Production of Bioethanol from Macroalgae *Gelidium* sp. Using Agarase Enzymes of Marine Bacteria

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**Abstract:** Red macroalgae *Gelidium* sp. has been used for production of agar and then bioethanol since it has high carbohydrate content. This study focused on hydrolysis of agar of *Gelidium* sp. by agarases enzyme that produced from marine bacteria that was collected from different genera of bacteria which found in seawater, marine sediments, and another microorganisms. The optimal enzyme stability concentration for *Gelidium* sp. hydrolysis was achieved in terms of total reducing sugar at 664 ppm, 10% concentration. Results of protein molecule measured by sodium dodecyl sulphate polyacrylamide gel (SDS PAGE) method was 37 kDa, 41 kDa and 52 kDa respectively. Hydrolysis of *Gelidium* sp. produce 1.04% bioethanol and species of bacteria identified using molecular 16 sDNA was *Pseudomonas stutzeri*. Marine bacteria showed potential to hydrolyse red macroalgae for producing bioethanol.

**Keywords:** Red macroalgae, *Gelidium* sp., agarase enzyme, hydrolysis, ethanol

1. Introduction

The need for fossil fuels such as gasoline or diesel has increased while on the other hand its availability is inversely proportional to the needs due to the non-renewable characteristic of fossil energy. It is becoming more important in driving the development of renewable energy sources...
Various studies have been conducted on biodiesel production from microalgae (Mata et al., 2010) but there are few reports on production of bioethanol from macroalgae. Macroalgae, in fact, are rich in polysaccharides and many of these are commercially important as phycocolloids (Chapman and Chapman, 1980; Therkelsen, 1993; Van de Velde and De Ruiter, 2005). However, their saccharification and fermentation are less commonly studied. Thus, special efforts are required to understand these processes with a view to identify the potential of such macroalgae for bio-ethanol production.

*Gelidium* sp. is one of the red macroalgae contain high carbohydrates and agar. High carbohydrate content in *Gelidium* sp. can be used as feedstock for bioethanol. It is also supported by the results in Nahak et al. 2011 using *Enteromorpha* sp. which has carbohydrate content reaches 70-72%. Research in Japan showed that *Gelidium* sp. produce 44% agar, with 67.85 - 76.15% carbohydrate content and low lignin content (Wi et al., 2009).

Until now, the feedstock utilization for bioethanol is using more cellulose than agar. Nevertheless, agar also has some compounds that can be used as feedstock. Agarase is one of the enzymes classified into two categories, namely α-agarase and β-agarase. Agarase basic structure consists of units of β-D-galactose and 3,6 anhydro-α-L-galactose. α-agarase that hydrolyzes α-1,3 bond of agarose will produce series of oligosaccharides called agarobiose. β-agarase that hydrolyzes β-1,4 bond of agarose will produce series of oligosaccharides called neoagarobiose (Chi et al., 2012). This hydrolysis result is galactose that will be fermented into bioethanol with yeast help.

The process to produce bioethanol is conducted in two ways: hydrolysis and fermentation. Hydrolysis or saccharification is also known as breaking polysaccharides into monosaccharides (Kim et al., 2010). Hydrolysis is generally divided into two, acid and enzyme hydrolysis. Enzyme hydrolysis is environmental friendly bioprocess technology to breakdown feedstock into simple sugars by using enzymes. Fermentation is the process of breaking organic compounds (especially sugar and fat) by microorganisms under anaerobic conditions to produce simpler organic products. Some bacteria live on macroalgae is estimated to produce enzymes and break down agarase isolates source to be nutrition growth source. The enzymes produced by these bacteria can hydrolyze and degrade agar into agarobiose oligosaccharides and galactose from agarase enzyme, which later can be used as material for bioethanol.

The purpose of this study was to determine the stability of agarase enzyme hydrolysis, molecular weight of enzyme agarase, levels of bioethanol, and to identify enzyme-producing bacterial species by conducting 16sDNA agarase sequence.
2. Materials and Methods

This study was conducted in April 2013 - February 2014 at Surfactant and Bioenergy Research Center (SBRC) Laboratory, Plant Genetic Laboratory - Biology Department, Faculty of Mathematics and Natural Sciences, Microbiology Laboratory - Department of Biology, Microbiology and Biological Resources Research Center of Biochemistry and Biotechnology Laboratory (PPSHB), Bogor Agricultural University, Indonesia.

2.1. Rejuvenation of Bacteria Isolate

Bacteria used here is one of SBRC collection that has been isolated. Bacteria was breed with inoculating it into nutrient agar. The bacteria were incubated for 1 x 24 hours at 37°C (Munifah et al., 2011). Each grown and multi-time purified colony was moved into the inclined agar medium MA (Marine Agar) in test tube using needle loop and incubated at 37°C for 24 hours. The bacteria were then transferred into 50 ml liquid NB medium (nutrient broth) then incubated at room temperature with agitation of 100 rpm, the growth of bacterial culture was measured by optical density (Optical Density) using spectrophotometer at 660 nm wavelength with 30 minutes interval to enter the phase stationary. Bacteria that have the best activity was input for further test by determining enzyme activity such as producing agarase enzyme. And then the characteristic of bacterial enzymes include pH and temperature will be tested until saccharification or hydrolysis process of Gelidium sp. with agarase.

2.2. Enzyme Stability

Quantitative assay was performed using produced enzyme with temperature and pH characteristic. Seaweed washed with water to remove the salt content and remaining substrate, then was dried under the sun and used for saccharification or hydrolysis processes. Saccharification was conducted in test tube. 15 g dried Gelidium sp. was cut up finely with distilled water and then put into autoclave for 30 minutes at 121°C and 1 atm. Furthermore, the result from autoclave was cooled and added with enzyme in optimum buffer using concentration of 5%, 10%, 15% and 20%. The incubation was carried out at known optimum temperature. Time observations were conducted every 24 hours for 72 hours. Glucose content from hydrolisis with enzyme agarase was based on absorbance at λ 540 nm.

2.3. Determination of Molecular Weight

Acrylamide concentration used in this analysis was 10% (w/v). Staining used was silver. SDS-PAGE detection was conducted by releasing electrophoresis results gel from the print and measured with blue bromphenol migration distance. The gel was dyed and soaked in fixation solution (25%...
methanol + 12% acetic acid) for 1 hour with constant shaking. Then it was immersed in 50% (v/v) ethanol for 2x20 minutes. The solution was replaced with developer solution and washed with akuabiddestilata. After being washed, it was added with silver nitrate for 30 minutes and then washed again with akuabiddestilata for 2x20 seconds and added with Na2CO3 and formaldehyde and last, with fixation solution (Rosenberg, 1996).

2.4. Fermentation

The best concentration results of enzyme stability was fermented into bioethanol by preparing 90 mL of juice that previously hydrolyzed using 10% enzyme and incubated at the optimum temperature (50°C) for 72 hours (the result of enzyme stability). Furthermore, YMGP added with Saccharomyces cerevisiae inoculum (previously incubated at 30°C for 24 hours) was input. YMGP (Yeast Malt Glucose peptone) composition for each glucose were 5g/L, 5g/L, 5g/L and 40 g/L. Hydrolysate from hydrolysis was then added with 0.5% urea and 0.06% NPK from sugar source as nutrient. The fermentation process was carried out for 5 days (Sari, 2013). Fermentation result was then distilled and the ethanol was measured using density meter.

2.5. Identification of Bacterial Isolates

Molecular identification of bacterial isolates was carried by encoding genes 16S-DNA. DNA analysis was performed using one primer (63F) CAG GCC TAA CAC ATG CAA GTC and primer (1387R) GGG CGG CAA GGC GTA WGC. Bacterial isolation method was according to Qiagen DNA Mini Kit instructions. The results was then blasted and compared with NCBI data base (http://www.ncbi.nlm.nih.gov/BLAST/) to know the species name of bacterial isolates (Marchesi et al., 1998).

2.6. Analysis of Data

Analysis of the data was presented in graphs and tables and then discussed based on the results and related literature.

3. Results and Discussions

3.1. The Enzyme Stability

The stability of the enzyme was conducted to determine the enzyme time works optimally at hydrolysis time. The result showed an increase occurs slowly from hour-24 and up to 72 hours at 10% concentration (Figure 1). Hydrolysis in Gelidium sp. with 10% concentration of enzyme produced reducing sugar of 667 ppm (w/v), while the concentration of enzyme treatment 15% and 20% at hour-
72 was decreased. This indicates that enzyme worked optimally to hydrolyze *Gelidium* sp. substrate for 72 hours at 10% concentration of enzyme. The increase in enzyme concentration did not affect the increase of produced sugar.

![Figure 1. The enzyme stability of agarase BSUC4](image)

The use of different enzyme concentrations to determine the optimum enzyme hydrolysis showed differences in concentration of reducing sugar produced. Enzyme concentration of 10% at hour-72 showed optimum results to hydrolyze *Gelidium* sp. than the concentration of 5%, 15% and 20%. These results indicated that hydrolysis process is best at 10% concentration of enzyme (Figure 1). The use of higher enzyme concentrations did not always lead to an increase in reducing sugar produced due to enzyme and substrate have worked at optimal concentrations (Suhartono, 1989). Saraswathi et al. (2011) reported that the enzyme stability time length is from hours 24 to 72. In those hours, agar was already hydrolyzed optimally. This hydrolysis process will produce galactose sugar that will be fermented with the help of *Saccharomyces cerevisiae* yeast.

### 3.2. Molecular Weight

The molecular weight was determined using SDS Page and SDS Page analysis results can be seen in Figure 2. Determination of the molecular weight was calculated based standard curve

\[ y = -1.5311x + 2.2827 \]

where \( y \) is molecular weight markers (kDa), and \( x \) is mobility relative protein (cm). The resulting protein bands from BSUC4 isolates contained three bands, namely 37 kDa, 41 kDa and 52 kDa.
Protein bands generated from isolates BSUC4 were three bands; 37 kDa, 41 kDa and 52 kDa. Based on the results of proteins classification, molecular weight of agarase enzyme from isolates BSUC4 was ≤ 60 kDa. The results of previous study conducted by Saraswati et al. (2011) showed that low molecular weight of agarase enzyme ≤ 100 enzyme molecular weight of 20 kDa Bacillus subtilis, 33 kDa of Psedualteromonas sp. (Oh et al., 2010), 39.5 kDa from Alteromonas sp. (Wang et al., 2005), 42 kDa species of Vibrio sp (Sugano et al., 1994) and 60 kDa of Pseudomonas sp (Liao et al., 1997).

3.3. Fermentation

Fermentation result from Gelidium sp. using agarase enzyme BSUC4 produced 1.04% ethanol content (Figure 3). Fermented ethanol using enzyme has higher yield than using H2SO4 acid that has been done by Saputra et al. (2013) which was using 0.5% acid and produced 0.7% ethanol and Sari (2013) using 1% acid and produced 0.5% ethanol.

Galactose is the largest monosaccharide component from the results of hydrolysis Gelidium sp. Galactose is sugar derivative of agar which is the major polysaccharide in Gelidium sp. Saccharomyces

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*cerevisiae* are known to use galactose to produce ethanol (Goh and Lee, 2010). After galactose was converted to ethanol through glycolytic pathway (Moat et al., 2002), the ethanol produced from the fermentation of *Gelidium* sp. was 1.04% (Figure 3). This suggests that the use of agarase enzyme in hydrolyzing *Gelidium* sp. for bioethanol production more effectively than using acid.

### 3.4. Identification of Isolates

BSUC4 is SBRC-IPB collection isolated from *Caulerpa* sp. environment in Pari Island, Seribu Islands, Jakarta. BSUC4 bacterial isolates was included in mesophilic bacteria living at temperature of 37°C on agar media, has yellowish white color and lives in substrate. Based on DNA sequence analysis, isolates BSUC4 have degree of similarity or homology of 99% with e value of 0, 91% query cover of the total score in 1380 types of *Pseudomonas stutzeri* (Figure 4). *Pseudomonas stutzeri* classification was included in kingdom Bacteria, phylum Proteobacteria, Class Gamma Proteobacteria, Order Psedomonadales, Family Psedomonadaceae, Genus Pseudomonas, Species *Pseudomonas stutzeri* (Lalucat et al., 2006). *Pseudomonas stutzeri* was bacteria that can produce bio-surfactant and has helped a lot in hydrocarbon pollution process. Several previous studies have shown that bacteria can produce agarase enzymes such research of *Psedoalteromonas* sp. (Vera et al., 1998), *Agarivorans* sp. (Hu et al., 2008), *Psedoalteromonas* sp. (Oh et al., 2010), *Acinotobacter* sp. (Laksmikanth et al., 2009), *Vibrio* sp. (Sugano et al., 1994), *Bacillus subtilis* (Saraswathi et al., 2011), *Alteromonas agaryticus* (Potin et al., 1993), *Pseudomonas* sp. (Thulasidas et al., 2012). *Pseudomonas stutzeri* is the first report that can produce agarase enzyme compared with research of Thulasidas et al. (2012) by using genera *Pseudomonas* sp. with unknown species.

![Phylogenetic tree of isolates BSUC4](image)

**Figure 5.** Phylogenetic tree of isolates BSUC4

The results of DNA sequence analysis showed that isolates BSUC4 have degree of similarity or homology of 100% with *Pseudomonas stutzeri* (Figure 4). Classification *Pseudomonas* was included in Bacteria kingdom, phylum Proteobacteria, Class Gamma Proteobacteria, Order Psedomonadales, Family Psedomonadaceae, Genus *Pseudomonas*, Species *Pseudomonas stutzeri* (Lalucat et al., 2006).
4. Conclusions

The stability of each different enzyme concentrations to hydrolyze Gelidium sp. showed that 10% concentration is the highest compared to the other concentrations with molecular weight of acetone precipitation results resulted 3 bands such as 37 kDa, 41 kDa and 52 kDa. Levels of bioethanol produced from hydrolysis using the enzyme was 1.04% and the species identified was Pseudomonas stutzeri.

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