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Screening and Identification of Cellulolytic Bacteria from Public Cemetery Soil

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Abstract

Background: A public cemetery (TPU) is where bodies are buried. Before being buried, the body is wrapped in shroud or full clothes and sometimes put in a coffin. These materials contain cellulose, a substrate for cellulolytic microorganisms, including bacteria, to decompose. Exploration of cellulolytic bacteria in TPU has not been found, even though TPU holds potential cellulolytic bodies. This study aims to determine the cellulolytic potential and identify bacterial isolates from TPU. **Methods:** 36 bacterial isolates tested for cellulolytic potential have been isolated from TPU Pracimaloyo Kartasura, Sukoharjo. Selection of cellulolytic bacteria used selective Carboxymethyl Cellulose (CMC) media dripped with Congo red 0.1%, while identification was based on colony morphology and Gram staining. **Results:** This study obtained one isolate (2.78%) showing high cellulolytic activity with a cellulolytic index (IS) 7, namely P14, 24 isolates (66.67%) in the "moderate" category, four isolates (11.11%) with category "low," and seven isolates (19.44%) did not show cellulolytic ability. Isolates cellulolytic positive have a shiny white colony color, entire edges, raised elevation, and belong to the Gram-negative coccus form. **Conclusions:** From the results of this study, it can be concluded that public burial sites (TPU) harbor potential cellulolytic bacteria.

Keywords: Cellulolytic bacteria, public burial place (TPU), Pracimaloyo

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Introduction

Public Cemetery Land (TPU) is an area intended for the burial of all people regardless of religion and class, and this area is usually managed by the Level II Local Government or Village Government (Diputra & Syaodih, 2017). The burial process was carried out at a depth of 200 cm to avoid wild animals (Afni, et al., 2022), very actively decomposes the body, occurring approximately 30 days after death to produce soil minerals needed for the growth of microorganisms, including bacteria. This follows a study by Putra et al (2023) that found abundant bacteria around TPU. In addition to the decomposition of bodies, cellulose materials are also decomposed at TPU, such as shrouds, wood coverings, coffins, and clothing. This material, including cellulolytic bacteria, becomes a substrate for cellulose-decomposing bodies (Agustriono & Hasaanah, 2016). The cellulose substrate is a source of cellulolytic microorganisms for their growth by producing cellulase enzymes. The cellulose substrate is a source of cellulolytic microorganisms for their growth by producing cellulase enzymes. Several cellulolytic bodies that have been identified from the fungal group are *Phanerochaete chrysosporium* (Rahayu et al., 2017), while from the bacterial group, there are several genera, including Bacillus, Streptomyces, and Acinetobacter (Khotimah et al., 2020; Karthika et al., 2020; Sharma et al., 2022).

Based on reference searches, no research has been found that discusses cellulolytic bacteria from public burial grounds. Cellulolytic bacteria are found in decaying leaves,

producing a fairly high cellulose content from waste soil (Murtiyaningsih & Hazmi, 2017). Cellulolytic bacteria from forest soil in Bali, which has a lot of litter and rotted wood, supports the existence of cellulolytic bacteria (Yusnia et al., 2019). This is the basis for the presence of cellulolytic bacteria in TPU with the support of the shroud substrate, wooden covering, coffin, and clothing included in the burial process.

Isolation and screening of cellulolytic bacteria have been carried out from several sources, including research (Yusnia et al., 2019), which selected cellulolytic bacteria from several forest soils in Bali. The results showed that 21 isolates had the potential to degrade cellulose, with the highest cellulolytic index of 5.41 and the highest cellulose degradation rate of 8.32%. Research Murtiyaningsih & Hazmi (2017), on the isolation and activity testing of cellulolytic bacterial cellulase enzymes from waste soil has the potential to degrade cellulose with a cellulolytic activity index of 0.875. In the study (Fauziah & Ibrahim, 2020), cellulolytic bacteria in peat soil obtained 24 isolates with a cellulolytic index belonging to the high category of 6 isolates. In Indonesia, similar studies in TPU have never been reported.

The cellulase enzyme is an extracellular enzyme produced in cells and then released into the growth medium (Kartika & Ibrahim, 2021). These enzymes have been widely used in various fields, such as paper, pharmaceutical, detergent, and food industries. Enzyme-based industries are increasingly important than chemical-based industries because of process safety, low refining costs, high yields, effective process management, and economics. Current promising applications of cellulase enzymes are in the beverage (wine), feed, and food industries (Ejaz et al., 2021), as well as handling organic waste containing cellulose (Mifbakhuddin et al., 2022). Research Puspitasari & Ibrahim (2020) states that cellulase enzymes are widely used in industrial applications, and market demand is quite high. This aligns with research by Akmala & Supriyo (2022), which used cellulase enzymes to make biodegradable foam to replace Styrofoam, primarily from tapioca flour. This enzyme is very important in degrading cellulose into glucose from cellulose-containing materials, such as shrouds and clothes, in the burial process.

In the previous study, soil bacteria were collected from Pracimaloyo TPU and obtained as many as 36 isolates but did not carry out further characteristics. Pracimaloyo Cemetery is one of the largest cemeteries managed by the Surakarta City government (DKP Surakarta), with 14.5 hectares consisting of 19 burial blocks. With so many graves, the cellulose substrate is also abundant, so it is estimated that the soil samples from TPU contain cellulolytic bacteria. This research is expected to find cellulolytic bacteria that can degrade cellulose. Cellulolytic bacteria are microbes with great potential to degrade cellulose because they have a faster growth rate than other microbial groups, so the time needed to produce cellulase enzymes is shorter (Yusnia et al., 2019). Therefore, this study aims to determine the potential of cellulolytic bacteria derived from TPU and identify them.

Method

The experimental study aimed to determine the potential of bacteria and the morphological characteristics of cellulolytic bacteria in Pracimaloyo TPU. The study was conducted from January to April 2023. The tools used in this study were Laminar Air Flow (LAF), autoclave (GEA LS35LJ), Erlenmeyer (Pyrex) 500 ml, Petri dish (Iwaki), incubator (Memmert N55), measuring cup (Pyrex) 100 ml, test tube (Iwaki), spatula, oven (Maspion), hot plate magnetic stirrer, vortex, micropipette (Socorex), stationery, and documentation tools. The samples used in this study were collections from the biology laboratory of Universitas Muhammadiyah Surakarta, with as many as 36 isolates. The materials used are nutrient agar (NA) (Merck) media, carboxymethyl cellulose (CMC), aquades, aluminum foil, congo red dyes, cotton, umbrella paper, NaCl, 70 % alcohol, and gloves.

Rejuvenation of isolates

The collection of bacterial isolates was subcultured into oblique nutrient agar (NA) using ose and then incubated at 37 °C for 48 hours. The bacterial culture is ready for use in cellulolytic activity testing.

Cellulolytic ability screening

The cellulolytic bacteria screening medium used was a CMC selective medium consisting of 2 g NaNO_3, 0.5 g K_2 HPO_4, 0.02 g MnSO_4, 0.02 g FeSO_4, 5 g CaCl_2 and added 0.5 % CMC. All ingredients are dissolved in 1 liter of distilled water. The ready selective media is then inoculated with the bacterial isolates by dabbing them on the surface of the media (Zubaidah et al., 2019). Incubation was carried out at 37 ℃ for 48 hours. After the colonies grew, the clear zone colonies were measured and followed by bacterial screening with congo red staining. Next, pour the 0.1 % Congo red dye solution into a petri dish that has been incubated for 48 hours, then leave it for 20 minutes and rinse with 1 M NaCl. The appearance of a clear zone means positive (+) degrades cellulose around the bacterial colonies. The clear zone can be seen after washing with 1 M NaCl solution. Congo red is a sodium salt of benzidinedazo-bis-1-naphthylamine-4 sulfuric acid (C_32 H_22 N_6 Na_2 O_6 S_2), which dissolves with NaCl washing to form a clear zone (Tearher & Wood, 1982; Hartanti, 2020). The red color outside the clear zone indicates residual cellulose that is not degraded (Murtiyaningsih & Hazmi, 2017). The cellulolytic activity was determined by calculating the cellulolytic index (IS) by comparing the diameter of the clear zone formed with the diameter of the bacterial colony (Shajahan et al., 2017). The IS value calculation formula is as follows:

$$Cellulolytic\ Index\ (CI) = \frac{Total\ Diameter\ (clear\ zone)}{Colony\ Diameter}$$

Identification of bacteria based on colony morphology and Gram staining

Identification of bacteria can be done by observing macroscopic and microscopic characteristics. Macroscopic features include the shape of the colony, namely round, point, irregular, like roots, as well as filaments or threads and circles. Colony margins can be wavy, split, serrated, threaded, and curly. Colony colors include white, yellow, reddish, brown, orange, pink, green, and purple. Colony elevations include flat, embossed, curved, and convex. Fine colony texture is shiny, rough, wrinkled, or dry powdery. In addition, the size varies; this can be done by measuring the diameter of the growing bacterial colonies (Irianto, 2014). The macroscopic characteristics of bacterial colonies vary according to cell shape, size, and staining. The shape of the bacterial cell is like a round (coccus) with each combination, and microscopic measurements can be made with a micrometer and the staining that was carried out, including Gram staining (Lengkong et al., 2022). Gram staining is performed by smearing bacteria on a sterile glass object. Bacteria taken from pure culture colonies on nutrient agar (NA) slant. After fixation, crystal violet was dripped onto the smear and left for 1 minute, then rinsed with running water. After cleaning, drip with Lugol for 1 minute, then rinse with running water. Then drip with 96% alcohol and leave for 20-30 seconds; wash with running water, add safranin for 1 minute, and rinse using running water. Dry over the fire and observe under a microscope. Gram-negative bacteria are characterized by a red color after Gram staining, while a purple color characterizes Gram-positive bacteria.

Result

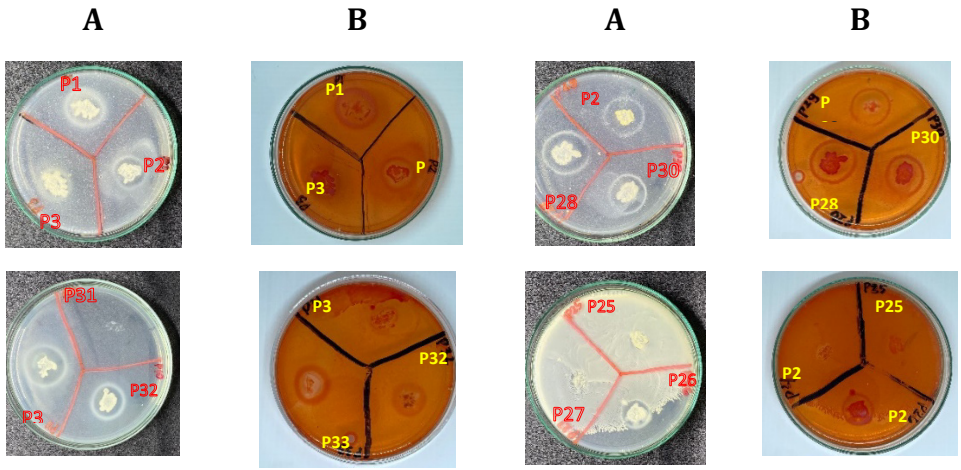
The selection of cellulolytic bacteria from TPU Pracimaloyo, Kartasura soil, using CMC selective media can be seen in Table 1.

Table 1. Cellulolytic bacteria screening results

Isolates	Diameter (cm)		Cellulotic Index (CI)	Category
	Clear Zone	Colony		
P1	3,15	3	1,05	Keep
P2	4	2	2	Keep
P3	6,6	4	1,65	Keep
P4	3	3	1	Low
P5	0	0	0	None
P6	3	3	1	Low
P7	5,25	3	1,75	Keep

P8	0	0	0	None
P9	5	5	1	Keep
P10	0	0	0	None
P11	3,5	1,69	2	Keep
P12	6	6	1	Low
P13	7,5	6	1,25	Keep
P14	6,3	0,9	7	Tinggi
P15	6,3	3,6	1,75	Keep
P16	4,2	4	1,05	Keep
P17	4	4	1	Keep
P18	7,5	6	1,25	Keep
P19	6	6	1	Keep
P20	5,9	3,1	1,9	Keep
P21	5,6	3,2	1,75	Keep
P22	0	0	0	None
P23	3,15	3	1,05	Keep
P24	4,2	2,1	2	Keep
P25	0	0	0	None
P26	5,9	4,7	1,25	Keep
P27	0	0	0	None
P28	2	2	1	Keep
P29	3,15	3	1,05	Keep
P30	2	2	1	Keep
P31	0	0	0	None
P32	5,9	4,7	1,25	Keep
P33	4,2	4	1,05	Keep
P34	3	3	1	Keep
P35	4	4	1	Keep
P36	2	2	1	Low

Table 1. shows that 2.78% of the isolates had a cellulolytic index (IS) in the "high" category, isolate P14. A total of 4 isolates (11.11%) with the cellulolytic index (IS) were in the "low" category, namely P4, P25, P27, and P31, while 24 isolates (66.67%) were in the "moderate" category, namely P1, P2, P3, P7, P9, P11, P13, P15, P16, P17, P19, P20, P21, P23, P24, P26, P28, P29, P30, P32, P33, P34, and P35. Seven isolates (19.44%) did not show cellulolytic ability, namely P5, P8, P10, P22, P27, P25, and P31. The clear zone produced with a diameter of above 4, which is produced by cellulolytic bacteria, is included in the "high" category. In contrast, the "low" category ranges from 0.5 - 1.9, and the “medium” category from 2.0 - 3.9 (Dar et al., 2015). Based on the cellulolytic bacteria screening test results, the six selected isolates were categorized as having a “high” degree of degradation.



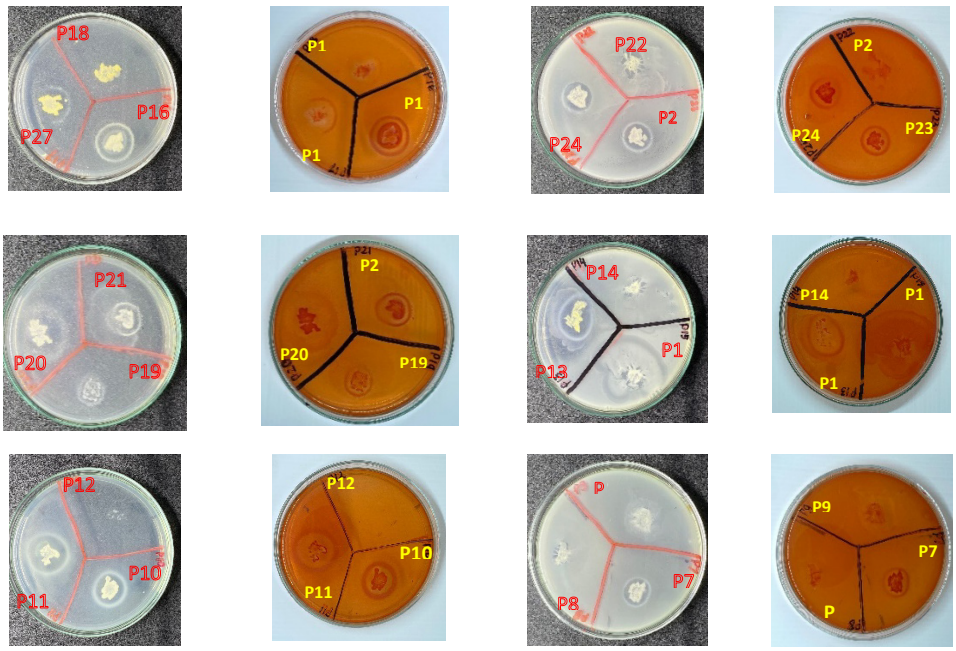


Figure 1. Clear zones formed due to the decomposition of CMCs as cellulose substrates. A) Colony before dripping congo red 1%; B) Bacterial colony after dripping congo red 1 %

The cellulolytic index (CI) is indicated by forming a clear zone around the bacterial colony after being dripped with 1% congo red (Figure 1.). The screening results showed varying CI values among the bacterial isolates tested.

Identification of bacteria based on colony morphology and Gram staining

Bacterial isolates that can degrade cellulose are then identified: colony morphology identification and Gram staining. Colony morphology results by observing colony shape characteristics, colony color, elevation, edges, optical properties, surface properties, cell shape, and biochemical testing in Gram staining, shown in Table 2. The ten potential isolates were then quantified into six isolates based on the final screening results by growing isolates in CMC media so that for each Petri dish, the potential isolate data became 16.7 %. A total of 6 isolates showed the degree of cellulose degradation and produced a cellulolytic bacterial index with the largest value of 7 (Table 1.). Furthermore, the six isolates were carried out with a Gram staining test.

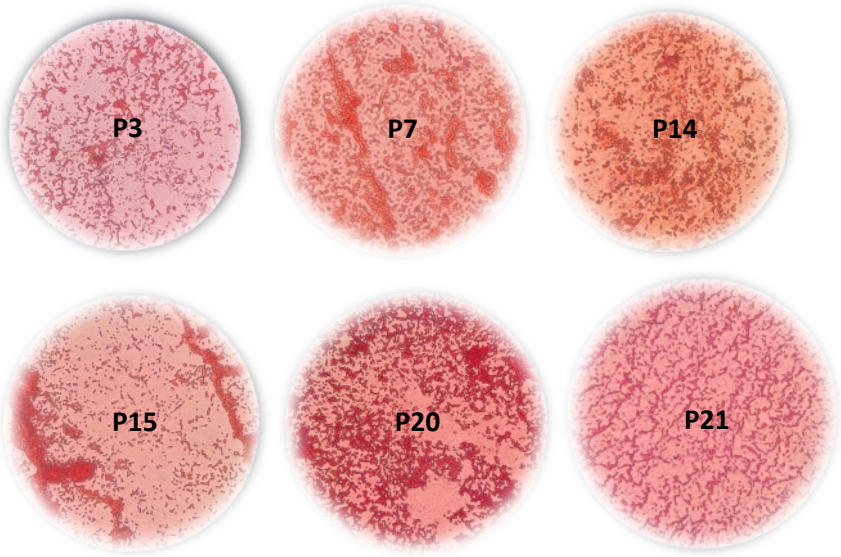


Figure 2. Gram staining results of isolates P3, P7, P14, P15, P20, and P21.

Gram staining of six soil bacterial isolates with a high cellulose index was gram-negative because they were reddish and coccus-shaped (Figure 2.). Gram-negative bacteria have a thin peptidoglycan layer but contain a lot of lipids, so they lose the crystal violet-Lugol complex when Gram staining using 1% Congo red so that the cells appear colorless. Staining the cells with red safranin solution makes the cells turn red (Hidayat, 2011).

Table 2. Morphological characteristics of cellulose-degrading bacteria in the soil of public cemeteries (TPU) Characteristic Isolates

Isolates	Characteristic							
	Colony morphology							
	Shape	Color	Elevation	Edge	Size	Surface	Cell Shape	Gram Staining
P3	Circular	White	Raised	Entire	small	Soft	Coccus	Negative
P7	Circular	White	Raised	Entire	small	smooth glossy	Coccus	Negative
P14	Circular	Milky White	Raised	Entire	small	Soft	Coccus	Negative
P15	Circular	White	Raised	Entire	small	Soft	Coccus	Negative
P20	Circular	White	Raised	Entire	small	smooth glossy	Coccus	Negative
P21	Circular	Milky White	Raised	Entire	small	smooth glossy	Coccus	Negative

Discussion

The cellulolytic bacteria screening results from Pracimaloyo TPU show great potential. Of the 36 isolates tested, 2.78 % were positive for cellulolytic (Figure 1.), even the largest cellulolytic index (IS) value reached 7. This CI value was higher than the isolates from the research results (Yusnia et al., 2019) using forest soil in Bali. This shows that Public Burial Ground (TPU) can potentially obtain cellulose-degrading microorganisms, including bacteria and fungi. This is because, in TPU, materials contain cellulose, such as shrouds, clothes, wood coverings, and coffins which are included in the burial process. The process of decomposing the bodies also adds soil minerals which will support the growth of other microorganisms so that their numbers are abundant (Putra et al., 2023). At TPU, there is a continuous change of substrate as new bodies are buried. Thus, decomposing microorganisms will maintain their activity.

The cellulolytic activity was demonstrated by forming clear zones on CMC selective media (Figure 2.) by extracellular cellulase enzymes secreted by bacterial isolates (Murtiyaningsih & Hazmi, 2017). The results of cellulose degradation are simple monosaccharide sugars, and complex bonds with Congo red do not occur. The red color around the clear zone indicates the presence of unhydrolyzed cellulose residue. A clear zone can be formed completely after rinsing with 1M NaCl. The higher the activity of the cellulolytic index produced by each isolate, the greater the ability of the isolate to degrade cellulose (Andriani et al., 2023). The screening results showed that none of the obtained isolates could hydrolyze the cellulose in the CMC agar medium. The ability of bacteria to grow on specific CMC media indicates that these bacteria can utilize cellulose as a nutrient source. This follows the research results Apun et al (2000), the diameter of the clear zone formed on CMC media indicates cellulolytic activity.

The identification results of the six isolates can be seen in Table 2, which shows the similarity of the colonies to all potential isolates. The similarity is shown from the shape of the bacteria, namely round (circular) white, raised colony elevation, and entire colony edge with the characteristics of a smooth, shiny bacterial surface and coccus-shaped cells. This is consistent with research Satwika et al. (2021) and Fauziah & Ibrahim (2020) showing cellulolytic bacteria characteristics from kitchen waste, livestock manure, and peat soil, respectively. The Gram staining results identified six isolates as Gram-negative and having the form of Coccus (Figure 2.). Gram-negative could not hold the dye after decolorization by Congored 1% with alcohol to produce a red color. Based on this identification, it is confirmed

that cellulolytic bacteria are commonly found in burial soils supported by a forming substrate. The cellulolytic potential of bacterial isolates in TPU adds information that these locations need to be explored by varying the types of TPU. Different burial methods will certainly provide different substrates for the decomposing corpse. This will certainly affect the diversity and potential of decomposers.

Conclusions

The TPU Pracimaloyo stores bacterial isolates with potential cellulolytic capabilities, namely P14, with a cellulolytic index reaching 7. The observations of colony morphology showed that the isolates formed circular white colonies, raised colony elevations, and entire colony edges with smooth, shiny surface characteristics. Gram staining results are Gram-negative and cocci shaped.

Declaration statement

The authors reported no potential conflict of interest.

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