Cellulase activity of bacteria isolated from water of mangrove ecosystem in Aceh Province

Irma Dewiyanti[1,2], Darmawi Darmawi[3,*], Zainal Abidin Muchlisin[4], Teuku Zahrial Helmi[5], Iko Imelda Arisa[4], Cut Nanda Defira[6], Fitriyani[4], Sawva Yura[4]

[1]Graduate School of Mathematics and Applied Science, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[2]Marine Science Department, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[3]Laboratory of Microbiology, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[4]Aquaculture Department, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[5]Laboratory of Biochemical, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia
[6]Biology Department, Faculty of Mathematic and Natural Science, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia

ABSTRACT

Cellulolytic bacteria that produce cellulase enzymes play an essential role in degrading cellulose in their habitat. The presence of cellulolytic bacteria strongly supports the fertility and productivity in mangrove waters. The objectives of the study are to analyze the activity of cellulase enzyme qualitatively through the cellulolytic index and quantitatively through the activity and specific activity of the cellulase enzyme from bacteria isolated from the water of mangrove ecosystems in Aceh Province. The qualitative experiment of enzyme activity was carried out at the Microbiology laboratory SKIPM Aceh, and a quantitative experiment of enzyme activity was conducted at the Microbiology Laboratory, Biology Department, IPB. Isolation of cellulolytic bacteria isolated from mangrove water used Carboxy Methyl Cellulose (1% CMC) selective media and carried out by spread plate method. The ability of bacteria to produce cellulase was tested qualitatively using the spot technique, this test was carried out using 1% Congo Red. Furthermore, the quantitative testing of cellulase enzymes activity adopted the DNS spectrophotometric method. The specific activity of the cellulase enzyme can be determined by using the Lowry method. There were 21 isolates that had a clear zone and had the ability to produce cellulase enzymes from 49 isolates that were successfully purified. The highest cellulolytic index (CI) produced using BAM421 isolate with the value of 5.50 was included in the high category, followed by BAM326 and BAM132 isolates, with values of 1.55 and 1.05 were categorized into the medium category. The other isolates were in the low cellulolytic index category. The isolate with the highest CI value was further tested using the quantitative enzyme activity test. The highest cellulase enzyme activity of BAM421 occurred at 24hr (0.0029 U/ml). The highest specific cellulase activity of BAM421 was at 24hr with the value of 0.210 U/mg. The result concluded that the qualitative test showed CI values can be categorized into low, medium, and high. Moreover, the value of the quantitative assay described that the cellulase enzyme and the specific enzyme activities of the bacteria were low in the study area.

Introduction

The mangrove ecosystem is one of the tropical marine ecosystems that has high primary and secondary productivity. Mangrove ecosystems are a source of various microbial that can produce important enzymes applied in agriculture, fisheries, animal husbandry, and industry (Dias et al., 2011; Dourado et al., 2012; Safika et al., 2018). Litter produced by mangrove vegetation is the main source of organic matter available as a food source for various kinds of aquatic organisms (Bouillon et al., 2008; Mchenga and Abdalla, 2017; Srisunont et al., 2017). Cellulose content is found in the litter that falls into the water and as a carbon source that can be utilized by cellulolytic microorganisms for their growth by producing cellulase enzymes. The

degradation of cellulose material undergoes various complex processes and requires the role of cellulase enzymes from microbes (Lynd *et al.*, 2002; Gupta *et al.*, 2011; Irfan *et al.*, 2012). Cellulase enzymes are produced by cellulolytic bacteria to degrade cellulose contained in their growth media (Acharya *et al.*, 2008). Cellulolytic bacteria can be obtained from anywhere, especially from sources containing cellulose such as plants (Menendez *et al.*, 2015).

Extracellular enzymes produced by microbes are used to break down the complex organic nutrient into simple component that can be transported into cells as a source of nutrients (Subagiyo *et al.*, 2017). Cellulose as an abundant source of glucose requires accelerating the utilization of cellulolytic bacterial decomposition (Kurniawan *et al.*, 2019). Generally, cellulase is the mixture of three enzymes, i.e., exoglucanases, endoglucanases, and β-glucanases (Cheng *et al.*, 2012). Furthermore, Andriani *et al.* (2012) reported that cellulolytic bacteria that secrete cellulase enzymes are useful as crude degradations of animal feed raw materials. Enzymes derived from microbes are relatively more stable and have more diverse properties than enzymes derived from plants and animals (Mohapatra *et al.*, 2003). In the field of fisheries, degradation of cellulose in fish feed raw materials can increase fish growth due to optimal feed digestibility (Kurniawan *et al.*, 2018). Microbial populations and their activities can be influenced not only by physical and chemical characteristics of the soil (Oller Costa *et al.*, 2012), but also by the oceanographic conditions of the waters (Pupin and Nahas, 2013).

The cellulose degradation test was carried out to qualitatively determine the presence or absence of cellulolytic activity and how much cellulose degradation power could be produced from each isolate. The isolates that had cellulase activity were indicated by the formation of a clear zone around the edges of the bacterial isolate colonies, while the amount of cellulose degradation produced was calculated using the cellulolytic index value. Quantification of microbial populations as phosphate solvents (Ghosh *et al.*, 2012), amylase producers (Kumar *et al.*, 2012), and cellulase enzyme producer (Meryandini *et al.*, 2009) can provide important information about the decomposition process of organic matter and mineralization of organic compounds to increase the fertility and productivity of mangrove ecosystem. Chantarasiri (2021) reported 3 genera of cellulolytic bacteria isolated from sedimentary water in the littoral zone, which are *Aeromonas*, *Bacillus*, and *Exiguobacterium*. Species of *Bacillus* sp. H1666 was cellulolytic bacteria

isolated from seawater sample recorded by Harshvardhan *et al.*, 2013. Several studies on cellulolytic bacteria associated with mangrove habitats have been carried out (Behera *et al.*, 2016; Naresh *et al.*, 2019; Rudiansyah *et al.*, 2017; Subagiyo *et al.*, 2017). However, there is no information about the presence and activity of cellulolytic bacteria in mangrove ecosystems, Aceh Province. Therefore, the objectives of the present study are to analyse the activity of enzyme cellulase qualitatively through the cellulolytic index and quantitatively through the activity and specific activity of the cellulase enzyme isolated from mangrove water in northern coast of Aceh Province.

Materials and Methods
Location and time of research

The present research was conducted in November 2020 to January 2021, then it was continued from April to October 2021. The research location was determined based on purposive sampling method. There are four research stations categorized based on the tidal action in the northern coast of Aceh Besar (Figure 1). Station 2 and 4 were always inundated not only in high tide but also in low tide, however station 1 and 3 were inundated only at high tide and exposed to the air at lowest tide. Each sample point was repeated three times. Analysis of research samples were carried out at the microbiology laboratory of Fish Quarantine Station, Quality Control and Safety of Fishery Products, Aceh (SKIPM), and the microbiology laboratory of Biology Department, IPB University.
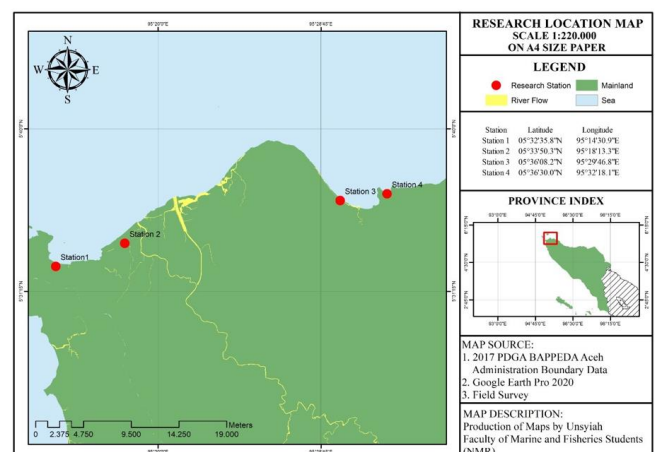

Figure 1. Red dots show the research location.

Materials

The materials used in this research were pure bacteria isolated from the waters of mangrove ecosystem, pure isolates of BAM421, *carboxy methyl cellulose* (CMC) agar media, $MgSO_4 .7H_2O$, K_2HPO_4,

CaCl$_2$, FeSO$_4$, KNO$_3$, yeast extract, glucose, agar, 1% Congo red, NaCl 1M, distilled water, and 70% alcohol, pipette tips, autoclave, LAF, and incubator. The tools used during the research were made of glass, especially water sample containers, petri disks, test tubes, and Erlenmeyer flasks were washed clean and then air dried. The equipment and containers were sterilized using an autoclave at 121^{0}C for 15 minutes.

Qualitative assay of cellulase activity

Sample testing was carried out on pure isolates obtained from water samples in mangrove ecosystems that have clear zones. Firstly, water samples were observed at the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences (FMIPA, USK) until bacterial characterization, and the observation was continued at the Microbiology Laboratory, Fish Quarantine Station, Quality Control and Safety of Fishery Products (SKIPM) Aceh to observe the formation of clear zones. The ability of bacteria to produce cellulase enzymes was tested qualitatively where isolates from agar stock was spotted on a petri dish containing CMC media. Incubation was carried out at 70^{0}C for 48 hours. Cellulolytic activity testing was carried out using 1% Congo Red. The isolate media was flooded by 1% Congo Red and left for 15 minutes to detect the clear zone, then washed with 1M NaCl. Bacteria isolates that were able to decompose CMC were indicated by the formation of a clear zone around the colony after being tested using the Congo red. The diameter of the formed clear zone was measured using a calliper. Cellulolytic index (CI) is required for each culture isolate obtained from water samples of mangrove ecosystems (Bradner *et al.*,1999). Cellulolytic index is the ratio between the clear zone diameter minus the colony diameter divided by the colony diameter. Qualitatively, the greater the value of the cellulolytic index, the greater the cellulase enzyme produced by bacteria. The grade of cellulase enzyme activities were classified based on the cellulolytic index value with a low category if the CI $\leq$ 1, medium if the CI value = 1 to 2, and high if CI $\geq$ 2 (Choi *et al.*, 2005). The cellulolytic index for each isolate culture obtained from water samples of the mangrove ecosystem was calculated using the following equation (Bradner *et al.*, 1999):

$$\text{Cellulolytic index} = \frac{\text{clear zone diameter (cm)-colony diameter (cm)}}{\text{colony diameter (cm)}}$$

Quantitative assay of cellulase activity

Cellulase enzyme activity was tested qualitatively using crude extract based on glucose level. Glucose level was measured using the DNS method (Miller, 1959) with soluble cellulose and starch as the substrates. Enzyme production was started by inoculating one loop of rejuvenated bacterial colonies on CMC into 100 ml of 1% liquid CMC media and they were incubated in a shaking incubator at 120 rpm for 24-30 hours. Enzyme harvesting was carried out every 6 hours, specifically at 12, 18, 24, 30, 36, 42, 48, and 54 hours by inoculating 3 ml of bacterial culture into sterile Eppendorf tube, the supernatant contained crude extract enzyme was obtained by centrifuging the bacterial culture at the speed of 10.000 rpm for 10 minutes. The supernatant formed was separated from the pellet, and it was used as crude extract enzyme (EEK) to observe the enzyme activity using DNS reagent. Furthermore, 2 ml of DNS reagent was added and vortexed, then heated at 100 ^{o}C for 10 minutes and followed with a cooling process. The absorbance values of the sample, control and blanks were measured at a wavelength of 540 nm using a spectrophotometer. Measurement of enzyme activity was carried out until 54 hours. The activity of cellulase enzymes can be calculated with the following equation.

$$\text{Cellulase activity (U/ml)} = \frac{\text{Xsample - Xcontrol x Diluent Factor x 1000}}{\text{Weight of glucose x incubation time}}$$

where: X sample is glucose concentration of sample (mg/mL); X control is glucose concentration of control (mg/mL); weight of glucose is 180.156 g/mol

The specific activity of the cellulase was calculated by comparing the enzyme activity to the total protein concentration as determined using the Lowry method (Lowry *et al.*, 1951) at a wavelength of 650 nm. The standard curve of protein was prepared using various Bovine Serum Albumin (BSA) concentrations. The specific activity was calculated using the following equation.

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme Activity (U/mL)}}{\text{Total protein (mg/ mL)}}$$

Results

Qualitative test of cellulase enzyme activity

Qualitative enzyme activity test was used to determine the bacteria that produce cellulase enzymes by showing the clear zone produced by each isolate. Cellulolytic activity was indicated by the ability of bacteria to hydrolyse *Carboxy Methyl Cellulose* (CMC) substrates, and it makes the area around the bacteria looking more contrasted and the formation of a clear zone (Yosmar *et al.*, 2013). Yoo *et al.* (2004)

which stated that colonies that were positive for extracellular cellulase production would be surrounded by a clear zone with a red background in areas not degraded by cellulase. According to Zhang *et al.* (2009) giving 1% Congo red solution in the cellulose degradation test to detect zones that can be degraded by the cellulase enzyme, while the 1M NaCl solution helps to make the clear zone that formed to appear clearer (Figure 2). Based on the results of qualitative cellulase testing, there were 21 isolates that had clear zones from 49 isolates that had been purified. The highest number of isolates producing cellulase enzymes were found at station 3 with as many as 7 isolates, followed by station 1, and station 2, where the numbers of isolates found were 6 and 4 respectively.
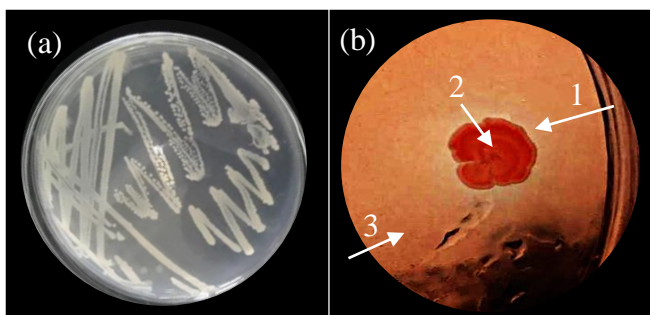

Figure 2. (a) Cellulolytic bacteria of BAM421 isolate, (b) cellulase enzymes produced by cellulolytic bacteria. Clear zone (1), Bacterial Colony (2), and CMC media was flooded with 1% Congo red (3).

Table 1. Colony diameters, clear zone diameters, and cellulolytic index values of bacteria isolates from mangrove water samples.

No.	Isolate code	Colony diameter (cm)	Clear zone diameter (cm)	Cellulolytic index (CI)
1	BAM111	0.27 ± 0.03	0.39 ± 0.06	0.43
2	BAM112	0.46 ± 0.05	0.59 ± 0.04	0.30
3	BAM123	0.20 ± 0.04	0.38 ± 0.06	0.88
4	BAM131	0.53 ± 0.02	0.73 ± 0.08	0.38
5	BAM132	0.24 ± 0.02	0.48 ± 0.22	1.05
6	BAM134	0.29 ± 0.04	0.37 ± 0.05	0.28
7	BAM211	0.54 ± 0.01	0.62 ± 0.03	0.15
8	BAM231	0.41 ± 0.03	0.51 ± 0.15	0.23
9	BAM233	0.17 ± 0.02	0.26 ± 0.07	0.49
10	BAM234	0.72 ± 0.04	0.80 ± 0.00	0.11
11	BAM311	0.34 ± 0.03	0.63 ± 0.14	0.88
12	BAM325	1.39 ± 0.02	1.53 ± 0.02	0.10
13	BAM326	0.10 ± 0.03	0.26 ± 0.05	1.55
14	BAM333	0.87 ± 0.13	1.00 ± 0.12	0.14
15	BAM334	0.45 ± 0.03	0.57 ± 0.00	0.27
16	BAM335	0.56 ± 0.06	0.78 ± 0.56	0.38
17	BAM336	0.24 ± 0.04	0.35 ± 0.01	0.47
18	BAM411	1.07 ± 0.28	1.28 ± 0.21	0.20
19	BAM412	0.94 ± 0.59	1.16 ± 0.47	0.24
20	BAM413	0.29 ± 0.04	0.47 ± 0.01	0.60
21	BAM421	0.10 ± 0.06	0.65 ± 0.28	5.50

Table 1 shows the cellulolytic index (CI) obtained from each isolate that produces cellulase enzymes.

The cellulolytic indices obtained was varied and ranges from 0.10 to 5.50 cm. The highest CI value was detected from isolate BAM421 (5.50) and followed by isolate BAM326 (1.55). Those isolates were isolated from non-rehabilitated mangrove sites. Stations 1 and 2 were in rehabilitated mangrove, while stations 3 and 4 were in non-rehabilitated mangrove. Both have different number of vegetation, mangrove species, abundance, and physical-chemical water parameters. The lowest CI value was BAM234 which was isolated from station 2, which was a rehabilitated mangrove site.

Quantitative Testing of Cellulase Enzyme Activity

Quantitative testing was done on measurements of enzyme activity from BAM421 isolates which had the highest cellulolytic index for 2.5 days of incubation (54 hours) to obtain the optimal time for producing cellulase enzymes. Measurements were carried out 9 times, namely at 6th hour, 12nd hour, 18th hour, 24th hour, 30th hour, 36th hour, 42nd hour, 48th hour, and 54th hour. Figure 3 shows that the highest activity (0.0029 U/ml) was measured at the 24th hour, it then decreased until the 30th hour observation and increased again at the 42nd hour with a lower value than before (0.0003 U/ml). This indicates that there are differences in the ability of the isolates to break down the substrate temporally. The curve indicates that cellulase enzyme production was started at the 6th hour, when the value was 0.0008 U/ml, then the enzyme produced decreases at the 48th hour and rises again. Enzyme activity levels go up and down due to the different cell growth phases.
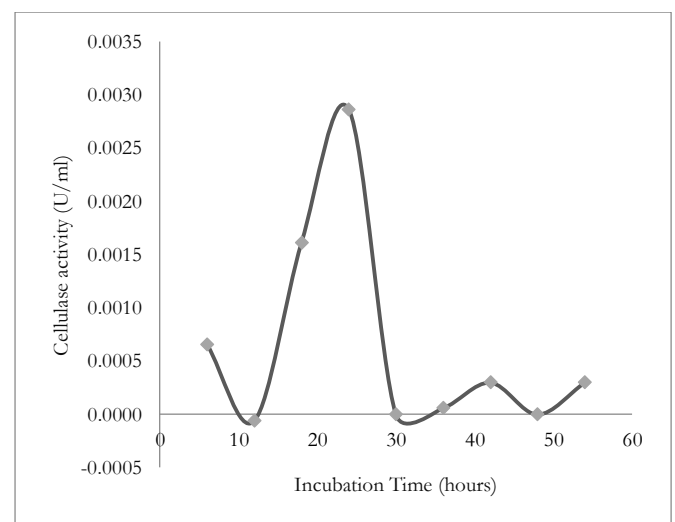

Figure 3. Cellulase activity of Isolate BAM 421 isolated from soil mangrove

The measurement of cellulase activity and specific cellulase activity is not only influenced by reduction in sugar concentration but also but also by dissolved

protein. Dissolved protein content was measured using the Bradford method. Dissolved protein test started with BSA standard curve, the absorbance of BSA obtained have value of equation $y = 3.2x + 0.021$ with a value of $R^2 = 0.982$. The protein concentration ranged from 0.009 to 0.014 mg/ml (Table 2). The result indicated that the enzyme sample does not only contain cellulase protein but also contains other proteins because the samples was still in a crude extract enzyme that has not been purified (Murtiyaningsi and Hazmi, 2017). The higher protein concentration, the higher specific cellulose activity to be obtained.

Table 2. Protein concentration of BAM421 isolate in different incubation time.

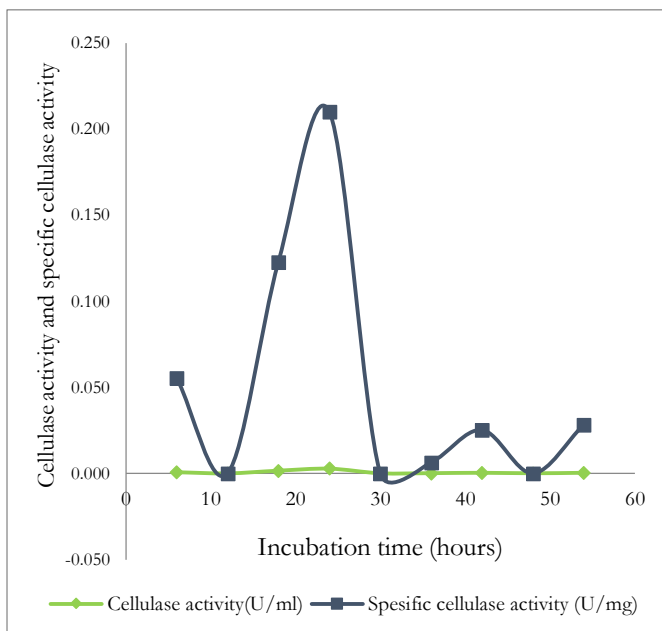| No.	Incubation time (hours)	Protein concentration (mg/ml)	Specific cellulase activity (U/mg)
1	6	0.012	0.055
2	12	0.011	0.000
3	18	0.013	0.123
4	24	0.014	0.210
5	30	0.010	0.000
6	36	0.009	0.006
7	42	0.012	0.025
8	48	0.012	0.000
9	54	0.011	0.028

Figure 4. Specific enzyme cellulase activity of BAM421 isolated from water

Figure 4 shows the specific cellulase activity while including protein concentration. Almost similarly with enzyme activity in Figure 2, the highest specific enzyme cellulase was recorded at the 24th hour as 0.210 U/mg and decreased sharply until the 30th h of

observation, while there is no specific cellulase in this observation time. The specific activity of the cellulase enzyme continued with slight increase starting at the 36th hour until the 42nd hour (0.025 U/mg), and from the 48th hour to the 54th hour (0.028 U/mg). Both the values of cellulase and specific cellulase activities fluctuated during the observation time.

Discussion

The activity of cellulase enzyme can be obtained by qualitative and quantitative measurements. There were 12 isolates that showed clear zones and they had the ability to produce cellulase enzymes from 49 samples isolated from the waters of mangrove ecosystem. Test of cellulase activity was obtained qualitatively by observing clear zones formed in CMC media after it was flooded by 1% Congo red. Clear zone formation around the bacterial colony is caused by the cellulose degradation process. Congo red cannot strap to the CMC media without β-1,4glycosidic bonds contained in cellulose because the presence of cellulase enzymes can bind hydrolysed cellulose polymers (Jo et al., 2011). Furthermore, Anand et al. (2009) mentions that Congo red will bind specifically to polysaccharides that have β-1,4 glycoside bonds, in this research the polysaccharides are contained in the CMC agar medium. While the red colour shows the remaining cellulose that is not hydrolysed, resulting in the formation of Congo Red cellulose. The clear zone formed can be seen clearly through washing using NaCl 1M.

The diameter of clear zone and the colony diameter are needed to calculate the Cellulolytic Index (CI). The CI values ranged from 0.11 to 5.50. The highest IC value was recorded at station 4 from BAM421 isolate (5.50) followed by BAM326, and BAM132 with the value 1.55 and 1.05, respectively. The CI value of BAM421 was categorized as high, whereas both BAM326 and BAM132 categorized as medium. Furthermore, the other isolates were categorized into low cellulolytic index. According to Choi et al. (2005), if the CI ≤ 1 is low categorized, CI from 1 to 2 is medium, and high if CI ≥ 2. Khalila et al. (2020) reported CI from bacterial cellulolytic isolated from Ie Seuum, Aceh Besar ranged from 0.50 to 2.20. Furthermore, Anuar et al. (2014) found there were 14 cellulolytic bacteria isolated from Dumai waters and the highest cellulase enzyme activity was 931-4A isolate with the value of 1.186 categorized as medium of CI.

The difference in cellulolytic index values may be caused by different types of isolates that can produce cellulase enzymes. According to Pitri et al. (2005), the

difference in cellulolytic index values was due to each isolate having different abilities in hydrolysing CMC media so that it affected the size of the clear zone formed. The ability of bacteria to degrade cellulose varies depending on the type of bacterial strain (Goenadi et al., 1993). Furthermore, according to Sari et al. (2014), protein synthesis will be influenced by DNA which has a certain base sequence, therefore some cellulase enzyme proteins produced from by the same genus can have different cellulase enzyme activities, and this would also be likely for bacteria of different genera.

The diameter of the clear zone is generally larger than the diameter of the colony, because the cellulase enzyme is secreted into the surrounding environment by cellulose-degrading bacteria. Bacteria can't enter cellulose molecules because the size of cellulose is larger than the size of bacterial cells (Zverlova et al., 2003). Meryandini et al. (2009) stated the size of the resulting clear zone indicates the potential of cellulolytic bacteria in the cellulose decomposition process. Potential cellulolytic bacteria were obtained with indications of forming the widest clear zone and the resulting brightness. Moreover, isolates that produced a clear zone diameter twice than the colony diameter were potential enzyme producers. Thus, the presence of bacterial cellulolytic activity can be characterized qualitatively by the formation of a clear zone around the bacterial colonies that grow on CMC selective media (Ochoa–Solano and Olmos–Soto, 2006). The higher the cellulase index of isolate, the greater the cellulolytic activity produced (Apun et al., 2000).

Of all isolates that have been qualitatively tested for their activity, not all isolates were tested quantitatively. The selection of the quantitatively tested isolates was based on the size of the clear zone formed. Only the isolate with the highest cellulolytic will be measured for enzyme activity. Measurement of cellulase enzyme activity of BAM421 in 54 hours incubation time was carried out by measuring the absorbance of reducing sugars. It aims to find out on at what hours does the isolate has its maximum cellulase activity. The highest cellulase activity was recorded at 24 hours with a value of 0.0029 U/ml and reducing sugar was 0.0077 mg/ml. Cellulase activity is proportional to the level of reducing sugar produced, where the higher the enzyme activity, the higher the reducing sugar produced. Iqbalsyah et al. (2019) reported the bacteria cellulase activity from Geobacillus isolate, which was isolated from a geothermal area in Jaboi, Weh Island was low at 14 hours (0.29 U/g) but increased significantly between 38 hour and 48 hour (0.57 U/g to 1.62 U/g) and the

highest cellulase activity was at 72 hour (1.74 U/g). While a study done by Sonia et al. (2015) recorded the highest cellulase activity at 24 hour (0.08 U/ml). The cellulase enzyme activity in the present study was lower compared to previous studies mentioned previously, but similarly the time of highest activity was recorded at 24 hours. The highest specific activity was recorded at 24 hours with the value of 0.210 U/ml, and the protein content was also highest at the same time (0.014 mg/m). Iqbalsyah et al. (2019) found that the highest specific cellulase activity produced at 48 hours with the value of 0.44 U/mg protein.

When the activity increases, it is possible that the cell will also experience an exponential growth phase, while when there is a decrease in activity, the cell has reached a stationary phase which is then followed by a death phase which causes a decrease in the activation of the enzymes produced. The cause of cellulase activity decrease is assumed to be the limitation of nutrients content in medium. Behera and Ray (2016) mentioned cellulase molecules are difficult to hydrolyse because they are clogged by lignin structurally. In addition, cells basically prefer simple substrates, when glucose runs out, cellulose will be used as a carbon source so that this breakdown causes glucose to accumulate and inhibit the active nature of the cellulase enzyme (*feedback inhibition*), and in addition, cellulase synthesis repression occurs because cells use glucose as a carbon source which influences the cell not producing cellulolytic enzymes (Abalos et al., 1997). It is important to note that during the stationary phase, the cell also produced protease enzymes that could damage the activity of the cellulase enzyme (Martina et al., 2002)

Figure 3 and 4 showed that in the early stage of incubation, cellulase was already increasing sharply from the 12th hour to the 24th hour. At the 0 to 12th hour was the lag phase of cellulase activity where the bacteria are still adapting to the new environment. Bacterial growth begins to have a significant change at 24 hours and this stage is called the log phase where bacteria begin to replicate DNA and then cells begin to grow, bacterial growth takes place rapidly up to twice the original number (Thiel, 1999). Furthermore, the increase of enzyme production was associated with the increase of cell growth indicated that cellulose was actively used by cellulolytic bacteria during the growth phase (Seo et al., 2013). There are some incubation times that showed no cellulase activity, this is probably because the level of enzyme purity. The enzyme used is the crude extract enzymes, therefore the enzyme still possibly contains

other components or other proteins that are inhibitors that can interfere with the function of the enzyme.

Conclusion

There were 21 of 49 bacterial isolates that had cellulolytic index values by producing a clear zone around the bacterial colonies isolated from mangrove water. The larger the clear zone, the higher the ability of bacteria to produce cellulase enzymes. The cellulolytic index is the result of qualitative testing of cellulase enzyme activity. BAM421 isolate had the highest cellulolytic index value, and it was categorized as having high cellulase activity. This isolate was further analysed using quantitative. The cellulase enzyme activity quantitative test showed that the isolate of BAM 421 produced the highest enzyme activity at the 24th hour of incubation time, as well as the specific enzyme activity. Isolates of cellulolytic bacteria isolated from mangrove water in both sites had low, medium and high ability to produce cellulase. Both cellulase activity and specific cellulase activity were lower than those recorded by previous studies.

Acknowledgments

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