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Antibacterial activity of *Proteus spp.* isolated from the rice-fish farming system cultivation area against *A. hydrophila*

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Abstract

Proteus spp bacteria is one of the bacteria that have antibacterial activity. Bacteria that produce antibacterial can suppress the growth of pathogenic bacteria such as *A. hydrophila*. This study aimed to determine the antibacterial activity of several strains of *Proteus spp*. against *A. hydrophila* from the rice-fish farming system cultivation area in the Banyumas district. Research stages included isolating bacteria from a sample, preparing the test bacteria, and testing the inhibition power of *Proteus spp*. in agar-well diffusion dishes. The results showed that the identified *Proteus* strains included *Proteus mirabilis* strain MRKMSEC 72 (BA1), *Proteus penneri* strain CPrp_RA24 (BA3), *Proteus* mirabilis strain BN7 (BA7), *Proteus mirabilis* strain BN7 (BU4), and *Proteus vulgaris* strain Siii (BS11). All strains obtained had an antibacterial activity that could inhibit Aeromonas. It was also found that most *Proteus spp*. Species are sensitive to antibiotics, especially amoxicillin. *Proteus* species with high levels of antibiotic sensitivity are expected to be able to fight *A. hydrophila* in cultivation environments and be used as biocontrol agents.

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Introduction

Bacteria in an ecosystem can produce metabolites that can inhibit other species of microorganisms (1-4). *Proteus* spp is one of the bacteria that produce antibacterial activity (5,6). Antibacterial activity of *Proteus spp.* Able to inhibit the growth of enteropathogenic bacteria such as Enterobacter, Klebsiella, and Salmonella (7). Drzewiecka (8) stated that *Proteus* sp bacteria positively affected aquatic animals. *Proteus* spp has probiotic properties, namely being able to produce bacteriocins and having antagonistic activity against many pathogenic bacteria. Several *Proteus* bacteria known to have bacteriocin activity are *P. mirabilis, P. vulgaris, P. penneri, P. genomospecies 4*, and *Proteus sp* (7). *Proteus* isolated from the rice-fish farming system area is expected to be able to deal with the problem of attack by *Aeromonas* pathogenic bacteria. It was further explained that apart from proteus, Pseud was another bacterium that was found and able to inhibit pathogens (9). *Aeromonas* sp. is a common Gram-negative pathogenic bacteria that attacks farmed fish and can cause huge losses of up to 100% (10,11). *Aeromonas sp*. is an opportunistic pathogen in fish but, under certain conditions such as stress and decreased immune function, can become a major pathogen in fish (12-14). The strain that is most known to be malignant in attacking fish is *A. hydrophila* which causes Motile Aeromonas Septicemia (MAS) disease (15-18). The attack of *A. hydrophila* is characterized by symptoms of redness to hemorrhagic on the surface of the fish's body (19). In addition to the body surface, *A. hydrophila* attacks were found to cause severe damage to internal organs, namely the spleen (20-22). Treatment of *A. hydrophila* in fish farming often uses chemicals such as antibiotics (23,24). However, this treatment is ineffective because it can cause resistance to *A. hydrophila* and other pathogenic bacteria. Antibacterial-producing bacteria is an alternative that can be used to suppress the growth of *A. hydrophila*. In the environment, these bacteria produce antibacterial compounds that can be

used as biocontrol agents to suppress pathogenic bacteria, especially *A. Hydrophila* (25,26).

In addition, antibiotics are often used to manage fish culture with the rice-fish farming system in a way that harms resistant bacteria, so farmers must use natural fertilizers to increase the fertility of the water. In this case, the aim of the antimicrobial test of *Proteus* bacteria as a probiotic agent in the fish cultivation of the rice-fish farming system is an intensive one to utilize fish waste as fertilizer for rice growth. This is very new and has not been widely applied, so conducting an in-depth study is necessary. Additionally, antagonistic bacteria-producing antibacterial compounds must be manufactured to address the problem of pathogenic bacteria attacks. This study aimed to determine the antibacterial activity of several strains of *Proteus* spp. against *A. hydrophila* from the rice-fish farming system cultivation area in the Banyumas district.

Materials and methods

Time, place and research method

The research was conducted in January-May 2022 at the Muhammadiyah University Purwokerto Integrated Laboratory. This research method uses a survey method. The collected samples included intestines (anterior, middle, and posterior), maintenance water media at three points, and sediment at three points.

Isolation and identification of bacteria

Bacterial isolation from samples (intestine, water, and sediment) was taken following the procedure of Nurhafid *et al.* (27). The tools used in this study included autoclaves, pipettes, Erlemeyer, Bunsen, Petri dishes, digital scales, measuring cups, needles, micro pastels, incubators, test tubes, vortexes, paper disks, and microwaves. The materials used in the study included TSA (*Trypcase Soya Agar*) agar media, 0.9% sterile NaCl, and distilled water. In the first step, 0.5 ml of water or other dissolved samples were transferred into a test tube containing 4.5 ml of 0.9% NaCl in a 10^{-1} dilution and homogenized using a vortex. Then taken from the 10^{-1} dilution, as much as 0.5 ml was put into the 10^{-2} dilution test tube and homogenized. The dilution step was repeated until the tube reached a 10^{-5} dilution. Furthermore, each dilution was cultured using the pour plate technique and incubated at 28°C for 48 hours. Bacteria that grow in single colonies are streaked to purify selected bacteria based on colony morphology.

The identification of bacteria was established on the similarity of the 16S rDNA sequence with the GenBank data. In short, the bacterial DNA was extracted using the Presto™ Mini gDNA Bacteria Kit following the manufacturing procedure (Geneaid) to obtain a pure DNA solution. The reaction mixture was prepared in a total volume of 350µl for the total sample (seven samples), including the positive control, the amplified sample, and the negative control (nucleus-free water). Each reaction sample contained a mixture of 1µl gDNA as a template, 1µl forward and reverse primer each (10pmol/µl), the reaction for amplification was 25µl Mytaq HS Redmix (Bioline), then added 22µl nuclease-free water to obtain the total volume of each sample as much as 50µl. The PCR amplification reaction was conducted using Primus 25 Thermocycler PCR (Peqlab). The primers used in amplification followed the research of Palkova *et al.* (28) 27f (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1392r (5'- GGT TAC CTT GTT ACG ACT T -3') with results 1500bp amplification (Figure 1). The results of the 16s rDNA gene amplification were sequenced. They analyzed using the Basic Local Alignment Search Tool (BLAST) to obtain sample similarity values with the National Center for Biotechnology Information (NCBI) database.

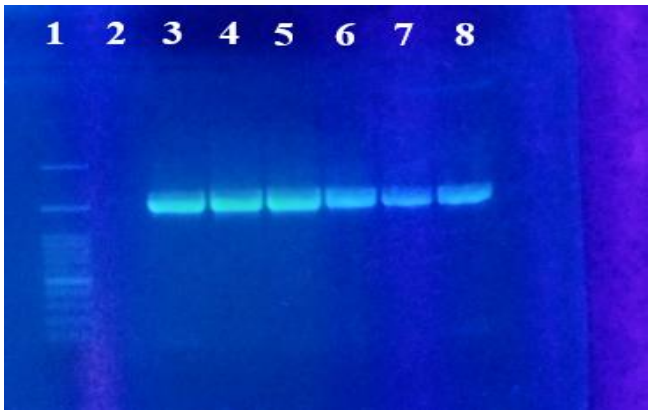


Figure 1: Visualization of the electrophoretic gel amplification results Note: 1. DNA marker, 2. Negative control, 3. Positive control, 4-8 DNA samples.

Bacteria tested

The test bacteria (*A. hydrophila*) used in this study was obtained from the Jenderal Soedirman University Research Laboratory of Fisheries and Marine Sciences. *Aeromonas* bacteria were activated by incubation for 24 hours in TSB (Tryptic Soy Broth) media.

Inhibitory power of Proteus spp. by the agar-well diffusion method testing

Bacterial isolates *Proteus spp.*, which have been identified, were tested for antibacterial activity against *A. hydrophila* using the agar well diffusion method. The culture was conducted by dropping 100 µl of *A. hydrophila* bacterial pellets and spreading them using L rods on the growth medium. A sterile paper disk was added to the agar's surface, and then dropped as much as 10 µl of the *Proteus* spp bacteria pellet was to be tested. Then incubated at 28°C for 36 hours. The inhibition zone was Observed and measured as a value of antimicrobial activity (29). The inhibition zones were divided into four categories, namely weak (≤5.0 mm),

moderate (6-10 mm), strong (11-20 mm), and very strong (≥20 mm) (30). In addition, the antimicrobial activity of bacteria was compared to that of antibiotics. Standard antibiotic disks used for control are as follows: Tetracycline (30 mcg), Amoxicillin (25 mcg), Chloramphenicol (30 mcg), and Gentamicin (10 mcg). The interpretation of the antibiotic inhibition zone was divided into three categories, namely Resistant (≤14 mm), Intermediate (15-18 mm), and Susceptible (≥19 mm) (31,32).

Results

Antimicrobial activity of Proteus spp.

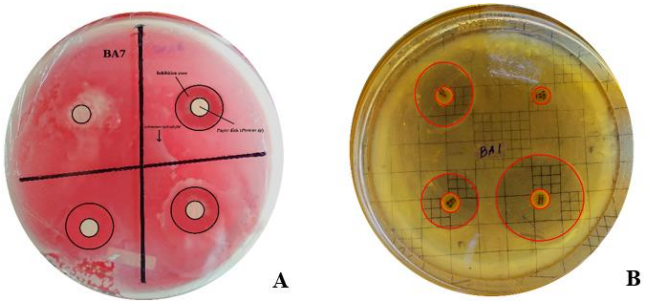
As a result of the identification test, Five of *Proteus spp.* Strains were identified as 3 *Proteus mirabilis*, 1 *Proteus penneri*, and 1 *Proteus vulgaris* (Table 1). This study examined the antimicrobial activity of *Proteus spp.* Strains against *A. hydrophila* bacteria (Figure 2). All *Proteus* strains obtained showed antibacterial activity against *A. hydrophila* (Table 2). Antibacterial activity began to appear in the 24th to 36th hour. The antibacterial activity produced by *Proteus* spp. The weak and strong categories were around 2-25 mm.

Proteus spp bacteria sensitivity to antibiotics

The several strains of *Proteus* spp. showed a different sensitivity level to several antibiotics. The diameter of the inhibition zone (mm) for standard antibiotic test bacteria is shown in Figure 3. In our study, it was found that *Proteus mirabilis* MRKMSEC 72 (BA1) had antibacterial activity in a weak category. However, the level of sensitivity to tetracycline antibiotics, chloramphenicol, and gentamicin is very high. Meanwhile, *Proteus mirabilis* BN7 (BA7) has very strong antibacterial activity. However, sensitivity to tetracycline antibiotics, amoxicillin, and chloramphenicol is very low. On the other hand, *Proteus mirabilis* BN7 (BU4) has moderate antibacterial activity. However, the level of sensitivity to tetracycline antibiotics, chloramphenicol, and gentamicin is quite high. In the *Proteus Penneri* CPrp_RA24 (BA3) strain, the antibacterial activity was included in the medium category with a high sensitivity to tetracycline, chloramphenicol, and gentamicin. In addition, the *Proteus vulgaris* Siii (BS11) shows moderate antibacterial activity. However, sensitivity to tetracycline, chloramphenicol, and gentamicin is very low. Most of the *Proteus* spp strains have a low sensitivity to the antibiotic amoxicillin (Tables 3 and 4).

Table 1: Similarity percentage matrix of 16s rRNA Proteus spp.

No	Code	BA3	BA7	BS11	BA1	BU4
1	BA3	100,00	99,72	99,77	41,13	43,05
2	BA7	99,72	100,00	100,00	40,57	43,12
3	BS11	99,77	100,00	100,00	39,09	43,75
4	BA1	41,13	40,57	39,09	100,00	76,95
5	BU4	43,06	43,12	43,75	76,95	100,00



A B

Figure 2: A. Antibacterial test, B. Antibiotic sensitivity test.

Table 2: Blast results isolate the strain and origin of the collection

No	Code	Strain	Similarity (%)	Source
1	BA1	*Proteus mirabilis* strain MRKMSEC 72	99,00	rice-fish farming, Panembangan
2	BA3	*Proteus penneri* strain CPrp_RA24	99,79	rice-fish farming, Panembangan
3	BA7	*Proteus mirabilis* strain BN7	100	rice-fish farming, Panembangan
4	BU4	*Proteus mirabilis* strain BN7	100	Intestine Fish, Panembangan
5	BS11	*Proteus vulgaris* strain Siii	100	rice-fish farming, Panembangan

Table 2: Antimicrobial activity of *Proteus* strains on *A. hydrophila* bacteria

Code	Strain	Inhibition zone diameter(mm)			Inhibition zone category
		12 hours	24 hours	36 hours	
BA1	*Proteus mirabilis* strain MRKMSEC 72	-	2	3	Weak
BA3	*Proteus penneri* strain CPrp_RA24	-	7	9	Medium
BA7	*Proteus mirabilis* strain BN7	-	25	25	Very strong
BU4	*Proteus mirabilis* strain BN7	-	4	7	Medium
BS11	*Proteus vulgaris* strain Siii	-	5	5	Medium

Table 3: Inhibition zone diameter (mm) for standard antibiotic test bacteria

Code	Strain	Antibiotics			
		Tetracycline	Amoxicillin	Chloramphenicol	Gentamicin
BA1	*Proteus mirabilis* strain MRKMSEC 72	31^{S}	0^{R}	21^{S}	20^{S}
BA3	*Proteus penneri* strain CPrp_RA24	25^{S}	0^{R}	14^{I}	13^{I}
BA7	*Proteus mirabilis* strain BN7	7^{R}	9^{R}	0^{R}	14^{I}
BU4	*Proteus mirabilis* strain BN7	14^{I}	9^{R}	23^{S}	15^{I}
BS11	*Proteus vulgaris* strain Siii	0^{R}	30^{S}	0^{R}	13^{R}

Description: Resistant (R), Intermediate (I), and Susceptible (S).

Discussion

In this study, it was found that *Proteus* spp bacteria had antibacterial activity against *A. hydrophila*. The formed antibacterial activity belongs to the weak to very strong category. Proteus mirabilis BN7 (BA7) obtained the highest activity, which was 25 mm. This value shows a relatively high value. According to the positive control, bacteria-producing antibacterial, namely *B. subtilis, S. aureus, V. cholera,* and *E. coli*, produced an activity of 26.7-28.8mm. The activity value produced by *Proteus mirabilis* BN7 (BA7) can be a probiotic candidate for environmental biocontrol agents. Based on research by Ravi *et al.* (33) found several types of bacteria that produce antibacterial compounds that can be used as biocontrol agents. Antibacterial-producing bacteria excrete antibacterial compounds useful as a balance control for pathogenic bacteria such as *A. hydrophila* (34). Antibacterial compounds that are excreted in the environment can cause the breakdown of the pathogenic bacteria's cell wall, reducing the population in the environment (35,36).

The results showed that *Proteus spp* bacteria produced optimal antibacterial products at 24 to 36 hours, the stationary phase. This is indicated by forming an inhibition zone around the paper disk with a high value. The inhibition zone was formed because *A. hydrophila* could not grow due to the antibacterial compounds produced by the test bacteria. According to Subagiyo and Djunaedi (37), the growth of pathogenic bacteria can be inhibited by types of bacteria that produce antibacterial compounds. The mechanism of *Proteus spp* bacteria as antibacterial producers inhibit the pathogen *A. hydrophila* by releasing secondary metabolite products into the environment. Naturally, bacteria compete for nutrients and space to survive. The presence of *Proteus* spp bacteria as antibacterial producers can dominate and suppress *A. hydrophila* because it has antibacterial compounds. These bacteria can be used as a control in the environment to suppress the growth of the pathogen *A. hydrophila* so that there is a balance in the cultivation environment (38,39).

The result showed that the level of antibiotic sensitivity of several strains of *Proteus spp*. is low, especially the sensitivity to the antibiotics amoxicillin, tetracycline, and chloramphenicol. This is due to the large number of bacteria that are resistant to antibiotics. According to Alqurashi *et al.* (40), 8.4% of *Proteus* spp strains, especially *Proteus mirabilis*, have resistance to various antibiotics such as ciprofloxacin, amoxicillin, gentamicin, amoxicillin/clavulanic acid, cefotaxime and chloramphenicol (8,41). In recent years, *Proteus* and other Enterobacter bacteria have experienced increasing resistance to antibiotics (42). This is caused by the frequent use of antibiotics against animals and the environment. According to our research, the area for sampling rice-fish farming system ponding passed through community settlements, and local people continue to put chemical fertilizers in fish ponds to minimize pest problems. According to Pepi and Focardi (43), the uncontrolled use of fertilizers and antibiotics can cause the spread of antibiotic residues in the environment, increase the level of antibiotic resistance in bacteria in the waters and indirectly transfer this resistance to human pathogens (44-46).

The antibacterial activity produced by *Proteus spp*. is a natural antibiotic that can replace and reduce the use of antibiotics. According to Nguyen *et al.* (7) Antibacterial or bacteriocin activity of *Proteus spp*., if cloned, purified, and recognized as safe, can be a promising candidate for alternative antibiotics that are environmentally friendly against pathogenic bacteria such as Enterobacter, Klebsiella, and Salmonella, besides having antibacterial activity, *Proteus spp*. It can also act as bioremediation. Drzewiecka (8) stated that *Proteus spp*. Those isolated from the environment can immobilize heavy metals (47), utilize toxic pollutants (48), and stimulate plant growth, as well as nematocidal (49) and fungicidal properties. This statement is supported by research by Zhang *et al.* (50), where the bacterium *Proteus mirabilis* isolated from the coastal waters of China was able to remove ammonia through an oxidation process. Reche and Fiuza (51) also stated that the presence of the bacteria *P. mirabilis, P. vulgaris,* and *P. penneri* could be indicators of environmental pollution in paddy field waters and irrigation.

Conclusion

Proteus bacteria identified in this study are strains of *Proteus mirabilis*, *Proteus penneri*, and *Proteus vulgaris*. All *Proteus* spp bacteria showed antibacterial activity against

Aeromonas hydrophilla. Several Proteus spp strains' antibacterial activity ranged from 2-25 mm with low to very strong categories. However, some *Proteus spp*. It has a low level of sensitivity to several antibiotics, especially amoxicillin. A high level of antibiotic sensitivity and antibacterial activity indicates that Proteus bacteria isolated from the rice-fish farming system cultivation area can be used as biocontrol agents for the cultivation environment to combat *A. hydrophila*.

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Conflict of interest

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

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النشاط المضاد لبكتيريا المتقلبات المعزولة حقول زراعة الرز المشغولة بالأسماك ضد جراثيم الايروموناس هايدروفيليا

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الخلاصة

بكتيريا *Proteus spp.* هي واحدة من البكتيريا التي لها نشاط مضاد للبكتيريا. يمكن للبكتيريا التي تنتج مضادا" للبكتيريا أن تثبط نمو البكتيريا المسببة للأمراض مثل *A. hydrophila*. هدفت هذه الدراسة إلى تحديد الفعالية المضادة للبكتيريا لعدة سلالات من *Proteus spp.* ضد *A. hydrophila* من منطقة زراعة نظام استزراع الأرز والأسماك في منطقة بانيوماس. تضمنت مراحل البحث عزل البكتيريا من عينة، وإعداد بكتيريا الاختبار، واختبار قوة تثبيط *Proteus spp.* في أطباق انتشار الأكار. أظهرت النتائج أن سلالات Proteus التي تم تحديدها شملت سلالة *Proteus* MRKMSEC 72 (BA1)، سلالة *Proteus mirabilis* CPrp_RA24 (BA3)، سلالة *Proteus mirabilis* BN7 (BA7)، وسلالة *Proteus mirabilis* BN7 (BU4)، وسلالة *Proteus vulgaris* Siii (BS11). جميع السلالات التي تم الحصول عليها كان لها نشاط مضاد للجراثيم التي يمكن لها أن تثبط الـ *Aeromonas*. كما وجد أن معظم *Proteus spp.* حساسة للمضادات الحيوية، وخاصة أموكسيسيلين. من المتوقع أن تكون أنواع البروتيوس ذات المستويات العالية من الحساسية للمضادات الحيوية قادرة على محاربة *A. hydrophila* في بيئات الزراعة واستخدامها كعوامل مكافحة حيوية.