APPENDICES

APPENDIX A

Media

1. Media composition and preparation

1.1. Nutrient broth

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

Medium was adjusted pH to 7.0 and sterilized at 121°C for 15 min.

1.2. Medium for screening appropriate nitrogen sources (M1)

Organic nitrogen source	0.3 g
K_2HPO_4	3.0 g
KH_2PO_4	1.0 g
$CaCl_2.2H_2O$	0.5 g
$ZnSO_4.7H_2O$	0.1 g
Inorganic nitrogen source	2.0 g
$MgSO_4.7H_2O$	2.0 g
Copra meal	5.0 g
Distilled water	1000 ml

Medium was adjusted pH to 6.8 and sterilized at 121°C for 15 min.

1.3. Medium for studying effect of carbon source on β-mannanase production (M5)

Soybean meal	5.0 g
K_2HPO_4	0.3 g
KH_2PO_4	1.0 g
$CaCl_2.2H_2O$	1.0 g
$ZnSO_4.7H_2O$	0.05 g

$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.2 g
Carbon source at the concentration of 0, 2, 4, 6, 8 and 10 g/l	
Distilled water	1000 ml

Medium was adjusted pH to 6.8 and sterilized at 121°C for 15 min.

1.4. Medium for studying time course of β-mannanase production (M6)

Soybean meal	5.0 g
K_2HPO_4	0.3 g
KH_2PO_4	1.0 g
$CaCl_2.2H_2O$	1.0 g
$ZnSO_4.7H_2O$	0.05 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.2 g
Carbon source at the optimal concentration	
Distilled water	1000 ml

Medium was adjusted pH to 6.8 and sterilized at 121°C for 15 min.

1.5. Medium for studying effect of growing temperatures, initial pH and bioreactor experiment on β-mannanase production (M7)

Soybean meal	5.0 g
K_2HPO_4	0.3 g
KH_2PO_4	1.0 g
$CaCl_2.2H_2O$	1.0 g
$ZnSO_4.7H_2O$	0.05 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.2 g
Copra meal	6.0 g
Distilled water	1000 ml

Medium was adjusted pH to 6.8 for studying effect of growing temperatures and bioreactor experiment. In case of studying effect of initial pH, medium was adjusted to various values including 4.0, 5.0, 6.0, 6.5, 6.8, 7.0, and 8.0, then sterilized at 121°C for 15 min.

APPENDIX B

Measurement activity enzyme

1. Enzyme assay and preparation substrate

DNS reagent

3, 5-Dinitrosalicylic acid	10 g
NaOH	16 g
Potassium sodium tartrate	300 g

After dissolving all ingredients in 600 ml distilled water volume was made up to 1 liter. The reagent was stored in a brown colored bottle.

Mannanase assay and calculation

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.

Example for mannanase activity calculation

A_{540} = absorbance at 540 nm

D = external dilution factor

d = internal dilution factor (V_t/ V_e)

V_e = enzyme volume (0.25 ml)

V_t = totol volume (0.50 ml)

MW = molecular weight of mannose (180 g/mol)

Incubation time = 10 min

Mannanase activity (U/ml) = $(A_{540} \times D \times d \times 1000)/(V_e \times MW \times 0.5523 \times 10)$

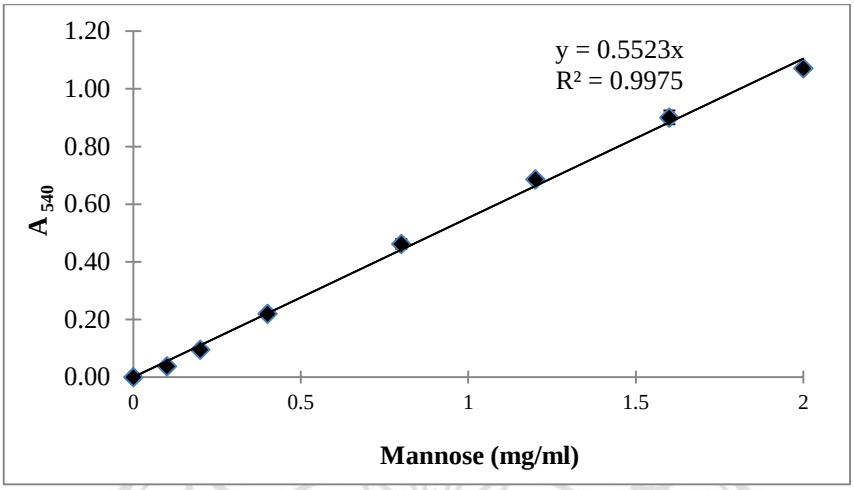

Figure A.1 Calibration curve of standard mannose concentration versus the absorbance at 540 nm.

APPENDIX C

Measurement protein

1. Bradford protein assay

Bradford solution

Coomassie brilliant blue G250	0.5 g
Ethanol	250 ml
Phosphoric acid	425 ml

After dissolving all ingredients in 600 ml distilled water volume was made up to 1 liter and filtered through Whatman filter paper No. 2. The solution was stored in a brown colored bottle and kept it in 4^{o}C.

Procedure

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 absorbances were measured at 595 nm.

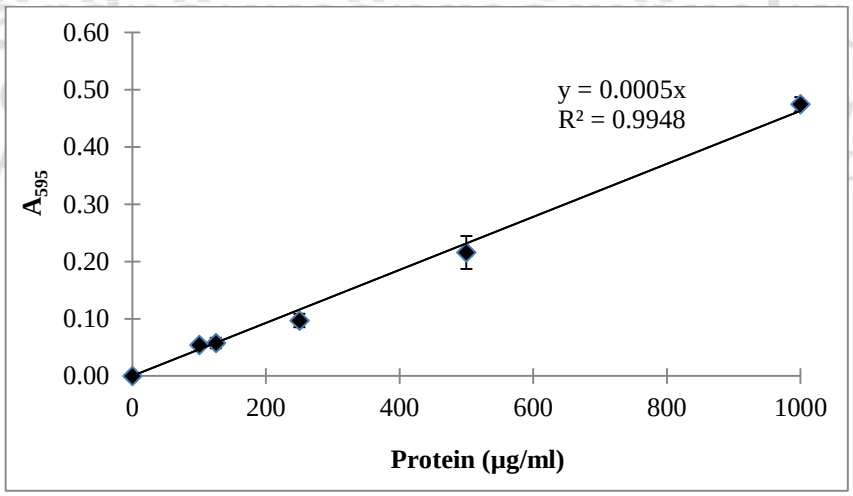

Figure A.2 Standard curves for protein assay.

APPENDIX D

Electrophoresis

Reagents

Acrylamide stock solution (30% (w/w) acrylamide, 2.7% (w/w) bisacrylamide)

- To dissolve 29.2 g of acrylamide and 0.8 g of bisacrylamide in 100 ml of distilled water.

1.5 M Tris-HCl, pH 8.8

- To dissolve 18.2 g of Tris base in distilled water and adjust to pH 8.8 with HCl before adding distilled water to a final volume of 100 ml.

0.5 M Tris-HCl, pH 6.8

- To dissolve 6.1 g of Tris base in distilled water and adjust to pH 6.8 with HCl before adding distilled water to a final volume of 100 ml.

10% (w/v) sodium dodecyl sulfate (SDS)

- To dissolve 10 g of SDS in 100 ml of distilled water.

10% (w/v) ammonium persulfate (APS)

- To dissolve 100 mg of APS in 1 ml of distilled water. Make the APS solution freshly daily.

TEMED (*N,N,N',N'*-tetramethylenediamine)

5X sample buffer

- 0.6 ml of 1 M Tris-HCl, pH 6.8
- 5 ml of 50% glycerol
- 2 ml of 10% SDS
- 0.5 ml of β-mercaptoethanol
- 1 ml of 1% bromophenol blue
- 0.9 ml of distilled water

5X electrode buffer

- To dissolve 72 g of glycine, 15 g of Tris base and 5 g of SDS in 1000 ml of distilled water.

Coomassie gel staining

- 0.1% (w/v) Coomassie brilliant blue G-250 in 40% methanol and 10% acetic acid.

Coomassie gel destaining

- 40% methanol and 10% acetic acid.

Procedure

1. Clean the glass plates, comb, spacers and upper buffer reservoir of the gel equipment with detergent and completely dry.
2. Assemble glass plate sandwich and spacers.
3. Prepare monomer solution for resolving gel by mixing all reagents in Table A.1. Deaerate the mixed solution for 15 min.
4. Softly mix the 25 µl of APS and 2.5 µl into deaerated monomer solution.
5. Carefully introduce the solution into glass plate sandwich using an autopipette to about 0.5 cm below the short plate.
6. Gently layer around 1 to 5 mm of distilled water on the top of gel solution and allow gel to polymerize for 30-60 min.
7. Prepare stacking gel monomer solution by mixing all reagents in Table A.2.
8. Deaerate monomer solution for 10 min.
9. Pour off the water covering stacking gel and dry the upper area with filter paper.
10. Insert comb into glass plate sandwich and tilt the comp at a little corner to provide a way for bubbles to get away.
11. Add 10 µl of TEMED and 50 µl of 10% APS to deaerated monomer solution and pour carefully stacking gel solution on top of resolving gel.
12. Place comb in its suitable position and allow the gel for polymerization for 30 to 45 min.

Table A.1 Formulation of resolving gel.

Component	Volume
Water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% SDS	0.1 ml
30% Acrylamide	4.0 ml
10% APS	50 µl
TEMED	5 µl

Table A.2 Formulation of stacking gel.

Component	Volume
Water	6.1 ml
0.5 Tris-HCl, pH 6.8	2.5 ml
10% SDS	0.1 ml
30% Acrylamide	1.3 ml
10% APS	50 µl
TEMED	5 µl

Sample preparation

1. Combine protein sample and 5X sample buffer (4:1) in an Eppendroff tube.
2. Heat at 100^{o}C for 8 min.

Reconstitution of protein marker

Add 2 ml of sample buffer, 1% (v/v) β-mercaptoethanol, 1% (w/w) SDS, 8 M Urea and 0.01 M Tris, adjusted to pH 6.8 with phosphoric acid, to the vial from Pharmacia manufacture. This gives 1 mg/ml protein concentration.

Electrophoresis

1. Place gel into electrophoresis chamber and attach both gels to electrode assembly.
2. Add electrophoresis buffer to inner and outer reservoir and remove carefully comb from stacking gel.
3. Load sample prepared into well in stacking gel by arranging them under electrode buffer.
4. Attach electrode plugs to power supply.
5. Turn on power supply to 70 volt, till Bromophenol blue loading dye front reached lower gel.

Dye staining with Coomassie brilliant blue G-250

1. Pick up the gel and transfer to a small container containing Coomassie gel stain.
2. Agitate for 30 min on a slow shaker.
3. Pour out stain.
4. Add Coomassie destain and continue slow shaking. Change destain solution many times, till the background is removed satisfactorily.

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