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Index of Proteolytic Bacterial Activity in Rice-Fish Farming System

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Abstract

Rice-fish farming system cultivation is one of the developments in fisheries cultivation technology that can increase the economic value of society. However, fish farming activities carried out in rice-fish farming system ponds will produce organic waste from metabolic waste and inedible feed. The organic waste in the water is used to grow and reproduce bacteria. This study aimed to determine the number of bacteria and the proteolytic bacterial activity index in the water of rice cultivation ponds. Purposive sampling method was employed in the study, namely by taking water samples in each rice-fish farming system cultivation pond at predetermined points and representing the population. Bacterial isolation was conducted on general bacterial media and skim milk agar media. Then Gram, catalase, and oxidase tests were conducted and the proteolytic activity index was calculated. The results obtained from this study were bacterial counts ranging from 0.18-0.8 x 10^4 CFU/mL. Pond A's proteolytic bacterial activity index at the inlet point was 0.22, the middle was 1, and the outlet was 0.54. The results of the proteolytic activity index for the Pond E at the inlet point was 0.84, middle, and it was 1.2, and outlet 0.84. Apart from that, the average proteolytic activity index, in Pond A it was 0.5, and in Pond E the average proteolytic activity index was 0.9.

Keywords: *Rice-fish farming system, Pond water, Total bacteria, Proteolytic bacteria, Activity index*

1. Introduction

Rice-fish farming system is a system of farming in rice fields along with fish cultivation, apart from that, the cultivation of the rice-fish farming system is an effort to utilize rice fields as a place for cultivating fish and also as an interval between rice planting seasons (Bobihoe *et al*., 2015; Idham Shilman *et al*., 2021). rice-fish farming system cultivation in Indonesia has a national-scale fish production value of around 1.2% of the total fish cultivation production value of 83 tons of fish (KKP, 2020). The total area of rice-fish farming system fields is 8,118,233 Ha, consisting of irrigated land of 3,170,996 Ha (39.06%) and non-irrigated land of 4,497,237 Ha (60.94%) (Direktorat Jenderal Perikanan Budidaya, 2018). In cultivating rice-fish farming system, the type of fish that is widely used in Indonesia is tilapia, but you can also use other types of fish such as goldfish, nilem, and catfish (Badriyah *et al*., 2020; Lestari & Rifai, 2017).

During fish farming activities in rice-fish farming system ponds, organic waste will be generated from metabolic waste and inedible feed (Khoirul Anam *et al*., 2017). The ability of fish to absorb feed is

only around 25-30% of the feed given so that the remaining 75% will accumulate in the pond as organic waste (Mustofa *et al*., 2018). This organic waste is used by bacteria as a source of nutrition to grow and reproduce. This is in accordance with the opinion of Sukenda & Harris (2006) who stated that the number of bacteria in the waters of fish cultivation ponds is higher due to the nutrient content due to the accumulation of organic material from leftover feed and fish metabolism. These bacteria will degrade carbon and nitrogen compounds into proteins through proteolytic enzymatic reactions (Rosmaniar, 2011 ; Utomo *et al*., 2019)

Proteolytic bacteria are bacteria that have extracellular enzymatic activity of proteases (Artha *et al*., 2019), these protease enzymes have a function in hydrolyzing proteins into amino acids (Zainuddin *et al*., 2017; Rizaldi *et al*., 2018). Several genera of bacteria known to produce protease enzyme compounds are *Bacillus*, *Lactobacillus*, *Eubacterium*, *Pyrococcus*, *Pseudomonas*, *Proteus*, and *Staphylococcus* (Arief *et al*., 2010; Hasanah, 2014; Ward *et al*., 2009; & Yahdiyani *et al*., 2021). Bacteria with proteolytic activity must be screened and selected before being used as probiotics.

Bacteria from rice-fish farming system cultivation pond water were isolated and selected for their proteolytic activity and pure cultures. As for previous research by Saidah (2014) who in her research succeeded in isolating proteolytic bacteria from hot springs, Istikhomah (2021) succeeded in obtaining proteolytic activity in vannamei shrimp pond water. Furthermore, in a research study conducted by Asril & Leksikowati (2019), they reported proteolytic activity from tofu waste. Listiowati *et al*. (2022) succeeded in reporting the proteolytic index in the digestive tract of Nilem fish. This is the basis for conducting research related to the screening of proteolytic bacteria from pond water of rice-fish farming system cultivation in Panembangan Village, Cilongok District, Banyumas Regency.

2. Materials And Methods

A This research was conducted from September to November 2022, at the Laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University. The object used in the study was water from the rice-fish farming system cultivation pond in Panembangan Village, Cilongok District, Banyumas Regency. The sampling method used is purposive sampling method, namely by taking water samples in each of the rice-fish farming system cultivation ponds at the inlet, middle and outlet points which represent the population. The main data in this study were the number of bacteria and the proteolytic activity index. The supporting data in this study were the morphology of the bacterial colonies, the proportion of gram-positive and negative bacteria, the catalase test and the oxidase test.

Sampling

Sampling was conducted in the waters of the rice-fish farming system cultivation pond in Panembangan village with three sampling points, in two different rice-fish farming system ponds (Purposive sampling). The samples were collected by placing sterile sample bottles in the Pond, closing them tightly, and labeling them, then putting them in cool boxes for transportation to the laboratory.

The Making of Cultural Media

TSA (Tryptic Soy Agar) media was prepared by pouring 4 grams of TSA powder into an Erlenmeyer and then adding 100 mL of distilled water. The TSA solution was heated using a hot plate magnetic stirrer for 5 minutes. TSA was sterilized using an autoclave at 121°C for 15 minutes. Then the TSA solution is stored until slightly warm and later used for further treatment. Media for the proteolytic activity test was prepared by pouring 4 grams of TSA powder into the first Erlenmeyer and then adding 100 mL of distilled water. 2 grams of skim milk powder was poured into the second Erlenmeyer then added 100 mL of distilled water. Afterward, both Erlenmeyers were heated for five minutes on a magnetic stirrer using a hot plate. Then each Erlenmeyer was autoclaved at 121°C for 15 minutes. After the autoclave process, the TSA media and skim milk were mixed slowly to avoid clumping.

Bacterial Isolation

Preparation Bacterial isolation was conducted using 0.5 mL of sample water suspended in 4.5 mL of NaCl solution then homogenized with a vortex and diluted serially. Each sample was serially diluted using a test tube containing 4.5 mL of physiological solution, put in a 10^{-1} dilution tube, then homogenized using a vortex. A total of 0.5 mL of sample was taken from a 10^{-1} dilution tube and then

homogenized in a second tube. Do the same steps until the dilution is 10^{-3}. The results of the 10^{-1} and 10^{-3} dilutions were then cultured in 0.5 mL of TSA media using the pour plate method. Place the TSA medium in a cup containing the bacterial sample and then incubate for 24-48 hours at 28°C.

Calculation of the Number of Colonies

Colonies that have grown are then counted using the TPC (Total Plate Count) calculation method using the Madigan & Martinko (2006) formula which is modified as follows:

$$\text{The Number of Bacteria} = \sum \text{colony} \times \frac{1}{\text{dilution}} \times \frac{1}{\text{culture volume (mL)}}$$

3% KOH Gram Test

The Gram KOH test aims to determine whether the bacteria belong to the group of Gram-positive bacteria or the group of Gram-negative bacteria. The Gram KOH test was conducted by adding one drop of 3% KOH solution to the glass slide. The bacterial isolate is taken using a sterile loop needle and then streaked thinly on the drop earlier. The gram KOH 3% test was conducted once for each isolate. A Gram-negative bacterium is characterized by mucus and stickiness when rubbed in KOH solution, which indicates a positive test result. A negative test result is characterized by no formation of bacterial mucus when scratched, these bacteria belong to the Gram-positive group (Suslow *et al*., 1982). Then the results of the percentage of positive Gram and negative Gram are calculated using the formula:

$$\text{Percentage of Gram positive (\%)} = \frac{\textit{total number of gram positive colonies}}{\textit{the total number of colonies observed}} \times 100$$

$$\text{Percentage of Gram negative (\%)} = \frac{\textit{total number of gram negative colonies}}{\textit{the total number of colonies observed}} \times 100$$

Catalase Test

The catalase test aims to determine catalase activity in bacteria. The catalase test was conducted by dropping one drop of H^2O^2 solution on the glass slide. Furthermore, the bacterial isolates were taken using a sterile loop needle and rubbed on a glass preparation that had been dripped with H^2O^2 solution. The catalase test was performed once for each isolate. Positive test results are indicated by the formation of bubbles, while negative test results do not form bubbles (Yuka *et al*., 2021).

Oxidase Test

The oxidase test aims to determine the activity of the oxidase enzyme in bacteria. The oxidase test was conducted by taking bacterial isolates using an ose needle, and then wiping them on a glass slide. After that, the glass slide was covered or coated with filter paper, then p-amino dimethylaniline oxalate reagent was added. The oxidase test was carried out once for each isolate. Positive results are indicated by blue filter paper, while negative results are indicated by no change in color (Anggraini *et al*., 2016).

Proteolytic Bacteria Isolation

Isolation of proteolytic bacteria was conducted by taking bacterial colonies from the purification results using an ose needle, then scratching a little on skim milk agar media. Bacteria were incubated for 48 hours at 28°C. Bacterial isolates that had positive proteolytic activity were indicated by the formation of a clear zone, after which the bacteria were preserved and stored using TSB (Tryptic Soy Broth) with 15% glycerol in a refrigerator at -20ºC.

The proteolytic activity index was calculated by measuring the diameter of the clear zone formed around the colony, and then dividing it by the diameter of the growing colony. The isolate with the largest proteolytic activity index had a high level of enzyme activity. Proteolytic bacterial isolates were tested for their ability based on the proteolytic index formula (Sumardi *et al*., 2010) :

$$\textit{Proteolytic activity index} = \frac{\textit{clear zone diameter (cm)}}{\textit{colony diameter (cm)}}$$

The proportion of proteolytic bacteria can be calculated using the formula (Sinatryani et al., 2014) :

$$Proportion\ of\ proteolytic\ bacteria\ (\%) = \frac{the\ number\ of\ proteolytic\ bacteria\ obtained}{the\ total\ number\ of\ colonies\ observed}\ x\ 100$$

Data Analysis

Data was analyzed descriptively to determine the number of bacteria, the proportion of gram bacteria, the proportion of bacterial catalase, and the proportion of bacterial oxidase, and a non-parametric test (Kruskal-Wallis test) was used to calculate the descriptive proteolytic activity index. The data is presented in the form of tables, pictures, and graphs and then compared with the literature.

3. Results and Discussion

A Number of Bacteria in Rice-Fish Farming System Pond Water

The number of bacteria in the rice-fish farming system pond water can be seen by culturing the bacteria from the rice-fish farming system pond water. The cultured bacteria were then counted using the Total Plate Count (TPC) method. The number of bacteria obtained in this study varied at each point which can be seen in Table 1.

Table 1. Number of Bacteria in Water Rice-Fish Farming System Cultivation Ponds

Sampling Point	Number of Bacteria (CFU/mL)	
	Pond A	Pond E
Inlet	0,6 x 10^4	0,4 x 10^4
Middle	0,18 x 10^4	0,5 x 10^4
Outlet	0,8 x 10^4	0,7 x 10^4

Source: Authors

Based on Table 1, the number of bacteria found in the rice-fish farming system pond water ranged from 0.18-0.8 x 10^4 CFU/mL. The highest number of bacteria in Pond A was found at the outlet point (0.8 x 10^4 CFU/mL), followed at the inlet point (0.6 x 10^4 CFU/mL) and the lowest at the outlet point (0.18 x 10^4 CFU /mL). In Pond E, the highest number of bacteria was found at the outlet point (0.7 x 10^4 CFU/mL), followed at the midpoint (0.5 x 10^4 CFU/mL), and the lowest at the inlet point (0.4 x 10^4 CFU/mL).

Proportion of Proteolytic Bacteria

Proteolytic bacteria were positively identified in rice-fish farming system cultivation pond water using TSA media supplemented with 2% skim milk powder. The proportion of proteolytic bacteria can be calculated by dividing the number of proteolytic bacteria by the total number of colonies observed and then multiplying by one hundred. The process of protein breakdown by bacterial colonies will produce a clear zone or hydrolysis zone around the bacterial colonies (Figure 1).

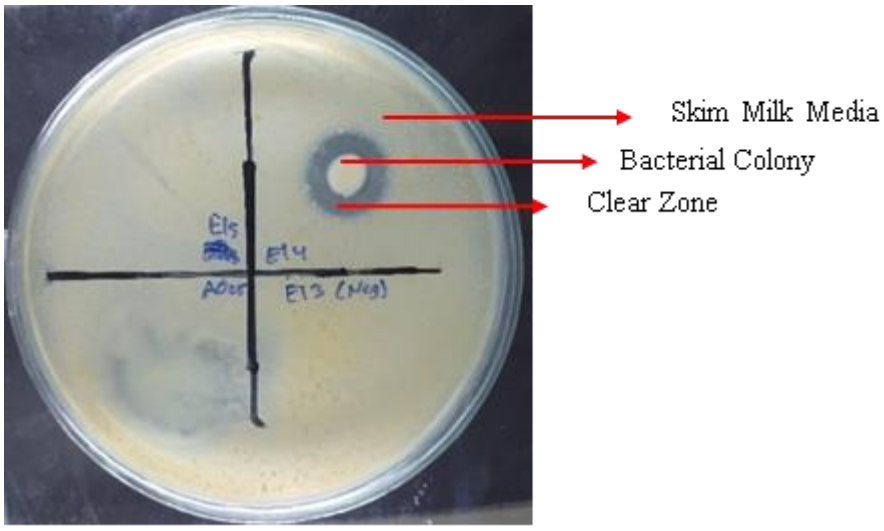

Figure 1. Proteolytic Activity in Skim Milk Media

The clear zone that forms around bacterial colonies on skim milk media indicates that the bacteria produce protease enzymes (Pramono *et al.*, 2019). Meanwhile, bacterial colonies that do not produce a clear zone are suspected to have little or no proteolytic activity at all. The formation of the clear zone is caused by the hydrolysis of casein in skim milk media into peptides and amino acids (Fitriadi *et al*., 2023). The calculation of the proportion of proteolytic bacteria in the rice-fish farming system pond water can be seen in Table 2.

Proteolytic Activity Index

The proteolytic bacterial activity index can be calculated by dividing the diameter of the clear zone by the colony diameter. The proteolytic activity test aims to determine the ability of bacteria to produce protease enzymes as indicated by the activity index of proteolytic bacteria. The proteolytic bacterial activity index in rice-fish farming system cultivation pond water can be seen in Table 2.

Table 2. Calculation of the proportion of proteolytic bacteria in rice-fish farming system pond water.

Sampling Point	Proteolytic Bacterial Activity Index	
	Pond A	Pond E
Inlet	0,22	0,84
Middle	1	1,2
Outlet	0,54	0,84
Average	0,5	0,9
Standard Deviation	0,357389	0,779241

Source: Authors

Based on Table 2, the results of measuring the activity index of proteolytic bacteria in Pond A at the inlet point were 0.22, the middle was 1, and the outlet was 0.54. The results of the proteolytic activity index of Pond E at the inlet point were 0.84, the middle was 1.2 and the outlet was 0.84. In addition, the average proteolytic activity index in Pond A was 0.5 and in Pond E the average proteolytic activity index was 0.9.

Proteolytic Bacterial Colony Morphology

Proteolytic bacteria were observed in the pond water of rice mine cultivation to determine their shape, elevation, edges, colors, and sizes. According to Sabdaningsih *et al*. (2013), morphological characterization aims to observe the morphology of colonies on bacterial isolates that have passed selection. (Wondal *et al*., 2019), also added that the morphological characterization of bacteria aims to see the physical characteristics of bacteria. Observation of the morphology of the bacterial colonies in the water of the rice paddy cultivation ponds can be seen in Table 3.

Table 3. Bacterial colony morphology of rice-fish farming system pond water

Shape	Elevation	Edge	Color	Isolate Code
Circular	Convex	Entire	Putih	AI18, AO14
Circular	Convex	Entire	Putih	EO10
Circular	Convex	Entire	Putih Kekuningan	ET11
Circular	Convex	Entire	Putih kekuningan	EI12
Circular	Convex	Entire	Putih Krem	ET21
Circular	Convex	Entire	Putih Krem	EO4
Circular	Convex	Entire	Putih susu	EO9
Circular	Crateriform	Entire	Putih Kekuningan	EO8
Circular	Crateriform	Undulate	Putih	AI14
Circular	Flat	Entire	Putih bening	ET12
Circular	Flat	Entire	Putih Kekuningan	ET15
Circular	Flat	Entire	Putih Kekuningan	AO2
Circular	Pulvinate	Entire	Putih	EI22
Circular	Pulvinate	Entire	Putih	AO19
Circular	Pulvinate	Entire	Putih Kekuningan	ET14

Circular	Raised	Entire	Putih	AO1
Circular	Raised	Entire	Putih Krem	EI21
Circular	Raised	Undulate	Kuning	EI9
Circular	Raised	Undulate	Putih susu	EI8
Circular	Umbonate	Entire	Putih krem	AO25, EO14
Filamentous	Convex	Filamentous	Putih	AT6
Filamentous	Flat	Filamentous	Putih	AO17
Filamentous	Umbonate	Lobate	Putih bening	AO15
Irregular	Convex	Entire	Putih susu	EI13
Irregular	Convex	Lobate	Putih kekuningan	EI2
Irregular	Convex	Undulate	Putih	AI25
Irregular	Crateriform	Lobate	Putih	ET17
Irregular	Crateriform	Undulate	Putih Krem	EO6
Irregular	Flat	Entire	Putih	AO3
Irregular	Flat	Entire	Putih	AI17
Irregular	Flat	Entire	Putih bening	AO5
Irregular	Flat	Lobate	Putih bening	AO13
Irregular	Flat	Undulate	Putih Krem	EO13
Irregular	Pulvinate	Entire	Putih kekuningan	ET7
Irregular	Pulvinate	Entire	Putih Kekuningan	ET10
Irregular	Pulvinate	Entire	Putih Kekuningan	ET13
Irregular	Raised	Lobate	Putih	ET16
Irregular	Raised	Lobate	Putih	AO24
Irregular	Umbonate	Lobate	Putih	ET8
Spindle	Convex	Entire	Putih	EO11
Spindle	Flat	Entire	Putih susu	EI16
Spindle	Pulvinate	Entire	Putih Kekuningan	EI19
Spindle	Raised	Entire	Putih susu	EI4

Description: AI: Pond A Inlet, AT: Pond A Middle, AO: Pond A Outlet; EI: Inlet E Pond, ET: Middle E Pond, EO: Outlet E Pond.

Based on Table 3, the morphological observations that have been carried out can be seen with varying results. From observations of the morphology of proteolytic bacteria in water, it was found that the most common characteristics were circular shape, convex elevation, entire edge, white color and small size. In general, bacterial colonies found in rice-fish farming system cultivation pond water have circular or irregular shapes, convex or pulvinate elevations, entire or lobate edges, white or translucent white, and varying sizes. This shows that each colony found in the water of the rice-fish farming system cultivation pond has different morphological characteristics. The different colony morphology is thought to indicate that each isolate comes from a different species.

Bacterial Biochemical Test

Observation of catalase using H2O2 reagent aims to determine the activity of the catalase enzyme in breaking down hydrogen peroxide into oxygen (Fadilah *et al*., 2022). The result of a positive catalase test is the formation of bubbles in the bacterial isolate, while a negative catalase test result does not form air bubbles (Sianipar *et al*., 2020). The oxidase test using p-aminodimethylaniline oxalate reagent has the aim of knowing the presence of oxidase enzymes in bacteria. A positive result from the oxidase test is the appearance of a purple color on the filter paper when the reagent is dropped on it and the absence of color indicates a negative oxidase test result (Arfiandi & Tumbol, 2020). The proportion of catalase and bacterial oxidase test results in the water of the rice-fish farming system cultivation ponds can be seen in Table 4.

Table 4. Proportion of catalase and oxidase tests for bacteria in rice-fish farming system pond water

Test Name	Pond A (%)			Pond E (%)		
	Inlet	Middle	Outlet	Inlet	Middle	Outlet
Catalase Test (+)	92	84	72	88	64	88

Catalase Test (-)	8	16	28	12	36	12
Oxidase Test (+)	72	84	84	80	88	88
Oxidase Test (-)	28	16	16	20	12	12

Based on Table 5, the highest proportion of positive catalase tests in Pond A was 92% at the inlet point, while Pond E was 88% at the midpoint and outlet. Meanwhile, the highest proportion of positive oxidase tests was in Pond A at 84% at the midpoint and outlet, then Pond by 88% at the midpoint and outlet.

Mine rice cultivation pond water has a different number of bacteria due to different types of bacteria. This is supported by colony morphology data (Table 4) which has been observed to also show different characteristics. Moreover, the amount of organic matter in rice-fish farming system cultivation ponds can affect the number of bacteria due to feed residues and fish metabolism. This is in accordance with the opinion of Kristiawan *et al*. (2014), who stated that the concentration of organic matter is related to the number of bacteria, the higher the concentration of organic matter, the higher the abundance of bacteria in these waters. The organic material in the rice-fish farming system pond water comes from leftover food and fish feces that accumulate in the waters. Bacteria in water utilize organic materials as a source of nutrients for their growth (Yuspita *et al*., 2018). The use of organic materials by bacteria is carried out by decomposing the nutritional content through a proteolytic enzymatic process (Rosmaniar, 2011; Prayogo *et al.*, 2018). The results of this study are in accordance with the research of Sunitha & Krishna (2016), the number of bacteria in rice-fish farming system pond water ranges from $1x10^4$ - $5.6x10^4$ CFU/mL. In this study, probiotics were added so that the number of nitrifying bacteria could be greater. While Arifudin (2019) reported the number of bacteria in the rice-fish farming system pond water ranged from $4x10^4$ - $10x10^4$ CFU/mL, these results were different because in that study there were additional treatments for cultivation media such as the use of ex-mining soil.

Table 5. Proportion of proteolytic bacteria in rice-fish farming system pond water

Sampling Point	Pond A			Pond E		
	Number of Isolates	Number of Proteolytic Isolates	Proportion (%)	Number of Isolates	Number of Proteolytic Isolates	Proportion (%)
Inlet	25	4	16	25	10	40
Middle	25	1	4	25	11	44
Outlet	25	11	44	25	8	32

Based on Table 5, the proportion of water proteolytic bacteria in Pond A and Pond E has different results. The highest proportion of proteolytic bacteria in Pond A was at the outlet point at 44% and the lowest at the midpoint at 4%. Furthermore, the highest proportion of proteolytic bacteria in Pond E was found at the midpoint at 44% and the lowest at the outlet point at 32%. These results indicate that proteolytic bacteria can be found in rice cultivation pond water. The highest proportion of proteolytic bacteria in Pond A and Pond E was at the outlet and middle points. The difference in the proportion of proteolytic bacteria is thought to be due to each bacteria's ability to hydrolyze proteins differently, not all bacteria can produce protease enzymes. This is supported by differences in the morphological characteristics of the bacteria obtained, meaning that not all bacteria have proteolytic activity. Said & Likadja (2012) stated that not all bacteria have the potential to produce protease enzymes. Apart from that, at the midpoint, there is a buildup of food waste and fish metabolic waste, while at the outlet point, there is a discharge of cultivation water which contains high levels of organic matter. Rice-fish farming system cultivation pond water contains organic materials such as protein caused by uneaten feed residue and fish metabolic waste. This is supported by Asril & Leksikowati (2019), who state that proteitic bacteria can be found in environments that contain high protein.

b

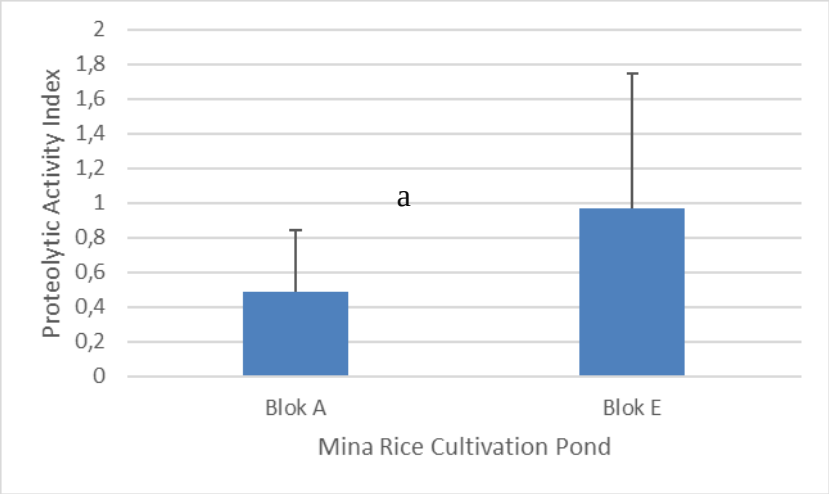

Figure 2. Proteolytic Activity Index Graph

Description: Different letter notations show significantly different results of the Kruskal-Wallis test (Non-Parametric Test) ($P<0.05$)

Several bacteria isolates produce different levels of proteolytic activity, which causes a difference in the index of proteolytic activity. This is evidenced by the different characteristics of the bacteria obtained in this study. In addition, it could also be due to the incubation time of the bacteria which affects the high or low index of proteolytic activity. According to Hengkengbala *et al*. (2021) who stated that the different indexes of proteolytic activity could be due to the ability of bacteria to produce protease enzymes (Prihanto *et al*., 2021). In addition, Ginting *et al*. (2018) stated that some bacterial isolates required a longer incubation time to hydrolyze protein. The average results of the proteolytic activity index were carried out by non-parametric tests which can be seen in Figure 2.

Based on Figure 2, the results of the non-parametric test (Kruskal-Wallis) showed that the differences in water sampling locations, namely Pond A and Pond E, were significantly different in terms of the activity index of proteolytic bacteria ($P<0.05$). The different proteolytic activity index is thought to be because each bacterial isolate has a different hydrolysis ability. An example of a bacterial isolate that has high proteolytic activity is an isolate with code EO9, with a circular morphology, convex elevation, entire rim, milky white in color and medium size, and has a Gram-negative, catalase positive and oxidase positive. Apart from that, the difference in proteolytic activity index values is also thought to be due to the different water sources used as media for rice-fish farming system cultivation (Figure 2). In Pond A, the water source enters the inlet without going through the residential area, while in Pond E, the water source that enters the inlet canal has already passed through the residential area. So it is suspected that the water used as a medium for cultivating the rice-fish farming system in Pond E contains a lot of organic matter produced from household waste around the water source. Apart from that, the quality of rice-fish farming system cultivation water from the two ponds is also different. Pond A has a temperature of 27°C, pH ranges from 6.5-7.5, and dissolved oxygen is 7.6-7.8 mg/L, while Pond E has a temperature of 28°C, pH ranges from 5. 8-7.7 and dissolved oxygen of 6.1-7.4 mg/L.

The different forms of bacterial colonies are influenced by several factors, such as environmental factors (biotic and abiotic), growth media factors and temperature (Safrida & Devira, 2012). Natsir Djide & Farid Samawi (2016), also argued that adaptation to the environment in which they live will affect the morphology and anatomical structure of bacteria to survive. The water quality in the two rice-fish farming system ponds is different. Pond A has a temperature of 27°C, pH ranges from 6.5-7.5, and dissolved oxygen is 7.6-7.8 mg/L, while Pond E has a temperature of 28°C, pH ranges from 5. 8-7.7 and dissolved oxygen of 6.1-7.4 mg/L. According to Cappuccino & Sherman (1987), the diversity of colony morphology indicates that each colony has different characters. However, the difference in morphology is not sufficient to be used as a parameter for identifying bacteria, so it is necessary to carry out microscopic observations and the biochemical properties of each of these isolates.

The proportion of Gram-positive and Gram-negative bacteria in the water of the rice paddy cultivation pond can be seen in Figure 3.

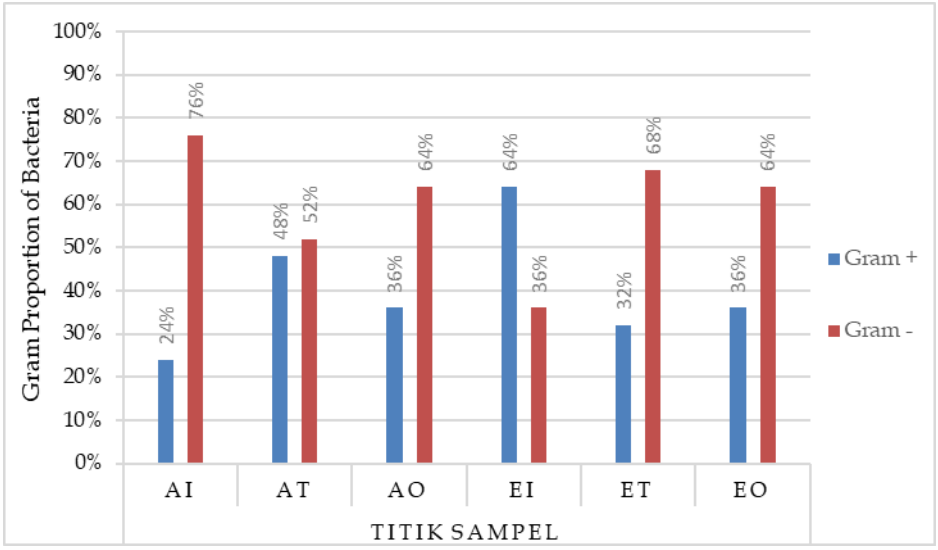

Figure 3. Gram Proportion of Bacteria

Description: AI: Pond A Inlet, AT: Pond A Middle, AO: Pond A Outlet; EI: Inlet E Pond, ET: Middle E Pond, EO: Outlet E Pond.

Based on Figure 5, Pond A has the highest proportion of Gram-positive bacteria at 48% at the midpoint, while the lowest at the inlet point was 24%. The highest proportion of Gram-negative bacteria was at the inlet point of 76% and the lowest was 52% at the midpoint. Pond E had the highest proportion of Gram-positive bacteria at 64% at the inlet point and the lowest at the midpoint of 32%. In addition, the highest proportion of Gram-negative bacteria was at the midpoint of 68% and the lowest at the inlet point of 36%. Gram-positive bacteria do not form mucus because they have a thick peptidoglycan layer (Hardiansyah *et al*., 2020). Meanwhile, Gram-negative bacteria are slimy because the addition of 3% KOH can destroy the bacterial cell wall (Suryadi *et al*., 2013). The number of Gram-negative bacteria in the rice-fish farming system cultivation pond water is greater than the Gram-positive bacteria. According to Tri Askar *et al*. (2018), the large number of gram-negative bacteria is caused by the high level of organic waste in the waters. Gram-negative bacteria are more commonly found in polluted environments. Widigdo *et al*. (2020) added that Gram-negative bacteria can be competitors for probiotic bacteria which maintain good water quality. Some examples of Gram-positive bacteria that can be found in aquaculture waters include the Genus *Bacillus, Lactobacillus, Streptococcus,* and *Staphylococcus* (Agustinus *et al*., 2010). Gram-negative bacteria are *Aeromonas, Pseudomonas* and *Escherichia* (Azhar & Ulkhaq, 2017). The dominance of bacteria with positive catalase and positive oxidase test results indicates that the rice-fish farming system cultivation pond water has many bacteria that can carry out aerobic respiration. This is supported by Hidayatun (2020), who states that aerobic bacteria will produce the enzymes catalase and oxidase. Rice-fish farming system cultivation environments that contain organic materials can also create high numbers of aerobic bacteria because the organic materials are used by bacteria to grow and reproduce (Jeanua *et al*., 2014).

The catalase enzyme is an enzyme that can catalyze or accelerate the decomposition of hydrogen peroxide into water and oxygen (Fadilah *et al*., 2022). Bacteria under certain conditions can produce H2O2, but if the bacteria do not break down H_2O_2 into other compounds then the bacteria can die. Solving these compounds can be done if there is a catalase enzyme (Pulungan & Tumangger, 2018). Susanti *et al*. (2017) added that H2O2 is a substance that is toxic to cells because it can deactivate enzymes in bacterial cells. The bacterial oxidase test was carried out by wiping the bacteria on an object glass and then covering it with filter paper, and then dropping the reagent p-aminodimethylaniline oxalate (Panjaitan *et al*., 2020). Positive oxidase test results will be indicated by the appearance of a purple color on the filter paper. The color change that occurs on the filter paper indicates that the bacteria have the oxidase enzyme (Djohari *et al*., 2019). According to Hederstedt

(2022), oxidase enzymes play a role in reducing oxygen to water and energy in aerobic bacteria in the process of electron transport. Several types of bacteria that have positive oxidase activity include *Pseudomonas* sp., *Aeromonas* sp., and *Vibrio* sp. (Mahmudah *et al*., 2016).

4. Conclusion

The number of bacteria in rice-fish farming system cultivation pond water ranges from 0.18-0.8 x 10^4 CFU/mL. The highest number of bacteria in Pond A was found at the outlet point (0.8 x 10^4 CFU/mL), followed by the inlet point (0.6 x 10^4 CFU/mL), and the lowest at the outlet point (0.18 x 10^4 CFU /mL). In Pond E, the highest number of bacteria was found at the outlet point (0.7 x 10^4 CFU/mL), followed at the midpoint (0.5 x 10^4 CFU/mL), and the lowest at the inlet point (0.4 x 10^4 CFU/mL). The proteolytic bacterial activity index at the inlet of Pond A was 0.22, the middle was 1, and the outlet was 0.54. The results of the proteolytic activity index of Pond E at the inlet point were 0.84, the middle was 1.2 and the outlet was 0.84. In addition, the average proteolytic activity index in Pond A was 0.5 and in Pond E the average proteolytic activity index was 0.9.

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