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Isolation and Identification of Soil Bacteria in Pracimaloyo Public Cemetery, Kartasura

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ABSTRACT

In The Pracimaloyo Public Cemetery, the body's decomposition occurs continuously, producing soil nutrients and minerals that affect the growth of bacteria. Bacterial population data from the cemetery area is still very limited, whereas cemeteries hold the risk of contamination with pathogenic bacteria. This research aims to find out how the population and diversity of bacteria in the Pracimaloyo Public Cemetery. Soil samples were taken from the Pracimaloyo cemetery at 2 locations (blocks 8 and 18), each at 20 and 50 cm depth. Soil samples are inoculated in Nutrient Agar media using the spread plate method. After 48 hours, colony counting, colony morphology, and gram staining observations were carried out. The rate of soil bacterial populations in blocks 8 and 18 at a depth of 20 cm was 4.23×10^7 CFU/g and 9.79×10^7 CFU/g, while at depths of 50 cm, it was 1.94×10^7 CFU/g and 1.92×10^7 CFU/g. The morphology of the bacterial colonies is dominated by circular shape, entire margin, flat elevation, and white color. 20 isolates are gram-negative and 16 isolates are gram-positive, the cell form is dominated by the bacillus.

1. INTRODUCTION

Soil is the top layer of the earth that is formed by the rock's corrosion over a long period. This process is due to the role of microorganisms. Bacteria are one of the most common microorganisms found in the soil and the most important elements in soil biological characteristics (Rahman et al., 2020). Soil bacteria also play a role in nitrogen decomposition cycles, soil fertility, and decomposers.

According to the Government Regulation of the Republic of Indonesia No. 9 of 1987, a public cemetery is land used for funeral needs, provided for each person without distinction of race, tribe, religion, and culture of an individual. In Indonesia, the size of land for the burial of bodies is provided at a maximum of 2.5 x 1.5 meters with a depth of at least 1,5 meters from the surface of the ground (Indriana, 2014). Soil in cemeteries can be a risk contributor of pathogenic bacteria causing the disease. Examples of pathogenic bacteria that can be found in the cemetery are *Bacillus* spp., *Escherichia* spp., *Enterococcus* spp., *Staphylococcus* spp., *Penicillium* spp., and *Aspergillus* spp. (Całkosiński et al., 2015).

In the cemetery, the body's decomposition process occurs actively. Decomposition is the PMI (Post-Mortem Interval) phase that occurs as a result of natural processes that cause tissue damage after death. In the process of body decomposition, the decomposition phase is divided into 5 based on changes in the body's physical, the fresh phase, the swollen stage, the active decomposition stage, and the skeletonization change phase (Putra et al., 2021).A healthy human body can release bacteria. Most of these bacteria are not pathogenic and can accelerate the

decomposition of organic matter (Żychowski & Bryndal, 2015). In the process of decomposition of bodies, bacteria play an important role in assisting the decomposition process and maintaining the functioning of soil ecosystems (Całkosiński et al., 2015). As a result of the decomposition carried out by the microorganisms, the organic material derived from the body's decomposition process will move to the soil and the bacteria will dominate the area around the decomposition process, so the bacterial diversity will increase.

The research is located at the Pracimaloyo Public Cemetery, one of the largest cemetery sites managed by the Surakarta City Government, with a total of 19 blocks of the cemetery with an altitude of 106 m. There have been many processes of decomposition of bodies at the cemetery. As a result of the body's decomposition process, nutrients, and minerals will be produced in the soil, which will affect the increase in soil bacterial populations. This research was carried out because of the limited research on soil bacteria in the cemetery. The cemetery can be a potential site for pathogenic bacteria that can contaminate soil and pose a threat to public health. (Abia et al., 2019).

In Indonesia, no research has been found on the population and diversity of soil bacteria in cemeteries. Several studies on the population and diversity of soil bacteria have been conducted in various countries, such as Poland (Całkosiński et al., 2015), South Africa (Abia et al., 2019), and Australia (Żychowski & Bryndal, 2015). This research aims to find out how the population and diversity of bacteria are present in the Pracimaloyo Public Cemetery. This research data is expected to be used as a reference related to the population and diversity of bacteria found in the Pracimaloyo Public Cemetery.

2. MATERIALS AND METHODS

2.1. Research Location

The research is located at Pracimaloyo Public Cemetery, one of the largest cemeteries managed by the Surakarta City Government, located at Makamhaji, Kartasura, Sukoharjo, Central Java. Pracimaloyo Public Cemetery has an altitude of 106 m and coordinates at 7°33'46.02" S, 110°46'25.92" E.

2.2. Tools and materials

The tools used in this research are autoclave (*GEA LS-35LJ)*, hot plate (*Cimarec*$^{+}$), magnetic stirrer, digital analytical balance (*Durascale DAB-E223)*, test tube (*Pyrex*), test tube rack, Laminar Air Flow (LAF), Erlenmeyer tube (*Pyrex*), incubator (*Memmert),* beaker glass (*Pyrex*), bunsen burner, disposable petri dish, stopwatch, sprayer, micropipette (*Socorex 10-100),* binocular microscope (*Olympus CX-21*), inoculating loops, object glass, dryer, vortex (*DLAB MX-S*), and *Colony Counter* (*Funke Gerber*). The main materials used in this research are Pracimaloyo Public Cemetery soil samples, Nutrient Agar (Merck 1.05450.0500), and a Gram stain kit.

2.3. Sampling

Sampling is carried out by digging the soil using a ground drill at blocks 8 and 18, respectively at 20 cm and 50 cm depth. The air temperature and pH soil in block 8 is 33.3°C and 5, then in block 18 is 34.8 °C and 6.5. Furthermore, soil samples are dried and mashed, then stored in plastic and marked according to the block of sampling and its depth. The soil sample is stored in the refrigerator for further use.

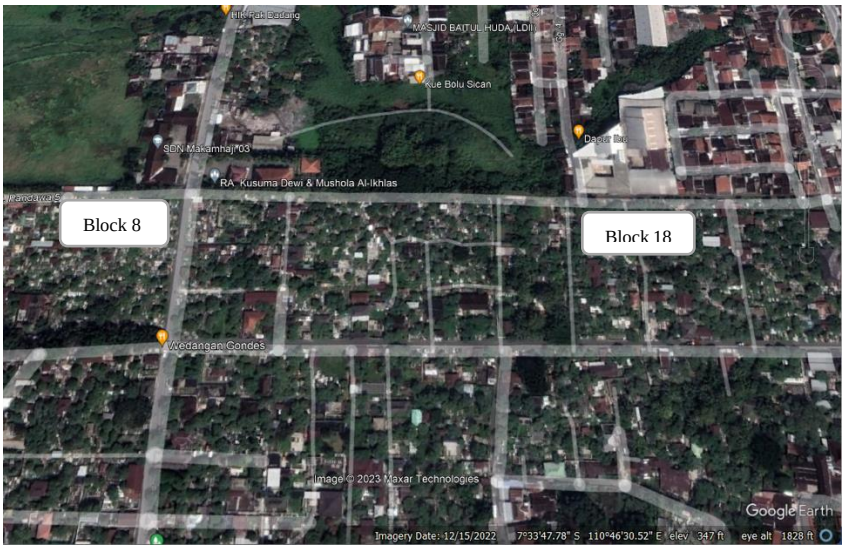

Figure 1. Sample location

2.4. Research Procedure

2.4.1. Serial dilution

Sampling dilution is one of the steps for bacterial suspension diluting intended at lowering the number of microorganisms in the suspension (Yunita et al., 2015). The dilution of the sample is carried out by adding 1 gram soil sample to the diluting reaction tube containing aquadest with a volume of 9 ml, and then the series diluting procedure is completed till the 10^{-7} dilution.

2.4.2. Bacterial isolation

Bacterial isolation is done by spread technique. The advantage of using the spread technique is that microorganisms that grow on the medium can spread evenly on the surface part of the medium (Putriawati et al., 2018). The 10^{-5}, 10^{-6}, and 10^{-7} dilution series were inoculated on a petri dish that already contained a solid nutrient-agar medium, 0.1 ml for each sample. The sample is spread using a drigalski spatula. Furthermore, the petri dish was incubated for 48 hours in the incubator at 37°C temperature (Nor et al., 2018) in reverse position.

2.4.3. Estimation of the bacterial population

The estimation of bacterial populations is done using the Total Plate Count (TPC) method by counting the colonies on the media using the colony counter. The number of bacterial populations is stated as colony-forming units (CFU) (Lestari et al., 2016). On the calculation of bacterial populations using the Total Plate Counter method, to determine the number of initial bacteria before dilution, apply the following formula (Irfan, 2014):

Colony Forming Unit (CFU) / ml = $\left(\frac{1}{sample\ volume}\right) x \left(\frac{1}{dilution\ factor}\right) x$ total $colonies$

2.4.4. Bacteria isolate purification

The purification of bacterial isolates aims to separate the outcomes of the inoculation consisting of various bacteria colonies of different types to produce the colony of pure bacteria isolate. The purification of bacteria isolates is done to obtain a desired type of pure bacteria and then be further grown on the agar slope (Marzuki et al, 2014). The bacterial isolate is purified by moving the bacteria suspension from the incubated petri dish into the agar slope. Bacteria taken

for pure bacterial culture have different morphology and color, resulting in a suspension of pure bacteria for further identification.

2.4.5. Bacteria identification

Identification of soil bacteria is done macroscopically and microscopically. The macroscopic identification of bacteria is done by observing the morphology of the bacterial colony, such as the shape, margin, elevation, and color of the colony. The microscopical identification is carried out by Gram staining. The process of Gram staining is carried out by making bacterial smears on the glass of objects, followed by adding gram A (violet crystal), gram B (mordan), gram C (alcohol), and gram D (safranin). Gram staining results were observed using a microscope to find the gram type and cell shape data from bacteria (Walida et al., 2019).

3. RESULTS AND DISCUSSION

3.1. Soil Bacteria Population

Table 1. Soil bacteria population

Block	Air temperature (°C)	Soil pH	Depth (cm)	Bacterial colonies population (*CFU/g*)
8	33.3	5	20	4.23×10^7
			50	1.94×10^7
18	34.8	6.5	20	9.79×10^7
			50	1.92×10^7

Based on Table 1, in Block 8, the highest soil bacteria population is found at a depth of 20 cm (4.23×10^7 CFU/g), and the lowest soil bacterial population is found at a depth of 50 cm (1.94×10^7 CFU/g). In Block 18, the highest soil bacteria population is found at a depth of 20 cm (9.79×10^7 CFU/g), and the lowest soil bacterial population is found at a depth of 50 cm (1.92×10^7 CFU/g).

Pracimaloyo Public Cemetery is one of the largest public cemeteries in Kartasura, there have been a lot of decomposition processes in that cemetery. Due to the process of body decomposition, the bacterial populations in the cemetery may increase with the availability of organic material. This is because most of the bacteria in the soil belong to anaerobic bacteria that rely on organic material in the soil to produce food and energy. Anaerobic bacteria play an important role in soil because they can degrade dead organic matter into elements returned to the soil such as N, P, K, Ca, Mg, and others (Zahidah & Shovitri, 2013).

Bacterial growth can be influenced by several environmental factors, such as soil temperature and soil pH (Mandala et al., 2021). Soil pH conditions determine the microorganism population in the soil. At a pH of 5.5 – 7, bacteria that decompose the organic material will grow optimally (Rukmana et al., 2020). Soil pH in the block 8 is 5 and in the block 18 is 6.5, based on this, bacteria will grow well in both blocks. The availability of organic material can also affect the bacterial populations found in the soil (Prasetya, 2021). Too much organic material in the soil will cause low oxygen levels because the decomposition process needs oxygen to happen. (Yuningsih et al., 2014). Based on this, the deeper the soil, the lower the oxygen levels in it, this is because in the cemetery there are many processes of decomposition of bodies that produce organic material. Because of the low levels of oxygen, anaerobic bacteria will be found more as they get deeper in

the soil, and aerobic bacteria will rarely be found.

The majority of bacterial populations are found on the surface of the soil with a depth of 20 cm. The least bacterial population is found in soil with a depth of 50 cm. In the research conducted by Irfan (2014), more soil bacterial populations were found on the surface (0 cm and 25 cm) compared to the depths of 75 cm and 100 cm. The use of inadequate bacterial isolation techniques often only grows the few anaerobic bacteria that are commonly found because anaerobe bacteria have significant differences in oxygen tolerance and low oxygen levels are essential for them to reproduce (Nagy et al. 2018).

Anaerobic bacteria are so sensitive to different levels of oxygen that they require excellent cultural conditions, such as anaerobe media and environments. Based on this, the reason why there is a significant difference in bacterial populations at a depth of 50 cm compared to a depth of 20 cm is that most bacteria present in the 50 cm depth soil layer belong to anaerobic bacteria and cannot be grown easily with simple bacteria isolation techniques. It requires a suitable environment for anaerobic bacteria to live and grow. In addition, the media also influences the growth of anaerobic bacteria. Mediums that do not correspond to the characteristics of anaerobic bacteria will cause inhibition of the growth of the bacteria (Ristiati et al., 2014).

3.2. Bacteria Identification

After the isolation of the bacterial colony, the result is 36 bacteria isolates that will be further observed to be identified, 30 isolates from a depth of 20 cm and 6 isolates from 50 cm. Identification of bacteria here includes observation of the morphology of bacterial colonies and gram staining. The results are shown in Table 2.

Table 2. Bacteria colonies morphology and gram staining observation result

Isolate	Colony shape	Margin	Elevation	Color	Gram type	Cell shape
P-1	rhizoid	rhizoid	flat	cream	positive	bacillus
P-2	rhizoid	rhizoid	flat	white	positive	coccus
P-3	circular	entire	flat	cream	negative	bacillus
P-4	irregular	undulate	flat	white	negative	bacillus
P-5	filamentous	lobate	flat	yellow	negative	bacillus
P-6	irregular	lobate	raised	white	negative	bacillus
P-7	irregular	entire	raised	yellow	positive	bacillus
P-8	circular	entire	raised	yellow	positive	coccus
P-9	circular	entire	flat	white	negative	bacillus
P-10	circular	entire	raised	white	positive	bacillus
P-11	circular	entire	flat	white	positive	coccus
P-12	filamentous	lobate	flat	yellow	negative	bacillus
P-13	circular	entire	raised	yellow	negative	coccus
P-14	irregular	entire	flat	white	negative	coccus
P-15	circular	entire	flat	yellow	negative	bacillus
P-16	circular	entire	raised	white	negative	coccus
P-17	circular	entire	flat	orange	negative	coccus
P-18	circular	entire	flat	yellow	negative	coccus
P-19	circular	entire	raised	cream	negative	coccus
P-20	circular	entire	flat	white	positive	bacillus
P-21	circular	entire	flat	yellow	positive	bacillus
P-22	filamentous	lobate	flat	white	negative	bacillus

Isolate	Colony shape	Margin	Elevation	Color	Gram type	Cell shape
P-23	irregular	erose	flat	white	positive	bacillus
P-24	irregular	lobate	flat	white	negative	bacillus
P-25	irregular	lobate	flat	white	positive	bacillus
P-26	rhizoid	rhizoid	flat	white	negative	bacillus
P-27	circular	entire	flat	yellow	positive	coccus
P-28	filamentous	filamentous	flat	white	positive	bacillus
P-29	circular	entire	flat	orange	negative	bacillus
P-30	circular	entire	convex	white	positive	bacillus
P-31	circular	entire	flat	white	negative	bacillus
P-32	circular	entire	raised	brown	positive	bacillus
P-33	circular	entire	flat	yellow	negative	coccus
P-34	irregular	lobate	flat	white	positive	bacillus
P-35	irregular	undulate	raised	white	negative	bacillus
P-36	circular	entire	flat	cream	positive	coccus

3.2.1. Observation of bacteria colonies morphology

Bacterial colonies are a group of bacterial cells that can be observed directly (Holderman et al., 2017). Observation of bacterial colony morphology is one of the stages in the isolation of bacteria that aims to facilitate the process of identification to determine the type of bacteria based on the characteristic of the bacterial colony. The morphology of the bacterial colony was observed by observing the shape, margin, elevation, and color of the selected bacteria colony.

In the observation of bacteria colony morphology, from the observation of the shape of the colonies, there were 20 circular colonies, 9 irregular colonies, 4 filamentous colonies, and 3 rhizoid shaped colonies. Based on the observation of the margin type of the colony, there were 21 colonies with entire margin type, 8 colonies with lobate margin type, 2 colonies with undulate margin type, 1 colony with filamentous margin type, 3 colonies with rhizoid margin type, and 1 colony with erose margin type. In the elevation type observation of the colony, there were 26 colonies with flat elevation, 9 colonies with raised elevation, and 1 colony with convex elevation. In the color morphology observation, 19 colonies were white, 10 colonies were yellow, 4 colonies were cream, 2 colonies were orange, and 1 colony was brown.

3.2.2. *Gram staining observation*

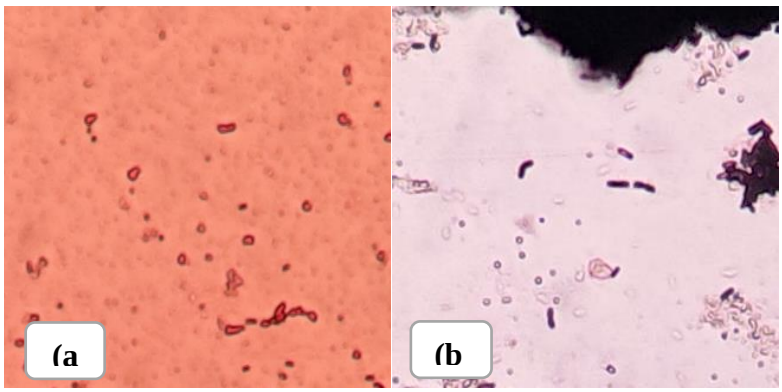

Figure 2. Gram staining result, (a) P-18 and (b) P-23

In the observation of gram staining, several variations of gram type and cell shape of soil bacteria were found. As can be seen in Figure 2, (a) is an isolate of P-18, which has a gram-negative bacterial type and coccus cell shape, and (b) is a P-23 isolate, which has a gram-positive bacteria type and bacillus cell shape. Based on Table 2, in the observation of the gram type of bacteria, 20 isolates are gram-positive bacteria and 16 isolates are gram-negative bacteria. This result is similar to Chairani et al. (2016) that there are more gram-negative bacteria found in soil than gram-positive bacteria. Furthermore, in research conducted by Fitriani et al. (2016), gram-negative bacteria are also dominant in soil from the gram staining process.

In the process of gram staining, the bacteria cells will become purple color when the bacteria are gram-positive bacteria and the bacteria cells will be red when they are gram-negative bacteria (Rohde, 2019). The difference between gram-positive and gram-negative bacteria is that gram-positive bacteria have thick cell walls and thin cell membranes. gram-negative bacteria have a thick layer of cell membrane without the presence of cell walls in the outer layer (Paquete, 2020).

Based on their shape, bacterial cells are divided into three basic forms, namely round (coccus), rod shape (bacillus), and spiral (spirilla) (Sohilauw, 2023). In the cell shape observation, there are 24 bacillus isolates and 12 coccus isolates. This result is similar to Li et al. (2022) *Bacillus* is the dominant form of bacteria found in the soil. In addition, the research of Dewi et al (2017) stated that bacillus is also the dominant form of bacterial cells found in gram staining. This can happen because the bacillus has the ability to form endospores as a form of adaptation to protect bacteria from extreme threats in the soil (Zeigler & Nicholson, 2017), such as changes in nutrient level, water, and temperature (Handayani et al., 2023).

4. CONCLUSIONS

The Pracimaloyo Public Cemetery had an average bacterial population of 4.47×10^7, with block 18 at a depth of 20 cm having the highest bacterial population (9.79×10^7). Different bacterial colony shapes, margins, elevations, and color morphologies were discovered based on the 36 isolates that were observed. Additionally, the majority of the isolates belong to the gram-negative type of bacteria at 55.56% percent and bacillus shape at 66.67 % percent. More research is required to understand more of the anaerobic bacterial population in the cemetery.

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