

Isolation and Culture Properties of a Thermophilic Agarase-Producing Strain, *Microbulbifer* sp. SD-1

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Abstract

An agar-degrading enzyme-producing strain was isolated from seawater. The isolate was identified as *Microbulbifer* sp. SD-1 by 16S rRNA sequencing analysis. The optimal pH and temperature for growth were 6.0 and 30°C, respectively, and growth was possible at pH 9.0 and 60°C. The isolate required 5% NaCl for optimal growth and showed 45% growth activity without NaCl. Agar concentrations of 0-0.4% in the medium did not affect growth. Thin-layer chromatography analysis revealed that this strain could degrade agar into a monosaccharide and oligosaccharide, which may have industrial applications.

Key words: Agar, Agarase, *Microbulbifer* sp., Polysaccharides

Introduction

Agar is a complex polysaccharide composed of agarose and agarpectin that originates from marine red algae such as *Gelidium latifolium*, *G. amansii* and *Gracilaria verrucosa* (Duckworth and Yaphe, 1970). Agar is an abundant natural resource and has been used as a food for several hundred years in oriental nations. At present, agar has a wide variety of uses due to its stabilizing and gelling characteristics. It has been used in microbiological media and for gel electrophoresis because agar is difficult for microorganisms to metabolize and because it forms clear, stable, and firm gels. Agar is also categorized as a “generally recognized as safe” (GRAS) food additive and is used in icings, glazes, processed cheese, jelly sweets, and marshmallows (Fu and Kim, 2010).

Agarolytic enzymes can be divided into two groups depending on their mode of action, α - and β -agarases, which produce agar-oligosaccharides and neoagar-oligosaccharides, respectively (Suzuki et al., 2002). Neoagar-oligosaccharides

possess various biological activities, including antitumor activity, macrophage-stimulating activity to increase immune function, anti-oxidizing activity, probiotic activity, and a skin moisturizing and whitening effect (Rhee et al., 2010). The agar-oligosaccharide produced by α -agarase possesses anti-oxidative and anti-cancer activities (Ohta et al., 2005).

Some researchers have investigated the possibility of obtaining agarolytic enzymes from bacteria (Ohta et al., 2005; Lee et al., 2007). Although many genera of agarolytic bacteria have been identified, mainly from marine environments, since the first isolation from seawater by Gran in 1902, novel and more active agarolytic enzymes are required for industrial applications (Rhee et al., 2010). Therefore, to investigate the possibility of valuable industrial applications, we isolated a novel agar-degrading bacterium from seawater and characterized its culture properties and enzymatic activity.

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Materials and Methods

Isolation of bacteria, media and chemicals

Seawater, seaweeds, and mud sampled from the seashore in Busan, Korea, were used for bacterial isolation. Media for isolation were marine broth 2216 and nutrient broth, which was prepared with 0.4 μm -filtered seawater and 1.5% agar. One hundred microliters of each sample was spread on an agar plate and incubated at 37°C for five days. Lugol's iodine solution (2 g KI and 1 g I₂ in 200 mL water) was then sprayed onto the agar medium. Bacterial colonies that formed a clear zone were selected as agarolytic bacteria. Selected colonies were stored at -80°C with glycerol until required. Media used in this study were obtained from Difco Co. (Detroit, MI, USA) and chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Identification of isolated strain

The isolate was identified by 16S rRNA sequencing and scanning electron microscopy. Chemicals and reagents for DNA manipulation were purchased from Takara Co. (Tokyo, Japan) and SolGent Co. (Daejeon, Korea). Chromosomal DNA purification for 16S rRNA sequencing was performed using the method of Berns and Thomas (1965). The DNA oligonucleotide primers were 27F (5'-AGAGTTTGATCCTGGCT-CAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') and were synthesized by SolGent Co. (Dunbar et al., 2000). The reaction mixture (final volume, 50 μL) contained *Taq* polymerase 0.5 μL (2.5 U), *Taq* polymerase buffer 5 μL ($\times 10$), 2.5 mM dNTP 4 μL , ddH₂O 34.5 μL , 20 pmol each primer 2 μL , and template DNA 2 μL . PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with a final 7 min extension at 72°C. The amplification products were examined by 1% agarose gel electrophoresis, purified with a QIAquick PCR cleanup kit (Qiagen Inc., Valencia, CA, USA), and directly sequenced with the same primers that were used for PCR (SolGent Co.). The resulting sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) program with the ribosomal RNA gene sequence from GenBank (www.ncbi.nlm.nih.gov/BLAST). Morphological observations were performed using a scanning electron microscope (Hitachi S-2400; Hitachi, Tokyo, Japan).

Optimal temperature and pH

Nutrient broth (Difco Co.) with an additional 3% NaCl was used to culture the isolate. To determine the optimal temperature and pH for growth, the strain was cultured at various temperatures between 20 and 80°C at pH 7, and at pH 5.0-10.0 at 37°C. Cultures were incubated at 150 rpm for 24 h and the absorbance at 600 nm was measured.

NaCl requirement

The isolate was cultured in Nutrient broth (pH 6.0) containing 0-10% (w/v) NaCl at 30°C for 24 h and the absorbance at 600 nm was measured.

Effect of agar concentration

The effect of agar concentration on growth of the isolate was determined. The isolate was cultured at 30°C for 30 h at 150 rpm in Nutrient broth containing 0-0.4% (w/v) agar with 3% NaCl at pH 6.0 and the absorbance at 600 nm was measured every 3 h.

Thin-layer chromatography

Thin-layer chromatography (TLC) of agar hydrolysate was performed on a Silica Gel 600 glass plate (F254; Merck Co., Darmstadt, Germany). To confirm the agarolytic activity of *Microbulbifer* sp. SD-1, culture supernatant containing agar hydrolysate from 24 h culture broth with 0.3% (w/w) agar was applied. Five microlitres of culture supernatant were spotted and developed with n-butanol-acetic acid-ddH₂O (2:1:1, v/v) for 1 h. Oligosaccharides were detected by spraying with ethanolic sulfuric acid (375 mL ethanol+100 mL sulfuric acid) followed by drying at 110°C for 10 min (Duckworth and Yaphe, 1970). Galactose as a monosaccharide and lactose as a disaccharide were used as standards.

Results and Discussion

Isolation of agarase-producing strain

A bacterial strain that produced an extracellular agarolytic enzyme was isolated from seawater near Busan, Korea. Bacterial cells that can hydrolyze agar create a shallow depression or liquefaction around colonies on a 1.5% agar plate. Additionally, Lugol's iodine solution was used to confirm the presence of haloes surrounding the colonies. Unstained haloes around colonies on agar plates treated with iodine show that the properties of the gel have been changed by the action of agarase diffusing out from the bacteria. The binding of iodine to agar gels to give a colored complex depends on the structure of the gel matrix. As shown in Fig. 1, the isolate produced extracellular agarase, resulting in a noticeable halo around the colony after staining with Lugol's iodine solution.

Identification of the agarolytic strain

For taxonomic identification, the 16S rRNA sequence (1,457 bp) was determined and compared with sequences available in the GenBank database using the BLAST search program. *Microbulbifer maritimus* strain TF-17 showed the

greatest homology (97% identity), followed by *M. salipaludis* and *M. elongatus* (95 and 94% identity, respectively) (Table 1). Scanning electron microscopy showed that the isolate is rod-shaped, non-flagellum, and 0.5-0.7×2.0-2.5 μm in size,

which are typical properties of *Microbulbifer* sp. (Fig. 2) (Jonnadula et al., 2009). Therefore, the isolate was renamed *Microbulbifer* sp. SD-1.

The genus *Microbulbifer* was first proposed by González

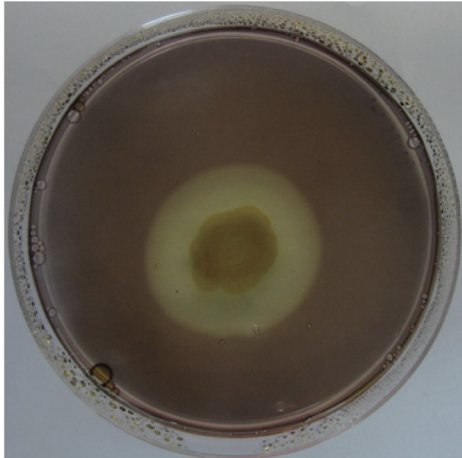


Fig. 1. Staining of agar using Lugol's iodine solution on Marine agar medium plate presenting the diffusion of extracellular agarase.

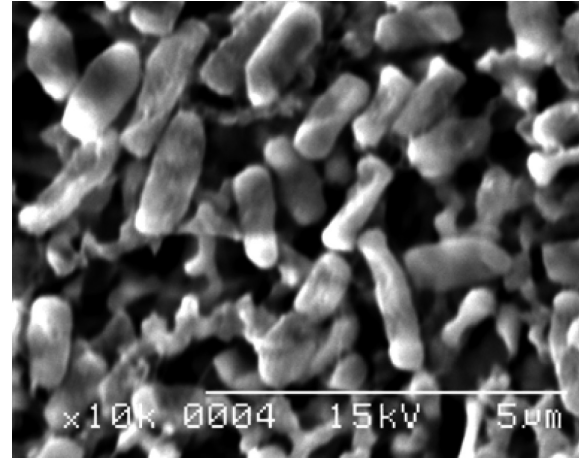


Fig. 2. Scanning electron microscopy of strain showing typical shape of *Microbulbifer* sp. SD-1.

Table 1. 16s rRNA sequence (1,457 bp) and identification of the isolated strain SD-1 by homology search based on 16S rRNA

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1 attgaacgct ggcggcaggc ctaacacatg caagtgcgagc gcgaaagtgc cttcgggaaat
61 aagtagagcg gcggacgggt gagtaacgcg tgggaaattg cccagtagtg ggggacaact
121 cggggaaact cgagctaata ccgcatacgc cctacggggg aaagcggggg atcttcggac
181 ctcgcgctat tggatatgcc cgcgtcggat tagctagttg gtgaggtaaa ggctcaccaa
241 ggcgacgac cgtagctggt ctgagaggat gatcagccac actgggactg agacacggcc
301 cagactccta cgggaggcag cagtggggaa tattggacaa tgggcgcaag cctgatccag
361 ccatgccgcg tgtgtgaaga aggccttagg gttgtaaac actttcagtc gggaggaaag
421 cctaattggt aataccattt aggattgacg ttaccgacag aagaagcacc ggcctaactc
481 gtgccagcag ccgcggtaat acggagggtg cgagcgtaaa tcggaattac tgggcgtaaa
541 gcgcgcgtag cgggttagtt aagctggatg tgaagcccc gggtcaacc tgggaactgc
601 attcagaact ggctggctag agtacgagag agggtagtgg aatttcctgt gtacggtga
661 aatgcgtaga tataggaagg aacaccagtg gccgaaggca ctgcctgctc cgatactgac
721 gctgagggtc gaaagcgtgg ggagcaaca ggattagata ccctggtagt ccacgccgta
781 aacgatgtct actagtcgta gggtccttg agggctttgt gacgcagcta acgcaataag
841 tagaccgctt ggggagtacg gcccaaggt taaaactcaa atgaattgac gggggcccgc
901 acaagcggtg gagcatgtgg ttaattcga agcaacgcga agaaccttac cagggcttga
961 catcctcgga actttccaga gatggattgg tgccctcggg aaccgagtga cagggtctgc
1021 atggctgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgtaacg agcgaacccc
1081 ttgtccctag ttgccagcac ttgggtggg aactctaggg agactgccgg tgacaacccg
1141 gaggaaggtg gggacgacgt caagtcatca tggcccttac gtccctgggt acacacgtgc
1201 tacaatggct ggtacagacg gttgcgagag cgcgagggtg agctaatccg agaaaaccag
1261 tcgtagtcgg gatcggagtc tgcaactcga ctccgtgaag tcggaatcgc tagtaatcgc
1321 gaatcagaat gtcgcggtga atacgttccc gggccttgta cacaccgccc gtcacacat
1381 gggagtgggt tgcctccaga gtggctagtc taaccttcgg ggggacggtc accacggagt
1441 gattcatgac tgggggt
    
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Reference strains	Accession no.	Identity (%)
<i>Microbulbifer maritimus</i> strain TF-17	NR_025772	97
<i>Microbulbifer salipaludis</i> strain SM-1	NR_025232	95
<i>Microbulbifer elongatus</i> strain DSM 6810	NR_025246	94
<i>Marinobacter lutaensis</i> strain T5054	NR_025116	92
<i>Pseudomonas thermotolerans</i> strain CM3	NR_028008	91

The sequence data was analyzed via ribosomal database (www.ncbi.nlm.nih.gov/BLAST).

et al. (1997) for *Microbulbifer hydrolyticus*, a Gram-negative, rod-shaped, strictly aerobic, and biopolymer-decomposing marine gammaproteobacterium. Since then, ten species of the genus have been isolated from various marine sources, including seawater, seaweeds, mud, sandstone, sediments, and solar saltern (Yoon et al., 2004; Jonnadula et al., 2009). One of the prominent features of these bacteria is their ability to degrade more than one insoluble complex polysaccharide, such as agar, carrageenan, cellulose, xylan, alginate, and chitin. *Microbulbifer degradans* 2-40, the only strain reported to degrade 10 polysaccharides, has been reclassified as *Saccharophagus degradans*, and various industrial applications are under development (Ensor et al., 1999).

Optimal growth temperature of *Microbulbifer* sp. SD-1

The growth rate of *Microbulbifer* sp. SD-1 at various temperatures was measured (Fig. 3). *Microbulbifer* sp. SD-1 showed maximum growth at 30°C, and 86, 81, 80, and 68% of this maximum growth rate at 37, 40, 50 and 60°C, respectively. No or little growth was observed at 20 and 70°C. The optimal growth temperature of *Microbulbifer* sp. SD-1 was

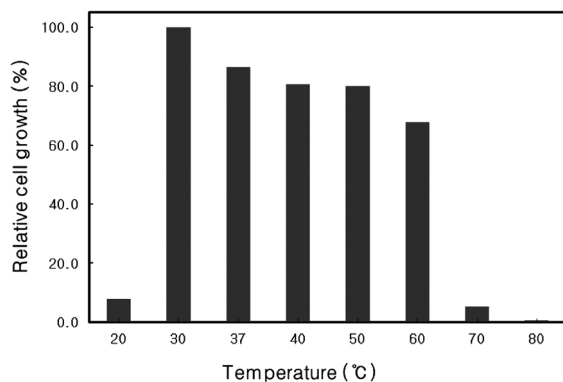


Fig. 3. Effect of temperature on the cell growth by *Microbulbifer* sp. SD-1.

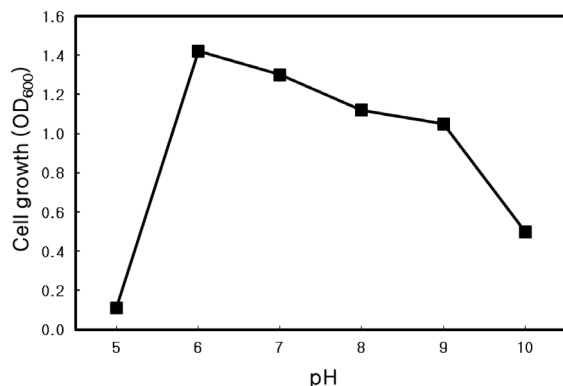


Fig. 4. Effect of pH on the cell growth by *Microbulbifer* sp. SD-1.

similar to that of *M. elongatus*, which was 25-30°C. The maximal temperature for growth of *Microbulbifer* sp. SD-1 was 60°C (68% of maximum growth rate), which is much higher than that in homologues. The maximal temperatures for growth of *M. maritimus* and *M. salipaludis*, which showed the highest identity based on 16S rRNA analysis, were 48 and 45°C, respectively. These data suggest that *Microbulbifer* sp. SD-1 has thermophilic properties and raises the possibility of thermophilic agarase production by this isolate (Jonnadula et al., 2009).

Optimal pH of *Microbulbifer* sp. SD-1

The optimal pH for growth of *Microbulbifer* sp. SD-1 was determined using culture media pH-adjusted to 5.0-10.0 (Fig. 4). The data indicated that pH 6.0 was optimal, and that 92-74% of the maximum growth rate occurred at pH 7-9. No or little growth was observed below pH 5.0 or over pH 10.0. These results are similar to those for *M. maritimus* (optimal pH 6.5-7.5), meaning that *Microbulbifer* sp. SD-1 is a neutrophilic bacterium (Yoon et al., 2004; Jonnadula et al., 2009). Accordingly, the growth medium was adjusted to pH 6.0.

NaCl requirement of *Microbulbifer* sp. SD-1

The effect of NaCl concentration on the growth of *Microbulbifer* sp. SD-1 was determined (Fig. 5). Growth rate increased with NaCl concentration from 0-5% and then rapidly decreased at NaCl concentrations of 6% or greater; little growth occurred at 9-10% NaCl. The optimum NaCl concentration for growth of *Microbulbifer* sp. SD-1 was 5%, which is similar to those (3-5% NaCl) of many halophilic marine bacteria. Around 45% of the maximum growth rate occurred in the absence of NaCl. The optimal NaCl concentration for growth of *M. maritimus* was 2-4% and no growth occurred in the absence of NaCl. However, growth was evident at 10% NaCl, illustrating the various NaCl tolerances of *Microbulbifer* species.

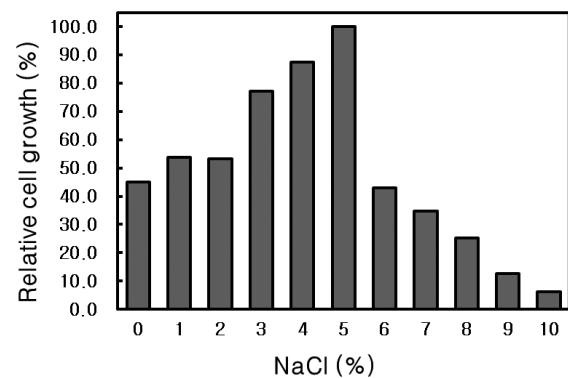


Fig. 5. Effect of NaCl on the cell growth by *Microbulbifer* sp. SD-1.

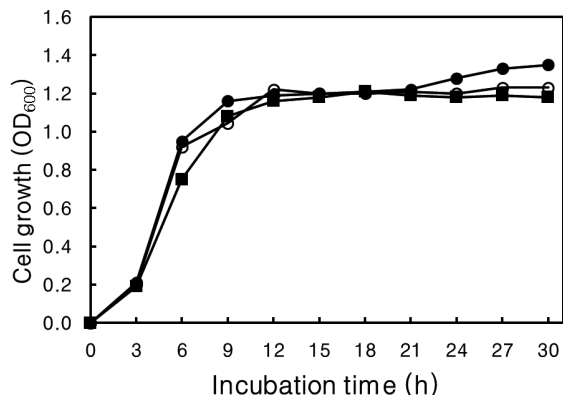


Fig. 6. Growth kinetics of *Microbulbifer* sp. SD-1 at various agar concentration in batch culture condition (●, without agar; ○, 0.2% agar; ■, 0.4% agar).

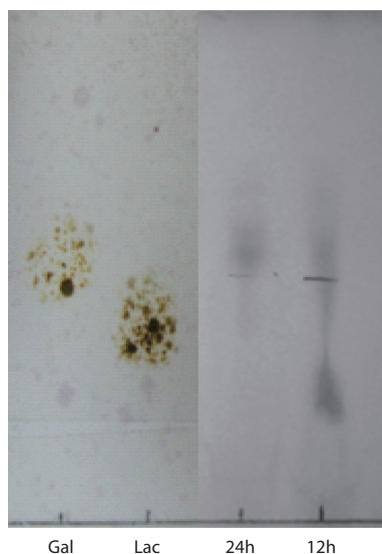


Fig. 7. Thin-layer chromatography analysis of agarolytic products hydrolyzed from agar by *Microbulbifer* sp. SD-1. Gal; galactose, Lac; lactose, 24 h; 24h culture supernatant, 12h; 12 h culture supernatant.

Effect of agar

Agar, a type of polysaccharide, can be a source of carbohydrate for strains that can hydrolyze agar into smaller saccharides. The effect of agar on growth rate was determined (Fig. 6). No effect on growth rate was evident at more than 30 h culture or at the time to reach the stationary phase at 12 h, with or without agar in the medium. These results suggest that *Microbulbifer* sp. SD-1 may use agar as a secondary carbon source, after other nutrients were exhausted. Most *Microbulbifer* strains, including *M. salipaludis* and *M. elongatus* but not *M. maritimus*, have been reported to exhibit agarase activity (Yoon et al., 2004; Jonnadula et al., 2009).

TLC analysis of agar hydrolysate

To confirm the agarolytic activity of *Microbulbifer* sp. SD-1, supernatant from a 24 h culture with 0.3% (w/w) agar was applied to a silica gel TLC plate along with galactose and lactose as standards (Fig. 7). When the plate was developed and visualized, two spots were detected. *Microbulbifer* sp. SD-1 produced a monosaccharide and an oligosaccharide longer than lactose after 12 h culture. After 24 h culture, it produced mainly a monosaccharide similar to galactose. These results are similar to those for *Cellvibrio mixtus* SC-22, in that production of monosaccharide increased with culture time, and different from those of *Agarivorans* sp. JA-1 which produced only a hexamer (Lee et al., 2006; Rhee et al., 2010). Whether *Microbulbifer* sp. SD-1 produces α - and/or β -agarase remains unclear; however, this novel strain may have industrial applications due to its rapid, thermophilic monosaccharide production. Research on the characteristics, over-expression, activity on other marine polysaccharides, and optimal monosaccharide production conditions of this strain and agarase will continue.

Acknowledgments

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References

- Berns KI and Thomas CA Jr. 1965. Isolation of high molecular weight DNA from *Hemophilus influenzae*. *J Mol Biol* 11, 476-490.
- Duckworth M and Yaphe W. 1970. Thin-layer chromatographic analysis of enzymic hydrolysates of agar. *J Chromatogr* 49, 482-487.
- Dunbar J, Ticknor LO and Kuske CR. 2000. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl Environ Microbiol* 66, 2943-2950.
- Ensor LA, Stosz SK and Weiner RM. 1999. Expression of multiple complex polysaccharide-degrading enzyme systems by marine bacterium strain 2-40. *J Ind Microbiol Biotechnol* 23, 123-126.
- Fu XT and Kim SM. 2010. Agarase: review of major sources, categories, purification method, enzyme characteristics and applications. *Mar Drugs* 8, 200-218.
- González JM, Mayer F, Moran MA, Hodson RE and Whitman WB. 1997. *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int J Syst Bacteriol* 47, 369-376.
- Jonnadula R, Verma P, Shouche YS and Ghadi SC. 2009. Characterization of *Microbulbifer* strain CMC-5, a new biochemical variant

- of *Microbulbifer elongatus* type strain DSM6810^T isolated from decomposing seaweeds. *Curr Microbiol* 59, 600-607.
- Lee DG, Kim NY, Jang MK, Lee OH and Lee SH. 2007. Isolation and characterization of a marine bacterium *Thalassomonas* sp. SL-5 producing β -agarase. *J Life Sci* 17, 70-75.
- Lee SA, Kim JU, Jung JG, Kim IH, Lee SH, Kim SJ and Lee JH. 2006. Production of β -agarase in batch and fed-batch culture by *Agarivorans* sp. JA-1. *Korean J Biotechnol Bioeng* 21, 389-393.
- Ohta Y, Hatada Y, Miyazaki M, Nogi Y, Ito S and Horikoshi K. 2005. Purification and characterization of a novel alpha-agarase from a *Thalassomonas* sp. *Curr Microbiol* 50, 212-216.
- Rhee YJ, Han CR, Kim WC, Jun DY, Rhee IK and Kim YH. 2010. Isolation of a novel freshwater agarolytic *Cellvibrio* sp. KY-YJ-3 and characterization of its extracellular β -agarase. *J Microbiol Biotechnol* 20, 1378-1385.
- Suzuki H, Sawai Y, Suzuki T and Kawai K. 2002. Purification and characterization of an extracellular α -neogaroooligosaccharide hydrolase from *Bacillus* sp. MK03. *J Biosci Bioeng* 93, 456-463.
- Yoon JH, Kim IG, Oh TK and Park YH. 2004. *Microbulbifer maritimus* sp. nov., isolated from an intertidal sediment from the Yellow Sea, Korea. *Int J Syst Evol Microbiol* 54, 1111-1116.