J. Fish. Sci. Technol. 9(2), 57-63, 2006

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Optimal Culture Conditions and Isolation of a ι-Carrageenase-producing Marine Bacterium

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A bacterial strain capable of hydrolyzing carrageenan was isolated from the coast of Busan in Korea. The isolated strain (HS5322) is aerobic, gram-negative, rod-shaped, and motile. Comparison of the 16S rDNA of the isolate with that of known *Pseudomonas* sp. showed that sequence similarity was at most 95%, implying that the isolate is a new *Pseudomonas* species. The organism grew optimally at NaCl concentrations of 2.0 to 2.5%. The optimum temperature and pH for carrageenase production in a 72-h flask culture containing 1% carrageenan was 20°C and pH 8.5, respectively. Of the synthetic substrates tested, the highest enzyme activity was obtained with *p*-nitrophenyl β-D-galactopyranoside.

Key words: Carrageenan, Carrageenase, *Pseudomonas* HS5322

Introduction

Many of the modified carbohydrate residues found in marine plants have properties that are of commercial interest. The major polysaccharides in marine plants, agar, alginate, and carrageenan, are used in the food, pharmaceutical, and cosmetic industries (De Ruiter and Rudolph, 1997). Carrageenans are the main cell wall components of several marine red algae (Rhodophyta), in which they play a variety of structural and signaling roles (Kloareg and Quatrano, 1988; Potin et al., 1999). They consist of a linear backbone of galactopyranose residues linked by alternating α(1→3) and β(1→4) linkages. This regular structure is modified by 3,6-anhydro bridges and substitution with sulfate ester or pyruvate acetyl groups (Smith, 2004). On the basis of the level and position of sulfate or pyruvate substitution, carrageenans are classified into three types, κ, λ, and ί-carrageenans. The reological properties of these carra-geenans are influenced by the substituted parts of the molecule (Therkelson, 1993).

The antiviral properties of several types of carrageenan oligosaccharides have been demonstrated in cell culture and animal models (Smith, 2004). Carrageenan oligosaccharides are produced from various carrageenans by acid hydrolysis (Yamada et al., 2000) and radiation (Relleve et al., 2005). Enzymatic hydrolysis of carrageenan by carrageenases provides an alternative method to prepare carrageenan oligosaccharides. Marine bacteria have been shown to possess at least three types of carrageenases. For example, κ-carrageenase (McLean and Williamson, 1979; Potin et al., 1995) and λ-carrageenase (Johnston and McCandless, 1973) have been isolated from *Alteromonas carrageenovora*, ί-carrageenase from unidentified marine gramnegative aerobic rod bacterium (Greer and Yaphe, 1984) and *Alteromonas fortis* (Gurvan et al., 2001), and κ-carrageenase and ί-carrageenase from *Cytophaga drobachiensis* (Potin et al., 1991) and *Zobellia galactanovorans* (Barbeyron et al., 1998).

In the present study, we report the isolation and identification of a novel marine bacterium producing ί-carrageenase activity and describe the culture conditions yielding optimal growth of the isolate and ί-carrageenase production.

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Materials and Methods

Chemicals

Peptone, yeast extract, and agar were purchased

from PC&S (Busan, Korea). *p*-nitro-phenyl β-D-galactopyranoside (NPG), ι-carra-geenan, and alginate were obtained from Sigma (St. Louis, MO, USA). κ-Carrageenan was purchased from DUCHEFA (Haarlem, Netherlands) and λ-carrageenan from Wako (Osaka, Japan). Substrates used to determine substrate specificity were obtained from Sigma.

Culture medium

The isolated bacteria were cultured on modified Zobell 2216E (MZ) plates containing 10 g bactopeptone, 10 g yeast extract, 20 g ι-carrageenan, 250 mL distilled water, and 750 mL sea water (pH 7.0). Carrageenase activity was assayed by culturing the bacteria in medium containing 5 g ι-carrageenan per liter.

Isolation of strains producing ι-carrageenase

A seaweed sample, *Gelidium amansii*, was collected from the coast of Busan in Korea and homogenized in five volumes of autoclaved seawater. The homogenate was diluted 100-, 1,000-, and 10,000-fold with sterilized seawater and spread onto MZ plates. Bacterial colonies that had formed a clear zone on the plate, indicating carrageenase activity, after 4 days of incubation at 25°C were picked and isolated as a single colony by the same plating method.

Phenotypic tests

Cell morphology of gram-stained culture smears was observed under an optical microscope. Gram staining was performed using a staining kit (Difco, Detroit, MI, USA) according to the manufacturer's instructions. The motility of the isolate was examined using wet mounts. Biochemical tests of the isolate were carried out using VITEK GNI (Biomerieux, Lyon, France).

DNA amplification and sequencing

Genomic DNA of the isolate was prepared by the method of Sambrook et al. (1989). To investigate the 16S rDNA of the isolate, a 1,474-bp PCR product, corresponding to position 8-1,512 (*Escherichia coli* numbering), was sequenced. The fD1 primer used in this experiment was 5'-AGAGTTTGATCCTGGCTCAG-3' and the rP2 primer was 5'-ACGGCTACCTTGTTACGACTT-3'. The forward primers used in this study were 5'-CCTACGGGAGGCAGCAG-3' (341-357) and 5'-CACAAGCGGTGGAGCATGT-3' (position 933-1,011, *E. coli* numbering), and the reverse primers were 5'-GGCCCGGGAACGTATTCACC-3' (position 1,389-1,370, *E. coli* numbering) and 5'-ATTACCGCGGCTGCTGG-3' (position 534-518, *E. coli* numbering). Reaction conditions consisted of 30 cycles at 95°C (1 min), 52°C (30 s) and 72°C (1 min), plus one additional cycle with a final 20-min chain elongation. Sequencing was performed using a DNA sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 310 DNA sequencer (Perkin-Elmer, Norwalk, CT, USA) at the Center for Gene Research (TaKaRa Co., Tokyo, Japan).

Phylogenetic analysis

The sequence of each PCR product was manually aligned with those of the genus *Pseudomonas* obtained from the GenBank databases. 16S rDNA nucleotide sequences of other *Pseudomonas* strains (*P. mendocina*, *P. pseudoalcaligenes*, *P. alcaliphila*, *P. stutzeri*, *P. nitroreducens*, *P. flavescens*, *P. anguilliseptica*, *P. andersonii*) that were similar to those of the isolate were searched using GENETYX-WIN (v8.0; Software Development Co. Ltd., Tokyo, Japan) software. *Escherichia coli* served as the out-group in these comparisons.

Determination of enzyme activity

ι-carrageenase activity was determined using NPG. The reaction mixture contained 250 μL of enzyme solution, 250 μL of 8 mM NPG, and 500 μL of 200 mM Tris-HCl (pH 7.0). After incubation for 20 min at 37°C, the reaction was stopped by adding 1 mL of 0.5 M NaOH. Enzyme activity was determined by mea-suring absorbance at 410 nm and was expressed as 1 μmol *p*-nitrophenol liberated per minute per milliliter of enzyme solution at 37°C.

Determination of culture conditions

Optimum incubation conditions for growth of the isolate and ι-carrageenase production were determined by culturing the cells in MZ medium. Erlenmeyer flasks (250 mL) containing 50 mL of the medium adjusted within a pH of between 3.0 and 9.5 were inoculated with 250 μL of a glycerol stock of the isolate and incubated at 25°C for 3 days. The effect of carbon source on cellular growth and enzyme activity was determined by growing the cells at 25°C for 3 days in MZ medium containing a final 0.5% concentration of one of the following carbon sources: ι-carrageenan, κ-carrageenan, λ-carrageenan, agar, or alginate. The effect of incubation temperature (10, 15, 20, 25, 30°C) on cellular growth and enzyme activity was determined at pH 7.0 following incubation of the culture for 3 days. The effect of ι-carrageenan concentration on cellular growth and enzyme activity was studied by culturing the isolate at 25°C for 3 days in MZ medium containing different concentra-

tions of ι-carrageenan (pH 7.0). The effect of incubation time (12, 24, 48, 72, 96, 120 h) on cellular growth and enzyme activity was determined at 25°C in MZ medium (pH 7.0). Enzyme activity was measured after or during incubation of the isolate by assaying the supernatant of a 5-mL aliquot of culture medium that had been centrifuged at 4°C and 8000 rpm for 20 min. Cell growth was determined by measuring the turbidity of the culture at 600 nm (Ultrospec 3000; Pharmacia Biotech, Tokyo, Japan).

Substrate specificity of the enzyme

Substrate specificities were determined by replacing the NPG in the assay mixture with one of the following substrates: 8 mM *p*-nitrophenyl α-L-fucopyranoside, *p*-nitrophenyl β-L-fucopyranoside, *p*-nitrophenyl β-D-fucopyranoside, *p*-nitrophenyl α-D-galctopyranoside, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl phosphate, or *p*-nitrophenol sulfate.

Results

Isolation of ι-carrageenase-producing bacteria

Five strains of bacteria capable of degrading carrageenan on MZ plates were isolated from seaweed samples. Each isolate was separated on the MZ plate and carrageenase activity was confirmed by hydrolysis of carrageenan after incubation at 25°C for 3 days. The isolate showing the highest carrageenase activity was chosen for further study. The activity from this isolate, named HS5332, was further confirmed by hydrolysis of NPG and ί-carrageenan.

Phenotypic and biochemical properties

The isolated strain was found to be gram-negative and rod-shaped (data not shown). The biochemical properties of strain HS5332 are summarized in Table 1. Results of tests included in the BIOLOG identifycation system suggested that the strain was motile, catalasepositive, and unable to oxidize carbon sources (Table 1).

Phylogenetic analysis

We analyzed the 16S rDNA sequence from strain HS5322 and performed a phylogenetic analysis using sequence data of nine *Pseudomonas* species type strains, with *E. coli* as the out-group (Felsenstein, 1985; Felsenstein, 1993; Fitch and Margoliash, 1967; Saitou and Nei, 1987). The phylogenetic analysis showed that strain HS5322 belonged to the genus *Pseudomonas* (Fig. 1). The similarity of HS5322 with

Table 1. Biochemical characteristics of *Pseudomonas* sp. HS5322

Test	Result
Acid production	
Glucose	-
Lactose	-
Maltose	-
Xylose	-
L-Arabinose	-
Enzyme activity	
Catalase	+
Urease	+
Tryptophan deaminase	-
β-galactosidase	-
Arginine digydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Oxidase	+
Carbon source	
Acetamide	-
Esculin	-
Citrate	-
Malonate	-
Glucose	-
Arginine	-
Lysine	-
Ornithine	-
Raffinose	-
Sorbitol	-
Sucrose	-
Inositol	-
Adonitol	-

P. mendocina was 95%. The sequence of 16S rDNA from the newly identified strain was registered in GenBank (accession no. AY443041) as *Pseudomonas* sp. HS5322.

Optimum culture conditions

Basal medium adjusted to pH 3.0-9.5 was inoculated with a glycerol stock of strain HS5322 and incubated at 25°C and 200 rpm for 72 h in rotary shaker. Higher cell growth and carrageenase activity were obtained under neutral and weak-alkaline pH ranges. Cell growth and enzyme production increased with increasing pH, up to 8.5, and decreased over the alkaline pH range (Fig. 2).

The isolated strain was inoculated in basal medium (pH 7.0) and incubated at 10, 15, 20, 25, and 30°C for 72 h under the same incubation conditions noted above. Cell growth and enzyme activity increased with increasing incubation temperature, reaching a maximum at 20°C (Fig. 3). Incubation of the cells at temperatures above 25°C retarded cell growth and enzyme activity.

Several γ-subclasses of *Proteobacteria* isolated in coastal areas require NaCl for growth. Similarly, the isolate had an absolute requirement of NaCl for

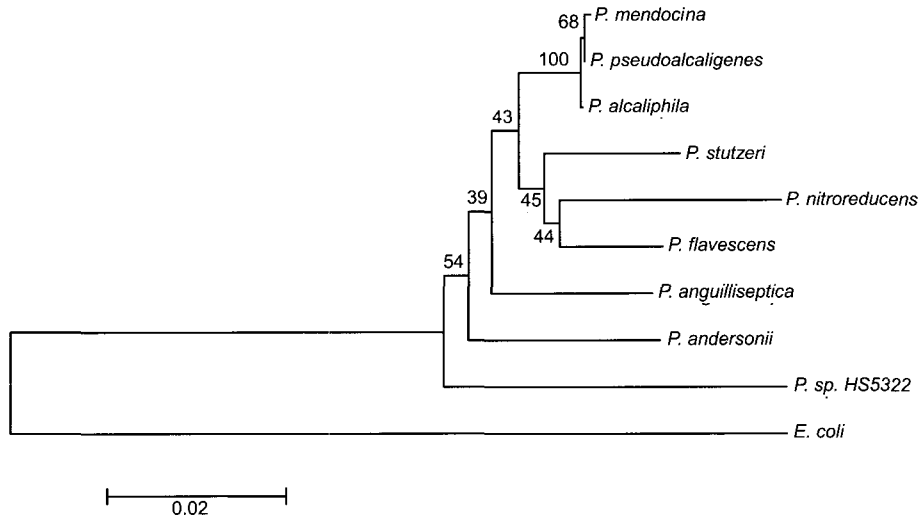

Fig. 1. 16S rDNA dendrogram showing the position of strain HS5322 among several phylogenetically closely related *Pseudomonas* species. Scale bar represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) are shown at the branching points.

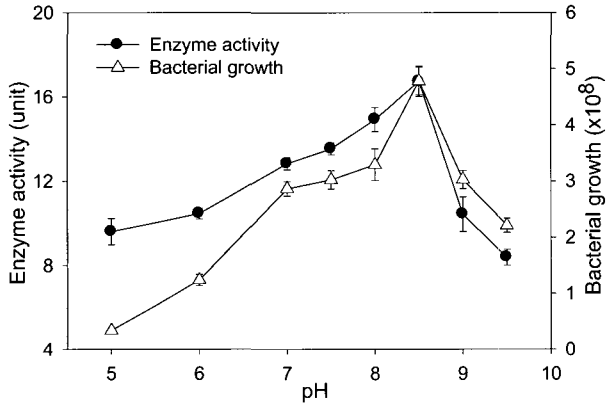

Fig. 2. Effect of pH on *Pseudomonas* sp. HS5322 growth and enzyme activity.

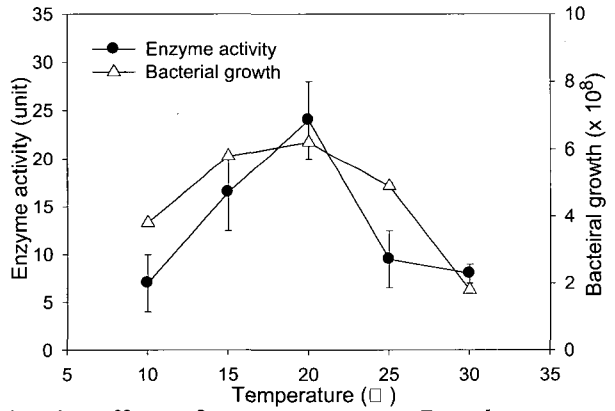

Fig. 3. Effect of temperature on *Pseudomonas* sp. HS5322 growth and enzyme activity.

growth and grew poorly when the concentration of NaCl was lower than 0.5%. The salt concentration producing optimal enzyme activity by strain HS5322 was 1.5-2.0% NaCl, while the concentration producing optimal growth was 2.0-3.0% (Fig. 4).

The optimal incubation time was prolonged until 5 days when strain HS5322 was grown in medium containing 2.0% NaCl at pH 8.5, 20°C. As shown in Fig. 5, the enzyme activity of the isolate was maximal after 72 h of incubation and decreased afterwards.

Substrate specificity of enzyme

As displayed in Table 3, cellular ι-carrageenase activity was highest on NPG and lower on other substrates. These results suggest the enzyme is an endohydrolase that cleaves the internal β(1→4) linkages of carrageenans. Carrageenan-degrading enzymes isolated from various marine bacteria display strict substrate specificity, distinctly recognizing the sulfating pattern of the digalactose repeating unit. This property provides an approach to investigating the structure-function relationships of hydrolases that degrade sulfated polysaccharides (Michel et al., 2001).

Discussion

Proteobacteria isolated from marine environments require salt for their growth (Gonzalez and Moran, 1997) and their abundance is reportedly reduced in the presence of decreased salinity. Strain HS5322 is a gram-negative and aerobic bacterium with an absolute requirement for NaCl. Indeed, the bacterium failed to grow when NaCl was absent from the medium. 16S rDNA sequence analysis suggested that the isolated strain belongs to the γ-subclass of the

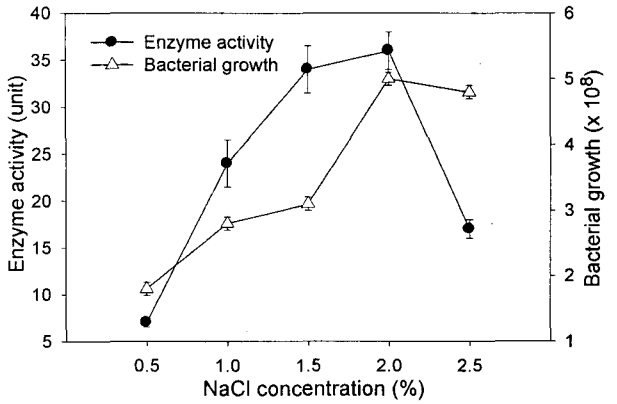

Fig. 4. Effect of NaCl concentration on *Pseudomonas* sp. HS5322 growth and enzyme activity.

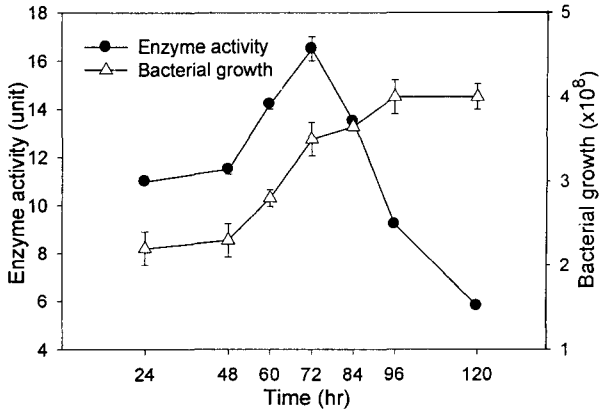

Fig. 5. Effect of incubation time on *Pseudomonas* sp. HS5322 growth and enzyme activity.

Table 2. Effect of carbohydrate sources on ι-carrageenase activity of *Pseudomonas* sp. HS5322

Carbohydrate source	Activity ($A_{410\,nm}$)
Alginate	0.060
Agar	0.003
K-carrageenan	0.021
λ-carrageenan	0.199
ι-carrageenan	0.279

Table 3. Substrate Specificity of the carrageenase

Substrate	Relative activity (%)
p-nitrophenyl α-L-fucopyranoside	15.8
p-nitrophenyl β-L-fucopyranoside	19.2
p-nitrophenyl β-D-fucopyranoside	19.2
p-nitrophenyl α-D-galactopyranoside	25.3
p-nitrophenyl β-D-glucuronide	14.0
p-nitrophenyl α-D-mannopyranoside	23.3
p-nitrophenyl β-D-mannopyranoside	23.2

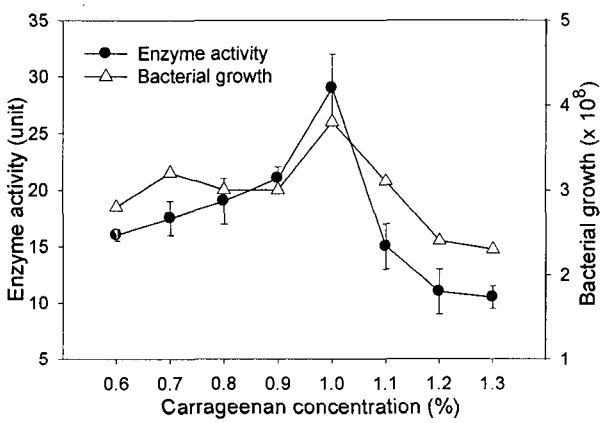

Fig. 6. Effect of ι-carrageenan concentration on *Pseudomonas* sp. HS5322 growth and enzyme activity.

Proteobacteria.

Information obtained from the 16S rDNA sequence of strain HS5322 provides powerful evidence for classifying bacterial species and genus (Woes, 1987). Microorganisms with less than 97% similarity in their 16S rDNA gene sequence do not yield DNA reassociation values of more than 60% and thus are considered to belong to different species (Wayne et al., 1987). To identify the isolate eventually named HS5322, its 16S rDNA sequence was analyzed and found to be closet (95% similarity) to that of *P. straminea*. Comparison of the 16S rDNA sequence of strain HS5322 with those from 11 different *Pseudomonas* species showed less than 97% 16S rDNA similarity; thus, the isolated strain in this study may represent a new species of the genus *Pseudomonas*.

Proteobacteria previously isolated from marine environments have diverse metabolic capabilities, including the ability to utilize ι-carrageenan (Greer and Yaphe, 1984; Gurvan et al., 2001), λ-carrageenan (Johnston and McCandless, 1973), and κ-carrageenan (McLean and Williamson, 1979; Potin et al., 1991). Proteobacteria inhabiting coastal seawater may be of particular interest for the study of organic sulfur cycling in the ocean; for example, the isolate from this study is also involved in the degradation of organic sulfur compounds, especially carrageenans, in marine environments.

The degradation of sulfonated or sulfated compounds by bacterial enzymes has been reviewed in detail (Kertesz, 1999). Sulfated polysaccharides are degraded by lyase to yield sulfated disaccharides, which are subsequently desulfated and then hydrolyzed by a glucosidase to yield monosaccharides. The carbohydrate sulfatase of the marine gram-negative aerobe *Alteromonas carrageenovora* has been proposed to play a role in the degradation of sulfated polysaccharides from marine plants (Barbeyron et al., 1995). Most bacterial carbohydrate lyases are involved in pathways related to the utilization of polysaccharides as carbon sources. Isolate HS5322 was

able to hydrolyze the glucoside bonds in ι-carrageenan (Table 3).

Most strains of marine bacteria producing carrageenases are closely associated with algae and mollusks. The marine proteobacterium *Pseudomonas* sp. HS5322, isolated from a seaweed sample, exhibited carrageenase activity. We analyzed the dynamics of bacterial growth and enzyme activity under various culture conditions to identify those conditions affecting the biosynthesis of carrageenases by the isolated strain.

Acknowledgments

This work was supported by a research grant from Pukyong National University, Korea.

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(Received May 2006, Accepted June 2006)