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Facile preparation of surface *N*-halamine chitin nanofiber to endow antibacterial and antifungal activities

A.K. Dutta [a], M. Egusa [a], H. Kaminaka [b], H. Izawa [a], M. Morimoto [a], H. Saimoto [a], S. Ifuku [a,*]

[a] Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, 4-101 Koyama-cho Minami, Tottori 680-8552, Japan
[b] Faculty of Agriculture, Tottori University, 4-101 Koyama-cho Minami, Tottori 680-8553, Japan

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ABSTRACT

N-halamine chitin nanofiber (NF) film was prepared by the reaction of chitin NF film with sodium hypochlorite solution to endow the film with antibacterial and antifungal activities. The amount of active chlorine content loaded on the chitin NF film depended on the sodium hypochlorite concentration and reaction time. FT-IR, UV–vis, XRD, and TG analyses showed that the N—H bond was substituted to the N—Cl bond and that the reaction took place at the chitin NF surface. After chlorination, the characteristic nanochitin morphology was maintained. Although the active chlorine content of the film gradually decreased by disassociation of the N—Cl bond, chlorine was rechargeable into chitin NF by another sodium hypochlorite solution treatment. The chlorinated chitin NF film showed strong efficacies against Gram-negative and -positive bacteria of *Escherichia coli* and *Staphylococcus aureus*, respectively. Moreover, the films showed 100% and 80% inhibition of spore germination when faced against *Alternaria alternata* and *Penicillium digitatum* fungi, respectively.

1. Introduction

Chitin is a natural carbohydrate polymer that abounds in the exoskeletons of crustaceans and in fungal cell walls. Despite chitin's enormous abundance, most crab and prawn shells are thrown away as industrial waste. Recently, we developed a method to isolate chitin nanofibers (NFs) from those shells by a simple mechanical disintegration (Ifuku et al., 2009; Ifuku, Nogi, et al., 2011; Muzzarelli, 2012). Although chitin is insoluble and precipitates in water, chitin NFs can disperse homogeneously. So the dispersion is easy to handle and to shape into desired forms for specific applications. The morphological characteristics of chitin NFs include a thickness of approximately 10 nm and efficient mechanical properties owing to its extended crystalline structure (Abe, Ifuku, Kawata, & Yano, 2014). By making use of these characteristics, we have prepared optically transparent chitin NF composites with acrylic resins (Ifuku, Morooka, Nakagaito, Morimoto, & Saimoto, 2011). Chitin NF could significantly increase the Young's moduli and the tensile strengths of acrylic resins, and decrease thermal expansion without losing transparency or flexibility by virtue of chitin NFs' reinforcement effect. Moreover, chitin NFs have powerful biological activities. For instance, they have anti-inflammatory actions via suppressing NF-κB and MCP-1 activations, and suppress fibrosis in an acute ulcerative colitis mouse model, indicating that chitin NF is potentially a novel medicine or functional food for patients with inflammatory bowel disease (Azuma, Osaki, Ifuku, et al., 2012; Azuma, Osaki, Wakuda, et al., 2012). Furthermore, application of chitin NF to skin improved the epithelial granular layer and increased granular density and resulted in a lower production of TGF-β, indicating that they can be incorporated into the manufacture of cosmetics or textiles (Ito et al., 2014). Consequently, we expect that nanochitin with the above-mentioned properties will have increased applicability as a novel bio-material (Ifuku & Saimoto, 2012). However, chitin NFs do not have antimicrobial properties.

N-halamine has received much attention owing to its attractive functions, such as its efficacy against microorganisms as well as its stability, rechargeability, and nontoxicity to humans (Kocer, Cerkez, Worley, Broughton, & Huang, 2011; Worley et al., 2003). A covalent bond forms between nitrogen and halogen atoms (N—X) in *N*-halamine-based material. In halamine, chlorine has generally been used to connect with amine, amide, or imide groups due to the ease of modifying chlorine as well as to its safety, its green reaction in water, and its low cost as a reagent (Cao & Sun, 2009; Lauten et al., 1992; Worley & Williams, 1998). Diluted sodium hypochlorite solution, common household bleach, is can be used for *N*-chlorination. The antimicrobial properties of *N*-chlorine are the result of the direct transfer of chlorine ions (Cl^+) to receptors in the

* Corresponding author. Tel.: +81 857 31 5592; fax: +81 857 31 5813.
E-mail address: sifuku@chem.tottori-u.ac.jp (S. Ifuku).

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cells of microorganisms. Thus, *N*-halamine can kill microorganisms without the release of free chlorine (Barnes et al., 2006).

Some researchers have applied *N*-chlorination to chitosan, which is produced by the deacetylation of chitin to obtain antimicrobial chitosan, although chitosan itself has antimicrobial activity in nature (Cao & Sun, 2008; Cheng, Ma, Li, Ren, & Huang, 2014; Li, Hu, Ren, Worley, & Huang, 2013; Shin et al., 2014). On the other hand, there has been no report about *N*-halamine-based chitin, although the acetamide group in chitin could be used for *N*-chlorination. This is because chitin is an insoluble powder and difficult to handle as a biomaterial. Since chitin NFs can homogeneously disperse in water, they will be useful for the reaction. Here, we demonstrated *N*-halamine chitin NFs for the first time and used them to endow chitin NF with antibacterial and antifungal properties.

2. Experimental

2.1. Materials

α-Chitin from crab shell was purchased from Koyo Chemical and used as received. The molecular weight determined by viscosity method and degree of acetylation determined by elemental analysis were 65 kDa and over 94%, respectively. Sodium hypochlorite solution with 5% active chlorine content was from Wako Pure Chemical Industries and was used throughout this study. The other chemicals were analytical grade and used without further purification.

2.2. Preparation of N-halamine chitin nanofiber film

Chitin NFs were prepared according to a previously reported method (Dutta et al., 2013). In brief, dry chitin powder was dispersed in water at 1 wt.%. Acetic acid was added to the chitin dispersion to adjust the pH to 3. It was passed through a high pressure water-jet system (Star Burst Mini, HJP-25001S, Sugino Machine Co., Ltd.) equipped with a ball-collision chamber for mechanical disintegration. The treatment was conducted for 30 cycles for nanofibrillation. The obtained chitin NF was dispersed in water at a fiber content of 0.1 wt.%. The water was poured into a glass petri dish and dried at 40 °C overnight. The resultant cast film had a uniform surface with a thickness of approximately 25 μm and a weight of 110 mg. For *N*-chlorination, the chitin NF film was soaked in a preset amount of sodium hypochlorite solution at room temperature for a predetermined period of time. After *N*-chlorination, the film was washed with deionized water thoroughly and air-dried. The washing water was tested with potassium iodide/starch to ensure that the free chlorine was completely removed.

To study the stability of the chlorinated chitin NF film, the film was stored at 25 °C and the chlorine content was tested periodically for a 30-day storage time. For the rechargeability test, the film was treated with 0.3% sodium thiosulfate aqueous solution at room temperature for 30 min to quench the active chlorine and then chlorinated again according to the above-mentioned chlorination procedure with sodium hypochlorite.

2.3. Measurement of active chlorine content

Iodometric titration was employed to determine the active chlorine content loaded onto the chitin NF film (Johnson et al., 2000). Approximately 0.05 g of chlorinated chitin NF was dispersed in 5 mL of deionized water containing 1 wt.% acetic acid. One gram of potassium iodide was added and the mixture was stirred vigorously for 1 h at room temperature. After then, the titer titrated against the titrant sodium thiosulfate, and 1 mL of 1% starch solution was added as an indicator near the end point. The oxidative chlorine in the film was calculated using the following equation:

$$[Cl^+] = \frac{V \times N \times 35.45}{W \times 2} \times 100$$

where $[Cl^+]$ is the wt.% of oxidative chlorine on the chitin NF, V is the volume of consumed sodium thiosulfate solution in one liter, N is the normality of the titrant (equiv./L), and W is the weight of the chlorinated chitin NF sample in grams.

2.4. Characterization of chlorinated chitin NF film

The infrared spectra of the chitin NF film and the chlorinated one were recorded with an FT-IR spectrophotometer (Spectrum 65, Perkin-Elmer Japan Co., Ltd.) equipped with an ATR attachment (diamond/ZnSe crystal) with a resolution of 4 cm^{-1}.

X-ray diffraction (XRD) profiles of the samples were obtained with Ni-filtered CuK_α from an X-ray generator (Ultima IV, Rigaku) operating at 40 kV and 30 mA. The diffraction profile was detected using an X-ray goniometer scanning from 5° to 35°.

The regular light absorbances of these samples were measured using a UV–vis spectrophotometer (V550; JASCO, Tokyo, Japan).

The thermal stability of the chlorinated chitin NF film was studied on a TG/DTA 6300 (Seiko Instruments). The sample weighed approximately 5 mg and was heated in argon at a rate of 10 °C min^{-1} up to 500 °C.

For field emission scanning electron microscopic (FE-SEM) observation, the chitin NF film was coated with an approximately 2 nm layer of Pt by an ion sputter coater and observed by FE-SEM (JSM-6700F; JEOL, Ltd.) operating at 2.0 kV.

2.5. Antibacterial activity test

Escherichia coli (*E. coli*, NBRC 3972, Gram-negative bacteria) and *Staphylococcus aureus* (*S. aureus*, NBRC 12732, Gram-positive bacteria) were used to study the antibacterial properties of the chlorinated chitin NF films. Both species were purchased from the NITE Biological Resource Centre (Chiba, Japan). We followed the "sandwich" system for antibacterial tests (Cao & Sun, 2008). Polypeptone-yeast broth solution (polypeptone 1%; yeast extract 0.2%; $MgSO_4$, 0.1%) was used to grow *E. coli* and *S. aureus* for 24 h at 37 °C. The bacteria were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS) (pH 7), and then resuspended in PBS to densities of 10^8–10^9 CFU/mL. The chlorinated chitin NF films were cut into small pieces (ca. 1 cm × 1 cm), and the films were sterilized for 15 min on each side under UV light. Ten microliters of an aqueous suspension containing 10^8–10^9 CFU/mL of *E. coli* or *S. aureus* was placed onto the surface of a film. Another, identical film was used for sandwiching to ensure adequate contact with the bacteria. After a certain period of contact time, the entire sandwich was transferred into 10 mL of 0.03 wt.% sodium thiosulfate aqueous solution. The mixture was vigorously shaken for 5 min and sonicated for 10 min. Cao et al. demonstrated that sodium thiosulfate solution at this concentration could quench the active chlorines in the films without affecting the growth of the bacteria (Cao & Sun, 2008). An aliquot of the solution was serially diluted, and 100 μL of each dilution was plated onto agar plates (Luria Bertani agar for *E. coli*, polypeptone-yeast agar for *S. aureus*). A similar procedure was applied to unchlorinated chitin NF films as positive controls. Bacterial colonies were counted after incubation at 37 °C for 24 h.

2.6. Antifungal activity test

Alternaria alternata (strain O-94) and *Penicillium digitatum* (strain P1), were selected as illustrative examples of fungi for the

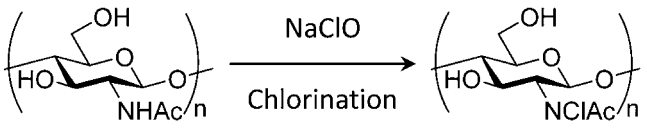

Fig. 1. Surface *N*-chlorination of chitin nanofiber.

antifungal activity study of the chitin NF films following the sandwich system. Both fungi were maintained on potato dextrose agar (Difco, Detroit, MI, USA) slopes. For antifungal activity tests, spores of *A. alternata* were prepared as previously described (Johnson et al., 2000). For sporulation, *P. digitatum* was grown on a PDA agar plate at 24 °C for 1 week. Upon suspension in PBS, the concentration of fungi was found to be 10^7 spores/mL. Ten microliters of spore suspension (10^7 spores/mL) was placed onto the surface of one of the chlorinated chitin NF films (1 cm^2) and sandwiched by the other. After 24 h of contact time, the entire sandwich was harvested in 5 mL sodium thiosulfate aqueous solution (0.03%) and sorted out as described above. Unchlorinated chitin NF films were challenged against the same fungal suspension under identical conditions as the positive and negative controls. After incubation for 24 h, the inhibition of spore germination was observed under a light microscope. Each antifungal test was repeated three times.

3. Results and discussion

3.1. Active chlorine content of N-halamine chitin nanofiber

Chitin NF film was treated with diluted sodium hypochlorite solution to introduce chlorine into the acetamide group at the C2 position (Fig. 1). Fig. 2 shows the effect of the sodium hypochlorite concentration on the amount of active chlorine content introduced in chitin NF film with 30 min chlorination time. After NaClO treatment with 0.25% and 0.50% concentrations, the active chlorine content increased steeply to 1.64% and 2.48%, respectively, by the loading of chlorine onto chitin NF. However, above 0.50% NaClO concentration, the reaction rate decreased, and chlorine content was almost saturated, reaching 2.69% by the 1.00% concentration treatment. Fig. 3 shows the effect of chlorination time on the active chlorine content loaded on chitin NF using 0.50% NaClO diluted solution. The active chlorine content rapidly increased to 1.35% after only 5 min chlorination time, reaching 2.48% after 30 min. However, chlorination times of more than 30 min did not increase chlorine content effectively. These saturation behaviors in chlorination are caused by the heterogeneous reaction of chitin NF crystal (Ifuku, Morooka, Morimoto, & Saimoto, 2010). First, the chitin NF surface was preferentially chlorinated with a higher reaction speed,

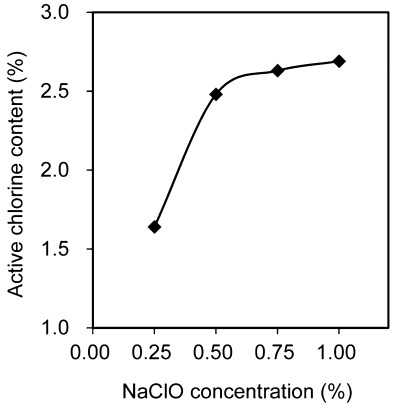

Fig. 2. Effect of sodium hypochlorite concentration on active chlorine content loaded on chitin nanofiber.

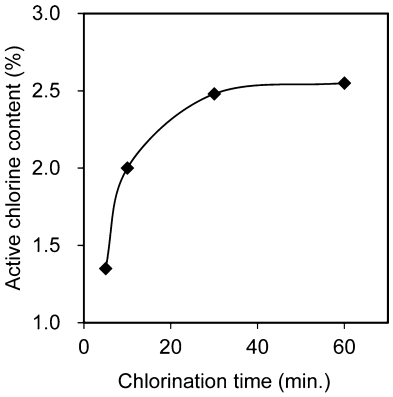

Fig. 3. Effect of chlorination time on active chlorine content in chitin nanofiber film.

and then the inside crystalline core part of chitin NF reacted gradually. From Figs. 2 and 3, the saturated active chlorine content corresponded to approximately 15% of the degree of substitution (DS). The chlorination DS seems reasonable to a certain extent, since the DS of a surface-modified chitin NF previously reported was approximately 20% (Ifuku, Suzuki, Izawa, Morimoto, & Saimoto, 2014).

3.2. Structural characterization of N-halamine chitin nanofiber

The FT-IR spectra of *N*-halamine chitin NF are studied (Fig. S1). After the chlorination reaction of chitin NF, the amide II band at 1554 cm^{-1} corresponding to the N—H bending vibration mode was decreased and slightly shifted to 1558 cm^{-1} (Gopalan & Dufresne, 2003). This is obviously due to the partial substitution of the N—H bond to the N—Cl bond (Ren et al., 2008).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.08.094.

The UV–vis spectra of *N*-halamine chitin NF film are shown in Fig. 4. After chlorination, a broad absorption peak at approximately 320 nm appeared. The peak is known to be caused by the disassociation of the N—Cl bond; the disassociation also provides evidence of *N*-halamine formation of chitin NF after sodium hypochlorite treatment (Czech, Fuches, & Antczak, 1961; Kleinberg & Audrieth, 1954).

An XRD study was carried out to investigate the effect of the chlorination treatment on the crystalline structure of chitin NF (Fig. S2). After chlorination, all diffraction peaks observed at 9.2°, 19.2°, 20.7°, and 22.8° coincided with those of the original chitin NF. They were typical antiparallel crystal patterns of α-chitin (Minke & Blackwell, 1978). Therefore, the chlorination did not change the

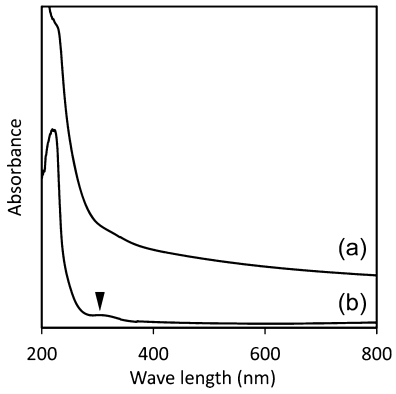

Fig. 4. UV–vis spectra of (a) chitin nanofiber film and (b) the chlorinated derivative with 2.63% active chlorine content.

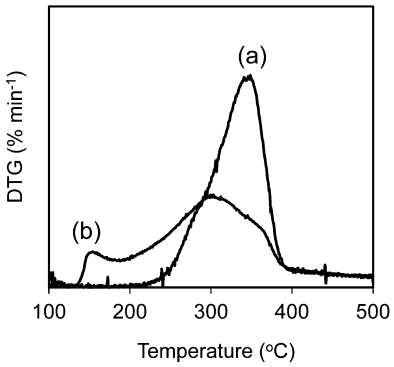

Fig. 5. Derivative TG analysis of (a) chitin nanofiber film and (b) the chlorinated derivative with 2.63% active chlorine content.

crystalline structure of chitin NF, thus indicating that chlorination took place on the surface of chitin NF, as mentioned above.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.08.094.

Fig. 5 displays derivative TGA curves of chitin NF films before and after sodium hypochlorite treatment. The original chitin NF film showed a single degradation peak at 348 °C. On the other hand, the chlorinated sample had a major broad degradation peak in the range of around 300–360 °C and a minor degradation peak at 155 °C. The major degradation would correspond to the decomposition of unchlorinated chitin NF. The minor degradation at 155 °C would be due to the decomposition of the *N*-halamine part on the chitin NF surface, since the association energy of the N—Cl bond is lower than that of the N—H bond (Sun & Sun, 2001, 2004). Thus, chlorination accelerated the thermal decomposition of chitin.

FE-SEM images of the chitin NF before and after chlorination are shown in Fig. 6. The characteristic nanofiber morphology was maintained after the reaction. The fiber width was also similar to that of the original chitin NF. Similar fiber width also indicates that balky chlorine was introduced on the chitin NF surface.

The stability and rechargeability of the *N*-halamine chitin NF film was studied (Fig. 7). The active chlorine content of freshly prepared *N*-halamine chitin NF film was 2.69%. The content gradually decreased with increasing storage time due to the disassociation of the N—Cl bond and became 2.0% after 15 days of storage. However, further storage up to 30 days total did not remarkably reduce the chlorine content. After 30 days of storage, we could introduce chlorine on the chitin NF surface again by another sodium hypochlorite treatment. After the second chlorination treatment under the same conditions, the active chlorine content was recovered to 2.60%. Thus, chlorinated chitin NF has excellent rechargeability to recover antimicrobial and antifungal activity to be as described next.

3.3. Antimicrobial and antifungal efficacy

Table 1 presents the biocidal efficacy results of unchlorinated and chlorinated chitin NF films against Gram-negative *E. coli* and Gram-positive *S. aureus*. Chitin NF films containing 2.69% of active chlorine were challenged with 10^8–10^9 CFU/mL each of *S. aureus* and *E. coli*, respectively. Data showed that the efficacy of the films increased as the contact time increased, and about a 100% kill rate for *E. coli* was achieved within just 10 min of contact time. However, all the microbes of the stated bacterial species died within 30 min, which was treated as a total kill, within the contact area of the chlorinated films. On the other hand, the unchlorinated chitin NF film did not show any antimicrobial activity against these pathogens, even after 60 min of contact time. This reminds us that chitin nanofibers have no antibacterial functions.

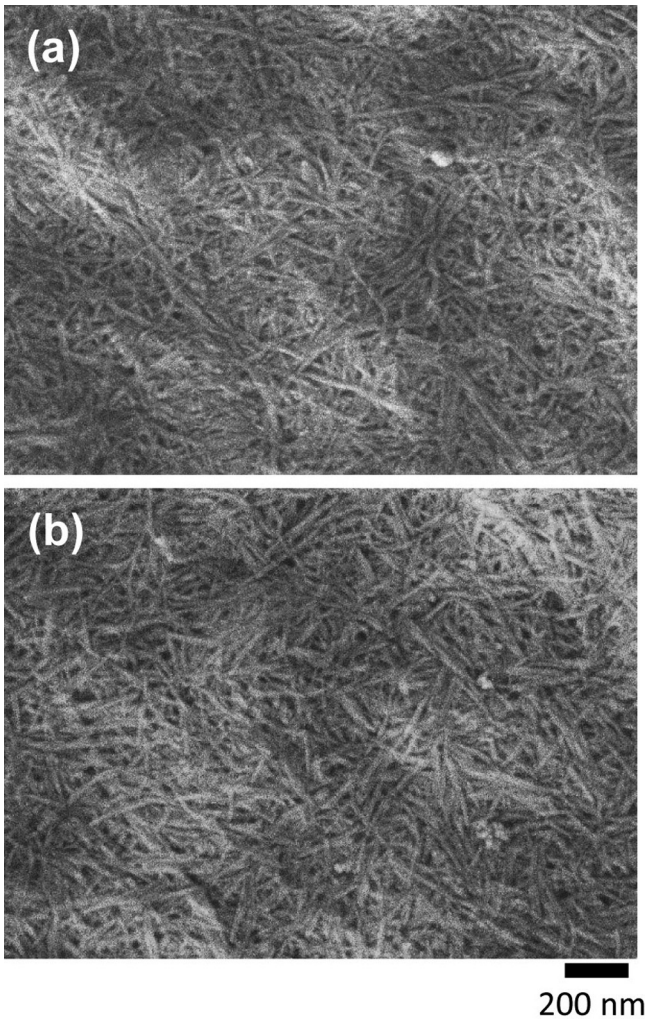

Fig. 6. SEM images of (a) chitin nanofiber film and (b) the chlorinated derivative with 2.63% active chlorine content.

A. alternata is a mainly saprophytic fungus, but it is also known as a plant pathogen and as an allergen in humans. On the other hand, *P. digitatum* is a common postharvest pathogen causing a green mold on citrus fruits. The antifungal activity of chlorinated chitin NF films was assessed by the percentage inhibition of spore germination (Fig. 8) of an individual fungus, where the original spore concentration was 10^7 spores/mL.

The most striking finding was that the chlorinated chitin NF film inhibited the growth of *A. alternata* spores by almost 100%

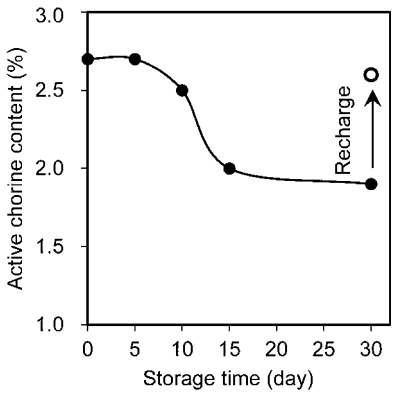

Fig. 7. Storage stability and rechargeability of chlorinated chitin nanofiber film.

Table 1
Percentage reduction of *E. coli* and *S. aureus*.

Contact time (min)	*E. coli*[a] (%)		*S. aureus*[a] (%)	
	Un-chlorinated CNF film	Chlorinated CNF film[b]	Un-chlorinated CNF film[b]	Chlorinated CNF film[b]
5	0	86.4	0	46.7
10	0	99.9	0	87.8
30	0	Total killed	0	Total killed
60	0[c]	Total killed	0[c]	Total killed

[a] *E. coli* and *S. aureus* concentration were 10^8–10^9 CFU/mL.
[b] The chlorinated CNF film contained 2.69% of active chlorine.
[c] Compared with the antibacterial result of cellulose membrane, which was used as the negative control in the antimicrobial study.

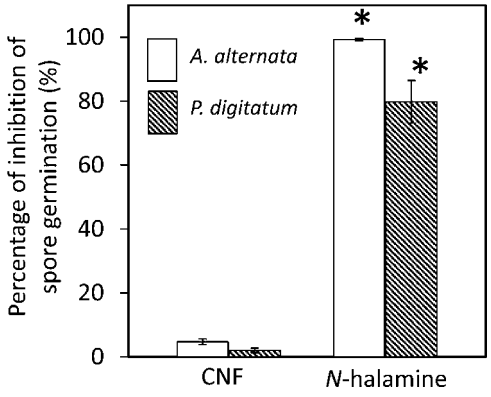

Fig. 8. Antifungal effect of chlorinated chitin NF films with 2.69% active chlorine content. Spores were sandwiched between chitin NF film for 24 h. Spores were harvested and placed on cellulose membrane for microscopic observation. Data represent the mean of three independent experiments and error bars. Asterisks indicate significant differences with Tukey's test between films ($P < 0.05$).

compared with the controls. In contrast, the germination of *P. digitatum* spores was more difficult to stop, 80% of the spores were inhibited. We stated earlier that spores were sandwiched between chitin NF films for 24 h and that the accuracy of the results was verified by Tukey's test ($P < 0.05$). These findings are fascinating, as the transfer of oxidative chlorine from the chitin NF films to the appropriate receptors in microorganisms could effectively destroy or inhibit the enzymatic or metabolic process, leading to the expiration of the microorganisms.

4. Conclusion

N-halamine-based chitin NF film was prepared successfully by using easily available diluted sodium hypochlorite. The concentration of sodium hypochlorite and the reaction time strongly affected the active chlorine content on the films. FT-IR, UV–vis, XRD, and TGA analyses showed the presence of a N—Cl bond on the chitin NF surface. Chlorine was rechargeable onto chitin NF by the same sodium hypochlorite treatment. All the microbes of *E. coli* and *S. aureus* died within 30 min of contact with the chlorinated chitin NF film. Moreover, 100% of *A. alternata* and 80% of *P. digitatum* fungal spore stopped their germination upon 24 h treatment with the chlorine-containing chitin NF films. Here, chitin NF acquired antibacterial and antifungal properties in addition to various characteristics, such as nanostructure, high surface area, excellent mechanical properties, stable dispersibility in water, facile formability, and several biological properties compared to conventional chitin powder. Chlorination will expand the range of commercial applications of chitin NF, especially in medical, food, and cosmetics fields.

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