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HYDROLYSIS OF COCONUT ENDOSPERM CARBOHYDRATE BY A MICROBIAL MANNANASE

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ABSTRACT

A crude mannanase was produced by a microbial isolate, which probably belongs to the genus *Micrococcus*, using coconut endosperm fibrous residue as substrate. Enzyme production was maximum at pH 7.5 and after four days of incubation at room temperature (~29°C). The optimal pH and temperature for enzyme assay were 6.4 and 50°C, respectively. The enzyme was stable in the pH range 6.0 - 6.8 and its activity rapidly decreased at a temperature greater than 50°C. It converted 40% of the dry weight of the coconut residue into reducing sugars at pH 6.4 and 50°C after one hour of incubation. Precipitation with ammonium sulfate at 60-70% saturation resulted in a 2.4 - fold purification of the enzyme.

INTRODUCTION

The solid moisture-free endosperm of the coconut (*Cocos nucifera* L.) consists mainly of carbohydrate, triglycerides and protein (1). Carbohydrate constitutes more than 90% of endosperm cell walls and contains polymers of mannose, galactose and glucose (2, 3, 4). Carbohydrate is the main constituent of copra meal, which is the fibrous residue obtained from the traditional copra process, as well as of the coconut aqueous process residue (1).

The complete hydrolysis of coconut endosperm carbohydrate requires the action of one or more enzymes of the beta-mannanase type [endo-β-D mannanase, (1-4)- β-D- mannan mannohydrolase, EC 3.2.1.78]. These enzymes have been detected in microorganisms, plants and animals (5,6). The production of suitable enzymes, preferably by microorganisms, should allow the efficient saccharification and utilization of coconut endosperm cell walls.

The present study was aimed at the optimal production of a microbial mannanase using as substrate the fibrous residue of coconut endosperm. The enzyme produced was characterized in terms of the optimal pH and temperature for the saccharification of coconut endosperm residue.

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MATERIALS AND METHODS

Preparation of coconut endosperm residue. Desiccated coconut meat, which was kindly given by the Red V Coconut Products of Lucena City, was washed exhaustively with tap water and then with distilled water and then dried at 105°C overnight. The dried material was comminuted in a Wiley mill to 100 mesh size or finer. It was extracted with petroleum ether (Mallinckrodt B.P. range 30-60°C) and then exhaustively washed with 70% (v/v) ethanol. The material was analyzed for total nitrogen and fat using AOAC procedures (7) and for total carbohydrate using the anthrone method (8).

Enzyme preparation- The microorganism, which was used as enzyme source, was isolated from the microflora of moist coconut endosperm residue which had been exposed to the atmosphere for two days. The isolation procedure consisted of streaking the microbial sample into sterile potato dextrose agar in a petri dish and transferring a single colony into each agar slant. The microbial isolate which was used in the present study was earlier found to produce the highest hydrolytic activity on coconut endosperm residue (M.C. Jorge II and E.J. del Rosario, unpublished data). The isolated microorganism was grown on a basal medium containing 0.5% (w/v) coconut residue (150 mesh size particles) as carbon source and the following weights of nutrients per liter of solution: KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.4 g; $MgSO_4.7H_2O$, 0.3g; $CaCl_2$, 0.3 g; Na_2HPO_4, 1.0g; and NaH_2PO_4, 1.0 g; The inoculated medium was placed in an Erlenmeyer flask and agitated in a rotary shaker at room temperature (~29°C). Aliquots were taken from the fermented medium after two, three, four and five days of incubation. The liquid was filtered and then centrifuged at 5000 rpm for ten minutes and the supernate was used for enzyme assay and protein determination.

Enzyme assay and characterization- Coconut endosperm fibrous residue, which was prepared as earlier described, was used as enzyme substrate and was added to the assay medium to a final solid-to-liquid ratio of 10% (w/v). The assay medium was prepared by adding equal volumes of crude enzyme extract and 0.05 M sodium citrate buffer (pH 4.8). The assay mixture was incubated at 30°C for an hour in an Eberbach water bath-shaker and then placed in a boiling water bath for 30 min. Reducing sugars produced from hydrolysis were determined by the dinitrosalicylate method (9) using glucose as standard. Soluble protein was analyzed by the Lowry method (10). Adjustments of pH were done using a Radiometer Model PHM 62 pH meter. Fractional precipitation of the enzyme with ammonium sulfate (Baker 'Analyzed' reagent grade) was done after concentrating the crude enzyme extract six-fold through an Amicon PM10 ultrafiltration membrane.

RESULTS AND DISCUSSION

The following is a partial analysis of coconut endosperm residue after exhaustive washing with water, petroleum ether and 70% (v/v) ethanol:

Moisture	-	18.44%
Ash	-	1.79%
Crude fat	-	0.58%
Crude protein	-	1.15%
Total carbohydrate		91.8 %

The percentages of ash, crude fat, crude protein and total carbohydrate were calculated on a moisture-free basis and were the results of two separate trials with two determinations per trial. The carbohydrate content obtained in this study, namely 92.3% on a water- and oil-free basis, is identical to that previously reported by Rajasekharan (11) and Hagenmaier *et al.* (3). The carbohydrate in coconut meat residue was found to consist of polymers of mannose, galactose and glucose in agreement with previously reported results. Balasubramaniam (2) had shown that galactomannans constitute 61% of the total polysaccharides in the mature kernel, followed by mannan (26%) and cellulose (13%). Treatments of coconut residue with 5% (w/v) sulfuric acid in the autoclave at 126°C, 3/1 acid-to-substrate ratio (v/w) for one hour resulted in 55.4% conversion of the initial weight of coconut residue into reducing sugars. The resulting sugars in the acid hydrolysate consisted of 44% mannose, 18% galactose, 11% glucose and 21% disaccharides and oligosaccharides (4). Complete acid hydrolysis of the carbohydrate in coconut residue was reported by Hagenmaier *et al* (3) to yield 76% mannose, 21% glucose and 5% unidentified. The above-mentioned results prove that mannose is the predominant monosaccharide of coconut endosperm residue followed by galactose and glucose and the predominant polysaccharides are heteromannans. The crude enzyme preparation, which was used in the present study, converted 40.4% of the initial dry weight of coconut residue into reducing sugars at pH 6.4 and 50°C after one hour of incubation. This shows that the crude enzyme extract had mannanse activity.

The microorganism, which was used for mannanase production, is unicellular and flocculent. It is aerobic and gram-positive and consists of colorless, non-motile spherical cells. It appeared white and smooth on agar slant and formed pellicles in nutrient broth. Microscopic estimation of cell dimensions using a micrometer slide indicated that the cells are less than two

microns in size. These preliminary observations show that the microorganism is a bacterium and probably belongs to the genus *Micrococcus*.

The microbial production of crude mannanase using coconut endosperm residue as sole carbon source was studied in terms of the optimal pH as shown in Figure 1. Using coconut residue also as enzyme substrate, a maximal value of enzyme activity equal to 0.61 I.U./mg protein was obtained at pH 7.5 after four days of incubation at ~29°C. One international unit (I.U.) of enzyme activity is defined as the amount of enzyme which liberates one micromole of reducing sugars (expressed as glucose) per minute at 30°C. The specific activities at pH 8.0 and 6.0 were approximately 60% and 40%, respectively, of the activity at pH 7.5. The optimal length of incubation for mannanase production at pH 7.5 was four days as shown in Figure 2. A specific activity of 0.132 I.U./mg protein was obtained after four days of enzyme production which abruptly decreased to 0.09 I.U./mg protein on the fifth day.

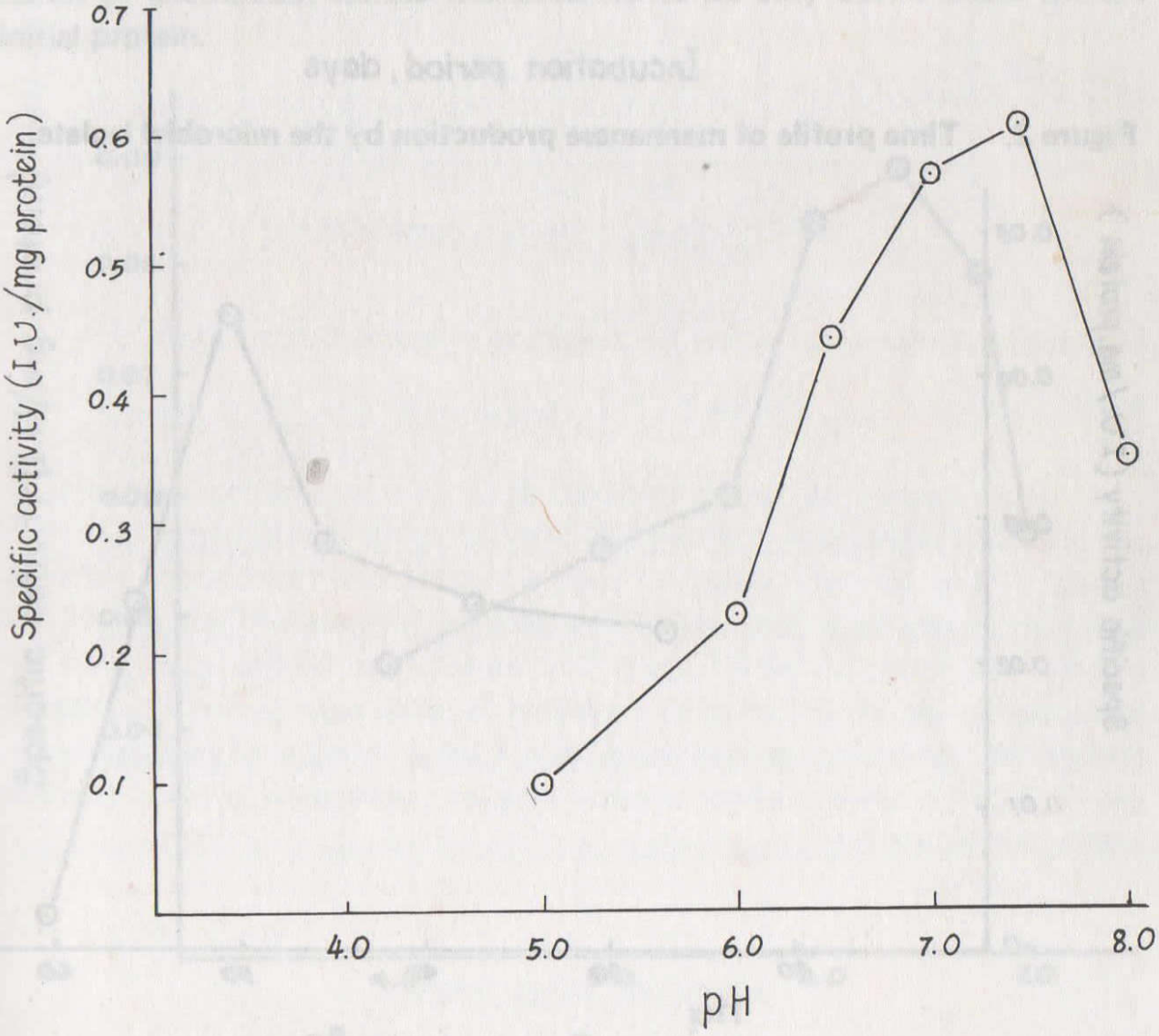

Figure 1. pH dependence of enzyme activity during mannanase production.

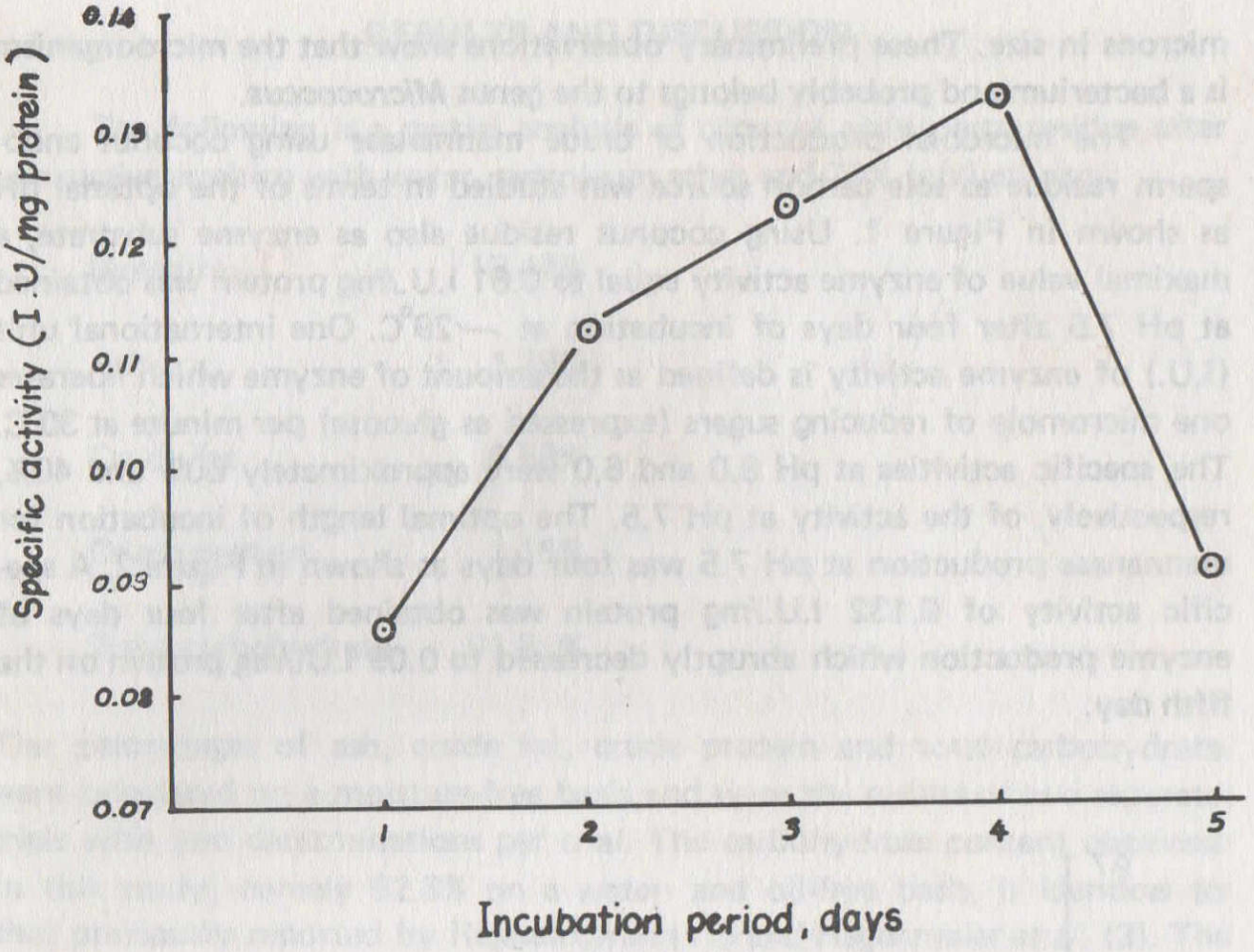

Figure 2. Time profile of mannanase production by the microbial isolate.

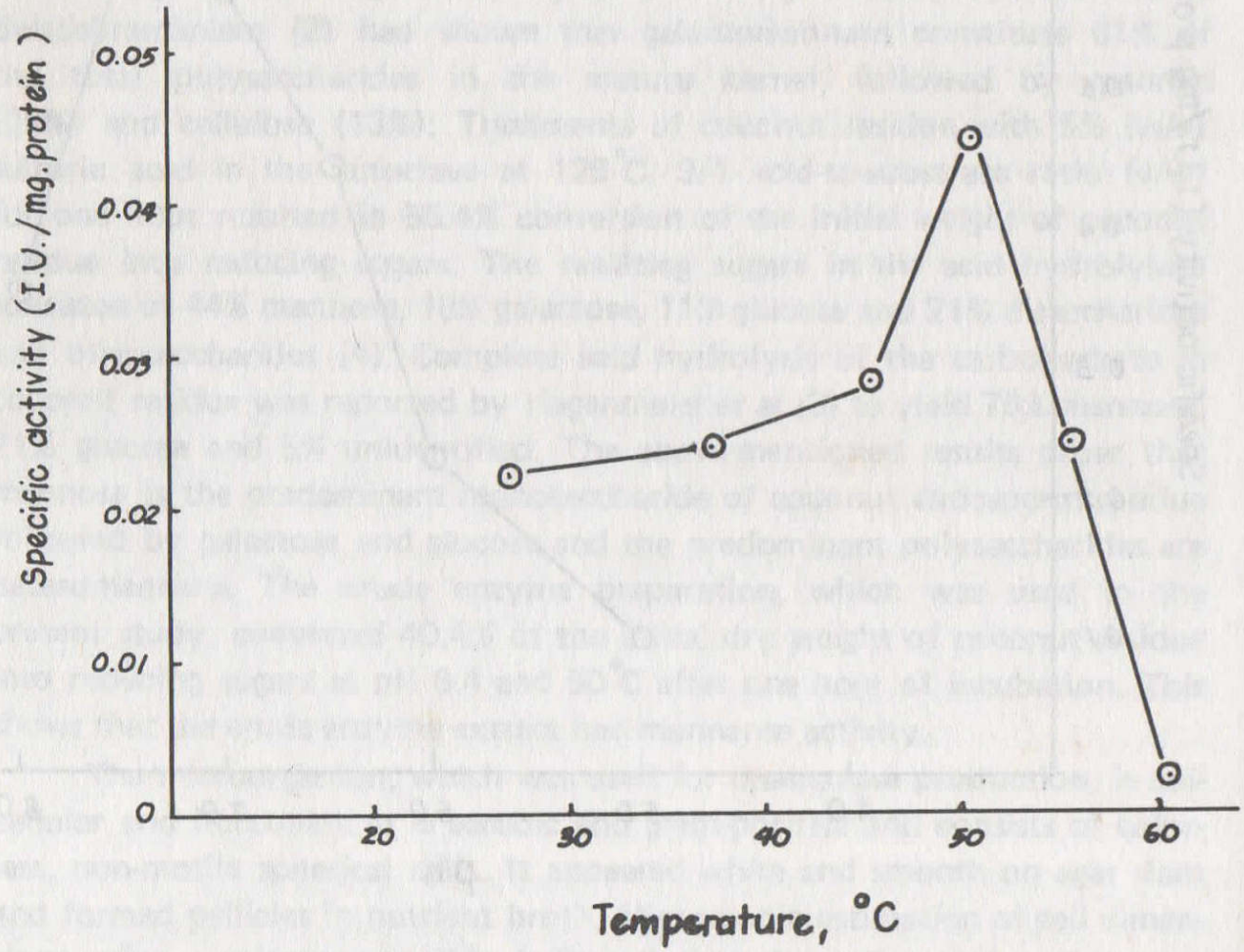

Figure 3. Effect of temperature on mannanase activity at pH 5.

The effect of temperature on the activity of mannanase at pH 5.0 is presented in Figure 3. The enzyme activity was maximal at 50°C and decreased to almost zero at 60°C. The optimal temperature observed in the present study is identical to that reported by Nakajima *et al* (12) for an endo-beta-mannanase from a soil bacterium. However, for fungal beta-mannanases an optimal temperature of 60°C was reported by Reese and Shibata (13) and 65°C by Eriksson and Winnel (14).

The pH dependence of the specific activity of the crude mannanase at 50°C is shown in Figure 4. Maximal enzyme activity was observed at pH 6.4. The optimal pH range is relatively narrow in contrast to the broad pH range which was found for two fungal beta-mannanases by Reese and Shibata (13).

Results of the fractional precipitation of the crude enzyme extract by ammonium sulfate are given in Figure 5. Mannanase activity was highest, namely 1.5 I.U./mg protein at 60-70% ammonium sulfate saturation. This specific activity is 2.4 times greater than that obtained for the crude enzyme preparation. However, protein recovery in the precipitate at the optimal saturation of ammonium sulfate was observed to be only 23.4% based on the initial protein.

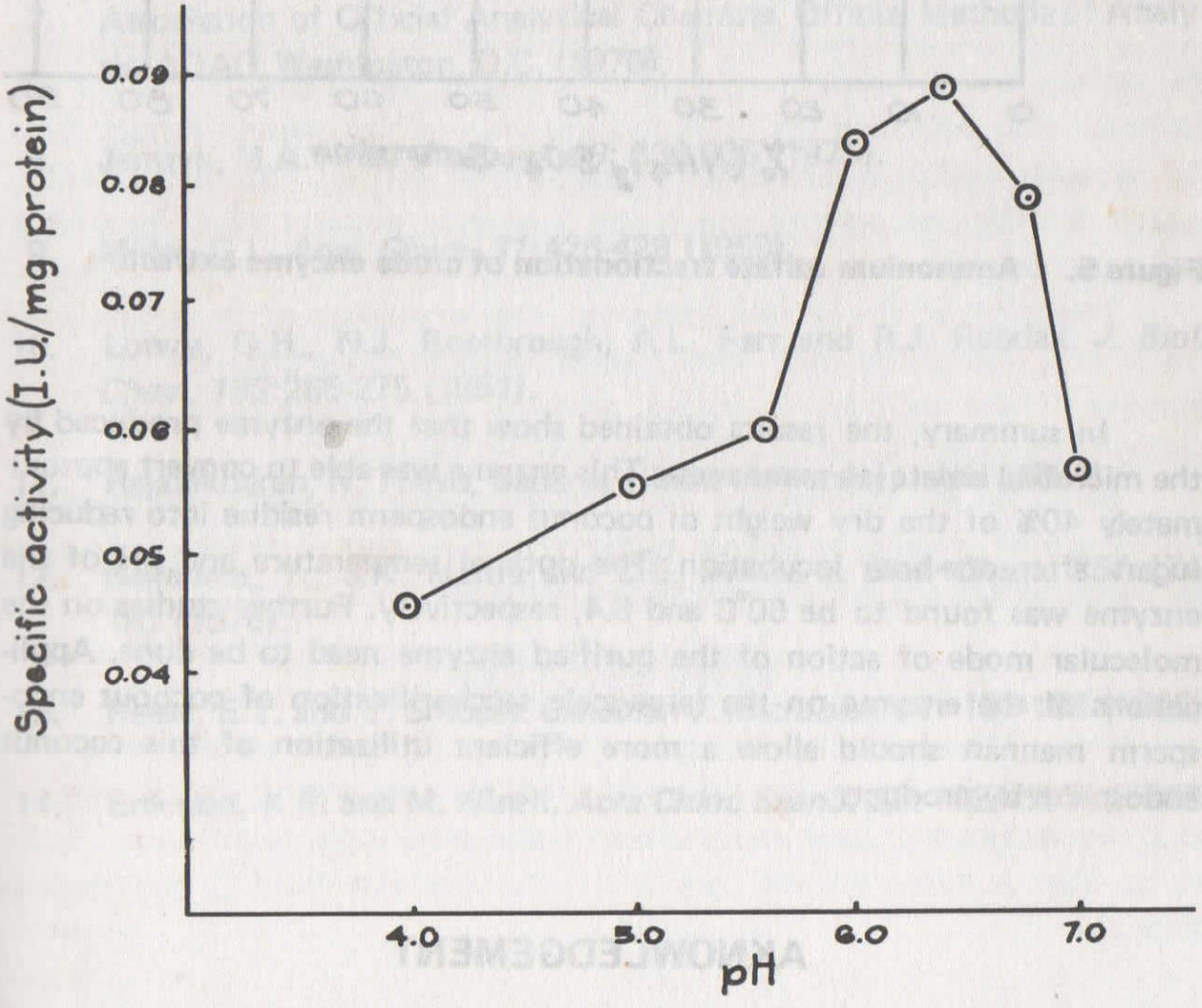

Figure 4. Effect of pH on mannanase activity at 50°C.

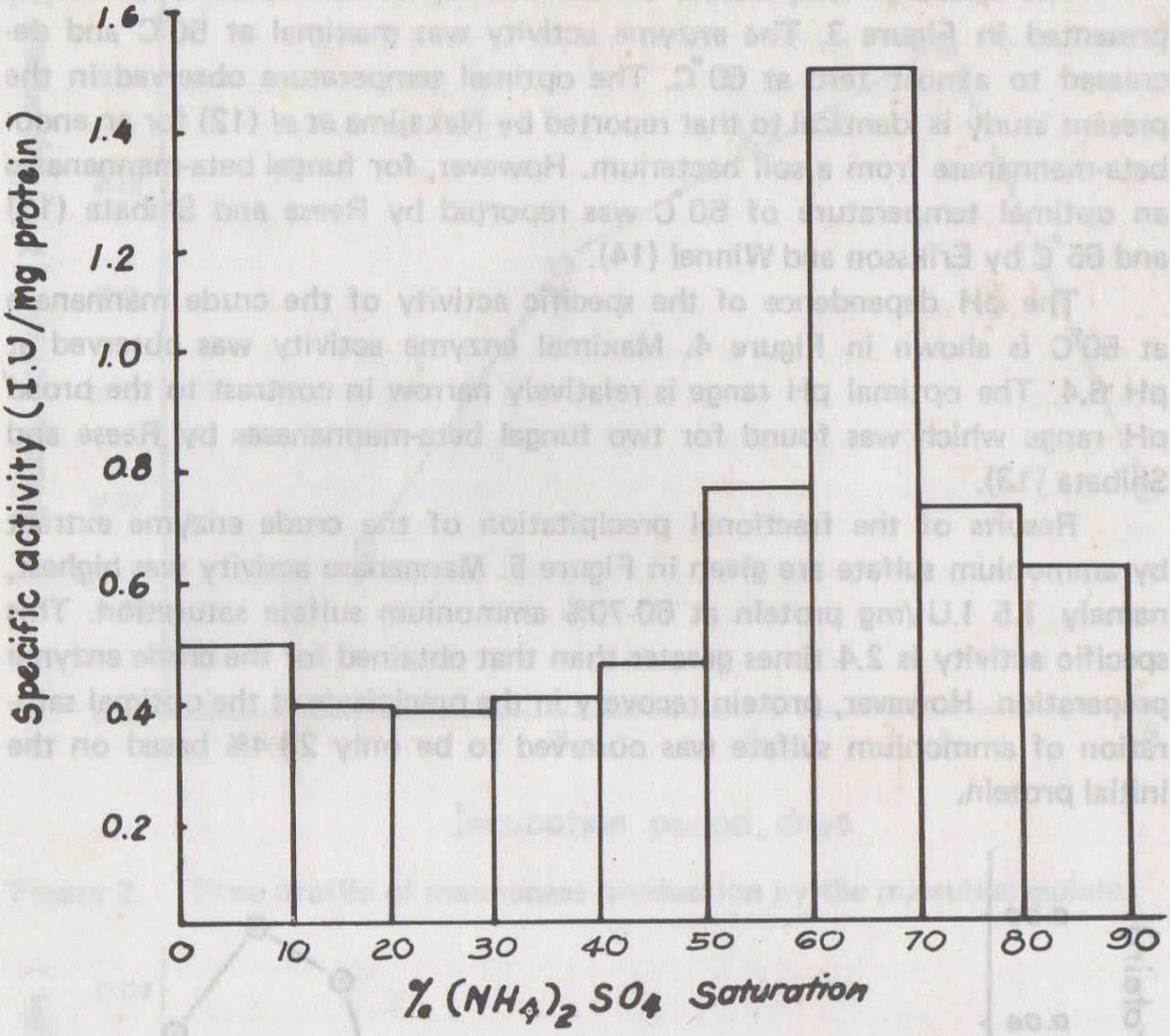

Figure 5. Ammonium sulfate fractionation of crude enzyme extract.

In summary, the results obtained show that the enzyme produced by the microbial isolate is a mannanase. This enzyme was able to convert approximately 40% of the dry weight of coconut endosperm residue into reducing sugars after one-hour incubation. The optimal temperature and pH of the enzyme was found to be 50°C and 6.4, respectively. Further studies on the molecular mode of action of the purified enzyme need to be done. Applications of the enzyme on the large-scale saccharification of coconut endosperm mannan should allow a more efficient utilization of this coconut endosperm by-product.

AKNOWLEDGEMENT

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