Immobilized lysozyme for the continuous lysis of lactic bacteria in wine: Bench-scale fluidized-bed reactor study

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Abstract

Lysozyme from hen egg white (HEWL) was covalently immobilized on spherical supports based on microbial chitosan in order to develop a system for the continuous, efficient and food-grade enzymatic lysis of lactic bacteria (*Oenococcus ~~O.~~ oeni*) in white and red wine. The objective is to limit the sulfur dioxide dosage required to control malolactic fermentation, via a cell concentration typical during this process.

The immobilization procedure was optimized in batch mode, evaluating the enzyme loading, the specific activity, and the kinetic parameters in model wine. Subsequently, a bench-scale fluidized-bed reactor was developed, applying the optimized process conditions. HEWL appeared more effective in the immobilized form than in the free one, when the reactor was applied in real white and red wine. This preliminary study suggests that covalent immobilization renders the enzyme less sensitive to the inhibitory effect of wine flavans.

Keywords: Hen egg white lysozyme; Microbial chitosan; Fluidized bed reactor; White wine; Red wine; Antimicrobial activity

1 Introduction

Lysozyme from hen egg white (HEWL-E.C. 3.2.1.17), is commonly used in soluble form to inhibit lactic acid bacteria in different foods. As a muramidase, HEWL catalyzes the hydrolysis of the β (1,4) glycosidi 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 (Lasanta, Roldán, Caro, Pérez, & Palacios, 2010), thu disrupting cellular structural integrity, which results in the lysis of the bacterial cells.

In winemaking, the traditional method applied to control malolactic fermentation is based on the use of sulfur dioxide, but this constitutes a health concern to sulfite-sensitive asthmatic consumers (Sonn Cejudo-Bastante, Chinnici, Natali, & Riponi, 2009). HEWL can be useful to control the spontaneous Gram-positive bacterial growth which often causes spoilage or stuck fermentation (Tirelli & De Noni, 2007), and thu its use is able to limit the necessary sulfur dioxide dosage (Liburdi, Benucci, & Esti, 2014) ~~(Esti et al., 2013)~~. Nevertheless, it has been already demonstrated (Weber et al., 2009) that the presence of free HEWL in wine ca itself cause allergic reactions. On account of this and in accordance with the recent European food legislation (Council Directive 2003/89/EC and 2007/68/EC; European Commission Regulation (EU) No. 1266/2010/EC) the use of lysozyme as an additive in wine must be declared on the ingredient label. To overcome this problem, the immobilization of HEWL on insoluble supports represents an interesting strategy for its application a it allows an easy recovery of the biocatalyst which can be reused in continuous process. In this context, HEWL has been covalently immobilized on different materials (Appendini & Hotchkiss, 1997; Zacchigna et al 1999), including chitosan from animal sources (Lian, Ma, Wei, & Liu, 2012). Recently, a membrane, based on microbial chitosan has been obtained for the first time for use as an enzyme immobilization carrier b

Zappino et al. (2015). However, to the best of our knowledge chitosan from *Aspergillus niger*, which is the only form approved for the food industry (EC No. 606/2009), has never been used for the production of spherica supports to be applied in a continuous bioreactor containing immobilized HEWL.

The properties of a carrier, especially its size, can influence the conformation and apparent activity of immobilized enzymes (Kahraman, Bayramoğlu, Kayaman-Apohan, & Gŭngör, 2007). Furthermore, becaus material modification is often easier, cheaper, and less detrimental than enzyme modification, techniques that alter material properties have been more frequently explored for immobilization technologies. Recently Talbert and Goddard (2012) reported that by manipulating the properties of a support (i.e. reducing the water content and the size), the immobilized enzyme conformation and/or apparent activity can be maintained o enhanced. Moreover, lipase from *Candida rugosa*, immobilized on two types (dry and wet) of chitosan beads, showed different properties, both in terms of enzyme stability and catalytic efficiency (Chiou & Wu, 2004 The low retained activity of immobilized HEWL that has been described by numerous authors (Jiang, Long, Huang, Xiao, & Zhou, 2005; Lian et al., 2012; Çetinus & Öztop, 2003) is mainly due to diffusion limitation an steric hindrance, since the biocatalytic system appears doubly heterogeneous: the enzyme attached to a solid support has to react with a substrate located in a separate solid material (cell wall). This problem could b largely overcome by using a fluidized bed reactor (FBR), which is especially recommended when the substrates are viscous or contain suspended particles (Gòmez et al., 2007). In this reactor, the flow of substrat keeps the immobilized enzyme particles in a fluidized state, thus obtaining a high catalytic surface area. Moreover, the liquid flow maintains the biocatalyst in a hydrodynamic balance between the force of gravity an drag forces derived by the upflow substrate stream (Van Zessen, Tramper, Rinzema, & Beeftink, 2005). Several studies have reported the widespread and successful application of FBRs in the food industry, fo example, for apple juice clarification (Diano et al., 2008), the production of fructo-oligosaccharides and invert sugars (Lorenzoni et al., 2015), lactose hydrolysis (Roy & Gupta, 2003), the flavour enhancement c beverages (Gueguen, Chemardin, Pien, Arnaud, & Galzy, 1997) and other catalytic reactions (Saponjic et al., 2010). The benefits derive from the continuous operational mode as well as improved mass transfe (Lorenzoni et al., 2015).

The aim of this work was to realize an efficient, food-grade and continuous bioprocess to control the spontaneous *Oenococcus* ~~*O.*~~ *oeni* bacteria growth in white and red wine, and to demonstrate the potential use c lysozyme immobilized on microbial chitosan beads in a fluidized-bed reactor.

2 Materials and methods

2.1 Materials

The hen egg-white lysozyme (HEWL, EC 3.2.1.17; systematic name: peptidoglycan *n*-acetylmuramic hydrolase) and the *O. oeni* lyophilised cells (lot No. E6003/3014/8032) were oenological preparations kindly supplied b Lallemand Inc. (Italy). The protein content of the lysozyme preparation was 8% (Bradford, 1976).

Chitosan powder from *A. niger*, purchased from KitoZyme S.A. (Herstal, Belgium), served to produce the beads used as carrier for HEWL immobilization.

The real Italian wines (vintage 2014) used in this study were: i) Sauvignon blanc white wine (pH: 3.3; Total acidity: 5.9 g l^{-1} tartaric acid; alcohol: 12.9% v/v; total SO_2: 83 mg l^{-1}; free SO_2: 13 mg l^{-1}; malic Acid: 0.9 g l^{-1}; tota polyphenol: 141 mg l^{-1} gallic acid) from Casale del Giglio winery (Le Ferriere, LT); ii) Sangiovese red wine (pH: 3.2; total acidity: 7.2 g l^{-1} tartaric acid; alcohol: 13.6% v/v; total SO_2: 67 mg l^{-1}; free SO_2: 21 mg l^{-1}; malic Acid: 0.5 g l^{-1} total polyphenol: 1640 mg l^{-1} gallic acid) from Gentili winery (Cetona, SI). All other chemicals were of analytical grade and were purchased from Sigma–Aldrich (Milano, Italy).

2.2 Chitosan bead preparation

Chitosan powder was dissolved in an aqueous solution of acetic acid (5 v/v%) and kept, under stirring conditions, until complete solubilisation was achieved. The solution was then added dropwise, through a peristaltic pum (Minipuls 3 Gilson, Italy), into a gently stirred coagulation liquid (2 M sodium hydroxide and 26 v/v% ethanol). The macrospheres obtained (wet beads) were filtered and washed with distilled water (Birò, Nemeth, Sisak, Feczko, & Gyenis, 2008). With the aim to improve the characteristics of this support, the carrier was dehydrated in a stove at 40 °C for 48 h (to produce dry beads).

2.3 Immobilization procedure

In separate experiments, 1 g of coupling agent (C_n) and 15 ml of 50% v/v ethanol solution was added to 1.0 g chitosan beads (either dry or wet) and gently shaken at 70 °C for 12 h. Then, the activated beads were collected an washed with distilled water in order to remove the excess of C_n.

The coupling agent (C_n), containing

CHO groups, can covalently cross-link with both chitosan and enzyme (Chen et al., 2010). Thus, the cross linking probably occurs through Schiff's base reaction between the

CHO groups of C_n and the

NH_2 groups of both chitosan and enzyme (Chen et al., 2010).

An increasing amount of enzyme in solution, which consisted of HEWL solubilised in distilled water (0.5–6 mg ml^{-1}), was mixed with 0.25 g of chitosan beads (dry weight) and 1 ml of acetate buffer 0.2 M (pH 5.6). After shakin at 30 °C for 8 h, unbound enzyme was removed from the supports by extensively washing with ammonium sulphate (2 M) and tartaric acid buffer (pH 3.2).

The amount of enzymatic HEWL protein bound to the chitosan beads was indirectly determined as the difference between the concentration of protein into the enzyme solution before and after immobilization. The protei concentration was determined by Bradford's method (Bradford, 1976), using Coomassie brilliant blue reagent and measuring absorbance at 595 nm. BSA was used as standard protein.

2.4 Immobilization efficiency determination

Immobilization efficiency (EI, %) was determined to provide a measure of the enzyme's specific activity after the immobilization process and allow conclusions about the best procedure:

$$EI = [A_{beads}/(A_{loaded} - A_{unbound})] \times 100 \quad (1$$

where A_{loaded} is the loaded activity, $A_{unbound}$ is the activity remaining in the supernatant, and A_{beads} is the activity bound to the support.

2.5 Muramidase activity in batch-mode

The activity of free and immobilized HEWL was investigated at 20 °C against model wine (tartaric buffer, pH 3.2, ethanol 12% v/v) with *O. oeni* added as substrate.

For the free form, the cell lysis was detected by measuring the decrease in OD_{600nm} *vs* time (Deckers, Vanlint, Callewaer, Aertsen, & Michiels, 2008; Esti, Liburdi, Palumbo, Benucci, & Garzillo, 2014) in 4-ml quartz cuvettes (1 cr light path), where the following reagents were mixed to reach a final volume of 3.5 ml: 0.5 ml of sucrose (0.27 M), for enzyme stabilisation, 0.5 ml of saline solution (NaCl 0.9%), 0.1 ml of lysozyme (1 mg ml^{-1}), model wine and increasin amounts of substrate ($5 \times 10^7 - 2 \times 10^8$ CFU/mL). The lytic reaction was monitored in continuous mode at 25 °C using a spectrophotometer (Shimadzu UV-2450) equipped with a thermostated cell (MPM Instruments Type M 900-T with magnetic stirring. One unit of HEWL activity (U) was defined as a decrease of 0.001 OD_{600} 1/min. Moreover, lysozyme specific activity (SA) was expressed taking into consideration HEWL protein content.

The activity of immobilized lysozyme was determined by adding 0.25 g (dry weight) of biocatalyst to the cell suspension (final volume 3.5 ml, as described previously) and stirring with end-over-end rotation for 30 min. The ce suspension was separated from the biocatalyst, at 5 min intervals, and the OD_{600nm} was detected for the activity determination. In order to evaluate the chitosan antimicrobial activity, a blank assay with the carrier beads without HEWL was done to account for the OD_{600nm} loss, due to non-enzymatic reactions.

2.6 Muramidase activity in the fluidized bed-reactor

Continuous lysis of *O. oeni* (2 mg ml^{-1}, corresponding to $7 \cdot 10^7$CFU) was carried out both in model and real wines in a fluidized bed-reactor (FBR), which consisted of a cylindrical glass column (volume: 232 cm^3, length: 37 cm inner diameter: 4.2 cm) equipped with an external water jacket for temperature control. Inside the FBR, which contained 2.8 g (dry weight) of HEWL immobilized on chitosan beads (dry or wet), the reaction mixture (280 ml) was drive by a N_2 upflow (1 bar); a peristaltic pump (Pumpdrive 5206 Heidolph) was used for feeding the model or real wine (Sauvignon blanc or Sangiovese) added along with the cell suspension, to obtain upward movement in the column a varying flow rates. Cell lysis, reflecting muramidase activity, was assessed by measuring the decrease in OD_{600nm} (Deckers et al., 2008). The optimal flow rate was identified after taking into account the effect of space velocity (S_V) on th fluidized-bed height (h) and on the substrate-lysis rate (r_S), since this velocity has to ensure a satisfactory contact time between enzyme and substrate (defined as residence time, τ) for the catalytic process.

2.7 Antimicrobial activity estimation

The suspension of lyophilized *O. oeni* cells was prepared, in model or real wines (white and red), to reach a final cell concentration of 10^7 CFU ml^{-1}. Free and immobilized lysozyme were prepared according to the abov mentioned procedures and then brought in contact with the bacterial suspension. The decrease in absorbance at 600 nm of the cell solution, which was continuously stirred (T = 20 °C), and kept in contact with the free or immobilize HEWL, was monitored until a constant value was reached. Preliminary trials were carried out in order to investigate the potential antimicrobial activity of the support bearing the HEWL, firstly immobilized and then deactivated with 2 M NaOH (data not show).

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 Rie (1990):

$$N(t) = K + A\left\{-\exp\left[\left(\frac{\mu_{max}e}{A}\right)(\lambda - t) + 1\right]\right\} \quad (2$$

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 of the cell density (CFU ml^{-1}), λ is the lag time (expressed in min), μ_{max} i the maximal decrease rate (expressed as min^{-1}). This parameter (μ_{max}) was taken as an effective measure of the free and immobilized HEWL antimicrobial activity, as determined in model and real wine, whereas Eq. (2) was fitted to th experimental data by a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.) and the quality of the regression was evaluated by the coefficient of determination (R^2) (Gil, Brandao, & Silva, 2006).

2.8 Statistical Analysis

The data, which were derived from the average of three replicate measurements, were analysed by one-way completely randomised Analysis of Variance (ANOVA) with an EXCEL® Add-in macro DSAASTAT (Onofri, 2006 followed by Tukey Honestly Significant Difference (Tukey HSD) post hoc test (P = 0.05) for multiple comparisons of samples.

2.9 Determination of kinetic parameters

The kinetic parameters (V_{max} and K_M) of free and immobilized HEWL were determined, according to the Michaelis–Menten equation, fitting experimental data by a non-linear regression procedure (GraphPad Prism 5.0, GraphPa 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 (the Michaelis–Menten constant) is equal to the substrate concentration whe the initial velocity is one-half of the maximum one (V_{max}), and is an indicator of catalytic efficiency.

2.10 Storage stability

As reported by Lian et al. (2012), the storage stability of free and immobilized enzyme was investigated by determining the residual activity (RSA) determined at intervals during the storage period. Immobilized HEWL wa maintained in tartaric acid buffer (pH 3.2) at 4 °C. At regular time intervals (weekly), biocatalysts were separated from the buffer solution by filtration and were used for the assay activity. RSA was expressed as reported below:

$$\text{RSA, } \% = (A_f/A_0) \times 100 \quad (3$$

where A_0 is the initial activity and A_f is the activity revealed weekly.

3 Results and discussion

3.1 Immobilization of HEWL

The method for HEWL immobilization on homemade chitosan beads (dry or wet), involves a covalent enzyme binding to the polymer. An initial experiment was carried out in order to determine the effect of the HEW concentration in the immobilization solution, in the range of 0.5–6 mg ml^{-1}, on the enzyme loading, as well as on the eventual activity of immobilized enzyme. The results (Fig. 1) show that, for both supports, enzyme loading was th same up to 2 mg ml^{-1} of HEWL solution, and then increased with increasing concentration of the immobilization solution, to finally reach the maximum value of 204 $\mu g_{protein}\ g^{-1}{}_{chitosan}$ and 528 $\mu g_{protein}\ g^{-1}{}_{chitosan}$ for dry and wet beads respectively. As ascertained for other enzymes (Saponjic et al., 2010), also for HEWL the covalent attachment to the chitosan supports is dependent on the enzyme concentration. In addition, it appears evident that lower specific activit was observed at higher enzyme loading, possibly due to mass transfer problems, which depend on the diffusion of substrate into the support particles and on the access to the active site (Lian et al., 2012; Saponjic et al., 2010).

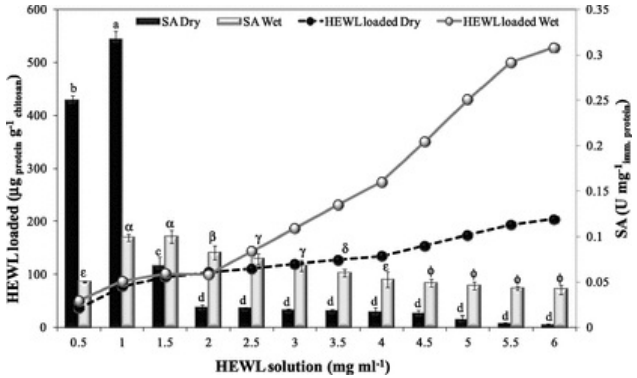

Fig. 1 Effect of enzyme solution (HEWL solubilised in distilled water) concentration (mg ml^{-1}) on lysozyme loaded (μg g^{-1}) and on the specific activity (SA, U $mg^{-1}{}_{imm.\ protein}$) of the biocatalysts immobilized on dry beads (Dry) and on wet beads (Wet).

Overall, these results are encouraging when compared with those reported in the literature for other enzymes, covalently immobilized on different supports, in terms of both enzyme loading and specific activity (Lian et al., 2012 Çelem & Önal, 2009). Therefore, the loading of 77 $\mu g_{protein}\ g^{-1}_{chitosan}$ and 86 $\mu g_{protein}\ g^{-1}_{chitosan}$ for dry and wet beads respectively, obtained using a coupling solution of 1 mg ml^{-1}, seems to be the most appropriate choice, since this als resulted in rather high activities corresponding to 0.32 U $mg^{-1}_{imm\ protein}$ and 0.09 U $mg^{-1}_{imm\ protein}$, respectively (Fig. 1), with a satisfactory amount of protein bound. Moreover, the immobilization efficiency (Eq. (1)), measured for dry an wet beads, was 36% and 4%, respectively. These EI values could be ascribable to the low amount of enzymatic HEWL protein immobilized on chitosan supports, as well as to the reduction of lysozyme activity resulting from th immobilization process. The low amount of protein loaded on the novel chitosan supports (77 and 86 μgprotein g^{-1} chitosan for dry and wet beads, respectively), appeared comparable to the results described by other authors (Chiou & Wu, 2004), who immobilized lipase on dry or wet chitosan beads, achieving a similar protein loading (71 and 109 μgprotein g^{-1} chitosan, respectively). Moreover, as described by Secundo (2013) the immobilization process often affect the catalytic activity of enzyme, through different mechanisms such as reduced accessibility of the substrate to the active site, loss of the enzyme dynamic properties and alteration of the conformational integrity of the biocatalyst.

3.2 Kinetic characterization and storage stability in batch-mode

Comparing the kinetic curves of HEWL (free and immobilized) on dry and wet beads, it appears evident that covalent binding significantly reduces the muramidase activity (data not shown). This reduction, after covalen immobilization, is a common phenomena and may be attributable to the low amount of enzyme immobilized on chitosan supports. However, it cannot be excluded that diffusion issues could have affected the lysozyme activity, a described for other enzymes (Jiang et al., 2005). In addition, during the immobilization process, HEWL may be obstructed and some active sites damaged during the coupling process (Lian et al., 2012). As illustrated in Table 1, kineti parameters, determined in batch-mode, were affected by the immobilization process, with a significant decrease of V_{max} observed for immobilized HEWL respect to the free form, as has been ascertained for other enzymes (Bayramoglu Yilmaz, & Arica, 2004).

Table 1 Kinetic parameters estimated in model wine (tartaric acid buffer pH 3.2 containing 12%, v/v ethanol) at 20 °C for free lysozyme (HEWL-Free) and HEWL immobilized on chitosan: Dry beads and Wet beads.

	V_{max} (U $mg^{-1}_{protein}$)	K_M (mg ml^{-1})	R^2	Half-life (days)
HEWL-Free	14.30 ± 1.13	6.88 ± 0.80	0.95	7
Dry beads	0.94 ± 0.23	6.93 ± 2.44	0.95	7
Wet beads	0.61 ± 0.18	16.66 ± 5.74	0.99	21

Otherwise, the K_M for the lysozyme bound on dry beads was found to be approximately the same with respect to the value observed for free HEWL, while the K_M of the enzyme immobilized on wet beads was higher. As reporte by Çetinus and Öztop (2003), an increase in K_M, after immobilization, indicates that the biocatalyst has an apparent lower affinity for its substrate than that of the free enzyme. This phenomena may be caused by the steric hindrance a the active site caused by the support, a loss of enzyme flexibility necessary for substrate binding, or diffusional resistance to solute transport near the support particles (Lian et al., 2012; Çetinus & Öztop, 2003).

Comparing the two immobilized biocatalysts (Table 1), HEWL covalently linked on dry beads presented a lower K_M value (about 3-fold) with respect to HEWL immobilized on wet beads, indicating a better affinity of the enzym toward its substrate. The higher substrate affinity revealed for HEWL immobilized on the dry beads with respect to wet beads, could be possibly ascribed to their different size differences (dia.: 2 mm and 3 mm, respectively). Numerou authors (Sang & Coppens, 2011; Talbert & Goddard, 2012) reported that the reduced size of the carrier tended to increase the activity of immobilized enzymes. Vertegel, Siegel, and Dordick (2004) reported that the protei conformational changes of HEWL were seen to be reduced after immobilization onto silica nanoparticles, indicating that the size of silica nanopores reduced the conformational changes imposed on the immobilized enzyme.

Stability is one of the most important parameters for immobilized enzymes in industrial applications, and an increased stability may render immobilized HEWL even more advantageous than its free form (Lian et al., 2012). Th stability of immobilized HEWL was evaluated in model wine over a period of 50 days, maintaining the biocatalyst in tartaric acid buffer (pH 3.2) at 4 °C. The free enzyme showed a RSA (Eq. (3)) of about 50% after only 7 days (Table 1 and then appearing completely deactivated after 21 days. Under the same storage conditions, the activity loss revealed for immobilized enzyme on dry beads was similar with respect to that observed for the free form. On the other hanc HEWL immobilized on wet supports preserved 90% of its initial activity after 7 days, and approximately 50% was maintained after 21 days (Table 1). Moreover, after 50 days the only enzyme activity still detectable was revealed fo HEWL bound on wet beads (18%).

3.3 Optimization of fluidized-bed reactor parameters

Based on the promising results already obtained in the batch system, a FBR for the continuous lysis of *O. oeni* in wine as been developed and reported here. In this system, microbial hydrolysis was achieved by HEWL covalentl immobilized on carriers (dry or wet chitosan beads) in a FBR, where the biocatalyst was suspended in dynamic equilibrium by the interaction of the upward flow of substrate and gravitational forces (Van Zessen et al., 2005). No enzym

leakage occurred during the treatment in the FBR, as demonstrated by electrophoretic analysis (data not shown).

The optimization of process parameters, essential for achieving an industrially feasible bioreactor, was carried out in model wine, with addition of a cell suspension (2 mg ml^{-1}, corresponding to 10^6–10^7 CFU ml^{-1}, a typica malolactic fermentation concentration), at 20 °C, and varying the flow rate [Q_v (56–1064 ml min^{-1})], as reported in Table 2.

Table 2 Process parameters of fluidized-bed reactor containing lysozyme immobilized on Dry or Wet chitosan beads, toward *O. oeni* as substrate (2 mg ml^{-1}).

Q_v (ml min^{-1})	S_v (min^{-1})	τ (min)	C_s (CFU)		r_S (CFU min^{-1})	
			Dry	Wet	Dry	Wet
56	0.24	4.14	3.23×10^6	4.92×10^6	1.81×10^8	2.75×10^8
224	0.97	1.04	8.89×10^6	1.90×10^7	1.99×10^9	4.26×10^9
392	1.69	0.59	1.54×10^7	5.05×10^7	6.02×10^9	1.98×10^{10}
560	2.41	0.41	8.22×10^6	2.96×10^7	4.6×10^9	1.66×10^{10}
728	3.14	0.32	5.26×10^6	1.83×10^7	3.83×10^9	1.33×10^{10}
1064	4.59	0.22	2.83×10^6	6.48×10^6	3.01×10^9	6.90×10^9

Q_V [Flow rate (ml min^{-1})]; S_V [Space velocity (min^{-1})]; τ [Residence time (min)]; C_S [Substrate concentration (CFU)]; r_S [Substrate lysis rate (CFU min^{-1})].

The effect of space velocity (S_V) on the fluidized-bed height and on the substrate-lysis rate (r_S), determined for HEWL immobilized on dry or wet chitosan beads is described in Fig. 2. The bed expansion increases with the liqui flow and the bed height of both carriers differed only at the intermediate values of S_V, whereas no significant difference was observed at the lowest and greatest S_V.

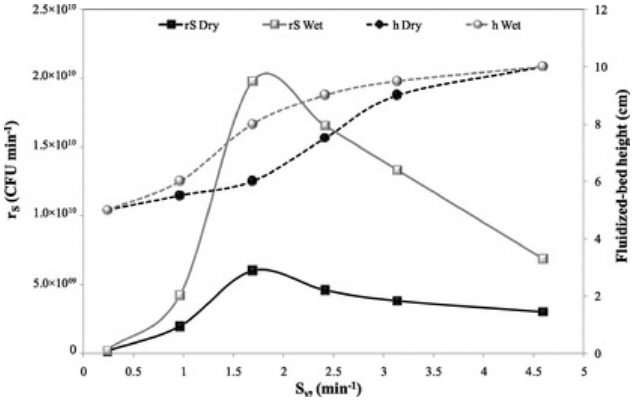

Fig. 2 Influence of the space velocity (S_v) on the substrate-lysis rate (r_S) and on the fluidized-bed height (h) in fluidized-bed reactor, containing lysozyme immobilized on dry beads (Dry) and on wet beads (Wet), toward *O. oeni* as substrate (2 mg ml^{-1}).

The data in Fig. 2 show that the relationship between space velocity (S_V) and substrate-lysis rate (r_S) is represented by a bell shape curve with a maximum r_S at S_V of 1.69 min^{-1}, which corresponds, for both biocatalysts, to th optimal flow rate (Q_v 392 ml min^{-1}) for *O. oeni* lysis. Increasing the S_V beyond the maximum, the substrate-lysis rate decreased, probably because of the ineffective contact of HEWL with cells, caused by the excess turbulence at hig flow velocity (Saponjic et al., 2010; Zhou, Chen, & Yan, 2014). Moreover, the highest r_S (1.98×10^{10} CFU min^{-1}) was observed for HEWL immobilized on wet beads when the fluidized bed height was 8 cm. For HEWL on dry carriers, th maximum r_S (6.02×10^9 CFU min^{-1}) was achieved at 6 cm bed height (Fig. 2).

3.4 Antimicrobial activity of immobilized HEWL in the fluidized-bed reactor

Applying the above mentioned optimized process conditions (S_V 1.69 min^{-1}, Q_v 392 ml min^{-1}) the continuous *O. oeni* lysis in a FBR containing immobilized HEWL was then investigated in both model and real white (Sauvigno

blanc) and red (Sangiovese) wine.

In order to quantify the respective free and immobilized HEWL antimicrobial activities, as measured in both model and real wines, the fitted curves, obtained as described, are presented in Fig. 3. The estimated parameters (A μ_{max}, and λ) are reported in Table 3 and the corresponding R^2 values revealed that the Gompertz function satisfactorily fit the experimental data. Therefore, they can be used to describe the *O. oeni* cell decrease *vs* time in presence o either free or immobilized HEWL.

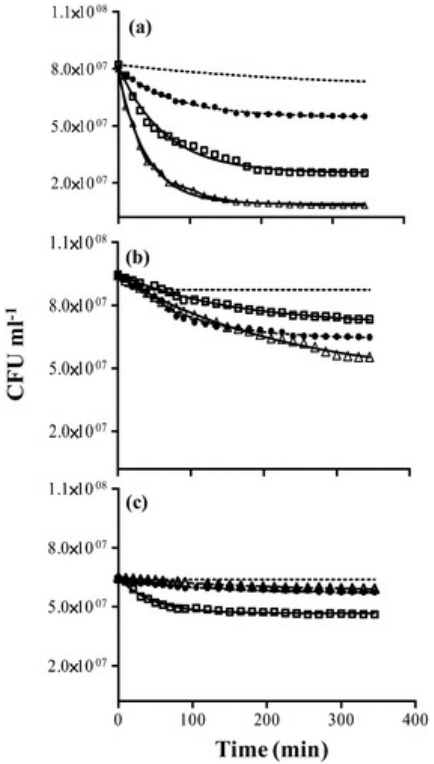

Fig. 3 Lysis of *O. ~~Oeni~~ oeni* cell (2 mg ml^{-1}) in fluidized-bed reactor, containing lysozyme free (△) or immobilized on Dry (●) or Wet (□) chitosan beads in: a) model wine, b) white wine (Sauvignon blanc) and c) red wine (Sangiovese). A control sample (traced line) represented by lysozyme firstly immobilized and then deactivated, was used in both model and real wines in order to ascertain any possible autolytic phenomena of *O. oeni* as well as the antimicrobial effect of sulphites contained in real wines.

Table 3 Parameters obtained fitting the modified Gompertz equation to the experimental values of lysozyme free (Free-HEWL) or immobilized on Dry (Dry-HEWL) or on Wet (Wet-HEWL) beads in fluidized-bed reactor, in model and real wines, toward *O. oeni* cell substrate (2 mg ml^{-1}).[a]

	k (CFU × ml^{-1})	A (CFU × ml^{-1})	μ_{max} (min^{-1})	λ (min)	R^2
Model wine					
Free-HEWL	7.80×10^7 (7.60×10^7, 8.0×10^7)	9.68×10^6 (9.01×10^6, 1.04×10^6)	2.62×10^{-2} (2.47×10^{-2}, 2.77×10^{-2})	26.47 (25.03, 28.10)	0.99
Dry-HEWL	7.99×10^7 ($7.92x10^7$, 8.1×10^7)	5.46×10^7 (5.42×10^7, 5.51×10^7)	1.28×10^{-2} (1.19×10^{-2}, 1.37×10^{-2})	54.24 (50.55, 58.52)	0.99
Wet-HEWL	8.10×10^7 (7.83×10^7, 8.38×10^7)	2.53×10^7 (2.39×10^7, 2.67×10^7)	1.53×10^{-2} (1.36×10^{-2}, 1.70×10^{-2})	45.18 (40.64, 50.86)	0.99
Sauvignon blanc white wine					

Free-HEWL	9.40×10^7 (9.31×10^7, 9.49×10^7)	4.40×10^7 (4.02×10^7, 4.79×10^7)	4.33×10^{-3} (3.70×10^{-3}, 4.96×10^{-3})	160.2 (139.7, 187.8)	0.99
Dry-HEWL	9.65×10^7 (9.48×10^7, 9.82×10^7)	6.41×10^7 (6.28×10^7, 6.53×10^7)	1.15×10^{-2} (9.88×10^{-3}, 1.32×10^{-2})	60.07 (52.52, 70.14)	0.98
Wet-HEWL	9.48×10^7 (9.41×10^7, 9.54×10^7)	7.05×10^7 (6.93×10^7, 7.18×10^7)	6.43×10^{-3} (5.63×10^{-3}, 7.23×10^{-3})	107.9 (95.90, 123.2)	0.99
Sangiovese red wine					
Free-HEWL	6.50×10^7 (6.48×10^7, 6.53×10^7)	5.80×10^7 (5.74×10^7, 5.86×10^7)	5.67×10^{-3} (4.62×10^{-3}, 6.70×10^{-3})	122.5 (103.5, 149.9)	0.99
Dry-HEWL	6.34×10^7 (6.31×10^7, 6.38×10^7)	5.72×10^7 (5.67×10^7, 5.76×10^7)	8.52×10^{-3} (6.74×10^{-3}, 1.03×10^{-2})	81.38 (67.30, 102.90)	0.98
Wet -HEWL	6.50×10^7 (6.39×10^7, 6.60×10^7)	4.66×10^7 (4.63×10^7, 4.70×10^7)	2.24×10^{-2} (1.98×10^{-2}, 2.50×10^{-2})	30.92 (27.70, 34.99)	0.98

[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}).

A control sample, represented by HEWL, firstly immobilized and then deactivated by 2 M NaOH, was used in both model and real wines. It was useful to evaluate any possible autolytic phenomena of *O. oeni* as well as th antimicrobial effect of sulphites contained in real wines. In model wine (Fig. 3a), a slight OD decrease was observed for the control sample, indicating an unimportant autolytic phenomena of *O. oeni*. In real wines, the cell conter appears nearly unchanged for both the control samples (Fig. 3b and ~~3~~c), proving that the low level of free SO_2 (13 and 21 mg l^{-1} in white and red wine, respectively) did not exert a significant antimicrobial effect toward *O. oeni*.

As is clear, the curves (Fig. 3) demonstrate the decrease of cell concentration *vs* time, proving the antimicrobial activity of both free and immobilized HEWL in the FBR system, for all the samples studied (model and real wines).

The values obtained for the parameters A and λ indicate higher cell lysis in a shorter time, needed to reach the plateau when free HEWL was added in model and white wine, with respect to red wine (Sangiovese), where th content of high molecular weight flavans was 40-fold greater than for white wine (Sauvignon blanc). This confirms the role of phenolic compounds in inactivating HEWL, as was recently described by Liburdi, Benucci, Palumbo, and Est (2016).

Also, as reported by Zwietering, Jongenburger, Rombouts, and Van't Riet (1990), the most meaningful parameter, with respect to the antimicrobial activity, is μ_{max}, which was found to be higher for the free form respect to bot biocatalysts in model wine; this phenomena may be attributable to the covalent immobilization of enzyme, which affected the immobilized HEWL activity. When the *O. oeni* cell lysis was evaluated in real wines using the FBR, both th immobilized forms appeared more active than the soluble enzyme, and thus achieved the highest μ_{max} values.

Moreover, the μ_{max} calculated for the two immobilized biocatalysts, demonstrated that the HEWL linked to wet beads appeared more effective in both model and red wine, whereas HEWL immobilized on dry beads was mor useful for the hydrolysis of *O. oeni* cell in Sauvignon blanc. This result could be explained considering that the hydrophobicity of poly- cationic support affects the interaction between the immobilized enzyme and substrate, as well a between the biocatalyst and the enzyme inhibitors which are present in real matrices (Talbert & Goddard, 2012), for example, the low molecular weight flavans (white wine) and higher molecular weight flavans (red wine).

Dry beads seemed to be a more appropriate support for the application of immobilized HEWL in white wine, probably because of their hydrophobic character, deriving from the lower moisture content (58% w/w, after swelling which limits the interaction with hydrophilic low molecular weight flavans. On the other hand, the greatest antimicrobial activity, revealed in red wine with HEWL immobilized on wet beads (moisture content 85% w/w), is probabl attributable to a pronounced hydrophilicity of the biocatalyst, which reduces the interaction with high molecular weight flavans.

4 Conclusion

A continuous, efficient and food-grade fluidized-bed reactor, containing HEWL immobilized on microbial chitosan beads, was applied for the first time to lyse lactic bacteria (*O. oeni* cell suspension) in white an red wine, a result that implies a decrease in the sulfur dioxide dosage required to control the malolactic fermentation.

The immobilization procedure was optimized varying the amount of HEWL in the coupling solution in order to maximize the catalytic performance of HEWL eventually immobilized on dry and wet chitosa beads. Based on the promising results obtained in the batch mode, a laboratory bench-scale FBR was developed and its operational parameters were investigated using model wine. Applying the optimized proces conditions indentified, immobilized HEWL appeared even more useful than the free form, in the continuous lysis of lactic bacteria in real white (Sauvignon blanc) and red (Sangiovese) wine.

Moreover, comparing the two immobilized biocatalysts, HEWL bound to dry beads was more useful for the hydrolysis of *O. oeni* cell in Sauvignon blanc, whereas HEWL immobilized on wet beads appeared mor

effective in red wine.

5 Uncited references

EC. No. 2676 (1990) and Liburdi, Benucci, and Esti (2014).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.089.

References

Appendini P. and Hotchkiss J.H., Immobilization of lysozyme on food contact polymers as potential antimicrobial films, *Packaging Technology and Science* **10**, 1997, 271–279.

Bayramoğlu G., Yilmaz M. and Arica M.Y., Immobilization of a thermostable α-amylase onto reactive membranes: kinetics characterization and application to continuous starch hydrolysis, *Food Chemistry* **84**, 2004, 591–599.

Birò E., Nemeth A.S., Sisak C., Feczko T. and Gyenis J., Preparation of chitosan particles suitable for enzyme immobilization, *Journal of Biochemical and Biophysical Methods* **70**, 2008, 1240–1246.

Bradford M.M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binging, *Analytical Biochemistry* **72**, 1976, 248–254.

Çelem E.B. and Önal S., Immobilization of phytase on epoxy-activated Sepabead EC-EP for the hydrolysis of soymilk phytate, *Journal of Molecular Catalysis B: Enzymatic* **61**, 2009, 150–156.

Çetinus S.A. and Öztop H.N., Immobilization of catalase into chemically crosslinked chitosan beads, *Enzyme and Microbial Technology* **32**, 2003, 889–894.

Chen H., Liu L., Lv S., Liu X., Wang M., Song A. and Ji X., Immobilization of *Aspergillus niger* xylanase on chitosan using dialdehyde starch as a coupling agent, *Applied Biochemistry and Biotechnology* **162**, 2010, 24–32.

Chiou S.H. and Wu W.T., Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups, *Biomaterials* **25**, 2004, 197–204.

Conte A., Buonocore G.G., Sinigaglia M. and Del Nobile M.A., Development of immobilized lysozyme based active film, *Journal of Food Engineering* **78**, 2007, 741–745.

Deckers D., Vanlint D., Callewaer L., Aertsen A. and Michiels C.W., Role of the lysozyme inhibitor ivy in growth or survival of Escherichia coli and *Pseudomonas aeruginosa* bacteria in hen egg white and in human saliva and breast milk, *Applied and Environmental Microbiology* **74**, 2008, 4434–4439.

Diano N., Grimaldi T., Bianco M., Rossi S., Gabrovska K., Yordanova G., … Mita D.G., Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors, *Journal of Agricultural and Food Chemistry* **56**, 2008, 11471–11477.

EC. No 1266/2010, European Commission Regulation (EC) No. 1266/2010 of the European Parliament and of the Council of 22 December 2010 amending Directive 2007/68/EC as regards labelling requirements for wines Text with EEA relevance, *Official Journal* **L347**, 2010.

~~EC. No. 2676, Commission Regulation (EC) No. 2676/90 of 17 September 1990 determining Community methods for the analysis of wines, *Official Journal of the European Communities* **L272**, 1990.~~

Esti M., Liburdi K., Palumbo F., Benucci I. and Garzillo A.M.V., Kinetic study of a hen-egg white lysozyme-based oenological preparation, *Food Science and Biotechnology.* **23** (1), 2014, 151–155.

Gil M.M., Brandao T.R. and Silva C.L., A modified Gompertz model to predict microbial inactivation under time-varying temperature conditions, *Journal of Food Engineering* **76** (1), 2006, 89–94.

Gòmez J.L., Bòdalo A., Gòmez E., Hidalgo A.M., Gòmez M. and Murcia M.D., Experimental behaviour and design model of a fluidized bed reactor with immobilized peroxidase for phenol removal, *Chemical Engineering Journal* **127**, 2007, 47–57.

Gueguen Y., Chemardin P., Pien S., Arnaud A. and Galzy P., Enhancement of aromatic quality of Muscat wine by the use of immobilized b-glucosidase, *Journal of Biotechnology* **55**, 1997, 151–156.

Jiang D.S., Long S.Y., Huang J., Xiao H.Y. and Zhou J.Y., Immobilization of *Pycnoporus sanguineus* laccase on magnetic chitosan microspheres, *Biochemical Engineering Journal* **25**, 2005, 15–23.

Kahraman M.V., Bayramoğlu G., Kayaman-Apohan N. and Gŭngör A., α-Amylase immobilization on functionalized glass beads by covalent attachment, *Food Chemistry* **104**, 2007, 1385–1392.

Lasanta C., Roldán A., Caro I., Pérez L. and Palacios V., Use of lysozyme for the prevention and treatment of heterolactic fermentation in the biological aging of sherry wines, *Food Control* **21**, 2010, 1442–1447.

Lian Z.X., Ma Z.S., Wei J. and Liu H., Preparation and characterization of immobilized lysozyme and evaluation of its application in edible coatings, *Process Biochemistry* **47**, 2012, 201–208.

Liburdi K., Benucci I. and Esti M., Lysozyme in wine: An overview of current and future applications, *Comprehensive Review in Food Science and Food Safety* **13**, 2014, 1062–1073.

Liburdi K., Benucci I., Palumbo F. and Esti M., Lysozyme immobilized on chitosan beads: Kinetic characterization and antimicrobial activity in white wines, *Food Control* **63**, 2016, 46–52.

Lorenzoni A.S.G., Aydos L.F., Klein M.P., Ayub M.A.Z., Rodrigues R.C. and Hertz P.F., Continuous production of fructooligosaccharides and invert sugar by chitosan immobilized enzymes: Comparison between in fluidize and packed bed reactors, *Journal of Molecular Catalysis B: Enzymatic* **111**, 2015, 51–55.

Onofri A., Enhancing Excel capability to perform statistical analyses in agriculture applied research, In: *Computational statistics and data analysis – Statistical Software Newsletters* 2006, International Association for statistical Computing.

REG. EC No. 606/2009, Annex I A, Appendix 13.

Roy I. and Gupta M.N., Lactose hydrolysis by Lactozym™ immobilized on cellulose beads in batch and fluidized bed modes, *Process Biochemistry* **39**, 2003, 325–332.

Sang L. and Coppens M., Effects of surface curvature and surface chemistry on the structure and activity of proteins adsorbed in nanopores, *Physical Chemistry Chemical Physics* **13**, 2011, 6689.

Saponjic S., Knežević-Jugović Z.D., Bezbradica D.I., Zuza M.G., Saied O.A., Bosković-Vragolović N. and Mijin D.Z., Use of *Candida rugosa* lipase immobilized on sepabeads for amyl caprylate synthesis: Batch and fluidized bed reaction study, *Electronic Journal of Biotechnology* **13**, 2010, 1–15.

Secundo F., Conformational changes of enzymes upon immobilisation, *Chemical Society Reviews* **42**, 2013, 6250–6261.

Sonni F., Cejudo-Bastante M.J., Chinnici F., Natali N. and Riponi C., Replacement of sulphur dioxide by lysozyme and oenological tannins during fermentation: influence on volatile composition of white wines, *Journal of the Science of Food and Agriculture* **89**, 2009, 688–696.

Talbert J.N. and Goddard J.M., Enzymes on material surfaces, *Colloids and Surfaces B: Biointerfaces* **93**, 2012, 8–19.

Tirelli A. and De Noni I., Evaluation of lysozyme stability in young red wine and model systems by a validated HPLC method, *Food Chemistry* **105**, 2007, 1564–1570.

Van Zessen E., Tramper J., Rinzema A. and Beeftink H.H., Fluidized-bed and packed-bed characteristics of gel beads, *Chemical Engineering Journal* **115** (1–2), 2005, 103–111.

Vertegel A., Siegel R. and Dordick J., Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme, *Langmuir* **20**, 2004, 6800–6807.

Weber P., Kratzin H., Brockow K., Ring J., Steinhart H. and Paschke A., Lysozyme in wine: A risk evaluation for consumers allergic to hen’s egg, *Molecular Nutrition & Food Research* **53**, 2009, 1469–1477.

Zacchigna M., Di Luca G., Lassiani L., Varnavas A., Pitotti A. and Boccù E., Improved activity in acidic media of immobilized lysozyme, *Applied Biochemistry and Biotechnology* **76**, 1999.

Zappino M., Cacciotti I., Benucci I., Nanni F., Liburdi K., Valentini F. and Esti M., Bromelain immobilization on microbial and animal source chitosan films, plasticized with glycerol, for application in wine-like medium: Microstructural, mechanical and catalytic characterisations, *Food Hydrocolloids* **45**, 2015, 41–47.

Zhou G.X., Chen G.Y. and Yan B.B., Biodiesel production in a magnetically-stabilized, fluidized bed reactor with an immobilized lipase in magnetic chitosan microspheres, *Biotechnology Letters* **36**, 2014, 63–68.

Zwietering M.H., Jongenburger I., Rombouts F.M. and Van’t Riet K., Modeling of the bacterial growth curve, *Applied and Environmental Microbiology* **56** (6), 1990, 1875.

Appendix A. Supplementary data

Multimedia Component 1

Supplementary data

Highlights

- Lysozyme from hen egg white (HEWL) was immobilized on microbial chitosan beads.
- A Fluidized bed reactor (FBR), containing immobilized HEWL, was realized.
- The food-grade FBR was applied for the continuous lysis of *O. oeni* cell in wine.
- FBR was useful to limit the SO_2 dosage to control the malolactic fermentation.

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