**Running title**: Improving Stress Tolerance in Wheat Using Thiourea

# Activity Of Amilolytic Bacteria Isolated From Rice-Fish Farming System Pond Sediments

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# Novelty statement

• Amylase enzyme activity is able to break down organic materials in the environment

• Amylolytic bacteria have potential as environmental probiotic agents

• High amylolytic index indicates potential amylase enzyme activity

• Amylolytic bacteria help speed up the process of breaking down food in the fish's body

• Amylolytic bacteria as environmental bioremediation agents

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# Abstract

Most of the bacteria present in rice-fish farming system sediments act as degraders of organic matter. Amylolytic bacteria are bacteria that act as starch degradation which is organic matter that accumulates in rice-fish farming system sediments. This study aimed to determine the activity of amylolytic bacteria found in rice-fish farming system sediments. The method used in this research is the observation method by means of purposive sampling on two rice-fish farming system ponds, namely Block A and Block E which consists of three sampling points, namely at the inlet, middle and outlet points of the pond. The results showed that of the 150 bacterial isolates obtained, 78 bacterial isolates had amylolytic activity. There were 23 isolates of amylolytic bacteria which had the best index of amylolytic activity, ranging from 1.8 to 4.0. The results also obtained 5 bacterial isolates that had the highest amylolytic activity, namely isolates with SAT.2 code; SAO. 1; SAO. 12; SEI.1; SEO 16 with amylolytic index range obtained from 3.0-4.0. The high activity index of the amylolytic bacteria obtained indicates that they are promising candidates as probiotics for degrading starch in organic matter extracted from the bottom of Rice-Fish Farming ponds.

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

# Introduction

Rice-fish farming system cultivation is an integrated cultivation system (Akbar et al., 2017) that combines fish and rice cultivation in one area (Rahmadi et al., 2019). Fish farming activities with the rice-fish farming system have been implemented in several countries such as Indonesia, Thailand, Vietnam, the Philippines, Bangladesh, Malaysia and other countries. Rice-fish farming system cultivation is very efficient and effective in rice fields that have water availability throughout the year. Rice-fish farming system makes 10% of rice fields as fish shelters in the form of ditches or ponds (Vromant et al., 2001). Cultivating fish with the rice-fish farming system can increase the productivity of paddy fields by increasing farmer income, increasing yield diversity, increasing soil and water fertility, reducing the use of chemical fertilizers by 30%, and reducing pests and diseases of rice plants (Ahmadian et al., 2021). Rice-Fish Farming cultivation systems tend to vary from region to region depending on topography and weather conditions, so there is still a lot of potential and opportunities to improve the technology (Astuti et al., 2020).

One of the potentials of the Rice-Fish Farming cultivation system that has not been optimally utilized is the bacteria found in pond sediments. Most of the bacteria present in pond sediments play a role in the process of biodegradation of organic matter. The abundance of organic matter that has accumulated in pond sediments such as leftover feed, fish feces, and parts of rice plants that fall on the body of the pond is used by bacteria as a source of nutrition (Burducea et al., 2022). In addition, nutrients such as phosphorus, carbon and nitrogen contained in Rice-Fish Farming sediments make Rice-Fish Farming sediments an ideal place for the growth of various types of bacteria, one of which is starch degrading bacteria or what are called amylolytic bacteria (Arunrat et al., 2022; Feng et al., 2016).

Amylolytic bacteria are bacteria that are capable of producing amylase enzymes. The amylase enzyme functions as a biocatalyst which is able to catalyze the hydrolysis process of starch to produce simpler molecules such as glucose, maltose and dextrin (Hanzen et al., 2017). Amylolytic-producing bacteria not only help in accelerating the process of decomposing food in the fish's body (Firmani, 2022), but are able to act as bioremediation agents for organic matter found in the environment (Artha et al., 2019). Several genera of bacteria capable of producing amylase enzymes include *Arthrobacter, Escherichia, Micrococcus, Proteus, Pseudomonas, Serratia, Streptomyces,* and *Bacillus* (Klinfoong et al., 2022). The activity of amylolytic bacteria is indicated by the formation of a clear zone around the colony on starch media (Kiti et al., 2020). The clear zone in the media can be observed with the addition of iodine (Wulandari et al., 2021). Amylolytic bacteria that are capable of producing high amylase enzymes can be used as probiotic bacteria (Sahoo et al., 2015). This is the reason why information is needed regarding the presence of amylolytic bacteria in the sediment of the rice fish farming pond.

# Materials and Methods

**Time and Place of Research**

This research was conducted from September to November 2022. Sediment sampling was conducted 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 with the purposive sampling method, namely in two Rice-Fish Farming ponds, namely Block A Rice-Fish Farming pond and Block E Rice-Fish Farming pond, with 3 sampling points in each pond, namely inlet sediment, middle sediment and outlet sediment (Figure 1). The sampling process was conducted with a sterile spoon, where sediment samples were taken from the top layer of the surface with a depth of 3 - 4 cm. Then, selected sediment samples were placed in sterile petri dishes, stored in cool boxes, and then processed in a laboratory under controlled conditions.

**Figure 1.** Rice-Fish Farming Pond Sediment Sampling Location

**Bacterial Isolation**

Bacterial isolation begins with serial dilution of sediment samples. Sediment samples were diluted by weighted 0.1g samples suspended in 0.5mL 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). A total of 0.5 mL of sample suspension was taken from the first tube and homogenized in the second tube (10-2 dilution), and this procedure was carried out until the third tube (10-3 dilution). The results of the 10-1 to 10-3 dilution were taken as much as 0.5 mL each and cultured using the pour plate method on TSA media. Followed by incubation for 18-24 hours at 28ºC.

Colonies that grew on each TSA medium, namely at dilution 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 then calculated using the total plate count (TPC) calculation method with 30-300 colonies. Calculation of the abundance of bacteria can be calculated using the formula Nurhafid *et al.* (2021) as follows:

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

**Bacterial Morphology Observation**

First, 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 taken as many as 25 isolates from each sample dilution taken. Sampling of bacterial colonies was based on visible morphological differences. The selected bacteria were then stocked using streak plate technique on TSA media.

**Amylolytic Activity Testing**

Amylolytic activity was determined by taking a looped needle culture on TSA media, streaking it on starch media, and incubating it at 28°C for 48 hours. Bacterial isolates that have amylolytic activity are characterized by the formation of a clear zone around the colony (Artha et al., 2019). Visualization of amylolytic activity was conducted by dripping with iodine solution. The results of the clear zone formed were measured by the diameter of the bacterial colony and the diameter of the clear zone to determine the amylolytic activity index formed. The measurement results are included in the amylolytic activity calculation formula as quantitative data. The formula for calculating the amylolytic activity index refers to the formula used (Melisha et al., 2016) is as follows:

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

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

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

**Gram Observation**

Gram observations were conducted with 3% KOH solution. Gram observations were conducted by dropping one drop of 3% KOH on a glass object. Then one ose of amylolytic bacterial isolates was taken from each culture stock and then stained with 3% KOH solution on a glass object. Then lift the ose slowly. Gram positive is characterized by no mucus formation when the loop is removed. While Gram negative is characterized by the formation of mucus (Kesaulya et al.,2021).

**Catalase Enzyme Activity Test**

Catalase tested was conducted with a solution of hydrogen peroxide (H2O2). The catalase test was conducted by dropping one drop of H2O2 solution on a glass object. Then one ose of amylolytic bacterial isolates was taken from each culture stock and then reviewed on a glass object containing H2O2 solution. Positive results are indicated by the formation of gas bubbles in the review results. The appearance of a few bubbles indicates a weak reaction. A negative result is indicated by the absence of gas bubbles formed (Nandi et al., 2019).

**Oxidase Test**

The oxidase test was conducted with the tetramethyl-blue reagent. The oxidase test was conducted by taking one ose of amylolytic bacterial isolates, then scanning them on glass objects. Cover the bacterial review using filter paper. Tetramethyl-blue reagent is added as much as 1-2 drops on the object glass. A positive oxidase result is indicated by a change in the color of the paper to dark blue or purple and a negative oxidase result is indicated by no color change on the paper (Ullah et al.,2021).

**Data Analysis**

The data obtained from the results of the study were in the form of data on the abundance of bacteria, colony morphology, activity index of amylolytic bacteria, proportion of amylolytic bacteria, gram characteristics, catalase and oxidase activity of amylolytic bacteria. The data is presented in the form of pictures, tables and graphs, then analyzed descriptively and compared with the literature

# Results

***Rice-Fish Farming Sediment Bacterial Abundance***

 The number of bacterial abundances in Rice-Fish Farming sediments shows quite varied values at each sampling point. Where the number of bacterial abundances found at the midpoint is higher than the inlet and outlet. The abundance of bacteria found in Rice-Fish Farming sediments can be seen in Table 1.

**Table 1.** Rice-Fish Farming Sediment Bacterial Abundance

|  |  |
| --- | --- |
| **Sample** | **Bacterial Abundance (CFU/g)** |
| **Block A** | **Block E** |
| Inlet | 5,7 x 105 | 2,0 x 105 |
| Middle | 7,0 x 105 | 9,3 x 105 |
| Outlet | 2,7 x 105 | 6,0 x 105 |
| **Average** | 5,1 x 105 | 5,7 x 105 |

## **Rice-Fish Farming Sediment Bacterial Morphology**

 The morphology of the bacterial colonies obtained in this study tended to vary greatly. There were 102 different types of bacteria among the 150 isolates obtained. The different morphological characteristics of bacteria are taken in reference to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994)which includes shape, elevation, edges, color, and size. In this study the colony forms obtained were circular, irregular, filemantous, rhizoid, spindle, and puntiform. The elevation consists of convex, crateriform, raised, pulvinate, umbonate, and flat. The edges consist of entire, undulate, lobate, and filamentous. The color of the bacterial colonies consists of milky white, translucent white, grayish white, white, yellowish white, yellow, creamy white, brownish white, brownish yellow, creamy brown, and light yellow. Colony size consists of small, medium and large.

## Proportion and Index of Amylolytic Bacteria in Rice-Fish Farming Sediment

The proportion of amylolytic bacteria obtained in this study was indicated by the activity of the amylase enzyme produced by the bacteria. This is indicated by the presence of a clear zone that forms around the bacterial colonies (Figure 2). The large clear zone formed indicates the high activity of the amylase enzyme produced by the bacteria.



**Figure 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

 The results of the amylolytic activity test of bacteria showed that out of 150 bacterial isolates isolated from Rice-Fish Farming sediments, 78 of them had amylolytic activity. The number of bacterial isolates with positive amylolytic activity illustrates the proportion of amylolytic bacteria present in the Rice-Fish Farming pond sediment. The proportion of amylolytic bacteria obtained in this study is presented in Table 2.

**Table 2.** Proportion of Amylolytic Bacteria in Rice-Fish Farming 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 |

***Rice-Fish Farming Sediment Amylolytic Bacteria Activity Index***

 The activity index of amylolytic bacteria in this study was obtained by dividing the diameter of the bacterial clear zone by the diameter of the bacterial colony. The amylolytic activity index value taken in this study was based on the incubation time which had the highest average amylolytic activity index value. In this study the highest average index value was obtained at 48 hours of incubation time. The amylolytic activity index is presented in Figure 3.

**Figure 3.** Rice-Fish Farming 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).

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

**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.

## **Gram Test Results, Catalase and Oxidase Activities of Amylolytic Bacteria**

In this study, the 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 study varied quite a bit between tests. The results of the Gram test, catalase and oxidase activity of amylolytic bacteria are presented in Table 4.

**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 |

Five isolates of the 23 bacterial isolates were purified based on amylolytic activity index values exceeding 3, resulting in a total of 23 isolates. Then, these bacteria were characterized based on their biochemical properties. Characteristics of bacterial isolates with the highest amylolytic activity can be seen in table 5 namely:

**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

# Discussion

 Across both blocks of the Rice-Fish Farming pond sediments, bacteria abundance is relatively high. These results indicate that sediment can become a substrate that contains nutrients so that bacteria can grow and live in the sediment. The factors that influence the difference in abundance are the flow of water and the texture of the sediment found in the pond. Block A is characterized by a sand sediment texture of the water flowing from the mountains. Whereas in block E the water flow has passed through settlements with a muddy sediment texture. The difference in the flow of incoming water with the texture of the sediment causes differences in the number of bacteria abundance present in Rice-Fish Farming ponds (Alfionota et al., 2019; Guan et al., 2015; Irene et al., 2020). Other factors that affect differences in bacterial abundance are culture media, conditions during the culture process, treatment before culture, isolation techniques, incubation time (Ayuningrum et al., 2021). The abundance of bacteria present in the pond allows for the potential for bioremediation of bacteria. The morphological differences obtained can be used to differentiate the bacterial species present in sediments based on their morphological differences (Sousa et al., 2013). The observed morphological differences could be due to the characteristics of each bacterium in forming colonies. The ability of bacteria to form colonies can be influenced by environmental factors and the adaptability of these bacteria (Joseph et al., 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 that comes from leftover feed, fish feces, and parts of rice plants that fall and accumulate in ponds is one of the factors that affects the proportion of amylolytic bacteria present in ponds. Further explained in the research of Ayuningrum et al., which states that the high proportion of amylolytic bacteria in ponds is influenced by various factors, one of which is the presence of organic matter containing starch. The content of organic matter containing starch is quite high in waters causing water quality to be disrupted which will have an impact on the bacterial community in sediments (Zhao et al., 2023). However, amylase-producing bacteria can reduce the starch content in the Rice-Fish Farming ponds in order to optimize aquaculture activities.

 During this study, amylolytic index values tended to be higher at 48 hours. This is caused by differences in the ability of bacteria to produce amylase enzymes. It was further explained in the research by Erfanimoghadam & Homaei (2023), which stated that each species of bacteria is capable of producing different amylase enzymes. In addition, the length of incubation time for bacteria also affects the ability of amylolytic bacterial species to produce amylase enzymes (Abo-Kamer et al., 2023). In this study the amylase enzyme produced tended to be higher at 48 hours of incubation time. This happened because at 48 hours the bacteria entered the logarithmic phase. In this phase, the ability of bacteria to produce amylase enzymes is at its peak. This was proven in the study of Abo-Kamer et al. (2023) which stated that the best ability of amylolytic bacteria was at an incubation time of 48 hours. In addition, in the research by Yassin et al. (2021) stated that this time was the peak for bacteria to produce amylase enzymes. Meanwhile, the initial 24-hour incubation time was indicated as a bacterial lag phase on new media. In this phase, bacteria tend to maintain their cells as an adaptation process by absorbing the nutrients contained in the media to form a colony (Gonzalez & Aranda, 2023). As a result, the clear zone formed at 24 hours of incubation is smaller than the clear zone at 48 hours.

 This study also examined the biochemical characteristics of amylolytic bacteria which included gram, catalase, and oxidase aimed to see 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 shows its ability to adapt to quite extreme environmental conditions (Garde et al., 2021). Bacteria with a thin peptidoglycan cell wall will be very susceptible to rupture or lysis, causing the death of the bacteria. In contrast to bacteria that have a thick peptidoglycan layer, the protection these bacteria tend to have is strong (Silhavy et al., 2010; Slavin et al., 2017). The results of the catalase test from amylolytic bacteria showed that 60 isolates were positive for catalase and 18 isolates were catalase negative. Positive catalase results indicate that amylolytic bacteria are able to degrade hydrogen peroxide, which is a toxic compound against bacterial cells (Arihantana & Puspawati, 2017; Glorieux & Calderon, 2017). he number of bacteria capable of producing catalase enzymes can be an indicator of the level of contamination of a waters (Eddine, 1963; Hosetti & Patil, 1992; Sridhar & Pillai, 1972). The results of the oxidase test of amylolytic bacteria showed that 48 isolates were positive for catalase and 30 isolates were negative. The results of the oxidase test showed the ability of bacteria to produce cytochrome oxidase enzymes. This cytochrome oxidase enzyme is not owned by all bacteria, so that a positive result can be the basis for identifying certain bacteria that are capable of producing this enzyme (Hederstedt, 2022).

 Five bacteria with the highest amylolytic activity were selected among 23 bacteria with the best amylolytic index, ranging from 3.0 to 4.0. The five isolates had different biochemical characteristics. These differences in characteristics indicate that the bacteria are of different species. Some of the bacteria obtained showed potential, where apart from being able to produce amylase enzymes, these bacteria were also able to produce catalase and oxidase enzymes. In addition to its high amylolytic index value, it also has the ability to produce catalase and oxidase enzymes, which should be further investigated.

# Conclusion

The number of bacteria that have amylolytic activity is around 78 isolates. There are 23 best isolates that have amylolytic index ranging from 1.8 to 4.0. There were also 5 isolates with the highest index of amylolytic activity, namely isolate SAT.2; SAO. 1; SAO. 12; SEI.1; SEO 16 with amylolytic index range obtained from 3.0-4.0. The five bacterial isolates have different biochemical characteristics. Consequently, the high index obtained from their biochemical characteristics indicates that these bacteria have probiotic potential.

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# Conflict of Interest

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

**Data Availability**

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

**Ethics Approval**

Not applicable to this paper

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