CHAPTER 2

MATERIALS AND METHODS

2.1 Chemical Reagents

Chemical Reagents	Production Company
Acetic acid	MERCK
Acrylamide	SIGMA-ALDRICH
Ammoniummolybdate	MERCK
Ammonium persulfate	MERCK
Avicel	ALDRICH
Beech wood xylan	SIGMA
Beef extract	MERCK
Bis-Acrylamide	SIGMA-ALDRICH
Bovine serum albumin fraction V	MERCK
Bromophenol blue	MERCK
Calcium carbonate	MERCK
Caesine	FLUKA
Citric acid	MERCK
CMC	-
Cobalt chloride	J.T. BAKER
Coomassie brilliant blue R	MERCK
Copper (II) sulfate	CARLO ERBA
Corncob powder	-
DEAE-cellulose	SIGMA
Dibasic sodium phosphate	MERCK
Dinitrosalicylic acid	SIGMA
Disodiumarsenate	SIGMA
Methanol	LAB-SCAN
Ferrous sulfate	J.T. BAKER

Chemical Reagents	Production Company
Folin-ciocalteau reagent	SIGMA
Glucose	FLUKA
Glycerol	-
Glycine	MERCK
Hydrochloric acid	MERCK
Magnesium sulfate	J.T. BAKER
Manganese sulfate	J.T. BAKER
Mercaptoethanol	SIGMA
Monobasic sodium phosphate	MERCK
Phenol	MERCK
Potassium dihydrogen phosphate	MERCK
Potassium nitrate	CARLO ERBA
Sodium acetate	MERCK
Sodium bicarbonate	CARLO ERBA
Sodium carbonate	CARLO ERBA
Sodium citrate	MERCK
Sodium dodecyl sulfate (SDS)	SIGMA-ALDRICH
Sodium potassium tartrate	MERCK
Sodium sulfate	BHD
Sodium sulfite	MERCK
Soluble starch	CARLO ERBA
Sugarcane baggase	Sugar Factory, Lampang
TEMED	SIGMA-ALDRICH
Tris-hydroxyaminomethane	MERCK
Xylose	WAKO
Yeast extract	MERCK
Zinc sulfate	MERCK

2.2 Equipments

Names of Equipments	Production Company
5-L Fermentor	B-Braun
Analytical balance	PRECISA
Autoclave Model ACV-3167	IWAKI
Controlled temperature shaker	-
Dialysis tube	Cellu Sep
Electrophoresis kit	E-C APPARATUS CORP.
Electrophoresis power supply	E-C APPARATUS CORP.
Fraction collector alpha 200	FRACTOMETTE
Glass column 1.8 X 150 cm	-
Glass column 1.8 X 30 cm	-
Gradient tank	-
Magnetic stirrer	PMC
Micro centrifuge	EPPENDROF
Micropipette	EPPENDROF
pH Meter	CYBERSCAN
Refrigerated centrifuge	SORVAL
Spectonic 21	GENESYS
Ultrasonic cleaner	METTLER ELECTRIC CORP.
UV/Vis spectrophotometer	JASCO
Vortex mixer	
Water bath	SHEL-LAB

2.3 Media

Basal medium (Harchand and Singh 1986)

K_2HPO_4	2 g
KH_2PO_4	1.5 g
$(NH_4)_2SO_4$	1.4 g
Yeast extract	2 g
Peptone	1 g
$MgSO_4 \cdot 7H_2O$	0.03 g
Tween 80	2 ml
Trace mineral solution*	1 ml
Carbon source	1% (w/v)

Adjust to a final volume of 1 l by adding distilled water: Sterile at 121 °C for 15 min.

Trace mineral solution*

$ZnSO_4 \cdot 7H_2O$	140 mg
$MnSO_4 \cdot H_2O$	160 mg
$FeSO_4 \cdot 7H_2O$	500 mg
$CoCl_2 \cdot 2H_2O$	200 mg

The pH of the solution should be in the range of 3.0-3.5: Adjust to a final volume of 1 l by adding distilled water.

Baggase and Corncob powder preparation

Baggase and Corncob were cut into small pieces by a scissor then milled with a blender. After that the Baggase and Corncob powder were sifted through a 200 mesh sieve. The fine Baggase and Corncob powder were kept in glass bottle until use.

Modified nutrient broth

Peptone	5.0 g
NaCl	5.0 g
Beef extract	1.0 g
Yeast extract	2.0 g

Adjust to a final volume of 1 l by adding distilled water: Sterile at 121 °C for 15 min.

Starch caesine agar

Soluble starch	2.0 g
K_2HPO_4	2.0 g
Caesine	3.0 g
KNO_3	2.0 g
$MgSO_4 \cdot 7H_2O$	0.05 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$CaCO_3$	0.02 g
Agar	15 g

Adjust to a final volume of 1 l by adding distilled water: Sterile at 121 °C for 15 min.

2.4 Methods

2.4.1 Microorganism

Streptomyces Ab106.3 was isolated from a teak plantation area in Chiangmai University campus, for using in agricultural waste utilization project. Ab106.3 isolate showed the highest CMCase activity among 840 isolates detected by plate assay

(Naiyatat *et al.*, 1998). From preliminary test in submerge culture; supplemented with CMC and various carbon sources, it was found that Ab106.3 also produced xylanases.

Streptomyces Ab106.3 was maintained on starch casein agar slant. After sub-culturing, the slant was maintained at 55 °C for 5 days, and subsequently stored at 4 °C. Subculture was done every 2 months.

2.4.2 Determination of Enzyme Activities

Enzyme activity was determined by the amount of reducing sugar released from beech wood xylan or CMC. For xylanase assay, the reaction mixture consisted of 500 µl of enzyme substrate; 0.5% (w/v) of beech wood xylan in 50 mM citrate buffer pH 6.0, and 500 µl of enzyme. For CMCase assay, the reaction mixture consisted of enzyme substrate; 0.5% (w/v) of CMC in 50 mM citrate buffer pH 6.0, and 500 µl of enzyme. After incubation for 30 min at 55 °C, the amount of reducing sugar was determined by Somogyi and Nelson method and DNS method (Appendix A). Enzyme activity was expressed as µmol of reducing sugar released per milliliter of enzyme solution (U/ml). Specific activity was expressed as enzyme activity per milligram of protein (U/mg.protein).

2.4.3 Determination of Protein

Protein determination was carried out by Lowry's method (Appendix B). In purification steps, A_{280} was used to monitor the trace of protein.

2.4.4 Study of Xylanase Production

2.4.4.1 Screening of Carbon Sources for Xylanase Production

The culture media were basal medium (Harchand and Singh,1997) and supplemented with 1% (w/v) of Avicel, CMC, untreated corncob powder or untreated sugarcane baggase in each experiments.

Seed culture was prepared by adding 1 loop of *Streptomyces* Ab106.3 spores into 50 ml of nutrient broth in 125-ml Erlenmeyer flask. The culture was incubated on a rotary shaker at 150 rpm, 37 °C for 24 h. To study the production of xylanases, seed cultures of 4%(v/v) were inoculated into 120 ml of basal medium supplemented with various carbon sources in 250-ml Erlenmeyer flasks. They were incubated in a temperature-controlled shaker at 55 °C 150 rpm. To study the time course of xylanase and CMCase productions, a sampling volume of 5 ml of each treatments were taken at 24 h interval for 168 h. Samples were centrifuged at 4,500 rpm for 15 min, the supernatant was collected and subsequently kept at –20 °C until the enzyme assays were carried out.

2.4.4.2 Xylanase Production in 5-l Fermentor

The culture medium was basal medium (Harchand and Singh, 1997) supplemented with 1% (w/v) of untreated sugarcane baggase, without tween 80 supplementation.

To produce xylanases, 3.5 l of culture medium in 5-l fermentor was inoculated with 4% (v/v) of seed culture. The fermentation parameters, agitation rate, aeration rate, pH, and fermentation temperature were 200 rpm, 1vvm, 7.0, and 55°C, respectively. To determine the time course of xylanase and CMCases productions, samples were taken 24 h interval for 240 h. After sampling, it was centrifuged at 4,500 rpm for 15 min; the supernatant was collected and subsequently kept at –20 °C until the enzyme assays were carried out.

2.4.5 Study of Partial Purification of Xylanases

2.4.5.1 Xylanase Production for Purification Studies

The fermentation parameters for xylanase production were the same as previously described. After 96 h of cultivation, the culture medium was filtrated

through a cloth filter, and was centrifuge at 10,000 X g for 15 min at 4 °C. The supernatant, crude enzyme, was collected and kept at 4 °C overnight.

2.4.5.2 Ammonium Sulphate Precipitation

Ammonium sulfate at 20-90% saturations were used to precipitate proteins from crude enzyme. The amount of ammonium sulfate used was chosen from Appendix D. The precipitation was done at 4 °C for 1 h. Precipitant was centrifuged at 10,000 X g for 10 min at 4 °C. Both supernatant and precipitant were assayed for xylanase activities.

2.4.5.3 Dialysis

Dialysis was done at 4 °C for 24 h. The dialysis buffer was 50mM Citrate buffer pH 6.0. During dialysis buffer were changed 6 h intervals.

2.4.5.4 Partial Purification of Xylanases by Ion-exchange Chromatography

To replace counter ion and remove of the contaminated protein in used DEAE-cellulose, the swelled DEAE-cellulose was filtrated through Whatman™ No. 4 filter paper. The cake of DEAE-cellulose was re-suspended in 3 volumes of 0.2 N NaOH, stirred gently for 10 min, and re-filtrated. This step was repeated twice by changing of 0.2 NaOH to 0.2 N HCl and distilled water, respectively. After washing with distilled water, the pH of DEAE-cellulose suspension was adjusted to 8.0 with NaOH. Treated DEAE-cellulose was packed with a flow rate of 0.5 ml/min into a glass column of 1.8X30 cm., the height of the gel was 20 cm. To equilibrate the column, 500-700 ml of 50mM Tris-HCl buffer pH 8.0 was applied to the column with a flow rate of 0.5 ml/min. The column equilibration was done at 4 °C.

Proteins in supernatant were precipitated by 80% saturation of ammonium sulfate. Precipitant was collected by centrifugation at 10,000 X g for 15 min at 4 °C. The precipitant was dissolved in 50mM Citrate buffer pH 6.0, and filled into dialysis tube. Dialysis was done as described in 2.4.5.3.

The pH of dialyzed enzyme was adjusted to 8.0 with NaOH before loading into the column. The loading flow rate was 0.5 ml/min. After the enzyme loading, 50mM Tris-HCl buffer pH 8.0 was applied into the column to elute the unbound enzyme. The effluent was sequentially collected every 10 ml. When the A_{280} of the effluent reached nearly zero, the gradient of 0-1.0 N of NaCl in 50 mM Citrate buffer pH 6.0 was applied. To draw a chromatogram, the A_{280} and xylanase activity of every 3 fractions of effluents were determined and plotted against fraction numbers.

2.4.5.5 Partial Purification of Xylanases by Gel Filtration Chromatography

The contaminated proteins in used gel were filtrated through Whatman ™ No.4 filter paper. The gel was re-suspended in 50mM citrate buffer pH 6.0, and re-filtrated in the same buffer twice, and was kept at 4 °C. The gel suspension was with a flow rate of 0.25 ml/min into a glass column, 180X1.7 cm, until the gel height was 120 cm. To equilibrate the column, 700 ml of 50 mM Citrate buffer pH 6.0 was applied into the column with a flow rate of 0.25 ml/min. The column equilibration was done at 4 °C. The partial purified concentrated xylanases obtained from DEAE-cellulose column chromatography were applied into the column with flow rate of 0.25 ml/min. After loading the enzyme, 50 mM Citrate buffer pH 6.0 was applied to the column with the same flow rate. The effluent was sequentially collected every 10 ml. To draw a chromatogram, A_{280} and xylanase activity of every 2 fractions was determined and plotted against fractions number.

2.4.5.6 Protein Determination by SDS-PAGE

Chemical reagents preparation and procedure of SDS-PAGE and Activity stain were described in appendix C.

2.4.6 Study of Some Properties of Xylanases

2.4.6.1 Determination of The Optimum Temperature for Xylanases

Enzyme samples were diluted, 100 folds for crude enzyme and 5 folds for partial purified enzyme, with 50 mM citrate buffer pH 6.0. Xylanase assay was carried out at 30, 40, 50, 60, 70, and 80 °C for 30 min; the reaction mixture consisted of 500 µl of enzyme substrate: 0.5 % (w/v) of beech wood xylan in 50 mM citrate buffer pH 6.0 and 500 µl of diluted enzyme. The amount of reducing sugar was determined by DNS method.

2.4.6.2 Determination of The Optimum pH for Xylanases

Enzyme samples were diluted, 100 folds for crude enzyme and 5 folds for partial purified enzyme, with various buffers of pH 4-11. Xylanase assay was carried out at 55 °C for 30 min. The reaction mixture and reducing sugar determination was the same as previously described in 2.4.6.1.

2.4.6.3 Determination of Temperature Stability of Xylanases

Enzyme samples were diluted, 100 folds for crude enzyme and 5 folds for partial purified enzyme, with 50 mM citrate buffer pH 6.0. Then, they were incubated at 50, 60, 70, and 80 °C. Sampling of 0.5 ml were taken intervals to determine the residual of xylanase activity.

2.4.6.4 Determination of pH Stability of Xylanases

Enzyme samples were diluted, 100 folds for crude enzyme and 5 folds for partial purified enzyme, with various buffers of pH4-11. They were kept at 4 ^{o}C. Sampling of 0.5 ml were taken 24 h intervals to determine the residual activity of the enzyme.