KIMIKA 10: 15-23 (1994)
Printed in the Philippines

Purification and characterization of mannanase from *Pseudomonas* Sp.

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(Accepted May 6, 1994)

Mannanase enzyme was produced by a local isolate of *Pseudomonas* sp. in batch culture using coconut endosperm residue "sapal" as carbon substrate. The optimal pH for enzyme production was 6.0 and enzyme activity of the culture suspension was maximal during the third day of fermentation.

Sequential column chromatography through DEAE-cellulose and Sephadex G-75 gave an enzyme recovery of 58.2 % and 12.3-fold purification. The enzyme was electrophoretically homogeneous, consisted of only one protein subunit and had a molecular weight of ~20 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The optimal pH and temperature for the purified enzyme were 5.0 - 6.5 and 45 - 50°C, respectively. The K_m and V_m values were 1.9 mM and 0.15 mM/min, respectively, for galactomannan from "makapuno" coconut; the corresponding values were 4.9 mM and 0.095 mM/min for locust bean gum galactomannan. Enzymatic hydrolysis of the latter galactomannan gave mannose and mannooligosaccharides as major products.

Key Words: Mannanase, Pseudomonas enzyme, galactomannan hydrolysis.

MANNANASE ENZYMES CATALYZE THE RANDOM HYDROLYSIS OF MANnans, galactomannans and glucomannans, which are the major carbohydrates of coconut endosperm cell walls. Enzymatic breakdown of these cell walls could facilitate extraction of oil and proteins from coconut endosperm (or meat) during wet processing. Thus, the availability of highly active mannanases at reasonable cost could provide a substantial cost reduction for coconut processing.

Mannanases have been isolated and purified from culture filtrates of *Streptomyces* sp. (1) and *Bacillus* sp. (2). The ß-mannanase from *Streptomyces* sp. had an optimum pH and temperature of 6.8 and 57°C, respectively, and had a molecular weight of 42.9 kD. *Bacillus* sp. ß-mannanase had optimum pH and temperature of 5.0 and 55°C, respectively and the estimated molecular weight was 38 kD. Purification of the microbial mannanase involved ion exchange and gel chromatography. An earlier study by Papa and del Rosario (3) involved partial characterization of crude mannanase from a local microbial isolate.

The present paper, which is based on the M.S. thesis work of Parayno (4), deals with production in shake flask culture of mannanase enzyme(s) by a local isolate of *Pseudomonas* sp. followed by chromatographic purification of the prepared enzyme. The purified enzyme was also characterized in terms of the optimal pH, heat stability, molecular weight, kinetic parameters and hydrolytic action on locust bean gum galactomannan.

Experimental

A. Preparation of Substrate and Fermentation Media

The fresh coconut meat, which was purchased from the Los Baños market, was grated and hand pressed in order to extract the coconut milk "gata". The coconut meat was washed extensively with several portions of tap water until the washings were almost clear. The coconut meat residue "sapal" was then air dried and ground to 20 mesh using a Wiley Mill. The coconut oil was extracted from the meat residue with petroleum ether in a Soxhlet apparatus. A local isolate of the bacterium *Pseudomonas* sp., which was the source of mannanase, was maintained by monthly transfers into TGYA (Tryptone-Glucose-Yeast Extract-Agar) slants. The organism was grown in a basal medium containing (per liter): KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.4 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2$, 0.3 g; Na_2HPO_4, 1.0 g. Coconut meat residue "sapal", which was prepared as described above, was used as carbon substrate at a final solids level or concentration of 1%.

B. Mannanase Assay

The assay medium was prepared by making appropriate dilutions of enzyme and citric acid buffer (0.5 M, pH 4.8). The substrate was added to a final concentration

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of 20 mM. Assay was conducted at 30°C and reducing sugar produced by enzymatic hydrolysis was determined by the method of Miller (5).

C. Optimum pH for Enzyme Production

The optimum pH for enzyme production was determined in shake flask cultures in the pH range 5.0 - 8.0 at 0.5 unit intervals. Agitation was done in a rotary shaker at 180 rpm. Aliquot samples were obtained every day for five days. The samples were centrifuged in order to separate the bacterial cells, after which the cell-free supernate was assayed for mannanase activity. The optimum temperature for mannanase assay was determined at 30 - 70°C (5°C interval). The optimum pH for assay was also determined using 0.2 M phosphate buffer and 0.2 M NH_4OH - NH_4Cl buffer for the pH range 4.5 - 8.0 and 8.5 - 10.0, respectively, at 40°C.

D. Mannanase Production in Fermenter

The time profile of mannanase production was determined using a 1L Bio-Flo stirred tank fermenter. The agitation rate and aeration rate used were 400 rpm and 1 vvm, respectively. Enzyme assay was done every 24 hours on aliquot samples. The substrate concentration was 1 % and pH was maintained at 6.0. After the time profile has been determined, batch fermentation was done for three days after which the culture was harvested and filtered through nylon cloth in order to separate the suspended solids. The extract was then centrifuged in a Sorval refrigerated centrifuge for 30 minutes at 10,000 rpm to remove the bacterial cells. The supernatant liquid was recovered and sodium azide was added to a final concentration of 0.02 %. Two liters of the crude enzyme extract were clarified by ultrafiltration (UF) in a tangential flow apparatus using polysulfone membranes with a molecular weight (M.W.) cut-off of 50 kD. The permeate was collected using a Watson Marlow pump and concentrated in a stirred cell UF apparatus using a 76 mm polysulfone membrane with a M.W. cut-off of 10 kD. The final volume of the concentrated enzyme was 150 ml.

E. Enzyme Purification

1. Ion-Exchange Chromatography

A pre-swollen Whatman DEAE-cellulose-ion exchanger was used to purify the crude enzyme extract. The ion exchanger (125 grams) was activated by suspension in 0.2 M pH 8.0 Tris-HCl buffer for 15 minutes with slight stirring and then allowed to settle after which the buffer was decanted. The same procedure was repeated with the pH adjusted to 8.0 and the exchanger was allowed to settle for 15 minutes before decanting off the buffer. The exchanger was washed with 20 L of 0.02 M pH 8.0 Tris-HCl buffer in order to reduce the concentration from 0.2 to 0.02 M. It was then packed in a 23.5 X 4.2 cm. column and equilibrated by continuous washing using 2 L of the buffer solution at 5°C.

Fifty-five ml of the concentrated enzyme were passed through the exchanger at a rate of 1.5 ml/min. The column was washed with 800 ml of pH 8.0 Tris-HCl buffer (0.02 M) at the same flow rate (1.5 ml/min) to completely remove the unadsorbed protein fraction from the column. The adsorbed proteins were removed from the column by salt gradient elution; the final salt concentration of the gradient was 1.0 M NaCl. One hundred fractions (10 ml each) were collected using a Cygnet fraction collector at a flow rate of 0.8 ml/min. The protein profile was determined in terms of the absorbance at 280 nm using a Hitachi UV-VIS spectrophotometer. Mannanase activity was determined for each fraction. The fractions with substantial mannanase activity were pooled and concentrated again to 20 ml in a stirred cell UF apparatus using polysulfone membranes with M.W. cut-off of 10 kD.

2. Gel Chromatography

Sephadex G-75 was swollen for 5 hours in a 20 mM, pH 7.5 phosphate buffer at 100°C, degassed prior to packing in a 55 x 2.2 cm column and then equilibrated in the buffer at 5°C.

The concentrated enzyme (15 ml) which was pooled from DEAE-cellulose fractions was introduced on top of the column and eluted at the rate of 0.25 ml/min. Forty 5-ml fractions were collected using a Cygnet fraction collector and the protein and mannanase elution profiles were determined.

The enzyme activity and protein concentration were determined for each purification step. Protein was analyzed by the Lowry method (6).

3. Electrophoresis

The purity of the enzyme and the number of protein subunits were determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) using a 8 x 10 cm Bio-Rad electrophoretic cell. A 12.5 % separating gel and 5 % stacking gel were prepared and allowed to polymerize in the cell. Fifty µl of sample was applied and the sample was allowed to migrate until the tracking dye reached the bottom of the gel. Then the gel was stained for 2 hours followed by destaining for 24 hours.

Molecular weight (MW) standards in the range 10-70 kD were used. The relative mobility of the standards was determined and plotted against the logarithm of molecular weight (MW); the MW of the sample was determined by interpolation.

F. Enzyme Characterization

The optimum pH for mannanase activity was determined in the pH range 2-10 using as buffers 0.02 M glycine-HCl, 0.02 M phosphate buffer and 0.02 M NH_4Cl-NH_4OH in the pH ranges 2 - 3 , 4 - 8 and 9 - 10, respectively.

The optimum temperature for enzyme activity was determined in the range 30 - 65°C for 30 minutes. The stability of the enzyme at different temperatures was also

determined in the range 30 - 60°C. Samples were obtained every 10 minutes in the first hour, every 30 minutes in the second hour and every hour in the last three hours of incubation.

G. Determination of Kinetic Parameters

Two types of galactomannans were used in the kinetic experiments, namely locust bean gum and coconut galactomannan ("makapuno" or sport variety). Kinetic experiments were done at 30°C. The substrate concentrations used were 10, 20, 40, 60, 80, and 100 mM. Samples were obtained every 10 minutes for locust bean gum galactomannan and every 2 minutes for coconut galactomannan. Values of the Michaelis-Menten parameters K_m and V_m were determined.

H. Hydrolysis of Locust Bean Gum Galactomannan

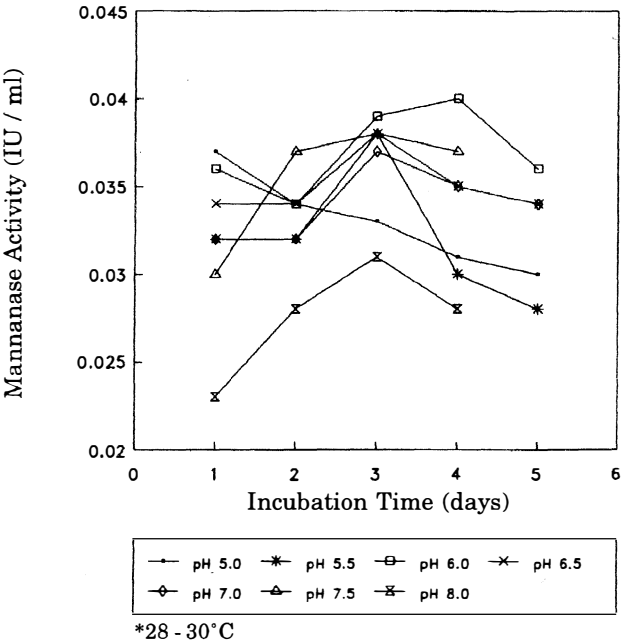
The locust bean gum solution was incubated at 30°C in a Yamato water bath shaker and aliquot samples were obtained after 30 min, 48 hours and 1 week. The sugars present after hydrolysis were determined by High Pressure Liquid Chromatography (HPLC).

Results and Discussions

A. Optimization of Fermentation Parameters

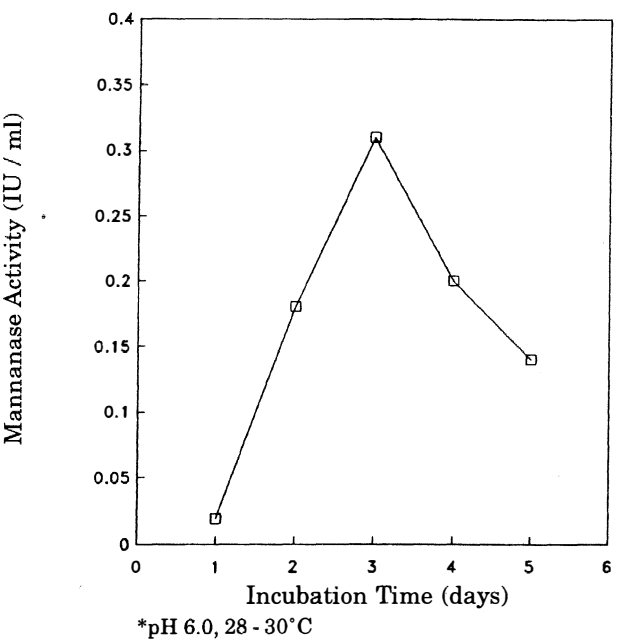
The optimum pH for mannanase production in shake-flask culture was found to be pH 6.0 as shown in Figure 1. The time profile of mannanase production in a stirred-tank batch fermenter is presented in Figure 2; mannanase production was maximal on the third day of fermentation. The fact that the time profiles for enzyme production at pH 6.0 are different as shown in Figures 1 and 2, may be explained by the different fermentation set-ups used. The much higher enzyme activities obtained using the stirred-tank fermenter (Figure 2) was due to the more efficient aeration and agitation for the fermenter compared to the shake-flask culture (Figure 1). Visual examination of the coconut endosperm residue substrate revealed that the particle size decreased after three days of fermentation. However, cellulose was found to be absent in the extract and indicates that the cellulosic component of the residue was largely undergraded. Balasubramaniam (7) had earlier reported that 13 % of the total polysaccharides in the coconut endosperm is cellulose.

The effect of temperature and pH on mannanase activity in the crude enzyme extract was determined. Initial assays were done at 30°C at pH 4.8; these conditions were adapted from a previous study (3) using a species of *Micrococcus*. The optimum temperature and pH would not be expected to be the same for *Pseudomonas* sp. which was used in the present study. Figure 3 shows that the optimum temperature for enzyme activity at pH 4.8 was found to be 40°C. Based on the average deviation, there were no significant differences in the enzymatic activity at 40, 45, and 50°C. The optimal pH range was found to be 7.5 - 9.0 at 40°C as shown in Figure 4 with the indicated error bars for the experimental data points. On this basis, and in order in minimize adverse effects of a very alkaline medium the enzyme assays were done at pH 7.5 and 40°C.



*28 - 30°C

Figure 1. Mannanase production in shake-flask culture of *Pseudomonas* sp. at different pH values*



*pH 6.0, 28 - 30°C

Figure 2. Time profile of mannase production by *Pseudomonas* sp. in stirred-tank (batch) fermenter*.

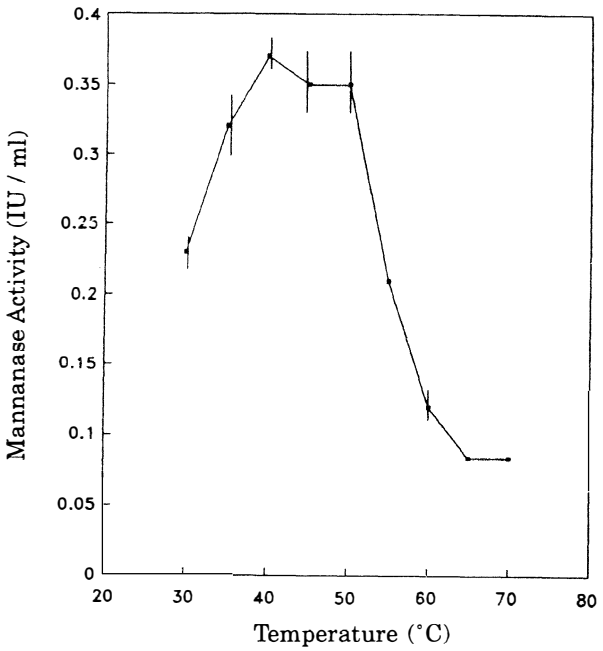


Figure 3. Effect of temperature on mannanse assay at pH 4.8.

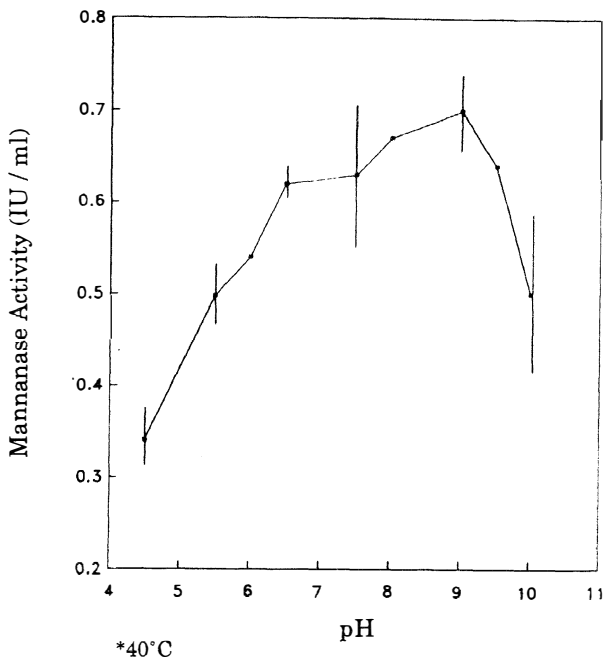


Figure 4. Effect of pH on mannanase assay*.

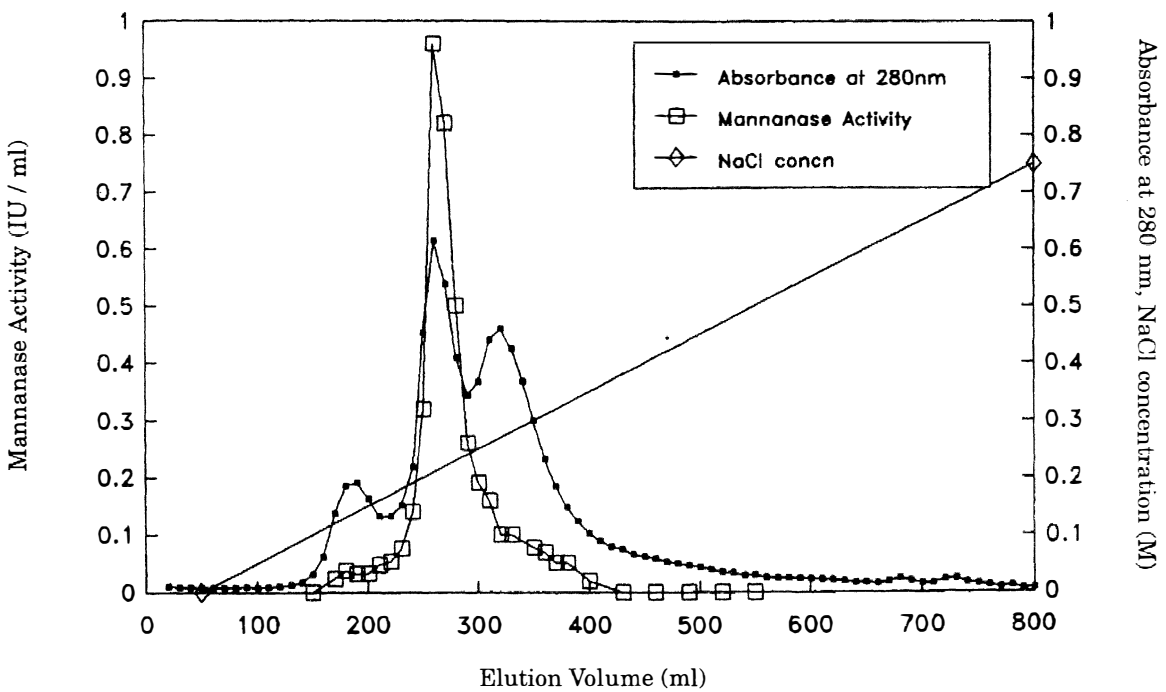


Figure 5. Ion exchange chromatography of mannanase from *Pseudonomas* sp.

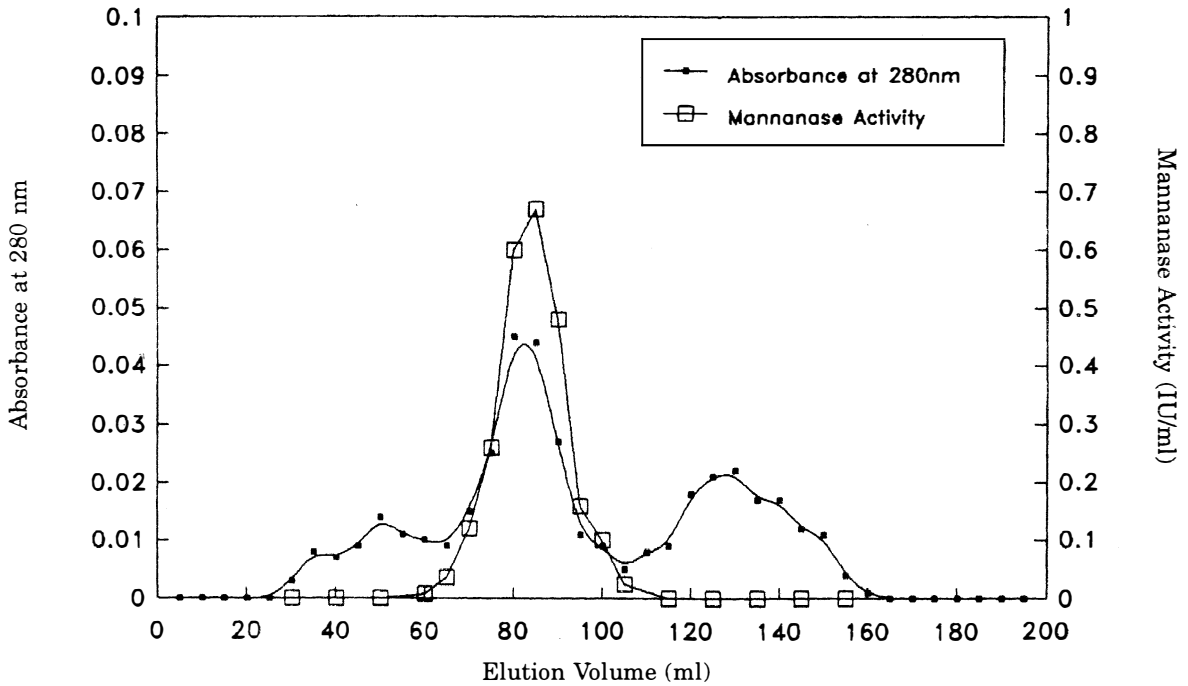


Figure 6. Elution profile of mannanase in Sephadex G-75.

B. Purification of Mannanase

The mannanase, which had been produced by fermentation, was first purified by DEAE-cellulose chromatography at pH 8.0. The elution profile showed only one mannanase fraction (Figure 5). The next purification step (Sephadex G-75 gel chromatography) also gave one mannanase fraction as shown in Figure 6. The enzyme purification results are summarized in Table 1. Fifty eight percent enzyme recovery and 12.3-fold purification were obtained; the latter was calculated from the ratio of the specific activity values.

Only one electrophoretic band under subunit- dissociating conditions using SDS-PAGE (Figure 7) was observed after gel chromatography. This means that the enzyme was pure and consists of only one subunit.

The calibration curve for SDS-PAGE is shown in Figure 8. Seven standards were used with molecular weight (MW) values ranging from 10 to 70 kD. The MW of the enzyme was found to be ~ 20 kD.

C. Characterization of Purified Mannanase

The effect of pH on mannanase activity is summarized in Figure 9; the optimum pH of mannanase was 5.5 at 40°C. However, the mannanase activity error bars indicate that there is no significant differences between the activities at pH 5.0, 5.5 and 6.5. The pH optima then, would be in the pH range 5.0 - 6.5.

Figure 10 shows that the optimum temperature for mannanase activity was in the range 45 - 50°C. The activity-time profiles of the enzyme at different temperatures

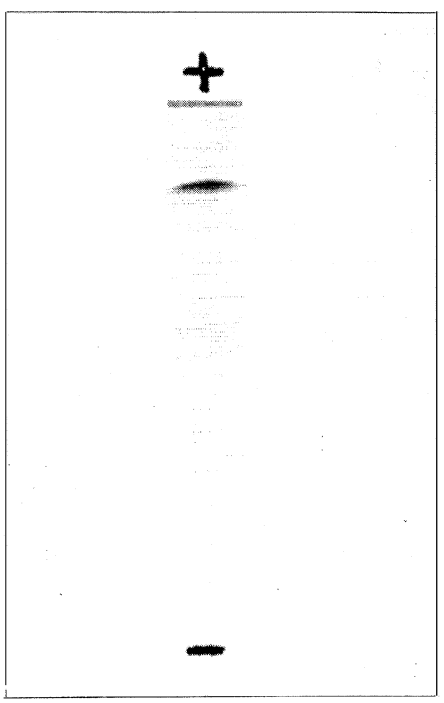


Figure 7. Electropherogram of purified mannanase on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1. Purification of mannanase from *Pseudonomas* sp.

Purification Step	Volume (ml) (IU/ml)	Volumetric Activity (IU/ml)	Total Activity (IU)	Protein concn. (mg/ml)	Total Protein (mg)	Specific Activity (IU/mg)	Fold Purification	Recovery (%)
Crude Extract	55	1.42	78.1	0.54	29.7	2.6	(1)	(100)
Ion-exchange Chromatography (DEAE-Cellulose)	84	0.54	45.4	0.16	13.4	3.4	1.3	58.1
Sephadex G-75 Chromatography	94.5	0.48	45.4	0.015	1.4	32	12.3	58.1

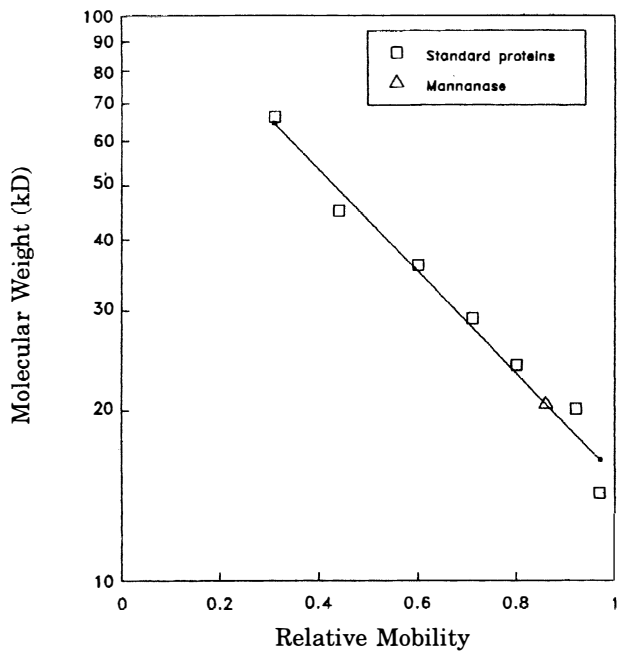
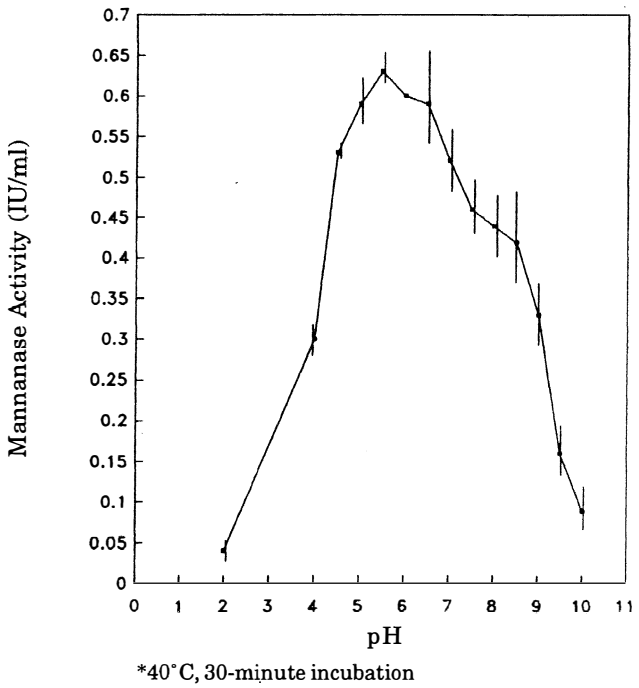


Figure 8. Calibration curve for SDS-PAGE electrophoresis.



*40°C, 30-minute incubation

Figure 9. Effect of pH on activity of purified mannanase*.

are shown in Figure 11. The activity of mannanase was maximal at 50°C for 10 minutes; however, at this temperature the activity gradually decreased with time. The enzyme was rapidly inactivated at 60°C. The limiting activity-time profiles were horizontal during five hours of incubation at 40 and 30°C, indicating long term stability of the enzyme at temperatures less than 40°C.

The effects of metal ion addition on mannanase activity are summarized in Table 2. Among the ions tested only Ca^{++} and K^{+} were found to have no significant effect on enzyme activity. As expected, heavy metal ions, which bind the sulfur atoms of cystine, reduced enzymatic activity. At a concentration of 5 mM, Na^{+} reduced the enzyme activity to 76.9%; this may be the reason for the low retention of enzyme activity (58.1%) during ion exchange chromatography. However, this specific effect on mannanase activity needs further studies and mechanistic explanation.

D. Determination of Kinetic Parameters

The plot of the ratio of substrate concentration to the initial reaction velocity [S]/v against the initial substrate concentration [S] using coconut galactomannan and locust bean gum galactomannan are given in Figures 12 and 13, respectively. The kinetic parameters were calcu-

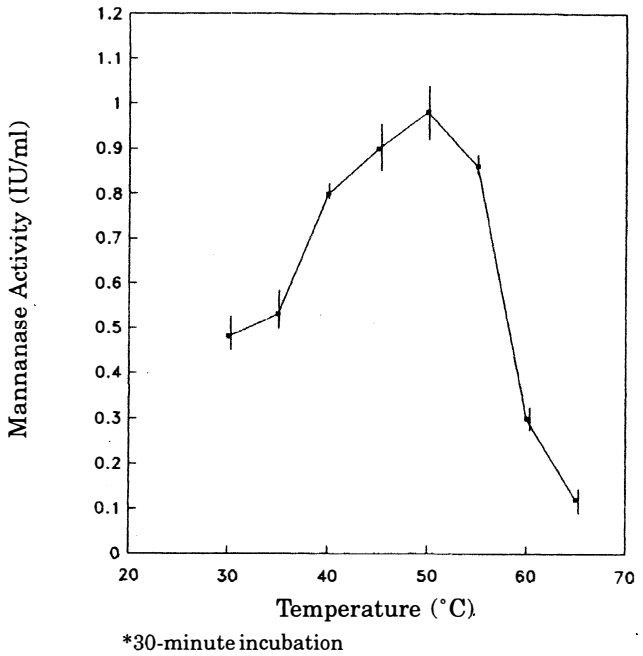


Figure 10. Effect of temperature on activity of purified mannanase at pH 5.5*.

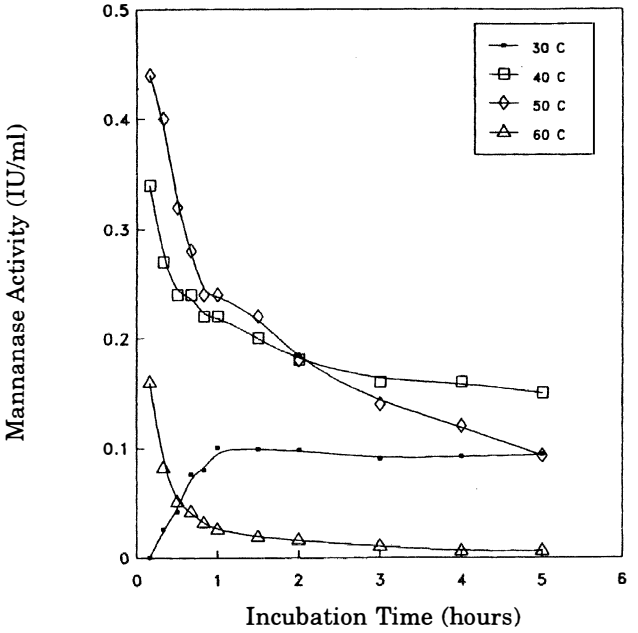


Figure 11. Stability of mannanase at different temperatures.

Table 2. Effect of metal ion addition on mannanase activity *

Ion	Fraction of original enzyme activity
Na^+	0.77
K^+	0.99
Mg^{2+}	0.88
Ca^{2+}	0.99
Ag^+	0.92
Hg^{2+}	0.11
Ni^{2+}	0.88
Pb^{2+}	0.75
Cu^{2+}	0.89
Zn^{2+}	0.85
Fe^{3+}	0.84
Fe^{2+}	0.85
Cd^{2+}	0.76
control (no added metal ion)	1.00

*final metal ion concentration, 5 mM

lated from the slope ($1/V_m$) and intercept (K_m/V_m) of this plot by linear regression. The values of K_m and V_m for coconut galactomannan are 1.9 mM and 0.15 mM/min, respectively; the corresponding K_m and V_m values for locust bean gum galactomannan are 4.9 mM and 0.095 mM/min, respectively. This means that the affinity of the enzyme for coconut galactomannan is 2.6 times greater than that for locust bean gum galactomannan. However, the maximal enzyme velocity was only slightly higher for coconut galactomannan. The kinetic parameters for *Bacillus stearothermophilus* ß-mannanase were determined by the Talbot and Sygusch (8); K_m and V_m were found 9.26 mM and 0.68 mM/min, respectively. However, these K_m and V_m values were determined at the optimum temperature of 70°C for *B. stearothermophilus*. This elevated temperature could explain the higher V_m value for the thermophilic bacterium compared to what was obtained for *Pseudomonas* sp., whose enzyme kinetic parameters were determined at 30°C.

E. Enzymatic hydrolysis of Locust Bean Gum Galactomannan

Figure 14 shows the HPLC chromatograms obtained after hydrolysis of galactomannan from locust bean gum. According to Baker and Whistler (9), locust bean gum galactomannan contains ß-1,4 linked mannose moieties in which an α-D-galactopyranosyl unit is attached at C-6 on every four mannopyranosyl residue. Based on the results, it can be seen that after 30 minutes a trisaccharide and mannooligosaccharides were formed as well as an unknown product; however, no mannose was yet formed. The unknown product did not give exactly the same retention time as mannobiose. Spiking with this disaccharide showed two adjacent peaks. The identity of this sugar, which is either a mono- or disaccharide, still needs to be established. After 48 hours, mannose was produced and the mannose peak height subsequently increased. The trisaccharide that was formed is probably mannotriose based on the fact that its retention time was close to those of raffinose and melezitose which are trisaccharides. Based on the studies of McCleary and Nurthen (10), hy

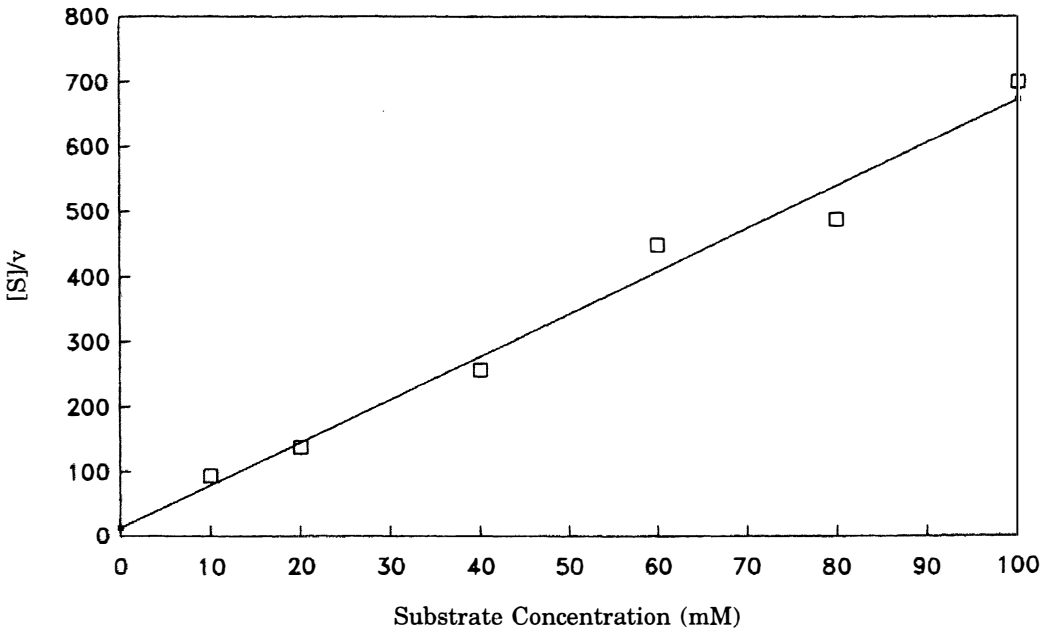


Figure 12. Plot of [S]/v versus [S] for the enzymatic hydrolisis of coconut endosperm galactomannon.

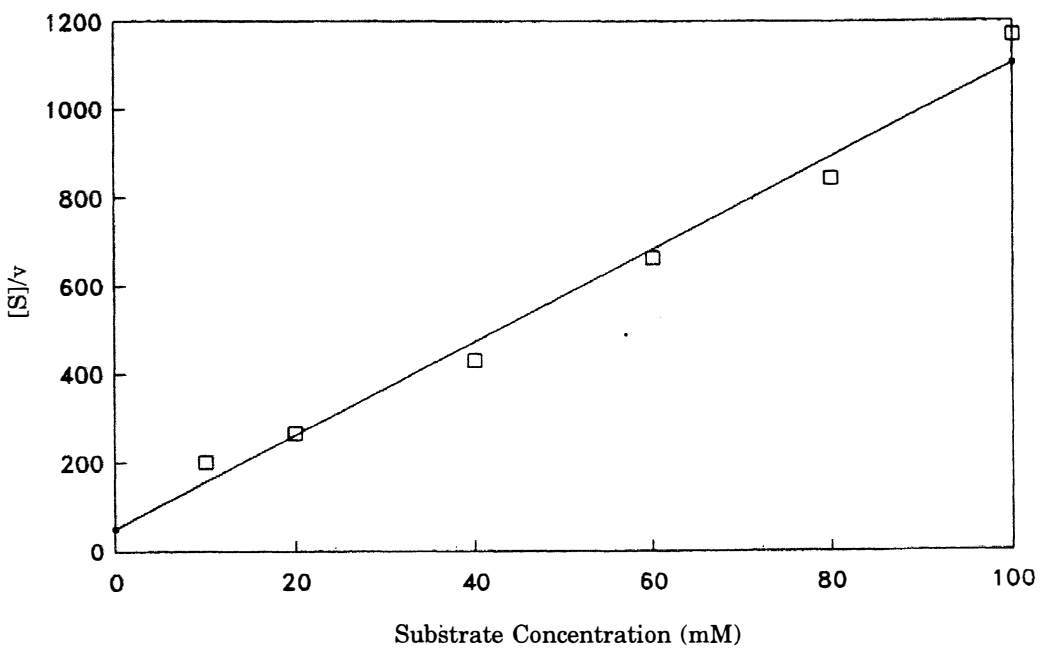


Figure 13. Plot of [S]/v versus [S] for the enzymatic hydrolysis of locust bean gum galactomannan.

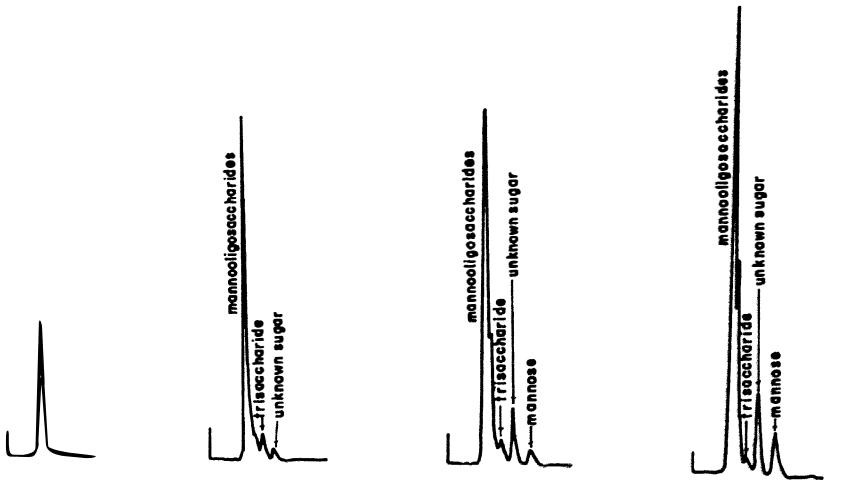


Figure 14. Chromatograms (HPLC) of locust bean gum galactomannan after hydrolysis by *Pseudomonas* mannanase.

drolysis of a water soluble galactomannan from carob gum by *A. niger* ß-D-mannanase gave as major products di-, tri-, tetra-, and octa-saccharides and minor quantities of D-mannose and longer oligosaccharides. The present study gave similar results for the hydrolysis of locust bean gum galactomannan. This indicates that the enzyme is probably an endo-ß-D-mannanase; however, further research is needed in order to establish the mechanism of action of the isolated mannanase.

Conclusion

Mannanase production by *Pseudomonas* sp. in shake flask culture was found maximal at pH 6.0. The enzyme activity of the culture medium in a stirred-tank fermenter peaked during the third day of batch fermentation.

Purification by DEAE-cellulose chromatography (pH 8.0) followed by Sephadex G-75 gel chromatography (pH 7.5) gave an enzyme recovery of 58.1% and 12.3 fold purification. The enzyme consisted of only one protein subunit and the molecular weight was ~ 20 kD by SDS-PAGE.

The optimum pH and temperature for mannanase activity were in the range 5.0 - 6.5 and 45- 50°C (30 minute incubation), respectively. K^+ and Ca^{2+} had no effect on enzyme activity while heavy metal ions reduced activity. Sodium ion reduced enzyme activity by 77%; this could explain the low recovery during ion exchange chromatography.

The K_m and V_m values were 1.9 mM and 0.15 mM/min, respectively, for galactomannan from "makapuno" coconut; the corresponding values were 4.9 mM and 0.095 mM/min for locust bean gum galactomannan.

Enzymatic hydrolysis of the locust bean gum galactomannan gave as major products mannose and mannooligosaccharides; this indicates that the isolated mannanase from *Pseudomonas* sp. is probably an endo-ß-D-mannanase.

Acknowledgements

The authors are grateful to the following: National Research Council of the Philippines (NRCP) for providing thesis support to D. A. Parayno, Prof. M. E. Flavier for the purified galactomannan from "makapuno" coconut, Dr. E. M. T. Mendoza and the UPLB Institute of Plant Breeding for use of the refrigerated centrifuge, UPLB-BIOTECH for use of the fraction collector and cold room, and J. J. S. Fama for valuable assistance.

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