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RESEARCH PAPER

Characterization of Two Thermostable β-agarases from a Newly Isolated Marine Agarolytic Bacterium, *Vibrio* sp. S1

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Abstract An agar-degrading bacterium, strain S1, was isolated from the coastal seawater of Jeju Island, Korea, and identified as a novel species of the genus *Vibrio*. The isolate, *Vibrio* sp. S1, produced at least five kinds of extracellular agarases in artificial sea water broth containing yeast extract and bacto peptone, and two of them were purified to homogeneity. Both agarases, AgaA33 and AgaA29, with apparent molecular weights of 33 k*Da* and 29 k*Da*, respectively, exhibited an optimum temperature and pH of 45°C and 7.0, respectively. AgaA33 and AgaA29 showed acidophilic properties and maintained 93% and 87% of the maximum agarase activity at 50°C, respectively, displaying their thermostability. Moreover, more than 80% activity was retained after heat treatment at 45°C for 1 h. Their agarase activities were inhibited by the presence of EDTA and remarkably stimulated by the presence of Mn^{2+} in a concentration-dependent manner, indicating that both agarases required the Mn^{2+} ion as a cofactor. The AgaA33 enzyme exhibited K_m and V_{max} values of 4.02 mg/mL and 27 U/mg, respectively. AgaA29 exhibited K_m and V_{max} values of 3.26 mg/mL and 200 U/mg, respectively. The instrumental analysis demonstrated that both are new β-agarases that can hydrolyze agarose and agaro-oligomers into neoagarotetraose and neoagarohexaose. In addition, AgaA33 coproduced neoagarooctaose as the major final product. Both thermostable enzymes are expected to be useful for the industrial application of agar.

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1. Introduction

Red algal biomasses, such as those in the genera *Gelidium*, *Gracilaria*, and *Gelidiella*, are important natural resources for producing traditionally hydrocolloid polysaccharides including agar. Hydrocolloid polysaccharides are technologically and economically important due to their broad application in the food, pharmaceutical, medicinal, cosmetic, and biotechnological industries depending on their physicochemical properties. Thus, the production of agar is estimated to be 10,600 tons per year [1]. Recently, agar has received much attention because it can be converted into more valuable chemicals such as ethanol and butanol [2]. Moreover, oligosaccharides prepared by the enzymatic hydrolysis of agar showed various biological functions. However, for the efficient use of agar, it is necessary to develop agar hydrolyzing enzymes that can work well under severe reaction conditions.

Agar is made up of repeating units of alternating 1,3-linked β-D-galactopyranose and 1,4-linked α-L-galactopyranose that allows it to form helical dimers. Agarose, which is a major constituent of agar, has 1,4-linked 3,6-anhydro-α-L-galactopyranose instead of L-galactopyranose [3]. Agar can be hydrolyzed by β-agarase at its β-(1,4)-linkage producing neoagarooligosaccharides accompanied by D-galactopyranose at the reducing ends. In contrast, agar is also hydrolyzed by α-agarase at its α-(1,3)-linkage and, thus, agarooligosaccharides with 3,6-anhydro-L-galactopyranose at the reducing ends are produced. The resulting oligosaccharides are further hydrolyzed by various enzymes into monomers, which are finally metabolized by the microorganisms [2].

Agar exhibits hysteresis; it melts close to the boiling point of water (85°C) and forms a gel when cooled to about 32–43°C in 1.5% solution [4]. The double helices of the agar molecules coalesce to form a three-dimensional structural framework during gel formation, and the water molecules are trapped between the gaps, limiting their free movement. Therefore, the agarase enzymes do not have efficient access the substrate in the gel state [5], and maintaining the sol state of the agar solution at high temperature is the most important factor in the agar hydrolysis process [6].

In this context, much effort has been made to overcome the low solubility of agar due to the sol–gel transition. The acid pretreatment method using acetic acid or sulfuric acid at high temperature followed by the agarases mixture-catalyzed hydrolysis has been very successful in avoiding the sol–gel transition at 45°C [7]. Recently, a newly developed chemical process using phosphoric acid as a catalyst was reported, which can be applied to a 30.7% (w/v) agarose loading for the hydrolysis of agarose into agarobiose [8]. However, there are still several problems to be solved such as the high concentration of salt due to the neutralization of acid hydrolysate and coproduction of the toxic substance 5-hydroxymethylfurfural (5-HMF), which is derived from heat-labile 3,6-anhydro-α-L-galactose at high temperature [7,8].

In order to overcome the disadvantages of chemical treatment, it is necessary to develop a complete decomposition process of agar using only the enzyme; thus, an agarase that can stably operate at 45°C or higher than the sol–gel transition temperature of the agar solution is required. Many agarases have been reported from various marine, soil, and intestinal microorganisms, which are primarily active at mesophilic temperatures [2]. Among them, Aga16B, originating from *Saccharophagus degradans* 2-40, was shown to be thermostable at temperatures up to 50°C and was, thus, successfully applied to the enzymatic liquefaction of agarose at 45°C [6]. However, it is absolutely necessary to find various agarases that can work more reliably at high temperatures.

For this purpose, we isolated and identified a marine agarolytic bacterium, *Vibrio* sp. S1, that can grow at 40°C and characterized two thermostable extracellular agarases, AgaA33 and AgaA29, which are produced by it.

2. Materials and Methods

2.1. Isolation of an agar hydrolyzing bacterium, strain S1

Seawater sample was collected from Jeju Island and serially diluted in artificial sea water (ASW) containing 6.1 g Tris base, 12.3 g $MgSO_4$, 0.74 g KCl, 0.13 g $(NH_4)_2HPO_4$, 17.5 g NaCl, and 0.14 g $CaCl_2$, at pH 7.2. The diluted solution (200 μL) was spread onto an ASW-YP agar plate containing ASW supplemented with 0.02% (w/v) yeast extract and 0.3% (w/v) bacto peptone [9]. The plate was incubated at 40°C for 24 h. Colonies showing agarase activity were selected after staining with Lugol's iodine solution (0.05 M iodine in 0.12 M KI). One bacterial colony, strain S1, showing the highest agar hydrolyzing activity, was selected to study.

2.2. Phylogenetic analysis based on the 16S rRNA gene sequence

The genomic DNA of strain S1 was extracted by using the Genomic DNA extraction kit (DyneBio, Korea), and it was used as the template for the amplification of the 16S rRNA gene by polymerase chain reaction using the bacterial universal primers 27F and 1492R [10]. The nearly complete 16S rRNA gene sequence (1,461 bp) of strain S1 was sequenced and registered as JF965428 in GenBank. The 16S rRNA gene sequence was compared to those in the GenBank database using the BLAST algorithm. The 16S rRNA gene sequences of related type strains were collected from the EzTaxon server (http://www.eztaxon.org [11]) to construct a phylogenetic tree. The sequences were aligned using the Clustal W software [12], and the 5' and 3' gaps were edited via the BioEdit program [13]. The neighbor-joining (NJ) method [14] from the Mega 6 program [15] was used for constructing the phylogenetic tree. The bootstrap values were calculated using data that was restructured close to 1,000 times and marked into branching points, and the evolutionary distance matrix was estimated according to Kimura's two-parameter model [16].

2.3. Chemotaxonomy analysis

Chemotaxonomic characteristics were analyzed from strain S1 grown on ASW-YP agar or in ASW-YP broth at 40°C for 48 h. Major respiratory quinones and the DNA G+C content were analyzed by reverse phase HPLC as described by Komagata and Suzuki [17] and Mesbah *et al.* [18], respectively. Cellular fatty acids from whole cells grown on the ASW-YP agar were extracted using the standard protocol of the microbial identification system MIDI and identified by gas chromatography [19].

2.4. Biochemical characteristics

Growth at different temperatures (4, 15, 25, 37, 40, 42, and 45°C), various pH values (4.0–11.0 at interval of pH 1), and different NaCl concentrations (0–20%, w/v) on ASW-YP agar plates at 40°C were determined. Biochemical tests were executed using the API 20NE, API Staph, and API ZYM kits (Biomérieux, France) according to the instructions with the exception that the bacterial suspension was

prepared in AUX or Staph medium supplemented with 1% (w/v) NaCl.

2.5. Determination of cell growth and agarase activity

The S1 cells were inoculated in ASW-YP broth containing 0.1% (w/v) agar as the carbon source and incubated at 40°C for 120 h with vigorous agitation. The culture broths were sampled at a regular interval and cell density was measured by a spectrophotometer at 600 nm. The sample was centrifuged at 10,000 × *g* for 20 min, and the cell-free supernatant was collected to measure agarase activity. The enzyme solution (100 μL) was mixed with 3.9 mL of buffer A (20 mM Tris-Cl, pH 7.0) containing 0.2% (w/v) agarose. After reaction at 40°C for 30 min, the agarase activity was measured at 600 nm by following the dinitrosalicylic acid (DNS) method as previously described [9]. One unit (U) of agarase was defined as the amount of enzyme that produced 1 μmole of D-galactose per min under the assay conditions. D-galactose was used as a reference reducing sugar for preparing the standard curve.

2.6. Purification of agarases from the culture broth of strain S1

The liquid culture was performed at 40°C with vigorous shaking. Strain S1 was cultured in ASW-YP broth containing 0.1% (w/v) agar for 24 h. The culture broth (10 mL) was inoculated to fresh medium (1 L) and further incubated for 72 h. The cell-free culture broth (870 mL) obtained after centrifugation at 10,000 × *g* for 20 min was used for $(NH_4)_2SO_4$ precipitation (75%, w/v). After centrifugation at 15,000 × *g* for 60 min, the precipitated protein pellet was resuspended with buffer B (10 mM Tris-Cl, pH 8.0) and dialyzed against the same buffer overnight, and then it was concentrated by ultrafiltration using a 10-k*Da* cut-off column (PALL, NY, USA) to 5 mL. The protein sample was loaded onto a Resource-Q column (Amersham Pharmacia Biotech, UK) equilibrated with buffer B. After washing with the same buffer, the proteins were eluted with a linear gradient of 0 to 1 M NaCl in buffer B at a flow rate of 1 mL/min. The active fractions were concentrated by ultrafiltration. Two active fractions, sample I (fraction numbers 5~6) and sample II (fraction numbers 9~10), were pooled and concentrated by ultrafiltration to a final volume of 0.5 mL. The protein samples I and II were loaded onto a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with buffer B and eluted with the same buffer at a flow rate of 0.5 mL/min. Each protein purified was designated as AgaA33 (from sample I) and AgaA29 (from sample II) and used for further analysis. The proteins were analyzed by 0.1% sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE). For zymogram, unboiled protein samples were applied to gels including 0.3% (w/v) agarose as the substrate. After electrophoresis, the gels were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in buffer A for 30 min, and incubated at 45°C for 60 min in the same buffer. Then, the gels were stained with Lugol's iodine solution.

2.7. Biochemical properties of the agarases

The effect of pH on agarase activity was measured at 45°C under the various pH conditions using the following buffers: 20 mM MOPS (pH 6–7), 20 mM tris-Cl (pH 7–9), and 20 mM glycine-NaOH (pH 9–10). The effect of temperature on agarase activity was measured in buffer A at temperatures ranging from 20 to 50°C. The thermostability was determined in buffer A at 45°C after incubation at temperatures ranging from 20 to 70°C for 1 h.

The effect of metal ions on agarase activity was measured in buffer A containing 5 mM or 10 mM of the metal salts, $CoCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $CaCl_2$, KCl, NaCl, and EDTA.

The kinetic parameters of AgaA33 (2.3 μg) and AgaA29 (1.5 μg) were determined at 45°C for 5 min in buffer A toward agarose (molecular mass, 120 k*Da*) at concentrations ranging from 0.5 to 25 mg/mL. The kinetic parameters were calculated from a Lineweaver-Burk plot of initial velocity data [20].

2.8. Structural analysis of the product

Each enzyme reaction for AgaA33 and AgaA29 was carried out at 45°C for 20 h in buffer A containing 0.2% agarose. The reaction mixtures were analyzed by thin layer chromatography (TLC) on a silica gel 60 plate (Merck, USA) and the spots corresponding to the neoagarotetraose were collected for NMR analysis as previously described [21]. ^{13}C-nuclear magnetic resonance (NMR) spectrum of the product was recorded using a Superconducting FT-NMR spectrometer (500 MHz, DRX-500; Bruker, USA).

3. Result

3.1. Isolation and characterization of an agar-hydrolyzing bacterium, strain S1

Strain S1 was isolated from the coastal seawater of Jeju Island located at 126°08'~126°58' east longitude and 36°06'~33°00' north latitude. The strain decomposed the agar and produced a clear halo around the colonies on the ASW-YP agar plate (Fig. 1A). Strain S1 was gram negative and formed circular, smooth, and white-colored colonies on ASW-YP agar and marine agar (Difco 2216) plates. Transmission electron microscopy revealed that it was rod shaped with flagella (Fig. 1B). Strain S1 showed 16S rRNA gene sequence similarity to the strains of the genus *Vibrio* such as *V. variabilis* R-40492^T (98.7%), *V. neptunius*

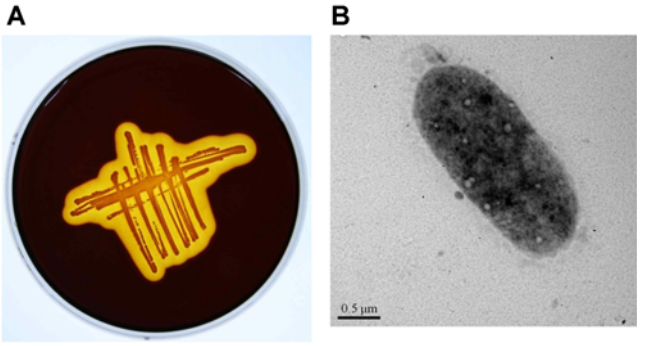

Fig. 1. Phenotypic characteristics of strain S1. (A) Detection of agarase production on the agar plate. Strain S1 was cultured on ASW-YP agar at 40°C for 1 day. The agar plate was stained with Lugol's iodine solution. (B) Transmission electron microscopy. Strain S1 grown on ASW-YP agar at 40°C for 1 day was negatively stained and observed using transmission electron microscopy (JEM1010, JEOL, Japan). Scale bar = 0.5 μm.

LMG20536^T (96.5%), and *V. marinus* R-40493^T (96.2%). In the NJ phylogenetic tree based on the 16S rRNA gene sequences, the isolate formed an independent lineage branching separate from the genetically close *V. variabilis* R-40492^T and the others (Fig. 2). The cutoff value distinguishing novel species was proposed to be 98.7% [22]. Thus, the phylogenetic and sequence similarity analyses based on the 16S rRNA gene suggested that strain S1 is a different species in genus *Vibrio* from other recognized species.

The strain S1 was able to grow at 15–42°C (optimum, 35–40°C), at pH 6.0–9.0 (optimum, pH 7.0–8.0), and at 0–6% (w/v) of NaCl (optimum, 2–3%). Strain S1 was positive for activities associated with nitrate reduction, alkaline phosphatase, arginine dihydrolase, urease (weak), esterase (C4 and C8), leucine arylamidase, valine arylamidase (weak), acid phosphatase, naphtol-AS-BI-phosphohydrolase, and α-glucosidase (weak), but it was negative for activities associated with acetyl-methyl-carbinol production, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminase, α-mannosidase, and α-fucosidase. Acid was produced from D-glucose, D-fructose, D-mannose, D-maltose, D-trehalose, D-mannitol, D-xylose, D-saccharose, and N-acetyl-glucosamine but not from D-lactose, xylitol, D-melibiose, D-raffinose, or methyl-α-D-glucopyranoside. The major fatty acids (> 10%) were summed feature 3 comprising $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH (34.7%), $C_{16:0}$ (24.7%), $C_{18:1}\omega7c$ (15.2%), $C_{14:0}$ (10.1%). The G+C content of the DNA was 50.79 mol %. The predominant respiratory quinone was Q-8. All these results were within the range of the biochemical and physiological characteristics of most species of the genus *Vibrio*. Based on phylogenetic and chemotaxonomic analyses, we named the novel strain S1 as *Vibrio* sp. S1 and deposited it in American Type Culture Collection (ATCC) as ATCC BAA-2322.

3.2. Cell growth and agarase production of *Vibrio* sp. S1

Cell growth started to increase from 12 h after incubation and reached its maximum on day 3, and then it gradually decreased until day 5 in ASW-YP broth containing 0.1% (w/v) agar as the carbon source at 40°C with vigorous shaking (Fig. 3A). The total agarase activity produced by *Vibrio* sp. S1 also sharply increased from 12 h and reached the maximum level on day 3, then it decreased until day 5

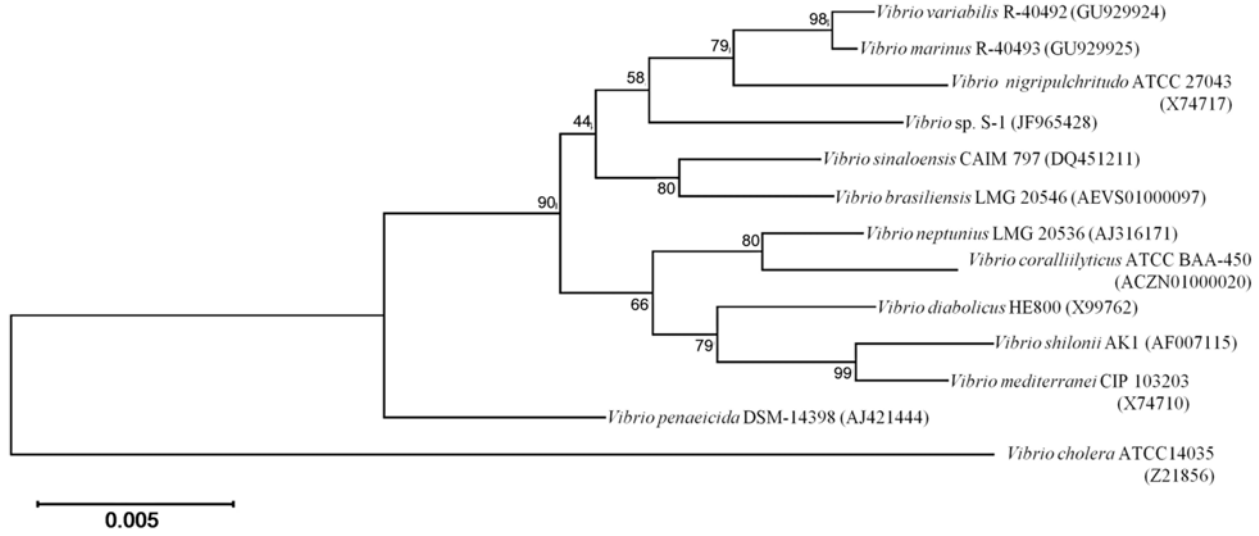

Fig. 2. Neighbor-joining phylogenetic tree analysis of strain S1 based on the 16S rRNA gene sequence. Distances were determined according to the Kimura's two-parameter model and bootstrap values (> 50%) based on 1,000 replicates are indicated as the percentages at the nodes. Nucleotide sequence accession numbers are given in parentheses. Scale bar = 0.005 substitutions per 100 nucleotides.

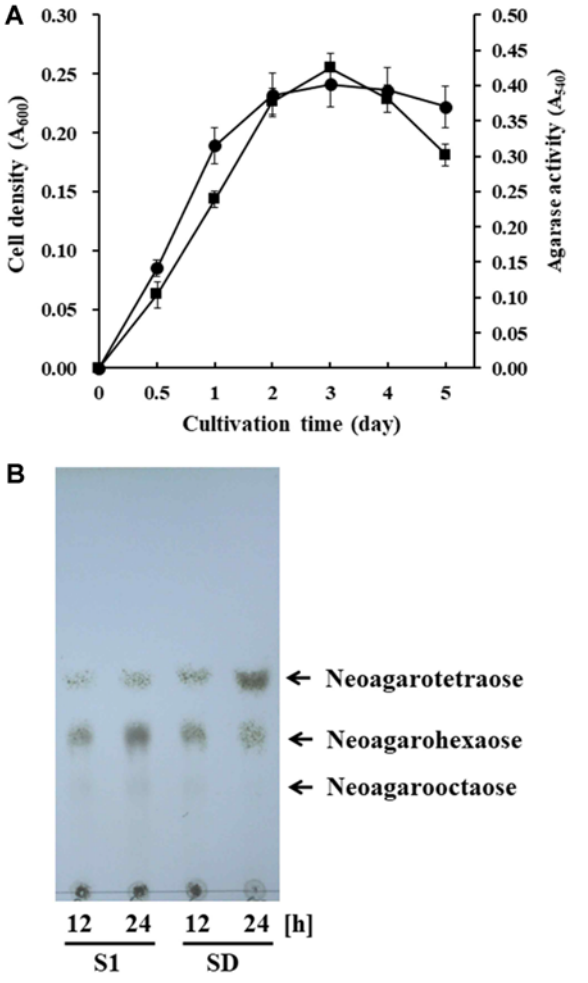

Fig. 3. Cell growth and agarase production of *Vibrio* sp. S1 in liquid culture. (A) Cell growth and agarase production. *Vibrio* sp. S1 was cultured in ASW-YP containing 0.1% (w/v) agar at 40°C. -●-, cell density; -■-, agarase activity. All shown data are mean values from at least three replicate experiments. (B) Thin layer chromatography analysis. The enzyme reaction was carried out at 40°C with 0.5% (w/v) agarose as the substrate. S1, crude enzyme prepared from the culture broth of *Vibrio* sp. S1 by $(NH_4)_2SO_4$ precipitation; SD, DagA β-agarase from *Streptomyces coelicolor* was used to prepare the standard oligosaccharides, neoagarotetraose, neoagarohexaose, and neoagarooctaose [21].

with a tendency similar to the cell growth (Fig. 3A). The hydrolysis product of the agarose by the cell-free culture broth of *Vibrio* sp. S1 was separated and detected by TLC (Fig. 3B). The complete hydrolysis of the agarose yielded two major spots, which had the same mobility as that of neoagarotetraose and neoagarohexaose.

3.3. Purification of two agarases from the culture broth of *Vibrio* sp. S1

Two agarases named as AgaA33 and AgaA29 were purified to homogeneity from the cell-free culture broth of *Vibrio* sp. S1 by three sequential purification steps, which were $(NH_4)_2SO_4$ precipitation, ion exchange column chromatography, and gel filtration column chromatography. AgaA33 and AgaA29 were separated from peak I and peak II, respectively, of the Resource-Q anion ion exchange column chromatography. The purification yields for each step are summarized in Table 1. The apparent MWs of AgaA33 and AgaA-29 were estimated as 33 k*Da* (Fig. 4A) and 29 k*Da* (Fig. 4B) by SDS-PAGE, respectively. The agarase activities of the proteins corresponding to AgaA33 and AgaA29 were confirmed by active staining (Fig. 4). The specific activities of purified AgaA33 and AgaA29 were 11.87 U/mg and 11.17 U/mg, respectively. For N-terminal amino acid sequencing, the purified agarases were electrophoretically transferred onto a polyvinylidene difluoride membrane (Merck Millipore, Burlington, MA, USA) after SDS-PAGE and then analyzed by the Edman degradation procedure. However, the N-terminal amino acid sequences of AgaA33 and AgaA29 were not determined because their N-terminal parts were blocked.

3.4. Biochemical properties of AgaA33

AgaA33 was found to exhibit maximum agarase activity at pH 7.0 (Fig. 5A). Around 72% of the maximum activity at pH 6.0 was observed, whereas only 57% and 9% of the maximum activity was observed at pH 8.0 and 10.0, respectively. The agarase activity increased until 45°C, at which the enzyme exhibited the maximum activity (Fig. 5B). AgaA33 still maintained 93% of its maximum activity at 50°C but lost most activity at 70°C. The enzyme was stable over the temperature range of 20 to 45°C but retained 12% of its maximum activity after heat treatment at 50°C for 1 h. The agarase activity was significantly

Table 1. Summary of purification for AgaA33 and AgaA29

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold
Culture supernatant	870.0	4,410.90	645.60	0.15	1.00
$(NH_4)_2SO_4$ precipitation and ultrafiltration	5.0	71.75	41.08	0.57	3.80
Ion-exchange chromatography and ultrafiltration (sample I)	0.5	0.47	5.58	8.81	58.73
Ion-exchange chromatography and ultrafiltration (sample II)	0.5	0.16	1.41	8.11	54.07
Gel filtration and ultrafiltration (Aga-33)	0.4	0.09	0.73	11.87	79.13
Gel filtration and ultrafiltration (Aga-29)	0.4	0.06	0.67	11.17	74.47

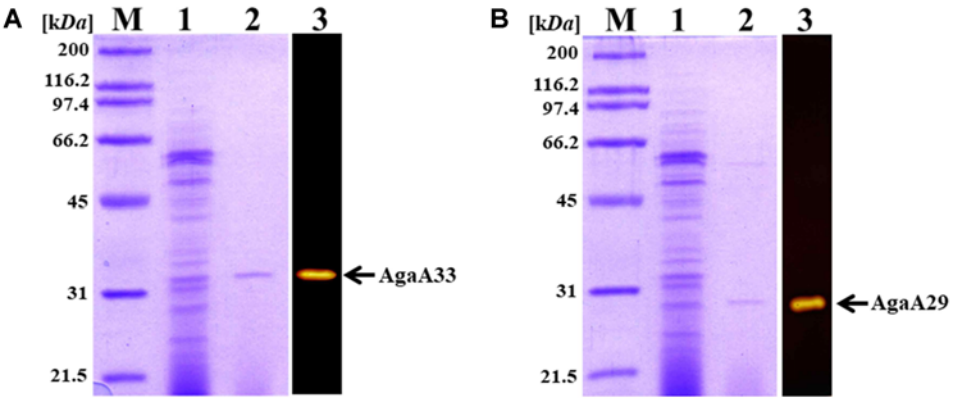

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of agarases purified from *Vibrio* sp. S1. Two agarases purified from the culture broth of *Vibrio* sp. were analyzed by 0.1% SDS-12% PAGE. (A) AgaA33 and (B) AgaA29. M, molecular weight standards; lane 1, total extracellular proteins after $(NH_4)_2SO_4$ precipitation; lane 2, purified protein; lane 3, zymogram for the purified agarase.

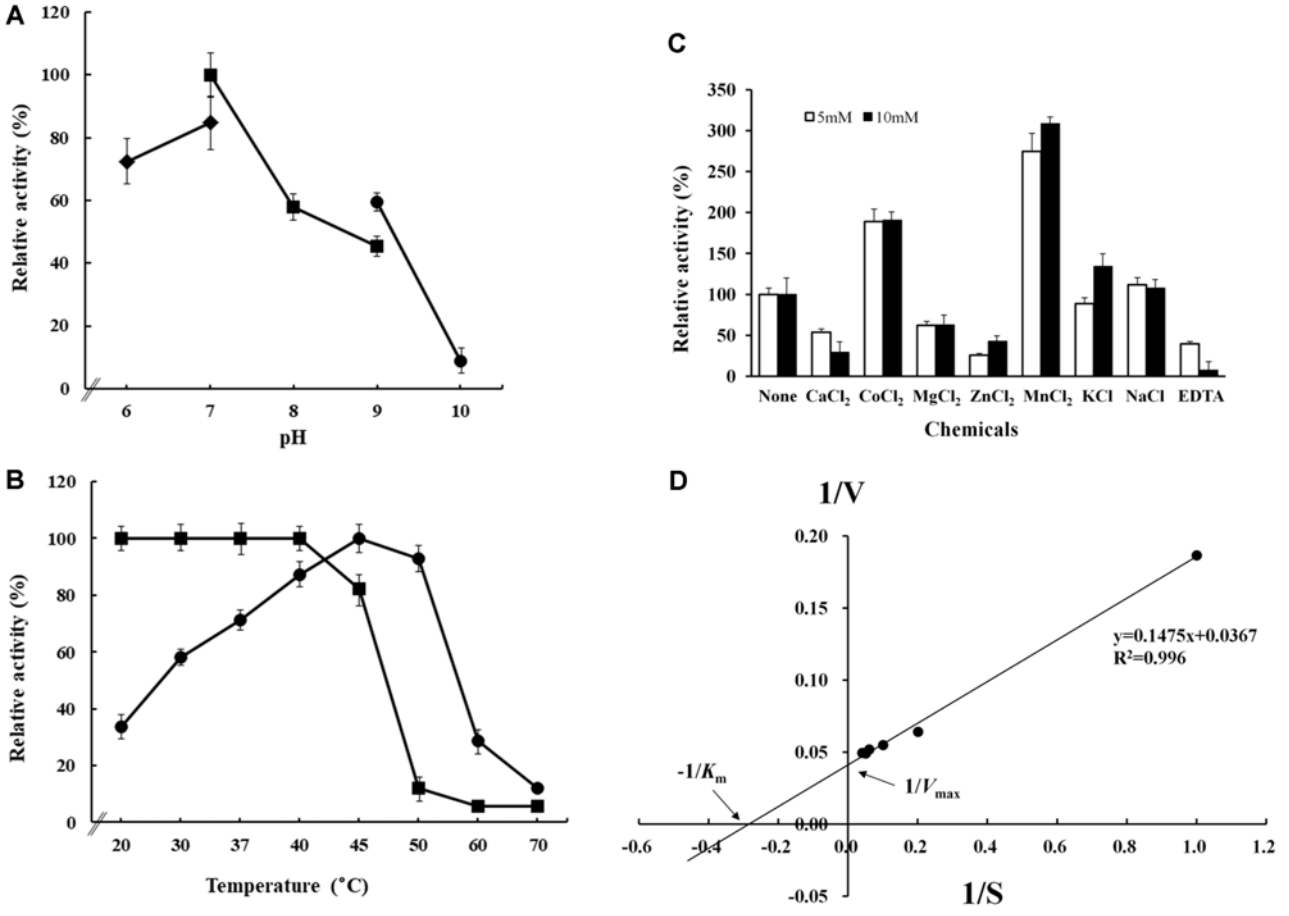

Fig. 5. Biochemical properties of agarase AgaA33. (A) Effect of pH. The agarase activity of AgaA33 was measured at pH 5.0 to 10.0 at 45°C. -◆-, 20 mM MOPS buffer; -■-, 20 mM tris-Cl buffer; -●-, 20 mM glycine-NaOH buffer. (B) Effect of temperature. The agarase activity of AgaA33 was measured at different temperatures ranging from 20 to 70°C at pH 7.0. Thermostability was measured at 45°C after preincubation at different temperatures ranging from 20 to 70°C for 60 min. All data shown are mean values from at least three replicate experiments. In (A) and (B), the highest agarase activity was considered 100% when calculating the relative activities. (C) Effect of metal ions and EDTA on the agarase activity of AgaA33. The final concentrations of the chemicals added were 5 and 10 mM. The agarase activity in reaction buffer only was considered 100% when calculating the relative activities. (D) Lineweaver-Burke plot to determine the kinetic parameter of Aga-33 acting on agarose.

inhibited by EDTA in a concentration-dependent manner, implying AgaA33 needed a metallic cofactor for enzyme activity (Fig. 5C). Although several divalent metal ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+} strongly inhibited the agarase activity, Mn^{2+} and Co^{2+} remarkably enhanced enzyme activity. In particular, Mn^{2+} could enhance the enzyme activity to a greater degree than Co^{2+} in a concentration-dependent manner (274% at 5 mM and 308% at 10 mM), strongly indicating that Mn^{2+} may be a cofactor for AgaA33. The K_m value and V_{max} of AgaA33 in response to agarose were 4.02 mg/mL and 27 U/mg, respectively (Fig. 5D).

3.5. Biochemical properties of AgaA29

The maximum pH at which AgaA29 functioned was pH 7.0 (Fig. 6A). The enzyme showed relatively low activity under alkaline conditions, whereas it showed nearly 92% of its maximum activity at pH 6.0. The enzyme had an optimum temperature at 45°C (Fig. 6B). At 40°C and 50°C, the enzyme showed 90% and 87% of its maximum activity, respectively, but lost most activity at 70°C. The enzyme was stable over the temperature range of 20 to 45°C but retained 13% of its maximum activity after heat treatment at 50°C for 1 h. Similar to AgaA33, the agarase activity of AgaA29 was inhibited by EDTA but significantly enhanced by Mn^{2+} in a concentration-dependent manner (Fig. 6C). The K_m value and V_{max} of AgaA29 in response to agarose were 3.26 mg/mL and 200 U/mg, respectively (Fig. 6D).

3.6. Mode of action of AgaA33 and AgaA29

The hydrolyzed products of agarose by AgaA33 and AgaA29 depending on reaction time were analyzed by TLC. At the initial stage of the reaction, AgaA33 and AgaA29 hydrolyzed agarose to generate a series of (neo)agarooligosaccharides. In the case of AgaA33, more

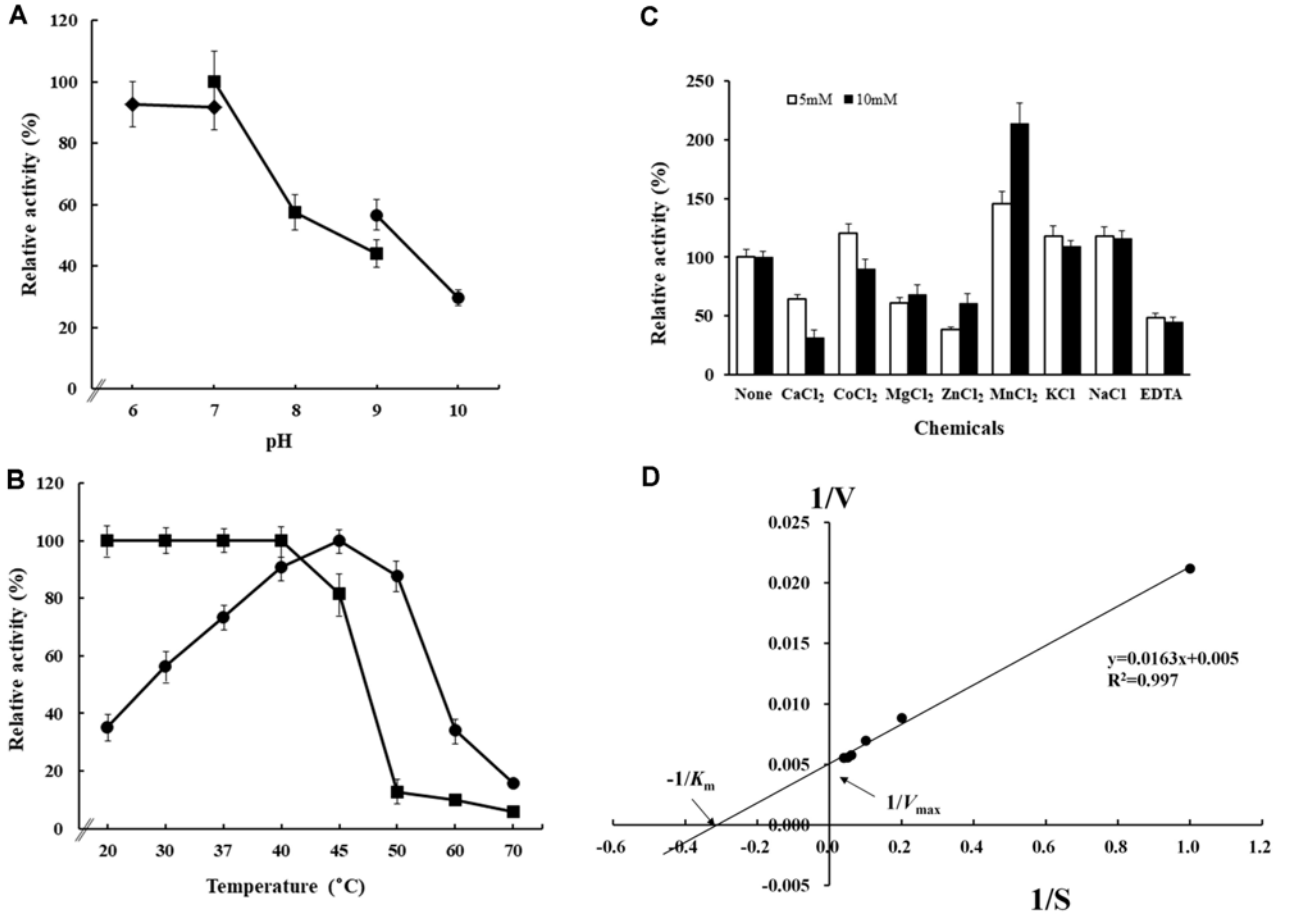

Fig. 6. Biochemical properties of agarase AgaA29. (A) Effect of pH. The agarase activity of AgaA29 was measured at pH 5.0 to 10.0 at 40°C. -◆-, 20 mM MOPS buffer; -■-, 20 mM tris-Cl buffer; -●-, 20 mM glycine-NaOH buffer. (B) Effect of temperature. The agarase activity of AgaA29 was measured at different temperatures ranging from 20 to 70°C at pH 7.0. Thermostability was measured at 45°C after preincubation at different temperatures ranging from 20 to 70°C for 60 min. All data shown are mean values from at least three replicate experiments. In (A) and (B), the highest agarase activity was considered 100% when calculating the relative activities. (C) Effect of metal ions and EDTA on the agarase activity of AgaA29. The final concentrations of chemicals added were 5 and 10 mM. The agarase activity in reaction buffer only was considered 100% when calculating the relative activities. (D) Lineweaver-Burk plot to determine the kinetic parameter of AgaA29 acting on agarose.

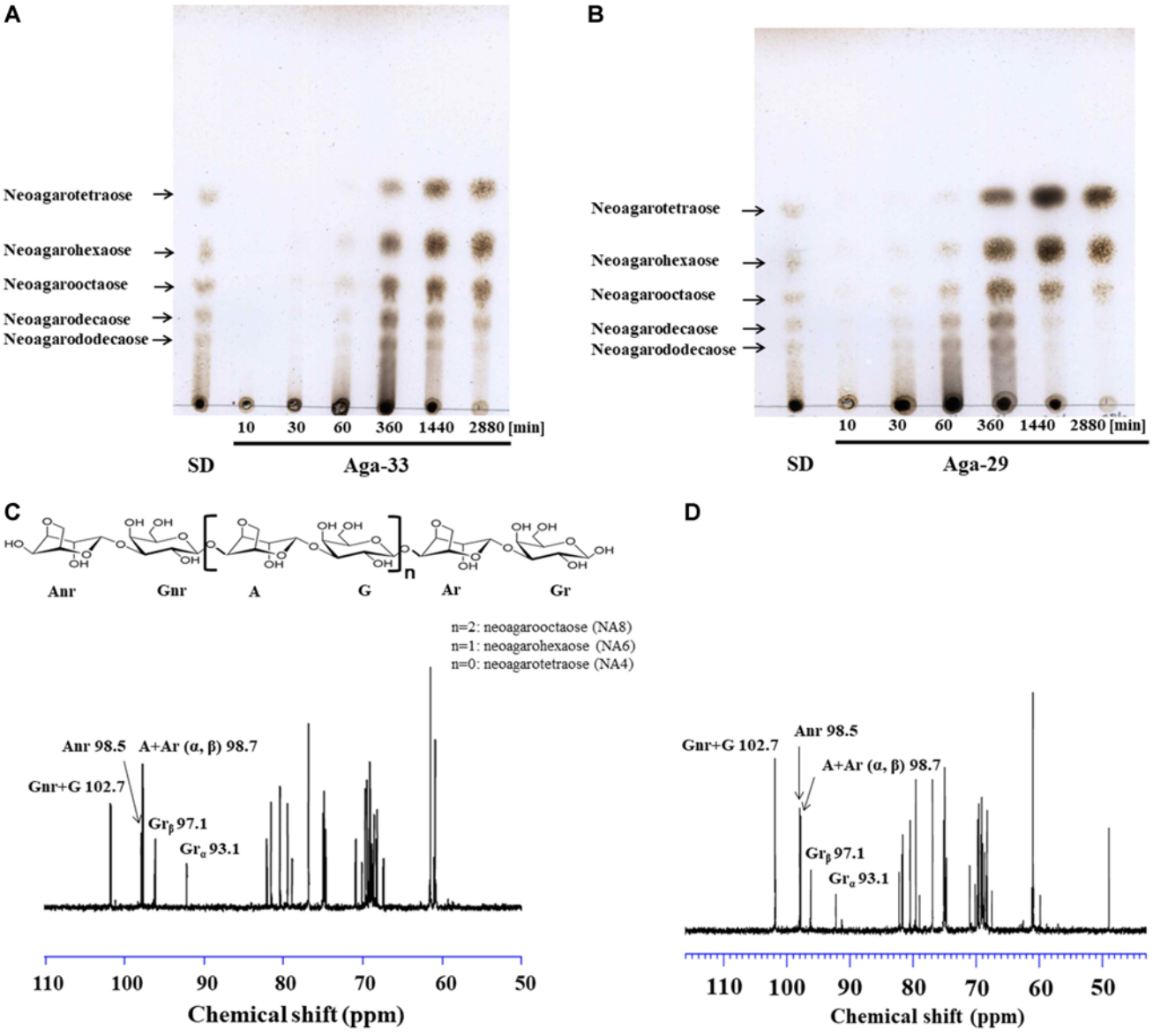

Fig. 7. Instrumental analysis of agarose hydrolysate by AgaA33 and AgaA29. (A, B) Thin-layer chromatogram of the agarose hydrolysate by AgaA33 (A) and AgaA29 (B) depending on the reaction time. SD, standards for neoagarotetraose, neoagarohexaose, and neoagarooctaose prepared by hydrolyzing agarose with DagA [21]). (C, D) ^{13}C-NMR spectrum of agarose hydrolysate (neoagarotetraose) by AgaA33 (C) and AgaA29 (D). For the sake of clarity, the chemical structure of neoagarooligosaccharides is presented in (C). G, β-D-galactopyranoside; A, 3,6-anhydro-α-L-galactopyranoside; r, reducing end; nr, non-reducing end; α, α anomer; β, β anomer.

than five major spots were accumulated over the course of the first 360 min. However, long oligosaccharides were further degraded as the reaction progressed, forming three short oligosaccharides at 48 h. (Fig. 7A). The major products were predicted as (neo)agarotetraose, (neo)agarohexaose, and (neo)agarooctaose by comparison to the standard neoagarooligosaccharides prepared from agarose hydrolyzed by DagA β-agarase as previously described [21]. In the case of hydrolysate by AgaA29, more than five major spots dominantly accumulated sequentially over the course of the first 360 min, and then larger oligosaccharides gradually hydrolyzed into two major products at 48 h (Fig. 7B). The two major products were predicted as (neo)agarotetraose and (neo)agarohexaose.

The ^{13}C-NMR spectrum analysis of the oligosaccharide mixtures revealed that the hydrolyzed products by AgaA33 and AgaA29 had the typical spectra of neoagarooligosaccharides [21,23]. Neoagarooligosaccharides are produced by the cleavage of β-(1,4)-linkages by β-agarase and, thus, have D-galactose at the reducing end. As shown in Fig. 7C and 7D, both hydrolyzed samples showed typical resonance signals at about 93.1 and 97.1 ppm, which are characteristic

of the α and β anomeric forms, respectively, of the reducing end (D-galactose) in neoagarotetraose. There was no signal at about 90.8 ppm corresponding to the anomeric form of 3,6-anhydrogalactose at the reducing end, which is produced by the cleavage of α-(1,3)-linkages by α-agarase [24]. This result demonstrated that the cleavage occurred by β-agarases.

All the results indicated that AgaA33 and AgaA29 are β-agarases that cleave the β-(1,4) linkages of agarose; thus, long neoagarooligosaccharides become neoagarotetraose and neoagarohexaose as the final product. Additionally, AgaA33 can also hydrolyze agarose into neoagarooctaose, which has never been studied for its biological function.

4. Discussion

A novel agarolytic marine bacterium was isolated from a coastal seawater sample of Jeju Island, Korea, and was named *Vibrio* sp. S1. At least five proteins were detected by SDS-PAGE and activity staining (zymogram) in the total extracellular protein sample prepared from strain S1 (data not shown). Two agarases were successfully purified from the cell-free culture broth of *Vibrio* sp. S1. The agarases had approximate MWs of 33 k*Da* (AgaA33) and 29 k*Da* (AgaA29) as estimated by SDS-PAGE. So far, there have been six agarases reported in the genus *Vibrio*, none of which have molecular weights similar to AgaA33 or AgaA29 (Table 2). In this regard, the two enzymes are presumed to be novel agarases that have never been reported.

AgaA33 and AgaA29 had optimum temperatures of 45°C and maintained 93% and 87% of their maximum agarase activities at 50°C, respectively. Moreover, both can withstand heat treatment at 45°C for 1 h and maintained their activity more than 80% of the control without heat treatment. In this respect, the two enzymes have considerable thermal resistance and, thus, will be applicable for agar hydrolysis even at 45°C, where 1.5% of the agar solution is not gelled. Due to the gelation properties of the agar solution, the enzyme reaction is usually limited to the use of agar solutions of about 0.3% to 1% [25]. However, the two enzymes studied here are considered to be applicable to the decomposition of agar solutions of 1.5% or more, which is economically very competitive.

To date, three thermophilic agarases have been reported (Table 2). The neoagarobiose-producing β-agarase from *Bacillus* sp. BI-3, a thermophilic bacterium isolated from a hot spring, had an optimum temperature of 70°C and retained more than 50% activity after heat treatment at 80°C for 15 min [26]. Another anaerobic thermophilic bacterium isolated from a hot spring, *Thermoanaerobacter wiegelii*, was also reported to produce an agarase that was active within the temperature range of 50 to 80°C (optimum, 70°C) and heat-stable; its activity remained after treatment at 90°C for 1 h [27]. A halophilic archaeon *Halococcus* sp. 197A, isolated from a solar salt, produced a thermophilic β-agarase Aga-HC with an optimum temperature of 70°C [28]. Aga-HC retained 50% activity after 1 h at 95°C. Unfortunately, other information on these three agarases including amino acid and genetic sequences are not

Table 2. Enzymatic properties of β-agarases characterized from Genus *Vibrio* and thermostable β-agarases from other bacterial genus

Strain	β-Agarase	Optimum condition Temp (°C)/pH	Molecular mass (kDa)	K_m (mg/mL)/ V_{max} (U/mg)	End products	Reference
Bacillus sp. BI-3	No name	70/6.4	58	ND/ND	NA2	[26]
Thermoanaerobacter wiegelii	No name	70/ND	67	ND/ND	ND	[27]
Halococcus sp. 197A	Aga-HC	70/6.0	55	ND/ND	NA2, NA4, NA6	[28]
Pseudoalteromonas hodoensis H7	AgaH71	45/6.0	33	28.33/88	NA2, NA4, NA6	[9]
Pseudoalteromonas sp. H9	AgaH92	45/6.0	50	59.56/156	NA4, NA6	[31]
Paenibacillus agarexedens BCRC 17346	AgaB-4	55/6.0	97	3.60/183	NA4	[30]
Saccharophagus degradans 2-40T	Aga16B	55/7.5	64	7.7/18	NA4, NA6, NA8	[6]
Cellulophaga omnivescoria W5C	Aga2	45/8.0	38	2.59/275	NA4, NA6	[32]
Vibrio sp. AP-2	No name	ND/5.5	20	ND/ND	NA2	[38]
Vibrio sp. JT0107	AgaA	30/8.0	107	ND/ND	NA2, NA4	[39]
Vibrio sp. PO-303	AgaA	45/7.0	87	ND/ND	NA4, NA6	[40]
	AgaB	55/7.5	115	ND/ND	NA2	
	AgaC	38/6.5	57	ND/ND	NA8, NA10	
Vibro sp. CN41	AgaA	40/7.5	110	3.54/3	NA4	[41]
Vibrio sp. S1	AgaA33	45/7.0	33	4.02/27	NA4, NA6,NA8	This study
	AgaA29	45/7.0	29	3.26/200	NA4, NA6	

ND, not determined; NA2, neoagarobiose; NA4, neoagarotetraose; NA6, neoagarohexaose; NA8, neoagarooctaose; NA10, neoagarodecaose

available; thus, no further research has been updated.

Among the marine mesophilic bacteria, no remarkable thermophilic enzymes have been reported. The optimum temperature for most enzyme reactions is in the range of 25 to 45°C, and the thermal stability is not very high (Table 2). Among them, the agarase that acts at the highest temperature was isolated from the deep-sequencing data sets of mangrove sediments [29]. The recombinant agarase, rAgaM1, maintained high activity from 30 to 60°C and more than 70% relative activity after incubation at 40°C for 1 h. However, other information on its producer strain and enzymes are not available. The β-agarases AgaB-4 from *Paenibacillus agarexedens* BCRC 17346 [30] and Aga16B from *S. degradans* 2-40T [6] were reported to have maximum activity at 55°C and be thermostable up to 50°C, and the latter could be successfully applied for enzymatic liquefaction of 1 % (w/v) agarose at 45°C. Other examples are β-agarases AgaH71 from *Pseudoalteromonas hodoensis* H7 [9], AgaH92 from *Pseudoalteromonas* sp. H9 [31], and Aga2 from *Cellulophaga omnivescoria* W5C [32] which showed maximum activity at 45°C. Especially, AgaH71 and AgaH92 retained more than 90% at 45°C and 85% at 50°C of their initial activities, respectively, after heat treatment for 1 h. In this sense, the two agarases described in this report have relatively very high thermal stability among the enzymes produced by marine microorganisms.

Comparing the kinetic parameters, Aga2 [32] and AgaA29 showed lower K_m values and higher V_{max} among reported thermostable enzymes including AgaA33 (Table 1). As a result, AgaA29 produced by *Vibrio* sp. S1 is considered to be an enzyme whose catalytic efficiency is extremely superior among these enzymes.

Agar-derived oligosaccharides have been reported to be nontoxic and have various physiological and biological effects, such as antiviral, antitumor, immune stimulator, and antioxidative stress activities [2]. Recently, important biological effects of neoagarooligosaccharides including antiobesity and antidiabetic activities [25], cholesterol homeostasis [33], antisepsis [34], antitumor immunity [35], and the meliorating activity of gut microbiota dysbiosis [36] were also reported. Moreover, the simultaneous enzymatic saccharification of agar and its bioconversion into ethanol was technically established in the recombinant strain of *Saccharomyces cerevisiae* [37]. Despite the availability of agar, the development of thermostable enzymes that can be used stably at high temperatures above the sol–gel transition temperature is an often unsurmountable limitation in agar processing. In this study, both of the enzymes were blocked at their N-terminus, so the corresponding gene information could not be studied. However, genomic sequencing is in progress to obtain genetic information. Thus, future studies will be carried out on the massive production and application of the thermostable enzymes of *Vibrio* sp. S1 for agar processing.

5. Conclusion

In order to efficiently utilize agar having various industrial applications, it is necessary to develop thermostable agarases which can act at a temperature at which the agar solution does not solidify. According to this necessity, a new marine microorganism, which has the maximum growth at 40°C using agar as a carbon source, was isolated. In addition, two agarases produced by *Vibrio* sp. S1 were characterized as novel thermostable enzymes showing maximum activity at 45°C. So far, plenty of agar hydrolytic enzymes have been reported from many microorganisms, but specific information on thermostable enzyme is scarce. Therefore, the strain *Vibrio* sp. S1 and the agarase produced by it are expected to be useful to the industrial application of agar.

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References

1. Rhein-Knudsen, N., M. T. Ale, and A. S. Meyer (2015) Seaweed hydrocolloid production: an update on enzyme assisted extraction and modification technologies. *Mar. Drugs.* 13: 3340-3359.
2. Chi, W. J., Y. K. Chang, and S. K. Hong (2012) Agar degradation by microorganisms and agar-degrading enzymes. *Appl. Microbiol. Biotechnol*. 94: 917-930.
3. Usov, A. I. (2011) Polysaccharides of the red algae. *Adv. Carbohydr. Chem. Biochem*. 65: 115–217.
4. Nussinovitch, A. (1997) *Hydrocolloid Applications*: *Gum technology in the food and other industries*. pp. 1-18. Springer, New York, NY, USA.
5. Brinker, C. J. and G. W. Scherer (2013) Sol–gel science: the physics and chemistry of sol–gel processing. pp. 2-10. *Sol–gel processing*. Academic Press, Cambridge, MA, USA.
6. Kim, J. H., E. J. Yun, N. Seo, S. Yu, D. H. Kim, K. M. Cho, H. J. An, J. H. Kim, I. G. Choi, and K. H. Kim (2017) Enzymatic liquefaction of agarose above the sol-gel transition temperature using a thermostable endo-type β-agarase, Aga16B. *Appl. Microbiol. Biotechnol.* 101: 1111-1120.
7. Kim, H. T., S. Lee, K. H. Kim, and I. G. Choi (2012) The complete enzymatic saccharification of agarose and its application to simultaneous saccharification and fermentation of agarose for ethanol production. *Bioresour. Technol.* 107: 301-306.
8. Kim, D. H., E. J, Yun, S. H. Lee, and K. H. Kim (2018) Novel two-step process utilizing a single enzyme for the production of high-titer 3,6-anhydro-L-galactose from agarose derived from

red macroalgae. *J. Agric. Food. Chem*. 66: 12249-12256.
9. Park, D. Y., W. J. Chi, J. S. Park, Y. K. Chang, and S. K. Hong (2015) Cloning, expression, and biochemical characterization of a GH16 β-agarase AgaH71 from *Pseudoalteromonas hodoensis* H7. *Appl. Biochem. Biotechnol.* 175: 733-747.
10. Baker, G. C., J. J. Smith, and D. A. Cowan (2003) Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods.* 55: 541-555.
11. Chun, J., J. H. Lee, Y. Jung, M. Kim, S. Kim, B. K. Kim, and Y. W. Lim (2007) ExTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* 57: 2259-2261.
12. Thomson, J. D., D. G. Higgins, and T. J. Gibson (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.
13. Hall, T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95-98.
14. Saitou, N. and M. Nei (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.
15. Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.
16. Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*. 16: 111-120.
17. Komagata, K. and K. Suzuki (1987) Lipid and cell-wall analysis in bacterial systematic. *Methods Microbiol.* 19: 161-207.
18. Mesbah, M., U. Premachandran, and W. B. Whitman (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int. J. Syst. Bacteriol.* 39: 159-167.
19. Sasser, M. (1997) Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note 101. MIDI Inc., Newark, DE, USA.
20. Lineweaver, H. and D. Burk (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc*. 56: 658-666.
21. Temuujin, U., W. J. Chi, S. Y. Lee, Y. K. Chang, and S. K. Hong (2011) Overexpression and biochemical characterization of DagA from *Streptomyces coelicolor* A3(2): an endo-type β-agarase producing neoagarotetraose and neoagarohexaose. *Appl. Microbiol. Biotechnol.* 92: 749-759.
22. Stackegrandt, E. and J. Ebers (2006) Taxonomic parameters revisited: tarnished gold standards. *Microbiol. Today.* 33: 152-155.
23. Morrice, L. M., M. W. McLean, F. B. Williamson, and W. F. Long (1983) Beta agarase I and II from *Pseudomonas atlantica* purifications and some properties. *Eur. J. Biochem.* 135: 553-558.
24. Rochas, C., P. Potin, and B. Kloareg (1994) NMR spectroscopic investigation of agarose oligomers produced by an alpha-agarase. *Carbohydr. Res.* 253: 69-77.
25. Hong, S. J., J. H. Lee, E. J. Kim, H. J. Yang, J. S. Park, and S. K. Hong (2017) Anti-obesity and anti-diabetic effect of neoagarooligosaccharides on high-fat diet-induced obesity in mice. *Mar. Drugs.* 15: 90.
26. Li, J., Y. Sha, D. Seswita-Zilda, Q. Hu, and P. He (2014) Purification and characterization of thermostable agarase from *Bacillus* sp. BI-3, a thermophilic bacterium isolated from hot spring. *J. Microbiol. Biotechnol.* 24: 19-25.
27. Bannikova, G. E., S. A. Lopatin, V. P. Varlamov, B. B. Kuznetsov, I. V. Kozina, M. L. Miroshnichenko, N. A. Chernykh, T. P. Turova, and E. A. Bonch-Osmolovskaia. (2008) The thermophilic bacteria hydrolyzing agar: characterization of thermostable agarase. *Prikl. Biokhim. Mikrobiol.* 44: 404-409.
28. Minegishi, H., Y. Shimane, A. Echigo, Y. Ohta, Y. Hatada, M. Kamekura, T. Maruyama, and R. Usami (2013) Thermophilic and halophilic β-agarase from a halophilic archaeon *Halococcus* sp. 197A. *Extremophiles.* 17: 931-939.
29. Di, W., W. Qu, and R. Zeng (2018) Cloning, expression, and characterization of thermal-stable and pH-stable agarase from mangrove sediments. *J. Basic. Microbiol.* 58: 302-309.
30. Chen, Z. W., H. J. Lin, W. C. Huang, S. L. Hsuan, J. H. Lin, and J. P. Wang (2018) Molecular cloning, expression, and functional characterization of the β-agarase AgaB-4 from *Paenibacillus agarexedens*. *AMB Express*. 8: 49.
31. Chi, W. J., C. R. Lee, S. Dugerjonjuu, J. S. Park, D. K. Kang, and S. K. Hong (2015) Biochemical characterization of a novel iron-dependent GH16 β-agarase, AgaH92, from an agarolytic bacterium *Pseudoalteromonas* sp. H9. *FEMS Microbiol. Lett.* 362: fnv035.
32. Ramos, K. R. M., K. N. G. Valdehuesa, G. M. Nisola, W. K. Lee, and W. J. Chung (2018) Identification and characterization of a thermostable endolytic β-agarase Aga2 from a newly isolated marine agarolytic bacteria *Cellulophaga omnivescoria* W5C. *N. Biotechnol*. 40: 261-267.
33. Yang, J. H., S. S. Cho, K. M. Kim, J. Y. Kim, E. J. Kim, E. Y. Park, J. H. Lee, and S. H. Ki (2017) Neoagarooligosaccharides enhance the level and efficiency of LDL receptor and improve cholesterol homeostasis. *J. Funct. Foods.* 38: 529-539.
34. Kang, D. R., G. Y. Yoon, J. Cho, S. J. Lee, S. J. Lee, H. J. Park, T. H. Kang, H. D. Han, W. S. Park, Y. K. Yoon, Y. M. Park, and I. D. Jung (2017) Neoagarooligosaccharides prevent septic shock by modulating A20- and cyclooxygenase-2-mediated interleukin-10 secretion in a septic-shock mouse model. *Biochem. Biophys. Res. Commun.* 486: 998-1004.
35. Lee, M. H., J. H. Jang, G. Y. Yoon, S. J. Lee, M. G. Lee, T. H. Kang, H. D. Han, H. S. Kim, W. S. Choi, W. S. Park, Y. M. Park, and I. D. Jung (2017) Neoagarohexaose-mediated activation of dendritic cells via Toll-like receptor 4 leads to stimulation of natural killer cells and enhancement of antitumor immunity. *BMB Rep.* 50: 263-268.
36. Zhang, N., X. Mao, R. W. Li, E. Hou, Y. Wang, C. Xue, and Q. Tang (2017) Neoagarotetraose protects mice against intense exercise-induced fatigue damage by modulating gut microbial composition and function. *Mol. Nutr. Food Res.* 61: 1600585.
37. Lee, J. S., S. K. Hong, C. R. Lee, S. W. Nam, S. J. Jeon, and Y. H. Kim (2019) Production of ethanol (agaro-bioethanol) from agarose by unified enzymatic saccharification and fermentation in recombinant yeast. *J. Microbiol. Biotechnol.* 29: 625-632.
38. Aoki, T., T. Araki, and M. Kitamikado (1990) Purification and characterization of a novel beta-agarase from *Vibrio* sp. AP-2. *Eur. J. Biochem.* 187: 461-465.
39. Sugano, Y., I. Terada, M. Arita, M. Noma, and T. Matsumoto (1993) Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. Strain JT0107. *Appl. Environ. Microbiol.* 59: 1549-1554.
40. Dong, J., Y. Tamaru, and T. Araki (2007) A unique beta-agarase, AgaA, from a marine bacterium, *Vibrio* sp. strain PO-303. *Appl. Microbiol. Biotechnol.* 74: 1248-55.
41. Liao, L., X. W. Xu, X. W. Jiang, Y. Cao, N. Yi, Y. Y. Huo, Y. H. Wu, X. F. Zhu, X. Q. Zhang, and M. Wu (2011) Cloning, expression, and characterization of a new beta-agarase from *Vibrio* sp. strain CN41. *Appl. Environ. Microbiol.* 77: 7077-7079.