CHAPTER III
EXPERIMENTAL

3.1 Equipment and Materials

3.1.1 Equipment

Table 3.1 Instruments and models used in this work

INSTRUMENT	MODEL
FTIR	thermo Nicolet Company, Nexus 470
NMR	Oxford 300
Particle Charge Detector	Herrsching, MÜtek PCD 03
Gel Permeation Chromatography	Pump: Waters 600E System Controller; Detector: Waters 410 Differential Refractometer
Tensile Tester	Lorentzena Wettre(L&W) Co.
Tear Tester	Lorentzena Wettre(L&W) Co.
Burst Tester	Lorentzena Wettre(L&W) Co.
Brightness Tester	Lorentzena Wettre(L&W) Co.
Forma Laminar Airflow Workstation	Thermo Electron Cor., Model 1849
Isotemp Incubator	Fisher Scientific, 500 series
Autoclave-Steam Sterilizer	Tuttnauer Autoclave-Steam Sterilizer model 1730M
Shaker	Fisher Scientific, Model 231
Water bath shaker	New Brunswick Scientific, Innova 3100

3.1.2 Materials

Low molecular weight and medium molecular weight chitosan; Glycidyltrimethylammonium chloride (GTMAC), 75% in water; Carboxymethyl cellulose sodium salt (CMC) with molecular weights of 90,000 and 250,000, DS. =

0.7 were all purchased from Sigma-Aldrich company. Also, Luria Bertani Bouillon, high salt (LB-Broth), final pH 7.5 at 25°C was bought from Fluka. LB Agar; Phosphate Buffered Saline (PBS) which obtain 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride for 1 tablet in 200 ml of water were produce from Sigma company. 3-(trimethylsilyl) propionic-2, 2, 3, 3 ,d_4 acid, sodium salt, 98 atom % D which is use as stardard chemical in NMR. was purchased from Adrich company. Anhydrous Ethyl Alcohol was a commercial alcohols inc. product (Canada). Furthermore, bleached pulp pad were obtained from Tembec company.

3.2 Methodology

3.2.1 Modification of Chitosan

3.2.1.1 Deacetylation of Chitosan

Chitosan was deacetylated by dispersed 20 grams of chitosan in 200 ml of 10% wt. NaOH solution which containing 2 grams of $NaBH_4$ as an antioxidant agent. After Stir the mixture in 3 necked flask at 110°C for 5 hours, the mixture was filtrated by glass filter and washed with the distilled water until neutral to pH paper. Further purified by washing with MeOH and acetone, then dried under vacuum oven at 70oC for 12 hours.

3.2.1.2 Synthesis of cationic chitosan

Deacetylated chitosan was dispersed in distilled water in 10%wt. concentration at 85°C. The mole ratios of chitosan to GTMAC have been varying, as shown in the Table 3.2, by divided into 3 portions GTMAC was added every 2 hour intervals. After 10 hours of reaction, the yellowish reaction solution was poured into cold acetone while stirring and keeps in the refrigerator overnight. Acetone was decanted and the remaining product was dissolved in MeOH. The solution was precipitated in mixture of acetone: ethanol (4:1 ratio). The white product was collected by filtration and further purified by washing with hot EtOH using a Soxhlet extractor for 24 hours. The final product was dried at 70°C overnight.

Table 3.2 The ratios of the chitosan to GTMAC added in the reaction

Ratio of chitosan to GTMAC	GTMAC amount per 6 g. of chitosan	
	Total (ml)	Each portion (ml)
1:1	7.14	2.38
1:1.5	10.71	3.57
1:4	28.55	9.52

3.2.1.3 Degree of acetylation and modifying confirmation

Deacetylation performance was evaluated by using ^{1}H-NMR. At first, Chitosan and deacetylated chitosan were dissolved in HCl containing 3-(trimethylsilyl) propionic-2, 2, 3, 3 ,d_4 acid and modified chitosan was dissolved in D_2O. Then, ^{1}H-NMR spectrum was recorded in D_2O at 25 °C on an Oxford 300 MHz spectrometer operating at 300.13 MHz for nuclei.

3.2.1.4 FITR

A Nexus 470 FTIR has been use to qualify the existing of quaternary ammonium group that has been graft on the chitosan polymer by mixing sample with KBr to form pellet using 64 number of scans.

3.2.2 Strength properties of paper

3.2.2.1 Fiber Adsorption Treatment

Modified chitosan and fibers were mixed based on oven dried weight in 125 ml. Erlen mier. Samples were shaken during experiment at 30°C and 200 rpm in a water bath shaker (Innova 3100, New Brunswick Scientific). The suspension was filtered after treating and the filtrate was collected for adsorption analysis. Control sample was provided for each sample accordingly by mixing of modified chitosan with deionized distilled water. Exactly similar volume was taken from each sample and its control to titrate with PVSK solution (0.5 mN) by Particle Charge Detector MÜtek PCD 03 (Herrsching, Germany). Finally, adsorption amount was calculated based on the concentration difference of modified chitosan in filtrate and

control sample. Three repeats were conducted to get an average value of adsorption for each sample.

3.2.2.2 Pulp preparation

Pulp pad was first soaked in water for 24 h. it was then, disintegrated by disintegrator with 15,000 revolutions. After that, it was filtrated and repulped by homogenizer. It was kept in refrigerator at 5°C for 2 days and finally its moisture content was measured according to TAPPI accordance with TAPPI T412 cm-94.

3.2.2.3 Fiber modification

Pulp fibers were first dispersed in distilled water into 1 L 3-neck glass flask at 3% consistency and 30 °C for 1 hour. Then, modified chitosan solution (1%) were added and mixed for 1 hour. Afterwards, CMC solution (0.5%) was added into the fibers and stirred for 1 hour. At last, pulp was washed thoroughly with distilled water. Depending on procedure, washing stage was conducted for some samples before adding of CMC.

Table 3.3 The variables in fiber modification

Variable	Range
Molecular weight of chitosan	Low (50,000-190,000), Medium (1900,000- 310,000)
Molecular weight of CMC	Low (90,000), High (250,000)
Charge ratio of chitosan:CMC	1:0.5, 1:1, 1:1.5
Amount of chitosan added	0.2%, 0.6%, 1%

3.2.2.4 Handsheet making and characterization of mechanical and optical properties

Hand-sheets were made from each pulp samples to measure optical and mechanical properties based on TAPPI T 205 om 88. Afterwards, hand-sheets

were kept in conditioning room specified in TAPPI T 402 om93. Light Scattering coefficient and brightness of hand sheets were tested according to TAPPI T om-91 and T 452 om 92 standard methods. Tensile strength and tear strength were measured in relevance with TAPPI T 494 om 88 and T 414 om 88, respectively, using a Lorentzena wetter (L&W) tensile strength tester.

3.2.3 Antimicrobial tests

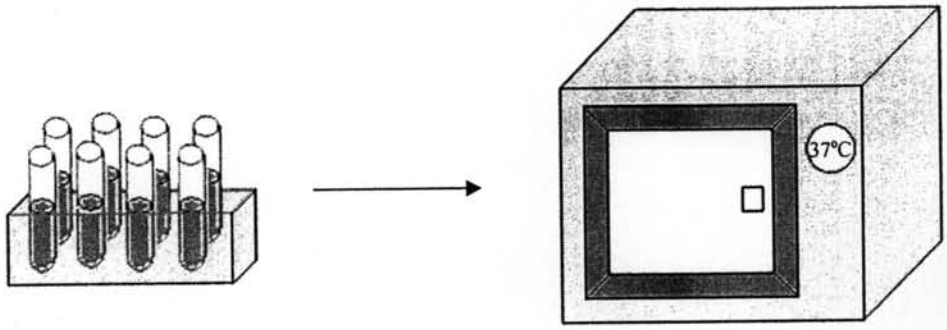
3.2.3.1 Minimum Inhibitory Concentration (MIC)

All the equipments used in this test have been sterilized by autoclave at 121°C for 15 minutes. LB-Broth solution was prepared in the concentration of suspend 25 grams in 1,000 ml of purified water then heat with frequent agitation and boil for one minute. The sample solutions were prepared in different concentration of modified chitosan in LB-Broth solution which indicates in Table 3.4. After obtain the determine concentration, the sample had been separated into 2 portions. First portion which has 2 ml solution were added with 0.2 ml of *E.Coli* (10^6 CFU/ml). The rest portion is reference to compare the change with the first portion after incubated at 37°C for 18 hours.

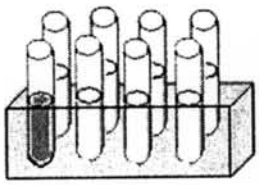
After testing all the items contact to the *E.Coli* should be sterilize and clean before throw away or storage.

Table 3.4 Sample preparation for MIC testing

Tube number	1	2	3	4	5	6	7	8
Chitosan concentration (ppm)	500	250	125	62.5	31.25	15.625	7.813	3.91
Volume of 1%chitosan solution(ml)	4	2	1	0.5	0.25	0.125	0.063	0.031
Volume of Broth (ml)	4	6	7	7.5	7.75	7.875	7.938	7.969
Total volume (ml)	8	8	8	8	8	8	8	8



(a) Preparation of the samples in different concentrations and incubated at 37°C for 18 hours.

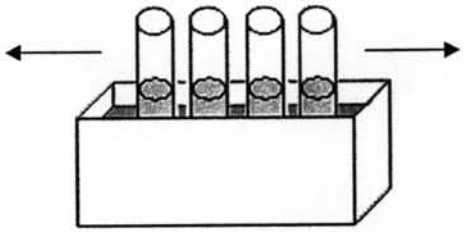


(b) Color qualification of the samples after incubated.

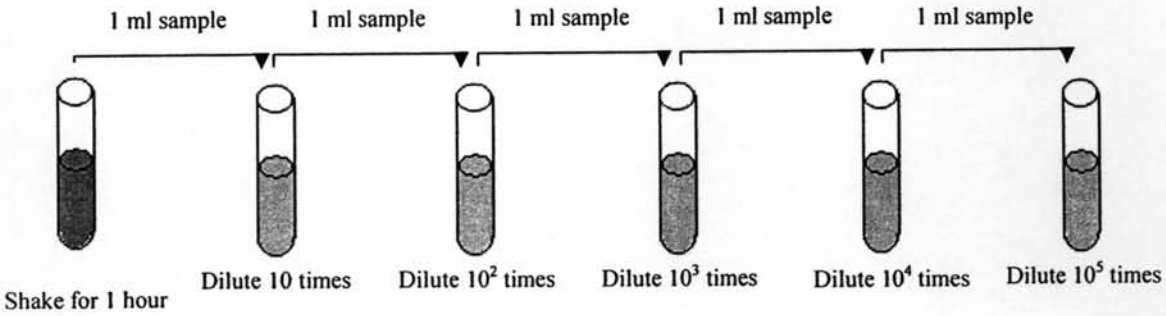
Figure 3.1 Schemes of biocide efficiency tests with MIC method.

3.2.3.2 Biocide efficiency test with shaking method

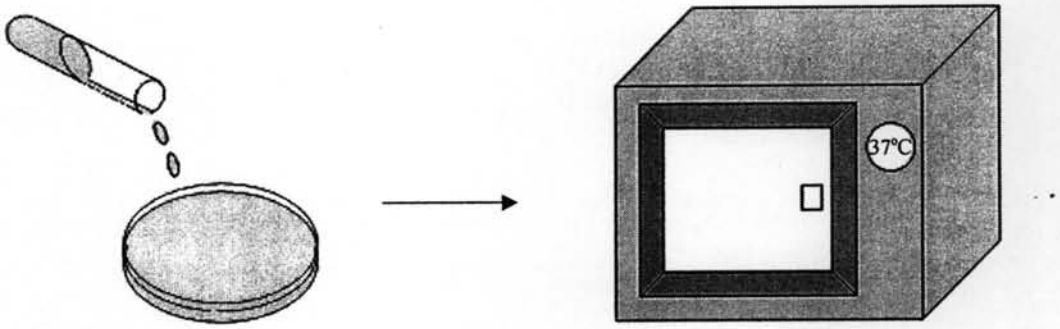
All the equipments used in this test have been sterilized by autoclave at 121°C for 15 minutes. Phosphate Buffered Saline (PBS) solution was obtained from dissolving 1 tablet in 200 ml. And the culture of 10^6 CFU/ml of *E.Coli* had been prepared. Agar plates were prepared by dissolve LB agar 35 g/l in deionized water and heat with frequent agitation and boil for one minute then put in the agar plates to cool down until the semi-solid of agar form. Paper sample 0.1 gram was marinated in PBS 4.5 ml. with 0.5 ml of *E.Coli* solution. Sample tube has been shaking at 200 rpm, 37°C for 1 hour. Then, sample solution has been taken out to dissolve in 5 tubes which are diluted 10 to 10^5 times. After that 0.1 ml of each sample was spread by the glass hockey in agar plate, and put in the incubator at 37°C for 18 hours. The number of bacterial colonies has been counted to verify the antimicrobial activity.



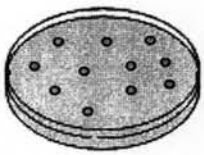
(a) Shaking at 200 rpm, 37°C for 1 hour.



(b) Dilution from 10 to 10^5 times.



(c) Spreading 0.1 ml of sample into an agar plate then incubated at 37°C for 18 hours.



(d) Counting the number of bacteria colonies in the agar plate.

Figure 3.2 Schemes of biocide efficiency tests with shaking method.