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Characterization of Blend Films of Chitosan/Poly-Saccharide from *Grifola frondosa*

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Films were prepared by the casting method using chitosan (CH) and polysaccharide from *Grifola frondosa* (GF) in different ratios. The concentration of GF ranged from 0-80 % (v/v). Films were evaluated for mechanical and swelling properties. Water vapour transmission rate and oxygen permeability of the films were also investigated. The water vapour transmission rate did not change significantly upon addition of GF. Films containing 20 % (v/v) GF showed very low oxygen permeability, good tensile and puncture strength. It indicated that blend films owned better properties against degradation *in vitro* than that of chitosan film. The antimicrobial activity of films containing 20 % (v/v) GF was comparable to CH films against *Staphylococcus aureus* (MRSA) (ATCC 25923). Composite films obtained from CH and GF may be potentially applied to a broader field in wound dressings.

Key Words: Chitosan *Grifola frondosa* dressing.

INTRODUCTION

The skin is considered the largest organ of the body and has many different functions. skin loss occurs frequently as a result of burn, trauma and diseases. It needed dressings for wound healing[1]. The field of wound healing has been major emphasis in chitosan-based medical applications research.

A number of researchers have examined the host tissue response to various chitosan-based implants. In general, these materials have been found to evoke a minimal foreign body reaction, with little or no fibrous encapsulation[2]. It observed the typical course of healing with formation of normal granulation tissue, often with accelerated angiogenesis. Chitosan (CH), have received much interest for their application in agriculture, biomedicine, biotechnology and food industry due to their biocompatibility, biodegradability and bioactivity[3]. Due to its antimicrobial activity[4], CH film is a promising biomaterial that can be using in wound healing[5]. However, functional properties of chitosan films can be improved by combining it with other hydrocolloids[6]. Thus importance is attached to the research on the combination of CH with other macromolecules.

Grifola frondosa (maitake) is an edible and medicinal mushroom. *Grifola frondosa* (Basidiomycetes, Aphyllopherales, Polyporaceae) is an edible mushroom that has recently received much attention, not only because of its nice almondy flavor but also because of its medicinal properties, which include antioxidant, hypotensor, antitumoral, immunomodulating, immunostimulating, antiinflamatory, hypocholesterolemic and hypoglycemiant activities[7-9]. Among the many bioactive compounds reported from the *Grifola frondosa* fruiting body, polysaccharide fractions have been shown to enhance immune cell activities and thus to be useful as an adjunct to cancer and human immunodeficiency virus therapy[10].

Vitamin C (ascorbic acid) played an important role during the course of wound healing. As an antibacterial oxidant, vitamin C can strengthen the body's immune ability and promote inflammatory chemotaxis function of all the tissue. All wounds can bring forth lots of oxygen free radicals under the polluted circumstance. The oxygen free radicals have the function of improving the permeability of capillary, injuring the endothehal cell of vas, agfravating ischemia, edema and hypoxia of the wound tissue. Vitamin C takes part in compounding the collagenous protein and interstitial histiocyte, changing permeability of the wall of capillary. It not only renews tissue and reduces exudation, but also accelerates the velocity of wound healing[11-13]. It can also help chitosan solving in water for complex forming.

In the present study, we investigated that properties of blend films of chitosan/polysaccharide from *Grifola frondosa*. It may be potentially applied to a broader field in wound dressings.

EXPERIMENTAL

Chitosan (M_η: 1.0×10^5-1.7×10^5, deacetylation degree: 75-85 %) was purchased from Sigma. The dried powder of polysaccharide from *Grifola frondosa* was purchased from

Tianrun Maitake Co. Ltd. (Qingyuan, China). Vitamin C was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Three-times recrystallized eGF-white lysozyme was purchased from Sigma Chemical Co. (USA) and used without further purification. Common burn-wound pathogens, namely methicillin-sensitive and resistant *Staphylococcus aureus* (MRSA) (ATCC 25923) was obtained from the American Type Culture Collection (Manassas, VA). Nutrient broth (NB) and nutrient agar were used as the culture medium of the bacteria.

Preparation of blend films of chitosan/polysaccharide from *Grifola frondosa*: 2 g of vitamin C was dissolved in 100 mL of distilled water at 25 ºC under vigorous agitation to form a clear solution. The chitosan/polysaccharide from *Grifola frondosa* ratios were 100/0; 90/10; 80/20; 70/30; 60/40; 40/60; 30/70:20/80(w/w), relative to dry chitosan, with a total mass of 4 g. The chitosan and polysaccharide from *Grifola frondosa* were both dissolved in solution containing of vitamin C. The mixture was stirred vigorously at 25 ºC for 1 h and degassed. Homogeneous solution were obtained. Finally, it was poured on to plates and water evaporated at room temperature for 2 days. Films formed could be easily removed from the glass plates and had an average thickness of 65 μm. The films were free standing as they did not roll over or break. Visually, all the films were translucent. The films of different ratios above were coded as CG0, CG10, CG20, CG30, CG40, CG50, CG60, CG70 and CG80, respectively. The blend films containing more than 80 % *Grifola frondosa* had little mechanical strength, so they were discarded. The pure *Grifola frondosa* film has little mechanical strength too.

Characterization of the CG films

FTIR of the blend films: IR spectra of the films were recorded using a Fourier transform infrared (FTIR) spectrometer (Nicolet 170SX, USA) with attenuated total reflection instruments for investigation intermolecular interaction. The films were taken on the flat sheet and data were collected over 64 scans with a resolution of 4 cm^{-1} at room temperature.

Microstructure observation of the blend films: Scanning electron microscopy (SEM) images of the films were taken with a microscope (JEOL 6700 F, Japan). The films were cut into pieces and snapped and then vacuum dried. The surface of the films were sputtered with gold and then observed and photographed.

Mechanical properties: Tensile strength (σb) and elongation (εb) at break of the films were measured on a versatile electron tensile tester (CMT-6503, Shenzhen SANS Test Machine Co. Ltd., China) with a tensile rate of 5 mm/min. The film samples were cut into strips that were 100 mm in length by 10 mm in width and fit to the tensile grips. The initial grip separation was set at 50 mm and the crosshead speed was set at 5 mm/min. The film specimens were mounted in the film-extension grips of the testing machine and stretched at a rate of 50 mm/min until breaking. Tensile strength was expressed in MPa and was calculated by dividing the maximum load (N) by the initial cross-sectional area (m^2) of the specimen. Puncture strength was evaluated using a needle probe of 2 mm in diameter at a constant rate of 1 mm/s and the results were expressed in terms of N. For both, tensile or puncture tests, each reported value corresponded to at least five determinations.

Water vapour transmission rate (WVTR) and oxygen permeability (OP): Water vapour transmission rate tests were carried out using an automatic water vapour permeability testing machine L80-5000 (PBI Dansensor, Denmark) at 37 ºC and 10/15 % relative humidity. Water vapour transmission rate of films was measured using aluminium sample cards (reduction to 5 % area). Oxygen permeability of the film was estimated with automated oxygen permeability testing machine OPT-5000 (PBI Dansensor, Denmark) at 23 ºC and 0 % RH. Sample was placed in sample holder having an exposed testing area of 50 cm^2. Oxygen permeability of each sample was averaged from three separate tests.

Water swelling properties: The membrane was immersed in distilled water/PBS (pH = 7.4)/HAc (pH = 3) for 72 h; then its surface moisture was wiped and the wet membrane weighed. Then this weighed wet membrane W_{wet} as dried at a fixed temperature (25 ºC) until constant weight as dry membrane, W_{dry}, was achieved. The membrane swelling can be calculated from dwelling $(Q_W) = [(W_{wet} - W_{dry})/W_{dry}] \times 100$ %.

***In vitro* degradation:** The blend films were immersed in 4 mg/mL lysozyme solution in 0.1 M PBS at pH 7.4 and 37 ºC. After determined intervals of time the films were taken out from the lysozyme solution, rinsed with double distilled water, dried and weighed. The extent of *in vitro* degradation was expressed as the percentage of the weight of the dried film after lysozyme treatment.

***In vitro* antibacterial test:** The antibacterial performance of the blend films were assessed by an *in vitro* bacteria culture. 100 μL MRSA-NB suspension with a concentration of 10^5-10^7 CFU/mL was plated on a nutrient agar plate. The plate was then supplemented with different kinds of blend films and incubated[14] further at 37 ºC for 12 h. The diameter of the antibacterial zone was then measured.

RESULTS AND DISCUSSION

ATR-IR analysis: FT-infrared is of importance to the study of the molecular structure. The width and intensity of spectral bands as well as position of the peaks are all sensitive to environmental changes and to conformations of macromolecules on the molecular level. Intermolecular interactions occur when different polymers are compatible. Therefore, the FTIR spectra of the blends are different from those of pure polymers, which is advantageous to the study of compatibility between two polymers. We explored the interaction between chitosan and polysaccharide from *Grifola frondosa* by ATR-IR. Fig. 1 shows the ATR-IR spectra of CG0, CG20, CG40, CG60 and CG80 in the 4000-400 cm^{-1} wave number range.

The characteristic absorption bands[15] of mannose in GF appeared at 890 and 870 cm^{-1}. The stretching vibration peaks of C-H of methyl at 2921 and 2894 cm^{-1} and the absorption band at 3400 cm^{-1} is assigned to the stretching of N-H groups bonded to -OH in chitosan[16]. The bands at 1674-1641 cm^{-1} was attributed to the characteristic absorptions of asymmetrical stretching vibration of NH_3^+ in chitosan[17]. The strong absorption band at 1641 cm^{-1} for *Grifola frondosa* film shifted to 1674 cm^{-1} and the bands at around 3633 cm^{-1} broaden and shifted to a higher wavelength with the increase of the *Grifola frondosa*

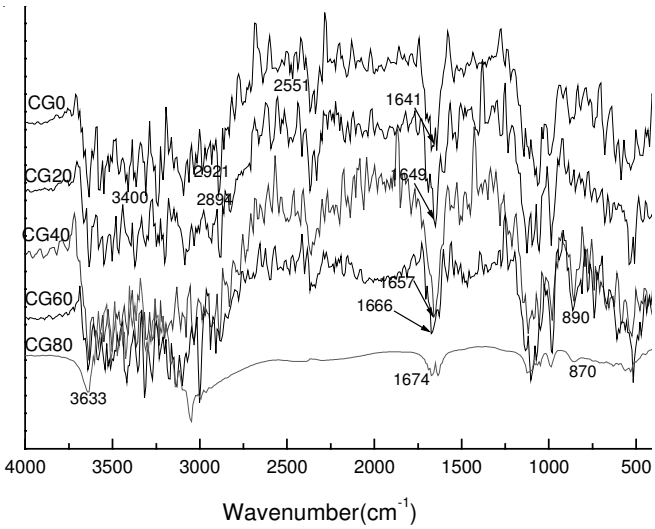

Fig. 1. FTIR of blend films

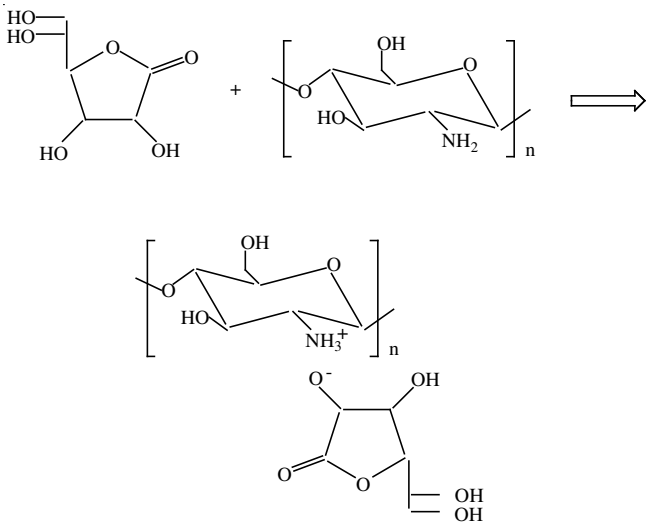

Fig. 2. Structure of complex of chitosan-vitamin-C

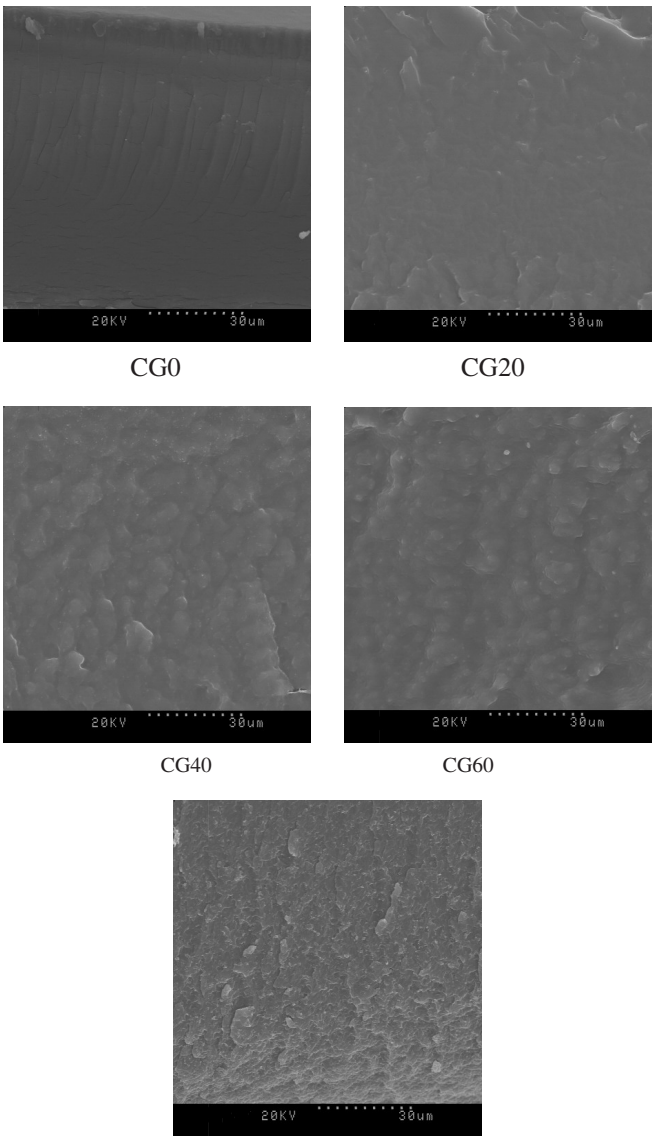

Fig. 3. SEM of blend films

content. Based upon these results, it seems that the NH_3^+ and OH groups in *Grifola frondosa* should participate in intermolecular hydrogen bonds with the OH and NH_3^+ groups in chitosan, respectively[18].

Morphological characteristics of the films: The surface exposure to air of the pure and blend films were examined by SEM. The morphology of the pure and blend films are shown in Fig. 3. The films of pure chitosan exhibit a uniform microstructure, which leads to the formation of homogenous films having densely packed molecules of chitosan. The blend films of CG20 and CG40 show smooth morphology, suggesting that high miscibility occurred when the content of the *Grifola frondosa* in the blend films was low. As the *Grifola frondosa* content increased, the morphology of the blend film surfaces changed from being smooth to becoming rough resulted from phase separation, which may lead to the decrease of mechanical strength of the blend films[16].

Mechanical properties: Adequate mechanical strength and extensibility are generally required for a film to withstand external stress and maintain its integrity as well as barrier properties during applications in biomaterials. Blends of polysaccharides with different ratios of polymers can be useful to improve tensile properties. The tensile strength (TS) of the different films was measured and is shown in Fig. 4. It can be seen from Fig. 3 that the TS increased with the incorporation of GF in CH films. Comparing the TS values between films of different ratio of CH-GF, it can be seen that there was a two-fold increase in tensile strength of CH films with a concentration of 20 % (v/v) GF. The high TS values of these films could be attributed to the formation of intermolecular hydrogen bonding between NH_3^+ of the CH backbone and OH^- of the GF. The amino groups (NH_2) of CH are protonated to NH_3^+ in the acetic acid solution, whereas the ordered structures of GF molecules are destroyed with the solubilization process, resulting in the OH^- groups being exposed to readily form hydrogen bonds with NH_3^+ of the CH. For pure CH films, TS values reported in literature were higher than those obtained in the present work and this may be attributed to CH composition, presence of plasticizer and method of film preparation[19,20]. However, further increase in concentration of GF (40-80 %, v/v) in blend, the tensile strength of films decreased significantly. This could

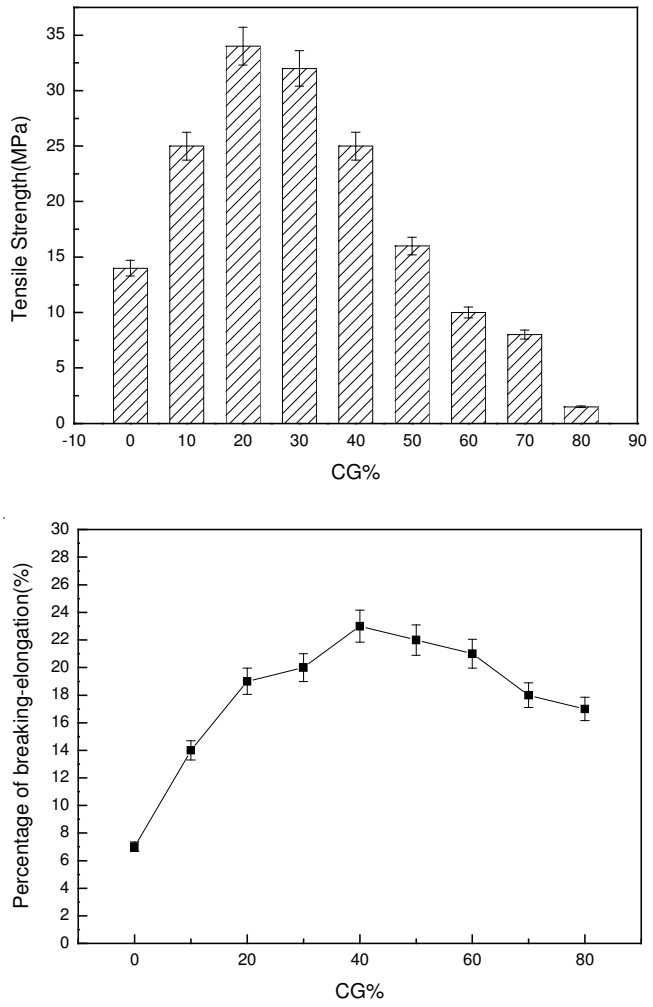

Fig. 4. Dependence of tensile strength and the elongation at break on the content of *Grifola frondosa* for the blend films

be attributed to thixotropic behaviour of GF solution which resulted in reduction in viscosities of film forming solution containin GF (40-80 %, v/v) in blend. Another explanation was that phase separation appeared gradually with the GF content. It resulted to the tensile strength of films decreased significantly with the increasing of GF content. The elongation at break showed similar trend as that of tensile strength. It may be attributed to GF could improve the plasticity of CH. It is well known that the dry pure chitosan film showed hard and brittle nature of film while pure GF film showed poor tensile strength.

The puncture force of films showed similar trend as that of tensile strength too. On addition of GF to CH films at concentration of 20 % doubled the puncture force. But further addition of GF led to decrease in the force (Fig. 5). These results indicate CH films with 20 % (v/v) GF had better mechanical properties as compared to other blends. It may act as a skin-barrier role in wound dressings for its proper mechanical strength and extensibility.

Water vapour transmission rate (WVTR) and oxygen permeability (OP): The water vapour transmission rate of films plays an important role in wound healing. Therefore, it is the most extensively studied property of films. Water vapour permeability is assumed to be independent of the water vapour

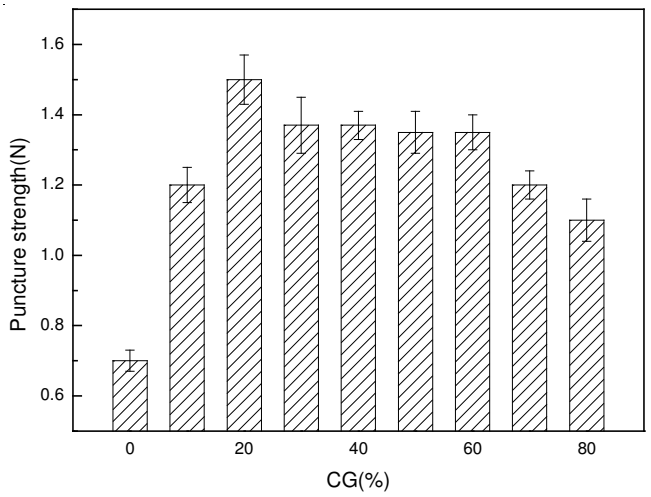

Fig. 5. Dependence of puncture strength on the content of *Grifola frondosa* for the blend films

pressure gradient applied across the films. However, hydrophilic materials, such as polysaccharide films, deviate from this ideal behaviour due to interactions of permeating water molecules with polar groups in the film's structure[21]. The WVTR values ranged from 4005-4125 g/m^2/day for the CH-GF films and did not change significantly with varying concentrations of GF (Table-1). Butler *et al.*[19] reported that the chitosan films have relatively poor water vapour barrier characteristics, which result from their hydrophilicity. Glycerol, through its plasticizing action, changes the polymer network creating mobile regions with larger interchain distances, promoting water clustering by competing with water at active sites of the polymer matrix and the formation of micro cavities in the polymer network structure. Water sorption by biopolymers often results in swelling and conformational changes. The absorbed water plasticizes the film matrix, leading to a less dense structure where chain ends are more mobile, thus increasing transmission rate[22].

TABLE-1
WVTR AND OP OF CH-GF FILMS

Film sample	WVTR (g/m^2/day) (%)	OP (mL/m^2/day) (%)
CG0	4110 ± 2.9	1840 ± 0.6
CG10	4060 ± 1.43	530 ± 1.9
CG20	4125 ± 1.95	230 ± 1.6
CG30	4005 ± 1.75	245 ± 1.7
CG40	4117 ± 0.87	190 ± 0.8
CG50	4080 ± 1.53	260 ± 1.2

Oxygen is the key factor for oxidation, which is responsible for changes O_2 and CO_2. Therefore, films that provide a proper oxygen barrier can help wound healing. Generally, hydrophilic biopolymer films show good oxygen barrier property. The oxygen permeability (OP) of films with different concentrations of CH and GF were compared (Table-1). Compared with GF, CH is more hydrophilic. Consequently, incorporation of GF to the polymer matrix at lower concentrations can reduce the oxygen barrier property of the films. When the volume ratio between CH and GF was 80:20, the blend film showed the lowest oxygen permeability. This result may be attributed to reducing compatibility and enhancing electrostatic repulsion between CH and GF. But further increase

in GF ratio in blend led to increase in oxygen permeability of films. It is known that the addition of GF contributes to the decrease of oxygen permeability, while it is normally accepted that a higher concentration of GF increases oxygen permeability[23]. Chitosan films with 20 % (v/v) GF films are more suitable for wound healing. This result indicates the potential of CH-GF films to be used as a wound dressing for its suitable oxygen permeability. It was close to the oxygen permeability of epidermis[24].

Water swelling properties: Owing to our interest in developing polymeric materials with improved properties, we have studied the degree of swelling of the blend films in double distilled water, PBS (pH = 7.4) and acid water(pH = 3). After 72 h, blend films has been solved in acid water(pH = 3) while not in double distilled water and PBS. As in shown in Fig. 6, with the *Grifola frondosa* content increased, swelling increased too. The swelling degree in PBS is lower than that in double distilled water. It couldn't be measured when the *Grifola frondosa* content beyond 50 % for it became scraps in water. It may be used as wound dressings for its appropriate swelling degree in PBS of CG20.

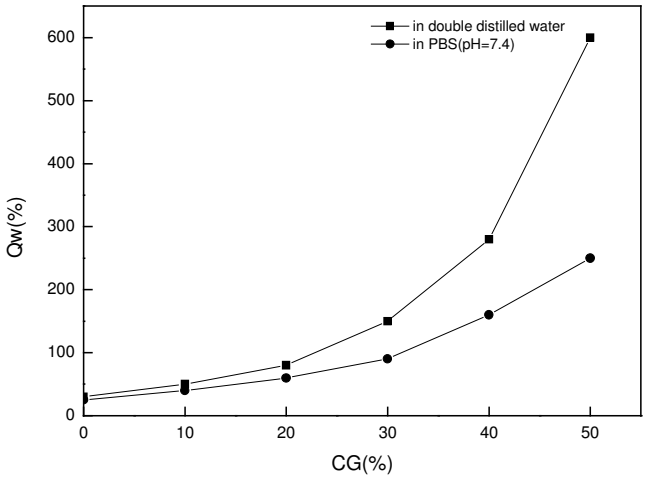

Fig. 6. Swelling degree of blend films

Degradation *in vitro*: In general, polysaccharides are degraded by enzymatic hydrolysis. chitosan is also reported to follow this principle. Indeed, they exhibit no appreciable degradation when brought into contact with aqueous neutral media containing no enzymes at room temperature[25].

Chitosan readily undergoes degradation in PBS containing lysozyme while GF not. The *in vitro* degradation of deacetylated derivatives with 4 mg mL^{-1} lysozyme is shown in Fig. 7. In contrast to chitosan, blend films are virtually resistant against the enzymatic hydrolysis at this lysozyme concentration.

***In vitro* antibacterial test:** The development of complementary methods to inhibit the growth of pathogenic bacteria such as biomaterial associated antimicrobial agents is an active area of research. A number of studies on the antimicrobial characteristics of films made from chitosan have been carried out by earlier workers[25]. Antibacterial activity of CH-GF films against methicillin-sensitive and resistant *Staphylococcus aureus* (MRSA) (ATCC 25923) is shown in Fig. 8. It can be seen from the figures that CH films were effective against the

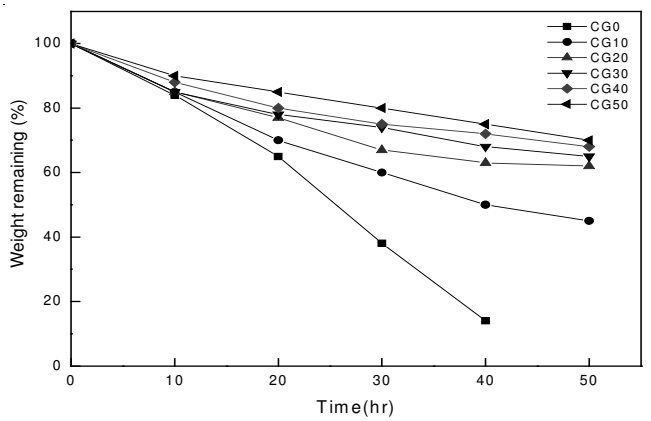

Fig. 7. *In vitro* degradation of chitosan and blend films in 4 mg mL^{-1} lysozyme solution of pH 7.4 at 37 ºC

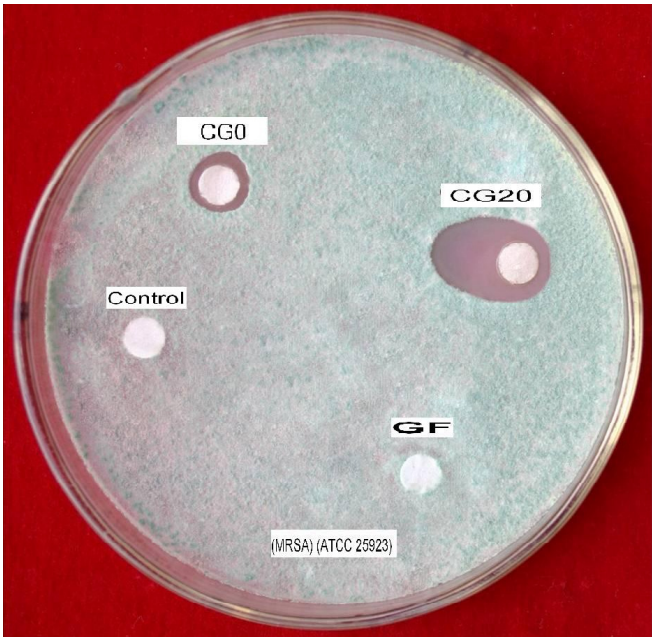

Fig. 8. Antibacterial activity of CG blend films against MRSA

organisms while GF not. Although, the exact mechanism of the antimicrobial action of CH and its derivatives is still imperfectly known, but different mechanisms for gram-positive and gram-negative bacteria have been proposed. One of the reasons for the antimicrobial character of CH is its positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms[26].

Conclusion

Chitosan and polysaccharide from *Grifola frondosa* can be used to make a composite film having desirable protective characteristics. The concentration of the two polysaccharides in the film affected mechanical and swelling properties of the films to various extents. The film prepared with the blend containing 80 % chitosan and 20 % *Grifola frondosa* (v/v) was found to be the best as it had lower oxygen permeability, better mechanical properties while retaining the similar antibacterial properties of chitosan, when compared with chitosan film not containing *Grifola frondosa*. Further studies would be required to determine the use of these films in commercial

medicine systems. It may be potentially applied to a broader field in wound dressings.

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