Evaluating the effect of different winemaking techniques on ethanol production

by

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Declaration

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Summary

Over the years, different techniques have been used to legally reduce the ethanol content of wines. Several physical processes are available for producing wines with less alcohol. Despite their efficacy, these treatments have a capital and operational cost influence. They can also affect the concentration of other wine components. On the other hand, vast amount of research has been conducted through genetic modification of wine yeast strains in order to reduce the ethanol yield of *Saccharomyces cerevisiae* by diverting sugar metabolism towards various by-products. However, genetically modified yeasts are not currently accepted in most wine industries worldwide, including South Africa. Therefore, other approaches need to be envisaged.

Commercial enzymes are commonly added during winemaking. Most enzymes essential for vinification naturally occur in grapes, but are inefficient under pH and sulphur levels associated with winemaking. Enzymes of fungal origin are resistant to such conditions. The most widely used commercial enzymes include pectinases, hemicellulases, glucanases and glycosidases. With the exception of glucanases, produced by *Trichoderma harzianium*, all the other enzymes are produced by *Aspergillus niger.*

In this study, the possibility of using Gluzyme Mono® 10.000 BG (Gluzyme) (Novozymes, South Africa) to reduce the glucose content of synthetic grape must and grape must before fermentation in order to produce wine with a reduced alcohol content was investigated.
Gluzyme is a glucose oxidase preparation from *Aspergillus oryzae*, currently being used in the baking industry. Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide in the presence of molecular oxygen.

Gluzyme was initially used in synthetic grape must where different enzyme concentrations and factors influencing its activity were investigated for its use in winemaking. The results showed that up to 0.5% v/v less alcohol were obtained using an enzyme concentration of 20 kU compared to the control. This reduction in alcohol was increased to 1 and 1.3% v/v alcohol at pH 3.5 and pH 5.5 respectively in aerated synthetic grape must using 30 kU enzyme.

Secondly, Gluzyme trials were carried out using Pinotage grape must. Gluzyme treated wines after fermentation contained 0.68% v/v less alcohol than the control samples at 30 kU enzyme. Colour and volatile flavour compounds of treated wine did not differ significantly from the untreated samples. Lower free anthocyanin and total phenol concentrations in treated than control samples were observed, possibly due to the hydrogen peroxide oxidation which could have led to polymerisation.

The present study has clearly demonstrated that Gluzyme may be used in winemaking to produce reduced-alcohol wine without affecting its colour and aroma compounds. The enzyme in its current form is however, not ideal for winemaking; other forms such as liquid or powder form should be considered if the enzyme is to be used under winemaking conditions.

Future work should focus on evaluating the potential new form of the enzyme and studying the effects of Gluzyme in various grape must in semi-industrial scale. A tasting panel should also evaluate its impact on the organoleptic properties and the overall quality of the resulting wines.

Opsomming

Oor die jare is verskillende tegnieke aangewend om die etanolinhoud van wyne op wettige maniere te verlaag. Daar is verskeie fisiese prosesse beskikbaar om wyn wat minder alkohol bevat, te produseer. Ondanks die doeltreffendheid van hierdie prosesse, word kapitale en operasionele koste daardeur beïnvloed. Die prosesse kan ook 'n invloed hê op die konsentrasie van ander komponente in die wyn. Daarteenoor is baie navorsing gedoen oor die genetiese verandering van wyngiste om die etanol-opbrengs van *Saccharomyces cerevisiae* te verminder deur die suikermetabolisme na verskeie byprodukte te analiseer. Tans word geneties veranderde gis egter nie in die meeste wynbedrywe wêreldwyd, ook in Suid-Afrika, aanvaar nie. Daarom moet ander benaderings in die vooruitsig gestel word.

Kommersiële ensieme word oor die algemeen gedurende die wynbereidingsproses bygevoeg. Die meeste ensieme wat noodsaaklik is vir wynbereiding kom natuurlik in druiwe voor, maar is ondoeltreffend op die pH- en swaelvlakke wat met wynbereiding geassosieer word. Swamagtige ensieme is bestand teen sulke toestande. Die kommersiële ensieme wat die meeste gebruik word, sluit in pektinase, hemisellulase, glukanase en glikosidase. Behalwe vir glukanase, wat deur *Trichoderma harzianium* geproduseer word, word al die ander ensieme deur *Aspergillus niger* geproduseer.

In hierdie studie is die moontlikheid ondersoek om Gluzyme Mono® 10.000 BG (Gluzyme) (Novozymes, Suid-Afrika) te gebruik om die glukose-inhoud van sintetiese mos te verminder voordat fermentasie geskied, om sodoende wyn met 'n verminderde alkoholinhoud te maak. Gluzyme is 'n glukose-oksidasepreparaat van *Aspergillus oryzae,* wat tans in die bakbedryf gebruik word. Glukose-oksidase dien as katalisator om die oksidasie van glukose na glukoon-suur en waterstofperoksied in die teenwoordigheid van molekulêre suurstof te bewerkstellig.

Gluzyme is oorspronklik in sintetiese druiwemos gebruik, waar verskillende ensiem-konsentrasies en faktore wat die ensieme se aktiwiteite beïnvloed, ondersoek is vir gebruik in wynbereiding. Volgens die uitkoms van die navorsing, is tot 0.5% v/v minder alkohol verkry wanneer 'n ensiemkonsentrasie van 20 kU gebruik is vergeleke met die kontrolegroep. Hierdie verlaging in alkohol is onderskeidelik tot 1 en 1.3% v/v alkohol met 'n pH van onderskeidelik 3.5 en 5.5 verhoog in belugte sintetiese druiwemos waar 30 kU ensieme gebruik is.

Tweedens is Gluzyme-proewe met Pinotage-druiwemos uitgevoer. Wyne wat met Gluzyme behandel is, het na afloop van fermentasie 0.68% v/v minder alkohol bevat as die kontrole-monsters met 30 kU ensieme. Kleur- en vlugtige geurverbindings van behandelde wyn het nie noemenswaardig van die onbehandelde monsters verskil nie. Daar is laer antosianien- en algehele fenolkonsentrasies by die kontrolemonsters waargeneem, moontlik weens die waterstofperoksiedoksidasie wat tot polimerisasie kon lei.

Die huidige studie het duidelik getoon dat Gluzyme in wynbereiding gebruik kan word om wyne met 'n verlaagde alkoholinhoud te maak sonder dat die kleur- en aromaverbindings beïnvloed word. Die ensiem in sy huidige vorm is egter nie ideaal vir wynbereiding nie; ander vorme daarvan, soos 'n vloeistof- of poeiervorm, behoort oorweeg te word as die ensiem onder wynbereidingsomstandighede gebruik gaan word.

Toekomstige werk behoort daarop te fokus om die potensiële nuwe vorm van die ensiem te evalueer en die invloed van Gluzyme op verskillende soorte druiwemos op 'n gedeeltelike industriële skaal te bestudeer. 'n Proepaneel sal ook die middel se invloed op die organoleptiese eienskappe en die algehele gehalte van die wyne wat voortvloei hieruit, moet evalueer.

This thesis is dedicated to my parents, family and friends for their continuous support, encouragement and enthusiasm.

Biographical sketch

Busisiwe was born in Melmoth, KwaZulu Natal. She matriculated at Masibumbane High School in Ulundi in 1997. She obtained her BSc degree in Agriculture (Viticulture and Oenology) at Stellenbosch University in 2003. In 2004, she worked at Fairview Estate in Paarl and in 2005; Busisiwe enrolled for a Master degree in Agriculture (Oenology) also at Stellenbosch University.

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Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal South African Journal of Enology and Viticulture to which Chapter 3 will be submitted for publication.

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Chapter 1

General introduction and project aims

1. General introduction and project aims

1.1 Introduction

Winemaking is one of the most ancient of man's technologies that has become one of the most commercially prosperous biotechnological processes. Advances in the second half of the 20th century have clearly shown that fermentation of grape must and the production of quality wines is not as simple a process as Pasteur, the founder of modern enology, suggested over a century ago. Considerable progress has been made over the last decade in understanding the biochemistry and interactions of yeast, lactic acid bacteria and other microorganisms during the winemaking process.

One of the major concerns in the South African wine industry is the production of wines with ever-increasing high alcohol content, posing serious problems in the international wine market. Thus, the immediate challenge for the wine industry is to produce wines containing reduced alcohol levels using traditional methods or yeast selection procedures that are acceptable to the consumer and which can be adapted by winemakers.

The most important biochemical transformation that occurs in grape must during winemaking is the fermentation of sugars, especially glucose and fructose, resulting in ethanol, carbon dioxide and energy production as well as the generation of a large number of sensorially important metabolites such as higher alcohols, organic acids and esters that will consequently influence the product quality (Romano *et al.*, 1998; Lambrechts & Pretorius, 2000). Ethanol production from sugar mixtures by microorganisms has been the subject of extensive research (Ingram & Doran, 1995). *Saccharomyces cerevisiae* has been the most widely used organism for ethanol production. The main pathway involved in ethanol production is the glycolytic pathway (Hatzimanikatis *et al.*, 1998). The complexity of glycolysis and its regulation has been the main obstacle in many experimental attempts to increase ethanol production and to manipulate its functions by metabolic engineering (Schaaff *et al.*, 1989; Boles *et al.*, 1993). However, the prime factors controlling ethanol production are sugar content, temperature and yeast strain (Jackson, 1994).

The ethanol content affects stability and organoleptic characteristics of a wine. Malolactic fermentation, performed by the bacteria, *Oenococcus oeni*, can become sluggish due to the presence of several factors including a high ethanol concentration (Osborne & Edwards, 2006). Ethanol inhibits the growth of lactic acid bacteria (Jackson, 1994). Of the lactic acid bacteria, species of *Lactobacillus* are more ethanol tolerant. The alcohol tolerance appears to decrease both with higher temperatures and lower pH values (Jackson, 1994). Furthermore, wines are taxed, in large part, based on their alcohol levels. Thus, careful monitoring of alcohol is critical in stylistic wine production and in carrying out accurate fortifications as well as in formulating blends for bottling.

A growing demand for wines containing lower alcohol content has resulted in a shift from full-bodied wines made from fully matured grapes, which give rise to wines with high alcohol content towards wines of a lower alcohol content. The consumption of alcoholic beverages such

as wine and beer with low alcohol content has also shown to increase over the past decade. This could be as a result of both increased awareness for health and stricter laws pertaining to drinking and driving, thus, indicating a growing market for low alcohol wines (Scudamore-Smith & Moran, 1997; Pickering *et al*., 1998).

The focus within the wine industry is to find production methods that can be used to produce wines with low- or reduced-alcohol content without any adverse effect on the wine quality in order to meet consumer's demand for these classes of wines. Commercial interest has also been stimulated by the potential for savings in taxes/tariffs on the reduced alcohol content of these wines (Pickering *et al*., 1998; Gladstones & Tomlinson, 1999; Gladstones, 2000).

South Africa with its warm climate wine producing regions tends to have higher grape sugar concentrations, resulting in the production of wines with higher levels of alcohol. Thus, for South Africa to be able to compete in the international market, it is crucial that possible methods or techniques be developed in order to reduce the alcohol content of the wine, that will be efficient, accurate and without adverse effects on the quality of wine.

Enzymes play a definitive role in the process of winemaking. Indeed, wine can be seen as the product of enzymatic transformation of grape sugar. Most of these enzymes originate from the grape itself, from indigenous microflora on the grapes and from the microorganisms present during winemaking. The endogenous enzymes of grapes, yeasts and other microorganisms present in must and wine are often neither efficient nor sufficient under winemaking conditions, to effectively catalyse the various biotransformation reactions (Moreno-Arribas & Polo, 2005). The use of commercial enzyme preparations for winemaking arose as a result of increased knowledge on enzymatic activities involved in the biotransformation of must into wine and the nature and structure of the macromolecules found in must and wines (Moreno-Arribas & Polo, 2005). These commercial enzymatic preparations favour the natural process by reinforcing the grapes' and yeasts' own enzymatic activities, giving winemakers more control over the process.

The addition of these commercial enzymes to resolve clarification and filtration problems (pectinases, xylanases, glucanases, proteases) or to release varietal aromas (glycosidases) is a common practice in vinification. The number and variety of products available, knowledge of their action mechanisms and their effects on wine quality has evolved dramatically over the last few years. Most commercial enzyme preparations are derived from different species of filamentous fungi, mainly *Aspergillus* spp., accepted as GRAS (Generally Recognised as Safe) and by the International Code for Enological Practices of the International Organisation of Vine and Wine (O.I.V.). Mixed enzyme preparations that fulfill more than one function in the process are often used.

The concept of treating grape must with glucose oxidase (GOX), to reduce glucose content of grape must, thereby producing a wine with a reduced alcohol content after fermentation, (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al*., 1998, 1999a, b, c), was introduced as an alternative approach to several physical processes that are used for the removal or reduction of alcohol in wine. When grape must is treated with GOX, the enzyme converts glucose into gluconic acid which cannot be metabolised by wine yeasts (Villettaz, 1987). The reaction takes place in the presence of molecular oxygen. The enzyme is expensive, which is a limiting factor for its use in the wine industry.

Peinado *et al.* (2004) has shown however, that *Schizosaccharomyces pombe* is able to reduce the gluconic acid content of wine as opposed to the findings by Villettaz (1987). *S. pombe* did not, however, completely deplete gluconic acid from treated wines (Peinado *et al.*, 2007); only up to 30 to 50% of all gluconic acid present in wine was removed.

As a result of pure GOX being expensive for use in wine production, an alternative to GOX, Gluzyme Mono® 10.000 BG (Novozymes, South Africa) hereinafter referred to as Gluzyme, was evaluated to produce wines with reduced alcohol content. Gluzyme is a glucose oxidase preparation from *Aspergillus niger*, produced by a genetically modified *Aspergillus oryzae* microorganism. Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. Gluzyme is currently used in the baking industry as the key enzyme for cost-effective gluten strengthening.

As an alternative to pure GOX, the possibility of using Gluzyme under winemaking conditions to reduce glucose content of synthetic or grape must was evaluated in order to produce a wine with reduced alcohol content.

1.2 Project aims

This study closely relates to the research programme on reduced alcohol wines at the Institute for Wine Biotechnology in which genetically modified wine yeast strains are being developed to achieve this goal. The aim of this study was to investigate the possibility of using Gluzyme, a commercial glucose oxidase preparation to reduce the glucose content of synthetic or grape must under winemaking conditions.

The specific aims of this study were as follows:

a) To establish Gluzyme dosage for use in winemaking and to evaluate the different enzyme concentrations on ethanol production.

b) To investigate the effect of different factors that could influence the activity of Gluzyme efficiency under winemaking conditions; such as pH, aeration, temperature and sulphur dioxide in synthetic grape must.

c) To perform Gluzyme trials in grape must and, to perform chemical analysis of wines produced from Gluzyme treated grape juice. The analyses included full analysis of the must before and after the enzyme treatment, ethanol content as well as phenolic composition of these wines at the end of alcoholic fermentation.

1.3 Literature cited

Boles, E., Heinisch, J. & Zimmermann, F.K., 1993. Different signals control the activation of glycolysis in the yeast *Saccharomyces cerevisiae*. Yeast 9, 761-770.

Gladstones, J. & Tomlinson, B., 1999. A proposal by the independent winemakers association for volumetric taxation based on alcohol content. Wine Ind. J. 14, 92-99.

Gladstones, J., 2000. Implications of lowering wine alcohol content. Wine Ind. J. 15, 45-46.

Hatzimanikatis, V., Emmerling, M., Sauer, U. & Bailey, J.E., 1998. Application of mathematical tools for metabolic design of microbial ethanol production. Biotechnol. Bioeng. 58, 154-161.

Ingram, L.O. & Doran, J.B., 1995. Conversion of cellulosic materials to ethanol. FEMS Microbiol. Rev. 16, 235-241.

Jackson, R.S., 1994. Wine Science. Principles and applications. Academic Press, San Diego. New York. pp. 184-186 & 264-265.

Lambrechts, M.G. & Pretorius, I.S., 2000. Yeast and its importance to wine aroma- a review. S. Afr. J. Enol. Vitic. 21, 97-129.

Moreno-Arribas, M.V. & Polo, M.C., 2005. Winemaking biochemistry and microbiology. Current knowledge and future trends. Crit. Rev. Food Sci. Nutrition 45, 265-269.

Osborne, J. & Edwards, C., 2006. Inhibition of malolactic fermentation by *Saccharomyces* during alcoholic fermentation under low- and high- nitrogen conditions: a study in synthetic media. Aust. J. Grape & Wine Res. 12, 69-78.

Peinado, R.A., Mauricio, J.C., Medina, M. & Moreno, J.J., 2004. Effect of *Schizosaccharomyces pombe* on aromatic compounds in dry sherry wines containing high levels of gluconic acid. J. Agric. Food Chem. 52, 4529-4543.

Peinado, R.A., Moreno, J.J., Maestre, O. & Mauricio, J.C., 2007. Removing gluconic acid by using different treatments with a *Schizosaccharomyces pombe* mutant: effect on fermentation by-products. Food Chem. 104, 457-465.

Pickering, G.J. & Heatherbell, D. A., 1996. Characterisation of reduced alcohol wine made from glucose oxidase treated must. Food Technol. 26, 101-107.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1998. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. Food Res. Int. 31, 685-692.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999a. The production of reduced alcohol wine using glucose oxidase treated juice. Part I: Composition. Am. J. Enol. Vitic. 50, 291-298.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999b. The production of reduced alcohol wine using glucose oxidase treated juice. Part II: Stability and SO_2-binding. Am. J. Enol. Vitic. 50, 299-306.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999c. The production of reduced alcohol wine using glucose oxidase treated juice. Part III: Sensory. Am. J. Enol. Vitic. 50, 307-316.

Romano, P., Paraggio, M. & Turbanti, L., 1998. Stability in by-product formation as a strain selection tool of *Saccharomyces cerevisiae* wine yeasts. J. Appl. Microbiol. 84, 336-341.

Schaaff, I., Heinisch, J., Zimmermann, F.K., 1989. Overproduction of glycolytic enzymes in yeast. Yeast 5, 285-290.

Scudamore-Smith, P. & Moran, J., 1997. A growing market for reduced alcohol wines. Wine Ind. J. 12, 165-167.

Villettaz, J.C., 1987. A new method for the production of low alcohol wines and better balanced wines. In: Lee, T. (Ed). Proc. 6th Aust. Wine Ind. Tech. Conf., July, Adelaide, Australia. pp. 125-128.

Chapter 2

Literature review

Techniques available for the production of reduced- and low-alcohol wines

2. Literature review

2.1 Introduction

Winemaking 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 a wine yeast and also proven itself as a reliable starter culture organism for inducing alcoholic fermentation. Its basic role is to convert grape sugar into alcohol, and its secondary metabolic activities result in the production of higher alcohols, fatty acids and esters, which are the important flavour and aroma compounds that are essential for consistent and predictable wine quality.

In an effort to produce wine of a good quality, grape must is typically prepared from optimally ripe grapes. This does not only give the high flavour intensity that is required, but can also result in a more than adequate concentration of sugar. A high concentration of sugar leads to the production of wines with high levels of alcohol, with some wines reaching ethanol concentrations above 15% v/v (Godden, 2000; Day *et al.*, 2002). The high alcohol content of wine has several implications. It can affect its organoleptic properties (Guth & Sies, 2002) and can mask its overall aroma and flavour.

Stuck fermentations are more common in musts with higher sugar concentrations. For example, higher temperatures and rapid ripening during the latter part of the season in 1998 meant that °Brix of higher than 23 were not unusual in South Africa (Ellis, 1999). Alcohol concentrations of higher than 13% v/v were common in some 1998 wines. Even much higher °Brix of up to 28 is common nowadays.

The reduction of ethanol content in alcoholic beverages especially, wine and beer, is of great commercial interest. Consumer demand for lower-alcoholic beverages is continuously increasing due to both increased health awareness and stricter laws pertaining to drinking and driving. This has therefore increased the demand for wines containing less alcohol, putting a great deal of pressure on wine producers, particularly those in wine-producing regions with a warmer climate where grape sugar levels can become very high.

This review presents the most relevant scientific contributions to the issue of high alcohol wines. It also gives an overview of the current technologies as well as some possible methods that can be used to obtain a wine with reduced-alcohol content and their influence on the quality and flavour composition of the resultant wine.

2.2 Demand for wines containing low- or reduced-alcohol content

There has been increased international interest and consumer demand for reduced-alcohol, low-alcohol and de-alcoholised wines (Schobinger & Dürr, 1983; Anon., 1988; Hees, 1990; Hoffmann, 1990; Simpson, 1990; Howley & Young, 1992).

Commercial interest has also been stimulated by the potential for savings in taxes/tariffs on the reduced alcohol content of these classes of wines. Furthermore, wines with a reduced alcohol content offer a number of potential social and health benefits for consumers (Pickering, 2000). Social benefits may include improved productivity and function after activities involving alcohol consumption, lower risk of prosecution or accidents while driving.

Health advantages may include reduced calorie intake and decreased risk for alcohol-related diseases.

2.3 Major chemical constituents of grapes and wine

2.3.1 Water

Water content of grapes and wine is seldom discussed. Nevertheless, as the predominant chemical constituent of grapes and wine, water plays critical roles in establishing the basic characteristics of wine (Jackson, 1994). The water also governs the basic flow characteristics of wine. It is an essential component in many of the chemical reactions involved in grape during growth, juice fermentation and wine aging (Jackson, 1994).

2.3.2 Sugars

Simple sugars may bind together to form polymers, like pectins, starches, hemicelluloses and celluloses, or can bind with other compounds, such as lactones and anthocyanidins, to form glycosides. Only some of the simple sugars taste sweet. The principal grape sugars are glucose and fructose (Jackson, 1994). Economically, they are also the most important products produced by grapevines, since they largely determine the edibility of the fruit and the final alcohol content of wine (Kliewer, 1967). Grape must usually contains approximately equal amounts of glucose and fructose at maturity (Amerine *et al.*, 1972; Zoecklein *et al.*, 1995; Fleet, 1997), whereas over-ripe grapes often have a higher proportion of fructose (Jackson, 1994). Sugars other than glucose and fructose do occur, but in relatively insignificant amounts.

S. cerevisiae is known to display a preference for glucose. Since fructose is almost twice as sweet as glucose (Lee, 1987), its presence as residual sugar has a much stronger effect on the final sweetness of the wine especially in the case of stuck fermentation (Boulton *et al.*, 1996), and residual fructose is thus the main cause of undesirable sweetness in dry wines.

High residual fructose also means a lower yield of ethanol and a higher risk for microbial spoilage of the finished wine. It has also been reported that stuck fermentations are frequently characterised by an unusually high fructose to glucose ratio (Gafner & Schütz, 1996).
Grape sugar content varies depending on the species, variety, maturity and health of the fruit. Grape sugar content is also critical to yeast growth and metabolism. *S. cerevisiae*, the primary wine yeast, derives most of its metabolic energy from glucose and fructose (Jackson, 1994). Sugar concentration can also increase the volatility of aromatic compounds (Sorrentino *et al.*, 1986).

2.3.3 Alcohols

Alcohols are organic compounds containing one or more hydroxyl group (-OH). Simple alcohols contain a single hydroxyl group, whereas diols and polyols contain two or more hydroxyl groups, respectively (Jackson, 1994). Alcohol is the result of the fermentation process, during which yeast converts sugar into alcohol and carbon dioxide. The alcohol content of a wine influences its stability and sensory properties. Wines are also taxed mainly on the basis of their alcohol content. Thus careful monitoring of alcohol is important in stylistic wine production and in carrying out accurate fortifications as well as in the formulation of blends for bottling (Zoecklein *et al*., 1995). Additional alcohols of importance in winemaking include glycerol and other polyhydric alcohols such as fusel oils. Individually and collectively, these may, on occasion, be of sensory or regulatory importance (Zoecklein *et al*., 1995).

2.3.3.1 Ethanol (ethyl alcohol)

Ethanol is the most important alcohol in wine. Although, small quantities are produced in grape cells during carbonic maceration, the primary source of ethanol is yeast fermentation (Jackson, 1994). Besides water, ethanol is the most plentiful compound in wine. A wine's strength is expressed in terms of alcohol content or the percentage of alcohol by volume. The alcoholic strength of wine in average is 12.6% v/v although it may exceptionally be as high as 16% v/v. (Ribéreau-Gayon *et al*., 2000).

Besides its significant physiological and psychological effects, ethanol is crucial to the stability, aging and sensory properties of wine (Jackson, 1994). During fermentation, the increasing alcohol content limits the growth of most microorganisms. Microbes that might produce off-flavours are generally inhibited. The inhibitory effect of ethanol, combined with the acidity of the wine, allows the wine to remain sound for years in the absence of air. The addition of ethanol to stabilise certain wines is a long-standing winemaking tradition (e.g. Port). However, ethanol is toxic for humans, affecting the nerve cells and liver. The lethal dose (LD_{50}) by oral consumption is 1 400 mg/kg body weight.

Ethanol acts as an important solvent in the extraction of pigments and tannins during red wine vinification. This capacity is involved in solubilising certain odoriferous molecules and certainly contributes to the expression of aroma in wine.

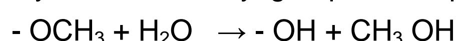
The chemical properties of ethanol are limited to its alcohol function. In particular, it esterifies with tartaric, malic and lactic acids. Ethanol may also react with aldehydes, especially acetaldehyde in free form. This is not usually the case in sulphited wines, as sulphur dioxide reacts very strongly with ethanal, producing an acetal (diethoxyethane) (Ribéreau-Gayon *et al*., 2000).

Ethanol can react with the hydrogen sulphide produced by fermenting yeast or resulting from the residues of some vineyard treatment products. This reaction generates ethanethiol, which has a very unpleasant smell. Since this compound is much less volatile than hydrogen sulphide, it is very difficult to eliminate. It is therefore advisable to rack wines as soon as

alcoholic fermentation is completed and again immediately after malolactic fermentation, since hydrogen peroxide may also be produced by lactic acid bacteria. The oxidation-reduction balance may also cause ethanethiol to form diethyl disulphide. This compound is even less volatile and has a very unpleasant smell that spoils the flavour of the wine (Ribéreau-Gayon *et al.*, 2000).

2.3.3.2 Methanol (methyl alcohol)

Methanol is always present in wine in very small quantities, usually between 30 and 35 mg/L (Ribéreau-Gayon *et al.*, 2000). It has no organoleptic impact. Methanol is not formed by alcoholic fermentation (Bertrand & Silberstein, 1950), but results exclusively from enzymatic hydrolysis of the methyl groups of the pectin during fermentation (Ribéreau-Gayon *et al.*, 2000):



$$- OCH_3 + H_2O \rightarrow - OH + CH_3OH$$

Since skin contact is often prolonged in the making of red wine, these wines show higher amounts of methanol (Sponholz, 1988). While grapes have relatively low pectin content, wine is the fermented beverage with the lowest concentration of methanol. The methanol content depends on the extent to which the grape solids, especially when the skins that have high pectin content are macerated (Ribéreau-Gayon *et al.*, 2000).

Red wines have a higher concentration (152 mg/L) than rosés (91 mg/L), while white wines have even less (63 mg/L) (Ribéreau-Gayon *et al.*, 1982; Linskens & Jackson, 1988). Wines made from hybrid grape varieties have higher methanol content than those made from *Vitis vinifera* due to the higher pectin content of the skins of the hybrid grapes. Addition of pectolytic enzymes to the wine in order to facilitate extraction or clarification, by breaking alpha (1→4) bond of the pectin polymer, also increases methanol content (Zoecklein *et al.*, 1995; Margalit, 1997).

The methanol content of wine is not influenced by the fermentation temperature, although, as mentioned above, pectin treatments as well as prolonged skin contact do have an influence on the methanol content (Gnekow & Ough, 1976).

Methanol is well known for its toxicity. Following ingestion, it oxidises to produce formic aldehyde and formic acid, which are both toxic to the central nervous system. Formic aldehyde deteriorates the optical nerve, causing blindness. Methanol never accumulates to toxic levels under legitimate winemaking process (Jackson, 1994). Wines that are produced from grapes infected with mould from *Botrytis cineria* often contain up to 364 mg/L of methanol (Sponholz, 1988) compared to wines produced from sound grapes. The methanol content of wines is very low and therefore will not contribute much to the fullness of the wines, but it is involved in aroma formation as part of the methyl esters of wine (Nykänen & Suomalainen, 1983).

2.3.3.3 Fusel oils (higher alcohols)

Higher alcohols found in wine occur as by-products of yeast catabolism, resulting from amino acids and contribute to the aroma of wine (Massel, 1969). Quantitatively, the most important

higher alcohols are iso-amyl, amyl, iso-butyl, propyl and methyl alcohol. Several of these are produced during fermentation and reach concentrations of 150 to 550 mg/L in wine (Ribéreau-Gayon *et al.*, 1982).

These alcohols and their esters have intense odours that play a major role in wine aroma. This group of alcohols may present problems in distillation, where they concentrate in the 'tails' fractions of distilled spirits (Zoecklein *et al.*, 1995). Depending on the production objectives, significant amounts may represent defects in the sensory interpretation of the distillate (Zoecklein *et al.*, 1995). The major source of higher alcohols is amino acids, which are transformed into alcohols by a sequential process of transamination, decarboxylation and reduction (Margalit, 1997).

Quantitatively and qualitatively, fusel oils represent an important group of alcohols that may affect the wine flavour. They may be present in wines at varying concentrations. Quantitatively, iso-amyl alcohol generally accounts for more than 50% of all fusel oil fractions (Muller *et al.*, 1993). When their concentrations exceed 400 mg/L, the higher alcohols are regarded as a negative influence on the quality of the wine (Rapp & Mandery, 1986). The higher fermentation alcohol content of wine varies according to fermentation conditions, especially the yeast strain.

In general, factors that increase the fermentation rates, such as yeast biomass, oxygenation, high temperature and the presence of matter in suspension, also increase the formation of higher alcohols (Ribéreau-Gayon *et al.*, 2000). Distillation techniques have a major impact on the concentration of higher alcohols (Boulton *et al.*, 1995).

2.4 Determination of ethanol content of wine

Alcohol is the major product of alcoholic fermentation. Quantitative analysis of ethanol is important for the control of fermentation and certification of alcoholic drinks. For this purpose, several physicochemical and chemical processes of ethanol determination in wines and fermented musts are used. The formal expression of ethanol concentration in alcoholic beverages is given as a percentage volume of alcohol per volume of liquid (% v/v).

Frequent, fast and accurate results are necessary in order to control the quality of the wine from the grape to the bottle. The principle for wine taxation is also based mainly on the alcohol content. The physical and sensory properties of wine are partly dependent on alcohol content. Blending which results in changes in the final alcohol content, may subsequently result in a change in wine stability (Zoecklein *et al.*, 1995). The common methods used for determination of ethanol concentration are as described below.

2.4.1 Ebulliometric determination

Ebulliometry is the most common procedure for the determination of the ethanol content of aqueous solutions (Zoecklein *et al.*, 1995). The method is fairly user-friendly but it is the least accurate of all the listed methods, with an accuracy of ±0.5% v/v (Jacobson, 2006). The analysis is based on the Raoult's Law relationship of boiling point depression. Although simple

in theory, several interferences may be encountered in the routine laboratory application of ebulliometry, the most important being the effect of sugars. According to the colligative properties of solutions, sugar molecules would be expected to cause a boiling point elevation (hence lower apparent ethanol levels). However, this is contradictory, as sweet wines usually boil at a temperature lower than expected, resulting in higher apparent ethanol concentration level. This is due to the sugar-water matrix squeezing out of the ethanol, thereby increasing its vapour pressure. To reduce errors attributed to sugar, sweet wines may be diluted with water to a sugar level of less than 2%, yielding a boiling point of 96˚C to 100˚C (Zoecklein *et al.*, 1995).

2.4.2 Enzymatic method

Enzymatic reagent kits are commercially available for the determination of ethanol in body fluids and have been modified for assaying ethanol levels in wine. The enzymatic methods of ethanol determination are mainly based on the monitoring of NADH produced in the reaction catalysed by NAD-dependent alcohol dehydrogenase (Jacobson, 2006). NADH consequently is easily detectable using a spectrophotometer at 340 nm (Jacobson, 2006).

The procedure has shown good recovery of ethanol from freeze-dried, de-alcoholised samples that were reconstituted with known amounts of ethanol. However, the enzyme assay is not precise for very accurate work, but it does offer speed and little sample preparation in estimating wine alcohol levels (McCloskey & Replogle, 1974).

Although this method is specific, it has some disadvantages associated with the necessity of using an expensive cofactor or acetaldehyde dehydrogenase, which are used to shift the equilibrium of the reaction towards ethanol oxidation. In addition, the molar extinction coefficient for NADH is low, which determines fairly low sensitivity of analysis.

2.4.3 Gas chromatography (GC)

A method is described for the specific quantitative analysis of ethanol in wine by gas chromatography. This method, which uses an internal standard and flame ionisation detector, is more accurate and more precise than the other methods commonly used (Stackler & Christensen, 1974).

Gas chromatography is a technique used to separate volatile components in the sample. Wine (juice or distillate) is injected into a heated tube that is packed with a specialised adsorbent through which an inert gas flows. Ethanol and other volatile components are vaporised and carried through the tube (also referred to as a GC column) toward a detector that senses their presence. Because of differences in their interaction with the adsorbent, different compounds migrate or travel through the column at different rates, and are separated by the time they reach the detector. To quantify ethanol, one has to prepare standards of known concentrations, inject them into the GC, and compare their detector responses to that of the unknown sample (Zoecklein *et al.*, 1995).

The same GC technique can be used to analyse fusel oils. For this particular analysis, a column with a different, specialised adsorbent and a different column temperature are used. The gas-liquid chromatography method determines ethanol separately from other wine components that interfere in other methods, and without distillation or chemical reaction. When large numbers of samples are to be analysed, advantages include a short time per sample and the potential for extensive automation (Stackler & Christensen, 1974).

2.4.4 Higher performance liquid chromatography (HPLC)

The determination of organic acids and alcohols is important for many disciplines, including food science, biotechnology, biochemistry and biomedicine (Castellari *et al.*, 2000). This technique can be used to quantify certain mould, yeast and bacterial metabolites in a juice sample (Zoecklein *et al.*, 1995).

In particular, in the wine industry, the analysis of sugars, organic acids, glycerol and ethanol is often required for the quality evaluation and characterisation of grapes, musts and wines. Filtered juice samples are injected into a special HPLC column that separates the components from each other and from any other matrix compounds. Quantification is accomplished by comparing component peak areas to those from standard solutions chromatographed in the same way.

The coupling of HPLC-FT-IR (Fourier Transformed Infrared) has been demonstrated as a new and versatile tool for the direct determination of the main components of wine including glucose, fructose, glycerol, ethanol, acetic, citric, lactic, malic, succinic and tartaric acid (Vonach *et al.*, 1998).

2.4.5 Fourier Transformed Infrared (FT-IR)

Since most compounds absorb in the infrared region, FT-IR spectroscopy can provide qualitative information about the compounds. This is of particular interest for analytes such as carbohydrates or alcohols that are not or only poorly detected by standard UV-spectroscopy.

Current instrumentation has optional software modules that contain ready-to-use calibrations for simultaneous determination of several components in a sample. One such instrument that has been introduced to the market is the Winescan FT 120 instrument (Foss Electric, Ltd, Hillerød, Denmark). Commercial calibrations with the instrument include those for quantifying ethanol, volatile acidity, total acidity, pH, malic acid, lactic acid, glucose, residual sugar, fructose, glycerol and Folin C index (Gishen & Holdstock, 2000).

FT-IR provides a precise measurement method which requires no external calibration. Possible limitations in the use of this technology include interference due to the absorbance of water, which decreases the accuracy of determination of some components, such as sulphur dioxide. In terms of concentration range, FT-IR is generally not considered to measure accurately below 0.1 to 0.2 g/L.

The detection limit can be improved when FT-IR instrumentation is used in conjunction with conventional analytical instruments such as HPLC (Vonach *et al*., 1998).

2.5 Influence of alcohol on the taste of wine

Quality is always associated with a certain harmony of tastes, where no taste dominates the other. The alcoholic component is an important ingredient in the overall taste of wine. It has a bitter/sweet sensation, described as a harsh or “burny” mouth feel. A high alcohol concentration can affect the sensory properties of the wine (Guth & Sies, 2002). Depending on the wine style, alcohol can make the wine to appear as unbalanced.

Furthermore, high alcohol content can mask the overall aroma and flavour of the wine (De Barros Lopes *et al*., 2003). It has been observed that the less ethanol in a complex wine model mixture, the greater the intensity of the fruity and floral odours. This could be due to an increased partial pressure of the odorants with reduced ethanol concentration (Grosch, 2001).

2.5.1 Acidity and balance

Although the acid character of wine is due to its hydrogen ion concentration, both pH and acidity play important roles in the total sensory perception of this stimulus. With equivalent acid concentration, the increasing order of perceived sourness of acids commonly found in wine acids is malic, tartaric, citric and lactic.
Ethanol is effective in increasing the acid perception thresholds, and this increase is even more dramatic with the inclusion of sucrose. Phenols may also be active in increasing the minimum detectable acid levels (Zoecklein, 2002).

2.5.2 Alcohol and balance

Alcohol provides a sense of sweetness. Thus, a wine with a high phenolic load frequently is better balanced, with both a lower acidity and higher alcohol content. Relatively small differences in the alcohol concentration can cause a difference in the structure and aroma (Zoecklein, 2002). Alcohol has a direct impact on the varietal aroma intensity. Too much alcohol provides a spirit-like character, reducing the perception of the varietal. This is a further reason beyond structural balance to attempt to regulate and control the alcohol concentration.

According to a study done by Fischer & Noble (1994), an increase in ethanol content raises the intensity of bitterness, but has only a slight effect on sourness. Furthermore, Mattes & DiMeglio (2001) have observed that ethanol itself has a bitter taste at a concentration near perception threshold.

Martin & Pangborn (1970) also observed that alcohol slightly enhanced the sweetness of sucrose and depressed the perceived intensity of saltiness and sourness. Alcohol, on top of possessing taste properties (sweet and bitterness) and thermal effects, may also play an important role as a taste and aroma enhancer.

With increasing de-alcoholisation, acidity, bitterness and astringency are heightened, often to the point of imbalance, as the softening and harmonising effect of alcohol are increasingly reduced (Pickering, 2000).

New technological advancements to control the ethanol concentration in the finished wine include reverse osmosis, spinning cone technology, as well as osmotic distillation (Pickering, 2000).

2.6 Metabolic pathways involved in ethanol production by yeast

Metabolism refers to the biochemical assimilation (anabolic pathways) and dissimilation (catabolic pathways) of nutrients by a cell (Feldmann, 2005). As in other organisms, these processes in yeast are mediated by enzymatic reactions, and the regulation of the underlying pathways has been studied to a great extent in yeast.

The major route of glucose and fructose utilisation in *S. cerevisiae* is called glycolysis. Glycolysis is the general pathway for the conversion of glucose to pyruvate (Bisson, 1993), whereby the production of energy in the form of ATP (adenosine triphosphate) is coupled to the generation of intermediates and reducing power in the form of NADH (nicotinamide adenine dinucleotide) for biosynthetic pathways. Two principal modes of the use of pyruvate in further energy production are respiration and fermentation (Fig. 2.1).

Yeasts can be categorised into several groups according to their modes of energy production, utilising either respiration or fermentation (Table 2.1) (Feldmann, 2005). These processes are regulated mainly by environmental factors; the best documented being the availability of glucose and oxygen. Yeasts can adapt to varying growth environments, and even within a single species, the prevailing pathways will depend on the actual growth conditions.

The major routes of carbon metabolism in *Saccharomyces* depends on the substrate available and growth conditions. Availability of oxygen plays a critical role in metabolism as molecular oxygen is required as the terminal electron acceptor during respiration, but it has a different role during high sugar, relatively anaerobic fermentation. The glycolytic pathway is operational under both fermentative and respiratory modes of metabolism. During fermentation, a carbon compound serves as terminal acceptor of the electrons that are generated in the pathway in the course of converting sugar metabolites to energy in the form of ATP. In *Saccharomyces*, pyruvate is converted to acetaldehyde, which serves as terminal electron acceptor generating ethanol (Boulton *et al.*, 1996).

During respiration, which may be important in the early phases of vinification (Boulton *et al.*, 1996) and in all phases of commercial yeast production, more of the energy is captured in the form of ATP. This is a result of the action of two metabolic pathways: the TCA (tricarboxylic acid) cycle and the electron transport chain. The generation of ATP during respiration is called oxidative phosphorylation and that resulting from glycolysis is called substrate level phosphorylation (Boulton *et al.*, 1996).

Enzymes of the TCA cycle and electron transport chain are localized in a subcellular organelle, the mitochondrion.

Since respiration is ultimately dependent upon oxygen, these enzymes are not synthesized constitutively, but only when required for metabolism.

In yeast, expression of the genes encoding these enzymes is controlled by the concentration of glucose or other fermentable sugar in the medium. The genes are repressed by high concentration of glucose, meaning that mRNA is not made; there is no transcription. This regulatory phenomenon is called glucose repression or the Crabtree effect (Crabtree, 1929; De Deken, 1966).

When the substrate is not limiting, yeasts rely upon fermentation or substrate level phosphorylation for ATP production. Thus fermentation is the preferred mode of metabolism even when molecular oxygen is available. As sugar concentration becomes limiting, yeast has to switch to respiratory metabolism in order to generate sufficient ATP for growth and metabolism. This metabolic switch does not take place if oxygen is not available.

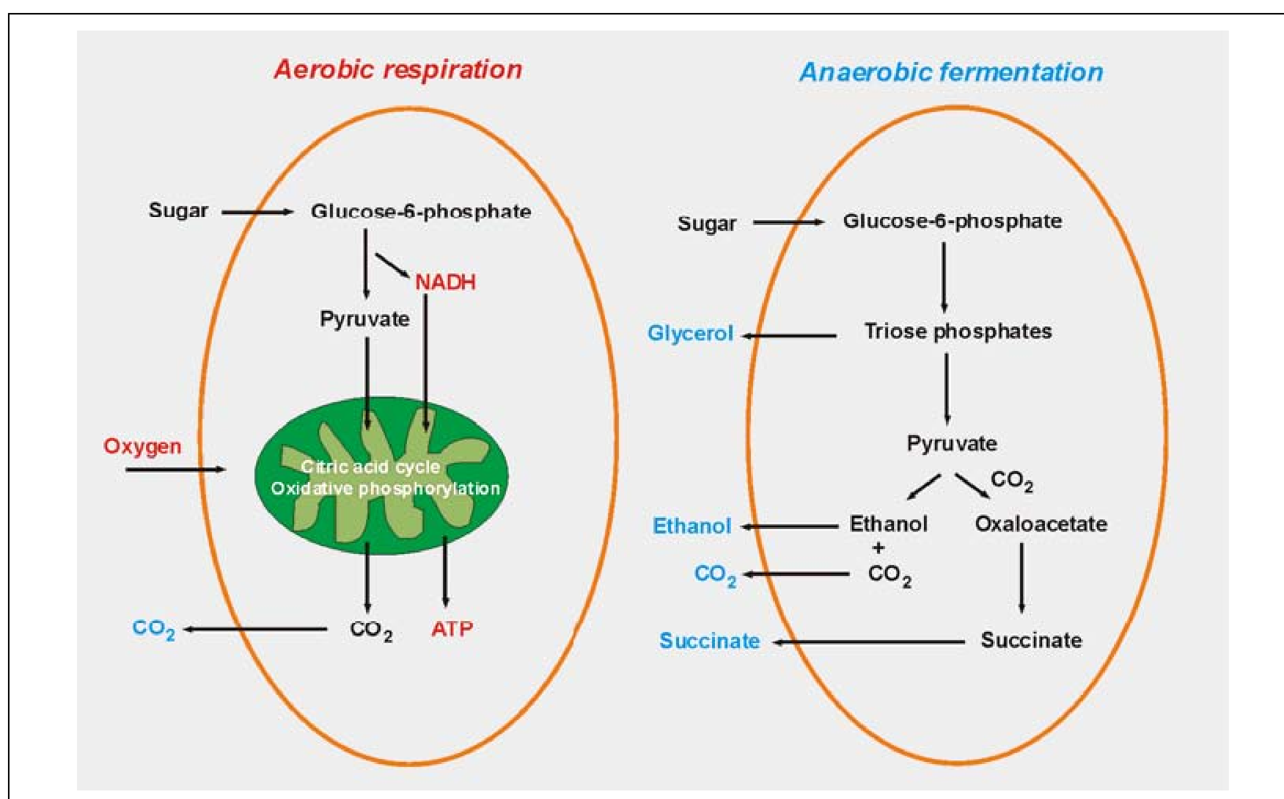


FIGURE 2.1

Metabolism of yeast under aerobic and anaerobic conditions (Feldmann, 2005).

TABLE 2.1

Principal modes of respiration in yeasts (Feldmann, 2005)

Types	Examples	Respiration	Fermentation	Anaerobic growth
Obligate respirers	*Rhodotorula* spp.	YES	NO	NO
Anaerobic respirers	*Cryptococcus* spp. *Candida* spp. *Kluyveromyces* spp.	YES	Anaerobic in pregrown cells	NO
Aerobic fermenters	*Pichia* spp. *Schizosaccharomyces pombe*	Limited	Aerobic and anaerobic	NO
Facultative aerobic fermenters	*Saccharomyces cerevisiae*	Limited	Aerobic and anaerobic	Facultative
Obligate fermenters	*Torulopsis* spp.	NO		YES

2.6.1 Effect of high alcohol on yeast

S. cerevisiae is widely used as a wine yeast starter culture. Most strains of *S. cerevisiae* are inhibited as the alcohol levels reach 14-15% v/v (Zoecklein *et al*., 1995). However, several strains are more alcohol tolerant.

Ethanol directly links to temperature. In other words, as the ethanol content of the fermenting yeast increases the sensitivity of yeast to ethanol increases.

2.6.2 Factors that influence ethanol production by yeast

The transformation of grape juice into wine is essentially a microbial process. As such, it is important for the oenologist to have an understanding of yeast and fermentation biochemistry as the fundamental basis of the winemaking process.

Alcoholic fermentation, which is the conversion of the principal grape sugars glucose and fructose to ethanol and carbon dioxide, is conducted by yeasts of the genus *Saccharomyces*, generally by *S. cerevisiae* (Boulton *et al*., 1996). Some factors strongly affect alcoholic fermentation, and thus the quality of the finished wine (Torija *et al*., 2003). The most important factors are clarification of grape juice, levels of sulphur dioxide, temperature of fermentation, composition of grape juice, yeast strain and the interaction with other microorganisms (Ribéreau-Gayon *et al*., 2000).

2.7 Effect of high alcohol on lactic acid bacteria

Malolactic fermentation (MLF) is carried out by lactic acid bacteria, mainly *Oenococcus oeni*. Among the parameters that determine the growth of LAB is the ethanol content. Other factors include pH, temperature and SO_2 (Osborne & Edwards, 2006).
LAB are sensitive to ethanol. *Oenococcus oeni* is inhibited in environments richer in ethanol and becoming more difficult at ethanol concentrations greater than 13% v/v. MLF is more optimal at the ethanol concentration less than 13% v/v.

2.8 Current technologies used to reduce the alcohol content of wine

2.8.1 Thermal and distillation methods

2.8.1.1 Vacuum distillation

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

The original pressure boiling pan and distilling vessel have been replaced by vacuum distillation apparatus, which enable the removal of ethanol at much lower temperatures. Until recently, the process of de-alcoholisation required heating and evaporation of 50 to 70% of the wine to reduce the alcohol content to below 0.5% v/v.

There have been considerable variations on and modifications of the distillation and evaporation principle, most of which are patented (Déglon, 1975; Thumm, 1975; Boucher, 1983, 1985, 1988; Schobinger *et al*., 1986; Trothe, 1990). These modifications mostly incorporate one or more of the non-thermal methods, shorter processing times, lower temperatures and improved aroma recovery techniques. They also include the addition of blended grape juice or concentrate to the reduced-alcohol wine, primarily to adjust the sensory properties of the wine.

2.8.1.2 Spinning cone column

Over the years, different techniques have been developed to legally reduce the alcohol content of wines (Theron, 2006; Goode, 2005). One such technique is the spinning cone column (SCC). The SCC is a modern, multi-stage strip column, which was first developed in the United State of America (USA) in the 1930s and modified more recently in Australia. It is currently being marketed world-wide by the Californian Company, ConeTech Inc. (Theron, 2006).

The SCC is a gas-liquid contacting device consisting of a vertical counter-current flow system that contains a succession of alternate rotating and stationary metal cones, where upper surfaces are wetted with a thin film of liquid (juice or wine) (Pickering, 2000). Wine is fed into the top of the column, where gravity and a vacuum pump pull it down through the first fixed cone and onto the first rotating cone. The movement of the rotating cone spins the wine into a thin liquid film, forcing it up and over the lip of the cone so that it drops onto the next stationery cone,

and so on down. As it exits the column, about half a percentage of the total volume of wine undergoing the process is converted into an inert stripping gas called ‘cold steam’, which is just above room temperature (Hay, 2001). This vaporous, cold steam feeds back into the bottom of the column and travels upward along the surface of the thin film of wine travelling downward.

Fins on the underside of each rotating cone whip the rising stream of vapour into a turbulent state, which combines with the spinning motion of the wine travelling downward to strip the wine of its volatile flavour and aroma compounds and capture them in a liquid form. The cone employs a two-stage process. On the first pass of the wine through the cone, it is stripped of its flavour and aroma essences. Then, it is run back down through the column, where the cold steam vapour removes the alcohol from the wine. The flavour and aroma compounds are then introduced back into the lowered-alcohol or de-alcoholised wine, which, when recombined into the winemaker’s total blend, lowers the overall alcohol content.

The SCC reduces alcohol to lower levels than does reverse osmosis. In either method, removing the alcohol inevitably reduces the volume of the wine being treated (Hay, 2001).

The cost of this treatment varies according to the volume of wine being treated, but the technology is generally expensive. A schematic representation of the process is given in Fig. 2.2.

The SCC is mostly chosen because it preserves essential flavours and aromas. Other advantages include high efficiency, low liquid residence times, low entrainment, minimal thermal damage, the ability to handle highly viscous juice and good energy efficiency (Sykes *et al.*, 1992; Gray, 1993; Pyle, 1994).

The SCC process is also used to finely adjust alcohol levels in full-strength premium wines. Furthermore, distillation and evaporation techniques have the advantage that extracts, minerals and other non-volatile components in the original wine are preserved.

The main technical disadvantage of the SCC is that some heating of the wine is required for the de-alcoholisation step, which is carried out at about 38°C (Pickering, 2000). The expected cost of treatment in South Africa is 23 cents per litre (excluding transport). When considering that only 10% of the wine has to be treated, the production cost of the total final volume is 2.3 cents per litre (Theron, 2006).

2.8.1.3 Freeze concentration

Another thermal method, which is used infrequently, is freeze concentration. The water in wine may be removed by freezing and the alcohol in the residual liquid can be removed by vacuum distillation. The wine can also be cooled until crystals are formed, and these can then be separated and thawed later. The resulting low-alcohol wine can be adjusted to any alcohol content with the separated alcohol fraction. The process is relatively delicate and expensive (Schobinger *et al.*, 1986; Villettaz, 1986).

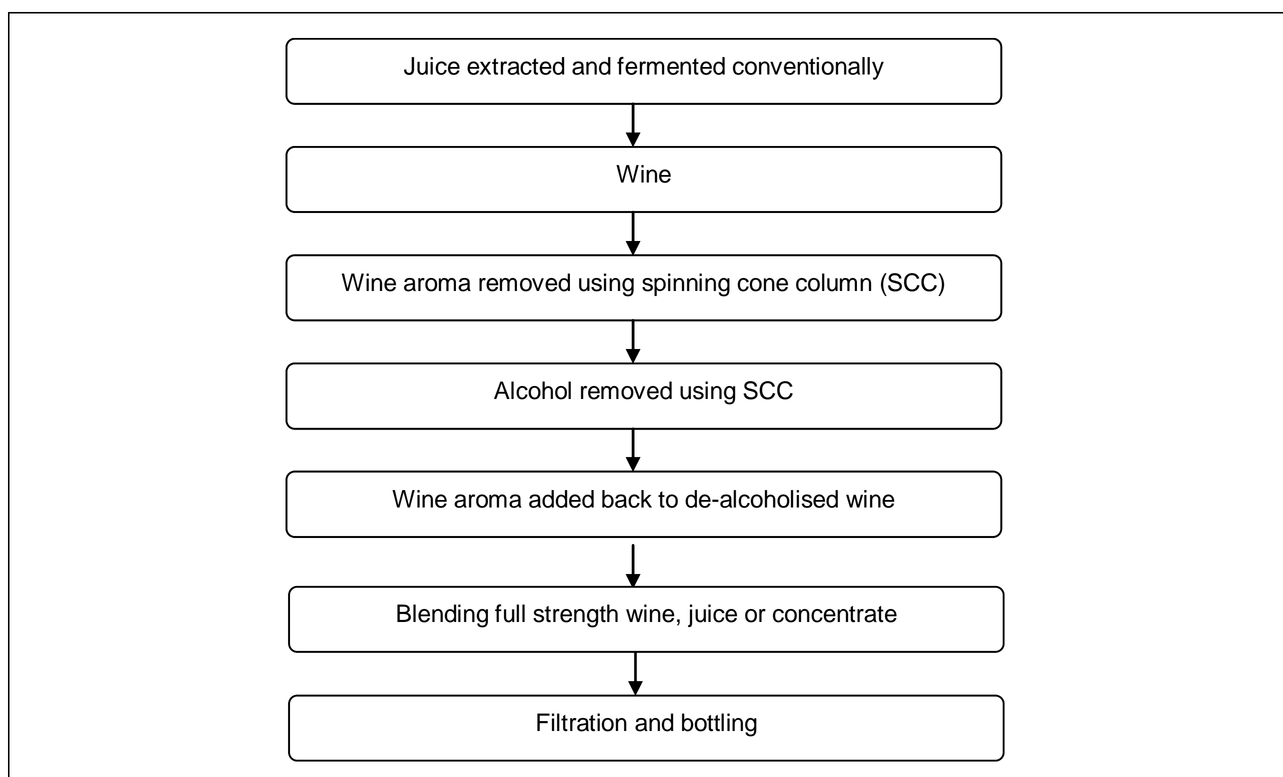


FIGURE 2.2
Wine-processing scheme using spinning cone column (SCC) (Pickering, 2000).

2.8.2 Membrane processes

2.8.2.1 Reverse osmosis

Reverse osmosis is currently the most widely used technique for reducing the alcohol content of wine (Pickering, 2000). The wine is pumped through the membrane at a pressure greater than the osmotic pressure, causing compounds with a smaller molecular weight, such as ethanol and water, to diffuse selectively through the membrane, thereby removing the alcohol from the wine. The process is illustrated in Fig. 2.3.

The membrane separates compounds based on their molecular weight as well as the membrane pore size. Since ethanol and water molecules are small in comparison to the wine component matrix, the larger compounds, such as organic acids and phenolics, are retained in the wine and are then concentrated. Water is added back to the concentrated wine to restore the initial balance of these materials and produce a pleasing, non-alcoholic or reduced-alcohol wine. This cold separation method is believed to be a superior technology, since there is no heating of the product and the wine therefore retains all of the natural flavours from the grapes.

The removal of alcohol is conducted under high pressure, with the temperature controlled at 7 to 13°C and using very small membrane pore sizes so that only alcohol and water pass through. If desired, the alcohol can be recovered from the alcohol-and-water permeate by standard steam distillation. With the use of a proper support system and sufficient pressure,

reverse osmosis can reduce the alcohol content of wine to almost any degree desired. Other advantages include the reductive environment that can be maintained during processing and good energy efficiencies (Pickering, 2000).

There are two reverse osmosis systems, one equipped with ethanol-permeable membranes and the other with selective ethanol-retention membranes. The permeate-exchange unit is necessary to ensure the water and ethanol balance of the system. The product intended for manufacture by the proposed technique remains 'wine' by its composition and organoleptic quality (Bui *et al.*, 1986).

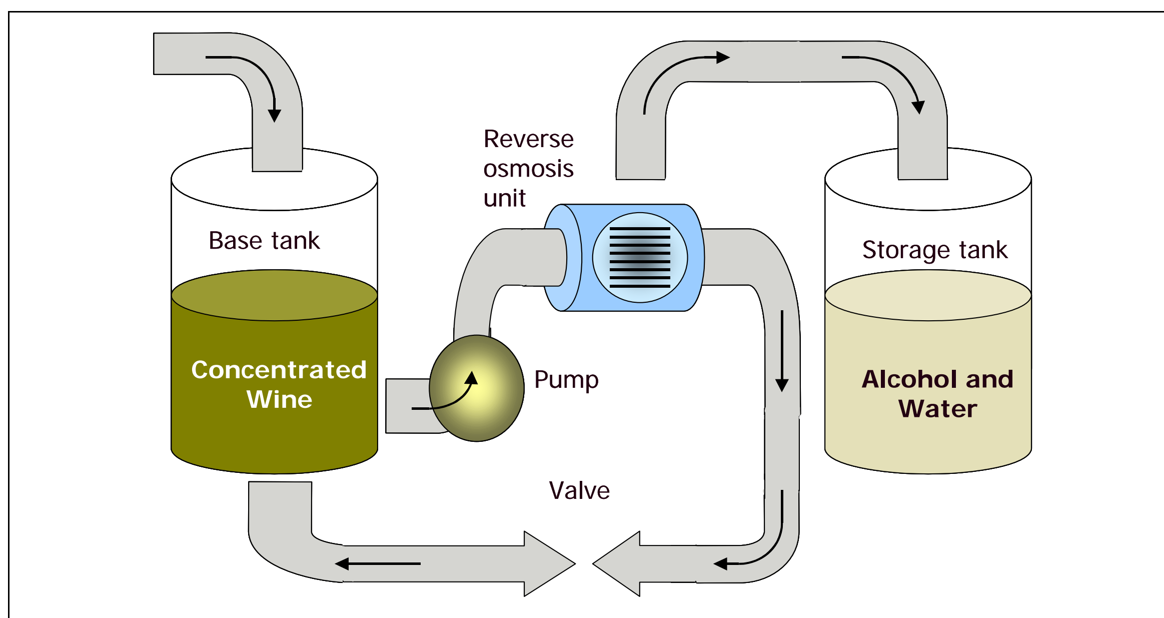


FIGURE 2.3
Schematic representation of the use of reverse osmosis to remove alcohol from wine (adapted from Mermelstein, 2000).

2.8.2.2 Dialysis

Dialysis uses differences in concentrations for substrate transport, in contrast to reverse osmosis, which uses hydrostatic pressure as the driving force (Pickering, 2000). In dialysis, water is used to provide the concentration gradient, which allows net movement of ethanol and compounds with a low molecular weight out of the wine and into the water.

The advantages include functioning without pressure- there is no need for increases in concentration, no cooling of the system and only a small loss of carbon dioxide (Schobinger *et al.*, 1986).

2.8.3 Low fermentable sugar

2.8.3.1 Early harvesting of grapes

Fermentable sugars consist mainly of hexoses and are normally found at varying concentrations in grape juice, depending on the grape variety and the growth region (Ferreira, 2004). Harvesting grapes at an early stage of development and subsequent vinification result in a reduced alcohol content in wine. However, unripe aromas and unacceptably high acid levels in the finished wine result in a product of inferior quality (Pickering, 2000).

2.8.3.2 Early arrest of fermentation

Early arrest of fermentation results in a reduced alcohol content wine. These wines will have some structure, even though the method is quite restrictive in terms of the styles that can be produced. It is best used when the product is the low-alcohol version of a wine style that is traditionally sweet (Pickering, 2000).

Wines produced by this method have high residual sugar content and therefore the wine has to be microbiologically stable. This is usually achieved by clarification and sulphur dioxide addition (Pickering, 2000). Moreover, early arrest of fermentation produces wines of low quality and stability, favouring the growth of spoilage microorganisms (Caridi *et al.*, 1999).

2.8.4 Rehydration of grapes

A high concentration of grape sugar can pose serious problems during primary and secondary fermentations. Stuck fermentations often occur because many yeast strains are inhibited at high alcohol levels. These conditions can give rise to wines with high levels of residual sugar. High alcohol levels also inhibit malolactic fermentation.

It has become a common practice in California wineries to add water to high-sugar grape must or juice prior to primary fermentation. The purpose of this is to dilute the sugar content to a more manageable level of about 24.5°Brix. Adding water to must or juice will not only dilute the sugar concentration, but will also dilute total acidity and all the other components. Therefore, unless the must or juice already has excessive acidity, it is important to use water that is acidulated with tartaric acid to perform the dilution.

The acidulated water will not only dilute the sugar concentration, but will keep the total acidity and pH constant. Usually seven grams of tartaric acid is added to a litre of distilled water to make up the acidulated water dilution solution. This solution of tartaric acid is then used to dilute high-sugar must or juice before fermentation.

This practice is nevertheless strictly forbidden in many countries, including South Africa.

2.8.5 Blending

Low-alcohol wines can be achieved by blending wines with a high alcohol concentration with wines with a lower alcohol concentration to reduce the alcohol content of the wine (Anelli *et al*., 1986; Maccarone *et al*., 1993).

2.9 Possible biological methods that can be used to reduce the alcohol content of wine

2.9.1 The use of non-*Saccharomyces* yeasts in combination with *Saccharomyces cerevisiae*

Besides *Saccharomyces cerevisiae*, other species of the same genus that can be isolated from grape juice and wine belong to the species *Saccharomyces uvarum* (Rainieri *et al.,* 1999). This species is capable of fermenting at low temperatures (6 to 10°C) and is often responsible for starting fermentations in cold-stored grape juices (Castellari *et al*., 1992).

The major oenological characteristics of these strains are their ability to synthesise malic acid. An increase in malic acid can contribute to improving the acidity of wines produced in areas with warmer climates, where grape juice acidity is usually insufficient (Castellari *et al*., 1994). These strains also produce low concentrations of acetic acid and high concentrations of glycerol and succinic acid which are important traits for the improvement of aroma profile of wine (Kishimoto *et al*., 1993, Castellari *et al*., 1994).

Some of the non-*Saccharomyces* yeasts respire and do not ferment sugars to alcohol, thus the sugar content can be reduced through the formation of by-products other than ethanol. *S. uvarum* strains produce wines with lower levels of ethanol than *S. cerevisiae* wine strains. They also produce high concentrations of higher alcohols, especially β-phenyl-ethanol (Bertolini *et al*., 1996).

Most of these non-*Saccharomyces* yeast species grow in the early stages of wine fermentation, but are eventually out-competed by *S. cerevisiae* due to their lower tolerance of increasing ethanol concentrations and decreasing levels of oxygen (Fleet & Heard, 1993; Boulton *et al*., 1995; Fleet 1997; Hansen *et al*., 2001). It has been shown that non-*Saccharomyces* yeast strains can be detected throughout wine fermentation (Jolly *et al*., 2003).

The non-*Saccharomyces* yeasts can therefore influence the course of fermentation as well as the character of the resultant wine (Jolly *et al*., 2003).

Previous studies have also revealed the potential of indigenous wine yeasts to produce extracellular enzymes of oenological significance that modify and improve the sensory properties of wine. Various secondary metabolic activities of the yeast during a spontaneous alcoholic fermentation can give more complexity to the wine, including a broader spectrum of aroma and flavours (Ciani & Ferraro, 1998; Egli *et al*., 1998, Soden *et al*., 2000).

Non-*Saccharomyces* fermentative genera found in grapes include *Kloeckera*, *Hanseniaspora*, *Debaryomyces*, *Hansenula* and *Metschnikowia*. During primary alcoholic

fermentation of sugar, the wine yeast *S. cerevisiae*, together with other indigenous non-*Saccharomyces* species, produces ethanol, carbon dioxide and a number of by-products. Some of these yeast-derived metabolites include alcohols, acetates and C_4-Cπ fatty acid ethyl esters, which are found in the highest concentration in wine (Lambrechts & Pretorius, 2000).

Although *S. cerevisiae* is responsible for the alcoholic fermentation, the presence of non-*Saccharomyces* species could play an important role by producing secondary metabolites, which can contribute to the final taste and flavour of wines (Esteve-Zarzoso *et al.*, 1998). This may be preferred, as mixtures of indigenous yeast species and *S. cerevisiae* strain starter cultures so as to reflect the biodiversity and stylistic distinctiveness of a given region. This could also help winemakers fulfil the consumer demands for individual wines with intact local character and ensure the survival of a wine's varietal aspects (Lambrechts & Pretorius, 2000).

Another potential aspect of non-*Saccharomyces* yeasts is that low alcohol-producing non-*Saccharomyces* yeast strains can be used in combination with *Saccharomyces* yeast, leading to a low-alcohol wine.

Smith (1995) studied the effects of temperature and aeration on the reduction of sugar content and the production of alcohol by selected yeast strains in Müller-Thurgau grape juice. This author also combined inoculation with selected yeast, short-term controlled aeration of the juice and an anaerobic fermentation using *S. cerevisiae* to produce reduced-alcohol wine. Using *Pichia stipitis* or *Candida tropicalis* as the aerobes, wines with 25 to 30% less alcohol were produced.

2.9.2 Screening for yeast strains with reduced ethanol production

Following a broad screening of yeast strains, Kolb *et al.* (1993) found that *Pichia stipitis* was particularly well suited to sugar removal from juice. Their claims included the elimination of more than 50% of juice sugar within 20 hours, no requirement for added nutritive or other substances and a minimum of adverse effects on the sensory and functional qualities of the juice.

2.9.3 The use of glucose oxidase enzyme

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 distillation or membrane techniques.

An alternative, and potentially less expensive process, involves enzymatic removal of some of the glucose from grape juice by treating the grape juice from mature fruit with glucose oxidase (GOX) enzyme to reduce the glucose content of the juice, which produces wine with a reduced alcohol content after fermentation (Villettaz, 1986, 1987; Heresztyn, 1987; Pickering *et al.*, 1993). The oxidation of glucose results in the formation of D-glucono-1, 5-lactone and hydrogen peroxide. The reactions are as shown in Fig. 2.4.

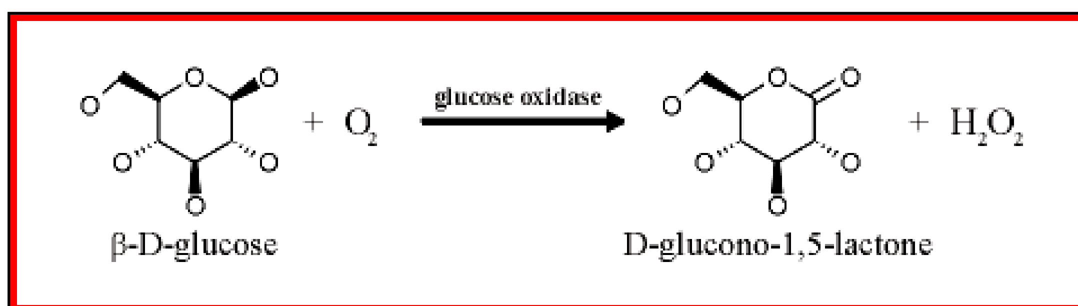


FIGURE 2.4
Glucose oxidase reaction that catalyses the oxidation of β-D-glucose to glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor.

For most large-scale applications, the two enzymatic activities are not separated. The glucose oxidase and catalase may be used together when net hydrogen peroxide is to be avoided, since hydrogen peroxide inhibits the enzyme activity. Hydrogen peroxide is an effective bactericide and may be removed after use by treatment with catalase, which converts it into water and molecular oxygen.

This technology was introduced by Villettaz (1986, 1987) and Heresztyn (1987), whereas Pickering *et al*. (1999a) reported on a number of trials designed to optimise glucose conversion by the glucose oxidase enzyme in grape juice. The trials have shown that the low pH of grape juice is the dominant factor in limiting glucose conversion and deacidification seems to raise the pH of the juice to within the optimal levels for the enzyme activity (Pickering *et al*., 1999b). Pickering *et al*. (1999c) have also reported on the sensory characteristics of these wines. With regard to sensory properties, wines treated with glucose oxidase have increased acidity due to the high levels of gluconic acid; sweetening these wines using unfermented juice or juice concentrate is recommended to counteract this imbalance.

Reduced-alcohol wine from glucose oxidase treatment has a significantly modified taste (showing an increased acidity) and appearance, although most aroma, aroma-by-mouth and mouth feel characteristics appear relatively unaffected (Pickering *et al*., 1999c). The exception is the fruity aromas, which become generally less intense in GOX-treated wines due to the aeration of the juice that is required for optimal enzyme activity.

The physical and chemical stability of white wine produced from GOX-treated juice and the SO_2-binding behaviour of the examined wines were investigated by Pickering *et al*. (1999c). Preliminary work with Riesling juice indicated a higher SO_2-binding affinity in GOX-treated wines compared to the wines made from conventionally processed juices. Wine treated with glucose oxidase showed an increased demand for SO_2-binding compared to the control wine, and the authors suggest that much of this is the result of a higher concentration of unidentified carbonyl compounds.

Chemical evaluation of white wine produced by using glucose oxidase showed a reduction of up to 40% in the potential alcohol yield that could be obtained (Pickering *et al*., 1999a), although high levels of gluconic acids are retained in the finished wine, with relatively little

change in other non-volatile compounds. A relatively high concentration of esters was also reported. The juice is normally treated with glucose oxidase before fermentation. Maximum activity has been observed during the first four to six hours, after which the activity decreases significantly in terms of the rate of gluconic acid formation and glucose degradation (Pickering *et al.*, 1998). The reaction mechanism for the glucose oxidase enzyme system is shown in Fig. 2.5.

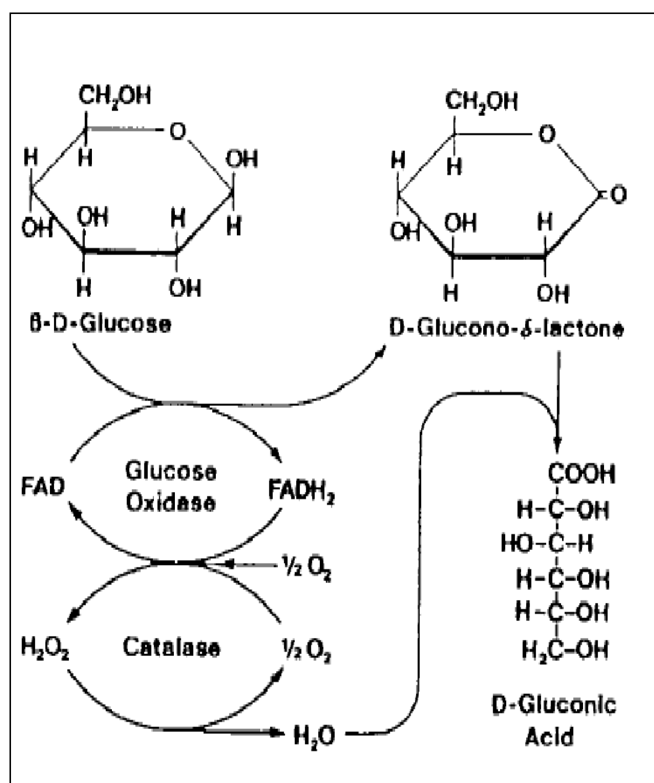


FIGURE 2.5

The reaction mechanism of the glucose oxidase-catalase system (Hartmeier & Willcox, 1981).

Through enzymatic treatment of the juice, glucose is converted to gluconic acid, which cannot be metabolised by yeast, and the wine that is produced after fermentation from the treated juice therefore contains less alcohol. The lower levels of alcohol could be a direct effect of the juice aeration that is carried out, as GOX requires oxygen for glucose conversion.

A simplified flow diagram for the production of reduced- or low-alcohol wine using glucose oxidase is given in Fig. 2.6. One of the limiting factors of the glucose oxidase system is that wines produced from GOX-treated juice show increased SO_2-binding power compared to wines made by conventional methods (Pickering *et al.*, 1999b). A higher concentration of carbonyl compounds may account for this increased SO_2 demand and, in addition, more sulphate is formed from sulphur dioxide in GOX wines.

The greater SO_2-binding capacity of GOX wines is a concern, given statutory regulations governing maximum permitted SO_2 levels in wine and the general trend towards lower SO_2 usage in the wine industry.

GOX wines have been shown to be more stable against browning and have a more golden colour, possibly as a result of increased quinone production and the regeneration of oxidisable phenolic substrates (Pickering *et al.*, 1999b). Another limitation of the glucose oxidase-catalase system is that the highest alcohol reduction obtainable corresponds to 50% of the potential alcohol formed, since the amount of glucose contained in grape juice accounts for only 50% of the total sugar fraction (Villettaz, 1987).

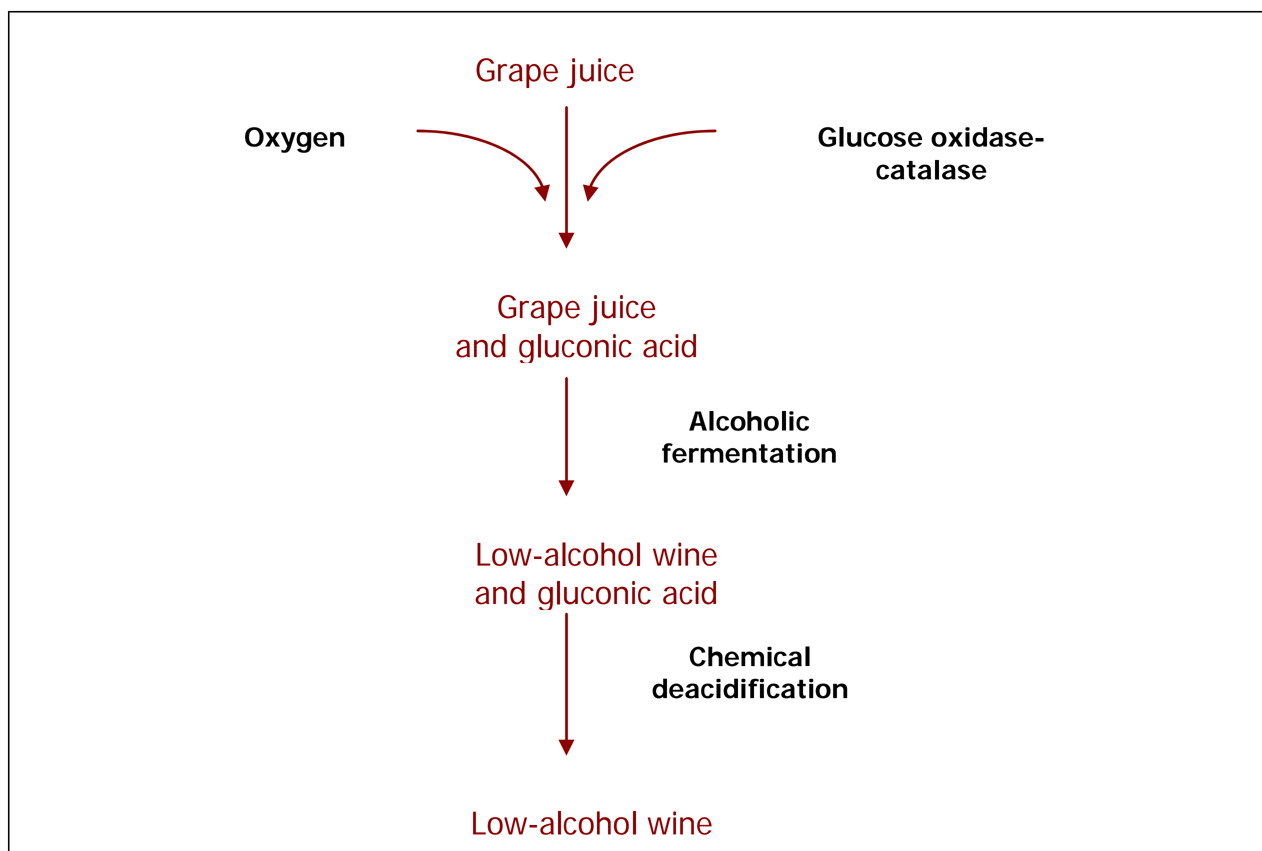


FIGURE 2.6
A simplified flow diagram for the production of reduced-alcohol wine using glucose oxidase (Pickering *et al.*, 1998).

2.9.3.1 Factors influencing glucose oxidase (GOX) pure enzyme (from *Aspergillus niger*) efficiency.

2.9.3.1.1 Enzyme dose

To a certain extent, the glucose conversion increases with increasing enzyme dose, as reported by Villettaz (1987) and Heresztyn (1987).

2.9.3.1.2 pH

The effects of pH on enzymatic reactions are largely caused by the reversible ionisation of the substrate or amino acid residues of the enzyme. These effects are manifested as changes in enzyme activity, stability or interaction (binding) with ligands.

The pH optima of enzymes can vary from pH 2 (pepsin) to pH 10 (alkaline phosphatase), with most enzymes exhibiting optima near neutral pH (Whitaker, 1972). Every enzyme requires a specific pH value or pH range for optimal activity (Uhlig, 1998).

Trials were undertaken by Pickering *et al.* (1998) to optimise glucose conversion by glucose oxidase enzyme in grape juice. These trials showed that the low pH of grape juice is the dominant factor limiting glucose conversion (Pickering *et al.*, 1998). Optimum pH of GOX activity has shown to be between 5.5 and 6.0 and the enzyme is stable between 4.5 and 7.0 (Whitaker, 1972). Pickering *et al.*, (1993) reported that deacidification of grape juice with calcium carbonate before treatment with GOX significantly increased both rate and extent of glucose conversion. Wardman (1995) successfully used base (5M KOH) prior to and during GOX treatment of grape juice to regulate the pH.

2.9.3.1.3 Aeration

Villettaz (1987) and Heresztyn (1987) noted that the optimum aeration rates for glucose oxidase treatment of grape juice had not been established. Improved glucose oxidase performance is suggested at the higher aeration rate although this effect becomes minimal or negligible by the end of the treatment period (Pickering *et al.*, 1998). The rate of disappearance of oxygen can be used as an assay of GOX activity (Whitaker, 1994). An optimal aeration regime was eventually established for Müller-Thurgau juice, namely aeration at 4 L. min^{-1} and mixing at 330 rpm (Pickering *et al.*, 1998).

2.9.3.1.4 Temperature

Most chemical reactions depend on temperature. Enzyme performance usually improves with increasing temperature. In general, the enzymatic conversion rate doubles for every increase in temperature of 10°C (Uhlig, 1998).

The optimum temperature for the glucose oxidase has previously been reported as being between 30 and 40°C (Whitaker, 1972; Merck Index, 1986; Novozyme, 1990), while immobilised glucose oxidase may retain 20 percent of its optimal activity between 0 and 5°C (Hartmeier & Willcox, 1981). For use in grape juice, a desirable temperature range of between 15 and 20°C in GOX has been suggested (Villettaz, 1986, 1987; Heresztyn, 1987). When comparing treatments with GOX at 20 and 30°C, the latter author noted reduced enzyme activity at higher temperature. A lower processing temperature will be advantageous with respect to undesirable microbial activity and the general quality of the juice.

In a subsequent trial conducted without temperature control, an increase of about 7°C above ambient (22°C) was noticed during processing over 10 hours (Pickering *et al.*, 1998). Both glucose oxidase and catalase reactions are exothermic, which may account for this increase in temperature.

2.9.3.1.5 Sulphur dioxide (SO_2)

Sulphur dioxide is the most common antioxidant and antimicrobial agent used in the vinification of grape juice. However, it has been suggested (Ough, 1960; Mc Leod & Ough, 1970) and reported as being inhibitory to the activity of the GOX system in wine (Merzhanian & Tagunkov, 1967).
Further research showed that, at the levels used in wines, SO_2 delays the GOX-mediated removal of oxygen, but does not completely inhibit it (White & Ough, 1973; Scott, 1975; Ough, 1975; Pickering *et al.*, 1998).

2.9.4 The use of glucose oxidase as a biological control agent

A number of inhibitory techniques with GRAS (Generally Regarded as Safe) status may be used for the biological preservation of foods. These may be more acceptable to consumers than the use of chemical additives. Some of these include bacteriocins, lysozyme and glucose oxidase (Geisen, 1998).

Since glucose oxidase oxidises glucose to gluconolactone, which is then converted to gluconic acid, the pH decreases and other secondary products, such as hydrogen peroxide, which inhibits the growth of spoilage organisms, are formed. Hydrogen peroxide is the major factor in the inhibitory effect (Yoo & Rand, 1995). The increased concentration of the hydrogen peroxide could result in hyperbaric oxygen toxicity, which is a direct result of the peroxidation of membrane lipids that could lead to increased permeability of the membrane. The hydrogen peroxide can also react with other compounds to form additional inhibitory compounds (De Vuyst & Vandamme, 1994).

The glucose oxidase system has been used to preserve fish, leading to improved refrigeration shelf life. It has also shown antimicrobial activity when used with several other seafood products. This system also inhibits microbial growth of a variety of meat spoilage microorganisms and some pathogens (Yoo & Rand, 1995).

Glucose oxidase production by genetic engineering is an effective means for introducing broad-spectrum antibacterial activity into fungal starter cultures or any other industrially important starter culture (Geisen, 1998; Schoeman *et al.*, 1999).

The use of glucose oxidase as a biological control agent for food preservation against a broad spectrum of spoilage microorganisms without the use of chemical additives may be satisfactory to some consumers. The overexpression of the GOX gene in certain microorganisms can enhance the inhibitory effect of glucose oxidase.

2.9.5 Commercial applications of glucose oxidase

The glucose oxidase from *Aspergillus niger* is of industrial importance. It is widely used for the detection and quantification of glucose in industrial solutions as well as in body fluids. It is also used for the stabilisation of various foods and beverages in order to improve their shelf life (Whittington *et al.*, 1990; De Baetselier *et al.*, 1991; Hammer, 1998; Park *et al.*, 2000; Kapat *et al.*, 2001), and to maintain flavour and colour stability (Ohlmeyer, 1957; Power, 1998; Pickering, 1998; Vemulapalli *et al.*, 1998).

The glucose oxidase has been used safely and effectively in the food and beverage industries since the 1950s. Although there are many applications of this enzyme, only a few have been commercially successful. Two major applications of glucose oxidase are glucose removal and oxygen removal. Other applications include gluconic acid production, breadmaking as well as in biosensors.

2.9.5.1 Glucose removal

An important application of the glucose oxidase-catalase system was developed by Scott (1953) for desaccharification in order to stabilise commercial liquid egg white. To increase the stability of a product with extended shelf life, the concentration of free glucose must be reduced to less than 0.1% (Uhlig, 1998).

The removal of glucose for the minimisation of browning (Millard reaction) is also performed commercially for other food ingredients, using procedures similar to those used for egg desugaring (Szalkucki, 1993). In these applications, the enzymatic process is preferred over fermentation because of the minimal change in the flavour of the treated product.

2.9.5.2 Oxygen removal

Citrus concentrates and citrus beverages are products containing solubilised oxygen, which yields peroxide on exposure to sunlight (Uhlig, 1998). These peroxides cause significant flavour changes in citrus beverages. Orange and grapefruit juice concentrates can also undergo oxidative changes and it is therefore desirable to remove the oxygen (Szalkucki, 1993; Lea, 1995). Glucose oxidase added to freshly expressed orange juice will cause a significant decrease in the potential for yeast growth (Uhlig, 1998). As a result, the shelf life is significantly improved (Sagi & Mannheim, 1988).

The glucose oxidase-catalase system can also stabilise white wine against both browning and changes in taste (Ough, 1960). It has also been used for the stabilisation of beer (Power, 1993) and the removing of oxygen from cheese packaging and in mayonnaise, thereby preventing changes in taste (Bloom *et al.*, 1956).

One of the major drawbacks was the unforeseen problem with hydrogen peroxide formed by the enzyme reaction, while others were mainly due to economics and the availability of equipment at the time (Power, 1998).

2.10 Genetically engineered wine yeast strains for the production of reduced-alcohol wine

2.10.1 Expression of *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential for reducing alcohol in wine

A recombinant yeast strain that can reduce the alcohol levels in wine has been engineered (Malherbe *et al.*, 2003). Another benefit of this strain is to reduce the risk of microbial spoilage and oxidation.

Using molecular techniques, a single copy of the *Aspergillus niger* glucose oxidase gene, integrated into the genome of a laboratory strain of *S. cerevisiae* was constitutively expressed under the control of the *PGK1* promoter. The yeast *MFa 1s* leader sequence directed the secretion of Gox1p enzyme. The production of extracellular glucose oxidase activity was found to be growth-related and the secreted glucose oxidase converts glucose into gluconic acid in the media before the yeast cells are able to metabolise the glucose to ethanol.

After vinification of Chardonnay grapes using a genetically modified yeast strain; the alcohol content was approximately 1.8% v/v less ethanol compared to the control strain (Malherbe *et al.*, 2003). This could be one way of developing wine yeast starter culture strains for the production of wine with low alcohol content and reduced levels of chemical preservatives like sulphur dioxide.

2.10.2 Reduction of ethanol formation through overproduction of glycerol

Apart from ethanol and carbon dioxide, glycerol is the most abundant product of yeast fermentation. It is non-volatile and does not contribute to wine aroma (Eustace & Thornton, 1987), but contributes to the smoothness and viscosity (Noble & Bursick, 1984). Glycerol concentrations vary between 1 and 10 g/L (Ough *et al.*, 1972; Goiffon *et al.*, 1980; Moruno & Di Stefano, 1989).

The amount of glycerol formed during fermentation is influenced by the yeast strain (Radler & Schutz, 1982); however, the chemical composition of the medium and factors including oxygen content, fermentation temperature and pH have been demonstrated to be relevant to the process (Ough *et al.*, 1972; Gardner *et al.*, 1993).

Glycerol is an economically important alcohol due to its wide application in the food, beverage, chemical and pharmaceutical industries (Scanes *et al.*, 1998). The formation of glycerol during the production process of beverages such as wine and beer has gained considerable attention because of the perception that glycerol contributes to the quality of these products. The glycerol present in these beverages can be ascribed mainly to the metabolic activity of microorganisms associated with the fermentation process. Among these microorganisms, *S. cerevisiae* has been the main focus of intense research and scientific data relating to glycerol formation during alcoholic fermentation have been generated from a number of scientific disciplines.

There has also been considerable interest in modifying glycerol metabolism in *S.cerevisiae* because of the perceived sensory importance of glycerol in wine and other beverages (Scanes *et al*., 1998). Furthermore, increasing glycerol formation by *S. cerevisiae* can divert the flow of carbon away from ethanol production during fermentation (Michnick *et al*., 1997; Remize *et al*., 1999; De Barros Lopes *et al*., 2000; Prior *et al*., 2000) towards the formation of various by-products. This is of particular importance, since harvesting fully ripened grapes is becoming a common practice that can lead to the production of wines that have, in addition to enhanced varietal character, a high concentration of alcohol that can be perceived as being out of balance.

In *S. cerevisiae*, glycerol is synthesised by reducing the glycolytic intermediate dihydroxy acetone phosphate (DHAP) to glycerol-3-phosphate dehydrogenase (G3P), catalysed by the glycerol-3-phosphate dehydrogenase (GPD), followed by dephosphorylation of G3P by glycerol-3-phosphatase (GPP). The two isoenzymes of GPD are encoded by the genes *GPD1* and *GPD2* for NAD-dependent glycerol-3-phosphate dehydrogenase, and *GPP1* and *GPP2* for glycerol-3-phosphates (Remize *et al*., 2003). These enzymes have different physiological roles; the Gpd1p-Gpp2p combination is involved mainly in glycerol production during osmotic stress, whereas the Gpd2p-Gpp1p combination is primarily involved in adjusting the NADH-NAD^+ redox balance under anaerobic conditions (Albertyn *et al*., 1994; Ansell *et al*., 1997; Pahlman *et al*., 2001).

The first step in glycerol synthesis is the most important as glycerol 3-phosphate dehydrogenase activity controls the amount of glycerol produced (Nevoigt & Stahl, 1996; Michnick *et al*., 1997; Remize *et al*., 1999). During alcoholic fermentation, the major role of glycerol formation is to maintain the cytosolic redox balance (Michnick *et al*., 1997), especially during anaerobic growth. As the ethanol formation from sugar is redox-neutral, the cytosolic NADH produced in surplus during other cellular processes, such as biomass production and the excretion of oxidised metabolites, is re-oxidised by GPD during glycerol formation.

Glycerol is also involved in the osmoregulation of *S. cerevisiae* and acts as the main compatible solute in this organism. Osmotic stress gives rise to the over-production of glycerol, which is controlled mainly by the induction of *GPD1*.

2.10.2.1 Glycerol production in response to osmotic stress

Once introduced into grape juice, yeast cells are exposed to a medium that contains a high concentration of osmotically active compounds, notably glucose and fructose. This could cause a rapid leakage of water from the cell to the surrounding medium (Scanes *et al*., 1998; Bauer & Pretorius, 2000). In order to prevent this leakage, *S. cerevisiae* produces and accumulates glycerol in an attempt to bring equilibrium between the internal osmotic pressure and the external medium. Glycerol is thus referred to as a compatible solute in this context, since it is compatible with enzyme and membrane functions.

The expression of genes involved in the synthesis of glycerol in response to osmotic stress, namely *GPD1* and *GPD2*, is partially under the control of the HOG (high osmolarity glycerol)

pathway (Albertyn *et al*., 1994; Norbeck *et al*., 1996) and all aspects of the sensing and transduction of the osmotic stress response are currently the main focus of intense research (Bauer & Pretorius, 2000; Hohmann, 2002).

2.10.2.2 Glycerol production in response to redox balancing

The preference of *S. cerevisiae* for NADH in dissimilatory reductions, such as the reduction of acetaldehyde to ethanol during growth on sugars, is very strong (Pronk *et al*., 1996; Bakker *et al*., 2001). NADH/NAD^+ is considered as a conserved moiety and only catalytic quantities of these pyridine nucleotides are present in the yeast. The yeast therefore maintains a balance between the amounts of NADH and NAD^+, and the reduction of NAD^+ must be matched by the oxidation of NADH and vice versa.

The dissimilation of sugar to pyruvate under strictly anaerobic conditions results in the conversion of glyceraldehyde-3-phosphate (GA-3-P) to glyceraldehyde-1, 3-biphosphate (G-1, 3-bP) is indicated in Fig. 2.7. Pyruvate is then decarboxylated to form acetaldehyde, which subsequently acts as an electron acceptor in the reoxidation of NADH.

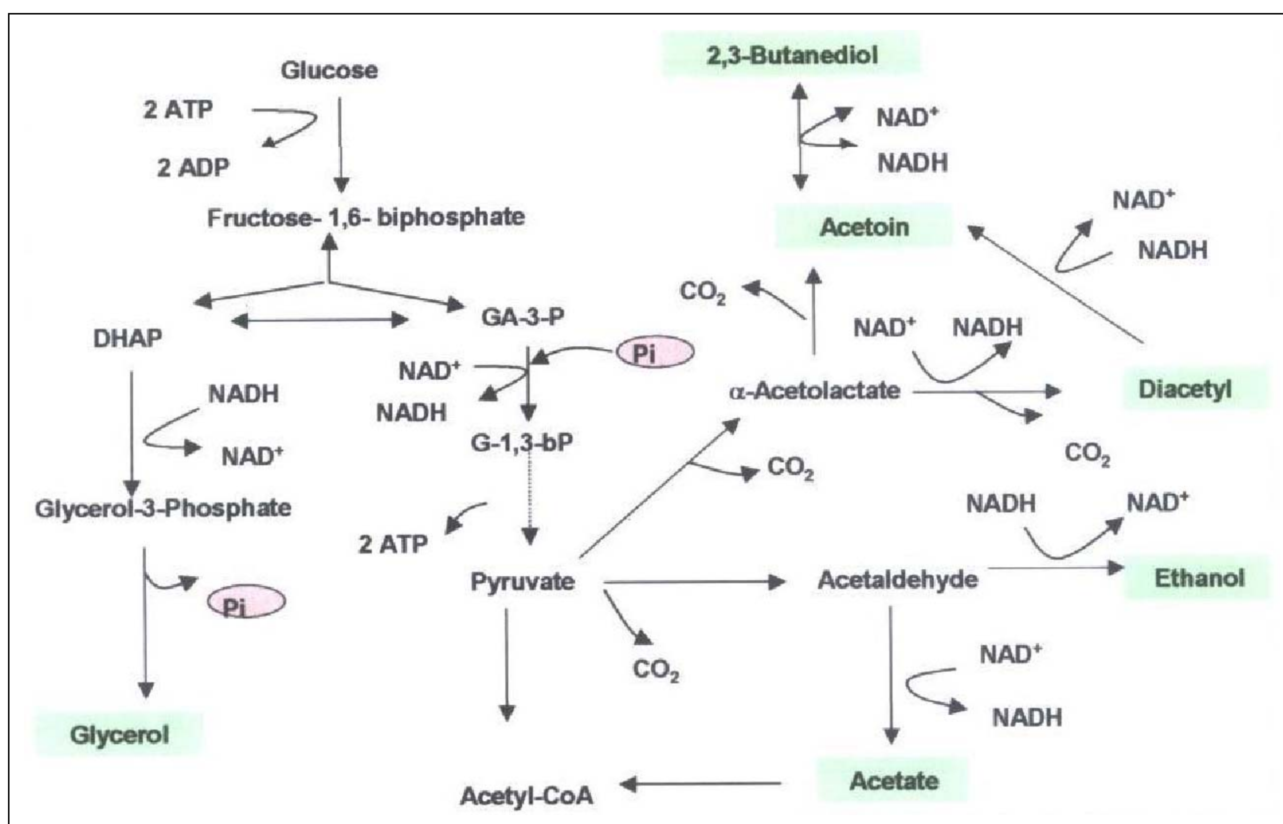


FIGURE 2.7
The formation of glycerol and other by-products in relation to redox balancing in *Saccharomyces cerevisiae* (adapted from Pronk *et al*., 1996).

The yeast formation of biomass during the early stages of fermentation results in a surplus of NADH (Pronk *et al.*, 1996; Bakker *et al.*, 2001). The reoxidation of NADH is achieved through the formation of secondary by-products such as glycerol and butane-2, 3-diol. Of these by-products, glycerol is predominant, and *ca.* 3.3% w/w of the sugar utilised under fermentative conditions is converted to glycerol (Oura, 1977).

The redox balance thus dictates that an increased specific rate of glycerol production will be balanced by an increased production of oxidised metabolites, such as acetaldehyde or acetic acid, pyruvate, succinic acid, acetoin, diacetyl and butane-2, 3-diol (Fig. 2.7). These components have the potential to influence the sensory and flavour attributes of wine (Nieuwoudt, 2004).

2.10.2.3 The manipulation of glycerol levels by genetic engineering

A slight increase in glycerol production in wine can be achieved by using yeast strains selected for high glycerol production and by optimising fermentation conditions. Increasing the level of glycerol has also been attempted by means of the selective hybridisation of wine yeast strains, leading to the construction of yeast producing 10 to 11 g/L glycerol (Eustace & Thornton, 1987) compared to the glycerol concentration usually produced by wine yeast strains.

More recently, genetic engineering approaches have been successful in redirecting the carbon flux towards glycerol formation.

It has been suggested that, although environmental factors have a significant influence on the levels of glycerol formed by the yeast, the variation may also be due to genetic diversity among yeast strains (Rankine & Bridson, 1971). The formation of glycerol by wine yeast is considered as a favourable attribute and has been identified as one of the main targets for genetic improvement (Thornton, 1983; Pretorius, 2000).

One of the aims is to optimise the glycerol production in wine and to develop a mechanism for redirecting the carbon flux away from ethanol towards glycerol formation in order to produce wine with a reduced ethanol concentration (Eglinton *et al.*, 2002).

There has also been increased interest in manipulating the glycerol levels that are formed by the yeasts by using recombinant DNA techniques (Michnick *et al.*, 1997; Remize *et al.*, 1999; De Barros Lopes *et al.*, 2000; Eglinton *et al.*, 2002).

Nieuwoudt (2004) showed that overexpression of the glycerol-3-phosphatase dehydrogenase-encoding gene of *S. cerevisiae* (*GPD2*) led to a substantial increase in the glycerol concentration formed in Chardonnay wine produced by the recombinant strain (16.5 g/L), compared to the amount of glycerol formed by the parental strain (7.9 g/L).

The overexpression of *GPD2* did not only result in an increased concentration of glycerol, but was also accompanied by an increase in acetic acid concentration (1.02 g/L) for the recombinant strain, as opposed to 0.58 g/L for the parental strain (De Barros Lopes *et al.*, 2000). It was also noted that the altered glycerol production of the recombinant strain reduced the ethanol concentration formed during the production of wine by 6 g/L (approximately 0.6% v/v), and this shows the potential for this technique to reduce the ethanol content in wine. The

increased in acetic acid was expected, since yeast glycerol metabolism serves, among other functions, as a redox sink for the surplus of NADH formed during assimilatory sugar metabolism (Nieuwoudt, 2004). However, stimulating glycerol formation could result in the formation of more oxidised metabolites such as acetic acid.

Subsequent efforts have been directed at decreasing acetic acid formation through the deletion of the aldehyde dehydrogenase gene (*ALD6*) that catalyses the conversion of acetaldehyde to acetic acid (Eglinton *et al.*, 2002). Thus, the concentration of ethanol in wine can be reduced by developing yeast strains in which the glycolytic flow of sugar is diverted away from ethanol formation towards the production of glycerol.

2.11 Conclusion

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. A high concentration of sugar leads to the production of high alcohol wines. The high alcohol content of wines can affect the organoleptic properties and mask the overall aroma and flavour of the wine. Stuck fermentations are common in musts with higher sugar concentrations.

Thus health conscious consumer and increasing social concerns have led to a demand for reduced alcohol levels in wine production.

Several physical processes that are currently used for the removal or reduction of alcohol in wine tend to involve expensive equipment and can be detrimental from a quality point of view. Regardless of which method is employed, increased aroma loss and modification of other flavour components are reported with increasing removal of alcohol.

A great deal of attempts through genetic engineering of yeast strains to reduce ethanol content of wine has been done, but the high alcohol wines remain the biggest challenge as genetically modified organisms are not yet permissible for use in the South African wine industry.

Fast, reliable and cost-effective methods should therefore be developed in order to produce wines with reduced-ethanol content.

This review highlighted on available physical processes as well as some other possible methods that can be used to reduce the alcohol content of wine and their influence on the aroma and flavour quality of these wines.

Thus 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.

2.12 Literature cited

Albertyn, J., Hohmann, S., Thevelein, J.M. & Prior, B.A., 1994. *GPD1*, which encodes glycerol-3-phosphate dehydrogenase is essential for growth under osmotic stress in *Saccharomyces cerevisiae* and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. 14, 4135-4144.

Amerine, M.A., Berg, H.W. & Cruess, W.V., 1972. The technology of winemaking. 3rd ed. Westport. CT: AVI Publishing Co.

Anelli, G., Massantini, R. & Contini, M., 1986. Kiwine: nuova bevanda a basso tenore alcolico a base di mosto dúva. Industrie delle bevande 180-184.

Anonymous, 1986. Low-alcohol drinks. Which? December, 576-577.

Anon, 1988. Low alcohol: A must for the future? Decanter. December, 85-86.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M. & Adler, L., 1997. The two iso enzymes for yeast NAD+- dependent glycerol-3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. 16, 2179-2187.

Bakker, B.M., Overkamp, K.M., Van Maris, A.J.A., Kötter, P., Luttik, M.A.H., Van Dijken, J.P. & Pronk, J.T., 2001. Stochiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae.* FEMS Microbiol. Rev. 25, 15-37.

Bauer, F.F. & Pretorius, I.S., 2000. Yeast stress response and fermentation efficiency: How to survive the making of wine- A review. S. Afr. J. Enol. Vitic. 21, 27-51.

Bertolini, L., Zamboelli, C., Giudici, P. & Castellari, L., 1996. Higher alcohol production by cryotolerant *Saccharomyces* strains. Am. J. Enol. Vitic. 47, 343-345.

Bisson, L.F., 1993. Yeast-metabolism of sugars. In: Wine Microbiology and Biotechnology. Fleet, G.H. (ed). Harwood Academic Publishers. Chur. 55-75.

Blackwelder, W.C., Yano, K., Rhoads, G.G., Kagan, A., Gordon, T. & Palesch, Y., 1980. Alcohol and mortality. The Honolulu Heart Study. Am. J. Medicine 68, 164-169.

Bloom, J., Scofield, G. & Scott, D., 1956. Oxygen removal prevents rancidity in mayonnaise. Food Packer 37, 16-17.

Boucher, A.R., 1983. Preparation of wines having a low calorie content and a reduced alcohol content. United States Patent 4 405 652, 20/9.

Boucher, A.R., 1985. Verfahren und Vorrichtung zur Herstellung eines alkoholfreien Weingetränks, Deutsche Offenlegungsschrift DE 3 429 777 A1, 28/2.

Boucher, A.R., 1988. Preparation of alcohol free wine. United States Patent US 4 775 538.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E., 1995. In: Principles and Practices of Winemaking. New York: Chapman & Hall. New York.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E., 1996. Yeast and biochemistry of ethanol fermentation. In: Principles and Practicers of Winemaking. New York: Chapman & Hall. London. pp.102-192.

Bui, K., Dick, R., Moulin, G. & Galzy, P., 1986. A reverse osmosis for the production of low ethanol content wine. Am. J. Enol. Vitic. 37, 297-300.

Caridi, A., Crucitti, P. & Ramondino, D., 1999. Winemaking of must at high osmotic strength by thermotolerant yeast. Biotechnol. Lett. 21, 617-620.

Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P. & Zambonelli, C. 1994. Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant *Saccharomyces* strains. Vitis. 33, 49-52.

Castellari, L., Pacchioli, G., Zambonelli, C., Tini, V. & Grazia, L., 1992. Isolation and initial characterization of cryotolerant *Saccharomyces* strains. Ital. J. Food Sci. 3, 179-186.

Castellari, M., Versari, A., Spinabelli, U., Galassi, S. & Amati, A., 2000. An improved HPLC method for the analysis of organic acids, carbohydrates and alcohols in grape musts and wines. J. Liq. Chrom. & Technol. 23, 2047-2056.

Ciani, M. & Ferraro, L., 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. J. Appl. Microbiol. 85, 247-254.

Crabtree, H.G., 1929. Observations on carbohydrate metabolism of tumors. Biochem. J. 23, 536-545.

Day, R.E., Gishen, M., Godden, P.W. & Hughes, J.B., 2002. Producing unique wine styles for overseas market. In: Blair, R. J., Williams, P. J. & Høj, P.B. (eds). Proc. 11th Aust. Wine Ind. Tech. Conf. October, 2002. Adelaide, Australia. pp. 27-32.

De Baetselier, A., Vasavada, A., Dohet, P., Ha-Thi, V., De Beukelaer, M., Erpicum, T., De Clerck, L., Hanotier, J. & Rosenberg, S., 1991. Fermentation of a yeast producing *Aspergillus niger* glucose oxidase: scale-up, purification and characterisation of the recombinant enzyme. Biol. Technol. 9, 559-561.

De Barros Lopes, M., Eglinton, J., Henschke, P., Høj, P. & Pretorius, I., 2003. The connection between yeast and alcohol reduction in wine. Managing the double-edged sword of bottled sunshine. Wine Ind. J. 18, 1-4.

De Barros Lopes, M., Rehman, A., Gockowiak, H., Heinrich, A.J., Langridge, P. & Henschke, P.A., 2000. Fermentation properties of a wine yeast over-expressing the *Saccharomyces cerevisiae* glycerol-3-phosphate dehydrogenase gene (*GPD2*). Aust. J. Grape Wine Res. 6, 208-215.

De Deken, R.H., 1966. The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol. 44, 129-156.

Déglon, H., 1975. Procédé pour la séparation des alcools et essences volatiles des autres produits dans les mélanges obtenus par fermentations. Schweizer Patent 564 603, 15.6.

De Vuyst, L. & Vandamme, E.J., 1994. Antimicrobial potential of lactic acid bacteria. In: Bacteriocins of lactic acid bacteria. L. De Vuyst & E.J. Vandamme (eds). New York. Blackie Academic & Professional. pp. 99-103.

Egli, C.M., Edinger, W.D., Mitrakul, C.M. & Henick-Kling, T., 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. J. Appl. Microbiol.85, 779-789.

Eglinton, J.M., Heinrich, A.J., Pollnitz, A.P., Langridge, P., Henschke, P.A. & De Barros Lopes, M., 2002. Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the *ALD6* aldehyde dehydrogenase gene. Yeast 19, 295-301.

Ellis, L.P., 1999. Stuck fermentation in red wines: causes, consequences and recommendations for the 1998 season. Wynboer, March. pp. 1-3.

Esteve-Zarzoso, B., Manzanares, P., Ramón, D. & Querol, A., 1998. The role of non-*Saccharomyces* yeasts in industrial winemaking. Int. Microbiol. 1, 143-148.

Eustace, R. & Thornton, R.J., 1987. Selective hybridization of wine yeasts for higher yields of glycerol. Can. J. Microbiol. 33, 112-117.

Feldmann, H., 2005. Yeast molecular biology: a short compendium on basic features and novel aspects. Adolf-Butenandt-Institute. Univ. of Munich. Munich, Germany.

Fischer, U. & Noble, A.C., 1994. The effect of ethanol, catechin concentration, and pH on sourness and bitterness of wine. Am. J. Enol. Vitic. 45, 6-10.

Fleet, G.H., 1997. Wine. In: Food microbiology: fundamentals and frontiers. Doyle, M.P., Beuchat, L.R. & Montville, T.J. (eds). Am. Soc. Microbiol. Washington DC. pp. 671-694.

Fleet, G.H. & Heard, G.M., 1993. Yeast-growth during fermentation. In: Wine microbiology and biotechnology. Fleet, G.H. (ed). Harwood academic Publishers. Singapore. pp. 27-54.

Gafner, J. & Schütz, M., 1996. Impact of glucose-fructose ratio on stuck fermentations: practical experiences to restart stuck fermentations. Vitic. Enol. Sc. 51, 214-218.

Gardner, N., Rodriguez, N. & Champagne, P.C., 1993. Combined effects of sulfites, temperature and agitation time on production of glycerol in grape juice by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 59, 2022-2028.

Geisen, R., 1998. Inhibition of food-related pathogenic bacteria by god-transformed *Penicillium nalgiovense* strains. J. Food Protect. 62, 940-943.

Gishen, M & Holdstock, M., 2000. Preliminary evaluation of the performance of the Foss Winescan FT 120 instrument for the simultaneous determination of several wine analysis. Australian Grapegrower Winemaker Ann. Tech. Issue. pp 75-81.

Gnekow, B. & Ough, C.S., 1976. Methanol in wines and musts: source and amounts. Am. J. Enol. Vitic. 27, 1-6.

Godden, P., 2000. Persistent wine instability issues. Aust. Grapegrower Winemaker. 443, 11-14.

Goiffon, J.P., Blachere, A., Perez, J.L. & Portal, E., 1980. Détermination du glycérol dans les vins par HPLC; comparaison avec les autres méthodes. Ann. Falsif. Exp. Chim. 73, 17-23.

Goode, J., 2005. The application of science in winemaking. Wine science. Mitchell Beazley. 109-114.

Gray, C., 1993. History of the spinning cone column, juice technology workshop, Special Report 67. Cornell University.

Grosch, W., 2001. Evaluation of the key odourants of foods by dilution experiments, aroma models and omission. Chem. Senses. 26, 533-545.

Guth, H. & Sies, A., 2002. Flavour of wines: towards an understanding by reconstitution experiments and an analysis of ethanol's effect on odour activity of key compounds. In: Blair, R.J., Williams, P.J. & Høj, P. B. (eds). Proc. 11th Aust. Wine Ind. Techn. Conf. October. Adelaide, Australia. pp. 128-139.

Hammer, F.E., 1998. Oxidoreductases. In: Enzymes in food processing. Nagodawithana, T. & Reed, G. (eds). Academic Press. New York, NY. pp. 251-254.

Hansen, E.H., Nissen, P., Sommer, P., Nielsen, J.C. & Arneborg, N., 2001. J. Appl. Microbiol. 91, 541-547.

Hartmeier, W. & Willcox, I.C., 1981. Immobilised glucose oxidase and its use for oxygen removal from beer. Technical Quarterly, Masters Brewers' Association of the America. 18, 145-149.

Hay, J., 2001. From isinglass to osmosis and a cone. Wine Business Monthly VIII, 1-8.

Hees, W., 1990. Marketing changes, legal questions and the production of dealcoholised wines. In Proc. 9th Int. Oen. Symp. Cascais, Portugal, May. pp. 154-165.

Hennekens, C.H., 1996. Alcohol and risk of coronary events. In: Alcohol and the cardiovascular system. Zakhari, S. & Wassef, M. (eds). NIAAA Research Monograph No. 31, NIH Pub. No. 96-4133. U.S. Govt., Washington, DC. pp. 15-24.

Henschke, P., 1997. Stuck fermentation- Causes, prevention and cure. ASVO Seminar pp. 30-38.

Heresztyn, T., 1987. Conversion of glucose to gluconic acid by glucose oxidase enzyme in Muscat Gordo juice. The Australian Grapegrower and Winemaker, April. pp. 25-27.

Hoffmann, D., 1990. The European wine market up to the year 2000 – trends and marketing efforts. In: Proc. 9th Int. Oen. Symp. Cascais, Portugal, May. pp. 138-153.

Hohmann, S., 2002. Osmotic stress signaling and osmoadaptation in yeasts. Mol. Microbiol. Rev. 66, 300-372.

Howley, M. & Young, N., 1992. Low alcohol wines: the consumer choice? Int. J. Wine Marketing 4, 45-46.

Jackson, R.S., 1994. Wine science – principles and applications. Academic Press, San Diego, New York. pp. 182-188.

Jacobson, J.L., 2006. Introduction to wine laboratory practices and procedures. Springer. New York, USA. 141.

Jolly, N.P., Augustyn, O.P.H. & Pretorius, I.S., 2003. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55-62.

Jolly, N. P., Augustyn, O. P. H. & Pretorius, I. S., 2003. The use *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine. S. Afr. J. Enol. Vitic. 24, 63-69.

Kapat, A., Jung, J.K. & Park, Y.H., 2001. Enhancement of glucose oxidase production in batch cultivation of recombinant *Saccharomyces cerevisiae*: optimisation of oxygen transfer conditions. J. Appl. Microbiol. 90, 216-222.

Kishimoto, M., Shinohara, T., Soma, E. & Goto, S., 1993. Identification and enological characteristics of cryophilic wine yeasts. J. Brew. Soc. Japan. 88, 708-713.

Kliewer, W.M., 1967. The glucose-fructose ratio of *Vitis vinifera* grapes. Am. J. Enol. Vitic. 18, 33-41. Department of Viticulture and Enology. University of California, Davis.

Kolb, E., Wiesenberger, A., Stahl, U. & Hawart, K., 1993. Biological processes for the partial reduction of sugar in fruit juices. Proc. Int. Fed. Fruit Juice Prod. Symp, May. Budapest, Hungary. pp. 7-20.

Lambrechts, M.G & Pretorius, I.S., 2000. Yeast and its importance to wine aroma – a review. S. Afr. J. Enol. Vitic. 21, 97-105.

Lea, A.G.H., 1995. In: Tucker, G.A. & Woods, L.F.J. (eds). Enzymes in food processing, (2nd Ed). Enzymes in the production of beverages and fruit juices. Blackie Academic & Professional, Chapman & Hall, New York. pp. 246.

Lee, C.K., 1987. The chemistry and biochemistry of the sweetness of sugars. Adv. Carbohydr. Chem. Biochem. 45, 199-351.

Linskens, H.L. & Jackson, J.F., 1988. Wine analysis. New Series 6, 151-152.

Lynch, L., 1988. Low-alcohol: a must for the future? Decanter, December. pp. 85-86.

Maccarone, E., Nicolosi Asmundo, C., Cataldilupo, M.C., Campisi, S. & Fallico, B., 1993. Oranwine. A new wine cooler with blood orange juice. Ital. J. Food Sc. 5, 397-408.

Malherbe, D.F., 2003. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential application in wine production. MSc. Thesis.

Malherbe, D.F., Du Toit, M., Cordero Otero, R.R., Van Rensburg, P. & Pretorius, I.S., 2003. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential application in wine production. Appl. Microbiol. Biotechnol. 61, 502-511.

Malherbe, D.F. & Van Rensburg, P., 2003. Let the yeast do the work for you: Less alcohol per bottle. Wynboer. June. pp. 1-4.

Margalit, Y., 1997. In: Concepts in wine chemistry. Chapter 1: Must and wine composition. Crum, J. (ed). The Wine Appreciation Guild. San Francisco. pp. 23-24.

Martin, S. & Pangborn, R.M., 1970. Taste interaction of ethyl alcohol with sweet, salty, sour and bitter compounds. J. Sc. & Food Agric. 21, 653-655.

Massel, A., 1969. Applied Wine Chemistry and Technology. Heidelberg Publishers, London. pp. 78-79.

Mattes, R.D. & Di Meglio, D., 2001. . Ethanol perception and ingestion. Physiol. & Behaviour. 72, 217-229.

Mattick, L.R. & Rice, A.C., 1970. Survey of the glycerol content of New York state wines. Am. J. Enol. Vitic. 21, 213-215.

Mc Leod, R. & Ough, C.S., 1970. Some recent studies with glucose oxidase in wine. Am. J. Enol. Vitic. 21, 54-60.

McCloskey, L.P. & Replogle, L.L., 1974. Evaluation of an enzymatic method for estimating ethanol in wines using an “Enzymatic Kit”. Am. J. Enol. Vitic. 25, 194-197.

Merck Index, 1986. (11th Ed). Merck & Co. Rahway, NJ.

Mermelstein, N.H., 2000. Removing alcohol from wine. Food Technol. 54, 89-92.

Michnick, S., Roustan, J.-L., Remize, F., Barre, P. & Dequin, S., 1997. Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for GPD1 encoding glycerol 3-phosphate dehydrogenase. Yeast. 13, 783-793.

Moruno, F.G. & Di Stefano, R., 1989. Determination of glycerol, 2-3 butanediol and glycols in wine. Vini Ital. 31, 41-46.

Muller, C.J., Fugelsang, K.C. & Wahlstrom, V.L., 1993. Capture and use of volatile flavour constituents emitted during wine fermentations. In: Beer and wine production: analysis, characterization and technological advances. Gump, B.H. (ed). Am. Chem. Society Series 536, 219-234. Washington, DC.

Nevoigt, E. & Stahl, U., 1996. Reduced pyruvate decarboxylase and increased glycerol-3-phosphate dehydrogenase (NAD+) levels enhance glycerol production in *Saccharomyces cerevisiae*. Yeast. 12, 1331-1337.

Nevoigt, E., Pilger, R., Mast-Gerlach, E., Schmidt, U., Freihammer, S., Eschenbrenner, M., Garbe, L. & Stahl, U., 2002. Genetic engineering of brewing yeast to reduce the content of ethanol in beer. FEMS. Yeast Res. 2, 225-232.

Nieuwoudt, H., 2004. Glycerol and wine. Ph.D. Thesis. University of Stellenbosch, South Africa.

Nieuwoudt, H., Prior, B., Pretorius, I.S. & Bauer, F.F., 2002. Glycerol and wine quality: Fact and fiction. Wynboer, November. pp. 96-101.

Noble, A.C. & Bursick, G.F., 1984. The contribution of glycerol to perceived viscosity and sweetness in white wine. Am. J. Enol. Vitic. 35, 110-113.

Norbeck, J., Påhlman, A.K., Blomberg, A. & Adler, L., 1996. Purification and characterisation of two isoenzymes of DL- glycerol 3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 271, 13875-13881.

Novozyme, 1990. Novozyme ® 358 Product Sheet, December. Novo Nordisk, Switzerland.

Nykänen, L. & Suomalainen, H., 1983. Aroma of beer, wine and distilled alcoholic beverages. In: Handbuch der Aromaforschung. Rothe, M. (ed). Akademie Verlag, Berlin. Holland, U.S.A. pp. 17-22.

Oberholster, A., 2003. Effect of viticultural and winemaking practices on the phenolic composition of grapes and wines. Part II. Wynboer, April. pp.1-14.

Ohlmeyer, D.W., 1957. Use of glucose oxidase to stabilise beer. Food Technol. October, 503-507.

Osborne, J.P. & Edwards, C., 2006. Inhibition of malolactic fermentation by *Saccharomyces* during alcoholic fermentation under low-and high-nitrogen conditions: a study in synthetic media. Aust. J. Grape & Wine Res. 12, 69-78.

Ough, C.S., 1960. Die Verwendung von glukose oxydase in trockenem weisswein. Mitt. Klosterneuburg, Aust. 10A, pp. 14-23. In Scott, D., 1975a. Oxidoreductases. In: Enzymes in food processing, (ed). G. Reed. Academic Press, New York. pp. 222-230.

Ough, C.S., 1975. Further investigations with glucose oxidase-catalase enzyme systems for use with wine. Am. J. Enol. Vatic. 26, 30-36.

Ough, C.S., Fong, D. & Amerine, M.A., 1972. Glycerol in wine: determination and some factors affecting. Am. J. Enol. Vitic. 23, 1-5.

Oura, E., 1977. Reaction products of yeast fermentations. Process Biochem. 12, 19-21 & 35.

Pahlman, A.K., Granath, K., Ansell, R., Hohmann, S. & Adler, L., 2001. The yeast glycerol 3-phosphates Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic and oxidative stress. J. Biol. Chem. 276, 3555-3563.

Park, E.H., Shin, Y.-M., Lim, Y.-Y., Kwon, T.-H., Kim, D.-H. & Tang, M.-S., 2000. Expression of glucose oxidase by using a recombinant yeast. J. Biotechnol. 81, 35-44.

Pearl, R.P., 1926. Alcohol and longevity. Knopf, New York.

Pickering, G.J., 2000. Low and reduced alcohol wine: a review. J. Wine Res. 11, 130-133.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999a. The production of reduced-alcohol wine using glucose oxidase treated juice. Part I. Composition. Am. J. Enol. Vitic. 50, 291-298.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999b. The production of reduced-alcohol wine using glucose oxidase treated juice. Part II. Stability and SO_2-binding. Am. J. Enol. Vitic. 50, 299-306.

Pickering, G. J., Heatherbell, D.A. & Barnes, M.F., 1999c. The production of reduced-alcohol wine using glucose oxidase treated juice. Part III. Sensory. Am. J. Enol. Vitic. 50, 307-316.

Pickering, G.J., 1998. The use of enzymes to stabilise colour and flavour in wine – an alternative to SO_2. Aust. Grapegrower and Winemaker, September. pp. 101-103.

Pickerin, G.J., Heatherbell, D.A. & Barnes, M.F., 1998. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. Food Res. Int. 31, 685-692.

Pickering, G.J., & Heatherbell, D.A., 1996a. Characterisation of reduced alcohol wine made from glucose oxidase treated must. Food Technol. 26, 101-107.

Pickering, G.J., & Heatherbell, D.A., 1996b. Recent research on the use of glucose oxidase for the production of reduced alcohol wines. Proc. 11th Int. Oen. Symp., June. Sopron, Budapest, Hungary. pp. 153-179.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1993. A new technology for the production of reduced-alcohol wine. Poster Abstract. In: Proc. New Zeal. Grape and Wine Symp., (ed). Jordan, D.T. Vol. 8, November, Auckland. pp. 131-134. New Zealand Society for Viticulture and Oenology. New Zealand.

Power, J., 1993. In: Enzymes in food processing (3rd Ed). Chapter 17: Enzymes in brewing: VIII. Extension of shelf life of packaged beer. Nagodawithana, T. & Reed, G. (eds). Academic Press, New York. pp. 455-456.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast. 16, 675-729.

Prior, B.A., Toh, T.H., Jolly, N., Baccari, C. & Mortimer, R.K., 2000. Impact of yeast breeding for elevated glycerol production on fermentative activity and metabolic formation in Chardonnay wine. S. Afr. J. Enol. Vitic. 21, 92-99.

Pronk, J.T., Yde Steensma, H. & Van Dijken, J.P., 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast. 12, 1607-1633.

Pyle, L., 1994. Processed foods with natural flavour: the use of novel recovery technology. Nutrit & Food Sci. 1, 12-14.

Radler, F. & Schutz, H., 1982. Glycerol production of various strains of *Saccharomyces*. Am. J. Enol. Vitic. 33, 36-40.

Rainieri, S., Zambonelli, C., Hallsworth, J.E., Pulvirenti, A. & Giudici, P., 1999. *Saccharomyces uvarum*, a distinct group within *Saccharomyces sensu stricto*. FEMS Microbiol. Lett. 177, 177-185.

Rankine, B.C. & Bridson, D.A., 1971. Glycerol in Australian wines and factors influencing its formation. Am. J. Enol. Vitic. 22, 6-12.

Rapp, A. & Mandery, H., 1986. Wine aroma. Experentia. 42, 873-884.

Remize, F., Cambon, B., Barnavon, L. & Dequin, S., 2003. Glycerol formation during wine fermentation is mainly linked to Gpd1p and is only partially controlled by the HOG pathway. Yeast. 20, 1243-1253.

Remize, F., Roustan, J.L., Sablayrolles, J.M., Barre, P. & Dequin, S., 1999. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. Appl. Environ. Microbiol. 65, 143-149.

Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D., 2000. Handbook of enology, Vol. II. The chemistry of wine stabilization and treatments. John Wiley & Sons, Ltd., Chichester. England. pp. 41-43.

Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D., 1998. The microbiology of wine and vinifications. Handbook of enology. Vol. II. (1st Ed). John Wiley & Sons, Ltd., New York, Toronto.

Ribéreau-Gayon, J., Peynaud, E., Sudraud, P. & Ribéreau-Gayon, P., 1982 Sciences et Techniques du vin, 1, Analyses et Contrôle des vins. (2nd Ed). Dunod, Paris.

Rowe, D., 1989. How low can you go? Decanter, March. 75-78.

Sagi, I. & Mannheim, C.H., 1988. Shelf life of enzymatically deaerated fresh chilled citrus juice. Proc. Int. Citrus Cong., March. Tel Aviv.

Scanes, K.T., Hohmann, S. & Prior, B.A., 1998. Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. S. Afr. J. Enol. Vitic. 19, 17-24.

Schobinger, U., 1986. Alkoholfreier Wein-Hestellungsverfahren und Sensorische Aspekte. Mitt. Gebiete Lebensm. Hyg. 77, 23-28.

Schobinger, U., Waldvogel, R. & Durr, P. 1986. Verfahren zur herstellung von alcohol-freiem wein oder fruchtwein, Schweizer Patent, CH 654 023 A5, in: Durr, P. and Cuénat, P. (1988) Production of dealcoholised wine. In: Smart, R. E., Thornton, R. J., Kodrigues, S. B. and Young, J. E. (Eds). Proc. 2nd Int. Symp. For Cool Climate Viticulture and Oenology, January 1988. Auckland, New Zealand. pp. 363-364.

Schobinger, U., & Dürr, P., 1983. Diatische und sensorische Aspekte alkoholfreier Weine. Alimenta 22, 33-36.

Schobinger, U., Dürr, P. & Waldvogel, R., 1986. Die Entalkoholisierung von Wein und Fruchtweinen Schweiz. Zeitschrift für Obst- und Weinbau 122, 98-110.

Schoeman, H. Vivier, M.A, Du Toit, M., Dicks, L.M.T. & Pretorius, I.S., 1999. The development of bactericidal yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (pedA) in *Saccharomyces cerevisiae*. Yeast. 15, 647-656.

Scott, D., 1975. Oxidoreductases. In; Reed, G. (ed). Enzymes in food processing. Academic Press, New York. pp. 222-230.

Scott, D.J., 1953. Glucose conversion in preparation of albumen solids by glucose oxidase-catalase system Agric. Food Chem. 1, 727.

Seigneur, M., Bonnet, J., Dorian, B., Benchimol, D., Drouillet, F., Gouverneur, G., Larrue, J., Crockett, R., Boisseau, M.R., Ribéreau-Gayon, P. & Bicaud, H., 1990. Effect of the consumption of alcohol, white wine and red wine on platelet function and serum lipids. J. Appl. Cardiol. 5, 215-222.

Simpson, A., 1990. Impact of anti-alcohol campaigns on the wine industry. Proc. 9th Int. Oen. Symp., May. Cascais, Portugal. pp. 126-137.

Smith, P.M., 1995. Biological processes for the reduction of alcohol in wines. PhD Thesis Lincoln University, New Zealand.

Soden, A., Francis, I.L., Oakey, H. & Henschke, P.A., 2000. Effects of co-fermentation with *Candida Stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape Wine Res. 6, 21-30.

Sorrentino, F., Voilley, A. & Richon, D., 1986. Activity coefficients of aroma compounds in model food systems. AIChE J. 32, 1988-1993.

Sponholz, W.R., 1988. 4 Der Wein 4.3. Fehlerhafte und unerwünschte Erscheinungen im Wein. In: Chemie des Weines. Würdig, G. & Woller, R. (eds). Ulmer, Stuttgart.

Stackler, B & Christensen, E.N., 1974. Quantitative determination of ethanol in wine by gas chromatography. Am. J. Enol. Vitic. 25, 202-207.

Sykes, S.J., Casimir, D.J. & Prince, R.G.H., 1992. Recent advances in spinning cone column technology. Food Aust. 44, 462-464.

Szalkucki, T., 1993. Applications of oxidoreductases. In chapter 10, Nagodawithana, T. & Reed, G (eds). Enzyme in food processing. 3rd (Ed). Academic Press, New York. pp. 279-285.

Theron, C., 2006. Current vineyard and cellar events: the use of spinning cone column (*Dunlaag vakuumdistillasie*) to reduce alcohol in wines. Wynboer, January. pp. 1-2.

Thornton, R.J., 1983. New yeast strains from old – the application of genetics to wine yeasts. Food Technol. Aust. 35, 46-50.

Thumm, H.J., 1975. Low alcohol wine, Australian Patent 66 366, 24.4, In: Schobinger, U., Durr, P. and Waldvogel, R. (1986). Die Entalkoholisierung von Wein und Fruchtweinen (eine neue Möglichkeit zur Herstellung von zuckerreduzierten Fruchtsaftgetränken), Torija, J.T., Rozès, N., Poblet, M., Guillamón, J.M. & Mas, A., 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 80, 47-53.

Uhlig, H., 1998. General characteristics of technical enzymes. In: Industrial enzymes and their applications. Translated and updated by Elfriede, M. Linsmaier-Bednar, Ph.D. John Wiley & Sons, New York. pp. 13-18 & 401-404.

Van Rensburg, P. and Pretorius I. S., 1999. Enzymes involved in wine production (Article 3). Wynboer, February. pp. 1-7.

Van Schalkwyk, H. & Archer, E., 2000. Determining optimum ripeness in wine grapes. Wynboer, May. pp. 1-5.

Vemulapalli, V., Miller, K.A. & Hoseney, R.C., 1998. Glucose oxidase in bread making systems. Cereal Chem. 75, 439-442.

Villettaz, J.C., 1987. A new method for the production of low alcohol wines and better balanced wines. In: Lee, T. (Ed). Proc. 6th Aust. Wine Ind. Tech. Conf., July, Adelaide, Australia. pp. 125-128.

Villettaz, J.C., 1986. Method for production of a low alcoholic wine and agent for performance of the method. European Patent no: EP 0 194 043 A1.

Vonach, R., Lendl, B. & Kellner, R., 1998. High-performance liquid chromatography with real-time Fourier-transform infrared detection for the determination of carbohydrates, alcohols and organic acids in wines. J. Chrom. A 824, 159-167.

Wardman, R., 1995. Determination and removal of gluconic acid in reduced alcohol wine and high acid grape juice. M. Appl. Sc. Thesis. Lincoln University, New Zealand.

Whitaker, J.R., 1994. Principles of enzymology for the food sciences. 2nd (Ed). M. Dekker. New York.

Whitaker, J.R., 1972. Principles of enzymology for the food sciences. [cited in Heresztyn, T., 1987. Conversion of glucose to gluconic acid by glucose oxidase enzyme in Muscat Gordo juice. The Aust. Grapegrower & Winemaker, April, 25-27].

White, B.B. & Ough, C.S., 1973. Oxygen uptake studies on grape juice. Am. J. Enol. Vitic. 24, 148-152.

Whittington, H., Kerry-Williams, S., Bidgood, K., Dodsworth, N., Peberdy, J., Dobson, M., Hinchliffe, E. & Balance, D.J., 1990. Expression of the *Aspergillus niger* glucose oxidase gene in *A. niger, A. nidulans* and *Saccharomyces cerevisiae.* Curr. Genet. 18, 531-536.

Yoo, W. & Rand, A.G., 1995. Antibacterial effect of glucose oxidase on growth of *Pseudomonas fragi* as related to pH. J. Food Sci. 60, 868-879.

Zimmermann, F.K., 1993. Different signals control the activation of glycolysis in the yeast *Saccharomyces cerevisiae*. Yeast 9, 761-770.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F.S., 1995. Wine analysis and production. Chapman & Hall, New York. pp. 91 & 101-112.

Chapter 3

Research results

The production of reduced-alcohol wines using Gluzyme Mono® 10.000 BG-treated grape juice

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3. Research results

The production of reduced-alcohol wines using Gluzyme Mono® 10.000 BG-treated grape juice.

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Abstract

High alcohol wines have become a major problem in the international wine trade. Several physical processes are used to produce wines containing less alcohol, all of which involve the selective extraction of ethanol based on volatility or diffusion. In this study, we investigated the possibility of Gluzyme Mono® 10.000 BG (Gluzyme) (Novozymes, South Africa) to reduce the glucose content of synthetic grape must before fermentation in order to produce wine with reduced alcohol content. Gluzyme is a glucose oxidase preparation from *Aspergillus oryzae*, currently used in the baking industry. Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2) in the presence of molecular oxygen. Gluzyme was initially used in synthetic grape must, where different enzyme concentrations and factors influencing its efficiency were investigated for its use in winemaking. The results showed that up to 0.5% v/v less alcohol using an enzyme concentration of 20 kU compared to the control samples. This reduction in alcohol was increased to 1 and 1.3% v/v alcohol at pH 3.5 and pH 5.5 respectively in aerated (8 mg/L O_2) synthetic grape must using 30 kU enzyme. Secondly, Gluzyme was used to treat Pinotage grape must before fermentation. Gluzyme-treated wines at 30 kU enzyme concentration, after fermentation contained 0.68% v/v less alcohol than the control wines. A lower acetic acid concentration was observed in treated compared to control wines. Lower free anthocyanin and total phenol concentrations were observed in treated than in control samples, possibly due to the H_2O_2 oxidation which could have led to polymerisation of phenolic compounds.

Abbreviations: kU: kilo units; O_2: oxygen; H_2O_2: hydrogen peroxide; SO_2: sulphur dioxide

3.1 Introduction

The production of wine is a biotechnological process marked with tradition. However, research in oenology aims to improve the understanding of the chemical and biochemical reactions that take place in grape juice during its transformation into wine. In the production of fermented beverages such as wine, yeast facilitates the biochemical conversion of sugars to ethanol and carbon dioxide and produces a number of sensorially important metabolites such as higher alcohols, organic acids and esters that will consequently influence product quality (Lambrechts & Pretorius, 2000; Romano *et al*., 1998).

Saccharomyces cerevisiae is widely used as a wine yeast starter culture. Yeast species and strains vary in their abilities to utilise carbohydrates in the formation of alcohol and other by-products, as well as in their ability to grow in varying concentrations of alcohol (Zoecklein *et al*., 1995). Most strains of *S. cerevisiae* are inhibited as alcohol levels reach 14 to 15% v/v (Zoecklein *et al*., 1995). However, several strains are more alcohol tolerant. The quantity of alcohol and CO_2 formed as well as the nature and concentration of by-products vary with yeast strain, temperature of fermentation and the extent of aeration. Ethanol inhibits the growth of lactic acid bacteria, which then inhibits malolactic fermentation (Jackson, 1994).

As a result of a growing demand worldwide for wines containing lower levels of alcohol, there is a continuous quest for new strategies and techniques that can be used to produce low-alcohol wines. However, in the new world, winemaking practices favour the production of wines with high flavour intensity, prepared from fully matured grapes. In most cases, the juice obtained from such grapes contains very high sugar concentrations, resulting in wines with high levels of alcohol. Furthermore, the high alcohol concentration may affect the quality of wine by altering the volatility of aroma compounds (Athes *et al*., 2004). Several physical processes are available for the production of wines with less alcohol. However, these processes involve selective extraction of ethanol based on volatility or diffusion (Pickering, 2000). Despite their efficacy, these processes are expensive and difficult to perform, and can also affect the flavour balance through the loss of aroma compounds (Heux *et al*., 2006). One biological alternative would be to use yeast strains that produce low ethanol yield, a method that promises to be faster and less expensive (Heux *et al*., 2006).

Several attempts have also been made through genetic engineering to reduce the ethanol yield of *S. cerevisiae* by diverting sugar metabolism into by-products other than ethanol, for instance yeast strains producing more glycerol and less ethanol (Nevoigt *et al*., 2002; Remize *et al*., 1999). Another strategy has been to express lactate dehydrogenase in yeast, resulting in the simultaneous conversion of pyruvate into ethanol and lactate, thereby reducing the ethanol yield (Dequin *et al*., 1999). In addition, other approaches have been based on the removal of fermentable sugar from grape must, which has been achieved by using glucose oxidase before fermentation to catalyse the oxidation of glucose to gluconolactone in the presence of molecular oxygen (Pickering *et al*., 1998,

1999a, b, c). A yeast strain able to produce glucose oxidase during fermentation and its potential to produce low-alcohol wine has been developed and produced lower ethanol (Malherbe *et al*., 2003).

Gluzyme Mono® 10.000 BG (Novozymes), hereinafter referred to as Gluzyme, is a glucose oxidase preparation from *Aspergillus niger*, produced by a genetically modified *Aspergillus oryzae* microorganism (see addenda B and C). Gluzyme catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. Gluzyme is used in the baking industry due to it being inexpensive compared to pure glucose oxidase. Its main application is in gluten strengthening in dough systems and is used by bread improvers to facilitate the baking process.

The present study aimed to investigate the effect of Gluzyme on the glucose content of synthetic grape must or grape juice under winemaking conditions in order to reduce the alcohol content of the resultant wine after fermentation. Different factors influencing Gluzyme's efficiency such as pH, aeration, sulphur dioxide and temperature in synthetic grape must were also assessed.

Finally, Gluzyme was used to treat grape juice before fermentation in order to confirm the tendencies as observed when using synthetic grape must.

3.2 Materials and methods

3.2.1 Gluzyme Mono® 10.000 BG treatment using synthetic medium: Laboratory-scale fermentation

3.2.1.1 Media preparation

Synthetic grape must was prepared according to the instructions as described by Bely *et al*. (1990). Initially, 40 g/L sugar (glucose: fructose ratio of 1:1) was used. In subsequent trials using synthetic grape must, the sugar concentration was increased to 200 g/L (glucose: fructose ratio of 1:1). The acid concentrations were adjusted as follows: 0.5 g/L citric acid, 1.0 g/L malic acid and 2.0 g/L tartaric acid. The pH of the synthetic grape must was adjusted to pH 3.3 using sodium hydroxide (10 M NaOH). All the trials performed using synthetic grape must were carried out in 500 mL Erlenmeyer flasks with each containing 200 mL of synthetic grape must in triplicate. The synthetic grape must was sterilised by filtration through 0.22 µm and all equipment was sterilised before use. The Erlenmeyer flasks were closed with fermentation airlocks.

3.2.1.2 Gluzyme Mono® 10.000 BG treatments

Gluzyme (Novozymes, (Pty) Ltd, Benmore, South Africa) as shown in Addenda B & C, currently available for the baking industry, was used to treat synthetic grape must before fermentation. Gluzyme Mono 10.000 BG is 10,000 Glucose oxidase units per gram (GODU/g). Its recommended dosage range for the baking industry is 0.25–5 g per 100 kilogram (kg) of flour, which corresponds

to 25–500 GODU per kg flour. Gluzyme optimum dosage varies depending on the flour quality, formulation and processing, and should therefore be determined through baking trials.

According to the manufacturer, like all other glucose oxidases, higher dosages of Gluzyme may cause off-flavours during a long fermentation. The active components of Gluzyme are readily soluble in water at all concentrations found under normal usage. However, water solutions will become turbid because the enzyme is encapsulated with wheat flour.

The effect of Gluzyme dosage on glucose content was initially investigated in a synthetic grape must at four different enzyme concentrations: 0, 5, 10 and 20 kilo units (kU) conducted at pH 3.3 using 40 g/L sugar (glucose: fructose ratio of 1:1).

The experiment was carried out in two sets. The first one consisted of enzyme treatment using synthetic grape must with aeration. The second experiment was carried out without aeration. The aim of this experiment was to investigate the role of oxygen in Gluzyme reaction, which converts glucose into gluconic acid and hydrogen peroxide. Secondly, it also aimed to compare the activity of the enzyme in aerated and non-aerated synthetic grape must treated with different concentrations of the enzyme.

The synthetic grape must was treated with Gluzyme before fermentation. The enzyme reaction was performed for a period of nine hours and after 24 hours with the first sampling one hour after enzyme addition and thereafter every three hours. In order to stop the enzymatic reaction, 200 µL of sulphuric acid (4 M H_2SO_4) was added to the sample (1.8 mL). At the end of the enzyme treatment, all samples were inoculated with *S. cerevisiae* VIN13 (Anchor Yeast Biotechnologies (Pty) Ltd, South Africa) ($1x10^6$ cells/mL) from pre-grown cultures at 30°C for 72 hours in a Yeast Peptone Dextrose (YPD, Merck, Johannesburg, South Africa) broth. The yeast ($1x10^6$ cells/mL) was spinned down at 5 000 rpm for five minutes and re-suspended in 20 mL sterile distilled water before inoculation. Fermentation was allowed to proceed to dryness. The fermentation process was monitored by recording weight loss on a daily basis. Ethanol concentration was determined at the end of fermentation by using high-performance liquid chromatography (HPLC).

Further experiments that were carried out in synthetic medium contained 200 g/L sugar (glucose: fructose ratio of 1:1). The nine hours of enzyme treatment was performed and thereafter all samples were inoculated with *S. cerevisiae* VIN13 ($1x10^6$ cells/mL) prepared according to the above-mentioned procedure. Ethanol was determined at the end of fermentation.

3.2.2 Factors influencing Gluzyme Mono® 10.000 BG efficiency

Factors such as pH, aeration, temperature and sulphur dioxide were investigated using Gluzyme-treated synthetic grape must containing 200 g/L sugar (glucose: fructose ratio of 1:1). Each factor was investigated as a separate experiment according to the methods as described below. The amount of enzyme used in all the experiments was 30 kU. All the experiments were carried out at laboratory temperature of 20 to 22 °C, except for the temperature experiment which was carried out at 15 and 25°C. The completion of alcoholic fermentation was confirmed by an estimation of the

sugar content using Clinitest tablets (Bayer Corporation, New York, USA 10591-5097). Ethanol concentration was determined as described in Section 3.2.4.

3.2.2.1 pH

Gluzyme treatment was carried out at different pH levels to evaluate the effect of pH on the enzyme activity in synthetic medium. The pH of the synthetic medium was adjusted to three different pH levels using 10 M NaOH: pH 3, pH 3.5 and pH 4. At each pH level, controls (without enzyme) and enzyme treatments were included. The enzyme was added to the medium and the reaction was performed for nine hours. At the end of the nine hours of enzyme treatment, all samples were inoculated with *S. cerevisiae* VIN13 ($1x10^6$ cells/mL). The fermentation process was monitored by recording weight loss on a daily basis. Further pH experiments were carried out under the same conditions, which included pH 3.5 and pH 5.5 with aeration, with the latter pH being regarded as within the optimum pH range for the enzyme activity (Pickering *et al.*, 1998).

3.2.2.2 Aeration

The effect of aeration on Gluzyme efficiency in synthetic grape must was evaluated. Air was introduced into the synthetic grape must by decanting the synthetic grape must to obtain three different dissolved oxygen levels, which was measured using a dissolved oxygen meter (Oxi 330 and Oxi 330i, Merck). The control synthetic grape must was sparged with nitrogen gas (N_2) (Afrox, Epping, Cape Town, South Africa) to lower the oxygen concentration to <1 mg/L O_2. In the second treatment, the oxygen level was 2 mg/L. In the third treatment, 4 mg/L oxygen was achieved. At each level of dissolved oxygen a control, without Gluzyme and Gluzyme-treated samples were included. Once the different oxygen levels have been achieved, the synthetic grape must was transferred into 500 mL Erlenmeyer flasks that had been sparged with N_2. These are the oxygen levels normally found under winemaking conditions in the grape must (Du Toit, 2006). The synthetic grape must was treated with Gluzyme for nine hours. All samples were then inoculated with VIN13 ($1x10^6$ cells/mL) and the fermentation was monitored by recording weight loss on a daily basis.

More trials with aeration included increased levels of dissolved oxygen up to 8 mg/L. The pH of the synthetic grape must was adjusted to pH 3.5 and pH 5.5. At each pH and at 8 mg/L O_2, a treated and a control samples were included. The enzyme was added to the synthetic grape must. The enzyme treatment was performed for nine hours. At the end of the nine-hour period, all samples were inoculated with VIN13 ($1x10^6$ cells/mL). Fermentation was monitored by recording the weight loss on a daily basis.

3.2.2.3 Temperature

Two different temperatures, 15 and 25°C were evaluated for their effect on Gluzyme efficiency in synthetic grape must adjusted to pH 3.3. No specific oxygen adjustments were made. Treatments consisted of a control (without enzyme) and Gluzyme-treated samples. The synthetic grape must was left overnight at its respective temperatures to achieve the desired temperature. It was inoculated with the enzyme the following day. The enzymatic reaction was performed for nine hours. After nine hours, all samples were inoculated with *S. cerevisiae* strain VIN13 ($1x10^6$ cells/mL). Fermentation was monitored by recording weight loss on a daily basis.

3.2.2.4 Sulphur dioxide (SO_2)

The effect of SO_2 on Gluzyme efficiency in synthetic grape must was investigated. The SO_2 was added to the synthetic medium at the following total concentrations: 0, 30 and 60 mg/L in triplicate, using 2.5% SO_2 stock solution, prepared from metabisulphate (Everywine, Stellenbosch, South Africa). The synthetic grape must was left overnight to stabilise at laboratory temperature. The following day, samples were taken for analysis of free (FSO_2) and total (TSO_2) SO_2 before the enzyme treatment, using the Aspiration method (VinLab, an accredited wine analyses laboratory, Stellenbosch, South Africa). The enzyme was added to all samples and the enzymatic reaction was performed for nine hours after which samples were again taken for FSO_2 and TSO_2 analysis at the end of the enzyme treatment. After inoculation, fermentation was allowed to proceed to dryness. The fermentation process was monitored on a daily basis by recording the weight loss. At the end of fermentation samples were analysed for ethanol concentration using HPLC.

Another SO_2 trial was carried out where three sets of fermentations were undertaken. The aim of these trials was to observe if gluconic acid has an influence on the SO_2 binding effect. The synthetic grape must composition and SO_2 levels were the same as mentioned above. The SO_2 was added to the grape must and left overnight to stabilise. The first treatment contained no Gluzyme and no gluconic acid (A). In the second treatment, Gluzyme was added (B). In the third treatment, gluconic acid (Sigma-Aldrich, Aston Manor, South Africa) was added at 8 g/L (C), the amount that was obtained from previous SO_2 trials performed. All samples were analysed for the FSO_2 and TSO_2 before enzyme and gluconic acid treatment. The enzyme and gluconic acid treatments were performed for nine hours. After nine hours, samples were taken to measure the FSO_2 and the TSO_2. Gluconic acid concentration was also determined using enzymatic kits. All samples were inoculated and fermentation was monitored on a daily basis by recording the weight loss. Ethanol concentration was determined at the end of fermentation.

3.2.3 Glucose and gluconic acid determination

D-Glucose and D-gluconic acid concentrations were measured enzymatically using enzymatic kits (R-Biopharm AG, D-64293 Darmstadt, Germany) with a 1 mL total assay volume in disposable cuvettes using spectrophotometer measurements at 340 nm.

3.2.4 Ethanol determination

Ethanol concentration was determined by HPLC (Castellari *et al.*, 2000). The standard solutions were prepared as follows: 1, 4, 8 and 12% v/v ethanol, using 96% ethanol (Merck) and diluted five times with Millipore/MilliQ water. Undiluted samples were filtered through a 0.22 µm sterile syringe filter and diluted five times with Millipore/MilliQ water for analysis on the HPLC to ensure that ethanol content was within the calibration range. The solvent, standard solutions and all samples were filtered through a 0.22 µm filter paper before running them on the HPLC. A Waters 717 auto sampler and refractive index detector was used with Agilent 1100 binary pump and Millennium software. Separation was achieved on a Biorad, Aminex HPX-87H ion exclusion column 300x7.8 mm at an injection volume of 5 µL. An isocratic flow rate of 5 mM H_2SO_4 was used at 0.6 mL/min at 45°C.

3.2.5 Statistical analysis

The ANOVA method was used for the statistical analysis of the data to determine if there were differences in control and treated samples, using Statistica version 8, statsoft. One-way ANOVA or two-way ANOVA, depending on the parameters of interaction, was used. Statistical analysis was performed for the treatments carried out in synthetic medium and not in Gluzyme-treated wines because replications limited this analysis.

3.2.6 Small-scale wine vinification of Gluzyme Mono® 10.000 BG-treated grape juice

3.2.6.1 Preparation of must

Pinotage grapes from the Stellenbosch area, South Africa, were used from the 2007 harvesting season. The grapes were crushed and destemmed, and the must was divided equally into 10 litre buckets. The skins were separated, mixed and divided equally in buckets to ensure that every treatment received equal amounts of skins and juice. The analysis of the must pH, sugar concentration (°Brix), titratable acidity (TA) and SO_2 was performed by using a Metrohm Titrino apparatus (702 SM Titrino, Swiss lab) equipped with a 722 stirrer (Swiss lab). No acid adjustment or SO_2 additions were made to the must before Gluzyme treatment. Full analysis of the juice was done using the Grape Scan FT120 instrument (Foss Electric, Denmark). The instrument utilises

Fourier transformed infrared (FT-IR) spectroscopy. All samples were degassed by filtration prior to the analysis using a filtration unit (type 79500, Foss Electric, Denmark) with filter paper circles graded at 20–25 µm with a diameter of 185 mm.

3.2.6.2 Treatment of grape juice with Gluzyme Mono® 10.000 BG

The effect of Gluzyme on the glucose conversion efficiency in Pinotage grape juice was investigated using 3 g/L (30 kU) enzyme. Mean composition parameters of the juice analysed were as follows: sugar concentration (°Brix), pH, acidity as well as FSO_2 and TSO_2. The juice was treated with Gluzyme prior to fermentation. These treatments were performed with two different batches of Pinotage grapes (A and B). The enzymatic reaction was performed for six hours in wine A and the sugar concentration was analysed after the enzyme treatment for both control (without enzyme) and treated samples. The treatments were carried out in triplicate. In wine B, Pinotage grape juice was treated with the enzyme prior to fermentation for 48 hours. In this case treatments were carried out in duplicate.

3.2.6.3 Wine fermentation

At the end of the Gluzyme treatments, all samples were inoculated with VIN13 strain at 0.3 g/L. All fermentations were conducted at 25°C fermentation in the experimental cellar at the Department of Viticulture and Oenology, Stellenbosch University. Pumping-over (the juice over the skins) was performed two to four times a day.
The sugar content of the fermenting must was monitored by using the Grape Scan FT120. The wines were fermented dry on the skins and pressed after the completion of alcoholic fermentation. A small-scale basket press was used, and the skins were pressed up to 1.5 bar. The wine from each treatment was collected after pressing and kept separately in 4.5-litre glass bottles. The wines did not undergo malolactic fermentation.

3.2.6.4 Bottling

Following the settling of yeast lees, SO_2 (50 mg/L) was added to the wines. The wines were then filtered, bottled and sealed in 750-mL screw-cap bottles (two to three bottles per treatment). The wines were stored at 15°C until analysed.

3.2.6.5 Analyses of standard parameters in wine

Wines A and B were analysed for pH, volatile acidity (VA), total acidity (TA), malic acid (MA), lactic acid (LA), glucose and fructose, ethanol and glycerol using the Winescan FT120. Further analyses including phenolics and volatile flavour compounds were carried out as described below.

3.2.6.5.1 Determination of ethanol content

The Winescan FT120 mid-infrared spectrometer (Foss Electric, Ltd, Hillerød, Denmark) equipped with a purpose-built Michelson interferometer was used for the quantification of the ethanol content of the wines. The apparatus has ready-to-use calibration models for quantification of the important wine parameters, including ethanol (Gishen & Holdstock, 2000; Nieuwoudt, 2004). Since CO_2 levels in wine can interfere with the accuracy of quantification, fermentation samples were degassed by filtration prior to analysis using a filtration unit (type 79500, Foss Electric, Denmark) connected to a vacuum pump. Filter paper circles graded at 20–25 µm with a diameter of 185 mm (Schleicher & Schuell, reference number 10312714) were used for filtration. The spectral measurements in the wave number range 929 cm^{-1} to 5011 cm^{-1} were generated by the spectrometer and used to quantify ethanol.

3.2.6.5.2 Phenolic and colour analyses

3.2.6.5.2.1 Colour density

Absorbance was measured in a Helios Alpha spectrophotometer (Thermo Electron Corp.). Undiluted wine (at actual wine pH and SO_2 levels) was used in a 1 mm path length glass cuvette. The sum of the absorbance readings (A_{420}, A_{520} and A_{620}) were noted and corrected (x10) to standard 10 mm path length values.
For the determination of modified wine colour density, wine samples were diluted using a model wine solution (10 g/L potassium bitartrate, 12% ethanol in 1-litre) and the pH was adjusted to pH 3.6 with 10 M NaOH. The sum of the absorbance readings (A_{420}, A_{520} and A_{620}) were noted and corrected (x10) to standard 10 mm path length values. These values describe the intensity of colour at a uniform pH and without any bleaching effects of SO_2. These colour analyses were based on the approaches of Somers & Evans (1977) and Bakker *et al.* (1986).

3.2.6.5.2.2 Total phenols and free anthocyanins

Five millilitres of 1 M HCl was used to dilute 50 µL of wine sample in a test tube for the determination of total phenols. After a three-hour equilibration at room temperature, the absorbance (A_{280}) was measured using a 1 cm quartz cuvette.
Free anthocyanins were analysed using SO_2-bleaching according to the method described by Ribereau-Gayon *et al.* (2000b).

3.2.6.5.2.3 Volatile flavour compounds

The volatile aroma profile of the wines (esters, higher alcohols and volatile acids) was determined by analysing a diethyl ether extract of the wine on the GC-FID. A Hewlett Packard 6890 Plus GC

(Little Falls, USA) equipped with a split/splitless injector and an FID detector was used. A J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 0.32 mm i.d. × 0.5 μm film thickness, was used for separation. The initial temperature programme was as follows: 33°C for 17 minutes then raised up 12°C/min to 240°C where it was held for 5 minutes. The carrier gas was H_2 at 3.3 mL/min, constant flow mode. Injection volume of 3 μL in split mode (split ratio 15:1) at 200°C was used. The split flow was 49.5 mL/min. The method has been described by Malherbe (2007) for analyses of volatile flavour compounds in wine.

The extracts for injection into the GC were prepared by extracting 5 mL of wine with internal standard, 4-Methyl-2-Pentanol (Fluka, ≥ 97%) and 100 μL of 0.5 mg/L solution in wine stimulant (water pH, 3.5; 2.5 g/L tartaric acid and 12% ethanol) with 1 mL of diethyl ether (99.5%, Merck).

The wine/ether mixture was followed by sonication for 5 minutes in an ultrasonic bath to facilitate the mixing of the diethyl ether layer and the wine. This was then followed by centrifugation for 3 minutes at 4 000 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) (99%, Merck). The dried diethyl ether extract was transferred to a vial insert and capped. This was then injected into the GC-FID. The concentrations of the volatile compounds were calculated by comparing their retention times and peak areas with those of known standards.

3.3 Results and discussion

3.3.1 Synthetic medium treated with Gluzyme Mono® 10.000 BG: Laboratory-scale fermentation

3.3.1.1 Enzyme dose

An effective Gluzyme dose was needed for use in winemaking to reduce the glucose content of grape must to gluconic acid and hydrogen peroxide in order to produce a wine with less alcohol. Several different enzyme concentrations were therefore investigated using synthetic grape must. These were as follows: 0, 5, 10 and 20 kU. The experiments were carried out in a synthetic grape must with and without aeration. Fig. 3.1 shows the estimated glucose and gluconic acid concentrations of treated and control samples.

A decrease in glucose and an increase in gluconic acid concentrations were observed. This decrease in glucose concentration was more pronounced in aerated (Fig. 3.1A) in comparison to non-aerated (Fig. 3.1B) synthetic grape must, which is possibly explained by the fact that the enzymatic reaction requires oxygen for effective conversion of glucose to gluconic acid. The enzymatic reaction seemed very slow and the researchers assumed that if the enzyme is encapsulated with wheat flour, then it might require longer contact time for the enzymatic reaction to occur, considering that these were not at the optimal conditions for the enzyme. Although an extrapolation was made from 9 to 24 hours, clearly gluconic acid was higher at higher concentrations of Gluzyme (20 kU). Therefore, if the concentration of the enzyme is increased,

more product will be formed. The initial sugar concentration was 40 g/L (glucose: fructose of 1:1 ratio), which corresponds to 0.1 mol/L glucose. At the end of the nine hours of Gluzyme treatment, 0.04 mol/L gluconic acid was obtained for a 20 kU enzyme concentration. This indicated that 0.06 mol/L glucose was converted leading to 0.04 mol/L gluconic acid that was formed directly from the action of the enzyme on glucose.

It has been shown that both rate and extent of glucose conversion by pure GOX increased with increasing enzyme dose (Pickering *et al.*, 1998), and this is consistent with results reported by Villettaz (1987) and Heresztyn (1987) up to the maximum dose (1 g/L) used.
Gluzyme seemed to follow the same pattern in trials carried out in synthetic grape must with up to 20 kU concentration which led to a 0.5% v/v decrease in ethanol concentration.

Fermentations were also carried out using 200 g/L sugar (glucose: fructose ratio of 1:1) and the accumulated weight loss was used to formulate the fermentation curves as shown in Fig. 3.2A. The control (without Gluzyme addition) fermented slower compared to the treated samples, however, all samples did ferment to dryness.

The wheat flour used in standardisation of this enzyme could act as nutrient to the yeast cell or as yeast solid matter resulting in more rapid fermentation in the treated than in the control. Ethanol concentration at different enzyme concentrations at the end of fermentation showed up to 0.5% v/v less ethanol compared to the control at an enzyme concentration of 20 kU (Fig. 3.2B) and also in Addendum A for statistical analysis (Fig. 5.1).

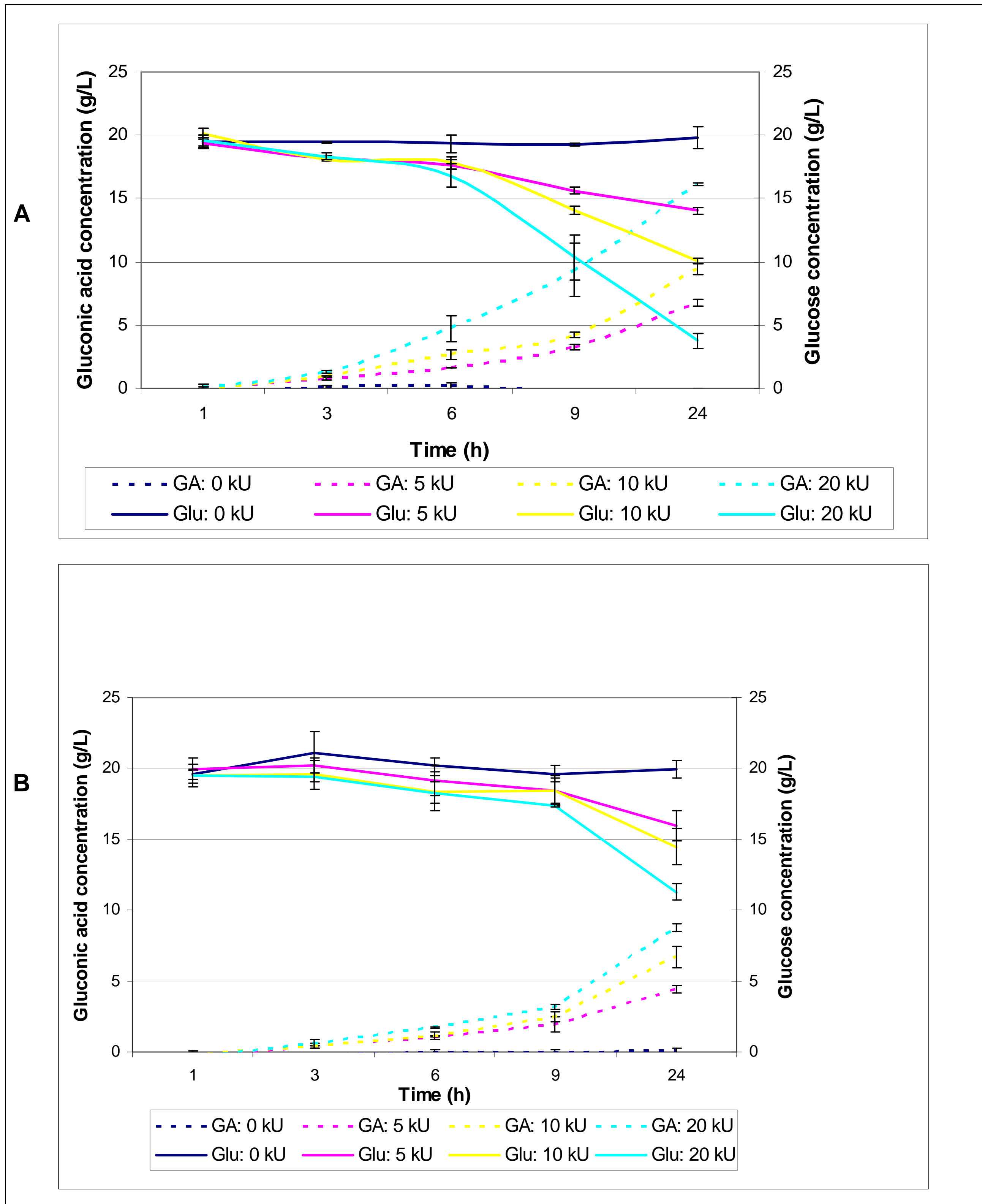

FIGURE 3.1
Glucose (Glu) and gluconic acid (GA) concentrations of Gluzyme-treated synthetic grape must (40 g/L sugar) before fermentation at different enzyme concentrations. A: with aeration and B: without aeration.

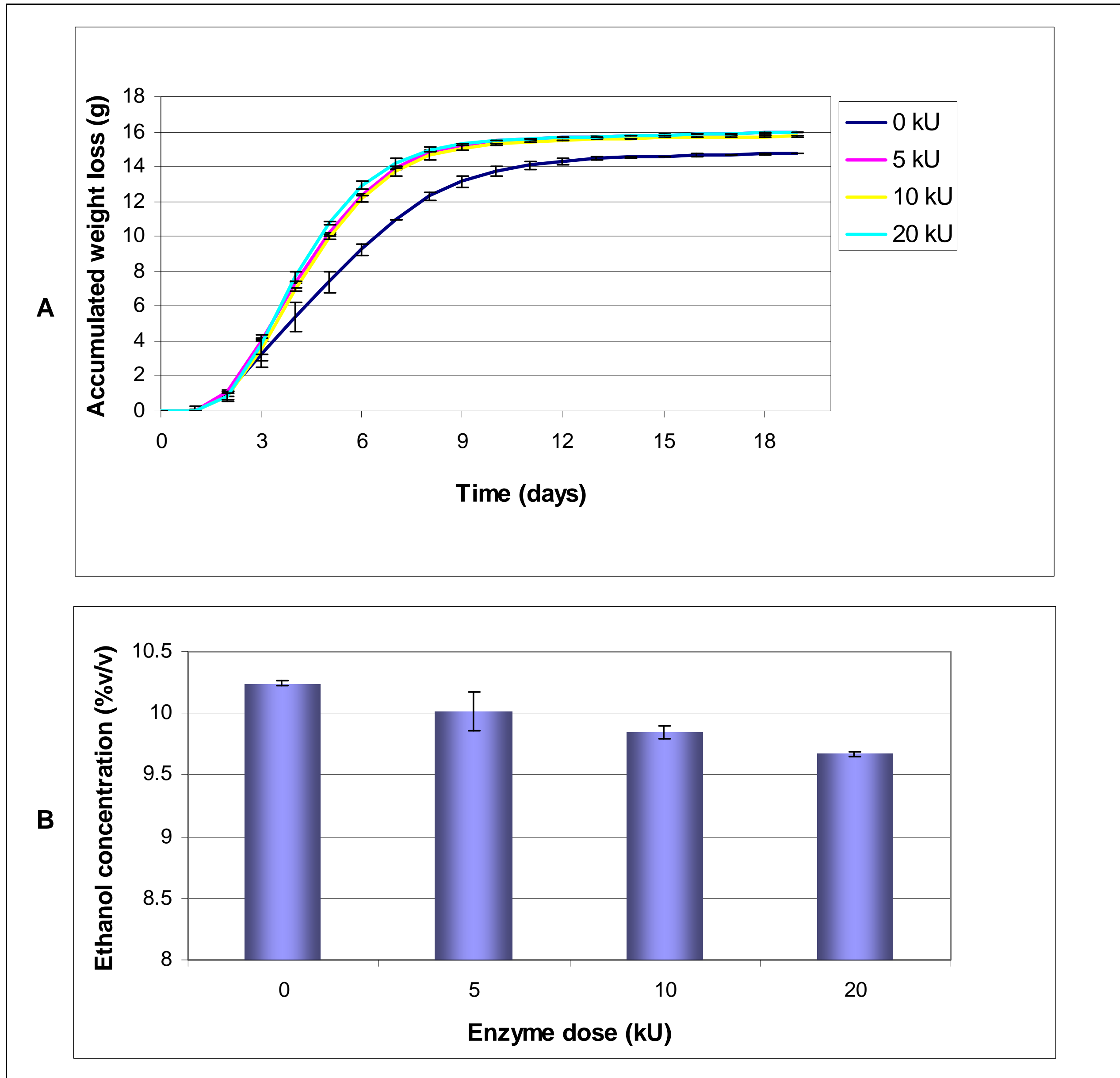

FIGURE 3.2
Accumulated weight loss of fermented synthetic grape must (200 g/L sugar) treated with Gluzyme (A). The bars on the graph represent ethanol (EtOH) concentration (%v/v) measured at the end of alcoholic fermentation (B).

3.3.1.2 Factors influencing Gluzyme Mono® 10.000 BG's efficiency

3.3.1.2.1 Aeration

The effect of different oxygen levels on the activity of Gluzyme and the resultant ethanol concentration is shown in Fig. 3.3. Oxygen was introduced into the synthetic grape must by means

of aeration before adding the enzyme, a recommended method by the manufacturer, since pure oxygen can give rise to oxidisation of the enzyme. Aeration showed minimal to no effect on ethanol concentration whereas the effect of enzyme addition showed significant differences ($p < 0.05$) as shown in Addendum A (Fig. 5.6) in ethanol concentration, especially at high oxygen levels. It has previously been shown that oxygen is important for optimal conversion of glucose into gluconic acid and hydrogen peroxide by pure GOX enzyme. According to Pickering *et al.* (1998), improved GOX performance is suggested at a higher aeration rate, but this effect becomes negligible by the end of the enzyme treatment period.

Temperature and oxygen were not optimally monitored in our experiments. This could have led to less effective aeration and therefore minimal effects on the enzyme. General trends of less ethanol being produced in the case of the control sample were observed as the O_2 levels were increased (data not shown).

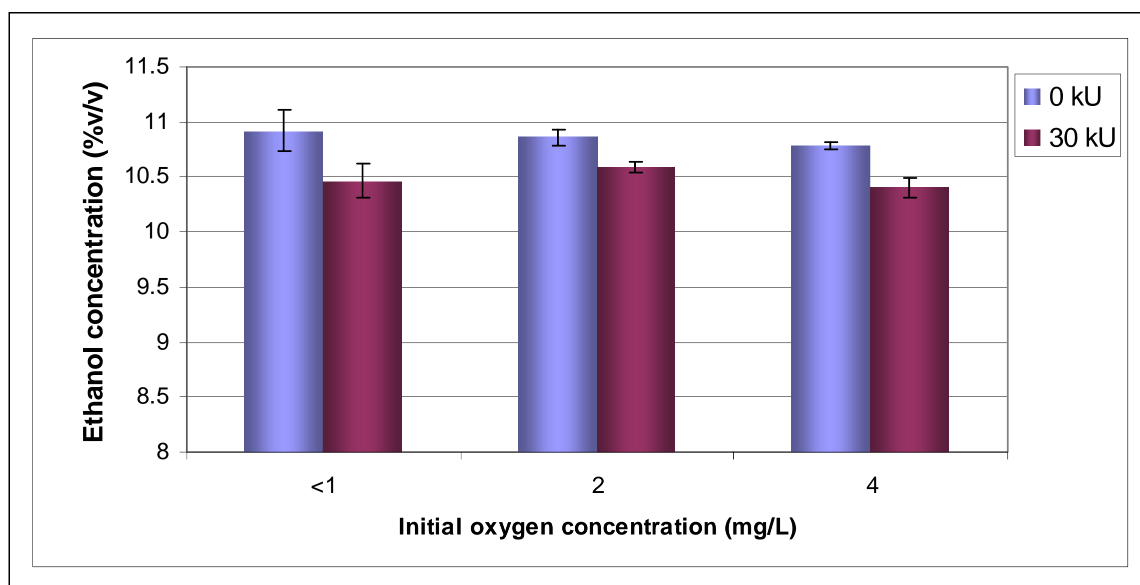

FIGURE 3.3

Ethanol concentration of fermented synthetic grape must treated with Gluzyme at different levels of aeration.

3.3.1.2.2 pH

Pure glucose oxidase has an optimum pH of 5.5 and a broad pH range of 4–7 (Borole *et al.*, 2005; Hanft & Koehler, 2006; Pickering *et al.*, 1998). Gluzyme is more stable at pH 5.5.

Gluzyme was investigated at the following different pH levels: pH 3, pH 4 (which normally occur in wine) and pH 5.5, the optimum pH for the enzyme. There were no differences observed in ethanol concentration at pH 3 and pH 4 between the control and treated samples, only trends of less ethanol being produced at pH 5.5 were observed ($p = 0.05$) in the treated, compared to the control sample (data not shown). The reason here could be that the enzyme dose of 30 kU might

have been insufficient for optimal glucose conversion at a lower pH than the optimal pH of the enzyme.

Therefore, a higher amount of enzyme dosage should be tested at different pH levels, including the optimal pH of the enzyme, in order to have valid conclusion on Gluzyme's efficiency for reducing ethanol content in wine.

In later trials using synthetic medium, pH was further tested with aeration at 8 mg/L at pH 3.5 and pH 5.5. A reduction of alcohol of about 1 and 1.3% v/v respectively was obtained using 30 kU enzyme concentration (Fig. 3.4). This indicated that both aeration and pH could have major effects on influencing Gluzyme activity under winemaking conditions. Improved pure GOX performance is suggested at a higher aeration rate, although this effect becomes negligible at the end of the treatment period (Pickering *et al*., 1998). Low pH has shown to be the dominant factor limiting the rate and extent of glucose conversion by pure GOX (Pickering *et al*., 1998). It seemed that Gluzyme follows the same pattern although more work still needs to be done to verify these results in grape must, considering that this was performed in synthetic medium. A significant advantage may be obtained if the must pH is first adjusted to the Gluzyme optimal pH before the treatment.

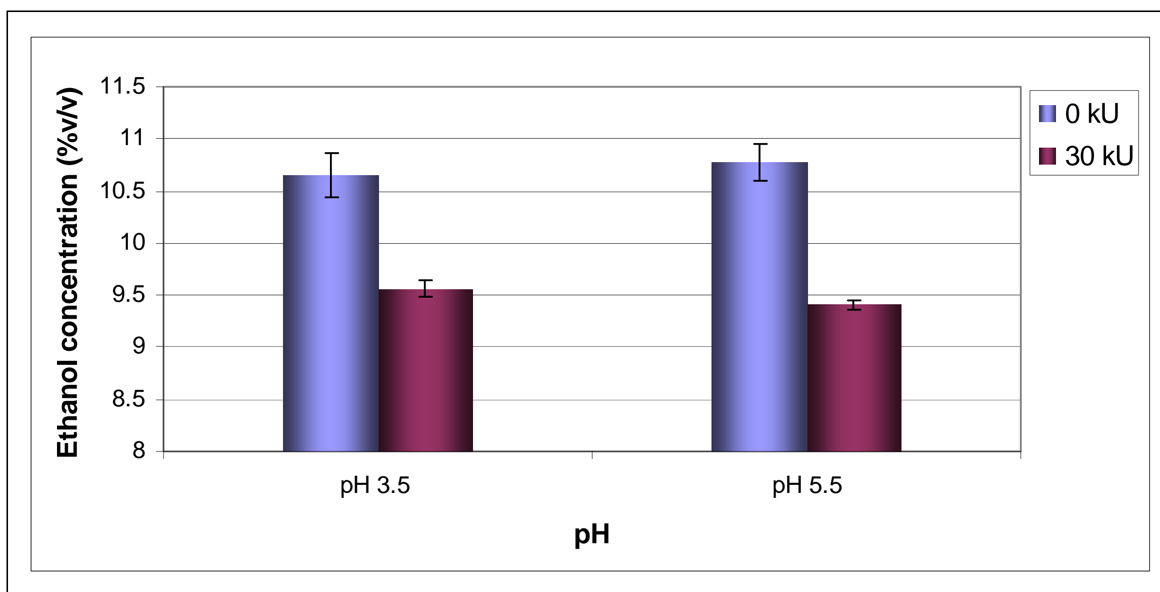

FIGURE 3.4

Ethanol concentration of fermented synthetic grape must treated with Gluzyme at different pH levels (pH 3.5 and 5.5) with aeration (8 mg/L O_2).

3.3.1.2.3 Temperature

According to the manufacturer, Gluzyme has 100% activity at 60°C whereas a pure GOX optimum temperature range has been reported to be between 30 and 40°C (Whitaker, 1972). For use in grape juice, a desirable temperature (pure GOX) range of between 15 and 20°C has been suggested (Heresztyn, 1987; Villettaz, 1986, 1987). There were no differences observed in GOX

performance by Pickering *et al.* (1998) between 20 and 30°C. In this study, Gluzyme was investigated at 15 and 25°C. No differences in Gluzyme performance were observed. As a result, no differences in ethanol concentration were obtained (data not shown). Very little ethanol was reduced at 25°C (whereas no reduction was observed at 15°C), but it was not significantly different as shown in Addendum A (Fig. 5.2). No significant differences could be observed in the control samples at 15°C and 25°C. The manufacturer commented that about 10 to 20% Gluzyme activity could be obtained at these temperatures in comparison to the 100% activity at 60°C. In GOX, authors have noted diminished enzyme activity at higher temperatures (Heresztyn, 1987). A change in temperature means a change in one of the reactants such as oxygen and decreased oxygen solubility at high temperatures may be offsetting the expected benefits of temperature rise (Scott, 1975), although this does not seem to have been the case in this study.

3.3.1.2.4 Sulphur dioxide (SO_2)

In the first SO_2 trial that was performed, a control (without enzyme addition) was not included and all samples were inoculated with Gluzyme Mono® 10.000 BG at 0, 30 and 60 mg/L SO_2. All the SO_2 present in the samples was consumed by the end of the enzyme treatment (Table 3.1). This may be due to rapid oxidation of free SO_2 to sulphate by H_2O_2 during pure GOX treatment as observed by Pickering *et al.* (1998) and Ough (1975). Glucose has a much lower binding power towards SO_2 whereas fructose and saccharose show virtually no reaction (Ribéreau-Gayon *et al.*, 2000).

In subsequent trials Gluzyme, gluconic acid and a control (with no enzyme and no gluconic acid) were included. Table 3.2 shows the SO_2 analysis before and after treatment with Gluzyme in synthetic medium. The aim here was to further investigate the complete depletion of SO_2 as observed in the previous experiment of this study and to observe the capability of gluconic acid to bind SO_2 as well its effect on the resultant ethanol content at the end of fermentation.

Once again, zero or negligible amounts of free SO_2 were observed by the end of the Gluzyme treatment period. Most of the SO_2 appears to have been bound. The control samples did not differ with regard to the SO_2 concentration. Gluconic acid is known to bind SO_2 easily (Barbe *et al.*, 2002) although its individual contribution is insignificant at the levels normally found in winemaking (Ribéreau-Gayon *et al.*, 2000). Further research is required on the effect of SO_2 on Gluzyme under winemaking conditions. Using pure GOX, it has been shown that after fermentation, zero or negligible amounts of free SO_2 remained (Pickering *et al.*, 1999b) and that could primarily be due to binding with acetaldehyde formed during fermentation. No SO_2 analysis was performed after fermentation in this study. Further research on this aspect using Gluzyme-treated must, still needs to be done.

Statistical differences in ethanol concentration were observed in A compared to B, while C did not differ greatly from either A or B (Fig 3.5). General trends of increased reduction in ethanol concentration were observed as the SO_2 level was increased in B.

According to the manufacturer, at SO_2 levels between 100 and 200 mg/L, the enzyme can become inhibited. At the beginning of vinification, these SO_2 levels are not yet encountered.

It has been suggested (McLeod & Ough, 1970) and reported by Merzhanian & Tagunkov (1967) that SO_2 can be inhibitory to the activity of the pure GOX system in wine. Previous research has shown that at the levels used in wine, SO_2 will delay the GOX-mediated removal of oxygen, but not completely inhibit it (Ough, 1975; White & Ough, 1973).

TABLE 3.1

SO_2 analyses before and after Gluzyme treatment of synthetic grape must without a control.

		BEFORE GLUZYME TREATMENT		AFTER GLUZYME TREATMENT	
Treatment	**Added SO_2 (mg/L)**	**FSO_2 (mg/L)**	**TSO_2 (mg/L)**	**FSO_2 (mg/L)**	**TSO_2 (mg/L)**
Gluzyme	0	1	1.3	1	1
	30	1.3	24.6	1	1
	60	3	54.3	1	1

TABLE 3.2

SO_2 analyses before and after Gluzyme treatment of synthetic grape must with gluconic acid as a control.

		BEFORE GLUZYME TREATMENT		AFTER GLUZYME TREATMENT	
Treatment	**Added SO_2 (mg/L)**	**FSO_2 (mg/L)**	**TSO_2 (mg/L)**	**FSO_2 (mg/L)**	**TSO_2 (mg/L)**
A	0	0	0	1	1.3
	30	2	15	1	12
	60	2	33.6	1	31.3
B	0	0	0	0.6	1.3
	30	0	14.6	0	0
	60	0	32	0	0
C	0	0	0	0.3	0.3
	30	0	15.3	1	10.6
	60	0	32.3	1	31.3

A: without Gluzyme and gluconic acid; B: Gluzyme-treated and C: Gluconic acid-treated

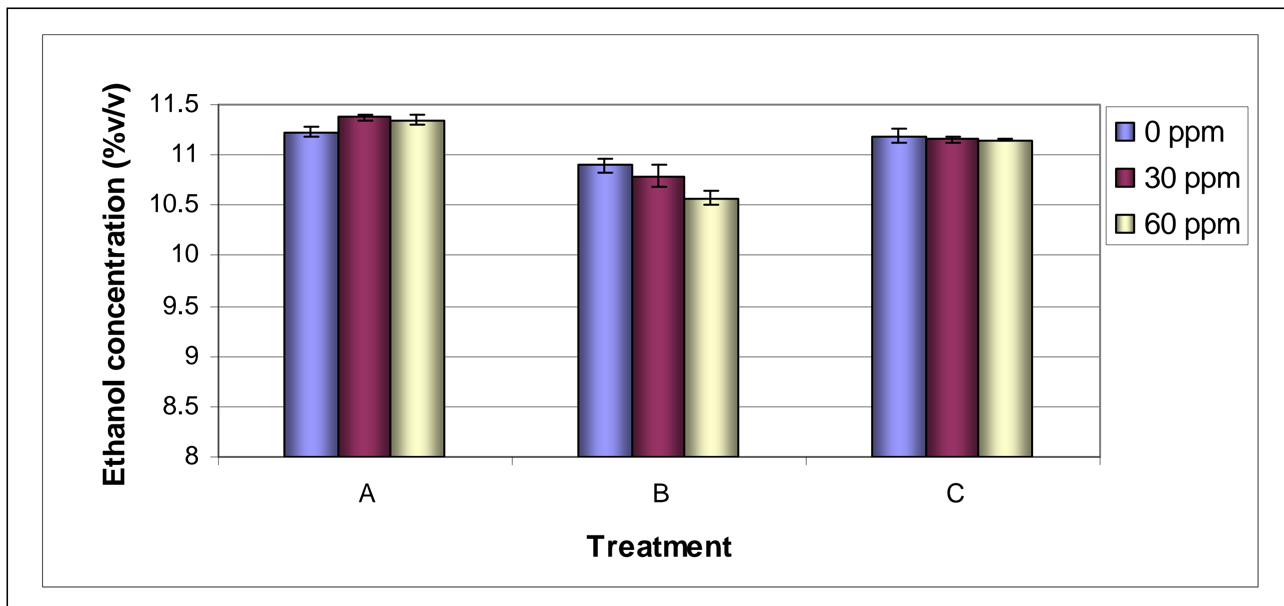

FIGURE 3.5

Ethanol concentration of fermented synthetic medium treated with Gluzyme at different SO_2 levels (mg/l). A: without Gluzyme and Gluconic acid; B: Gluzyme-treated and C: Gluconic acid-treated samples.

3.3.2 Small-scale wine vinification using Gluzyme Mono® 10.000 BG-treated grape juice

3.3.2.1 Treatment of grape juice with Gluzyme Mono® 10.000 BG

During fermentation, the sugar content of wine A was monitored by recording a decrease in °Brix of the fermenting must. Gluzyme-treated samples fermented between 1 and 1.5 °Brix faster than the control (Table 3.3). The fermentation process of wine A was completed within three days. It should also be considered that other factors could play a role, such as the yeast strain that was used, temperature, etc.

The grape juice analyses at the end of the enzyme treatment (wine B) are shown in Table 3.4. A reduction in sugar content of about 11 g/L, which corresponded to approximately 0.68% v/v less ethanol, was obtained at the end of the alcoholic fermentation compared to the control wine. This corresponded with the lower ethanol levels obtained in the final wine. The pH of the enzyme-treated wine dropped with an increase in total acidity (Table 3.5). This can be attributed to the production of gluconic acid, which has also been observed in pure GOX-treated wines (Pickering *et al.*, 1998, 1999a). Although gluconic acid concentration was not determined from these wines, the increase in total acidity, decrease in pH as well as the reduction in sugar content indicates that Gluzyme has the potential of reducing the glucose content of wine, as shown in Table 3.5 (wine B). It would be interesting if SO_2 measurements were performed on the finished wines since gluconic acid binds SO_2. The six-hour treatment (wine A) did not show differences in ethanol concentration whereas a 48-hour treatment (wine B) was about 0.68% v/v less ethanol compared to the control wines.

Longer processing time could have been necessary in the case of wine A, considering the low pH of the grape juice as compared to the optimal pH of the enzyme as well as the complex composition of the red grape juice that could possibly influence the enzyme efficiency. However, ethanol concentration of wine A after fermentation did not differ from the control although the pH and acidity of the wine seemed to have been slightly affected by the enzyme treatment. Since wine A was treated with Gluzyme for six hours, the researchers assumed that the complex composition of red grape could have caused a delay in the enzymatic reaction, considering that these were not the optimal conditions of the enzyme. The enzyme is also encapsulated with wheat flour, which could mean that longer contact time might be necessary for effective enzymatic performance.

TABLE 3.3
Mean values of °Brix (in triplicate) monitored during fermentation of wine A.

Fermentation	0 kU	30 kU
Day 1	24.46	23.16
Day 2	10.8	8.96
Day 3	0.6	<0

The standard deviation of control and Gluzyme-treated wines is ≤ 0.5.

TABLE 3.4
Analyses of Pinotage grape juice at the end of Gluzyme treatment using Foss Winescan (average of duplicate samples) (wine B).

Parameters	0 kU	30 kU
Glucose-Fructose (g/L)	235.5	224
°Brix	23.7	23.25
Density	1.099	1.097
TA (g/L)	3.835	4.795
pH	3.555	3.43

TABLE 3.5

Analyses performed after fermentation of Gluzyme-treated grape juice on the Foss Winescan. Wine A is the 6-hour Gluzyme treatment and Wine B is a 48-hour treatment of separate batches.

	Wine A		**Wine B**	
Parameters	**0 kU**	**30 kU**	**0 kU**	**30 kU**
pH	3.95	3.84	3.77	3.63
VA (g/L)	0.36	0.30	0.42	0.33
TA (g/L)	5.73	6.32	6.21	6.93
MA (g/L)	2.27	2.38	2.06	1.79
LA (g/L)	0.17	0.20	0.32	0.65
Glucose (g/L)	0.97	0.95	0.81	0.91
Fructose (g/L)	1.0	0.94	0.74	0.66
Ethanol (% v/v)	14.34	14.30	13.30	12.62
Glycerol (g/L)	10.33	11.46	10.78	10.92

VA: volatile acidity; TA: total acidity; MA: malic acid; LA: lactic acid

3.3.2.2 Phenolic and colour analyses

3.3.2.2.1 Colour density

There was a slight decrease in colour and modified colour densities of the enzyme treated compared to the control wines (Fig. 3.6).

3.3.2.2.2 Total phenols and free anthocyanins

Trends of lower concentrations of total phenols (Table 3.6) and free anthocyanins (Fig. 3.7) of treated, compared to control wines, were observed and this could possibly be due to hydrogen peroxide oxidation which could have led to polymerisation. pH also influences the phenolate anions that would participate in the regenerative polymerisation reaction to form re-oxidisable hydroquinone with quinones (Du Toit, 2006). Phenolic changes in pure GOX-treated wines have been studied (Pickering *et al*., 1999a). A decrease in hydroxycinnamate content has been observed during vinification in pure GOX-treated wines in comparison to control wines. Hydroxycinnamates appear to be the primary phenolic class responsible for browning reactions in grape must (Cheynier *et al*., 1990). Their decrease during processing and the concurrent colour decrease suggest that they may be important in influencing the final colour of pure GOX wines (Pickering *et al*., 1999a).

3.3.2.2.3 Volatile flavour compounds

General trends of a decrease in acetic acid concentration were observed in the enzyme treated wine B (Table 3.7) compared to the control wines with an increase in ethyl lactate. Wine A did not show differences (data not shown).

Lower acetic acid levels in pure GOX wines have been observed by Pickering *et al*. (1999a). This decrease in acetic acid concentration could possibly result from the antimicrobial activity of the H_2O_2 that is produced by the GOX reaction. The antimicrobial activity of the GOX system is due to the cytotoxicity of the H_2O_2 that is formed, although lowering of the pH by the gluconic acid that is formed may influence the growth of some microorganisms (Fugelsang *et al*., 1995).

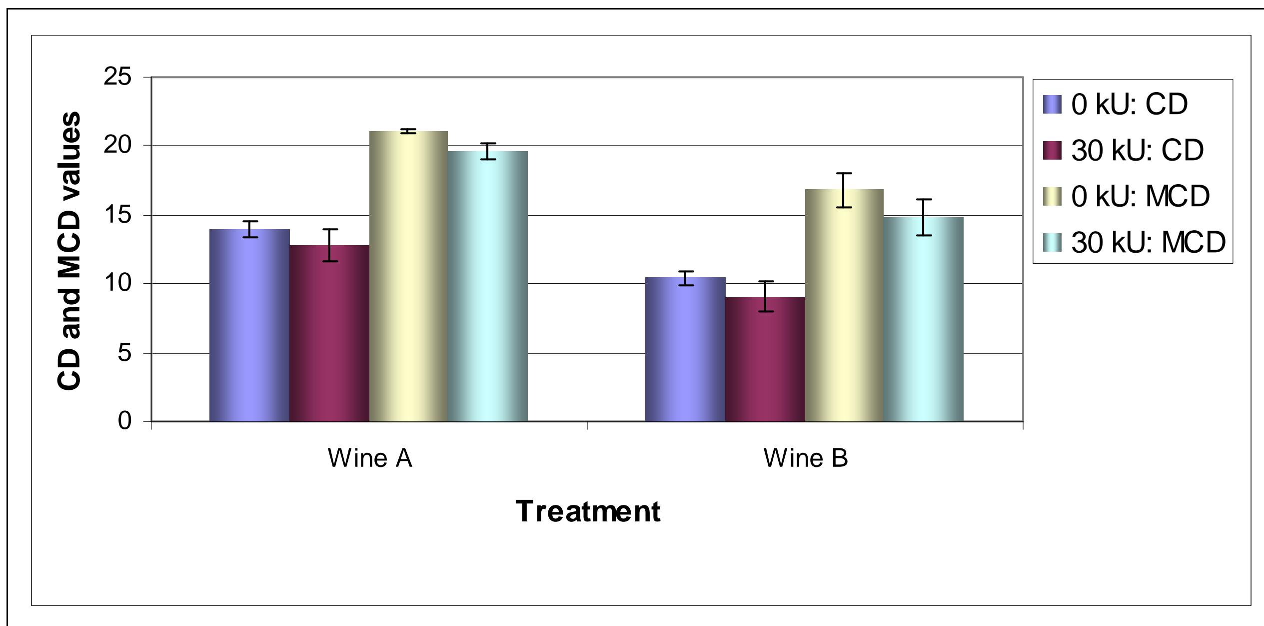

FIGURE 3.6
Colour density (CD) and modified colour density (MCD) of Pinotage wines made from Gluzyme-treated grape juice.

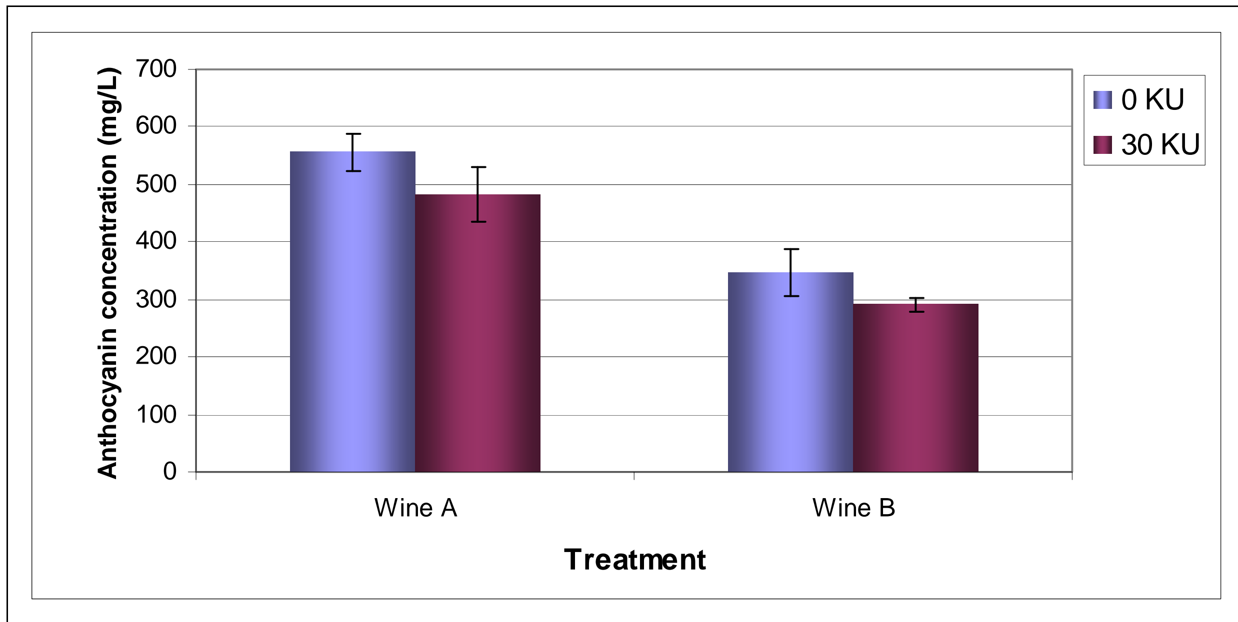

FIGURE 3.7

Total anthocyanin concentration of Pinotage wines made from Gluzyme-treated grape juice (Wine B).

TABLE 3.6

Phenolic content of wines made from Gluzyme-treated grape juice.

Sample	0 kU	30 kU
Wine A	40.722	37.383
Wine B	36.655	35.966

TABLE 3.7

Volatile flavour compounds of wines made from Gluzyme-treated grape juice.

	Wine B	
Volatile compounds (mg/L)	**0 kU**	**30 kU**
Ethyl Acetate	125.351	90.329
Propanol	126.033	107.909
Isoamyl alcohol	217.836	220.043
Ethyl Lactate	109.121	136.455
Acetic Acid	646.802	591.717

3.4 Conclusion

This study was the first investigation into using Gluzyme in winemaking to reduce the ethanol content. A number of trials were conducted in synthetic grape must and grape must using Gluzyme to determine the effective dose of the enzyme that would reduce the glucose content of the grape must. Furthermore, the researchers determined certain factors that may affect the enzyme efficiency, and how to optimise these factors for the winemaking process. All trials carried out with Gluzyme in synthetic grape must and grape must under resulted in complete alcoholic fermentations.

The results showed up to 0.5% v/v less ethanol being obtained in enzyme treated synthetic medium at an enzyme concentration of 20 kU. About 0.68% v/v less ethanol was obtained from wines made from Gluzyme-treated grape juice at 30 kU enzyme concentration. The effects of aeration in combination with pH seemed to have differences in ethanol concentration of the treated compared to the control samples. Raising the pH of the synthetic must at high aeration rate prior to treatment with Gluzyme appeared to be effective in reducing the glucose content of the must. SO_2 did not show significant differences in ethanol concentration between the control and the treated samples. Different temperatures did not lead to major differences in ethanol concentration. The results presented here are an indication of how Gluzyme efficiency would be influenced by these factors. However, more work is required to perform specific enzyme activities for each factor and to optimise these factors for use in winemaking to produce low-alcohol wine.

A six–hour Gluzyme treatment did not show differences in ethanol concentration and 24 – hour treatment showed up to 0.68% v/v less ethanol compared to the control wines. An increased total acidity and concurrent but slight decrease in pH were observed in treated compared to the control wines. This was attributed to the gluconic acid production. The colour and modified colour densities did not differ drastically from that of the control wines. Lower concentrations of total phenols and free anthocyanins were observed in the treated than in the control wines, possibly due to the H_2O_2 oxidation which could have led to polymerisation. It seemed that Gluzyme treatment also led to lower levels of acetic acid.

As a preliminary study, the researchers aimed at investigating the effect of Gluzyme on the glucose content under winemaking conditions. The enzyme in its current form is however, not ideal for winemaking; other forms such as liquid or powder should be considered if the enzyme is to be used under winemaking conditions. The effects of Gluzyme treatment on stability and ageing potential of the wines as well as its impact on the overall quality of the wines still need to be investigated further.

3.5 Literature cited

Athes, V., Pena y Lillo, M., Bernard, C., Perez-Correa, R. & Souchon, I., 2004. Comparison of experimental methods for measuring infinite dilution volatilities of aroma compounds in water/ethanol mixtures. J. Agric. Food Chem. 52, 2021-2027.

Bakker, J., Preston, N.W. & Timberlake, C.F., 1986. Ageing of anthocyanins in red wines: Comparison of HPLC and spectral methods. Am. J. Enol. Vitic. 37, 121-126.

Barbe, J.C., de Revel, G. & Bertrand, A., 2002. Gluconic acid, its lactones and SO_2 binding phenomena in musts from botrytised grapes. J. Agric. Food Chem. 50, 6408-6412.

Bely, M., Sablayrolles, J. & Barre, P., 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferm. Bioeng. 70, 246-252.

Borole, D.D., Kapadi, U.R., Mahulikar, P.P. & Hundiwale, D.G., 2005. Glucose oxidase electrodes of a terpolymer poly (aniline-co-o-anisidine-co-o-toluidine) as biosensors. European Polymer J. 41, 2183-2188.

Castellari, M., Versari, A., Spinabelli, U., Galassi, S. & Amati, A., 2000. An improved method for the analysis of organic acids, carbohydrates and alcohols in grape musts and wines. J. Liq. Chromatogr. & Rel. Technol. 23, 2047-2056.

Cheynier, V., Rigaud, J., Souquet, J., Duprat, F. & Moutounet, M., 1990. Must browning in relation to the behaviour of phenolic compounds during oxidation. Am. J. Enol. Vitic. 41, 346-349.

Dequin, S., Baptista, E. & Barre, P., 1999. Acidification of grape must by *Saccharomyces cerevisiae* wine yeast strains genetically engineered to produce lactic acid. Am. J. Enol. Vitic. 50, 45-50.

Dequin, S. & Barre, P., 1994. Mixed lactic acid-alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei* L(+)-LDH. Biotechnol. 12, 173-177.

De Vuyst, L. & Vandamme, E.J., 1994. Antimicrobial potential of lactic acid bacteria. In: L. de Vuyst and E.J. Vandamme (eds.). Bacteriocins of lactic acid bacteria. New York. Blackie Academic & Professional. pp. 99–103.

Du Toit, W.J., 2006. The effect of oxygen on the composition and microbiology of red wine. PhD thesis. Stellenbosch University, South Africa.

Fugelsang, C.C., Johansen, C., Christgau, S. & Adler-Nissen J., 1995. Antimicrobial enzymes: Applications and future potential in the food industry. Review. Trends in Food Sc. & Technol. 6, 390-396.

Geisen, R., 1999. Inhibition of food-related pathogenic bacteria by god-transformed *Penicillium nalgiovense* strains. J. Food Protect. 62, 940-943.

Gishen, M. & Holdstock, M., 2000. Preliminary evaluation of the performance of the Foss Winescan FT 120 instrument for the simultaneous determination of several wine analyses. The Aust. Grapegrower & Winemaker, Annual Techn. Issue, pp. 1-10.

Hanft, F. & Koehler, P., 2006. Studies on the effect of glucose oxidase in bread making. J. Sc. Food Agric. 86, 1699-1704.

Heresztyn, T., 1987. Conversion of glucose to gluconic acid by glucose oxidase enzyme in Muscat Gordo juice. Aust. Grapegrower and Winemaker, April, pp. 25-27.

Heux, S., Sablayrolles, J.M., Cachon, R. & Dequin, S., 2006. Engineering a *Saccharomyces cerevisiae* wine yeast that exhibits reduced ethanol production during fermentation under controlled microoxygenation conditions. Appl. Environ. Microbiol. 72, 5822-5828.

Jackson, R.S., 1994. Wine Science. Principles and applications. San Diego. New York. Academic Press. pp. 264-266.

Lambrechts, M.G. & Pretorius, I.S., 2000. Yeast and its importance to wine aroma: A review. S. Afr. J. Enol. Vitic. 21, 97-129.

Malherbe, D.F., Du Toit, M., Cordero Otero, R.R., Van Rensburg, P. & Pretorius, I.S., 2003. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. Appl. Microbiol. Biotechnol. 61, 502-511.

Malherbe, S., 2007. Industry-wide assessment and characterisation of problem fermentations: MSc. thesis. Stellenbosch University, South Africa. pp 49-51.

McLeod, R. & Ough, C.S., 1970. Some recent studies with glucose oxidase in wine. Am. J. Enol. Vitic. 21, 54-60.

Merzhanian, A.A. & Tagunkov, Yu. D., 1967. Action of glucose oxidase in grape wines and must. Chem. Abstracts 68, 5652.

Michnick, S., Roustan, J.L., Remize, F., Barre, P. & Dequin, S., 1997. Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for *GPD1* encoding glycerol 3-phosphate dehydrogenase. Yeast 13, 783-793.

Nevoigt, E., Pilger, R., Mast-Gerlach, E., Schmidt, U., Freihammer, S., Eschenbrenner, M. Garbe, L. & Stahl, U. 2002. Genetic engineering of brewing yeast to reduce the content of ethanol in beer. FEMS Yeast Res. 2, 225-232.

Nevoigt, E. & Stahl, U. 1996. Reduced pyruvate decarboxylase and increased glycerol-3-phosphate dehydrogenase [NAD^+] levels enhance glycerol production in *Saccharomyces cerevisiae*. Yeast 12, 1331-1337.

Nieuwoudt, H., 2004. Glycerol and wine. PhD thesis. Stellenbosch University, South Africa.

Ough, C.S., 1975. Further investigations with glucose oxidase-catalase enzyme systems for use with wine. Am. J. Enol. Vitic. 26, 14–20.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1998. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. Food Res. Int. 31, 685-692.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999a. The production of reduced alcohol wine using glucose oxidase treated juice. Part I: Composition. Am. J. Enol. Vitic. 50, 291-298.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999b. The production of reduced alcohol wine using glucose oxidase treated juice. Part II. Stability and SO_2- binding. Am. J. Enol. Vitic. 50, 299–306.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999c. The production of reduced alcohol wine using glucose oxidase treated juice. Part III. Sensory. Am. J. Enol. Vitic. 50, 307-316.

Pickering, G.J., 2000. Low- and reduced-alcohol wine: a review. J. Wine Res. 11, 129-144.

Remize, F., Roustan, J.L., Sablayrolles, J.M., Barre, P. & Dequin, S., 1999. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationery phase. Appl. Environ. Microbiol. 65, 143-149.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000a. Conditions of yeast development. In: Handbook of Enology, Vol.1: The microbiology of wine and vinifications. John Wiley & Sons. New York. pp. 75-106.

Ribéreau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D., 2000b. Chemical properties of anthocyanins and tannins. In: Handbook of Enology, Vol 2: The chemistry of wine stabilisation and treatments. John Wiley & Sons. New York. pp 139-164.

Romano, P., Paraggio, M. & Turbanti, L., 1998. Stability in by-product formation as a strain selection tool of *Saccharomyces cerevisiae* wine yeasts. J. Appl. Microbiol. 84, 336-341.

Scott, D., 1975. Applications of glucose oxidase. In G. Reed. (ed.). Enzymes in food processing. New York Academic Press. pp. 519–547.

Somers, T.C. & Evans, M.E., 1977. Spectral evaluation of young red wines: Anthocyanin equilibria, total phenolics, free and molecular SO2 “chemical age”. J. Sc. Food Agric. 28, 279-287.

Villettaz, J.C., 1986. Method for production of a low alcoholic wine and agent for performance of the method. European Patent no: EP 0 194 043 A1.

Villettaz, J.C. 1987. A new method for the production of low alcohol wines and better balanced wines. In: T. Lee (ed.). Proceedings of the. 6th Aust. Wine Ind. Tech. Conf. Adelaide Australian Industrial Publishers. pp. 125-128.

Whitaker, J.R., 1972. Principles of enzymology for the food sciences. [cited In Heresztyn, T. (1987). Conversion of glucose to gluconic acid by glucose oxidase enzyme in Muscat Gordo juice. Aust. Grapegrower and Winemaker, April, 25-27].

White, B.B. & Ough, C.S., 1973. Oxygen uptake studies on grape juice. Am. J. Enol. Vitic. 24, 148-152.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F.S., 1995. Alcohol and extract. Chapman & Hall. Wine analysis and production: Chapman & Hall. pp. 97-114.

Chapter 4

General discussion and conclusions

4. General discussion and conclusions

There is a continuous demand for higher quality products in order to meet consumer's expectations (Bisson *et al*., 2002). Of these is the increased international interest and consumer demand for low- and reduced-alcohol wines (Schobinger & Dürr, 1983; Heess, 1990; Hoffmann, 1990; Simpson, 1990; Howley & Young, 1992). Commercial interest has also been stimulated by the potential for savings in taxes/tariffs on the reduced alcohol content in these classes of wines (Pickering *et al*., 1998). This has put a great deal of pressure on wine producers, particularly those in warm climate wine-producing regions, as well as countries that export wine to Europe such as South Africa and Australia, where grape sugar content can become very high. High sugar concentrations invariably lead to the production of wines with high levels of alcohol, with some wines reaching ethanol concentration above 15% v/v (Godden, 2000; Day *et al*., 2002). High ethanol concentration can affect the sensory properties of the wine (Guth & Sies, 2002) and, depending on the wine style, alcohol can be perceived as a burning sensation on the palate, making the wines to appear unbalanced. Furthermore, the higher alcohol content can mask the overall aroma and flavour of the wine.

Several physical processes are used for the removal or reduction of alcohol in wine (Pickering, 2000); all of which involve the selective extraction of alcohol based on volatility or diffusion. Despite their efficacy, these processes require expensive equipment, and they can affect the flavour balance through loss of aroma compounds. Extensive research has also been done through genetically engineering wine yeast strains to reduce the alcohol content of wine (Heux *et al*., 2006; Malherbe *et al*., 2003; Nevoigt *et al*., 2002).

The aim of this study was to evaluate the possibility of using Gluzyme to reduce the glucose content of grape must before fermentation in order to produce wine with reduced-alcohol level.

Gluzyme is currently being used in the baking industry. The enzyme catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. Gluzyme was used initially in synthetic grape must where different enzyme concentrations and factors influencing its activity were investigated. The enzyme dose that can be used in winemaking to reduce the glucose content had to be established through trials in synthetic must. We established that 20 kU of enzyme is necessary to reduce the ethanol concentration by 0.5% v/v, when the enzyme preparation is incubated nine hours before fermentation. It was also shown that the reduction in ethanol concentration increased with an increasing amount of enzyme which is consistent with the results reported by Pickering *et al.* (1998) when using pure GOX. However, like all glucose oxidases, higher concentrations of Gluzyme can cause off-flavours during long hours of incubation, because, long-term exposure of a food product to H_2O_2 could cause rancidity of the food as a result of lipid oxidation (Fugelsang *et al*., 1995).

In further trials, the enzyme concentration was increased to 30 kU, and different factors influencing the enzyme activity were investigated using synthetic must. This resulted in ethanol reduction of about 1 and 1.3% v/v in aerated synthetic must at pH 3.5 and pH 5.5 respectively. Increasing the pH and aeration rate of the must seemed to be more effective in favouring the

enzymatic reaction. Similar results were obtained by Pickering *et al.* (1998) showing that low pH of grape juice was found to be a dominant limiting factor in the rate and extent of glucose conversion by GOX. When considering the high pH of South African red wine, Gluzyme could possibly be active in converting glucose under these pH conditions, and if so, then acid additions could be reduced, since the enzyme increases the acidity of the wine as a result of gluconic acid being produced.

Sulphur dioxide treatment did not have a major influence on enzyme activity, but the effect of Gluzyme treated samples differed significantly from the control (without Gluzyme and gluconic acid) and gluconic acid treated samples. No differences in ethanol concentration were obtained between the control and gluconic acid treated samples. According to the manufacturer, only SO_2 levels between 100 and 200 mg/L have inhibitory effects on the enzyme activity, but not under the SO_2 levels usually found in winemaking. It was also observed that by the end of the enzyme treatment, almost zero or negligible amounts of total SO_2 were left. This has potential implications with regard to antimicrobial and antioxidant activities conferred by SO_2 during juice aeration and to the inhibition of polyphenol-oxidase (Pickering *et al.*, 1998) in GOX treated must. Rapid oxidation of free SO_2 to sulphate by H_2O_2 that is produced by the enzymatic reaction could be a possible reason for quick disappearance of SO_2 during processing. Using H_2O_2 as a control in SO_2 trial could provide more precise answers to this. It must also be kept in mind that the rate of oxygen consumption in must declines drastically with the addition of SO_2 as the enzyme requires oxygen for its optimal glucose conversion. Thus, SO_2 levels have to be monitored very closely if Gluzyme is to be used in winemaking to ensure that SO_2 functions are not minimized by the enzyme treatment.

The pH is one of the key factors affecting the function of SO_2. The pH plays a role, firstly, at higher pH levels; more total SO_2 is needed to get the same level of free molecular SO_2. Secondly, and as a consequence, SO_2 is more effective as an antimicrobial agent at a lower pH.

There were no differences in ethanol concentration between control and treated samples at 15°C and 25°C. This result seemed to be in agreement with what has been observed using pure GOX when comparing the enzyme performance at 20°C and 30°C (Pickering *et al.*, 1998). A desirable temperature range for use in grape juice has been suggested to be between 15°C and 20°C for pure GOX (Villettaz, 1986, 1987; Heresztyn, 1987). The optimum temperature range for Gluzyme is between 40 and 60°C as recommended by the manufacturer for use in the baking industry. However, it is not known how efficient Gluzyme is under winemaking conditions.

Finally, Gluzyme was used to treat Pinotage grape must before fermentation using 30 kU enzyme concentration. No acid or SO_2 additions were performed in grape must before treatment with Gluzyme. One batch of Pinotage grapes was treated for 6 hours and another batch for 48 hours respectively. Differences in ethanol concentration of the treated and untreated samples were obtained at 6 and 48 hours of Gluzyme treatment. These results showed that more ethanol was reduced (0.68% v/v) at 48 than 6 hours of enzyme treatment. The decrease in sugar content of Gluzyme treated juice directly corresponded to the amount of ethanol that was reduced. The reason for no differences in 6 hours of Gluzyme treatment could be that the complex composition of red grape must might have delayed the enzymatic reaction, considering

that other factors were also not optimal for the enzyme. It could also be that longer contact time may be necessary for the enzyme to effectively convert glucose to gluconic acid and H_2O_2 under winemaking conditions. The pH of the treated samples did not change drastically compared to the control samples. However, total acidity increased with 1 g/L in the treated samples at the end of enzyme treatment. This could be attributed to an increase in gluconic acid concentration of the treated samples as previously been observed by other authors (Pickering *et al.*, 1998, 1999).

The simultaneous presence of δ-gluconolactone together with gluconic acid produced by glucose oxidation has been known for a long time (King & Cheldelin, 1958; Fewster, 1958). This lactone is able to bind SO_2 used as a wine preservative thereby diminishing its activity. The gluconolactone form occurs in equilibrium with gluconic acid but at wine pH, this equilibrium favours the gluconic acid formation (McCloskey, 1974). Gluconic acid affects wine stability during aging and storage, and poses a high risk of alteration, mainly through catabolism of the acid by various microorganisms such as lactic acid bacteria (Pérez *et al.*, 1991). This problem can only be solved by reducing the gluconic acid content in wine. *Schizosaccharomyces pombe* is able to utilise D-gluconate as a growth substrate (Peinado *et al.*, 2004). Recently, a wild *S. pombe* yeast strain has been used to reduce the gluconic acid content in wine (Peinado *et al.*, 2004) and the effect on fermentation by-products has been studied by Peinado *et al.* (2007). The results showed that the amounts of volatile compounds were similar with the treatments where gluconic acid was previously depleted. Amino acids were used in large amounts by *S. pombe* during removal of gluconic acid; this affected subsequent fermentation by *S. cerevisiae* and the formation of by-products.

One of the concerns about the use of Gluzyme in winemaking is the 1:1 ratio of glucose to fructose that becomes shifted by the enzyme treatment. As the fermentable sugar fraction of grape juice is approximately 50% glucose and 50% fructose (variation exists due to variety, season and ripeness), fermenting the glucose-depleted juice after treatment with pure GOX produces a wine with approximately half of the potential alcohol content (Pickering, 2000). On the other hand, it raises some questions concerning stuck fermentation as there can be more fructose remaining after some of the glucose has been converted to gluconic acid by Gluzyme. During fermentation with most yeast strains, the consumption of glucose is faster than that of fructose and toward the end of fermentation; most of the residual sugar is fructose (Margalit, 1996).

In this study, we aimed at reducing the alcohol level by at least 0.5 to 2.0% v/v and, not so much that it causes a major shift in the ratio of glucose to fructose in grape must during fermentation or that it imparts negatively on the aroma and flavour characteristics of the wine.

The colour and modified colour densities of wine did not differ drastically from the control. It was expected that there would be differences in colour of treated wines because of the decreased pH by the enzyme treatment; but the pH of the treated wines did not differ significantly. Low pH of Gluzyme treated wines could increase colour hue and microbial stability of these wines since a lower number of microorganisms is active at low pH. Lower concentrations of total phenols and anthocyanins were observed in treated than control wines,

possibly due to the H_2O_2 oxidation which could have led to polymerisation. Acetic acid and ethyl acetate were found to be lower in treated than control wines, which is in accordance with the results obtained by Pickering *et al.* (1999).

One of the health implication posed by Gluzyme in its current form is the presence of gluten. The diluent for Gluzyme is wheat flour. Wheat flour contains about 5 to 13% gluten.

Gluten is the elastic water-insoluble protein found in wheat and other grains. For people suffering from celiac disease, all forms of gluten are toxic to the digestive system. The protein causes an immunological reaction in the small intestine, resulting in the disintegration of the finger-like villi that facilitate the absorption of nutrients.

The present study has clearly demonstrated that Gluzyme may be used in winemaking to produce reduced-alcohol wine without affecting its colour and aroma compounds. The enzyme in its current form is however, not ideal for winemaking; other forms such as liquid or powder form should be considered if the enzyme is to be used under winemaking conditions.

Future work should focus on evaluating potential new form of the enzyme and studying the effects of Gluzyme in various grape must in semi-industrial scale. Proper monitoring of Gluzyme activity on a time frame in order to show its efficiency under winemaking is required. A tasting panel should also evaluate its impact on the organoleptic properties and the overall quality of the resulting wines.

Literature cited

Athes, V., Pena y Lillo, M., Bernard, C., Perez-Correa, R. & Souchon, I., 2004. Comparison of experimental methods for measuring infinite dilution volatilities of aroma compounds in water/ethanol mixtures. J. Agric. Food Chem. 52, 2021-2027.

Bisson, L.F., Waterhouse, A.L., Ebeler, S.E., Walker, M.A. & Lapsley, J.T., 2002. The present and future of the international wine industry. Nature 418, 696-699.

Day, R.E., Gishen, M., Godden, P.W. & Hughes, J.B., 2002. Producing unique wine styles for overseas markets. Blair, R.J., Williams, P.J. & Høj, P.B. (eds). In: Proc. 11th Australian wine industry technical conference. Adelaide, SA. Australian, Wine Industry Technical Conference Inc. pp. 27-32.

Fewster, J.A., 1958. Growth of *Acetobacter suboxydans* and the oxidation of aldoses, related carboxylic acids and aldehydes. Biochem. J. 69, 582-595.

Fugelsang, C.C., Johansen, C., Christgau, S. & Adler-Nissen J., 1995. Antimicrobial enzymes: Applications and future potential in the food industry. Review, Trends in Food Sc. & Technol 6, 390-396.

Godden, P., 2000. Persistent wine instability issues. Aust. Grapegrower Winemaker 443, 11-14.

Guth, H. & Sies, A., 2002. Flavour of wines: towards an understanding by reconstitution experiments and an analysis of ethanol's effect on odour activity of key compounds. Blair, R.J., Williams, P.J. & Høj, P.B. (eds). In: Proceedings of the 11th Australian wine industry technical conference. Adelaide, SA. Australian, Wine Industry Technical Conference Inc. pp. 128-139.

Hanft, F. & Koehler, P., 2006. Studies on the effect of glucose oxidase in bread making. J. Sci. Food Agric. 86, 1699-1704.

Heess, W., 1990. Marketing changes, legal questions and the production of dealcoholised wines. Proc. 9th Int. Oenol. Symp., Cascais, Portugal, May. 154-165.

Heresztyn, T., 1987. Conversion of glucose to gluconic acid by glucose oxidase enzyme in Muscat Gordo juice. The Australian Grapegrower and Winemaker April, 25-27.

Heux, S., Sablayrolles, J.M., Cachon, R., & Dequin, S., 2006. Engineering a *Saccharomyces cerevisiae* wine yeast that exhibits reduced ethanol production during fermentation under controlled micro oxygenation conditions. Appl. Environ. Microbiol. 72, 5822-5828.

Hoffmann, D., 1990. The European wine market up to the year 2000- trends and marketing efforts. Proc. 9th Int. Oenol. Symp., Cascais, Portugal, May. 138-153.

Howley, M. & Young, N., 1992. Low alcohol wines: the consumers choice? Int. J. Wine Marketing 4, 45-46.

King, T.E. & Cheldelin, V.H., 1958. Multiple pathways of glucose oxidation in *Acetobacter suboxydans*. Biochem. J. 68, 31.

Malherbe, D.F., Du Toit, M., Cordero Otero, R.R., Van Rensburg, P. & Pretorius, I.S., 2003. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. Appl. Microbiol. Biotechnol. 61, 502-511.

Margalit, Y., 1996. Winery technology & operations. A handbook for small wineries. The Wine Appreciation Guild, San Francisco. pp. 2-5.

McCloskey, L.P., 1974. Gluconic acid in California wines. Am. J. Enol. Vitic. 25, 198-201.

Miller, K.A & Hoseney, R.C., 1999. Effect of oxidation on the dynamic rheological properties of wheat flour-water doughs. Cereal chem. 76, 100-104.

Müller, D., 1928. Studies on the new enzyme glucose oxidase. I. Biochem. Z. 199, 136-170.

Pazur, J.H. & Kleppe, K., 1964. the oxidation of glucose and related compounds by glucose oxidase from *Aspergillus niger*. Biochem. 35, 578-583.

Nevoigt, E., Pilger, R., Mast-Gerlach, E. & Schmidt, U., 2002. Genetic engineering of brewing yeast to reduce the content of ethanol in beer. FEMS Yeast Res. 2, 225-232

Peinado, R.A., Moreno, J.J., Maestre, O., Ortega, J.M., Medina, M. & Mauricio, J.C., 2004. Gluconic acid consumption in wines by *Schizosaccharomyces pombe* and its effect on the concentration of major volatile compounds and polyols. J. Agric. Food Chem. 52, 493-497.

Peinado, R.A., Moreno, J.J., Maestre, O. & Mauricio, J.C., 2007. Removing gluconic acid by using different treatments with a *Schizosaccharomyces pombe* mutant: effect on fermentation by-products. Food Chem. 104, 457-465.

Pérez, L., Valcárcel, M.J., González, P. & Domecq, B., 1991. Influence of Botrytis infection of the grapes on the biological aging process of Fino Sherry. Am. J. Enol. Vitic. 42, 58-62.

Pickering, G.J., Heatherbell, D.A., & Barnes, M.F., 1998. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. Food Res. Int. 31, 685-692.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999. The production of reduced alcohol wine using glucose oxidase treated juice. Part I: Composition. Am. J. Enol. Vitic. 50, 291-298.

Pickering, G.J., 2000. Low- and reduced-alcohol wine. J. Wine Res. 11, 129-144. A review.

Schobinger, U. & Dürr, P., 1983. Diatetische und sensorische aspekte alkoholfreier weine. Alimenta 22, 33-36.

Simpson, A., 1990. Impact of anti-alcohol campaigns on the wine industry. Proc. 9th Int. Oenol. Symp. Cascais, Portugal, May. pp. 126-137.

Villettaz, J.C., 1986. Method for production of a low alcoholic wine and agent for performance of the method. European Patent no: EP 0 194 043 A1.

Villettaz, J.C., 1987. A new method for the production of low alcohol wines and better balanced wines. Proc. 6th Aust. Wine Industry Techn. Conf., Adelaide, Australian Industrial Publishers.

Chapter 5

Addenda

5. Addenda

A: Statistical analysis of Gluzyme Mono® 10.000 BG-treated synthetic grape must

5.1 Enzyme dose

One-way ANOVA method was used to find differences between treated and control samples for analysis of ethanol concentration. According to the Benforri test, significant differences were observed when comparing treatment A (0 kU) and C (10 kU), and A and D (20 kU) but there were no differences observed between A and B (5 kU). The statistical analysis for ethanol concentration at different enzyme concentrations is shown in Fig. 5.1.

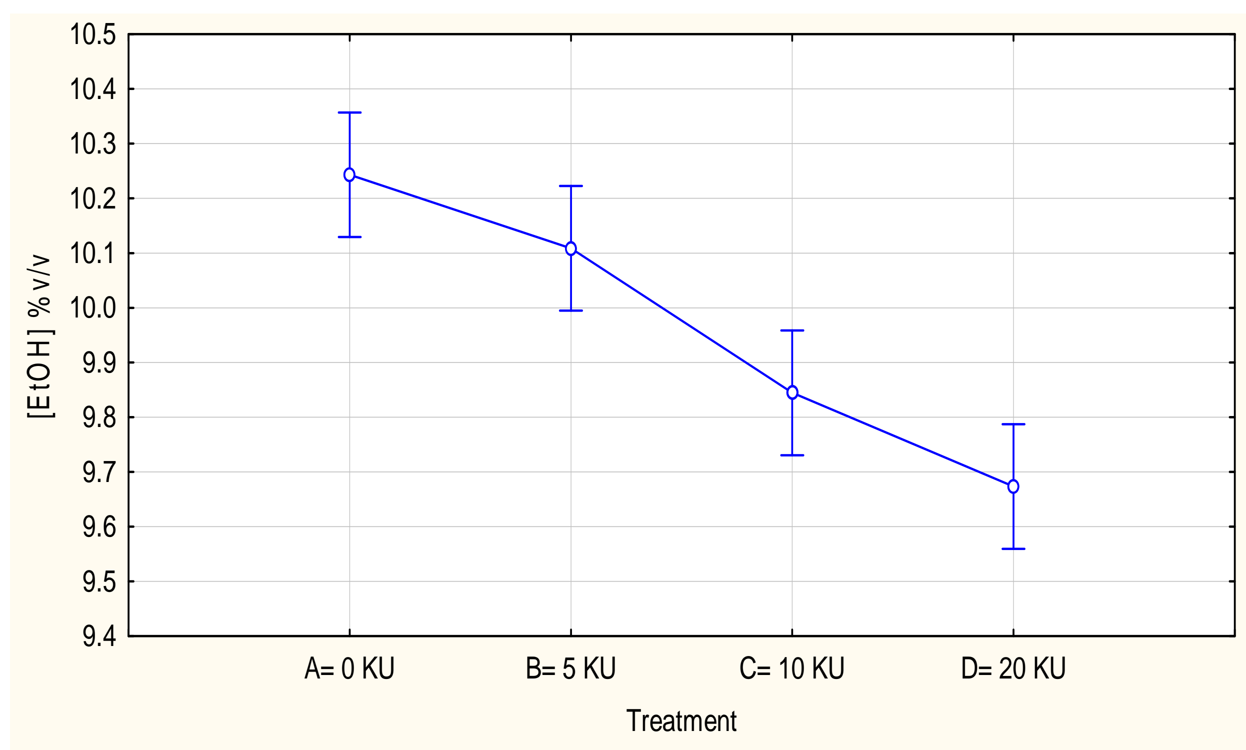

FIGURE 5.1

Ethanol concentration of Gluzyme-treated synthetic grape must (40 g/L sugar at pH 3.3) using different enzyme concentrations.

5.2 Factors influencing Gluzyme activity

A Two-way ANOVA was used to analyse differences between the treatments. The statistical analysis that was performed on the treatments for different factors influencing Gluzyme efficiency is given below. The synthetic grape must was prepared using 200 g/L sugar and was adjusted according to various treatments performed.

5.2.1 Temperature

No statistical differences were obtained for the temperature experiments. However, general trends of increased ethanol reduction at 25°C than 15°C were observed in treated than control samples (Fig. 5.2).

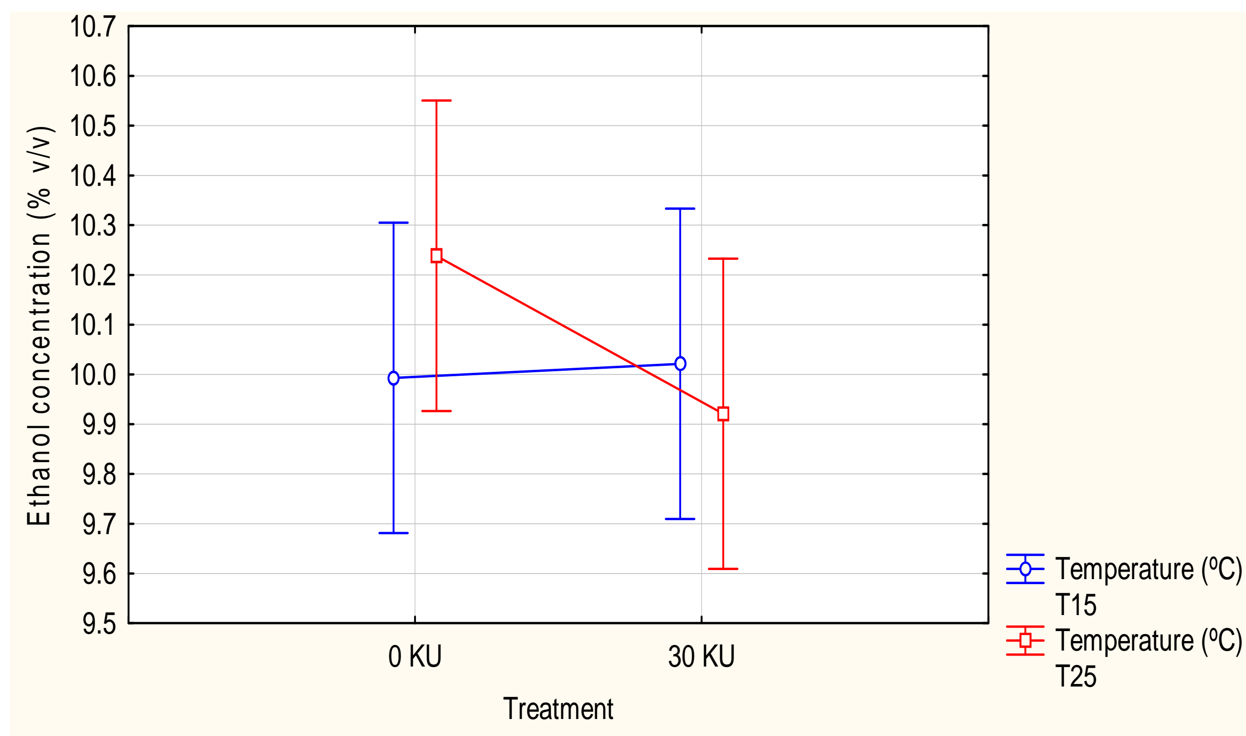

FIGURE 5.2
Ethanol concentration of Gluzyme-treated synthetic grape must at 15°C and 25°C.

5.2.2 pH

There were no significant differences in ethanol concentration observed at different pH levels tested. Trends of less ethanol being produced at pH 5.5 were observed ($p= 0.05$) in treated than control samples (Fig. 5.3), shows the pH effect on enzyme efficiency to reduce ethanol. There were no differences between pH 3 and pH 4, but Gluzyme treatment was significantly different (Fig. 5.4).

5.2.3 Oxygen

There were no differences as a result of oxygen treatment that were observed (Fig. 5.5). Significant differences were obtained when comparing a control versus the treated samples ($p<0.05$) as shown in Fig. 5.6.

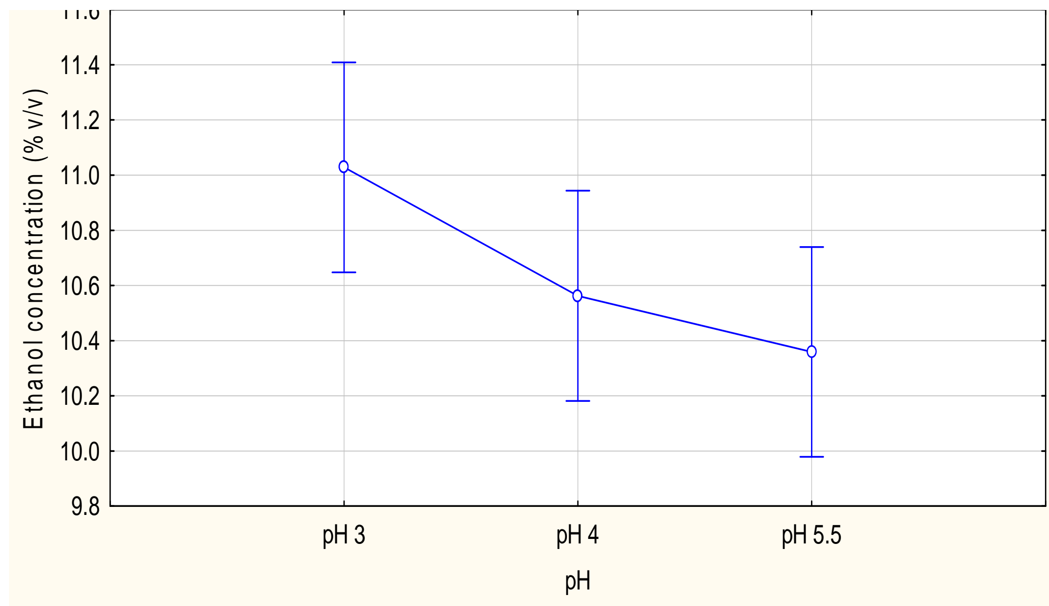

FIGURE 5.3
Ethanol concentration of Gluzyme-treated synthetic grape must at different pH levels (pH effect).

5.2.4 Sulphur dioxide

Sulphur dioxide treatments did not have any influence on ethanol concentration (Fig. 5.7). Although Gluzyme treated samples differed significantly from the control (A) (without Gluzyme and gluconic acid) and C (gluconic acid treated) no differences were observed between the control and gluconic acid treated samples (Fig. 5.8).

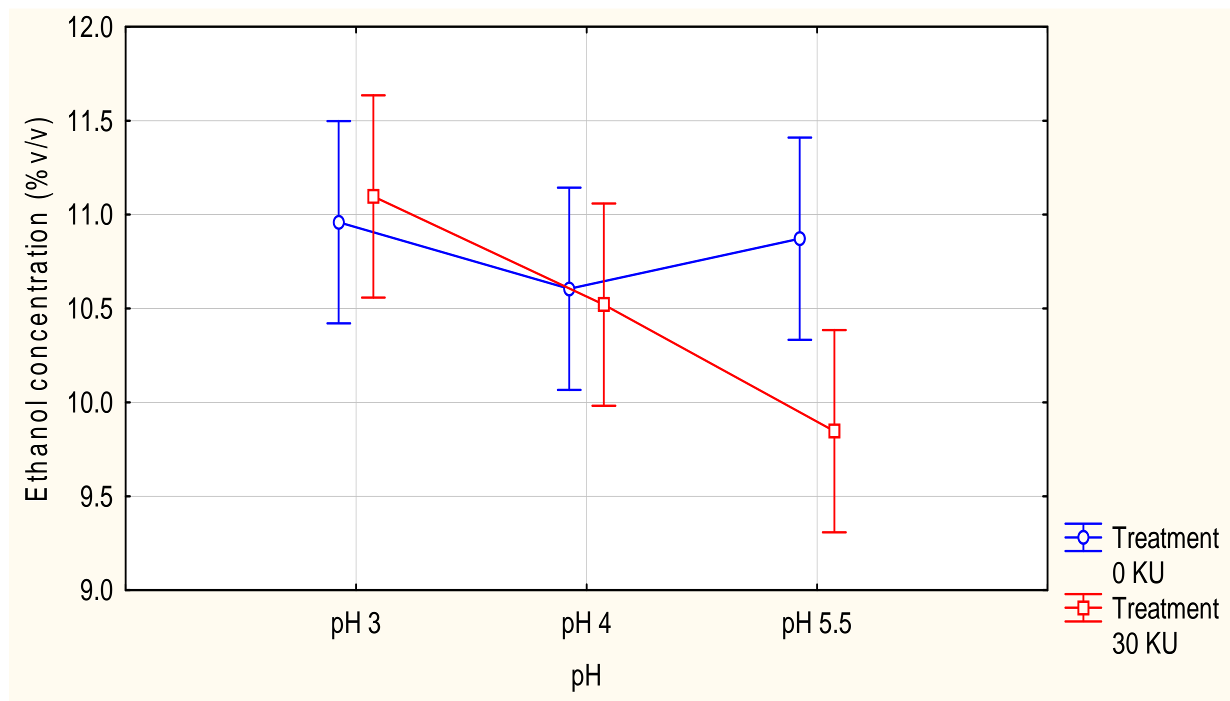

FIGURE 5.4
Ethanol concentration of Gluzyme-treated synthetic grape must at different pH (Gluzyme treatment effect).

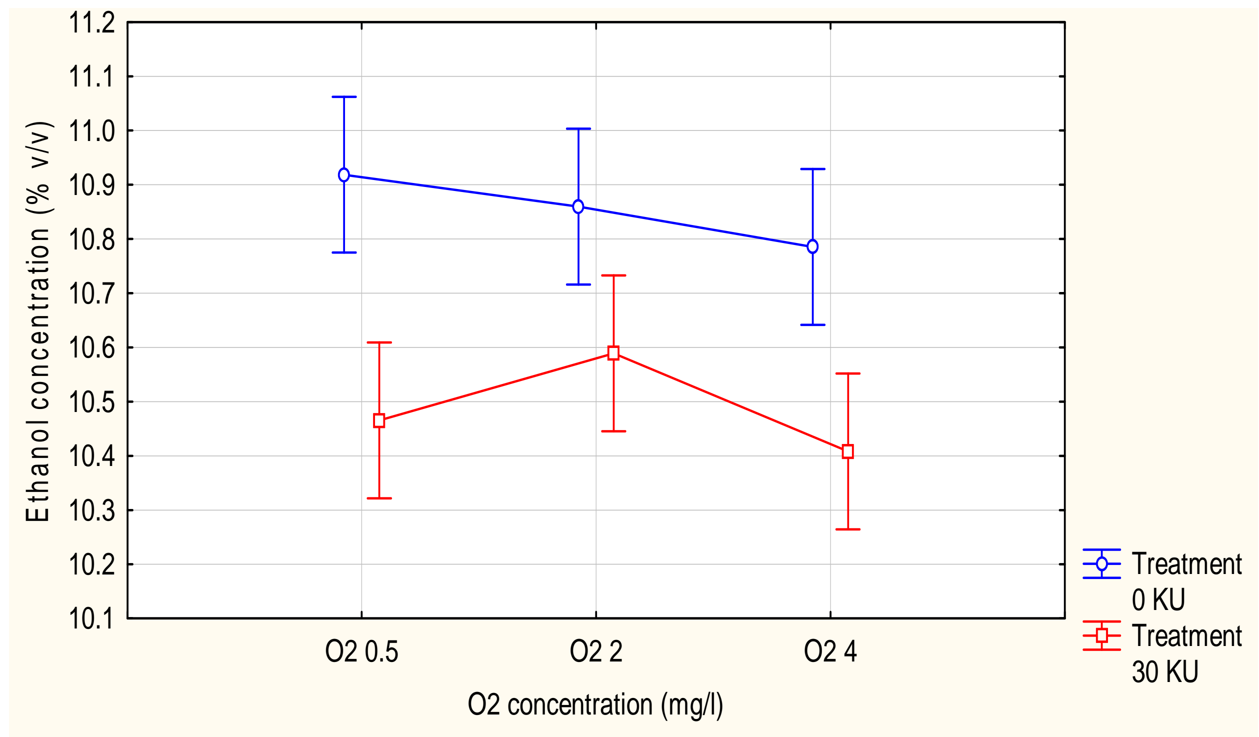

FIGURE 5.5

Ethanol concentration of Gluzyme-treated synthetic grape must at different aeration rates.

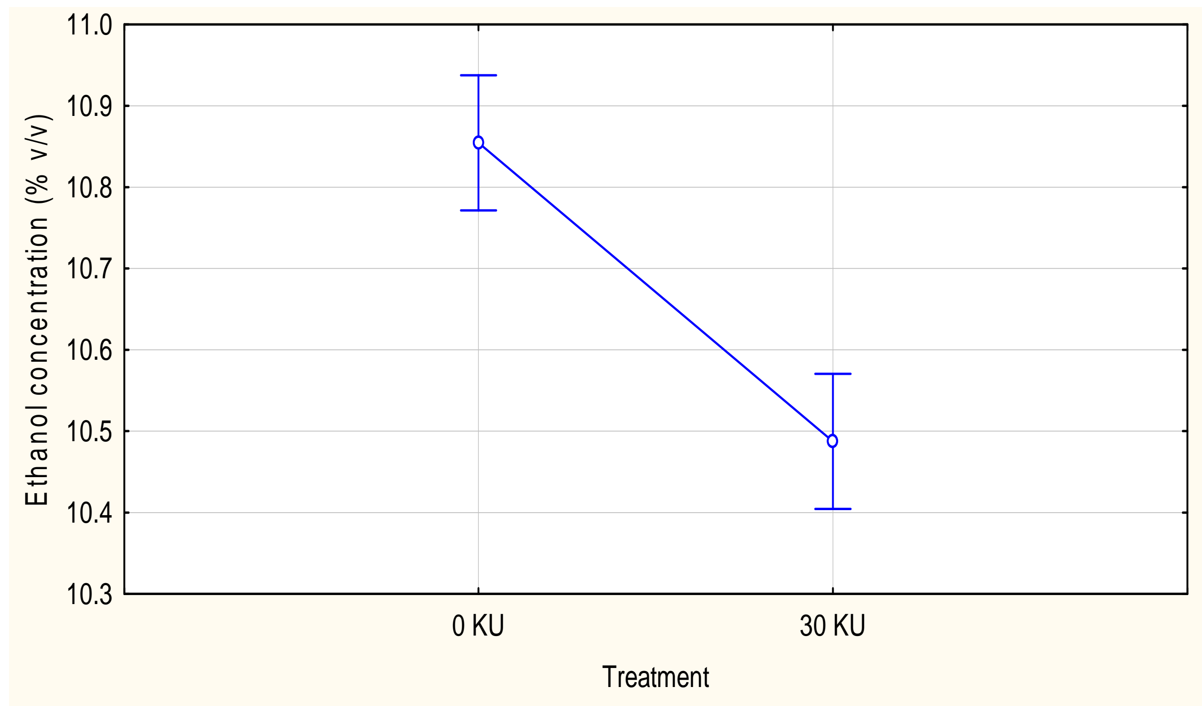

FIGURE 5.6

Ethanol concentration of Gluzyme-treated synthetic grape must at different aeration rates (differences observed as a result of Gluzyme treatment).

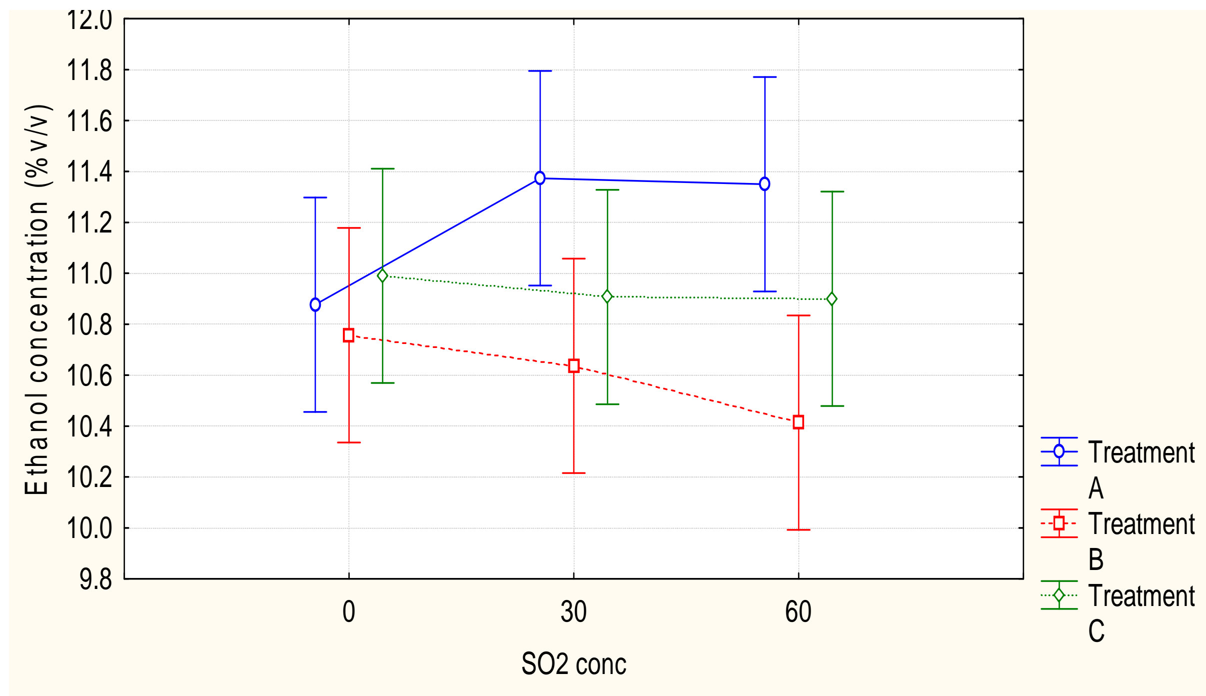

FIGURE 5.7

Ethanol concentration of Gluzyme-treated synthetic grape must at different SO_2 levels: A: without Gluzyme and gluconic acid; B: Gluzyme treated and C: Gluconic acid treated samples.

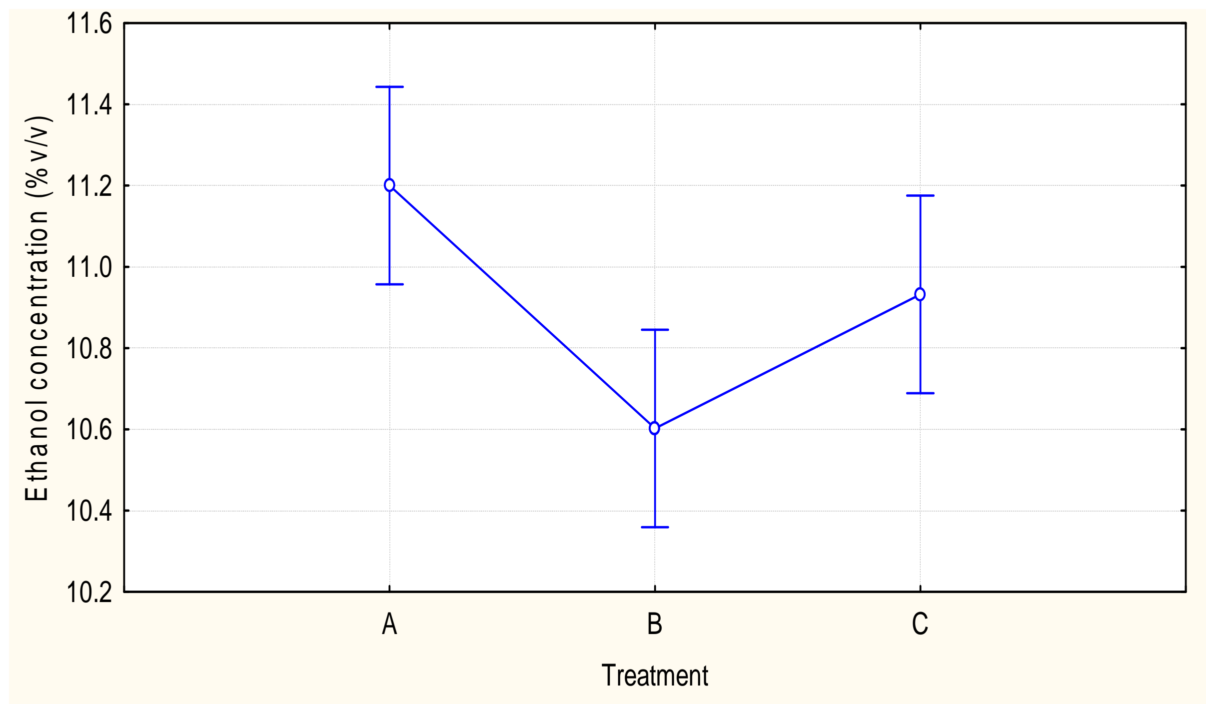

FIGURE 5.8

Ethanol concentration of Gluzyme-treated synthetic grape must (significant difference observed at B due to Gluzyme treatment): A: without Gluzyme and gluconic acid; B: Gluzyme treated and C: Gluconic acid treated samples.

B: Product data sheet: Gluzyme Mono® 10.000 BG

Gluzyme® Mono 10000 BG

Valid From **2007-09-28**

Product Characteristics:

Declared Enzyme	Glucose-oxidase
Declared Activity	10000 GODU/g
Colour	Yellow to light brown
Physical form	Granulate
Particle size	Approx. 50-212 microns
Diluents	Wheat flour
Stabilisers	Sodium chloride
	This product is standardized by Documented Addition in a process controlled by Novozymes ISO 9001 quality system. See Documented Addition Info Sheet for further information.
Solubility	Active component is readily soluble in water at all concentrations that occur in normal usage. Standardisation components can cause turbidity in solution.
Production organism	Aspergillus oryzae
Production Method	Produced by submerged fermentation of a genetically modified micro organism. The enzyme protein, which in itself is not genetically modified, is separated and purified from the production organism.

1 / 2

Product Specification:

	Lower Limit	Upper Limit	Unit
Glucose Oxidase Units GODU	10000		/g
Total Viable Count	-	50000	/g
Coliform Bacteria	-	30	/g
Enteropathogenic E.Coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before: When stored as recommended, the product is best used within 6 months from date of delivery.

Storage at customer's warehouse: 0-10°C (32°F-50°F)

Storage Conditions: In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

Safety and Handling Precautions

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product has been developed to resist light mechanical effects. However,excessive mechanical wear and tear or crushing may create dust. All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes. A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.

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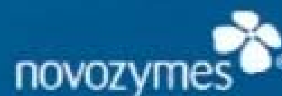
C: Product sheet: Gluzyme Mono® 10.000 BG

Cereal Food / 2002-27688-01.pdf

Product Sheet



Gluzyme Mono® 10.000 BG

Description

Gluzyme Mono 10.000 BG is a glucose oxidase preparation from *Aspergillus niger* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and H_2O_2.

Product properties

Product type

Gluzyme Mono 10.000 BG is standardized using a special wheat flour with a narrow particle size distribution. Gluzyme Mono 10.000 BG is a mix of yellowish-grey, free-flowing, non-dusting, agglomerated granulates and flour. It has an average particle size of 150 microns within the range 50-212 microns.

Activity

Gluzyme Mono 10.000 BG is available as:

Gluzyme Mono 10.000 BG ....10,000 GODU/g

GODU = Glucose Oxidase Units.

The product is standardized with wheat flour by Documented Addition in a strict ISO-controlled process. See the Analytical Method for further information. The product contains a non-standardized amount of catalase side activity which neither hinders nor helps its functionality in the bread-making process.

Solubility

The active components of Gluzyme Mono 10.000 BG are readily soluble in water at all concentrations occurring in normal usage. However, water solutions will be turbid due to the wheat flour used for standardization of the enzyme.

Food-grade status

Gluzyme Mono 10.000 BG complies with the recommended purity specifications for food-grade enzymes given by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

Other characteristics

Gluzyme Mono 10.000 BG is stable in the pH range 3.5-7.0 and up to at least 50°C (122°F); it can probably be used at up to at least 60°C (140°F).

Packaging

See the standard Packaging List for more packaging information.

Application

Gluzyme Mono 10.000 BG can be used to strengthen gluten in dough systems. The oxidation of glucose results in the formation of gluconic acid and hydrogen peroxide (1). The formed hydrogen peroxide is capable of oxidizing free sulphydryl groups in gluten protein, thereby forming disulphide linkages (2).

The catalase side activity does not affect the performance of glucose oxidase. The addition of Gluzyme Mono 10.000 BG results in stronger and more elastic dough with greater resistance to mechanical shock, better ovenspring and larger loaf volume. Gluzyme Mono 10.000 BG is active in the dough but will be inactivated during baking.

Reaction Parameters

Dosage

The recommended dosage of Gluzyme Mono 10.000 BG is within the range 0.25-5 g per 100 kg flour (i.e. 2-50 ppm, 0.1-2.2 g/cwt), corresponding to 25-500 GODU per kg flour. The optimum dosage of Gluzyme Mono 10.000 BG can vary depending on flour quality, formulation and process and should be determined through baking trials. In bread-baking procedures using overnight fermentation, overdosing or dosing at higher than the recommended levels may result in off-flavour. For application in non-yeast-raised dough systems, a higher dosage is recommended, e.g. 100-500 ppm.

Safety

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product has been developed to resist mechanical effects. However, excessive mechanical wear and tear or crushing may create dust.

All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes.

Handling precautions

Gluzyme Mono 10.000 BG can easily be mixed with flour or starch. Preparing a 1:10 pre-mix can facilitate its use.

A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information on how to handle the product safely.

Storage

Recommended storage conditions are 0-10°C (32-50°F) in unbroken packaging, dry and protected from the sun. The product has been formulated for optimum stability. However, enzymes gradually lose activity over time. Extended storage and/or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.
When stored under the recommended conditions, the product is best used within 6 months.

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