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Isolation of Proteolytic Bacteria from Pond Sediments for the Maintenance of an Integrated System Fish and Rice

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ABSTRACT

Proteolytic bacteria play an important role in the ecosystem, especially as probiotic and bioremediation agents. Isolation of proteolytic bacteria is very necessary to separate proteolytic bacteria from other bacteria that come from a mixture of various bacteria. The purpose of this practical work is to determine the proteolytic bacteria found in the sediment of the Mina padi pond using the streak plate isolation technique. Bacterial isolation techniques started from bacterial sampling, preparation of tools and materials, manufactureof culture media, inoculation of bacteria, isolation of bacteria on TSA media, calculation of the number of bacterial colonies, observation of bacterial morphology and bacterial purification, and isolation of proteolytic bacteria. The results of the isolation using the streakplate technique obtained 8 bacterial isolates that had proteolytic activity with different morphology, namely isolates with codes S1, S4, S5, S6, S7, S9, S10, and S12. The isolates with codes S1, S4, S9, S10 and S12 were suspected to belong to the genus *Bacillus* Isolates with code S5 were suspected to belong to the genus *Staphylococcus* Isolates with code S6 were thought to belong to the genus *Pseudomonas* Isolates with code S7 were suspected to belongto the genus *Acinetobacter.* The average total number of bacteria obtained in this practical work was 1.8 x 10^5 CFU/g.

Keywords: *Proteolytic bacteria, bacterial isolation, streak plate, fish and rice integration, pond sediments*

INTRODUCTION

Maintenance integrated fish and rice is system combines fish farming and rice plants in one area (Rahmadi *et al.,* 2019 ; Akbar, 2017). The fish and rice system is inseparable from the soil and sediment at the bottom of the pond. Sediment in the integrated fish and rice system is caused by fish excrement and fish food residues. However, this sediment is very beneficial because it mixes with the soil so that it can fertilize the soil and become fertilizer for plants (Herlambang *et al.,* 2021).

Sediments in ponds are formed due to the deposition process of organic matter particles both from feed residues, fish feces, plankton, mud particles carried by water flows and other organisms that die (Suwoyo *et al.,* 2014). Pond sediments contain an abundant population of microorganisms with high diversity (Jumiarni, 2010). The abundant content of organic matter, inorganic and minerals makes sedimentsthe most ideal place of life for decomposer bacteria and various species of bacteria (Hanzen *et al.,* 2017).

Bacteria are microorganisms that have a simple structure (Artati and Oman, 2019). Bacteria in general are able to produce extracellular enzymes thatplay an important role in the ecosystem, especially as probiotic and bioremediation agents. One of the bacteria capable of producing extrcellular enzymes is proteolytic bacteria. Proteolytic bacteria are bacteria that are able to produce the protease enzyme. Protease not only plays a role in

cellular metabolic processes, but can also be applied in the industrial field (Rizaldi *et al.,* 2018). Proteolytic bacteria are found in soil, water, mud and certain environmental strains (Hamdani *et al.,* 2019). Observation of proteolytic bacteria can be made if they are separated from the environment and other bacteria . The separation of these bacteria can be done with isolation techniques (Jufri, 2020).

Isolation is a bacterial separation technique that is carried out to determine the type, study culture, morphology, physiology, and characteristics accompanied by purification. The principle of bacterial isolation is to separate one type of bacteria from another that comes from a mixture of various bacteria (Sabbathini *et al.,* 2017). Bacterial isolation generally has several techniques, one of which is the *streak plate* technique. The *streak plate* technique has the advantage of being able to produce a single colony with easily distinguishable contaminant bacteria (Dahlia *et al.,* 2017). Bacterial isolation techniques are very important to study, this is because bacteria are one of the microbes that are difficult to observe, so by isolating bacteria, it will make it easier to see and observe the forms of bacterial growth.

MATERIALS AND METHODS

2.1 Bacterial Sampling

Sediment sampling is carried out using *the purposive sampling* method , namely at the *outlet* of the pond. Sampling is carried out using a sterile spoon on the upper layer of the surface, after the sediment sample is taken, it is then put in a sterile petri dish and stored in *a cool box* for further processing in the laboratory.

2.2 Preparation of Tools and Materials

Tools and materials to be used in the isolation of proteolytic bacteria are sterilized first using an autoclave with a temperature of 121ºC and a pressure of 2 atm for 15 minutes.

2.3 Creation of Cultural Media

A. *Triptic Soy Agar* Media (TSA)

The culture medium used to calculate the total number of bacteria and isolate bacteria is TSA (*Triptic Soy Agar)* media. TSA media is made by means of every 4 gr of TSA powder poured into the erlenmayer then added 100 ml of aquades . Furthermore, it is heated until medidih using *a hot plate magnetic stirrer* with a temperature of 300ºC. Then the media is sterilized using an autoclave with a temperature of 121ºC with a pressure of 2 atm for 15 minutes. Culture media is stored until it will be used.

B. Skim *milk media*

The culture medium used for the isolation of proteolytic bacteria is *skim milk.* The *skim milk medium* is made by means of 2 gr *of skim milk powder* put into the erlenmeyer and added 50 mL of aqueous. A total of 4 gr of TSA powder is put into the erlenmayer then 50 ml of aquades is added. *Skim milk powder* solution and TSA media are heated to medidih using *a hot plate magnetic stirrer* with a temperature of 300ºC. Then the media is sterilized using an autoclave with a temperature of 121ºC with a pressure of 2 atm for 15 minutes. After sterility, *skim milk powder* solution and TSA media solution are mixed and homogenized. *Skim milk media is* stored until it will be used.

2.4 Bacterial Inoculation

The bacterial inoculation procedure is carried out by weighing 0.1 grams of pond sediment samples which are suspended into 0.5 ml of physiological solution then

homogenized with *a vortex* and diluted gradually. Dilution of the sample was carried out serially using three test tubes containing 4.5 mLof sterile physiological (dilution of 10-1-10-3). A sample of 0.5 mL was taken and homogenized with 4.5 mL of physiological solution in the first tube (dilution of 10-1). A total of 0.5 mL of sample suspension was taken from the first tube and homogenized in the second tube (dilution 10-2), and the procedure is carried out up to the third tube (dilution 10-3).

The sample dilution results are then grown on TSA media using the *pour plate* method , as much as 0.5 mL of sample solution from each dilution tube is taken and put into a sterile empty dish. Then the TSA media in a warm condition is poured on the saucer and homogenized. Incubation is carried out for 18-24 hours at a temperature of 28ºC.

2.5 Bacterial Isolation on TSA media

Colonies that grew separately on TSA media were taken several isolates selected based on different morphologies to be isolated. Bacterial isolation is carried out using *the streak plate* technique. Then the bacteria that have been isolated are incubated for 18-24 hours at a temperature of 28ºC. The results of the isolation of the growing bacteria are carried out morphological observations and purification of bacteria.

2.6 Calculation of the Number of Colonies

Colonies that grow on each TSA medium i.e. at 10 retailers-1 to dilution 10-3 calculated in its entirety. After obtaining the number of colonies from each dilution, the total sediment bacteria grown is then calculated by multiplying the number of colonies by one per dilution factor used. The results of dilution of the sample are then grown on TSA media by *the pour plate* method. The growing colonies are then calculated by the *total plate count* (TPC) calculation method using the formula (Michael and John, 2006 *in* Nurhafid *et al.,* 2021) as follows:

$$\text{Number of bacteria} = \text{Number of colonies} \times \frac{1}{Dilution} \times \frac{1}{Culture\ volume} \times \frac{1}{Sample\ weight}$$

CFU/g

2.7 Bacterial Morphological Observations and Bacterial Purification

Bacteria that grow on *Triptic Soy Agar* media are first observed in the macroscopic morphologyof their colonies, namely in terms of color, shape, elevation, edges, and size. A single colony on the results of bacterial isolation on subsequent TSA media carried out the purification stage . Purification of sediment bacteria is carried out by *streakplate* technique on TSA oblique media .

2.8 Isolation of Proteolytic Bacteria

Isolation of proteolytic bacteria is carried out by taking bacterial cultures usingose needles on oblique media then scratched on *skim milk* media and incubated for 48 hours at a temperature of 28ºC. Observations were made at 12, 24, 36 and 48 hours to determine the differences in the activity of the hydrolysis zone that occurred. Bacterial isolates that have proteolytic activity show the presence of clear zones around the colony (Zainuddin *et al.,* 2017). The results of the clear zone formed are measured by colony diameter and total diameter to determine the proteolytic activity index and are included in the proteolytic activity calculation formula as quantitative data (Nurhafid *et al.,* 2021). The calculation formula of the proteolytic activity index referring to the formula used (Durham *et al.,* 1987 *in* Hengkengbala *et al.,* 2021) is as follows:

$$\text{Proteolytic Activity} = \frac{Total\ diameter\ of\ clean\ zone\ (cm)}{Diameter\ of\ bacterial\ colony\ (cm)}$$

2.9 Data Analysis

The data obtained are further analyzed descriptively with the help of tables. The primary data that has been analyzed are then compared with the secondary data taken based on scientific articles .

RESULTS AND DISCUSSION

3.1 Bacterial Morphology

Based on bacterial isolation, 15 strains of bacteria were suspected. This conjecture is based on differences in the morphological features of bacteria seen macroscopically . The morphology is viewed based on the color, shape, elevation and margins of bacterial colonies growing on TSA media. The fifteen bacterial isolates were coded S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15. This code is based on the morphological differencesof the colonies of each bacterial isolate .

Table 1. Morphology of bacterial colonies derived from rice mina sediments

Sample Code	Morphology				
	Shape	Elevation	Edge	Color	Size
S1	Circular	Convex	Entire	Brownish-white	Big
S2	Irregular	Flat	Undulate	Brownish-yellow	Keep
S3	Rhizoid	Flat	Filamentous	Brownish-white	Big
S4	Circular	Convex	Entire	Clear white	Big
S5	Irregular	Flat	Undulate	Clear white	Big
S6	Irregular	Flat	Undulate	Brownish-yellow	Big
S7	Circular	Umbonate	Entire	Grayish-white	Keep
S8	Circular	Convex	Entire	brownish-white	Small
S9	Circular	Convex	Entire	Clear white	Small
S10	Circular	Convex	Entire	Grayish-white	Keep
S11	Circular	Pulvinate	Entire	Yellowish-white	Big
S12	Circular	Convex	Entire	Clear white	Small
S13	Circular	Convex	Entire	yellowish-white	Keep
S14	Circular	Pulvinate	Entire	white	Keep
S15	Circular	Flat	Entire	yellowish-white	Small

Based on the morphological observations of colonies above, several forms of bacterial colonies were obtained, namely circular, irregular and rhizoid. The edges of the bacterial colonies there are entire, undulate, and filamentous. The elevations observed from the sides there are convex, flat, umbonate and pulpinate. The color of the observed bacterial isolates of there is a brownish-white, brownish-yellow, brownish-yellowish-yellowing, clear-white, yellowish-white- white. The size of the colonies of acquired bacteria includes small, medium and large. The morphology of bacterial colonies obtained in this practical work is the same as the morphology of bacteria in general. According to Rizaldi, (2016) the form of bacterial colonies in general is circular, irregular, filamentous and rhizoid. The edges of the colonies formed are entire, undulate, filamentous, curled, and lobate. Colony elevations are raised, convex, flat, umbonate, and pulvinate.

3.2 Proteolytic Bacteria

Based on the isolation of peroteolytic bacteria, 8 bacterial isolates that have proteolytic activity were obtained, namely isolates with the codes S1, S4, S5, S6, S7, S9,

S10, and S12. The presence of proteolytic activity in bacterial isolates is indicated by the presence of clear zones around the colony. The diameter of the clear zone is measured to calculate the resulting clear zone index (Maziah, 2009).

Table 2. Activity of proteolytic bacteria derived from rice mina sediments

Isolate Code 7	tivity of proteolytic enzymes	Clear zone index
S1	+	1,5
S2	-	0
S3	-	0
S4	+	1,2
S5	+	1,8
S6	+	1,3
S7	+	1,5
S8	-	0
S9	+	1,3
S10	+	1,3
S11	-	0
S12	+	1,3
S13	-	0
S14	-	0
S15	-	0

Description: Exists (+), None (-)

Isolates with codes S1, S4, S9, S10 and S12 are thought to belong to bacteria of the genus *Bacillus.* This is based on the morphology of the colony obtained , namely circular shape , convex relevance, entire edge, brownish-white color, clear white, grayish-white with small, medium, and large kolini sizes. According to Delia *et al.* (2018) *Bacillus* bacteria have the characteristics of *a* circular or punctiform bacterial colony, margin entire or lobate, dull white, medium-large sized colonies not slimy, gram-positive bacteria, have endospores, are phlegm and are partially motile (Diarti *et al.,* 2017). Holt *et al.* (2000) stated that the color of the colony *of Bacillus* bacteria cannot be known, has positive catalase, negative oxidase and is anaerobic aerobic or facultative .

Isolates with the code S5 are thought to belong to bacteria of the genus *Staphylococcus*. This is based on the morphology of the colony obtained, namely irregular shape, flat relevance, undulate edges, clear white color with a large size. According to Suyasa (2019) *Staphylococcus* bacteria have the characteristics of irregular bacterial colonies, margins of entire and undulate, flat and convex relevance. *Staphylococcus* bacteria are gram-positive bacteria, cocci-shaped, non-motile, non-spore, facultative anaerobic, posistive catalase and negative oxidation (Lifitriyah, 2020). Holt *et al.* (2000) state that the color of the colonies of *Staphylococcus* bacteria is usually opaque, white or cream and sometimes yellow.

Isolates with the code S6 are thought to belong to the genus *Pseudomonas*. This is based on the morphology of the colony obtained, namely irregular shape, flat relevance, undulate edges, brownish-yellow color with a large size. According to Holt *et al.* (2000) in Adiathy *et al*. (2017) *Pseudomonas* bacteria have a characteristic irregular colony shape, flat surface (flat), and are yellowish-white-cream in color. *Pseudomonas* bacteria

are gram-negative short rod-shaped cell size 3 µm, motile, positive catalase, positive SCA, oxidase and indole negative.

Isolates with the code S7 are thought to belong to the genus *Acinetobacter*. This is based on the morphology of the colony obtained, namely circular shape, umbonate relevance, entire edge, grayish-white color with medium size. According to Puspadewi et al. (2020) bacteria of the genus *Acinetobacter* have the characteristics of an embossed colony, white in color, smooth edges, round in shape, including gram-negative bacteria and aerobic bacteria. According to Barrow and Feltham (1993) in Setyati *et al.* (2016) *Acinetobacter* is non-motile, catalase is positive, oxidase is negative, and oxidative degradation of sugars or not at all.

Bacteria of the genus *Bacillus, Staphylococcus, Pseudomonas*, and *Acinetobacter* are proteolytic bacteria that are quite often found in sediments. Rizaldi *et al.* (2018) and Pamaya et al. (2018) stated that *Staphylococcus, Pseudomonas and Bacillus* bacteria are proteolytic bacteria that are often found in sediments or soils. Setyati *et al*. (2019) also stated that *Acinetobacter and Bacillus* bacteria are types of proteolytic bacteria that are often found in sediments. According to Su *et al*. (2020) protease-producing bacteria are generally dominated by the genus *Bacillus*. Proteolytic activity in bacteria differs depending on the type of bacteria. Rizaldi *et al*. (2018) stated that each isolate of a different type of bacteria has a different proteolytic activity. The activity is shown from the difference in the diameter of the clear zone that is visible.

The activity of the protease enzyme is influenced by environmental factors such as temperature, growth pH, incubation time, protein substrate (Saranraj *et al*., 2017), influence of inhibitors, and activators (Laili, 2021). Temperature affects the activity of enzymes to determine the appropriate conditions for degrading the substrate. Incubation time affects the activity of proteolytic enzymes. pH affects enzymatic processes and the process of transporting various components across cell membranes to support cell growth and enzyme production. The substrate affects the proteolytic activity because the substrate is an inducer for the protease enzyme (Herawati, 2020). Inhibitors have an effect on reducing the speed of enzymatic reactions. Inhibitors can inhibit the work of enzymes temporarily or permanently (Laili, 2021).

3.3 Total Number of Bacteria

The calculation of total bacteria using the Total Plate Count (*TPC*) method is a method that is often used to grow live microbial cells on the media so that these cells can live well and form colonies that can be seen directly with the eye without using a microscope. Colonies of bacteria can be counted using hand counters. The calculation of bacterial colonies in petri dishes can be done by dividing the petri dish into four parts on the petri dish cover with a permanent marker to facilitate the calculation of bacteria growing on the media (Tyas *et al.*, 2018).

Tabel 3. The number of bacteria from each dilution

Dilution	Number of colonies	Number of bacteria (CFU/gram)
10^{-1}	375	$5{,}4 \times 10^{4}$
10^{-2}	102	$1{,}5 \times 10^{5}$
10^{-3}	24	$3{,}4 \times 10^{5}$
Average		$1{,}8 \times 10^{5}$

The average total number of bacteria obtained in this practical work is 1.8 x 10^5 CFU / g. The total number of bacteria is thought to be influenced by the application of the rice mina cultivation system, the type of feed used during maintenance, soil fertility and water quality in rice mina ponds. Herlambang *et al*. (2021) stated that fish farming systems, fish feed types and changes in soil and water quality, modulate the number of bacterial communities in ponds. Susilawati *et al*. (2013) also stated that variations in the number of bacteria are influenced by type, depth, structure, texture and soil moisture, as well as soil environments such as aerobic and anaerobic.

Fitriana and Asri (2022) stated that the population growth of proteolytic bacteria is also influenced by humidity, temperature and soil pH which is influenced by the presence of climate which will have an impact on the physiological properties of the soil so that the population and diversity of bacteria in the soil are increasingly abundant. In addition, the increase in the number of bacteria in the pond is influenced by organic matter as a nutrient for growth. Bacterial growth is influenced by several parameters such as temperature, pH and dissolved oxygen. Bacteria will die if the nutrients in the growing medium have been exhausted (Arfiatia *et al*., 2020).

CONCLUSION

Based on the results of the practical work carried out, it can be concluded that the bacterial isolation technique with the streak plate method is a recommended technique to find out the proteolytic bacteria present in rice mina sediments. This is evidenced by obtaining 8 bacterial isolates that have proteolytic activity, namely isolates with codes S1, S4, S5, S6, S7, S9, S10, and S12. In isolates with codes S1, S4, S9, S10 and S12 are suspected to belong to bacteria of the genus *Bacillus*. Isolates with the code S5 are thought to belong to bacteria of the genus *Staphylococcus*. Isolates with the code S6 are thought to belong to the genus *Pseudomonas*. Isolates with the code S7 are thought to belong to the genus *Acinetobacter*. The average total number of bacteria obtained in this practical work is 1.8 x 10^5 CFU / g.

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