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# Isolation and molecular identification of proteolytic bacteria from vaname shrimp (*Lithopenaeus Vannamei*) ponds as probiotic agents

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**Abstract**

Vannamei shrimp culture in intensive and traditional ponds is developing rapidly in Indonesia. The development starts with technology, high density, and feeding systems. It results in very high organic material produced in the aquaculture ponds, and this water quality management is necessarily maintained. This study aims to isolate and identify the molecular of proteolytic bacteria from Vannamei shrimp aquaculture ponds using a different system that is as probiotic agents. This study used a survey method and random sampling technique from traditional and intensive shrimp aquaculture. The results showed that the proportion of proteolytic bacteria in traditional ponds was higher than that in intensive ponds. The screening based on colony morphology and highest activity index obtained 4 bacterial isolates of *Vibrio* sp. and *Pseudoalteromonas* white an identity value of 98.23-99.80%. The value of this activity was observed at 7.0 cm, subsequently inhibiting *Vibrio* sp. by 1.5 cm. The bacteria were also found to develop at pH 7 and a temperature of 28°C adequately. Additionally, the maximum proteolytic activity at a 48-hours incubation indicated their potential relevance as a biotechnological probiotic. Further studies on the potential of *Pseudoalteromonas ganghwensis* strains found that the bacteria have antimicrobial activity and are sensitive to antibiotics that can be used as aquaculture probiotic agents.

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## Introduction

Indonesia reportedly has abundant waters as an archipelagic state and has also potentially developed in health, food, energy, and industry. One of the biodiversity of these waters is the waters and sediment contents of the *Vannamei* shrimp ponds, which have lots of technological development from the traditional, intensive, semi-intensive, and super-intensive reservoirs (1). However, the differences among these technologies are the cultivation treatment and shrimp production. *Vannamei* shrimp ponds are rich in nutrients and contain many bacteria, which produce various primary and secondary metabolites, such as enzymes (2). Shrimp production in intensive ponds uses probiotic technology to maintain water quality allowing these bacteria to degrade the feed waste and organic material that settles in the pond. These bacteria are expected to live and develop properly to produce hydrolytic enzymes in the pond waters, Such a condition results in a rich-in-nutrients pond letting the Vanvamei shrimp grow and develop well (3). The enzymes derived from these microorganisms include cellulose, galactosidase, protease, lipase, lactase, and amylase (4,5).

This proteinous metabolite functions as a biocatalyst for chemical reactions in living things, with protease found to be primarily utilized (6-8). As a biocatalyst, protease is found to hydrolyze peptide bonds from proteins, with the number of compounds produced depending on the enzyme-producing bacteria (9). This enzyme is primarily vital for

industrial purposes, including the food, pharmaceutical, cosmetic, and feed probiotic industries (10,11). Based on (12), protease was also produced from the thermophilic fungi isolated from water. This was in line with (13), which indicated that a producer isolated from the marine sediments was the *Bacillus halodurans*. According to Mahajan and Badgujar (14), the sources of protease derivation were analyzed in plants 43.85%, bacteria 18.09%, fungi 15.08%, animals 11.15%, algae 7.42%, and viruses 4.41%. Moreover, several studies were conducted on the production of bacteria-based proteases, such as the *Rhodovulum sulfidophilum* PS342, which was potentially used in shrimp cultivation due to being a fine producer of proteolytic enzymes and antivibration compounds (15). *Lactococcus latis* was also found to increase proteolytic content from 40.6-94.3% (16), with *Bacillus* having high enzymatic activity between 24.6-15.9 U/ml (17).

Based on being reused in similar locations, the microbes obtained from local isolation are expected to be in line with their environment and habitat, for the optimal performance to produce proteases (18). The microbial proteases are also expected to function adequately when the environmental condition chemically, including acidic, neutral, or base, and catalytically involving serine, aspartyl, thiol, or metal meet their needs (19,20). It was further explained that proteolytic enzyme-producing bacteria are also expected to have actinobacteria against pathogenic bacteria such as antivibrio. According to Salamone *et al.* (21), several types of bacteria produced proteolytic enzymes of *Vibrio* sp. Enterococcus faecium MA002, Enterococcus lactis MA056, Leuconostoc mesenteroides MA064, Enterococcus lactis MA068, dan Enterococcus lactis MA084 (22). This might help the degradation process of organic material in shrimp ponds; however, it would be dangerous when *Vibrio* dominates the bacterial population in the pond, and hence the number must be limited (23).

Water and shrimp pond sediments are often rich in bacteria due to the high technology in providing aquaculture probiotics (24,25), which allows the emergence of many potential microorganisms. Besides intensive ponds, there are also traditional reservoirs containing local bacteria. Several previous studies have reportedly evaluated the various type of proteolytic-producing bacteria from shrimp ponds, such as (26), which showed that *Lactobacillus* sp., *Pseudomonas* sp. (27), *Bacillus* sp. *Lactobacillus* sp. *and Pseudomonas* sp. had amylolytic, cellulolytic, and lipolytic activities in the digestive tract of the *Vannamei* crustaceans (shrimps). *Bacillus subtilis E20* (28) and *Bacillus* sp. were also found to produce very high content of proteases and biofilms (29,30). These bacteria help the degradation process of organic material in shrimp ponds maintain water quality. The more bacteria that can produce hydrolytic enzymes, the pond will produce good shrimp production.

The problem of shrimp culture is currently found in the constant presence of pathogenic bacteria in poor water conditions. Controlling pathogenic bacteria using antibiotics in aquaculture is ineffective and even prohibited since it produces residues and the pathogenic bacteria become resistant. Probiotics are biotechnology developed from microorganisms that can produce extracellular enzymes. The ability of microorganisms such as bacteria produces primary metabolite products such as proteases, amylase, lipase, and other enzymes. Moreover, some bacteria have secondary metabolite activity to compete in the environment. This ability needs advanced study to be developed as a biocontrol agent in suppressing the growth of pathogenic bacteria. In addition, the characteristics of probiotic agents against antibiotics need to be examined. Bacteria exposed to antibiotics can transfer genes to pathogenic bacteria so that they will transform resistant characteristics. According to Lerminiaux and Cameron (31), the spread of bacterial resistance was significantly faster in the horizontal spread between bacteria through natural transformation. Some bacteria were identified to be able to transfer resistant genes, so-sensitivity tests and antibiotic resistance tests need to be performed on the probiotic agents.

As potential protease producers, the local bacteria originating from Indonesian waters, especially the *Vannamei* shrimp ponds, have reportedly not been identified, prompting the need for further reports. Therefore, this study aims to obtain local bacterial isolates as probiotic production agents in Vannamei shrimp cultivation.

## Materials and methods

### Place and time of study

This study was carried out between January-December 2021, where the isolation and characteristics of proteolytic-producing bacteria were conducted at the Research Laboratory of the Fishery and Marine Science Faculty, Jenderal Soedirman University. The data obtained were divided into two parts, namely primary and secondary information, which includes the results and parameters to be performed and analyzed, respectively. The study was conducted using a purposive random sampling technique and a survey method. The sampling point was determined based on the sample variation, the type of aquaculture pond and random sampling.

### Sampling location

Bacterial isolation was taken from the shrimp pond waters of the Pangandaran coast. It was located at latitude (5°34'18, 32'N, 102°48'25, 86'E). The sampling location was an area of Vannamei shrimp culture employing different cultivation systems, namely traditional and intensive. The ponds used as sampling locations were recorded to have successful harvests in the last 5 cycles. The traditional pond was built with soil bottom and walls, while intensive ponds have plastic-covered bottoms and walls.

**Sampling of shrimp pond water and sediment**

Sampling was carried out on the water and sediments of the pond, as the bacterial isolates were obtained from the traditional and intensive reservoirs. This was carried out at 7 sampling points within the intensive (sediments, the inlet, middle, and outlet waters) and traditional (inlet and outlet waters and the sediment) ponds. Water sampling was done at a depth of 60 cm at each point, and the sediment was taken from the bottom of the vannamei shrimp ponds. Especially for water intake at the inlet, it was carried out in the reservoir pond before the water was poured into the shrimp pond. The top sediment layer of 20 cm was collected with a sterile shovel (box-corer), then put in a sterile container (sample bottle), and stored in an iced cool box. Before the isolation process, all samples were stored in a refrigerator at 4°C in the Laboratory of Fisheries and Marine Sciences, Jenderal Soedriman University.

**Bacterial isolates**

The bacterial isolates were obtained from the specified water and sediment location points and subsequently diluted based on the modified procedure (32). Each sample was serially diluted using five test tubes, which contained 4.5 mL of physiological sterile (dilution $10^1$-$10^5$). Moreover, a 0.5 mL sample was obtained and homogenized with a 4.5 mL physiological solution in the first tube (dilution $10^1$). This suspension was subsequently obtained and homogenized in the second tube (dilution $10^2$), with the procedure being continuously performed until the fifth apparatus (dilution $10^5$). The results were then grown on a marine agar through the pour plate method. This indicated that the sample solution of 0.5 mL was obtained from each dilution tube and placed into an empty sterile plate where homogenization was carried out with the addition of a marine agar media (±40°C), leading to the performance of incubation at 28°C for 24 h. The culture resulting from the dilution grew on marine agar media. It was then isolated using an ose needle randomly selected for 32 isolates, and the morphology of the colonies formed was recorded, including shape, edge, elevation, color, and size of the colony. Each of the 32 isolates was streaked on marine agar media to obtain a single colony. Bacterial purification was carried out using the streak method. The bacterial isolates selected based on the colony morphology were rubbed on marine agar media and incubated for 24 hours at 28ºC. The purified bacteria were then rubbed on marine agar on an inclined tube as stock for the next stage.

**Isolation and measurement of proteolytic index**

Isolation of proteolytic bacteria was carried out by taking bacterial culture using an ose needle on inclined media, then was streaked on skim milk media following (33) and incubated for 48 hours at 28ºC. A clear zone around the colony indicated thepresence of proteolytic bacteria. The proportion of proteolytic bacteria in the sample was calculated by comparing the number of proteolytic with non-proteolytic bacteria. The proteolytic bacteria were then tested by taking one ose and scraping it on a new skim milk medium. It was then incubated for 48 hours at 28ºC. Observations were made at 24, 36, and 48 hours to determine the differences in the hydrolysis zones presented. The activity index of proteolytic bacteria is calculated by measuring the total hydrolysis area divided by the colony area (34).

**Antibiotics sensitivity test**

An antibacterial test was performed using the paper disk method. Pathogen *Vibrio parahaemolyticus* was cultured on marine agar using a spread plate. A paper disk was then put on the media surface and dripped with 10µL of sample bacterial culture; it then was incubated for 48 hours. The ability to produce antibacterial is indicated by forming a clear zone around the paper disk. The paperdisk method was used to test the sensitivity level of bacteria to antibiotics. A total of 100µL of sample inoculum was spread on solid marine media using the spread plate technique. Afterward, 4 paperdisks containing different types of antibiotics, namely tetracycline (30mcg), amoxicillin (25mcg), clorophenicol (30mcg), and gentamicin 10mcg were put on the marine media. They were incubated at 28ºC for 24 hours. Sensitivity was indicated by a clear zone around the paper disk (35).

**Bacterial DNA extraction**

Bacterial DNA extraction was carried out using the Genomic DNA Mini Kit following the instructions (Geneaid Biotech). Several stages of DNA extraction are preparation, lysis, binding, washing, and elution. The preparation stage is growing bacteria on TSB media for 24 hours and then washing using sterile distilled water. The cell lysis stage is by adding GT buffer and Proteinase K and then incubation at 60 C for 30 minutes in a water bath. GBT buffer was added and incubated for 20 minutes to ensure cell lysis. The next step is binding and washing using absolute ethanol. DNA separation was carried out using the spin column technique by adding W1 and wash buffer and then centrifuged. The last step, elution, is done by adding elution buffer, which has been heated with a volume of 50µL and then stored at a-20C.

**Amplification PCR**

PCR amplification was carried out using Primus 25 Thermocycler PCR (Peqlab). The primers used in amplification follow the research of (36) 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3'), and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') resulted in amplification of 1500bp. The PCR program used for amplification was denaturation at 94ºC for 2 minutes, followed by 35 cycles of denaturation at 94ºC for 20 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 20 seconds, followed by a final extension at 72ºC for 5 minutes and storage at 25ºC for 1 minute.

**16s rDNA sequence analysis**

Analysis of Basic Local Alignment Search Tool (BLAST) was performed. The sequencing results were aligned with sequences in the GenBank which resulted in identity values to determine bacterial species; it was then continued by describing the bacterial samples in the branching of the phylogenetic tree (37).

**Data analysis**

Data on proteolytic bacteria proportion, proteolytic index, antibacterial, and sensitivity to antibiotics are presented to determine the potential of bacteria and compare the systems used in the shrimp culture. Bacterial identification data were analyzed by comparing the sample sequence homology with the sequence in GenBank and presented in an evolutionary tree.

**Results**

Isolation of bacteria from the sample was carried out using the pour plate culture method from the dilution results. Cultured bacteria are usually isolated and purified based on morphological characteristics as the initial standard in distinguishing bacteria. However, in this study, the technique was carried out by randomly taking as many as 32 isolates from the culture without ignoring the morphological characteristics of the colony (data not shown). This number is considered representative of all bacteria that have been successfully cultured on the media. This technique was carried out to maximize isolation in obtaining bacteria as probiotic agents and suppress errors in distinguishing colony morphological characteristics that might occur due to the similarity of characters.

**Proteolytic bacteria isolation**

A total of 224 bacteria isolates were found at 7 sample points within the intensive (inlet and outlet waters, as well as sediments) and traditional (inlet, middle, and outlet waters, as well as sediments) ponds. The ability of those bacteria isolates to produce extracellular protease enzymes was detected, leading to the foundation of proteolytic bacteria proportion to be compared between ponds. Data on the number of isolates and the proportion of proteolytic bacteria are presented in table 1.

Table 1: The number of isolates and the proportion of proteolytic bacteria

| Type of pond | Sampling point | Number of isolates | Positive | Negative | Percentage (%) |
|---|---|---|---|---|---|
| Traditional | Water | 32 | 12 | 20 | 37.5 |
| | Inlet | 32 | 28 | 4 | 87.5 |
| | Outlet | 32 | 29 | 3 | 90.6 |
| | Sediment | 32 | 32 | 0 | 100.0 |
| Average | | 32 | 25.2 | 6.7 | 78.9 |
| Intensity | Inlet | 32 | 26 | 6 | 81.2 |
| | Outlet | 32 | 28 | 4 | 87.5 |
| | Sediment | 32 | 17 | 15 | 53.1 |
| Average | | 32 | 23.6 | 8.3 | 73.9 |
| | Total | 224 | 172 | 52 | |

Several bacterial isolates were taken randomly at each point for 32 isolates to identify the proportion of proteolytic bacteria. The values of proteolytic bacteria proportion varied among sampling points in the traditional and intensive ponds. The proportions of proteolytic bacteria at the sampling points of traditional ponds (water, inlet, outlet, and sediment) were 37.5%, 87.5%, 90.6%, and 100%, respectively. While those at the point of intensive pond sampling (inlet, outlet, and sediment) were 81.2%, 87.5%, and 53.1%, respectively. The proportion of proteolytic bacteria among sampling points in intensive and traditional ponds was higher than the bacteria without proteolytic activity, namely 53.1 - 100%. However, water sample in traditional ponds was observed to have a lower proportion of proteolytic bacteria than non-proteolytic bacteria, namely 37.5%. Remarkably, the proportion of proteolytic bacteria isolated from traditional pond sediments was 100% producing extracellular protease enzymes. Based on the average value, traditional ponds have a higher proteolytic proportion than intensive ponds, namely 78.9 and 73.9%.

**Activity index of proteolytic bacteria**

A total of 172 proteolytic bacteria were distinguished based on the colonys morphological characteristics, and the highest proteolytic activity index was identified from 4 selected isolates of proteolytic bacteria. The proteolytic activity index was measured every 12 hours after 24 hours to determine the optimum activity of bacteria in producing protease enzymes in the growth cycle. Observation results of proteolytic activity index at different hours are displayed in table 2.

It was identified that the activity index of proteolytic bacteria reached a high value in 36 and 48 hours compared to that in 24 hours. The proteolytic activity index was generated by bacteria of isolate codes II15 and II12. The two isolates showed the same activity index at every hour of

observation, i.e., 1.3 at the 24th hour, 7.0 at the 36th hour, and 7.0 at the 48th hour. Meanwhile, bacteria of isolate codes TT17 and SI09 showed different activity indexes. The activity index of isolate TT17 at the 24th, 36th, and 48th hours of observation were 1.8, 4.5, and 4.5, respectively. While those of isolates SI09 at the 24th, 36th, and 48th hour of observation was 2.0, 4.3, and 4.3.

**Molecular identification of proteolytic bacteria**

Four bacterial isolates were selected based on the highest activity index identified molecularly based on the 16s rDNA gene. The identification was carried out using amplification of 16s rDNA gene supported by PCR machine to obtain a 1500bp amplicon product. The results of amplicon products were sequenced to obtain sequences for blast analysis on GenBank. The analysis of blast results is displayed in table 3 and figure 1.

Analysis of blast sequence of proteolytic bacterial 16s rDNA gene aligned with the sequence in GenBank was identified in 2 different groups, namely *Vibrio* sp. and *Pseudoalteromonas* sp. The analysis resulted in the similarity of identity percentages of 98.23 - 99.80%. Bacteria SI09, TT17, and II12 were identified as *Vibrio* sp., namely *Vibrio parahaemolyticus* and *Vibrio navarensis*. Meanwhile, isolate II15 was identified as *Pseudoalteromonas ganghwensis*. The evolutionary analysis found stable branching in both groups with a bootstrap value of 99%.

Table 2: Observation of proteolytic activity index at different hours

| Isolate code | Proteolytic Activity Index (Hour) | | |
|---|---|---|---|
| | 24 | 36 | 48 |
| TT17 | 1.8 | 4.5 | 4.5 |
| SI09 | 2.0 | 4.3 | 4.3 |
| II15 | 1.3 | 7.0 | 7.0 |
| II12 | 1.3 | 7.0 | 7.0 |
| Average | 1.6 | 5.7 | 5.7 |

Proteolytic activity index is measured on a centimeter (cm) scale.

Table 3: Blast analysis data of 16s rDNA gene sequence of proteolytic bacteria

| Isolate code | Reference Sequence (blast) | Query cover | Length (bp) | Accession number | Identity (%) |
|---|---|---|---|---|---|
| I09 | *Vibrio parahaemolyticus* MC32 | 99 | 1189 | MT5340261.1 | 98.23 |
| TT17 | *Vibrio parahaemolyticus* BpShHep 36 | 100 | 1020 | MF949061.1 | 99.80 |
| II12 | *Vibrio navarensis Strain* 34 | 98 | 1012 | MT974084.1 | 98.90 |
| II15 | *Pseud. ganghwensis* WAB2121 | 99 | 1178 | MH169282.1 | 98.56 |

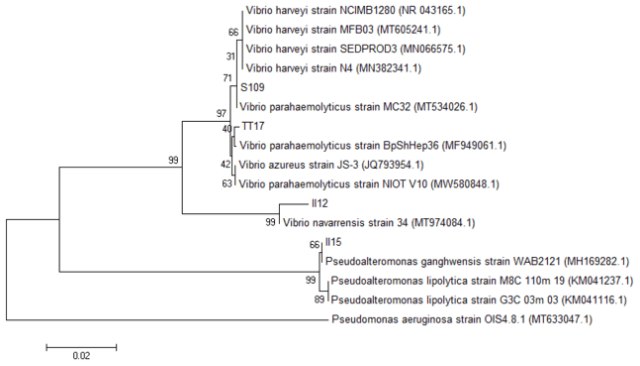


Figure 1: Phylogenetic tree of proteolytic bacteria and closely related strains based on 16s rDNA sequences. NCBI access numbers are shown in parentheses. The tree was constructed using evolutionary techniques based on Neighbor-joining and bootstrap values applied in 1000 replications. Bacterial strains without an accession number belong to sample bacteria. Branching of *Pseudomonas aeruginosa* strain OIS4 8.1 is an out-group.

**Test of antibacterial activity and sensitivity to antibiotics**

Isolate II15 is a bacterium isolated from the intensive inlet pond. There are several types of bacteria from intensive ponds, but those with enzymatic activity are isolates II15. Isolate II15 identified as *Pseudoalteromonas ganghwensis*, was detected for its ability to produce antibacterial compounds against the pathogen *Vibrio parahaemoliticus*. Antibacterial compounds can be detected by paperdisk method dripped with sample culture. Antibacterial activity against pathogens was indicated by forming a clear zone around the paper disk. The sensitivity of II15 to several types of antibiotics was tested to determine the bacteria sensitivity to antibiotics. Tests of antibacterial and antibiotic sensitivity are shown in Figure 2.

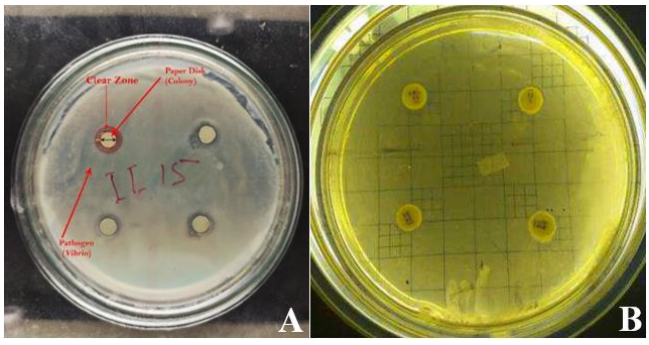


Figure 2: A. Antibacterial test, B. Antibiotic sensitivity test.

The antibacterial activity test of isolate II15 against the pathogenic bacterium *Vibrio parahaemolyticus* showed positive antibacterial activity. Isolate II15 was able to inhibit the growth of pathogens as indicated by a clear zone around the paper disk in each culture replication. In addition to antibacterial, isolate II15 is very sensitive to several types of antibiotics. The clear zone formed in the antibiotic test showed very high sensitivity to each type of antibiotic.

**Discussion**

Intensive vannamei shrimp culture technology with high stocking densities requires more selective control. One of the controls is probiotics, which can maintain environmental balance and shrimp health (38). The initial steps to obtain potential strains to be probiotic agents are isolation and screening of several products from bacterial metabolism that can benefit the host and the shrimp culture environment. Traditional ponds can be used as probiotics as the right environment for potential bacterial strains. Traditional ponds have natural environmental factors leading to various complex biotic and abiotic interactions. Factors in a complex environment can form bacterial communities with abilities to produce potential metabolites compounds such as biocontrol in aquaculture (39). Out of 224 isolates successfully isolated on marine media, 172 bacterial isolates were identified to produce extracellular protease enzymes. The percentages of proteolytic bacteria proportion varied at each sampling point and type of aquaculture pond. The proportion of proteolytic bacteria varied due to differences in the concentration of organic matter in pond waters and sediments. The concentration of organic material in sediment is higher in culture water because sediment is the lower layer as a place for organic material accumulation. In general, the proportion of proteolytic bacteria is higher than other bacteria. A considerable number of proteolytic bacteria in aquaculture ponds is due to abundant protein sources allowing proteolytic bacteria domination to carry out their function as protein degraders (40). Various studies found that protease enzyme-producing bacteria are abundant in water, sediment, shrimp intestines, and agricultural wastes (41,42).

These results were in line with (43), which stated that soil was a protein-rich organic material enabling peptidase bacteria to play an essential role in recycling nitrogen. However, the functions of these bacteria varied from one another, depending on the sediment being utilized as a growth medium (40). The isolates also developed between 5-37°C, with protease induced in the end log, stationary, or death phase (44). Other evidence was the sampling from the hot springs at 50, 60, and 70°C, where 1, 5, and 0 isolates were observed for each location. Another evidence also showed that 4 isolates produced proteolytic enzymes in the index value of 3.8 U/mL (45). At a temperature of 60-65 $^{0}$ C, the highest proteolytic-producing bacteria was *G. thermoglucosidasius SKF4*, subsequently indicating that the soil sediments contained the carbohydrates to increase the activity of suitable specific ectoenzymes including β-glucosidase, -N-acetylglucosamine, and lipase (46).

Traditional ponds have a higher average percentage than intensive ponds. Results of the study showed a percentage of 100% in the traditional pond sediment samples. However, the sediment in intensive ponds was 53.1%. The high percentage of proteolytic bacteria in traditional ponds was because the pond was constructed by soil wall and bottom, causing complex interactions in the pond. This can trigger the diversity of bacteria inhabiting the environment. In addition, feeding activities will lead to the accumulation of high protein organic matter since the remaining feed of the shrimp accumulates at the bottom of the pond (47). This is under the opinion of that soil is an accumulation of protein organic matter, so peptidase bacteria play an essential role in reforming the nitrogen cycle. It contrasted with the results in intensive pond sediments, which tend to be lower. This is presumably because the ponds bottom was coated with plastic and the sediment sample was the accumulated residual sediment that had been cleaned, resulting in unsatisfactory observation results. Based on this present study, the highest amount of enzymatic production was found at the sediment point of traditional ponds, where 32 isolates containing extracellular proteolytic activities were observed. The lowest point was also found in the middle waters of the traditional ponds, where 11 isolates were observed (43).

This was in line with (48), which stated that aquaculture ponds (aquatic and sedimentary) had high organic and inorganic contents, due to the remaining uneaten feed and shrimp excrement. The study was conducted by isolating 215 isolates from the pond sediments in the Phu vhang area, where the results showed that 25 bacteria had extracellular proteolytic, amylolytic, and cellulolytic activities, with the most active microbe identified as the *Bacillus* sp. *W12*. Also, the SO4 and W04 strains obtained from the sediment and water had proteolytic indexes of 17.24 ± 0.89 and 21.67 ± 0.95 U/mL, respectively. This indicated that proteolytic bacteria were produced through thermophilic growth at 45-80°C. These results were subsequently in line with (49), which obtained isolates from the digestive tract of a catfish, where the highest type of extracellular-producing bacteria was *Bacillus* sp. with a proteolytic index value of 2.09 ± 0.41 U/mL. The highest extracellular production was due to the natural utilization of the soil base, which led to very high carbon and nitrogen contents. This indicated that the increase in protease was influenced by the contents of xylose and urea (50), nitrogen (51), and ammonium sulfate (13,52). Therefore, the maximum protease production (141.46 U/mg) with a C-N source and 1 M NaCl was obtained for 24 h, at a pH 8 below 250 rpm. This indicated that the isolation of bacteria from mangrove sediments increased the weight of vannamei shrimp by 141.9%, showing that the isolates had good extracellular properties in degrading protein (53).

The proteolytic activity index showed the optimum activity at 36 and 48 hours. Additionally, the bacteria had entered the stationary phase at 36$^{th}$ and 48$^{th}$ hours, and the stationary phase is the peak phase of bacterial growth. This phase is indicated by a similar amount of growth and dead cells, resulting in a stable curve. Bacterial growth consists of 4 phases: the lag phase, log phase, stationary phase, and death phase. According to Santhi (54), bacteria produce protease enzymes maximally when the water condition meets several factors; pH 7, glucose carbon source, nitrogen peptone source, and incubation time of 36 - 48 hours. This research used growing media in which the nitrogen source was peptone pH 7, which is the exact pH, to find the optimal stationary phase. Unfortunately, these studies did not run tests on those factors. Identification of proteolytic bacteria based on blast analysis of 16s rDNA gene sequence had similarities with 2 groups of bacteria, namely *Vibrio* sp. and *Pseudoalteromonas* sp. The identity value obtained was very high, i.e., 98.23 - 99.80%. The isolates identified from the *Vibrio* sp., II12 as *Vibrio navarensis*, SI09, and TT17 as *Vibrio parahemolyticus*. While from the group of *Pseudoalteromoas* sp., it was found one isolate of II15 identified as *Pseudoalteromonas ganghwensis*. *Vibrio* sp. is the most common pathogen found in brackish and marine waters. More than 20 species of *Vibrio* were found in brackish waters as potential pathogens on shrimp (55,56). One of the most frequently found strains and even has the highest frequency is *Vibrio parahemolyticus* (55,35). The disease caused by this strain is Acute Hepatopancreatic Necrosis Disease (AHPND), also known as Early Mortality Syndrome (EMS), which attacks the larvae and causes death up to 100%. The mechanism of this infection is that pathogenic bacteria produce toxins that damage all cells and are usually detected in the hepatopancreas (57).

*Pseudoalteromonas* sp. is one bacteria that can adapt and thrive in the digestion of shrimp and sea eels (58,59). This bacterium is likely to have activity as a probiotic agent because it has several metabolic products used in aquaculture. Several tests have been carried out that *Pseudoalteromonas* has proteolytic, amylolytic, floc formation, and antibacterial activity (several data are not presented). The same study was conducted by (60,61) that *Pseudoalteromonas ganghwensis* produces an extracellular protease enzyme, amylase, and can form floc. In addition, this bacterium has a cellulolytic activity used as an aquaculture waste bioremediation agent (48). This study further tested the ability of antibacterial tests against the pathogen *Vibrio parahaemolyticus*, sensitivity, and resistance tests to several types of antibiotics. The results showed that *Pseudoalteromonas ganghwensis* could inhibit the growth of pathogenic bacteria that commonly attack shrimp ponds. According to Desriac *et al.* (62), *Pseudoalteromonas* sp. has secondary metabolite activity able to inhibit the growth of pathogenic bacteria even in low concentrations and has excellent opportunity to be developed as aquaculture probiotics. Using 16s rDNA, the sequencing analysis showed that the percentage of similarity ranged between 99-100% of the target bacterium (*Pseudoalteromonas Ganghwensis WAB2121*) with the GenBank database. All strains were saved to the GenBank with accession number, MH169282.1.

Furthermore, the bacterial species producing proteolytic activity were abundantly isolated from the water (41), sediment, shrimp gut, agricultural waste (42), and intestinal worms (59). Besides having good proteolytic activity, *Pseudoalteromonas ganghwensis WAB2121* also had antivibrio, certifying its suitability in maintaining the balance of aquacultural environments, especially vannamei shrimp culture. In this study, the microorganism was isolated from the inlet of the intensive pond, with bacterial growth observed to be ordinarily and increasingly influential at 24 and 48 h, at a pH and temperature of 7 and 28°C, respectively. This was the optimal point for bacteria to produce proteolytic activity. Besides having proteolytic activity, *Pseudoalteromonas* also formed biofilms to reduce non-biodegradable proteins in shrimp pond sediments (60). These bacteria were subsequently identified in the intestine of the isolated vannamei shrimp and found to have the ability to utilize carbon and nitrogen sources. They also could adequately degrade starch and casein (61) and polysaccharides to oligosaccharides.

The potential of bacteria as probiotics needs to be developed to improve productivity and the cultivation environment. A study on *Pseudoalteromonas ganghwensis* is necessary since it has many abilities that can be used as probiotics. Research by (48) found that *Pseudoalteromonas* has cellulosic capabilities as a biodegradation agent for aquaculture waste. Screening results of research by (60) obtained 7 *Pseudoalteromonas* sp. producing protease enzymes and very high floc formation in the environment, which can be used as natural feed for aquaculture fish. Research by (63) found that several strains of *Pseudoalteromonas* produce bioactive compounds used in health industry. This study found that *Pseudoalteromonas* has antimicrobial activity as a biocontrol agent against *Vibrio* pathogenic bacteria and the results of sensitivity tests for several types of antibiotics show very high sensitivity so that they can be used as probiotic candidates. Tests on sensitivity to antibiotics in probiotic bacteria need to be carried out to determine the resistance characteristics. Bacteria resistant to antibiotics have resistant genes that can be transferred to other bacteria through several mechanisms. This is very risky for increasing resistance in horizontal control of pathogenic bacteria. The presence of resistant genes in probiotic bacteria is not recommended to be used in aquaculture since its possibility of resistant genes spread in pathogenic bacteria (64). This study obtained isolates of *Pseudoalteromonas ganghwensis* bacteria tested for sensitivity to several types of antibiotics. The test results

show that *Pseudoalteromonas ganghwensis* has very high sensitivity and can be used as aquaculture probiotics.

## Conclusions

From the isolation of proteolytic bacteria from shrimp ponds with different aquaculture systems, it was found that the traditional culture system had a higher proportion than the intensive aquaculture system. Screening based on morphology and the highest proteolytic index obtained 4 isolates identified from the *Vibrio* sp. and *Pseudoalteromonas* with identity 98.23 - 99.80%. *Pseudoalteromonas ganghwensis* strain has antibacterial activity that can inhibit pathogen *Vibrio parahaemolyticus* and very high antibiotic sensitivity. These two characteristics make it probiotic bacteria.

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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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# العزل والتوصيف الجزيئي للجراثيم المحللة للبروتين من أحواض روبيان فانام كعوامل معززة حيوية

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**الخلاصة**

تطورت عملية استزراع روبيان فانام في الأحواض المكثفة والتقليدية بشكل سريع في إندونيسيا. بدأ التطور من خلال التقنيات والكثافة العالية وانظمة التغذية. ان هذا التطور تسبب بانتاج عالي للمواد العضوية في أحواض الاستزراع المائي، والذي تحتم ضرورة المحافظة على ادامة جودة المياه. تهدف هذه الدراسة إلى العزل والتوصيف الجزيئي للجراثيم المحللة للبروتين باعتبارها معززات حيوية وذلك من احواض استزراع روبيان فانام باستخدام تقنات متعددة. إستخدمت هذه الدراسة طريقة المسح وتقنية أخذ العينات العشوائية من احواض الاستزراع التقليدية والمكثفة للروبيان. أظهرت النتائج أن نسبة البكتيريا المحللة للبروتين في الأحواض التقليدية كانت أعلى من تلك الموجودة في الأحواض المكثفة. وأظهر المسح المعتمد على المظهر المستعمري و أعلى مؤشر نشاط أن ٤ عزلات جرثومية تعود الى *Vibrio* sp. و *Pseudoalteromonas* بقيمة مطابقة ٩٨,٢٣-٩٩,٨٠٪. ولوحظت قيمة هذا النشاط عند ٧,٠ سم ، ولاحقا أدى إلى تثبيط *Vibrio* sp. بمقدار ١,٥ سم. كما لوحظ نمو الجراثيم عند درجة حموضة ٧ ودرجة حرارة ٢٨ درجة مئوية بشكل كافٍ. بالإضافة إلى ذلك ، أشار الحد الأقصى من نشاط التحلل للبروتين في فترة حضانة لمدة ٤٨ ساعة إلى أهميتها المحتملة كمعزز حيوي. ووجدت دراسات أخرى حول إمكانية امتلاك عتر من *Pseudoalteromonas ganghwensis* لنشاط مضاد للأحياء المجهرية وانها حساسة للمضادات الحيوية والتي يمكن استخدامها كمعززات حيوية في المزارع المائية.

Live feeds used in the larval culture of red cusk eel, *Genypterus chilensis*, carry high levels of antimicrobial-resistant bacteria and antibiotic-resistance genes (ARGs). Anim. 2020;10(3):1-23. DOI: 10.3390/ani10030505

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