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FILMS OF CHITOSAN AND CHITOSAN-OLIGOSACCHARIDE NEUTRALIZED AND THERMALLY TREATED: EFFECTS ON ITS ANTIBACTERIAL AND OTHER ACTIVITIES

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ABSTRACT:

The present study focuses on the effects of heat and neutralization treatments on solubility, water vapour permeability and antimicrobial activity of chitosan (Ch) and chitosan/chitooligosaccharide (ChO)-based films. ChO films showed stronger antimicrobial activity against *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Serratia liquefaciens and Lactobacillus plantarum* than Ch films, indicating that this effect is attributed to the presence of chitooligosaccharides (COS) in the films. Heat and neutralization treatments decreased significantly the solubility of chitosan films and gave rise to a sharp loss in their antimicrobial activity. The incorporation of COS in chitosan films increased the inhibitory effect against the studied microorganisms without affecting significantly the water vapour permeability of the films. Thus, it is possible to get a more insoluble chitosan film with high antimicrobial activity by means of incorporation of COS combined with heat or neutralization treatments.

KEYWORDS:

Chitosan, chitooligosaccharide, antimicrobial activity, heat treatment, neutralization

1. Introduction

Nowadays there is an increasing interest in biodegradable/compostable polymers from renewable sources due to environmental problems caused by conventional food packaging materials (Leceta, Guerrero & de la Caba, 2013). The problems in disposing of huge quantities of waste generated by non-biodegradable food packaging have led to the study of biopolymers as materials to be used as films and coatings in food packaging (Azeredo, Miranda, Ribeiro, Rosa & Nascimento, 2012).

Development of materials from biopolymers for different applications have been a hot topic for several years, due to increasing prices of petroleum, a non-renewable resource with diminishing quantities (Ruban, 2009; Souza, Cerqueira, Martins, Casariego, Teixeira & Vicente, 2010), and increasing environmental concerns. This approach will continue playing an important role in the food industry (Satyanarayana, Arizaga & Wypych, 2009).

Today, the use of polymers from renewable sources in food packaging is growing. The tendency is to use natural compounds to enlarge the shelf-life of all types of food increasing the preservation and protection from oxidation and microbial spoilage.

The natural polymers used in food packaging have the advantages to be available from replenishable resources, biocompatible, biodegradable, and all these characteristics lead to ecological safety (Prashanth & Tharanathan, 2007).

In this context, chitosan and its chitooligosaccharides (COS), which are known to possess multiple functional properties, have attracted considerable interest due to their biological activities and potential applications in the food, pharmaceutical, agricultural and environmental industries. Both have inherent antimicrobial activity owing to the fact that long positively charged chitosan molecules interact with negatively charged bacteria (Zivanovic, Chi & Draughon, 2005). Chitosan is a versatile material with

proved antimicrobial activity. Three antibacterial mechanisms have been proposed (Goy, de Britto & Assis, 2009): i) the ionic surface interaction resulting in wall cell leakage (Liu, Du, Wang & Sun, 2004). In this model, the interaction is mediated by the electrostatic forces between the protonated NH_3^+ groups and the negative residues; ii) the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms (Sebti, Martial-Gros, Carnet-Pantiez, Grelier & Coma, 2005). The chitosan molecules are assumed to be able to pass through the bacteria cell wall and reach the plasma membrane; and iii) the formation of an external barrier chelating metals and provoking the suppression of essential nutrients to microbial growth (Cuero, Osuji & Washington, 1991). It is well known that chitosan has excellent metal-binding capacities where amine groups are responsible for the uptake of metal cations by chelation. It is likely that all events occur simultaneously but at different intensities.

Besides, chitosan is a non-toxic compound and another fascinating advantage of this compound is the film-forming capacity that it presents, which allows its application directly as a film or as a coating without the necessity of a carrier matrix (Fernandez-Saiz, Soler, Lagaron & Ocio, 2010). Chitosan films are regarded as biofunctional material, well tolerated by living tissues, particularly applicable as edible films/coatings to prolong shelf-life and preserve quality of fresh foods.

Moreover, there is a growing interest to develop materials with antimicrobial properties in order to prevent alterations in food caused by microbial spoilage.

On the other hand, as food quality and safety are major concerns in the food industry, there is also a need for an efficient method for the delivery of preservatives into foods. Addition of compounds directly into food is an established practice with some disadvantages. Instant addition of antimicrobials in formulation often results in instant inhibition of non-desired microorganisms. However, the surviving microorganisms will continue growing, especially when the concentration of antimicrobials added to the formulation will get depleted. This may be due to complex interactions with the food

matrix, or by natural degradation over time causing short shelf-life. To overcome this issue, antimicrobial packaging can be considered a modern technology that could have a significant impact on shelf-life extension and food safety. Use of antimicrobial agents in food packaging can control the microbial population and targets specific microorganisms to provide higher safety and quality products. Many classes of antimicrobial compounds have been evaluated in film structures, synthetic polymers and edible films. Among them, COS have received much more interest because they are not only water-soluble, but also possess distinctive biological activity such as antifungal and antibacterial activity, immuno-enhancing effects, and antitumor effects. Studies on the biological activities of chitosan and its oligomers have been increasing, as no single type of chitosan or its oligomers exert all of the above mentioned activities. Moreover, different chitosan derivatives and enzymatic products have different structures and physicochemical properties, which may result in novel bioactivities or novel findings in known bioactive compounds (Xia, Liu, Zhang & Chen, 2011).

Several reports discuss the antimicrobial activity of chitosan, demonstrating different results depending on source of chitin, molecular weight, deacetylation degree, and the experimental methodologies used, but they all confirm that chitosan and its oligosaccharides have strong antimicrobial effects and are safe for human use. Hence, the antimicrobial characteristics of chitosan and its oligosaccharides present a profitable potential for developing natural food packaging materials and functional food-additives.

Chitosan is known to be a very hydrophilic material with very low water resistance. The biggest drawback in use of chitosan films is their hygroscopicity. In fact, this material may virtually dissolve in the presence of high moisture products. In food packaging, the dissolution of the biopolymer could compromise packaging structure, physical integrity and organoleptic or microbiological food quality aspects. Therefore, there are a number of strategies that have been used in literatures, such as crosslinking or blending with a more water resistant material, to reduce its water sensitivity (Fernandez-Saiz, Lagaron

& Ocio, 2009; Tang, Du & Fan, 2003). However, these alternatives to reduce the water effect on the polymer do also adversely alter its biocide properties, suggesting that both effects may well often be opposed. Therefore, further investigations on this issue are needed in order to develop formulations of chitosan with a proper balance of water resistance and antimicrobial properties.

Taking into account that there are not many reports about the effect of high temperatures and neutralization treatments on the functional properties of chitosan and its depolymerisation products (COS), we found interesting to study the effect of heat and neutralization treatments on antimicrobial activity of chitosan films alone, and with a COS incorporated in the formulation. Five representative bacteria, *Escherichia coli* and *Serratia liquefaciens* (Gram-negative), *Lactobacillus plantarum*, *Bacillus cereus*, and *Staphilococcus aureus* (Gram-positive), which are common spoilage bacteria for food contamination, have been tested.

Thus, the aim of this work is focused on analysing the addition of depolymerisation products, COS, and the effect of heat and neutralization treatments on functional properties and antimicrobial activity of chitosan-based films.

2. Materials and Methods

2.1. Materials

Commercial food-grade chitosan (PubChem CID: 21896651) with a molecular weight of 169 kDa and a degree of deacetylation of 84% purchased from TRADES, S.A. (Barcelona, Spain) was utilized to obtain the films. Acetic acid (PubChem CID: 176, min. 99.8%, reagent grade, Scharlau, Spain) was used to fix the solution pH, and sodium hydroxide (PubChem CID: 14798, PA-ACS-ISO) used for neutralization, was provided by Panreac, Spain. All reagents were used as received.

Chitosan (DA 86%, *Mw* 180 KDa) from fresh North Atlantic shrimp shells (*Pandalus borealis*) (Primex, Iceland) was purified and hydrolysed according to enzymatic depolymerisation (Mengíbar, Mateos-Aparicio, Miralles & Heras, 2013) using chitosanase from *Streptomyces griseus* (EC 3.2.1.132) (Sigma-Aldrich, St. Louis, MO,

USA). COS (DA 83%, *Mw* 8.6KDa) were separated by tangential ultrafiltration system Vivaflow 200 (Sartorius-Stedim Biotech, Goettingen, Germany) using polyetersulfone (PES) membranes with different molecular weight cut off size.

2.2. Films preparation

Chitosan films (Ch) were prepared by casting, formed by solvent evaporation and the conversion of gelled solution rapidly to a solid film. A 10 g/L chitosan solution was prepared in a 10 g/L acetic acid aqueous solution. The chitosan solution was stirred at room temperature until it was completely dissolved, and then poured into multiwall plates and dried. The films used in the subsequent experiments were dried at 45 ℃ and 50% relative humidity and then peeled from the plates.

Chitosan-chitooligosaccharide films (ChO) were prepared in the same conditions but starting from two solutions: A 20 g/L chitosan solution in a 10 g/L acetic acid and a 20 g/L COS solution in 10 g/L acetic acid, mixed at ratio 1:1 to get final solution of Chitosan 10 g/L-COS 10 g/L.

Two treatments were applied to the previous formed films: 1) heat treatment at 105 ℃ overnight and, 2) neutralization by spraying with 13µl/cm^2 of NaOH 0.25 mol/L.

2.3. Total soluble matter (TSM)

Total soluble matter was measured by immersion in 25 mL of distilled water, with slight stirring at ambient temperature for 24 hours. After this time, samples were dried in an oven at 105 ºC for 24 h. TSM was calculated in relation to the dry mass and it was expressed as the percentage of the film dry matter solubilized.

2.4. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectra of all films were carried out on a Performer SpectraTech spectrometer using ATR diamond crystal. A total of 64 scans were performed at 4 cm^{-1} resolution. The measurements were recorded between 400 and 4000 cm^{-1}. The samples were measured at three points to check for film homogeneity and they yielded similar spectra.

2.5. DSC measurements

Differential scanning calorimeter (DSC) measurements were performed using a TA Instruments (model DSC Q1000, New Castle, USA). The samples were scanned under a N_2 atmosphere from ambient temperature to 100 ℃ at a constant heating rate of 10 ℃ /min. The weight film was 5-10 mg.

2.6. Scan Electron Microscopy (SEM)

The microstructure of films was observed by scanning electron microscopy (SEM). The samples were examined using a scanning electron microscope (JEOL JSM-6335, JEOL, Tokyo, Japan).

2.7. X-ray Diffraction (XRD)

X-ray diffraction patterns were obtained using an X-ray diffractometer (PHILIPS X′PERT SW) with a copper anode. The samples were scanned continuously from 0° to 50° (2 θ) at 45 kV and 40 mA.

2.8. Water Vapour Permeability (WVP)

WVP measurements were performed in a PERMATRAN W3/33 (Mocon), at 23 ℃ and 50% relative humidity. The sample films were cut into a circle of 4 cm diameter and the test area was 5 cm^2.

WVP was calculated as:

$$WVP = WVTR\ x\ /\Delta P$$

Where WVTR is defined as:

$$WVTR = changing\ in\ weight\ (g)/time\ (h) x\ test\ area\ (m^2)$$

and ΔP is the partial pressure difference of the water vapour across the film.

2.9. Antimicrobial activity

The antimicrobial activity of all chitosan films was tested against the growth of five typical food spoilage bacteria; two Gram-negative: *E. coli* CECT45, *S. liquefaciens* CECT483, and three Gram-positive: *S. aureus* ATCC12599, *B. cereus* CECT148, and *L. plantarum* CECT220, obtained from the Spanish Type Culture Collection and the American Type Culture Collection. These strains were stored in nutritive broth with

20% glycerol at -80 ℃ until needed. For experiment al use, the stock cultures were maintained by regular subculture to Plate Count Agar (PCA) or Man Rogasa and Sharpe (MRS) Agar at 4 ℃ and transferred monthly.

The antimicrobial effect of all Ch and ChO films was evaluated by determining the bacterial growth in nutrient broth. Mueller Hilton Broth (MHB) was used for *E. coli*, *B. cereus*, *S. aureus* and *S. liquefaciens* and Man Rogasa and Sharpe (MRS) Broth for *L. plantarum*. The assay was conducted in 2 mL of bacterial culture in exponential phase (10^5 ufc/ml) with films pieces (sterilized with UV light) containing 0.01 g of chitosan in Ch films, and 0.01 g of chitosan and 0.01 g of COS in ChO films. Cell cultures were incubated for 24 hours at 37 ℃ for *E. coli*, *B. cereus*, *S. aureus*, and *L. plantarum* and at 26 ℃ for *S. liquefaciens*.

After incubation, serial decimal dilutions were prepared and spread onto fresh plates of PCA or MRS Agar. The number of colony-forming units (CFU) was assessed after plates had been incubated for 48 hours. Results were expressed as percentage of growth (Log (ufc/mL)) inhibition of bacteria respect to control without films.

2.10. Statistical analysis

Statistical analysis was performed with R program. Analysis of variance (ANOVA) and the Tukey´s multiple range test were performed to detect significant differences in the film properties. The significance level used was 0.05.

3. Results and Discussion

3.1. Total soluble matter

Chitosan and COS formed homogeneous solutions separately. When both solutions were mixed, the resulting liquid was completely clear. It would seem that chitosan solution is not affected by the incorporation of the mixture of COS. As Chitosan and its oligosaccharides are similar molecules with different molecular weights, the mix would only increase the polydispersity of the sample minimally, which may lead to a reorganization of the matrix network in solution. DSC thermograms (Figure 1) showed similar structures for both Ch and ChO films. Similar endothermic peaks attributed to

water loss represented the energy required to vaporize water present in both films. have not provided any relevant information about miscibility. Table 1 shows the total soluble matter (TSM) of Ch and ChO films, heat treated and neutralized. It can be seen that the films without any treatment were totally soluble.

However, when the films were heat-treated or neutralized, TSM values decreased significantly, indicating a change in the chemical structure of the film. Temperature promotes Maillard reaction which brings the browning of compounds due to the interactions between carbonyl groups and amino compounds. The increase of insoluble matter was related to the decrease of free amino groups (Umemura and Kawai, 2007), as it was observed by FTIR results shown below. On the other hand, heat can change the physical properties of chitosan (Leceta, Guerrero, Ibaburu, Dueñas & de la Caba, 2013; Leceta, Guerrero & de la Caba, 2013) affecting its aqueous solubility, rheology, and appearance. The formation of chitosan films reduced the crystallization of chitosan, showing amorphous behaviour as it can be observed in Figure 2, where a wide diffraction peak at 2Θ=20.2 º for heat treated and non-treated chitosan films can be seen. According to Rivero et al. (Rivero, Garcia & Pinotti, 2012), heat curing of chitosan films led to a structural change characterized by peaks located at 2Θ = 15 º and 20 º, but in this study, heat treatment had not effect on the crystallinity of the films. For neutralized films, the insolubility was lower compared with the heat-treated films. Because of the treatment with NaOH, some of the protonated amine groups ($-NH_3^+$) were neutralized causing partial insolubility of the films (Fernandez-Saiz, Lagaron & Ocio, 2009).

3.2. FTIR spectroscopy

In order to study the interactions between functional groups in Ch and ChO films, FTIR analysis was carried out. Chitosan´s typical structure shows in FTIR spectra characteristic absorption bands at 1594 and 1650 cm^{-1}, attributed to amide II (N-H) and amide I (C=O) respectively, at 1380 cm^{-1} due to the distorting vibration of $C-CH_3$, and at 3441 cm^{-1} which indicates the –OH stretching vibration and the intramolecular

hydrogen bonding of chitosan molecules. Absorption bands of chitosan powder at 1594 and 1650 cm^{-1} usually shift to a lower wavenumber, at 1634 and 1546 cm^{-1} approximately, in the chitosan films, due to the relaxation of the chains. These are the wavenumbers where the bands can be approximately observed in Figure 3 for the films of this study. The two dominant bands centred at 1546 and 1405 cm^{-1} are associated to carboxylate ions ($-NH_3^+$ $^-OOCH^-$) (Lagaron, Fernandez-Saiz & Ocio, 2007). The amine groups in this chemical form are referred to as "activated" or protonated amine groups, and are responsible for the biocide character of chitosan. It can be observed that the intensity of the band at 1634 cm^{-1} (amide I) was always lower than the intensity of the band between 1546-1558 cm^{-1} (amide II) for the films without treatment, as consequence of the presence of available protonated amine groups ($-NH_3^+$) produced in the evaporation of solvent to form the films (Fernandez-Saiz, Ocio & Lagaron, 2006). However, when the films were submitted to treatment the difference in the intensity of those two bands became smaller for both studied processes (Figure 3). This result could indicate that crosslinking and Maillard reaction between carbonyl and amine group in the same chitosan chain could be promoted by temperature in the case of heat treated films, and also that there has been a decrease of the number of biocide groups ($-NH_3^+$) as a consequence of neutralization treatment. This is in agreement with the decrease of solubility observed for treated films. According to Leceta et al. (Leceta, Guerrero, Ibaburu, Dueñas & de la Caba, 2013), temperature and relative humidity promote crosslinking and chemical reactions, such as the Maillard reaction. The early stage of Maillard reaction involves the formation of conjugates between the carbonyl group of the carbohydrate ends with the amine group in chitosan, producing a Schiff base, which subsequently cyclizes to produce the Amadori compound and insoluble polymeric compounds, referred as melanoidins. In the treatment with NaOH, the neutralization effect is joined to the formation of sodium salt of acetic acid.

When the COS were introduced in the films (Figure 3), it was observed that in the heat treated films, the decrease in relatively intensity bands centred at 1546 and 1405 cm^{-1}

were less marked with respect to the neutralized ChO films, indicating the minor capacity to stablish crosslinking in presence of short chains of polymer. However, the presence of COS in the films increases the number of protonated amine groups and carboxylate ions which are neutralized and form salts, reducing the intensity of these bands.

3.3. Water Vapour Permeability

Water vapour permeability is a key property for the films intended to be used as food packaging. The WVP values are shown in Table 1.

WVP was not affected by heat treatment, as it was also stated by Leceta et al. (Leceta, Guerrero, Ibaburu, Dueñas & de la Caba, 2013), who also reported changes in TSM but not in WVP for heat-treated chitosan films. Although heat treatment can reduce hygroscopicity, through crosslinking and Maillard reaction, and subsequently water vapour permeability, both the temperature and duration of heat treatment influenced the degree of heat induced changes in the films. As it was shown in X-ray Diffraction analysis (Figure 2), no changes in the microstructure of the films were observed. SEM analysis (Figure 4) also revealed that the internal microstructure of Ch and ChO films was homogenous, smooth and with relatively roughness and, in the heat-treated films, no irregularities caused by the crosslinking were observed.

On the contrary, for chitosan films, WVP was significantly increased due to neutralization, but in all cases, the values remained in the same range as the values reported by Park et al. (Park, Marsh & Rhim, 2002) at the same test conditions (25ºC and 50% RH). Higher values reported by other authors (Leceta, Guerrero & de la Caba, 2013) can be attributed to the higher temperature and relative humidity of the measures (38ºC and 90% RH).

Incorporation of COS in the film composition increased notably the WVP value, which can be attributed to the hydroxyl groups, which are hydrophilic and less resistant to water vapour transmission, since the polar groups attract migrating water molecules and thereby facilitate water transport, and also due to the shorter length of the COS

chains, which would facilitate the diffusion of water vapour through the film (Sun, Wang, Kadouh & Zhou, 2014).

3.4. Antimicrobial activity

Antimicrobial activities of Ch and ChO films in liquid culture medium are shown in Table 2.

As it can be seen, the presence of Ch films affected the cell viability of the tested microorganisms inhibiting their growth. However, the inhibitory effect differed depending on the type of bacterium although the present study showed that there was not a clear difference between the Gram-positive and Gram-negative bacteria studied. The activity of chitosan and its derivatives or oligomers against different bacteria and fungi has already been widely reported. The mode of antibacterial activity is a complex process that differs among bacteria due to different cell surface characteristics. In several studies, stronger antibacterial activity was apparent against Gram-negative bacteria than Gram-positive, while in another study Gram-positive bacteria were more susceptible, perhaps as a consequence of the Gram-negative outer membrane barrier. Many works have already demonstrated that there were no significant differences between the antibacterial activities against the bacterium. Various initial reaction materials and conditions could contribute to the diverse results (Kong, Chen, Xing & Park, 2010).

The heat treated and neutralized Ch films presented lower antimicrobial activity than the non-treated ones. These results are in good agreement with FTIR results, which showed a reduction of the absorption bands at 1546 and 1405cm^{-1}, attributed to the carboxylate groups, which in the literature (Leceta, Guerrero, Ibaburu, Dueñas & de la Caba, 2013; Lagaron, Fernandez-Saiz & Ocio, 2007) have been related to the antimicrobial character of chitosan. Chitosan shows optimum performance only in gelled or viscous acid solution form, when the amine groups are allegedly protonated or “activated” (Fernandez-Saiz, Ocio & Lagaron, 2006).

335 ChO films showed stronger antimicrobial activity than Ch films, indicating that this
336 effect is caused by the presence of the COS into the films, which denotes the
337 reinforcing effect of more active groups that produce inhibition. Since such
338 antimicrobial mechanism is supposed to be based on electrostatic interaction, it
339 suggests that the greater the number of cationized amines, the higher will be the
340 antimicrobial activity. Therefore, COS increased the number of cationized amines and
341 improved the antimicrobial activity of ChO films. As it has been reported by other
342 authors (Goy, de Britto & Assis, 2009; Kong, Chen, Xing & Park, 2010), this would not
343 be possible by increasing the concentration of chitosan, because the amount of
344 chitosan available to bind to a charged bacterial surface is apparently reduced as the
345 concentration of chitosan increases. A possible explanation is that in the presence of a
346 larger number of charged sites, the chains tend to form clusters by molecules
347 aggregation. Observations have confirmed that, at higher concentrations, chitosan, due
348 to its filmogenic character, tends to form a coating over the bacteria, no necessarily
349 attached to the surface, and independently of the bacteria type. Other possible
350 mechanism reported is the penetration of low molecular weight chitosan in the cell,
351 blocking the transcription of RNA from DNA due to adsorption (Fernandez-Saiz, Ocio &
352 Lagaron, 2006). In this sense, the addition of COS to the film could favour also this
353 mechanism, as it can be observed in the results of inhibition.
354 The effect of COS incorporation produced more differences in antimicrobial activity in
355 the case of neutralized films than in heat treated ones. Heat treated ChO films showed
356 higher antimicrobial activity (except for *L. plantarum*) with respect to Ch films treated at
357 the same temperature. As it was shown, the solubility of these films was lower than that
358 of the neutralized films, due to the strong interactions produced by the crosslinking and
359 the Maillard reaction. However, the conditions of antimicrobial growth medium when
360 the COS are present in the film, would make that the small molecules could be
361 redissolved, being presented in the medium low molecular weight chitosan and more

amine groups activated available to give the possible actuation mechanism to inhibit the different bacteria tested.

The percentage of inhibition of all the tested bacteria was significantly higher for ChO neutralized films with respect to Ch ones. In this case, the results does not seem to be in agreement with the intensity of the bands at 1546 and 1405 cm^{-1} associated to carboxylate ions with biocide action, which showed less intensity in presence of COS according the ATR_FTIR spectroscopy experiments. The process of neutralization would be higher in presence of higher number of amine groups causing a decrease in the percentage of these protonated groups and therefore, in the bands corresponding to carboxylate ions, due to the formation of sodium salt of acetic acid. In the same way, the conditions of the antimicrobial experiments in which a liquid culture medium with pH 6 is used, the amine groups would start to be protonated, and redisolution of small size chain of COS could be favoured, which was demonstrated by the higher solubility of these films observed by TSM experiments.

The lack of biocide properties of neutralized chitosan films showed by Ouattara et al. (Ouattara, Simard, Piette, Bégin & Holey, 2000) when they were applied onto the surface of processed meat, was attributed to the inactivation of amine groups by titration with an alkaline component. But, in apparent opposition to these results, Tang et al. (Tang, Du & Fan, 2003) also obtained bactericidal effect toward *S. aureus* and *E.coli* when suspensions of both neutralized chitosan films and neutralized cross-linked chitosan films were measured. Among some feasible reasons for this behaviour could be the incomplete neutralization process of the tested films (Fernandez-Saiz, Lagaron & Ocio, 2009).

These results show that it is possible to reduce the solubility of chitosan films while their antimicrobial properties are maintained, by incorporation of antimicrobial COS.

4. Conclusions

The results of this work showed that the incorporation of chitooligosaccharides (COS) in neutralized or heat treated chitosan films led to obtain films with reduced solubility

and with stronger antibacterial activity due to the reinforcement of more functional active groups.

When the chitosan films were neutralized or heat treated, they became more water insoluble but lost their antibacterial properties. Heat treatment decreased further the solubility due to the crosslinking and the Maillard reaction. Besides, in the neutralization treatment, some of the neutralized protonated amine groups with the corresponding formation of sodium salt of acetic acid caused partial insolubility of the films. The incorporation of COS to chitosan films increased the inhibitory effect against the studied microorganism.

In view of these results, it can be concluded that incorporation of COS combined with heat or neutralization treatments has been proved to be a viable method to get a final chitosan film with decreased solubility, without affecting their water vapour permeability, and antibacterial activity with higher efficiency depending on the microorganism.

Further experiments are necessary to know about the behaviour of the film in different food matrices but, according to the results ChO, films seem to be a promising material which can be used for food packaging applications.

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Table 1. Total soluble matter (TSM) and water vapour permeability (WVP) average values and standard deviations of non-treated chitosan (Ch) and chitosan-oligosaccharide (ChO) films, heat-treated (Ch105, ChO105) and neutralized (ChNaOH, ChONaOH).

FILM SAMPLE	TSM (%)	WVP. 10^8 (g·mm/m^2·s·Pa)
Ch	100^a	2.573±0.111^a
ChO	100^a	4.037±0.117^b
Ch105	31.3±1.0^b	2.330±0.086^a
ChO105	41.6±3.6^b	3.934±0.202^b
ChNaOH	57.4±8.6^c	4.421±0.084^b
ChONaOH	78.6±1.5^d	3.879±0.820^b

$^{a-d}$ Two values followed by the same letter are not significant (p>0,05) different thought the Tukey´s multiple range test. Measurements were made in triplicate and quadruplicate, respectively

Table 2. Average % inhibition values and standard deviations of non-treated chitosan (Ch) and chitosan-oligosaccharide (ChO) films, heat-treated (Ch105, ChO105) and neutralized (ChNaOH, ChONaOH) on the viable count of *E. coli, S. liquefaciens, B. cereus, S.aureus and L.plantarum.* Data are expressed as % inhibition with respect to control.

	% Inhibition				
	E. Coli	S. liquefaciens	B. cereus	S. aureus	L. plantarum
Ch	>88.2 ± 0.0^a	48.9 ± 4.3^a	82.8 ± 6.3^a	72.48 ± 7.0^a	>89.6 ± 0.0^a
Ch105	63.4 ± 4.3^b	9.5 ± 1.4^b	56.6 ± 4.1^b	17,4 ± 2.4^b	75.7 ± 0.3^b
ChNaOH	38.5 ± 7,2^c	3.7 ± 0.2^c	11.9 ± 0.9^c	2.2 ± 0.3^c	64.3 ± 1.5^c
ChO	>88.2 ± 0.0^a	61.9 ± 1.3^d	>87.2 ± 0.0^a	>85.3 ± 0.0^d	>89.6 ± 0.0^a
ChO105	>87.7 ± 0.0^a	27.0 ± 1.2^e	75.7 ± 3.1^a	30.1 ± 2.7^e	61.2 ± 1.0^c
ChONaOH	>89.9 ± 0.0^a	54.7 ± 2.4^f	>86,8 ± 0.0^a	>88,6 ± 0.0^d	>89.6 ± 0.0^a

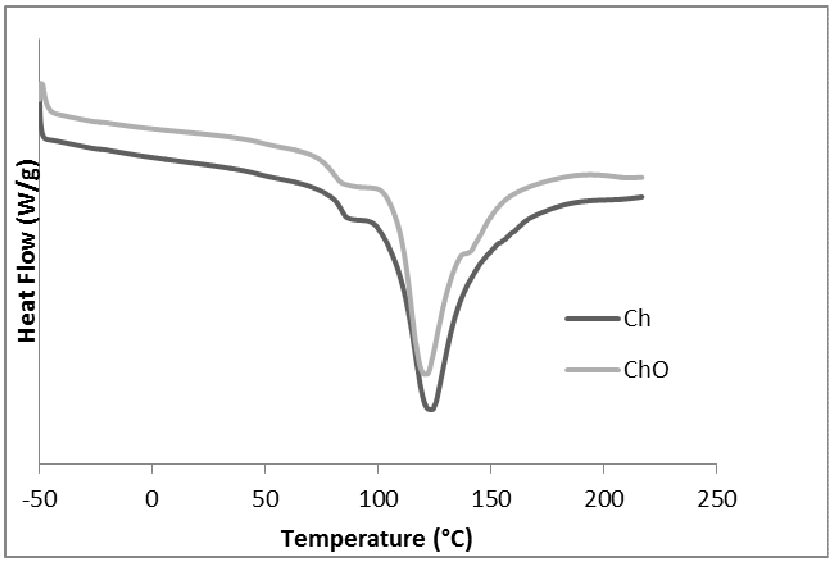
$^{a-f}$ Two values followed by the same letter for each microorganism are not significant (p>0,05) different thought the Tukey´s multiple range test. Experiments were made in triplicate.

Figure 1. DSC of non-treated Ch and ChO films.

Figure 2. X-ray diffractograms of heat treated and non-treated chitosan films (Ch) and Chitosan-oligosaccharide films (ChO).

Figure 3. FTIR spectra of treated and non-treated chitosan films (Ch) (top) and Chitosan-oligosaccharide films (ChO) (bottom).

Figure 4. SEM spectra of heat treated and non-treated chitosan films: a) Ch, b) Ch105, c) ChO and d) ChO105.



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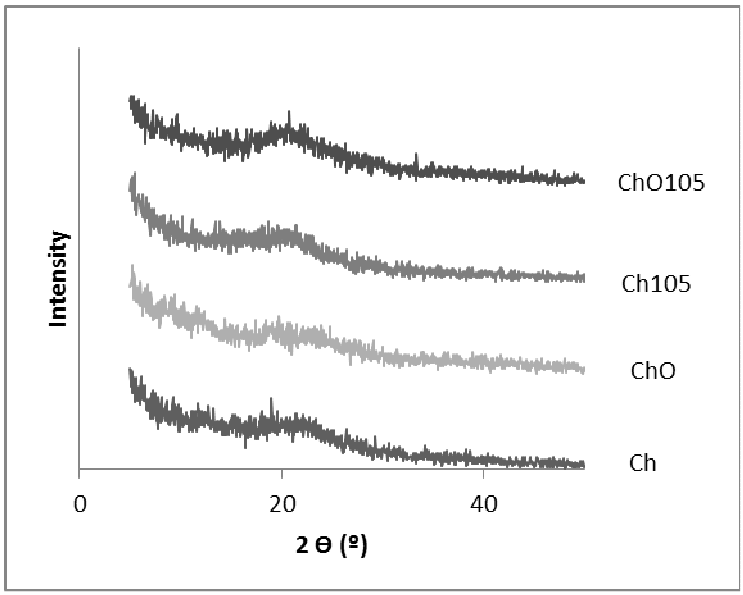

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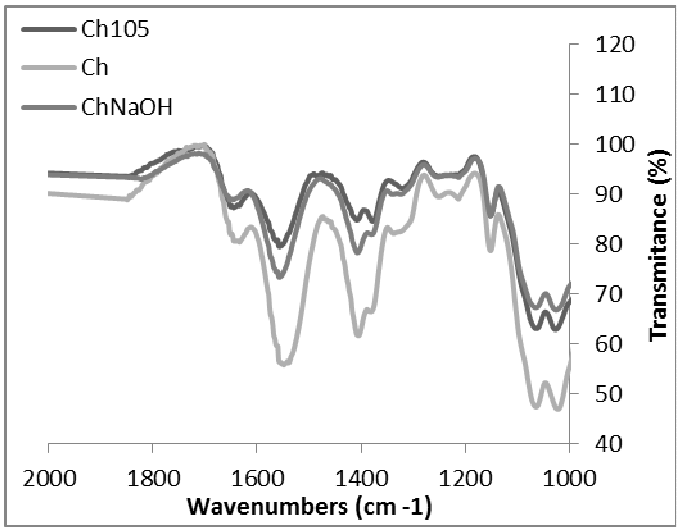

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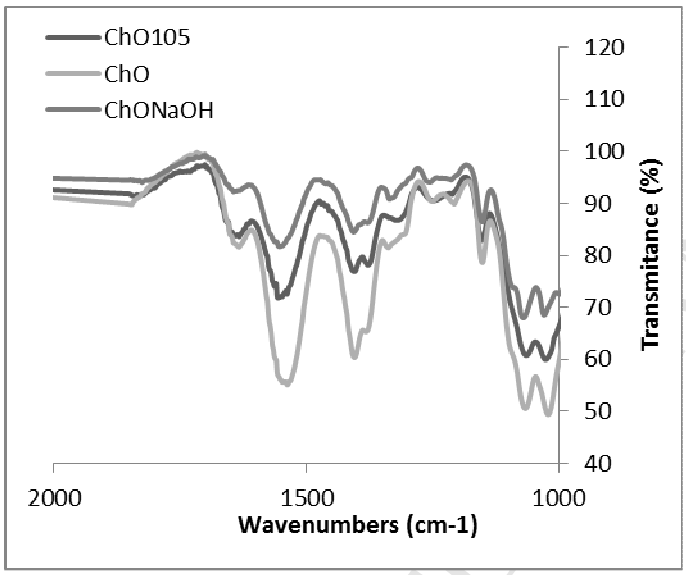

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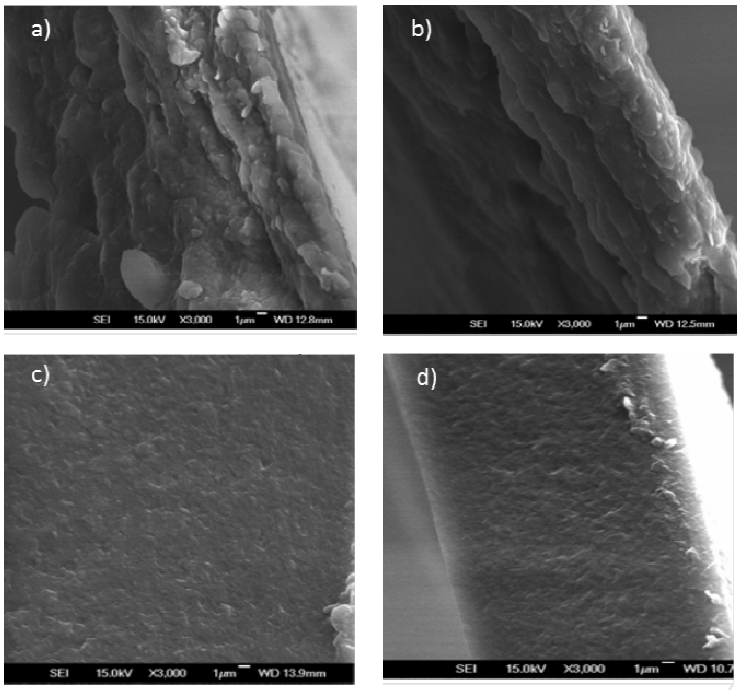

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- A new chitosan-based insoluble film with antimicrobial activity has been developed
- Heat or neutralization treatments decreased the solubility of chitosan films but caused a lost in their antimicrobial activity
- Incorporation of COS in chitosan films increased their antimicrobial activity