

Development of Antibacterial Nano-cellulose – Chitosan Films Activated with Nisin for Food and Medicine Application

M. Mirhosseini^{1*}, and R. Dehghan²

ABSTRACT

In this research, biodegradable Chitosan–Nano-Cellulose–Nisin (CH-NC-N) film was synthesized and utilized for antibacterial application in medicine and food packaging. The antibacterial chitosan–nano-cellulose–nisin film was characterized using various techniques such as mechanical and physical properties analysis, Scanning Electron Microscopy (SEM), X-Ray Diffraction (XRD), and Fourier Transform Infrared Spectroscopy (FTIR) techniques. The film's ability to inhibit growth of pathogenic bacteria including *Escherichia coli*, *Escherichia coli* XDR, *Klebsiella pneumonia* XDR, *Listeria monocytogenes*, and *Staphylococcus aureus* was examined. Furthermore, the film was used for meat packaging at a temperature of 4°C for a duration of 26 days. Data analysis revealed an improvement in the mechanical properties and water absorption of the film following the addition of nano-cellulose and nisin. The presence of nisin in the CH-CN film was confirmed through analysis of FTIR, XRD, and SEM data. Antimicrobial analysis of film determined the high potential of nisin as an antimicrobial agent in CH-CN-N film. Compared to the control, the CH-CN-N film successfully inhibited the growth of spoilage bacteria in meat for 26 days. Additionally, the sensory properties of meat packaged with this film were minimally affected. These results indicate that the chitosan-nano-cellulose-nisin film is suitable for utilization in food systems and medical applications.

Keywords: Antibacterial activity, Biocomposite, Food packaging, Shelf life of food, Spoiled meat.

INTRODUCTION

Preventing contamination by microorganisms and increasing the shelf life of food have become an essential issue due to the increase in world population and the globalization of the food market (Pirsa and Asadi, 2021). The utilization of spoiled meat or canned food endangers human health and can lead to hospitalization or even death. Antimicrobial packaging has attracted much interest among scientists and industry because of its capacity to prevent the growth of microorganisms on the surface of food products. However, directly applying

antimicrobials to the surface of food could help limit the effects caused by the movement of effective ingredients into the food tissue. A severe lack of antimicrobial action might also appear because of the reaction or deactivation of the effective ingredients by food ingredients (Huq *et al.*, 2014; Khan *et al.*, 2016). Bioactive technology offers another resolution to limit bacterial growth in food products. In this type of packaging, inhibition of bacterial growth is possible by the targeted release of bioactive ingredients from the film substance to the food product overlays. The controlled released packaging is a novel group of effective ingredients that develop food safety and

¹ Department of Biology, Payame Noor University, Tehran, Islamic Republic of Iran.

² Department of Biology, Science and Research Branch Islamic Azad University, Tehran, Islamic Republic of Iran.

*Corresponding author; e-mail: m.mirhosseini@pnu.ac.ir



quality throughout storage (Salmieri *et al.*, 2014). The synthetic polymers are utilized in food packaging because of their exceptional thermal-mechanical, economic, and protective barrier properties. Today, it is known that synthetic polymers used in food packaging have compounds that are very dangerous for human health and the environment. Synthetic polymers cause serious damage to the environment due to their long decomposition time. Therefore, a serious problem in the world is the wide use of synthetic polymers in food packaging (Dehnad *et al.*, 2014a). However, the interest in using biodegradable biopolymers is expanding, especially in food packaging (Pirsa, 2021). However, application of natural polymers in food packaging is limited due to their inadequate mechanical and barrier properties. Therefore, degradable natural polymers are in high demand for fillers that enhance their mechanical and barrier properties. They also need bioactive ingredient that increase their antibacterial properties (Baysal *et al.*, 2023; Meydanju *et al.*, 2022; Pirsa and Mohammadi, 2021). Additionally, due to increasing consumer demand for the use of renewable materials in the production of biodegradable packaging films, many researchers in textile chemistry and fiber science have begun replacing petroleum-derived synthetic materials with environmentally friendly alternatives. (Meydanju *et al.*, 2022; Shabkhiz *et al.*, 2021). Bio-nanocomposites can be introduced as a new group of films containing a polymer matrix strengthened with nanofiber. Nano-cellulose reinforced films have attracted much interest today due to their great capacity in food packaging and their renewable nature (Lu *et al.*, 2021; Pirsa and Mohammadi, 2021). Chitosan, is the most abundant polymer after cellulose in nature. Chitosan, because of its biocompatibility, degradability, and harmless properties, has been used in food packaging (Baysal *et al.*, 2022; Khan *et al.*, 2016; Khan *et al.*, 2012). Nisin is a bacteriocin generated by *Lactococcus lactis* strains and is generally regarded as a safe substance by the USA FDA (Gedarawatte *et al.*, 2021; Huq *et al.*, 2014; Khan *et al.*, 2016). The present study aimed to

provide the antimicrobial film of CH-NC-N for the first time. Loading CH-NC by nisin can prepare antimicrobial active CH-NC. The mechanical and wettability characteristics of the film were investigated. The film was characterized by FTIR, XRD, and SEM. The antimicrobial activity was evaluated against spoilage and pathogenic bacteria.

MATERIALS AND METHODS

Nano-Cellulose Synthesis

Nano-cellulose was prepared by acid hydrolysis from microcrystal cellulose (MCC, Sigma-Aldrich, USA). Quickly, sulfuric acid solution (95-97% Merck, Germany) (64 v/v%) was added to the aqueous MCC suspension (10 g 100 mL⁻¹) and kept at 44°C for two hours. The resulting suspension was centrifuged at 14,000 rpm for 15 minutes. The precipitate was then washed several times with distilled water to neutralize its acidity, and then sonicated (Chrom Tech Ultrasonic Processor, Korea) for 30 min in an ice bath and dried with a freeze dryer (Celebi and Kurt, 2015; García *et al.*, 2017). The nano-cellulose was characterized by XRD (Bruker, D8 Advance, Germany), FTIR (Bruker, Tensor27, Germany), and Dynamic light scattering (DLS) (Brookhaven, 90plus/BI-MAS USA) techniques.

Antimicrobial Films Synthesis

Chitosan (medium molecular weight, deacetylation degree 75–85%, Sigma-Aldrich, USA), solution with different concentrations (1, 1.5, and 2%, w/v) in 1% acetic acid (100% Merck, Germany) was prepared for 24 h. Different concentrations of NCs (0.5, 0.65, 0.8, and 1 w/v%) were added to the chitosan solution and mixed for 30 min. Then, this solution was sonicated in an ice bath for 15 min. Finally, 0.6% (v/v) glycerol was added to the solutions and homogenized according to the above-mentioned conditions. Then, the solutions were poured into glass plates and

dried at 30 °C for 20 h. Finally, after several times optimization- the film with 1.5% (w/v) chitosan, 0.65 (w/v) nano-cellulose, and 0.6% (v/v) glycerol (Merck, Germany) were selected for antimicrobial films synthesis (Celebi and Kurt, 2015; Dehnad *et al.*, 2014a; Dehnad *et al.*, 2014b). Nisin (Sigma–Aldrich, USA)–EDTA (Merck, Germany) mixture (60 or 30 $\mu\text{g mL}^{-1}$ of nisin, and 30 mM of disodium ethylene diamine tetraacetate) was added to the CH-NC solution and homogenized according to the above-mentioned conditions. Then, the solutions were poured into glass plates and dried at 30 °C for 20 h (Celebi and Kurt, 2015; Dehnad *et al.*, 2014b).

Nanocomposite Film Characterization

SEM images of film surfaces were obtained with a MIRA III Tescan (Czechia) microscope. The samples were covered by a thin gold layer, then, their images were determined at an accelerating voltage of 10 to 20 kV at different magnifications. The X-Ray pattern extents were prepared with a Philips PW1730 diffractometer by Cu K_α radiation. The organic groups on the nanocomposite films were determined by FTIR-ATR (Fernandes *et al.*, 2010).

Air–Water Contact Angle Investigation

A drop of deionized water was placed on the surface of the films. Water droplet images on the surface of films were recorded by a digital camera (Sony, Model F707). The images were managed with Adobe Photoshop 6.0 software to gain contact angle information (Leceta *et al.*, 2013).

Mechanical Characteristics

The film mechanical characteristics were determined by a Sherli Micro350 (UK) under the ASTM manner D882. Primary grip departure and crosshead speed were adjusted at 50 mm and 5 mm/min,

respectively. Young's Modulus (YM), and Tensile Strength (TS) were determined from curves of Stress-Strain (Szymańska-Chargot *et al.*, 2019).

Antibacterial Activity

The agar diffusion procedure was applied for defining antibacterial activity. One-centimeter squares (1 cm^2) were prepared from the films. Films were disposed on TSA (Tryptic Soy Agar, Merck, Germany), which was previously inoculated separately with 10^6 CFU mL^{-1} of *Escherichia coli* PTCC1394, *Staphylococcus aureus* PTCC1431, *Listeria monocytogenes* PTCC1074, *Escherichia coli* XDR, and *Klebsiella pneumonia* XDR (from Milad laboratory, Yazd, Iran), respectively. Plates took place for 24 hours at 37°C. The inhibition zone round samples were applied to determine the antimicrobial activity (Dehnad *et al.*, 2014a; Firouzabadi *et al.*, 2014). Also, CH-NC-N film was employed in a TSB (Tryptic Soy Broth, Merck, Germany) medium and inoculated separately with 10^7 CFU mL^{-1} of *L. monocytogenes*, *E. coli*, *S. aureus*, *E. coli* XDR, and *K. pneumonia* XDR, respectively. The tubes were incubated at $25\pm 1^\circ\text{C}$ at a speed of 50 rpm. Samples (1 mL) were subsequently collected from the tested tubes over a period of 24 hours. Dilution series were prepared from these samples (10^{-1} to 10^{-9}) and cultured on TSA plates 37°C for 24 hours (Mirhosseini and Afzali, 2016).

Shelf-Life Investigation in Raw Meat

Freshly slaughtered meat was purchased from a butcher near Payam Noor University (Yazd, Iran). The meat sample was disinfected by immersing it in 70% ethanol and passing it through a flame. The 10 gr slabs of purified meat sample were wholly packaged with CH, CH-NC, CH-NC-N, or nylon films. The packaged meat samples were then placed on separate plates and



stored for 26 days at 4°C. A physical and microbiological investigations were performed on days 1, 3, 5, 13, 20, and 26. Physical assessment of the meat was accomplished to evaluate its shelf-life operating a standardized method for color and odor. The meat samples were managed for bacterial counts using a standard method. One gram of sterile packaged meat was removed each time and placed in a 400 mL homogenization package containing 9 mL of 0.1% (w/v) bacto-peptone (Merck, Germany) and massaged with a Stomacher 400 laboratory blender (Seward, UK) at high speed for 120 sec. Suitable 10-fold dilutions of the homogenate were spread plated onto TSA and MRSA (DE MAN, ROGOSA, and SHARPE Agar, Merck, Germany), for enumeration of the whole bacteria and Lactic Acid Bacteria (LAB). Then, plates were incubated at 25°C for 72 hours before counting bacteria (Dehnad *et al.*, 2014b; Mirhosseini and Arjmand, 2014).

Statistical Analysis

All experiments were accomplished in triplicate. Data presented are the average of three replicates. The information were exposed to one-way Analysis Of Variant

(ANOVA) by an SPSS software (SPSS Statistic 19.0). Post hoc multiple comparisons were defined by the Tukey's test with the significance level set at $P < 0.05$. SigmaPlot 12.3 and Excel 2017 software were used to draw curves and charts.

RESULTS

Nano-Cellulose Characteristics

The size of NCs was evaluated by DLS analysis. Data analysis showed that the particle size ranged from 16.1-101.2 nm, and the mean particle size was 40.76 nm (Figure 1). The XRD pattern of NC displayed peaks about nearby $2\theta = 15, 16.5,$ and 22.5° designated as the crystal planes with Miller indications of -110, 110, 200, which signify the standard cellulose-I construction (Figure 2) (Celebi and Kurt, 2015). The FTIR spectra of C and NC presented an extensive band in the area $3,500-3,200 \text{ cm}^{-1}$ that illustrated the free "O-H" stretching vibration of the "OH" groups in cellulose molecules. In addition, the spectra result of all samples indicated the specific "C-H" stretching vibration around $2,894 \text{ cm}^{-1}$

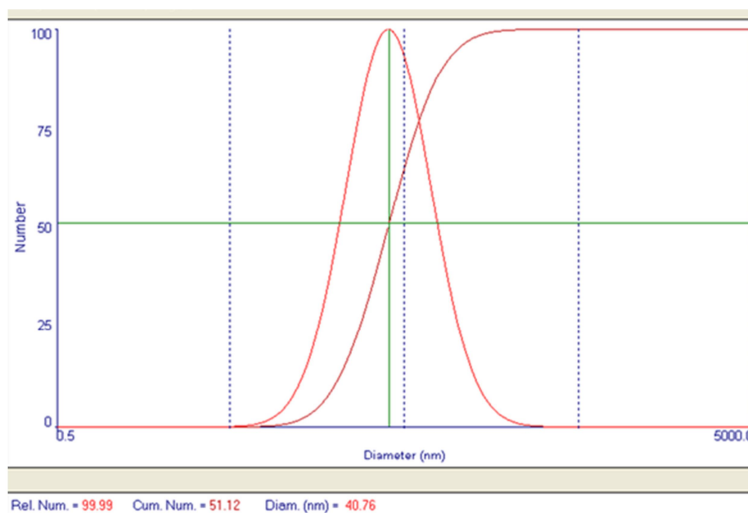


Figure 1. Light scattering diagram of nano-cellulose particles.

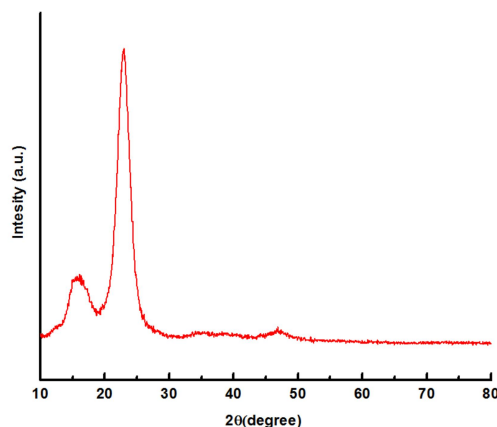


Figure 2. Investigation of X-Ray diffraction pattern of nano-celluloses.

(Celebi and Kurt, 2015; Khalil *et al.*, 2001). Moreover, the maximum vibration observed at $1,365\text{ cm}^{-1}$ in all samples originated from the "C–O" and "C–H" bond bending vibration in the polysaccharide aromatic rings (Celebi and Kurt, 2015; Le Troedec *et al.*, 2008). The peak studied in the spectra of total samples located at the $1,054\text{ cm}^{-1}$ point is because of the "C–O–C" stretching vibration in the pyranose ring. The greatest big absorption band that frequently enhances NC is that of 902 cm^{-1} , which withstands cellulose II, the matter of which gradually enhances from cellulose to nano-cellulose (Pappas *et al.*, 2002). The "C–O–C" glycosidic ether band at $1,105\text{ cm}^{-1}$ and

"C–C" ring breathing band at $\sim 1,155\text{ cm}^{-1}$ of them result from the polysaccharide constituent is becoming progressively missing in nano-cellulose due to hydrolysis, and decreasing in the molecular weight (Figure 3) (Garside and Wyeth, 2003).

Chitosan/Nano-cellulose/Nisin Film Characterization

The XRD spectra of CH and CH-NC and CH-NC-N films presented typical sharp peaks at around $2\theta = 8^\circ$ and $2\theta = 11^\circ$ and a broad halo at $2\theta = 20^\circ$. The two sharp peaks showed a hydrous crystalline construction,

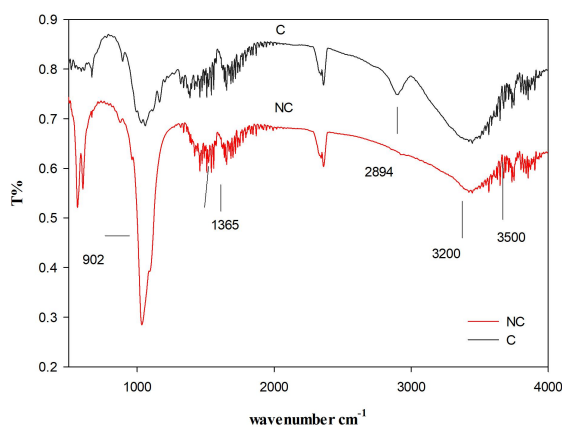


Figure 3. Infrared spectroscopy of cellulose and cellulose nanoparticles.

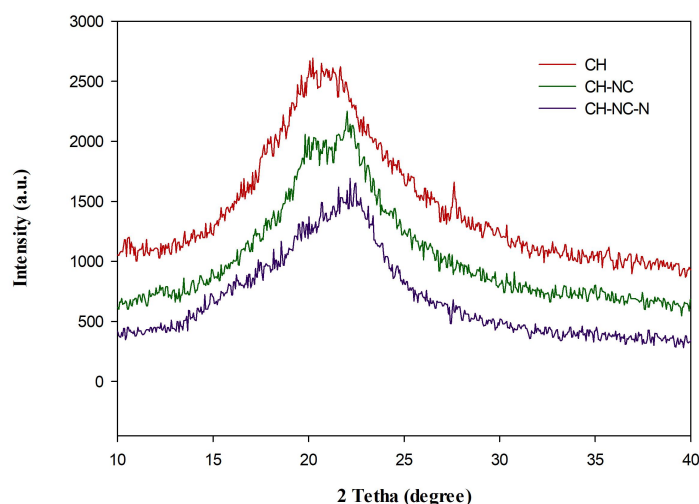


Figure 4. XRD photographs for chitosan–nano-cellulose- nisin biocomposite.

while the broad halo peak designated an amorphous construction of chitosan (Dehnad *et al.*, 2014a). The diffraction diagram for NC displayed three significant peaks at $2\theta = 15$, 16.5, and 22.5 degrees, which were assigned to [101], [101], and [002] atomic planes of cellulose I, respectively. The typical peak of NC at $2\theta = 22.5^\circ$ showed on the XRD pattern of CH-NC and CH-NC-N nanocomposite films. Also, the peak strength amplifies with the addition of NC in the film as a result of the trans-crystallization effect, which is described as the orientation of crystals of a semicrystalline matrix perpendicularly to the nano-celluloses (Celebi and Kurt, 2015; Dehnad *et al.*, 2014b). When, the chitosan peak is compared with the nanocomposite film peak, it could be observed that adding NCs and nisin replaced the situation of the chitosan peak to the more significant positions (nearby to the bounds of the NC peak), but reduced the peak strength and the spaces among the sheets (Figure 4) (Dehnad *et al.*, 2014b).

The FTIR pattern of the CH films is essentially transferrable to the stretching of intra- and intermolecular "OH" and "CH₂OH" vibrations at "3,500–3,250 cm⁻¹", overlaid

with stretching "NH₂" (3,500–3,400 cm⁻¹) and "NH" secondary amides vibrations (3,300–3,280 cm⁻¹). Also, "2,960–2,870" cm⁻¹ agrees to asymmetric and symmetric "CH" vibrations (Khan *et al.*, 2012). Amide I's vibrational peak at "1633" cm⁻¹ and, moreover, amide II at "1,538" cm⁻¹ have been detected. Due to the incorporation of NC into the chitosan, particular alterations can be noticed in the FTIR pattern of CH films. An intense peak showed at "3,342" cm⁻¹, not displayed in the control CH films. Moreover, the strength of the peak "3,342" cm⁻¹ raised hydrogen bonding between CH and NC. Other bands at "1,538" and "1,340" cm⁻¹ had their intensity increased after NC addition. Moreover, there was an extreme amplification in the strength of the absorption peaks at "1,054" and "1,032" cm⁻¹ because of NC integration. Nevertheless, other variations created by the supplement of NC are minimal, as guessed from the small quantity of NC combined to formulate the CH-NC films (Khan *et al.*, 2012). Several changes can be identified in the full FTIR spectrum after adding nisin to the CH-NC film. The absorption peak was observed at "3,150–3,450" cm⁻¹, following the addition of nisin. This peak is due to the "OH" band of the peptides. Other bands seen with the addition of

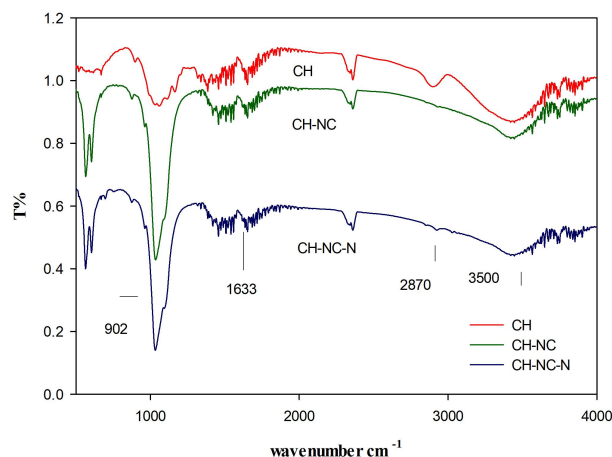


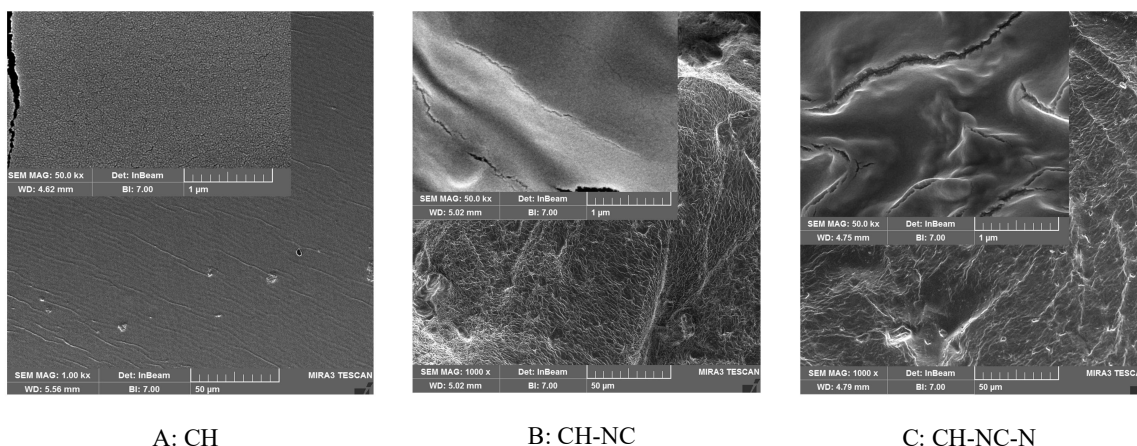
Figure 5. Infrared spectroscopy of Chitosan (CH), Chitosan-Nano-Cellulose (CH-NC), and Chitosan-Nano-Cellulose–Nisin (CH-NC-N) films.

nisin are: at "1,656", and "1,537" cm^{-1} , which are mainly related to the amide I and amide II bands, respectively, and both are related to the peptide bond (Figure 5) (Salmieri *et al.*, 2014).

The chitosan SEM image (Figure 6-a) indicates a relatively smooth surface without phase separation, signifying complete chitosan dissolution in the acidic solution. By adding nano-cellulose to the chitosan film, NCs shows as white areas in the CH film. The nanocomposite film has shown a dense and uniform structure, which is indicative of proper distribution and uniform

distribution of nano-celluloses. It also shows strong adhesion between nano-celluloses and chitosan in the nanocomposite film (Figure 6-b) (Celebi and Kurt, 2015). With the addition of nisin to nanocomposite films, the structure of the film has become more uniform and homogeneous (Figure 6-c).

Data analysis showed that the contact angle was around 86.16 for chitosan film. However, with the addition of NC, the value of the contact angle (81.71) was reduced. The addition of nisin to the nanocomposite reduced the contact angle (76.88) (Figure 7).



A: CH

B: CH-NC

C: CH-NC-N

Figure 6. SEM of Chitosan (CH), Chitosan-Nano-Cellulose (CH-NC), and Chitosan-Nano-Cellulose–Nisin (CH-NC-N) films.

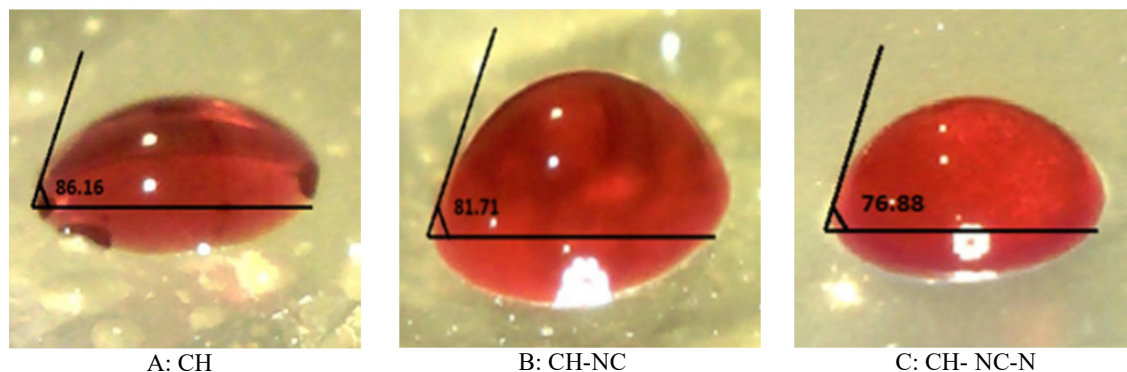


Figure 7. Contact angle values of Chitosan (CH), Chitosan-Nano-Cellulose (CH-NC), and Chitosan-Nano-Cellulose-Nisin (CH-NC-N) films.

Mechanical properties of films show that by applying a force of up to 56.67 N, the elongation to breakpoint reached 14.78 mm, and with more force, the film tore. Adding nano-cellulose and nisin to the chitosan film increases the tensile strength and length to a rupture point of 1.9 to 14.78 mm. The YM also increases from 2.178 to 39.172 MPa, indicating that the tensile strength of the CH-NC-N film is higher than that of the control film (Figure 8).

Antibacterial Assay in Medium

The results of the investigation of the antimicrobial property in the solid medium are presented in Table 1. Data analysis showed

that the films prepared with $30 \mu\text{g mL}^{-1}$ of nisin-nano-cellulose-chitosan had no antimicrobial effect on any of the tested bacteria. By increasing the nisin concentration to $60 \mu\text{g mL}^{-1}$, the nanocomposite film had an antimicrobial effect on all the tested bacteria.

In this research, CH-NC-N film had inhibitory activity on *S. aureus*, *L. monocytogenes*, *E. coli*, *E. coli XDR*, and *K. pneumonia XDR* in a liquid medium. The number of colonies of *S. aureus*, *L. monocytogenes*, *E. coli*, and *K. pneumonia XDR* in the medium containing CH-NC-N film are drastically reduced ($P \leq 0.05$). The results also showed that adding nisin and nano-cellulose to the chitosan film reduced the growth of *E. coli XDR* ($P \geq 0.05$) (Figure 9).

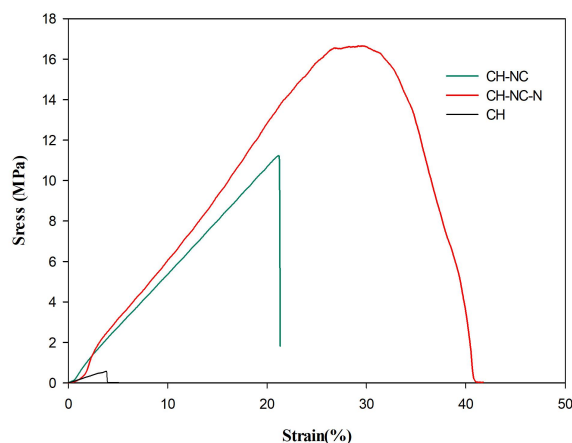


Figure 8. Mechanical properties of Chitosan (CH), Chitosan-Nano-Cellulose (CH-NC), and Chitosan-Nano-Cellulose-Nisin (CH-NC-N) films.

Table 1. Inhibition zone of agar disc diffusion tests for chitosan-nano-cellulose-nisin biocomposites.

Strains	Treatment				
	Control (mm)	Chitosan (mm)	Chitosan Nano-cellulose (mm)	Chitosan-Nano-cellulose-Nisin (30 µg mL ⁻¹ of nisin) (mm)	Chitosan-Nano-cellulose-Nisin (60 µg mL ⁻¹ of nisin) (mm)
<i>Escherichia coli</i>	- ^a	-	-	-	20±0
<i>Staphylococcus aureus</i>	-	-	-	-	20±0
<i>Escherichia coli XDR</i>	-	-	-	-	20±0
<i>Klebsiella pneumonia XDR</i>	-	-	-	-	30±0.1
<i>Listeria monocytogene</i>	-	-	-	-	19.5±0.2

^a -: No inhibition zone.

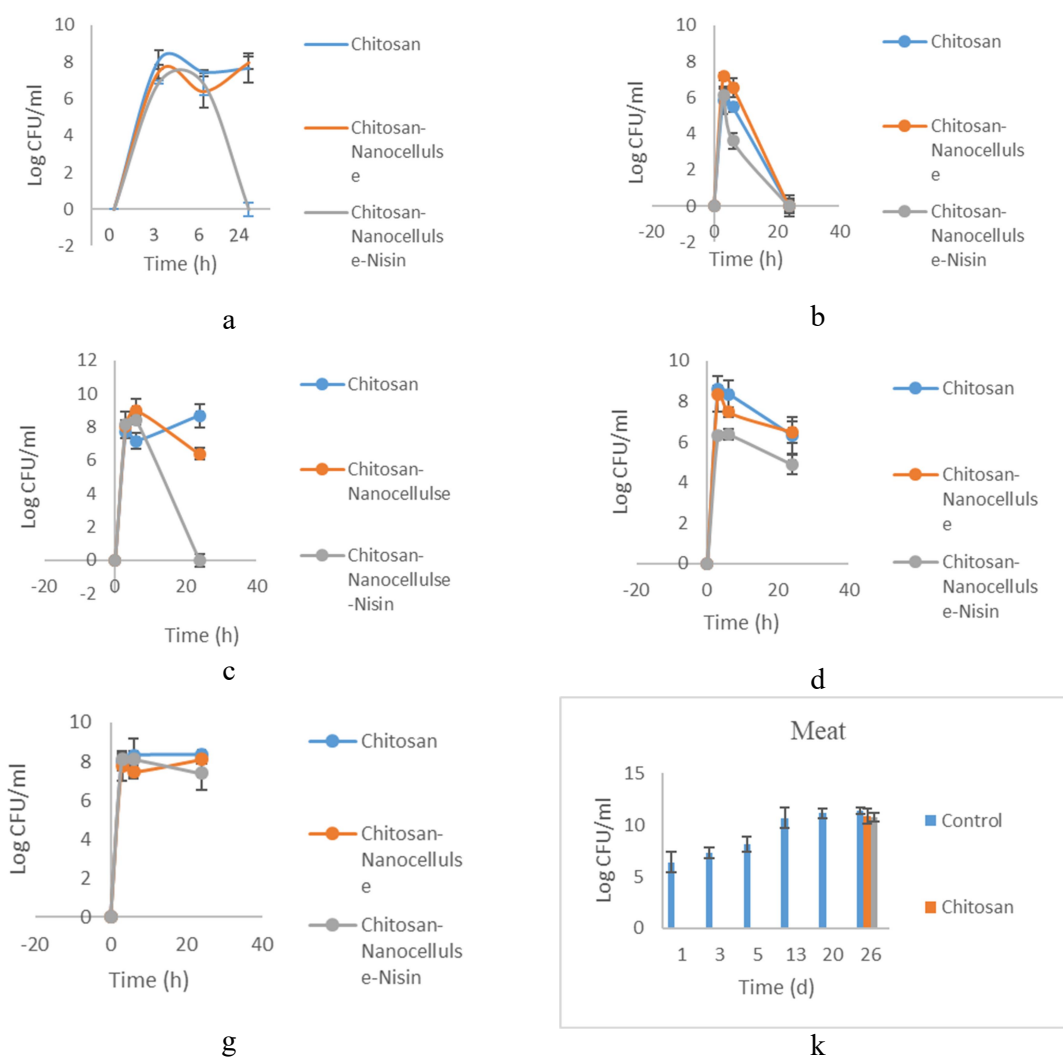


Figure 9. Inhibitory effect of chitosan-nano-cellulose nanocomposite against bacteria in liquid medium and bacteria initially present on meat samples. a: *S. aureus*, b: *L. monocytogenes*, c: *E. coli*, d: *E. coli XDR*, g: *Klebsiella pneumonia XDR*, and k: Meat.

**Table 2.** Discoloration and putrid odor scores over time for packaged meat samples.^a

Treatment	Days						
	0	1	3	5	13	20	26
Control	-	±	+	+	+	++	+++
Chitosan	-	-	-	-	-	±	+
Chitosan - Nano-cellulose	-	-	-	-	-	±	+
Chitosan- Nano-cellulose- Nisin	-	-	-	-	-	-	-

^a -: No discoloration and no putrid odor, +: No discoloration and putrid odor.

Shelf-Life Investigatin in Raw Meat

The results showed that after 26 days, only the CH-NC-N film prevented the growth of bacteria and preserved the meat sample compared with the control ($P \leq 0.05$). Also, the results showed that no LABs were isolated from any of the samples during the 26 days (Figure 9).

Furthermore, on the 26th day, small discoloration and a slight off-odor were observed in the packaged meat samples, indicating the initiation of spoilage (Table 2).

DISCUSSION

Newly, nano-cellulose composite packaging materials with antimicrobial activity are an excitable subject in a recent investigation. A nanocomposite packaging material prepared from natural antimicrobials, chitosan, or nano-cellulose has the possibility to be widely operated in the food manufacturing (Dehnad *et al.*, 2014a; Dehnad *et al.*, 2014b; Lu *et al.*, 2021). However, we can investigate more into the combination of chitosan, and nano-cellulose with other antimicrobial agents.

In this research, the CH-NC-N biocomposite with $30 \mu\text{g mL}^{-1}$ of nisin had no inhibitory effect on the tested bacteria. By increasing the nisin concentration to $60 \mu\text{g mL}^{-1}$ due to the increase in the release

rate of nisin in the culture medium, they showed an inhibitory effect on all tested bacteria. Ce *et al.* (2012) stated the antibacterial activity of chitosan films, including peptide p34, nisin, and natamycin, on *L. monocytosis*, *S. aureus*, *E. coli*, *B. cereus*, and *L. acidophilus*. These films showed excellent antimicrobial properties against all tested bacteria (Cé *et al.*, 2012). The nisin penetrates the bacterium's cytoplasm and creates a hole in this membrane, which causes essential substances to leak out of the cell, which finally leads to bacterial death (Gedarawatte *et al.*, 2021). Nisin displays an antibacterial effect versus a wide variety of Gram-positive bacteria, but has slight activity versus fungi, yeasts, and Gram-negative bacteria (Gedarawatte *et al.*, 2021; Khan *et al.*, 2016). It is also able to kill such microorganisms in combination with other antimicrobials. Therefore, in this study, EDTA was used together with nisin. The combination of nisin and EDTA causes the inhibitory effect of the produced nanocomposite on Gram-negative bacteria. When EDTA is added to the environment, 30-50% of lipopolysaccharide and other proteins of the outer membrane of Gram-negative bacteria are released immediately. Oxidation of magnesium and calcium ions in the lipopolysaccharide layer of the bacterial outer membrane also causes instability of the lipopolysaccharide layer and increases cell permeability. Therefore, the access of nisin to the cytoplasmic membrane in Gram-

negative bacteria is facilitated and it causes the permeability and leakage of substances from the cytoplasmic membrane and, finally, cell death. Khan *et al.* (2016) described that a nisin and EDTA formulation had a synergistic influence versus *E. coli* and *L. monocytogenes*.

In the present study, nanocomposite containing nisin was used to evaluate the antimicrobial effect on the meat. The results indicated that only the nanocomposite containing nisin effectively inhibited bacterial growth and preserved the meat sample after 26 days.

Salmieri *et al.* (2014) conducted a study on the inhibition of *L. monocytogenes* in hams cooked with nanocomposites made from polylactic acid–nano-cellulose–nisin. Their results determined nisin as a robust antimicrobial agent in the produced nanocomposite.

Treatment of the ground meat by chitosan–nano-cellulose composite reduced lactic acid bacteria compared with nylon-packaged ground meat at 3 and 25°C afterward six days of storing, respectively (Dehnad *et al.*, 2014b). Pattanayaiving *et al.* (2015) incorporated lauric arginate and nisin Z in pullulan films. The findings from this report designate the LAE- and LAE–nisin Z-containing pullulan films exhibited outstanding inhibition versus foodborne bacteria on fresh and extra-managed muscle foods (Pattanayaiving *et al.*, 2015). Yang *et al.* (2020) produced sugarcane bagasse nano-cellulose/nisin hybrid films. Data analysis showed that the nisin concentration had considerable effect on the antibacterial activity, light transmission, gas barrier, and mechanical properties of these films. This film was employed as a lining on low-molecular-weight polyethylene plastics for hamburger packaging. The results showed that the film entirely prevented *L. monocytogenes* through 7 days of storing at 4°C (Yang *et al.*, 2020). Polyvinyl alcohol/chitosan/modified bacterial nano-cellulose (mBNC) films were produced by incorporating 4-hexyl resorcinol (4HR) for food packaging purposes. The results

showed that the integration of 4-HR and mBNCs enhanced the film's yellowness and redness but reduced the film's transparency and brightness. The water vapor permeability, moisture content, and elongation at the break of the films were developed by adding 4-HR and mBNCs, while tensile strength and water solubility were decreased. The antioxidant properties of the films were significantly developed by adding 4-HR. The active films presented outstanding antimicrobial influence versus spoilage bacteria on vacuum-packed frozen raw beef (Choo *et al.*, 2021). Gedarawatte *et al.* (2021) conducted a study on the antibacterial activity of nisin-loaded bacterial cellulose nanocrystals versus certain meat spoilage lactic acid bacteria. The results showed that the nisin-loaded BCNs can be applied as antimicrobial ingredients in active food packaging (Gedarawatte *et al.*, 2021).

FTIR and XRD analysis described the molecular interaction after adding nano-cellulose and nisin. Nisin, being a cationic peptide, exhibits a negative interaction with chitosan due to their positive charges in acidic conditions (Ibarguren *et al.*, 2017; Saini *et al.*, 2016). The SEM images also show that the nano-cellulose, and nisin are evenly distributed inside the nanocomposite.

Measuring the contact angle on a film is a suitable marker of the degree of hydrophilicity and hydrophobicity. The concluding shape of the water drop on the film surface can be captured as an indicator of the surface wettability (Leceta *et al.*, 2013). Chitosan, due to having the functional groups of amines, acetamides, or hydroxyl groups, are hydrophilic polymer. Though, with the addition of NC and nisin, the contact angle value was reduced, which could be ascribed to the hydrophilic properties of cellulose particles. Nano-celluloses have more hydrophilic properties than cellulose polymers. The NC hydrophilicity could be the consequence of the incorporation of polar sulfate bands, the revealing of hydroxyl bands, and the prohibition of non-polar constituents from



the cellulose construction throughout the production procedure (Celebi and Kurt, 2015; Bahar *et al.*, 2012). In all instances, films made by microfluidics had lower contact angle values, signifying modifications in the conformation of molecules, and the revealing of the hydrophilic bands to the outside (Celebi and Kurt, 2015; Leceta *et al.*, 2013). The addition of nisin to the nanocomposite reduced the water contact angle value. This indicates an increase in water absorption in the film due to the hydrophilicity of nisin.

Food coatings must resist scratches and brittleness and show flexibility. Therefore, the mechanical properties of the bio-nanocomposite were investigated. The results of the CH-NC-N film show that Young's Modulus (YM), tensile strength (TS), and uniform elongation (UE) increase. The increase in breaking point after the addition of nano-cellulose may be due to the coordinated relationships between the polymer and nano-cellulose, the effective transfer of force through the polymer and nano-cellulose layers. Bonding between anionic sulfate groups of nano-cellulose, and cationic amide groups in chitosan could create better interaction between the matrix and the filler. This can conduct superior TS amounts of the nanocomposite films (Dehnad *et al.*, 2014a).

CONCLUSIONS

In this research, chitosan-based nanocomposite film was produced, including chitosan, nano-cellulose, and nisin. Nano-cellulose was synthesized from cellulose particles with acid hydrolysis. The value of TS and YM of bio-nanocomposite are considerably enhanced with the increase of nisin and nano-cellulose.

The development of mechanical and physical properties of the CH-NC-N films was mainly because of the filling effect of nano-cellulose. The presence of nisin and nano-cellulose in the synthesized bio-nanocomposite has been proven by XRD

and FTIR spectra. SEM image of the CH-NC-N films showed a uniform construction, specifying appropriate spreading of nano-cellulose and nisin into the chitosan film. Furthermore, the chitosan-nano-cellulose-nisin bioactive composite considerably inhibited all the tested pathogenic bacteria. However, examination of all information showed that the CH-NC-N film had an extraordinary possibility for utilization in the antimicrobial meat packaging. The evidence can assist the food industry in implementing the necessary processing requirements to ensure food safety while producing products with enhanced sensory properties and reduced energy costs.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge the generous cooperation of Nano Structured Coatings Institute, Yazd Payame Noor University, PO Code 89431-74559 Yazd, Iran.

REFERENCES

1. Bahar, E., Ucar, N., Onen, A., Wang, Y., Oksüz, M., Ayaz, O., Ucar, M. and Demir, A. 2012. Thermal and Mechanical Properties of Polypropylene Nanocomposite Materials Reinforced with Cellulose Nano Whiskers. *J. Appl. Polym. Sci.*, **125**: 2882-2889.
2. Baysal, G., Demirci, C. and Özpınar, H. 2023 Properties and Synthesis of Biosilver Nanofilms for Antimicrobial Food Packaging. *Polymers*, **15**(3): 1-12.
3. Baysal, G., Olcay, H. S., Keresteci, B. and Özpınar, H. 2022. The Antioxidant and Antibacterial Properties of Chitosan Encapsulated with the Bee Pollen and the Apple Cider Vinegar. *J. Biomater. Sci. Polym. Ed.*, **33**: 995-1011.
4. Cé, N., Noreña, C. P. Z. and Brandelli, A. 2012. Antimicrobial Activity of Chitosan Films Containing Nisin, Peptide P34, and Natamycin. *CyTA - J. Food*, **10**: 21-26.
5. Celebi, H. and Kurt, A. 2015. Effects of Processing on the Properties of

- Chitosan/Cellulose Nanocrystal Films. *Carbohydr. Polym.*, **133**: 284-293.
6. Choo, K. W., Dhital, R., Mao, L., Lin, M. and Mustapha, A. 2021. Development of Polyvinyl Alcohol/Chitosan/Modified Bacterial Nanocellulose Films Incorporated with 4-Hexylresorcinol for Food Packaging Applications. *Food Packag. Shelf Life*, **30**: 100769.
 7. Dehnad, D., Emam-Djomeh, Z., Mirzaei, H., Jafari, S. -M. and Dadashi, S. 2014a. Optimization of Physical and Mechanical Properties for Chitosan–Nanocellulose Biocomposites. *Carbohydr. Polym.*, **105**: 222-228.
 8. Dehnad, D., Mirzaei, H., Emam-Djomeh, Z., Jafari, S.-M. and Dadashi, S. 2014b. Thermal and Antimicrobial Properties of Chitosan–Nanocellulose Films for Extending Shelf Life of Ground Meat. *Carbohydr. Polym.*, **109**: 148-154.
 9. Fernandes, S. C. M., Freire, C. S. R., Silvestre, A. J. D., Pascoal Neto, C., Gandini, A., Berglund, L. A. and Salmén, L. 2010. Transparent Chitosan Films Reinforced with a High Content of Nanofibrillated Cellulose. *Carbohydr. Polym.*, **81**: 394-401.
 10. Firouzabadi, F. B., Noori, M., Edalatpanah, Y. and Mirhosseini, M. 2014. ZnO Nanoparticle Suspensions Containing Citric Acid as Antimicrobial to Control *Listeria Monocytogenes*, *Escherichia Coli*, *Staphylococcus Aureus* and *Bacillus Cereus* in Mango Juice. *Food Control*, **42**: 310-314.
 11. Garcia, A., Labidi, J., Belgacem, M. N. and Bras, J. 2017. The Nanocellulose Biorefinery: Woody Versus Herbaceous Agricultural Wastes for Ncc Production. *Cellulose*, **24**: 693-704.
 12. Garside, P. and Wyeth, P. 2003. Identification of Cellulosic Fibres by Ftir Spectroscopy - Thread and Single Fibre Analysis by Attenuated Total Reflectance. *Stud. Conserv.*, **48**: 269-275.
 13. Gedarawatte, S. T. G., Ravensdale, J. T., Al-Salami, H., Dykes, G. A. and Cooray, R. 2021. Antimicrobial Efficacy of Nisin-Loaded Bacterial Cellulose Nanocrystals against Selected Meat Spoilage Lactic Acid Bacteria. *Carbohydr. Polym.*, **251**: 117096.
 14. Huq, T., Riedl, B., Bouchard, J., Salmieri, S. and Lacroix, M. 2014. Microencapsulation of Nisin in Alginate-Cellulose Nanocrystal (Cnc) Microbeads for Prolonged Efficacy against *Listeria Monocytogenes*. *Cellulose*, **21**: 4309-4321.
 15. Ibarguren, C., Audisio, M. C., Sham, E. L., Müller, F. A. and Farfan Torres, E. M. 2017. Adsorption of Nisin on Montmorillonite: A Concentration Strategy. *J. Food Process. Preserv.*, **41**: e12788.
 16. Khalil, H. P. S. A., Ismail, H., Rozman, H. D. and Ahmad, M. N. 2001. The Effect of Acetylation on Interfacial Shear Strength between Plant Fibres and Various Matrices. *Eur. Polym. J.*, **37**: 1037-1045.
 17. Khan, A., Gallah, H., Riedl, B., Bouchard, J., Safrany, A. and Lacroix, M. 2016. Genipin Cross-Linked Antimicrobial Nanocomposite Films and Gamma Irradiation to Prevent the Surface Growth of Bacteria in Fresh Meats. *Innov. Food Sci. Emerg. Technol.*, **35**: 96-102.
 18. Khan, A., Khan, R. A., Salmieri, S., Le Tien, C., Riedl, B., Bouchard, J., Chauve, G., Tan, V., Kamal, M. R. and Lacroix, M. 2012. Mechanical and Barrier Properties of Nanocrystalline Cellulose Reinforced Chitosan Based Nanocomposite Films. *Carbohydr. Polym.*, **90**: 1601-1608.
 19. Le Troedec, M., Sedan, D., Peyratout, C., Bonnet, J. P., Smith, A., Guinebretiere, R., Gloaguen, V. and Krausz, P. 2008. Influence of Various Chemical Treatments on the Composition and Structure of Hemp Fibres. *Compos. - A: Appl. Sci. Manuf.*, **39**: 514-522.
 20. Leceta, I., Guerrero, P., Ibarburu, I., Dueñas, M. T. and De La Caba, K. 2013. Characterization and Antimicrobial Analysis of Chitosan-Based Films. *J. Food Eng.*, **116**: 889-899.
 21. Lu, Q., Yu, X., Yagoub, A. E. A., Wahia, H. and Zhou, C. 2021. Application and Challenge of Nanocellulose in the Food Industry. *Food Biosci.*, **43**: 101285.
 22. Meydanju, N., Pirsá, S. and Farzi, J. 2022. Biodegradable Film Based on Lemon Peel Powder Containing Xanthan Gum and Tio2–Ag Nanoparticles: Investigation of Physicochemical and Antibacterial Properties. *Polym. Test.*, **106**: 107445.
 23. Mirhosseini, M. and Afzali, M. 2016. Investigation into the Antibacterial Behavior of Suspensions of Magnesium Oxide Nanoparticles in Combination with Nisin and Heat against *Escherichia Coli* and *Staphylococcus Aureus* in Milk. *Food Control*, **68**: 208-215.



24. Mirhosseini, M. and Arjmand, V. 2014. Reducing Pathogens by Using Zinc Oxide Nanoparticles and Acetic Acid in Sheep Meat. *J. Food Prot.*, **77**: 1599-1604.
25. Pappas, C., Tarantilis, P. A., Daliani, I., Mavromoustakos, T. and Polissiou, M. 2002. Comparison of Classical and Ultrasound-Assisted Isolation Procedures of Cellulose from Kenaf (*Hibiscus Cannabinus* L.) and Eucalyptus (*Eucalyptus Rodustrus* Sm.). *Ultrason. Sonochem.*, **9**: 19-23.
26. Pattanayaiying, R., H-Kittikun, A. and Cutter, C. N. 2015. Incorporation of Nisin Z and Lauric Arginate into Pullulan Films to Inhibit Foodborne Pathogens Associated with Fresh and Ready-to-Eat Muscle Foods. *Int. J. Food Microbiol.*, **207**: 77-82.
27. Pirsas, S. 2021. Nanocomposite Base on Carboxymethylcellulose Hydrogel: Simultaneous Absorbent of Ethylene and Humidity to Increase the Shelf Life of Banana Fruit. *Int. J. Biol. Macromol.*, **193**: 300-310.
28. Pirsas, S. and Asadi, S. 2021. Innovative Smart and Biodegradable Packaging for Margarine Based on a Nano Composite Polylactic Acid/Lycopene Film. *Food Addit. Contam. Part A*, **38**: 856-869.
29. Pirsas, S. and Mohammadi, B. 2021. Conducting/Biodegradable Chitosan-Polyaniline Film; Antioxidant, Color, Solubility and Water Vapor Permeability Properties. *Main Group Chem.*, **20**: 133-147.
30. Saini, S., Sillard, C., Belgacem, M. N. and Bras, J. 2016. Nisin Anchored Cellulose Nanofibers for Long Term Antimicrobial Active Food Packaging. *RSC Adv.*, **6**: 12422-12430.
31. Salmieri, S., Islam, F., Khan, R. A., Hossain, F. M., Ibrahim, H. M. M., Miao, C., Hamad W. Y. and Lacroix, M. 2014. Antimicrobial Nanocomposite Films Made of Poly(Lactic Acid)-Cellulose Nanocrystals (Pla-Cnc) in Food Applications: Part A—Effect of Nisin Release on the Inactivation of *Listeria Monocytogenes* in Ham. *Cellulose*, **21**: 1837-1850.
32. Shabkhiz, M. A., Khalil Pirouzifard, M., Pirsas, S. and Mahdavinia, G. R. 2021. Alginate Hydrogel Beads Containing Thymus *Daenensis* Essential Oils/Glycyrrhizic Acid Loaded in B-Cyclodextrin. Investigation of Structural, Antioxidant/Antimicrobial Properties and Release Assessment. *J. Mol. Liq.*, **344**: 117738.
33. Szymańska-Chargot, M., Chylińska, M., Pertile, G., Pieczywek, P. M., Cieślak, K. J., Zdunek, A. and Frąc, M. 2019. Influence of Chitosan Addition on the Mechanical and Antibacterial Properties of Carrot Cellulose Nanofibre Film. *Cellulose*, **26**: 9613-9629.
34. Yang, Y., Liu, H., Wu, M., Ma, J. and Lu, P. 2020. Bio-Based Antimicrobial Packaging from Sugarcane Bagasse Nanocellulose/Nisin Hybrid Films. *Int. J. Biol. Macromol.*, **161**: 627-635.

توسعه فیلم های ضد میکروبی نانو سلولز - کیتوزان فعال شده با نیزین برای کاربردهای غذایی و پزشکی

م. میرحسینی، و ر. دهقان

چکیده

در این تحقیق فیلم کیتوزان زیست تخریب پذیر - نانو سلولز - نیزین (CH-NC-N) سنتز شد و برای کاربرد آنتی باکتریال در پزشکی و بسته مواد غذایی مورد استفاده قرار گرفت. فیلم ضد باکتری کیتوزان-نانو-سلولز-نیزین با استفاده از تکنیک های مختلفی مانند آنالیز خواص مکانیکی و فیزیکی، میکروسکوپ الکترونی

رویشی (SEM)، پراش اشعه ایکس (XRD) و طیف‌سنجی مادون قرمز تبدیل فوریه (FTIR) بررسی شد. فعالیت ضد باکتریایی فیلم کیتوزان - نانو سلولز - نیزین علیه باکتری‌های عامل بیماری مثل: اشریشیا کلی، استافیلوکوکوس آرنوس، لیستریا مونوسیژنوز، اشریشیا کلی XDR و کلبسیلا پنومونیه XDR مورد بررسی قرار گرفت. همچنین از این فیلم برای بسته بندی گوشت در دمای ۴ درجه سانتی گراد به مدت ۲۶ روز استفاده شد. تجزیه و تحلیل داده‌ها نشان داد که خواص مکانیکی و جذب آب فیلم پس از افزودن نانو سلولز و نیزین بهبود یافته است. تجزیه و تحلیل داده‌های FTIR، XRD و SEM امکان توصیف حضور نیزین در فیلم CH-CN را فراهم کرد. تجزیه و تحلیل ضد میکروبی فیلم، پتانسیل بالای نیزین را به عنوان یک عامل ضد میکروبی در فیلم CH-CN-N در برابر همه باکتری‌های پاتوژن آزمایش شده مشخص کرد. فیلم CH-CN-N رشد کل باکتری‌های عامل فساد طبیعی را در گوشت به مدت ۲۶ روز در مقایسه با شاهد مهار کرد. همچنین نتایج نشان داد که خواص ظاهری و فیزیکی گوشت بسته بندی شده با این فیلم تغییر چندانی نداشته است. نتایج نشان می‌دهد که فیلم کیتوزان - نانو سلولز - نیزین برای استفاده در سیستم‌های غذایی و کاربرد پزشکی مناسب است.