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Selection of Proteolytic Bacteria from Bonoloyo Public Burial Place (PBP), Banjarsari, Surakarta, Central Java

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Abstract: *Public Burial Place (PBP) is a cemetery for everyone. In PBP, protein decomposition occurs actively because the human body is made up of 16.4% protein, so it is most likely that proteolytic bacteria can be found. Previous research has isolated as many as 45 isolates from PBP Bonoloyo, Surakarta, but has not selected their proteolytic capabilities. The aim of the study was to isolate proteolytic bacteria from the Bonoloyo Public Cemetery and identify them. The bacterial isolate of PBP Bonoloyo is a collection from the FKIP Biological Laboratory of Muhammadiyah University of Surakarta that selected its proteolytic capabilities using the milk agar scheme (SMA). The parameters measured are the diameter of the colony and the lymphatic zone formed to obtain the value of the proteolytic index (IP). For bacterial isolates showing a positive proteolytic activity, simple identification is carried out through the observation of colonial morphology and Gramme colouring. The results of the study showed that as many as 28 isolates (62.2%) were positive for proteolytic activity, with IP values >2 for B1 and B3. Bacterial proteolytic isolates are dominantly white to yellow with irregular, filamentary, spindle, and circular colonial forms. The results of Gramme colouring show that bacterial isolates that have proteolytic abilities are a group of Gramme negative bacteria that are basil shaped. The bacterial isolate that shows the highest IP value is the B1 isolate, which is suspected to be of the genus Bacillus. This research obtained data showing that in the public cemetery (PBP), Bonoloyo stores potential as a proteolytic bacterial habitat for a group of Gramme negative bacteria.*

Keywords: *Proteolytic Bacteria; Proteolytic Index; Public Burial Place (PBP); Milk Agar Scheme (SMA); Bacterial Colonies*

1. INTRODUCTION

Public Burial Place (PBP) is an area for burial for everyone, regardless of religion and certain groups, management is carried out by local government level II or village level government (Nurfazri et al., 2021). Each religion has its own rules regarding the order of funeral processes that have been adjusted to the teachings of their respective religious beliefs. In Islam, the body is wrapped in a shroud and buried into a grave 1.5-2 m deep. Christian bodies are given 70% formalin injections to kill bacteria and are fully clothed and then put in coffins and buried (Winarni, 2018). Ethnic Chinese believe in death ceremonies by cremation or burial.

The human body consists of 40% solid substances such as proteins, carbohydrates, minerals, fats, organic and inorganic substances. According to Brochek J, protein and fat make up the human body by 16.4% and 15.3% (Yuliasih &; Nurdin, 2020). The process of decomposition of the corpse occurs in less than 48 hours so that it experiences damage to body structure (decomposition) due to enzymes, bacteria in the body, and organisms from outside the body of the corpse (Try Upayogi, 2019). After death, the body

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temperature of the corpse is cold and stiff ranging from 28-29°C. Decay begins with the spread of bacteria in the digestive system and visible signs of decay in the lower right abdomen are turquoise (Parinduri, 2020). The process of decomposition of the corpse body can be observed starting from 5 physical changes, namely in the form of a fresh phase (*autolysis*), extended swelling (*Bloat*), decay in the active body (*active decay*), decay in advanced stages (*advanced decay*), and bone decay (Brilliana Putra et al., 2020). The decomposition of the corpse produces gases such as methane, carbon dioxide, hydrogen sulfide, and nitrogen. In addition, in the form of fatty acids, sulfur, amino acids, methane, ammonium compounds, mercaptans, ammonia, and nitrates included in organic matter (Fineza et al., 2014). When a living creature has died, it can cause its basic composition to disintegrate and decompose into a simpler composition (Indrayani et al., 2021).

The results of the decomposition of the corpse in the form of organic compounds and minerals will enrich soil nutrients so that the population of Cambodian tree rhizosphere soil bacteria at PBP Pracimoloyo is abundant. The population of Cambodian rhizosphere bacteria in PBP Pracimaloyo ranges from 1.9-10.4 x 106 CFU/g (Putra et al., 2023). Because at PBP it was reported that there was an active decomposition of the body (Majgier et al., 2014), then there must be decomposing microorganisms such as fungi and bacteria, among others. Decomposing microorganisms that may be found in PBP are lipolytic and proteolytic microorganisms due to the presence of lipids and proteins that make up the human body as substrates.

Proteolytic bacteria are bacteria that can degrade proteins because they are able to produce extracellular protease enzymes (Puspitasari et al., 2012). Proteolytic bacteria can grow at pH conditions between 2.5-8.5 and can survive at a salinity of 35 ppt (Oktavia et al., 2022). In previous studies, protease enzyme sources came from animals, bacteria, and plants. The largest source of protease enzymes is plants (43.85%), followed by bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%), and viruses (4.41%) (Mahajan & Badgujar, 2010). Thus, these advantages make plant proteases a top choice for medical, food, pharmaceutical, as well as biotechnology (Mehrnoush et al., 2011). The application of protease enzymes is very wide because it has high economic value in industries including leather, textiles, detergents, food industry, dairy processing, collagen production, and industrial waste treatment (Razzaq et al., 2019). The advantages of using bacteria to become protease producers are due to affordable production costs, the use of environmentally friendly sources, and easy productivity increases.

Research aimed at exploring proteolytic bacteria has been carried out by calculating the Proteolytic Index (IP), including derived from peat soils (Mahdiyah, 2015), mangrove forest land (Hastuti Sri et al., 2017), Tofu liquid waste (Asril &; Leksikowati, 2019), and on a 72-hour post-fermentation red oncom (Lestari et al., 2018). From several studies, potential proteolytic bacteria with varying IP values were obtained. Five isolates derived from peat soils have proteolytic capabilities with IP 3 (Mahdiyah, 2015), from mangrove forest soil 1.62-2.94 and one of them was identified from the species *Vibrio parahaemolyticus* (Hastuti Sri et al., 2017), from tofu liquid waste 3.20 (Asril &; Leksikowati, 2019). The formation of a clear zone with a diameter of 78 mm around the bacterial colony showed that protease activity of bacterial isolates from red oncom samples after 72 hours of fermentation (Lestari et al., 2018).

From previous studies, bacterial isolation has been carried out from PBP Bonoloyo and 45 pure bacterial isolates have been collected. These isolates have never been further characterized, including their proteolytic potential. Exploration of proteolytic bacteria originating from PBP in Indonesia has never been found based on searching for publication articles, even though in PBP there is active protein decomposition.

PBP Bonoloyo is one of the largest cemeteries in Surakarta City managed by the City Government with a land area of 28 ha. This PBP has uneven soil contours that vary between 112-143 meters above sea level and are divided into several blocks. The potential stored in PBP related to the presence of proteolytic bacteria is very necessary because of its utilization value in various fields. Thus, based on the description above, a study was conducted that aimed to select isolates of soil bacteria from PBP Bonoloyo that have the ability to decompose proteins (proteolytics) and carry out simple identification.

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2. RESEARCH METHODS

This study used a non-experimental type to select bacterial isolates from burial grounds that have proteolytic abilities. The research place is in the Microbiology Laboratory of the Biology Education Study Program, FKIP, University of Muhammadiyah Surakarta. The research period was conducted from January 2023 to April 2023.

Materials and Instruments

The tools needed in this study include petri dishes, *hot plates* (Cimarec*), ovens (Maspion*), sprayers, incubator ovens *(Memmert*), oses, vortexes, erlenmeyer *(Pyrex), matches (*Tokai), *micropipettes* (Socorex 10-100*), digital analytical balances* (Durasclae DAB-E223*), spirtus* burners, autoclaves (*GEA LS-35LJ),* measuring cup (*Pyrex Iwaki),* mini box studio, refrigerator *(Sharp*), Laminar Air Flow *(LAF),* blue tip, *driglaski*, *pH meter*, Olympus CX21 binocular microscope, *object glass*, test tube rack, label paper, *test tube*, *stationery*, and documentation tools while the main material in this study is bacterial isolate from PBP Bonoloyo as many as 45 isolates were collected by the Biology Laboratory of the Biology Education Study FKIP University of Muhammadiyah Surakarta, aquades, nutrient agar media (*Merck*), *Milk Agar Scheme* (SMA), and Gram staining dyes.

Proteolytic Bacteria Screening

The screening process on proteolytic bacteria is carried out with media *Milk Agar Scheme* (SMA). Bacterial isolates that have been subcultured are then inoculated on a proteolytic selective medium, each carried out 2 repetitions. After incubation for 2 x 24 hours at 37°C. The ability to decompose proteins is characterized by the formation of a clear zone around the colony. The calculation to obtain the value of the Proteolytic Index (IP) uses the following formula: (Hastuti Sri et al., 2017; Ibrahim et al., 2015).

$$PI = \frac{diameter\ of\ clear\ zone}{diameter\ of\ bacterial\ colonies}$$

Bacteria Characterization

Macroscopic observation of proteolytic bacterial isolates can be observed by looking at the shape, edges, surface, and color of colonies in bacteria while microscopic observations can see from the shape, size, and coloring of cells, namely Gram staining and endospores (Muharni et al., 2013). Gram-positive bacteria can retain the purple color of the crystalline substance violet iodine even after washing with alcohol. In contrast, Gram negative bacteria are red because the crystalline substance violet iodine can dissolve in alcohol solution and produce the red substance safranin (Nurhayati &; Martindah, 2015).

3. RESULTS AND DISCUSSION

Based on selection testing of proteolytic bacteria using *Milk Agar Scheme* (SMA) media showed as many as 28 isolates (62.2%) positive for proteolytics (Table 1) indicating the presence of a clear zone around the colony (Figure 1).

Table 1. Results of Proteolytic Bacteria Capability Screening from Bonoloyo Public Burial Place (PBP), Banjarsari, Surakarta City, Central Java

Bacterial Codes	Diameter of Colony (cm)	Diameter of Clear Zone (cm)	Proteolytic Index (PI)
B1	1,4	5,9	4,2
B2	1,55	3,25	2,4
B3	3,85	4	3,9
B4	1,4	3,25	2,3
B5	2,15	3,3	2,7
B6	2,3	3,4	2,8
B8	1,8	3,7	2
B9	1,75	3,25	1,8
B10	0,35	1,2	3,4
B11	1,25	3,35	2,7
B12	1,5	3,9	2,6
B13	2	2,45	1,2
B14	1,4	2,85	2
B15	2,55	3,75	1,5
B16	1,7	3,35	2
B20	2,15	2,55	1,2
B24	0,9	3,15	3,5
B26	2,65	3,6	1,3
B27	2,6	3,85	1,5
B28	1,75	3,25	1,8
B30	1,9	3,25	1,7
B31	1,65	2,6	1,5
B32	0,8	1,55	1,9
B37	2,85	3,15	1,1
B39	2,05	3,4	1,6
B40	2	2,2	1,1
B41	0,75	1,75	2,3
B42	0,6	1,2	2

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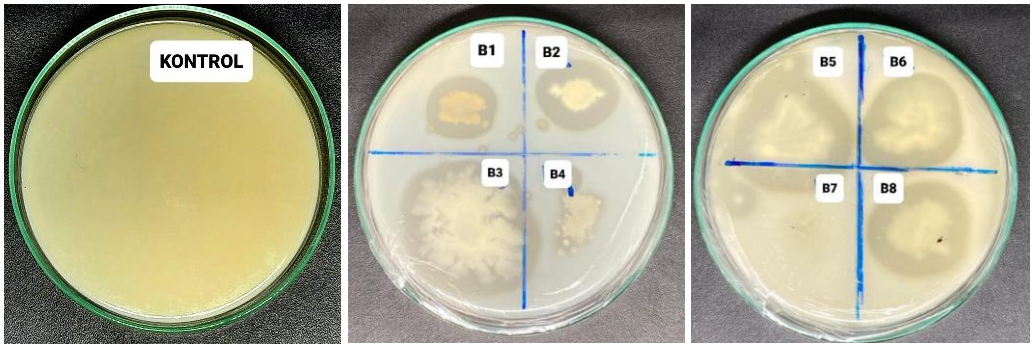
Comparison of the size of the clear zone formed with the bacterial colony will be obtained Proteolytic Index (PI) which shows the potential of the bacterial isolate in hydrolyzing proteins. The resulting Proteolytic Index (PI) value ranges from 1.1-4.2. In isolates of bacteria B1 and B3 showed PI> 2 values, 4.2 and 3.9 respectively (Table 1).

A total of 62.2% of bacterial isolates have shown proteolytic activity. This percentage is more than the percentage of bacterial proteolytic activity from mangrove soil in Margomulyo, Balikpapan (50%) (Hastuti Sri et al., 2017) and from red oncom after 72 hours of fermentation (Lestari et al., 2018). The large number of isolates that have been detected as proteolytic bacteria from PBP is likely related to the condition of the presence of substrates, namely the human body containing protein by 16.4% (Yuliasih &; Nurdin, 2020). In addition, in common burial places active processes of protein decomposition occur. This happens because the body's decay process lasts less than 48 hours as the bacteria spread through the digestive system and the lower right corner of the stomach shows visible signs of decay in turquoise color (Parinduri, 2020). In PBP ecosystems, these proteolytic bacteria break down proteins into oligopeptides and amino acids.

The media used for screening proteolytic abilities are: *Milk Agar Scheme* (SMA). This media contains casein as a source of nitrogen and lactose as a source of carbon. *Milk Agar Scheme* (SMA) which is bone white and yellowish indicates that the casein contained is quite high. Casein is a milk protein consisting of phosphoproteins that are bound to calcium to form calcium caseinate (Linda et al., 2015). Bacteria that have proteolytic ability in media *Milk Agar Scheme* (SMA) will form a clear zone around the bacterial colony (Figure 1). The clear zone formed will increase during the incubation period. The presence of a clear zone indicates the results of bacterial isolation can use protein as a source in the media (Badriyah et al., 2013). This is because proteolytic bacteria produce proteases that can hydrolyze casein. In the process of casein hydrolysis, protease and peptidase enzymes are used to be able to detect the activity of these hydrolytic enzymes.



(a) **(b)** **(c)**

Figure 1. Screening results of proteolytic ability of bacterial isolates from Bonoloyo Public Burial Place (PBP): (a) Control of *Milk Agar Scheme (*SMA) medium, (b) Clear zone between isolates, (c) Differences in the size of the clear zone of each isolate.

Proteolytic Index (PI) values indicate variations in the ability of bacterial isolates in the process of breaking down protein substrates into amino acids. The calculation of PI values from 28 isolates is classified into the high category. Proteolytic index measurements can be successfully measured well if they fall into the high (>3.1 mm), medium (2.1-3.1 mm), and low (<2.1 mm) categories (Ahmad et al., 2013). Based on PI values, B1 and B3 isolates have higher PI values of 4.2 and 3.9 respectively than isolates from Mangroves on Neolbaki Beach by 1.9 mm (Lobo et al., 2022). The proteolytic ability of B1 and B3 isolates is also stronger than bacterial isolates as a starter in livestock waste treatment which has an PI value between 0.40-0.57 (Yahdiyani et al., 2021). PI values can experience significant changes or differences due to activity

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during the incubation period of bacteria on the test media.

Positive proteolytic bacterial isolates were observed with colony morphology (Table 2) and cell morphology and bacterial staining (Table 3)

Table 2. Morphology of Proteolytic Bacterial Isolate Colonies from Bonoloyo Public Burial Place (PBP) Banjarsari Area, Surakarta City, Central Java

Isolate Codes	Shape	Edge	Elevvasis	Color
B1	Irregular	Undulate	Umbonate	Yellowish
B2	Irregular	Undulate	Convex	White
B3	Filamentous	Lobate	Umbonate	White
B4	Irregular	Lobate	Convex	White
B5	Irregular	Undulate	Convex	White
B6	Irregular	Undulate	Convex	White
B8	Irregular	Undulate	Convex	White
B9	Irregular	Undulate	Pulvinate	White
B10	Irregulate	Undulate	Convex	Yellowish
B11	Irregular	Undulate	Convex	Yellowish
B12	Spindle	Entire	Convex	Yellowish
B13	Irregulate	Lobate	Convex	White
B14	Irregular	Lobate	Convex	Yellowish
B15	Irregular	Undulate	Umbonate	White
B16	Spindle	Entire	Convex	White
B20	Irregular	Entire	Pulvinate	White
B24	Circular	Entire	Convex	White
B26	Irregulate	Undulate	Umbonate	White
B27	Irregulate	Undulate	Convex	White
B28	Irregular	Undulate	Flat	White
B30	Irregular	Undulate	Convex	White
B31	Irregulate	Lobate	Convex	Yellowish
B32	Irregulate	Undulate	Flat	White
B37	Irregular	Entire	Convex	White
B39	Irregular	Undulate	Convex	White
B40	Irregular	Undulate	Convex	White
B41	Circular	Entire	Convex	White
B42	Circular	Entire	Convex	Yellowish

The morphology of bacterial colonies is dominated by white to yellowish color with colony shape *irregular*, *filamentous*, *spindle*, and *circular* While at various elevations such as *umbonate*, *convex*, *pulvinate*, and *flat* (Table 2). Microorganisms that have grown in different media, will show different macroscopic shapes when growing. Culture characteristics are used as the basis for the division of microorganisms into taxonomic group categories (Sabdaningsih et al., 2013). The results showed as many as 28 isolates of bacteria macroscopically, most of which were shaped *irregular* and isolates that have been isolated show bone-white characteristics. This is due to high levels of casein in the media *Milk Agar Scheme* (SMA) with characteristic bone white and yellowish color (Linda et al., 2015)

Table 3. Results of Gram Staining of Soil Proteolytic Bacteria Isolate in Bonoloyo Banjarsari Public Burial Land (PBP), Surakarta City, Central Java

Isolate Codes	Cell Shape	Gram
B1	Bacillus	-
B2	Bacillus	-
B3	Bacillus	-
B4	Bacillus	-
B5	Bacillus	-
B6	Bacillus	+

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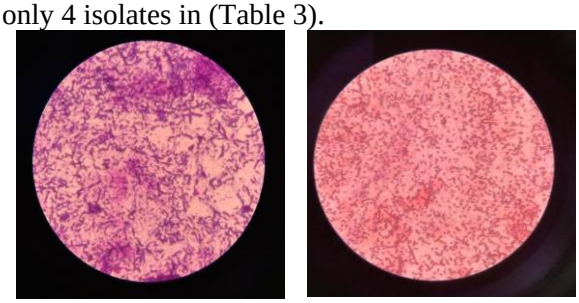
Isolate Codes	Cell Shape	Gram
B8	Bacillus	-
B9	Bacillus	+
B10	Bacillus	-
B11	Bacillus	-
B12	Bacillus	-
B13	Bacillus	-
B14	Coccus	-
B15	Bacillus	+
B16	Bacillus	-
B20	Coccus	-
B24	Coccus	-
B26	Bacillus	-
B27	Coccus	-
B28	Bacillus	-
B30	Bacillus	-
B31	Coccus	-
B32	Bacillus	+
B37	Coccus	-
B39	Bacillus	-
B40	Coccus	-
B41	Coccus	-
B42	Coccus	-

Microscopic observations of Gram staining obtained isolates of proteolytic bacteria from PBP Bonoloyo which is a group of Gram-negative bacteria so that it shows the cells are red (Figure 2. b). Gram negative bacteria can form a color due to cell wall components composed of lipid layers so that the main dye gram C cannot be maintained when washed, which is crystal violet (Gram A). This group of bacteria gives the red color of the second dye, safranin (Gram D) and at the end of the Gram stain (Rahmadian et al., 2018). The bacterial isolates identified as Gram positive were only 4 isolates in (Table 3).



(a) **(b)**

Figure 2 Gram Staining Results of Proteolytic Bacteria Isolate from Bonoloyo Public Burial Ground: (a) gram positive (b) gram negative.

Proteolytic bacterial isolates from soil at PBP Bonoloyo are mostly shaped *irregular* and includes the Gram-negative group (Table 3). Plastic degrading waste soil bacteria are PPs 3 which is a Gram negative group *Trees* (Ainiyah & Shovitri, 2014). Proteolytic bacterial isolates from PBP Bonoloyo with high IP namely B1 have colony morphology *irregular*edge *undulate*, elevated *umbonate* while B3 has colony morphology *filamentous*edge *lobate*, Elevation *umbonate* which belongs to the Gram-negative shaped group *basil.* The bacterial isolate is thought to be a genus *Bacillus* and *Pseudomonas*. This is in accordance with the results of characterization that proteolytic bacteria from septic tanks originating from genera include: *Bacillus, Pseudomonas, Streptococcus, Proteus, Streptobacillus, dan Staphylococcus* (Puspitasari et al., 2012).

4. CONCLUSION

Bonoloyo Public Burial Ground is a habitat for soil bacteria that have the potential to be proteolytic as much as 62.2% with the highest Proteolytic Index (PI) value of 4.2 (B1 isolate). Proteolytic potential bacteria have irregular colony character, *undulate edge*, umbonate *elevation* and are Gram negative *bacilli.*

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