RESEARCH ARTICLE

Characterization of Agarolytic Bacterium *Microbulbifer elongatus* PORT2 and Its GH16 Agarase

Santi Rukminita Anggraeni[1,2,*] and Marion B. Ansorge-Schumacher[3]

OPEN ACCESS

[1] Research Center for Marine Conservation and Maritime Studies, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Bandung 45363, Indonesia

[2] Research Collaboration Center for Biomass and Biorefinery, Universitas Padjadjaran-National Research and Innovation Agency of Indonesia

[3] Molecular Biotechnology Research Group, TU Dresden, 01062 Dresden, Germany

*Corresponding Author:
santi.rukminita@unpad.ac.id

Received: 23 May 2022

Accepted: 25 July 2022

Published: 30 August 2022

Academic Editor: Dr. Dewi Seswita Zilda

 Accreditation Number:148/M/KPT/2020. ISSN: 2089-5690, e-ISSN: 2406-9272. https://doi.org/ 10.15578/squalen.653

Abstract

Sugars from agar exhibit potential biological activities and comprise a sustainable source of third-generation biofuel. Their provision through enzyme-assisted bioconversion of agar is considered a mild and environmentally-friendly process. This work reports a mesophilic-agarolytic bacterium characterization from Batu Karas, West Java-Indonesia coastal seawater. Analysis 16S rRNA showed a phylogeny relationship of the bacterium to the genus *Microbulbifer*, especially to *M. elongatus*. A gene coding for agarase AgaF16A was cloned and expressed in *E. coli* BL21(DE3) using a genome mining approach. The protein sequence of AgaF16A showed 86.79% similarity to the catalytic domain of glycoside hydrolase (GH) 16 β-agarase from *Microbulbifer thermotolerans* JAMB-A94. The enzyme cleaved agarose and agar oligomers larger than neoagarooctaose into neoagarohexaose and neoagarotetraose. It also showed activity on natural agar extracted from Indonesian agarophytes. The AgaF16A was active in wide pH and temperature ranges. It retained more than 80% activity after one hour incubation at 50 °C, indicating thermostability, favorable for technical application.

Keywords: *microbulbifer*, agarase, GH16, thermostability, genome mining

Introduction

Macroalgae are important sources of marine polysaccharides with unique properties and structures depending on species and ecological factors. One of them is agar which has industrial applications, mainly as a stabilizer and gelling agent. Prominent producers of agar (agarophytes) are red algae such as *Gracilaria* sp. and *Gelidium* sp. (Yun et al., 2017; Yoon et al., 2010). Agar is the primary matrix polysaccharide present in the cell walls of agarophytes. It is composed of agarose and agaropectin. Agarose is a neutral polysaccharide made up of linear chains of alternating residues of 1,3-O-linked b-D-galactopyranose and 1,4-O-linked 3,6-anhydro-α-L-galactose (Araki, 1956). It forms thermo-reversible gel aggregates. Agaropectin, on the other hand, is the branching part with the same linear backbone as agarose, but some hydroxyl groups of 3,6-anhydro-α-L-galactose residues substituted by sulfoxy or methoxy or pyruvate residues (Lahaye & Rochas, 1991).

Nowadays, macroalgae are considered potential resources for third-generation biofuels and feedstock of interesting bioactive-oligosaccharides. Agar-derived saccharides can be prepared either using chemical or biochemical methods. The latter involves the action of agarolytic enzymes, which deliver more specific products and is generally considered a greener method. Agarolytic enzymes, also named agarases, can be classified into different families of glycoside hydrolases (GH). Glycoside hydrolases that are exclusively active as agarases belong to the families GH50, GH86, GH96, and GH117. Some are also known within the family GH16 (Michel & Czjzek, 2013). A combination of agarases from different families conveys agar polysaccharide degradation into oligomers and/or monomers in several steps (Ekborg et al., 2006).

The action mode of agarases classifies them into endo- and exo-agarases. Endo-agarases use agarose or agar as the main substrate, while exo-agarases accept only smaller agar oligo- or neoagarooligosaccharides. Another classification is

based on the type of glycosidic-bond cleavage, dividing the enzymes into α-agarases and β-agarases. α-Agarases hydrolyze α-1,3 linkages, whereas β-agarases work on β-1,4 linkages in the agar molecules (Yun et al., 2017). The majority of agarases described to date belong to the β-agarases. Diverse physical and biochemical properties have been described for these enzymes (Fu & Kim, 2010).

The marine environment is the main source for agarase producers such as *Flammeovirga* sp. OC4 (Chen et al., 2016), *Catenovulum* sp. X3 (Xie et al., 2013), *Cellulophaga omnivescoria* W5C (Ramos et al., 2018) and some *Microbulbifer* spp. (Ohta et al., 2004a; Ohta et al., 2004b). Microorganisms from other niches such as soil and the human gut also produce a few agarases (Mai et al., 2016; Su et al., 2017; Temuujin et al., 2011).

This study reports the characterization of an agarolytic bacterial isolate from Batu Karas, West Java, Indonesia seawater, *M. elongatus* PORT2, identification and heterologous expression of a GH16 agarase from this organism. Previously, three heterologous GH50 agarases from *M. elongatus* PORT2 were successfully expressed and characterized (Anggraeni & Ansorge-Schumacher, 2021). *M. elongatus* PORT2 is a mesophilic bacterium. Nevertheless, its agarases adapt to a wide range of pH and show thermostability, which is particularly attractive for further industrial application.

Material and Methods

Chemicals and Reagents

Unless otherwise stated, all chemicals, buffer reagents and microbiological media were from Roth or Sigma (Germany). D-galactose and agarose were purchased from VWR (Germany). Neoagarotetraose, neoagarohexaose and neoagarooctaose were from Qingdao BZ Oligo Biotech (China).

Bacterial isolate and Growth Conditions

The isolation of PORT2 had been described in the previous study (Anggraeni & Ansorge-Schumacher, 2021). Briefly, the isolate PORT2 was obtained from seawater of coastal area Batu Karas Pangandaran, West Java, Indonesia (7° 452 03 S, 108° 302 03 E). A seawater sample was taken using a sterile bottle and directly transported and processed for isolation using serial dilution on sterile KNO_3 agar (yeast extract 1 g, KNO_3 0,.2 g, agar 15 g, seawater 1 L, pH 8). The colony formed a pit on the surface of the medium and was further purified with the streak plate method on KNO_3 agar. Incubation was performed for 24-48 h at 28-30 °C.

Characterization of the Isolate

The isolate was maintained on Yeast Extract Agar Artificial Seawater Media (YEASW) (yeast extract 3 g, tryptone 6 g, agar 15 g, NaCl 30 g, KCl 0.55 g, $NaHCO_3$ 0.16 g, NH_4NO_3 0.016g, $MgCl_2.6H_2O$ 12.58 g, Na_2HPO_4 0.008 g, $FeSO_4.7H_2O$ 0.02 g, $CaCl_2.2H_2O$ 2.38 g, distilled water 1 L). The isolates were grown at 28 or 30 °C. The liquid culture was grown without agar at 30 °C on a rotary shaker at a speed of 150-200 rpm, overnight to 24 h. The stock culture was stored at -80 °C in 20% (v/v) of sterile glycerol.

Gram staining was performed on a 24-h culture, and the motility of cells was examined in marine broth (Roth) at different growth phases (lag, exponential and stationary) using light microscopy with immersion oil (100x scale of the objective). Catalase was assayed using 0.3% hydrogen peroxide with an overnight colony grown on a YEASW plate. The ability to utilize starch, amylopectin, laminarin, agar, glucose, sucrose, and galactose was assayed using each compound as a sole carbon source of media (carbon source 0.2%; peptone 0.25%; K_2HPO_4 0.2%; NaCl 3%; 100 mL dH_2O) (Cortes-Tolalpa et al., 2017). Qualitative agarase activity was assayed by Lugol's iodine dispersion onto the colony that grew on YEASW media (Liu et al., 2016). API 20 NE (Biomerieux) was used for assimilation tests. The temperature response was determined by growing the colony at different temperatures (4, 28, 30, 37, and 45 °C). The growth response to NaCl was measured in marine broth (with the addition of NaCl to a concentration of 8% (w/v) and 10% (w/v), respectively).

Analysis of 16s Ribosomal DNA

The genome of PORT2 had been sequenced in a previous study (Anggraeni & Ansorge-Schumacher, 2021). The draft genome sequences were analyzed using RNAmmer 1.2 server (http://www.cbs.dtu.dk/services/RNAmmer/) to obtain the 16S rRNA sequence. The sequence was submitted to RDP Release 11 (https://rdp.cme.msu.edu/) (Cole et al. 2014) and the NCBI database for similarity analysis. Multiple sequence alignments and bootstrap for phylogeny analysis were performed with the MEGA 6 package using the maximum likelihood heuristic method with 1000 bootstrap (Jeong et al., 2019; Wang et al., 2021). The tree was generated using the best-fit substitution model Tamura Nei with a uniform rate and pattern with complete deletion for a gap or missing data and contained different outgroups for comparison.

Cloning, Expression of the *agaF16A* Gene, and Purification of Recombinant AgaF16A

The AgaF16 protein sequence was obtained from the draft genome annotation of PORT2 (Anggraeni & Ansorge-Schumacher, 2021). Protein sequence homology was further analyzed using BLASTp against the non-redundant protein database (nr) and pdb on the NCBI web platform. The presence of a signal peptide was predicted using the web platform of SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP).

The genome mining approach used the draft genome sequence from the previous study (Anggraeni & Ansorge-Schumacher, 2021) was used in the genome mining approach to amplify *agaF16,* excluding the signal peptide and carbohydrate-binding modules sequences. The exclusion aimed to increase the possibility of intracellular overexpression of the catalytic domain designated as AgaF16A. Briefly, the *agaF16A* gene was amplified from the total genomic DNA of PORT2. The amplification was performed in 50 µL of PCR mix composed of Q5 Taq polymerase (NEB) (3 U/µL), primer pair (10 µM of each), dNTPs (25 mM) and genomic DNA as a template (50-100 ng). Primer pairs for the amplification of *agaF16A* were OSA9-F (5'-TTTTTTGGATCCGCGGACTGGGA TGGCATCCCG-3'; *Bam*HI) and OSA16-R(5'-AAAAAAGAATTCTTAGGAGCCGCCACCG GTCGCC-3'; *Eco*RI). The PCR product was purified using the Qiaquick PCR Purification Kit (Qiagen) and cloned into the expression vector pFO4. The plasmid was designated to generate a 6-His tag at the N-terminus of the recombinant protein. The recombinant plasmid (pMeagaF16A) was transformed into *Escherichia coli* NEB5α cells. The validity of recombinant plasmid was confirmed by the HincII restriction map. After validation, the plasmid was transformed into *E. coli* BL21 (DE3) cells for protein production. The *E. coli* BL21 (DE3) cells harboring the plasmid were cultured in 250 mL auto-induction medium ZYP 5052 (Studier, 2005) supplemented with ampicillin (final concentration 100 µg/mL) for 48 h at 20 °C.

Unless specified, protein purification was performed at 4 °C. The cells were harvested by centrifugation at 8,000 *g* for 15 min and then resuspended in cold lysis buffer (20 mM Tris HCl pH 8.0; 500 mM NaCl; 1 mM EDTA, 0.1% Triton X-100, 5 mM $MgCl_2$) with the addition of freshly added DNAseI 10 mg/µL. The cell suspension was disrupted by sonication (LabSonic M Sartorius) (100% amplitude, three cycles, 1 min/cycle). After centrifugation, the soluble fraction was collected and loaded onto HisTrap FF crude 5 mL (GE) according to the manual. The partial purification was performed isocratically by using washing buffer (HEPES 50 mM pH 8, Imidazole 10 mM and 500 mM NaCl) and elution buffer (HEPES 50 mM pH 8, Imidazole 250 mM, 500 mM NaCl). SDS-PAGE was performed using 10% (w/v) polyacrylamide gels and detected with Coomassie brilliant blue R-250 staining (Laemmli 1970). Protein concentration was determined by the Bradford method using Rotinanoquant reagent (Roth) and bovine serum albumin as a standard according to the manufacturer's instructions.

Characterization of Recombinant *agaF16A*

An enzymatic mixture was prepared in a total volume of 400 µL containing 0.2% (w/v) agarose in HEPES (50 mM, pH 8). The enzymatic reaction was investigated for the initial reaction rate at 50 °C. The reaction was stopped by adding dinitrosalicylic acid (DNS) reagent (1% 3,5-dinitrosalicylic acid; 0.2% phenol; 1% NaOH; 20% potassium sodium tartrate tetrahydrate) (w/v) into the solution (Miller, 1959). The ratio of DNS reagent to sample was 1:1. The mixtures were incubated in 96 wells on a thermal cycler (Advanced Primus 96) at 98 °C, 10 min and 4 °C for at least 15 min and measured at 540 nm absorbance (Tecan Infinite 200). The amount of reducing sugars in the sample was calculated from a calibration curve with D-galactose (0 to 10 mg/mL in buffer). Appropriate controls of substrate and enzyme were used during the experiment. All measurements were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme produced by 1 µmol D-galactose per minute at standard conditions. The enzyme activity was reported as % relative to activity. The activity of AgaF16A in HEPES 50 mM pH 8; 50 °C, 20 min of reaction represented 100% activity.

Enzyme characterization included determination activity at different pH, temperatures and in the presence of certain chemicals. The range of pH was determined with buffer salts at a concentration of 50 mM using sodium acetate (pH 4); KPi (pH 6-7.5); HEPES (pH 8): Tris (pH 9) and CAPS (pH 10), respectively. Temperature range and stability were measured at 20 to 80 °C. Influences of $MgCl_2$, EDTA, beta-mercaptoethanol and DTT (dithiothreitol) were determined at a concentration of 1 mM in appropriate buffer, NaCl had a concentration of 5 mM, $CaCl_2$ of 0.5 mM and glycerol of 0.1% (v/v). Kinetic parameters were examined using agarose as substrate (1-10 mg/mL).

Protein modeling for AgaF16A was performed as described in Anggraeni & Ansorge-Schumacher (2021). A 3D-model structure of AgaF16 was built using template recommendations from the SWISS-model server. The quality of the model was inferred from GMQE and QMEAN values (Waterhouse et al., 2018).

Further structure homology and visualization analysis were performed using UCSF Chimera (Pettersen et al., 2004).

Analysis of Substrate and Product Specificity

As described in Anggraeni and Ansorge-Schumacher (2021), substrate and product specificities were analyzed. Different types of polysaccharides and agar-oligosaccharides such as agarose, agar, amylose, iota carrageenan, neoagarooctaose (NA8), neoagarohexaose (NA6), neoagarotetraose (NA4), alcohol insoluble residue (AIR) of *Gracilaria* sp. (AIRG), *Gelidium* sp. (AIRS), and *Ulva* sp. (AIRU) were tested as substrates. Two artificial substrates, *para*-nitrophenyl β-D-galactopyranoside (β-*p*npg) and *para*-nitrophenyl α-D-galactopyranoside (α-*p*npg) were used to determine the type of glycosidic bond cleavage.

Nucleotide Sequence Accession Number

The sequence of 16S rRNA was deposited in GenBank under accession number MH622756 (Anggraeni & Ansorge-Schumacher, 2021). The nucleotide sequence of *agaF16* was deposited in GenBank under accession number MH996639.

Results and Discussion

Characteristics of Microbulbifer Elongatus PORT2

The isolate PORT2 was identified as a non-sporulating gram-negative bacterium. The characterization showed long rod-shaped cells in the logarithmic growth phase. These cells were 2-13 µm in length and 0.8-1 µm in width (Figure 1a). The bacterium was non-motile. The isolate had morphological plasticity and could change shape from long rod to coccoid, dependent on the nutritional condition (Figure 1b). This characteristic is called pleomorphism and has been described for some other *Microbulbifer* spp. (Nishijima et al., 2009; Wakabayashi et al., 2012). The colony showed opaque color on marine agar (Roth) and brownish color on Zobell Agar after 48 h. After five days, the isolate liquified YEASW media indicates its agarolytic ability (Figure 1c).

PORT2 grew at temperatures between 28-37 °C and pH values of 6-8. The optimum conditions were 30 °C and pH 8. The bacterium had tolerance for sodium chloride presence from 2-10% (w/v). It also reduced nitrate to nitrite (denitrification). It produces neither indole nor H_2S during growth on SIM (Sulfide, Indole, and Motility) media. PORT2 showed a positive reaction for catalase, agarase and amylase. It utilized esculin, ferric citrate, glucose, starch, laminarin, amylopectin, agar and agarose as sole carbon sources but not L-arginine, urea and gelatin, fructose, fucose, lactose, sucrose and mannose. These characteristics highlight PORT2 as a mesophilic heterotroph marine bacterium.

Phylogenetic Analysis of Isolate PORT2 Based on 16S rRNA

The 16S rRNA sequence of isolate PORT2 was extracted from the draft genome information (Anggraeni & Ansorge Schumacher, 2021). The RDP analysis classified the isolate within the genus *Microbulbifer*. BLAST analysis of the 16S rRNA showed 99% similarity of PORT2 with *Microbulbifer elongatus* DSM 6810^T/ATCC 10144^T, 98% with *M. hydrolyticus* DSM11525^T, *M. salipaludis* SM-1, and 97% with *M. mangrovi* DD-13.

The strain DSM6810^T is the type strain of *M. elongatus*. Isolate CMC-5 (Jonnadula et al., 2009) and isolate HZ11 (Sun et al., 2014) represent two strains with a close relationship to the type strain. Phylogeny construction based on 16S rRNA clustered the isolate PORT2 together with *M. elongatus* DSM6810^T, *Microbulbifer* sp. CMC-5 and *Microbulbifer* sp. HZ11 with bootstrap value 100 (Figure 2).

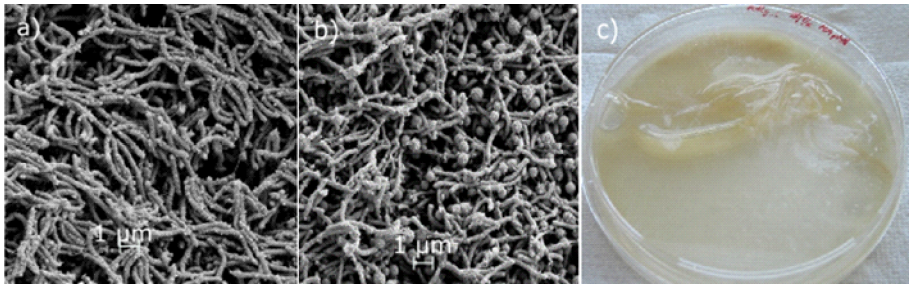

Figure 1. Image from a culture of PORT2 on Marine Agar (Roth): Scanning Electron Microscopy: a) overnight, b) after 72h; c) Hydrolysis of Yeast Extract Agar Artificial Seawater Media (YEASW) after five days of incubation of PORT2 at 30 °C.

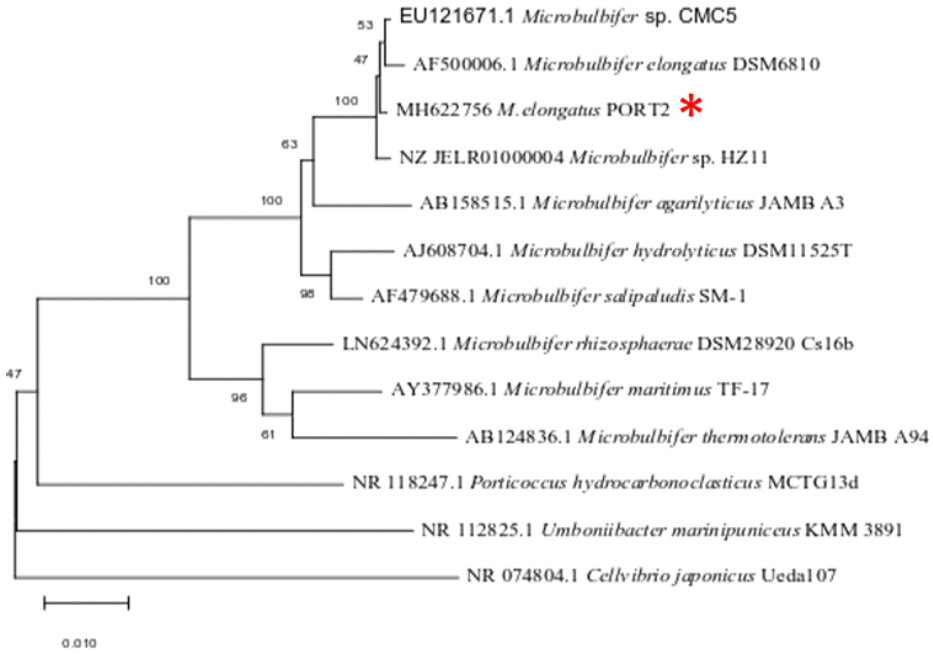

Figure 2. 16S rRNA-based maximum likelihood phylogeny tree of PORT2. Numbers at branch points indicate the bootstrap value (%) of 1000 replications. Some other *Gammaproteobacteria* from the order *Cellvibrionales* such as *Porticoccus hydrocarbonclasticus* MCTG (accession number NR118247), *Umboniibacter marinipuniceus* KMM (accession number NR112825) and *Cellvibrio japonicus* Ueda (accession number NR074804) were used as outgroups. The branch length indicates the expected number of nucleotide substitutions per 100 nucleotides.

Distinct characteristics of PORT2 in comparison to DSM6810^T and CMC-5 were observed. PORT2 was immotile. It tolerated 10% NaCl and hydrolyzed neither gelatin nor sucrose. On the other hand, DSM6810^T is motile and able to produce H_2S. It hydrolyzes gelatin, & sucrose and also tolerates NaCl of 3% (Yoon et al., 2003). *M. elongatus* CMC-5 also hydrolyzes gelatin and tolerates NaCl up to a concentration of 8% (Jonnadula et al., 2009). However, no information on the biochemical characteristics of HZ11 was available. Characteristics of *M. elongatus* PORT2 were summarized in Table S1.

Based on this morpho-biochemical and 16S rRNA phylogenetic analysis, PORT2 was proposed as a new strain of *M. elongatus* and designated as *M. elongatus* strain PORT2. Previously, *M. elongatus* was named *Pseudomonas elongata* because of its sharing phenotypic similarities with an aerobic rod shape-gram-negative bacteria. It is an agarolytic bacterium hydrolyzed agarose into neoagarotetraose, noagarohexaose and neoagarooctaose (Vattuone et al.,1975). However, its agarolytic enzymes have not yet been studied in detail. *P. elongata* was reclassified as a new species namely *M. elongatus*, after an extensive study involving polyphasic, chemotaxonomy, DNA-DNA hybridization, and 16S rRNA analysis. The type strain species is *M. elongatus* ATCC 11044^T / DSM6810^T /LMG2182^T (Anzai et al., 2000; Yoon et al., 2003). Several other *Microbulbiferaceae* also showed agarolytic activities, such as AgaA7 from *M. agarilyticus* JAMB A7 (Ohta et al., 2004a), ID256 from *Microbulbifer* Q7 (Su et al., 2017), AgaA from *M. thermotolerans* JAMB-A94 (Ohta et al., 2004b), N3–1 from *Microbulbifer* sp. BN3 (Li et al., 2018).

Characteristics of AgaF16A

The AgaF16 complete protein sequence deduced from the genomic data showed an N-terminal signal peptide type I preceding a GH16 catalytic domain. The catalytic domain was connected to two C-terminals-carbohydrate binding modules 6 (CBMs 6) (Figure 3a). The presence of SPI and 2 CBMs in AgaF16 indicates extracellular localization of the enzyme for either degradation of polymeric substrates or cell wall-targeting (Arnal et al., 2019). The catalytic domain of AgaF16 showed 86.79 % identity to a GH16 beta-agarase from *M. thermotolerans* JAMB-A94 (Takagi et al., 2015). The AgaF16 was successfully produced in *E.coli* BL21(DE3) by excluding the signal peptide and CBM sequences and designated as AgaF16A. Recombinant AgaF16A had a protein size of around 31 kDa (Figure 3b.).

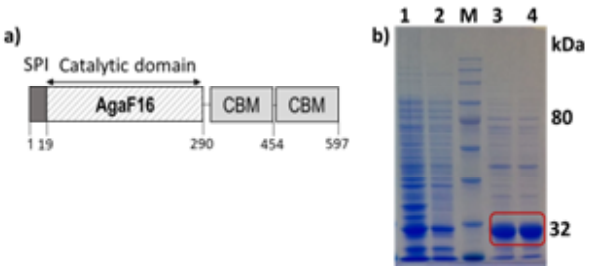

Figure 3. a) Modularity of AgaF16. Signal Peptide I (SPII) (*gray*); catalytic domain (*diagonal line*); carbohydrate-binding modules (CBMs) (*light gray*); numbers below the bars represent positions of amino acids, b) SDS PAGE of recombinant agarase AgaF16A: 1&2) Cell lysate and soluble protein of AgaF16A; 3&4) AgaF16A after Ni-NTA purification and Amicon ultrafiltration, respectively. M) Marker.

The enzyme was active in a wide pH range between 6 and 9 (Figure 4a) and at temperatures between 20 °C to 70 ˚C (Figure 4b). The enzyme also retained more than 90% of its activity after incubation at 50 ˚C for one h (Figure 4c). The AgaF16A activity increased due to certain chemicals such as Mg^{2+}, EDTA, β-mercaptoethanol, and DTT at 1 mM of each and in 10% glycerol Low concentrations of strong reducing agents, DTT and β-mercaptoethanol, enhanced the enzyme activity almost twice. This was an astonishing result since, normally, reducing agents play a role in maintaining or enhancing enzyme activity by protecting thiol residues or disulfide bridges from oxidation.

In contrast, such residues can be found neither in AgaF16 nor AgaF16A. The enzyme activity also increased along with the substrate concentration. It did not reach substrate saturation until 10 mg/mL agarose (Figure 4d), indicating low substrate binding affinity and concomitantly a high K_M. The activity of AgaF16A was negatively affected by extreme pHs (pH 4 and 10) and by 150 mM NaCl and 5 mM $CaCl_2$ (Figure 4e).

In particular, the observed temperature stability of AgaF16A contrasts known agarases from similar organisms. Agarases from agarolytic *Microbulbiferaceae* were primarily active at a temperature below 50 °C, except for AgaA7 from *Microbulbifer* sp. JAMB A7 and AgaA from *M. thermotolerans* JAMB A94 (Ohta et al., 2004a; Ohta et al., 2004b). However, both species were isolated from a deep-sea environment, not from coastal water, with growth temperature ranges between 20–52 °C.

Substrate Specificity and Product Analysis

Qualitative screening on *para*-nitrophenyl β-D-galactopyranoside (β-*p*npg) and *para*-nitrophenyl α-D-galactopyranoside (α-*p*npg) indicated β-glycosidase

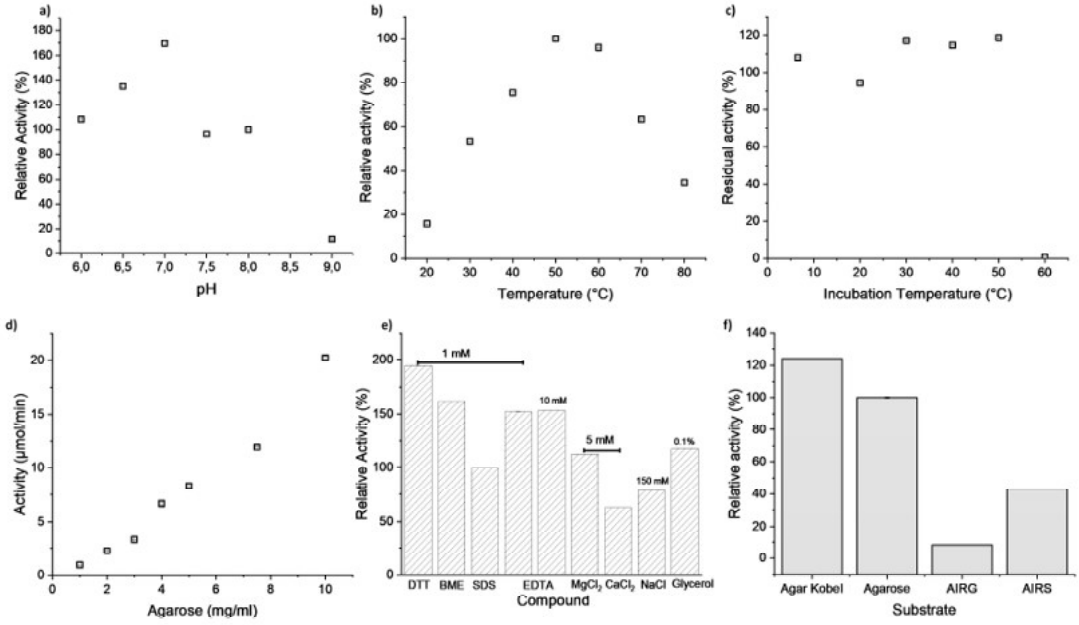

Figure 4. a) pH range, b) Temperature range, c) Temperature stability of AgaF16A. Preincubation time for temperature stability test: 1 h, d). The activity of AgaF16A at different substrate concentrations, e) Activity of AgaF16A in the presence of additives. The enzymatic reaction was performed using 0.2% agar or agarose in HEPES (50 mM, pH 8) at 50 °C, f) Substrate preference of AgaF16A; AIRG and AIRS were alcohol insoluble residues of *Gracilaria* sp. and *Gelidium* sp., respectively. All data are the mean values of triplicate measurements. Error bars represent the deviation from the mean value.

activity of AgaF16A. The enzyme showed specificity on agar-like substrates such as agarose, agar, and alcohol insoluble residues of *Gracilaria* sp. (AIRG) and *Gelidium* sp. (AIRS) as substrates (Figure 4f). It was inactive on amylose, starch, κ-carrageenan and sodium alginate. It released products with retention times using HPLC similar to neoagarohexaose (NA6) and neoagarotetraose (NA8) (Figure 5a). The AgaF16A used neoagarooctaose as the smallest substrate and converted it to NA4 and NA6 (Figure 5b). The gradual release of mixed products indicated an endo-β agarase action (Ohta et al., 2004a) (Figure 5c). Agar-derived saccharides such as neoagarohexaose, neoagarotetraose and neoagarobiose have potential bioactivities as whitening agent (Lee et al., 2008), anti-diabetic and anti-obesity (Hong et al., 2017), antitumor agents (Lee et al., 2017) as well as biofuel production (Offei et al., 2018).

Homology modeling was performed to visualize the substrate-binding site and mode of action of AgaA16A. This method is considered a viable method for predicting the structural and molecular function of an unknown protein (Jabeen et al., 2018). The best template was generated using a SWISS-model server from a catalytic domain of the thermostable endo β-agarase AgaA from *M. thermotolerans* JAMB A94 (PDB id: 3WZ1.1.A) (MtAgaA) (Takagi et al., 2015). AgaF16A showed 87% identity to this template with a GMQE score of 0.98 and QMEAN Z-score: -0.09 indicating good model reliability (Benkert et al., 2011).

In the model, AgaF16A displayed two antiparallel β-sheet jelly rolls forming a concave cleft active-site typical for the GH16 family (Figure 5d). Superposition of AgaF16A with AgaBE189D (pdb id: 4ATF) from *Zobellia galactanivorans* DsiJ (ZgAgaB) (Hehemann et al., 2012) visualized the binding sites (Figure 5e). MtAgaA and ZgAgaB are endo β-agarases from the GH16 family with retaining mechanism (Takagi et al., 2015; Jam et al., 2005). The multiple sequence alignments displayed catalytic residues conservation among AgaF16A and these templates. The substrate-binding residues in AgaF16A were more similar to MtAgaA than to ZgAgaB. The binding site followed a conserved β-bulge motif, EXDXXE. The motif of the catalytic sequence in AgaF16A was E^{127}[I]D[V][I]E^{132} with E127 and E132 as putative catalytic residues (Figure 5e.). AgaF16A had neither a loop bridge nor a second substrate binding site in ZgAgaB (Jam et al., 2005).

Conclusion

This study elucidates the previously uncharacterized agarase of *P. elongata* in 1975. In addition, it provided preliminary information on AgaF16A from *M. elongatus*

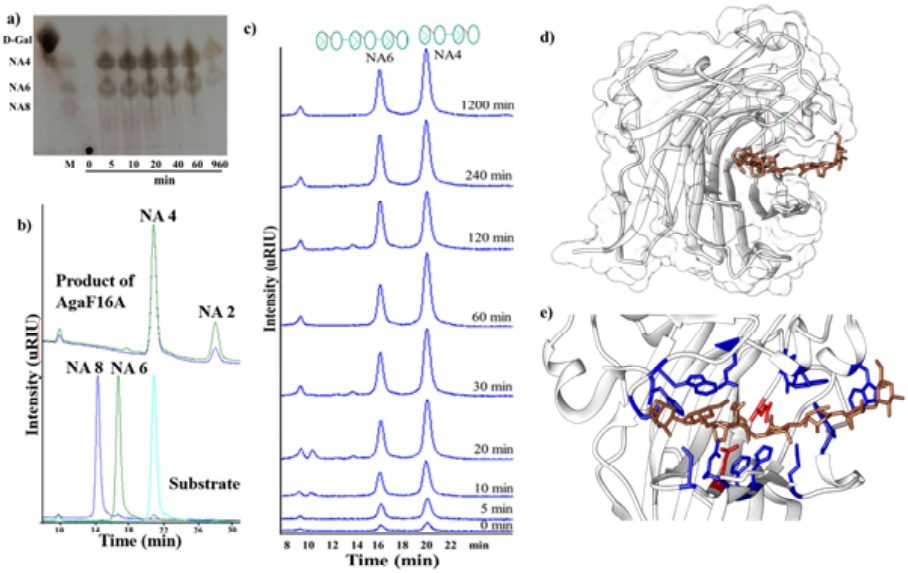

Figure 5. a) AgaF16A activity on 0.2% agarose (in minutes); b-c) AgaF16A activities on neoagarooligosaccharides and agarose, respectively, monitored by HPLC-RID (each substrate: 0.5 mg/mL in ultrapure water; The reaction was performed at 50 °C), The retention times of neoagaroligosaccharide standards are: 14.6 min (NA8); 16.9 min (NA6); 20.9 min (NA4); 28 min (NA2) and 33.7 min (D-galactose); d) Prediction of protein-substrate model of AgaF16A (*white*); neoagarooctaose (NA8) (*brown*), e). Prediction of substrate binding residues (*blue*) and catalytic residues (*red*). Abbreviations: neoagarohexaose (NA6); neoagarotetraose (NA4); neagarobiose (NA2); and D-galactose (D-gal).

PORT2 as a promising biocatalyst for agar biorefinery processes. The enzyme showed tolerance to pH and temperature and adaptability to use different types of agar substrates. The generated products need further study to elucidate their potential application as active compounds for cosmetics and pharmaceuticals.

Acknowledgment

This research was funded by a Ph.D. Scholarship of the Indonesia Endowment Fund for Education (LPDP) (Contract Number: PRJ-319/LPDP/2015) and supported by a short-term research stay fellowship at Marine Glycobiology Group and Station Biology Roscoff by the Graduate Academy of TU Dresden (Grant Number: PSP-Element:F-003661-553-Ü2A-2330000). Deep appreciation is given to Mirjam Czjzek, Christoph Loderer, Francois Thomas, and Robert Laroque for sharing their valuable knowledge and experiences.

Abbreviations Used

SDS: sodium dodecyl sulfate; PAGE: polyacrylamide gel electrophoresis; DNS: 3,5 dinitro salicylic acid; TLC: thin layer chromatography; EDTA: ethylene diamine tetraacetic; DTT: Dithiothreitol; BME: α-mercaptoethanol.

Supplementary Material

Supplementary material is available on the Journal's website.

References

Anggraeni, S. R., & Ansorge-Schumacher, M. B. (2021). Characterization and Modeling of Thermostable GH50 Agarases from *Microbulbifer elongatus* PORT2. *Marine Biotechnology (New York, NY)*, *23*(5), 809. https://doi.org/10.1007/s10126-021-10065-0

Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H., & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology*, *50*(4), 1563-1589.

Araki, C. (1956) Structure of the agarose constituent of agar-agar. *Bulletin of the Chemical Society of Japan*, 29:4: 543-544. doi.org/10.1246/bcsj.29.543.

Arnal, G., Stogios, P. J., Asohan, J., Attia, M.A., Skarina, T., Viborg, A.H., Henrissat, B., Savchenko, A., & Brumer, H. (2019). Substrate specificity, regiospecificity, and processivity in glycoside hydrolase family 74. *Journal of Biological Chemistry*, 294(36), 13233–13247. https://doi.org/10.1074/jbc.RA119.009861.

Benkert, P., Biasini, M., & Schwede, T. (2011). Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics*, 27, 343-350.

Chen, X. L., Hou, Y. P., Jin, M., Zeng, R. Y., & Lin, H. T. (2016). Expression and characterization of a novel thermo-stable and pH-stable α-agarase from deep-sea bacterium *Flammeovirga* sp. OC4. *Journal of Agricultural and Food Chemistry*, *64*, 7251–7258.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., ..., Tiedje, J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, *42*(D1), D633-D642.

Cortes-Tolalpa, L., Salles, J. F., & van Elsas, J. D. (2017). Bacterial synergism in lignocellulose biomass degradation–complementary roles of degraders as influenced by complexity of the carbon source. *Frontiers in Microbiology*, *8*, 1628.

Ekborg, N.A., Taylor, L.E., Longmire, A.G., Henrissat, B., Weiner, R.M., & Hutcheson, S.W. (2006). Genomic and proteomic analyses oft the agarolytic system expressed by *Saccharophagus degradans* 2-40. *Applied and Environmental Microbiology*, *72*(5), 3396–3405.

Fu, X.T., & Kim, S.M. (2010). Agarase: review of major sources, categories, purification method, enzyme characteristics and applications. *Marine Drugs*. *8*(1), 200-218. https://doi.org/10.3390/md801020.

Hehemann, J.H., Correc, G., Thomas, F., Bernard, T., Barbeyron, T., Jam, M., Helbert, W., Michel, G., & Czjzek, M. (2012). Biochemical and structural characterization of the complex agarolytic enzyme system from the marine bacterium *Zobellia galactanivorans*. *Journal of Biological Chemistry*, *287*, 30571. https://doi.org/10.1074/jbc.M112.377184.

Hong, S.J., Lee, J.H., Kim, E.J., Yang, H.J., Park, J.S., & Hong S.K. (2017). Antiobesity and anti-diabetic effect of neoagarooligosaccharides on high-fat diet-induced obesity in mice. *Marine Drugs*, *15*(4),90.

Jabeen, A., Mohamedali, A., & Ranganathan, S. (2018). *Protocol for protein structure modelling*. In M. Cannataro, B. Gaeta, & M. Asif Khan (Eds.), Encyclopedia of bioinformatics and computational biology (Vol. 3, pp. 252-272). Amsterdam; Oxford; Cambridge: Elsevier. https://doi.org/10.1016/B978-0-12- 809633-8.20477-9.

Jam, M., Flament, D., Allouch, J., Potin, P., Thion, L., Kloareg, B., Czjzek, M., Helbert, W., Michel, G., & Barbeyron, T. (2005). The endo-beta-agarases AgaA and AgaB from the marine *bacterium Zobellia galactanivorans*: two paralogue enzymes with different molecular organizations and catalytic behaviours. *Biochemical Journal*, *385*(Pt 3), 703–713. https://doi.org/10.1042/BJ20041044.

Jeong, S. E., Kim, K. H., Lhee, D., Yoon, H. S., Quan, Z. X., Lee, E. Y., & Jeon, C. O. (2019). *Oceaniradius stylonematis* gen. nov., sp. nov., isolated from a red alga, Stylonema cornucervi. *International Journal of Systematic and Evolutionary Microbiology*, *69*(7), 1967-1973.

Jonnadula, R., Verm,a P., Shouche, Y.S., & Ghadi, S.C. (2009). Characterization of *Microbulbifer* strain CMC-5, a new biochemical variant of *Microbulbifer elongatus* type strain DSM6810^T isolated from decomposing seaweeds. *Current Microbiology*, 2009. *59*(6), 600-607. https://doi.org/10.1007/s00284-009-9480-1

Lahaye, M., & Rochas, C. (1991). Chemical structure and physico-chemical properties of agar. *Hydrobiologia*, 221, 137-148.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, Vol 227.

Lee, D.G., Jang, M.K., Lee, O.H., Kim, N.Y., Ju, S.A., & Lee S.H. (2008). Overproduction of a glycoside hydrolase family 50 α-agarase from *Agarivorans* sp. JA-1 in *Bacillus subtilis*

and the whitening effect of its product. *Biotechnology Letter, 30*(5), 911–918.

Lee, M.H., Jang, J.H., Yoon, G.Y., Lee, S.J., Lee, M.G., Kang, T.H., Han, H.D., Kim, H.S., Choi, W.S., Park, W.S., Park, Y.M., & Jung I.D. (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 Reports, 50*(5), 263–268.

Li, R.K., Chen, Z., Ying, X.J., Ng, T.B., & Ye, X.Y. (2018). A novel GH16 beta-agarase isolated from a marine bacterium, *Microbulbifer* sp. BN3 and its characterization and high-level expression in *Pichia pastoris*. *International Journal of Biological Macromolecule, 119*, 1164-1170.

Liu, G., Wu, S., Jin, W., & Sun, C. (2016). Amy63, a novel type of marine bacterial multifunctional enzyme possessing amylase, agarase and carrageenase activities. *Scientific Reports, 6*, 18726. https://doi.org/10.1038/srep18726

Mai, Z., Su, H., & Zhang, S. (2016) Isolation and characterization of a glycosyl hydrolase family 16 beta-agarase from a mangrove soil metagenomic library. *International Journal of Molecular Sciences, 17*, 1360; https://doi.org/10.3390/ijms170813607.

Michel, G., & Czjzek, M. (2013). Polysaccharide-degrading enzymes from marine bacteria. In *Marine Enzymes for Biocatalysis* (pp. 429-464). Woodhead Publishing.

Nishijima, M., Takadera, T., Imamura, N., Kasai, H., An, K-D., Adachi, K., Nagao, T., Sano, H., & Yamasato, K. (2009). *Microbulbifer variabilis* sp. nov. and *Microbulbifer epialgicus* sp. nov., isolated from Pacific marine algae, possess a rod-coccus cell cycle in association with the growth phase. *International Journal of Systematic and Evolutionary Microbiology, 59*(Pt 7), p. 1696-707.

Offei, F., Mensah, M., Thygesen, A., & Kemausuor, F. (2018). Seaweed bioethanol production: A process selection review on hydrolysis and fermentation. *Fermentation*, *4*(4), 99.

Ohta, Y., Hatada, Y., Nogi, Y., Miyazaki, M., Li, Z., Akita, M., Hidaka, Y., Goda, S., Ito, S., & Horikoshi, K. (2004a) Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from a novel species of deep-sea *Microbulbifer*.*Applied Microbiology and Biotechnology, 64*(4), 505-514. https://doi.org/10.1007/s00253-004-1573-y.

Ohta, Y., Nogi, Y., Miyazaki, M., Li, Z., Hatada, Y., Ito, S., & Horikoshi, K. (2004b). Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from the novel marine isolate, JAMB-A94. *Bioscience, Biotechnology, and Biochemistry*, *68*(5), 1073-1081. https://doi.org/ 10.1271/bbb.68.1073.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., & Ferrin, T.E. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, *25*(13),1605-12.

Ramos, K. S. M., Valdehuesa, K. N. G., Nisola, G. M., Lee, W. K., & Chung, W. J. (2018). Identification and characterization of a thermostable endolytic α-agarase Aga2 from a newly isolated marine agarolytic bacteria *Cellulophaga omnivescoria* W5C. *New Biotechnology, 40*, 261–267.

RNAmmer-1.2. Predictions of ribosomal RNA sub units. http://www.cbs.dtu.dk/services/RNAmmer/

SignalP-4.1. Signal peptide and cleavage sites in gram+, gram- and eukaryotic amino acid sequences. http://www.cbs.dtu.dk/services/SignalP/)

Sun, C., Chen, Y.J., Zhang, X.Q., Pan, J., Cheng, H., & Wu, M. (2014). Draft genome sequence of *Microbulbifer elongatus* strain HZ11, a brown seaweed-degrading bacterium with potential ability to produce bioethanol from alginate. *Marine Genomics, 18*, 83-85.

Studier, F.W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expression and Purification, 41*(1): 207-234.

Su, Q., Jin, T., Yu, Y., Yang, M., Mou, H., & Li, L. (2017). Extracellular expression of a novel beta-agarase from *Microbulbifer* sp. Q7, isolated from the gut of sea cucumber. *AMB Express, 7*(1), p. 220 https://doi.org/10.1186/s13568-017-0525-8.

Takagi, E., Hatada, Y., Akita, M., Ohta, Y., Yokoi, G., Miyazaki, T., Nishikawa, A. & Tonozuka, T.(2015). Crystal structure of the catalytic domain of a GH16 beta-agarase from a deep-sea bacterium, *Microbulbifer thermotolerans* JAMB-A94. *Bioscience, Biotechnology, and Biochemistry, 79* (4), 625-632.

Temuujin, U., Chi, W.J., Lee, S.Y., Chang, Y.K., & Hong, S.K. (2011). Overexpression and biochemical characterization of DagA from *Streptomyces coelicolor* A3(2): an endo-type beta-agarase producing neoagarotetraose and neoagarohexaose. *Applied Microbiology and Biotechnology, 92*(4), p. 749-59.

Vattuone, M.A., De Flores, E.A., & Sampietro, A.R. (1975) Isolation of neoagarobiose and neoagarotetraose from agarose digested by *Pseudomonas elongata*. *Carbohydrate Research, 39*,164-167.

Wakabayashi, M., Sakatoku, A., Noda, F. Noda M., Tanaka, D., & Nakamura, S. (2012). Isolation and characterization of *Microbulbifer* species 6532: a degrading seaweed thalli to single cell detritus particles. *Biodegradation*, *23*, 93-105. doi:/10.1007/s10532-011-9489-6.

Wang, D., Wang, J., Shui, B., Zhu, L., Wang, J., Jin, L., & Qu, W. (2021). Complete genome sequence of Microbulbifer sp. YPW1 from mangrove sediments in Yanpu harbor, China. *Archives of Microbiology*, *203*(10), 6143-6151.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., ... & Schwede, T. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research*, *46*(W1), W296-W303.

Xie, W., Lin, B. K., Zhou, Z. R., Lu, G. Y., Lun, J. S., Xia, C. Y., Li, S., & Hu, Z. (2013). Characterization of a novel α-agarase from an agar-degrading bacterium *Catenovulum* sp. X3. *Applied Microbiology and Biotechnology*, *97*, 4907–4915.

Yoon, J.H., Kim, H., Kang, K.H., Oh, T.K., & Park, Y.H. (2003). Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology, 53*, 1357-1361. https://doi.org/10.1099/ijs.0.02464-0

Yoon, J. J., Kim, Y. J., Kim, S. H., Ryu, H. J., Choi, J. Y., Kim, G. S., & Shin, M. K. (2010). Production of polysaccharides and corresponding sugars from red seaweed. In *Advanced Materials Research* (Vol. 93, pp. 463-466). Trans Tech Publications Ltd.

Yun, E.J., Yu, S., & Kim, K.H. (2017). Current knowledge on agarolytic enzymes and the industrial potential of agar derived sugars. *Applied Microbiology and Biotechnology*, 101(14): 5581-5589.