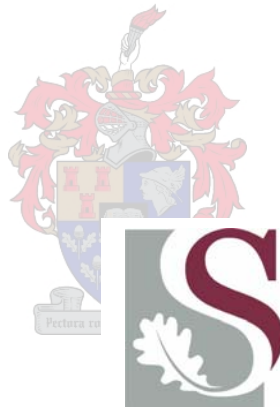# The effect of exogenous protease on the relative enzyme activity of β-glucosidase in oenological conditions.

by

Elsa Marita Swart

Thesis presented in partial fulfilment of the requirements for the degree of Master of Agricultural en Forestry Sciences at Stellenbosch University.
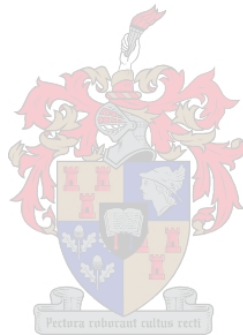
December 2004

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DECLARATION

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

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Name of candidate   **Date**

SUMMARY

The distinctive varietal flavour of wines is a combination of absolute and relative concentrations of chemical compounds. Volatile compounds are responsible for the odour of wine and non-volatiles cause the sensation of flavour. Accompanying these senses, a third, tactile, sense of 'mouth-feel' is recognizable. This forms the complete organoleptic quality of wine.

Several hundred different compounds are simultaneously responsible for the odour release in wine, and since there is no real character impact compound, the aroma of wine can be described as a delicate balance of all these compounds. One of the most important groups of volatiles is the monoterpenes, which play a role in both aroma and flavour. This is especially significant for the Muscat varieties, but these flavour compounds are also present in other non-muscat grape varieties, where they supplement the varietal aroma. Monoterpenes occur in wine as free, volatile and odorous molecules, as well as flavourless non-volatile glycosidic complexes. The latter slowly releases monoterpenes by acidic hydrolysis, but the impact on varietal aroma is considered insufficient for wines that are consumed young. It is therefore important to supplement the release mechanism, in order to enhance the varietal aroma of the wine. The enzymatic hydrolysis mechanism functions in two successive steps: firstly, depending on the precursor, the glycosidic linkage is cleaved by α-L-arabinofuranosidase, α-L-rhamnosidase, β-D-xylosidase or β-D-apiosidase. The second step involves the liberation of the monoterpene alcohol by a β-glucosidase. This enzymatic hydrolysis does not influence the intrinsic aromatic characteristics of the wine, as opposed to acid hydrolysis.

Pectolytic enzymes play an important role in cell elongation, softening of tissue and decomposition of plant material. These enzymes are used to improve juice yields, release colour and flavour compounds from grape skins, as well as improve clarification and filterability. Pectolytic enzymes work synergistically to break down pectins in wine. Protopectinase produce water-soluble and highly polymerised pectin substances from protopectin, it acts on non-methylated galacturonic acid units. Pectin methylesterase split methyl ester groups from the polygalacturonic chain. Polygalacturonase break down the glycosidic links between galacturonic acid units. Pectin and pectate lyases have a β-eliminative attack on the chain and it results in the formation of a double bond between C4 and C5 in the terminal residues.

From the above it can be seen that enzymes play a pivotal role in the winemaking process. Unfortunately, in winemaking a lot of factors can influence the effects of enzymes. One possible factor in the wine medium is the presence of acid-protease, from yeast and/or fungal origin. This type of enzyme utilizes other enzymes as substrates and renders them useless. Pure enzyme preparations were used to study the interactions of a yeast acid-protease and a report activity (β-glucosidase) *in vitro*. A bottled wine and a buffer were used as *in vitro* conditions. Enzyme assays

were performed to determine the relative activity over a number of days. The results indicated that even though both enzymes showed activity in both the media, the yeast protease did not have any significantly affect on the report activity. Subsequently wine was made from Sauvignon blanc grapes, with varying enzyme preparation additions. Enzyme assays were performed during the fermentation; and chemical, as well as sensory analysis were done on the stabilized wine. The results confirmed that the yeast protease did not have any significant affect on the report activity in these conditions. The protease's inability to affect the report activity seems unlikely due to the fact that it is active at a low pH range and has been suggested as the only protease to survive the fermentation process. It seems possible that a wine-related factor, possibly ethanol, is responsible. Thus it seems that yeast protease does not threaten the use of commercial enzymes in the winemaking process in any significant way.

Future work would entail more detailed enzyme studies of interactions between protease, both from yeast and fungal origin, and other report activities in specified conditions. The degradation capability could be directed towards unwanted enzyme activities that cause oxidation and browning of the must. The characterization of interactions between protease and β-glucosidase activities may hold key to producing wines with enhanced aroma and colour potential, as well as the elimination of unwanted enzyme activities.

OPSOMMING

Die herkenbare kultivar karakter van wyn is 'n kombinasie van absolute en relatiewe konsentrasies van verskeie chemiese komponente. Vlugtige komponente is verantwoordelik vir die geur, of aroma, van wyn en die nie-vlugtige komponente veroorsaak die sensasie van smaak. 'n Derde, fisiese sensasie, die 'mondgevoel', is ook herkenbaar. Dit vorm die omvattende organoleptiese kwaliteit van die wyn.

'n Paar honderd verskillende komponente is gelyktydig verantwoordelik vir die aroma vrystelling in wyn en omdat daar geen werklike karakter 'impak' komponent is nie, kan die aroma van wyn beskryf word as 'n delikate balans van al die betrokke komponente. Een van die mees belangrike groepe vlugtige komponente is die monoterpene wat 'n rol speel in beide aroma en smaak. Dit is veral belangrik by Muskaat kultivars, maar hierdie aroma komponente is ook teenwoordig in nie-muskaat druif kultivars, waar hulle bydra tot die kultivar karakter en aroma. Monoterpene kom in wyn voor as vry, vlugtige en aromatiese molekules en in geurlose, nie-vlugtige glikosidies-gebonde komplekse. Die gebonde vorm word stadig vrygestel deur 'n suurhidrolise, maar dit word as onvoldoende beskou vir wyne wat vroeg gedrink word. Dit is dus belangrik dat die vrystelling van geurstowwe verhoog word om die kultivar karakter van die wyn te versterk. Die ensiematiese hidrolise proses behels twee opeenvolgende stappe: eerstens, afhangende van die aard van die voorloper, word die glikosidiese verbinding deur α-L-arabinofuranosidase, α-L-ramnosidase, β-D-xilosidase, of β-D-apiosidase gebreek. In die tweede stap word die monoterpeen-alkohol deur β-glukosidase vrygestel. Hierdie ensiematiese afbraak proses verander nie die intrinsieke aromatiese kenmerke van die wyn, soos met suurhidrolise die geval is nie.

Pektolitiese ensieme speel 'n fundamentele rol in selverlenging, sagwording en afbraak van plant materiaal. Hierdie ensieme word gebruik om sap opbrengs te verhoog, aroma en smaak komponente vry te stel uit die doppe, asook om sapverheldering en filtrasie te verbeter. Die pektolitiese ensieme werk op 'n sinergistiese wyse om pektien in wyn af te breek. Protopektinase produseer water-oplosbare en hoogs gepolimeriseerde pektien uit protopektien, slegs uit nie-gemetileerde galakturoonsuur eenhede. Pektien metielesterase verwyder metiel-ester groepe van die poligalakturoonsuurketting. Die glikosidiese bindings tussen galakturoonsuur eenhede word deur poligalakturonase afgebreek. Pektien- en pektaat-liase het 'n β-eliminasie aanslag op die ketting en as gevolg daarvan word dubbelbindings tussen C4 en C5 in die terminale residue gevorm.

Vanuit bogenoemde is dit dus duidelik dat ensieme 'n kardinale rol speel in die wynbereidingsproses. Ongelukkig is daar 'n verskeidenhied van faktore wat die werking van ensieme in die wynbereidingsproses kan beïnvloed. Een moontlike faktor is die teenwoordigheid van 'n suur-protease, van fungisidiese en/of gis oorsprong, in die wynmedium, omdat dit ander ensieme as substraat kan benut en

degradeer. Suiwer ensiem preparate is gebruik om die ensiem interaksie tussen 'n gis suur-protease en 'n verslag aktiwiteit (β-glukosidase) *in vitro* te ondersoek. 'n Gebotteleerde wyn en 'n buffer is gebruik om die *in vitro* kondisies na te boots. Relatiewe ensiem aktiwiteit is ontleed oor 'n aantal dae. Beide die ensieme het aktiwiteit getoon in die media, maar gis protease het geen statisties beduidende invloed gehad op die aktiwiteit van die verslag ensiem nie. Daaropvolgend is wyn berei van Sauvignon blanc druiwe, met verskillende ensiempreparaat toevoegings. Die ensiemaktiwiteit is deurlopend tydens fermentasie gemeet. Na afloop van stabilisasie is chemiese, sowel as sensoriese ontledings op die wyn gedoen. Die resultate het bevestig dat gis protease, onder hierdie kondisies, geen beduidende invloed op die verslag aktiwiteit gehad het nie. Die protease se onvermoë om die verslag aktiwiteit beduidend te beinvloed blyk onwaarskynlik aangesien die suur-protease aktief is by lae pH vlakke en dit as die enigste protease voorgestel is wat die fermentasie proses kan oorleef. Dit blyk asof 'n wyn-verwante faktor, moontlik etanol, hiervoor verantwoordelik kan wees. Dus hou protease geen gevaar in vir die gebruik van kommersiële ensieme in wynbereiding nie.

Navorsing kan in die toekoms fokus op meer gedetailleerde ensiem interaksie studies tussen protease en ander ensiem aktiwiteite, in gespesifiseerde kondisies. Die degradasie kapasiteit kan moontlik aangewend word om ongewenste ensiem aktiwiteite, wat byvoorbeeld oksidasie en verbruining veroorsaak, te verminder. Die karakterisering van die interaksies tussen protease en β-glukosidase kan dus die sleutel wees tot die produksie van wyne met verhoogde aroma potensiaal, asook die eliminasie van ongewenste ensiematiese aktiwiteite.

This thesis is dedicated to my family and friends.
Hierdie tesis word opgedra aan my familie en vriende.
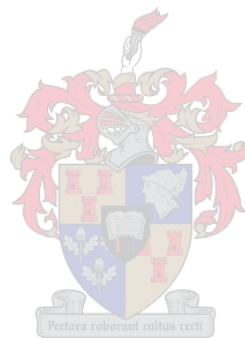
BIOGRAPHICAL SKETCH

Elsa M. Swart was born in Malmesbury in the Western Cape province of South Africa. She completed her schooling at Bellville High School in 1995. After a finishing year in Northern Switzerland, she returned to South Africa to begin her tertiary studies at the Department of Oenology and Viticulture, University of Stellenbosch. Elmari Swart obtained a B. Sc. Oenology and Viticulture degree in 2000. She free-lanced as winemaker in Barossa Valley, Australia. In 2001, she enrolled for a M. Sc. degree in Oenology at the University of Stellenbosch.

Elmari lives in Cape Town with her partner in life.
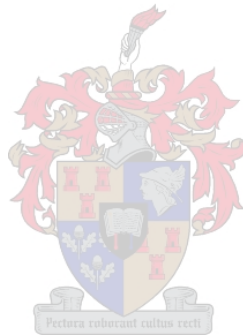
ACKNOWLEDGEMENTS

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

DR. PIERRE VAN RENSBURG, Department of Oenology, Stellenbosch University, who acted as my supervisor and accepted me as one of his students; for his guidance, enthusiasm and his devotion throughout this project;

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MY FAMILY AND FRIENDS, for their love and support;

MY PARTNER IN LIFE, without you I could never do this;
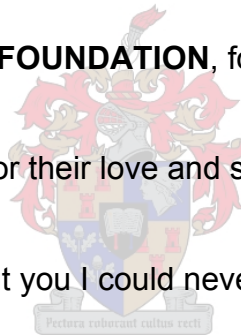
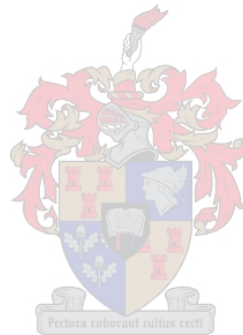**THE ALMIGHTY**, for this opportunity.

PREFACE

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of The South African Journal of Enology and Viticulture to which Chapter 3 will be submitted for publication.

Chapter 1   **General Introduction and Project Aims**

Chapter 2   **Literature Review**
Biocatalysts and Wine - A Review

Chapter 3   **Research Results**
The Effect of Exogenous Protease on the Relative Enzyme Activity of β-glucosidase in Oenological Conditions.

Chapter 4   **General Discussion and Conclusions**

CONTENTS

GENERAL INTRODUCTION

AND

PROJECT AIMS

CHAPTER 1: GENERAL INTRODUCTION AND PROJECT AIMS

1.1 ENZYMES IN WINE PRODUCTION

Until the early 17th century, wine was considered to be the only wholesome, readily storable product, and this accounted for the rapid global improvement in wine fermentation technology. Today, wine is consumed as a first choice lifestyle product of moderation. It has become synonymous with culture and style; and plays a major role in the economies of many nations. Annually about 26 billion litres of wine are produced from about 8 million hectares of vineyards across the world (Cape Wine Academy, 2001). There is, however, a decline in consumption and a steady rise in production. This has lead to a current worldwide oversupply of 15-20% (Cape Wine Academy, 2001) which creates fierce competition in the market place. Another determining factor is the shift in consumer preference from basic commodity wine to premium and ultra-premium wines (Pretorius, 2000). These are driving forces for the transformation of the wine industry from a production-orientated industry to a market-driven industry (Cape Wine Academy, 2001). It has resulted in increased diversity and innovation, much to the benefit of the consumer. Wine quality is defined as "sustainable customer and consumer satisfaction" and for this reason there is an urgent demand for further improvements of wine quality, purity, uniqueness and diversity (Pretorius, 2000). Fundamental innovations in various aspects of the winemaking process are revolutionizing the wine industry, while the market pull and technology push continue to challenge the tension between tradition and innovation. Now there are new, and for the moment controversial, ways of innovation – genetic engineering (Stidwell *et al.*, 2001), protein engineering (Van den Burg & Eijsink, 2002) and the use of enzyme kinetics (Van Rensburg & Pretorius, 2000). This study will focus on the latter.

Enzyme kinetics is of great importance during grape maturation (Rapp & Mandery, 1986) when the potential aroma profile of the "wine" is established, and is sensitive to damage. During the fermentation stage enzymes are of importance in the following areas: partial release of potential flavour and aromas, enhancement of colour and clarification. Enzymes also play an important role in the natural stabilization of the wine before bottling.

It has become apparent that the release of monoterpene alcohols from their glycones could increase the aroma of wine to a great extent (Ribéreau-Gayon *et al.*, 1975; Marais, 1983; Rapp & Mandery, 1986); therefore this subject has become a focal point on wine-related research. Pectolytic enzymes are used to improve juice yields, release of colour and flavour compounds from the grape skins, as well as improve clarification and filterability (Blanco *et al.*, 1994; Gainvors *et al.*, 1994; Kotoujansky, 1987). Cellulases consisting of endoglucanases, exoglucanases and cellobiases act in a synergistic manner to increase clarification and prevent cloudiness in wine (Eriksson & Wood, 1985). It is used in conjunction with pectolytic enzymes to improve filterability and stabilization of wine against haziness (Van Rensburg & Pretorius, 2000) and other visual problems caused by *Botrytis cinerea* infections (Verhoeff & Warren, 1972). The development of protein haze in white wine is considered the next most common physical instability after the precipitation of potassium bitartrate and enzymes could possibly be used in future to address this problem.

There is however, an enzyme that could destroy all the possible benefits of other enzymes in wine. Yeast and fungal acid protease uses other enzymes (protein-based) as substrates and renders them useless (Aschteter & Wolf, 1985; Babayan & Bezrukov, 1985; Behalova & Beran, 1979). This will limit the efficiency of any enzyme application in the wine making process, as well as having a major economic impact on the production costs. Therefore it is of great importance that this enzyme's kinetics are well documented and understood, in order to limit its possible devastating effects and possibly apply it to reduce haze formation and protein instability in wine.

1.2   ENZYMES IN INDUSTRIAL PROCESSES

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. Processes that incorporate high temperatures and/or high pressures to drive the reaction, may lead to high energy costs and require large volumes of cooling water downstream (Anonymous, 2000). Harsh and hazardous processes involving high temperatures, pressures, acidity or alkalinity need high capital investment, specially designed equipment and control systems; and the process may result in poor yields. There

may be production of unwanted or harmful by-products that are costly to dispose of and may have a negative impact on the environment (Anonymous, 2000).

These drawbacks can be virtually eliminated by using enzymes. Enzyme reactions are carried out under mild conditions and they are highly specific (Van Rensburg & Pretorius, 2000). Their working involves very fast reaction rates and is carried out by numerous enzymes with different roles (Underkofler, 1976). As industrial enzymes originate from biological systems, they contribute to sustainable development through being isolated from micro-organisms, in fermentations using primarily renewable sources (Anonymous, 1999). In addition only small amounts of the specific enzyme is required to carry out chemical reactions even on industrial scale (Pretorius, 1999). These preparations are available in both liquid and solid form and take up very little storing space. Developments in genetic and protein engineering have led to improvements in both stability and overall application of industrial enzymes.

While the reactions catalysed by a single enzyme are relatively few, their numbers are high. This is due to their most important characteristic: specificity, which is the capacity of acting on one substance only or on a limited group of substances. There are various types and degrees of specificity:

Chemical groups' specificity: the enzyme breaks down only a specific chemical group or link; in turn such specificity can be absolute or relative. In the first instance a small modification of the molecule is sufficient to inactivate the enzyme; in the second one several similar substances can be acted upon (Van Rensburg & Pretorius, 2000).

Specificity of substrate: the enzyme act on certain compounds and not on others which are also susceptible to undergo the same reaction (Van Rensburg & Pretorius, 2000).

Enzymes have applications in both the food and non-food industries. The non-food applications include textile finishing for silk, cotton, denim and wool; leather preparation; processing of pulp and paper, animal feed, oil and gas drilling, biopolymers and fuel alcohol. Table 1 presents a selection of enzymes currently used in industrial processes listed accordingly to class.

TABLE 1:   Typical enzymes used in industrial processes.

Class	Industrial enzymes
Oxidoreductases	Peroxidases Catalases Glucose oxidases Laccases
Transferases	Fructosyl-transferases Glucosyl-transferases
Hydrolases	Amylases Cellulases Lipases Pectinases Proteases Pullulanases
Lyases	Pectate lyases Alpha-acetolactate decarboxylases
Isomerases	Glucose isomerases

Applications in the food industry are common. Enzymes are used for sweetener production, sugar processing, baking, dairy product preparation, brewing, winemaking, distilling, protein hydrolysis for food processing and extractions from plant material. Hydrolases are used in the industry, especially in the manufacturing of food products. Here again, the possible advantages can be nullified if an acid protease was to destroy the required enzyme activity.

1.3 AIMS OF STUDY

Saccharomyces cerevisiae is credited as the "wine yeast", but it is in actual fact the enzymes, from whichever origin, that are responsible for the conversion of grape juice to the incredibly complex liquid called wine. These enzymes have many different functions within this biotransformation, and if correctly exploited, can be of an even greater influence in the winemaking process. It can enhance the natural flavour and aroma that are locked up in the non-volatile state, as well as reduce instabilities in the wine. Health benefits can also be increased through an enzymatic application. The primary aim of this study was to determine the interactions between a *S. cerevisiae* (yeast) acid protease and *Aspergillus* sp. β-glucosidase in wine-related conditions. Characterizing and quantifying their interactions as expressed in two different *in vitro* conditions and during fermentation.

We aimed to establish the nature and scale of any affects the protease might have on the report activity, by quantifying whether any significant increase/ decrease or synergy would occur in the relative enzyme activities. Also we aimed to establish how long the protease enzyme would show activity during fermentation conditions.
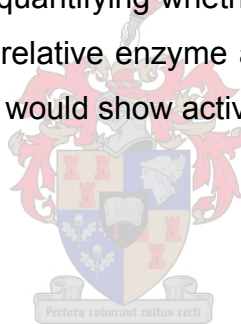
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LITERATURE REVIEW

BIOCATALYSTS AND WINE – A REVIEW

CHAPTER 2: BIOCATALYSTS AND WINE – A REVIEW

2.1 PLANT CELL WALL AND GRAPE BERRY POLYSACCHARIDES

The composition and structure of the grape berry cell walls are of interest because of their importance in wine production technology. Each berry consists of a thin, elastic epicarp (the skin), a juicy and fleshy mesocarp (the pulp) and an endocarp, which is indistinguishable from the pulp that surrounds the carpels containing the seeds (Jackson, 1994; Peynaud & Ribéreau-Gayon, 1971). The plant cell wall is the source of most of the polysaccharides found in wine. Although the flesh of the grape berry contributes greatly to the volume of juice yield, extraction is primarily from the berry skin. The alcohol-insoluble residues obtained from grape berry pulp consist of predominantly cellulose, hemicellulose, xyloglucan and the pectic polysaccharides homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Saulnier & Thibault, 1987; Nunan *et al.,* 1997).

The hemicellulose and pectin polysaccharides, as well as the aromatic compound lignin, interact with the cellulose fibrils, creating a rigid structure strengthening the plant cell wall (De Vries & Visser, 2001). They also form covalent cross-links, which are thought to be involved in limiting the cell growth and reducing cell wall biodegradability (De Vries & Visser, 2001). Two types of covalent cross-links have been identified between plant cell wall polysaccharides and lignin (Fry, 1986). The first is a linked formed by diferulic acid bridges, which occur between arabinoxylans, between pectin polymers and between lignin and xylan (Ishii, 1991; Bach Tuyet Lam *et al.*, 1992; Oosterveld *et al.*, 1997). The second type of cross link is formed between lignin and glucuronic acid attached to xylan (Imamura *et al.*, 1994). Recently indications of a third type of cross-linking have been reported involving a protein- and pH-dependant binding of pectin and glucaronoarabinoxylan to xyloglucan (Rizk *et al.*, 2000). These polysaccharides all contribute to some extent to the composition and physical characteristics of the juice and thus have an influence on the final product (wine). Their structure and degradation is therefore of great importance to the winemaker.

2.1.1   Structural Features of Pectins

Pectic substances are structural heteropolysaccharides and are the main constituents of middle lamella and primary cell walls of higher plants (Whitaker,1990). Pectin is responsible for lubricating or cementing cell walls, thus insuring integrity and coherence of plant tissue (Rombouts & Pilnik, 1978). They are also involved in plant host and pathogen interactions (Collmer & Keen, 1986).

Pectic substances are divided into four main groups (American Chemical Society; Kertesz, 1987): protopectins, pectinic acids, pectins and pectic acids. Protopectin is considered the parent compound and is water-insoluble. The other three are totally or partially soluble in water. The reasons for insolubility is diverse and includes binding of polyvalents ions, secondary valency bonding between pectin and cellulose, salt-bridging carboxyl-groups of pectin and other cell wall constituents (Sakai, 1992).

Pectic substances consist mainly of α-D-1,4-galacturonic acid molecules (pectate) or its methylated ester (pectin) (Pretorius, 1997) as is illustrated in Figure 1. In pectin more than 75% of the carboxyl-groups are methylated and free carboxyl-groups occur in clusters along the chain. The primary chain consists of a "smooth" region (Figure 6) of α-1,4-D-galactoronic acid units and are β-1,2 and β-1,4-linked to rhamnose units with side chains. This gives the chain a "hairy" character. These "hairy" regions, as identified by Schols *et al*. (1996) consist of three different sub units. Subunit I is a xylogalacturonan (xga) (a galacturonan backbone substituted with xylose), subunit II is a short section of a rhamnogalacturonan backbone that has many long arabin, galactan, and/or arabinogalactan side chains, and subunit III is a rhamnogalacturonan composed of alternating rhamnose and galacturonic acid residues. It is suspected that subunit III connects the other two subunits. The basic linear chain is composed of the same repeating building unit; partially methylated α-D-1,4-linked galactopyranosiduronic acid residues (Chesson, 1980). The fact that there are usually few rhamnose residues present means that long chains of galacturonan are linked together by rhamnose-rich blocks (Pilnik & Voragen, 1970). The galacturonosyl residues can be esterified with methanol and/or O-acetylated at C2 or C3 (McCready & McComb, 1954).

In rhamnogalacturonan I, the D-galacturonic acid residues in the backbone is interrupted by α-1,2-linked L-rhamnose residues, to which long arabinan and galactan chains can be attached at O4. The arabinan chain consist of a main chain of

α-1,5-linked L-arabinose residues that can be substituted by α-1,3-linked L-arabinose residues and by feruloyl residues, attached terminally to O2 of the arabinose residues (Colquhoun *et al.,* 1994; Guillon & Thibault, 1989). The galactan side chain contains a main chain of β-1,4-linked D-galactose residues, which can be substituted by feruloyl residues at O6 (Colquhoun *et al.,* 1994; Guillon & Thibault, 1989). Rhamnogalacturonan I also contains acetyl groups ester-linked to O2 or O3 galacturonic acid residues of the backbone (Scholz & Voragen, 1996; 1994). Rhamnogalacturonan II is a polysaccharide of approximately 30 monosaccharide units with a backbone of galacturonic acid residues that is substituted by four side chains. The structure of these side chains have been shown to contain several common sugars (Mazeau & Perez, 1998).

Vidal *et al.* (2001) determined that there is three-fold more rhamnogalacturonan I and II in the skin tissue than in the pulp tissue. These results are consistent with the fact that more grape polysaccharides are present in red wines than in white wines. Rhamnogalacturonan II is also a prominent polysaccharide in juices that are obtained by enzymatic liquefaction of fruits and vegetables (Doco *et al.*, 1997). Arabinogalactan proteins are a quantitively major grape polysaccharide in wines. They are released as soon as the berry is crushed and pressed (Vidal *et al.*, 2000). Rhamnogalacturonan I is a quantitively minor component in wine, even though its concentration in the cell wall is three fold higher than that of rhamnogalacturonan II. Homogalacturonan, which accounts for 80% of the pectic substances in grape berry cell walls, has been detected at the initial stage of berry processing and its concentration has been estimated at < 100 mg/L in the must (Vidal *et al.*, 2000).
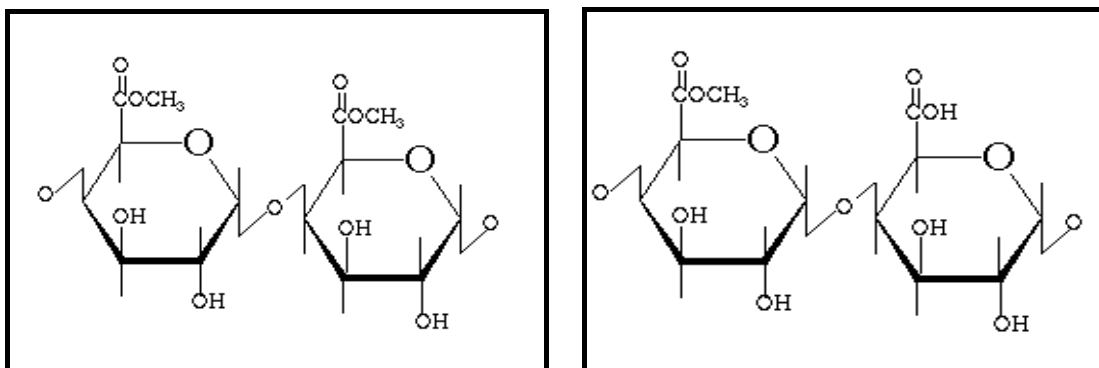
FIGURE 1: Examples of pectin models (Internet http://class.fst.ohio-state.edu/fst621/Gums/Imag16.gif , 2003).

Pectins are generally soluble in water, where they form viscous solutions, depending on the molecular weight and degree of esterification, pH and electrolyte concentration (Deuel & Stutz, 1958). Grape pectins together with other polysaccharides such as cellulose and hemicellulose greatly influence the clarification and stabilization of must and wine. They are responsible for turbidity, viscosity and filter blockages and are present at levels of 300 to 1000 mg/L (Van Rensburg & Pretorius, 2000).

2.1.2 Structural Features of Cellulose

Cellulose is the major polysaccharide in woody and fibrous plants and therefore is the most abundant polymer in the biosphere (Mathew & Van Holde, 1990). It constitutes 40-50% of cell wall substances and this percentage is relatively constant between species (Coughlan, 1990).

Cellulose is a polyalcohol of D-anhydroglucopyranose units linked by β-1,4-glucosidic bonds (Lamed & Bayer, 1988). It consists of a linear polymer of glucose units, with each glucose unit rotated 180° with respect to it neighbour along the main axis of the chain (Coughlan, 1990). The size of a cellulose molecule can be given as a number of repeating units or the degree of polymerization (Figure 2).
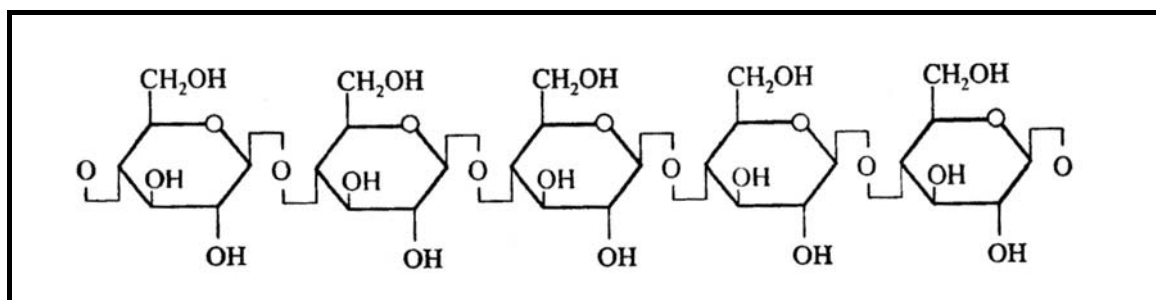
FIGURE 2: The primary structure of cellulose (Cowling & Kirk, 1976).

The degree of polymerization ranges from 30 to 15 000 units (Coughlan, 1990). The chains associate through interchain hydrogen bonds and van der Waals interactions to form microfibrils that aggregate to form insoluble fibers (Pretorius, 1997). There are areas of order, i.e. crystalline areas, and also less-ordered, or amorphous, areas within the cellulose fibers. Bohinski (1987) found that the basis of the water

insolubility is the high hydrogen bonding capacity between individual chains, which gives a degree of strength.

Two major types of xyloglucans have been identified in the plant cell wall. According to De Vries & Visser (2001), xyloglucan type XXXG consists of repeating units of three β-1,4-linked D-glucopyranose residues, substituted with D-xylopyranose via an α-1,6-linkage, which are separated by an unsubstituted glucose residue. In xyloglucan type XXGG, two xylose-substituted glucose residues are se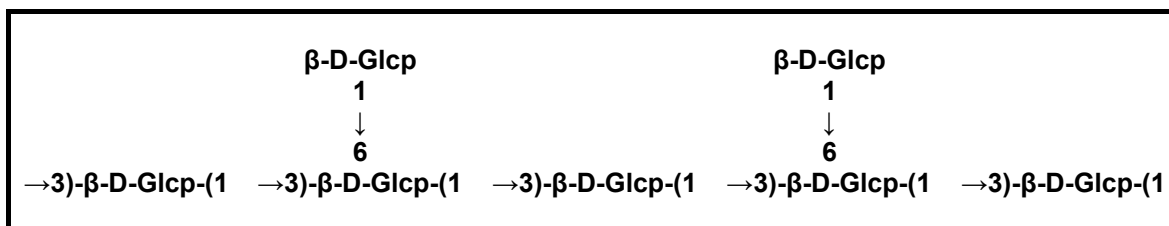parated by two unsubstituted glucose residues. According to Hantus *et al.* (1997) and Vincken *et al.* (1997), the xylose in xyloglucan can be substituted with α-1,2-L-fructopyranose-β-1,2-D-galactopyranose and α-1,2-L-galactopyranose-β-1,2-D-galactopyranose disaccharides. L-Arabinofuranose has been detected α-1,2-linked to main-chain glucose residues or xylose side groups (Hisamatsu *et al.*, 1992; Huisman *et al.*, 2000). In addition, the xyloglucans can contain O-linked acetyl groups (Ring & Selvendran, 1981; York *et al.*, 1996). The xyloglucans are strongly associated with cellulose and thus add to the structural integrity of the cell wall.

Glucans are a major cell wall component in most yeast, according to Duffus *et al.* (1982), forming more than 50% of the cell wall. These can be divided into two groups. The first and major group have a linear chain of D-glucose units with β-1,3-links containing β-1,6-branchings (Fleet & Phaff, 1981). The second group is a β-1,6-glucan with β-1,3-linked lateral chains. These glucans are released into the wine during fermentation and cell autolysis. They prevent natural sedimentation of cloud particles and cause filter blockages (Van Rensburg & Pretorius, 2000). Fining agents such as bentonite can remove the cloudiness, but filter problems remain. Alcohol induces polymerization of glucans and thus aggravates the problem towards the end of fermentation (Van Rensburg & Pretorius, 2000).

Botrytis cinerea is another cause of increased concentrations of glucans. Generally grape β-glucans consist of short areas of β-1,4-linked glucose moieties, interrupted by single β-1,3-linkages. In contrast the high molecular weight β-glucan produced by *B. cinerea* consists of a β-D-1,3-backbone with β-D-1,6-side chains (Dubourdieu *et al.*, 1981; Villettaz *et al.*, 1984), see Figure 3.

```
                    β-D-Glcp                              β-D-Glcp
                        1                                     1
                        ↓                                     ↓
                        6                                     6
→3)-β-D-Glcp-(1    →3)-β-D-Glcp-(1    →3)-β-D-Glcp-(1    →3)-β-D-Glcp-(1    →3)-β-D-Glcp-(1
```

FIGURE 3: The structure of the β-glucan of *Botrytis cinerea* (Dubourdieu *et al.*, 1981).

2.1.3 Structural Features of Hemicellulose

After cellulose, hemicellulose is the most abundant renewable polysaccharide in nature. It is mostly found in plant cell walls. It can be classified according to chemical composition and structure and therefore has been divided into four main groups: xylans, which are the major group, mannans, galactans and arabinans (Puls & Schuseil, 1993). Hemicellulose may be linear or branched and have a degree of polymerization (DP) of up to 200 units. The monomers are linked by β-1,4-glycosidic bonds. The exception to this rule is D-galactopyranose residues, which are β-1,3-linked. The predominant hemicellulose, β-1,4-xylan, has a high degree of polymerisation and is highly branched (Thomson, 1993). β-1,4-linked D-xylopyranosyl residues carry acetyl, arabinosyl and glucanosyl as most common substituents.

There are two types of hemicellulose, namely homopolysaccharides and heteropolysaccharides. Most hemicellulose in nature occurs as heteroglucans. The heteroglucans in hardwood can contain two or more of the following: D-galactose, D-glucose, D-glucuronic acid, 4-O-methylglucuronic acid, D-mannose, D-xylose, L-arabinose and D-galacturonic acid (Coughlan *et al.*, 1993). In grasses hemicellulose is comprised of D-xylose, L-arabinose, D-glucose and D-galactose. This causes a great extent of different structures to be possible. Therefore unique combinations of hemicellulolytic enzymes are needed for effective and total degradation (Puls & Schusiel, 1993).
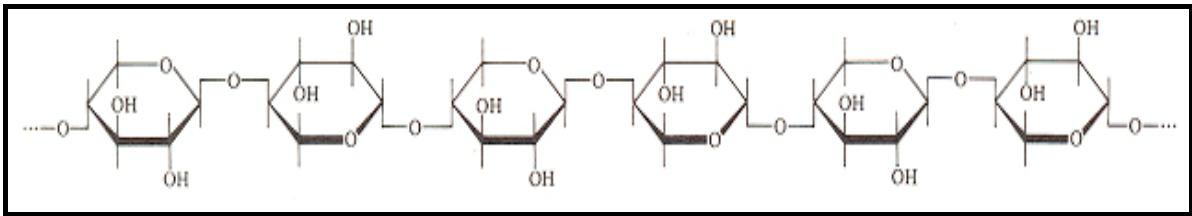
FIGURE 4: Example of a typical xylan model (Macgregor & Greenwood, 1980).

Xylan is composed of β-1,4-linked xylose units, see Figure 4, forming a xylan backbone with side chains connected to the backbone (Christov & Prior, 1993). In hardwoods and grasses, the main chain contains an O-acetyl group at the C2 and/or C3 positions, whereas in softwoods and annual plants it can be substituted with arabinose at the C3 position.

According to De Vries & Visser (2001), the arabinose can be connected to the main chain via α-1,2- or α-1,3-linkages either as single residues or as short side chains. The side chain can also contain xylose β-1,2-linked to arabinose, and galactose, which can be either β-1,5-linked to arabinose or β-1,4-linked to xylose. Glucuronic acid and its 4-O-methyl ether are attached to the xylan backbone via an α-1,2-linkage, whereas aromatic residues (feruloyl and *p*-coumaroyl) residues have so far been found attached only to O5 of terminal arabinose residues (Saulnier *et al.*, 1995; Smith & Hartley, 1983; Wende & Fry, 1997). Also, xylan can be esterified with phenolic acids. The phenolic acids facilitate intermolecular cross-linking between xylan and lignin in the cell wall matrix (Strauss *et al.*, 2003).

Galactomannans and galactoglucomannans form a second group of hemicellulolytic structures present in plant cell walls. These compounds are the major hemicellulose fraction in gymnosperms (Aspinall, 1980). It consist of a backbone of β-1,4-linked D-mannose residues, which can be substituted by D-galactose residues via a β-1,6-linkage. Two different structures can be identified within this group of polysaccharides (Timell, 1967). Both consist of a β-1,4-linked D-mannose backbone, which can be substituted by α-1,6-linked D-galactose. The galactoglucomannan backbone also contain β-1,4-linked D-glucose residues. Water-soluble galactoglucomannan has higher galactose content than water-insoluble galactoglucomannan, and in addition contains acetyl residues attached to the main chain (Timell, 1967).

2.2   AROMATIC RESIDUES IN PLANT CELL WALL POLYSACCHARIDES

Wine flavour is a very complex interaction of chemical compounds that are collectively responsible for specific and general wine aroma. Each contributes to a smaller or larger extent to the final organoleptic whole, as perceived by the consumer. The chemical composition of the wine can be roughly divided into two groups, volatile and non-volatile (Stidwell *et al.*, 2001). The sensation of smell can be attributed to the volatile compounds, whereas the non-volatiles are responsible for the perception of taste or flavour.

The basic taste sensations that are perceived are sourness, bitterness, sweetness and saltiness. The sugars, organic acids, polymeric phenols and minerals in the wine are responsible for these tastes (Stidwell *et al.*, 2001). These compounds posses different organoleptic thresholds, but generally have to be present at levels of 1% (in total) or more to have an influence on the flavour of the wine (Rapp & Mandery, 1986). One of the key differences between the two groups is that volatiles can be perceived at much lower concentrations than the non-volatiles (Gaudigni *et al.*, 1963).

The organoleptic properties of the wine are divided according to their origin. The first grouping namely 'primary aroma' originates from the grape itself, including any and all changes that the grapes themselves experience. Secondary aroma or 'fermentation aroma' includes all stages of processing and fermentation (Stidwell *et al.*, 2001). The 'tertiary aroma' is derived from maturation and is described as the bouquet of the wine (Ribéreau-Gayon, 1978). This can be achieved in wooden casks and/or the bottle.

All of these influences play a role, to varying degrees, and directly influence the quality of the final product. It is important to begin the process of winemaking with a raw product of the highest quality, and then to maintain protective surroundings to produce a balanced final product.

All grapes posses a generic grape aroma that forms the basis of the varietal aroma. The cultivar then also incorporates a distinctive aroma that sets it apart from other varieties. The basic generic aroma of wines consist of a combination of these cultivar-related compounds, as well as fermentation products such as esters, aldehydes, ketones, alcohols, phenols, organic sulphurs, and acetates (Stidwell *et al.*, 2001). The absolute concentrations of the cultivar-related compounds vary between cultivars, but the relative distribution is similar for groups of cultivars, e.g.

Muscat varieties. The compounds that are responsible for distinctive aromas are often referred to as 'impact odorants'. None of these are solely responsible for an aroma; it is rather a collective effort of chemically closely related compounds (Marais, 1983). Here the absolute concentrations are important, as it will influence whether an aroma is just perceived or indeed recognized (Marais, 1983). But even more importantly is the influence of synergistic working, where compounds that are chemically related will enhance a certain aroma, without increased concentrations of the individual components (Ribéreau-Gayon *et al.*, 1975). Thus even though some chemical compounds have distinctive smells, they are not the only ones responsible for that smell in the wine.

The varietal flavour of grapes is mainly due to the profile of volatile secondary metabolites. There are a few compounds that are either present in such low concentrations that they cannot be detected in the grape must, or their water solubility is so poor it effectively prevents them from making an impact on the aroma of the must. These include aliphatic n-alkanes and some aromatic hydrocarbons like toluene, xilene and alkylbenzenes (Schreier *et al.*, 1976; Stevens *et al.*, 1957; 1967; 1969). These compounds precipitate with the must slurry during wine making, rendering them even more insignificant. Very few esters are present in the *Vitis vinifera* species. According to Rapp & Knipser (1980) they are mainly acetate esters of short chain alcohols and acetates of some monoterpene alcohols. (E)-methyl geranoate are found in Muscat grape varieties (Schreier *et al.*, 1976).

According to Rapp & Mandery (1986) aldehydes play a significant role in the aroma of wine, as enzymatic processes that form C6-aldehydes and alcohols take over at the moment of grape cell destruction. These compounds are quantitively dominant, so the aroma of the grape must will be highly dependant on which of these compounds were present.

Also present in the grape must is small fractions of ketones, with 2- and 3-n-alkanones occurring in the highest concentrations. n-Alcohols with a chain length of four to 11 carbons compromise the alcohol fraction (Schreier, 1979). In general these alcohols do not significantly contribute to the aroma impact imparted by the alcohol fraction in the final product, but according to Welch *et al.* (1982) they could play a role in the varietal aroma of Muscadine grapes. This can be due to the presence of isoamyl alcohol, hexanol, benzaldehyde and 2-phenylethanol and its derivatives.

Particularly the monoterpene alcohols are of great importance in the muscat-type cultivars as well as the non-muscat types (Marais, 1983; Rapp & Mandery,

1986). These terpenols can be either in the free and volatile state (odorous), or in the flavourless, non-volatile state bound in glycosidic complexes, see Table 2. Glycosylesters of monoterpenes have been observed by Mulkens (1987). β-D-glucose is the most common feature for glycosidically bounded volatiles (Williams, 1993). The most common terpenols are geraniol, nerol and linalool (Günata *et al.*, 1988). Ribéreau-Gayon *et al.* (1975) found that linalool and geraniol are the most aromatic within the terpene fraction. The other monoterpenes generally have a much higher perception threshold than that of linalool, which is quite low at 100 µg/L, as illustrated in Table 2.

TABLE 2: Properties of monoterpenoids-aroma and sensory threshold data in water (from Van Rensburg & Pretorius, 2000).

Compound	Aroma	Sensory threshold (µg/L)
Geraniol	Floral, rose-like, citrus	132
Citronellol	Sweet, rose-like, citrus	100
Linalool	Floral, fresh, coriander	100
Nerol	Floral, fresh, green	400
α-Terpineol	Lilac	460

The monoterpenes (Figure 5) do not only impart the muscat-like aromas, but range from spicy, smoky and peppery to grassy. It is possible to distinguish between different cultivars according to their unique terpene profiles (Stidwell *et al.*, 2001). It contributes largely to the aroma of Muscat cultivars, such as Muscat d'Alexandrie, Mario Muscat, and Muscat de Fontignan. It can also support the varietal aromas of other cultivars, such as Chardonnay, Cape Riesling and Sauvignon blanc (Rapp & Mandery, 1986). Sauvignon blanc can, however, attribute most of its distinctive aroma to the methoxypirazines that imparts the grassy and vegetative odours.

Oxides of these monoterpene alcohols are also common, especially those of linalool and nerol. Their perception thresholds are high (3000-5000 µg/L). However, although they would not be detected on their own, the perception threshold for the group is much lower than for a single component because of the synergistic effect of the constituents (Ribéreau-Gayon *et al.*, 1975).

**TABLE 3:** The fractions of the three most common terpenols as they occur in the grape berry (Marais, 1985).

	mg/100g berries			% of each alcohol		
	Linalool	Nerol	Geraniol	Linalool	Nerol	Geraniol
Skins	14,2	15,2	100	26%	95,6%	94,6%
Flesh	13,5	0,45	3,5	24%	2,7%	3,3%
Juice	27,5	0,30	2,5	50%	1,7%	2,5%

The monoterpene fraction is located mostly in the skin of the grape berry, and to a lesser degree in the juice, as is illustrated in Table 3. The distribution for different monoterpenes differ, with 95% of geraniol and nerol being concentrated in the skin of Muscat d'Alexandrie, whereas linalool is distributed almost equally between the juice, skin and cellular debris (Rapp & Mandery, 1986; Cordonnier & Bayonove, 1978; 1981).

The aroma profile does not change significantly during fermentation. This is due to the fact that the yeast cannot synthesize monoterpenes and are generally unable to break the glycosidic bounds. The most common changes that are observed are the conformation shifts and oxidation. Linalool can, however, undergo drastic changes during aging; it can be transformed to other terpenes, radically changing the relative amounts of individual compounds. Ribéreau-Gayon (1978) observed that it remains very difficult to predict the aroma changes during aging as it has been found that the perception threshold for monoterpenes in wine ranges from 100 µg/mL to 700 µg/mL. Also an increase in certain compounds may have little or no effect (e.g. linalool oxide) while others like α-terpineol may have a negative influence (Güntert, 1984; Rapp *et al.*, 1985c).

Ferulic acid can be linked to both hemicellulose (Smith & Hartley, 1983) and the pectin (Rombouts & Thibault, 1986) fractions of plant cell walls and is able to cross-link these polysaccharides to each other as well as to the aromatic polymeric compound lignin (Ishii, 1997; Lam *et al.*, 1994). This cross-linked structure results in an increased rigidity of the cell wall. It has been suggested that these cross-links may play a role in preventing biodegradability of the plant cell wall by micro organisms.

Additionally, the antimicrobial effects of these aromatic compounds (Aziz *et al.*, 1998) may contribute to the plant defence mechanism against phytopathogenic micro organisms (De Vries & Visser, 2001).
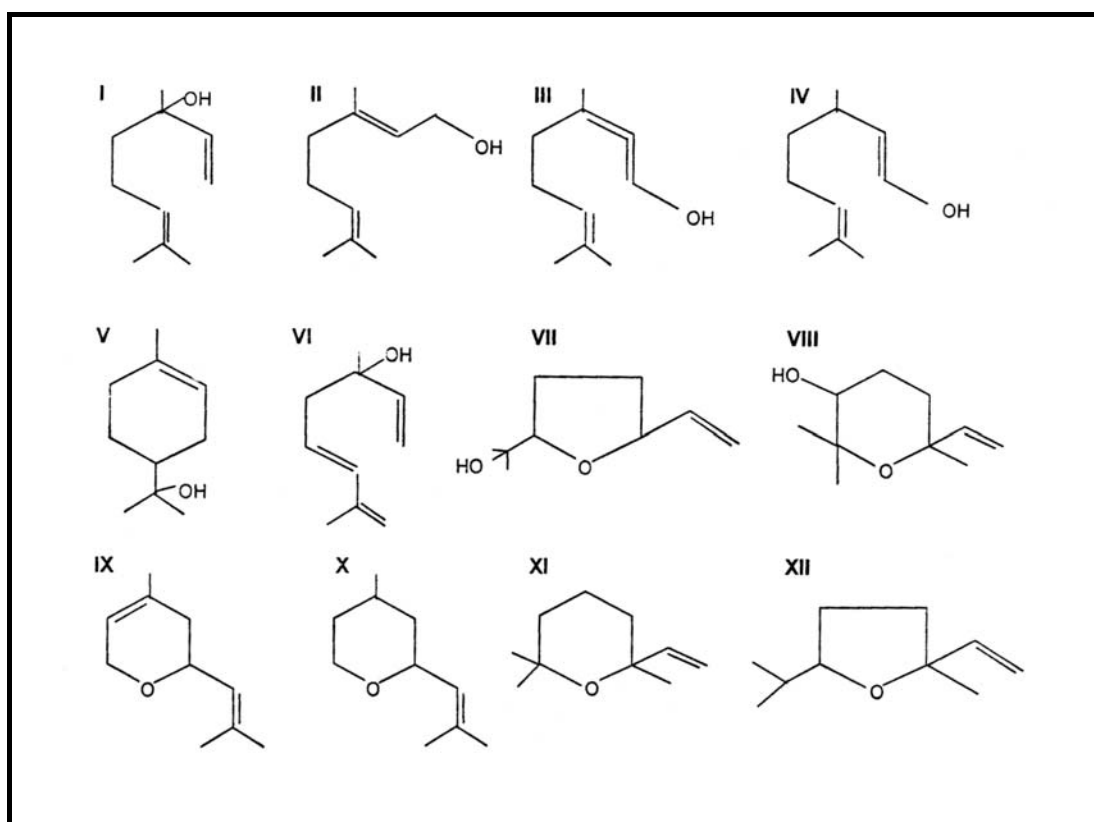
FIGURE 5: Volatile monoterpenes found in wine. I – linalool, II – geraniol, III – nerol, IV – citronellol, V – α-terpineol, VI – hotrienol, VII & VIII – linalool oxides, IX – nerol oxide, X – rose oxide, XI& XII – ethers (Rapp & Mandery, 1986).

2.3   THE AROMA OF WINE (FERMENTATION AND MATURATION)

The secondary aroma or bouquet of wine is derived by all processing of the grape matter, but mainly the fermentation process. Tertiary aroma is derived from ageing of the wine in wooden containers (barrels) and/or in the bottle.

According to Ribéreau-Gayon (1978) there can be distinguished between two types of bouquet: the bouquet of oxidation, due to the presence of aldehydes and acetyls, and the bouquet of reduction which is formed after bottle ageing. Fine red wines in particular benefit from storage in a wooden barrel. A large number of aromatic elements are extracted from the wood during storage (Puech, 1987; Singleton, 1987; Vivas & Glories, 1996), adding to the complexity of the wine without

diminishing the fruit aromas. The main compounds extracted from wooden barrels are flavonols and lactones (Puech, 1987; Singleton, 1987).

Ribéreau-Gayon (1971) has shown increases in the levels of volatile acidity and ethyl acetate occur in wines during the ageing in wood barrels. The major acid contributing to volatile acidity is acetic acid (Onishi *et al.*, 1977). Direct hydrolysis and extraction from wood contributes only a fraction, while alkaline and strong acid hydrolysis from hemicellulose is the major sources for the increase in acetic acid (Nishimura *et al.*, 1983).

Acetates are produced enzymatically in excess of their perception thresholds and contribute to the pleasant, fruity aroma of wine. According to Simpson (1978a) and Marais & Pool (1980) these are hydrolyzed during storage until their levels are similar to that of their corresponding acids and alcohols. In contrast to the decrease of acetate levels, the ethyl esters of diprotic acids show a constant increase caused by the chemical esterification during the course of ageing.

According to Rapp *et al.* (1985a; b) various chemical reactions occur during bottle maturation which plays an important role in the change of the aroma of the wine. These chemical reactions can be divided into four groups:

(i)   changes in concentrations of esters (increase of ethyl esters, decrease of acetates);
(ii)   formation of compounds from carbohydrate degradation;
(iii)   formation of compounds from carotinoid degradation;
(iv)   changes in terpeniod concentration.

Vitispirane (a compound resulting from carotinoid degradation) has a camphoraceous eucalyptus-like odour, increases during storage (Simpson, 1978b; Simpson *et al.*, 1977) and can result in an off-flavour. A decrease in the levels of acetate esters during bottle ageing severely depletes the wine's fruitiness. However, the levels of other compounds may increase, and not always with pleasant results. 1,1,6-trimethyldihydronaphtalene (TDN), a hydrocarbon arising from carotinoid degradation, causes a petrol-like character in older wines, especially in the Riesling cultivar (Stidwell *et al.*, 2001). Damascenone is another product of carotene degradation, but shows a decline in concentration during storage (Güntert, 1984). Furane derivatives are examples of carbohydrate degradation. Furfural and ethyl furoate are formed in young wines, and furfural amounts increase during wine storage (Rapp *et al.*, 1985c).

Some of the monoterpenes undergo drastic changes during wine maturation. Linalool is transformed to other terpene compounds, with the main reaction occurring via α-terpineol to 1,8-terpin, a compound which is only formed during wine ageing. The reactions that take place can be summarized as follows:

    (i)   a decrease in monoterpene alcohols, like linalool, geraniol and citronellol,

    (ii)   an increase in: linalool oxides, nerol oxide hotrienol, hydroxylinalool and hydroxycitronellol;

    (iii)   formation: 2,6,6-trimethyl-2-vinyl-tetrahydropyran, the anhydrolinalool oxides, 2,2-dimethyl-5-(1-methylpropyl)-tetrahydrofuran and cis-1,8-menthandiol (Hennig & Villforth, 1942; Buttery *et al.*, 1971).

Changes in the average concentration of terpenes have a dramatic effect on the aroma profile of the wine. For example, it is estimated that linalool decreases to 10% of its original amount after 10 years of storage. If it estimated that an un-aged wine has an average of 400 μg/L linalool, it is clear that within a few years the concentration would be well below the perception threshold for this compound (Güntert, 1984; Rapp *et al.*, 1985b). An increase in α-terpineol could have a negative effect on the maturing wine's aroma profile. This problem occurs mainly in white wines, as monoterpenes do not significantly contribute to the flavour of red wines.
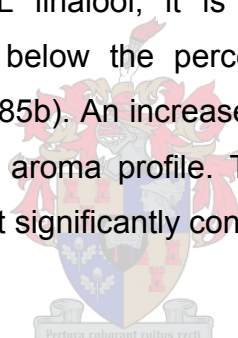
2.4   BREAKDOWN OF POLYSACCHARIDES BY BIOCATALYSTS

The reduction in the molecular weight of a polymer saccharide is called polysaccharide degradation. According to Gowariker *et al.* (1986) this can be induced by four different types of mechanisms: chemical (acid or alkali), physical (thermal), microbial and enzymatic degradation. All of these have oenological and ecological advantages and disadvantages. Hydrolysis by acids and alkali result in toxic by-products which are expensive to treat. Opposite to this mechanism, hydrolysis by naturally occurring microbial populations is inexpensive, extremely stable and does not cause pollution problems (Kubicek *et al.*, 1993; Pretorius, 1997).

Enzymes originate from a multitude of habitats. Grapes produce enzymes, as well as yeasts and other microbes (such as fungi and bacteria) associated with vineyards and cellar equipment (Van Rensburg & Pretorius, 2000). Certainly the most

noteworthy fact about enzymes is their specificity. They can act on only one or a limited number of substances, recognising a specific chemical group (Uhlig, 1998). Another advantage is the ability to carry out single-step or multi-step transformations of organic compounds that are not easily accomplished by conventional methods (Strauss *et al.,* 2003). This as well as their activity levels are poorly understood, but is still of greatest importance in the fermentation process, see Table 4.

Hydrolytic enzymes have to be secreted or expressed on the cell surface because high molecular weight oligomers are unable to enter microbial cells (Warren, 1996). In eukaryotes, the enzymes secretary pathway starts from the endoplasmic reticulum and moves through the Golgi bodies and vesicles to the membrane (Kubicek *et al.*, 1993), where it stays confined to the microbe's cell surface in eukaryotes and prokaryotes or is secreted into the growth media (Biely, 1993).

There are four major areas where enzymes are of particular use to improve the winemaking process:

(i)   juice clarification, must processing, colour extraction (pectinases, glucanases, xylanases, protease);

(ii) reduction of ethylcarbamate formation (ureases);

(iv) release of varietal aromas from the precursor compounds (glucosidases);

(v)   reducing alcohol content (glucose oxidases).
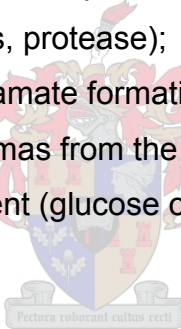
TABLE 4: Enzymes derived from grapes and wine associated microbes involved in winemaking (adapted from Van Rensburg & Pretorius, 2000).

Enzyme	Remarks
Grapes (*Vitis vinifera*)	
Glycosidases	Hydrolyse sugar conjugates of tertiary alcohols; inhibited by glucose; optimum pH 5-6
Protopectinases	Produce water-soluble, highly polymerized pectin substances from protopectins
Pectin methylesterases	Split methyl ester groups of polygalacturonic acids, release methanol, convert pectin to pectate; thermo-stable; opt. pH 7-8
Polygalacturonases	Hydrolyse α-D-1,4-glycosidic bonds adjacent to free carboxyl groups in low methylated pectins and pectate; optimum pH 4-5
Pectin lyases	Depolymerise highly esterified pectins
Proteases	Hydrolyses peptide bonds between amino acid residues of proteins; inhibited by ethanol; thermo stable; optimum pH 2
Peroxidases	Oxidation metabolism of phenolic compounds during grape maturation; activity limited by peroxide deficiency and SO_2 in must
Tyrosinases (oxidoreductases)	Oxidize phenols to quinines, resulting in browning
Fungi (*Botrytis cinerea*)	
Glycosidases	Degrades all aromatic potential of fungal infected grapes
Laccases	Broad specificity to phenolic compounds, cause oxidation and browning
Pectinases	Saponifying and depolymerising enzymes, cause degradation of plant cell walls and grape rotting
Cellulases	Multi-component complexes : endo-, exoglucanses and cellobiases; synergistic working, degrade plant cell walls
Phospholipases	Degrades phospholipids in cell membranes
Esterases	Involved in ester formation
Proteases	Aspartic proteases occur early in fungal infection, determine rate and extent of rotting caused by pectinases; soluble; thermo stable
Yeast *Saccharomyces cerevisiae*	
β-Glucosidases	Some yeast produce β-glucosidases which are not repressed by glucose
β-Glucanases	Extra cellular, cell wall bound and intracellular, glucanases; accelerate autolysis process and release mannoproteins
Proteases	Acidic endoprotease A accelerates autolysis process.
Pectinases	Some yeast degrade pectic substances to a limited extent; inhibited by glucose levels < 2%
Bacterial (Lactic acid bacteria)	
Malolactic enzymes	Convert malic acid to lactic acid
Esterases	Involved in ester formation
Lipolytic enzymes	Degrades lipids

2.4.1 Pectinases

The pectolytic enzymes in fruits play an important role in cell elongation, softening of tissue during maturation and decomposition of plant material (Whitaker, 1990). Apart from the grape itself, other micro flora that are associated with grapes also produce pectinases. The mould *Botrytis cinerea* is responsible for grey or noble rot, and it produces various extracellular enzymes including pectinases (Verhoeff & Warren, 1972). It could produce pectolytic enzymes in concentrations 200 times higher than in healthy grapes.

Classification of Pectolytic enzymes:

Pectolytic enzymes are classified on their mode of attack on the pectin molecule. They either de-esterify or depolymerise specific substrates (Collmer & Keen, 1986), as illustrated in Figure 6. Four enzymes that are closely related and work in a synergistic manner achieve this.

Protopectinase: produce water-soluble and highly polymerized pectin substances from protopectin (insoluble). It reacts at sites having three or more non-methylated galacturonic acid units and hydrolyses the glycosidic bond (Sakai, 1992). Type A acts on the actual chain, while type B acts on the polysaccharide chains connecting the primary chain to the cell wall.

Pectin methylesterase: splits the methyl ester groups of polygalacturonic acids (Whitaker, 1972). It converts pectin to pectate and produces methanol (McKay, 1988), but does not reduce the chain length. The hydrolysis of these methyl ester groups is thought to proceed in a linear fashion along the galacturonan chain, requiring at least one free carboxyl adjacent to the methyl group under attack (Solms & Deuel, 1955).

Polygalacturonase: the most commonly encountered pectic enzyme. It breaks down the glycosidic link between galacturonic acid units with the absorption of a molecule of water (Blanco *et al.*, 1994). Also, it works synergistically with pectin methylesterases in acting only on molecules with free carboxyl groups (Gainvors *et al.*, 1994). Exopolygalacturonases break down distal groups of the chain, resulting in slow reduction of chain length. Endopolygalacturonases act randomly with faster results on chain length and viscosity.

Pectin and pectate lyases: the β-eliminative attack of lyases on a chain results in the formation of a double bond between C4 and C5 in the terminal residues at the non-reducing end, and generates an oligomer with a 4,5-unsaturated galacturonosyl at the end (Kotoujansky, 1987). Different lyases can be distinguished on the basis of their preference for highly esterified pectinic acid (pectin lyase) or pectic acid (pectate lyase) and on the average randomness in the eliminative depolymerisation and behaviour towards oligomeric substrates (Pilnik & Rombouts, 1979). Enzyme activity is suppressed by chelating agents such as EDTA, but reinstated by the addition of calcium ions (Moran *et al.*, 1968; Garibaldi & Bateman, 1971; Chesson & Codner, 1978).
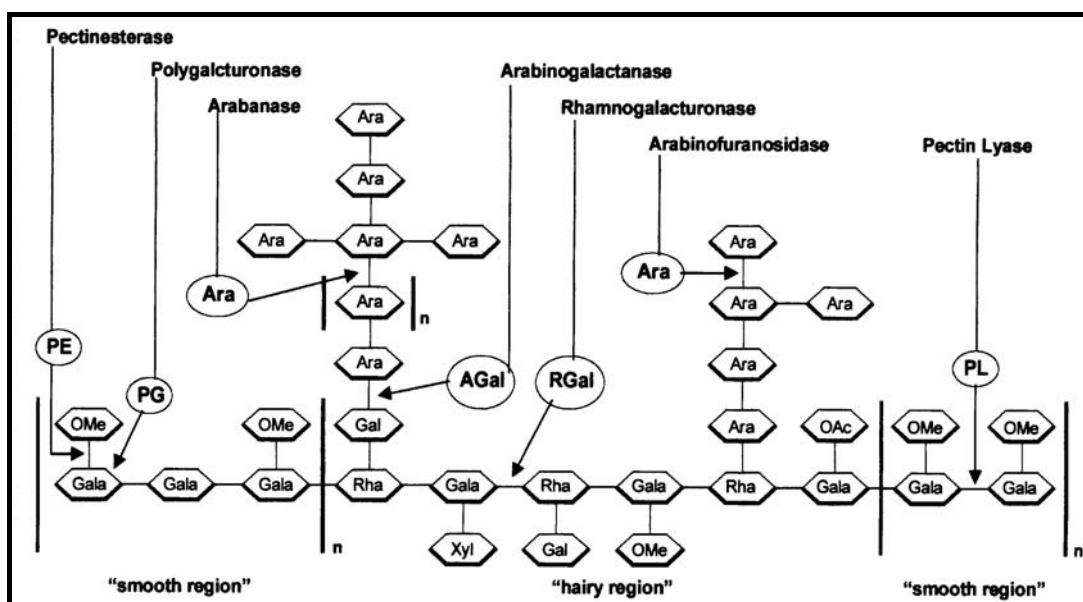
FIGURE 6: The proposed pectin model and enzymatic degradation thereof (from Van Rensburg & Pretorius, 2000).

It has recently been suggested that calcium content in the grape berry may be involved in the grape derived polygalacturonase activity. According to Cabanne & Donéche (2001) this enzyme activity is absent during the herbaceous growth period and increases during ripening. They found that calcium content decreased during ripening and that these trends were diametrically opposed. Thus it seems that polygalacturonases' activity increases with degree of maturity and decreases with calcium content. Takayanagi *et al.* (2001) also investigated polygalacturonase activity. Their results showed the enzyme activity markedly increased within 24 hours after the addition of yeast to crushed grapes, whereas no enzyme activity was detected throughout fermentation in must made from the juice alone. They stated that

this indicated the yeast produced the polygalacturonase and that the skin fraction (seeds and skins) were necessary for production of the enzyme.

Synergism has been reported between pectinolytic enzymes. Pectin methyl-esterase from *Aspergillus aculeatus* strongly enhanced the degradation and depolymerisation of pectin by polygalacturonases (Christgau *et al.*, 1996). Similarly, rhamnogalacturonan acetylesterase (RGAE) from *Aspergillus aculeatus* had a positive effect on the hydrolysis of the backbone of pectic hairy regions by rhamnogalacturonase A and rhamnogalacturonase lyase from *A. aculeatus* (Kauppinen *et al.*, 1995). Pectin lyase positively influenced the release of ferulic acid from sugar beet pectin by a feruloyl esterase from *Aspergillus niger* (De Vries *et al.*, 1997). Synergy also occurs among pectinolytic enzymes as demonstrated by the release of ferulic acid from pectin by a second *A. niger* feruloyl esterase that is positively affected by endoarabinase and arabinofuranosidase from *A. niger* (Kroon & Williamson, 1996). Recently, synergy in the degradation of hairy regions from sugar beet pectin was studied using six accessory enzymes and a main-chain-cleaving enzyme (De Vries *et al.*, 2000). The positive effect of RGAE on the degradation of the hairy-region backbone also positively affected the activity of feruloyl esterase A, β-galactosidase, and endogalactanase from *A. niger*. Additionally, synergistic effects among these three enzymes, an endoarabinase, and an arabinofuranosidase from *A. niger* were detected.
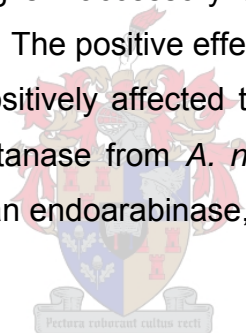
2.4.2 Glucanases (Cellulases)

Cellulolytic enzymes are produced by a wide variety of micro organisms, such as bacteria, actinomycetes and fungi, by higher plants, as well as invertebrate animals (Finch & Roberts, 1985). The degradation of cellulose can be achieved by thermal, chemical or biochemical processes (Alén, 1990; Blazej *et al.*, 1990). Although enzymatic degradation is a much slower process, it is the preferred method of cellulose hydrolysis as it is more environmentally friendly.

The crystalline cellulose fibers are embedded in a matrix of hemicellulose, lignin and pectin that is held together by hydrogen bonds. Enzymatic hydrolysis of cellulose is complicated by the compactness of the molecules which reduce the accessibility of the enzymes (Béquin, 1990). The first step is absorption of the enzyme to the surface of the fibre. Temperature and the type of cellulose used does

influence the absorption, but it is largely dependant on pH. Ghose & Bisaria (1979) put the maximum absorption at 50°C and pH of 4,8.

A "C1-Cx" hypothesis was proposed in an attempt to explain the enzymatic mechanisms involved in cellulose degradation (Reese *et al.*, 1950; Eriksson *et al.*, 1990). It was postulated as follows:

$$C_1 \qquad\qquad\qquad C_x$$

Crystalline cellulose $-\!-\!\rightarrow$ Amorphous cellulose $-\!-\!\rightarrow$ Soluble products

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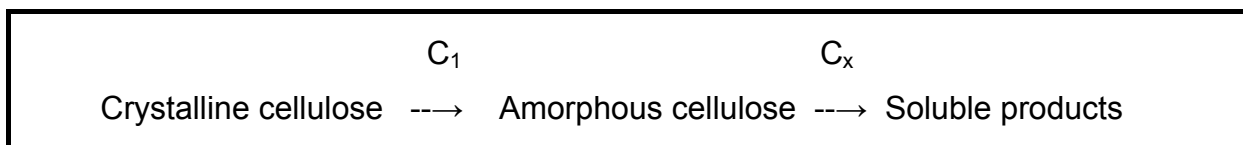
This hypothesis proposed that crystalline cellulose is modified by the activity of C_1 (Reese, 1976) and that the modified products are hydrolyzed by other enzymes. It was suggested that C_1 is a non-hydrolytic chain-separating enzyme that separates the cellulose chains by disrupting the hydrogen bonds (Eriksson *et al.*, 1990). C_x represents several randomly acting enzymes that can hydrolyze non-crystalline cellulose and β-1,4-oligomers of glucose. The development of separation methods has led to the discovery of individual enzymes. Cellulases consist of endoglucanases, exoglucanases and cellobiases, see Figure 7. These act in a synergistic manner based on research showing that the individual enzymes do not degrade cellulose, but a mixture of the three cause extensive hydrolysis (Eriksson & Wood, 1985).

β-glucanases, classified as endo- and exoglucanases, hydrolyse the β-O-glycosidic linkages of the β-glucan chains, leading to the release of glucose and oligosaccharides (Nebreda *et al.*, 1986). These enzymes are not only important for the removal of haze-forming glucans, but also the release of mannoproteins during aging on yeast lees (Ribéreau-Gayon *et al.*, 2000

Classification of Cellulases:

Endoglucanases: attack glucans randomly at regions of low crystallinity and split the β-1,4-glucosidic bonds. According to Finch & Roberts (1985) they have the following general characteristics:

(i) they occur commonly in multiple forms that differ in molecular weight, thermo stabilities and mode of attack;

(ii) they display acidic pH optimums;

(iii) the purified enzyme shows little activity against the native cellulose;

(iv)   they display transferase activity against cellodextrins;

(vi) turnover numbers are comparable to amylases for starch.

Exoglucanases: release cellobiose from the reducing end of the chain. These enzymes show a preference for low molecular weight cellulolytic substrates and, while not involved in primary attack on cellulose, they can catalyse further degradation of oligosaccharides. The cellulolytic systems are acidic and the enzymes show highest activity and stability under these conditions. Endoglucanases have broader substrate specificity than exoglucanases, because they can accommodate the bulky side chains of the substrate (Penttilä *et al.*, 1986).

Cellobiases: these are a member of the β-glucosidases and are substrate specific exoglucanases (Finch & Roberts, 1985) and are capable of hydrolysing a broad spectrum of β-glucosides (Wright *et al.*, 1992). Each of these enzyme classes consists of a number of iso-enzymes and they act synergistically to degrade glucans. The end product of endo- and exo-glucanases, is cellobiose, which is then hydrolyzed by cellobiases (Coughlan, 1990; Pretorius, 1997).

Finch & Roberts (1985) suggest that there are two forms of synergism. The first is between exo- and endo-enzymes. The endoglucanases act randomly and produce non-redusive ends that become the substrate for exoglucanases (Ladisch *et al.*, 1983). This form of synergism appears only on crystalline substrates and is absent on soluble derivates (Eriksson *et al.*, 1990). In this case exoglucanases is the limiting enzyme. The second form of synergism is where the degradation products are inhibitory to the cellulases (cellobioses) and these products are then removed by hydrolytic and oxidative enzymes. In addition to the two major types of synergism, other unusual types have been observed, including endo-endo and exo-exo synergism (Coughlan, 1990; Kubicek *et al.*, 1993).
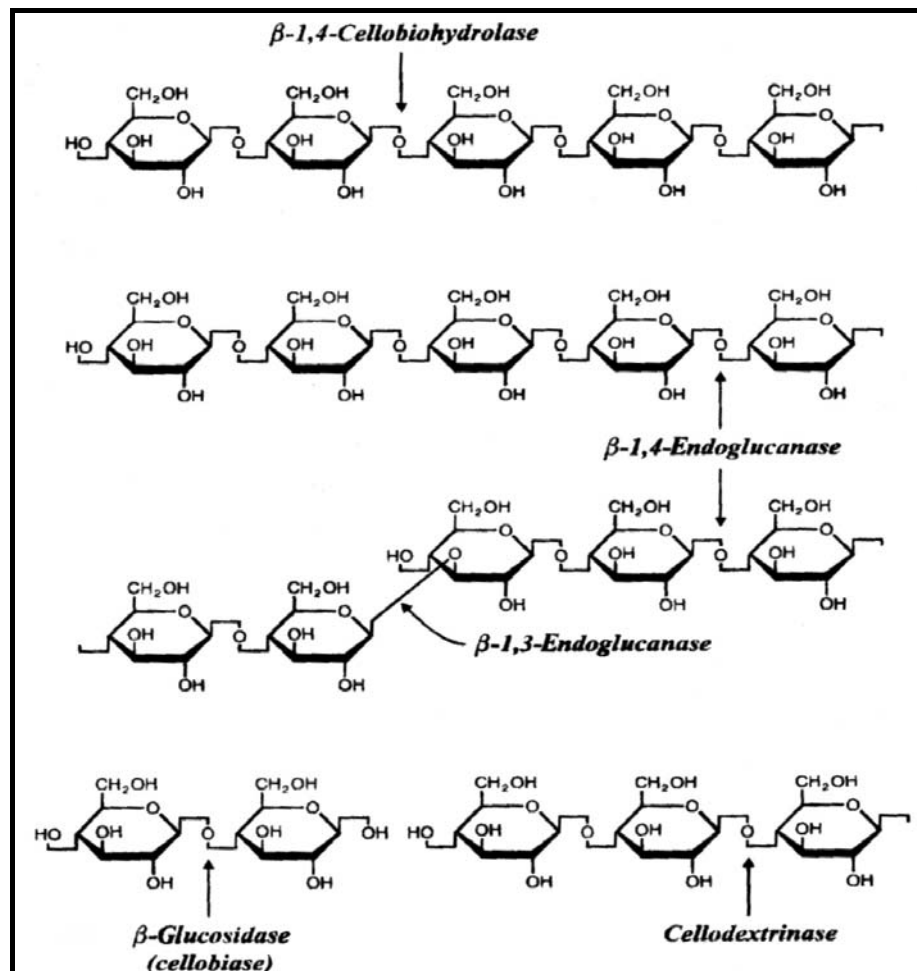
FIGURE 7: Schematic representation of the enzymatic degradation of glucan and cellulose (from Van Rensburg & Pretorius, 2000).
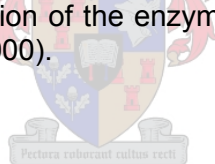
2.4.3 Xylanases (Hemicellulases)

Plant heteroxylan is a complex structure that requires several hydrolytic enzymes to facilitate complete breakdown. Hemicellulases include β-D-galacturonases, β-D-mannases and β-D-xylanases, see Figure 8.

Endo-1,4-β-xylanase attacks the xylan backbone and generates non-substituted or branched xylo-oligosaccharides. Endoxylanases are often prevented from cleaving the xylan backbone due to the presence of substituents (Thomson, 1993; Pretorius, 1997). Thus it acts synergistically with acetylesterases, exoglycosidases and esterases to liberate the substituents from the xylan backbone (Tenkanen & Poutanen, 1992). Synergistic action has been observed between endoxylanse, β-xylosidase, arabinoxylans, arabinofuranohydrolase and acetyl-xylan esterase in the degradation of different xylans (Kormelink & Voragen, 1993). Synergy has also been observed between these enzymes and other xylanolytic enzymes.
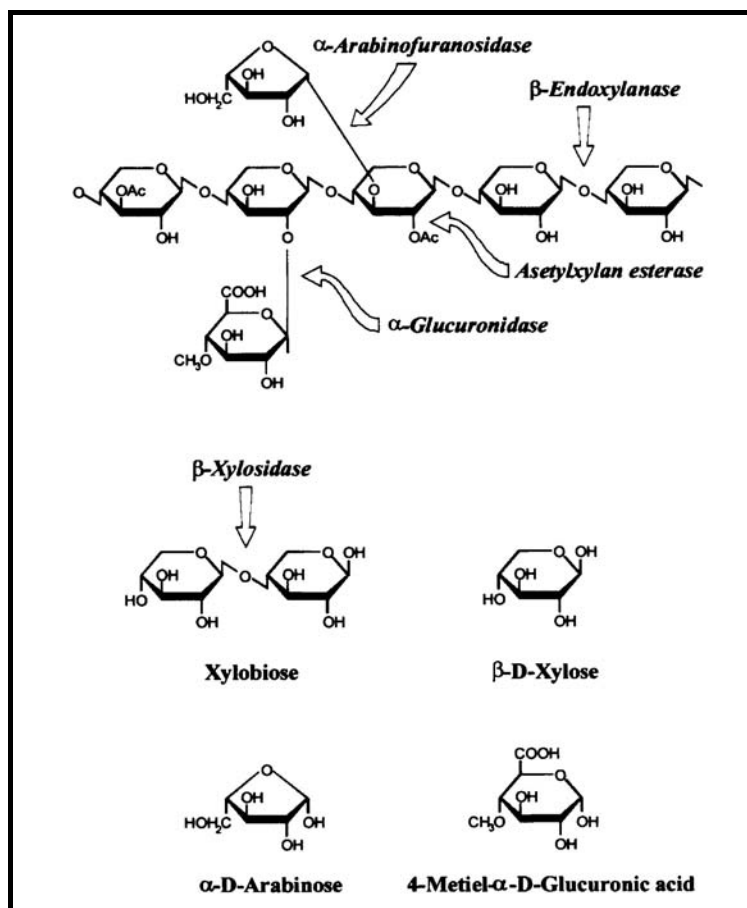
FIGURE 8: Schematic representation of the enzymatic degradation of hemicellulose (from Van Rensburg & Pretorius, 2000).

The release of ferulic acid from xylan by a feruloyl esterase from *Aspergillus niger* was strongly enhanced by the addition of endoxylanses (Bartolome *et al.*, 1995; De Vries *et al.*, 2000). Similarly, both endoxylanse and β-xylosidase positively influenced the release of 4-O-methylglucoronic acid from birch wood xylan by an α-glucuronidase (De Vries *et al.*, 1998). Recent studies revealed that synergistic action in degradation of xylan does not only occur between main-chain-cleaving enzymes and accessory enzymes, but also among accessory enzymes; and nearly all accessory enzymes are positively influenced by the activity of main-chain-cleaving enzymes (Denison, 2000). A strong synergistic effect has been observed for the role of *A. niger* acetylxylan esterase in the hydrolysis of birch wood xylan by three endoxylanases from *A. niger* (Kormelink *et al.*, 1993).

The activity of xylans and β-xylosidases depend on the chain length of xylo-oligosaccharides, the former decreasing with decreasing length, the latter increasing (Thompson, 1993). Exo-xylanase and 1,4-β-xylosidase are able to produce D-xylose through their specific activities (Biely, 1993).

31

Endoxylanases are classified as debranching and non-debranching enzymes according to their ability of attacking glucoronoxylans (Dekker, 1985). Both these types are able to attack glucoronoxylans and unsubstituted 1,4-β-D-xylans. The non-debranching group are the most common and degrade heteroxylans at random (Poutanen, 1988).

A synergistic effect was observed in the degradation acetyl-galactoglucomannan. The presence of galactose and acetyl residues on the backbone severely hindered the activity of β-mannase (Puls *et al.*, 1992). The presence of acetylmannanesterase and to a lesser extent α-galactosidase significantly increased the β-mannase activity on this substrate. Additionally, the action of β-mannase and α-galactosidase on acetylgalactoglucomannan was positively influenced by the removal of acetyl residues from the main chain by acetylgalactoglucomannan esterase (Tenkanen *et al.*, 1993; 1995).

2.4.4 Glycosidases

The hydrolysis of precursor compounds is very important, as it utilises the potential pool of aroma in the wine. This can be achieved by acid hydrolysis or by enzymatic liberation. Williams *et al.* (1982) suggested an acid hydrolysis, but due to its nature is not preferred, as it can alter the aromatic character of the wine. Günata *et al.* (1988) proposed enzymatic hydrolysis (see Table 5) as an alternative as the enzymes are able to split the β-glycosidic bonds without modifying the aromatic characteristics of the wine. The liberation occurs in two steps: the first is the enzymatic cleavage of the 1,6-intersugar-linkage, which requires the action of a glycosidase (α-arabinofuranosidase, α-rhamnosidase, β-xylosidase or a β-apiosidase) which acts on various sugars. This results in a monoterpenyl glucoside. The second step involves the liberation of the terpene alcohol via a β-glucosidase enzyme (Sánchez-Torres *et al.*, 1996) which acts only on the glucose sugar.

Glycosidases may adversely affect the colour of the wine. They cleave the sugar from the anthocyanin, leaving an unstable aglycon that will spontaneously transform into a stable colourless from (Huang, 1956). Wightman *et al.* (1997) investigated this effect on colour in Pinot noir and Cabernet Sauvignon. They found a destruction of total monomeric anthocyanins as well as individual pigments. The

presence of acylating groups on malvidin-3-glucoside did not appear to affect the enzyme activity. Preparations that caused the most anthocyanin degradation also produced wines with higher amounts of polymeric anthocyanin. They also found that increased enzyme concentration magnified these effects. These treatments had a pronounced effect on other phenolics as well. This study confirms that the use of enzyme preparations must be carefully considered, as they may alter the composition of the wine significantly. Endogenous grape glycosidases are inhibited by glucose. It has poor stability at low pH and at high ethanol levels. Therefore these *Vitis vinifera* enzymes would be virtually inactive during winemaking conditions. They are also aglycone specific, and are incapable of hydrolysing the conjugates of tertiary alcohols. Thus, some of the most important monoterpenes, e.g. linalool, are not released by the enzyme (Grossman *et al.*, 1990). Certain processing actions, such as clarification, considerably reduce the activity of β-glucosidase.

TABLE 5: Enzymes for aroma extraction (adapted from Van Rensburg & Pretorius, 2000).

Enzyme	Company	Activities	Time of addition
Expression[20]	Darleon	Pectinase + β-glucosidase	Add to fermentation with residual sugar below 10g/L
Endozym Cultivar	AEB Africa	Pectinase	To grapes or must
Endozym Rouge	AEB Africa	Pectinase + hemicellulase + cellulase	During red grape maceration before SO_2
Vinozym	Novo Nordisk	Pectinase + side	Directly into crusher
Vinozym FCE	Novo Nordisk	Pectinase, hemicellulase, cellulase	Maceration of white grapes
Novoferm 12	Novo Nordisk	Pectinase + side	Towards end of alcoholic fermentation
Novarom	Novo Nordisk	Glycosidases	At end of alcoholic fermentation
Novarom G + Novarom L	Novo Nordisk	Pectinase + β-glucosidase	At end of fermentation during racking
Vinozym G	Novo Nordisk	Pectinase	Into crusher, for colour phenolic extraction
Trenolin Bukett	Erbslöh	Pectinase + β-glucosidase	Towards end of alcoholic fermentation
Rapidase X-Press	DSM	Pectinase	To grapes or mash
Rapidase AR2000	DSM	Pectinase + Glycosidases	At end of alcoholic fermentation
Lafazym Extract	Laffort	Pectinases	On crushed grapes

Canal-Llaubères (1993) suggested that glycosidase preparations from *Aspergillus* sp. could reinforce the varietal aroma if added once the yeast has depleted the glucose in the juice. Park (1996) investigated the changes in free and glycosidically bound monoterpenes as a function of fermentation, wine aging and enzyme treatment. Muscat d'Alexandrie and Gewürztraminer wines were treated with crude pectinase, Rohapect 7104 (Röhm, Darmstadt, Germany), containing β-glucosidase activity, after aging for 8 months. The results indicated that the Rohapect 7104 enzyme preparation could effectively be used in hydrolysing bound monoterpenes. However, it is considered that in normal wines the monoterpenes are very stable and are hydrolysed slowly, therefore releasing the floral aroma over a long period of time during aging. Conversely, the enzyme-treated wines released all the desirable monoterpenes at once, leaving no pool to maintain a constant supply of terpenic-floral aroma in the wine. In addition, free monoterpenes (which have desirable floral aromas in wine) can be interconverted to other, more chemically stable but less desirable compounds, such as α-terpineol. The low pH causes the monoterpenes to be unstable and promotes hydrolysis, rearrangement or oxidation of the compounds. Park (1996) also suggested it is very important that the commercial preparations never contain cinnamyl esterase, as this enzyme can lead to the formation of vinyl-phenols with a very undesirable animal odour.

Grossman *et al.* (1987) have suggested that yeast glycosidases are not inhibited by glucose. Although the activity was lower in must than in wine, it was still capable to release monoterpenes. Yanai & Sato (1999) used a purified intracellular β-glucosidase from *Debaromyces hansenii* Y-44 to ferment Muscat juice. The treated wines showed a considerable increase in the concentration of monoterpenols produced. Linalool and nerol increased by 90% and 116% respectively. Darriet *et al.* (1988) and Dubourdieu *et al.* (1988) reported that certain strains of *Saccharomyces cerevisiae* also possess a β-glucosidase located in the periplasmic space of yeast cells. However this activity seems to be very limited. Grimaldi *et al.* (2000) identified and partially characterized a glycosidic activity from commercial strains of lactic acid bacterium, *Oenococcus oeni*, which are utilized for the malolactic fermentation of wine. In evaluating the potential of these activities as liberators of glyco-conjugated grape aromas, responses to oenological pH values, glucose, fructose and ethanol concentrations were determined. The optimal pH was observed at pH 5,5; but in the pH range of 3,5 to 4,0; 12% to 43% of the activity was retained.

2.5 UTILIZATION OF BIOTRANSFORMATION FOR CHANGES IN CHEMICAL COMPOUND PROFILE

Grape processing can be divided into three stages: (a) pre-fermentation, (b) fermentation and (c) post fermentation. The pre-fermentation stage is the determining stage for the quality of the raw grape material. It includes harvesting and transport of the grapes, crushing, cold soaking and pressing; as well as settling of the juice. It could also include any other processing steps that are taken before the juice is allowed to begin fermentation. During this stage the most important areas of enzyme influence are clarification of juice and release of varietal aroma. During harvest it is an advantage to pick the grapes by hand, as it is easier to clear the bunches and remove poor quality grapes. This is also a preventative measure to minimize the pathogen related or PR-proteins in the wine (as a result of fungal attack), which cause great problems with clarity and filtration. The method of grape processing influence the activity of native enzymes, for example, heat treatment to enhance colour extraction deactivates enzymes and certain fining agents such as bentonite remove it from the medium. Also sulphur-dioxide and other microbes may cause the native enzymes to be ineffective.

Most of the native enzymes (originating from the grape) are either insufficient in quantity or ineffective because of other influences as mentioned before. Therefore it is very common that the native enzyme activity is supplemented by industrial preparations in order to achieve the desired effect. These industrial preparations are obtained from organisms such as *A. niger*, which is cultivated under optimal cell growth and enzyme production conditions. Of the 2500 different enzyme-catalysed reactions recognised by the International Union Handbook of Enzymes Nomenclature (Gacesa & Hubble, 1998) only about 30 are currently used in industrial preparations.

The pectolytic enzymes were the first commercial enzyme preparation used in the wine industry (Rombouts & Pilnik, 1980). Commercial pectinases are used to improve juice yields, release of colour and flavour compounds from grape skins and to improve clarification and filterability. See Table 6 and 7 for examples of various commercially available preparations. Most commercial enzyme preparations are obtained from fungal sources (Alkorta *et al.*, 1994), with most strains belonging to *Aspergillus* species. The preparation of deliberately mixed enzymes is very useful as it performs multiple functions. The liquefaction enzymes are an example, containing cellulases and hemicellulases in addition to pectinases.

TABLE 6: Commercial pectinase preparations to improve clarification, filtration and yield of juice and wine (adapted from Van Rensburg & Pretorius, 2000).

Enzyme	Company	Activities	Time of addition
RapidaseVino Super	DSM	Pectolytic	To juice before settling
Rapidase Filtration	DSM	Pectolytic + β-glucanase	Add at end of fermentation
Rapidase X-press L	DSM	Pectolytic + side activities	To white grapes or mash
Rapidase CB	DSM	Pectolytic	To juice before settling
Endozyme Active	AEB Africa	Pectolytic	To juice before settling
Pectizym	AEB Africa	Pectolytic	To juice
Pectocel L	AEB Africa	Pectolytic	To grapes or juice
Endozym Éclair	AEB Africa	Pectolytic	To musts with high quantities of solids
Endozym Pectoflot	AEB Africa	Pectolytic	To must, 4h before flotation initiation
Glucanex	Novo Nordisk	β-glucanase	Between first racking and filtration
Ultrazym	Novo Nordisk	Pectolytic	To white and red mash
Novoclair FCE	Novo Nordisk	Pectinases	To grape must
Pectinex Superpress	Novo Nordisk	Pectolytic + hemicellulases	Directly into destemmer / crusher
Vinoflow	Novo Nordisk	Pectinases + β-glucanase	At end of fermentation
Influence	Darleon	Pectolytic + side activities	In red wine during fermentation
Lafazym CL	Laffort	Pectolytic	Prior to fermentation
Lafase 60	Laffort	Pectolytic	In barrel for thermo-vinification musts
Extractalyse	Laffort	Pectolytic + glucanases	In barrel for aging and filtration improvement

TABLE 7: Commercial pectinase preparations to improve extraction and stabilization of colour during winemaking (adapted from Van Rensburg & Pretorius, 2000).

Enzyme	Company	Activities	Time of addition
Enzym'Colour Plus	Darleon	Pectolytic + Proteolytic	To juice or must
Endozym Contact Pelliculaire	AEB Africa	Pectolytic	To juice or must
Endozyme Rouge	AEB Africa	Pectolytic + side activities	During maceration (Before SO_2)
Vinozym EC	Novo Nordisk	Pectolytic, arabinase + cellulase	Into crusher or mash tank for colour and aroma extraction
Rapidase Ex Colour	DSM	Pectolytic + side activities	Before maceration
Lallemand OE	Lallemand	Pectinase + hemicellulase + cellulase	To grapes before pressing
Lallemand EX	Lallemand	Pectinase + hemicellulase + cellulase	To grapes before pressing
Lafase He Grand Cru	Laffort	Pectolytic	On crushed grapes at start of fermentation
Lafase HE	Laffort	Pectolytic	During pre-fermentation maceration

Inhibiting factors are very important to consider with the use of any enzyme preparation, as it will influence activity and the amount needed for completion of the reaction. This in turn could have economic implications. The optimum pH for pectinases originating from grapes and associated micro flora usually varies between pH 2 and pH 8. They are therefore not notably inhibited by wine pH (pH 3 – pH 4), as illustrated in Table 4 (Van Rensburg & Pretorius, 2000). However, temperature significantly influences enzyme activities. Below 10°C their activity drops to levels that are too low for effective degradation of pectic substances in grape juice or wine. As the temperature rises, the reaction rate doubles with each increase of 10°C (Van Rensburg & Pretorius, 2000). In one theory it was stated that one eighth of enzyme concentration could be sufficient if the juice was processed at 55°C (Hagan, 1996), but it would have detrimental effects on the wine aromas and flavours.

The commercial preparations contain the active proteins (enzymes), as well as sugars, inorganic salts and preservatives to stabilize and standardize the product (Hagan, 1996). These compounds are important in protecting the protein during sub-optimal storage conditions and exposure to light, which decreases activity (Hagan, 1996). If the enzyme were for example stored at 50°C for 1 hour, the activity loss could increase to 30%. There is, however, no loss of activity upon thawing.

Because these enzymes are essentially proteins, factors inhibiting proteins in general will decrease their effectiveness. This includes juice clarification using bentonite, which adsorbs the proteins and settles them out. Alcohol levels above 17% v/v and SO_2 concentrations over 500 mg/L also inhibit pectinases (Van Rensburg & Pretorius, 2000). Wines which are high in tannins will show reduced enzyme activity as tannins react with the proteins and render them useless.

The point in time at which the additions are made is, as mentioned before, of extreme importance. Pectolytic enzyme preparations based on pectinase activity are recommended for clarification of musts after pressing. It's pectinmethyl-esterasic and endogalacturonic activity causes hydrolysis of pectic chains and facilitates the draining of juice from the pomace (Brown & Ough, 1981) with an increase yield of a free-run juice with a lower viscosity. The addition of this enzyme lowers viscosity and causes cloud particles to aggregate into larger units, which settles as sediment (Chesson, 1980). The speeding up of the clarification process also produces more compact lees. When it is applied to pulp before pressing, it increases juice yield and colour extraction (Ough et al., 1975). Pectolytic enzyme preparations for so-called liquefaction comprise a mixture of pectinases with cellulases. During maceration, pectin degradation affects only the middle lamella pectin, and organized tissue is transformed into a suspension of intact cells (Van Rensburg & Pretorius, 2000). At the concentration of 2-4 g/hL, 15% increase in juice has been recorded over a time period of 4-10 hours (Ribéreau-Gayon et al., 2000).

James et al. (1999) suggested that the juice yields of Muscadine grapes (Vitis rotundifolia Michx.) could be increased with the use of its native cellulase enzyme. The characterization of this enzyme indicated that it has optimum activity at an pH of 4,0 and a maximum temperature of 45°C. Zn^{2+} and Mg^{2+} inhibited cellulase activity 32% and 30% respectively, at a concentration of 10 mM, while Cu^{2+} and Fe^{2+} stimulated enzyme activity. The enzyme degraded the substrates as an endoglucanase and cleaved glycosidic bonds as an exoglucanase. Thus it seems

likely to increase juice yields from Muscadine grapes by enhancing the conditions for enzyme action during juice manufacture.

Sims *et al.* (1988) compared a macerating enzyme (Macerating Enzyme GC219; Genecor) with a standard pectinase (Pectinol 60G; Genecor) on a *Vitis rotundifolia* cultivar and a *Euvitis* hybrid. The first is hard to press and the second has difficulty with clarification. The macerating enzyme used consisted of a mixture of pectinases, cellulases and hemicellulases. The maceration enzyme was only slightly more effective in increasing the free-run juice, but total yields were similar for the two enzymes. However, it greatly improved the degree of settling of the *Euvitis* hybrid, as compared to the standard pectinase.

With the use of pectinases, increases in methanol content were recorded (Massiot *et al.*, 1994; Servili *et al.*, 1992). Revilla & Gonzälez-SanJosé (1998) evaluated methanol production of different commercial preparations of pectolytic enzymes during the fermentation of red grapes. They used two clarifying pectolytic enzymes, Zimopec PX1 (Perdomini; 0,03 g/L) and Rapidase CX (DSM; 0,05 g/L), and two colour extraction enzymes, Pectinase WL extraction (Wormser Oenologie; 0,01 g/L) and Rapidase Ex Colour (DSM; 0,05 g/L). Their results indicated that the enzymatic treatment enhanced the methanol content during the initial phases of fermentation and it remained largely constant during storage.

Early research conducted by Ough *et al.* (1975) indicated that pectolytic enzyme treatment of red grape musts could accelerate the extraction of colour pigments and phenols. They concluded that the only significant effect on wine quality was the increased intensity of wine colour. The faster extraction of colour allows the pomace to be pressed earlier and is an advantage when tank space is in short supply. The shorter skin contact time results in wines of equal colour, but lower tannin content. Brown & Ough (1981) tested two commercial enzymes, Clarex-L and Sparl-L-HPG (supplied by Miles Laboratories), on grape musts of eight different white wine varieties. This study indicated an increase in total juice yields, clarity of wine, filterability, methanol production, wine quality, browning capacity and amount of settled solids. In contrast Wightman *et al.* (1997) indicated that some pectinase preparations are capable of reducing red wine colour through pigment modification and subsequent degradation. Further research showed an increase in colour, but with enhanced bitterness and astringency in the wine. Watson *et al.* (1999) investigated two enzymes preparations, Scottzyme Colour Pro and Colour Pro (Scott Laboratories) and found that both produced wines with higher concentrations of

anthocyanins, higher concentrations total phenols, greater colour intensity and better visual clarity than found in the untreated wines. Furthermore the enzyme-treated wines had increased aroma and flavour intensity, including enhanced spicy, cherry, raspberry aromas and flavours. It also had enhanced astringency and bitter characteristics. In further trails by Scott Laboratories, Watson *et al.* (1999) found that wines produced with enzyme treatments were higher in polymeric anthocyanins, polymeric phenols and catechin than the control wines, but not in the monomeric anthocyanin content.

In 1994 the Australian Wine Research Institute conducted a review into the performance of a range of commercial pectolytic enzyme preparations with respect to effect on red must and wine colour (Leske, 1996). This investigation sought to access the validity of the hypotheses that the use of pectic enzymes results in (i) greater colour extraction during red wine fermentation; (ii) faster colour extraction during maceration and fermentation of red grapes; (iii) greater colour extraction from red wines at pressing; and (iv) improved clarification. They used various products from a range of producers, including macerators and red colour extractors, along with several clarifiers in an attempt to determine differences between the groups. The results of the enzyme-treated musts showed no significant increase in any of the measured parameters at any stage of the processing when compared to the control samples. Leske (1996) concluded that the use of pectic enzyme preparations for improvements in colour extraction is unnecessary on the basis of these above-mentioned results.

Zimman *et al.* (2002) investigated the effect of a colour extraction enzyme as part of a maceration study using Cabernet Sauvignon grapes. They reported an increase in total proanthocyanidins due to an increase in specific fractions, but did not find an increase in colour intensity. They suggested, however, that these increased proanthocyanidins could favour coloured proanthocyanidin formation in the long term.

In stark contrast, Bakker *et al.* (1999) obtained totally different results in a study involving port wine. Two commercial pectolytic enzyme preparations were used on pilot scale to evaluate the effect on colour extraction during the short processing of crushed grapes prior to fortification to make port wine. The results showed that both enzyme preparations enhanced colour extraction during vinification. In the young wines the enzymes gave a darker hue to the wine. Maturation led to a general reduction in colour, but the differences in colour between the two sets of wines were

maintained. Sensory analysis showed that the wines produced with the enzymes had significant higher colour index, aroma and flavour intensity than the control.

Yeasts of the *Brettanomyces* type are found in red wines and are responsible for the production of volatile phenols characterized by animal, leather, ink, horse stable or barnyard odours. Certain winemaking techniques that favour extraction of phenolic compounds also indirectly favour the production of volatile phenols in wines that have been contaminated with *Brettanomyces*. The use of oenological enzymes could potentially cause an excess production of volatile phenols. Cinnamyl-esterase, a secondary activity seen in most enzymatic preparations produced by *Aspergillus niger* (pectinases), is the cause of this problem. The use of a purified enzyme without cinnamyl-estarase does not seem to induce an overproduction of these volatiles. Thus it is of great importance that the preparations used are of a very high quality and in a purified state (Gerbaux *et al.*, 2002).

Glucanex was one of the first commercial preparations to be tested on wines made from botrytised grapes (Villettaz *et al.*, 1984). The preparation consists mainly of exo-β-glucanase, endo-β-1,3-glucanase, exo-β-1,6-glucanase and an unspecific β-glucosidase activity. The enzyme treatment improved filtration, but did not cause any significant changes in the chemical composition of the wine. The treated samples showed a higher residual sugar level, but it could be partly due to the degradation of *Botrytis* glucans to glucose. Miklósy & Pölös (1995) conducted a study where glucans were added to Traminer must after skin contact. Three commercial enzyme preparations, Glucanex (Novo Nordisk), Novoferm 12L (Novo Nordisk) and Trenolin Buckett (Erbslöh), were used. Sensory analysis of the wines took place six months after fermentation. Wines treated with Trenolin Buckett was considered by more than 85% of a tasting panel to have a more desirable aroma, fruity taste and improved overall quality than the control. The wines treated with the other enzymes were again preferred to the control samples, although the differences were not as pronounced as with the Trenolin Buckett. At the present moment commercial β-glucanases are available for applications of clarification, filtration and aging of young wines (Canal-Llaubères, 1998). These enzymes are sourced from *Trichoderma* species. The preparations are active between 15 - 50°C and at pH 3 – 4. The influences of alcohol at elevated levels have not been researched, but concentrations up to 14% v/v have no reported negative effect. An SO_2 level of up to 350 ppm also has no apparent negative influence on relative enzyme activity.

Enzymes also have important applications as far as health benefits are concerned. There is a worldwide trend to consume less alcohol, stimulating the search for low alcohol wines. There are various physical treatments available involving expensive equipment such as reverse-osmosis (Canal-Llaubères, 1993). It can however non-specifically change the sensorial properties of the wine (Pretorius, 2000). Alternatives have been suggested, including redirecting grape sugars to glycerol at the expense of ethanol production, or the use of enzymes such as glucose oxidase and catalase (Van Rensburg & Pretorius, 2000). These enzymes convert glucose to gluconic acid, which is not metabolized by the yeast. This method results in wines with reduced alcohol and elevated acidity. Ethyl carbamate (urethane) is a suspected carcinogen that occurs in most fermented food and beverages (Van Rensburg & Pretorius, 2000). Thus as a health hazard there is a demand for reduced levels in wine. Acid ureases were investigated as a means to reduce urea, which is one of the substrate compounds for production of ethyl carbamate, in wine. The genes for expression of this enzyme were sourced from *Lactobacillus fermentatum*, and successfully expressed in *Saccharomyces cerevisiae* but the secretion was very low and thus not very successful (Visser, 1999).

Proteases have proven to be beneficial in the laundering and automatic dishwashing. It increases the effectiveness of detergents, especially for use at lower temperatures and lower pH levels. Proteases is also used in conjunction with glycoamylase and glucose oxidase to prevent plaque, it gives better results in a shorter time of application to the dentures (Anonymous, 2000). Wool is made of proteins and therefore wool treatments involve protease, which modifies the fibres. It reduces the 'facing up' of fibres that are caused by the dyeing process, and improves the pilling performance and increases softness. Proteases are also used to treat silk. The threads of raw silk must be de-gummed to remove sericin, a proteinaceous substance that covers the silk fibre. The traditional treatment used a harsh alkaline solution; however the use of proteolytic enzymes performs this duty without attacking the actual fibres. It is also used in the soaking, bating and de-hairing steps of leather preparation.

2.6 STABILITY OF BIOCATALYSTS

Catalysts act by reducing the energy barrier of chemical reactions, therefore producing a dramatic increase in reaction rates, ranging from 10^6 to 10^{24} fold (Illanes, 1999). The catalysts of cell metabolism are referred to as biocatalysts, i.e. the enzymes. However, in broader terms, biocatalysts are any biological entity capable of catalyzing the conversion of substrate into a product. Accordingly, biocatalysts can be divided in cellular (growing, resting or non-living cells) and non-cellular (enzymes that have been removed from the cell system that produced them) (Illanes, 1999).

Potential advantages of biocatalysts are their high specificity, high activity under mild environmental conditions and high turn-over rate; their biodegradable nature and their label as natural product (Polastro, 1989). Drawbacks are inherent to their complex molecular structure, which makes them costly to produce and intrinsically unstable.

Biocatalysts are inherently labile; therefore their operational stability is of paramount importance for any bioprocess. Recently the potential of extremophiles has been recognized, the cloning of termophilic genes into more suitable mesophilic hosts is now at hand to produce stable biocatalysts (Illanes, 1999).

Other approaches being utilized presently are:
(i)   site-directed mutagenesis to code for more stable proteins;
(ii)   immobilization and crystallization;
(iii) reaction media engineering;
(iv) selection for mutants with increased protein stability (Illanes, 1999).

Different agents, such as extreme temperatures and chemicals, promote enzyme inactivation. The latter can often be easily avoided by keeping the chemicals in question out of the reaction medium. Temperature, however, produced opposed effects on enzyme activity and stability; and is therefore a key variable in any biological process (Wasserman, 1984). As indicated by Marshall (1997) and Somkuti & Holsinger (1997) enzymes active at both low temperatures and stable at high temperatures are of great technological importance. Biocatalyst stability, i.e. the capacity to retain activity over time, is undoubtedly the limiting factor in most bioprocesses, biocatalyst stabilization being a central issue of biotechnology.

Enzyme stability is dictated by its three-dimensional configuration, which is in turn determined by genetic (primary structure) and environmental (interactions with the surroundings) factors. Research on extremophiles (organisms that does not just survive, but also thrive in extreme environmental conditions), as promising sources for highly stable enzymes, is a subject of great interest at the present moment (Herbert, 1992; Haard, 1998; Davis, 1998). Two of the most prominent properties of naturally occurring "hyper stable" enzymes are the presence of large surface area networks of electrostatic interactions and a tendency to be multimeric (Sterner & Liebl, 2001; Vieille & Zeikus, 2001; Szilagyi & Zavodsky, 2000). Biocatalyst thermo-stability allows for a higher operational temperature, which has the following advantages: higher reactivity (higher reaction rate, lower diffusional restrictions); higher stability; higher process yields (increased solubility of substrates and products); lower viscosity and fewer contamination problems (Mozhaev, 1993).

Daniel (1996) claimed that enzymes from thermophiles are stable even at temperatures more than 20°C higher than the optimum growth temperature for such organisms. It is interesting to note that mesophiles exhibit the same pattern. Although a high number of thermo-stable enzymes from thermophiles have been reported, their technological use still faces several challenges since knowledge on physiological and genetics of such organisms are poor; they are fastidious; grow slowly and are not recognized as safe. Thus commercial enzymes from thermophiles are still scarce and the thermo-stable enzymes used in industry are still produced from mesophiles, as illustrated in Table 8.

Thermo-stability is the result of differences in specific amino acid sequences and it has been ascribed to a more rigid configuration and to the high number of hydrophobic interactions. The sequences possibly linked to the thermo-stable phenotype organism can be identified by examining the primary sequences of termophilic and their mesophilic counterparts (Illanes, 1999). Imanaka *et al.* (1988) illustrated that this opens up the possibility of protein engineering techniques to produce point mutations in the mesophilic structural gene, resulting in the corresponding amino acid substitution in the primary structure of the encoded protein (Daniel, 1996).

TABLE 8: Industrial thermo-stable enzymes, commercial enzymes from thermophiles (from Illanes, 1999; Kristjánsson ,1989; Coolbear *et al.*,1992).

Industrial Thermo-stable Mesophilic Enzymes			Commercial Enzymes from Thermophiles	
Thermo-stable enzyme	Mesophile Producer	T_{op} °C	Thermo-stable Enzyme	Thermophile Producer
α-amylase	*Bacillus*	95	omalate dehydrogenase	*T. termophilus*
gluco-amylse	*Aspergillus*	60	β-amylase	*C. thermo-sulphuricum*
pullulanse	*Aerobacter*	60	α-galactosidase	*B. stearo-termophilus*
gluc isomerase	*Actinoplanes*	60	DNA polymerase	*P. furiosus*
pectinase	*Aspergillus*	60	α-amylase	*P. furiosus*
alcalase	*Bacillus*	60	glutamate dehydrogenase	*P. furiosus*
lipase	*Aspergillus*	60	cellulase	*R. marinus*
acid protease	*Mucor*	50		
lactase	*Aspergillus*	50		
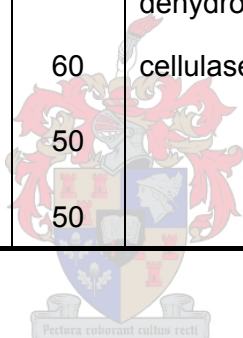
Protein engineering is also being used to obtain improved biocatalysts. Thermo-stable proteases capable of withstanding conditions of high pH and high concentrations of strong oxidants, are products of protein engineering produced by point amino acid substitutions in the most labile region of the molecule (Anonymous, 1997; Anonymous, 1998). The improvement of the hydrophobic core packing, introduction of disulphide bridges, stabilization of α-helix dipoles, engineering of surface salt bridges and point mutations are all aimed at reducing the entropy of the unfolded state of the protein (Van den Burg & Eijsink, 2002).

According to Mozhaev (1993) water acts as a reactant in inactivation reactions and also a lubricant in conformational changes associated with protein unfolding. Therefore, biocatalyst stabilization under operational conditions is a key issue. Stabilizing additives is a customary practice employed to improve shelf life of enzyme products, however its use as operational stabilizer has little significance and poor predictability (Ye *et al.*, 1988).

In non-conventional media the use of additives has proved to enhance enzyme activity and stability (Triantafyllou *et al.*, 1997). Chemical modification has also been used in that hydrophilic groups have been introduced in the surface on the enzyme molecule that reduces the contact of hydrophobic regions with water, thereby preventing incorrect refolding after reversible denaturation (Mozhaev, 1993). Derivatization with polymers is increasingly being proposed for stabilization of soluble enzymes. Sundaram & Venkatesh (1998) indicated that by modifying proteases with carbohydrate polymers, like polymeric sucrose and dextran, the enzyme is stabilized against inactivation by temperature and chaotropic agents.

Immobilization to solid carriers is perhaps the most utilized strategy to improve operational stability of biocatalysts. Among the methods available, multi-point covalent attachment is the most effective in terms of thermal stabilization (Guisán *et al.*, 1993), although thermal stabilization has also been reported for gel-entrapped enzymes (Gianfreda *et al.*, 1985). Illanes *et al.* (1988) observed a dramatic increase in thermal stability by immobilizing different enzymes to glutaraldehyde-activated chitin matrices, where multiple Shiff-base linkages are established between free amino acids in the protein and the aldehyde group in the glutaraldehyde linker. Cross-linked enzyme crystals (CLEC) are a highly stable novel type of biocatalyst. They are produced by stepwise crystallization and molecular cross-linking to preserve the crystalline structure. CLEC are extremely stable to temperature and organic solvents. The enzyme molecules are compacted to the theoretical limit, stabilization being a consequence of intense polar and hydrophobic interactions. CLEC are represented in the market with examples of lipases, thermolysin, glucose isomerase and penicillin acylase (Margolin, 1996). Medium engineering, the manipulation of the reaction medium (Gupta, 1992), is a completely different approach where the total substitution of water might be beneficial for biocatalyst stability (Bell *et al.*, 1995). It is thus clear that many options are available to improve stability of biocatalysts in various media and conditions.

2.7   PROTEASES AND WINE

Yeast proteases may have intra- or extracellular origin. The extracellular proteases are of importance during the autolysis process, where the wine is aged in contact with the yeast lees. Intracellular or cytoplasmic proteases serve to degrade cellular macromolecules and are confined to the vacuole (Rothman & Stevens, 1986), but can be released upon cell lyses. Only a few proteases however are active under the specific conditions of fermentation (Lurton, 1987). It has been reported that with prolonged storage on the lees, wine becomes more protein stable due to the action of protease A and the release of mannoproteins during autolysis.

As the enzyme in itself is hazardous to the yeast cell, an inherent protection mechanism is in place whereby the endoprotease is synthesized as a pre-protein. The pre-peptide is cleaved early in the secretory pathway and the pro-peptide is cleaved upon entry of the vacuole (Pretorius, 2000). This is to keep the protease A inactive during transport in the cell. According to Luo & Hofmann (2001), for the protease to become active, the regulatory-domain (auto-inhibitor) is cleaved by the protein or prevented from blocking the active site by other means.

Yeast autolysis represents an enzymatic self-degradation of cellular constituents, which occurs after cell death or causes cell death. The main events during autolysis are the breakdown of cell membranes, which allow for the release of hydrolytic enzymes, and subsequently, accumulation of degradation constituents in the medium (Babayan & Bezrukov, 1985). Therefore, hydrolytic enzymes are a major concern during autolysis and among the enzymes involved (phospholipases, glucanases, and nucleases), proteases have been studied extensively (Aschteter & Wolf, 1985; Babayan & Bezrukov, 1985; Behalova & Beran, 1979). According to Behalova & Beran (1979) the relative protease activity could serve as an indicator of the rate of autolysis. Various studies on differing yeast autolysis conditions have led to contradictory results. The pH of wine is unfavourable for most yeast proteases, so that autolysis in wine is a specific case. Protease A is the only acid protease found in yeast, which explains its role in nitrogen release. Because protease A is an endoprotease, its action should result in peptide release rather than the release of amino acids.

In order to better characterize autolysis during wine aging on lees, Alexandre *et al.* (2001) followed the evolution of protease A activity and nitrogen fractions, focusing on amino acids and peptides, during alcoholic fermentation. They reported

for the first time protease A activity in yeast cells during alcoholic fermentation and autolysis in synthetic must under oenological conditions. It was shown that the enzyme activity significantly increase after sugar exhaustion. Previous research has shown that protease A activity or its expression is induced during stress conditions, especially nutritional stress such as nitrogen starvation (Nakamura *et al.*, 1997). A study by Harsen *et al.* (1977) supported the possibility that the synthesis of protease appears to be repressed by glucose.

Alexandre *et al.* (2001) noted no extracellular protease activity during alcoholic fermentation. Also, they found that extracellular activity is 3- to 30- fold lower than intracellular activity measured during alcoholic fermentation and autolysis, respectively. The very low extracellular activity raised the question of whether or not protease activity can diffuse outside the cell, and whether autolysis awaits breakdown of the vacuolar membrane in order to release the enzyme. It seems probable that the walls of dead cells remain unbroken during autolysis (though thinner than in living cells) and it could still be an efficient barrier (Charpentier & Feuillat, 1993). They reported that after alcoholic fermentation, the amino acid content increased constantly during autolysis. The kinetics of the amino acid liberation has been described as follows: the first stage is a passive diffusion from the intracellular pool, but the second stage is linked to the action of protease activity (Lurton *et al.*, 1989), especially exoprotease like carboxypeptidase (Sato *et al.*, 1997). Peptide release during autolysis is an important oenological factor for various reasons:

(i)   peptides as nitrogen compounds favour malolactic fermentation in wines;

(ii)   the peptides could interact with phenolic compounds and improve natural fining in the wine medium;

(iii) they contribute to the organoleptic properties of the wine.

The development of haziness (protein instability) in white wine is considered the next most common physical instability after the precipitation of potassium bitartrate (Van Rensburg & Pretorius, 2000). High ethanol and low pH levels induce this instability occurring after bottling and during storage (Van Rensburg & Pretorius, 2000).

Thus far, bentonite, montmorrelite clay, has been used for its absorption qualities in the removal of protein hazes. Bentonite adsorption is not specific for only proteins; it removes a variety of charged species and aggregates. As a result, large amounts of added bentonite can decrease the organoleptic properties of the wines by removal of important aroma and flavour components (Voiliey *et al.*, 1990). It also

generates large volumes of lees and causes a 5-20% loss of wine (Canal-Llaubères, 1993).

Attention has been directed towards proteolytic enzymes, specifically acid proteases, as an alternative protein depletion technique to remove excess wine protein. These enzymes effectively hydrolyze the peptide linkages between amino-acid units and could be very effective to reduce haze formation and improve clarification and stabilization. The enzymatic hydrolysis of proteins into small peptides and their component amino acids could also serve to provide low molecular weight peptides and amino acids that may be utilized as substrates by the micro organisms in fermentation (Alexandre *et al.*, 2001). Experiments were conducted on proteolytic enzymes included the use of grape proteases (Cordonnier & Dugal, 1968), yeast proteases (Feuillat *et al.*, 1980; Ledoux *et al.*, 1992) and exogenous proteases (Modra, 1989) and the experiments have been performed at low temperatures to appropriate wine making. It has become clear that treatment of juices with proteolytic enzymes does not confer protection against protein precipitation, because the wine proteins are not hydrolyzed under these conditions (Heatherbell *et al.*, 1984; Modra & Williams, 1988; Waters *et al.*, 1992; 1995).

These problems could be overcome by the use of a vacuolar protease A, encoded by the PEP 4 gene that is active at low pH levels (Pretorius, 2000). It could be possible that the wine becomes more stable due to the action of protease A and the release of mannoproteins. On the other hand, secretion of proteins and other compounds by the yeast may raise the protein content of the wine and increase the haze formation. The search for fungal enzymes that degrade these haze-forming grape proteins has thus far remained unsuccessful (Pretorius, 2000). Research has shown that the protein-instability is not dependent on the total protein content of the wine, but rather on specific protein fractions. These fractions are grape derived and their size and iso-electrical points are such that they are susceptible to solubility limitations (Boulton *et al.*, 1996). It is normally associated with pathogen related (PR)-proteins, its production is induced in the grape during fungal attack.

Therefore, it seems unlikely that protease will replace bentonite fining at this point. This is not due to the inactivity or insufficient concentration of enzymes, but the inherently resistance of haze-forming proteins to proteolysis. As these specific proteins form part of the defence system of the plant against fungal protease, their degradation will unlikely to be achieved by an acid protease. This resistance to degradation is not due to protection by other components, covalently bonded sugars,

or associated phenolic compounds. It seems that protein conformation is responsible; the interactions with juice components serve to mask the protease sensitive sites (Van Rensburg & Pretorius, 2000). It appears that correct viticultural practices hold the key to controlling these PR proteins.

Protease preparations are highly active on exogenous protein substrates, thus the 'ineffectiveness' of proteolytic enzyme treatment of wines can not be due to the presence of protein inhibitors (Modra & Williams, 1988; Waters *et al.,* 1992). These observations suggest that grape and wine proteins are extremely resistant to proteolytic attack under winemaking conditions (Waters *et al.*, 1992). Furthermore, this proteolytic resistance is not due to phenolic association or glycosylation (Waters *et al.*, 1995), indicating that it is an inherent property of these polymers.

Non-specific proteolysis is important not only for protein degradation and amino acid recovery, but also for processes such as generation of peptides for antigen presentation. Cells and organisms employ targeted proteolysis for the purposes of receptor activation, cell-cycle regulation, apoptotic signalling and transcriptional regulation, amongst others (Luo & Hofmann, 2001).

Landbo & Meyer (2001) found that protease addition to black currant pomace greatly increased extraction of antioxidative phenols. Their research showed protease to significantly increase plant cell wall breakdown in the pomace, and suggested it as another application for the industrial enzyme preparations. Perl *et al.* (2000) attempted to establish the effect of protease on plant material necrogenesis and speculated that protease technology could in future be used to improve transient *GUS* expression in plants by interfering with different stages in which the hypersensitive-like response (HR) might be modulated. Thus it may in future be possible to decrease the concentration of proteins in wine and reduce haze-formation using protease technology.

2.8   CONCLUSION AND PROSPECTS

Wine producers today face intensifying competition brought about by a widening gap between production and consumption. There is a shift of consumer preference away from basic commodity wine to top quality wine and economic globalisation. Thus there is a need for revolutionising the whole winemaking process. The industry needs to transform from being production-orientated to being market-driven, and this creates an increasing dependence on, amongst other, biotechnological innovation.

The single most important factor in winemaking is the organoleptic quality (appearance, aroma and flavour) of the final product. The bouquet of the wine is determined by the presence of a well-balanced ratio of flavour compounds and metabolites (Lambrechts & Pretorius, 2000). There is an 'untapped pool' of aroma and flavour compounds in glycosidically bond precursors, or their substrates are re-routed to a different metabolic pathway, which leads to a lesser expression of desirable characteristics. Significant progress has been made in the construction of yeasts producing colour- and aroma-liberating enzymes (pectinases, glycosidases, glucanases and arabinofuranosidases) and ester-modifying enzymes (alcohol acetyl transferases, esterases and isoamyl acetate hydrolyzing enzymes) (Van Rensburg & Pretorius, 2000; Laing & Pretorius, 1993; Pèrez-González et al., 1993; Lambrechts & Pretorius, 2000; Lilly et al., 2000). Furthermore, several yeasts have been developed that produce optimized levels of glycerol (Eglinton et al., 2002; Remize et al., 1999), those who produce fusel oils and those producing phenolic acids. The accumulation of compounds that enhance viability and vigour are also targeted, e.g. sterols, threhalose and glycogen (Bauer & Pretorius, 2000). Another aim is to expand the capacity of *S. cerevisiae* to use nitrogen sources such as proline (Henske, 1997).

Fermentation problems have commercial implication such as wine spoilage, waste of fermentation space or aroma loss. Among the targets for improving fermentation performance are increased resilience and stress resistance of active dried-yeast cells (Ivorra et al., 1999; Kim et al., 1996; Tanghe et al., 2000); improved grape sugar and nitrogen uptake and assimilation; enhanced resistance to ethanol and other microbial metabolites and toxins; resistance to sulphite, heavy metals and agrochemical residues; and reduced foam forming (Pretorius & Van der Westhuizen, 1991; Pretorius, 1999; Pretorius, 2000; Pretorius, 2001; Pretorius, 2002). The enhancement of yeast cell resistance could reduce stuck or sluggish fermentations,

which often leads to wine spoilage. Loss of wine due to spoilage impacts enormously on the economics of the wine industry each year.

The fining and clarification of wine includes some expensive and laborious practises that generate large volumes of lees for disposal and cause aroma and flavour loss to the wine. As an alternative to physical treatments; yeast are being developed to secrete proteolytic and polysaccharolytic enzymes that would remove haze-forming proteins and filter-clogging polysaccharides (Querol & Ramon, 1996; Pretorius, 1999; Van Rensburg & Pretorius, 2000; Gognies *et al.*, 2001; Laing & Pretorius, 1993; Pèrez-González *et al.*, 1993; Van Rensburg *et al.*, 1998).

The uses of enzymes in winemaking have been proven to be highly beneficial in various aspects, and it has caused great advances in the quality of wine. However, the application of enzymes is still in its infancy. Many problems have to be solved before their full potential can be reached. An understanding of the interactions between enzymes is needed to in order to explore the diverse advantages this technology holds. There is an urgent need for the improvement of enzyme-application at the following target areas:

(i)   high activity levels (substrate turnover rates per unit of protein) under wine-specific conditions;

(ii)   high levels of resistance to inactivation by heat treatment;

(iii)   low pH conditions;

(iv)   proteolytic attack.
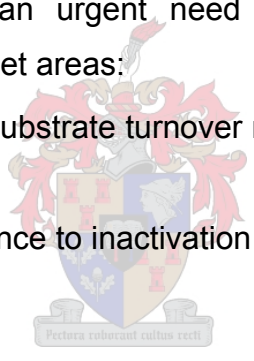
Also important is the stabilization of production costs and an extended shelf life under ambient conditions, as these have proven to be the most common problems at application level.

Commercial enzyme preparations are frequently used to supplement the endogenous enzyme activity. The production process of these types of preparations makes it impossible to obtain a pure enzyme product. The result is a mixture or cocktail of enzymes, which include a variety of different activities, such as glucosidases, glucanases, pectinases and proteases. One of the biggest threats in these enzyme cocktails is the presence of acid protease. This enzyme is potentially detrimental to all enzyme activities, as it uses other enzymes as its substrate, and may render them useless.

The exploration of enzyme potential will undoubtedly help the wine industry meet the technical and consumer challenges of the 21st century. Tremendous progress has been made over the past few years, generating a wide range of possibilities. However there are scientific, technical, economic, marketing, safety, legal and cultural issues that have to be addressed. At the present moment the deeply rooted concerns of consumers and traditionalists give the perception that it may border on 'commercial suicide' if any winery should prematurely pioneer the first wine fermented with recombinant yeast. It would however be self-crippling to the industry at large if the phenomenal potential of enzyme technology, which could propel the wine industry into the era of 'designer' products, would be ignored. The vast potential on a multitude of levels and applications will be realized, however, only if the application is judicious, systematic and achieved with high regard for the unique nature of the product. The diversity, which is the heart of the wine industry, should never be threatened by the use of enzyme applications. In fact, it should rather be applied as a protection mechanism in order to expand the diversity of high-quality wines.
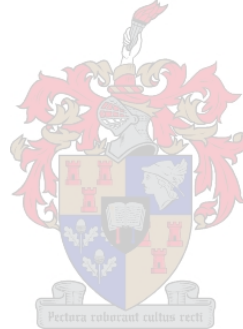
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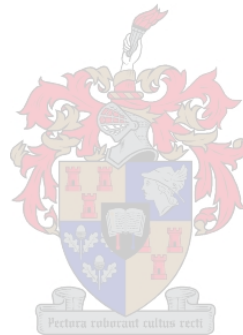
RESEARCH RESULTS

The effect of exogenous protease on the relative enzyme activity of β-glucosidase in oenological conditions.

The effect of exogenous protease on the relative enzyme activity of β-glucosidase in oenological conditions.

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The use of enzymes in winemaking has been proven to be highly beneficial, but the application is still in its infancy. Many problems have to be solved before their full potential can be reached. There is a need for the improvement of enzyme-application at the following target areas: high activity levels (substrate turnover rates per unit of protein) under wine-specific conditions, high levels of resistance to inactivation by heat treatment, low pH and proteolytic attack. Also important is the stabilization of production costs and a extended shelf life under ambient conditions.

The distinctive varietal flavour of wines is affected by the absolute and relative concentrations of many compounds, including monoterpenols. These occur as free volatile and odorous molecules, or glycosidically bound, odourless, non-volatile complexes. β-glucosidases are able to hydrolyze these glycosidic bonds and thereby releasing the aromatic terpenols. However grape-endogenous β-glucosidases are inhibited by glucose, exhibit poor stability at low pH and high ethanol levels typical in wine medium.

Commercial enzyme preparations are frequently used to supplement the endogenous enzyme activity. The production process of these types of preparations makes it impossible to obtain a pure enzyme product. The result is a mixture or cocktail of enzymes, which include a variety of different activities, such as glucosidases, glucanases, pectinases and proteases. One of the biggest threats in these enzyme cocktails is the presence of fungal acid protease. Also yeast protease poses a threat when the juice is inoculated with a yeast culture or when spontaneous fermentation occurs. This enzyme is potentially detrimental to all enzyme activities, as it uses other enzymes as its substrate, and may render them useless. Therefore in this study, we looked at the interactions between a yeast acid protease and a report activity (β-glucosidase), in order to quantify their interactions and influences in different matrixes. We aimed to establish the interactions between the enzymes in two *in vitro* conditions as well as during the fermentation of wine. Using pure enzyme preparations and enzyme assays, the *in vitro* studies indicated that protease did not significantly affect the β-glucosidase activity. Subsequently wine from Sauvignon blanc grapes were made, with varying enzyme applications. The data from the fermentation study indicated that protease did not significantly affect the β-glucosidase activity. Based on our data, we suggest that even though protease may potentially inhibit a desired enzyme activity, it does not seem to pose a threat of enzyme inhibition of β-glucosidase enzyme during the fermentation of natural wines.

3.2 INTRODUCTION

Enzymes play a distinctive role in the complex process of winemaking. From pre-fermentation through fermentation, aging and stabilization, the enzymes present are the driving force in this biotransformation. Thus, from a technical and chemical point of view, wine can be considered as a product of enzymatic transformation of grape juice.

Fungi obtain their nutrients by absorption of compounds from their environment. Therefore most fungi are secretors of hydrolases which could serve to degrade extracellular macromolecules to low molecular weight substrates, readily transported into the cell (Garraway & Evans, 1984). These substrates are then used to support and sustain growth and metabolism. In contrast to many types of yeast, members of the genus *Saccharomyces* do not normally secrete external hydrolases (Ogrydziak, 1993) although some mutants have been found that secrete vacuolar hydrolases to the external environment (Rothman & Stevens, 1986; Schaffner & Weissman, 1973).

The term enzyme is derived from the Latin words meaning "in yeast". Thought to be living things themselves, the opposite was proven by Eduard and Hans Buchner in 1897 when they discovered "zymase" (Walker, 1998). They prepared a cell-free extract for medical purposes, and preserved it with sugar. The mixture began to bubble and produce foam, thus cell-free fermentation took place.

Enzymes originate from a multitude of habitats. The grapes itself produce enzymes, as well as yeasts and other microbes (such as fungi and bacteria) associated with vineyards and cellar equipment. Certainly the most noteworthy fact about enzymes is their specificity. They can act on only one or a limited number of substances, recognising a specific chemical group. This, as well as their activity levels are poorly understood, but is still of greatest importance in the fermentation process. With the understanding and quantification of enzyme kinetics, it is possible to optimise preferred interactions to favour specific applications and decrease or even eliminate unwanted or negative influences. Most of the enzymes originating from the grape are either insufficient in quantity or ineffective because of other influences, such as sulphur dioxide, temperature or microbes. It is therefore only natural that commercial enzyme preparations are used to enhance the reactions. These preparations are obtained from organisms such as *Aspergillus niger,* which is cultivated under optimal conditions for growth and production. Of the 2500 different

enzyme-catalysed reaction recognised by the International Union Handbook of Enzymes Nomenclature (Gacesa & Hubble, 1998) only about 30 are used in industrial preparation. There are four major areas where enzymes are of particular use to improve the winemaking process:

(i)   juice clarification, must processing (e.g. pressing);

(ii)   reduction of ethyl carbamate;

(iii)   release of varietal aromas from the precursor compounds;

(iv)   reducing alcohol content.

Grape processing can be divided into three stages: (i) Pre-fermentation, (ii) Fermentation and (iii) Post-fermentation. There are various enzymes that are of particular importance during specific stages of the grape's processing.

Monoterpenes and monoterpene alcohols play an important role in the flavour of grapes and wines. Although occurring in all cultivars, it is less pronounced in non-muscat varieties, existing as a subtle support for flavour, whereas it is very important in the distinctive aromas of muscat varieties. The flavours they impart are not only linked to perfume-like and floral, but also include characters described as spicy, peppery, smoky and grassy. The major fractions of these compounds occur in the grape as glycosidically bound forms (Günata *et al.*, 1985; Voirin *et al.*, 1992; Williams *et al.*, 1982), which render them non-volatile and therefore flavourless.

There are various sugar moieties to which these terpenols are bound. Glycosidic bonds can be liberated by two methods: the first, acid hydrolysis has the disadvantage of possibly changing the varietal aroma of the wine. The second method is via enzymatic hydrolysis, which is attracting a lot of attention as an easier and more efficient means to improve varietal aroma and flavour. The hydrolysis mechanism is now well established (Günata *et al.*, 1985; 1988) and entails specific glycosidases active in two successive steps. In the first step, the action of α-L-arabinofuranosidase, α-rhamnosidase, β-xylosidase or β-apiosidase is necessary to cleave the inter-sugar linkage (Günata *et al.*, 1988), and this is followed by the second step in which a β-glucosidase liberates the aglycone. In cases where the disaccharide moiety consists of two glucose units, only a β-glucosidase is needed to facilitate the complete reaction (Haisman *et al.*, 1967). The release does not necessarily consist of a terpenol; it could have other beneficial properties, especially the release of resveratrol, a reported anti-oxidant (Vrhovsek *et al.*, 1997).

The use of enzymes have attracted more interest in recent times, as enzyme systems in native organisms are sometimes not efficient enough for flavour release under the conditions that apply, for instance the low pH and high glucose environment of juices. Glycosidases occur nearly in every organism, however, not all of these enzymes are suitable for expression in other organisms, for example those from plants exhibit high pH optima (pH 5 for *Vitis vinifera*), and the enzyme is virtually inactive at pH 3 - 4, the pH of juices and wine (Aryan *et al.*, 1987). Also the glycosidases from fungi are notoriously inhibited by glucose concentrations even as low as 9% (Riou *et al.*, 1989). These enzymes are also more active at high pH values (Woodward & Wiseman, 1982). Bacterial enzymes generally have the disadvantage of being active at high temperatures, 50°C and higher (Woodward & Wiseman, 1982). Yeast glycosidases exhibit the most favourable characteristics, and have therefore become the focal point of characterization and use of heterologous expression in other strains. These β-glucosidases have been studied intensively for future applications (Ranyal & Guerineau, 1984; Kuranda & Robbins, 1987: Machida *et al.*, 1988; Günata *et al.*, 1990; Gueguen *et al.*, 1994; Rosi *et al.*, 1994; Gueguen *et al.*, 1995; Riou *et al.*, 1988).

In this study we have aimed to establish the interactions between protease and a β-glucosidase, in order to quantify their interactions and influences in different media. Of equal importance is the individual and overall effect on wine quality. Here we present the results of *in vitro* studies and a fermentation study.

3.3   MATERIALS AND METHODS

3.3.1   *IN VITRO* STUDIES

The *in vitro* study was set up using two media (a wine and a buffer) as is indicated in Table 9. The samples were labelled as follows: Control, β-glucosidase and Protease, according to the enzyme additions made to each. All of these samples were done in triplicate to ensure statistical validity of the data. Samples were incubated in a water bath at 25°C in 2 mL sterilised eppendorfs with a volume of 1,5 mL, to which the enzyme additions were made.

Wine: The wine used was a Sauvignon blanc 1999, from the Paarl area. It had the following analysis: pH 3,4; total acidity 6,23 g/L (measured as tartaric acid

equivalent); residual sugar 2,2 g/L (measured as glucose equivalent), alcohol 12,0% v/v. The wine was stored at 15°C until used.

Citric buffer: The buffer was prepared using varying volumes of two stock solutions (0,1 M citric acid and 0,1 M sodium citrate), blended to a pH of 3,4 to correspond to that of the wine medium (Lillie, 1948). 150 µL of buffer at 0,1 M was placed in 2 mL eppendorfs and filled to a volume of 1,5 mL with distilled water.

Cellobiose: Cellobiose was used as a substrate for the β-glucosidase enzyme assays. 40 µL of a 16% stock solution was added. The stock solution was prepared by dissolving 1,6 g of cellobiose in 100 mL of distilled water.

TABLE 9: Experimental set up of *in vitro* studies

Medium	Control	β-glucosidase	Protease
Buffer	+cellobiose	+cellobiose + β-glucosidase	+cellobiose+ β-glucosidase +protease (3 concentrations)
Wine	+cellobiose	+cellobiose + β-glucosidase	+cellobiose + β-glucosidase +protease (3 concentrations)

β-glucosidase enzyme: Megazyme β-glucosidase. The enzyme addition was made at 10 g/hL, as per manufacturer suggestion.

Assay for β-glucosidase activity: The Sigma Glucose Trinder assay kit was used to determine the activity of the β-glucosidase enzyme. The Glucose Trinder reagent was prepared according to the instructions. Spectrophotometer set to 505 nm, with the absorbance reading set to zero with redistilled water as reference. A series of kuvettes was set up and labelled: reagent blank, standard, controls and samples (wine and buffer). 1,0 mL of glucose trinder reagent is pipetted into the tubes and left to warm up to assay temperature (25°C), as the stock solution is kept in refrigeration at 4°C. At timed intervals 5 µL of deionised water, standard, control and samples were added to the labelled kuvettes. It was gently mixed using a vortex. The kuvettes were incubated for exactly 18 minutes at ambient temperature (18-26°C). The absorbance (A) reading was taken at 505 nm at the same time intervals as for the additions. The glucose concentration was determined as follows:

A(sample) – A(blank) / A(standard) – A(blank) x Concentration of standard. It relates to a glucose concentration in mg/dL. To convert the results to SI units, the values are multiplied by 0,5555 to give it in units of mmol/L.

Protease enzyme: Protease A from Sigma. It is an endopeptide EC 3.4.23.6 from a bakers yeast origin. It is presented as a mass of 1 mg, with 34 units per mg. As it is presented in a solid form, it was suspended in 5 mL of distilled water, to prevent interference from proteins or DNA. Aliquots of 100 uL were stored at 4°C. Additions were done in 3 different concentrations as follows:

  (i)   Concentration 1 = 1 unit of protein,
  (ii)   Concentration 2 = 5 units of protein
  (iii) Concentration 3 = 10 units of protein

Assay for protease activity: An assay by Roche was used to determine activity, using a universal protease (casein and resorufin-labelled substrate) as is indicated in Table 10. Absorbance was read at 574 nm, using 2 mL plastic kuvettes. The reagents are stipulated as follows:

  (I) Substrate solution 0,4% casein w/v in redistilled water.
  (II) Incubation buffer 0,2 M Tris-HCl pH 7,8 and 0,02 M $CaCl_2$.
  (IV) Sample solution (wine or buffer medium).
  (V) Stop reagent 5% Trichloroacetic acid (w/v) in redistilled water.
  (VI) Assay buffer 0,5 M Tris-HCl, pH 8,8.

TABLE 10: Protease enzyme assay

Pipette into reaction vessel	Sample blank	Sample
(I) Substrate solution	20 µL	20 µL
(II) Incubation buffer	50 µL	50 µL
(III) Redistilled water	120 µL	20 µL
(IV) Sample solution	-	100 µL
Incubate at 37°C, for 60 minutes. Stop the reaction by addition of Stop Reagent		
(IV) Stop reagent	480 µL	480 µL
Incubate for 10 minutes at 37°C, subsequently centrifuge for 5 minutes and pipette supernatant into kuvettes		
Supernatant	400 µL	400 µL
(VI) Assay buffer	600 µL	600 µL
Mix and immediately read absorbance of sample against blank at 15-25°C, 547 nm.		

3.3.2   FERMENTATION STUDY

Experimental scale winemaking: Sauvignon blanc juice was used from grapes of origin Paarl. The grapes were harvested, destemmed, crushed and then pressed. The juice sample was taken at the initiation of pressing, to which 30 parts per million (ppm) SO_2 was added. It can therefore be considered as 'free-run' juice. The temperature of the juice at crushing was 22°C. The juice analysis was as follows: ±220 g/L sugar, pH 3,8; total acidity 8,4 g/L. Overnight settling, without bentonite or enzyme additions then took place at 15°C. The juice was transferred to glass fermenters with a volume of 4,5 L using N_2-gas (0,5 kPa pressure) to prevent oxidation.

The 4 sets of samples were done in triplicate to ensure statistical validity of the data and the samples labelled as follows:

> **Control** – normal fermentation, no additions made
>
> **β-glucosidase** – β-glucosidase enzyme added at 5 units/wine
>
> **Protease** – protease A addition at 3 units/wine
>
> **β-glucosidase** + **Protease** – β-glucosidase at 5 units/wine and protease A at 3 units/wine

Fermentation caps (S-shaped, filled with distilled water) were used to prevent oxidation. Fermentations were carried out at 15°C in temperature controlled rooms. Samples were taken from each fermenter using sterilized Pasteur pipettes and stored in autoclaved eppendorfs at 4°C. Sampling was done every 3 days. The wines were fermented dry (less than 5 g/L residual sugar, as per law) and cold stabilized at -4°C without being racked off the lees. The wines were not filtered prior to bottling. Wines were bottled separately using N_2-gas into sterilized bottles of 750 ml capacity and ring capped. Wines were stored at 12ºC in a temperature and humidity controlled room until analyzed.

Enzyme additions: Proteinase A from Sigma and Megazyme β-glucosidase were used for the enzyme additions; which is the same enzymes used for the additions in the *in vitro* experiments. The enzymes were handled as specified by the supplier.

Fermentation yeast: After the enzyme additions were made, the juice was inoculated with a *Saccharomyces cerevisiae* strain, VIN13, with a final concentration of $1x10^6$ cells/mL. The active dry yeast culture (ADYC) was rehydrated as specified by the supplier.

Enzyme activity: Protease activity was measured with the enzyme assay as described for the *in vitro* studies. The β-glucosidase activity was indirectly measured by organoleptic evaluation of the wines, see below.

Chemical composition: The levels of ethanol (% v/v), residual sugar, pH, total acidity, malic acid and lactic acid in the finished wines were determined using the methods described by Iland *et al.* (2000). The values obtained were confirmed using the Foss Wine Scan (Institute of Wine Biotechnology, Stellenbosch University). The Wine Scan method uses infra red light to determine the concentrations of various wine compounds.

Organoleptic evaluation: Organoleptic evaluation of the wines were done in a professional wine tasting facility (Department of Oenology, Stellenbosch University), which ensured the correct lighting and sufficient air circulation. The tasting panel was selected from South African Wine and Spirits Board accredited tasters, who have successfully completed the board's wine judging examination, and have extensive training and experience in the local industry and on the Veritas competition. Samples were arbitrarily numbered to ensure a good statistical spread. The samples were given in random order, with repetitions of samples to ensure consistency; as well as prevent palate fatigue, over-sensation or blunting of the palate. Line scales with a range of 0 – 10 were used to evaluate the wine. Line scales were provided in three categories, with labels for each factor to be evaluated:

1) Overall Quality: the quality, flavour concentration, palate weight and general intensity of aroma and flavour were judged;

2) Floral and Fruit: typical Sauvignon blanc flavours such as green pepper and grassiness, were judged alongside some other fruit and floral aromas and flavours;

3) Wine Chemical: alcohol, acidity and sweetness were evaluated, and scales were provided to indicate any problems such as oxidation, volatile acidity, etc.

Then the tasters were required to draw up a preference list including all the wines, listing which wines they considered to be of highest quality. Results were analysed to be presented in a radar graph.

3.4 RESULTS AND DISCUSSION

3.4.1 *IN VITRO* STUDIES

The *in vitro* studies were done to establish possible interactions between the β-glucosidase activity and acid protease. As the enzyme kinetics of these two enzymes have been studied in detail, we hoped to detect clear trends related to a defined set of conditions and to make conclusions based on the results, in order to suggest the possible interactions in a fermentation medium. Therefore a buffer and a wine medium were used to modulate the in vitro conditions.
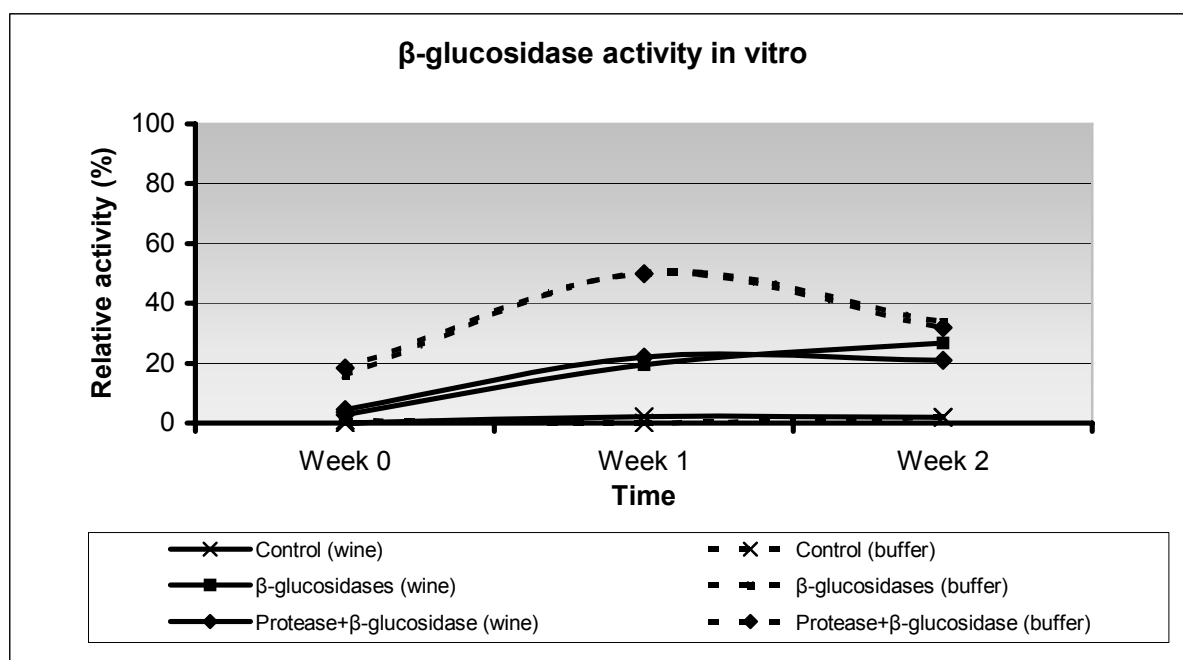
FIGURE 9: Relative β-glucosidase activity *in vitro*. The scale of relative activity (%) indicates the percentage of experimental values in two different reaction media, wine (solid line) and buffer (dash line), relative to the maximum value of the glucose release by the enzyme in each condition. The values shown here are the means from the assays done in triplicate ± 5% standard deviation. —✕— Control, —■— β-glucosidase, —◆— Protease + β-glucosidase. Only the data from two higher protease concentration samples were used (an average of the two), as these showed greater stability over the time period.

β-glucosidase activity was observed in the samples where the enzyme was added as opposed to the sample containing only the substrate (cellobiose), as is illustrated in Figure 9 (the standard deviation was < 5%). In the control buffer sample, the substrate, cellobiose, was not degraded; as no glucose were detected. This result supports the statement that an active β-glucosidase enzyme is needed to degrade the cellobiose. Figure 9 shows no significant difference between the relative activity

of the β-glucosidase enzyme when it is the single addition to the medium, or when protease is present. Based on these results we suggest that over the period of enzyme treatment, the presence of an active yeast acid protease had no significant inhibiting effect on β-glucosidase activity.

No significant change was detected in the glucose concentration in the control wine sample, but a significant increase in glucose was detected in both the enzymatic samples. It is again clearly shown by the data presented that the sample containing the protease has similar reported activity (expressed as glucose release) as the sample without protease, thus no significant affect on the β-glucosidase activity was detected. From this data we conclude that the exogenous yeast protease does not significantly inhibit the β-glucosidase activity in a wine medium, nor in buffer medium.

It is interesting to note that the β-glucosidase activity increased to an average of 50% in the buffer medium over the first week; thereafter the activity was reduced to an average of 35% at the end of the second week. In the wine medium an increase in β-glucosidase activity is seen in the first week to an average of 20%, with a further increase in the second week to an average of 30%.

In both the media the effect of protease on the β-glucosidase activity is insignificant; therefore the smaller increase of the β-glucosidase activity in the wine medium seems to be linked to an inhibitor which is present only in the wine medium. From these results it could be suggested that it is a wine-related compound that is responsible. It could further be suggested that the presence of ethanol (ethyl alcohol), a well-known enzyme inhibitor, could be responsible.

Although β-glucosidase activity shows a greater increase over time in the buffer medium, the absolute value for glucose present was still greater in the wine. This can be attributed to the fact that the wine had a residual sugar (RS) of 2,2 g/L as stated before. The presence of this sugar residue, inflates the values obtained from glucose analysis. The majority of this sugar residue is yeast unfermentable sugars; however, a small percentage of glucose and fructose could be present in dry wines. But, as all the wine samples were obtained from a single wine, this RS was reduced to zero for data analysis.
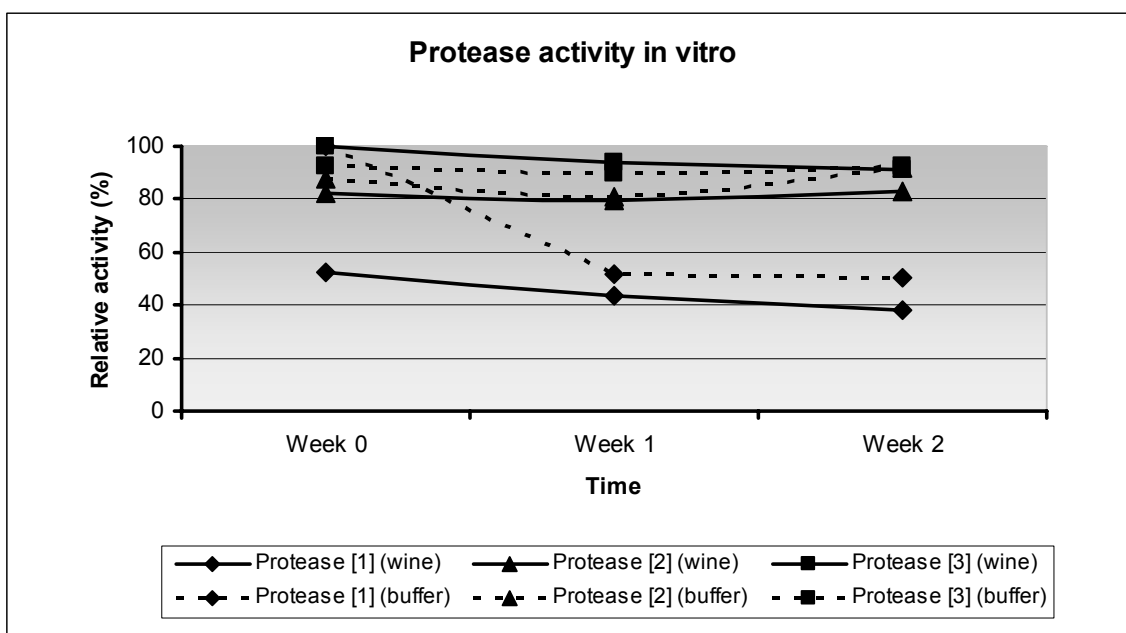
FIGURE 10: Relative protease activity *in vitro.* The scale of relative activity (%) indicates the percentage of experimental values in two different reaction media, wine (solid line) and buffer (dash line), relative to the maximum value of the absorbance obtained by the enzyme in each condition. The values shown here are the means from the assays done in triplicate ± 5% standard deviation. —◆— Protease [1], —▲— Protease [2], —■— Protease [3]. The data from all the protease concentration samples are presented, to indicate the stability of the two higher concentrations as compared to the lowest.

Protease activity was detected only in the samples containing additions of the protease enzyme preparation, but not in the control or β-glucosidase activity samples, as is shown in Figure 10. It is interesting to note that for protease Concentration 1, the measured relative activity decreased with an average of 20% after 2 hours, and with about 50% after 1 week in the buffer. This trend was observed in both media, however it was more pronounced in the buffer. This trend of initial decrease however, was not detected for the two higher protease concentrations, which showed no significant change during the time intervals mentioned.

When the protease activity values in wine is compared to that in buffer, it is very interesting to note that the relative protease activity in wine is on average only 20% of that measured in the buffer medium (data not shown). This level of relative activity may, however, be too low to affect (inhibit) other enzyme activities in the wine medium. This data also correlates with the results found in the relative activity assay for β-glucosidase. The lower relative protease activity reported in the wine medium (as opposed to buffer) supports the finding of higher activity of the report enzyme (β-glucosidase) in the wine medium.

These results indicate that the relative protease activity is consistently higher in the buffer medium, and that although the higher protease concentrations does not relate to a significant activity increase over time, it has potentially greater inhibiting power than the lower Concentration 1 which shows a significant decline over the time period.

3.4.2 FERMENTATION STUDIES

With the interactions between the β-glucosidase and the protease activities defined in the conditions of the *in vitro* studies, fermentation studies were done to establish whether the results reported correlated with that of the fermentation conditions, or if the interactions were in fact very different.
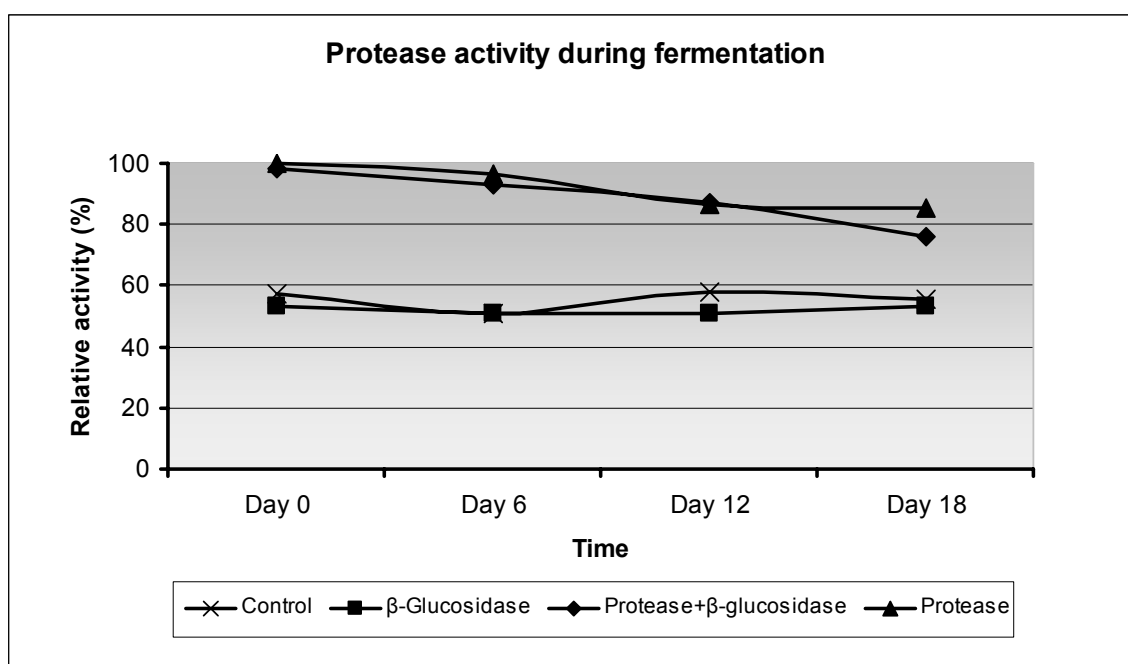
FIGURE 11: Relative protease activity during fermentation. The scale of relative activity (%) indicates the percentage of experimental values in the fermentation media (solid line), relative to the maximum value of the absorbance obtained by the enzyme. The values shown here are the means from the assays done in triplicate ± 5% standard deviation. —✕— Control, —■— β-glucosidase, —◆—β-glucosidase + protease and —▲— Protease.

As the results in Figure 11 indicate, a protease activity was detected in the control and β-glucosidase samples during fermentation. Thus it can be suggested that a endogenous acid protease activity was present. The origin of this activity may be the yeast responsible for the alcoholic fermentation or the grapes themselves. The

protease activity in the samples containing protease additions decreased only to about 80% after 15 days, which is still a significant factor in terms of inhibition potential in the medium.

From these results we have shown that acid protease is active even after 18 days of fermentation. However, this is the first report to indicate that the protease does not significantly affect the β-glucosidase activity during fermentation. Neither synergism, nor inhibition was detected over the monitored time period.

When the fermentation data is compared with that of the *in vitro* studies, we can conclude that the acid protease shows relative activity in all the media tested and it can be concluded that for at least 18 days of fermentation, protease is active in the medium. From our data we have shown that protease exhibits a more stable (constant over time) relative activity in buffer, and a declining relative activity in fermentation. It might be related to inhibiting factors such as alcohol levels and CO_2 formation in the fermentation. It is also interesting to note that the absolute activity in the fermentation was ± 25% of that recorded in the *in vitro* conditions. The difference is significant, but the relative activity in the wine fermentation is still capable of degradation of proteins, thus still a possible influence on β-glucosidases.

Sensory evaluations were conducted at the end of the maturation period, 8 months after the initiation of fermentation. There were only slight differences detected between the different wines, but no significant differences between the repetitions of applications (triplicate). Most of the tasting panel felt that the control wines and wine treated with only protease tended to be thin and watery compared to the wines treated with β-glucosidase enzymes. The wines which had any protease additions (both as only addition and in combination with β-glucosidase) exhibited a medicinal character (Figure 14) and it subsequently scored lower for the overall quality assessment, see Figure 12.

The wines made with only β-glucosidase enzyme scored slightly higher compared to the wines treated with both enzymes (β-glucosidase and protease), and significantly higher than the control and the protease sample. The β-glucosidase treated wines were rated as having a good balance and fresh and crisp aromas. It also strongly exhibited typical Sauvignon blanc aromas such as grassy tones, green pepper and tropical fruit aromas, see Figure 13.

From these indications it can be suggested that β-glucosidase was active during the fermentation and as a result enhanced the organoleptic profile of the all the wine samples by strengthening the terpenol-related aromas. It seems that the protease additions had a slightly negative effect on the aroma profile, in that it exhibited a medicinal character; but it did not seem to significantly affect the activity of the β-glucosidase enzyme in releasing aromatic compounds.
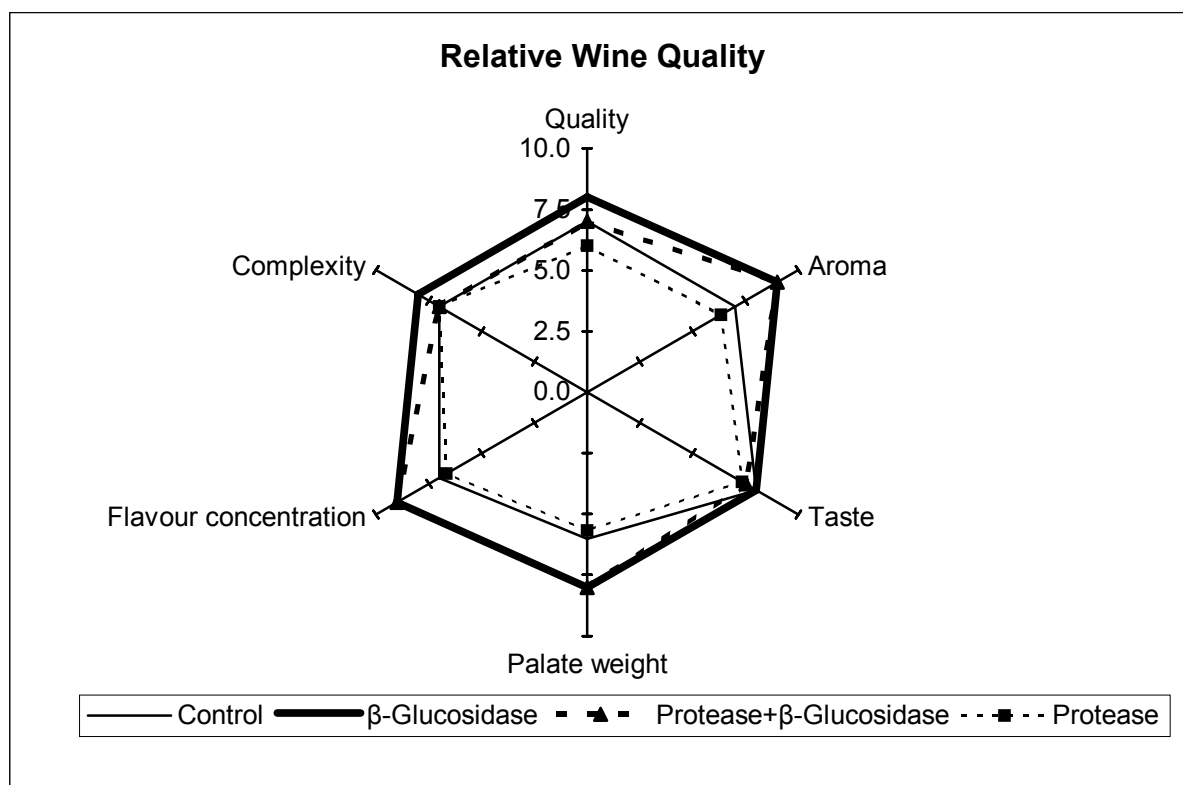

FIGURE 12: Relative wine quality (organoleptic perception).

From our data, we suggest that the protease had no significant affect on the β-glucosidase activity in the fermentation. These results are strongly supported by the results found in the *in vitro* studies, where protease was reported to have no significant affect on the β-glucosidase enzyme activity.

The wines were chemically analysed by methods described by Iland *et al.* (2000), standard analysis required for market ready wines were done, and results similar to the industry standard were recorded. The values obtained were confirmed using the Foss Wine Scan (Institute of Wine Biotechnology, Stellenbosch University), these values are stated in Tables 11 & 12.
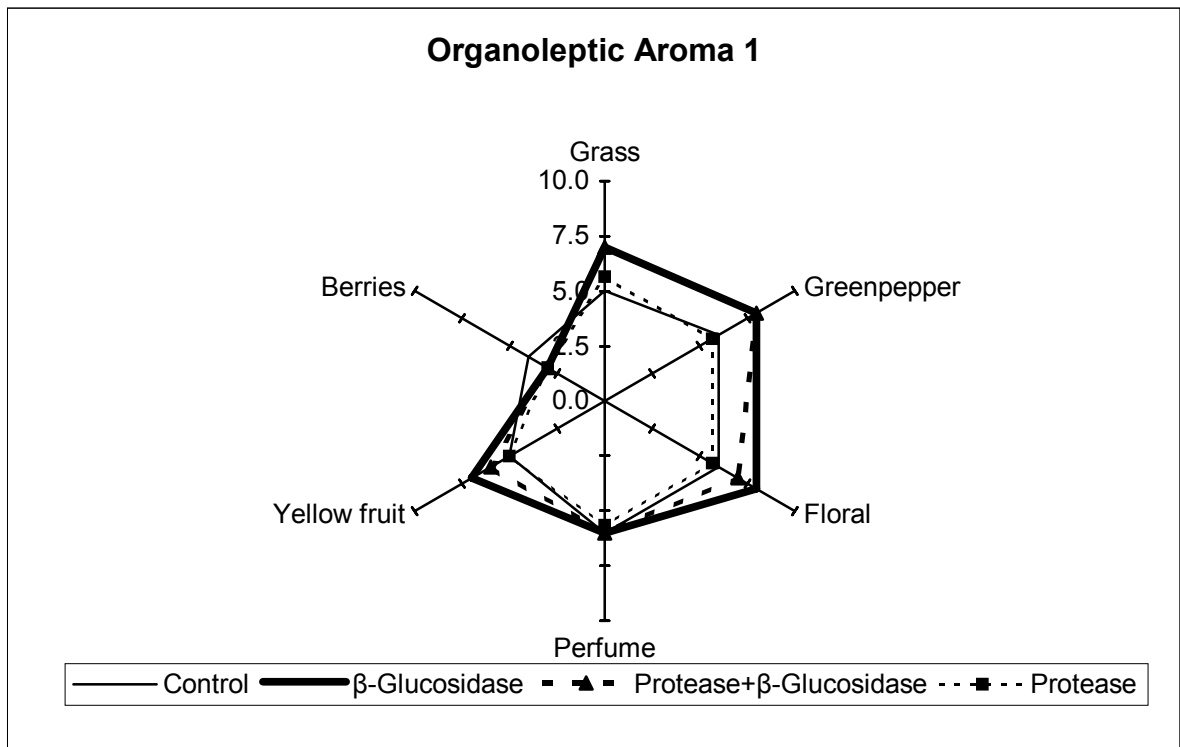
FIGURE 13: Relative fruit and floral aromas.
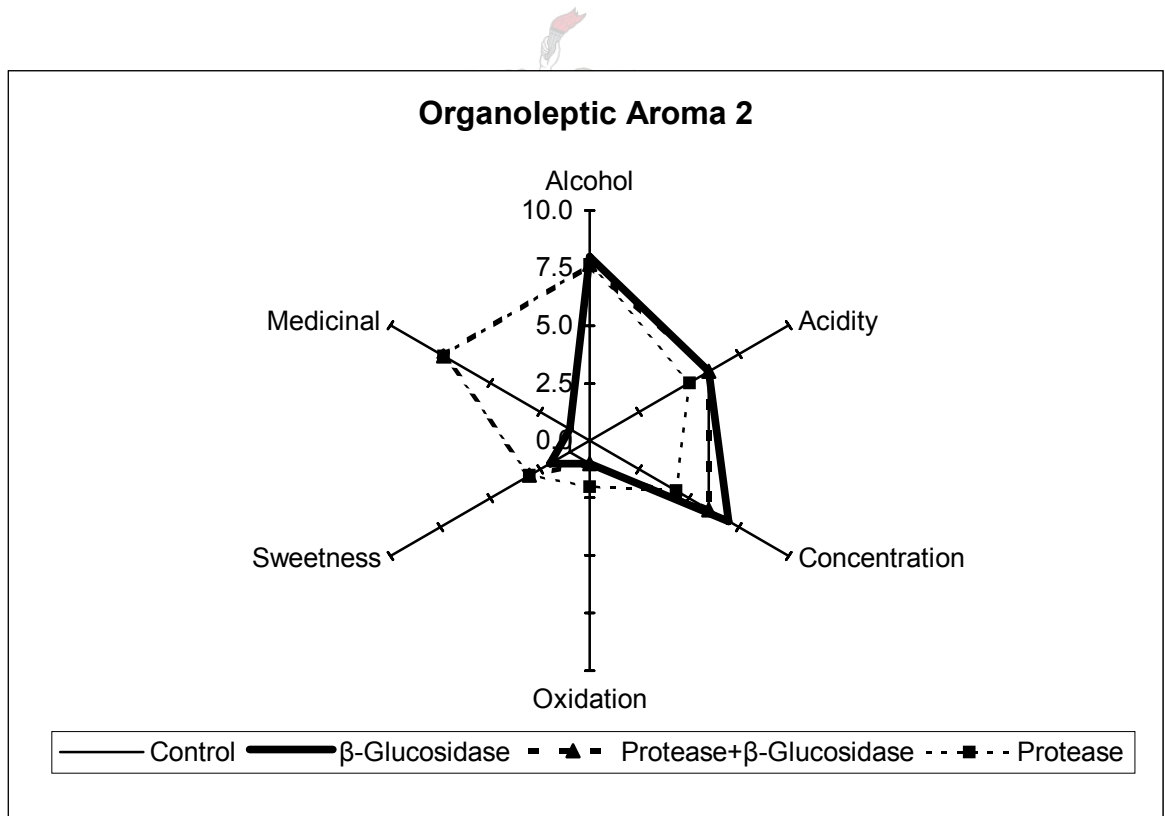
FIGURE 14: Wine concentration and other taste perceptions.

The wines treated with both enzymes showed a residual glucose content of 0,85 g/L. None of the other treatments showed any residual glucose. Residual fructose were however present in all the wines, with an average of 3,98 g/L. The ethanol content of the wines treated with β-glucosidase was above 13,0% (v/v), whereas the other wines had very similar analysis, with values between 12,0 and 13,0% (v/v). This may be partly explained by the release of glucose molecules by β-glucosidase and these may be fermented to ethanol. However, it seems unlikely that the entire volume of glucose increase could be attributed to enzyme activity.

TABLE 11: Wine Scan analysis 1

Sample	pH*	Total acidity** g/L	Glucose g/L	Ethanol % v/v
Control	3.22	6.11	0.01	12.82
β-glucosidase	3.19	6.19	0.03	13.43
Protease	3.21	6.26	0.03	12.95
Protease + β-glucosidase	3.16	6.14	0.85	12.56

*pH measured as standard SI unit.
**Total acidity measured as tartaric acid equivalent

TABLE 12: Wine Scan analysis 2

Sample	Volatile acidity* g/L	Malic acid g/L	Lactic acid g/L	Fructose g/L
Control	0.29	3.10	0.05	4.33
β-glucosidase	0.26	3.14	0.00	2.78
Protease	0.30	3.20	0.01	4.00
Protease + β-glucosidase)	0.22	3.07	0.00	4.60

*Volatile acidity measured as acetic acid equivalent

Our data also included measurements at 1 and 2 hours after the additions of the enzymes (data not shown). The reason for this is to establish a 'benchmark reading', if the enzyme reactions were taking place in a matter of hours, it would prevent the study continuing for an extended period of time. But to focus on the results after 1 and 2 weeks, is more closely related to the actual winemaking conditions in the industry. Another approach would be to wait during fermentation while protease inhibits the other enzyme activities present in the medium and then allow β-glucosidase to perform after fermentation has been completed. But as we have shown, protease does not significantly affect the β-glucosidase activity, and therefore the time of application is irrelevant. Thus we can conclude that the natural release of components by an enzyme activity over a period of time delivers the best quality wine. The reason for this is that other unwanted enzymes present in the medium are losing activity over time, which will result in fewer breakdowns of the newly formed components, and also the stability of newly formed components is enhanced when released over time.
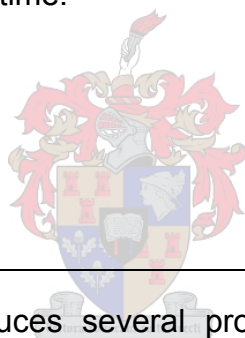
3.5   CONCLUSION

Yeast (*Saccharomyces*) produces several protease activities, of which the acid protease is the only one active in an acidic medium. It is the only protease from yeast origin that is active in a low pH medium that could survive the winemaking process. It is from a vacuolar origin (Ribéreau-Gayon *et al.*, 2000) to protect the cell from the enzymes destruction. It plays an essential role in the turn over of cellular proteins. In addition, the protease A is indispensable in the maturation of other vacuolar hydrolases as it converts the precursor forms into active enzymes (Ribéreau-Gayon *et al.*, 2000). It is released upon cell death and autolysis (Behalova & Beran, 1979), which is the degradation of the cell by its own proteases. This enzyme activity is used in the production of Champagne wines, where the mannoproteins released by β-glucanases (Ribéreau-Gayon *et al.*, 2000) during autolysis contribute to the organoleptic properties of the wine.

From our data we have shown that acid protease is active in a wine fermentation for at least 18 days. Also we suggest for the first time that protease does not significantly affect β-glucosidase during the fermentation of wine. This was

confirmed by *in vitro* studies where the protease had no significant affect on the β-glucosidase activity in either of the two media used.

We conclude from our data that protease does not significantly inhibit the β-glucosidase enzyme during fermentation. Future work might be dedicated to more detailed studies on the interactions between acid protease and other enzymes, as protease's kinetics is very well documented. The characterization of these enzymes' interactions may lead to applications in the elimination of unwanted enzyme activity, reduction of physical instability and enhanced varietal aroma. Studies are underway in our laboratory to continuously screen enzymes and their possible application in winemaking.

3.6   ACKNOWLEDGEMENTS
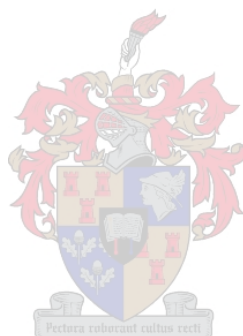
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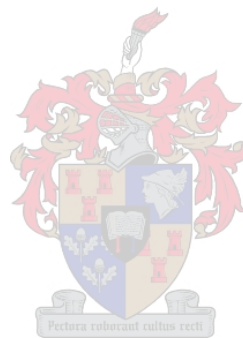
GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1   PERSPECTIVES

Wine is one of the most complex beverages known to humankind. It is one substance consisting of a wide spectrum of flavours, aromas and other organoleptic experiences. These flavours stem from a very complex, non-linear system of interactions between hundreds of compounds originating from the grape, the fermentation process and the aging of the product. The aroma of wine is a cumulative result of absolute amounts and specific ratios of many interactive compounds, rather than being attributed to a single 'impact' odorant (Noble, 1994; Cole & Noble, 1995).

The secondary aroma and flavour of the wine is the result of the influences of the yeast and fermentation conditions. This is because the flavour is derived from secondary metabolites of the grapes that undergo various changes in biosynthetic pathways. During fermentation the complexity of the wine is increased through the extraction of compounds from the solids present in the must, by modifying grape derived compounds, and producing a substantial amount of yeast metabolites (Scheier, 1979; Rapp & Versini, 1991).

A high percentage of the metabolites occur in their respective, non-volatile O-glycoside forms, rendering them odourless. This, however, creates a vast pool of aroma compounds that could be released over time, thus sustaining the aroma of the wine over a time period. Several studies have showed that the enzymatic hydrolysis can increase the amount of 'free' aroma compounds and thus drastically intensify the varietal character (Canal-Llaubères, 1993; Williams *et al.*, 1982). An example of this potential aroma pool is the monoterpene alcohols that naturally occur as glycosidically bound structures in grapes.

β-glucosidases are enzymes able to cleave these glycosidically bound aroma precursors. Indigenous grape glucosidases (Canal-Llaubères, 1993) are, however, inhibited by low pH and high ethanol levels. In contrast, the β-glucosidases of *Aspergillus* and other fungal species are mostly tolerant of the pH, glucose and ethanol levels in wine. These enzymes have been used as components in

commercial preparations, which are added to the fermentation or to young wines (Canal-Llaubères, 1993).

Chapter 1 of this thesis sketches the importance and place of wine in the society today as a first choice lifestyle product of moderation. Fundamental innovations in various aspects of the winemaking process are revolutionizing the wine industry, while the market pull and technology push continue to challenge the tension between tradition and innovation. Now there are new, and for the moment controversial, ways of innovation – genetic engineering, protein engineering and the use of enzyme kinetics. This chapter elaborates on the importance of enzyme kinetics in various areas concerning the winemaking process. It is involved in the grape during maturation for the production of the potential aroma profile, in the fermentation process, the reduction of potential hazardous components and stabilizing of the wine during maturation. It shows that enzymes can be employed to reduce the inherent drawbacks of industrial (chemical) transformation processes (Van Rensburg & Pretorius, 2000; Underkofler, 1976). The various enzymes currently used in industrial processes are mentioned alongside the different types of specificity (Van Rensburg & Pretorius, 2000). The last section of this chapter states clearly the danger of an acid protease, which can destroy all the possible advantages of enzyme kinetics. This motivates the research presented in the thesis; to identify and characterize the effects of an acid protease on one of the most commonly used enzymes, β-glucosidase, in the winemaking process.

From Table 4 it is clear that enzymes have various applications in the winemaking process, and are highly beneficial in enhancing the fermentation performance, strengthening the varietal aroma, broadening stability, as well as adding certain health benefits (Van Rensburg & Pretorius, 2000). Thus it seems inevitable that if the multitude of reactions that these enzymes catalyse is going to be exploited, the activity in question will have to be enhanced by an enzymatic preparation.

The first section of **chapter 2** focuses on plant cell wall and grape berry polysaccharides. It describes the structural features of the most important polysaccharides in terms of their basic structure as well as oenological significant substituents. The aromatic residues in plant cell walls are discussed. Only a small fraction of the odorous compounds (monoterpene alcohols) are in a free and volatile state and can contribute to the aroma of the wine. The larger fraction is bound to

saccharide moieties in the wine, rendering them non-volatile and odourless (Günata *et al.*, 1985; Voirin *et al.*, 1992; Williams *et al.*, 1982). Thus as large part of the possible aroma could be "lost" as it can not be detected by the consumer. The aroma of fermentation and maturation are equally important, as many chemical shifts between related compounds occur (Hennig & Villforth, 1942; Buttery *et al.*, 1971). Aging of wine in wood contribute significantly to the extraction of different aromatic compounds and very slow, controlled oxidation stabilises many compounds as well as the wine colour (Puech, 1987; Vivas & Glories, 1996; Singleton, 1987). Carbohydrate degradation, carotinoid degradation, formation of esters and shifts in terpeniod concentration are the most important chemical changes that occur during bottle maturation (Rapp *et al.*, 1985a; b).

We are introduced to the properties and characteristics of the various classes of enzymes that are presently used during winemaking processes. The method of degradation of the substrate by the specific enzyme is discussed. It looks at the possibility of releasing these bound monoterpenes alcohols and thus increasing the flavour of the wine through enzymatic hydrolysis (Günata *et al.*, 1988). The importance of pectinases and cellulases in the management of clarification and filterability of the wine, especially concerning *Botrytis cinerea* infected grapes, is highlighted (Blanco *et al.*, 1994; Ribéreau-Gayon *et al.*, 2000; Bailey *et al.*, 1993; Pretorius, 1997). Glycosidases have the ability to unlock the great aroma potential that lies hidden in the glycosidic bounded complexes (Günata *et al.*, 1988).

The second part of **chapter 2** focuses on the utilisation of biotransformation for changes in the chemical profile of wine. It emphasizes the importance of commercial enzymes and their applications in the industry. This concept is taken further when we look at the inhibiting factor for biocatalyst utilisation: stability. However, various methods are now available to improve the stability and activity of enzymes over the entire specificity range.

In the final section of this **chapter (2)** the relationship between wine and protease is detailed. This enzymes' kinetics have both the possibility to destroy other biocatalyst's advantages, as well as the possibility to promote peptide release and protein instability (Alexandre *et al.*, 2001). The increased recovery of peptides and amino acids during autolysis is an important oenological factor as peptides and nitrogen compounds favour malolactic fermentation in wines; the peptides could interact with phenolic compounds and improve natural fining in the wine medium and

they contribute to the organoleptic properties of the wine (Alexandre *et al.*, 2001). The physical instability of haze formation caused by pathogen-induced proteins could indirectly be minimized by the use of a vacuolar protease A, encoded by the *PEP4* gene, that is active at pH levels (Pretorius, 2000). It seems that wine becomes more stable due to the action of protease A and the release of mannoproteins during autolysis.

There is a shift in global trends in the industry regarding the use of commercial enzyme preparations and genetic engineering. It has become clear that if the wine industry wants to remain a player in this highly competitive market, the phenomenal potential that gene technology poses cannot be ignored. The vast potential on a multitude of levels and application will be realized, however, only if the application is judicious, systematic and achieved with high regard for the unique nature of the product.

Chapter 3 describes the research into the effect of an exogenous yeast acid protease on an *Aspergillus* sp. β-glucosidase in wine-related conditions. Identifying and characterizing their interaction as expressed in various conditions, including two different *in vitro* media and in fermentation of wine would achieve this.

In the *in vitro* studies the activity of the respective enzymes was determined in a wine and a buffer medium. The conditions in the two media were set to closely resemble each other in pH and substrate availability. Thus it was possible to determine if there are other influences within the wine medium than could affect enzyme activity, such as ethanol or other chemical compounds. Specific influences were not investigated. The effect of the protease on the report activity was established by quantifying whether the relative activity of β-glucosidase was significantly changed. These results were compared with a control and a sample containing only a β-glucosidase addition. The data showed a β–glucosidase activity increased over the monitored time period. The protease did not affect the β-glucosidase activity significantly. There was also no difference between the different protease concentrations in terms of effect on the β-glucosidase activity, showing that increased protease concentration did not affect β–glucosidase activity. It would rather seem to be a wine related factor that is responsible for the differences between relative activities of the β-glucosidase in the two media.

The fermentation studies were aimed at the establishment of the relative activity of, as well as the interactions between the two enzymes during fermentation

conditions. Fermentation is considered to be the crucial stage in the development of the wine's aroma profile and chemical composition. For this reason, enzymes are added specifically at this stage to reinforce the enzymatic release of aroma compounds and the stabilization of the aroma profile and colour. Experimental scale fermentations were set up with various enzyme additions and monitored by enzyme assays throughout the process of alcoholic fermentation. The wines were stabilized and bottled separately, and sensory and chemical analyses were performed. We report that both of the enzymes showed a relative activity during the fermentation of the wines.

The addition of protease to the fermentation did not significantly affect the β–glucosidase activity when compared to the wine made with only a β–glucosidase addition. It was postulated that protease might be able to inhibit or decrease the activity of β–glucosidase, and that the addition of β–glucosidase in the presence of this protease would therefore be useless. Even though the protease activity was shown to be higher in both the wine and buffer medium, when compared to the fermentation, it did not seem to adversely affect the activity of β–glucosidase to a significant degree.

From the data presented in this **chapter (3)** it can therefore be stated that both protease and β–glucosidase enzyme preparations are active in different media to varying degrees. From our data we have shown that protease is active for at least 18 days during the fermentation process. It has been shown for the first time that the protease did not significantly affect the report activity in the *in vitro* or the fermentation conditions.

Future work would entail more detailed studies of interactions between protease and enzymes in specified oenological conditions. The degradation capability of protease could be directed towards unwanted enzyme activities such as browning through oxidation of the must. The characterization of proteases' interaction with other enzymes may thus hold the key to producing wines with enhanced aroma potential, more stable colour; as well as the elimination of unwanted enzyme activities.

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