TABLE II. XYLANASE PRODUCTION IN VARIOUS MEDIA

Salts added to a basal medium		Initial pH	Xylanase (unit/ml)
None		7.2	undetectable
NaCl[a]	1.0%	10.5	undetectable
KCl[a]	1.0%	10.5	undetectable
KCl	1.0%	7.2	undetectable
Na_2CO_3	0.5%	9.7	2.8
Na_2CO_3	1.0%	10.2	5.1
Na_2CO_3	1.5%	10.5	3.5
$NaHCO_3$	1.0%	9.0	2.0
$NaHCO_3$	2.0%	9.3	3.1
K_2CO_3	1.0%	10.2	4.1

[a] pH was adjusted with NaOH. Basal medium is the bran medium minus Na_2CO_3.

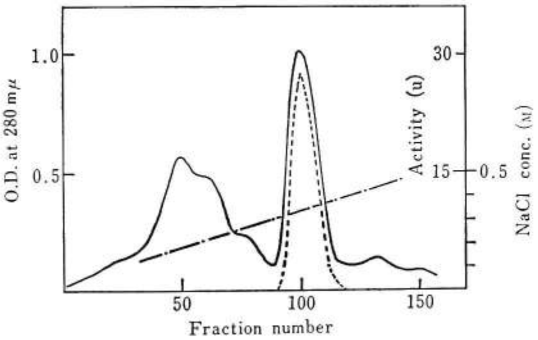

FIG. 1. Column Chromatogram on CM-cellulose. Column, 3×30 cm; load, about 270 mg of protein; flow rate, 50 ml/hr; O.D. at 280 nm was monitored by an Ohtake UV analyser. Further details are described in the text. Solid line, O.D. at 280 nm; dotted line, xylanase activity.

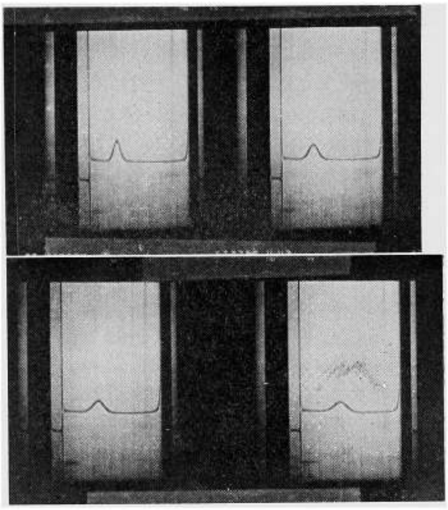

FIG. 2. Ultracentrifuge Run of Xylanase. The photographes were teken from left to right at 10 min intervals after reaching the full speed, 55,430 rpm. The enzyme concentration was 4 O.D. unit at 280 nm in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 N NaCl.

then dialyzed against tap water for 24 hr at 4°C. The dialysate was loaded onto a CM-cellulose column (3×30 cm) which was previously equilibrated with 0.02 M phosphate buffer (pH 4.5). After the column was successively washed with the same buffer, the enzyme was eluted with the buffer having a linear gradient increasing of NaCl at a flow rate of 50 ml/hr. A linear gradient was obtained by using 400 ml each of 0.05 M and 0.5 M NaCl in 0.02 M phosphate buffer (pH 4.5); 7-ml fractions were collected and each fraction was assayed. A typical chromatogram is shown in Fig. 1. Fractions containing xylanase were collected and dialyzed against 0.01 M phosphate buffer (pH 7.0) for 24 hr. The dialysate was loaded on a hydroxyl apatite column (1.5×30 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.0) and elution was carried out stepwisely increasing the concentration of phosphate buffer (pH 7.0). Over 90% of the enzyme activity was detected in the fraction eluted with 0.1 M phosphate buffer. This fraction concentrated

TABLE III. PURIFICATION OF XYLANASE

Steps	Volume (ml)	Total activity (unit)	Protein[a] (mg)	Specific activity (units/mg protein)	Recovery (%)
Supernatant fluid	1,000	3,500	2,050	1.7	100
$(NH_4)_2SO_4$ precipitation	50	3,000	273	11	86
CM-cellulose	100	1,500	70	25	50
Hydroxyl apatite	40	1,420	47	30	40
Sephadex G–75	80	1,200	35	35	35

[a] Protein concentration was measured by the method of Warburg and Christian.[9]

by varying the incubation temperature. In Fig. 7 the activity at 30°C is expressed as 100%. The activity at 60°C was about 500%. Thermal stability was examined as follows: the enzyme solution in 0.05 M Tris-HCl buffer (pH 7.0) was heated at the indicated temperatures for 10 min. And the residual activity was measured. Heat treatment was performed in the presence or absence of $CaCl_2$ (5 mM). As shown in Fig. 8, the residual activity was 100% after heated at 30~60°C in the presence of $Ca.^{2+}$

Effect of metals and inhibitors

The enzyme solution (0.02 ml) was mixed with 0.1 ml of 0.05 M Tris-HCl buffer (pH 7.0) containing the test reagents, pre-incubated at 40°C for 30 min, and then 0.1 ml of the substrate (xylan, 1%) was added. The residual activity was measured. As shown in Table IV, Hg^{2+}, Ag^{2+} and Cd^{2+} showed inhibitory effect on the enzyme.

Mode of action of the enzyme on xylan

About 10 mg of xylan were dissolved in 1 ml of water and its pH was adjusted to 7 with NH_4OH. To the solution was added 0.1 ml of the enzyme (11 U) and the mixture was incubated at 40°C. Aliquots of the reaction mixture were periodically withdrawn and chromatographed on Whatman No. 1 paper. The reducing sugars were detected by spraying with ammoniacal silver nitrate. As shown in Fig. 9, no xylose was detected and *Rf* values of three spots on the chromatogram were identical with those of xylobiose, xylotriose and xylotetraose in the tested solvent system.

TABLE IV. EFFECT OF METALS AND SOME REAGENTS ON XYLANASE ACTIVITY

Reagents added	Concentration (M)	Relative activity (%)
$HgCl_2$	10^{-3}	12
$Pb(CH_3COO)_2$	10^{-3}	75
$AgNO_3$	10^{-3}	55
$MnSO_4$	10^{-3}	83
$MgCl_2$	10^{-3}	102
$CuSO_4$	10^{-3}	83
$CoCl_2$	10^{-3}	110
$FeSO_4$	10^{-3}	115
$Fe_2(SO_4)_3$	10^{-3}	100
$CaCl_2$	10^{-3}	112
KCl	10^{-3}	100
$AlCl_3$	10^{-3}	99
$CdCl_2$	10^{-3}	50
$ZnSO_4$	10^{-3}	100
NaCl	10^{-3}	110
PCMB	10^{-4}	103
EDTA	10^{-2}	99
None		100

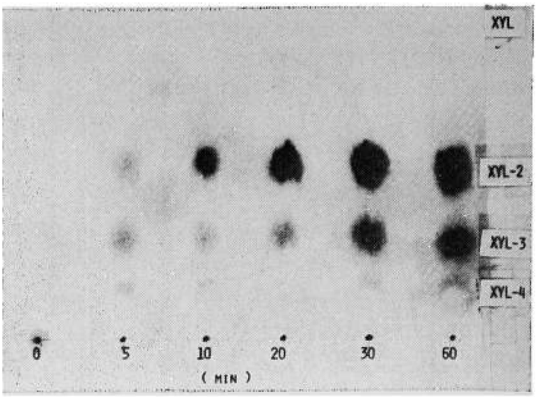

FIG. 9. Paper Chromatogram of the Hydrolyzate of Xylan. About 11 U of the enzyme were added to 1 ml of 1% xylan solution (pH 7, with NH_4OH), and incubated at 40°C. The hydrolyzates were chromatographed on Whatman No. 1 paper, and sprayed with ammoniacal silver nitrate.

DISCUSSION

The strain of *Bacillus* No. C-59-2 isolated from soil produced a xylanase in alkaline media. This strain, as we had reported about other alkalophilic bacteria in the previous papers,[1~5] grows well in alkaline media containing 1% Na_2CO_3 rather than neutral media. The morphological and cultural characteristics of this strain will be compared with other strains elsewhere. It is noteworthy that xylanase was only produced in alkaline media containing carbonate or bicarbonate salts. Action of these salts are not clear, although possibly due to the buffer action by these salts.

Homogeneity of the purified enzyme was supported by the following experiments: ultracentrifugal analysis, gel filtration and