CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

Three strains of *Acetobacter xylinum* were used in this study ; DK strain obtained from Department of Agro-Industry,Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, THAILAND ; AGR60 strain obtained from Division of Chemical Agriculture, Ministry of Agriculture and Coorperatives, Bangkok, THAILAND; and ST strain obtained from PANA Food Industry Com.Nonthaburi, THAILAND. The stock cultures of these three strains were maintained on YE agar slant. All cultures were stored at 4°C. These strains produced extracellular cellulosic gel. They show maximal activity at pH 4 to 5 and 28°C to 32°C.

3.1.2 Chemical substances

Chemical substances used in this experiment were as follows :

$(NH_4)_2SO_4$	ammoniumsulphate
KH_2PO_4	potassiumdihydrogenphosphate
$MgSO_4.7H_2O$	Magnesiumsulphate heptahydrate
$Co(C_2H_3O_2)_2.4H_2O$	Cobaltacetate
CH_3COOH	Acetic acid
$UO_42(C_2H_3O_2).2H_2O$	Uranilacetate
$AgNO_3$	Silvernitrate
$Cu(NO_3)_2.3H_2O$	Coppernitrate trihydrate
H_2O_2	Hydrogen peroxide
$ClCH_2COOH$	Chloroacetic acid
$CuSO_4.5H_2O$	Copper sulphate pentahydrate

NaOH	Sodiumhydroxide
NaCl	Sodiumchloride
HNO_3	Nitric acid
H_2SO_4	Sulfuric acid
$HOCH_2COOH$	Glycolic acid
MeOH	Methanol
EtOH	Ethanol
2,7 dihydroxynathaleindiol	
Acetone	
Glucose	
Fructose	
Sucrose	
Maltose	
Lactose	

3.1.2 Medium

The medium given below was used for propagating of *A. xylinum*

YE medium : sucrose 50 g, yeast extract 5 g, $(NH_4)_2SO_4$ 5 g

KH_2PO_4 3 g, $MgSO_4.7H_2O$ 0.05 g, distilled water 1 litre.

3.1.3 Coconut water

Coconut water was used as fermentation substrate. Fresh coconut water, obtained from Hua Ta Khae Market, was supplemented with 6% of sugar, sterilized at 121°C for 15 min., cooled to room temperature and, then, added with 4% of 5% acetic acid.

3.1.4 Nata cellulose powder

Nata cellulose powder , used for carboxymethylcellulose production, was obtained from Institute of Food Research and Development (IFRD), Kasetsart University, Bangkok, THAILAND.

3.2 Experimental equipments

Type of equipment	Model	Producer
Shaker	RO-30	Gerhardt, Germany
Spectrophotometer	CE-292	Cecil, Germany
Automatic autoclave	SS-320	Tomy, Japan
Hot air oven	700	Memert, Switzerland
pH meter	SP-701	Suntex, Taiwan
Chromameter	CR-300	Minolta, Japan
Incubator	Series B/E	Binder, Germany
Viscometer	RVG	Brookfield, USA
Balance	AE-50	Mettler, Switzerland
Balance	PE 3000	Mettler, Switzerland
Incubation chamber	-	local made, Thailand

3.3 Experimental methods

3.3.1 Studies on the optimum conditions for nata cellulosic gel formation by *A. xylinum*

3.3.1.1 Effect of *A. xylinum* strain on nata cellulosic gel formation ability

One loop of stock culture of strain AGR60, DK and ST was transferred to 250ml Erlenmeyer flask containing 100 ml YE medium. Flasks were shaked for 3 days and used as starter inoculum.

Fermentation conditions : Each strain of *A. xylinum* was cultivated in 4L of coconut water medium in fermentation tray ($15 \times 45 \times 15$ cm^3) covered with a piece of sterile paper, under static conditions. After 14 days of fermentation at 32°C, the nata cellulosic gel were harvested to measure gel formation. The cellulosic gel formation was shown as gel thick and gel weight expressed as its wet and dry weight.

3.3.1.2 Effect of sugars on nata cellulosic gel formation of *A. xylinum*

Three strains of *A. xylinum* were cultured in two media ; YE medium and coconut water medium ; and using various type of sugars as carbon source consisting of sucrose, maltose, lactose, glucose and fructose, in the fermentation jar (50.24 cm^2 surface area) containing of 150 ml of medium, then covered with a piece of sterile paper. Fermentation were carried on the static condition for 14 days at 32°C. Gel were harvested to measure nata cellulosic gel formation.

3.3.1.3 Effect of surface area of fermentation tray on nata cellulosic gel formation

Effect of surface area on nata cellulosic gel formation was studied in static cultures. Different surface area of fermentation trays were obtained by using various size of trays ; 7.5 x 11.25 cm^2 ,11.25 x 15.0 cm^2,15.0 x 22.5 cm^2, 22.5 x 30.0 cm^2, 30.0 x 45.0 cm^2. These tray contained media of 200, 400, 800, 1600 and 3200ml.,respectively. The suitable kind of media and strain of *A. xylinum* were selected by the experimental described in section 3.3.1.1 and 3.3.1.2. After 14 days of fermentation, the cellulosic gel were lift to measure cellulosic gel formation ability and cellulose content.

3.3.1.4 Analytical procedure

Gel forming ability

Gel were lift from the surface of fermentation mash, washed thoroughly in running water, hung freely to drain away the excess water and, then, weighed. The nata cellulosic gel thickness were determined from measuring four points and eight points of gel in case of using 150ml. fermentation jar and 4L of fermentation tray, respectively. In addition, the nata cellulosic gel harvested from tray contained media of 200, 400, 800, 1600 and 3200ml. were determined gel thickness by measuring four, six, eight, twelve and fourteen points, respectively and then calculated to get average gel thick for each nata cellulosic gel. The nata cellulosic gel was cut into 2.5 x 2.5 cm^2. and dried at 80°C for 6 hours, its moisture content was determined.

Determination of cellulose

The method of cellulose analysis was followed by TAPPI(1961) as described in APPENDIX I

Statistic Analysis

The Completely Randomized Design (CRD) was used in this studies(Gomez and Gomez, 1984). Within treatment, the means were compared by using the Duncan's New Multiple Range Test (DMRT).

3.3.2 Studies on factors influencing on carboxymethylcellulose (CMC) production from nata cellulosic gel by *Acetobacter xylinum*

3.3.2.1 Effect of nata cellulosic gel materials on CMC production

The cellulosic materials used in this experiment consisted of blended nata cellulosic gel, nata cellulose powder and wood cellulose powder, as control. The blended nata cellulosic gel was washed thoroughly in running water, hung freely to drain away the excess water. The 180 g of nata cellulosic gel, which contained 4.5 % cellulose, was blended for 3 minutes. For nata cellulose powder, the nata cellulosic gel was dried by drum drier, the 8.1 g of nata cellulose powder was used. The wood cellulose powder (commercial grade) of 8. 9 g was used as control. The cellulosic material were mixed with 12.8 g of sodiumhydroxide which was already dissolved in 20.25 ml of distilled water. Then, the reaction mixture was added with 5.94 g of sodiumchloroacetate (modified method of Dhariyal and Chipalkatti, 1958).

All ingredients were mixed for 5 hours and incubated at room temperature (25-30 $^{\circ}$C) for 24 hours. After 24 hours of aging, both reaction mixtures from all treatments were washed in hot water (vol. of hot water = 10 x wt. of mixture). The mixture then was neutralized by glacial acetic acid and precipitated by ethanol at the final concentration of 70%. The precipitates were dried at 65 $^{\circ}$C for 3-5 hours. Dried product was devided to determine its moisture content, degree of substitution (DS), viscosity and % impurities (sodiumchloride and sodiumglycolate).

3.3.2.2 Effect of ratio of nata cellulosic material to distilled water on CMC production

The selected nata cellulosic material was obtained from the section 3.3.2.1. The ratio of chosen cellulosic material to distilled water used were 1.0 : 1.0 , 1.0 :1.5, 1.0 : 2.0 and 1.0 : 2.5 (w/v). Other experimental details followed as described in previous experiment.

3.3.2.3 Effect of ratio of nata cellulosic material to sodiumhydroxide on CMC production

The selected cellulosic material and the optimum ratio of cellulosic gel to distilled water were obtained from previous experiments. The ratio of cellulosic gel to sodiumhydroxide used were 1.0 : 1.5, 1.0 : 3.0, 1.0 : 4.5 and 1.0 : 6.0 (w/w). Other exprimental procedures were performed in previous experiments.

3.3.2.4 Effect of ratio of cellulosic material to sodiumchloroacetate on CMC production

The chosen cellulosic material, the optimum amount of distilled water and sodiumhydroxide were obtained from previous sections. The ratio of cellulosic material to sodiumchloroacetate were as followed ; 1.0 : 0.36, 1.0 : 0.72, 1.0 : 1.08, 1.0 : 1.44 and 1.0 : 1.8 (w/w). Other experimental details were described in section 3.3.2.1.

3.3.2.5 Mixing time

The optimum ratio's of various reactants for CMC production obtained from section 3.3.2.1 - 3.3.2.4. The all ingredients were mixed for 4, 5, 6, 7 and 8 hours. Other experimental details were presented in previous experiments.

3.3.2.6 Effect of incubation time on CMC production

The optimum amount of compositions for CMC production were obtained previously. The suitable mixing time were obtained from experiment 3.3.2.5. These reaction mixture were incubated at room temperature. Sampling every 12 hours ; 0 , 12, 24, 36, 48 hrs. After sampling, each sample was immediately analysed unreacted sodiumchloroacetate. Further step of CMC productions and analyzations were shown previously.

3.3.2.7 Effect of ratio of reaction mixture to volume of hot water on CMC production

The suitable amount of ingredients for CMC preparation including appropiate mixing time and incubation time were determined previously. For product purification, reaction mixture reacted completely must be dissolved in hot water to get rid of impurities. These impurities were sodiumchloride and sodiumglycolate. The ratio of reaction mass to volume of hot water were as followed;1.0 : 5.0, 1.0 : 7.5, 1.0 : 10.0 and 1 : 12.5 (w/v). Other experimental determinations followed as shown in previous experiments.

3.3.2.8 Effect of final concentration of ethanol for CMC precipitation

The optimum conditions were determined in section 3.3.2.1-3.3.2.7. After dissolving reaction mass in hot water, CMC product was precipitated from reaction mixture by ethanol. Final concentration of ethanol were used as followed : 50%,60%,70% and 80%. Determination procedures were presented in previous section.

3.3.3 Analysis of product

The CMC which obtained from the all duplicated treatments in the experiments were analysed. The CMC obtained were determined degree of substitution (DS), viscosity (cP), L-value (whiteness) and the impurities. The DS determination was follwed by method of Green(1963) as described in APPENDIXI. The viscosity(cP) was determined by Brookfield viscometer model RVG spindle number 2 and speed 50 rpm.

The chromameter was used for determined L-value of products. The impurities, sodiumglycolate and sodiumchloride, were determined by spectrophotometer and by titration, respectively. Both analytical procedures were described in APPENDIX I.

3.3.4 Properties of CMC produced from cellulosic gel by *A. xylinum*

3.3.4.1 Effect of temperature on viscosity of CMC solution

The CMC powder obtained from the optimum condition of previous section was used in this study. The CMC from nata cellulose powder and two types of commercial CMC ; low and high viscosity, were prepared to 1% solution. All types of CMC were determined the viscosity as temperature of 20, 30, 40, 50, 60, 70 and 80 ^{o}C, respectively.

3.3.4.2 Effect of pH of CMC solution on its viscosity

The CMC produced from nata cellulose powder and two types of commercial CMC ; low and high viscosity, were prepared to 1% solution. Adjusted pH of all CMC solution to 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively. Determined its viscosity by Brookfield viscometer.

3.3.4.3 Relationship between CMC concentration and its viscosity

The CMC from nata cellulose powder and two grades of commercial CMC ; low and high viscosity, were prepared to make concentration of 1, 2, 3, 4, 5 and 6%, respectively. Determined its viscosity by Brookfield viscometer.