Journal of Aquaculture and Fish Health Vol. 13(1) - February 2024
P-ISSN: 2301-7309 / E-ISSN: 2528-0864
doi : 10.20473/jafh.v13i1.39210

Screening of Amylolytic Bacteria from Mina Padi Aquaculture in Panembangan Village, Cilongok District, Banyumas, Central Java

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Received : 2022-09-22
Accepted : 2024-02-02

Keywords :
Amylolytic bacteria, biochemical test, minapadi water

Abstract

Amylolytic bacteria play an important role in the ecosystem, especially as probiotic and bioremediation agents in cultivation, as examples can be found in minapadi culture. The purpose of this study was to determine the amylolytic bacteria in the waters of the minapadi pond. Bacterial isolation began with bacterial sampling, inoculation and isolation of bacteria, calculation of the total abundance of bacteria, observation of bacterial morphology and bacterial purification, isolation of amylolytic bacteria, and biochemical testing. Three isolates of amylolytic bacteria were successfully obtained, namely BA5, BA6, and BA7 with a weak to moderate activity index category. The amylolytic bacterial isolates obtained were successfully grouped into 3 different groups, namely *Proteus* sp., *Staphylococcus* sp., and *Micrococcus* sp. Amylolytic bacteria have a biodegradation role in the minapadi cultivation environment.

INTRODUCTION

Minapadi is an integrated cultivation system that combines fish cultivation and rice cultivation. Usually, fish rearing is carried out on the sidelines of rice crops to fill the gap between two rice crop seasons or as a substitute for secondary crops in rice fields. Fish commodities that can be grown in the minapadi cultivation system include goldfish, tilapia, Mozambique tilapia, catfish, carp, and tawes fish (Harahap, 2017). However, most minapadi cultivators use goldfish and tilapia, because these fish can grow well even in shallow waters and get a lot of sunlight (Akbar, 2017). Fish maintenance in the minapadi cultivation system is strongly influenced by the quality of pond waters, where the nutrients contained in the water can affect the growth of rice plants and fish growth.

Minapadi pond waters play an important role in the fish and rice plants' survival. In general, minapadi waters are inundated with parallel water circulation. Parallel circulation in minapadi waters makes the physical and chemical quality of the water fluctuate and difficult to control. However, microorganisms in the minapadi ecosystem can overcome this problem. The presence of minerals and other nutrients makes it an ideal place for

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microorganisms such as plankton and bacteria (Ahmadian *et al*., 2021).

One of the microorganisms that are needed in the growth of fish is probiotic bacteria. Probiotic is a live microorganism in fish farming that can be used as environmental biocontrol and increase the effectiveness of the feed given (Elumalai *et al*., 2002). Probiotic bacteria produce enzymes that can increase the breakdown of complex compounds into simple compounds. The bacteria contained in probiotics have a mechanism to produce several enzymes, one of which is the amylase enzyme. Amylolytic bacteria can produce amylase enzymes (Karel *et al*., 2019).

Amylolytic bacteria are bacteria that can break down starch into glucose (Putri *et al*., 2021). Amylolytic bacteria generally produce extracellular enzymes, where enzymes produced inside the cell are then released out of the cell (Nangin *et al*., 2015). There are 3 types of enzymes produced by amylolytic bacteria, namely ɑ- amylase, β-amylase, and ɤ- amylase (Widyati, 2008). Amylase enzyme produced by amylolytic bacteria can catalyze starch hydrolysis into glucose, maltose, and dextrin. Amylolytic bacteria themselves can come from soil and water. For example, bacteria that have been isolated from water are studied by *Bacillus* sp. and *Serratia* sp. (Hanzen *et al.*, 2017). According to Silaban and Simamora (2018), Amylolytic bacteria that live in water habitats are *Bacteriods, Lactobacillus, Clostridium, Micrococcus, Thermus,* and *Actinomycetes*.

The use of amylolytic bacteria should come from bacteria that grow in the area, because even if the bacteria type is the same, the bacteria strain may be different. In the microbial world, bacteria will mutually limit population growth with other microbes. The introduction of a new type of microbe, if the environment supports it can cause the loss of certain microorganisms that may be useful in other ways or can cause the environment to change. So, the purpose of this journal was to determine amylolytic bacteria from the minapadi waters in Panembangan Village, Cilongok District, Banyumas, Central Java.

METHODOLOGY

Ethical Approval

All research procedures are carried out according to standards without damaging or polluting the environment and testing laboratory.

Place and Time

This research was conducted from January 20 to June 20, 2022, in Panembangan Village, Cilongok District, Banyumas Regency, Central Java Province. Isolation of amylolytic bacteria was conducted at Muhammadiyah University Purwokerto Laboratory and Laboratory of Fisheries and Marine Sciences, Jenderal Soedirman University.

Research Materials

Research materials used include Petri dish (90x15mm, Anumbra), ose needle, Hotplate stirrer (IKA C MAG HS7, Germany), Tryptic Soy Agar (Merck 500gr) Tapioca (Rose Brand, 250gr). Triple Sugar Iron Agar (Himedia), MR-VP media (Merck), KOH Gram, H202, Sulfide Indole Motility (Merck), Test Tube (10ml, Iwaki), and Incubator (Memmert IN55, Germany).

Research Design

This research uses a survey method. Water samples were taken from several different locations in the fish-rice farm. The first location is taken from the water inlet, the second is taken from the middle of the cultivation pond, and the third is taken from the pond water outlet. Then, the samples were taken to the laboratory for observation inoculation and isolation of amylolytic bacteria.

Work Procedure

Bacteria Sampling

Water sampling was conducted using a survey method with a random sampling technique where water samples were

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taken at 3 points, namely 1) inlet, 2) middle, and 3) outlet. Water samples were put in a sterile container (Erlenmeyer). The Erlenmeyer tube was closed using aluminum foil and plastic wrap. Erlenmeyer tube was put into a cool box with a temperature of 4 - 8°C for observation in the laboratory.

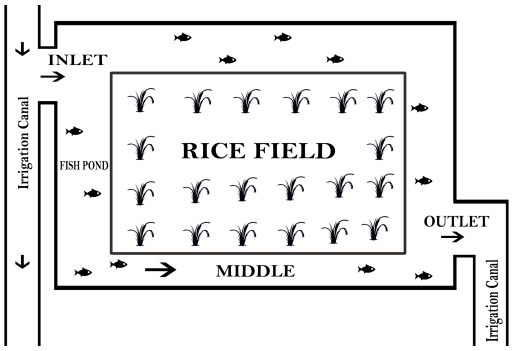

Figure 1. Illustration of the rice-fish farming system from the aerial view.

Bacterial Inoculation and Isolation

The bacteria inoculation procedure followed the procedure of Nisa (2022) which was to take 0.5 ml of pool water sample suspended into 0.5 ml of physiological solution in a test tube, then homogenized with a vortex and diluted gradually. Sample dilutions were conducted in series using two test tubes containing 4.5 mL of sterile physiological (10^{-1} - 10^{-2} dilution). 0.5 mL of water sample was taken and homogenized with 4.5 mL of physiological solution in the first tube (10^{-1}). A total of 0.5 mL of sample suspension was taken from the first tube and homogenized in the second tube (10^{-2}). After the dilution, take 0.5 ml of each dilution and put it into a petri dish that already contains TSA media using the pour plate method. Furthermore, the mixture between the sample and TSA media was homogenized by rotating the petri dish horizontally. The media was incubated for 24-48 hours at 28 °C.

Then, the bacterial colonies that had grown on TSA media were selected based on different morphologies for purification. Bacterial isolation was conducted to obtain a single isolate of bacteria using the streak plate method. Single bacterial isolates were separated based on their morphological characteristics ranging from size, shape, color, and elevation. Then, the isolated bacterial isolates were grown on inclined TSA media to serve as stock. All research processes were conducted aseptically using a Bunsen fire and carried out in Laminar Air Flow (LAF) to prevent contamination from other microorganisms.

Total Bacterial Abundance

To calculate the bacterial population, we counted the colonies on the growth media using the TPC method. Colonies were counted using a hand counter, and the results found in each dilution were recorded. The number of bacteria is calculated according to the formula (Kadri *et al.*, 2015) as follows:

$$\text{Total bacteria} = \text{number of colonies} \times \frac{1}{\text{dilution} \times \text{vol.planted (mL)}} \text{ (CFU/ml)}$$

Bacterial Morphology Observation and Bacterial Purification

First, bacterial colonies growing on Tryptic Soy Agar media were observed macroscopically in the morphology of the colonies, namely by color, shape, elevation, edge, and size. Then, single colonies on the results of bacterial isolation on TSA media were conducted in the purification stage. Sediment bacteria were purified by streak plate technique on TSA-inclined media.

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Amylolytic Bacteria Isolation

Isolation of amylolytic bacteria was conducted by taking bacterial cultures from an inclined tube using an osseous needle. The bacteria were streaked on growth media enriched with 1% starch and incubated for 48 hours at 28 °C. Observations were made at 12, 24, 36, and 48 hours to determine the maximum activity by showing the hydrolysis zone (clear zone) that occurred. Visualization of amylolytic activity was conducted with drops of iodine solution. To determine the amylolytic activity index, the colony diameter and the total diameter of the clear zone were measured. The index of amylolytic activity was measured using the following formula (Melisha *et al*., 2016):

$$\text{Amylolytic Index} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diameter}}$$

Biochemical Tests

A simple biochemical test was used to determine the type of bacteria that have amylolytic activity when isolated from bacteria that possess amylolytic activity. Some of the biochemical tests carried out were Gram KOH, catalase, oxidase, endospore, oxidation-fermentation, triple sugar iron agar, motility, indole, Methyl red, Voges Proskauer, and Simmons citrate. The results of these biochemical tests are the basis for grouping according to Bergey's manual of determinative bacteriology.

Data Analysis

Morphological data, bacterial abundance, and amylolytic bacteria activity obtained were analyzed descriptively. The data was presented in the form of tables and photos and discussed. The results obtained were compared with several studies and scientific references to enrich the studies.

RESULTS AND DISCUSSION

Description of Minapadi Cultivation

The location of minapadi pond cultivation is strategic as a fish cultivation area. Minapadi cultivation location in Panembangan village is located at a height of around 200-300 meters above sea level with an area of 25 ha and is 27 km from Banyumas city. This height eliminates the possibility of cultivation being affected by flooding, making it safe to use as a cultivation location. The water source used for cultivation comes from the Cipendok waterfall which flows through a river that passes through residential areas. Several previous studies have shown that the waters at the minapadi pond cultivation location have varying degrees of diversity, both plankton and gastropods (Palupi *et al.*, 2023). Based on water quality, the waters in the minapadi area have good quality characteristics as a cultivation medium. As a result of information obtained from farmers, the types of fish cultivated in minapadi ponds are mostly tilapia and several types of catfish. This study seeks to study the description of bacteria, especially amylolytic bacteria, which can degrade waste from agricultural activities.

Bacterial Inoculation and Abundance

Bacterial inoculation was conducted using the pour plate technique. Bacteria were grown on TSA media based on the dilutions. The results of bacterial inoculation from minapadi pond that have been incubated for 24 hours at a temperature of 28 °C can be seen in Figure 2.

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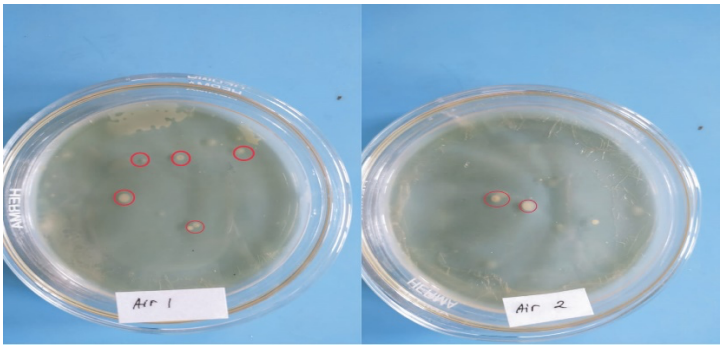

Figure 2. Bacterial inoculation results of water samples. Air 1 and 2 (dilution 10^{-1} and 10^{-2}).

The inoculation results showed that the bacteria could grow on TSA media. Pour plate culture produces colonies of bacteria that grow on agar media, showing the success of the process. According to Mikdarullah and Nugraha (2017), the selection of the pour plate technique was because it was able to make it easier to obtain pure cultures. Pure culture is very important to study the growth properties of each type of microorganism, so microorganisms need to be separated from one another. Pour plate was done by mixing the media that is still liquid with the stock of bacterial culture, so the cells were spread evenly on the agar medium. Bacterial purification is a very important step because the growing bacteria will be separated to see the physiological and biochemical characteristics of the bacteria.

The inoculation results showed that the abundance of colonies in the 10^1 dilutions was 8.4 x 10^2 CFU/mL, and in the 10^2 dilutions was 4.2 x 10^3 CFU/mL with an average bacterial abundance in the overall dilution of 2.5 x 10^3 CFU/mL. The number of bacteria obtained in this study showed low results. The research Putra *et al* (2014) regarding the abundance of bacteria in intensive shrimp pond water with a semi-biofloc system obtained 2.0 x 10^5 CFU/mL. The research Isroni *et al*. (2019) showed the lowest bacterial abundance in cultured water using the RAS system was 1.4x10^3. A low number of bacteria in this study have been caused by the use of chemical disinfectants in the cultivation of minapadi, especially in rice plants, to suppress the bacterial population in the aquatic environment. According to Widiastuti *et al*. (2019) the use of disinfectants and antibiotics can kill or inhibit susceptible (sensitive) bacteria. However, this can cause bacteria to become resistant, and the use of antibiotics becomes ineffective. In addition, chemical compounds such as disinfectants can affect the population of environmental bacteria. In addition, the low number of bacteria can be influenced by the characteristics of the bacteria on the growth media used. According to Nurhafid *et al.* (2021) bacteria that can grow in growth media are only 0.1% of the total bacteria in the environment so the composition and conditions in the growth media need to be considered.

Bacterial Colony Morphological Characteristics

The morphological characteristics of the colonies observed included shape, edge, elevation, size, and color. Colonies growing on growth media were observed for colony morphology to distinguish one bacterial strain from another. The morphological characteristics of the colonies are presented in Table 1.

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Table 1. Bacterial Colony Morphology Observation.

Sample Code	Bacterial Colony Morphology				
	Shape	Elevation	Edge	Color	Size
BA1	Round	Entire	Convex	Dark white	Small
BA2	Irregular	Lobate	Raised	Creamy white	Big
BA3	Entire	Entire	Crateriform	Creamy white	Small
BA4	Round	Entire	Pulvinate	Creamy white	Small
BA5	Filamentous	Filamentous	Flat	White	Small
BA6	Round	Entire	Convex	White	Medium
BA7	Irregular	Undulate	Flat	Yellow white	Small

Based on the bacterial colony morphology observation, seven types of bacterial isolates were distinguished. These differences can be seen from the appearance of the colony morphology indicating that each isolate came from a different species. In the bacterial screening stage, this is the basis for distinguishing. According to Anbari *et al*. (2022), the colony, morphological characteristics can be used as a first step in differentiating the type of bacteria. The results of the observation of colony morphology, bacterial isolates in general had a round shape, entire elevation, varying edges, creamy white, and small size.

This is followed by Suyasa (2019), that the morphology of bacterial colonies is generally circular, irregular, filamentous, and rhizoid. Elevation in the form of raised, convex, flat, umbonate, crateriform. The edges are entire, undulate, filiform, curled, and lobate. Bacteria have different colors according to the pigment they contain. Several types of bacteria are white, yellow, and even red (Arlita *et al*., 2013). Characteristics of the appearance of color in bacterial colonies are usually influenced by environmental factors. Safrida *et al*. (2012) suggest that color differences are influenced by several factors including environmental factors (biotic and abiotic), and food factors (growth medium).

Amylolytic Bacteria Isolation

Bacterial isolation was conducted on a growth medium with 1% starch. Then, the selected bacteria based on colony morphology were cultured on a specific amylolytic medium. Visualization of amylolytic bacteria is shown in Figure 3. The zone formed around the colony was calculated with a ruler to determine the activity produced by amylolytic bacteria. The measurement results of amylolytic bacterial isolates are shown in Table 2.

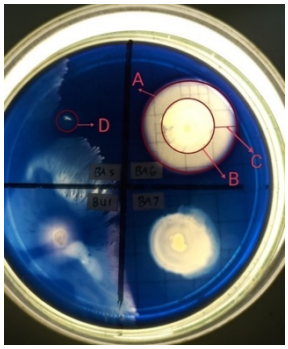

Figure 3. Amylolytic Activity Test Results. Description: (A) diameter of clear zone and bacterial isolates (B) bacterial isolates (C) clear zone (D) isolates of bacteria that do not produce amylase enzymes.

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Table 2. The activity of amylolytic bacteria derived from minapadi sediments.

Sample Code	Amylolytic Activity	Amylolytic index (cm)	Category Index
BA1	-	-	-
BA2	-	-	-
BA3	-	-	-
BA4	-	-	-
BA5	+	0,3	Weak
BA6	+	2,3	Medium
BA7	+	1,6	Medium

Description: Yes (+), No (-).

The index of amylolytic bacterial activity was derived from the isolates BA5, BA6, and BA7. The index of amylolytic activity in this study was included in the weak category for BA5, which was 0.3 cm, and the medium category for BA6 and BA7, which was 1.6-2.3 cm. Dar *et al*. (2015) stated that the bacterial hydrolysis zone was divided into three categories, namely weak (≤1.0 cm), medium (1.1-2.9 cm), and strong (≥3.0 cm). Measurements of the amylolytic index were conducted with an interval of 12 hours, namely at the 12th, 24th, 36th, and 48th hours. Based on the incubation results, amylolytic bacteria produced the enzyme amylase at 24, 36, and 46 hours. The exponential phase of amylolytic bacteria was obtained at 36th. The exponential phase is said to be quite long. According to Dwidjoseputro (1994), bacteria enter the exponential phase at 24 hours, in the exponential phase, the bacteria undergo constant cell division, and the number of cells increases. The exponential phase of bacteria generally varies. This is evidenced by the research of Setyati *et al*. (2015) which obtained the exponential phase of bacteria at 6-36 hours, while in the study of Sari *et al*. (2017) the exponential phase of bacteria occurs at 16-39 hours.

The amylase enzyme helps amylolytic bacteria process waste and organic materials that contain a great deal of starch in the water. Amylase enzyme can catalyze the hydrolysis reaction of starch and glycogen into maltose, maltotriose, isomaltose, and glucose. Also, amylase enzymes can break down starch polymer bonds into shorter oligosaccharides and simpler sugar molecules (Silitonga *et al*., 2019; Novitasari *et al*., 2021) or monosaccharide sugars, and then the monosaccharides will be degraded back into energy (Prayogo *et al*., 2018). Several bacteria capable of producing amylase enzymes have been found including the genera *Bacillus*, *Bacteroid*, *Lactobacillus*, *Clostridium*, *Micrococcus*, *Thermus*, *Actinomycetes* (Reddy *et al*., 2003), *Enterobacter*, *Klebsiella* (Hasanah *et al*., 2021), *Staphylococcus*, *Corynebacterium* (Kurniasih *et al*., 2014) *Proteus*, *Escherichia coli*, *Pseudomonas*, and *Streptomyces* (Pokhrel *et al*., 2015).

The activity of amylolytic enzymes produced by bacteria can be seen from the hydrolysis ability of amylolytic bacteria, by the formation of a clear zone on the starch media. The clear zone around the colony indicates that starch has been degraded to glucose or maltose (Susilawati *et al*., 2015). The clear zone formed due to the ability of bacteria to degrade starch was used as a determinant of amylase production by microbes in the iodine reaction. Microbes that grow on starch agar medium use starch as a substrate (Silaban and Simamora, 2018). The activity of the amylase enzyme produced by bacteria generally varies depending on the habitat and substrate. However, the production of amylase enzymes by bacteria can also be influenced by nutrient content, media pH, osmotic pressure, oxygen, temperature, and contamination during incubation (Susilawati *et al*., 2015; Sundari *et al*., 2019) the ability of bacteria, and bacterial characteristics (Zuraidah *et al*., 2020).

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Biochemical Test

The biochemical testing carried out in this research aims to initiate identification based on the biochemical characteristics of bacteria. Several parameters were used to differentiate bacterial isolates.

Table 3. Biochemical test.

No	Biochemical Test	Isolate code		
		BA 5	BA 6	BA 7
1	Grams	+	+	-
2	Catalase	+	-	+
3	Oxidase	-	-	+
4	Endospores	-	-	-
5	O/F	F	F	F
6	TSIA	A/A	A/A	A/A
7	Motility	-	-	-
8	Indole	-	-	-
9	MR	-	+	-
10	VP	-	-	-
11	Simmons Citrate	+	-	+
	Group	*Proteus* sp.	*Staphylococcus* sp.	*Micrococcus* sp.

Where: F= Fermentation, A/A= Acid, fermentation, (+) = positive, (-) = negative.

The results showed that amylolytic bacteria were detected from three different groups, namely *Proteus* sp., *Staphylococcus* sp., and *Micrococcus* sp. Several studies show that these bacteria are bacteria that are often detected in water and can interact in the digestive tract of fish (Adamu *et al.*, 2020). Several information from previous research shows that the *Micrococcus* sp. and *Proteus* sp. groups. This is a group of bacteria that can suppress fish pathogens. Apart from that, *Micrococcus* sp. is a lactic acid bacteria that is widely used as a probiotic (Pereira *et al.*, 2022). The *Staphylococcus* sp. group is known to be a pathogen in fish and can carry out symbiosis with other pathogenic bacteria to form biofilms on the surface of fish (Musharrafieh *et al.*, 2014).

Amylolytic is one of the activities of bacteria in breaking down organic material in the form of starch using the enzyme amylase. The minapadi ecosystem is thought to have abundant starch compounds since rice is an integrated agricultural object in the ecosystem. Starch is a compound that plants use as a nutrient reserve in the form of amylopectin and amylose granules (Basitoh *et al.*, 2019). Meanwhile, the organic material content in the minapadi ecosystem is very abundant, which comes from fertilizers provided in agricultural activities and accumulated waste. The amylolytic bacteria have an important function in breaking down organic compounds so that they do not become toxic and can be used by other organisms (Dat *et al.*, 2019). Currently, the ability of bacteria to produce the enzyme amylase is one of the considerations regarding their potential as probiotic bacteria so that in the future probiotics can be used as an alternative substitute for chemicals in rice cultivation.

CONCLUSION

The results of the isolation of amylolytic bacteria showed that there were 3 isolates, namely BA5, BA6 and BA7 with the activity index category being low to medium. Amylolytic bacteria were successfully subjected to biochemical testing and grouped into 3 different genera, namely *Proteus* sp. *Staphylococcus* sp. and *Micrococcus* sp. In the minapadi environment, amylolytic bacteria can perform biodegradation.

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CONFLICT OF INTEREST

There is no conflict of interest in this manuscript between all authors upon writing and publishing this manuscript.

AUTHOR CONTRIBUTION

The contribution of each author is as follows: Kasprijo, Ren Fitriadi, and Dini Ryandini collected and analyzed data, drafting, and manuscript preparation. Mohammad Nurhafid and Reza Muhammad Riady participated in the conception and experimental design.

ACKNOWLEDGMENT

This research was funded by the Directorate General of Higher Education, Research and Technology, Ministry of Education, Culture, Research and Technology of the Republic of Indonesia through *Penelitian Terapan - Jalur Hilirisasi* Funding in 2023.

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