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Isolation and characterization of marine bacteria from macroalgae *Gracilaria salicornia* and *Gelidium latifolium* on agarolitic activity for bioethanol production

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Abstract. *Gracilaria salicornia* and *Gelidium latifolium* have high content of agar and potential to be use as raw material for bioethanol. In bioethanol production, one of the processes level is enzyme hydrolysis. Various microorganisms, one of which is bacteria, can carry out the enzyme hydrolysis. Bacteria that degrade the cell walls of macroalgae and produce an agarase enzyme called agarolytic bacteria. The purpose of this study was to isolate bacteria from macroalgae *G. salicornia* and *G. latifolium*, which has the highest agarase enzyme activities, and to obtain agarase enzyme characteristic for bioethanol production. There are two isolates bacteria resulted from *G. salicornia* that are N1 and N3 and there are two isolates from *G. latifolium* that are BSUC2 and BSUC4. The result of agarase enzyme qualitative test showed that isolates bacteria from *G. latifolium* were greater than *G. salicornia*. The highest agarolitic index of bacteria from *G. salicornia* produced by isolate N3 was 2.32 mm and isolate N3 was 2.27 mm. Bacteria from *G. latifolium* produced by isolate BSUC4 was 4.28 mm and isolate BSUC2 was 4.18 mm, respectively. Agarase enzyme activities from isolates N1 and N3 were optimum working at pH 7 and temperature 30 °C, while from isolates BSUC4 was optimum at pH 7 and temperature 50 °C. This is indicated that the four bacteria are appropriate to hydrolyze macro alga for bioethanol production.

1. Introduction

Biomass is a potential raw material that can be used to produce bioethanol and one of biomass is macroalgae. Macroalgae has several advantages, ie. Low lignin content, do not compete with terrestrial biomass, not a major food source, have high sugar content, and in Indonesia, cultivation productivity is quite high [1].

G. salicornia and *G. latifolium* are macroalgae producer of gelatin that potential to be use as raw material for bioethanol. Both macroalgae are suitable for cultivation because of several factors ei. easily obtain and contain high levels of agar that can be hydrolyzed into oligosaccharides.

The content of agar in *G. salicornia* in Indonesia generally range between 32-48% and the carbohydrate content of *G. latifolium* reach 67.85-76.15% [2].

Research on bioethanol from terrestrial biomass has long been done, but the use of macroalgae (*G. salicornia*.) as raw material for bioethanol production is still not a done deal. One study evaluating the crude production of bioethanol from *G. latifolium* and *G. verrucosa* done by Kawaroe et al [3], which produces crude bioethanol in the fifth and sixth day. Difficulties experienced is due to several factors that greatly affect the bioethanol production process at every stage that must be passed. Stages of the process is the process of hydrolysis (by acid and enzym) and fermentation. Factors that influence the hydrolysis process including the carbohydrate content of the raw material used, the conditions of pH and temperature, while the fermentation process is a type of microorganism, sugar resulting from the hydrolysis process, time, pH, and temperature.

Bacteria that degrade the cell walls of macroalgae and produce agarase enzyme called agarolitic bacteria and enzymes produced called agarase enzyme. The existence and characteristics of agarolitik bacteria can be determined by agarase activity test in qualitative and quantitative. Qualitative agarase activity performed by dripping Lugol reagent. This reagent can indicate the activity of the bacteria in producing agarase above culture vessels [4]. Agarase enzyme quantity is generally measured by spectrophotometry value of the increased concentration of reducing sugars by the method of Nelson or DNS method that uses D-galactose as standard. The enzyme activity (U/mL) was defined as the amount of enzyme required to release 1 mol of D-galactose per minute [5]. Agarase activity test is also necessary to know the characteristics of the bacteria to produce bioethanol optimum. It is the background that the potential of bacteria agarolitik of macroalgae for bioethanol needs to be developed further.

This study aims to investigate the characteristics of agarase enzyme activity of macroalgae *G. salicornia* and *G. latifolium* to produce bioethanol.

2. Materials and Method

This study was conducted on May 2015-March 2016 at Surfactant and Bioenergy Research Center (SBRC) and the Microbiology Laboratory of the Department of Marine Sciences and Technology, IPB.

2.1. Rejuvenation of bacteria

Bacteria cultivated by applying bacteria into nutrient agar. The bacteria were incubated in an incubator for 1 x 24 hours at 30°C. Colonies that have been purified subsequently inoculated into an agar medium slant NA in-use as stock.

2.2. Qualitative Test of Agarase Enzyme

Qualitative test performed with Lugol staining method. Bacterial isolates dripped onto an agar medium YPA. The bacteria were incubated for 3 days at 30 °C [3], then agarolitic activity tested by adding Lugol dye as many as 2 mL and allowed to stand for 15 minutes. Diameter of clear zone and the diameter of colonies formed were measured. Measuring the diameter of clear zone and the diameter of the colony did in two repetitions. Potential agarolitic bacteria in producing agarase enzyme can be seen qualitatively by calculating the index of agarolitic. Agarolitic index equation (1) is the ratio between the diameter of the clear zone and colony. The larger the index agarolitic generated, the greater the enzyme produced by the bacterial isolates. Agarolitic Index is an index of the ability of bacteria to degrade the media or produce agarase enzyme.

$$IA = \frac{dzb - dk}{dk} \quad (1)$$

Where:
IA: Agarolitic index (mm)
Dzb: Clear zone diameter (mm)
Dk: Colony diameter (mm)

2.3. Determination of Optimum Time Enzyme Production Agarase
The timing of the inoculum be done by culturing two loops of isolates in 5 mL of nutrient broth and incubated for 620 minutes. The cultures were incubated at room temperature in a shaker with agitation speed of 85 rpm. Sampling was performed during 15 hours of incubation with the sampling time span of 1 hour and measured values Optical Density (OD) at a wavelength of 600 nm.

A total of 5 mL nutrient broth containing cell cultures was inoculated in 500 mL production medium and incubated in a water bath. Inoculum pouring time seen from the exponential growth of bacteria at growth curve. Sampling was done every 6 hours for 42 hours during incubation. 1 mL of the reaction and 3 mL DNS reagent was centrifuged for 15 minutes, then T stirred using vortex and heated in boiling water for 5 minutes. Agarase activity is expressed in International Units U/mL. One unit is the amount of enzyme required to break one mol agarase be reducing sugars per minute in test conditions. Glucose levels resulting from hydrolysis by enzymes agarase based on the absorbance at λ 550 nm. Then, agarase activity was calculated based on the equation of the agarase enzyme activity in the modification (2) [3].

$$\text{Agarase enzyme activity (U/mL)} = \frac{glukosa\ (mg/L)\times 1000}{BM\times V\times t} \times fp \qquad (2)$$

Where
BM: Glucose molecule weight (0.18 mg/µmoll); *V*: Enzyme volume (1 mL); *T*: incubation time (30 minute)

2.4. Crude enzyme production Agarase
Production of agarase enzyme was done according to the procedures and incubation time of the highest agarase activity that was known from agarase activity curves. Media production were incubated at 30°C in a water bath with agitation speed of 100 rpm, then harvested during the agarase enzyme production time highs that have been obtained previously. During the incubation period, sampling was done for the calculation of reducing sugars do as much as 1 mL with the addition of 3 mL. Cell culture on production media that containing extracellular agarase enzyme was centrifuged at 2500 rpm for 15 min at 4°C to separate the enzyme solution with bacterial pellets. Supernatant resulted from centrifugation then stored at 10°C as the crude extract enzyme.

2.5. Agarase Enzyme characteristic
Adding 0.2 mL of enzyme reacted with 1.8 mL of the substrate-tested effect of pH on enzyme activity. Substrates made by a mix of 1.8 g from 0.5% bacto agar into a buffer with different pH levels at 3,4,5,6,7,8 and 9. Each enzyme was incubated at 30° C for 30 minutes. Agarase enzyme activity was measured according to the test procedures before.

The influence of temperature on the enzyme activity carried out by reacting 0.2 mL to 1.8 mL enzyme substrate wherein the substrate is made by mixing 1.8 g bacto that the optimum pH buffer. Enzymes that have been mixed with the substrate and then incubated at a temperature level of between 20-80 ° C with an interval of 10 ° C for 30 minutes of incubation time. Agarase enzyme activity was measured according to the test procedures before.

3. Results and Discussion

3.1. Agarase Activity

Clear zone formed demonstrated that the enzyme activity of agarase extraseluler issued by N1 and N3 isolates from *G. salicornia* and BSUC2 and BSUC4 of *G. latifolium* (Table 1).

Table 1 Agarolitic index

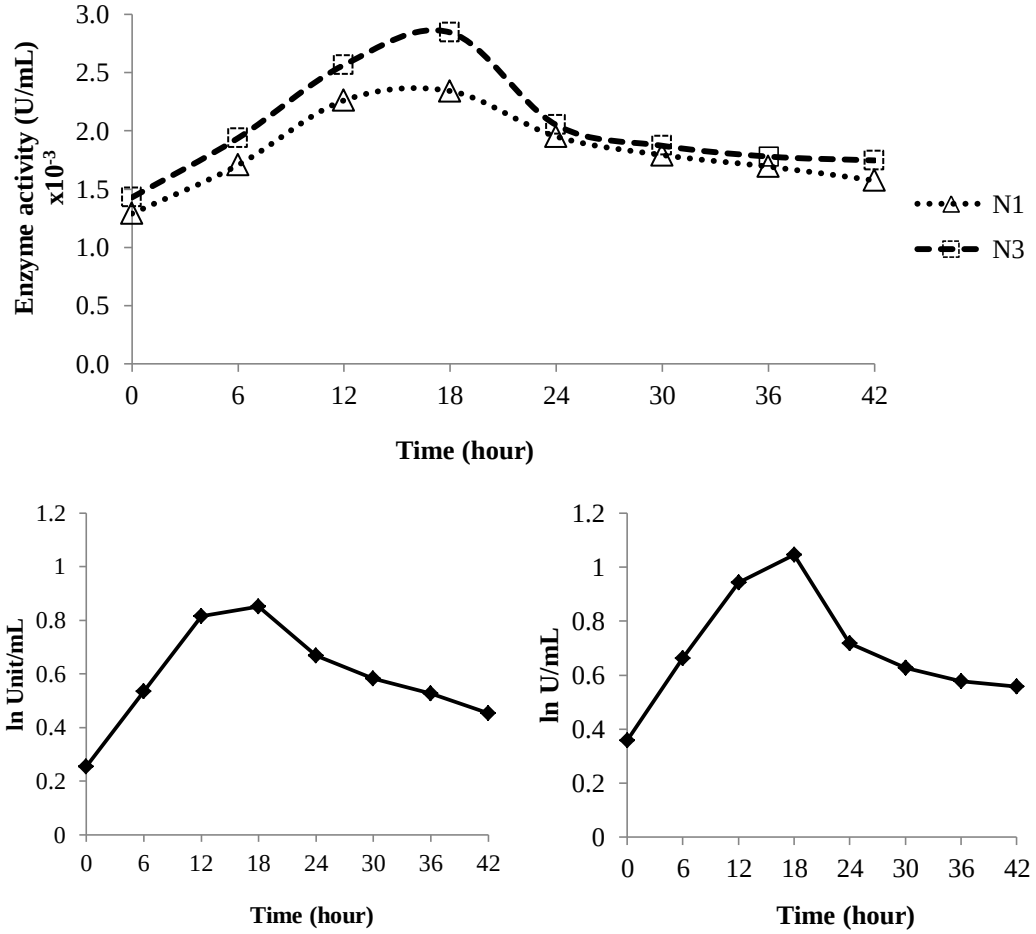
Isolate	Agarolitic Index (mm)
N1[1]	2.27
N2[1]	2.32
BSUC2[2]	4.18
BSUC4[2]	4.28

[1]Bacteri isolated from *G. salicornia*
[2]Bacteria isolated from *G. latifolium* [6]

Potential of agarolitic bacteria in producing agarase enzyme can be seen qualitatively by calculating their agarolitic index. Table 1 showed the agarase enzyme activity of bacterial isolates from *G. latifolium* greater than from *G. salicornia*. The big agarolitic index formed from isolates BSUC4 (*G. latifolium)* was 4.28 mm.

3.2. Optimum time of Agarase Enzyme Production

The peak time of growth can be used as a determination of the best time for pouring the inoculum media to the production media for the optimum number of cells that are expected to generate curves in corresponding enzyme activity. Isolates N1 and N3 had the best time for pouring inoculum at the same time, i.e. at the 13^{th} hour. While, isolates BSUC2 and BSUC4 of *G. latifolium* optimum at the 14^{th} hour and 8^{hr} all through the 30 minutes [3]. Once known, the next step is to determine the best enzyme production time and can be seen from the curves of enzyme activity (Figure 1).



(b) (c)

Figure 1. (a) Agarase enzyme activity and rate of enzyme activity from (b) isolate N1 and (c) isolate N3.

The rate of agarase enzyme activity that isolated from bacterial can be written with the equations N_t/N_0 = exp (0.0009t) to isolate N1 and N_t/N_0 = exp (0.0006t) to isolate N3. Isolate N1 and N3 have had the highest enzyme activity time at the 18th hour. The high enzyme activity of isolate N1 was 2.34×10^{-3}U/mL and N3 was 2.84×10^{-3}U/mL. The enzyme activity of isolate BSUC4 also optimum at the 18th hour with the value of the enzyme activity that was 2.14×10^{-3} U/mL. The enzyme activity was not seen in BSUC2 isolate that cause these isolates did not include in further tested [6].

3.3. Characteristic of Agarase Enzyme

3.3.1. Optimum of pH

The enzyme activity is influenced by the pH where the pH or pH changes that do not fit will cause the area and catalytic enzyme conformational changes. In addition to changes in pH also causes the denaturation of enzymes causing loss of enzyme activity [7]. N1 isolates agarase enzyme activity optimum at pH 7 buffer with agarase activity value of 1.38 × 10-3 U/mL and the optimum pH N3 isolates were also obtained at pH 7 buffer with agarase activity value of 1.31 × 10-3 U/mL (Figure 2).

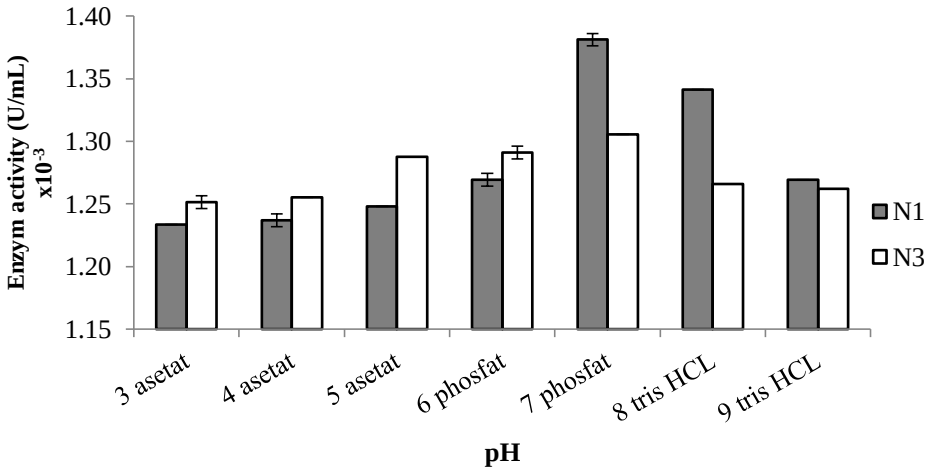


Figure 2. Effect of pH on agarase activity

Changes in pH effect on enzyme activity will happen if the conditions of low or high pH can also cause denaturation. The enzyme activity will drop drastically at a pH greater than 8.5 [6]. Energy reserves begin to run out and nutrients can lead to reduced enzyme activity decreased. Both isolates showed maximum yield at pH 7 phosphates, this shows that the pH is pH optimum for producing the agarase enzyme. These results do not differ much from the results of previous studies that the research carried out used the bacterium Bacillus subtilis and found that pH 8 is an optimum pH for growth [3] and agarase enzyme activity and research conducted by Kawaroe *et al*. [3] using bacterial isolates from *G. latifolium* shows the optimum results at pH 7 [3].

Characteristics of enzymes measured in this study were the effect of pH on the activity of the agarolitic enzyme with pH interval of 3-9. Yu et al**.** [7] mention that the pH is good for agarase enzyme activity at 3-8.5 [8] and Fu et al. [5] stated that the optimum pH at a range 6.5-7.5. Changes in pH can effect on enzyme activity if the conditions of pH is low, or high pH can also cause denaturation and will be clicking causes enzyme activity to decrease [8]. The enzyme activity will drop drastically at a pH greater than 8.5 [7]. Energy reserves begin to run out and nutrients can lead to reduced enzyme activity decreased. Both isolates showed that maximum yield at pH 7 phosphate. This indicates that this pH level is optimum for producing the agarase enzyme. These results do not differ much from the results of previous studies used the bacterium *Bacillus subtilis* [4] and found that pH 8 is an optimum pH for growth and agarase enzyme activity and research conducted by [6] using bacterial isolates from *G. latifolium* shows the optimum results at pH 7 [6].

3.3.2. Temperature

The enzyme activity will increase as the temperature rises until it reaches maximum activity and subsequently the enzyme activity decreases when the temperature is getting hotter due to denaturation of proteins [8]. The highest agarase enzyme activity generated by both isolate N1 and N3 obtained at 30^0C with a value of enzyme activity were 1.31×10^{-3} U/mL and 1.32×10^{-3} U/mL respectively (Figure 3).

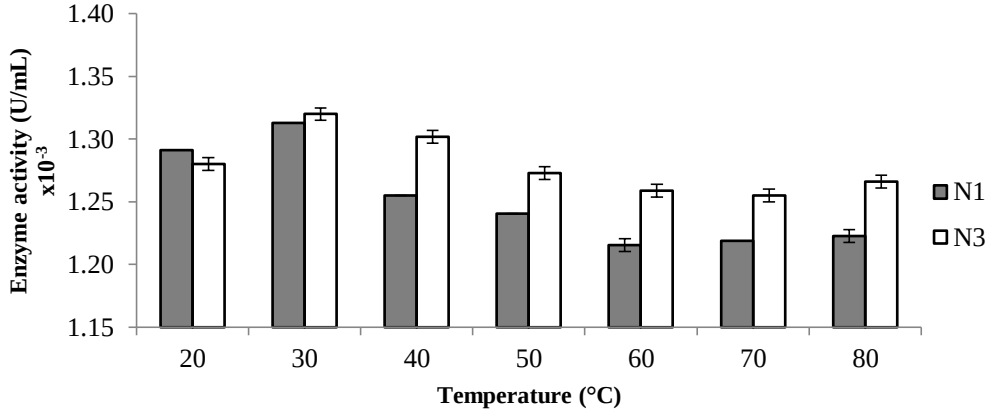


Figure 3. Temperature of Agarase Enzym Activity

The growth of bacterial cultures and production of enzymes optimum at temperature range of 20-50^{0}C in the basal medium [4]. The influence of higher temperatures can inhibit enzyme activity through a process of agarase enzyme protein when denaturation. Temperatures are getting warmer will decide the secondary bonding enzymes and may alter or eliminate the secondary and tertiary structure of the enzyme so that the enzyme activity will decline. The results showed that the optimum temperature suitable for enzyme production process was at a temperature of 30^{0}C. Research conducted by [6] with isolates from *G. latifolium* showed that the highest agarase activity occurred at 50^{0}C with enzyme activity value at 1.33 × 10^{-3} U/mL [8]. This suggests that the effect of temperature on the enzyme activity of agarase optimum at temperature range of 30-50^{0}C.

4. Conclusions

Agarase enzyme activity from bacterial that was isolated from *G. latifolium* is greater than isolated from *G. salicornia*. Characteristics of culture bacterial were indicated from the agarolitic index of isolates N1 (2.32 mm) and N3 (2.27mm) as well as from bacteria that isolated from *G. latifolium* that is BSUC2 at 4.18 mm and BSUC4 at 4.28 mm. Agarase enzyme activity from isolate N1 and N3 were optimum at pH 7 and a temperature of 30 ^{0}C, while the isolate BSUC4 optimum at pH 7 and a temperature of 50^{0}C. The whole of these characteristics indicated that there were bigt potential for bacterial culture of macroalgae *G. salicornia* and *G. latifolium* as raw material for bioethanol.

References

[1] Kawaroe M, Sunuddin, A, Hwangbo J and Shaumi A. 2015 Characteristics and selulotic activities of endophytic fungi in macroalgae (*Sargassum* sp., *Gracilaria* sp., *Gelidium* sp., and Caulerpa sp.) from seagrass habitat in Pari Island, Thousand Islands, Jakarta *Int. J. Sci: Basic Appl. Res*. **22** 149-160

[2] Wi G W, Kim H J, Mahadevan S A, Yang D J, Bae H J 2009 The potential value of the seaweed Ceylon moss (*Gelidium amansi*) as an alternative bioenergy resource *Bioresource Technology.* **100** 6658–6660

[3] Kawaroe M, Wulansari D, W. S, Hwangbo J and Santoso J. 2015 The optimum fermentation periode to produce bioethanol from Gelidium *latifolium* and *Gracilaria verrucosa J. Eng. and Tech.* **47** (6) 650-663

[4] Suzuki H, Sawai Y, Suzuki T, Kawai K 2003 Purification and characterization of an extracellular β-agarase from *Bacillus* sp. MK03 *J Biosci Bioeng*. **95** (4): 328-334

[5] Fu XT, Kim S M 2010 Agarase: review of major sources, catacories, purification method, enzyme characteristic and applications *Marine Drugs.* **8** 200-218

[6] Kawaroe M, Rusmana I and Nurafni 2014 Production of bioethanol from macroalgae gelidium sp. using agarase enzymes of marine bacteria *Int J. Environ. Bioenerg*. **9** 243-251.

[7] Yu F S, Hui C Y, Yen L 2009 The discovery of agarolytic bacterium with agarase gene containing plasmid, and sone enzymology characteristics *Int J Appl Sci Eng.* **7** (1) 25-41
[8] Fu W, Han B, Duan D, Liu W, and Wang C 2008 Purification and characterization of agaraes from marine bacterium *Vibrio* sp. F-6. *J Ind Microbiol Biotechnol.* **8** 915-922.