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Public Cemetery's Potency as The Source of Proteolytic Bacteria

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Abstract: *Cemetery is an area provided for burial and acts as an active bodies disposal site. One group of bacteria which is likely to be found with a high population is the proteolytic bacteria because the human body is made up of 46.85% protein. The exploration of proteolytic bacteria from public cemetery in Indonesia has not been conducted as so this research is necessary and become the main objective of this study. This research is a non-experimental study to isolate soil bacteria from Pracimaloyo public cemetery in the district of Kartasura. Skim Milk Agar (SMA) is used as selective media. Proteolytic capacity was determined using the proteolytic index (PI). Potential proteolytic bacteria isolates were identified based on morphology features and PI values > 2 were isolated from P23 and P22 isolates. Bacterial proteolytic isolates are dominantly colonies of white to yellowish with irregular and circular shapes. Gram-staining results indicated that bacterial isolates with proteolytic abilities are belong to Gram-negative bacili bacteria. The highest PI value of bacterial isolates exhibited by P23 isolates and possibly belong to the genus Bacillus. The study has discovered that Pracimaloyo holds the potential as a proteolytic soil bacteria habitat dominated by the Gram-negative group.*
Keywords: *Bacteria, proteolytic, cemetery*

1. INTRODUCTION

Public cemetery is a ground area provided as burial site for every citizen of any religion and class (PP Republik Indonesia, 1987). Corpse burial procedures in Indonesia are diverse. Corpses of Moslem are wrapped in cotton fabric, buried in 1.5 m depth and 2.1x1.5 wide (the wide of the burial site is customized to the corpse body size). For Christians, the corpse is preserved with formalin 70%, wrapped in a full wardrobe and laid inside a coffin. Buddhist is similar to Christian but without formalin preservation, meanwhile Hindu is wrapped in brand new fabric or wardrobe and being cremated (Winarni, 2018).

Human body is composed of 50.54% water, 46.85% protein, 13.50% fat, 0.14% calcium, 6.7% phosphor and 25.51% dry materials (Forbes *et al*., 1953). Corpse decomposition by bacteria happens less than 48 hours after time of death, starting with the dispersal of bacteria inside digestive system to promote decaying and later shows green coloration at lower part of abdomen (Di Maio, 1993). The decomposition of corpse happens in five stages which are fresh (autolysis), bloat, active decay, advanced decay and skeletonisation. It takes 8-12 years in general to decompose human corpse buried in the ground and 50 years if it's kept inside a coffin (Dent et al., 2004). Another report related to corpse decomposition inside a coffin in US informed that skeletonisation happens after two years of burial (Alfsdotter et al., 2022). Skeletonisation stage is highly affected by the burial depth, temperature, rainfall, and sunlight exposure (Spaulding, 2020).

Public cemetery has been reported as an active decomposition site (Majgier *et al*., 2014). One group of decomposer bacteria which is possible to be found in high population is the

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proteolytics, since human body is composed of 46.85% protein (Forbes *et al*., 1953). This protein becomes proteolytic bacteria subsrate for its protease activity.

Protease secreted by proteolytic bacteria is beneficial in industry, pharmacy, and food processing. Protease is applied in the industry of detergent, leather, textile, and food processing (Susanti *et al*., 2019). In pharmacy industry, protease is applied as wound cleanser, cosmetic, collagen production and other chemicals industry (Li *et al*., 2013). In food processing, protease is applied in milk processing such as cheese production (Ningsih, 2021). This type of protease is applied to coagulate milk protein before it's being fermented into cheese. Among the benefits of using bacteria as the producer of protease are affordable, quick, and saving place.

Many researches have been calculating Proteolytic Index (PI) in order to explore proteolytic bacteria, some are isolated from peat soil (Mahdiyah, 2015), mangrove soil (Hastuti, 2017), rhizosphere soil of Talango village (Utomo, 2014) and soil from bean farming (Fitriana, 2021). Some potential proteolytic bacteria with various PI values were successfully isolated as the result of mentioned researches. The highest PI value of peat soil isolates is 3 (Mahdiyah, 2015), mangrove soil highest PI value is 2.94 and identified as *Vibrio parahaemolyticus* (Hastuti, 2017), rhizosphere soil of Talango village's highest PI value is 3.3 and belong to genus *Aeromonas* (Utomo, 2014) and the highest PI value of rhizospheric bacteria isolated from bean farming soil is 2.54 (Fitriana, 2021). Azotobacter is also known as the protein-degrading bacteria (Firdausi and Zulaika, 2015).

An exploration on proteolytic bacteria isolated from public cemetery has not been conducted. Public cemetery is one of many active organic decomposition sites, particularly protein decomposition and has a potency of storing potential proteolytic bacteria. Pracimaloyo Public Cemetery is the second largest public cemetery (14.5 ha) after Bonoloyo Public Cemetery which is managed by the City Government of Surakarta. Proteolytic bacteria are very useful and beneficial in many scopes so that further exploration of this group of bacteria is necessary. The objective of this research is to select soil bacteria collected from Pracimaloyo Public Cemetery with proteolytic ability and identify them using simple methods.

2. RESEARCH METHOD

This research considers as exploration to select bacterial isolates collected from cemetery soil with proteolytic potency. Soil samples were collected from 20cm and 50cm depth. Research was conducted at Microbiology Laboratory of Biology Education Department, Faculty of Teaching Training and Education Universitas Muhammadiyah Surakarta from Desember 2022 to March 2023.

Materials and Instruments

Instruments : hot plate (*Cimarec*), sprayers, petri dishes, oven (*Maspion*), incubator (*Memmert*), vortex, erlenmeyers flask (*Pyrex*), micro pipette (*Socorex 10-100*), digital scale (*Durascale DAB-E223*), matches(*Tokai*), rubbing alcohol burner, ose, autoclave (*GEA LS-35LJ*), measuring glass (*Iwaki*), mini studio, refrigerator (*Sharp*), *Laminar Air Flow* (LAF), blue tip, drygalski, binocular microscope (*Olympus*), object glass, pH meter, test tubes and test tubes rack. The main material for this research is 36 bacterial isolates collected from Pracimaloyo Public Cemetery, district of Kartasura (collection of Biology Laboratory FKIP UMS), Nutrient Agar (*Merck*), Skim Milk Agar (*Merck*) and Gram staining dyes.

Proteolytic Bacteria Screening

Proteolytic bacteria screening was conducted using Skim Milk Agar (SMA) media. Purified bacterial isolates are then transferred into petri dishes containing SMA media using ose needle. Incubation was conducted at 37^0C for 48 hours. Proteolytic ability was identified with the formation of clear zones around the bacterial colonies. Calculation of Proteolytic Index was using the formula below (Ibrahim *et al*., 2015).

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

Bacterial Characterization

Bacterial characterization was performed using morphological and microscopic observation. Observation on colonies morphology including margin, elevation and colors. Microscopic observation was performed with standard Gram Staining procedure (Cappucino & Welsh, 2019). Gram staining results were observed under the microscope using 1000x magnification. Purple

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bacterial cells were classified as Gram positive, while red or pink bacterial cells were classified as Gram negative.

3. RESULTS AND DISCUSSION

This research succeeded in isolating 36 bacterial isolates collected from Pracimaloyo Public Cemetery to examine its proteolytic ability. Screening on proteolytic ability on Skim Milk Agar media was resulting with 21 bacterial isolates (58.3%) were able to hydrolyses protein which was identified with the formation of clear zone around the colonies (Fig.1). The comparison between clear zone size and bacterial colonies size was used to calculate the Proteolytic Index to show bacterial isolate potency in hydrolyzing protein. PI values range from 1.08 to 2.08. P22 isolates and P23 isolates showed PI value > 2, which are 2.03 and 2.08 respectively (Table 1).

Table 1. Screening result on proteolytic ability of soil bacteria isolated from Pracimaloyo Public Cemetery, Kartasura

Bacterial codes	Diameter of colony (cm)	Diameter of clear zone (cm)	Proteolytic Index (PI)	Annotate
P1	3.15	3.75	1.19	20
P3	2.1	3	1.42	20
P4	1.5	2.35	1.56	20
P5	4.2	4.55	1.08	20
P6	2.75	1.65	1.66	20
P7	2.9	3.5	1.20	20
P10	2.5	3.75	1.5	20
P11	1.6	2.8	1.75	20
P12	1.45	2.65	1.82	20
P13	3.25	3.75	1.15	20
P14	1	1.8	1.8	20
P15	2.85	3.3	1.15	20
P16	2.4	3.95	1.64	20
P21	2.2	3	1.36	20
P22	1.35	2.8	2.03	20
P23	1.32	2.75	2.08	20
P24	2.4	3.2	1.33	20
P26	1.49	2.9	1.95	20
P28	3	3.6	1.20	20
P34	2.4	3.7	1.54	50
P35	2	2.9	1.45	50

Ann : 20 (20cm depth); 50 (50cm depth)

Skim Milk Agar was applied as the media for proteolytic ability screening. This type of media is composed of the main nutrient, lactose as the source of carbon and casein as the source of nitrogen. Casein level on SMA media was high enough to appear yellowish or broken white. Casein is a milk protein composed by phospho-protein (Susanti *et al*., 2019). Bacteria which performed proteolytic ability on SMA media would form clear zones around the colony (Fig. 1). The clear zone represented the ability of bacterial isolates to utilize protein of the media as its nutrient source (Asril & Leksikowati, 2019). This phenomenon happened due to the hydrolysis of casein by protease produced by proteolytic bacteria. Hydrolysis of casein was used as the indicator of hydrolytic activity of protease and peptidase.

As many as 58.3% of bacterial isolates performed proteolytic activity, which is higher compared to proteolytic activity of peat soil at Margomulyo, Balikpapan for only 50% (Hastuti, 2017) and rhizospheric bacteria of bean plant for only 53.8% (Fitriana, 2021). The number of isolates detected as proteolytic bacteria collected from cemetery soil was possibly related to the presence of protein containing substrate. It is also reported that the decomposition of the body is highly active in Public cemetery (Majgier *et al*., 2014) since the time human was declared dead, the bacterial decomposition will soon take place in less than 48 hours (Di Maio, 1993). The presence of proteolytic bacteria is very important for the Public Cemetery ecosystem for its role on decomposing protein into olygopeptides and amino acids (Agus *et al*., 2014).

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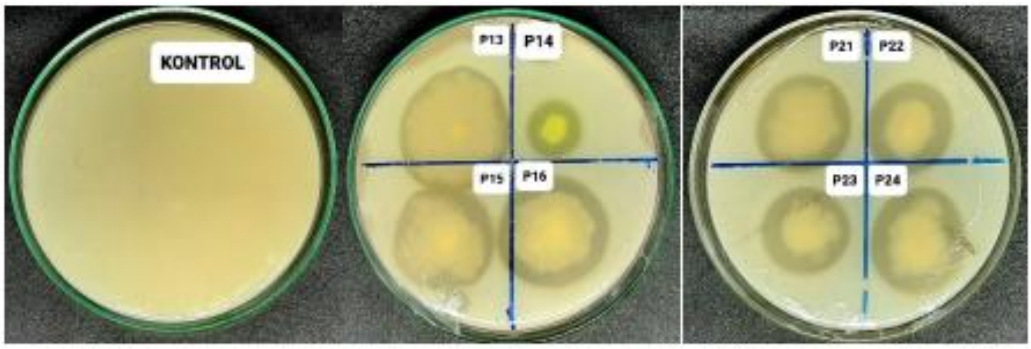

Figure 1. Screening result on proteolytic ability of soil bacteria isolated from Pracimaloyo Public Cemetery: (a). control medium Skim Milk Agar (SMA), (b) clear zone among isolates, (c) the size different of clear zone performed by each isolate.

PI value shows various ability of bacterial isolates in breaking down protein into amino acids. The calculation of PI value from 21 isolates was categorized low (<2.1) to medium (2.1-3.1) (Ahmad *et al*., 2013). This activity or PI value was lower than the PI value of bacteria isolated from bean plant farming soil which is 2.54 (Fitriana, 2021) and bacteria isolated from mangrove soil which is 2.94, identified as *Vibrio parahaemolyticus* (Hastuti, 2017). The PI value of P23 and P22 are higher than NS-3 and NS-6 isolates, which were isolated from cow feces and mixture of hay as organic fertilizer, with PI value 0.57 and 0.49 respectively (Yahdiyani *et al*., 2021). PI value was altered according to the activity of bacteria during incubation on culture media.

There are 19 selected proteolytic bacteria isolated from 20cm depth and 2 were isolated from 50cm depth (Table 1). This result could not be a basis for drawing a conclusion that the isolates closer to the corpse performed higher proteolytic activity. The 20cm depth samples showed more numbers of population compared to those from 50cm depth (data has not been published yet). The discovery is aligned with the result of Lisa, 2020 research on peat soil.

Bacterial isolates which positively perform proteolytic ability have been observed for its morphological features and through Gram staining. The results are shown in Table 2.

The morphology of bacterial colonies was dominated by white to yellowish color, irregular and circular shape, and various elevations which are flat, raised, convex and pulvinate (Table 2). Microscopic observation through Gram staining showed that bacterial isolates with proteolytic ability are Gram negative bacili and only 2 isolates are Gram positive (Table 2).

Table 2. Morphological features of the colony and Gram staining result of soil bacteria isolated from Pracimaloyo Public Cemetery

Isolate Codes	Shape	Margin	Elevation	Colors	Cell shape	Gram
P1	Irregular	Undulate	Convex	White	Bacil	-
P3	Irregular	Undulate	Flat	White	Coccus	-
P4	Irregular	Undulate	Flat	White	Coccus	-
P5	Irregular	Undulate	Flat	White	Bacil	-
P6	Irregular	Undulate	Flat	White	Coccus	-
P7	Irregular	Undulate	Flat	White	Bacil	+
P10	Circular	Entire	Flat	White	Coccus	-
P11	Circular	Entire	Flat	White	Coccus	-

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Isolate Codes	Shape	Margin	Elevation	Colors	Cell shape	Gram
P12	Irregular	Entire	Flat	White	Bacil	-
P13	Irregular	Undulate	Pulvinate	Yellowish	Coccus	-
P14	Irregular	Undulate	Flat	Yellowish	Coccus	-
P15	Irregular	Undulate	Raised	Yellowish	Bacil	-
P16	Irregular	Undulate	Raised	Yellowish	Coccus	-
P21	Circular	Entire	Flat	White	Coccus	-
P22	Irregular	Lobate	Flat	White	Coccus	-
P23	Circular	Entire	Flat	White	Bacil	-
P24	Irregular	Undulate	Flat	White	Bacil	-
P26	Irregular	Entire	Flat	Yellowish	Coccus	-
P28	Irregular	Undulate	Flat	Yellowish	Coccus	-
P34	Irregular	Entire	Flat	Yellowish	Bacil	+
P35	Irregular	Entire	Flat	White	Coccus	-

The highest PI value was shown by P23 isolates with circular shape, entire margin, flat elevation, white color, bacillus shape, Gram negative and possibly belong to genus Bacillus. Study on bacterial community at cemeteries in South Africa using Illumina ® Miseq platform showed the dominant genus of Pseudomonas, Bacillus, Corynebacterium, Mycobacterium, Staphyllococcus and Streptococcus (Abia et al., 2019). Those bacteria are also classified as proteolytic bacteria (Rizaldi *et al*., 2018). Proteolytic bacteria also act as probiotics (Anbari *et al*., 2022) that support human health (Widiyaningsih, 2011). For further research, identification on potential proteolytic isolates should be conducted down to species level based on 16S rRNA.

4. CONCLUSION

Pracimaloyo Public Cemetery is inhabited by 58.3% of potential proteolytic soil bacteria with the highest PI value of 2.08 (P23 isolates). Morphological features of proteolytic bacteria colonies isolated from Pracimaloyo Public Cemetery are irregular and circular shape, flat; raised; convex and pulvinate elevation, white to yellowish color and belonging to Gram negative group.

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