CHAPTER 2

METHODOLOGY

A part of this chapter has been published in the following articles:

1. D. K. Manna, A. K. Nandi, M. Pattanayak, P. Maity,S. Tripathy, A. K. Mandal, S. Roy, S. S. Tripathy, N. Gupta, S. S. Islam. Carbohydrate Polymers; 134, 375–384, 2015.

2. D. K. Manna, A. K. Mandal, I. K. Sen, P. K. Maji, S. Chakraborti, R. Chakraborty, S. S. Islam. International Journal of Biological Macromolecules; 80, 455–459, 2015.

3. D. K. Manna, Ashis K. Nandi, M. Pattanayak, P. Maity, A. K. Mandal, N. Gupta and S. S. Islam. Carbohydrate Polymer. 157, 1657-1565, 2017.

2.1. Structural analysis of polysaccharides

The structure determination of the polysaccharides (PS) the prime importance was attributed on the purity of the PS. Different purification techniques like precipitation and re-precipitation with different solvent systems, ultra centrifugation, dialysis, chromatography, etc. were adopted for this purpose. The structures of polysaccharides are determined by combinations of two methods: **1. Chemical method** which includes total acid hydrolysis, methylation, Smith degradation, periodate oxidation studies. **2. Spectroscopic method** which comprises 1D- NMR (^{1}H, ^{13}C, DEPT-135) and 2D-NMR (DQF-COSY, TOCSY, NOESY, ROESY, HSQC, HMBC) analyses. A schematic diagram of extraction, purification and structural analysis of a PS by chemical and NMR spectroscopic methods has been presented in the following diagram (**Figure 2.1).**

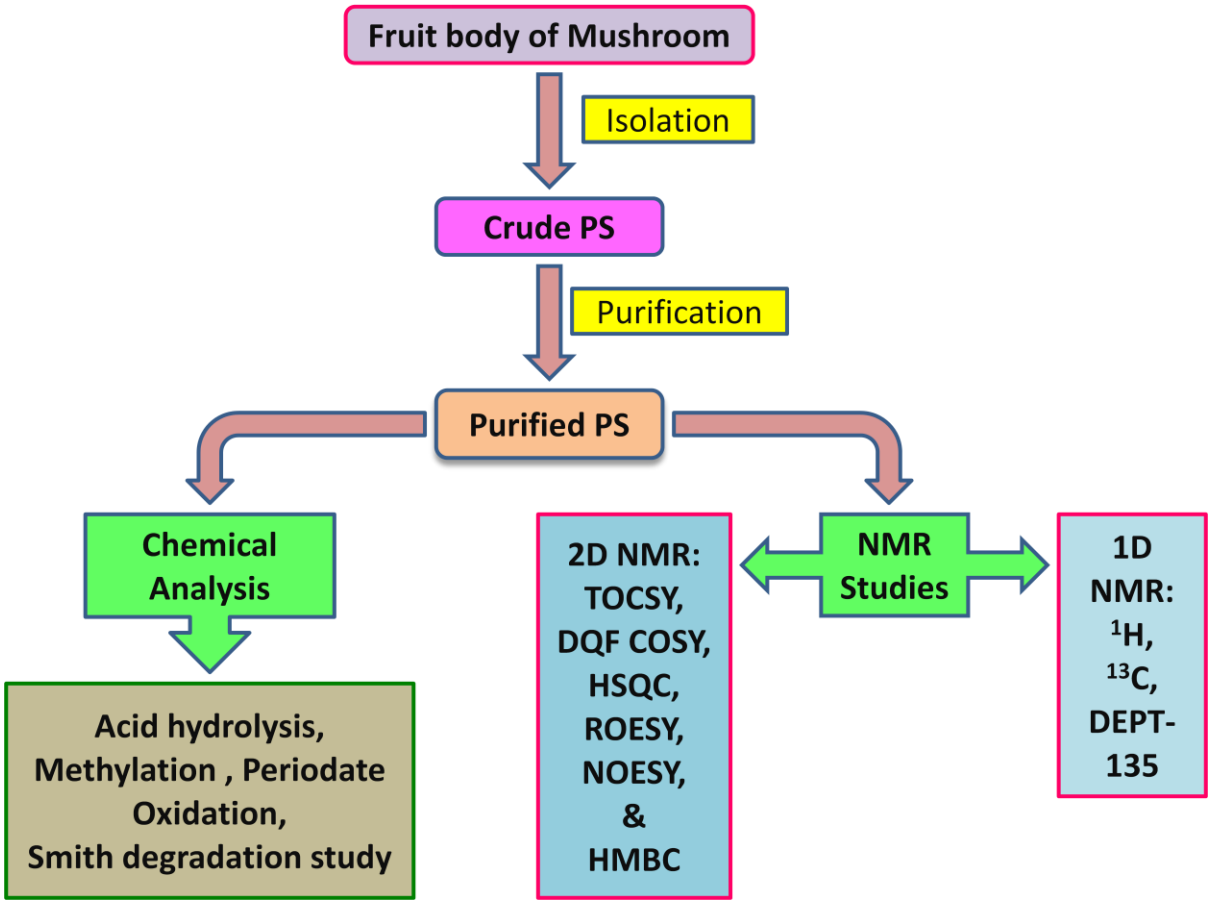

Figure 2.1. Schematic diagram of structural analysis of polysaccharides

2.2. Isolation and Purification of polysaccharides from fruit bodies of mushroom

2.2.1. Isolation and Purification of polysaccharides (PS-I and PS-II) from alkaline extraction of an edible mushroom, *Termitomyces heimii*

The dried fruit bodies of the mushroom *Termitomyces heimii* (120 g) were boiled with 4% NaOH solution for 1 h. The reddish-brown mixture was then reserved at 4°C for overnight and filtered. The filtrate was centrifuged (in Heraeus Biofuge stratus centrifuge) at 8000 rpm at 4 °C for 50 min. EtOH (1:5 v/v) was added with the supernatant to precipitate out the polysaccharide. The precipitated was dissolved in minimum volume of distilled water and dialyzed for 24 h through a cellulose bag (D9652, Sigma–Aldrich) in distilled water to retain high molecular weight materials (>12,400 Da) within the bag. The materials was collected and freeze-dried to get crude polysaccharide (600 mg).

Purification of crude polysaccharides was made by gel permeation chromatography (GPC) which works on the basis of size relative to the pores in the packing materials. The crude polysaccharide (30 mg) was passed through Sepharose 6B column (90 cm × 2.1 cm, fractionation range 10,000-10,00,000 Da), water used as eluant with a flow rate of 1 ml/2min, then collected using Redifrac fraction collector and monitored by the phenol-sulfuric acid procedure [203] at 490 nm using Shimadzu UV-vis spectrophotometer, Model-1601. Two fractions, PS-I (test tube 16–26) and PS-II (test tube 33–39) were obtained and freeze-drying yielded 12 mg and 2 mg of pure PS-I and PS-II respectively. Further purification of PS-I through GPC produced only a single homogeneous fraction and the structural and biological study of this molecule has been incorporated in this thesis. The fractionation and purification steps are depicted in **Figure 2.2.**

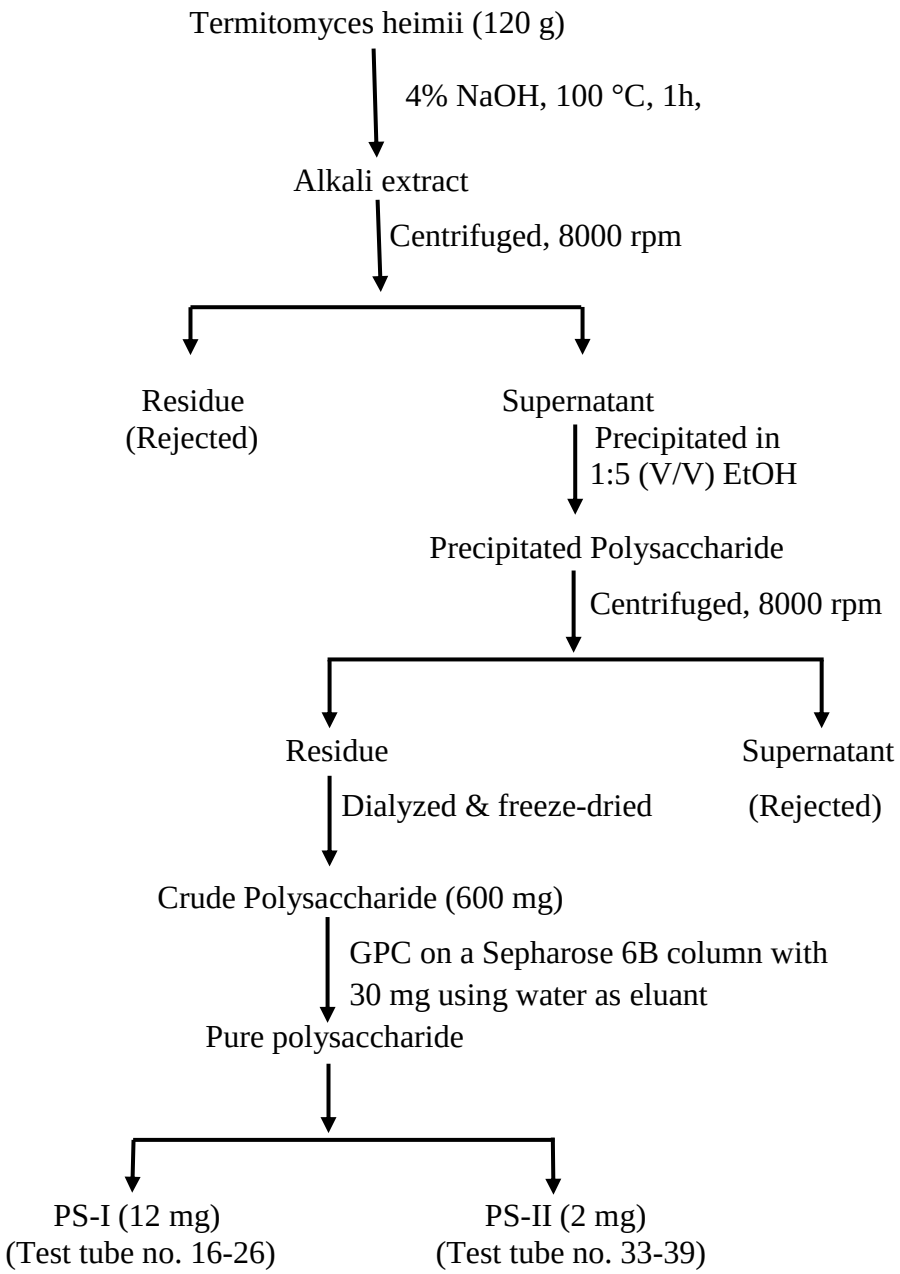

Figure 2.2. Schematic diagram of isolation and purification of polysaccharides from alkaline extract of the fruit bodies, *Termitomyces heimii*

2.2.2. Isolation and Purification of polysaccharides (PS-I and PS-II) from aqueous extraction of an edible mushroom, *Lentinus fusipes.*

The dried fruit bodies of the mushroom *Lentinus fusipes* (50 g) were boiled with distilled water for 10 h. Then filtration, followed by centrifugation, precipitation, dialysis, isolation of crude polysaccharide (2.1 g) and purification of crude polysaccharide (25 mg) by Sepharose 6B column were performed by following the procedures as discussed above in the Section 2.2.1 of this chapter.

Two fractions, PS-I (test tube 30–42) and PS-II (test tube 54–68) were obtained and freeze-drying yielded 9 mg and 11 mg of pure PS-I and PS-II respectively. Further purification of PS-I through GPC produced only a single homogeneous fraction and the structural and biological study of this molecule has been incorporated in this thesis. The fractionation and purification steps are depicted in **Figure 2.3.**

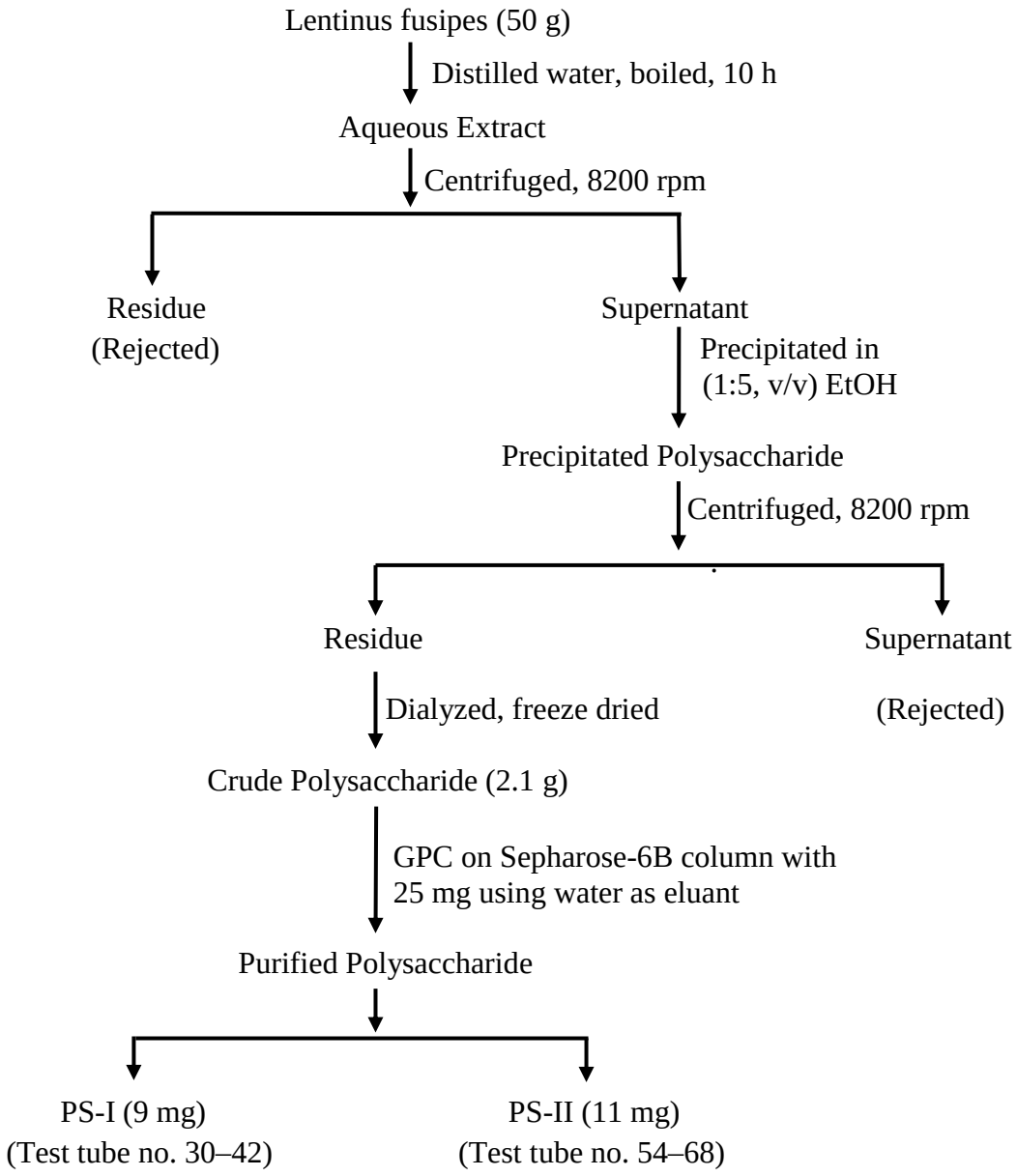

Figure 2.3. Schematic diagram of isolation and purification of polysaccharides from aqueous extract of *Lentinus fusipes.*

2.3. Determination of physical properties

2.3.1. Measurement of optical rotation

The pure polysaccharide (5 mg) was dissolved in 5 ml of distilled water and then optical rotation was measured using a Jasco Polarimeter model P-1020 at 31 ^{o}C.

2.3.2. Molecular weight determination

The molecular weight was determined by adopting gel-chromatographic technique [204]. The elution volumes of standard dextrans (T-200, T-70, T-40 possessing molecular weight 200,000; 70,000 and 40,000 respectively) through Sepharose-6B column were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide through same column and with the same flow rate was then plotted on the same graph and then graphically the average molecular weight was determined.

2.4. Chemical analysis

2.4.1. Monosaccharide analysis

For structural analysis it is essential to know the kinds of monosaccharides present in the polysaccharide. The pure PS (3.0 mg) was totally hydrolyzed with TFA (CF_3COOH;) (2 M, 2 ml) in boiling water bath for 18 h. Then it was reduced with $NaBH_4$ (7 mg), afterwards addition of dilute AcOH and distillation with MeOH to remove boric acid. The alditol acetates of reduced sugars were prepared with Py-AcOH (1:1) in a boiling water bath for 1 [205] and were analyzed by gas chromatography (GC) in Hewlett-Packard model 5730 A

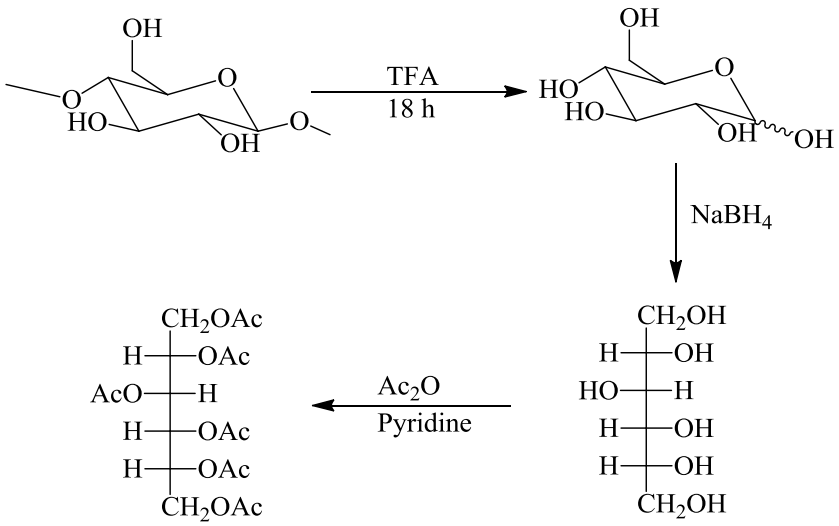

Figure 2.4. Schematic diagram of preparation of alditol acetates.

2.4.2. Determination of absolute configuration

In polysaccharide, monosaccharides usually exist as cyclic hemiacetal which can adopt either the D or L configuration. The monosaccharides were configured by the method of Gerwig et al., 1978 [206]. PS (1.0 mg) was treated with TFA, then with a volume of 250 µl of HCl (0.625 M) solution in R-(-)-2-butanol and heated at 80 ^{o}C for 16 h. Then TMS-derivatives of dry reactants were prepared with N,O-bis(trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GC using a capillary column SPB-1 (30 m x 0.26 mm) with a temperature program 3 ^{o}C/min from 150 to 210 ^{o}C. The (+)-2-butyl-2,3,4,6-tetra-*O*-TMS-glycosides were recognized by comparison with the prepared standard D- and L-enantiomers of different monosaccharides.

2.4.3. Linkage analysis

The different mode of connection of monosaccharide units in a polysaccharide is determined by methylation analysis which is finally confirmed by periodate oxidation study.

2.4.3.1. Methylation analysis

Methylation analysis predicts mode of linkage among monosaccharide units. This experiment includes conversion of all free hydroxyl groups present in PS into methoxyl groups followed by cleavage of only inter-glycosidic linkages by acidic hydrolysis, leaving the methyl ether bonds intact. The monomers are reduced and acetylated to produce volatile partially methylated alditol acetates (PMAA) (Figure 2.5). The position of *O*-acetyl groups in PMAA reflects the linkage patterns of the corresponding sugars in the polyasaccharide. 3 mg of PS was methylated by the method of Ciucanu and Kerek, 1984 [207]. The methylated PS was treated with 90% HCOOH (1 ml) at 100 °C for 1 h and the excess HCOOH was reoved. After that the product was reduced with $NaBH_4$ and alditol acetates were prepared with Py-Ac_2O. GLC–MS analysis of alditol acetates was performed on Shimadzu GLC-MS Model QP-2010.

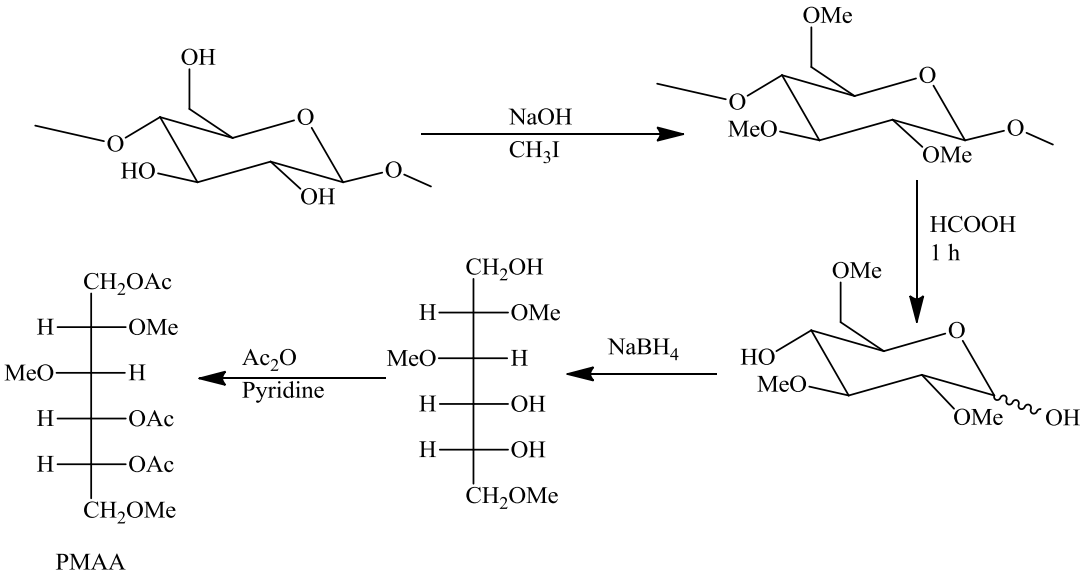

Figure 2.5. Schematic diagram of preparation of PMAA of polysaccharides.

2.4.3.2. Periodate oxidation study

Presence of free hydroxyl groups in vicinal position in polysaccharides makes them to react with periodic acid or it's salt to form two aldehydes. Non-reducing sugar residues and (1→6)-linked hexopyranose residues with three adjacent hydroxyl groups can

consume two molar equivalent of periodate which leads to double decomposition to form one molar equivalent of formic acid as presented in Figure 2.6. (1→2)-and (1→4)-linked hexopyranose units consume one equivalent of periodate per mole yielding a dialdehyde. Whereas (1→3)-linked hexopyranose residues will not be affected by this reaction because of absence of vicinal hydroxy groups.

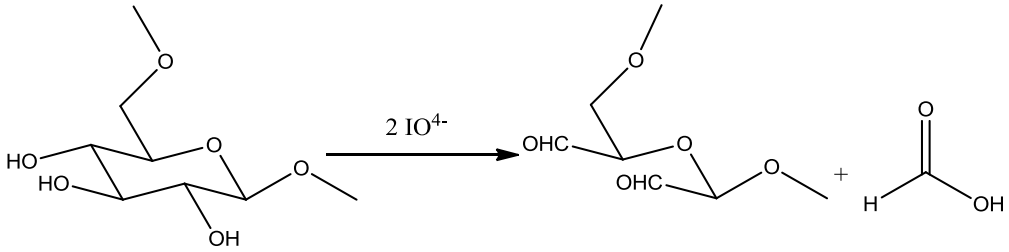

Figure 2.6. Schematic diagram of periodate oxidation of (1→6)-linked hexopyranoside.

PS was treated with 0.1 M (20 ml) sodium metaperiodate solution for 3 days in the dark. The excess periodate was destroyed by ethylene glycol. Then it was dialyzed in water, followed by reduction with $NaBH_4$, neutralisation with AcOH and again dialysis in water and finally freeze dried. This material was divided into three parts in a ratio of 1:1:4. From first part alditol acetates were prepared for GLC analysis and from second part PMAA were used for and were prepared for GLC–MS analysis.

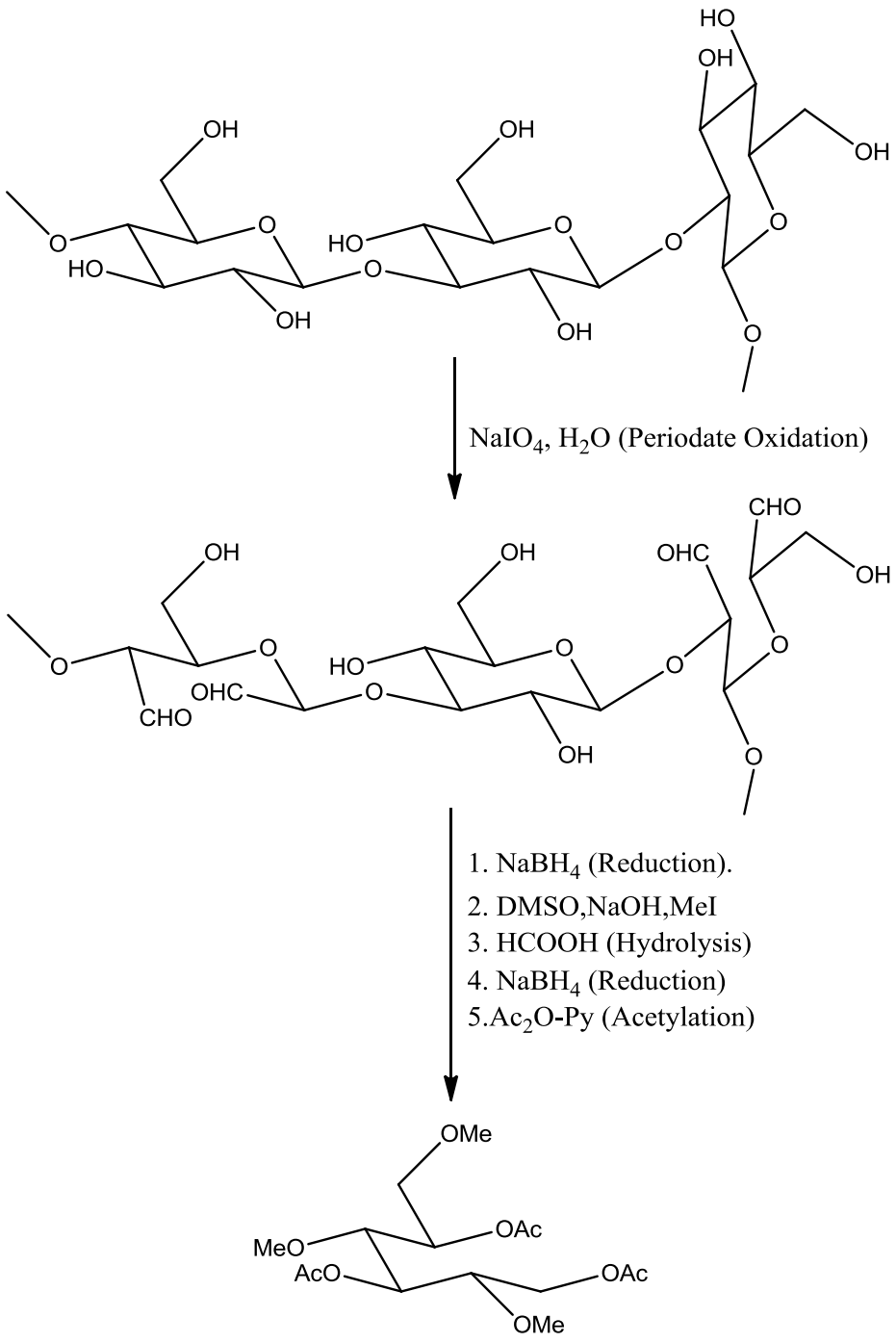

Figure 2.7. Schematic diagram of periodate oxidation study

2.4.4. Smith degradation

Smith Degradation is in fact combination of periodate oxidation, reduction and mild acid hydrolysis. This study offers plenty of information about the structure of the polysaccharide. It can selectively degrade a polysaccharide to either an oligosaccharide or a polysaccharide with a less number of repeating unit. The mild hydrolysis of the third part obtained from periodate oxidation was performed with 0.5 M TFA for 15 h at 25 ^{o}C to release the attached residual part of the oxidized material [208-209]. The excess acid was removed by. The material was freeze dried repeatedly and purified on Sephadex G-25 column for ^{13}C NMR analysis.

2.4.5. Sequence analysis: Partial acid hydrolysis

Partial acid hydrolysis of the polysaccharides also provides detailed information about the linkage types and sequence of different sugar residues in polysaccharide. 30 mg of polysaccharide was hydrolyzed in TFA (6 ml, 0.1 M) at 100 °C for 1 h. The addition of ethanol (3:1 v/v) to the acid free aqueous solution of hydrolyzed product produces a precipitate which was separated, washed with ethanol and freeze-dried (P1) for methylation and ^{13}C NMR analysis. The fragmented parts of polysaccharide present in supernatant were also recovered for methylation analysis.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is broadly used for structural elucidation of polysaccharides i.e.; for identification of monosaccharide residues, analysis of anomeric configurations (α or β), confirmation of linkages and sequence of the sugar units in the repeating unit. The complete establishment of the structure of different polysaccharides is possible by the application of two types of NMR techniques, 1D-NMR that includes ^{1}H, ^{13}C, DEPT-135 and 2D-NMR that includes TOCSY, DQF-COSY, NOESY, ROESY, HMQC, HMBC experiments.

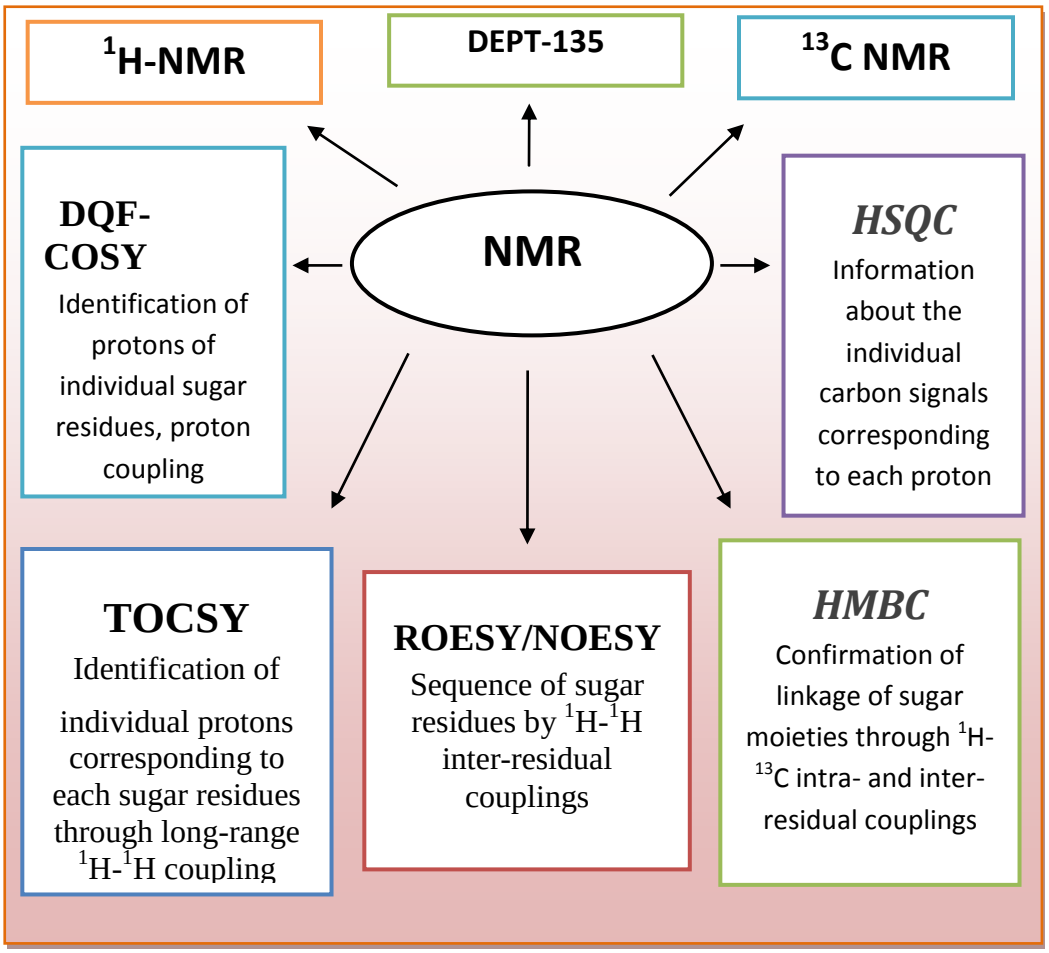

Figure 2.8. Schematic diagram of NMR spectroscopic methods for the determination of structure of a polysaccharide.

2.5.1. Preparation of NMR sample and instrumentation

In 1H NMR a major problem is that the signals of OH protons of sugar units and the proton from residual water, both appear in the approximately same region of the ring proton signals and thereby creates a definite interference for peak identification. Therefore, proper sample preparation is very important before NMR experiments. In this thesis work all polysaccharide samples were made free from water by keeping it over P_2O_5 in vacuum for several days and deuterium exchanged [210] by repeated lyophilization with D_2O (99.96%. atom ^{2}H, Aldrich) and then the NMR experiments were performed in D_2O with Bruker ASCENT-700 and Bruker Avance DPX-500

spectrometer at 30 °C. The 1H NMR spectrum was recorded using presaturation method which suppresses the HOD signal at 4.70 ppm. For NOESY and ROESY mixing delay time was 300 ms but, that was 80 ms in HMBC.

2.5.2. 1D or One-dimensional NMR

The numbers as well as relative proportion of monomeric units present in a polysaccharide and their anomeric configuration are determined by ^{1}H-NMR. Generally, the anomeric protons resonate between 4.4-5.5 ppm and remaining all ring protons resonate between 3–4.2 ppm. In case of deoxy sugars the methyl protons appear in between 1.1-1.3 ppm. The anomeric protons from each residues give distinct recognizable signals depending upon their α or β anomeric configurations. Normally, the α-anomer resonates downfield by 0.3-0.5 ppm compared to the β-anomer in D-pyranoses. Thus, the α-anomeric protons produced signals in between 4.8-5.5 ppm, while the β-anomeric protons produced signals in between 4.4-4.8 ppm. The vicinal coupling constant between anomeric H-1 and H-2 ($J_{1\text{-}2}$) indicates the relative orientation of two protons; an axial in pyranose form large coupling constant ($J_{1\text{-}2}$ ~ 8 Hz), whereas an equatorial-axial orientation produced a smaller ($J_{1\text{-}2}$ ~ 4 Hz) i.e., decreased by approximately 4 Hz [11]. Different sugars are identified from their characteristic coupling constant.

Due to low natural abundance of ^{13}C, it has much weaker signals than ^{1}H, but, ^{13}C NMR signals are spread out over a 20 times wider region than ^{1}H-NMR signals, that made it advantageous over the ^{1}H-NMR. In ^{13}C NMR spectra, anomeric carbons resonate in the range of 90-110 ppm and the non-anomeric carbons in the range of 60-90 ppm. The α-anomeric carbon generally resoate in the range of 95-103 ppm whereas most of the β-anomeric carbons resonate in the region of 101-105 ppm. Unsubstituted ring carbons usually appear in the region of δ 65-75 ppm. In case of deoxy sugars the methyl carbons appear in the region of 15-20 ppm. [212]. If there is any linkage at any carbon, the signal for that carbon will suffer a downfield shift by 4-10 ppm. and the carbon next to that one will appear in a little upfield region by 0.7-4.7 ppm. Signals for carbonyl carbons are generally observed between 165-185 ppm. [212-213].

Sometimes the ^{1}H J_{1-2} values are not sufficient to assign the anomeric configurations of the sugars. If the glycosyl residue has the *manno*-configuration, the distinction between the two anomeric forms is very difficult since the coupling costant values for α (J_{1-2}~ 1.8 Hz) and β (J_{1-2} ~ 1.5 Hz) are close enough to be differentiated. The one bond ^{13}C-^{1}H-coupling constants are useful to establish the anomeric configuration of sugar residues. For D-sugars a $^{1}J_{C1,H1}$ ~ 170 Hz indicates an α-anomeric sugar configuration whereas $^{1}J_{C1,H1}$ ~ 160 Hz indicates a β-anomeric sugar configuration [214]. This is interchanged for the L-sugars. C-1, H-1 coupling constants were determined from proton coupled ^{13}C NMR experiment.

In addition to ^{13}C NMR spectroscopy DEPT-135 (Distortionless enhancement by polarization transfer-135) technique is also very useful. In a DEPT-135 spectrum only -CH_2 groups exhibit a inverse signal at the same region of 13^{C} NMR, whereas, both, -CH_3 and -CH group exhibit positive signals. The signals for the C-6 atoms of various sugar residues appear as -CH_2 and therefore are identified by negative signals between 60-70 ppm in DEPT-135 [212].

2.5.3. 2D or Two-dimensional NMR

Multi-pulse sequences in 2D NMR experiments can reveal additional informations like intra-residual connectivities as well as inter-residual cross connectivities between the different nuclei. In the present thesis different 2D NMR experiments such as TOCSY, DQF-COSY, NOESY, ROESY, HMQC and HMBC have been used.

2.5.3.1. TOCSY or Total Correlation Spectroscopy

TOCSY is very powerful tool to confirm the assignments of ^{1}H spectrum and the scalar connectivity of the proton signals within a spin system. **TOCSY** spectrum provides the correlation among the protons that have same spin system. It yields both, short and long-range correlations among all geminal and vicinal protons. In this thesis work, TOCSY experiments have mixing time of 300 ms.

2.5.3.2. DQF-COSY or Double Quantum Filtered Correlation Spectroscopy

DQF-COSY is a modified version of the basic COSY experiment. It provides the information about the protons of an individual sugar residue through a three-bond coupling. An anomeric proton is selected as starting point and coupling constants are measured from DQF-COSY spectrum. The anomeric configuration of all sugar residues was confirmed by the $J_{H\text{-}1,H\text{-}2}$ value.

2.5.3.3. NOESY or Nuclear overhauser enhancement spectroscopy

A **NOESY** spectrum provides homonuclear (^{1}H-^{1}H) coupling correlations between spatially proximal protons through space rather than bond. NOE connectivies are often observed between protons, attached to glycosidically link two carbon atoms. The sequence of sugar residues and their position of linkages in a polysaccharide were determied from NOESY experiment. The NOESY mixing delay time was 300 ms.

2.5.3.4. ROESY or Rotating frame Overhauser Enhancement Spectroscopy

Like NOESY spectrum, a ROESY spectrum is also provides information about the position of glycosydic linkages and the sequence of monosaccharide residues in the polysaccharide. A ROESY spectrum correlates the closely spaced protons of two different sugar residues, but, not connected by a chemical bond via the Rotational nuclear overhauser effect (ROE). ROESY is especially useful where NOESY signals are weak The ROESY experiment also yields cross peaks raised from chemical exchange. The ROESY mixing delay time was 300 ms.

2.5.3.5. HSQC or Heteronuclear single quantum coherence

A HSQC spectrum correlates each carbon with the directly bonded proton(s) to it. The number of monosaccharides can more easily be determined in an HSQC spectrum than in a 1H-NMR. All the ^{13}C signals of the monosaccharide residues are assigned from the individual proton signals assigned from DQF-COSY and TOCSY spectrum as they are directly correlated.

2.5.3.6. HMBC or hetero multiple bond coherence spectroscopy

A HMBC spectrum correlates proton and carbon which are two or three bonds away with great sensitivity. It is very helpful to establish the linkage between monosaccharide units as well as sequence of them. HMBC experiment confirms the NOESY and ROESY connectivities of a polysaccharide.

2.6. Biological activities

2.6.1. Isolation of lymphocytes from peripheral blood mononuclear cells (PBMCs)

Fresh blood samples were collected from all groups of individuals (total 30 blood samples, 6 samples for each group) satisfying the Helsinki protocol and layered with phosphate buffered saline (PBS) (pH 7.0) in a ratio of 1:2 (histopaque 1077). Centrifugation at 1400 rpm for 45 min resulted a white milky layer consisting of mononuclear cells which was isolated and cultured in RPMI 1640 medium under 5% CO_2 and 95% humidified atmosphere at 37 ºC for 2 h [215]. The lymphocytes were isolated from the non adherent layer of the cultured cells by washing with PBS and centrifugation at 2000 rpm for 10 min. The depletion of the macrophages and B cells in PBMC were performed by passing through a nylon wool column.

2.6.2. Cell lysate preparation

The cell suspension was centrifuged at 1500 rpm for 5 min and the supernatants were collected and kept at −20 ^{o}C. The cell pellets were suspended in ice cold PBS and freeze–thawed at least four times followed by sonication (Ultrasonic Processor, Tekmar, Cincinnati, OH, USA) for 20 s. The cellular debris was discarded from the lysates by centrifugation at 12,000 rpm for 20 min at 4 ^{o}C. Bovine serum albumin was used as standard for estimation of protein content in lysate [216].

2.6.3. Cell viability

The in-vitro cellular toxicity of polysaccharides was studied on PBMC which were seeded into 96 wells of culture plates having 180 µl of complete media and incubated for 48 h. Polysaccharide was added to PBMC at varied concentrations and incubated at 37 °C in a humidified CO_2 incubator for 24 h. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported earlier [217].

2.6.4. Determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) level

Glutathione, a tripeptide (L-γ-glutamyl-L-cysteinylglycine), considered as an important anti-oxidant. Glutathione exist in both, reduced (GSH) and oxidized (GSSG) forms in cells. Oxidative stress decreases the level of GSH. Estimation of GSH of the cell lysates were carried out by centrifugation of the mixture of cell lysate and 25% of TCA at 2,000X g for 15 min to precipitate out settled proteins [218]. The supernatant was diluted with sodium phosphate buffer (1 ml, 0.2 M, pH 8.0). Later, 2 ml of 0.6 mM Ellman's reagent or DTNB (5-5'- dithiobis [2-nitrobenzoic acid]) was added which forms a yellow complex with GSH. The optical density of this complex measured at 405 nm. The levels of GSH were expressed as µg of GSH/mg protein.

The reaction of GSH of cell lysate (0.5 ml) with 2-vinylpyidine (2 µl) [219] and incubation for 1 hr at 37 °C, followed by deprotenization with sulfosalicylic acid (4%) and centrifugation at 1,000X g for 10 min settled the precipitated proteins. The supernatant was used for estimation of GSSG level via optical density measurement after reacting with DTNB at 412 nm.

2.6.5. Determination of lipid peroxidation (MDA)

Lipid peroxidation is an important determinant to assess the cellular damage. Several toxic by-products especially malondialdehyde (MDA) is released by lipid peroxidation inlymphocytes cells. MDA lavel indicates the extent of lipid peroxidation in lymphocytes. MDA lavel was measured according to the method of Ohkawa et al., 1979 [220]. A mixture of cell lysate, tert-butyl hydroperoxide (BHP) (500 µM in ethanol), Tris-HCl buffer (50 mM, pH 7.4) and $FeSO_4$ (1 mM) incubated at 37 °C for 90 min. The reaction was quenched by of sodium dodecyl sulfate (SDS) (0.2 ml, 8%) and acetic acid (1.5 ml, 20%, pH 3.5). Then TBA (1.5 ml, 0.8%) added and heated at 95 °C for 45 min and centrifuged. The MDA level was examined by measuring the optical density of TBA reactive substances (TBARS) in supernatants at 532 nm. The levels of lipid peroxidation were expressed in terms of n mol/mg protein.

2.6.6. Protective role on cell viability

Human lymphocytes ($4x10^6$) were seeded into 96 wells plate and stimulated for 6 h with lethal dose of nicotine (10mM) at 37 ^oC [219]. Then, cells were washed with PBS (50 mM) for 3 times and again incubated with different doses of polysaccharide for 24h at 37 ^oC in culture media. The cell viability was estimated by MTT assay [217].

2.6.7. Effect on ROS and NO generation

Nicotine induced lymphocytes release several factors like ROS and nitric oxide (NO). The treatment with different doses of polysaccharides in the nicotine induced lymphocytes diminishes the generation of both. The generation of intracellular ROS from cells has been measured by using the $DCFH_2$-DA (2', 7'-dichlorodihydrofluorescein diacetate) to detect and quantify the ROS [218]. The trapped fluorescent dye (DCF) inside the cells was used to evaluate and detection of ROS. The antioxidant effect of polysaccharides on nicotine-induced lymphocytes was investigated by flow cytometry (FACS CALIBUR, Becton Dickinson, USA) assay [61] and the data were analysed by CellQuest software.

The NO concentration was investigated by a microplate assay using Griess reagent (1% sulfanilamide, 0.3% naphthylethylenediaminedihydrochloride, 7.5% H_3PO_4). The nitrite concentration in the mixture of culture supernatants (100 µL) and 100 µL of the Griess reagent was measured at 550 nm [221].

2.6.8. DPPH radical-scavenging assay

In presence of an antioxidant molecule the violet ethanolic solution of DPPH radical becomes colourless due to electron-transfer from an antioxidant molecule to a stable free-radical like DPPH (2, 2-diphenyl-1-picrylhydrazyl; Sigma Aldrich) radical. The radical scavenging action of the polysaccharide on was determined by spectro-photometrically using the procedure as described earlier [222]. Various concentrations of the polysaccharide were mixed with DPPH (2 ml, 0.1 mM) in 50% ethanol. Then the mixture was shaken strongly and kept for 30 min and the absorbance was observed at 517 nm using UV-vis spectrophotometer, model-1601. Ascorbic acid was chosen as positive control.

The DPPH radical scavenging percentage (P) was determined as follows:

$$P= (A_0 - A_c)/A_0 \times 100\%$$

Where, P is the % of reduction of the DPPH, A_0 the initial absorbance of DPPH, A_c is the absorbance after added polysaccharide concentration 'c'. All experiments were performed in triplicate and mean absorbance was used in calculation.

2.6.9. Nitric oxide assay for the test for macrophage activity

The RAW 264.7 cells were grown at Dulbecco's modified Eagle's medium (DMEM) at 37 °C and seeded in 96 well tissue culture plates with a concentration of 5 x 10^5 cells/ml (180 µl) [223]. The cells were treated with varied concentrations of polysaccharide and kept for 48 h. The supernatant from each well was collected and NO production was estimated using Griess reagent as adopted by Green et al,. 1982 [224]. LPS from L651 Salmonella enteric serotype typhimurium, 4 µg/ml (Sigma, St. Louis, USA) was used as positive control and soluble starch 100 µg/ml (Merck, India) as negative control [61].

2.6.10. Splenocyte proliferation assay

A single cell suspension of spleen of normal mice was prepared by homogenization in Hank's balanced salt solution (HBSS) under aseptic conditions. The cell pellets were collected from suspension and the red blood cells (RBC) were removed using hemolytic Gey's solution. The cells were washed with HBSS and then suspended in complete RPMI (Roswell Park Memorial Institute) medium. Cell concentration was fixed to 1 x 10^6 cells/ml and the viability of splenocytes was tested by trypan blue dye exclusion assay. Now, the cells (180 µl) were plated in 96-wells tissue culture plates and incubated with 20 µl of various concentrations of polysaccharide. PBS (10 mM, pH 7.4) was used as carrier control and soluble starch (Merck, India, 100 µg/ml) as negative control [61]. LPS (L6511 of S. Enteric, serotype typhimurium) 4 µg/ml was as positive control. All cultures were set up at 37 °C for 72 h in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes (% of Splenocyte Proliferation Index or % SPI) was calculated by the sulforhodamine B (SRB) assay as adopted by Mandal et al., 2015 [225]. The data were reported as the mean of ± standard deviation of six different observations and compared against control (PBS) [224, 226].

2.6.11. Statistical analysis

The data were expressed as mean ± the standard error of the mean (SEM), n = 4. Comparisons of the means of control, and experimental groups were made by one way ANNOVA tests (using a statistical package, Origin 6.1, Northampton, MA 01060 USA), $P < 0.05$ as a limit of significance. The correlation analysis was performed using Statistica software version 8.0.

2.7. Synthesis of silver nanoparticles

Polysaccharides have hemiacetal end to reduce metal salt precursors and lot of hydroxyl group and other functionalities which act as template for stabilization of nanoparticles, this made polysaccharides, among many possible biomolecules, most efficient for synthesis of metal nanoparticles. Neutral polysaccharides like starch, cellulose, dextran,

cationic polysaccharide like chitosan and anionic polysaccharides like heparin, hyaluronic acid, alginic acid and also their chemically modified analogues were used for the synthesis and stabilization of Ag-NPs [112,124-129,134-137,138,227-228]. In the present thesis, a heteroglycan consisting of glucose, fucose and galactose was used both as reducing and surface capping agent for AgNPs synthesis, the detailed procedure is discussed in chapter 5, section 5.B.1.

2.8. Characterization of nanoparticles (NPs)

2.8.1. UV-vis spectral analysis

Metallic particles having diameter typically in the nanometer range elastically scatter optical light with efficiency because of SPR. The intensity, wavelength and width of SPR band dependent on the size, shape and material composition of the nanoparticles (NPs). The SPR band of Ag-NPs is shown by absorption maximum near at 420-430 nm. Formation of SPR band in desired range of UV-vis spectrum indicates the formation of nanoparticles. UV-vis spectra of NPs solution was recorded in a 1cm quartz cuvette with Shimadzu UV-vis 1601 spectrophotometer.

2.8.2. High resolution-transmission electron microscopy (HR-TEM) analysis

In TEM, the samples interact with the electron beam by diffraction. TEM images reveal the size distribution and dispersion of NPs in polymer matrices of nano-composites. HR-TEM is an imaging mode of the TEM that permit the imaging of the crystallographic structure of any sample at an atomic scale, which is helpful to study nanoscale properties of crystalline material. TEM were recorded in a JEOL–JEM-2100 HRTEM operated at 200 kV. For TEM analysis a droplet of aqueous solution of NPs was spread onto a carbon coated copper grid (300 meshes) and dried under IR lamp. Micrographs were taken in both in the transmission mode and diffraction mode.

2.8.3. X-ray diffraction (XRD) analysis

XRD data of NPs generally provide information about crystallinity, crystallite size, orientation of the crystallites. For XRD analysis, a thin film of the NPs was prepared on a

microscopic glass slide using the aqueous solution of heteroglycan capped NPs and dried in vacuum. The measurements were carried out with a Rigaku Miniflex II X-ray diffractometer, using Ni-filtered Cu Kα (λ=0.15406 nm) radiation. The diffraction intensities were recorded from 30° to 80° 2θ angle.

2.8.4. Fourier transform-infrared spectra (FT-IR) analysis

FT-IR spectroscopy is widely used to characterize nanoparticles. The FT-IR spectra of the nanoparticles, which contain some capping agent, exhibit additional peaks in comparison with the FT-IR pattern of a bare nanoparticle. So the property change with different capping agent can easily be detected with FT-IR spectroscopy. FT-IR analysis of the dried NPs-PS conjugates was performed in FTIR-8400S (Shimadzu) instrument between 4000 and 400 cm^{-1} using KBr pellet technique.

2.9. Antibacterial activity of AgNPs-PS conjugates

2.9.1. Growth inhibition study in presence AgNPs- PS conjugates

The antibacterial activity was studied with *E. coli.* MREC33 [229]. Monodispersed suspensions of AgNPs–PS conjugates of different concentrations in deionised water were prepared by means of ultrasonic vibration (100 W, 30 kHz) for 10 min. Bacterial cultures were inoculated from freshly prepared slants into 100 ml of Mueller Hinton (MH) broth (M391 Himedia, India). 10 µl of mid-log cells was then inoculated in 100 ml of freshly prepared MH broth supplemented with varied concentration (5-40 µg/ml) of AgNPs–PS conjugates. MH broth containing bacterial inoculums without AgNPs–PS conjugates and containing AgNPs–PS conjugates without bacterial inoculums were used as negative and positive control, respectively. All the flasks were then incubated at 37 ^{o}C in a rotary shaker (150 rpm). The bacterial growth was monitored at different time intervals for determination of both minimum inhibitory concentration (MIC) and LD_{50} concentration (dosage that will cause 50% reduction in bacterial population) of AgNPs–PS conjugates by measuring the absorbance or OD of the culture media at λ_{600} nm.

2.9.2. Study of DNA degradation in presence of AgNPs-PS conjugates

DNA degradation study was carried out with AgNPs-PS conjugates to understand the mechanism of antibacterial activity. Ag-NPs–PS conjugate treated MREC33 cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and finally subjected to flow cytometry (FACS CALIBUR, Becton Dickinson, USA) observation to measure DNA fragmentation with respect to control MREC33 cells (without any Ag-NPs treatment).

2.10. Hemolysis assay and blood aggregation studies

EDTA stabilized human blood sample was collected and centrifuged at 1600 rpm for 5 min and the plasma, buffy coat, and the top layer of cells were discarded. The remaining packed red blood cells (RBCs) were washed four times with PBS, then 0.2 ml of packed RBC was diluted to 4 ml with PBS [230]. The diluted RBC suspension (0.2 ml) was then mixed with Ag NPs-PS conjugates in PBS (0.8 ml) at various concentrations to establish hemocompatibility [231] of Ag NPs-PS conjugates. Diluted RBC suspension mixed with 0.8 ml PBS and double distilled water respectively, was used as negative and positive control respectively. The mixture was gently vortexed and incubated at room temperature for 2 h. After centrifugation (1600 rpm, 5 min) of the incubated mixture, absorbance of the supernatant was measured by UV-visible spectrophotometer at 541 nm. The percent hemolysis was calculated by using the following formula.

Percent hemolysis = $(A_S-A_N) / (A_P-A_N) \times 100$

Where, A_S is the sample absorbance, A_N is the absorbance of the negative control and A_P is the absorbance of the positive control.

2.11. Growth inhibition studies to evaluate synergistic effects of AgNPs-PS conjugates and antibiotics

The test bacterium, E. coli MREC33 was resistant to ampicillin (50 µg/ml), azithromycin (10 µg/ml), kanamycin (10 µg/ml) and netilimicin (20 µg/ml). 10 µl of mid-log cell culture was inoculated in 100 ml of freshly prepared MH broth, to which 5 µg/ml AgNPs-

PS conjugates and 50 µg/ml ampicillin or 10 µg/ml azithromycin or 10 µg/ml kanamycin or 20 µg/ml netilimicin were added. Control sets were: a) cells incubated with any single antibiotic (ampicillin/azithromycin/kanamycin/netilimicin) without AgNPs-PS conjugates, b) cells incubated with AgNPs-PS conjugates (LD_{50} dosage) without any antibiotic, and c) cells incubated in absence of both AgNPs-PS conjugates and antibiotics. All the flasks were then incubated at 37 °C in a rotary shaker (150 rpm). Growth was measured in terms of O.D. at λ_{600} nm.