ISOLATION AND IDENTIFICATION OF ANAEROBIC RUMEN BACTERIUM, *ACTINOMYCES* SP. 40 AND ENZYMATIC PROPERTIES OF β-1, 4-ENDOGLUCANASE

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Summary

A bacterial strain No. 40, which produced extracellular endoglucanase, was isolated from the rumen of Korean native goats and identified to be a genus of *Actinomyces* sp. The optimum conditions for endoglucanase production in PY-CMC medium were initial pH of 7.0 and 4 days of cultivation at 39℃. When localization of endoglucanase activity of *Actinomyces* sp. was determined, 68% of the enzyme activity was found in the extracellular fraction, 11% of the activity was detected in the periplasmic space and the remaining activity was in the intracellular and cell-bound fractions. The maximal endoglucanase activity was observed at pH 5.0 and it was most stable at pH 5.0. The optimum temperature of this enzyme activity was 55℃, but enzyme activity was gradually lost at temperature above 60 ℃. The crude enzyme was activated by addition of 10 mM cysteine and 10 mM DTT. But it was inhibited by addition of 10 mM Cu^{++} and Fe^{++}. This crude enzyme could digest carboxymethylcellulose (CMC), and degrade xylan, avicel, pNPG, and pNPC to a less extent.

(Key Words : *Actinomyces* sp. Endoglucanase, Korean Native Goat)

Introduction

Cellulosic materials are most abundant polymer in nature, but mammals do not produce enzymes which can degrade these materials. Ruminant animals have developed the capacity for cellulose digestion by a number of species of cellulolytic bacteria, and to a lesser extent, by cellulolytic fungi and protozoa (Bryant, 1973). The rumen is one of the most abundant sources of cellulolytic bacteria. *Ruminococcus flavefaciens, Ruminococcus albus, Butyrivibrio fibrisolvens,* and *Bacteriodes succinogenes* are the four major cellulolytic bacteria found in the rumen (Bryant, 1973).

A number of cellulolytic systems in the bacteria have been partially characterized. *Ruminococcus flavefaciens* is one of the most important species of rumen bacteria and is capable of degrading plant cell walls (Pettipher and Latham, 1979). Also, *Ruminococcus flavefaciens* degrades crystalline cellulose more efficiently than *Ruminococcus albus* (Pettipher and Latham, 1979). *Bacteriodes succinogenes* posseses the ability to hydrolyze cellulosic materials such as cotton fibers and straw (Groleau and Forsberg, 1981).

In addition to four major cellulolytic rumen bacteria, *Bacteriodes ruminicola* secretes two carboxymethylcellulases (CMCase) into the culture supernatant (Matsushita et al., 1991). These enzymes degrade xylan, but they have very low activity on native cellulose.

A series of experiments were conducted to obtain bacteria with high cellulolytic activity from various ruminant animals. As a results of screening tests, *Actinomyces* sp. 40 has been isolated from Korean native goat, containing the highest cellulolytic activity of isolates tested.

This paper deals with the isolation and identification of *Actinomyces* sp. 40 and characterization of enzymatic properties of endoglucanase from the microorganism.

Materials and Methods

Chemicals

Whatman No. 1 filter paper was obtained from Whatman International Ltd. (Maidstone, England).

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Avicel was purchased from Fluka Co. Ltd. CMC, xylan (from cat spelts), p-nitrophenyl-β-D-cellobioside (pNPC), p-nitrophenyl-β-D-glucopyranoside (pNPG), glucose assay kit, cysteine, dithiothreitol (DTT), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and dinitrosalicylic-acid (DNS) were obtained from Sigma Co. (St. Louis, MO, USA). Other chemicals were obtained commercially and were of the purest grade.

Sample collection from rumen content

The experiment was conducted with four male Korean native goats weighing between 30 to 35 kg. The animals were fed with rice straw and concentrates in a ratio of 7:3 and water *ad libitum* throughout the experimental period. Rumen contents were collected from Korean native goats through a rumen fistular. The solid contents were removed through two layers of cheese cloth and the filtrates were used as innocula. The filtrates were placed in a plastic bottle, flushed with CO_2 gas and cultured as soon as possible.

Media and anaerobic techniques

All media and anaerobic techniques used in this work were prepared by technique of Hungate (1950) and a modification of the technique of Bryant and Burkey (1953). The enrichment media used for isolation and maintenance of cellulolytic rumen bacteria were a modification of Scott and Dehority (1965) with Whatman No. 1 filter paper strip (1.0 cm^2) as a sole source of carbohydrate and the stock culture was maintained by periodical transfer to anaerobic test tube with filter paper strip in the same medium. Fermentation of substrates from isolated bacteria was examined by PYG medium (Holdeman et al., 1977).

Enrichment, isolation, and identification of cellulolytic bacteria

One milliliter of rumen sample was inoculated into 9 ml of the enrichment medium (MSD) in anaerobic test tube and cultured at 39℃ for 5 days under anaerobic conditions. Each of one milliliter of diluted culture broth was inoculated in the roll tube with Whatman No. 1 medium and supplemented with 0.025% (W/V) cellobiose, 0.05% (W/V) of starch and glucose instead of filter paper as carbon sources. For bacterial isolation, colonies in roll tubes were cultured in liquid broth with 0.025% (W/V) each of cellobiose and glucose and 0.05% (W/V) of starch as carbon sources. After cultivation, cup diffusion method by 0.1% (W/V) Congo-red solution (Teather and Wood, 1982) with the cultured supernatant were used for the detection of extracellular CMCase activity.

In order to identify the isolated strain, morphological and physiological characteristics were examined according to the method of Holdeman et al. (1977). Also identification of the bacterium by fatty acid methyl esters (FAMEs) analysis was accomplished by the Microbial Identification System. The strain used for FAMEs analysis was grown on PYG medium for fatty acid analysis. Identification of the FAMEs and subsequent data management was done as described previously (Stead, 1989; Vauterin et al., 1991). Data management of the FAME profiles was accomplished by the Microbial Identification System software package (MIS version No. 3.6) (Microbial ID, Inc., Newark, Delaware; MIDI).

Crude enzyme preparation

Cultures were centrifuged at 10,000Xg at 4℃ for 10 min and the supernatant was removed. Harvested cells were washed, centrifuged and resuspended in 10 ml of potassium phosphate buffer (0.5 M, pH 6.5). Cell-free extracts were obtained by sonication (20 kHz, 1 min). The sum of the supernatant and cell-free extract fractions were used as the crude enzyme solution.

Enzyme assays

CMCase activity

One milliliter of the crude enzyme solution was mixed with 1 ml of 1% CMC solution in 0.5 M potassium phosphate buffer (pH 6.5). The reaction was proceeded for 2 h at 40℃, then the reaction was stopped by boiling for 5 min. Reducing sugar produced was measured by the DNS reagent (Miller et al., 1960). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmole of glucose equivalent of reducing sugar per minute under the above conditions.

Avicelase and xylanase activity

Avicelase and xylanase activity were assayed with 1 ml of 2% avicel (W/V) and xylan (W/V) in 0.5 M potassium phosphate buffer (pH 6.5). Reducing sugar produced was determined by the same method as mentioned above.

To find the substrate specificity, enzymatic hydrolysis of pNPC and pNPG were measured by the amount of released p-nitrophenol as described by Deshpande et al. (1984).

Distribution of enzyme

The fractionations of extracellular, periplasmic, cell-bound, and intracellular enzymes were performed by a modification of the methods of Cornelis et al. (1982). The culture broth was centrifuged at 8,000Xg for 10 minutes at 4℃ and washed twice with an equal volume of original broth containing 0.9% NaCl. The cells harvested at 4℃ were washed in 0.5 M potassium phosphate buffer (pH 6.5) and suspended in a half volume of 25% (W/V) sucrose solution in potassium phosphate buffer (pH 6.5) - 1 mM ethylenediaminetetraacetic acid (EDTA) at room temperature. After 10 minutes of slow shaking, the cells were sedimented by centrifugation. It was then suspended in the same volume of ice-cold distilled water and shaken for another 10 minutes. The cells were centrifuged and the supernatant fluid was used as the periplasmic fraction. The cells sonicated in the same buffer were centrifuged and the supernatant was used as the intracellular fraction. The suspended cell debris was used as the cell-bound fraction. The extracellular fraction was calculated from the sum of the culture broth supernatant, the two washes, and the EDTA treatment supernatant.

Results and Discussions

Isolation and identification of the CMC-degrading bacterium

Ten CMC-degrading bacteria were isolated from the rumen of Korean native goats. Among ten isolated strains, No. 40 showed the highest CMCase activity in the culture supernatant (figure 1). Therefore, strain No. 40 was selected as an CMC-degrading enzyme producer which secreted efficiently into the culture supernatant.

A photomicrograph of strain No. 40 is shown in figure 2 and the physiological and biochemical characteristics are listed in table 1. From the data in table 1, strain No. 40 was rod shaped (0.5-0.8 by 0.8-2.5 μm) in PYG broth, strictly anaerobic, gram positive, and catalase negative. The fermentation products produced in PYG both were acetic acid, lactic acid, and succinic acid. Once strain No. 40 was identified as an *Actinomyces* sp., according to Holdeman et al. (1977) and Park (1986), we performed FAMEs analysis for the detailed identification of the isolated strain No. 40. Cellular fatty acid profile of strain No. 40 is shown figure 3. FAMEs and DMAs were present in the microorganism, with $C_{18:1\ cis\ 9}$ FAME being the major component (33.66%), followed by $C_{16:0}$ FAME (16.31%), $C_{18:1\ cis}$ DMA (15.45%), $C_{18:0}$ FAME (6.47%), $C_{12:2}$ FAME (6.45%), $C_{14:0}$ FAME (3.18%), $C_{18:0}$ 12-OH (2.49%), $C_{16:1\ cis}$ FAME (2.31%) and about 6.24% unknown.

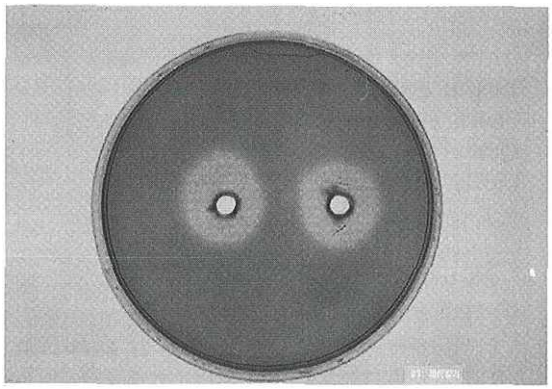

Figure 1. CMC-degrading activity of strain No. 40. After cultivation, 50 ul of culture supernatant was loaded on the CMC plate. The plate was incubated at 37℃ for 24 h for the crude enzyme reation and then 1 mg/ml of Congo red solution was added.

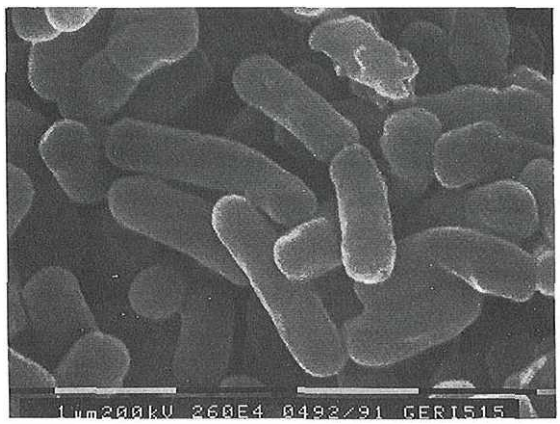

Figure 2. Scanning electron micrograph of strain No. 40 (X 26,000). A strain No. 40 was cultured in the PYG broth for 24 h at 39℃.

From the results of the physiological, biochemical characteristics and FAMEs analysis, strain

No. 40 were similar to those of genus *Actinomyces*. Therefore, this strain was named as *Actinomyces* sp. 40.

Park (1986) reported that this baterium was found predominantly in the rumen of Korean native goats and secreted high CMCase activity into the culture supernatant.

TABLE 1. PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES OF STRAIN NO. 40

Morphologic properties			
Form	Rod shape		
Size (μm)	0.5-0.8 by 0.8-2.5		
Gram staining	Positive		
Biochemical properties			
Esculin hydrolyzed	−	Fermentation products from	
Starch hydrolyzed	+	PYG broth;	
Gelatin digested		Acetic acid	+
Indole produced	−	Lactic acid	+
H_2S produced	−	Succinic acid	+
Catalase	−		
Hemolysis			
Growth in bile	+		
Growth condition	Strictly anaerobic		
Fermentation of substrates[a]			
Adonitol	−	Lactose	−
Amygdalin	−	Maltose	+
Arabinose		Mannitol	
Cellobiose	−	Mannose	−
Dextrin	d	Melezitose	−
Dulcitol	−	Melibiose	+
Erythritol		Raffinose	+
Esculin	−	Rhamnose	−
Fructose	+	Ribose	−
Galactose	+	Salicin	
Glucose	+	Sorbitol	−
Glycerol	−	Starch	+
Glycogen	+	Sucrose	+
Inositol	−	Trehalose	
Inulin	−	Xylose	−

[a] Basal medium was cultured with Peptone Yeast extract (PY) medium at 39℃ for 24 h.
d: Diversity.

Culture conditions

(a) Effects of carbon source and initial pH

To obtain the culture conditions showing the highest CMCase activity, media containing different carbon sources were examined. Table 2 shows the effects of various carbon sources on the production of extracellular CMCase. As shown in table 2, CMC was the most effective as a substrate for the production of CMCase. But cellobiose, filter

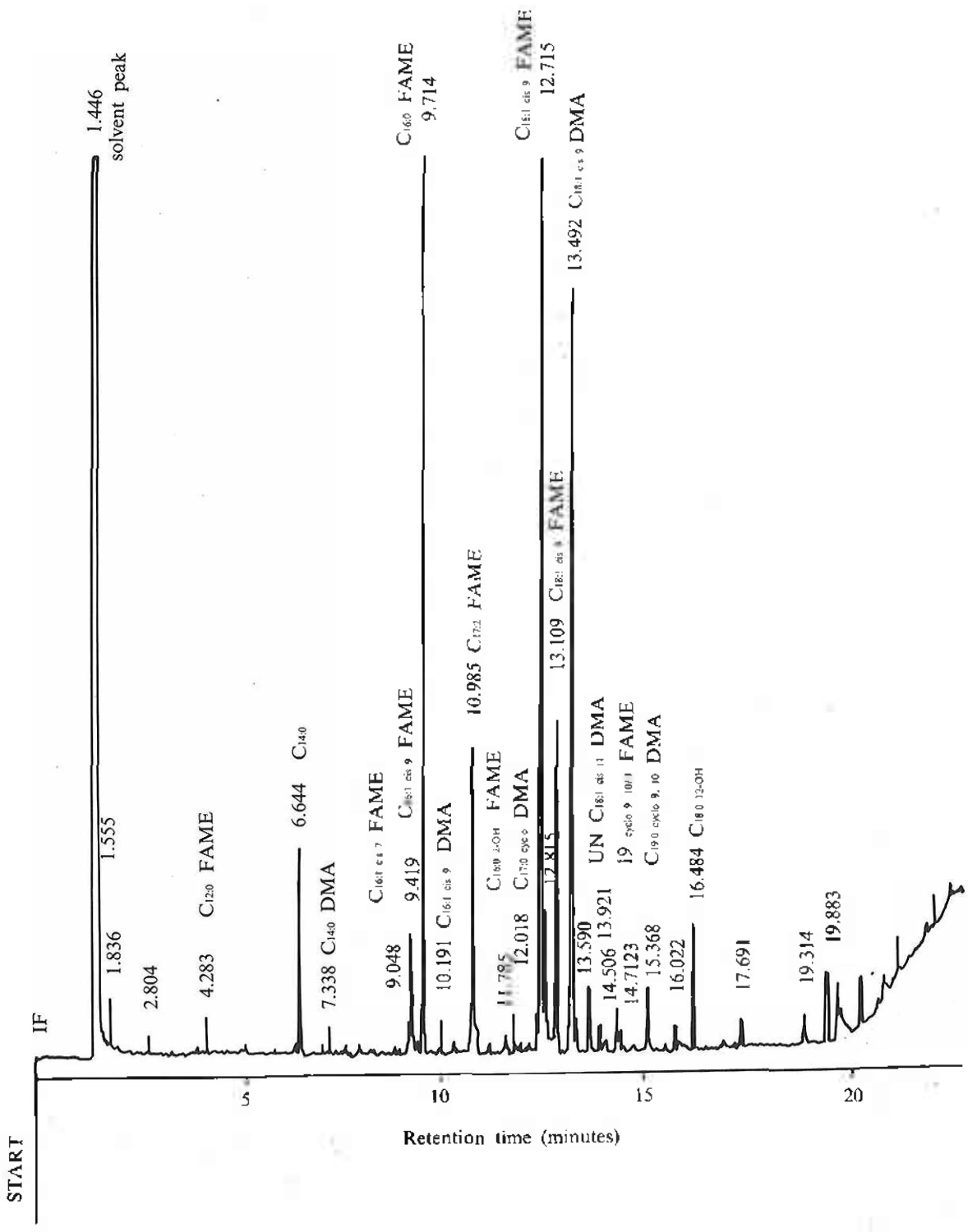

Figure 3. Gas chromatographic of fatty acid methyl esters (FAMEs) from strain No. 40. DMA, dimethyl acetyl: UN, Unknown.

paper, and xylose were not effective for cell growth and the CMCase production.

To study the effect of initial pH on CMCase activity, the strain was cultivated in PY CMC medium at 39℃ with various initial pH. As shown in figure 4, the maximal cell growth and CMCase activity were observed at pH 7.0 and decreased markedly above pH 9.0.

TABLE 2. EFFECT OF CARBON SOURCES ON CMCase PRODUCTION

Carbon sources	Relative activity (%) for CMC
None	0
CMC	100[a]
Sucrose	85
Starch	82
Maltose	32
Glucose	29
Cellobiose	4
Filter paper	0
Xylose	0

Actinomyces sp. 40 was cultured at 39℃ for 4 days in Peptone-Yeast extract (PY) medium containing 1% of each CMC, filter paper, and xylose, and for 24 h in PY medium containing 1% of each other carbon sources.
[a] 0.565 × 10^{-1} units/ml.

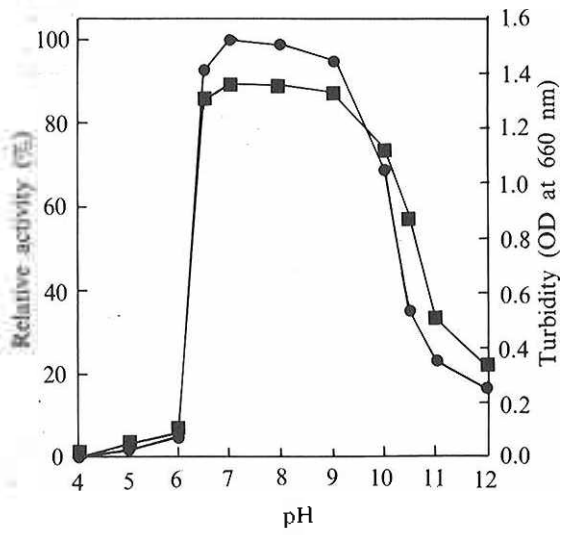

Figure 4. Effect of initial pH on the CMCase activity of *Actinomyces* sp. 40. Symbols: ●, bacterial growth; ■, enzyme activity. OD, Optica density.

(b) **Time courses on CMCase production**

The strain was cultured in PY-CMC medium at 39℃ for 5 days under anaerobic condition. Ten ml of culture broth was withdrawn at suitable time intervals and the enzyme activity was measured from the supernatant fluid. Figure 5 shows the changes on CMCase activity during cultivation. The CMCase activity reached a maximum on 4 days, when the culture entered the stationary phase, and then decreased slightly.

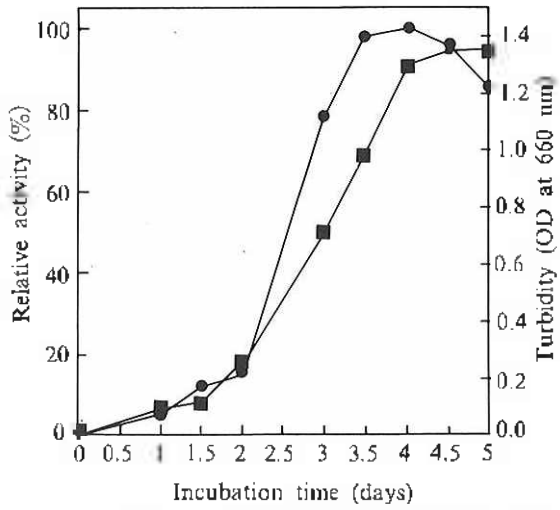

Figure 5. Effects of incubation time on growth and CMCase activity of *Actinomyces* sp. 40. Symbols: ●, bacterial growth; ■, enzyme activity. OD, Optica density.

(c) **Localization of CMCase**

The distribution of CMCase activities in cultures of the strain was examined. The CMCase activity found in the extracellular, periplasmic, intracellular, and cell-bound fractions were 67.7, 11.3, 10.5, and 10.5%, respectively. The CMCase was mainly secreted into extracellular portion. This result indicates that the enzyme from *Actinomyces* sp. 40 was secreted more powerfully in comparison with other rumen bacteria. Generally, bacterial enzyme seems to be retained in or on the cells, whereas the fungal enzyme is released from the cells. For example, Groleau and Forsberg (1981) reported that 30% of total endoglucanase activity of *Bacteriodes succinogenes* was present in extracellular fraction. β-Glucosidases from rumen bacteria such as *Bacteriodes succinogenes* (Groleau and Forsberg, 1981), *Ruminococcus flavefaciens* (Pettipher

and Latham, 1979), and *C. thermocellum* (Ait et al., 1982) were found to be cell associated.

Properties of the crude enzyme

(a) Effect of pH on enzyme activity and stability

To study the effect of pH on endoglucanase activity, the crude enzyme was incubated with 1 % CMC in various buffers ranging from pH 3.0 to 10.0, and the reducing sugars were measured. Figure 6 shows a pH optimum of 5.0, with 90% of its activity retaining between pH 5.0 and 7.0. The optimal pH for the enzyme activity was similar to the results reported for cellulolytic rumen bacterium, *Bacteriodes ruminicola* (Matsushita et al., 1990), but slightly lower than those of *Ruminococcus albus* (Ohmiya et al., 1987), *Bacteriodes succinogenes* (Taylor et al., 1987), and *Clostridium josui* (Fujino et al., 1990).

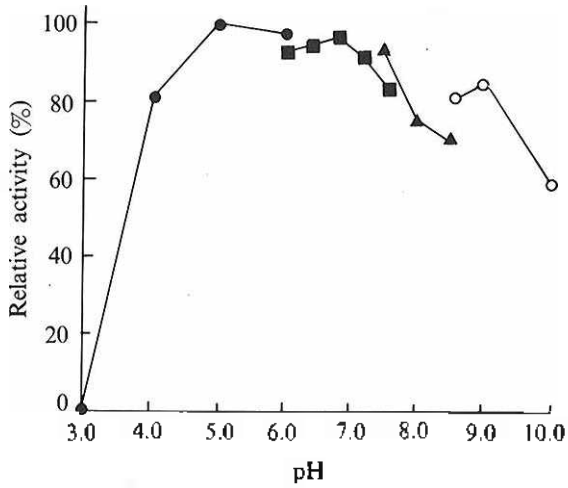

Figure 6. Effect of pH on the CMCase activity of *Actinomyces* sp. 40. The buffers used were 0.5 M citrate buffer (pH 3.0-6.0), 0.5 M potassium phosphate buffer (pH 6.0-7.6), 50 mM HEPES (pH 7.5-8.5), and 0.05 M borate buffer (pH 8.5-10.0).
Symbols: ●, citrate buffer; ■, potassium phosphate buffer; ▲, HEPES; ○, borate buffer.

To investigate the effect of pH on enzyme stability, samples of the crude enzyme were incubated in buffers of various pH at room temperature for 24 h and then the remaining activities were assayed. As shown in figure 7, the activities for hydrolysis of CMC were stable between pH 4.5 and 8.0. The pH range for enzyme stability in present study is broad in comparison with the CMCase produced from *Cellulomonas uda* (Nakamura et al., 1982).

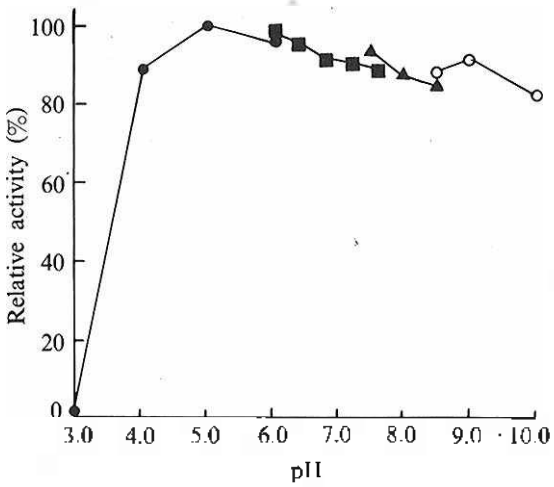

Figure 7. Effect of pH on the CMCase stability of *Actinomyces* sp. 40. The buffers used were 0.5 M citrate buffer (pH 3.0-6.0), 0.5 M potassium phosphate buffer (pH 6.0-7.6), 50 mM HEPES (pH 7.5-8.5), and 0.05 M borate buffer (pH 8.5-10.0).
Symbols: ●, citrate buffer; ■, potassium phosphate buffer; ▲, HEPES; ○, borate buffer.

(b) Effect of temperature on enzyme activity and stability

The enzyme activity was assayed at various temperatures. Figure 8 shows the temperature-activity profile for CMCase. The optimum temperature for hydrolyzing CMC was 55℃. The optimum temperature value is similar with *Clostridium josui* (Fujino et al., 1990) but rather higher than the optimal temperature of 44-47℃ observed in *Ruminococcus albus* (Ohmiya et al., 1987) and *Bacteriodes succinogenes* (Taylor et al., 1987).

The stability was tested by incubation crude enzyme at various temperatures for 3 h and then measuring the remaining activity. As shown in figure 9, this enzyme was stable at temperatures below 50℃ for 3 h (5% loss). However, residual activity was 50% at 65℃ after 20 min. A complete loss of activity was observed at 65℃ after 30 min, and at 70℃ after 10 min (data not shown). When

endoglucanase from *Ruminococcus albus* incubated at pH 6.8 for 10 min, the enzyme was stable up to 50℃ and lost its activity completely at 70℃ (Ohmiya et al., 1987).

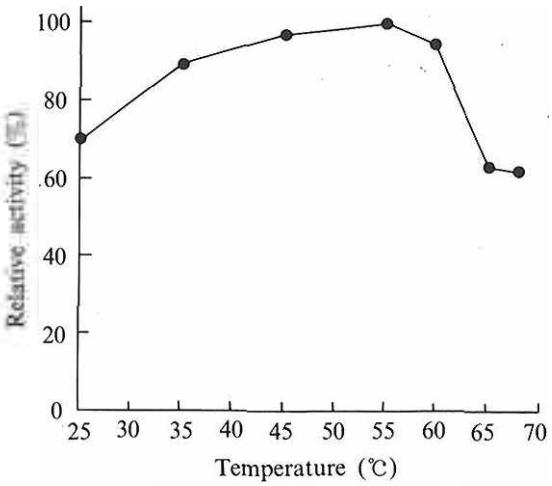

Figure 8. Effect of temperature on the CMCase activity of *Actinomyces* sp. 40.

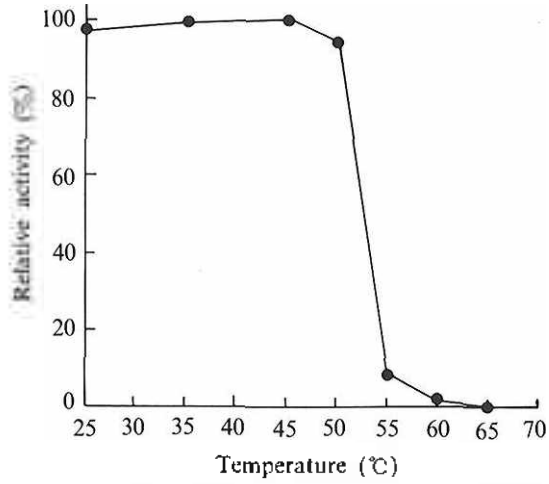

Figure 9. Effect of temperature on the CMCase stability of *Actinomyces* sp. 40.

(c) Effect of various chemicals on enzyme activity

The effects of various chemicals on enzyme activities are shown in table 3. The activity for hydrolyzing CMC was increased by addition of 10 mM cysteine or 10 mM DTT and markedly decreased by addition of 10 mM Fe^{++} or Cu^{++}. Reducing reagents such as cysteine and DTT brought about slight activation as well as stabilization of the enzyme. The activating effect of reducing reagents such as DTT and cysteine was the same as shown in *Ruminococcus albus* (Watanabe et al., 1992). However, the CMCase was not affected by addition of 1 mM of Cu^{++}, Zn^{++}. Taylor et al. (1987) reported that *Bacteriodes succinogenes* was sensitively inactivated by 0.1 mM of same metal ions. Also, endoglucanase from *Ruminococcus albus* was inhibited by addition of sulfhydryl-reacting reagents such as P-chloromercuibenzoate, N-ethylmaleimide, and iodoacetamide. This susceptibility to inactivation indicated that the enzyme may be a thio enzyme (Ohmiya et al., 1987).

TABLE 3. EFFECT OF VARIOUS REAGENTS ON ENZYME ACTIVITY

Reagents	Relative activity (%) for CMC	
	1 mM	10 mM
None	100	100
$CaCl_2$	102	99
Cysteine	102	125
$FeSO_4 \cdot 7H_2O$	91	48
$CuSO_4 \cdot 5H_2O$	102	20
DTT	105	132
$MgSO_4 \cdot 7H_2O$	96	97
$ZnSO_4 \cdot 7H_2O$	102	91

(d) Substrate specificity

The activity of the enzyme on various substrates was assayed. The results summarized in table 4 shows that the enzyme could hydrolyze CMC to the largest extent, while hydrolyzing activity for xylan and avicel was not significant. The amount of released glucose from hydrolysis of the substrates was also measured. The result is estimated that most of the end products released from CMC was cellodextrins. Therefore, it seems that β-glucosidase enzyme is not present in the crude enzyme from *Actinomyces* sp. 40. All cellulolytic bacteria have been known to secrete a variety of endoglucanases, most of which show little activity toward crystalline cellulose.

To examine the nature of the enzyme, we used chromogenic substrates, such as pNPC and pNPG. Agluconic bond and holosidic bond cleavage activities were estimated by spectrophotometric mea-

surement of the p-nitrophenol and glucose produced, respectively. The results are shown in table 4. The enzyme showed hydrolytic activity on agluconic bonds of both pNPG and pNPC, and on holosidic linkage of pNPC. Deshpande et al. (1984) reported that none of exoglucanases from *Trichoderma reesei* and *Sporotrichum pulverulentum* showed any hydrolytic action on the holosidic bond of pNPC, while both endoglucanases and β-glucosidase from same strains were active on both agluconic and holosidic linkages. Thus we concluded that the enzyme produced from *Actinomyces* sp. 40 is endo-β-1, 4-glucanase.

TABLE 4. ENZYME ACTIVITIES ON VARIOUS SUBSTRATES OF *ACTINOMYCES* SP. 40

Released product	Enzyme activity (Unit/ml crude enzyme solution)				
	Xylan	Avicel	CMC	pNPC[a]	pNPG[b]
Reducing Sugar	0.43×10^{-2}	0.64×10^{-2}	0.16	N.D.[c]	N.D.
Glucose	0	0.45×10^{-2}	0.67×10^{-2}	1.27×10^{-2}	N.D.
p-nitrophenol	N.D.	N.D.	N.D.	0.33×10^{-3}	0.59×10^{-2}

[a,b] Action on agluconic bond is measured by the release of p-nitrophenol, and the action on holisidic bond by estimating the glucose produced.
[c] N.D.; Not determined.

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