Chitosan beads from microbial and animal sources as enzyme supports for wine application

Ilaria Benucci [a], Claudio Lombardelli [a], Ilaria Cacciotti [b, *], Katia Liburdi [a], Francesca Nanni [c], Marco Esti [a]

[a] Dept. for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy
[b] Dept. of Engineering, University of Rome "Niccolò Cusano", INSTM RU, Via Don Carlo Gnocchi, 3, 00166 Rome, Italy
[c] Dept. of Enterprise Engineering, University of Rome "Tor Vergata", INSTM RU "Rome-Tor Vergata", Via del Politecnico, 1, 00133 Rome, Italy

ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form
12 May 2016
Accepted 14 May 2016
Available online 15 May 2016

Keywords:
Chitosan beads
Aspergillus niger
Stem bromelain
Enzyme immobilization
White wine
Packed-bed reactor

ABSTRACT

Chitosan beads from a microbial source, i.e. *Aspergillus niger* (An), were produced by precipitation method for the first time and compared with supports prepared from an animal source, i.e. shellfish derived chitosan with three different molecular weights (low (LMW), medium (MMW) and high (HMW)). The produced beads were used as enzyme carriers to be applied in a continuous packed-bed reactor (PBR) for white wine protein stabilization.

For this purpose, the beads were crosslinked with glutaraldehyde (GDH) and stem bromelain was immobilized on the carrier surface as a model enzyme, following two different procedures (i.e., cross-linking with glutaraldehyde (GDH) and direct linkage (DL)).

Drop-like beads with an average diameter of 3.0–3.5 mm and a moisture content of 86–94% were obtained. The morphology of the produced beads in dried state, in terms of shape and surface, was studied by means of a scanning electron microscope (SEM), evidencing the obtainment of nearly spherical or oval particles. The efficacy of the crosslinking procedure and of bromelain immobilization was demonstrated by means of SEM investigation and infrared spectroscopy (FT-IR) analysis, revealing a rougher surface. Various initial protein concentrations ranging between 0.45 and 18.00 mg_{BSAeq} mL^{-1} were tested in order to identify the optimal amount and to evaluate the influence of the initial concentration on the total protein loading.

Stem bromelain proved to be more active when immobilized by DL on An beads and was efficient in reducing white wine hazing potential continuously, as verified with a laboratory bench-scale PBR.

1. Introduction

Chitosan (CS), a cationic polymer combined by β 1-4 glicosidic linkage, is obtained from chitin, a major structural component of the invertebrates exoskeleton and of the fungi cell wall (Santos, Veiga, Pina, Podczeck, & Sousa, 2002). Commercially available CS is usually produced from shrimp and crabshell chitin by N-deacetylation, using strong alkali. There are various limitations in using this procedure industrially: (i) the seasonality in resource availability, (ii) the high processing costs, (iii) the complexity of the procedure, (iv) the varying physico-chemical characteristics of CS (White, Farina, & Fulton, 1979). Moreover, the presence of shrimp antigen as well as heavy metal contaminants (nickel and copper) in the final product could cause allergic reactions, as described by other authors (Tan, Tan, Wong, & Khor, 1996). In order to overcome these limitations, the production of CS from fungal mycelia is an alternative which enables to obtain a CS free of heavy metals and standardized in terms of acetylation degree, molecular weight, viscosity, charge distribution (Nwe, Furuike, & Tamura, 2010).

In the food industry CS appears to be a good support for enzyme immobilization (Shellfish Wastes; Talbert and Goddard, 2012), since it is non toxic, user-friendly, available in various forms (powder, gel, fibers and membranes) and demonstrating high protein affinity (Zappino et al., 2015). CS beads, which vary in molecular weight and deacetylation degree, generally prepared by the precipitation method, have been widely studied as carriers for enzyme immobilization (Biró, Németh, Sisak, Feczkó, & Gyenis, 2008; Krajewska, 2004).

* Corresponding author.
E-mail address: ilaria.cacciotti@unicusano.it (I. Cacciotti).

In this work, CS beads from a microbial source, i.e. *Aspergillus niger* (An), were produced for the first time by precipitation method, and compared with CS beads prepared from shellfish CS powder with three different molecular weight polymers (i.e. low (LMW), medium (MMW) and high (HMW)). In fact, at present the European food law only permits the application of microbial CS from *Aspergillus niger* in the food industry (REG. EC No. 606/2009). CS membrane based on chitosan from *A. niger* (An) was obtained for the first time as immobilization support by Zappino et al. (2015).

The aim of this study was to produce biopolymeric enzyme carriers with optimal properties to be applied in a packed-bed reactor (PBR), for white wine protein stabilization. To date there is still a need to develop specific and effective methods alternative to the addition of fining agents (i.e. bentonite), which represents the common but unsustainable enological practice to achieve stable white wines. The enzymatic hydrolysis of wine proteins via proteases could represent a useful and specific tool to prevent haze formation in white wine, without affecting the organoleptic properties (Benucci et al., 2016). Among proteases, bromelain from pineapple stem, categorized as a food additive by the U.S. Food and Drug Administration and included in the list of substances generally recognized as safe (U.S. Food and Drug Administration, 2016), was tested.

In order to achieve this purpose, the obtained CS beads were used as supports for immobilizing pineapple stem bromelain using glutaraldehyde (GDH) as cross-linker, or by direct linkage. Their thermal properties were studied by differential scanning calorimetry (DSC) before and after crosslinking with GDH. The functional chemical groups were detected by infrared (FT-IR) spectroscopy measurements. Moreover, the morphology of the produced carriers was analyzed by means of a scanning electron microscope (SEM) before and after enzyme immobilization, in order to evaluate the influence of the surface topography on immobilization efficiency. The proteolytic activity of the biocatalysts was evaluated and the best biocatalyst was selected for white wine protein stabilization treatment in laboratory bench-scale PBR.

It is important to highlight that, to the best of our knowledge, microbial CS has not yet been used for the production of spherical supports to be applied in a continuous bioreactor.

2. Materials and methods

2.1. Materials

Stem bromelain (EC 3.4.22.32) was provided by Sigma Aldrich (Milan, Italy). Beads as supports for enzyme immobilization were produced using CS powders obtained from 2 different sources: i) microbial from *A. niger* (An; Lot#12121611L4; percentage of deacetylation: 70%, supplied by KitoZyme, Herstal, Belgium); ii) animal, consisting in shellfish polymers with three different molecular weights (i.e., low 50–190 kDa (LMW; Lot#MKBG3334V; percentage of deacetylation: 75%), medium 190–310 kDa (MMW; Lot#MKBH1108V; percentage of deacetylation: 75–85%) and high 310–375 kDa (HMW; Lot#MKBP9232V; percentage of deacetylation: 75%), supplied by Sigma Aldrich, Milan, Italy).

The selected synthetic tripeptide chromogenic substrate, Bz-Phe-Val-Arg-*p*-nitroaniline (pNA), was purchased from Bachem (Bubendorf, Switzerland). All the other chemicals were analytical grade (Sigma Aldrich, Milan, Italy). The unfined Sauvignon blanc wine (pH: 3.3; total acidity: 5.9 $g_{tartaric\ acid}\ L^{-1}$; L(−)-Malic acid: 0.9 g L^{-1}; ethanol 12.9% v/v; total SO_2 83 mg L^{-1}; free SO_2 13 mg L^{-1}; total phenols 141 $mg_{gallic\ acid\ eq}\ L^{-1}$), produced during the 2014 vintage, was supplied by the winery Casale del Giglio (Le Ferriere (LT), Italy).

2.2. Chitosan based supports preparation and characterization

CS beads were prepared according to the precipitation method (Biró et al., 2008). Animal (5 w/v %) and microbial ((15 w/v %) CS powders were dissolved in an aqueous solution of acetic acid (2 v/v %). The obtained solutions were added dropwise through a capillary into a gently stirred coagulation liquid (2 N sodium hydroxide and 26 v/v % ethanol). The obtained macrospheres were filtered and washed with distilled water until neutrality.

2.2.1. Moisture content

The moisture content was determined immediately after CS beads making. Approximately 10 g were dried at 90 °C for 24 h in a hot air oven (Memmert, Germany) for each CS carrier. The moisture content (MC, %) was calculated according to the Eq. (1), where m_d is the dried mass and m_w is the wet mass:

$$MC(\%) = \frac{m_w - m_d}{m_d} \times 100 \quad (1)$$

In order to obtain an average value, the experiment was repeated three times for each CS sample.

2.2.2. Spectroscopic characterization and morphology

Infrared spectra (Fourier Transform Infrared Spectroscopy, FTIR Perkin Elmer) were recorded in the region 400–4000 cm^{-1} using KBr pellets (1%wt/wt), spectral resolution of 4 cm^{-1}, scans number 32. The normalization of the acquired spectra was obtained by assigning a 100% value to the most intense peak.

The morphology of the CS beads was analyzed by observation at field-emission gun scanning electron microscope (FEG-SEM, Cambridge Leo Supra 35, Carl Zeiss), after drying at 40 °C for 48 h in an oven. The dried samples were analyzed after sputter-coating with gold under argon atmosphere (25 mA, 120 s).

2.2.3. Thermal properties

The thermal properties of the prepared beads, before and after crosslinking with GDH, were investigated by means of differential scanning calorimetry (DSC, TAInstruments Q2000) in the following conditions: sample weight ~5 mg, nitrogen atmosphere (N_2 flow rate 50 cc min^{-1}), range of temperature 20–400 °C, heating rate 10 °C/min.

2.3. Enzyme immobilization

Stem bromelain was immobilized on CS beads by applying two previously mentioned procedures (Benucci, Esti, Liburdi, & Garzillo, 2012): i) direct immobilization linkage (DL) which involves the protease carboxyl groups of Asp or Glu residues and the amino groups of the support (Benucci et al., 2012) and ii) cross-linking using glutaraldehyde (GDH) through the Schiff's base formation (Çetinus & Öztop, 2003).

The cross-linking procedure was performed by dipping 1 g of beads into 10 mL of glutaraldehyde solution 3% (v/v), maintaining the obtained suspension under constant agitation for 1 h at room temperature, and then profusely washing it with distilled water.

The coupling of the enzyme was carried out by immersing the unmodified or activated beads in stem bromelain solutions, which were prepared by adding appropriate amounts of enzyme powder to the immobilization buffer (tartaric acid/sodium tartrate 0.03 M, pH 3.2).

Different initial protein concentration in the range of 0.45–18.00 $mg_{BSAeq}\ mL^{-1}$ were tested. The components were shaken (150 rpm) overnight at 20 °C; the CS beads with immobilized bromelain were then filtered and washed thoroughly with 2 M NaCl in order to

remove all non-covalently bound enzymatic proteins, rinsed three times with immobilization buffer and left to stand for 20 min in a 0.1 M glycine solution.

2.4. Immobilization yield determination

All supernatants and washing solutions were collected and diluted with buffer solution in order to reach a constant final volume for determining the bound protein. The immobilization yield (IY, %) was evaluated 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 protein. The percentage of bound protein was indirectly determined as the difference between the amount of protein in solution before and after immobilization.

2.5. Enzymatic activity assay

Proteolytic activity toward the selected substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM) was tested in model wine (0.03 M, tartaric acid/sodium tartrate solution pH 3.2, containing 12% v/v of ethanol) at 20 °C. Stem bromelain cleaves synthetic substrate via the hydrolysis of the ester bond between amino acids in the N-terminal position and pNA, whose release was detected spectrophotometrically at 410 nm.

Protease activity was determined by measuring the change in absorbance *vs* time using an UV–visible spectrophotometer (Shimadzu UV 2450, Milan, Italy). Specific activity calculated in IU of pNA produced ($\varepsilon_{mmol} = 8.480\ mM^{-1}\ cm^{-1}$ for pNA, as reported by Hale, Greer, Trinh, and James (2005)) was expressed as IU mg^{-1} of immobilized protein (IU $mg^{-1}{}_{IP}$). A blank correction was carried out using a sample without enzyme. All measurements were taken in triplicate.

2.6. Wine protein content determination

Total protein content of the Sauvignon blanc wine, before and after enzymatic treatment with protease immobilized on CS beads, was determined using the potassium dodecyl sulphate method (Vincenzi, Marangon, Tolin, & Curioni, 2011). Proteins were precipitated from 1 mL of wine by adding 10 μL of 10%w/v sodium dodecyl-sulphate and 250 μL of 1 M KCl. The pellet obtained after centrifugation was then dissolved in 1 mL of distilled water by ultrasonic bath and protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's instructions, using BSA as standard for the calibration curve.

2.7. Optimization of packed-bed reactor parameters

A PBR containing bromelain immobilized on CS beads was used to achieve the protein stabilization of Sauvignon blanc wine. Firstly, it was necessary to optimize the operational parameters such as flow rate (Q_v), space velocity (S_v) and residence time (τ), using model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM).

The PBR consisted in an unbreakable glass tube with an internal volume of 7.29 cm^3 (length of 11.46 cm and inner diameter of 0.9 cm). The column was packed with 13 g of biocatalyst and glass beads (0.5 cm in thickness) were placed at both ends of the tube. The PBR was connected to the rest of the apparatus and a peristaltic pump (Minipuls 3. Gilson, Milan, Italy) was used for feeding the model wine, fortified with the synthetic substrate, into the tube, which moved upwards through the column at varying flow rates (from 1.08 to 12.00 mL min^{-1}). Samples of the effluent stream were then collected and analyzed spectrophotometrically at 410 nm in order to evaluate the concentration of the reaction product (C_p), represented by free pNA, as well as the product-formation rate (r_p).

2.8. Wine treatment in packed-bed reactor

50 mL of Sauvignon blanc wine were treated in a bench-scale PBR packed with CS beads with immobilized bromelain, by feeding the wine at the optimised flow rate (4.86 mL min^{-1}). Treatment was carried out in triplicate in three different PBRs in order to obtain three independent replicates. The treated wines were then analyzed in triplicate in order to determine both the hazing potential and the total protein content.

2.9. Heat test

Heat test was carried out on untreated wine and on wine samples treated in PBR (20 °C) containing bromelain immobilized on An CS beads. The hazing potential of white wine was determined by a heat test: the wine samples were incubated at 80 °C for 6 h and then kept at 4 °C for 16 h (Pocock & Rankine, 1973; Vincenzi et al., 2011). After equilibration at room temperature (approximately 25 °C), haze formation was measured using an HD 25.2 turbidimeter (Delta Hom) calibrated with Formazin turbidity standard, ranging from 0 to 800 NTU (Delta Hom) and turbidity was expressed in nephelometric turbidity units (NTU). Wine stability was calculated as the difference in the wine NTU after and before the heat test, that is proportional to hazing potential. Moreover, wine is considered stable if this difference does not exceed 2 NTU (Moine-Ledoux & Dubourdieu, 1999). The turbidity removal yield (TRY(%)) by PBR was calculated as the percentage of turbidity removed from the treated wine.

2.10. Statistical analysis

Data, obtained from the average of three replicate measurements, were analyzed by a one-way completely randomized Analysis of Variance (ANOVA) with an EXCEL® Add-in macro DSAASTAT (Onofri, 2006), followed by a Tukey Honestly Significant Difference (Tukey HSD) post-hoc test ($P = 0.05$) for multiple comparisons of samples.

3. Results and discussion

The thermal properties of the obtained dried beads, before and after crosslinking with GDH, were investigated by DSC. As an example, the heating scan DSC curves of An beads before and after GDH crosslinking are compared in Fig. 1.

A broad endothermic peak at approximately 100 °C was detected and may be due to the residual solvent removal, but mainly to the dissociation process of interchain hydrogen bonding of chitosan (Chuang, Young, Yao, & Chiu, 1999), having the bead been previously dried at 40 °C for 48 h, whereas the exothermic band at around 270–300 °C is attributed to chitosan decomposition (Sakurai, Maegawa, & Takahashi, 2000; Zeng, Fang, & Xu, 2004).

As expected, the decomposition temperature is shifted at higher values in the case of crosslinked beads (e.g., 278 °C *vs* 288 °C for uncrosslinked and crosslinked An beads, respectively).

The occurred cross-linking and activation with GDH were further confirmed by comparing FT-IR spectra of native and activated beads (Fig. 2A and B). In all acquired FT-IR spectra, the typical vibration modes of chitosan were revealed (Chen et al., 2004). The infrared spectra of GDH cross-linked chitosan beads are very similar to that of pristine chitosan, since the functional groups of GDH are also present in chitosan. However, the occurred cross-

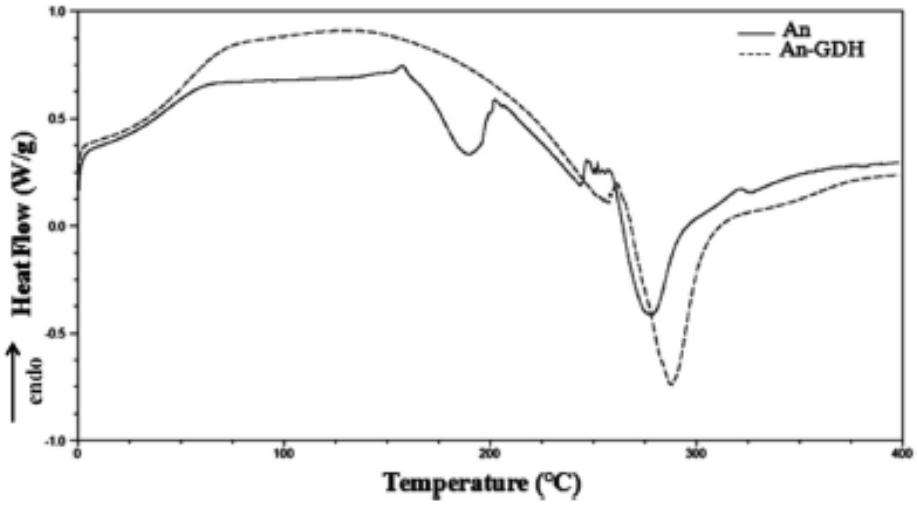

Fig. 1. DSC curves of *Aspergillus niger* chitosan beads (first heating scan) before (An) and after crosslinking with glutaraldehyde (An-GDH).

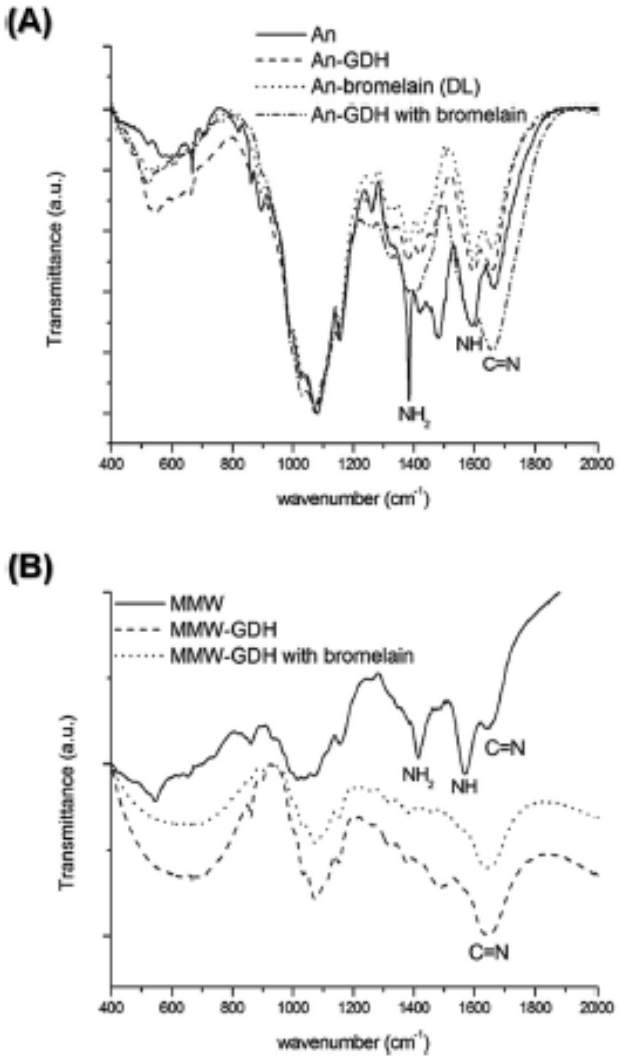

Fig. 2. FT-IR spectra of chitosan beads from: A) *Aspergillus niger* (An) before and after GDH crosslinking and bromelain immobilization by the two different immobilization techniques (crosslinking (GDH) and direct linkage (DL)); B) shellfish with Medium Molecular Weight (MMW) before and after glutaraldehyde crosslinking (GDH) and bromelain immobilization.

linking reaction was suggested by the presence of the peak around 1655 cm^{-1} which can be due to both amide I group of chitosan and C═N stretching band of the formed Schiff's base (Knaul, Hudson, & Creber,1999; Monteiro & Airoldi, 1999; Vieira & Beppu, 2006). In fact, the GDH molecule was crosslinked with two chitosan units: the covalent bond is achieved by the Schiff's base formation (Çetinus & Öztop, 2003), which involves the reaction of the two aldehydic groups of GDH and the free amine groups of the CS beads with the consequent conversion of the CS amino groups into an imine group (C═N) (Alves & Mano, 2008).

Moreover, the absence of the peak at 1715 cm^{-1}, ascribed to the free aldehydic groups of GDH (Rao, 1963), testified the complete crosslinking occurrence and/or the efficacy of the carried out water washings to remove the unreacted molecules.

By comparing the FT-IR spectra of An chitosan derived beads, before and after GDH crosslinking, it is interesting to note that for the activated beads the relative intensity of the peak at 1385 cm^{-1} (CH_3 in amide groups and primary amino group ($-NH_2$)) was remarkably decreased with respect to the band at 1655 cm^{-1}, suggesting that most of the primary amino groups were involved in the cross-linking process (Fig. 2A). On the other hand, for activated MMW chitosan beads, the peak at 1655 cm^{-1}, ascribed to C═N stretching, is very evident and associated to the absence of the peak at 1593 cm^{-1}, related to the NH_2 vibrational mode in amino group (amide II), suggesting an efficient GDH crosslinking also for shellfish carriers (Fig. 2B).

Finally, the FT-IR spectra of the bromelain immobilized on the An beads are comparable (Fig. 2A), independently of the applied immobilization technique (DL and GDH), taking into account that the first procedure (DL) involves the bond between the protease carboxyl groups of Asp or Glu residues and the amino groups of the support, and the second one (GDH) the bond between the GDH and the free amino groups of lysine residue of the enzyme.

The occurred GDH crosslinking was also corroborated by observing the SEM micrographs of all the bead surfaces.

In Fig. 3 low magnification SEM micrographs of all the produced dried beads, before and after crosslinking and bromelain immobilization, are compared. As expected, the average diameter significantly decreased after drying, considering that the drop-like and nearly spherical or oval wet beads presented an average diameter of 3.0—3.5 mm (Table 1), as determined by observation at optical microscope (data not shown). This decrement was particularly evident in the case of shellfish derived chitosan beads, due to their

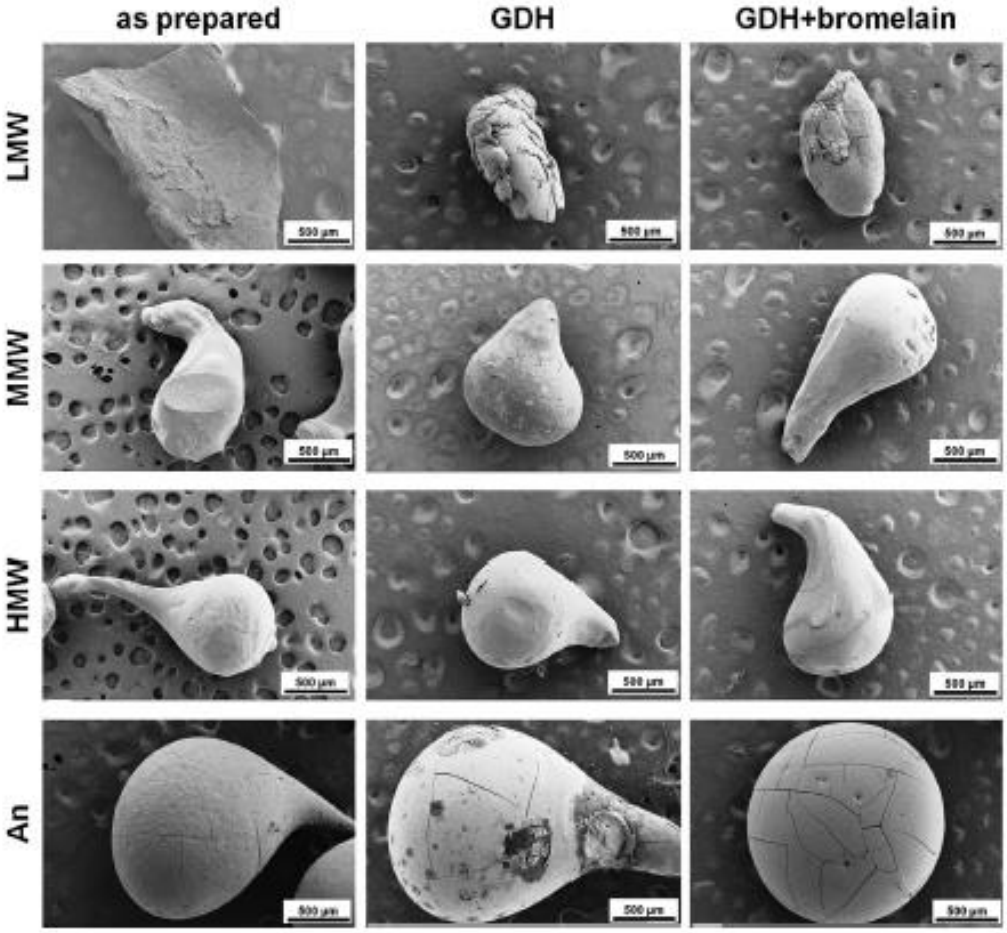

Fig. 3. SEM micrographs of dried chitosan beads before and after glutaraldehyde crosslinking (GDH) and bromelain immobilization. Chitosan beads were obtained from shellfish with low (LMW), Medium (MMW) and High (HMW) Molecular Weight, or from *Aspergillus niger* (An).

Table 1
Average diameter and moisture content of chitosan beads obtained from shellfish with Low (LMW), Medium (MMW) and High (HMW) Molecular Weight, or from *Aspergillus niger* (An).

Carrier	Diameter (mm)	Moisture content (%)
LMW	3.5 ± 0.2	94 ± 1
MMW	3.0 ± 0.3	94 ± 1
HMW	3.5 ± 0.2	93 ± 1
An	3.0 ± 0.2	86 ± 1

higher moisture content compared to *Aspergillus niger* carriers (94% vs 86%, Table 1). The samples maintained their original shape even after drying, with the exception of the LMW chitosan ones. In fact, LMW chitosan beads looked like discs with a hollow centre before GDH crosslinking, while the crosslinked LMW beads preserved their oval shape being more robust and strong (Fig. 3).

Moreover, by comparing the SEM micrographs of the shellfish derived chitosan beads, it is evident that the increase of molecular weight enabled to obtain compact and solid drop-like beads in both wet and dried states. In fact, it is well known that the bead shape is usually affected by polymer molecular weight, the used cross-linker, the cross-linker concentration and the cross-linking time (Shu & Zhu, 2002).

High magnification SEM micrographs of all beads allowed for the analysis of the native and activated bead surfaces. It is important to note that An chitosan derived beads have a rough surface both before and after GDH crosslinking (Fig. 4). In the case of the shellfish chitosan derived beads, the native chitosan beads are characterized by a relatively smooth surface with few wrinkles, whereas the roughness increased in the GDH grafted samples due to the insertion of GDH chains on the bead surface (Fig. 4). On the other hand, the An beads presented a remarkably rough surface also prior to GDH crosslinking that did not seem to significantly influence An surface morphology.

3.1. *Effect of enzyme amount on protein loading and activity of immobilized protease*

Both immobilization methods (DL and GDH) were optimized by evaluating the influence of the initial protein concentration in the range of 0.45–18.00 mg_{BSAeq} mL^{-1} on the total protein loading, in order to obtain an industrially feasible biocatalyst. As shown in Fig. 5a, protein loading on the four different CS beads significantly increased with the increment of the initial protein content up to 2.25 mg_{BSAeq} mL^{-1} and then remained constant. This observation has also been reported in literature by other authors (Monier, Ayad, Wei, & Sarhan, 2010), who proved that any excess of enzymatic protein was lost in the filtrate and washing solution following saturation. Furthermore, similar protein loading values were obtained for all the supports tested and for all initial protein contents, with a protein loading of approximately 5.6 mg_{BSAeq} g^{-1}. On the other hand, a remarkable influence of the initial protein content on

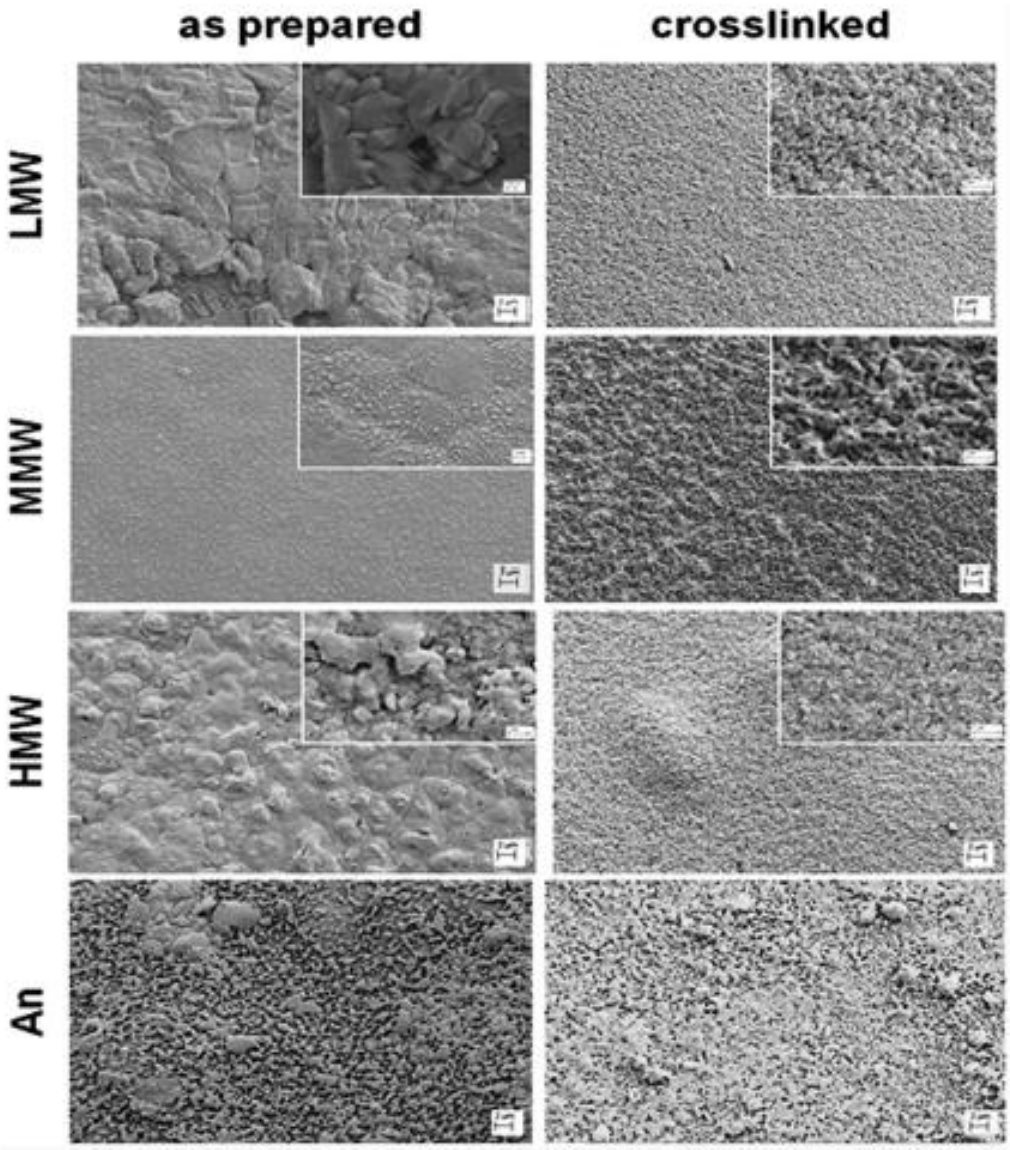

Fig. 4. SEM micrographs of the dried chitosan beads surface before and after glutaraldehyde crosslinking. Chitosan beads were obtained from shellfish with Low (LMW), Medium (MMW) and High (HMW) Molecular Weight, or from *Aspergillus niger* (An).

the protein loading was observed in the case of the GDH immobilization procedure (Fig. 5b). Moreover, the highest protein loading was achieved when protease was immobilized on HMW beads by GDH (13.6 mg_{BSAeq} on 1 g beads), probably due to their rougher surface characterized by a finer microstructure (Fig. 4). However, in all cases, the bromelain immobilization obtained for all tested bead surfaces was further confirmed by the SEM observation of the bead surface after crosslinking and bromelain immobilization, evidencing a very rough surface due to bromelain attachment (Fig. 6), in agreement with previous reports (Monier et al., 2010; Singh, Singh, Suthar, & Dubey, 2011).

Taking into account these data, the IY and the activity of immobilized bromelain toward the synthetic tripeptide chromogenic substrate as well as toward the wine proteins, were compared for the biocatalysts obtained when the initial protein concentration ranged from 1.80 to 4.50 mg_{BSAeq} mL^{-1} (Table 2). However, it was not possible to determine the activity of bromelain immobilized by DL on animal CS beads (LMW, MMW and HMW) due to the solubility of the unmodified supports in the acidic model wine (pH 3.2). Data reported in Table 2 show that IY was in the range of 10–30% and 16–60% for DL and GDH, respectively. It appears that the bromelain activity toward the synthetic substrate, as well as toward wine proteins, is inversely related to the IY, possibly due to the close packing of the enzyme on the support surface which could limit the access of substrate. It is generally acknowledged that the catalytic efficiency of immobilization process decreases when enzyme loading exceeds a certain value and an optimum activity level should be selected (Knezevic, Milosavic, Bezbradica, Jakovljevic, & Prodanovic, 2006). Bromelain proved to be more active toward the synthetic substrate (1.409 ± 0.015 IU mg^{-1}_{IP}), as well as having the highest wine protein removal rate (0.57 ± 0.08 mg $L^{-1}min^{-1}mg^{-1}_{IP}$), when an initial protein concentration of 1.80 mg_{BSAeq} mL^{-1} was immobilized by DL on microbial chitosan. Therefore, bromelain directly linked on An-CS beads was selected for further studies, including the optimization of PBR parameters and the stabilization treatment of Sauvignon blanc wine in continuous mode.

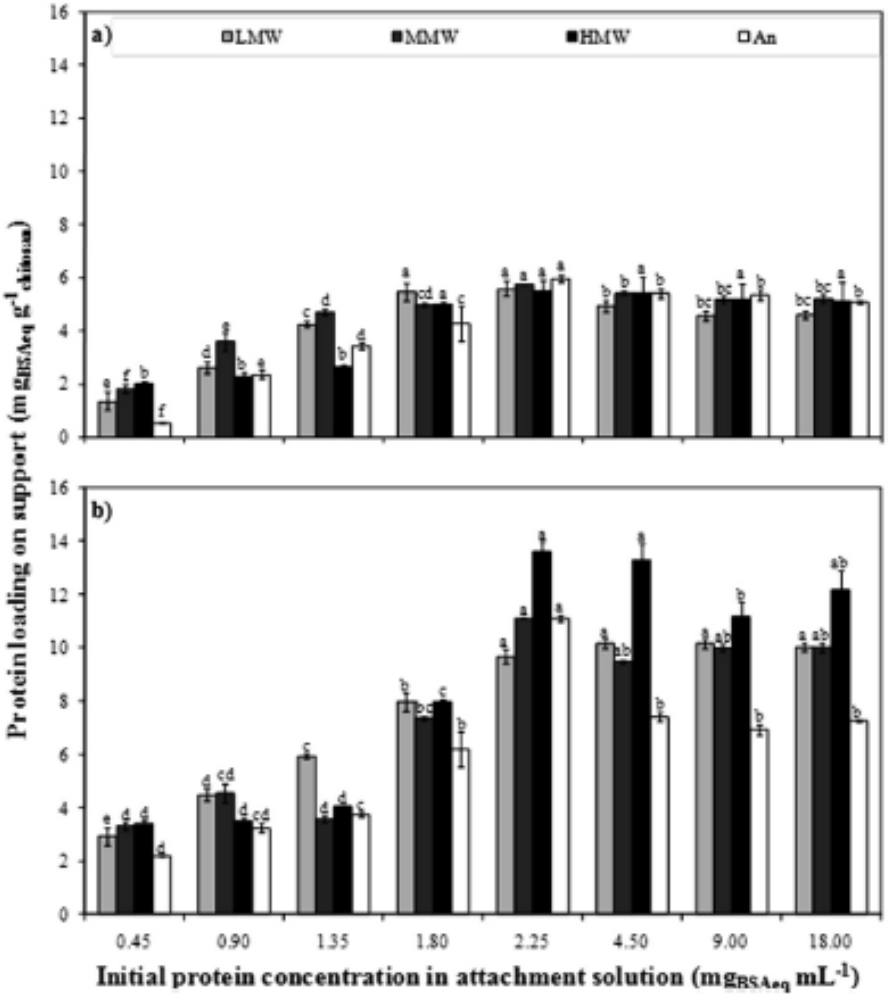

Fig. 5. Effect of the initial protein concentration (0.45–18 mg$_{BSAeq}$ mL^{-1}) in the immobilization solution, containing stem bromelain, on the protein loading, applying two different immobilization procedures: a) direct linkage (DL); b) cross-linking (GDH). Protease was immobilized on beads based on: chitosan from shellfish with Low (LMW), Medium (MMW) and High (HMW) Molecular Weight and from *Aspergillus niger* (An). Reported data are mean ± 95% confidence interval of triplicate analysis. For each support (LMW, MMW, HMW or An) different letters indicate significant differences in the protein loading achieved using different initial protein concentration (Tukey's test, $P \leq 0.05$).

3.2. Wine stabilization treatment in packed-bed reactor

Hereafter, the proteolytic activity of bromelain directly linked on An-CS beads was tested in a PBR with the aim of reducing the hazing potential of white wine in continuous-mode. Firstly, it was necessary to optimize the operational parameters such as flow rate (Q_v), space velocity (S_v) and residence time (τ). For this purpose, 50 mL of model wine, fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM), was fed through the PBR loaded with 13 g of bromelain immobilized on An-CS beads at various flow rates (ranging from 1.08 to 12.00 mL min^{-1}). Data reported in Fig. 7 show that the relationship between r_p and S_v is represented by a bell shape curve with a maximum of free pNA formation at S_v of 0.32 min^{-1} (Q_v 4.86 mL min^{-1}) with a τ of 3.1 min. The free pNA yield, obtained with higher S_V, significantly decreased, indicating that the increase of Q_v reduced the contact time between the substrate and biocatalyst surface during passage through the column (Table 3), as reported by other authors (Laudani, Habulin, Kneza, Della Porta, & Reverchon, 2007), who proved a reciprocal relationship between Q_v and τ (Formuso & Akoh, 2002; Laudani et al., 2007; Watanabe, Miyawaki, Adachi, Nakanishi, & Matsuno, 2001; Xia et al., 2004).

Sauvignon blanc wine was treated in the packed-bed reactor, containing bromelain immobilized on An-CS beads, at the optimized flow rate (Q_v 4.86 mL min^{-1}). The same wine was recycled in the PBR for eight passages, in order to obtain a stabilized wine (Table 4). Proteolytic treatment in continuous mode resulted in a significant reduction of wine hazing potential, as well as of the protein content until the fourth passage in the PBR, when the maximum TRY was achieved (approximately 80%). Further passages in the same PBR did not significantly reduce either the wine hazing potential or the protein content. In light of these observations the treatment of Sauvignon blanc was carried out in five different PBRs in series, with the aim of achieving the full stabilization of wine (Table 5). As previously observed, both hazing potential and protein content remarkably decreased up to the fourth passage, thus achieving a reduction of approximately 72 and 63%, respectively, after which the following passage did not fully stabilize the Sauvignon blanc (Table 5). These results are in accordance with the observations of Marangon, Van Sluyter, Robinson, Muhlack, and Holt (2012), who stated that the stability test may result in false positives. The heat test is carried out at 80 °C, temperature that causes the precipitation of all wine proteins, even those that are known to be heat stable (i.e. invertase) and would not

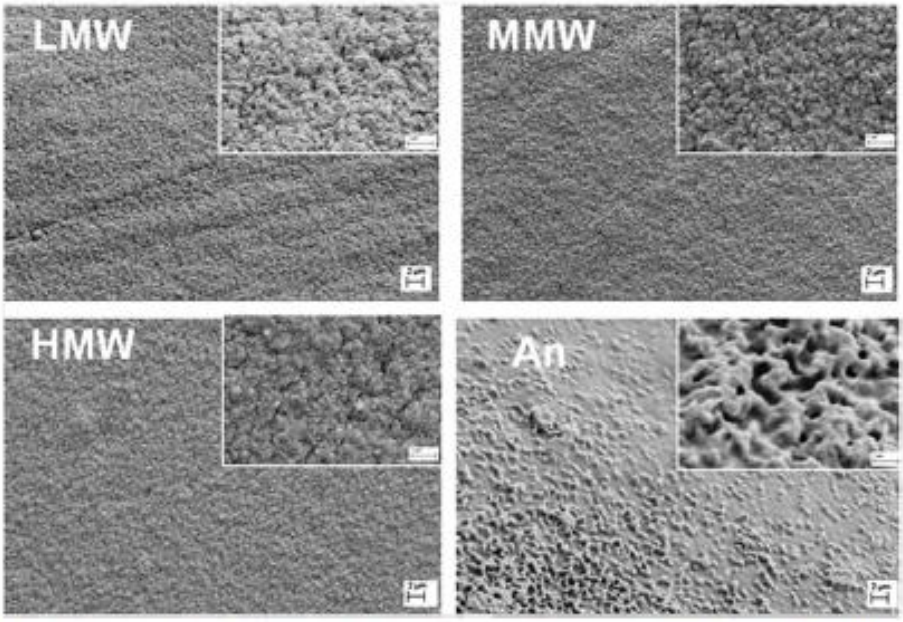

Fig. 6. SEM micrographs of chitosan beads surface after bromelain immobilization. Chitosan beads were obtained from shellfish with Low (LMW), Medium (MMW) and High (HMW) Molecular Weight, or from *Aspergillus niger* (An).

Table 2
Effect of the immobilization procedure (direct linkage (DL) or cross-linking (GDH) on beads by: chitosan from shellfish with Low (LMW), Medium (MMW) and High (HMW) Molecular Weight and from *Aspergillus niger* (An)) and initial protein concentration of the immobilization solution on the immobilization yield (IY) and on protease activity (20 °C) toward synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM, tested in model wine) and Sauvignon blanc wine proteins.

Chitosan	Immobilization procedure	Initial protein concentration ($mg_{BSA_{eq}}$ mL^{-1})	IY (%)	Specific activity (IU mg^{-1}_{P})	Proteins removal rate (mg L^{-1} min^{-1} mg^{-1}_{P})
LMW	DL	1.8	30 ± 2	nd	nd
		2.2	25 ± 1	nd	nd
		4.5	11 ± 1	nd	nd
	GDH	1.8	43 ± 1	0.086 ± 0.007	0.18 ± 0.03
		2.2	44 ± 1	0.093 ± 0.009	0.20 ± 0.04
		4.5	23 ± 1	0.187 ± 0.005	0.34 ± 0.02
MMW	DL	1.8	28 ± 1	nd	nd
		2.2	26 ± 1	nd	nd
		4.5	12 ± 1	nd	nd
	GDH	1.8	41 ± 3	0.075 ± 0.006	0.17 ± 0.03
		2.2	49 ± 4	0.079 ± 0.001	0.18 ± 0.02
		4.5	21 ± 2	0.191 ± 0.001	0.29 ± 0.02
HMW	DL	1.8	28 ± 1	nd	nd
		2.2	24 ± 2	nd	nd
		4.5	12 ± 1	nd	nd
	GDH	1.8	44 ± 3	0.061 ± 0.007	0.23 ± 0.02
		2.2	60 ± 3	0.045 ± 0.004	0.23 ± 0.03
		4.5	29 ± 2	0.111 ± 0.024	0.34 ± 0.02
An	DL	1.8	24 ± 4	1.409 ± 0.015	0.57 ± 0.08
		2.2	26 ± 1	0.986 ± 0.148	0.54 ± 0.06
		4.5	12 ± 1	0.839 ± 0.089	0.42 ± 0.08
	GDH	1.8	34 ± 4	0.086 ± 0.0 8	0.35 ± 0.06
		2.2	49 ± 3	0.047 ± 0.005	0.25 ± 0.05
		4.5	16 ± 1	0.114 ± 0.003	0.20 ± 0.04

precipitate in bottles (Esteruelas et al., 2009; Falconer et al., 2010; Pocock & Waters, 2006).

Numerous studies investigated alternative fining treatments aimed to achieve the protein stabilization of white wines, since it represents an essential stage in their production. Among adsorbent materials, zirconium oxide (powder and pellets), packed in a column (Pashova, Guell, & López, 2004), as well as polysaccharides (i.e. agar, carrageenan, and alginic acid) extracted from seaweeds (Cabello-Pasini, Victoria-Cota, Marcias-Carranza, Hernadez-Garibay, & Muniz-Salazar, 2005), reduced the total protein content of about 50–70%. These adsorbent materials showed an efficiency similar to that achieved using the PBR containing bromelain on An-CS beads. Moreover, our data appears in accordance with Marangon et al. (2012), who achieved 80% reduction in the total protein content of wine, combining a flash pasteurization process with a proteolytic treatment.

4. Conclusions

Chitosan beads were produced starting from two different

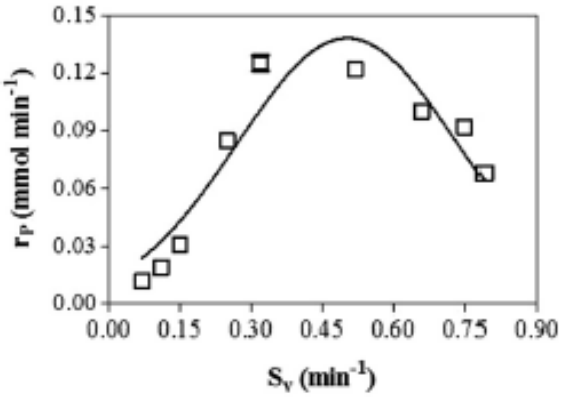

Fig. 7. Relationship between space velocity (S_V) and product-formation rate (r_p) in a packed-bed reactor (PBR-br), containing bromelain immobilized on An-(*Aspergillus niger*) chitosan beads, and fed with model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM) at different flow rates (range 1.08–12.00 mL min^{-1}).

Table 3
Process parameters of the packed-bed reactor, containing bromelain immobilized on An-(*Aspergillus niger*) chitosan beads, fed at varying flow rates with model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM).

Q_V (mL min^{-1})	S_V (min^{-1})	τ (min)	C_p (mmol)	r_p (mmol min^{-1})
1.08	0.07	13.9	0.011 ± 0.002	0.012
1.64	0.11	9.2	0.012 ± 0.000	0.019
2.20	0.15	6.9	0.014 ± 0.002	0.031
3.78	0.25	4.0	0.023 ± 0.003	0.085
4.86	0.32	3.1	0.026 ± 0.004	0.125
7.83	0.52	1.9	0.016 ± 0.002	0.122
10.00	0.66	1.5	0.010 ± 0.002	0.100
11.25	0.75	1.3	0.008 ± 0.001	0.092
12.00	0.79	1.3	0.006 ± 0.001	0.068

Legend: Q_V: flow rate; S_V: space velocity; τ: residence time; C_p: concentration of the reaction product; r_p: product-formation rate.

Table 4
Protein stability data on Sauvignon blanc wine treated for eight passages in the same packed-bed reactor, containing bromelain immobilized on An-(*Aspergillus niger*) chitosan beads, at the optimized flow rate (4.86 mL min^{-1}). Mean values are shown (n = 3).

Treatment	Net haze after heat test		Residual protein content	
	NTU	Reduction (TRY, %)	mg $_{BSAeq}$ L^{-1}	Reduction (%)
Untreated wine	72^{a}	–	211^{a}	–
1° passage	49^{b}	33	151^{b}	28
2° passage	37^{c}	49	131^{c}	38
3° passage	33^{c}	55	114^{d}	46
4° passage	17^{d}	76	82^{e}	61
5° passage	15^{d}	80	72^{e}	66
6° passage	17^{d}	76	68^{e}	68
7° passage	16^{d}	78	64^{e}	70
8° passage	16^{d}	78	63^{e}	70

Values with different roman letters are significantly different (Tukey's test, $P \leq 0.05$).

chitosan sources, i.e. microbial (*Aspergillus niger*) and animal (shellfish, with three different molecular weights), and were used as enzyme carriers to be applied in a continuous packed-bed reactor for white wine protein stabilization, both as prepared and crosslinked with glutaraldehyde (GDH).

The occurred GDH crosslinking was demonstrated by carrying out DSC, FT-IR and SEM analyses, evidencing the shift of the chitosan decomposition temperature to higher values, the presence of the C=N typical vibrational modes and the increased roughness,

Table 5
Protein stability data on Sauvignon blanc wine treated in series in five different packed-bed reactors, containing bromelain immobilized on An-(*Aspergillus niger*) chitosan beads, at the optimized flow rate (4.86 mL min^{-1}). Mean values are shown (n = 3).

Treatment	Net haze after heat test		Residual protein content	
	NTU	Reduction (TRY, %)	mg $_{BSAeq}$ L^{-1}	Reduction (%)
Untreated wine	73 ± 3^{a}	–	207 ± 3^{a}	–
1° passage	53 ± 1^{b}	27	158 ± 1^{b}	24
2° passage	48 ± 5^{bc}	35	115 ± 3^{c}	44
3° passage	27 ± 4^{e}	64	101 ± 3^{d}	51
4° passage	20 ± 1^{d}	72	77 ± 3^{e}	63
5° passage	18 ± 6^{d}	76	73 ± 1^{e}	65

Values with different roman letters are significantly different (Tukey's test, $P \leq 0.05$).

respectively.

Stem bromelain was successfully immobilized on all the tested supports by following the two procedures (i.e., crosslinking with GDH and DL), which were optimized by varying the amount of protein in the coupling solution in order to maximize the catalytic performance of the bound enzyme. Despite the highest immobilization yield was always achieved with GDH linkage, stem bromelain proved to be more active in model wine toward synthetic substrate as well as in real matrix toward wine proteins, when it was immobilized by DL on microbial chitosan.

Considering these promising results, a laboratory bench-scale packed-bed reactor was developed and its operational parameters were investigated in model wine. Applying the optimized process conditions, bromelain immobilized by DL on microbial chitosan proved to be a useful tool for the continuous reduction of white wine hazing potential.

References

Alves, N. M., & Mano, J. F. (2008). Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications. *International Journal of Biological Macromolecules, 43*, 401–414.

Benucci, I., Esti, M., Liburdi, K., & Garzillo, A. M. V. (2012). Pineapple stem bromelain immobilized on different supports: catalytic properties in model wine. *Biotechnology Progress, 28*, 1472–1477.

Benucci, I., Lombardelli, C., Liburdi, K., Acciaro, G., Zappino, M., & Esti, M. (2016). Immobilised native plant cysteine proteases: packed-bed reactor for white wine protein stabilisation. *Journal of Food Science and Technology, 53*, 1130–1139.

Biró, E., Németh, Á., Sisak, C., Feczkó, T., & Gyenis, J. (2008). Preparation of chitosan particles suitable for enzyme immobilization. *Journal of Biochemical and Biophysical Methods, 70*, 1240–1246.

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

Cabello-Pasini, A., Victoria-Cota, N., Marcias-Carranza, V., Hernadez-Garibay, E., & Muniz-Salazar, R. (2005). Clarification if wines using polysaccharides extracted from seaweeds. *American Journal of Enology and Viticulture, 56*, 52–59.

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

Chen, S. C., Wu, Y. C., Mi, F. L., Lin, Y. H., Yu, L. C., & Sung, H. W. (2004). A novel pH-sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. *Journal of Controlled Release, 96*, 285–300.

Chuang, W. Y., Young, T. H., Yao, C. H., & Chiu, W. Y. (1999). Properties of the poly(vinyl alcohol)/chitosan blend and its effect on the culture of fibroblast in vitro. *Biomaterials, 20*, 1479–1487.

Esteruelas, M., Poinsaut, P., Sieczkowski, N., Manteau, S., Fort, M. F., Canals, J. M., et al. (2009). Characterization of natural haze protein in sauvignon white wine. *Food Chemistry, 113*, 28–35.

Falconet, R. J., Marangon, M., Van Sluyter, S. C., Neilson, K. A., Chan, C., & Waters, E. J. (2010). Thermal stability of thaumatin-like protein, chitinase, and invertase isolated from Sauvignon blanc and Semillon juice and their role in haze formation in wine. *Journal of Agricultural and Food Chemistry, 58*, 975–980.

Formuso, L. B., & Akoh, C. C. (2002). Lipase-catalysed acidolysis of olive oil and caprylic acid in a bench-scale packed bed bioreactor. *Food Research International, 35*, 15–21.

Hale, L. P., Greer, P. K., Trinh, C. T., & James, C. L. (2005). Proteinase activity and

stability of natural bromelain preparations. *International Immunopharmacology, 5*, 783–793.
Knaul, Z. J., Hudson, S. M., & Creber, A. M. K. (1999). Crosslinking of chitosan fibers with dialdehydes: proposal of a new reaction mechanism. *Journal of Polymer Science Part B: Polymer Physics, 37*, 1079–1094.
Knezevic, Z., Milosavic, N., Bezbradica, D., Jakovljevic, Z., & Prodanovic, R. (2006). Immobilization of lipase from Candida rugosa on Eupergit® C supports by covalent attachment. *Biochemical Engineering Journal, 30*(3), 269–278.
Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology, 35*, 126–139.
Laudani, C. G., Habulin, M., Kneza, Z., Della Porta, G., & Reverchon, E. (2007). Immobilized lipase-mediated long-chain fatty acid esterification in dense carbon dioxide: bench-scale packed-bed reactor study. *Journal of Supercritical Fluids, 41*, 74–81.
Marangon, M., Van Sluyter, S. C., Robinson, E. M., Muhlack, R. A., Holt, H. E., et al. (2012). Degradation of white wine haze proteins by aspergillopepsin I and II during juice flash pasteurization. *Food Chemistry, 135*, 1157–1165.
Moine-Ledoux, V., & Dubourdieu, D. (1999). An invertase fragment responsible for improving the protein stability of dry white wines. *Journal of the Science of Food and Agriculture, 79*, 537–543.
Monier, M., Ayad, D. M., Wei, Y., & Sarhan, A. A. (2010). Immobilization of horseradish peroxidase on modified chitosan beads. *International Journal of Biological Macromolecules, 46*(3), 324–330.
Monteiro, O. A. C., Jr., & Airoldi, C. (1999). Some studies of crosslinking glutaraldehyde interaction in a homogeneous system. *International Journal of Biological Macromolecules, 26*(2–3), 119–128.
Nwe, N., Furuike, T., & Tamura, H. (2010). Production of fungal chitosan by enzymatic method and applications in plant tissue culture and tissue engineering: 11 Years of our progress, present situation and future prospects. *Biopolymers, 7*, 135–162.
Onofri, A. (2006). Enhancing Excel capability to perform statistical analyses in agriculture applied research. In *Computational statistics and data analysis – Statistical software newsletters*. International Association for statistical Computing.
Pashova, V., Guell, C., & López, F. (2004). White wine continuous protein stabilization by packed column. *Journal of Agricultural and Food Chemistry, 52*, 1558–1563.
Pocock, K. F., & Rankine, B. C. (1973). Heat test for detecting protein instability in wine. *Australian Wine, Brewing and Spirit Review, 91*(5), 42–43.
Pocock, K. F., & Waters, E. J. (2006). Protein haze in bottled white wines: How well do stability tests and bentonite fining trials predict haze formation during storage and transport? *Australian Journal of Grape and Wine Research, 12*, 212–220.
Rao, C. N. R. (1963). *Chemical application of infrared spectroscopy*. London: Academic Press New York.
REG. EC No. 606/2009, Annex I A, Appendix 13.
Sakurai, K., Maegawa, T., & Takahashi, T. (2000). Glass transition temperature of chitosan and miscibility of chitosan/poly(N-vinyl pyrrolidone) blends. *Polymer, 41*, 7051–7056.
Santos, H., Veiga, F., Pina, M., Podczeck, F., & Sousa, J. (2002). Physical properties of chitosan pellets produced by extrusion – spheronisation: influence of formulation variables. *International Journal of Pharmaceutics, 246*, 153–169.
Shellfish Wastes. MIT Sea Grant Report. MISG 77-3: MIT Cambridge, MA.
Shu, X. Z., & Zhu, K. J. (2002). Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure. *International Journal of Pharmaceutics, 233*, 217–225.
Singh, A. N., Singh, S., Suthar, N., & Dubey, V. K. (2011). Glutaraldehyde-activated chitosan matrix for immobilization of a novel cysteine protease, procerain B. *Journal of Agricultural and Food Chemistry, 59*, 6256–6262.
Talbert, J. N., & Goddard, J. M. (2012). Enzymes on material surfaces. *Colloids and Surfaces B: Biointerfaces, 93*, 8–19.
Tan, S. C., Tan, T. K., Wong, S. M., & Khor, E. (1996). In W. F. Stevens, M. S. Rao, & S. Chandrkrachang (Eds.), *Proceedings of the second Asia Pacific Chitin and Chitosan Symposium, Bangkok* (pp. 50–57).
U.S. Food and Drug Administration. (2016). *Partial list of enzyme preparations that are used in foods*. Washington, D.C.: U.S. Food and Drug Administration, Center of Food Safety and Applied Nutrition, Office of Food Additive Safety. Available at http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/EnzymePreparations/default.htm Accessed 22.04.16.
Vieira, R. S., & Beppu, M. M. (2006). Interaction of natural and crosslinked chitosan membranes with Hg(II) ions. *Colloids and Surfaces A, 279*, 196–207.
Vincenzi, S., Marangon, M., Tolin, S., & Curioni, A. (2011). Protein evolution during the early stages of white winemaking and its relations with wine stability. *Australian Journal of Grape and Wine Research, 17*, 20–27.
Watanabe, Y., Miyawaki, Y., Adachi, S., Nakanishi, K., & Matsuno, R. (2001). Continuous production of acyl mannoses by immobilized lipase using a packed bed reactor and their surfactant properties. *Biochemical Engineering Journal, 8*, 213–216.
White, S. A., Farina, P. R., & Fulton, I. (1979). Production and isolation of chitosan from Mucor rouxii. *Applied and Environmental Microbiology, 38*(2), 323–328.
Xia, Y. M., Fang, Y., Xu, Z. G., Hu, X. Y., Shen, Y. A., & Brown, J., Jr. (2004). A novel process for enzymatic synthesis of N-lauroyl–amino propionitrile using a packed bed reactor coupled with on-line separation. *Journal of Molecular Catalysis B: Enzymatic, 31*, 111–115.
Zappino, M., Cacciotti, I., Benucci, I., Nanni, F., Liburdi, K., Valentini, F., et al. (2015). Bromelain immobilization on microbial and animal source chitosan films, plasticized with glycerol, for application in wine-like medium: microstructural, mechanical and catalytic characterizations. *Food Hydrocolloids, 45*, 41–47.
Zeng, M., Fang, Z., & Xu, C. (2004). Effect of compatibility on the structure of the microporous membrane prepared by selective dissolution of chitosan/synthetic polymer blend membrane. *Journal of Membrane Science, 230*, 175–181.