KARAKTERISTIK MORFOLOGI DAN KEMAMPUAN BAKTERI PROTEOLITIK LAHAN GAMBUT DALAM MENGHAMBAT *Salmonella typhi* dan *Escherichia coli*

SKRIPSI

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MEDAN
2021

ABSTRACT

This study aims to determine the characteristics of proteolytic bacteria isolates from peatlands from the Meranti Panam Garden area, Labuhanbatu, North Sumatra and their potential in inhibiting *Salmonella typhi* and *Escherichia coli*. The method used is an experimental method on a laboratory scale. The results of the casein hydrolysis test on *Skim Milk Agar* media were continued with the inhibition test for the growth of *Salmonella typhi* and *Escherichia coli* bacteria. The results showed that the proteolytic bacteria isolates of peatlands had colony morphological characteristics of round, irregular, and wavy shape, white and cream colony colors, with Gram test results including Gram positive and Gram negative with stem and *coccus* shapes. There were 6 proteolytic bacteria in peatlands which were able to produce clear zones in the growth of *E. coli* bacteria with the largest clear zone found in PSp10 isolate, namely 14.88 mm and the lowest clear zone found in PSp9 isolate, namely 10.1 mm. *S.typhi* shows that not all bacteria are able to produce clear zones. PSp9 isolate produced the largest clear zone diameter, namely 10.25 mm and PSp8 isolate produced the lowest clear zone, namely 8.50 mm. PSp7 and PSp8 isolates did not have clear zones.

Keywords : Proteolytic, Peatland, Antimicrob, *Salmonella typhi*, *Escherichia coli*, Hydrolysis.

CHAPTER I

INTRODUCTION

1.1 Background of Study

Based on data from Global Wetlands, Indonesia has the second-largest peatland in the world covering an area of 22.5 million hectares (Katadata, 2019). Approximately 5,241,473 ha or 35.17% of Indonesia's peatlands are classified as shallow peatlands (Wahyunto et al., 2014) across Papua (2,425,523 ha), Sumatra (1,767,303 ha), and Kalimantan (1,048,611 ha) islands. In addition to its use in agricultural development, peatlands containing microorganisms of bacteria have the potential to be used in the health sector (Mahdiyah, 2015). Peat soil brings a very high C-organic content, comprising 47.08% - 50.01% as it is included in organic soil (Salma et al., 2019).

Bacteria have an active role in dissolving organic materials, making them easy to be found in peat soils as the media are formed from the decomposition of organic materials under anaerobic conditions. Peat soil is acidic because it is modified by the content of organic acids incorporated in peat colloids. Decomposition of organic matter under anaerobic conditions causes the formation of phenolic and carboxylic compounds which cause high peat acidity. In addition, the nutrient content in peat also allows abundant microorganisms to live and serve roles such as proteolytic, cellulolytic, and nitrogen-fixing abilities (Mahdiyah, 2015).

Proteolytic bacteria are bacteria that can deteriorate proteins, producing extracellular protease enzymes. Generally, bacteria producing proteases are those

from the *Bacillus, Pseudomonas, Proteus Streptobacillus, Staphylococcus, and Streptococcus* (Puspitasari, 2012). In a study by Mahdiyah (2015), five isolates of proteolytic bacteria were obtained from peat soil from Banjarmasin, South Kalimantan. Proteolytic bacteria producing proteolytic enzymes could generate bacteriocins that will inhibit the growth of pathogenic microbes. The results of the study by Afriani et al., (2009), stated that *Lactobacillus Brevis* is a proteolytic bacterium that can inhibit the growth of *Staphylococcus aureus* with an inhibitory power of 21.3 mm. In the research by Apriyani et al., (2017), rhizosphere bacteria with proteolytic potential were also able to impede the growth of pathogenic bacteria isolated from the genus *Erwinia* with an inhibitory power of 11.91 mm. Additionally, in research by Erlindawati et al. (2015), three bacterial isolates in peat soil inhibited the growth of *Escherichia coli* bacteria, namely *Enterobacter cloacae*, *Enterobacter gergoviae,* and *Proteus rettgeri*.

Several proteolytic bacteria from the *Bacillus* genus produce protease enzymes used on an industrial scale, especially in the detergent, pharmaceutical, leather products, meat tenderization, protein hydrolysate, food products, and industrial waste treatment industries (Yusriah and Kuswytasari, 2013). Moreover, proteases are used to process silkworm scleroproteins before the spinning process of yarn, for a mixture of scar repair ointments, and digestive aids (Titin, 2011).

Escherichia coli and *Salmonella typhi* are bacteria that may harm humans, forming various diseases. *Escherichia coli* can cause pneumonia, endocarditis, wound infection, and abscesses in parts of organs (Entjang, 2003). *Salmonella typhi* is a source of several infections, ranging from mild to severe gastroenteritis such as typhoid fever and bacteremia (Jawetz et al., 2010).

It is necessary to conduct this research to study the potential of proteolytic bacteria originating from peatlands, understanding their morphological characteristics and their antimicrobial activity against *Salmonella typhi* and *Escherichia coli.*

1.2 Formulation of Study

The formulation of the problem in this study is the unknown characteristics and potential of peatland proteolytic bacteria in the Meranti Panam Plantation, Labuhanbatu, North Sumatra in inhibiting *Salmonella typhi* and *Escherichia coli*.

1.3 Objectives of Study

The purpose of this study was to determine the characteristics of isolates of peatland proteolytic bacteria in Meranti Panam Plantation, Labuhanbatu, North Sumatra, and their potential to inhibit *Salmonella typhi* and *Escherichia coli*.

1.4 Significance of Study

The benefit of the research is scientific information regarding the characteristics of isolates of peatland proteolytic bacteria in Meranti Panam Plantation, Labuhanbatu, North Sumatra, and their potential to inhibit *Salmonella typhi* and *Escherichia coli*.

CHAPTER II

LITERATURE REVIEW

2.1 Peatland

Peat is the organic matter or material that naturally accumulates in an excessively wet state, is incompressible, or has only undergone a slight restructure. Peat is formed by layered plant material over a very long period of time (Bintoro et al., 2010).

According to Noor et al., (2015), peat formation is a transformation process and translocation. The transformation process is forming biomass with the support of dissolved nutrients, water, air, and solar radiation. The translocation process is the transfer of material by the water stream from a higher place to lower ground facilitated by wind (air) due to pressure differences. Because the process of biomass formation from local plant residues is faster than the reshuffle process, a layer of organic matter is built from time to time.

Peat soil has a unique character due to its high acidity. Peat is an organic soil formed from the remains of decomposed plants and is degraded into organic deposits employing aerobic and anaerobic bacteria (Subiksa and Wahyunto, 2011). Peat soil has a high organic matter content of more than 85%, with a C-organic content of 12-18%, depending on the clay fraction with a peat thickness of more than 40 cm with a BD above 0.1 g cm^3. Peat soil produced from tropical swamp forest vegetation has a heterogeneous composition, consisting of logs, twigs, and unkempt roots comparable to the original plant (Zulman, 2015).

Resistant peat has multifunction, including hydrological, production, and ecology which is vital for human survival. Peatland productivity is highly

dependent on human management and actions (Masganti, 2013). Peat characteristics can alter due to human actions in the form of land clearing, land burning, drainage canals, and mining (Hirano et al., 2014).

Peat soil generally has a relatively high level of acidity with a pH range of 3-5. The high acidity of peat soils is prompted by the high levels of humic and fulvic phenolic acids created in the decomposition process (Bintoro et al., 2010). Peat soil is composed of 65% organic compounds consisting of lignin, cellulose, hemicellulose, wax, tannins, suberin, protein, and humic compounds. Besides ensuring the availability of water, peat also plays a significant role in maintaining environmental quality (Suriadikarta, 2012). Microorganisms play an important role in restructuring organic matter (Andersen et al., 2013). Peat soil is a habitat that is difficult for living things to occupy. However, several types of bacteria can exist and grow in peat soil, such as *Enterobacter cloacae, Enterobacter gergoviae, and Proteus rettgeri* (Erlindawati et al., 2015), and *Bacillus* sp *(*Rossa & Dini, 2017).

2.2 Proteolytic Bacteria

Proteolytic bacteria are bacteria capable of degrading proteins and producing extracellular protease enzymes. Protease is a proteolytic enzyme that catalyzes the disintegration of peptide bonds in proteins. In the growth medium, proteolytic bacteria are supplemented with skim milk containing casein for bacteria to secrete proteases that can degrade protein. Casein is the main milk protein, a macromolecule composed of amino acid subunits linked by peptide bonds. Casein serves as a substrate for protease enzymes. Commonly, proteolytic bacteria are those from the genus *Bacillus, Pseudomonas, Proteus Streptobacillus,*

Staphylococcus, and *Streptococcus* (Puspitasari, 2012). Protease is an enzyme that functions to hydrolyze peptide bonds in proteins into oligopeptides and amino acids. Proteases (serine proteases, cysteine/thiol proteases, aspartate proteases, and metal proteases) are enzymes widely used in industries, such as pharmaceutical, leather, detergent, food, and sewage treatment. Proteases used in the industry account for about 60% of enzyme sales in the world. Proteases can be isolated from various organisms such as bacteria 44.78%, plants 43.85%, and animals 11.15%. Protease from bacteria is the highest number compared to other sources (Baehaki et al., 2011).

Several proteolytic bacteria from the *Bacillus* genus provide protease enzymes used on an industrial scale, especially in the detergent, pharmaceutical, leather products, meat tenderization, protein hydrolysate, food products, and sewage treatment (Yusriah and Kuswytasari, 2013). The use of microorganisms as enzyme producers is also simpler and more efficient effort thanks to their fast growth and flourishing on easily available substrates. The ability of proteolytic activity in bacteria is indicated by a clear zone around the bacterial colony, which is the hydrolysis of casein in skimmed milk on the media (Badriyah & Ardyati, 2013).

2.3 Antimicrobial

Antimicrobials are substances or components that can inhibit the growth of bacteria/mold (bacteriostatic or fungistatic) to eliminate bacteria or molds (bactericidal or fungicidal) (Zheng et al., 2013). The categories of antimicrobial strength are presented in Table 1 below.

Document Accepted 11/5/22

Diameter of Clear Zone (mm)	Inhibition Power
>20	Very strong
11-20	Strong
6-10	Medium
<5	Weak

Table 1. Category of antimicrobial strength by clear zone diameter

Source: Susanto, Sudrajat, and Ruga (2012)

2.3.1 Classification of Antimicrobials Based on the Mechanism of Action

Classifications of antimicrobials based on their action mechanism are as follows (Setiabudy, 2011):

1. Inhibit the synthesis or damage to the bacterial cell wall. The bacterial cell wall consists of polypeptidoglycan, which is a primary complex of mucopeptides (glycopeptides). They generally are bactericidal.
2. Modify or inhibit protein synthesis. Bacterial cells synthesize various proteins that occur on the ribosomes assisted by mRNA and tRNA. Inhibition arises through interaction with the bacterial ribosome. They generally are bacteriostatic.
3. Inhibit essential enzymes in folate metabolism. They generally are bacteriostatic.
4. Affect the synthesis or metabolism of nucleic acids.
5. Affect the permeability of bacterial cell membranes.

Based on the spectrum of action, they are divided into two groups; broad-spectrum activity and narrow-spectrum activity.

1. Broad-spectrum antimicrobial works against more bacteria, either gram-negative or gram-positive, and fungi.

2. Narrow spectrum antimicrobial works against some types of bacteria only.

2.3.2 Antimicrobial Power Test Method

The antimicrobial power test method aims to determine the concentration of an antimicrobial substance to obtain an effective and efficient treatment system. There are two methods to test antimicrobial activity; diffusion and dilution (Setiabudy, 2011).

1. The diffusion method is the measurement and observation of the diameter of the clear zone that forms around the disk, in which the measurements are taken after left sit for 18-24 hours and measured using a caliper (Sari et al., 2013)

a. Disk diffusion method or Kirby Bauer method uses paper disks containing antimicrobial substances placed on agar media that has been planted with test bacteria.

b. The E-Test method is to determine the MIC (Minimum Inhibitory Concentration), which is the minimum concentration of antimicrobial substances in restraining the growth of the test bacteria. This method uses a plastic strip containing an antibacterial agent and is placed on an agar medium.

c. The ditch-plate technique is a process where antimicrobial substances are placed in a ditch made by cutting the agar medium in a petri dish in the middle longitudinally and the test bacteria are streaked in the ditch.

d. Cup-plate technique, this method is almost identical to the disk diffusion method, however, the difference is that it does not use paper disks. In the agar media, a well is made and is given an antimicrobial agent.

e. In the gradient-plate technique, the agar medium is melted and the test solution is added, then the mixture is poured into a petri dish and placed in a slant position.

2. Dilution Method

The dilution method is the method used to measure the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the tested antibiotics.

In this method, the series of test tubes will be filled with liquid media and several bacterial cells to be examined, then serial dilutions are performed with a certain concentration and be filled with antibiotics to be tested, next the series of tubes are incubated at 37ºC for 18-24 hours, and the turbidity that occurs in the serial tube should be observed (Setiabudy, 2012).

The MIC results will show the lowest concentration if the tube observed is the tube with the best clarity (an indicator of no bacterial growth). Furthermore, the culture results from all the clear tubes are inoculated on the agar media. Subsequently, the media are incubated and observed to see whether or not bacterial colonies grow. Meanwhile, the results of MBC are concluded based on the presence or absence of bacterial colonies that grow on the incubated agar media (Setiabudy, 2012).

2.4 *Salmonella typhi*

Salmonella typhi is a rod-shaped bacterium, gram-negative, non-sporing, has a width of 0.7 – 1.5 m and a length of 2.0 – 5.0 m, the average colony size is 24 mm, dominantly swims with flagella peritrichous (Batt and Tortotello, 2014).

The cell wall consists of murein, lipoprotein, phospholipid, protein, and lipopolysaccharide (LPS) which are arranged in layers (Jawetz, 2016).

The lipopolysaccharide moiety can function as an endotoxin and plays an important role in determining organism virulence. This macromolecular endotoxin complex encompasses three components; an outer O-polysaccharide, a middle part (R core), and an inner layer lipid A. In general, organisms in Salmonella are the cause of various types of infections, ranging from mild to severe gastroenteritis, such as typhoid fever and bacteremia. Salmonella is the causative agent of salmonellosis, which is an endemic disease and leads to immense losses in Indonesia (Jawetz et al., 2010).

S. Typhi can survive for several months to a year if it is attached to feces, butter, milk, cheese, and frozen water (Yatnita, 2011). If it is found in feces outside the human body, it can survive 1-2 months. Meanwhile, the milk can help it to reproduce and live longer because proteins, fats, and sugars are provided, which are saprophytic substrates (Monica et al., 2013).

S. typhi is a facultative intracellular parasite that can live in macrophages and promote gastrointestinal symptoms only at the end of the disease course, usually after a long fever, bacteremia, and finally localization of infection in the submucosal lymphoid tissue of the small intestine (Yatnita, 2011).

2.5 *Escherichia coli*

Escherichia coli is a gram-negative, rod-shaped facultative anaerobic bacterium commonly found in the large intestine of warm-blooded creatures. In general, strains of E. coli are harmless, yet some serotypes can provoke serious

food poisoning and food product recall due to contamination with these bacteria (CDC, 2014b). *Escherichia coli* can normally be present in the small intestine of humans and warm-blooded animals, including poultry. These bacteria in the intestine can reach millions per gram of intestinal contents, hence it has long been used as an index organism for fecal contamination, as well as the presence of enteric pathogens in food and beverages. Accumulation of *E.coli* can cause diarrhea, especially in infants (Tatang and Wardah, 2014).

Escherichia coli has several toxins with certain serotypes. These serotypes entail several specialized adaptations and lead to disease through different mechanisms (Irianto, 2014).

1. Enterotoxigenic *Escherichia coli* (ETEC)

   This type produces LT and ST toxins. This toxin acts on enterocytes to stimulate fluid secretion, causing diarrhea. LT toxin has 70% homology with cholera toxin. This toxin is heat-labile and increases localized cyclic adenosine monophosphate (cAMP) in enteric cells. ST toxin is heat-stable and stimulates cyclic guanosine monophosphate. *E.coli* containing this type of enterotoxin is associated with traveler's diarrhea, and brief watery diarrhea.

2. Enteroaggregative *Escherichia coli* (EAggEC)

   Some strains of *E. coli* can adhere to enteric cells and cause cell aggregation. These bacteria do not invade cells and are known as EAggEC and can cause acute diarrhea. These bacteria are enveloped in fibril structures that are assumed to mediate attachment. The strain expresses an ST-like toxin or a hemolysin-like toxin.

3. Enteropathogenic *Escherichia coli* (EPEC)

   It resembles *E. coli* which was first recognized as the primary pathogen causing diarrheal epidemics in children. Adherence is associated with loss of microvilli and is caused by rearrangement of host actin.

4. Enterohemorrhagic *Escherichia coli* (EHEC)

   This strain generates verotoxin which is active on Vero cells in vitro. Dysentery can be complicated by hemolysis and acute renal failure. A corresponding toxin (Shiga toxin) is a major virulence determinant in Shigella dysenteriae.

CHAPTER III

RESEARCH METHOD

3.1 Time and Site

This research was conducted from August to November 2020 at the Microbiology Laboratory, State University of Medan.

3.2 Tool and Material

3.2.1 Tool

The tools used in this study are Petri dish, test tube, test tube rack, knife, spatula, analytical balance, dropper pipette, magnetic stirrer, sterile cotton bud, inoculation loop, microscope, object glass (slide), cover glass, tweezers, vortex, Erlenmeyer flask, Bunsen burner, caliper, stirring rod, Laminar Air Flow (LAF), autoclave, and incubator.

3.2.2 Material

The materials used are peat soil, Skim Milk Agar (SMA), Nutrient Agar (NA), blank disk (oxoid), physiological NaCl, aquades, spiritus, disinfectant, alcohol 70%, the culture of *Salmonella typhi* and *Escherichia coli*, Safranin, crystal violet, Lugol's solution, cotton, tissue, aluminum foil, frosted paper, label paper, heat-resistant plastic, and plastic wrap.

3.3 Research Procedure

3.3.1 Research Sample

Peat soil samples were taken as much as 100 g from 5 points in an area of 20 m^2 of peatland Meranti Panam Plantation, PTPN IV, PT Cisadane Sawit Raya Negeri Lama, Labuhanbatu, North Sumatra using a purposive sampling method. Therefore, 5 soil samples were obtained at the same depth of 40 cm. The soil

selected as samples was the soil around the circle weeding. The location of the sample from which the soil was taken was cleaned of litter (Irfan, 2014). Sampling was carried out in a sterile condition. The sample was placed in a polyethylene bag and then taken to the laboratory for isolation.

3.3.2 Isolation of Proteolytic Bacteria

One gram of soil sample was put into a 250 ml Erlenmeyer containing 9 ml of NaCl 0.9% and a series of dilutions were carried out from 10-1 to 10-5 (Mahdiyah, 2015). The diluted bacteria were then isolated on SMA (Skim Milk Agar) media. Isolation of proteolytic bacteria using Skim Milk Agar (SMA) media were incubated at 37°C for ± 48 hours (Dawn Diah, 2012). The bacterial population in each sample was calculated. The calculation of the bacterial population (CFU/ml) was carried out using the formula according to Omar et al., (1996).

$$\frac{1}{X.Y} . x\ mean\ of\ number\ of\ colonies$$

Explanation :

X = Dilution Factor

Y = Volume of sample added (ml)

3.3.3 Characterization of Proteolytic Bacteria

The proteolytic bacteria that grew on the media were inoculated on the slanted agar medium as a stock of bacteria, then they were identified macroscopically and microscopically. Macroscopic identification of bacteria includes bacterial colony shape, colony color, colony edge, and colony elevation

(A. L. Putri & Kusdiyantini, 2018). Microscopic identification was carried out by Gram staining (Waidil et al., 2014).

3.3.4 Proteolytic Activity Test

The ability of proteolytic bacteria to produce protease enzymes can be determined by inoculation on Skim Milk Agar media. The purified bacterial isolates were then cultured on Skim Milk Agar media which had been frozen in a petri dish by creating a circle with a diameter of ±1 cm. The bacterial cultures were then incubated at 37ºC for 24 hours. Following that, the diameter of the clear zone around the bacterial colonies was measured using a caliper (Badriyah and Ardyati, 2013). Furthermore, isolates that produced a clear zone in the proteolytic activity test were used in the antimicrobial test (Apriyani et al., 2017).

3.3.5 Proteolytic Bacterial Antimicrobial Test against Test Microbes

Antimicrobial test of proteolytic bacteria applied the Disk Diffusion Method (Kirby-Bauer Test), which is the measurement and observation of the diameter of the clear zone formed around the paper disk, and was evaluated after being sitting for 18-24 hours using a caliper (Sari et al., 2013).

The test bacteria used are *Salmonella typhi* and *Escherichia coli*. The two cultures were then made into a suspension and applied to the surface of the Nutrient Agar media using a sterile cotton swab. A total of 0.1 ml of proteolytic bacterial suspension was dropped onto a blank paper disk (oxoid). The test paper disk was positioned on the side of the media that had been streaked with the test bacteria and incubated for 24 hours at 37ºC. The inhibition zone formed was measured and recorded (Suryani et al., 2017).

CHAPTER V

CONCLUSION AND SUGGESTION

5.1 Conclusion

In this study, the following conclusions were drawn:

5. Peatland proteolytic bacteria had morphological characteristics of colonies in the form of round, irregular round, and wavy, white, and cream colors, with Gram test results including Gram-positive and Gram-negative with rod and coccus shapes.
6. Peatland proteolytic bacteria had antimicrobial activity against *Escherichia coli* and *Salmonella typhi*
7. Antimicrobial activity against *Escherichia coli* bacteria had the highest clear zone diameter of 14.88 mm on PSp10 isolates and the lowest clear zone of 10.1 mm on PSp9 isolates.
8. Antimicrobial activity against *Salmonella typhi* bacteria had the highest clear zone diameter of 10.25 mm on PSp9 isolates and the lowest clear zone of 8.50 mm on PSp8 bacterial isolates.
9. Not all proteolytic bacteria exhibited antimicrobial activity against *Salmonella typhi* bacteria, two isolates of proteolytic bacteria did not show any antimicrobial activity against *Salmonella typhi* bacteria, including PSp7 and PSp11 bacteria, as shown by the absence of a clear zone around the paper disc.

5.2 Suggestion

Further research is recommended to examine extracts of proteolytic bacteria from peatlands to see the most effective concentration in producing inhibitory power against pathogenic bacteria.