Book Chapter

Incorporating Microbial and Commercial Enzymes into Beverage Manufacturing Procedures

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Published **October 27, 2023**

This Book Chapter is an excerpt of an article published by Fernanda Cosme, et al. at Fermentation in April 2023. (Cosme, F.; Inês, A.; Vilela, A. Microbial and Commercial Enzymes Applied in the Beverage Production Process. Fermentation 2023, 9, 385. https://doi.org/10.3390/fermentation9040385)

How to cite this book chapter: Fernanda Cosme, António Inês, Alice Vilela. Incorporating Microbial and Commercial Enzymes into Beverage Manufacturing Procedures. In: Advances in Food Science. Hyderabad, India: Vide Leaf. 2023.

Funding: This research was funded by the financial support provided to CQ-VR—Chemistry Research Centre—Vila Real (UIDB/00616/2020 and UIDP/00616/2020) by FCT—Portugal and COMPETE.

Acknowledgments: The authors would like to thank the CQ-VR—Chemistry Research Centre—Vila Real for its financial support.

Abstract

Enzymes are highly effective biocatalysts used in various industrial processes, particularly in winemaking and other fermented beverages. These enzymes can be found naturally in fruits and the microbiota present during the beverage-making process. Commercial preparations containing a blend of enzymes (such as glucosidases, glucanases, pectinases, and proteases) are also commonly used. The performance of these enzymes has been significantly improved over the years, resulting in increased production levels and the introduction of new technological innovations to enhance efficiency and wine quality. While enzymes have been traditionally used in the beverage industry, recent studies have focused on optimizing their performance under processing conditions, including the use of immobilized enzymes. This review aims to provide a detailed overview of the potential of endogenous enzymes in wine microorganisms, as well as enzymes obtained from grapes or commercial preparations already in use in the beverage industry. Additionally, future trends in enzyme production and application will be presented.

Keywords

Enzymes; Glucosidases; Glucanases; Pectinases; Grape-Berry; Yeasts; Lactobacillus

Introduction

Enzymes are biocatalysts that accelerate biochemical reactions in living organisms. They can also be extracted from cells to catalyze several critical industrial processes [1]. Enzymes were discovered in the second half of the nineteenth century. In 1878, the German physiologist Wilhelm Kühne was the first scientist to use the word 'enzyme' when explaining the process of sugar fermentation by yeast. The word derives from the Greek words *en* (meaning 'within') and zume (meaning 'yeast') [1].

Enzymes are, typically, globular proteins and consist of long linear chains of amino acids that fold to produce a three-dimensional structure. Enzymes are incredibly efficient and highly specific biocatalysts, pivotal in beverage production. For example, many biotransformations occurred in wine production by employing the enzymes produced in the indigenous microflora on the grape and in the microorganisms present in the winemaking [2,3].

With the advance of biotechnology and the understanding of the function of enzymes, commercial enzyme preparations are used at different stages of the process in the beverage industry (alcoholic and non-alcoholic beverages) to obtain better quality products [3–5]. For example, they accelerate solid settling and clarification and improve color extraction. They can also achieve better extraction performances by increasing juice yield and processing efficiency, such as pressing [6].

Commercial enzyme preparations act like the natural enzymes from grapes and yeast but with improved selectivity, activity, and stability under operational conditions [3]. In the beverage industry, applying enzymes is a gainful option, which improves yields and, at the same time, reduces carbon footprint, energy consumption, and environmental pollution [7–9].

Enzymes can be used as food additives and processing aids. Most are used as processing aids, whereas others, such as lysozyme and invertase, are used as additives [10]. Among all

groups of food and beverage enzymes, amylases, pectinases, and cellulases are extensively used in the beverage industry [8].

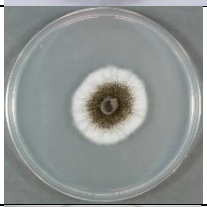
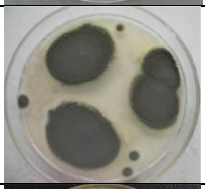
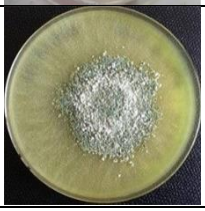
In this review, we will provide a comprehensive analysis of the various sources of enzymes used in the production of alcoholic and non-alcoholic beverages, including those derived from grapes, wine microorganisms, and commercial preparations. Furthermore, we will discuss upcoming trends in enzyme production and their potential applications.

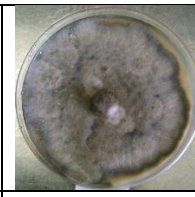
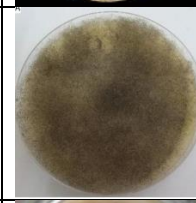
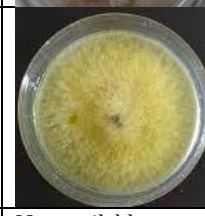
Enzymes Produced by Fungi (Molds and Yeasts) in the Beverage Industry

According to a report by Markets and Markets [11], the food enzymes market is expected to grow by $914.04 million between 2022 and 2027. This growth is projected to accelerate at a CAGR of 6.28% during the forecast period due to several factors, including advancements in R&D, environmental concerns, and urbanization. Additionally, technological advancements such as enzyme engineering and the introduction of genetically engineered enzymes have contributed to the growth of the food industry. Enzymes are used in various food applications such as baking, brewing, dairy, oil/fats, beverages, juice, and wine [12]. Most commercial enzymes used in food and drinks are mass-produced using fungal hosts, including yeasts (unicellular) and filamentous fungi (multicellular), as shown in Table 1.

The yeast *Saccharomyces cerevisiae* has been widely used for food and alcoholic drinks due to its ability to ferment sugars into ethanol and carbon dioxide. Filamentous fungi or molds have also been used for food and beverage production. For example, miso is a traditional Japanese seasoning processed using *Aspergillus oryzae* and *A. sake* for the fermentation of soybean, barley, or rice. However, fungi can also be an essential component of the final food product (*Penicillium roqueforti* strains used in blue cheese) [13]. In 1894, the manufacturing of an enzyme complex from *A. oryzae* was developed to produce different enzyme products in submerged fermentation [13]. However, the development of commercial sources of glucoamylase was the basis for the enzyme revolution in the starch industry [14].

Table 1: List of approved enzyme activity for use as processing aids in food in Europe. For each product, fungal producers species and their colonies formed on Potato-Dextrose Agar-PDA in Petri dishes are shown. Adapted from [13].

Enzyme Activity	Production Microorganism	Morphology of Colonies
Aminopeptidase, Arabinofuranosidase, Catalase, Cellulase, Glucanase (endo-beta), Glucanase (exo-beta), Glucanase (beta), Glucoamylase, Glucose oxidase, Lactase or galactosidase (beta), Lysophospholipase, Pectinase, Xylanase	*Aspergillus niger*	
Aminopeptidase (leucyl), Protease, Tannase	*Aspergillus oryzae*	
AMP deaminase, Protease (exopeptidase)	*Aspergillus melleus* and *Aspergillus usamii*	
Cellulase, Ribonuclease	*Penicillium funiculosum*	
Cellulase, Glucanase (exo-beta), Xylanase	*Trichoderma reesei*	

Dextranase	*Chaetomium erraticum*	
Glucanase (beta)	*Talaromyces emersonii*	Not available
Lipase triacylglycerol	*Candida rugosa*	
Lipase triacylglycerol, Glucanase (endo-beta), Glucanase (exo-beta)	*Rhizopus niveus*	
Lipase triacylglycerol	*Rhizopus oryzae*	
Protease (mucorpepsin)	*Rhizomucor miehei*	
Glucanase (beta), Xylanase	*Humicola insolens*	Not available

From Table 1, we can exemplify some fungal enzymes, such as (i) Glucoamylases (EC 3.2.1.3) that are exo-acting enzymes that catalyze the hydrolysis of polysaccharide starch from the non-reducing end, releasing β-glucose. These enzymes are produced by *Aspergillus niger*, *A. awamori*, and *Rhizopus oryzae* and are widely used for industrial applications [15]. They are found in various applications in the beverage industry, such as sweet syrups (high-glucose and high-fructose), and play an essential role in producing sake, soya sauce, and light beer [16]. (ii)

Acidic protease from *Aspergillus usamii* has been employed for the enhancement of functional properties of wheat gluten, allowing the release of peptides and amino acids for proper fermentation, not only for bread making but also for beer making, as they are efficient even at low pH by balancing the amino acid profile of beer [16,17]. (iii) Lipases, produced by *A. niger* or *A. oryzae*, are used in wine production to modify wine aroma [16]. (iv) Pectinolytic enzymes (polygalacturonase and pectase) are produced by *Aspergillus* spp. and are widely used for juice clarification, increasing juice yield before the grapes' fermentation [18]. Invertase is produced from *Saccharomyces* spp—cells by applying a proteolytic enzyme [18].

Enzymes Produced by Lactic Acid Bacteria in the Beverage Industry

Lactic acid bacteria (LAB) play a crucial role in the production and quality of various types of food worldwide. They are known for their complex metabolic activity, making them a valuable enzyme source. These enzymes have various industrial applications, but the slow growth of certain LAB strains, especially those isolated from wines, can hinder their large-scale production. However, using whole cells of LAB instead of purified enzymes offers several advantages. Whole cells provide a natural environment for enzymes, which helps maintain their activity in non-conventional media. Additionally, whole cells can regenerate co-factors effectively, enhancing their enzymatic activity. Overall, LABs are crucial in the food industry due to their enzymatic capabilities, and their safety status (GRAS – Generally Recognized as Safe) makes them suitable for various applications [19,20].

The main enzymes of wine LAB strains are the Malolactic enzymes, Glycosidases, Esterases, Lipases, Proteases, and Tannases.

Malolactic Enzyme-EC. 4.1.1.101– Malolactic Fermentation (MLF) is defined as the bioconversion of L-malic acid to L-lactic acid and carbon dioxide by the malolactic enzyme that exists in all wine LAB strains of (formerly *Lactobacillus, Pediococcus, Leuconostoc,* and predominantly *Oenococcus)* isolated from

grapes, must, and wine samples. This secondary fermentation, typically after alcoholic fermentation, results in significant physicochemical and sensory modifications necessary in red or other specific wines [21,22]. Beyond deacidification (the immediate effect of a decrease in acidity by the transformation of a dicarboxylic acid (L-malic acid), which is characterized by a rough taste, into a monocarboxylic acid (L-lactic acid) with a milder taste) of the wine, with simultaneous change of its olfactory and gustatory perception, MLF contributes significantly to microbial stability (by the removal of L-malic acid, a potential carbon source for some spoilage microorganisms [23,24]) and often to the improvement of the sensory profile of wines. Modifications in wine aroma by LAB result from less aggressiveness to the palate of L-lactic acid and also from a vast number of other compounds, such as diacetyl, acetoin, 2,3-butanediol, ethyl lactate, and diethyl succinate esters, and some higher alcohols and aromatic aglycones released by the action of LAB β-glucosidases [25–28]. Glycosidases-EC 3.2.1.21 β-D-Glucoside glucohydrolases; (Glycosidasic activity)— The varietal aroma of wine is determined by volatile compounds, which are usually present in grapes as non-volatile, odorless molecules linked to monoglycosides (β-D-glucose) and diglycoside (β-D-glucose and a second sugar unit of α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, or β-D-apiofuranose) [29]. Different classes of volatiles, including monoterpenes and C13-norisoprenoids, are the aglycon moiety that constitutes these glycoconjugate compounds. Although the acidic hydrolysis during wine aging can transform them into free volatile aroma compounds, a slow process and faster enzymatic hydrolysis of these glycoconjugates are performed by wine microorganisms. Thus, the release of the odorous aglycon is achieved by the action of the β-D-glucosidase enzyme, being necessary for diglycosides before the activity of an appropriate exo-glycosidase [30]. According to Grimaldi et al. [31], *O. oeni* strains possess β-D-glucopyranosidase, α-D-glucopyranosidase, and β-D-xylopyranosidase activities but also minimal α-L-rhamnopyranosidase and α-L-arabinofuranosidase activities. *Lactobacillus* spp. and *Pediococcus* spp. also have varying degrees of β-D-glucopyranosidase and α-D-glucopyranosidase

activities, although approximately one order of magnitude less than those seen for *O. oeni*.

All species capable of performing MLF possess β-glucosidase activity, although strain-dependent. Many research works have shown the capacity of many *Oenococcus oeni* strains to hydrolyze grape glycoconjugate aroma precursors with differences in the extent and specificity of β-glucosidase activity [31–34]. According to [32], the variability observed in β-glucosidase activity in different strains is a consequence of the influence of wine's sugar, pH, and ethanol content.

Lactiplantibacillus plantarum [35] (formerly *Lactobacillus plantarum*) strains isolated from South African wines [36] and Italian wines [37] showed β-glucosidase activity and the ability to release odorant aglycones from odorless glycosidic aroma precursors. Due to a more diverse enzyme profile observed in *Lactiplantibacillus plantarum* when compared with *O. oeni* strains, particularly with regards to the presence of the aroma-modifying enzymes β-glucosidase and phenolic acid decarboxylase (PAD), Krieger-Weber et al. [38] suggest and recommend the future use of this species in the modification of wine aroma profile and use as a commercial starter culture.

However, it is essential to emphasize that sometimes, glycosidase activity can also harm the final product quality by the increase in smoke-taint-associated aromas [39,40] when some volatile phenols are the glycoconjugate compounds or impacting wine color when anthocyanin glucosides are involved [40].

Esterase-EC 3.1.1.6 acetyl ester hydrolases (Esterase activity)—Wine esters are secondary or tertiary aroma compounds that contribute significantly to wine aroma [40,41]. These compounds are produced by yeasts during alcoholic fermentation and by LAB during MLF [41] via ester hydrolysis through esterase activity and also by slow chemical esterification [41] between alcohol and acids during wine aging [42]. The primary esters in wine include ethyl esters of organic acids (e.g., ethyl lactate), fatty acids (e.g., ethyl hexanoate, ethyl octanoate, ethyl

decanoate), and acetates of higher alcohols (e.g., ethyl acetate, isoamyl acetate) [41]. The role of LAB in the ester composition profile of wines produced worldwide after MLF is well documented in many references [43–46]. Although all wine LAB species of *Lactobacillus*, *Pediococcus*, and *Oenococcus* hold esterase activity, strains of *O. oeni* have shown the highest activity [47]. The degree of LAB contribution to the ester profile of the wine is strain-specific [28,48] and is conditioned by factors such as pH, temperature, and ethanol concentration [47]. Sumby et al. [28] realized that *O. oeni* esterases could hydrolyze and synthesize esters of short-chained fatty acids, the degree of each activity being conditioned by strain and wine composition. Additionally, according to Lasik-Kurdy et al. [49] results, the MLF inoculation strategy can affect the quantity and quality of esters released by the bacteria, with the co-inoculation technique showing an increase in the release of ethyl esters (ethyl lactate, diethyl succinate, and ethyl acetate), that may enhance the wine with floral and fruity notes, depending on their concentration.

Lipase-EC 3.1.1.3 triacylglycerol acylhydrolases (Lipase activity)—By their action, wine lipids are cleaved, rendering different volatile compounds (esters, ketones, aldehydes) and fatty acids. While the former may positively affect wine flavor, the odors of fatty acids are usually not desirable [50]. Lipase activities are present in all genera of wine LAB. In a survey performed by Matthews et al. [51] in LAB isolated from Australian wines, lipase activity was restricted to three *Lactobacillus* isolates for enzymes of interest in enology. From palm wine, Nkemnaso [52] recovered six LAB isolates—*Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, *Levilactobacillus brevis*, *Limosilactobacillus fermentum* [35], formerly *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactococcus* spp., and *Leuconostoc* spp. with lipase activity.

Proteases and peptidases (Proteolytic activity)— Although *O. oeni* and other wine strains of different species LAB responsible for MLF have complex nutritional requirements, they cannot utilize inorganic nitrogen sources, unlike the fermenting yeast *Saccharomyces cerevisiae*. Instead, sufficient amounts of organic

nitrogen in the form of amino acids and peptides must be supplied or present in the musts or wines [38]. Thus, some works have characterized proteolytic enzymes in *O. oeni* strains [53–56] and in wine *Lactobacillus* strains [57] that may help to gain access to rare nitrogen sources during MLF [56]. According to the results obtained by Aredes Fernández et al. [5], the utilization of *Oenococcus oeni* with proteolytic activity to carry out the MLF would contribute to enhancing the beneficial biological activities (antioxidant and antihypertensive activity) of the final product and provide an additional value to regional wines. Wine LAB proteolytic activities could be exploited in winemaking and could potentially replace or reduce the use of fining agents such as bentonite to remove unwanted wine proteins [38].

Tannase-EC 3.1.1.20 (Tannase activity)—The action of taninoacil hydrolase enzyme, commonly termed tannase, reduces wine astringency and turbidity and may increase the quality of wine and result in a better and pleasant sensory perception for consumers. This activity was observed in strains of *Lactiplantibacillus plantarum* [35] (formerly *Lactobacillus plantarum*) [5,58–60] and *Limosilactobacillus frumenti* [35] (formerly *Lactobacillus frumenti*) [59] *Oenococcus oeni* [50,61] and *Pediococcus* [51]. Other enzymes (Enzymatic activity)—Also critical is the ability of some LAB strains to produce enzymes that degrade biogenic amines (BAs) and mycotoxins. Aromatic amines such as tyramine, phenylethylamine, tryptamine, or serotonin are another class of compounds that can be oxidized by laccases [4,50]. Two laccases (multicopper oxidases) extracted from a wine strain *Lactiplantibacillus plantarum* J16 and a cereal strain *Pediococcus acidilactici* CECT 5930 were identified and characterized [62,63], revealing BA degradation. Olmeda et al. [64] also described *Pediococcus* laccases of *P. acidilactici* 5930 and *Pediococcus pentosaceus* 4816 strains. A *Lactiplantibacillus plantarum* strain (CAU 3823) could degrade biogenic amines in culture media conditions and Chinese rice wine at the end of post-fermentation [65]. Thus, the authors suggest using similar strains as an efficient method to decrease the biogenic amine contents in traditional fermented food made by multiple microbes, such as wine, rice wine,

sausages, vinegar, cheese, and kimchi, among others. Regarding detoxification activity, LAB is at the top of the list of microorganisms for the degradation of mycotoxins due to their GRAS status, as mentioned before. For detoxification of mycotoxins in foods, LAB may use two mechanisms: (i) using the viable cells of the microorganisms and (ii) using the enzymes produced by certain LAB strains [66]. In a group of LAB isolated from Douro wines and identified as belonging to *O. oeni*, *Lactobacillus plantarum*, *Pediococcus parvulus*, Abrunhosa et al. [67] perceived biodegradation of ochratoxin A (OTA) in a synthetic medium only in *Pediococcus parvulus* strains able to degrade OTA. Due to the ability of these strains to degrade OTA, potential biotechnological applications could be used to reduce the health risks associated with this mycotoxin not only in the wine industry but also in other food industries (sausage, beer) and the animal feed industry [68].

Enzymes found in Grapes and Commercial Preparations Used in Wines Production

In wine production, enzymes are essential for the biotransformation processes that convert grape juice into wine. These processes are carried out by enzymes from microorganisms (yeasts, fungi, bacteria) and grapes and by commercially available enzymatic preparations added externally [3,69], Figure 1.

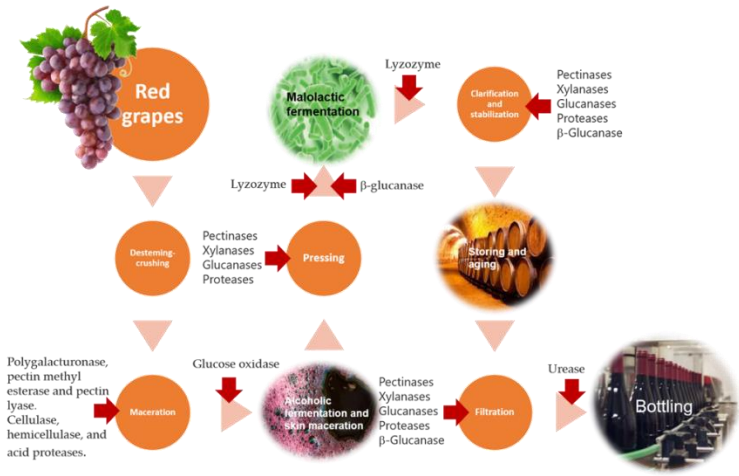


Figure 1: Schematic representation of the conventional red wine's production process, indicating the stages at which enzymes could be introduced. Adapted from Ray et al. [70].

Hence, enzymes can be applied across the entire winemaking process. Their utilization enhances the extraction of anthocyanins from the skins of red berries, consequently augmenting the color intensity of the resultant wine, a crucial quality determinant in red wines. During this phase, commercial enzyme preparations primarily encompass the activities of pectolytic enzymes, including polygalacturonase, pectin methylesterase, pectin lyase, cellulases, hemicellulases, and acid proteases [70,71], as depicted in Figure 1.

Pectinases, xylanases, glucanases, and proteases are employed to enhance clarification and filtration processes. These enzymes can improve pressing efficiency and juice extraction [72], as illustrated in Figure 1. These enzymes' disruption of grape-cell structures promotes the extraction of compounds present in both the pulp and the skin. This improves the extraction and clarification stages of grape must and results in the extraction of compounds influencing the aroma and color of the eventual wine [4,72].

Once the alcoholic fermentation is completed, the interaction with lees occurs. Wine lees refer to the sediment that forms at the bottom of the vessel containing wine after alcoholic fermentation during storage or following authorized treatments.

It also includes the residue obtained after the filtration or centrifugation of this product by EEC Regulation No. 337/79. Lees predominantly consist of deceased yeasts, bacteria, tartaric acid, and inorganic substances [73]. Yeast autolysis, which takes place after the death of cells, involves the breakdown of biopolymers through the action of hydrolytic enzymes such as β-glucanases and proteases. This process releases peptides, amino acids, fatty acids, and nucleotides from the yeast cytoplasm to the wine. Additionally, glucans and mannoproteins from the yeast's cell wall are released, significantly influencing the wines' stability and final sensory attributes. To minimize wine's contact with lees and mitigate risks of oxidation and microbial contamination, enzymatic preparations enriched with β-glucanases can be employed. These exogenous glucanases can catalyze the hydrolysis of β-(1-3) and β-(1-6)-glycosidic bonds in the cell wall β-glucan chains. This gradual degradation of the cell wall accelerates yeast lysis [73,74].

Alternative enzymes can enhance the desired aromatic qualities of wine varieties. These enzymes release glycosidic aroma precursors, which remain stable throughout the winemaking process. This is particularly important as the glycosidase activity of *S. cerevisiae* is somewhat restricted [75]. Commercial enzymatic products containing ample glycosidases are accessible from *Aspergillus niger* and can be used in winemaking [76].

Commercial enzyme preparations typically comprise blends of various activities, including glucosidases, glucanases, pectinases, and proteases [77]. The search for enzymes with more precise characteristics will persist. A significant objective of ongoing research is to explore the substantial inherent enzyme capabilities of microorganisms involved in winemaking.

At the pH levels and concentrations of sulfur dioxide commonly encountered in the winemaking process, grape enzymes often

exhibit low activity [78]. As a result, commercial enzymatic preparations are employed at various stages of winemaking for multiple purposes. These include reducing viscosity and increasing juice yield, enhancing color extraction, releasing varietal aromas from precursor compounds, improving clarification and filterability, and reducing the formation of ethyl carbamate, among other benefits. These commercial enzymatic preparations are more tolerant and active under winemaking conditions [68,79,80]. Enzymatic preparations of fungal origin, particularly those produced by *Aspergillus* spp., are generally considered safe (GRAS). However, it's worth noting that glucanases produced through direct fermentation (e.g., by *Trichoderma harzianum*, *Trichoderma longibrachiatum* or *T. reesei*, and *Penicillium funiculosum*), *Lactobacillus fermentum* for urease (EC 3.5.1.5), and hen egg white for Lysozyme are also used [81]. These preparations include pectinases, hemicellulases (EC 3.2.1.7 8), β-glucanases (EC 3.2.1.5 8), xylanases, and glycosidases (EC 3.2.1.2 0), which are specified in the International Code for Enological Practices of the International Organization of Vine and Wine [81]. Furthermore, Arabinanases (EC 3.2.1.9 9), Cellulases (EC 3.2.1.4), Glucosidases (EC 3.2.1.2 1), Galactanases (EC 3.2.1.8 9), Pectinlyases (EC 4.2.2.1 0), Pectinmethylesterases (EC 3.1.1.1 1), and Polygalacturonases (EC 3.2.1.1 5) are also encompassed in the International Code for Enological Practices of the International Organization of Vine and Wine [81].

Enzymes found in Grape Berry

The grape berry contains two of the three primary pectolytic enzymes utilized in enology: a pectin-methylesterase (EC 3.1.1.11) mainly localized in the berry skin [82]. Its activity is influenced by the grape variety and increases with grape maturity [83]. Additionally, an endo-polygalacturonase (EC 3.2.1.15) is present [82]. These activities diminish towards the end of grape maturation and under winemaking conditions such as alcohol content and pH [78]. These grape pectinases are named based on their mode of action on the pectin molecule. These enzymes either de-esterify (pectinesterase) or

depolymerize (polygalacturonases, polymethylgalacturonases, pectin, and pectate lyases) specific pectic substrates [84].

The most significant glycosidase activity is β-D-glucopyranosidase (EC 3.2.1.21). However, α-L-arabinofuranosidases (EC 3.2.1.55) and α-L-rhamnospyranosidase (EC 3.2.1.40) are also present in grape berries, and their activities increase as the berries mature [85,86]. Nonetheless, grape β-D-glucosidase exhibits weak activity towards grape terpene glycosides under winemaking process conditions characterized by low pH, high levels of sugars (glucose and fructose), polyphenols, and ethanol [85,87,88].

Grapes also possess polyphenol oxidase (EC 1.14.18.1) activity and peroxidase (EC 1.11.1.7) activity that triggers enzymatic browning when grapes are crushed or pressed during the wine production process [89]. Polyphenol oxidase functions as a cupric oxidoreductase, catalyzing the hydroxylation of monophenols into *ortho*-diphenols (cresolase activity) and the oxidation of *ortho*-diphenols into their corresponding *ortho*-quinones (catecholase activity) [90]. The subsequent polymerization of *ortho*-quinones results in the formation of brown-red-black pigments [91]. The nomenclature of polyphenol oxidase is based on specific substrate specificity, such as phenolase, laccase, cresolase, catecholase, tyrosinase, diphenolase, or monophenolase. Grapes contain tyrosinases, catecholase, or catechol oxidase. However, grapes infected with *Botrytis cinerea* have laccase, which lacks monohydroxylase activity and oxidizes various substrates [92]. Peroxidase, another oxidoreductase enzyme, also contributes to enzymatic browning. This is due to the ability of diphenols to act as reducing substrates in this reaction, and the involvement of this enzyme in browning has been documented by numerous researchers [93].

Commercial Enzymatic Preparations Used in Wines Production

The utilization of commercial enzymatic preparations is necessary due to the insufficient concentration and activity of enzymes naturally found in grapes under winemaking conditions,

preventing the attainment of anticipated outcomes. These grape-derived enzymes are not viable under low temperatures, within the pH range of grape must (2.8–3.8), and they are significantly inhibited by high levels of glucose, sulfur dioxide, phenolic compounds, and alcohol [69]. As a result, commercial enzyme preparations (including pectinases, glucanases, hemicellulases, glycosidases, xylanases, ureases, and lysozyme) are introduced into the wine production process.

Pectinases

Pectinases, also known as pectinolytic enzymes, constitute a group of enzymes that facilitate the breakdown of pectin—a high molecular weight acidic heteropolysaccharide primarily composed of α (1−4) linked D-galacturonic acid residues. These enzymes are categorized based on their mechanism of action and play a crucial role in various processing stages, such as juice extraction and clarification [94,95]. Commercial enzymatic pectinase preparations used in winemaking encompass different pectolytic enzyme activities, including polygalacturonases, pectin esterases, pectinlyases, and rhamnogalacturonan [85]. Among these, polygalacturonases are the most commonly encountered pectic enzymes. They catalyze the hydrolysis of glycosidic bonds randomly across all chain links (endopolygalacturonase E.C. 3.2.1.15) or from the nonreducing end of homogalacturonan, releasing galacturonic or digalacturonic acid residues (exopolygalacturonases E.C. 3.2.1.67) [96]. Lyases are further categorized into pectate lyases (E.C. 4.2.2.9 and E.C. 4.2.2.2) or pectin lyases (E.C. 4.2.2.10) based on their preference for highly esterified pectinic acid or pectate and the extent of randomness in eliminative depolymerization and actions on oligomeric substrates. Pectin lyase exhibits specificity for very esterified pectin. In contrast, endopectate lyase, specific for the penultimate bond at the reducing end of the galacturonan chain, targets pectates, releasing unsaturated digalactosiduronate as the sole end-product [79]. Pectin methylesterases (E.C. 3.1.1.11) remove methoxy groups from polygalacturonic acids. This process releases methanol and transforms pectin into pectate [85].

Cellulases and Hemicellulases

These enzymes catalyze the degradation of cellulose-type polysaccharides within grape cell walls, primarily through the action of endo-(1-4)-D-glucanases. Multiple enzymatic activities are necessary for the hydrolysis of cellulose, including endoglucanases (EC 3.2.1.4), exoglucanases (cellobiohydrolases, EC 3.2.1.91), exo-glucohydrolases (EC 3.2.1.74), and β-glucosidases (EC 3.2.1.21) [97]. Endo-glucanases catalyze the hydrolysis of osidic bonds within cellulose randomly. They also contribute to the degradation of xyloglucans, classified as hemicelluloses. Exo-glucanases release cellobiose and glucose as intermediaries for the fragments produced by endo-glucanase activity. β-glucosidase breaks down cellobiose into glucose [98]. This enzyme also participates in the release of aroma precursors. Additionally, two prominent families of xylanases are responsible for the hydrolysis of β-1,4 bonds, constituting the principal component of hemicellulose. These families include endo-β-1,4-xylanase, EC 3.2.1.8, which generates xylose and xylobiose by acting randomly within the chains, and exo-xylanases, predominantly yielding D-xylose from the non-reducing ends of the xylans [99].

Glycosidases and Glucosidases

Enzymatic preparations encompass activities that catalyze the hydrolysis of the saccharide portion of glycosylated aromatic compounds in grapes. These enzymes are specific for sugars (such as glucosidase and galactosidase) and a particular type of glycosidic bond (e.g., β-glucosidase). They can hydrolyze glycosylated aroma precursors (which are not odor-active), thereby making the compound volatile. Examples of grape varietal volatile compounds that exist in glycosylated precursor forms include monoterpenes (such as linalool, citronellol, nerol, and geraniol) and C13-norisoprenoid derivatives (like β-ionone, β-damascenone), which contribute to floral and fruity aromas (such as rose, violet, citrus). When these aroma molecules are linked to sugar molecules (termed glycosylated aroma precursors), they lack odor intensity. However, the compounds become odor-active once the sugar molecules are detached from

these glycosylated aroma precursors [100]. The enzymes involved in this process include α-L-arabinofuranosidase, which catalyzes the hydrolysis of the interosidic bond within the precursor arabinofuranosyl glucosides of volatile compounds. Furthermore, α-L-rhamnosidase enables the sequential hydrolysis of rhamnosyl glucoside precursors of volatile compounds, while β-D-glucosidase releases glucose from the volatile compounds. Among these, ß-D-glucosidase serves as the primary glycosidase enzyme [87].

Glucanases

Glucanases (EC 3.2.1) are categorized as endo- and exo-β-1,3 glucanases, which can hydrolyze glucans produced by *Botrytis cinerea.* These glucans are extracted into the grape juice and wine from grapes affected by grey or noble rot [101,102]. β-glucans are polysaccharides with a high molecular weight (800 kDa), composed of β-1→3-linked glucose units with β-1→6-linked side chains [101]. The presence of glucans in wine increases viscosity, often complicating clarification and filtration processes. Exo-β-1,3-glucanases break down β-glucan chains by gradually cleaving glucose residues from the non-reducing end, releasing glucose as the sole hydrolysis product. On the other hand, endo-β-1,3-glucanases catalyze the intermolecular hydrolysis of β-glucan, liberating oligosaccharides [103] as a result. Consequently, these enzymes catalyze the hydrolyzing of β-*O*-glycosidic linkages within β-glucan chains, leading to the liberation of glucose and oligosaccharides [104,105].

Urease

Urea, originating from yeast activity in wine, serves as the primary precursor of ethyl carbamate, a compound with recognized carcinogenic properties as established by the Food and Drug Administration [106]. As a result, certain importing countries, including Canada, have regulations specifying the maximum permissible ethyl carbamate content in wines, set at 30 µg/L. Enzymatic degradation of urea has emerged as a potential solution to mitigate the presence of ethyl carbamate. Urease (urea amidohydrolase EC 3.5.1.5), produced by the

Lactobacillus fermentum strain, facilitates the cleavage of ethyl carbamate into carbon dioxide and ammonia [107,108].

Lysozyme

Lysozyme is an enzyme found in hen egg whites, functioning as a peptidoglycan N-acetylmuramide glycanhydrolase (EC 3.2.1.17). Its mechanism of action is related to hydrolyzing the β-1,4-bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine [109,110]. This process destabilizes the bacterial peptidoglycan cell wall, leading to cell lysis. The primary application of lysozyme in winemaking is to prevent or delay malolactic fermentation (MLF) by inhibiting the growth of lactic acid bacteria, especially those belonging to the genera *Lactobacillus* and *Pediococcus*. Additionally, it aids in reducing the amount of sulfur dioxide required to be added to the grape must or wine [111,112].

Utilization of Enzymes at Various Stages of the Winemaking Process

The sensory attributes of wine, including color, especially red wines, are closely related to the volatile and phenolic compounds and aroma precursors found within the cell walls of grape skin cells [112]. As a result, the technological application of enzymes is carried out across various stages of wine production: pre-fermentation–juice extraction (pectolytic enzymes, cellulases, hemicellulases), sugar reduction (glucose oxidases), color extraction (cellulases, hemicellulases, pectolytic enzymes), aroma extraction (glycosidases), polyphenol reduction (phenol oxidases), fermentation, post-fermentation– a clarification (proteases), filtration (β-glucanases, cellulases, pectolytic enzymes), microbial stabilization (lysozyme), and aging–mouthfeel extraction (β-glucanases, cellulases) [113]. These enzymes are incorporated to enhance grape juice yields and color extraction, elevate the expression of varietal aroma compounds, selectively manage or inhibit gram-positive bacteria (such as lactic acid bacteria), and improve the process of clarification, stability, and filterability. The principal target of these enological enzymes is the macromolecular colloids present in grape juice or

wine, with a specific focus on polysaccharides. These polysaccharides can originate from the grape berry (such as pectin) or be synthesized by microorganisms (like glucan), subsequently introduced into the wine during winemaking. Pectolytic enzymes find use in breaking down the cell wall polysaccharides in grape berries (pectins, which are linear polysaccharides consisting of galacturonic acid units linked through α-1,4-glycosidic bonds). Consequently, they facilitate the release of juice, aromatic precursors, and phenolic compounds [114]. Applying these enzymes leads to a higher juice yield (with an increase in extraction rate of 5 to 10%). Additionally, this enzymatic activity aids in extracting aromatic precursors from the grape skins into the juice. The impact of this process varies based on grape varieties, maturity levels, and the duration of enzymatic action [115,116]. Following extraction, the grape must appear naturally cloudy and might contain residues in varying amounts, influenced by the intensity of mechanical treatments employed. As a result, clarification becomes essential to enhance the aromatic quality of the wine. While settling and grape must clarification often occur spontaneously, certain grape varieties may necessitate the addition of pectinases. The efficiency of the enzyme at this stage relies on factors like grape variety, grape maturity, and the conditions of harvesting and pressing [116]. Research has demonstrated that adding pectolytic enzymes enhances clarification and positively influences viscosity and filterability. This improvement is attributed to these enzymes breaking down intricate carbohydrate structures into more soluble oligomers, particularly in grape varieties characterized by high pectin levels [117]. In evaluating red wine, its color is one of the most crucial sensory attributes, significantly impacting its market acceptance. The red wine color and its stability throughout aging are primarily governed by anthocyanins and their interactions with other phenolic compounds. Among grape skin phenols, there are cell-wall phenols that bind to polysaccharides via hydrophobic interactions and hydrogen bonds and non-cell-wall phenols, encompassing phenols confined within cell vacuoles and those associated with the cell nucleus [112,118]. Anthocyanins accumulate in grape skins, and their extraction transpires during the maceration process. However, in traditional winemaking,

only about 40% of the anthocyanins are extracted from the skins during classic maceration [119,120]. The grape skin cell walls hinder the diffusion of phenolic and aroma compounds from grapes into the grape must or wine. It comprises roughly 30% neutral polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan, and mannan), 20% acidic pectin substances (of which 62% are methyl esterified), approximately 15% insoluble proanthocyanidins, and less than 5% structural proteins [99,112,121]. Consequently, the degradation of cell-wall polysaccharides is a pivotal stage for augmenting the release of phenols from grape skins [112]. Pectinases, cellulases, and hemicellulolases can catalyze the hydrolysis of bonds within cell-wall polysaccharides. Consequently, the inclusion of pectolytic enzymes (such as polygalacturonase, pectin methyl esterase, and pectin lyase), cellulolytic enzymes, and hemicellulolytic enzymes during the maceration process of red grapes enhances the extraction of anthocyanins from the skin of the berries. This results in an intensified color for the red wine [4,120,122-124]. Enzyme treatment has several benefits, including an elevated polyphenol content, intensified color, and enhanced color stability. The treatment fosters co-pigmentation reactions involving anthocyanins, tannins, and catechins, contributing to color stabilization. The primary goal of employing these commercial enzymatic preparations is the efficient breakdown of the grape cell wall, a significant barrier for the diffusion of aroma precursors, pigments, tannins, and other constituents. Nonetheless, the extent of hydrolysis is greatly influenced by factors such as the grape variety the type, concentration, and purity of the enzyme(s) applied [125,126]. Volatile compounds originating from grapes encompass monoterpenes, C13-norisoprenoids, and benzene derivatives, with many of these compounds exhibiting floral and fruity aromas. However, these compounds can exist as free, volatile odor forms or be bound to sugars, rendering them as odorless, non-volatile glycoconjugates [127,128]. These glycosidic aroma precursors can transform into aromatic volatiles through the action of glycosidase enzymes, which hydrolyze employing β-glucosidase activity [127,129–131]. Applying β-glucosidase can amplify the aromatic profiles of specific wines by breaking the β-1,4 bond, thereby releasing the volatile component from its

conjugated form [132]. Given the limited glycosidase activities found in grape, yeast, and bacterial enzymes, exogenous commercial enzymatic preparations possessing β-glycosidase activities are employed within the wine production process to enhance the release of volatile compounds bound to glycosides. These compounds, set free from glycosidic precursors, are profoundly influential in determining wine's aroma and varietal character, recognized as pivotal aspects of wine quality [133]. Another concern pertains to the instability of protein in white wine, which is linked to grape proteins, including those with defensive and pathogenesis-related (PRP) functions for the grape berry. These proteins encompass chitinases (28–32 kDa) and thaumatin-like proteins (20–25 kDa) [134–140]. Over time, the potential application of exogenous proteolytic enzymes to degrade unstable proteins and stabilize the wine has been under scrutiny [141]. However, these pathogenesis-related proteins resist enzymatic action under winemaking conditions [141–143]. Various researchers have explored the use of microbial proteases [144], such as those from *Aspergillus niger* [145], *Botrytis cinerea* [146], and *Saccharomyces cerevisiae* [147] to mitigate wine haze potential. Nonetheless, these endeavors were not uniformly successful due to the significant resistance of wine proteins to proteolysis. Marangon et al. [148] utilized an acid protease sourced from *Aspergillus niger*, particularly abundant in aspergillopepsin I and aspergillopepsin II, in combination with flash pasteurization (at 75°C) to eliminate unstable proteins. The heat treatment was necessary for protein degradation, rendering them susceptible to protease action. Although this approach proves effective technologically, using heat treatment comes with limitations, as it demands specialized equipment and is often avoided due to the potential for negative sensory impacts. In another study, Van Sluyter et al. [149] investigated a protease from *B. cinerea*, known as BcAP8, which demonstrated efficacy against chitinases during grape juice fermentation without necessitating heat treatment. Comuzzo et al. [150] demonstrated that the concurrent utilization of heat and proteases with Greek grape varieties holds promise for stabilizing wine proteins. It's important to note that the effectiveness of commercial enzyme preparations hinges on the purity of their enzymatic activities. Using poorly characterized products can lead to limitations and

inaccurate conclusions when comparing them with other treatments. Due to the inherent nature of commercial enzyme preparations, confirming the specific primary activity and potential side activities isn't always feasible, which complicates comparing commercial products and assessing their efficacy and possible adverse effects [113].

Future Trends in Enzyme Production and Usage

Due to growing environmental concerns, environmentally friendly and sustainable production is gaining attention in the enzyme bioprocessing industries. Sustainable technologies also need to be competitive in terms of efficiency and cost. As a result, the use of microbial enzymes in various processes is becoming a trend for adding value, conservation, and improving quality in the wine and beverage industry [151, 152]. However, natural enzymes, whether derived from raw materials or microbial sources, have limitations as biocatalysts. For instance, enzyme denaturation can occur due to low pH and other factors, such as the conditions in large-scale industrial fermenters and lower catalytic efficiency at ambient conditions [153]. Consequently, enzymes extracted from natural sources or used in native forms often fall short of meeting consumer requirements. To address these limitations, native enzymes are frequently engineered to function under non-physiological reactions, paving the way for designing novel biochemical pathways and producing new metabolites [153,154]. Enzyme engineering, or enzyme technology, offers a sustainable and environmentally friendly approach to industrial processes. With continuous advancements, creating more effective and efficient enzymatic systems that contribute to a cleaner and greener environment will become feasible. Novel enzyme activity and stability must be designed and developed to withstand challenging processing conditions associated with rapid and efficient enzyme purification strategies [8]. Therefore, in the upcoming years, there is significant potential for developing enzyme systems that combine high effectiveness and efficiency, requiring fewer resources and less energy to achieve optimal performance and maximum product yield. Thanks to advancements in molecular biotechnology, the manipulation of existing enzymes and the

creation of new ones have become increasingly achievable, making advanced enzyme systems more accessible than ever before [155]. Enzymes from non-GRAS (Genetically Recognised as Safe) microorganisms can also be cloned into microorganisms that are recognized as safe genetically, enabling their production at high levels [156]. Moreover, adopting enzyme immobilization technology has provided scientists with cost-effective alternatives for enzyme applications. This technology can enhance product yield, maximize profitability, and minimize environmental risks by attaching enzymes to solid supports [157,158]. Enzyme optimization in the wine industry involves employing various biotechnological tools, including immobilized enzymes. This approach effectively improves enzyme performance by anchoring enzymes to solid materials, enhancing operational stability, boosting activity, and reducing costs [159]. This presents a promising avenue for optimizing enzymes in the wine industry. Considering wine processing, using immobilized enzymes is safer and environmentally friendly. Immobilized enzymes are easily detachable and do not leave protein residues in the wine. Simultaneously, they can be reused multiple times [113,157]. Furthermore, immobilized multi-enzymes or co-immobilized cross-linked enzyme aggregates (combi-CLEAs) can be employed when the winemaking process involves the sequential engagement of multiple enzymes [160]. Another potential avenue is enhancing grape and microbial enzyme activities at various stages of winemaking [113]. Another crucial consideration is the substantial generation of by-products and waste in the form of spent grains and pomace within the beverage industry. These materials hold valuable bioactive compounds such as polyphenols, dietary fiber, and essential oils. Through enzymatic bioprocessing, these valuable components can be efficiently recovered for further utilization in food applications [161]. This approach facilitates the integration of by-products from beverage production back into the food chain, fostering an environmentally friendly and sustainable food system. However, it's important to acknowledge that biotechnology and microbial engineering tools may face negative consumer perceptions due to limited awareness of these technologies. The bioprocessing of extremozymes (enzymes produced by organisms thriving under

extreme conditions) can broaden the application range of enzymes and expand their market presence. Extremozymes originate from extremophilic microorganisms that can flourish and grow under extreme conditions [162]. These microorganisms can be isolated from diverse extreme environments such as hot springs, hydrothermal vents, saline lakes, and Antarctic/Polar regions [163], producing enzymes with distinctive properties pertinent to food and beverage applications. Other sources of extremoenzymes (including proteases, lipases, β-galactosidase, α-galactosidase, α-amylase, and esterase) encompass hydrocarbon-contaminated soil, superficial saline soils, acidic creeks and pools, acid mine drainage, brine pools, industrial wastewaters, refineries, exploration sites, mud sediments, and sewage treatment plants [163].

Concluding Remarks

Enzymes have gained increasing attention in the beverage industry in recent decades for their technological and economic advantages. They address deficiencies in the activity of inherent enzymes and are widely used. Various enzyme preparations are available on the commercial market. Most of these enzymes are used in beverages manufactured on a large scale using fungal hosts, such as yeasts and filamentous fungi. Lactic acid bacteria (LAB) are also a significant source of enzymes. However, certain strains exhibit slow growth, which is an obstacle to their industrial production. To overcome this, a prevalent approach is the direct application of LAB in wines to achieve the desired biochemical transformations. Enzymes play a crucial role in enhancing various stages of the production process. Pectinases and glycosidases particularly stand out for their role in clarification and aroma release.

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