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Inspecting the genome sequence and agarases of *Microbulbifer pacificus* LD25 from a saltwater hot spring

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Neoagaro-oligosaccharides prepared by agar hydrolysis have various application fields, including the pharmaceutical, cosmetic, and food industries. In this study, an agarolytic strain was isolated from a saltwater hot spring and identified as *Microbulbifer pacificus* LD25 by 16S rRNA. The whole genome sequence of *M. pacificus* LD25 was obtained. It had a size of 4.27 Mb and comprised 3062 predicted genes in 37 contigs with a G+C content of 58.0%. Six agarases were annotated and classified into three families, namely, GH16 (AgaL1), GH86 (AgaL2, AgaL3), and GH50 (AgaL4, AgaL5, AgaL6), which shared 75–96% identities with unpublished hypothetical proteins and agarases. AgaL1, AgaL4, and AgaL6 can be successfully expressed and purified in *Escherichia coli*. AgaL1 and AgaL4 displayed a significantly agarolytic capability, whereas AgaL6 exhibited a rarely detectable enzymatic activity. The optimal temperature and pH required for the activity of AgaL1 and AgaL4 was 50°C and 60°C, respectively, at pH 7. The specific activities of AgaL1 and AgaL4 were achieved at 16.8 and 9.6 U per mg of protein. Both agarases were significantly inhibited in the presence of EDTA, MgO, $ZnCl_2$, and H_2O_2. However, AgaL1 was resistant to 0.1% SDS and AgaL4 was slightly activated by $CaCl_2$. Substrate hydrolysis detected by LC-MS/MS analysis indicated that neoagarobiose was the main product during AgaL1 and AgaL4 catalysis. Furthermore, AgaL4 was thermostable and retained over 93% of its relative activity after pre-incubation at 70°C for 180 min. Consequently, *M. pacificus* LD25 has a potential for agarase production in *E. coli* and industrial applications.

[**Key words:** *Microbulbifer pacificus*; Genome sequence; Agarase; Neoagarobiose; Thermostability]

Several agarolytic bacteria have been identified in marine environments. Thus, agar can be catalyzed into agaro-oligosaccharides (AOSs) and neoagaro-oligosaccharides (NAOSs) by α-agarase and β-agarase, respectively, from agar-degrading bacteria. β-Agarases are divided into four families, namely, glycoside hydrolase-16 (GH16), glycoside hydrolase-50 (GH50), glycoside hydrolase-86 (GH86), and glycoside hydrolase-118 (GH118), in the Carbohydrate-Active Enzymes database. They are capable of hydrolyzing agarose into neoagaro-oligosaccharides (1). A novel cold-adapted β-agarase belonging to GH39 derived from a marine bacterium, *Gayadomonas joobiniege*, was recently reported (2). NAOSs can be further cleaved into 3,6-anhydro-L-galactose (L-AHG) and D-galactose by neoagarobiose hydrolase. L-AHG and NAOSs exhibit various physiological activities, including anti-inflammation, skin whitening, and moisturizing properties, and are therefore used as pharmaceutical and cosmetic ingredients (3). Moreover, NAOSs can potentially serve as prebiotics for stimulating the cell growth of bifidobacteria and lactobacilli (4). Recent works have demonstrated the crucial roles played by different NAOSs in immunology and anti-fatigue function. Zhang et al. (5) suggested that neoagarotetraose may alleviate intense exercise-induced fatigue in a mouse model. Lee et al. (6) indicated that neoagarohexaose can promote the maturation of dendritic cells through a Toll-like receptor and activation of natural killer cells for antitumor treatment.

The genus of *Microbulbifer* has been recorded for 25 species with validly published names. These species are typically found in high salinity environments, such as marine sediments, mangrove forests, and halophyte rhizosphere (7–9). Nine species of *Microbulbifer*, namely, *Microbulbifer agarilyticus*, *M. aggregans*, *M. donghaiensis*, *M. elongatus*, *M. mangrovi*, *M. marinus*, *M. thermotolerans*, *M. variabilis*, and *M. yueqingensis* with whole genome sequences have been submitted to the NCBI database. Meanwhile, the properties of agarase in different *Microbulbifer* species have been extensively investigated. Four agarases from *Microbulbifer elongates*, *M. thermotolerans*, and *M. maritimus* have been characterized and published (10–13). Two β-agarases belonging to GH16 in *M. elongates* and *M. thermotolerans* and one β-agarases belonging to GH86 in *M. thermotolerans* were identified based on the distinguishing feature of the glycoside hydrolase family, and they yielded neoagarotetraose and neoagarohexaose as the main products, respectively (10–12). However, the classification of

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agarase from *M. maritimus* remains unclear because of inadequate genetic sequences (13). Furthermore, the crystal structure of β-agarase (GH16) designated as *Mt*AgaA from *M. thermotolerans* consisted of a β-jelly roll fold conserved in other members of the GH16 family (14). This report further suggested that the thermostability of *Mt*AgaA may be attributed to the high hydrogen bonds, salt bridges, short length of the surface loops and the residues of Pro and Arg.

In this study, a marine strain was isolated from a saltwater hot spring of Zhaori Spring, Green Island, Taiwan, and characterized with agarolytic functionality. This strain was identified as *Microbulbifer pacificus* LD25 through 16S rRNA sequencing. The whole genome sequence of *M. pacificus* LD25 was obtained by Illumina Solexa analysis. The genome was 4.27 Mb in size and comprised 3062 predicted genes. This is the first genome sequence of *M. pacificus* submitted to the NCBI database. To examine the agarolytic capability of *M. pacificus* LD25, six agarases (AgaL1-6) were cloned and overexpressed in *Escherichia coli*. Then, the agarolytic activities and major hydrolysis products of the recombinant agarases were determined and described.

MATERIALS AND METHODS

Bacterial growth and 16S rRNA analysis The bacterial LD25 strain was isolated and purified from Zhaori Spring, Green Island, Taiwan, a world-class saltwater hot spring with temperature of approximately 60–70°C. LD25 was cultivated in marine agar medium (BCRC Medium 61) at 30°C for 3 days. The agarase, protease, gelatinase, esterase, cellulose, and xylanase, activities of LD25 were determined by agar plate method. To observe the clear zone of enzymatic activities, the agar media were utilized as follows. (i) Agarase: Observation of marine agar degradation with Gram's iodine solution (0.203 g of Iodine and 5.2 g of potassium iodide in 100 mL aqueous solution) was performed. (ii) Protease: Commercial nonfat dry milk (1%) was added in marine agar medium for 2 days of bacterial cultivation, and a clear zone was further observed. (iii) Gelatinase: Gelatin (1%, w/v) was added in marine agar medium for 2 days of bacterial cultivation. HgCl (5%, w/v) was used to wash medium, and a clear zone was further observed. (iv) Cellulase: Sodium carboxymethylellulose (1%, w/v) was added in marine agar medium for 2 days of bacterial cultivation. Congo red solution (0.1% Congo red in 20% ethanol) was used for the staining of the medium for 30 min, then 1 M NaCl was added for 15 min, and 1 N HCl was finally added for clear zone observation. (v) Xylanase: Xylan (1%, w/v) was added in marine agar medium for 2 days of bacterial cultivation, and the stained method was consistent with cellulase. (vi) Esterase: Tween 80 (0.45%, w/v) was added in the marine agar medium for 2 days of bacterial cultivation, and a turbid zone around the colony was observed. To determine the amylase activity, LD25 was added into the SM medium (10 g yeast extract, and 10 g soluble starch in 1 L aqueous solution). Then, Gram's iodine solution was mixed with the SM broth, and a clear solution was further observed. Furthermore, LD25 was grown at different temperature and salinity ranging from 15°C to 50°C and 2–10% NaCl for optimal condition. Antibiotic susceptibilities including ampicillin, carbenicillin, and tetracycline were used. Analysis of the acid production was performed using API 50 CHE (bioMérieux, Marcy l'Etoile, France).

After cultivation, the LD25 strain was centrifuged and harvested at 8000 ×*g* for 10 min. The bacterial pellet was harvested for genomic extraction. The chromosome was amplified with the primers F8 (5′-AGTTTGATCCTGGCTCAG-3′) and R1492 (5′-ACCTTGTTACGACTT-3′) for 16S rRNA, and sequenced by using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The phylogeny of 16S rRNA was conducted by using the neighbor-joining method of MEGA software with 1000 bootstrap replicates (15). Accession numbers for the different *Microbulbifer* species were compared.

Genome sequencing and annotation For the comprehensive examination of the *Microbulbifer* sp. LD25 genome, draft genome sequencing was conducted through the next generation sequencing (NGS) method. The LD25 chromosome DNA with OD 260/280 in a range of 1.8–2.0, and quantity ratio using Qubit versus NanoDrop over 0.7 was achieved for Illumina Solexa analysis (Illumina, San Diego, CA, USA). The library construction, whole genome sequencing, and genome de novo analysis were performed by Welgene Biotech (Taipei, Taiwan). DNA was sonicated to the size between 400 and 500 bp by Misonix 3000 sonicator (Misonix. Inc., Farmingdale, NY, USA) and analyzed by bioanalyzer DNA 1000 chip (Agilent Technologies GmbH, Waldbronn, Germany). The library was constructed using a TruSeq DNA Preparation protocol of Illumina. The DNA sequencing reads were trimmed and screened with the ConDeTri software for acquisition of qualified reads (16). Celera assembler was used for the de novo assembly of the cleaned and filtered reads (17). Genes were annotated in MAKER2 (18) by using a GeneMark (19) and searched against NCBI non-redundant database by BLASTP. Putative open reading frames for agarases were employed for phylogenetic tree construction by using the neighbor-joining method of MEGA software with 1000 bootstrap replicates (15). The different agarase genes of glycoside hydrolases including GH16, GH39, GH50, GH86, and GH118 were compared.

Agarase expression plasmids construction For the amplification of the agarase genes including AgaL1-6 from *M. pacificus* LD25, different primers for AgaL1 (forward primer, 5′-CACCATGAACCGTTTAACTATCACCC-3′; reverse primer, 5′-TCGTAAATCCGCAGTGAGC-3′), AgaL2 (forward primer, 5′-CACCATGAAACTCCC-CTACCCCA-3′; reverse primer, 5′-GTTGCGCACCGGCACGGT-3′), AgaL3 (forward primer, 5′-CACCATGAAGCCACATTCCCTGC-3′; reverse primer, 5′-TTCACGGGC GCCGCGCAC-3′), AgaL4 (forward primer, 5′-CACCGTGGCCGCGGAAAATAACG-3′; reverse primer, 5′-GTGAGCCTCGCTACTGGAAC-3′), AgaL5 (forward primer, 5′-CACCATGCGCTCAGCACTGAGCC-3′; reverse primer, 5′-CTCGACAGACTTCACATCGC-3′), and AgaL6 (forward primer, 5′-CACCGTGCTGCTGCCTGCCTGTG-3′; reverse primer, 5′-ATCATTTTTTTGCCCATAGCG-3′) (the underlined sequence was the adaptor for ligation with the pET151/D-TOPO plasmid) were designed for polymerase chain reaction (PCR). For the PCR reaction, 250 ng DNA, 10 μL of 10× PCR buffer, 2 μL of 10 μM forward and reverse primers, 8 μL of 2.5 mM dNTP, and 5 U of *Taq* enzyme (Takara Bio Inc., Shiga, Japan) were mixed. PCR conditions were performed as follows: 5 min at 95°C for 1 cycle; 30 s at 95°C, 30 s at 50–60°C, and 2–4 min at 72°C per cycle for 30 cycles; and 10 min at 72°C for 1 cycle and maintained at 4°C. PCR products were ligated with a Champion pET151 Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA, USA) for the overexpression plasmids and subsequent transformation of *E. coli*.

Overexpression of agarases in *E. coli* and recombinant agarase proteins purification The six overexpression plasmids were transformed into competent cells of *E. coli* C43(DE3). The transformants were incubated on an Luria–Bertani (LB) agar plate with 0.5 mM isopropy-β-D-thiogalactoside (IPTG), and 100 μg mL^{-1} ampicillin. A single colony was picked and cultivated overnight in LB broth. *E. coli* cells were transferred into fresh LB broth and grown at 37°C until OD_{600} reached 0.5. Culture was induced by adding IPTG to 0.5 mM and incubated at 25°C for overnight. An *E. coli* pellet was harvested and suspended in buffer (25 mM Tris-HCl, 150 mM NaCl, and pH 7). The cells were disrupted by JY92-IIN sonicator (Ningbo Scientz Biotechnology, Ningbo, China) and centrifuged at 10,000 ×*g* for 15 min at 4°C. The supernatant was purified and followed by a Ni-NTA purification system (Invitrogen). A 3-kDa Amicon Ultra-15 Centrifugal Filter (Millipore, Bedford, MA, USA) was further used for concentrating and removing imidazole. Protein content was monitored using SDS-PAGE and colloidal Coomassie blue staining. The purified protein was determined by Bradford protein assay kit (Bio-Rad, Hercules, CA, USA).

Enzymatic activity assay of the agarases Enzymatic activity was measured by a spectrophotometric assay wherein reducing sugars obtained from the digestion of bacteriological agar with agarases were estimated. The mixture used in the enzyme activity assay contained the purified agarase and 0.3% agar (dissolved in 25 mM Tris-HCl, 150 mM NaCl, pH 7) at a constant temperature for 1 h incubation. The reaction was interrupted by 100°C incubation. For determining the reducing sugars, the mixture was added into DNS solution (1% 3,5-dinitrosalicylic acid, 30% potassium sodium tartrate, and 1.5% NaOH). The resulting mixture was then heated in boiling water for 5 min. A spectrophotometer was used for the detection of reducing sugars at OD_{540}. To optimize the enzymatic activity, different temperatures in a range of 30–70°C was performed. The pH profile of agarase activity was detected between 4 and 9. For the assays at pH 4.0–5.0, 50 mM sodium acetate/acetic acid buffer was used. For the assays at pH 6.0–8.0 and pH 9.0, 100 mM sodium phosphate and glycine/NaOH buffers, respectively, were used. To determine the effect of different ion and metal on agarases, various chemical compounds such as KCl (50 mM), $CaCl_2$ (50 mM), MgO (50 mM), $ZnCl_2$ (50 mM), NaCl (50–600 mM), EDTA (0–4.8 mM), SDS (0.1%) and H_2O_2 (0.1%) were utilized. The thermostability of enzyme was carried out by incubating enzyme at 50–70°C from 0 to 180 min. The enzyme was immediately chilled, and the activity was assayed using DNS method.

Liquid chromatographic mass/mass spectrometry of the reaction products The mixture with the purified agarase and bacteriological agar was incubated at a constant temperature for 1 h. After incubation, reaction was centrifuged at 10,000 ×*g* under 4°C for 10 min to remove the undigested agar debris. The supernatant was detected using a Silica Gel 60 thin layer chromatography (TLC) plate (Merck, Darmstadt, Germany). The TLC plate was monitored by UV, and the major hydrolysis product was cut off. The corresponding product was extracted by methanol and concentrated by a pressure-reducing method. The products were carried out by Mithra Biotechnology Inc. (Taipei, Taiwan) and identified by liquid chromatographic mass/mass spectrometry. Liquid chromatography was fitted with a C18 column (Biobasic 18, 1.0 × 150 mm, 2 μm) (Thermo Fisher Scientific, Waltham, MA, USA), and the condition was as follows: solvent A, 0.1% (v/v) formic acid in H_2O, and solvent B, 0.1% (v/v) formic acid in 95% acetonitrile. Gradient parameter was set as 1% solvent B, linear increase from 1% to 55% solvent B at 7 min, further linear increase from 55% to 90% at 11 min for keeping 3 min and then linear decrease to 1% B at 16 min for 4 min. The mass spectrometry was performed by the Q-Exactive mass spectrometer coupled with Ultimate 3000 RSLC system (Thermo Fisher Scientific). The desolvation temperature was 275°C, and the capillary voltage was 3.5 kV.

Nucleotide sequence accession number *M. pacificus* LD25 (BCRC81085) was deposited at the BCRC, Food Industry Research and Development Institute, Taiwan (accession number: BCRC81085). The accession numbers of expression plasmids including AgaL1–AgaL6 have been submitted to the NCBI. The accession numbers are MH621328–MH621333. This Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession no. PREV00000000. The version described in this paper is version PREV01000000.

RESULTS

Identification of LD25 by 16S rRNA LD25 was isolated and purified from a saltwater hot spring in Green Island, Taiwan. Several enzymatic activities including agarase, amylase, cellulase, esterase, gelatinase, protease, and xylanase were determined by agar plate and broth method. The results revealed that LD25 exhibited significant activities in agarase, cellulase, and gelatinase, followed by xylanase, amylase, esterase, and protease.

To realize the LD25 strain, 16S rRNA sequencing and phylogeny were conducted (Fig. S1). The 16S rRNA sequences were analyzed by the BLAST program of NCBI to interpret the bacterial relationships. The result indicated that the LD25 strain shared 99% identity with *M. pacificus* SPO729^T (DQ993341.1) and 98% identities with *Microbulbifer gwangyangensis* GY2^T (JF751045.2) and *Microbulbifer okinawensis* ABAB23^T (AB500893.1). The phenotypic characteristics of LD25 were further analyzed and compared with the phylogenetic neighbors of the genus *Microbulbifer* (Table S1). LD25 can be grown at 45°C and 7% NaCl; however, its growth was arrested at 50°C and 10% NaCl. Acid production from carbon sources and antibiotic susceptibility indicated that LD25 was similar to *M. pacificus* SPO729^T which cannot utilize lactose, D-mannose, and L-rhamnose as sole carbon and was resistant to ampicillin, but sensitive to carbenicillin. Therefore, LD25 was designated as *M. pacificus* LD25.

Draft genome sequence of *M. pacificus* LD25 The draft genome sequence of *M. pacificus* LD25 was obtained through next generation sequencing of the Illumina HiSeq platform. A total of 9,853,958 reads and 2,237,993,400 bp were obtained (Table S2). De novo assembly was performed by a Celera assembler and generated 37 contigs with a G+C content of 58%. A mapping rate of 97% was achieved. The total genome size was 4.27 Mb, and 3062 predicted genes were revealed. The Clusters of Orthologous Groups of proteins (COGs) database was utilized for the functional annotation of the sequenced genome (20). The results indicated that 3056 genes of *M. pacificus* LD25 were functionally assigned in the COGs database (Fig. S2).

Bioinformatic analysis of agarases To further examine the profile of agarases in *Microbulbifer* sp. LD25, database search and open reading frame (ORF) prediction were performed to gain information on the putative agarase genes. Six putative ORFs encoding agarase proteins in the range of 1794 bp to 4077 bp were identified. They were designated as AgaL1-6, which had deduced amino acids of 598, 1359, 810, 747, 793, and 744 in length, and predicted molecular weights of 64.3, 146.9, 89.0, 84.6, 88.7, and 82.7 kDa, respectively (Table S3). The deduced amino acids of AgaL1-6 genes were determined by the BLAST program of NCBI and had 75–96% identities with hypothetical proteins and agarases of *Microbulbifer* species. These homologous genes with AgaL1-6 remain uncharacterized and unpublished. Moreover, a phylogeny was constructed using the deduced amino acids to evaluate of the relationship of the glycoside hydrolase family among the six genes and known agarases (Fig. 1). The results suggested that the six genes were divided into three families: GH16 (AgaL1), GH86 (AgaL2 and AgaL3), and GH50 (AgaL4, AgaL5, and AgaL6).

By using *in silico* analysis (SignalP 4.1), putative signal peptides were predicted and observed at the N-terminal sequences of AgaL1, AgaL2 and AgaL3 with 19, 29 and 20 amino acids, respectively (21). This finding was consistent with the agar plate analysis of *M. pacificus* LD25, thereby confirming the significant agar degradation and agarase diffusion from the colonies demonstrated by staining with iodine reagent. Furthermore, 3D structures of β-agarase from the GH16 and GH50 families have been reported (14,22). In the research described here, the catalytic glycoside hydrolase module was examined by comparing the conserved domain of the deduced amino acid sequences and predicting the 3D structures by the SWISS-MODEL (23). Glutamate and aspartate residues are typically involved as accepting and donating protons in the catalytic activity during hydrolysis (14,22). Several consensus residues were identified, and the following catalytic sites were hypothetically recognized: 146th Glu, 148th Asp, and 151th Glu for AgaL1; 725th Asp, 840th Glu, and 1015th Glu for AgaL2; 221th Asp, 344th Glu, and 480th Glu for AgaL3; 479th Asp, 481th Glu, and 610th Asp for AgaL4; 529th Asp, 531th Glu, and 660th Asp for AgaL5; 470th Glu, 472th Glu, and 613th Asp for AgaL6 (Fig. S3). Moreover, the non-catalytic carbohydrate-binding module (CBM) of β-agarase for agar or neoagaro-oligosaccharide binding function was assumed to be located at the C-terminus for AgaL1 and AgaL2 by the NCBI Conserved Domain Architecture Retrieval Tool search. No significant CBM domain of β-agarase was matched with AgaL3-6. This may result from few studies about the analysis of 3D protein structure responding to different family of agarase.

Construction and overexpression of recombinant agarases in *E. coli* Six agarase genes were cloned and overexpressed in *E. coli* to characterize the AgaL1-6 proteins. Several primers were designed to amplify the *agaL1-6* genes, and the pET151/D-TOPO plasmid with a His-tag fusion of the N-terminus triggered by the T7 promoter was used for the DNA transformation in *E. coli* C43(DE3). The results showed that overexpression proteins were not obviously produced in the transformants harboring *agaL2*, *agaL3*, and *agaL5* genes. However, AgaL1, AgaL4, and AgaL6 appeared as a single band on the SDS-PAGE after protein purification in a nickel column (Fig. 2). In addition, the molecular masses of recombinant proteins fused with His-tag were in agreement with the predicted values.

Characterization of recombinant agarases The purified proteins of AgaL1, AgaL4, and AgaL6 were utilized to determine the enzymatic activities by using bacteriological agar and agarose as substrates at 37°C for 1 h. The results showed that the enzymatic activity of AgaL6 was nearly undetectable. Conversely, AgaL1 and AgaL4 displayed remarkable activities, and bacteriological agar exhibited a higher enzymatic activity than agarose. Thus, bacteriological agar was employed for the subsequent optimal activity and thermostability detection of AgaL1 and AgaL4.

Fig. 3 presents the optimal temperature and pH profiles of AgaL1 and AgaL4 activities. The optimal temperatures of AgaL1 and AgaL4 were 50°C and 60°C, respectively. AgaL1 maintained residual activities of 73% and 70% at 30°C and 60°C, respectively. The activity of AgaL4 decreased to 51% at 70°C. The AgaL1 and AgaL4 activities had similar pH profiles. The optimal pH of the AgaL1 and AgaL4 activities was the same at pH 7. The residual activities of AgaL1 and AgaL4 were 77%–79%, and 60%–76% at pH 6–8 with sodium phosphate buffer. With acetic buffer, the agarase activities of AgaL1 and AgaL4 were 33%–64%, and 52%–57% at pH 4–5. The relative activities of AgaL1 and AgaL4 were 37% and 58%, respectively, at pH 9.0 when glycine/NaOH buffer was used. To calculate the specific activity of agarases, D-galactose was used as the standard for reducing sugar. One unit (U) of agarase activity represented the production of 1 μmole of reducing sugars per min. Under the optimal condition, the specific activities of AgaL1 and AgaL4 were 16.8 and 9.6 U per mg of protein, respectively.

Effects of various reagents on AgaL1 and AgaL4 Fig. 4 shows the effects of different metal ions and chemical reagents on the recombinant agarase activities. Various sodium chlorides

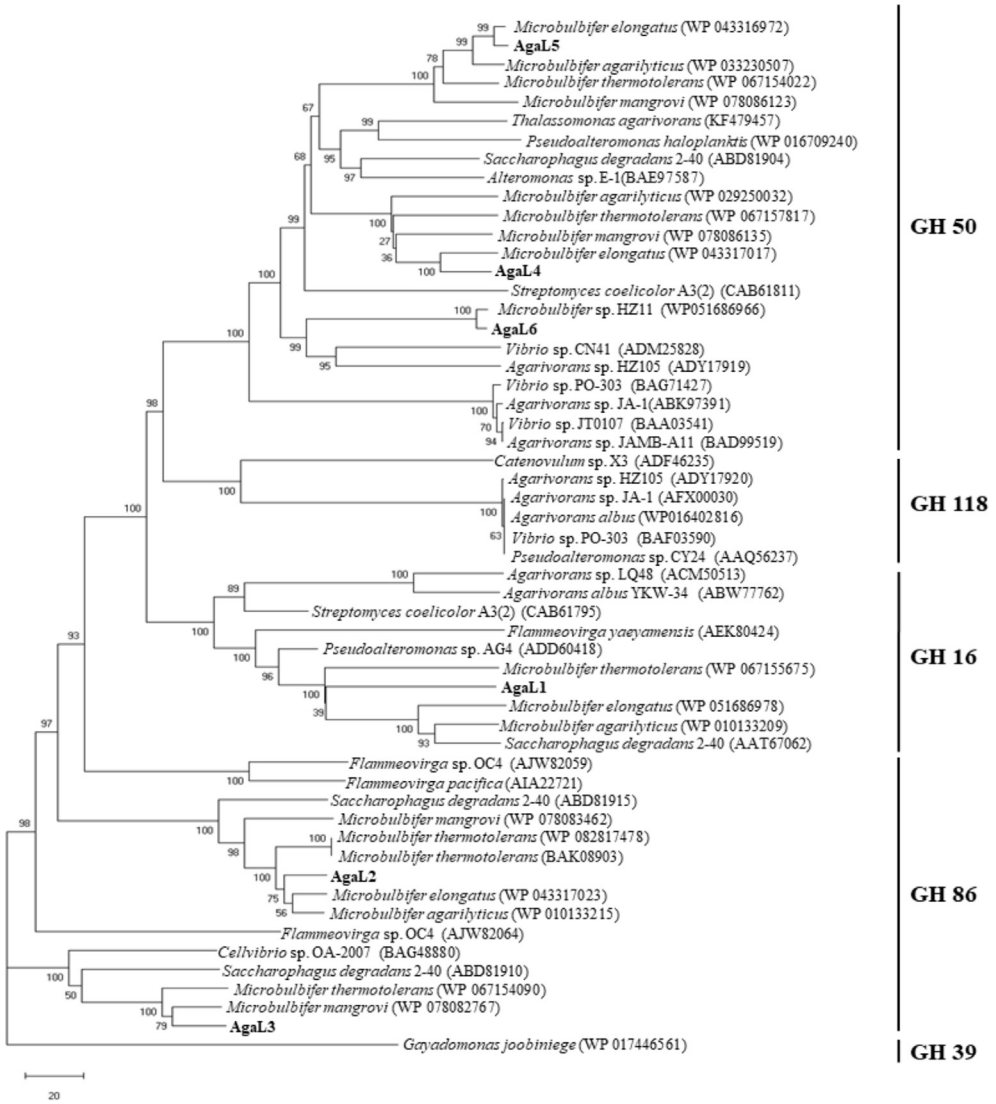

FIG. 1. Phylogenetic tree of deduced amino acids of β-agarases. The phylogeny was carried out by AgaL1-6 from *Microbulbifer pacificus* LD25 and various β-agarases from GH16, GH39, GH50, GH86, and GH118 families. The tree was constructed using the neighbor-joining method by MEGA software. Bootstrap values of 1000 replicates are shown in the nodes.

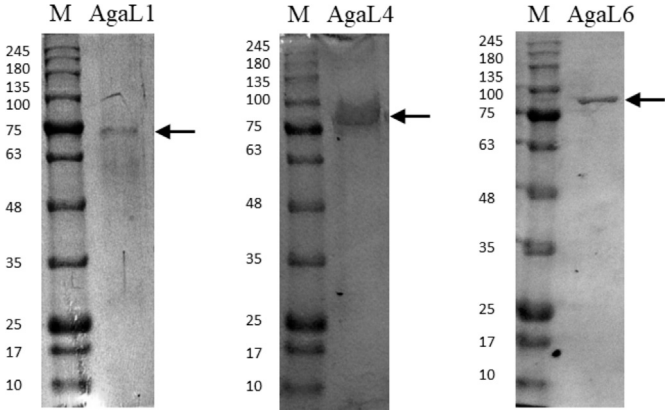

FIG. 2. SDS-PAGE analyses of recombinant AgaL1, AgaL4, and AgaL6 proteins stained with Coomassie Brilliant Blue. Major band on the SDS-PAGE gel was observed, suggesting β-agarase purification through the nickel column. The arrow and M indicate the purified proteins and the protein marker, respectively.

from 50 to 600 mM were used to explore the agarase activities of AgaL1 and AgaL4 because of the isolation of *Microbulbifer* sp. LD25 from the saltwater hot spring. The maximal activity of AgaL1 was detected at 240–360 mM, whereas AgaL4 activity was found at 240 mM. This result suggested that AgaL1 had a higher tolerance for salinity than AgaL4. The relative activity of AgaL1 was increased to over 94% from 50 to 600 mM NaCl. Nevertheless, the residual activities of AgaL1 and AgaL4 were gradually decreased when EDTA was added from 0 to 4.8 mM. The activity of AgaL1 sharply decreased to 40% at 4.8 mM, whereas AgaL4 maintained a 75% relative activity. In addition, AgaL4 was significantly inhibited by MgO, $ZnCl_2$, SDS, and H_2O_2. A similar result was also observed in AgaL1. However, 0.1% SDS had no effect on AgaL1 activity. The enzymatic activity of AgaL4 was slightly activated by $CaCl_2$.

Identification of hydrolysis product of agarases by LC-MS/MS analysis The hydrolysis products from the catalysis of AgaL1 and AgaL4 were obtained by incubation with 0.3% bacteriological agar at 50°C and 60°C for 1 h, respectively. The mixtures were subjected to TLC analysis, and only a single spot was clearly perceived in the

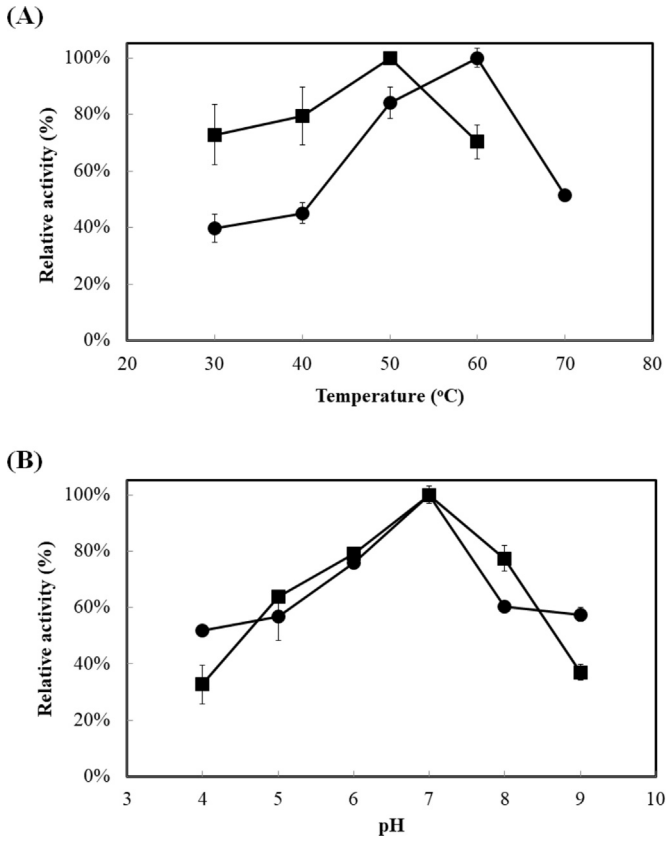

FIG. 3. Optimal temperature and pH analyses of recombinant AgaL1 and AgaL4 activities. (A) The temperature profiles of AgaL1 and AgaL4 were measured at 30°C–70°C and pH 7 with Tris–HCl buffer for 1 h using 0.3% agar. (B) The pH profiles of AgaL1 and AgaL4 were measured at 50°C and 60°C, respectively, with 50 mM sodium acetate/acetic acid buffer (pH 4–5), 100 mM sodium phosphate buffer (pH 6–8), and glycine/NaOH buffer (pH 9) for 1 h using 0.3% agar. Squares and circles indicate AgaL1 and AgaL4, respectively. All values are the average of three determinations.

AgaL1 and AgaL4 reactions. To confirm the hydrolysis products of AgaL1 and AgaL4, the main products were extracted from the TLC plates and then subjected to LC-MS/MS. A major peak was observed at retention times of 1.88 and 1.54 min in the AgaL1 and AgaL4 catalysis, respectively. The mass spectrum presented a high intensity of *m/z* (mass-to-charge ratio) 365 ions in both AgaL1 and AgaL4 reactions (Fig. 5). The *m/z* 365 ion was presumed to be [neoagarobiose + CH_3CN]$^+$ because of two major fragments, namely, *m/z* 185 for [3,6-anhydro-α-L-galactose + Na]$^+$ and *m/z* 203 for [galactose + Na]$^+$. This result verified that neoagarobiose was the main hydrolysis product of AgaL1 and AgaL4.

Thermostability of AgaL1 and AgaL4 To reduce the cost for industrial application, previous studies have investigated the thermal resistance of enzymes (24). The thermostable analysis of AgaL1 and AgaL4 was conducted from 0 to 180 min at 50 and 70°C (Fig. 6). The result revealed that the AgaL1 activity was completely lost after 120 min of incubation at 50°C. The half-life, the time over which the 50% activity is lost, of AgaL1 was approximately maintained for 65 min at 50°C according to the regression calculation ($y = -0.0085x + 1.0499$, $R^2 = 0.975$). Nevertheless, AgaL4 was more thermostable than AgaL1, which corresponded with the optimal temperature result of enzymatic activity. The AgaL4 activity can be attained over 93% relative activities for 180 min of incubation at 50°C and 70°C.

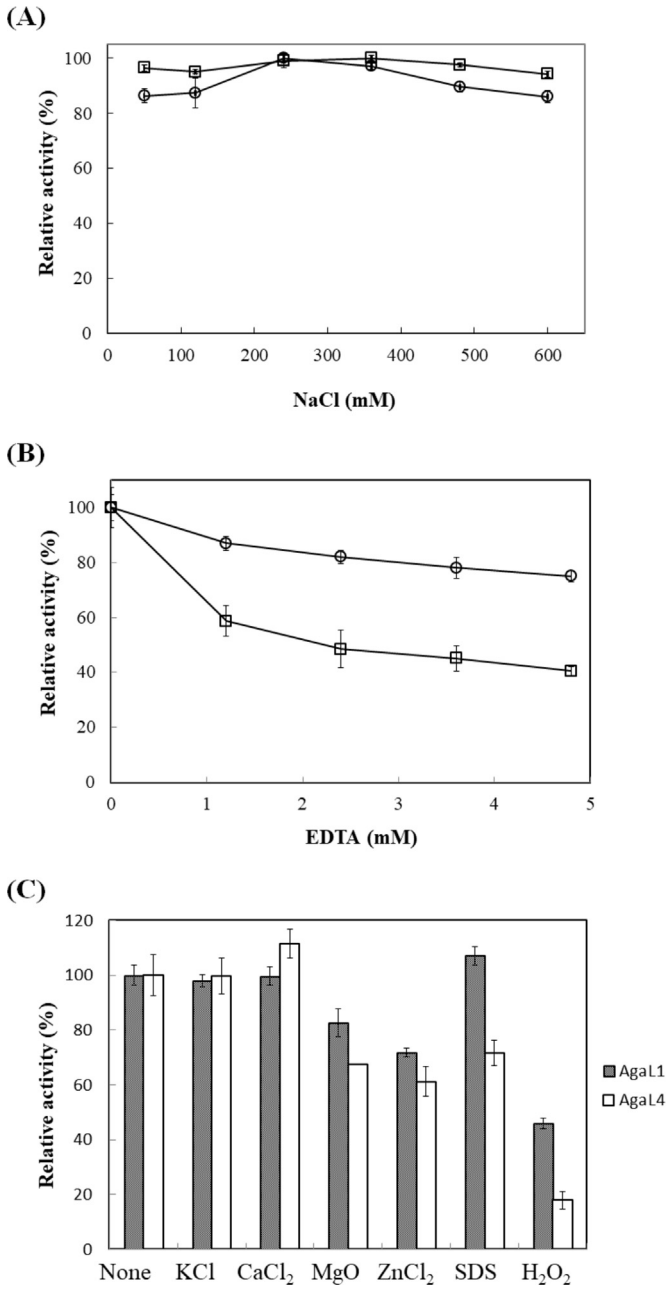

FIG. 4. Effect of various reagents on AgaL1 and AgaL4 activities. (A) NaCl, (B) EDTA, and (C) various reagents (50 mM KCl, $CaCl_2$, MgO, and $ZnCl_2$; 0.1% SDS and H_2O_2) were added into the pH 7 sodium phosphate buffer for AgaL1 and AgaL4 activity analysis at 50°C and 60°C. Squares and circles indicate AgaL1 and AgaL4, respectively.

DISCUSSION

Several studies on the analysis of *Microbulbifer* species including the characterizations of agarase, chitinase, esterase, alginate lyase, amylase, and carrageenase have been published (14,25–28). In this study, *M. pacificus* LD25 was isolated from a saltwater hot spring and exhibited various enzymatic activities. The draft genome sequence of *M. pacificus* LD25 was carried out and submitted to the NCBI database for further inspection of its functional proteins. Compared with the different genome sequences of *Microbulbifer* species, *M. pacificus* LD25 (4,271,795 bp) was proximate to the reported draft genome size of *M. elongatus* (4,223,108 bp) (Table S1) (25,29–32). However, genomic annotation suggested that *M. pacificus* LD25 had the least genes related to the published

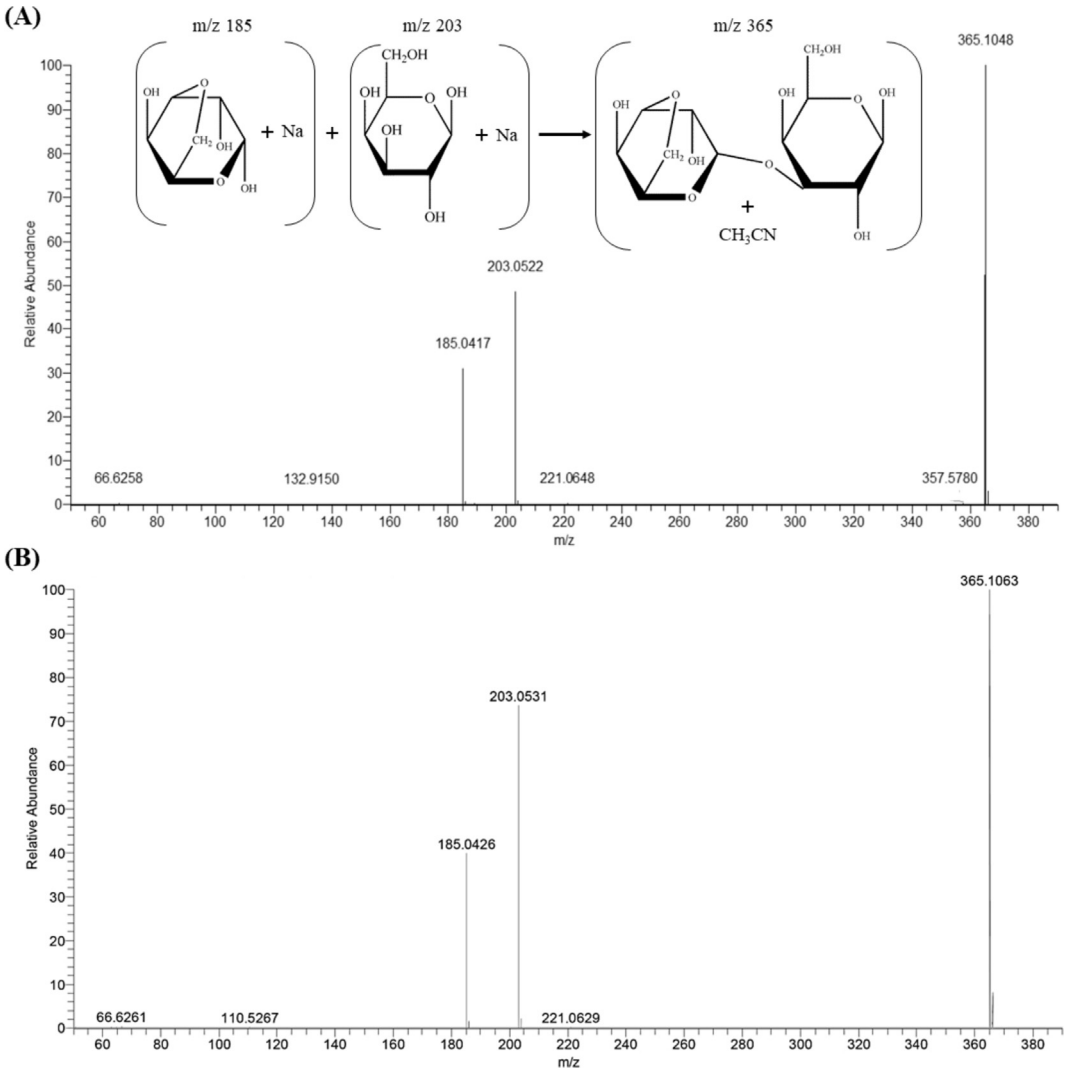

FIG. 5. LC-MS/MS spectrum of the neoagarobiose catalyzed by (A) AgaL1 and (B) AgaL4. The major product indicated that both spectrums show the fragment ions of *m*/*z* 365. Two major product fragments, *m*/*z* 185 for [3,6-anhydro-α-L-galactose + Na]$^+$ and *m*/*z* 203 for [galactose + Na]$^+$, were perceived together with the parent *m*/*z* 365 in MS/MS spectrum.

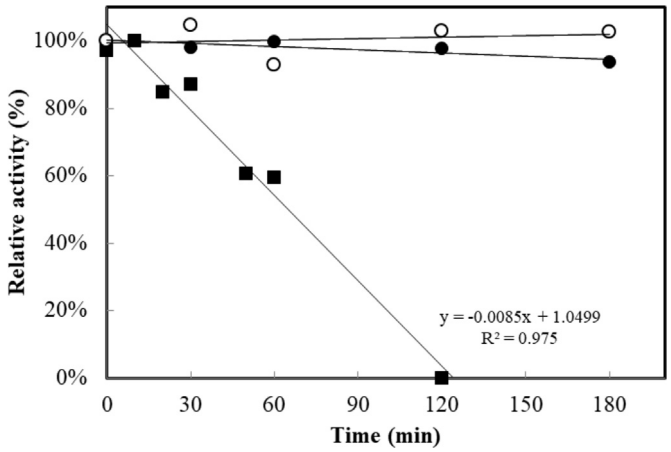

FIG. 6. Thermostable analyses of AgaL1 and AgaL4 relative activities. The AgaL1 and AgaL4 were incubated at 50–70°C by the time course from 0 to 180 min. The remaining AgaL1 and AgaL4 enzymatic activities were measured at 50°C and 60°C, and pH 7 for 1 h, respectively. Closed squares indicate AgaL1 activity at 50°C, and open circles and closed circles show AgaL4 activity at 50°C and 70°C, respectively.

genomes of the *Microbulbifer* species. In addition, agar plate determination indicated that *M. pacificus* LD25 can degrade various polysaccharides including the different activities of agarase, cellulose, gelatinase, xylanase, amylase, esterase, and protease. Moreover, genomic annotation of *M. pacificus* LD25 revealed the presence of six agarases, four alginate lyases, eight amylases, one carrageenase, seven chitinases, three gelatinases, two pullulanases, one α-glucanase, two β-glucanases, three endo-glucanases, five α-glucosidaes, five β-glucosidaes, two xylanases, and 46 relative proteases and esterases.

Considerable research effort has been directed at the heterologous overexpression of agarases in *E. coli* and *B. subtilis* (2,12). To investigate the properties of AgaL1-6, *E. coli* C43(DE3) was used to overexpress the recombinant agarases. The SDS-PAGE analysis results indicated that AgaL1, AgaL4, and AgaL6 belonging to GH16 and GH50 were successfully expressed in *E. coli* C43(DE3) through nickel column purification (Fig. 2). By contrast, no overexpressed proteins were detected in AgaL2, AgaL3, and AgaL5 belonging to GH86, and GH50. This phenomenon may be ascribed to the codon usage bias in *E. coli* or the protein misfolding with additional signal peptides or larger molecular weight (33). Additionally, a rare

activity of AgaL6 can be detected. Further studies on the active sites or binding domains with different substrates or metal ions such as point mutation experiments would clarify the catalytic capacity of AgaL6. AgaL1 and AgaL4 displayed higher digestibility when bacteriological agar was used rather than agarose for electrophoresis. According to our review of relevant literature, the optimal temperature of AgaL4 for agarase activity was as high as those reported by many previous studies on agarases from *Cellulophaga omnivescoria*, *Flammeovirga* sp., *Pseudoalteromonas* sp., *Aquimarina agarilytica*, and *Microbulbifer* sp. and was only lower than that from a thermophilic bacterium, *Bacillus* sp. BI-3 (Fig. 3) (10,34–38). Moreover, the maximal activities of these agarases were close to a neutral pH between 6 and 8. Consistent with other agarases, AgaL1 and AgaL4 were inhibited by EDTA (Fig. 4) (2,10). Nevertheless, different metal ions and reagents exerted different effects on the agarases. The properties of AgaL1 were similar to those of β-agarase from *Microbulbifer* sp. Q7, which was inhibited by Zn^{2+} and Mg^{2+} and resistant to SDS (39). By contrast, Ca^{2+} slightly improved AgaL4 activity. Besides, neoagarobiose was determined by LC-MS/MS as the main product in both AgaL1 and AgaL4 (Fig. 5). This finding agreed with the hydrolysis product catalyzed by other β-agarase of GH16 and GH50 families (22,40).

Although several thermostable characterizations containing non-polar residues (Val, Ala, Leu and Ile) and aromatic residues (Phe, Trp and Tyr) for elevating hydrophobicity, and polar charged residues (Arg and Lys) for electrostatic interaction have been explained, improving the enzymatic thermostability by alternating the appropriate sites of amino acids remains difficult (41). Furthermore, increased helix stability, additional salt bridges, decreased chain flexibility, and decreased number of cavities also contributed to the enzymatic stabilization of highly thermostable proteins (41,42). In this study, the relative activity of AgaL4 was maintained at over 93% for 180 min of pre-incubation at 70°C (Fig. 6) which is a higher thermostability than those reported in many previous studies (35–38). Comparison of the amino acid compositions between AgaL1 and AgaL4 revealed that the frequency of polar charged residues in AgaL4 was higher (9.51%) than that in AgaL1 (5.85%). In addition, on the basis of the predicted 3D protein structure, the α-helix content of thermostable AgaL4 was markedly higher than that of AgaL1 and played an important role in thermal resistance. These factors may reinforce the interactions of protein residues to facilitate thermostability.

In conclusion, the whole genome sequence of *M. pacificus* LD25 was successfully obtained and submitted to the NCBI database. Six agarases, namely AgaL1-6, were observed in the genome annotation whereas only three agarases, namely, AgaL1, AgaL4 and AgaL6, could be overexpressed and purified from *E. coli*. Recombinant AgaL1 and AgaL4 could hydrolyze agar, and neoagarobiose was the main product in AgaL1 and AgaL4. The catalytic activity of AgaL6 was nearly undetectable. By contrast, AgaL4 exhibited a thermostable enzymatic activity. Helix contents and polar charged residues may be critical factors influencing enzymatic thermostability. Overall, our study revealed that *M. pacificus* LD25 had a potential for agarase production in *E. coli* and other industrial applications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.10.001.

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