CHAPTER 3

Materials and Methods

3.1 Material and chemical reagents

3.1.1 Media and chemical reagents

Media

Nutrient broth (NB medium) (Appendix A)

Names of chemical reagents	Company
Ammonium chloride	AJAX FINECHEM
Ammonium nitrate	AJAX FINECHEM
Ammonium sulfate	AJAX FINECHEM
Beef extract	HIMEDIA
Bovine serum albumin	MERCK
Bradford reagent	(See Appendix C)
Butanol	LAB SCAN
Calcium chloride dihydrate	LOBA CHEMIE
Casein	SIGMA
Citric acid	RANKEM
Coomassie brilliant blue G-250	LIFE SCIENCE
DEAE-Sephadex A-50	PHARMACIA
Di-ammonium citrate	PANREAC
Di-potassium hydrogen phosphate	AJAX FINECHEM
Di-sodium hydrogen phosphate	RANKEM
DNS reagent	(See Appendix B)
Ethanol	MERCK
Hydrochloric acid	MERCK
Konjac flour	NOKKOO

Locust bean gum	SIGMA
Magnesium sulfate heptahydrate	LAB SCAN
Peptone	HIMEDIA
Potassium dihydrogen phosphate	AJAX FINECHEM
Sephacryl S-100	PHARMACIA
Sodium chloride	AJAX FINECHEM
Sodium dihydrogen phosphate dihydrate	AJAX FINECHEM
Sodium hydroxide	LAB SCAN
Sulfuric acid	MERCK
Thymol	LOBA CHEMIE
Tris (hydroxymethyl) aminomethane	FISHER
Tryptone	KEMMAR
Yeast extract	HIMEDIA
Zinc sulfate heptahydrate	LOBA CHEMIE

3.1.2 Equipment

Names of equipment	**Company**
Autopitpette	LAB SOLUTIONS
Autoclave	ALL AMERICAN
Dialysis bag	WAKO
Hot air oven	MEMMERT
GradiFrac System	AMERCHAM
Larminar air flow	MICROTECH
pH meter	CYBERSCAN
Refrigerated Centrifuge Sorvall Super T21	KENDRO
Shaking incubator	KUHNER
Spectrophotometer	SHIMADZU
TLC aluminium sheets	MERCK
TLC developing tank	CAMAG
Ultrafiltration (Vivaspin concentrator)	VIVASCIENCE
Vortex-2 Genie	SCIENTIFIC INDUSTRIES
Water bath	MEMMERT

3.2 Methods

3.2.1 Microbial strain and maintenance

Bacillus subtilis M7, the mutant obtained by X-ray mutagenesis of *B. subtilis* MR10, was obtained from Laboratory of Microbial Resources and Enzyme Technology, Faculty of Agro-Industry, Chiang Mai University. The strain was grown on nutrient agar (NA) at 37^{o}C for 24 h and stored at 4^{o}C.

3.2.2 Copra meal preparation

The residue from coconut milk extraction was boiled for 2 h with 2 volumes of distilled water (DW), and then it was filtered through fine-grained cloth. The copra meal was suspended again with the same volume of DW and left at 4^{o}C overnight. The solidified coconut oil was removed and the defatted copra meal was dried at 60^{o}C until the free water was completely removed. The dried sample was ground with a hammer mill and sieved (30 mesh) before keeping in a vacuum bag at room temperature (15-30^{o}C).

3.2.3 Enzyme production

1) Culture conditions and enzyme production experiment

B. subtilis M7 was transferred to basal medium containing gram per liter of 5 copra meal, 0.3 yeast extract, 3 K_2HPO_4, 1 KH_2PO_4, 0.5 $CaCl_2.2H_2O$, 0.1 $ZnSO_4.7H_2O$, 2.0 $(NH_4)_2SO_4$ and 2.0 $MgSO_4.7H_2O$ and incubated with shaking at 180 rpm and 37^{o}C for 30 h. The cells were removed from culture broths by centrifugation with 13,000 rpm for 10 min, and the supernatant was assayed for β-mannanase as described below.

2) Determination of β-mannanase activity

β-Mannanase activity was determined as described by Khanongnuch *et al*. (1998). Mixing 0.25 ml of 0.5% (w/v) locust bean gum in 0.1M phosphate buffer pH 7.0 with 0.25 ml of appropriately diluted enzyme sample, incubated for 10 min at 50°C. The reaction was stopped by adding 0.5 ml DNS reagent and then boiling in water bath for 10 min. After cooling, 5 ml of distilled water was added and mixed well. The samples were measured the absorbance at 540 nm (Miller, 1959). One unit of enzyme

activity was defined as the amount of enzyme required to liberate 1 μmole of reducing sugar per minute under the assay condition.

3) Determination of protein

Protein concentration was determined by the dye binding method (Bradford, 1976) using bovine serum albumin (BSA) as standard protein. The protein concentration was determined by mixing 0.015 ml of sample with 0.6 ml of Bradford solution, and then incubated for 10 min at room temperature. Afterwards, the sample absorbance was measured at 595 nm.

4) Screening for appropriate nitrogen sources

The effects of different inorganic and organic nitrogen sources on β-mannanase production were investigated by selecting different inorganic nitrogen sources including ammonium nitrate, ammonium chloride, ammonium sulfate, and di-ammonium citrate. All treatments were investigated at the same concentration of 2 g/l nitrogen source in the basal medium containing gram per liter of 5 copra meal, 0.3 yeast extract, 3 K_2HPO_4, 1 KH_2PO_4, 0.5 $CaCl_2.2H_2O$, 0.1 $ZnSO_4.7H_2O$ and 2.0 $MgSO_4.7H_2O$, then pH was adjusted to 6.8. Each flask was incubated with 180 rpm shaking and 37^oC for 30 h. In similar way, effect of different organic nitrogen sources on enzyme production was also evaluated by comparing the activity obtained from peptone, tryptone, yeast extract, beef extract, casein, corn steep liquor and soybean meal at the concentration of 0.3 g/l. The inorganic and organic nitrogen sources giving the highest β-mannanase activity were selected for further study.

5) Screening for the factors affected on β-mannanase production

Totally 8 ingredients composed in the enzyme production medium including soybean meal, K_2HPO_4, KH_2PO_4, $CaCl_2.2H_2O$, $ZnSO_4.7H_2O$, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, copra meal were screened for the influenced nutritional factor on enzyme production using Plackett and Burman experimental design. Fifteen treatments were generated. The minus symbol stands for low level while plus symbol is high level (Table 3.1). The Plackett and Burman code values of each variable in each treatment were presented in Table 3.2. β-Mannanase activity was determined as described

previously and the factors influenced on β-mannanase production were indicated by regression analysis using the Design Expert program version 7.0.

In the similar way, when locust bean gum and konjac flour were used as carbon sources, the ingredients were also screened for the influenced nutritional factor on enzyme production using Plackett and Burman experimental design. The concentration of each variable at high and low level used in the experiment was shown in Table 3.3 and 3.5 when locust bean gum and konjac flour were used as the carbon source, respectively. The Plackett and Burman code values of each variable in each treatment were presented in Table 3.4 and 3.6 when locust bean gum and konjac flour were used, respectively.

6) Effect of carbon source on β-mannanase production

Effect of carbon source on β-mannanase production was investigated by supplementation of copra meal to the optimized medium (containing gram per liter of 5 soybean meal, 0.3 K_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$) at the concentration of 0, 2, 4, 6, 8 and 10 g/l, then incubated with 180 rpm, 37°C for 30 h. β-Mannanase activity was determined.

Similarly, effect of locust bean gum and konjac flour on enzyme production were investigated by supplementation of locust bean gum and konjac flour to the optimized medium at the concentration of 0, 2, 4, 6, 8 and 10 g/l locust bean gum, and to that at the concentration of 0, 2, 4, 6, 8 and 10 g/l konjac flour, respectively. The concentration of each carbon source giving the highest β-mannanase activity was selected for further study.

7) Time course of β-mannanase production

B. subtilis M7 was cultivated in the optimal medium containing gram per liter of 5 soybean meal, 0.3 K_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$ and 6 copra meal, pH 6.8 and incubated with shaking at 180 rpm and 37°C for 48 h. β-Mannanase activity was determined every 6 h.

In similar way, *B. subtilis* M7 was cultivated in the optimal medium with 10 g/l locust bean gum and in that with 4 g/l konjac flour when locust bean gum and konjac flour were the carbon source, respectively.

Table 3.1 The concentration of each variable at high and low level used in the Plackett and Burman design while copra meal is a carbon source.

Variables	Concentration (g/l)	
	High (+)	Low (-)
Soybean meal	5	1
K_2HPO_4	3	0.3
KH_2PO_4	1	0.1
$CaCl_2.2H_2O$	1	0.1
$ZnSO_4.7H_2O$	0.25	0.05
$(NH_4)_2SO_4$	2	0.2
$MgSO_4.7H_2O$	2	0.2
Copra meal	5	0.5

Table 3.2 Treatments generated based on Plackett and Burman design while copra meal is a carbon source.

Treatments	A	B	C	D	E	F	G	H
1	+	+	-	+	-	-	+	-
2	-	+	+	-	-	-	-	+
3	+	-	+	+	+	-	-	-
4	-	+	-	+	+	+	-	-
5	-	-	+	-	+	+	+	-
6	-	-	-	+	-	+	+	+
7	+	-	-	-	+	-	+	+
8	+	+	-	-	+	+	-	+
9	+	+	+	-	-	+	+	-
10	-	+	+	+	+	-	+	+
11	+	-	+	+	-	+	-	+
12	-	-	-	-	-	-	-	-
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0

A = Soybean meal, B = K_2HPO_4, C = KH_2PO_4, D = $CaCl_2.2H_2O$, E = $ZnSO_4.7H_2O$, F = $(NH_4)_2SO_4$, G = $MgSO_4.7H_2O$, H = Copra meal

Table 3.3 The concentration of each variable at high and low level used in the Plackett and Burman design while locust bean gum is a carbon source.

Variables	Concentration (g/l)	
	High (+)	Low (-)
Soybean meal	5	1
K_2HPO_4	3	0.3
KH_2PO_4	1	0.1
$CaCl_2.2H_2O$	1	0.1
$ZnSO_4.7H_2O$	0.25	0.05
$(NH_4)_2SO_4$	2	0.2
$MgSO_4.7H_2O$	2	0.2
Locust bean gum	5	0.5

Table 3.4 Treatments generated based on Plackett and Burman design while locust bean gum is a carbon source.

Treatments	A	B	C	D	E	F	G	H
1	+	+	-	+	-	-	+	-
2	-	+	+	-	-	-	-	+
3	+	-	+	+	+	-	-	-
4	-	+	-	+	+	+	-	-
5	-	-	+	-	+	+	+	-
6	-	-	-	+	-	+	+	+
7	+	-	-	-	+	-	+	+
8	+	+	-	-	+	+	-	+
9	+	+	+	-	-	+	+	-
10	-	+	+	+	+	-	+	+
11	+	-	+	+	-	+	-	+
12	-	-	-	-	-	-	-	-
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0

A = Soybean meal, B = K_2HPO_4, C = KH_2PO_4, D = $CaCl_2.2H_2O$, E = $ZnSO_4.7H_2O$, F = $(NH_4)_2SO_4$, G = $MgSO_4.7H_2O$, H = Locust bean gum

Table 3.5 The concentration of each variable at high and low level used in the Plackett and Burman design while konjac flour is a carbon source.

Variables	Concentration (g/l)	
	High (+)	Low (-)
Soybean meal	5	1
K_2HPO_4	3	0.3
KH_2PO_4	1	0.1
$CaCl_2.2H_2O$	1	0.1
$ZnSO_4.7H_2O$	0.25	0.05
$(NH_4)_2SO_4$	2	0.2
$MgSO_4.7H_2O$	2	0.2
Konjac flour	5	0.5

Table 3.6 Treatments generated based on Plackett and Burman design while konjac flour is a carbon source.

Treatments	A	B	C	D	E	F	G	H
1	+	+	-	+	-	-	+	-
2	-	+	+	-	-	-	-	+
3	+	-	+	+	+	-	-	-
4	-	+	-	+	+	+	-	-
5	-	-	+	-	+	+	+	-
6	-	-	-	+	-	+	+	+
7	+	-	-	-	+	-	+	+
8	+	+	-	-	+	+	-	+
9	+	+	+	-	-	+	+	-
10	-	+	+	+	+	-	+	+
11	+	-	+	+	-	+	-	+
12	-	-	-	-	-	-	-	-
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0

A = Soybean meal, B = K_2HPO_4, C = KH_2PO_4, D = $CaCl_2.2H_2O$, E = $ZnSO_4.7H_2O$, F = $(NH_4)_2SO_4$, G = $MgSO_4.7H_2O$, H = Konjac flour

8) Effect of growing temperatures on β-mannanase production

To examine the effect of growing temperatures, the culture of *B. subtilis* M7 in the optimal medium containing gram per liter of 5 soybean meal, 0.3 K_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$ and 6 copra meal, pH 6.8 was incubated with 180 rpm for 30 h at various temperatures including 30, 37 and 45ºC. The supernatant of the culture was separated and assayed for β-mannanase activity.

9) Effect of initial pH on β-mannanase production

To examine the effect of initial pH, the initial pH of optimized medium containing gram per liter of 5 soybean meal, 0.3 K_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$ and 6 copra meal was adjusted to various values including 4.0, 5.0, 6.0, 6.5, 6.8, 7.0, and 8.0 using 1.0 N NaOH or 1.0 N HCl solution. *B. subtilis* M7 was cultivated in optimized medium and incubated with shaking at 180 rpm and 37ºC for 30 h. The extracellular β-mannanase activity found in the culture broth was determined.

10) Bioreactor experiment

The fermentation was carried out in a 5-L bioreactor (4L working volume) using the optimized medium containing gram per liter of 5 soybean meal, 0.3 K_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$ and 6 copra meal, pH 6.8. The effect of agitation rate was investigated within the range of 200 and 400 rpm at 37ºC, 1 vvm for 48 h. β-Mannanase activity was determined every 6 h.

11) Storage stability of the β-mannanase

Crude enzyme from *B. subtilis* M7 was mixed with 5 stabilizers including maltodextrin (Belghith *et al*., 2001; Alloue *et al*., 2007), sorbitol, NaCl (Selivanov, 2005), $(NH_4)_2SO_4$ and starch (Lian *et al*., 2002) while that without stabilizer was used as control. All treatments were incubated at 4ºC for 42 days. Thereafter, the residual activity was determined.

3.2.4 Enzyme purification

1) Purification of *B. subtilis* M7 β-mannanase

Crude enzyme was precipitated by 80% ammonium sulfate. The ammonium sulfate was gradually added in crude enzyme solution and stirred at 4°C. The solution was stood for 1 h after the complete addition of ammonium sulfate. The precipitated proteins were harvested by centrifugation at 6,000 rpm, 4°C for 30 min and dissolved with 20 mM sodium phosphate buffer pH 7.0. This solution was then dialyzed with the same buffer. The equilibrium solution was applied into DEAE-Sephadex column (4.5× 19 cm) equilibrated with 20 mM sodium phosphate buffer pH 7.0. After that, 20 mM sodium phosphate buffer pH 7.0 was applied in order to remove unbound protein prior to elute with stepwise gradient of 0-0.5 M NaCl. The eluted fractions were collected to measure absorbance at 280 nm and determined enzyme activity. The active fractions were pooled, desalted and concentrated using ultrafiltration with MWCO of 10 kDa. The concentrated enzyme was applied to Sephacryl S-100 column (2.5× 54.5 cm) equilibrated with 20 mM sodium phosphate buffer pH 7.0. The enzyme was eluted by the same buffer. The eluted fractions were evaluated for their purity by SDS-PAGE. The purified enzyme was stored at -20°C for further characterization.

2) Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was done by the method of Laemmli (1970) in 12% resolving gel and 4% stacking gel with 0.1% of SDS. Denaturing condition was carried out for SDS-PAGE by heating the protein under the presence of β-mercaptoethanol at 100°C for 8 min. The protein was stained with Coomassie brilliant blue G-250.

3.2.5 Characterization of purified β-mannanase

1) pH optimum and stability

The optimum pH of purified β-mannanase activity was examined at pH values of 4.0-9.0 under otherwise standard assay conditions. A locust bean gum solution (0.5%, w/v) in the appropriate buffers, each at 20 mM—citrate phosphate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 8.0–9.0)—was used to determine β-mannanase activity. The relative activity was calculated compared to that of the highest

activity. Enzyme stability was determined using the same buffer systems in the range of 4.0–9.0 by incubating purified β-mannanase in the various buffer solutions at 4°C for 24 h. Then, the remaining enzyme activity was measured under standard assay conditions.

2) Temperature optimum and stability

The temperature optimum of purified β-mannanase activity was determined by performing the standard activity assay at temperatures ranging from 20-65°C. The relative activity was calculated compared to that of the highest activity. For the determination of thermal stability, purified β-mannanase was incubated at various temperatures for 1 h. Thereafter, the remaining enzyme activity was measured.

3) Substrate specificity

Various substrates including locust bean gum, guar gum, konjac flour and copra meal, each at a concentration of 0.5% (w/v), were used for the determination of β-mannanase activity under standard assay conditions. The relative activity was calculated compared to that obtained from locust bean gum.

4) Effect of various metal ions and chemicals

The effects of EDTA, SDS and mercaptoethanol as well as various metal ions (K^+, Na^+, Li^+, Co^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+}, Mn^{2+}, Ca^{2+}, Pb^{2+}, Fe^{3+}, and Al^{3+}), each at a concentration of 5 mM, on β-mannanase activity were determined under standard assay conditions. The relative activity was calculated compared to that without any metal ion and chemical.

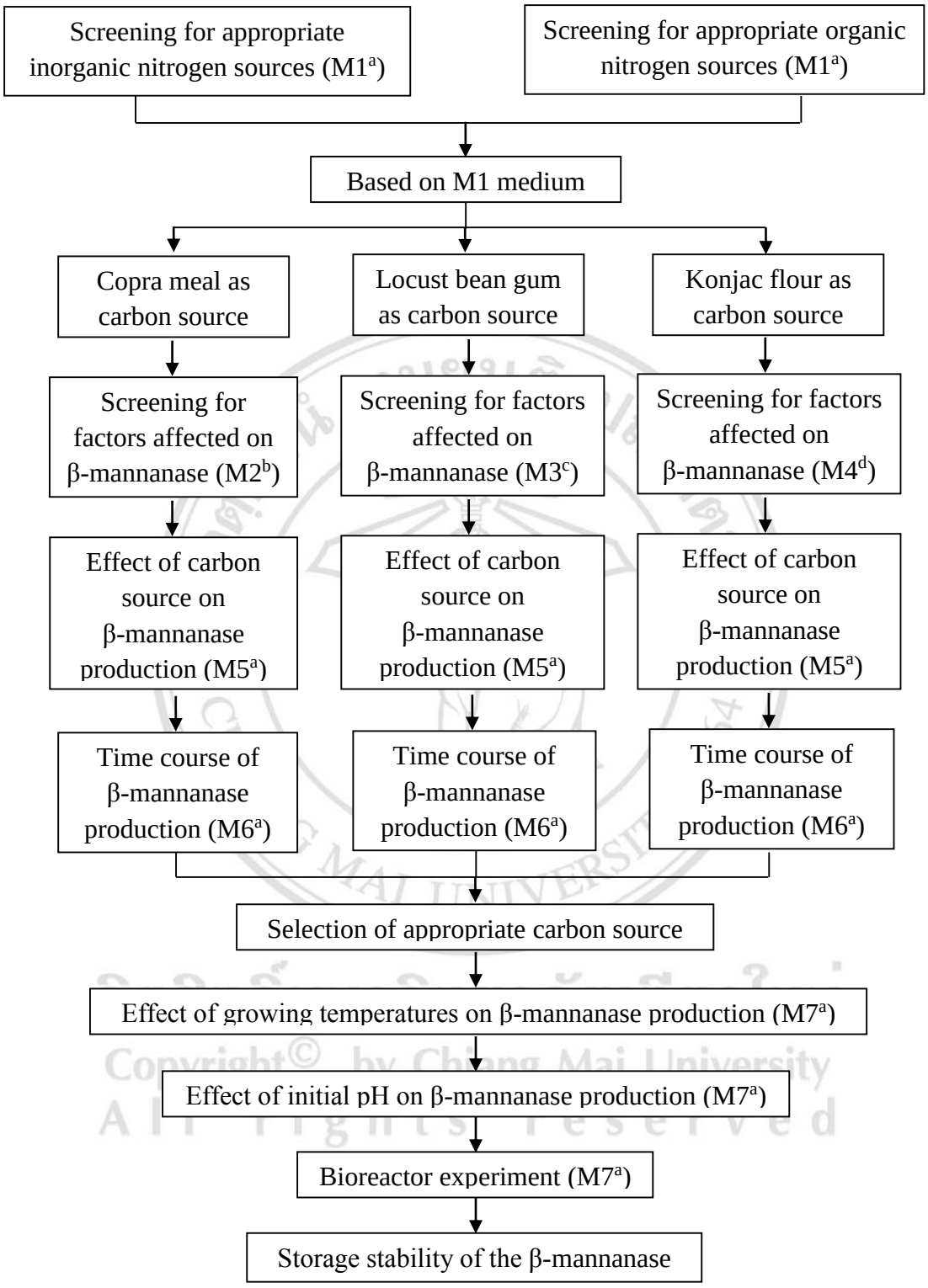
5) Determination of kinetic parameters

The Michaelis-Menten kinetic parameters, K_m and V_{max}, were determined by using locust bean gum as substrates at concentrations ranging from 0.1–10 g/l. The condition was carried out at pH 7.0 and 50°C. The K_m and V_{max} were calculated from Lineweaver-Burk plot created by SigmaPlot 12.0 (Sysstat Software, Inc., San Jose, CA, USA).

6) Hydrolysis experiments

The purified β-mannanase (2 U) was incubated with 1 ml of 0.5% (w/v) locust bean gum to achieve the final ration of substrate of 0.4 U purified enzymes per

milligram substrate. The reaction mixture was incubated at 50°C for 6 h. The reaction was stopped by heating at 100°C for 10 min. The hydrolysates were analyzed by thin-layer chromatography (TLC). The solvent used as mobile phase was composed of *n*-butanol: ethanol: water in the ratio of 5:3:2, respectively. Two microliters of sample was applied onto Merck classical silica TLC plate and developed for 4 h in developing solvent. The TLC plates were then developed by dipping in 0.5% (w/v) thymol prepared in 5% (v/v) H_2SO_4 in ethanol and heating at 105^{o}C for 5 min and sugars appeared as pink spots. The similar experiment was conducted using copra meal as substrate. The hydrolysis products were compared with those of locust bean gum.



Note: [a] the composition of the medium descripted in Appendix A; [b] Table 3.1 and 3.2; [c] Table 3.3 and 3.4; [d] Table 3.5 and 3.6, respectively

Figure 3.1 Summary of experimental procedure for β-mannanase production.

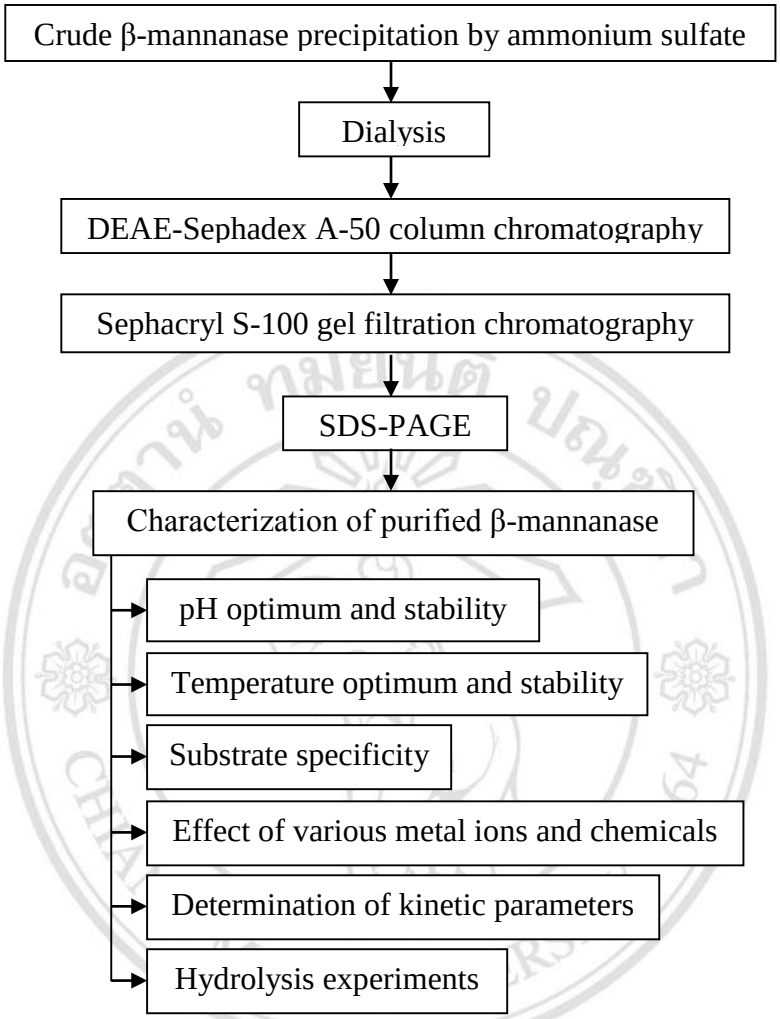


Figure 3.2 Summary of experimental procedure for purification and characterization of β-mannanase.