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Shelf Life Extension of Package's Using Cupper/(Biopolymer nanocomposite) Produced by One-Step Process

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ABSTRACT: The use of new compounds to increase the shelf life of more perishable foods based on nanoparticles and biodegradable polymers have been developed in packaging. Chitosan (Cts) is a natural biopolymer with excellent biodegradability and nontoxicity and antibacterial effect. In this research antibacterial copper nanoparticles (CuNPs) were incorporated in the biodegradable chitosan matrix for applications as packaging materials. The properties of Cu/Cts bionanocomposites (BNCs) were studied as a function of the CuNPs concentration. Surface morphology and elemental composition of the film was investigated by scanning electron microscopy attached to EDXRF. Mechanical analysis and water vapor barrier properties of CuNPs/Cts composites were analyzed. It was observed that mechanical and water vapor barrier properties of the films were improved by the concentration of CuNPs. The antibacterial activity of CuNPs/Cts thin films were evaluated based on the diameter of the inhibition zone in a disk diffusion test against gram positive bacteria i.e., *Staphylococcus aureus* and *Bacillus cereus* and gram negative bacteria i.e., *S.epidermidis* and *Streptococcus A.* that are responsible in food deterioration, using Mueller Hinton agar at different concentration of CuNPs. The results revealed a greater bactericidal effectiveness for nanocomposite films containing 0.15mol/L of $CuSO_4$ solution. Packages prepared from Cts/Cu nanocomposite films were used for meat packaging. The films were filled with meat and then stored at 4 ºC. Microbial stability of the meat was evaluated after 3, 7, 10 and 15 days of storage. The results showed that microbial growth rate significantly reduced as a result of using this nanocomposite packaging material.

Keywords: *Active Packaging, Antibacterial, Biodegradable, Bionanocomposite, Chitosan*.

Introduction

A coordinated system of preparing foods or drugs and their packaging is necessary for transport, distribution, storage and retailing. Preservation of foods and drugs against microorganism, oxygen, light and water vapor influence, poses major challenges in new active scavenging packaging to shelf life extension and increase the quality. Antibacterial packaging is a complimentary method to the existing preservation methods to control the undesirable microorganisms in packages. Controlled migrations of the active compound from the packaging material into the food or drug enable not only the initial inhibition of undesirable microorganism's presence, but also create a residual activity over time, during transport, storage and distribution.

Many technologies were improved to produce biodegradable packaging material, which can be consumed by microorganisms and return to compounds found in nature. Approximated time for plastic bags and soft plastics to biodegrade in marine environment is more than twenty years.

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Biodegradable waste can often be used for composting and may well be a resource for heat, electricity and fuel.

Natural antimicrobial agents are good candidates for active packaging which occur in nature or isolated from microbial, plant, or animal sources. Chitosan is an example of natural antimicrobial polymer obtained by deacetylation of chitin obtained commercially from shrimp and crabshell. Chitosan is cellulose-based natural polymers with advantages including edibility, biocompatibility, barrier properties, attractive appearance, non-toxicity, non-polluting and low cost (Imran *et al*., 2010). It has been extensively investigated for many different applications due to its excellent bioactivity, biodegradability, and multifunctional groups, as well as its solubility in aqueous medium and being as an excellent barrier to oxygen. Consequently, they have a variety of current and potential applications in biomedical products, food packaging film, bone substitutes, and artificial skin (Darder *et al*., 2005).

Nanomaterials as a chemical antibacterial agents offer antimicrobial properties due to their high surface area/volume ratio. Gold and silver nanoparticles are the most widely studied particles. Different inorganic antibacterial materials, including Au and Ag nanoparticles have been expanded and some are in commercial use (Kawashita *et al*., 2000). In recent years, copper nanoparticles (CuNPs) have attracted great interest due to their potential applications, such as, conductive films, lubrication, nanofluids, ink, metallic coatings and catalysis (Tilaki *et al*., 2007; Zhu *et al*., 2005; Larsen *et al*., 2004; Wang *et al*., 2004; Patel *et al*., 2005). The antimicrobial activity of CuNPs is a new features issued against different microorganism (SCI Finder; Saniusman *et al*., 2013). Since the copper has been known as an antibacterial agent (Faundez *et al.*, 2004; Mary *et al*., 2009), higher disinfecting effects are expected in nano scales. However, no study has been conducted on the application of the CuNPs for food packaging.

Development and design of an efficient antimicrobial packaging requires an interdisciplinary approach which involves advances in food technology, microbiology, biotechnology, chemistry and packaging and material sciences. As well as an appropriate incorporation method should be developed according to the selected antimicrobial agent and food or drug application. Several silver-ion containing zeolite or glass systems have been incorporated into many polymers, such as polyethylene, polypropylene, and polyamide and become commercially available (Chaudhry *et al*., 2008).

Regarding the importance of production of natural antibacterial and biodegradable material for food packaging, an interdisciplinary approach was developed. For this purpose, the spherical structure of copper nanoparticle with an antimicrobial activity and biocompatibility incorporated in chitosan as a natural biodegradable polymer was synthesized using simple and low cost method and at room temperature. The capability of the prepared Cu/Cts. NBCs film was evaluated for antibacterial active packaging. The antibacterial, mechanical and chemical properties for food packaging were investigated. Investigation concerned with the shelf life of meat in this packaging was evaluated during the time of storage.

Materials and Methods

- *Chemicals*

Copper sulphate (99.98), was obtained from Merck chemical company (Darmstadt, Germany) and was used as copper precursor.$NaBH_4$ (98.5%), low-molecular-weight chitosan and glacial acetic acid (99%) were obtained from Sigma-Aldrich (St Louis, MO).

Gram positive: *Streptococus A.* (ATCC 19615), *S.epidermidis* (ATCC 12228), *S.aureus* (ATCC 25922) *B.cereus* (ATCC 11788), bacteria used for antibacterial assay were obtained from the BaharAfshan Ltd, Iran.

- Synthesis of (CuNPs)/Chitosan film

For the synthesis of Cu/Cts BNCs, chitosan solution (100 mL, 0.5 wt%) was prepared by solubilization in 1.0 wt% of acetic acid solution (pH ~3.53) under constant stirring for 90 minutes. For synthesis of CuNPs, $CuSO_4$solutions were added to each soluble Cts sample under constant stirring. The aqueous Cts-acetic acid solution thickened after the addition of $CuSO_4$ solution. The $CuSO_4$ concentration was 0.015, 0.03, 0.06and 0.15(mol/L) in the samples. Freshly prepared $NaBH_4$ (4×10^{-2} M) solution was then added to the suspensions under continuous stirring to reach a constant $CuSO_4/NaBH_4$molar ratio (1:4). After the addition of the reducing agent, stirring was continued for another hour. The suspensions of Cu/Cts BNCs were kept closed during 4 hr to prevent micro bubble formation, then centrifuged and washed four times using double-distilled water to remove the copper ion residue. Films were obtained at a wet thickness of 0.5 mm (measured by caliper) using casting bars and the plates were placed on a leveled surface at room temperature and let to dry for 25 hr. After drying, the films were removed and conditioned in sealed plastic bags, stored at room temperature.

- Evaluation of antibacterial activity

The in vitro antibacterial activity of the samples was evaluated by the well diffusion method using Mueller Hinton agar with determination of diameter of the inhibition zone formed around the well, which conformed to the recommended standards of the National Committee for Clinical Laboratory Standards (NCCLS, 2000). Petriplates containing 20ml Muller Hinton medium were seeded with 24hr culture of bacterial strains. Wells were cut and 20 µl of the BNC solution were added. The plates were then incubated at 37°C for 24 hours. Streptomycin antibiotic was used as a positive control and Dimethyl sulfoxide was used as a negative control.

Minimum inhibitory concentration (MIC) was determined for synthesized Cts/CuNPs solution showing antimicrobial activity against test bacteria by serial dilution method. Broth microdilution method was followed for determination of MIC values. 1ml of media was taken in a test tube, then 1ml of test solution (100µg/ml) was added. Thereafter, 0.1ml of the microbial strain (bacterial) prepared in 0.9% NaCl was added to the test tube containing media and test solution. Serial dilutions were carried out eight times (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 ml). The test tubes were stoppard and incubated at 37^oC for 24hrs. The MIC values were taken as the lowest concentration of the BNC in the test tube that showed noturbidity after incubation. The turbidity of the contents in the test tube was interpreted as visible growth of microorganisms. The minimum bacterial concentration (MBC) was determined by subculturing 50µl from each test tube showing no apparent growth. Least concentration of test substance showing no visible growth on subculturing was taken as MBC.

- Characterization methods and instrument

The structures of the Cu/Cts BNCs produced were examined by powder X-ray diffraction using the Siemens-D5000-1. Scanning electron microscopy (SEM)and energy dispersive x-ray (EDX) analysis were performed using the Jeol JXA-840 instrument attached with EDX to study the morphology and elemental composition of the film. The water vapor permeability (WVP) was determined using a Labthink WVP system to determine the relative humidity (RH) at the film underside and consequently water vapor permeability according to Angles (Angles *et al.*, 2000). For this purpose, three samples from each formulation were chosen and allowed to dry for 24 hours. Then cut into (23× 23× 0.1mm) diameter. After initial weighting, samples moved to desicator at 25°C with 75% relative humidity. Weights were taken periodically after steady state was achieved and used to calculate the percentage of RH at the film underside using the following relation:

$$RH(\%) = \frac{W_t - W_0}{W_0} \times 100 \quad (1)$$

Where W_0 is initial weight of the sample and W_t is the weight after t seconds. The mechanical properties of the composites were evaluated in rectangular pieces of the film with dimensions chosen in accordance to ASTM (1997) and conditioned at 24°C for 48 hr before measurements. An INSTRON tension test instruments was used to determine the maximum tensile strength (TS) and elongation at break. Films were stretched up using a speed of 5 mm min^{-1}. Tensile properties were calculated from the plot of stress (tensile force/initial cross-sectional area) versus strain (extension as a fraction of the original length). The mechanical properties were analyzed as a function of nanoparticles ratio by weight in the film.

- *Preparation and storage of meat in packages*

To prepare minced sample, 300 g of fresh fatty beef meat were purchased from the local market in Tabriz, Iran. They were grinded using a semi-industrial meat grinder (Buffalo CB943 Meat Grinder**,** China), thoroughly washed with detergent and hot water. Mincing was repeated two times for perfect mixing of fat and meat. The minced was immediately transferred into a sterile glass container under sanitized conditions, sterilized in an autoclave at 121 ºC for 15 min and refrigerated at 4 ºC until processed. Packages were prepared by a hand heat sealer using Cu/Cts BNC films and pure chitosan films 15 × 10 cm in size. The packages were immediately wrapped in aluminum foil and sanitized at 95 ºC for 2 min. After cooling and under a sterile laboratory hood, 10 g of minced sample was poured into each package and sealed by the heat sealer. Packages containing minced samples were stored in dark and cool conditions (4 ºC) and microbial counts of the samples were evaluated in duplicate order by pour plate method using MRS agar and incubation for 48 h at 37 ºC under CO_2 atmosphere (5%), immediately after packaging and after 3, 7, 10 and 15 days of storing.

Results and Discussion

- *X-ray diffraction analysis*

Figure 1 presents the X-ray diffraction pattern of Cu/Chitosan BNCs and pure Chitosan films. The X-ray diffraction pattern of the chitosan showed a broad scattering peak which indicated its highly amorphous structure and arise from its chains close to the interplanar Vander Waals distance for aromatic groups (Mitchell *et al*, 1987; Kassim *et al.*, 1992).

The Miller indices are shown above the diffractions. The diffraction patterns for Cu/Chitosan BNCs composite showed peaks of CuNPs (JCPDS file 004-0873) phase corresponding to (111), (200) and (220) crystallographic planes of the face-centered cubic copper crystals, respectively. For all the samples, the main crystalline phase was copper, and no other obvious phases were found as impurities in the XRD patterns. The intensities of (111), (200) and (220) reflections due to the CuNP phase, were also found to increase along with the increased CuNPs in the solid support matrix.

The crystallite size of CuNPs incorporated in the Cu/Cts BNCs film was calculated by using Scherrer's formula for the (111) peak at 2θ of 43.37ºand was found to be 30 nm (Ebrahimiasl *et al*., 2010).

- Morphology and composition

The surface morphology of CuNPs/Cts film is evaluated by SEM. Figure 2 shows the SEM image of the Cu/Cts BNCs. It is observed that the films are uniform and the nanoparticles are homogeneously distributed in the polymer matrix. The EDX spectra for the Cu/Cts BNCare shown in Figure 2. The peaks at around 0.3 and 0.5keV are related to the binding energies of carbon and oxygen in chitosan structure. The peak around 0.95keVis related to Cu^{2+} element (Nasimul Alam *et al*., 2013). Therefore, the EDX spectra and composition (Table 1) of the Cu/Cts BNCs confirmed the presence of elemental compounds in the film without any impurity peaks. The intensity of the peaks is in agreement with elemental composition of synthesized BNC.

- Mechanical analysis

Mechanical stability of the film during processing and flexibility during shipping and handling is the main characteristic of the film used in packaging. Tensile strength, elastic modulus, and elongation at break analyses describe how the mechanical properties of such materials relate their chemical structures (Ebrahimiasl *et al*., 2010). Figure 3 shows the tensile strength of the Cu/Cts BNCs in different concentration of $CuSO_4$. The result revealed that the mechanical stability of the film increase by $CuSO_4$ concentration. Elongation at break for the film was presented at Figure 4.

Elongation of the film in the break time is related to its elasticity. It is observed that elongation at the break time and consequently the elasticity of the film, as well, increase by $CuSO_4$concentration. The incorporation of CuNPs in polymer matrix increased the mechanical resistance and elasticity of the film for all concentrations. This is due to increase interaction of the nanoparticles and polymer chain and intermolecular bonding accordingly (Ebrahimiasl *et al*., 2015).

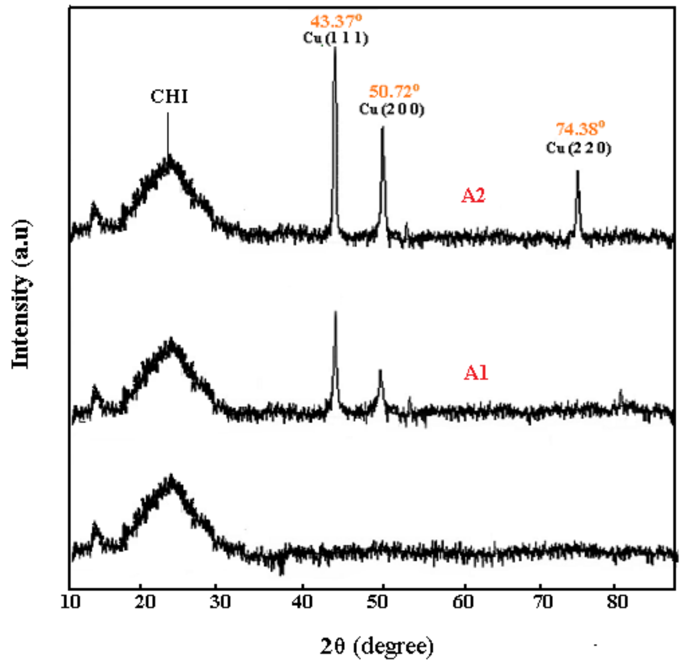
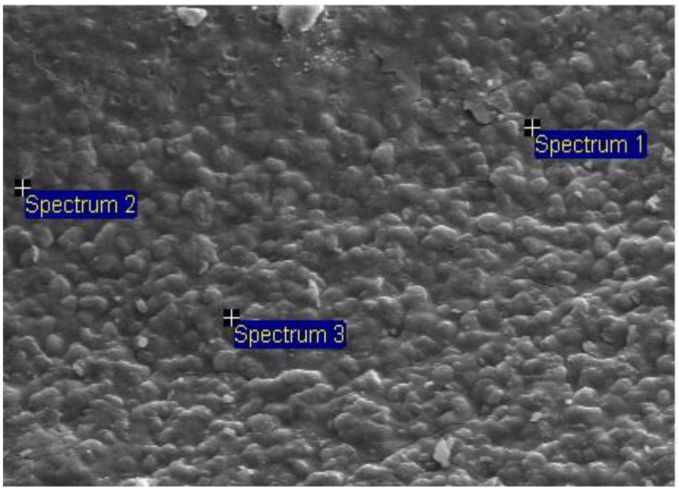


Fig. 1. Powder X-ray diffraction patterns of chitosan and copper/chitosan BNCs for determination of copper crystals at different $CuSO_4$ concentrations: (A1) 0.06mol/L, (A2) 0.15mol/L.



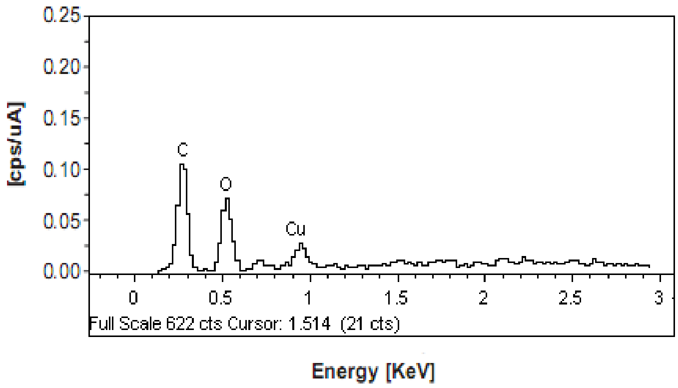


Fig. 2. Scanning electron microscopy micrograph and related EDX analysis for prepared CuNPs/ Cts BNCs film.

Table 1. EDX analysis result of the Cts/CuNPs film in different area

	C	O	Cu	Total
Spectrum 1	50.44	33.98	15.58	100
Spectrum 2	57.19	31.9	10.91	100
Spectrum 3	53.82	31.91	14.27	100
Mean	53.82	32.60	13.59	-
Std. deviation	3.37	1.20	2.41	-

- *Water vapor permeability*

Moisture is one the most important factors affecting food spoilage, with regard to the role of water in creating the conditions for enzymatic chemical and biological activities. Higher water activity in materials supports more microorganisms' growth. Bacteria usually require at least 0.91, and fungi 0.7 water activity to exist. For many years, researchers tried to prevent bacterial growth with moisture content control. Consequently, a need developed for information on the water resistant characteristics of many packaging materials in use. The hydrophilic natures of polysaccharide films make them poor water barriers. The CuNPs effect on the relative humidity at film underside for the synthesized Cts/CuNPs BNC is shown in Figure 5 for different concentration of$CuSO_4$.

Upon addition of nanoparticles in chitosan matrix, a decrease in the WVP values and relative humidity at film underside was observed. The RH value was 98% for the native chitosan film. The addition of nanoparticles induced a decrease in WVP values and RH at film underside. The RH values and WVP varied from 93 ± 0.9%and 76 ± 0.1% for chitosan films containing 0.015 (mol/L) $CuSO_4$ to 75 ± 0.9 and 62± 0.5 % for films containing 0.15 (mol/L) $CuSO_4$ respectively.The presence of nanoparticles reduced the intermolecular spacing within the films and increase in cohesion structures, thus reducing the water vapor permeability through films.

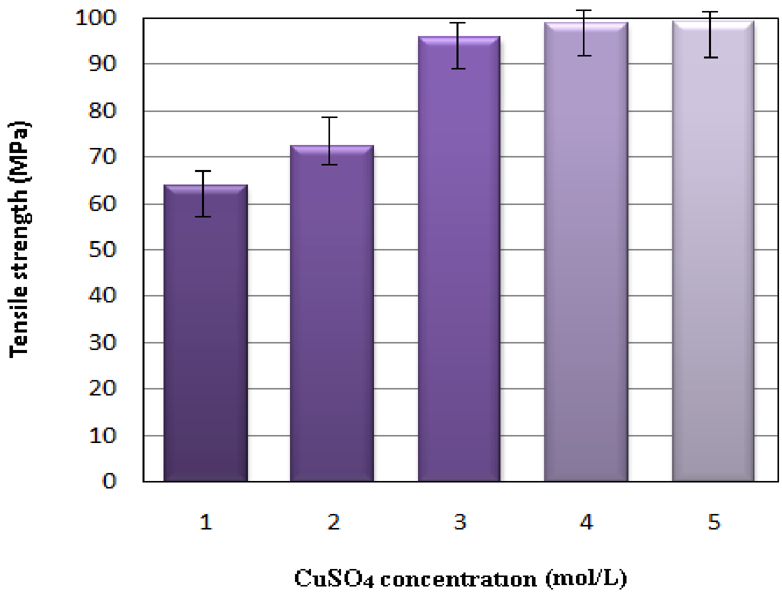


Fig. 3. Tensile strength of net chitosan films, and chitosan films with different concentration of $CuSO_4$.

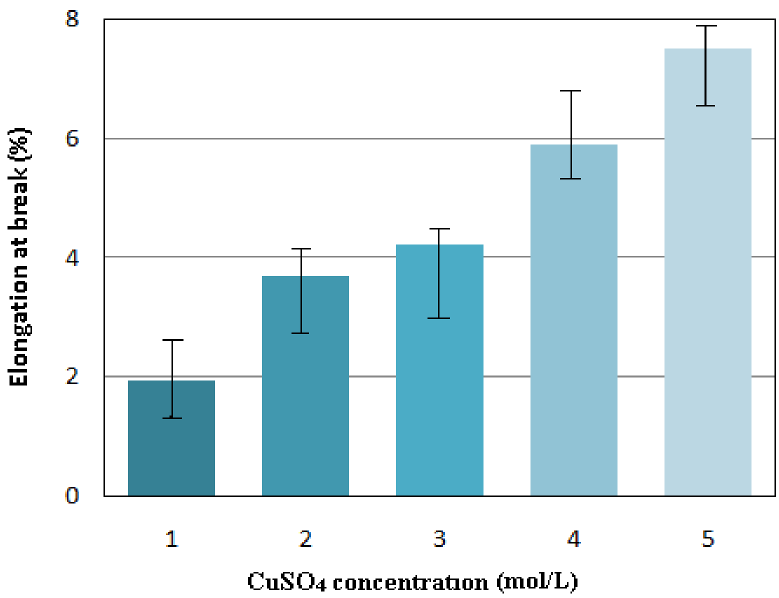
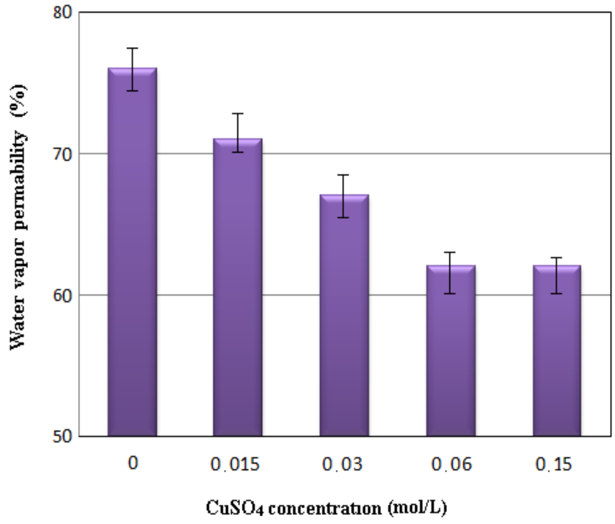


Fig. 4. Elongation at break for net chitosan films, and chitosan films with different concentration of $CuSO_4$



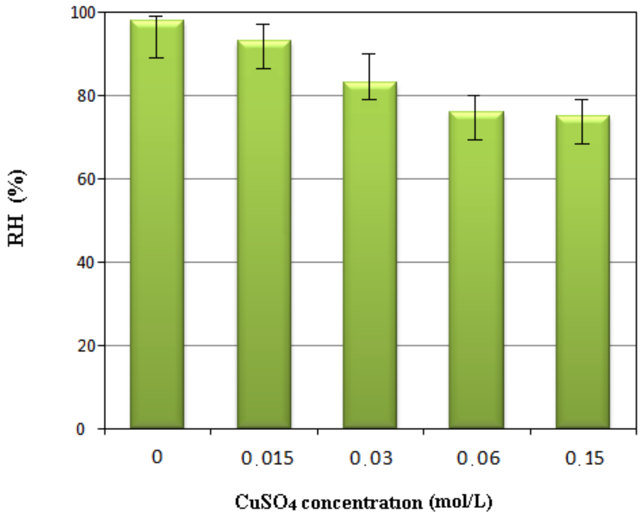


Fig. 5. Relative humidity at film underside and water vapor permeability of Cts/CuNPs BNCs in different concentration of $CuSO_4$.

- *Antibacterial activity*

B.cereus and *Streptococcus* can cause the deterioration of meats and variety of foods, particularly rice and leftovers, as well as sauces, soups, pigs and other prepared foods. Therefore the inhibitory effect of the packaging film against this bacterial, could be effective in prolong time of the food safety. Inhibition zone values were obtained for the synthesized Cts/CuNPsBNCs tested against gram positive bacteria listed in Table 2. The tests were repeated three times for each treated sample, and are presented as average values in Table 2 and Figure6, respectively. The result shows that the CuNPs in chitosan suspension had high antibacterial activity against gram-positive bacteria. The antibacterial activity increased by the concentration of $CuSO_4$solution. Several mechanisms argue for the antibacterial activity of nanoparticles, including generation of oxygen species to degradation of cell structure or release of ions from the surface of nanoparticles to binding cell membrane (Ninnemann *et al*., 1968; Lee *et al*., 2005; Lok *et al*., 2006; Sawai *et al*., 2003). The result reinforces the idea that the lipopolysaccharide protein in

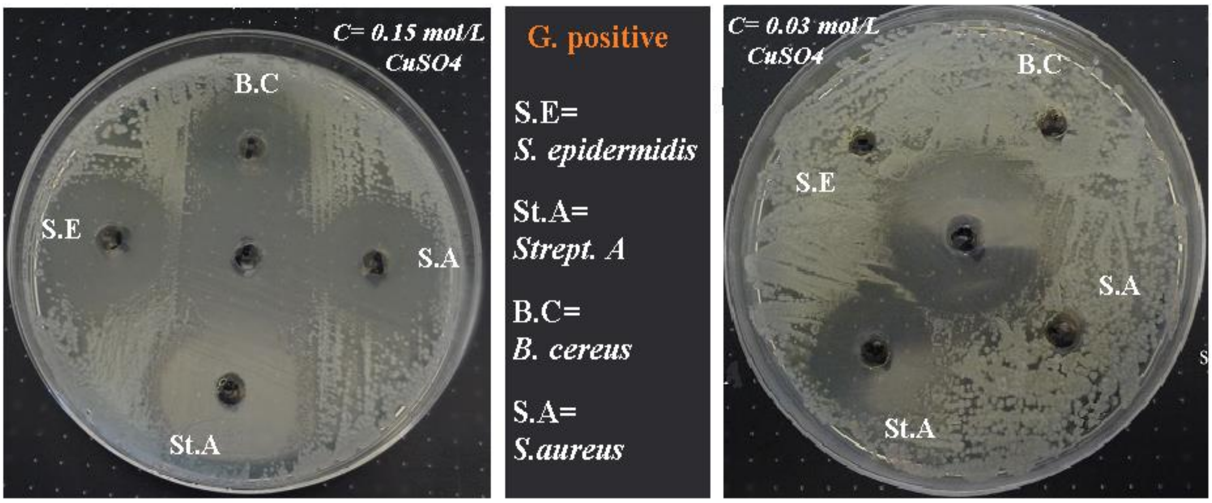


Fig. 6. The antibacterial activity of Cts/Cu NPsBNC at different concentration of $CuSO_4$ evaluated by well diffusion method.

Table 2. Average inhibition zone and MIC & MBC values for Cts/Cu NPsBNCs tested against four bacteria.

Bacteria	Inhibition zone (mm)		Control positive (mm)	MIC	MBC
	Cts/CuNPs (0.015 mol/L)	Cts/CuNPs (0.15mol/L)	*SM*	(µg/ml)	(µg/ml)
S.aureus	NA	12.2 ± 0.061	21.2 ± 0.020	615	615
B.cereus	NA	9.3 ± 0.078	21.2 ± 0.058	931	931
S.epidermidis	NA	15.3 ± 0.141	29.1 ± 0.045	229	448
Strept A	12.6 ± 0.030	21.1 ± 0.043	29.1 ± 0.028	154	154

NA=not appear

the cell wall of bacteria is the reason for different antibacterial effect (Yoon *et al*., 2007). The interaction of CuNPs with the sulfhydryl groups (C-SH) in the cell wall, derange cellular respiration and due to cell death (Siva Kumar *et al*., 2004).

Bacteriostatic (MIC) and bactericidal activity (MBC) of the synthesized BNC were presented in Table 2. MIC and MBC values of Cts/CuNPs solution were observed very low, indicating well bacteriostatic and bactericidal activity of the composite. The composite showed inhibitory activity against *Staphylococcus aureus*, *Bacillus cereus*, *S.epidermidis* and *Streptococcus A*. The least MIC value was observed against Strept. A (154 µg/ml) and the least MBC value was observed against Strept. A (154 µg/ml). MIC of ZnO against the three bacterias, Escherichia coli, Staphylococcus aureus and Bacillus subtilis were found to be 6.25µg/ml, 6.25µg/ml, 12.5 µg/ml, respectively (Singh *et al*., 2013). AgNPs stabilized by Tulsi leaf extract were found to have enhanced antimicrobial activity against well- known pathogenic strains, namely Staphylococcus aureus and E. coli (Ramteke *et al*., 2013).

With the regard to the excessive antibacterial effect of Cts/CuNPs film against gram positive bacteria that is responsible to deterioration of foods and clinical infections, it can be used as a substitute for commercial chemical antibiotics.

- *Effect of packaging on storage of meat*

The synthesized film was used for packaging of meat. Mean initial microbial population immediately after packaging was determined to be 3.7 log cfu/mL in minced samples. The variations in the microbial population during storage are shown in Figure 7. In all packages, the mean population increased after 3, 7 and 10 days of storage. According to Figure 7, the level of microbial population increased to 8.91 log cfu/mL after 7 days of storage in chitosan pure packages which is higher than that of Cu/Cts BNC 3% and 15%. In all the samples (0, 3 and 15% Cu NPs), any significant increase was not observed over 10 days of storage. The microbial population decreased with an increase in Cu NPs ratio from 3 to 15%. Copper ions released from the surface of these nanoparticles can interact with thiol groups in protein to induce bacterial inactivation, condensation of DNA molecules, and loss of their replication ability (Feng *et al*., 2000). Based on electron spin resonance (ESR) measurements, the antimicrobial mechanism of nanoparticles, are related to the formation of free radicals and the subsequent free radical-induced membrane damage (Kim *et al*., 2007).

Conclusion

Biodegradable Cts/CuNPsBNCs were successfully prepared at different $CuSO_4$ concentrations by using $NaBH_4$ as a chemical reducting agent without any heat treatment. The XRD analysis confirmed that the crystallographic planes of the copper crystals were of the face-centered cubic type. SEM and EDX results indicated that the external morphology of Cu/Cts BNCs is uniform and confirmed the presence of elemental compounds in the film without any impurity. Tensile strength (mechanical stability) and elongation at break (elasticity) of the synthesized Cu/Cts BNCs is increased by the presence of CuNPs to 99.1 ± 1.0 MPa and 75.2 ± 2.0 % respectively. Decreased values were observed in the WVP for Cts/CuNPs film, in comparison to the net chitosan film. The antibacterial activities of Cu/Cts BNCs at different concentrations of $CuSO_4$showed strong antibacterial activity against gram-positive bacteria. These results indicate that Cts/CuNPs composites can be used in packaging for certain bacterial inactivation and control. The prepared Cts/CuNP BNCs packages showed a significant antimicrobial activity as compared to pure chitosan in minced samples packaging.

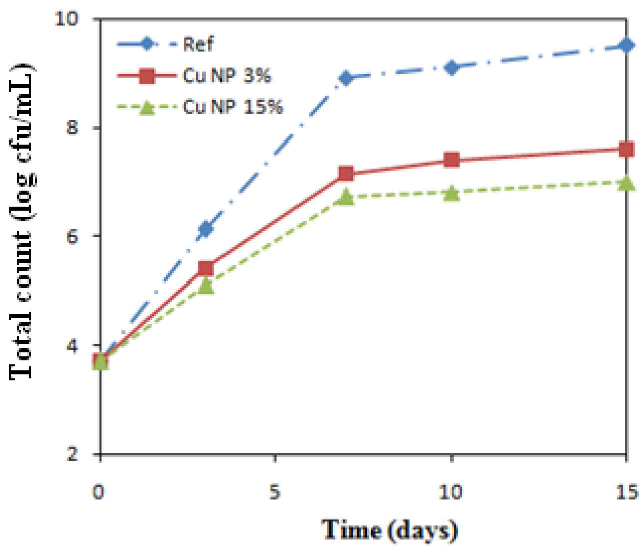


Fig. 7. The effect of packaging containing Cu NPs on the microbial population during 15 days of storage at 4 ºC.

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