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Isolation of a Novel *Tenacibaculum* sp. JS-1 and Characterization of Its β-Agarase

Jin Sun Kim[1], Young Min Woo[2], Dong-Geun Lee[1,2], Andre Kim[1,2], and Sang-Hyeon Lee[1,2]*

[1]*Department of Pharmaceutical Engineering, Silla University, Busan 46958, Republic of Korea*
[2]*Department of Bioscience, Graduate School, Silla University, Busan 46958, Republic of Korea*

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This study reports the isolation of a bacterium capable of degrading agar and the characterization of its agarase. An agar-degrading marine bacterium JS-1 was isolated using Marine agar 2216 media from seawater collected from the seashore of Angolpo, Changwon, Gyeongnam Province, Republic of Korea. An agar-degrading bacterium was named as *Tenacibaculum* sp. JS-1 by phylogenetic analysis based on 16S rRNA gene sequence. The extracellular crude agarase was prepared from the culture media of *Tenacibaculum* sp. JS-1 and used for characterization. Relative activities at 20, 30, 40, 50, and 60℃ were 39, 73, 100, 74, and 53%, respectively. Relative activities at pH 5, 6, 7, and 8 were 46%, 67%, 100%, and 49%, respectively. Its extracellular agarase showed maximum activity (164 U/l) at pH 7.0 and 40℃ in a 20 mM GTA buffer. The residual activities after heat treatment at 20, 30, and 50℃ for 30 min were 84, 73, and 26% or more, respectively. After 2 h heat treatment at 20, 30, 40, and 50℃, the residual activities were 80, 64, 52 and 21%, respectively. Thin layer chromatography analysis suggested that *Tenacibaculum* sp. JS-1 produces extracellular β-agarases that hydrolyze agarose to produce neoagarooligosaccharides, including neoagarohexaose (12.3%), neoagarotetraose (65.1%), and neoagarobiose (22.6%) at 6 h. *Tenacibaculum* sp. JS-1 and its β-agarase could be valuable for producing neoagarooligosaccharides with a variety of functional properties. These properties include inhibiting bacterial growth, slowing down starch degradation, and whitening, which are of interest for pharmaceuticals, food, cosmeceuticals, and nutraceuticals.

Keywords: **β-agarase, marine bacterium, neoagarooligosaccharides, *Tenacibaculum* sp. JS-1, thin layer chromatography (TLC)**

Introduction

Korea is surrounded by the sea on three sides, and the domestic production of red algae, which is the raw material for agar, amounts to several thousand tons per year. However, only about 6.5% of the red algae is utilized, leaving the majority unattended. Agar is used in the food industry, such as in jelly and diet foods [1, 2]. In addition, due to its strong coagulation power and resistance to decomposition, it is widely utilized in molecular biology experiments and microbial media [3].

Agar is a polysaccharide that is a major component of algae, such as *Grateloupia filicina*, and is composed of agarose and agaropectin [4]. Agarose, a neutral polysaccharide, accounts for about 70% of agar and is a conjugate of β-D-galactose linked in a 1→3 bond [5, 6]. Agaropectin, an acidic polysaccharide, is a conjugate of 3,6-anhydro-α-L-galactose linked in a 1→4 bond [5, 6]. Agar-derived oligosaccharides have been reported to exhibit numerous functional properties in living organisms. Agar oligosaccharides have been reported to inhibit bacterial growth, prevent starch aging, whitening, and provide antioxidant benefits. They are considered a high-value-added material that can be utilized in diverse areas such as new drug development [7].

Methods for producing agar-derived oligosaccharides include enzymatic hydrolysis and acid hydrolysis. The acid hydrolysis method generates by-products after the

***Corresponding author**
Phone: +82-51-999-5624, Fax: +82-51-999-5628
E-mail: slee@silla.ac.kr

reaction, requires a neutralization process, and faces challenges related to the poor functionality and stability of the produced oligosaccharides [8]. The enzymatic hydrolysis method can produce agar-derived oligosaccharides more easily because agar-degrading enzymes act specifically on polysaccharides under mild conditions, omitting the neutralization process [8]. Agarase, an enzyme that decomposes agar, includes α-agarase and β-agarase. α-Agarase can produce agarooligosaccharides [9], while β-agarase can produce neoagarooligosaccharides by decomposing agar or agarose [8]. Most agarases originate from marine bacteria, and their characteristics differ according to their bacterial sources [5, 8]. New strains could also offer new agarase genes for the large-scale production of functional agarooligosaccharides or neoagarooligosaccharides [10, 11]. Agarase could be useful in the industrial sector [12]. Therefore, it is important to search for novel agar-degrading strains and characterize the enzymes produced by these strains, as they are essential for producing high-value-added products using underutilized agar. Accordingly, this study isolated and identified a strain from seawater collected from Angolpo, Changwon, Gyeongnam Province, Republic of Korea. The study reported on the enzymatic activity of a novel strain that produces β-agarase.

Materials and Methods

Isolation and identification of an agar-degrading strain

Samples isolating of bacteria with agar-degrading activity were collected from Angolpo, Gyeongnam Province, Republic of Korea. Samples were spread on Marine broth 2216 medium (Difco), with which 1.5% (w/v) agar added. The samples were then cultured at 30℃. After culturing, the strain JS-1, which decomposes Marine agar 2216 medium through agar-decomposing activity, was isolated in pure form. An isolated bacterial strain was cultured, and genomic DNA was extracted using the Wizard Genomic DNA Isolation Kit (Promega, Cat.#: A1120). Subsequently, the 16S rDNA gene fragment was amplified. As PCR primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') were used. DNA sequencing was performed by Bionics (Republic of Korea). The analyzed DNA sequence was reviewed for similarity to reported strains using BLAST, and a neighbor-joining tree was created using phylogeny analysis in the Mega program (ver. 11) and analyzed using the bootstrap method (n = 1,000).

Measurements of growth and enzyme activity of an agar-degrading strain over different incubation periods

The strain JS-1 was inoculated into 4.0 ml of Marine broth 2216 medium and cultured with shaking at 30℃ and 250 rpm for one day. Afterward, 4 ml of the culture medium was inoculated into an Erlenmeyer flask containing 50 ml of Marine broth 2216 medium supplemented with 0.2% (w/v) agar and cultured with shaking at 30℃ and 250 rpm for 6 days. During the shaking culture, 1.0 ml of culture medium was collected every 24 h to measure the growth and agar-degrading enzymatic activity over time. The growth of the strain was measured spectrophotometrically at 600 nm.

Preparation of crude enzyme solution

The JS-1 strain was cultured for days, and with of enzymatic data was recorded at different to incubation times. After centrifuging the culture broth at 3000 ×*g* for 15 min at 4℃ to remove the bacterial cells, 15 ml of the resulting supernatant was placed in Snake Skin Dialysis Tubing (Thermo Fisher Scientific, USA). Dialysis was conducted in a beaker containing 900 ml of 20 mM Tris-HCl (pH 7.0) buffer at 4℃ for 2 h twice, and then for more than 12 h once. A new buffer solution was used for each dialysis. The crude enzyme solution, after dialysis, was filtered through a membrane (0.45 μm, Millipore, USA) and subsequently stored in a refrigerator at 4℃.

Enzyme assay

Agarase activity was determined by the enzymatic production of reducing sugars from agarose [8]. The DNS solution was prepared by dissolving 13.2 g of NaOH, 7.07 g of 3,6-dinitrosalicylic acid, 5.53 g of sodium sulfate, 204 g of potassium tartrate (Rochelle salt), and 5.07 g of phenol in 1000 ml of distilled water. The crude enzyme solution (0.5 ml) was incubated in 1.0 ml of a buffer solution containing 0.2% (w/v) melted agar at each reaction temperature for 30 min. The reaction mixture was then boiled for 10 min and cooled to room temperature. Subsequently, 1.5 ml of the DNS solution was added. The amount of reducing sugar liberated was measured using D-galactose as a standard.

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One unit of enzyme activity was defined as the amount of protein that produces 1.0 mmol of reducing sugar per minute under assay conditions.

Measurements of enzyme activities at different temperatures

To compare agarase activities at different temperatures using a crude enzyme solution, a substrate solution was prepared by dissolving 0.2% (w/v) agarose in a buffer containing 20 mM GTA buffer (3,3-dimethylglutamic acid, tris (hydroxymethyl) aminomethane, and 2-amino-2-methyl-1,3-propanediol) at pH 7.0. After heating 1.0 ml of the substrate solution, 0.5 ml of crude enzyme solution was added and reacted at temperatures of 20, 30, 40, 50, and 60℃ for 30 min. Subsequently, the enzyme activity was measured.

Measurements of enzyme activities based on pHs

To compare agarase activities based on pHs, substrate solutions were prepared using 20 mM GTA (pH 4.0–10.0) buffer containing 0.2% (w/v) agarose. After heating 1.0 ml of each substrate solution, 0.5 ml of crude enzyme solution was added and reacted at 40℃ for 30 min. Subsequently, the enzyme activity was measured.

Measurements of the thermal stabilities of the enzyme

To measure the thermal stability of agarase, a substrate solution was prepared using 20 mM GTA (pH 7.0) buffer containing 0.2% (w/v) agarose. Crude enzyme (0.5 ml) solution was heat-treated at 20, 30, 40, 50, and 60℃ for 0, 0.5, 1.0, and 2.0 h. Subsequently, 1.0 ml of melted substrate solution was added, and the mixture was incubated at 40℃ for 30 min. Subsequently, the enzyme activity was measured.

Chromatographic analysis of the products of agarose hydrolysis

Hydrolyzed products of agarose were identified using thin-layer chromatography (TLC). Enzymatic hydrolysis of 1.0% (w/v) agarose (USB Inc., USA) was carried out at 40℃ in a 20 mM GTA (pH 7.0) buffer for 0, 0.5, 1, 2, and 6 h. The reaction mixtures were applied to silica gel 60 TLC plates (Merck, Germany) [13, 14]. The plates were developed using a solvent system composed of n-butanol:acetic acid:H_2O (2:1:1, v/v). The spots were visualized by spraying with 10% (v/v) H_2SO_4 and heating to 80℃. D-Galactose (Sigma Chemical Co., USA) and neoagarooligosaccharides were used as standards [15].

Results and Discussion

Isolation and identification of an agar-degrading bacterium

Agar-decomposing colonies were selected. The selected strain was purified more than three times. After culturing the pure isolated strain and collecting the cells by centrifugation, the 16S rRNA gene of the extracted genomic DNA was analyzed. The analysis revealed the highest similarity of 98% to *Tenacibaculum mesophilum*, leading to its designation as *Tenacibaculum* sp. JS-1. The phylogenetic position of the strain is depicted in Fig. 1.

Agar-degrading *Tenacibaculum* strains were isolated from China, to the best of the authors' knowledge. Xu *et al*. focused on the phylogenetic positions of an isolated *Tenacibaculum* strain, while Liu *et al.* emphasized the isolation and culture of agar-degrading strains [16, 17]. The characteristics of the agarase from *Tenacibaculum* strains have not been reported. This study, however, focuses on elucidating the characteristics of the agarase from a *Tenacibaculum* strain. Xu *et al.* [16] isolated agarolytic *Tenacibaculum* species from marine algae and characterized the isolate along with other *Tenacibaculum* strains. *Tenacibaculum* genus is gram-negative, rod-shaped, and catalase-positive. However, other phenotypic characteristics varied among *Tenacibaculum* species, including pigmentation, oxidase activity, growth temperature, agarase activity, and gliding motility [16]. *Tenacibaculum agarivorans* showed growth at 1.0–5.0%

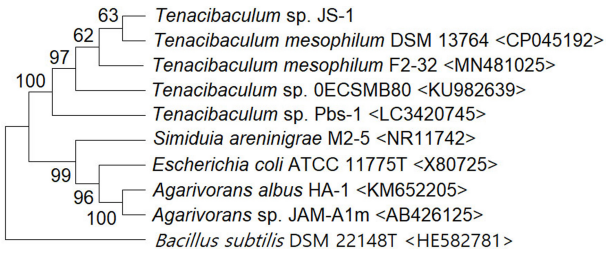

Fig. 1. Neighbor-joining phylogenetic tree based on the 16S rDNA sequence, comparing the isolated *Tenacibaculum* sp. JS-1 strain interacts with other bacteria. The numbers at the branch node represent percentages of bootstrap values (n = 1,000), and the numbers in parentheses correspond to the numbers in GenBank.

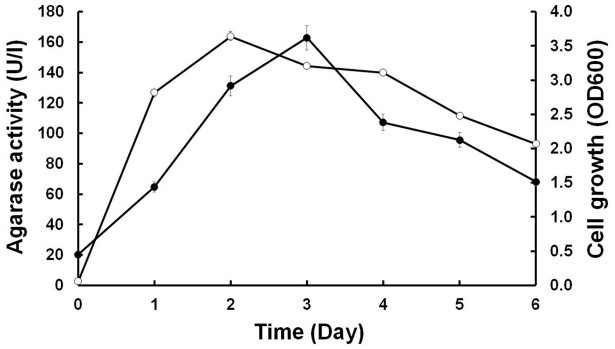

Fig. 2. Cell growths and agarase activities of *Tenacibaculum* sp. JS-1 with culture time. ○ Agarase activity (U/l), ● Cell growth (OD_{600}).

(w/v) NaCl, with an optimum range of 2.0–3.0% [16].

Bacterial growths and enzyme activities of the isolated strain over time

The agarolytic enzyme activity and growth curves of *Tenacibaculum* sp. JS-1 are shown in Fig. 2. It was found that bacterial growth peaked on the 3rd day and decreased from the 4th day. Enzyme activity was highest on the 2nd day and decreased significantly from the 3rd day. Therefore, in subsequent studies, culture medium cultured for 2 days was used. Xu *et al.* reported that the growth of *Tenacibaculum* occurs between 15 and 37℃, with the optimal growth temperature being 28℃ [16].

Enzyme activities at different temperatures

The activities of the agarase produced by *Tenacibaculum* sp. JS-1 according to temperature are shown in Fig. 3A. The enzyme activity increased as the temperature rose to 40℃ and reached its peak at 40℃. Above 40℃, there was a significant decrease in enzyme activity as the temperature rose. Considering the activity at 40℃ as 100%, the relative activities were 73% at 30℃, 74% at 50℃, and 53% at 60℃. There was no report on the optimal temperature and pH for agarase of the genus *Tenacibaculum*. When checking the optimal temperatures of agarases from other strains, such as *Agarivorans* sp. JS-1 [18] showed the highest activity at 50℃, and *Alteromonas* sp. SH-1 [19] and *Cellulophaga* sp. J9-3 [20] showed the highest activities at 30℃ and 35℃, respectively.

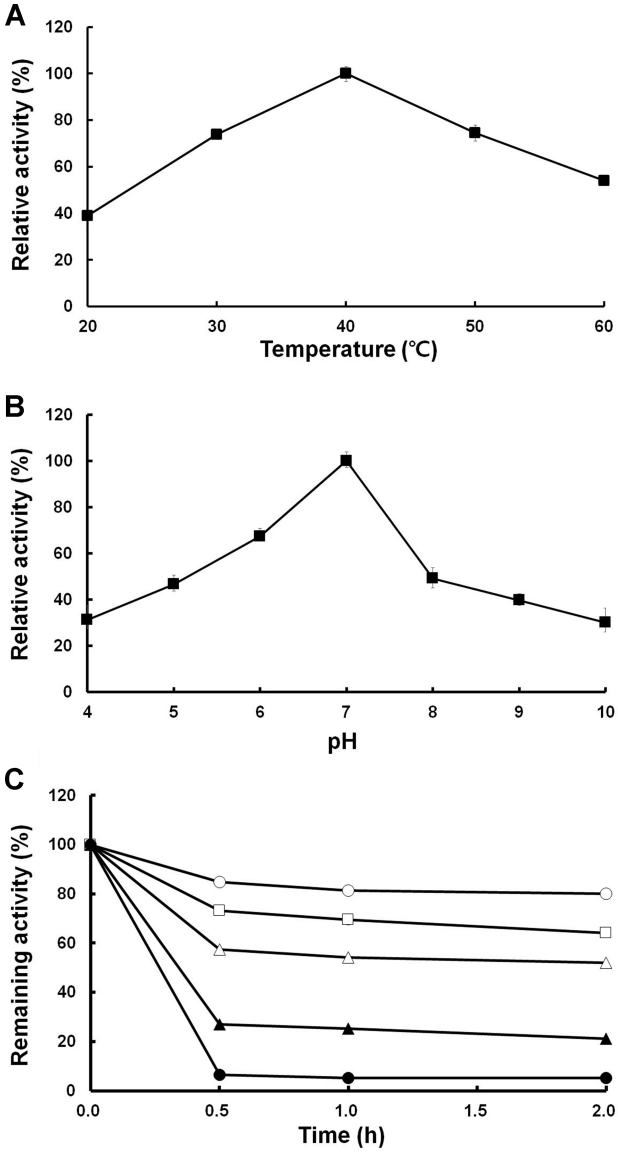

Fig. 3. Enzymatic characteristics of the agarase. (**A**) The effects of reaction temperatures on agarase activity. The reactions were carried out at 20, 30, 40, 50, and 60℃ in 1.0 ml of 20 mM GTA (pH 7.0) buffer containing 0.2% (w/v) agarose and 0.5 ml of raw enzyme solution for 30 min. (**B**) The effects of pHs on agarase activity. The reactions were carried out at 40℃ in 1.0 ml of the corresponding GTA buffer containing 0.2% (w/v) agarose and 0.5 ml of raw enzyme solution for 30 min. (**C**) Remaining activities of the agarase after heat treatments. The raw enzyme solutions were pre-incubated at 20 (○), 30 (□), 40 (△), 50 (▲), and 60℃ (●) for 0, 0.5, 1.0, 1.5, and 2.0 h. The reactions were then carried out at 40℃ in 1.0 ml of 20 mM GTA (pH 7.0) buffer containing 0.2% (w/v) agarose and 0.5 ml of heat-treated raw enzyme solution for 30 min.

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Enzyme activities based on pHs

The activities of the agarase produced by *Tenacibaculum* JS-1 according to pHs are shown in Fig. 3B. The enzyme exhibited the highest activity at pH 7.0, and when the activity at pH 7.0 was considered as 100%, the relative activities were 67% at pH 6.0, and 30% at pH 4.0 and pH 10.0. Additionally, it exhibited activities of 46% and 49% at pH 5.0 and pH 8.0, respectively. When checking the optimal pHs of agarases from other strains, *Agarivorans* sp. JS-1 [18] showed the highest activity at pH 8, and *Alteromonas* sp. SH-1 [19] and *Cellulophaga* sp. J9-3 [20] showed the highest activities at pH 6 and pH 7.0–7.5, respectively.

Thermal stabilities of the enzyme

The thermal stabilities of *Tenacibaculum* sp. JS-1 derived agarase are shown in Fig. 3C. Considering that the enzyme activity measured without heat treatment at the optimal temperature and pH was 100%, it was observed that more than 80% of the residual activity remained after exposure to 20℃ for 2 h. At temperatures above 30℃, residual activity remains lower after 30 min of exposure than after 2 h of exposure at 20℃. Even when exposed to temperatures up to 40℃ for 2 h, there was approximately 52% residual activity. However, when heat treated above 50℃, the residual activity was observed to decrease to about 20% after 2 h. Enzymes typically exhibit higher activity when substrates are in a solution state rather than in a gel or solid state. Agar has a gelling temperature of 32–36℃ and a melting temperature of 85–86℃ [21]. The productivity of functional agar-derived oligosaccharides would be high when agar is in a solution state at 40℃, where the optimal temperature for agarase activity of *Tenacibaculum* sp. JS-1. In addition, approximately 52% of its residual activity was maintained after 2 h exposure at 40℃.

Chromatographic analysis of the hydrolysis products

The time-course of hydrolyzed products from agarose was examined at 40℃ for up to 6 h (Fig. 4), and hydrolyzed products were quantified using by NIH image software. After 1 h of incubation, the main products were neoagarotetraose (53.9% of total products) and neoagarobiose (24.2% of total products). These results suggest that the enzyme is an endo-type β-agarase. With the passage of time (2 to 6 h), the amounts of neoagarotetraose showed little variation (66.1 to 65.1% of total products), while the amounts of neoagarobiose increased (17.1 to 22.6% of total products) and the amount of neoagarohexaose decreased (16.8 to 12.3% of total products). The main product is neoagarotetraose, while neoagarobiose and neoagarohexaose are also produced from agarose. These results indicate that the enzyme hydrolyzes β-1,4 linkages in agarose. Neoagarooligosaccharides produced from agarose or agar exhibit a variety of functional properties that are of interest for pharmaceuticals, food, cosmeceuticals, and nutraceuticals [22]. Hence, β-agarase of *Tenacibaculum* sp. JS-1 would be useful for a diverse range of areas.

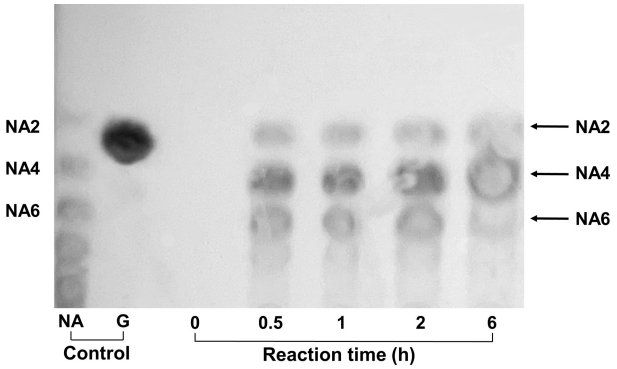

Fig. 4. TLC analysis of the hydrolyzed products of agarose using agarase. The reactions were carried out at 40℃ in 1.0 ml of 20 mM GTA (pH 7.0) buffer containing 0.2% (w/v) agarose and 0.5 ml of raw enzyme solution for 0, 0.5, 1, 2, and 6 h. (NA, neoagarooligosaccharides; D, D-galactose; NA6, neoagarohexaose; NA4, neoagarotetraose; NA2, neoagarobiose).

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Jo HG, Lee SJ, Lee DG, Lee SH. 2016. Characterization of agarase produced from the isolated marine bacterium *Marinomonas* sp. SH-2. *J. Life Sci.* **26**: 198-203.
2. Lim DJ, Kim BJ, Bae SK, Kim J, Kong JY. 1999. Immobilization of agarase for the agarooligosaccharide production. *Korean J.*

Micriobiol. Biotechnol. **27**: 208-214.
3. Lee CE, Lee SJ, Lee DG, Lee SH. 2016. Isolation of a new agar degrading bacterium, *Maribacter* sp. SH-1 and characterization of its agarase. *Microbiol. Biotechnol. Lett.* **44**: 156-162.
4. Cha JA, Kim YJ, Seo YB, Yoon MH. 2009. Isolation of an agarolytic bacteria, *Cellvibrio mixtus* SC-22 and the enzymatic properties. *J. Appl. Biol. Chem.* **52**: 157-162.
5. Chi WJ, Lim JH, Park DY, Kim MC, Kim CJ, Chang YK, *et al*. 2013. Isolation and characterization of a novel agar degrading bacterium, *Alteromonas macleodii* subsp. GNUM08120, from red macroalgae. *Korean J. Microbiol. Biotechnol.* **41**: 8-16.
6. Park GT, Lee DG, Kim NY, Lee EJ, Jung JG, Lee JH, *et al.* 2005. Isolation and characterization of a marine bacterium producing thermotolerant agarase. *J. Life Sci.* **15**: 884-888.
7. Lin F, Ye J, Huang Y, Yang Y, Xiao M. 2019. Simple preparation of diverse neoagaro-oligosaccharides. *Prosesses* **7**: 267.
8. Lee DG, Lee SH. 2012. The classification, origin, collection, determination of activity, purification, production, and application of agarases. *J. Life Sci*. **22**: 266-280.
9. Chi WJ, Park JS, Kwak MJ, Kim JF, Chang YK, Hong SK. 2013. Isolation and characterization of a novel agar-degrading marine bacterium, *Gayadomonas joobiniege* gen, nov, sp. Nov., from the southern sea. *Korean J. Microbiol. Biotechnol*. **23**: 1509-1518.
10. Jeon MJ, Kim AR, Lee DG, Lee SH. 2012. Cloning, expression, and characterization of a novel GH-16 β-agarase from *Agarivorans* sp. JA-1. *J. Life Sci.* **22**: 1545-1551.
11. Min KC, Lee CE, Lee DG, Lee SH. 2018. Isolation of *Agarivorans* sp. KC-1 and characterization of its thermotolerant β-agarose. *J. Life Sci*. **28**: 1056-1061.
12. Chandrasekaran M, Kumar S. 1997. The industrial enzymes derived from marine bacteria include α-amylase, α-glucosidase, agarase, α-galactosidase, cellulases, chitinase, lipase, pp. 47-79. *In* Chandrasekaran M., Kumar S., editors. *Biotechnology*.
13. Duckworth M, Yaphe W. 1970. Thin-layer chromatographic analysis of enzymic hydrolysate of agar. *J. Chromatogr.* **49**: 482-487.
14. Groleau D, Yaphe W. 1977. Enzymatic hydrolysis of agar: purification and characterization of β-neoagarotetraose hydrolase from *Peudomonas atlantica*. *Can. J. Microbiol.* **23**: 672-679.
15. Jang MK, Lee HO, Yoo KH, Lee DG, Lee SH. 2007. Secretory overexpression of β-agarase in *Bacillus subtilis* and antibacterial activity of enzymatic products. *J. Life Sci*. **17**: 1601-1604.
16. Xu Z, Yu P, Mu D, Liu Y, Du Z. 2017. *Tenacibaculum agarivorans* sp. nov., an agar-degrading bacterium isolated from marine alga *Porphyra yezoensis* Ueda. *Int. J. Syst. Evol. Microbiol.* **67**: 5139-5143.
17. Liu MYF, Zhao WTP, Zhou YJZ, Huang KDY, Xiao JNW, Wang X. 2024. Diversity of culturable bacteria from the coral reef areas in the south China sea and their agar-degrading abilities. *Microorganisms* **12***:* 187.
18. Kim JS, Lee DG, Yeo GW, Park MJ, Lee SH. 2023. Isolation of *Agarivorans* sp. JS-1 and characterization of its β-agarase. *J. Life Sci.* **33**: 357-362.
19. Lee SJ, Shin DY, Kim JD, Lee DG, Lee SH. 2016. Characterization of α-agarase from *Alteromonas* sp. SH-1. *Korean J. Biotechnol. Bioeng.* **31**: 113-119.
20. Kim DS, Kim JH, Chi WJ. 2021. Purification and biochemical characterization of β-agarase produced by marine microorganism *Cellulophaga* sp. J9-3. *Korean J. Micriobiol. Biotechnol.* **49**: 329-336.
21. Timothy JF. 2023. Statistics to Use. Available from https://www.linkedin.com/pulse/gelling-together-why-agar-gel-strength-matters-settle-dr-tim. Accessed Mar. 22, 2024.
22. Torres MD, Flórez-Fernández N, Domínguez H. 2019. Integral utilization of red seaweed for bioactive production. *Mar. Drugs* **17**: 314.

http://dx.doi.org/10.48022/mbl.2403.03012