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Full Length Article

Exploration and Molecular Identification of Potential Amylolytic Bacterial Probiotics, Environmental Biodegradation of Rice-fish Farming

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Abstract

Amylolytic bacteria is one of the bacteria that has high potential and plays an important role in the aquaculture environment. The purpose of this study was to explore and identify amylolytic bacteria from rice fish farming sediments as probiotic bacteria. The research was conducted by survey in Banyumas Regency, Central Java, Indonesia. Amylolytic bacteria were detected using growth media enriched with starch. Then, the Amylolytic bacteria were explored with various tests such as antibacterial activity against *Aeromonas hydrophila*, proteolytic activity, synergism, detection of *Aeromonas* sp. using specific Glutamate Starch Phenile (GSP) medium and sensitivity test to several types of antibiotics for their potential as probiotics. The results showed that 10 isolates were detected to have amylolytic activity. Two bacterial isolates were successfully explored with several tests including detection of amylolytic activity, inhibition activity of *A. hydrophila* bacteria, synergism and sensitivity to several types of antibiotics. Molecular identification results based on 16s rRNA gene, isolates S10 and S11 were similar to *Bacillus velezensis* (99,65%) and *Proteus mirabilis* (100%). In the future, it is hoped that the strains found can become probiotics in rice fish farming.

Keywords: *Bacillus velenzensis*; Enzymatic activity; Potential bacteria; Rice fish farming

Introduction

Rice fish farming is an integrated cultivation of rice and fish in one ecosystem to increase the efficiency of the use of paddy fields. In Indonesia, especially in Central Java, the rice-fish farming system has begun to become a concern of the government to improve the welfare of the people of the Banyumas Regency. Rice fish farming several commodities, including tilapia, catfish, and carp, in an integrated manner. This commodity is an ideal commodity because it is known to have environmental tolerance characteristics suitable for rice-fish farming (Nurhayati *et al.* 2016; Rozen *et al.* 2019).

Probiotics are one of the developments in biotechnology that can be used as decomposing agents in the environment and for fish digestion. In several development methods in cultivation, probiotics are an important factor that needs to be added to the environment and feed to accelerate biochemical processes in digestion and the environment. Some of the important roles of probiotics include maintaining digestive stability, host health, and the environment (Azhar *et al.* 2021; Butt *et al*. 2021; Diwan *et al*. 2022). An aquaculture probiotic can take advantage of enzymes produced by metabolic processes such as breakdown of molecules (proteins, carbohydrates, fats, and fiber), the ability to inhibit pathogens, as well as the ability to form symbiosis in complex environments (Yukgehnaish *et al*. 2020).

Bacterial exploration is needed to determine the possibilities for exploiting extracellular activities. Amylase is one of the potentials of various enzymes produced by bacteria (Khiftiyah *et al*. 2018; Artha *et al*. 2019; Pramono *et al.* 2019). The amylase enzyme plays a very important role in the environment, namely the biodegradation of organic waste that settles at the bottom of the environment (Suciati *et al*. 2016; Wahyuni *et al*. 2021). Aquaculture waste is relatively higher in the aquatic environment because composition of some feeds that are not digested by fish

enters water as waste; thus, amylolytic bacteria play an important role in decomposing these molecules. In addition, waste in the environment needs to be decomposed to prevent a decrease in the quality of the cultivation environment (Das *et al*. 2014; Dutta *et al*. 2018). Several bacteria have the ability to suppress the growth of pathogens to maintain the balance of bacterial community, and to be able to work together in a complex environmental balance, which needs to be explored. In addition, antibiotic contamination in the aquatic environment makes some bacteria resistant against them. Hence, it is very necessary to carry out special studies on bacteria that degrade these antibiotics.

Bacteria that have potential in aquaculture are expected to be identified to facilitate the grouping of the characters possessed by each strain. Identification based on the 16s rRNA gene is a valid technique for identifying bacteria, therefore this technique can be used as a basis for molecular identification (McNichol *et al.* 2021). Various descriptions showed the importance of this research to be carried out to explore amylolytic bacteria to determine their potential so that they can be for used as probiotic bacteria in degrading aquaculture waste.

Materials and Methods

General experimental details

This research was conducted using a survey method. Samples were taken from pond sediments the rice-fish farming in Panembangan Village, Cilongok District, Banyumas Regency, Central Java, Indonesia. This research was conducted from January to April 2022 at the Research Laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University, and the Microbiology Laboratory of Muhammadiyah University, Purwokerto.

Research design

The design of this research is based on the results of the isolation of amylolytic bacteria from the sediment of Mina Padi ponds. Samples were taken from the outer sediment adjacent to the roots of rice plants. Amylolytic bacterial isolates were subjected to exploratory observations using several tests to obtain bacteria that have the potential to biodegrade organic materials. Bacterial isolates that have the potential to be developed as probiotics in the future.

Isolation of amylolytic bacteria

Bacterial samples were obtained from the sediment in the rice-fish farming. Isolation was carried out using Tryptone-Soy-Agar (TSA) as a medium for bacterial growth in general. Testing the activity of amylolytic bacteria was carried out using TSA media enriched with 1% starch and visualized by adding Lugol's solution to the surface of the media after incubation (Bairagi *et al*. 2002; Ni'matuzahroh *et al*. 2021). The amylose-degrading bacteria obtained were explored for their potential in plant development. Several tests to determine the potential of bacteria as probiotics, namely amylolytic activity, antibacterial activity against *Aeromonas hydrophila* (Lawalata *et al.* 2011; Fitriadi *et al*. 2023a), proteolytic activity (Susanti *et al*. 2021), synergism, detection of *Aeromonas* sp. using specific Glutamat Starch Phenile (GSP) medium (Lee and Wendy 2017) and sensitivity tests to several types of antibiotics (Fitriadi *et al*. 2023a).

Antibacterial test

The pathogen, *A. hydrophila*, was tested for antibacterial activity using the paper disk method. Test and pathogenic bacteria were cultured in liquid media separately. Suspensions of pathogenic bacteria were cultured using the spread plate method on the surface of solid media in 100 µL dishes. Then the paper disk was placed on the surface of the agar on which the pathogen has been cultured. A 10 µL of the test bacterial suspension was added onto the paper disk and incubated the culture medium at 30ºC for 48 h. The clear layer that formed around the paper disk showed the ability of the test bacteria to inhibit pathogenic bacteria (Lawalata *et al.* 2011; Fitriadi *et al*. 2023a).

Proteolytic activity

Proteolytic activity was carried out using doting on solid growth media enriched with 2% skim. Bacteria that grow and produce a hydrolysis zone around the colony show the bacteria's ability to degrade protein. Bacteria were incubated at 30ºC for 48 h (Susanti *et al*. 2021).

Bacterial synergism

Bacterial synergism was carried out using the cross-culture method on solid growth media. The test bacteria were cultured using the cross-scratch method with other test bacteria. Bacteria were incubated at 30ºC for 48 h. Bacteria that grow and stuck to the meeting point between the isolates showed that these bacteria have synergistic properties (Fitriadi *et al*. 2023a).

Detection of the pathogen *Aeromonas* sp.

Detection of *Aeromonas* sp. was carried out using the culture method on specific GSP media. GSP specific media will show a yellow color if the test bacteria are bacteria from the *Aeromonas* sp. group after incubation for 48 h at 30ºC (Lee and Wendy 2017).

Sensitivity antibiotic test

Antibiotic sensitivity was detected using the Kirby-Bauer disk diffusion test method (Uma and Rebecca 2018;

Wang *et al*. 2020). The test bacteria were cultured in liquid media for 24 h and then spread in 100 µL on the surface of the solid media plate. The antibiotic disk is placed on the surface of the culture medium and then incubated for 48 h at 30ºC. Large clear zone that forms around the disk shows that the bacteria were sensitive to antibiotics. The antibiotics tested were Tetracycline, Amoxicillin, Chloramphenicol and Gentamicin (Lee and Wendy 2017).

Molecular identification

The selected bacteria indicated the potential as probiotic bacteria by molecular identification of the selected bacteria based on the 16srRNA gene. The initial stage in this step was purification of gDNA according to the Presto™ Mini gDNA Bacteria Kit (Geneaid) procedure according to the sing kit (https://www.geneaid.com/data/download/attached/1602745908822784327.pdf). Then PCR amplification was done using primers 27f and 1392R with Taq PCR namely MyTaq HS Red Mix eith PCR conditions according to the manufacturer's instructions. (https://www.bioline.com/mwdownloads/download/link/id/2687/mytaq_hs_red_mix_product_manual.pdf). The annealing temperature was 55ºC for 30 sec. The PCR program used for amplification was pre-denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 20 sec, annealing at 55ºC for 30 sec, extension at 72ºC for 20 sec, followed by a final extension at 72ºC for 5 min and storage at 25ºC for 1 min and the PCR results were visualized using agarose gel electrophoresis. The DNA bands were sequenced and analyzed by BLAST on GenBank (NCBI) to compare the reference sequences. The bacterial strains obtained were constructed using Mega software version 11 to determine the strains on the evolutionary tree (Hardiyanti *et al*. 2022).

Data analysis

In general, the data obtained were analyzed descriptively. Afterwards, bacteria with amylolytic activity were examined for their potential as probiotics. Exploration data were supported by several previous studies to strengthen the research results obtained (Fitriadi *et al.* 2023b). Identification results were visualized in the form of a phylogenetic tree to place the strains of each species.

Results

Based on the results of culture on growth TSA media, a total of 501 colonies were obtained. The observation of the colony morphology was carried out to distinguish bacteria from the total colony 501. Based on the morphological observations of the colonies, 15 different bacterial isolates were obtained. Colony morphological observations were based on the shape, edges, elevation, size, and color of the colonies (Table 1). Results revealed that 10 positive bacterial isolates had an amylolytic activity which was as indicated by a clear zone round the colony (Table 2). Clear zone formed was calculated to obtain the activity value of the amylase enzyme produced by the bacteria. Activity measurements were carried out at 12, 24, 36 and 48 h to see the maximum phase of the excreted enzyme. The activity and measurement time of each amylolytic bacteria were variable. Based on the average value, bacterial isolates showed relatively the same activity. The highest average activity value was shown at the 24^{th} h followed by 36^{th}, 48^{th} and 12^{th} h. The highest activity was shown by bacteria with the codes S09 and S03, namely 3.25 (24^{th} and 36^{th} h) and 3.3 (36^{th} h). In addition to the two bacteria with the isolated code, the activity value was classified as moderate, namely S01, S04, S06 and S07 (Table 2; Fig. 1).

The exploration of amylolytic bacteria was supported with several tests, such as antibacterial activity against the *A. hydrophila*, ability to break down proteins, synergism, sensitivity to antibiotics, as well as other biochemical tests to verify identification. Ten isolates with amylolytic activity were evaluated for their potential as probiotics if they possessed antibacterial and proteolytic properties. Tests on positive GSP media showed that the isolate was a pathogenic-bacteria and was not included in the potential category (Table 3).

The test results on several tests showed varying results. Ten isolates of amylolytic bacteria were detected; four of them had the ability to inhibit the growth of the *A. hydrophila* pathogen with different inhibiting abilities. S08, S10, and S11 were classified as intermediate, and S13 was classified as weak. In addition, as many as seven isolates of amylolytic bacteria were also detected to have the ability to break down proteins with moderate activity categories, namely S01, S04, S05, S06, S07, S10 and S11. Five of the ten amylolytic bacteria isolates tested on GSP media were positive as *Aeromonas* sp., namely S01, S06, S07, S09 and S12. Based on the exploration and screening, the bacteria were grouped based on their probabilities as probiotic bacteria. The grouping results showed that there were two bacterial isolates that had the opportunity to become probiotics, namely S10 and S11 (Table 4). To meet the requirements as probiotic bacteria, testing of the synergism properties of bacteria needs to be done to ensure that these bacteria can live in symbiosis and live together in an environment (Table 4).

Based on antibiotic tests, the two bacterial isolates showed different properties for each type of antibiotic. Two isolates showed high resistance to chloramphenicol type antibiotics, and intermediate response to gentamicin. Sensitivity to tetracycline type antibiotics was shown by S10 and Amoxicillin by S11. However, most of them are resistant to antibiotics. The synergism test of two bacterial isolates showed synergistic properties, meaning that the bacteria could live together in one environment without harming one another (Table 4).

Table 1: Bacterial colony morphology

Sample code	Bacterial colony morphology				
	Shape	Elevation	Side	Colour	Size
S01	Circular	Convex	entire	Brownish White	Big
S02	Irregular	Flat	undulate	Brownish Yellow	Medium
S03	Rhizoid	Flat	filamentous	Brownish White	Big
S04	Circular	Convex	entire	Clear White	Big
S05	Irregular	Flat	undulate	Clear White	Big
S06	Irregular	Flat	undulate	Brownish Yellow	Big
S07	Circular	Umbonate	entire	Gray White	Medium
S08	Circular	Convex	entire	Brownish White	Small
S09	Circular	Convex	entire	Clear White	Small
S10	Circular	Convex	entire	Gray White	Medium
S11	Circular	Pulvinate	entire	Yellowish White	Big
S12	Circular	Convex	entire	Clear White	Small
S13	Circular	Convex	entire	Yellowish White	Medium
S14	Circular	Pulvinate	entire	White	Medium
S15	Circular	Flat	entire	Yellowish White	Small

Table 2: Area measured (mm) in terms of amylolytic bacterial activity

Sample code	Incubation time (h)			
	12	24	36	48
S01	1.60	2.80	2.33	2.33
S02	1.50	1.50	1.50	1.67
S03	-	2.00	3.33	1.71
S04	2.00	2.75	2.40	2.20
S06	-	-	2.00	1.05
S07	2.00	2.00	1.20	1.07
S09	2.66	3.25	3.25	2.60
S10	-	1.50	1.50	1.50
S11	1.23	1.62	1.62	1.31
S12	1.57	1.75	1.56	1.33
Average	1.51±0.17	1.82±0.45	1.58±0,38	1.56±0.43

Description: Big (≥4 mm), medium (≥2-3.9 mm), small (≤1.9 mm)

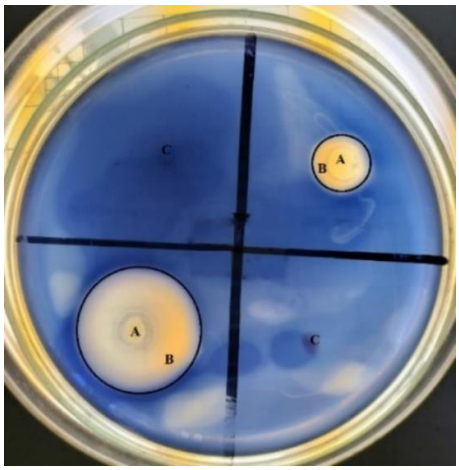

Fig. 1: Amylolytic activity; (A) bacterial Isolate; (B) inhibition zone; (C) Isolate that does not have amylolytic activity

Two selected isolates from several tests showed several abilities that could be used as aquaculture probiotic agents. Next, molecular identification is necessary to place it into a phylogenetic tree as a branching line. The results of alignment comparisons in GenBank and phylogenetic tree construction (Table 5 and Fig. 2). Based on the blast sequences obtained at the GenBank, the bacteria with the code S10 had 99.62% similarity with *Bacillus velezensis* strain PDW335. The bacteria with the code S11 had 100% similarity with *Proteus mirabilis* strain BN7 which is suspected to be a group of pathogenic bacteria in humans. The results of phylogenetic tree construction showed that bacterial isolates have stable branches in *B. velenzensis* and *P. mirabilis* with zero or parallel values. This indicated that the sequence similarity was relatively the same with the group of species being compared (Fig. 2).

Table 3: Antibacterial, proteolytic and detection tests of *Aeromonas* sp.

Sample code	Antibacterial	Protease	GSP
S01	-	++	+
S02	-	-	-
S03	-	-	-
S04	-	++	-
S06	-	++	+
S07	-	++	+
S09	-	-	+
S10	++	++	-
S11	++	++	-
S12	-	-	+
S13	+	-	-

Description: Strong (+++), Intermediate (++), Weak (+)

Table 4: Test on antibiotics and bacterial synergism

Isolate code	Antibiotics				Synergism
	Tetracycline (30 mcg)	Amoxicillin (25 mcg)	Chloramphenicol (30 mcg)	Gentamicin (10 mcg)	
S10	S	R	R	I	Positive
S11	R	S	R	I	Positive

Description: Sensitive (S), Intermediate (I), Resistant (R)

Table 5: Blast analysis data of 16s rDNA gene sequence of amylolytic bacteria

Isolate code	Blast results	Query cover (%)	Identity (%)	Number access
S10	*Bacillus velezensis* strain PDW335	100	99.65	MZ642485.1
S11	*Proteus mirabilis* strain BN7	100	100.00	MN560008.1

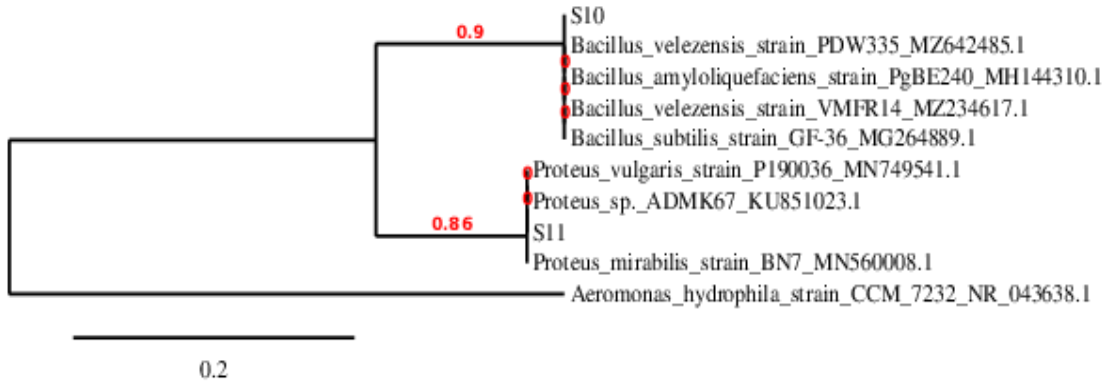

Fig. 2: Phylogenetic tree of isolate S10 and S11. Branching on *Aeromonas hydrophila* as out-group

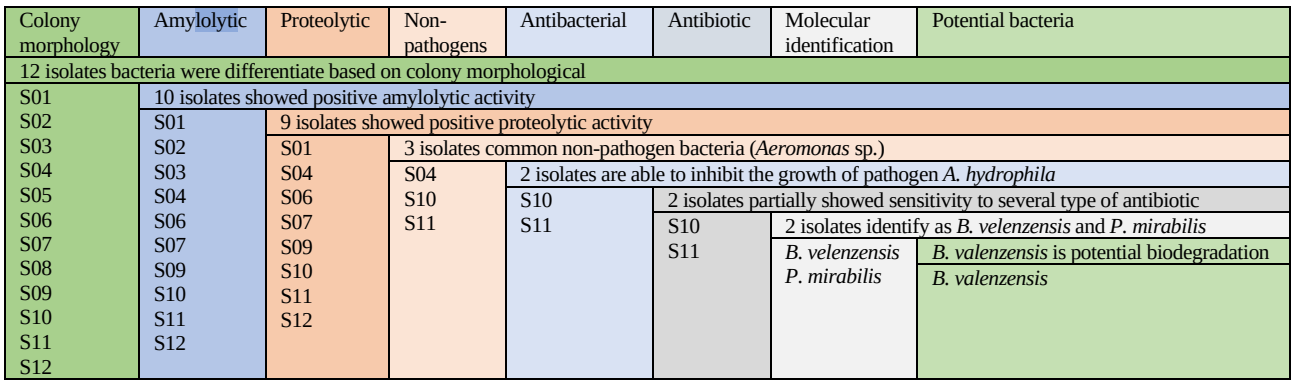

Fig. 3: Exploration of potential biodegrading bacteria

Discussion

Rice fish farming is a complex ecosystem because it has several factors in diversity. In this study, bacteria were isolated from sediments taken from three different parts, namely the bottom, walls, and roots of rice plants in rice-fish farming ponds and homogenized in one container. This aims to obtain bacterial isolates that represent the entire rice-fish farming pond. In addition, this study used TSA growth medium, which is a common medium for bacterial growth. According to Wang *et al.* (2018) only a small portion of the bacterial community can be cultured on bacterial growth media. In this study, the total number of colonies obtained was very low. A total of 501 colonies, and 15 isolates were differentiated based on their colony morphology (Sousa *et al.* 2013). A total of 10 bacterial isolates were detected to have amylolytic activity. However, if converted in percentage terms, amylolytic bacteria were detected in 66.6% of the bacterial isolates (Fitriadi *et al*. 2023a). This proportion is quite high. So, it is very important to study to deal with the degradation of organic waste by bacteria (Satyantini *et al*. 2020).

Amylase enzyme activity can be measured in the stationary phase or the exponential phase. This study examined the amylase enzyme activity in starch-enriched growth media. However, this technique is able to describe the maximum amylolytic activity produced at 24 h (Fitriadi *et al*. 2023a). This can describe conditions in an environment where carbohydrates are maximally broken down by amylolytic bacteria in that range of time. This time period is considered significant because some amylolytic bacteria enter the stationary phase for more than 48 h or up to 72 h (Schubert 2020). This shows that carbohydrates in the environment are likely to be broken down starting from the 12 h and reaching maximum in the 24th h so that they do not cause contamination in the environment.

Exploration of amylolytic bacteria in this study was carried out with several tests. These included. 1) Amylolytic activity. This plays a role in breaking down protein in digestion so that it can be used optimally by fish. 2) Antibacterial compound against *A. hydrophila*. *A. hydrophila* is a common pathogen in fresh waters. In the cultivation environment, these bacteria are also commonly found so that the ability to inhibit these pathogens is needed as a biocontrol agent in the environment so that the microorganisms in the environment remain balanced. 3) Synergism. Digestive bacteria have several interactions; one of which can synergize in the fish environment. This ability involves a complex interaction to carry out its role. 4) Sensitivity to antibiotics. The level of sensitivity and resistance of antibiotics shows the level of environmental pollution caused by the use of antibiotics. This study found that bacteria are sensitive, intermediate and resistant to certain antibiotics. The sensitive and intermediate nature of bacteria is one of the characteristics that probiotic bacteria need to have. However, it does not rule out the possibility that resistant bacteria are also good for use as probiotic bacteria because these bacteria are thought to be able to degrade the contamination from these antibiotics. Several studies have shown that resistant bacteria, with certain treatments, can be used as bacteria that decompose antibiotic compounds in the environment (Rojewska *et al*. 2021; Yang *et al*. 2021), so this research will be interesting if two bacteria that have the potential as probiotic candidates are developed. widely used as biodegradable agents, especially antibiotics. The existence of beneficial bacteria is needed to synergize with other bacteria so that the community in the environment remains stable. Several studies have shown that the bacteria found in this study have a very high potential to be developed into probiotic bacteria. Especially in isolates from *Bacillus* group. Which have been widely found to have beneficial characteristics in aquaculture environment (Thurlow *et al*. 2019; Nayak 2021).

The results of bacterial identification based on the 16s rRNA gene showed bacteria from the genus *B. velezensis* and *P. mirabilis.* Molecular identification based on the 16s rRNA gene is a standard identification that targets universal genes from prokaryotic microorganisms (Fig. 2). This 16s rRNA partial genome has a unique locus and has standard criteria for variable and conservative properties in making phylogenetic trees (McNichol *et al*. 2021). The two bacterial strains in this study are common bacteria often found in aquatic environments (Sunny *et al.* 2016; Mahestri *et al.* 2021). *Bacillus* sp. group is a group of bacteria that play a crucial role in the environment, including sediments. The breakdown of organic material that settles at the bottom of the pond will be degraded by the *Bacillus* group through aerobic pathways (Alegbeleye *et al.* 2017; Lahiri *et al.* 2018). Furthermore, *P. mirabilis* bacteria are bacteria found in the human urinary tract. However, these bacteria also live naturally in nature and play a role in accelerating plant growth and some isolates work together to create opportunities for other bacteria to carry out biodegradation (Drzewiecka 2016). Until now, it has not been reported that the presence of these bacteria is pathogenic for fish.

The candidates for probiotic bacteria have certain criteria before they are designated as probiotic bacteria. For the tests carried out in this study, the first step is the process of determining probiotic bacteria (Saarela *et al*. 2000). The very interesting thing to develop from the results of this research is the use of these bacteria in the rice-fish farming system. One of the important things about probiotic bacteria is that bacteria come from their native habitat so that the bacteria will carry out their roles and functions to their fullest (Wuertz *et al.* 2021; Husain *et al.* 2022). Several studies on indigenous bacteria have been carried out (Prayogo *et al*. 2018; Husain *et al*. 2022; Fitriadi *et al*. 2023a), but the isolation of probiotic bacteria for the development of rice-fish farming has not been carried out. Rice-fish farming is very complex system. The aquatic environment for fish and rice plants uses a lot of chemicals as fertilizers. The probiotic bacteria obtained are expected to be developed given the

very complex interactions in the environment. Not only the environment for rice-fish farming, the application of fertilizer to rice plants is strongly suspected to contain chemical compounds, which can also cause the environment to contain high waste material and contamination so that probiotic bacteria are to break down these compounds.

Conclusion

Amylolytic bacteria were successfully detected by 10 isolates on starch media. The results of exploration based on amylolytic activity, antibacterial activity against *A. hydrophila*, proteolytic activity, synergism and sensitivity to several types of antibiotics test, two isolates emerged as potential probiotics. The two isolates, namely S10 and S11, were identified as *B. velezensis* and *P. mirabilis*. These probiotic bacteria can be developed to degrade antibiotics and to break agricultural waste in rice-fish farming activities.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Data Availability

Not applicable.

Ethics Approval

Not applicable.

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