Chapter 2:

Experimental and characterization techniques

2.1. Materials

Biodegradable polymer

Chitosan (from shrimp shells) with 75% degree of deacetylation was bought from LOBA Chemie and was used as received.

Reagent

2-Bromopropionyl bromide (97%) was bought from Aldrich, India and was used as received.

Clay

Aluminium silicate hydrate (trade name kaolin) was purchased from Oxford Lab Fine Chem LLP. Bentonite also known as montmorillonite and aluminium silicate hydrate, was bought from Sisco Research Laboratories Pvt. Ltd. Silica gel (SiO_2) was obtained from Thermo Fisher Scientific India Pvt. Ltd. All the clays were used exactly as they were supplied.

Solvents

Triethylamine (99%) from Merck, India, N, N- Dimethylformamide (DMF) (99%) from Emplura, India, Glacial acetic acid ≥ 99% from EMPLURA, India, Sodium Hydroxide (NaOH) from EMPLURA, India and distilled water were used as received.

Bacterial strain

Two tested bacterial species one gram-negative and gram-positive pathogen (MTCC-739-*Escherichia coli* and MTCC-441-*Bacillus subtilis* respectively) were supplied by MTCC, Chandigarh, Punjab, India.

2.2. Characterization techniques

2.2.1. Instrumental measurements

2.2.1.1. Fourier transform infrared spectroscopy

The Agilent Cary 630 Fourier transform infrared (FT-IR) spectrometer was employed to record FT-IR spectra for all the samples, covering a range of 4000-700 cm^{-1} with a resolution of 4 cm^{-1}. KBr pellets were utilized in the process, and each spectrum was obtained by accumulating 8 scans.

Figure 2.1. Agilent Cary 630 FT-IR spectrometer.

(Source: https://www.researchgate.net/figure/Agilent-Cary-630-FTIR-Spectrometer-equipped-with-MicroLab-application_fig1_364566684)

2.2.1.2. Ultraviolet/Visible spectroscopy

Ultraviolet/Visible (UV/Vis) analyses were conducted on both pure CS and MCS using a UV-2600 UV/Vis spectrophotometer, Model UV-2600 (A12595880048ML), with a resolution of 2 nm. For the CS/clay biocomposite films, recordings were performed on an Agilent Cary 60 UV/Vis spectrophotometer with a resolution of 1 nm. All sample recordings were carried out within the wavelength range of 200–800 nm.

Figure 2.2. UV-2600 UV/Vis spectrophotometer.

(Source: https://m.indiamart.com/proddetail/uv-2600-uv-2700-uv-vis-spectrophotometer-21015225891.html)

2.2.1.3. Nuclear magnetic resonance spectroscopy

The ^{1}D nuclear magnetic resonance (NMR) spectra of CS and MCS were recorded on 600 MHz NMR spectrometer (Advance III, Bruker, Biospin, Switzerland) operating at 150.154 MHz for ^{13}C frequencies with Bruker's 3.2 mm Efree probe.

Figure 2.3. 600 MHz NMR spectrometer.

(Source: https://www.uochb.cz/en/instrumentation/4/bruker-avance-iiitm-hd-600-mhz)

2.2.1.4. X-ray diffraction pattern

The X-ray diffraction (XRD) pattern of the CS/KAO biocomposite films was recorded using an X-ray diffractometer (Rigaku Ultima IV) at room temperature. Cu Kα radiation (λ = 1.5418 Å) was emitted from a broad focus Cu tube operated at 40 kV and 40 mA for sample measurement. Other samples were analyzed using a Bruker AXS X-ray diffractometer (model D8 Focus, Germany) with Cu-Kα radiation (wavelength λ = 0.154 nm). The instrument operated at 30 kV and 30 mA, with a scanning rate of 0.05 degrees per second, covering a 2θ range from 10° to 70°. The step size for the investigation was set at 0.1 (2θ).

Figure 2.4. X-ray diffractometer (Rigaku Ultima IV).

(Source: https://www.rigaku.com/de/products/xrd/ultima)

2.2.1.5. Scanning electron microscopy

Scanning electron microscopy (SEM) images of CS and MCS were obtained using an analytical scanning electron microscope (JSM 6390LV, JEOL, Japan) operating at an accelerating voltage of 20 kV. The SEM images of CS/KAO biocomposite films were captured by an analytical scanning electron microscope (Zeiss SIGMA 300) operated at an accelerating voltage of 5.00 kV. Additionally, SEM images of CS/BNTN and CS/Si biocomposite films were taken using an analytical scanning electron microscope (JSM-7610F) with an operating accelerating voltage of 5.00 kV. To prepare the samples, all biocomposite films were cut into small pieces and mounted onto the SEM holder using double glue tape. Subsequently, a mixture of palladium (Pd) and gold (Au) was plasma-sputtered over the surface of the samples.

Figure 2.5. Scanning electron microscope.

(Source: https://www.researchgate.net/figure/JEOL-JSM-6390-LV-scanning-electron-microscope_fig8_330279057)

2.2.1.6. Universal testing machine

The tensile properties of some biocomposite films were evaluated using a Zwick/Roell universal testing machine (UTM). The grip separation was set at 10 mm, and a cross-head speed of 10 mm min^{-1} was applied. Tensile strength and elongation measurements were conducted on three specimens cut from each film sheet. Thus, the measurements were

performed on a total of three specimens for each film type, with mean values calculated for both tensile strength and elongation from a single sample.

Additionally, the texture analyzer (TA-HDPlus, Stable Microsystems, UK) was utilized to determine the tensile properties of some biocomposite films. Thin strips, 10 mm in width, were cut from each biocomposite film to assess their textural characteristics. These strips were secured in the grips of the testing machine with an initial gap of 20 mm between the grips. The tests were carried out with specific parameters: a pre-test speed of 2 mm s^{-1}, a test speed of 3 mm s^{-1}, a post-test speed of 10 mm s^{-1}, a distance of 75 mm, and a trigger force of 10 g. The testing probe was attached to a 5 kg load cell. All measurements were conducted in triplicate, and the average values were reported.

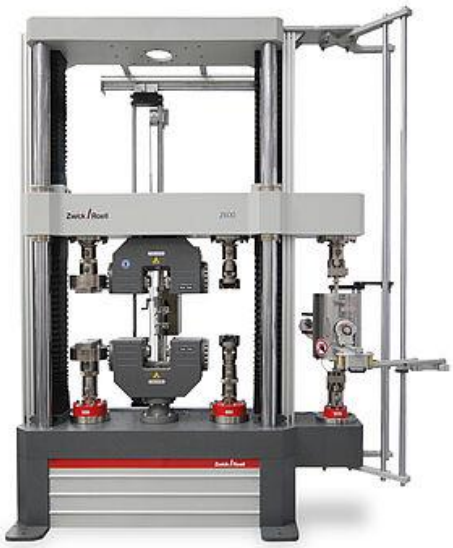

Figure 2.6. Zwick/Roell UTM.

(Source: https://www.zwickroell.com/products/static-materials-testing-machines/universal-testing-machines-for-static-applications/)

2.2.1.7. Thermogravimetric analysis

Thermogravimetric analysis (TGA) analysis of CS/KAO biocomposite films (~ 3 mg) was carried out using a Netzch instrument (Model: STA449F3A000). The samples were placed in the balance system and heated from 20 °C to 600 °C at a rate of 10 °C min^{-1} under an argon atmosphere. TGA results for other samples were obtained using a Netzsch instrument (Model: STA2500A-0297-N). The samples (~3 mg) were loaded into the machine's balance system and subjected to heating in the temperature range of 20 °C to 600 °C. The heating

rate employed was 10 °C min^{-1}, and the analysis was conducted under a nitrogen atmosphere with a constant flow rate of 30 mL min^{-1}. Subsequently, the samples were cooled under an airflow, and the analysis was performed with a dwelling time of 58 min.

2.2.1.8. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis of CS/KAO biocomposite films (~ 6 mg) was conducted using a Netzch instrument (Model: STA449F3A000). The samples were heated from 20 °C to 600 °C at a rate of 10 °C min^{-1} under an argon atmosphere. DSC analysis for other samples (~ 6 mg) was performed using a NETZSCH DSC 214 Polyma (DSC21400A-0438-L). These samples underwent heating, starting from 30 °C and reaching 400 °C, at a controlled heating rate of 10 °C min^{-1}. The DSC analysis was carried out under a nitrogen atmosphere with a constant flow rate of 20 mL min^{-1}.

Figure 2.7. Instrument for TGA and DSC analyses.

(Source: https://www.cif.iastate.edu/mass-spec/tga)

2.2.2. Antibacterial activity

2.2.2.1. By agar well diffusion method

The agar well diffusion assay of the samples was conducted following the methods outlined by Magladi *et al.* in 2004 and Valgas *et al.* in 2007, with slight modifications [98, 99]. To initiate the investigation, aseptically, three (3) wells were created for CS/KAO biocomposite films, four (4) wells for CS/BNTN and CS/Si biocomposite films, and two (2) wells for CS and MCS, each with a diameter of 6 mm, on nutrient agar plates using a cork borer. Subsequently, agar cylinders were removed using a sterile loop.

Next, the test organism (bacterial species) was swabbed onto the prepared agar plate using a sterile streak. Following the swabbing of the test organism, the wells were filled with

different volumes (60, 100, and 140 µL mg^{-1}) of stock solutions for the CS/KAO biocomposite films. For other test samples, each well was loaded with 140 µL. Amoxicillin served as the standard, and varying volumes (60, 100, and 140 µL mg^{-1}) of a 1 mg mL^{-1} concentration were used. The wells were allowed to dry by passing a blower in a laminar air flow (LAF) environment. Once completely dry, the plates were aseptically removed from the LAF and then incubated at 37 °C for 18 h. Inhibition zones were recorded for both the standard and control in the test.

2.2.2.2. By microdilution method

The minimal inhibitory concentration (MIC) was determined using solely MTCC-739-*Escherichia coli* bacteria through this method. In a test tube, 2 mL of a pre-prepared CS sample with a concentration of 10 mg mL^{-1} was combined with 2 mL of nutrient agar solution and thoroughly mixed. Subsequent 1:1 serial dilution was carried out by incorporating agar solution to achieve concentrations spanning from 10 mg mL^{-1} to 0.31 mg mL^{-1}. Subsequently, 100 µL of 739 bacteria was introduced into each test tube containing the sample (CS) and a sterility control. All test tubes, including the sterility control and a growth control (containing nutrient agar without an antimicrobial substance), were incubated at 37 °C for 18 h. The detection of bacterial growth in the test samples was accomplished by measuring the optical density.

2.2.3. Swelling Study

The swelling properties of all biocomposite films were investigated by immersing them in distilled water with a pH of 7.0 at room temperature for 1, 3, 6, 9, and 12 h. Prior to immersion, the samples were dried in an oven, and their initial weight (w_i) was measured. Subsequently, the samples were placed into bottles filled with distilled water, and the initial immersion time was recorded as t = 0. Measurements were then taken at specific time intervals until a stable weight value (w_f) was achieved. Afterward, the samples were filtered using filter paper. This entire procedure was repeated three times to ensure consistent results.

The swelling percentage (S_w) of each sample was calculated using the following equation [79]:

$$S_w = \frac{w_f - w_i}{w_i} \times 100 \ldots\ldots\ldots (2.1.)$$

2.2.4. Computational methods

The molecular geometry optimization and frequency calculations of the reactant's CS monomer, BPB, and possible products were performed using the Becke 3-parameter Lee Yang Parr (B3LYP) [100] functional in conjunction with the 6-31G(d,p) [101] basis set implemented in the Gaussian 16 software [102]. The optimized structures were visualized using the Gauss View 6.0 software [103]. The frequency calculation was performed to confirm that the optimized geometries of the reactants and products do not have imaginary frequencies. Further, the presence of only one imaginary frequency confirmed geometry corresponds to the transition-state (TS) structures. The intrinsic reaction coordinates (IRC) path was obtained to generate the energy profile connecting the reactant and the product via the TS. Further, using the same level of theory and basis set the molecular electrostatic potential (MEP) and frontier molecular orbitals were obtained etc. [104]. The reactants' MEP maps were generated using the Multiwfn 3.8 [105] and visual molecular dynamics (VMD) [106] program. The single-point calculations were performed by using the domain-based local pair natural orbital coupled-cluster theory with single, double, and perturbative triple excitations [DLPNO-CCSD(T)] [107] implemented in the ORCA software [108]. The relative energy profile has been plotted for the proposed reaction mechanism.