Enzymes in Winemaking: Harnessing Natural Catalysts for Efficient Biotransformations - A Review

P. van Rensburg and I.S. Pretorius

Institute for Wine Biotechnology and Department of Viticulture & Oenology, University of Stellenbosch, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa

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Enzymes play a definitive role in the ancient and complex process of winemaking. From a scientific and technical point of view, wine can be seen as the product of enzymatic transformation of grape juice. From the pre-fermentation stage, through fermentation, post-fermentation and aging, enzymes are the major driving forces catalysing various biotransformation reactions. These biocatalysts originate not only from the grape itself but also from yeasts and other microbes (fungi and bacteria) associated with vineyards and wine cellars. Through better understanding of these enzymatic activities, winemakers have come to learn how to control the unwanted enzymes while optimising the desired activities. Today, winemakers reinforce and extend the action of these endogenous enzymes by the judicious application of an ever-increasing spectrum of commercial enzyme preparations. These enzyme preparations are applied to winemaking with the aims of improving the clarification and processing of wine, releasing varietal aromas from precursor compounds, reducing ethyl carbamate formation and lowering alcohol levels. This review article summarises the most important enzymes applied to winemaking, the nature and structure of their substrates, and the reactions catalysed by these enzymes. This paper also reviews the limitations of the endogenous enzymes derived from grapes and microbes present in must and wine, along with the effects of commercial enzyme preparations on process technology and the quality of the final product. Prospects of developing wine yeast strains expressing tailored enzymes are also highlighted.

INTRODUCTION

The term "*enzyme*" is derived from the Latin words meaning "*in yeast*". Enzymes were once thought to exist at organism level, until in 1926 Sumner demonstrated that enzymes are in fact proteins which act as biological catalysts. Apart from facilitating reactions they are also able to accelerate reactions without undergoing any permanent structural change (Underkofler, 1976). While the types of reactions catalysed by enzymes are limited (hydrolyses, oxidations, reductions, *etc.*) their numbers remain very high. This is due to one of the most intriguing characteristics of enzymes, their specificity. Enzymes have the capacity to act on one substance, or a limited number of substances, by recognising only a specific chemical group or substrate. This absolute specificity may be broadened or removed completely by a small modification of the enzyme which is sufficient to render it inactive. While these characteristics may limit the field of application, they also provide for targeted interventions, which could not normally be achieved in any other way.

Enzymes play a pivotal role in the winemaking process. In addition to enzymes which occur in pre- and post-fermentation practices, there are at least ten different enzymes driving the fermentation kinetics that convert grape juice to wine. It is therefore of key importance to understand the nature and behaviour of these enzymes and to create the optimal conditions to exploit those enzymes which are beneficial, while inhibiting those which may be detrimental to wine quality. Many of these enzymes originate from the grape itself, the indigenous microflora on the grape and the microorganisms present during winemaking. Since the endogenous enzymes of grapes, yeasts and other microorganisms present in must and wine are often neither efficient nor sufficient under winemaking conditions to effectively catalyse the various biotransformation reactions, commercial enzyme preparations are widely used as supplements.

All these commercial enzyme preparations are obtained from microorganisms cultivated on substrates under conditions that optimise their production and facilitate their purification at a competitive cost. Research in this field is very active and continually expanding. The number of enzymes produced on an industrial scale (approximately 30) represents only a fraction of the total number of enzymes that have been discovered: there are 2500 different enzyme-catalysed reactions listed in the International Union Handbook of Enzymes Nomenclature (Gacesa & Hubble, 1998).

This article summarises the most important enzymes that act in winemaking to improve (i) the clarification and processing of wine (pectinases, glucanases, xylanases, proteases), (ii) the release of varietal aromas from precursor compounds (glycosidases), (iii) the reduction of ethyl carbamate formation (urease), and (iv) the reduction in alcohol levels (glucose oxidase). For a better understanding of the reactions catalysed by these enzymes, some background information is presented on the nature and structure of various substrates transformed by the enzymes. This paper also reviews the limitations of the endogenous enzymes derived from grapes or microbes in must and wine (Table 1), along with the advantages resulting from application of industrial enzyme preparations. Prospects for developing wine yeast strains expressing these heterologous enzymes are also highlighted.

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THE IMPORTANCE OF PECTINASES TO WINE CLARIFICATION AND PROCESSING

Structure of pectic substances

Pectic substances are structural heteropolysaccharides, and are the major constituents of the middle lamellae and primary cell walls of higher plants (Whitaker, 1990). They are responsible for the integrity and coherence of plant tissues. Apart from their function as "lubricating" or "cementing" agents in cell walls of higher plants, pectic substances are also involved in the interactions between plant hosts and their pathogens (Collmer & Keen, 1986).

The American Chemical Society classifies pectic substances into four main types: protopectins, pectinic acids, pectins and pectic acids (Kertesz, 1987). Protopectin is considered to be the parent compound of pectic substances, serving as the glue that holds the cells together. Whereas protopectins are water-insoluble, the other three are either totally or partially soluble in water. The reasons for insolubility in plant tissues are diverse and include the binding of pectin molecules with polyvalent ions (Ca^{2+}, Mg^{2+} and Fe^{2+}), secondary valency bonding between pectin and cellulose and/or hemicellulose, salt-bridging between carboxyl groups of pectin molecules and other cell wall constituents as well as the basic groups of proteins (Sakai, 1992).

Pectic substances consist mainly of α-D-1,4-linked galacturonic acid residues (pectate) or its methyl ester (pectin) (Pretorius, 1997). In pectin, at least 75% of the carboxyl-groups are esterified with methanol, with the free carboxyl-groups occurring in clusters along the chain. One can distinguish between acid pectic substances, also called pectins (homogalacturonans, rhamnogalacturonans), and neutral pectic substances (arabinans, galactans, arabinogalactans). Primary chains consist of "smooth" α-1,4-D-galacturonic acid units and are β-1,2 and β-1,4 linked to L-rhamnose units (at about one every 25 galacturonic acid units) with side chains varying in composition and length (Fig. 1). Neutral sugars are concentrated in blocks of highly substituted rhamnogalacturonate regions (giving the rhamnogalacturonan portion of the pectin backbone a "hairy" character) separated by un-substituted areas comprising almost exclusively D-galacturonate units (Whitaker, 1990). Rhamnogalacturonans are the major constituents of the pectic substances. Highly branched arabinogalactans or predominantly linear chains of β-D-1,4-galactopyranosyl residues are associated with some rhamnose-rich regions, covalently linked through a terminal galactopyranosyl residue. The chemical structure and proportions of pectin substrates vary considerably depending on the source, portion and age of the plant material from which it is isolated.

Together with other polysaccharides such as glucan (cellulose) and xylan (hemicellulose), grape pectins influence the clarification and stabilisation of must and wine. These polysaccharides are found in wines at levels between 300 and 1000 mg/L and are often responsible for turbidity, viscosity and filter stoppages.

Enzymatic hydrolysis of pectic substances

The presence of pectins in all fruits is accompanied by an equally extensive spread of enzymes capable of breaking them down. The pectolytic enzymes derived from plants play a role in cell elongation, softening of some plant tissues during maturation and storage, and decomposition of plant materials (Whitaker, 1990). Specifically, pectolytic enzymes in grapes make an important contribution to the changes that occur to pectic substances during grape ripening.

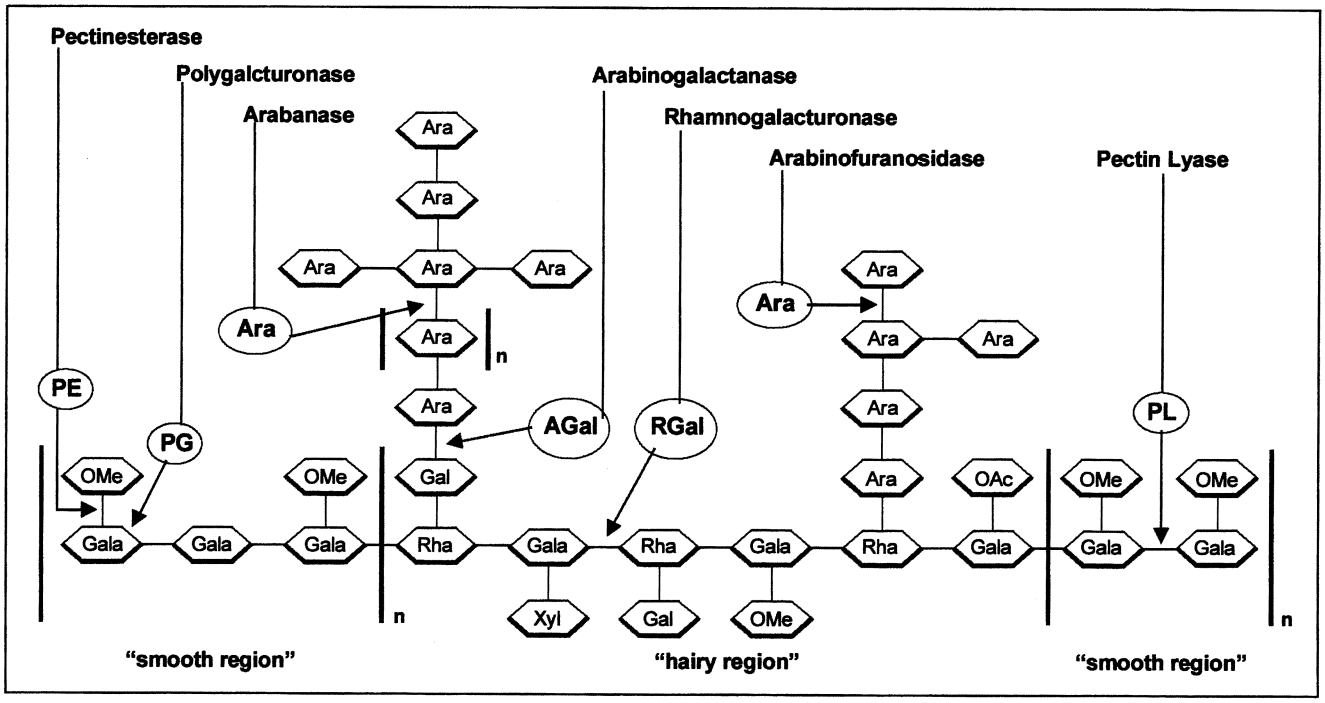

FIGURE 1

The new proposed pectin model and enzymatic pectin degradation.

Apart from the pectinases produced by the grape itself, several pectolytic enzymes that end up in must and wine originate from the microflora associated with the grape berries. One of the most important fungi infecting grapes, the grey mould *Botrytis cinerea*, is responsible for grey or noble rot. Various extracellular enzymes, including pectinases, are produced by this fungus (Verhoeff & Warren, 1972; Shepard & Pitt, 1976; Dubernet *et al.*, 1977; Dubourdieu, 1978; Verhoeff & Liem, 1978). The concentration of pectolytic enzymes in the *Botrytis*-infected grapes is about 200 times higher than in the healthy grapes.

These grape and microbial pectinases are classified according to their mode of attack on the pectin molecule. These enzymes de-esterify (pectinesterases) or depolymerise (polygalacturonases, polymethylgalacturonases, pectin and pectate lyases) specific pectic substrates as can be seen in Fig. 1 (Rexová-Benková & Marcovic, 1976; Laing & Pretorius, 1992; Gainvors *et al.*, 1994; González-Candelas *et al.*, 1995; Pretorius, 1997).

***Protopectinases*:** The term "protopectinases" refers to enzymes producing water-soluble and highly polymerised pectin substances from protopectin (which is insoluble) by reacting at sites having three or more non-methylated galacturonic acid units and hydrolysing the glycosidic bond (Sakai, 1992). Type A protopectinase reacts with polygalacturonic acid regions of protopectin, while the B-type acts on the polysaccharide chains connecting the polygalacturonic acid chain and the cell wall constituents.

***Pectin methylesterases*:** Pectin methylesterases (EC 3.1.1.1.11) split the methyl ester group of polygalacturonic acids, proceeding in a linear fashion along the chain, and thereby freeing methanol and converting pectin into pectate (McKay, 1988). Its action does not reduce the length of the pectic chains. The esterases require at least one free carboxyl group adjacent to the methyl group under attack. They attack the chain from the reducing end, transforming pectin to low methoxyl pectin, pectic acid and methanol.

***Polygalacturonases*:** Polygalacturonases are the most commonly encountered pectic enzymes. They break down the glucosidic links that connect the molecules of the galacturonic acid to one another, with the absorption of one molecule of water (Blanco *et al.*, 1994). Since they act on molecules with free carboxylic groups, they have little effect on highly methylated pectin in the absence of pectin methylesterases; therefore polygalacturonases function synergistically with pectin methylesterases (Gainvors *et al.*, 1994). The increase in reducing end groups is accompanied by a strong reduction in viscosity of the substrate solution (Whitaker, 1990). There are two types of polygalacturonases with widely differing technological influence. The exopolygalacturonases break down the distal groups of the pectic molecule, resulting in a relatively slow reduction of the chain's length. The endopolygalacturonases act randomly on all the chain's links, with faster and more incisive consequences with regard to molecular dimensions and reduction of viscosity.

Pectin and pectate lyases: The β-eliminative attack of the lyases on the galacturonan chain results in the formation of a double bond between C_4 and C_5 in the terminal residue at the non-reducing end, thereby generating an oligomer with a 4,5-unsaturated galacturonosyl at the end. Different lyases can be distinguished on the basis of their preference for highly esterified pectinic acid or pectate and on the degree of randomness in the eliminative depolymerisation and behaviour towards oligomeric substrates. Pectin lyase is specific for highly esterified pectin, whereas pectates and low-methoxyl pectins are the best substrates for endopectate lyase. Exopectate lyase is specific for the penultimate bond at the reducing end of the galacturonan chain, liberating unsaturated digalactosiduronate as the sole end-product.

Industrial pectinase preparations

Limitations of pectinases derived from grapes and wine-related microbes: The optimum pH of the pectinases originating from the grape berries and associated micoorganisms usually varies between pH 2 and 8 (Table 1). Most of these pectinases are therefore not notably inhibited at pH values which are normally found in grape juice, must and wine (pH 3.0 — 4.0). Furthermore, these endogenous pectolytic enzymes are active within wide limits of temperature, but at very different rates. Pectolytic activity drops to negligible levels at low winemaking temperatures. Other factors that reduce the effectiveness of endogenous pectinases in must and wine clarification include the levels of sulfur dioxide (SO_2), tannins and alcohol as well as bentonite treatment. Owing to these inhibiting factors and concomitant inadequate degradation of pectic substances in must and wine by the endogenous pectinases, commercial pectinase preparations are most often added to assist juice extraction and wine clarification.

Characteristics of commercial pectinase preparations: Winemakers are concerned with only a very small proportion of all commercial enzymes. The first commercial enzyme preparations used in the wine industry consisted of pectinases (Rombouts & Pilnik, 1980). Today, pectic enzymes alone account for about one-quarter of the world's food enzyme production. In winemaking, commercial pectinase preparations are used to improve juice yields by degrading structural polysaccharides that interfere with juice extraction, the release of colour and flavour compounds entrapped in grape skins, and with the clarification and filtration of wine.

Sources and activities of commercial pectinase preparations: Most commercial preparations of pectic enzymes are obtained from fungal sources (Alkorta *et al.*, 1994). In fact, although for obvious economic reasons it is very difficult to find reliable information about commercial production of pectic enzymes, all producer strains probably belong to *Aspergillus* species. Over the years these became available in a variety of names, effectiveness and purity (Tables 2 and 3). Early samples were sometimes contaminated with less desirable enzymes such as polyphenol-oxidases, whereas the newer products are purer. The preparation of deliberately mixed enzymes is gaining in popularity because the products have more than one function; the composite enzyme mixtures of pectinases, cellulases, hemicellulases and glycosidases are an example. Enzyme preparations containing cellulases and hemicellulases, in addition to pectinase activities, are known as liquefaction enzymes.

The activity of commercial pectinase preparations are usually reported in one of the following activity units: (i) as apple juice depectinising activity (AJDU), based on the reciprocal time required to clarify fresh apple juice at pH 3.5 and 45°C (Brown & Ough, 1981); (ii) as polygalacturonase activity (PGU), based on the reduction in viscosity of polygalacturonate substrate at

TABLE 1

Enzymes derived from grapes and wine associated microbes involved in winemaking.

Enzyme	Remarks
Grapes (*Vitis vinifera*)	
Glycosidases	Hydrolyse sugar conjugates of tertiary alcohols; inhibited by glucose; optimum pH 5-6
Protopectinases	Produce water-soluble and highly polymerised pectin substances from protopectin
Pectin methylesterases	Saponifying enzymes that split metyl ester groups of polygalacturonic acids thereby releasing methanol and converting pectin into pectate; thermostable; optimum pH 7-8
Polygalacturonases	Hydrolyse α-D-1,4-glycosidic linkages adjacent to a free carboxyl group in low methylated pectins and pectate; optimum pH 4-5
Pectin lyases	Depolymerise highly esterified pectins
Proteases	Hydrolyse the peptide linkages between the amino acid residues of proteins; inhibited by ethanol; thermostable; optimum pH 2
Peroxidases	Play an important role in the oxidation metabolism of phenolic compounds during grape maturation; activity is limited by peroxide deficiency and sulphur dioxide in must
Tyrosinases (oxido-reductases)	Oxidise phenols into quinones resulting in undesirable browning
Fungi (*Botrytis cinerea*)	
Glycosidases	Degrades all aromatic potential of fungal infected grapes
Laccases	Broad specificity towards phenolic compounds and cause serious oxidation and browning problems
Pectinases	Saponifying and depolymerising enzymes causing the degradation of plant cell walls and grape rotting
Cellulases	Multicomponent complexes comprising endoglucanases, exoglucanases (cellobiohydrolases) and cellobiases (a member of β-glucosidases) that act synergistically in a stepwise process to degrade plant cell walls thereby causing grape rotting
Phospholipase	Degrades phospholipids in cell membranes
Esterases	Involved in ester formation
Proteases	Aspartic proteases are produced at the early stage of fungal infection of grapes and determine the subsequent rate and extent of rotting caused by pectinases; soluble; thermostable
Yeast (*Saccharomyces cerevisiae*)	
β-Glucosidases	Some yeasts produce β-glucosidases which are not repressed by glucose
β-Glucanases	Consist of extracellular, cell wall bound and intracellular, sporulation specific glucanases; accelerate autolysis process and release mannoproteins
Proteases	Acidic endoprotease A accelerates autolysis process
Pectinases	Some yeasts degrade pectic substances to a limited extent; inhibited glucose levels higher than 2%
Bacterial (Lactic acid bacteria)	
Malolactic enzymes	Convert malic acid to lactic acid
Esterases	Involved in ester formation
Lipolytic enzymes	Degrade lipids

TABLE 2
Commercial pectinase preparations that improve the clarification, filtration and yield of juice and wine.

Enzyme	Company	Activities	Time of addition
Rapidase Vino Super	Gist-brocades/Anchor	Pectolytic	To juice before settling, to mechanical harvester or press
Rapidase Filtration	Gist-brocades/Anchor	Pectolytic + β-glucanase	Add at the end of fermentation
Rapidase X-Press	Gist-brocades/Anchor	Pectolytic	To grapes or mash
Rapidase CB	Gist-brocades/Anchor	Pectolytic	To the debourbage step
Endozym Active	AEB Africa	Pectolytic	To juice before settling
Pectizym	AEB Africa	Pectolytic	To juice before settling
Pectocel L	AEB Africa	Pectolytic	To grapes or juice
Glucanex	Novo Nordisk	β-Glucanase	Between the first racking and filtration
Ultrazym	Novo Nordisk	Pectolytic	To white and red mash and to debourbage
Pectinex Superpress	Novo Nordisk	Pectolytic + hemicellulases	Directly into mill
Influence	Darleon	Pectolytic + side activities	To debourbage and in red wine during fermentation

TABLE 3
Commercial pectinase preparations that improve the extraction and stabilization of colour during winemaking.

Enzyme	Company	Activities	Time of addition
Enzym'Colour Plus	Darleon	Pectolytic + Proteolytic	To juice or must
Endozyme Conatct Pelliculaire	AEB Africa	Pectolytic	To juice or must
Endozyme Rouge	AEB Africa	Pectolytic + side activities	During maceration (before SO_2)
Vinozym EC	Novo Nordisk	Pectolytic, arabinase and cellulases	Into crusher or mash tank
Rapidase Ex Color	Gist-brocades/Anchor Yeast	Pectolytic + side activities	Before maceration

pH 4.2 and 30°C; (iii) as pectin methylesterase activity (PMEU), based on the amount of enzyme required to liberate a micromole of titrable carboxyl groups per minute at pH 3.5 and 37°C.

Factors influencing the activity of pectinase preparation: A number of inhibiting factors are important to consider when commercial pectinases are added to juice and wine. Though the pH of must and wine do not inhibit the activity of most commercial pectinase preparations, other factors such as temperature can reduce the efficiency of the pectolytic activity significantly. For instance, below 10°C their activity drops to levels which are too low to effectively degrade pectic substances in must and wine. As the temperature rises, the rate of the pectolytic reaction doubles every 10°C. In theory at least, one could use eight times less enzyme if the must and juice could be processed at 55°C (Hagan,

1996). However, at temperatures above 50-55°C commercial pectinases are rapidly inactivated.

The temperature stability of commercial pectinase preparations is another crucial factor influencing their activity. Commercial pectinase preparations contain not only the active protein (2-5%), but also sugars, inorganic salts and preservatives to stabilise and standardise the specified activity of the final products (Hagan, 1996). These compounds act as important protectants during sub-optimal storage conditions and exposure to temperature fluctuations. Nevertheless, factors such as high storage temperatures can still have detrimental effects on the activity of commercial pectinase preparations; the higher the storage temperature, the higher the rate of enzyme loss (Hagan, 1996). For example, if a commercial pectinase preparation was stored at 50°C for 1 hour, the enzyme loss could increase 30%. However, most suppliers produce their products with a higher-than-specified activity to take into account minor temperature fluctuations and the effects of storage. Liquid pectinase preparations in general will lose about 10% of their activity per year if unopened and stored at less than 10°C. Powdered products are more stable, with expected loss of activity of between 5 and 10%. Interestingly, pectinases can also be frozen without any loss of activity upon thawing.

Sulfur dioxide and alcohol only exert an inhibiting action above concentrations of 500 mg/L for SO_2 and over 17% (v/v) for ethanol. Bentonite may be used only after the pectolytic enzymes have carried out their action. Very tannic wines should first be treated with suitable doses of gelatine in order to remove the tannins that would react with the proteins.

***Effects of pectinase additions on wine processing and quality*:** The point in the winemaking process at which enzymes are applied is very important. Normally pectinases are applied after pressing to clarify the juice. (Examples of commercial enzymes for general use are listed in Table 2.) Over the last three decades a considerable amount of research has been conducted to illustrate the advantage of using these commercial enzymes in winemaking.

Effect on juice extraction and clarification: The addition of pectinases lowers the viscosity of grape juice and causes cloud particles to aggregate into larger units, which sediment and are removed easily by settling. If pectinases are applied to the pulp before pressing, one can improve juice and colour yield. The enzymatic pectin degradation yields thin free-run juice and a pulp with good pressing characteristics. Pectolytic enzyme preparations for so-called liquefaction comprise a mixture of pectinases with cellulases. During maceration, pectin degradation affects only the middle lamella pectin, and organised tissue is transformed into a suspension of intact cells. When pectinases are used at a concentration of 2-4 g/hl, 15% more juice can be obtained during a settling period of 4-10 hours (Ribéreau-Gayon *et al.*, 2000).

Sims *et al.* (1988) compared a macerating enzyme (Macerating Enzyme GC219; Genencor) with a standard pectinase (Pectinol 60G; Genencor) on a hard-to-press *Vitis rotundifolia* cultivar and a hard-to-clarify *Euvitis* hybrid. The macerating enzyme consisted of a mixture of pectinases, cellulases and hemicellulases. The macerating enzyme was slightly more effective than the standard pectinase in increasing free run, but not total yields, of both the muscadine and *Euvitis* hybrid. However, the macerating enzyme greatly improved the degree of settling of the hard-to-clarify hybrid as compared to the pectinase preparation.

Effect on methanol levels: Some researchers have found that the addition of pectolytic enzymes induces an increase of methanol levels in different fermented products, such as ciders (Massiot *et al.*, 1994) and wine (Servili *et al.*, 1992; Bosso, 1992; Bosso & Ponzetto, 1994). Nicolini *et al.* (1994), however, pointed out that many other factors, such as grape variety, oenological practices and the yeast strain used, can influence methanol production.

Revilla & González-SanJosé (1998) evaluated methanol production by different commercial preparations of pectolytic enzymes during the fermentation process of red grapes, Tinto fino (*Vitis vinifera*). Four different commercial preparations of pectolytic enzymes were used at the maximum doses suggested by the manufacturers. They used two clarifying pectolytic enzymes, Zimopec PX1 (Perdomini; 0.03 g/L) and Rapidase CX (Gist-brocades; 0.05 g/L), and two colour extracting enzymes, Pectinase WL extraction (Wormser oenologie; 0.01 g/L) and Rapidase Ex Colour (Gist-brocades; 0.05 g/L). The results showed that the enzymatic treatments enhanced the methanol content from day one of fermentation for three of these four enzymes, and from day three for all of them. Every enzymatic treatment produced higher methanol levels than the control in the final wine, but this was statistically significant only for Rapidase CX. During storage the methanol levels remained largely constant.

Effect on the extraction of pigments and phenols: Early research conducted by Ough *et al.* (1975) indicated that pectolytic enzyme treatment of red grape musts could accelerate the extraction of pigments and phenols. They concluded that the only significant effect on wine quality was the increased intensity of wine colour. They also stated that when fermentation tanks are in short supply, the advantage of enzyme treatment is obvious, since the faster colour extraction will allow the pomace to be pressed up to 24 hours earlier. This shorter skin contact time results in wines of equal colour, but lower tannin content. Subsequently, Brown & Ough (1981) tested two commercial enzymes, Clarex-L and Sparl-L-HPG (supplied by Miles Laboratories), on the grape must of eight different white varieties. These treatments resulted in an increase in total juice yields, clarity of the wine, filterability, methanol production, wine quality, browning capacity and amount of settled solids. Table 3 lists a few examples of commercial enzymes which can be used for extraction and stabilisation of colour.

Wightman *et al.* (1997) conducted research on the use of commercial pectinase enzyme preparations in Pinot noir and Cabernet Sauvignon wines. In contrast to the findings of Ough *et al.* (1975), Wightman *et al.* (1997) indicated that some pectinase preparations are capable of reducing red wine colour through pigment modification and subsequent degradation. Subsequently, Scott Laboratories investigated the effects of two enzyme preparations, Scottzyme Color Pro and Color X (Watson *et al.*, 1999). Both enzymes produced wines with higher concentrations of anthocyanins and total phenols, and greater colour intensity and visual clarity than untreated control wines. The enzyme-treated wines also had increased aroma and flavour intensity, including enhanced spicy, cherry, raspberry aromas and flavours, and enhanced bitterness and astringency characteristics. Further trials

by Scott Laboratories included the addition of five enzyme preparations using both a low and a high level of addition as recommended by the suppliers (Watson *et al.*, 1999). These enzyme preparations included Scottzyme Color Pro and Scottzyme Color X added at a rate of 12 and 20 g/hL, Lallzyme EX (Lallemand) at 4 and 8 g/hL, Rapidase EX (Gist-brocades) at 4 and 8 g/hL, and Vinozyme G (Cellulo) at 3 and 6 g/hL. All five of the commercial enzyme preparations produced wines with greater total phenolic content than untreated controls. Wines produced by enzyme treatment were higher in polymeric anthocyanins, polymeric phenols and catechin than the control wines, but not in monomeric anthocyanin content. A panel was able to differentiate the wines produced by the lower enzyme treatments more clearly from the control wines than those produced with the addition of the lower dosage rates of the enzymes, which tended to produce wines with greater purple and red colour, increased colour intensity and enhanced fruity, floral, spicy, vegetative, earthy and body characteristics. At the higher treatment levels, the trends in colour, appearance and aroma characteristics were similar to the lower enzyme treatments. However, in flavour, the wines were described as having generally enhanced acidity, bitterness and astringency characteristics.

In 1994 the Australian Wine Research Institute conducted a review into the performance of a range of commercial available pectic enzyme preparations with respect to effect on red must and wine colour (Leske, 1996). This investigation sought to assess the validity of the hypotheses that the use of pectic enzymes results in (i) greater colour extraction during red wine fermentation; (ii) faster colour extraction during maceration and fermentation of red grapes; (iii) greater colour extraction from red wines at pressing; and (iv) improved wine clarification.

All macerators and red colour extractors were used in all trials with red must, along with several selected clarifiers, in an attempt to determine any differences among the groups (Table 4). All the preparations were added at the suppliers' median specified rates. Trial 1, in which the effect during maceration only was assessed, evaluated the different preparations listed in Table 5 added to Cabernet Sauvignon must. Trial 2 (the fermentation trial) was performed using 11 of the 15 products listed.

TABLE 4
Details of the enzyme preparations used.

Company	Product	Concentration added	Classification
Concept Chemical Corp.	Succozym	0.03 mL/L	C
	Uvazym	15 mg/L	C
Lallemand Australia	Peclyve V	20 mg/L	C
	Peclyve VC	15 mg/L	R
	Peclyve VEP	20 mg/L	M
Solvay Biosciences	Clarex ML	0.0225 mL/L	M
	Clarex P150	7.5 mg/L	C
	Optivin	0.0075 mL/L	C
	Pectinase AT	0.0075 mL/L	C
Gist brocades	Rapidase ex Color	30 mg/L	R
	Rapidase Vinosuper	15 mg/L	C
Novo Nordisk	Pectinex Ultra SP-L	0.0221 mL/L	M
	Pectinex 3 XL	0.0375 mL/L	C
Chr. Hansen's Lab	Pectiflora V	0.015 mL/L	C
Bleakley Foods	Cytolase M219	0.25 mL/L	R
	Cytolase M102	0.214 mL/L	M
	Cytolase PCLS	0.03 mL/L	C
Enzymes Australia	Rohapect VRC	85 mg/L	R
	Rohapect DSL	0.017 mL/L	C
Quest International	Biocellulase W	0.086 mL/L	M
	Biopectinase plus	0.086 mL/L	C
	Bioredase	0.114 mL/L	R
	Biopectinase 200AL	0.114 mL/L	C

Note: C denotes clarifier; M: macerator; R: red colour extractor

TABLE 5
Details of the enzymes used in red must trials.

Product	Classification	Trial 1	Trial 2	Trial 3
Peclyve V	C	X	X	X
Peclyve VC	R	X	X	
Peclyve VEP	M	X	X	
Clarex ML	M	X		X
Clarex P150	C	X		
Rapidase ex color	R	X	X	
Pextinex Ultra SP-L	M	X		
Pectiflora V	C	X		
Cytolase M219	R	X	X	X
Cytolase M102	M	X	X	
Cytolase PCL5	C	X	X	
Rohapect VRC	R	X	X	
Biocellulase W	M	X	X	
Biopectinase plus	C	X	X	
Bioredase	R	X	X	X

The trials were conducted with 2 kg samples of must in screw-capped plastic vessels. The enzymes were added at the appropriate concentrations, the samples were mixed and the headspace of each vessel was flushed with carbon dioxide before sealing. For Trial 1, the samples were left to stand at 15°C for 24 hours before pressing. The fermentation trial samples were inoculated with 500 mg/L wine yeast strain Mauri AWRI 796 and 200 mg/L diammonium phosphate and fermented on the skins for seven days at 20°C, with twice-daily mixing by inversion of the temporarily sealed vessel. It was necessary to repeat Trial 2 (with smaller numbers of enzyme products, Table 2), filtering the samples through 0.8 mm filters before spectral analysis. The results of the enzyme-treated musts showed no significant increase in any of the measured parameters at any stage of processing when compared to that of the control samples. Leske (1996) concluded that the use of pectic enzyme preparations for improved rate and extent of colour extraction during maceration and fermentation of red musts is unnecessary on the basis of the above mentioned trials.

In stark contrast, totally different results were obtained in a study on the effect of enzymes during vinification on colour and sensory properties of port wines by Bakker *et al.* (1999). Two commercial pectolytic enzyme preparations, Vinozyme G and Lafase H.E., were used in an experiment carried out on a pilot scale (850 kg grapes/tank) to evaluate the effect on colour extraction during the short processing of crushed grape prior to fortification to make port wine. Results showed that both enzyme preparations enhanced colour extraction during vinification, although Vinozym G was more effective than Lafase H.E. Instrumental analysis of the young finished wines showed that the enzyme treatments gave darker wines (Bakker *et al.*, 1999). Maturation for 15 months led to a general reduction in colour for all wines, but differences in colour between the wines resulting from enzyme treatment were maintained. Sensory analysis after nine months maturation showed that Vinozym G treatment produced wines with significantly higher colour, aroma and flavour intensity scores than the control.

Development of pectolytic wine yeast strains

Pectinases produced by Saccharomyces cerevisiae: Pectic enzymes are mainly found in moulds and bacteria, but are also present in some yeasts. Significant pectolytic activity was found in *Saccharomyces fragilis* (*Kluyveromyces fragilis*) and *Candida tropicalis*, whereas *Saccharomyces thermantitonum*, *Torulopsis kefyr* and *Torulopsis lactosa* have weaker activity (Luh & Phaff, 1951). Pectinesterases were detected in *Debaryomyces membranaefaciens* var. *hollandius*, *Endomycopsis olmeri* var. *minor*, *Candida krusei*, *Hansenula*, *Rhodotorula* and *Zygopichia* (Bell & Etchells, 1956). Polygalacturonase activity was found in *Candida silvae*, *Candida norvegensis*, *Geotrichum candidum*, *Pichia guilliermondii*, *Pichia membranaefaciens*, *Torulopsis candida* and *Trichosporum cutaneum* (Call & Emeis, 1978; Sanchez *et al.*, 1984; Ravelomanana *et al.*, 1986). Furthermore, several *Saccharomyces* species were also reported to have polygalacturonase activity, including *S. carlsbergensis*, *S. chevalieri*, *S. cerevisiae*, *S. oviformis*, *S. uvarum* and *S. vini* (Kotomina and Pisarnitskii, 1974; Sanchez *et al.*, 1984). Bell & Etchells (1956) reported weak pectolytic activity for *S. cerevisiae*, whereas Luh

& Phaff (1951) reported that *S. cerevisiae* cultures tested had no noticeable effect on pectin.

It was later claimed that certain strains of *S. cerevisiae* have the ability to degrade polygalacturonic acid in the presence of glucose (McKay, 1990). Recently, a single culture of *S. cerevisiae* was isolated that supposedly produces pectinesterase, polygalacturonase and pectin lyase (Gainvors *et al.*, 1994). None of these enzymes have been purified nor their genes cloned. Blanco *et al.* (1994) reported that at least 75% of oenological strains tested showed limited pectolytic activity. Endopectate-degrading enzymes occurred primarily in the growth medium, as is the case for most other yeast species. Synthesis of pectic enzymes was reported to be constitutive, providing the glucose concentration in the medium did not exceed 2%. A higher concentration of glucose led to the total inhibition of these pectolytic activities. Interesting enough, the pectolytic activity was found to be significantly lower with growth on glucose as carbon source than with galactose.

Subsequently, Blanco *et al.* (1998) speculated that all *S. cerevisiae* strains contain a promoter-less polygalacturonase gene or else a non-functional one. This structural polygalacturonase-encoding gene (*PGU1*) from *S. cerevisiae* IM1-8b was eventually cloned and sequenced. The predicted protein comprises 361 amino acids, with a signal peptide between residues 1 and 18 and two potential glycosylation points in residues 318 and 330. The putative active site is a conserved histidine in position 222. This *S. cerevisiae* polygalacturonase shows 54% homology with the fungal polygalacturonases and only 24% homology with its plant and bacterial counterparts. *PGU1* is present in a single gene copy per haploid genome and it is detected in all strains, regardless of their phenotype. The expression of *PGU1* gene in several strains of *S. cerevisiae* revealed that the polygalacturonase activity depend on the plasmid used and also on the genetic background of each strain but in all cases the enzymatic activity increased.

Expression of pectinase-encoding genes in S. cerevisiae: The first heterologous pectinase genes expressed in *S. cerevisiae* were derived from the soft-rot causing plant pathogenic bacteria *Erwinia chrysanthemi* and *Erwinia carotovora*. To complement the limited pectolytic activity in *S. cerevisiae*, the pectate lyase (*pelE*) and polygalacturonase (*peh1*) genes from *E. chrysanthemi* and *E. carotovora* were inserted into different expression-secretion cassettes, comprising novel combinations of yeast and bacterial gene promoters, secretion signal sequences and gene terminators, and expressed in *S. cerevisiae* (Laing & Pretorius, 1992; 1993a). Transcription initiation signals present in the expression/secretion cassette were derived from the yeast alcohol dehydrogenase I ($ADH1_P$) and mating α-factor ($MF\alpha1_S$) gene promoters, and the *Bacillus amyloliquefaciens* α-amylase gene ($AMY1_P$), whereas the transcription termination signals were derived from the yeast tryptophan synthase gene terminator ($TRP5_T$). Secretion signals were derived from the yeast $MF\alpha1_S$, the *B. amyloliquefaciens* α-amylase, *E. chrysanthemi* pectate lyase and *E. carotovora* polygalacturonase leader sequences. The $ADH1_P$-$MF\alpha1_S$ expression-secretion system proved to be the most efficient control cassette for the expression of *pelE* and *peh1*, and the secretion of pectate lyase and polygalacturonase in *S. cerevisiae*. A pectinase cassette comprising $ADH1_P$-$MF\alpha1_S$-*pelE*-$TRP5_T$ (designated *PEL5*) and $ADH1_P$-$MF\alpha1_S$-*peh1*-$TRP5_T$ (designated *PEH1*) was constructed and expressed in *S. cerevisiae* (Laing & Pretorius, 1993b). The co-expression of *PEL5* and *PEH1* synergistically enhanced pectate degradation.

Subsequently, the *PEL5*, *PEH1* and *END1* (endo-β-1,4-glucanase) constructs were also co-expressed in wine and distillers' yeast strains of *S. cerevisiae* (Van Rensburg *et al.*, 1994). Carboxymethylcellulose and polypectate agarose assays revealed the production of biologically active pectate lyase, polygalacturonase and endo-β-1,4-glucanase, by the *S. cerevisiae* transformants. Interestingly, although the same expression-secretion cassette was used in all three constructs, time course assays indicated that the pectinases were secreted before the glucanase. It was suggested that the bulkiness of the *END1*-encoded protein and the five alternating repeats of Pro-Asp-Pro-Thr(Gln)-Pro-Val-Asp within the glucanase moiety could be involved in the delayed secretion of the glucanase.

In a similar, but independent, study a cDNA copy of the endo-polygalacturonase gene of *Aspergillus niger* was successfully expressed in *S. cerevisiae* under the control of the *ADH1* promoter (Lang & Looman 1995). Plasmid stability was significantly improved by the removal of most of the bacterial vector sequences resulting in no measurable effect on copy number. Expression was further increased by removal of single-base repeats at both termini of the gene. The natural secretion signal functioned well in the yeast. Exchanging the natural leader sequence with that of *MFα1* led to a reduction in the amount of secreted protein. The protein was correctly processed, even though the cleavage site for the *KEX2*-protease only partly fits the consensus sequence.

In another attempt to construct a pectolytic wine yeast strain, the *pelA* gene (cDNA) of *Fusarium solani* F. sp. *pisi* was fused to the *S. cerevisiae* actin gene promoter and this expression cassette was introduced into an industrial wine yeast strain (González-Candelas *et al.*, 1995). It was found that pectate lyase was secreted into the culture medium only during the stationary phase. Experiments proved that cell lysis could not account for the apparent activity. The delay could be attributed to protein folding problems due to the presence of 12 cysteine residues. However, the recombinant wine yeast was able to produce a wine with the same physico-chemical characteristics as that produced by the untransformed strain.

In contrast to the yeast pectinase gene cassettes, the pectin lyase gene (*pnlA*) of *Glomeralla cingulata* was initially poorly expressed in yeast under the control of the *GAL10* promoter (Templeton *et al.*, 1994). Expression was later improved after changing the sequence surrounding the start codon CACC*AUG*, which was poorly recognised in *S. cerevisiae*. The consensus sequence was changed to the more conventional CAAA*AUG*, contributing to a 6 to 10-fold increase in pectolytic activity in *S. cerevisiae*.

It is hoped that the development of pectolytic wine yeast strains will facilitate clarification of the must and wine during fermentation without adding expensive commercial enzyme preparations. Such wine yeast strains might also lead to colour and flavour enhancement.

THE IMPORTANCE OF GLUCANASES TO WINE CLARIFICATION AND PROCESSING

Structure of β-glucans

The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, glucans (the major component of cellulose), and to a lesser extent, hemicellulose (mainly xylans) (Pretorius, 2000). Cellulose and hemicellulose, together with lignin, are the major polymeric constituents of plant cell walls and form the largest reservoir of fixed carbon in nature. Cellulose is a condensation polyalcohol consisting of D-anhydroglucopyranose units linked by β-1,4-glycosidic bonds. It consists of a linear polymer of glucose units with each glucose residue rotated 180° with respect to its neighbours along the main axis of the chain (Fig. 2). The degree of polymerisation of cellulose ranges from 30 to 15 000 units (Coughlan, 1990). Sixty to seventy adjacent unipolar chains associate through interchain hydrogen bonding and van der Waals interactions to form ordered crystalline microfibrils that aggregate to form insoluble fibres (Pretorius, 1997). Whereas cellulose is a homopolymer, hemicellulose is a heteropolysaccharide that is closely associated with cellulose in plant material. The predominant hemicellulose, β-1,4-xylan, has a high degree of polymerisation and is highly branched (Thomson, 1993). The common substituents found on the β-1,4-linked D-xylopyranosyl residues are acetyl, arabinosyl and glucanosyl residues (Fig. 3).

Of all polysaccharides, the β-glucans produced by *B. cinerea* in botrytised grape juice can be regarded as the strongest influence on the clarification and stabilisation of must and wine. Generally, β-glucans consist of short stretches of β-1,4 linked glucose moieties, interrupted by single β-1,3 linkages. By contrast, the high molecular weight β-1,3-1,6-glucan secreted by the *B. cinerea* consists of a β-D-1,3-linked backbone with very short β-D-1,6-linked side chains (Dubourdieu *et al.*, 1981; Villettaz *et al.*, 1984). This glucose polymer is released into the grape juice and later found in the wine.

Glucan prevents the natural sedimentation of cloud particles in the grape must and causes filter stoppages. This negative effect can be overcome by using fining agents such as bentonite, or by centrifugation. Such treatment will force the sedimentation of the cloud but will not remove the glucan, and filtration problems remain. Alcohol induces polymerisation of the glucan molecules, thus more severe problems occur at the end of alcohol fermentation.

Enzymatic hydrolysis of glucans

Cellulases are multicomponent complexes, often consisting of endoglucanases, exoglucanases and cellobiases, that act in a stepwise and synergistic process to achieve efficient degradation of cellulose (Fig. 2). The major end product of concerted endoglucanase and exoglucanase activity is cellobiose, that is then hydrolysed to glucose by cellobiases (a member of the β-glucosi-

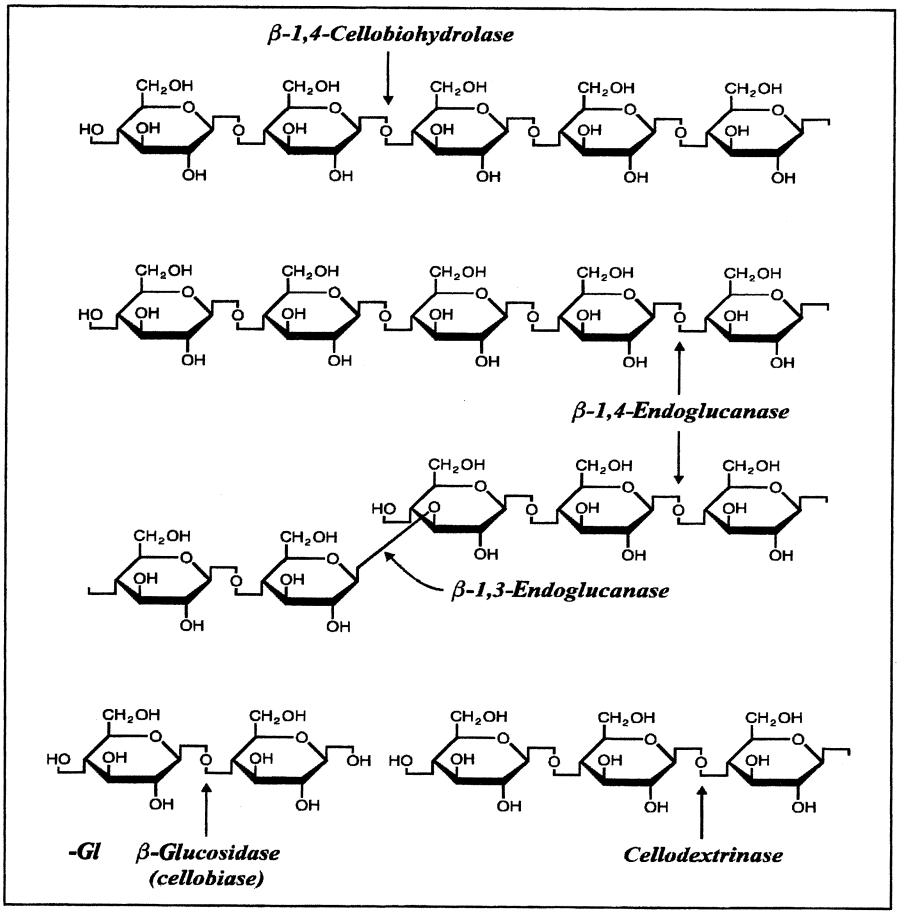

FIGURE 2

Schematic representation of the enzymatic degradation of glucan and cellulose.

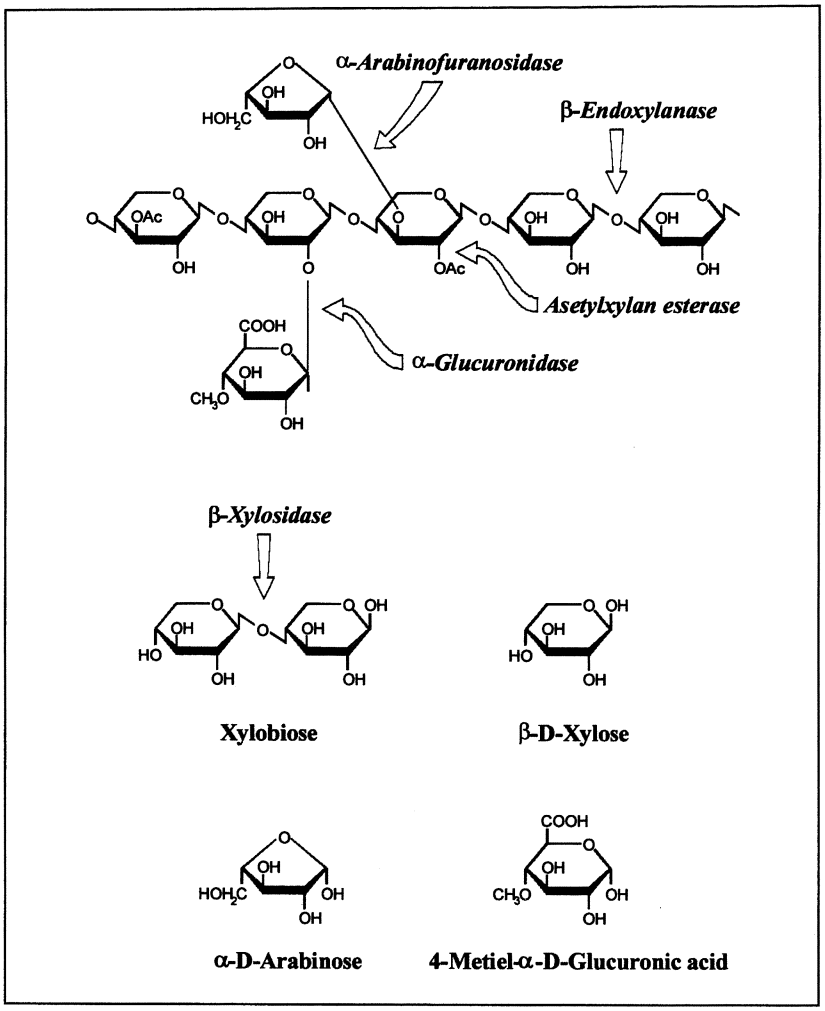

FIGURE 3
Schematic representation of the enzymatic degradation of hemicellulose.

dases) (Coughlan, 1990; Pretorius, 1997). Likewise, hemicellulases which specifically degrade the backbone of hemicellulose, include β-D-galactanases, β-D-mannases and β-D-xylanases (Fig. 3). Endoxylanases (EC 3.2.1.8) are often prevented from cleaving the xylan backbone by the presence of substituents (Thomson, 1993; Pretorius, 1997). Therefore, in many cases, these must be removed before extensive degradation of the backbone can occur. The enzymes involved include acetylesterases (EC 3.1.1.6), α-L-arabinofuranosidases (EC 3.2.1.55) and α-glucuronidases (EC 3.2.1). Once endoxylans have released small xylooligosaccharides, the β-xylosidases (EC 3.2.1.37) cleave the oligomeric fragments to mainly xylose. The activity of xylans and β-xylosidases also depends on the chain length of xylooligosaccharides, the former generally decreasing with decreasing lengths, and the latter increasing (Thomson, 1993).

Owing to the pivotal role they play in the clarification of grape must and wine, glucan-degrading cellulases will be the main focus of this section. β-Glucanases, classified as endo- and exoglucanases hydrolyse the β-*O*-glycosidic linkages of β-glucan chains, leading to the release of glucose and oligosaccharides (Nebreda *et al.*, 1986). These enzymes are important not only to remove haze-forming glucans from wine; they also play an important role in the release of mannoproteins during aging on yeast lees.

Endoglucanases: Endoglucanases attack the glucan (cellulose) chain randomly and split β-1,4-glucosidic linkages. According to Finch & Roberts (1985), endoglucanases (β-1,4-D-glucan glucano hydrolase, EC 3.2.1.4) have the following general characteristics: (i) they commonly occur in multiple forms with different molecular weights, carbohydrate contents, thermostabilities and modes of attack; (ii) they display acidic pH optima; (iii) purified endoglucanases generally show little activity towards native cellulose; (iv) many endoglucanases display transferase activity towards cellodextrins; and (v) the turnover numbers are comparable to those of amylases for starch.

Exoglucanases: Exoglucanases release cellobiose (two glucose units) from the non-reducing end of glucan and cellulose (Bisaria & Mishra, 1989). Exoglucanases (β-1,4-D-glucan cellobiohydrolases, EC 3.2.1.91) show preference for low molecular weight cellulolytic substrates and, while not involved in the primary attack on cellulose, can catalyse further degradation of oligosaccharides. Most exoglucanases are glycoproteins and exist as single polypeptides, with a remarkably narrow range of molecular weights (Coughlan, 1985). As is the case with the other components of cellulolytic systems, these enzymes are acidic and are

most active and most stable under these conditions. Endoglucanases have a broader substrate specificity than cellobiohydrolases, because they can accommodate the bulky side chains of the substrate (Penttilä *et al.*, 1986).

***Cellobiases*:** Cellobiases (β-1,4-D-glucoside glucohydrolase, EC 3.2.1.21) are substrate (cellobiose) specific exoglucanases (Finch & Roberts, 1985) and belong to a diverse family of enzymes (β-glucosidases) capable of hydrolysing a broad spectrum of β-glucosides (Wright *et al.*, 1992). Each of these enzyme classes consists of a number of isoenzymes and they act in a synergistic manner to degrade glucans.

Industrial glucanase preparations

Sources and characteristics of commercial glucanase preparations: The commercial β-glucanase preparations authorised for use in winemaking are produced by species of the *Trichoderma* (e.g., *T. harzianum*). One β-glucanase unit (BGXU) corresponds to the quantity of enzyme required to produce 1 mMol of reducing sugars per minute using *Botrytis* glucan and incubating at 30ºC for 10 minutes (Canal-Llaubères, 1998). Commercialised glucanases are normally active between 15-50ºC at pH 3 to 4. An alcohol concentration of up to 14% (v/v) has no negative effect on these enzyme preparations. The level of SO_2 has no negative effect on the enzyme up to 350 ppm.

Effects of glucanase additions on wine processing and quality: Glucanex (Novo Nordisk) was one of the first commercial glucanase preparations to be tested on wines made from botrytised grapes (Villettaz *et al.*, 1984). Glucanex mainly contains an exo-β-glucanase, an endo-β-1,3-glucanase, an exo-β-1,6-glucanase and an unspecific β-glucosidase activity. Except for the improvement of filtration, this enzyme treatment did not induce any major changes in the chemical composition of the wine. The enzyme-treated sample showed a higher residual sugar level than the control wine, but part of this difference was due to the enzymatic hydrolysis of the *Botrytis* glucan to glucose (about 50 mg/L). No significant organoleptic differences could be noted between the treated and untreated samples.

In a later study glucanases were added to Traminer must after skin contact (Miklósy & Pölös, 1995). Three commercial enzyme preparations, Glucanex (Novo Nordisk), Novoferm 12L (Novo Nordisk) and Trenolin Buckett (Erbslöh), were used. The sensory analysis of these glucanase-treated wines took place after six months. Wine treated with Trenolin Buckett was considered by more than 85% of a tasting panel to have a more desirable aroma, fruity taste and improved overall quality, than the control one. The differences were not as strong in wines treated with the other commercial enzymes. More than 70% of the wine tasters found the overall quality and flavour of the Novoferm 12L treated wines superior to the control, and in the case of Glucanex, the preference was only more than 60%.

Today, commercial β-glucanases are widely available for clarification, filtration and aging of young wines (Canal-Llaubères, 1998).

Development of glucanolytic wine yeast strains

The glucanase multigene family of S. cerevisiae: Many yeasts secrete endo- and exoglucanases to the cell surface (Chambers *et al.*, 1993; Table 6). *S. cerevisiae* synthesises several enzymic

TABLE 6
Properties of β-glucanase systems of several yeast species (adapted from Nombela *et al.*, 1988).

Yeast	Type	Substrates	Mol. Wt	Glycoprotein
Schizosaccharomyces versatilis	Endo-β-1,3-glucanase	L, OL	97 000	
	Exo-β-1,3-1,6-glucanase	L, P, PNPG	43 000	ND
Schizosaccharomyces pombe	Endo-β-1,3-glucanase (I)	L, OL	160 500	ND
	Endo-β-1,3-glucanase (II)	L, OL	75 000	-
Candida utilis	Endo-β-1,3-glucanase	L, PNPG	20 000	+
	Exo-β-1,3-1,6-glucanase	L, P, PNPG	20 000	+
	Endo-β-1,3-glucanase	L, OL	21 000	+
Candida albicans	Endo-β-1,3-glucanase	L, OL, P	49 000	+
	Exo-β-1,3-glucanase	L	107 000	ND
Kluyveromyces phaseolosporus	Endo-β-1,3-glucanase (I)	L, OL	180 000	+
	Endo-β-1,3-glucanase (II)	L, OL	45 000	ND
	Exo-β-1,3-1,6-glucanase (III)	L, P	18 500	+
	Exo-β-1,3-1,6-glucanase (IV)	L, OL, P	8 700	+
Pichia polymorpha	Endo-β-1,3-glucanase (I)	L, OL	47 000	+
	Exo-β-1,3-1,6-glucanase (II)	L, OL, P, PNPG	40 000	+
	Exo-β-1,3-glucanase (III)	L, PNPG	30 000	+

L, Laminarin; OL, Oxidized laminarin; P, Pustulan; PNPG, p-nitro-phenyl-β-D-glucoside; ND, not determined

forms with β-1,3-glucanase activity. The following β-1,3-glucanase genes from *S. cerevisiae* were cloned and characterised: *EXG1*, *EXG2*, *BGL1*, *BGL2*, *SSG1* and *SPR1* (Nebreda *et al.*, 1986; Kuranda & Robbins, 1987; Klebl & Tanner, 1989; Muthukumar *et al.*, 1993; San Segundo *et al.*, 1993). When their restriction maps, nucleotide sequences and chromosomal map positions were compared, it became evident that *EXG1* is identical to *BGL1*, and *SSG1* is identical to *SPR1*.

The *EXG1* (*BGL1*) gene codes for two main differentially glycosylated extracellular exo-β-1,3-glucanases present in the culture medium, whereas a related gene, *EXG2*, encodes a minor exo-β-1,3-glucanase (Farkas *et al.*, 1973; Nebreda *et al.*, 1986; Larriba, 1993). The *BGL2* gene encodes a cell-wall-bound endo-β-1,3-glucanase, while *SSG1* (*SPR1*) encodes a sporulation-specific exo-β-1,3-glucanase.

Expression of glucanase-encoding genes in S. cerevisiae: Since the endogenous glucanolytic activities of wine yeast strains are not sufficient to avoid clarification and filtration problems, several cellulases have been produced by *S. cerevisiae* as heterologous proteins (Table 7). These glucanase genes were obtained from bacteria, yeasts and moulds.

The first heterologous glucanase genes to be introduced in yeast were cloned from the soil bacterium *Bacillus subtilis*. The *B. subtilis* gene (*beg1*), encoding endo-β-D-1,3-1,4-glucanase, was expressed in *S. cerevisiae* under the control of its own promoter and signal sequences (Hinchliffe and Box, 1984; Hinchliffe, 1985). In this case, the β-glucanase activity was low and could be detected only in crude cell-extracts. The fact that no extracellular endo-β-D-1,3-1,4-glucanase activity could be detected in cultures of *S. cerevisiae* may be indicative of the inability of yeast to process the bacterial protein so as to promote secretion. Higher intracellular levels of β-glucanase were achieved by Cantwell *et al.* (1985) by placing the endo-β-D-1,3-1,4-glucanase gene from *B. subtilis* under the control of the *ADH1* promoter on a high copy number 2μm-based plasmid vector. However, when we fused this *B. subtilis* endo-β-D-1,3-1,4-glucanase gene to the $ADH1_P$-$MF\alpha1_S$ expression-secretion cassette (designated *BEG1*) in a single-copy centromeric plasmid, we could still not detect any extracellular enzyme activity in the culture fluids of yeast transformants (unpublished data). By contrast, when the *beg1* gene was inserted into the $ADH2_P$-$MF\alpha1_S$ expression-secretion cassette ($ADH2_P$-$MF\alpha1_S$-*beg1*-$ADH2_T$, designated *BEG1*) in a multi-copy number plasmid, high levels of β-glucanase activity were secreted by yeast transformants (Van Rensburg *et al.*, 1996).

In another attempt to develop a glucanolytic *S. cerevisiae* strain, an endo-β-D-1,4-glucanase gene (*CenA*; carboxymethylcellulase or CM-cellulase) was cloned from the cellulolytic bacterium *Cellulomonas fimi* and expressed in *S. cerevisiae* (Skipper *et al.*, 1985). Secretion of active endo-β-1,4-glucanase by the recombinant yeast cells was greatly increased when the leader sequence of a secreted yeast protein, the K_1 killer toxin, was inserted in-frame immediately upstream of the bacterial cellulase sequence. Likewise, Curry *et al.* (1988) expressed the *C. fimi* exoglucanase-encoding gene (*cex*) in *S. cerevisiae* by using the *MEL1* promoter and the α-galactosidase signal peptide. Subsequently, Wong *et al.* (1988) co-expressed the endoglucanase- and exoglucanase-encoding genes from *C. fimi* in *S. cerevisiae*. The cellulase mixture secreted by the *S. cerevisiae* transformants was able to hydrolyse filter paper and pretreated aspen wood chips in a reaction stimulated by β-glucosidase.

Encouraged by these results, Nakajima *et al.* (1993) fused a DNA segment encoding a signal peptide from yeast invertase (*SUC2*) in-frame to the *Bacillus circulans* β-1,3-glucanase gene (*bglH*). This construct was expressed in *S. cerevisiae* under the control of the yeast galactokinase gene (*GAL1*) promoter (Nakajima *et al.*, 1993). However, due to the eroding of the yeast cell wall, the bacterial β-1,3-glucanase inhibited the growth of the *S. cerevisiae* transformants. There was also a decrease in cell size and expansion of vacuoles during the expression of the *bglH* gene. However, it was shown that this toxic effect could be reduced by culturing the yeast transformants at low temperatures (16°C). Demolder *et al.* (1993) also used the *GAL1* promoter and *MFα1* prepro-sequence to express the *Nicotiana plumbaginifolia* β-1,3-glucanase in *S. cerevisiae*. The expressed β-1,3-glucanase was also found to be toxic to the yeast cells as reflected by strong growth inhibition. This glucanase could only be detected inside the cells. The glucanase interfered with the cell wall structure from within the cell; after induction of glucanase the recombinant yeast lost up to 20% of some periplasmic enzymes.

Several cellulase genes (*egl1*, *egl3*, *cbh1* and *cbh2*) from the fungus *Trichoderma reesei* were also expressed in yeast (Knowles *et al.*, 1985; Lehtovaara *et al.*, 1986; Van Arsdell *et al.*, 1987; Penttilä *et al.*, 1988; Bailey *et al.*, 1993). Penttilä *et al.* (1987b) transformed two brewer's strains with a recombinant plasmid containing a *T. reesei* endo-β-1,4-glucanase gene by using the marker gene for copper resistance, *CUP1*. During primary fermentation, the β-glucans of the wort were almost completely removed and the filterability of the beer was also significantly improved. When Penttilä *et al.* (1987a) expressed the two endo-β-1,4-glucanase genes, *egl1* and *egl3*, in *S. cerevisiae* under the control of the yeast phosphoglycerate kinase (*PGK1*) gene promoter, neither enzyme affected the growth rate of the yeast strains. However, both endoglucanases clearly affected the morphology and size of the yeast cells. Subsequently, Penttilä *et al.* (1988) also expressed two cellobiohydrolases (*cbh1* and *cbh2*) from *T. reesei* in *S. cerevisiae*. Both enzymes were efficiently secreted into the culture medium when the *PGK1* promoter was used and the *T. reesei* signal sequences were maintained. Although the production levels of both cellulases by the yeast transformants were low compared to the production by *T. reesei*, the concentrated and purified enzymes were active against their natural substrates (Bailey *et al.*, 1993). Despite overglycosylation of these yeast-derived cellulases compared to native *T. reesei* enzymes (Penttilä *et al.*, 1987a, 1988), the specific activity of the yeast-made endo-β-1,4-glucanase was not markedly altered in comparison with that of the native enzyme (Zurbriggen *et al.*, 1991). By contrast, a decrease in the specific activity of yeast-made *T. reesei* celbiohydrolase II was observed compared to the native enzyme (Pentillä *et al.*, 1988). Strong exo-endo- and exo-exo-synergism has also been reported between the *Trichoderma* cellulases (Bailey *et al.*, 1993).

A cellulase from a different fungus, *Aspergillus aculeatus*, was also expressed in *S. cerevisiae* (Ooi *et al.*, 1994). The cDNA for FI-carboxymethyl cellulase was combined with the yeast *GAP*

to proteolysis. Their resistance is not due to protection by other components in wine nor is it due to covalently bound sugars (glycosylation) or associated phenolic compounds. It appears that protein conformation bestows stability to these PR-proteins and that appropriate viticultural practices, rather than post-harvest processing, may hold the key to controlling the concentrations of protein in wine.

THE IMPORTANCE OF GLYCOSIDASES TO WINE AROMA AND FLAVOUR

Terpenoid-derived aromas and flavours in wine

The varietal flavour of grapes is determined mainly by the accumulation and profile of volatile secondary metabolites. Terpenols play an important role in the determination of flavour and aroma of grapes and wines. This is especially applicable to wines of muscat varieties, but it also holds true with related cultivars and other non-muscat varieties (Marais, 1983; Rapp & Mandary, 1986). These terpenols can be found in grapes as free, volatile and odorous molecules, as well as in flavourless, non-volatile glycosidic complexes (Table 8). These complexes most often occur as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides and 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides of mainly geraniol, nerol and linalool (Günata *et al.*, 1988). The precursors are, however, hydrolysed slightly during the fermentation process. Essentially, the hydrolysis process functions in two steps. First, depending on the precursors, the glycosidic linkages are cleaved by either an α-L-arabinofuranosidase, α-L-rhamnosidase or a β-D-apiosidase. The second step involves the liberation of the monoterpenols by β-glucosidase (Sánchez-Torres *et al.*, 1996).

Industrial β-glucosidase preparations

***Limitations of endogenous glucosidases*:** β-Glycosidases occurs naturally in a wide variety of plants, fungi and yeasts. Endogenous glycosidases, however, show little or no activity towards grape terpenyl-glycosides in the must and wine. Grape glycosidase activities are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine. Therefore, these *V. vinefera* enzymes would be virtually inactive during winemaking. A further constraint to the effectiveness of these endogenous glycosidases stems from their aglycone specificities. These enzymes were all found to be incapable of hydrolysing sugar conjugates of tertiary alcohols. Thus, glycosides of some of the most flavour-important monoterpenes (e.g., linalool) are unaffected by these glycosidases, even under ideal conditions. Furthermore, Grossmann *et al.* (1990) showed that certain processing steps (e.g., clarification) considerably reduced β-glucosidase activity.

***Characteristics, disadvantages and advantages of commercial glycosidase preparations*:** Commercial enzymes are typically crude fungal preparations, containing impurities such as extraneous enzymes, proteins, mucilage and melanoidins (Martino *et al.*, 1994). Some of these activities can adversely affect the colour of wine, while selected β-glucosidases liberate bound terpenols from terpenyl-glycosides, thereby enhancing varietal flavours.

Anthocyanin-destroying β-glucosidase activity: Some β-glucosidase activities can cleave the sugar from anthocyanin, leaving the unstable aglycon that spontaneously transforms into a colourless form (Huang, 1956). Evidence of anthocyanin-destroying β-glucosidase activity and β-1,2-glucosidase activity in commercial preparations has been reported in the production of fruit juices such as raspberry and strawberry juices (Blom & Thomassen, 1985; Jiang *et al.*, 1990). Therefore, Wightman *et al.* (1997) investigated whether the same effects could be observed on grape and wine pigments. In this study four commercial enzyme preparations were tested in Pinot noir and Cabernet Sauvignon wines. The following enzymes were evaluated at their highest recommended dosages (w/v) for wine: AR2000 (0.005%); Cytolase PCL5 (0.005%); Rapidase EX Color (0.005%; Gist-brocades) and Rohapect VR Super L (0.01%; Rohm). The enzyme treatments appeared to have similar effects on both Pinot noir and Cabernet Sauvignon wines. Two enzyme preparations, AR2000 and Cytolase PCL5, had pronounced effects on wine colour. Both caused significant destruction of total monomeric anthocyanin as well as individual pigments. The presence of acylating groups on malvidin-3-glucoside did not appear to inhibit this enzymatic effect. Preparations that caused the most anthocyanin degradation also produced wines with higher amounts of polymeric anthocyanin. Increasing the enzyme concentration magnified these effects. The enzyme preparations had a marked effect on the other phenolics as well. This study showed that the use of enzyme preparations must be closely examined, since they may alter the wine composition.

Enhanced liberation of grape terpenoids: With the elucidation of the enzymatic mechanisms of hydrolysis of terpenyl-glycosides, several laboratories across the world searched for fungal enzymes capable of enhancing wine aroma (Günata *et al.*, 1988; Grossmann & Rapp, 1988; Cordonnier *et al.*, 1989). Table 9 lists some enzymes for aroma extraction.

Trials conducted with an experimental β-glucosidase preparation from *Aspergillus* indicated that it could indeed reinforce the varietal aroma and bouquet of certain wines if used in fermented juice as soon as the glucose has been depleted by the yeast cells

TABLE 8

Properties of monoterpenoids - aroma and sensory threshold data in water (adapted from King & Dickinson, 2000).

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

TABLE 9
Enzymes for aroma extraction.

Enzyme	Company	Activities	Time of addition
Expression[20]	Darleon	Pectinase + β-glucosidase	Add to fermenting wines with residual sugar below 10g/L
Endozym Cultivar	AEB Africa	Pectinase	To grapes or must
Endozym Rouge	AEB Africa	Pectinase + Hemicellulase +cellulase	During red grapes maceration before SO_2
Vinozym	Novo Nordisk	Pectinase + side	Directly into crusher
Novoferm 12	Novo Nordisk	Pectinase + side	Towards the end of alcoholic fermentation
Trenolin Bukett	Erbslöh	Pectinase + β-glycosidase	Towards the end of alcoholic fermentation
Rapidase X-Press	Gist-brocades/Anchor	Pectinase	To grapes or mash
Rapidase AR2000	Gist-brocades/Anchor	Pectinase + side	Add at the end of alcoholic fermentation

(Canal-Llaubères, 1993).

Park (1996) investigated the changes in free and glycosidically bound monoterpenes as a function of fermentation, wine aging and enzyme treatment. Muscat of Alexandria and Gewürztraminer grapes were used in this study. About 0.33 g of crude pectinase, Rohapect 7104 (Röhm, Darmstadt, Germany), containing β-glucosidase activity was directly added to one liter of wine; the enzyme preparation was not added until the wines were aged for 8 months. The enzyme-treated wines were stored immediately in a cellar (9.5°C) for about 14 months. The results indicated that the Rohapect 7104 enzyme preparation could effectively be used in hydrolysing bound monoterpenes. However, according to Park (1996), several factors should be considered in using enzymes. In normal wines, the bound monoterpenes are relatively stable and are hydrolysed slowly, therefore they release the floral aroma over a long period of aging. Conversely, the enzyme-treated wines released all of the desirable monoterpenes at once, so there was nothing left to maintain a constant level of terpenic-floral aroma in the wine. In addition, freed monoterpenes such as linalool, nerol and geraniol, which are generally considered desirable floral aromas in wine, can be interconverted to other, more chemically stable but less desirable compounds, such as α-terpineol. This interconversion occurs because the major freed monoterpenes are relatively unstable and the low pH of wine promotes the hydrolysis, rearrangement or oxidation of these compounds. Park (1996) speculated that bound monoterpenes may be beneficial because they provide a constant aroma through "naturally controlled release" of monoterpenes. He also suggested it is important that the commercial preparations never contain cinnamate decarboxylase, as this enzyme can lead to the formation of ethyl-phenols with a very disagreeable animal odour.

Development of wine yeast strains with aroma-liberating glycosidases

β-Glucosidases produced by yeasts: In contrast to the grape glycosidase, yeast glucosidases are not inhibited by glucose. It was reported that although the β-glucosidase from *Hansenula* sp., isolated from fermenting must, was capable of liberating aroma substances in wine, it was less effective in must (Grossmann *et al.*, 1987). An intracellular β-glucosidase from *Debaryomyces hansenii* Y-44 was purified and used in the fermentation of Muscat juices (Yanai & Sato, 1999). A wine with a considerable increase in the concentration of monoterpenols was produced. Especially the linalool and nerol contents increased by 90 and 116%, respectively. According to some reports, certain strains of *S. cerevisiae* also possess a β-glucosidase that is located in the periplasmic space of yeast cells (Darriet *et al.*, 1988; Dubourdieu *et al.*, 1988). This activity appears to be very limited and therefore recent studies have rather focused on non-*Saccharomyces* yeasts such as *Hanseniaspora vinea* (Vasserot *et al.*, 1989) and *Candida* species (Günata *et al.*, 1990). Results obtained so far from studies on yeast glycosidases indeed suggest that specific yeast strains can affect the varietal aroma of wines (Laffort *et al.*, 1989).

Expression of β-glucosidase and α-L-arabinofuranosidase: The addition of exogenous aroma-liberating enzyme preparations to wine is an expensive practice, and is viewed by many purists as an "artificial" or "unnatural" intervention by the winemaker. This has led to renewed interest in the expression of heterologous enzymes in wine yeasts.

When the β-1,4-glucanase gene from *Trichoderma longibratum* was expressed in wine yeast the aroma intensity of wine was shown to have increased, presumably due to the hydrolysis of

lycosylated flavour precursors (Pérez-González *et al.*, 1993). ikewise, we have expressed the β-glucosidase gene of *S. fibugera*, the α-L-arabinofuranosidase (*ABF2*) of *A. niger* and a gluanase gene cassette consisting of several glucanase genes 3*EG1*, *END1* and *EXG1*) (Pretorius, 2000). Trials are underway) determine the effects of these recombinant yeasts on the varital character of wine.

'HE IMPORTANCE OF ACIDIC UREASES TO THE WHOLEOMENESS OF WINE

'he demand for a reduction in ethyl carbamate levels of wine

thyl carbamate (also known as urethane) is a suspected carcinoen that occurs in most fermented foods and beverages. Given the otential health hazard, there is a growing demand from conımers and liquor control authorities to reduce the allowable limıs of ethyl carbamate in wines and related products. Although oung wines do not contain measurable levels (< 10 µg/L) of thyl carbamate, the required precursors are present which can enerate a considerable amount of this mutagenic compound 'hen wine is aged or stored at elevated temperatures (Ough *et al.*, 988b). High-alcohol beverages such as sherries, dessert wines ıd distilled products also tend to contain much higher levels of thyl carbamate than table wine. It is believed that ethyl carbaıate forms in aging wines, fortified wines and brandies by reacon between urea and ethanol (Ough *et al.*, 1988a). For this reaon, excessive application of urea-containing fertilisers to vines ıd spraying of urea shortly before harvest to remove leaves are ot recommended. Furthermore, the use of urea-containing nutriıt supplements for yeast during wine fermentations to avoid uck or sluggish fermentations is also prohibited. Apart from ıese factors that could lead to high urea levels and concomitant ansgression of ethyl carbamate limits, *S. cerevisiae* strains also ary widely with regard to their urea-forming ability (Ough *et al.*, 991).

'he formation of urea and ethyl carbamate during yeast ferıentation

ı *S. cerevisiae* urea is formed during the breakdown of arginine, ne of the main amino acids in grape juice, by the *CAR1*-encodd arginase. During this reaction, arginine is converted to rnithine, ammonia and carbon dioxide, while urea is formed as ı intermediate product. Certain yeast strains secrete urea into ine and, depending on fermentation conditions, may be unable) further metabolise the external urea. Although all *S. cerevisiae* rains secrete urea, the extent to which they re-absorb the urea iffers (An & Ough, 1993). *S. cerevisiae* secretes more urea at igher fermentation temperatures, whereas high ammonia conentrations suppress the re-absorption of urea by the yeast. It is ıerefore important to inoculate grape must with a low-urea proucing wine yeast strain when the juice has a high arginine con-ıt.

'evelopment of a urease-producing wine yeast strain

train selection is only one way of reducing the accumulation of rea in wine. As an alternative means of curbing ethyl carbamate ɔrmation in the end product, successive disruption of the *CAR1* ginase gene in an industrial saké yeast proved to be successful ı eliminating urea accumulation in rice wine (Kitamoto *et al.*, 991). This arginase deletion mutation resulted in a yeast strain ıat could not metabolise arginine, but it also impeded growth, thereby limiting the commercial use of such a strain.

Another possibility is adding commercial preparations of acidic urease, enabling the hydrolysis of urea in wine (Ough & Trioli, 1988). This practice has recently been approved by the OIV and is used in some wine-producing countries to lower ethyl carbamate levels in their wines and related products. A less expensive route to lower levels of ethyl carbamate would be to develop a wine yeast that produces an extracellular, acidic urease. In one such attempt a novel urease gene was constructed by fusing the α, β and γ subunits of the *Lactobacillus fermentum* urease operon (Visser, 1999). In addition, jack bean urease linker sequences were inserted between the α and β, as well as the β and γ subunits. Both gene constructs were successfully expressed under the control of the *S. cerevisiae PGK1* promoter and terminator signals in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*. Although the level of transcription in *S. cerevisiae* was much higher than in *S. pombe*, the secretion of urease peptides was extremely low (Visser, 1999). Unlike the *S. pombe* urease, the *S. cerevisiae*-derived urease was unable to convert urea into ammonia and carbon dioxide. The absence of recombinant urease activity in transformed *S. cerevisiae* cells is probably due to the lack of the essential auxiliary proteins present only in urease-producing species such as *S. pombe*. Without these proteins, *S. cerevisiae* is unable to assemble the various subunits into an active urease. It seems, therefore, that accessory genes of *L. fermentum* will also have to be cloned and expressed in addition to the structural urease genes to enable *S. cerevisiae* to express an active urease.

THE IMPORTANCE OF GLUCOSE OXIDASE TO THE PRODUCTION OF LOW-ALCOHOL WINE

The quest for low-alcohol wines

There is a decreasing trend worldwide in the consumption of alcohol, stimulating research into the production of low-alcohol wines. Low-alcohol wines can be produced by various physical treatments involving expensive equipment such as reverse osmosis (Canal-Llaubères, 1993). Current physical ethanol-removing processes non-specifically alter the sensorial properties of the final product (Villettaz, 1986; Pretorius, 2000). Proposed alternatives include redirecting more of the grape sugars to glycerol at the expense of ethanol during fermentation, or the use of enzymes such as glucose oxidase (GOX) and catalase. Treatment of grape juice with glucose oxidase could convert glucose into gluconic acid which is not metabolisable by wine yeast. Wines produced in such a way should have reduced levels of ethanol and higher acidity. Furthermore, this technology could also be employed to produce a reserve of acidic musts or wines for blending purposes (Canal-Llaubères, 1993).

The application of glucose oxidase in the production of low-alcohol wines

Pickering *et al.* (1999a) conducted experiments on the production of reduced-alcohol wine by using glucose oxidase treated juice. In this study grape juice was converted to gluconic acid by glucose oxidase, and conversion efficiencies of 87 and 74% were obtained for Müller-Thurgau and Riesling juices, respectively. The alcoholic fermentation of these GOX-treated juices proceeded without incident, and final ethanol concentrations of 6.3 to 6.5% (v/v) were obtained, corresponding to 36 to 40% reduction

in alcohol compared to the control wines. At bottling the pH of GOX wines were very similar to those of control wines, although titratable acidity remained significantly higher.

The physical and chemical stability of the wine was also investigated by Pickering *et al.* (1999b). The GOX-treated wines showed increased SO_2-binding power compared to control wines, above that which can be explained by the juice aeration process required for enzymatic activity. The GOX-treated wines had a more golden colour, but were more stable against browning after six months of bottle aging, whereas control wines continued to brown throughout the two-year period of monitored aging. Although, GOX significantly modified the taste, the aroma and mouthfeel characteristics were relatively unaffected (Pickering *et al.*, 1999c), except for aromas such as "lime", "apple" and "fruity/floral," which are generally less intense due to the juice aeration required for the enzyme.

These initial results are very encouraging, but it is clear that further research is necessary to gain more knowledge into the possible application of glucose oxidase in the production of low-alcohol wines. Once this concept has been researched to the stage of implementation, the possibility exists to develop wine yeast starter cultures that can produce glucose oxidase during fermentation, simplifying the production of low-alcohol wines.

CONCLUSIONS AND PROSPECTS

The ancient process of winemaking is completely dependent on the activity of several enzymes. The best wines are produced when the desired enzymatic activities are optimally reinforced and the negative effects restricted to a minimal level. Over the last two decades commercial enzyme preparations have gained enormous popularity in the wine industry to supplement and/or complement the endogenous enzymes derived from grapes and microbes present in the must and wine. They are effective, specific and convenient to use, and it can be expected that the search for enzymes with improved characteristics will continue. To comply with the ever-increasing demands of modern winemakers and consumers for the best quality wine at every price point, it is also inevitable that novel enzymes will be designed for specific purposes and then tailored through protein engineering technologies. These "designer" enzymes will then be added to the must and wine, or their genes introduced into specialist wine yeast starter culture strains. There is tremendous potential benefit to the wine producer and consumer alike in the application of these exciting new technologies and developments; however, that benefit will be realised only if the application is judicious and done with high regard for the unique nature of the product.

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