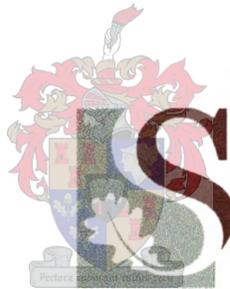


The evaluation of β -glucosidase activity produced by wine-isolated yeasts

by

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*Thesis presented in partial fulfillment of the requirements for the degree of
Master of Science at Stellenbosch University.*

April 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.

Nydia Potgieter

SUMMARY

β -Glucosidases constitute a major group of biologically important enzymes that catalyze the hydrolysis of glycosidic linkages in β -glucosides, as well as in glycosides that contain only carbohydrate residues, e.g. cellobiose. These enzymes occur in all living kingdoms and perform a variety of functions in organisms ranging from bacteria to highly evolved mammals. Three different types of β -glucosidases are found in humans, each with its own function: glucocerebrosidase (a deficiency causes Gaucher disease), lactase-phlorizin hydrolase (a deficiency results in lactose intolerance) and cytosolic β -glucosidase (responsible for the hydrolysis of β -glucosides ingested with foods of plant and animal origin). In plants, the functions of β -glucosidases include pigment metabolism, biomass conversion and cyanogenesis, a function it shares with insect β -glucosidases. Microbial β -glucosidases, as part of the cellulase enzyme system that is responsible for the hydrolysis of cellobiose and short-chain oligosaccharides into glucose, play a role in the conversion of cellulosic biomass to liquid fuel. These microbial β -glucosidases also play a very important role in the enhancement of fruit and wine aromas through the liberation of monoterpenols.

Monoterpenols play an invaluable role in the flavor and aroma of grapes and wine, and are present as free, volatile and odorous molecules, as well as flavorless, non-volatile glycosidic complexes. These complexes most often occur as 6-O- β -D-xylopyranosyl- β -D-glucopyranosides, 6-O- β -D-glucopyranosyl- β -D-glucopyranosides, 6-O- α -L-arabinofuranosyl- β -D-glucopyranosides, 6-O- α -L-rhamnopyranosyl- β -D-glucopyranosides, or 6-O- β -D-apiofuranosyl- β -D-glucopyranosides of mainly linalool, geraniol, nerol, α -terpineol and hotrienol. Two mechanisms exist for the release of monoterpenes from glycosidically bound non-volatile precursors: acid hydrolysis and enzymatic hydrolysis. As high temperature acid hydrolysis causes a rearrangement of the monoterpene aglycones, the focus has shifted towards the more efficient enzymatic hydrolysis that does not result in modifications of the intrinsic aromatic character of the wine.

The endogenous β -glucosidases of grapes (*Vitis vinifera*), as well as of the wine yeast *Saccharomyces cerevisiae*, exhibit very low activity towards the glycoside precursors, and thus the focus has increasingly fallen on the addition of exogenous β -glucosidases to enhance wine flavor. Fungal, bacterial and some yeast β -glucosidases have been indicated as effective aroma liberators, but these enzymes are not always suitable for use under the harsh conditions that prevail during winemaking (i.e. low pH, low temperatures, and high ethanol and glucose concentrations). The limited enzyme activities of the abovementioned microorganisms have resulted in a search among non-*Saccharomyces* yeasts for β -glucosidases that can withstand these conditions.

The β -glucosidase activities of 20 wine-associated non-*Saccharomyces* yeasts were quantified, characterized and assessed to determine the efficiency with which they could liberate monoterpenols from their terpenyl-glycosides. The *Debaryomyces*

pseudopolymorphus β -glucosidase from intracellular crude cell extracts exhibited the most suitable combination of properties in terms of functionality at wine pH, resistance to wine-associated inhibitory compounds (glucose, ethanol and sulfur dioxide), high substrate affinity and large aglycone-substrate recognition. This yeast strain was also used, in conjunction with *S. cerevisiae* VIN13, for the small-scale fermentation of Chardonnay juice. The results indicated that the β -glucosidase of *D. pseudopolymorphus* had definite potential as a wine aroma-enhancing enzyme, as the concentrations of free terpenols (nerol, geraniol and citronellol) were significantly increased during fermentation.

Future experimental work would include an in-depth study of the kinetic characteristics of the β -glucosidases (both cytosolic and cell-associated) exhibiting the highest terpenol-liberating activity under winemaking conditions. The next step would then be the cloning and expression of the most efficient β -glucosidase gene in a commercial wine yeast. Such a recombinant wine yeast would release grape-derived aroma compounds from their non-volatile precursors during single culture fermentations, thereby increasing the sensorial quality of wine.

OPSOMMING

β -Glukosidases vorm deel van 'n groot groep biologies belangrike ensieme wat die hidrolise van glikosidiese bindings binne β -glukosiede, sowel as binne glikosiede wat slegs uit koolhidraatresidue bestaan, soos bv. sellobiose, kataliseer. Hierdie ensieme kom in alle koningkryke van lewende organismes voor en verrig 'n wye verskeidenheid funksies binne organismes wat wissel van bakterieë tot hoogs ontwikkelde soogdiere. Drie verskillende tipes β -glukosidases, elk met sy eie funksie, kom in mense voor: glukoserebrosidase ('n gebrek hieraan lei tot Gaucher-siekte), laktaseflorizinhidrolase ('n gebrek hieraan gee aanleiding tot laktose-intoleransie) en sitosol β -glukosidase (verantwoordelik vir die hidrolise van β -glukosiede wat saam met voedsel van plant en dier oorsprong ingeneem word). Die funksies van β -glukosidase binne plante sluit in pigmentmetabolisme, biomassa-omsetting en sianogenese, wat ook 'n funksie van insek β -glukosidases is. Mikrobiiese β -glukosidases, as deel van die sellulase-ensiemstelsel wat verantwoordelik vir die hidrolise van sellobiose en kortketting-oligosakkariede na glukose is, speel 'n rol in die omsetting van sellulosebiomassa na brandstof. Hierdie mikrobiiese β -glukosidases speel ook 'n baie belangrike rol in die verbetering van vrugte- en wynaroma deur die vrystelling van monoterpenole.

Monoterpenole speel 'n belangrike rol in die geur en aroma van druiwe en wyn, en kom voor as vry, vlugtige en aromatiese molekules, asook geurlose, nie-vlugtige glikosidies-gebonde komplekse. Hierdie komplekse is meestal in die vorm van 6-O- β -D-xilopiranosiel- β -D-glukopiranosiede, 6-O- α -L-arabinofuranosiel- β -D-glukopiranosiede, 6-O- β -D-glukopiranosiel- β -D-glukopiranosiede, 6-O- α -L-ramnopiranosiel- β -D-glukopiranosiede, of 6-O- β -D-apiofuranosiel- β -D-glukopiranosiede van hoofsaaklik linalool, geraniol, nerol, α -terpineol en hotrienol. Monoterpenole kan op een van twee maniere van hul suikermolekules vrygestel word: suurhidrolise of ensimatisiese hidrolise. Die hoë temperatuur waarby suurhidrolise plaasvind veroorsaak 'n herrangskikking van die monoterpeen aglikone, en die fokus het gevolglik verskuif na die meer effektiewe ensimatisiese hidrolise wat nie verandering van die intrinsieke aromatiese karakter van die wyn tot gevolg het nie.

Die endogene β -glukosidases van druiwe (*Vitis vinifera*) en die wyngis *Saccharomyces cerevisiae*, toon baie lae aktiwiteit ten opsigte van die aromatiese voorlopers, en dus word daar toenemend op die toevoeging van eksogene β -glukosidases tot die wyn gefokus om meer geur vry te stel. Daar is bevind dat β -glukosidases van fungiese, bakteriële en gis oorsprong effektiewe aromavrystellers is, maar hierdie ensieme is nie altyd gepas vir gebruik in wyn nie, aangesien dit 'n omgewing is met 'n lae pH, lae temperatuur, en hoë etanol- en glukosekonsentrasies. Die beperkte ensiemaktiwiteit van bogenoemde mikroorganismes het gelei tot 'n soeke onder nie-*Saccharomyces* giste na β -glukosidases wat in die wynomgewing kan funksioneer.

Die β -glukosidase-aktiwiteit van twintig wyn geassosieerde nie-*Saccharomyces* giste is gekwantifiseer en gekarakteriseer om te bepaal tot watter mate dit monoterpenole van hul terpeniel glikosiede kan vrystel. Die intrasellulêre β -glukosidase teenwoordig in the selekstrak van *Debaryomyces pseudopolymorphus*, het die belowendste resultate getoon ten opsigte van funksionaliteit by wyn se pH, weerstand teen wyn geassosieerde inhibeerders (glukose, etanol en swaweldioksied), hoë substraataffiniteit en breë aglikoon-substraat herkenning. Hierdie gisras is ook in kombinasie met *S. cerevisiae* VIN13 gebruik vir die kleinskaalse fermentasie van Chardonnay sap. Die resultate het getoon dat die β -glukosidase van *D. pseudopolymorphus* wel potensiaal het om wynaroma te verhoog, aangesien die konsentrasie van ongebonde terpenole (nerol, geraniol en citronellol) aansienlik tydens fermentasie toegeneem het.

Toekomstige eksperimentele werk sluit in, onder meer, 'n in-diepte studie van die kinetiese eienskappe van die β -glukosidasies (beide sitosolies en sel-geassosieerd) wat die meeste terpenole onder wynmaakkondisies vry stel, asook die klonering en uitdrukking van die enkele β -glukosidasegeen met die hoogste aktiwiteit, in 'n kommersiële wyngis. Só 'n rekombinante wyngis sal die vrystelling van druif-gebaseerde aromakomponente van hul nie-vlugtige, geurlose voorlopers tydens enkel-kultuur fermentasies teweeg bring.

This thesis is dedicated to my parents and Dewald.
Hierdie tesis is aan my ouers en Dewald opgedra.

BIOGRAPHICAL SKETCH

Nydia Potgieter was born in Johannesburg, South Africa on 24 January 1977. She attended Aliwal North Primary School and matriculated at Aliwal North High School in 1995. Nydia enrolled at the University of the Free State in 1996 and obtained a BSc degree in Genetics, Biochemistry and Physiology in 1998. In 1999 she obtained a BScHons degree in Genetics from the same university.

ACKNOWLEDGEMENTS

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

JESUS CHRIST, because this study would not have been possible without His love and strength;

DEWALD VAN DYK, for invaluable discussions and continuous support and encouragement;

PROF SAKKIE PRETORIUS, The Australian Wine Research Institute, for critical reading of the dissertation and the opportunities made possible by him;

DR PIERRE VAN RENSBURG, for critical reading of the dissertation and for acting as my supervisor;

DR RICARDO CORDERO OTERO, for invaluable discussions and critical reading of the dissertation;

DR JUAN UBEDA IRANZO, **DR ANA BRIONES-PEREZ** and **MARIA AREVALO VILLENA**, for invaluable technical assistance;

JUDY CRONJE, for her assistance and support;

MY FELLOW RESEARCHERS at the Institute for Wine Biotechnology, for their assistance and invaluable discussions; and

The **NATIONAL RESEARCH FOUNDATION** and **WINETECH**, for financial support.

PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the *Journal of Food Science*, in which Chapter 3 was published.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
 β -Glucosidase: An overview.

Chapter 3 **Research Results**
Characterization of the β -glucosidase activity produced by enological strains of non-*Saccharomyces* yeasts.

Chapter 4 **General Discussion and Conclusions**

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

β -Glucosidases (β -D-glucoside glucohydrolase, EC 3.2.1.21) constitute a major group of well-characterized, biologically important enzymes that catalyze the hydrolysis of glycosidic linkages in aryl- and alkyl- β -glucosides, as well as in glycosides containing only carbohydrate residues (e.g. cellobiose). These enzymes are also involved in the synthesis of oligosaccharides and glucoconjugates through the processes of reverse hydrolysis or transglycosylation. β -Glucosidases belong to families 1 and 3 of the 88 families classified under the glycosyl hydrolase category and occur in all living kingdoms. Structural and catalytic properties shared by all β -glucosidases include the absolute requirement for a β -glycoside as substrate, an acid pH optima (pH 5-6), and subunit molecular weights of 55-65 kDa. Similarities in substrate specificity for the glycone (glucose), as well as for some non-physiological aglycones (e.g. nitrophenols), have also been observed.

1.1.1 GENERAL OCCURRENCE AND APPLICATIONS

β -Glucosidases are ubiquitous enzymes that perform a variety of functions in organisms ranging from bacteria to highly evolved mammals. In microorganisms, β -glucosidases mainly form part of the cellulase enzyme system and are responsible for the hydrolysis of cellobiose and short-chain oligosaccharides into glucose. This enzyme system is of great industrial importance, as it is responsible for the conversion of cellulosic biomass to liquid fuel (Saha and Bothast 1996; Cho and others 1999). Microbial β -glucosidases are also involved in the enhancement of fruit and wine aromas through the liberation of monoterpene alcohols (Gueguen and others 1997). In plants, the functions of β -glucosidase include pigment metabolism, seed development, biomass conversion and the hydrolysis of phytohormone precursors. This enzyme also plays a major role in a defense mechanism found in both plant and insect systems, namely cyanogenesis, during which hydrogen cyanide is released from cyanogenic glucosides upon attack (Esen 1993). Human acid β -glucosidase (glucocerebrosidase) has potential in the development of therapeutic and diagnostic procedures that will be useful in the treatment of Gaucher disease (a lysosomal storage disease caused by a deficiency in glucocerebrosidase). One such treatment method entails the intravenous injection of purified human placental β -glucosidase (Esen 1993). The two other human β -glucosidases, lactase-phlorizin hydrolase and the cytosolic β -glucosidase, are responsible for the hydrolysis of dietary lactose and dietary xenobiotics (β -glucosides ingested with foods of plant and animal sources) respectively (Harvey and others 1995; Berrin and others 2002).

1.1.2 THE ROLE OF β -GLUCOSIDASES IN AROMA LIBERATION IN WINE

A variety of foods, such as apples, grapes, fruit juices and wines, are known to contain monoterpenoids, 10-carbon compounds with strong sensorial qualities that

play an invaluable role in the flavor and fragrance industries. Extensive research on the aromatic potential of different fruits and their fermenting products (Günata and others 1985; Vasserot and others 1993; Chyau and others 2003) has revealed that, in addition to a free fraction of volatile terpenoids, naturally non-odorous and non-volatile glycoside precursors occur that represent an important source of fragrant compounds. These non-odorous precursors consist of an aglycone moiety that can be linked to β -D-glucose or the disaccharides α -L-arabinofuranose, α -L-rhamnopyranose and β -D-apiofuranose. The aglycone part contains mainly monoterpenols, such as nerol, linalool, geraniol, citronellol and α -terpineol, and aromatic alcohols such as benzyl- and 2-phenylethyl alcohol (Günata and others 1985). It is often found that the glycosidically bound non-odorous fraction exceeds the amount of free aroma compounds at a ratio ranging from 1:1 (in non-muscat grape varieties such as Chardonnay) to 5:1 (Muscat of Alexandria grapes) (Williams and others 1982).

Two mechanisms exist for the release of monoterpenes from glycosidically bound non-volatile precursors: acid hydrolysis and enzymatic hydrolysis. Studies have shown that high temperature acid hydrolysis causes rearrangement of the monoterpene aglycones (Usseglio Tomasset and Di Stefano 1980; Williams and others 1982), and the focus has therefore shifted towards the more efficient enzymatic hydrolysis that does not result in modifications of the aromatic character. The enzymatic hydrolysis of glycosidically bound monoterpenes is a two-step process consisting of (i) cleavage of the glycosidic linkages by an α -L-arabinofuranosidase, α -L-rhamnopyranosidase or β -D-apiosidase (depending on the structure of the aglycone moiety), followed by (ii) the liberation of the monoterpenols by β -glucosidase (Gueguen and others 1996). The efficiency of hydrolysis by β -glucosidases has been found to be dependent on, among others, the origin of the enzyme and the structure of the aglycone. The hydrolysis of glucosidic linkages present in must and wine by endogenous grape β -glucosidases is generally a slow process, due to the fact that plant β -glucosidases are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine. These enzymes are also unable to hydrolyze the sugar conjugates of tertiary alcohols, such as linalool and α -terpineol, and are thus unable to liberate the entire flavor reservoir (Günata and others 1985; Aryan and others 1987).

Supplementation with β -glucosidases from external sources has been studied extensively as a method to overcome the abovementioned constraints. Trials conducted with fungal β -glucosidases indicated that these enzymes are very effective as aroma liberators, but only if introduced into the wine after all the glucose has been depleted by the yeast cells, as they are strongly inhibited by glucose (Aryan and others 1987; Günata and others 1993). Yeasts show more promise as sources of exogenous enzymes. Certain strains of *Saccharomyces cerevisiae* have been reported to possess β -glucosidase activity, but the activity towards glycoside precursors appears to be very limited, even though they are less sensitive to glucose

(Delcroix and others 1994; Hernández and others 2002). This limited activity has resulted in a search for non-*Saccharomyces* yeasts containing novel β -glucosidases with the desired characteristics (high affinity for grape-derived terpenoid aglycones, optimal activity at wine pH, resistance to glucose inhibition and high tolerance to ethanol). In-depth studies have been performed on the β -glucosidases of, among others, species of *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora* and *Pichia* (Vasserot and others 1989; Rosi and others 1994; McMahon and others 1999; Fernández and others 2000; Garcia and others 2002).

1.2 PROJECT AIMS

The need for more efficient aroma-liberating enzymes has led us to search for novel β -glucosidases that can withstand the harsh conditions of alcoholic fermentation (low pH, high ethanol levels, high glucose concentrations, and sometimes rather high sulfur dioxide levels).

The specific aims and approaches of this study were as follows:

- (i) screening of wine-related non-*Saccharomyces* yeasts for β -glucosidase activity under different growth conditions;
- (ii) determination of the cell localization of the β -glucosidases;
- (iii) optimization of the β -glucosidase liquid assays;
- (iv) assessment of the effects of wine-associated inhibitory compounds on enzyme activity; and
- (v) microvinification with the yeast exhibiting the best enzymatic properties to determine its effect on the terpenic glycosides in wine.

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CHAPTER 2

LITERATURE REVIEW

β -glucosidases: An overview

2.1 INTRODUCTION

Glycosidases are responsible for the catabolism of a variety of carbohydrate-containing compounds. These hydrolases catalyze chemical transformations at the C₁ position of carbohydrates, and a number of metabolic processes depend on these enzymes for their efficiency, selectivity and regulation. β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are a widespread group of enzymes hydrolyzing a broad variety of glycosides, including alkyl- and aryl-β-D-glucosides (e.g. methyl-β-D-glucoside and *p*-nitrophenyl-β-D-glucoside), and glycosides containing only carbohydrate residues (e.g. cellobiose). The physiological function of β-glucosidases varies greatly, depending on their origin (plants, fungi, bacteria, animals and humans) and substrate specificity.

It is expected that, because of the omnipresence of β-glucosides and β-glucosidases in nature, some structural and catalytic properties will be shared by all β-glucosidases. Nearly all have an acid pH optimum (pH 5-6), subunit molecular weights of 55-65 kDa, and an absolute requirement for a β-glycoside (i.e. glucoside, and to a lesser extent fucoside and galactoside) as substrate. Remarkable similarity in substrate specificity for glycone (glucose), as well as for some non-physiological aglycones (e.g. nitrophenols), has also been observed. However, these β-glucosidases may have widely different physiological glucosidic substrates with different aglycone moieties. In general, β-glucosidases from different orders and kingdoms appear to differ in their specificities for the aglycone (an alkyl or aryl group) linked to the glucosyl group by a β-glucosidic bond. The β-glucosidases of humans, fungi, bacteria and dicotyledonous plants have been shown to be glycosylated, while those of monocots are not.

The localization of the enzyme differs between sources. In plants, the β-glucosidases of dicots are localized to the cell wall (Kakes 1985; Frehner and Conn 1987) or protein bodies (Swain and others 1992), while the β-glucosidases of monocots are localized to the plastids (Thayer and Conn 1981; Nisius and Ruppel 1987; Esen 1992). In mammals (e.g. humans and mice), acid β-glucosidase is localized to the lysosome, while its neutral counterpart is a soluble protein found in the cytosol (Price and Dance 1967; Glew and others 1993). Generally, the cloned bacterial enzyme occurs in the cytosol; however, recombinant β-glucosidase from *Ruminococcus* sp. is extracellular, while Bgl of *Pyrococcus herikoshii* expressed in *Escherichia coli* is bound to the membrane fraction (Takano and others 1992; Matsui and others 2000). Fungal β-glucosidases are mostly extracellular, but a few, such as recombinant BglA encoded by *Aspergillus kawachii*, are localized in the periplasmic space of the host *Saccharomyces cerevisiae* (Iwashita and others 1999). Yeast β-glucosidases can be located either intracellularly (*Dekkera intermedia*, *Kloeckera* sp., *Hanseniaspora* sp.), extracellularly, or to the cell membrane, or they can be located in a combination of the three cell areas (*Debaryomyces hansenii*, *Candida*

molischiiana) (Blondin and others 1983; Gondé and others 1985; Vasserot and others 1990; Rosi and others 1994).

The various β -glucosidases have been the focal point of numerous studies over the years and the current research has significant scientific, medical and economical implications. Plant β -glucosidases have been implicated in a variety of growth, productivity, defense and food and feed toxicity-related reactions, such as cyanogenesis. Fungal and bacterial β -glucosidases appear to be ideal candidates for the engineering of a β -glucosidase to be used as part of the cellulase complex in the industrial-scale conversion of cellulose to glucose. Human acid β -glucosidase has potential in the development of therapeutic and diagnostic procedures that will be useful in the treatment of Gaucher disease, and the cytosolic neutral β -glucosidase is implicated in the hydrolysis of β -glucosides ingested with foods of plant and animal origin. The β -glucosidases found in insects are also believed to be involved in a form of cyanogenesis (the release of hydrogen cyanide upon the hydrolysis of cyanogenic glucosides).

The focal point of the opening section of this literature review is the different roles played by the β -glucosidases found in the various kingdoms, including their biochemical characteristics (substrate specificity, stability, inhibitors, etc.). Finally, the biotechnological and industrial applications of β -glucosidases are discussed.

2.2 β -GLUCOSIDASE: CLASSIFICATION AND MODE OF ACTION

β -Glucosidases are known for having dual activity, namely the cleavage and synthesis of glycosidic bonds, and it is these activities that make β -glucosidases so important in a number of applications. An understanding of the structure and mode of action of these enzymes is necessary for the development of economically viable biotechnological and industrial applications.

2.2.1 CLASSIFICATION

β -Glucosidases are a heterogeneous group of enzymes occurring in all living kingdoms. At present there is no single well-defined method for the classification of these enzymes. Two methods for classification appear in the literature, based on (i) substrate specificity and (ii) nucleotide sequence identity (NSI) (Henrissat and Bairoch 1996). When substrate specificity is used as the basis for classification, β -glucosidases will be classified as being either (i) aryl β -glucosidases, which act on aryl-glucosides, (ii) true cellobiases, which hydrolyze cellobiose to release glucose, or (iii) broad substrate specificity enzymes, which act on a wide spectrum of substrates. The majority of β -glucosidases characterized to date fall in the last category (Bhatia and others 2002). One of the first classification schemes based on the available sequences proposed the grouping of β -glucosidases into two types, namely Type I and Type II (Beguin 1990). Rojas and others (1995) proposed an alternative scheme, in which these enzymes are divided into two subfamilies, subfamily A (BGA) and

subfamily B (BGB). These earlier methods have largely been replaced by the currently acceptable NSI scheme, proposed by Henrissat and Bairoch (1996), which is based on sequence and folding similarities (hydrophobic cluster analysis, HCA) between these enzymes. This nomenclature system is widely used and continuously updated at <http://afmb.cnrs.mrs.fr/~pedro/CAZY/dt.html> (Henrissat 1991; Henrissat and Davies 1997).

The classification scheme proposed for all glycosylhydrolases has resulted in the recognition of 88 families, and the β -glucosidases are categorized in either family 1 or 3, with the exception of glucosylceramidase, which is a member of family 30. Both these families consist of retaining enzymes that hydrolyze their substrates, with a net retention of an anomeric configuration that occurs via a double-displacement mechanism (Withers and Street 1989; Withers 2001). Family 1, comprising nearly 62 β -glucosidases from archaeobacteria, plants and mammals, also includes 6-phosphoglycosidases and thioglucosidases. Most family 1 enzymes are known to have significant β -galactosidase activity (Bhatia and others 2002). The crystal structures have already been determined for numerous family 1 β -glucosidases. These include the cyanogenic β -glucosidase (linamarase) from *Trifolium repens* (Tolley and others 1993), BGLA from *Paenibacillus (Bacillus) polymyxa* (Sanz-Aparicio and others 1998) and *Bacillus circulans* subspecies *alkalophilus* (Hakulinen and others 2000), CelB from *Pyrococcus furiosus* (Kaper and others 2000) and, most recently, ZmGLU1 (Czjzek and others 2000) and Zm-p60.1 (Zouhar and others 2001) from maize. Family 1 β -glucosidases are also classified as members of the 4/7 super family. This family has a common (β/α)₈-barrel motif consisting of similar amino acid sequences at the active site, and also includes enzymes such as family 2 β -galactosidase, family 5 cellulases, family 10 xylanase and family 17 barley glucanases (Jenkins and others 1995).

Family 3 of the glycosylhydrolases consists of nearly 44 β -glucosidases and hexosaminidases of bacterial, mold and yeast origin. The crystal structure has been determined only for a related β -D-glucan (exo- \rightarrow 3, 1 \rightarrow 4) glucanase (Exo 1) from *Hordeum vulgare*, which catalyzes the hydrolysis of cell-wall polysaccharides (Varghese and others 1999). At molecular level, the genes of family 3 β -glucosidases consist of five distinct regions, the N-terminal residues, a N-terminal catalytic domain, a nonhomologous region, a C-terminal domain of unknown function, and the C-terminal residues (Bhatia and others 2002).

2.2.2 MODE OF ACTION

β -Glucosidase catalyzes the hydrolysis of glycosidic linkages formed between the hemiacetal -OH group of a cyclic aldose or glucose and the -OH group of another compound, such as sugar, amino-alcohol, aryl-alcohol, or primary, secondary and tertiary alcohols (Bhatia and others 2002). During glycosylation, an enzymatic nucleophile attacks the anomeric (C₁) center of the substrate glycoside (1), resulting in the formation of a covalently linked α -glycosyl enzyme intermediate (2) through an

oxocarbenium ion-like transition state (Withers and Street 1988). The anomeric configuration at C₁ is thus reversed, as shown in **Figure 2.1**.

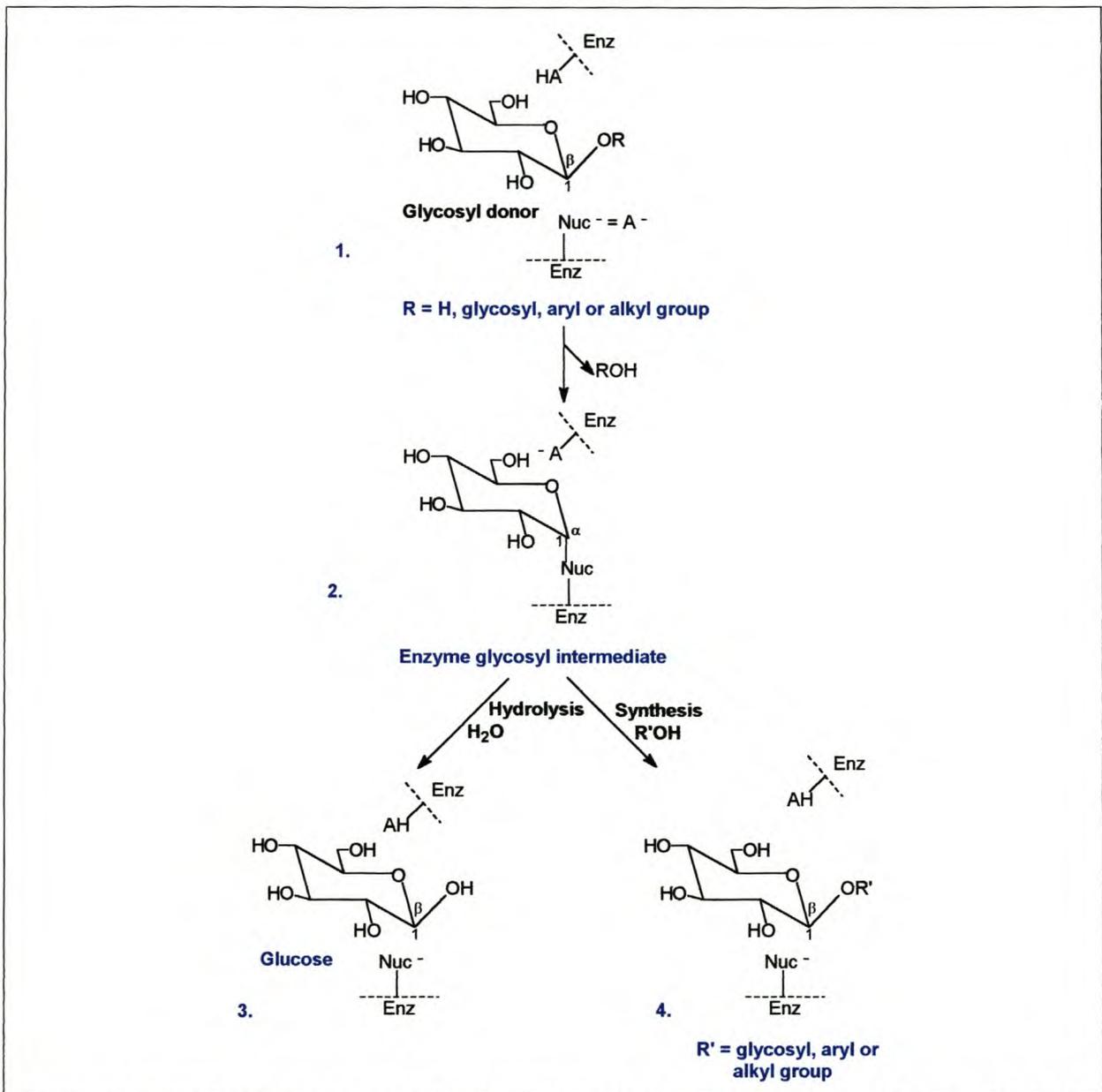


Figure 2.1. The proposed reaction mechanism of β -glucosidase. Nucleophilic attack by the enzyme functional group (A⁻, in 1) leads to inversion of the anomeric configuration of the β -glucosidic bond in the enzyme-glycosyl intermediate (2). Subsequent reactions in the presence of H₂O or sugars (aryl- or alkyl-glucosides) lead to retention of the β -form, resulting in hydrolysis (3) or synthetic reactions (4), respectively (adapted from Bhatia and others 2002).

A second active residue of the enzyme serves as the acid base catalyst and donates a H⁺ to the glycosidic oxygen, thereby assisting in the departure of the aglycone (or other glycone, as in disaccharides) group (Bhatia and others 2002). The glycosyl-enzyme intermediate (2) is hydrolyzed via general base-catalyzed attack by water at the anomeric center to release β -glucose as the product (3). The trans-addition of an -OH group results in the net retention of the β -anomeric configuration. The nucleophilic residue also acts as the leaving group in the deglycosylation step.

Both the formation and hydrolysis of the enzyme-glycosyl intermediate occur via an oxocarbenium ion-like transition state (Bhatia and others 2002).

The biosynthesis of glycoconjugates can occur either by reverse hydrolysis under thermodynamic control, or by transglycosylation under kinetic control (Bhatia and others 2002). In reverse hydrolysis, the substrate (1) has an H instead of an R (Figure 2.1). The enzyme-glycosyl intermediate is intercepted by R'OH, where R' is a second sugar molecule, yielding a disaccharide product (4). In the transglycosylation method, the substrate (1) has an R instead of an H and is an "activated" donor. The enzyme-glycosyl intermediate can be trapped by a nucleophile other than water, such as aryl or alkyl alcohol (as R'OH), to yield a new glycoside. Here, the efficiency of the formation of the product is determined by competition between water and the acceptor R'OH for the enzyme-glycosyl intermediate (Bhatia and others 2002).

2.3 SOURCES AND CHARACTERISTICS

Over the past two decades, considerable progress has been made with the molecular biology and biochemistry of β -glucosidase enzymes. Research has been focusing on the application of these enzymes with respect to specific problems (biomass conversion, cyanogenesis, host-parasite interactions and Gaucher disease). In order to have a better understanding of these applications, insight into the functioning of the enzymes in different organisms (humans, plants, insects and microorganisms) is of great importance.

2.3.1 MAMMALIAN β -GLUCOSIDASES

Three native β -glucosidase enzymes have been identified in humans (Hays and others 1996). Two of these, the glucocerebrosidase and lactase-phlorizin hydrolase, are membrane bound and have been shown to have specific substrate activities (Hays and others 1996; Auricchio and others 1963). The third β -glucosidase is a broad-specificity cytosolic enzyme found abundantly in the liver, kidney and small intestines of mammals (Mellor and Layne 1971; Daniels and others 1981).

2.3.1.1 Human acid β -glucosidase

β -Glucosidic linkages are rare in mammalian cells. The only known endogenous substrate for β -glucosidases is glycosphingolipid glucosylceramide (glucocerebroside), which is purported to be catabolized specifically in lysosomes. Nevertheless, various β -glucosidase activities are observed in human tissues and cell types. Primarily because of its role in Gaucher disease (see section 2.3.1.1.2), the enzyme that has been studied the most and characterized the best, both at protein and gene level, is the acid β -glucosidase (glucocerebrosidase; EC 3.2.1.45) (Mikhaylova and others 1996). The fact that glucocerebrosidase is a tetramer in its active form (Pentchev and others 1973) and that it requires an activator protein (saposin C) makes it unique.

Glucocerebrosidase is active not only towards the natural lipid substrate glucosylceramide, but also to a number of artificial β -D-glucosidic and β -D-xylosidic substrates, such as 4-methylumbelliferyl- β -D-glucoside (4-MUGlc) and *p*-nitrophenyl- β -D-glucoside (PNPGlc) (Mikhaylova and others 1996). It is irreversibly inhibited by conduritol B-epoxide (CBE) (Grabowski and others 1985) and activated by sodium taurocholate (TCh) (Raghavan and others 1980), as well as by phosphatidylserine in combination with the sphingolipid activator protein 2 (SAP-2) (Basu and others 1984). Alkyl-glycons, i.e. the N-alkyl-deoxynojirimycins and N-alkyl- β -glucosylamines, were also found to be extremely potent inhibitors (Grabowski and others 1984; Osiecki-Newman and others 1988).

Deficient β -glucocerebrosidase activity leads to the systemic accumulation of its substrate (glucocerebroside), mainly within cells of the monocyte/macrophage lineage (Germain and others 2001), which is characteristic of Gaucher disease. The existence of a second, non-lysosomal, tightly membrane-bound glucocerebrosidase has been reported in many tissues and cell types (Van Weely and others 1993). This enzyme seems to be identical to the enzyme previously described as a membranous non-specific β -glucosidase (Yaqoob and Carroll 1980; Maret and others 1981) and, like the lysosomal enzyme, is also able to hydrolyze glucocerebroside and 4-MUGlc. However, it differs from lysosomal glucocerebrosidase in several properties: it is not located in the lysosomes, has a more neutral pH optimum, is markedly inhibited by detergents and is not deficient in Gaucher disease (Van Weely and others 1993).

2.3.1.1.1 Human saposins: activators of glucocerebrosidase

Several lysosomal hydrolyses in the catabolic pathway of glycosphingolipids require low molecular weight proteins for optimal activity *in vitro* and *in vivo*. These heat-stable glycoproteins have been variously called sphingolipid activator proteins or saposins. Five of these "activators" have been characterized at the protein and molecular genetic levels and have been designated saposins A, B, C, D and G_{M2} gangliosidase activator (O'Brien and others 1988; Fürst and Sandhoff 1992). Saposins A, B, C and D are encoded by a single locus, termed prosaposin, on chromosome 10q (O'Brien and others 1988). A single precursor polypeptide, prosaposin, is synthesized first, and the individual saposins are released by proteolytic processing in the lysosome (Fujibayashi and Wenger 1986) (**Figure 2.2**).

Saposin C has been designated heat stable factor, co- β -glucosidase and SAP-2. The physiological importance of saposin C is evident in glucocerebroside storage and a Gaucher-like phenotype in patients with a deficiency of saposin C. Recently, a second protein activator of *in vitro* acid β -glucosidase activity was described (Morimoto and others 1989), and this protein was designated saposin A. Very high levels of saposin D and A have been found in Gaucher disease tissues (Morimoto and others 1990). Mutations at the prosaposin locus on chromosome 10q produce a nonfunctional activator and cause Gaucher-like symptoms.

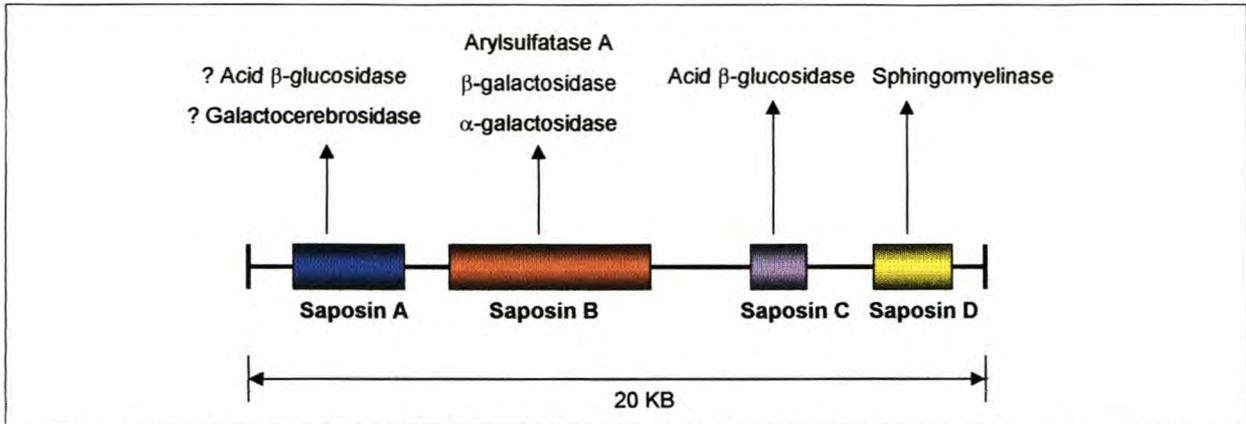


Figure 2.2. Schematic representation of the precursor polypeptide prosaposin (adapted and modified from Qi and others 1994).

2.3.1.1.2 Glucocerebrosidase-associated diseases: Gaucher disease

Gaucher disease, the most frequent lysosomal storage disorder, is an autosomal recessive disease characterized by a deficiency in glucocerebrosidase (Brady and others 1965). This enzymatic defect leads to the systemic accumulation of glucocerebroside in macrophages throughout the body, resulting in clinical manifestations. Major organs that are affected include the liver, spleen (which may become enlarged) and bones (which may be subject to fractures). Marrow involvement often results in anemia and a lowered platelet count (Beutler 1993).

Gaucher disease is conventionally classified into three clinical types on the basis of the absence (type 1) or presence and severity (types 2 and 3) of primary central nervous system involvement (Germain and others 2001). Type 1 (MIM 230800) is the most common form, with markedly heterogeneous clinical expression with respect to age of onset, progression and severity of clinical manifestations. Common features are splenomegaly, frequent concurrent liver enlargement, anemia and thrombocytopenia. Orthopedic complications occur in severe cases. The much rarer clinical types 2 (MIM 230900) and 3 (MIM 231000) differ from type 1 in that patients show neurological involvement; type 2 (infantile type) has infantile onset of severe central nervous system involvement and death in early childhood, whereas type 3 has onset of mild central nervous system involvement in adolescence or early adulthood and has a more indolent course (Brady and others 1993).

The full-length cDNA and genomic sequences encoding human glucocerebrosidase have been isolated and characterized (Horowitz and others 1989; Beutler and others 1992) and, in 1994, Grace and others expressed its structural gene in *Spodoptera frugiperda*. Cloning of the active gene *GBA*, which is located on the long arm of chromosome 1, has allowed the identification of the nature of mutations underlying the enzyme deficiency in patients with Gaucher disease. A glucocerebrosidase pseudogene has been identified about 16 kb downstream from the functional gene. There is a high degree of sequence similarity between the pseudogene and the functional gene, and the promoter of the pseudogene is still active (Horowitz and others 1989). The major difference between these two genes is

the presence of four large intronic deletions in the pseudogene (Grabowski and others 1993). Various studies have demonstrated the molecular heterogeneity of acid β-glucosidase deficiency, and more than 100 gene alterations responsible for the disease have been identified (Beutler and Gelbart 1996; Germain and others 1998). These include point mutations in exons and in a splice site, a nucleotide insertion, crossovers between the gene and the pseudogene, and gene conversions (Beutler 1992).

Disorders due to inherited enzyme deficiencies can be treated at several different levels, including mere symptomatic treatment, substrate deprivation, supply of a missing product, exogenous administration of the defective enzyme, allogeneic transplantation with genetically normal tissue and, potentially, replacement of the defective gene.

2.3.1.2 Lactase-phlorizin hydrolase

Lactase-phlorizin hydrolase (LPH; EC 3.2.1.108) is a glycosylated membrane-bound enzyme found in the brush-border of the small intestine and is primarily responsible for the hydrolysis of dietary lactose. It has been established that, except in Northern European adults and a few small, predominantly pastoral populations, a deficiency of this enzyme commonly causes lactose intolerance (Harvey and others 1995; Wang and others 1995). LPH is responsible for the hydrolysis of pyridoxine-5'-β-D-glucopyranoside, a common dietary form of vitamin B₆, and plays a role in vitamin B₆ bioavailability (Mackey and others 2002). It also plays an important role in the hydrolysis of (iso)flavonoid glycosides (section 2.3.1.4), such as quercetin-3-glucoside and quercetin-4'-glucoside (Day and others 2000).

The pattern of expression of LPH among other brush-border hydrolases is quite unique. Its activity is only high during the suckling period and decreases markedly to low levels upon weaning. Exceptions to this developmental pattern occur in North Europeans and their descendants, as well as in some isolated African groups (Jacob and others 1994). LPH contains two separate active sites located on two physically similar family 1 glycosyl hydrolase domains: domains III and IV (Zecca and others 1998). The lactase site is selective towards glycosides with hydrophilic moieties, such as lactose, and the phlorizin site is more selective for hydrophobic substrates, such as phlorizin and glycosylceramides (Arribas and others 2000). Amino acid sequence analysis has indicated that these two domains, respectively, show 67.5% and 66.2% sequence similarity with the cytosolic β-glucosidase from guinea pig liver (Hays and others 1996).

2.3.1.3 Cytosolic β-glucosidase

The cytosolic β-glucosidase (CBG; EC 3.2.1.21) is one of the three best-characterized native β-glucosidase enzymes found in the mammalian system. It is active as a single polypeptide chain. There are wide differences between species and tissues in the distribution and content of the CBG. In general, the richest source

of the β -glucosidase in most vertebrates appears to be the liver, but it is also abundant in the kidneys, spleen and small intestine (Glew and others 1993). The enzyme has been purified from the livers of human, cow and guinea pig, and also from pig kidney and intestine (Daniels and others 1981; LaMarco and Glew 1986).

A distinguishing feature of CBG is its ability to hydrolyze many common dietary xenobiotics, including glycosides of phytoestrogens, flavonoids, simple phenolics and cyanogens (Daniels and others 1981; Berrin and others 2002). It also catalyzes the hydrolysis of a broad range of aryl glycosides, including glucosides, galactosides, xylosides, arabinosides and fucosides, and exhibits high affinity for the 3- and 17-glucosides of 17 α -estradiol, estrone-3-glucoside and 17 β -estradiol-3-glucoside (Daniels and others 1981). The enzyme is inhibited by sodium taurocholate at very low concentrations (Peters and others 1975; Legler and Bieberich 1988) and, unlike glucocerebrosidase, it is not inhibited by conduritol B epoxide (Daniels and others 1981; McMahon and others 1997). Guinea pig CBG shares several catalytic properties with domain III of LPH, including the ability to hydrolyze β (1-6) disaccharides (Gopalan and others 1992a) and poor activity towards glucosylceramide (Daniels and others 1981). Mantei and others (1988) have hypothesized that the CBG may have evolved from a partial duplication of the LPH gene, similar to the duplications that resulted in the contemporary structure of LPH.

The human CGB belongs to family 1 of glycosyl hydrolases (Henrissat 1991; Henrissat and Davies 1997) and catalyzes the hydrolysis of O-linked β -glycosidic bonds at the non-reducing end of carbohydrates. It also catalyzes transglycosylation reactions in which a sugar residue is transferred from a substrate molecule to an acceptor other than water to form a new glycoside (Gopalan and others 1992b). These properties are consistent with the fact that CBG is a configuration-retaining glycosidase (Legler and Bieberich 1988). The physiological function of the broad-specificity CBG has eluded investigators for decades. Evidence that L-picein (a plant phenolic glucoside) was an excellent substrate for and dhurrin (a naturally occurring cyanogenic β -D glucoside) a potent inhibitor of CBG led LaMarco and Glew (1986) to believe that this enzyme might play a role in the detoxification of toxic plant glucosides that find their way into the diets of man or animals. Experiments performed by Gopalan and others (1992a) have confirmed this hypothesis by demonstrating that guinea pig CBG catalyzes the hydrolysis of the cyanogenic glucosides amygdalin and prunasin. It has also been established that amygdalin, prunasin and vicine, another plant glucoside, are transported rapidly and without hydrolysis across the gut. This enables the liver to be involved in the biotransformation of these glycosides.

CBG satisfies at least three of the four characteristics of detoxification enzymes enumerated by Jakoby and Zeigler (1990): (i) it has affinity for amphipathic xenobiotics owing to the presence of polar and hydrophobic domains in its catalytic center, (ii) it displays a broad specificity with regard to the glycone and can tolerate variations in the aglycone moieties of its monosaccharide substrates, albeit to a

limited extent, and (iii) it is present in significant amounts in the liver and intestine (detoxification enzymes are usually concentrated in a specific organ). While CBG may play a protective role in the biotransformation pathway, as described above, the possibility that it may contribute to the pathogenesis of toxic plant glucosides must, however, also be considered. In the case of vicine, as well as other cyanogenic glucosides, the toxicity of these compounds only manifests after the glucose residues have been removed (Gopalan and others 1992a). It is unclear if varying levels of the enzyme in humans could underlie variations in the susceptibility of different individuals to these toxic plant glucosides.

It has been established that the process of dietary flavonoid glycoside metabolism in humans is shared by CBG and LPH (Németh and others 2003). The *in vivo* function of the broad-specificity CBG has yet to be confirmed.

2.3.1.4 The deglycosylation of flavonoid glycosides

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. These (iso)flavonoids are potent antioxidants *in vitro* and have been shown to be effective anti-carcinogenic and anti-atherogenic agents in various animal and cell models (Birt and others 2001). It is a popular belief that these polyphenols, as part of dietary fruits and vegetables, provide protection against disease by functioning as antioxidants *in vivo* (Yang and others 2001).

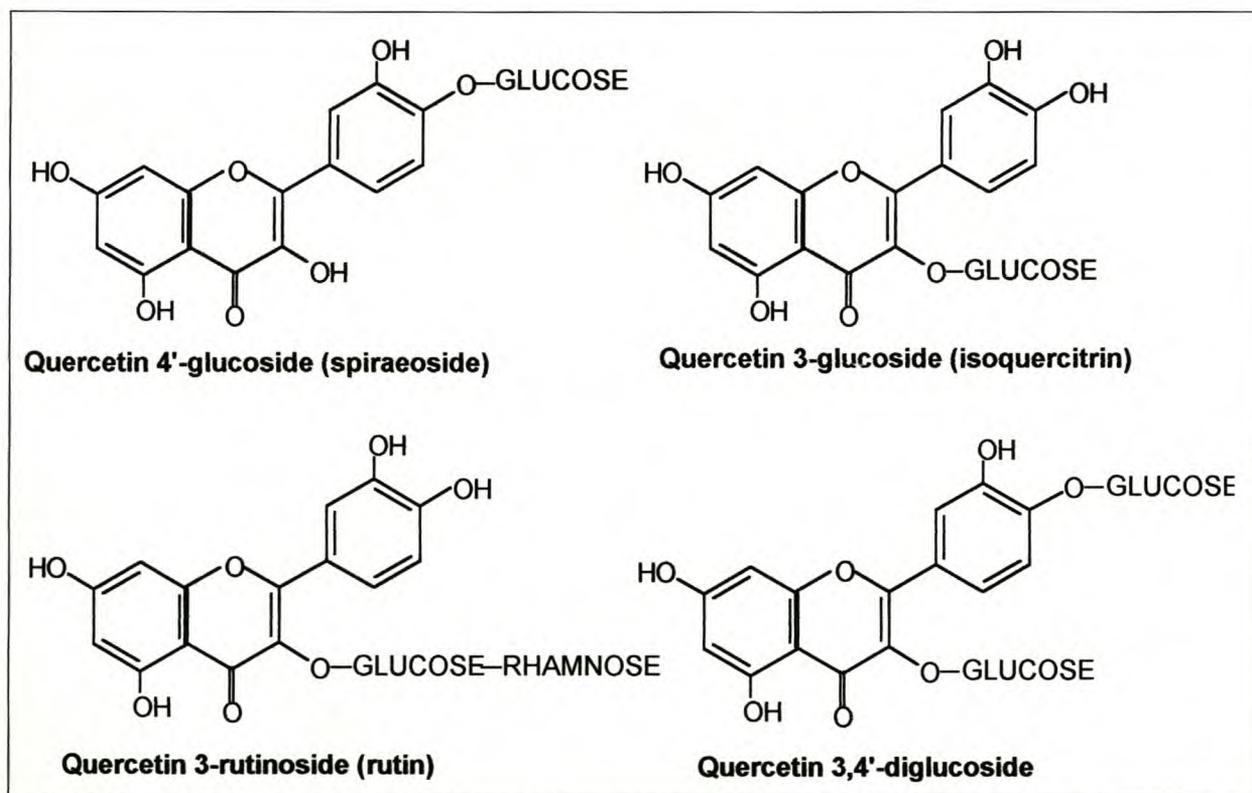


Figure 2.3. The structure of quercetin and several examples of its common glycosylated derivatives that are present in plants and food (adapted from Németh and others 2003).

The dietary intake of quercetin, the major representative of the flavonol subclass of flavonoids, mainly occurs through the ingestion of tea, onions and apples. Earlier

studies on the flavonoid content of onions (*Allium cepa* L.) have shown that the main flavonoids are quercetin, quercetin-4'-glucoside, quercetin-3,4'-diglucoside, quercetin-7,4'-diglucoside and isorhamnetin glycoside (**Figure 2.3**). The minor flavonoids have been identified as quercetin-3-glucoside and quercetin-5-glucoside (Fossen and others 1998).

In plants, flavonoids exist almost exclusively as β-glycosides. Flavonols are found mainly as 3-O-glycosides, although the 7 and 4' positions may also be glycosylated, as in onions (Fossen and others 1998). Other classes of flavonoids, such as flavones, flavanones and isoflavones, are mainly glycosylated at the 7 position. Most industrial and domestic food-processing procedures do not lead to cleavage of the glycosidic linkage (Price and others 1997) and the flavonoids in foods are therefore generally present as glycosides. Until recently, it was generally accepted that flavonoid glycosides remained intact until they reached the colon, where they underwent deglycosylation mediated by the microbial enzymes. However, recent studies have demonstrated that the primary site of absorption of some of these glycosides is the small intestine (Hollman and others 1997; Erlund and others 2000). Biological activity depends on the presence or absence of the glycoside residue. The position and nature of the sugar residue may increase the uptake of the compound in the small intestine (Williamson and others 1996).

There is considerable evidence to support the hypothesis that deglycosylation is a prerequisite for the absorption of dietary (iso)flavonoid glycosides. Research done by Németh and others (2003) has demonstrated that only two of the β-glucosidases present in the human small intestine are capable of hydrolyzing flavonoid glycosides: LPH and the broad-specificity CBG. Whereas CBG is located intracellularly and would require active transport of the hydrophilic glucosides into the cells, LPH is exposed to the lumen and its action would release aglycones into the lumen, where passive diffusion across the membrane could occur. A model for the absorption of dietary flavonoid glycosides that highlights the role of human small intestinal β-glucosidases is presented in **Figure 2.4**. The polyphenol glycosides (PP-sugar) reach the small intestine, since these compounds are not absorbed in the stomach (Crespy and others 2002). In the small intestine they may be hydrolyzed by LPH, with its catalytic sites exposed to the lumen, or transported into the enterocytes by glucose transporters such as SGLT-1 and then hydrolyzed by CBG to give the aglycone (PP), which will be further metabolized into conjugates.

Based on the results of the study done by Németh and others (2003), it is now possible to predict the absorption kinetics for (iso)flavonoids from a variety of foods. Polyphenol glycosides that are substrates for LPH and/or CBG exhibit enhanced absorption kinetics and increased bioavailability. The glycosides that are not substrates for LPH or CBG, for example rutin found in tea, are generally not absorbed in the small intestine and will travel further down the gastrointestinal tract and reach the colon, where exposure to the colonic microflora will result in rapid

degradation. It has been established that CBG and LPH play an integral part in the deglycosylation, and subsequent absorption, of dietary flavonoid glycosides.

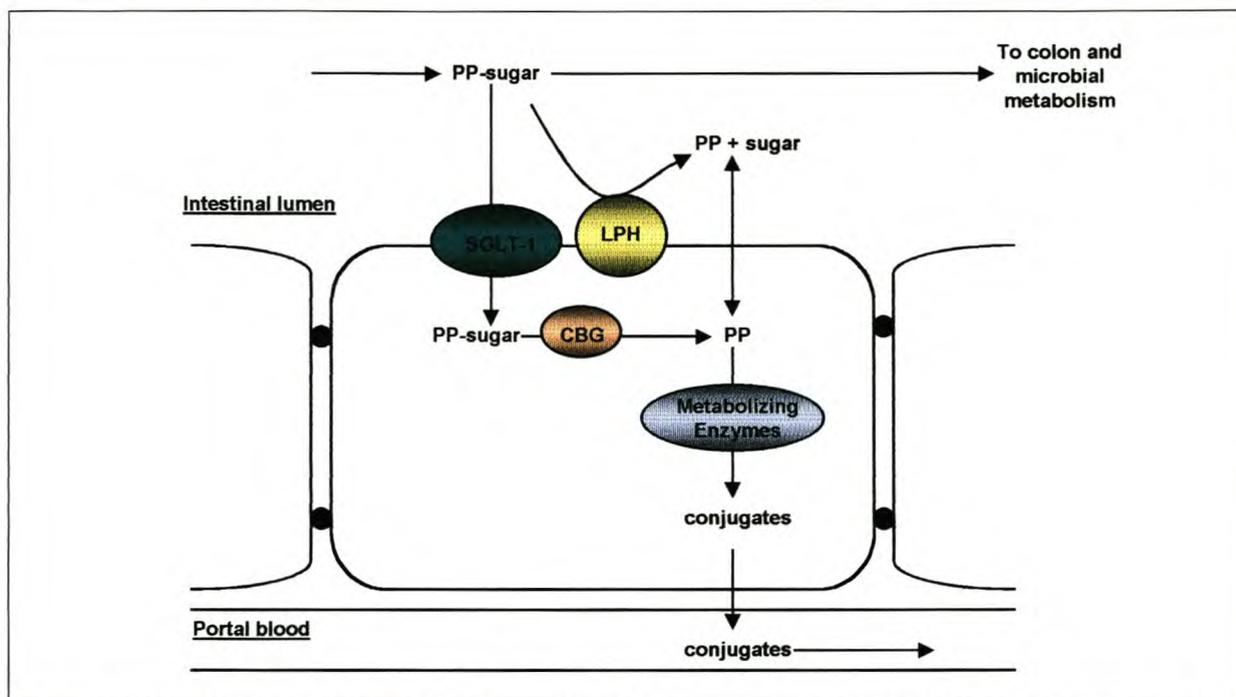


Figure 2.4. Diagram highlighting the role of human small intestinal β -glucosidases in the absorption of dietary flavonoid glycosides (adapted from Németh and others 2003).

2.3.2 β -GLUCOSIDASES FOUND IN INSECTS

In insects, digestive β -glucosidases are important for the hydrolysis of di- and oligo- β -saccharides derived from hemicelluloses and cellulose, and are involved in insect-plant interactions. The first classification of insect digestive β -glucosidases, comprised of three classes, was based on substrate aglycone specificity (Terra and Ferreira 1994). Class 1 consists of enzymes with glycosyl β -glucosidase and aryl- (or alkyl) β -glucosidase activity. These enzymes hydrolyze cellobiose, lactose, PNPGlu, β -*p*-nitrophenylgalactoside (PNPGal), β -*p*-nitrophenylfructoside (PNPFru) and other disaccharides. Class 2 includes the enzymes with only glycosyl β -glucosidase activity, therefore only substrates such as cellobiose and lactose can be hydrolyzed. Class 3 is composed of enzymes with only aryl- or alkyl- β -glucosidase activity and, due to the fact that they preferentially hydrolyze monosaccharides linked to a hydrophobic aglycone, for example glycolipids such as glycosylceramides, it is highly probable that class 3 β -glucosidases are glycosylceramidases (Terra and Ferreira 1994).

Evolutionary divergence resulted in insect β -glucosidases varying from multiple enzymes with different substrate specificities to a single enzyme able to hydrolyze all β -glycosides at the same active site. The presence of multiple enzymes with different specificities allows the insect to avoid plant glycoside intoxication by repressing the enzyme directly related to toxic aglycone production (Ferreira and others 1998). Insects for which multiple β -glucosidases were reported include the sugar cane borer

(*Diatraea saccharalis*), which has three (Ferreira and others 1997), the fall armyworm (*Spodoptera frugiperda*) with two (Marana and others 2000), and the yellow mealworm (*Tenebrio molitor*), which has four (Ferreira and others 2001). Insects with only a single enzyme include *Rhynchosciara americana* and *Erinnyis ello* (Ferreira and Terra 1983; Santos and Terra 1985).

As the aglycone portion of plant glycosides is much more toxic than the glycoside itself, the specificity of digestive β -glucosidases may play a very important role in the ability of the insect to use a specific plant as food source. Ferreira and others (1997) showed that neither the development nor the digestive β -glucosidases of *S. frugiperda* larvae fed with an artificial diet containing amygdalin was affected. In contrast, the performance of sugar cane borer larvae was affected and the activity of one of their three β -glucosidases decreased when they were fed a similar diet. Another example of the role of β -glucosidase in this interaction is the response of cabbage plants to *Pieris brassica* herbivory. The insect β -glucosidase elicits the liberation of a plant compound that attracts wasps, which prey upon the insect (Mattiacci and others 1995).

In 1986, Low and others detected β -glucosidase activity in honey. It was hypothesized that this enzyme activity could originate from the honey bee (*Apis mellifera*), and this led to the purification and characterization of the β -glucosidase from *A. mellifera* by Pontoh and Low in 2002. They found β -glucosidase activity in the hypopharyngeal glands, honey sac, ventriculus and hindgut of the honey bee. The enzyme is secreted into the mouth during feeding, passes to the honey sac and from there on to the honeycomb cells and ventriculus. It shows resistance to protease activity and has no activity towards cellobiose, which indicates that it is a class 3 β -glucosidase. These results indicate that the role of β -glucosidase in the alimentary canal of honey bees is not for the digestion of cellulosic material such as pollen, but is most likely present to hydrolyze glycoside toxins ingested by the honey bee (Terra and Ferreira 1994).

Ferreira and others (2001) did extensive research on the four β -glucosidases found in the midgut of the yellow mealworm (*T. molitor*). The likely function of β Gly1 and β Gly2 involves the digestion of oligosaccharides derived from hemicellulose digestion. These two enzymes have a preference for oligosaccharides linked with β -1,3 bonds (Ferreira and others 2001). β Gly3 is inhibited by high concentrations of amygdalin, cellotriose, cellotetraose and cellopentaose. It hydrolyzes β -galactosides and β -mannosides in varying degrees of efficiency, and its high activity towards β -glucosides (hydrolyzes β -1,3 and β -1,4 bonds with similar efficiency) makes it the main enzyme responsible for the hydrolysis of β -glucoside links in disaccharides in *T. molitor*. β -Gly4 is a β -galactosidase that poorly hydrolyzes lactose and has high activity when hydrophobic substrates are used. Its physiological role is speculated to be the hydrolysis of galactolipids that are found in vegetal tissues (Ferreira and others 2003).

Ferreira and others (2002) found that the β Gly1 and β Gly3 of the sugar cane borer (*D. saccharalis*) are similar to *T. molitor* β Gly3 and β Gly1 respectively, and that *D. saccharalis* β Gly2 is in some aspects similar to *T. molitor* β Gly4. Since each of the three *D. saccharalis* β -glycosidases has a similar enzyme in *T. molitor*, it is tempting to speculate that insects with the same number of glycosidases will have similar enzymes. Only the characterization of more insect β -glycosidases will be able to substantiate the validity of such a hypothesis.

2.3.3 β -GLUCOSIDASES IN THE PLANT KINGDOM

In plants, β -glucosidases have been implicated in a variety of key metabolic events and growth-related responses, ranging from defense against some pathogens and herbivores through the release of coumarins, thiocyanates, terpenes and cyanide, and the hydrolysis of conjugated phytohormones (e.g. glucosides of gibberellins, auxins, abscissic acid and cytokinins), to scent production in flowers and aroma liberation in fruit. These β -glucosidases vary greatly in their specificity for the aglycone: some have broad substrate specificity (Selmar and others 1987; Cicek and others 2000), while others are highly specific and react only with a narrow range of aglycones (Hösel and Conn 1982; Babcock and Esen 1994; Cicek and others 2000).

Two types of β -glucosidases are found in plants, namely β -D-glucosidases, which hydrolyze O-linked β -glycosidic bonds, and β -S-glucosidases (Myrosinase), which catalyze the cleavage of S-linked β -glucosidic bonds (Bhatia and others 2002). Only a few β -glucosidase genes have been cloned from plant sources (Table 2.1).

Table 2.1. Some β -glucosidases cloned from plant sources (NA = not available).

Source	Gene name	Host	Enzyme location	Reference
Barley	BGQ60	<i>E. coli</i>	NA	Leah and others 1995
<i>Catharanthus roseus</i> Strictosidine	BGL	<i>S. cerevisiae</i>	NA	Geerlings and others 2000
<i>Costus speciosus</i>	BGL	<i>E. coli</i>	NA	Inoue and others 1996
Maize isozymes	GLU1, GLU2	<i>E. coli</i>	NA	Cicek and Esen 1999
<i>Trifolium repens</i>	NA	NA	NA	Barrett and others 1995
<i>Manihot esculenta</i>	NA	NA	NA	McMahon and Sayre 1998
<i>Prunus dulcis</i>	NA	NA	NA	Legler and Harder 1978

2.3.3.1 Plant cyanogenic β -glucosidases

Cyanogenesis is one of the functions reported for plant β -glucosidases. This process, characterized by the release of hydrogen cyanide (HCN) upon the hydrolysis of cyanogenic glucosides (CGs), only becomes apparent after damage to the cyanogenic organs or tissues of the plant. The tissue level compartmentalization of

CGs and their hydrolyzing enzymes prevents large-scale hydrolysis in intact plant tissue (Vetter 2000).

CGs can be defined chemically as glycosides of α -hydroxynitriles that are β -linked to mostly D-glucose and belong to the secondary metabolites of plants. The distribution of cyanogenic glucosides in the plant kingdom is relatively wide, with at least 2500 CG-containing taxa. Both the Dicotyledonopsida and Monocotyledonopsida classes have plant families with cyanogenic compounds, but most families belong to the dicots. The following plant families have all been reported to contain CGs: Compositae, Euphorbiaceae, Linaceae, Papaveraceae, Fabaceae, Rosaceae, Polypodiaceae, Mimosaceae, Poaceae and Oleaceae (Vetter 2000).

One of the most probable explanations of the biological role of CGs in some plants is its participation in defense mechanisms against different phytopathogens, even though both plants and animals possess the ability to detoxify cyanide. Studies have shown that CGs can act either as feeding deterrents or phagostimulants, depending on the insect species. Research done by Brattsen and others (1983) showed that the larvae of the southern armyworm (*Spodoptera eridania*) prefer to graze on CG-containing plants and grow better when cyanide is present in their diet. Malagon and Garrido (1990), on the other hand, concluded that bitter almond plants are resistant to the larvae of buprestid *Capnodis tenebrionis*, owing to the high concentration of CGs. Extensive research has been done on the two distinctly different cyanogenic enzyme systems, the amygdalin and linamarase systems (Hughes 1993; Poulton 1993; Vetter 2000).

2.3.3.1.1 Cyanogenesis in black cherry

The kernels of *Prunus* species (Rosaceae) are a rich source of the cyanogenic diglycoside (*R*)-amygdalin (the β -gentiobioside of (*R*)-mandelonitrile), and of the complex enzyme system commonly known as emulsin, which attacks a wide variety of α - and β -glycosidic bonds. Work done by Poulton (1993) indicated that three enzymes are involved in the degradation of amygdalin to HCN and benzaldehyde, and he subsequently separated the emulsin of black cherry (*Prunus serotina*) into three different enzyme activities: (i) amygdalin hydrolase (AH), which catalyzes the breakdown of amygdalin to glucose and prunasin, (ii) prunasin hydrolase (PH), which catalyzes the hydrolysis of prunasin to glucose and mandelonitrile, and (iii) mandelonitrile lyase (MDL), which catalyzes the dissociation of mandelonitrile to the toxic products HCN and benzaldehyde (**Figure 2.5**).

AH, PH and MDL not only catalyze distinct reactions sequentially, but are also present in different subcellular compartments. AH and PH are both confined to the procambium, but AH is distributed throughout the whole procambium, whereas PH is restricted to the peripheral cells of this meristematic tissue. MDL, even though present in the procambium in small amounts, is primarily located in protein bodies of the cotyledonary parenchyma cells. None of these enzymes is found in the bundle sheath and endosperm (Poulton 1993). As in other *Prunus* species, young leaves

and immature fruits contain prunasin, but amygdalin predominates in mature fruits (Nahrstedt 1973). During cotyledon development, the levels of AH, PH and MDL increase rapidly, specifically within the seeds (Poulton 1993). Some cyanogenic forage plants accumulate the highest level (which is or can be poisonous for the animals consuming them) in the phase of active growth, when the cells are in the enlargement phase and have very intensive metabolic activities.

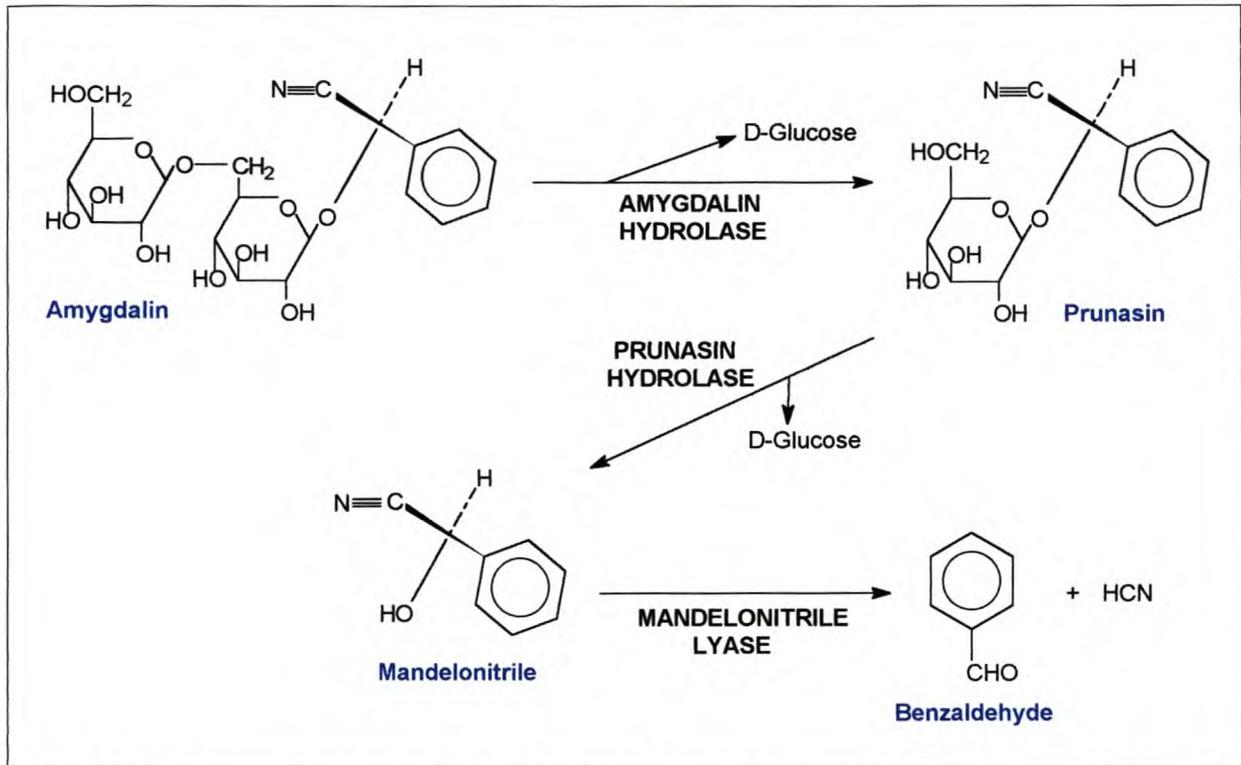


Figure 2.5. Catabolic pathway for (*R*)-amygdalin by almond emulsin and homogenates of mature *Prunus serotina* seeds (adapted and modified from Poulton 1993).

During the purification and characterization of AH, PH and MDL, it was found that all three of these enzymes consist of different isoforms. Kuroki and Poulton (1986) purified the four isoenzymes of AH (designated AH I, I', II, II'). These isoenzymes are all monomeric glycoproteins. Investigations done on the substrate specificity of AH I and II indicated that, among the naturally occurring glycosides tested, the endogenous cyanogenic diglycoside (*R*)-amygdalin was hydrolyzed most rapidly. The aglycone specificity was demonstrated by the high activity toward 4-MUGlc, PNPGlc and *o*-nitrophenyl- β -D-glucoside (ONPGlc), and the lower activity toward PNPGal and the aliphatic cyanogenic diglycosides linustatin and neolinustatin (Kuroki and Poulton 1986). Mixed substrate experiments indicated that amygdalin, 4-MUGlc and PNPGlc were hydrolyzed at the same active site. No activity was noted toward β -gentobiosides, cellobiose and the cyanogenic monoglucosides (*R*)-prunasin, (*S*)-sambunigrin, (*S*)-dhurrin and linamarin. Cations are not required for activity, and the addition of chelators, thiol agents and sulfhydryl reagents had no significant effect. However, AH is competitively inhibited by the pyrrolizidine alkaloid castanospermine, and by prunasin (Kuroki and Poulton 1986).

The three isoforms of PH (designated PH I, IIa and IIb) were also reported by Kuroki and Poulton (1987). In this case it was found that PH IIa was a dimeric glycoprotein, while the other two were monomeric. These isoforms exhibit a narrow specificity towards (*R*)-prunasin and efficiently utilize 4-MUGlc, PNPGlc, ONPGlc and PNPGal as substrates. They are, however, inactive towards the diglucosides (*R*)-amygdalin, linustatin and neolinustatin, and the monoglucosides (*S*)-dhurrin and linamarin. Mixed substrate studies showed that prunasin, PNPGlc and ONPGlc were hydrolyzed at the same active site. Like AH, PH activity is unaffected by the addition of cations, chelators, thiol agents and sulfhydryl reagents, and is also inhibited by castanospermine (Kuroki and Poulton 1987).

Yemm and Poulton (1986) identified the five monomeric multiple forms (two major, three minor) of MDL. Forms 4 and 5, which constitute the two major forms, were used in detailed comparative kinetic studies. Neither lyase form exhibits a metal ion requirement and the addition of sulfhydryl reagents is ineffective, except for iodoacetic acid and iodoacetamide, which inhibit these proteins in black cherry and almond respectively. Both forms are inhibited by unsubstituted and *o*- and *p*-hydroxy-substituted aromatic substrate analogs, as well as by hexanoic acid (Yemm and Poulton 1986).

This microheterogeneity shown by AH, PH and MDL in the *Prunus* cyanogenic system is also found in analogous enzymes from other rosaceous stone fruits, such as almonds (Shibata and Nisizawa 1965; Grover and others 1977). Poulton (1993) has speculated that such multiplicity might well result from allelic variance, because, as an enzyme source, seeds are collected from many individual trees or, at best, a single tree.

2.3.3.1.2 Cyanogenesis in white clover and cassava

In white clover (*Trifolium repens*) and cassava (*Hevea brasiliensis*), cyanogenesis is caused by the action of a cyanogenic β -glucosidase on two cyanogenic substrates: linamarin and lotaustralin. The generation of cyanide from linamarin is a two-step process involving the initial deglycosilation of linamarin, and the cleavage of linamarin to acetone cyanohydrin to form acetone and cyanide. These reactions are catalyzed by a homodimeric β -glucosidase (linamarase) and by α -hydroxynitrile lyase (HNL) (Figure 2.6) (Vetter 2000).

All cyanogenic plant species that have been investigated in detail show variation in the amount of HCN produced. This variation reflects differences in both the production of CGs as well as the enzymes responsible for its degradation. In 1942, Corkill reported that two genes control the discrete form of variation found in white clover. The presence or absence of the glycosides is regulated by alleles of a single gene, designated *Ac*, whereas the presence or absence of the enzyme linamarase is governed by alleles of another, independently inherited gene, designated *Li*. Only plants which possess dominant functional alleles of both genes liberate HCN when damaged. Both genes also affect the vegetative and reproductive characteristics of

the plants, especially seed and flower production. Noitsakis and Jacquard (1992) demonstrated that acyanogenic plants have a competitive advantage over cyanogenic ones with regard to relative fitness (i.e. biomass production and the number of flowers per plant are higher) under experimental conditions.

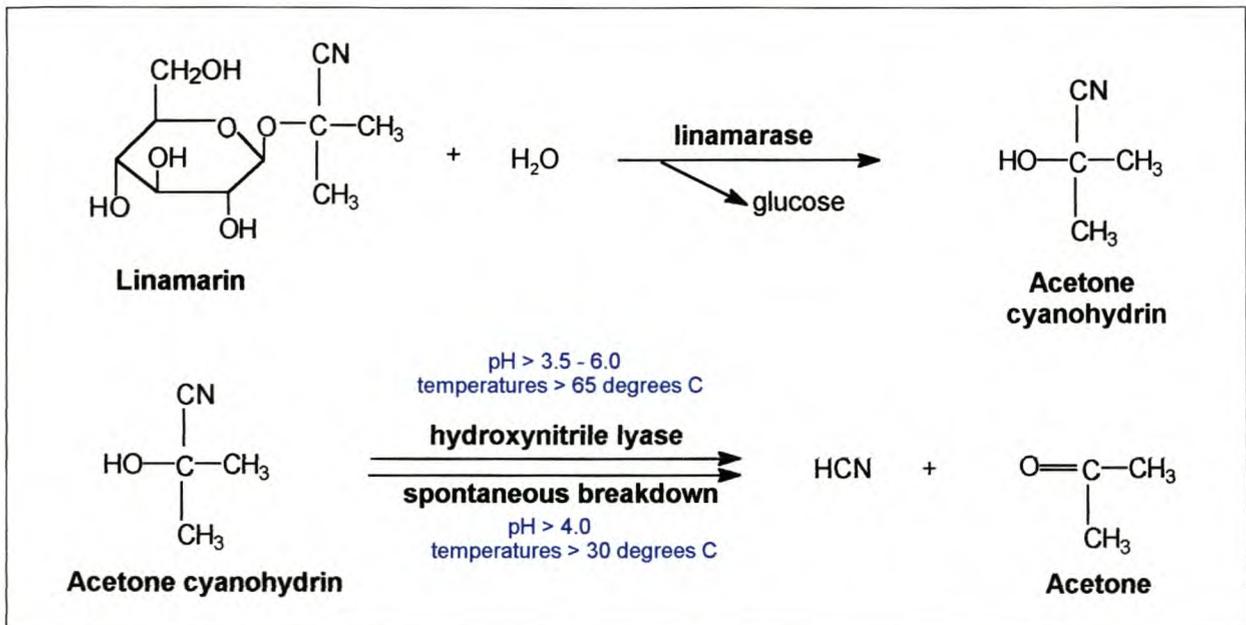


Figure 2.6. Cyanogenesis from linamarin (adapted and modified from Vetter 2000).

The cyanogenic enzymes can be located in different parts of the plant. Hughes (1993) has reported that the linamarase from *T. repens* is found extracellularly, whereas *H. brasiliensis* linamarase is localized in the cell wall (Mkpong and others 1990). The distribution of linamarase activity and linamarin varies between different organs and tissues of the same plant and, in addition, there are tissue-specific differences in linamarase levels within the same plant organ. The distribution of linamarin in cassava roots is 12-fold higher in the root peel than in the inner parenchymatic tissue (Bradbury and others 1991). The tissue and subcellular localization of HNL (responsible for the final step of cyanogenesis) were determined in *Sorghum* by Wajant and others (1994). The highest level of the enzyme was found in the cytoplasm, with lower levels in the organelles and no enzyme being detected in the cell wall or vacuoles.

The linamarases of some plants have such broad substrate specificities that the term generalized β -glucosidases apply rather than linamarase. An example of a plant with broad substrate specificity is cassava: its β -glucosidase is the only of its kind present in the plant and will hydrolyze a variety of β -linked glycoses and aglycones (Tull and others 1991; Yeoh and Yeoh 1994). The linamarase of *T. repens* also has broad substrate specificity, depending on the aglycone and on the type of glucosidic linkage (Pocsi and others 1989). Kinetic data on the linamarase purified from flax seed (*Linum usitatissimum* L.) revealed that this enzyme rapidly hydrolyzes PNPGlc and PNPGal, is only slightly active towards (S)-dhurrin, and is inactive toward linustatin, β -gentobioside and amygdalin (Fan and Conn 1985).

In *H. brasiliensis*, linamarin is stored in large quantities in the endosperm. During germination and early seedling development, the total linamarin content rapidly decreases, without any apparent release of HCN, suggesting that linamarin is converted to noncyanogenic substances. The linustatin pathway (**Figure 2.7**) provides a convincing explanation for this decrease. Selmar (1993) proposed that, in all plants with apoplastic β -glucosidases, the stored monoglucosides (e.g. linamarin and lotaustralin) are mobilized and converted to the corresponding diglucosides (e.g. linustatin and neolinustatin) for transport to the cotyledons and primary leaves, where they are converted back to CGs and other substances. This would suggest that β -glucosides are metabolized to other compounds and have other functions in the plant besides cyanogenesis.

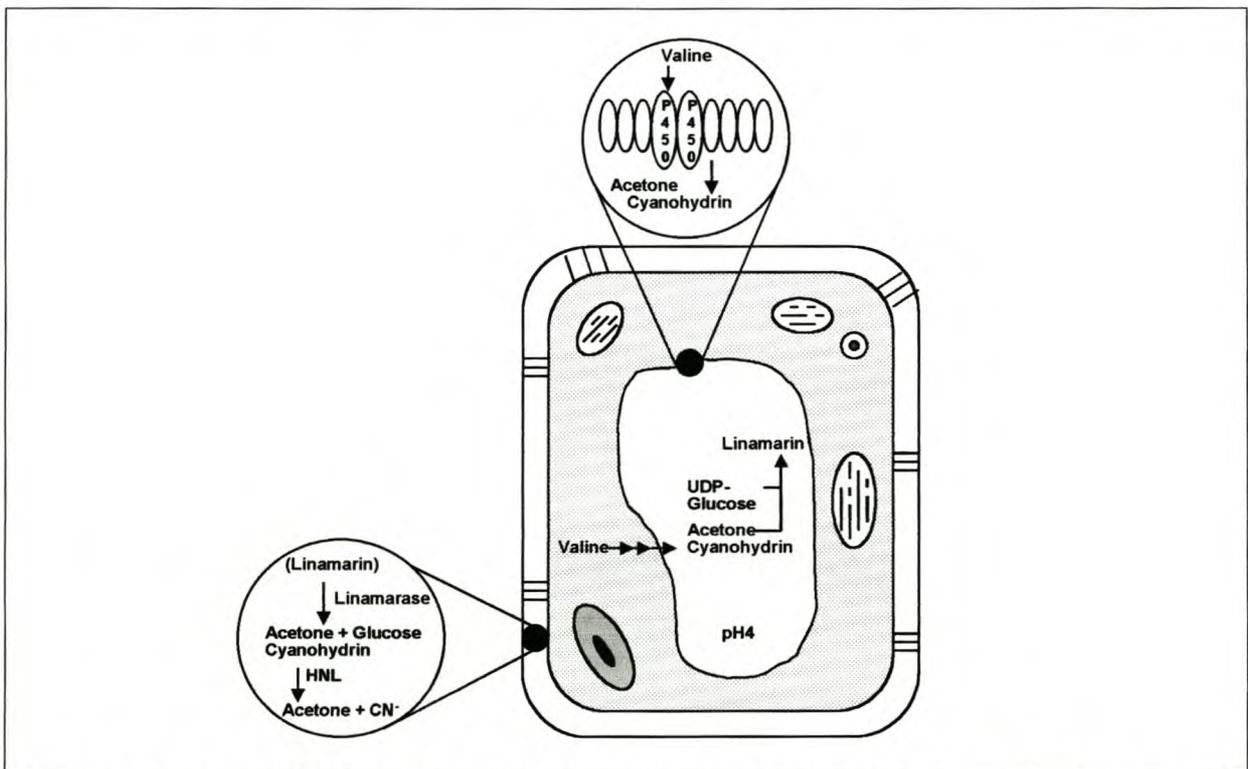


Figure 2.7. A model for linamarin synthesis and cyanide production following the rupture of a cassava leaf mesophyll cell (adapted and modified from Vetter 2000).

It is clear that the protection of seedlings by the presence of CGs has a selective advantage, but the situation is different for linamarase. Research has shown that linamarase is not necessary for the deterrence of slugs and snails (Dirzo and Harper 1982; Kakes 1993). The enzyme mixture in the gut of snails has a high linamarase activity. It is possible that linamarase is necessary for defense against other herbivores lacking linamarase activity in their guts, but the evidence for selective eating by organisms other than mollusks is weak.

2.3.3.2 Scent production in flowers

Little is known about the enzymology and biochemistry of fragrance production, or of the mechanisms of storage and secretion of these compounds. Many volatile

compounds are found in floral tissue in non-volatile forms, such as glycoside precursors (Loughrin and others 1992). The sugar moiety has to be removed in the flower where these compounds are secreted. This necessitates activity of glucosidases in floral tissue that is correlated with fragrance production (Watanabe and others 1993).

Narcissus has a strong fragrance and hundreds of its aromatic volatile compounds have been isolated and classified (Ehret and others 1992), but little is known about the enzymes involved in scent production. The involvement of deglycosylation in fragrance compound production has led to the assumption that narcissus would have high β -glucosidase activity in its flowers as the final step in scent emission. Research was done by Reuveni and others (1999) to determine whether this was indeed the case. Even though β -glucosidase activity was observed in the closed narcissus buds, a significant increase of about 60% in activity was reported as the scent emission increased to reach its maximal intensity when the buds were fully open. Enzyme activity was detected in both membrane and soluble fractions of the buds and open flowers, but the increase in activity after flower opening was mostly observed in the soluble fraction. No significant difference was found between the activity in the flower parts, the petals and the corona (Reuveni and others 1999).

The results further indicated that the pH of the assay solution had a significant influence on the stability of this particular β -glucosidase. The enzyme activity increased with decreasing pH, but was very unstable at pH 5.2. The inhibition of the enzyme by gluconic acid lactone was also dependent on the pH of the solution: where it usually inhibits the β -glucosidase activity, no inhibition was noted at pH 5.3. The influence of gluconic acid lactone on whole flower volatile emission was also investigated in the same study, and a decrease of 25% in emission was detected after an incubation period of 24 hours. The observation that β -glucosidase activity correlated with the increase in volatile emission, and that inhibition of the enzyme activity *in vivo* reduced volatile emission, support the original hypothesis that β -glucosidase activity is involved in scent production in narcissus flowers during flower development.

2.3.3.3 Effect of denaturants on plant β -glucosidases

Studying the activity and stability of enzymes under conditions that denature and inactivate typical proteins provides considerable information about their structure and can lead to the development of novel or unconventional conditions to carry out enzyme-catalyzed reactions in the laboratory or on an industrial scale. Esen and Gungor (1993) performed studies on the effects of ionic detergents sodium dodecyl sulfate (SDS) and deoxycholate (DOC), chaotropic agents (urea), and organic solvents dimethylformamide (DMF), ethylene glycol and methanol on maize and almond β -glucosidases.

Maize β -glucosidase was found to be very stable and active in the presence of SDS and DOC, but the stability of the enzyme in SDS appeared to be strongly dependent upon pH, with the enzyme being most susceptible to inactivation at acidic pH values, most notably pH 4. Ionic detergents are thought to disrupt hydrophobic interactions (Strambini and Gonnelli 1986; Mashino and Fridovich 1987) and possibly hydrogen bonding, making the hydrophobic groups and hydrogen bonds of the enzyme more accessible to a small molecule like urea. Inactivation of maize β -glucosidase was shown to occur rapidly, even at low concentrations (<2 M) of urea.

The enhancement of enzyme activity by low concentrations of certain organic solvents has been well documented (Veronese and others 1984; Guagliardi and others 1989). It is suggested that organic solvents loosen the secondary and tertiary structure by disrupting hydrogen bonding and hydrophobic interactions, resulting in a more relaxed and flexible conformation which can activate enzymes that have a compact and rigid structure (Veronese and others 1984; Guagliardi and others 1989). In the case of methanol, it was found that concentrations of between 2.5 and 10% had a slight stimulatory effect on maize β -glucosidase activity. Higher concentrations (20-30%) had only a slight inhibitory effect, with maximum inhibition (30%) reported for 30% methanol.

2.3.4 MICROBIAL β -GLUCOSIDASES

Potentially, microorganisms are very suitable enzyme factories, since it is relatively easy to increase enzyme yield by altering the environmental parameters (e.g. temperature, pH, O₂ transfer, medium composition, growth rate and stage of growth), or through genetic manipulation (e.g. enzyme induction, catabolite repression and gene cloning). Microbial β -glucosidases share many functions, including the conversion of cellulose to fuel-ethanol, aroma liberation in fruit and wine, and the degradation of organic matter in soil and marine ecosystems. Extensive research is being done on the optimization of these enzyme systems for industrial application.

Bhatia and others (2002) studied the properties and applications of β -glucosidases from a variety of microbial sources. In general, yeast β -glucosidases were shown to be highly active on aryl- β -D-glucosides, to possess low activity towards cellobiose, and to be found either intracellularly or located at the cell surface. The pH optimum ranges from pH 5.7-6.8 and the enzymes are unstable at temperatures $\geq 50^{\circ}\text{C}$. Yeast β -glucosidase genes have been expressed in eukaryotic hosts such as *Saccharomyces cerevisiae* or *Candida* sp., with the exception of β -glucosidases from *C. wickerhamii* and *Pichia etchellsii*, for which the genes were expressed in a prokaryotic expression system, *Escherichia coli* (Pandey and Mishra 1995; Skory and Freer 1995; Sethi and others 2002). In general, the enzymes expressed in *E. coli* were either localized intracellularly, as in BgII from *P. etchellsii* (Pandey and Mishra 1995), or to the periplasmic space, as in BgIII from the same yeast (Sethi and others 2002). *BGL1* and *BGL2* of *Saccharomycopsis fibuligera*

cloned into *S. cerevisiae* were, however, secreted into the extracellular medium (Machida and others 1988).

Brettanomyces, the nonsexual and nonsporulating form of *Dekkera*, is well known as a wine spoilage organism. This yeast produces 4-ethyl phenol and 4-ethyl guaiacol during fermentation through the decarboxylation of the hydroxycinnamic acids *p*-coumaric and ferulic acid (Chatonnet and others 1992). These phenols, considered as natural components in wine, become spoilage compounds when present in excessive amounts (Heresztyn 1986). Even though the vineyard (Beech and Davenport 1970), air (Beech and Davenport 1970; Beech 1993) and improperly sanitized cellar equipment (Van der Walt and Van Kerken 1961) are all sources of *Brettanomyces* contamination, wine barrels are the most frequently cited source of contamination (Fugelsang and others 1993). Some evidence suggests that new barrels are more susceptible to the growth of *Brettanomyces* than old barrels (Larue and others 1991). Many *Brettanomyces* species have β -glucosidases that are highly active towards cellulose and hemicellulose, enabling them to thrive on the large amounts of these compounds found in new barrels (Larue and others 1991; Humphries and others 1992).

Ait and others (1979) characterized the β -glucosidase of the anaerobic bacterium *Clostridium thermocellum*, which is located in the periplasmic space and has great affinity for the aryl β -D-glucoside substrate. The pH and temperature optima of this enzyme are 6 and 65°C, respectively. Bacteria with higher enzyme activity towards cellobiose include *Bacteroides succinogenes* (Groleau and Forsberg 1981). Most recombinant bacterial β -glucosidases are proteins with a relatively low molecular weight that are located intracellularly in the native organism. These enzymes are generally found in the cytosol, although recombinant β -glucosidase from *Ruminococcus* sp. (Takano and others 1992) was extracellular, while BgxA of *E. chrysanthemi* (Vroemen and others 1995), BglA of *E. herbicola* (Marri and others 1995), and SalA and SalB of *A. irakense* (Faure and others 1999) were localized in the periplasmic space. *Pyrococcus horikoshii* Bgl expressed in *E. coli* was bound to the membrane fraction (Matsui and others 2000).

The β -glucosidase from *Aspergillus* is highly active on cellobiose, has a pH optimum of 4.0-5.0, and can be found intra- or extracellularly. High thermal stability is observed for the *A. phoenicis* and *A. niger* enzymes, since they are inactivated only at temperatures $\geq 65^\circ\text{C}$ (Sternberg and others 1977). Other fungal β -glucosidases include those from thermophilic microorganisms. The enzyme from *Thermoascus aurantiacus* has a pH optimum of 5.0 and a temperature optimum of 70°C (Tong and others 1980). Fungi are known to secrete many forms of the same enzyme, depending on the strain and environmental conditions. One example is the two forms of *A. oryzae* (BGI and HGT-BG) that were identified by Riou and others (1998). These two forms were produced in different total and relative amounts, depending on the carbon source used for cultivation. Fungal β -glucosidases have been expressed in eukaryotic expression systems such as *T. reesei* (Barnett and others 1991),

Aspergillus sp. (Takashima and others 1999), *S. cerevisiae* (Dan and others 2000) and *P. pastoris* (Dan and others 2000), with the exception of the β-glucosidase from *Talaromyces emersonii* (Morrison and others 1990) and *Phanerochaete chrysosporium* (Li and Ranganathan 1998), which were expressed in *E. coli*. Recombinant fungal β-glucosidases were mostly localized extracellularly, but a few, such as recombinant BglA from *A. kawachii*, were localized in the periplasmic space of the host *S. cerevisiae* (Iwashita and others 1999).

2.3.4.1 Substrate specificity, inducers and inhibitors

β-Glucosidases with very broad substrate specificity have been isolated from many fungi (Kwon and others 1992; Copa-Patino and Broda 1994; Gueguen and others 1995; Yan and Lin 1997). Substrates include cellobiose, (1→3)-, (1→4)- and (1→6)-β-diglucosides, aryl- and alkyl-β-glucosides, cello-oligosaccharides and PNPGlc (Saha and Bothast 1996; Riou and others 1998). Surprisingly, the HGT-BG from *A. oryzae* can also quite efficiently hydrolyze maltose and other diglucosides with (1→3)-, (1→4)- and (1→6)-α linkages (Riou and others 1998). The only other fungal β-glucosidase reported to have the ability to hydrolyze both β- and α-glucosides is a β-glucosidase from *Botrytis cinerea* (Gueguen and others 1995).

Inhibition by various metal cations, such as Ag⁺, Cu²⁺, Hg²⁺, Zn²⁺ and Fe²⁺, has been reported for the β-glucosidases from *Aspergillus* spp., including *A. oryzae* and *A. niger* (Yan and Lin 1997; Riou and others 1998). Other well-known inhibitors of β-glucosidase activity include castanospermine, deoxynojirimycin, methyldeoxynojirimycin and glucono-δ-lactone (Ridruejo and others 1989). Activation by organic solvents has been observed for β-glucosidases from *A. niger* (Yan and Lin 1997), *A. oryzae* (Riou and others 1998), *B. cinerea* (Gueguen and others 1995), *C. peltata* (Saha and Bothast 1996) and *Fusarium oryzae* (Christakopoulos and others 1994). It has been proposed that the alcohol activation of these enzymes may be due to their glycosyltransferase activities.

Substrate inhibition by cellobiose and competitive inhibition by glucose are common properties of microorganisms that place an important constraint on the industrial use of these enzymes. This kind of inhibition has been reported for *T. reesei*, *Sporotrichum thermophile*, *Neocallimastix frontalis* and *B. cinerea* (Schmid and Wandrey 1987; Li and Calza 1991; Bhat and others 1993; Gueguen and others 1995). Microorganisms with β-glucosidases that exhibit high glucose tolerance are particularly favorable for application in the enzymatic hydrolysis of cellulose to glucose. β-Glucosidases with high glucose tolerance have been reported for *C. peltata*, *A. oryzae* and *A. niger* (Saha and Bothast 1996; Yan and Lin 1997; Riou and others 1998).

2.3.4.2 β-glucosidase activity in soil

The decomposition of plant litter is an essential process in the conversion of organic matter into humus, and therefore in the biogeochemical cycles of elements such as

carbon, nitrogen, phosphorus and sulfur. It is well established that enzyme activities in decomposing litter in both soil and water environments are directly related to rates of litter mass loss (Sinsabaugh and Linkins 1988; Sinsabaugh and others 1992; Sinsabaugh and Findlay 1995). Once released, the enzyme activity is primarily regulated by chemical and physical factors, such as litter chemistry, substrate availability and temperature (Sinsabaugh and Linkins 1987; Sinsabaugh 1994). Changes in soil chemistry (e.g. pH), nutrient availability (e.g. NO_3^- and NH_4^+ concentrations) and litter quality can result in different enzyme activities in the decomposing litter, which in turn can result in increased or decreased rates of litter decomposition (Carreiro and others 2000). Cellulose and hemicellulose are the most abundant carbohydrates among the chemical components of soil organic matter (Sinsabaugh and Moorhead 1994).

Soil enzymes have been suggested as potential indicators of soil quality because of the ease of measurement, microbial ecological significance, sensitivity to environmental stress and rapid response to changes in land management (Yakovchenko and others 1996; Dick 1997). Moreover, soil enzymes can be distinguished as exoenzymes (produced by living cells) or endoenzymes (released during disintegration of cells) (Tabatabai and Fu 1992) and, even though primarily of microbial origin, these enzymes can also originate from plants and animals (Tabatabai 1994). β -Glucosidase plays an important role in the degradation of cellulose, the main component of plant polysaccharides. It completes the hydrolysis process by catalyzing the cleavage of cellobiose to release two molecules of glucose per mole of cellobiose and, therefore, regulates the supply of an important energy source for microorganisms that are unable to take up cellobiose directly. β -Glucosidase is derived predominantly from soil microbial heterotrophs, in particular members of the mucorales (fungi), such as *Actinomucor* or *Mortierella* (Hayano and Tubaki 1985). Research has shown that β -glucosidase is the most abundant and easily detected of the three enzymes involved in cellulose degradation in soil and is rarely substrate limited, making it ideal for examining the importance of physico-chemical controls in the turnover of soil organic matter (Eivazi and Tabatabai 1988; Debosz and others 1999).

The effects of different compounds on soil enzyme activities have been investigated in earlier studies of natural soil systems (Sparling and others 1981; Martens and others 1992). Soil amended with sources of organic carbon such as poultry manure, sewage sludge, barley straw or fresh alfalfa showed doubled or quadrupled enzyme activity for a year after the addition (Martens and others 1992). Increased enzyme activity has also been observed in soil amended with glucose (Sparling and others 1981). Different responses to nitrogen amendments have been reported for different ecosystems. β -Glucosidase activity in natural systems increased with the addition of nitrogen to the soil (Carreiro and others 2000; Saiya-Cork and others 2002), but no change was observed in agricultural plots (Bandick and Dick 1999). In 2002, Lee and others investigated the effect of accumulated

heavy metals found at a shooting range on soil microbial activity. Their results were consistent with the findings of Kuperman and Carreiro (1997), who reported that β -glucosidase activity is markedly reduced as heavy metal concentrations increase. It is clear from these studies that high concentrations of heavy metals have a negative impact on the structure and function of soil communities and ecosystems.

Evidence suggests that cellulolytic enzymes are inducible (Ali and Sayed 1992; Heupel and others 1993), and that cellobiose, sophorose and lactose are good inducers. Busto and others (1995) investigated the induction and location of β -glucosidases produced by fungal and soil bacterial cultures grown on cellobiose. It was found that cellobiose is an active inducer in β -glucosidase synthesis in soil enrichment cultures by promoting the specific proliferation of both *Pseudomonas* and *Aspergillus* cultures. The enzyme location was determined to be intracellular in the bacteria and extracellular in the fungi. Their findings confirmed previous results that indicated that some cellulases, particularly those from bacterial origin, perform the catalytic function *in vivo* in an immobilized state, and that fungal cellulases are extracellular enzymes (Klyosov 1990). These results may suggest that there are different induction mechanisms for the intracellular bacterial cellulases and the extracellular fungal cellulases.

2.4 THE APPLICATIONS OF β -GLUCOSIDASES

The effective application of β -glucosidases requires large-scale production of the enzymes and a detailed knowledge of their reaction mechanisms. A substantial amount of work has been done on cloning and expressing the structural genes of β -glucosidases from a variety of microorganisms and plants into high-yielding mesophilic expression systems, such as *E. coli*, *S. cerevisiae* and filamentous fungi. The dual activity of β -glucosidases, which includes the cleavage and synthesis of glycosidic bonds, plays a pivotal role in a number of biotechnological applications. These applications can broadly be classified into two classes: (i) applications based on the hydrolytic activity of the enzyme, and (ii) applications based on the synthetic activity of the enzyme. The following discussion presents an overview of the applications of β -glucosidases in various biotechnological sectors, such as medicine, industry and food.

2.4.1 APPLICATIONS BASED ON HYDROLYTIC ACTIVITY

The increasing shortage of petroleum resources has necessitated the development of bioprocesses for the conversion of renewable resources such as starch, cellulose and hemicellulose to liquid fuels. The contribution of β -glucosidase to cellulose hydrolysis is significant, since cellobiose is an inhibitor of both endo- and exo-glucanases and needs to be removed to allow efficient and complete saccharification of cellulose. Most microbial β -glucosidases are very sensitive to end-product (glucose) inhibition, which limits activity (Gueguen and others 1995; Saha and others

1995). To minimize the accumulation of cellobiose and glucose, for example, is to simultaneously saccharify and ferment (SSF) the substrate (Grohmann 1993; Philippidis and others 1993). *S. cerevisiae*, which is well known for its superior ethanol-producing capabilities, is unable to utilize or transport cellobiose and its β -glucosidase gene is poorly expressed (Raynal and Guerineau 1984; Machida and others 1988). It is therefore necessary to develop a cellobiose-utilizing *S. cerevisiae* strain that can be used with SSF. β -Glucosidase genes that have been cloned for expression in *S. cerevisiae*, leading to ethanol production from cellobiose, include the genes from *S. fibuligera* (Machida and others 1988), *Aspergillus aculeatus* (Takada and others 1998), *C. wickerhamii* (Skory and others 1996) and *C. peltata* (Saha and Bothast 1996). In 1999, Cho and others developed a recombinant yeast strain that produced a full set of cellulolytic enzymes. This strain, *S. cerevisiae* L2612 δ GC, can be used for the production of ethanol from cellulose in a single step.

β -Glucosidase supplementation with a commercial cellulase preparation of *T. reesei* was beneficial for single-stomached animals, such as pigs and chickens (Leclerc and others 1987; Zhang and others 1996), as cellulose degradation was enhanced by the enzyme, leading to better nutrient utilization. In the food industry, the application of gellan, an exopolysaccharide produced by *Sphingomonas paucimobilis*, is very limited because of its high viscosity and low solubility. The intracellular β -glucosidases produced by *Bacillus* sp. were shown to catalyze cleavage of the trisaccharide glycosyl-rhamnosyl-glucose to release glucose and rhamnosyl-glucose, thereby reducing viscosity. The structural genes encoding the β -glucosidase activities in *Bacillus* were subsequently cloned (Hashimoto and others 1998). β -Glucosidases are also associated with the removal of bitterness from citrus fruit juices by catalyzing the hydrolysis of naringin to prunin (Roitner and others 1984), and with the enhancement of fruit and wine aromas by the liberation of monoterpene alcohols (Gueguen and others 1997).

Among other candidates for hydrolytic attack by β -glucosidases are flavonoid and isoflavonoid glucosides. Ridgway and others (1997) described the hydrolysis of phloridzin to liberate its aglycone moiety, which is a precursor of melanin. The latter is known to reduce the risk of skin cancer and promotes dark hair color. Similarly, the deglycosylation of betacyanin (betalains) by β -glucosidases in *Beta vulgaris* is the first step toward the degradation of these compounds to release the bioactive cellular metabolite, which has anti-tumor activity. In a recent application of the hydrolytic activity of β -glucosidases, the bioconversion of oleuropein, which causes bitterness in unripe olives, was performed in a bioreactor using immobilized β -glucosidase from the thermophile *Sulfolobus solfataricus*. The aglycone moiety released as a result of the cleavage step is a pharmacologically-active compound that is useful in the prevention of coronary heart disease and cancer (Briante and others 2000).

Interestingly, β -glucosidases from bacterial sources, such as *Cellovibrio mixtus* (Sakellaris and others 1997), *Thermoanaerobacter brockii* (Breves and others 1997) and *Thermotoga neopolitana* (Zverlow and others 1997), also act as laminaribiases.

This property is significant in the production of yeast extract and the conversion of algal biomass to fermentable sugars. The role of certain plant β -glucosidases is noteworthy in pigment metabolism and isolation. Dried saffron (*Crocus sativus*) flower florets were treated with β -glucosidases in order to isolate precarthamine pigment (Saito 1993). **Figure 2.8** summarizes the action of β -glucosidases on different types of glucosidic compounds that result in the generation of useful products or properties.

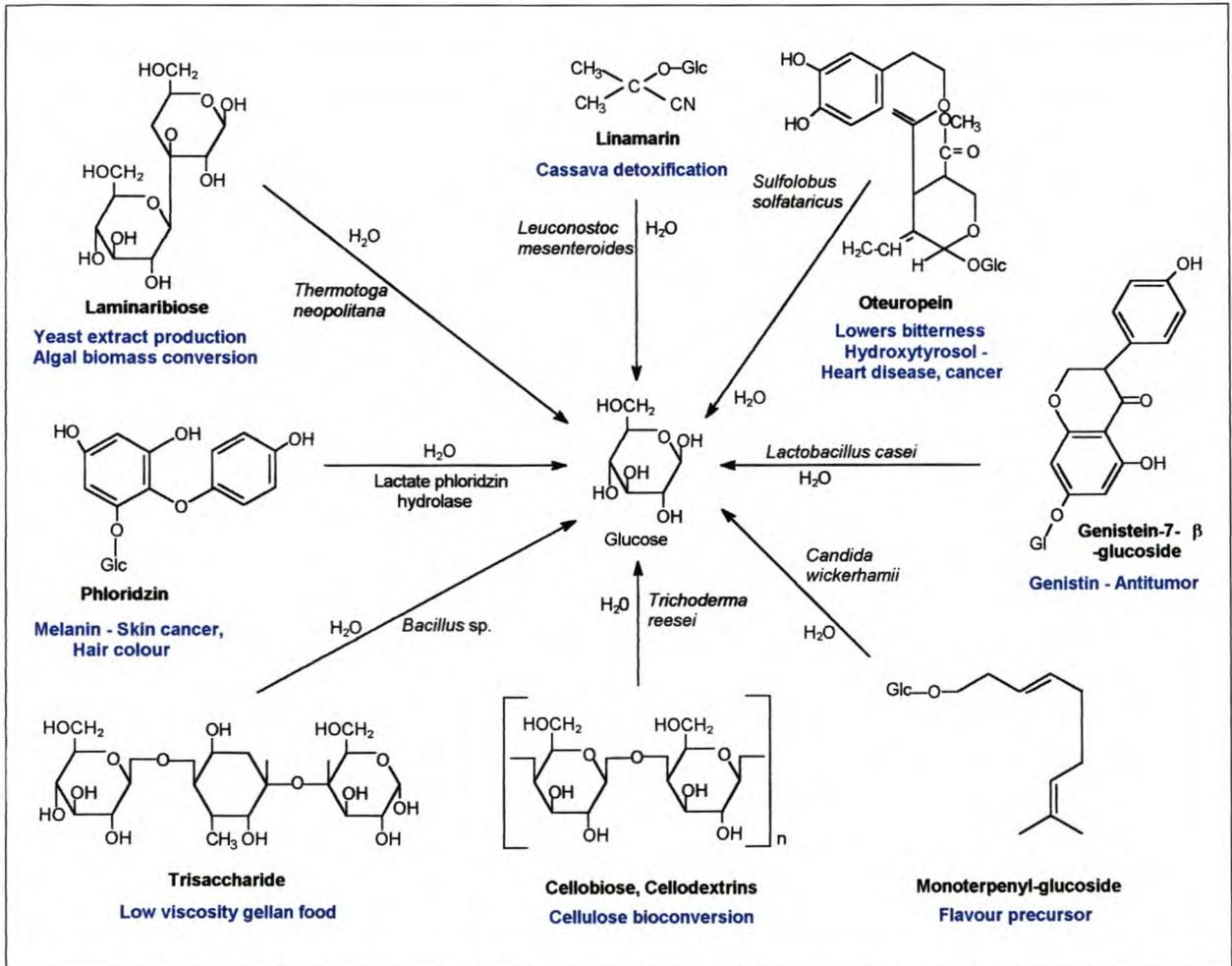


Figure 2.8. Applications based on hydrolytic activities of β -glucosidase. The enzyme source for each reaction is shown on the arrow. The useful products or properties are indicated in blue (adapted and modified from Bhatia and others 2002).

2.4.2 APPLICATIONS BASED ON SYNTHETIC ACTIVITY

The transferase activity of β -glucosidases may be used in the synthesis of a variety of compounds, such as oligosaccharides and glycoconjugates. Biotransformations are a well-established means for the manufacture of pharmaceuticals, fine chemicals and food ingredients, owing to the high selectivity of enzymes and the use of mild reaction conditions. Both reverse hydrolysis and transglycosylation reactions have been used for this purpose (Bhatia and others 2002).

Alkyl-glucosides, a new generation of biodegradable, non-ionic surfactants with good emulsifying and antimicrobial agents, are synthesized by the use of organic solvents. These compounds have been used as drug carriers and as solubilizing

agents for biological membranes (Kiwada and others 1985; Shinoyama and others 1991). Esterification of butyl-glucoside in the presence of phenyl butyric acid in a coupled β -glucosidase/*Candida* sp. lipase reaction resulted in the synthesis of an aromatic *n*-alkyl glucoside ester that was effective in the treatment of fever, rheumatism, headache and other ailments (Otto and others 1998). The role of methyl-glucoside as a precursor for the synthesis of methyl-laminario oligosaccharides was also established and was furthermore shown to be useful in AIDS therapy (Dainippon-Ink-Chem 1996).

The glucosides of organosilicon alcohols, synthesized by free and immobilized *P. furiosus* enzymes, have potential applications as agrochemicals and drugs (Fischer and others 1996). Apart from primary alcohols, the secondary, tertiary, monoterpene and aryl alcohols, or even diols, may also serve as acceptors of the glycosyl group in β -glucosidase-catalyzed biotransformations. The market for the enzymatic synthesis of glucosides of monoterparyl alcohols is growing due to public concern for the safety of food ingredients (Günata and others 1994). Tricone and Pagnotta (1995) used thermostable β -glucosidases from *S. solfataricus* to synthesize natural compounds, such as aryl-glucosides, that possess repellent and antifeedant properties.

2.4.3 OTHER APPLICATIONS

When De Duve suggested in 1964 that enzyme replacement therapy (ERT) might be a successful approach to the treatment of lysosomal storage diseases, type 1 Gaucher disease appeared to be a promising candidate for such therapy. Early attempts to treat patients by administering glucocerebrosidase were disappointing (Brady and others 1974; Pentchev and others 1975), but new attempts have been made to target glucocerebrosidase to macrophages by modifying the oligosaccharides of the enzyme (Furbish and others 1981; Doebber and others 1982). Patients respond well to ERT with purified macrophage-targeted glucocerebrosidase derived either from human placenta, alglucerase (Ceredase, Genzyme Corporation, Cambridge, Mass), or from recombinant DNA production methods, imiglucerase (Cerezyme, Genzyme Corporation) (Barton and others 1991; Grabowski and others 1995; Zimran and others 1995). ERT is extremely expensive and, even though the occurrence of IgG antibodies against alglucerase in patients with Gaucher disease receiving ERT has been reported, it is uncommon. In the majority of patients in whom non-neutralizing anti-alglucerase antibodies occurred, these receded spontaneously without an adverse effect on the patients' response to ERT (Richards and others 1993; Rosenberg and others 1999). Research is being done on β -glucosidase gene transfer as an alternative method of treatment (Kohn and others 1991; Correll and others 1992).

The levels of serum glycosylhydrolases, β -glucosidase and β -galactosidase increase after liver injury and have been used as a diagnostic tool to detect premalignant and malignant lesions of oral mucosa in hamsters (Balasubramanian

and others 1996). The activities of these enzymes are markedly elevated only in the carcinoma stage. The H-antigen of *Histoplasma capsulatum* (a pathogenic fungus causing respiratory disease) was found to exhibit β-glucosidase activity. Since this H-antigen could elicit cell-mediated immunity and humoral immunity, it was used for serodiagnosis of histoplasmosis (Fischer and Woods 2000).

The study of glycosidase inhibitors is an interesting area of applied research. Glycosidases are involved in a variety of metabolic disorders, such as diabetes, viral attachment, bacterial infection and cancer formation, and potent and selective glycosidase inhibitors may potentially have an application in AIDS, diabetes, cancer and crop protection.

2.5 β-GLUCOSIDASES IN WINEMAKING

One of the most important characteristics of a good quality wine is its aroma, which is the result of complex interactions between the substances produced from grapes during fermentation and arising during ageing. The chemical composition of wine is determined by many factors, including grape variety, the geographical and viticultural conditions of grape cultivation, the microbial ecology of the grape and the fermentation processes, and winemaking practices (Cole and Noble 1997).

Microorganisms play a prominent role in determining the chemical composition of wine. They affect the quality of the grapes prior to harvest and, during fermentation, they metabolize grape sugars and other components into ethanol, carbon dioxide and hundreds of secondary end-products that, collectively, contribute to the subtlety and individuality of wine character (Nykänen 1986; Lambrechts and Pretorius 2000; Fleet 2003). Yeast, bacteria and filamentous fungi all contribute to the microbial ecology of wine production and the chemical composition of wine, although yeasts have the predominating influence because of their role in conducting the alcoholic fermentation (Fleet 1993, 2003). Several researchers have shown that, in contrast to *Saccharomyces* species, non-*Saccharomyces* yeasts produce and secrete a variety of enzymes (esterases, glycosidases, lipases, β-glucosidases, proteases, cellulases etc.) into the periplasmic space and the medium, where interaction with grape precursor compounds can occur to produce aroma active compounds, and thus play an important role in varietal aroma (Charoenchai and others 1997).

2.5.1 THE ROLE OF MONOTERPENOIDS IN WINE AROMA

Monoterpenoids, which are 10-carbon compounds with strong sensory qualities, are widely found in nature as the chief constituents of many essential oils, making them invaluable compounds in the flavor and fragrance industries. They are produced from a common precursor, geranyl pyrophosphate (GPP), by higher plants, algae, fungi and, in some cases, even yeast (King and Dickinson 2000). Two of the plant species that produce monoterpenoids are *Vitis vinifera* (grapes) and *Humulus lupulus* (hops), which indicates that these compounds may have significant value to the winemaker

and brewer. Some fungal, *Penicillium* (Larsen and Frisvad 1994, 1995), and yeast species are also able to produce monoterpenoids. Yeast species that produce terpenoids include *Kluyveromyces lactis* (Drawert and Barton 1978), *Torulaspora delbrueckii* (formerly *Saccharomyces fermentati*) (Fagan and others 1981) and *Ambrosiozyma monospora* (Klingenberg and Sprecher 1985).

The study of the aromatic potential of some fruits, such as grape (Günata and others 1985), passion fruit (Engel and Tressl 1983), papaya (Schwab and others 1989), raspberry (Pabst and others 1991), mango (Sakho and others 1997), pineapple (Wu and others 1991), strawberry (Roscher and others 1996) and lychee (Chyau and others 2003), as well as of their fermented products (juice and wine) (Günata and others 1985; Vasserot and others 1993), has revealed that, besides a free fraction of volatile terpenoids, naturally non-odorous and non-volatile precursors exist that represent an important source of fragrant compounds (Cordonnier and others 1986). Chemically, the aglycone moiety of the precursor glucoside can be linked to β -D-glucose or to the disaccharides 6-O- α -L-arabinofuranosyl- β -D-glucopyranose, 6-O- α -L-rhamnopyranosyl- β -D-glucopyranose and 6-O- β -D-apiofuranosyl- β -D-glucopyranose (Günata and others 1985; Voirin and others 1990). This aglycone component is frequently formed by terpenols such as linalool, nerol, geraniol, α -terpineol, citronellol, and in some cases by linalool oxides and terpene diols and triols. Other possible precursors include aliphatic or cyclic alcohols, such as hexanol, 2-phenylethanol, benzylalcohol, C₁₃-norisoprenoids and phenol acids, and volatile phenols such as vanillin (Günata and others 1985; Park and Noble 1993).

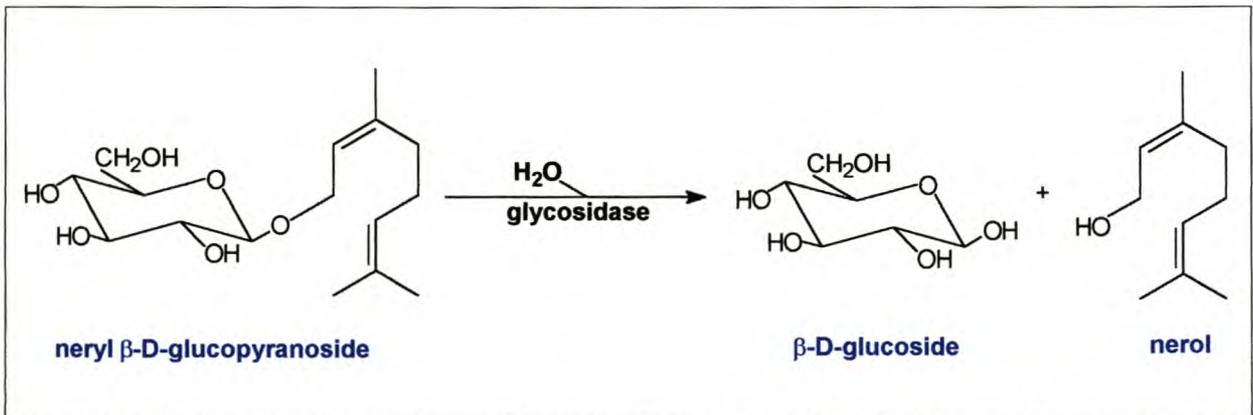


Figure 2.9. The hydrolysis of neryl β -D-glucoside to nerol and glucose (Hemingway and others 1999).

Bound terpenoids can be released during the vinification process by glycosidase enzymes produced by the grapes themselves, or by the microorganisms taking part in the process (Park and Noble 1993; Delcroix and others 1994; Zoecklein and others 1997). Enhancing this activity has been suggested as a method for increasing the levels of terpenoids in wines (Ganga and others 1999). **Figure 2.9** shows the hydrolysis of one of the grape glycosides (neryl β -D-glucoside) and the formation of its flavor active molecule, nerol.

Monoterpenes are particularly abundant in aromatic grape varieties, such as Muscat, Riesling and Gewürztraminer (Günata and others 1990), and it has been observed that most have their own recognizable flavor. Simpson (1979) described the aroma of geraniol and nerol as rose-like, that of linalool as coriander, linalool oxides as camphorous, and nerol oxide as vegetative. Generally, the bound glycosides are more abundant than the free terpenoids (Dimitriadis and Williams 1984; Günata and others 1985), and the ratios of bound to free terpenoids can also vary between different grape cultivars, i.e. Muscat of Alexandria grapes have a ratio of 5:1, whereas some non-muscat varieties have a ratio of 1:1 (Williams and others 1984).

2.5.2 β -GLUCOSIDASES IN AROMA LIBERATION

There are two possible mechanisms that can be used for the release of the monoterpenes from their glycosidically bound, non-volatile precursors, i.e. acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis was thought to be a very efficient method for monoterpene liberation, but studies have shown that high temperature acid hydrolysis causes a rearrangement of the monoterpene aglycones (Usseglio Tomasset and Di Stefano 1980; Williams and others 1982). Enzymatic hydrolysis, on the other hand, is highly efficient and does not result in modifications of the aromatic character (Günata and others 1985). Studies have shown that enzymatic hydrolysis occurs in two stages, following a sequential mechanism. First, an α -L-rhamnosidase, and an α -L-arabinofuranosidase, or a β -D-apiofuranosidase (depending on the structure of the aglycon moiety) cleave the (1 \rightarrow 6) osidic linkage, and then the monoterpenols are liberated from the monoterpenyl β -D-glucosides by the action of a β -glucosidase (Günata and others 1988, 1990).

The efficiency of the hydrolysis of monoterpenyl β -D-glucosides by β -glucosidases has been found to be dependent on, among others, the origin of the enzyme and the structure of the aglycon. Aromatic potential is naturally revealed during fruit maturation by endogenous grape β -glucosidases, but these enzymes exhibit little or no activity towards grape terpenyl-glycosides in must and wine. Grape glycosidase activities are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine (Bayonove and others 1984; Aryan and others 1987). Grossmann and others (1990) also showed that certain processing steps, such as clarification and centrifugation, considerably reduce β -glucosidase activity. A further constraint on the effectiveness of endogenous glycosidases stems from their inability to hydrolyze sugar conjugates of tertiary alcohols such as linalool and α -terpineol (Günata and others 1985; Aryan and others 1987). Once these tertiary alcohols are glycosylated in the fruit, they are irreversibly lost for flavor purposes, and only the action of an exogenous non-specific glucosidase or chemical hydrolysis will release the free flavor compounds from their aroma-inactive glycosidic forms.

Research focusing on β -glucosidases of fungal origin has indicated that these enzymes can indeed be used to enhance the varietal aroma of certain wines. They

are, however, only effective if introduced into the wine after the yeast cells have depleted the glucose, since they are strongly inhibited by glucose (Aryan and others 1987; Günata and others 1993). According to some reports, certain strains of *S. cerevisiae* also possess β -glucosidase activity and, even though they are less sensitive to glucose, the activity towards glycoside precursors seems to be very low (Günata and others 1986; Delcroix and others 1994; Hernández and others 2002). The addition of exogenous β -glucosidase during or following fermentation has been found to be the most effective way to improve the hydrolysis of the glycoconjugated aroma compounds in order to enhance wine flavor (Aryan and others 1987; Shoseyov and others 1990; Vasserot and others 1993; Gueguen and others 1997).

The need for more suitable enzymes has resulted in a search among non-*Saccharomyces* yeasts such as *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora* and *Pichia* for novel β -glucosidases with the desired properties (Vasserot and others 1989; Rosi and others 1994; McMahon and others 1999; Fernández and others 2000; Garcia and others 2002). For glycosidases to be exploited in the enhancement of wine aroma, they must satisfy a few criteria: high affinity for grape-derived terpenoid aglycones, optimal activity at wine pH (pH 2.5–3.8), resistance to glucose inhibition, and high tolerance to ethanol (Riou and others 1998). Cordero Otero and others (2003) have shown that the β -glucosidase from *D. pseudopolymorphus* is suitable for use under wine conditions. Its optimum pH lies within the wine spectrum (pH 2.5–3.8), it exhibits resistance to wine-associated inhibitory compounds, such as glucose, ethanol and sulfur dioxide, and it has high substrate affinity and large aglycone-substrate recognition. In microvinification trials, this enzyme was also found to increase the concentrations of citronellol, nerol and geraniol during the fermentation of Chardonnay juice.

The addition of exogenous aroma-liberating enzyme preparations to wine is an expensive practice and is viewed by many purists as an “unnatural” intervention by the winemaker. This has led to renewed interest in the expression of heterologous enzymes in wine yeast. The aroma intensity of wine was shown to increase when the β -1,4-glucanase gene from *T. longibratum* was expressed in *S. cerevisiae*. This was presumably due to the hydrolysis of glycosylated flavor precursors (Pérez-González and others 1993). Similarly, the β -glucosidase gene of *S. fibuligera*, the α -L-arabinofuranosidase (*ABF2*) of *A. niger* and a glucanase-encoding gene cassette consisting of several glucanase genes (*BEG1*, *END1* and *EXG1*) were expressed (Pretorius 2000).

2.6 CONCLUSION

β -Glucosidases play a pivotal role in many crucial biological pathways, a few of which are the biosynthesis and degradation of structural and storage polysaccharides, cellular signaling, oncogenesis and host-pathogen interactions. β -Glucosidases also have a wide range of applications in the medical and biotechnological sectors: the

treatment of lysosomal storage diseases such as Gaucher disease, the enzymatic hydrolysis of cellulose to produce bio-fuel, the degradation of cyanogenic glucosides for detoxification, and the liberation of bound monoterpenols to enhance the flavor of wines and fruit juices. The synthesis of oligosaccharides, alkyl glucosides and glyco-conjugates by the transferase activity of β-glucosidase is another area of commercial interest: many of these carbohydrate molecules can serve as potent pharmaceuticals and targets for drug design.

The use of commercial enzyme preparations for the enhancement of wine flavor has gained enormous popularity over the past two decades. Even though effective and convenient to use, a few unresolved issues regarding the application of these enzyme preparations still exist, such as the time of application (i.e. during fermentation, after fermentation, etc.) and aglycone specificity. The use of these commercial products is sometimes considered to be “unnatural” and future research should therefore focus on the expression of heterologous enzymes from non-*Saccharomyces* yeasts (shown in some cases to have unique β-glucosidase activities) in *S. cerevisiae*.

This literature review has focused on the description of the properties, physiological functions and applications of native β-glucosidases from various plant, animal and microbial sources. Considering the importance of β-glucosidase in all aspects of life, this topic will continue to be a focal point in future research.

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CHAPTER 3

RESEARCH RESULTS

Characterization of the β -glucosidase activity produced by enological strains of non-*Saccharomyces* yeasts

Article published in *Journal of Food Science*

Characterization of the β -glucosidase activity produced by enological strains of non-*Saccharomyces* yeasts

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3.1 ABSTRACT

The β -glucosidase activities of 20 wine-related non-*Saccharomyces* yeasts were quantified, characterized and assessed for the efficiency with which they could release aroma-enhancing compounds during the winemaking process. Of these enzymatic activities, the β -glucosidase activity of *Debaryomyces pseudopolymorphus* revealed the most suitable combination of properties in terms of functionality at wine pH, resistance to wine-associated inhibitory compounds (glucose, ethanol and sulfur dioxide), high substrate affinity and large aglycone-substrate recognition. Its potential as a wine aroma-enhancing enzyme was confirmed by significantly increasing the concentrations of free volatiles (citronellol, nerol and geraniol) during the fermentation of Chardonnay juice inoculated with both *D. pseudopolymorphus* and a widely used commercial starter culture strain of *Saccharomyces cerevisiae*, VIN13.

3.2 INTRODUCTION

Terpenes are a class of compounds that contribute to the varietal aroma of a number of fruits and their fermented products. Previous studies on wine have revealed that, in addition to a free fraction of volatile terpenols, there are non-odorous and non-volatile precursors that represent an important source of fragrant compounds (Cordonnier and others 1986; Williams and others 1995). These non-odorous precursors consist of terpenols that are linked to sugars such as 6-O- α -L-ramnopyranosyl-, 6-O- α -L-arabinofuranosyl-, and 6-O- β -D-apiofuranosyl- β -D-glucosides. The aglycone part mainly contains monoterpenols and aromatic alcohols, such as benzyl- and 2-phenylethyl alcohol (Günata and others 1985). In general, bound glycoside forms are more abundant than the free ones (Dimitriadis and Williams 1984; Günata and others 1985) and, in grapes, the ratio between the potential (bound) and free monoterpenes varies between 5:1 in Muscat of Alexandria and 1:1 in some non-muscat varieties such as Chardonnay (Williams and others 1982).

Acid hydrolysis of grape glycosides has been studied as a method for the release of bound monoterpenes with a view to enhancing the aroma of grape juice through the formation of free volatiles. This method, which is promoted by heating, causes a rearrangement of the monoterpene aglycones (Williams and others 1982). As an alternative, enzymatic hydrolysis has attracted much interest with regard to the enhancement of wine flavor. Unlike acidic hydrolysis, enzymatic hydrolysis is highly efficient and does not alter the aglycone (Günata and others 1990). β -Glucosidase (1,4- β -D-glucosidase; EC 3.2.1.21) is a key enzyme in the enzymatic release of these bound monoterpenols from their glycosidic precursors (Shoseyov and others 1990; Gueguen and others 1996), and functions through the sequential hydrolysis of the glycosidic bonds. Depending on the precursors, the glycosidic linkages are firstly cleaved by an α -L-arabinofuranosidase, α -L-rhamnopyranosidase or a β -D-apiosidase. The second step involves the liberation of the monoterpenols by β -glucosidase (Günata and others 1988; Gueguen and others 1996).

Limited hydrolysis of glycosides occurs during berry maturation by endogenous fruit β -glucosidases (Cordonnier and others 1986). These plant-derived β -glucosidases are characterized by a restricted specificity with respect to aglycone, and are inhibited by a glucose concentration higher than 1% (Bayonove and others 1984; Aryan and others 1987). Since these enzymes cannot liberate all of the aromatic potential in grape must, hydrolytic experiments were performed with exogenous β -glucosidases (Shoseyov and others 1990; Rosi and others 1994; Saha and Bothast 1996). Trials conducted with β -glucosidases of fungal origin have indicated that these enzymes can indeed enhance the varietal aroma of certain wines. These enzymes can only be introduced into the wine after the yeast cells have depleted the glucose, as they are strongly inhibited by glucose (Aryan and others 1987; Günata and others 1993). According to some reports, certain strains of *Saccharomyces cerevisiae* also possess β -glucosidase activity (Delcroix and others 1994; Hernández and others 2002). This activity appears to be very limited and therefore recent studies have rather focused on non-*Saccharomyces* yeasts, such as *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora* and *Pichia* (Vasserot and others 1989; Rosi and others 1994; McMahon and others 1999; Fernández and others 2000; Garcia and others 2002). Results obtained from studies on yeast glycosidases suggest that specific yeast strains can affect the varietal aroma of wines (Rosi and others 1994). For glycosidases to be exploited in the enhancement of wine aroma, they must satisfy a few prerequisites. These include a high affinity for grape-derived terpenoid aglycones, optimal activity at wine pH, resistance to glucose inhibition, and high tolerance to ethanol.

The need for more efficient aroma-liberating enzymes has led us to search for novel β -glucosidases that fit the abovementioned criteria. With this in mind, the purpose of this investigation was to screen wine-related non-*Saccharomyces* yeasts (species belonging to *Brettanomyces*, *Candida*, *Debaryomyces*, *Kloeckera* and *Zygosaccharomyces*) for the appropriateness of their β -glucosidases by quantifying,

characterizing and comparing these activities. *Debaryomyces pseudopolymorphus* was identified as the strain with the highest β -glucosidase activity and with a high tolerance of both glucose and ethanol. The effect of co-fermentation with *D. pseudopolymorphus* and *S. cerevisiae* (VIN13) on the terpenic glycosides in wine was also investigated.

3.3 MATERIALS AND METHODS

3.3.1 YEAST STRAINS

The names and origins of the 20 yeast strains that were used in this study are summarized in **Table 3.1**. Four of the strains are from the yeast collection of the Department of Microbiology, University of the Free State (UFS), South Africa, while the remaining 16 strains were obtained from the Wine and Fermentation Technology Division, ARC Infruitech-Nietvoorbij, Stellenbosch, South Africa.

Table 3.1. Yeast strains used in this study.

Species	Isolates	Institution	Origin
<i>Candida colliculosa</i>	C2/5	Nietvoorbij ^a	Cellar
<i>C. guilliermondii</i>	M1/30	Nietvoorbij	Vineyard
<i>C. hellenica</i>	O1/16	Nietvoorbij	Vineyard
<i>C. lambica</i>	M2/4	Nietvoorbij	Cellar
<i>C. oleophila</i>	O1/29	Nietvoorbij	Vineyard
<i>C. pelliculosa</i>	R1/17	Nietvoorbij	Vineyard
<i>C. sorbosa</i>	R2/29	Nietvoorbij	Cellar
<i>C. stellata</i>	C2/20	Nietvoorbij	Cellar
<i>C. valida</i>	O1/22	Nietvoorbij	Vineyard
<i>Debaryomyces hansenii</i>	R2/15	Nietvoorbij	Cellar
<i>D. maramus</i>	Y-0896	UFS ^b	N.A.
<i>D. polymorphus</i> var. <i>africanus</i>	Y-0064	UFS	N.A.
<i>D. pseudopolymorphus</i>	Y-0541	UFS	N.A.
<i>Kloeckera apiculata</i>	R2/21	Nietvoorbij	Cellar
<i>Kloeckera</i> sp.	R2/30	Nietvoorbij	Cellar
<i>Pichia carsonii</i>	Y-0895	UFS	N.A.
<i>P. farinosa</i>	R1/15	Nietvoorbij	Vineyard
<i>P. kluyveri</i>	R2/11	Nietvoorbij	Cellar
<i>Zygosaccharomyces bailii</i>	O1/25	Nietvoorbij	Vineyard
<i>Brettanomyces</i> sp.	O1/33	Nietvoorbij	Cellar
<i>Saccharomyces cerevisiae</i>	VIN13	IWBT ^c	Anchor Yeast

^aARC Infruitech-Nietvoorbij, ^bUniversity of the Free State (UFS), and ^cInstitute for Wine Biotechnology (IWBT).

These indigenous strains were isolated from different wine production regions in the Western Cape, South Africa, namely Constantia (represented by C), Robertson (represented by M), Slanghoek (represented by O) and Stellenbosch (represented by R) (Strauss and others 2001). The strain collection used in this study was compiled from vineyard and winery isolates. Yeast strains were grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30°C for 48 h.

3.3.2 ISOLATION OF CELL-ASSOCIATED PROTEIN

YPD broth (50 mL) was inoculated at an optical density at 600 nm (OD_{600}) of 0.1 from an overnight culture and grown at 30°C for 48 h. Cells were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 5 mL of 50 mM Tris (pH 7.5, containing 10 mM NaCl) buffer. Glass beads (amount, 0.1 g; diameter, 0.2 mm) were added and the cells were vortexed vigorously for 3 min. After centrifugation at 6000 rpm for 2 min, the supernatant, containing the crude cell protein extract, was carefully removed and used for the enzyme assays.

3.3.3 PROTEIN DETERMINATION AND ENZYME ASSAYS

Protein concentration was determined by using Pierce protein reagent with bovine serum albumin as the standard (Bradford 1976). β -Glucosidase activity was assayed using a modified protocol from Lloyd and Whelan (1969). Since the β -glucosidase of most tested yeasts was cell-associated, all enzymatic tests were conducted on total crude cell extracts. Crude cell extract (40 μ L) was mixed with 40 μ L of a 0.4% (w/v) cellobiose solution in 0.1 M citrate-phosphate buffer (pH 3.4). Water was added to the reaction mixture to obtain a total volume of 100 μ L. The mixture was incubated at 25°C for 30 min and the amount of glucose released from the hydrolyzed cellobiose was determined using the Glucose Trinder Kit (Sigma), according to the specifications of the supplier. Enzyme and reagent blanks were included and subtracted from the absorbance obtained for the enzyme-substrate reaction mixture (A_{505} - reaction blank/ Standard - reaction blank \times 100 = [glucose] in mg/100 mL). The specific amount of glucose released was expressed as mg/100 mL/mg of protein/30 min. The effect of ethanol, SO_2 and glucose on β -glucosidase activity was assessed through the addition of different concentrations of these substances to the reaction mixtures prior to incubation at 25°C. Cultures were grown in duplicate and assays were conducted in quadruplicate. The standard deviations ranged from 3% to 5%. One unit of β -glucosidase activity was defined as the amount of crude enzyme required to produce 1 μ mol of glucose under the assay conditions employed. The calculation of the specific activity (U/mg) was based on the level of enzymatic activity divided by the protein concentration of the crude enzyme preparations.

3.3.4 MICROVINIFICATION

Microvinifications were carried out with single-strain samples of either *D. pseudopolymorphus* or *S. cerevisiae* (VIN13), as well as with a mixed-culture

sample (*D. pseudopolymorphus* plus *S. cerevisiae* VIN13). Chardonnay must (700 mL for each sample) with an SO₂ concentration of 35 mg/L was inoculated with either *D. pseudopolymorphus* or *S. cerevisiae* (VIN13) to cell concentrations of 1 × 10⁷ cells/mL and 1 × 10⁶ cells/mL, respectively. The same concentration of inocula was used on the first day of fermentation for the mixed-culture samples. The musts were fermented in 1 L glass bottles for 10 d at 25°C ± 2°C, and the enzymatic assays were carried out in quadruplicate.

3.3.5 STANDARD WINE ANALYSIS

Fourier Transform Infrared Spectroscopy (FTIR), using the GrapeScan 2000 instrument (FOSS Electric, Denmark), was used to determine the chemical characteristics of the fermented wines. The commercial calibrations for the analysis of the wine were provided by FOSS, and all calibrations were validated.

3.3.6 DETERMINATION OF MONOTERPENE CONCENTRATIONS

The determination of monoterpene concentrations was performed using a modified protocol from Ferreira and others (1993). A total of 10 mL of wine was mixed with a solution of 2 µL of 2,6-dimethylheptenol (400 mg/L in ethanol as internal standard) before liquid-liquid extraction with 200 µL of Freon 113. The Freon extracts of the terpenes were analyzed by gas chromatography (Hewlett-Packard model 5890 series II) on a Supelco SPB5 column using HE as carrier gas. Three standard solutions were prepared for all the terpenes. The pure chromatographic standards of analytical quality were obtained from Fluka and Sigma-Aldrich (Germany). 1,1,2-Trichloro-1,2,2-trifluoroethane (Freon 113) of HPLC quality was obtained from Aldrich. The compounds were identified by a flame ionization detector and compared with the retention times of the pure chemical standards. The amount of each terpene was quantified by plotting the heights of peaks on the chromatograms versus the terpene concentration in wine. Five calibration points were used for each of the terpene compounds.

3.4 RESULTS AND DISCUSSION

The role of microbial β-glucosidases in the liberation of monoterpenols from glycoside precursors has been reported in numerous studies (Gueguen and others 1996; Iwashita and others 1999; Yanai and Sato 1999). Therefore, the potential of β-glucosidases to release flavor compounds from glycosidically bound non-volatile precursors is of great significance to the wine industry. Previous work has shown that, among yeasts, different strains of *Candida* (Günata and others 1990), *Debaryomyces* (Rosi and others 1994), *Metschnikowia* (Fernández and others 2000) and *Brettanomyces* (McMahon and others 1999) exhibit activity towards various β-glucosides.

In the present study, a total of 20 yeast strains isolated from vineyards and wine cellars (**Table 3.1**) were tested for β -glucosidase activity. A preliminary evaluation of β -glucosidase activity on different protein fractions (extracellular, intracellular cytosol and membranes) for all the strains showed that the activity was primarily cell-associated, and extracellular activity was detected only for *D. pseudopolymorphus* and *C. oleophila* (data not shown). **Figure 3.1** shows that *C. lambica*, *C. valida*, *D. hansenii* and *K. apiculata* have very low β -glucosidase activity, with none of the strains releasing more than 0.03 mg/100 mL of glucose from cellobiose. The only *Brettanomyces* strain (a yeast usually associated with wine spoilage) used in this study showed the highest activity, viz. 4.84 mg/100 mL of glucose released from cellobiose. The other species with high β -glucosidase activity were *D. polymorphus* (3.69 mg/100 mL of glucose), *D. pseudopolymorphus* (3.79 mg/100 mL of glucose) and, to a lesser extent, the two strains *C. oleophila* (2.58 mg/100 mL of glucose) and *Z. bailii* (2.66 mg/100 mL of glucose). The four yeasts with the highest activity were selected to investigate the effect of ethanol, glucose and SO₂ on their β -glucosidase activities.

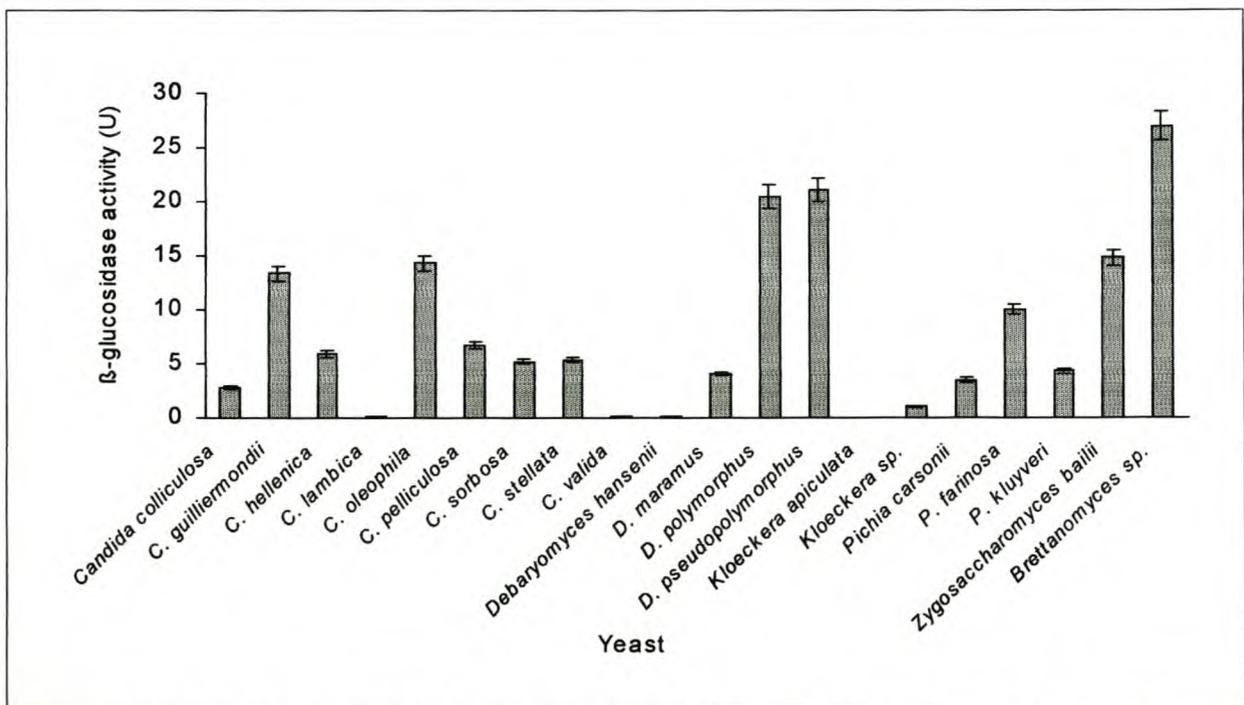


Figure 3.1. Cellobiose hydrolysis by yeast β -glucosidases. Cellobiose degradation by yeast was analyzed using the glucose oxidase/peroxidase method to determine the formation of glucose from the substrate. Glucose was assessed after incubation of cell crude extract for 30 min at 25°C in the presence of 8 mg of cellobiose in 100 mM citrate-phosphate buffer pH 3.4. Cultures were grown in duplicate and the assays repeated four times. The specific activity of the enzymes was expressed as 1 μ mol of glucose produced per mg of protein, equivalent to U/mg. The standard deviations were between 3% and 5%.

Previously, it has been found that alcohol stimulates β -glucosidase activity in many fungal and yeasts species (Delcroix and others 1994; Yan and Lin 1997). Pemberton and others (1980) proposed that alcohol activation of some β -glucosidases might be due to their glycosyltransferase activity. However, some other

yeast β -glucosidases (Mateo and Di Stefano 1997), as well as grape β -glucosidases (Aryan and others 1987), have been reported to be inhibited by ethanol. As shown in **Figure 3.2A**, we also observed that ethanol inhibited β -glucosidase activity at all concentrations tested for the four different crude extracts evaluated. The most prominent inhibition was observed for the β -glucosidase activity of *C. oleophila*, which exhibited a 21% (compared to the total activity of the untreated samples) reduction in enzyme activity at 14% ethanol.

Inhibition by glucose, which, although there are some exceptions (Rosi and others 1994; Saha and Bothast 1996; Riou and others 1998), is a common characteristic of β -glucosidases (Gueguen and others 1995; Spagna and others 2002), is an important constraint for the industrial use of these enzymes in winemaking. Our results on the effect of different glucose concentrations (4, 8 and 15%) on β -glucosidase activity are presented in **Figure 3.2B**. The results depicted in **Figure 3.2B** show that the β -glucosidase of the *Brettanomyces* sp. was strongly inhibited by the presence of glucose, whereas the β -glucosidase activities derived from the other yeasts were, to varying degrees, more resistant to glucose inhibition. At a glucose concentration of 15%, the *Brettanomyces* β -glucosidase activity was only 0.12% (ratio of treated reaction mixture/untreated reaction mixture) of the maximum activity. The *D. polymorphus* β -glucosidase activity appeared to be insensitive to glucose, whereas the activity of both *C. oleophila* and *D. pseudopolymorphus* were markedly increased throughout the whole glucose concentration spectrum that was tested. The *D. polymorphus* β -glucosidase showed an initial 30% increase in activity, which was maintained throughout the glucose series tested. At a glucose concentration of 15%, the *D. polymorphus* β -glucosidase displayed a five-fold increase in activity. *C. oleophila*, showing a three-fold increase in β -glucosidase activity at a concentration of 15%, exhibited a gradual increase in activity, which correlates to an increase in glucose concentration. The most outstanding increase in β -glucosidase activity was observed in *D. pseudopolymorphus*, which exhibited a marked increase of four-fold at 8% glucose and five-fold at 15% glucose.

Little is known about the influence of sulfur dioxide on β -glucosidase activity. Delcroix and others (1994) found that an SO_2 concentration of 50 mg/L had no effect on enzyme activity. As shown in **Figure 3.2C**, our data is in agreement with this observation. None of the SO_2 concentrations tested had a marked effect on the β -glucosidase activity of the four crude protein extracts tested. The only exception was in the case of *D. polymorphus*, which showed a significant increase in activity throughout the entire SO_2 series tested. The *D. polymorphus* β -glucosidase exhibited an overall increase in activity throughout the series tested, reaching a three-fold induction at 60 ppm. At 60 ppm, the *C. oleophila* and *Brettanomyces* β -glucosidases maintained 87.4% and 86.9% of their maximum activity, respectively. *D. pseudopolymorphus* showed a 20% increase in β -glucosidase activity at 30 ppm SO_2 , and still maintained its maximum activity at 60 ppm. No explanation for this

increase can be offered at this point in time, and this aspect needs further investigation.

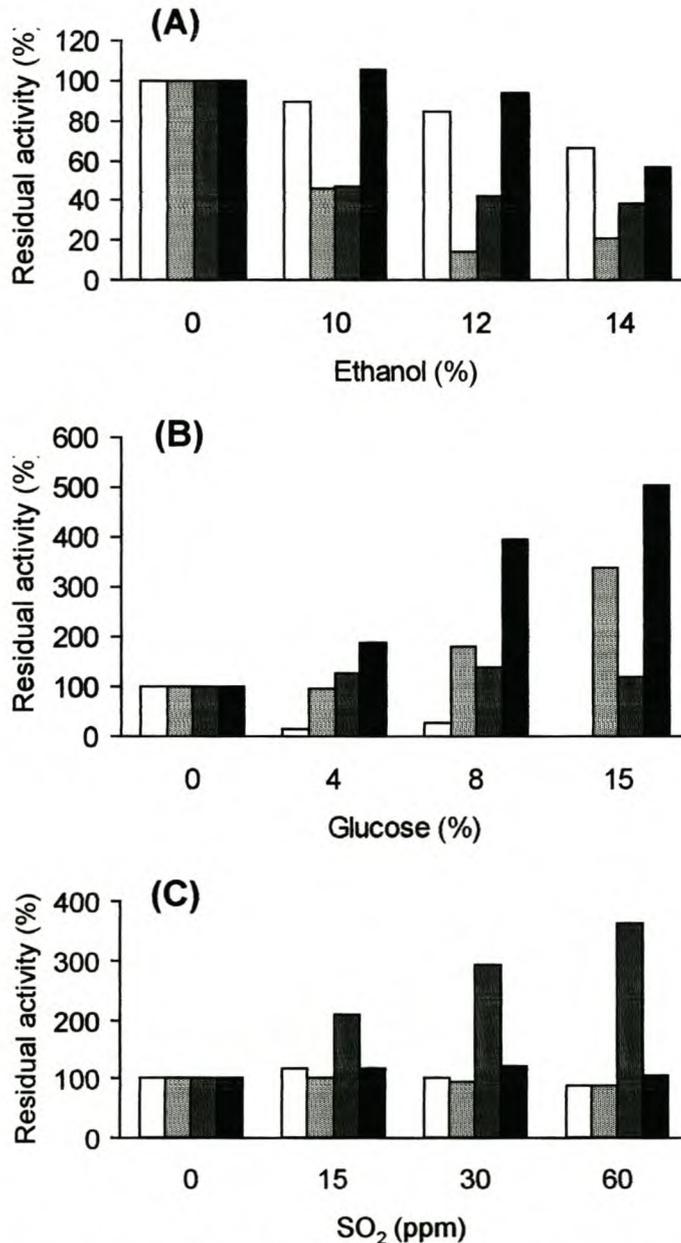


Figure 3.2. Effects of ethanol (A), glucose (B) and SO₂ (C) on β -glucosidase activity of *Brettanomyces sp.* (white bars), *C. oleophila* (grey bars), *D. polymorphus* (dark grey bars), and *D. pseudopolymorphus* (black bars). Residual activity was assayed after incubation of cell crude extract for 30 min at 25°C in 100 mM citrate-phosphate buffer pH 3.4, complemented with different values of ethanol, glucose and SO₂. The scale of residual activity indicates the percentage of the experimental value for various enzyme reactions relative to the maximum value (100%) of the non-spiked reaction mixture of each enzyme. The values are means of triplicate experiments, and the relative error was less than 5%.

Microorganisms of enological origin do not always produce enzymes that can be used in wine conditions (Mateo and Di Stefano 1997; Spagna and others 2002). However, the enzymatic properties exhibited by *D. pseudopolymorphus*, i.e. high activity at higher glucose and sulfur dioxide concentrations and relative resistance to high ethanol values, suggest that this yeast has an appropriate kinetic profile to be

used in the wine industry. Therefore, microvinifications were performed with single-strain samples consisting of either *D. pseudopolymorphus* or *S. cerevisiae* (VIN13; a commercial wine starter culture strain), as well as with a mixed-culture sample consisting of both these yeasts. No obvious differences in the pH and density of the wines were observed between the single-strain and mixed-culture inocula after 10 d of fermentation (Table 3.2). *D. pseudopolymorphus*, as a single-strain inoculum, is not an effective fermenting wine yeast under the fermentation conditions tested. After 10 d of fermentation, the wine still contained a reducing sugar content of 200.19 g/L, as well as 187.39 g/L of glucose and fructose, and an ethanol content of 0.42% (v/v). The volatile acid and total acid content of the single-culture fermentations were slightly lower than that of the mixed-culture fermentation (Table 3.2). Furthermore, it appears that the mixed-culture inoculum was more effective in driving these particular fermentations than the single-strain *S. cerevisiae* VIN13 inoculum, since the wine had a reducing sugar content of 43.46 g/L, 44.33 g/L of glucose and fructose, and an ethanol content of 10.22 % (v/v) after 10 d of fermentation.

Table 3.2. Analysis of wines at 10 days of fermentation.

Inoculated strain	pH	Volatile acid ^a	Total acid ^a	Reducing sugar ^a	Malic acid ^a	Glucose+ Fructose ^b	Ethanol ^b	Density
<i>D. pseudopolymorphus</i>	2.91±	0.22±	5.58±	200.19±	3.65±	187.39±	0.42±	1.08±
	0.25	0.04	0.40	16.04	0.34	15.33	0.03	0.01
<i>S. cerevisiae</i>	2.92±	0.52±	6.24±	50.88±	4.02±	52.07±	9.82±	1.02±
	0.01	0.01	0.02	0.35	0.02	0.19	0.01	0.01
<i>D. pseudopolymorphus</i> and <i>S. cerevisiae</i>	2.85±	0.56±	6.47±	43.46±	4.01±	44.33±	10.22±	1.01±
	0.01	0.01	0.01	0.19	0.03	0.11	0.01	0.01

^a g /L, ^b % v/v. Fermentation assays were carried out in quadruplicate using 700 mL of Chardonnay must for each sample. The juice was sulfited at 35 mg/L. *D. pseudopolymorphus* and *S. cerevisiae* VIN13 strain samples were inoculated at a level of 1×10^7 cells/mL and 1×10^6 cells/mL, respectively. The same value of the inoculums applied for the single-culture samples was used on the first day of fermentation for the mixed-culture samples. The juices were fermented in 1 L glass bottles at $25 \pm 2^\circ\text{C}$ for 10 d.

Whether *D. pseudopolymorphus* produces any off-flavors in wine was not determined in this study, since a comprehensive analysis of the alcohol, acid and phenolic compounds was not done. Rather, the influence of the co-fermentation of *D. pseudopolymorphus* and *S. cerevisiae* on the concentration of free volatiles in Chardonnay wine was studied (Table 3.3). In general, the concentration of the primary alcohols obtained with the single-strain *S. cerevisiae* fermentation was slightly higher than that obtained with the single-strain *D. pseudopolymorphus* fermentation, except for the concentration of geraniol, which was seven-fold higher in

the latter. No significant differences were found in the linalool and α -terpineol concentrations produced with either the single-strain or mixed-culture fermentation. The results clearly indicate that fermentation with the mixed-culture inoculum resulted in a significant increase in the concentration of citronellol (37.2 $\mu\text{g/L}$) and geraniol (21.2 $\mu\text{g/L}$) after 10 d. In the case of nerol, fermentation with the mixed-culture inoculum resulted in only a 2 to 3 $\mu\text{g/L}$ increase in concentration in comparison to the fermentation that was performed with the two single-strain inocula. This could be explained by the fact that certain β -glucosidases display selectivity to specific aglycones (Williams and others 1995; Mansfield and others 2002). Grape β -glucosidases, for example, act on precursors that have a primary alcohol as aglycone, such as geraniol and citronellol, but do not cleave glycosides bonding a tertiary alcohol, such as linalool or α -terpineol (Günata and others 1985). Citronellol, in particular, was produced in high concentrations by the co-fermentation. Di Stefano and others (1992) suggested that this compound could originate from the reduction of geraniol and nerol by yeast during fermentation. Chardonnay is one of the varieties known to contain low levels of terpenes or their glycosides (Dimitriadis and Williams 1984; Sefton and others 1993) and is not dependent on monoterpenes for flavor (Strauss and others 1986). The limited presence of flavor precursors, such as neryl β -D-glucopyranoside, can explain the low concentration of nerol produced during wine fermentation.

Table 3.3. Influence of co-fermentation with *D. pseudopolymorphus* and *S. cerevisiae* VIN13 strain on the concentration of free volatiles in Chardonnay wine.

Volatile compound	Inoculated yeast strain		
	<i>D. pseudopolymorphus</i>	<i>S. cerevisiae</i> VIN13	<i>D. pseudopolymorphus</i> and <i>S. cerevisiae</i> VIN13
Linalool	34.7 \pm 1.2 ^a	34.9 \pm 1.4 ^a	33.6 \pm 1.5 ^a
Terpineol	36.4 \pm 1.1 ^a	35.7 \pm 1.3 ^a	33.3 \pm 1.1 ^a
Citronellol	3.1 \pm 0.2 ^a	7.2 \pm 0.3 ^a	37.2 \pm 1.1 ^a
Nerol	4.6 \pm 0.3 ^a	5.6 \pm 0.2 ^a	7.9 \pm 0.2 ^a
Geraniol	7.4 \pm 0.2 ^a	1.3 \pm 0.1 ^a	21.2 \pm 1.0 ^a

^a $\mu\text{g/L}$. The numbers represent mean values and deviations from the mean of quadruplicate fermentation experiments.

3.5 CONCLUSIONS

This study makes an important contribution to an extensive survey aimed at the exploitation of the hidden enological potential of the untapped wealth of non-*Saccharomyces* yeasts and their enzymes (Pretorius and others 1999; Fernández and others 2000; Strauss and others 2001; Fernández-González and others 2003; Jolly and others 2003a, 2003b, 2003c). The data presented here suggest that the β -

glucosidase activity of *D. pseudopolymorphus* possess suitable properties in terms of optimum pH, resistance to wine-associated inhibitory compounds (glucose, ethanol and sulfur dioxide), high substrate affinity and large aglycone-substrate recognition. The potential of this newly discovered β -glucosidase to increase the aromatic varietal character of wines through the hydrolysis of flavor glucosidic precursors is underpinned by its capability to increase the concentrations of citronellol, nerol and geraniol during the fermentation of Chardonnay juice inoculated with both *D. pseudopolymorphus* and a commercial wine yeast, *S. cerevisiae* VIN13. However, additional work is required to determine the influence of *D. pseudopolymorphus* on wine aroma, as well as the effectiveness of the β -glucosidase enzyme in large-scale winemaking trials. This study also lays the foundation for the cloning and expression of the *D. pseudopolymorphus* β -glucosidase gene in a commercial wine yeast. Such a recombinant wine yeast would release grape-derived varietal aroma compounds from the non-volatile, non-odorous precursors during single culture fermentations, thereby increasing the sensorial quality of wine - the single most important aspect in winemaking.

3.6 ACKNOWLEDGEMENTS

This research was funded by grants from the National Research Foundation (NRF), the South African wine industry (Winetech) and Secretaria de Estado de Educación y Universidades de España.

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The screening and characterization of the β -glucosidase activity produced by wine, whiskey and beer yeast strains

The need for highly efficient aroma-liberating enzymes has led to the search for novel β -glucosidases amongst different yeast strains of enological origin. The cellular localization of the β -glucosidase enzyme, as well as the influence of wine-associated inhibitory compounds (glucose, ethanol and sulfur dioxide) on enzyme activity, are all essential factors to consider when choosing an exogenous β -glucosidase for use in winemaking. The following section provides a summary of some of the experiments that have not been included in the previously presented publication.

A.1 INTRODUCTION

Monoterpenols, which are 10-carbon compounds with strong sensorial qualities, are present in food in two different forms: (i) a free fraction of volatile terpenols, and (ii) a bound fraction of naturally non-volatile glycoside precursors. The influence of these bound and free terpenol fractions on the aroma of fruit, as well as on their fermented products (e.g. wine and beer), has been studied extensively over the last two decades (Günata and others 1985; Cordonnier and others 1986; Vasserot and others 1993; Stucky and McDaniel 1997; Chyau and others 2003).

Two monoterpenol-producing plant species that are of great interest to the beverage industry are grapes (*Vitis vinifera*) and hops (*Humulus lupulus*). The main terpenoids present in grapes are linalool, nerol, geraniol, α -terpineol and citronellol (Park and Noble 1993), whilst in brewing, studies have identified geraniol and linalool as terpenols present in hops and beer (Peacock and Deinzer 1981). It has been established in previous studies (Bayonove and others 1984; Aryan and others 1987; Günata and others 1990) that monoterpenols can be released from their glycosidic complexes through the action of β -glucosidases. Exogenous enzymes of yeast origin have been shown to be the most effective in releasing bound monoterpenols from grapes, since plant and fungal β -glucosidases are strongly inhibited by glucose.

The limited activity of *Saccharomyces cerevisiae* β -glucosidases has resulted in a search for more efficient enzymes among non-*Saccharomyces* yeasts. Cordero Otero and others (2003) screened a large number of yeasts of enological interest (grape berry yeasts, *sensu stricto* wine yeasts, wine spoilage yeasts) to determine their β -glucosidase activities and found that *Debaryomyces pseudopolymorphus* was the strain with the highest β -glucosidase activity. This strain has a high tolerance of both glucose and ethanol, and was also able to increase the concentrations of some monoterpenols during fermentation.

This section presents some of the more interesting preliminary data obtained during the investigation of the β -glucosidase activities of the different non-*Saccharomyces* yeasts. Experiments were performed to determine the localization of the β -glucosidase activity and to determine the effect of different growth and

environmental conditions on enzyme activity. The results of these experiments formed the basis for the experiments described in detail in section 3.

A.2 MATERIALS AND METHODS

A.2.1 YEAST STRAINS

The names and origins of the 19 whiskey and six beer yeast strains that were used in this study are summarized in **Table 3.4**.

Table 3.4. Whiskey and beer yeasts used in this study.

Strain reference	Name/Origin
WH 299	HE1000; Lalvin (Canada)/Brewer's strain
WH 300	DCL Scotland
WH 301	DCL Scotland
WH 302	DGI 338; ex Anchor Yeast
WH 303	Anchor Yeast; ex Maurivin
WH 304	No. 269; ex NCP
WH 305	No. 270; ex NCP
WH 306	No. 271; ex NCP
WH 308	DGI 227; ex Anchor Yeast
WH 309	DGI 335; ex Anchor Yeast
WH 310	DY10; ex Anchor Yeast
WH 311	NCYC ^a 87; NRRL ^b Y-567
WH 312	NCYC 431; NRRL Y-132
WH 313	NRRL Y-978
WH 314	NRRL Y-567
WH 315	NRRL Y-684
WH 316	NRRL Y-635
WH 317	CBS ^c 1311
WH 319	M15; ex Anchor Yeast
ISP 115	MB6; ex South African Breweries
ISP 117	MB17; ex South African Breweries
ISP 154	2266 O'Keef (Spencer)
ISP 157	2269 Labott (Spencer)
ISP 160	2272 Brit Brew (Spencer)
ISP 163	2275 Brit Brew (Spencer)

^aNational Collection of Yeast Cultures, England, ^bARS Culture Collection, USA, ^cCentraalbureau voor Schimmelcultures, Netherlands.

The names and origins of the 27 wine-related yeast strains that were used in this study are summarized in **Table 3.5**. Four of the strains are from the yeast collection of the Department of Microbiology, University of the Free State (UFS), South Africa,

six of the strains are industrial strains used for experimental purposes at the Institute for Wine Biotechnology, Stellenbosch, South Africa, while the remaining 17 strains were obtained from the Wine and Fermentation Technology Division, ARC Infruitech-Nietvoorbij, Stellenbosch, South Africa. These indigenous strains were isolated from different wine production regions in the Western Cape, South Africa, namely Constantia (represented by C), Robertson (represented by M), Slanghoek (represented by O) and Stellenbosch (represented by R) (Strauss and others 2001).

Table 3.5. Wine yeast strains used in this study.

Species	Isolates	Institution	Origin
<i>Brettanomyces</i> sp.	O1/33	Nietvoorbij ^a	Cellar
<i>Candida colliculosa</i>	C2/5	Nietvoorbij	Cellar
<i>C. guilliermondii</i>	M1/30	Nietvoorbij	Vineyard
<i>C. hellenica</i>	O1/16	Nietvoorbij	Vineyard
<i>C. lambica</i>	M2/4	Nietvoorbij	Cellar
<i>C. oleophila</i>	O1/29	Nietvoorbij	Vineyard
<i>C. pelliculosa</i>	R1/17	Nietvoorbij	Vineyard
<i>C. sorbosa</i>	R2/29	Nietvoorbij	Cellar
<i>C. stellata</i>	C2/20	Nietvoorbij	Cellar
<i>C. valida</i>	O1/22	Nietvoorbij	Vineyard
<i>Debaryomyces hansenii</i>	R2/15	Nietvoorbij	Cellar
<i>D. maramus</i>	Y-0896	UFS ^b	N.A.
<i>D. polymorphus</i> var. <i>africanus</i>	Y-0064	UFS	N.A.
<i>D. pseudopolymorphus</i>	Y-0541	UFS	N.A.
<i>Kloeckera apiculata</i>	R2/21	Nietvoorbij	Cellar
<i>Kloeckera</i> sp.	R2/30	Nietvoorbij	Cellar
<i>Pichia carsonii</i>	Y-0895	UFS	N.A.
<i>P. farinosa</i>	R1/15	Nietvoorbij	Vineyard
<i>P. kluyveri</i>	R2/11	Nietvoorbij	Cellar
<i>Rhodotorula glutinis</i>	R1/23	Nietvoorbij	Vineyard
<i>Saccharomyces cerevisiae</i>	VIN13	IWBT ^c	Anchor Yeast
<i>S. cerevisiae</i>	VIN7	IWBT	Anchor Yeast
<i>S. cerevisiae</i>	WE372	IWBT	Anchor Yeast
<i>S. cerevisiae</i>	WE14	IWBT	Anchor Yeast
<i>S. cerevisiae</i>	228	IWBT	Anchor Yeast
<i>S. cerevisiae</i>	N96	IWBT	Anchor Yeast
<i>Zygosaccharomyces bailii</i>	O1/25	Nietvoorbij	Vineyard

^aARC Infruitech-Nietvoorbij, ^bUniversity of the Free State (UFS), and ^cInstitute for Wine Biotechnology (IWBT).

A.2.2 SCREENING METHOD

Yeasts strains were grown in 100 mL YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30°C for 48 h. Screening was carried out on agar plates containing arbutin as sole carbon source. The media consisted of (g/L): 6.7 yeast nitrogen base (YNB, Difco), 5 arbutin, and 20 agar. The pH of the media was adjusted to 5.0 before autoclaving. Two milliliters of a filter-sterilized 1% ammonium ferric citrate solution were added to 100 mL of media before pouring the plates. After the plates were dry, 10 μ L of each liquid culture suspension were dropped onto the plates. Each plate was inoculated with four different cultures, incubated at 25°C and examined after 2, 4, 6 and 8 days. An uninoculated plate served as the control. Strains with β -glucosidase activity hydrolyze the substrate and a dark red/brown color develops in the agar.

A.2.3 SUPERNATANT PREPARATION AND ENZYME ASSAY

The wine yeast strains were inoculated in YPD broth (10 mL) and grown overnight at 30°C to an optical density (OD_{600}) of 2. Two milliliters of each culture were centrifuged at 5 000 rpm for 5 min and the supernatant was carefully removed and retained. A cocktail of protease inhibitors (Roche) was added to each supernatant (40 μ L/mL of supernatant) to inhibit any protease activity. The activities of the β -glucosidases present in the supernatants were determined using different environmental conditions (pH and temperature) and different substrates (cellobiose or arbutin) (Table 3.6).

Table 3.6. Conditions used for β -glucosidase activity determination in the supernatants.

Test	Substrate	pH	Temperature
i	Cellobiose	3.5	25°C
ii	Cellobiose	7	37°C
iii	Arbutin	3.5	25°C

Supernatant (50 μ L) was mixed with 5 μ L of a 0.01% (w/v) (i/ii) cellobiose or (iii) arbutin solution and 10 μ L of 10 mM citrate-phosphate buffer (i/iii) at pH 3.5 or (ii) pH 7. Water was added to the reaction mixture to obtain a total volume of 100 μ L. The mixture was incubated at (i/iii) 25°C or (ii) 37°C for 1 h, and the amount of glucose released from the hydrolyzed substrate was determined using the Glucose Trinder Kit (Sigma) according to the specifications of the supplier. Enzyme and reagent blanks were included and subtracted from the absorbance obtained for the enzyme-substrate reaction mixture (A_{505} - reaction blank/ Standard - reaction blank \times 100 = [glucose] in mg/100 mL). The specific amount of glucose release was expressed as mg/mL of supernatant. The assays were conducted in triplicate and the standard deviations were usually less than 6% and never more than 15%. The calculation of

the specific activity (U/mL) was based on the level of enzymatic activity divided by the amount of supernatant (mL) present in the enzymatic reaction.

A.2.4 CELL PREPARATION AND ENZYME ASSAY

The wine yeast strains were inoculated in YPD broth (100 mL) at an optical density of 600 nm (OD_{600}) to 0.2 from a 24 h culture and grown at 30°C for 72 h. The OD_{600} of 1 mL of culture was taken, the amount (mL) of culture to be used to have the same optical density (OD_{600}) per reaction was determined and the dry biomass of the specified amount was determined. Cells were harvested by centrifugation at 5 000 rpm for 5 min and washed twice with 10 mM citrate-phosphate buffer (pH 3.4). The pellet was used for the enzymatic assays and mixed with 40 μ L of a 16% (w/v) cellobiose solution in 10 mM citrate-phosphate buffer (pH 3.4). Water was added to the reaction mixture to obtain a total volume of 100 μ L. The mixture was incubated at 25°C for 19 h and the amount of glucose released from the hydrolyzed substrate was determined using the Glucose Trinder Kit (Sigma) according to the specifications of the supplier (see 3.2.2.3). The specific amount of glucose released was expressed as mg/100 mL/mg of cells. The effect of ethanol, SO_2 and glucose on β -glucosidase activity was assessed through the addition of different concentrations of these substances to the reaction mixtures prior to incubation at 25°C. Cultures were grown in duplicate and assays were conducted in triplicate. The standard deviations were usually less than 6% and never more than 15%. One unit of β -glucosidase activity was defined as the amount of crude enzyme required to produce 1 μ mol of glucose under the assay conditions employed. The calculation of the specific activity (U/mg) was based on the level of enzymatic activity divided by the amount of cells (mg) present in the enzymatic reaction.

A.3 RESULTS AND DISCUSSION

The potential of microbial β -glucosidases to release flavor compounds from glycosidically bound non-volatile precursors is of great significance to the wine industry. Numerous studies have been performed to identify microorganisms with β -glucosidases that can be used for the efficient liberation of monoterpenols under the harsh conditions of alcoholic fermentation (Aryan and others 1987; McMahon and others 1999; Hernández and others 2002). Results have indicated that, among yeasts, different strains of *Candida* (Günata and others 1990), *Debaryomyces* (Rosi and others 1994) *Metschnikowia* (Fernández and others 2000) and *Brettanomyces* (McMahon and others 1999) exhibit activity towards various β -glucosides.

A total of 52 yeast strains of varying origin and field of application (27 wine yeasts, 19 whiskey yeasts and six beer yeasts) were tested for β -glucosidase activity. A preliminary evaluation of β -glucosidase activity was performed on solid screening media containing arbutin as sole carbon source. The hydrolysis of arbutin, resulting in red/brown-colored colonies, was considered an indication of β -glucosidase activity.

None of the whiskey and beer yeast strains tested showed any β -glucosidase activity (data not shown). The results of the wine yeast screenings (Table 3.7) indicated that all of the non-*Saccharomyces* yeast strains, except *C. lambica*, *C. sorbosa*, *D. hansenii* and *R. glutinis*, were able to hydrolyze arbutin to a varying degree. Of the six strains of *S. cerevisiae* tested, only one strain (VIN7) was able to hydrolyze arbutin slightly. Figure 3.3 shows the enzyme activity of *S. cerevisiae* VIN7, *C. lambica*, *C. oleophila* and *Brettanomyces* sp. as seen on the screening plates.

Table 3.7. Yeasts tested for β -glucosidase activity on solid media.

Species	Isolates	Activity*
<i>Brettanomyces</i> sp.	O1/33	+
<i>Candida colliculosa</i>	C2/5	+
<i>C. guilliermondii</i>	M1/30	+
<i>C. hellenica</i>	O1/16	+
<i>C. lambica</i>	M2/4	-
<i>C. oleophila</i>	O1/29	+
<i>C. pelliculosa</i>	R1/17	+
<i>C. sorbosa</i>	R2/29	-
<i>C. stellata</i>	C2/20	+
<i>C. valida</i>	O1/22	+
<i>Debaryomyces hansenii</i>	R2/15	-
<i>D. maramus</i>	Y-0896	+
<i>D. polymorphus</i> var. <i>africanus</i>	Y-0064	+
<i>D. pseudopolymorphus</i>	Y-0541	+
<i>Kloeckera apiculata</i>	R2/21	+
<i>Kloeckera</i> sp.	R2/30	+
<i>Pichia carsonii</i>	Y-0895	+
<i>P. farinosa</i>	R1/15	+
<i>P. kluyveri</i>	R2/11	+
<i>Rhodotorula glutinis</i>	R1/23	-
<i>Saccharomyces cerevisiae</i>	VIN7	+
<i>S. cerevisiae</i>	VIN13	-
<i>S. cerevisiae</i>	WE372	-
<i>S. cerevisiae</i>	WE14	-
<i>S. cerevisiae</i>	228	-
<i>S. cerevisiae</i>	N96	-
<i>Zygosaccharomyces bailii</i>	O1/25	+

* Activity is expressed as hydrolysis of arbutin resulting in red/brown-colored colonies.

The screening of the different yeast strains for β -glucosidase activity on solid media gave a general indication of the yeast enzyme activities. As arbutin is a

substrate that can be hydrolyzed by β -glucosidases as well as glucanases, it was necessary to do additional screenings using a substrate that is exclusively hydrolyzed by β -glucosidases, i.e. cellobiose. All wine yeast strains previously tested on solid media were used to determine whether β -glucosidase activity is located extracellularly. After growth for 24 h on basal medium with glucose as the sole carbon source, β -glucosidase activity was assayed on the culture supernatant and the effect of different environmental conditions (pH, temperature and substrate) on enzyme activity was determined.

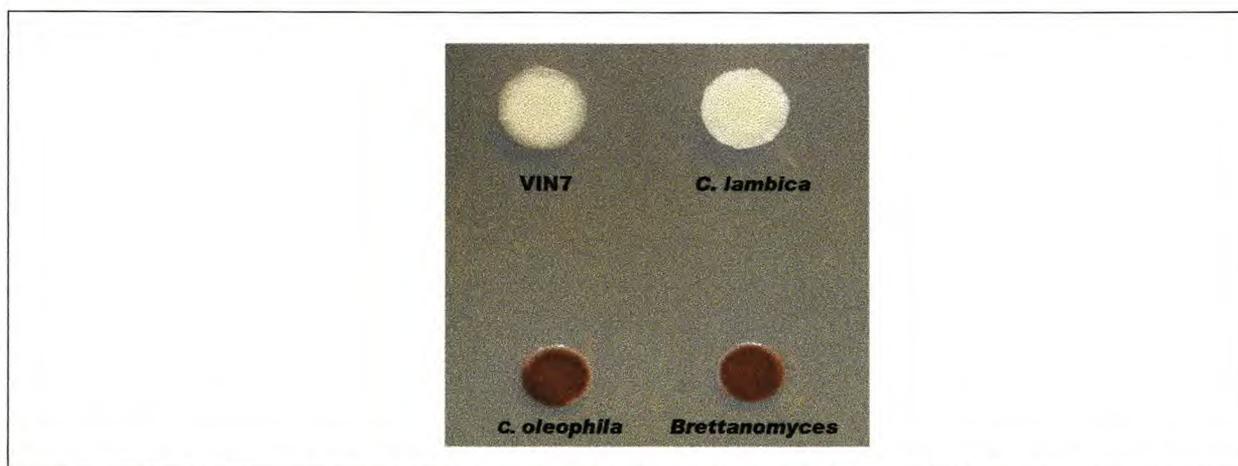


Figure 3.3. Screening for β -glucosidase activity on solid media. Strains were spotted onto screening plates and incubated for eight days. The following strains are shown above: *S. cerevisiae* VIN7, *C. lambica*, *Brettanomyces* sp. and *C. oleophila*. Colored colonies are indicative of arbutin hydrolysis.

Table 3.8. The variability of β -glucosidase activity (U/mL).

Species	Isolates	Cellobiose, pH 3.5, 25°C	Cellobiose, pH 7, 37°C	Arbutin, pH 3.5, 25°C
<i>Brettanomyces</i> sp.	O1/33	51.20 ± 3.68	43.50 ± 6.36	51.70 ± 2.69
<i>Candida colliculosa</i>	C2/5	36.87 ± 3.11	43.00 ± 1.70	102.00 ± 7.07
<i>C. guilliermondii</i>	M1/30	368.33 ± 29.13	323.67 ± 18.03	364.20 ± 6.51
<i>C. hellenica</i>	O1/16	35.53 ± 2.00	67.10 ± 0.42	30.10 ± 1.27
<i>C. lambica</i>	M2/4	459.73 ± 53.56	172.80 ± 14.94	293.90 ± 29.84
<i>C. oleophila</i>	O1/29	94.60 ± 7.69	63.70 ± 9.48	67.70 ± 1.56
<i>C. pelliculosa</i>	R1/17	243.33 ± 32.80	221.27 ± 5.71	197.80 ± 23.19
<i>C. sorbosa</i>	R2/29	31.60 ± 3.11	48.70 ± 3.54	36.20 ± 5.37

Table 3.8 (continued)

Species	Isolates	Cellobiose, pH 3.5, 25°C	Cellobiose, pH 7, 37°C	Arbutin, pH 3.5, 25°C
<i>C. stellata</i>	C2/20	44.87 ± 0.64	25.50 ± 0.99	286.50 ± 1.56
<i>C. valida</i>	O1/22	80.80 ± 2.60	91.13 ± 13.73	45.10 ± 3.54
<i>Debaryomyces hansenii</i>	R2/15	4925.67 ± 168.91	7068.87 ± 80.77	7586.80 ± 517.89
<i>D. maramus</i>	Y-0896	4860.20 ± 38.90	6181.07 ± 311.20	5850.50 ± 84.71
<i>D. polymorphus</i> var. <i>africanus</i>	Y-0064	95.30 ± 2.40	160.07 ± 20.03	61.60 ± 2.55
<i>D. pseudopolymorphus</i>	Y-0541	41.20 ± 5.09	54.40 ± 4.33	47.20 ± 6.51
<i>Kloeckera apiculata</i>	R2/21	24.50 ± 2.97	99.50 ± 13.72	24.60 ± 3.11
<i>Kloeckera</i> sp.	R2/30	43.80 ± 5.37	39.00 ± 2.36	61.90 ± 5.23
<i>Pichia carsonii</i>	Y-0895	4783.13 ± 200.96	6279.53 ± 263.18	5456.30 ± 312.68
<i>P. farinosa</i>	R1/15	28.10 ± 0.14	105.80 ± 16.80	57.60 ± 8.49
<i>P. kluyveri</i>	R2/11	31.07 ± 2.08	26.80 ± 3.96	38.90 ± 4.10
<i>Rhodotorula glutinis</i>	R1/23	26.60 ± 3.70	46.00 ± 5.89	91.40 ± 8.20
<i>Saccharomyces cerevisiae</i>	VIN7	475.13 ± 20.97	170.20 ± 10.18	329.00 ± 42.99
<i>S. cerevisiae</i>	VIN13	212.47 ± 4.99	66.07 ± 6.93	125.90 ± 16.26
<i>S. cerevisiae</i>	WE372	631.80 ± 23.05	166.00 ± 2.95	452.90 ± 12.59
<i>S. cerevisiae</i>	WE14	467.53 ± 23.51	181.60 ± 19.50	381.00 ± 25.46
<i>S. cerevisiae</i>	228	308.60 ± 35.09	93.53 ± 2.66	205.60 ± 20.93
<i>S. cerevisiae</i>	N96	20.54 ± 20.54	177.93 ± 15.92	461.20 ± 28.85
<i>Zygosaccharomyces bailii</i>	O1/25	65.10 ± 9.48	55.80 ± 4.24	60.10 ± 8.91

The results indicate that the highest β -glucosidase activities are obtained when the enzymatic reactions are performed under the pH and temperature conditions (pH 3.5; 25°C) generally found during alcoholic fermentation (Table 3.8). It was also shown that β -glucosidases are more efficient in hydrolyzing cellobiose than arbutin as substrate. Overall, *C. hellenica*, *C. sorbosa*, *K. apiculata* and *P. kluyveri* had the lowest β -glucosidase activity, with none of the strains releasing more than 2 mg/100 mL of glucose from either cellobiose or arbutin under winemaking conditions. It is possible that the β -glucosidase activities of these strains are cell-associated.

Contrary to the results obtained from the screening on solid media, *D. hansenii* was the strain that showed the highest activity under winemaking conditions in the liquid screening assays, releasing 246.28 mg/100 mL of glucose from cellobiose and 379.34 mg/100 mL of glucose from arbutin. The other species with high β -glucosidase activity were *D. maramus* (243.01 mg/100 mL of glucose from cellobiose; 292.53 mg/100 mL of glucose from arbutin) and *P. carsonii* (234.16 mg/100 mL of glucose from cellobiose; 272.82 mg/100 mL of glucose from arbutin). Five yeasts strains with different levels of β -glucosidase activity in culture supernatant were selected to determine their enzyme activity in whole cells (Figure 3.4). The effects of glucose, ethanol and SO₂ on these whole cell β -glucosidase activities were also investigated.

Figure 3.4 shows that *D. maramus* has the highest parietal β -glucosidase activity of all five strains tested, with 1.17 mg/100 mL of glucose being released from cellobiose. *S. cerevisiae* WE372 and *S. cerevisiae* VIN7, releasing only 0.09 mg/100 mL and 0.29 mg/100 mL of glucose from cellobiose respectively, are the strains with the lowest activity. These results, in conjunction with the results depicted in Table 3.8, indicate that the β -glucosidase activities of the *S. cerevisiae* strains tested are mainly extracellular.

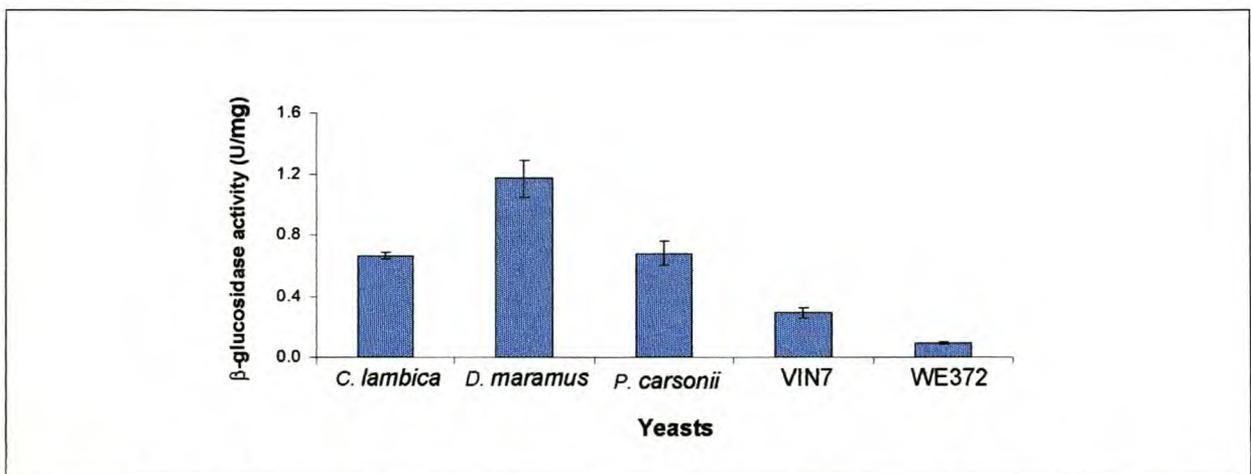


Figure 3.4. Cellobiose hydrolysis by yeast β -glucosidases. Glucose released as a result of substrate hydrolysis was assessed after incubation of whole cells for 19 h at 25°C in the presence of 16% (w/v) cellobiose in 10 mM citrate-phosphate buffer at pH 3.5. The assays were performed in triplicate and the standard deviations were never higher than 15%. The specific activity of the enzymes was expressed as 1 μ mol of glucose produced per mg of whole cells, equivalent to U/mg.

Although there are some exceptions (Rosi and others 1994; Saha and Bothast 1996; Riou and others 1998), the inhibitory effect of glucose is a common characteristic of β -glucosidases (Gueguen and others 1995; Spagna and others 2002) and an important constraint on the industrial use of these enzymes in winemaking. The results on the effect of different glucose concentrations (2, 12 and 24°B) on β -glucosidase activity are presented in **Figure 3.5A**. The results clearly indicate that the β -glucosidase activities of all five strains were markedly increased throughout the entire glucose concentration spectrum tested. *D. maramus* exhibited the most gradual increase in β -glucosidase activity, ranging from 150-fold at 12°B to 190-fold at 24°B. *S. cerevisiae* VIN7, showing only a five-fold increase in β -glucosidase activity at 2°B, displayed a 600-fold induction at 24°B. The largest increase in enzyme activity was observed in *S. cerevisiae* WE372, which exhibited a marked increase of 16×10^2 -fold at 12°B and 40×10^2 -fold at 24°B.

Several studies performed on β -glucosidases of fungal and yeast origin has indicated that alcohol has a stimulatory effect on enzyme activity (Delcroix and others 1994; Yan and Lin 1997). However, grape β -glucosidases (Aryan and others 1987), as well as some other yeast β -glucosidases (Mateo and Di Stefano 1997), have been reported to be inhibited by ethanol. As shown in **Figure 3.5B**, general stimulation of β -glucosidase activity was also observed for the five different yeast strains evaluated at all ethanol concentrations tested. The *D. maramus* β -glucosidase activity appeared to be insensitive to ethanol, whereas *S. cerevisiae* VIN7 exhibited a slight overall increase in activity throughout the series tested, reaching a two-fold induction at 14%. *C. lambica* displayed an initial three-fold increase in β -glucosidase activity, which was maintained throughout the ethanol series tested. The most prominent induction was observed for the β -glucosidase activity of *P. carsonii*, which exhibited a 50-fold increase in enzyme activity at 14% ethanol. The β -glucosidase of *S. cerevisiae* WE372 was the only enzyme that did not exhibit a linear increase in enzyme activity as the ethanol concentration increased. An initial 15-fold increase in activity was observed at 10% ethanol, but, even though the activity decreased again as the ethanol concentration increased, a three-fold increase in activity was still observed at 12% and 14%.

Little is known about the influence of sulfur dioxide on β -glucosidase activity. Delcroix and others (1994) found that an SO_2 concentration of 50 mg/L had no effect on enzyme activity. As shown in **Figure 3.5C**, the data for *C. lambica* are in agreement with this observation. None of the SO_2 concentrations tested had a marked effect on the β -glucosidase activity of this yeast strain. The *D. maramus* β -glucosidase exhibited an initial two-fold increase in activity, which was maintained throughout the series tested. The most prominent inhibition was observed for the β -glucosidase activity of *P. carsonii*, which exhibited a 50% and 65% (compared to the total activity of the untreated samples) reduction in enzyme activity at 30 ppm and 60 ppm respectively. *S. cerevisiae* WE372, exhibiting an initial increase in β -glucosidase activity to reach a four-fold induction at 30 ppm, was strongly inhibited by a SO_2

concentration of 60 ppm, retaining only 50% of its original activity at this concentration.

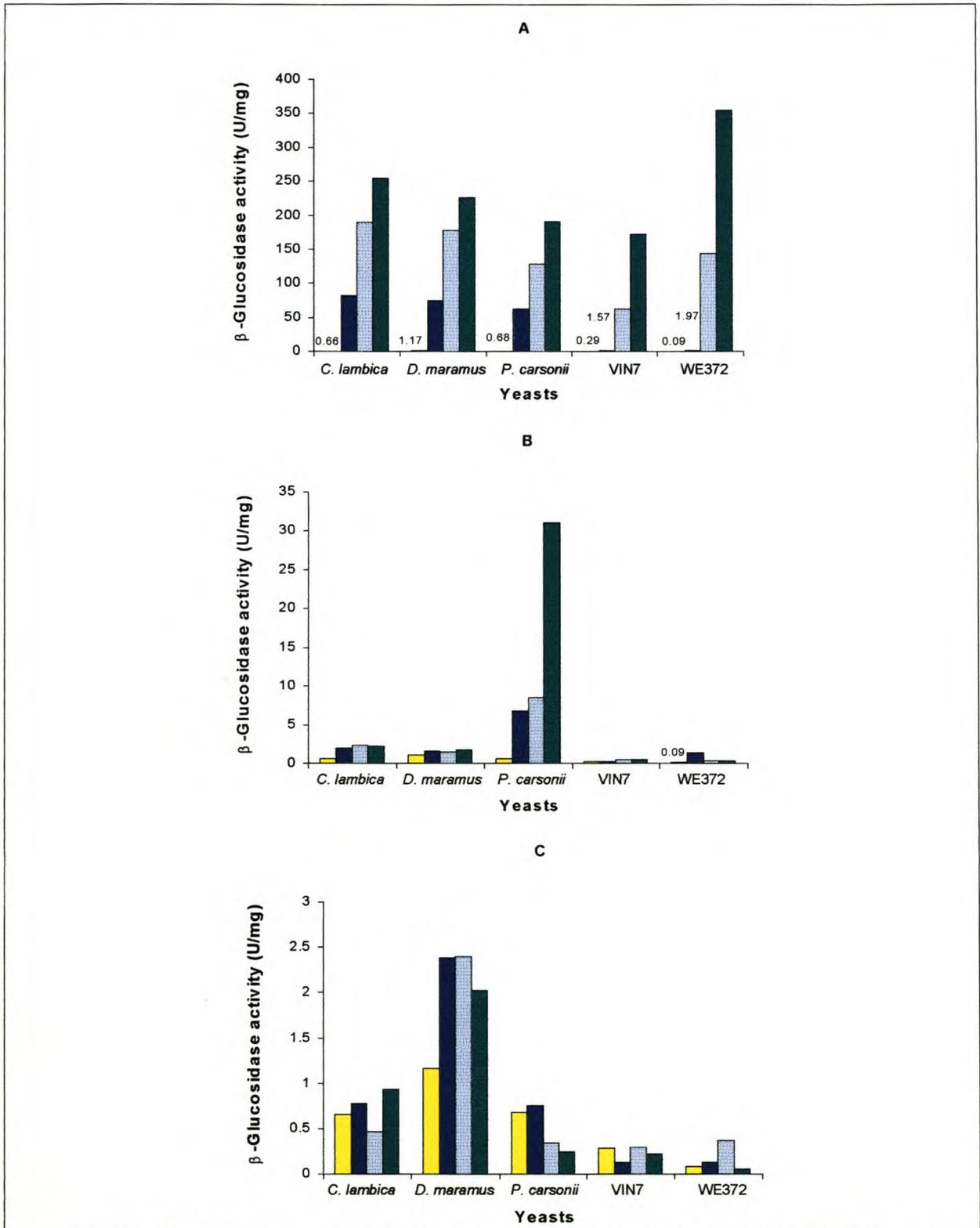


Figure 3.5. Effects of glucose (A), ethanol (B) and SO₂ (C) on the β -glucosidase activities of different yeast strains. (A) Glucose concentrations tested include 2°B (blue bars), 12°B (purple bars) and 24°B (green bars); (B) ethanol concentrations tested include 10% (blue bars), 12% (purple bars) and 14% (green bars); (C) SO₂ concentrations tested include 15 ppm (blue bars), 30 ppm (purple bars) and 60 ppm (green bars). In all cases, the yellow bars indicate the non-spiked reaction mixture of each enzyme. The values are means of triplicate experiments and the relative error was less than 15%.

The results obtained from the above experiments make an important contribution to an extensive survey aimed at the exploitation of the hidden enological potential of the untapped wealth of non-*Saccharomyces* yeasts and their enzymes (Pretorius and others 1999; Fernández and others 2000; Strauss and others 2001; Cordero Otero and others 2003). The data suggest that the β -glucosidase activity of *D. maramus* possesses suitable properties in terms of optimum pH and resistance to wine-associated inhibitory compounds (glucose, ethanol and sulfur dioxide). However, additional work is required to determine the efficiency of the *D. maramus* β -glucosidase enzyme to increase the aromatic varietal character of wines through the hydrolysis of flavor glycosidic precursors.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

4.1 CONCLUDING REMARKS AND PERSPECTIVES

Wine is one of the most complex beverages known to man. Its aroma stems from a complex, non-linear system of interactions between substances from grapes, substances produced during fermentation, and those that arise during ageing. Yeast and fermentation conditions are claimed to be the most important factors influencing wine flavor, as the principal flavor of wine is derived from the secondary metabolites of the grapes. Fermentation increases the chemical and flavor complexity of wine by assisting in the extraction of compounds from solids present in the grape must, modifying some grape-derived compounds, and producing a substantial amount of yeast metabolites.

In grapes, a high percentage of aromatic compounds occur as their respective non-volatile *O*-glycosides. Several studies have shown that the varietal character of wine can be intensified by the enzymatic hydrolysis of the aroma precursors present in the wine through the action of β -glucosidases (Günata and others 1990; Canal-Llaubères 1993). Some β -glucosidases are not active in the wine environment, however, as they are inhibited by the low pH and high ethanol and glucose levels that prevail. The endogenous β -glucosidases from grapes and the wine yeast *Saccharomyces cerevisiae* are both examples of such β -glucosidases (Canal-Llaubères 1993). Fungal, bacterial and certain yeast β -glucosidases have been shown to be very effective aroma liberators, but they are mostly also inhibited under winemaking conditions. The limited enzyme activity found in the abovementioned microorganisms has resulted in a search amongst non-*Saccharomyces* yeasts for unique β -glucosidases that are very effective under winemaking conditions.

This thesis commenced with the emphasis on monoterpene alcohols (monoterpenols) and their important contribution to the varietal aroma of some wines. As mentioned before, a small fraction of monoterpenols found in juice and wine is free, and therefore volatile, whereas a larger fraction is bound to saccharide moieties that render them non-volatile and odorless (Williams and others 1982; Günata and others 1985). Chapter 1 explored the possibility of releasing these monoterpenols from their glucoconjugated aroma precursors through enzymatic hydrolysis, thereby enhancing the aroma of the wine. β -Glucosidase is a key enzyme in the enzymatic release of bound monoterpenols, and this chapter provided a broad overview of the enzymatic properties of some of the β -glucosidases (grape, fungal and yeast) that, to a certain extent, play a role in monoterpene liberation.

The first section of Chapter 2 introduced the properties and characteristics of β -glucosidases. Emphasis was placed on the origin of these enzymes, as well as their individual functions and modes of action. The β -glucosidases discussed in this section include the three β -glucosidases found in humans (human acid β -glucosidase, lactase-phlorixin hydrolase and cytosolic β -glucosidase), insect β -glucosidases, β -glucosidases found in plants and, lastly, microbial β -glucosidases.

As these enzymes are responsible for the hydrolysis and synthesis of oligosaccharides, they play a pivotal role in a number of biotechnological applications. An overview of these applications in the medical, industrial and food sectors was also provided in this chapter.

The second part of Chapter 2 focused in greater depth on the influence of monoterpenols on wine aroma, and especially on the use of β -glucosidases to liberate these monoterpenols from their terpenyl-glycosides. A classic wine environment is generally described as an environment in which low pH and temperature, and high ethanol and glucose concentrations prevail. These factors are all inhibitors of β -glucosidase activity, and not all β -glucosidases are thus suitable for use as aroma liberators under wine conditions (Aryan and others 1987; Günata and others 1990). In this section of Chapter 2, various β -glucosidases from different sources were discussed and evaluated to determine which enzymes can successfully overcome the abovementioned inhibition.

Chapter 3 described the screening of wine-related non-*Saccharomyces* yeasts to determine the efficiency with which they could liberate monoterpenols from their terpenyl-glycosides. The first section of this chapter focused on yeasts with primarily cell-associated β -glucosidases, whereas the second section focused more on cytosolic β -glucosidase activities. The experimental data clearly indicate that the efficiency of β -glucosidases to hydrolyze glycosidic bonds is dependent on the cell-fraction used in the experiment. *Candida lambica*, for instance, exhibits poor β -glucosidase activity when assays are performed using crude cell extracts, but it is one of the yeasts with the highest enzyme activity when assays are performed with the supernatant, which indicates that the β -glucosidase activity of this yeast is primarily extracellular.

Due to the higher reproducibility of the liquid assays performed with the crude cell extracts, it was decided to focus mainly on the β -glucosidases located in this cell fraction. The four yeast strains exhibiting the highest cell-associated β -glucosidase activity were selected to investigate the effect of ethanol, glucose and SO_2 on their activity. *Debaryomyces pseudopolymorphus* was identified as the yeast strain that exhibited the most suitable combination of properties in terms of functionality at wine pH, resistance to wine-associated inhibitory compounds (ethanol, glucose and SO_2), high substrate affinity and large aglycone-substrate recognition (Cordero Otero and others 2003). Microvinifications were performed with a mixed-culture sample consisting of *D. pseudopolymorphus* and *S. cerevisiae* VIN13 (a commercial wine starter culture strain), and the wines were analyzed. The results indicated that the concentrations of free terpenols (nerol, geraniol and citronellol) were significantly increased during fermentation, an indication of precursor-hydrolysis by the β -glucosidase enzyme of *D. pseudopolymorphus*.

Future experimental work would entail an in-depth study of the kinetic characteristics of the non cell-associated β -glucosidases exhibiting the highest terpenol-liberating activity under winemaking conditions. The next step would be the

cloning and expression of the most efficient β -glucosidase gene in a commercial wine yeast. Such a recombinant wine yeast would release grape-derived aroma compounds from their non-volatile precursors during single culture fermentations, thereby increasing the sensorial quality of wine.

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