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ANTIMICROBIAL EFFECT OF DIFFERENT CHITOSAN PREPARATIONS AGAINST SELECTED FOOD BORNE PATHOGENS

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ABSTRACT

The antimicrobial effect of the polyamide 6/66-chitosan blend obtained by extrusion and chitosan-coated plastic films was compared with pure chitosan films and chitosan solution against *Escherichia coli*, *Listeria monocytogenes* and *Bacillus cereus*. The films from polyamide 6/66-chitosan blend had no antibacterial activity, and the chitosan plastic film coating (bilayer system) showed only contact inhibition, without increase of contact area. The pure chitosan films did not show inhibition halos, however, contact inhibition was observed. During the assays, this films absorbed moisture, increasing the diameter of the discs at least 30%, causing contact inhibition on the area in all microorganisms tested. The best inhibition effect was observed with chitosan solution, reaching the total inhibition for *Listeria monocytogenes* and a log reduction of 3.6 and 2.3 for *Escherichia coli* and *Bacillus cereus,* respectively. Chitosan has an antimicrobial effect, although this property was reduced by the incorporation of a plastic matrix.

KEYWORDS: Antimicrobial films, chitosan, inhibition halos, physicochemical properties, coating.

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INTRODUCTION

Food quality and safety are major concerns in the food industry as consumers prefer fresh and minimally processed products. In particular, bacterial contamination of ready-to-eat products is the main risk to human health [1]. Traditionally, antimicrobial additives are mixed into initial food formulations to control microbial growth and extend shelf-life; however, this strategy is not always effective due to the fact that the protective ability of the antimicrobial agent can be neutralized by reactions and/or interactions in the complex food system [2]. Antimicrobial packaging is the most promising active packaging system that has been found to be highly effective in killing or inhibiting spoilage and pathogenic microorganisms that contaminate foods [3]. In recent years there has been remarkable development in the polymeric and edible packaging films made with biopolymers such as cellulose, chitosan, alginate and starch. Furthermore, antimicrobial agents have been added to improve the preservation of packaged food. These films possess the potential for improving microbial stability in food by acting on the food surface [4]. However, edible and biodegradable bio-based films are not always intended to completely replace the conventional packaging material [2]. Chitosan is a polysaccharide composed of *β*-1,4 linked glucosamine and *N*-acetyl glucosamine randomly distributed, is prepared by deacetylation of chitin, which is the second most abundant polysaccharide found in nature after cellulose [5]. Chitosan has been found to be non-toxic, biodegradable, biofunctional, biocompatible, and to have antimicrobial characteristic. One of the reasons for the antimicrobial character of chitosan is the protonation of the amino group of the chitosan chain in acid solution, causing chitosan to be positively charged and interact with negatively-charged microbial cell membranes, leading to the leakage of protein and other intracellular constituents of the microorganisms [6,7]. Chitosan was thus used as a polymeric matrix to produce, from renewable resources, films that exhibit potential antimicrobial properties with different groups of microorganisms, such as bacteria (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes)* and fungi (*Botrytis cinerea, Fusarium oxysporum, Piricularia oryzae)* [8,9]. However, the antimicrobial activity of chitosan will depend on several factors such as the kind of chitosan (deacetylation degree, molecular weight) used the pH of the medium, and the temperature and presence of several food components [10]. Chitosan may be used in various food preservation applications, such as direct addition of chitosan to food, direct application of chitosan films or coatings to food surface, addition of chitosan sachets to packages, and use of chitosan incorporated into packaging materials [11]. In particular, modification of chitosan by blending it with other polymers is a convenient and effective method of improving physical properties for practical utilization [12]. Numerous researches have reported the antimicrobial activity of chitosan in solutions and films blended with other polymers such as cellulose, poly(vinyl alcohol), or poly(lactic acid) [13], but there are no published reports on testing the feasibility of chitosan-based antimicrobial packaging films through incorporation of chitosan into polyamide 6/66 by extrusion. The aim of this study was the preparation of chitosan-based films in order to study their antimicrobial activity to improve food preservation.

MATERIALS AND METHODS

The raw material was shrimp waste (cephalothoraxes and exoskeleton) obtained from local shrimp processing factories in Sonora, Mexico. Chitin was obtained by lactic fermentation of shrimp waste, according to the procedure of [14]. Chitosan with low molecular weight was obtained according to [15] with some modification. In the first step; the chitin obtained was immersed in 4.5% (w/v) NaOH solution at 65 °C for 4 h in order to remove proteins. Then, the precipitate solid was immersed in 3.6% (w/v) HCl solution at room temperature for 4 h in order to eliminate minerals, rendering chitin as

the main product. Finally, the second step consisting of an alkaline deacetylation was accomplished by immersion in a 45% (w/v) NaOH solution at 120°C for 2 h, followed by rinsing with abundant water, and drying was accomplished at 40 °C for 12 h. Prior to its use, chitosan was milled using a hammer mill (LM 3100, Perten Instrument, Hägersten, Sweden) to obtain a fine powder of a 180 µm particle size.

(i) Chemical and physical properties of chitosan

For moisture content determination, the samples were heated overnight in an electric oven at 60 °C. For ash content, ground dried samples were heated for 5 h in an electric oven at 525 °C. Total nitrogen content was calculated by micro-Kjeldahl [16]. The deacetylation degree of chitosan was measured by UV/vis spectrophotometer (Varian, Cary 50 Bio, USA) using dual standard according to the procedure carried out by [17] with modification. The standard curve was obtained by N-acetylglucosamine and D(+) glucosamine hydrochloride standards to represent the two monomers of chitosan. Specifically, 11 mg of chitosan was dissolved in 50 mL of 0.1 N HCl and its absorption intensity was recorded on a UV spectrophotometer at 201 nm. The shear viscosity of chitosan was measured at room temperature by a digital viscometer (Brookfield, Middleboro, MA. USA) using a cylindrical spindle (LV #1) at 30 rpm. The viscosity molecular weight (M_w) was investigated by using an Ubbelohde viscosimeter (Cannon-Fenske, Pittsburgh, PA, USA) at 25 ± 1°C. Five chitosan concentrations of 0.0014, 0.0012, 0.001, 0.0008 and 0.0006 g/mL were dissolved in a 0.3 M CH_3COOH/0.2M CH_3COONa solution, and filtered through membranes with a 0.45 µm pore size. The viscosity M_w was calculated based on Mark Houwink Equation according to the following formula.

$$[\eta] = KM^a \qquad (1)$$

Where [η] is the intrinsic viscosity, *M* is the relative molecular weight and *K* and *a* are empirical constants that depend of the polymer nature, the solvent and the temperature. In this paper, *K* = 0.074 mL/g and *a* = 0.76 [18].

(ii) Preparation of chitosan based films

Pure chitosan films

The pure chitosan films were made using the solvent evaporation technique according to [19] with some modification. Four chitosan solutions (1, 2, 2.5 and 3% w/v) were prepared by dissolving chitosan in 1% (v/v) acetic acid and stirred for 1 h at room temperature. A film-forming solution was then poured on polystyrene plates and left to dry for 24 h at 40 °C on a previously leveled surface, until complete evaporation of the solvent. Finally, the dried films were peeled off the plate and maintained at 25 °C in a polystyrene bag.

Polyamide 6/66-chitosan-based blend

The incorporation of chitosan powder into polyamide 6/66 Ultramid, uncolored (BASF, Germany) was made by an extrusion technique with an extruder (Beutelspacher, Mexico, Mexico D.F). The pre-dried polymers were mixed using a single screw extruder operating at a constant speed of 155 rpm. The mixing temperature was 215 °C, and the residence time was 3.2 min. Four different concentrations were prepared in ratios of 100:5, 100:6, 100:8 and 100:10 (polyamide 6/66:chitosan, w/w). A control film was made by polyamide 6/66 without incorporated chitosan [18].

Chitosan-coated plastic films (bilayer system)

The coating of plastic films with chitosan was made according to the method described by [12] with some modification. Specifically, polyamide 6/66 films were taped to 15 x 7 cm (221 cm^2) glass plates and 10 mL of chitosan-coating solution was cast at different concentrations 1, 2, 2.5 and 3% (w/v). Afterwards, the coated films were dried for 24 h at 40 °C on a previously leveled surface. Then chitosan-coated films contained 0.45, 0.9, 1.13 and 1.35 mg of chitosan per cm^2 of film surface. A control film was made with polyamide 6/66 without chitosan solution. Finally, the films were maintained at 25 °C in a polystyrene bag.

(iii) Antimicrobial Activity assay

Three pathogenic bacteria in food that are typical meat product contaminants were used in this study. *Escherichia coli* ATCC 10536, *Bacillus cereus* ATCC 10876 and *Listeria monocytogenes* ATCC 7644, were obtained from MediMark Europe (Florida, USA), and all culture media used were obtained from BD Bioxon (Estado de México, México). The bacterial cultures were grown on nutrient agar slant and kept at 4 °C. Every 25 days, subculture was carried out to maintain bacterial viability. In the preparation of seeding culture for the antimicrobial test of pure chitosan films, a loopful of each strain from the agar slant was taken and re-inoculated into plates with nutrient agar and incubated at 35 °C for 24 h. Then a loopful of these bacterial cultures were inoculated in sterile saline solution (0.9%) (BD Vacutainer Serum, D.F., Mexico) reaching a concentration of 1.5 x 10^8, obtained by comparing the concentration with the Mcfarland turbidity standard No. 0.5. For testing of chitosan solution, the microorganisms were inoculated in peptone water having an absorbance value of 0.2 reaching (10^5-10^6 CFU/mL), obtained at an optimal density of 600 nm by UV/vis spectrophotometer according to [13].

(iv) Antimicrobial test of films

Antimicrobial activity test of films was carried out by using an agar-diffusion method according to the methodology described by the National Committee of Clinical Laboratory Standards with some modifications [20]. All films were cut into disc forms with a 15 mm diameter. Film discs were placed on Mueller Hinton agar (BD Bioxon, USA) plates that had been previously seeded with 0.1 mL of inocula containing *Escherichia coli, Listeria monocytogenes or Bacillus cereus* at a concentration of 1.5 x 10^8 CFU/mL. The plates were then incubated at 35 °C for 24 h. The diameter of the contact area of the films with the agar surface was measured. The antimicrobial acetic acid effect was evaluated with the preparation of 15 mm discs, Whatman # 1, impregnated by submersion for 30 s. in 1% (v/v) acetic acid and dried at 40 °C for 24 h.

(v) Antibacterial activity of chitosan solution

The antimicrobial activity of the chitosan solution was evaluated according to [21] with some modification. Specifically, a stock solution of chitosan was prepared by dissolving 1000 mg/l in 1% (v/v) acetic acid. The chitosan solution was aseptically mixed with Mueller Hinton broth (MHB) to formulate concentrations of 20, 100, 150 and 200 mg/l. The pH of the dilutions was adjusted to 5.9 using NaOH (1N) and then sterilized. Then 0.1 mL of inocula (10^{-5}-10^{-6} CFU/mL) of the indicator microorganisms were added to a tube with 10 mL of MHB containing chitosan solution and incubated at 36 °C for 24h. Viable cells counts (CFU/mL) were made on trypticase soy agar (BD, Bioxon, USA) using the plate count method and incubated at 36°C for 24 h.

(vi) Statistical analysis

All experiments were performed in triplicate and data expressed as mean value ± standard deviation. The statistical significance of differences between means ($p < 0.05$) was estimated by ANOVA using Statgraphic plus for Windows version 4.0 (Statpoint Technologies, Inc. Warrenton, Virginia).

RESULTS AND DISCUSSION

Physicochemical characterization of chitosan

Table 1 shows the physicochemical characterization of chitosan. Moisture and ash are lower than the 6.78 and 6.84% reported by [22]. However, the ash and moisture content is similar to those reported by [23] of 0.08 and 4.75% respectively. The low ash content is due to the demineralization process and, according to [19], a high-quality chitosan must contain □1% of residual ash.

Table 1
Physicochemical characterization of chitosan.

Parameters	Value
Moisture, (%)	5.6 ± 0.054
Ash, (%)	0.2 ± .020
Nitrogen, (%)	6.2 ± 0.11
DA, (%)	84 ± 2.7
Viscosity, (cps)	152 ± 0.30
Molecular weight (kDa)	135

Means values of n = 3, triplicate determination ± standard deviations. Means in each colum with different superscript letter are significantly different (P 0.05).

In similar studies, [24] mentioned that chitin and chitosan are interesting compounds due their high nitrogen content (6.89%). Also, [25] showed a 6.2% of nitrogen content of in purified chitin and values of 8.8-9.5% for chitosan. These results are similar to those obtained in this study due to the fact that nitrogen in chitin was mainly distributed in protein and chitin. However, after alkaline treatment the protein was removed; this process guarantees the exact determination of nitrogen content in the chitosan [26]. The chitosan obtained is of low molecular weight (~135 kDa). The low molecular weight of chitosan obtained may be related to the biological treatment applied to chitin or the depolymerization during the removal of protein and minerals in the silage, and the purification procedures and subsequent deacetylation [19]. This result is similar to those mentioned by [27], who obtained low-molecular-weight chitosan (34 and 263 kDa, respectively). The deacetylation degree obtained in the work at hand is similar to commercial chitosan (Fluka and Sigma) with 81 to 91.4% [28]. It is also within the range of values (75-85%) reported by [25]. Also, [29] reported a chitosan with a 90% level of deacetylation resulting in high-quality chitosan. According to [30] the acetyl group of chitin cannot be removed in the presence of alkalis without the degradation of polysaccharide chains, due to the reagents, high temperature and the reaction times required to obtain a complete deacetylation. It was demonstrated that highly deacetylated chitosan showed more antimicrobial activity, due to increased solubility and charge density [3]. Variations in chitosan preparation processes affect parameters such as degree of deacetylation, distribution of acetyl groups, chain length, and conformational structure of chitosan [9]. Similarly, viscosity is influenced by these factors. According to [31] with high temperature (140-150 °C) chitosan is obtained with a viscosity range of 311-511 cps. Moreover, [32] reported values with a range of 106-6370 cps, which are within the range of the chitosan obtained in this study.

Antimicrobial activity tests

1. Pure chitosan films

The results show only contact inhibition without inhibition halos against *Escherichia coli*, *Bacillus cereus* and *Listeria monocytogenes*. However, the pure chitosan films absorbed moisture, increasing the diameter of the discs at least 30%, causing contact inhibition on the area in all microorganisms tested. This confirmed that chitosan is incapable of diffusing through the adjacent agar and its biocide effect is related to its release of protonated glucosamine fractions into the culture medium [13]. Several authors have pointed the antimicrobial activity of chitosan and its application in food packaging due to its capacity to form films [33]. [34] found that pure chitosan films showed contact inhibition against *Listeria monocytogenes,* with inoculum levels from 10^4 to 10^6 CFU/mL. In the same way, [10] mentioned the effect of chitosan against *Listeria monocytogenes* showing contact inhibition with a concentration of 0.7%, and also mentioned that *Bacillus cereus* does not show contact inhibition at any concentration. However when antimicrobial agents such as nisin, potassium sorbate and garlic oil are added, inhibition halos are present [3]. Also, [1] mentioned

the effect of pure chitosan films against *Escherichia coli,* showing a decrease in the number of colony forming units with the increasing chitosan concentration. The absence of inhibition halos in the test can be attributed to chitosan's inability to migrate through the pores of the agar, so that only organisms in direct contact with the active sites of chitosan are inhibited [4]. [2] showed that moisture content of pure chitosan films had a significant impact on their antimicrobial effect, which reduced with decreasing film moisture content. This behavior was observed in this study due to an increase in the diameter of the films discs caused by moisture absorption, thus helping the mobility of chitosan.

2. Polyamide 6/66 films with chitosan

Nowadays the development of active packaging based on new materials obtained by blending methods is one of most important techniques in food applications, and the most commonly used materials are polyamide and polyvinyl alcohol [11]. However, the antimicrobial test of polyamide 6/66 films with chitosan showed no inhibitory effect at any evaluated chitosan concentration against *Escherichia coli*, *Listeria monocytoges* and *Bacillus cereus*. Previous work reported the preparation of polyamide 6/66 with chitosan by the method of solvent evaporation and determined a strong interaction of chitosan with polyamide 6/66 through hydrogen bridges (NH2--- OH, OH----OH, C=O---OH). Those films showed good packaging but limiting the mobility of chitosan on the matrix [11], however they do not reported the antimicrobial capacity of these films. A similar study made for [10], developed low-density polyethylene films with chitosan reported an inhibitory effect on *Escherichia coli*, *Listeria monocytogenes,* and *Salmonella enteriditis,* achieving the complete inhibition with higher doses (1.4 to 2.1%) after 12 h on fresh red meat. This study agrees with several authors showing that the efficiency of chitosan depends on the application technique because better effects can be obtained with chitosan solution (coating) while chitosan matrix (solid state) reduces the antimicrobial effect, showing only contact inhibition [3] Thus, [35] mentioned that the mechanism of antimicrobial action of chitosan is not well defined; however, it is related to the positive charge of the amino group at pH□6, providing at the same time solubility to the polymer. In this study, chitosan was incorporated in powder form into the plastic matrix; therefore, the amino groups were not protonated and behaved as an inert polymer, unable to inhibit microbial colonies.

3. Chitosan-coated plastic films

These films showed only inhibition by contact without inhibition halos against the three bacteria studied. These results are similar to those reported for pure chitosan films because it allows the direct contact of pure chitosan with the bacteria. Then, for the potentialization of this films could be necessary the incorporation of additives as essential oils or preservatives [36]. Also, [3] mentioned that the films obtained by the bilayer technique are efficient in terms of mechanical properties, however, there is no industrial application due the additional steps of preparation (spray and dry), while films made by blended (bio-composite films) are more attractive for application.

4. Chitosan solution

The chitosan solution shows a higher inhibition at the time that increases the concentration of chitosan (Table 2). These results are similar to those reported by [1] who mentioned a decreasing number of colony forming units as the concentration of chitosan increased. In this way, [37] tested the antimicrobial effects of chitosan against *Listeria monocytogenes* and pointed that *Listeria monocytogenes* was completely inhibited. Also, [38] mentioned the complete inhibition of *Listeria monocytogenes* with 250 mg/l of chitosan solution. These results agree with the obtained in this investigation, evidencing the high susceptibility of *Listeria monocytogenes* to chitosan solution.

Table 2
Results of growth inhibition by chitosan solution a different concentration.

Concentration (mg/ L)	*Escherichia coli* Log count (CFU/ml)	*Bacillus cereus* Log count (CFU/ml)	*Listeria monocytogenes* Log count (CFU/ml)
0	7.98 ± 0.05[a]	6.54 ± 0.68[a]	7.39 ± 0.01[a]
20	7.43 ± 0.02[a]	6.35 ± 0.02[a]	5.86 ± 0.02[b]
50	7.14 ± 0.02[a]	6.35 ± 0.01[a]	1.2 ± 0.01[c]
100	6.97 ± 0.014[b]	5.95 ± 0.07[a]	0
150	5.11 ± 0.51[c]	4.31 ± 0.02[b]	0
200	4.31 ± 0.21[d]	4.09 ± 0.04[b]	0

The results are the means of three experiments. Treatment means were separated using the Tukey test (P 0.05).

Similarly, [8] mentioned an inhibitory effect with chitosan concentrations of 100, 20 and 20 mg/l for *Bacillus cereus, Escherichia coli* and *Staphylococcus aureus,* respectively. Meanwhile, [39] reported that 100 mg/l achieved a reduction of 99% on *Escherichia coli* and *Staphylococcus aureus.* These results are similar to those reported in this work and the difference between the effective concentrations of chitosan can be attributed to the physicochemical properties of the polymers, such as molecular weight, degree of acetylation, pH of the chitosan solution and the evaluated microorganism [7,13].

CONCLUSION

This work evaluated the antimicrobial effect of chitosan in three different films against *Escherichia coli*, *Listeria monocytogenes* and *Bacillus cereus*, which are considered a health risk in food. The films of the polyamide 6/66-chitosan blend obtained by the extrusion technique have no antimicrobial activity. However, the chitosan films showed inhibition by contact, and a similar effect was obtained with the chitosan-coated plastic films. Additionally, the chitosan solution showed a logarithmic reduction against the three microorganisms, achieving the complete inhibition of *Listeria monocytogenes*. The antimicrobial activity of chitosan is limited by their incorporation in plastic matrices, however, could be used in foods as a solution or coating to preserve its antimicrobial effect.

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