

Research Article

GENOME MINING AND EXPRESSION OF A β -AGARASE FROM MARINE BACTERIUM *Microbulbifer* sp. ARAS-458.1 ISOLATED IN VIETNAM

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ABSTRACT

Agarolytic microorganisms and their enzymes are of growing interest for producing bioactive agaro-oligosaccharides. From 169 environmental samples collected in Vietnam, 507 agarolytic bacterial strains were isolated. Among them, *Microbulbifer* sp. ARAS-458.1, obtained from red algae (*Rhodophyta*) in Quang Binh Province, exhibited remarkable agar-degrading activity. Genome sequencing revealed a 4.0 Mb draft genome containing 3,350 predicted protein-coding genes and a GC content of 57.1%. Comparative analysis showed 85.84% average nucleotide identity with *Microbulbifer elongatus* DSM 6810^T, indicating that ARAS-458.1 represents a distinct species. Six putative agarase genes were identified, including MARAS458A, a β -agarase of the glycoside hydrolase family 16 (GH16). MARAS458A was cloned and expressed in *Escherichia coli* and *Pichia pastoris*. In *E. coli*, the enzyme formed insoluble inclusion bodies, whereas expression in *P. pastoris* yielded an active, extracellular enzyme with a specific activity of 3.25 U/mg. The enzyme hydrolyzed agar to produce neoagarotetraose (NA4) and neoagarohexaose (NA6) as final products, confirming its endo-agarase function. MARAS458A exhibited optimal activity at 45°C and pH 7–10, was stable below 40°C, and showed thermolability at 45°C. The dual production of NA4 and NA6, together with its alkaline nature, distinguishes MARAS458A from previously reported *Microbulbifer* β -agarases, suggesting structural variations affecting substrate processing and product specificity. These findings highlight MARAS458A as a promising biocatalyst for sustainable production of functional agaro-oligosaccharides for food and health applications.

Keywords: β -agarase, *Microbulbifer*, marine, agar, oligosaccharides.

INTRODUCTION

Agar is an essential gelling agent widely used in the food, cosmetic, and medical industries. It is primarily extracted from

marine red algae of the genera *Gelidium* and *Gracilaria*. Agar consists mainly of two components, namely agarose and agaropectin. Agarose is a non-ionic linear polysaccharide with repeating units of D-

galactose and 3,6-anhydro-L-galactose (L-AHG), linked by alternating β -1,4 and α -1,3 glycosidic bonds (Chi *et al.*, 2012; Hehemann *et al.*, 2012). Meanwhile, agaropeptin is a charged polysaccharide with similar repeating units attached with $-\text{OSO}_3^-$, $-\text{OCH}_3$, glucuronate, or pyruvate residues (Chumsook *et al.*, 2023; Jiang *et al.*, 2023).

Because of its gelling properties and relative resistance to microbial hydrolysis, agar has traditionally been used in microbiology as a solid cultivation medium. The ability of certain bacteria to degrade agar and utilize as the carbon source for growth was first reported in 1902 (Gran, 1902). Since then, numerous studies have identified a wide range of agarolytic bacteria, including Gram-negative genera such as *Pseudomonas*, *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Alterococcus*, *Microbulbifer*, *Agarivorans*, *Thalassomonas*, *Flavobacterium*, *Cellulophaga*, and *Zobellia*, as well as Gram-positive members of the phyla *Actinobacteria* and *Firmicutes* (Fu and Kim, 2010). Agarolytic bacteria have been isolated primarily from marine but also from non-marine environments (Fu and Kim, 2010; Jahromi and Barzkar, 2018).

Agarases are classified into α -agarases and β -agarases based on the type of glycosidic bond they cleave. α -Agarase cleaves α -1,3-glycosidic linkages in agarose, producing oligosaccharides of various lengths that contain L-AHG at the reducing end, such as agarobiose (A2), agarotetraose (A4), and agarohexaose (A6) (Wang *et al.*, 2020; Yu *et al.*, 2020). To date, only slightly more than ten α -agarases have been reported, and all belong to glycoside hydrolase family 96 (GH96). In contrast, β -agarases cleave β -1,4-glycosidic bonds and belong to multiple glycoside hydrolase families, including GH16, GH39, GH42, GH50, GH86, and

GH118, with the majority belonging to GH16 and GH50. The hydrolysis generates neoagarooligosaccharides of various lengths, such as neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), neoagarooctaose (NA8), neoagarodecaose (NA10), and neoagarododecaose (NA12) (Long *et al.*, 2024; Yu *et al.*, 2020; Zhu La *et al.*, 2024).

Agarases have diverse applications in biotechnology and research. They are primarily used to produce functional agarooligosaccharides (AOSs) with potential prebiotic, antioxidant, and therapeutic properties (Hong *et al.*, 2017a; Hong *et al.*, 2017b; Yun *et al.*, 2013; Zhu La *et al.*, 2024). L-AHG and AOSs recently have been identified as non-cariogenic sweeteners with the potential to replace xylitol, given their broad availability (Yu *et al.*, 2019). Agarases support the bioconversion of red algal biomass into value-added products (Jiang *et al.*, 2020). In molecular biology, β -agarase enables the gentle recovery of DNA from agarose gels (Chi *et al.*, 2012; Hehemann *et al.*, 2012). In this study, we report the genome analysis, cloning, and expression of a putative β -agarase gene from a novel marine bacterium isolated from the coastal area of Vietnam and discuss its potential application in agarooligosaccharide production.

MATERIALS AND METHODS

Materials

Marine agar 2216, yeast extract, and peptone were purchased from TM Media, India. Agar, NaCl, K_2HPO_4 , $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , CaCl_2 , I₂, KI, sodium citrate, KH_2PO_4 , and 3,5-dinitrosalicylic acid (DNS reagent) were purchased from Samchun Chemicals, South Korea. Tris-HCl and glycine-NaOH were

obtained from Biobasic, Canada. Phenol, chloroform, n-butanol, acetic acid, and methanol were purchased from Scharlau, Spain. The Silica Bead DNA Gel Extraction Kit, GeneJET Genomic DNA Purification Kit, and GeneJET Plasmid Miniprep Kit were obtained from ThermoFisher Scientific, USA. The TruSeq Nano DNA Library Prep Kit was purchased from Illumina, USA. Restriction enzymes *Xho*I, *Bam*HI, *Eco*RI, *Xba*I, *Mss*I, and T4 DNA ligase were obtained from ThermoFisher Scientific, USA. The pET28a(+) vector was obtained from Novagen, USA, and the pPICZαA vector was obtained from Invitrogen, USA. Zeocin, kanamycin, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Invitrogen, USA. Biotin was purchased from HiMedia, India. YNB was obtained from Sigma-Aldrich, USA. DEAE FF ion-exchange resin was purchased from Cytiva, USA, and Vivaspın ultrafiltration units (10 kDa MWCO) were obtained from Vivascience, Germany. Agarose M and D-galactose were obtained from Biobasic, Canada, while NA4 and NA6 were obtained from Biosynth Ltd., UK. TLC silica gel 60 plates were purchased from Merck, Germany. *Escherichia coli* BL21 (DE3) was obtained from Novagen, USA. *E. coli* DH5α and *Pichia pastoris* X33 were purchased from Invitrogen, USA.

Isolation of agarolytic bacteria

For the isolation of agarolytic bacteria, various types of samples from both marine and non-marine environments were collected across Vietnam. Marine-related samples included red algae, seaweeds, seawater, sea sediment, and waste sludge from agar-processing factories. Non-marine samples consisted of paddy soils and plant roots.

As direct isolation of agarolytic bacteria was not feasible, the samples were enriched by incubating approximately 1 g of each sample in 50 mL of liquid mineral medium containing agar as the sole carbon source (0.1% agar, 2.5% NaCl, 0.1% K₂HPO₄, 0.1% (NH₄)₂SO₄, 0.05% MgSO₄, 0.01% CaCl₂, pH 7.0-7.5) for 24–36 h at 30°C. The enriched suspensions were then spread onto mineral medium agar (MSA) plates and incubated at 30°C for 24–36 h. Colonies forming visible depressions on the agar surface, indicating agar degradation, were purified on MSA. Marine agar 2216 was used for routine maintenance of the strains. The collection of agarolytic bacteria was preserved in liquid nitrogen at the Vietnam Collection for Industrial Microorganisms (VCIM), Food Industries Research Institute, Hanoi, Vietnam.

Identification of bacteria by 16S rDNA sequencing

DNA was extracted using phenol-chloroform (1:1, v/v) from a loop-full of 24-h-old culture grown on Marine agar 2216 and purified using a Silica Bead DNA Gel Extraction kit. PCR amplification and sequencing of the 16S rRNA gene were performed using universal primers 27F and 1492R (James, 2010). The amplicons were sequenced at First BASE Laboratories Sdn Bhd (Selangor, Malaysia). The obtained sequences were compared with reference sequences using the BLAST algorithm via the EZBioCloud database (<https://www.ezbiocloud.net/>) to determine phylogenetic affiliation.

Whole-genome analysis

Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit according to the manufacturer's instructions.

Whole-genome sequencing was performed using the Illumina paired-end platform with a library prepared using the TruSeq Nano DNA Library Prep Kit. The raw sequencing reads were assembled using SPAdes (St. Petersburg genome assembler), a robust *de novo* genome assembler tailored for single-cell and multicell bacterial genomes. The genome annotation was conducted using Prokka, an automated software pipeline for rapid prokaryotic genome annotation, enabling the identification of coding sequences (CDS), rRNA, and tRNA genes. The protein sequence of each strain was uploaded to the dbCAN3 (Zheng *et al.*, 2023) server based on integrating three state-of-the-art tools/databases for automated CAZyme annotation.

Cloning and expression of *MARAS458A* in *Escherichia coli*

The *MARAS458A* gene, putatively encoding β -agarase, was amplified from *Microbulbifer* sp. ARAS-458.1 by PCR using primers MARAS458A-*Xho*I-F (5'-CTCGAGGTTGCTCAACGTAAATTTGT-3') and MARAS458A-*Bam*HI-R (5'-GGATCCGCCGACTGGGACGGCACT-3'), based on the predicted signal peptide-free sequence. The PCR product was digested with *Xho*I and *Bam*HI, and then ligated into the pET28a(+) vector, and then transformed into *E. coli* BL21 (DE3) using standard protocols (Sambrook *et al.*, 1989). Transformants were selected on LB-kanamycin (25 μ g/mL) plates and cultured at 30°C. Protein expression was induced with 1 mM IPTG and incubated for 20 h. Cultures were centrifuged at 13000 rpm for 15 min to separate supernatants and cell pellets. The pellets were lysed, sonicated, and insoluble proteins were solubilized in 8 M urea. Denatured proteins were refolded by stepwise dialysis (from 0 M to 8 M urea) in

Tris-HCl buffer (pH 7.0) at 4°C (Zhai *et al.*, 2016). After removing aggregates by centrifugation, the supernatant containing refolded β -agarase was collected for further analysis.

Cloning and expression of *MARAS458A* in *Pichia pastoris*

MARAS458A was amplified using the primers MARAS458A-*Eco*RI-F (5'-TGAATTCGACTGGGACGGCACT-3') and MARAS458A-*Xba*I-R (5'-TTCTAGAAAGTTGCTCAACGTAAATTGT-3'). The PCR product was digested with *Eco*RI and *Xba*I, purified, and ligated into the pPICZ α A vector using T4 ligase. The ligation mix was transformed into *E. coli* DH5 α via heat shock and plated on low-salt LB agar with 50 μ g/mL zeocin, followed by incubation at 37°C for 24 h. The recombinant pPICZ α A-*MARAS458A* plasmid was linearized with *Mss*I and transformed into *P. pastoris* X33 using the Micropulser electroporator (BioRad, USA). Transformants were selected on YPD agar containing 100 μ g/mL zeocin. For extracellular expression, selected clones were grown in BMM medium (100 mM phosphate buffer, pH 6.0, 1.34% YNB, 0.4 ppm biotin) supplemented with 0.5% glycerol for the initial 19 h at 28°C. Methanol induction was then initiated by adding 0.5% methanol (0 h methanol induction), and the methanol concentration was gradually increased to 5% at the end (96 h methanol induction). Cultivation was continued for a total of 144 h. The culture broth was centrifuged at 4500 rpm for 15 min, and the supernatant was obtained. It was further concentrated using a crossflow filtration system with a 10 kDa MWCO membrane. Protein purification was performed using DEAE FF ion-exchange chromatography with a 5 mL sample load

and 1 mL/min flow rate. Elution was achieved via a linear gradient of 0–1 M NaCl in 50 mM Tris-HCl (pH 7.0) over 20 min. Protein concentration was determined using the Lowry method (Waterborg, 2009).

Electrophoresis and zymography

Zymography was performed based on conventional denaturing SDS-PAGE using a 12% polyacrylamide gel containing 0.1% agarose. After electrophoresis, to renature the protein fractions, the gel was rinsed twice in 2% Triton X-100 for 30 min at room temperature, and then twice with ice-cold 50 mM Tris-HCl buffer, pH 7.0 for 15 min. In-gel hydrolysis was carried out by incubating the gel in 50 mM Tris-HCl buffer, pH 7.0 at 37°C for 1 h. The gel was then stained with Lugol's solution.

Agarase activity

For the agarase activity assay, 100 μ L of testing solution was mixed with an equal volume of 0.8% [w/v] melted agarose M. The reaction mixture was incubated at 40°C for 20 min, and the amount of reducing sugars produced was determined using the DNS method (Miller, 1959). One unit of enzymatic activity was defined as the amount of enzyme that produces reducing sugar equivalent to 1 μ mol of D-galactose per minute. The effect of temperature on enzymatic activity was assessed in triplicate by incubating the enzyme in a 50 mM Tris-HCl buffer (pH 7.0) for 30 min over a temperature range of 20–80°C. The effect of pH was evaluated across a pH range of 2–11 using the following buffers: Na-citrate for pH 2–5, potassium phosphate for pH 5–7, Tris-HCl for pH 7–10, and glycine-NaOH for pH 10–11. For temperature and pH stability assays, enzyme aliquots were preincubated at various temperatures and pH values for the indicated times, and relative

enzymatic activity was subsequently measured.

Thin-layer chromatography

Thin-layer chromatography (TLC) was used to identify the hydrolysis products generated by MARAS458A agarase over time. The reaction was performed in a mixture containing 0.4% (w/v) melted agarose in 50 mM Tris-HCl buffer (pH 7.0) and 0.1 U/mL MARAS458A agarase. At different time intervals, 3 μ L of the reaction mixtures were applied to silica gel 60 TLC plates. The plates were developed using a solvent system of *n*-butanol–acetic acid–water (2:1:1, v/v/v). For visualization, the plates were air-dried, sprayed with 10% (v/v) H₂SO₄, and heated at 110 °C for 10 min. D-galactose, NA4, and NA6 were used as reference standards.

RESULTS AND DISCUSSION

Agarolytic bacteria

From 169 samples, 507 strains of agarolytic bacteria were obtained. Among them, 223 were isolated from marine-related environments, and 284 from paddy soils and plant roots. Based on differences in whole cell SDS-PAGE patterns, 181 strains were selected for species identification by 16S rRNA gene sequencing. The strains were assigned to 39 species of *Paenibacillus*, *Microbulbifer*, *Vibrio*, *Marinimicrobium*, and *Metabacillus*. The phylogenetic relationships and ecological traits of the agarolytic bacteria isolated in Vietnam will be described in detail elsewhere.

Among the obtained strains, ARAS-458.1, a Gram-negative, rod-shaped, aerobic bacterium, exhibited remarkable agarolytic activity. The strain was isolated from red algae (*Rhodophyta*) collected in the coastal region of Quang Binh, Vietnam

(17.498161°N, 106.628066°E, 2020). The 16S rRNA gene sequence showed the highest similarity (99.36%) to *Microbulbifer elongatus* DSM 6810^T (Figure 1A). Owing

to its strong agar-degrading ability, the strain ARAS-458.1 was selected for genome mining to identify agarolytic enzymes.

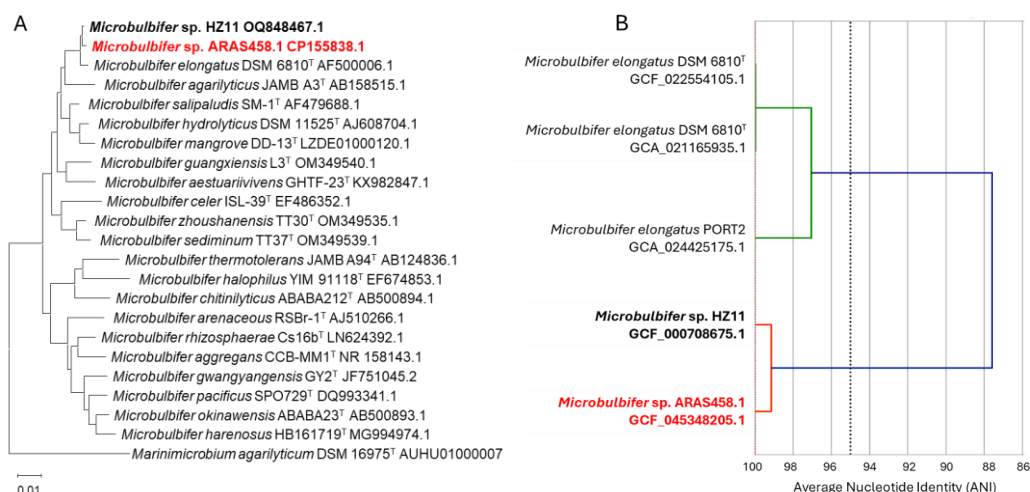


Figure 1. Phylogenetic analysis of *Microbulbifer* sp. ARAS458.1 and closely related species. (A) Phylogenetic tree based on 16S rDNA sequences constructed using the Neighbor-Joining method and Maximum Likelihood model in MEGA12 (Kumar *et al.*, 2024). An alignment of 1377 positions was used for analysis, and all ambiguous positions were removed for each sequence pair. GenBank accession numbers of the sequences are indicated after the taxon names. The scale bar represents 1% sequence divergence; (B) A phylogenomic tree based on average nucleotide identity (ANI) comparison using the fastANI method and average clustering.

Genome analysis

The draft genome of ARAS-458.1 comprises 4,011,081 base pairs distributed across 254 contigs, with lengths ranging from 78 bp to 469,244 bp and a GC content of 57.1%. Genome annotation predicted 3,350 protein-coding genes, accounting for 89.85% of the total genomic content, along with 44 tRNA genes. OrthoANIu analysis (<https://www.ezbiocloud.net/tools/ani>) revealed an average nucleotide identity (ANI) value of 85.84% between ARAS-458.1 (GCA_045348205.1) and its closest relative, *M. elongatus* (GCA_021165935.1), indicating limited genomic similarity and supporting their classification as distinct species (lower than 95%) (Figure 1B). Here and elsewhere, the strain ARAS-458.1 will be assigned to

Microbulbifer sp. Via Prokka, six putative agarase coding genes were predicted in the *Microbulbifer* sp. ARAS-458.1 genome. Among them, *MARAS458A* was identified as encoding a β -agarase that belongs to glycoside hydrolase family 16 (GH16), which is known for efficient agar-degrading activity (Jiang *et al.*, 2020). *MARAS458A* was therefore selected for expression analysis.

Sequence analysis of *MARAS458A*

Comparison of the deduced amino acid sequence of *MARAS458A* with the InterPro Database (<https://www.ebi.ac.uk/interpro/>) indicated that the domain 18-291 belongs to GH16 β -agarase, and the domain 311-441 belongs to the CBM6 carbohydrate-binding module. SignalP 6.0 (DTU Health Tech)

identified a signal peptide of 20 amino acids located at the N-terminal of MARAS458A. In this study, we will utilize the signal peptide encoded by the vector backbone, therefore, only the sequence encoding the mature protein will be cloned. The predicted protein would have 536 amino acids and an estimated molecular weight of 59.37 kDa, and pI of 4.54. The overall sequence identity between MARAS458A and agarase from *M. elongatus* BAC99022.1 was 99%. The β -agarase MARAS458A also shares 64.98%, 64%, and 63.04% similarity with that of marine agarolytic bacteria *Microbulbifer agarilyticus* BAE06228.1, *Microbulbifer* sp. BN3 AXY54922.1, and *Microbulbifer thermotolerans* 3WZ1_A, respectively. Multiple sequence alignments revealed that the conserved regions among all agarases correspond to residues Ala20–Val293 in MARAS458A.

Cloning and expression of MARAS458A

The *MARAS458A* gene was cloned and expressed in both *E. coli* and *P. pastoris*. When expressed in *E. coli*, a strong protein band of approximately 51 kDa, corresponding to the predicted size of the target protein, was observed. Agarase activity

was also detected by zymogram (Figure 2). Further characterization and application of the recombinant enzyme were limited by its precipitation, which required solubilization and refolding for recovery (data not shown). Only minimal agarase activity was detected in the soluble fraction following cell disruption. Such aggregation into inclusion bodies is a well-documented consequence of high-level heterologous expression in *E. coli* (Palmer and Wingfield, 2012).

High agarase activity was obtained when *MARAS458A* was expressed extracellularly in *P. pastoris* using the pPICZ α A expression system. After 96 h of cultivation, agarase activity in the fermentation broth reached 1.06 U/mL, with a specific activity of 0.78 U/mg. Partial purification using ion-exchange chromatography increased the specific activity to 3.25 U/mg. Upon expression in *P. pastoris*, the recombinant protein did not display discrete bands on SDS-PAGE or zymogram analysis (Figure 2). Such smear-like or diffuse band patterns are frequently observed in *P. pastoris* due to varying degrees of glycosylation among expressed protein molecules (Eskandari *et al.*, 2023).

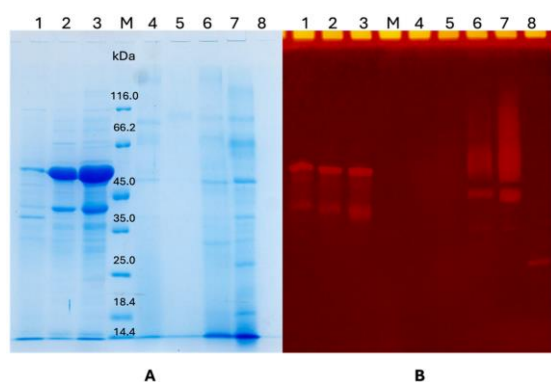


Figure 2. SDS-PAGE (A) and Zymogram (B) analyses of MARAS458A expressed in *E. coli* and *P. pastoris*. 1, 2, 3 – MARAS458A expressed in *E. coli* BL21; M – Pierce™ Unstained Protein MW Marker (ThermoFisher Scientific, USA); 4 – *P. pastoris* X33 (no insert); 5, 6 – MARAS458A expressed in *P. pastoris* X33 after 0 and 96 h of methanol induction; 7 – partially purified MARAS458A β -agarase; 8 – agarase from *Pseudomonas atlantica* (Sigma-Aldrich).

Characterization of MARAS458A agarase

Partially purified MARAS458A agarase expressed in *P. pastoris* was used for enzyme characterization. Thin-layer chromatography analysis of the reaction mixture containing melted agarose and MARAS458A agarase over a 24-h time

course is presented in Figure 3. After the initiation of hydrolysis (5 min), the reaction mixture contained both the final products, NA4, NA6, and high-molecular-weight oligosaccharides. Hydrolysis was completed after 3 h of incubation, yielding NA4 and NA6 as the final products. This hydrolysis pattern confirmed that MARAS458A functions as an endo- β -agarase.

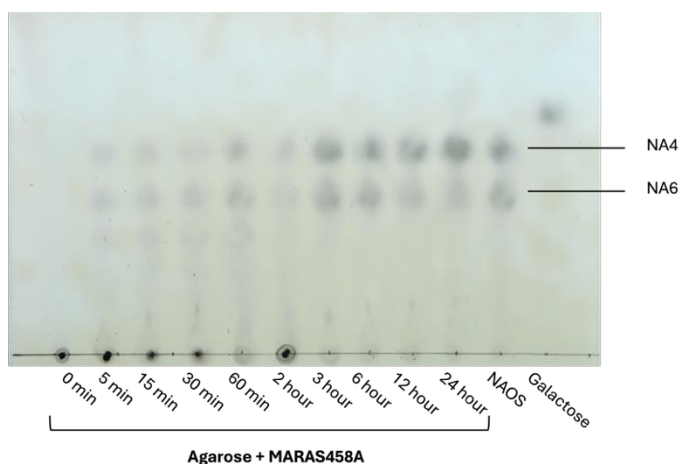


Figure 3. Thin-layer chromatography showing the time course hydrolysis of melted agarose by MARAS458A agarase. NAOS - a neoagarooligosaccharide mixture containing NA4 and NA6.

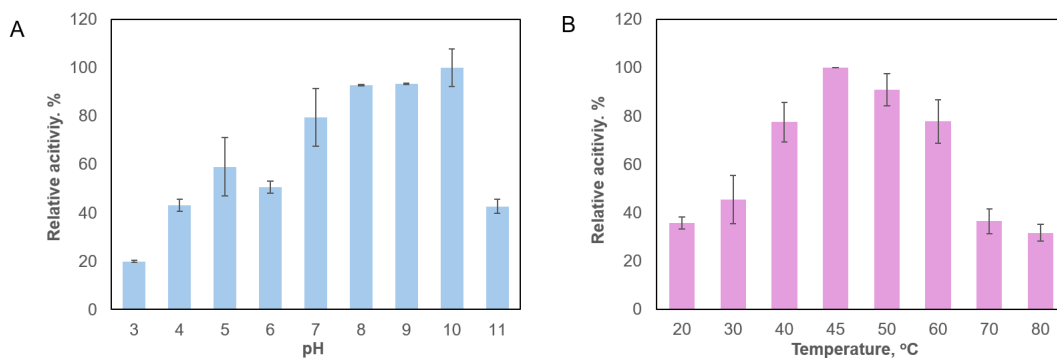


Figure 4. Enzymatic activity of recombinant MARAS458A β -agarase at different pH (A) and temperatures (B).

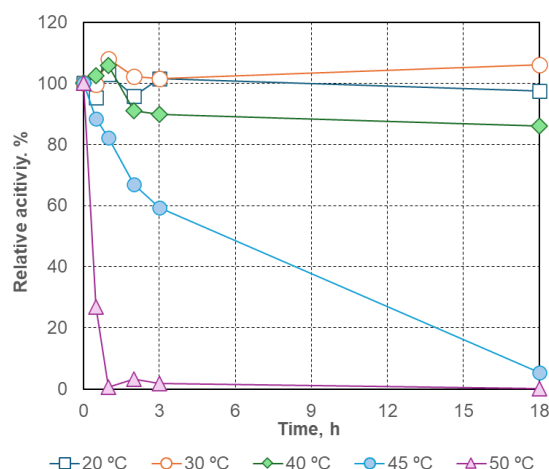


Figure 5. Thermostability of recombinant MARAS458A β-agarase at different temperatures during 18 h of incubation.

MARAS458A is an alkaline β-agarase exhibiting high activity in the pH range of 7–10. At pH 11, the activity decreased drastically (Figure 4A). This property differs markedly from other β-agarases reported in *Microbulbifer* species (Table 1), which typically exhibit optimal activity under neutral pH conditions. When assayed at pH 7, the enzyme showed maximum activity at 45°C (Figure 4B). MARAS458A is relatively thermolabile. At 45°C, the enzyme activity declined rapidly, with 60% remaining after 3 h and nearly complete loss after 18 h. The enzyme was stable at temperatures up to 40°C, retaining 90% and 86% of its initial activity after 3 h and 18 h of incubation, respectively (Figure 5).

The production of NA4 and NA6 as the final hydrolysis products distinguishes MARAS-

458A from other β-agarases reported in *Microbulbifer* species (Table 1). Although MARAS458A differs from the β-agarase of *M. elongatus* (BAC99022.1) by only four amino acids, these substitutions appear to account for the formation of NA6 among the final hydrolysis products. Notably, two of the four substitutions occur within the catalytic domain, involving a change from valine (hydrophobic) to glutamine (polar) and from aspartic acid (negatively charged) to arginine (positively charged). Such alterations may strongly influence structural flexibility and the electrostatic interactions with the substrate (Cronin and Kirsch, 1988; Strohmeier *et al.*, 2004). We also do not exclude the possibility that glycosylation associated with protein expression in *P. pastoris* may contribute to the enzyme's inability to hydrolyze NA6.

Table 1. Comparison of agarase from *Microbulbifer* sp. ARAS-458.1 with the most similar agarases.

Protein	Classification	Source organism	Differences (aa)	Hydrolysis products	Optimum condition	Reference
MARAS458A	β-agarase	<i>Microbulbifer</i> sp. ARAS-458.1	This study	NA4, NA6	pH 7-10, 45°C	This study

BAC99022.1	β -agarase	<i>M. elongatus</i> JAMB-A7	4	NA4	pH 7, 50°C	(Ohta <i>et al.</i> , 2004a)
BAE06228.1	agarase	<i>M. agarilyticus</i> JAMB-A3	37	NA4	pH 7, 50°C	(Ohta <i>et al.</i> , 2004b)
AXY54922.1	agarase	<i>Microbulbifer</i> sp. BN3	78	NA2, NA4	pH 6, 50°C	(Li <i>et al.</i> , 2018)
3WZ1_A	β -agarase I	<i>M. thermotolerans</i> JAMB-A94	85	NA4	pH 7, 55°C	(Takagi <i>et al.</i> , 2015)

In this study, among a large collection of agarolytic bacteria isolated in Vietnam, *Microbulbifer* sp. ARAS-458.1 was identified as one of the most active strains capable of efficient agar degradation. Whole-genome sequencing and analysis revealed six putative agarase-related genes, one of which was confirmed by cloning and heterologous expression. The recombinant β -agarase MARAS458A, expressed in *P. pastoris*, exhibited high catalytic activity and hydrolyzed agar to yield NA4 and NA6 as the final products. The dual production of NA4 and NA6, together with its alkaline nature, distinguishes MARAS458A from previously reported β -agarases of *Microbulbifer* species, suggesting subtle structural variations that influence substrate processing and product specificity. Collectively, these findings provide new insights into the diversity of agarolytic systems in *Microbulbifer* and highlight the potential of MARAS458A as a biocatalyst for the sustainable production of functional agaro-oligosaccharides with applications in food and health industries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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