3. Methodology

The aim of this thesis is to develop synthetic routes for quaternary structural variants in different bio-materials. Chemical modifications of biomaterial broadens the spectrum of its applications, as biocide. Alkylation is done for providing a perfect balance in hydrophobic and hydrophilic properties. For hydrophobicity, the quaternary structure is integrated via choline chloride (CC). Choline chloride (CC) is a natural quaternary functionality for green modification of materials in quaternary structure. Quaternization makes the polymer water soluble and also enhances its biological applications. QACs are widely used in various fields because of their biocompatibility and the possibility to tailor the new biomaterials.

Polysaccharides are structurally diverse. Sucrose, chitosan and cyclodextrin being biogenic can be explored as a raw materials to replace the current oil-based and non-renewable products for developing novel biomass based bioactive materials or biocides. Additionally, biopolymer has also additional advantage of easy modification by different types of covalent linkages. Sucrose, chitosan and cyclodextrin are modified in alkylated structure by alkylation reaction with alkyl halides. Alkylated product is then reacted with ECH and CC in order to obtain quaternary ammonium compound. The synthesized quaternary ammonium compound is then tested for antibacterial and antifungal activity.

3.1. Materials

Sucrose (CDH, New Delhi), Cyclodextrin (HIMEDIA, Mumbai), Chitosan (HIMEDIA, Mumbai), Ethyl iodide (SIGMA-ALDRICH, USA), Iododecane (SIGMA-ALDRICH, USA), Iodohexadecane (SIGMA-ALDRICH, USA), Epichlorohydrin (CDH, New Delhi), Choline chloride (HIMEDIA, Mumbai) and all other reagents were of analytical grade.

3.2. Methods

3.2.1. Alkylation reaction

The alkylation reaction was conducted in two necked round bottom flask equipped with stirrer and condenser. Chitosan 1.0 g in 2.0 mL NaOH (50%) and 8 mL isopropanol were placed in two neck round bottom flask. The reaction mixture was kept for one hour at room temperature. Furthermore, 1.5 molar equivalent of alkyl halide (ethyl iodide) was added drop wise in 20 minutes. The reaction mixture was stirred at 50 °C for 8h. Then the reaction mixture was cooled, neutralized with 0.1 N HCl and acetone was added in adequate amount for complete
precipitation. The resulted precipitates were filtered and washed with alcohol or ether to remove impurities. The same procedure was repeated with other alkyl halides i.e. iododecane and iodoheptadecane and cyclodextrin and sucrose to obtain alkylated products of chitosan.

3.2.2. Quaternary product

Cationic polymers of alkylated products were synthesized according to Mansourpour et al. (2015) with minor modifications. The reaction was performed in three necked flask fitted with stirrer and condenser. The alkylated product was dissolved in 20 mL mixture of isopropanol and H₂O (1:1 ratio) and 20 mL HCl (0.1 N). Afterward, 1.5 mole equivalent of choline chloride (in 10 mL of 10% NaOH) was added. Spontaneously, 1.5 mole equivalent of ECH was also added drop-wise. The temperature of solution was kept at 50 °C for 24 h. After completion of the reaction, the reaction mixture was neutralized with HCl and then acetone was added for complete precipitation. The precipitates were washed with ethanol or ether to remove impurities. Similar procedure was repeated with other alkylated compounds in different molar ratio 1:3:3 of alkylated chitosan/CC/ECH. Similar procedure was repeated for sucrose and cyclodextrin.

3.3. Reaction efficiency

The efficiency of reaction was calculated gravimetrically as follows:

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\% \text{ Efficiency} = \frac{W_s}{W_t} \times 100
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Where, \(W_s\) is the actual yield of reaction and \(W_t\) is the theoretical yield of the reaction for 100% conversion of the hydroxyl groups.

3.4. Analytical determination

3.4.1. Degree of quaternization

The synthesized QACs were characterized for degree of quaternization (DQ) by the precipitation titration. Sample was dissolved in distilled water and titrated with 0.1 M aqueous silver nitrate using 5.0% aqueous potassium chromate as indicator. The DQ was calculated according to the following equation:
DQ = \frac{cV}{1000} - \frac{m}{1000} \times \frac{CV}{M_1} + \frac{CV}{1000} \times \frac{M_2}{M_1}

Where, c (mol/L) is the concentration and V (mL) is the volume of aqueous silver nitrate, m (g) is the weight of quaternary product, M_1 (mol/g) is the molar mass of quaternary compound and M_2 (mol/g) is the molar mass of ammonium group in quaternary compound.

3.5. Characterization

Characterization of quaternary products will be carried out by FT-IR, elemental analysis (CHN) and Scanning Electron Microscopy (SEM) in order to evaluate the efficiency and uniformity of the applied protocol. FTIR analysis was carried in the wavenumber ranges 400 cm\(^{-1}\) to 4000 cm\(^{-1}\) using Infrared spectrophotometer (Agilent technologies, Cary 630). The morphology and shape of synthesized polymer was examined through SEM studies using Scanning Electron Microscope, JSM-6100. Elemental analysis (CHN) was performed for the determination of %age of C, H and N in the sample. The elemental analysis (CHN) was determined using elemental analyzer CHNS (Thermo Finnigan, Italy).

3.6. Application characterization

The quaternary ammonium compounds has wide spectrum of applications and characterized for different biological activities. Gram positive bacteria (S. aureus), gram negative bacteria (P. aeruginosa) and fungi (C. albicans) were used for the evaluation of antibacterial activity of synthesized products by agar well diffusion method. Colony forming unit (CFU) and minimum inhibitory concentration (MIC) was also calculated for the synthesized compounds with two strains of bacteria i.e. S. aureus and P. aeruginosa.

3.6.1. Antibacterial activity

(a) Micro-organisms

Gram positive bacteria S. aureus and gram negative bacteria P. aeruginosa were used for the evaluation of antibacterial activity of synthesized biogenic quaternary ammonium compounds. The strains of bacteria were collected from Microbiology Lab of Shoolini University.
(b) Inoculums preparation

Stock culture was maintained at 4 °C on slopes of nutrient agar. An active culture for experiment was prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton agar (MHA) for bacteria and they were incubated without agitation for 24 h at 37 °C. 0.2 mL of culture was inoculated and incubated till it reached the turbidity equal to that of the standard solution.

(c) Antibacterial assay

The effect of synthesized biogenic biocides on the both bacterial strains (S. aureus and P. aeruginosa) was assayed by agar well diffusion method.

(d) Agar well diffusion method

Test solutions of each sample (10 mg of quaternary ammonium compound in 10 mL acetate buffer of pH 5.6) were prepared for biological activity determination. Specified media were prepared and sterilized by an autoclave. For antibacterial activity, the MHA (Mueller Hinton Agar) plates were prepared by pouring 25.0 mL of molten media into sterile plates. The plates were allowed to solidify for 10 min and 0.1% inoculums suspension was swabbed uniformly. The inoculum was allowed to dry for 10 min in an aseptic room. This provided the uniform surface for the growth of bacterium and was used for antibacterial activity studies. The wells were shaped on the solidified media with the help of sterile glass borer in such a way that there was no overlap for zone of inhibition. 2.0 µL of the synthesized compound were added to the wells. Petri plates were kept at room temperature for half an hour for diffusion of the sample in agar media. Petri plates were then incubated at 27 °C for 24 h.

3.6.2. Antifungal activity

The effect of synthesized biogenic biocides on the fungal strain (C. albicans) was assayed by agar well diffusion method.

(a) Micro-organisms

Antifungal activity of the synthesized quaternary ammonium compounds was evaluated against C. albicans. The effect of quaternary ammonium compounds on the fungal strain was assayed by agar well diffusion method.
(b) Inoculums preparation

Stock culture was maintained at 4 °C on slopes of media containing glucose (1.0 gm), peptone (0.5 gm) and yeast (2.0 gm). Active culture for experiments was prepared by transferring a loopful of spores from the stock cultures to test tubes of media for fungi and they were incubated without agitation for 42 h at 37 °C. 0.2 L of culture was inoculated and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution.

(c) Antifungal assay

(d) Agar well diffusion method

Test solution of each sample (10 mg of quaternary ammonium compound in 10 mL acetate buffer having pH 5.6) were prepared for biological activity determination. Specified media were prepared and sterilized by an autoclave. For antifungal activity, the PDA (Potato dextrose agar) plates were prepared by pouring 15.0 mL of molten media into sterile plates. The plates were allowed to solidify for 5 min and 0.1% inoculums suspension was swabbed uniformly and the inoculums was allowed to dry for 10 min in an aseptic room. This provided the uniform surface for the growth of fungi and was used for antifungal activity studies. The wells were shaped in the solidified media with the help of sterile glass borer in such a way that there is no overlapping of zone of inhibition. 2.0 µL of the extracts were added to the wells made. Petri plates were kept at room temperature for half an hour for diffusion of the sample into agar media. Petri plates were then incubated at 37 °C for 48 h.

3.7. Minimum inhibitory concentration (MIC) by micro dilution method

MIC was determined by broth micro dilution method (CLSIM7/-A7). The assay was done using 96 well plates in Mueller Hinton broth. Minimum effective antibacterial concentration (70 µg/mL) was chosen from the previous assay (well method) for the further study. In micro-dilution study, the concentration was varied for the confirmation of MIC values in different dilutions for series of compounds in micro-titer plate wells. Culture (bacteria and fungus) was added in micro-titer plate wells and was incubated for 24 h. Resazurin was used as indicator after 24 h incubation and kept for 2-4 h. Lowest concentration that prevented colour change was taken as the minimum inhibitory concentration.
3.8. Antibacterial assay by colony forming unit (CFU) method

The synthesized compounds were screened against *P. aeruginosa* and *S. aureus* for anti-microbial activity which were grown overnight in nutrient broth (NB) medium in incubator shaker at 37 °C. Nutrient broth solution was prepared by dissolving 1.3 g of solid NB in 100 mL of distilled water in a 250 mL flask. The flask containing the NB and all the apparatus were autoclaved before use. For each sample estimation, three test tubes were taken. In one test tube 10 mL of NB and 1 mL of bacterial culture were added. This test tube was considered as control in which the normal bacterial growth took place. In other test tube 5.0 mL of NB, 1.0 mL bacterial culture and 1000 µg/mL of samples were added. Same concentration of sample was also added with 10 mL of NB. These test tubes were considered as reference of the samples. Dilution was done in apendoff with distilled water. All these test tubes were kept in the incubator shaker at 30 °C. Colonies were counted at 24 h by seeding the aliquot of incubated sample on nutrient agar plates. To determine cfu/mL, number of microorganisms in the plates was multiplied by dilution factor and divided by the volume used to seed the plate (Allen et al., 2004). This procedure was repeated for three days to count the colonies after 24. CFU was calculated using following formula:

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CFU = \frac{\text{No. of colonies}}{\text{Dilution}} \times \text{Volume of inoculum plated}
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