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Isolation an agar degradation *Bacillus* sp. AT6 and preliminary application for seaweed saccharification

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Abstract

Background: Agar is a common polysaccharide found in nature. However, agar is strongly resisted to the degradation processing, leading to limitation of its application in various areas. Thus, finding an effective solution for agar saccharification significantly improves the economically effects of agar based substrates.

Methods: Soil samples were collected from TienPhong Forestry Ltd. Company, ThuyXuan District, ThuaThien Hue province, Vietnam. Potential agar degrading bacteria were screened on a mineral salt agar medium. The isolate was identified based on 16S rRNA nucleotide sequence, morphological, physiological, and biochemical characteristics. Agarase production was evaluated by modification culture conditions including incubation time, shaking speed, and initial inoculum size. Molecular mass of extracellular agarase was determined by native SDS-PAGE. The effect of pH, temperature, metal ions, and organic solvents were conducted for enzyme characterization. Application of enzyme was investigated on seaweed saccharification.

Result: An agar degrading bacterial strain was isolated from soils and identified as *Bacillus* sp. AT6. Maximal agarase accumulation obtained in the culture containing an inoculum size of 10% (v/v), shaking speed of 210 rpm, and 96 hours incubation. The agarase revealed a single band on zymogram analysis with an apparent molecular weight of 180 kDa. The optimal temperature and pH were 40°C and pH 8.0, respectively. All tested metal ions and organic solvents partially decreased enzyme activity. Treatment seaweed by agarase resulted in reducing sugars release present in the reaction, indicating the saccharification of seaweed was succeeded.

Conclusion: *Bacillus* sp. AT6 is a new report of agarolytic bacteria that produces extracellular agarase enzymes. The present results promise strain AT6 is a great candidate for agar saccharification for industrial application.

Introduction

Agars are the most importance component contributing to cell walls structure of genera *Gelidium* and *Gracilaria* red algae. The *Gracilaria* genus plays economic importance as the most abundant and promising resource for agar production. This alga is commercially cultivated in various countries in subtropical regions such as Indonesia, Argentina, South Korea, Vietnam, Philippines, Chile, and Namibia [1]. Agars consist of the matrix between agaroses and agaropectin backbones, accounting for 70% and 30% dry weight, respectively [2,3]. Agarose is linked by the backbones of (l-3)-β-d-galactose and (l-4)-3,6-anhydro-α-l-galactose units. Meanwhile, agaropectin polysaccharide chain consists of 3,6-anhydro-l-galactose units in integration with pyruvate and sulfoxy/methoxy residues [4]. Agars have widely applied in food, pharmaceutical, cosmetics industries [5]. Basically, agar structure can be degraded by different methods including enzymatic saccharification and chemical hydrolysis. However, enzymatic saccharification is a preferable strategy for agar-oligosaccharides preparation due to high efficiency, less contamination and environment friendly [6].

Agarases (EC 3.2.1.81) belong to the glycoside hydrolase (GH) family which catalyze the hydrolysis of agar. According to the distinct cleavage pattern or production formation, agarases contain α-agarase (E.C. 3.2.1.158) and β-agarase (E.C. 3.2.1.81). α-agarase acts on α-1,3-linkages, whereas β-agarase catalyzes β-1,4-linkages, resulting in agaro-oligosaccharides and neoagaro-oligosaccharides products, respectively [7-9]. Agarases and their hydrolysis products have extensive applications in the cosmetic, pharmaceutical and food industries [10]. These agaro-oligosaccharides exhibit a great physiological activities including moisturizing effect [11], skin whitening effects, anti-inflammatory [12], macrophage-stimulating, antioxidant, and immunogenic activities [13], anti-diabetic and anti-obesity effects [14], and can be used as prebiotics [11].

Up to date, most agar degrading bacteria have been reported living in marine habitats such as marine sediments, marine algae, and seawater including *Vibrio* sp. [15], *Ammoniibacillus* sp. [16], *Acinetobacter junii* [17], and *Flammeovirga* sp. [18]. However, a few studies have reported several species of agarase enzyme-producing bacteria are deriving from freshwater and terrestrial environments [19]. In this study, we investigated the agarolytic bacteria isolated from soils in local forestry farm. The effect of culture conditions on agarases production and biochemical properties of extracellular agarases by isolate was also evaluated. Finally, the isolate was further applied for seaweed (*Gracilaria verrucosa*) hydrolysis.

Methods

Samples collection

Soil samples were collected from TienPhong Forestry Ltd. Company, ThuyXuan District, ThuaThien Hue province, Vietnam and stored in plastic bags. Then, the samples were delivered and stored at 4°C in laboratory for further analysis.

Screening and isolation agarase producing bacteria

One gram of soil was suspended in distilled water with ratio 1:10 and diluted by 10-fold serial dilution method to obtain 10^{-4}, 10^{-5}, and 10^{-6} diluted solutions. Samples (0.1 mL) of each dilution were then plated on Mineral Salt Agar (MSA) containing (g/L): K_2HPO_4 0.5; $(NH_4)_2SO_4$ 0.5; $MgSO_4.7H_2O$ 0.5; $CaCl_2$ 0.1; NaCl 0.5; agar 15 and pH 7.0. Plates were inoculated at 37°C for 7 days and daily qualified for agarolytic activity. The depression or liquefaction around colonies was assessed as a signal of agarase activity. After 7 days, plates were stained using iodine solution. The appearance of pale-yellow halo zones were referred to as agar-degrading activity [20]. All colonies showing liquefaction, depressions and clearance zone in the agar were picked up, transferred to new LB medium plates and purified by repeating sub-culture on new LB medium. The colony with the highest potential agarolytic activity was selected for further investigation.

Identification of the bacterial isolates

The isolate was identified morphological characteristics including shape, colour, transparency, margin by Gram staining techniques and observed using a microscopy [21]. Catalase assay was conducted by standard protocol [22].

The total DNA of isolate was extracted based on description of Sambrook et al. (2001) [23]. The total DNA was diluted in 30 µL sterile water. The 16S rRNA nucleotide sequence was amplified using a primer pair consisting of 27-F (5'-AGAGTTTGATCCTGGCTCAG–3') and 1492-R (5′-TACCTTGTTACGACTT-3′). The amplification was carried out in a Thermal Cycler (Eppendorf, Germany). The amplification protocol consisted of an initial denaturation step for 5 min at 95°C, 30 cycles of denaturation step for 60 s at 95°C, annealing step for 30 s at 55°C, extension step for 90 s at 72°C. The reaction was extended for 10 min at 72°C. Amplicon products were qualified by electrophoresis on 1 % (w/v) agarose gel. The 16S rRNA nucleotide sequence was obtained by a DNA sequencing (Firstbase, Malaysia). The nucleotide sequence was analyzed to remove errors and aligned with nucleotide sequences on GenBank database using BLAST tool. Sequence similarities between the isolates and closest relatives were determined and recorded. Isolate was classified by

conducting a phylogenetic analysis by Maximum Likehihood methodusing MEGAsoftware (Ver. 11) [24].

Agarase production

The single colony of isolate was inoculateed in 5 mL LB medium for overnight (approximately 16 h). The overnight culture (1%, v/v) was transferred into 50 mL MSA medium containing 0.2% (w/v) agar and continuously cultured at 37°C for 120 h, shaking speed of 180 rpm. The supernatnat cultures were hargested every 24 h by elimininationcells pelet with centrifuging at 10.000 rpm, 4°C, 15 min. Agarase activity was qualified according to the enzymatic assay [17].

Enzymatic assay

Agarase activity was determined by 3,5-dinitrosalicylic acid (DNSA) reagent procedure with some modifications [25]. One hundred microliters of the enzyme solution was mixed a volume of 400 µL containing 50 mM phosphate buffer (pH 7.0) and 0.2% (w/v) agarose [26]. The reaction was carried out at 40°C for 15 min [27]. Then, a volume of 500 µL DNSA solution was added to the reaction mixture. The reaction was terminated by boiling for 10 minutes. The reducing sugars release was measured at the absorbance of 540 nm. Finally, the reduced sugars were calculated based on D-galactose concentration. Enzyme activity was expressed as unit and was assessed as an enzyme amount required for producing 1 µmol of D-galactose per minute through agarose hydrolysis.

Effect of culture conditions on enzyme production

The culture conditions affecting on agarase production were conducted by culture the isolate on 50 mL MSA containing 0.2% (w/v) agar, pH 7.0. The culture was carried out at 37°C for 5 days. Inoculum sizes of 1, 5, and 10% were selected to evaluate agarase accumulation in medium culture. Meanwhile, shaking speeds were performed at values of 150, 180 and 210 rpm. One milliliter of culture was harvested daily and removed the cells pellet by centrifuging at 10.000 rpm, 4°C for 15 min. The supernatants were used for enzymatic qualification assay as described above.

Zymogram analysis

Zymogram analysis was carried out to estimate the molecular mass of agarase using a 5% polyacrylamide stacking gel and a 12% polyacrylamide separating gel containing 2% agar. After electrophoresis, the gel was sunk in 2% (v/v) Trixton X-100 for 30 min at room temperature and washed three times using distilled water. Gel was incubated in 50 mMTris-HCl buffer (pH 8) for 4 hat 37°C. To visualize agarase activity, the gel was stained using Lugol's iodine solution for 10 minutes. The appearance of pale-yellow zones against a brown-violet background was assessed as agarase activity [28].

Agarase characterization

The effect of pH on enzyme activity was evaluated using different pH buffer solution. Glycine-HCl was used to generate buffers with pH 2-3, sodium acetate was prepared for pH 4-5 buffers, sodium phosphate was carried out for pH 6-8 buffers and glycine -NaOH was conducted for pH 9-12 buffers. Enzymatic assay was conducted as mentioned above. The effect of temperature was performed in a temperature ranging from 20 to 70°C. The residue activity was accessed as the activity percentage in compared to the maximum enzyme activity.

The effects of metal ions and organic solvents on enzyme activity were carried out by incubation enzyme solution in the presence of 1 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} ions. The enzyme solution was incubated for 1 h at 40°C, pH 8.0. Then the retained agarase activities were recorded. To evaluate the effect of organic solvents, the retained enzyme activities were determined after 1 h of incubation enzyme solution at 40°C, pH 8.0 in the presence of various chemical detergents (acetone, methanol, ethanol, hexan and isopropanol) at 30% concentration. Reactions without metal ions or organic solvents were used as control.

Evaluation of seaweed hydrolysis

The extracellular agarase was harvested after 96 h of culture in MSA and centrifuging for 15 min at 10.000 rpm, 4°C. The seaweed (*G. verrucosa*) was purchased in local market and grinded into powder. Seaweed powder was mixed with 50 mM of pH 8.0 phosphate buffer to final concentrations of 0.1, 0.3 and 0.5% (w/v) and incubated at 40°C. The enzyme was added and the reactions were conducted for 24 hours. The reducing sugars release was measured as described above.

Data analysis

The experiments were repeated with three replicates and the data were analyzed using MS excel software (Ver. 2016).

Results

Screening and isolation agar degrading bacteria

Potential agarolytic bacteria in the soils were preliminary screened MSA medium. After 24 hours inoculation, potential agarolytic bacteria grew on surface of agar plate and formed a clearing liquefaction and depression zone around colonies. Total of 7 isolates showing different morphology were transferred into new MSA agar plate. Figure 1 showed the agar hydrolytic by supernatant culture of the isolate AT6, indicating a clearance zone with diameter of 39 ± 1 mm

after 24 h of incubation at 37°C. Thus, the isolate was selected for further investigation on agarolytic activity.

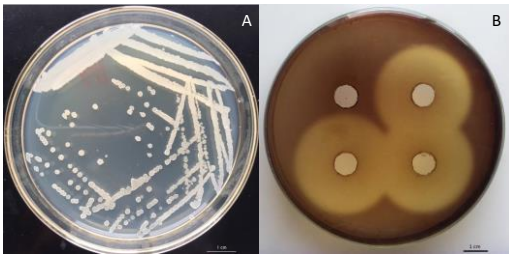

Figure 1: Colony characteristics and extracellular agarase activity of the isolate AT6. (A) Colony characteristics of the isolate AT6 grows on LB medium. (B) Agarolytic activity visualization as clear zones around wells. The enzyme activities were determined using free-cells supernatant culture.

Bacteria identification

The identification of strain AT6 was based on morphological and physiological characteristics. The morphological characteristics are shown in Figure 1A and Table 1. AT6 isolate formed cream colonies and ranged in size 1.0 mm. The bacterium was rod under the microscope observation. Gram staining indicated the isolate was gram-positive. Meanwhile, the isolate exhibited catalase positive.

Isolate Code	Morphology characterization				Biochemical test	
	Colony color	Shape	Margin	Size	Gram	Catalase
AT6	Cream	Rod	Entire	1 mm	+	+

Notes: (+): Positive; (-): Negative

Table 1: Morphological and biochemical properties of agarolytic bacterial isolates AT6.

The 16S rRNA region was sequenced and the nucleotide sequence analysis clearly demonstrated that strain AT6 belongs to *Bacillus* genus and exhibited maximum similarity with *Bacillus* sp. 72 and *Bacillus* sp. PEX-1 (100% similarity). The phylogenetic tree between strain AT6 and other *Bacillus* species showing in Figure 2 confirmed the genetic relationship. Thus, the AT6 strain was identified as *Bacillus* sp. AT6. The nucleotide sequence has been deposited on GenBank databases under accession number of ON227004.

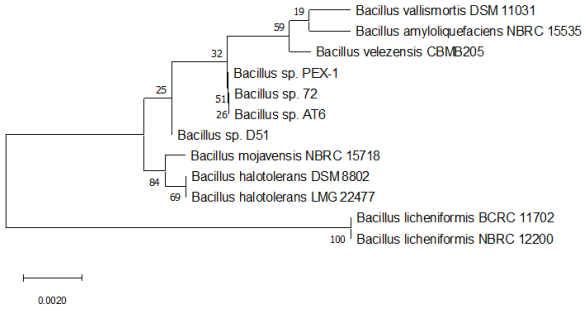

Figure 2: Phylogenetic tree of the isolate *Bacillus* sp. AT6 and other *Bacillus* species using 16S rRNA nucleotide sequences. The tree was built by the Maximum Likelihood method and Tamura-Nei model. The associated taxa clusters are shown as branches.

Agarase production

The effect of incubation time on agarase production is shown in Figure 3A. Particularly, the enzyme activity gradually increased after 24 hours of incubation (8.17 U/mL) and reached maximal activity (14.96 U/mL) at 96 hours of incubation. Extension culture after this optimum period did not increase the activity of the enzyme, whereas the activity dropped to 14.15 U/mL after 120 hours of incubation. Meanwhile, agarase enzyme activities of *Bacillus* sp. AT6 at different inoculum sizes are shown in Figure 3B. The results demonstrated that the size of the inoculum had a strong relation with enzyme activity. At the inoculum size of 1% (v/v), agarase activity was lowest of 14.96 U/mL after 96 hours culture, while a higher agarase activity occurred with 16.89 U/mL at the inoculum size of 5% (v/v). The highest enzyme activity (17.92 U/mL) observed at 10% (v/v) inoculum size.

Oxygen is an essential factor for the growth of aerobic bacteria and typically depends on the shaking speed of the culture process. The effect of shaking speed on agarase accumulation was examined by varying the shaking speed from 150 to 210 rpm. Maximal agarase activity of 21.15 ± 0.23 U/mL obtained when the shaking speed was 210 rpm. The agarase activity reached 16.03 ± 0.13 and 17.93 ± 0.39 U/mL at 150 and 180 rpm, respectively (Figure 3C).

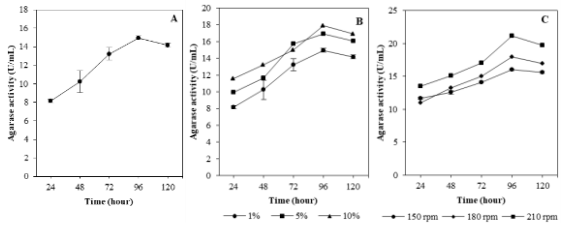

Figure 3: Effect of incubation time (A) inoculum sizes (B) and shaking speeds (C) on the activity of agarase produced by *Bacillus* sp. AT6. All data are represented for the average of three experiments and the standard deviations are represented as error bars.

Zymogram analysis

Molecular mass and agarase activity were identified by SDS-PAGE using 12% polyacrylamide gels contained with 2% agar. Figure 4 showed an apparent clear band on a brown-violet background with molecular mass of approximately 180 kDa. This result indicated the *Bacillus* sp. AT6 secreted extracellular agarase at very high molecular mass.

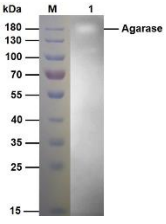

Figure 4: Zymogram analysis of extracellular agarase produced by *Bacillus* sp. AT6. Lane M: Lane M: PageRuler™ Prestained Protein (Thermo Scientific, USA); Lane 1: Free-cells suppernatant culture.

Characterization of agarase

The effect of pH on agarase activity was investigated at different pH from 2.0 to 12.0. The obtained results in Figure 5A shows agarase from *Bacillus* sp. AT6 was active in wide pH ranging of 7-10 with peak activity at pH 8.0. Enzyme activity maintained 93.26%, 96.42%, and 87.05% at pH 7.0, 9.0, and 10.0, respectively. Meanwhile, the enzyme strongly inhibited in acidic conditions whereas the retained enzyme activities were >60% at the pH below 5.0. Thermal dependence of agarase was evaluated by qualifying the enzyme activity variation at temperatures from 20 to 70°C. The results in Figure 5B showed the influence of temperature on agarase activity secreted by *Bacillus* sp. AT6. The enzymatic activity gradually increased with increasing temperature up to the optimum temperature at 40^{0}C and rapidly decreased at the temperature above 50°C. Effect of metal ions on agarase activity are shown in Figure 6A. Fe^{2+} and Zn^{2+} ions exhibited strong inhibitory effects on the agarase activity. In comparison, Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Mg^{2+} ions slightly exhibited a suppressive effect. Figure 6B showed the effects of different organic solvents on agarase activity, indicating the agarase activity significantly affected by organic solvents at final concentration of 30%. The enzyme activities were 80.09 ± 1.25%, 70.71 ±1.37%, 75.55 ± 0.08%, 79.35 ± 0.33%, and 65.64 ± 1.15 % in the presence of acetone, ethanol, methanol, hexan, and isopropanol, respectively compared to control.

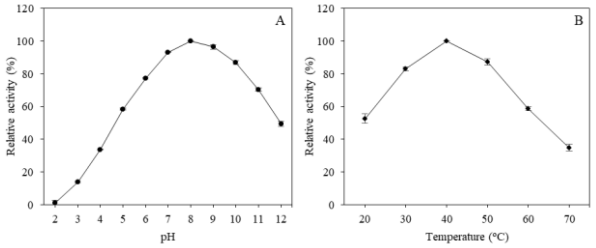

Figure 5: Effect of pH (A) and temperature (B) on agarose activity. All data are represented for the average of three experiments and the standard deviations are represented as error bars.

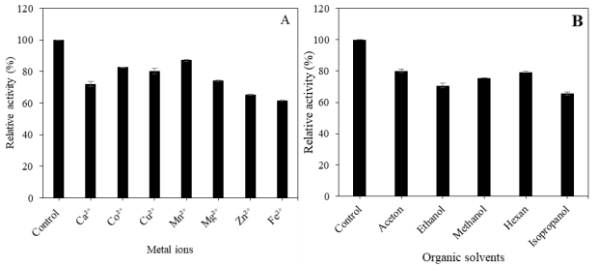

Figure 6: Effect of metal ions (A) and organic solvents (B) on agarose activity. All data are mean values from triplicate experiments and the error bars represent the standard deviation.

Evaluation of seaweed hydrolysis

Agar is major component of seaweed. In this study, the ability of using extracellular agarase from *Bacillus* sp. AT6 to hydrolyze the seaweed was examined at various concentrations of 0.1, 0.3, and 0.5% (w/v). The results in Figure 7 indicated the highest amount of reducing sugars release was 503.02 ± 27.57 µg/mL after 24 hours of incubation at 40°C when incubated the enzyme with seaweed at concentration of 0.1%. Meanwhile, the agarase produced 432.86 ± 38.26 and 404.58 ± 13.97 µg reducing sugars at seaweed concentration of 0.3% and 0.5%, respectively.

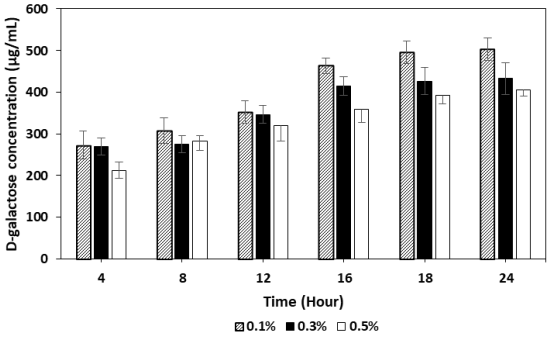

Figure 7: Reducing sugars release from seaweed saccharification by extracellular agarase of *Bacillus* sp. AT6. All data are mean values from triplicate experiments and the error bars represent the standard deviation.

Discussion

To date, most agar- degrading bacteria have been detected in marine habitats, where red algae act as a substrate or only source of carbon. However, a few studies have focused on bacteria degrading agar from non-marine environments, such as soils. Previous studies isolated agar-degrading bacteria including *Alteromonas* sp. and *Cytophaga* sp. from freshwater; *Spirochaetaalkalica* from a soda lake; *Paenibacillus* sp. SSG-1 [29], *Bacillus* sp. [30], *Cytophaga* sp. [31]; *Steroidobacter agariperforans* from vegetable crop fields [32,33]; and *Streptomyces coelicolor* from soils [2]. In this study, a agar-degrading bacterium strain was successfully isolated and screened from soils. The molecular identification based on 16s rRNA nucleotide sequence, morphology, physiological and biochemical characteristic analysis indicated the isolate belongs to *Bacillus* genus.

Modification culture conditions affected on enzyme accumulation in which the optimal initial inoculum size, shaking speed, and incubation time were 10% (v/v), 210 rpm, and 96h, respectively. A smaller inoculum size necessitates more time for the cells to proliferate to a sufficient quantity to consume the substrate and produce the enzyme. A higher cells count in the optimum inoculum would assure fast division and biomass synthesis.Meanwhile, a low agitation

intensity significantly impacts on enzyme accumulation by bacteria due to reducing oxygen supply and poor mixing of nutrients in medium fermentation culture [34].

Bacillus sp. AT6 produced a single extracellular agarasewith molecular mass of approximately 180 kDa.The reported molecular mass of agarasesvarried from 20 to 360 kDa. Among them, the largest agarase is found in *Alteromonas agarlyticus* GJ1B [35], while the smallest agarase secreted by *Vibrio* sp. AP-2 [15]. *Agarivorans* sp. BK-1 produced three agar-degrading enzymes with molecular masses of 110, 90, and 55 kDa. The size of the agar-degrading enzyme with the highest activity was confirmed to be 110 kDa [36]. Meanwhile, soil bacteria including *Acinetobacter* sp. AGLSL-1, *Bacillus* sp. MK03, and *Alteromonas* sp. E-l produced agarase with molecular mass of 100 kDa, 113 kDa and 180 kDa, respectively [30,37,38].

The extracellular agarase exhibited maximal activity at pH 8.0 and temperate of 40^{o}C. These findings are similarly to agarase from *Antarctic psychrophilic*, *Pseudomonas aeruginosa* ZSL-2, and *Thalassospira profundimonas* with pH peaks at 8.0 [39,40, 41]. This optimum pH was higher compared to that of agarase from *Alteromonas* sp. C-1 of pH 6.5 [42], *Vibrio* sp. AP-2 of pH 5.5 [15]. The thermal dependent profiles of agarase from *Bacillus* sp. AT6 are consistent with the previous reportssuch as *Alteromonas* sp. E1 [38], *Acinetobacter* sp. AGLSL-1 [13], *Microbulbifer* sp. Q7 [6]. Meanwhile, agarase produced by *Aquimarina agarilytica* ZC1 exhibited lower optimal temperature of 25^{o}C optimum pH at 7.0 [26]. Other studies reported higher optimal temperature for agarase including agarase from *Alterococcus agarolyticusa* [43], *Halococcus* sp. 197A [44], and *Acinetobacter* sp. [13]. In the present study, agarase produced by *Bacillus* sp. AT6 had moderate temperature optimum that suggests some advantages such as degrading the agar process at room temperature, reducing the cost of establishing conditions for enzyme activity. All tested metal ions and organic solvents partially inhibited enzyme activity. These effects were similar to previous report, in which Ca^{2+}, Mg^{2+}, Na^{+}, Cu^{2+} and K^{+} ions slightlyinhibited the activity of β-agarase [41].

Agar products delivered from red algae have numerous applications for human. The hydrolysate products contain high bioactive activities including anti-inflammatory, antioxidant, and immunogenic activities [5]. Thus, agar hydrolysates are importance sources for various industries including cosmetic, pharmaceutical and food [45]. Moreover, the applied of microbial enzymes for agar hydrolysis process positively contributes to enhance the economic value chain of red algae. The present data demonstrated that extracellular agarase from *Bacillus* sp. AT6 efficiently hydrolyzed agar powder in which released reducing sugar reached 503.02 ± 27.57 µg/mL. However, the hydrolysis efficiency reduces when increasing the agar powder concentration. Thus, the present study suggests that enzymes and substrates only work optimally at an appropriate ratio, whereas increasing the enzyme or substrate does not increase the efficiency but inhibits the reaction. This study is enabling the application capacity of using extracellular agarase for treatment of seaweed or enriched agar substrates to higher value products.

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Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

Dang Thi Thanh Ha: Methodology, Investigation, Data analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration. Trinh Thi Phuong Thao: Investigation, Data analysis, Writing - original draft, Writing - review & editing, Visualization. Le Thi Kim Thoa: Validation, Data analysis, Writing - review & editing. Tran Trung Dung: Writing - review & editing, Project administration. Tran Thi Minh Ha: Validation, Writing - review & editing. Phan Thi Thuc Quyen: Writing - review & editing, Funding acquisition. Bui Thi Khuyen: Investigation, Writing - review & editing. Nguyen Duc Huy: Conceptualization, Methodology, Writing - review & editing, Supervision.

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