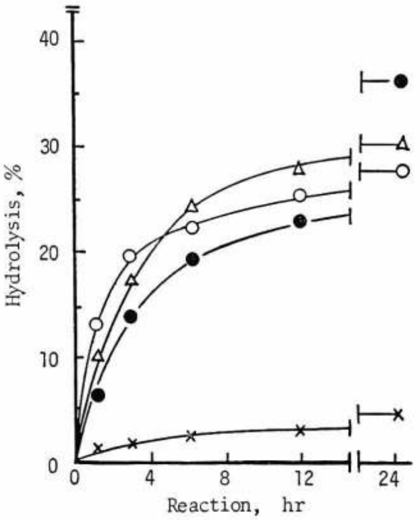

FIG. 11. Time Course of Hydrolysis of Several Mannans by Bacterial Mannanase.

6 ml of 0.5% mannan solution containing M/50 acetate buffer, pH 6.0, reacted with 1 ml of enzyme (activity, 1 U/ml).

○—○, soybean; ●—●, ceffee bean; △—△, konjak; ×—×, guar gum.

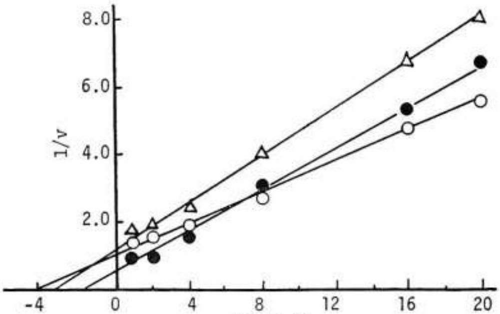

FIG. 12. V_{max}- and Km-Values of Bacterial Mannanase on Several Mannans.

○—○, coffee bean; ●—●, soybean; △—△ konjak.

calculated to be 0.28%, 0.59% and 0.34%, respectively.

5. *Action patterns of the mannanase*

The thin-layer chromatograms which were obtained with the mannan hydrolysates after a twenty-four hour incubation with the enzyme (Fig. 11) are presented in Fig. 13. Of the hydrolysis products from soybean galactomannan, the oligomers symbolized as 2 and 3 were mannobiose and mannotriose, respectively, from the facts that their *Rf* values on the thin-layer chromatogram were consistent with those of the authentic samples and that they yielded only mannose in the theoretical quantity on hydrolysis by either β-mannosidase or acid. The spots numbered 2′ of the hydrolysate of konjak glucomannan was an oligomer consisting of glucose and mannose. Number 3 of the hydrolysate was mannotriose.

The main products of coffee bean galactomannan were manno-triose, -tetraose and -biose in the decreasing order (Fig. 14). They were identified by hydrolysis with β-mannosidase and acid as described above. The spots marked A and B in Fig. 14 were hetero-

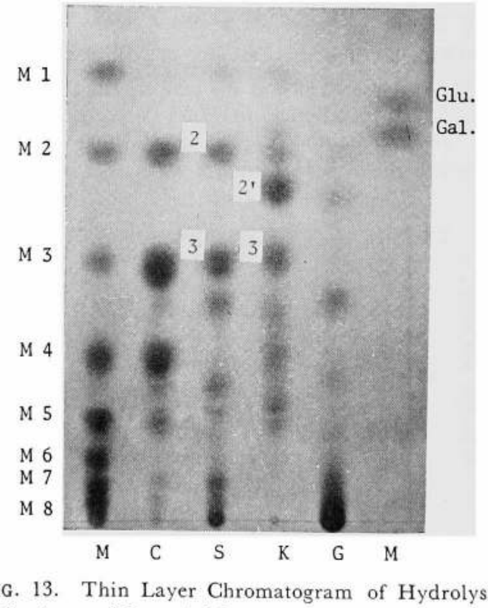

FIG. 13. Thin Layer Chromatogram of Hydrolysis Products of Several Mannans by the Mannanase.

M, markers: M 1～M 8, mannose～mannooctaose; Glu, glucose; Gal, galactose; C, coffee bean galactomannan; S, soybean galactomannan; K, konjak glucomannan; G, guar gum galactomannan; locator, anis-aldehyde-sulfuric acid.

oligomers consisting of galactose and mannose, the ratio of galactose to mannose being 1 : 3 for A and 1 : 4 for B (Fig. 15).

The action mode of the enzyme on several β-1,4-mannosidic oligomers was investigated. The substrates were prepared from a partial hydrolysate of coffee bean galactomannan with the bacterial mannanase. A 2 g of galac-

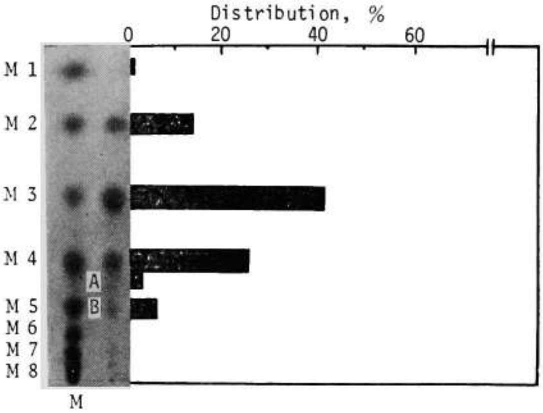

FIG. 14. Quantitative Thin Layer Chromatograms of Hydrolysis Products of Coffee Bean Mannan by the Mannanase.

Hydrolysis degree, 36%; M, markers; M 1∼M 8, mannose∼mannooctaose; locator, anis-aldehyde-sulfuric acid.

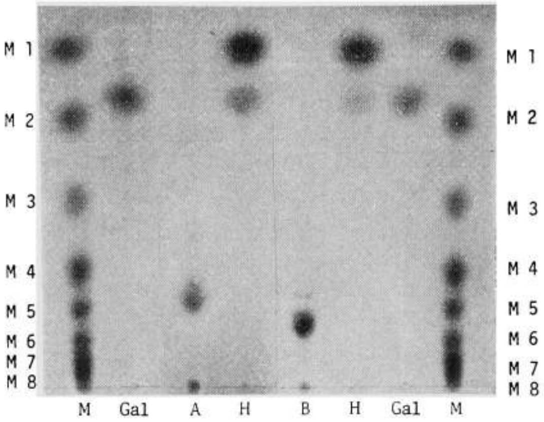

FIG. 15. Constituent Sugars of Heterooligosaccharide A and B Formed in Digestion of Coffee Bean Galactomannan with Bacterial Mannanase.

M, markers; M 1∼M 8, mannose∼mannooctaose; Gal, galactose; A, oligosaccharide A in Fig. 14; B, oligosaccharide B in Fig. 14; H, hydrolysate of oligosaccharide A and B with 1 N HCl, 2 hr; locator, anis-aldehyde-sulfuric acid.

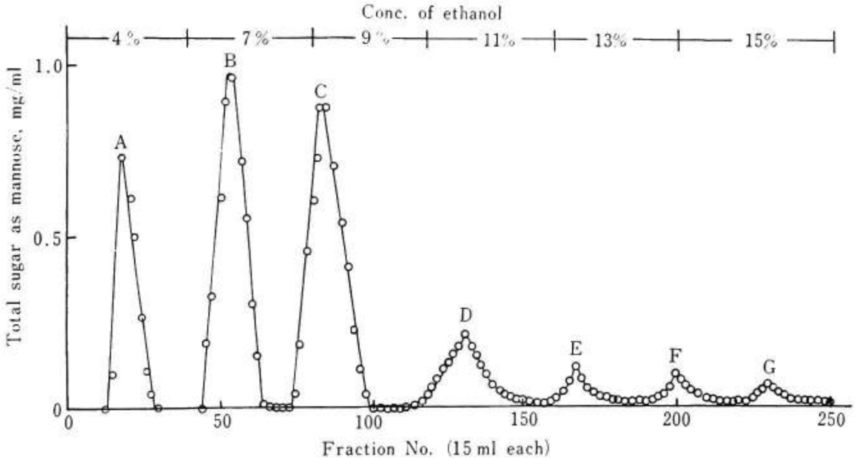

FIG. 16. Isolation of Mannooligosaccharides by a Charcoal Column.

Column, 1.8×45 cm; charcoal, activated charcoal for chromatography (Wako Pure Chemical Industries, Ltd.); oligosaccharides applied, 20 ml (reducing sugar as mannose, 144 mg; total sugar after acid hydrolysis, 650 mg), mannose (2.5 mg) appeared in the breakthrough solution, flow rate, 50 ml/hr; 20°C.

tomannan was suspended in 200 ml of M/50 acetate buffer, pH 6.0, containing 50 units of the mannanase and the suspension was incubated at 40°C. Four hours later, the suspension (hydrolysis degree, 15%) was mixed with two volumes of ethanol and the supernatant was concentrated under reduced pressure. An adequate quantity of the concentrate was subjected

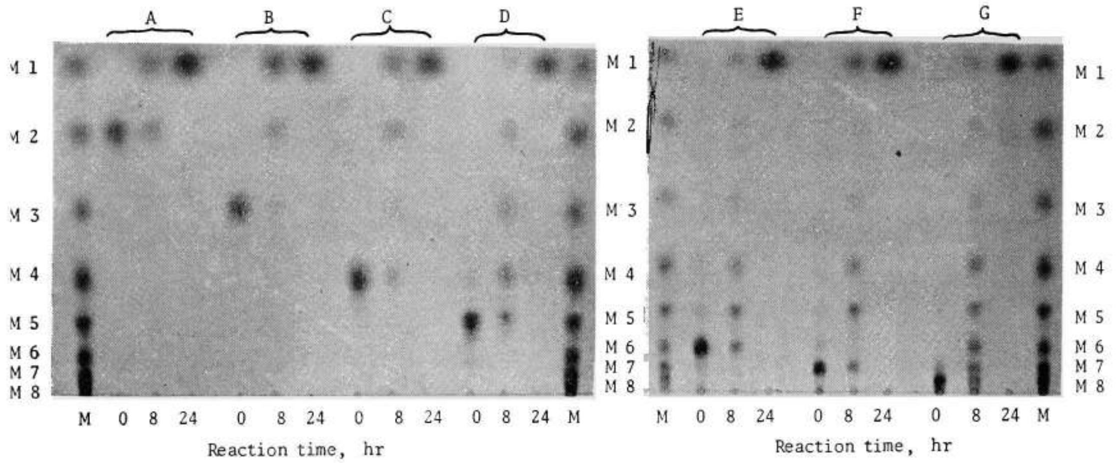

FIG. 17. Thin Layer Chromatograms of Hydrolysis Products of Several Mannooligosaccharides with β-Mannosidase of *Rhizopus niveus.*

The oligosaccharide, 2.0 μmoles, was reacted with 2 ml of enzyme solution (activity, 1.0 U/ml) containing M/50 acetate buffer, pH 5.0, at 40°C; M, markers; M 1∼M 8, mannose∼mannooctaose; locator, anis-aldehyde-sulfuric acid.

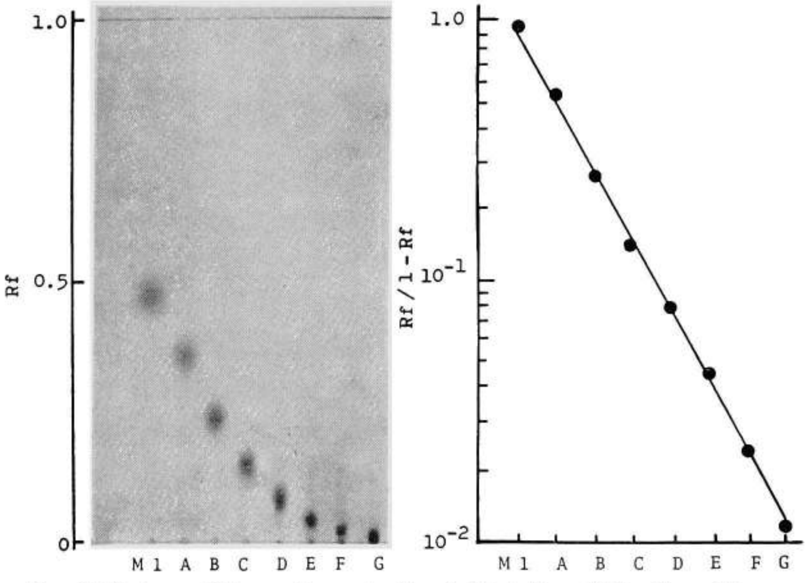

FIG. 18. *Rf* Values of Mannooligosaccharides Isolated from Coffee Bean Mannan.

M 1, mannose; locator, anis-aldehyde-sulfuric acid.

to a charcoal column chromatography (Fig. 16) and each fraction was chromatographed by the thin layer method. As shown in Figs. 17 and 18, each fraction was found to contain a sole sugar component consisting only of mannose. Polymerization degree of the mannooligomers was determined by the graphical method reported by French[20)] (Fig. 18) and from the ratio of reducing sugar to total sugar content (Table II).

To 0.5 ml of 1.0% solution of each of mannooligomers was added 0.5 ml of the mannanase solution (total activity, 0.5 units) in M/50 acetate buffer, pH 6.0, and at certain intervals of time

TABLE II. RATIOS OF REDUCING SUGAR[a)] TO TOTAL SUGAR[b)] CONTENT OF THE ISOLATED MANNOOLIGOMERS

Symbols in Fig. 18	Ratio
A	2.02 (M 2)
B	3.00 (M 3)
C	3.90 (M 4)
D	5.05 (M 5)
E	5.85 (M 6)
F	6.75 (M 7)
G	7.85 (M 8)

[a), b)]; determined by the Somogyi-Nelson method and by the phenol-sulfuric acid method, respectively.

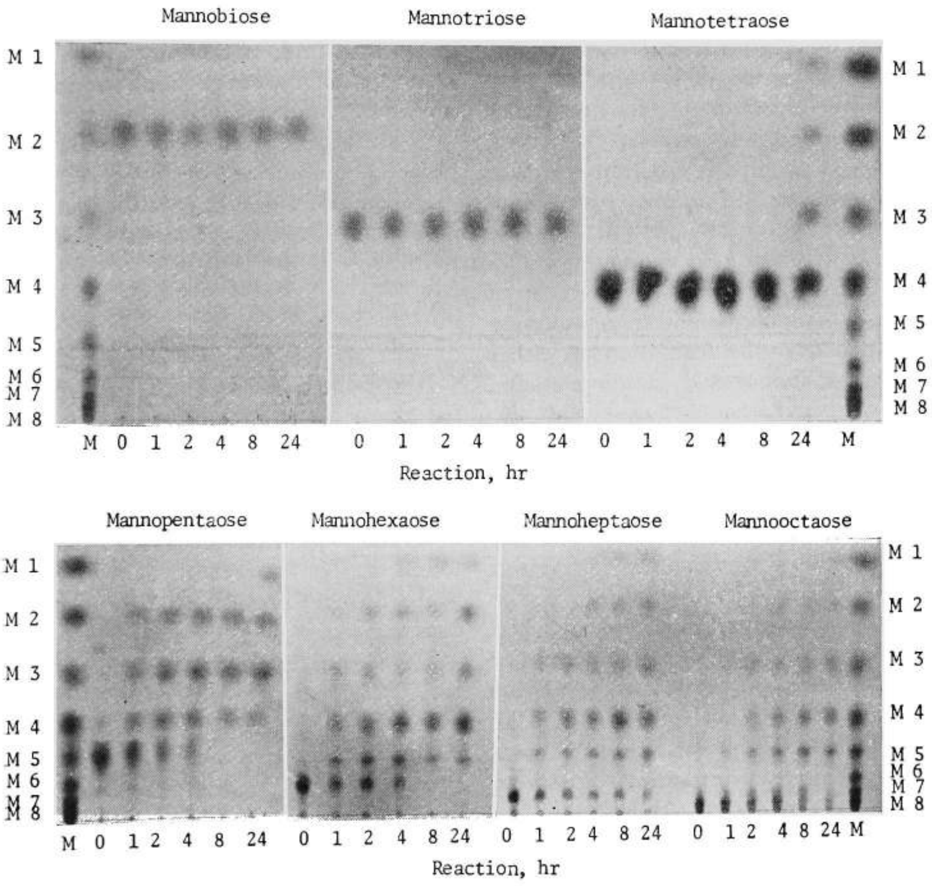

FIG. 19. Action of the Mannanase on Several β-1,4-Mannosidic Oligomers.

M 1 ~ M 8, mannose ~ mannooctaose as the markers; locator, anis-aldehyde-sulfuric acid.

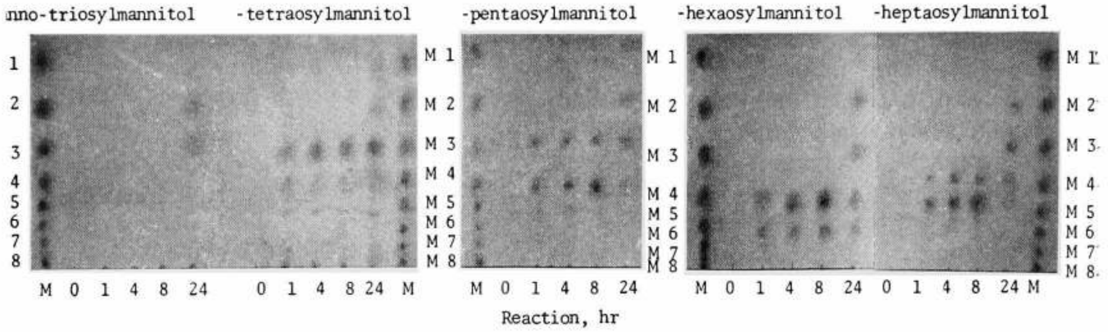

FIG. 20. Action of Bacterial Mannanase on Manno-triosyl-, -tetraosyl-, -pentaosyl-, -hexaosyl- and -heptaosyl-mannitol.

M, markers: M 1 ~ M 8, mannose ~ mannooctaose.

t 40°C an aliquot of the reaction mixture vas subjected to thin-layer chromatography. n Fig. 19 are presented the results, showing hat the bacterial mannanase does not attack }-1,4-mannobiose and mannotriose. However, nannotetraose was hydrolyzed to produce nannose, mannobiose and mannotriose. Man-iopentaose was hydrolyzed in the ways to produce either mannose and mannotetraose or nannobiose and mannotriose. In the case of nannohexaose, mannobiose and mannotetraose ather prevailed over the formation of either nannotriose or mannose and mannopentaose. Mannoheptaose and mannooctaose were also iydrolyzed to mannotetraose and mannopen-aose, the former being produced in larger juantities.

The action pattern of the enzyme was also nvestigated on several mannooligosylmannitols vhich were prepared by reduction of the nannooligomers with sodium borohydride. The hydrolysis products were detected by etrazolium blue whose reaction was positive only for reducing sugars. As shown in Fig. 20, he enzyme produced mannotriose and man-iobiose from mannotriosylmannitol, indicating hat the enzyme hydrolyzed the substrate to orm either mannobiose and mannosylmannitol or mannotriose and mannitol. The main diges-ion products by the enzyme were as follows: Mannotriose from mannotetraosylmannitol, manno-triose and -tetraose from mannopentaosyl-mannitol, manno-tetraose and -pentaose from mannohexaosyl- and mannoheptaosyl-mannitol, respectively.

The above results clearly indicate that the bacterial mannanase preferentially attacks the β-1,4-mannosidic linkages situating at the third or fourth from the non-reducing end of the mannose chain.

DISCUSSION

It appears quite reasonable from the ecological character that *Bacillus subtilis* secretes several hemicellulolytic enzymes. Our previous paper[1)] has reported that arabinogalactanase of the bacterium is secreted inducibly. However, the mannanase in the present paper was found to be formed in the culture medium without the enzyme substrates or their related compounds.

The mannanase purified in the present paper was stabilized by the addition of calcium ions, especially at high temperatures. As the enzyme was active even in the presence of metal chelators at low temperatures, calcium ion may act only to protect the enzyme from heat inactivation.

From the action patterns, it is quite clear that the enzyme attacks mannan endowise. However, when reacted with β-1,4-mannosidic