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Original research

Immobilized lysozyme onto bacterial cellulose nanofibers as active and reinforcing agent of sodium caseinate based films: physical characteristics and antimicrobial activity

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A B S T R A C T

The aim of this research was to compare the effect of free and immobilized Lysozyme on the physicochemical and antimicrobial properties of sodium caseinate (SC) based active films. Lysozyme was immobilized onto bacterial cellulose nanofiber (BCNF) and decreasing of its activity was approved after immobilization. Free and immobilized enzymes were incorporated into SC films at the concentrations of 0.5 and 1 mg.100ml^{-1} and the films were characterized. Addition of BCNF and lysozyme diminished significantly ($p<0.05$) the moisture absorption and water vapor permeability of SC films and immobilized enzyme had higher effect than free enzyme on decreasing of these parameters. The tensile strength and Young's modulus were increased and elongation to break was decreased by incorporation of BCNF. An adverse effect was observed for lysozyme addition but the effect of immobilized enzyme on the weakening of tensile properties was lower than free lysozyme. According to X-ray diffraction (XRD) results, the crystallinity of films increased by incorporation of BCNF and lysozyme. However, the immobilization reduced the crystalline regions of BCNF. Antimicrobial activity of lysozyme increased after immobilization and SC films containing immobilized enzyme exhibited considerable activity against Gram-positive bacteria *S. aureus*, *L. monocytogenes*. Gram-negative bacteria *E. coli*, *Y. enterocolitica*, mold of *A. niger* and yeast of *S. cerevisiae*.

Keywords: Active film; Lysozyme immobilization; Sodium caseinate; Physical properties; Antimicrobial activity

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1. Introduction

Active packaging is a novel technology that besides having main inhibition properties of the usual packaging (such as inhibition properties against air, vapor, and mechanical stresses), by changing conditions of packaging improves safety and shelf life of food and preserves its quality and sensory properties during distribution chain. Active packaging can have multiple roles which are not present in usual and common packages, for example, releasing flavors, antioxidants or antimicrobials and absorbing of oxygen, humidity or ethylene (Ghanbarzade & Almasi, 2011).

In recent years, researches on food packaging have mostly focused on biodegradable films such as films made of proteins with animal and plant origin (wheat gluten, corn zein, whey protein, meat protein etc.), polysaccharides (pectin, cellulose, chitosan) or a combination of them (Akhondzade et al., 2011). There are advantages to these films such as renewability, having agriculture origin, being environment friendly, ability to carry antimicrobial substances, and not being harmful for human health (Ghanbarzadeh et al., 2015). One of the most important types of protein films is films based on sodium caseinate (SC). Due to complex intermolecular bonds, films produced from milk proteins have good features of prevention from gas transition (Atares et al., 2014). SC films have poor mechanical strength and high water vapor permeability (Salgado et al., 2010). There are many reports on the fabrication and characterization of SC based biodegradable films (Chevalier et al., 2018a,b; Picchio et al., 2018).

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Nowadays, nanomaterials are used in biopolymeric films as nanoreinforcing agents in order to improve mechanical features and survivability against water vapor which are two main defects of these films (Perada et al., 2011a). Bacterial cellulose nanofiber (BCNF) is one the most important and highly applicable nanomaterials that has been studied as a nanoreinforcing agent in different polymers and biopolymers (Ghanbarzadeh et al., 2015).

BCNF also provides a proper surface area for absorbing or covalent bonding to various enzymes (Uddin Khan et al., 2017). Antimicrobial enzymes are one of the substances which are used in food active packaging (Wang et al., 2018). One of the advantages of enzymes as antimicrobial agents in developing of active packaging is having large molecular structure, in addition, they can affect microbe cellular membrane when attached to packaging material and they can stay active for a long time (Abouhmad, 2017). One of these important antimicrobial enzymes is lysozyme. In food packaging industry, lysozyme enzyme is used to produce antimicrobial films in order to develop shelf life of foods (Nakimbugwe et al., 2005). Lysozyme, a glycosidase enzyme, has higher antimicrobial activity on gram positive bacteria since their exterior membrane doesn't not exist peptidoglycan layer (Safarik, 2016). Antimicrobial role of this enzyme has led to wide application in different food, pharmacology, medicine, and cosmetics industries (Coma, 2008).

In modern biotechnology science, immobilization has been considered as proper method for developing of prolonged utilizing of enzymes because immobilizing of enzymes can improve stability of enzymes, also enzyme can be reused (Homaei et al., 2013). Beside easy separation and reusability, enzyme immobilization has other advantages such as increased storage stability, increased stability against temperature variations, increased stability in organic solvents and other severe and intolerable operating conditions (Sirisha et al., 2016).

There are some reports on the immobilization of lysozyme enzyme onto various substrates such as silicon rubber films (Flores et al., 2017), magnetic nanoparticles (Chen et al., 2018) and calcium alginate film (Wang et al., 2018). The immobilization of this enzyme onto plant cellulose based nanomaterials has been also investigated (Abouhmad et al., 2017; Khan et al., 2017; Liu et al., 2018).

In our previous research (Bayazidi et al., 2018), the immobilization of lysozyme onto BCNF using physical absorption method was investigated. Antimicrobial activity of lysozyme in different ranges of pH and temperatures were studied against various bacteria. Results indicated that storage stability of lysozyme was increased after immobilization. Moreover, the immobilized enzyme exhibited more than 70% of its initial activity after 9 cycles of reusing.

To the best of our knowledge, there is no report on the using of immobilized enzymes in the fabrication of SC based active films. As the second step of the previous research, we aimed to use free and immobilized lysozyme as antimicrobial agent in the fabrication of SC based active films. The physical, morphological and antimicrobial properties of the films were studied.

2. Material and Methods

2.1. Materials

SC powder with protein content of 85% purchased from Iran Caseinate Co. (I.I.C). BCNF in the form of white gel (3%) was kindly provided by Nano Novin Polymer Co. (Sari, Iran), Glycerol as plasticizer and lysozyme enzyme were produced from Merck (USA) and Sigma (Germany), respectively. All other chemicals were of analytical grade. All microorganisms (*Staphylococcus aureus* (ATCC-19111), *Listeria monocytoogenesis* (ATCC-19114), *Escherichia coli* (ATCC-11775), *Yersinia enterocolitica* (ATCC-27729), *Micrococcus Lysodeikticus* (ATCC-4698), *Aspergillus niger* (ATCC-10577), and *Saccharomyces cerebellum* (ATCC-2540)) were purchased from culture collection of the Industrial Microorganisms Collection (Iran).

2.2. Immobilization of lysozyme enzyme

Enzyme immobilization was performed by physical adsorption method according to our previous research (Bayazidi et al., 2018). 2.5 ml of prepared lysozyme solution (with concentrations 0.5 and 1 mg) was incubated with 3 gr BCNF at 25 ºC for 12 h. Then, by centrifuge with 4000 rpm for 5 min, the unbound enzyme was separated.

2.3. Determination of free and immobilized lysozyme activity

Enzyme activity was determined using the lysis of *Micrococcus lysodeikticus* cells by monitoring the turbidity reduction at 450 nm. A 2.5 ml dispersion (OD_{450}=1.3) of the *M. lysodeikticus* was prepared using phosphate buffer 0.1 M (pH=6.24); then 0.5 ml and 1 ml soluble free and immobilized enzyme were prepared in phosphate buffer 0.1 M (pH=6.24). A 0.5 ml of bacteria was mixed into curette with 0.5 ml enzyme (both free and immobilized, separately) and the turbidity reduction of each soluble were measured with UV-Vis spectrophotometer at 450 nm for 60 second at every 10 s. Lysozyme activity was calculated through the following formula (Lian, 2012; Sun et al., 2016; Wang et al., 2018).

$$\text{Activity (U/mg)} = \frac{\Delta OD_{450}}{0.001\ \text{m}} \quad (1)$$

where ΔOD_{450} is the difference in absorbance of *M.lysodeikticus* solution for the wavelength of 450 nm after 5 min; m refers to the mass (mg) of lysozyme in 0.5 ml of free enzyme solution or immobilized enzyme solution.

2.4. Preparation of SC active films

In order to produce biodegradable active films, solvent casting method was used according to Schou et al. (2005), with some modifications. 4 g dry SC powder was added to 100 ml distilled water and was constantly stirred. Stirring continued up to one hour in 30±5 °C with speed of 1100 rpm by using magnetic stirrer. After that, glycerol with amount of 40% of dry matter was added and stirred for 10 mins. For producing biocomposite active films (SC-BCNF), BCNF at concentration of 2.5 % w/w of SC was added to 50 ml distilled water and was stirred for 12 h at 30±5 °C and was sonicated for 30 mins by ultrasonic bath (ASONE model 4R USD, Korea) at frequency of 40 KHz. In the next step, SC solution and sonified BCNF dispersion were mixed and 37.5 % w/w glycerol was added as plasticizer.

For producing active films containing free enzyme, 0.5 and 1 mg concentrations of lysozyme enzyme were prepared with phosphate buffer (pH=6.25) and was added to film solution. These films were coded as F.Lys.0.5 and F.Lys.1. In the films containing

immobilized lysozyme, the BCNF immobilized enzyme was added at final concentrations of lysozyme equal to 0.5 and 1 mg/ml. these two films were coded as Im.Lys.0.5 and Im.Lys.1 samples. Neat SC film was prepared as control sample. From each film solution, 100 ml was slowly dispersed in polystyrene petri dishes with 10 cm diameter and were dried at 20±5 °C and in relative humidity of 50±5 % for 48 h. Finally, the dried films were peeled and were kept in polyethylene bags.

2.5. Characterization of films

2.5.1. Moisture absorption (MA)

In order to measure the MA of films the method of Angles and Dufresne (2000) was used. Samples of films were cut in 20×20 mm^2 and then, they were kept in desiccator containing saturated solution of calcium sulfate with relative humidity (RH) of 0%. After that, all the samples were weighed with 0.0001 accuracy and then they were transferred to the desiccator containing a saturated solution of calcium nitrite at a temperature of 20-25 °C (RH=55%). Then, samples were weighed in different time intervals until their weight reaches an equilibrium state and the rate of MA was calculated through the following equation:

$$\text{MA}\ (\%) = \frac{W_t - W_0}{W_t} \times 100 \qquad (2)$$

where W_t and W_0 are the weights of the sample after t time at 55% RH and the initial weight of the sample, respectively. All measurements were performed in three replicates.

2.5.2. Water vapor permeability (WVP)

Permeability of film to water vapor was determined according to ASTM standard (ASTM, 2005 E96-05) with some changes (Almasi et al., 2015a). First, the films were conditioned for 24 h in 55% RH which were made by calcium nitrite. In order to conduct the test, special vials with a diameter of 2 cm and a height of 4.5 cm were used. The cap on these vials had vent with a diameter of 8 cm on which a piece of the conditioned film was placed. Next, 3g of calcium sulfate were placed into vials which it is made 0% relative humidity and a piece of film was cut and were placed in vial cap. Vials were weighed and were placed in desiccator containing potassium sulfate saturated solution. Saturated potassium sulfate in temperature of 25 ºC produces RH equal to 97%. The desiccator was adjusted at 25±1 ºC and the weight of the vials was measured every few hours for 3 days. Increased weight curve of vials was drawn in time and after calculating linear regression, slope of the resulted line was calculated. By dividing line slope related to each film into the surface which is exposed to water evaporation transfer, the water vapor transition rate (WVTR) was obtained (Equ. 3). Then, through the Equ. 4, water vapor permeability (WVP) was calculated.

$$\text{WVTR} = \frac{\text{Slope}}{A} \qquad (3)$$

$$\text{WVP} = \frac{\text{WVTR} \times L}{\Delta P} \qquad (4)$$

where WVTR represents water vapor transmission rate, defined as the slope of weight gain versus time ($g.s^{-1}$) divided by the transfer area (m^2), L shows the average film thickness (m), and ΔP is the partial water vapor pressure difference (Pa) between inside and outside of the film. The WVP was measured via testing each specimen in triplicate.

2.5.3. Moisture absorption (MA)

In order to determine samples' tensile strength (TS), the percentage of elongation to break (ETB) and Young's modulus (YM), SANTAM series of Universal Testing Machines/STM-20 (South Korea) was used. This test was performed in environment temperature according to ASTM-D88210 standard method (ASTM, 2010). First, films were conditioned under 25 ºC temperature and 50±5% RH, and then, two samples from each film were cut in form of dumbbell with 0.5×8.5 cm^2 size. The initial grip separation and the cross-head speed were adjusted to 40 mm and 0.5 $mm.min^{-1}$, respectively.

2.5.4. X-Ray diffraction (XRD) analysis

X-ray patterns of SC film were recorded using a XRD diffractometer (labx XRD-6000 Shimadzu, Japan), equipped with CuKα radiation at a wavelength of 0.1546 nm. The voltage and the current used were 40 kV and 30 mA, respectively. The scanning of the samples was performed over the range of diffraction angle 2θ=1-40° with a scanning rate of $1°.min^{-1}$ and step interval of 0.02° at ambient temperature (Pourjavaher et al., 2017).

2.5.5. Antimicrobial activity measurements

Antimicrobial activity of all produced films was examined against microbial growth of Gram-positive bacteria *S. aureus*, *L. monocytogenes* and Gram-negative bacteria *E. coli*, *Y. enterocolitica* and mold of *A. niger* and yeast of *S. cerevisiae*. Commercial strains of microorganisms with a little change were incubated by using the method of Arrieta et al. (2014) in 20 ml broth nutrient in 37 °C for 48 h under sterile conditions. After that, strains of microorganisms were activated, an amount of colony was cultivated on agar nutrient culture by sterile loop and was incubated for 48 h in incubator at 37 °C. The isolated colony from each microorganism was placed in physiology serum until they reached 0.5 McFarland opacity (in which 0.1 optical density in 600 nm corresponded with 1.5×10^8 cell in ml). After that, 4 serial dilutions were performed in order to reach 10^4 cfu/ml. 1 ml from each liquid of film forming solution was poured in flask containing 20 ml broth nutrient, then 1 ml from each of 10^4 cfu/ml microbe was added to each flask containing film and culture and was vibrated for one minute. Flasks containing samples were incubated at 37 ºC and for each microbial sample, one flask containing each of microorganism inside culture without film was prepared as a control. Optical density in 600 nm (OD 600 nm) was recorded as indicator of cell viability and microbial growth during time.

2.6. Statistical analysis

The analysis of the statistical data was carried out using SPSS software (Version 22; SPSS, Chicago IL, USA) via one-way analysis of variance (ANOVA). Duncan's multiple range tests were performed to study the difference between mean values of film specimens' characteristics at a statistical level of 0.05.

3. Results and Discussion

3.1. Free and immobilized lysozyme activity

Results of enzyme's activity are shown in Table 1. Free lysozyme in compare to immobilized enzyme exhibits less activity, even though by increasing enzyme's concentration the relative activity was increased in both forms of enzymes. At concentration of 1 mg activity of free and immobilized enzyme were recorded as 79.20% and 69.6% respectively. Usually after immobilization the specific activity is reduced. It is reported in previous researches on immobilization of lysozyme on chitosan substrates (Graebin et al., 2016; Cappanella et al., 2016). The reduction of immobilized enzyme efficiency may cause by the diffusional limitations resulting from the diminished molecular flexibility of the enzyme and hindering and damaging of some active sites during immobilization (Lian et al., 2012; Bayazidi et al., 2018; Uddin khan et al., 2017).

Table 1. Relative activity of free and immobilized lysozyme.

Lysozyme dosage (mg/ml)	Lysozyme activity (%)	
	Free form	Immobilized form
0.1	37.5±0.65^{c}	18.57±0.00^{a}
0.15	42.85±0.01^{d}	32.14±0.00^{b}
0.25	54.65±0.00^{e}	42.15±0.43^{d}
0.5	68.75±0.76^{f}	55.54±1.21^{e}
1	79.20±1.76^{g}	69.6±0.57^{f}

Values are given as mean ± standard deviation.
Means with same superscripts are not significantly different ($p > 0.05$).

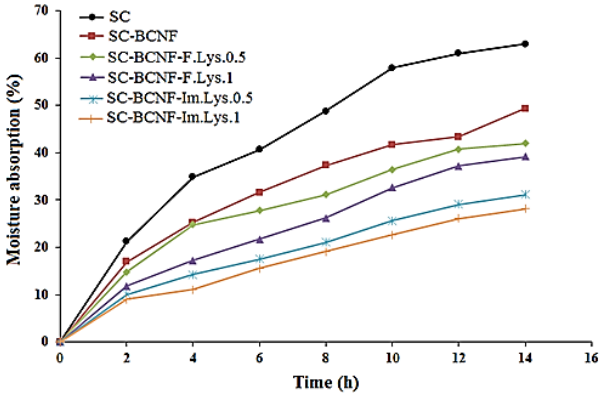

Fig. 1. Moisture absorption of SC based films containing free and BCNF-immobilized lysozyme.

3.2. Moisture absorption (MA)

The MA of produced films is shown in Fig. 1. The neat SC film had the highest MA which reached to 13.002±0.005% after 48 h that is most likely due to its high hydrophilic nature. By adding BCNF to SC film, the rate of MA was significantly decreased to 9.330±0.031% ($p<0.05$). BCNF besides of its hydrophilic nature, causes the decrease in MA of nanocomposite films due to its highly crystallinity and rigidity (Chaichi et al., 2017). Similar results were reported by Perda et al. (2011). By adding lysozyme enzyme to films containing BCNF, the rate of MA decreased more and in the films containing BCNF-immobilized enzyme, the lowest MA was achieved. There was a significant difference between rate of MA in films containing immobilized enzymes and films containing free enzyme. In equal enzyme concentration, the MA decreasing was higher in immobilized enzyme which shows that the enzyme immobilization has higher reducing effect on MA of BCNF reinforced SC films which is probably due to the opining the 3D structure of BCNF and increasing its surface area that causes to increase its interactions with free hydrophilic residues of SC and leading to decrease MA. In a similar research by Kim et al., (2008) on nanobiocatalysts, it was found that immobilizing of enzymes on nanofibers causes to increase the porosity and free specific surface of nanofibers. Also, the findings of Park et al. (2013) were in agreement with our results.

3.3. Water vapor permeability (WVP)

The amounts of WVP of films are shown in Fig. 2. According to the results, by adding BCNF-immobilized lysozyme with 1 mg enzyme, WVP of film decreased from 1.05×10^{-8} to 7.87×10^{-9} g/m.h.Pa in comparison to free BCNF loaded film. In dead, by adding BCNF, WVP decreased significantly (Chaichi et al., 2017). In the film containing 1 mg free lysozyme, WVP decreased significantly in comparison to the neat SC films. Also, there is a significant difference between film containing immobilized lysozyme and free lysozyme with the same concentrations. It is observable that in lower concentrations of immobilized enzyme (0.5 mg) the significant difference can be seen while there is no significant difference in film containing 0.5 mg free lysozyme and the film containing pure BCNF. In general, WVP was decreased significantly except for samples of 0.5 mg free lysozyme and the film containing pure CNF. The amount of enzyme had a decreasing effect and immobilized enzyme had higher effect on decreasing of WVP.

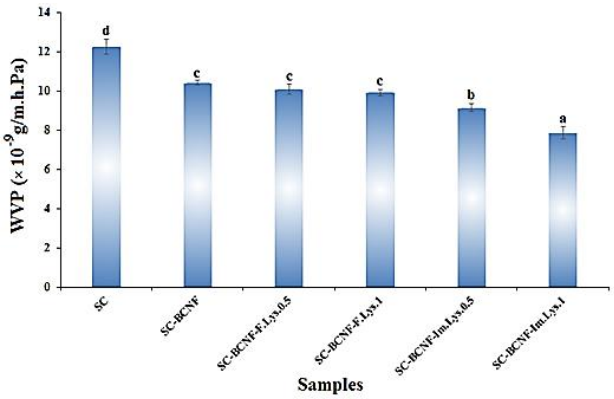

Fig. 2. WVP of SC based films containing free and BCNF-immobilized lysozyme. Data are the means ± standard deviation. Mean values with different letters are significantly different ($p < 0.05$).

The decrease in WVP in films containing immobilized lysozyme in comparison to free lysozyme is due to opening the structure of BCNF and its better distribution in SC matrix. Consequently, the diffusion of water vapor molecules into more tortuous matrix will be more difficult (Almasi et al., 2015b; Pereda et al., 2011a). Also, as it can be seen in Fig. 2, the WVP of the neat SC film decreased from 1.22×10^{-8} to 1.05×10^{-8} after addition of BCNF. In general, WVP in hydrophilic films depends on the diffusion and solubility of water vapor molecules inside film

matrix. When the structure of nanocomposite is formed, impervious layers of nanoparticles make a long zigzag pathway for water molecules to pass through and increase the length of pathway to prevent water molecules from passing (Pereda et al., 2011a). In a similar research, Pereda et al. (2011b) produced SC film with CNF and the results confirm the present observation.

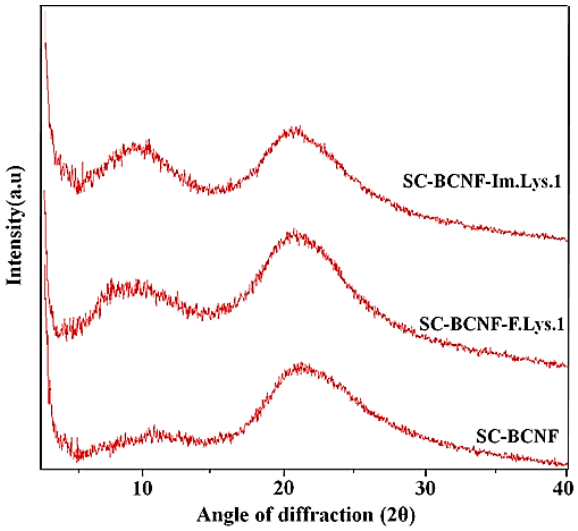

Fig. 3. XRD patterns of BCNF loaded SC films and films containing free and BCNF-immobilized lysozyme.

3.4. Mechanical properties

Table 2 shows the mechanical properties of SC active films. As it is seen, neat SC film has low mechanical strength and high flexibility and showed the lowest TS and the highest ETB. By adding 2.5% BCNF, mechanical strength of film was significantly increased from 3.45 to 6.81 MPa and it was nearly doubled. Also, its elongation was decreased. This shows the improving effect of BCNF on increasing mechanical strength of SC film. By placing cellulose chains between casein macromolecules and by generating new hydrogen bonds among them, the integrity of polymer was increased and its resistance against strain and break increased. Also, Young's modulus (YM) of film was approximately tripled as a result of adding BCNF. Pereda et al. (2011b) and Achaby et al. (2018) found same result about the effect of adding cellulose nanofibers to SC films. The improving effect of cellulose based nanoreinforcements on the mechanical properties of other biodegradable films has been also approved (Zhang et al., 2013; Chen et al., 2018; Fu et al., 2015; Kalia et al., 2014; Zhang et al., 2018; Chaichi et al., 2017; Hamou et al., 2018).

Regarding the Table 2, by adding free and immobilized lysozyme, the mechanical strength of the film was slightly decreased and its flexibility was increased. In fact, this effect about immobilized enzyme wasn't significant and these samples didn't have significant changes in both concentrations with neat SC film and with film containing BCNF. However, free enzyme decreased TS and YM and increased ETB. The reason is that probably free lysozyme enzyme molecules act as plasticizer and by being placed between casein chains, and thus, the decreasing of cross linking between SC chains causes to decrease the mechanical strength. However, in the immobilized form, enzyme is placed in BCNF chains and lower plasticizing effect is seen. Therefore, it can be concluded that enzyme in immobilized form on BCNF doesn't have negative effect on mechanical properties of SC film, but free enzyme weakens these features (Benucci et al., 2018).

3.5. X-ray diffraction (XRD) analysis

Fig. 3 shows patterns obtained from the XRD test. The neat SC film had no observable peak indicating its amorphous nature. The film containing BCNF showed a distinct peak around 2θ=22.5º which is related to crystalline cellulose structure type *I* in BCNF matrix (Almasi et al., 2015b). By regarding the significant intensity of this peak, the SC-BCNF film can be considered as a semi-crystalline film (Cao et al., 2018; Chaichi et al., 2017; Shabanpour et al., 2018). Moreover, a very small peak at an angle of about 10° for the case of casein containing BCNF was observed, and by adding the enzyme, the intensity of this peak increased. Even in the case of adding free enzyme, this peak was well detected. Given that no specific crystalline structure has been reported for lysozyme enzymes, therefore, the appearance of new crystalline regions in the presence of this enzyme can be attributed to the formation of new bonds between enzyme and casein chains or cellulose fibers, and as a result of the formation of more regular crystalline regions in the film's structure, the crystallinity of films has been increased. In the film containing 1 mg of enzyme immobilized on CNF, the intensity of this peak increased slightly in the 2θ=10º region, which confirmed the formation of more joints and, consequently, more regular crystalline structure. A remarkable point about the XRD spectrum of the film containing immobilized enzyme is the decreasing of BCNF peak at 2θ=22.5º after lysozyme immobilization. By fixing the enzyme on BCNF and during different treatments used for this process, the BCNF crystal structure changes and part of its crystalline area is reduced. Therefore, it can be concluded that by adding the lysozyme enzyme to the SC film, new crystalline regions are formed in the film, but all these crystalline areas are not BCNF-related regions, since by the enzyme immobilization on the BCNF, the new peak intensity is increased; however, BCNF-related peak intensity is reduced. The results of the effect of cellulose nanofibers on the casein film are consistent with the results of Pereda et al. (2011a). However, no study has ever been conducted on the effect of lysozyme enzymes or any other enzymes on the structural properties of edible films to compare the results of this study.

3.6. Antimicrobial properties

The antimicrobial activity of the films was investigated against *S. aureus*, *E. coli*, *L. monocytogenes*, *Y. enterocolitica*, *A. niger*, and *S. servisiae*. Regarding Fig. 4, as it was expected, the neat SC film showed any antimicrobial activity and the obtained values for this film had significant difference with BCNF loaded films and BCNF-immobilized lysozyme films. This investigation indicates that the lysozyme after immobilization into BCNF revealed a high antimicrobial activity (Liu et al., 2018). Films containing both free and immobilized lysozyme had different antimicrobial activity against Gram-positive and Gram-negative bacteria, and the strongest effect of films was on Gram-positive *S. aureus* and *L. monocytogenes* bacteria. It is believed that Gram-negative bacteria have external lipopolysaccharide membrane in addition to the peptidoglycan layer whereas Gram-positive bacteria only have cell membrane and peptidoglycan layer (Park et al., 2013; Shankar et al., 2014; Shankar et al., 2015).

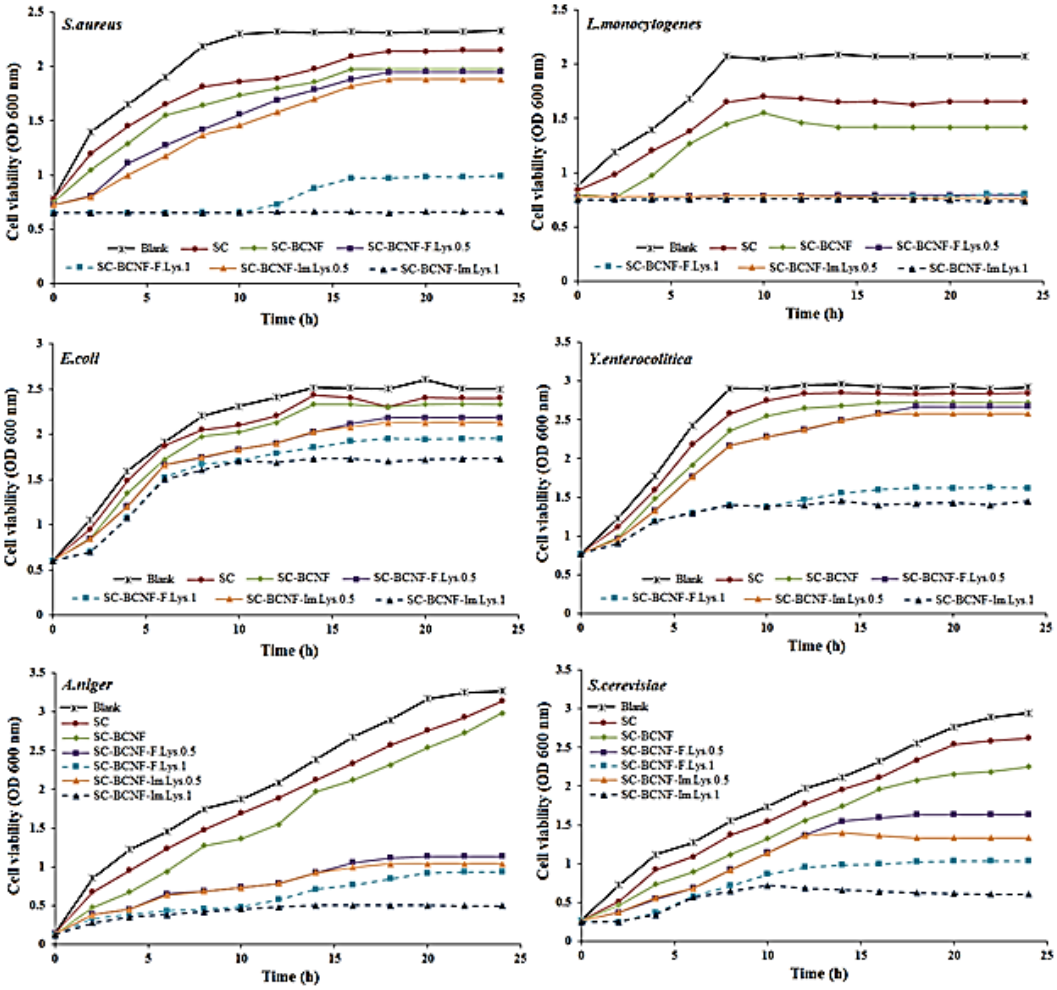

Fig. 4. Antimicrobial activity of SC based films containing free and BCNF-immobilized lysozyme against Gram-positive bacteria (*S. aureus* and *L. monocytogenes*), Gram-negative bacteria (*E. coli*, and *Y. enterocolitica*) mold (*A. niger*) and yeast (*S. cerevisiae*).

Table 2. Mechanical properties of free and immobilized lysozyme loaded SC films.

Films	Tensile strength (MPa)	Young's modulus (MPa)	Elongation to break (%)
SC	3.45±0.43^{a}	400.76±5.65^{a}	21.32±5.65^{c}
SC-BCNF	6.81±0.76^{c}	1111.87±3.76^{e}	14.54±0.78^{a}
SC-BCNF-F.Lys.0.5	6.00±0.02^{c}	902.87±0.78^{c}	15.50±0.65^{a}
SC-BCNF-F.Lys.1	5.32±0.21^{b}	866.65±3.45^{b}	17.87±0.76^{b}
SC-BCNF-Im.Lys.0.5	6.65±0.98^{c}	954.65±5.98^{c}	14.65±1.00^{a}
SC-BCNF-Im.Lys.1	6.15±0.91^{c}	1004.15±4.14^{d}	13.87±1.90^{a}

Values are given as mean ± standard deviation.
Means with same superscripts are not significantly different ($p > 0.05$).

It must be mentioned that the immobilized enzyme has more bactericidal effect on both Gram-positive and Gram-negative bacteria compared to free enzyme (Uddin Khan et al., 2017). As it is shown in the Fig. 4, the immobilized enzyme stopped the growth of Gram-positive bacteria after 10 h while in Gram-negative bacteria, after 16 h the growth was reached to equilibrium. In free enzyme, in the same concentration, after 18 h the growth was stopped. Lysozyme enzyme is one the most important preservatives and antimicrobial agents (Akhondzade et al., 2007). Lysozyme has strong inhibiting effect on mold and yeast. As it is see in the Fig. 4, enzyme has prevented the growth of *Aspergillus* and *Saccharomyces* from the early hours. These results are consistent with the results obtained by Wagh et al. (2013) on the using of lysozyme in cheddar cheese packaging conducted a study on immobilized lysozyme and investigated its effects on *E. coli* and gained similar results. Also Kuorwel et al. (2012) did a research on cheddar cheese packaging with an edible coating containing lysozyme on viability of *A. niger* and achieved similar results. The

results also are consistent with the results obtained by Park et al. (2013) and Hanusove et al. (2013).

4. Conclusion

This research was one of the rare reports on the using of immobilized enzymes in the matrix of food active packaging materials. It demonstrated that although the activity of lysozyme would be decreased after immobilization on BCNF, however, the films containing immobilized lysozyme had a good antimicrobial potential against various pathogens. This research approved that BCNF is a suitable substrate for immobilization of lysozyme that can be used simultaneously as immobilizing support and reinforcing agent. SC films containing immobilized lysozyme exhibited good mechanical and barrier properties and can be used as active food packaging material. Further investigations focused on the final using of these active films for shelf life extension of various foods are required in order to explore the importance of these prepared biodegradable active films.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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