Enzymes for Wine Fermentation: Current and Perspective Applications

Harald Claus [1],* and Kiro Mojsov [2]

[1] Institute of Molecular Physiology, Microbiology and Wine Research, Johannes Gutenberg-University, 55099 Mainz, Germany

[2] Faculty of Technology, University "Goce Delčev" Štip, Krste Misirkov No. 10-A, P.O. Box 201, 2000 Štip, Macedonia; kiro.mojsov@ugd.edu.mk

* Correspondence: hclaus@uni-mainz.de

Abstract: Enzymes are used in modern wine technology for various biotransformation reactions from prefermentation through fermentation, post-fermentation and wine aging. Industrial enzymes offer quantitative benefits (increased juice yields), qualitative benefits (improved color extraction and flavor enhancement) and processing advantages (shorter maceration, settling and filtration time). This study gives an overview about key enzymes used in winemaking and the effects of commercial enzyme preparations on process engineering and the quality of the final product. In addition, we highlight on the presence and perspectives of beneficial enzymes in wine-related yeasts and lactic acid bacteria.

Keywords: wine clarification; extraction; pectinase; glycosidase; protease; phenoloxidase; color; aroma; non-*Saccharomyces* yeasts

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1. Introduction

Over the last decades, commercial enzyme preparations have gained increasing popularity in the wine industry [1–5]. They offer many advantages such as accelerated settling and clarification processes, increased juice yield, and improved color extraction (Table 1).

Table 1. Enzymes used for winemaking and their function.

Application/Process	Enzymatic Activity	Aim
Enhancement of filtration/clarification of must	Pectinolytic enzymes	Degradation of viscosity (pectin)
Mash fermentation/heating (red wine)	Pectinase with side activities (cellulases, hemicellulases)	Hydrolysis of plant cell wall polysaccharides. Improvement of skin maceration and color extraction of grapes, quality, stability, filtration of wines
Late phase of fermentation (white wine)	Glycosidases	Improvement of aroma by splitting sugar residues from odorless precursors
Young wine	Glucanases	Lysis of yeast cell walls, release of mannoproteins
Contaminated juice	Glucanases	Lysis of microbial exopolysacharides to improve clarification
Wine	Urease	Hydrolysis of yeast derived urea, preventing formation of ethyl carbamate
Must, wine	Lysozyme from hen egg	Control of bacterial growth
Must, wine	Proteases	Wine stabilization by prevention of protein haze; Reduction of bentonite demand

Technical enzyme preparations are usually obtained from fungi, which are cultured under optimal conditions on substrates to facilitate their preparation and purification. In contrast to grape-derived

enzymes, which are often inactive under wine conditions (low pH, presence of ethanol, phenolic compounds, sulphite etc.), fungal enzymes are resistant. The production of oenological enzymes for use in the European Union is regulated by the International Organization of Vine and Wine (OIV), which has ruled that *Aspergillus niger* and *Trichoderma* sp. may be used as source organisms (i.e., have GRAS, "generally regarded as safe" status) [1,2]. Selected strains from *A. niger* are fermented under aerobic conditions in optimized growth media for production of pectinases, hemicellulases and glycosidases, *Trichoderma* species are used for production of glucanases and *Lactobacillus fermentum* for urease.

　　Current commercial enzyme preparations are usually cocktails of different activities, such as glucosidases, glucanases, pectinases and proteases [5]. The search for enzymes with improved and more specific characteristics will continue. In this respect, the study and exploration of the high endogenous enzyme potential of wine and grape-associated microorganisms (Table 2) will assist the wine industry to meet prospective technical and consumer challenges. In contrast to filamentous fungi, yeasts with beneficial enzymatic endowment, could be directly used as starter cultures, without application of enzyme preparations.

Table 2. Microbial enzymes with relevance for winemaking.

Enzyme	Remarks
Fungi (*Botrytis cinerea*)	
Glycosidases	Influence aromatic potential of infected grapes by release of volatile aroma compounds
Laccases	Broad specificity to phenolic compounds, cause oxidation and browning
Pectinases	Depolymerizing enzymes, cause degradation of plant cell walls and grape rotting
Cellulases	Multi-component complexes: endo-, exoglucanases and cellobiases; synergistic working, degrade plant cell walls
Lipases	Degrade lipids (e.g., in cell membranes)
Esterases	Involved in ester formation and degradation
Proteases	Aspartic proteases occur early in fungal infection, determine rate and extent of rotting caused by pectinases
Yeasts	
Glucosidases	Some yeasts produce β-glucosidases which are not repressed by glucose and are resistant to ethanol and low pH; positive influence on wine flavor
Glucanases	Occur extracellular, cell wall associated and intracellular, accelerate autolysis process and release of mannoproteins
Proteases	Acidic endoproteases accelerate autolysis process and degradation of grape proteins
Pectinases	Degrade pectin in grape cell walls
Lactic acid bacteria	
Malolactic enzymes	Convert malic acid to lactic acid
Esterases	Involved in ester formation and degradation
Glycosidases	Deliberate flavor compounds
Lipases	Degrade lipids
Lichenases, Glucanases, Cellulases, Xylanases	Degradation of polysaccharides
Proteases	Hydrolysis of proteins
Tannases	Hydrolysis of tannins (polymeric phenolic compounds)
Laccases	Oxidation of phenolic compounds

　　In the following paragraphs we give an overview on widely used enzyme preparations for wine fermentation and a special focus on wine-associated microorganisms as alternative enzyme sources.

2. Pectinases

　　The grape cell wall consists of cellulose microfibrils linked together by a matrix of xyloglucan, mannan, xylan (hemicellulose) and pectin, all of which is stabilized by a protein network. The high viscosity of pectin, which is dissolved after berry crushing impedes juice extraction, clarification and filtration. In addition, pectin prevents diffusion of phenolic and aroma compounds into the must during wine fermentation.

2.1. Commercial Pectinases

The complete degradation of pectin needs cooperation of several enzymes to break the complex molecule into small fragments [1,2]. They include different enzymatic activities:

- Polygalacturonase (homogalacturonan-hydrolase) (PG): hydrolytic depolymerization of the polygalacturonic acid chain. One can differentiate enzymes that cleave either single galacturonic acid units from the chain end (exo-activity, exoPG, EC 3.2.1.67), or in the middle of the chain (endo-activity, endoPG, EC 3.2.1.15).
- Pectinlyase/pectate lyase (EC 4.2.2.2 and 4.2.2.9): nonhydrolytic cleavage of the polygalacturonic acid chain.
- Pectinesterase (EC 3.1.1.11): hydrolytic cleavage of methanol from the D-galacturonic acid chain, causing drastic viscosity reduction in the liquid portion of the mash and better must flow.
- Acetylesterase (EC 3.1.1.6): cleaves acetyl residues from D-galacturonic acid with release of acetic acid. By this way the interfering acetyl residues at the connecting points of the side chains of the "hairy regions" are removed which facilitates further enzymatic degradation.

Most commercial preparations are derived from fungal sources [1–5] and are more or less well-defined enzyme mixtures (Table 3). The application of bulk enzyme preparations is advantageous as it fulfills several functions. Examples are liquefaction enzymes, which contain cellulases and hemicellulases in addition to pectinases.

Commercially available pectinase preparations contain the active enzymes (2–5%) and additives (sugars, inorganic salts, preservatives) which stabilize and standardize the specificities of the products [1]. Factors that generally inhibit proteins will reduce effectiveness of the enzymes. These include juice clarification with bentonite, which adsorbs and deposits the proteins. Alcohol levels above 17% (*v/v*) and SO_2 levels above 500 mg/L inhibit pectinases [4]. Tannin-rich wines show reduced enzyme activity as phenolic polymers react with the proteins and render them useless.

Table 3. Examples of commercial pectinase preparations used for winemaking modified from [3,4].

Supplier	Enzyme Preparation	Purpose of Application
AEB, South Africa	*Pectocel L*	Improvement of clarification, filtration and product yield
	Endozym Pectoflot	Improvement of clarification, filtration and product yield
	Endozym Contact Pelliculaire	Enhancement of extraction and color stabilization
	Endozym Rouge	Enhancement of extraction and color stabilization
	Endozyme Active	Improvement of clarification, filtration and product yield
Begerow, Germany	*Siha Panzym Extract G*	Enhanced extraction and release of color and aroma
	Siha Panzym Clair Rapide G	Improvement of clarification, filtration and product yield
	Siha Panzym Fino G (β-Glucanase)	Improvement of clarification, filtration and sensory
	Siha Panzym Arome G (β-Glucosidase)	Enhanced aroma development
Darleon, South Africa	*Influence*	Improvement of clarification, filtration and product yield
	Enzym' Color Plus	Enhancement of extraction and color stabilization
DSM, Switzerland	*Rapidase Filtration*	Improvement of clarification, filtration and product yield
	Rapidase Vino Super	Improvement of clarification, filtration and product yield
Enartis, Italy	*Uvazym 1000S*	Clarification of white juices—facilitation of fining and filtration
	Progress Quick	Must flotation
	Uvazym couleur	Enhanced extraction during short macerations
Erbslöh, Germany	*Trenolin bukett DF (β-Glycosidase)*	Enhanced aroma development—Improvement of clarification
	Trenolin Super DF	Improvement of clarification, filtration and product yield
	Trenolin Flot DF	Must flotation
	Trenolin 4000 DF	Enhancement of sugar yield
	Trenolin Filtro DF (β-Glucanase)	Improvement of clarification and filtration; Hydrolysis of *Botrytis cinerea* exopolysaccharide slime
	Trenolin Bukett DF	Enhance of color and aroma release from red grapes
Laffort, France	*Lafazym press*	Enhanced color and tannin extraction—Facilitation of fining and filtration
	Lafazym CL	Improvement of clarification, filtration and product yield
	Lafase 60	Improvement of clarification, filtration and product yield
	Lafase HE	Enhancement of extraction and color stabilization
Lallemand, France	*Lallemand EX*	Enhancement of extraction and color stabilization
	Lallemand OE	Enhancement of extraction and color stabilization
Novo Nordisk, Denmark	*Novoclair FCE*	Improvement of clarification, filtration and product yield
	Vinozym EC	Enhancement of extraction and color stabilization
	Glucanex (β-Glucanase)	Improvement of clarification and filtration; Hydrolysis of *Botrytis cinerea* exopolysaccharide slime
	Ultrazym	Improvement of clarification and filtration
	Pectinex Superpress	Improvement of clarification and filtration
Valley Research, USA	*Crystalzyme*	Rapid clarification–Color improvement–Increased complexity–Process efficiency

2.1.1. Effect on Juice Extraction, Clarification and Filtration

The pulp of the grape varieties is rich in pectin compounds. The incomplete hydrolysis of these molecules by the endogenous enzymes can therefore cause problems in processing. If pectinases are applied to the pulp prior to pressing, they can improve juice and color yield. For the clarification of musts after pressing, pectinase-based enzyme preparations are recommended. Its pectin methyl esterase and endogalacturonic activities cause hydrolysis of the pectin chains and facilitate the drainage of juice from the pomace with an increased yield of a free-flowing juice with lower viscosity [6–8]. In addition, it causes cloud particles to aggregate into larger units that deposit as sediment. The acceleration of the clarification process also produces more compact lees. When applied to the pulp before pressing, it increases juice yield and color yield [6–8].

2.1.2. Effect on Color Extraction

Anthocyanidins are the red grape pigments, which mainly occur in the grape skin [9]. The chemical structure, commonly referred to as "flavylium cation", is characterized by two benzene rings linked by an unsaturated cationic heterocycle. Normally, the dye molecule is linked to a glucose monomer, which improves water solubility and stability. Pelargondin, cyanidin, delphinidin, peonidin, petundin and malvidin are the main variants identified in grapes and wine.

Flavonols are light yellow pigments found in the skins of both red and white grapes [9]. These are mainly the glycosylated forms of kaempferol, quercetin and myricetin. In red wine concentrations are in the range of 100 mg/L, in white wines 1–3 mg/L.

Under natural conditions, solubilization of phenolic compounds from grapes is facilitated by increased ethanol concentrations in the course of alcoholic fermentation. However, the extraction is uncomplete as the grape skin forms a physical barrier against the diffusion of anthocyanins, tannins and flavors from the cells. Therefore, various oenological techniques have been developed that result in wines that have good visual characteristics and are as stable as possible [10]. Especially wines made by pectinase treatment showed higher concentrations of anthocyanins and total phenols, as well as greater color intensity and optical clarity compared to untreated control wines [6–8].

2.1.3. Immobilization

Immobilization is a commonly used strategy to conserve the desirable properties of enzymes for biotechnological applications. In addition to improved stability, immobilization offers a number of advantages, such as reusability, ease of product separation, and better control of catalysis. Various methods have been described for the immobilization of pectinases, such as inclusion in alginate [11], physical adsorption to anionic resins [12], and covalent bonding to supports such as porous glass [13] and nylon [14]. A pectinase from *Aspergillus niger* immobilized on chitosan-coated carriers retained 100% of its original activity after several cycles of reuse [15].

2.2. Yeast Pectinases

S. cerevisiae strains, despite their genetic ability to secrete an endo-polygalacturonase, usually show no or only weak pectinase activity. In contrast, many so-called "wild" yeasts have been identified to be pectinase producers [16–20].

Grape fermentations at low temperatures (15–20 °C) are believed to protect the volatile compounds, thereby improving the aromatic profile of the wines. Therefore, cold-active enzymes are required for both extraction and clarification [21]. Psychrophilic yeasts are natural sources for such biocatalysts [22]. The pectinolytic enzymes of *Cystofilobasidium capitatum* and *Rhodotorula mucilaginosa* are effective under oenological pH (3.5) and temperature conditions (6.0 °C and 12 °C). Also, pectinases from several *A. pullulans* strains remain active at wine-relevant concentrations of glucose, ethanol or SO_2, bearing the potential as processing aids for low-temperature wine fermentations [23].

3. Lipases

Lipids in wine originate directly from the grape berry [24] and by autolysis of wine yeast [25]. It has been reported that lipid composition undergoes considerable changes during wine fermentation [26].

Authentic lipases (E.C. 3.1.1.3) are mainly active at the oil–water interface of emulsified substrates with long fatty acid chains. Triglycerides are cleaved rendering glycerol and fatty acids. In contrast, carboxylic ester hydrolases (see below) hydrolyze soluble esters with relatively short fatty acid chains [27]. However, the transition between both activities appears somewhat fluid.

Lipolytic activities have been detected only in few wine-relevant *Lactobacillus* strains [28], but in different genera of yeasts isolated from natural environments [29,30]. In theory, lipases could be used for winemaking for decomposition of lipoid cell membranes, thereby improving color extraction from red grape berries (Figure 1).
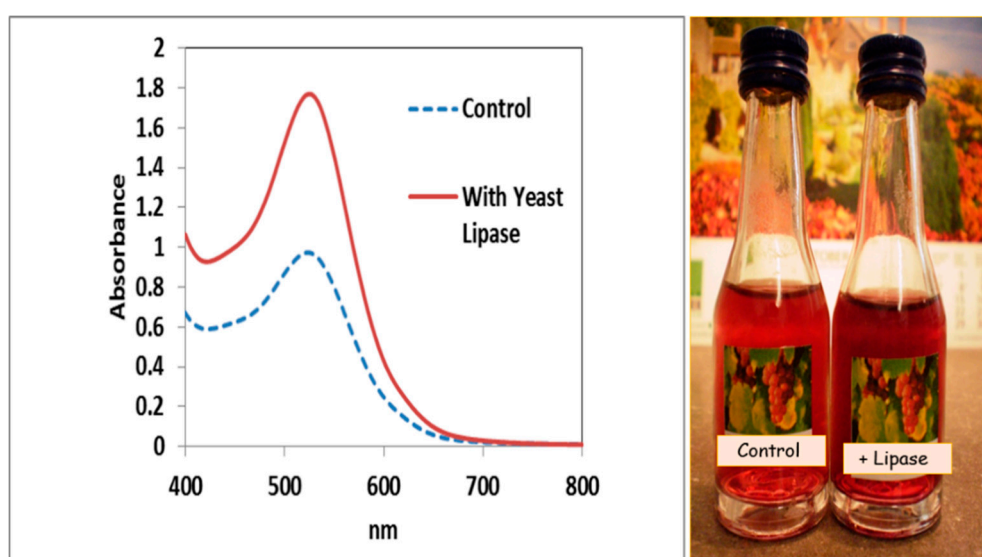

Figure 1. Color extraction from red grapes after a model fermentation without and in presence of a yeast lipase (Claus, unpublished data).

4. Glucanases

4.1. Commercial Glucanases

Polysaccharides in must and wine originate from the grape berries (cellulose, hemicellulose, pectins) and from cell walls of yeasts during growth and autolysis (beta-glucans, chitin). Several strains of lactic acid bacteria (especially *Pediococcus* spp.) and the grape fungus *Botrytis cinerea* produce viscous capsular or extracellular polysaccharides impairing wine filtration [31]. The colloidal polysaccharides cannot be removed from wine by flocculants, adsorbents or filtration. Thus, commercial products with glucanase activities e.g., those from *Trichoderma* sp., and *Taleromyces versatilis* are useful to reduce viscosity of musts and wine caused by microbial contaminations [5] (Table 3).

Two types of glucanases are relevant for wine fermentation: (i) exo-β-1,3-glucanases split β-glucan chains by sequentially cleaving glucose residues from the non-reducing end and releasing glucose as the sole hydrolysis product and, (ii) endo-β-1,3-glucanases catalyse the intramolecular hydrolysis of β-glucans with release of oligosaccharides.

Some yeast cell constituents, in particular the wall, can exert a significant impact on the technological and sensory properties of wine. The cell wall consists of β-glucans (~60%), mannoproteins (~40%) and chitin (~2%). In particular, the mannoprotein fraction has attracted increasing interest in wine fermentation to stabilize tartaric acid and protein, improve mouthfeel and reduce astringency [32–34]. The use of mannoproteins of the cell wall and chitin as binding elements for the removal

of undesirable compounds such as ochratoxin A and toxic heavy metals has been proposed. The mannoprotein fractions present in the yeast cell walls are highly variable between species and even strains, providing opportunities for the development of alternative fining products that could replace conventional proteinaceous animal preparations with allergenic potential.

Commercial enzyme preparations (Table 3) facilitate the release of these components. As a prerequisite they have to be effective under acid wine conditions, moderate temperatures and in presence of ethanol [35].

4.2. Microbial Glucanases

A major microbial source of polysaccharide-degrading exoenzymes are non-*Saccharomyces* yeasts belonging to the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces*, *Hanseniapora*, *Kluyveromyces* and *Wickerhamomyces* (Table 4). Glucanolytic enzyme activities were also detected in wine-relevant lactic acid bacteria [28] (Table 2).

Table 4. Glucanases of non-*Saccharomyces* yeasts with possible use for winemaking modified from [4,18].

Species	Specificity	Substrate	MW(kDa)
Candida albicans	Endo-β-1,3-	L, OL, P	49
	Exo-β-1,3-	L	107
Candida hellenica	nd	G	nd
Candida lambica	nd	G	nd
Candida pulcherrima	nd	G, Li	nd
Candida stellata	nd	G, Li	nd
Candida utilis	Endo-β-1-3-	L, PNPG	20
	Exo-β-1,3-1,6-	L, P, PNPG	20
	Endo-β-1,3-	L, OL	21
Kloeckera apiculata	nd	G, Li	nd
Kluyveromyces phaseolosporus	Endo-β-1,3-(I)	L, OL	180
	Exo-β-1,3-(II)	L, OL	45
	Exo-β-1,3-1,6-(III)	L, P	18.5
	Exo-β-1,3-1,6-(IV)	L, Ol, P	8.7
Pichia polymorpha	Endo-β-1,3-(I)	L, OL	47
	Exo-β-1,3-1,6-(II)	L, OL, P, PNPG	40
	Exo-β-1,3-(III)	L, PNPG	30
Schizosaccharomyces pombe	Endo-β-1,3-(I)	L, OL	160
	Endo-β-1-3-(II)	L, OL	75
Schizosaccharomyces versatilis	Endo-β-1,3-	L, OL	97
	Exo-β-1,3-1,6-	L, P, PNPG	43
Wickerhamomyces anomalus AS1	Exo-β-1,3-	L, PNPG	47.5

L: laminarin, OL: oxidized laminarin, P: pustulan, G: β-glucan (Barley), Li: lichenan, PNPG: p-nitro-phenyl-β-D-glucoside; nd: not determined.

5. Glycosidases

The organoleptic properties of wine are determined by a variety of different compounds that are already present in the grape (aroma) or arise during fermentation or storage (bouquet). Acids such as tartaric acid or citric acid affect the taste, but the characteristic odor and taste is mainly due to volatile organic substances such as esters, alcohols, thiols or terpenes [36–38].

Due to their low odor threshold, particularly terpenes determine wine flavor. Like other aroma active compounds (C13 norisoprenoids, benzene derivatives, aliphatic alcohols, phenols), they are secondary metabolites mainly derived from the grape skin. Approximately 90% of these compounds do not exist in a free form, but are conjugated to mono- or disaccharides, thereby forming water-soluble and odourless complexes. The most frequently occurring aroma precursors in grape varieties such as *Muscat* and *Riesling* are the glycosidic bound terpenes linalool, nerol and geraniol.

The sugar residues consist of rutinoside (rhamnose-glucose), arabinoside (arabinose-glucose) or apioside (apiose-glucose) [38].

Enzymatic hydrolysis of sugar-conjugated precursors release very aromatic, volatile terpenes (aglycones). Usually, the terminal sugars are first cleaved off by a rhamnosidase, an arabinosidase or an apiosidase. In a second step, the terpenes are released by a β-D-glucopyranosidase. This means that the latter activity alone can release only terpene compounds bound to a single glucose residue. In addition to a stepwise reaction, some glucosidases are able to hydrolyze the glycosidic bond to the aglycone, regardless of the number of sugar residues [39,40]. Nowadays commercial enzyme preparations are available that can hydrolyze the disaccharide directly from the terpene in a single step [5]. In many bulk enzyme preparations, glycosidase activities occur as side activities along with pectinase and glucanase activities (Table 3).

An important microbial source of wine-related enzymatic activities are lactic acid bacteria [28] (Table 2). Perez-Martin et al. [41] studied >1000 isolates for glycosidases. The β-glucosidase activities were only found in cells, but not in the supernatants of the cultures. Four *O. oeni* isolates retained their enzymatic activity under the conditions of winemaking. In a similar study, cell-bound glucosidase and arabinosidase activities from *O. oeni* strains released high levels of monoterpenes from natural substrates under optimal conditions [42]. The enzymes showed broad substrate specificities (release of both primary and tertiary terpene alcohols) and remained active in grape juice.

Glycosidase activities have been also detected in various non-*Saccharomyces* yeasts (*Candida*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Rhodotorula*, *Trichosporon*, *Wickerhamomyces*) [43–52] (Table 5). Several experiments on the technical application of yeast glycosidases to improve organoleptic quality of wines gave positive results [40,44,46,49,50].

Polyphenols in red wine, such as resveratrol, have gained increasing public and scientific interest due to their supposed beneficial effects on human health [53]. A majority of the polyphenols in nature are conjugated to sugars or organic acids, making them more hydrophilic and less bioavailable to humans. The amount of glycosylated forms of resveratrol, known as piceid or polydatin, has been found to be up to ten times higher in red wines. Since these modified forms are less bioactive, experiments with β-glucosidases from various fungal sources have been undertaken to increase the trans-resveratrol content in wines by hydrolysis of glycosylated precursors. The multifunctional glucanase WaExg2 of *W. anomalus* AS1 released the aglycones from the model compounds arbutin, salicin, esculin and polydatin [52]. WaExg2 was active under typical wine conditions such as low pH (3.5–4.0), high sugar concentrations (up to 20% *w/v*), high ethanol concentrations (10–15% *v/v*), presence of sulphites and various cations. Therefore, this yeast strain could be useful in wine production for several purposes: to increase the levels of sensory and beneficial compounds by cleaving glycosylated precursors or reducing the viscosity by hydrolysis of glycan slurries. In this context Madrigal et al. [29] underlined that glucose- and ethanol-tolerant enzymes from *Wickerhamomyces* are of great interest to the wine industry.

Table 5. Aroma enhancing enzymes of non-*Saccharomyces* yeasts with possible use for wine fermentation modified from [54].

Species	Enzymatic Activities*				
	β-D-Glucosidase	α-L-Arabino-Furanosidase	α-L-Rhamnosidase	β-D-Xylosidase	Carbon-Sulfur Lyase
Aureobasidium pullulans	+	+	+		
Brettanomyces anomalus	+				
Candida guillermondii	+		+	+	
Candida molischiana	+				
Candida stellata	+		+	+	
Candida utilis				+	
Candida zemplinia					+
Debaryomyces castelli	+				
Debaryomyces hansenii	+				
Debaryomyces polymorphus	+				

Table 5. *Cont.*

Species	Enzymatic Activities*				
	β-D-Glucosidase	α-L-Arabino-Furanosidase	α-L-Rhamnosidase	β-D-Xylosidase	Carbon-Sulfur Lyase
Debaryomyces pseudopolymorphus	+				
Debaryomyces vannjii	+				
Hanseniaspora guillermondii	+				
Hanseniaspora osmophila	+			+	
Hanseniaspora vineae	+	+	+	+	
Hanseniaspora uvarum	+	+	+	+	
Issatschenkia terricola	+				
Kluyveromyces thermotolerans	+				+
Metschnikowia pulcherrima	+			+	+
Pichia angusta			+		
Picha anomala	+	+	+	+	
Pichia capsulata		+			
Pichia guilliermondii			+		
Pichia kluyveri					+
Pichia membranaefaciens	+			+	
Saccharomycodes ludwigii	+				
Schizosaccharomyces pombe	+				
Sporidiobolus pararoseus	+				
Torulasporus delbrueckii	+				+
Torulasporus asahii	+				
Wickerhamomyces anomalus	+	+		+	
Zygosaccharomyces bailii	+				

* Activity detected (+).

6. Esterhydrolases and -Synthetases

Esters (e.g., ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) contribute to the most desirable fruity wine flavors [36,37]. They are synthesized by the grapes but are also produced by yeasts in course of alcoholic fermentation [55]. During malolactic fermentation, significant changes in the concentration of individual esters were observed [56]. Presence of alcohol acyltransferases (ester synthesis) and esterases (ester hydrolysis) in wine yeasts [37] and lactic acid bacteria [57] is well documented.

Depsides are esters of aromatic hydroxycarboxylic- or phenolic acids with each other or with other carboxylic acids of the grape, such as tartaric acid [5]. These compounds can be hydrolyzed by cinnamoyl esterases ("depsidases"), which often appear as side activities in enzyme preparations made from *A. niger* [5] The fission products can have a negative influence on wine quality. Enzymatic deliberated phenolcarboxylic acids, such as caffeic acid or coumaric acid, can be converted by the yeast metabolism to the volatile phenol derivatives 4-vinylguajacol and 4-vinylphenol, which are unpleasant side-tastes in the wine [5]. Therefore, commercial pectinase preparations should be free of depsidase side activities. In a recent study of 15 commercial enzyme preparations, approximately half of the samples yielded significant cinnamoyl esterase activities [58].

7. Proteinases

Proteins in must in wine are derived from the grapes, and from microbial cells (yeasts, lactic, acid bacteria) and their activities [59]. Another important source are protein-based wine additives (e.g., lysozyme, ovalbumin, gelatin, casein) which could pose allergenic-like reactions to consumers [60–62]. Most of these proteins have vanished after termination of wine fermentation and subsequent fining procedures. However so-called pathogen-related (PR) proteins (β-glucanases, chitinases, thaumatin-related proteins) can still be present. They are synthesized by the plants for defence against bacterial or fungal infections and in response to abiotic stress [63]. Due to their compact structures, they are resistant against acid wine conditions, heat, and proteolysis [59].

In combination with other wine ingredients, PR proteins can cause undesirable turbidity especially during cold storage of white wines with negative economic consequences [59]. Currently, protein removal is mainly achieved by bentonite addition [64], a process that can be associated with decreased

wine quantity and quality. Bentonite acts essentially as a cation exchanger, and individual wine proteins adsorb to different degrees on the clay [64]. Proteins that are negatively charged at wine pH (about 3.5) and/or are highly glycosylated as laccases of *Botrytis cinerea* are less bound by bentonite. Thus, new fining agents are desired to remove proteins from wine.

7.1. Proteases from Fungal and Plant Sources

Enzymatic degradation of wine proteins seems an attractive alternative to bentonite treatment as it would minimize losses of volume and aroma. As a prerequisite, suitable proteases have to be active under specific wine conditions (acid pH, presence of ethanol, sulphites, phenolics) and preferably act at low temperatures. Another challenge is the resistance of PR proteins against proteolysis due to their special molecular features like disulfide-bonds and glycosylations. Nevertheless, other grape proteins might be more susceptible, and thus proteases may help to reduce effective bentonite dosages. Currently, proteases from plants (papain, bromelain) have been tested with some promising results [65,66]. A fungal protease from *Aspergillus* sp. (aspergilloglutamic peptidase) has already approved for Australian winemaking [67]. The enzymatic procedure involves flash-pasteurization of grape must and is thus limited to specialized wineries. In this context, a protease of *Botrytis cinerea* BcAp8 has been described to hydrolyze grape chitinase at moderate temperatures [68].

7.2. Microbial Proteases

Microbial proteases can be an alternative or supplement to bentonite treatment for removal of unwanted wine proteins. Most *Saccharomyces cerevisiae* strains show no extracellular protease activity on diagnostic agar media [16–19,69]. However, a 72 kDa extracellular pepsin-like aspartic protease was characterized from a PIR 1 strain [70,71]. The enzyme was active during grape juice fermentations, although it did not affect turbidity-inducing proteins, unless the wine was incubated at 38 °C for extended time.

Proteinase A (PrA, saccharomycin; EC 3.4.23.25) is the major vacuole protease of *S. cerevisiae* encoded by the *PEP4* gene. As result of yeast autolysis, PrA enters wine in course of alcoholic fermentation. Far more, it has been found that under stress conditions (e.g., nutrient limitations) PrA is not targeted to the vacuole, but is misdirected to the cell membrane and secreted in the medium [72]. This would be advantageous for winemaking in view of haze reduction. Interestingly, the same situation is undesirable for beer brewery as PrA degrades proteins (e.g., lipid transfer protein 1), necessary for foam formation. Apart from PrA, *S. cerevisiae* expresses different cell-bound proteases, some of which are not fully characterized [73].

Non-*Saccharomyces* yeasts are important sources of extracellular enzymes including proteases (Table 6). Strains of *Metschnikowia pulcherrima* and *Wickerhamomyces anomalus* secreted aspartic proteases and degraded a model protein (bovine serum albumin) during growth in grape juice [74]. In a recent study, heterologous expressed aspartic protease MpAPr1 from *M. pulcherrima* [75] was added to a Sauvignon Blanc must. It was shown that the enzyme was active during fermentation and degraded wine proteins to some extent [76]. An alternative strategy would be to perform wine fermentations with appropriate protease-positive starter cultures. In addition to cost reductions, there are no administrative restrictions for yeast applications in must and wine, which must be taken into account with enzyme preparations.

Occurrence of proteolytic activities in lactic acid bacteria is also well-documented [28] (Table 2). Growth of *Oenococcus oeni* depends on the presence of amino acids in the culture medium because of deficiency in corresponding synthetic pathways. This bacterium secretes several proteases which may help to gain access to rare nitrogen sources during malolactic fermentation [77].

Table 6. Extracellular proteases of non-*Saccharomyces* yeasts with possible use for wine fermentation.

Species	Mode of Identification	Characterization	Reference
Candida apicola	Skim milk agar (pH 3.5), Gene Sequencing	Aspartic protease CaPR1 (39.2 kDa)	[78]
Candida stellata	Casein agar	nd	[18]
Hanseniaspora guelliermondii Hansenispora valbyenis Hanseniapora occidentalis	Casein agar and broth (pH 6.0)	nd	[79]
Hanseniaspora uvarum	Skim milk agar (pH 3.5)	nd	[16]
	Casein agar	nd	[18]
Kloeckera apiculata	Enzymatic; Inhibitor studies	Acid endopeptidase	[80]
Metschnikowia pulcherrima	Skim milk agar (pH 3.5)	nd	[16]
	Azocasein hydrolysis during fermentation of grape must	nd	[81]
	Skim milk agar (pH 3.5), Sequencing of protease gene, Purification	Aspartic protease pAPR1 (40.8 kDa)	[75,78]
	Casein agar	nd	[18]
	Skim milk agar (pH 4.5), Enzymatic; Inhibitor studies, LC-MS/MS	Aspartic protease	[74]
Wickerhamomyces anomalus	Skim milk agar (pH 3.5)	nd	[16]
	Skim milk agar (pH 4.5), Enzymatic; Inhibitor studies, LC-MS/MS	Aspartic protease WaAPR1 (47 kDa)	[74]

nd: not determined.

8. Phenoloxidases

Spontaneous and enzymatic oxidations exert dramatic effects on the final phenolic composition from the grape berry to bottled wine [82,83]. Once the integrity of the berries is destroyed, oxidative enzymes (phenoloxidases) and their phenolic substrates are exposed to the air, resulting in enzymatic browning (Figure 2).

Figure 2. Wine browning by *Botrytis*-laccase (right).

There are two classes of copper enzymes responsible for these reactions [82,83]: Tyrosinase (E.C. 1.14.18.1) hydroxylates monophenols to ortho-diphenols and oxidizes the latter to ortho-quinone intermediates which easily react further to polymeric, mostly colored products. Laccase (E.C. 1.10.3.2) has no monohydroxylase activity, and oxidizes a broad spectrum of different phenols and other compounds by a radical mechanism. Tyrosinase originates from grape berries [84], whereas laccases in must and wine are derived from epiphytic fungi, particularly *Botrytis cinerea* [82]. Phenolic compounds as caffeic acid, gallic acid, vanillic acid, ferulic acid, or especially resveratrol are known for their beneficial effects on human health. In addition to be radical scavengers, they are activators of the human's intrinsic cellular antioxidant system and have antimicrobial properties. Tyrosinase and laccase oxidize phenolic wine compounds and thus alter their antioxidant and antimicrobial properties [85–87]. In this context, it is an interesting observation that gallic acid oxidation by a laccase from *Trametes versicolor* was higher at 30 °C than at 45 °C. Although fungal laccases are usually more active at higher temperatures, the effect can be explained by reduced oxygen solubility under the experimental conditions [88].

Laccase is generally not very welcome in wine, but several studies have ruled out that controlled laccase treatments could promote wine stabilization and even improve sensory properties [89–93].

Volatile phenols particular produced by *Brettanomyces/Dekkera* sp. yeasts are associated with a serious "Brett" taste defect in wine. Lustrata et al. [94] used a laccase from *T. versicolor* to reduce concentrations of 4-ethylguaiacol and 4-ethylphenol in a synthetic model wine.

Biogenic amines (BA) are another class of undesirable compounds in wine [95–97]. They originate from the grape berries or are formed during fermentation by activities of decarboxylase-positive microorganisms [98–102]. Although more common in foods such as cheese, BA have received much attention in wine, as ethanol can enhance the negative effects on human health by inhibiting the enzymes responsible for the detoxification of these compounds [101].

Enzymatic degradation of BA is usually catalyzed by various classes of oxidases [103,104]. Depending on the type of prosthetic group, they can be classified into FAD-dependent (E.C. 1.4.3.4) and copper-containing amine oxidases (CAOs, E.C. 1.4.3.6). The latter have been detected in various yeasts such as *Kluyeromyces marxianus* or *Debaryomyces hansenii* [105,106]. These enzymes belong to the class of type 2 or "non-blue" copper proteins which convert primary amines to the corresponding aldehydes with an equimolar consumption of molecular oxygen and formation of hydrogen peroxide and ammonia.

Aromatic amines such as tyramine, phenylethylamine, tryptamine or serotonin are another class of compounds that can be oxidized by laccases [19]. Callejónet et al. [107] detected enzymatic

activities responsible for BA degradation in lactic acid bacterial strains isolated from wine. Responsible enzymes have been isolated and purified from *Lactobacillus plantarum* J16 and *Pediococcus acidilactici* CECT 5930 strains and have been identified as intracellular laccase-like multicopper oxidases. When the *L. plantarum* J16 laccase was overexpressed in *Escherichia coli*, it oxidized some BA, mainly tyramine [108].

9. Urease

Increased amounts of urea in wine can originate from yeast activities and then converted by a chemical reaction into the carcinogenic substance urethane (ethylcarbamate). During malolactic fermentation, lactic acid bacteria can produce other precursors of ethyl carbamate, such as arginine-derived citrulline and carbamyl phosphate. Especially at higher temperatures fermented wines may contain excessive amounts of urethane [109]. Therefore appropriate precautions should be taken to prevent the production of urethane. These include, for example, the selection of suitable starter cultures for malolactic fermentations and the reduction of arginine concentrations in the grape. Urease was introduced in 1997 by the EU as a new enzymatic wine treatment agent and can be used in exceptional cases. The enzyme splits urea into ammonia and carbon dioxide, preventing urethane formation. The commercial urease from *Lactobacillus fermentum* is effective on urea at doses of 50 mg/L in red wines and 25 mg/L in white wines [32].

10. Lysozyme

Yeasts, lactic acid and acetic acid bacteria have a significant influence on wine quality [110]. Microbial growth in musts and wines is conventionally controlled by the addition of sulfur dioxide. However, presence of sulphites in alcoholic beverages, particularly in wines, can cause pseudo-allergic responses with symptoms ranging from gastrointestinal problems to anaphylactic shock [32,33]. Other antimicrobials such as sorbic acid and dimethyl carbonate are primarily active against yeasts, but have limited activity against bacteria [32,34].

Lysozyme (EC 3.2.1.17) is a muramidase widely used to control microbial growth in foods such as cheese and wines [111,112]. Extensive enzymatic hydrolysis of the bacterial cell wall peptidoglycan, a polymer of *N*-acetyl-D-glucosamine units which are β-1,4-linked to *N*-acetylmuramic acid, results in cell lysis and death in hypoosmotic environments. Some lysozymes can kill bacteria by stimulating autolysin activity. In addition, bactericidal mechanisms involving membrane damage without enzymatic hydrolysis of peptidoglycan has been reported for c-type lysozymes, such as hen egg white lysozyme [113]. Gram-negative bacteria (i.e., acetic acid bacteria) are rather resistant against lysozyme, because the outer membrane acts as a barrier.

Lysozyme commercially produced from hen's egg white has been approved for winemaking by the International Organization of Vine and Wine in 2001 [114]. The amount added normally ranges between 250–500 mg/L. Four main applications and dosages are: (a) prevention of the onset of malolactic fermentation (early addition of 100–150 mg/L); (b) total inhibition of bacteria activity and malolactic fermentation (500 mg/L); (c) protection of wine during suboptimal alcoholic fermentation (250–300 mg/L); (d) stabilization of wine after malolactic fermentation (250–300 mg/L). Lysozyme can be eliminated by addition of fining agents, among which bentonite and metatartaric acid are the most efficient.

It has been reported that various Gram-positive strains of *Pediococcus* sp., *Lactobacillus* sp. and *Oenococcus oeni* [113] were not efficiently hydrolyzed by hen's egg white lysozyme. Reasonable explanations are structural modifications of the peptidoglycan, like *N*-deacetylation and *O*-acetylation of the glycan chains or amidation of free carboxyl groups of amino acids in the peptide chains [113]. As a possible alternative to hen's egg white lysozyme, exoenzymes (protease and muramidase) from *Streptomyces* species showed a broad bacteriolytic spectrum under winemaking conditions [98,113].

It should be mentioned that hen's egg lysozyme can display pH-dependent chitinase side activities. Under adverse conditions yeast cell walls (containing 2–4% chitin primarily in the bud scar regions)

can be weakened by lysozyme with significant effects on vitality and stress response of *Saccharomyces cerevisiae* during wine fermentation [114].

11. Legislative Regulations

The use of enzymes in wine production in the European Union is regulated by the International Organisation of Vine and Wine (OIV). Specified resolutions define general aspects of enzymes in winemaking, the permitted enzyme activities, mode of application and enzyme activity measurements. The USA, Canada and China have national regulations in winemaking [5].

Genetic engineering. Today's enzyme production is based either on special selected wildtype strains or on genetically modified organisms (GMOs). The use of GMO production strains has considerable advantages: the product yields with GMOs is much higher than with wild strains and undesirable side activities become minimized. This makes it more efficient to produce and to guarantee the purity of the enzyme products. The labelling is regulated by the resolution OIV-OENO 485-2012.

GMOs for must fermentations. Although increasing numbers of *Saccharomyces* yeasts have been improved by genetically engineering [115,116], only two GMOs have been allowed for winemaking in three countries. The first recombinant strain to get official approval by appropriate food safety authorities (in the USA and Canada) was the malolactic wine yeast ML01. The GMO carries the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mleA*). The second strain ECMo01 expresses the urease gene constitutively to prevent formation of urethane [115]. Whether yeast strains obtained by protoplast fusion should be considered as GMO is in legal limbo.

12. Conclusions

Nowadays, the use of technical enzymes is a well-established strategy to improve wine quantity and quality. Currently they are mainly produced by *Aspergillus* species and applied as bulk preparations with several side activities. In view of consumer safety, more defined activities and alternative biological producers seem to be preferable. Yeasts, naturally occurring on grapes, have been found to be a rich source of oenological interesting enzymes. Their activities can be exploited in form of new enzyme products or directly as starter cultures for wine fermentation. This would satisfy the increasing trend to produce more individual wines with the aid of non-*Saccharomyces* yeasts [117].

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