Chapter 4

Preparation and characterization of chitosan/kaolin clay biocomposites

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Published as cover page

Abstract

CS is a naturally occurring polycation known for its numerous favourable characteristics. KAO clay, on the other hand, serves as a natural inorganic filler and finds applications in various fields such as medicine, ceramics, food additives, etc. Hence, combining these two biomaterials — CS and the natural filler (KAO clay) — can result in the formation of a biocomposite known as CS/KAO biocomposite, showcasing a range of intriguing properties. This research involves the preparation of composites consisting of CS and KAO clay by blending a solution of CS in dilute acetic acid with KAO clay at different weight ratios. The comprehensive investigation of these biocomposites involves the utilization of various analytical techniques, including FT-IR, UV/Vis, XRD, SEM, UTM, TGA, and DSC analyses. The antibacterial activities of varying concentrations of CS-KAO against both gram-negative (*Escherichia coli*) and gram-positive (*Bacillus subtilis*) bacteria have been assessed using the agar well diffusion method. Swelling assessments are also performed on the biocomposites.

4.1. Introduction

One of the major possibilities to eradicate the global environmental contamination is extensive applicability and availability of biodegradable plastics. In this contrast biodegradable polymers are found to be an excellent candidate. It has been observed that biodegradable plastics playing a great role in enhancing the quality of living standards, and industrial development throughout the globe [124]. By implementation of biodegradable films can enable us the final replacement of plastic packaging bags which are basically environment hazard and not recyclable. The prime goal of this current research is to create biodegradable, environmentally friendly materials with improvised characteristic which can mitigate the issue [69].

In the present scenario, various composites made from synthetic polymers derived from petroleum have a negative impact on the ecosystem of the planet as they are non-biodegradable. Moreover, hazardous or toxic properties of these polymers make them a worse contender. In this contrast an urgent eco-friendly green composite material of natural origin must be come into effect [34]. As it is known that biopolymer films are eco-friendly substitutes for synthetic, non-biodegradable films, hence researchers taking a great interest in developing such materials [125]. Generally, the syntheses of bio-composites are an

interdisciplinary field that composed of basic science, material science, and engineering which adds new dimensions to the properties of biopolymers [126].

Several industrial applications of bio-composites are basically made from various waste and naturally occurring materials [127]. In this regard, biodegradable polymer CS is a wonderful option due to its unique qualities, such as its low cost, wide availability, biocompatibility, biodegradability, hydrophilicity, lack of toxicity, ease of chemical modification, good adhesion, ion-exchange and adsorption properties, etc [35, 128, 129]. Naturally occurring polysaccharides cellulose, dextran, pectin, alginic acid, agar, agarose, and carrageenan are examples of neutral or acidic polysaccharides, in contrast to chitin and CS, which are examples of extremely basic polysaccharides. As a result of these exceptional properties, numerous CS-composite based products have been developed extensively [130] viz. in wastewater treatment [131], tissue engineering [77], agriculture [132], biomedicine [133], drug delivery [134] etc. The lone CS-based products are very few as it doesn't have such exceptional properties like CS-composite materials [36].

To overcome these issues various kind of CS-based composites have been developed in the recent years [67]. In this purpose CS immobilization on clay minerals has received magnificent attention [68]. They have a higher selective gas permeability ratio CO_2/O_2 than traditional synthetic films and are also excellent fat and oil barriers [135]. However, they generally have poor water vapor barrier properties. Polymer/clay composites have drawn a lot of recognition in the recent years due to their extraordinary potential to enhance the barrier properties of thin films [125]. Due to their high aspect ratios and high surface area, these composites are a class of hybrid materials made of organic polymer matrices and micro/nanoscale organophilic clay fillers. If clay particles are properly dispersed in the polymer matrix at a loading level of 1% to 5% (w/w), unique combinations of physical and chemical properties will be obtained, making these composites appealing for making films and coatings for a variety of industrial applications [125, 136].

Clay minerals have been identified as natural inorganic substances with distinct structural adsorption, rheological, and thermal properties by recent research [137]. Due to the presence of their surface hydroxyl (–OH) groups, which may easily connect with water molecules, these materials naturally have a hydrophilic nature [138]. The use of clay minerals for metal binding [137], dye removal [139], and fruit packaging [140], either alone or in combination with other natural or manufactured polymers, has a long and enchanted history. To improve

clay's compatibility with other polymers, however, purification and modification may occasionally be required [141]. Because of their smaller particle size, larger surface area, favourable aspect ratio, and superior dispersion capabilities, clay minerals can greatly enhance the properties of CS [38]. The acidity of the $-NH_3^+$ group is primarily responsible for the CS's electrolytic nature and chelating properties. CS can be intercalated with KAO through cationic exchange and hydrogen bonding due to the polycationic character of this biopolymer in acidic conditions, and the resultant composites exhibit fascinating features [45].

In addition, clay minerals have certain wonderful qualities including strong biocompatibility, non-toxicity, and excellent controlled release prospects, which support their use in food, medicine, pharmacy, cosmetics, and other industries [39]. To the best of our knowledge, however, the KAO-based CS biocomposite has received minimal attention and has received fewer scholarly articles [34, 44, 70-72]. To the best our knowledge no report has been done on antibacterial activities for one gram negative and one gram positive bacteria using CS/KAO clay biocomposite films in different weight ratios so far. Moreover, swelling tests of the same are also never been reported. We think the novelty of our work lies in the biological application, swelling property as well as comparative study of different physico-chemical properties of CS/KAO clay biocomposite films of different weight ratios.

We were therefore highly interested in synthesizing some CS/KAO-based green bio-composite materials which must be environmentally benign. The purpose of the current study is to create CS/KAO biocomposite films in which KAO is combined with a CS solution in acetic acid. By using various characterizing techniques viz. FT-IR, UV/Vis, XRD, SEM, UTM, TGA, and DSC, the structure of the produced CS/KAO biocomposites films were successfully investigated. Moreover, the present work is designed to investigate the antibacterial effects of different CS/KAO ratio against two pathogenic bacteria- *Escherichia Coli* and *Bacillus subtilis*. Also, the swelling tests of the biocomposites have been observed. From our literature survey analysis, it has been observed that CS/KAO biocomposite films of different weight ratios received minimal attention towards various physicochemical properties viz. mechanical, thermal, swelling tests etc. as compared to pure CS film. Furthermore, no antibacterial properties have been investigated and reported till now for the same. We thought there have been lots of scopes to study on this area and this is the basic reason why we are interested for the development of this work. Moreover, these

biocomposites may find some applications in the field of analytical and environmental science.

4.2. Synthesis

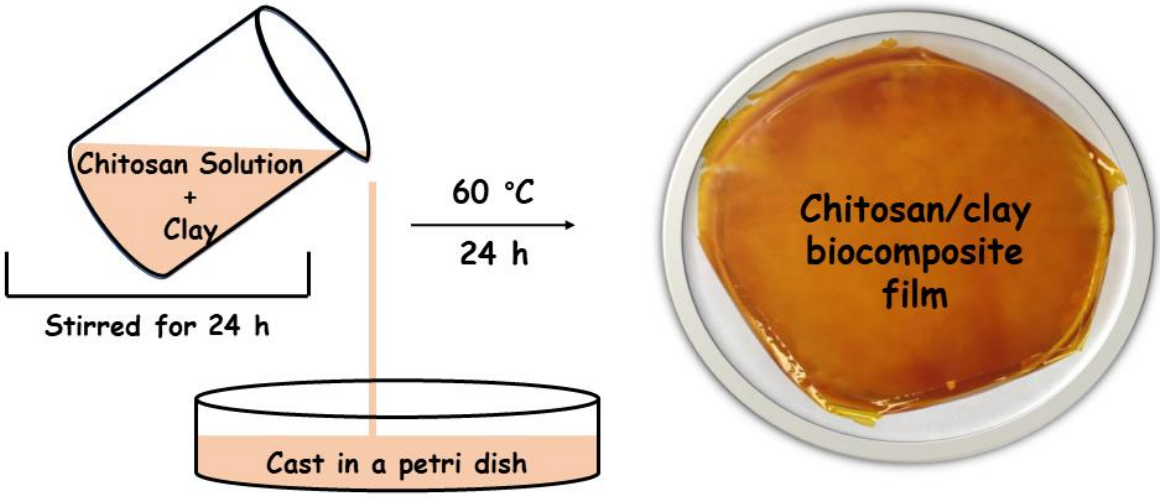
4.2.1. Chitosan film

CS solution was prepared by dissolving 1 g of CS powder in 100 mL of aqueous acetic acid solution (2%, v/v), which was then stirred at 40 °C for 5 h followed by vacuum filtration to remove the insoluble residue (Scheme 4.1.). The solution thus formed were cast into petri dish and dried at 60 °C for 24 h to evaporate the solvent. The film thus formed was soaked with an aqueous solution of 0.05 M NaOH to remove residual acetic acid. Further film was neutralized by rinsing with distilled water and then dried at room temperature.

Scheme 4.1. Reaction scheme for preparing CS film.

4.2.2. Chitosan/kaolin biocomposite films

First 2% CS solution was prepared by procedure mentioned in the section 4.2.1. After that, 0.1 g KAO clay was added to the CS solution and stirred at room temperature for 24 h with ≈ 700 rpm. The solution thus formed was cast into petri dish and dried at 60 °C for 24 h to evaporate the solvent and films were formed thereof (Scheme 4.2.). Following the same procedure applied for CS film, the dried films were soaked with an aqueous solution of 0.05 M NaOH to remove residual acetic acid and further films were neutralize by rinsing with distilled water and then dried at room temperature. In the similar manner, other films were obtained by varying the amount of the KAO clay (viz. 0.2 g, 0.3 g, and 0.4 g). Table 4.1. summarizes the details of CS/KAO clay biocomposites.



Scheme 4.1. Reaction scheme for preparing CS/clay biocomposite film.

The pictorial presentation of the CS and CS/KAO biocomposite films was shown below (Figure 4.1):

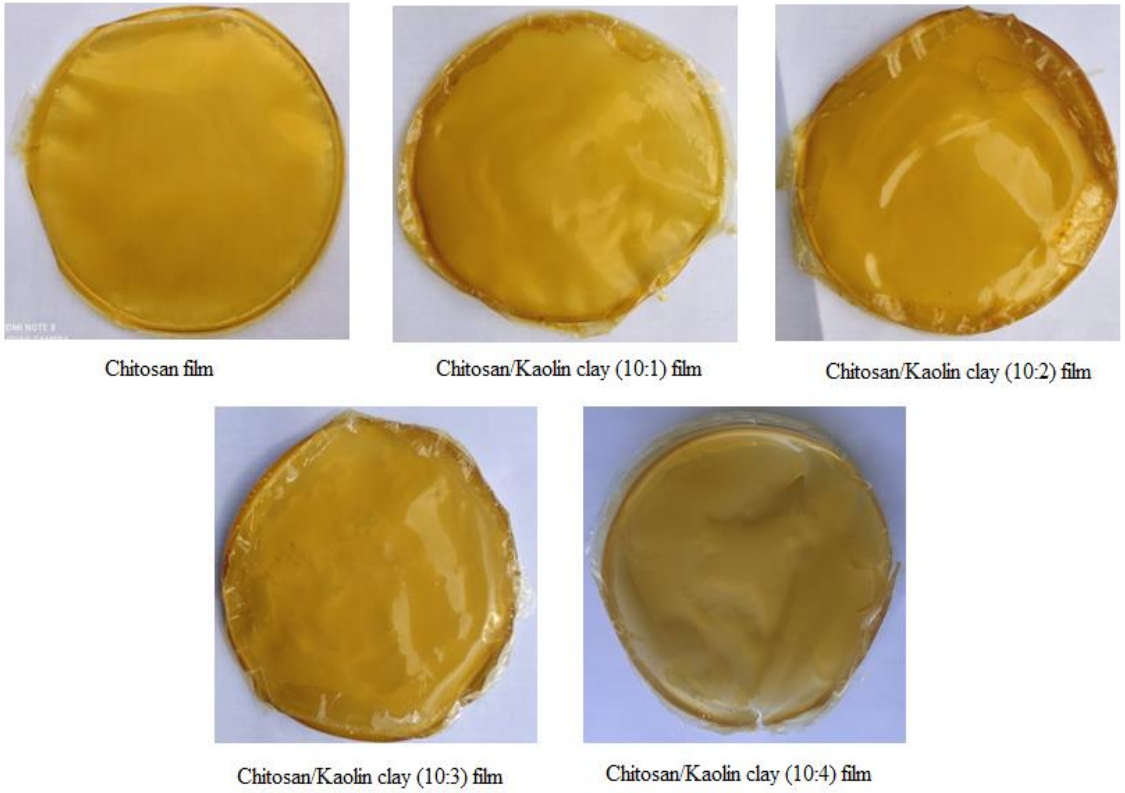


Figure 4.1. Images of CS and CS/KAO clay biocomposite films.

Table 4.1. The details of the CS/KAO biocomposite films.

Sample name	% of CS	% of KAO	Thickness of the film (mm)
CS	100	0	0.11
CS/KAO-1	100	10	0.13
CS/KAO-2	100	20	0.14
CS/KAO-3	100	30	0.16
CS/KAO-4	100	40	0.18

CS indicates for chitosan and CS/KAO-1, CS/KAO-2, CS/KAO-3 and CS/KAO-4 for chitosan/kaolin biocomposite films with 10%, 20%, 30%, 40% respectively.

4.2.3. Crude sample for antibacterial study

Different concentrations of dry CS and CS/KAO biocomposite films were dissolved in 2% acetic solution at 10 mg mL^{-1} concentration. The dissolved mixtures were strewed at 40 °C at constant rotation for 5 h. Then, the samples were used for antibacterial activity.

4.3. Results and discussion

4.3.1. Structural evaluation

4.3.1.1. Analysis via FT-IR spectroscopy

FT-IR spectra were recorded in the region 4000–700 cm^{-1} for the CS/KAO biocomposite films in the ratios of CS: KAO 10:01, 10:02, 10:03, and 10:04 as depicted in the Figure 4.2. The band at 3675 cm^{-1} corresponds to the stretching frequencies of the -OH group of the KAO clay whereas the band at 3384 cm^{-1} was observed for the main functional group of CS, i.e., O-H stretching vibrations. The bands of the biocomposites (i.e., from a-d) at 2924 and 1068 cm^{-1} are consistent with bands those observed for the pure CS film. The bands at 2924 and 1068 cm^{-1} are corresponds to C-H symmetric stretching and C-O stretching respectively. The band at 796 cm^{-1} corresponding to the vibration bands of the silicate remain unaffected in the biocomposite. In the spectrum of CS, the absorption band at 1639 cm^{-1} corresponds to the in-plane N-H bending vibration. From the Figure 4.2. it has been evident that the absorption band at 1639 cm^{-1} for CS film was shifted to 1624 cm^{-1} in the biocomposite films which shows close agreement with the results reported by Dey *et al.*

[34]. In their investigation they have confirmed that the bicomposite films prepared was not a physical mixture for which absorption band was observed at 1633 cm^{-1} but for biocomposite films it was at 1624 cm^{-1}. The electrostatic interaction between the protonated amine groups ($-NH_3^+$) of CS and negatively charged sites of KAO clay might be the reason for this frequency shifting [69, 142].

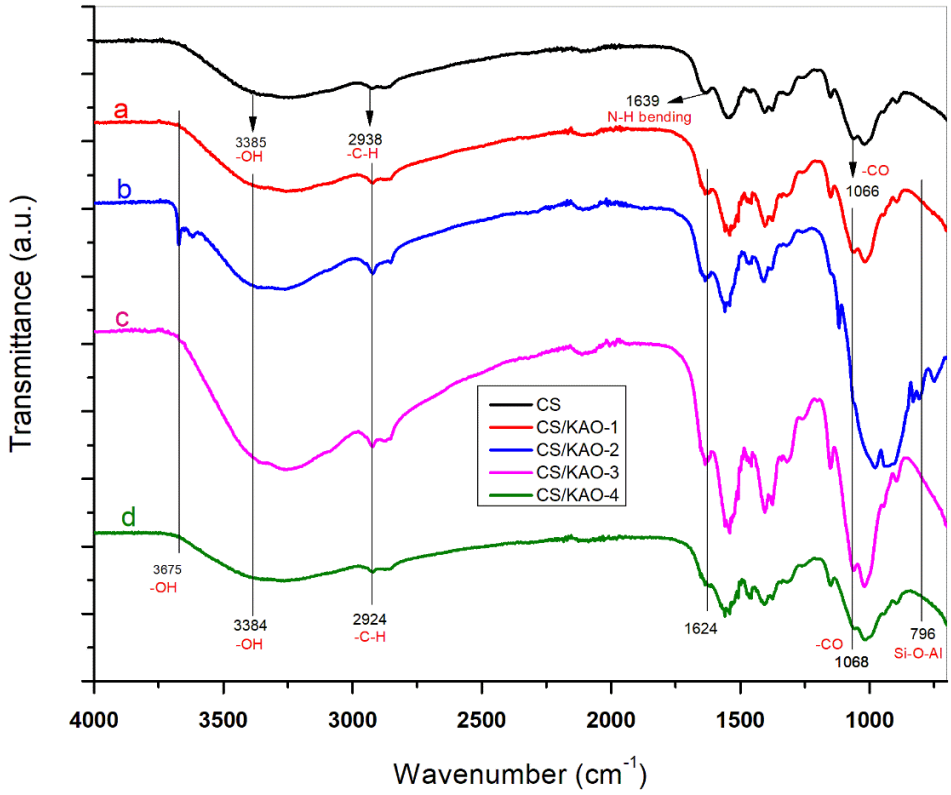


Figure 4.2. FT-IR spectra of CS and CS/KAO biocomposite films (a–d).

4.3.1.2. Analysis via UV/Vis spectroscopy

The UV/Vis spectra of the CS and CS/KAO biocomposite films were taken at room temperature as shown in Figure 4.3. The absorption bands were observed in the range of 300–400 nm. The wavelength of CS film was found to be at 354 nm, while for the CS/KAO biocomposite films assigned as CS/KAO-1, CS/KAO-2, CS/KAO-3, and CS/KAO-4 were found to be at 383 nm, 395 nm, 368 nm, and 373 nm respectively. The absorption bands observed at 300–400 nm is due to the direct electronic transition from d-π* orbital's which is also termed as the Soret band. The concentration of KAO clay on addition to the CS affects the position and shape of the UV absorption bands. The increases in concentration of the clay in the biocomposites are directly proportional to the intensity of light absorption, thus

influencing the position and shape of the wavelength in the spectrum. At high concentration molecular interaction occurs, which alters the shape and position of the bands. As a result, the peak shift happens in the different CS/KAO biocomposite films. From the Figure 4.3., it has also been observed that the maximum absorption for CS/KAO-4 film signifies the highest concentration of the KAO clay presents in the film. From the UV/Vis analysis we are basically want to investigate the interaction of KAO clay on the pure CS matrix. Moreover, we also like to observe how does the different weight ratios of CS/KAO biocomposite films influence on absorption band. Another evidence of this analysis is to obtain the Soret band at 300–400 nm which were observed in all the cases of our synthesized biocomposites as well as for the pure one. Furthermore, this analysis also provides a supporting structural information obtained from other spectroscopic methods, especially FT-IR analysis.

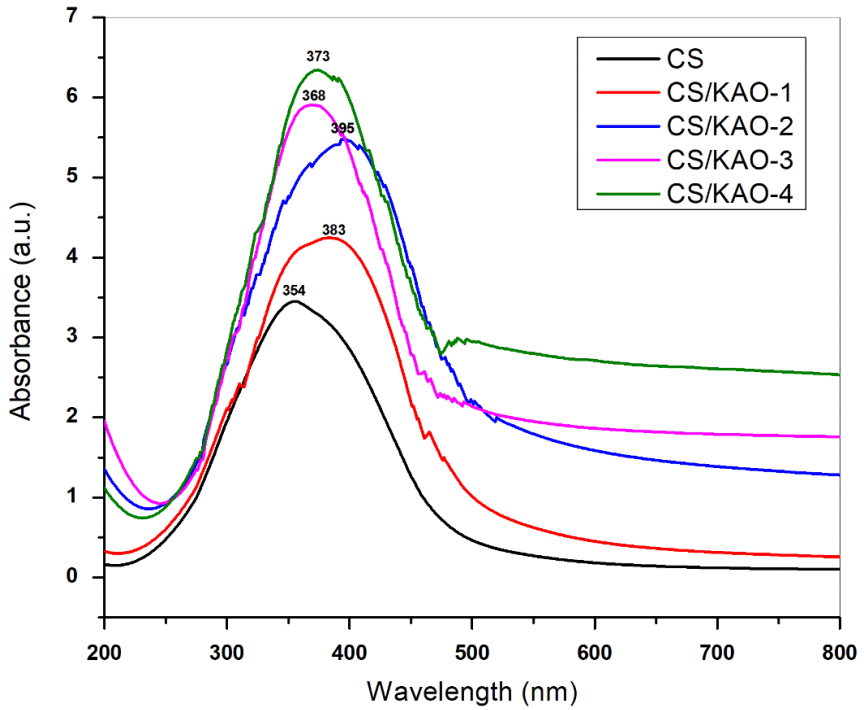


Figure 4.3. UV/Vis spectra of CS and CS/KAO biocomposite films.

4.3.2. Morphological study

4.3.2.1. Analysis via XRD

XRD analysis of the CS and CS/KAO biocomposite films were investigated as shown in Figure 4.4.(a). Peaks thus obtained for CS film was compared with CS/KAO biocomposite films to know the preliminary information regarding the presence KAO clay in the

biocomposites. It has been found that CS has a semi crystalline nature whereas KAO clay has crystalline nature. In the 2θ range of about 10°-70°, six characteristics peaks were obtained for CS/KAO biocomposite films. The XRD pattern of CS showed broad diffraction peaks at 2θ around 22.4° corresponding to the typical fingerprints of semicrystalline CS [34]. From the Figure 4.4.(a) it is clear that the crystallinity of CS was disappeared in the CS/KAO biocomposite films as the peak of CS at 2θ = 22.4° is absent in the CS/KAO biocomposite films. The investigation resembles the results obtained by Biswas *et al.* which indicate a pretty good dispersion of the CS and KAO clay on CS [71]. It has been observed that in the biocomposites CS/KAO-1 to CS/KAO-4 with increasing concentration of the clay particle the intensities of the peaks were also increases and small broadening takes place. Therefore, it can be assumed that the KAO clay with different concentration was successfully incorporated into the CS. From the Figure 4.4.(a) it is cleared that from CS/KAO-4 to CS/KAO-1 the peaks are slightly shifted to higher diffraction angle indicating the lattice contraction and suggested smaller crystalline size. A broad peak was observed in 2θ = 26.60°, 26.68°, 27.10°, and 27.30° in samples CS/KAO-1 to CS/KAO-4 and from this peak using Scherer formula crystallite size was calculated. The position of the peaks was calculated from Origin graphic software. The corresponding peaks are 2θ = 26.60°, 26.68°, 27.10°, and 27.30° in samples CS/KAO-1, 2θ = 26.58°, 26.48°, 27.07°, and 27.29° in samples CS/KAO-2, 2θ = 26.28°, 26.18°, 26.70°, and 27.16° in samples CS/KAO-3, 2θ = 26.60°, 26.68°, 27.10°, and 27.30° in samples CS/KAO-4. The average size of crystallite was determined using Scherer formula (Equation 4.1.) [143].

$$D = \frac{kl}{Vw_{2q}cosq_B} \ldots\ldots\ldots (4.1.)$$

where, q_B is the Bragg angle in radian and K = 0.9 for spherical shape. It was found that particle size increases from sample CS/KAO-1 to CS/KAO-4 and ranges from 18 nm to 21 nm. For CS/KAO-1 particle size were 18.72 nm, for CS/KAO-2 particle size were 19.46 nm, for CS/KAO-3 particle size were 21.32 nm, for CS/KAO-4 particle size were 21.97 nm. Since we used in thin film form within capping material so possibility of agglomeration can be neglected. Therefore, XRD peak broadening was considered only for size. The lattice parameter "a" was determined from three prominent peak 2θ = 26.60°, 29.06°, and 45.18° and systematic errors in 2θ were eliminated by Nelson and Riley plot from three peaks. The corrected value of lattice constant "a" is calculated by F(θ) to zero. The lattice constant was explained in Figure 4.4.(b)

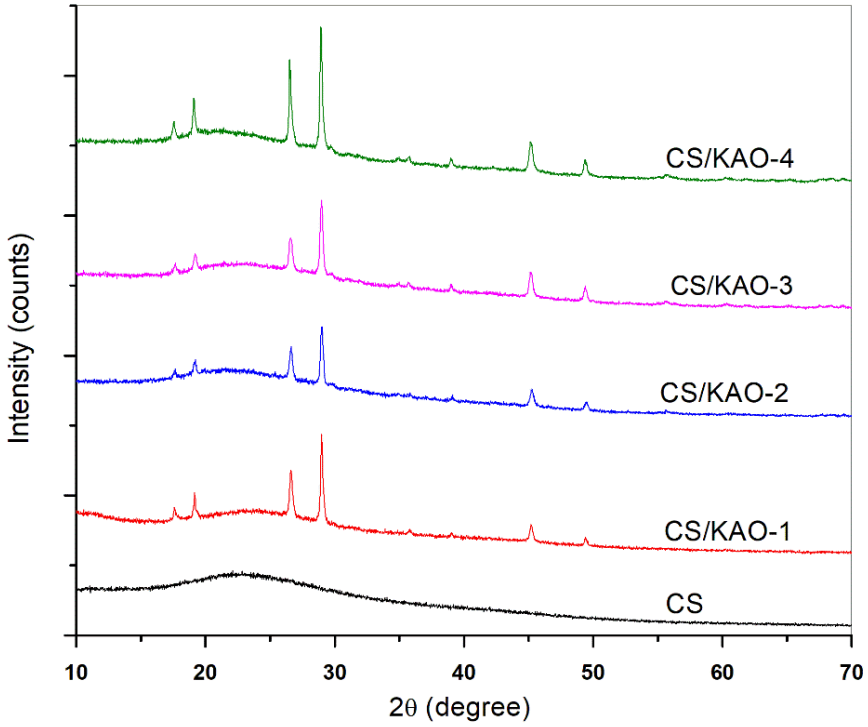


Figure 4.4. a) XRD patterns of CS and CS/KAO biocomposite films.

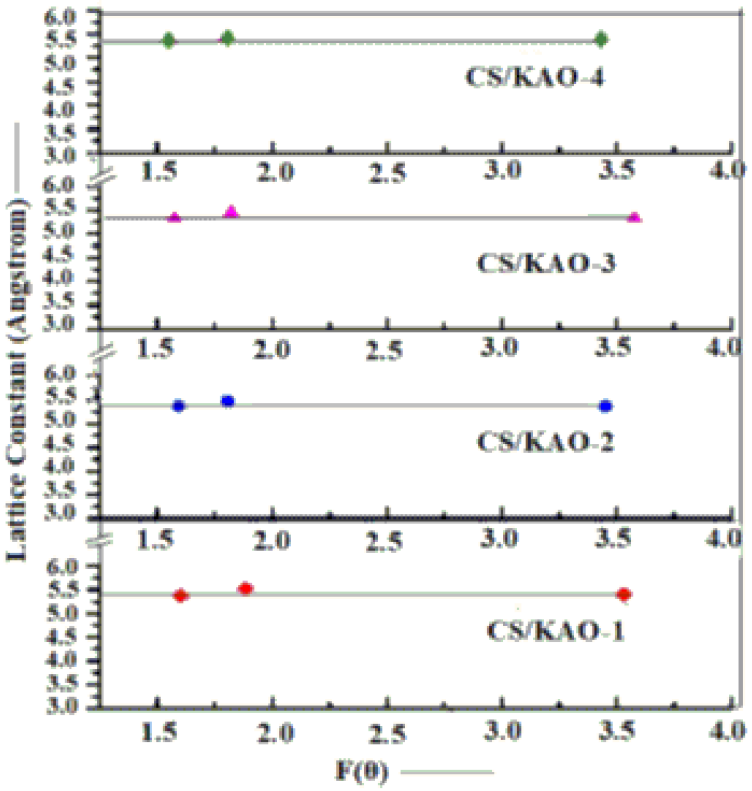


Figure 4.4. b) Nelson and Rilay plots of CS and CS/KAO biocomposite films.

4.3.2.2. Analysis via SEM

SEM images were obtained for the film of CS and CS/KAO biocomposite films to know the morphologies of the same. For better resolution, images were taken at the 5.00 KX magnifications. The micrographs in Figure 4.5. show the changes in the morphologies of CS and biocomposites CS/KAO-1 to CS/KAO-4. SEM micrographs of biocomposite films show how the clay particles were dispersed in the CS matrix. The surface of CS was smooth and irregular, while in case of biocomposite films the clay particles were dispersed in the polymer matrix. Figure 4.5.(a) shows a fibrous network of CS matrix while in case of CS/KAO-1 biocomposite, the clay particles were exfoliated and intercalated in the CS matrix as shown in the Figure 4.5.(b). This also shows that the clay particles were dispersed throughout the CS matrix [71]. However, the micrographs of CS/KAO-2 and CS/KAO-3 as shown in the Figure 4.5.(c,d) revealed that the homogeneous dispersion of KAO clay in the CS matrix. The micrographs also affirm that the CS/KAO-2 possessed a more uniform and smoother surface than CS/KAO-3 [79]. The composite CS/KAO-4 shows a fibrous network with rough surface which may be due to the presence of higher amount of KAO clay in the CS matrix. From the analysis it can be understand that with the increase in the amount of clay in the biocomposite films the surface getting rougher which shows close agreement with the results reported by Biswas *et al.* in their investigation [71].

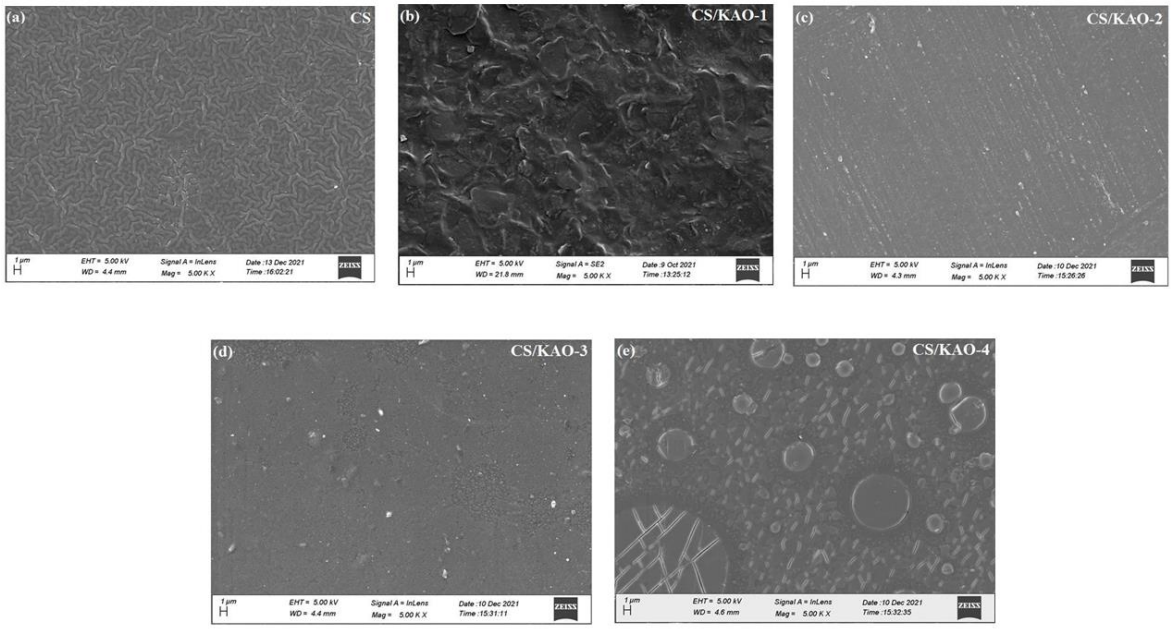


Figure 4.5. SEM images of a) CS b) CS/KAO-1 c) CS/KAO-2 d) CS/KAO-3 and e) CS/KAO-4.

4.3.3. Mechanical property

From our literature analysis it was found that the tensile properties of CS/KAO biocomposite films gain minimal attention. Basically, the mechanical properties of polymers are one of the features that distinguish them from small molecules. For better understanding the physical behaviours of the CS and CS/KAO biocomposite films, mechanical properties viz. tensile strength, Young's modulus and elongation at break were taken into consideration. The properties were investigated listed in Table 4.2. Table 4.2. showed that tensile strength and Young's modulus of the composites were increased whereas the elongation at break decreased as the amount of KAO clay in the biocomposite films increased. From the UTM analysis, it was observed that on increasing the amount of KAO clay in the biocomposite films the values of tensile strength increase which indicates the increase in the rigidity of the biocomposite. A higher Young's modulus values for the biocomposites indicates the characteristics of lower toughness. Higher Young's modulus value also indicates the brittleness for the same. Thus, high value of Young's modulus for CS/KAO-4 biocomposite indicates lower toughness and more brittleness than the other synthesized biocomposite films. Moreover, the lower elongation at break values indicates low ductility. As a result, it may be concluded that among all the biocomposites, CS/KAO-4 has low ductility and higher rigidity, which indicates the higher brittleness of the material. The enhanced tensile strength and modulus observed for the biocomposite films reflect that there was better polymer-filler interaction. In an investigation reported by Laaraibi *et al.* observed some similar trends on addition of bentonite clay to the CS matrix [124]. As the biocomposite films shows significant tensile strength which is considered as an interesting mechanical property therefore it can be used as a food packaging material. We are planning to explore this application in our upcoming project.

Table 4.2. Mechanical properties of the CS film and CS/KAO biocomposite films.

Film type	Tensile strength (MPa)	Young's modulus (MPa)	Elongation at break (%)
CS	29.6	5.1	6.2
CS/KAO-1	26.1	6.1	5.8
CS/KAO-2	29.1	6.6	5.6
CS/KAO-3	31.4	6.7	5.5
CS/KAO-4	34.7	7.6	5.3

4.3.4. Thermal analysis

4.3.4.1. TGA

TGA analyses of the synthesized films of CS and CS/KAO biocomposite films were carried out to investigate the thermal stability of the materials. TGA and DTG (derivative thermogravimetry) curves of the CS, CS/KAO-1 and CS/KAO-3 were depicted in the Figure 4.6.(a,b) respectively.

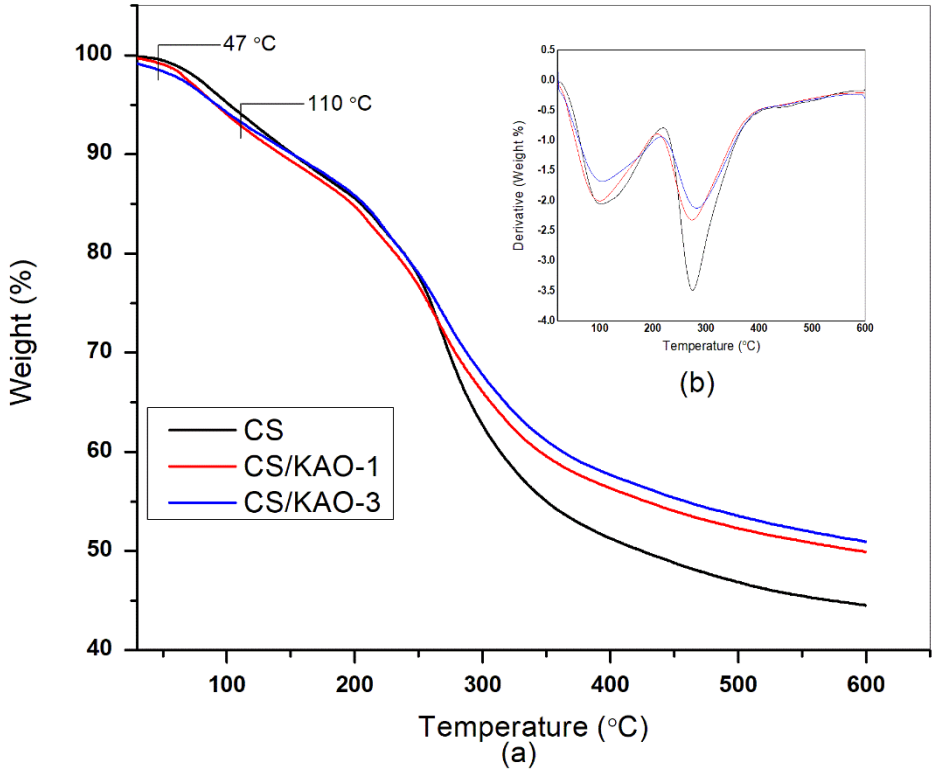


Figure 4.6. a) TGA and b) DTG plots of CS and CS/KAO biocomposite films.

The degradation processes of the same were undergone through two steps. All biocomposite films were degraded in the range of 47–110 °C. This is considered as the first range of degradation, which occurred due to loss of water molecules. Pure CS starts breakdown mostly at temperature about 245°C and degradation almost completed at about 457 °C. The oxidative break down of the carbonaceous residue occurred in the temperature range of between 457 and 550 °C which is considered to be the second stage of degradation [124, 144]. Table 4.3. summarizes the both degradation temperature ranges of CS and CS/KAO biocomposite films. From the data's it is observed that CS/KAO clay biocomposite films have superior thermal stability than the pure CS. It is also observed that on increasing the

clay proportion in the biocomposites, there is an enhancement in the stability of CS/KAO clay biocomposite films which expected to be close agreement with the assumption we try to draw. In an investigation reported by Biswas *et al.* shows good resemblance with the results obtained in this work [71] This observation is due to the chemical impregnation of the KAO clay particles to the matrix of the CS.

Table 4.3. Thermal properties of CS and CS/KAO biocomposite films.

Sample	Kaolin loading (% weight w.r.t. polymer weight)	First degradation		Second degradation	
		T_{onset}	T_{max}	T_{onset}	T_{max}
CS	Nil	47	107	245	457
CS/KAO-1	10	48	108	250	487
CS/KAO-3	30	48	110	258	506

4.3.4.2. Analysis via DSC

DSC measurements were carried out to ascertain the glass transition temperature (T_g) of the CS and CS/KAO biocomposite films. To the best our knowledge, T_g of CS/KAO clay biocomposites with different weight ratios have never been reported. During the investigation it was found that the T_g values for CS, CS/KAO-1, and CS/KAO-3 were found to be 130, 136, and 149 °C respectively. The T_g value increased with the increase of KAO clay content in the CS matrix. This indicates that there is increase in crystallinity of the biocomposite films. A notable difference in the DSC thermograms of the CS and CS/KAO clay biocomposite films were observed, as illustrated in the Figure 4.7. It was found that CS has an exothermic degradation peak between 328–329 °C, whereas biocomposites have a degradation peak between 340–343 °C. These results indicate that the degradation was getting slowed down in the composites due to its chemical interaction of the KAO clay with CS matrix. Hence it is observed that the CS/KAO biocomposite films have improved the thermal stability than that of CS which shows close agreement with the results reported by S. C. Dey *el al.* [34]

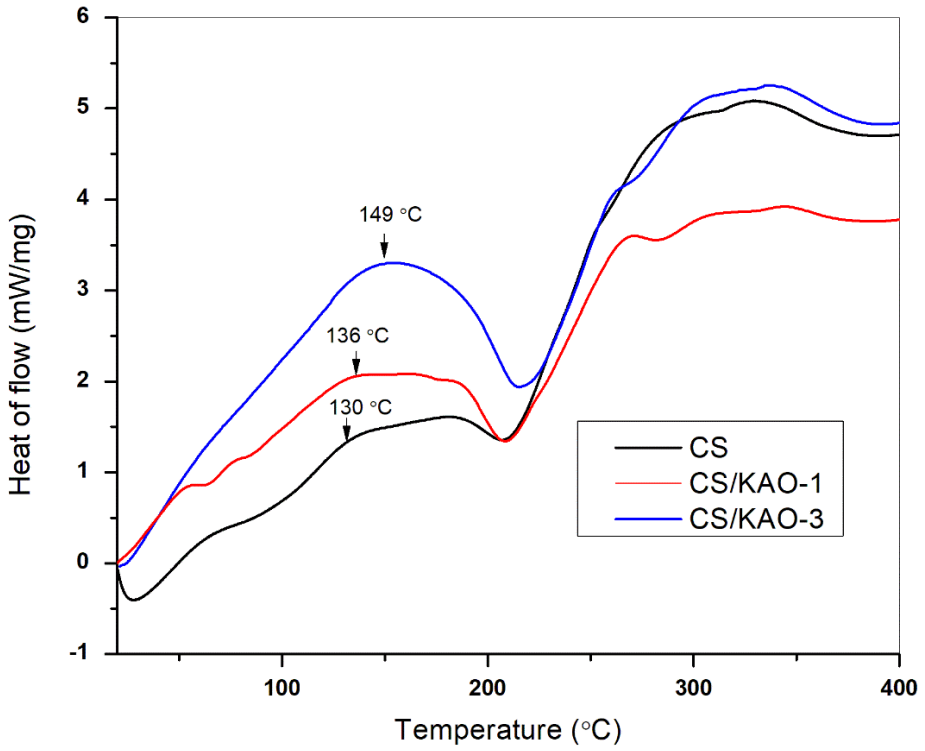


Figure 4.7. DSC plots of CS and CS/KAO biocomposite films.

4.3.5. Study of antibacterial property

The agar well diffusion test [98,99] results for CS and CS/KAO biocomposite films are shown in Figure 4.8. The test samples, i.e., CS, CS/KAO-1, CS/KAO-2, CS/KAO-3, and CS/KAO-4 were evaluated for antibacterial assay against *Escherichia coli* and *Bacillus subtilis* in concentration of 10 mg mL^{-1}. MIC was tested against bacterial pathogens *Escherichia Coli* and *Bacillus subtilis* at different concentrations of the test samples (10, 5, 2.5, 1.25, 0.625, and 0.31 mg mL^{-1}). In vitro susceptibility tests showed that test samples had antibacterial effects against both the bacteria at MIC 10 mg ml^{-1}. Therefore 10 mg mL^{-1} concentration was selected for antibacterial activity to determine the zone of inhibition using agar well diffusion method. The solution used for dissolving the test samples (2% acetic acid) also showed some inhibition properties. The highest zone of inhibition was seen up to 21 mm diameter by CS/KAO-2 followed by 18 mm in both CS/KAO-1 and CS, 13 mm on CS/KAO-4 and lowest inhibition 10 mm diameter was seen on CS/KAO-3 against *Escherichia coli* at 140 µg mL^{-1}. In case of gram-positive bacteria, the highest zone of inhibition was seen up to 17 mm diameter on CS/KAO-3 followed by 15 mm on CS/KAO-

1 and CS/KAO-2 each, 13 mm on CS and lowest inhibition 12 mm diameter was seen on CS/KAO-4 against *Bacillus subtilis* at 140 µg mL^{-1}.

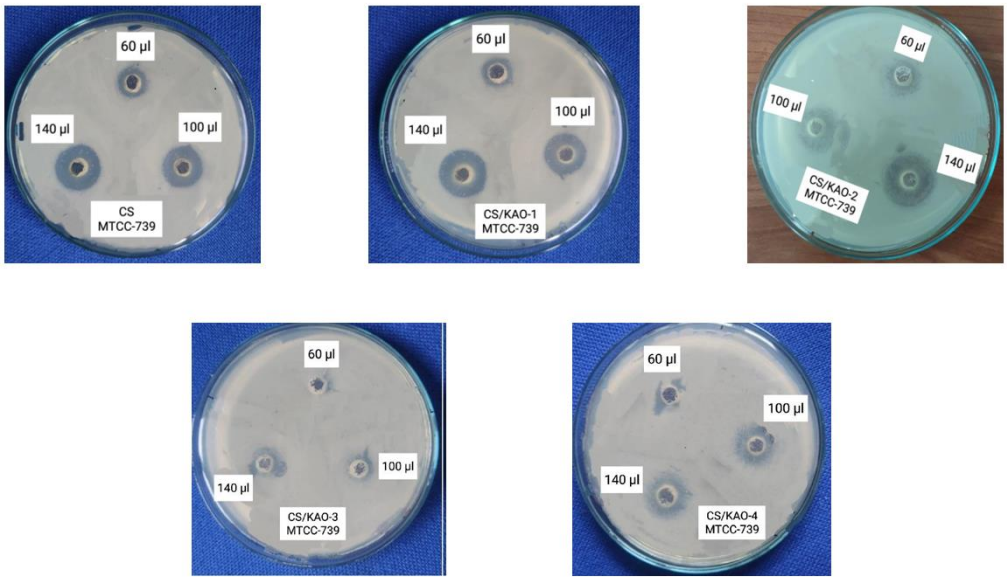


Figure 4.8. a) Antibacterial activities of CS and CS/KAO clay against gram-negative bacteria.

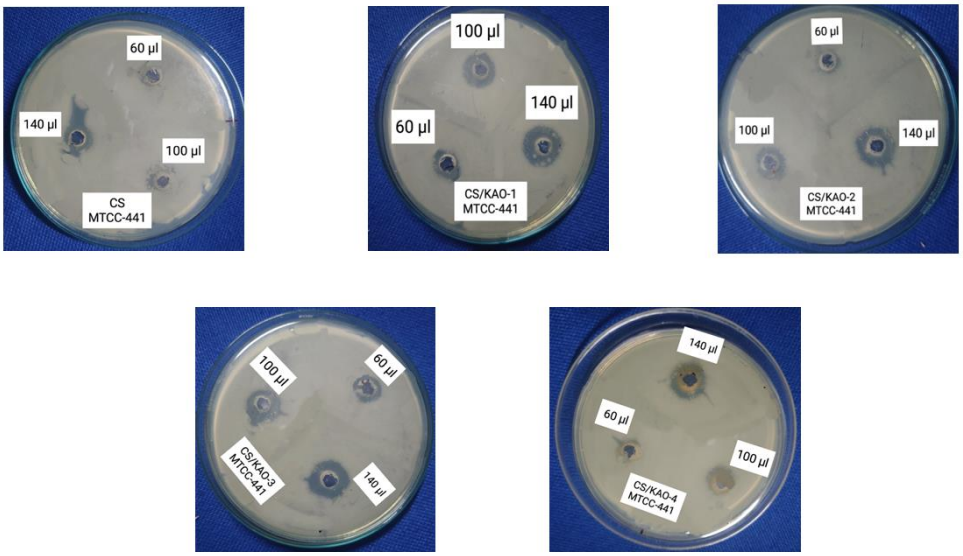


Figure 4.8. b) Antibacterial activities of CS and CS/KAO clay against gram-positive bacteria.

CS is the only test sample which showed antibacterial activity on all concentration against gram-negative bacteria but only one concentration against gram-positive bacteria. According to our literature analysis, the antibacterial study of CS/KAO clay biocomposite

films for different weight ratios by agar well diffusion method has never been reported. The biocomposite films shows significant inhibitory effect against gram-negative bacteria as well as gram positive bacteria. It was observed that this was somewhat more effective against gram-negative bacteria than gram positive bacteria. This is due to higher hydrophilicity in gram-negative bacteria than in gram-positive, CS is more sensitive to gram-negative bacteria which exhibits increased morphological changes on treatment when compared to gram-positive [123]. Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, whereas gram-positive bacteria are surrounded by layers of peptidoglycan many times thicker than is found in the gram-negatives. In the presence of a thin peptidoglycan layer in gram-negative bacteria than gram-positive, the low molecular weight CS can easily cross the cell wall of gram- negative bacteria, while high molecular weight CS acts as a barrier interfering with the proper absorption of nutrients by the microbial cell [118]. Moreover, CS is reported to be polycationic in nature, so it can easily interfere with negatively charged residues at the cell surfaces causing cell wall disruption and alteration of membrane permeability which results in the inhibition of DNA replication and subsequently cell death [145]. It is also reported that cationic analogous can easily bind the surface membrane of gram- negative bacteria due to the presence of anionic structures such as lipopolysaccharides and proteins [146]. Cankaya *et al*. reported the antibacterial properties for CS/Na^+ Montmorillonite, CS/Nanoclay 1–135 and CS/Nanoclay 1–140 and observed good antimicrobial activities for those biocomposites [79]. The zone of inhibition of crude extract against bacterial strain MTCC-739 and MTCC-441 are summarized in Table 4.4.

Table 4.4. Zone of inhibition (diameter in mm) in different concentration against MTCC-739 and MTCC-441 bacterial strain.

Sl. No.	**Sample (test & standard)**	**Zone of inhibition (diameter in mm) in 140 µl solution concentration**	
		Gram negative bacteria	**Gram positive bacteria**
1.	CS	18	13
2.	CS/KAO-1	18	15
3.	CS/KAO-2	21	15
4.	CS/KAO-3	10	17
5.	CS/KAO-4	13	12
6.	Amoxycillin	23	23

4.3.6. Analysis of swelling test

To the best of our knowledge, the swelling test of the CS/KAO biocomposite films with various weight ratio have never been reported. Swelling tests were performed for all the synthesized CS and CS/KAO biocomposite films. It has been observed that the highest swelling property was observed for CS/KAO-1 while the least was observed in case of CS/KAO-4 (Table 4.5). The following hierarchy was obtained by comparing the swelling behavior of CS and CS/KAO biocomposite films (Figure 4.9):

CS > CS/KAO-1 > CS/KAO-2 > CS/KAO-3 > CS/KAO-4

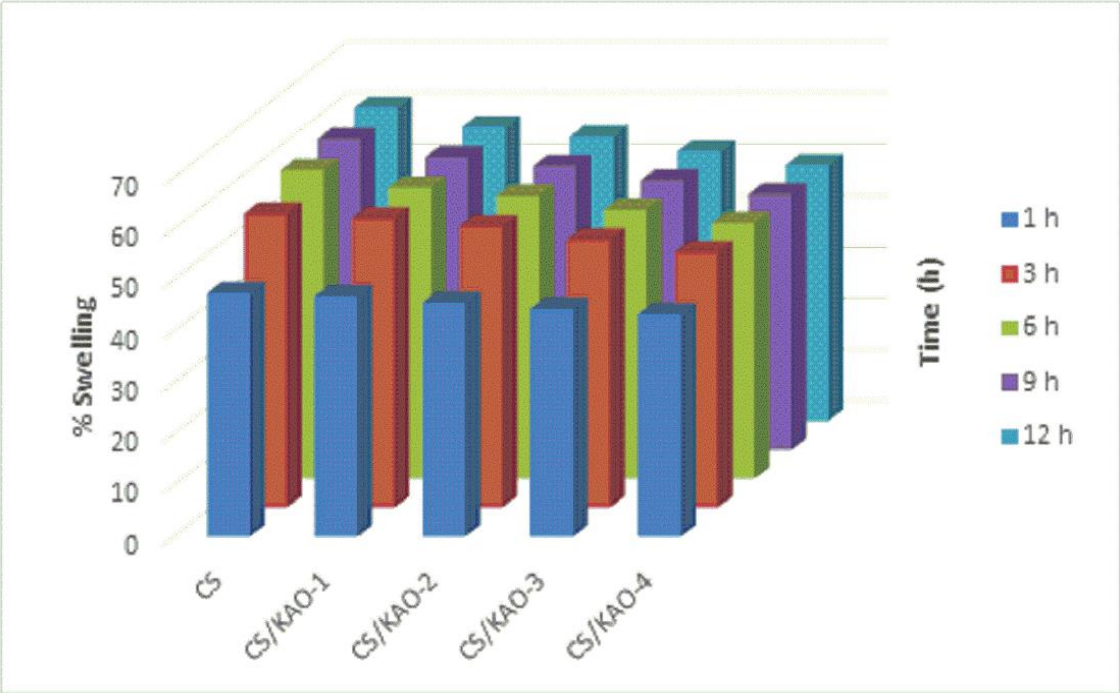


Figure 4.9. Swelling test plots of CS and CS/KAO biocomposite films.

Swelling experiment shows that the CS and CS/KAO biocomposite films have different levels of water absorption capacity. It was observed that on increase in the KAO clay amount to the CS matrix shows an inverse effect to the swelling of the films. The hydrophilic property provided by groups -OH and $-NH_2$ in the structure of CS matrix with the clay particles would be the reason of this behavior. The results agree well with the investigation reported by Cankaya *et al.* using CS/Na^+ Montmorillonite, CS/Nanoclay 1–135 and CS/Nanoclay 1–140 [79].

Table 4.5. Percentage of swelling for all the biocomposites.

Sample	Swelling %				
	1 h	**3 h**	**6 h**	**9 h**	**12 h**
CS	47.4	57.2	60.1	60.6	61
CS/KAO-1	46.8	56.3	56.7	57	57.2
CS/KAO-2	45.5	54.8	55.1	55.3	55.4
CS/KAO-3	44.3	52.2	52.4	52.5	52.5
CS/KAO-4	43.2	49.7	49.8	49.8	49.8

4.4. Conclusion

In this work, four films of CS/KAO biocomposite films were prepared using CS and KAO clay at different weight ratios in a 2% acetic acid solution. The ratios of the biocomposite films were maintained at about 10:01, 10:02, 10:03, and 10:04 respectively. All the biocomposite films were characterized by various physico-chemical methods viz. FT-IR, UV/Vis, XRD, SEM, UTM, TGA/DTG, and DSC. Two tested bacteria's viz. *Escherichia coli* and *Bacillus subtilis* were used to determine the antibacterial activities of the biocomposite films. The biocomposite films were also performed with swelling tests. FT-IR analysis reveals that absorption band at 1639 cm^{-1} for CS film was shifted to 1624 cm^{-1} in the biocomposites. CS/KAO biocomposite films showed a high UV/Vis absorption than pure CS matrix. XRD pattern confirmed that the KAO clay was effectively incorporated into the CS with four different concentrations. SEM micrographs showed that in case of CS/KAO-1 biocomposite, the clay particles were uniformly dispersed into the matrix and thus showed an exfoliated structure whereas in case of CS/KAO-2 and CS/KAO-3 the filler and matrix interaction was more prominent. The fibrous network and rough surface in CS/KAO-4 was due to the high concentration of KAO clay in the CS matrix. The gradual addition of filler is the reason of enhancement in the mechanical properties of biocomposites. The biocomposite films showed greater thermal stability than that of the CS matrix. The thermal stabilities improved with the increase of clay amount into the CS matrix. Further DSC suggests that the thermal stability of CS was improved due to composite formation. Antibacterial activity of biocomposite films was investigated in which the test materials had significant inhibitory impact against *Escherichia coli* than gram-positive bacteria *Bacillus subtilis*. Swelling experiment shows that the CS and CS/KAO clay biocomposites have

different levels of water absorption capacity and increase of the clay is inversely related to the swelling of the films. From our literature analysis it was observed that the CS/KAO clay biocomposites has several applications. As the synthesized biocomposites shows good tensile strength as well as significant antibacterial properties it may be used in the food packaging purposes. We can believe that all biocmposites have the ability to perform well as food packaging material but since CS/KAO-2 shows highest zone of inhibition against *Escherichia coli*, so that among all the biocomposites CS/KAO-2 may be the best material for food packaging applications. Also, it may be observed that among all the biocomposites, CS/KAO-2 possessed a more uniform and smoother surface which is desirable for its industrial applications. It was also observed that, as the biocomposites were rich in clay it may shows some good performance in the absorption for various cationic dye and heavy metals. On the basis of the literature investigation, we can say that CS/KAO-4 may have possibly better performance for cationic dye uptake because CS/KAO-4 biocomposite contain highest amount of KAO clay whereas CS/KAO-1 may adsorb heavy metals because lowest amount of KAO clay is present. The findings suggest that CS/KAO clay biocomposites have exceptional properties with wide applications, hence able to occupy a potential market across the globe.