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K. Liburdi, I. Benucci, F. Palumbo, M. Esti

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LYSOZYME IMMOBILIZED ON CHITOSAN BEADS: KINETIC CHARACTERIZATION AND ANTIMICROBIAL ACTYIVITY IN WHITE WINES

Liburdi K.[a], Benucci I.[a*], Palumbo F.[a], Esti M.[a]
[a]Department for Innovation in Biological, Agro-food and Forest systems (DIBAF), Tuscia University, via S. Camillo de Lellis, 01100 Viterbo, Italy.
*Corresponding author: phone:+39 0761357426, Fax:+39 0761 357498; e mail: ilaria.bei@unitus.it

Abstract

In order to be able to use lysozyme as an anti-microbial agent during the winemaking process, hen egg-white lysozyme (HEWL) was covalently immobilized on chitosan beads. A cell suspension of *Oenococcus oeni,* an oenological strain involved in the winemaking process, was utilized as enzyme substrate. Both a kinetic study and evaluation of antibacterial activity, of the free and immobilized HEWL, were performed in model and real white wines. The catalytic parameters V_{max} and k_{cat} turned out to be higher for free-HEWL in model and white wines, demonstrating that covalent immobilization affected cell lysis velocity. However the K_m values were similar for the free and immobilized enzyme in wine model and only slightly lower in white wines for the immobilized biocatalyst. Moreover, the covalent immobilization also reduced the lysozyme antimicrobial efficiency, although it is worth noting that, in white wine, the antimicrobial activity of immobilized HEWL is not affected by the concentrations of free SO_2 and total phenols. Even though the proposed covalent immobilization strongly affected the lysozyme catalytic and antimicrobial efficiency, it improved the stability of immobilized HEWL in white wine, thus demonstrating its potential for use as an efficient antimicrobial agent in wine-making applications.

Key words: Hen egg-white lysozyme; White wine; Kinetic study; Enzyme immobilization; Antibacterial activity.

1. Introduction

In winemaking processes the hen egg-white lysozyme (HEWL), an enzyme with muramidase activity, is used to control the growth of *Oenococcus oeni* (Gerbaux, Villa, Monamy, & Bertrand, 1997; Gerbaux et al., 1999; Delfini et al., 2004; Cejudo-Bastante et al., 2010; Lasanta, Roldán, Caro, Pérez, & Palacios, 2010). HEWL is a 129-amino acid enzyme and as a muramidase it has the ability to hydrolyze the beta (1,4) glycosidic bond between the N-acetylmuramic acid and the N-acetyl-D-glucosamine of peptidoglycan, the major component of the cell walls of Gram-positive bacteria (Losso, Nakai, & Charter, 2000; Hashemi, Aminlari, & Moosavinasab, 2014). Use of lysozyme in winemaking was approved by OIV in 1997 (resolution OENO 10/97), while the successful control of lactic acid bacteria (LAB) growth using HEWL has been well demonstrated in

both white and red wines (Gao et al., 2002; Bartowsky, 2009; Lòpez et al., 2009). However, over the last decade several case studies have revealed severe allergic reactions due to the presence of lysozyme in wines (Weber, Steinhart, & Paschke, 2007; Weber et al., 2009). Thus, wines treated with lysozyme must be subject to specific labelling in accordance with the European Commission Regulation (EU) No. 1266/2010 of 22 December 2010, which amends directive 2007/68/EC as to the labeling requirements for wines. In light of HEWL's usefulness and its simultaneous allergenic properties, lysozyme immobilization could represent an interesting compromise for its use in wine. Having the lysozyme covalently immobilized on solid supports could allow for its removal from the wine so as to exclude the need for its presence in the label.

As reported by Liburdi, Benucci, and Esti (2014), HEWL has been successfully immobilised on various materials, including organic and inorganic polymers, using physical and chemical mechanisms. Generally chemical immobilization, such as covalent binding method, is mainly used when a reaction process does not require enzyme in the final product (Nisha, Arun Karthick, & Gobi, 2012). To date, few studies have addressed the development of covalent immobilized HEWL for winemaking applications (Zacchigna et al., 1999; Liburdi, Straniero, Benucci, Garzillo & Esti, 2012) and no study have been conducted in real wine measuring the kinetic and antimicrobial properties of insoluble lysozyme. In the last decades, many studies have been conducted relative to HEWL immobilised on different chitosan-based materials, especially for food packaging applications (Park, Daeschel, & Zhao, 2004; Duan, Park, Daeschel, , & Zhao, 2007; Yuceer & Caner 2014; Wen et al., 2016;). Chitosan and its derivatives, have proven to be excellent and safe candidates for food enzyme immobilization (Spagna, Barbagallo, Greco, Manenti, & Pifferi, 2002; Krajewska, 2004; Ge, Zhao, Mo, Li, & Li, 2012), mainly due to their non-toxic, biocompatible and biodegradable properties.

Moreover chitosan is considered as an antimicrobial agent when the high density of free amino group are present in the polymer structure (Liu, Du, Yang, & Zhu, 2006).

In this work, lysozyme was covalently immobilized on chitosan, the kinetic parameters and the antibacterial activity of both the free and immobilized HEWL were studied in model and real white wines using *O. oeni* as substrate.

2. Materials and Methods

2.1 Materials

The lysozyme-based (E.C.3.2.1.17) oenological preparation from hen egg white (HEWL) and *Oenococcus oeni* lyophilised cells, were kindly donated by Lallemand Œnologie (Blagnac Cedex,

France). The chitosan beads, named Chitopearl BCW-3010 (BCW), were obtained from Wako Chemicals GmbH (Neuss, Germany).

All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Chemical characterization of white wines

3 white wines, from different grape cultivars (Chardonnay, Moscato and Sauvignon *blanc*), were collected in an Italian market and used for the kinetic and antimicrobial study of free and immobilized lysozyme. The wine chemical parameters, reported in Table 1, were determined with officially endorsed tools such as the European Official Methods (Regulations (EU) No 2676/90). All samples were analyzed in triplicate, and the mean and standard deviation (±SD) were reported in Table 1.

2.3 Cell substrate characterisation

Oenococcus oeni cells (0.2 mg ml^{-1}), were rehydrated, washed in saline solution (NaCl 0.9%), and grown in 50 ml of modified ATB (Acidic Tomato Broth): casein peptone tryptic digest (10 g l^{-1}); yeast extract (5 g l^{-1}); glucose (10 g l^{-1}); $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}); $MnSO_4 \cdot H_2O$ (0.05 g l^{-1}); cysteine-HCl (0.5 g l^{-1}), and filtered tomato juice (25 % v/v; Oxoid LTD., England). The cell concentration was spectrophotometrically monitored by measuring the optical density at 600 nm (OD_{600nm}; Shimadzu 2450 UV/VIS) to describe the *O. oeni* growth curve until the steady state was achieved (28 hours at 37 °C and shaking at 170 rpm). At regular time intervals (1 h), cell culture aliquots were collected and diluted to obtain measurable OD_{600nm} values (below 2 Unit Absorbance, UA). At the same time, 0.1 ml of conveniently diluted culture was plated on agarised ATB medium (agar 15 g l^{-1}), and bacterial cell viability was measured as the number of colony forming units (CFU) per ml. The equation of the straight line ($y=2*108x$, where y: CFU/mL, x: OD_{600} and R^2: 0.99) was then used to calculate CFU/mL values corresponding to the different absorbance values and cell substrate concentrations used in the enzymatic assay. Moreover, cell stability was tested by monitoring OD_{600nm} for 300 sec in the absence of lysozyme. The experiment was performed three times with triplicates during the spectrophotometric measuring.

2.4 Immobilization procedure

The HEWL immobilization on BCW was performed using glutaraldehyde as crosslinker (Oliveira et al., 2001). These biocatalysts (GDA-HEWL) were incubated, with slow stirring, for 2 h at room temperature. At the end of the incubation, samples were washed three times with immobilization phosphate buffer (Na_2HPO_4/NaH_2PO_4 0.1 M, pH 7) and then let stand for 20 min in a 0.1-M glycine solution.

At the end of immobilization time, supports were washed three times with 2 M NaCl solution to remove all non-covalently bound proteins. Immobilization yield (IY, %) was determined by Bradford's method (Bradford, 1976), using *Coomassie* brilliant blue reagent and measuring absorbance at 595 nm. Bovine serum albumin (BSA) was used as standard.

2.5 Assay of HEWL catalytic activity

Kinetic characterization of free or immobilized lysozyme was carried out at 25° C, both in model wine (tartaric buffer, pH 3.2, ethanol 12% v/v) and in 3 different white wines (Chardonnay, Moscato and Sauvignon blanc) using *O. oeni* as substrate. The cell lysis was detected by measuring the decrease in optical density at 600 nm (OD_{600}) using a Perkin-Elmer Lambda 25 UV/VIS (Beaconsfield Buks, B).

The assay mixture contained increasing amounts of cell substrate (1×10^8- 4×10^8 CFU mL^{-1}), 0.5 mL of sucrose (0.27 M) for cells membrane stabilization , 0.5 mL of saline solution (NaCl 0.9 %), 0.1 mL of lysozyme (2 mg ml^{-1}) and tartaric buffer (pH 3.2) or real wine to reach a final volume of 3.5 mL.

The lytic reaction was carried out in 4-mL cuvettes with a 1 cm light path for 300 seconds at 600 nm with stirring at 25°C in a SHIMADZU UV 2450 spectro-photometer with a thermostated cell (MPM Instruments Type M 900-TI). One unit of lysozyme activity (U.) was defined as a decrease of 0.001 OD_{600} units/min. For each trial a blank correction was made, using a sample not containing enzyme.

The activity of immobilized lysozyme (GDA-HEWL) was determined by adding 1 g of lysozyme immobilized chitosan beads to the cell suspension (final volume 3.5 ml, as described previously) and agitating at 50 rpm with end-over-end rotation. After 30 min, the cell suspension was separated from the biocatalyst. The OD_{600} was measured before and after the treatment to calculate the activity. In order to consider the chitosan antimicrobial activity, a blank assay with the carrier beads without LYZ, was done to account the OD_{600} loss due to non-enzymatic reactions.

2.6 Determination of kinetic parameters

Kinetic parameters (V_{max}, K_M and $k_{cat)}$ were determined according to the Michaelis–Menten equation, fitting experimental data by a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.). The goodness-of-fit of each data set to its best-fit theoretical kinetic curve was assessed as the square of the correlation coefficient (R^2). K_m (Michaelis–Menten constant) is equal to the substrate concentration when the initial velocity is one-half of the maximum one (V_{max}), indicating catalytic efficiency. The k_{cat} (turnover number) refers to the number of substrate molecules converted into product, in a unit of time, by an enzyme molecule fully saturated with substrate.

2.7 Antimicrobial activity

The suspension of lyophilized *O. oeni* cells was prepared, in model or real white wines, to reach a final cell concentration of 10^7 CFU ml^{-1}. The free-HEWL and GDA-HEWL biocatalysts were prepared according to the above mentioned procedures and then brought in contact with the bacterial suspension (3.5 ml). The decrease in absorbance at 600 nm of the cell solution, which was continuously stirred and kept in contact with the free and immobilized lysozyme was monitored until a constant value was reached. In order to consider the antimicrobial activity of chitosan beads, a blank assay with the carrier beads was done. Each test was conducted in triplicate.

As reported by Conte, Buonocore, Sinigaglia, and Del Nobile (2007), the lysozyme antimicrobial efficacy can be determined by using the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts, and Van't Riet (1990):

$$N(t) = K + A\left\{-\exp\left[\left(\frac{\mu_{max}\, e}{A}\right)(\lambda - t) + 1\right]\right\} \quad (1)$$

where $N(t)$ (expressed as CFU ml^{-1}) is the microbial cell density at a certain process time (t), K is the initial value of $N(t)$ (CFU ml^{-1}), A is the maximum decrease of the cell density (CFU ml^{-1}), λ is the lag time (expressed in s), μ_{max} is the maximal decrease rate (expressed as s^{-1}) and this parameter was taken as a measure of the free and immobilized HEWL antimicrobial activity in real and model wine. Eq. (1) was fitted to the experimental data by a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.), where the quality of the regression was evaluated by the coefficient of determination (R^2) (Gil, Brandao, & Silva, 2006).

3. Results and Discussions

3.1 HEWL catalytic activity

HEWL was covalently linked onto chitosan beds activated with GDA, obtaining an IY of 70%.

Lysis of the bacterial *O. oeni* cell wall has been taken as a measure of the activity of lysozyme. In Figure 1 are reported the theoretical and real trends of the HEWL kinetic assays, for which all curves followed the hyperbolic behavior of the Michaelis–Menten equation. However, for both free-HEWL and GDA-HEWL, the curves indicate an excess-substrate inhibition in model and white wines. Kinetic parameters were calculated according to the Michaelis-Menten equation excluding the part of the curve wherein this substrate inhibition occurs (Table 2). Nevertheless, the experimental variability resulting from the use of a cell substrate did not allow us to estimate the K_i value according to the kinetic model of excess-substrate inhibition (Verhamme, Van Dedem, & Lawers, 1988). It has been reported previously that the use of *O. oeni*, as cell substrate causes a

pronounced lysozyme substrate inhibition at wine pH (Esti, Liburdi, Palumbo, Benucci, & Garzillo, 2014). From a microbiological point of view, it should be noted that the concentration of substrate at which inhibition starts is on the order of 10^8 CFU ml^{-1}, that is higher than the typical MLF one (10^6-10^7 CFU ml^{-1}, Bouix & Ghorbal, 2013). As a consequence we can exclude the possibility that inhibition happens during MLF.

As reported in Table 1, V_{max} and k_{cat} were always higher for free-HEWL in model and white wines, demonstrating that covalent immobilization reduced cell lysis velocity. However, thus far all reported immobilization systems for lysozyme were prone to unacceptable activity loss. Covalent binding appears to have been the major coupling method employed by various research groups, and the retained enzyme activities against *M. lysodeikticus* and *O. oeni* in these systems fell in the range of 0.8% to 14% of the activity of the free enzyme so as to probably preclude practical application (Liburdi, Straniero, Benucci, Garzillo, & Esti, 2012). Collectively, diffusion limitation, steric hindrance, excessive coupling, and coupling via amino acid residues essential for catalytic activity were concluded to be the main reasons for the very low retained activities (Wu & Daeschel 2007). The diffusion limitation of immobilized HEWL likely results because of the insoluble particulate nature of the microbial cell wall substrate. This system is doubly heterogeneous since the enzyme attached to a solid support must react with a substrate located in a separate solid material, namely, the cell wall. Additionally, steric factors may be more significant in this configuration than when the substrate is completely soluble. Moreover, Datta, Arminger, and Ollis (1973) first observed a reciprocal relationship between binding yield and the retained activity of immobilized protein, which has been repeatedly confirmed by subsequent reports (Crapisi, Lante, Pasini, & Spettoli, 1994; Chen & Chen 1996.).

The K_m values found were similar for the free and immobilized enzyme in the wine model. Nevertheless, GDA-HEWL appeared to demonstrate better catalytic properties in Chardonnay, Moscato and Sauvignon blanc, in terms of having better substrate affinity (lower K_m) than free-HEWL. This tendency, not very pronounced, could probably be due to non-covalent interactions (hydrogen bonds or van der Waals interactions) between the immobilized biocatalyst surface and the substrate (Longo & Combes, 1995). Similar results were reported by different researchers studying covalently immobilized HEWL on solid supports (Zacchigna et al., 1999; Wu & Daeschel 2007; Liburdi, Straniero, Benucci, Garzillo, & Esti, 2012).

As is well known, kinetic studies performed using the growing cell density give useful information about the HEWL catalytic efficiency in real wine. Nevertheless, these data don't provide useful information on *O. oeni* lysis when the lysozyme treatment is being used to prevent MLF. For this

reason, the modified Gompertz equation has been applied in order to better describe the lytic behaviour of *O. oeni* at the cell density exhibited during the MLF.

3.2 Antimicrobial study

Often the effectiveness of HEWL activity against different bacterial strains is assayed by simply recording the optical density decrease of the cell suspension (Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile, 2006; Conte, Buonocore, Sinigaglia, & Del Nobile, 2007; Conte et al., 2008; Esti, Liburdi, Palumbo, Benucci, & Garzillo, 2014). In this study, since non-growing of *O. oeni* suspensions was recovered during the rehydration step (data not shown), it is possible to affirm that the observed OD decrease was due to the HEWL antibacterial activity.

The antimicrobial efficiency of free HEWL has been widely described in the literature, whereas data concerning to antibacterial action of covalently immobilized lysozyme remain very limited. In order to quantify the respective Free-HEWL and GDA-HEWL antimicrobial activities, in both model and real wines, Eq. (1) was fitted to the experimental data using the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts, and Van't Riet (1990). The fitted curves obtained are reported in Fig. 2, while the Gompertz parameters (A, μ_{max}, and λ) were also estimated and are reported in (Table 3). In Fig. 2 the curves are shown, and the difference in cell decrease is evident, in that for model wine, Chardonnay and Sauvignon b. the antimicrobial activity of the free form was more effective respect to that of the immobilized one, whereas in Moscato, the curves of Free-HEWL and GDA-HEWL, having the same behavior, clearly demonstrate the inhibition effect of phenolic compounds on free lysozyme efficiency. In Moscato wine, a higher polyphenol content was measured, being 2 to 3 times greater than for Chardonnay and Sauvignon b., respectively. As reported by Zwietering, Jongenburger, Rombouts, and Van't Riet (1990), the most meaningful parameter with respect to the antimicrobial activity is μ_{max}. As can be observed in Table 3, R^2 values reveal that the Gompertz function satisfactorily fits the experimental data; therefore, it can be used with confidence to describe the *O. oeni* cell decrease in the presence of either free or immobilized lysozyme. The value obtained for the parameter A, showed the high cell lysis achieved with the free-HEWL in both real and model wine. A similar behavior was observed for lambda value which can be explained as the time needed to reach a plateau (A).

On the basis of the kinetic parameters reported (V_{max} and k_{cat}), the lysozyme antimicrobial activity (μ_{max}) was more highest for the free form in model wine, since the covalent immobilization of enzyme affected the HEWL activity. However, the μ_{max} of the soluble enzyme was decreased in the three real wines, although to differing degrees. On the other hand, the lower antimicrobial value, that was found for immobilized lysozyme, was not so strongly affected by the presence of chemical

components in the real wine. In figure 3, the calculated values of μ_{max} are reported as a function of the free SO_2 and phenol content to show the dependence of the antimicrobial activity on these compounds. As can be inferred from the data, the effectiveness of free-HEWL decreased, in a linear manner, with the increasing concentration of the two wine inhibitors considered. Thus, it can be assumed that the HEWL antimicrobial activity was clearly correlated with the free-SO_2 concentration and the total phenolic content of the three different white wines. The efficacy of HEWL in inhibiting undesirable lactic flora varies with the lysozyme structure (Ibrahim, Higashiguchi, Juneja, Kim, & Yamamoto, 1996) when condensed tannins are present in the medium (Guzzo, Cappello, Azzolini, Tosi, & Zapparoli, 2011). Indeed, procyanidinic tannin–protein interactions, as affected by ethanol, SO_2, and pH (Serafini, Maiani, & Ferro-Luzzi, 1997; Tirelli & De Noni 2007), are responsible for the inhibition of several enzymes in wine, including lysozyme (Rawel, Meidtner, & Kroll, 2005; Gonçalves, Soares, Mateus, de Freitas, 2007; Prigent et al., 2009). To the best of our knowledge, sulfur dioxide inhibition of lysozyme in wine-like conditions is still unknown, although its inhibitory effect on the activity of various enzymes, such as trypsin and plant metabolic enzymes, has been studied (Wedzicha, 1987; Malhotra & Hocking, 1976). It was demonstrated that sulfur dioxide is able to inactivate many enzymes by splitting their disulfide linkages; in particular, both sulfur dioxide and H_2SO_3 are able to convert disulfide bonds of enzymes or proteins to thiosulfonates and thiols (Malhotra & Hocking, 1976).

On the contrary the μ_{max}, calculated for GDA-HEWL, was not affected by the presence of free SO_2 and total phenols. Therefore, it is important that covalent immobilization seemed to render the enzyme less sensitive to the presence of these compounds. These results suggest that multi-point covalent immobilization of the enzyme reduces catalytic inactivation where chemical inhibitors are present. The mechanism for such process is not clear, although increased conformational stability is likely to be implicated (Mateo, Abian, Fernandez–Lafuente, & Guisan, 2000). However, an extensive literature on the useful dose of HEWL for controlling lactic acid bacteria during winemaking is available. Pitotti, Bo & Boschelle (1991) stated that 200-300 mg/L of HEWL provide a useful lytic effect in white wine. In must and new wine, the addition of 500 mg/L (distributed in 1 or 2 doses) of lysozyme is necessary in order to control the lactic bacteria spoilage (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). In our case, the protein immobilized was about 0,6 $mg_{protein}/g_{support}$ (± 0.01), and given that in white wine the useful quantity of carrier could be 120-180 $g_{support}$/L while in must and new wine this amount could be 800 $g_{support}$/L, this consideration represents a limit as to the multi-point covalent immobilized HEWL application during winemaking.

4. Conclusions

The present study, focused on the catalytic and antimicrobial efficiency of immobilized lysozyme in white wines, is useful in evaluating the use of the immobilized biocatalyst as anti-microbial agent during the winemaking process. The kinetic parameters, in model and real white wines, showed that after covalent immobilization K_m values did not change, whereas V_{max} was considerably altered. This may be explained in terms of the substrate's inability to reach the enzyme active site after immobilization on the solid support, in spite of the substrate affinity being maintained. As can be inferred from the catalytic study, the covalent immobilization also reduced the lysozyme antimicrobial efficiency. However the improved stability of the immobilized enzyme represents an interesting result, which even if it does not balance out the lose of catalytic efficiency, still provides a good basis for discussion about the application of lysozyme covalent binding procedures in order to prevent the allergenic protein release in food medium.

In conclusion, the present study shows that the proposed multi-point covalent immobilization cannot be readily applied for lysozyme since it results in an increase of steric hindrance to the active site and, as a consequence higher amount of carrier that should be used during winemaking to control lactic acid bacteria. However, the results reported here relative to the possibility of minimizing immobilized enzyme inactivation in white wine, suggest that it is worthwhile to investigate new and different immobilization mechanisms in order to create better lysozyme-based biocatalysts having antimicrobial activity in wine.

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432

Table 1. Chemical parameters measured in the three white wines: Chardonnay, Sauvignon b. and Moscato.

Wine	**pH**	**Titratable acidity** (Tartaric acid, g*L^{-1})	**Alcoholic degree** (ethanol, % v/v)	**Total SO_2** (mg*L^{-1})	**Free SO_2** (mg*L^{-1})	**Malic acid** (g*L^{-1})	**Polyphenols** (mg*L^{-1})
Chardonnay	3.4 (± 0.02)	5.6 (± 0.4)	13.5 (± 0.7)	90 (± 3.2)	18 (± 0.9)	1.3 (± 0.2)	191 (± 7.2)
Moscato	3.5 (± 0.01)	5.4 (± 0.6)	12.9 (± 0.6)	109 (± 4.5)	24 (± 1.5)	0.5 (± 0.03)	423 (± 12.3)
Sauvignon blanc	3.3 (± 0.01)	5.9 (± 0.3)	12.7 (± 0.8)	83 (± 2.1)	13 (± 1.1)	0.9 (± 0.06)	140 (± 11.8)

Table 2. Kinetic variables (Mean 6 SD) of free and immobilized HEWL toward *O. oeni* cell substrate in Model Wine and in three different white wines.

	V_{max} (U $mg^{-1}_{protein}$)	K_m (CFU ml^{-1})	k_{cat} (min^{-1})
Model Wine			
Free-HEWL	5309 (± 661)	$5*10^8$ (± $8*10^7$)	$8*10^6$(± 661)
GDA-HEWL	30 (± 1.2)	$5*10^8$ (± $1*10^8$)	$2*10^3$ (± 1.2)
Chardonnay			
Free-HEWL	3525 (± 558)	$1*10^9$ (± $2*10^8$)	$5*10^6$ (± 558)
GDA-HEWL	13 (± 2)	$2*10^8$ (± $6*10^7$)	$7*10^2$ (± 2)
Moscato			
Free-HEWL	773 (± 127)	$5*10^8$ (± $5*10^7$)	$1*10^6$ (± 127)
GDA-HEWL	3 (± 0.4)	$3*10^8$ (± $8*10^7$)	$3*10^2$ (± 0.4)
Sauvignon Blanc			
Free-HEWL	2098 (± 99)	$9*10^8$ (± $1*10^7$)	$3*10^6$ (± 99)
GDA-HEWL	10 (± 0,7)	$1*10^8$ (± $2*10^7$)	$1*10^3$ (± 0.7)

Table 3. Parameters obtained fitting the modified Gompertz equation to the experimental values of free and immobilized HEWL, in Model Wine and in three different white wines, toward *O. oeni* cell substrate[a].

[a] Values within parentheses indicate the confidence limits of the antimicrobial parameters. *K*, initial content of bacterial cell (CFU ml^{-1}); *A* maximum decrease in the cell density (CFU ml^{-1}); λ, lag time (expressed as s); μ_{max} maximal decrease rate (expressed as s^{-1}).

	k (CFU*ml^{-1})	**A** (CFU*ml^{-1})	μ_{max} (s^{-1})	λ (s)	R^2
Wine model					
Free-HEWL	$6.39*10^7$ ($6.02*10^7$, $6.07*10^7$)	$2.61*10^6$ ($1.31*10^6$, $3.91*10^6$)	$2.85*10^{-2}$ ($2.47*10^{-2}$, $3.23*10^{-2}$)	24.32 (21.46, 28.04)	0.99
GDA-HEWL	$5.36*10^7$ ($5.30*10^7$, $5.42*10^7$)	$3.37*10^7$ ($3.18*10^7$, $3.56*10^7$)	$2.90*10^{-3}$ ($1.59*10^{-3}$, $4.21*10^{-3}$)	239 (164.6, 435.9)	0.99
Chardonnay					
Free-HEWL	$5.49*10^7$ ($5.24*10^7$, $5.74*10^7$)	$6.45*10^6$ ($6.47*10^6$, $8.25*10^6$)	$1.47*10^{-2}$ ($12.49*10^{-3}$, $16.85*10^{-3}$)	47.25 (41.13, 55.50)	0.98
GDA-HEWL	$5.56*10^7$ ($5.34*10^7$, $5.85*10^7$)	$3.06*10^7$ ($2.85*10^7$, $3.27*10^7$)	$2.11*10^{-3}$ ($1.46*10^{-3}$, $2.77*10^{-3}$)	327.80 (250.31, 479.92)	0.98
Moscato					
Free-HEWL	$4.58*10^7$ ($4.64*10^7$, $4.70*10^7$)	$3.69*10^7$ ($3.55*10^7$, $3.82*10^7$)	$9.17*10^{-3}$ ($4.87*10^{-3}$, $13.47*10^{*-3}$)	75.62 (51.47, 142.40)	0.90
GDA-HEWL	$5.06*10^7$ ($4.92*10^7$, $5.19*10^7$)	$4.43*10^7$ ($4.27*10^7$, $4.58*10^7$)	$1.9*10^{-3}$ ($0.48*10^{-3}$, $3.33*10^{-3}$)	364 (208, 1466)	0.95
Sauvignon Blanc					
Free-HEWL	$6.53*10^7$ ($6.00*10^7$, $7.05*10^7$)	$8.43*10^6$ ($5.83*10^6$, $1.10*10^7$)	$1.96*10^{-2}$ ($15.55*10^{-3}$, $23.70*10^{-3}$)	35.32 (29.25, 44.58)	0.94
GDA-HEWL	$5.00*10^7$ ($4.77*10^7$, $5.24*10^7$)	$3.11*10^7$ ($2.57*10^6$, $3.65*10^7$)	$1.22*10^{-3}$ ($0.33*10^{-3}$, $2.10*10^{-3}$)	568.1 (239.3, 2066)	0.98

Figures and Captions

Figure 1. Specific activity (U. $mg^{-1}_{protein}$) of free and immobilized lysozyme (free-HEWL and GDA-HEWL, respectively) towards *Oenococcus oeni* substrate measured in wine model and in three different white wines (Chardonnay, Moscato and Savignon blanc). Dotted lines represent the theoretic Michaelis - Menten behavior.

Figure 2. Effectiveness of the free and immobilized lysozyme (-□- free-HEWL and -△- GDA-HEWL, respectively) against an *O. oeni* suspension in wine model and in three different white wines (Chardonnay, Moscato and Sauvignon blanc).

Figure 3. Antimicrobial activity (μ_{max}), of free and immobilized lysozyme (-□- free-HEWL and -△- GDA-HEWL, respectively) plotted as a function of the polyphenols (mg L^{-1}, **I**) and free SO_2 (mg L^{-1}, **II**) concentrations wines.

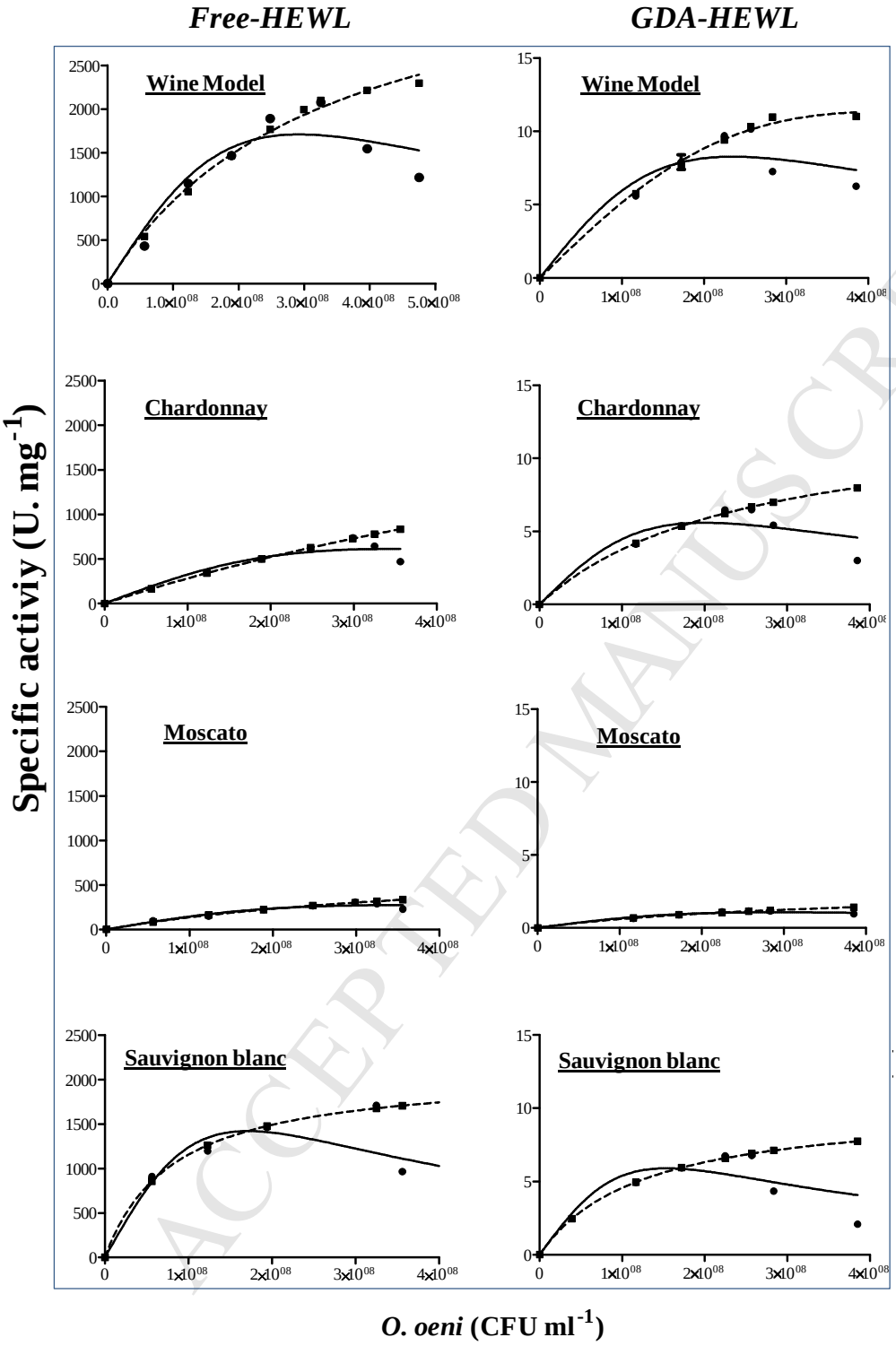

Fig. 1

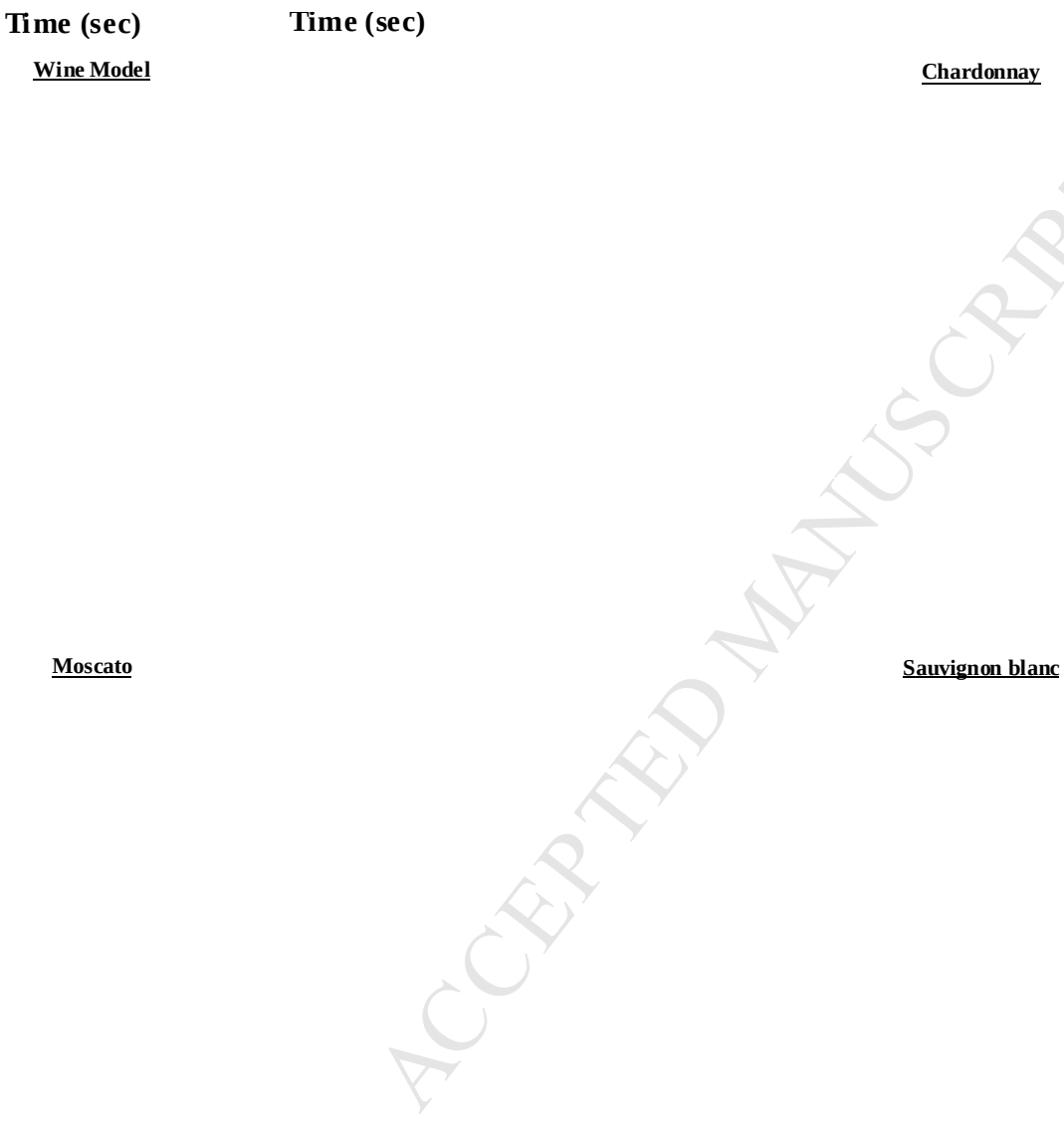

Fig. 2

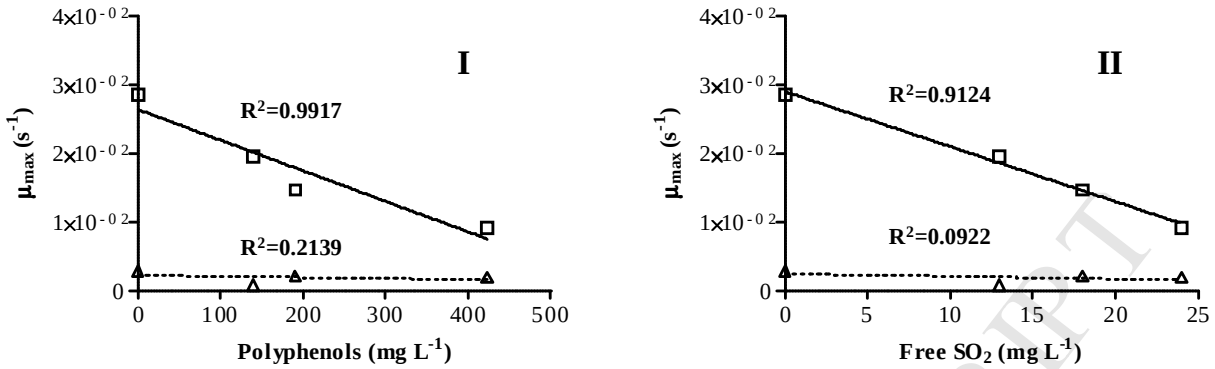

Fig. 3

- Catalytic and antimicrobial efficiency of lysozyme, covalently immobilized on chitosan beads, was tested in white wines.
- The covalent immobilization of lysozyme caused the loss of its catalytic and antimicrobial efficiency.
- The antimicrobial activity of immobilized biocatalyst, in white wines, is not affected by the wine inhibitors.