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Screening and Enzyme Activity of Cellulase-Producing Bacteria Isolated from *Kemiri Sunan* (*Reutealis trisperma* (Blanco) Airy Shaw) and Empty Fruit Bunches of the Palm Oil (*Elaieis guineensis*)

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Abstract— **Cellulase enzymes have an important role in biocatalyst technology, especially in the pulp industry. Cellulase is synthesized by a large type of microorganisms, including cellulose-producing bacteria isolated from *Kemiri Sunan* (*Reutealis trisperma*) shell and empty fruit bunches of the palm oil (EFBPO). In this study, the screening of cellulase-producing bacteria isolated from *Kemiri Sunan* shell and EFBPO were carried out in order to evaluate its enzyme activity. The method used was an experimental method followed by descriptive analysis using four treatments with two repetitions. This study includes; (1) Lignocellulosic biomass analysis, (2) Isolation of cellulase-producing bacteria, (3) Qualitative assay of cellulase-producing bacteria, (4) Enzyme activity assay. Both cellulose and hemicellulose contents in *Kemiri Sunan* shell were 27.38% and 44.46%, respectively, and 48.55% and 28.06% in empty fruit bunches of palm oil. Qualitative assay of cellulase-producing bacteria from *Kemiri Sunan* shell resulted in the widest clear zone accounting of 77.19% (isolate K2) and 73.06% (isolate T3) from EFBPO. Screening continued by measuring the cell growth with an interval time of 8, 24, 32 and 48 h. The enzyme activity showed the highest cellulase activity (isolate K3) from *Kemiri Sunan* shell was 4.3 × 10-2 U/mL after 48 h incubation. However, isolate T4 from EFBPO resulted in higher cellulase activity than that *Kemiri Sunan* shell, accounting of 7.2 × 10-2 U/mL. Our study suggests that cellulase-producing bacteria from *Kemiri Sunan* and EFBPO has been screened to synthesize cellulase enzyme.**

Keywords—***Kemiri Sunan***; **cellulose; screening; enzyme activity; palm oil.**

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I. INTRODUCTION

The industry needs cellulases that can work optimally and have high activity. Nowadays, there is a lot of research on selecting cellulase-producing microbes that can be applied in the industrial world. Bacteria and fungi can produce cellulase enzymes. Bacteria that have a high growth rate compared to fungi have good potential for use in cellulase production. However, the application of bacteria in producing cellulases is not widely used. Bacterial cellulases usually do not have one of three cellulases, i.e., FPase. However, cellulases produced by bacteria are often more effective catalysts.

Cellulase is a type of extracellular enzyme that can hydrolyze the bonds of β-1,4-glycosidic to cellulose and produce glucose products [1]. Cellulase is produced by fermentation methods, both solid-state fermentation (SSF) and sub-merged fermentation methods. The isolation process of cellulase-producing bacterial enzymes from *Kemiri Sunan* shell more suitable to use the liquid phase fermentation method because the bacteria grow in high humidity conditions. However, fungi must be treated in conditions with low humidity levels. The longer interval time of the fermentation process can provide opportunities for microbes to break down the components in the substrate into components that are simpler and easier to digest [2].

Kemiri Sunan (*Reutealis trisperma*) is one of the most potential plants in Indonesia for producing vegetable oil. The existence of *Kemiri Sunan* is still rare because the cultivation of *Kemiri Sunan* is still new, and *Kemiri Sunan* is well-known as a poisonous plant. *Kemiri Sunan* is still less developed compared to the type of candlenut commonly used as a kitchen spice. The production *Kemiri Sunan* ranges from 7.5 tons/ha/year. *Kemiri Sunan* consists of fruit, shell, seeds, and kernel. *Kemiri Sunan* seeds contain about 50% oil. The high oil content can be an alternative to fossil oil because it can be processed into biodiesel [3]. *Kemiri Sunan* shell becomes

agricultural waste, which can still be utilized for substrate in the production of the cellulose enzyme so that it can increase the value of the *Kemiri Sunan* shell.

Indonesia is one of the biggest oil palm producers in the world. In 2017, oil palm plantations in Indonesia increased to 12.3 million ha with total palm oil production of 35.3 million tons [4]. Palm oil processing by various companies leaves solid and liquid waste. Oil palm empty fruit bunches are one of the oil palms wastes. Oil palm empty fruit bunches originating from mills have very abundant availability or are almost the same as crude palm oil (CPO) yield [5]. Palm empty fruit bunches, such as in wood or other plants, contain chemical elements of fat, cellulose, lignin, and hemicellulose that can be an alternative source of raw material for non-wood fibers for pulp and paper.

Cellulase-producing bacteria can be produced from agricultural waste, which is isolated from the Empty Fruit Bunch of the Oil Palm (EFBPO) and *Kemiri Sunan* shell. The screening process of cellulase-producing bacteria from the *Kemiri Sunan* shell and oil palm empty fruit bunches were carried out by liquid phase fermentation through grow the bacteria on selected media and tested for its activities. Based on the explanation above, this study was conducted to screen and test the ability of cellulase-producing bacteria from *Kemiri Sunan* shell and oil palm empty fruit bunch samples qualitatively and quantitatively.

II. Materials and Methods

A. Pretreatment of Kemiri Sunan Shells and EFBPO

Physical treatment on *Kemiri Sunan* shells and the Empty Fruit Bunch of the Palm Oil (EFBPO) was conducted by cleaning the surfaces. The water content reduction process was carried out by drying the shells under the sun for 12 hours until *Kemiri Sunan* shell, and EFBPO turns to brownish-yellow. Color changes indicated that water content has decreased, and respiration has been inhibited. The drying process was also to prevent the decay process of *Kemiri Sunan* shell due to the development of destruction microbes. Shell size reduction into 2 cm to simplify the process using disc mill. The wet shell caused the shell stick to the disc mill pieces when it ground. *Kemiri Sunan* and EFBPO powder from the milling was heated at 105°C for 4 hours until the powder did not clot and simplified the separation process during sieving. The sieving process was done using a 100-mesh sieve [6].

B. Lignocellulosic Biomass Analysis

Lignocellulose biomass analysis of *Kemiri Sunan* shell and EFBPO refer to the Chesson method. The formula calculated for the calculation of lignocellulosic content is presented in equation (1) below.

$$\text{Hemicellulose content} = a\text{-}b \times 100\% \quad (1)$$

$$\text{Cellulose content} = b\text{-}c \times 100\% \quad (2)$$

$$\text{Lignin} = c\text{-ashweight} \times 100\% \quad (3)$$

C. Isolation of Cellulase-Producing Bacteria

Kemiri Sunan shell and EFBPO were decomposed and the size was reduced to 40 mesh. Each 1 g of sample added 20 mL of BSM (Basal Salt Medium) + CMC (Carboxymethyl Cellulose) +antifungal steril media and filtered by Whatman type filter paper no. 1. After that, samples incubated at room temperature (30°C) and stirred at 150 rpm for 10 days. A growing bacterial colony which was able to use cellulose as a single carbon source furthermore isolated using BSM-CMC-CR (Congo Red) medium and incubated for 24-48 hours [7].

D. Qualitative Assay of Cellulase-Producing Bacteria

After 24-48 hours, bacterial colonies showed clear zones on BSM-CMC-CR indicated the potency to produce cellulase. The following colonies were tested qualitatively by calculating the ratio of diameter on clear zones. The culture of pure isolates was taken and subculture in BSM-CMC-CR medium by a point streak method in order to calculate the ratio of the diameter of the clear zone produced. The best value qualitative test result would be taken for calculating cellulose activity in further steps. Qualitative testing or hydrolysis capacity test was conducted to determine the potential for cellulose degradation from the isolates qualitatively estimated by calculating hydrolysis capacity (HC), i.e., the ration clearing zone and colony diameter [8]. Qualitatively, the amount of cellulose activity can be expressed as a cellulosic index or cellulose activity index (IAS) obtained by using the following formula:

$$Z = (r1\text{-}r2)/r1 \times 100\ \% \quad (4)$$

Description:
Z = percentage of cellulose income qualitatively
r1 = clear zone radius (cm)
r2 = radius of bacterial colonies (cm)

E. Enzyme Activity Assay

Cellulase activity assay used the DNS method following [9]. As much as 1 mL of cellulase-producing bacterial culture was inoculated into 20 mL of 1% CMC Broth media, incubated at 37oC and 120 rpm aeration. Followed by taking 1.5 mL of inoculum then centrifuged at 10,000 rpm for 10 minutes at 4oC to get crude enzyme extracts. 1 ml of crude enzyme added with 1 ml of 1% CMC containing citrate buffer with pH 4.8. After that, incubated at a temperature of 55 degrees for 15 minutes, followed by the addition of 1 ml of DNS and heated 100 degrees for 5 minutes, then added 1 mL of tartrate and cooled. Aquabidest was added until the solution reached 10 ml and was tested by spectrometry using a wavelength of 540 nm. One unit of cellulase enzyme activity was expressed as the amount of µmol of glucose products resulting from the hydrolysis of cellulase enzymes per minute. The value of cellulase activity was determined according to the formula:

$$EA = \frac{GC}{MW\ glucose\ x\ t}\, x\, \frac{H}{E} \quad (5)$$

Description:
EA : enzyme activity (U/mL)
GC : glucose concentration (
MW : molecule weight (180 g/mol)
t : incubation time (minute)
H : enzyme-substrate total volume (mL)
E : enzyme volume (mL)

III. RESULTS AND DISCUSSIONS

A. *Lignocellulose Content*

Kemiri Sunan shell and EFBPO known as agricultural waste containing lignocellulose. Lignocellulose is an abundant organic component in nature and consists of three types of polymers, namely cellulose, hemicellulose, and lignin [10]. Characterization chemical composition the *Kemiri Sunan* shell and EFBPO were conducted include testing hemicellulose levels, cellulose, and lignin, as presented in Table 1.

TABLE I
LIGNOCELLULOSE BIOMASS (%) IN *KEMIRI SUNAN* SHELL AND EFB OF THE PALM OIL

Lignocellulose Biomass	*Kemiri Sunan* Shell (%)	EFBPO (%)
Hemicellulose	44.49±7.81	28.06±0.41
Cellulose	27.38±2.06	48.55±7.64
Lignin	28.13±9.87	23.39±8.05

The cellulose content needs to be analyzed to determine the potential of the *Kemiri Sunan* shell and EFBPO as a substrate for cellulase production. The *Kemiri Sunan* shell contains 28% of cellulose, while EFBPO contains a higher of cellulose, accounting of 48%. The high cellulose content in the EFBPO has been used as an alternative non-wood fiber raw material for the pulp and paper industry [26]. The screening process on cellulase-producing bacteria can be observed from how much the cellulose content of the material is used. Cellulose, a polysaccharide, acts as an inducer which could increase cellulase production. Cellulase is a multienzyme complex that hydrolyzes cellulose into glucose. Furthermore, microorganisms use cellulose as a carbon source to be used as a fermentation substrate by converting it to organic acids, ethanol, single-cell proteins [11]. Cellulose enzyme produced by microorganisms which degrade cellulose has a potency of their application in various industries [12].

Hemicellulose is a non-cellulosic component of both primary and secondary cell walls, and it follows cellulose in abundance. Whereas cellulose is formed from units of glucose, different monomer units constitute hemicellulose [13]. The content of hemicellulose in the *Kemiri Sunan* shell and EFBPO were 44,49 % and 28.06%, respectively. At the same time, lignin content was 28.13% and 23.39%, respectively.

Lignin is a non-carbohydrate fraction that is complex and difficult to separate. Lignin content of *Kemiri Sunan* shell reaching 28% need to be further analyzed to determine the inhibition of lignin in the enzymatic hydrolysis process. Lignin is a heterogeneous aromatic polymer with a branched tissue system and does not have a fixed shape [14]. Lignin, which protects cellulose, is resistant to *hydrolysis* due to *arylalkyl* bonds and ether bonds [15].

B. *Qualitative Assay*

The extracellular activity of cellulase enzymes produced by cellulase-producing bacteria could be identified qualitatively by observing the clear zone produced in the media of BSM-CMC-CR. Carboxymethyl Cellulose (CMC) media is an anionic polymer that is commonly used in cellulase activity testing [16]. Cellulase-producing bacteria growth on BSM-CMC-CR media containing potassium phosphate which plays a role in increasing the growth and density of bacterial cells [17] while *magnesium sulfate* has a role as a nutrient for bacteria to grow and develop. CMC is the most appropriate substrate that able to induce bacteria to produce cellulase enzymes. The clear zone formed shows the potential for cellulose degradation in the media by bacteria qualitatively estimated by calculating the hydrolysis capacity (HC), i.e. the ratio of the diameter of the clearing zone around the colony. The results of the calculation of the clear zone within five days of incubation at 37°C shown in Fig 1.

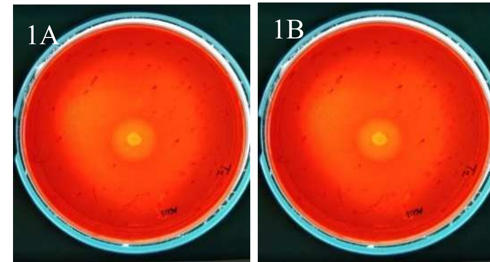

Fig. 1 The clear zone formation by cellulose-producing bacteria isolated from (1A*) Kemiri Sunan* shell; (1B) EFB of palm oil

The clear zone formed was measured using scientific calipers by calculating the Cellulase Activity Index (IAS) formula. The area of the clear zone showed the potential for degradation by isolates grown on cellulose media containing Carboxymethyl Cellulose qualitatively, which was estimated by calculating the hydrolysis capacity (HC).

C. *Cellulose Activity Index*

The calculation of cellulase index activity showed that K2 code from *Kemiri Sunan* shell and T3 code from EFBPO had the highest percentage of cellulase producing with a value of 77.53% and 73.06%, respectively (Fig.2A, 2B).

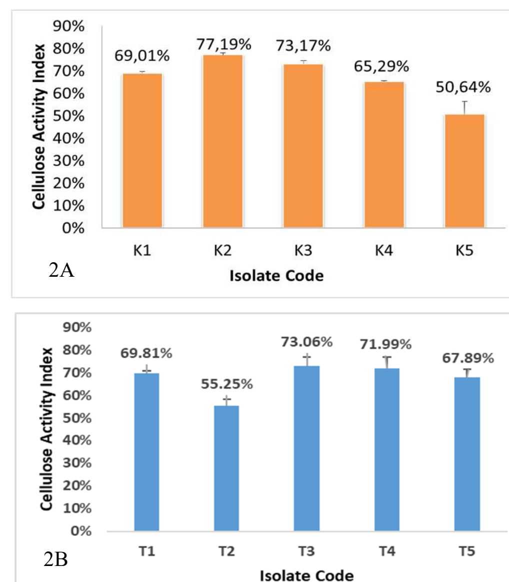

Fig. 2 Cellulose activity index of cellulose-producing bacteria isolated from (2A) *Kemiri Sunan* shell; (2B) EFB of palm oil.

Both of those two isolates, K2 and T3, have the potential to produce the most optimal cellulose enzyme. According to [18], the greater the cellulosic index or clear zone produced, the greater the cellulose enzyme secreted by the bacteria.

Clear zone formation indicates that polysaccharide has been degraded into saccharides with shorter chains so that it unable to absorb congo red dyes. The cellulose activity index value produced by microbes could be increased by extending the incubation time. Therefore, the enzyme possible to degrade more CMC substrates.

The five isolates from *Kemiri Sunan* shell and EFBPO could decompose the substrate used. The presence of clear zones resulting from several isolates had the potential for endoglucase or CMCase activity. CMC as a substrate unable to be transported into bacterial cells or molds due to the high molecular weight of CMC. Therefore, cellulose-degrading enzymes were released outside the cell or only retained on the surface of the bacterial colony cell walls and diffuse to the media [19]. The cellulose activity index in each isolate showed differences that were influenced by the concentration of agar medium and substrates used. According to [20], the smaller media pores which cause inhibition of the enzyme degradation process was influenced by the media content of CMC.

D. Cellulase-Producing Bacteria Cell Growth

The highest growth of cellulase-producing bacteria from *Kemiri Sunan* shells was K4 code at 32 hours, with an absorbance value of 1.14. In comparison, the highest growth of cellulose-producing bacteria from EFBPO (T5) was reached at 30 hours (Fig.3A and 3B). This was influenced by adequate nutrition, optimum pH, and temperature that supports bacterial growth to be high. After incubation past 30 hours, cellulase-producing bacteria growth undergoes a stationary phase where the number of bacterial cells no longer increased. All cellulase-producing bacteria isolates began entering the death phase at 48 hours incubation because cell growth had stopped, and the bacteria had used up energy reserves to respire.

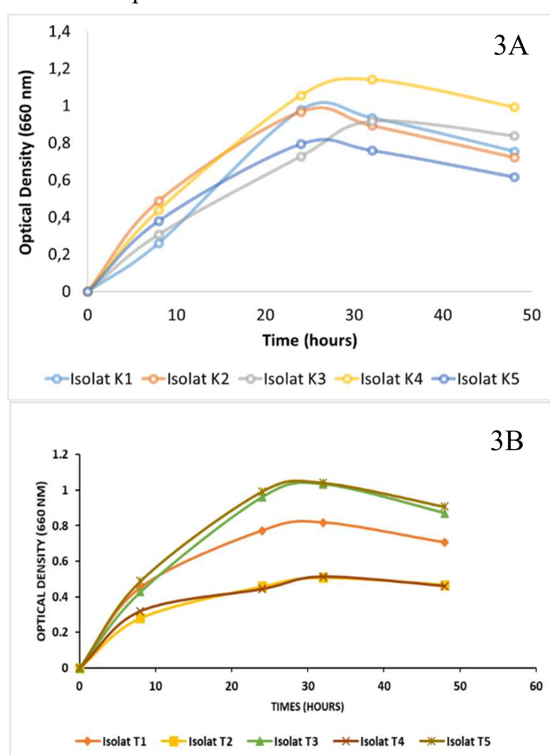

Fig. 3 Cellulose-producing bacteria growth isolated from (3A) *Kemiri Sunan* shell; (3B) EFB of palm oil

Bacterial growth patterns obtained by observing growth curves in bacterial liquid culture on Nutrient Broth (NB) media. The composition of the media Nutrient Broth (NB) is a beef extract and peptone that needed for bacterial growth. Peptone contributes to organic nitrogen in the form of amino acids and long-chained fatty acids. Beef Extract provides additional vitamins, carbohydrates, salts, and other organic nitrogen compounds [21].

During the incubation period, cellulase-producing bacteria were incubated at 37°C. Temperature is a physical factor that affects the rate of bacterial growth on chemical reactions and the stability of the structure of protein molecules. Chemical reactions will increase at a certain temperature due to an increase in reactant kinetic energy. Metabolic microbial growth is a chemical reaction that takes place in the cell and is catalyzed by enzymes [22].

E. Enzyme Activity Assay

The measurement of enzyme activity was carried out using the DNS method based on the estimated amount of glucose (reducing sugar) as a result of hydrolysis cellulose [23]. DNS is an aromatic compound that will react with reducing sugars, which form 3-amino-5-dinitrosalicylic acid, which is a compound that is able to absorb electromagnetic radiation waves strongly at a wavelength of 540 nm [24].

The level of reducing sugars was determined by preparing a glucose standard curve that serves to determine the concentration of the solution with its absorbance value. Glucose solution is used as a solution for making standard curves because glucose includes reducing sugars produced from cellulose hydrolysis by cellulase enzymes. Standard curves were made with variations in the concentration of glucose 0; 25; 50; 75; 100; 125; 150; (mg/mL). The results of the standard glucose curve have a linear equation y= 0.2542x - 0.0041 with a correlation value (R2) of 0.9927 (Fig. 4). This equation is used to determine the glucose concentration in the sample. If there is a reduction of sugar in the sample, the addition of a yellow DNS solution will initially react with the reducing sugar so that it turns reddish-orange.

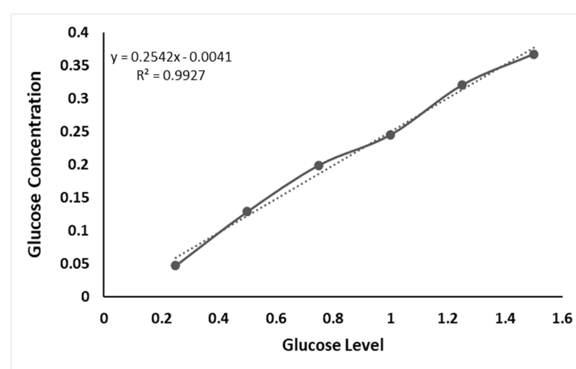

Fig. 4 Standard Glucose Curve

The highest value of cellulase activity described the most optimal time to produce cellulase enzymes. Five cellulase-producing bacteria isolates from each sample had different enzyme activity values (Figure 5A and 5B). The highest enzyme activity from the *Kemiri Sunan* shell was isolated K3 after incubation at 48 hours with a value of 4.32 x 10^{-4} U/mL. The highest enzyme activity from EFBPO was in the isolate

T4 with a value of 7.2×10^{-4} U/mL after 48 h of incubation. Fluctuating value could be caused by glucose levels absorbed at a certain time and the availability of substrate decreased with time [25]. Therefore, a decrease in enzyme activity occurred.

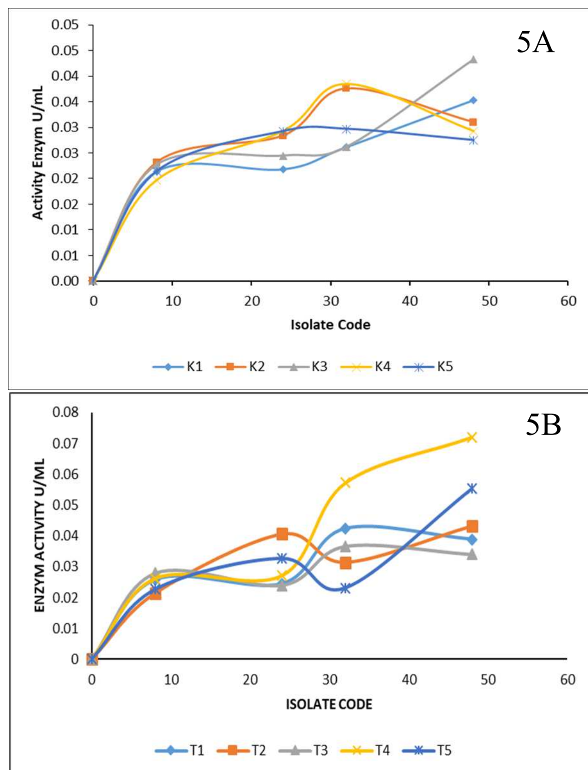

Fig. 5 Enzyme activity value of cellulose-producing bacteria growth isolated from (5A) *Kemiri Sunan* shell; (5B) EFB of palm oil.

As seen in figure 5A, K3 and K4 code from *Kemiri Sunan* shell has reached the highest cellulase activity at 32 h with values of 3.75 x 10-4 U/mL and 3.84 x 10-4 U/mL, respectively. However, after 32 h, there was a decrease in activity due to the presence of glucose in a certain time, and the availability of substrate decreased, resulting in a decrease in the value of cellulase enzyme activity. On the other hand, T4 code from EFBPO had a more extended period to the reached maximum condition than other isolates at 48 hours with an enzyme activity value of 7.2×10^{-4} U/mL (fig. 5B).

The enzyme activity graphs above showed that each cellulase-producing bacterial isolate has a different optimum activity based on the unit of time. Several factors affect enzyme activity, including temperature, pH, substrate concentration, enzyme concentration, inhibitors, activators, and contact time. Absorbance values obtained were plotted on a standard curve to determine the concentration of glucose products in the sample.

F. Morphology Analysis of Cellulase-Producing Bacteria

Gram staining in cellulose-producing bacterial cells produced red color and basil-shaped bacterial cells. Characteristics of Gram-negative bacteria have a thick lipid bilayer, a thin peptidoglycan layer, and a high permeability. It causes the bacteria cell layer unable to absorb violet crystalline dyes but able to absorb safranin so that the cell turns red when observed under a microscope, as shown in fig. 6. In contrast to Gram-negative bacteria, the Gram-positive bacteria cell layer remains purple because the cell wall is composed of thick peptidoglycan that does not fade with alcohol [26]. Based on the staining and morphology analysis results, cellulose-producing bacterial isolated from *Kemiri Sunan* shell and EFBPO was indicated to the genus *Pseudomonas* sp. *Pseudomonas* sp is one of the bacteria known to produce cellulose [27][28].

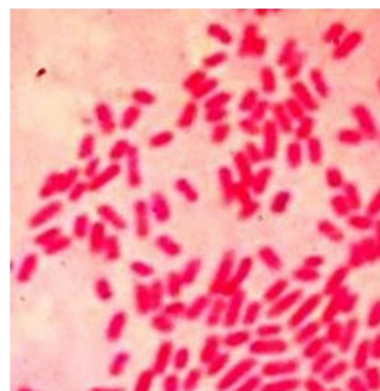

Fig. 6 Gram-staining of Cellulase-Producing Bacteria

In addition, colony morphology observations were also carried out, such as color, shape, surface texture of the single bacterial colony (Table 2). Based on the colony's morphological observations, all the cellulose-producing bacteria isolates, both from *Kemiri Sunan* shell (K1-K5 code) and from EFBPO (T1-T5 code), have the same characteristics including turbid color, round shape, slimy texture, and the surface has elevation or convex, and the colony does not produce zones.

TABLE II
COLONY MORPHOLOGY ANALYSIS OF CELLULOSE-PRODUCING BACTERIA

Isolate code	Colony Morphology Analysis				
	Color	Shape	Texture	Surface	Colony
K1	Turbid	Round	Slimy	Elevation	No zone
K2	Turbid	Round	Slimy	Elevation	No zone
K3	Turbid	Round	Slimy	Elevation	No zone
K4	Turbid	Round	Slimy	Elevation	No zone
K5	Turbid	Round	Slimy	Elevation	No zone
T1	Turbid	Round	Slimy	Elevation	No zone
T2	Turbid	Round	Slimy	Elevation	No zone
T3	Turbid	Round	Slimy	Elevation	No zone
T4	Turbid	Round	Slimy	Elevation	No zone
T5	Turbid	Round	Slimy	Elevation	No zone

IV. CONCLUSION

In conclusion, cellulose-producing bacteria could be isolated and selected from *Kemiri Sunan* shell and EFBPO of the palm oil with vary enzyme activity value. Isolate K2, from *Kemiri Sunan* shell showed the highest clear zone with percentage of 77.19% ± 0.0084 and given enzyme activity value accounting of 4.32×10^{-4} U/mL after incubation time of 48 h. In addition, the isolate T3 from EFBPO of the palm oil resulted the widest clear zone percentage (73.06±0.0386). Other isolate from EFB (T4) showed the highest enzyme activity value accounting of 7.2×10^{-4} U/mL.

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