CHAPTER 2
MATERIALS AND METHODS

2.1 Materials and Chemical Reagents.

Media and chemical reagents	Company
Acetic acid	MERCK
Beef extract	MERCK
Bovine serum albumin fraction V	MERCK
Calcium carbonate	MERCK
Caesine	FLUKA
Carboxy methly cellulose	-
Citric acid	MERCK
Cobalt chloride	J.T. BAKER
Coomassi brilliant blue G	MERCK
Copper (II) sulfate	CRALO ERBA
Dibasic sodium sulfate	MERCK
3,5-dinitro salicylic acid	FLUKA
Ferrous sulfate	J.T. BAKER
Glucose	FLUKA
Glycine	FLUKA
Hydrochloric acid	FLUKA
Hydrogen peroxide	-
Magnesium sulfate	J.T. BAKER
Manganese sulfate	J.T. BAKER
Monobasic sodium sulfate	MERCK
Oat spelt xylan	SIGMA
Oxalic acid	MERCK
Potassium dihydrogen phosphate	MERCK
Potassium nitrate	CRALO ERBA
Sodium acetate	MERCK
Sodium bicarbonate	CRALO ERBA
Sodium carbonate	CRALO ERBA

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Media and chemical reagents	Company
Sodium citrate	MERCK
Sodium hydroxide	MERCK
Sodium potassium tratrate	CRALO ERBA
Sodium sulfate	MERCK
Soluble starch	CRALO ERBA
Tween 80	FLUKA
Xylose	WAKO
Yeast extract	MERCK
Zinc sulfate	MERCK

2.2 Equipments.

Equipments	Company
Analytical balance	PRECISA
Autoclave model ACV-3167	IWAKI
Colorimetric Hunter Lab	
Incubator shaker	-
pH meter	CYBERSCAN
Refrigerated centrifuge	SORVAL
Spectornic 21	GENESYS
Spectrophotometer model 530	JASCO
Ultrasonic cleaner	METTLER ELETRIC CORP.
Vortex mixer	-
Water bath	SHEL-LAB

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2.3 Media.

Modified medium (Techapun *et al* , 2001)

KH_2PO_4	1.5	g.
K_2HPO_4	2.0	g.
$(NH_4)_2SO_4$	2.0	g.
Yeast extract	0.075	g.
Peptone	0.075	g.
Tween 80	0.075	ml.
Trace elements	5.2	ml.
Cane bagasses	10.0	g.

Adjust to a final volume 1 L with deionized water. Sterile at 121°C for 15 min.

Trace element solution

$ZnSO_4.7H_2O$	140	mg.
$MnSO_4.H_2O$	160	mg.
$FeSO_4.7H_2O$	500	mg.
$CoCl_2.2H_2O$	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 deionized water.

Starch caesine agar

Soluble starch	2.0	g.
K_2HPO_4	2.0	g.
Caesine	3.0	g.
KNO_3	2.0	g.
$MgSO_4.{}_7H_2O$	0.05	g.
$FeSO_4.\ {}_7H_2O$	0.01	g.
$CaCO_3$	0.02	g.
Agar	15.0	g.

Adjust to a final volume 1 L with deionized water. Sterile at 121°C for 15 min.

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2.4 Methods.

2.4.1 Microorganism.

Streptomyces sp. Ab106.3 was employed. This strain was isolated from the soil in Chiangmai University, Thailand. It was chosen as a potential thermotolerant xylanase producer (Techapun *et al* , 2001).

Streptomyces sp. Ab106.3 was maintained on starch caesine agar slant. After subculturing, the slant was maintained at 55°C for 5 days, and subsequently stored at 4°C. Subculturing was done once a month.

2.4.2 Culture Media.

Similar to Modified medium on the previous page.

2.4.3 Inoculum Preparation.

One loop of *Streptomyces* sp. Ab106.3 was inoculated into the modified medium supplemented with 0.1 per cent (w/v) oat splet xylan, pH 7.0 . The incubation temperature, 55°C and 250 ml medium in 1 L flask on a reciprocal shaker (100 rpm) was carried out for 1-2 days. The inoculum size was 5 per cent (v/v) (Techapun *et al* , 2001).

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2.4.4 Experimental Design Setup.

Temperature and pH were the most important factors for xylanase production (Techapun *et al* , 2001).. In the present study, the levels of variables chosen for trials were shown in Table 2.1. The Central Composite design for two independent variables was estimated and shown in Table 2.2.

Table 2.1. Maximum and Minimum Levels of Temperature and pH Used in the Central Composite Experimental Design.

Independent variables	Levels				
	$-\alpha$	-1	0	+1	$+\alpha$
X_1 ; temperature,(^{o}C)	28	35	50	65	71
X_2 ; pH	3.6	4.5	6.5	8.5	9.3

Table 2.2. The Central Composite Design for the Two Independent Variables, Factors 1 and 2.

Treatment	Factor1 (X1)	Factor2 (X2)	Temperature (X1)	pH (X2)
1	-1	-1	35	4.5
2	-1	1	35	8.5
3	1	-1	65	4.5
4	1	1	65	8.5
5	-1.414	0	28	6.5
6	1.414	0	71	6.5
7	0	-1.414	50	3.6
8	0	1.414	50	9.3
9	0	0	50	6.5
10	0	0	50	6.5
11	0	0	50	6.5

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2.4.5 Enzyme Production.

Cane bagasses was added into basal medium (200ml in 500 ml Erlenmeyer flasks). The varieties of pH as were adjusted described in Table 2.2. Inoculum size of 5 per cent was used. Then, it was incubated at various temperatures as previously described on a rotary shaker at 100 rpm. Fermentation took 7 days. Then, the activities of cellulase, mananase and xylanase were assayed. The enzyme activities were measured as described by Techapun *et al* , 2001.

2.4.6 Enzyme Activity Measurement.

The fermented medium was centrifuged at 10,000 rpm for 10 min, at 4 ^{o}C. The supernatant was diluted to the optimal concentration with sodium phosphate buffer pH6.0. After that, the supernatant was incubated at 55 ^{o}C with 0.5 per cent CMC (for endo-glucanase activity) , 0.5 per cent locus bean gum (for mannanase activity) and 0.5 per cent oat spelt xylan (for xylanase activity). Reducing sugar was estimated by dinitrosalicylic method (Miller, 1959). One international unit of enzyme activity (IU) was defined as 1μ *mole* of reducing sugar produced per ml per min at a standard condition (Techapun *et al.* 2001).

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2.4.7 Quadratic Model Analysis.

In this study, the variables were temperature and pH, while the observed response was the xylanase activity. The second degree quadratic model (Box *et al* 1978.) was established by the method of least square as following:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

Where, Y = predicted response (xylanase yield), X_1 and X_2 are coded forms of the input variables (temperature and pH, respectively), b_0 is a constant ; b_1, b_2 are linear coefficients; b_{12} is cross product coefficient ; b_{11}, b_{22} are quadratic coefficients. The relation between coded forms of the input variables and actual value of pH and temperature are described as following:

$$A_i = (X_i - X_0^*) / \Delta X$$

Where, X_i is uncoded value or actual value of temperature or pH; X_0^* is actual of the same variable at center point; ΔX is the step change of the variable. Table 2.2, represents the design matrix of the variables under consideration in both coded and uncoded values (e.g. uncoded form of pH, +1.0 is equal to pH 8.5). Quadratic model analysis by "SX. Version 7 " program (Analytical Software) was employed to find out the quadratic mathematical model and for investigation of the effects of pH and temperature on xylanase production. The three dimension contour plot (Statistica version 5 , SiEGE Production Inc. USA) was employed.

2.4.8 Test for the Accuracy and Precision of the Model.

To test the model accuracy and precision, *Streptomyces* sp. Ab106.3 was cultured in the optimized condition (50°C at constant pH 7.2) on rotary shaker for 7 days.

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2.4.9 Characterization of Crude Xylanases from *Streptomyces* sp. Ab106.3.

2.4.9.1 Temperature Profile.

The effect of temperature on the activity of xylanases was investigated in the range of 35° - $75\ ^{\circ}C$. The crude enzyme (14 IU) was diluted with 50 mM phosphate buffer to obtain the final pH level of 7. The final activity of enzyme was 4-5 IU. The samples were incubated with substrate at 35°, 45°, 55°, 60°, 65°, 70° and $75^{\circ}C$ for 20 min. Xylanase activities were determined as previously described.

2.4.9.2 pH Profile.

The reaction was performed at the optimal temperature to determine the optimal pH of enzyme . The activity was measured with 0.5 per cent w/v oat spelt xylan in 50mM acetate buffer in the range of pH 4-5, 50mM phosphate buffer in the range of pH 6-7, 50mM Tris-HCl buffer in the range of pH 8-9. Xylanase activities were determined as previously described.

2.4.9.3 Stability of Xylanases.

Reaction mixtures of 10 ml (2 ml culture supernatant and 8 ml buffer) were incubated at 55° , 65° and $75^{\circ}C$ under pH 6 , 7 , 8 and 9 . Remaining of xylanase activities were determined as previously described.

2.4.9.4 Effect of Bleaching Reagent on Xylanase Stability.

Effects of bleaching reagents such as hydrogen peroxide and sodium hypochloride on xylanase stability were determined by adding various concentrations of hydrogen peroxide (0.1 – 0.5 per cent w/v) and sodium hypochloride (0.1 – 0.7 per cent w/v) to the reaction mixture of 10 ml and incubated at 45° , 55° and $65^{\circ}C$. Xylanase activities were measured according to the standard assay as previously described.

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2.4.9.5 Enzyme Kinetic Studies.

Enzyme kinetics were investigated by Lineweaver –Burk plot. The crude enzyme (14 IU) was diluted with phosphate buffer pH 7 to obtain the final activity of enzyme 5 IU which was equivalent to the total soluble protein of 0.132 µg/ml. The hydrolysis of xylan was studied by mixing 4 ml of crude enzyme with 6 ml of different concentrations of oat spelt xylan (1 – 20 g/l) in 50mM phosphate buffer pH7, and incubating at 65 ^{o}C for 20 min. Samples were centrifuged to removed insoluble solid. The supernatant was analyzed for the liberated reducing sugar by the DNS method. Effect of hydrogen peroxide on kinetic parameters were studies by adding hydrogen peroxide to the final concentrations of 0.1 – 0.3M to the reaction mixture which contained 4 ml of crude enzyme and 6 ml of different concentrations of oat splet xylan (2 – 15 g/l). Then, it was incubated at 65 ^{o}C for 20 min. Reducing sugar was determined by DNS method.

2.4.10 Prebleaching and Bleaching of Kraft Pulp.

2.4.10.1 Biobleaching Pulp with Xylanases.

The enzymatic bleaching studies were performed at pulp concentration of 5 per cent ,pH 7, 55 ^{o}C . Different doses of xylanases obtained from *Streptomyces* sp. Ab106.3 were 5 to 15 IU/g of pulp (dry basis). The samples were taken after 3, 24, 36 and 48h after incubation. The pulp was filtered though filter paper (Whatman NO.1) and washed twice with 200 ml deionized water. The filtrate (before washing) was determined for reducing sugar by DNS method. Lignin derived compounds (LDCs) and chromophore released from pulp were monitored by measuring the increase in absorbance of the filtrate at 280 nm and 465 nm, respectively. The pulp was dried at 55 ^{o}C for 3h and brightness of dried pulp was measured by Color Quest II machine.

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2.4.10.2 Hydrogen Peroxide Bleaching of Enzyme Pretreated Pulp

Hydrogen peroxide was applied in order to evaluate the bleachability of the enzyme-pretreated pulps, as proposed by Christov and Prior, 1997. The bleaching conditions were 1.5 per cent H_2O_2; 0.7per cent NaOH; 0.5 per cent $MgSO_4$; 70 °C, 3 h, and 5 per cent pulp consistency. Control was conducted under the same conditions without the enzyme pretreatment step. The pulps were filtered though filter paper (Whatman NO.1) and washed twice with 200 ml deionized water. The pulp was dried at 55 °C for 3h.Then, the brightness of dried pulp was measured by Color Quest II machine.

2.4.10.3 Enzyme Bleaching of Hydrogen Peroxide Pretreated Pulp

The bleaching sequences of pulp were performed by treating pulp with hydrogen peroxide as described in 2.4.11.2. After bleaching, pulp was neutralized with 0.1 N sulphuric acid, washed with deionized water, dried at 55°C and analyzed. After that, the pulp was treated with enzyme as described in 2.4.11.1.Control sample was conducted under the same conditions without enzyme. The reducing sugar, released lignin and properties of pulp were determined as previous described.

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