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Activity of Cellulolytic Bacteria fom Sediment of Mangrove Forest Ecotourism Pesawaran Lampung

(Aktivitas Bakteri Seluloltik Asal Sedimen Kawasan Wisata Hutan Mangrove Pesawaran Lampung)

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Abstrak

Bakteri selulolitik mampu menghidrolisis substrat yang mengandung selulosa, seperti serasah mangrove dalam sedimen mangrove, dengan menghasilkan enzim selulase untuk mengubah selulosa menjadi senyawa yang lebih sederhana. Penelitian ini bertujuan untuk mengisolasi bakteri dari sedimen mangrove yang mampu menghasilkan enzim selulase dan mengukur aktivitas enzim tersebut dalam mendegradasi substrat selulosa. Metode yang digunakan meliputi isolasi bakteri selulolitik dari sedimen mangrove, uji aktivitas enzim secara kualitatif, pembuatan kurva standar dan kurva pertumbuhan bakteri selama 24 jam, kurva standar glukosa, serta uji aktivitas enzim secara kuantitatif menggunakan spektrofotometer. Hasil isolasi menghasilkan 18 kandidat bakteri selulolitik dengan karakteristik makroskopis dan mikroskopis yang beragam. Uji aktivitas enzim secara kualitatif selama 4 hari pada media Zobell dengan tambahan 0,5% CMC menunjukkan indeks aktivitas dari 5 isolat. Indeks aktivitas tertinggi untuk masing-masing isolat adalah: isolat M2 (1,96 pada hari ke-3), isolat M3 (4,29 pada hari ke-4), isolat M4 (2,02 pada hari ke-4), isolat M6 (4,39 pada hari ke-4), dan isolat M13 (3,87 pada hari ke-4). Tiga isolat potensial dengan rata-rata indeks aktivitas enzim tertinggi dipilih untuk uji kuantitatif. Uji kuantitatif ekstrak kasar enzim selulase selama 7 hari pada substrat CMC 0,5% menunjukkan aktivitas hidrolisis tertinggi pada hari ke-5 oleh isolat M6 sebesar 0,15 U/mL, diikuti oleh isolat M13 (0,14 U/mL) dan isolat M3 (0,13 U/mL).

Kata kunci: Bakteri selulolitik, Selulase, Sedimen Mangrove

Abstract

Cellulotyc bacteria can hydrolyze cellulose rich substrates such as mangrove litter found in mangrove sediments by producing cellulase enzymes that break cellulose into simpler compounds. This study aimed to isolate bacteria from mangrove sediments capable of producing cellulase enzymes and to evaluate the enzymatic activity of these isolates in degrading cellulose substrates. The research methods included isolating cellulotyc bacteria from mangrove sediments conducting qualitative enzyme activity tests creating a standard curve of bacterial growth, monitoring bacterial growth over 24 hours, preparing a standard glucose curve, and performing quantitative enzyme activity tests using a spectrophotometer. A total of 18 cellulotyc bacterial candidates were isolated, each displaying distinct macroscopic and microcospic characteristics. Qualitative enzyme activity tests were conducted over four days on Zobell medium with 0.5% CMC, yielding activity indices from five isolates. The highest activity indices were observed as follows: isolate M2 (1.96 on day 3), isolate M3 (4.29 on day 4), isolate M4 (2.02 on day 4), isolate M6 (4.39 on day 4), and isolate M13 (3.87 on day 4). Three isolates with the highest average enzyme activity indices were selected for further quantitative analysis. Quantitative tests of cellulase enzyme crude extracts on 0.5% CMC substrate over seven days revealed that isolate M6 exhibited the highest hydrolysis activity on day 5 (0.15 U/mL), followed by isolate M13 (0.14 U/mL) and isolate M3 (0.13 U/mL)

Keywords: Cellulolytic bacteria, Cellulase, Mangrove sediment

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INTRODUCTION

The mangrove ecosystem in Indonesia is 31,890 km^2 which is one of the largest mangrove ecosystems in Southeast Asia. Mangrove forests on the island of Sumatra are widespread in most of the eastern coastal areas, especially coastal areas that are protected from the crashing waves of the Indian Ocean. One of these areas is in the Lampung sea environment [1]. Ecologically, mangrove roots are able to bind sediment and prevent coastal erosion and play a role in absorbing accumulated heavy metals [2]. Organic material originating from piles of fallen mangrove litter will be trapped in the roots of mangrove vegetation so that it mixes with mangrove sediment [3]. The litter is decomposed by microorganisms into minerals and nutrients which are distributed by environmental factors in the waters to be utilized by aquatic organisms and the mangrove vegetation itself [4].

Microbes play an important role in the process of decomposing litter from mangrove forests. Microbes produce extracellular enzymes (cellulase, protease, lipase and amylase) to break down complex organic materials contained in mangrove litter into simpler molecules that can be transferred into cells as nutrients. The condition of mangrove vegetation which adapts to fluctuating environments requires microbes to produce extracellular enzymes that are able to work in complex environmental conditions so that enzymes obtained from mangrove sediment bacterial isolates have high tolerance to environmental factors such as high salinity and tidal conditions [5].

Cellulose is the main component of mangrove, so the presence of cellulase enzymes produced by cellulolytic bacteria is needed to break down cellulose into simple sugars in the form of oligosaccharides and glucose [5]. The glucose obtained can be used as raw material for fermentation and bioethanol production. Cellulase can also be used in the pulp and paper industry for fiber modification and color cleaning. In the textile sector, cellulase is used as a biopolishing fabric to soften and brighten fabrics [3]. On the other side, cellulase can also be used to increase the digestibility of fish feed raw materials that contain high fiber or cellulose [6].

Based on previous studies, regarding the potential of the mangrove ecosystem as a source of extracellular enzymes produced by bacteria, the cellulase enzyme activity from mangrove sediments on the North coast of Semarang 225.40 mm^2/ml [5]. Primary forest soil bacterial isolates in Lake Kalimpa'a, Central Sulawesi, were able to produce cellulase enzymes with an enzyme activity of 0.30 U/ml [7]. Indonesia has many mangrove ecosystem areas that have not been explored as a source of isolation for cellulase-producing bacteria. Mangrove Forest Ecotourism in Pesawaran, Lampung is one of the potential areas that can be utilized as a source of isolation of cellulolytic bacteria. Therefore, this study aims to isolate cellulolytic bacteria from Mangrove Forest Ecotourism Pesawaran, Lampung sediments and test their cellulase activity capabilities.

METHODS

Sample Preparation

Sampling was carried out from three locations in the Petengoran Lampung Mangrove Forest using the random sampling method. Mangrove sediment samples were taken from the top layer of the surface using a shovel and then placed in a plastic ziplock, put into an ice box and taken to the laboratory [8]. The medium used for bacterial isolation is Zobell agar medium containing 10 grams of CMC (Carboxymethyl Cellulose), Zobell media (15 grams of bacterial agar, 5 grams of bacterial peptone, 1 gram of yeast extract), and 1000 ml of seawater.

Isolation of Cellulolytic Bacteria and Characterization

A 10 grams sediment sample was placed in an Erlenmeyer flask containing 90 ml of sterile seawater and incubated in a water bath shaker for 24 hours and to obtain a dilution of 10^{-1}. Next, a series of dilutions up to 10^{-7} was made by taking 1 ml of the suspension and then placing it in 9 ml of sterile seawater to obtain a 10^{-2} dilution and so on until a 10^{-7} dilution was obtained, then vortexed [9]. From the dilution factors 10^{-5} and 10^{-6}, 0.1 ml of bacterial suspension was taken and inoculated into each petri dish containing Zobell agar medium using the spread plate method using an L rod on the surface of the Zobell agar medium. After completion of inoculation, the petri dish was coated with a plastic seal to minimize contamination and incubated in an incubator for 48 hours at a temperature of 37°C [10]. After the bacteria grow, purification is carried out using the streak plate method.

Macroscopic observation of bacterial colony morphology on Zobell agar media includes color, shape, elevation and edge of the colony [11]. Colony shapes observed from above can be round, threadlike, rooty, and irregular. Observation of bacterial cells under a microscope was carried out using the Gram staining method.

Qualitative Cellulolytic Enzyme Assay

The purified bacterial isolate was inoculated into a Petri dish by spotting it on Zobell agar medium which was added with 0.5% CMC and incubated for 5 days at 37°C. Next, every 24 hours the enzyme activity index was measured by pouring a 2% iodine solution into the media and leaving it for 15 minutes. After that, the diameter of the clear zone formed was measured using a caliper [12]. The cellulase activity index is calculated based on the ratio of the clear zone diameter to the colony diameter [13]. Bacterial isolate with the largest enzyme activity index will be rejuvenated. Rejuvenation of bacterial isolates was carried out by taking 1 ose of the isolate and then growing it on Zobell agar slant media.

Enzyme activity index:

$$\frac{clear\ zone\ diameter\ \ (mm)\ -\ colony\ diameter\ (mm)}{colony\ diameter\ (mm)}$$

Standard Curve of Bacterial Growth

Cellulolytic bacterial isolates that had been rejuvenated were taken in 1 dose and inoculated in 100 ml of Zobell broth media, then incubated on a water bath shaker for 16 hours at a temperature of 37°C and a speed of 120 rpm. Next, the bacterial culture in the media was first diluted with ratio 1:1, 1:2, 1:4, 1:8, and 1:16 in Zobell media with 0.5% CMC added, then the absorbance value was measured for each dilution. Using a spectrophotometer with a wavelength of 600 nm. After that, 1 ml of the original culture was taken and diluted in 9 ml of distilled water with a dilution factor of 10^{-1} to 10^{-8}. Next, 0.1 ml of dilution factors 10-6 and 10-7 were taken and then spread into each petri dish containing Zobell agar medium added with 0.5% CMC and incubated for 24 hours, then the growing colonies were counted.

Bacterial Growth Curve

A total of 1 batch of bacterial isolates was inoculated into 50 ml of Zobell broth media and then incubated in a shaker at a speed of 120 rpm for 24 hours. 1 ml of culture was taken and put into 50 ml of Zobell broth media with the addition of 0.5% CMC and then incubated using a shaker for 24 hours. An inoculum of 2 ml was taken every 2 hours for 24 hours then the optical density (OD) value was measured using a spectrophotometer at a wavelength of 600 nm. Next, growth curve was created by plotting the absorbance value against incubation time. Growth curve used for knowing the growth phases of bacteria, so the optimum time of certain bacteria to produce enzymes can be determined. The inoculum sample was also serially diluted to 10^{-4}. At the 10^{-4} dilution, 100 µL was taken to calculate the total plate count using the pour method. [14].

Glucose Standard Curve

A glucose stock solution is prepared by adding 1 gram of glucose (1000 mg) into 100 ml of sterile distilled water. The concentration used in the glucose standard curve is 1 mg/ml, so it is made by dissolving 1 ml of stock solution in 9 ml of sterile distilled water. Then dilution is carried out with concentrations of 0, 50, 100, 150, 200, 250, 300 ppm. A total of 1 ml of each concentration of glucose solution was added with 2 ml of DNS (3,5-Dinitro salicylic acid) solution then vortexed to make it homogeneous. The mixture then incubated for 15 minutes at 100°C. Further, the absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The results were analyzed using Microsoft Excel with the absorbance value on the x-axis and the concentration value on the y-axis, to create the curve's regression equations [15].

Quantitative Enzyme Activity Test

The bacterial isolate was inoculated in 100 ml of Zobell broth media with 0.5% CMC substrate and then incubated using a water bath shaker at room temperature at a speed of 150 rpm for 24 hours. Then 1 ml of the culture was taken and inoculated in a new Zobell broth medium with 0.5% CMC substrate. Next, it was incubated on a water bath shaker at room temperature at a speed of 150 rpm. The enzyme crude extract is harvested during the highest production time based on the optimum incubation time, namely at the end of the exponential phase. Crude enzyme harvesting was conducted until 7 data points were obtained. A volume of 4 ml of culture was taken and put into a falcon tube then centrifuged at 4,000 rpm for 30 minutes at 4°C to separate the supernatant (enzyme) from the pellet (bacterial cells) [16]. A volume of 2 ml of the supernatant was taken and 1 ml of 0.5% CMC broth was added, then vortexed and incubated for 60 minutes at room temperature. As a negative control, the supernatant was replaced with sterile distilled water. Then 2 ml of DNS solution was added and incubated at 100°C for 10 minutes then cooled. After cooling, the sample was measured for absorbance using a spectrophotometer at a wavelength of 540 nm [15]. Cellulase enzyme activity can be calculated using the following formula [17].

$$Enzyme\ Activity\ \left(\frac{U}{mL}\right) = \frac{C}{Molecular\ weight\ Glucose \times t} \times \frac{H}{E}$$

C	: Glucose concentration (ppm)
BM	: Molecular weight (g/mol)
t	: Incubation time (minute)
H	: Volume enzyme-substrate (mL)
E	: Volume enzyme (mL)

RESULTS AND DISCUSSION

Characterization of Cellulolytic Bacteria

Bacteria from mangrove sediments that were successfully isolated on Zobell selective media with the addition of 1% CMC amounted to 18 bacterial isolates with various characteristics. A number of 17 bacterial isolates were in the form of coccus and 1 isolate was in the form of a bacill. The Gram staining results of the isolates obtained were dominated by Gram positive bacteria (14 isolates) and 4 isolates were Gram negative bacteria. These results are not much different from previous studies. Cellulolytic bacterial isolates originating from mangrove mud in Sungailiat, Bangka [6] are dominated by Gram-negative bacteria in the form of coccus. The results of the isolation of cellulolytic bacteria from mangrove sediments in the Dumai Marine Station [9], all isolates were Gram positive bacteria in the bacilli form.

Table 1. Characteristics of candidate cellulolytic bacteria from mangrove sediments

No	Isolate	Colony shape	Colony colour	Colony edge	Colony elevation	Gram	Colony shape
1	M1	Circular	Milky white	Entire	Flat	+	Coccus
2	M2	Circular	Cream	Entire	Flat	+	Coccus
3	M3	Rhizoid	Yellow	Rhizoid	Flat	-	Coccus
4	M4	Circular	Cloudy white	Undulate	Flat	-	Coccus
5	M5	Circular	Cloudy white	Entire	Raised	+	Coccus
6	M6	Circular	Cloudy white	Entire	Convex	+	Coccus
7	M7	Circular	Milky white	Entire	Flat	+	Coccus
8	M8	Circular	White	Entire	Flat	-	Coccus
9	M9	Irregular	Milky white	Undulate	Convex	-	Coccus
10	M10	Circular	White transparent	Entire	Raised	+	Coccus
11	M11	Circular	White transparent	Entire	Raised	+	Coccus
12	M12	Circular	Cloudy white	Entire	Raised	+	Coccus
13	M13	Irregular	Cream	Undulate	Flat	+	Coccus
14	M14	Irregular	Cloudy white	Undulate	Flat	+	Coccus
15	M15	Circular	White transparent	Entire	Flat	+	Coccus
16	M16	Circular	White transparent	Entire	Flat	+	Coccus
17	M17	Irregular	Yellowish white	Undulate	Raised	+	Coccus
18	M18	Circular	Cloudy white	Entire	Flat	+	Bacill

Qualitative Cellulolytic Enzyme Assay

All bacterial isolates were tested for qualitative enzyme activity on Zobell agar media with 0.5% CMC added. The results showed that there were 5 bacterial isolates that could form clear zones around colonies. The five potential bacterial isolates are M2, M3, M4, M6, and M13. The formation of a clear zone indicates that the bacterial isolate is capable of producing cellulase enzymes which can hydrolyze cellulose on the CMC substrate into glucose [18]. The results of cellulose degradation in CMC by cellulolytic bacterial isolates were clearly visible after adding 2% iodine dye (Figure 1).

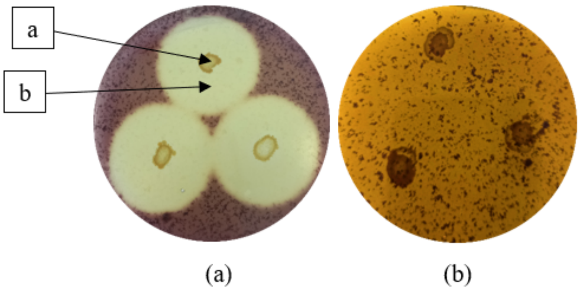

Fig. 1. Comparison of enzyme activity in 2 different isolates (a) Hydrolysis activity by bacterial isolate M13 on day 3: a) Colonies, b) Clear zone. (b) Isolate M18 has no hydrolysis activity

Carboxymethyl Cellulose (CMC) is a compound derived from cellulose with the basic structure of β-1,4-glucopyranose which forms a cellulose polymer. Cellulose in CMC has a shorter chain structure than natural cellulose so it is easily hydrolyzed by cellulolytic bacteria [19]. Cellulolytic bacteria use the CMC substrate as a carbon source to produce cellulase enzymes which can hydrolyze cellulose in CMC into glucose [13]. Cellulase is an extracellular enzyme with an endo-β-1,4-glucanase complex which works actively in hydrolyzing amorphous chains of cellulose into glucose and cellobiose [20].

The clear zone cannot be colored by iodine because the glucose, as the result of hydrolysis of cellulose in CMC by the cellulase enzyme, cannot bind with iodine [21]. The β-1,4-glycoside bonds that connect the D-glucose monomers in CMC to form amorphous cellulose chains have been broken, resulting in a clear zone forming around the colony, which indicates hydrolysis activity by the cellulase enzyme [18]. The blackish brown part of the media shows that cellulose is not hydrolyzed and forms a cellulose-iodine complex. This is because the iodine dye binds strongly to the cellulose in the β-1,4-glycoside region which produces a brownish color around it [11]. The qualitative cellulase enzyme activity test is conducted to determine the cellulose hydrolysis power of each isolate based on the enzyme activity index obtained from measuring the clear zone diameter and colony diameter.

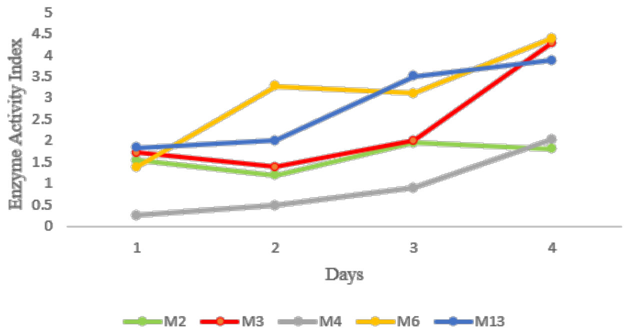

Fig. 2. Cellulase enzyme activity index for 4 days incubation

The enzyme activity index shows the ability of cellulolytic bacterial isolates to produce cellulase enzymes which can be determined by calculating the ratio of the clear zone diameter to the colony diameter. Based on Figure 2, it shows that there was an increase and decrease in the enzyme activity index over 4 days. Enzyme activity index measurements were only carried out for 4 days because on the following day the clear zone formed was so large that it covered almost the entire surface of the media so it could not be measured.

Cellulolytic activity is considered high when the enzyme activity index is ≥ 2, moderate when the index ranges between 1–2, and low when the index is ≤ 1 [22]. Each cellulolytic bacterium exhibits varying capabilities in producing cellulase enzymes to hydrolyze substrates in the media, leading to differences in cellulase activity indices among bacterial isolates [23]. The variations in enzyme production are influenced by the bacteria's genetic composition and the carbon source used [24]. Based on the highest average enzyme activity indices, isolates M3, M6, and M13 were selected for quantitative enzyme activity analysis.

Quantitative Cellulolytic Enzyme Assay

Enzyme activity was measured by determining reducing sugar levels using the glucose standard curve equation with the DNS method. Reducing sugars are sugars capable of donating electrons to electron-accepting compounds due to the presence of a free aldehyde or ketone group. These include all monosaccharides (glucose, fructose, galactose) and most disaccharides (maltose, lactose), but not sucrose or polysaccharides [25]. Glucose was selected as the standard solution for curve preparation because it is one of the reducing sugars produced during substrate hydrolysis by cellulase enzymes [26]. The reaction between reducing sugars and the DNS reagent involves a redox process, where the aldehyde group in the sugar is oxidized to a carboxyl group, and 3,5-dinitrosalicylic acid (DNS reagent) is reduced to 3-amino-5-nitrosalicylic acid. This reaction causes the DNS solution to change color from yellow to reddish-orange, indicating the presence of reducing sugars [27].

The production of crude enzyme extracts aims to assess the cellulase activity of potential bacterial isolates (M3, M6, M13) by measuring reducing sugar levels in the samples using the DNS method. The enzyme production timing was determined based on the growth curves of the three cellulolytic bacterial isolates. As a primary metabolite, the cellulase enzyme is produced by cellulolytic bacteria during the transition from the exponential (logarithmic) phase to the early stationary phase. According to the growth curve results (Figure 3), the end of the exponential phase for the three isolates occurs at the 18th hour of incubation. Therefore, cellulase enzyme harvesting was performed every 18 hours, resulting in seven data points collected over the experimental period.

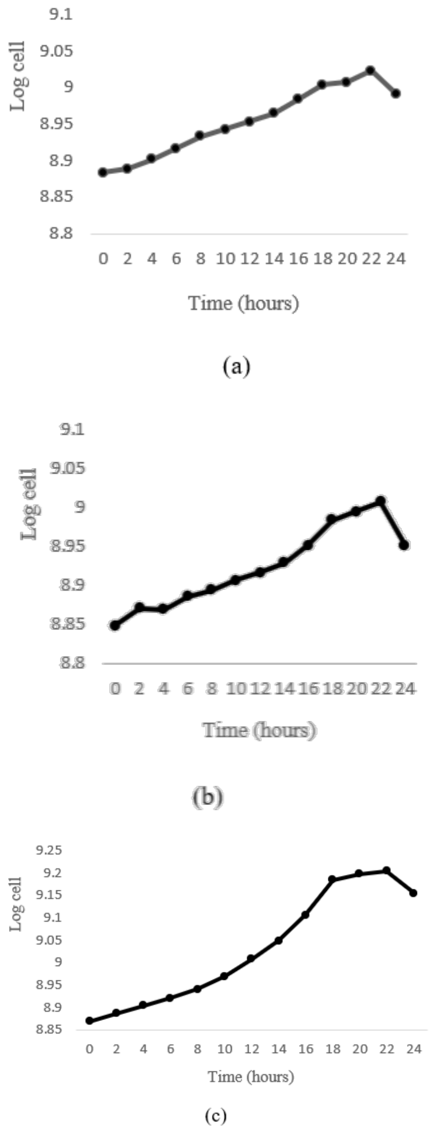

Fig. 3. Growth curve of cellulolytic bacterial isolates (a) Isolate M3, (b) Isolate M6, (c) Isolate M13

In the exponential phase, bacterial cell growth increases twice as fast, causing the nutrients in the media to be actively used by the bacteria. The cellulase enzyme will be produced by bacteria when nutrient sources other than the CMC substrate begin to run low so that the bacteria will utilize the CMC substrate in the media as a carbon source to support their growth [28]. The Zobell media used contains yeast extract and CMC substrate which can be utilized by cellulolytic bacteria as a carbon source.

The results of the quantitative cellulase enzyme test showed that on the 5th day all bacterial isolates had the highest enzyme activity and then experienced a decrease in enzyme activity on the 6th to the 7th day. Isolate M6 had the highest cellulose hydrolysis activity on 0.5% CMC substrate among the other two isolates, with enzyme activity 0.15 U/mL. The highest cellulose hydrolysis activity of M3 is 0.13 U/mL, while M13 isolate had the highest cellulose hydrolysis activity at 0.14 U/mL. These results confirm the results of the qualitative enzyme activity test that was previously carried out, where isolate M6 had the highest enzyme activity (Fig.2).

In research by [31], the highest cellulase enzyme activity produced by bacteria from mangrove swamp mud sediments in Thailand was 1.78 U/mL using a CMC substrate concentration of 2%. Sediment sampling was taken near the mangrove roots at a depth of 15 cm. According to [3], the sampling area in mangrove roots contains a lot of organic material that comes from piles of litter and becomes a growth substrate for bacteria. The highest enzyme activity test result in this study was 0.151 U/mL, relatively low compared to previous studies. This is possibly caused by the low purity of the enzyme because the enzyme used is a crude extract enzyme so the enzyme still contains protein or other cell components [26].

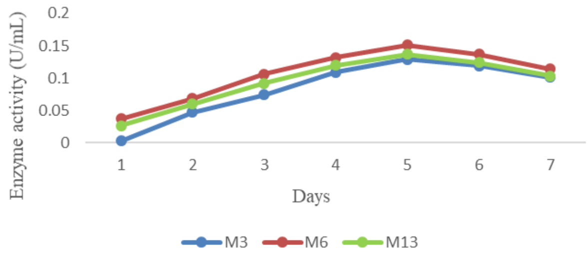

Fig. 4. Cellulase enzyme crude extract activity

Carboxymethyl Cellulose (CMC) is a pure cellulose substrate with an irregular (amorphous) chain polymer structure. CMC is used by cellulolytic bacteria as a carbon source and inducer to produce cellulase enzymes. An inducer is a substrate for an enzyme that is induced to deactivate the repressor and start the transcription process so that gene expression occurs and an enzyme corresponding to the substrate is formed [20]. The cellulase enzyme that plays a role in the hydrolysis of CMC substrates is endo-β-1,4-glucanase (CMC-ase) which randomly cuts β-1,4-glycoside bonds on the surface of cellulose to produce oligosaccharides [29].

The substrate hydrolysis activity by the cellulase enzyme is influenced by the length of hydrolysis time to produce the enzyme. The longer the hydrolysis time, the higher the levels of reducing sugar (glucose) produced due to increased cellulase enzyme production activity by cellulolytic bacteria. There was a decrease in enzyme activity on days 6 to 7 because the substrate in the growth medium began to decrease and the products from the hydrolysis of the cellulose substrate increased. Decreased enzyme activity can occur because bacterial cells use glucose as a simple carbon source, thereby affecting cellulase enzyme production to decrease. In addition, the substrate hydrolysis process that occurs causes glucose to accumulate in the media so that glucose can inhibit the work of the cellulase enzyme. Glucose is known to be an inhibitor that can directly inhibit the work of the β-glucosidase enzyme. Glucose will bind to the active site of β-glucosidase because it has a similar shape to the substrate so it can inhibit enzymatic hydrolysis activity. The inhibited work of the β-glucosidase enzyme causes an increase in the concentration of cellobiose in the media because it cannot be hydrolyzed into glucose [30].

CONCLUSION

There are 18 isolates of cellulolytic bacteria obtained in this study with different macroscopic and microscopic characteristics. The results of quantitative cellulase enzyme activity tests on 0.5% CMC substrate showed that the highest hydrolysis activity occurred on day 5 by isolate M6 at 0.151 U/mL. The other two cellulolytic bacterial isolates, namely isolate M3 and isolate M13, had the highest cellulose hydrolysis activity of 0.129 U/mL and 0.137 U/mL.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest in this study.

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