ISOLATION OF AN AGRICULTURAL SOIL AGAROLYTIC *FLAVOBACTERIUM* SP. AT2 AND ITS AGARASE CHARACTERIZATION

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Abstract. This study aimed to isolate a novel agarase-producing soil bacterium and characterize its agarose, which is known to produce biofunctional agarooligosaccharides or neo-agarooligosaccharides. A novel agar-degrading bacterium, namely AT2, was isolated from the agricultural soil and the enzyme activity was identified on a specific medium. The 16S rRNA nucleotide sequence represented 99% identity with that of the members of the *Flavobacterium* genus; hence, the isolated bacterium was named as *Flavobacterium* sp. AT2. The extracellular agarase reached maximum activity at 22.79 U/mL under the following culture conditions: 10% (v/v) inoculum size, shaking rate 210 rpm after 72 h incubation. The optimum conditions of the agarase activity were pH 7.0 and 60°C in 50 mM sodium phosphate buffer. The molecular mass of the purified agarase was 180 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The extracellular agarase hydrolyzed seaweed with the highest yield of 423.58 ± 33.75 μg/mL reducing sugar released after 24 h. Thus, *Flavobacterium* sp. AT2 and its enzyme could be practical for applications in food, cosmetic, and medical studies.
Keywords: *agarolytic bacterium, agarooligosaccharide, agar degradation, enzyme production, seaweed hydrolysis*

Introduction

Agar is the main glycan component that is segregated from the cell wall of red algae, for instance, *Gracilaria* sp. and *Gelidium* sp., and it consists of up to 60% of the algae dry weight (Hong et al., 2017). As the major biopolymer and supporting structure in seaweed, a cocktail of agarose and agaropectin constitutes the molecular structure of agar (Mostafavi and Zaeim, 2020). Agarose is formed by the repetition of β-d-galactose and 3,6-anhydro-α-L-galactose. Agaropectin has the same basic building blocks as agarose, but the hydroxyl groups of the 3,6-anhydro-α-L-galactose units are partially substituted by sulfoxy, methoxy, or pyruvate residues (Park et al., 2020).

Agarases, a class of enzymes that catalyze the hydrolysis of agar or agarose to produce oligosaccharides, are classified into α- or β-agarases based on the cleavage pattern (Zhang et al., 2018). The α-agarase (EC 3.2.1.158), which acts on the α-1,3 glycoside bonds and yields agarooligosaccharides with 3,6-anhydro-α-L-galactose at the reducing ends of products, and β-agarase (EC 3.2.1.81), which cleaves the β-1,4

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glycoside bonds to generate neo-agarooligosaccharides with β-D-galactose at the reducing ends of products (Chen et al., 2016). To date, phylogenetically diverse marine bacteria, isolated mostly from seawater and marine sediments, including *Alteromonas* (Chi et al., 2014), *Pseudomonas* (Hsu et al., 2015), and *Vibrio* (Liu et al., 2020), have been reported to produce agarases with diverse catalytic activities and biotechnological application potentials.

Flavobacterium is a genus consisting of approximately 100 species found in different habitats including sea ice, fresh water, sediments and soil (Bernardet and Bowman, 2011). *Flavobacterium* species have showed ability on production of value products including extracellular hydrolysis enzymes for utilization the biocatalysts in bioremediation or wastewater treatment (Huang et al., 2013). There has been a report on secretion of agarases by an isolate belonging to *Flavobacterium* genus (Kim et al., 2014). These enzymes have been widely applied for saccharification of biomass for bioproduction of biofuels and biochemicals from renewable sources.

In the present study, we described the identification of a novel agricultural soil agarolytic bacterium, *Flavobacterium* sp. AT2, and investigated the production and characterization of its extracellular agarase. Further, the hydrolysis efficiency of the enzyme was also determined using seaweed biomass as a substrate.

Materials and methods

Isolation of agarase-producing bacterial strain

The agarase-producing strain was isolated from agriculture soils collected from Thua Thien Hue Province, Vietnam. The soils were located at the treatment pots of the wastes from a plant *in vitro* propagation farm. The strain was inoculated on a Mineral Salt Agar (MSA) containing (g/L): $CaCl_2$ 0.1; $MgSO_4.7H_2O$ 0.5; $(NH_4)_2SO_4$ 0.5; K_2HPO_4 0.5; NaCl 0.5; agar 1.5% and pH 7.0, and incubated at 37°C for 7 day. After 7 days, plates were stained with iodine solution. Pit or clear zone-forming colonies were picked out and purified on Luria-Bertani medium (1% yeast extract, 2% peptone, 2% NaCl, 2% agar).

Identification of agarase-producing bacterial strain

Morphological, physiological, biochemical characteristics and 16S rRNA phylogenetic analysis were used to identify the isolated strain to genus. The chromosomal DNA of the agarase-producing strain was extracted according to the method of Sambrook et al. (2001). Using the chromosomal DNA as the template, the 16S rRNA region was amplified with primers 27F and 1492R (Thao et al., 2021), and the PCR products were sequenced by Apical Scientific Sdn. Bhd. (Malaysia). The analyzed nucleotide sequence was compared with known strains using BLAST of the NCBI GenBank database. Phylogenetic trees were constructed using MEGA 11 software, using the Maximum Likelihood method and Tamura-Nei model.

Enzymatic activity assay

Agarase activity was measured using 3,5-dinitrosalicylic acid (DNSA) method with some modifications (Miller, 1959). One hundred microliters of the enzyme solution was added to 400 μL of 50 mM phosphate buffer (pH 7.0) with the addition of agarose of 0.2% (w/v), followed by incubation at 40°C for 15 min. A volume of 500 μL of DNSA reagent solution was added to the reaction mixture. Then, the reaction was terminated

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by boiling in water bath for 10 min. After cooling to room temperature, the reducing sugar released was measured at the absorbance of 540 nm. Finally, the reduced sugars were calculated using D-galactose as a standard substrate. One unit (U) of enzyme activity was defined as the amount of enzyme capable of generating 1 μmol of D-galactose per minute through agarose hydrolysis under the conditions of the assay.

Effect of culture conditions on agarase production

The ability on agarase production by the isolate was conducted on 50 mL MSA containing 0.2% (w/v) agar, pH 7.0 with different input parameters including inoculum sizes and shaking rates. Briefly, the isolate was pre-cultured in 5 ml LB medium for overnight with shaking rate of 180 rpm. The overnight cultures were centrifuged at 10.000 rpm for 10 min and the cell pellets were washed in distilled water. The cells were resuspended in 5 ml MSA medium after removing the water and transferred into new shaking flask containing MSA medium with initial inoculum sizes of 1-10% (v/v). Meanwhile, shaking rates of 150, 180 and 210 rpm were chosen to identify the best condition for agarase productivity. The culture processes were carried out at 37°C for 5 days. The enzyme activities in supernatant culture were measured according to the agarase activity assay**.**

Effect of pH, temperature, metal ions and organic solvents

In each experiment, 0.2% agar solution and agarase were mixed and incubated at various durations and temperatures. The relative agarase activity was determined using the DNS method. The optimum temperature of agarase activity was determined by monitoring the relative enzymatic activity at temperatures ranging from 20°C to 70°C at pH 7. The optimum pH was tested at a pH range of 2.0-12.0 with pH intervals of 0.5 at 60°C. A glycine-HCl, sodium acetate, sodium phosphate buffer and glycine-NaOH buffer were used to generate the pH ranges of 2.0-3.0, 4.0-5.0, 6.0-8.0, and 9.0–12.0, respectively. The relative activity was accessed as the percentage of activity in compared to the maximum enzyme activity.

The effects of various metal ion salts on the agarase activity were tested by determining the enzyme activity in the presence of various ions ($CaCl_2$, $CoCl_2$, $CuSO_4$, $MnCl_2$, $MgSO_4$, $ZnSO_4$ and $FeSO_4$) at a final concentration of 1 mM, incubated at 60°C for 1 h. To evaluate the effect of organic solvents, the retained enzyme activities were determined after 1 h of incubation enzyme solution at 60°C, pH 7.0 in the presence of various chemical detergents (acetone, methanol, ethanol, hexane and isopropanol) at 30% concentration. The assay mixture without the addition of metal ion salts or organic solvents was used as the control.

Zymogram analysis

The molecular weight of agarase was evaluated on native SDS-PAGE. The gel was consisted of a stacking layer (5% polyacrylamide) and a separating layer (12% polyacrylamide) supplemented with 2% agar. After the electrophoresis complete, the gel was sliced into two pieces. The first piece of the gel was sunk in 2% (v/v) Trixton X-100 solution for 30 min at room temperature. The gel was then washed with distilled water for three times before incubation in 50 mM Tris-HCl buffer pH 8, 37°C for 4 h. The agarase activity was determined by staining the gel in Lugol's iodine solution for 10 min (Su et al., 2017). The pale-yellow zone on the gel was observed as agarase

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activity. The second piece of the gel was staining in coomassie brilliant blue for protein identification (Kwon et al., 2020). The protein band corresponds to the pale-yellow zone was assessed as agarase.

Hydrolysis of seaweed by extracellular agarase

The isolate was inoculated in MSA medium for 72 h and the supernatants were collected by centrifuging the culture at speed of at 10.000 rpm, 4°C for 15 min. The free-cells supernatants were determined agarase activity as described above before examining for seaweed (*Gracilaria verrucosa*) hydrolysis. The powder of seaweed was prepared by milling the raw seaweed and suspended in phosphate buffer (50 mM, pH 7.0) to final concentrations of 1, 3 and 5% (w/v). The hydrolysis reactions were carried out in 1.5 mL tube containing volume of 100 μL of extracellular agarase (22.8 U/mL), 100 μL of seaweed solution and incubated at 60^{o}C for 24 h. The reducing sugar released was measured as described above.

Data analysis

Each experiment was conducted in three replicates. The statistical analysis was performed using SPSS version 16.0 software. The data were evaluated using one-way analysis of variance (ANOVA) with Duncan's *post hoc* test within the experimental groups and expressed as mean ± standard error. Statistical significance was assessed at a P value < 0.05 as a 95% confidence interval.

Results and discussion

Isolation and identification of agarase-producing bacteria strain

Through the screening of agarolytic bacteria from agriculture soils, one of the colony exhibited strong agar hydrolysis as forming a soft pits on the agar surface with clear haloes around the bacterial colonies was named as strain AT2 and selected for further investigation. *Figure 1* showed the agar-degrading activity of supernatant of strain AT2 producing in MSA liquid medium, which is reflected by a bright clear zone around the wells. The strain forms colonies with a white color and circular in shape on LB agar plates. Biochemical analysis indicated strain AT2 is gram-negative, rod-shaped bacteria and exhibits catalase positive.

The partial 16S rRNA nucleotide sequence of strain AT2 (1394 bp, GenBank accession no. ON227000) was compared with nucleotide sequences available from GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/) using the Blast function. The results showed that the 16S rRNA nucleotide sequence of AT2 isolate had the closest relative (99.64% similarity) to that of *Flavobacterium tistrianum* N15127 (accession no. MK389327). Meanwhile, the isolate exhibited a 16S rRNA nucleotide similarity of 98.64% to both *Flavobacterium tagetis* GN10 (accession no. NR_175551) and *Flavobacterium* sp. TISTR 1602 (accession no. AB465580), respectively. Therefore, strain AT2 is being classified as species belonging to the genus *Flavobacterium*, named *Flavobacterium* sp. AT2. The phylogenetic tree is shown in *Figure 2*.

As agar is the major component of red algae. It is more likely that agar degrading bacteria are isolated from marine sources but a few agar degrading bacterial isolates are also obtained from other sources like *Ammoniibacillus agariperforans* from compost

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(Sakai et al., 2015), *Paenibacillus* sp., *Pseudomonas* sp., and *Klebsiella* sp. from plants (Song et al., 2015), *Steroidobacter agariperforans* (Sakai et al., 2014) and *Paenibacillus sp.* (Song et al., 2014) from soils. These bacteria are capable of utilizing agar as the only source of carbon and energy. Soils act as a habitat for a wide variety of micro-organisms as compared to other environments. Thus, it was chosen as a source for isolating agarolytic bacteria in the presence study. To date, there have been other two agar-degrading strain that belong to the genus *Flavobacterium* reported, strain *Flavobacterium faecale* sp. nov. WV33 (Kim et al., 2014) and *Flavobacterium faecale* INACH002 (Lavín et al., 2016). However, no characterization information about these agarase was reported. Thus, the characterization of agarase from *Flavobacterium* presetting in the study would be contributed to knowledge about this carbohydrate hydrolysis class.

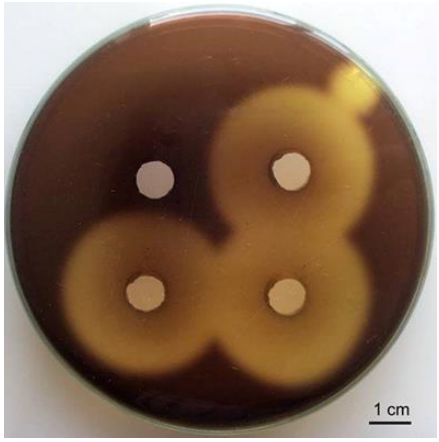

Figure 1. *Agar degradation by extracellular agarase representing as clear haloes after staining with Lugol's iodine solution*

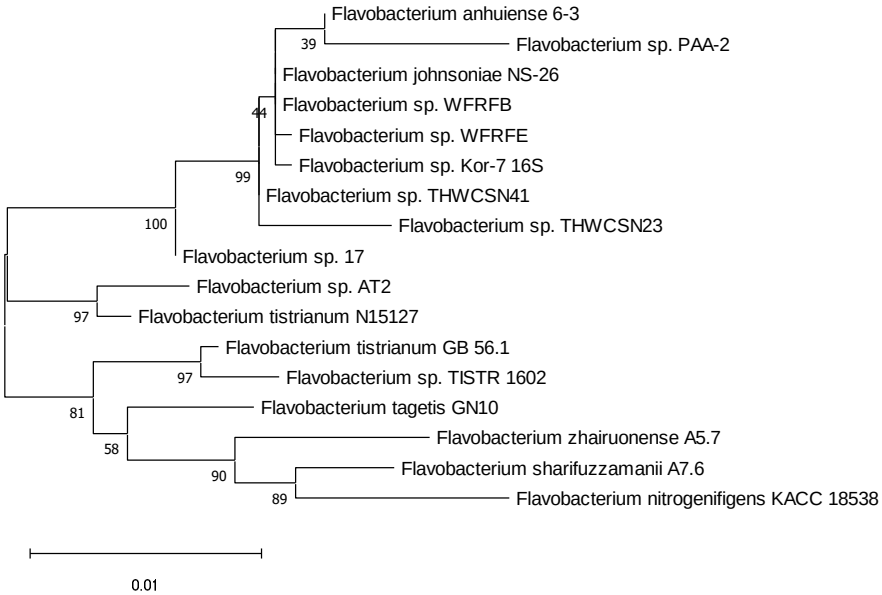

Figure 2. *Phylogenetic tree based on 16S rRNA sequences comparison between Flavobacterium sp. AT2 strain and other bacteria. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches*

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Agarase production by strain AT2

To clarify the level of agar degradation, the AT2 cells were cultured in the Erlenmeyer flask to produce agar-degrading enzyme and the change of agar- degrading enzyme activity in the supernatants of the culture broth were investigated. *Figure 3A* shows the production of agarase in the presence of 0.2% (w/v) agar. Generally, modification culture time, inoculum sizes, and shaking rate significantly affected on agarase production ($p > 0.05$). The agar-degrading enzyme activity was 10.79 U/mL after cultivation for 120 h and the maximum activity was 16.83 U/mL at 72h after incubation. The inoculum concentration plays an important role in agarase production. The optimum inoculum size was 10% (v/v) with a significant higher specific activity of 21.66 U/mL ($p > 0.05$) (*Fig. 3B*). Lower inoculum size requires longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. An increase in the number of cells in the inoculums would ensure a rapid proliferation and biomass synthesis (Singh and Kaur, 2012). The effect of shaking rate on agarase production was determined by modifying the shaking rate from 150 to 210 rpm. Maximal agarase activity of 22.8 U/mL attained when the shaking rate was 210 rpm. The agarase activity reached 17.7 and 21.7 U/mL at 150 and 180 rpm, respectively (*Fig. 3C*).

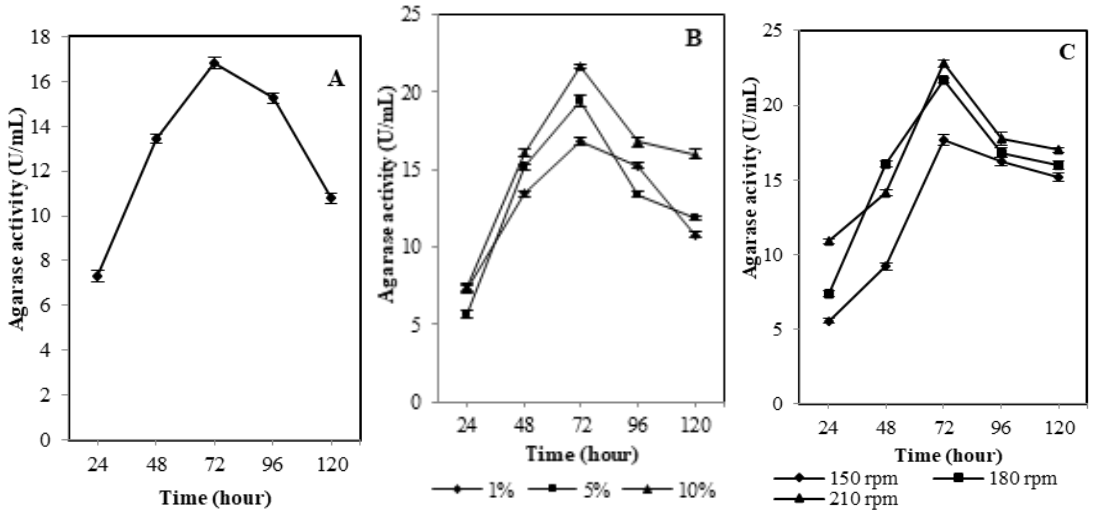

Figure 3. *Effect of incubation time (A) inoculum size (B) and shaking rate (C) on the agarase activity by Flavobacterium sp. AT2. All data are mean values from triplicate experiments and the error bars represent for the standard deviation*

Characterization of agarase

The effects of temperature, pH, metal ions and organic solvents on agarase activity were also investigated. Temperature and pH are considered to be decisive parameters for enzyme activity. The optimal temperature of various agarases are higher than the gelling temperature of agar because compact bundles of gelled agar hinder enzyme action (Jonnadula and Ghadi, 2011). Most of the agarases known to date are thermolabile enzymes incapable of displaying their activities at temperatures exceeding 50–60°C (Li et al., 2014a). The agarase was exposed to temperatures ranging from 20°C to 70°C. As shown in *Figure 4A*, the activity of agarase from *Flavobacterium* sp. AT2 significantly influenced by temperature, agarase activity gradually increased from 20°C

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to 60°C, exhibiting the highest agarolytic activity at 60°C. Its activity dramatically decreased at temperatures above 70°C. The agarase showed maximum activity at 60°C and was stable below the same temperature with strain *Catenovulum agarivorans* YM01T (Cui et al., 2014).

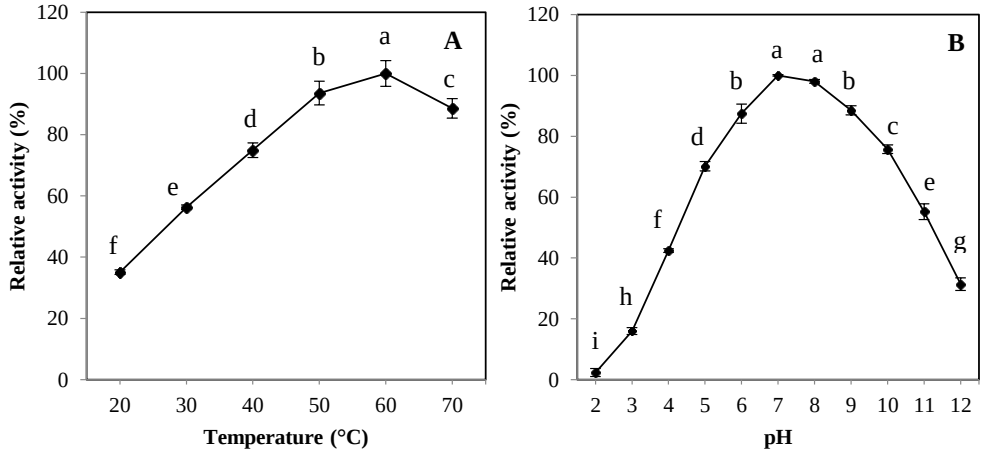

Figure 4. *Effect of reaction temperature (A) and pH on agarase activity. All data are mean values from triplicate experiments and the error bars represent the standard deviation. Different letters indicate significant differences ($p > 0.05$)*

The effect of pH on agarase activity was tested using pH values ranging from 2.0 to 12.0, in four different buffers as shown in *Figure 4B*. The data indicated the enzyme activity significantly affected by modification the pH values. Agarase of *Flavobacterium* sp. AT2 exhibited over 80% activity in the pH range of 6.0-9.0, with its highest activity observed at pH ranges of 7.0 to 8.0 ($p < 0.05$). No activity was detected at pH 2.0 or 12.0. These results are similar to some previous reports, agarase from *C. agarivorans* YM01 (An et al., 2018), *Aquimarina agarilytica* ZC1 (Lin et al., 2017), *Flammeovirga* sp. MY04 (Di et al., 2018) has an optimal pH at 7.0.

To determine the effect of metal ions, the enzyme assay was carried out in the presence of 1 mM $CaCl_2$, $CoCl_2$, $CuSO_4$, $MnCl_2$, $MgSO_4$, $ZnSO_4$, $FeSO_4$ and EDTA (*Fig. 5A*). The relative activity of agarase activity was significant inhibited by $CuSO_4$, $FeSO_4$, $CoCl_2$, $MgCl_2$, $ZnSO_4$ and EDTA ($p > 0.05$). In contrast, enzyme was partially activated by 1 mM $MnCl_2$ and $CaCl_2$. Meanwhile, Mn^{2+} ion did not significant change enzyme activity ($p < 0.05$). In particular, $CaCl_2$ significant increased more than 15% agarase activity ($p > 0.05$). The promotion of Ca^{2+} on the activity of agarases has been observed in other reports (Zeng et al., 2016; Lee et al., 2021). The reduction of enzyme activity might be caused by the structural change of a catalytic domain resulting from the interactions between the metal ions and the functional groups of the enzyme (Gupta et al., 2013). The inhibitory effect of chelator EDTA along with other metal ions was also found in different agarases such as α-agarase from *Thalassomonas* sp. LD5 (Zhang et al., 2018), *Catenovulum maritimum* STB14 (You et al., 2022).

This study also investigated the influence of various organic solvents on enzyme activity. In particularly, organic solvents significantly reduced agarase action ($p > 0.05$). The results demented that the enzyme retained > 50% activity in the presence of acetone and methanol with relative activities of 87.7 and 55.4%, respectively. Meanwhile, others organic solvents showed negative effects on activity (*Fig. 5B*).

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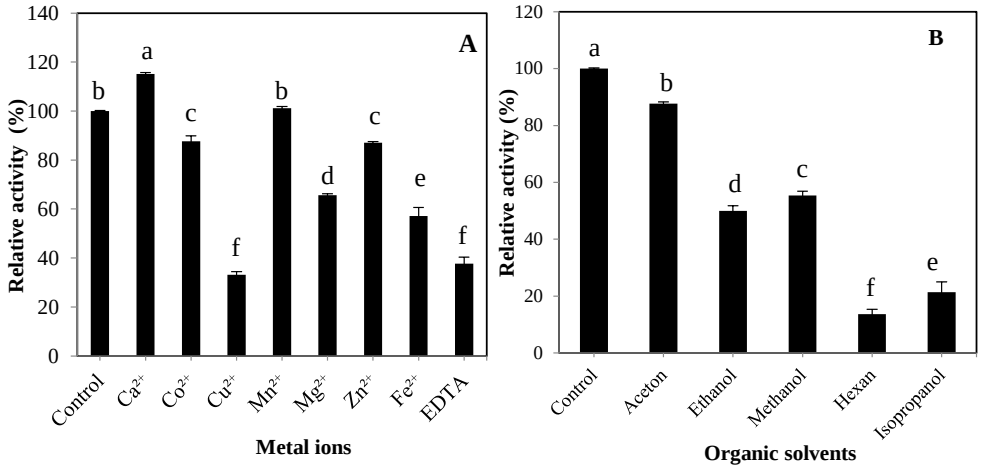

Figure 5. *Effect of metal ions (A) and organic solvents (B) on agarase activity. All data are mean values from triplicate experiments and the error bars represent the standard deviation. Different letters indicate significant differences (p > 0.05)*

Zymogram analysis

The molecular weight and agarase activity of the agarase was confirmed by SDS-PAGE containing 2% agar. The molecular mass of the agarase was 180 kDa, as determined by a clear band on a brown-violet background when comparison with the standard protein markers (*Fig. 6*), which was similar to the reported molecular weight of Cm-AGA and AgaD (You et al., 2022; Zhang et al., 2018). The molecular mass of the agarase from *Flavobacterium* sp. AT2 appeared to be larger than those of β-agarase (66.2 kDa) from a *Thalassospira profundimonas* (Zeng et al., 2016), agarase (75 kDa) from *Gilvimarinus agarilyticus* JEA5 (Lee et al., 2021), β-agarase (51 kDa) from *Pseudoalteromonas* sp. H9 (Chi et al., 2015), β-agarase (97.32 kDa) from *Paenibacillus agarexedens* BCRC 17346 (Chen et al., 2018), β-agarase (132 kDa) from *Gayadomonas joobiniege* G7 (Jung et al., 2017).

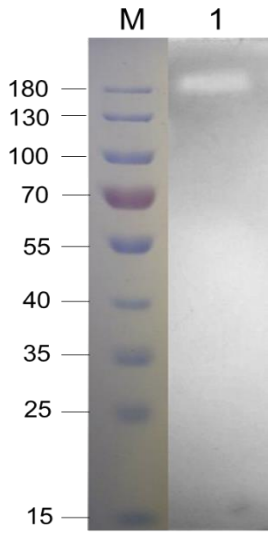

Figure 6. *Zymogram analysis of crude agarase. Lane M: Lane M: PageRuler™ Prestained Protein (Thermo Scientific, USA); Lane 1: crude enzyme*

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Evaluation of seaweed hydrolysis

The results in *Figure 7* showed the ability of using extracellular agarase from *Flavobacterium* sp. AT2 to hydrolyze the seaweed was examined at various concentrations of 0.1, 0.3, and 0.5% (w/v). The highest hydrolysis process observed at seaweed concentration of 0.1%. The reducing sugar released accumulated higher when increase the incubation time. The highest reducing sugar released reached after 20 h incubation with maximal value of 423.58 ± 33.75 μg/mL after 24 h. There was no significant different between 20 h and 24 h incubation ($p < 0.05$). Similar, treatment seaweed at concentration of 0.3% by extracellular agarase indicated that the reducing sugar released dramatically increased and reach highest amount of 322.54 ± 23.56 μg/mL after 24 h of incubation. However, the increasing of reducing sugar released after 12 h inoculation was not significant different ($p < 0.05$). The data on the seaweed hydrolysis at concentration of 0.5% showed the maximal reducing sugar released occurred at 247.63 ± 13.68 μg/mL after 24 h hydrolysis but the sugar concentration did not significantly change after 8 h inoculation ($p < 0.05$). These data suggest that the enzyme effectively hydrolyzes seaweed at lower concentration. Increasing substrate concentration is not only significantly reducing the treatment time but also decreasing reducing sugar released accumulation.

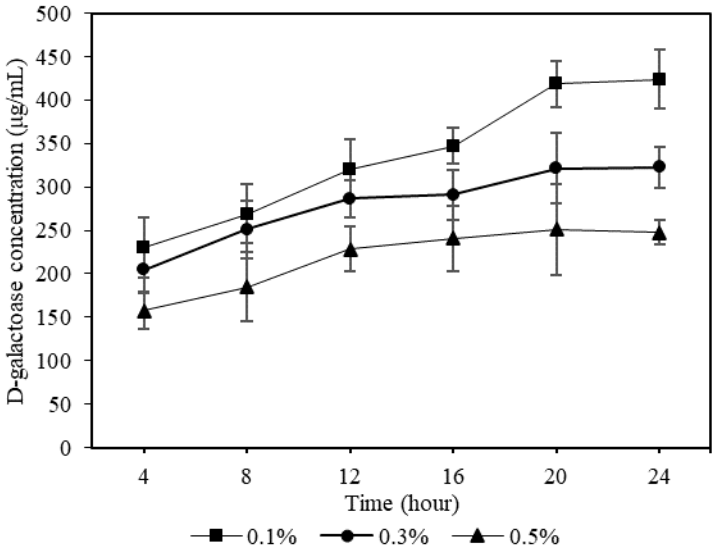

Figure 7. *Reducing sugar released from seaweed powder by extracellular agarase. All data are mean values from triplicate experiments and the error bars represent for the standard deviation*

The important application of agarase is to produce bioactive oligosaccharides from agar for industry. Agarase has been reported with ability to yielding galactose amount of 20% dry weight of agar-based biomass (Gupta et al., 2013). Similarly, agarase from *Flavobacterium* sp. AT2 produced high reducing sugar released from seaweed substances. There has been reported that such as the agar hydrolyzed products exhibit strong anti-inflammatory activity by suppressed nitrite production (Yun et al., 2013; Higashimura et al., 2013). Meanwhile, agarase hydrolyzed products also show high antioxidant activity (Leema Roseline and Sachindra, 2018), prebiotic potential (Li et al., 2014b) and anti-tumor activity (Enoki et al., 2012). Thus, production agarase

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hydrolyzed oligosaccharides through enzymatic hydrolysis processing to replace the chemical processing is preferred to produce an eco-friendly and safety material for human usage. *Flavobacterium* sp. AT2 exhibited strong ability on hydrolysis of seaweed to produce oligosaccharides could be a value candidate for industrial application.

Conclusion

A novel agar-degrading bacterium was successfully isolated from the agricultural soils. The isolate was identified as *Flavobacterium* sp. AT2 based on the 16S rRNA nucleotide sequence comparison. The isolate produced high agarase amount in culture conditions consisting of inoculum size at 10% (v/v), shaking rate of 210 rpm for 72 h of incubation. The enzymatic properties of agarase showed optimum activity at pH 7.0 and 60°C. Agarase activity increased in the presence of Ca^{2+} and Mn^{2+} metal ions. The isolate secreted extracellular agarase at a high molecular weight of 180 kDa. Extracellular agarase hydrolyzed seaweed with the highest efficiency of 423.58 ± 33.75 μg/mL reducing sugar after 24 h. These features suggest that agarase from *Flavobacterium* sp. AT2 is a good candidate for industrial applications in the cosmetic, pharmaceutical and food industries.

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