Potential Marine Fungi *Hypocreaceae* sp. as Agarase Enzyme to Hydrolyze Macroalgae *Gelidium latifolium*

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Abstrak

Potensi Jamur Hypocreaceae sp. sebagai Enzim Agarase untuk menghidrolisis Makroalga Gelidium latifolium

Agarase dapat mendegradasi agar ke oligosakarida dan memiliki banyak manfaat untuk makanan, kosmetik, dan lain-lain. Banyak spesies pendegradasi agar adalah organismelaut. Beberapa agarase telah diisolasi dari genera yang berbeda dari mikroorganisme yang ditemukan di air dan sedimen laut. Hypocreaceae sp. diisolasi dari air laut Pulau Pari, Kepulauan Seribu, Jakarta, Indonesia. Berdasarkan hasil identifikasi gen 16S rDNA dari 500 basis pasangan, isolat A10 memiliki 99% kesamaan dengan Hypocreaceae sp. Enzim agarase ekstraseluler dari Hypocreaceae sp. memiliki pH dan suhu optimum pada 8 TrisHCl (0,148 µ.mL^{-1}) dan 50°C (0,182 µ.mL^{-1}), masing-masing. Enzim Agarase dari Hypocreaceae sp. mencapai kondisi optimum pada aktivitas enzim tertinggi selama inkubasi dalam 24 jam (0,323 µ.mL^{-1}). SDS page mengungkapkan bahwa ada dua band dari protein yang dihasilkan oleh agarase dari Hypocreaceae sp. yang berada di berat molekul 39 kDa dan 44 kDa dan hidrolisis Gelidium latifolium diperoleh 0,88% etanol.

Kata kunci: *enzim agarase, Hypocreaceae sp., hidrolisis, fungi, rDNA.*

Abstract

Agarase can degradedagarto oligosaccharide and has a lot of benefits for food, cosmetics, and others. Many species of agar- degrader are marine-organism. Several agarases have been isolated from different genera of microorganisms found in seawater and marine sediments. Hypocreaceae sp. was isolated from sea water of Pari Islands, Seribu Islands, Jakarta, Indonesia. Based on the results of the 16S rDNA gene identification of 500 base pairs, A10 isolates had 99 % similarity toHypocreaceae sp. The extracellular agarase enzyme from Hypocreaceae sp. have optimum pH and temperature at 8 TrisHCl (0.148 µ.mL^{-1}) and 50 °C (0.182 µ.mL^{-1}), respectively. Agarase enzyme of Hypocreaceae sp. reach an optimum condition at the highest enzyme activity during incubation in 24 hours (0.323 µ.mL^{-1}). SDS Page revealed that there are two bands of protein produced by agarase of Hypocreaceae sp. which are at molecular weight of 39 kDa and 44 kDa and hydrolisis of Gelidium latifolium obtained 0,88% ethanol.

Key words*: agarase enzym, Hypocreaceae sp., hydrolysis, marine fungi, rDNA*

Introduction

Agarase is one of the enzymes that is classified in two categories namely α-agarase and β-agarase. So far, agarase have been isolated from several genera of microorganisms derived from sea water, sediment and marine environment (Fu and Kim, 2010). Agarase can be used in cosmetics and food industry. Another benefit of agrase is that it can hydrolyze agar into oligosaccharides. Agar is widely available in red seaweed (Rhodophyceae) including *Gelidium spp*. and *Gracilaria spp* (Kawaroe *et al.,* 2015). Agar compositions found are around 44% for *Gelidium spp.* and 53% for *Gracilaria spp.* (Nguyen *et al.,* 2012). In addition to the high content of agar, seaweed has a carbohydrate content ranges from 70-72% (Nahak *et al.,* 2011).

G. latifolium has benefit as agar, raw material of paper and can also used as a raw material of bioethanol degraded by acid (Kawaroe *et al.,* 2014). Use of *G.latifolium* as raw material for bioethanol

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has been mostly utilizing its content of starch, cellulose and hemicellulose, whereas biomass components such as agar which also have the potential to produce bioethanol has not been fully utilized. This is due to the hydrolysis process only uses cellulase enzymes that are only able to hydrolyze the cellulose fraction. Agar has potential of bioethanol raw material sincetheir components are composed by β-D-galactose and 3,6-anhydro-α-L-galactose (Fu and Kim, 2010). The success of conversion *Gelidium spp.* as bioethanol raw material is determined by several different processes, hydrolysis and fermentation (Nahak *et al.,* 2011). One of the hydrolysis processes is by using enzymes produced by micro-organisms.

Several studies have been conducted regarding agarase enzyme produced by marine microorganisms such as *Bacillus megaterium* (Khambhaty *et al.,* 2008), *Acinetobacter sp.* (Lakshmikanth *et al.,* 2006), *Pseudomonas sp.* (Gupta *et al.,* 2013) and *Alteromonas sp.* (Wang *et al.,* 2006). So far, bacteria are the only microorganisms studied and yet not researches about fungi as agarase producer. Gosh and Gosh (1992) mentioned that marine fungi has good enzyme activity in the degradation process of a compound. This study was the first research of agarase produced by a marine fungi*Hypocreaceae sp.* to hydrolyze *G. latifolium.*

Material and Methods

Fungi (A10) were isolated from Pari Island, Seribu Island, Jakarta, Indonesia and obtained from microbiology laboratory of Surfactant and Bioenergy Research Center, Bogor Agricultural University, Indonesia. Isolates rejuvenation was conducted by growing isolates fungi on Potato dextrose agar (PDA) medium. Furthermore, the molds were incubated at 28^{0}C for 7 days (Pervez *et al.,* 2012).

Optimum time to produce enzyme

The optimum time to produce enzym started with determining pouring timing of inoculum. Timing of inoculum was determined by culturing 2 isolates loops in 15 mL of liquid Potato dextrose (PDL) medium and incubated in 3-5 days, then poured into 135 mL of starter medium. The cultures were incubated at 50^{0}C in a stirrer with agitation speed of 100 rpm. Sampling was carried out for 7 days incubation with a 24-hour time span to measure the total amount of spores. Afterwards, isolates growth curve was developed to determine the best time pouring of inoculum in media production.

In order to determine the optimum time of enzyme activity, 15 mL of potato dextrose liquid medium containing cell cultures was inoculated into 135 mL of media production. Inoculum pouring time was observed in isolates exponential growth time (logarithmic growth phase) which has been known from fungi growth curve. Sampling was done every day in 4 days during incubation, then supernatant was tested its enzyme activity by using a modified Miller method based on maximum absorbance of reagent solution (Wood and Saddler, 1988).

Agarase activity is expressed in International Units μmL^{-1}. One unit is the amount of enzyme needed to break down to 1 µmol of cellulose into reducing sugar per minute in test conditions. Glucose levels resulting from agar hydrolysis with agarase enzyme value based on absorbance value at λ 550 nm.

Identification of Fungi

Identification of fungi isolates (A10) based onITS1-5.8S-ITS2 rDNA genes. DNA analysis was done by using primary internal transcribed spacer 1 (ITS1) 5'- CTT GGT CAT GTAA TTA GAG GAA-3 'and internal transcribed spacer 4 (ITS4) 5'- TCC TCC GCT TAT TGA TAT GC-3'. Isolation method is in accordance with the instructions of DNA Extraction Kit. The 16S rDNA gene sequence was compared with nucleotide sequences in the database by using the BLAST algorithma the NCBI site (Nursid *et al*., 2011).

Productionof agarase enzyme

Enzyme production was done based on highest production time on the curve that has been known previously. Media production of the enzyme was incubated in waterbath with a temperature of 50^{o}C and agitation speed of 100 rpm. The duration of incubation was determined based on the highest production time has been obtained previously. Production medium that contained enzyme was centrifuged at a speed of 2,500 rpm and a temperature of 4^{0}C for 30 minutes to separate enzyme solution or supernatant with pellets. The results of centrifugation supernatant were then stored at temperature of 10^{0}C as enzyme crude extract (Fu and Kim, 2010; Kawaroe *et al.,* 2014).

Acid and temperature characteristic

Acid characteristic was known through measuring optimum pH by adding as much as 0.2 mL enzyme which was reacted with 1.8 mL of substrate. The substrate was prepared by mixing 1.8 g of seaweed powder into buffer with various levels of pH 3-9, among others, 0.05 M acetate buffer (3, 4, 5), 0.05 M citrate phosphate buffer (5, 6, 7) and 0.05 M trisHCl buffer (7, 8, 9). Each enzyme was

incubated at 28°C for 30 minutes. Agarase enzyme activity was measured according to previous test procedures (Fu and Kim, 2010).

Optimum temperature was measured by reacting 0.2 mL enzyme with 1.8 mL substrate in which substrate was prepared by mixing 1.8 g of macroalgae *G. latifolium* powder into optimum pH buffer. Enzyme that has been mixed with substrate then incubated at temperature between 28°C to 90°C with 10°C range for 30 minutes of incubation time. Agarase enzyme activity was measured according to previous test procedures (Fu and Kim, 2010).

Enzyme stability test

A total of 15% (w/v) *G. latifolium* was dried and further added with distilled water in autoclave for 30 minutes at temperature of 121°C and a pressure of 1 atm. Furthermore, after it was cool, enzyme in the optimum buffer was added with a concentration of 5%, and incubation conducted at optimum temperature by measuring the time span in every 24 hours along 72 hours.

Determination of molecular weight (SDS page)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS *page*) of agarase was conducted in 12,5% gel as per method described by Sambrook and Russel (2001) along with standard molecular weight protein markers.

Fermentation process

Initial fermentation was done by preparing 90 mL hydrolysate and hydrolysis was performed by adding enzyme as much as 5% and then incubated at optimum temperature of this enzyme for 24 hours (taken from the enzyme stability yield). Furthermore, *Saccharomyces cerevisiae* inoculum was prepared in 10 mL YMGP (Yeast Malt Glucose Peptone) medium with the composition of 5 $g.L^{-1}$, 5 $g.L^{-1}$, 5 $g.L^{-1}$ and 40$g.L^{-1}$ then incubated at 30°C for 24 hours (Yanagisawa *et al*., 2011). Fermentation was done in anaerobic conditions at temperature of 50°C. Hydrolysate that has been hydrolyzed then added to *Saccharomyces cerevisiae*inoculum and added with 0.5% urea and 0.06% NPK from sugar as a source of nutrients (Setyaningsih *et al.,* 2012). Fermentation process was done in 5 days. Fermentation yield was distilled, then the yield of ethanol were measured using density meter (Anton Paar).

Results and Discussion

Fungi identification

Results of ITS1-5.8S-ITS2 rDNA gene identification of A10 was a species of *Hypocreaceae sp*. with the degree of homology of 99% (Figure 1.).

Fungi strain A10 that resulted from agarase screeningwith the highest activity was isolated from sea water of Pari Island, Seribu Islands, Jakarta. The isolates morphology is green color, massive growth on live media and has a conidia shape like a branching stem. DNA identification results showed that the isolates has similarity with the species *Hypocreaceae sp.* which a member of kingdom of fungi, division of Ascomycota, class of Sordariomycetes, order of Hypocreales, family of Hypocreaceae. This is a freely living species in soil environment.

Acid and temperature characteristics

Acid and temperature characteristics are parameters that can affect the activity of enzymes in hydrolysis process.Effects of pH and temperature in agarase enzyme activity can be seen in Figures 2 and 3. Figure 2 shows that agarase activity was gradually rising at pH 3-5 acetate, then decreased quite sharply at pH 5-7 phosphate and increased back sharply at pH 7-9 TrisHCl. Theoptimum agarase enzyme activity was found at pH 8.0 Tris HCl, which indicates that the agarase enzyme produced from *Hypocreaceae sp.* having activity at alkaline pH condition.

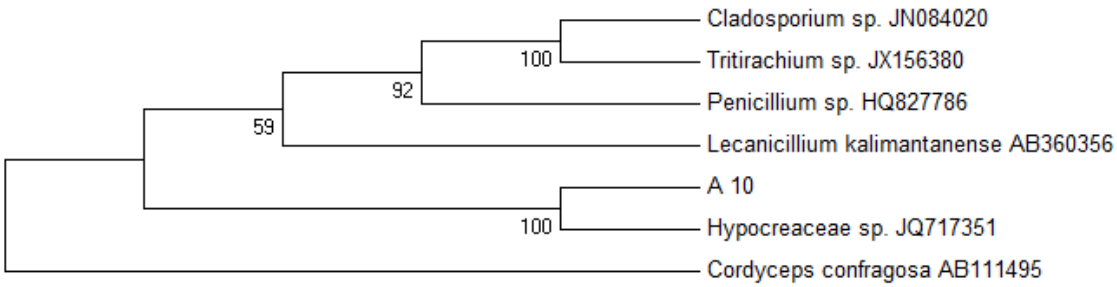

Figure 1. Phylogenic tree based on 16S rDNA sequence analysis showing the position of *Hypocreaceae* sp.and A10

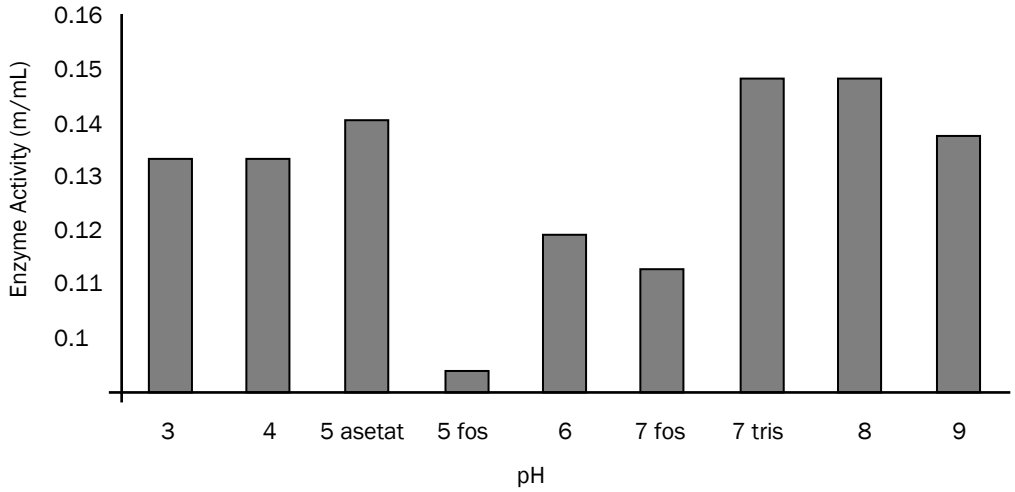

Figure 2. Effect of pH on agarase activity isolated from *Hypocreaceae* sp.

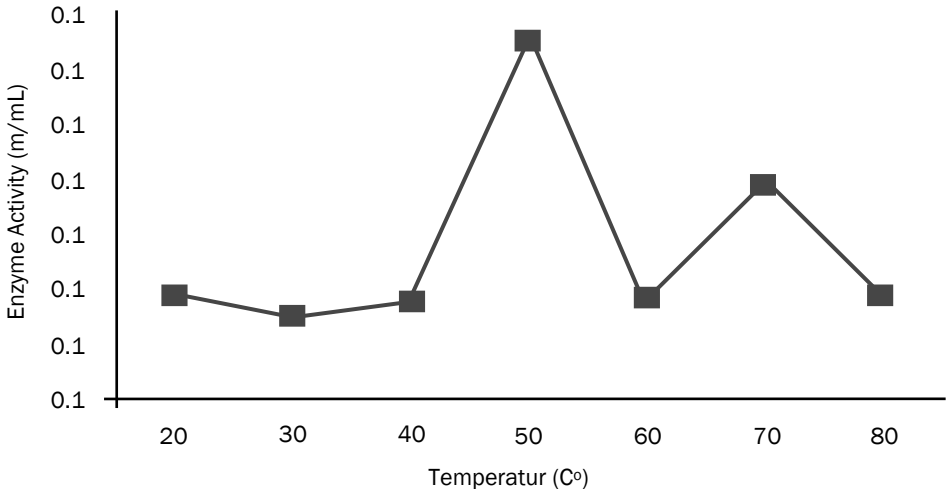

Figure 3. Effect of temperature on agarase activity isolated from *Hypocreaceae* sp

Some studies show that optimum agarase activity of microorganisms are in the alkaline pH, 7.0 for *B. cereus* (Suzuki *et al.,* 2002) and 9.0 for *Pseudomonas sp.* (Gupta *et al.,* 2013). Lakshmikanth *et al*. (2006) stated that the average agarase will have optimal activity at pH ranging from 6.5 to 9.0. Enzymes will show the maximum catalytic activity at a certain of pH range. Changes in pH will affect the amino and carboxyl groups of the enzyme protein. Extreme pH values can cause enzyme to be denatured that causes the enzyme loses its biological activity.

Effect of temperature on agarase activity can be seen in Figure 3. The figure shows that there is a slow decrease of anzyme activity at a temperature of 20-40^{o}C, then increase very sharp at 50^{o}C and decline at 60^{o}C. Optimum activity of agarase enzyme isolated from *Hypocreaceae sp.* occurs at temperature of 50^{o}C. So that maximum activity of agarase enzyme was obtained at a temperature of 50^{o}C. Jonnadula and Ghadi (2011) stated that the average marine microorganisms has agarase activity at the optimum temperature range of 30-50^{o}C at intervals of 5^{o}C. Optimum agarase enzyme activity produced by *B. cereus* (Suzuki *et al.,* 2002), *B. megaterium* (Khambhaty *et al.,* 2008), and *Acinetobacter sp.* (Lakshmikanth *et al.,* 2006) are all at a temperature between 30-50^{o}C. Temperatures can affect the enzyme activity since increased temperatures can accelerate a reaction. Decreased in enzymes activity that occurred above a temperature of 50^{o}C was due to interruption of the secondary bond enzyme because of the large kinetic energy of the molecules of the enzyme, resulting in the loss of the secondary and tertiary structure of

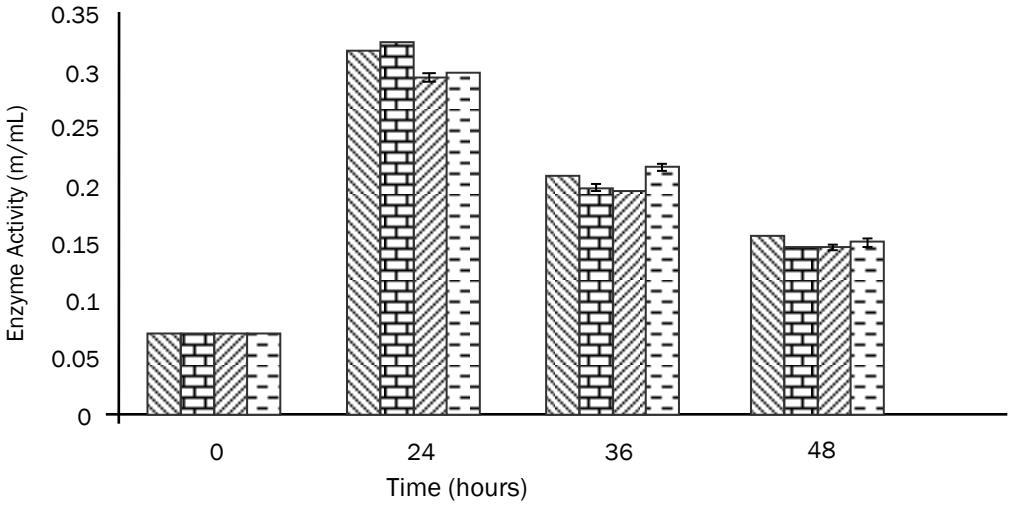

Figure 4. Stability of agarase activity isolated from *Hypocreaceae* sp.
Note : ▨ = 5 %, ▭ = 10 %, ▨ =15 %, ▭ = 20 %

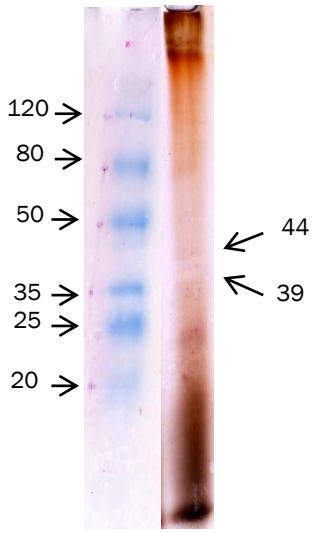

Figure 5. SDS page of agarase isolated from *Hypocreaceae* sp.

the enzyme. Heat can also cause the loss of most of a less strong bond on the structure of the enzyme protein. While the decline in enzyme activity at temperatures below 50^{o}C was due to the low affinity between enzyme with substrate so that the hydrolysis process was not running perfectly and enzyme activity was decreased.

Enzyme stability

The enzyme stability measurement was done to see how long the enzymes can work optimally.The stability of enzymes is highest at 24th hour and have a slow decline at 48th and 72nd hour. It was indicated that the enzyme will work optimally to hydrolyze a substrate at 24th hours (Figure 4.). The best concentration of enzyme that produced highest activity was 10%. Faster stability time of agarase enzyme work has been fulfilled by the substrate at 24th hour, so that the enzyme activity will decrease after 24th hour. Increasing the enzyme concentration does not affect activity of enzyme produced. This is due to very high concentration of enzyme would not be meaningful because all substrate molecules have been hydrolyzed by lower concentrations.

Molecular weight (SDS page)

Determination of molecular weights was conducted based on the SDS standard curve with the equation of y= -1,5311x + 2.2827, where y= log molecular weight marker (kDa), while x= relative

mobility of proteins (cm). Molecular weight was determined by taking the anti-log y value. Based on the results visualization on a gel it was known that there were two bands, 39 kDa and 44 kDa (Figure 5). Molecular weights obtained in this research are low. Several studies have shown that the agarase enzyme having such a low molecular weight of 39.5 kDa from *Alteromonas sp.* (Wang *et al.,* 2006), 33 kDa from *Pseudoalteromonas antarctica*, 32 kDa from *Pseudomonas atlantica* (Morrice *et al.,* 1983), and 20 kDa from *Vibrio sp.* (Aoki *et al.,* 1990). Based on molecular weight classification agarase enzyme it was known that agarase produced by *S. cucurbitacearum* is included in the classification 2 (≤50 kDa) (Jonnadula and Ghadi, 2011).

Ethanol fermentation

Fermentation of *G.latifolium* thatwas previously hydrolyzed using agarase enzyme showed that ethanol content are 0.88% compare to acid hydrolize (Figure 6.). Obtained ethanol was the result of distillation process, so it is not yet purify.The result of fermentation showed that ethanol content are 0.88% and it is higher than result of fermentation of *G. latifolium* by using acid (1% v/v) that produce ethanol only 0.50% (Kawaroe *et al.,* 2015). This indicates that the use of agarase enzymes to hydrolyze *G. latifolium.* in the bioethanol production process is more effective than acid.In hydrolysis process, agarase role is to break down the molecules so that the β-1,4 position gives a neo-agarobiose and converted into galactose. For β it istemporary, while for α will break at the α-1,3 positions resulting agarobiose and converted into galactopyranose (Khambhaty *et al.,* 2008; Fu and Kim 2010; Chi *et al.,* 2012). Results of the enzymatic hydrolysis then fermented by *S. cerevisiae* to produce bioethanol. Nguyen *et al*. (2012) stated

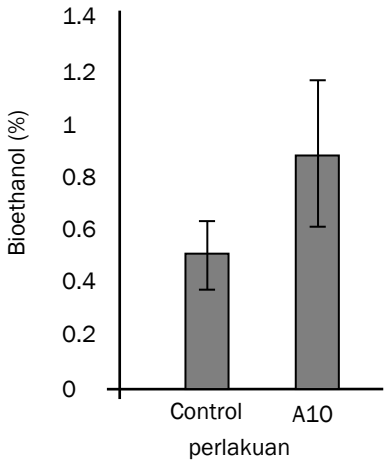

Figure 6. Concentration of Bioethanol

that bioethanol can be produced from the fermentation of all materials that contain sugar. The fermentation process is a biological process in which sugar molecules are converted into cellular energy and also produces ethanol as a byproduct with the help of yeast. Fermentation is basically breaking down one molecule of glucose into two molecules of pyruvate. Pyruvic acid molecules produced will be used by the yeast to produce energy. In anaerobic condition, pyruvic acid is converted to acetaldehyde and then to ethanol (Fardiaz, 1989). The fermentation process is basically influenced by medium, temperature, microorganisms, nutrients and pH substrate (Saroso, 1998).

Conclusion

Conclusion from this research is crude extract of agarase enzyme from *Hypocreaceae sp.* isolates has optimum activity at pH 8.0 (Tris-HCl), temperature of 50^{o}C, and enzyme stability at 24th hour. Adding agarase enzyme to fermentation process is capable to produce bioethanol from *G. latifolium* up to 0.88% and higher than the fermentation using acid 0.50%.

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References

Aoki, T., T. Araki & M. Kitamikado. 1990. Purification and characterization of a novel β-agarase from Vibrio sp. AP-2. Eur. J. Biochem. 187:461–465. doi:10.1111/j.1432-1033.1990.tb15326.x

Chi, W.J., Y.K. Chang & S.K. Hong. 2012. Agar degradation by microorganisms and agar-degrading enzymes. *Appl. Microbiol. Biotechnol*. 94:917–930. doi:10.1007/s00253-012-402 3-2

Fardiaz, S. 1989. Fisiologi Fermentasi.PAU Institut Pertanian Bogor, Bogor. [Indonesian]

Fu, X.T. & S.M. Kim. 2010. Agarase: Review of Major Sources, Categories, Purification Method, Enzyme Characteristics and Applications. Mar. Drugs 8:200-218.doi: 10.3390/md8010200

Gosh, B.K. & A. Gosh. 1992. Degradation of Cellulose by Fungal Cellullase. In G. Winkelmann (eds) Microbial Degradation of Natural Products. VCH Publishers-Inc, New York.

Gupta, V., N. Trivedi, M. Kumar, C.R.K. Reddy & B. Jha. 2013. Purification and characterization of exo-b-agarase from an endophytic marine bacterium and its catalytic potential in

bioconversion of red algal cell wall polysaccharides into galactans. *Biomass Bioenerg*. 49:290-298. doi:10.1016/j.biombioe.2012.12.027

Jonnadula, R. & S.C. Ghadi. 2011. Purification and Characterization of β-agarase from Seaweed Decomposing Bacterium Microbulbifer sp. Strain CMC-5. *Biotechnol. Bioprocess Eng.* 16:513-519. doi:10.1007/s12257-010-0399-y

Kawaroe, M,, K. Rusmana & Nurafni. 2014. Production of Bioethanol from Macroalgae Gelidium sp. using Agarase Enzymes of Marine Bacteria. *Int J. Environ. Bioenerg*. 9:243-251.

Kawaroe, M., A. Sunuddin, J. Hwangbo & A. Shaumi. 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.

Kawaroe. M., D.W. Sari, J. Hwangbo & J. Santoso. 2015. The optimum fermentation periode to produce bioethanol from *Gelidium latifolium* and *Gracilaria verrucosa. J. Eng. and Tech.* [In Review]

Khambhaty, Y., K. Mody & B. Jha. 2008. Purification, Characterization and Application of a Novel Extracellular Agarase from a Marine *Bacillus megaterium*. *Biotechnol. Bioprocess. Eng.* 13:584-591.doi:10.1007/s1225700800263

Lakshmikanth, M., S. Manohar, Y. Souche & J. Lalitha. 2006. Extracellular agarase LSL-1 producing neoagarobiose from a newly isolated agar-liquefying soil bacterium, *Acinetobacter* sp. AG LSL-1. *World J. Microb. Biot.* 22:1087-1094. doi:10.1007/s11274-006-9147-z

Morrice, L.M., M.W. McLean, F.B. Williamson & W.F. Long. 1983. Beta-agarases I and II from *Pseudomonas atlantica*: Purifications and some properties. *Eur. J. Biochem.* 135:553–558. doi:10.1111/j.1432-1033.1983.tb07688.x

Nahak, S., N. Gayatri, P. Itishree & R.K. Sahu. 2011. Bioethanol from Marine Algae: A Solution to Global Warming Problem. *J. Appl. Environ. Biol. Sci.* 1:74-80.

Nguyen, T.H.M. & V.H. Vu. 2012. Bioethanol production from marine algae biomass: prospect and troubles. *J. Vietnam Environ.* 3: 25-29. doi:10.13141/jve.vol3.no1.pp25-29

Nursid, M,. E. Chasanah, Murwantoko & S. Wahyuono. 2011. Penapisan kapang laut penghasil senyawa sitotoksik dari beberapa perairan di Indonesia. *J. Pascapanen dan Bioteknol. Kel. Perik*. 6:45-56.

Pervez, M.R., M. Musaddiq & P.V. Thakare. 2012. In-vitro antimicrobial studies of isolated *Myrotheciums*pp mrp001 against human pathogens. *Aust. J. Basic Appl. Sci.* 2:228-236.

Sambrook, J. & D.W. Russell. 2001. Commonly Used Techniques in Molecular Cloning Appendix 8. In Molecular Cloning Vol. 3 (3rd edition). Cold Spring Harbor Laboratory Press, New York.

Saroso, H. 1998. Pemanfaatan kulit pisang dengan cara fermentasi untuk pembuatan alkohol. Majalah Bistek 06: 20-28. [Indonesian].

Setyaningsih, D., S. Windarwati, I. Khayati, N. Muna & P. Hernowo P. 2012. Acid hydrolysis technique and yeast adaptation to increase red macroalgae bioethanol production. *Int. J. Environ. Bioenerg.* 3:98-110.

Suzuki, H., Y. Sawai, T. Suzuki & K. Kawai. 2002. Purification and characterization of an extracellular α-neoagarooligosaccharide hydro lase from *Bacillus* sp. MK03. *J. Biosci Bioeng* 93:456-463. doi:10.1016/S1389-1723(02)80092-5

Wang, J.X., H.J. Mou, X.L. Jiang & H. Guan. 2006. Characterization of a novel β-agarase from marine *Alteromonas* sp. SY37–12 and its degrading products. *Appl. Microbiol. Biotechnol*. 71:833–839. doi:10.1007/s00253-005-0207-3

Wood, T.M. & J.N. Saddler. 1988 Increasing the availability of cellulose in biomass materials. In Wood WA, Kellogg ST (eds) Methods in Enzymology. Academic Press, New York.

Yanagisawa, M., N. Kanami, A. Osamu & N. Kiyohiko. 2011. Production of high concentrations of bioethanol from seaweeds that contain easily hydrolyzable polysaccharides. *Process Biochem.* 46:2111-2116. doi:10.1016/j. procbio.2011.08.001