Characterization of Acidic Carboxymethylcellulase Produced by a Marine Microorganism, *Psychrobacter aquimaris* LBH-10

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A microorganism hydrolyzing carboxymethylcellulose (CMC) was isolated from seawater, identified as *Psychrobacter aquimaris* by analysis of 16S rDNA sequences, and named *P. aquimari* LBH-10. This strain produced an acidic carboxymethylcellulase (CMCase), which hydrolyzed carboxymethylcellulose (CMC), cellobiose, curdlan, filter paper, *p*-nitrophenyl- β -D-glucopyranoside (pNPG), pullulan, and xylan, but there was no detectable activity on avicel and cellulose. The optimal temperature for CMCase produced by *P. aquimari* LBH-10 was 50°C and more than 90% of its original activity was maintained at broad temperatures ranging from 20 to 50°C after 24 hr. The optimal pH of the CMCase was 3.5, and more than 70% of its original activity was maintained under acidic conditions between pH 2.5 and 7.0 at 50°C after 24 hr. The optimal pH of CMCase produced by *P. aquimaris* LBH-10 seems to be lower than those produced by any other bacterial and fungal strain. CoCl₂, EDTA, and PbCl₂ at a concentration of 0.1 M enhanced CMCase-produced *P. aquimaris* LBH-10, whereas HgCl₂, KCl, MnCl₂, NiCl₂, and SrCl₂ inhibited it.

Key words : Carboxymethylcellulase, *Psychrobacter aquimaris*, marine microorganism, rice bran, acidophilic CMCase

Introduction

Cellulose is considered to be the most important reservoir of carbons to convert glucose, which can be used as a substrate for production of ethanol, organic acids, and other chemicals [3,8]. Cellulose is a linearly condensed polymer consisting of D-anhydroglucopyranose joined together by β -1,4-glycosidic bonds with a degree of polymerization (DP) from 100 to 20,000 [39]. Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials [20]. Enzymatic process to hydrolyze cellulosic materials could be accomplished through a complex reaction of three enzymes; endoglucanase (carboxymethylcellulase), exocellobiohydrolase (avicelase), and β -glucosidase [38]. The enzymatic saccharification of cellulosic materials such as rice hulls was performed by commercial cellulases, in which the major cellulase was carboxymethylcellulase [4]. Most of commercial cellulases have been produced by *Aspergillus* and *Trichoderma* species with solid state cultures [11,12,27]. Bacterial cellulase systems of

Clostridium, *Cellomonas*, *Bacillus*, *Thermonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, and *Acetivibrio* species have been reported [31,37].

Hydrolysates of cellulosic biomass contain mainly glucose and xylose, which can be used as substrates for the production of ethanol by microbial fermentations [3]. Production of ethanol from lignocellulosic biomass by simultaneous saccharification and fermentation (SSF) was first reported in 1977 [33]. Recently, cellulases have been used for removing fuzz from the surface of cellulosic fibers, enhancing the softness, and brightening cotton fabrics in textile and detergent industries [2,6,13]. Cellulases are also used in animal feeds for improving the nutritional quality and digestibility [24].

Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the severe and wide range of environments [30]. Activities of enzymes produced by psychrophilic organisms are reported to be much higher at low temperature than those of its mesophilic and thermophilic counterparts [15]. A gene coding for a cold-active lipase produced by *Psychrobacter* sp, isolated from Antarctic seawater, was cloned and expressed in *E. coli* [30]. We first described characterization of the car-

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boxymethylcellulase (CMCase) produced by *P. aquimaris* isolated from seawater and identified in this study.

Materials and Methods

Isolation of a marine microorganism producing cellulases

To isolate microorganisms producing cellulases, seawater of the seashore in the Kyungsang Province of Korea was suspended with 0.85% (w/v) NaCl and then the suspension was cultivated on the marine agar plate at 30°C for 3 days. Isolated cultures were prepared by transferring cells from the agar plate to 200 ml medium containing 20 g/l carboxymethylcellulose (CMC), 2.5 g/l yeast extract, 5 g/l K_2HPO_4 , 1.0 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.6 g/l $(NH_4)_2SO_4$ at in 500 ml Erlenmeyer flasks and cultured at 30°C for 3 days under aerobic conditions. Basis on the productivity of carboxymethylcellulase (CMCase), one microorganism was selected for the production of CMCase and identified by nucleotide sequencing of 16S rDNA.

Analysis of 16S rDNA sequences

For the nucleotide sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Co., Madison, USA). Two primers annealing at the 5' and 3' end of the 16S rDNA were 5'-AGAGTTTGTATCCTGGCTCAG-3' (positions 8 to 27, *E. coli* 16S rDNA numbering) and 5'-AAGGAGGTGATCCAGCCGCA-3' (positions 1541 to 1522, *E. coli* 16S rDNA numbering), respectively [36]. PCR amplification was performed as described in the previous report [14]. The PCR reaction was run for 35 cycles in a DNA thermal cycler (Model No. 9700, Perkin-Elmer Co. Wellesley, USA). Amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, and purified. Purified products were cloned into pGEM-T Easy vector (Promega Co., Madison, USA) and subsequently sequenced using ALF Red automated DNA sequencer (Pharmacia, Sweden). The 16S rDNA sequence of the isolate was aligned with those in the GenBank database. Multiple alignment of sequences and calculations of levels of sequence similarity were performed by using CLUSTAL W [34]. The neighbor-joining phylogenetic analysis was carried out with MEGA program [16].

Scanning electron microscopic observations

Samples of cultured *P. aquimaris* LBH-10 were fixed by

3.0% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 0.1% (w/v) $MgSO_4$ at 4°C for 30 min. Fixed samples were post-fixed by 1.0% (v/v) osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) at 4°C for 24 hr and serially dehydrated in ethyl alcohol. After critical-point drying, they were coated with gold using a sputter-coating system (sputter-coater 5150A, Edward High Vacuum International, Crawley, England) and then examined with a scanning electron microscope (JSM-35CF, JEOL, Japan) operating with an accelerating voltage of 5.0 KV. Images were digitized and stored as tagged image file format files in the microscope computer.

Production and purification of carboxymethylcellulase (CMCase)

The isolated microorganism was grown at 30°C in the marine broth. The main culture was carried out in the medium containing 20 g/l rice bran, 2.5 g/l yeast extract, 5 g/l K_2HPO_4 , 1.0 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.6 g/l $(NH_4)_2SO_4$ at 30°C for 48 hr under aerobic conditions. After cultivation, cells were removed from the culture broth by centrifugation at $12,000 \times g$ for 20 min and supernatants were concentrated by ultrafiltration. Concentrated supernatants were brought to 70% (w/v) saturation with $(NH_4)_2SO_4$, left at 4°C for 24 hr, and centrifuged at $12,000 \times g$ for 20 min. The precipitate was dissolved in 10 mM sodium phosphate buffer (pH 6.5) and dialyzed overnight against the same buffer. The crude enzyme solution was filtered through 0.45 μm membrane filter. Purification of the CMCase from the crude enzyme solution was followed as described in the previous report [14,19].

Analytical methods

Cell growth, as a dry cells weight, was measured by directly weighing the biomass after drying to constant weight at 100-105°C after collection of cells centrifugation at $12,000 \times g$ for 10 min. The CMCase activity was measured by mixing 0.1 ml of enzyme solution with 0.1 ml of 1.0% (w/v) CMC in 10 mM sodium phosphate buffer, pH 6.5 at 50°C for 60 min. The reaction was stopped by adding the 3,5-dinitrosalicylic acid (DNS) reagent. The mixture was boiled for 10 min, cooled in ice, and its optical density at 550 nm was determined [25]. Glucose (Sigma-Aldrich, UK) was used for preparation of a calibration curve. One unit of CMCase was defined as the amount of enzyme that released 1 μmol of glucose per min at 50°C.

Substrate specificity

The substrate specificity of the purified enzyme was determined by performing the assay with avicel (microcrystalline cellulose), CMC, cellobiose, cellulose, curdlan, filter paper, *p*-nitrophenyl- β -D-glucopyranoside (pNPG), pullulan, and xylan. The activity for hydrolyzing avicel, CMC, cellulose, cellobiose, curdlan, pullulan, and xylan was determined as described in a previous report [14,21]. The activity for hydrolyzing filter paper carried out by mixing 0.5 ml of enzyme solution in 0.5 ml of 10 mM sodium phosphate buffer (pH 6.5) and one strip of Whatman filter paper No.1 (size 1 cm x 6 cm). The mixture was incubated at 50°C for 60 min. The amount of reducing sugar produced by the reaction was measured by DNS method [25]. The activity for hydrolyzing pNPG as a β -glucosidase activity was measured by the amount of releasing *p*-nitrophenol. The reaction mixture contained 50 μ l of 0.1 M pNPG solution, 100 μ l of enzyme solution in 10 mM sodium phosphate buffer (pH 6.5). The reaction mixture was incubated at 50°C for 60 min and the absorbance of *p*-nitrophenol released was measured at 410 nm. A standard curve was prepared using *p*-nitrophenol.

Effect of temperature on activity and thermal stability

The optimal temperature for enzymatic reaction of the purified enzyme for hydrolyzing CMC in 10 mM sodium phosphate buffer (pH 6.5) was determined by incubating the mixture of the purified enzyme and 1.0% CMC for 60 min at different temperatures ranging from 20 to 90°C. The reaction was stopped by addition of DNS solution. Thermal stability was performed by incubation of the purified enzyme in 10mM sodium phosphate buffer (pH 6.5) at different temperatures ranging from 20 to 90°C for 24 hr. The residual activity of each sample for hydrolysis of CMC was then quantified at 50°C for 60 min with the DNS method.

Effect of pH on activity and pH stability of CMCase

The optimal pH for enzymatic reaction of the purified enzyme for hydrolyzing CMC was examined by incubating the mixture of the purified enzyme and 1.0% CMC in the presence of appropriate buffers; 10 mM citrate buffer (pH 2.5 to 6.0), 10 mM sodium phosphate buffer (pH 6.0 to 7.0), 10mM Tris-HCl (pH 7.0 to 9.0), and 10 mM glycine-NaOH (pH 9.0 to 11.0), at 50°C for 60 min. The reducing sugars from the reaction were measured by the DNS method. The pH stability of the purified enzyme was determined by incubating the mixture of the purified enzyme and 1.0% CMC

in various pHs of buffers described above for 24 hr. The residual activity of each sample for hydrolyzing CMC was then quantified at 50°C for 60 min with the DNS method.

Effect of additives on CMCase activity

To examine effects of various additives on the purified enzyme, the enzymatic reaction for hydrolyzing CMC was performed by the presence of additives. The additives used in this study were CoCl₂, EDTA, HgCl₂, KCl, MnCl₂, NiCl₂, PbCl₂, and SrCl₂ at a final concentration of 0.1 M. The reaction mixture with each additive were incubated at 50°C for 60 min. The CMCase activity was measured by DNS method. The activity assayed in the absence of additives was defined as control.

Results and Discussion

Identification of a microorganism isolated from seawater

A microorganism hydrolyzing carboxymethylcellulose (CMC) was isolated from seawater of the Kyungsang Province in Korea and named as a strain LBH-10. The phylogenetic analysis of a strain LBH-10 using its 16S rDNA nucleotide sequence data showed that this strain had more than 98% of homology with *Psychrobacter aquimaris*, as shown in Table 1. Based on the evolution distance and the

Table 1. Similarity of the isolated microorganism from seawater with *Psychrobacter* species based on the 16S rDNA sequences

Strain	Identities (%)	Nucleotide differences/compared
<i>P. aquimaris</i> AY722804	98.1	22/1157
<i>P. proteolyticus</i> AJ272303	97.3	31/1153
<i>P. arcticus</i> AY444823	97.1	34/1160
<i>P. fozi</i> AJ430827	97.1	34/1161
<i>P. okhotskensis</i> AB094794	97.0	34/1160
<i>P. namhaensis</i> AY722805	96.8	36/1160
<i>P. cryohalolentis</i> AY660685	96.6	40/1162
<i>P. luti</i> AJ430828	96.6	39/1160
<i>P. urativorans</i> AJ609555	96.4	42/1162
<i>P. gacincta</i> AJ312213	95.8	49/1161
<i>P. submarinus</i> AJ309940	95.8	49/1162
<i>P. frigilicola</i> AJ609556	95.6	51/1162
<i>P. marincola</i> AJ309941	95.6	51/1162
<i>P. faecalis</i> AJ421528	95.4	53/1161
<i>P. jeotgali</i> AF441201	94.6	62/1165
<i>P. pacificensis</i> AB016057	94.6	63/1167
<i>P. immobilis</i> U39399	94.5	64/1154

phylogenetic tree resulting from 16S rDNA sequencing and the neighbour-joining method [7], this strain was identified as *Psychrobacter aquimaris* and named as *P. aquimaris* LBH-10, as shown in Fig. 1. The scanning electron microscopic observations show that *P. aquimaris* LBH-10 is a round-shaped bacterium, as shown in Fig. 2. *Psychrobacter* is a genus within gamma-proteobacteria. These bacteria live in extremely cold

Fig. 1. Neighbour-joining tree based on 16S rDNA sequences of *P. aquimaris* LBH-10. Numbers at nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled dataset. Scale bar indicates 0.1 nucleotide substitution per nucleotide position.

habit as well as on deep sea environments, and these are oxidase-positive, with a strictly oxidative metabolism, and demonstrate a moderate halotolerance [29].

Substrate specificity

Substrate specificity of CMCase produced by *P. aquimaris* LBH-10 was investigated by assaying the activity hydrolyzing various cellulosic substrates such as avicel, CMC, cellobiose, cellulose, filter paper, *p*-nitrophenyl- β -D-glucopyranoside (pNPG), and xylan [10]. The CMCase degraded CMC, cellobiose, curdlan, filter paper, pNPG, pullulan, and xylan, whereas there was no detectable activity on avicel and cellulose, as shown in Table 2. Significance of each value was analyzed by MYSTAT software (Systat Software, Inc., Chicago, USA). The CMCase had significantly higher activity towards CMC, a soluble cellulosic substrate with β -1,4-linkages, than any other substrates. Relative hydro-

Table 2. Substrate specificity of the carboxymethylcellulase produced by *P. aquimaris* LBH-10

Substrate	Activity (U/ml)	Relative activity (%)
Avicel	ND	-
CMC	269.7 $\pm$ 5.1 ^a	100.0 $\pm$ 1.9
Cellobiose	43.7 $\pm$ 9.2 ^c	16.2 $\pm$ 3.4
Cellulose	ND	-
Curdlan	28.3 $\pm$ 6.5 ^d	10.5 $\pm$ 2.4
Filter paper	49.6 $\pm$ 7.0 ^c	18.4 $\pm$ 2.6
pNPG	31.8 $\pm$ 8.1 ^d	11.8 $\pm$ 3.0
Pullulan	12.7 $\pm$ 4.0 ^e	4.7 $\pm$ 1.5
Xylan	58.3 $\pm$ 5.4 ^b	21.6 $\pm$ 2.0

ND: not detectable
Values with different letters are significantly different at $p < 0.05$.

(A)

(B)

Fig. 2. Scanning electron microscopic observations of *P. aquimaris* LBH-10 with magnifications of 5,000 times (A) and 15,000 times (B).

lyzing activities of cellobiose, which is a disaccharide with β -1,4-linkage produced from the partial hydrolysis of cellulose, and xylan containing mainly of β -1,4-linked xylose, were 16.2 and 21.6%, respectively. It also hydrolyzed curdlan with β -1,3-linkages, and pullulan, which consists of maltotriose and maltotetraose units with both $-(1\rightarrow)$ and $-(1\rightarrow)$ linkages [32]. Furthermore, pNPG as a substrate for β -glucosidase was also degraded by CMCase produced by *P. aquimaris* LBH-10 unlike other endoglucanases [23]. CMCase produced by *P. aquimaris* LBH-10 could not hydrolyze crystalline type of cellulosic materials such as avicel and cellulose powder. This is probably due to the low affinity of the purified enzyme for crystalline cellulose. From these results, it seems more appropriate to refer to this enzyme as an endo type of cellulase having relatively low β -glucosidase activity.

Effect of temperature on activity and thermal stability of CMCase

The effect of temperature on CMCase produced by *P. aquimaris* LBH-10 was examined at various temperatures ranging from 20 to 90°C for 60 min at pH 6.5. Relative activities of CMCase at 20, 30, 40, 50, 60, 70, 80, and 90°C were 68.4 ± 5.3 , 82.3 ± 4.3 , 89.9 ± 5.1 , 100.0 ± 4.1 , 99.6 ± 3.2 , 45.9 ± 6.4 , 23.1 ± 6.1 , and $22.0\pm5.7\%$, respectively. The optimal temperature for CMCase produced by *P. aquimaris* LBH-10 was 50°C at pH 6.5, as shown in Fig. 3A. The optimal temperature of CMCase produced by a marine bacterium, *P. aquimaris* LBH-10, is similar to those produced by another marine bacterium, *B. subtilis* subsp. *subtilis* A-53 as well as *B. amylolique-*

faciens DL-3 isolated from soil [14,21], however, it is lower than those produced by fungal strains such as *A. japonicus* and *A. niger*, which optimal temperatures for CMCase are 65°C and 70°C, respectively, as shown in Table 3 [1,17].

The thermal stability of CMCase produced by *P. aquimaris* LBH-10 was also determined at various temperatures ranging from 20 to 90°C for 24 hr at pH 6.5. The residual activity in aliquots withdrawn at various temperatures was assayed at 50°C for 60 min under standard conditions. After 24 hr incubation at pH 6.5, more than 90% of its original activity of the CMCase was maintained at broad temperatures ranging from 20 to 50°C, whereas less than 20% of its original activity was observed at temperature higher than 50°C, as shown in Fig. 3B. Due to its psychrophilic characteristics, CMCase produced by *P. aquimaris* LBH-10 seems to be very stable at relatively low temperature [29].

Effect of pH on activity and pH stability of CMCase

The effect of pH on CMCase produced by *P. aquimaris* LBH-10 was examined at various pHs ranging from pH 2.5 to pH 11.0. The optimal pH for CMCase was found to be 3.5, and more than 70% of its original activity was maintained under acidic conditions between pH 2.5 and 7.0, as shown in Fig. 4A. Optimal pHs of CMCases produced by *B. subtilis* subsp. *subtilis* A-53 and *B. amyloliquefaciens* DL-3 were 6.5 and 7.0, respectively [14,21], whereas those of acidic CMCases produced by *Aspergillus*, *Clostridium*, *Paecilomyces*, and *Trichoderma* species ranges from 4.0 to 6.0 [17,31,22,28]. The optimal pH of CMCase produced by *P. aquimaris*

Fig. 3. Optimal temperature (A) and thermal stability of the CMCase produced by *P. aquimaris* LBH-10. For the optimal temperature of CMCase, the enzyme was incubated at pH 6.5 with 1.0% (w/v) CMC at 20, 30, 40, 50, 60, 70, 80, and 90. For the thermal stability of CMCase, the enzyme was incubated at 20 (●), 30 (■), 40 (▲), 50 (◆), 60 (○), 70 (□), 80 (△), and 90°C (◇) for 24 hr. Each of means and standard deviation was obtained from triplicated experiments.

Table 3. Optimal temperature and pH of various carboxymethylcellulases produced by bacterial and fungal microorganisms

Microorganism	Substrate	Temperature (°C)	pH	Reference
<i>B. amyloliquefaciens</i> DL-3	CMC	50	7.0	[21]
<i>B. subtilis</i> subsp. <i>subtilis</i> A-53	CMC	50	6.5	[14]
<i>B. subtilis</i>	CMC	60	5.0-6.0	[31]
<i>Cellomonas</i> sp.	CMC	50	6.0	[9]
	Cellobiose	50	5.5	
	Filter paper	45	6.0	
<i>Clostridium thermocellum</i>	CMC	60	6.0	[31]
<i>Erwinia chrysanthemi</i> 3665	CMC	52	6.2-7.5	[5]
<i>Psychrobacter aquimaris</i> LBH-10	CMC	50	3.5	This study
<i>Aspergillus japonicus</i>	CMC	65	4.5	[17]
<i>Aspergillus niger</i>	CMC	70	6.0-7.0	[1]
<i>Paecilomyces</i> sp.	CMC	60	4.0	[28]
<i>Rhizopus oryzae</i>	CMC	55	5.0-6.0	[26]
<i>Trichoderma viride</i>	CMC	50	4.2	[22]
	Chitosan	60	5.2	

Fig. 4. Optimal pH (A) and pH stability (B) of tCMCase produced by *P. aquimaris* LBH-10. For the optimal pH of CMCase, the enzyme was incubated at 50°C for 1 h with 1.0% (w/v) CMC dissolved in citrate buffer (● pH 2.5, 3.0, 3.5, 4.0, 5.0, and 6.0), sodium phosphate buffer (■ pH 6.0 and 7.0), Tris-HCl buffer (▲ pH 7.0, 8.0 and 9.0) and glycine-NaOH buffer (◆ pH 9.0, 10.0, and 11.0). For pH stability of CMCase, the enzyme was incubated at 50°C with 1.0% (w/v) CMC at pH 3.0 (●), 4.0 (■), 5.0 (▲), 6.0 (◆), 7.0 (▼), 8.0 (○), 9.0 (□), 10.0 (△), and 11.0 (◇) for 24 hr.

LBH-10 seems to be lower than those produced by any other bacterial and fungal strains.

The pH stability of CMCase produced by *P. aquimaris* LBH-10 was also examined at various pHs ranging from pH 3.0 to pH 11.0 for 24 hr. More than 80% of its original activity was observed at pH 3.0 and more than 50% of the original activity was maintained at acidic pHs ranging from 3.0 to 6.0 after 24 hr at 50°C, as shown in Fig. 4B. Less than 20% of the original activity of the CMCase produced by *B. subtilis* subsp. *subtilis* A-53 was maintained at acidic pH less than

6.0 [14]. The pH stability of the CMCase produced by *P. aquimaris* LBH-10 under acidic pH seems to be a special characteristic of this species isolated from marine [35].

Effect of additives on activity of CMCase

The effect of additives on CMCase produced by *P. aquimaris* LBH-10 was investigated in the presence of various additives at the final concentration of 0.1 M. The CoCl_2 , EDTA, and PbCl_2 enhanced the CMCase produced by *P. aquimaris* LBH-10, whereas HgCl_2 , KCl, MnCl_2 , NiCl_2 , and SrCl_2

inhibited the CMCase, as shown in Table 4. Relative activities of CMCase in the presence of 0.1 M CoCl₂, EDTA, HgCl₂, KCl, MnCl₂, NiCl₂, PbCl₂, and SrCl₂ were 134.2±6.9, 144.9±4.5, 69.52±7.6, 77.2±4.0, 64.2±2.1, 84.3±2.6, 118.3±2.2, and 89.0±2.3%, respectively. HgCl₂ and MnCl₂ strongly inhibited CMCase produced *P. aquimaris* LBH-10. It has been suggested earlier that the inhibition by Hg²⁺ ions is not just related to binding the thiol groups, but may be the result of interactions with tryptophan residue or the carboxyl group of amino acids in the enzyme [18].

Unlike a previous report on inhibition of CMCase produced by a bacterial strain isolated from soil [21], CoCl₂, EDTA, and PbCl₂ enhanced CMCase produced by *P. aquimaris* LBH-10. CMCase produced by a marine bacterium, *B. subtilis* subsp. *subtilis* A-53 was reported to be enhanced by 0.01 M EDTA, KCl, and NiCl₂ [14]. It seems that its tolerance against some metals as well as salts is one of distinctive characteristics of marine microorganisms [29]. Most of additives at a concentration of 0.05 M inhibited activity of CMCase produced by *B. amyloliquefaciens* DL-3 isolated from soil [21]. To determine their positive effect, CoCl₂, EDTA, and PbCl₂ were added to CMCase at final concentrations ranging from 0.0 to 1.0 M. Its activity was enhanced by CoCl₂, EDTA, and PbCl₂ with a concentration of 0.25 M, however, CMCase produced by *P. aquimaris* LBH-10 was also inhibited at more than 0.5 M, as shown in Fig. 5. Due to its specificity for substrates, thermal stability at low temperature, optimal acidic pH of 3.5, and higher tolerance for salts and heavy metals, CMCase produced by *P. aquimaris* LBH-10 can be used for hydrolyzing cellulosic materials in severe conditions at low temperatures, acidic environments, and high concentration of metal ions such as in textile, detergent, and animal feed industries. Our next study will be focused on

Table 4. Effect of additives on CMCase produced by *P. aquimaris* LBH-10

Additives	Activity (U/ml)	Relative activity (%)
Control	245.8±15.5 ^d	100.0±6.3
CoCl ₂	329.9±20.0 ^b	134.2±6.9
EDTA	368.5±11.1 ^a	149.9±4.5
HgCl ₂	170.8±18.7 ^g	69.5±7.6
KCl	189.8±9.8 ^f	77.2±4.0
MnCl ₂	157.8±5.2 ^g	64.2±2.1
NiCl ₂	207.2±6.4 ^e	84.3±2.6
PbCl ₂	290.8±5.4 ^c	118.3±2.2
SrCl ₂	218.8±5.4 ^e	89.0±2.2

Fig. 5. The effect of various additives with final concentrations ranged from 0 to 1.0 M on activity of the CMCase produced by *P. aquimaris* LBH-10: ●, EDTA; ■, CoCl₂; and ▲, PbCl₂.

optimization for the production of CMCase by this marine bacterium with low cost of carbon and nitrogen sources.

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초록 : 해양미생물 *Psychrobacter aquimaris* LBH-10가 생산하는 산성 carboxymethylcellulase의 특성에 대한 연구

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Carboxymethylcellulose (CMC)를 분해하는 미생물을 해수에서 분리하였으며, 16S rDNA의 염기서열을 분석하여 동정한 결과, *Psychrobacter aquimaris*로 확인 되어 *P. aquimaris* LBH-10로 명명하였다. 이 균주는 CMC, 셀로바이오스, 커드란, 여과지, *p*-nitrophenyl- β -D-glucopyranoside (pNPG), 폴루란 및 자일란을 분해하였으나, avicel 및 섬유소는 분해하지 못하였다. *P. aquimaris* LBH-10이 생산하는 carboxymethylcellulase (CMCase)의 최적 반응 온도는 50°C이었으며, 20°C에서 50°C의 온도 범위에서 24시간이 경과한 후에도 90% 이상의 활성을 유지하였다. 또한, 이 균주가 생산하는 CMCase의 최적 반응 pH는 3.5이었으며 pH 2.5에서 pH 7.0 사이의 산성 조건하에서 24 시간이 경과한 후에도 70% 이상의 활성을 유지하였다. *P. aquimaris* LBH-10이 생산하는 CMCase의 최적 반응 pH는 지금까지 발견된 섬유소 분해효소 중에서 가장 낮은 pH로 판단된다. 제한된 농도의 CoCl₂, EDTA, 및 PbCl₂은 *P. aquimaris* LBH-10가 생산하는 CMCase의 활성을 증가시켰으나, HgCl₂, KCl, MnCl₂, NiCl₂, 및 SrCl₂는 이 균주가 생산하는 CMCase의 활성을 감소시켰다.