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Isolation and Identification of *Pseudomonas* sp. CMC-50 Producing Carboxymethyl Cellulase and Characterization of Its Crude Enzyme

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A strain that produces a high level of carboxymethyl cellulase was isolated from rotten leaves. The isolated strain was revealed to be gram-negative, oxidase-positive, and catalase-negative. From the electron microscopic features, it was identified as a rod-shaped bacterium with peritrichous flagella and did not form spores. Morphological and biochemical characteristics of the strain were found to be similar to the *Pseudomonas* species. However, carbon utilization test showed different results. Based on the results, this new strain was identified as *Pseudomonas* sp. CMC-50. CMCase produced by this strain showed a strong activity in neutral and weak acidic conditions and maximum activity at 50°C.

Key words: Pseudomonas *sp., carboxymethyl cellulase, cellulose.*

Cellulose is a polymer of glucose in β-1,4-glycosidic linkages and constitutes the most abundant organic substance on earth. However, human and some animals are unable to digest it due to the absence of cellulolytic enzymes. In order to be used as food or energy sources, cellulose must be hydrolyzed into glucose units. Cellulase produced from fungi and bacteria hydrolyzes cellulose into glucose or oligosaccharide units.[1]

Cellulase consists of three cellulolytic enzymes, with each enzyme hydrolyzing cellulose in a different manner. The first enzyme, 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91), removes cellobiose or glucose units from nonreducing ends of cellulose chains and is also known as exoglucanase, cellobiosidase or avicellase. The second enzyme, 1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4), hydrolyzes internal 1,4-β-D-glycosidic linkages in cellulose and is also known as endoglucanase or CM-cellulase. The third enzyme, β-D-glucoside glucohydrolase (EC 3.2.1.21), hydrolyzes cellobiose and removes glucose from non-reducing ends of cello-oligosaccharide. This enzyme is commonly known as β-glucosidase. These three cellulolytic enzymes function synergistically to hydrolyze cellulose or its chemically modified polymers and exist in multi-component enzyme system.[1-4]

Bacteria such as *Cellulomonas* sp.,[5] *Pseudomonas* sp.,[6,7] *Clostridium* sp.,[8] *Acetobacter* sp.,[9] and *Bacillus* sp.[10] have been reported to produce cellulase, and *Trichoderma reesei* and other fungi [1,11] are used for the industrial production of cellulase. Cellulase has been especially useful for such purposes as silage inoculation to increase digestion for the feed, ethanol production using cellulolytic biomass, management of byproduct of agricultural products or waste, and clarification of fruit juice in food industry.[1,2] Studies on specific cellulase such as alkaline or thermophilic cellulase are in progress to further exploit their applications.[12-14]

Cellulase has a very important significance in basic researches due to the its industrial importance of its products such as glucose, xylose, and oligosaccharide. Presently, 15% of the world enzyme market is cellulase-related such that cellulase represents one of the most important enzymes in the food industry.[3] Therefore, it is essential to find new strains producing stable and high cellulase activity in specific conditions. In this study, we report on the identification of a new CMCase-producing microorganism isolated from rotten leaves and the characterization of its CMCase enzyme activity.

Materials and Methods

Screening and isolation of CMCase-producing microorganism. To isolate novel CMCase-producing microorganisms, we collected rotten leaves, soil, and fecal materials from domestic animals from 12 different regions in Korea. One gram of each sample was mixed with 10 ml of 0.8% NaCl solution. The supernatant of the solution was diluted and spread on 1% CMC medium agar plate (pH 7.6). The composition of CMC medium (standard medium) was as follows: 10 g CMC (Wako), 2.5 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, 7.6 g Na_2HPO_4, 0.2 g NaCl, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 1.0 g yeast extract in 1 L distilled water. After incubation at 30 for 3 days, colonies grown on the CMC medium were isolated and transferred to new plates.

Using Congo red reagent (0.1% Congo red in distilled

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Abbreviations: CMC, carboxymethyl cellulose; CMCase, carboxymethyl cellulase.

water), we selected CMCase-producing strains from colonies. Congo red reagent was placed for 30 min on the colonies incubated at 30℃ for 5 days on the CMC medium to stain CMCase-producing strains, and plates were washed with 1 M NaCl. The strains producing clear zone were inoculated in 5 ml of 1% CMC medium in test tube and incubated at 30℃ for 5 days with shaking. The CMCase activities of selected strains were measured using DNS reagent[15] at 550 nm using a spectrophotometer.

Electron microscopy. Scanning electron microscopy: Cells grown on 1% CMC plates were fixed with 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) solution and then cooled for 2 h in cold chamber. Subsequently, cells were washed twice with 0.05 M cacodylate buffer. After washing, specimens were fixed with 1% OsO_4, and then dehydrated in a graded ethanol series from 30 to 100%. Specimens were critical-point dried through hexamethyldisilazane (HMDS) substitution for 15 min three times, coated with gold, and examined under a JSM 5410 LV SEM (JEOL Lt, Japan).

Transmission electron microscopy: Cells grown on 1% CMC broth was suspended in 0.85% saline solution. A small drop of the suspension was placed on a carbon-coated copper grid and allowed to settle down for 10 min. Cells were negatively stained with 2% phosphotungstic acid. The specimens were examined under a transmission electron microscope JEOL-1010 (JEOL Lt, Japan).

Biochemical test. To determine the biochemical characteristics using an API 20 E kit (Analytab, France), the isolated colonies were swabbed from the CMC agar plate and suspended in 0.85% saline with a turbidity equivalent to 0.5 on the Macfarland standard. Two hundred microliters of suspension were distributed to the ampoules to test for biochemical characteristics, and the ampoules were incubated at 30℃ for 24 h.

Assay of CMCase activities. CMCase activity was determined using the modified method of Miller.[15] The crude enzyme solution from culture was mixed with 1 ml of 1% CMC medium (pH 7.6) in a test tube. After 30 min of incubation at 50℃, released reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) solution. The activity of one unit (U) of CMCase activity was defined as the amount of enzyme, which produced 1.0 μmol of reducing sugar per minute under the assay conditions. Protein concentration was measured using the Bradford method[16] with bovine serum albumin as standard.

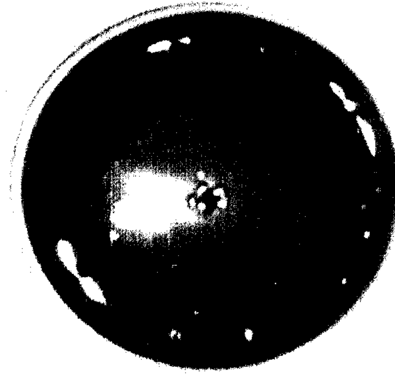

Fig. 1. Clear circle of *Pseudomonas* sp. CMC-50 with Congo-red reagent.

Fig. 2. Scanning electron microscopy of *Pseudomonas* sp. CMC-50.

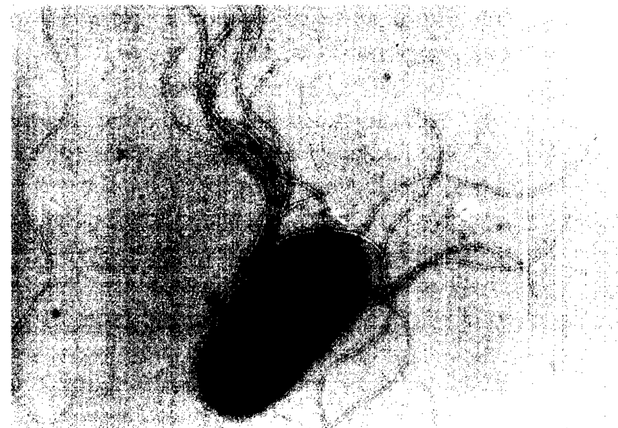

Fig. 3. Transmission electron microscopy of *Pseudomonas* sp. CMC-50.

Results and Discussion

Isolation of the strains producing CMCase. Over 400 strains were obtained from soil, rotten leaves, and domestic animal secretions. Of these strains, 20 were shown, using Congo red reagent to have enough CMCase activities to display more than 1.0 cm diameter of clear circle in CMC agar plate. These strains were isolated and inoculated in 5 ml of 1% CMC medium at 30℃ for 5 days, and the CMCase activities using DNS reagent were measured. Subsequently, we isolated a strain possessing the highest CMCase activity and named it CMC-50. CMC-50 showed a clean circle 5.0 cm in diameter on the 1% CMC plate with Congo red reagent. (Fig. 1)

Table 1. Morphological properties of *Pseudomonas* sp. CMC-50.

Form	Rods	Spore	negative
Size(μm)	0.5~1.0 × 2.5~3.0	Motility	positive
Gram staining	negative	optimum temperature	30°C
Color of colony	weak yellow	Utilization of O_2	facultative aeroic

Table 2. Result of API 20 E kit of *Pseudomonas* sp. CMC-50.

Test	Results	Test	Results
Beta-Galactosidase	+	Glucose	-
Argnine dihydrolase	-	Mannitol	-
Lysine decarboxylase	-	Inositol	-
Ornithine decarboxylase	-	Sorbitol	-
Simmons citrate	+	Rhamnose	-
H_2S production	-	Sucrose	-
Urease	+	Melibiose	-
Tryptophane deaminase	-	Amygdaline	-
Indole	-	Arabinose	-
Voges proskauer	-	Catalase	-
Gelatine hydrolysis	+	Oxidase	+

+ : Positve - : Negative

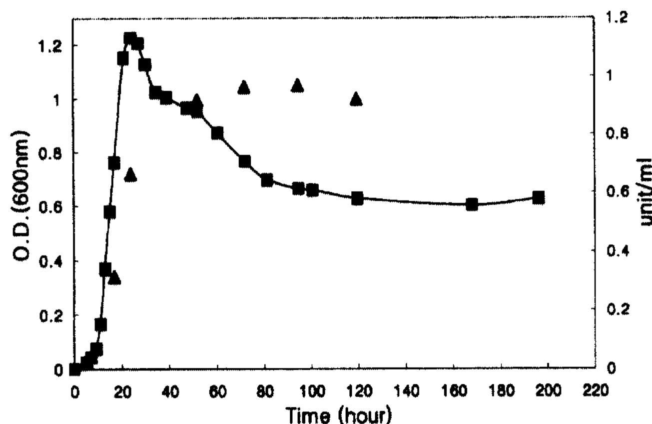

Fig. 4. Cell growth and production of CMCase of *Pseudomonas* sp. CMC-50.
■: Cell growth; ▲: unit/ml

Table 3. Effects of carbon sources on the CMCase production.

Carbon source	% Relative activity
Cellulose(CMC)	100
Xylose	50
Starch	6
Sucrose	0
Fructose	7
Glucose	10
Cellobiose	32

Identification of the CMC-50 strain. The identification of CMC-50 strain was based on the morphological, physiological, and biochemical characteristics. The electron microscopic features are shown in Figs. 2 and 3. The results obtained from morphological and physiological tests are summarized in Table 1. The isolate, CMC-50, is an aerobic, non spore-forming, gram negative, motile, and rod-shaped bacterium with peritrichous flagella. The size was 0.5~1 μm 2.5~3.0 μm, and the optimal growth temperature was 30°C.

Biochemical characteristics of CMC-50 were measured using API 20 E kit (Table 2). CMC-50 was catalase-positive, oxidase-negative, and urease-positive. It showed no fluorescent activity and, when compared to *Pseudomonas fluorescens* var. *cellulosa*[6,17)], different characteristics in CMCase activity and some carbon utilization were observed. Furthermore, it did not utilize all of the carbon sources such as sucrose, mannitol, arabinose, inositol, and glucose using API 20E. Based on these results, the isolated strain was identified as *Pseudomonas* sp. and labelled as *Pseudomonas* sp. CMC-50.[18)]

Growth curve and CMCase production. To determine the relatedness of cell growth and CMCase activity, *Pseudomonas* sp. CMC-50 was cultured in standard media at 30°C for 6 days with vigorous shaking. Figure 4 shows the growth curve of *Pseudomonas* sp. CMC-50 and CMCase production at various times. Cell growth was reduced significantly after reaching the stationary phase. The enzyme activity of crude CMCase was not detected until after 20 h, by which time growth reached the middle logarithmic phase. Subsequently, enzyme level rose rapidly, reaching maximum activity after 50 h, corresponding to the early stationary phase. Thus, the production of CMCase of *Pseudomonas* sp. CMC-50 was not related to the cell mass. Maximum enzyme activity of crude culture was 0.97 unit/ml (pH 7.6).

Effect of carbon sources on CMCase production of *Pseudomonas* sp. CMC-50. To determine the optimal carbon source, *Pseudomonas* sp. CMC-50 was cultured with 2% of each carbon source in standard media at 30°C for 6 days with vigorous shaking. CMC was found to be the optimum carbon source in CMCase production (Table 3). As in the case of API 20E test where a general carbon source was not used, both *Pseudomonas* sp. CMC-50 and *Bacillus* sp. NO. 1139 did not produce CMCase using sugars such as glucose, fructose, sucrose, and starch. But, while *Pseudomonas* sp. CMC-50produced CMCase with xylan, *Bacillus* sp. NO. 1139 could only produce the enzyme in the presence of CMC and was inhibited in the presence of 0.2 % glucose.[19)] Yamane et al. reported that extracellular cellulase of *Pseudomonas fluorescens* var. *cellulosa* was inducible, and the cellulase formation was enhanced in the presence of cellulose or sophorose.[6)] *Pseudomonas* sp. CMC-50 showed increasing CMCase production as CMC % in media, and the optimal CMC percent was 4%.

Effect of pH on the activity and stability of CMCase. The effect of pH on the CMCase activity in the crude extract of CMC-50 was determined in buffers of different

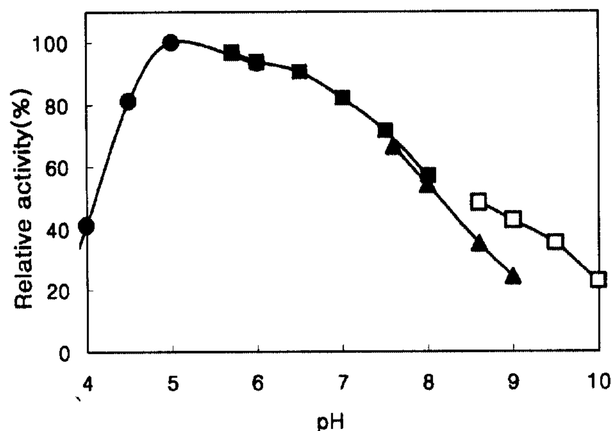

Fig. 5. Effects of pH on the activity of CMCase produced by *Pseudomonas* sp. CMC-50.
●, Citrate-phosphate buffer (pH 2.6-6.0); ■, Phosphate buffer (pH 5.7-8.0); ▲, Tris-HCl buffer (pH 7.6-9.0); ❑, Glycine-NaOH buffer (pH 8.6-10.6).

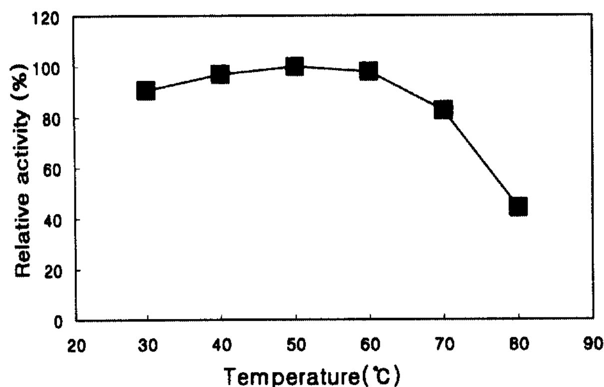

Fig. 6. Effects of temperature on the activity of CMCase produced by *Pseudomonas* sp. CMC-50.

pHs. This crude enzyme was active over a broad pH range. (Fig. 5) The strongest activity was observed at pH 4.5 to 7.5 with more than 70% of the maximum activity at 37°C. The stability of CMCase was investigated at different pHs. After preincubation at 30°C for 1 h, the residual activity of crude enzyme was measured at pH 5. The enzymatic activity was not inactivated over pH 4 to 8. (Data not shown) This results was similar to CMCase produced by other bacteria.[1,3] As observed, the CMCase of *Pseudomonas* sp. CMC-50 could be used in neutral pH condition by the industry.

Effect of temperature on the activity and stability of CMCase. The effect of temperature on the activity of CMCase was measured by varying incubation temperature from 30 to 80°C at intervals of 10°C. The crude enzyme was active at 30 to 70 with at least 80% maximum activity (Fig. 6). The optimum temperature of the enzyme activity was approximately 50°C, which was higher than those observed for CMCase from other bacteria.[1,20] The thermal stability of CMCase was measured by incubating the enzyme solution at different temperatures for 20 min. The CMCase activity was stable up to 60°C (Fig. 7). This CMCase from *Pseudomonas*

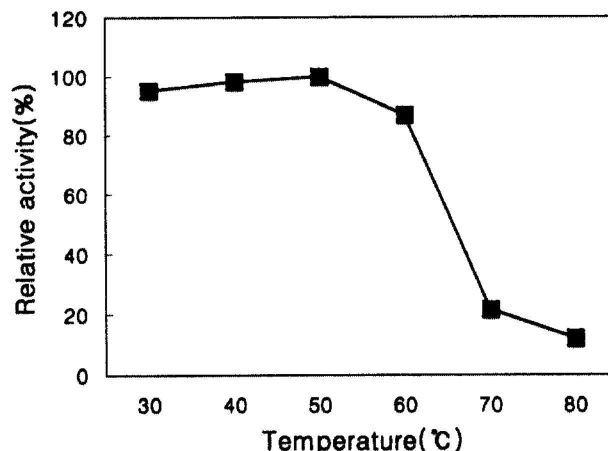

Fig. 7. Effects of temperature on the stability of CMCase produced by *Pseudomonas* sp. CMC-50.

sp. CMC-50 showed a strong thermal stability compared to those of other strains reported previously.[10] In order to further characterize CMCase from *Pseudomonas* sp. CMC-50, we are currently in the process of purifying the enzyme.

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References

1. Son, Y. J., Sul, O. J., Chung, D. K., Han, I. S., Choi, Y. J. and Jeong, C. S. (1997) Isolation and Characterization of *Trichoderma* sp. C-4 producing cellulase. *Kor. J. Appl. Microbiol. Biotechnol.* **25**, 346-353.
2. Hahm, B. K., Kim, Y. K., Yu, J. H. and Bai, D. H. (1997) Isolation and Identification of cellulase-producing microorganism, and determination of optimal culture condition. *Kor. J. Food Sci. Technol.* **29**, 1028-1032.
3. Yoon, K. H., Jung, K. H. and Park, S. H. (1997) Isolation and enzyme production of a cellulase-producing *Bacillus* sp. 79-23, *Kor. J. Appl. Microbiol. Biotechnol.* **25**, 546-551.
4. Okoshi, H., Ozaki, K., Shikata, S., Oshino, K., Kawai, S. and Ito, S. (1990) Purification and Characterization of Multiple Carboxymethyl Cellulase from *Bacillus* sp. KSM-522. *Agric. Biol. Chem.* **54**, 83-89.
5. Halsall, D. M. and Gibson, A. H. (1986) Comparison of two *cellulomonas* strains and their interaction with *Azospirillum brasilense* in degradation of wheat straw and associated nitrogen fixation. *Appl. Environ. Microbiol.* **51**, 855-861.
6. Yamane, K., Suziki, H. and Nisizawa, K. (1970) Purification and properties of extracellular and cell-bound cellulase components of *Pseudomonas fluorescens* var. *cellulosa*. *J. Biochem.* **67**, 19-35.
7. Scott, T. C., Cosgrove, J. M., Coon, C. L., Kenney, J. A.

and Scott, C. D. (1995) Use of cellulase from *Pseudomonas fluorescens* for the hydrolysis of waste paper in an attrition bioreactor. *Appl. Biochem. Biotechnol.* **51/52**, 537-543.
8. Bayer, E. A. and Lamed, R. (1986) Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. *J. Bacteriol.* **167**, 828-836.
9. Tahara, N., Yano, H. and Yosinaga, F. (1997) Two types of cellulase activity produced by a cellulose-producing *Acetobacter* strain. *J. Fermen. Bioeng.* **83**, 389-392.
10. Kawai, S., Okoshi, H., Ozaki, K., Shikata, S., Ara, K. and Ito, S. (1988) Neutrophilic *Bacillus* strain, KSM-522, that produces an alkaline carboxymethyl cellulase. *Agric. Biol. Chem.* **52**, 1425-1431.
11. Ilmen, M., Saloheimo, A., Onnela, M. L. and Penttila, M. (1997) Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei. Appl. Environ. Microbiol.* **63**, 1298-1306.
12. Shikata, S., Saeki, K., Okkoshi, H., Yoshimatsu, T., Ozaki, K., Kawai, S. and Ito, S. (1990) Alkaline cellulase for laundry detergents: production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. *Agric. Biol. Chem.* **54**, 91-96.
13. Horikoshi, K. (1971) Production of alkaline enzyme by alkophilic micro organism. Part I. Alkaline protease produced by *Bacillus* No. 22. *Agric. Biol. Chem.* **35**, 1407-1414.
14. Horikoshi, K., Nakao, M., Kurono, Y. and Sashihara, M. (1984) Cellulase of an alkalophillic *Bacillus* strain isolated from soil. *Can. J. Microbiol.* **30**, 774-779.
15. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal.Chem.* **31**, 426-428.
16. Bradford, M. (1959) A rapid and sensitive method for the quantitation of microgram-quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* **72**, 248-254.
17. Dees, C., Ringelberg, D., Scott, T. C. and Phelps, T. J. (1995) Characterization of cellulose-degrading bacterium NCIMB 10462. *Appl. Biochem. Biotechnol.* **51/52**, 263-273.
18. Krieg, N. R. and Holt, J. G. (1984) Grem-negative aerobic rods and cocci. In *Bergey's Manual of Systematic Bacteriology*, pp. 141-199, Williams & Wilkins, Baltimore.
19. Fukumori, F., Kudo, T. and Horikoshi, K. (1985) Purification and properties of a cellulase from alkalophilic *Bacillus* sp. No. 1139. *J. Gen. Microbiol.* **131**, 3339-3345.
20. Ito, S., Shikata, S., Ozaki, K., Kawai, S., Okamoto, K., Inoue, S., Takei, A., Ohata, Y. and Satoh, T. (1989) Alkaline cellulase for launder detergents: production *Bacillus* sp. KSM-635 and enzymatic properties. *Agric. Biol. Chem.* **53**, 1275-1281.