Chitosan/clay nanocomposite films as supports for enzyme immobilization: An innovative green approach for winemaking applications

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ABSTRACT

Protein haze formation in white wines during storage is considered the most important instability phenomenon of non-microbial origin. The use of proteolytic enzymes, covalently immobilized on solid supports, has recently proved to be a promising approach for reducing the haze potential of white wines. In this study, supports based on chitosan and nanoclays were produced by solvent casting technique, and investigated for use as a covalently linked bromelain carrier in wine-like medium. Various kinds of nanoclays were tested, including montmorillonite, sepiolite and bentonite, in different amounts (1–5% w/w with respect to chitosan). More specifically, unmodified and organically modified clays and an activated bentonite authorized for contact with food (i.e. OPTIGEL CK) were considered, and their effect on the final microstructural, thermal and mechanical properties of the obtained composite systems was investigated, on the basis of their different chemical composition, morphology, hydrophilicity/hydrophobicity and surface charge, resulting in different interactions with the polymeric matrix and a different enzyme loading.

The nanocomposite films were used as innovative supports for the covalent immobilization of pineapple stem bromelain, selected as reference enzyme, and the kinetic parameters of the immobilized bromelain were analyzed.

1. Introduction

Protein haze formation is the most important instability phenomenon of non-microbial origin, which occurs in white wines during storage (Batista, Monteiro, Loureiro, Teixeira, & Ferreira, 2009; Waters et al., 2005). Bentonite is the most commonly used fining agent for stabilizing white wines, despite its unspecificity toward haze forming proteins and its detrimental effect on wine quality (Ferreira, Picarra-Pereira, Monteiro, Loureiro, & Teixeira, 2002; Sauvage, Bach, Moutounet, & Vernhet, 2010). For this reason, various authors have evaluated the effectiveness of proteolytic enzymes, either in free form (Marangon et al., 2012; Younes, Cilindre, Jeandet, & Vasserot, 2013) or covalently immobilized on solid supports, for reducing the haze potential of white wines (Benucci, Esti, & Liburdi, 2014; Liburdi, Benucci, & Esti, 2010), as a promising alternative to bentonite fining.

In recent years, many inorganic (i.e. glass, mineral kissiris, γ-alumina, polygorskite, montmorillonite, hydromica, porous porcelain, pumice stone) and organic materials (i.e. alginate, cellulose, carrageenan, agar, pectic acid and chitosan) commonly found in nature, have been proposed as immobilization supports to be used in winemaking (Colagrande, Silva, & Fumi, 1994; Divies, Cachon, Cavin, & Prevost, 1994; Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). Among the organic materials, thanks to its hydrophilicity, biocompatibility and biodegradability, chitosan (CS), a natural polyaminosaccharide obtained by *N*-deacetylation of chitin, is deemed to be ideal for producing supports in various forms (particles, gels, spheres, fibres and membranes) for the immobilization of food enzymes (Altun & Cetinus, 2007; Benucci et al., 2016; Boadi & Neufeld, 2001; Dwevedi & Kayastha, 2009; Krajewska,

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2004; Malmiri, Ghaz Jahanian, & Berenjian, 2012; Noda, Furuta, & Suda, 2001; Spagna, Barbagallo, Casarini, & Pifferi, 2001; Zappino et al., 2015). In a previous study, membranes based on CS from animal (shell fish) and microbial (*Aspergillus niger*) sources have been obtained by solvent casting method, using glycerol as plasticizer (Zappino et al., 2015), for applications in acidic medium mimicking wine. However, several authors have affirmed that CS films have low mechanical properties and thermal stability (Cengiz, Çavaş, & Yurdakoç, 2012; Tang et al., 2009; Wang, Shen, Zhang, & Tong, 2005; Chang & Juang, 2007a, b), and that adding proper nanofillers (i.e. carbon nanotubes, nanoclays and silica) to the polymeric matrix may be an effective way of improving the mechanical and thermal properties (Panzavolta et al., 2014).

In particular, nanoclays are widely used for the preparation of CS based composites, due to their low cost, high availability, high cation exchange capacity, strong adsorption ability and high surface area (Chang & Juang, 2007b; An, Zhou, Zhuang, Tong, & Yu, 2015; Bertolino et al., 2016; Daraei et al., 2013). Among the clays, the most widely studied ones are montmorillonite (MMT, a hydrated 2-1 layered alumina-silicate, composed of two silica tetrahedral sheets attached to a central Al octahedral sheet), and sepiolite (SP, a microfibrous hydrated magnesium silicate, composed of two tetrahedral silica sheets enclosing a central magnesia sheet), employed in numerous industrial and environmental applications (i.e. oil refining, wastewater treatment, odour removal, pharmaceuticals, and pesticide carriers, paper and detergent industries) (Hsu, Wang, & Lin, 2012; Lewandowska, Sionkowska, Furtos, Grabska, & Michalska, 2015; Sedaghat, Ghiaci, Aghaei, & Soleimanian-Zad, 2009). Moreover, several studies have been devoted to the use of organomontmorillonite (oMMT), produced by exchanging inorganic cations of MMT with organic ammonium ions, in order to make the MTT more hydrophobic and suitable for several applications, such as clay-based nanocomposites (Ray & Okamoto, 2003) and absorbents of organic pollutants (Adebajo, Frost, Kloprogge, Carmody, & Kokot, 2003). Indeed, the surface is characterized by a stable negative charge and by hydrophilicity, due to the presence of inorganic cations (e.g. Na^+ and Ca^{2+}). Therefore, attempts have been made to modify the clay surface with cationic surfactants, such as alkyl ammonium salts (Gupta & Suhas, 2009), since replacing exchangeable cations with cationic surfactants changes the clay surface from hydrophilic to hydrophobic, originating organoclays (Gupta & Suhas, 2009; He, Duchet, Galy, & Gérard, 2006).

In particular, CS films loaded with MMT/oMMT and SP have also been used to produce supports for enzyme immobilization (Azeredo, 2009; Gopinath & Sugunan, 2007) and as green plastics (Darder, Aranda, & Ruiz-Hitzky, 2012; Wang, Chen, & Tong, 2006). In fact, in the absence of other organic modifiers, this biopolymer is able to intercalate into the pristine clay via the cationic exchange process, originating intercalated and exfoliated nanostructures, which can provide strong interactions between the clay and CS (Tang et al., 2009). To the best of our knowledge, in literature there is a lack of systematic studies about the influence of several clays on the final properties of composite CS based supports as enzymatic carriers for application in winemaking. Therefore, the aim of this study was to produce biopolymeric nanocomposite films as carriers for the covalent immobilization, via glutaraldehyde (GDH) cross-linking, of a proteolytic enzyme (bromelain from pineapple stem) to be applied in winemaking process. In order to achieve this goal, on the basis of a previous study by the same authors (Zappino et al., 2015), low molecular weight CS blended with 25% glycerol was selected as biopolymeric matrix and different kinds of nanoclays were added as fillers to the polymeric matrix. More specifically, organically modified montmorillonites, bentonites (unmodified and modified) and sepiolite were tested in different amounts (1–5% w/w with respect to CS). We decided to use, as immobilization procedure, the covalent coupling of the protease to glutaraldehyde-activated supports, being the most commonly applied (also for food applications), quite simple and efficient (Garavand, Rouhi, Razavi, Cacciotti, & Mohammadi, 2017). The chemical, thermal, microstructural and mechanical properties of the obtained systems were analysed by infrared spectroscopy (FTIR/ATR), differential scanning calorimetry (DSC), scanning electron microscopy (SEM) and uniaxial tensile tests.

2. Materials and methods

2.1. Materials

Films, to be used as supports for enzyme immobilization, were produced using CS powder obtained from an animal source, composed of shellfish powder with low molecular weight (Lot#MKBG3334V; 50–190 kDa; percentage of deacetylation: 75%; Sigma Aldrich). Five different nanoclays, kindly provided by various suppliers (Table 1), were added to the CS-based films as fillers: i) high purity organically modified montmorillonites (DK1N and DK2); ii) selected and activated bentonite authorized for contact with food (OPT); iii) high purity modified bentonite (SMP); iv) high purity unmodified sepiolite (PAN). The characteristics of the used clays are summarized in Table 1.

The organoclays DK1N and DK2 were modified by different levels of cation exchange reaction with octadecylammonium salt in order to broaden their interlayer spacings, according to the method described in literature (Kornmann, Lindberg, & Berglund, 2001), and had an average thickness of approximately 25 nm (aspect ratio 100–1000).

The tripeptide chromogenic substrate, Bz-Phe-Val-Arg-*p*-nitroaniline (pNA), used for evaluating protease activity, was purchased from Bachem (Bubendorf, Switzerland). Stem bromelain (EC 3.4.22.32), which was selected as referred enzyme, glycerol (≥99.5%), glutaraldehyde (GDH) (25%), and all of the other reagents were obtained from Sigma-Aldrich (Milan, Italy).

2.2. Preparation of chitosan/clay nanocomposite films

Films were prepared by solvent casting technique, using low molecular weight CS (1% w/v) blended with glycerol in the weight ratio CS:glycerol 75:25, selected on the basis of previous results by Zappino et al. (2015). The five different nanoclays were added in various w/w percentages with respect to CS (i.e. 1, 3, 5% w/w), thus obtaining three different compositions for every clay (DK1N-x, DK2-x, OPT-x, SMP-x, PAN-x, where x is the clay w/w percentage).

Firstly, the nanoclays were ultrasonicated in aqueous solution (Ultrasonicator Sonics Vibracell CV33, 750 W, 20 kHz, Amplitude 30%, time 30′), subsequently acetic acid (2% v/v), low molecular weight CS (1 %w/v) and glycerol were added. The obtained suspensions were magnetically stirred overnight. The CS solutions were poured onto plastic Petri dishes, which were placed in a fume hood at room temperature for 48 h to allow for solvent evaporation and film formation.

Moreover, as a reference, the clay-free film, composed of CS and glycerol, was produced following the same procedure.

2.3. Chemical, thermal, mechanical and morphological characterization of chitosan/clay nanocomposite films

The typical functional groups of the produced films were investigated by means of FT-IR spectrometer (Jasco, FT/-6600), equipped with an attenuated total reflectance (ATR). Spectra were acquired by pressing the samples into contact with the ATR cell, in

Table 1
Characteristics of the used clays.

Clay	Supplier	Density	Interlayer spacing[a] (nm)	Clay kind
DK1 N	Zhejiang Fenghong New Material Co., ltd. (China)	1.8	2.29	Organomontmorillonite
DK2	Zhejiang Fenghong New Material Co., ltd. (China)	1.8	2.25	Organomontmorillonite
OPTIGEL CK (OPT)	BYK Additives GmbH (Germany)	2.5	n.a.	Pure bentonite
SMP	Zhejiang Fenghong New Material Co., ltd. (China)	2.2	–	Modified bentonite
PANGEL S9 (PAN)	Tolsa S. A. (Spain)	2.1	–	Sepiolite

[a] The interlayer spacing corresponds to Bragg reflection peak (d001) from raw organoclay powders using X-ray diffraction (XRD) scan.

the following conditions: wavenumber range 600–4000 cm^{-1}, spectral resolution 4 cm^{-1} and scans number 32.

The thermal properties of the produced films were studied by means of differential scanning calorimetry (DSC, TAInstruments Q2000) under the following conditions: sample weight ~5 mg, nitrogen atmosphere (N_2 flow rate 50 cc/min), range temperature 20–400 °C, heating rate 10 °C/min.

The mechanical behaviour of all the prepared samples was investigated by performing uniaxial tensile tests on dog-bone specimens (width 4.8 mm, length 22.25 mm) with an electromechanical apparatus (Lloyd LRX Lloyd Instruments), on the basis of ASTM D882 standard, under the following conditions: test speed 1.2 mm/min, load cell 50 N, number of specimens per type 8 (in order to obtain average values). The mechanical properties were calculated considering the nominal specimen cross-section. In order to evaluate the nanoclay distribution within the polymeric matrix and the CS/nanoclay interface, the fracture surfaces of some selected stress-strain films were observed at Field-Emission Gun Scanning Electron Microscope (FEG-SEM, Cambridge Leo Supra 35, Carl Zeiss).

2.4. Protease immobilization on CS/clay nanocomposite films

Before enzyme immobilization, the CS-film samples were neutralized by overnight shaking in 26% (v/v) ethanol-NaOH 2 M solution and then cut into squares (10 mm × 10 mm) with a razor blade. The immobilization procedure was performed using GDH as cross-linker, activating the surface of each CS-film square with 1 mL of 3% GDH (v/v). The suspension was kept at room temperature under constant agitation for 2 h, and then thoroughly washed with distilled water. Thereafter, 1 mL of bromelain preparation (2.2 $mg_{protein}$ mL^{-1}), previously solubilised in the immobilization buffer (tartaric acid/sodium tartrate 0.03 M, pH 3.2), was added to the active CS-film square and shaken (150 rpm at 20 °C) overnight. At the end of the incubation time, the samples were washed three times with immobilization buffer and then left to stand for 20 min in a 0.1 M glycine solution.

At the end of the immobilization procedure, the biocatalysts were washed three times with 2 M NaCl solution to remove all non-covalently bound proteins. After collecting the supernatant, the films were rewashed in the immobilization buffer. All supernatants were collected and diluted with buffer solution to a constant final volume in order to determine the bound protein.

The immobilization yield (IY, %) was indirectly determined as the difference between the protein concentration in the enzyme solution before and after immobilization. The protein concentration was determined using the Bradford method (Bradford, 1976) with Coomassie brilliant blue reagent and measuring absorbance at 595 nm. Bovine serum albumin (BSA) was used as standard protein.

2.5. Proteolytic activity assay

Proteolytic activity toward the tripeptide chromogenic substrate (Bz-Phe-Val-Arg-pNA) 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 with an UV–visible spectrophotometer (Shimadzu UV 2450, Milan, Italy). Specific activity, calculated in IU (International Unit) of pNA produced (μmmol = 8.480 mM^{-1} cm^{-1} for pNA, as reported by Hale, Greer, Trinh, & 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. Kinetic characterization of immobilized protease

A kinetic study of protease immobilized on CS/clay nanocomposite films was carried out in model wine, fortified with Bz-Phe-Val-Arg-pNA substrate (0–750 μM). Kinetic parameters were determined according to the Michaelis-Menten equation using a non linear regression procedure (GraphPad Prism 5.01, GraphPad Software, Inc.).

The K_M (Michaelis-Menten costant) value reflects the enzyme-substrate complex formation, whereas k_{cat} (turnover number) measures the number of substrate molecules turned over per enzyme per minute. Moreover, k_{cat} is indicative of the product release velocity, representing the maximum number of moles of substrate converted into the product per number of moles of catalyst per unit time. In addition, as K_a (affinity constant) is the ratio k_{cat}/K_M and reflects the affinity of the enzyme toward the substrate, it is indicative of both reaction steps and expresses the overall catalytic efficiency. The goodness of-fit for each data set (to their best-fit theoretical kinetic curves) was assessed as the square of the correlation coefficients (R^2).

2.7. Statistical analysis

The data, obtained from the average of three replicate measurements, were analysed 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

3.1. Chemical, thermal, mechanical and morphological properties of chitosan/clay nanocomposite films

In order to investigate the possible chitosan/clays interaction, FTIR/ATR spectra were acquired on clay-free film and films loaded with 5% w/w of all used nanoclays, revealing the typical chitosan vibration modes (Chen et al., 2004) in all cases (Fig. 1). However, a slight shift of the peaks at 1574 cm^{-1}, ascribed to the vibrational mode of chitosan protonated amine group (δ_{NH3}) (Darder, Colilla, &

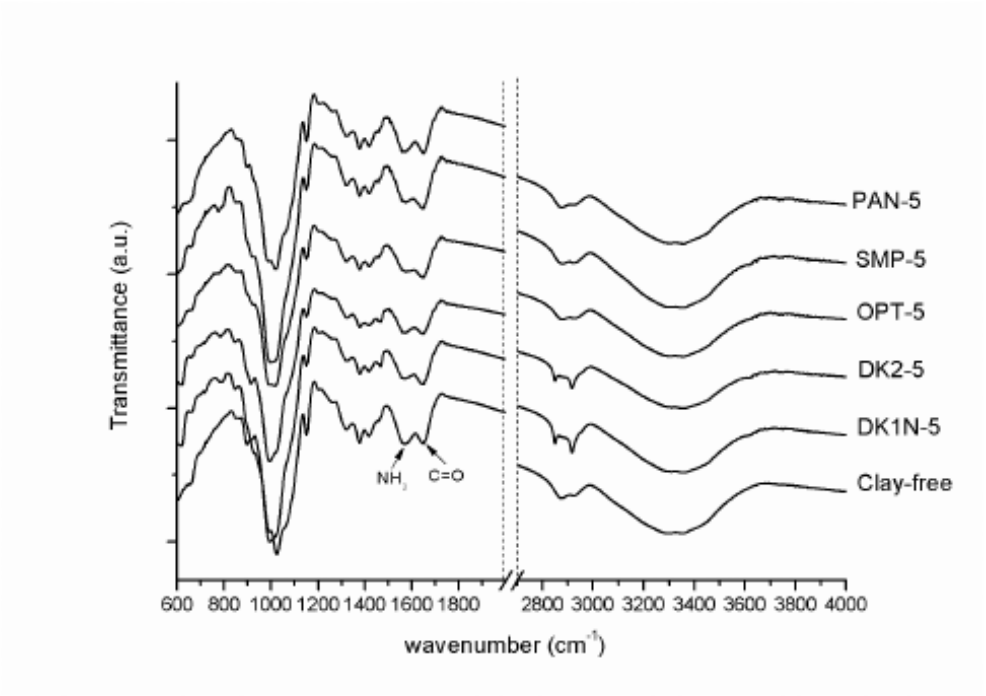

Fig. 1. FTIR/ATR spectra of clay-free chitosan film and chitosan/clay nanocomposite films loaded with 5% w/w of all used clays.

Ruiz-Hitzky, 2003), was recorded in the case of composite films. The δ_{NH3} shift towards a lower frequency testifies the occurred electrostatic interaction between NH_3^+ and the negatively charged sites of the clay (Darder et al., 2003).

It is also interesting to note that in the case of DK1N-5 and DK2-5 films, the peaks at 2920 cm^{-1} and 2875 cm^{-1}, ascribed to the –CH vibrational modes, are more defined and intense with respect to the other samples, due to the contribution of the used organomodifiers for the modification of the used montmorrilonite powder.

Furthermore, DSC measurements were acquired in order to study the thermal properties of the produced films. Fig. 2a shows the comparisons of the DSC first run curves of the films, unloaded and loaded with 5% w/w of all the used nanoclays. In most of samples a broad endothermic peak was observed at approximately 110–120 °C, caused by the dissociation process of interchain hydrogen bonding of CS (Chuang, Young, Yao, & Chiu, 1999), as well as an exothermic band at approximately 285–290 °C, ascribed to CS decomposition (Sakurai, Maegawa, & Takahashi, 2000; Zeng, Fang, & Xu, 2004). It is interesting to note that the composite films loaded with organo-montmorillonite (DK1N-5 and DK2-5) were characterised by a decreased hydrogen bonds dissociation temperature value compared to the clay-free sample (121 °C), whereas those loaded with sepiolite (PAN-5) and bentonite, both pure (OPT-5) and modified (SMP-5), showed comparable values (120 °C, 119 °C and 120 °C, respectively). In fact, the presence of organoclays (DK1N and DK2), whose chemical structure is schematized in Fig. 2b (adapted from Mamun, Soutome, Meng, & Fujimori, 2015), led to a significant decrement of the hydrogen bonds dissociation temperature and of the related enthalpy, particularly for DK1N-5, due to their hydrophobic behaviour and, thus, lower interaction with the water molecules. Moreover, DK1N-5 presented a lower dissociation temperature with respect to DK2-5, since this clay is more hydrophobic, as reported in the manufacturer's (Zhejiang Fenghong New Material Co., Ltd. (China)) data sheet.

As regards to chitosan degradation temperature, comparable values were measured in the case of DK1N-5 and DK2-5 in respect to clay-free film, while an increased value was recorded for the film loaded with PAN (290 °C *vs* 287 °C for clay-free CS), thus suggesting

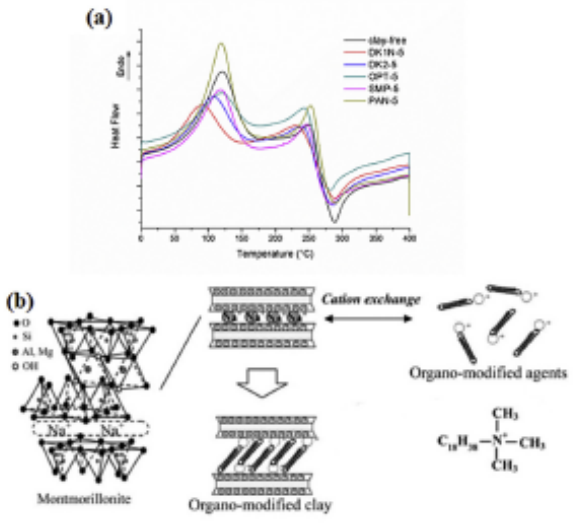

Fig. 2. (**a**) DSC first heating curves of clay-free chitosan film and chitosan/clay nanocomposite films loaded with 5% w/w of all used clays; (**b**) chemical structure of organo-modified montmorrilonite (adapted from Mamun et al., 2015 with permission).

a good dispersion of these fillers within the polymeric matrix and their action as thermal barrier. Finally, a slight decrement of CS thermal decomposition was evidenced in the case of SMP-5 and OPT-5 samples, recording a value of 283 °C.

The mechanical properties of the produced nanocomposite films were analysed in order to investigate the effect of the addition of different nanoclay kinds, at various amounts (1, 3, 5% w/w with respect to the chitosan), on the films behaviour.

Adding PAN and SMP nanoclays resulted in significantly higher σ_{max} values compared to the clay-free film, characterised by a slightly progressive increase with the nanoclay content. These experimental findings suggested the formation of intercalated and/or exfoliated composite structures, due to the strong interaction between the PAN or SMP clays, characterised by a negative charge and hydrophilic surface, and the cationic and hydrophilic CS chains. Indeed, intercalation occurs when a small amount of polymer is inserted between the clay layers, thus expanding the interlayer spacing and forming a well-ordered multilayer structure, whereas exfoliation occurs when the clay layers are completely separated and the individual layers are distributed throughout the polymer matrix (Xu, Ren, & Hanna, 2006). The increase in σ_{max} value for the composite films containing SMP can be attributed to the high rigidity and aspect ratio of the nanoclay, in addition to the high affinity between the biopolymer and the used nanoclay, as reported in the case of chitosan/bentonite films by Laaraibi, Charhouf, Bennamara, Abourriche, & Berrada, M. (2015). Similar results are reported by Darder et al. (2006) who highlighted that the addition of sepiolite to chitosan is able to play an evident reinforcing effect, suggesting a good dispersion of the used nanoclays (in forms of fibrils) within the chitosan matrix. According to them, this finding has to be ascribed to the electrostatic interactions between the positively charged amine groups of the biopolymer and the negatively charged silanol groups of the silicate, as well as to the hydrogen bonding interactions (Darder et al., 2006).

On the other hand, DK1N, DK2 and OPT based nanocomposite supports presented σ_{max} values comparable to the value recorded for the clay-free film (Fig. 3). This different behaviour may be justified by the bad filler/polymeric interface due to the hydrophilic nature of chitosan and the hydrophobic surface of the organo-modified clays, in the case of DK1N and DK2, thus originating tactoid structures. Indeed, it is well known that in the case of organoclays, micro-scale composites are usually obtained, presenting a tactoid organization where the clay particles are dispersed within the polymer matrix and the layers do not separate (Xu et al., 2006).

A similar trend was observed for Young modulus (E), which appeared to be significantly improved when SMP and PAN were added as fillers, regardless their concentration. Moreover, the inclusion of the other nanoclays, i.e. DK1N, DK2 and OPT, did not significantly affect the E value with respect to the clay-free film (Fig. 4). As expected, a sharp decrease in elongation at break was observed for all nanocomposites compared to the clay-free film (Fig. 5), due to the presence of an inorganic component and consequent restricted mobility of macromolecular chains (Laaraibi, Charhouf, Bennamara, Abourriche, & Berrada, 2015).

It is interesting to evidence that, in all cases, independently of the kind of nanoclay, the amount of filler, added within the considered range (1–5 %w/w), did not seem to significantly affect the mechanical properties of the obtained nanocomposite films.

Moreover, these data suggest that PAN and SMP clays were characterized by better disagglomeration and reinforcement actions due to their hydrophilic nature and, therefore, their better dispersion in water medium and tendency to form stronger polar interactions with CS chains, as supported by SEM observation of the stress-strained fracture surface for the samples loaded with 5% w/w of SMP and PAN nanoclays (Fig. 6d–e). In both cases the used fillers were well and homogeneously distributed within the polymeric matrix, presenting a good wettability and compatibility, especially in the case of PAN which is characterized by needle-like nanoclays oriented in the load direction (Fig. 6e, inset), resulting in an improved mechanical behaviour ascribable to their typical morphology.

Based on the collected mechanical data (Figs. 3–5) and SEM micrographs of the fracture surfaces of the stress-strained films (Fig. 6), the different behaviour may be due to the distinct chemical composition and morphology of the added nanoclays, as PAN is needle-like and the others are platelet-like, and to the different agglomeration tendency.

3.2. Kinetic properties of protease immobilized on chitosan/clay nanocomposite films

The nanocomposite films, characterised by the presence of various nanoclays in different amounts (1, 3, 5% w/w with respect to the chitosan), were used as innovative supports for the covalent

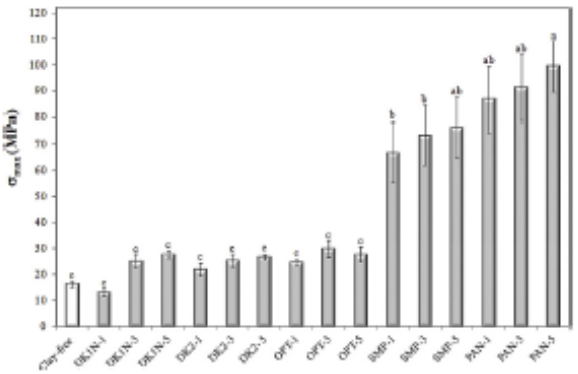

Fig. 3. Ultimate tensile stress (σ_{max}) of chitosan/clay nanocomposite films, obtained by adding nanoclays at various concentration (1, 3 and 5% w/w with respect to the chitosan), compared to the clay-free chitosan film. Reported values are mean ± 95% confidence interval of triplicate measurements. The used roman letters (i.e. a, b, c and ab) indicate significant differences (Tukey's test, $P < 0.05$) in the mechanical results among the investigated samples.

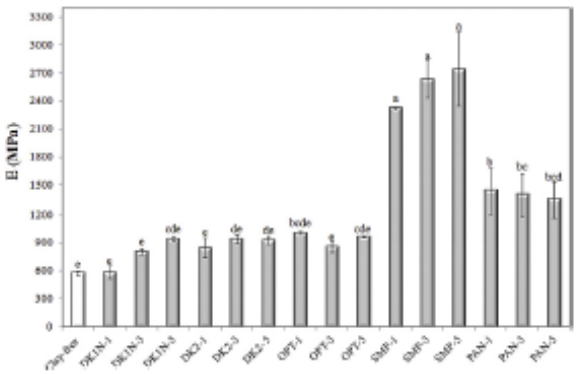

Fig. 4. Tensile modulus (E) of chitosan/clay nanocomposite films, obtained by adding nanoclays at various concentrations (1, 3 and 5% w/w with respect to the chitosan), compared to the clay-free chitosan film. Reported values are mean ± 95% confidence interval of triplicate measurements. The used roman letters (i.e. a, b, e, bc, de, bcd, cde, bcde) indicate significant differences (Tukey's test, $P < 0.05$) in the mechanical results among the investigated samples.

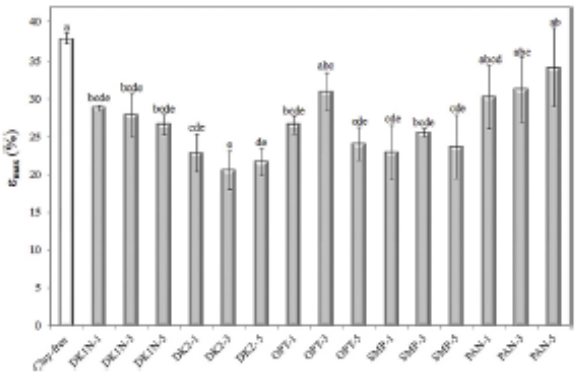

Fig. 5. Elongation at break (ε_{max}) of chitosan/clay nanocomposite films, obtained by adding nanoclays at various concentrations (1, 3 and 5% w/w with respect to the chitosan), compared to the clay-free chitosan film. Reported values are mean ± 95% confidence interval of triplicate measurements. For each value, means with different roman letters are significantly different ($P \leq 0.05$).

immobilization of pineapple stem bromelain, which was selected as reference enzyme.

The inclusion of fillers within the chitosan matrix affected the IY as well as the kinetic parameters of the immobilized bromelain, resulting in noticeable differences among the samples (Table 2). The amount of immobilized protein significantly increased for all the nanocomposite films compared to the clay-free support, with the exception of SMP which showed comparable IY values to those observed for the clay-free film (Table 2). The highest IY values were observed for the PAN supports, regardless the amount of added nanoclays, with values approximately 2-fold higher than the control film. Similar results were reported by Başak, Aydemir, Dinçer, and Becerik (2013), who proved that adding bentonite into chitosan beads significantly increased the catalase immobilization yield.

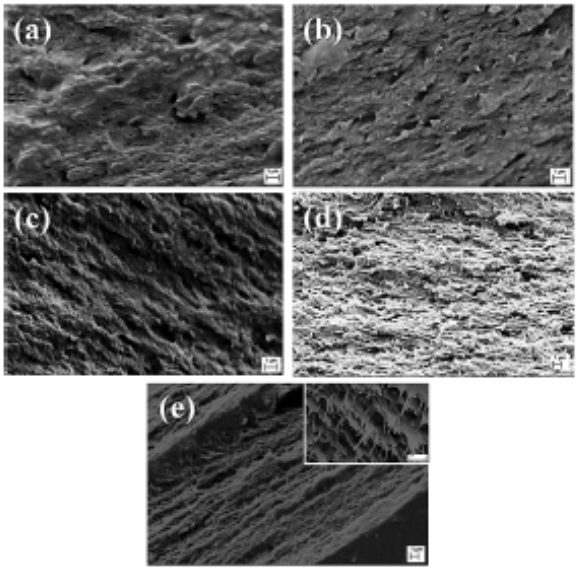

Fig. 6. SEM micrographs of the fracture surfaces of stress-strained fracture surfaces for the films: (a) DK1N-5, (b) DK2-5, (c) OPT-5, (d) SMP-5, (e) PAN-5 (inset: higher magnification).

Table 2
Immobilization yield (IY), maximum velocity (V_{max}), Michaelis-Menten constant (K_M), turnover number (k_{cat}) and affinity constant (K_a) of bromelain, immobilized on clay-free chitosan film (control support) and on chitosan/clay nanocomposite films loaded with clays at various concentrations (1, 3 and 5% w/w with respect to the chitosan), toward Bz-Phe-Val-Arg-pNA substrate (0–750 μM), in model wine (tartaric acid/sodium tartrate solution 0.03 M, pH 3.2, containing 12% v/v of ethanol at 20 °C). Immobilized enzyme parameters are mean of triplicate measurements.

Sample	IY (%)	V_{max} ($mIUmg^{-1}_{ip}$)	K_M (μM)	k_{cat} (min^{-1})	K_a ($min^{-1}\mu M$)	R^2
Clay-free	23^{e}	3.91^{a}	28.70^{e}	139.00^{a}	4.90^{a}	0.99
DK1N-1	34^{d}	0.58^{gf}	102.20^{cd}	17.95^{fgh}	0.18^{bcd}	0.97
DK1N-3	33^{d}	0.57^{gf}	109.30^{cd}	16.92^{fghi}	0.15^{cd}	0.97
DK1N-5	36^{cd}	0.59^{gf}	125.30^{cd}	18.17^{fg}	0.14^{cd}	0.96
DK2-1	35^{d}	0.68^{fg}	94.35^{d}	19.50^{f}	0.21^{bcd}	0.98
DK2-3	35^{d}	0.63^{gf}	82.54^{de}	21.04^{f}	0.25^{bcd}	0.98
DK2-5	36^{d}	0.59^{gf}	120.00^{cd}	17.52^{fgh}	0.15^{cd}	0.97
OPT-1	38^{bcd}	1.15^{bc}	198.90^{ab}	43.80^{d}	0.22^{bcd}	0.97
OPT-3	41^{abc}	0.93^{d}	127.40^{cd}	39.96^{e}	0.29^{bcd}	0.94
OPT-5	36^{d}	1.13^{bc}	145.40^{abcd}	45.71^{d}	0.31^{bcd}	0.96
SMP-1	24^{e}	1.22^{bc}	139.20^{abcd}	59.26^{b}	0.43^{bc}	0.97
SMP-3	24^{e}	1.27^{b}	135.00^{bcd}	59.74^{b}	0.44^{bc}	0.96
SMP-5	25^{e}	1.12^{c}	98.06^{d}	52.80^{c}	0.54^{b}	0.96
PAN-1	45^{a}	0.70^{efg}	200.60^{a}	12.70^{i}	0.06^{d}	0.95
PAN-3	42^{ab}	0.85^{de}	163.70^{abc}	15.07^{ghi}	0.09^{cd}	0.95
PAN-5	43^{ab}	0.78^{ef}	193.10^{ab}	13.79^{hi}	0.07^{cd}	0.95

For each parameter, values with different roman letters are significantly different (Tukey's test, $P < 0.05$).

A kinetic study of the obtained biocatalysts was carried out in order to investigate the effect of support modification, achieved through the inclusion of fillers, on the behaviour of immobilized enzyme. All the biocatalysts followed the hyperbolic behaviour described by the Michaelis-Menten equation (Fig. 7) and their catalytic properties are summarized in Table 2. As reported by other authors (Bai, Li, & Wang, 2006), the kinetic parameters of immobilized enzymes are affected by different factors (i.e. change in enzyme conformation, steric hindrance and diffusion limitations) which often cause an increase in K_M values. In general, although the inclusion of nanoclays improved the mechanical properties of the supports and increased IY, it negatively affected the catalytic properties of the immobilized protease. Therefore, in all cases bromelain on nanocomposite films showed lower V_{max} (maximum initial velocity) and k_{cat} values than the enzyme linked to the clay-free support, thus suggesting a reduced product release velocity. The simultaneous increase of K_M (Table 2) shows a limited enzyme–substrate complex formation, which may be caused by steric hindrance due to the higher amount of proteins bound to the nanocomposite films than to the clay-free support. These data appear to be in accordance with the findings described by other authors (Jiang, Long, Huang, Xiao, & Zhou, 2005; Knezevic, Milosavic, Bezbradica, Jakovljevic, & Prodanovic, 2006), who proved that the activity yield is usually inversely related to the amount of bound enzyme. Thus, a large amount of enzyme molecules immobilized on the carrier could limit the accessibility of the substrate to the active sites, due to diffusion limitation, resulting in a decrease of biocatalyst activity (Jiang et al., 2005). Moreover, the decrease in K_a, observed for all the biocatalysts obtained by immobilizing bromelain on nanocomposite films, indicated an apparent lower affinity of the protease for the synthetic substrate. These results could be attributed to the increased rigidity of the composite supports, which results from the addition of clay in chitosan matrix (Chang & Juang, 2005) and that could limit the mobility of immobilized enzyme (Barbosa et al., 2013). Comparing the nanocomposite samples, although the PAN supports linked the greatest amount of protein, the catalytic activity of bromelain appeared significantly compromised and showed the highest K_M and the lowest K_a values. Contrastingly, when protease was

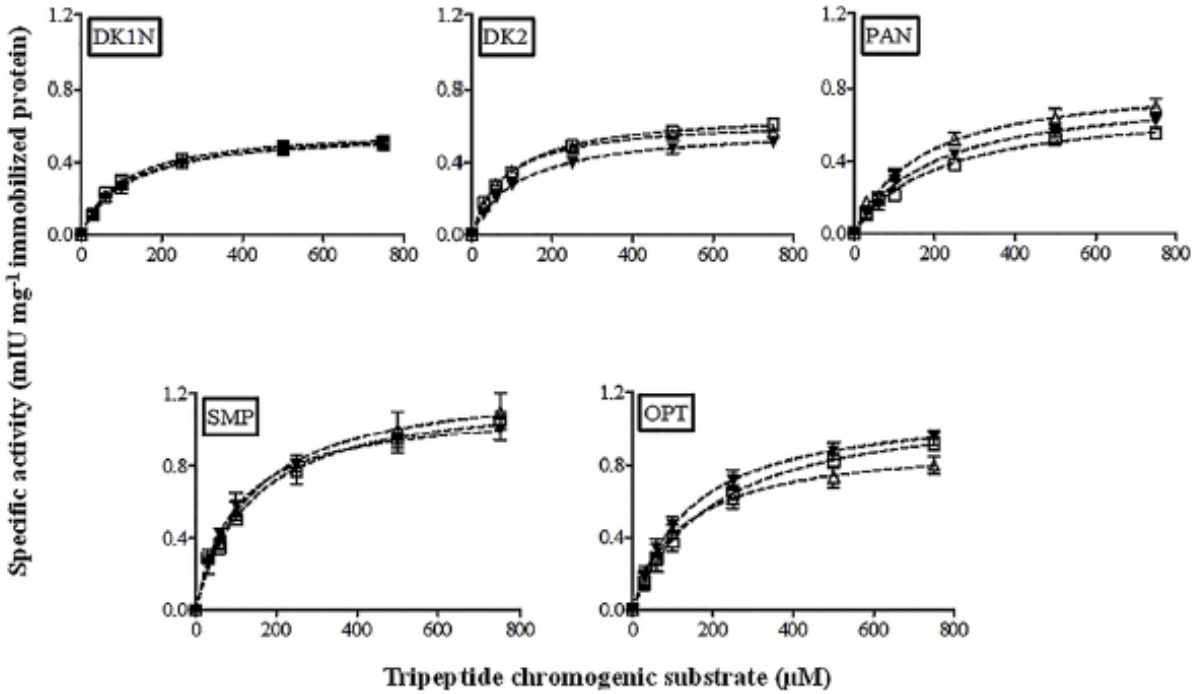

Fig. 7. Kinetic curves of bromelain, immobilized on chitosan/clay nanocomposite films loaded with different nanoclays (DK1N, DK2, PAN, SMP and OPT) at various concentrations: 1 (□), 3 (Δ) and 5 (▼) % w/w. Proteolytic activity was determined toward Bz-Phe-Val-Arg-pNA substrate (0–750 μM), in model wine (tartaric acid/sodium tartrate solution 0.03 M, pH 3.2, containing 12% v/v of ethanol at 20 °C).

immobilized on SMP films, characterized by the lowest IY, the estimated V_{max}, k_{cat} and K_a appeared to be significantly higher than the other samples (Table 2).

Despite different immobilized biocatalysts have been obtained using various chitosan composite supports, no unequivocal effect has been observed in terms of enzymatic kinetic parameters. Catalase showed a higher V_{max} when it was immobilized on chitosan/clay composite beads with respect to neat chitosan ones, with similar K_M values (Başak et al., 2013). Chang and Juang (2007a) revealed the highest V_{max} value but also the greatest K_M when acid phosphatase was immobilized on chitosan–ZrO_2 beads compared to neat chitosan ones.

4. Conclusions

Composite films, based on chitosan and nanoclays, to be applied as innovative supports for the covalent immobilization of proteolytic enzymes, were produced using different kinds of nanoclays, i.e. organomodified montmorillonite, bentonite (both unmodified and modified), and sepiolite.

According to the collected data, nanoclay inclusion led to enhanced mechanical properties, particularly in the case of films based on SMP and PAN; however it reduced the proteolytic activity of bromelain covalently immobilized onto the carrier. Indeed, the formation of intercalated and/or exfoliated composite structures in the case of chitosan loaded with PAN and SMP was confirmed by mechanical tests which showed a significant increase in both σ_{max} and E values compared to the clay-free film and a slightly progressive increase with the nanoclay content. On the other hand, the obtainment of tactoid structures in the case of DK1N, DK2 and OPT based nanocomposite supports was suggested by comparable σ_{max} and E values with respect to the clay-free film.

Furthermore, the inclusion of fillers within the chitosan matrix affected the IY as well as the kinetic parameters of the immobilized bromelain. More specifically, the amount of immobilized protein was remarkably higher for all the nanocomposite films compared to the clay-free support, with the exception of SMP which showed comparable values. However, despite the enhanced mechanical properties and increased IY, the clays addition negatively affected the catalytic properties of the immobilized protease. The lower values of V_{max}, k_{cat} and K_a suggest a reduced product release velocity and an apparent lower affinity of the protease for the synthetic substrate compared to the clay-free support, whereas the simultaneous increase of K_M shows a limited enzyme–substrate complex formation.

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