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Isolation and Characterization of *Bacillus* sp. KD1014 Producing Carboxymethyl-Cellulase

Kyung-Dong Lee, Jungho Kim, and Hoon Kim*

Department of Agricultural Chemistry, Sunchon National University, Sunchon 540-742, Korea

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A microorganism producing carboxymethyl-cellulase (CMCase) was isolated from 300 soil and compost samples. The isolate was identified as *Bacillus* sp. by Biolog™ test and fatty acid analysis, and named as *Bacillus* sp. KD1014. The isolate could degrade, in addition to CMC, various kinds of polysaccharides such as levan, xylan, starch, and filter paper but hardly degrade microcrystalline Avicel. The optimum growth and CMCase production of the isolate was observed between 16- and 25 hr-culture at 45℃ and pH 5.0. The maximum CMCase activity was observed at pH 4.5 and 60℃. The CMCase was found to bind to Avicel. The CMCase was internally cleaved as growth continued. When crude supernatant was used for activity staining, three major bands were detected on a native gel, however, only one major band was detected on a denaturating gel after removal of the detergent.

Key words: CMCase, *Bacillus* sp. KD1014, internal cleavage, binding to Avicel, activity staining

Bacillus species produce and secrete many hydrolytic enzymes, such as α-amylase, proteases, glucanases, glucose isomerase, and restriction endonucleases, into culture media (17). Among these enzymes, glucanases have been used to produce edible sugars from naturally abundant biomass. Their accessibility to cellulosic substrates has been improved by mechanical and chemical pretreatment of cellulosic materials with the aid of hemicellulases. Many *Bacillus* species have been found to produce cellulases and xylanases and their genes were cloned and characterized (21). Bacterial cellulases from *Cellulomonas fimi* have been intensively investigated (4). Many cellulases were found to be composed of at least two domains, cellulose-binding domain and catalytic domain (21). Recently, cellulases of *Bacillus*, including *B. subtilis* BSE616 (9, 15, 16) and *B. subtilis* IFO 3034 (14), were found to be able to bind to cellulosic materials which they could not hydrolyze.

In this paper, we describe isolation and identification of a microorganism producing cellulase, and some properties of the crude CMCase.

Materials and Methods

Media

PBYC, PBY (1.0% polypeptone, 0.5% beef extract, 0.2% NaCl, 0.05% yeast extract) supplemented with 0.5% carboxymethyl-cellulose (CMC) was used for isolation of bacteria degrading CMC. LBC, LB (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) supplemented with 0.5% CMC was routinely used for production of CMCase.

Isolation of CMC-degrading microorganisms

CMC-degrading microorganisms were isolated from 300 soil and compost samples collected from regions of Chonnam and Kyungbuk. After incubation at 55℃ for 24 hr, a series of decimal dilutions of each sample was made with 0.9% NaCl, and appropriate volumes of each dilution was spread onto PBY agar plates. After overnight incubation at 55℃, the colonies were tooth-picked onto PBYC plates and grown. After growth, the plates were stained with Congo red by flooding with 0.1% dye solution and positive colonies were selected as primary candidates. The candidates were tested for their ability to hydrolyze various polysaccharides such as xylan, starch, levan, filter paper, and microcrystalline Avicel by the same procedure. Colonies with high hydrolyzing activities on agar plates were selected and tested for their ability to produce CMCase in broth culture using *B. subtilis* BSE616 as a positive control, and an isolate with the highest activity was used for further studies.

Identification of the isolate

* To whom correspondence should be addressed

General characteristics of the isolate were determined according to the Manual of the Methods for General and Molecular Bacteriology (3) and Bergey's Manual of Systematic Bacteriology (20). Utilization of different carbon sources was tested by Biolog MicroStation™ 2 System (Biolog, Inc., Hayward, CA) using BUGM™ (Biolog Universal Growth Medium) supplemented with 1.0% glucose and Biolog GP Microplate™ as in the manufacturer's instruction manual (2). Methyl esters of cellular fatty acids of the isolate was analyzed by a gas liquid chromatography (Hewllet-Packard 5890A) and MIDI Microbial Identification System.

Assay of CMCase activity

CMCase activity was determined by measuring reducing sugars released from CMC (grade of medium viscosity) by the dinitrosalicylic acid method (13). One unit of the enzyme was defined as the amount catalyzing liberation of 1 μmol glucose equivalent per min at 50°C.

Binding of CMCase produced by the isolate to microcrystalline cellulose (Avicel)

Binding of CMCase to Avicel was carried out by mixing an appropriate amount of the culture supernatant with 50 mg of Avicel swollen in 50 mM sodium phosphate buffer (pH 6.9) and gently agitating for 1 hr at 4°C. After centrifugation at 3,000 rpm for 3 min in a microcentrifuge, the supernatant was collected and used for quantitation of binding ability. Debinding was carried out by adding distilled water to the CMCase-bound Avicel and gently shaking for 20 min at room temperature. Protein concentrations were determined according to Lowry *et al.* (12).

Western blot analysis

Proteins in 8 and 18 hr culture supernatant of the isolate were precipitated with 60% (v/v) cold acetone, electrophoresed by SDS-PAGE according to Laemmli (11), and used for Western blotting. A rabbit antiserum was prepared as described previously (8, 10) using the purified cytoplasmic endo-β-1,4-glucanase (CMCase) from *Escherichia coli* (pBS1) transformant (15). Western blotting was performed according to Harlow and Lane (5) using alkaline phosphatase-conjugated secondary antibody (Promega, USA).

Activity staining of the culture supernatant

Activity staining of CMCase of the isolate was carried out by staining electrophoresed gels with Congo red. The isolate was grown at 45°C for 24 hr, proteins in the culture supernatant were precipitated with 30~90% ammonium sulfate on ice. The pellet was dissolved in 50 mM citrate buffer (pH 5.5) and dialyzed against the same buffer. The proteins were then separated on a native and on a SDS polyacrylamide gel. SDS was removed by washing the SDS gel twice in a buffer containing 25% (v/v) isopropanol for 1 hr at room temperature (1). Renaturation of the proteins was done by soaking the gel in two changes of the buffer for 1 hr at room temperature. The gels were then transferred onto 1.5% (w/v) agar gels containing 1% (w/v) CMC in the buffer, and incubated for 1 hr at 55°C. The agar gels were then stained with 0.1% Congo red solution and destained with 1 M NaCl.

Results and Discussion

Isolation of microorganisms producing CMCase

About 1,000 positive colonies were isolated from 300 preincubated (1 day at 55°C) soil and compost samples collected from 100 sites in the primary screening. One hundred isolates among them showing large halos were tested for their ability to hydrolyze various polysaccharides such as xylan, levan, filter paper, starch, and Avicel at 37 and 50°C. Three isolates with higher hydrolyzing activity than the control strain, *Bacillus subtilis* BSE616, for all the substrates tested were selected and named KD1014, KD1040, and KD1133 (Table 1). Among them, KD1014 showing higher extracellular saccharolytic activities than other isolates was used for further studies.

Table 1. Extracellular saccharolytic activities of the isolates

Isolates \ Substrates	CMC		Levan		Xylan		Filter paper		Starch		Avicel	
	37°C	55°C	37°C	55°C	37°C	55°C	37°C	55°C	37°C	55°C	37°C	55°C
KD1014	1.1#	1.1	1.4	1.2	1.4	1.1	1.2	1.2	0.5	0.3	-*	-
KD1040	0.9	1.0	1.1	1.0	1.2	1.0	1.1	1.0	0.8	0.4	-	-
KD1133	1.1	1.0	1.3	1.1	1.2	1.1	1.2	1.1	0.6	0.4	-	-
BSE616	0.9	0.8	1.0	1.0	1.0	0.9	1.0	1.1	0.4	0.3	-	-

#Numbers represent diameter (cm) of clear zone.

*; not detectable.

Table 2. General characteristics of the *Bacillus* sp. KD1014

Tested characteristics	Result	Tested characteristics	Result
Length (μm)	0.8	Growth at 20℃	+
Gram stain	+[a]	40℃	+
Motility (growth at 50℃)	+	70℃	-
Cell morphology	Rod	Hydrolysis of	
Spore formation	+	Starch	+
Growth in air	+	Gelatin	+
Gas from glucose	-	Casein	+
Catalase activity	+	Urease	-
Oxidase activity	+	Nitrate reduction	-
Citrate utilization	-	Sulfate reduction	-
Temperature optimum	50~65℃	Indole formation	-

[a]Symbols; +, positive; -, negative

Table 3. Utilization of various carbon sources by the isolate *Bacillus* sp. KD1014[a]

Tested carbon sources	Result	Tested carbon sources	Result
D-trehalose	-[b]	Citric acid	+
Methyl pyruvate	+	Formic acid	+
Glycerol	*	D-galactonic acid	+
Dextrin	+	α-keto butyric acid	+
Glycogen	+	α-keto glutaric acid	-
Tween 40	+	D, L-lactic acid	*
L-arabinose	*	Propionic acid	-
L-arabtiol	*	Succinic acid	*
D-fructose	-	D-alanine	+
L-fucose	-	L-alanine	+
D-galactose	+	L-asparagine	-
α-D-glusose	-	L-glutamic acid	*
m-inositol	*	L-histidine	-
Cellobiose	-	Hydroxy-L-proline	-
i-erythritol	+	L-leucine	*
α-lactose	-	L-ornithine	-
Maltose	+	L-phenyl alanine	+
D-mannitol	+	L-proline	+
D-mannose	-	D-serine	*
D-melibiose	-	L-serine	*
D-raffinose	*	γ-amino butyric acid	+
L-rhamnose	*	Thymidine	+
D-sorbitol	+	Phenyl ethylamine	+
Sucrose	*	2,3-butanediol	-
Acetic acid	-	L-aspartic acid	+
cis-aconitic acid	-		

[a]Carbohydrate utilization test was performed by Biolog MicroStation™ 2 Sytem (Biolog, Inc., Hayward, CA) as described in Materials and Methods.

[b]Symbols; +, positive; *, positive/negative; -, negative in 4 hr.

Identification of the isolate KD1014

Morphological and physiological characteristics of the isolate KD1014 are shown in Table 2. KD1014 is an aerobic, Gram-positive, spore-forming, rod-shaped bacterium. The ability of KD1014 to utilize various carbon sources determined by Biolog test was found to be similar to that of *B. subtilis* with a similarity of 0.542 and to that of *B. alcalophilus* ss. *halodurans* with a similarity of 0.311 (Table 3). The cellular fatty acid composition of KD1014 was also similar to that of *B. subtilis* with a similarity of 0.508. Major cellular fatty acids of KD1014 were branched chain fatty acids such as 40.64% of 15:0 anteiso, 18.69% of 15:0 iso, 14.79% of 17:0 anteiso and 11.40% of 17:0 iso (Table 4). From the results, the isolate was identified as a *Bacillus* species and named *Bacillus* sp. KD1014.

Table 4. Composition of the cellular fatty acids of the isolate KD 1014

Fatty acids	Composition (%)
14:0 iso	2.37
15:0 iso	18.69
15:0 anteiso	40.64
16:0 iso	7.88
16:1 wll	0.55
16:0	3.13
iso 17:1 w10c	0.58
17:0 iso	11.40
17:0 anteiso	14.79

Effect of temperature and pH on growth, CMCase production, and CMCase activity of *Bacillus* sp. KD 1014

Bacillus sp. KD1014 grew at temperatures between 20℃ and 60℃ and the optimum growth temperature was 30℃ (Fig. 1A). When the *Bacillus* sp. KD1014 was grown at 30℃, the cell density was increased until 30 hr and then decreased drastically thereafter. The optimum temperature for CMCase production by the isolate was 45℃ (Fig. 1B). The CMCase activity in the culture broth reached its maximum after 15 hr of growth and remained at the level until 30 hr. At 50℃, CMCase activity in the culture broth reached its maximum after 12 hr of growth and then decreased sharply thereafter. The CMCase activity of KD1014 was about 20% higher than that of the control strain, *B. subtilis* BSE616, in both the broth and solid culture (data not shown).

The growth of *Bacillus* sp. KD1014 was almost same at three different initial pHs, 5.0, 7.0, and 9.0 (Fig. 2A). However, the CMCase production at initial pH 5.0 was higher than at pHs 7.0 and 9.0 (Fig. 2B). At initial pHs 5.0 and 7.0, the level of CMCase activities in culture broths reached their maxima after 15 hr of growth and remained at the level until 30 hr. At initial pH 9.0, the CMCase activity was increased until 15 hr and then decreased thereafter.

CMCase, in the supernatant of *Bacillus*sp. KD1014 cultured for 24 hr at 45℃ was relatively stable in acidic condition, pH 4.0 to 6.0, and showed a maximal activity at pH 4.5 (data not shown). The optimum temperature for the CMCase was 60℃ (data not shown).

Binding of the CMCase produced by *Bacillus* sp. KD1014 to Avicel

Although the CMCase did not hydrolyze Avicel, about 65% of the CMCase could be bound to Avicel under the experimental condition and 70% of the CMCase bound to Avicel was released from Avicel with the addition of distilled water. The CMCases produced by *B. subtilis* BSE616 (9) and *B. subtilis* IFO 3034 (14), which also did not hydrolyze Avicel, could be bound to Avicel under the same experimental condition.

Changes in the sizes of the CMCase during the growth of *Bacillus* sp. KD1014

Changes in the sizes of the CMCase during the growth of *Bacillus* sp. KD1014 was determined by Western blotting using a rabbit antiserum against cytoplasmic endo-β-1, 4-glucanase from *E. coli* (pBS1) transformant. When the CMCase from 8 hr-cultured supernatant was analyzed by Western blotting, a major band, corresponding to about 35, 000 daltons, was found along with three minor bands (Fig. 3). With 18 hr-cultured sample, the major band of the 8 hr sample almost disappeared. Even though the quantity

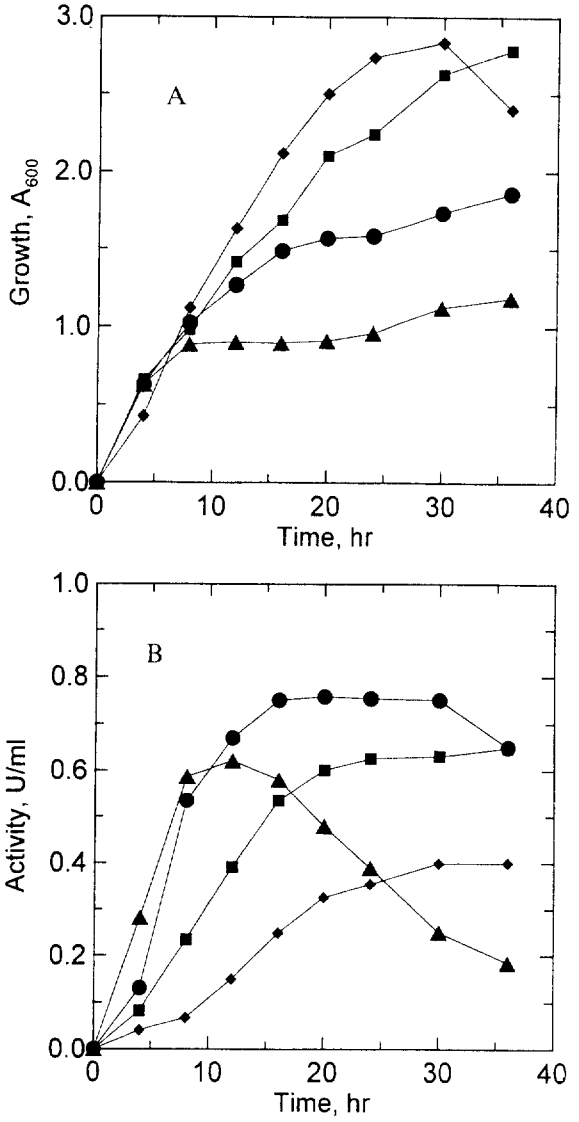

Fig. 1. Growth (A) and production of CMCase (B) by *Bacillus* sp. KD1014 at different temperatures. Symbols: ◆, 30℃; ■, 37℃; ●, 45℃; ▲, 50℃.

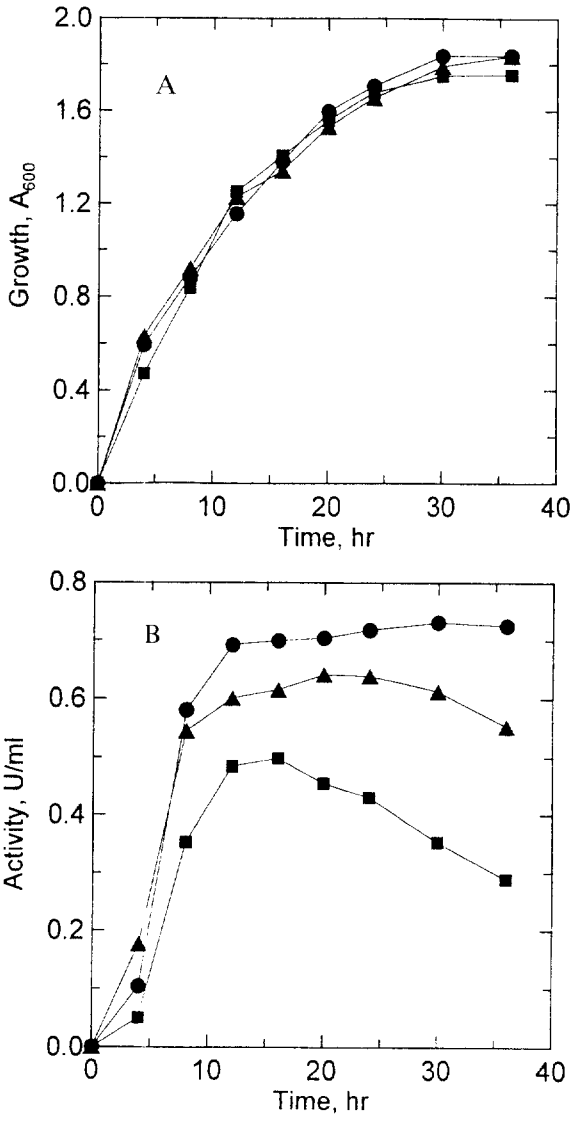

Fig. 2. Growth (A) and production of CMCase (B) by *Bacillus* sp. KD1014 at different initial pHs. Symbols: ●, pH 5.0; ▲, pH 7.0; ■, pH 9.0.

of each band could not be accurately determined on a nitrocellulose membrane, it was thought to be reasonable to state that the proteins of the major band was being converted to smaller protein molecules. Similar phenomenon was also observed with *B. subtilis* BSE616 (8), *B. megaterium* (pCK98) transformant (6, 7, 10), harboring the structural gene for the endo-β-1,4-glucanase, and *B. subtilis* DLG (18), although the protein sizes of the major bands were different from that of *Bacillus* sp. KD1014.

Activity staining of the CMCase

When a crude enzyme concentrate of *Bacillus* sp. KD 1014 was electrophoresed on a native gel, three major active bands were detected on a replica agar gel containing CMC (Fig. 4A) indicating that at least three kinds of native CMCases were existing in the concentrate of the isolate culture. However, only one band corresponding to 32,000 daltons was observed on a denaturating gel (Fig. 4B). It might be possible that some of the native CMCase of the isolate exist in dimeric, oligomeric, or aggregated form as in the case of α-amylase from *B. licheniformis* (6, 19). This possibility is being further analyzed in this laboratory and detailed information will be published elsewhere.

Acknowledgement

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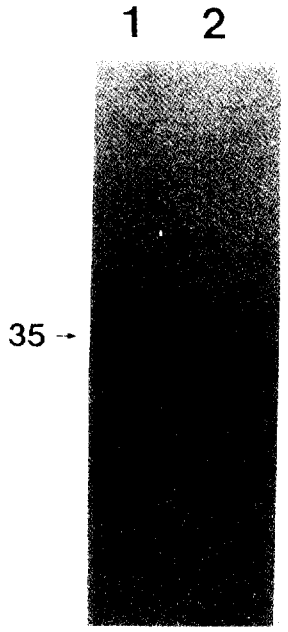

Fig. 3. Western blot analysis of the CMCase produced by *Bacillus* sp. KD1014. Lane 1, 8 hr-cultured sample; lane 2, 18 hr-cultured sample. The number in the left margin indicates molecular weight of the protein in kilodaltons.

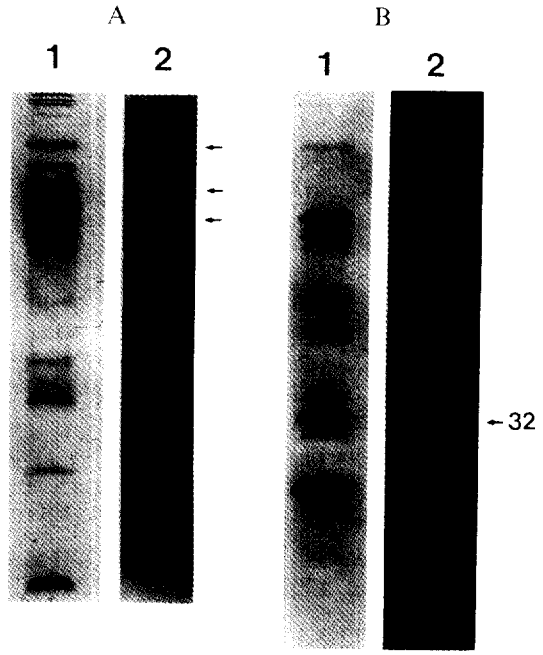

Fig. 4. Activity and protein staining of the CMCase produced by *Bacillus* sp. KD1014 after native (panel A) and SDS-PAGE (panel B). Lane 1, protein staining; lane 2, activity stained zymogram. The arrows indicate CMCase-active bands. The number in the right margin indicates molecular weight of the protein in kilodaltons.

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