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Biocatalysis in the winemaking industry: Challenges and opportunities for immobilized enzymes

Carminna Ottone | **Oscar Romero** | **Carla Aburto** | **Andrés Illanes** | **Lorena Wilson**

Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

Correspondence
Lorena Wilson, Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Av. Brasil 2085, Valparaíso, Chile.
Email: lorena.wilson@pucv.cl

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Abstract

Enzymes are powerful catalysts already being used in a large number of industrial processes. Impressive advantages in enzyme catalysts improvement have occurred in recent years aiming to improve their performance under harsh operation conditions far away from those of their cellular habitat. Production levels of the winemaking industry have experienced a remarkable increase, and technological innovations have been introduced for increasing the efficiency at different process steps or for improving wine quality, which is a key issue in this industry. Enzymes, such as pectinases and proteases, have been traditionally used, and others, such as glycosidases, have been more recently introduced in the modern wine industry, and many dedicated studies refer to the improvement of enzyme performance under winemaking conditions. Within this framework, a thorough review on the role of enzymes in winemaking is presented, with special emphasis on the use of immobilized enzymes as a significant strategy for catalyst improvement within an industry in which enzymes play important roles that are to be reinforced paralleling innovation.

KEYWORDS

biotransformations, enzymatic reactors, immobilized enzymes, industrial application, winemaking

1 | INTRODUCTION

Wine is the product of the biochemical transformations of the compounds present in grape juice by means of a controlled alcoholic fermentation. Yeasts convert sugars into ethanol and other metabolites, as well as into a wide range of volatile and nonvolatile compounds that significantly contribute to the sensory properties of wine: color, flavor, bitterness, sourness, and aroma. *Saccharomyces cerevisiae* is the most common yeast used for winemaking. In addition, molecules that have a positive impact on human health are synthesized through the fermentation of the must. (Samoticha, Wojdyło, Chmielewska, Politowicz, & Szumny 2017; Soyollkham et al., 2011.)

Traditionally, must fermentation was performed by cultivating the natural yeasts present in grapes skin, and the biotransformations occurred by means of the enzymes produced by such yeasts (Pretorius, 2000). This situation has radically changed: the advances in biotechnology and the better understanding of the function of enzymes make modern winemaking a high-tech industry (Delcroix, Günata, Sapis, Salmon, & Bayonove, 1994; Fernández, Ubeda, & Briones,

Nomenclature: 3-MH, 3-Mercaptohexan-1-ol; 3MHA, 3-Mercaptohexyl acetate; 4-MMP, 4-Mercapto-4-methylpentan-2-one; API, β-D-Apiofuranosidase; ARA, α-L-Arabinofuranosidase; CLEAs, Crosslinked enzyme aggregates; CSTR, Continuous stirred tank reactor; FEMR, Free enzyme membrane reactor; GOX, Glucose oxidase; k_d, Inactivation constant; K_m, Michaelis constant; MT, Monoterpene; NIP, Norisoprenoids; PBR, Packed-bed reactors; RAM, α-L-Rhamnopyranosidase; SF, Stabilization factor; STR, Stirred tank reactors; TLPs, Thaumatin-like proteins; V_{max}, Maximum reaction rate; βG, β-D-Glucosidase.

2000; Pretorius, 2000). Nowadays, the use of enzymes at different stages of the process is a well-established practice in the large-scale production of wine, and even in boutique wineries.

Indeed, several commercial enzyme preparations, with different activities, are available for their use in different steps of winemaking (Guérin, Sutter, Demois, Chereau, & Trandafir, 2009). Commercial enzymes act like the natural enzymes in grapes and yeast, but with enhanced activity, selectivity, and stability under operational conditions (Dequin, 2001; Pretorius, 2000). Commercial enzymes can be used in several steps of the winemaking process, going from postharvesting to postfermentation and aging. They have the benefit of accelerating the winemaking process, by reducing maceration (Ducasse et al., 2011), settling, and clarification times (Merín, Mendoza, Farías, & De Ambrosini, 2011), and by speeding up the release of aromatic compounds, which is a very important aspect in the sensorial appraisal of wine (Delcroix et al., 1994).

The use of commercial enzymes has proved to be quite advantageous in modern winemaking (Van Rensburg & Pretorius, 2000). When choosing a commercial enzyme, it is important to take into consideration if it was specifically designed for the requirements of each wine variety. The selection of the enzymes is important because off-flavors may appear or the varietal typicality may be masked when the enzyme selected is inadequate. Moreover, the timing and amount of enzyme addition at the different stages of the process are also important for having a good-quality wine; in many cases, that information is delivered by the manufacturer. However, both powder and liquid commercial preparations are based on free enzymes and, therefore, further separation of the enzymatic protein from the wine is necessary, which is usually achieved by the addition of bentonite (Pretorius, 2000). Limitations on the use of enzymes in the wine industry arise from their low stability under the operation conditions required in winemaking and the low efficiency of use of the soluble enzymes that cannot be reused (Ahumada, Martínez-Gil, Moreno-Simunovic, Illanes, & Wilson, 2016).

Nowadays, impressive advances in the field of enzyme immobilization make immobilized enzymes most promising catalysts in industrial biotechnology (Barbosa et al., 2015; Mateo, Maicas, & Thieβen, 2015; Mohamad, Marzuki, Buang, Huyop, & Wahab, 2015; Rodrigues et al., 2019). After more than four decades of development, enzyme immobilization is still facing many challenges with ample room for future opportunities in different fields of applications. Some industrial examples of the use of immobilized enzymes are found in the fields of sweeteners (DiCosimo, McAuliffe, Poulose, & Bohlmann, 2013), interesterification of edible oils and fats (Macrae & Hammond, 1985; Samoylova, Sorokina, & Parmon, 2016), prebiotics (Vera et al., 2016), and antibiotics (Illanes & Valencia, 2017). From all the advantages associated to immobilized enzymes as process catalysts, two are particularly relevant to their application in winemaking, namely, the production of a catalyst-free product and the development of a continuous process (Bolivar, Eisl, & Nidetzky, 2016; Poppe, Fernandez-Lafuente, Rodrigues, & Ayub, 2015). In this context, the use of immobilized enzymes in winemaking is only recent, but several reports have appeared in the last 3 years with the main purpose of process automation (Ahumada et al., 2016; Benucci et al., 2016; Cappannella et al., 2016; Liburdi, Benucci, Palumbo, & Esti, 2016; Lu et al., 2017).

This review presents a thorough review on the immobilization of enzymes and their use in modern winemaking. Most common immobilization methods and enzyme carriers for food applications will be compared and critically analyzed considering the specific requirements for winemaking. Mechanisms of immobilization of pectinases and glycosidases especially developed for clarification and aroma release, respectively, will be reviewed and analyzed. Also, recent advances in the immobilization of proteases, lysozyme, and urease for novel applications in the stabilization and polishing operations in winemaking will be reviewed. Finally, future research opportunities and challenges in the scale-up and automation of the enzymatic reactions in winemaking will be appraised.

2 | ENZYMES USED IN WINE PROCESSING

2.1 | Brief description of the winemaking process

Wine production considers four main stages schematized in Figure 1. First, the grapes are crushed by pressing and kept in maceration with the purpose of extracting as much as juice as possible for must formation. Here, the use of enzymes is considered as a pretreatment step, which precedes winemaking. Second, the alcoholic fermentation takes place, where the main specific features of the wine are obtained; in this part, the aroma release is an important issue that can be enhanced by the use of some specific enzymes, as explained below. Third, clarification step has the purpose of reducing the turbidity and viscosity of wine, as well as avoiding operational issues such as filter stoppages due to the high concentration of polysaccharides. The addition of pectic enzymes facilitates the clarification and filtration process. Lastly, aging and stabilization operations aim at obtaining the physicochemical properties of the final product, which can be improved by the use of suitable enzymes. The specific functions that a wide variety of enzymes have in winemaking will be detailed in the following sections.

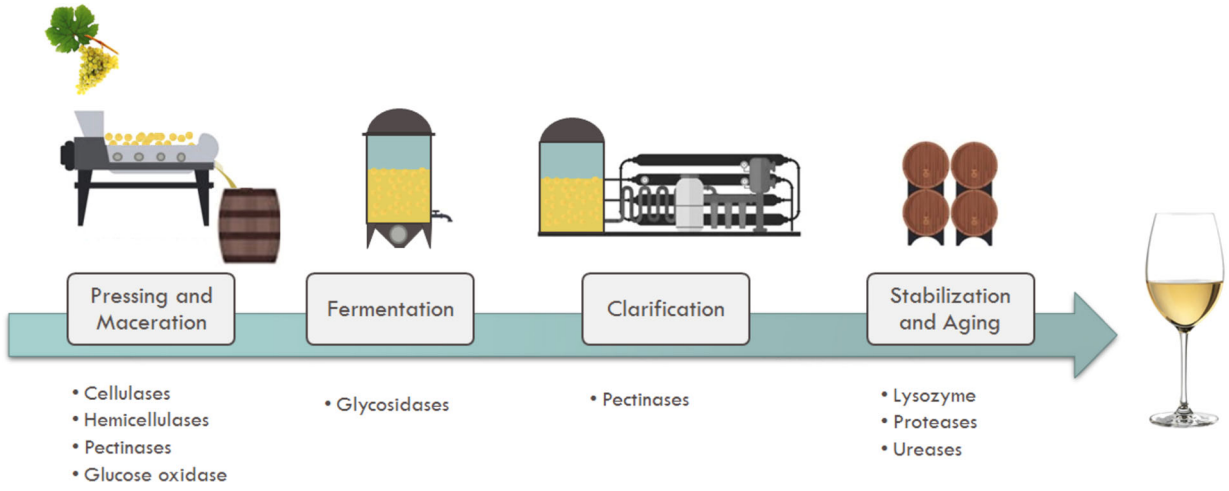

FIGURE 1 Diagram of the main steps in the winemaking process

2.2 | Source of the enzymes for winemaking applications

Wine industry, being considered as a part of the food industry, is therefore subjected to the same regulations. A large number of commercial enzymes used for wine processing are extracted from *Aspergillus niger* because it is a GRAS microorganism. In addition, approximately 90% of the enzymes produced in *Aspergillus* spp. are secreted into the culture medium, are resistant to the conditions of fermentation, and are accepted without restrictions for the elaboration of products destined for people consumption (Gummadi & Panda, 2003). However, other safe microorganisms (such as *Trichoderma reesei*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis*) have been also studied for high performance at winemaking conditions, which is of interest for all endogenous enzymes, including glycosidases, pectinases, proteases, and urease.

The use of enzymes, as well as the use of other additives in the winemaking industry, is regulated by the International Organization of Vine and Wine (OIV), which deals with different aspects related to winemaking. OIV defines issues related to the use of enzymes in winemaking, such as allowable enzyme activities, mode of application, and protocols for determination of activity. Most of the producing countries are regulated by OIV, except the United States, Canada, and China, which have their own national regulation (Hüfner & Haßelbeck, 2017).

2.3 | Enzymes added in the maceration step

It has been observed that the maceration of grapes with certain enzymes results in improved characteristics of wine (Garg et al., 2016; Revilla & José, 2003). The addition of enzymes during the extraction and maceration steps has four main objectives: (a) increase of the antioxidant activity, (b) extraction of aromatic precursors, (c) extraction of color, and (d) control of the alcohol content.

By increasing juice extraction efficiency, mixtures of pectinases, hemicellulases, and cellulases accomplish the first three objectives stated above (Garg et al., 2016). During the extraction and maceration of the must, cellulases and hemicellulases facilitate the rupture of grape cells and pectinases degrade the structural polysaccharides that hinder juice extraction: those compounds bound to the cells of the skin, pulp, and seeds, such as phenolic derivatives (with antioxidant activity) and the precursors of fragrances, are released and solubilized. The major components of red wine color are released as well.

Ethanol produced by fermentation is strongly related to the glucose concentration. Therefore, for controlling the content of ethanol in wine, glucose concentration is adjusted by adding glucose oxidase (GOX; EC 1.1.3.4) to the must. GOX oxidizes part of the glucose in the must using molecular oxygen as electron acceptor, as shown in Equation (1).

$$\mathrm{D-Glucose} + \mathrm{H_2O} + \mathrm{O_2} \xrightarrow{\text{glucose oxidase}} \mathrm{D-Gluconic\ \ Acid} + \mathrm{H_2O_2} \quad (1)$$

Conversions up to 40% have been obtained with this procedure (Ozturk & Anli, 2014). However, hydrogen peroxide is obtained as by-product that may oxidize other wine components, such as phenolic substrates (Pickering, Heatherbell, & Barnes, 1998). To solve this problem, a multienzyme process was proposed by including catalase (EC 1.11.1.6) in the reaction medium for reducing hydrogen peroxide to water. However, at the industrial level, the enzymatic approach to reduce ethanol content in wine is seldom used because dissolved oxygen is required for the reaction catalyzed by GOX. In white wine production, the presence of dissolved O_2 is undesirable because it is involved in side reactions that

alter their organoleptic properties (that is, color, aroma, and flavor) of the wine (Pickering et al., 1998).

2.4 | Enzymes added in the fermentation step

The concentration and proportion of volatile compounds determine the type of wine flavor (Samoticha et al., 2017). The fragrances of wine are classified into four types: varietal, prefermentative, fermentative, and postfermentative aromas. Varietal aromas are the result of the grape's own metabolism, and are influenced by the variety, degree of ripening, soil, climate, and terroir. Prefermentative aromas are produced by oxidation and hydrolysis reactions during the juice extraction and maceration steps. Herbaceous flavors caused by molecules, such as aldehydes and C6 alcohols, are mainly responsible for the prefermentative aromas. Fermentative aromas are produced by the yeast metabolism during the alcoholic and malolactic fermentations, where alcohols, acids, esters (giving a fruity, floral, or herbal aroma), carbonylated, and sulfur compounds are produced (Samoticha et al., 2017). The maturation of wine and the use of wooden barrels for wine aging are responsible for the postfermentative aromas.

Varietal aroma compounds in grapes are mainly conjugated with other molecules that render them nonvolatile. The aroma can be perceived only after the volatilization of such aromatic compounds (Lu et al., 2017). Hence, the role of the enzymes in wine aroma enhancement is the release of the volatile precursors, which can be a cysteinylated or glycosylated derivatives. Table 1 shows the enzymes that are involved in the release of varietal aromas in different types of red and white wines. Sauvignon Blanc and Muscat are the two more studied wine types with respect to the aroma release mechanism during winemaking because of their high concentration of cysteinylated and glycosylated derivatives, respectively.

Sauvignon Blanc wines are divided into two classes: "green" (vegetative, grassy, herbaceous, asparagus, green pepper, capsicum, and tomato leaf) and tropical (gooseberry, grapefruit, and passion fruit) (Coetzee & du Toit, 2012). Methoxypyrazine compounds are the most important compounds responsible for the "green" character in Sauvignon Blanc grapes and wines (Lund & Bohlmann, 2006). Those compounds are found in the grape and in the must conjugated with cysteine (Swiegers et al., 2007). Among them, 4-mercapto-4-methylpentan-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH), and 3-mercaptohexyl acetate (3MHA) have been identified. The olfactory threshold of these compounds is very low: 20 ng/L for 3-MH and 0.8 ng/L for 4-MMP, whereas the concentration in wines can reach values as high as 1,200 and 44 ng/L, respectively (Darriet, Tominaga, Lavigne, Boidron, & Dubourdieu, 1995; Tominaga, Baltenweck-Guyot, Des Gachons, & Dubourdieu, 2000). Tominaga et al. (2000) demonstrated that the formation of the volatile thiols 3-MH and 4-MMP involves an enzymatic pathway with β-lyase and their respective precursors (Cys-4-MMP and Cys-3-MH), as shown in Equation (2). Swiegers et al. (2007) showed that 3-MH can be transformed into 3-MHA via alcohol acetyltransferase from *Saccharomyces cerevisiae*:

$$\mathrm{S-3-(hexan-1-ol)-L-cysteine} \xrightarrow{\beta\text{-lyase}} \mathrm{3-mercaptohexan-1-ol + pyruvic\ acid} \quad (2)$$

Wine yeast strains have limited capacity for producing aroma-enhancing thiols from their nonvolatile counterparts in grape juice (Howell et al., 2004). To overcome this drawback, several studies have focused on the development of novel yeast strains with enhanced aroma release properties. Swiegers et al. (2007) developed a modified wine yeast with an overexpressed carbon–sulfur lyase activity. They found out that the recombinant yeast released up to 25 times more 4-MMP and 3-MH than the control host strain.

The aroma component in the glycoside is known as aglycone and the sugar moiety as glycone. Different molecules such as terpenoids, C6 compounds, aliphatic alcohols, benzenic derivatives, and volatile phenols can be aglycone constituents. The sugar part is represented by glucose or disaccharides (rhamnose–glucose, arabinose–glucose, and apiose–glucose) (Jibin, Xiao-Lin, Niamat, & Yong-Sheng, 2017).

With respect to aroma, terpenoids represent the most interesting constituents in white wine. Terpenoids can be divided into terpene alcohols and terpene aldehydes. The name of terpenoids is based on their number of isoprene subunits (Piñeiro, Palma, & Barroso, 2004). The most abundant are monoterpenes, sesquiterpenes, and C13-norisoprenoids. Terpenoids have a low olfactory detection threshold (Agosin, Belancic, Ibacache, Baumes, & Bordeu, 2000), and this property makes them the most studied components in the aromatic spectra of *Vitis vinifera*. Some important monoterpenes are limonene, 4-terpineol, terpinolene, citronellol, α-terpineol, linalool, hotrienol, and nerol oxide, and the most common terpene aldehydes are geranial, neral, and citronellal (Maicas & Mateo, 2005).

Monoterpene glycosides are nonvolatile compounds, so they are known as aroma precursors because they need to be released from the sugars before being perceived. The enzymatic hydrolysis of these compounds occurs by a two-step reaction mechanism, as shown in Figure 2 that schematizes the role of glycosidases for the volatilization of terpenes in wines by using a multienzyme approach.

In the first step, α-L-arabinofuranosidase (ARA; EC 3.2.1.55), α-L-rhamnopyranosidase (RAM; EC 3.2.1.40), β-D-xylopyranosidase (XYL; EC 3.2.1.37), and β-D-apiofuranosidase (API) dissociate the monoterpene from the respective residual sugar—arabinose, rhamnose, xylose, and apiose, respectively. In the second step, the monoterpene is released by hydrolysis with β-D-glucopyranosidase (βG; EC 3.2.1.21), known as β-glucosidase, with the concomitant

TABLE 1 Enzymes used for aroma release in wine

Enzymatic activity	Aroma classification / Volatile compounds	Aroma attribute	Wine type	References
Glycosidases: β-glucosidase; α-arabinosidase; α-rhamnosidase; β-apiosidase.	Terpenes/ α-terpineol; geraniol; nerol; linalool; citronellol.	Fruity and floral	Muscat	Cabaroglu et al., 2003; Jesus, Campos, Ferreira, and Couto, 2017
			Gewûrztraminer	Cabaroglu et al., 2003; Malherbe, Moine, and Kramer, 2014
			Riesling	Cabaroglu et al., 2003; Michlmayr et al., 2012
	Others/ C6 compounds, aliphatic alcohols benzenic derivatives; volatile phenols	Herbaceous flavors	White wines	Cabaroglu et al., 2003; Samoticha et al., 2017
	Norisoprenoids/ β-damascenone; 3-hydroxy-β-damascone; dihydro-α-ionona; 3-oxo-7,8-dihydro-α-ionol.	Grassy, tea, lime, and honey	Chardonnay, Syrah	Cabaroglu et al., 2003; Wang, Tao, Wu, An, and Yue, 2017
			Merlot, Cabernet Sauvignon	Cabaroglu et al., 2003; Murat, Tominaga, and Dubourdieu, 2001
	Norisoprenoids/ 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN); (E)-1-(2,3,6-trimethylphenyl); buta-1,3-diene (TPB).	Petrol aroma	Riesling	Cabaroglu et al., 2003; Sacks et al., 2012
β-lyase Alcohol acetyltransferase	Thiols/ 4-mercapto-4-methylpentan-2-one (4-MMP); 3-mercaptohexan-1-ol (3-MH); 3-mercaptohexyl acetate (3MHA).	Grapefruit, passion fruit, black currant, and box-hedge	Sauvignon blanc, Muscat Gewûrztraminer Riesling Chardonnay	Coetzee and du Toit, 2012; Darriet et al., 1995; Peyrot des Gachons, Tominaga, and Dubourdieu, 2000

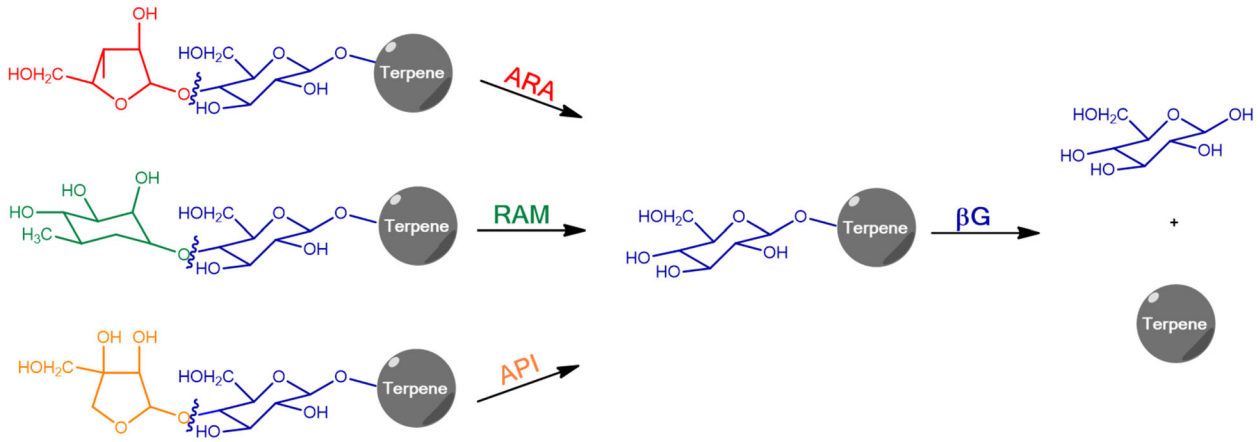

FIGURE 2 Scheme of the cascade reaction mechanism for the release of the glycosylated precursor molecules catalyzed by four different glycosidases: α-L-arabinofuranosidase (ARA), α-L-rhamnopyranosidase (RAM) and β-D-apiofuranosidase (API), and β-D-glucopyranosidase (βG). Modified from (Ahumada et al., 2016)

release of a second sugar—glucose. At this point, the released terpene is volatile and will be part of the wine aroma profile.

Previous works demonstrated that the enological conditions (pH, ethanol content, temperature, and presence of sodium metabisulfite) have an inhibitory effect on endogenous glycosidases from grape yeasts, such as *Saccharomyces cerevisiae* (Van Rensburg & Pretorius, 2000). Other yeasts isolated from enological ecosystems have been studied: for instance, βG from *Issatchenkia terricola* has shown high glycosidase activity with promising application for aroma enhancement of Muscat wine (González-Pombo, Fariña, Carrau, Batista-Viera, & Brena, 2011). Glycosidases used for aroma release come mainly from filamentous fungi (such as *A. niger*), performing well in the hydrolysis of glycoside flavor precursors (Cabaroglu, Selli, Canbas, Lepoutre, & Günata, 2003; Palmeri & Spagna, 2007).

2.5 | Enzymes added in the clarification step

Pectinases (E.C.3.2.1.15) are a heterogeneous enzyme group that break down high-molecular-weight pectic substances. They have been used for decades in the food and winemaking industries for fruit juice processing (Duvetter et al., 2009; Mohnen, 2008; Rajdeo, Harini, Lavanya, & Fadnavis, 2016; Ribeiro, Henrique, Oliveira, Macedo, & Fleuri, 2010). Pectic substances are heteropolysaccharides with a net negative charge that play an important role in plant structure, being the major constituents of the middle lamellae and primary cell walls of higher plants (Whitaker, 1990). Different enzyme activities are involved in the degradation of pectin; a scheme of the main activities is shown in Figure 3. Polygalacturonase hydrolyzes the α-D-1,4-linked galacturonic acid residues; pectin methylesterase hydrolyzes the bond between the galacturonic acid residues with their methyl esters; pectin lyase (blue) cleaves the bond between the α-l,4-D-galacturonic acid units with the β-1,2- or β-l,4-L-rhamnose units. Together with other polysaccharides, such as glucan (cellulose) and xylan (hemicellulose), grape pectins hinder the clarification and stabilization of must and wine. These polysaccharides are found in wines at levels between 300 and 1,000 mg/L and are often responsible for turbidity, viscosity, and filter stoppages (Van Rensburg & Pretorius, 2000).

The following classification has been proposed for pectinases, according to their function (Mojsov, 2016):

1. The hydrolysis of pectic acid, belonging to the hydrolase family, namely, endopolygalacturonase (EC 3.2.1.15) and exopolygalacturonase (EC 3.2.1.67);
2. The degradation of pectic acid by means of an elimination type reaction, belonging to the lyase family, namely, endopolygalacturonase lyase (EC 4.2.2.2), exopolygalacturonase lyase (EC 4.2.2.9), and endopolymethyl-D-galactosiduronate lyase (EC 4.2.2.10);
3. The cleavage of the methyl ester bond in pectin with esterase activity, namely, pectin methyl esterase (EC 3.1.1.11).

Exogenous pectinases are always needed for the clarification step, because they are not produced by the yeasts used for the fermentation. Pectinases are widely distributed in nature and are produced by bacteria, yeasts, fungi, and plants (Lang & Dörnenburg, 2000). In technical and economic terms, pectinases from microbial origin are the choice. Mostly used are pectinases from molds, particularly *Aspergillus* spp. The commercial pectic enzymes are regulated in the European Union by the OIV and usually their formulations contain polygalacturonase, pectin lyase, and pectin methyl esterase. The *Aspergillus* strain, the nature of the fermented substrate, the culture conditions, and the degree of purification lead to mixtures with varying proportions of these activities. Several companies offer different products with pectic enzymes in different proportions to suit the needs of each process (Ugliano, 2009; Van Rensburg & Pretorius, 2000). Commercial preparations are not always optimal for each particular process, and their use involves side effects, being this a matter of controversy. Pectin methyl esterase activity in pectinase preparations will be required for polygalacturonase to act, but when highly esterified pectin is used, an unacceptable increase of methanol in the products may occur (Vilanova et al., 2000). Pectic enzymes are the most abundant in so-called "pectinase" commercial preparations, but they contain other enzyme activities that are undesirable in winemaking, as is the case of polyphenol oxidases and cinnamyl esterases (Mantovani, Geimba, & Brandelli, 2005; Van Rensburg & Pretorius, 2000).

Generally, pectic enzymes are used in the clarification step to aid in the extraction process, maximize juice yield, facilitate filtration, and intensify flavor and color (Martín & Morata de Ambrosini, 2014). The addition of pectinase in winemaking reduces the viscosity and turbidity of the must. The turbidity is assumed to occur mainly via electrostatic destabilization of suspended, negatively charged pectin particles (Endo, 1965). Once this occurs to a significant extent, particles will be agglomerated and removed by centrifugation or filtration (Grassin, 1996). If the enzymes are added to the pulp before pressing, must yield increases, facilitating the pressing and enhancing the color. A high level of polygalacturonase is very effective for clarification but may require the prior action of pectin lyase. In order to transfer wine must between container vessels, racking should be fast not to leave sediment, so avoiding oxidation, growth of exogenous microorganisms, and loss of nutrients. To that purpose, enzyme preparations should have a high activity of pectin lyase. The use of pectinases in grape treatment will also aid in greatly reducing filtration time (Blanco, Sieiro, Díaz, Reboredo, & Villa, 1997).

Few studies have focused on the recovery of the soluble enzyme, which could have an economic implication in the

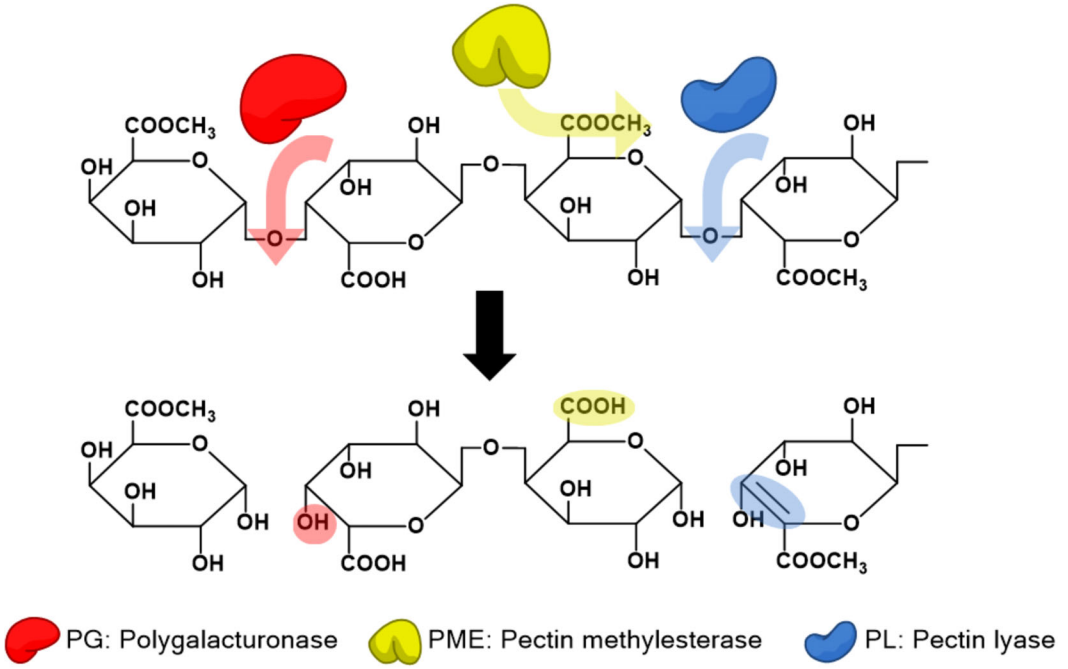

FIGURE 3 Hydrolysis of pectin chains by different enzyme activities: polygalacturonase (red); pectin methylesterase (yellow); pectin lyase (blue)

process (Grześkowiak-Przywecka & Słomińska, 2007). An alternative for the recovery of the enzyme is the use of a free enzyme membrane reactor (FEMR), whose main advantages are the reuse of the enzyme, the reduction of product inhibition, the high efficiency, and the low labor costs (Rodriguez-Nogales, Ortega, Perez-Mateos, & Busto, 2005; 2008). The major drawbacks of a membrane reactor are the rapid decreases in permeate flux due to fouling and the concentration polarization effect (Grześkowiak-Przywecka & Słomińska, 2007), although the use of hydrolytic enzymes helps to reduce those effects.

2.6 | Enzymes added for wine stabilization and aging

Wine is prone to spoilage by oxidation, secondary fermentation or the presence of excess protein. Therefore, it needs to be stabilized and polished, which may be accomplished by treatment with bentonite and the addition of sulfur dioxide. The development of an effective and cheap alternative to such methods has been searched for intensively. Addition of proteolytic enzymes to eliminate the excess of protein, lysozyme for microbial control, and urease addition for the removal of urea are promising options to conventional methods.

2.6.1 | Proteases

Protein haze is a problem that can affect white and rose wines due to the aggregation and precipitation of unstable proteins under postbottling conditions. Although this protein haze has no effect on wine taste or flavor, most consumers reject cloudy wines. The most abundant classes of haze-forming proteins that occur in grape juice and white wines are chitinases and thaumatin-like proteins (TLPs), which are small and compact proteins with a globular structure, stable and positively charged at wine pH (Van Sluyter et al., 2015).

The use of bentonite is by far the most common method to remove the proteins before wine bottling, but this laborious process produces wine losses and can negatively affect its sensory quality (Van Sluyter et al., 2013). An alternative to bentonite for removing these haze-forming proteins is their enzymatic hydrolysis with proteases. Several proteases from different sources have been applied to avoid haze formation. However, conventional and commercially available acid proteases, such as bromelain or papain, have shown low activities at winemaking conditions (15 to 18 °C and low pH). Moreover, several components of wine, such as phenols, tannins, and free sulfur dioxide, are strong inhibitors of proteases (Benucci, Esti, & Liburdi, 2015). Haze-forming proteins are known to be resistant to hydrolysis by exogenous proteases (Marangon et al., 2012).

In that direction, efforts for finding new and better sources of proteases have been fruitful (Mateo et al., 2015). Proteases from the yeast *Metschnikowia* spp. (Chasseriaud et al., 2015; Schlander, Distler, Tenzer, Thines, & Claus, 2017; Theron, Bely, & Divol, 2017) and the fungus *Botrytis cinerea* (Van Sluyter et al., 2013) have been successfully applied in preventing protein formation in white wines. Also commercially available proteolytic enzymes have been studied (Pocock, Høj, Adams, Kwiatkowski, & Waters, 2003). Due to the strong resistance to hydrolysis by chitinases and TLPs,

the enzymatic hydrolysis needs to be combined with heat treatment (90 °C for 1 min) to achieve a total removal of the unstable proteins (Pocock et al., 2003).

2.6.2 | Lysozyme

Lysozyme (*N*-acetylmuramide glycanhydrolase; EC 3.2.1.17) is added mostly in soluble form for the control of lactic acid bacteria in several foods. Its mechanism of action is related to the destabilization of the peptidoglycan cell wall structure in Gram-positive bacteria. Lysozyme acts by hydrolyzing the β-1,4 bonds between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid (Proctor & Cunningham, 1988). Lysozyme is present in almost all secretions, body fluids, and tissues of animals, but the commercial preparations are almost exclusively isolated from chicken egg white. There are economic and technical reasons for this choice: egg white is an abundant and inexpensive source of lysozyme (Kovacs-Nolan, Phillips, & Mine, 2005), and the obtained enzyme is more soluble, stable, and has a wider pH and temperature operational range than lysozymes from other sources (Abeyrathne, Lee, & Ahn, 2013; Salazar & Asenjo, 2007). In winemaking, lysozyme is used to prevent the growth of lactic acid bacteria, particularly from the genera *Lactobacillus* and *Pediococcus*, without inhibiting wine yeasts (Bartowsky, 2009; Liburdi, Benucci, & Esti, 2014). Lactic acid bacteria can produce substances leading to quality defects in wine, such as mousy taint, volatile acidity, and overt buttery characters (Liburdi et al., 2014).

Lysozyme also acts on malolactic bacteria like *Oenococcus oeni*, and besides the benefits of the malolactic fermentation (MLF) in some grape varieties and wine styles, further microbial control against post-MLF is needed. Sulfur dioxide is normally added to the wine for inhibiting MLF and preventing the development of other spoilage microorganisms. In recent years, much effort has been devoted to reduce the concentration of sulfur dioxide used in winemaking (Bartowsky, 2009). Alternatives to the use of lysozyme for wine stabilization have been explored, and actually several commercial enzyme preparations based on the action of lysozyme are available (Claus & Mojsov, 2018).

The main issue for lysozyme application in winemaking is the high dosages frequently used (250 to 500 ppm) due to the low activity of the enzyme at winemaking conditions (Cappannella et al., 2016). Furthermore, the utilization of lysozyme preparations from egg white implies adding a mandatory labeling on the final product indicating the presence of food allergens in it (Liburdi et al., 2014).

2.6.3 | Urease

Ethyl carbamate, which is formed from the reaction of urea and ethanol, Equation (3), is a naturally occurring compound that can develop in wine during storage and aging.

$$\left(NH_2\right)_2CO + C_2H_5OH \rightarrow NH_2COOCH_2CH_3 + NH_3 \quad (3)$$

The potent carcinogenic activity of ethyl carbamate has been demonstrated, so the FDA recommends several precautionary actions such as the degradation of urea by enzymatic hydrolysis (Gowd, Su, Karlovsky, & Chen, 2018; Pozo-Bayón, Monagas, Bartolomé, & Moreno-Arribas, 2012).

The feasibility of acid urease (EC 3.5.1.5) application for the removal of urea from several types of wines was demonstrated (Fidaleo, Esti, & Moresi, 2006; Ough & Trioli, 1988). However, the effectiveness of this treatment depends on the type of wine, the content of inhibitory factors, and usage conditions (Cerreti et al., 2016). As in other cases, the low activity of the commercial enzyme preparations at winemaking conditions makes necessary to look for better-suited enzymes or new strategies of application.

3 | IMMOBILIZATION OF ENZYMES FOR WINEMAKING APPLICATIONS

3.1 | Enzyme immobilization

Immobilized enzymes are powerful heterogeneous catalysts for industrial processes, whose main advantages are their robustness under operation conditions and the enhanced efficiency of use associated with catalyst recovery and the option of developing long-term continuous reactor operations. There is now an ample spectrum of immobilization methodologies (Barbosa et al., 2015; Betancor & Luckarift, 2008; Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007; Mohamad et al., 2015) but no general guidelines are applicable for optimizing the catalyst, which will depend on the specific reaction catalyzed, so that immobilization should be optimized in each particular case. Immobilization methods can be classified as carrier-bound or carrier-free (Figure 4).

Enzyme immobilization to a support can be done by establishing covalent bonds or noncovalent interactions between the (activated) support and the enzyme. Multipoint covalent attachment is the one conferring higher stabilization to the enzyme protein (Barbosa et al., 2015; Carlsson et al., 2014; Guisán, 1988; Gupta & Raghava, 2011; Jesionowski, Zdarta, & Krajewska, 2014; Mateo, Fernández-Lorente, Abian, Fernández-Lafuente, & Guisán, 2000; Mohamad et al., 2015). Support materials are many, chitosan, agarose, and silica being among the most used (Guisán, 1988; Mateo et al., 2006; Urrutia et al., 2014). Enzymes can also be immobilized by entrapment in a porous matrix of different materials, usually polymers. Among them, polyvinyl alcohol (PVA) outstands by offering many advantages (Cattorini et al., 2009; Durieux, Nicolay, & Simon, 2000; Illanes, 2011; Liu, Chen, & Shi, 2018; Lozinsky & Plieva, 1998; Porto et al., 2019). So, PVA has been thoroughly used for cell immobilization (Durieux et al., 2000; El-Naas, Mourad, & Surkatti, 2013; Lozinsky & Plieva, 1998; Nonthasen, Piyatheerawong, & Thanonkeo, 2015; Rebroš, Rosenberg, Stloukal, & Krištofíková, 2005;

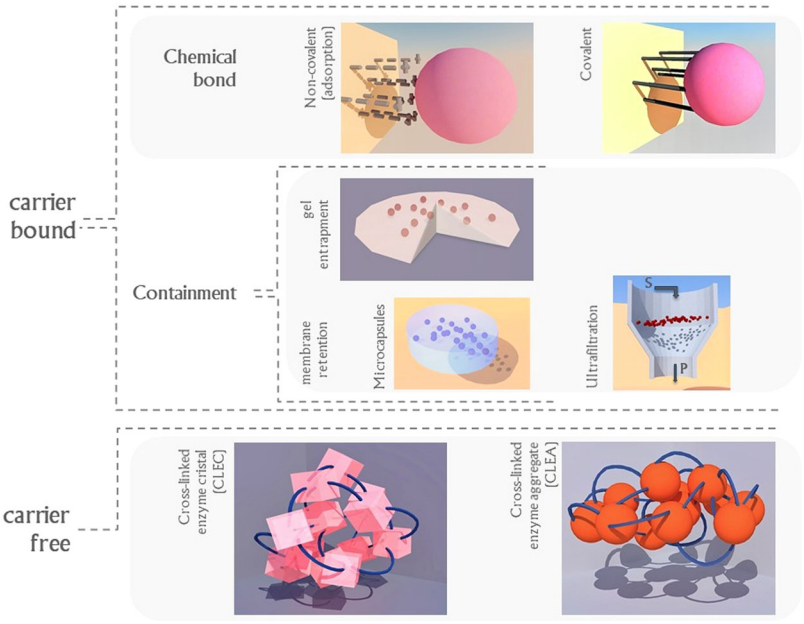

FIGURE 4 Scheme of carrier-bound and carrier-free immobilization methods

Wittlich, Capan, Schlieker, Vorlop, & Jahnz, 2004; Zhu et al., 2014). However, enzyme leaching out of the matrix is a major drawback, which can be avoided by encapasulation of the previously crosslinked enzyme, as illustated by the case of penicillin acylase immobilization (Wilson et al., 2004). Enzymes can also be entrapped by containment within a semipermeable membrane (Jochems, Satyawali, Diels, & Dejonghe, 2011).

Carrier-free systems consist in the immobilization of enzymes without support using the same protein structure as matrix, being crosslinked enzyme aggregates (CLEAs) quite appealing because of its simple preparation and lack of inert support. The enzyme protein is first precipitated under nondenaturing conditions and then crosslinked using a bifunctional reagent, without requiring the use of a highly pure enzyme preparation and avoiding the dilution of the enzyme in the solid supports (Gupta & Raghava, 2011; Sheldon, 2007, 2011; Sheldon & van Pelt, 2013; Sheldon, Schoevaart, & Van Langen, 2005).

Several reviews have been published regarding the most commonly used crosslinking agents, including glutaraldehyde, dextran-aldehyde, and PEI/carbodiimides (Sheldon, 2019; Xu, Wang, Li, Gao, & Zhang, 2018). Among them, glutaraldehyde is by far the most used because of its commercial availability and low cost in addition to its high reactivity (Migneault, Dartiguenave, Bertrand, & Waldron, 2004). Salient properties of CLEAs are their high specific activity and stability, low cost of production, and possible reuse (Sheldon et al., 2005; Wilson et al., 2006). Many reactions have been carried out using CLEAs of different enzymes (Gruškienė, Kairys, & Matijošytė, 2015; Gupta, Jana, Kumar, & Jana, 2015; Illanes et al., 2007; Sekhon et al., 2014; Vafiadi, Topakas, & Christakopoulos, 2008; Van Pelt et al., 2008; Velasco-Lozano et al., 2014; Wilson et al., 2006; Zerva et al., 2018). This strategy has allowed stabilizing the complex structure of multimeric enzymes (Wilson et al., 2004) and couple several enzymes within one catalyst particle (combi-CLEAs) (Ahumada et al., 2016; Araya, Urrutia, Romero, Illanes, & Wilson, 2019). Combi-CLEAs have been used in sequential or cascade biocatalytic processes with the advantage that the enzymes are confined in a delimited space and close to each other, unlike when solid supports are used (Chmura et al., 2013; Dalal, Sharma, & Gupta, 2007; Sheldon, 2007; Talekar et al., 2013; Wilson et al., 2006).

3.2 | Characteristics of immobilized enzymes for use in wine production

Some advantages of using immobilized enzymes in the wine industry as process catalysts are listed below:

1. Product free of catalyst: Using immobilized enzymes, additional operations for enzyme inactivation and removal from the product stream are unnecessary, being the presence of the enzyme protein in the final product objectionable in most of the cases. For instance, lysozyme cannot be present in wine, but if immobilized it can be easily separated from the wine, and therefore an allergen labeling on the final product is avoided (Liburdi et al., 2016).
2. Better reaction control: The point of maximum product yield at which reaction must be stopped is much easily attainable when the enzyme is immobilized and quickly removed from the product stream. A good example of this is the reaction of hydrolysis of glycosylated terpenes in young wines where a fraction of the aroma needs to be preserved for being released with time in the bottle; only a limited number of terpenes need to be released during the

wine processing (Ahumada, Urrutia, Illanes, & Wilson, 2015).

3. Better enzyme stability: This feature of immobilized enzymes is a major asset for enzymes used in winemaking, because operation conditions in the different stages of the process are harsh enough for enzyme performance, that is, low pH and ethanol (Benucci et al., 2015). The stabilization of the three-dimensional structure of the enzyme due to its immobilization favors the expression of its activity under process conditions.
4. Better management: The immobilization of the enzyme allows to manage the effects on the activity due to the presence of compounds such as phenols, tannins, sulfur dioxide, and strong enzyme inhibitors (Benucci et al., 2015), because higher enzyme doses can be added to compensate for the inhibition effects, without altering the properties of the product.
5. Continuous operation: Enzyme immobilization allows conducting operations in continuous mode, which is advantageous with respect to conventional batch in terms of productivity, and in the case of winemaking a high degree of automation in plant operation can be envisaged (Benucci et al., 2016).

The design of the biocatalyst is subjected to several requirements, as schematized in Figure 5. The biocatalyst needs to be active and stable at wine making conditions, not showing side activities and comply with GRAS standards, and hopefully with green chemistry principles. In this case, the selection of a suitable catalyst and support is very important because, given its application, it is necessary that the immobilized catalyst be adapted to the conditions of wine production, rather than the other way around.

Most works dealing with the use of immobilized enzymes have used the carrier-bound strategy. The most reported materials as enzyme supports for winemaking applications are polymer-based beads. Some commercially available acrylic-based materials have been proposed (Bortone, Fidaleo, & Moresi, 2012; González-Pombo et al., 2011). However, the most promising supports are made of biodegradable polymers, such as alginate (Busto, García-Tramontín, Ortega, & Perez-Mateos, 2006; Rehman et al., 2013) and chitosan (Benucci et al., 2016). Chitosan and alginate are nontoxic, inexpensive, and readily available in different formats (powder, gel, fibers, and membranes), so they are particularly suited for the immobilization of enzymes used in the food processing industry (Spagna et al., 1998; Urrutia et al., 2014).

Physical entrapment faces the problem of enzyme leakage, but this does not occur with covalent bonding (Tapias, Rivero, López-Gallego, Guisán, & Trelles, 2016). In fact, multipoint covalent attachment has demonstrated very strong enzyme stabilization and retention, which allows long-term continuous operation and obtaining a product free of catalyst, both aspect being quite relevant in food processing (Li, Li, Wang, & Tain, 2008) and particularly so in winemaking. Reports of carrier-free immobilized enzymes in connection with winemaking are scarce, the recovery of the catalyst from the product stream being a major hurdle because of the small and nonuniform size of CLEAs (Ahumada et al., 2015; Magro et al., 2016).

Even though there are many advantages of using immobilized enzymes, it is not always possible to use them and one has to rely on the use of soluble enzymes. This being the case, advances in the field are oriented to the bioprospecting of novel sources of more stable and active enzymes, and their improvement by genetic manipulations.

Next, recent advances on the use of immobilized enzymes in the different operations of the winemaking process are reviewed.

3.3 | Immobilization of enzymes added in the maceration step

Pectinases, cellulases, and hemicellulases are recommended to be used in free form during the maceration of the must. Free enzymes can work at the interfaces of the solid and liquid phases, which allow a higher interaction of the substrates (for example, cellulose and pectin) with the catalytic site of the enzyme than in the case of immobilized enzymes. The immobilization of pectinases will be addressed in the section of immobilized enzymes for clarification.

In this stage, it may be interesting to study the immobilization of GOX because GOX catalyzes the oxidation of glucose, which is a small substrate present in the liquid phase of the macerated must. Ruiz et al. (2018) studied the encapsulation of GOX in hollow alginate beads. The purpose of the work was to obtain an active biocatalyst at the pH of the must, because soluble GOX needs basic conditions to efficiently reduce the sugar content. The hollow beads had a semipermeable layer that offers a protective environment to the enzyme with an inner pH different from the pH in the must. The optimal pH of immobilized GOX showed a shift of one unit toward acidic pHs with respect to the soluble enzyme. The reusability of the immobilized GOX was studied in eight consecutive cycles.

3.4 | Immobilization of enzymes added in the fermentation step

Research on different immobilization approaches for the enzymes involved in wine aroma release reactions (βG, ARA, RAM, XYL, and API) has increased in recent years. In some cases, the co-immobilization of two or more glycosidases was evaluated. The research focused on the necessity of having a good interaction among the enzymes in order to

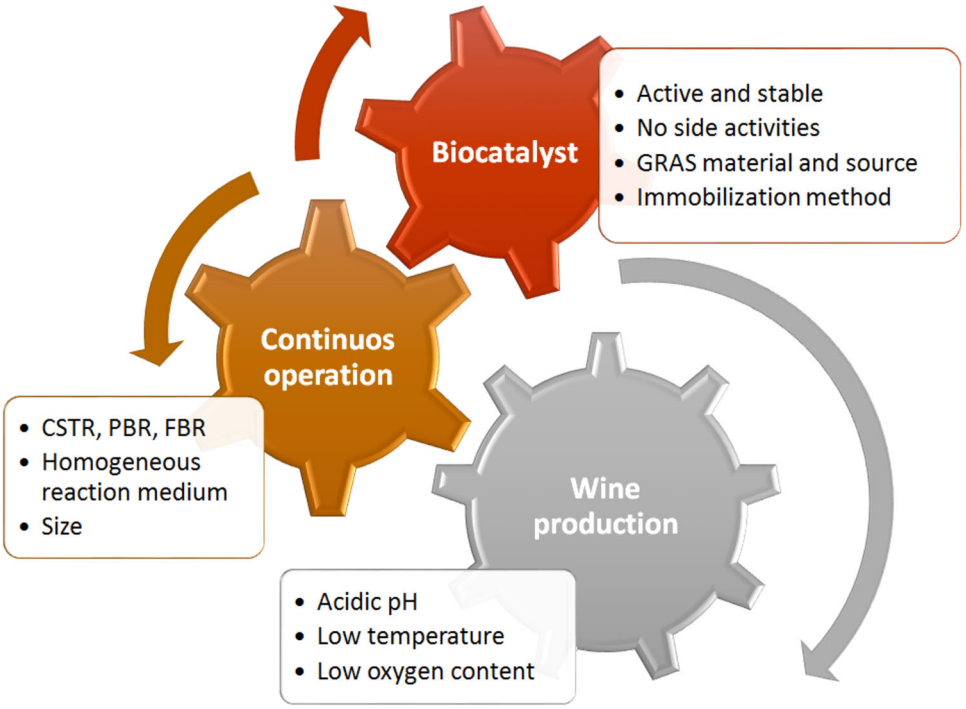

FIGURE 5 Diagram of the interconnections between the design of the biocatalyst and the operational conditions to achieve a continuous operation in wine production

increase the efficiency of the overall hydrolytic reaction. A summary of the different strategies reported for glycosidase immobilization is shown in Table 2.

Considering the conditions of the fermentation step, the biocatalysts are usually characterized in terms of thermal stability and residual activity at different pHs and ethanol concentrations. Several works reported results using a model wine solution, which consisted in a solution at pH 3.5 containing ethanol (ranging from 10% to 14% v/v), tartaric acid, malic acid, and sodium metabisulfite (Ferner et al., 2016; González-Pombo, Fariña, Carrau, Batista-Viera, & Brena, 2014).

Gallifuoco et al. (1998) studied the hydrolysis of terpenes with glycosidases supported on chitosan particles. They demonstrated that the catalysts were sensitive to the presence of ethanol in the medium. The first-order deactivation rate constant increased linearly with the ethanol concentration in the range from 3% to 12% w/v. Figueira, Sato, and Fernandes (2013) tested different supports for the immobilization of a purified β-glucosidase (βG), best results in terms of immobilization yield being obtained with sol–gel microparticles and PVA.

Glycosidases immobilized on chitosan particles were characterized in terms of optimum pH and kinetic parameters (that is, K_m and V_{max}) and were contrasted with the corresponding parameters of the free enzyme. For βG activity, a shift toward lower pH (from 4.5 to 4.0) was observed, whereas no variation of optimum pH was found for ARA (Spagna et al., 1998). The K_m of ARA increased with respect to the free enzyme, whereas the K_m of βG showed no variation. V_{max}, both for ARA and βG, decreased after immobilization. The authors attributed the change in the kinetic parameters to the presence of diffusional restrictions for the substrate. The immobilized enzymes were evaluated using a wine model solution containing 10% of ethanol, 9 g/L tartaric acid, and adjusted to pH 3.3 with potassium carbonate and were added with the aromatic precursors extracted and isolated from Muscat skin grapes. The authors observed a different trend of the activity toward tertiary and primary alcohol glycoside precursors between immobilized and free enzymes. In the case of free enzymes, the highest activity was observed with linalool, a tertiary alcohol glycoside precursor, whereas the contrary occurred by using immobilized enzymes, where activity on nerol and geraniol, both primary alcohol glycoside precursors, was higher than on linalool. This behavior was explained in terms of the impaired accessibility of linalool to the catalytic site in the immobilized enzyme.

The use of commercially available supports was also investigated. A βG from *Candida molischiana* 35M5N was immobilized in Duolite A568 resin and used for increasing the aroma in Muscat wine verifying the liberation of terpenes and an increase in enzyme stability due to immobilization (Gueguen, Chemardin, Pien, Arnaud, & Galzy, 1997). Similarly, Vila-Real et al. (2011) immobilized a naringinase preparation containing βG and ARA activities in ionic liquid sol–gel matrices. Epoxy-activated acrylic beads (commercially marketed as Eupergit C), among other commercially

TABLE 2 Immobilized glycosidases used for aroma release in wine

Enzymes	Source	Support	Biocatalyst parameters	Aroma released	Reference
ARA βG	*Aspergillus niger* Commercial preparation: Rapidase® AR 2000 (DSM, Deft, The Netherlands)	Crosslinked enzyme aggregate (CLEA)	$SF_{ARA} = 8.9$[a] $SF_{\beta G} = 33.8$[a] ARV = 18%[b]	Linalool Nerol Geraniol	Ahumada et al., 2016
		Chitosan	$SF_{\beta G} = 18$[a] $SF_{ARA} = 5.4$[a] ARV = 233%[b] $K_{m\ \beta G} = 0.60$ mM $K_{m\ ARA} = 1.3$ mM	Linalool Nerol Geraniol	Spagna et al., 1998
βG	*Aspergillus niger* Commercial preparation: Cytolase PCL5 (DSM, Deft, The Netherlands)	Chitosan	$k_{d\ \beta G}$ (12% w/v ethanol) $= 5.03 \times 10^{-4}$/hr $k_{d\ \beta G}$ (glucose 0.5% w/v) $= 6.6 \times 10^{-4}$/hr	–	Gallifuoco, Alfani, Cantarella, Spagna, & Pifferi, 1999
βG ARA RAM		Epoxy-activated acrylic beads (Eupergit C Rhon; Darmstadt, Germany)	$SF_{\beta G} = 1.22$[a] $SF_{ARA} = 5.44$[a] $SF_{RAM} = 1.14$[a] $ARV_{MT} = 186\%$[b]	Linalool Geraniol α-Terpineol norisoprenoids (vomifoliol and 3-oxo-a-ionol))	González-Pombo et al., 2014
βG	*Issatchenkia terricola*	Epoxy-activated acrylic beads (Eupergit C Rhon; Darmstadt, Germany)	$SF_{\beta G} > 5.5$[a] $ARV_{MT} = 35\%$[b] $ARV_{NIP} = 366\%$[b]	Monoterpenes norisoprenoids	González-Pombo et al., 2011
βG ARA RAM XYL	*Aspergillus niger*	polyvinyl alcohol–based magnetic particles	$K_{m\ \beta G} = 0.509$ mM	–	Ferner et al., 2016

Abbreviations: βG, β-D-glucosidase; ARA, α-L-arabinofuranosidase; RAM, α-L-rhamnopyranosidase; k_d, Inactivation constant, K_m, Michaelis constant; MT, monoterpene; NIP, norisoprenoids.

[a]Stabilization factor (SF) = $t½_{\text{soluble enzyme}}/t½_{\text{immobilized enzyme}}$; in wine or in simulated wine, at pH 3.5 and 16 to 23 °C.

[b]Aroma release variation: Enhancement of the aroma concentration in wine obtained with the immobilized enzyme with respect to the wine control.

available supports, have a remarkable stabilization effect on the protein conformation, which is attained by multipoint attachment. González-Pombo et al. (2014) developed a procedure for the co-immobilization of βG, ARA, and RAM onto Eupergit C starting from a commercial enzyme preparation from *A. niger*. Esterase activity can produce unwanted side reactions; therefore, glycosidases are usually purified before being added to the must. According to the method proposed by González-Pombo et al. (2014), the esterase activity present in the soluble enzyme preparation remained in the supernatant and thus the immobilized catalyst showed no esterase activity. A panel of expert wine testers analyzed the enzymatically treated wine. All judges (10/10) found significant differences between the control and the treated wine in favor of the latter; nine of them indicated that the fruit and floral flavors (related with the terpene release) accounted for such difference.

Magnetic beads are an alternative to commercially available supports having the advantage of easy separation from the reaction medium. Four enzymes, including βG, ARA, RAM, and XYL, were immobilized on PVA-based magnetic particles, functionalized with carboxyl groups on the surface for protein anchoring (Ferner et al., 2016). The authors observed no difference between the K_m of the immobilized and the free enzyme, suggesting that the enzyme conformation was not affected by the immobilization, which is in contrast to results reported in other studies (Spagna et al., 1998).

Recently, we proposed for the first time the co-immobilization of *A. niger* βG and ARA by aggregation and crosslinking generating the so-called combi-CLEAs (Ahumada et al., 2016; Ahumada et al., 2015). Combi-CLEAs are produced by nondenaturing protein precipitation followed by crosslinking with a bifunctional reagent, being an attractive

option to conventional immobilization to solid inert supports. The advantages of using combi-CLEAs rely on its simplicity, high specific activity, low production cost, and use of nonpurified enzymes. The authors performed enzyme stability experiments with combi-CLEAs in Muscat wine and evaluated the performance of such biocatalyst on the release of glycoside-based aroma compounds: terpenes, alcohols, and esters. Stabilization factors (SF = half life time of soluble enzyme/half life time of immobilized enzyme) for βG and ARA in combi-CLEAs incubated at simulated winemaking conditions were 33.8 and 8.9 for βG and ARA, respectively (Ahumada et al., 2015). The stability of βG there reported is the highest to date (Table 2). Regarding the aroma enhancement, the authors observed a higher release of total terpenes in wine treated with combi-CLEAs, being their concentration 18% higher than in the control wine. Because cascade reactions produce the liberation of terpenes in wine, the use of combi-CLEAs may result in a better performance because all the enzymes are in close proximity to each other (Sheldon & van Pelt, 2013).

Immobilization of glycosidases may allow a more controlled cascade reaction by means of an optimal balance of the activities of each immobilized enzyme. In addition, the proper design of the catalyst can assure a high activity of the different enzymes at the operational conditions throughout the reaction and thus increase the productivity of terpene release.

3.5 | Immobilization of enzymes added in the clarification step

Traditional technology for the clarification of juices and wine is characterized by enzyme being used only once (Grześkowiak-Przywecka & Słomińska, 2007). Despite the excellent catalytic properties of pectinases, using them in free format presents some drawbacks such as poor stability under operational conditions and low efficiency of use because recovery and reuse is not feasible (Sheldon, 2007). Therefore, immobilization of pectinase in a wide variety of carriers and methods is being considered interesting for clarification and depectinization, because of the increase in operational stability and biocatalyst reuse. Table 3 shows several methods developed for the preparation of immobilized pectinases, having each of them advantages and disadvantages. Several supports have been used to immobilize pectinases by entrapment. They include natural supports, such as alginate (Bustamante-Vargas et al., 2016; Busto et al., 2006; Rehman et al., 2013), agar-agar (Rehman, Aman, Zohra, & Qader, 2014), and synthetic supports, such as PVA (Cerreti et al., 2017; Esawy, Gamal, Kamel, Ismail, & Abdel-Fattah, 2013). The major advantage of entrapment, the simplest method of enzyme immobilization, is that catalytic particles of regular size can be produced. However, due to the high porosity and small size of the enzyme molecules, they may leak out of the polymer matrix.

There are several studies of carrier bound immobilization by covalent attachment. Lei and Bi (2007) immobilized pectinase covalently into silica-coated chitosan support and reported that the catalyst exhibited an increased resistance against thermal and pH denaturation exhibiting a wide optimal pH range of activity, between 3.0 and 4.5. Lei and Bi (2007) and Fang, Chen, Zhang, and Chen (2016) immobilized pectinase onto amino silica-coated magnetic nanoparticles and reported that the reaction ranges of pH and temperature were broadened, and the thermal stability, storage stability, and reusability were enhanced.

The immobilization on an inert support implies additional processing and materials costs, and the large amount of inert mass leads to a low specific activity of the biocatalyst. By contrast, crosslinked enzymes are insoluble stable catalysts without the dilution effect of an inert support, being obtained by a simple, rapid, and economic procedure. Magro et al. (2016) used combi-CLEAs of pectinase and cellulase that were 2.36 times more thermally stable than the free enzyme, being reusable for four cycles of operation. Combi-CLEAs produced a turbidity reduction of 56.7%, higher than for the soluble enzyme (47.9%), improving the clarification of grape juice.

Although several methods of immobilization have been used for pectinases, supports suitable for producing biocatalysts for technical applications should maintain a high level of expressed enzyme activity, have pore and particle sizes suitable for the diffusion of pectin into the enzyme niche, be stable at operational conditions and innocuous.

3.6 | Immobilization of enzymes added for wine stabilization and aging

Several immobilized enzymes, mainly covalently attached to a solid support, have been used for wine stabilization. Table 4 summarizes the different enzymes and supports used in wine stabilization.

3.6.1 | Immobilization of enzymes added for preventing protein haze

Stabilizing white wines by acid proteases is of great interest, and such enzymes have been used and commercialized for such purpose (Van Sluyter et al., 2013). Nevertheless, enzyme application in free form is limited by the stringent regulations enforced in European countries (Feijoo-Siota & Villa, 2011). Also, the low activity and stability of commercially available proteases at winemaking conditions has driven the attention to the use of them in immobilized format.

Because proteases have been utilized in a large number of industrial applications, several efficient strategies of immobilization have been carried out (Ataide, Gérios, Mazzola,

TABLE 3 Immobilized pectinases used for juice or wine clarification

Enzyme	Source	Support	Reference
Pectinases	Commercial preparations: Rapidase C80 (Gist Brocades, Deft, The Netherlands), Biopectinase CCM (Quest International, Naarden, The Netherlands), Grindamyl 3PA (Danisco, Copenhagen, Denmark) and Pectinex 3XL (Novozymes, Copenhagen, Denmark)	Alginate	Busto et al., 2006
	Commercial preparations: Pectinex Ultra Color and Pectinex BE XXL (Novozymes, Copenhagen, Denmark); Klerzyme 150 and Rapidase (DSM, Deft, The Netherlands); Panzym Smash XXL and Panzym YieldMASH (Begerow, Darmstadt, Germany)	PVA	Cerreti et al., 2017
	Commercial preparations: Pectinex Ultra SP-L, Pectinex Ultra Color, Pectinex Smash XXL, Novozym 33095, Pectinex Ultra Clear, Pectinex BE XXL (Novozymes, Copenhagen, Denmark)	Crosslinked enzyme aggregate (CLEA)	Dal Magro et al., 2016
	Commercial preparation	Activated glyoxyl agar-gel	Jeykumari and Narayanan, 2008
Alkaline pectinases	Commercial preparation: Qingdao Vland, 324 U/mL (Biotech Group Company Limited, Qingdao, China)	Fe_3O_4@SiO_2–NH_2	Fang et al., 2016
Polygalacturonase	Commercial preparation (Fluka Chemical Co., NY, USA)	Silica-coated chitosan	Lei and Bi, 2007
		Fe_3O_4/SiO_2@Carboxymethyl chitosan (CMCS)	Lei, Hu, Yang, and Lei, 2015
Pectinases	*Aspergillus niger* NRC1ami	PVA	Esawy et al., 2013
	Aspergillus niger ATCC 9642	Biomimetic matrix of alginate, gelatin/calcium oxalate	Bustamante-Vargas et al., 2016
	Aspergillus niger Commercial preparation: Rohapect®DA6L (AB Enzymes, Darmstadt, Germany)	Rigid polyurethanefoam	Bustamante-Vargas et al., 2015
	Aspergillus sp. Macer8 FJ	Glass microspheres, nylon 6/6 pellets, and PAN beads	Diano et al., 2008
	Aspergillus aculeatus	Epoxy activated polymer Dilbead	Rajdeo et al., 2016
		Florisil® and nano silica (activated with aldehyde groups)	Alagöz, Tükel, and Yildirim, 2016
	Bacillus licheniformis KIBGE-IB21	Alginate	Rehman et al., 2013
		Agar-agar and chitosan bead (activated with formaldehyde)	Rehman et al., 2014
Polygalacturonase	*Bacillus licheniformis* KIBGE-IB21	Alginate, polyacrylamide and agar-agar	Rehman et al., 2016
	Streptomyces halstedii ATCC 10897	Agarose (MANAE, PEI, and glyoxyl groups)	Tapias et al., 2016
	Mucor circinelloides ITCC-6025	Silica	Sharma, Rathore, and Sharma, 2013

TABLE 4 Immobilized enzymes used in wine stabilization and aging

Application	Enzyme	Source	Support	Reference
Protein haze removal	Bromelain	Native plant cysteine proteases (Sigma–Aldrich, St. Luis, USA)	Chitosan beads	Benucci et al., 2016; Benucci, Esti, Liburdi, and Maria Vittoria, 2012
		Pineapple stem (Sigma–Aldrich, St. Luis, USA)	Chitosan films	Zappino et al., 2015
		Pineapple stem (Sigma–Aldrich, St. Luis, USA)	Glass beads and acrylic beads	Benucci et al., 2012; Liburdi et al., 2010
	Papain	Native plant cysteine proteases (Sigma–Aldrich, St. Luis, USA)	Chitosan beads	Benucci et al., 2016
	Protease	*Aspergillus saitoi* (Sigma–Aldrich, St. Luis, USA)	Glass beads and acrylic beads	Liburdi et al., 2010
Microbial control	Lysozyme	Hen egg white	Chitosan beads	Cappannella et al., 2016; Liburdi et al., 2016
		Hen egg white	Magnetic polystyrene microparticles	Liburdi et al., 2012
		Micrococcus lysodeikticus (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China)	Chitosan nanowhiskers	Jiang et al., 2017
Urea removal	Urease	*Lactobacillus fermentum* (Nagase Europa GmbH, Düsseldorf, Germany)	Chitosan beads	Andrich et al., 2010b
			Acrylic beads	Andrich, Esti, and Moresi, 2009; Bortone et al., 2012

& Souto, 2018; Fernández-Lucas, Castañeda, & Hormigo, 2017). Examples of immobilized proteases applied in winemaking are few though different materials have been tested as support for covalent immobilization, such as chitosan, acrylic, and glass beads (Benucci, Esti, Liburdi, & Maria Vittoria, 2012; Liburdi, Benucci, & Esti, 2010). Stem bromelain immobilized on chitosan beads was successfully applied in a laboratory-scale stirred reactor, revealing a high capacity to reduce wine haze (by approximately 70%), without affecting its composition (Benucci, Esti, & Liburdi, 2014). Further studies showed that the immobilized enzyme was significantly more resistant to different inhibitors (ethanol, SO_2, skin, and seed tannins) than the soluble enzyme (Esti, Benucci, Liburdi, & Garzillo, 2015). Chitosan-immobilized bromelain was tested in a continuous packed-bed reactor (PBR). This immobilized enzyme was able to reduce the concentration of the protein in white wines by 68%, and the turbidity removal yield, calculated as the percentage of turbidity removed from the treated wine, was up to 96% (Benucci et al., 2016). These encouraging results showed that immobilized proteases can be applied as a sound alternative to conventional bentonite treatment for wine protein stabilization. However, this chitosan-immobilized bromelain showed a moderate stability when compared to other proteases immobilized by multipoint covalent attachment to glyoxyl agarose, used in other applications (Bahamondes et al., 2016). Although the use of immobilized proteases for white wine stabilization is promising, there is still room for improvement, including the exploration of other methodologies of immobilization.

3.6.2 | Immobilized lysozyme for wine microbial stabilization

Lysozyme has been successfully applied in winemaking, being an interesting alternative to sulfur dioxide in the control of lactic acid bacteria (Lisanti, Blaiotta, Nioi, & Moio, 2019). However, the high doses needed and the concern about allergic reactions are problems that can be tackled by lysozyme immobilization. Lysozyme covalently linked to a solid support can be easily and efficiently removed from the wine avoiding any concern regarding the allergenic properties of lysozyme that may impair wine labeling.

Despite that lysozyme has been immobilized in several support materials for microbial control (Bosio, Islan, Martínez, Durán, & Castro, 2016; Nahar, Mizan, Ha, & Ha, 2018), reports on immobilized lysozyme referred to its application in wine stabilization are few. Immobilization by covalent attachment to solid supports has been the preferred strategy, as in the case of other enzymes used in winemaking. Lysozyme was immobilized in chitosan, kinetically characterized and tested in different white wines (Liburdi et al., 2016). The immobilized enzyme showed a considerably lower V_{max} (maximum reaction rate) than the soluble enzyme, which can

be explained by an increase in steric hindrances due to the covalent linkage to the support. However, the antimicrobial activity of immobilized lysozyme was not affected by the concentrations of free SO_2 and total phenols at concentrations that produced 75% reduction of the activity of the soluble enzyme (Liburdi et al., 2016). The performance of the same immobilized enzyme was evaluated in a continuous fluidized-bed reactor (FBR) (Cappannella et al., 2016). The antimicrobial activity of soluble and immobilized lysozyme was measured in both model and real wines. The soluble enzyme showed a higher antimicrobial activity in the model wine; however, the immobilized enzyme showed a higher activity and better performance in real wine. The latter was even more noteworthy in red wine, probably due to its high concentrations of phenol and free SO_2. Immobilization has rendered the enzyme less prone to inhibition.

The immobilization of lysozyme on magnetic microparticles was reported by Liburdi, Straniero, Benucci, Garzillo, and Esti (2012). The enzyme was immobilized covalently to nonporous polystyrene microparticles and its kinetic behavior at wine pH was studied. Kinetic parameters of the immobilized enzyme differ from those of the soluble counterpart: the specificity constant (k_{cat}/K_m) decreases sevenfold after the immobilization. This difference is probably related to the structural modifications caused by the covalent attachment to the supports. The magnetic biocatalyst particles were easily separated from the reaction medium containing the product by applying a magnetic field, but despite this advantage, to the best of our knowledge, the use of immobilized lysozyme in magnetic microparticles in winemaking has not been reported yet. The main drawback of this system is the low activity of the immobilized lysozyme, mainly associated to diffusion limitations and steric hindrances. The substrate was located in a separate solid material (cell wall). However, this steric problem is associated with the immobilized enzyme, which can be reduced by applying the proper immobilization strategy (Guisán et al., 1997; Tavano, Berenguer-Murcia, Secundo, & Fernandez-Lafuente, 2018).

3.6.3 | Urea degradation with immobilized enzymes

Ureases are highly desirable in immobilized form for a number of applications, such as urea removal in fermented foods and beverages, analysis, and mineralization processes; therefore, the immobilization of urease has been extensively studied (Krajewska, 2009). Regarding the use of immobilized urease for urea degradation in fermented beverages, several examples in rice wine (sake) can be found (Zhang, Zha, Zhou, & Tian, 2016). Immobilization of urease on an inert carrier has the potential advantages of significant cost savings and process intensification. Consequently, continuous urea removal from sake with immobilized urease has been applied in Japan since 1988 (Matsumoto, 1993).

Even though the enzymatic hydrolysis of urea to NH_3 and CO_2 is a sound approach to avoid ethyl carbamate formation, the use of urease in grape wines has been barely reported. The effectiveness of this treatment is highly dependent on the type of wine, on the content of inhibitors, and other factors (Cerreti et al., 2016; Esti, Fidaleo, Moresi, & Tamborra, 2007). An alternative to overcome inhibition is the immobilization of urease. For example, urease immobilized into the chitosan-based support, Chitopearl, was more tolerant to the inhibitory effect of tannins extracted from grape seeds than its soluble counterpart (Andrich, Esti, & Moresi, 2010b). The same immobilized enzyme was tested in the degradation of urea in white wines (Andrich, Esti, & Moresi, 2010a). In white wines with low concentration of tannins, the difference in the rates of removal between soluble and immobilized enzymes was not so significant. However, soluble preparations of commercial enzymes usually have a low content of protein so that, even at maximum dosage allowed by regulations, the rate of urea degradation is low. An alternative to accelerate the process could be the use of immobilized urease, which can circumvent the concentration regulation because the biocatalyst is insoluble in wine and can be easily removed from it.

4 | OPPORTUNITIES AND CHALLENGES OF USING IMMOBILIZED ENZYMES IN THE WINE INDUSTRY

4.1 | Enzymes co-immobilization

Today many exogenous enzymes are important components in modern winemaking, and many preparations suitable for industrial use are now commercially available. However, many of these commercial enzymes were not specifically designed for application in wine, and being produced for other applications, they are not necessarily well-suited for performing under winemaking conditions, being in some cases strongly inhibited at such conditions. Most of the problems associated to the use of soluble enzymes can be solved by enzyme immobilization, because robust and more stable catalysts are produced and the catalyst can be recovered after use and utilized again with the advantage of delivering a product free of catalyst avoiding undesirable modifications of the organoleptic properties of the wine.

Co-immobilization of enzymes is certainly a more complex task than single enzyme immobilization and this explains why it is a rather recent subject of research that has been triggered by the advances in immobilization engineering. In general, different opportunities underline enzyme co-immobilization: reactions with coenzyme-requiring

enzymes that need coenzyme regeneration, product-inhibited reactions requiring product withdrawal from the reaction medium, and cascade reactions (Betancor & Luckarift, 2010).

Enzyme co-immobilization requires adequate immobilization methods considering a judicious selection of the support and mechanism of immobilization, and a thorough characterization of the enzymes and the kinetics of the reactions involved (Arana-Peña et al., 2019; El-Zahab, Meza, Cutright, & Wang, 2004; Hwang & Lee, 2019; Lopez-Gallego & Schmidt-Dannert, 2010; Ren et al., 2019; Sun et al., 2014; Torres & Batista-Viera, 2019; Yang, Dai, Wei, Zhu, & Zhou, 2019). It is expected that the activity of the enzymes can be preserved during co-immobilization while increasing their stability, hopefully attaining similar operational half-life values of each enzyme partner (Betancor & Luckarift, 2010). Several applications of enzyme co-immobilization have been reported both in the field of biosensors (Jeykumari & Narayanan, 2008; Niemeyer, Koehler, & Wuerdemann, 2002; Salinas-Castillo, Pastor, Mallavia, & Mateo, 2008) and in process development (Betancor, Berne, Luckarift, & Spain, 2006; Nouaimi-Bachmann, Skilewitsch, Senhaji-Dachtler, & Bisswanger, 2007; Wang & Zhang, 2015).

Combi-CLEAs is a promising strategy for the immobilization of commercial enzyme preparations containing several enzyme activities (Dalal, Kapoor, & Gupta, 2007; Sheldon, 2007; Talekar et al., 2013). Co-immobilization of enzymes in carrier-free systems was recently reported at the productive scale in winemaking (Ahumada et al., 2016). In this context, the co-immobilization of commercial preparations containing different activities stems out as an excellent opportunity for developing a sound technology for the winemaking industry by offering a simple procedure of constructing robust immobilized enzyme catalysts not requiring any purification of the enzymes present in the commercial preparation, so that a significant contribution to reduce the impact of catalyst cost on production cost is envisaged.

Even though combi-CLEAs may be recovered by centrifugation, the manipulation of particles whose size is small and hard to control is a major drawback for the application of CLEAs in industrial reactors, because a minimum particle size of 10 μm is required in order to separate the particles by filtration or sedimentation (Liese & Hilterhaus, 2013). This disadvantage certainly applies to combi-CLEAs; therefore, an alternative is its encapsulation or additional immobilization in a solid support to obtain a more robust and easy-to-handle catalyst to perform at a productive level. It is necessary to take into consideration the mechanical integrity of the CLEAs support during reactor operation. Gel beads from alginate and chitosan, may break down because of the shear forces produced by the agitator in stirred tank reactors (STRs), or by compression in long-term operations in PBRs (Martins dos Santos et al., 1997).

4.2 | Reactor configurations for winemaking

Maintaining the quality of the product is essential for the wine industry and for the consumers, so that there is a strict control for avoiding the contact of oxygen with the must during the different steps of the winemaking process (Cejudo-Bastante, Castro-Vázquez, Hermosín-Gutiérrez, & Pérez-Coello, 2011). Oxygen can react with phenols, darken the white wine, and react with some aroma components. Therefore, no mechanical stirring is used to homogenize the must and currently the must is subjected to a remounting step to improve mass transport during fermentation. Because good mixing is required for the unrestricted expression of the activity of immobilized enzymes, there is little option of using them in the same reactor configuration in which free enzymes are used, but they may well be used in other configurations. For example, in the case of the maceration and clarification steps, the addition of pectinases has improved the extraction and clarification efficiencies, respectively. The hydrolysis of pectin produces a transparent final product, which satisfies the consumer expectation. The requirement for a standardized product quality from batch to batch has prompted the investigation of using supported enzymes in winemaking, using a continuous mode of operation, which is unfeasible with free enzymes (Martín & Morata de Ambrosini, 2014).

Several configurations have been proposed and used for conducting enzyme-catalyzed processes (Illanes & Altamirano, 2008). However, reactors working under continuous operation are preferred for an automated process. The options for continuous operations are many, but the most used are PBRs and STRs with retention of the immobilized enzyme.

Cross-flow reactors (Lozano, Manjón, Romojaro, Canovas, & Iborra, 1987) and continuous stirred tank reactor (CSTR) (Biz et al., 2014; Iwasaki, Inoue, & Matsubara, 1998) have also been proposed as alternative modes of reactor operation at this processing step. Due to the product characteristics, STR is inadequate in winemaking because the agitation required to maintain the immobilized enzyme suspended promotes undesirable aeration. In this sense, PBR and FBR seem more adequate, because good substrate catalyst contact is achievable, and mass transfer rates (a key issue in heterogeneous catalysis) are good enough without resorting to agitation. FBR is frequently used, being especially recommended for highly viscous or insoluble substrates, as occurs in the different stages of wine production (Gómez et al., 2007). However, few studies have been conducted on the subject.

In the clarification stage, PBR and FBR were compared, showing that the latter was more efficient in pectin hydrolysis (De Oliveira, Dias, Da Silva, & Porto, 2018; Diano et al., 2008; Mahesh et al., 2016). Authors claimed that at a low circulation rate, the diffusion rate of the pectin into the catalytic site is high enough not to limit the activity of the enzyme and depectinization occurred at high yield, whereas

at a high circulation rate, substrate diffusion was limiting and depectinization yield was low.

Protein stabilization of white wine by treatment in a PBR containing immobilized proteases has been considered as a specific and mild alternative to the usual bentonite polishing operation (Benucci et al., 2016). A reduction of 68% of the protein content in white wines was obtained without affecting the content of phenol and mineral compounds, nor the sensory quality of the wine.

FBR with immobilized lysozyme was used in the enzymatic lysis of lactic acid bacteria in white and red wine to decrease the sulfur dioxide dosage required to control the malolactic fermentation, being quite efficient for such purpose (Cappannella et al., 2016).

FBR is a promising configuration for an automated winemaking process with immobilized enzymes. With this reactor configuration, selection of the support material is less restricted, because there are no compression effect, as in PBR, and no attrition, as in CSTR. FBR seems to be well in compliance with the requirements for preserving the organoleptic properties of wine.

Continuous reactors are necessary for developing an automated and standardized process. However, few studies have been focused on the study of the operational conditions of immobilized enzymes comparing different reactor types and modes of operation, which at the end will allow determining the best compromise among biocatalyst operational requirements, product quality, reactor investment, and operating costs.

4.3 | Challenges of using immobilized enzymes in winemaking

Enzyme immobilization has proven to have enormous technical advantages in the wine industry, but economic issues are fundamental requirements to take into consideration. This could be a particularly restrictive problem in the food processing sector, because most products have a low commercial value (Yushkova et al., 2019). With respect to immobilized enzymes, the carrier may represent an important fraction of the manufacturing cost of an immobilized enzyme (Tufvesson, Lima-Ramos, Nordblad, & Woodley, 2011). So, design and selection of the proper immobilized catalyst are crucial with respect to biocompatibility, chemical and thermal stability, reusability, and cost efficiency. Because immobilized enzymes are intended for reuse, it is necessary to take into consideration their mechanical integrity during reactor operation.

Different materials have been used for enzyme immobilization, including a variety of organic, inorganic, and hybrid materials. An important issue is the operational performance of immobilized enzymes at a large scale. On the one hand, gel beads, such as alginate and chitosan, have the advantages of being biodegradable and biocompatible. However, they have poor mechanical stability and may break down because of the shear forces produced by the agitator in STRs, or by compression in long-term operations in PBRs (Martins dos Santos et al., 1997). On the other hand, silica supports, having good mechanical properties, are usually small-sized particles whose recovery is cumbersome and expensive. Another important consideration is that the enzyme and also its carrier have to comply with GRAS standards and be approved by FDA, or similar regulatory agencies, to be used as processing aids (Andler & Goddard, 2018).

The wine industry uses reactors in the thousands-of-liters scale, therefore it needs technological solutions that focus on solving their particular problems. Even though there are many reports on the potential use of biocatalysts in the winemaking process, they are mostly referred to lab scale production. Novel materials and improvement of existing materials as carriers need to be developed for the design of more efficient, stable, biocompatible, and inexpensive biocatalysts (Zdarta, Meyer, Jesionowski, & Pinelo, 2018).

5 | CONCLUSIONS

Wine industry makes use of a wide variety of enzymes that are added in free form to the must. Advances in the production of enzymes as process catalysts, especially by enzyme immobilization, offer a very promising future for enzyme use in winemaking. Enzymes are being used for the improvement of several stages of the process, and pectinases and glycosidases stand out in clarification and aroma release, respectively. These enzymes perform well in terms of thermal, chemical, and mechanical stability at conditions of winemaking. Proteases, lysozyme, and urease have been used effectively for the prevention of protein haze, the control of lactic acid bacteria, and urea degradation, respectively. Reports considering the potential of immobilized enzymes in winemaking are many because catalyst stability is increased, catalyst can be recycled, and product free of catalyst is obtained. The co-immobilization of the enzymes that are present in commercial enzyme preparations currently used in the wine industry is a powerful strategy to build-up robust catalysts for the different stages of winemaking. Such co-immobilized catalysts will allow cascade reactions to be conducted, making the catalyst recoverable with a significant impact in the viability of the process by reducing enzyme expenditure, without interfering in the quality of the product. Presently, automated winemaking processes exist where the control of the enzymatic reactions involved will be favored by their use in immobilized form in those stages of the process where this is feasible. Potential use will be restricted though to the operational conditions and reactor configurations in

which immobilized enzyme can be used, which will have a significant effect on processing cost and product quality.

In summary, despite the obvious advantages of using immobilized enzymes, their use in the production of a quite sophisticated product such as wine still needs to solidly prove its benefit for the wine industry to adopt it.

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Carminna Ottone, Oscar Romero, and Carla Aburto jointly designed the structure of review and wrote the manuscript draft. Lorena Wilson and Andrés Illanes supervised the overall writing of the manuscript and critically revised and finalized it. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Carminna Ottone https://orcid.org/0000-0003-2713-2997
Oscar Romero https://orcid.org/0000-0002-0223-5167
Carla Aburto https://orcid.org/0000-0002-8886-8642
Lorena Wilson https://orcid.org/0000-0002-0791-920X

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