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

R. Fitriadi¹, M. Nurhafid¹, K. Kasprijo¹, D. Ryandini², R.M. Riady¹, P. Sukardi¹

1Faculty of Fisheries and Marine Science, 2Biology Department, Faculty of Biology, Jenderal Soedirman University, Purwokerto, Indonesia

**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 Sii (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.

**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 (*Tryptcase 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 10⁻³ 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 (Pqlab). 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 GGT 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.](Image 325x374 to 566x526)

**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.

<table>
<thead>
<tr>
<th>No</th>
<th>Code</th>
<th>BA3</th>
<th>BA7</th>
<th>BS11</th>
<th>BA1</th>
<th>BU4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BA3</td>
<td>100,00</td>
<td>99,72</td>
<td>99,77</td>
<td>41,13</td>
<td>43,05</td>
</tr>
<tr>
<td>2</td>
<td>BA7</td>
<td>99,72</td>
<td>100,00</td>
<td>100,00</td>
<td>40,57</td>
<td>43,12</td>
</tr>
<tr>
<td>3</td>
<td>BS11</td>
<td>100,00</td>
<td>99,77</td>
<td>100,00</td>
<td>39,09</td>
<td>43,75</td>
</tr>
<tr>
<td>4</td>
<td>BA1</td>
<td>41,13</td>
<td>40,57</td>
<td>39,09</td>
<td>100,00</td>
<td>76,95</td>
</tr>
<tr>
<td>5</td>
<td>BU4</td>
<td>43,06</td>
<td>43,12</td>
<td>43,75</td>
<td>76,95</td>
<td>100,00</td>
</tr>
</tbody>
</table>

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

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

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

<table>
<thead>
<tr>
<th>Code</th>
<th>Strain</th>
<th>Inhibition zone diameter(mm)</th>
<th>Inhibition zone category</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>12 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>BA1</td>
<td>Proteus mirabilis strain MRKMSEC 72</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>BA3</td>
<td>Proteus penneri strain CPrp_RA24</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>BA7</td>
<td>Proteus mirabilis strain BN7</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>BU4</td>
<td>Proteus mirabilis strain BN7</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>BS11</td>
<td>Proteus vulgaris strain Siii</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 3: Inhibition zone diameter (mm) for standard antibiotic test bacteria

<table>
<thead>
<tr>
<th>Code</th>
<th>Strain</th>
<th>Antibiotics</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Tetracycline</td>
</tr>
<tr>
<td>BA1</td>
<td><em>Proteus mirabilis</em> strain MRKMSEC 72</td>
<td>31(^R)</td>
</tr>
<tr>
<td>BA3</td>
<td><em>Proteus penneri</em> strain CPtp RA24</td>
<td>25(^S)</td>
</tr>
<tr>
<td>BA7</td>
<td><em>Proteus mirabilis</em> strain BN7</td>
<td>7(^R)</td>
</tr>
<tr>
<td>BU4</td>
<td><em>Proteus mirabilis</em> strain BN7</td>
<td>14(^I)</td>
</tr>
<tr>
<td>BS11</td>
<td><em>Proteus vulgaris</em> strain Siii</td>
<td>0(^R)</td>
</tr>
</tbody>
</table>

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. The antibacterial compounds produced by this strain were found to be effective against *A. hydrophila* and *E. coli*, producing an activity of 26.7-28.8 mm. The activity varied with the strain and was highest in *Proteus mirabilis* strain 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 produce excretory 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 other 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 metabolites 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 Alquarashi et al. (40), 8.4% of *Proteus* spp strains, especially *P. 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
Oreochromis. 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.

**Acknowledgment**

The authors want Kemdikbudristek and LPDP to provide funding through the Higher Education Research Grant Scheme: Independent Lecturer Research Grant with No. SK DIRUT LPDP No: KEP-2/LPDP/LPDP.4/2021.

**Conflict of interest**

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

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resistance and virulence factors of
bacteria producing antibacterial compound that associated with corals
and its potential application in NH4+


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lactic acid bacteria producing antimicrobial agents from Bakasang, an Indonesian
traditional fermented fish product. IJ

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