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Research article

PREPARATION AND CHARACTERIZATION OF CHITOSAN NANOCOMPOSITE FILMS IN DIFFERENT SOLVENT AND ITS ANTIMICROBIAL ACTIVITY

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ABSTRACT: In this present research programme,chitosan nanocomposites were prepared by using C 30B which is an organomodified nanoclay by solvent casting method and were characterized by FTIR, XRD and SEM. Different chitosan films like acetate, formate, citrate, lactate and ascorbate were also prepared by taking different types of solvent like acetic acid, formic acid, citric acid, lactic acid and ascorbic acid respectively. The properties like thickness, density and transparency of those films were studied. The antimicrobial activity of those films were also investigated against various food borne pathogens like *S.aureus, B.cereus, S.typhimurium and E.coli* and it was found that all chitosan citrate films showed higher inhibitory activity against all the types of pathogens.
Key words: Chitosan, Nanoparticle, Antimicrobial activity

INTRODUCTION

Naturally renewable biopolymers have attracted much research interest in recent yearsbecause of their potential use as edible and biodegradable films and coatings for food packaging.Chitosan, which can be derived from abundantly available chitin sources such as crustacean shellwastes, has excellent film-forming ability and inherent antimicrobial properties suitable for the development of edible antimicrobial films (Ralston and others 1964; Allan and Hadwiger, 1979;Stossel and Leuba, 1984; Kendra and others 1989; Sudarshan and others 1992; Yalpani andothers 1992; Fang and others 1994; No and others 2002). Moreover, the possibility of enhancingantimicrobial properties of chitosan by irradiation (Matsuhashi and Kume 1997), partialhydrolyzation (Davydova and others 2000), using different organic solvents (No and others2002), chemical modifications (Nishimura and others 1984; Tanigawa and others 1992),synergistic enhancement with preservatives (Chen and others 1996; Roller and others 2002), orby combing with other hurdle technologies, make them ideal for use as antimicrobial ediblepackaging materials.

Developing chitosan-layered silicate nanocomposites by inserting chitosan chains into interlayers of silicate can improve its mechanical properties. In recent years, polymer nanocomposites have received considerable interest because of their superior thermal and mechanical properties, as compared with the polymer itself(Sahoo D and Nayak P.L. 2011). Polymer-clay nanocomposites are a class of hybrid materials composed of organic polymer matrices and nanoscaleorganophilic clay fillers (Boninaet *al.* 2004). Of the nanoscale clays, montmorillonite (MMT) is of particular interest and has been studied widely. MMT is a hydrated alumina-silicate layered clay made up of two silica tetrahedral sheets fused to an edge-shared octahedral sheet of aluminum hydroxide. Its advantages of high surface area, large aspect ratio (50–1000), and platelet thickness of 10 Å make it suitable for reinforcement purposes (Bergeraet *al.*2004). The inorganic surface of MMT has also been modified by organic replacement of the interlayer sodium ions by various organic cations to make the platelets more compatible with polymers (Nayak P. L. and Sahoo D. 2011) .When nanoclay is mixed with a polymer, three types of composites (tactoids, intercalation, and exfoliation) can be obtained (Fig. 1). In the case of tactoids, complete clay particles are dispersed within the polymer matrix and the layers do not separate. Mixing a polymer and organoclay forms a micro-scale composite, with the clay serving only as a conventional filler. Intercalation and exfoliation are two ideal nano-scale composites. Intercalation occurs when a small amount of polymer is inserted between the layers of the clay, thus expanding the interlayer spacing and forming a well-ordered multilayer structure. In exfoliation, the layers of the clay are separated completely and the individual layers are distributed throughout the polymer matrix (Sahoo D. and Nayak P. L. 2012a. and Sahoo D. and Nayak P. L. 2012b). The formation of intercalation or exfoliation depends on the types and amounts of nanoclay used.

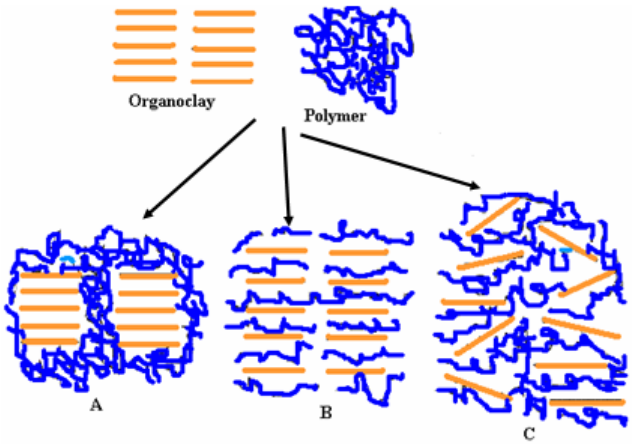

Figure 1. Schematic of possible composite structures obtained when mixing polymer with organoclay (A) Tactoids, (B) Intercalation and, (C) Exfoliation

Chitosan was used to form various flexible and transparent films resembling plastic packaging materials (Nadarajah and Prinyawiwatkul 2002). Properties of chitosan films are reported to be affected by the chitosan extraction process (Rout 2001; Nadarajah and Prinyawiwatkul 2002). Furthermore, various organic acids used to dissolve chitosan affect resultant film properties (Kienzle-Sterzer and others 1982; Caner and others 1998; Rhim and others 1998; Park and others 1999; Park and others 2002). Since a very limited literature is available on physicochemical properties of chitosan films, therefore, a variety of chitosan films can be developed using different film casting (organic acid) solvents and different amounts of addedplasticizer (glycerol).
The nanoclay used in this study for the preparation of chitosan nanocomposite films was Cloisite 30 B (C 30B) which is an organically modified Sodium in MMT with quaternary ammonium salts and the organic modifier in Cloisite 30B is methyl, tallow, bis-2 hydroxyethyl, quaternary ammonium, where tallow is 65% C18, 30% C16, and 5% C14.

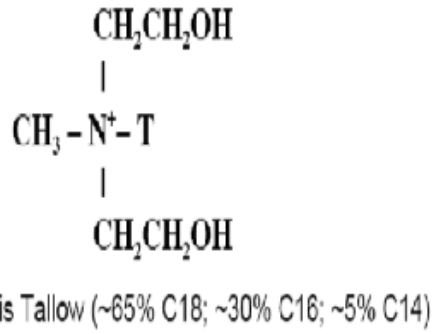

Figure 2. Structure of C 30B

EXPERIMENTAL

Materials

Chitosan was purchased from India Sea Foods, Kerala. Nano clay Cloisite 30B (C 30B), was purchased from Southern Clay Co.(USA). Acetic acid, Formic acid, Citric acid and other chemicals were used as analytical grade and purchased from Sigma–Aldrich Company.

Preparation of Chitosan Nanocomposite Films

Chitosan was separately dissolved with 1% of film casting solvents (acetic, formic, citric, lactic or ascorbic acids). Each solution was vigorously agitated for 30 minutes, immersed in boiling water for 10 minutes, cooled down to room temperature, and filtered through glass-wool to remove undissolved particles. Resultant film casting solution was divided into two batches.

One batch contained glycerol as a plasticizer at a ratio of 0.10, 0.20, 0.30, 0.40, and 0.50 (chitosan: glycerol, w/w), and the other batch was without a plasticizer. Nanoclay solutions with three clay compositions (1 wt %, 2.5 wt %, and 5 wt % based on chitosan) were prepared by dispersing appropriate amounts of clays into 10 ml of 1% acetic acid solution and vigorously stirring for 24 h. Afterwards, 200 ml of chitosan solution was added slowly into pretreated clay solutions. The mixtures were stirred continuously for 4 hrs and then cast onto a 31 × 31 cm Teflon coated plate and allowed to dry at room temperature (23^{o}C) for 48 hours. Dried films were carefully peeled out and stored in desiccators containing saturated NaBr until further tested.

Table 1: The properties of chitosan films formed by different casting solvent

Film Casting Solvent	Film Properties
Acetic acid	A yellow tinted, flexible, transparent, non-sticky film with smooth shiny surface and slight acidic odour
Formic acid	A yellow tinted, flexible, transparent, non-sticky film with smoothshiny surface without any acidic odour
Citric acid	A yellowish, flexible, transparent, non-sticky film with slight brittle and grainier surface without any acidic odour
Lactic acid	Highly sticky films which shrink upon peeling, becoming a stickymass/clump
Ascorbic acid	A brown coloured, highly brittle film

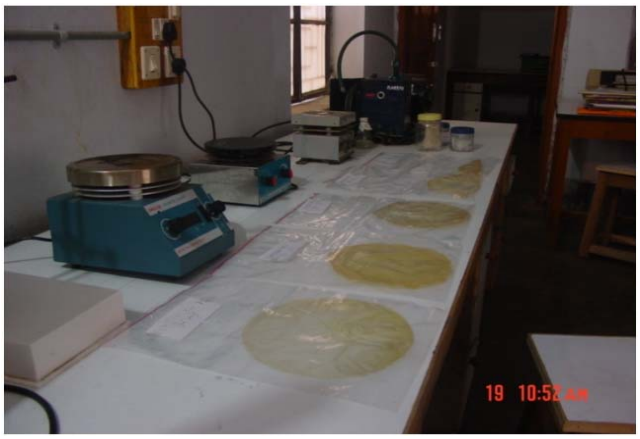

Figure 3. Chitosan films prepared by different casting solvent

Swelling of Chitosan Films

Water sorption capacities of chitosan films were determined by soaking them in phosphate buffered saline (PBS at pH 7.4) at room temperature. A known weight of chitosan film was placed in the PBS media for 30 minutes. The wet weight of the film was determined by first blotting the surface of the chitosan film with filter paper to remove excess water, and the film weighed immediately. The percentage of water adsorption in the medium (Wsw) was calculated from the equation:

$$W_{sw} = \frac{W_{30} - W_0}{W_0} \times 100$$

Where W_{30} represents the weight of the chitosan film after 30 minutes of sorption and W_0 is the initial weight of the chitosan film. The W_{sw} values were expressed as an average of 3measurements and standard deviation.

Solubility of Chitosan Films

The film solubility (S%) expresses the percentage of film's dry matter solubilized afterimmersion in water for 24 hours at 25°C, according to Gontard and others (1992). Theexperiment was performed under slow agitation. Film solubility (S%) was calculated from thefollowing equation:

$$S\% = \left(\frac{wt_{initial} - wt_{final}}{wt_{initial}} \right)$$

Where $Wt_{initial}$is the initial weight of chitosan film and Wt_{final} is the weight of chitosan film after immersion.

Fourier Transmission Infrared Spectra (FT–IR) Analysis

The Fourier Transmission Infrared Spectra (FT–IR) was obtained from the chitosan film through a Perkin Elmer Spectrum RX1 FT–IR spectrometer at Hanyang University, South Korea.

X-ray Diffraction (XRD)

The change in gallery height of the blend was investigated by WAXD experiments, which were carried out using X-ray diffractometer (BEDE D-3 system) with Cu Ka radiation at a generator voltage of 40 kV and a generator current of 100 mA. Samples were scanned from $2\theta = 1–10^o$ at a scanning rate of 2^o/min.

Scanning Electron Microscopy (SEM)

The chitosan films (taking acetic acid as a solvent) and their nano composites were characterized using SEM (440, Leica Cambridge Ltd, Cambridge, UK). The powdered specimens were placed on the Cambridge standard aluminium specimen mounts (pin type) with double-sided adhesive electrically conductive carbon tape (SPI Supplies, West Chester, PA). The specimen mounts were then coated with 60% Gold and 40% Palladium for 30 s with 45 mA current in a sputter coater (Desk II, Denton Vacuum, Moorestown, NJ). The coated specimens were then observed on the SEM using an accelerating voltage of 20 kV at a tilt angle of 30^o to observe the microstructure of the chitosan/ C 30B blends.

Antimicrobial Activity of Chitosan Films

The antimicrobial activity of chitosan film was assayed against different food borne bacteria like *S.aureus, B.cereus, S. typhimurium, and E.coli*by disk diffusion method according to Pelczar*et al* (1957).

The bacteria were grown on nutrient agar (NA) (peptone 5 g, beef extract 3 g, NaCl 3 g, agar 18 g, water 1000 ml, pH 7) plates for 12h at 30 ± 0.10C in a BOD incubator. A loopful of each bacteria was suspended in sterile distilled water separately and vortexed. The bacterial suspensions were adjusted to about 10^8 cells/ml with sterile distilled water, 1 ml was mixed with 100 ml NA medium, plated onto seven plates, allowed to solidify and then put into a refrigerator for 2h for hardening. The disks of films were cut into 5 mm diameter and then it was kept on the media. The plates were incubated at 30 ± 0.1°C for 12h and the inhibitory zones formed around the disk were recorded.

RESULTS AND DISCUSSIONS

Film-forming Ability of Chitosan

Chitosan is known for its film-forming ability. Chitosan filmed with various organicacids vary in their physiochemical properties. Flexible and transparent films prepared fromchitosan, resembling plastic films, were reported earlier (Nadarajah and Prinyawiwatkul2002). The solvent casting method of film formation, in which chitosan is first dissolved inacidic solvent, cast on plates, and air dried to form films, is often used owing to its simplicity. Inthis study, the film-forming ability ofchitosan was greatly influenced by the casting solvents. The chitosan films formed with lactic acid were highly sticky and hygroscopic. These films also exhibiteda high degree of shrinking and deformed instantly upon peeling. Although the films made withlactic or malic acid were less sticky when a plasticizer was avoided, they still exhibited shrinkage and deformation upon peeling. Chitosan films with ascorbic acid; however, resultant films were very brittle. They became far too fragile to handle when no plasticizer was added. Furthermore, all the ascorbate films developed deep brown coloration, presumably due tobrowning reactions, and thus became unappealing for packaging purposes. The film-forming ability of chitosan was desirable when acetic orcitric acid was used, resulting in highly flexible and transparent films. However, all plasticized films with acetic and citric acids exhibited high stickiness and hygroscopic nature, evenin the presence of the lowest plasticizer content. The high degree of hydrophilicity of chitosan is attributed to the deacetylated amino groups present in the polymeric chain, favouring considerable migration of water molecules to these sites. Addition of plasticizers minimizes oreliminates brittleness of the films. However, they also adversely affect film properties by making them more hygroscopic and contributing to greater absorption of moisture (Lawton 1992).Lawton (2004) demonstrated that addition of hydrophobic plasticizers can yield edible films thatare minimally hydrophilic.

This indicates the possibility of producing a variety of plasticizedchitosan films if compatible hydrophobic plasticizers are identified. Films made without plasticizers using acetic, formic and citric acids exhibited neitherstickinessnor hygroscopic nature, and they resembled plastic films. However, the unplasticized films formed with citric acids were slightly brittle in nature. This suggests that flexible and transparent films that resemble plastic films can be produced from chitosan usingacetic acid without any plasticizers. The film-forming ability of unplasticized chitosan with different organic acids and their film properties are described in Table 1. Therefore, after this finding, we go for the preparation of Chitosan nanocomposite films using acetic acid as a solvent.

Thickness and Density of Chitosan Films

We observed that the thickness of chitosan films was highly influenced by the type of film casting solvent used. Chitosan formed thicker films with citric acid compared to all other solvents used in the present study. This is in agreement with Begin and Calsteren (1999), who reported that pronounced increase in thickness of film cast with lactic or citric acid. According to Begin and Calsteren (1999), the citric acid chitosan-film solution gelled before the molecules could align and pack, which resulted in a thicker film. They also stated that the ability of citric acid to form multiple salt bridges with amino groups could act as a reticulating agent top romote gel formation.

The thickness of films varied from 0.03 mm for acetate and formate films to 0.08 mm for citrate films (Table 2). There was no difference in thickness between acetic and formic acid films. Citrate films showed significantly ($p<0.05$) greater thickness than acetate films. The density of films also varied significantly ($p<0.05$) depending on the acid used. Theformate films had relatively higher density than citrate and acetate films. However, no correlation between thickness and density of chitosan films was evident.

Table 2. Thickness and density of unplasticized crawfish chitosan films

Solvent	Thickness† (mm)	Density†† (g/cm3)
Acetic acid	0.030	1.46 ± 0.11
Formic acid	0.033	1.50 ± 0.12
Citric acid	0.079	1.32 ± 0.05
Lactic acid	0.052	1.19 ± 0.01

† Means of 10 random measurements and standard deviation.
†† Means of 3 measurements and standard deviation

Transparency of Chitosan Films

Transparency is one of the common optical properties of light permeable materials.Spectrophotometry is used to measure the transparency of a material by light-transmittance or absorbance using the Beer-Lambert's law relating the amount of light absorbed or transmitted bya material to the nature of the light absorbing material (Chang 1981 Han and Floros 1997).Development of transparent packaging materials which allow product visibility is a general trend and requirement in packaging films.

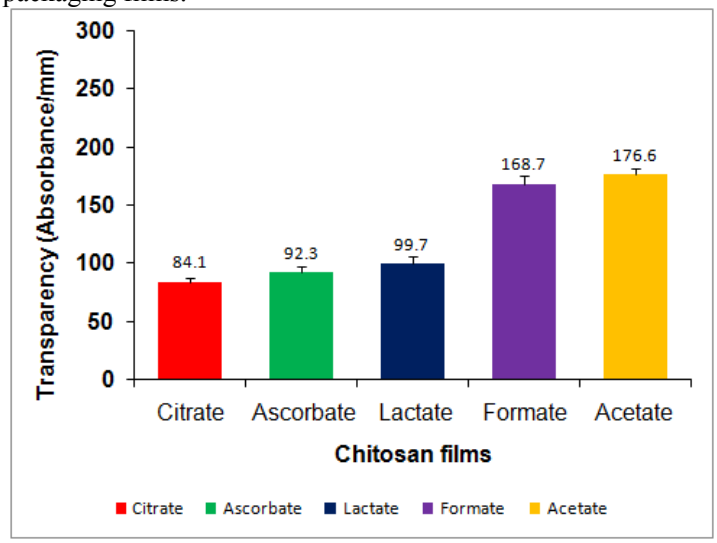

Figure 4. Transparency of unplasticized chitosan films

The type of acid used to form the film significantly ($p<0.05$) affected transparency of chitosan films (Figure 4). In general, the acetate films were more transparent with a mean transparency value (absorbance/mm) of 176.6 followed by formate with 168.7 and citrate films with 84.1. The lowest transparency values obtained for the citric acid films can be attributed to the higher tint of yellowness observed. However, compared to conventional low density polyethylene films with the transparency values of 20 - 30 (Han and Floros 1997), all chitosan films were exceptionally more transparent, thus may be used as see through packaging or coating materials.

Fourier transmission infra-red spectroscopy (FTIR)

The structure of the chitosan nano composite film was analyzed by using FTIR spectroscopy. Figure 5.shows FTIR spectra of chitosan /C 30B nano composites. A characteristic band at 3450 cm^{-1} is attributed to $–NH_2$ and –OH groups stretching vibration and the band for amide I at 1655 cm^{-1} is seen in figure which is the infrared spectrum of chitosan. Peaks at 1612 cm^{-1} (N–H bending),1566 cm^{-1} (N–H bending), 1450 and 1425 cm^{-1} (C–H bending), and also absorbencies due to structural O–H stretching at 3450 cm^{-1} , H–O–H deforming (absorbed water) at 1655 cm^{-1} , and Al–O vibrations at 915, 624 842, and 792 cm^{-1} confirm the presence of C 30B in the dispersion. The Si–O stretching peaks can be seen at 1086 and 1034 cm^{-1} and finally Si–O bending peaks at 520 and 467 cm^{-1}.

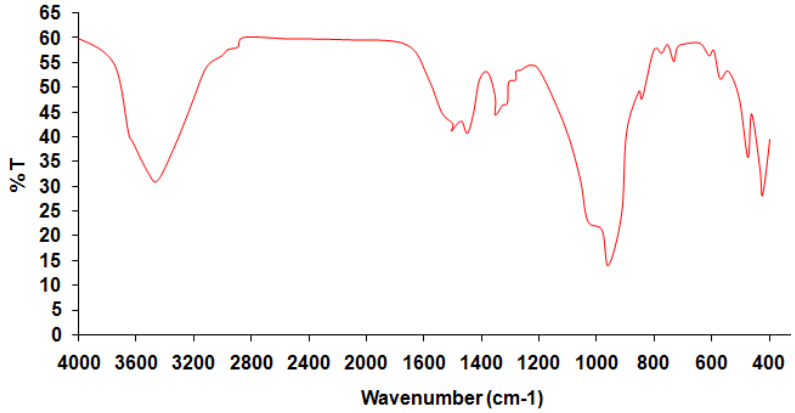

Figure 5.FTIR spectra of C 30B /chitosan nanocomposites

Scanning Electron Microscopy (SEM)

SEM has been employed for the observation of the surface morphology of the chitosan blended with different concentrations like 1%, 2.5% of C 30B. The microstructure obtained by SEM for the chitosan and its nanocomposites prepared by solvent casting, showed that particles are relatively well dispersed in the chitosan matrix. Figure 6.showed that as the concentration of the nanoclay increases from 1% to 2.5% the homogeneity of the surfaces also increases. In particular, 2.5% C30B was superior to individual polymers.

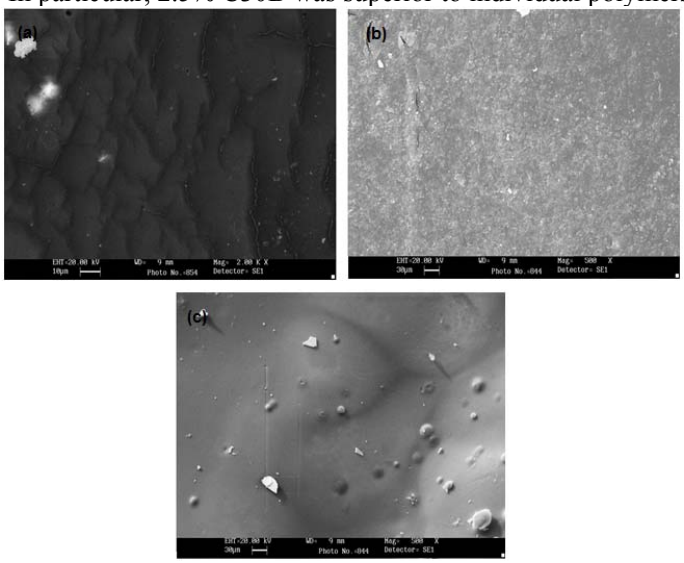

Figure 6. SEM of chitosan nanocomposite film

X-ray diffraction analysis

When C 30B was added to the chitosan solution, irrespective of amount, the peaks remained at the same position (2θ = 4.8°) (Figure 7.), indicating that no intercalation had occurred, and that microscale composite-tactoids were formed. It was very difficult to disperse C 30B in the chitosan aqueous solution and to form an intermolecular reaction between clay and chitosan in spite of the presence of the hydroxyl group in the gallery of C30B. Strong polar interactions, especially hydrogen bonding, critically affected the formation of intercalation and exfoliated hybrids.

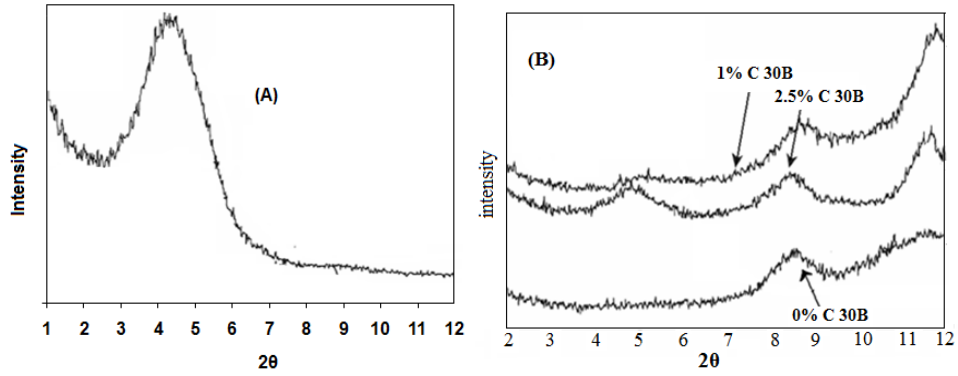

Figure 7. XRD of (A) C 30B and (B) chitosan/C 30B nanocomposite film

Swelling of Chitosan Films

Chitosan, being a hydrophilic polymer, shows high affinity towards water. Hence, uponhydration, chitosan films absorb water and swell. The swelling behaviors of unplasticized chitosan film formed with acetic, formic and citric acid are presented in Figure 8. The citrate films formed exhibited complete solubility in water and hence demonstrated no swelling effect. Chitosan films formed with formic acids showed higher degree ofswelling i.e. 18673.58 % followed by acetic acid 16534.23% .The formate film had significantly ($p<0.05$)greater swelling than the acetic film.

The chitosan formate films showing extremely high swelling may be desirable for development of absorbent pads such as those used in meat packages to absorb exudates from meat. In such case, the chitosan films can be directly used as absorbent pads without any plastic lining materials that are normally used to prevent desorption. The inherent antimicrobial property of chitosan films can also be beneficially utilized to improve shelf life of package contents. This would also help to replace currently used plastic wrapped absorbent pads.

Despite their extreme swelling behaviour, all formate and acetate films retained their integrity and showed no trend of dissolving in water, unlike the citrate films. Therefore, the acetate films which showed lowest swelling are recommended for packaging purposes. The citrate films which dissolve in water may be a good candidate to develop edible films and packaging materials which require complete solubility upon hydration.

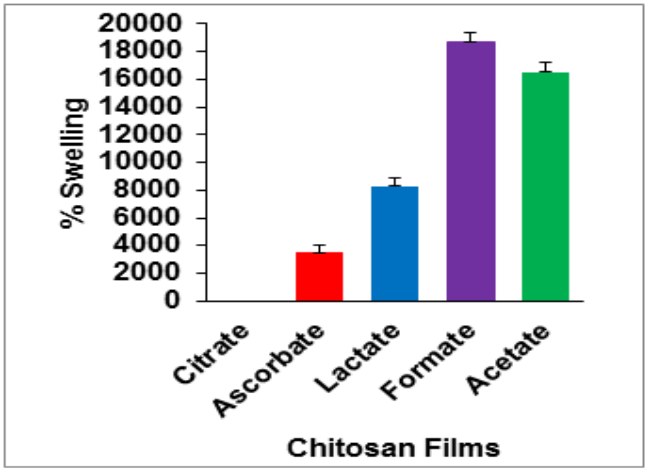

Figure 8. The % swelling of chitosan films formed with acetic, citric and lactic acids. Black bars indicate Standard deviation. The citric acid film which dissolved upon hydration is indicated as 0 swelling.

Solubility of Chitosan Films

Solubility of chitosan films is presented in Table 3. All citrate films werecompletely soluble and, hence, accounted for highest solubility. Formate films were moresoluble than acetate films. Data showed that solubility of chitosan was influenced by the type of acid used.

Table 3. Solubility of unplasticized chitosan films

Solvent	Solubility† (S%)
Acetic acid	0.27 ± 0.05
Formic acid	0.43 ± 0.02
Citric acid	1.00

Antimicrobial assay

Antibacterial activity of chitosan film formulations against 4 pathogenic bacteria was expressed in terms of zone inhibition. The zone inhibition assay revealed primarily three types of observations namely; defaced films without any clear or inhibition zones which could be attributed to the absence of any inhibitory activity, clear zones without inhibitory zones which could be attributed to bacteriostatic activity, and clear inhibition zone representing bactericidal inhibition by films. The diameter of the inhibition zones measured is given in Table 4.

Among the films studied, the chitosan ascorbic acid films showed minimum inhibitory activity. Chitosan acetate films defaced with *Bacillus cereus* and *Salmonella typhimurium* lawns indicating they were ineffective in controlling these bacteria. However, all chitosan acetate films showed bacteriostatic effects against *Staphylococcus aureus* and *Escherichia coli* as indicated by their clear zones in lawns. The chitosan formate films were also ineffective in controlling *Bacillus cereus* as indicated by defaced films by these bacterial lawns. Nevertheless, the chitosan formate films showed inhibitory effects against *Staphylococcus aureus, Salmonella typhimurium,* and *Escherichia coli.* However, compared to the chitosan citrate films, the inhibitory effect of chitosan formate films were lower and the thickness of the inhibitory zone was in the range of 11 to 12mm compared to 16 to 30 mm of chitosan citrate films. Chitosan lactic acid films and ascorbic acid films showed similar type of inhibitory effect against all the four types of bacteria. From the result it can also be seen that both lactate and ascorbate films were also effective against *S. Aureus* and *S.typhimurium* but both of them were ineffective against *Bacillus cereus* and *E. coli*. Among the chitosan films studied, all chitosan citrate films exhibited prominent inhibitory effect on all 4 pathogenic bacteria (Table 4.). All chitosan citrate films showed distinctive inhibition zones against all pathogenic bacteria tested and the inhibition zones were considerably thicker than those produced by chitosan formate films. Also, the inhibitory effects of chitosan citrate films were remarkably higher for *E.Coli* as indicated by larger inhibition zones accounting for more than 13 mm than chitosan formate film. The chitosan citrate films were the only films with antimicrobial effects against *Bacillus cereus*. The higher inhibitory activity shown by all chitosan citrate films can be attributable to complete solubility of chitosan which could make them more reactive against bacterial cells.

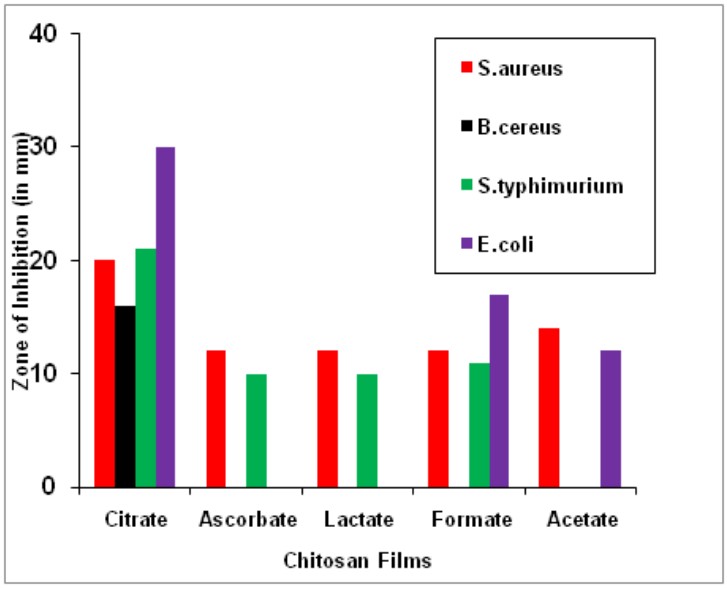

Figure 9. Graph showing the antimicrobial activity of chitosan against different microorganisms

Table 4.Antimicrobial activity of chitosan films against different pathogens

Diameter of Inhibition Zone				
Film Casting Solvent	*S.aureus*	*B.cereus*	*S.typhimurium*	*E.coli*
Acetic acid	14	-	-	12
Formic acid	12	-	11	17
Lactic acid	12	-	10	-
Citric acid	20	16	21	30
Ascorbic acid	12	-	10	-

CONCLUSION

In this study chitosan film s were prepared by using different types of solvents like acetic, formic, lactic, citric and ascorbic acids. C 30B which is organomodified nanoclay was used to prepare Chitosan nanocomposites by solvent casting method and were characterized by FTIR, XRD and SEM. From the FTIR data the presence of different functional groups for both chitosan and C 30B were confirmed. XRD data gave the idea that only tactics were formed. Among the chitosan films studied, all chitosan acetate films showed very good and favourable properties for using as packaging materials as compared to other chitosan films, because of its less swelling and high transparency, because of extremely high swelling behaviour of chitosan format films it may be used for the development of absorbent pads such as those used in meat packages to absorb exudates from meat and as citrate films completely dissolved in water it may be a good candidate to develop edible films and packaging materials which require complete solubility upon hydration, though further investigations are also required. Among antimicrobial activity all citrate films exhibited prominent inhibitory effect against all 4 pathogenic bacteria. All chitosan citrate films showed distinctive inhibition zones against all pathogenic bacteria tested and the inhibition zones were considerably larger than those produced by chitosan acetate, formate, ascorbate and lactate films. Also, the inhibitory effects of chitosan citrate films were remarkably higher for *E.Coli* and *S.typhimurium* as indicated by larger inhibition zones accounting for more than 13 mm. The chitosan citrate films were the only films with antimicrobial effects against *Bacillus cereus*. The higher inhibitory activity shown by all chitosan citrate films can be attributable to complete solubility of chitosan which could make them more reactive against bacterial cells.

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