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Full Length Article

Activity of Amilolytic Bacteria Isolated from Rice-Fish Farming System Pond Sediments

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Abstract

The majority of bacteria present in rice-fish farming system pond sediments are functioning as degraders of organic matter. Amylolytic bacteria also play a crucial role, functioning as starch degradation, organic matter that accumulates in rice-fish farming system pond sediments. Therefore, this research aimed to determine the activity of amylolytic bacteria found in rice-fish farming system pond sediments. An observation method was applied through purposive sampling on two rice-fish farming system ponds, namely Block A and Block E, which were sampled at inlet, middle and outlet. The results showed that among 150 bacterial isolates obtained, 78 had amylolytic activity. Further analysis identified 23 isolates of amylolytic bacteria which had the best index of amylolytic activity, ranging from 1.8 to 4.0. A total of 5 isolates with SAT.2 code, namely SAO. 1, SAO. 12, SEI.1 and SEO 16 had the highest amylolytic index ranging from 3.0 to 4.0. The high activity index of the amylolytic bacteria obtained showed potential as promising candidates for probiotics to degrade starch in organic matter extracted from the bottom of rice-fish farming system pond sediments. © 2024 Friends Science Publishers

Keywords: Correlations; Foliar spray; Grain yield; Root growth; Stress tolerance; Wheat

Introduction

Rice-fish farming system cultivation is an integrated agricultural method (Akbar *et al.* 2017), combining fish and rice cultivation in one area (Rahmadi *et al.* 2019). This innovative farming system has been successfully implemented in several countries such as Indonesia, Thailand, Vietnam, the Philippines, Bangladesh, Malaysia, and others. Specifically, rice-fish farming system cultivation is highly efficient and effective in areas with water availability throughout the year. In rice-fish farming system, approximately 10% of rice fields are designed as fish shelters in the form of ditches or ponds (Vromant *et al.* 2001). Cultivating fish using this innovative farming system increases the productivity of rice fields by improving farmer income, yield diversity, soil, and water fertility, as well as reducing the use of chemical fertilizers by 30%, pests, and diseases of rice plants (Ahmadian *et al.* 2021). Rice-fish farming system tend to vary from one region to another depending on topography and weather conditions, thereby requiring potential opportunities to improve the technology (Astuti *et al.* 2020).

An aspect of rice-fish farming system that has not been optimally explored is the bacteria found in pond sediments. The majority of bacteria present in pond sediments play a role in the process of biodegradation of organic matter (Satyantini *et al.* 2020). Organic matter, including leftover feed, fish feces, and parts of rice plants that settle at the body of the pond, serves as a nutritional source for bacteria (Utomo *et al.* 2019; Burducea *et al.* 2022). Additionally, nutrients such as phosphorus, carbon, and nitrogen contained in rice-fish farming system pond sediments are ideal for the growth of various types of microorganisms, including amylolytic bacteria (Feng *et al.* 2016; Arunrat *et al.* 2022).

Amylolytic bacteria are capable of producing amylase enzymes, which function as biocatalysts facilitating hydrolysis process of starch into simpler molecules such as glucose, maltose, and dextrin (Hanzen *et al.* 2017; Prayogo *et al.* 2018). Furthermore, amylolytic bacteria accelerate process of decomposing food in the fish's body (Firmani *et al.* 2022), acting as bioremediation agents for organic matter found in the environment (Artha *et al.* 2019). Several genera of bacteria

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capable of producing amylase enzymes include *Arthrobacter*, *Escherichia*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Serratia*, *Streptomyces* and *Bacillus* (Klinfoong *et al.* 2022). The activity of these bacteria is showed by the formation of a clear zone around the colony on starch media (Suciati *et al.* 2016; Kiti *et al.* 2020), which can be observed with the addition of iodine (Ni'matuzahroh *et al.* 2021; Wulandari and Purwaningsih 2021). Consequently, information regarding the presence of amylolytic bacteria in the sediment of the rice-fish farming system is crucial to use their potential benefits in agricultural practices.

Materials and Methods

Time and place of research

This research was conducted from September to November 2022 and sediment sampling was carried out at the rice-fish farming pond in Panembangan Village, Cilongok District, Banyumas Regency. Isolation of bacteria to Gram, catalase, and oxidase testing of amylolytic bacteria was conducted at the Microbiology Laboratory of the Muhammadiyah University in Purwokerto and the Research Laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University.

Sampling

Sediment sampling was conducted using the purposive sampling method, specifically targeting two rice-fish farming ponds, namely Block A and Block E, which were sampled at inlet, middle and outlet, as shown in Fig. 1. The sampling process was conducted using a sterile spoon, where sediment samples were taken from the top layer of the surface with a depth of 3 - 4 cm. Subsequently, selected sediment samples were placed in sterile petri dishes, stored in cool boxes, and processed in a laboratory under controlled conditions.

Bacterial isolation

Bacterial isolation commenced with serial dilution of sediment samples. Initially, 0.1 g of sediment samples were diluted and suspended in 0.5 mL physiological solution and homogenized using a vortex. The dilution process was conducted with three test tubes containing 4.5 mL of physiologically sterile (10^{-1}-10^{-3} dilution). A sample of 0.5 mL was taken and homogenized with 4.5 mL of physiological solution in the first tube (10^{-1} dilution). Subsequently, 0.5 mL of sample suspension was taken from the first tube and homogenized in the second tube (10^{-2} dilution), and the procedure was carried out until the third tube (10^{-3} dilution). The results of the 10^{-1} to 10^{-3} dilution were collected and cultured using the pour plate method on TSA media, followed by incubation for 18-24 h at 28ºC.

Colonies that grew on each TSA medium, namely at 10^{-1} to 10^{-3} dilution, were counted using a colony counter. After obtaining the number of colonies from each dilution, the abundance of sedimentary bacteria was calculated using the total plate count (TPC) calculation method with 30-300 colonies, using the formula expressed by Nurhafid *et al.* (2021):

$$\text{Number of bacteria} \left(\frac{\text{CFU}}{\text{g}}\right) = \text{Number of colonies} \times \frac{1}{\text{Dilution}} \times \frac{1}{\text{Culture volume}} \times \frac{1}{\text{Sample weight}}$$

Bacterial morphology observation

The bacteria growing on TSA media were observed for the macroscopic morphological characteristics of the colony consisting of color, shape, elevation, edges and size (Sabbathini *et al.* 2017). Bacterial colonies that grew separately were collected, consisting of 25 isolates from each sample dilution. Sampling of bacterial colonies was based on visible morphological differences. The selected bacteria were stocked using the streak plate technique on TSA media.

Amylolytic activity testing

Amylolytic activity was determined by taking a looped needle culture on TSA media and streaking it on starch media, followed by incubation at 28°C for 48 h. Bacterial isolates that have amylolytic activity were characterized by the formation of a clear zone around the colony (Khiftiyah *et al.* 2018; Artha *et al.* 2019). Visualization of amylolytic activity was conducted by dripping with iodine solution. The results of the clear zone formed were measured using the diameter of the bacterial colony and clear zone to determine the amylolytic activity index formed (Pramono *et al.* 2019; Fitriadi *et al.* 2023). Subsequently, the measurement results were included as quantitative data in the calculation of amylolytic activity index using the formula expressed below (Melisha *et al.* 2016).

$$\text{Amylolytic Activity} = \frac{\text{total diameter of the clear zone} - \text{diameter of the bacterial colony}}{\text{diameter of the bacterial colony}}$$

The proportion of amylolytic bacteria was calculated using the Sinatryani (2014) formula namely:

$$\text{Proportion of amylolytic bacteria (\%)} = \frac{\text{number of amylolytic bacterial colonies obtained}}{\text{total number of colonies observed}} \times 100$$

Gram observation

Gram observations were conducted by dropping one drop of 3% KOH on a glass object. Subsequently, a loopful of amylolytic bacterial isolates was taken from each culture stock and stained with 3% KOH solution on a glass object, followed by gradual lifting. Based on observations, gram positive is characterized by no mucus formation when the loop is removed, while the presence of mucus indicates gram negative (Kesaulya *et al.*2021).

Catalase enzyme activity test

Catalase testing was conducted with a solution of hydrogen peroxide (H_2O_2) by dropping one drop of H_2O_2 solution on a glass object. Subsequently, a loopful of amylolytic bacterial isolates was taken from each culture stock and reviewed on a glass object containing H_2O_2 solution. Positive results were showed by the formation of gas bubbles in the review. The appearance of a few bubbles indicated a weak reaction, while negative results were identified in the absence of gas bubbles (Nandi *et al.* 2019).

Oxidase test

The oxidase test was conducted with the tetramethyl-blue reagent by taking one ose of amylolytic bacterial isolates, followed by scanning on glass objects. Bacterial review was covered using filter paper and 1-2 drops of tetramethyl-blue reagent was added to the object glass. Positive oxidase results were showed by a change in the color of the paper to dark blue or purple, while negative results were shown by absence of color change (Ullah *et al.* 2021).

Data analysis

The data obtained from the results were in the form of abundance of bacteria, colony morphology, activity index of amylolytic bacteria, proportion of amylolytic bacteria, gram characteristics, catalase and oxidase activity of amylolytic bacteria. Subsequently, these data were presented in the form of pictures, tables, and graphs, which were analyzed descriptively and compared to the literature.

Results

Rice-Fish Farming System Pond Sediments Bacterial Abundance

The number of bacterial abundance in rice-fish farming system sediments is presented in Table 1, showed significantly varied values at each sampling point. Based on the results, bacterial abundance at midpoint was higher compared to inlet and outlet.

Rice-Fish farming system pond sediments bacterial morphology

The morphology of the bacterial colonies obtained in this research tended to vary greatly. Based on observations, there were 102 different types of bacteria among the 150 isolates obtained. The different morphological characteristics of bacteria were taken in reference to Bergey Manual of Determinative Bacteriology (Holt *et al.* 1994), which included shape, elevation, edges, color, and size. In this research, the colony forms obtained were circular, irregular, filemantous, rhizoid, spindle and puntiform. The elevation consisted of convex, crateriform, raised, pulvinate, umbonate, and flat, while edges comprised entire, undulate, lobate and filamentous. The color of the bacterial colonies consisted of milky white, translucent white, grayish white, white, yellowish white, yellow, creamy white, brownish white, brownish yellow, creamy brown and light yellow. Moreover, colony size ranges from small, medium and large.

Proportion and index of amylolytic bacteria in rice-fish farming system pond sediments

The proportion of amylolytic bacteria obtained was indicated by the activity of the amylase enzymes produced. This was indicated by the presence of a clear zone that forms around the bacterial colonies, as shown in Fig. 2. The large clear zone formed indicated the high activity of the amylase enzymes produced by the bacteria.

The results of the amylolytic activity test of bacteria showed that among 150 bacterial isolates from rice-fish farming sediments, 78 had amylolytic activity. The number of bacterial isolates with positive amylolytic activity showed the proportion of amylolytic bacteria present in the Rice-Fish Farming pond sediments, as presented in Table 2.

Rice-fish farming system pond sediments amylolytic bacteria activity index

The activity index of amylolytic bacteria was obtained by dividing the diameter of the bacterial clear zone by that of bacterial colony. The amylolytic activity index value was obtained based on the incubation time with the highest average index value. In this research, the highest average index value was obtained at 48 h of incubation time, as shown in Fig. 3.

Among 76 bacterial isolates with amylolytic activity indexes, 23 with the highest amylolytic activity index were selected for further analysis. The best isolate was taken based on the high index of amylolytic activity produced. According to Dar *et al.* (2015), bacterial hydrolysis zone is divided into three categories, namely weak ($\leq$ 1.0 cm), moderate (1.1 - 2.9 cm), and strong ($\geq$ 3.0 cm). The best bacterial amylolytic activity index values are presented in Table 3.

Gram test results, catalase and oxidase activities of amylolytic bacteria

In this research, gram, catalase, and oxidase tests were conducted to add to the characteristics of amylolytic bacteria. Amylolytic bacterial isolates with gram, catalase and oxidase characteristics obtained in this research varied quite a bit between tests. The results of the gram test, catalase and oxidase activity of amylolytic bacteria are presented in Table 4.

A total of 5 isolates of the 23 bacterial isolates were purified based on amylolytic activity index values exceeding

3. These bacteria were characterized based on biochemical properties. Characteristics of bacterial isolates with the highest amylolytic activity can be seen in Table 5 namely.

Discussion

Across Block A and Block E of the rice-fish farming sediments, there is a relatively high bacteria abundance. These results show that sediments can become a substrate containing nutrients facilitating the growth and survival of bacteria. However, the factors influencing the difference in abundance include the flow of water and the texture of the sediments found in the pond. Block A is characterized by a sand sediments texture of the water flowing from the mountains, while Block E has water flow passing through settlements with a muddy sediments texture. The difference in the flow of incoming water with the texture of the sediments causes variations in the number of bacteria abundance present in rice-fish farming ponds (Guan *et al.* 2015; Alfionita *et al.* 2019; Irene *et al.* 2020). Other factors include culture media, conditions during the culture process, treatment before culture, isolation techniques, and incubation time (Ayuningrum *et al.* 2021). The abundance of bacteria present in the pond offers an opportunity for bioremediation efforts (Prihanto *et al.* 2021). The morphological differences obtained can be used to differentiate the bacterial species in sediments (Sousa *et al.* 2013) due to the characteristics of each bacterium in forming colonies. The ability of bacteria to form colonies can be influenced by environmental factors and adaptability (Joseph 2021; Palma *et al.* 2022). Variations in the characteristics of the bacterial colonies obtained make the initial screening for further identification processes.

Organic matter containing starch originating from leftover feed, fish feces, and parts of rice plants that fall and accumulate in ponds serves as one of the factors affecting the proportion of amylolytic bacteria. According to Ayuningrum *et al.* (2021) the high proportion of amylolytic bacteria in ponds is influenced by various factors such as organic matter containing starch. The high starch content causes water quality to be disrupted, impacting bacterial communities in sediments (Zhao *et al.* 2023). However, amylase-producing bacteria can reduce starch content in rice-fish farming system pond sediments to optimize aquaculture activities.

During this research, amylolytic index values tended to be higher at 48 h due to differences in the ability of bacteria to produce amylase enzymes. According to Erfanimoghadam and Homaei (2023), each species of bacteria is capable of producing different amylase enzymes. The length of incubation time also affects the ability of amylolytic bacterial species to produce amylase enzymes (Abo-Kamer *et al.* 2023). In this research, the amylase enzyme produced tended to be higher at 48 h of incubation time, as bacteria entered the logarithmic phase. The higher incubation time is observed due to the ability of bacteria to produce amylase enzymes. This was proven by Abo-Kamer *et al.* (2023), where the best ability of amylolytic bacteria was at an incubation time of 48 h. Yassin *et al.* (2021) also stated that 48 h of incubation time was the peak for bacteria to produce amylase enzymes. Meanwhile, the initial 24-h incubation time was indicated as a bacterial lag phase on new media, where cell maintenance is carried out as an adaptation process by absorbing the nutrients contained to form a colony (Gonzalez and Aranda 2023). Consequently, the clear zone formed at 24 h of incubation is smaller compared to 48 h.

Table 1: Rice-Fish farming system pond sediments bacterial abundance

Sample	Bacterial Abundance (CFU/g)	
	Block A	Block E
Inlet	5.7×10^5	2.0×10^5
Middle	7.0×10^5	9.3×10^5
Outlet	2.7×10^5	6.0×10^5
Average	5.1×10^5	5.7×10^5

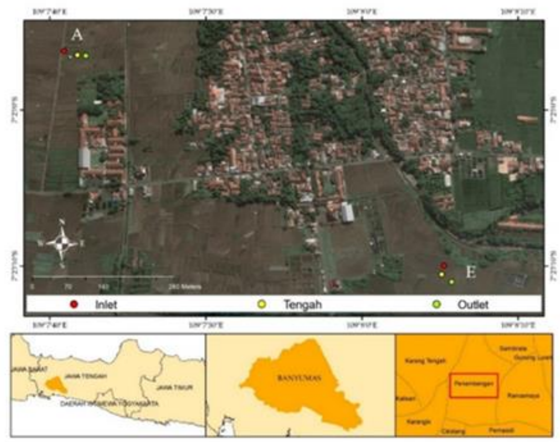

Fig. 1: Rice-Fish farming system pond sediments sampling location

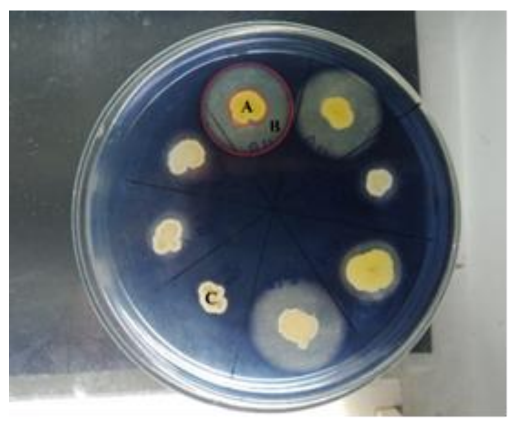

Fig. 2: Activity of the amylase enzyme produced by amylolytic bacteria. (**A**) Bacterial isolates (**B**) clear zones (**C**) bacterial isolates that do not produce amylase enzymes

Table 2: Proportion of amylolytic bacteria in rice-fish farming system pond sediments

Sample	Number of Isolates		Number of Amylolytic Isolates		Proportion (%)	
	Block A	Block E	Block A	Block E	Block A	Block E
Inlet	25	25	13	18	52	72
Middle	25	25	14	13	56	52
Outlet	25	25	10	10	40	40

Table 3: Highest amylolytic bacterial activity index

Block	Point	The best isolate	Amylolytic Index	Index Category
Block A	Inlet	SAI.8	2.0	Medium
		SAI.2	1.8	Medium
		SAI.13	2.1	Medium
	Middle	SAT.2	4.0	Strong
		SAT.10	1.7	Medium
		SAT.11	1.8	Medium
		SAT.12	2.7	Medium
		SAT.13	2.5	Medium
		SAT.15	1.9	Medium
		SAT.19	1.8	Medium
		SAT.21	2.3	Medium
		SAT.24	2.1	Medium
		SAT.25	2.3	Medium
	Outlet	SAO.1	4.0	Strong
		SAO.2	2.5	Medium
		SAO.12	3.0	Strong
		SAO.13	1.8	Medium
		SAO.16	2.5	Medium
		SAO.24	2.0	Medium
Block E	Inlet	SEI.1	3.4	Strong
		SEI.9	2.0	Medium
		SEI.11	1.9	Medium
	outlet	SEO.16	3.0	Strong

Description: SAI.8: Sediment block A inlet isolate 8, *etc.*; SAT.1: Middle block A sediment isolate 1, *etc.*; SAO.2: Sediment block A outlet isolate 2, *etc.*; SEI.25: Sediment block E inlet isolate 25; SET.1: Middle block E sediment isolate 1, *etc.*; SEO. 5: Sediment block E outlet isolate 5

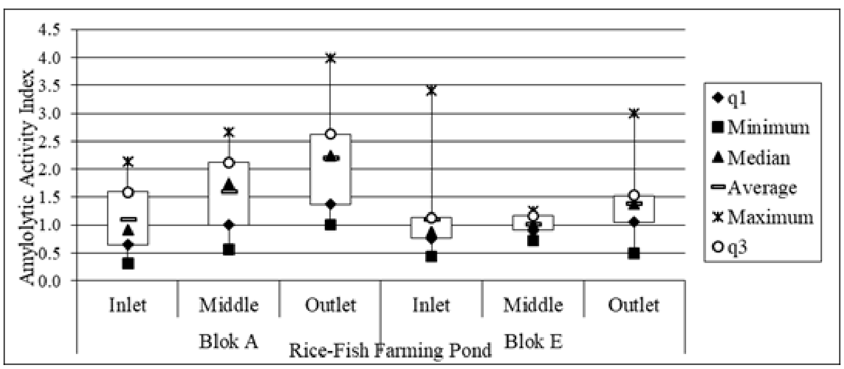

Fig. 3: Rice-Fish farming system pond sediment amylolytic bacteria activity index (Quartile 1 (q1): Data distribution of the lowest amylolytic index; Minimum: Lowest amylolytic index value; Median: Middle amylolytic index value; Average: Average amylolytic index value; Maximum: Highest amylolytic index value; Quartile 3 (q3): Distribution of the highest amylolytic index data; Box plot: amylolytic index data set)

This research examined the biochemical characteristics of amylolytic bacteria, which included gram, catalase, and oxidase to determine the advantages and potential of the amylolytic bacteria obtained. The results of the gram test for amylolytic bacteria showed that 34 isolates were gram positive and 44 isolates were gram negative. Differences in the gram of a bacterium showed the ability to adapt to significantly extreme environmental conditions (Garde *et al.* 2021). Bacteria with a thin peptidoglycan cell wall showed high susceptibility to rupture or lysis, resulting in death. In contrast to bacteria with a thick peptidoglycan layer, the protection of amylolytic bacteria tends to be strong (Silhavy *et al.* 2010; Slavin *et al.* 2017). Based on catalase test, amylolytic bacteria showed that 60 isolates were positive and 18 isolates were negative. Positive catalase results showed that amylolytic bacteria degraded hydrogen peroxide, a toxic compound against bacterial cells (Arihantana and Puspawati 2017; Glorieux and Calderon

Table 4: Gram test results, catalase and oxidase amylolytic bacteria

Block	Point	Gram Test		Catalase Test		Oxidase Test	
		Positive (+)	Negative (-)	Positive (+)	Negative (-)	Positive (+)	Negative (-)
Block A	Inlet	3	10	11	2	2	11
	Middle	8	6	9	5	7	7
	Outlet	5	5	8	2	6	4
Block E	Inlet	10	8	11	7	14	4
	Middle	4	9	12	1	13	0
	Outlet	4	6	9	1	6	4

Table 5: Characteristics of potential amylolytic bacteria isolates

Isolate Code	Form	Elevation	Edge	Gram	Catalase	Oxidase	IA
SAT.2	*Circular*	*Convex*	*Entire*	+	+	+	4.0
SAO.1	*Filamentous*	*Flat*	*Filamentous*	+	+	-	4.0
SAO.12	*Circular*	*Raised*	*Undulate*	-	+	+	3.0
SEI.1	*Filamentous*	*Umbonate*	*Filamentous*	-	-	+	3.4
SEO.16	*Irregular*	*Raised*	*Undulate*	+	+	-	3.0

Description: SAT.2: Middle block A sediment isolate 2, SAT.7: Middle block A sediment isolate 7, SAT.11: Middle block A sediment isolate 11, SAT.12: Middle block A sediment isolate 12, SET.10: Block sediment Middle E isolate 10; IP: Amylolytic Index

2017). Moreover, the number of bacteria capable of producing catalase enzymes could be an indicator of the level of water (Eddine 1963; Sridhar and Pillai 1972; Hosetti and Patil 1992). The results of the oxidase test of amylolytic bacteria showed that 48 isolates were positive and 30 isolates were negative, indicating the ability to produce cytochrome oxidase enzymes. Since cytochrome oxidase enzymes are not commonly found, a positive result can be the basis for identifying certain bacteria capable of producing this enzyme (Hederstedt 2022).

A total of five bacteria with the highest amylolytic activity were selected among 23 bacteria, ranging from 3.0 to 4.0, characterized by different biochemical properties. These differences indicate that various species of bacteria are capable of producing amylase, catalase, and oxidase enzymes. In addition to high amylolytic index value, amylolytic bacteria also can produce catalase and oxidase enzymes, which required further investigation.

Conclusion

In conclusion, this research showed that the number of bacteria with amylolytic activity was approximately 78 isolates. Furthermore, there were 23 best isolates with amylolytic index ranging from 1.8 to 4.0. A total of 5 isolates with different biochemical characteristics had the highest amylolytic index, namely isolate SAT.2, SAO.1, SAO.12, SEI.1 and SEO 16, with values ranging from 3.0 to 4.0. Consequently, the high index obtained from biochemical characteristics showed the presence of probiotic potential.

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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

Data presented in this research will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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