Journal of Microbiology and Biotechnology
Vol. 7. No. 5, 305-309, 1997

Purification and Characterization of Carboxymethyl Cellulase from *Bacillus stearothermophilus* No. 236

KIM, SOHNG-HWAN, SSANG-GOO CHO, AND YONG-JIN CHOI*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Bacillus stearothermophilus No. 236, an effective xylanolytic bacterium, produced an extracellular carboxymethyl cellulase when the strain was grown on xylan. The carboxymethyl cellulase was purified to homogeneity as judged by SDS-PAGE and zymogram. The carboxymethyl cellulase had a pI of 4.0, and a molecular mass of 95 kDa. The highest level of enzyme activity was observed at pH 6.5 and 60°C. The K_m and V_{max} values of the enzyme to carboxymethyl cellulose were 20.8 mg/ml and 0.63 μmole/min/mg protein, respectively. The enzyme was found to act also on filter paper and xylan as well as carboxymethyl cellulose. Therefore, it is expected that this xylanolytic strain isolated from soil could be efficiently used for xylan biodegradation.

Xylan is a major component of plant cell walls, representing up to 30% of the dry weight of plants (4). Unlike cellulose, xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with arabinosyl, glucuronosyl, acetyl, and glucosyl side chains (2). Therefore, the complete hydrolysis of the xylan molecule requires the cooperative actions of various xylanolytic enzymes including endo-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase, esterase, and glucosidase (19).

We have isolated a strain of *Bacillus stearothermophilus* No. 236 which produces a highly active xylan-degrading system (17). This bacterium was found to produce extracellularly the major xylanolytic enzymes such as endo-xylanases and β-xylosidases, and also to synthesize the debranching enzymes of acetylxylan esterases, α-arabinofuranosidases, and α-glucuronidase. These xylanolytic enzymes from *B. stearothermophilus* No. 236 were all purified and characterized in our laboratory. Furthermore, the genes encoding the enzymes were, with the exception of a α-glucuronidase all cloned in *E. coli* strains and their molecular details have been studied (3, 5, 8, 9, 11, 14, 15).

Recently, we found that the strain *Bacillus stearothermophilus* No. 236 also produced a carboxymethyl cellulase (CMCase), a cellulolytic enzyme. Interestingly, this CMCase was observed to have both cellulolytic activity and xylanolytic activity. Together, these observations raise some intriguing questions; what effect might the bifunctional enzyme have on the biodegradation of xylan by xylanolytic enzymes? And what is the physiological role of CMCase in this xylanolytic organism? As a first step to shed light onto the above mentioned questions, we purified and characterized CMCase from *Bacillus stearothermophilus* No. 236.

*Corresponding author
Phone: 82-2-920-1356. Fax: 82-2-923-9923.
E-mail: ssangoo@kuccnx.korea.ac.kr
Key words: *Bacillus stearothermophilus* No. 236, carboxymethyl cellulase, purification, cross-specificity

MATERIALS AND METHODS

Bacterial Strain and Chemicals

B. stearothermophilus No. 236 was used in this investigation. The strain was grown in optimal medium for the production of xylanase as described elsewhere (17).

Carboxymethyl cellulose, oat spelts xylan, DEAE-Sepharose CL-4B, and Sephacryl S-200 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used in this work were of analytical grade.

Enzyme and Protein Assay

For preparation of the substrate for CMCase, CM cellulose was dissolved in 0.05 M phosphate buffer (pH 6.5) at a concentration of 1.0%. CMCase activity was measured by incubating 0.2 ml of the appropriately diluted enzyme solution with 0.2 ml of the substrate solution for 30 min at 60°C. The enzyme reaction was stopped by adding 3 volumes of dinitrosalicylic acid solution (13). After the reaction mixture was boiled for 5 min, the reducing sugar released was measured by reading A_{540} (Beckman DU-64 spectrophotometer).

One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing

sugar as a glucose equivalent/min at 60°C.

Protein from column chromatography was measured by reading A_{280} and the protein concentration of the other enzyme samples was determined by Lowry's method using bovine serum albumin as a standard (12).

Purification of CMCase

The culture broth was spun with a Beckman JA10 rotor at 5000 rpm for 20 min at 4°C. The supernatant was used as the source of CMCase for purification. It was fractionated with ammonium sulfate at 35~55% saturation. The fractionated protein was precipitated by centrifugation at 7000 rpm for 40 min at 4°C.

The precipitate was redissolved in a minimum volume of 0.5 M phosphate buffer (pH 6.5), and the solution obtained was dialyzed for 1 day against 0.05 M of the same buffer. The dialyzed protein solution was chromatographed on a column of DEAE-Sepharose CL-4B previously equilibrated with 0.05 M phosphate buffer (pH 6.5). The enzyme was eluted with 450 ml of the same buffer followed by 500 ml of NaCl gradient (0 to 0.5 M). Fractions showing CMCase activity were collected and concentrated by adding ammonium sulfate to 60% saturation. The precipitate was redissolved with 0.05 M phosphate buffer (pH 6.5) and was run on a Sephacryl S-200 column equilibrated with the same buffer at a flow rate of 20 ml/h (2 ml/tube).

Determination of Enzyme Properties

The effect of temperature on enzyme activity was assessed by incubating the reaction mixtures at different temperatures in the range from 20 to 75°C. Thermostability was monitored by incubating the enzyme solution at a fixed temperature and removing aliquots at intervals of 1 h to test CMCase activity. The pH effect study was done in sodium acetate (pH 4~6), phosphate (pH 6~8), or Tris (pH 8~9) buffer at 60°C.

The effect of substrate concentration on CMCase activity was studied by measuring the activity in various concentrations of CM cellulose ranging from 5 to 20 mg/ml in phosphate buffer (pH 6.5).

CMCase was incubated with 1 mM metal ions at 37°C for 1 h. Residual activity was measured under enzyme assay conditions.

Gel Electrophoresis, Zymogram, and Analytical Isoelectric Focusing

SDS-PAGE (8% polyacrylamide) was performed according to the method of Laemmli (10). Proteins were stained with Coomassie brilliant blue R.

A zymogram was created according to the method of Béguin (1). The electrophoresed gel was washed four times each for 30 min in phosphate buffer (the first two washes contained 25% isopropanol). The gel was laid on a 2% agarose replica gel containing 0.5% CM cellulose and then incubated for 60 min at 60°C. The replica gel was introduced into a 0.1% Congo red solution and gently rocked for 15 min. Excess dye was decanted, and the gel was washed with 1 M NaCl until excess stain was totally removed from the active bands.

Isoelectric focusing was carried out with Phamalyte 3~10 and Phamalyte 2.5~5, as described previously (14).

Substrate Specificity

20 mg/ml of substrates and ten 0.1×1 cm strips of filter paper were tested to analyze the substrate specificity of CMCase. The hydrolytic products were quantitated by the dinitrosalicylic acid method.

CMCase activity against nitrophenyl derivatives was also tested. Each of 0.1 ml of 10 mM nitrophenyl derivatives was reacted with the same volume of CMCase for 30 min at 60°C. The enzyme reaction was stopped by adding 2 ml of 0.4 M Na_2CO_3, and the absorbance was monitored at 405 nm.

RESULTS AND DISCUSSION

Purification of CMCase

CMCase fractionated with ammonium sulfate at 35~55% saturation was applied onto a DEAE-Sepharose CL-4B column, and eluted with a linear gradient of 0~0.5 M NaCl in 0.5 M phosphate buffer (pH 6.5). The CMCase was eluted at about 0.3 M NaCl (Fig. 1). The fractions containing CMCase were pooled and concentrated by the addition of ammonium sulfate to 60% saturation. The concentrated sample was loaded onto a Sephacryl S-200 column (Fig. 2). The fraction showing CMCase activity from column chromatography was electrophoresed on an 8% polyacrylamide gel and a single protein band was identified on the gel (Fig. 3). Enzyme activity was observed to comigrate with the protein band, and the specific activity of the purified enzyme was 3.80 U/mg (Table 1).

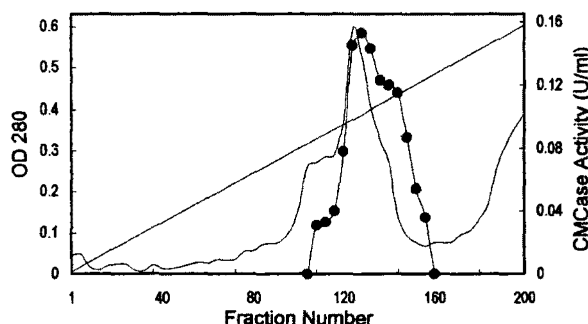


Fig. 1. Ion exchange chromatogram of the ammonium sulfate precipitate on DEAE-Sepharose CL-6B.

The ammonium sulfate precipitate was loaded on the DEAE-Sepharose CL-6B column (2.6×20 cm). After washing with 0.05 M potassium phosphate buffer (pH 6.5), the column was eluted with linear gradient of 0~0.6 M NaCl dissolved in the above phosphate buffer. Aliquots of each fraction were assayed to detect the CMCase activity. ——, absorbance at 280 nm; —●—, CMCase activity (U/ml).

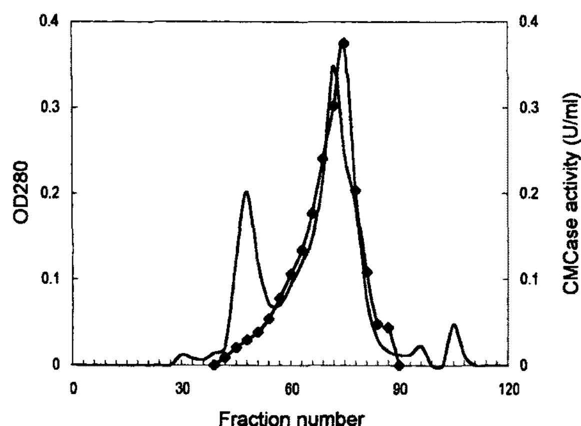


Fig. 2. Gel filtration chromatogram of the pooled fraction from ion-exchange chromatography on Sephacryl S-200.

The fractions obtained from DEAE-Sepharose CL-6B chromatography was applied to the gel filtration column (1.6×92 cm). Gel filtration was done with 0.05 M phosphate buffer (pH 6.5) at a flow rate of 12 ml/h (2 ml/tube). ——, absorbance at 280 nm; —●—, CMCase activity (U/ml).

Physicochemical Properties

The molecular mass and pI of CMCase were estimated to be approximately 95 kDa by SDS-PAGE (Fig. 3) and pH 4.0 by IEF (data not shown), respectively. These properties are similar to those reported for the enzyme of *Bacillus* strain 1139, which has a molecular mass of 92 kDa and a pI of pH 3.1 (7).

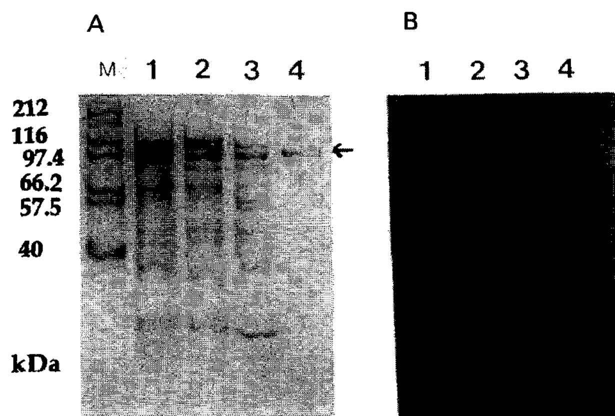


Fig. 3. SDS-PAGE monitoring of the major steps of CMCase purification.

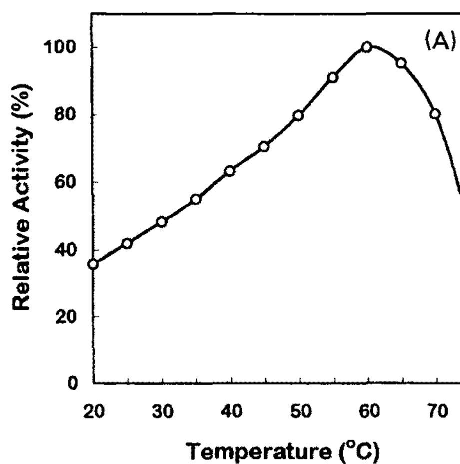
(A) SDS-PAGE was done on a 8% polyacrylamide gel. An arrow indicates the purified CMCase. (B) Zymogram stain of CMCase. Aliquots of protein solutions from each purification step were electrophoresed on the 8% SDS-PAGE followed by staining for CMCase activity as described under Materials and Methods. Transfer and CM cellulose hydrolysis were allowed to occur for 60 min at 60°C before staining. Lane 1, culture broth; lane 2, ammonium sulfate precipitate; lane 3, DEAE-Sepharose CL-6B column effluent; lane 4, Sephacryl S-200 gel filtrate; lane M, protein molecular weight size markers. Numbers in the left margin indicate mass (in kDa) of molecular weight markers.

Table 1. Summary of the purification of CMCase from *Bacillus stearothermophilus*.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture media	232.2	1922.4	0.121	100	1.00
Ammonium sulfate fractionation	129.1	257.3	0.502	55.6	4.15
DEAE-Sepharose CL-6B	45.3	46.2	0.981	19.5	8.11
Sephacryl S-200	31.2	8.20	3.80	13.4	31.4

General Properties

The optimum temperature for CMCase action was determined by varying the reaction temperature at pH 6.5



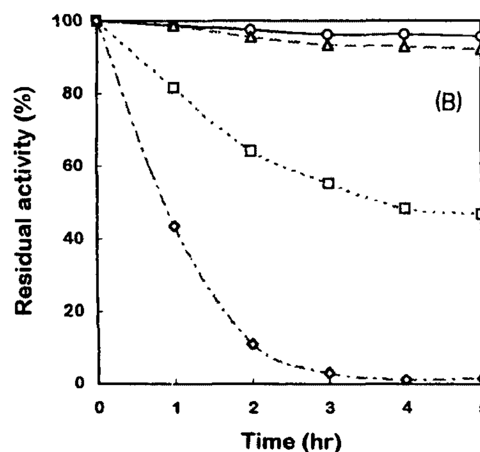


Fig. 4. Effect of temperature on the activity (A) and the stability (B) of CMCase.

(A) Enzyme reaction was carried out for 30 min at various temperatures as in the figure. Relative activity was represented as the percentage of the enzyme activity at 60°C. (B) Enzyme activity was measured at 60°C for 30 min allowing the enzyme solution to stand at various temperatures in 1-h interval for 5 h. —○—, 40°C; —△—, 50°C; ···□···, 60°C; —◇—, 70°C.

(Fig. 4A). CMCase was the most active at 60°C. At 70°C, the enzyme showed about 80% of the maximum activity. Similarly, at temperatures lower than 40°C, CMCase also showed poor activity against CM cellulose. The enzyme solution was heat-treated for 5 h at temperatures in the range of 40~70°C and the residual activity was measured at intervals of 1 h under standard assay conditions (Fig. 4B). CMCase had about 50% residual activity after 5 hr heat treatment at 60°C. Whereas, it showed nearly full activity at 50°C. CMCases from *Bacillus* can be divided into two major groups according to their optimum temperature. One has an optimum temperature at 40~45°C, and the other at 55~60°C. The CMCase from *Bacillus stearothermophilus* No. 236, therefore, could be assigned to the latter group, as it acted at high temperatures.

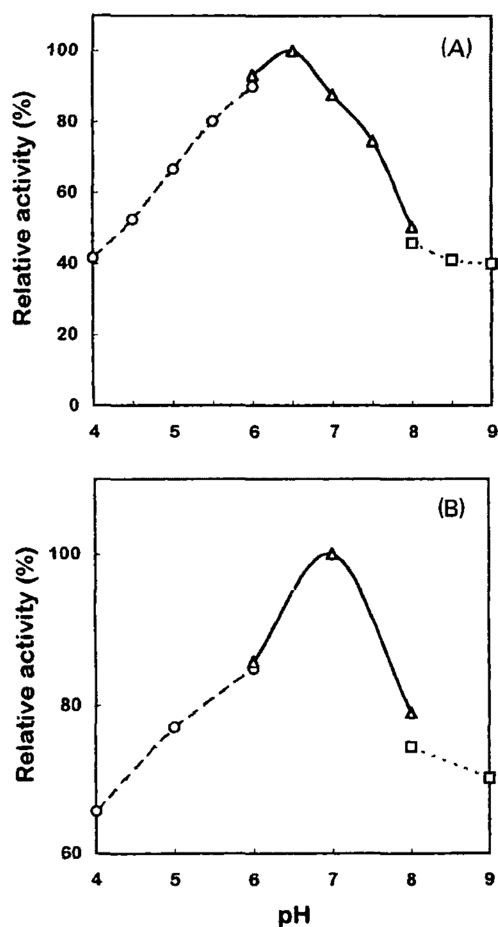


Fig. 5. Effect of pH on the activity (A) and the stability (B) of CMCase.

(A) Enzyme reaction was done at 60°C for 30 min at each pH. Relative activity was determined relative to the CMCase activity at pH 6. (B) Enzyme solutions were placed in various pH buffers (0.1 M instead of 0.05 M) at 4°C for 24 h. Enzyme reaction was carried out at 60°C for 30 min. ···○···, Sodium acetate; —△—, Potassium phosphate; ···□···, Tris·Cl.

The activities and stabilities of CMCase under various pH conditions were measured (Fig. 5). The enzyme had an optimum pH of 6.5, and the highest stability at pH 7. It had a narrow pH range of activity and stability at near neutral pH.

CMCase activity was examined in the presence of 1 mM of various metal ions (Fig. 6). The addition of Fe^{2+} or Fe^{3+} increased enzyme activity by about 20~30% of its original activity, but it was completely inhibited by the presence of Ag^{+} or Hg^{2+}.

Reaction velocity of the hydrolysis of CM cellulose by CMCase was analyzed, and the Michaelis constant (K_m) for the substrate was estimated from a Lineweaver-Burk plot. The K_m and maxiamal velocity (V_{max}) for CM cellulose were calculated to be 20.8 mg/ml and 0.63 μmole/min, respectively.

Substrate Specificity

The ability of CMCase to hydrolyze various polysaccharide substrates was tested (Table 2). The enzyme was most active on CM cellulose. In addition, CMCase showed very high activities against filter paper and xylan. However, this enzyme could not act on α-cellulose and cellobiose. These results indicate that CMCase cannot attack the crystalline region of native cellulose but hydrolyzes native cellulose by acting on the amorphous region in an endo-acting fashion.

It is very interesting that CMCase could hydrolyze various xylan substrates. Activities of CMCase against birchwood, larchwood, and oat spelts xylan were 0.97, 0.92, and 0.78 U/mg protein, respectively.

This cross-specificity has been reported in many organisms such as *Bacillus* sp. (18), *Clostridium acetobutylicum* (11), and *Trichoderma viride* (16). In *T. viride*, Shi-

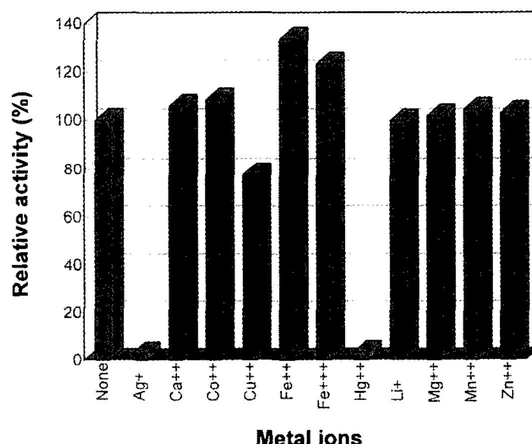


Fig. 6. Effect of metal ions on CMCase activity.

Each enzyme solution was preincubated at 37°C for 1 h in the presence of various metal ions (final concentration of 1 mM) before measuring CMCase activity. Enzyme reaction was carried out under the standard enzyme assay condition.

Table 2. Substrate specificity of the CMCase.

Compound	Specific activity (U/mg)
Cellobiose	0
Maltose	0
Sucrose	0
Dextran	0
Dextrin	0
Soluble starch	0
Birchwood xylan	0.97
Larchwood xylan	0.92
Oat spelts xylan	0.78
α-Cellulose	0
CM cellulose	3.90
Filter paper	0.48
o-Nitrophenyl α-D-glucose	0
p-Nitrophenyl α-D-glucose	0
p-Nitrophenyl β-D-glucose	0
o-Nitrophenyl β-D-xylose	0

kata and Nishzawa (1975) reported that CM cellulose and xylan reacted competitively with the same active site as cellulase. On the other hand, Lee *et al.* (1987) suggested that the cross-specificity was due to the non-specific nature of the xylanase toward the β-1,4 linkage in *C. acetobutylicum.*

In *B. stearothermophilus* No. 236, the ratio of CMCase activity to xylanase activity was 3.9~4.8 : 1. This value is similar to that for the endo-xylanase A of *C. acetobutylicum*. Further studies are required to shed light onto the precise action of CMCase of *B. stearothermophilus* No. 236 on both CM cellulose and xylan. On the basis of the results obtained in this work, it is highly probable that *B. stearothermophilus* No. 236 could be effectively used for the biodegradation of native xylan.

Acknowledgement

This work was supported by "the R & D program for Alternative Energy" of the Ministry of Trade, Industry, and Resources, Korea.

REFERENCES

1. Béguin, P. 1983. Detection of cellulase activity in polyacrylamide gels using Congo red-stained agar replicas. *Anal. Biochem.* **131**: 333-336.
2. Biely, P. 1985. Microbial xylanolytic systems. *Trends Biotechnol.* **3**: 286-290.
3. Cho, S. G., S. S. Park, Y. I. Park, and Y. J. Choi. 1992. Molecular cloning and expression of an endo-xylanase from *Bacillus stearothermophilus* into *Escherichia coli. Kor. J. Appl. Microbiol. Biotech.* **20**: 271-279.
4. Dekker, R. F. H. and G. N. Richards. 1976. Hemicellulose, their occurence, purification, properties and applications. *Adv. Carbohydr. Chem. Biochem.* **32**: 277-352.
5. Eom, S. J., H. S. Kim, S. G. Cho, and Y. J. Choi. 1995. Molecular cloning and expression of the α-L-arabinofuranosidase gene of *Bacillus stearothermophilus* in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **22**: 608-613.
6. Ferrari, E., A. S. Jarnagin, and B. F. Schmidt. 1993. Commercial production of extracellular enzymes, p. 917-937. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.) *Bacillus subtilis and other Gram-positive bacteria: Biochemistry, physiology, and molecular biology.* American Society for Microbiology, Washington D.C.
7. Fukumori, F., T. Kudo, and K. Horikoshi. 1985. Purification and properties of a cellulase from alkalophilic *Bacillus* sp. No. 1139. *J. Gen. Microbiol.* **131**: 3339-3345.
8. Kim, H. S., S. J. Eom, S. G. Cho, and Y. J. Choi. 1995. Molecular cloning and expression of the acetyl xylan esterase gene (*est*II) of *Bacillus stearothermophilus* in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **22**: 599-606.
9. Kim, I. S., S. G. Cho, and Y. J. Choi. 1993. Molecular cloning and expression of the acetyl xylan esterase gene of *Bacillus stearothermophilus* in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **21**: 542-548.
10. Laemmli, U. K. 1970. Cleavage of structure protein during the assembly of the head of bacteriophage T4. *Nature* (London) **227**: 680-685.
11. Lee, S. F., W. Forsberg, and J. B. Rattray. 1987. Purification and characterization of two endoxylanases from *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* **53**: 644-650
12. Lowry, O. H., N. J. Rasebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
13. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**: 426
14. Mun, A. R. and Y. J. Choi. 1992. Purification and characterization of exo-xylanase form *Bacillus stearothermophilus. Kor. J. Appl. Microbiol. Biotechnol.* **22**: 134-142.
15. Oh, S. W., S. S. Park, Y. I. Park, and Y. J. Choi. 1992. Molecular cloning and expression of *Bacillus stearothermophilus* β-D-xylosidase gene in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **20**: 136-142.
16. Shikata, S. and K. Nisizawa. 1975. Purification and properties of an exo-cellulase component of novel type *Trichoderma viride. J. Biochem.* **78**: 499-512.
17. Song, H. S. and Y. J. Choi. 1989. Production of xylanase by *Bacillus stearothermophilus. Kor. J. Appl. Microbiol. Technol.* **17**: 289-294.
18. Uchino, F. and T. Nakane. 1981. A thermostable xylanase from a thermophilic acidophilic *Bacillus* sp. *Agric. Biol. Chem.* **45**: 1121-1127.
19. Wong, K. K. Y., U. L. Larvy, and J. N. Saddler. 1988. Multiplicity of β-1,4-xylanase in microorganisms: function and application. *Microbiol. Rev.* **52**: 305-317.

(Received June 4, 1997)