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Chitosan composite films: Thermal, structural, mechanical and antifungal properties

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ABSTRACT

Based on colony spreading, chitosan from shrimp waste in agar media inhibited the growth of *Aspergillus niger* by 47.26%; there were not differences ($P > 0.05$) with respect to commercial chitosan (Fluka, BioChemika) (56.16%). All chitosan films showed similar glass transition temperatures ($P > 0.05$) with respect to cellophane control; however, chemically, all chitosan films showed an increase in the T_g values that could be related with the decrease ($P \leq 0.05$) in elongation percentage with respect to the control film. According to FT-IR spectroscopic analysis of chitosan films, the fungistatic activity can be related to the hydrogen bonds' formation between the amino groups of chitosan with the hydroxyl groups from polymer or sorbitol. The plasticizer addition increased ($P \leq 0.05$) the elongation of chitosan films. The Young's module, *E*, was lower ($P \leq 0.05$) for all chitosan films comparing with cellophane. When incorporated into the composite films elaborated by casting, chitosan retained its fungistatic activity. Even though the activity against fungi growth was lower (15.66% for non-plasticized silage chitosan films, pC_S), a reduction ($P \leq 0.05$) in the hyphae diameter of *A. niger* was observed. Results suggest that it is feasible to elaborate antifungal chitosan films, with good thermal stability and acceptable mechanical properties for food packaging.

1. Introduction

Chitosan is a natural polycationic polysaccharide derived from chitin, which is found in the crustacean's shells, insect's cuticle and cell wall of fungi. Chitosan possesses antimicrobial activity and filmogenic properties, besides being biocompatible and biodegradable. Chitosan films have been successfully probed at an experimental level on food such as eggs, fruits, vegetables, dairy products and meat (Bhale et al., 2003; Chien, Sheu, & Yang, 2007; El Ghaouth, Arul, & Asselin, 1992; Wu et al., 2000), where it has been observed that the chitosan treatment offers protection against contamination and microbial spoilage, increasing the food quality and shelf life.

In culture media, chitosan has an antimicrobial effect on bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae* and *Vibrio cholerae* (Coma, Deschamps, & Martial-Gros, 2003; Tsai, Su, Chen, & Pan, 2002), and fungi such as *Sclerotinia sclerotium*, *Botrytis cinerea*, *Monilinia fructicola*, *Rhizopus stolonifer* and *Aspergillus niger* (Li & Yu, 2001; Sebti, Martial-Gros, Carnet-Pantiez, Grelier, & Coma, 2005; Vargas, Albors, Chiralt, & Gónzalez-Martinez, 2006).

Three mechanisms have been proposed as an explanation to chitosan's antimicrobial properties. In the first one, the positive charges present in the polymeric chain of chitosan, due to its amino group, interact with the negative charges from the residues of macromolecules (lipopolysaccharides and proteins) in the membranes of microbial cells, interfering with the nutrient exchange between the exterior and interior of the cell. These charges can also compete with calcium for the electronegative sites in the membrane, compromising its integrity and causing the release of intracellular material, resulting in cellular death (Möller, Grelier, Pardon, & Coma, 2004; Rodríguez, Albertengo, Debbaudt, & Agulló, 2005).

The second mechanism proposes that chitosan acts as a chelating agent, creating compounds from traces of metals essential to the cell (Roller & Covill, 1999), while the third mechanism establishes that chitosan of low molecular weight is capable of entering the cell's nucleus itself, interacting with the DNA, interfering with the messenger RNA synthesis, affecting the synthesis of proteins

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and inhibiting the action of various enzymes (Rabea, El Badawy, Stevens, Smagghe, & Steurbaut, 2003).

Such mechanisms are based on results from previous investigations where chitosan has been added to culture media, whether in a liquid or a solid medium. In the case of films, the inhibition mechanism has not been explained; also, there are other external factors such as anaerobic conditions or asphyxia which should be considered.

Chitosan films are biodegradable, biocompatible, flexible, durable, strong, tough and hard to break, have moderate values of water and oxygen permeability, decrease the respiratory rate of food and also inhibit the microbial growth (Agulló, Rodríguez, Ramos, & Albertengo, 2003). Most of the mechanical properties of chitosan films are comparable to those of commercial polymers of medium strength such as cellulose (Jeon, Kamil, & Shahidi, 2002). The mechanic and permeable properties of chitosan films can be controlled by selecting molecular weight, a suitable solvent system (Park, Marsh, & Rhim, 2002), and the addition of plasticizer agents, dispersants and compatibilizers, among others (Chillo et al., 2008; Fernández-Cervera et al., 2004; Suyatma, Tighzert, & Copinet, 2005); however, the presence of such compounds can affect the antimicrobial activity of chitosan films.

The goal of this study was to assess the thermal, spectroscopic and mechanical properties, as well as the antifungal activity of chitosan films, plasticized and non-plasticized, prepared by *casting*.

2. Materials and methods

2.1. Chitosan

2.1.1. Silage chitosan, C_S

Chitosan was obtained through heterogeneous alkaline deacetylation (NaOH 60% (w/v), at 35 °C for 6 days) of the chitin obtained from fermented silage made of shrimp heads. For the silage preparation, ground shrimp heads were mixed with 10% (w/w) sucrose, 6% (v/w) lactic acid solution 10% (v/v) and 10% (v/w) of inoculum of *Lactobacillus* sp., and incubated at 30 °C for 48 h (Jaime-Quijada, Almendariz, Rodríguez, Jáuregui, & Plascencia-Jatomea, 2008). The solid fraction obtained was washed, oven dried at 40 °C, deproteinized with a solution of NaOH at 2% (w/v) and demineralized with a solution of HCl at 2% (v/v). The resulting solid corresponds to chitin.

2.1.2. Commercial chitosan, C_C

Commercial grade chitosan (Fluka, Biochemika, Japan) with medium viscosity (200–400 mPa s) and obtained by chemically treating crab waste, was used for comparative proposes.

2.2. Physicochemical characterization of chitosan

2.2.1. Protein and ashes content

The content of residual protein and ashes of chitosan was quantified according to the AOAC techniques (AOAC, 2005).

2.2.2. Deacetylation degree (%DD)

For each type of chitosan (flakes and films), the percentage of the amino groups was determined by Fourier Transform Infrared Spectroscopy, FT-IR (Perkin Elmer FT-IR Spectrum GX) analysis. Chitosan pellets prepared with potassium bromide, KBr (Aldrich, spectroscopic grade) were analyzed using 16 scans, at 4000–400 cm^{-1}. The analyses of the spectra were done according Khan, Peh, and Ch'ng (2002) and the deacetylation degrees were determined using Eqs. (1) and (2):

$$\mathrm{DD}\ (\%) = 100 - \left[\left(\frac{A_{1655}}{A_{3450}}\right) \times \frac{100}{1.33}\right] \quad (1)$$

$$\mathrm{DD}\ (\%) = 100 - \left[\left(\frac{A_{1655}}{A_{3450}}\right) \times 115\right] \quad (2)$$

where A_{1655} is the absorbance at 1655 cm^{-1} of the amide I band, A_{3450} is the absorbance at 3450 cm^{-1} of the hydroxyl band and 1.33 is the value of the A_{1655}/A_{3450} proportion for a completely acetylated chitosan.

2.2.3. Molecular weight

The molecular weight of chitosan was determined by using the viscometric method, with each biopolymer solutions at concentrations ranging from 0.25 to 6.0 g/L (w/v). The intrinsic viscosity of chitosan in 0.3 M acetic acid/0.2 M sodium acetate solution was measured by using an Ubbelohde capillary viscometer in a constant temperature water bath (25 °C), by triplicate (Hwang et al., 2002; Mathew, Brahmakumar, & Abraham, 2006). The molecular weight values were determined using the Mark–Houwink–Sakurada equation (Eq. (3)):

$$[\eta] = \kappa MV^{a} \quad (3)$$

where $[\eta]$ is the intrinsic viscosity, MV is the average viscometric molecular weight and both κ and a are empirical constants that depend of the polymer nature, the solvent and the temperature. κ and a values were 3.04×10^{-5} and 1.26, respectively (Rinaudo, Milas, & Le Dang, 1993).

2.3. Fungistatic activity of chitosan on Aspergillus niger

2.3.1. Microorganism and growth conditions

A strain of high occurrence food spoilage fungi, *A. niger* (NRRL 3), was activated in potato dextrose agar media, PDA (Difco, USA), and incubated at 30 ± 2 °C for 5 days. Spores were harvested by pouring a sterile solution of 0.1% (v/v) Tween 80 into the flask and stirring with a sterile magnetic bar for 5 min. The spore concentration of the suspension was determined using a Neubauer chamber (Brand, Germany) and adjusted at a final concentration of 1×10^5 spores/mL.

2.3.2. Inoculation techniques

Two inoculation techniques were used: (a) By puncture. A 2.4 μL volume of the inoculum containing 2×10^4 spores/mL was placed in the center of the Petri plate containing the agar, for determinations of radial growth. (b) By spread on plate. A 12 μL volume of the spore suspension containing 1×10^5 spores/mL was placed in the center of the Petri plate and spread onto the agar surface with a sterile glass rod. This technique was used for the spore germination determinations (Plascencia-Jatomea, Viniegra, Olayo, Castillo-Ortega, & Shirai, 2003). All the inoculated plates were incubated at 25 °C.

2.3.3. Chitosan solutions and media preparation

Each C_S and C_C chitosan solutions containing 20 g of biopolymer flakes per L (w/v) were prepared by stirring in 0.1 M of acetic acid solution for 24 h, at 25 °C. The obtained solutions were autoclaved at 121 °C for 15 min and added to sterile PDA agar media (Difco, USA), to get a chitosan final concentration of 2.82 g/L (w/v) (Martínez-Camacho et al., 2007), and a final pH of 4.5. An agar containing no chitosan with pH adjusted to 4.5 with 0.1 M of acetic acid solution was used as control.

2.3.4. Radial growth

The radial extension growth of the fungi colony at 25 °C was measured each 24 h and compared to the control media until the control reached the plate border (Plascencia-Jatomea et al., 2003). The fungistatic index was calculated using Eq. (4):

$$\text{Fungistatic inhibition}\ (\%) = 1 - \left[\frac{R_i}{R_c}\right] \times 100 \quad (4)$$

where R_c was the mean value of the colony radius of control media and R_i was the colony radius of the chitosan amended media (Guo et al., 2006). All the measurements were carried out in triplicate.

2.3.5. Spore's germination

The agar plates were inoculated by spreading 12 μL of a solution containing 1×10^5 spores/mL onto the agar surface and incubated at 25 °C. Samples were taken at different times and 200 spores (germinated or non-germinated) were randomly counted using light microscope (Carl Zeiss, USA) until the control reached the 100% of germinated spores. The number of germinated spores per plate was determined; a spore was considered as germinated when the length of its germinal tube reached one-half of the spore diameters (Plascencia-Jatomea et al., 2003). Each germination experiment was made in duplicate.

Fungistatic index was determined at 25 °C using Eq. (5), where S_i represented the percentage of spores germinating in the chitosan treated samples and S_c was the percentage of spores germinating in the control (Guo et al., 2006).

$$\text{Fungistatic inhibition } (\%) = 1 - \left[\frac{S_i}{S_c}\right] \times 100 \quad (5)$$

2.4. Film elaboration

The chitosan films were elaborated using the solvent evaporation technique or *casting*. For each type of chitosan, C_S and C_C, film forming solutions of 1% (w/v) were prepared by dispersing chitosan flakes in 0.1 M acetic acid. Chitosan films were prepared by the addition of 20% (w/w with respect to the amount of chitosan) of sorbitol as a plasticizing agent. Both mixtures, with and without plasticizer, were stirred with a sterile magnetic bar for 24 h at 25 °C; subsequently, they were degassed, left standing for 12 h at 25 °C and centrifuged at 7957 × *g* for 10 min.

Film forming solutions were then poured on polystyrene plates and left to dry for 24 h at 25 °C on a previously leveled surface, until the total evaporation of the solvent. The dried films were peeled from the plate and maintained at 25 °C at a relative humidity of 45%. The physical properties of the pure chitosan films, plasticized with sorbitol (pC_SS and pC_CS) and non-plasticized (pC_S and pC_C), were compared to those of commercial cellophane film (Control).

2.5. Physicochemical characterization of chitosan films

2.5.1. Thickness

The thickness of the chitosan films was determined by using a Mitutoyo micrometer (PB-1 JIS.B.7502, USA), reporting the average value of 10 measurements taken at different points at random.

2.5.2. Stress–strain mechanical properties

The strength and elongation of the films were evaluated by using an United equipment (SSTM 5KN model, USA) according to the conditions set by ASTM D882-94. Average values from at least three measurements were reported

2.5.3. Thermal analysis

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were carried out on an equipment SDT 2960 simultaneous DSC–TGA TA instruments. Samples of 6 mg approximately were taken and warmed up until 650 °C at a heating and cooling rate of 10 °C/min under an air flow of 23 mL/min.

2.5.4. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

The chitosan films were analyzed directly using FT-IR spectrophotometry equipment, in a spectral range from 4000 to 400 cm^{-1}.

2.6. Fungistatic activity

Once prepared, the films were neutralized by immersion in NaOH solution at 0.1% (w/v) for 5 s and were left to dry at 25 °C. Before being inoculated, they were cut into pieces of 6 mm of diameter, and sterilized by exposure to ultraviolet light for 5 min on each side. Radial growth and diameter of spores and hyphae of *A. niger* were determined.

2.6.1. Radial growth

To evaluate the effect of the films on the fungi radial growth, the sterile film pieces were placed on the agar, then the fungi inoculum was placed over the film surface. This inoculation system was called "agar/film/fungi" (above the film). Another inoculation system called "agar/fungi/film" (below the film) was carried out by placing the fungi inoculum on the agar, then the inoculum was covered with the film. In each case, an inoculum with a concentration of 1×10^3 spores/mL was deposited and incubated at 25 °C. Every 24 h, the diameter of the colonies was manually measured until the control reached the edge of the plate. Fungistatic index was obtained by Eq. (4).

2.6.2. Morphometric analysis of hyphae and spores

The diameter of the fungi spores and hyphae was determined by image analysis using Image-Pro Plus version 6.3 software (2008 Media Cybernetics Inc., USA), using an optical microscope (Olympus CX31, Japan) connected to an Infinity 1 camera (Media Cybernetics, USA), and using a 40× objective.

2.7. Scanning electron microscopy (SEM) of chitosan films

Observations on SEM of the chitosan films surfaces inoculated with *A. niger* were made. Samples were fixed in 5% (v/v) of glutaraldehyde solution for 24 h at 4 °C and then in 1% (w/v) of osmium tetroxide solution for 2 h at 4 °C. Dehydration by gradients of methanol solutions (at 30, 40, 50, 70, 80, 90 and 100%, v/v) was performed. The fixed and dried samples were coated with gold before observation in a scanning electron microscope (JEOL JSM 5410LV, Japan) (Echlin, 2009; Plascencia-Jatomea et al., 2003).

2.8. Statistical analysis

Statistics on a completely randomized design were determined using the one-way analysis of variance (ANOVA) procedure in the JMP software (JMP version 5.0, SAS Institute Inc., USA), at a level of significance set at $P = 0.050$. Means for groups in homogeneous subsets were carried out using the Tukey multiple comparisons test (Tukey's post hoc test), at 95% confidence interval. All data were presented as mean value with their standard error indicated (mean ± SE). Differences were accepted as significant when ($P \leq 0.05$).

3. Results and discussion

3.1. Physicochemical characterization of chitosan

3.1.1. Protein and ashes content

The yields of chitin and chitosan obtained from fresh shrimp waste were of 2.38 and 1.59%, respectively. Also, the calculated yield of chitosan from extracted chitin (dry basis) was 66.81%, consistent with values reported by Cira, Huerta, Hall, and Shirai (2002). It is possible that during fermentation depolymerization of chitin occurs (Rao & Stevens, 2005) by enzymatic action (Kumar, Gowda, & Tharanathan, 2004), causing a decrease in particle size of solid matter and the possible loss of chitin in the liquid fraction of silage

Table 1
Physicochemical characterization of chitosans.

Chitosan	Ash (%)	Protein (%)	DD (%)	[ɳ] (dL/g)	MW (kDa)
C_S	0.57 ± 0.045[a]	0.67 ± 0.001[a]	76.2 ± 0.36	2.58 ± 0.042	~100
C_C	0.10 ± 0.064[a]	0.66 ± 0.008[a]	64.5 ± 3.79	9.46 ± 0.077	~480

C_S: Chitosan obtained from chitin (of shrimp waste silage); C_C: commercial chitosan (Fluka, BioChemika, Japan); DD: deacetylation degree; [ɳ]: intrinsic viscosity; MW: molecular weight. Data, followed by their standard errors, are means of three experiments. Treatment means were separated using the Tukey test ($P > 0.05$).

(Zakaria, Hall, & Shama, 1998), with the consequent decrease in performance.

The content of inorganic matter and residual protein was higher ($P > 0.05$) in the C_S with respect to C_C (Table 1), but in all cases values <0.7% were observed. The agitation of material allows a higher removal of minerals (Rao & Stevens, 2005), however, considering that a high-quality chitosan must contain <1% of residual ash (No & Meyers, 1995), the C_S obtained in this study (using a simple static fermentation process) is of good quality and comparable to commercial C_C.

3.1.2. Deacetylation degree (%DD)

The degree of deacetylation (%DD) of C_S and C_C was of 76.2 ± 0.36 and 64.5 ± 3.79%, respectively (Table 1). These values are lower than those reported by Sini, Santhosh, & Mathew (2007), who obtained a 81% DD chitosan from chitin obtained by the fermentation of shrimp heads with *Bacillus subtilis*; they are also lower than those reported by Beaney, Lizardi-Mendoza, and Healy (2005), who obtained a 82.2% DD chitosan from chitin obtained by prawns' shells fermentation and *Lactobacillus salvarius*, *Enteroccus facium* and *Pediococcus acidilactici*; the authors conducted the alkaline thermal deacetylation (NaOH 50%, w/w) of chitin at 100 °C, while in our experiment the temperature used was of 35 °C. This may explain the differences in the values of %DD, which also can be affected by factors such as type of deacetylation (biological or chemical), concentration of reagents, chitin ratio:solvent and reaction time.

Another important factor in determining %DD is the base line used to correct the readings. Our study encompassed the proportion in A_{1655}/A_{3450} bands, which has been reported suitable for samples of crystalline chitin and chitosan which are perfectly dry. However, there are two approximated peaks, to 1655 and 1625 cm^{-1}, corresponding to the stretching C=O and C=N of the amide I, respectively, which may overlap, being observed a single peak. Another drawback in using this band, is the proximity to the peak around 1650 cm^{-1} corresponding to OH groups of polysaccharides and water molecules (Kasaai, 2008) as a result an increase in water content of the sample can increase the absorption of the band making it wider and interfering in the analysis.

It is advisable to maintain moisture-free samples and conduct a standard method of testing using samples of chitosan with a known deacetylation degree as a control. In this experiment, a slight widening of the band located at 3450 cm^{-1} for C_C was observed (data not shown), attributable to the increased presence of O–H bonds, mainly of hydrogen bridges caused by a greater amount of moisture in relation to C_S.

3.1.3. Molecular weight

The C_S presented a low intrinsic viscosity compared with C_C (Table 1), indicating that C_S has a lower polymerization degree, which is consistent with the low molecular weight (~100 kDa) of C_S. These results are similar to those reported by Beaney et al. (2005) and Sini et al. (2007), who obtained low molecular weight chitosan (70.3 and 256 kDa, respectively), both obtained from chitin extracted by lactic fermentation of shellfish waste.

The low molecular weight of chitosan obtained can be attributed to greater susceptibility to degradation of chitin and/or depolymerization during the removal of proteins and minerals in the silage, and in the purification procedures and subsequent deacetylation. The presence of residual minerals, specifically calcium, may have influence in the viscosity of chitosan, and hence on the calculated molecular weight. In this experiment, the C_S presented a higher ($P > 0.05$) percentage of residual inorganic material compared with C_C, which may explain the low molecular weight.

3.2. Fungistatic activity of chitosan on Aspergillus niger

3.2.1. Radial growth

In culture media amended with chitosan, C_C and C_S had higher ($P \leq 0.05$) inhibitory effect on the average colony radius with respect to control at 96 h (Table 2). None of the chitosans completely inhibited the fungus, so the effect is fungistatic and not fungicidal. In previous studies, Plascencia-Jatomea et al. (2003) found that medium molecular weight chitosan (3 and 5 g/L) inhibited the radial growth of *A. niger* by 73% compared with the control, without observing complete inhibition. Similarly, it has been reported that chitosan and its derivatives are not able to inhibit 100% the growth of fungi as *B. cinerea*, *Fusarium oxysporum* and *R. stolonifer* (Guo et al., 2006; Hernández-Lazaurdo et al., 2008; Rabea et al., 2006).

In this study both chitosans have a similar %DD, which may explain having similar fungistatic activity, as the %DD shows the proportion of free amino groups in the polymer that are directly related to the antimicrobial activity of chitosan.

3.2.2. Spore's germination

C_S and C_C presented lower ($P \leq 0.05$) values of germinated spores at 18 h with respect to controls (Table 2). Similar inhibition values have been reported for nematophagous fungi grown in media with low molecular chitosan, which could be associated with their high extracellular chitosanolytic activity (Palma-Guerrero, Jansson, Salinas, & Lopez-Llorca, 2007). Inhibition in the first stage of fungal growth is a great advantage since the fungus requires more time to absorb nutrients from the medium and develop colonies, allow-

Table 2
Colony radial extension and spore's germination of *Aspergillus niger* grown on PDA agar media with added chitosan (2.82 g/L), at 25 °C.

Treatment	Colony radial growth (cm) at 96 h	Fungistatic index on CR (%)	Germinated spores (%) at 18 h	Fungistatic index on GE (%)
C_S	1.28 ± 0.10[c]	47.26[a]	57.5 ± 3.5[b]	38.75[a]
C_C	1.07 ± 0.17[c]	56.16[a]	46.7 ± 2.7[b]	39.50[a]
CA	1.72 ± 0.10[b]	29.11[b]	80.0 ± 3.0[a]	1.25[b]
C	2.19 ± 0.10[a]	–	87.0 ± 1.0[a]	–

CR: Colony radial growth; GE: germinated spores; C_S: silage chitosan; C_C: commercial chitosan (Fluka, BioChemika, Japan); CA: acid control (PDA agar media, pH adjusted with acetic acid); C: PDA control. Data, followed by their standard errors, are means of three experiments. Treatment means were separated using the Tukey test ($P > 0.05$).

Table 3
Physicochemical properties of the chitosan films prepared by the Casting technique.

Film	Thickness (mm)	Strain (%)	T_g (°C)
pC_S	0.03 ± 0.01[a]	2.47 ± 0.10[a]	170.9[a]
pC_SS	0.02 ± 0.01[a]	5.06 ± 0.11[b]	169.2[a]
pC_C	0.04 ± 0.01[a]	1.96 ± 0.06[a]	170.0[a]
pC_CS	0.06 ± 0.02[a]	1.97 ± 0.05[a]	172.2[a]
C	0.03 ± 0.00[a]	6.68 ± 0.11[c]	163.9[a]

pC_S: Silage chitosan film; pC_SS: silage chitosan + sorbitol film; pC_C: commercial chitosan film; pC_CS: commercial chitosan + sorbitol film; C: commercial cellophane film. Data, followed by their standard errors, are means of three experiments. Treatment means were separated using the Tukey test ($P > 0.05$).

ing chitosan to be suitable for use as a preservative in solutions or coatings for food.

3.3. Physicochemical characterization of chitosan films

3.3.1. Thickness

The films of each chitosan (pC_S and pC_C) were homogeneous, transparent and slightly brittle. The addition of sorbitol did not change significantly ($P > 0.05$) the average thickness of the films (Table 3). The pC_C films were thicker (plasticized and non-plasticized), which may be caused by compacting differences of the chitosan chains. Likewise, it is possible that the mixture gels during the process of film formation, which can affect the alignment, sorting and compacting of the molecules not only from chitosan, but from anion, from the plasticizer and their interactions. The use of compounds, plasticizers or antimicrobial agents, with a molecular volume greater than the anion, in this case acetic acid, allows for the obtention of softer films that can be used to produce multilayer or covered films.

3.3.2. Stress–strain mechanical properties

In assessing the mechanical properties of pure chitosan films, it was observed that the addition of sorbitol increased significantly ($P \leq 0.05$) the elongation of pC_SS films with respect to cellophane control (Table 3). These results are consistent with those reported by Srinivasa, Ravi, & Tharanathan (2007), where it was observed that the addition of sorbitol increased the percentage elongation (from 10.8 to 42.5%) in films of chitosan, which were more flexible than chitosan films plasticized with glycerol and polyethylene glycol.

In determining the tensile strength (TS) it was found that the pC_S and pC_SS films presented lower ($P \leq 0.05$) TS values with respect to cellophane control, whereas those of pC_C and pC_CS were similar (Fig. 1). The plasticizer addition increased the Young module value, E, of chitosan films (data not shown), however, lower ($P \leq 0.05$) E values were obtained for all chitosan films, plasticized and non-plasticized, with respect to cellophane control.

The higher E value of pC_SS comparing with pC_CS can be explained in terms of molecular weight of chitosan, which can influence the type and number of polymer–polymer, polymer–plasticizer and polymer–solvent interactions. In general, the presence of the plasticizer in the films made with medium molecular weight chitosan reduces the necessary effort for the deformation, as well as the deformation of the films before their rupture (Fig. 1).

3.3.3. Thermal analysis

The glass transition temperature (T_g) of the chitosan films was measured by differential scanning calorimetry and the thermal decomposition temperature of the films was measured by thermogravimetric analysis. Fig. 2 shows diagrams of TGA–DSC of chitosan films.

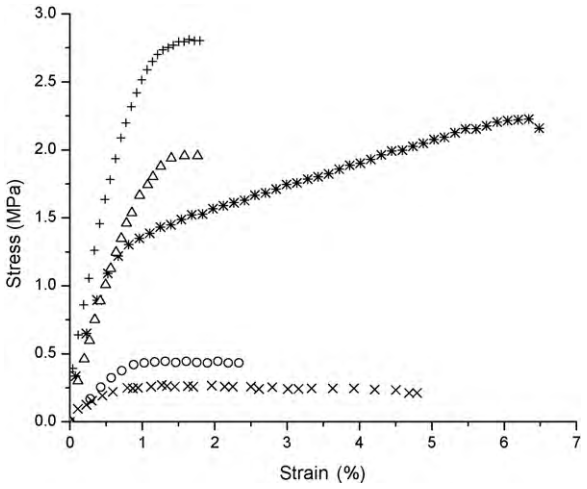

Fig. 1. Stress–strain graph of chitosan composite films: (○) silage chitosan film, pC_S; (×) silage chitosan + sorbitol film, pC_SS; (+) commercial chitosan film, pC_C; (△) commercial chitosan + sorbitol film, pC_CS; (*) commercial cellophane film (control).

The initial weight loss up to 135 °C is due to the removal of moisture and volatile material present in the films. A weak endothermic peak in DSC at around 200 °C corresponds to this degradation. Less weight loss at this temperature showed films with sorbitol. This may indicate stronger interaction between water molecules present in the films and sorbitol. These results are consistent with those reported by Suyatma, Tighzert, & Copinet (2005) for chitosan films plasticized with glycerol, ethylene glycol, polyethylene glycol and propylene glycol.

The onset temperature of thermo-oxidative degradation of chitosan films is observed at 135 °C (Fig. 2), accompanied with a massive weight loss up to 100% around 600 °C leaving behind no residue, and three simultaneous exothermic peaks in DSC are presented. The second significative weight loss is in the range of 135–300 °C, this event may be related to the decomposition of amine units (Simionatto & Gomes, 2006), an exothermic peak is observed in DSC around at 285 °C for this degradation of the material. The third stage of mass loss starts at 300 °C and continues up to 400 °C due to the degradation of the–CH_2OH group. Finally, total degradation of chitosan ring is close to the 600 °C.

Miscibility of chitosan and plasticizer in the amorphous phase was determined by the glass transition (T_g), whose value did not present statistic difference ($P > 0.05$), in none of the treatments (Table 3). It is possible that being in the film form, the amino groups of chitosan molecules are protonated, making the films to show greater affinity for water molecules compared with chitosan powder (Fernández-Cervera et al., 2004), this may indicate stronger interaction between water molecules present in the films and sorbitol.

Because of the potential effect of moisture content in determining the T_g of chitosan in the films, it is advisable to avoid the thermal degradation temperature of chitosan when making the first heating cycle, which is estimated between 200 and 250 °C (Dong, Ruan, Wang, Zhao, & Bi, 2004; Peesan, Supaphol, & Rujiravanit, 2005; Sakurai, Maegawa, & Takashi, 2000; Suyatma et al., 2005).

Pure chitosan films (>91% GD, 90 kDa) in acetic acid have presented T_g values of 203 °C (Sakurai et al., 2000) and 104 °C (Dong et al., 2004), whereas in chitosan/polyhydroxybutyrate films there has been a single T_g of 103 °C (Cheung, Wan, & Hu, 2002), which is attributed to the complete miscibility of the compounds.

The degree of deacetylation of chitosan does not significantly affect the glass transition temperature of chitosan films. In this experiment, we observed a single value of T_g (Table 3, Fig. 2), so

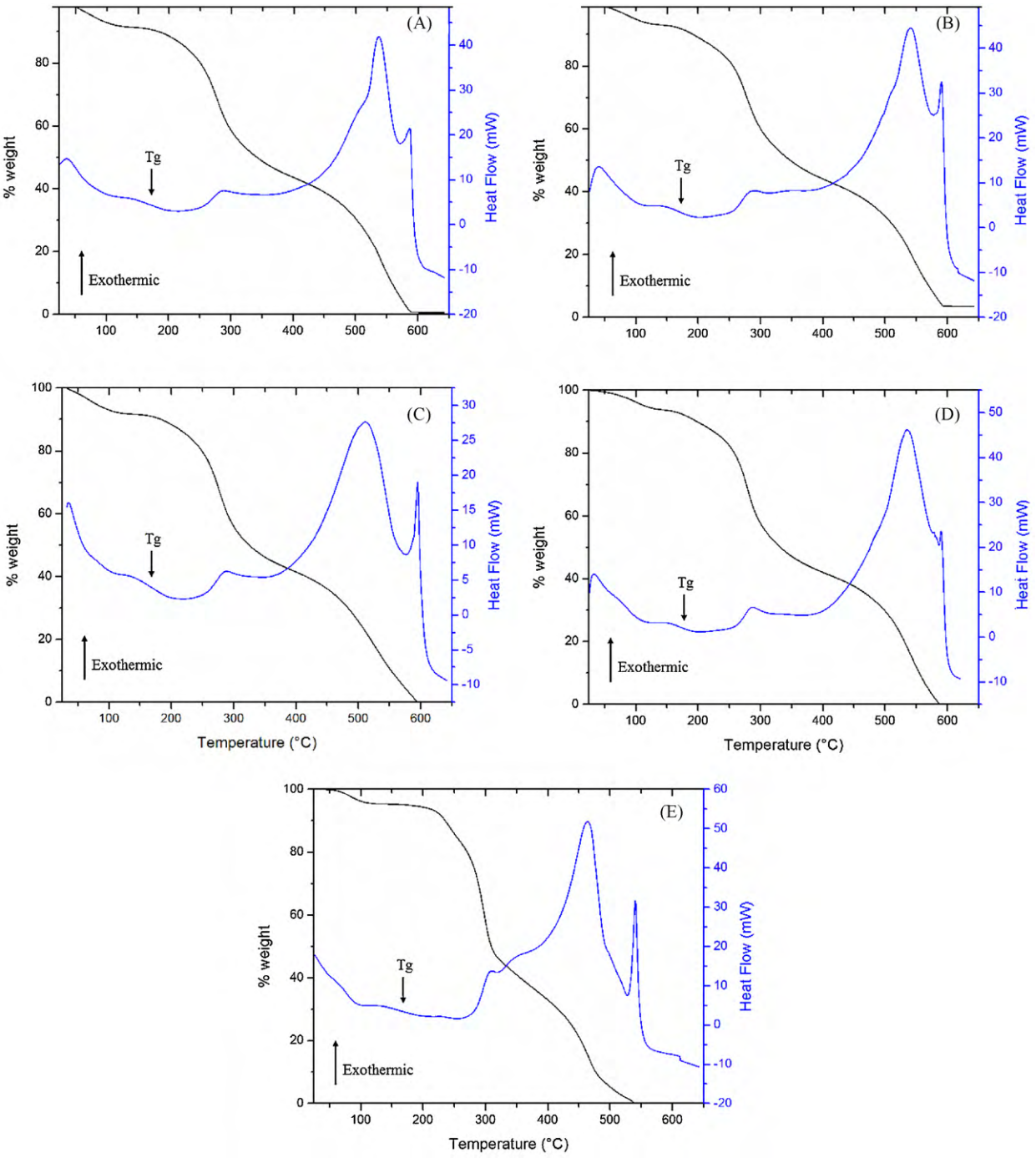

Fig. 2. Thermogravimetric analysis of chitosan films: (A) silage chitosan film, pC_S; (B) silage chitosan + sorbitol film, pC_SS; (C) commercial chitosan film, pC_C; (D) commercial chitosan + sorbitol film, pC_CS; (E) control (commercial cellophane film).

it can be assumed that sorbitol and chitosan are miscible and that the value of T_g does not vary significantly ($P > 0.05$) in films obtained from chitosan of low and medium molecular weight. Subsequent studies using high molecular weight chitosan will allow for the confirmation of the effect of molecular weight over thermal stability of the obtained films. Similar T_g values were reported by Suyatma, Tighzert, & Copinet (2005) for chitosan films plasticized with glycerol (178 °C). The plasticizer may have contributed to an ordering of the polymer chains according to their degree of polymerization favoring the interactions between it and increasing its crystalline character.

3.3.4. FT-IR analysis

FT-IR spectra of chitosan films (Fig. 3) show the emergence of a broad peak between 3200 and 3570 cm^{-1}, attributed to stretching of OH bonds in the molecule and to the hydrogen bridges built in films with water and sorbitol. It was found that pC_SS and pC_S presented widening further in that region, possibly as a result of strong interaction between the hydroxyl groups of the polymer–plasticizer, polymer–water and plasticizer–water. This result agree to those reported by Rueda, Secall, & Bayer (1999) who observed a broadened peak around 3400 cm^{-1}, even in chitosan films undergoing a drying process.

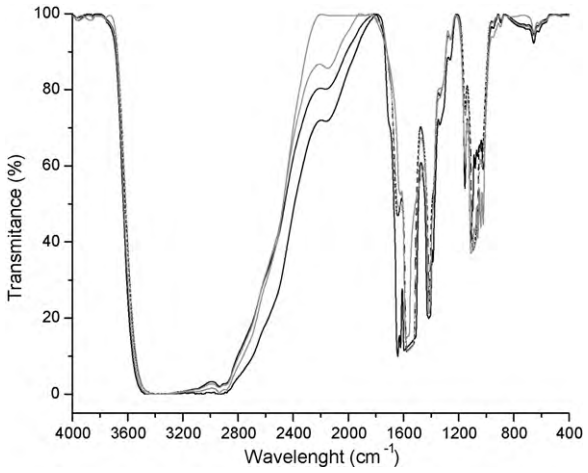

Fig. 3. FT-IR spectra of chitosan films, plasticized and non-plasticized: (—) silage chitosan film, pC_S; (- - -) silage chitosan + sorbitol film, pC_SS; (—) commercial chitosan film, pC_C; (- - -) commercial chitosan + sorbitol film, pC_CS.

At 1650 cm^{-1} the corresponding peak for the stretching of the chitosans amide I group whose appearance has been previously reported at about 1570 cm^{-1} in chitosan films, was observed (Park, Lee, Jung, & Park, 2001). In this study, the rightward shift of the peak (except in the pC_S film) suggests an increase in the number of hydrogen bonds formed between the chitosans $-NH_2$ groups and sorbitol –OH groups. Although the overall structure of the spectrum did not changed markedly with the addition of sorbitol, it can be deduced a good miscibility of the plasticizer with chitosan, which is confirmed according to the results obtained by thermal analysis.

3.4. Fungistatic activity of chitosan films

3.4.1. Radial growth

Whatever the type of inoculation used (systems agar/fungi/film and/or agar/film/fungi), no significant differences ($P > 0.05$) in fungistatic activity of chitosan films plasticized and non-plasticized were observed (Table 4). Being hydrophilic materials, films of chitosan can be semi-permeable to gases such as O_2, CO_2 and water vapor (Lacroix, 2009). Wet chitosan membranes exhibit large gas permeability and this high permeation rate is caused when chitosan is swollen by water vapor; in addition, the permeability rate of chitosan membranes is affected by the membrane preparation conditions and the acetic acid concentration in the cast solution (Akira, Makoto, & Tomotoshi, 1997).

In chitosan-containing films without alkaline treatment, chitosan chains are likely to be present as acetate salts. Tanabe, Okitsu, Tachibana, & Yamauchi (2002) tested the swelling behavior of chitosan films after neutralization by immersing films in 1% NaOH solution, observing that neutralized films also dissolved at pH 4.0, but swelled to the lesser extent at pH 6.3. Thus, neutralization of chitosan-containing films give decreased swelling under neutral aqueous environment.

The neutralization of chitosonium acetate films (prepared by dissolving chitosan in acetic acid) by rinsing with 0.1 M NaOH solution and with deionized water, restore the free amino groups and produce chitosan films that remained uniform in color at constant relative humidity (Murray & Dutcher, 2006). This fact impacts on the antimicrobial activity of the films and of course when used as food packing materials, since it is possible that microorganisms may be delayed or inhibited.

In this study, the fungistatic activity of the films was moderately higher than that observed for the chitosan solution (in PDA agar media); higher values may be due to the synergistic effect of chitosan and low permeability of the films, which limits the growth of fungus. Pathogenic isolates of *Aspergillus* spp., including *A. niger*, are clearly not capable of growth at an oxygen concentration of 0%; however, are capable of growth at low oxygen concentrations (0.5 and 2.5% in PDA agar, and at 0.1% on Hall's medium), showing that the growth rate is slowed and conidiation is delayed or absent (Hall & Denning, 1994).

Using the agar/fungi/film inoculation system, the colonies developed in the presence of films had a smaller radius (Table 4), which can be attributed to the limited availability of oxygen. Depending on the type of acid and the molecular weight of chitosan, the oxygen permeability of chitosan films varied from 0.4 to 5.89×10^{-8} cc/cm^2 day atm, which is comparable with commercial polyvinylidene chloride (PVDC) or ethylene vinyl alcohol copolymer films (Park et al., 2002). Low oxygen permeability of chitosan films can be exploited for food and medical packaging materials.

Sebti, Martial-Gros, Carnet-Pantiez, Grelier, & Coma (2005) found complete inhibition of spore germination of *A. niger* at 10 days of incubation by inoculating the fungus below of chitosan films (agar–fungi–film system). Using the same inoculation system, Sébastien, Stéphane, Copinet, & Coma (2006) found a total inhibition of *Fusarium moniliforme*, *F. proliferatum* and *Aspergillus ochraceus* in chitosan composite films with polyethylene glycol (PEG) and polylactic acid (PLA), which was attributed largely to the antimicrobial activity of chitosan.

Likewise, it has been reported significant inhibition in the growth of bacteria such as *E. coli*, *S. aureus* and *L. monocytogenes*, among others (Mi et al., 2006; Möller et al., 2004), in both types of inoculation. This effect may be due to the ability of chitosan to alter the cell wall structure and permeability of the membrane (Chung et al., 2004), delaying the microbial growth or killing.

Table 4
Fungistatic activity of chitosan films on the radial growth of *Aspergillus niger*, at 25 °C.

Blend film	Inoculation system	Fungistatic index (%) at 72 h	Hyphae diameter (μm) at 48 h	Spores diameter (μm) at 10 h
C_C	A	33.47 ± 8.09^{a}	5.43 ± 0.78^{d}	4.05 ± 0.17^{a}
	D	46.22 ± 24.9^{a}	5.87 ± 0.67^{cd}	4.11 ± 0.57^{a}
C_CS	A	27.08 ± 3.56^{a}	5.56 ± 0.88^{d}	3.87 ± 0.11^{a}
	D	64.15 ± 0.0^{a}	6.94 ± 1.21^{b}	5.67 ± 1.02^{a}
C_S	A	15.66 ± 1.78^{a}	4.72 ± 0.61^{e}	4.18 ± 0.23^{a}
	D	53.00 ± 8.31^{a}	5.77 ± 0.58^{cd}	3.99 ± 0.13^{a}
C_SS	A	22.04 ± 6.22^{a}	5.88 ± 0.92^{cd}	4.54 ± 0.22^{a}
	D	56.40 ± 10.8^{a}	6.66 ± 0.67^{b}	4.30 ± 0.41^{a}
C	A	25.40 ± 6.56^{a}	8.48 ± 1.74^{a}	4.98 ± 0.33^{a}
	D	39.44 ± 8.31^{a}	6.26 ± 1.77^{bc}	3.98 ± 0.34^{a}

A: Agar/film/fungi inoculation system; D: agar/fungi/film inoculation system; C_C: commercial chitosan film; C_CS: commercial chitosan + sorbitol film; C_S: silage chitosan film; C_SS: silage chitosan + sorbitol film; C: commercial cellophane film. Data, followed by their standard errors, are means of three experiments. Treatment means were separated using the Tukey test ($P > 0.05$).

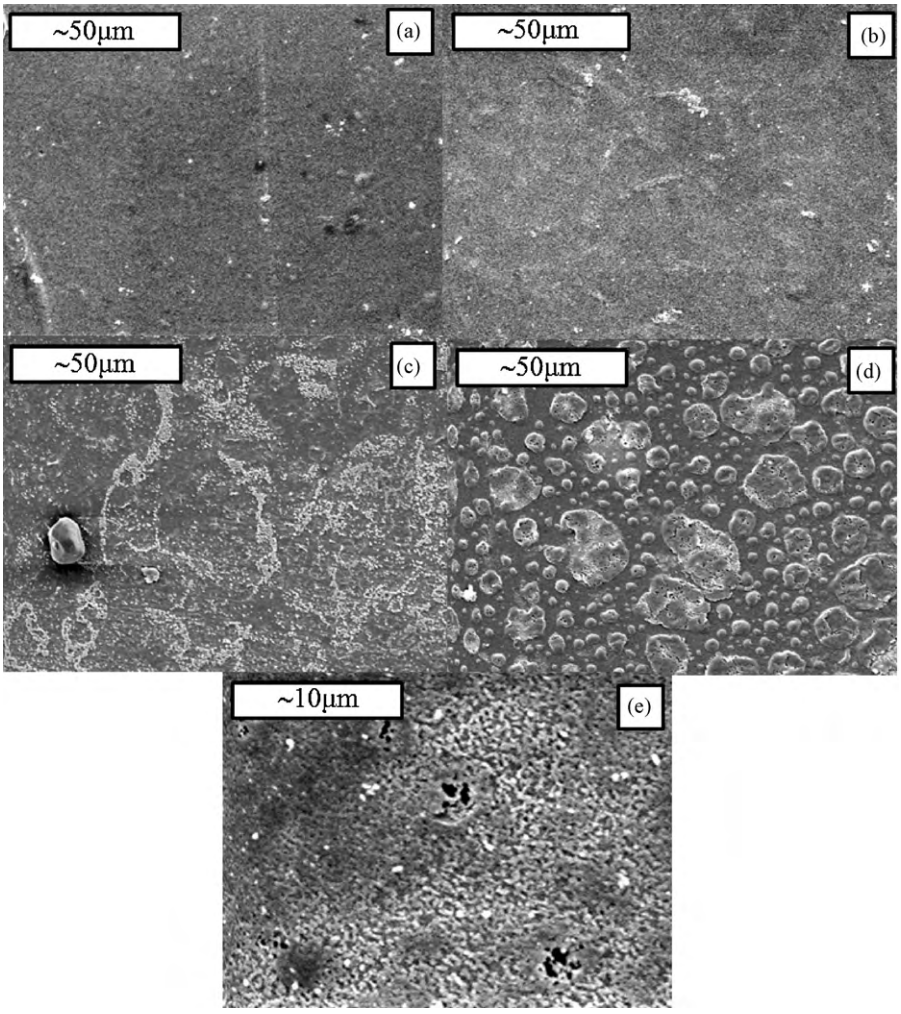

Fig. 4. Scanning electron micrography (SEM) analysis of chitosan films after 18 h of inoculation with *Aspergillus niger* (agar/film/fungi inoculation system): (a) control film (1000×); (b) pCSS film (1000×); (c) pCC film (1000×); (d) pCCS film (1000×); (e) pCS film (5000×).

In *in vitro* studies on solid culture media, filamentous fungi have shown that chitosan causes aggregation of the spores, affects the output of germinal tubule and interferes with the passage of nutrients through the membrane. Several research groups have hypothesized that an electrostatic interaction takes place between chitosan and either (i) negatively charged cell membrane components (i.e. phospholipids or proteins), (ii) amino acids in the Gram-positive bacterial cell wall, or (iii) various lipopolysaccharides in the outer membrane of Gram-negative bacteria, thereby affecting membrane integrity and permeability (Raafat & Sahl, 2009).

Direct contact of fungi with chitosan was reported to produce weakening and swelling of the hyphae, therefore the fungistatic properties of chitosan are related to its ability to induce morphological changes in the cell wall (Rabea et al., 2003). Examination of ultrathin sections of the conidia from the plant pathogenic fungus *F. oxysporum* f. sp. *radicis-lycopersici* exposed to chitosan (0.01 mg/mL) showed that they had suffered severe damage; cytoplasm appeared completely disorganized, plasma membranes retracted and most cell contents were lost (Palma-Guerrero et al., 2007).

Epifluorescence microscopy examinations have shown a probable action of chitosan on nucleic acids (Plascencia-Jatomea et al., 2003; Sebti et al., 2005). Chitosan-coated surfaces resist biofilm formation by bacteria and yeast; using time-lapsed fluorescence microscopy and fluorescence-dye-loaded *Staphylococcus epidermidis*, the permeabilization of these cells was observed as they alighted on chitosan-coated surfaces, which suggest chitosan disrupts cell membranes as microbes settle on the surface (Carlson, Taffs, Davison, & Stewart, 2008). This may explain the strong inhibitory effect on the spore's germination since it is possible that such effects occur when using chitosan as a functional composite film; however, more detailed studies are needed to determine the mechanism of action of such composite films on the growth of filamentous fungi, and the implications associated with its use in the control of food spoilage microorganisms.

3.4.2. Morphometric analysis of hyphae and spores

Chitosan is able to increase the number and depth of the grooves of spores of *R. stolonifer* (Hernández-Lazaurdo et al., 2008), and induce the aggregation of spores of *A. niger*, which can modify the time of emergence or termination of dormancy of the spore. Singha, Vesentinia, Singha, and Daniel (2008) found that increasing concentrations of chitosan induce excessive branching, vacuolation, and a reduction in hyphal diameter of *Sphaeropsis sapinea* and *Trichoderma harzianum*, suggesting that the plasma membrane may be the primary target of chitosan action, and that the two fungi differ in the extent to which they are affected.

In this study, image analysis showed that the diameter of the spores (Table 4) of *A. niger* was not affected significantly ($P > 0.05$) when the development of fungus on the films of chitosan, having also a small amount of visible spores. This may be because the

hydrophobic characteristics of spores prevent from adhering firmly to the film, causing difficulty in locating enough spores on the surface of the material. Similarly, it is possible that the hydrophilic properties of chitosan films increase the absorption capacity of water present in the culture medium, which may modify the three-dimensional arrangement of chitosan due to the repulsion between carbohydrate chains that causes the appearance of pores in the material. Conformational changes in the microstructure of chitosan films caused by swelling not only increase moisture sorption, but also create channels in the polymeric structure to allow the increase in permeant flow (Miranda, Garnica, Lara-Sagahon, & Cárdenas, 2004).

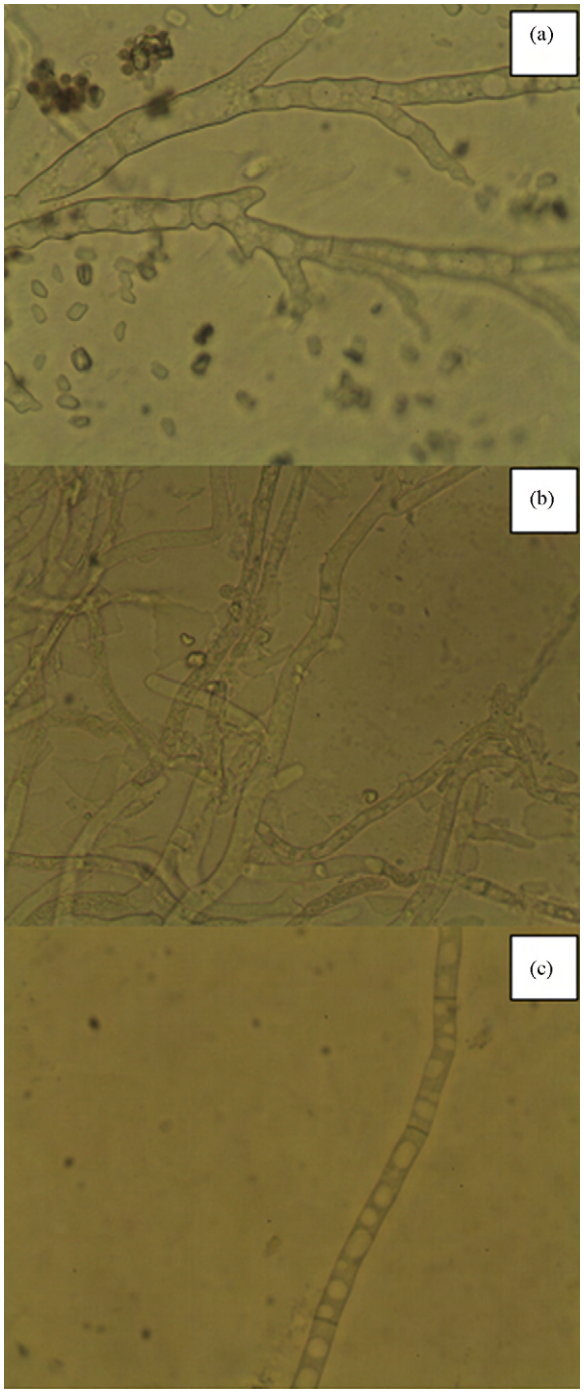

Fig. 5. Mycelium of *Aspergillus niger* grown on chitosan films, at 10 h after incubation at 25 °C, at 40×: (a) commercial cellophane film; (b) pC_S film; (c) pC_SS film.

Chitosan can be hydrophobically modified to possess both meso- and macropores (approximately 10 nm to greater than 100 nm in diameter) that provide the kind of hydrophobic microchemical environment that stabilizes enzyme activity for long periods of time (Klotzbach, Watt, Ansari, & Minteer, 2006). Mass and heat transfer, besides process variables such as acetic acid and chitosan concentrations, influence the final pore structure of porous chitosan scaffold that was immobilized with an NAD-dependent glucose dehydrogenase (Cooney et al., 2008).

Being the physical integrity of the material compromised, it is possible that the presence of these pores eventually favors the mass transference, with the consequent growth of the fungus.

By analyzing the morphology of *A. niger* in the apical growth phase, a decrease was observed ($P \leq 0.05$) in the average diameter of the hyphae (Table 4) developed in direct contact with chitosan plasticized films (pC_SS and pC_CS), which may be caused for changes in membrane permeability due to the pressure and strain of the fungal cell wall. Previous studies reported that, added in PDA agar, chitosan causes deformation and damage on the surface of the hyphae of *A. niger*, besides an increase in the average diameter of the hyphae, which augments with increasing concentration of polymer (Plascencia-Jatomea et al., 2003).

3.5. SEM analysis of chitosan films

The electron microscopy analysis of films (Fig. 4) corroborated the existence of pores in pure chitosan films (Fig. 4e). Equally, it is possible to observe a small number of spores adhered to the surface of the films, which could be due to the dragging of the spores during the fitting process. Future studies with transmission electron microscopy and scanning electron microscopy using different fixation techniques for biological samples, will allow for the confirmation of this theory.

It was noted that in the presence of chitosan, hyphae were smoother and thinner compared to those developed in the cellophane control film (Fig. 5), showing a wide range of bodies, probably vacuoles within the hyphae developed in direct contact with pC_SS films (Fig. 5c). Muzzarelli et al. (2001) reported that chemically modified chitosan caused distortion and shrinkage of hyphae of *Saprolegnia parasítica*, also severely disrupted the cytoplasmic content probably due to dissociation or damage to the wall and fungal cell membrane.

4. Conclusion

The results of this study suggest that even in plasticized or non-plasticized films, chitosan shows fungistatic activity, which makes possible the development of active packaging based on mixtures of chitosan, with good thermal stability. Factors like storage temperature, type of chitosan and the modification of mechanical and barrier properties, given by the addition of plasticizers or antimicrobial agents, may potentiate the antimicrobial effect of the films. Detailed studies on the effect of chitosan films on the process of mitotic divisions and morphogenesis of food important spoilage fungi, will allow knowing the activity of the biopolymer on the cell structure and physiology. In addition it will help to explain and to elucidate a possible action mechanism, which will establish the impact related to the use of active natural materials.

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