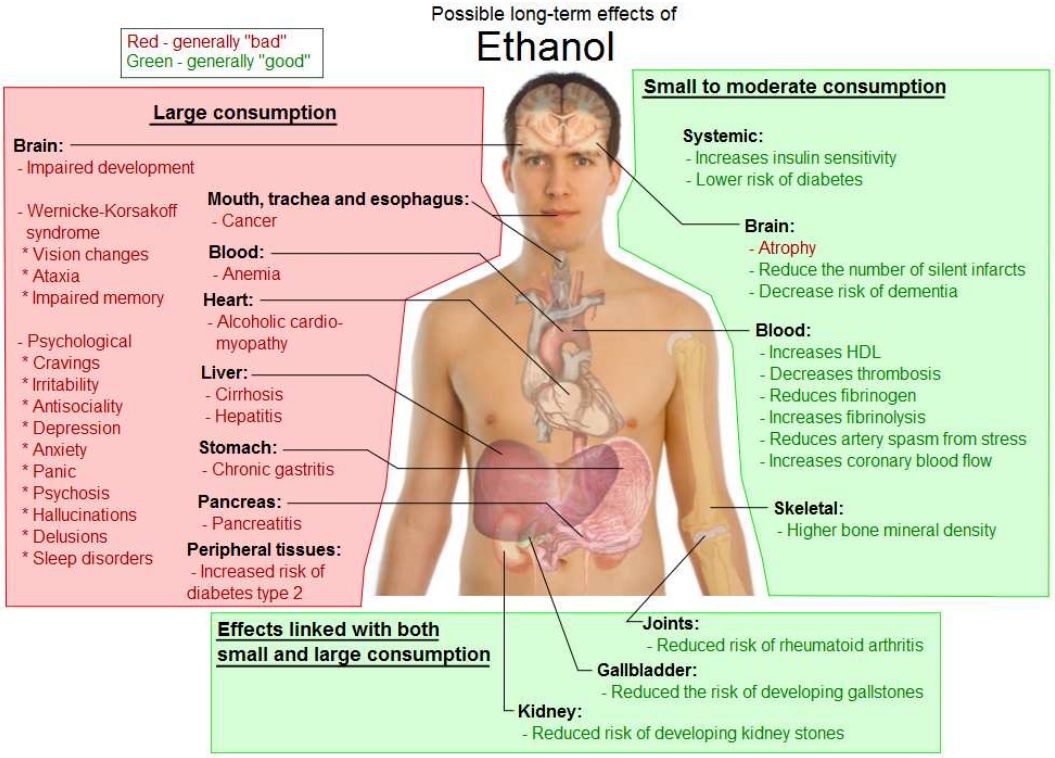


Figure 2.8: Possible long-term effects of excess and moderate alcohol consumption.

premises (Casswell and Gilmore, 1989; Room *et al.*, 2005).

2.5.2.2 Life style changes and consumer preferences

A trend, which can be related to social issues and changing attitudes towards lifestyle and health, has recently emerged with the growth in the health consciousness of drinking wine as opposed to hard spirits or beer (Stockley and Høj, 2005; Johansen *et al.*, 2006; Jenster *et al.*, 2008). The recent association of wine as a nutritional healthy beverage is likely to continue to influence consumption choices favoring wine over other alcoholic beverages, and this is a long term factor. For a number of years, there has been a move to lower alcohol content beverages (Jenster *et al.*, 2008) and serves as an indication that there is a growing market for low alcohol products. Therefore it comes as no surprise that the focus within the wine industry has also shifted towards low alcohol, reduced alcohol and de-alcoholized products as a result of the increased international interest and consumer demand (Pickering and Heatherbell, 1996; Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998*a*).

These changes occur and become a driving factor to merge synergistic responses to increasing homogeneous consumer behavior and the opportunity of newer markets to take place. This brings advantages to larger scale international retailers and further contributes to the globalization of wine markets (Jenster *et al.*, 2008).

2.6 The link between wine alcohol content, regulatory and trade issues

2.6.1 Alcohol legislation and regulations

All commodities are subjected to certain regulations, and wine is no exception. However, wines are probably subjected to more regulations because of their diversity which may cover aspects ranging from how grapes are grown, to when and where wine may be consumed (Jackson, 2000*e*).

We will not discuss the whole range of aspects that can contribute to the regulatory issues of wine, but instead will only focus on the impact of alcohol (ethanol).

2.6.1.1 Societal changes regarding alcohol consumption

Despite association of wine as a nutritional healthy beverage to be consumed with food, a recent study indicated that youth who saw more alcohol advertisements on average, drank more. In addition, after each additional alcohol advertisement seen, the observed number of drinks consumed increased as well (Snyder *et al.*, 2006). This is a serious problem as we all know the amount of alcohol advertisements during sports events. This could be enough reason to follow suit and ban alcohol advertisements during sports events, as was the case for tobacco advertisements.

In the day and age we live in, there are numerous stress factors, and it was found that repeated use of alcohol as a coping strategy to reduce anxiety or discomfort increases one's risk of developing alcohol dependence. Previous studies have found alcohol outcome expectancies (AOE; what people believe the effects of consuming alcohol is) strongly predict drinking behavior, in general, and also are related to many individuals drinking to cope with social situations (Carrigan *et al.*, 2008).

These examples show that it is important to regulate alcohol on all levels, whether

it is production, distribution or marketing. This should be controlled and enforced in such a manner as to reduce the negative impact on human society in general as well as societal changes.

2.6.1.2 Road safety legislation pertaining to alcohol consumption

Alcohol in the bloodstream results in impaired judgement and slowed reaction-times in drivers because of a delayed transmission of nerve signals from one nerve cell (i.e., neuron) to the next (Roehrs and Roth, 2001). Rates of alcohol-related casualties can and have been reduced in many countries by a combination of counter-measures, such as the adoption in much of the world of "per-se laws" forbidding driving above a stated blood-alcohol concentration. This can be further reduced by the subsequent lowering of the accepted level. The effectiveness of such laws is dependent on the perceived probability of being caught driving at greater than the allowed level. Furthermore, evidence suggests that having sobriety random check-points where people are asked to perform breath-testing as well as sustained police attention to drink-driving has an effect in lowering the number of alcohol-related casualties (Shults *et al.*, 2001; Room *et al.*, 2005).

Most countries define how much alcohol can be consumed in the form of "drinks" or "units". Unfortunately, there is no common standard, or set of rules, to define a standard drink measure among countries or in the scientific literature (Turner, 1990). Most countries do not use any standard definition for drinks, and, where serving sizes are defined, these measures depend to a great extent on local culture and customs. Furthermore, where standard units have been implemented, they may vary according to the type of beverage alcohol–spirits, wine, or beer (Dufour, 1999; ICAP, 2009). Globally, 1 unit is recognized as 0.02 g (0.015 g) in 100 mL blood or 0.10 mg per 1000 mL in breath, and it takes approximately one hour for your body to process this unit out of your system. This is influenced by the individual's weight and gender. For instance, a beer (340 mL) with an alcohol content of 4.5 % (v/v) contains 15.3 mL alcohol which is equivalent to 1.5 units when considering that a standard drink/unit size is equal to 10 g of ethanol in South Africa.

In South Africa the legal blood alcohol concentration is < 0.05 g per 100 mL ($\approx$ 2.5 units), and < 0.24 mg per 1000 mL for breath alcohol concentration. The law is even stricter for professional drivers with the legal blood alcohol concentration

is < 0.02 g per 100 mL (≈ 1 unit), and < 0.10 mg per 1000 mL for breath alcohol concentration (SADD, Online).

2.6.2 Alcohol taxation and trade

Various countries have tax systems which penalize wines (or alcoholic beverages in general) with higher alcohol levels. This practice is expected to become more widespread, as governments seek higher tax revenues – but seek also to motivate greater consumer awareness of alcohol levels and moderation in alcohol consumption.

2.6.2.1 Governmental controls and taxation according to wine alcohol content

In South Africa excise tax is based on volume of wine and not the alcohol concentration of the product. In some other jurisdictions, reducing the tax payable is often cited as a reason to reduce alcohol (Hay, 2001). Therefore, the main reason to remove alcohol in South Africa would seem to be precisely to change the vinous character ("wine" aroma without any elements that really stand out for description) of the wine. From a practical perspective, there are some legal considerations to take into account. Current EU regulations do not allow South African wine that has been subjected to removal of alcohol to be sold in the EU, although exports to the USA are allowed (Matthee, 2006). Alcohol removal can therefore be considered for correcting wines with excessive alcohol levels, but only for wines destined for local consumption in South Africa, or for those where the USA is the only envisaged export market. Even so, it seems worthwhile to be aware of the different commercial options (Salamon, 2006).

A Swedish study examining the effects on alcohol sales within the three beverage classes (beer, wine and spirits) revealed that consumers responded to these tax changes by shifting away from beverage brands that became relatively more expensive (Ponicki *et al.*, 1997).

While many South Africans either consume alcohol in moderation or do not drink at all, a large proportion of current drinkers consume alcohol at risky levels. The misuse of alcohol potentially places a heavy burden on the health, welfare, and private economic sectors of the country (Dh Parry *et al.*, 2003). Therefore, a better

method to manage total alcohol consumption in South Africa, would be to reset the taxes for alcohol sold in stores according to the absolute alcohol content of alcoholic beverages.

This form of taxation will force both consumers and producers to take into account not only the private costs, but the total costs related to their activities, i.e. the social costs (Dh Parry *et al.*, 2003). This strategy along with the new stricter drinking driving legislation should reduce alcohol abuse and the number of alcohol-related casualties.

2.6.2.2 Implications of regulations on wine trade, producer profitability and consumer affordability

Utilizing an alcohol taxation system, thus increasing the price of alcoholic beverages, is probably one of the most effective measures a country has for reducing alcohol-related harm and problems for both individuals and communities. A review of 112 studies recently examined the relationships between alcohol tax or price levels and alcohol sales or self-reported drinking. This review concluded that policies that raise the price of alcoholic beverages are an effective means of reducing alcohol consumption because alcohol price and tax increases are related inversely to drinking levels (Wagenaar *et al.*, 2009).

Stockwell and Crosbie (2001) investigated the aspects of alcohol supply and demand relationships in relation to the two main beverage varieties in Australia, wine and beer. He argued that the case study illustrates how the 'supply side' is able to create and protect demand for alcohol through both taking advantage of and influencing government regulation of the market for alcohol.

This is advantageous to the consumer, because this stimulates wine market growth which gives the consumers satisfaction because they have available a much greater wine supply with a rising level of wine quality and low prices. On the other hand, producers suffer dissatisfaction and difficulties in connection with the increasing competition level and the bargaining position of consumers. There is an excess supply in the world wine market as well as in Europe. According to the available statistics, world wine production is approximately 275 million hectoliters per year in average and consumption is about 220 million hectoliters (Pyšný *et al.*, 2007).

2.7 Modulation of wine alcohol content

Wine describes a diverse commodity class that consists mostly of yeast fermentation products and in some instances the interaction of additional microorganisms with must, or juice, pressed from grapes. Over the past decade a drop in the consumption of high alcohol beverages (10–13%) has become apparent and at the same time, the consumption of beverages with a lower alcohol content has shown an increase. Furthermore, as mentioned in section 2.5, it is clear that wine contains properties beneficial to human health if consumed in modest amounts and used to supplement healthy food. These benefits for health are mostly associated with the polyphenols, that are absorbable from wine but poorly from unfermented grape juice. Wines containing less alcohol provide all the benefits without the toxicity, and are much more affordable because of reduced taxation on these products (Pickering *et al.*, 1998*b*; Gladstones and Tomlinson, 1999; Gladstones, 2000; Halpern, 2008; Varavuth *et al.*, 2009).

This is a clear indication that there is a growing market for wines containing less alcohol (Scudamore-Smith and Moran, 1997; Halpern, 2008; Varavuth *et al.*, 2009). Therefore it comes as no surprise that as a result of the increased international interest and consumer demand, the focus within the wine industry has also shifted towards developing methods to produce reduced-alcohol (1.2% to 5.5–6.5% v/v), low-alcohol (0.5–1.2% v/v) and de-alcoholized (<0.5% v/v) products (Pickering and Heatherbell, 1996; Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998*a*; Jenster *et al.*, 2008). This classification system differ between countries and is loosely based on their labelling and legislative requirements. Therefore, interpretation and descriptions of these products will vary between countries.

Several methods exists to reduce ethanol production as mentioned by Malherbe *et al.* (2003); Biyela *et al.* (2009). These include time of harvest, choices prior to onset of alcoholic fermentation, during alcoholic fermentation or removal after fermentation. Some of the most popular techniques will be discussed below.

2.7.1 Physicochemical de-alcoholization of wine

Several physicochemical processes have been designed and used for the removal or the reduction of alcohol in wine, sometimes in combination. Some of these include

distillation and osmotic distillation, reverse osmosis and nanofiltration, spinning cone column, thermal gradient processing, membrane separation and membrane extraction, and pervaporation and thin-film evaporation under reduced pressure (Bui *et al.*, 1986; Scudamore-Smith and Moran, 1997; Scott, 1996; Mermelstein, 2000; Leeper, 2001; Pickering, 2000*a*; Baker, 2004; Varavuth *et al.*, 2009). Some of the processes that attempt to selectively remove alcohol while minimizing the loss of wine quality parameters are discussed below.

2.7.1.1 Distillation and osmotic distillation

Distillation using either evaporators or distillation columns is the most common thermal-based method for removing alcohol from wine.

Distillation uses the difference in boiling temperatures to separate various compounds and was the first method used by Carl Jung in Germany in 1920 to produce de-alcoholized wines. The product did not tast very good, because this process not only removes the alcohol, but also removes the flavor volatiles (Mermelstein, 2000).

Osmotic distillation (OD) is another membrane process of de-alcoholising wine which operates at low temperature and atmospheric pressure (Gryta, 2005). Wine is passed through a hydrophobic hollow-fiber membrane and degassed water is passed along the other side. The difference in vapor pressure results in some of the alcohol in the wine evaporating into the water. The transport mechanism of ethanol in de-alcoholization by the OD process can be divided into three steps: (i) evaporation of ethanol at the membrane pores on the feed side, (ii) diffusion of ethanol vapor through the membrane pores, and (iii) condensation of ethanol vapor in the stripping solution at the membrane pore exit (Varavuth *et al.*, 2009). This is done at room temperature without elevated pressures, except to gently pump the wine, whereas reverse osmosis uses high pressure and vacuum distillation uses elevated temperatures, both being conditions that may be detrimental to organoleptic wine quality (Mermelstein, 2000).

However, the mass transfer that takes place during the de-alcoholization of wines by OD is different from that of fruit juices. During the concentration of fruit juices by OD, water is the major volatile component transferred through the membrane from the feed side to the stripping side. But, in the de-alcoholization of wines, there is a simultaneous transport of both alcohol and water. The direction of water transport depends on the types of stripping solutions and this can affect the performance. Water is a good stripping solution for the de-alcoholization of wine

by OD. Furthermore, aditional advantages of water is: it is cheap, convenient, and non-corrosive (Varavuth *et al.*, 2009).

A disadvantage of the process is that it generates a lot of stripping water containing alcohol, which must be taken into consideration by any company that wants to use this process. However, the alcohol can be recovered by traditional distillation (Mermelstein, 2000). Alcohol can be reduced around 35% of the initial concentration. The important advantages of this process are avoiding thermal damage to components as well as aroma and flavor loss. Furthermore it does not consume a lot of energy (Varavuth *et al.*, 2009).

2.7.1.2 Reverse osmosis and nanofiltration

Membrane filtration has been applied to wine for a long time. Reverse osmosis (RO) and nanofiltration are two membrane processes that are applied to winemaking (Pilipovik and Riverol, 2005; Massot *et al.*, 2008).

Reverse osmosis derives its name from the reversal of the normal flow of water in osmosis. Osmosis is the diffusion of water across a differentially permeable membrane, from a region of higher to lower concentration. If sufficient pressure is exerted on the more concentrated solution, the diffusion of water and other permeable substances such as ethanol is reversed, with net movement occurring across the membrane into the dilute solution. The membrane's permeability determine whether compounds, based on their molecular weight and the membrane pore size, will pass through. The membranes used for RO are very selective and only the smallest molecules or ions will pass through them. For example, RO wine permeate contains water, alcohol, acetic acid (60% of the initial concentration), ethylic acetate (40% of the initial concentration) and lactic acid (15% of the initial concentration) (Mietton-Peuchot *et al.*, 2002; Massot *et al.*, 2008). Thus, the retention rates are a function of the selected membrane characteristics and the level of concentration is limited by osmotic pressure generated by the process itself and by the externally applied pressure (Massot *et al.*, 2008). Since ethanol and water are small compounds relative to the wine matrix (grape juice or must), the larger compounds, such as organic acids and phenolics, are retained in the wine at a higher concentration, thus retaining some of the natural flavors (Jackson, 2000*c*; Mermelstein, 2000).

However, as water is removed along with the ethanol, water must either be added back to the concentrated wine after reverse osmosis or added before the process is

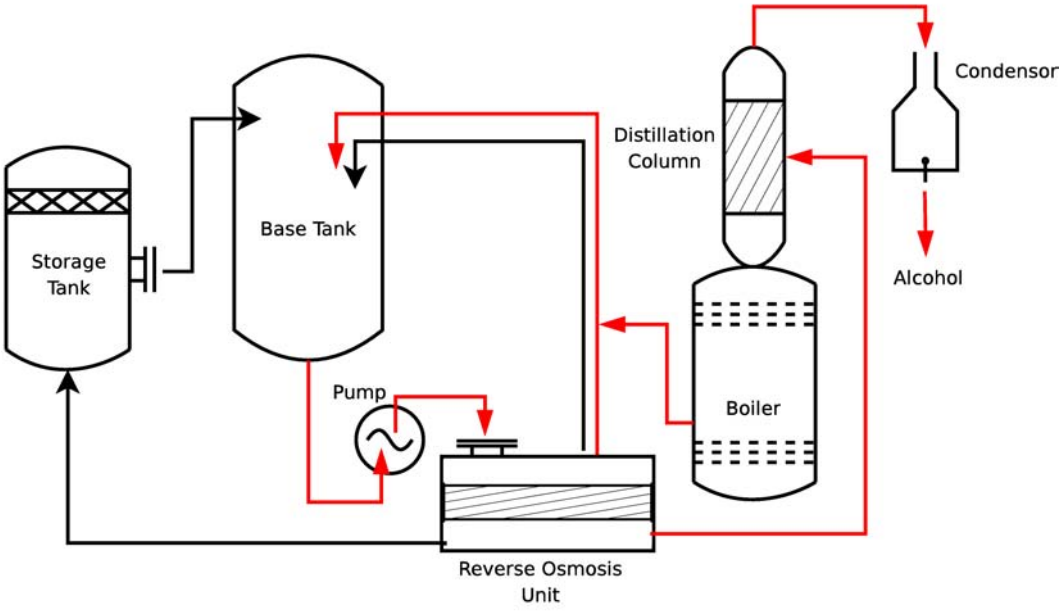


Figure 2.9: Reverse osmosis (RO), system for alcohol reduction. The base tank which is initially filled with wine. A pump pushes the wine into the reverse osmosis unit. Cylinders containing membranes are used to separate a syrupy concentrate from the alcohol and wine. In the RO system water and alcohol are pumped/flow into a storage tank and the concentrate is returned to the base tank. This process lasts 10 to 20 cycles (Mermelstein, 2000). In the double RO system the concentrate is returned to the base tank and can be cycled through the RO unit again. The water and alcohol are pumped into a distallation coulumn where alcohol and water are separated. Water originally removed by reverse osmosis is added back to the concentrated wine to restore the initial balance. Figure adapted from Vinovation (online).

started. This may create legal problems in countries where the addition of external water to wine is strictly prohibited. This legal dilemma of water addition from an external source can be circumvented by an ingenious system involving double reverse osmosis (Bui *et al.*, 1986) which produces alcohol-reduced and alcohol-enriched wines simultaneously. By interconnecting the two systems, water originally removed by reverse osmosis is added back to the concentrated wine to restore the initial balance of these materials. Therefore, no external water needs to be added to the alcohol-reduced concentrate nor is there a licencing problem created by the production of an alcohol distillate. Figure 2.9 presents a diagram depicting the reverse osmosis system for alcohol reduction. Care should however be taken in countries (e.g. South Africa) where mobile RO units are employed and where

legislation prohibits the use of mobile distillation units.

Unfortunately, this system cannot produce completely de-alcoholized wines, and is not economically feasible for the production of beverages with an alcohol percentage lower 0.45% (Pilipovik and Riverol, 2005).

The membrane separation process known as nanofiltration (NF) is essentially a liquid phase one, because it can separate a range of organic and inorganic compounds from a solution such as wine. NF membranes permeate water and ethanol but retain more substances than micro- and ultrafiltration membranes (Banvolgyi *et al.*, 2006). The separation is done by diffusion through a membrane, under pressure differentials that are considerably less than those used for RO, but still significantly greater than those for ultrafiltration (Sutherland, 2008). So NF is much more economical. Futhermore, NF can be carried out at room temperature, thus it protects the heat-sensitive and volatile compounds so that the wine does not lose its marked character (Banvolgyi *et al.*, 2006; Rosa Santos *et al.*, 2008).

Unfortunately, the complex separation mechanisms that occur in NF (e.g. physical, chemical and electrical interaction between the solvent, solutes and membrane) make the number of the operating parameters that control separation efficiency very long. Furthermore, different results for the same feed/run and the same membrane further complicate usage (Massot *et al.*, 2008).

Membranes are key to the performance of nanofiltration systems (Sutherland, 2008) and the large choice of membranes should facilitate their application and increase possibile coupling with other membrane techniques (e.g. reverse osmosis–nanofiltration, ultra-filtration–nanofiltration) for the purpose of correcting must or wine by reducing or increasing the concentration of a given solute. For example; a combination of RO–NF instead of a sole reverse osmosis stage, can reduce the quantity of must that have to be treated, to reduce the concentration sugar/ethanol (Massot *et al.*, 2008).

2.7.1.3 Spinning cone column

The spinning cone column (SCC) is a thin-film, multistage stripping column that uses centrifugal force to enhance low temperature distillation under vacuum. The column, 1 m in diameter and 5 m high, contains a series of approximately 40 alternating, stationary and rotary truncated cones. Wine is fed into the top of the column, flows down the upper surface of the first stationary cone with the assis-

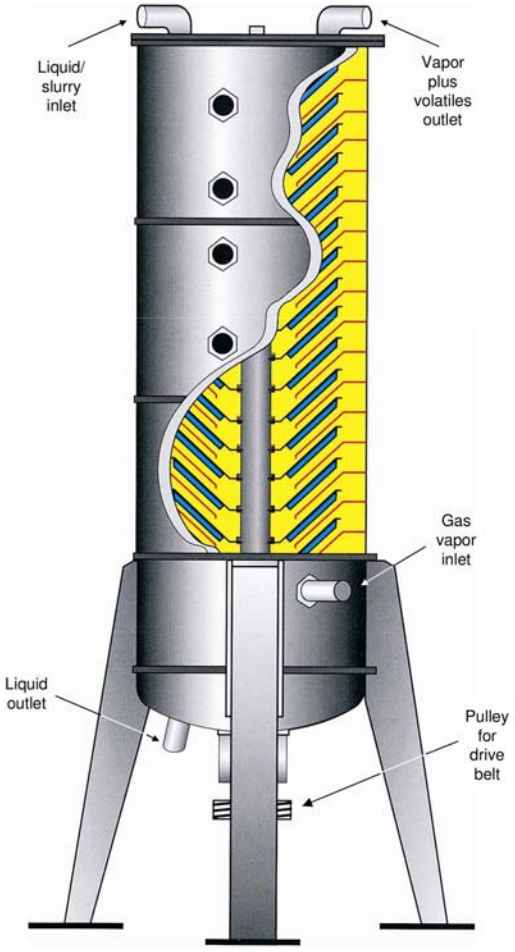


Figure 2.10: A graphical presentation of the spinning cone column (SCC).

tance of gravity and onto the surface of the first spinning cone, where centrifugal force gently spreads it into a thin liquid film that flows off the cone and drops onto the next stationary cone. This process repeats itself as the wine makes its way down the bottom of the column. Figure 2.10 presents the layout of a SCC.

This system does not use external steam as the stripping agent for wine. About half a percentage of the total volume of wine undergoing the process is converted into a form of low temperature vapor, which is just below room temperature (23–24°C). This cool vapor created from the wine itself rises from below and caries off the lightest molecules (volatile compounds) in the liquid as it passes across the surface of the thin film of wine. Fins on the underside of the rotating cones creates a high degree of turbulence into the rising vapor stream. The turbulence in the vapor

stream, thin film of liquid and the long vapor and liquid path lengths, leads to the highly efficient transfer of volatiles from the liquid to the vapor stream, in only a few seconds without causing any thermal damage to the wine. With the first pass through the column all the ultra light molecules, consisting of the delicate volatile compounds, flow out of the top of the column and passes through a condensing system which captures the volatiles as a concentrated colorless liquid which is kept separately.

The remaining liquid or slurry is pumped out of the bottom of the column. The process through the column is then repeated for a second time at $\pm$38°C and ethanol is reduced to $\pm$2.5% (v/v). The volatile compounds that was captured during the first run are then added back to the alcohol-reduced wine. This alcohol-reduced wine can then be blended with the original batch to produce a low-alcohol/reduced alcohol wine, which retains its original flavor (Gentis, 2009). Figure 2.11 summarizes the methodology of a spinning cone column (SCC) in the form of a short video clip.

2.7.1.4 Thermal gradient processing

In this method, wine is cooled to form ice crystals, which float to the top of the tank, increasing the alcohol concentration in the liquid. About half of the contents are then drained from the bottom of the tank. The tank is then heated to melt the ice crystals, essentially diluting the liquid in the tank and thereby producing wine with reduced alcohol concentrations. This is a very energy-intensive method and is not currently being used commercially (Mermelstein, 2000).

2.7.1.5 Membrane extraction (perstraction)

Membrane extraction, also known as perstraction, is similar to liquid-liquid extraction, except that a membrane is used as a barrier between the feedstream and the solvent stream. The feedstream contains a solute or liquid to be recovered. A solvent stream flows on the permeate side of the membrane. The desired solute or liquid in the feedstream selectively permeates across the membrane and into solvent stream. The chemial potential difference across the membrane creates the driving force (Leeper, 2001).

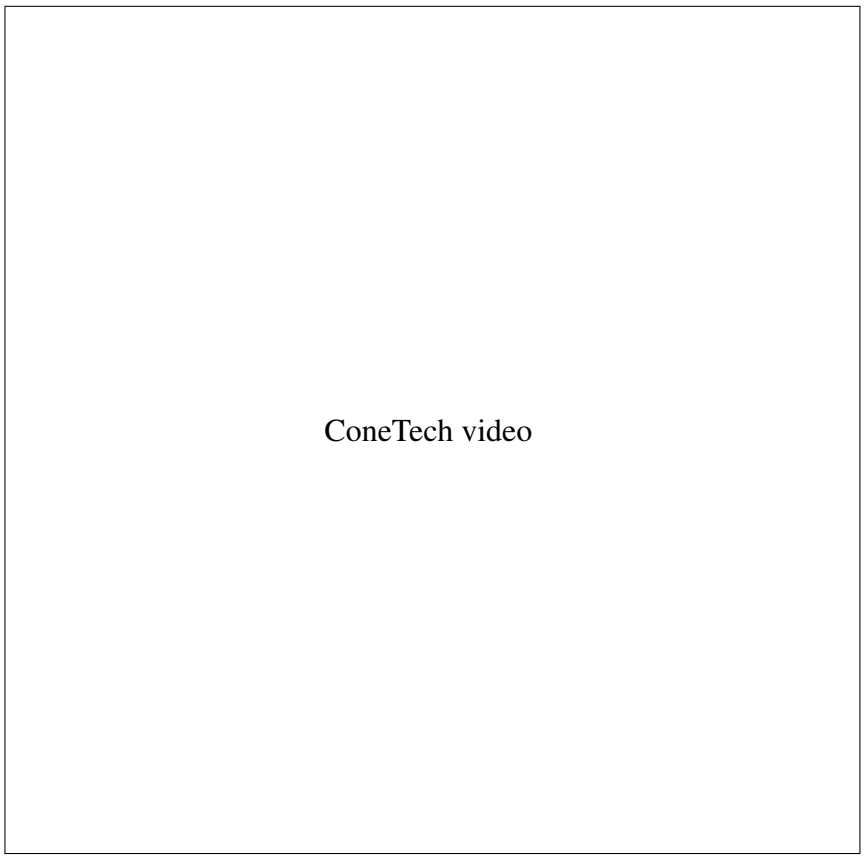


Figure 2.11: Methodology of a spinning cone column (SCC) presented as a video. *If the video does not start automatically, it can be viewed by opening the video file that is attached to this document and play it with your local media-player.*

2.7.1.6 Pervaporation

Pervaporation (PV) is a relatively new process that has elements in common with reverse osmosis (RO) and gas separation. Intrest in this process is growing due to the practical limitations of RO in many potential separations where otherwise extreme pressures would be required (Baker, 2004).

In PV the feed stream is a liquid mixture and a partial vapor pressure difference is maintained across the membrane. Separation is achieved by applying a lower pressure (vacuum) to the permeate side of the membrane whilst the other side is exposed to the liquid to be separated. Thus, the feed material is a liquid but the permeate is a gas. That is, the temperature and pressure of the permeate are such that the permeated components exist in the gaseous phase. The partial pressure of the permeate is thus kept lower than the saturation pressure and provides the necessary force for separation. Some compounds preferentially permeate across the mem-

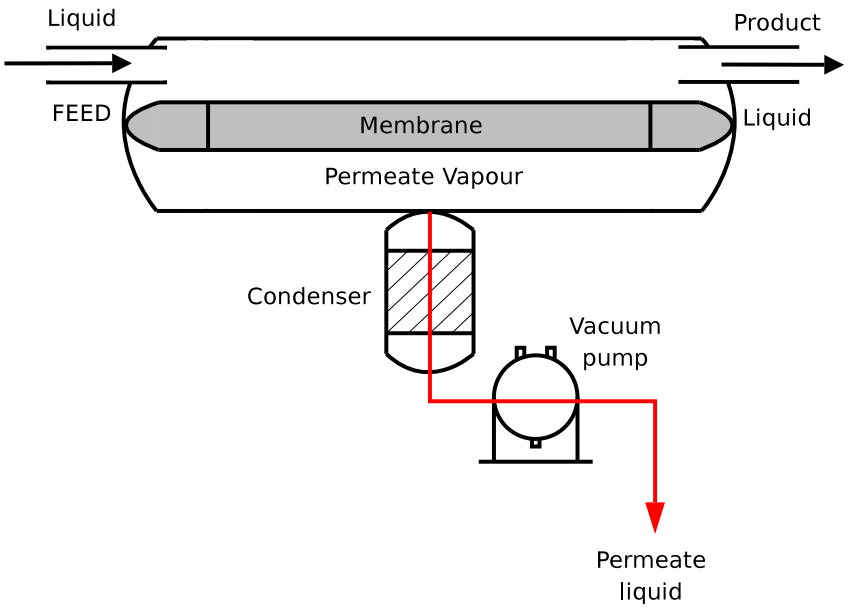


Figure 2.12: Pervaporation (PV) process using a condenser to generate the permeate vacuum. Figure adapted from from Scott (1996).

brane and vaporize on the low-pressure (permeate) side. The permeate side vapor is condensed and recovered. The less permeable components are concentrated in the retentate stream (Leeper, 2001). The low partial pressure can also be produced by using an inert carrier gas as an alternative (e.g. nitrogen) (Scott, 1996; Baker, 2004). A representation of a simple PV process using a condenser to generate the permeate vacuum is shown in Figure 2.12.

The attraction of pervaporation is that the separation obtained is proportional to the rate of permeation of the components of the liquid mixture through the selective membrane. Therefore, pervaporation offers the possibility of separating closely boiling mixtures or azeotropes that are difficult to separate by distillation or other means (Baker, 2004).

2.7.2 Biological de-alcoholization of wine

All these physicochemical de-alcoholization methods raise the concern as to the sensory quality of finished wines (Halpern, 2008) as well as the overall production cost as the processes also tend to involve expensive equipment and can also be intensive from a processing point of view (Villettaz, 1987; Pickering and Heatherbell, 1996; Pickering *et al.*, 1998*a*).

There is a number of ways alcohol production could be limited during alcoholic fermentation. The simplest method would be to harvest the grapes before ultimate ripeness has been achieved. Fermentation with immature grapes containing less sugar will result in reduced levels of ethanol, but can be responsible for wine with less flavor and aroma (Pickering *et al.*, 1998*b*,*a*). Another method is the dilution of the grape juice with the addition of water. It is important to know that the addition of water is illegal in many countries around the world (viz. South Africa, Australia, New Zealand and the EU).

The use of biological de-alcoholization methods has also been explored. Some of these methods can be implemented during or after alcoholic fermentation and will be discussed below.

2.7.2.1 Pre-fermentation enzymatic treatment of grape juice

An alternative approach to using expensive equipment is the concept of treating grape juice from the mature fruit with the enzyme glucose oxidase (Mcleod and Ough, 1970; Ough, 1975; Pickering, 2000*a*,*b*).

Glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4), is purified from the fungus *Aspergillus niger*, and has a well-established history in the food and beverage industry (Wong *et al.*, 2008; Bankar *et al.*, 2009). Both *A. niger* and GOX have GRAS status (Generally Regarded As Safe). During the enzymatic reaction, β-D-glucose is converted to D-glucono-δ-lactone and hydrogen peroxide (H_2O_2) in the presence of oxygen, which is non-enzymatically reduced to gluconic acid (GA). The basic net enzymatic reaction is shown in Figure 2.13.

When grape juice is treated with GOX prior to alcoholic fermentation, the total amount of glucose that can be metabolized to form ethanol is reduced. Furthermore, the end product of the GOX reaction is gluconic acid which *S. cerevisiae* is unable to metabolize. This results in wine with a reduced alcohol level (Villettaz, 1987; Pickering and Heatherbell, 1996; Pickering *et al.*, 1998*a*, 1999*a*,*b*,*c*; Biyela *et al.*, 2009). The overall acidity of these wines increases because of the excess GA that remains after fermentation, but can be adjusted, e.g. by chemical deacidification, until satisfactory organoleptic properties are obtained (Villettaz, 1987; Pickering

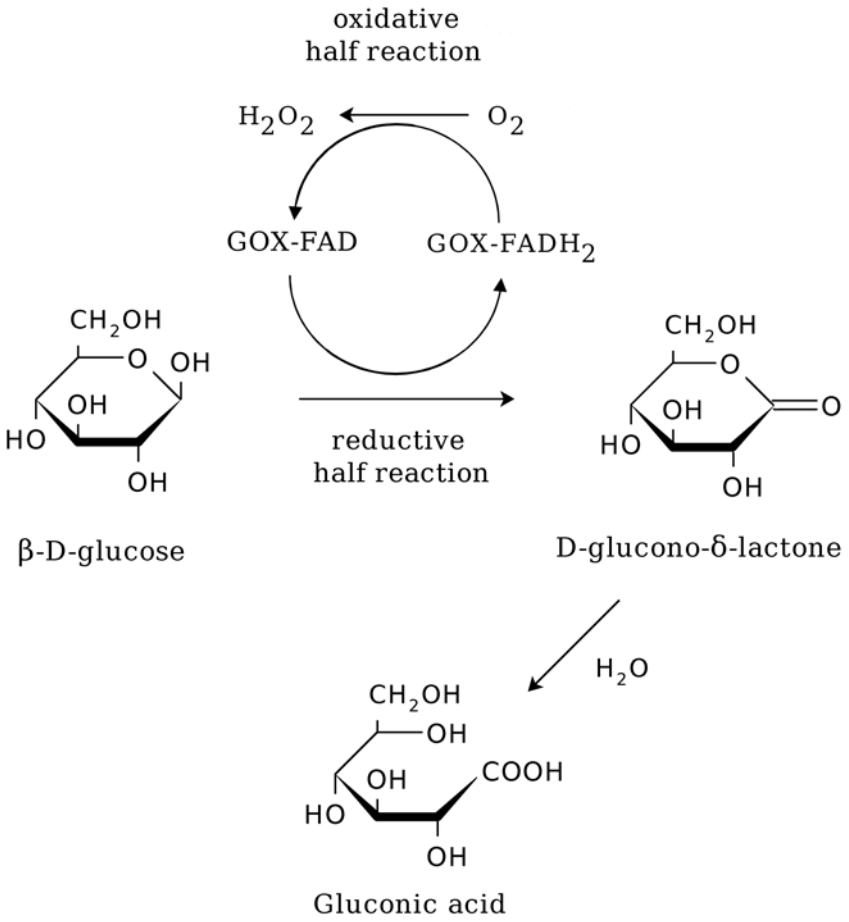


Figure 2.13: Glucose oxidase (GOX) enzymatic reaction.

et al., 1999*a*,*b*,*c*; Pickering, 2000*a*).

Another by-product of the GOX enzymatic reaction is hydrogen peroxide (H_2O_2). H_2O_2 might affect the wine negatively by oxidizing some of the chemical compounds and even inhibit enzyme activity. H_2O_2 is however an effective bacteriocide and could protect the wine against bacterial spoilage. The enzyme catalase, can be used to remove H_2O_2 by converting it to water and molecular oxygen. Therefore, glucose oxidase and catalase can be used together as a system to reduce glucose and control net H_2O_2 (Pickering *et al.*, 1999*a*).

The aroma, aroma-by-mouth and mouthfeel characteristics of GOX pre-treated wines are relatively unaffected, except for the fruity aromas, which are generally

less intense. This could be as a result of juice aeration that is required for optimal GOX activity and efficiency. The length of the flavor is also reduced and the high acidity is a detracting characteristic. In addition to chemical deacidification, the organoleptic properties could be corrected by adding sweet reserve wine, a common practice in the production of low-alcohol wines. Further research could determine the optimum ratio and composition required to maximize the sensory quality (Pickering *et al.*, 1999*c*, 2001).

One of the technological limits of the glucose oxidase-catalase system is that these GOX wines have an increased SO_2-binding capacity compared to wines made by conventional methods. SO_2 binds to gluconic acid (GA), and as a result less SO_2 is available for microbial stability. This makes the wine more susceptible to spoilage (Barbe *et al.*, 2002). This high SO_2-binding capacity is a cause for concern, given the statutory regulations governing the maximum permitted SO_2 levels in wine, as well as the general trends towards lower SO_2 use in the wine industry. The higher concentration of carbonyl compounds may account for this increased demand for SO_2. More sulphates are also formed in GOX-treated wines. These wines, however, are more stable against browning and have a more golden color, which is possibly a result of increased quinone production and the regeneration of oxidizable phenolic substrates (Pickering *et al.*, 1999*b*).

2.7.2.2 Restriction of alcohol production during fermentation

With all the commercial wine yeast strains available today, the winemaker can reduce the amount of alcohol that will be produced by using a yeast strain that have lower ethanol yields per gram of sugar utilized as compared to other strains. Erten and Campbell (2001) suggested that aerobic yeasts could be used for the production of low alcohol wines and produced acceptably flavoured wines with an alcohol content < 3% by agitation and aeration during fermentation.

Another strategy to reduce alcohol during alcoholic fermentation is to divert glucose metabolism away from ethanol production. This was accomplished by engineering a yeast to produce increased levels of glycerol. A significant increase in extracellularly accumulated glycerol and an associated decrease in ethanol concentration (up to 2% [v/v]) have been achieved by the over-expression of either of the authentic *GPD1*- or *GPD2*-encoded glycerol-3-phosphate dehydrogenase isozymes

of *S. cerevisiae*. The biosynthesis of glycerol requires the oxidation of NADH to NAD^+. This redox reaction, unlike that of ethanol synthesis, is not balanced by the oxidation of sugar during glycolysis. Under fermentative conditions an alternate pathway(s) must be used to perform the oxidation and offset the increased NAD^+ produced when *GPD2* is overexpressed. The yeast accomplishes that with the oxidation of acetaldehyde to acetic acid. Unfortunately, this led to increased concentrations of acetic acid that affected the wine's sensory character negatively. The biosynthesis of acetic acid from acetaldehyde is catalysed by aldehyde dehydrogenase (Ald) enzymes. It was thought that by deleting the *ALD6* gene, the wine's baclance could be restored. The compound that was most affected by deleting the *ALD6* gene was ethyl acetate. Its concentration decreased almost three-fold. Ethyl acetate is the main ester occurring in wines and, depending on its concentration, could contribute either to a fruity or solvent (varnish) odour. Further modification of this strain led to an unsatisfactory aroma profile. This strategy was not successful, but brings us closer to understanding how to possibly engineer a yeast in such a manner as to be able to control ethanol production (Michnick *et al.*, 1997; De Barros Lopes *et al.*, 2003; Cambon *et al.*, 2006).

Malherbe *et al.* (2003) genetically engineered a *S. cerevisiae* strain successfully to produce glucose oxidase (GOX) during alcoholic fermentation. GOX that was secreted into the grape juice was able to convert some of the glucose into GA and reduced the total ethanol by the end of fermentation by 1.8–2%. The advantage of this method over above mentioned molecular strategies is that the reaction takes place out side the yeast and thus, does not affect the redox balance inside the yeast. Further research would be necessary to test an industrial wine yeast, engineered to produce GOX, under small-scale cellar condition. If successful, this yeast could potentially provide an effective means of bio-adjusting the alcohol content to appropriate levels in commercial wines. This could meet consumers' demands for affordable low-alcohol wine, and have financial implication in savings on wine taxation.

It is important to realize that thus far only two genetically modified commercial wine strains are currently being employed for winemaking, and that the wine industry will not use such strains as starter cultures unless both the industry and the consumers are satisfied that they are safe and beneficial (Pretorius, 2000, 2001; Vivier and Pretorius, 2002).

Partial or incomplete fermentation can reduce alcohol levels, but have the inherent problem of excess residual sugar not only results in sweeter wines, but could initiate potential spoilage of wine by microbial invasion and growth (Pickering *et al.*, 1998*b*; Bartowsky and Henschke, 2004). Good microbial stability can be obtained by removing spoilage microorganisms (Jackson, 2000*c*).

2.7.2.3 Post-fermentation de-alcoholization of wine

The easiest way in correcting a too high alcohol wine and produce a low alcohol wine can be achieved by blending wines with a high alcohol concentration with wines with a lower alcohol concentration. This will reduce the alcohol content of the resulting wine. The percentage alcohol that will be reduced can be controlled by the alcohol concentrations of the wines that will be use for blending.

There are only a very few post-fermentation de-alcoholization methods of wine, specifically with regard to enzymatic treatments, and research in this area can contribute to the production of low- and reduced alcohol wines.

2.8 The need for further research

Today, there is a much stronger emphasis on the development of wine yeasts for the cost-effective production of wine with minimized resource inputs, improved quality and low environmental impact. Viticulture and oenology is about producing and presenting a good quality product consistently each year to the consumer. This is difficult as there is so many variables that can change and have to be taken into consideration. However, vinification techniques have evolved over the past decade and winemaking has now little in common to this “art” described even 20 years ago. How new technologies are received and can assist winemakers world-wide depends on how well potential new technologies are being researched as well as how well it is communicated to the wine industry.

2.8.1 Proposed strategies for further research

One way to achieve successful outcome of an alcoholic fermentation is by selecting and using strains with improved performance. Much effort has for instance been devoted to exploring biochemical/physiological determinants of ethanol tolerance in

yeast (Hu *et al.*, 2007). Thus, when considering a wine fermentation and the accumulation of ethanol during alcoholic fermentation, adaptation and survival of yeasts to increasing concentrations of ethanol are important criteria in selecting the correct wine strain. But, when selecting strains with reduced alcohol production the opposite would apply, and the selection would target strains that produce less ethanol during alcoholic fermentation and are more sensitive to increasing concentrations of alcohol.

As a non-recombinant means of strain improvement, adaptive evolution is a technique with great potential. By means of adaptive evolution, McBryde *et al.* (2006) demonstrated that an isolate of a commercial wine yeast had altered production of metabolites, including ethanol, glycerol, and succinic as well as acetic acid. Additionally, this new strain was able to more rapidly catabolize all available sugars under the set conditions. These results endorse the potential of adaptive evolution as a tool for the non-recombinant modification and optimization of industrial yeast strains (McBryde *et al.*, 2006).

The use of techniques such as mutagenesis, hybridization and recombinant DNA methods have significantly increased the genetic diversity that can be introduced into *S. cerevisiae* strains (Pretorius, 2001). We still have limited knowledge of industrial wine yeasts' complex genomes. But new ongoing research increases our knowledge about these strains and give us a glimpse of its genetic blueprint (Borneman *et al.*, 2008*b*,*a*). Using molecular techniques to directly modify a strain that enhance certain abilities, or remove those that are detrimental to wine quality could result in better and faster methods for strain differentiation. Applying new technologies, e.g. transcriptomics and proteomics, during a wine fermentation have and can increase our understanding of novel fermentation stress responses (Marks *et al.*, 2008; Salvadó *et al.*, 2008).

However, when considering the staggering potential advantages of improved wine yeasts to both winemaker and consumer, research is necessary to comply with strict statutory regulations and consumer demands regarding the future use of genetically modified strains in the food and beverage industries (Pretorius, 2000; Fleet, 2003; Varela *et al.*, 2005).

Uncovering the genetic regulation of variation for understanding the evolution of a fermentation to understand ethanol tolerance in *Saccharomyces* wine yeasts is

essential. This would enable us to see how certain species gain dominance in a fermentation, and ultimately improving the efficiency of how we select a new wine yeast strain that possesses higher tolerance to ethanol toxicity (Hu *et al.*, 2007). These types of selection could also be employed by using mixed cultures, which would enable us to select new yeasts species to assist older more traditional wine yeasts in flavor and aroma development during the winemaking process (Clemente-Jimenez *et al.*, 2005).

Additionally, as yeast cells have to be produced industrially prior to inoculation, it is worth noting that there are other conditions during the process of biomass production and drying (such as oxidative and desiccation stresses) to which yeast cells are subjected that may have adverse effects that should also be taken into consideration (Carrasco *et al.*, 2001). The physiological and biochemical changes that the yeast cells undergo during production is possibility an field that could be studied more indepth, as it is sometimes looked over when only the problems of fermentation is considered. Research in this field could be physiological or genetic/molecular by nature.

Most of the conventional methods used for the production of low and reduced alcohol wine on a commercial scale involve the removal of alcohol from fully fermented wines using physicochemical techniques which are expensive. The use of enzymes to assist and/or enhance a fermentation is nothing new, and winemakers use them regularly. Glucose oxidase is probably the enzyme showing the greatest potential to reduce alcohol, by either treating wine prior to fermentation (Pickering, 2000*b*,*a*; Pickering *et al.*, 2001; Biyela *et al.*, 2009) or during fermentation (Malherbe *et al.*, 2003). However, further research could focus on the development or evaluation of GOX preparations with higher activity at wine pH. Furthermore, the use of immobilized GOX may have some processing advantages in grape juice and could be evaluated. In addition, it would also be sensible to investigate the reduction in free SO_2 levels that are observed in GOX-treated wines compared to wines made by conventional methods. SO_2 binds to gluconic acid, and as a result less SO_2 is available for microbial stability. This makes the wine more susceptible to spoilage (Barbe *et al.*, 2002).

Another focal area for research are the actual fermentation, its on-line moni-

toring and control by new technologies that are now available. Very few industrial fermentations are currently monitored on-line, but this situation may change in the near future. Computers and chemical sensors that was unavailable 10 to 15 years ago or to expensive are now a promising method for improving fermentation control because: (i) it is much more accurate than manual density measurements and (ii) it makes possible new control strategies in which winemaking operating conditions are adapted to actual fermentation behavior in real time (Sablayrolles, 2009). This, astute monitoring of the fermentation can assist the winemaker in early identification of problem fermentations, and contribute to economic savings by reducing wasted time of having to first diagnose the problem followed by monetary expenditures to rectify a stuck and/or sluggish fermentation (Bisson and Butzke, 2000; Roger *et al.*, 2002; Lourens and Reid, 2003).

New processes and methodologies in thermal and membrane processes are being researched and developed. It is possible that where conventional yeast breeding methods, molecular yeast genetics and modern biotechnology are unable to perform the task at hand or are limited by strict statutory regulations, these processes can be employed with satisfactory results. One such a technology is ConeTech. Furthemore, as the different processes are better understood, they can sometimes be used in combination, to even further increase their performance. Membrane processes could for example also be coupled with ion exchange treatment or with vacuum evaporation (Massot *et al.*, 2008; Sutherland, 2008).

The stakes of success in meeting consumer expectations world-wide are high, wine markets change and have to adapt to these changes, but that is not enough. The pressure is on the wine industry to put the correct tools in the winemakers hand to deliver good quality product consistently each year. The only way to ensure that this is possible, is by performing research on all levels of oenology and viticulture.

2.8.2 Concluding remarks

A major challenge in warmer climate regions is the rapid accumulation of grape sugars, which can impose a premature harvest even though the grapes have not reached phenolic ripeness. Furthermore, high concentration of sugar leads to the production of high alcohol wines. The high alcohol content of wines can affect the organolep-

tic properties and mask the overall aroma and flavor of the wine. Therefore, fast, reliable and cost-effective methods should be developed in order to produce wines with reduced-ethanol content.

Stuck fermentations are more common in musts with higher sugar concentrations. These problem fermentations occur as a result of osmotic intolerance at the start of alcoholic fermentation or ethanol toxicity at a later stage during fermentation. Astute on-line monitoring of the fermentation in real time can assist the winemaker by identifying potential problem fermentations earlier, thus reducing overall costs by reducing time of having to first diagnose the problem followed by monetary expenditures to rectify a stuck and/or sluggish fermentation (Bisson and Butzke, 2000; Roger *et al.*, 2002; Lourens and Reid, 2003; Sablayrolles, 2009).

Recent years have brought substantial advances in our understanding of the risk relations of alcohol consumption and specific disorders (Room *et al.*, 2005; Jernigan, 2006) as well as the association of wine as a nutritional 'healthy' beverage. This is further emphasized by consumers changing their attitude in the health consciousness of drinking wine as opposed to hard spirits or beer (Stockley and Høj, 2005; Johansen *et al.*, 2006; Jenster *et al.*, 2008). This trend is likely to continue to influence consumption choices favoring wine over other alcoholic beverages. This is a long term factor as there has been a move to lower alcohol content beverages for a number of years (Jenster *et al.*, 2008). This serves as an indication that there is a growing market for low alcohol products. Low- and reduced alcohol wines as well as de-alcoholized wine is providing all the benefits without the toxicity of excess ethanol. However, organoleptic quality of de-alcoholized wine(s) are a current challenge, and requires further investigation (Halpern, 2008).

New processes and methodologies in thermal and membrane processes are being researched and developed that could assist in the reduction of ethanol with reduced financial costs and limited impact on wine flavor and aroma components. Furthemore, as the different processes are better understood, they can sometimes be used in combination (Massot *et al.*, 2008; Sutherland, 2008), to even further increase their performance and reduce monetary expenditures.

A lot of research has been done using molecular and biotechnology techniques

to genetically engineer a yeast strain that can reduce the ethanol content of wine successfilly. There are several yeasts that are able to reduce the ethanol content, but all these strains also produce chemical compounds that have a negative impact on the wine sensory profile (Michnick *et al.*, 1997; De Barros Lopes *et al.*, 2003; Cambon *et al.*, 2006). The most successful of these was a yeast secreting the enzyme glucose oxidase into the wine (Malherbe *et al.*, 2003). However, one should be aware that genetically modified organisms are not yet permissible for use in the South African wine industry. The successful commercialization of transgenic wine yeasts will depend on a multitude of scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues. Thus, it would be foolish to entertain unrealistic expectations over rapid commercialization and short-term benefits, but will be equally unwise to deny the potential advantages of genetically improved wine yeasts to both the winemaker and consumer (Pretorius, 2001).

Currently, the best non-physicochemical method is the use of GOX prior to alcoholic fermentation. Alcohol levels were successfully adjusted by as much as 40%. Unfortunately the overall acidity of these wines increase because of the excess GA that remains after fermentation. The excess acid can be adjusted by chemical deacidification, until satisfactory organoleptic properties are obtained (Villettaz, 1987; Pickering *et al.*, 1999*a*,*b*,*c*; Pickering, 2000*a*). One limitation of the GOX system is that these GOX-treated wines show increased SO_2-binding power. SO_2 binds to gluconic acid, and as a result less SO_2 is available for microbial stability. This makes the wine more susceptible to spoilage (Barbe *et al.*, 2002).

This review highlighted the effects of increasing ethanol concentrations on yeast cells and how this might induce suboptimal fermentation performance during winemaking. A discussion followed on the implications of high alcohol wines on human health as well as consumer preference, and the financial implications from a commercial point of view by wine taxation. Finally, we discussed possible solutions to address these high ethanol levels and means to reduce them to more acceptable levels employing physicochemical or biological methods. Therefore, the information provided here should assist winemakers in evaluating the relative merits and limitations of the available methods as well as provide an overview for producers and consumers of factors influencing the quality of these wines and highlighted the positive effects of moderate alcohol consumption with a balanced diet.

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Chapter 3

Evaluating the Production and Secretion of Glucose oxidase by *Saccharomyces cerevisiae* Yeast Strains

Abstract

Popular wine styles prepared from fully-ripened, more mature grapes are characterized by intense fruitiness and varietal flavors. However, lengthy maturation of grapes in the vineyard does not only translate into higher flavor intensity but also into higher sugar levels, which, in turn, lead to wines with higher concentrations of alcohol. Excessive alcohol levels can compromise wine flavor and render wine unbalanced. This, along with health issues and anti-social behavior linked to high-risk alcohol consumption patterns, stricter legislation and increased tax rates associated with high-alcohol wines, has increased demand for wines with reduced alcohol concentrations, but without loss of the intense fruity aromas. Although low-alcohol wines can be made using physical post-fermentation processes, such approaches are often expensive and can impact adversely on wine flavor. As an alternative strategy, yeast strains are being developed by several research groups to convert some of the grape sugars into metabolites other than ethanol. Based on promising results from previous preliminary work, this study focused on the development of *Saccharomyces cerevisiae* strains producing glucose oxidase (GOX;

β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4). GOX oxidizes glucose to D-glucono-δ-lactone and gluconic acid prior to its entry into glycolysis, thereby diverting a portion of the sugar carbon away from ethanol. The *GOX*-encoding gene from a food-grade fungus, *Aspergillus niger* was used to construct three cassettes (*GOX1*, *GOX2* and *GOX2LOX*). In these gene cassettes, the *A. niger GOX* gene was placed under the regulation of the *S. cerevisiae* phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). To facilitate secretion, in *GOX1* the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) was fused to the *GOX* gene, and in *GOX2* the native *A. niger* secretion signal of GOX was used. These gene cassettes were each integrated into the genome of two laboratory yeast strains (BY4742 and Σ1278b) and one industrial wine yeast strain (VIN13). An additional integration cassette, designated *GOX2LOX*, was constructed to knock out the *IME1* gene in *S. cerevisiae*. In *GOX2LOX*, *GOX2* was fused to a loxP cassette. This loxP cassette makes use of the proven Crelox-system for repetitive gene integration and genetic marker recovery, and contains two loxP sites that flank a G418 sulfate resistant marker gene (*kan*MX). VIN13-Δ1 was obtained by integrating a single copy of *GOX2LOX* into the *IME1* locus. To generate an asporogenic, GOX-producing wine yeast, VIN13-Δ2 was created by sporulation, micromanipulation and re-diploidisation of VIN13-Δ1. Comparative analysis indicated that (i) *GOX2* resulted in higher levels of extracellular glucose oxidase activity than *GOX1*; and that (ii) the levels of secreted glucose oxidase activity in the wine yeast transformants were sufficiently high to conduct follow-up small-scale wine fermentation trials for the production of low-alcohol wines.

3.1 Introduction

The challenge for grape and wine science in the 21^{st} century is to maximize the potential of technological innovation to future-proof the wine industry's success in meeting the aspirations of grapegrowers, winemakers and consumers alike. For producers, innovation is needed to maximize profitability and environmental sustainability in viticultural and winemaking practices, and to efficiently produce grapes and wine in line with changing market expectations. For consumers, innovation means effectively responding to (if not anticipating) shifting tastes and preferences for such overarching attributes as consistent yet affordable quality wine and more specific sensory characteristics, such as more fruitiness and lower alcohol levels.

The conundrum is that riper, more mature grapes generally deliver greater fruity flavor complexity but also result in increased sugar concentrations, particularly in warm climatic conditions. In turn, high sugar levels in grape must lead to wines with increased levels of alcohol. Although ethanol over a narrow range of concentrations (say, 11 to 15%) does not appear to have a noticeable influence on wine aroma, high alcohol levels can have a negative effect on the palate, increasing hotness, bitterness, drying, roughing and metallic sensations in some wine styles.

Besides the sensory-driven imperative to reduce alcohol levels in negatively affected wine styles, there are also health and tax related reasons for producing wines with lower levels of alcohol. Excessive consumption of alcohol is often associated with health issues and anti-social behavior. Therefore, worldwide, stricter legislation (e.g., drink-driving laws) and regulations (e.g., mandatory labeling) are constantly being reviewed and introduced to limit binge drinking and high-risk consumption patterns. Other mechanisms governments the world over are focusing on include increases in so-called 'sin' taxes and duties, i.e. the higher the alcohol content, the higher the tax and price to be paid by producers and consumers.

It is therefore clear that there is a range of interconnected legislative measures, production factors and consumer demand aspects that is driving research in developing and evaluating strategies to reduce alcohol content in some wine styles.

Viticultural strategies include investigations into earlier harvest dates (shorter 'hang times') linked to canopy management that would increase leaf area:yield ratios and change vine balance in such a way that sugar accumulation is delayed while flavor ripeness is preserved. However, the impact of viticultural practices on the secondary metabolite profiles of grapes and their derived wines remains to be established. Breakthroughs and novel viticultural solutions are unlikely in the short term.

Pre-fermentation strategies include dilution of high-sugar musts with low-strength juice, condensate or water, within the constraints of wine regulations. Commonly used post-fermentation techniques involve blending high-alcohol wines with low-alcohol wines and physical methodologies (which are sometimes used in combination), such as adsorption, centrifugation, distillation, thermal evaporation, thin-film evaporation under reduced pressure (also called vacuum distillation), freeze concentration, membrane filtration, reverse-osmosis, spinning cone-column technology (which is probably most effective and well known) and thermal gradient processing (Bui *et al.*, 1986; Scudamore-Smith and Moran, 1997; Mermelstein, 2000; Picker-

ing, 2000). However, there are significant product quality and/or cost disadvantages associated with these pre- and post-fermentation practices. These techniques often depend on the use of expensive equipment, and, in some countries, there are restrictions on the use of some of these technologies in commercial winemaking. Loss or modification of aroma and flavor compounds during processing is another important consideration for several of these techniques (Swiegers and Pretorius, 2005).

It is therefore no surprise that yeast fermentation-based solutions are being sought by several research groups around the world. For example, a significant decrease in ethanol concentration (up to 2%) and a concomitant increase in extracellularly accumulated glycerol have been achieved by the overexpression of either of the native *GPD1*- or *GPD2*-encoded glycerol-3-phosphate dehydrogenase isozymes of *Saccharomyces cerevisiae* (Michnick *et al.*, 1997; Remize *et al.*, 1999; De Barros Lopes *et al.*, 2000). However, it was found that these high-glycerol producing prototype strains also increased acetic acid concentrations to unacceptable levels. This negative side effect was circumvented by deleting the *ALD6*-encoded acetaldehyde dehydrogenase activity, the main contributor to the oxidation of acetaldehyde during fermentation. For example, a laboratory strain of *S. cerevisiae* over-expressing *GPD2* and lacking *ALD6* had the desired effect of producing more glycerol and less ethanol, without an increase in acetic acid (Remize *et al.*, 2000; Eglinton *et al.*, 2002). However, while the significance of this research to the wine industry represents a major advance in innovation, excitement was tempered by the knowledge that the use of genetically modified organisms (GMOs) in commercial winemaking has so far been prohibited in most countries. Thus the 'high-glycerol-low-alcohol' yeast must remain 'on the shelf' until it has not only cleared all the stringent food safety tests pertaining to products produced by GMOs but, more importantly, wait for the time when consumers are ready to reconsider the anti-GMO sentiment of some advocacy groups and accept the quality-enhancing benefits (Pretorius, 2000; Pretorius and Høj, 2005).

Another biological approach was introduced with the concept of treating grape must with glucose oxidase (GOX) to reduce the glucose content of the must, thereby producing a wine with reduced alcohol content after fermentation (Villettaz, 1987; Pickering and Heatherbell, 1996; Pickering *et al.*, 1998). GOX from the food-grade fungus, *Aspergillus niger*, metabolizes glucose to glucono-δ-lactone and gluconic acid (Bankar *et al.*, 2009). *A. niger*, GOX and gluconic acid have GRAS (generally regarded as safe) status (Wong *et al.*, 2008). This method proved successful,

and showed no detrimental effects on wine quality. However, the use of enzyme preparations introduces additional steps to the winemaking process and increases production cost significantly (Pickering *et al.*, 1999*a*,*b*,*c*).

To avoid these drawbacks, Malherbe *et al.* (2003) introduced an *A. niger* GOX-encoding gene into *S. cerevisiae*. The preliminary results obtained with this GOX-producing yeast strain indicated a decrease of 1.5% in laboratory-scale wine fermentations. Based on these promising results, the objective of the present study was to improve the GOX-encoding gene construct and to explore ways to increase the expression, production and secretion of GOX in various genetic backgrounds.

During the course of this study, three new GOX-encoding gene cassettes (*GOX1*, *GOX2* and *GOX2LOX*) were constructed and expressed in two laboratory yeast strains (BY4742 and Σ1278b) and a widely-used industrial wine yeast (VIN13). In these constructs, secretion of GOX was facilitated by either the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) or the native *A. niger* secretion signal of GOX, thereby allowing comparative studies.

Integration of the *GOX2LOX* gene cassette into the genome of the diploid VIN13 strain was targeted at the master regulator locus of meiosis, *IME1*, by using the CreloxP-system for repetitive gene integration and genetic marker recovery (Güldener *et al.*, 1996, 2002; Hegemann *et al.*, 2006). By replacing *IME1* with the *GOX2LOX* gene cassette, it was hoped to generate an asporogenic, GOX-producing wine strain, thereby limiting this GM strain to transfer genetic material to ambient yeasts (Ramírez and Ambrona, 2008).

3.2 Methods and Materials

All standard biological and molecular protocols and techniques used in this study are described in either Sambrook and Russell (2001) or Ausubel *et al.* (2003), unless otherwise specified.

3.2.1 Microbial strains, media and culturing conditions

All microbial strains used and generated in this study are listed in Table 3.1.

Escherichia coli strain DH5α was routinely cultured at 37°C and used for transformation and amplification of plasmid DNA. Lysogeny broth (LB) and agar (Bio-

lab, Merck, South Africa), containing the antibiotic ampicillin (100 mg/mL), were used to select ampicillin-resistant (Ap^R; bla) bacterial transformants.

S. cerevisiae yeast strains were cultivated aerobically at 30°C either in yeast extract peptone dextrose (YPD) medium [containing 1% yeast extract (w/v), 2% peptone (w/v) and 2% glucose (w/v), (Biolab, Merck, South Africa)] or a synthetic complete drop-out (SCD) medium [containing 2% glucose, 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI, USA)]. SCD media were supplemented with essential amino acids, lacking either uracil (SCD^{-ura}) or leucine (SCD^{-leu}), from a 0.13% amino acid stock solution. To maintain cell viability and selective pressure for plasmids or gene cassettes introduced into the yeast cells uracil and leucine (stock solutions consisted of 7.2 g/L leucine and 2.4 g/L uracil) were added to media according to the specific requirements of each laboratory strain. For the selection of G418 resistant (*kan*MX) yeast transformants, either YPD or SCD media were supplemented with 50–250 μg/mL Geneticin® (Gibco BRL, Germany; the activity of this chemical may be batch dependent) dissolved in distilled water (dH_2O).

VIN13-Δ1 was sporulated in liquid sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose). The cells were incubated at room temperature for 5–6 days.

3.2.2 PCR procedures for amplification of respective fragments

All primers used to amplify respective fragments by polymerase chain reaction (PCR) method during this study are listed in Table 3.2. Primer pairs and PCR programs used to amplify each fragment are summarized in Table 3.3. All the PCR fragments with their expected sizes are listed in Table 3.4.

Plasmid DNA from *E. coli* DH5α and genomic DNA from *S. cerevisiae* strains that served as templates for PCR amplifications were extracted using standard laboratory protocols (Ausubel *et al.*, 2003).

PCR reactions were carried out using a MJ Research PTC-100 (Bio-Rad, California, USA) or a PCR Express (Hybaid) thermal cycler. The reaction mixtures

Table 3.1: Micro-organisms used and constructed.

Micro-organisms/Strains	Genotype/Description	Source or reference
Escherichia coli		
DH5α	*supE*44Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gry*A96 *thi*-1 *re1*A1	Sambrook and Russell (2001)
Saccharomyces cerevisiae		
Σ1278b	JT4500 *ura3*	This laboratory
ΣpGOXi	JT4500 *URA3* $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$	Malherbe *et al.* (2003)
ΣpDMYIPgox1	JT4500 *URA3* $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$	This work
ΣpDMYIPgox2	JT4500 *URA3* $PGK1_P$-GOX-$PGK1_T$	This work
ΣpGOX1i	JT4500 *URA3* $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$	This work
ΣpGOX2i	JT4500 *URA3* $PGK1_P$-GOX-$PGK1_T$	This work
ΣpDMPgox2lox	Episomal pDMPgox2lox	This work
Σ-*IME1*Δ0	*IME1*Δ1::*GOX2LOX*	This work
BY4742	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (same genetic background as S288c)	EUROSCARF [a]
BYpDMPgox1	BY4742 *LEU2* $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$	This work
BYpDMPgox2	BY4742 *LEU2* $PGK1_P$-GOX-$PGK1_T$	This work
BYpGOX1i	BY4742 *LEU2* $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$	This work
BYpGOX2i	BY4742 *LEU2* $PGK1_P$-GOX-$PGK1_T$	This work This work
BYpDMPgox2loxi	BY4742 *LEU2* *GOX2LOX*	This work This work
VIN13	*MATa/MAT*α (Industrial wine yeast strain)	Anchor Yeast [b]
VIN13 pDMPgox2lox	VIN13 Episomal pDMPgox2lox	This work
VIN13-Δ1	VIN13 *IME1*Δ1::GOX2LOX (single deletion of IME1)	This work
VIN13-Δ2	VIN13 *IME1*Δ2::GOX2LOX (double deletion of IME1)	This work

The 'i' at the end of some yeast strains, indicate that an integration occurred in that yeast's genome.

[a] EUROSCARF : EUROpean Saccharomyces Cerevisiae ARchive for Functional analysis
[b] Anchor Yeast : Anchor Yeast (Cape Town, South Africa)

consisted of 1 μL template DNA (6 ng/μL), 6 μL of each primer (1.5 pmol/μL), 8 μL of dNTP mixture (1.25 mM, final concentration), 0.5 μL (1.75 units) of Expand DNA polymerase (Roche Diagnostics, Mannheim, Germany) and 5 μL of PCR reaction buffer (10x) without $MgCl_2$ [10 mM Tris-HCl pH 8.3, 50 mM KCl and 0.01% gelatin (w/v)]. A concentration range was used to determine the optimum amount (results not shown) of $MgCl_2$ (25 mM stock solution) to add to individual PCR reaction for fragment generation. Sterile dH_2O was used to adjust subsequent PCR reaction mixture to a final volume of 50 μL.

Two sets of microsatellite primers, RMP2MW and RMP2MC (Table 3.2), were used to discriminate between haploid and diploid strains of transformed VIN13 strains. PCR (program no. 17, Table 3.3) of *MATa* cells produced a 544 bp PCR product, and *MAT*α cells, a 404 bp product. Diploid cells contained both products (Huxley *et al.*, 1990; Field and Wills, 1998).

PCR products were separated in 0.8% agarose gels by gel electrophoresis and the correct size of each PCR fragment was confirmed by visualizing its banding pattern with an AlphaImage™ 2200 camera and AlphaEase™ software (Analytical and Diagnostic Products, Weltevrede Park, South Africa). For PCR products smaller than 1 kb, 1% agarose gels were prepared.

Table 3.2: Primers used to PCR respective fragments.

Primer name	Sequence (5' ⟶3')	Enzyme site(s) [a]	Size (bp)	Tm (°C)
Ani-*MFα$_S$1gox*-F	GATC AGA TCT ATG AGA TTT CCT TCA ATT TTT	*Bgl*II	31	55
Ani-*gox*-F	GATC AGA TCT **ATG**[b] CAG ACT CTC CTT GTG AGC TCG	*Bgl*II	34	66
Ani-*gox*-R	GATC CTC GAG TCA CTG CAT GGA AGC ATA ATC TTC	*Xho*I	34	64
PGK1$_P$-BamHI-F	GATC GGA TCC AAG CTT TCT AAC TGA TCT ATC CAA	*Bam*HI, *Hin*dIII	34	60.4
PGK1$_T$-BamHI-R	GATC GGA TCC AAG CTT TAA CGA ACG CAG AAT	*Bam*HI	31	61.9
PGK1$_P$-F	GATC GAT ATC AAG CTT TCT AAC TGA TCT ATC CAA	*Eco*RV, *Hin*dIII	34	60
PGK1$_T$-R	GATC GCG GCC GCA AGC TTT AAC GAA CGC AGA AT	*Not*I, *Hin*dIII	33	67
mMCS-F	GATC AGA TCT GCT AGC GCG GCC GCG GGA ATT CGA TA	*Bgl*II	36	70
mMCS-R	GATC CTC GAG ACT AGT CAT ATG GTC GAC CTG CAG GC	*Xho*I	36	69
loxPcas-F	GAT CGC ATG CAT AAC TTC GTA TAA TGT ATG CTA TA	*Sph*I	35	60
ClaIb-R	ATC GAT AGA TTG TCG CAC CTG ATT GCC CGA CAT TA	*Cla*I	35	64
ClaIb-F	ATC GAT TGT ATG GGA AGC CCG ATG CGC CAG AGT TG	*Cla*I	35	68
loxPcas-R	GAT CGG ATC CAT AAC TTC GTA TAG CAT ACA TTA TA	*Bam*HI	35	60
IME1-2nd-F	GAT CGA TAT CTC ACC TAC AGA GAA ACA AAT TCC TA	*Eco*RV	35	58.7
IME1-2nd-R	GAT CGG ATC CTT TGT TTG TGG GGA GAG GAA	*Bam*HI	30	63
IME1-end-F	GAT CGG ATC CTG CTG CGG CTT ACT CCT TCA	*Bam*HI	30	65.9
IME1-end-R	GAT CGA TAT CTT AAG AAT AGG TTT TAC TAA ACT TGT AGG A	*Eco*RV	40	57.5

Table 3.2 *continues on next page...*

Table 3.2 – *Continued*

Primer name	Sequence (5' ⟶3')	Enzyme site(s) [a]	Size (bp)	Tm (°C)
GOXLOX-F	GAT CGG ATC CTC TAA CTG ATC TAT CCA AAA CTG A	*Bam*HI	34	67
GOXLOX-Rb2	GGA TCC CCC GGG GAT CCA TAA C	*Bam*HI	22	68.2
PhR332-F	GATC CAA TTG GGT ACC CGG GGG ATC	*Mun*I, *Kpn*I	25	63
PhR332-R	GATC CAA TTG AAG CTT GCA AAT TAA AGC C	*Mun*I	29	57.7
Leu2-F	TAG GAT AAT TAT ACT CTA TTT CTC AAC AAG TAA TTG	-	36	71.4
Ura3-F	GGC TGT GGT TTC AGG GTC C	-	19	54
PGK-ter-F	ATC AAT TTT TTT CTT TTC TCT TTC CCC ATC	-	30	55.1
VIIup-F	CAA AAA ATC GGA ATT ACA GAC ACA CCA TTT	-	30	56.8
VIIup-R	ATG TAT TTC TTG CAT TGA CCA ATT TAT GCA	-	30	56.4
VIIdown-F	ATG GGT AAG GAA AAG ACT CAC GTT TCG	-	27	58.8
VIIdown-R	ACA CCT TGA TTT GCT TCT CCT TCT GTT ATT A	-	31	58.1
IME1-F702	AGA TCC TCT TCA TTA TCA TCT CTT TTT TCT A	-	31	54.2
IME1-R702	TTA AGA ATA GGT TTT ACT AAA CTT GTA GGA T	-	31	52.9
RMP2MW	GCT TCA CCT ATT CCA CTC TCG G	-	22	54.5
RMP2MC	CAA GCC TCT TCA AGC ATG AC	-	20	49.9

[a] Enzyme site(s) : Restriction enzyme sites added to primers are underlined in the second column.

[b] ATG : Recovered codon (ATG) for transcription initiation of *GOX* gene. The codon was removed in the work by Malherbe *et al.* (2003).

Table 3.3: PCR primer pairs and amplification programs used in this study.

Program number	Primer pair [a]	Template	Initial denaturation		Number of cycles	Amplification cycles		Final elongation		Fragment name/size [b]
			Temp (°C)	Time (s)		Temp (°C)	Time (s)	Temp (°C)	Time (s)	
1	Ani-*MFα$_S$1gox*-F Ani-*gox*-R	ΣpGOXi [g]	94	120	30	94 45 72	60 60 180	72	300	go1
2	Ani-*gox*-F Ani-*gox*-R	ΣpGOXi [g]	94	120	30	94 45 72	60 60 180	72	300	go2
3	PGK1$_P$-BamHI-F PGK1$_P$-BamHI-R	pDMPgox1, [p] pDMPgox2 [p]	94	120	30	54 72 94	60 180 60	72	300	gox1, gox2
4	loxPcas-F ClaI-R	pUG6 [p]	94	120	25	94 55 72	60 60 120	72	30	KanMXa
5	ClaIb-F loxPcas-R	pUG6 [p]	94	120	25	94 55 72	60 60 120	72	300	KanMXb
6	mMCS-F mMCS-R	pGTE-EcoRV [p]	94	120	30	94 50 72	60 60 20	72	60	mmcs

Table 3.3 *continues on next page...*

Table 3.3 – *Continued*

Program number	Primer pair [a]	Template	Initial denaturation		Number of cycles	Amplification cycles		Final elongation		Fragment name/size [b]
			Temp (°C)	Time (sec)		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	
7	IME1-2nd-F IME1-end-R	VIN13 [g]	94	120	30	55 72 94	60 90 60	72	300	*IME1*
8	IME1-2nd-F IME1-2nd-R	VIN13 [g]	94	120	30	94 50 72	60 60 20	72	60	IME1-2nd
9	IME1-end-F IME1-end-R	VIN13 [g]	94	120	30	94 50 72	60 60 20	72	60	IME1-end
10	GOXLOX-F GOXLOX-Rb2	pDMPgox2lox [p]	94	120	30	58 72 94	60 360 120	72	420	GOX2LOX
11	PhR332-F PhR322-R	pUT332 [p]	94	120	30	50 72 94	60 90 60	72	180	PhRcas
12	PGK-ter-F Leu2-F	BYpGOX1 [g] BYpGOX2 [g]	94	120	30	50 72 94	60 90 60	72	210	3343 bp

Table 3.3 *continues on next page...*

Table 3.3 – *Continued*

Program number	Primer pair [a]	Template	Initial denaturation		Number of cycles	Amplification cycles		Final elongation		Fragment name/size [b]
			Temp (°C)	Time (sec)		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	
13	PGK-ter-F	ΣpGOX1i [g]	94	120	30	50	60	72	210	1844 bp
	Ura3-F	ΣpGOX2i [g]				72	90			
						94	60			
14	VIIup-F	VIN13-Δ1 [g]	94	120	30	48	60	72	120	2323 bp
	VIIup-R					72	120			
						94	60			
15	VIIdown-F	VIN13-Δ1 [g]	94	120	30	48	60	72	120	1929 bp
	VIIdown-R					72	120			
						94	60			
16	IME1-F702	VIN13-Δ1 [g]	94	120	30	45	60	72	120	1929 bp
	IME1-R702	VIN13-Δ2 [g]				72	120			
						94	60			
17	RMP2MW	VIN13-Δ1 [g]	95	300	30	59	30	72	300	544 bp
	RMP2MC	VIN13-Δ2 [g]				72	40			404 bp
						94	30			

[a] See Table 3.2 for additional information on individual primers

[b] See Table 3.4 for detailed information regarding each PCR fragment

[g] Genomic DNA isolated from *Saccharomyces cerevisiae* strain (Table 3.1)

[p] Plasmid DNA (Table 3.5) isolated from *Escherichia coli* DH5α strain

Table 3.4: Fragments generated by PCR for cloning purposes.

Fragment name	Expected size (bp)	PCR program [a]	Name of plasmid [b]
go1	2135	1	pGTEgo1
go2	1850	2	pGTEgo2
gox1	3907	3	pGTEgox1
gox2	3622	3	pGTEgox2
kan-a	550	4	pGTEkan-a
kan-b	993	5	pGTEkan-b
mmcs	60	6	pGTEmmcs
IME1	1000	7	pGTE-*IME1*
IME1-2nd	108	8	pGTE-IME1-2nd
IME1-end	104	9	pGTE-IME1-end
GOX2LOX	5039	10	pGTE-GOX2LOX
PhRcas	1264	11	pGTE-PhRcas

[a] Refer to PCR program numbers in Table 3.3 for individual PCR program details.
[b] Name of plasmid: pGEM®-T Easy vector (pGTE) with cloned fragment. Complete plasmid description in Table 3.5.

3.2.3 DNA manipulations and vector constructions

Plasmids used and constructed in this study are listed in Table 3.5.

PCR fragments were excised from agarose gels and purified using a Qiaquick Gel Extraction Kit (Qiagen, South Africa), whereafter it was cloned into the pGEM®-T Easy vector (Promega, Whitehead Scientific, Cape Town, South Africa). The QIAprep Spin Miniprep Kit (Qiagen, South Africa) was used to isolate and clean plasmid DNA for cloning purposes. Standard laboratory techniques were used for restriction enzyme digestions, ligation reactions and *E. coli* transformations. Enzymes and ligases were obtained from Roche (Randburg, South Africa).

Fragments go1 and go2 were excised from pGTEgo1 and pGTEgo2 and sub-cloned into *Bgl*II and *Xho*I linearized vector pHVX2 (Volschenk *et al.*, 1997). This resulted in the construction of pDMPgox1 and pDMPgox2. Fragment kan-b was released from pGTEkan-b and sub-cloned into *Cla*I and *Bam*HI prepared vector pGTEkan-a. This resulted in the construction of pGTE-loxPcas, containing the loxPcas cassette of 1543 bp. The loxPcas cassette was excised from pGTE-loxPcas,

and sub-cloned into *Sph*I and *Bam*HI prepared pDMPgox1 and pDMPgox2 which yielded pDMPgox1lox and pDMPgox2lox, respectively.

A 3907-bp fragment was released from plasmid pDMPgox1 and sub-cloned into a *Pvu*II linearized YIPlac211 vector, thereby generating pDMYIPgox1. A 3622-bp DNA fragment carrying *GOX2*, was excised from pGTEgox2 and subcloned into *Bam*HI prepared YIPlac211, generating the integration vector pDMYIPgox2.

A new shuttle vector, pDMPM, was constructed to increase the number of restriction sites available for further cloning procedures. This was accomplished in several steps. Firstly, the vector pGEM®-T Easy was digested with the enzyme *Eco*RI, and then re-circularised by overnight ligation. Secondly, the multiple cloning site (*mcs*) of the now modified pGEM®-T Easy vector (named pGTE-EcoRV due to the loss of the *Eco*RV restriction site and subsequently also the 'T' site for PCR cloning) was used as template for PCR reaction number 6 (see Table 3.3). A fragment 'mmcs' was generated, and cloned into pGEM®-T Easy resulting in the vector pGTEmmcs. It was sub-cloned into the vector pHVX2, between the *PGK1* promoter and terminator, into *Bgl*II and *Xho*I restriction sites.

In addition, the loxPcas cassette was excised from pGTE-loxPcas with *Sph*I and *Bam*HI and sub-cloned into pDMPM at corresponding restriction sites directly behind the *PGK1* terminator. This new shuttle vector was named pDMPL and has multiple cloning sites behind the constitutive *PGK1* promoter that offer more restriction sites to clone into. The *KanMX* gene inside the loxPcas cassette will give any transformed yeast strain resistance to G418, and the two loxP sites flanking *KanMX* can be used with the CreloxP-system to recover the marker gene.

Sequence analysis was performed on the integration cassettes and plasmids containing *A. niger gox* gene, either at the Australian Genome Research Facility Ltd (AGRF; Adelaide, South Australia) or the Central DNA Sequencing Facility located on the Stellenbosch University campus (Stellenbosch, South Africa) to confirm there were no mutations. The *IME1* gene (cds) and flanking regions of the industrial yeast (VIN13) were cloned into the pGEM®-T Easy vector after successful PCR and sequenced. The sequence of the coding region of *IME1* differed slightly from the sequence found in the Saccharomyces Genome Database (SGD). However, regions flanking the gene were identical and primers were designed and

fragments were generated for cloning and integration into *IME1*.

The plasmid pGTE-IME1-end was digested with restriction enzymes *Bam*HI and *Sca*I, yielding a 1298-bp fragment. Plasmid pGTE-IME1-2nd was digested with restriction enzymes *Bam*HI and *Sca*I, yielding a fragment of 1946 bp. These two fragments were ligated together and resulted in the construction of pGTE-IME1-2nd-kout. Fragment GOX2LOX was released from pGTE-GOX2LOX and sub-cloned into *Bam*HI prepared pGTE-IME1-2nd-kout. This resulted in the construction of pGTE-Final2 containing the final knock-out cassette: 'Final2', a 5239-bp fragment.

The fragment 'PhRcas' was excised from pGTE-PhRcas and sub-cloned into the *Nsi*I and *Kpn*I prepared plasmid pSH47 (Güldener *et al.*, 1996). This resulted in the construction of pDMcrePhR, which contained the phleomycin resistance marker gene (Ph^R) and the gene coding for the enzyme cre-recombinase that is used in the CreloxP-system for marker recovery.

3.2.4 Yeast transformation and genomic integration

The *S. cerevisiae URA3* gene was cleaved by a restriction digestion with *Stu*I thereby linearizing plasmids pDMYIPgox1 and pDMYIPgox2. Linearized plasmid DNA was transformed into *S. cerevisiae* Σ1278b laboratory yeasts and positively transformed yeasts were observed as colonies growing on selective media (SCD^{-ura}) when the previous ura3 auxotroph phenotype changed to prototrophy. Similarly, plasmids pDMPgox1 and pDMPgox2 were individually transformed into *S. cerevisiae* BY4742 laboratory yeasts after they were linearized by a restriction digests within *LEU2* with enzyme *Eco*RV. Homologous recombination events were observed when the linearized plasmid carrying short terminal regions homologous with the chromosome integrated successfully and colonies appeared on SCD^{-leu} plates. Plasmids pDMYIPgox1, pDMYIPgox2, pDMYIPgox1 and pDMYIPgox2 were also transformed into *S. cerevisiae* Σ1278b and BY4742 strains as episomal plasmids.

These laboratory yeast strains were transformed using the lithium acetate procedure (Gietz and Schiestl, 2007) and resulted in the generation of eight new yeast strains: ΣpGOX1i, ΣpGOX2i, ΣpDMYIPgox1, ΣpDMYIPgox2 and BYpGOX1i,

Table 3.5: Plasmids used and constructed.

Name	Genotype	Source or reference
pHVX2	2μm *bla LEU2 PGK1$_P$-PGK1$_T$*	Volschenk *et al.* (1997)
YIplac211	2μm *bla lacZ URA3*	Gietz and Sugino (1988)
pUG6	*bla loxP-kanMX-loxP*	Güldener *et al.* (1996)
pUT332	2μm *bla URA3* Ph^R	Gatignol *et al.* (1990)
pSH47	2μm *bla URA3* Cre	Güldener *et al.* (1996)
pDMPM	2μm *bla LEU2 PGK1$_P$*-mmcs-*PGK1$_T$*	This work
pDMPL	2μm *bla LEU2 PGK1$_P$*-mmcs-*PGK1$_T$ loxP-kanMX-loxP*	This work
pDMcrePhR	2μm *bla URA3* Ph^R Cre	This work
pDMPgox1	2μm *bla LEU2 PGK1$_P$-MFα1$_S$-gox-PGK1$_T$*	This work
pDMPgox2	2μm *bla LEU2 PGK1$_P$-gox-PGK1$_T$*	This work
pDMYIPgox1	2μm *bla lacZ URA3 PGK1$_P$-MFα1$_S$-gox-PGK1$_T$*	This work
pDMYIPgox2	2μm *bla lacZ URA3 PGK1$_P$-gox-PGK1$_T$*	This work
pDMPgox1lox	2μm *bla LEU2 PGK1$_P$-MFα1$_S$-gox-PGK1$_T$–loxP-kanMX-loxP*	This work
pDMPgox2lox	2μm *bla LEU2 PGK1$_P$-gox-PGK1$_T$–loxP-kanMX-loxP*	This work
pGEM®-T Easy	hereafter pGEM®-T Easy is refered to as pGTE	Promega [a]
pGTE-EcoRV	pGEM®-T Easy digested with *Eco*RI and re-ligated	This work
pGTEmmcs	modified mcs of pGTE-EcoRV	This work
pGTEgo1	*MFα1$_S$-gox*	This work
pGTEgo2	*gox*	This work
pGTEgox1	*PGK1$_P$-MFα1$_S$-gox-PGK1$_T$*	This work
pGTEgox2	*PGK1$_P$-gox-PGK1$_T$*	This work
pGTEkan-a	kan-a (first 550 bp of KanMX cassette, 5'-side to *Cla*I restriction site)	This work
pGTEkan-b	kan-b (last 993 bp of KanMX cassette, from *Cla*I restriction site to 3'-site end)	This work
pGTEloxPcas	*loxP-kanMX-loxP*	This work
pGTE-IME1	The *IME1* gene of wine yeast VIN13, with 100 bp upstream of the start codon (ATG)	This work
pGTE-IME1-2nd	100 bp fragment (100 bp upstream of *IME1* ATG)	This work
pGTE-IME1-end	100 bp fragment (last 100 bp of *IME1* cds, on 3'-side)	This work
pGTE-IME1-2nd-kout	IME1-2nd and IME1-end joined together	This work
pGTE-GOX2LOX	*PGK1$_P$-gox-PGK1$_T$–loxP-kanMX-loxP*	This work
pGTE-Final2	IME1-end–GOX2LOX–IME1-2nd	This work
pGTE-PhRcas	*TEF1$_P$*-Ph^R-*CYC1$_T$*	This work

[a] Promega : Distributed in South Africa by Whitehead Scientific.

BYpGOX2i, BYpDMPgox1, BYpDMPgox2 (see Table 3.1).

The following transformations were performed using electroporation and a modified protocol of Volschenk *et al.* (2004). Yeast cells were pre-cultured overnight in 10 mL YPD at 30°C followed by inoculation into 500 mL YPD in a 2 L flask to an optical density at 600 nm (O.D.600) of 0.1. The culture was shaken vigorously at 30°C until the mid-logarithmic growth phase was reached (O.D.600 of 1.3–1.5). Yeast cells were harvested by centrifugation at 3000 rpm at 4°C and re-suspended in 80 mL sterile doubled-distilled water (ddH_2O). While swirling, 10 mL 10 X TE buffer (pH 7.5) was added, followed by 10 mL 1 M lithium acetate. After incubation for 45 min at 30°C with gentle agitation, 2.5 mL fresh 1 M DTT was added to the yeast suspension, while swirling, with a continued incubation for 15 min at 30°C with gentle agitation. The yeast suspension was subsequently diluted to a volume of 500 mL with ddH_2O, washed and concentrated three times at 3000 rpm, 4°C. Cell pellets were re-suspended each time as follow: first in 250 mL ice-cold ddH_2O, then in 30 mL ice-cold 1 M sorbitol and finally in 0.5 mL ice-cold 1 M sorbitol. This yielded a final volume of 1–1.5 mL cells with an approximate O.D.600 of 200. After the cell pellet was re-suspended, 40 μL of the concentrated yeast cells were mixed with 5 μL linear DNA (approximately 500 ng) in a sterile, ice-cold 1.5 mL tube. The cell-DNA admixture was transferred to an ice-cold 0.4 cm gap electroporation cuvette (Biorad, South Africa) and subjected to a pulse of 1.5 kV, 25 mF and 201 ohms using an Easy-jecT1450 V Twin pulse electroporation apparatus (EquiBio, Ashford, UK). Immediately after the pulse was administered, 1 mL ice-cold YPD was added to the cuvette, followed by a gentle mix for 2 to 4 h at 30°C. Aliquots of 250 μL yeast suspension were plated directly onto YPD-G418 agar plates with a concentration range of 50–250 μg/mL Geneticin® to eliminate false positives. Transformants were incubated for 3 to 4 days at 30°C, whereafter yeast colonies that appeared were selected and evaluated to identify those colonies that produce and secrete biologically active GOX.

The laboratory strains of *S. cerevisiae* Σ1278b and BY4742 as well as the industrial wine yeast VIN13, were transformed with the plasmid pDMPgox2lox. Gene cassette *GOX2LOX*, containing *GOX2* and *kan*MX, was declared ready for transformation after colonies were observed that produce biologically active GOX while growing on media containing Geneticin®. No further studies were performed on

the strains: ΣpDMPgox2lox, BYpDMPgox2lox and VIN13pDMPgox2lox (Table 3.1). However, pDMPgox2lox was also integrated into the genomes of *S. cerevisiae* Σ1278b, BY4742 and VIN13 at the *LEU2* locus after the plasmid was digested with the restriction enzyme *Eco*RV and linearized to initiate homologous recombination upon transformation. These new strains (ΣpDMPgox2loxi, BYpDMPgox2loxi and VIN13pDMPgox2loxi (Table 3.1)), were used in further experiments.

The integration cassette, 'Final2', was excised from plasmid pGTE-Final2, and the 5239-bp fragment was integrated into the genome of VIN13 and Σ1278b. Integration into Σ1278b resulted in strain Σ-*IMEI*Δ0, which does not have any copies left of *IME1*. VIN13 strain integrations took place by '1-step' replacement of the *IME1* gene (*IME1*Δ1::GOX2LOX). This resulted in the generation of the strain VIN13-Δ1, which showed resistance to the antbiotic Geneticin® after it was cultured on YPD-G418 agar plates, containing an optimum concentration of 200 g/mL.

Plasmid pDMcrePhR was transformed into VIN13-Δ1 by means of electroporation. Selection of phleomycin sulfate-resistant (Ph^R) VIN13-Δ1 transformants were performed on YPD agar supplemented with 100–300 μg/mL Phleomycin® (InvivoGen, Cayla, Germany) and the optimum concentration for selection was 200 μg/mL.

3.2.5 Sporulation, ascospore digestion and hybridization of VIN13-Δ1

VIN13-Δ1 was grown to an optical density (O.D.600) of 2.5–3.0 in YPD media, whereafter 1 mL of the culture was transferred to a sterile, disposable 15 mL tube and centrifuge for 5 min at 3000 rpm. The supernatant was removed, the pellet resuspended in 5 mL ddH_2O, and vortexed. Cells were centrifuged a second time and after the removal of the supernatant, resuspended in 1 mL of liquid sporulation medium. It was not necessary to supplement the medium with nutritional media because, no selective pressure was required for VIN13-Δ1. The cells were shaken at 30°C for 3–6 days, and evaluated for sporulation microscopically.

Tetrads were centrifuged for 5 min at 5000 rpm after sporulation took place. The pellet was washed in ddH_2O, and centrifuged again. The tetrads were then suspended in 150 μL lysis buffer [25 mg/mL Zymolase (ICN Immuno Biologicals),

1 M sorbitol] and incubated overnight at 30°C. The tetrads were inspected under a microscope for cell wall degradation that would facilitate easier dissection of each tetrad. The dissections were performed with the use of a Nikon micromanipulator and individual spores from each tetrad were placed in a horizontal line. Different tetrads were dissected and each tetrad's spores were placed below each other in a horizontal line. The spores were then incubated on YPD plates and incubated at 30°C for 5 days to allow self-diploidization (Ono *et al.*, 1990; Spencer and Spencer, 1996). This resulted in the generation of VIN13-Δ2.

3.2.6 Glucose oxidase (GOX) plate assays

This adapted assay from Hodgkins *et al.* (1993) confirmed the production and secretion of active GOX by yeast colonies that were previously selected on SCD^{-ura}, SCD^{-leu} and YPD-G418 plates and identified as positive transformants.

Yeast colonies were spotted onto YPD plates and incubated for 2–3 days at 30°C, giving time for the yeast strains to grow and secrete glucose oxidase. The plates were then overlaid with 10 mL of 0.1 M McIlvaine buffer, pH 7.0 [1% (w/v) agarose, 10 g/L glucose, 100 mg/L σ-dianisidine dihydrochloride (Sigma, South Africa) and 15 U/mL horseradish peroxidase type II (Sigma, South Africa)]. The overlay was allowed to set and the plates were incubated at 37°C for 1 hour.

As glucose oxidase converts glucose into gluconic acid, hydrogen peroxide (H_2O_2) is formed as a byproduct. The H_2O_2 is then used by horseradish peroxidase to oxidize σ-dianisidine dihydrochloride and a colour change was visible on the agar plates. Transformed yeast colonies secreting active glucose oxidase were surrounded by a brown halo. *S. cerevisiae* ΣpGOXi (Malherbe *et al.*, 2003) was used as positive control and untransformed yeast strains were used as negative control.

3.2.7 Verification of transformations and integrations

3.2.7.1 PCR confirmation of integration

Integration of plasmids and/or integration cassettes into laboratory and industrial strains were confirmed by PCR. Regions were identified where a primer would bind either upstream or downstream of the integration, as well as an area inside the DNA that was introduced into the genome. See Tables 3.2 and 3.3 for additional

information regarding the primers and PCR programs.

PCR with primers Leu2-F and PGK-ter-F yielded a fragment of 3343 bp and confirmed integrations into strains BYpGOX1i and BYpGOX2i. Primers, Ura3-F and PGK-ter-F generated a 1844 bp fragment and confirmed integrations into strains ΣpGOX1i and ΣpGOX2i. Integrations into VIN13 were confirmed by two sets of primers. The first set, VIIup-F and VIIup-R was used to generate an 2323-bp upstream fragment containing part of the integration cassette and part of the genomic sequence upstream of the *IME1* ATG. The second set of primers, VIIdown-F and VIIdown-R was used to generate a 1929-bp fragment that included part of the integrated cassette as well as genomic DNA downstream of the *IME1* gene.

3.2.7.2 Southern blot analyses

Genomic DNA was isolated from individual yeast strains. DNA isolated from strains BYpGOX1i and BYpGOX2i, where the *LEU2* locus was the target for integration, were digested with *Mlu*NI. In strains ΣpGOX1i and ΣpGOX2i where the *URA3* locus was the target of integration, genomic DNA was digested with *Ksp*I and *Nae*I. Genomic DNA isolated from industrial strains VIN13-Δ1, VIN13-Δ2 and where the *IME1* locus was the target of integration, was digested with *Mlu*NI and *Nar*I. Enzymatically digested DNA were separated on a 0.8% [w/v] agarose gel. Standard procedures (Sambrook and Russell, 2001) were used to depurinate, denature and neutralize the gel before the DNA fragments were transferred to a positively charged nylon membrane (AEC-Amersham, South Africa). The glucose oxidase gene (*GOX*) was used as a probe, prepared by PCR (no. 2) and the digoxigenin (DIG) nonradioactive nucleic acid labeling and detection system was used for Southern hybridization to verify integrants. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany).

3.2.8 Small-scale fermentations

A glucose rich medium (YPD) was used to perform comparative analysis of enzyme production between different yeast strains containing the different GOX cassettes. All fermentations were carried out at 30°C in 250 mL Erlenmeyer flasks, and with a volume of 100 mL. The sugar concentration was adjusted to 100 g/L and the pH was adjusted to 5. Fermentations were performed in triplicate. Pre-inoculums

were prepared overnight in similar media and all fermentations were inoculated to 10^6 cells/mL. In all the fermentations, untransformed yeast strains served as the control. Samples were taken at 0 h, 3 h, 6 h, 9 h and 24 h after inoculation. The following parameters were monitored: optical cell density (O.D.600), viable cell counts expressed as colony forming units (CFU/mL), and concentrations of glucose (g/L), gluconic acid (g/L) and glucose oxidase activity (units/L) intracellularly and extracellularly.

3.2.9 Spectrophotometric assay

3.2.9.1 Recovery and purification of enzymes

Samples (1.5–2 mL) from individual yeast fermentations were harvested at 5000 rpm for 5 min in an Eppendorf 5415 D benchtop centrifuge. The supernatant containing the extracellular enzyme was removed from the pelleted cells, and used without further purification to determine the amount of secreted active glucose oxidase. The cells collected during the first step were resuspended in 5 mL of 50 mM Tris (pH 7.5, containing 10 mM NaCl) buffer. Approximately 0.1 g of 0.2 mm acid washed glass beads (Sigma, South Africa) were added and the cells were mixed vigorously for 3 min. After centrifugation at 6000 rpm for 2 min, the supernatant containing the intracellular protein extract, was carefully removed and the samples stored until enzyme analysis could be performed.

3.2.9.2 Glucose oxidase spectrophotometric assay

A glucose oxidase (Catalogue Number: K-GLOX) kit, from Megazyme International Limited (Ireland), was used to perform spectrophotometric assays confirming the presence and activity of glucose oxidase in liquid cultures. One activity unit (U) of GOX, decomposes 1 mol of glucose in one minute. Samples of intra- and extracellular fractions were prepared as, described in above.

3.2.9.3 Gluconic acid spectrophotometric assay

Samples (1.5–2 mL) from individual yeast fermentations were harvested at 5,000 rpm for 5 min in a Eppendorf benchtop centrifuge. The supernatant was used in the D-gluconic acid/D-glucono-δ-lactone kit (Catalogue Number: K-GATE) from

Megazyme International Limited (Ireland), to perform spectrophotometric assays confirming the production and presence of gluconic acid in liquid cultures.

3.2.10 Statistical data analysis

Two-way repeated measures analysis of variance (ANOVA) was performed to determine the influence of yeast strain on time course production of extracellular GOX and GA during laboratory fermentations in synthetic media. Results are expressed as mean $\pm$ SEM. The level of significance was set at $P < 0.05$. *STATISTICA 9* (statistical software by Statsoft) was used to perform the analysis.

3.3 Results and Discussion

3.3.1 Construction of new multi-purpose vectors

Three new cloning vectors, pDMPM, pDMPL and DMcrePhR were constructed. The first, pDMPM, is a bacteria-yeast shuttle vector with the *bla* and *LEU2* marker genes, and an enhanced multiple cloning site flanked by the constitutive phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). pDMPL was essentially the same plasmid as pDMPM, except that it also contained a loxP cassette, containing the *kan*MX marker gene, which would enable any transformed yeast to have resistance to G418 sulfate. Finally, plasmid pDMcrePhR, which contains the Phleomycin® resistance marker gene (Ph^R) and the gene coding for the enzyme cre-recombinase (*Cre*). This plasmid can be used incombination with the CreloxP-system for marker recovery in any yeast strain (especially industrial strains) that would require resistance to Phleomycin®.

3.3.2 Generation of *S. cerevisiae* transformants

3.3.2.1 Cloning and selection of *GOX1*, *GOX2* and *GOX2LOX* expressing yeasts

Two gene cassettes (*GOX1* and *GOX2*) were constructed. The two cassettes both contained a structural *A. niger* GOX gene under the regulation of the phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). To facilitate secretion, in *GOX1* the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) was fused to the GOX gene (Malherbe *et al.*, 2003), and in *GOX2* the native *A.*

niger secretion signal of GOX was used. *GOX1* and *GOX2* were cloned into different vectors and cassettes (Table 3.5) and transformed in laboratory strains and an industrial wine strain of *S. cerevisiae* (Table 3.1).

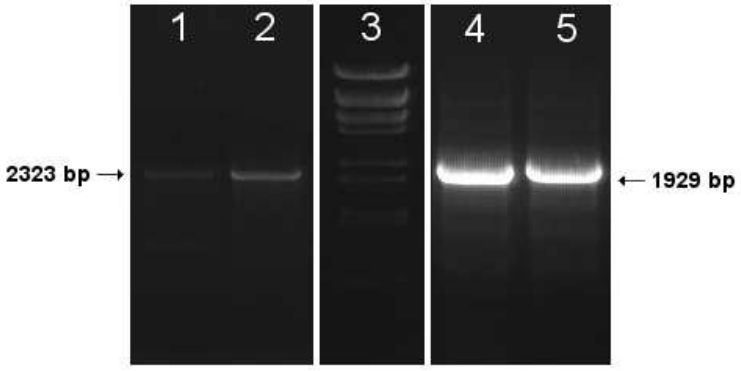
An additional integration cassette, designated *GOX2LOX*, was constructed to knock out the *IME1* gene in industrial *S. cerevisiae*VIN13. *GOX2LOX* consists of *GOX2* fused to a loxP cassette. This loxP cassette makes use of the proven CreloxP-system for repetitive gene integration and genetic marker recovery (Güldener *et al.*, 1996, 2002; Hegemann *et al.*, 2006), and contains two loxP sites that flank a G418 sulfate resistant marker gene (*kan*MX).

The Leu^+, Ura^+ and $G148^+$ integrants were screened for the secretion of biologically active GOX by selecting the colonies surrounded by a brown halo in GOX agar plate assays (results not shown).

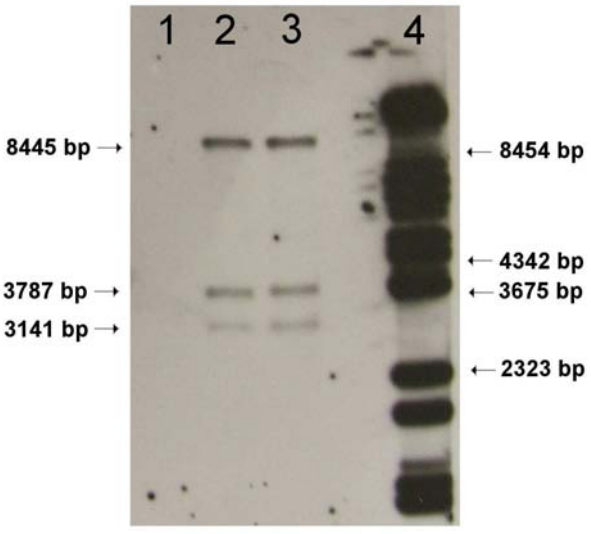
3.3.2.2 Confirmation of integrations by PCR and DIG Southern blot

PCR was used to confirm the successful integration of *GOX1* and *GOX2* into either the leu2 marker of BY4742 or the ura3 marker of Σ1278b. PCR was also used to verify integration of *GOX2LOX* into *IME1* of both BY4742 and Σ1278b. The *GOX2LOX* cassette was integrated successfully into VIN13 as a one-step integration of *IME1* and yielded VIN13-Δ1. Banding patterns consisting of a 2323-bp upstream fragment, and a 1929-bp downstream fragment were generated by PCR and this was indicative of the integration and orientation of *GOX2LOX*. As an example, Figure 3.1 (a) presents an electrophoresis gel photo containing banding patterns of PCR confirming integration of *GOX2LOX* into the genome of the industrial yeast strain VIN13.

A DIG Southern blot confirmed the integration of respective vectors and cassettes into the genome of the targeted industrial and laboratory yeast strains. Hybridization signals representing a single copy of GOX, with the exception of the double integration in VIN13-Δ2 were observed. As an example, a DIG southern blot autoradiogram confirming the integration of *GOX2LOX* as a single copy into VIN13-Δ1 and double copy into VIN13-Δ2 is presented in Figure 3.1 (b). Signals that confirm integration into the genome of VIN13 were detected in the form of banding patterns (8445 bp, 3787 bp and 3141 bp).



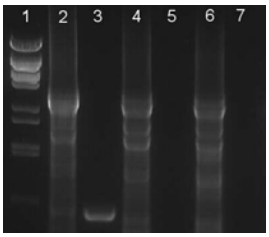
(a) An electrophoresis gel presenting banding patterns of PCR confirming integration of *GOX2LOX* into VIN13-Δ1. Each PCR fragment consists of either genomic DNA downstream or upstream of the *IME1* gene and part of the *GOX2LOX* cassette. Lanes 1–2 indicates a 2323-bp upstream fragment, lane 3 is the genetic marker λ *Bst*EII and lanes 4–5 a 1929-bp downstream fragment.



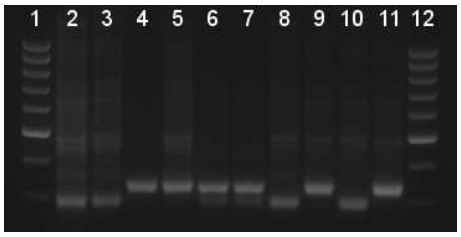
(b) A DIG Southern blot autoradiogram confirming the integration of *GOX2LOX* as a single copy into VIN13-Δ1 (lane 2) and double copy into VIN13-Δ2 (lane 3). Lane 1 contains the negative control (VIN13) and lane 4 the genetic marker λ *Bst*EII. Signals that confirm integration into the genome of VIN13 are presented in the form of banding patterns (8445 bp, 3787 bp and 3141 bp).

Figure 3.1: Confirmation of *GOX2LOX* integration into industrial wine yeast strain VIN13 by PCR and DIG Southern blot.

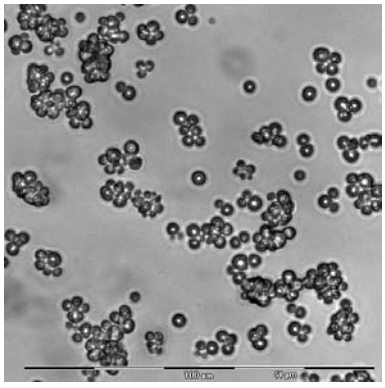
(a) Glucose oxidase secreted by recovered haploid VIN13-Δ2 yeasts, first and second from the left.



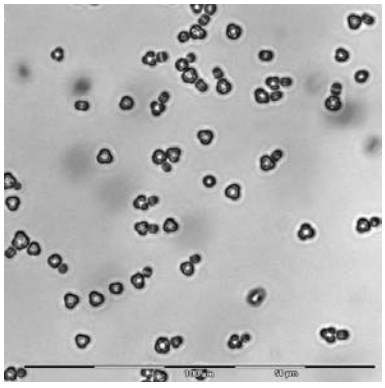
(b) PCR confirms VIN13-Δ2 strains (lanes 5 and 6) are *IME1*Δ0. GOX2 present in all strains (lanes 2, 4 and 6). VIN13-Δ1 in lane 3 still have one copy of *IME1*. Marker in lane 1 is λ *Bst*EII.



(c) Micro-satelite PCR to show the difference between haploid and diploid VIN13 strains. The GeneRuler™100 bp DNA Ladder (Fermentas Life Sciences, Glen Burnie) was used to identify correct band sizes (lane 1 and 12). Lanes 2–5 and 8–11 are haploid yeasts. Lanes 6 and 7 are diploid strains, because two bands are visible.



(d) New VIN13-Δ2 are *IME1*Δ0 and unable to sporulate.



(e) New re-diploidized single spore culture of GOX-none-producing VIN13-Δ1 (GOX2LOXΔ0) have recovered *IME1* genes, are able to sporulate, and form spores.

Figure 3.2: (a) GOX plate assay indicating recovered haploid strains of VIN13-Δ1 that secrete GOX. (b) PCR verifying the loss of the *IME1* and presence of *GOX2LOX*. (c) Double bands after PCR of micro-satelite DNA confirm diploid strains of VIN13-Δ2. (d) Non-sporulating VIN13-Δ2 *IME1*Δ0 and (e) Sporulating strains of re-diploidized single spore culture of VIN13-Δ1 (GOX2LOXΔ0) yeasts.

3.3.3 Construction and verification of diploid VIN13-Δ2 2n*IME1*Δ0

Industrial yeast VIN13-Δ1 was subjected to sporulation and yielded four-spore asci. These asci were dissected and each of the four haploid spores was placed on a YPD agar plate to stimulate growth. Recovered colonies were screened with the GOX plate assay to determine which colonies secreted active GOX [Figure 3.2 (a)]. The GOX-producing colonies were spotted onto rich medium (YPD) for re-diploidization (Huxley *et al.*, 1990; Ono *et al.*, 1990).

Genomic DNA was isolated and PCR was employed to confirm the presence of the *GOX2LOX* cassette and the absence (loss) of the *IME1* gene (Figure 3.2 (b)).

The recovered strains were further subjected to PCR to confirm their ploidy. *MATa*- and *MATα*-specific primers (Huxley *et al.*, 1990) were used to amplify *MATa* cells that produced a 544 bp PCR product, and *MATα* cells, a 404 bp product. Diploid cells contained both products [Figure 3.2 (c)].

This new diploid yeast strain, VIN13-Δ2 (*IME1*Δ0), secreted active GOX, and was unable to sporulate again [Figures 3.2 (d) and 3.2 (e)].

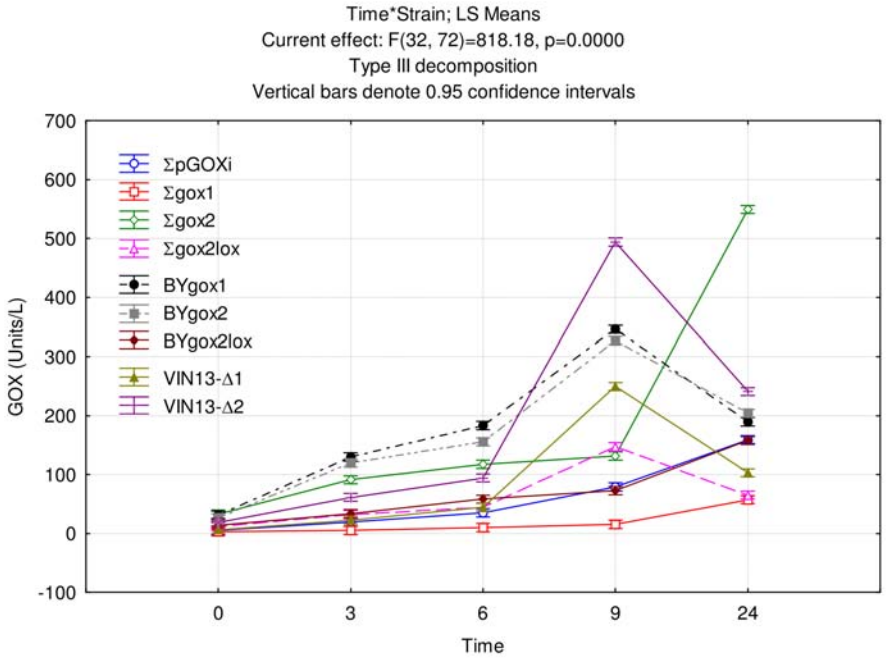
3.3.4 Laboratory fermentation evaluations

Three different yeast strains (two haploid laboratory strains, BY4742 and Σ1278b and one diploid wine strain, VIN13) transcribing either the *GOX1*, *GOX2* or *GOX2-LOX* gene cassettes were evaluated to elucidate the effect of different genetic backgrounds on the production and secretion of GOX.

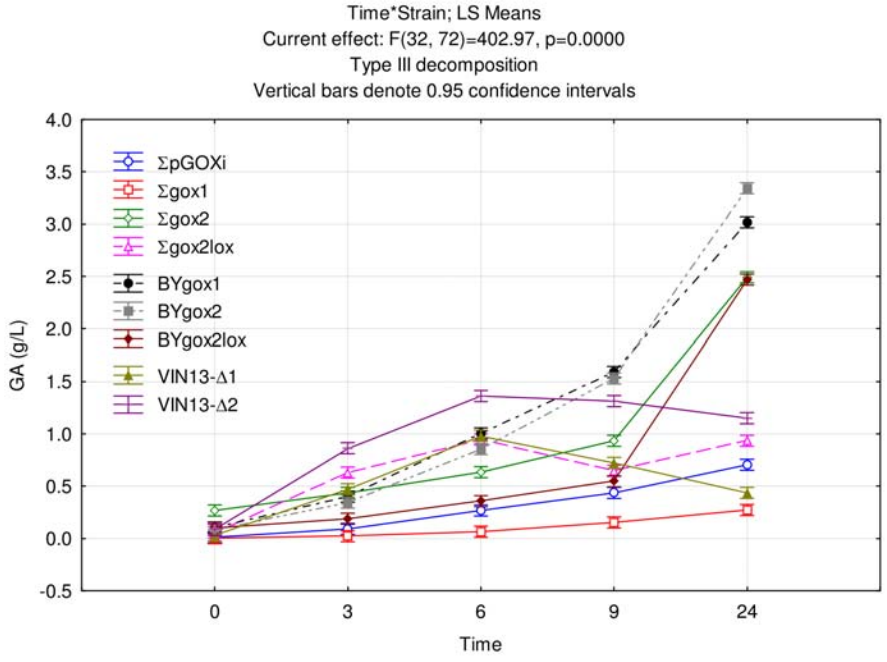
Time course production of both intra- and extracellular GOX were monitored spectrophotometrically alongside the acumulation of extracellular GA and growth of transformed strains BYpGOX1i, BYpGOX2i, BY-GOX2LOX, ΣpGOXi (Malherbe *et al.*, 2003), ΣpGOX1i, ΣpGOX2i, Σ-GOX2LOX, VIN13-Δ1 and VIN13-Δ2.

3.3.5 Statistical analysis of GOX and GA in synthetic media

The statistical significance of the effects of yeast strain on time course production of extracellular GOX and GA during laboratory fermentations was evaluated by two-way repeated measures analysis of variance (ANOVA). Strain–time interaction was significant ($P < 0.001$) and the results of the analysis of variation of both glucose oxidase (GOX) and gluconic acid (GA) are presented in Figures 3.3 (a) and (b).



(a) The statistical significance of the effects of yeast strain on the production of extracellular glucose oxidase (GOX).



(b) The statistical significance of the effects of yeast strain on the production of extracellular gluconic acid (GA).

Figure 3.3: The statistical significance of the effects of yeast strain on time course production of extracellular glucose oxidase and gluconic acid during laboratory fermentations evaluated by two-way repeated measures analysis of variance (ANOVA).

Table 3.6: Increase in glucose oxidase activity (Units/L) measured after 9 h.

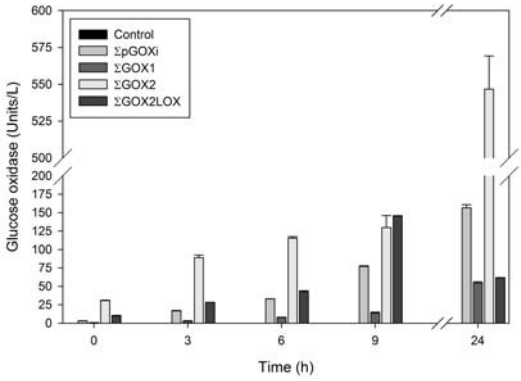
Yeast strain	Intracellular GOX	Extracellular GOX	Total GOX	% secretion
Σ1278b [a]	-	-	-	-
ΣpGOXi	14,815	74,019	88,834	83
ΣpGOX1i	1,447	13,128	14,575	90
ΣpGOX2i	3,762	99,194	102,956	96
Σ-GOX2LOX	5,963	135,646	141,609	96
BY4742	-	-	-	-
BYpGOX1i	14,309	316,541	330,850	96
BYpGOX2i	9,469	302,010	311,479	97
BY-GOX2LOX	808	60,152	60,960	99
VIN13	-	-	-	-
VIN13-Δ1	15,220	243,055	258,275	94
VIN13-Δ2 [b]	25,860	475,000	500,860	95

[a] ΣpGOXi was used as a reference for levels of secreted GOX (Malherbe *et al.*, 2003).
[b] VIN13-Δ2 : Contain two copies of *GOX2LOX*.

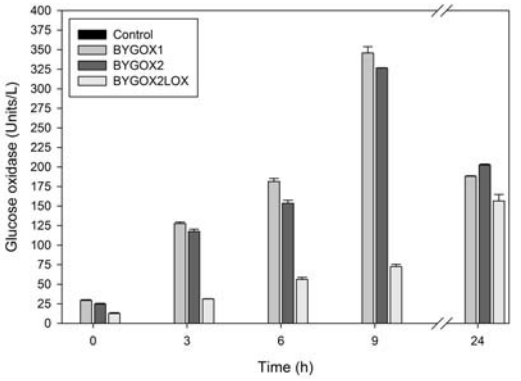
3.3.5.1 Production of glucose oxidase by *GOX1*, *GOX2* and *GOX2LOX* transformants

The ability of the newly-constructed strains to transcribe and secrete active glucose oxidase is shown in Table 3.6 and plotted in Figure 3.4. In all the transformed strains high amounts of GOX were produced and secreted into the culture medium during the first 9 h, which corresponds to the exponential growth phase (data not shown) of the yeast. In addition, although low levels of GOX were measured intracellularly the largest percentage of GOX activity was measured in the extracellular fraction, and consisted on average of 94% of all detected GOX. The low levels of GOX that were measured intracellularly could have been because the gene was transcribed constitutively, producing more enzyme that could be secreted at one time, and thus delayed secretion and an accumulation of GOX intracellularly.

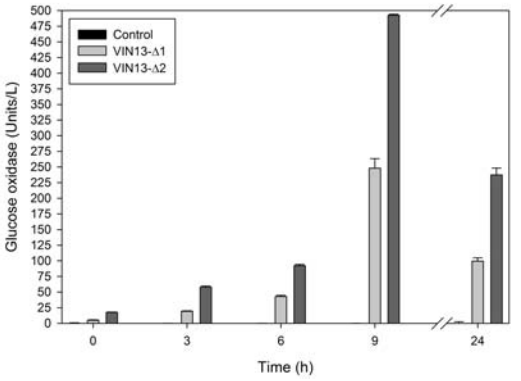
Of all the strains evaluated, VIN13-Δ2 containing two copies of *GOX2LOX* secreted the greatest quantity of active enzyme in 9 h, 475,000 Units/L. VIN13-Δ1, containing only one copy of *GOX2LOX* produced half the amount of GOX compared to VIN13-Δ2. The strains that secreted the second largest, but similar amounts, of GOX were BYpGOX1i (316,541 Units/L) and BYpGOX2i (302,010 Units/L). The Σ1278b strains, which were the slowest fermenters (data not shown) also produced and secreted the smallest quantity of GOX were ΣpGOXi (74,019



(a) Glucose oxidase secreted into the media by Σ1278b strains.



(b) Glucose oxidase secreted into the media by BY4742 strains.



(c) Glucose oxidase secreted into the media by VIN13 strains.

Figure 3.4: Extracellular levels of glucose oxidase (GOX) measured in Units/L after production and secretion by yeast strains (a) Σ1278b, (b) BY4742 and (c) VIN13. The statistical significance of the effects of yeast strain on time course production of GOX is presented in Figure 3.3 (a).

Units/L) and ΣpGOX1i (13,128 Units/L). ΣpGOX2i (99,194 Units/L) secreted more enzyme than ΣpGOXi and ΣpGOX1i.

Both Σ-GOX2LOX (135,646 Units/L) and BY-GOX2LOX (60,152 Units/L) produced average amounts of GOX compared with the rest of the strains that were evaluated. This could possibly be contributing to the fact that these strains were also transcribing the *kan*MX gene confirming resistance to G418 sulfate (Geneticin®). This could have caused an additional metabolic burden on the cells because, additional energy would have been necessary to produce aminoglycoside 3'-phosphotransferase (APH 3' II), and would have resulted in a lower production rate of GOX. This aspect will require further investigation.

The same levels of GOX activity were detected at 9 h and 24 h for ΣpGOXi and confirmed results obtained by (Malherbe *et al.*, 2003). This enabled the levels of activity of the new GOX producing strains to be rated. The general trend is also that all strains containing *GOX2* (GOX with its native secretion signal) retained less GOX intracellularly, and showed higher levels of extracellular GOX activity (Frederick *et al.*, 1990). In Figure 3.4 a decrease in GOX activity is visible for some of the strains. A possible explanation could have been that these strains were already reaching the end of the stationary phase and were dying/lysing, releasing compounds/chemicals (Kleppe, 1966) and/or proteases that can bind to GOX, render it inactive, or degrade the enzyme reducing overall activity.

3.3.5.2 Gluconic acid production by GOX producing strains of *S. cerevisiae*

The levels of D-gluconic acid (GA) were monitored in all the fermentations and all the recombinant strains contributed to the production of GA. The presence of GA showed that the secreted GOX was active and that D-glucose was converted to GA acid efficiently while the fermentation was running. Figure 3.5 presents the concentration of GA measured during the fermentations, and Table 3.7 summarizes the concentrations after 9 h and 24 h.

Despite the fact that there were large quantities of secreted GOX and that the fermentations were not strictly anaerobic, the enzymes were unable to convert large concentrations of D-glucose to GA in the media before the yeast cells were able to metabolize D-glucose through glycolysis. This indicated that the rate of conversion of glucose to gluconic acid is limited not only by the ratio of substrate:enzyme, but also dependent on the presence of molecular oxygen (O_2) (Bankar *et al.*, 2009).

The strains derived from BY4742 showed the highest GA concentration. These

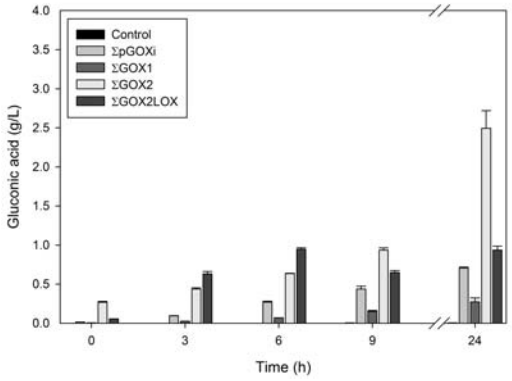
Table 3.7: Increase in D-gluconic acid (g/L) measured after 9 h and 24 h.

Yeast strain	9 h	24 h
Σ1278b [a]	-	-
ΣpGOXi	0.44	0.71
ΣpGOX1i	0.15	0.27
ΣpGOX2i	0.93	2.50
Σ-GOX2LOX	0.65	0.94
BY4742	-	-
BYpGOX1i	1.60	3.02
BYpGOX2i	1.53	3.34
BY-GOX2LOX	0.55	2.50
VIN13	-	-
VIN13-Δ1	0.72	0.44
VIN13-Δ2 [b]	1.32	1.15

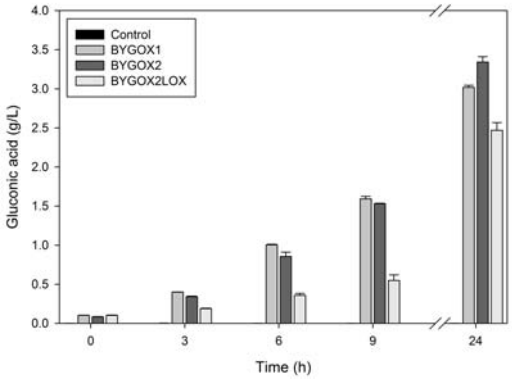
[a] ΣpGOXi was used as a reference for levels of GA produced (Malherbe *et al.*, 2003).
[b] VIN13-Δ2 : Contain two copies of *GOX2LOX*.

strains increase in biomass was relatively slow and as a result the uptake of D-glucose would also have been slower. This could have given the secreted enzyme more time to convert D-glucose to GA. Transformants containing the *GOX2* cassette (GOX with native secretion signal), showed higher levels of GA, possibly because more GOX was secreted by these strains. VIN13 strains converted less GA compared with *GOX2* strains. VIN13 strains were the fastest fermenters. They depleted the carbon source faster and completed the fermentation earlier than the other strains.

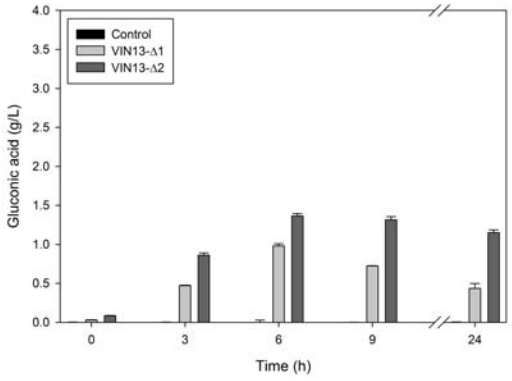
To explain the low levels of O_2, it is necessary to look at the fermentation itself. The conditions favored the yeast, and not necessarily the GOX enzyme. The fermentations were run at 30°C slightly higher than the enzyme's optimum of 25°C (Frederick *et al.*, 1990; Whittington *et al.*, 1990; Bankar *et al.*, 2009). It could be that the fermentations were progressing too fast, and did not allow enough time for the enzyme to perform proper conversions. Another explanation of the low amounts of O_2 in the fermentations, could be that the rate of CO_2 release was so high that it created a 'blanket' between the medium and the atmosphere and did not allow enough time for O_2 to undergo diffusion into the media. Furthermore, it is possible that the release (gas bubbles) of CO_2 also hindered the proper diffusion of O_2 into the media, and, where diffusion did take place, it was only in minute concentrations.



(a) Gluconic acid produced by Σ1278b strains.



(b) Gluconic acid produced by BY4742 strains.



(c) Gluconic acid produced by VIN13 strains.

Figure 3.5: Extracellular levels of gluconic acid (GA) measured in g/L after production and secretion by yeast strains (a) Σ1278b, (b) BY4742 and (c) VIN13. The statistical significance of the effects of yeast strain on time course production of GA is presented in Figure 3.3 (b).

GA concentrations were lower after 24 h in the fermentations completed by VIN13-Δ1 and VIN13-Δ2 (Table 3.7 and Figure 3.5). A possible explanation could be that these strains have already reached the end of the stationary phase and were dying and lysing, releasing compounds/chemicals that can bind to GA. Another possibility could be that VIN13-Δ1 and VIN13-Δ2 were using GA as a carbon source, as some strains of *Saccharomyces* are known to do (Van Dijken *et al.*, 2002; Peinado *et al.*, 2007). Diploid wine strain VIN13 is a cross between two haploid strains, N96 and WE228 (Van der Westhuizen and Pretorius, 1992), and although all three strains can be classified as *S. cerevisiae* it could be possible that these industrial wine strains have the ability to to metabolize GA. This needs to be confirmed.

3.4 Conclusions

Glucose oxidase from *A. niger*, is of considerable industrial importance and has previously been produced heterologously in host organisms such as *S. cerevisiae* (Frederick *et al.*, 1990; De Baetselier *et al.*, 1991; Hodgkins *et al.*, 1993; Kapat *et al.*, 2001). In this study, three new GOX-encoding gene cassettes (*GOX1*, *GOX2* and *GOX2LOX*) were constructed and expressed in two laboratory yeast strains (BY4742 and Σ1278b) and for the first time, a in widely-used industrial wine yeast (VIN13). In these constructs, secretion of GOX was facilitated by either the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) or the native *A. niger* secretion signal of GOX, thereby allowing comparative studies.

Integration of the *GOX2LOX* gene cassette into the genome of the diploid VIN13 strain was targeted at the master regulator locus of meiosis, *IME1*, by using the CreloxP-system for repetitive gene integration and genetic marker recovery (Güldener *et al.*, 1996, 2002; Hegemann *et al.*, 2006). By replacing *IME1* with the *GOX2LOX* gene cassette, it was hoped to generate an asporogenic, GOX-producing wine strain. Unfortunately, it was not possible to successfully recover the genetic marker and use the same *GOX2LOX* cassette for the second deletion/integration event. Various factors were tried that can effect the CreloxP-system, none of which yielded positive results. Therefore the specific reason for not recovering the genetic marker cannot be stated with certainty. After personal communication with the developer of the CreloxP-system (Güldener *et al.*, 1996, 2002), it can be concluded that the problem is probably strain related. The system does not always work in all yeast strains, and is more difficult to use in industrial yeast strains. It is suggested that research is

needed and further trials are called for.

However, by means of sporulation, micromanipulation and re-diploidization (Huxley *et al.*, 1990; Ono *et al.*, 1990), it was possible to generate a new yeast strain, VIN13-Δ2 (*IME1*Δ0), that secretes active GOX, is diploid and asporogenic. Thus, the possible transfer of genetic material to ambient yeasts from this newly constructed asporogenic yeast should be limited (Ramírez and Ambrona, 2008).

Comparative studies between newly-constructed GOX-producing strains showed that all strains produce biologically-active glucose oxidase as early as the beginning of the exponential growth phase and throughout fermentation to high levels. More enzyme was produced from BY4742 and VIN13 yeast strains containing *GOX2*/*GOX2LOX* containing the native *A. niger* secretion signal of GOX. D-gluconic acid was also measured during the fermentations, which indicates that conversion of β-D-glucose to D-gluconic acid took place.

Both industrial strains, VIN13-Δ1 and VIN13-Δ2 secreted GOX to high levels, approximately 3–6.4$\times$ higher as reported previously (Malherbe *et al.*, 2003). This is the first time that GOX was produced and secreted by industrial wine yeasts strains. Industrial VIN13-Δ1 and VIN13-Δ2 strains would be ideal strains to use in future winemaking trials to evaluate levels of reduced alcohol production. In conclusion, this study has resulted in the preparation of wine yeast starter culture strains that produce and secreted biologically-active GOX to high levels that can potentially provide an effective means of bio-adjusting the alcohol content to appropriate levels in commercial wines. This might offer a viable way to meet consumer demands for affordable low-alcohol wine, and have financial implications in savings on wine taxation.

One final general consideration can be made regarding the enormous contribution of gene technology to our basic understanding of the yeast. It will be unwise to entertain unrealistic expectations about rapid commercialization and short-term benefits for recombinant DNA technology in the wine industry (Pretorius and van der Westhuizen, 1991). This is clearly reflected the fact that to date there are only two recombinant yeast strains used on a commercial scale in the wine industry. They are ML01 commercialized by BioSpringer, a division of Lesaffre (Cebollero *et al.*, 2007) and 522^{EC-} (Coulon *et al.*, 2006). There are enormous benefits to both the wine consumer and the industry in the application of this exciting new technology and the first recombinant wine products should therefore unmistakably demonstrate organoleptic, hygienic and economic advantages for the wine producer

and consumer. Credible means must be found to effectively address the concerns of traditionalists within the wine industry and the negative overreaction of some consumer groups (Pretorius, 2000, 2001; Vivier and Pretorius, 2002; Deng *et al.*, 2008; Tamis *et al.*, 2009). Thus, the successful application, as well as the commercialization of transgenic wine yeasts, should not affect the wine's most enchanting and fascinating aspects, namely its diversity of style, wholesomeness and sensory qualities (Pretorius, 2003).

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Chapter 4

Evaluation of a Glucose-oxidase Producing Wine Yeast in Small-scale Winemaking Conditions Imploring Chemometrics

Abstract

Previous studies have shown that glucose oxidase can be used in winemaking in order to convert glucose to gluconic acid, thereby obtaining a reduced amount of ethanol at the end of alcoholic fermentation. In this work, the efficiency of glucose oxidase (GOX;β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* expressed and secreted from an genetically engineered industrial wine yeast (VIN13-Δ1) was monitored during small-scale vinifications. The *A. niger* structural glucose oxidase (*GOX*) gene including its native secretion signal was cloned into an integration vector under the regulation of a constitutive promoter. The enzyme secretion and activity were verified previously (Chapter 3, Section 3.3.5.1) in synthetic medium. Wines were made from white and red cultivars, to compare the enzyme activity under different winemaking conditions. Finished wines were analyzed by FT-MIR and GC-FID to determine their chemical composition and the levels of gluconic acid were determined by enzymatic assays. Multivariate data analysis (PCA and PLS1-discrim) was applied to the different sets of chemical data to discriminate between the wines made with the wild-type (VIN13) and VIN13-Δ1

strains. Results from this work indicated that glucose oxidase was produced and secreted by the strain VIN13-Δ1, but also that the enzyme was not highly active under the evaluated winemaking conditions. Consequently, no significant decrease in ethanol concentrations was observed in the wine made from VIN13-Δ1 when compared to that from VIN13. Chemometric projections of the score plots of both t1-t2 as well as t1-t3 plots for all results allowed insight into all significant variation up to three principal components (PCA) or PLS components, which showed very clearly that GA is a key factor in evaluating the effect of GOX in VIN13-Δ1 fermentation with regard to VIN13 fermentations. The VIN13-Δ1 effect manifestations were best shown on PLS1-discrim score plots that revealed that of the restricted variable subsets the MIR-compounds and GC-compounds yielded better results, with the GC-compounds displaying greater discriminability between cultivars and VIN13 / VIN13-Δ1. One can conclude from these results that the greatest influence of VIN13-Δ1 produced wines would be observed in the aroma components, but as there also was discriminability effects discernable in the MIR-compounds, the flavor component were also affected. The results suggest that additional oxygen, possibly in the form of micro-oxygenation, would be necessary to increase the enzyme activity in order to lead to a significant decrease in the total amount of ethanol.

4.1 Introduction

Over the last decade there has been a global rise in the temperature worldwide. More sunshine leads to an overall increase in the ripeness of grape berries especially in countries residing in the Southern hemisphere. As a consequence more fermentable sugars are present in grape juice before alcoholic fermentation. As more sugar enters glycolysis, more ethanol is produced and a higher than usual alcohol content in finished wines resides. Too much alcohol can affect wine quality negatively by interacting with certain color and aroma (phenolic) molecules. Such a wine would take longer to mature (Clarke and Bakker, 2004). Furthermore, following modern consumer trends, ever changing alcohol taxation policies in the beverage industry and stricter drinking-driving legislation, a demand has arisen for methods to produce reduced alcohol (1.2% to 5.5–6.5% v/v), low alcohol (0.5–1.2% v/v) and even de-alcoholized (not above 0.5% v/v) wines (Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998; Gladstones and Tomlinson, 1999; Gladstones, 2000; Pickering, 2000). These wines can be produced using methods such as: cen-

trifugation, reverse osmosis, thermal evaporation, membrane filtration and spinning cone column (Bui *et al.*, 1986; Pickering *et al.*, 1999*a*; Mermelstein, 2000). All of these methods are based on physical phenomena, are labour intensive and add to the overall production costs, making the finished product more expensive.

An alternative approach was introduced with the concept of treating grape must with glucose oxidase (GOX) to reduce the glucose content of the must, thereby producing a wine with a reduced alcohol content after fermentation (Villettaz, 1987; Pickering and Heatherbell, 1996; Pickering *et al.*, 1998). This approach provided satisfactory results and the alcohol content was reduced significantly in the wines treated by direct inoculation with GOX. The overall acidity of these wines increase because of the excess gluconic acid that remains after fermentation which affected the organoleptic properties of the wine. The acidity can be adjusted with additional steps, e.g. by chemical deacidification, until satisfactory organoleptic properties are obtained, but is again creating a multi-step process (Pickering *et al.*, 1999*a*,*b*,*c*; Biyela *et al.*, 2009).

Based on this approach, the GOX-encoding gene was overexpressed in *Saccharomyces cerevisiae* (Malherbe *et al.*, 2003) and the activity of the corresponding enzyme in grape must was confirmed. Consequently, reduced ethanol concentrations were obtained after completion of alcoholic fermentation. This genetically engineered strain produces and secretes active GOX. Since the enzymatic reaction is extracellular, it does not directly affect the intracellular redox balance, causing the yeast to produce unwanted metabolites. This work was performed under laboratory conditions using domesticated laboratory yeasts. It is worth investigating this strategy under winemaking conditions, in larger volumes, using industrial *Saccharomyces cerevisiae* wine yeasts that are also genetically engineered to produce and secrete active GOX (Chapter 3, Section 3.3.2).

The objective of this study was to evaluate the ability of a genetically engineered industrial wine strain to produce and secrete glucose oxidase, under winemaking conditions. Furthermore, enzyme efficiency was correlated to the final ethanol concentrations measured. The concentrations of flavor and aroma compounds was also measured in order to evaluate the impact of the enzyme on wine bouquet.

4.2 Methods and Materials

4.2.1 Microbial strain selection and origin of grapes

The yeast strains used to perform the experimental fermentations are summarized in Table 4.1. Wine fermentations were carried out using the following white varieties: Sauvignon blanc and Chardonnay, as well as the following red varieties: Merlot, Pinotage, Cabernet Sauvignon and Shiraz. All grapes came from Wolwedans Vineyards (Stellenbosch wine region, South Africa), with the exception of Merlot and Pinotage which came from Nelson Wine Estate (Paarl wine region, South Africa). See Table 4.2 for grape juice composition.

4.2.2 Vinification procedures, fermentation treatment and sampling

White grapes were collected in 220 L containers after crushing and destemming using a Bucher crusher/destemmer. The crushed berries and free-flow juice were treated with Pectazina H, a liquid pectolytic enzyme (3 mL/L as specified by the manufacturer; Dal Cin Gildo spa, Italy), and 30 ppm SO_2 were added. Maceration took place at 15°C overnight. The following day the crushed berries were pressed using a 128 L Tico 40 motorized hydrolic press with wooden cage (Enotecnica Pillan, Italy). The juices were collected in 220 L drums and left overnight at 4°C to clarify and the pomaces were thrown away. The clear grape juices were divided into 20 L stainless steel canisters. Each batch fermentation was conducted in 10 L. All experimental fermentations were inoculated to a final concentration of 1 x 10^6 cells/mL, and performed in triplicate by inoculating three separate samples of each white cultivar with either VIN13 (control) or VIN13-Δ1 (GM) industrial yeast

Table 4.1: Micro-organisms used during vinification of grape juice.

Yeast strains	Genotype	Source or reference
VIN13	MATa/MATα (Industrial wine yeast strain)	Anchor Yeast [a]
VIN13-Δ1	VIN13 IME1Δ1::GOX2LOX	see Chapter 3

[a] Anchor Yeast : Anchor Yeast (Cape Town, South Africa)

Table 4.2: Grape must composition.

Grape Cultivar	Sauvignon blanc	Chardonnay	Merlot	Pinotage	Cabernet Sauvignon	Shiraz
Sugar [a] (Brix)	21.6	20.6	22.9	22.5	25.5	25.3
YAN [b] (g/L)	334	340	188	381	262	242
pH [c]	3.51	3.46	3.89	3.69	3.72	3.76
TA [c] (g/L)	7.53	8.01	3.08	6.45	6.40	5.51

[a] Sugar concentration was determined using a Saccharometer (CDS, South Africa).
[b] The YAN (yeast assimable nitrogen) consisted of free ammonia and alpha amino nitrogen, and was determined with FT-MIR (FOSS).
[c] Total Acidity and pH was determined by titration using a 702 SM Titrino (Metrohm, South Africa) potentiometric endpoint titrator.

strains. Weight loss was measured every day by weighing individual canisters on a BM-150 platform scale (UWE, South Africa). On day 5 of the fermentation, 0.5 g/L fermentation supplement (Nutrivin, Anchor Yeast, South Africa) was added to ensure no stuck or sluggish fermentations. Wines were left to ferment until no more weight loss was observed (approximately 13–17 days). Fermentation vessels were not opened and no samples were taken during this time.

The red cultivars were crushed and destemmed (using a Bucher crusher/ destemmer). Grapes were pressed using a 128 L Tico 40 motorized hydrolic press with wooden cage (Enotecnica Pillan, Italy). Grape skins were then separated from the juice collected after pressing. Juice and skins were mixed separately until homology was achieved. Fermentations were conducted in a 25 L bucket and consisted of 8-10 L of juice and 10–12 kg of grape skins (cultivar dependant), and 30 ppm SO_2 were added. All experimental fermentations were inoculated to a final concentration of 1 x 10^6 cells/mL, and performed in triplicate by inoculating three separate samples of each red cultivar with either VIN13 (control) or VIN13-Δ1 (GM) industrial yeast strains. Sugar consumption (reduction) was measured every day using a Saccharometer (CDS, South Africa). The skins were punched down once a day. On day 3 of the fermention, 0.5 g/L fermentation supplement (Nutrivin, Anchor Yeast, South Africa) was added to ensure no stuck or sluggish fermentations.

Merlot and Pinotage fermentations were performed at 25–28°C. On day 4, the skins were taken off the Merlot and Pinotage must as the sugar concentration was less than 5 Brix. There was no pressing of the grape skins, and only free-run wine was collected. Fermentations proceeded in 4.5 L glass bottles (3 bottles for each original bucket fermentation). CO_2 loss was monitored until no loss was observed, and the fermentation was finished (10 days). Fermentations were not opened and no samples were taken during this time.

The Shiraz and Cabernet Sauvignon were left to ferment at 21°C on the skins for 5 days until the sugar content was below 5 Brix. No pressing of the grape skins took place, and only free-run wine was collected. Batch fermentations were reduced to 15 L and proceded in 20 L stainless steel fermentation vessels. CO_2 loss was monitored until no loss was observed, indicating the end of fermentation (5 days). Fermentations were not opened and no samples were taken during this time.

Samples (50 mL) were taken of all the wines after alcoholic fermentation, analyzed using FT-MIR (FOSS). The wines were cold-stabilized for a further 14 days after which they were racked, 50 ppm SO_2 were added, and filtered using K300 (3–4 μm) sheet filters (Columbit (Pty) Ltd, South Africa) and diatomaceous earth (also known as kieselguhr or celite) in a stainless steel filtration unit (custom made to the specifications of the experimental cellar). Wines were bottled in 750 mL green glass bottles (Console Glass, South Africa) and capped with an André Zalkin capper (MGC Industries, South Africa), using screw caps containing Teflon® seals (MGC Industries, South Africa). Wines were stored at 15°C for future chemical analysis.

4.2.3 Enzyme assays

4.2.3.1 Glucose oxidase plate assays

This assay was performed before and after all fermentations to confirm production and secretion of active glucose oxidase by VIN13Δ1 strains. Wine samples were spotted on YPD plates and incubated for 2–3 days at 30°C. The plates were then overlaid with 10 mL 0.1 M McIlvaine buffer, pH 7.0 [containing 1% (w/v) agar, 10 g/L glucose, 100 mg/L σ-dianisidine dihydrochloride (Sigma, South Africa) and 15 U horseradish peroxidase type II/mL (Sigma, South Africa)]. The overlay was

allowed to set and the plates were incubated at 37°C for 1 hour.
As glucose oxidase metabolises glucose to gluconic acid, H_2O_2 is formed as a byproduct. H_2O_2 is then used by horseradish peroxidase to oxidise σ-dianisidine dihydrochloride and a color change is visible on the agar plates. *S. cerevisiae* ΣpGOXi (Malherbe *et al.*, 2003) was used as positive control and untransformed VIN13 strain was used as negative control.

4.2.3.2 Determination of gluconic acid concentration

The D-gluconic acid/D-glucono-δ-lactone kit (K-GATE) of Megazyme International Limited (Ireland) was used to perform spectrophotometric assays confirming the production of gluconic acid in different experimental wines. D-gluconic acid/D-glucono-δ-lactone concentrations were determined in mg/L.

4.2.4 Chemical analysis of experimental wines

Chemical analyzes were performed on experimental wines using Fourier transform mid-infrared spectrophotometry (FT-MIR) analysis and Gas Chromatography (GC). Each instrument and method of sampling is described in the following paragraphs, and a list of compounds analyzed by each technique is provided in Tables 4.3 and 4.4.

4.2.4.1 Sample preparation and data acquisition using Fourier-transform mid-infrared (FT-MIR) analysis

A WineScan FT120 spectrometer (software version 2.2.1) equipped with a purpose build Michelson interferometer (FOSS Analytical, Denmark) was used for the generation of spectra in the wavenumber region 929–5011 cm^{-1}. The quality of mid-infrared spectra can be negatively influenced by high levels of carbon dioxide potentially present in wine, especially samples that are still in active fermentation stages. Must and wine samples were filtered using filter paper with a grading of 20–25 μm and a diameter of 185 mm (Schleicher & Schuell, catalogue number 10312714) and a filtration unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump. Two successive filtrations were performed to reduce the CO_2 content below 300 mg/L, as prescribed for FT-MIR (Malherbe, 2007).

Table 4.3: List of components analyzed by FT-MIR.

Grape must	Wine (post fermentation)
Alpha amino nitrogen	Ethanol
Ammonia	D-Fructose
Brix	D-Glucose
Ethanol	Glycerol
Glucose-Fructose	Lactic acid
pH	L-Malic acid
Tartaric acid	pH
Total acid	Tartaric acid
Tartaric acid	Total acid
	Volatile acidity

Various instrument settings have been pre-selected by the manufacturer and can not be changed by the user. A list of the compounds that were analyzed with the WineScan FT120 is summarized in Table 4.3.

Duplicate scans were obtained of each sample immediately after sample preparation. Although the whole spectral range (929–5011 cm^{-1}) is stored for each sample, only the wavenumbers 964–1532 cm^{-1}, 1716–2731 cm^{-1} and 3300–3500 cm^{-1} were used for further analysis and were selected to exclude spectral noise largely caused by the absorption of water (Nieuwoudt *et al.*, 2004).

4.2.4.2 Sample preparation and data acquisition of volatile flavor compounds by Gas Chromatography with Flame Ionization Detection (GC-FID)

Analysis of volatile higher alcohols, esters and carbonyl compounds was performed in triplicate with a Hewlett Packard 6890 Plus Gas Chromatograph (Little Falls, USA) equipped with a split or splitless injector and an FID detector. A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length x 0.32 mm internal diameter x 0.5 μm film thickness, was used for separation. The initial oven temperature was 33°C held for 17 minutes after which the temperature was increased to 240°C at 12°C/min and held for 5 minutes. The injection volume was 3 μL, at an injector temperature of 200°C. The split ratio was 15:1 and the split flow rate 49.5 mL/min. The column flow rate was 3.3 mL/min and the total run time was 50 minutes per sample. The detector temperature was 250°C. After each sample run, a post run of 5 minutes at oven temperature

Table 4.4: List of components analyzed by GC-FID.

Alcohols and higer alcohols	Acids and fatty acids	Acetate esters	Esters
2-phenylethanol	Acetic acid	2-phenylethyl acetate	Ethyl butyrate
Isoamyl alcohol	Butyric acid	Diethyl succinate	Ethyl decanoate
Isobutanol	Decanoic acid	Ethyl acetate	Ethyl hexanoate
Methanol	Hexanoic acid	Hexyl acetate	Ethyl octanoate
Butanol	Isobutyric acid	Isoamyl acetate	
Hexanol	Isovaleric acid		
Propanol	Octanoic acid		
	Propionic acid		
	Valeric acid		

240°C was performed with a gas flow of 6 mL/min to clean the column. After every 30 samples the column was heated and chemically treated by injecting hexane at oven temperature 220°C and holding it for 10 minutes to ensure propper cleaning (Smit, 2007).

All samples were centrifuged for 3 minutes at 4000 rpm to remove solid particles in the wine. The extracts for injection into the chromatographer were then prepared by extracting 5 mL of wine with 1 mL of diethyl ether (99.5%, Merck) after the addition of 10 mg/L internal standard (4-methyl-2-pentanol) (Fluka, $\geq$97%). This was followed by sonication for 5 minutes to facilitate mixing of the diethyl ether layer and the wine and centrifugation for 3 minutes at 4000 rpm to separate the diethyl ether layer from the wine. The diethyl ether layer was removed from the wine and dried on anhydrous sodium sulphate (Na_2SO_4) (Merck, 99%). The dried diethyl ether extract was transferred to a vial insert and capped. The carrier gas was hydrogen at a flow rate of 30 mL/min.
Concentrations of flavor compounds (listed in Table 4.4) were calculated by comparing their retention times and areas with those from calibration standard curves on HP-Chemstation software (Revision A.07.01 [682]) (Malherbe, 2007).

4.2.5 Statistical data analysis

Two-way analysis of variance (ANOVA) was performed to determine the influence of yeast strain and cultivar on the total amount of ethanol (% v/v) produced during alcoholic fermentations in experimental wines. Results are expressed as mean $\pm$ SEM. The level of significance was set at $P < 0.05$. *STATISTICA 9* (statistical software by Statsoft) was used to perform the analysis.

4.2.6 Chemometric data analysis

4.2.6.1 Data processing

Data obtained from FT-MIR, GC-FID and enzymatic assays were imported into the chemometric software Unscrambler (version 9.2, CAMO, Norway) for the purpose of multivariate data analysis. A master data matrix was constructed with rows representing wine samples (three independent replicates) [*objects*] of the six different cultivars and columns containing spectral data, and chemical compounds [*variables*]. All data were pretreated by so-called *auto-scaling* in order to avoid detrimental effects from inherent differences in measurement units. Auto-scaling is a widely used operation in multivariate data analysis; the result is a set of transformed variables with zero mean and a unit standard deviation, (Kowalski and Bender, 1972; Esbensen, 2002). The maset data matrix for this study comprised 60 objects, 1783 spectral variables (mid-infrared wavenumbers) and 36 non-spectral chemical variables (9 MIR chemical components, 26 GC chemical components and gluconic acid [GA]).

4.2.6.2 Multivariate data analysis

Following the objective of the thesis, focus will be on the specific discrimination features in the model documentations, especially the so-called "scores plot" and the "predicted vs. measured" validation plot.

The *scores plot* is a projection onto a particular sub-space, allowing optimal appreciation of the inter-sample relationships in two- or three projection dimensions only instead of the full variable FT-MIR-space (or GC-FID-space). In this plot, assessment of the discrimination between the samples takes place in precisely the two or three dimensions representing the largest variance differences between all samples, which is also maximally correlated to the [-1, +1] contrast in the Y-space (Esbensen, 2002).

In oder to optimize variable selection for the different wine fermentations (cultivars and teast strains), a PLS1-discrim analysis were performed. The "predicted vs. measured" validation plot summarizes the prediction performances of the particular PLS-discrim models. When a significant, centred, gap in the prediction Y-direction has been obtained by a properly validated model, this is also a reflection of a significant discrimination (the larger this gap, the more consistent and reliable

the discriminations observed).

In addition to PLS-discrim modelling, the patterns within the different sets of data were also investigated by principal-component analysis (PCA), while the correlations between different sets of data were determined by using partial least-squares (PLS) regression. *Loading plots* were not evaluated both due to the pre-processing of the FT-MIR spectra, but mainly because of the main intrest in the inter-object relationships.

4.2.6.3 Principal component analysis (PCA)

Principal component analysis (PCA) was used to extract information from multivariate data (Kettaneh *et al.*, 2005; Esbensen, 2002) and summarize the data (Wold *et al.*, 1987).

PCA is a multivariate technique that reduces the dimensionality of the original data matrix, containing numerous variables, to a more visually understandable low-dimensionality model, while retaining the maximum amount of variability. This allows for the main sources of variation in the data set to be detected directly even while being expressed by hundreds or thousands of variables. These models therefore allow for the identification of possible groupings of samples with similarities and relating them to specific variables or groups of variables, thus enabling us to interpret sample groupings, similarities or differences, as well as to evaluate the relationships between the different variables (Wold *et al.*, 1987, 2001; Esbensen, 2002).

Complex relationships between samples were investigated and expressed with the use of these models. The data structure was explored with this technique and possible outliers detected.

4.2.6.4 Discriminant analysis (PLS and PLS1-DISCRIM)

PLS regression is a bilinear modeling method for identifying the variations in a data matrix for explanatory or predictive purposes. By plotting the first PLS components one can view main associations between X variables and Y variables and also relationships within X data and within Y data (Wold *et al.*, 1987, 2001; Esbensen, 2002; Abdi, 2003). For predictive purposes, PLS1 models were constructed for individual Y variables to increase model-specificity and reliability. The data were analyzed by

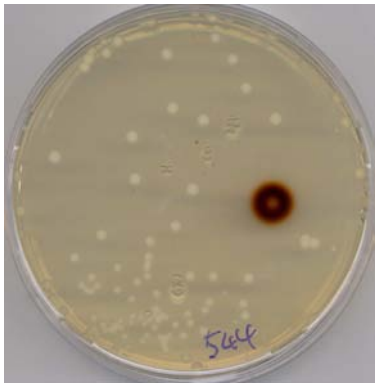
using test-set validation always with auto-scaled data.

PLS1-discriminant analysis was used to discriminate between VIN13 (commercial industrial non-genetically modified (non-GM) yeast) fermentations and VIN13-Δ1 (genetically modified (GM) yeast) fermentations. For that reason the PLS-Discrim models use a non metric, so-called *dummy* Y-variable. The dummy variable assigns a category "number" for a sample which belongs to a particular group. Specifically, a dummy variable was created representing VIN13 fermentations (signified by: -1) and VIN13-Δ1 fermentations (signified by: +1) to test the ability of the method to discriminate between VIN13 and VIN13-Δ1 fermentation samples. These dummy variables were then modelled with regards to the X-variables/matrix (FT-MIR spectra, FT-MIR compounds, GC compounds and Gluconic acid (GA) respectively).

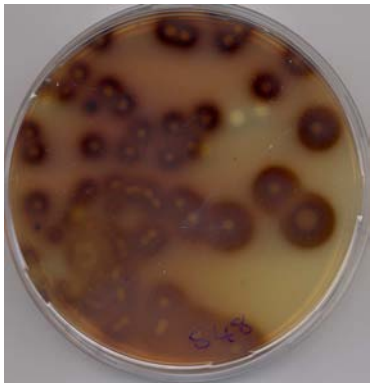
4.3 Results and Discussion

4.3.1 Expression and secretion of active GOX from stable industrial yeast integrations

An enzymatic assay was used to evaluate the genetically engineered VIN13-Δ1 strain to produce and secrete glucose oxidase, under winemaking conditions. After inoculation, a sample of each fermentation was plated onto YPD plates and the GOX plate assays were performed to confirm that all wines inoculated with VIN13-Δ1 contained GOX producing yeasts. The assay was again performed after alcoholic fermentation, in order to confirm that the enzyme is still produced and secreted by VIN13-Δ1, as well as to indicate that the inoculated yeast dominated the fermentation after vinification. VIN13-Δ1 yeast colonies, secreting active recombinant glucose oxidase, were surrounded by a brown halo. *S. cerevisiae* ΣpGOXi (Malherbe *et al.*, 2003) was used as positive control and untransformed yeast strains was used as negative control. Figures 4.1 (a) and 4.1 (b) show representative examples of the GOX plate assay carried out on the wine batches in which alcoholic fermentation was performed by VIN13 and VIN13-Δ1 strains respectively. None of the colonies had the ability to produce GOX in the VIN13 fermentations and 98% of the colonies were able to produce GOX in the VIN13-Δ1 fermentations. This confirmed that the expected strain performed alcoholic fermentation in all the batches and that the integrated *GOX* gene was stable.



(a) Yeast population from VIN13 wines. Non of the yeasts produce GOX, with the exception of the spotted positive control.



(b) Yeast population from VIN13-Δ1 wines. More than 98% of the colonies showed GOX activity.

Figure 4.1: Glucose oxidase plates assays indicating no GOX activity in (a) VIN13 wines and secreted active GOX at the end of alcoholic fermentation in (b) VIN13-Δ1 wines. The above plates from the Shiraz wines serve as a representative example for all the plate assays performed before and after alcoholic fermentation.

4.3.2 Chemical composition of experimental white wines

A total of three experimental white wines were made using two different cultivars, Sauvignon blanc and Chardonnay. Two batches of Chardonnay wine were made, of which one did not receive sulfur dioxide (SO_2) at crushing. The differences between these batches were evaluated for the potential influence of SO_2 on either GOX activity or the SO_2-binding power as a consequence of the production of gluconic acid (Barbe *et al.*, 2002).

4.3.2.1 Formation of gluconic acid (GA)

Table 4.5 indicates the levels of gluconic acid (GA) and glucono-δ-lactone after alcoholic fermentation in the experimental white wines. There was 80 mg/L more GA in the Sauvignon blanc wines made by VIN13-Δ1. This is only slightly higher as compared to the control wines, and not as high as expected. The fact that there was only a little increase in the levels of GA, indicates that either the enzymes activity was sub-optimal (affected by wine pH, and fermentation temperature), that perhaps there was not enough molecular oxygen present in the wine or the time of

oxygen exposure in the wine was too short to drive the conversion of glucose to GA by glucose oxidase. No significant differences were observed in the Chardonnay wines. However the GA content in wines made with VIN13-Δ1 was consistently higher. This would suggest that there was some activity and production of GA in the VIN13-Δ1 wines. In all the wines, most of the glucono-δ-lactone concentrations was much lower than that of GA. Glucono-δ-lactone and glucono-γ-lactone are in equilibrium with GA, representing, respectively, 5.8 and 4.1% of the acid level at pH 3.6–4.0 (Barbe *et al.*, 2002). The presence of low levels of GA in Sauvignon blanc wine controls and slightly higher levels observed in Chardonnay VIN13 wines can be explained by the presence of *Botrytis cinerea* (a necrotrophic fungus that produces GA from grape sugars) that was visible on some of the grapes before crushing. It is further possible that as a result of the SO_2-binding power less GA was detected in the Chardonnay wines that received SO_2 at crushing (Barbe *et al.*, 2002).

Table 4.5: Concentrations of gluconic acid and glucono-δ-lactone in white wines.

Chemical Compounds [a,b]	Sauvignon blanc		Chardonnay No SO_2 [c]		Chardonnay	
	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1
Gluconic acid	30	110	120	140	190	200
Glucono-δ-lactone	30	20	0	0	40	40

[a] The relative standard deviations (RSD) of all the samples were less than 5%

[b] Concentrations measured in mg/L.

[c] No SO_2 was added at crushing.

4.3.2.2 Analysis by FT-MIR (FOSS)

Table 4.6 shows the chemical compounds analyzed in experimental white wines after alcoholic fermentation. Analysis were done using FT-MIR (FOSS).

Table 4.6: Components analyzed by FT-MIR in white wines.

Chemical Compounds [a,b]	Sauvignon blanc		Chardonnay No SO_2 [c]		Chardonnay	
	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1
Ethanol	12.76	12.73	12.07	12.09	12.37	12.36
Fructose	1.21	0.96	0.82	0.91	1.36	2.13
Glucose	0.48	0.00	0.00	0.00	0.00	0.00
Glycerol	5.94	5.84	5.52	5.36	5.76	5.81
Lactic Acid	0.02	0.00	0.09	0.05	0.00	0.00
Malic Acid	3.08	3.03	3.25	3.27	3.33	3.38
pH	3.67	3.64	3.72	3.69	3.73	3.72
Total Acid (TA)	5.77	5.75	6.10	5.97	5.99	6.09
Volatile Acidity	0.28	0.26	0.29	0.29	0.34	0.34

[a] The relative standard deviation (RSD) of all the samples were less than 5%

[b] Measured units: Ethanol in % (v/v), pH in pH units, other compounds in g/L

[c] No SO_2 were added at crushing.

All the fermentations were completed and all wines were regarded as dry (sugar concentration below 5 g/L). No glucose remained in the finished wines, except for the Sauvignon blanc VIN13 wine which still had $\approx$ 0.5 g/L remaining. The average concentration of fructose remaining in the finished wines was $\approx$ 1 g/L. The highest level of fructose was detected in the Chardonnay VIN13-Δ1 wine (2.13 g/L). The only difference in ethanol was between the Sauvignon blanc VIN13 and VIN13-Δ1 wine. The VIN13 wine had 0.03% more ethanol. All the VIN13-Δ1 wines had less glycerol, except the Chardonnay VIN13-Δ1 wine, which had 0.05 g/L more.

Malolactic fermentations (MLF) were not conducted after the alcoholic fermentations. Almost no lactic acid was detected, with the exception of the Chardonnay wines that did not receive SO_2 at crushing. The low levels of lactic acid could possibly derived from lactic acid bacteria (LAB) present on the grapes before crushing ('acetic' smell during crush), as non were detected after alcoholic fermentation on GOX plates. There were no significant differences between the levels of malic acid measured between VIN13 and VIN13-Δ1 wines. The Chardonnay VIN13-Δ1 wine had the highest concentration of malic acid (3.38 g/L). The pH of all the white wines ranged between 3.64–3.73, with no significant differences. Volatile acidity measured the same between VIN13 and VIN13-Δ1 wines of the same cultivar. Chardonnay VIN13-Δ1 wines had the highest total acid (TA, 6.09 g/L). The total

acid was the lowest in Sauvignon blanc wines. 0.3 g/L more total acid was measured in Chardonnay wines.

4.3.2.3 Analysis by GC-FID

All the experimental white wines were analyzed by GC-FID to determine if any distinction could be made by comparing the chemical compounds that constitute the aromatic component of the wine.

The specific concentration ranges for the aromatic compounds were determined for South African wine cultivars in a study by Louw *et al.* (2009). These values were used as reference values to determine if detected concentrations for this study were above or below the average concentrations expected in South African cultivars (ACSAC). Furthermore the levels were also compared to odor thresholds (OTH) for each compound as reported in literature (Louw *et al.*, 2009).

Results obtained for the alcohols and higher alcohols are plotted in Figure 4.2. Although 2-phenylethanol, butanol and hexanol were detected in the wines, their respective concentrations were below the ACSAC and also below the OTH. They would not have an effect on the wine bouquet. Isoamyl alcohol concentrations were also below the ACSAC, but only by $\approx$ 35 mg/L in Sauvignon blanc wines (SB Ctrl and SB GM) and $\approx$ 20 mg/L in the Chardonnay wines (Ch Ctrl, Ch GM, Ch-S Ctrl and Ch-S GM). The average isoamyl alcohol concentration is however $\approx$ 100 mg/L above the OTH, and would contribute a banana aroma to the wines. The levels of isoamyl alcohol were lower in all the GM wines, and the banana aroma would be less. In Sauvignon blanc wines isobutanol concentrations were below the ACSAC and OTH. In Chardonnay wines the measured concentrations were $\approx$ 9 mg/L higher than the ACSAC, but still below the OTH, and would not have an influence on the aroma. The levels of methanol in Sauvignon blanc wines were equal to the ACSAC, $\approx$ 75 mg/L. In both Chardonnay wines methanol concentrations were below the ACSAC, $\approx$ 105 mg/L. Propanol concentrations were detected below the ACSAC, and well below the OTH. Of all the alcohols and higher alcohols, isoamyl alcohol and methanol will have the greatest influence on the aroma component.

The aroma profile, representing the acid and fatty acid component, is presented in Figure 4.3. Acetic acid, concentrations were detected below the ACSAC. In Sauvignon blanc (SB Ctrl and SB GM) wines and Chardonnay (Ch-S Ctrl and Ch-S GM) wines the measured concentrations were below the OTH, but in Chardonnay

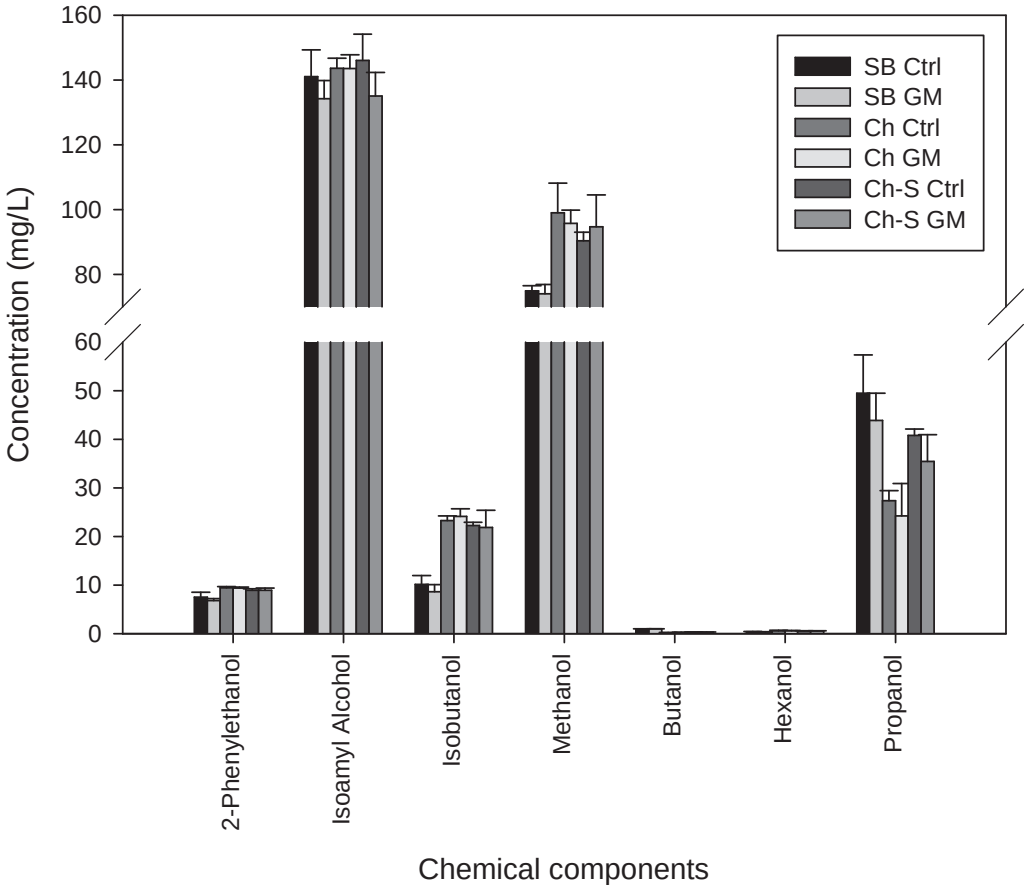


Figure 4.2: Alcohols and higher alcohol composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) white wines – Cultivars: Sauvignon blanc (SB), Chardonnay (Ch) and Chardonnay with no SO_2 added at crush (Ch-S).

(Ch Ctrl and Ch GM) wines the acetic acid concentrations were $\approx$ 50 mg/L above the OTH, and would contribute to a vinegar aroma. The acetic acid contribution is higher in the GM wine. Butyric acid and isovaleric acid concentrations were both below the ACSAC, but were higher then the OTH value, and would contribute to the aroma. Decanoic acid and octanoic acid concentrations were measured exceeding both ACSAC and OTH values. Decanoic acid levels were on average 4 $\times$ higher than the OTH (1 mg/L) and octanoic acid levels were on average 7 $\times$ higher than the OTH (0.5 mg/L). GM wines in general showed higher levels, with the exception of Ch-S Ctrl and Ch-S GM for octanoic acid levels. Hexanoic acid concentrations were similar as ACSAC, but were $\approx$ 10 $\times$ higher than the OTH (0.42 mg/L). This

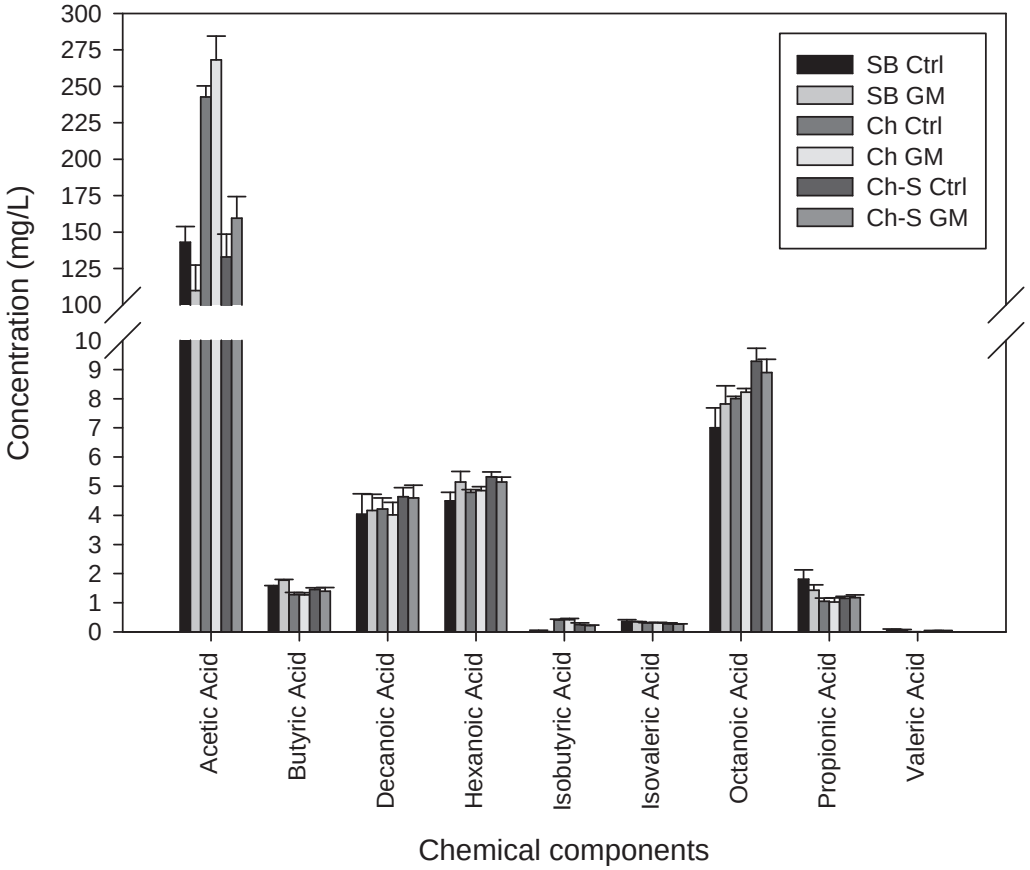


Figure 4.3: Acids and fatty acids composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) white wines – Cultivars: Sauvignon blanc (SB), Chardonnay (Ch) and Chardonnay with no SO_2 added at crush (Ch-S).

acid compound would have a large impact on the aroma. Isobutyric acid, propionic acid and valeric acid concentrations were all detected below the ACSAC and OTH values, and would have no effect on the aroma profile.

Figures 4.4 and 4.5 indicate the levels of esters present in the experimental wines. 2-phenylethyl acetate concentrations were higher for both ACSAC, and OTH in all the wines. There were no differences between the control and GM wines. Diethyl succinate was not detected. In Sauvignon blanc (SB Ctrl and SB GM) wines ethyl acetate measured higher than the average concentrations expected in South African cultivars (ACSAC), and also $\approx 8 \times$ higher than the OTH (12.26 mg/L). The concentration were also $\approx$ 10 mg/L higher compared to the control

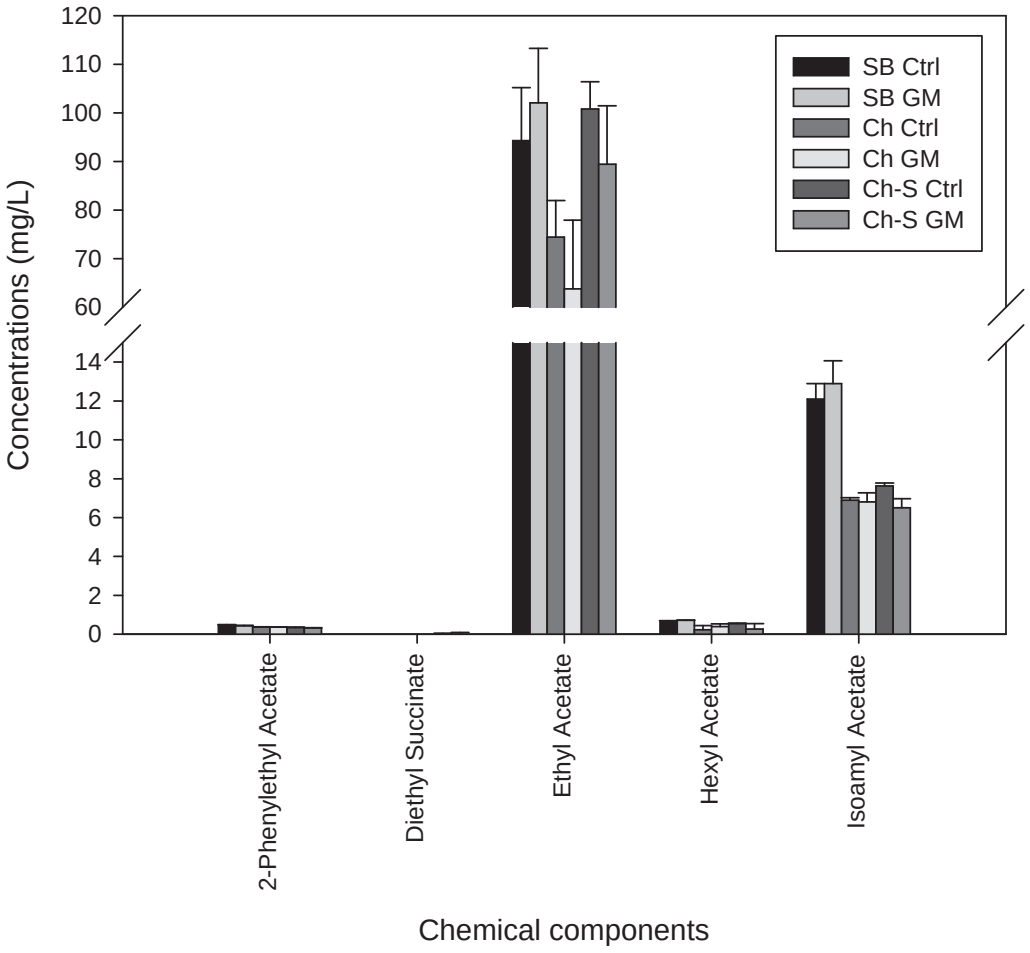


Figure 4.4: Acetate ester composition after alcoholic fermentation determined by GC-FID of of experimental control (Ctrl) and genetically modified (GM) white wines – Cultivars: Sauvignon blanc (SB), Chardonnay (Ch) and Chardonnay with no SO_2 added at crush (Ch-S).

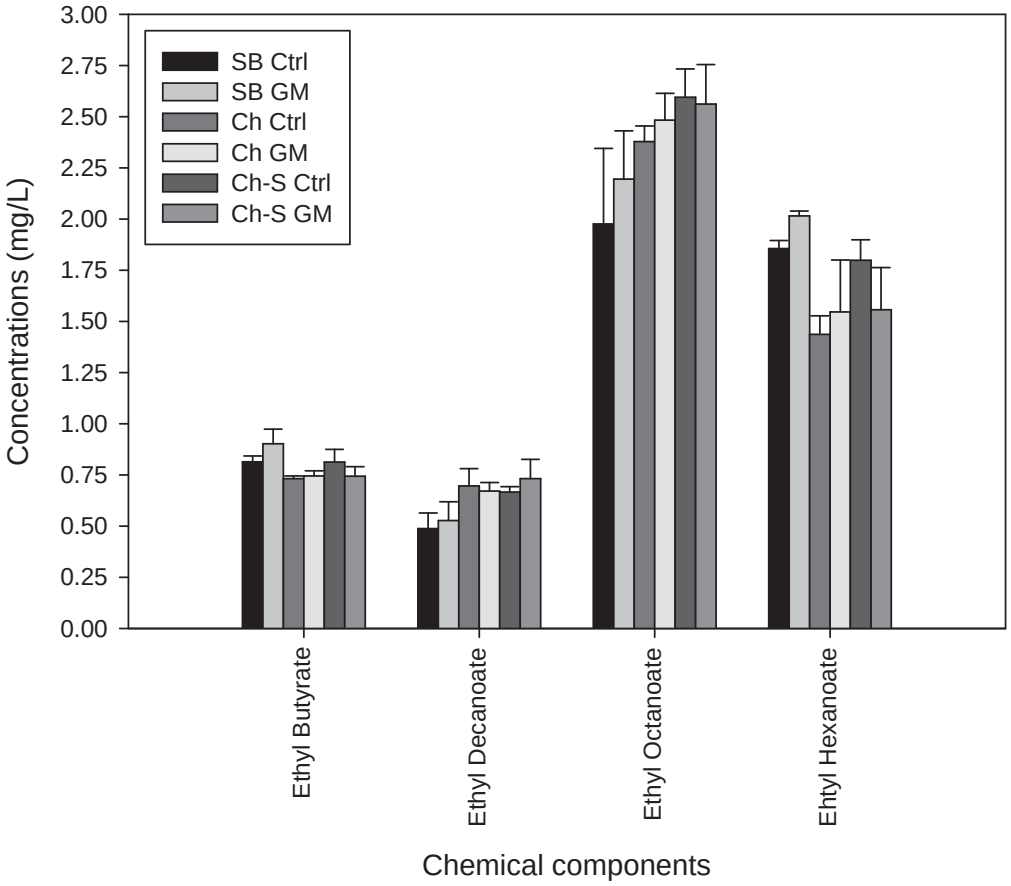


Figure 4.5: Ester composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) white wines – Cultivars: Sauvignon blanc (SB), Chardonnay (Ch) and Chardonnay with no SO_2 added at crush (Ch-S).

wine. In Chardonnay (Ch Ctrl and Ch GM) wines the concentration was lower than the ACSAC, but still higher than the OTH ($\approx 6 \times$). Ch GM had $\approx$ 10 mg/L less than Ch, the control wine. In Chardonnay (Ch Ctrl and Ch GM) the concentration was the same as the ACSAC, and also lower than the OTH. Hexyl acetate measured higher than the ACSAC, but lower than the OTH, and would not contribute to the wine aroma. Concentrations determined for isoamyl acetate were higher for both the ACSAC and the OTH values. In Sauvignon blanc wines the levels were $\approx$ 120 $\times$ higher and $\approx 7 \times$ higher in Chardonnay wines. Ethyl butyrate concentrations were higher in both Sauvignon blanc and Chardonnay wines, for ACSAC and OTH (0.02 mg/L) values. The last three compounds that were measured ethyl decanoate,

ethyl octanoate and ethyl hexanoate, measured higher concentrations for both ACSAC and OTH. The concentrations were also higher in all the VIN13-Δ1 wines, compared to the control wines.

Of all the aroma compounds measured the esters contributed the most to the aroma component.

4.3.3 Chemical composition of experimental red wines

A total of four experimental red wines were made using four different cultivars: Merlot, Pinotage, Cabernet Sauvignon and Shiraz. These wines were evaluated to determine their chemical composition and the effect of gluconic acid, if any, on the individual components contributing to their overall chemical composition.

4.3.3.1 Formation of gluconic acid

Table 4.7 indicates the levels of gluconic acid (GA) and glucono-δ-lactone after alcoholic fermentation in the experimental red wines.

All the wines made with VIN13-Δ1 showed higher levels of GA compared to the VIN13 wines. The largest increase were observed in Merlot (350 mg/L). In Pinotage and Shiraz wines an increase of ≈190 g/L and 140 g/L were observed respectively. The smallest increase was observed in Cabernet Sauvignon wines. This would indicate that there was some activity by the secreted glucose oxidase to convert glucose to GA in the VIN13-Δ1 wines. As in the white wines, only a small increase in the levels of GA was observed. However, the GA concentrations were slightly higher in the red wins as compared to the white wines. We know from work done in the previous chapter that the enzyme is secreted and active. The low activity indicated that the enzyme is affected by either the must (pH or temperature) or how the fermentation was carried out. Because the red wines were fermented on the skins in open buckets and the skins were punched down during the first 3 days of alcoholic fermentation, it would have increasing the must's exposure to molecular oxygen and resulting possibly in better enzyme activity.

In all the wines glucono-δ-lactone was converted to GA. This could be as a result of the slightly higher pH as was observed in the white wines as described by Barbe *et al.* (2002). The presence of low levels of GA wine controls can be explained by the presence of *B. cinerea* that was visible on some of the grapes

before crush. The fungus *B. cinerea* in known to produce gluconic acid (Barbe *et al.*, 2002).

4.3.3.2 Analysis by FT-MIR

Table 4.8 lists chemical compounds in experimental red wines as determined by FT-MIR (FOSS) subsequent to alcoholic fermentation.

All wine fermentations were completed by the yeast and wines were regarded as dry (sugar concentration below 5 g/L). No glucose remained in the finished wines, except in both the Shiraz VIN13 and VIN13-Δ1 wines. Remaining glucose measured $\approx$ 0.5 g/L. Fructose measured 0.62–1.02 g/L. No fructose was detected in Merlot wines. No difference was detected between the ethanol content of VIN13 and VIN13-Δ1 Pinotage wines. The Merlot fermented with VIN13-Δ1 had 0.04% more ethanol as compared to the VIN13 fermented wines. In both Cabernet Sauvignon and Shiraz the ethanol content was lower in the VIN13-Δ1 wines. 0.15% and 0.07% respectively. All wines had similar levels of glycerol, except the Merlot VIN13 wine, which had 0.19 g/L more. The higher level of glycerol could explain why there was less ethanol in the VIN13 compared to the VIN13-Δ1 wine.

Almost no lactic acid was detected in the Merlot. This was expected as non of the wines underwent MLF. The rest of the red wines had 0.1–0.2 g/L of lactic acid present, but there were no differences between VIN13 and VIN13-Δ1 wine levels. The low levels of lactic acid could come from the metabolic activity of low populations of lactic acid bacteria (LAB) coming from the grapes, as none were detected after alcoholic fermentation. There were no significant differences between the levels of malic acid measured between VIN13 and VIN13-Δ1 wines, however different levels of malic acid were observed between the different cultivars. Merlot had the least amount, $\approx$ 2 g/L, and the highest amount was detected in Pinotage wines, $\approx$ 3.63 g/L. The pH of all the white wines ranged between 3.87 and 3.94, with no significant differences between VIN13 and VIN13-Δ1 wines, with the exception of Cabernet Sauvignon wines which had levels of $\approx$ 4.05 g/L malic acid. Volatile acidity measured the same between VIN13 and VIN13-Δ1 wines of the same cultivar. Pinotage and Shiraz wines had the highest concentrations, $\approx$ 0.33 g/L and 0.25 g/L respectively. The total acidity showed significant differences between VIN13 and VIN13-Δ1 wines. The highest levels were detected in Cabernet Sauvignon wines, and measured 6.38 g/L. The lowest levels were detected in Merlot wines.

Table 4.7: Concentrations of gluconic acid and glucono-δ-lactone in red wines.

Chemical Compounds [a,b]	Merlot		Pinotage		Cabernet Sauvignon		Shiraz	
	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1
Gluconic acid	110	460	90	280	190	220	180	320
Glucono-δ-lactone	0	0	0	0	0	10	0	10

[a] The relative standard deviation (RSD) of all the samples were less than 5%

[b] Concentrations measured in mg/L.

Table 4.8: Components analyzed by FT-MIR in red wines.

Chemical Compounds [a,b]	Merlot		Pinotage		Cabernet Sauvignon		Shiraz	
	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1	VIN13	VIN13-Δ1
Ethanol	12.59	12.63	12.09	12.10	14.59	14.44	14.63	14.56
Fructose	0.06	0.04	0.62	0.62	1.02	1.00	0.90	0.86
Glucose	0.00	0.00	0.00	0.00	0.00	0.00	0.52	0.45
Glycerol	9.02	8.83	7.80	7.83	10.69	10.73	10.76	10.70
Lactic Acid	0.05	0.02	0.22	0.19	0.10	0.10	0.10	0.13
Malic Acid	2.08	1.99	3.61	3.65	2.83	2.82	2.12	2.07
pH	3.94	3.93	3.92	3.92	4.08	4.07	3.90	3.87
Total Acidity (TA)	5.13	5.18	5.59	5.59	6.38	6.38	5.95	5.97
Volatile Acidity	0.10	0.12	0.33	0.32	0.06	0.07	0.25	0.25

[a] The relative standard deviation (RSD) of all the samples were less than 5%

[b] Measured units: Ethanol in % (v/v), pH in pH units, other compounds in g/L

4.3.3.3 Analysis by GC-FID

All the experimental red wines were analyzed by GC-FID to determine the contribution of each chemical compound that contributes to the aromatic profile of the wine.

As for the analysis of major volatile compounds in white wines (Section 4.3.2.3) the specific concentration ranges determined for South African wine cultivars by Louw *et al.* (2009) were used as reference for the analysis of these compounds in red wines.

Results obtained for the alcohols and higher alcohols are plotted in Figure 4.6. The concentration of 2-phenylethanol in Pinotage was below the ACSAC, as well as below the OTH. In both Cabernet Sauvignon and Shiraz wines the concentration of this compound was below the ACSAC, but $\approx 3 \times$ higher than the OTH (14 mg/L). The Merlot control wine levels of 2-phenylethanol were higher than the ACSAC, but equal in the GM wine. Both the control and the GM wines had concentrations exceeding the OTH level $\approx 5\times$. Isoamyl alcohol concentrations below the ACSAC, $\approx$ 40 mg/L in Pinotage wines. In all the other wines the ACSAC were higher. In all the wines, isoamyl alcohol concentrations exceeded the OTH. The largest difference were observed in Cabernet sauvignon. Isoamyl alcohol was $\approx$ 80 mg/L above the OTH. The GM wine showed lower levels of isoamyl alcohol. Isobutanol concentrations were higher than the OTH in Merlot, Cabernet sauvignon and Shiraz wines, with the greatest increase, $\approx$ 25 mg/L, observed in Cabernet Sauvignon and Shiraz. The levels of methanol in all the wines measured below the ACSAC. Butanol and hexanol were detected in the wines, but their respective concentrations were below the ACSAC and also below the OTH. They would not have an effect on the wine's aroma. Propanol concentrations were detected below the ACSAC and OTH values for Pinotage and Merlot wines. In the Cabernet sauvignon and Shiraz wines propanol measured higher than the ACSAC. In Shiraz the concentrations exceeded the OTH by $\approx$ 10 mg/L, and would contribute to the wine aroma.

The aroma profile component, constituting acid and fatty acid, is presented in Figure 4.7. Acetic acid concentrations were detected below the ACSAC for all the wines. In Pinotage the concentrations were equal to the OTH. The rest of the wines measured below, and Merlot wines the lowest ($\approx$ 120 mg/L). In all the GM wines the concentration of acetic acid was higher. Butyric acid was detected in Pinotage

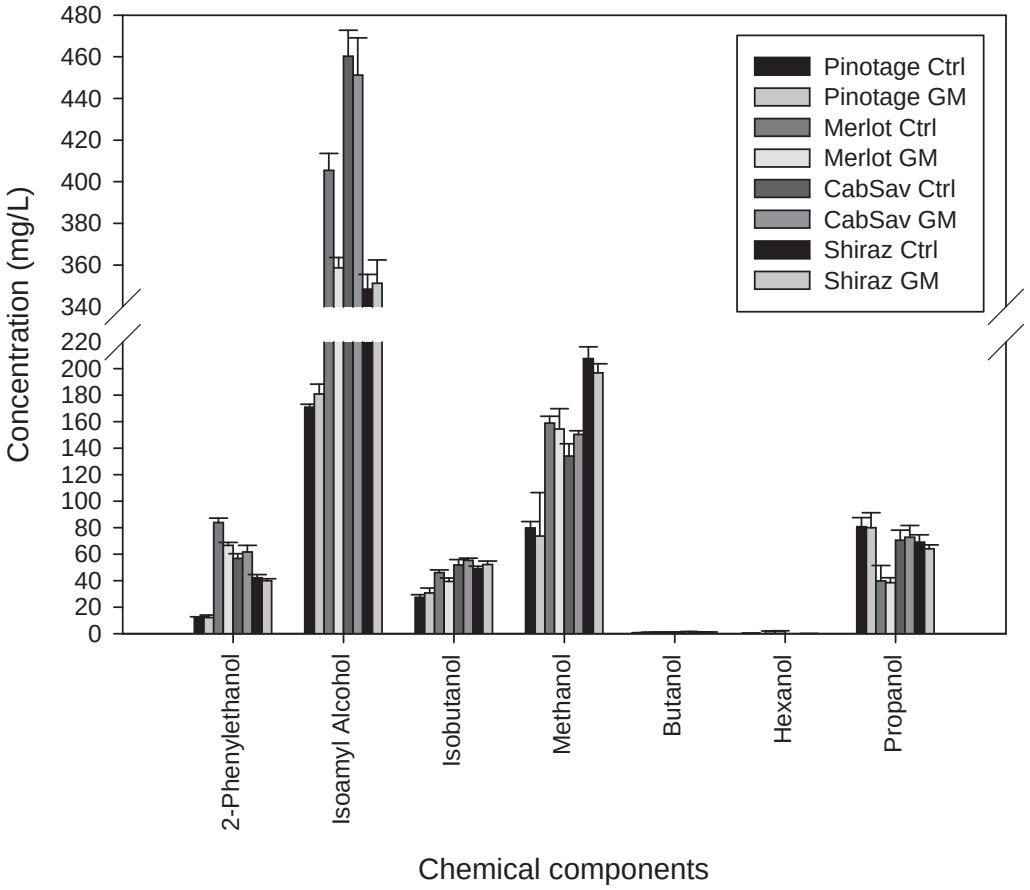


Figure 4.6: Alcohols and higher alcohol composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) red wines.

control wine, but were below the OTH. In Merlot the concentrations were higher for both the ACSAC and the OTH values. Butryic acid levels were higher than the OTH in the Cabernet Sauvignon control wine. No butyric acid was detected in Shiraz wines. Decanoic acid and Octanoic acid concentrations were measured exceeding both ACSAC and OTH values. Decanoic acid levels were on average double the OTH (1 mg/L), and octanoic acid levels were on average 4–7 $\times$ higher than the OTH (0.5 mg/L). The concentrations for these compounds were only slightly higher in the GM wines, but not significantly higher. Isovaleric acid concentrations measured below the ACSAC, but were higher then the OTH value in all the wines, and would contribute to the aroma. Isobutyric acid and propionic acid concentrations were

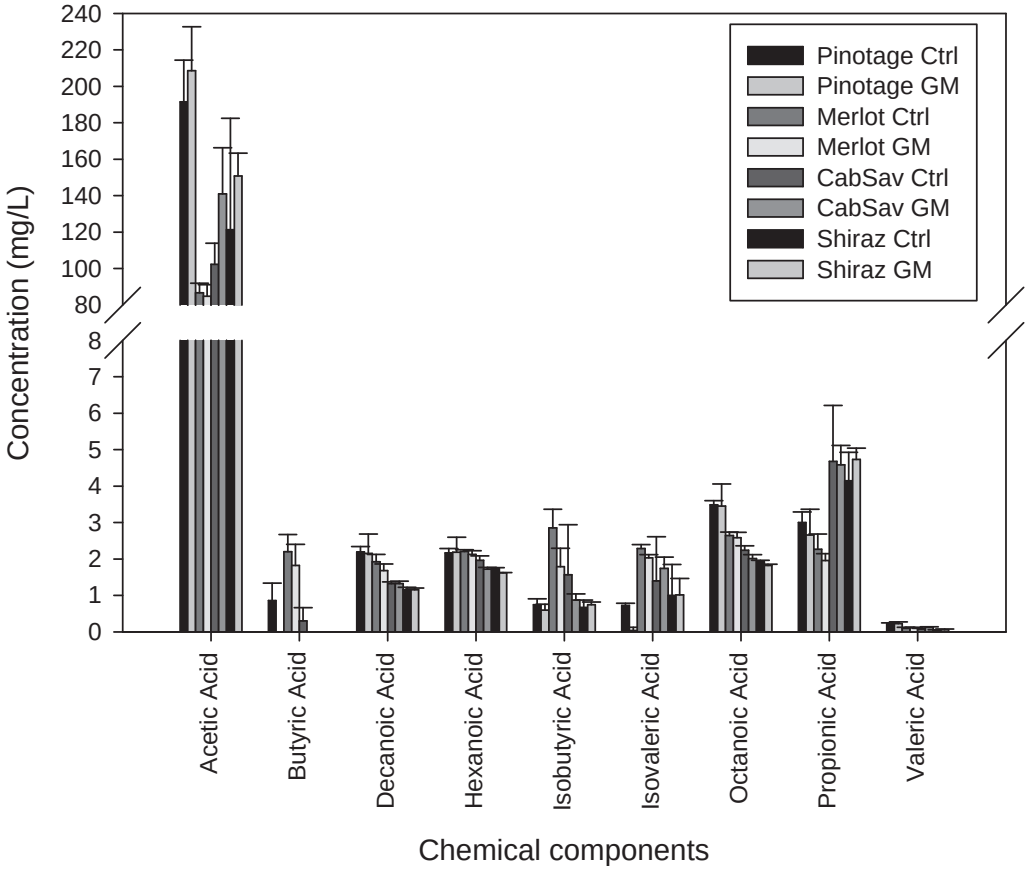


Figure 4.7: Acids and fatty acids composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) red wines.

all detected below the ACSAC and OTH values (with the exception of isobutyric acid in the Merlot control which were slightly higher), and would have no effect on the aroma profile. In Pinotage and Merlot wines, hexanoic acid concentrations were higher than the ACSAC but lower in Cabernet Sauvignon and Shiraz wines. In all the wines hexanoic acid will contribute to the aroma profile, and the greatest contribution would be observed in Pinotage and Merlot wines. Concentrations were slightly lower in GM wines. Valeric acid was detected, but will have a neutral effect.

Figures 4.8 and 4.9 indicate the levels of esters present in the experimental wines. 2-phenylethyl acetate concentratins were only detected in Pinotage control wines and Merlot control and GM wines. The compound have no influence on the aroma. Diethyl succinate was detected, but in extremely low concentrations,

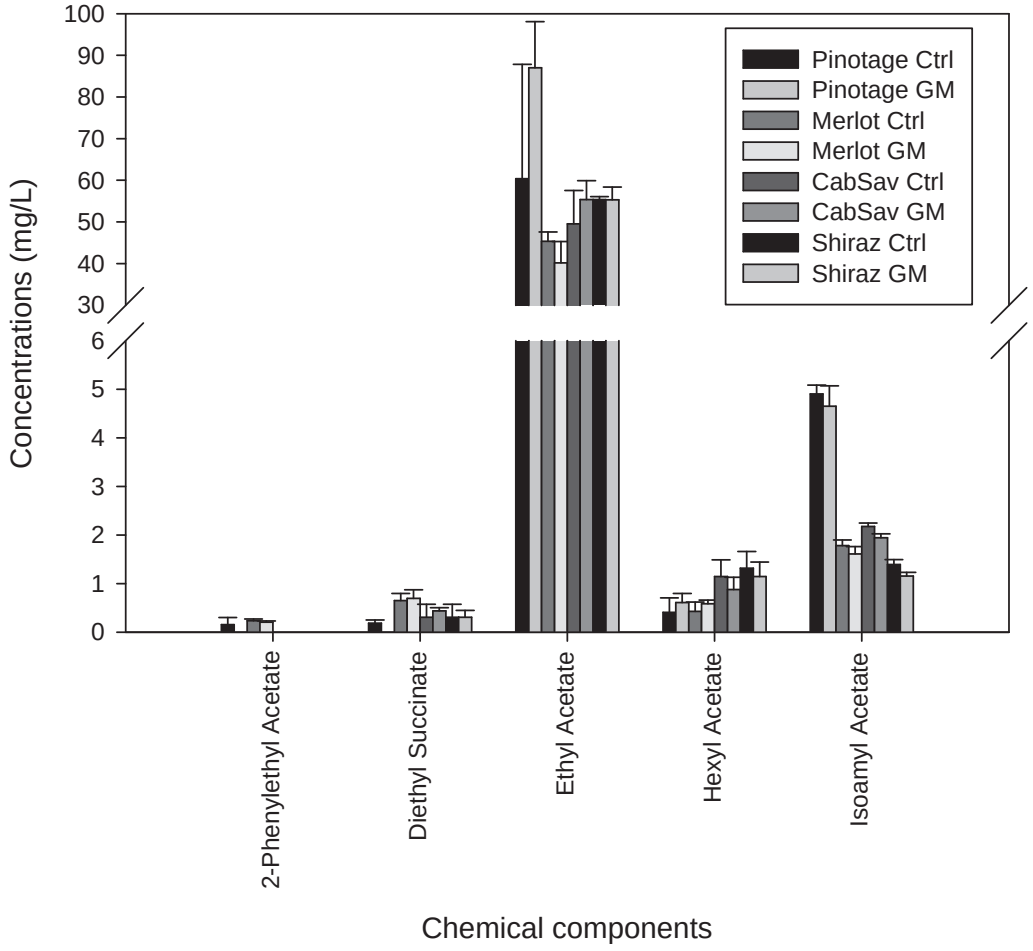


Figure 4.8: Acetate ester composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) red wines.

and as 2-phenylethyl acetate, does not contribute to the aroma. Ethyl acetate measured lower than the ACSAC in all the wines, higher than the OTH value. The concentration was the highest in Pinotage wines, and specifically the Pinotage GM wine. It was determined at $\approx 8 \times$ higher than the OTH (12.26 mg/L). Hexyl acetate will have no influence on the aroma profile of all the wines. It measured higher in Cabernet Sauvignon and Shiraz wines, but the GM wine concentrations were lower compared to the control wines. The exact opposite was observed in Pinotage and Merlot wines. Isoamyl acetate concentrations exceeded the ACSAC of 1.39 mg/L, with the exception of the Shiraz wines, which had concentrations of 1.39 mg/L and 1.16 mg/L respectively. Pinotage wines showed the highest concentrations. Despite

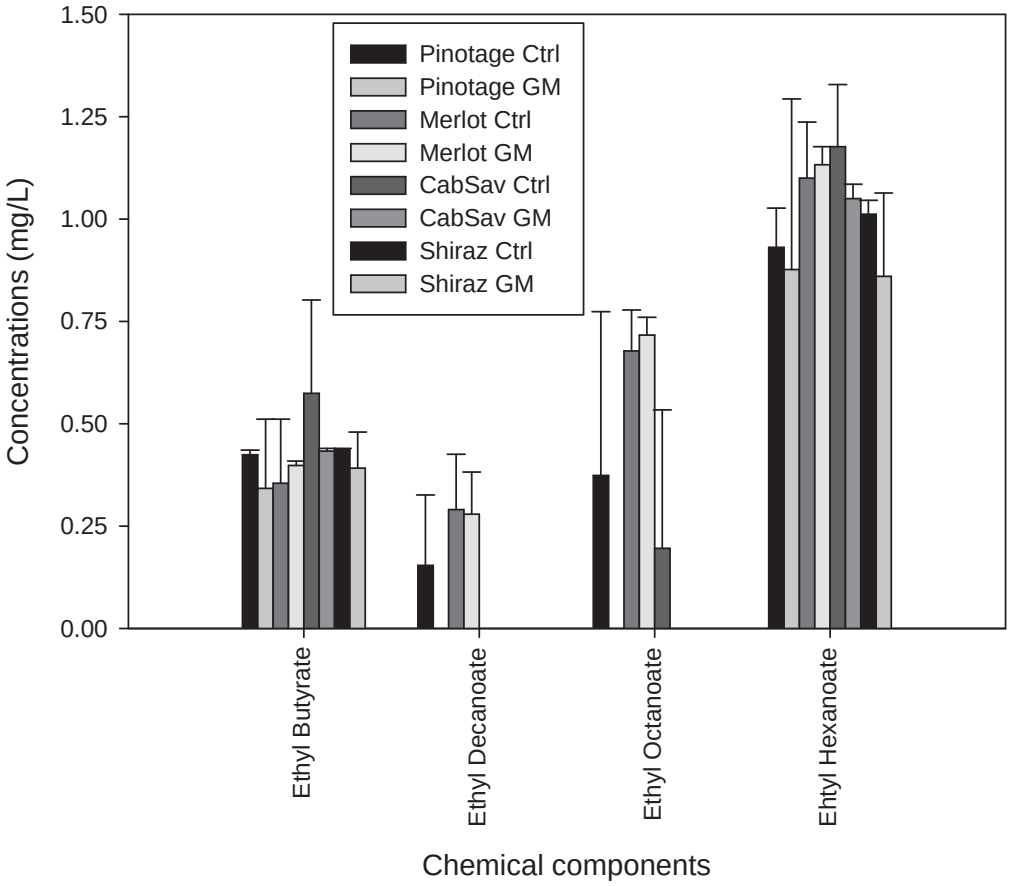


Figure 4.9: Ester composition after alcoholic fermentation determined by GC-FID of experimental control (Ctrl) and genetically modified (GM) red wines.

the fact that all the GM wines conained less isoamyl acetate, concentrations were higher than the OTH (0.03 mg/L).

Ethyl butyrate concentrations were higher in all the wines, for ACSAC and OTH (0.02 mg/L) values, and should contribute to the aroma profile. Merlot wines were the only wines that were affected by higher concentrations of ethyl decanoate. Merlot, Pinotage control and Cabernet Sauvignon control wines were the only wines to show concentrations of ethyl octanoate. The ethyl octanoate concentrations were higher than the OTH (0.005 mg/L) and would have an influence on the aroma profile. Ethyl hexanoate, measured higher concentrations for both ACSAC and OTH (0.014 mg/L). Unfortunately, with the large error bars, the individual components contribution to the aroma profile should only serve as an estimate. The concentra-

tions were also lower in all the GM wines, with the exception in Merlot.

Of all the aroma compounds measured the acid and fatty acid along with the alcohols and higher alcohols contributed the most to the aroma component.

4.3.4 Statistical analysis of ethanol in experimental wines

The statistical significance of the effects of yeast strain and cultivar on the total amount of ethanol (% v/v) produced during alcoholic fermentations was evaluated by two-way analysis of variance (ANOVA). The results of the analysis of variation is summarized in Table 4.9 and shown graphically in Figure 4.10. The results of the analysis of variation is summarized in Table 4.9 and shown graphically in Figure 4.10. Strain–cultivar interaction was significant (F = 3, P = 0.03) and from Figure 4.10 we can see a slight decrease from VIN13 (14.7) to VIN13-Δ1 (14.3) in wine made from the cultivar Cabernet Sauvignon. This confirms the reduction in alcohol measured by FT-MIR. None of the other cultivars displayed any reduction between strain–cultivar interactions.

Table 4.9: Two-way ANOVA indicating the significance of strain–cultivar interaction on ethanol % (v/v).

Effect	SS	Degrees of Freedom	MS	F	p
Strain	0.01	1	0.01	4	0.06
Cultivar	47.65	5	9.53	2494	0.00
Strain-Cultivar	0.52	5	0.01	3	0.03

4.3.5 Multivariate data analysis of experimental wines

Multivariate data analysis was employed as a tool to visualize the effects in experimental wines of VIN13-Δ1 fermentation with regard to VIN13 fermentations.

4.3.5.1 PCA and PLS1-DISCRIM

The data analysis presented below was organized to allow easy comprehension of the six major data analytical results. In addition, interaction between the main four experimental factors (F1–F4) that were evaluated in this study are summarized as follow:

`F1` [2 levels]: Control yeast (VIN13) / genetically modified yeast (VIN13-Δ1)

`F2` [6 levels]: Cultivars

`F3` [4 levels]: MIRspectra, MIRcompounds, GCcompounds, Gluconic acid (GA)

`F4` [2 levels]: PCA / PLS1-DISCRIM

`F1` encodes the main experimental feature; to which degree can the effects of genetically modified (GM) yeast fermentations be quantified as expressed by `F2`, and as

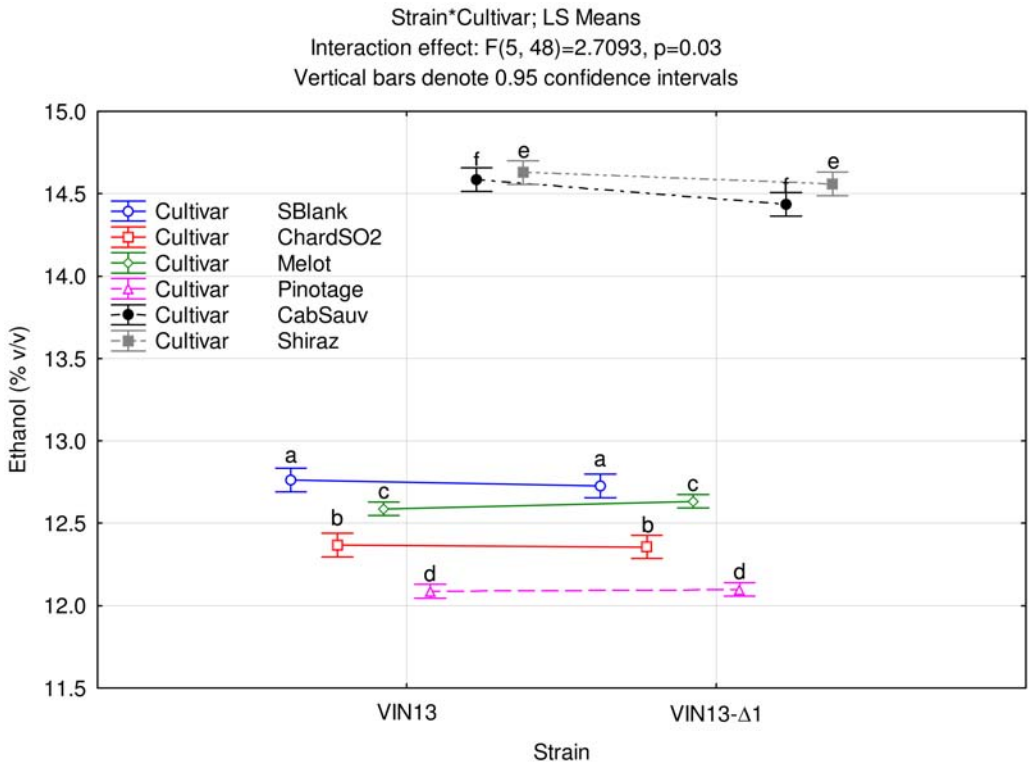


Figure 4.10: Two-way ANOVA analysis of variation indicating the influence of yeast strain and cultivar on ethanol % (v/v).

analyzed by F3. F4 signifies two data analytical alternatives; whether to use a supervised (PLS1-DISCRIM), or an unsupervised approach (PCA), to study whether GM-expression can be predicted by standard wine and must chemistry parameters alone (in this study enhanced by the presence of GA), or whether a data analysis approach designed actively to incorporate the *priori* knowledge of which data relate to the control fermentation as contrasted to the GM-effected counterpart (PLS1-DISCRIM) is helpful.

For PLS1-DISCRIM the DUMMY-variable is used as the Y-space, which takes on only the two-fold values: -1 (control/VIN13) and +1 (GM/VIN13-Δ1). PLS1-DISCRIM models are used here to evaluate the cultivar/variable relationships exclusively in the X-space (i.e. in scores plots only). This mode is termed "passive" PLS1-DISCRIM. The -1 designation, as well as the +1 designation, representing control and GM-affected fermentations respectively is known; we were not in any sense interested in the possible "prediction" of the value of the DUMMY-variable. For the same reason validation issues are neither of interest here.

B. cinerea (a necrotrophic fungus that affects wine grapes and produces GA) was present on some of the grapes that were crushed for the wine trials. Concluding section 4.3.5.1 (data analysis) a standard PLS1 was carried out for modelling of the GA concentration based on the MIR-spectra. This was performed in order to determine to which degree the GA concentration can be modelled from MIR-spectra alone; this will indicate which fermentations and/or cultivar replicates are significantly affected by *B. cinerea* in addition to the levels of gluconic acid that was generated in the fermentation process.

Following this logic, seven ($\times 2$) individual data analysis sessions are presented below. They have been organized in a logical order as presented graphically in Figure 4.11.

4.3.5.2 Data analytical objective

Multivariate data analysis was employed as a tool to visualize the effects of VIN13-Δ1 fermentation with regard to VIN13 fermentations, taking into account the available alternative chemical parameters; MIRspectra, MIRcompounds, GCcompounds and Gluconic acid (GA) alone, or in various combinations. F3 is illustrated in in-

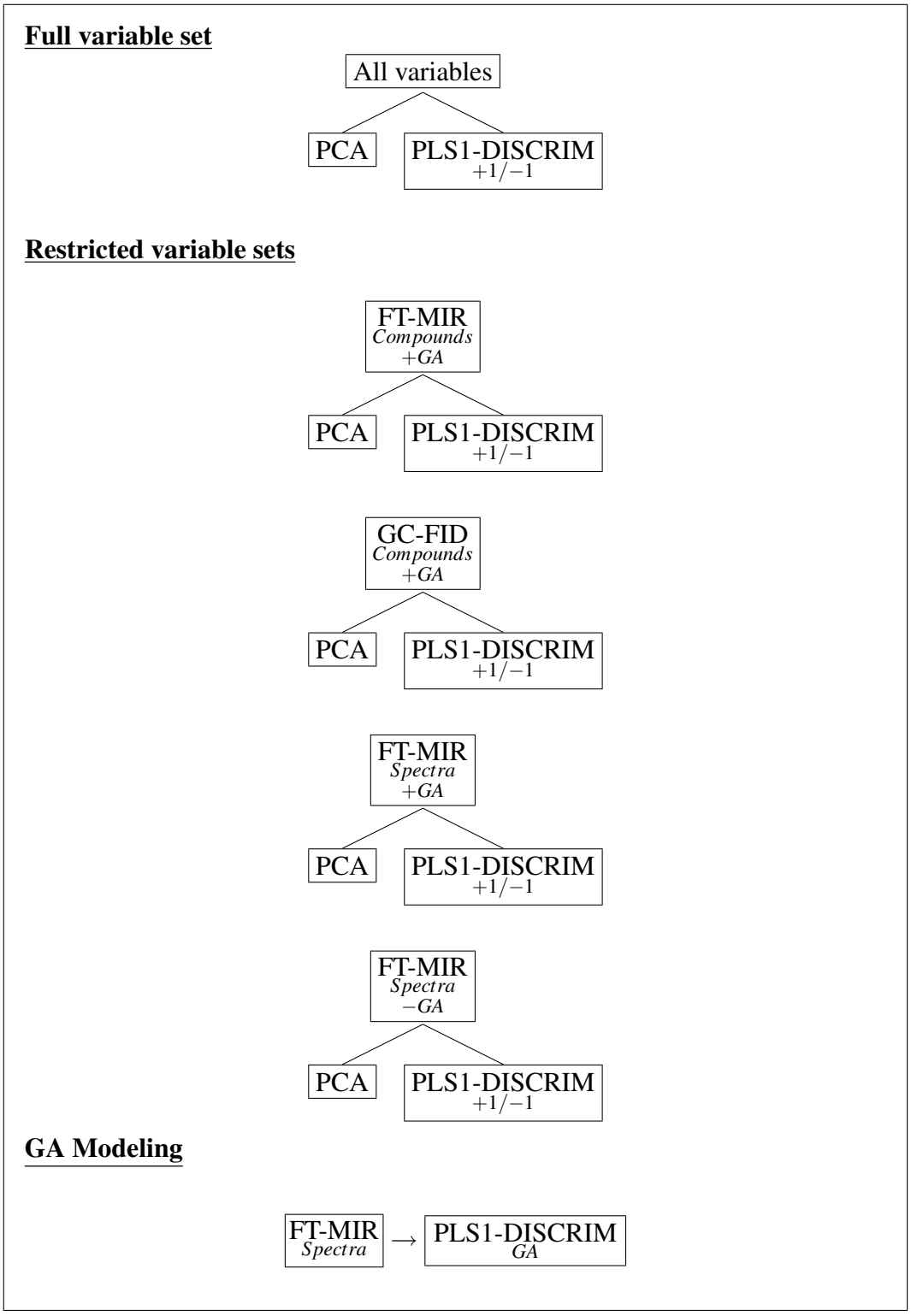


Figure 4.11: A graphic presentation of the logic order in which results of individual analytical data sets were organized.

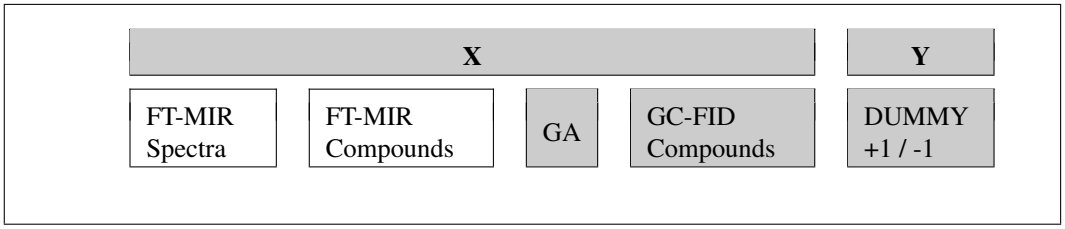


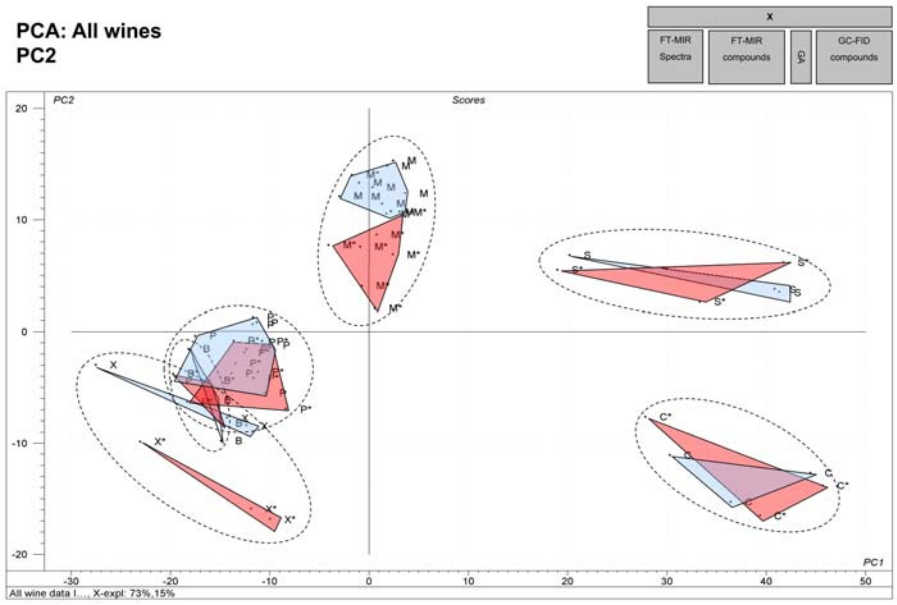
Figure 4.12: Example of the icon that accompanies each data analytical figure. This example presents an PLS1-DISCRIM of GC-compounds + Gluconic acid (GA) including the DUMMY (-1 / +1).

dividual plots after data analyses in Figures 4.14–4.25 and are accompanied by a small icon (see Figure 4.12 for example) at the top and righthand side of each plot for easy reference. Use is primarily made of chemometric projections as score plots. For consistency, use is made of both t1-t2 as well as t1-t3 plots for all results, allowing insight into all the significant variation up to three principal components (PC) or PLS components as the case may be. Each component is responsible for a certain % fraction of the total data variance in the X-space; this is indicated on the plots as well.

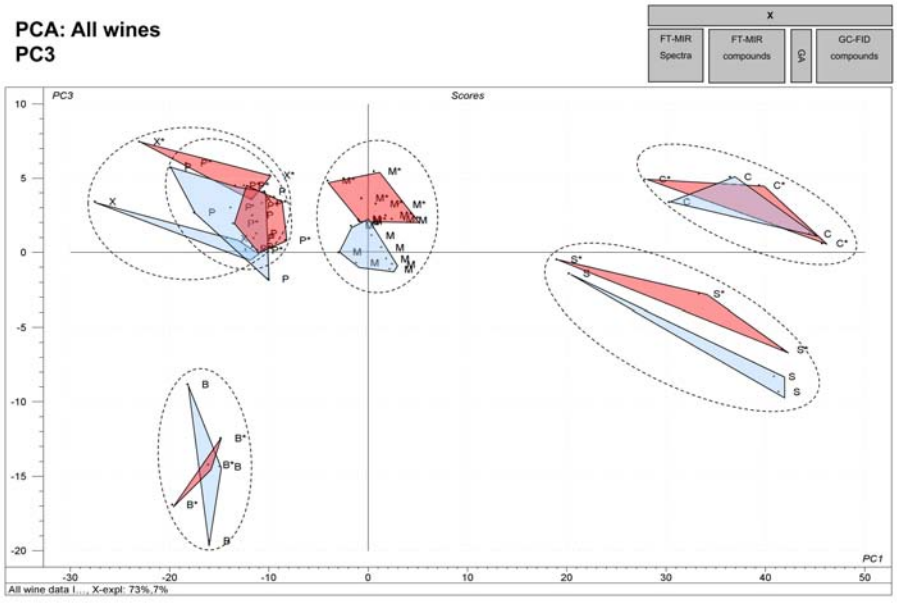
Selected data analyses (modelling gluconic acid (GA), Figure 4.24) also present loading plots, allowing insight into which variables most impact the VIN13-Δ1-discriminations.

These compound results will be further analyzed below in a series of partial data analysis each focusing on a specific chemical parameter set alone [F3]. To better comprehend the cultivars/variable relations based on these individual chemical parameters, the VIN13-Δ1-effect will be visualized alone for maximal understanding as can be seen in Figures 4.13 and 4.14 or 4.15 (different representation of Figure 4.14). In particular, the observed effect of the added information carried by the GA (as per inclusion/exclusion of this special chemical variable) would be better explained in this manner.

Annotations on score plots represent fermentation triplicates, and are shown by "triangular" shapes (one for control/VIN13 samples (blue) and VIN13-Δ1-samples (red) respectively). The VIN13-Δ1 effect manifestations are also shown by dotted eclipses from VIN13 to VIN13-Δ1.

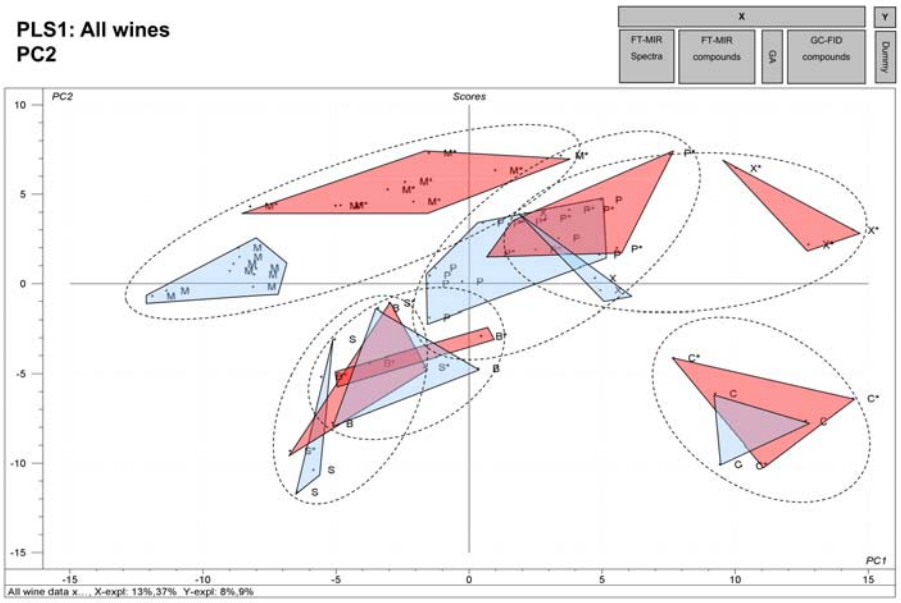


(a) PCA (full data set), PC1–PC2

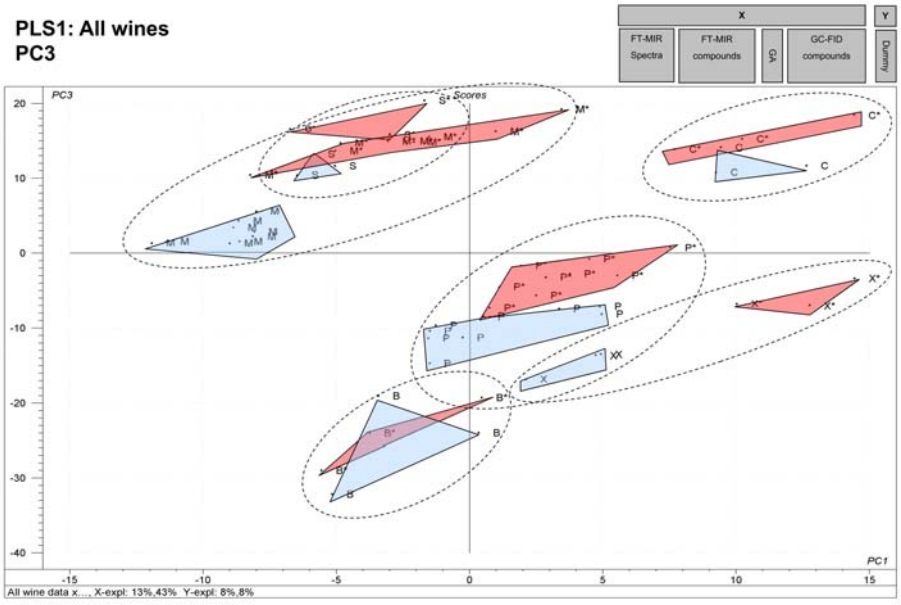


(b) PCA (full data set), PC1–PC3

Figure 4.13: PCA, (full data set). The score plots reveal the full impact of the total chemical parameter set on the discriminability between control and VIN13-Δ1-yeast fermentations. There are clear differential cultivar expressions for some varieties only (Merlot [M], Shiraz [S] and Chardonnay [X]) to no discernable effects (Cabernet Sauvignon [C], Pinotage [P] and Sauvignon blanc [B]).

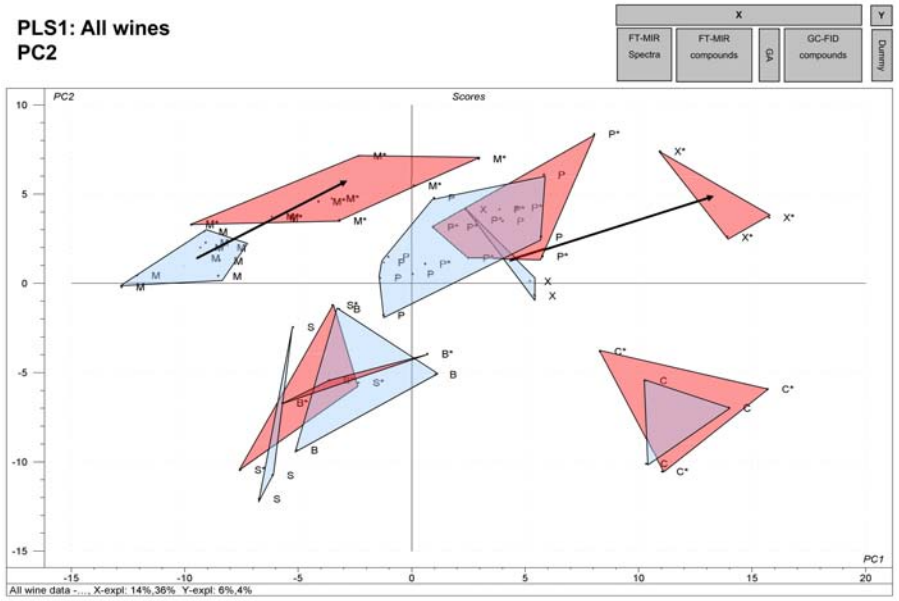


(a) PLS1-DISCRIM (full data set), PC1–PC2

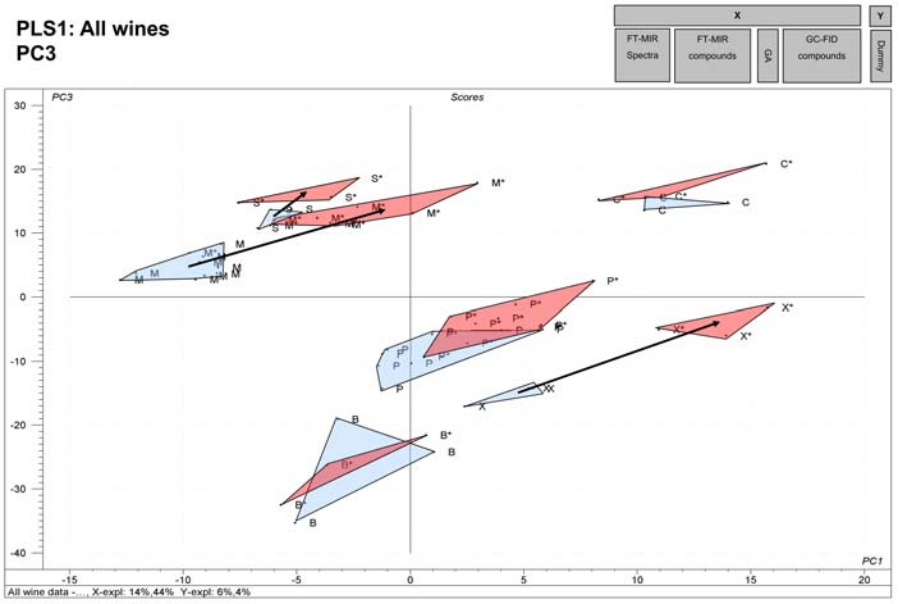


(b) PLS1-DISCRIM (full data set), PC1–PC3

Figure 4.14: PLS1-DISCRIM, full data set. Figure 4.14 and 4.15 uses two different annotation schemes to illustrate the following findings. There are now larger differential cultivar effects to be observed for all varieties when analysed by PLS1-DISCRIM. The largest differential cultivar effects were observed for Merlot and Chardonnay. Compare with Figure 4.13.

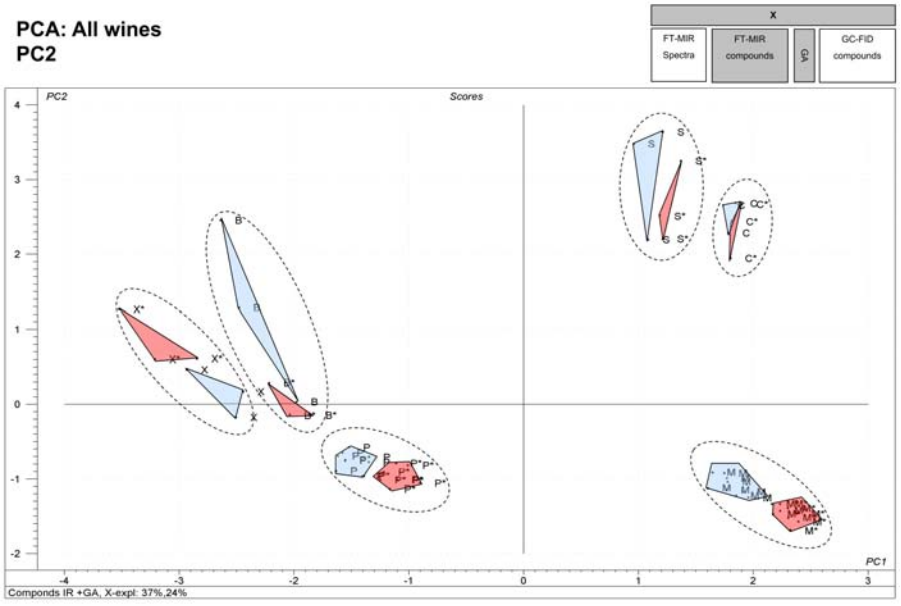


(a) PLS1-DISCRIM (full data set), PC1–PC2

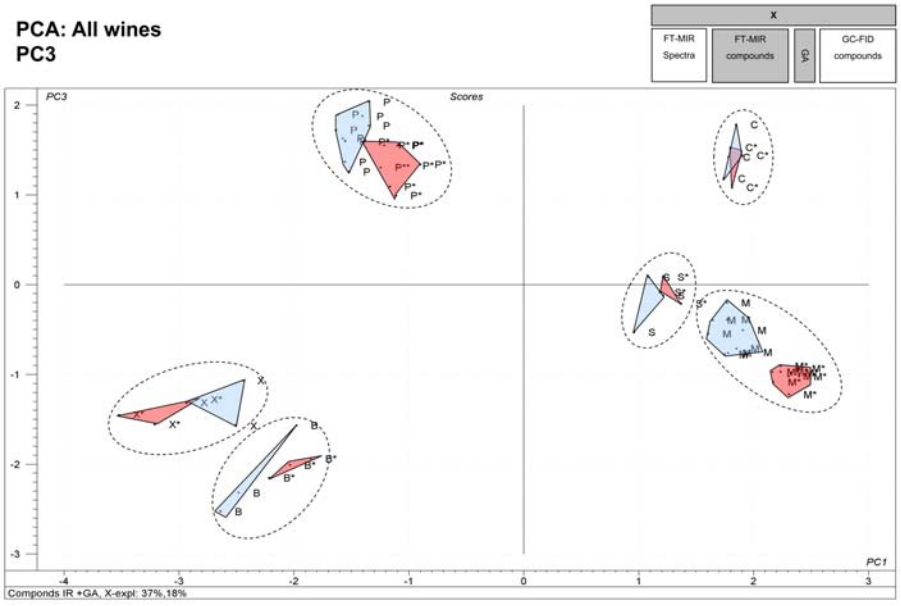


(b) PLS1-DISCRIM (full data set), PC1–PC3

Figure 4.15: PLS1-DISCRIM, full data set. Figure 4.14 and 4.15 uses two different annotation schemes to illustrate the following findings. There are now larger differential cultivar effects to be observed for all varieties when analysed by PLS1-DISCRIM. The largest differential cultivar effects were observed for Merlot and Chardonnay. Compare with Figure 4.13.

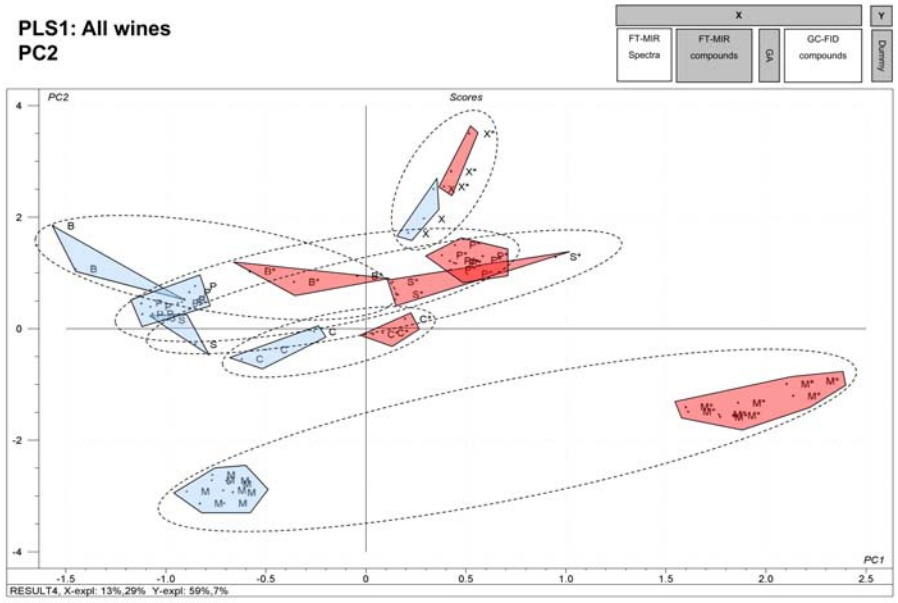


(a) PCA: MIR-compounds + GA, PC1–PC2

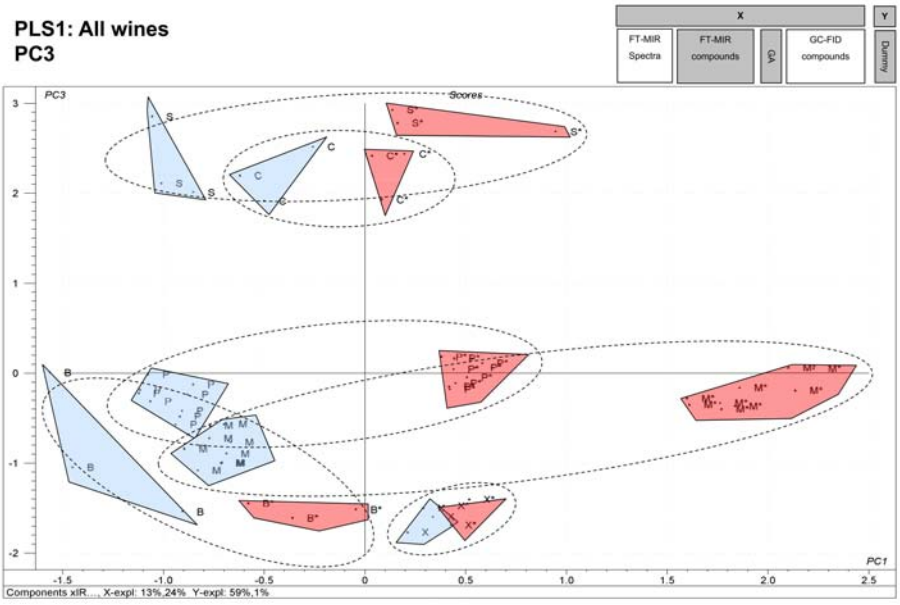


(b) PCA: MIR-compounds + GA, PC1–PC3

Figure 4.16: PCA: MIR-compounds + GA. Clear cultivar delineations, but only small discriminability effects between VIN13 and VIN13-Δ1 fermentations.

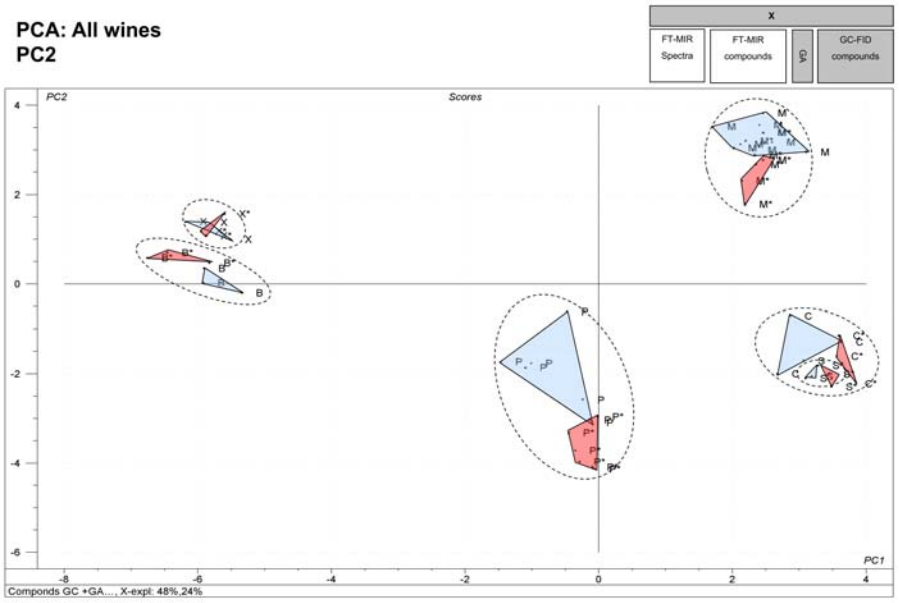


(a) PLS1-DISCRIM: MIR-compounds + GA, PC1–PC2.

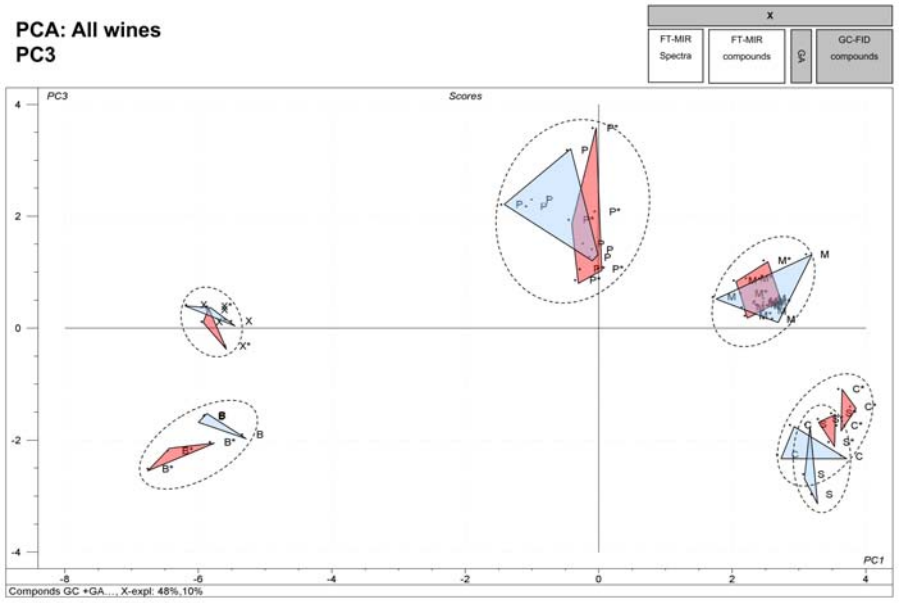


(b) PLS1-DISCRIM: MIR-compounds + GA, PC1–PC3.

Figure 4.17: PLS1-DISCRIM: MIR-compounds + GA. Clear cultivar discriminations with very noticeable increased discriminability between VIN13 and VIN13-Δ1 fermentations. Greatest F1 discrimination was observed for Merlot.

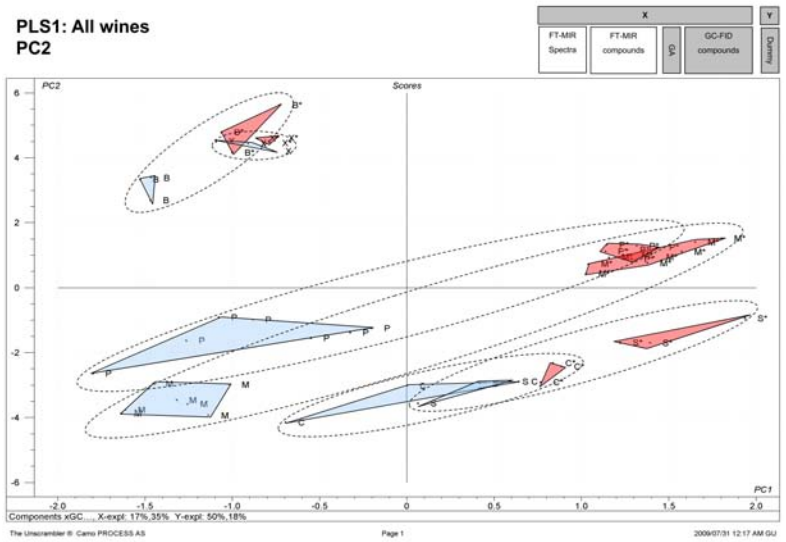


(a) PCA: GC-compounds + GA, PC1–PC2

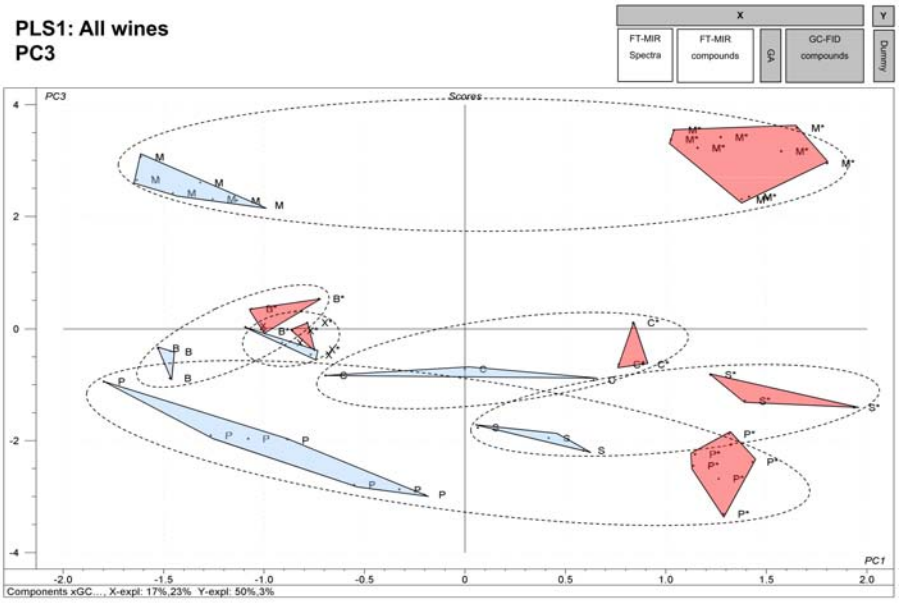


(b) PCA: GC-compounds + GA, PC1–PC3

Figure 4.18: PCA: GC-compounds + GA. Clear cultivar delineations, but only small VIN13 and VIN13-Δ1 fermentation discriminability effects.

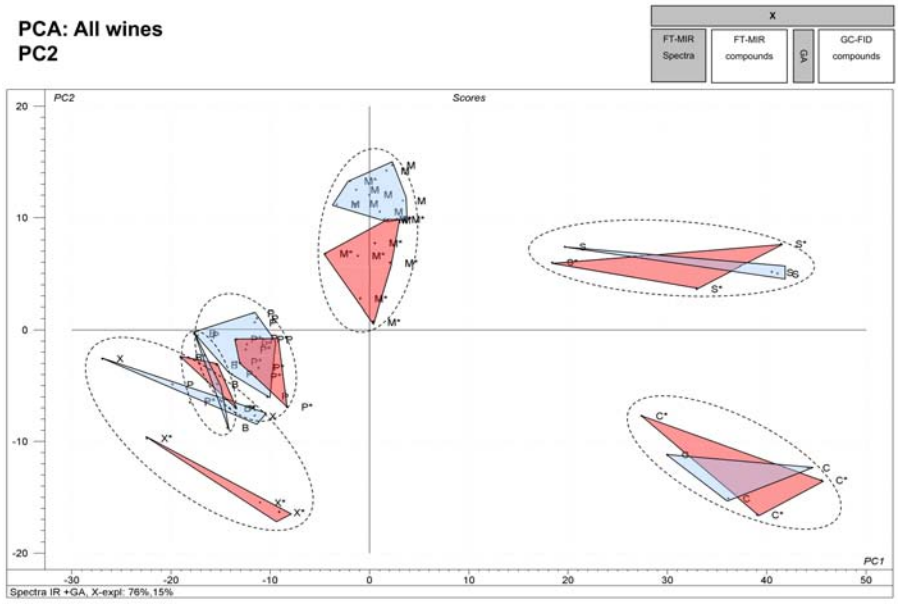


(a) PLS1-DISCRIM: GC-compounds + GA, PC1–PC2

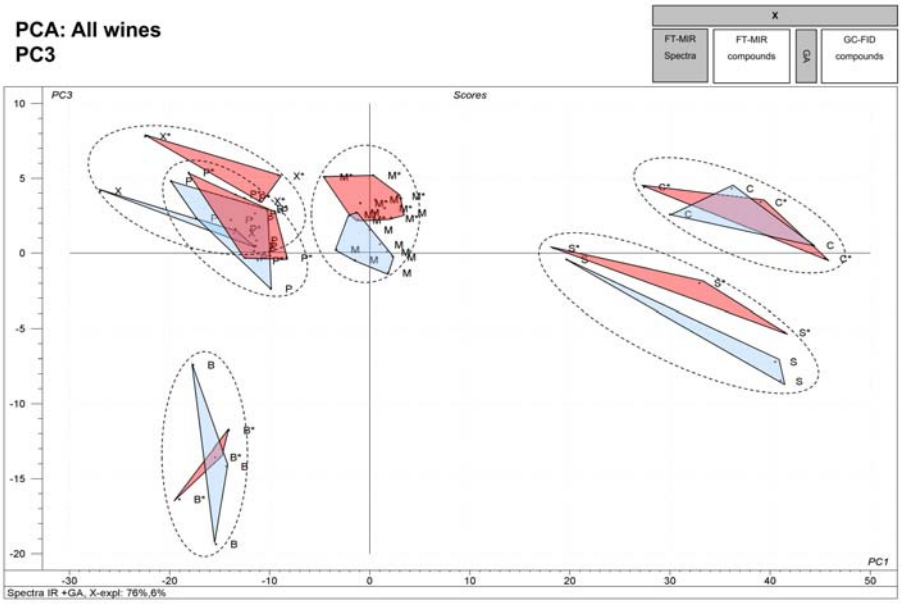


(b) PLS1-DISCRIM: GC-compounds + GA, PC1–PC3

Figure 4.19: PLS1-DISCRIM: GC-compounds + GA. Clear cultivar discriminations with very noticeable increased discriminability between VIN13 and VIN13-Δ1 fermentations. Greatest discriminability for `F1` was observed in cultivars Merlot and Pinotage, followed by Shiraz and Chardonnay.

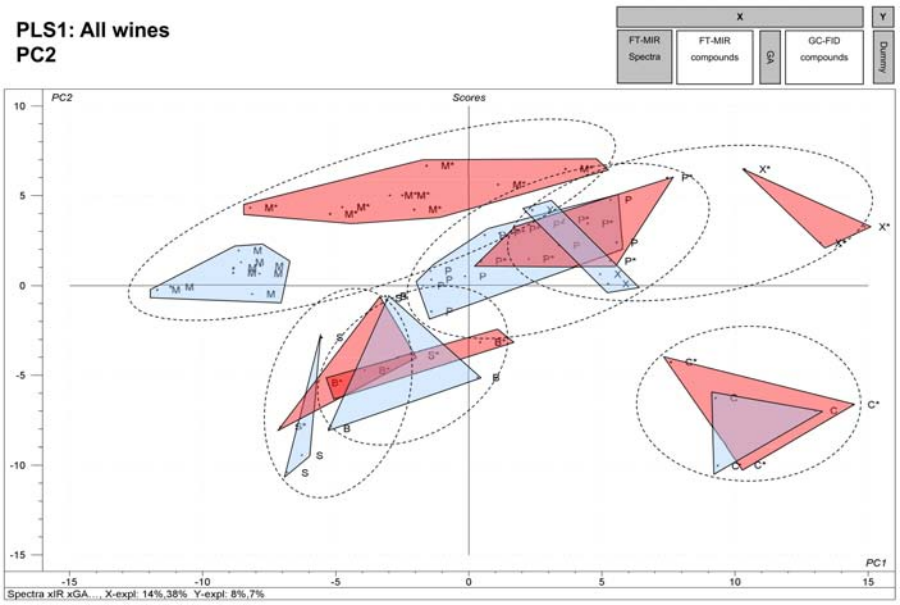


(a) PCA: MIR-spectra + GA, PC1–PC2

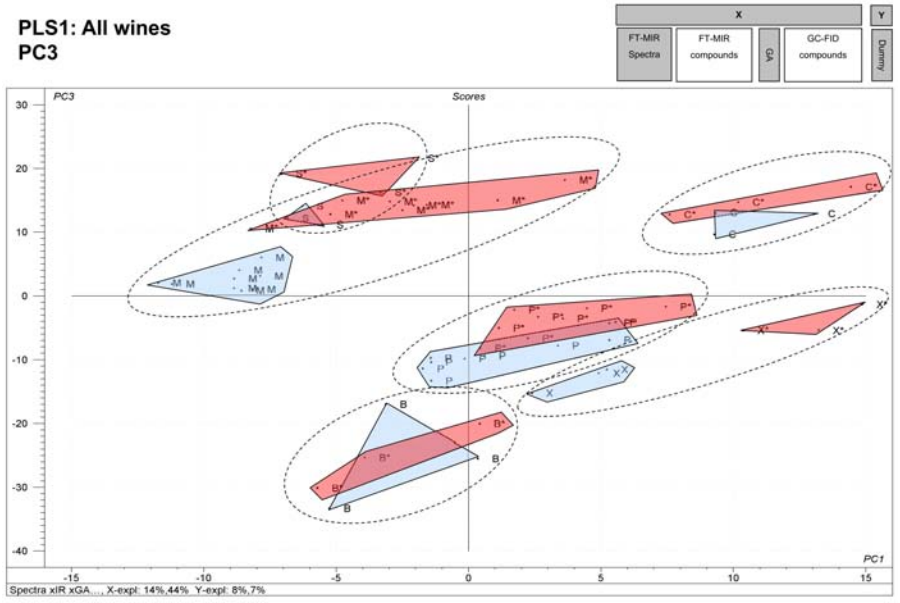


(b) PCA: MIR-spectra + GA, PC1–PC3

Figure 4.20: PCA: MIR-spectra + GA. Relatively clear cultivar delineations, less clear than Figures 4.16 and 4.18, and only small discriminability effects in `F1`.

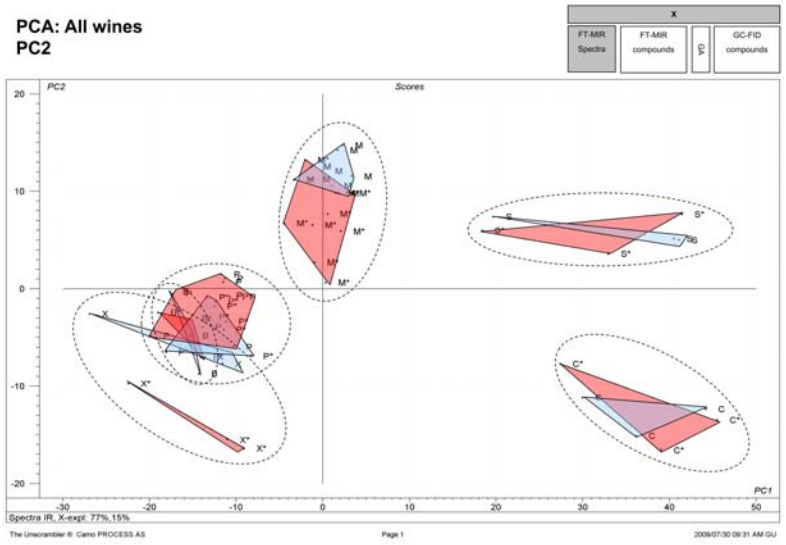


(a) PLS1-DISCRIM: MIR-spectra + GA, PC1–PC2

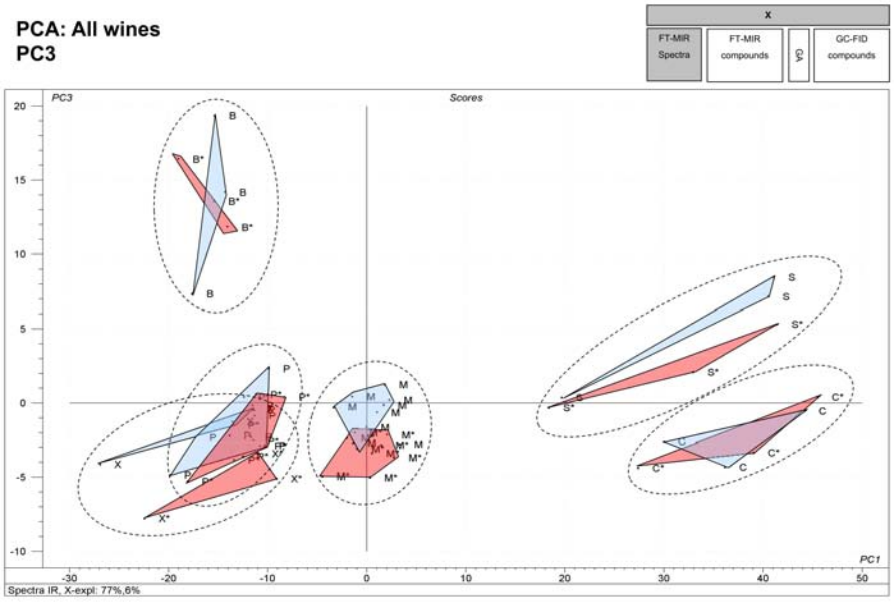


(b) PLS1-DISCRIM: MIR-spectra + GA, PC1–PC3

Figure 4.21: PLS1-DISCRIM: MIR-spectra + GA. Relatively clear cultivar discriminations. A little less clear than is observed in Figures 4.17 and 4.19, but with noticeably increased discriminability between VIN13 and VIN13-Δ1 fermentations. Compare with Figures 4.22 and 4.23 which duplicates Figures 4.20 and 4.21 but did not include GA in the model.

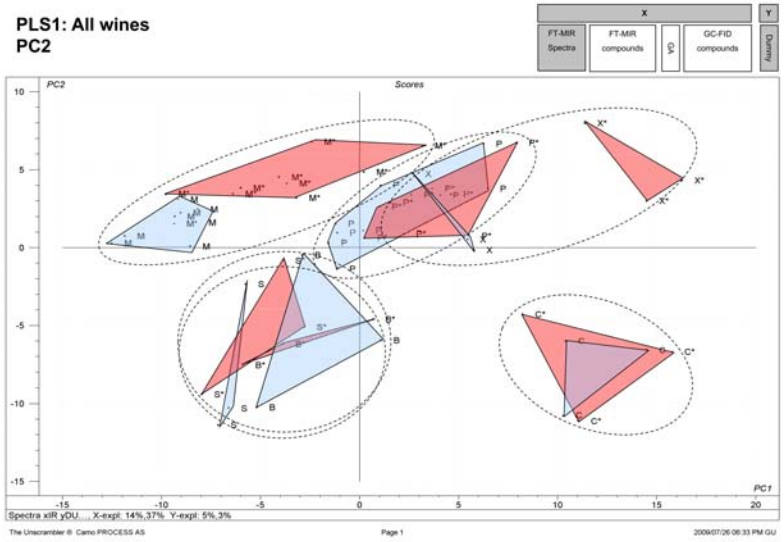


(a) PCA: MIR-spectra, PC1–PC2

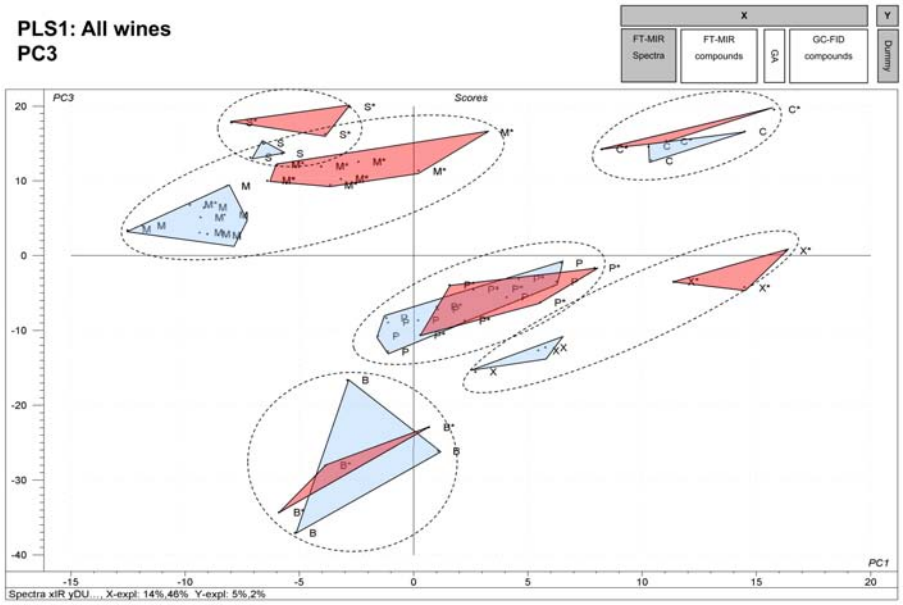


(b) PCA: MIR-spectra, PC1–PC3

Figure 4.22: PCA: MIR-spectra. Relatively clear cultivar delineations, but less clear than Figures 4.16 and 4.18), and only small VIN13-Δ1 fermentation discriminability effects.



(a) PLS1-DISCRIM: MIR-spectra, PC1–PC2



(b) PLS1-DISCRIM: MIR-spectra, PC1–PC3

Figure 4.23: PLS1-DISCRIM: MIR-spectra. Relatively clear cultivar discriminations, but less clear than can be seen in Figures 4.17 and 4.19). The discriminability between VIN13 and VIN13-Δ1 (F1) fermentations is present, but with noticeably less distinct than when GA is involved (included in the model). Compare with Figures 4.22 and 4.23 where include GA was included in the model.

4.3.5.3 Gluconic acid (GA) PLS calibration model (attempt)

Figure 4.25 shows the X-space projection from the GA model in Figures 4.24 (a) and (b). The score is annotated in a fashion similar to Figures 4.13–4.23. Rather poor cultivar (F2) discrimination as well as poor VIN13 / VIN13-Δ1 fermentation (F1) discriminability is observed.

4.3.5.4 Ethanol PLS calibration model (attempt)

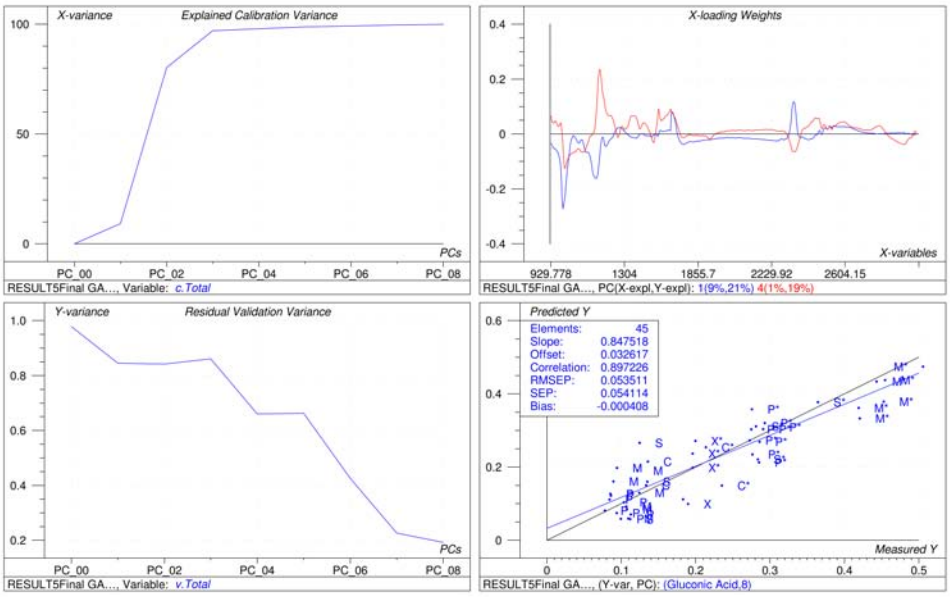
Figure 4.26 shows a multivariate calibration model of Ethanol (Y) based on MIR-spectra (X) to quantify whatever ethanol reduction that may, or may not, result from VIN13 / VIN13-Δ1-fermentation. Only the Pred/Meas plot is presented in the figure, as superposed on a trivial alcohol prediction model, there are virtually no VIN13-Δ1 discriminability effects observable.

4.3.5.5 Contention of chemometric output

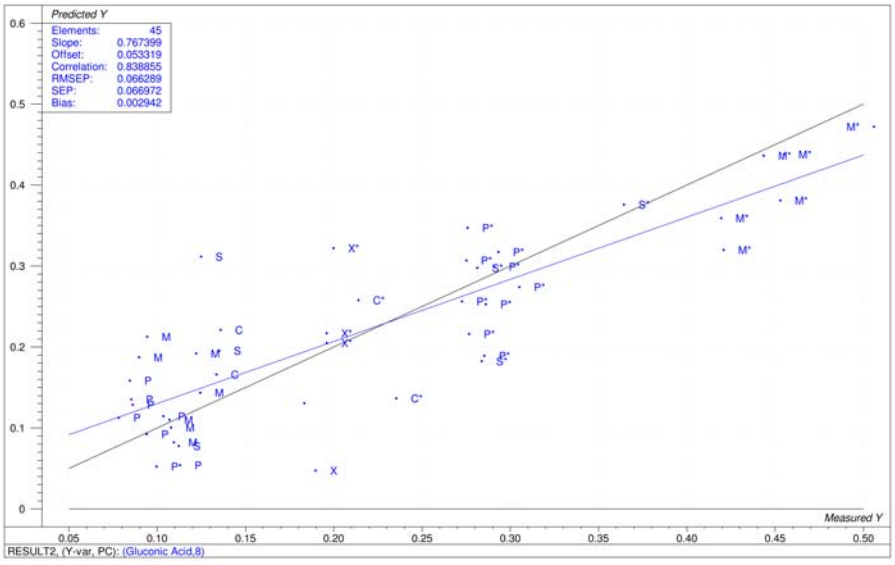
It is clearly visible that no satisfactory model of the GA concentration can be established based on the MIR spectra alone. Detailed inspection of this model, Figure 4.24 (a) (all four panels of the standard PLS documentation) reveals that only 61% of the y-variance can be modeled (using only 25% of the X-variance). The interpretation of these quantitative features is that the information residing in the GA variable cannot be acquired from the MIR spectral information.

There are no significant VIN13 / VIN13-Δ1 fermentation discriminability effects discernable in the similar ethanol PLS-model (Figure 4.26). The increase that can be observed is an increase in the total amount of sugar that was available at the onset of alcoholic fermentation (Table 4.2), rather than an increase in the total amount of ethanol produced during fermentation relative to the other fermentations.

In addition to the individual VIN13 / VIN13-Δ1 fermentation-effects (F1) visualized for the variable subsets, there is a major common cultivar (F2) effect. Merlot, Pinotage and Chardonnay consistently show the largest effect, which in turn must reflect the largest sensitivity to the presence of glucose oxidase (GM-modification) by VIN13-Δ1 strains. Parallel to this, across all alternative chemical subsets (F3), Shiraz and Sauvignon blanc display a correlated reduction in the GM-modification effect, and Cabernet Sauvignon almost a total absence, the interpretation of which

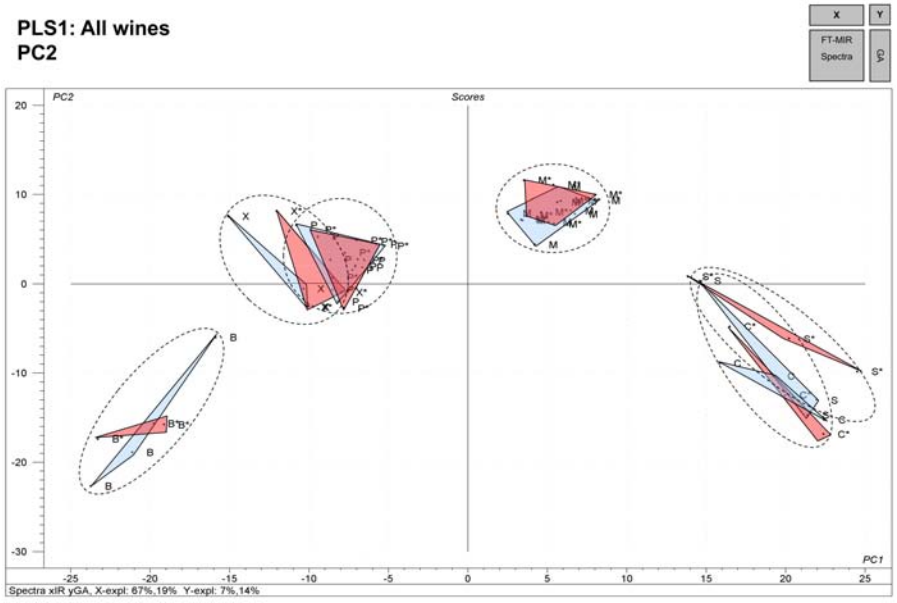


(a) PLS1 model of Gluconic acid based on MIR-spectra alone.

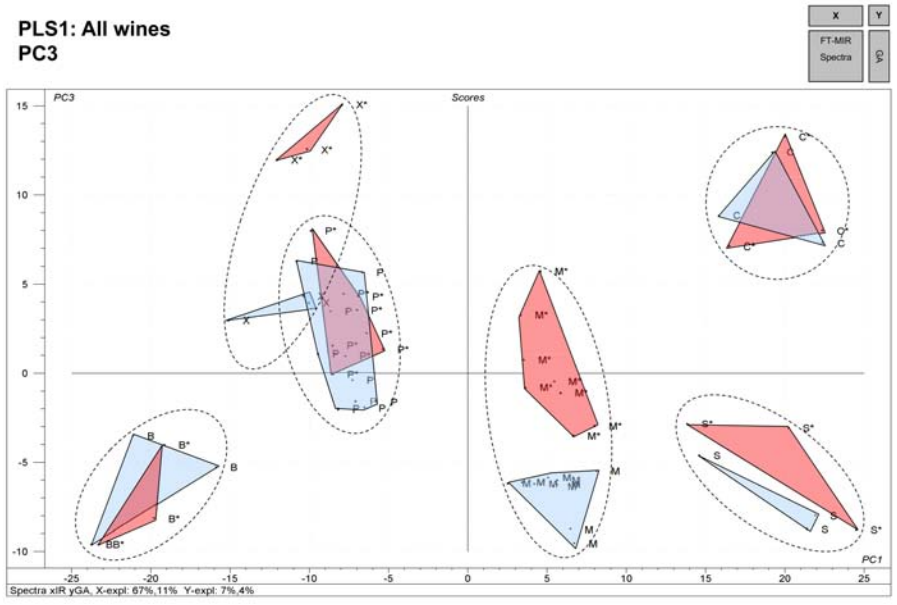


(b) Cross validated (full cross validation) GA model. Compare with the above PLS1 model of GA. This Pred/Meas validation shows that any attempt to derive GA concentration directly from MIR-spectra alone is not possible. RMSEP is far too large for acceptance in any analytical chemical context.

Figure 4.24: (a) PLS1 model of Gluconic Acid based on MIR-spectra alone. 15 outliers were deleted (sic). Leverage correction was applied for outlier deletion. (b) shows the Pred/Meas plot based on cross-validation. See text for detailed interpretation of modeling attempt.



(a) PLS1-DISCRIM: MIR-spectra, PC1–PC2.



(b) PLS1-DISCRIM: MIR-spectra, PC1–PC3.

Figure 4.25: PLS1-DISCRIM: The X-space projection from the GA. model in Figure 4.24 (a) and (b). The score is annotated in a fashion similar to Figures 4.13–4.23. Rather poor cultivar discrimination and poor GM discriminability.

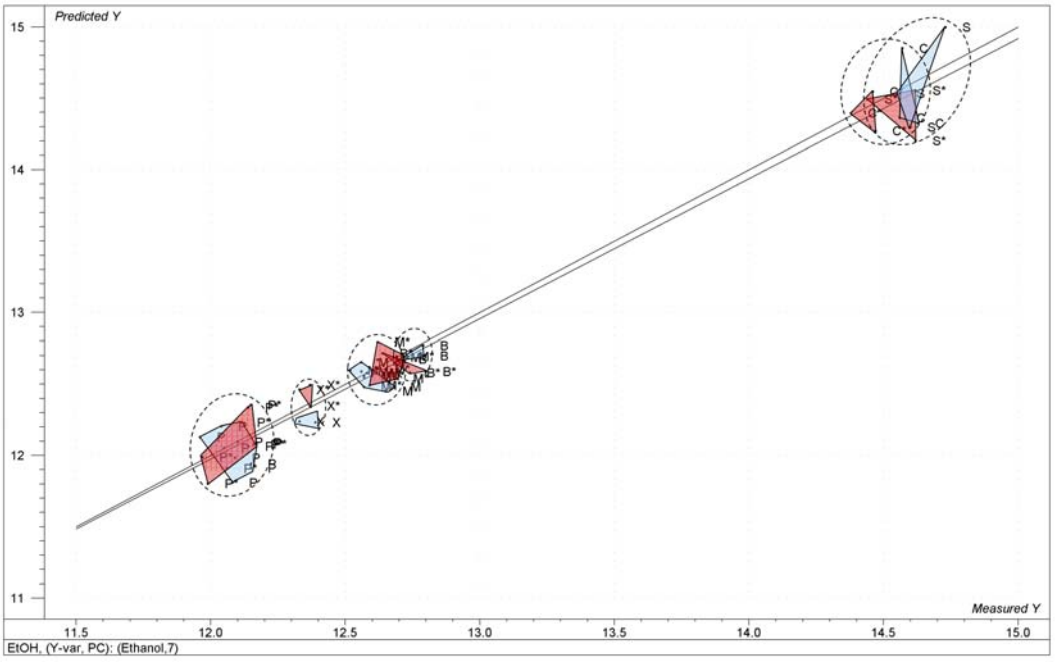


Figure 4.26: PLS1 multivariate calibration of Ethanol (Y) based on MIR-spectra (X). Only the Pred/Meas plot is shown here. Superposed on a trivial alcohol prediction model, there are virtually no VIN13 / VIN13-Δ1 (`F1`) discriminability effects observable.

signifies little to no glucose oxidase effect for these cultivars.

Of the restricted variable subsets the MIRcompounds and GC-compounds yielded better results, with the GC-compounds displaying greater discriminability between cultivars and VIN13 / VIN13-Δ1 fermentations. One can conclude from this result that the greatest influence of VIN13-Δ1 produced wines would be observed in the aroma component, but as there was some discriminability effects discernable in the MIR-compounds also, the favour component is also be affected.

Two major conclusions are evident:

1. The PLS1-DISCRIM approach consistently improves the discriminability versus PCA. PCA alone shows *some* of the same features only, but not as clearly. Since one always knows the "before – after" status of the experimental data, it is always possible to use the information encoded in the DUMMY Y-variable; thus the optimal PLS1-DISCRIM approach is always available for multivariate visualization.

2. Inclusion of GA in any of the three alternative variable sub-set greatly improves the discriminability for GM-modification-effect visualization and allows better quantification in the full multivariate displays. This effect is of course also present for the combined full variable set. Gluconic acid (GA) is thus concluded to constitute a significant chemical variable for the GM-modification-effect evaluation.

4.4 Conclusions

Based on the work done previously by Malherbe *et al.* (2003), the GOX-encoding gene was integrated into the genome of VIN13-Δ1, an industrial wine strain, and the production and activity of the corresponding enzyme was confirmed (Chapter 3). Consequently, the objective of this study was to evaluate the ability of a genetically engineered industrial wine strain to produce and secrete the glucose oxidase enzyme, under winemaking conditions. Furthermore, enzyme efficiency was correlated to the final ethanol concentrations measured. The concentrations of flavor and aroma compounds were also measured in order to evaluate the impact of the enzyme on wine bouquet.

We showed by means of the GOX plate assay that alcoholic fermentation was performed by VIN13 and VIN13-Δ1 strains respectively and that 98% of the colonies tested from VIN13-Δ1 fermentations were still able to produce GOX at the end of alcoholic fermentation. This confirmed that the expected strain performed alcoholic fermentation in all the batches and that the integration was stable.

Increased levels of gluconic acid was detected in all the VIN13-Δ1 fermentations, indicating that there was GOX activity. The higher levels of gluconic acid detected were lower as what was expected, and a shortage of molecular oxygen in the must to drive the conversion of glucose to GA by GOX would explain this observation. The largest increase in GA was observed in Sauvignon blanc (80 mg/L) and Merlot (350 mg/L).

In all the white wines, most of the glucono-δ-lactone was converted to GA. Glucono-δ-lactone and glucono-γ-lactone are in equilibrium with GA, representing, respectively, 5.8 and 4.1% of the acid level at pH 3.6–4.0 (Barbe *et al.*, 2002). The small amounts present in some wines could be as a result of the low pH. In all

the red wines glucono-δ-lactone was converted to GA. This could be as a result of the slightly higher pH than that observed in the wite wines. The reason that higher levels of GA were measured in the red wines could be correlated to the fact that the wines were fermented on the skins in open buckets. Furthermore the skins were punched down during the first 3 days of alcoholic fermentation, increasing the exposure of the wines to molecular oxygen and resulting in better activity of the GOX enzyme.

The presence of low levels of GA in VIN13 (control) wines can be explained by the presence of *B. cinerea* (a necrotrophic fungus, that produces GA from grape sugars) that was visible on some of the grapes before crushing. It is further possible that as a result of SO_2 binding to GA, less 'free' GA was detected in the wines that received SO_2 at crushing. The reduction of GA because of SO_2-binding has been described by Barbe *et al.* (2002).

Insignificant levels of reduced ethanol concentrations were obtained after completion of alcoholic fermentation. Cabernet Sauvignon wines showed the greatest reduction (0.15% (v/v) which coresponds to 2.34 g/L glucose [0.064 % (v/v) ethanol = 1 g/L glucose]). This was interesting, because Cabernet Sauvignon did not show the highest levels of GA, however the decrease in ethanol corresponds to the approximate increase in GA (3 g/L). The lower levels of GA could possibly be a result of SO_2-binding power and the reason that insignificant levels of reduced ethanol were detected could be because the differences in ethanol concentrations were to small and the method used to detect the differences were not sensitive enough.

In addition, Two-way ANOVA analysis of variation was performed to determine the influence of yeast strain and cultivar relationships on the total amount of ethanol (% v/v) produced during alcoholic fermentations. The results of the analysis of variation indicated that there was no differences in the strain–cultivar relationships.

Results obtained from GC-FID indicated that of all the aroma compounds measured in white wines, the esters contributed the most to the aroma component, and that the acid and fatty acid component along with the alcohols and higher alcohols contributed the most to the aroma component in red wines. Despite these conclusions, the overall aroma profiles for the different wines were very similar.

Multivariate data analysis was employed as a tool to provides a holistic picture

to visualize the effects of VIN13-Δ1 fermentation with in comparison to VIN13 fermentations (excluding the Chardonnay that did not received SO_2 at crushing), taking into account the alternative chemical parameters (MIR-spectra, MIR-compounds, GC-compounds and Gluconic acid (GA) alone, or in various combinations).

Chemometric projections of the score plots of both t1-t2 as well as t1-t3 plots for all results allowed insight into all significant variation up to three principal components (PCA) or PLS components, which showed very clearly that GA is a key factor in evaluating the effect of GOX in VIN13-Δ1 fermentation with regard to VIN13 fermentations.

The VIN13-Δ1 effect manifestations were best shown on PLS1-discrim score plots that revealed that the full impact of the 'GC-compounds + GA' parameter set on the discriminability between VIN13 and VIN13-Δ1-yeast fermentations. Clear cultivar differences with very noticeable increased discriminability between VIN13 and VIN13-Δ1 fermentations was observed with the greatest discriminability in cultivars Merlot and Pinotage, followed by Shiraz and Chardonnay. PLS1 discriminability for 'MIR-compounds + GA' indicated the greatest discrimination towards the cultivar Merlot. Of the restricted variable subsets the MIR-compounds and GC-compounds yielded better results, with the GC-compounds displaying greater discriminability between cultivars and VIN13 / VIN13-Δ1. One can conclude from these results that the greatest influence of VIN13-Δ1 produced wines would be observed in the aroma components, but as there also was discriminability effects discernable in the MIR-compounds, the favour component are also affected.

From the results it is also clear that no satisfactory model of the GA concentration can be established based on the MIR spectra alone. Detailed inspection of our model reveals that only 61% of the y-variance could be modeled (using only 25% of the X-variance). The interpretation of these quantitative features is that the information residing in the GA variable cannot be obtained from the MIR spectral information alone. It is thus necessary to actively measure the GA level.

Finally, the PLS1-discrim approach consistently improves the discriminability versus PCA. PCA alone shows some of the same features only, but not as clearly. Inclusion of GA in any of the three alternative variable sub-set greatly improves the discriminability for VIN13-Δ1 (GOX-effect) visualization and allows better quantification in the full multivariate displays. Gluconic acid (GA) is thus concluded to constitute a significant chemical variable for the discrimination of VIN13-Δ1 fermentations and evaluation of the GOX-effect.

This work has shown that a genetically engineered industrial wine strain were able to produce and secrete the glucose oxidase enzyme, under winemaking conditions. Furthermore, we have shown that the enzyme efficiency, correlated to the final ethanol concentrations, was sub-optimal and that molecular oxygen is of uttermost importance for the activation of the enzyme and would play a key part to the understanding and successful production of reduced alcohol wines. Lastly, we presented results that indicate that the aroma and flavor compounds were influenced and this could possibly impact the wine bouquet.

The authors would like to suggest micro-oxygenation as a possible solution to the activation of VIN13-Δ1 produced GOX during alcoholic fermentation. The specific concentration of oxygen that would be necessary during a fermentation to reduce ethanol by a specific margin would have to be determined carfully to evaluate oxidation of other chemical compounds that might have a negative impact on flavor and aroma. If successful, fermentation with VIN13-Δ1 can potentially provide an effective means of bio-adjusting the alcohol content to appropriate levels in commercial wines. This might offer a viable way to meet consumers' demands for affordable low-alcohol wine, and have financial implication in savings on wine taxation.

One final general consideration should be made considering the acceptance of genetically modified organisms (GMOs) in the wine industry. Further research is essential to ensure that such a yeast would not compromise the safety and sensory quality of the wine. Furthermore, it is important to realise that only two commercial wine strains is currently being employed which are genetically modified (Coulon *et al.*, 2006; Cebollero *et al.*, 2007), and that the wine industry will not use such strains as starter cultures unless both the industry and the consumers are satisfied that they are safe and beneficial (Pretorius, 2000, 2001; Vivier and Pretorius, 2002).

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Chapter 5

Impact of Oxygen on Glucose oxidase Activity Evaluated Using Model Fermentations

Abstract

Previous studies have successfully shown that glucose oxidase (GOX) can be used in small-scale fermentations with grape juice to convert glucose to gluconic acid, thereby obtaining a reduced amount of ethanol at the end of alcoholic fermentation. However, under winemaking conditions, active GOX secreted from VIN13-Δ1, a wine strain of *S. cerevisiae* genetically engineered to produce and secrete glucose oxidase, were relative unsuccessful, and levels of ethanol were reduced only by a small fraction (Chapter 4). In this study, we have successfully demonstrated that glucose oxidase secreted from VIN13-Δ1 is active in grape juice as well as during alcoholic fermentation, and that oxygen plays a key-role in enzyme activation in wine. We further confirmed that GOX is active under aerobic conditions, inactive under anaerobic conditions, and can be activated instantaneously when an anaerobic culture is switched to aerobic conditions (simulated micro-oxygenation). Finally, we showed with the help of a simplified model, that under ideal conditions, GOX secreted from VIN13-Δ1, can be employed to reduce the ethanol concentration by a predefined concentration.

5.1 Introduction

An increase in the temperature globally over the last decade led to an overall increase in the ripeness of grape berries in viticultural regions, especially in countries residing in the Southern hemisphere. Consequently a higher concentration of fermentable sugars is present in grape juice. This increased sugar concentration leads to a proportional increase in ethanol as the sugar is metabolized by the yeast via the glycolytic pathway during alcoholic fermentation. Moreover, a higher concentration of sugar increase the osmotic pressure and leads to the formation of secondary products which are not always desired (Pigeau and Inglis, 2007; Tofalo *et al.*, 2009). In addition to this, the fermentation can always become sluggish or stuck which increases the risk of spoilage by other micro-organisms.

The production of too much alcohol can effect wine quality negatively by its interaction with certain color and aroma (phenolic) molecules. For example; wine could take longer to mature (Clarke and Bakker, 2004). In addition to the effect on the chemical composition of the wine; modern consumer trends, ever changing alcohol taxation policies in the beverage industry and stricter drinking driving legislation, have resulted in a demand for methods to produce reduced alcohol (1.2% to 5.5–6.5% v/v), low alcohol (0.5–1.2% v/v) and even de-alcoholized (not above 0.5% v/v) wines (Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998; Gladstones and Tomlinson, 1999; Gladstones, 2000; Pickering, 2000). These demands can, to a certain extent be met with methods such as: centrifugation (filtration), reverse osmosis, thermal evaporation, membrane filtration and spinning cone column (Bui *et al.*, 1986; Pickering *et al.*, 1999*a*; Mermelstein, 2000). Unfortunately, all of these methods are based on physical phenomena, are labour intensive, and their utilization adds to the overall production costs, rendering the finished product more expensive.

Treating grape must prior to alcoholic fermentation with glucose oxidase (GOX) to reduce the glucose content of the must was introduced as an alternative approach (Villettaz, 1987; Pickering and Heatherbell, 1996; Pickering *et al.*, 1998, 1999*a*,*b*,*c*; Biyela *et al.*, 2009). This treatment resulted in wine with a reduced alcohol content after fermentation and provided satisfactory results. The alcohol content was reduced significantly in the wines treated by direct inoculation with GOX, but ad-

ditional steps were required to deacidify the wines, again creating a multi-step process.

Based on this approach, the GOX-encoding gene was overexpressed in a laboratory strain of *Saccharomyces cerevisiae* (Malherbe *et al.*, 2003) and the activity of the corresponding enzyme was confirmed in grape must. The ethanol yield was 1.8–2% lower when compared to control wines after alcoholic fermentation.

A genetically engineered industrial wine yeast strain (VIN13-Δ1) that can successfully produce and secrete active GOX was constructed (Chapter 3). Since the enzymatic reaction takes place extracellularly, it does not directly affect the intracellular redox balance, preventing the production of unwanted metabolites by the yeast as a result of the GOX enzymatic reaction.

VIN13-Δ1 was evaluated under winemaking conditions to reduce the alcohol content and the results showed that this genetically engineered industrial wine yeast strain was able to produce and secrete GOX. Furthermore, results showed that the enzyme efficiency (assessed by the final ethanol concentrations) was sub-optimal and suggest that molecular oxygen (micro-oxygenation) would be necessary for the activation of the enzyme under winemaking conditions and would play a key part in the understanding and successful production of reduced alcohol wines (Chapter 4).

The objective of the current study was to verify that in synthetic media, as well as grape must, GOX secreted by VIN13-Δ1 is active under aerobic conditions, inactive under anaerobic conditions and can be activated under anaerobic conditions by the addition of molecular oxygen (simulated micro-oxygenation). Finally, we wanted to show with the help of a simplified model, that under 'ideal' conditions GOX, secreted from VIN13-Δ1, can be employed to reduce the ethanol content of wine by a predefined amount and to determine in which conditions this strain could be used during winemaking.

5.2 Materials and Methods

5.2.1 Microbial strains, fermentation media and culturing conditions

A genetically engineered (GM) industrial strain of the wine yeast VIN13 (designated VIN13-Δ1) was used in this work. This industrial yeast was modified to express the *Aspergillus niger* structural glucose oxidase (*gox*) gene, and is regulated by the phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). The production and secretion of active glucose oxidase by VIN13-Δ1 has been demonstrated previously (Chapter 3, Section 3.3.2).

VIN13-Δ1 yeast strains were maintained on yeast peptone dextrose, YPD agar [containing 1% yeast extract (w/v), 2% peptone (w/v), 2% glucose (w/v) and 2% agar (w/v)] and cultivated at 30°C. Inocula for fermenter cultivations in synthetic media were prepared in YPD broth, however the sugar concentration was adapted to 10% glucose (w/v), which resembled the sugar concentration that would be used in the fermenter cultivations. Wine fermenter cultivations were performed in Shiraz must with a pH of 4.05 and sugar concentration of 25.3°B. The must was treated with 250 mg/L dimethyl dicarbonate (Velcorin®, Bayer AG) after it was thawed, but prior to inoculation, to eliminate all natural micro-organisms that could potentially still be present in the grape juice.

5.2.2 Precultivation

A small aliquot of frozen VIN13-Δ1 cell suspension was plated onto a YPD plate and incubated at 30°C for 2–3 days. The inoculum was grown in two stages. First a single recombinant VIN13-Δ1 colony was inoculated from a plate into a test tube containing 5 mL YPD broth and maintained at 30°C for 24 h at ≈150 rpm on a TC7 wheel (New Brunswick Scientific Inc., NJ, USA). This culture was then transferred to 100 mL YPD shaking at 80 rpm on a L.E.D. orbital shaker (Lab-Line Instruments Inc., IL. USA). Each Erlenmeyer was closed with a cottonwool plug and covered with foil, ensuring enough aeration for growth and enzyme production. Incubation was under the above described conditions for 2 days.

5.2.3 Fermentation setup

Batch fermentations were carried out in duplicate with a working volume of 800 mL using a high-performance BioFlo 110 benchtop fermenter (New Brunswick Scientific, Edison, NJ) with a heat blanket to regulate the temperature. The bioreactor was further equipped with a dissolved oxygen (DO) and pH probe. As a precaution, a foam trap was attached to the unit to manage potential foaming by the industrial strain. After fermenter vessel preparation the units containing rich media were autoclaved.

When grape must was used the units were autoclaved with deionized water (dH_2O) whereafter the water was removed and Velcorin® treated must was added under sterile condition within a Labotec laminar-flo unit. Each benchtop fermenter was computer controlled using the manufacturer's BioCommand software. The temperature and agitation speed were maintained at 25°C and 100 rpm, respectively. Each unit that was set up for an anaerobic fermentation was flushed with nitrogen gas (N_2) prior to inoculation to ensure a complete anaerobic environment, and each unit that was used for aerobic fermentation was flushed with oxygen (O_2) prior to each aerobic fermentation. During the courses of each run either O_2 or N_2 airflow was maintained at 1 L/min. Precultures were used to inoculate the bioreactors at 10^6 cells/mL.

5.2.4 Cell growth and sugar utilization

Samples with a volume of 5 mL were taken at 30 minute intervals. The first sample was taken after inoculation (0 h) and the last sample was taken when the fermentation was stopped after 9 hours in synthetic media or 7 days in must fermentations. These samples were used to perform individual measurements and assays.

Cell proliferation (i.e. growth) was determined spectrophotometrically throughout the fermentations by measuring the optical density (at 600 nm) of the suspensions using a Jenway 6100 spectrophotometer (Analytical Instrument Recycle, Inc, USA). Dilutions were made where necessary.

Spectrophotometric assays were performed to determine glucose and fructose concentrations in the must using a D-Fructose/D-Glucose Assay kit (Catalogue Number: K-FRUGL) of Megazyme International Limited (Ireland).

5.2.5 Confirming pure cultures and glucose oxidase production

Samples were taken after each fermentation and plated evenly onto YPD plates and incubated for 2–3 days at 30°C, providing the yeast strains with enough time for growth and glucose oxidase production and secretion. The plates were then overlaid with 10 mL of 0.1 M McIlvaine buffer, pH 7.0 [containing 1% (w/v) agarose, 10 g/L glucose, 100 mg/L σ-dianisidine dihydrochloride (Sigma, South Africa) and 15 U horseradish peroxidase type II/mL (Sigma, South Africa)]. The overlay was allowed to set and the plates were incubated at 37°C for 1 hour, whereafter the yeast colonies were inspected for production and secretion of glucose oxidase.

Glucose oxidase metabolizes glucose into gluconic acid, and H_2O_2 is formed as a by product. The H_2O_2 is used to measure glucose oxidase activity as it is used as a co-factor by horseradish peroxidase to oxidize σ-dianisidine dihydrochloride and a color change is visible on the agar plates. *S. cerevisiae* ΣpGOXi (Malherbe *et al.*, 2003) was used as positive control and an untransformed VIN13 wine yeast strain was used as negative control.

5.2.6 Analysis of gluconic acid

1.5–2 mL samples from individual bioreactor fermentations were harvested at 5000 rpm for 5 minutes in an Eppendorf 5415 D benchtop centrifuge. The supernatant containing D-gluconic acid and D-glucono-δ-lactone was removed from the pelleted cells, and used without further purification in the D-gluconic acid/D-glucono-δ-lactone kit (Catalogue Number: K-GATE) of Megazyme International Limited (Ireland), to perform spectrophotometric assays confirming the production and presence of gluconic acid in the bioreactors.

5.3 Results and Discussion

The objectives of this study were to use synthetic media, as well as grape must, to investigate whether GOX secreted by VIN13-Δ1 was active under aerobic conditions, inactive under anaerobic conditions and could be activated when inactive under anaerobic conditions by the addition of molecular oxygen (simulated micro-oxygenation). These objectives were met and will be discussed in the various sections that follow.

5.3.1 GOX production and secretion throughout fermentation

VIN13-Δ1 colonies that were grown from samples taken immediately after inoculation and again at the end of fermentation and subjected to the GOX plate assay were all surrounded by a brown halo. This confirmed that glucose oxidase was produced by the starter culture. Furthermore, it demonstrates that GOX was still produced by VIN13-Δ1 after 9 h and that VIN13-Δ1 dominated the fermentations.

5.3.2 Fermentation in synthetic media

5.3.2.1 Growth of VIN13-Δ1 under different oxidative conditions

Figure 5.1 (A) illustrates the growth measured as optical density at 600 nm of three fermentations during different anaerobic (U0) and aerobic (U1) fermentations in YPD. Also displayed on the plot in Figure 5.1 (A) is the optical density measured

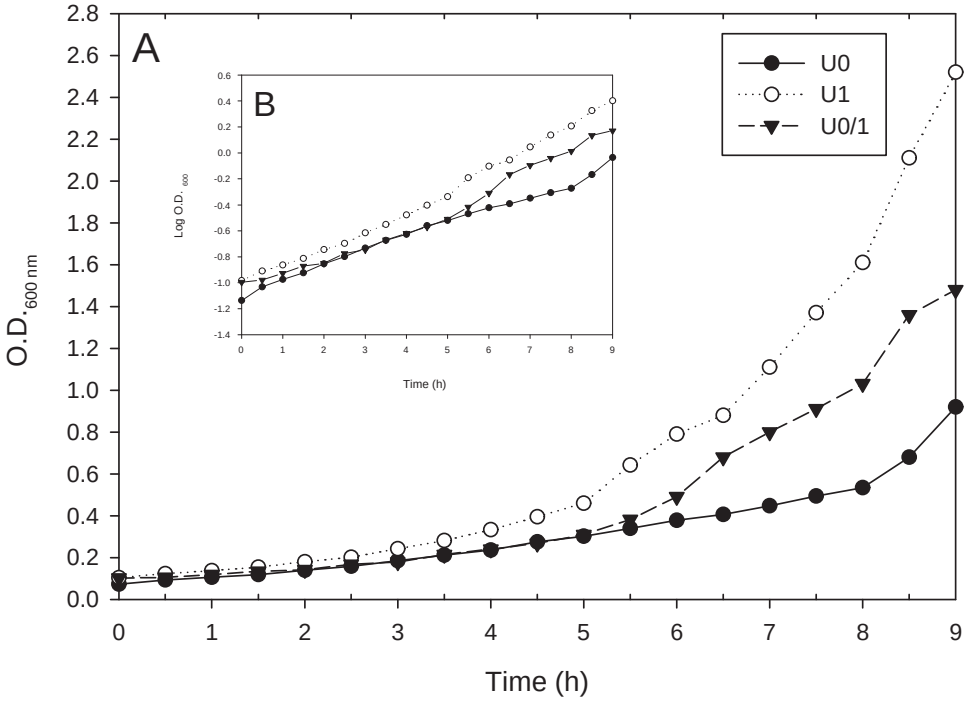


Figure 5.1: **A**. Growth as optical density (at 600 nm) in three fermentations during anaerobic (U0, •), aerobic (U1, ○) and micro-oxygenated (U0/1, ▼) fermentations in synthetic media. **B**. The specific rate of growth for VIN13-Δ1 during the different states of oxygenation.

in an anaerobic (U0) fermentation that received micro-oxygenation (U0/1). Figure 5.1 (B; insert) present the specific rate of growth for VIN13-Δ1 during the different states of oxygenation. Fermentation U0 was kept anaerobic for the full 9 hours, and reached a cell density of ≈ 0.9. Fermentation U1 was kept aerobic, and cell grew to a much higher density (≈ 2.55) in 9 hours compared to the cell densities of the anaerobic fermentations and reduced lag period. In the bioreactor, U0/1, where micro-oxygenation was performed between hours 3 to 6, cells grew to a higher density (≈ 1.5) than in the anaerobic fermentations, but not to such a cell density as what was observed during the aerobic fermentations. These results indicate that as soon as molecular O_2 is added to an anaerobic fermentation by means of micro-oxygenation, the rate of cell growth increases and a higher cell density is reached.

The increase in cell density was expected as the respiratory pathways would be enabled and more ATP produced. This additional energy is consumed when a more vigorous growth phase leads to additional biomass production. The increased growth rate will result in an increased GOX production rate as they are linked (Bankar *et al.*, 2009).

5.3.2.2 Gluconic acid production under complete anaerobic or aerobic conditions

Figure 5.2 (a) illustrates gluconic acid (GA) production over a period of 9 hours under complete anaerobic or aerobic conditions. Only a slight increase, 0.084 g/L, was observed in the levels of GA during anaerobic conditions. One would not expect any increase as glucose oxidase (GOX) needs molecular O_2 to perform the conversion. The slight increase suggests however that there are extremely low levels of O_2 present. Silicone pipes were used on the fermenter and are oxygen permeable to some extent. This could possibly explain the presence of minute amounts of molecular O_2 in the bioreactor and the slight increase in GA concentration that was observed.

The aerobic fermentation, also presented in Figure 5.2 (a), illustrates that when molecular O_2 is present in a fermentation, glucose oxidase is active and glucose can easily be converted to GA. The GA concentration measured 9.7 $\times$ higher under aerobic conditions (0.817 g/L) compared to anaerobic (0.084 g/L) conditions over a period of 9 hours. In addition, the pH also decreased by 0.65 units in the aerobic fermentation. The increase in GA could contribute to the overall acidity and thus the decrease in the pH. No significant changes in the pH was observed under anaerobic

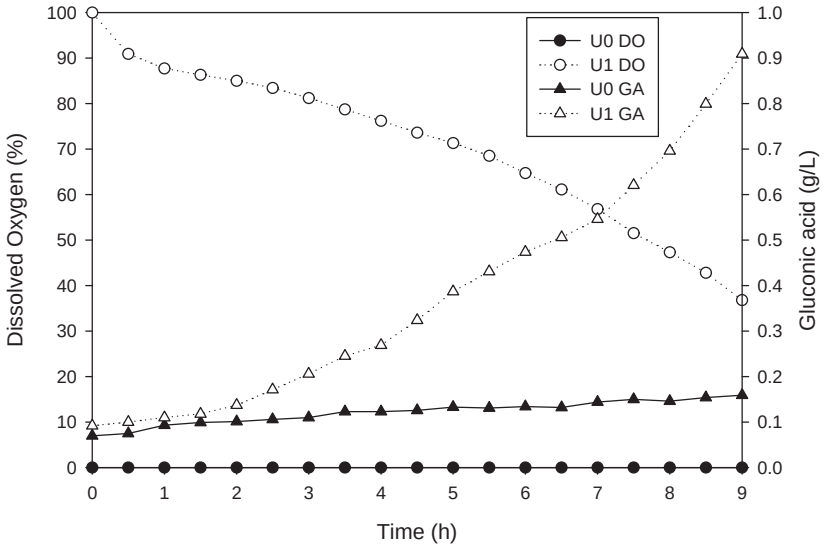
fermentation.

5.3.2.3 Gluconic acid production under anaerobic conditions and micro-oxygenation

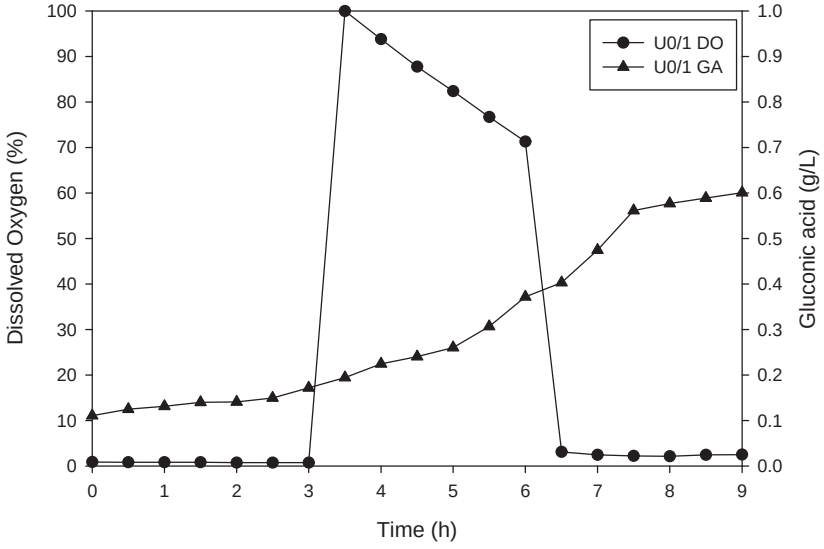
Figure 5.2 (b) illustrates gluconic acid production during an anaerobic fermentation where micro-oxygenation was performed for a period of 3 h. The fermentation was kept anaerobic at first, but after 3 hours micro-oxygenation was performed for a period of 3 h, where after the fermentation was left to return to an anaerobic state. To simulate micro-oxygenation, molecular O_2 was bubbled through the fermentation at 1 L/min. After molecular O_2 entered the fermentation, a sudden increase (Figure 5.3) in the concentration of gluconic acid (0.31 g/L) was observed. After 3 h, when micro-oxygenation was stopped, there was still an increase in the concentration of gluconic acid until all molecular O_2 is depleted, whereafter the level of gluconic acid seems to level off at 0.6 g/L. This indicates that the glucose oxidase enzyme can be activated during a anaerobic fermentation by the addition of molecular O_2, and that glucose will be converted to gluconic acid as long as there is molecular O_2 present within the fermentation.

5.3.2.4 Specific gluconic acid production rate

Figure 5.3 illustrates the specific rate at which gluconic acid (GA) is produced during fermentations in synthetic media under different oxidative conditions. The specific rate at which GA is produced under either aerobic or anaerobic conditions remained constant. However, Figure 5.3 clearly demonstrates that when micro-oxygenation (U0/1) was applied to an anaerobic fermentation between 3–6 h, GOX can be activated and the rate at which GA is formed is similar to the rate under which GA is produced in an aerobic fermentation. Another observation is as soon as micro-oxygenation is stopped, the rate at which GA is formed returns to its previous state, before activation of GOX, that is similar to that observed under anaerobic conditions. These results suggests that, it is possible to increase the rate at which GA accumulates similar to that of an aerobic fermentation by means of micro-oxygenation for a limited time span at a time of your own choosing.



(a) Gluconic acid (GA) production and dissolved oxygen (DO) utilization during a complete anaerobic (U0: ▲, ●) and aerobic (U1: △, ○) fermentation in synthetic media (YPD).



(b) Fermentation received micro-oxygenation (U0/1) from 3–6 h. Dissolved oxygen (DO, ●) and GA (▲) concentrations are displayed on this plot. The increase in GA concentration is noticeable.

Figure 5.2: Fermentations performed in synthetic media (YPD). (a) U0 represents a complete anaerobic fermentation and U1 represents a complete aerobic fermentation. (b) presents the fermentation (U0/1) that has undergone micro-oxygenation.

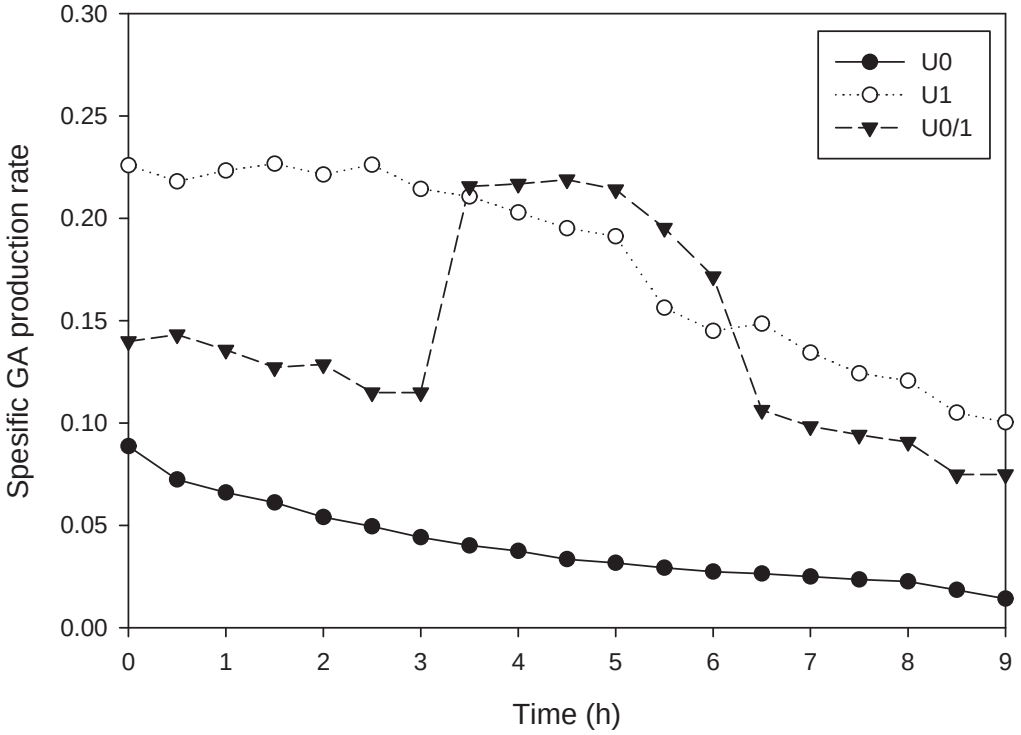


Figure 5.3: Specific gluconic acid production rate during different fermentations in synthetic media. U0 represents an anaerobic (●) fermentation and U1 an aerobic (○) fermentation. U0/1 started anaerobicaly, but received micro-oxygenation (▼) for a 3 h period, between 3–6 h. It is clearly visible that as soon as molecular oxygen is available, GOX is activated and the rate at which GA is formed is similar to the rate under aerobic conditions.

5.3.3 Fermentation in grape must

The accumulation of gluconic acid (GA), related to the activity of glucose oxidase (GOX) during alcoholic fermentation by VIN13-Δ1, was further investigated under controlled circumstances in a bioreactor using grape juice as substrate. The same conditions were applied as for fermentations performed in synthetic media (YPD). A set of batch fermentations, consiting of one anaerobic (U0) and one aerobic (U1) fermentation, was performed on two separate days. The data generated from the four wine fermentations (WF1-U0, WF2-U1 and WF3-U0, WF4-U1) will be discussed below, and duplicates are summarized in Figures 5.4 and 5.5.

Cell density expressed as optical density at 600nm (O.D.600), was measured, but showed no significant differences as compared to optical cell densities obtained

from the fermentations in rich media (results not shown).

5.3.3.1 Sugar consumption and gluconic acid production under anaerobic conditions

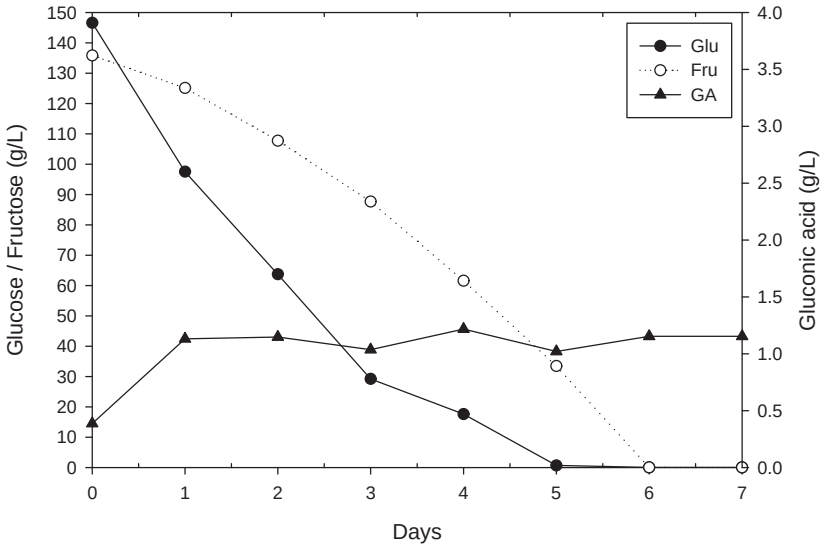
Duplicate fermentations are conferred in Figures 5.4 (a) and 5.4 (b) and present the rate at which both glucose and fructose are metabolized as well as the rate at which gluconic acid (GA) is produced during anaerobic conditions. In Figure 5.4 (a) $\approx$ 146 g/L glucose was consumed in 5 days and $\approx$ 135 g/L fructose was consumed in 6 days. During this time there was only an increase of 0.75 g/L GA, whereafter no increase was observed, and the levels of GA was stable at 1.15 g/L. As molecular O_2 is necessary for the enzymatic reaction, the fermentations were kept anaerobic by bubbling N_2 through the must at 1 L/min for the duration of the fermentation. This result indicates that in the absence of molecular O_2 no additional GA is formed in the grape must. The slight increase that can be observed during the first day of alcoholic fermentation was confirmed as GOX activity, GOX activated by molecular O_2 residing in the inoculum. The reaction takes place immediately, within a matter of minutes (results not shown).

Figure 5.4 (b) presents similar results as Figure 5.4 (a). Although the fermentation took one day longer to complete, glucose was depleted one day earlier than fructose. A similar increase in GA ($\approx$ 0.6 g/L) was observed, and again the increase was observed only during the first day that can be explained by GOX activated by molecular O_2 residing in the inoculum. During the remaining 6 days (until the fermentation were completed) the levels of GA remained the same ($\approx$ 0.95 g/L).

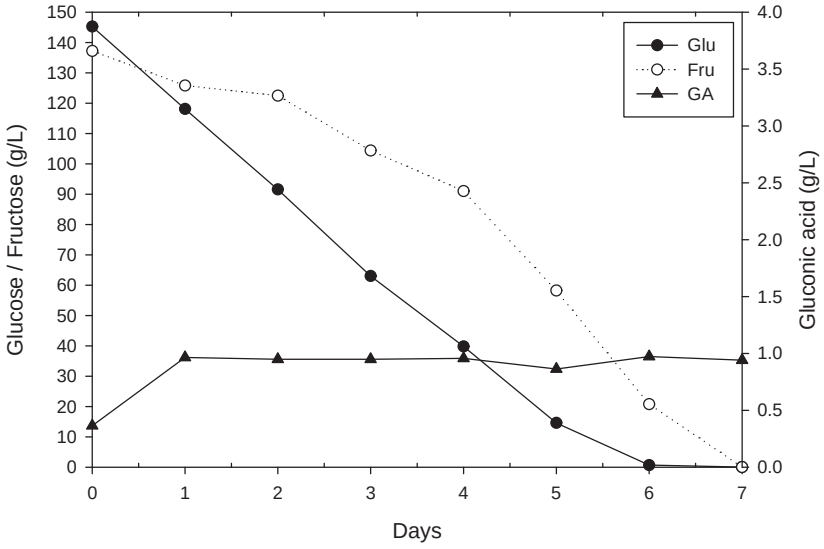
These anaerobic fermentations, performed in grape juice, indicate that in the presence of high concentrations of glucose, but in the absence of molecular O_2, the enzyme glucose oxidase cannot convert glucose to GA.

5.3.3.2 Sugar consumption and gluconic acid production under aerobic conditions

Figures 5.5 (a) and 5.5 (b) represent duplicate fermentations and indicate the rates at which glucose and fructose are consumed as well as the rate at which GA is produced during aerobic (U1) conditions. In Figure 5.5 (a) $\approx$ 139 g/L glucose was depleted in only 3 days and $\approx$ 131 g/L fructose was consumed in 4 days. During the course of the fermentation there was an overall increase of $\approx$ 3.1 g/L GA. All

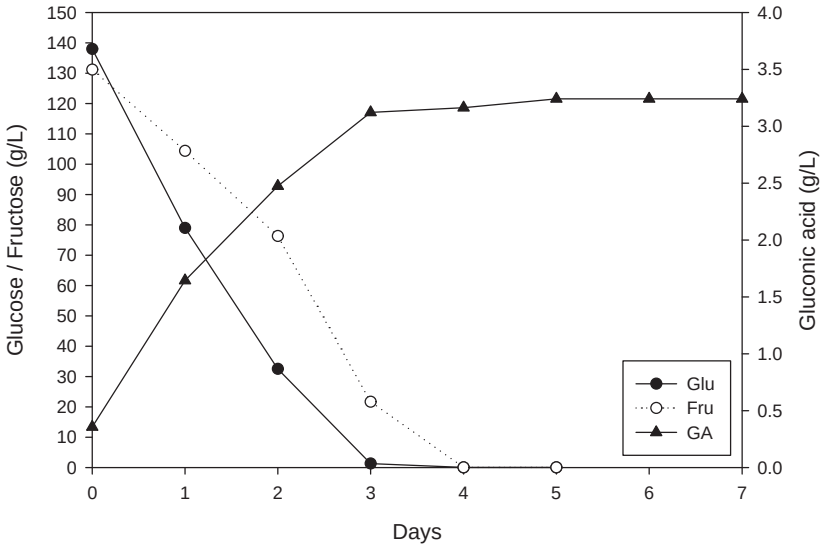


(a) Anaerobic wine fermentation (WF1-U0).

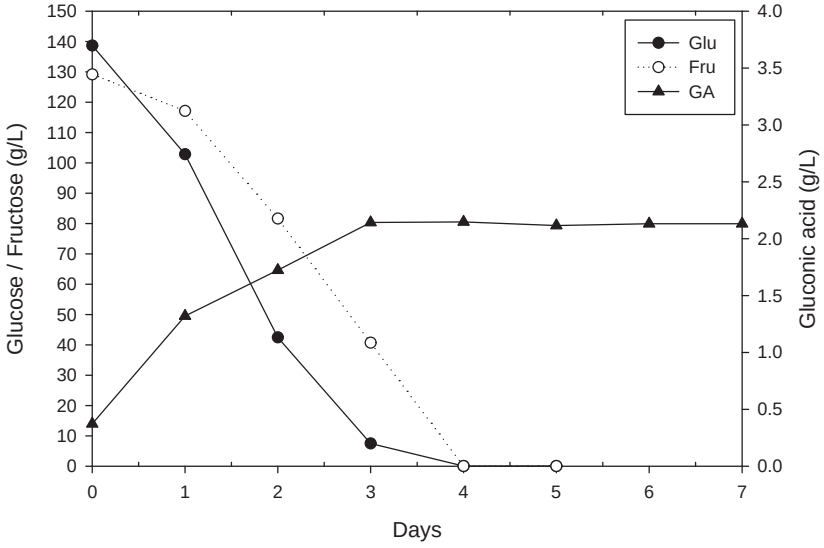


(b) Anaerobic wine fermentation (WF3-U0).

Figure 5.4: Anaerobic batch fermentations in the bioreactor using grape juice. (a) Wine fermentation (WF1-U0) and (b) Wine fermentation (WF3-U0).



(a) Aerobic wine fermentation (WF2-U1).



(b) Aerobic wine fermentation (WF4-U1).

Figure 5.5: Aerobic batch fermentations in the bioreactor using grape juice. (a) Wine fermentation (WF2-U1) and (b) Wine fermentation (WF4-U1).

the GA was produced during the first 3 days, whereafter the levels stabilized and no additional GA were formed. What can clearly be observed on the plot in Figure 5.5 (a) is that GA is only formed as long as there is glucose available. After three days no GA is produced despite the availability of molecular O_2. This result indicates that both glucose and molecular O_2 must be present in the grape must, for GA to be formed.

Fermentation WF4-U1 in Figure 5.5 (b) presents similar results as fermentation WF2-U1 in Figure 5.5 (a). Again glucose was depleted in approximately 3 days and all the fructose was depleted in 4 days. Although less GA ($\approx$ 1 g/L) was formed during this aerobic fermentation, the same trend is observed as in WF2-U1, Figure 5.5 (a). GA is only formed as long as there is glucose present, despite of the availability of an unlimited amount of molecular O_2.

These aerobic must fermentations indicate that in the presence of high concentrations of glucose, and in the presence of molecular O_2, the enzyme glucose oxidase can convert glucose to GA in grape must during alcoholic fermentation.

5.3.4 Model for reduced alcohol adjustments

To demonstrate our ability in modeling ethanol reduction in wine we will consider a wine fermentation carried out with grape juice containing 261 g/L sugar (26 Brix). A calculation that winemakers use to determine the potential level of alcohol in finished wines suggests that approximately 18 g/L of sugar will be converted to 1% (v/v) of ethanol during alcoholic fermentation (Ribéreau-Gayon *et al.*, 2006), resulting in a wine with an alcohol concentration of 14.5% (v/v). This calculation takes into account all alcohols, and not only ethanol.

5.3.4.1 Over-simplified model under 'ideal' circumstances

Considering the high alcohol wine (14.5% v/v), and the positive experimental results indicating that the VIN13-Δ1 wine yeast's active GOX were able to convert glucose to GA in grape must during alcoholic fermentation using glucose as substrate and micro-oxygenation, we could use the VIN13-Δ1 wine yeast to reduce the levels of alcohol. An alcohol reduction of 2% (v/v) in final alcohol concentration in 1L wine will serve as an example.

The theoretical conversion of 1 mol (180g) of sugar into 2 mol (92g) of ethanol and 2 mol (88g) of CO_2 could only be expected in the absence of any yeast growth

and loss of ethanol as vapor. Equation 5.3.1 presents the stoichiometric reaction for this conversion.

$$C_6H_{12}O_6 \longrightarrow 2C_6H_{12}O_6 + 2CO_2 \tag{5.3.1}$$

To reduce the alcohol by 2% (v/v), also equivalent to 15.78 g/L (0.343 mol/L) alcohol, we have to convert 30.78 g/L (0.1715 mol/L) sugar to GA.

The kinetic parameters for GOX from *A. niger* are the following: The Michaelis constant (K_m) is 33 mM (Swoboda and Massey, 1965) and the maximal limiting rate velocity (V_{max}) is 458 U/mg (Kalisz *et al.*, 1991; Bankar *et al.*, 2009). Furthermore, one GOX unit will oxidize 1.0 μmol of β-D-glucose to D-glucono-δ-lactone and H_2O_2 per min at pH 5.1 at 35°C, equivalent to an O_2 uptake of 22.4 μL per min. If the reaction mixture is saturated with oxygen, the activity may increase by up to 100% (Sigma-Aldrich). The main enzymatic reaction for the conversion of glucose to GA is shown in Equation 5.3.2.

$$C_6H_{12}O_6 \xrightarrow[O_2]{GOX} C_6H_{12}O_7 + H_2O_2 \tag{5.3.2}$$

From the above information, it is clear that one GOX unit will take 171500 minutes (or 120 days) to converted 30.78 g/L (0.1715 mol/L) sugar to 30.78 g/L GA. In chapter 3 results indicate GOX activity of $\approx$ 250,000 Units/L (after 9 h). If this level of units are present, it will take only 1 minute (41.16 seconds) to convert 30.78 g/L (0.1715 mol/L) sugar to 30.78 g/L GA.

This demonstrates the potential advantage of using VIN13-Δ1 during a fermentation to adjust final alcohol levels.

5.3.4.2 Considerations for wine parameters

The above over-simplified model can only be expected to work in the absence of a number of factors that contribute to the complexity and dynamics of a wine fermentation. Table 5.1 lists some of the factors that could contribute to the complexity of a GOX-fermentation. Oxygen will have the greatest influence on the enzymes ability to successfully perform the conversion. To saturate wine, approximately 6–8 mg/L of O_2 is necessary. Boulton *et al.* (1996) reported on the levels of O_2 required to

Table 5.1: Factors that can contribute to fermentation complexity and GOX enzyme reaction

Must composition	Yeast strain (VIN13-Δ1)
- Initial sugar concentration - Yeast assimilable nitrogen (YAN) - Temperature - pH - Glucose:Fructose ratio - Oxygen in must at start of fermentation - Free SO_2 concentration	- Inoculum size - Growth rate (increase in biomass) - Respiration - The rate at which additional GOX is produced during fermentation
Fermentation kinetics	**GOX enzyme kinetics**
- Fermenter size (volume) and shape - Temperature - Agitation - Carbon dioxide (CO_2) release - Aeration	- Enzyme concentration at start of fermentation - Increase in enzyme concentration during fermentation - pH - Temperature - Oxygen availability (time) - Substrate (glucose) availability

produce certain wine styles. Different amounts of saturation are also necessary for white and red wines. 10 Saturations can lead to white wine becoming oxidized, but it is well known that even fewer additions may lead to a reduction in the fruitiness of wine. In red wine however, 10 saturations can improve the quality of the wine (Du Toit *et al.*, 2006). The concentration of O_2 available for the GOX-reaction will in addition be influenced by the amount that dissolves into the wine during fermentation. This concentration will be influenced by the amount of CO_2 that is released because of alcoholic fermentation. Furthermore, the yeast can also compete with the other factors for the dissolved O_2. SO_2 that is added to the must at the beginning of fermentation to prevent spoilage organisms can also bind the molecular O_2, thus further reducing the availability of O_2 in the must. Finally, some unknown chemical components in the must might also reduce enzyme efficiency.

5.4 Conclusions

Strain development is no longer limited to the primary role of wine yeasts, namely to catalyze the rapid and complete conversion of grape sugars to alcohol and car-

bon dioxide without distorting the flavor of the final product. Today, there is a much stronger emphasis on the development of wine yeasts for the cost-effective production of wine with minimized resource inputs, improved quality and low environmental impact (Pretorius, 2003).

This work has shown that GOX produced and secreted from a genetically engineered industrial wine strain could be activated by micro-oxygenation and successfuly convert glucose to GA. Furthermore, the enzyme activity could be inhibited by stopping micro-oxygenation. This demonstrate the potential advantage of using VIN13-Δ1 during an wine fermentation to bio-adjust final alcohol levels to appropriate levels in commercial wines. This might offer a viable way to meet consumers' demands for affordable low-alcohol wine and have financial implication in savings on wine taxation.

Further work is required however, to investigate the organoleptic quality of wines that have under gone micro-oxygenation and in addition have increased levels of gluconic acid. The specific kinetics of the glucose oxidase enzyme and its activity after it is produced by VIN13-Δ1 should also be investigated as well as the specific fermentation parameters that will improve or hinder optimal enzyme activity during a fermentation in grape must. In addition, it would be worth while to include a purified form of the VIN13-Δ1 produced GOX to assist the understanding and modeling of reduced alcohol levels.

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Chapter 6

Overview, Final Discussion, Concluding Remarks and Strategies for Future Research

6.1 Overview

Like most other consumer products, yeast fermented beverages such as wine and beer are continuously subjected to the forces of market-pull and technology-push. Supply of wine to the consumer has therefore become intriguingly complicated as the wine marketplace has become more competitive than ever with improved wine quality and marketing sophistication. Furthermore, local markets are also influenced by global competitor countries' wine markets as trends indicate that the health benefits of wine can be found in the balance of a good diet that is supplemented with moderate levels of good quality wine (Peregrin, 2005; Paganini-Hill *et al.*, 2007; Walzem, 2008). In addition, wines containing less alcohol provide all the benefits without the toxicity, and is much more affordable because of reduced taxation (in certain countries) of these products (Halpern, 2008).

Therefore it comes as no surprise that over the past decade a drop in the consumption of high alcohol beverages (10–13%) was observed and at the same time, an increase in the consumption of beverages with a lower alcohol content. Factors driving this phenomena are stricter drink-driving legislation, the awareness of health risks arising from excessive alcohol intake (Shults *et al.*, 2001; Erten and Campbell, 2001; Room *et al.*, 2005), and to some extend new 'fashion' trends. These obser-

vations contribute to the development of new international and domestic consumer markets, which can benefit the wine industry by increasing the demand and consumption for wines with reduced alcohol concentrations in the final product. Such wines can be categorized as reduced alcohol (1.2% to 5.5–6.5% v/v), low alcohol (0.5–1.2% v/v) and even de-alcoholized (not above 0.5% v/v) wines (Scudamore-Smith and Moran, 1997; Pickering *et al.*, 1998; Gladstones and Tomlinson, 1999; Gladstones, 2000; Pickering, 2000).

As new trends in the upper end of these highly competitive and sophisticated wine markets continue to call for a diversity of innovations and due to the demanding nature of modern winemaking practices, there is an ever-growing quest for specialized wine yeast strains possessing a wide range of optimized, improved or novel enological properties (Pretorius, 2000; Pretorius and Bauer, 2002; McBryde *et al.*, 2006). Strain development is no longer limited to the primary role of wine yeasts, namely to catalyze the rapid and complete conversion of grape sugars to alcohol and carbon dioxide without distorting the flavor of the final product. Today, there is a much stronger emphasis on the development of wine yeasts for the cost-effective production of wine with minimized resource inputs, improved quality and low environmental impact (Pretorius, 2003). Research in this field is continually expanding, very active and the prospect of developing wine yeast starter culture strains expressing heterologous enzymes is also available (Whittington *et al.*, 1990; Park *et al.*, 2000; Van Rensburg and Pretorius, 2000; Kapat *et al.*, 2001; De Barros Lopes *et al.*, 2003; Cambon *et al.*, 2006; Nevoigt, 2008).

The yeast *Saccharomyces cerevisiae* is the most widely used industrial microorganism with GRAS (Generally Regarded As Safe) status and has long been considered one of the most popular organisms for molecular genetic studies. With the announcement of the complete nucleotide sequence of the yeast genome on 24 April 1996, there was a renewed interest in the genetic improvement of commercial yeast strains (Goffeau, 2000). The traditional use of the yeast *S. cerevisiae* in alcoholic fermentation has, over time, resulted in a substantial accumulated knowledge base concerning genetics, physiology, and biochemistry as well as genetic engineering and fermentation technologies. *S. cerevisiae* has become a platform organism for developing metabolic engineering strategies, methods and tools (Marks *et al.*, 2008; Nevoigt, 2008). This knowledge was further increased with the discovery that in-

dustrial strains contain additional DNA that may encode proteins that are unique to spesific industrial strain (Borneman *et al.*, 2008*a*,*b*).

Although gene technology has already contributed enormously to our basic understanding of the yeast, it will be unwise to entertain unrealistic expectations about rapid commercialization and short-term benefits for recombinant DNA technology in the wine industry (Pretorius and van der Westhuizen, 1991). The fact that to date there are only two recombinant yeast strains used on a commercial scale in the wine industry proofs that people are still hesitant to embrace this 'new' technology. The two recombinant yeast strains that were accepted are ML01 commercialized by BioSpringer, a division of Lesaffre (Cebollero *et al.*, 2007) and 522^{EC-} (Coulon *et al.*, 2006). There are enormous benefits to both the wine consumer and the industry in the application of this exciting technology and the first recombinant wine products therefore should unmistakably demonstrate organoleptic, hygienic and economic advantages for the wine producer and consumer. Credible means must be found to effectively address the concerns of traditionalists within the wine industry and the negative overreaction of some consumer groups (Pretorius, 2000, 2001; Vivier and Pretorius, 2002; Deng *et al.*, 2008; Tamis *et al.*, 2009). Thus, the successful application, as well as the commercialization of transgenic wine yeasts, should not affect the wine's most enchanting and fascinating aspects, namely its diversity of style, wholesomeness and sensory quality (Pretorius, 2003).

A major challenge in warmer climate regions is the rapid accumulation of grape sugars, resulting in a premature harvest and picking of grapes before they have reached phenolic ripeness. The sugar content increases even more when grapes are left longer on vines to obtain optimum (phenolic) ripeness and allow for enhanced flavor and aroma compound synthesis. Such wine fermentation could start with about 25–28% sugar (25–28 Brix) and in these circumstances the high sugar concentrations may cause problematic sluggish or stuck fermentations, either in prefermented grape juice with an inhibitory effect of sugar concentration (glucose:fructose), or towards the end of alcoholic fermentation with high ethanol concentration (Bisson, 2005*a*,*b*; Snyman, 2006; Malherbe *et al.*, 2007). Astute monitoring of the fermentation can assist the winemaker in early identification of problem fermentations, and contribute to economic savings by reducing wasted time of having to first diagnose the problem followed by monetary expenditures to rectify a stuck and/or sluggish fermentation (Bisson and Butzke, 2000; Lourens and Reid,

2003). Unfortunately, high sugar must lead to finished wines containing between 14 and 16% alcohol (v/v) which can be regarded as high alcohol wines. These types of problems and consumer demands call for research in methods to reduce or remove excess alcohol.

There are a number of physicochemical and biological de-alcoholization methods available, of which some are rather expensive, labour intensive and/or result in wines with spoiled organoleptic properties.

Several physicochemical processes exist that are used specifically for the removal or the reduction of alcohol in wine, sometimes in combination (Massot *et al.*, 2008; Sutherland, 2008). These processes tend to involve expensive equipment and can also be intensive from the view of processing (Bui *et al.*, 1986; Scudamore-Smith and Moran, 1997; Scott, 1996; Mermelstein, 2000; Leeper, 2001; Pickering, 2000; Baker, 2004; Varavuth *et al.*, 2009). Concerns have also been raised about the sensory quality of the finished product (wine) and, in addition, partial or incomplete fermentation and the fermentation of immature grapes with a low sugar content can have the inherent problem of excess residual sugar and a lack of flavor development in the resulting wine (Pickering *et al.*, 1999*c*, 2001).

The concept of treating grape must with the enzyme glucose oxidase (GOX), which has GRAS status, to reduce the alcohol level in wine after fermentation (Villettaz, 1987; Pickering and Heatherbell, 1996; Pickering *et al.*, 1999*a*,*b*,*c*, 1998), was introduced as an alternative approach to the expensive and controversial physicochemical methods. During the enzymatic reaction, β-D-glucose is converted to D-glucono-δ-lactone and hydrogen peroxide (H_2O_2) in the presence of oxygen, which is non-enzymatically reduced to gluconic acid (GA). Thus, after GOX treatment, the total amount of glucose that will be metabolized to form ethanol is reduced. Furthermore, gluconic acid can not be metabolized to form ethanol by *S. cerevisiae*. Wines produced in this way should have reduced levels of ethanol and higher acidity. This technology could also be employed to produce a reserve of acidic musts or wines for blending purposes (Canal-Llaubères, 1993).

A lot of research has been done using molecular techniques and genetic engineering to attempt engineering of a yeast strain that can reduce the ethanol content

of wine successfully. There are several yeast that are able to reduce the ethanol content, but all these strains produce in addition, chemical compounds that have a negative impact on wine sensory (Michnick *et al.*, 1997; De Barros Lopes *et al.*, 2003; Cambon *et al.*, 2006). Malherbe *et al.* (2003) successfully genetically engineered a *S. cerevisiae* strain to produce GOX during alcoholic fermentation. GOX that was secreted into the grape juice was able to convert some of the glucose into GA and reduced the total ethanol at the end of fermentation by 1.8–2%. The advantage of this method over other molecular strategies is that the reaction takes place outside the yeast cell and thus, does not affect the redox balance inside the cell leading to the production of unwanted metabolites.

6.2 Final Discussion

This study forms part of a comprehensive research program on reduced alcohol wines within the Institute for Wine Biotechnology (Malherbe *et al.*, 2003) and outlines the investigation of industrial yeast starter cultures to produce reduced alcohol wines. The industrial yeast VIN13 was genetically modified to encode the *Aspergillus niger* glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) for the production of reduced-alcohol wines.

In the first phase of this study we have successfully integrated a stable copy of the *GOX* gene encoding the *A. niger* glucose oxidase into the genome of two laboratory yeast strains (*S. cerevisiae* BY4742 and Σ1278b) and one industrial yeast strain (VIN13). Secretion of GOX, was facilitated by either the yeast mating pheromone α-factor secretion signal (MF$\alpha 1_S$) or the native *A. niger* secretion signal of GOX. To perform integrations into industrial strains, which are usually diploid or polyploid, the CreloxP-system for repetitive gene integration and genetic marker recovery (Güldener *et al.*, 1996, 2002; Hegemann *et al.*, 2006) was used. The site picked for integration into VIN13 was the *IME1* gene locus. The Ime1p is the master regulator of meiosis, and ensures the survival of diploid cells under nutritional limiting conditions by producing spores (Kassir *et al.*, 2003).

Successful genomic integrations were confirmed in all strains by either PCR or Southern Blot. By means of assays, it was determined that GOX is indeed tran-

scribed, secreted into the medium and biologically active. The transformed yeast expressed the highest amount of GOX during its exponential growth phase. It was also observed that the transformed yeasts secrete most of the total enzymes produced and that only a small fraction remained intracellular. More enzyme was produced from BY4742 and VIN13 yeast strains where secretion was facilitated by the native *A. niger* secretion signal of GOX. This demonstrated that the *A. niger* native GOX secretion signal was indeed recognized by *S. cerevisiae* and that it initiated secretion that was more efficent compared to using the yeast mating pheromone α-factor secretion signal. In addition, industrial strains secreted GOX to high levels, approximately 3–6.4 times more than reported previously (Malherbe *et al.*, 2003). Low levels of D-gluconic acid was also measured during the fermentations, indicating that conversion of β-D-glucose to D-gluconic acid took place. This is the first time that GOX is produced and secreted by industrial wine yeasts strains.

Integration of the *GOX2LOX* gene cassette into the genome of the diploid VIN13 strain was targeted at the master regulator of meiosis locus, *IME1*, by using the CreloxP-system for repetitive gene integration and genetic marker recovery (Güldener *et al.*, 1996, 2002; Hegemann *et al.*, 2006). By replacing *IME1* with the *GOX2LOX* gene cassette, it was hoped to generate an asporogenic, GOX-producing wine strain. Unfortunately, it was not possible to successfully recover the genetic marker and use the same *GOX2LOX* cassette for the second deletion/integration event. Various factors that could influence or have an effect on the CreloxP-system were investigated, none of which yielded positive results. Therefore the specific reason for not recovering the genetic marker cannot be stated with certainty. A personal communication with the developer of the CreloxP-system (Güldener *et al.*, 1996, 2002) proved helpful, and it can be concluded that the problem is probably strain related. The system does not always work in all yeast strains, and is more difficult to use in industrial yeast strains. It is suggested that research is needed and further trials are called for.

VIN13-Δ2 was obtained by sporulation, micromanipulation and re-diploidization of VIN13-Δ1. This resulted in the loss of the remaining *IME1* allele generating a *IME1*Δ0 genotype. This strain also transcribed and secreted active GOX, was diploid but was unable to sporulate. This sterilized industrial strain should be unable to sporulate and survive under nutritional limiting conditions, thereby also limiting unintended exchange of genetic material with ambient sporulating yeasts, as

demonstrated by Ramírez and Ambrona (2008). We have therefore generated an industrial wine yeast secreting GOX to high levels, that is unable to reproduce sexually. This alternative strategy is inconvenient as VIN13-Δ2 is not a 'real' VIN13 anymore, but re-diploidized single spore culture of VIN13-Δ1, and should be evaluated under winemaking conditions to obtain its organoleptic profile.

In the second phase of this study, we evaluated the ability of the genetically engineered industrial wine strain VIN13-Δ1 to produce and secrete the glucose oxidase enzyme, under small-scale fermentations of grape juice made from white and red cultivars, to compare the enzyme activity under different winemaking conditions.

We showed by means of the GOX plate assay that alcoholic fermentation was performed by VIN13 and VIN13-Δ1 strains, respectively, and that 98% of the colonies tested from VIN13-Δ1 fermentations were still able to produce GOX. This confirms that the expected strain performed alcoholic fermentation in all the batches and that the integration was stable.

Increased levels of gluconic acid was detected in all the VIN13-Δ1 fermentations, indicating that there was GOX activity. GA concentrations were determined by enzymatic assays and the higher levels of gluconic acid detected were still lower than expected. A shortage of molecular oxygen in the must to drive the conversion of glucose to GA by GOX would explain this observation. The largest increase in GA was observed in Sauvignon blanc (80 mg/L) and Merlot (350 mg/L). Higher levels of GA were measured in the red wines and can be correlated to the fact that the wines were fermented on the skins in open buckets. Furthermore the skins were punched down during the first 3 days of alcoholic fermentation, increasing the exposure of the wines to molecular oxygen and resulting in better activity of the GOX enzyme. In addition, it is possible that as a result of the SO_2-binding power, less GA was detected in wines that received SO_2 at crushing. The assay that we used to detect levels of gluconic acid in the various wines measures the 'free' GA, thus we were unable to determine the levels of SO_2 bound GA.

Finished wines were analyzed by FT-MIR and GC-FID to determine its chemical composition. Minimal levels of reduced ethanol concentrations were obtained after completion of alcoholic fermentation. Cabernet Sauvignon wines showed the greatest reduction (0.15% ethanol (v/v) which coresponds to 2.34 g/L glucose consumed [0.064 % (v/v) ethanol = 1 g/L glucose]). Cabernet Sauvignon did not show the highest levels of GA, but the loss in ethanol corresponds to the approximate

increase in GA (3 g/L).

Two-way ANOVA analysis of variation was performed to determine the influence of yeast strain and cultivar on the total amount of ethanol (% v/v) produced during alcoholic fermentations. The results of the analysis of variation indicated that there were no differences in the strain-cultivar relationships.

Results obtained from GC-FID indicated that of all the aroma compounds measured in white wines, the esters contributed the most to the aroma component, and that the acid and fatty acid component along with the alcohols and higher alcohols contributed the most to the aroma component in red wines. Despite these conclusions, the overall aroma profiles for the different wines were very similar.

Multivariate data analysis was employed as a tool to provide a holistic picture to visualize the effects of VIN13-Δ1 fermentation with compared to VIN13 fermentations (excluding the Chardonnay that did not received SO_2 at crushing), taking into account the alternative chemical parameters (MIR-spectra, MIR-compounds, GC-compounds and Gluconic acid (GA) alone, or in various combinations).

Chemometric projections of the score plots of both t1-t2 as well as t1-t3 plots for all results provided insight into all significant variation up to three principal components (PCA) or PLS components, which showed very clearly that GA is a key factor in evaluating the effect of GOX in VIN13-Δ1 fermentation in comparison to VIN13 fermentations.

The VIN13-Δ1 effect manifestations were best shown on PLS1-discrim score plots that revealed that the full impact of the 'GC-compounds + GA' parameter set on the discriminability between VIN13 and VIN13-Δ1-yeast fermentations. Clear cultivar differences with very noticeable increased discriminability between VIN13 and VIN13-Δ1 fermentations was observed with the greatest discriminability in cultivars Merlot and Pinotage, followed by Shiraz and Chardonnay. PLS1 discriminability for 'MIR-compounds + GA' indicated the greatest discrimination towards the cultivar Merlot. Of the restricted variable subsets the MIR-compounds and GC-compounds yielded better results, with the GC-compounds displaying greater discriminability between cultivars and VIN13 / VIN13-Δ1. One can conclude from these results that the greatest influence of VIN13-Δ1 produced wines would be observed in the aroma components, but as there was discriminability effects discernable in the MIR-compounds, the favor component are also affected.

From the results it is also clear that no satisfactory model of the GA concentration can be established based on the MIR spectra alone. Detailed inspection of our model reveals that only 61% of the y-variance could be modeled (using only 25% of the X-variance). The interpretation of these quantitative features is that the information residing in the GA variable cannot be gotten from the MIR spectral information alone. It is thus necessary to actively measure the GA level.

Finally, the PLS1-discrim approach consistently improved the discriminability versus PCA. PCA alone shows some of the same features only, but not as clearly. Inclusion of GA in any of the three alternative variable sub-set greatly improves the discriminability for VIN13-Δ1 (GOX-effect) visualization and allows better quantification in the full multivariate displays. Gluconic acid (GA) is thus concluded to constitute a significant chemical variable for the discrimination of VIN13-Δ1 fermentations and evaluation of the GOX-effect.

In the final phase of this study we performed various fermentations in bioreactors and have demonstrated that glucose oxidase secreted from VIN13-Δ1 is active in synthetic medium as well as grape juice during alcoholic fermentation, and that oxygen plays a key-role in enzyme activation.

Experiments performed in synthetic medium showed that it is possible to obtain a higher cell density under aerobic conditions compared to an anaerobic environment. Furthermore, we demonstrated that with the addition of molecular O_2 during an anaerobic fermentation by means of micro-oxygenation, the rate of cell growth increases and a higher cell density can be obtained as compared to a complete anaerobic fermentation. The increase in cell density was expected as the respiratory pathway was enabled and more ATP is produced. This additional energy is used to produce additional biomass through more vigours cell growth. This increased growth rate should result in an increased GOX production rate as they are linked (Bankar *et al.*, 2009).

In addition, no gluconic acid was produced during anaerobic conditions in synthetic medium, whereas production of GA was enhanced and measured 9.7 $\times$ higher under aerobic conditions. The specific rate at which GA is produced during either aerobic or anaerobic conditions remained constant. However, it was clearly observed that when micro-oxygenation was applied to an anaerobic fermentation GOX can be activated and the rate at which GA is formed is similar to the rate under aerobic conditions. Furthermore, as soon as micro-oxygenation was stopped,

the rate at which GA was formed returns to the previous state, before activation of GOX, that was similar as that observed under anaerobic conditions. This demonstrates that as long as there is glucose, micro-oxygenation could be used to activate GOX at any time during a fermentation.

In grape juice, anaerobic fermentations indicate that in the presence of high concentrations of sugar, specifically glucose, but in the absence of molecular O_2, GOX cannot convert glucose to GA. Aerobic must fermentations indicate that in the presence of high consentrations of sugar (glucose) and in the presence of molecular O_2, GOX can convert glucose to GA in grape must during alcoholic fermentation. This indicates that GOX can convert glucose to GA during alcoholic fermentation in grape must as long as there is substrate (glucose), and molecular O_2 that is necessary to drive the enzymatic reaction.

Finally we were able to show with the help of a over-simplified model that under 'ideal' conditions GOX, secreted from VIN13-Δ1, can be employed to reduce the ethanol by a predefined concentration.

6.3 Concluding Remarks

Glucose oxidase from *A. niger* is of considerable industrial importance and has previously been produced in heterologous host organisms (e.g. *S. cerevisiae*) (Frederick *et al.*, 1990; De Baetselier *et al.*, 1991; Hodgkins *et al.*, 1993; Kapat *et al.*, 2001; Malherbe *et al.*, 2003). In this study, we integrated the GOX-encoding gene from *A. niger* into an industrial wine strain (VIN13) for the first time. The resulting strain VIN13-Δ1 was able to transcribe and secrete active enzyme to a high concentration.

We obtained a second industrial wine strain, VIN13-Δ2, by sporulation, micromanipulation and re-diploidization of VIN13-Δ1 which also secrete active GOX to high concentrations. This resulted in the loss of the remaining *IME1* gene, and a *IME1*Δ0 genotype. This strain is diploid and unable to sporulate. This sterilized industrial strain should be unable to sporulate and survive under nutritional limiting conditions, thereby also limiting unintended exchange of genetic material with ambient sporulating yeasts. We therefore obtained an industrial wine yeast is unable to reproduce sexually and secrete active GOX to high concentrations.

This work has also shown that a genetically engineered industrial wine strain was able to produce and secrete the glucose oxidase enzyme, under winemaking

conditions. Furthermore, we have shown that the enzyme efficiency, correlated to the final ethanol concentrations, was sub-optimal and that molecular oxygen is of utmost importance for the activation of the enzyme and would play a key part to the understanding and successful production of reduced alcohol wines. Lastly, we presented results that indicated the aroma and flavor profiles of VIN13/VIN13-Δ1 wines differed only slightly. To determine the specific level where GA could possibly have an impact on the wine bouquet in fermentations with VIN13-Δ1, the GOX-reaction should be optimized to produce more GA under winemaking conditions.

We performed various fermentations performed in bioreactors and have demonstrated that glucose oxidase secreted from VIN13-Δ1 is active in synthetic medium as well as grape juice during alcoholic fermentation, and that oxygen plays a key-role in enzyme activation. We further confirmed that GOX is active under aerobic conditions, inactive under anaerobic conditions, and can be activated instantaneously when an anaerobic culture is switched to aerobic conditions (simulated micro-oxygenation).

Finally, we showed with the help of a simplified model, that under ideal conditions, GOX secreted from VIN13-Δ1, can be employed to reduce the ethanol by a predefined concentration.

6.4 Strategies for Future Research

The use of a genetically engineered yeast that produce and secrete GOX for reduction of ethanol by converting glucose to gluconic acid is at the moment the best genetic engineering strategy. The reason is that the reaction takes place outside the yeast and does not affect the redox balance which result in the production of unwanted metabolites. However, this strategy does not work under standard winemaking conditions. Additional molecular oxygen is necessary to activate GOX. We suggest semi-industrial wine fermentations with micro-oxygenation to activate the enzyme. Furthermore, wines will have to be analyzed to determine the effect of additional oxygen on color, flavor and aroma compounds in finished wines.

A pure form of the enzyme could assist in these experiments. It would be even better if GOX produced by VIN13-Δ1/2 could be isolated, purified, characterized

and used in these experiments. It would mean that the optimization of the GOX-system is for the yeast produced enzyme, and also reduce cost as pure enzyme bought, could be more expensive.

A proper model should be employed to test and verify that it is possible to predict ethanol reduction by a predefined concentration. This model should try taking into consideration most of the parameters of a wine fermentation. These could include volume, temperature, oxygen, agitation, shape and size of fermentation unit, GOX concentration, time exposure of enzyme to oxygen, yeast affinity for oxygen, yeast growth rate and biomass formation. Once the model works, trail fermentations on a larger scale could be performed to evaluate ethanol reduction.

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