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Isolation and Characterization of Cellulolytic Bacteria from Bonoloyo, Cemetery

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ABSTRACT

A public cemetery (TPU) is land provided by the government to bury bodies. The culture of funerals in Indonesia is varied but generally involves being buried in the soil with wrapped clothes, covered wooden boards, and tombs. The material contains cellulose that will be broken down by cellulolytic microorganisms, including bacteria. The exploration of the cellulolytic bacteria of TPU in Indonesia has not been carried out, so this study aims to investigate and identify cellulose bacteria isolates from TPU. This research is a non-experimental study for the isolation of bacteria in the soil of TPU Bonoloyo, Surakarta. A selection of cellulite bacteria is grown in CMC media. Isolates that show cellulolytic activity are identified by observing colonial morphology and Gram coloring. Selection results of cellulolytic bacteria obtained isolates with cellulolytic index (IS) including a high category of 1 isolate (B14), a medium category of 3 isolates (B16, B27, and B45), and 9 low category isolates. The study concluded that 28.8 % showed cellulolytic activity with irregular morphological forms of white dyeing on flat surfaces with flat edges and dominated by gram-negative coccyx-shaped..

1. INTRODUCTION

The public cemetery land (TPU) is the land area provided for the burial of bodies for each person without distinction of religion or group (Diputra & Syaodih, 2017), whose management is regulated in Government Regulation Number 9 of 1987. Funerals in Indonesia have a diverse culture. Muslim bodies are buried in cloth (Ubaidillah et al., 2018). The bodies of Christians and Catholics were buried in clean clothes along with coffins (Dan Dare Arradhika, 2012). The culture of slaughtering bodies in Indonesia is generally buried in the soil.

In the cemetery there is an active process of body decomposition. The decomposition causes changes in the soil. According to (Majgier et al., 2014) the soil layer in the cemetery area underwent degradation and caused chemical changes such as increased organic carbon content, nitrogen, and soil alkaline reactions. However, the degree of change depends on the depth of the grave. Various types of soil microbes are found in large numbers, one of which is soil bacteria capable of degrading cellulose (Maravi & Kumar, 2020). The TPU has the possibility of finding cellulose degradation bacteria because the clothes, wooden boards, and coffins are buried. The buried material contains a high cellulose content of about 42-47% (Fengel & Wejener, 1995).

Cellulolytic bacteria are one of the microbes that break down cellulose into a simpler compound (Yusnia et al., 2019; Nofu et al., 2014). Cellulolytic bacteria are said to be potentially cellulose-degrading because they have a faster growth rate than other microbes and are easily controlled. (Arsyad et al., 2018; Yusnia et al., 2019). Cellulase enzymes play a major role in everything from agriculture to medicine and are also used in academic and industrial research as they are easily obtainable (Pal et al., 2021; Harjuni et al., 2020). Cellulase enzymes are widely used in various types of industries, including the food and beverage industry, the textile industry, the detergent industry, the pulp and paper industry, the livestock feed industry, and agriculture (Harjuni et al., 2020). In agriculture, the enzyme cellulase can process coffee peel waste (Nurfitriani & Handayanto, 2017), and banana peel waste (Sukmawati, 2018).

From the search that has been done, cellulite bacteria have a lot of potential in various fields. Research related to the isolation of cellulolytic bacteria in Indonesia has been done a lot, among others, from the forest land in Bali (Yusnia et al., 2019), from yellow sugarcane waste (Nofu et al., 2014), from mangrove soil (Remijawa et al., 2020), from fermented weft (Raharjo & Isnawati, 2022), and from garbage soil (Murtiyaningsih et al., 2017). The results of the research on bogor subsoil grass obtained a cellulolytic index between 0.15 and 2.12 and a characterization of the morphology of the round-shaped colony, a glossy fine surface, a flat margin, as well as the elevation of the grass with the colony colors of white, cream, and lymph (Azizah, 2021), and in the study (Murtiyaningsih et al., 2017) of the garbage soil, a cellulolytic index of 0.85 with a predominant colony morfology of white and yellow color and smooth edges.

Publication articles on the isolation of cellulolytic bacteria have not been found after searches on the insulation of cellulitic bacteria from the cemetery. In the previous study, the bacteria were isolated from the cemetery of Bonoloyo, Surakarta, and 45 isolates were obtained (Laspartriana, 2023, not published), but the cellulolytic capabilities were not screened. Therefore, this study aims to select and identify cellulolytic bacterial isolates from TPU Bonoloyo, Surakarta. The results of this study are expected to yield bacterial isolates that have potential cellulolytic abilities. Furthermore, such isolates can be used for a variety of purposes, among them the treatment of organic waste, the alcohol industry, and the paper industry.

2. MATERIALS AND METHODS

1.1. Material

The tools used in this study include: Elenmeyer (*Pyrex)*, reaction tube (*Pyrex*), *autoclave (GEA LS-35LJ)*, *petri dish (Iwaki)*, *incubator (Memmert N55)*, oven (*Maspion*), a digital scale (*Durascale DAB-E223*), spatula, *Laminar Air Flow* (LAF), *micropipette* (*Socorex*), *hot plate magnetic stirrer* (*Ciramec+*), reaction tube shelf, a micropipette (*Socorex*).

The materials used in this study include: 45 bacterial isolates from TPU Bonoloyo, which are from the collection of Biology Laboratory FKIP UMS, 70% alcohol, aquades, gloves, bacteriological agar (*Oxoid*), $NaNO_3$, K_2HPO_4, $MgSO_4$, $MnSO_4$, $FeSO_4$, $CaCl_2$. $2H_2O$. *Congo red*, *Carboxymethyl Cellulose* (CMC).

1.2. Methods

The research was conducted at the Biology Laboratory, Faculty of Education and Education Sciences, Muhammadiyah University of Surakarta, from March 2023 until April 2023. This research is a non-experimental study for the isolation of soil bacteria from TPU Bonoloyo, Surakarta.

1.2.1. The rejuvenation in Nutrient Media (NA)

The rejuvenation is done using NA media dissolved in aquades. The medium is sterilized by autoclave at a temperature of 121 ^oC at a pressure of 1 atm for 15 minutes. Each bacterial isolate is taken in as much as 1 loop and occulted on the NA medium, incubated at room temperature for

48 hours (Arifin et al., 2019). Successful rejuvenation is characterized by colonies growing on the media (Wahyuningsih, N.,& Zulaika, 2018).

1.2.2. The production of a solid cellulolytic

The production of a solid cellulolytic medium for the isolation and characterization of cellulose bacteria is achieved by dissolving $NaNO_3$ 2 g; K_2HPO_4 0,5 g; $MnSO_4$. $7H_2O$ 0,02 g; $MgSO_4$ 0,02 g; $FeSO_4$ 0,02 g; $CaCl_2.2H_2O$ 5 g; CMC 0,5 %; and bacteriological agar 10 g in 1 L aquades (Dar et al., 2015). It is then mixed using a magnetic stirrer on a hot plate, adjusted to a neutral pH and sterilized at a temperature of 121 ^oC for 15 minutes.

1.2.3. Testing of Cellulolytic bacterial activity

Bacterial isolates that have grown are then incubated at a temperature of 30 ^oC for 48 hours (Murtiyaningsih et al., 2017). After 48 hours, the bacterial isolate was vaccinated on a 1 % selective CMC medium using the point method (Yusnia et al., 2019) and incubated for 48 hours, after which it was given 0.1 % congo red for 15 minutes and rinsed with NaCl 1 M.

The qualitative index of cellulolytic bacteria is determined by looking at the presence of the clear zone. The value of the cellulolytic index (IS) is calculated by comparing the values of the clear zone diameter and the colony diameter value of bacteria. The cellulolytic index can be calculated using the following formula: (Nababan et al., 2019)

$$IS = \frac{clear\ zone\ diameter - the\ colony\ diameter\ value\ of\ bacteria}{the\ colony\ diameter\ value\ of\ bacteria}$$

1.2.4. Characteristics of Morphology

Macroscopic and microscopical observations carry out morphological characterization. Macroscopic observations include colony shape, elevation, edge, and color of bacteria (Raharjo & Isnawati, 2022). Microscopic observations are carried out with a Gram color test. Gram coloring is done by taking as much as 1 ounce of bacterial insulate diluted in 3 ml of sterile aquades and taking 10 µl then placed on the glass of the object and inspected. Add 1 drop of violet crystal for 1 minute, then wash with running water and dry. After drying, add 1 drop of iodine solution. After 1 minute, wash with running water. The bacteria are then isolated with 95 % ethyl alcohol for 30 seconds and washed with running water. Then the bacterial isolate is added to saffranin for 2 minutes and washed with running water. The preparation is dried by sticking the tissue on the side of the review, then dried, and then the preparation is observed using a light microscope with an enlargement of 1000×. Gram-positive bacteria are marked with purple-colored cells, and Gram-negative bacteria are marked with red cells (Kurniawan et al., 2019).

3. RESULT AND DISCUSSION

1.3. The Cellulolytic Index

According to the findings of the research that has been carried out, TPU Bonoloyo obtained 28.8 % (13 isolates) of cellulolytic bacteria among the 45 collections of bacteria found in the laboratory. The results of cellulite activity from the isolation of CMC-enriched bacteria can be seen in Table 1.

Table 1. The cellulite index of 13 isolates.

Isolated code	Colonial diameter (cm)	Clear zone diameter (cm)	Cellulite index (cm)
B1	0,7	1,9	1,71
B4	0,65	1,7	1,62
B12	1,55	2,9	0,87
B14	0,45	2,5	**4,56**
B16	1	3,55	2,55
B20	1,4	3,35	1,39
B25	1,6	3,75	1,34
B26	1,55	3,15	1,03
B27	1,1	3,65	2,32
B28	1,7	3,4	1,00
B31	0,7	1,75	1,50
B43	1,5	3,3	1,20
B45	0,75	3,25	3,33

1.4. Testing of Cellulolytic Bacteria Activity

Qualitative cellulolytic bacteria test with CMC media was carried out on previously isolated bacterial isolates and 13 isolates were obtained. Based on table 1 it can be seen that B14 isolate has the largest cellulolytic index of 4.56. This indicates that B12 isolates have the ability to degrade cellulose in CMC media to be higher than other isolates. The cellulolytic index value obtained in this study is included in the medium to high category.

The value of the cellulolytic index obtained from this study has a relatively greater value than other studies. In research (Harjuni et al., 2020) showed that cellulolytic enzyme activity from mangrove sediments obtained the highest cellulolytic index of 2.21±1.04 and the lowest cellulolytic index of 0.67±0.29. Cellulolytic bacteria from rice straw isolated by (Azizah, 2021) obtained a cellulolytic index of around 0.15±2.12. And the cellulolytic index value of 0.87 was obtained from fermented feed mixed with water hyacinth, corn cobs, and rice bran (Raharjo & Isnawati, 2022). This shows that bacterial isolates that have the highest cellulase enzyme activity can hydrolyze cellulose to glucose and show a large clear zone around the colony (Harjuni et al., 2020).

Carboxymethyl Cellulose (CMC) is the best substrate for cellulase production because it can induce bacteria to produce cellulase enzymes (Idiawati et al., 2015). The clear zone shows hydrolytic activity by the extracellular enzyme cellulase excreted by bacterial isolates of a certain diameter. The hydrolysis product is in the form of monosaccharide simple sugars and there is no complex bond with *congo red* (Azizah, 2021). Isolates that are able to decompose CMC are shown by the formation of a clear zone around the colony after staining using *Congo red* (**Fig. 1**).

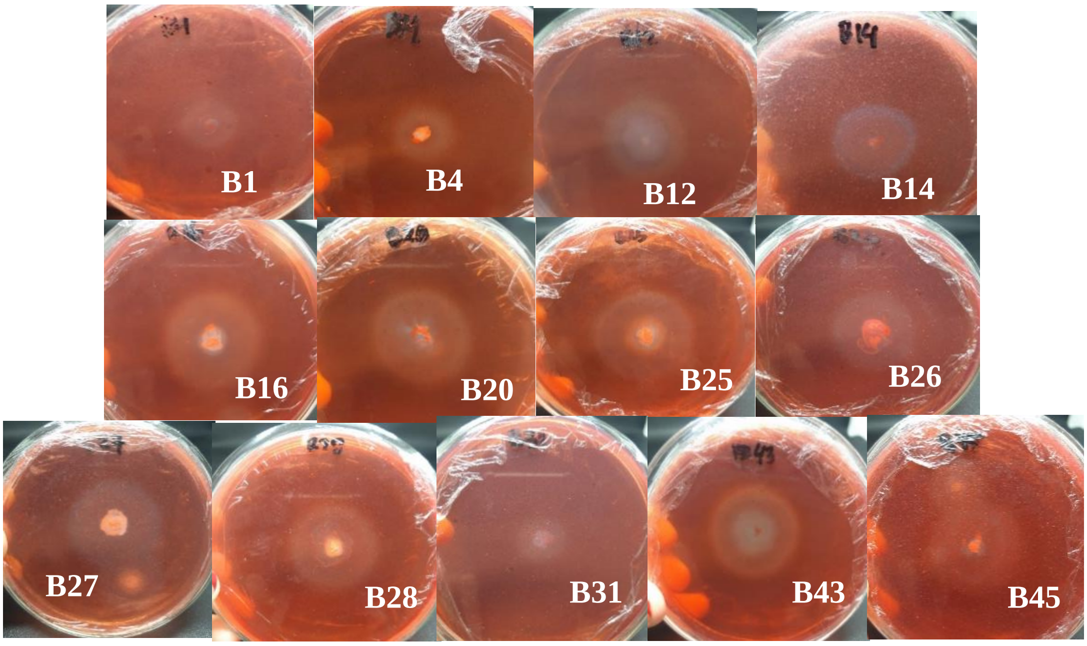

Figure 1. Qualitative cellulolytic bacteria activity test results (*Congo red*)

From the test results of cellulolytic bacteria activity shows different clear zone diameters. The ability of bacteria to break down cellulose can be seen from the difference in the speed of cellulose decomposition which is known to form clear zone areas. The higher the area of the clear zone produced can be assessed, the greater the ability of bacteria to degrade cellulose (Kurniawan et al., 2019). According to (Dar et al., 2015) the clear zone produced by cellulolytic bacteria that have a diameter above 4 cm can be categorized as high degradation rates while low degradation is in the range of 0.5–1.9 cm and medium 2.0–3.9 cm.

Based on the clear zone test results obtained, it shows that B12 isolate has the smallest cellulolytic index value with a value of 0.87 and B14 isolate has the largest cellulolytic index value of 4.56. The results of cellulolytic index testing and measurement can be seen in Table 1. It can be seen that IS (Cellulolytic Index) as many as 9 isolates obtained low degradation rate and as many as 3 isolates obtained medium degradation rate 1 isolate obtained high degradation rate at B14 which produces the largest cellulolytic index. According to (Upadhyay et al., 2012) that for each colony has a different production of clear zone diameters. (Azizah, Nur et al., 2014) added that the greater the cellulase activity index in isolates, the greater the cellulolytic activity produced.

Bacterial isolates with the highest degree of degradation obtained a cellulolytic index value of 4.56 and have the potential to be utilized in the needs of human life. Cellulose-degrading bacteria are needed in human life (Thomas et al., 2018) states bacteria are an important source of cellulase with various industrial and biotechnological applications. The potential of cellulolytic bacteria as cellulose degraders can be utilized for fish feed needs (Khulud, Janatul et al., 2021). Research (Kurniawan et al., 2018) added that cellulolytic bacteria from nature can be a solution to increase the digestibility of feed raw materials containing high fiber or cellulose.

1.5. Characterization of Cellulolytic Bacteria

Cellulolytic bacterial isolates were then observed colony morphology and Gram painting. The morphological features of the colonies of the 13 isolates can be seen in table 3.

Table 3. Morphological characters of cellulolytic bacteria

Isolation Code	Morphological Characters of Bacterial Isolate Colonies					Cell Characters	
	Color	**Shape**	**Size**	**Edges**	**Elevation**	**Shape**	**Gram**
B1	white	circular	small	entire	flat	*Coccus*	-
B4	milky white	irregular	small	undulate	flat	*Coccus*	-
B12	white	circular	medium	entire	Flat	*Coccus*	-
B14	white	circular	small	Entire	flat	*Coccus*	-
B16	milky white	irregular	medium	undulate	flat	*Coccus*	-
B20	white	irregular	medium	undulate	flat	*Coccus*	-
B25	milky white	irregular	medium	undulate	flat	*Coccus*	-
B26	white	circular	medium	entire	flat	*Coccus*	-
B27	yellowish white	irregular	large	undulate	flat	*Coccus*	-
B28	milky white	irregular	medium	undulate	raised	*Coccus*	-
B31	milky white	irregular	small	undulate	flat	*Coccus*	-
B43	white	circular	small	entire	flat	*Coccus*	-
B45	white	circular	small	entire	flat	*Coccus*	-

Table 2 shows that cellulolytic bacterial isolates obtained have a diversity of characters in terms of colonies, but not in terms of cell morphology. Based on cell morphological characters, 13 isolates of cellulolytic bacteria obtained are gram-negative coccus bacteria. Several previous studies supported these results, in a study (Azizah, Nur et al., 2014) showed that cellulolytic bacterial isolates obtained from vermicomposting empty oil palm bunches were gram-negative bacteria.

Colony morphological characterization in 13 isolates had varying colors, shapes, elevations and edges. The morphological characters of the obtained colonies are dominated by white colony color, irregular colony shape, flat colony elevation and entire colony edges. Differences in colony morphology in bacterial isolates are influenced by the isolation method used. This is in line with research (Satwika et al., 2021) that the isolation method used affects the characteristics of the isolates obtained. Bacterial isolates obtained from cemetery soil prove that the soil has potential as a cellulose degrader. However, the bacterial isolate is not yet known species identity, so further tests need to be carried out to determine its identity.

4. CONCLUSIONS

Bonoloyo burial ground, Surakarta is inhabited by bacteria that have potential cellulolytic activity. The morphology of the bacterial colony is dominated by white colony color, irregular colony shape, flat colony elevation and entire colony edge. As a result of Gram staining, the isolate is included in the gram-negative in the form of *coccus*.

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6. SUGGESTION

This study obtained potential cellulolytic bacteria isolates, but the species identity is not yet known so further tests need to be carried out to determine the species of cellulolytic bacteria in the burial ground.

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