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# ISOLATION AND MOLECULAR IDENTIFICATION OF AMYLOLITIC BACTERIA FROM VANNAMEI SHRIMP (*LITHOPENAEUS VANNAMEI*) PONDS AS PROBIOTIC AGENTS

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***Abstract***

Probiotics are bacteria that have the potential to be developed as biocontrol agents in aquaculture. Several probiotic abilities have been known such as amylum breakdown and antibacterial which are relevant to suppressing the growth of pathogens. This study aimed to isolate and identify the molecular amylolytic bacteria from vannamei shrimp aquaculture ponds with different systems as probiotic agents. The method used were a survey and random sampling technique from traditional and intensive shrimp farming. Parameters analyzed included the proportion of amylolytic bacteria, amylolytic index, and molecular identification. The results showed that the proportion of amylolytic bacteria in intensive ponds was higher than in traditional ponds. Screening based on colony morphology and the highest activity index obtained four identified bacterial isolates from the *Vibrio* sp, *Staphylococcus* sp, and *Pseudoalteromonas* groups with an identity value of 98.23%-99.80%. *Pseudoalteromonas* sp. has the highest amylotlytic content and generlly has antimicrobial activity which can be used as probiotic agents.

**Keywords:** *Amylolytic, Probiotic agent, Pseudoalteromonas, 16S rRNA gene, Aquaculture probiotic*

## Introduction

The fisheries sector is one sector that has a significant contribution to national economic development. This is based on the contribution of the fishery sector which plays a role in absorbing labor, increasing people's income, contributing to the country's foreign exchange, and food security in meeting domestic protein consumption. One of the productions of fish farming that has a good impact is the production of vannamei shrimp culture. Indonesia vannamei shrimp exports in 2020 recorded at 1.26 billion kg. Shrimp export volume increased by 28.96% compared to 2019 which was 207.70 million kg [1]. Shrimp also contributed to the total export volume of fishery products by 18.95% last year. Production of shrimp farming in Indonesia reached 911.2 thousand tons in 2020. Of that amount, as many as 157.4 thousand tons of shrimp farming production is in West Java as the largest nationally. This increase is because shrimp farming is now growing with intensive technology with high density [2-4].

The application of an intensive cultivation system for vannamei shrimp culture is conducted using a high stocking density [5]. This causes several obstacles in intensive system cultivation, including decreased water quality, increased stress, and low feed efficiency [6]. In

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addition, intensive vannamei shrimp culture has a greater chance of disease occurring because environmental conditions and shrimp physiological conditions are not balanced [7]. The method generally used to treat disease in white shrimp is the use of antibiotics [8, 9]. However, the use of antibiotics has a detrimental impact because it causes bacterial resistance in the waters and antibiotic residues can accumulate in the shrimp body [10]. Therefore, other alternatives are needed that can improve growth performance and prevent the potential for disease spread in vannamei shrimp culture [11]. One of the efforts that can be done to overcome these problems is by giving probiotics [12-17].

Several problems encountered by shrimp cultivators ensure that probiotics used by vannamei shrimp cultivators are uncontrolled and not yet effective. First: The shrimp farmers do not know whether the probiotic isolates used are suitable for brackish or fresh water usage. Currently, most farmers use probiotics that are commonly used in freshwater, fresh, brackish, and marine [18]. Second: The isolate should be used for aquatic or feed probiotics. Currently, cultivators use feed and water probiotics simultaneously, which significantly reduces the effectiveness of probiotics. Third: Isolates used were not derived from vannamei shrimp ponds, but from other environments, so they could not be developed after becoming probiotics. This of course will affect the effectiveness of the probiotic itself. Where Probitoik will be very susceptible to changes in pH, temperature, and other environments [19]. This is very important to do so that the effectiveness of the use of probiotics is maximized. On this basis, this research is the first step to overcoming some of the problems of shrimp farmers by isolating probiotic agent bacteria in terms of the quality and quantity of the amylolytic bacteria. This step is expected to be the first step in the production of a better probiotic agent.

## Materials and Methods

### *Place and Time of Research*

This research was conducted from January-December 2021. Isolation of bacteria and the characteristics of bacteria that produce protease and amylase secondary metabolites were performed at the Research Laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University. This study used a survey method where sampling locations are taken in two different vanname shrimp cultivation systems, namely traditional and intensive systems

### *Sampling Location*

Bacterial isolates were taken from the waters of shrimp ponds on the coast of Pangandaran (5° 34'18.32'N, 102° 48'25.86'E). The vannamei shrimp pond used for sampling has 2 cultivation systems, namely intensive and traditional with good yields for the last 5 cycles. Intensive ponds apply high stocking density with the bottom and walls covered by plastic while the traditional ponds have soil base and walls.

### *Sampling*

The samples taken in this study were water and pond sediments. Sampling was conducted at 7 points from traditional ponds and intensive ponds. In traditional and intensive ponds, samples were taken from the inlet, outlet, middle and pond sediments. Water samples were taken as much as 50 mL then put into sterilized sample bottles, and pond sediments were taken from the bottom of the waters. Samples were placed in a coolbox, then taken to the lab for analysis.

### *Bacteria Isolation*

Water and sediment samples were prepared using dilution. The dilution series followed the modified Mandigan et al, [20] procedure. Dilutions of $10^{-1}$ to $10^{-5}$ were conducted and each sample was diluted using five test tubes containing 4.5mL of sterile physiological. A total of 0.5mL of water sample and 0.1g of sediment sample were taken and put into the first test tube for $10^{-1}$ dilution and then homogenized. The test tube was homogenized using a vortex. Samples of water and sediment at a dilution of $10^{-1}$ were taken as much as 0.5mL and then inserted into the second test tube using a measuring pipette and a propipet as a $10^{-2}$ dilution and then homogenized

using a vortex. Then the same mechanism was carried out with $10^{-1}$ and $10^{-2}$ dilutions to obtain a $10^{-5}$ dilution. Each dilution series was then taken as much as 0.5mL and put into a sterile petri dish and poured with marine agar media (temperature ±40ºC) with the pour plate method. The sample was incubated for 24 hours at a temperature of 28ºC following the temperature of aquaculture ponds.

Bacterial colonies growing on marine media were isolated using an ose needle and then 32 isolates randomly selected the morphology of the colonies formed including shape, edge, elevation, color, and size of the colony. The bacterial isolates were isolated and purified by the streak plate method on marine agar media. It aimed to get a single colony of bacteria. The bacterial isolates selected based on the colony morphology were streaked on marine agar media and incubated for 24 hours at 28ºC. The purified bacteria were streaked on marine agar on an inclined tube as stock for the bacterial amylolytic test.

***Amylolytic Index Isolation and Measurement***

Isolation of amylolytic bacteria was conducted by isolating bacteria based on the morphological characteristics of the colony including shape, size, color, edge, and elevation of the diluted culture, then streaked on starch media and incubated for 48 hours at 28ºC. The presence of a clear zone around the colony indicates the presence of amylolytic bacteria after iodine drops [21]. The proportion of amylolytic bacteria in the digestive tract was calculated by comparing the number of positive and negative activities. Bacteria with positive amylolytic activity were then tested by taking one ose and scraping it on starch medium and then incubating it for 48 hours at 28ºC. The activity of amylolytic bacteria was calculated by dividing the total diameter of the hydrolysis zone (clear zone) by the diameter of the bacterial colony [22, 23]

***Bacterial DNA Extraction***

The bacterial DNA extraction method used was the Genomic DNA Mini Kit with a procedure following the instructions (Geneaid). Bacteria from Marine Broth (MB) media stock were cultured and incubated for 24 hours. The next step was to take as much as 1.0mL of bacterial culture results from MB that have been vortexed and then transferred to a 1.5mL microtube and spin down to form a pellet. Pellet washing which aimed to wash pellets from bacterial media is done by removing and replacing the supernatant on the microtube with sterile distilled water and then vortexing and spindown. Washing was repeated three times. The bacterial pellets were homogenized with 200L GT Buffer and 20L Proteinase K. The samples were vortexed and spindown and then incubated at 60°C for 30 minutes using a water bath. Next is to homogenize the tube every 5 minutes during incubation. The next stage is cell lysis. The sample was given 200L of GBT Buffer solution, then vortexed for 10 seconds and incubated again at 60ºC for 20 minutes. This is to ensure that the cell is completely lysed. The microtubes were homogenized every 5 minutes during the incubation process. Then, the Elution Buffer which will be used in the DNA elution stage is also incubated together with the sample. The next step is binding and washing DNA. This is done by adding 200µL of absolute ethanol into the sample and then homogenizing using a vortex and spindown for 10 seconds each.

The next mechanism is to prepare and attach the GS column to the 2mL collection tube. Then, transfer the sample into a GS column and centrifuge at 12,000rpm for 2 minutes. The collection tube was discarded and the GS column was transferred to a new 2mL collection tube. The next step was to add 400L of W1 Buffer to the GS column and then centrifuged at 6,000rpm for 30 seconds. A total of 600L of wash buffer was added to the GS column and then centrifuged at 6,000rpm for 30 seconds and the solution collected in the collection tube was discarded. To dry the matrix column, the solution collected in the collection tube was discarded and the GS column was centrifuged for 3 minutes at 6,000rpm. DNA elution is the next step which is carried out by transferring the dried GS-column to a new and sterile 1.5 microtube and then adding 100L of warmed Elution buffer to the center of the column matrix. The next step is to let the sample sit for 5 minutes. This is to ensure that the Elution buffer is fully absorbed. The sample was then

centrifuged at 6,000rpm for 30 seconds and the DNA sample solution was stored at -20°C until further processing.

***PCR Amplification***

PCR amplification was performed on bacterial 16S rRNA gene using Primus 25 Thermocycler PCR (PEQLAB). The primers used in the amplification of 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') with a product length of 1500bp, amplification following the study of *J.R. Marchesi et al.* [24] Predenaturase 94ºC for 2 minutes, followed by 35 cycles of denaturation 94ºC for 20 seconds, annealing 55ºC for 30 seconds, extension 72ºC for 20 seconds followed by final extension 72ºC for 5 minutes, and storage at 25ºC for 1 minute.

***16s rRNA Gene Sequence Analysis***

Basic Local Alignment Search Tool (BLAST) analysis is a way to find sequences that are closely related to our sequence data or that have similar identities. Sequences are obtained from the results of the sequencing and are aligned with the existing sequences in GenBank. Furthermore, the identity value will be obtained to determine the bacterial species and then proceed with describing the bacterial sample on the branching of the phylogenetic tree using the phylogeny.fr web [25].

***Data Analysis***

Data on amylolytic bacteria isolation, proportion of amylolytic bacteria, the best of amylolytic activity were then compiled and presented on table. The data presentation aims to determine the potential of bacteria and compare the systems used in shrimp farming. Analysis of bacterial identification data was carried out by comparing the sample sequence homology with the sequence in GenBank and presented in an evolutionary tree. The next discussion is to discuss descriptively the data analysis results that are supported and compared using previous research.

## Results

***Amylolytic Bacteria Isolation***

The results were obtained from 7 sampling points located in intensive and traditional pond areas. Based on these results, as many as 224 bacterial isolates were isolated. In traditional ponds, sampling points include an inlet, middle, outlet, and sediment waters. While in intensive ponds, sampling points include an inlet, outlet, and sediment waters. To determine the proportion of amylolytic bacteria which were then compared between ponds, the ability of 224 bacterial isolates to produce amylase secondary metabolites were detected. Table 1 shows the number of isolates and the proportion of amylolytic bacteria data.

**Table 1.** Number of Isolates and Proportion of Amylolytic Bacteria

| Pond Type | Sampling Point | Number of Isolates | Positive | Negative | Proportion (%) |
|---|---|---|---|---|---|
| Traditional | Inlet | 32 | 26 | 6 | 81,2 |
| | Outlet | 32 | 22 | 10 | 68,7 |
| | Sediment | 32 | 31 | 1 | 96,8 |
| Average | | 32 | 26,3 | 5,6 | 82,2 |
| Intensive | Inlet | 32 | 28 | 4 | 87,5 |
| | Outlet | 32 | 32 | 0 | 100 |
| | Sediment | 32 | 31 | 1 | 96,8 |
| Average | | 32 | 30,3 | 1,6 | 94,7 |
| Total | | 224 | 170 | 22 | |

A total of 32 bacterial isolates were taken randomly at each point to observe the proportion of amylolytic bacteria. Various proportions value of amylolytic bacteria were obtained from sampling points in traditional and intensive ponds. The values of the proportion of amylolytic bacteria at the sampling points of traditional ponds (inlet, outlet, and sediment) were 81.2, 68.7

and 96.8%, respectively. The value of the proportion of amylolytic bacteria at the sampling point of intensive ponds was 87.5, 100 and 96.8%, respectively. Compared to bacteria that did not have amylolytic activity, the proportion of amylolytic bacteria between sampling points in traditional and intensive ponds got a higher value, namely 68.7 - 100%. Based on the average value, intensive ponds get a higher amylolytic proportion than traditional ponds, which was 94.7%. While the amylolytic proportion value of traditional ponds based on the average value was 82.2%.

***Amylolytic Bacterial Activity Index***

A total of 170 amylolytic bacteria were distinguished based on the morphological characteristics of the colony. Six bacterial isolates were selected for determining their amylolytic activity. The amylolytic activity index was measured every 12 hours after 24 hours to determine the optimal activity of bacteria-producing amylase secondary metabolites in the growth cycle. Table 2 shows the results of the amylolytic activity index observations at different times.

**Table 2.** Observation of Amylolytic Activity Index at different hours

| Isolate Code | Amylolytic Activity Index (Hour) | | |
|---|---|---|---|
| | 24 | 36 | 48 |
| II08 | 1,2 | 5,0 | 3,8 |
| TT29 | 1,4 | 4,0 | 4,3 |
| TT22 | 0,0 | 5,5 | 4,4 |
| IO25 | 0,0 | 2,0 | 4,0 |
| II14 | 0,0 | 0,0 | 4,0 |
| II26 | 1,7 | 4,0 | 4,0 |
| Average | 0,7 | 3,4 | 4,1 |

Information: Measurement of amylolytic activity index is measured in centimeter (cm) scale

The activity index of amylolytic bacteria with the highest value was obtained at 36 hours, namely 5.5 with the isolate code TT22. Bacteria with isolate codes IO25, II14, and II26 at 48 hours of observation showed the same activity index, namely 4.0. Bacteria with isolate codes TT29 and II26 at 36 hours of observation showed the same activity index, namely 4.0. At the 24-hour observation, bacteria with isolate codes TT22, IO25, and II14 did not show any index of bacterial activity. The same thing was also obtained from bacteria with isolate code II14 at the 36th-hour observation which did not show any index of bacterial activity. Bacteria with isolate code II08 at the 24th, 36th, and 48th hours of observation, respectively, 1.2, 5.0 and 3.8. At the 24th hour observation, bacteria with isolate codes TT29 and II26 were 1.4 and 1.7, respectively. Bacteria with isolate code IO25 at 36 hours of observation got 2.0. Bacteria with isolate codes TT29 and TT22 at 48 hours of observation, respectively, 4.3 and 4.4. The average obtained from all bacterial isolates at the 24th, 36th, and 48th hour observations, respectively, was 0.7, 3.4 and 4.1.

***Molecular Identification of Amylolytic Bacteria***

The highest activity index was identified molecularly based on the 16s rRNA gene and 6 bacterial isolates were selected. Identification was conducted using amplification of the 16s rRNA gene with the help of a PCR machine to obtain a 1500bp amplicon product. Then the results of the amplicon products were sequenced to obtain sequences for blast analysis on GenBank. Table 3 and figure 1 shows the result of blast analysis.

The blast sequence analysis of the amylolytic bacterial 16s rRNA gene aligned with the sequence in GenBank identified 3 different groups, namely *Vibrio* sp., *Staphylococcus* sp., and *Pseudoalteromonas* sp. Based on the analysis, obtained the similarity of identity percentage was 97.91%-99.91%. The bacteria were identified as a group of *Vibrio* sp. is a bacterium with isolate codes TT29 and II08, namely *Vibrio parahaemolyticus*. Bacteria with isolate codes IO25, TT22 and II14 were identified as *Pseudoalteromonas* sp. namely *Pseudoalteromonas ganghwensis* and *Pseudoalteromonas profundi*. While isolate II26 was identified as a group of *Staphylococcus* sp. namely *Staphylococcus gallinarum*. The evolutionary analysis found stable branching in the three groups with a bootstrap value of 99%.

**Table 3.** Data analysis of amylolytic bacterial 16s rRNA blast gene sequences

| Isolate Code | Blast Result | Query Cover (%) | *E. Value* | Identity (%) | Accession Number |
|---|---|---|---|---|---|
| IO25 | Pseudoalteromonas ganghwensis strain WAB2121 | 100 | 0.0 | 99.58 | MH169282.1 |
| II26 | Staphylococcus gallinarum strain E2 | 99 | 0.0 | 98.30 | MH371285.1 |
| TT22 | Pseudoalteromonas profundi strain TP162 | 99 | 0,0 | 98.22 | NR152699.1 |
| TT29 | Vibrio parahaemolyticus strain TY-41 | 100 | 0,0 | 99.91 | MT550813.1 |
| II08 | Vibrio parahaemolyticus strain VP24 | 100 | 0,0 | 97.91 | MN536754.1 |
| II14 | Pseudoalteromonas ganghwensis strain WAB2121 | 100 | 0,0 | 99.66 | MH169282.1 |

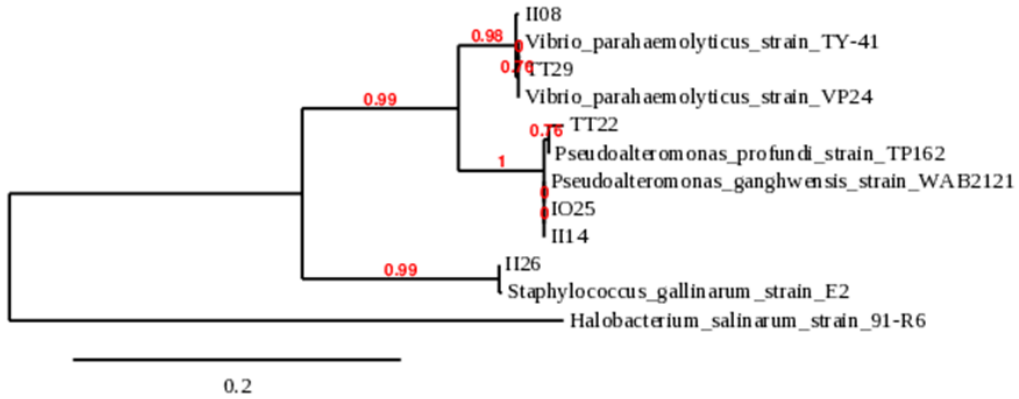


**Fig. 1.** Amylolytic bacterial phylogenetic tree constructed using Phylogeny.fr online program. Branching of Halobacterium salinarum strin 91-R6 is an out-group

## Discussion

Probiotics, which have the ability to maintain the culture environment and shrimp health, are one of the controls that can be used to increase more selective control in intensive vannamei shrimp culture technology [26, 27]. Bacterial communities that can produce metabolites that have potential as biocontrols in aquaculture can be formed from complex cultural environmental factors [28, 29]. Isolation and screening of products from bacterial metabolism is the first step to obtaining strains that have potential as probiotic agents. A total of 224 isolates were successfully isolated in marine media to obtain 32 isolates of bacteria that produce amylase secondary metabolites. At each sampling point based on the type of aquaculture ponds, the proportion of amylolytic bacteria that has varying percentages is obtained. The varying percentage of amylolytic bacteria was due to differences in the concentration of organic matter in the waters and pond sediments. In pond sediments, the concentration of organic matter is higher because sediment is the bottom layer used as a place for the accumulation of organic matter.

Intensive ponds have a higher average percentage than traditional ponds. Intensive ponds have the highest proportion of bacteria producing amylase enzymes of 94.7% compared to 82.2% in traditional ponds. Intensive ponds apply the provision of probiotics, prebiotics, and symbiotics in the cultivation process, this is also what makes intensive ponds further increase the amount of bacterial diversity in them [30]. Outlet samples in intensive ponds get a percentage of 100% while in traditional ponds get a percentage of 68.7%. A total of 32 amylolytic isolates were obtained from water and sediment samples in traditional ponds and intensive with selected isolates obtained a high amylolytic index of 6 isolates. The selected isolates have the potential to be used

as candidates for a probiotic consortium for the bioremediation of organic waste in ponds. According to *D.A. Anggorowati et al.* [31], six isolates of amylolytic bacteria obtained from traditional and intensive ponds can be used as probiotic candidates. Testing amylolytic activity in the identification of bacteria as probiotic candidates is important to determine the ability of bacteria to produce amylase enzymes used in starch hydrolysis. It was further explained that amylolytic activity in the digestive process is needed by bacteria producing amylolytic enzymes to produce exogenous enzymes for breakdown and digestion [32].

The amylolytic activity index showed optimum activity at 36 and 48 hours. The optimum activity was at 36 and 48 hours because at that hour the bacteria had entered the stationary phase. The stationary phase is the peak phase in the bacterial growth phase [33]. This phase is characterized by cells that grow the same as cells that die so that a stable curve is obtained. Bacterial growth has four phases, namely the lag phase, log phase, stationary phase, and death phase [34, 35]. Amylolytic bacteria obtained a starch higher than water bodies derived from the accumulation of organic matter from feed residues and shrimp metabolism at the bottom of the pond. *Sukenda et al*. [36] reported that the addition of sucrose as a carbon source in vannamei shrimp culture media could increase the total population of heterotrophic bacteria up to $10^7$cfu/mL. *B. Krishnan et al*. [37] proved that amylase production was maximized in the presence of starch as a carbon source from *Bacillus* sp.

Based on blast analysis of 16s rRNA gene sequences, identification of amylolytic bacteria obtained three groups of bacteria, namely *Vibrio* sp, *Staphylococcus* sp., and *Pseudoalteromonas* sp. Based on the analysis, obtained the similarity of identity percentage was 97.91%-99.91%. The bacteria were identified as a group of *Vibrio* sp. is a bacterium with isolate codes TT29 and II08, namely *Vibrio parahaemolyticus*. Bacteria with isolate codes IO25, TT22 and II14 were identified as *Pseudoalteromonas* sp. namely *Pseudoalteromonas ganghwensis* and *Pseudoalteromonas profundi*. While isolate II26 was identified as a group of *Staphylococcus* sp. namely *Staphylococcus gallinarum*. In brackish and marine waters, the most common and harmful pathogens for shrimp are the *Vibrio* sp. [38-40]. One of the most frequently found strains and even has the highest frequency is *Vibrio parahemolyticus* (38, 41, 42]. The disease caused by this vibrio strain is Acute Hepatopancreatic Necrosis Disease (AHPND) which is often also called the Early Mortality Syndrome (EMS) which attacks the larvae and causes up to 100% mortality [43, 44]. The mechanism of this infection, pathogenic bacteria will produce toxins that are known to damage all cells and are usually detected in the hepatopancreas [45].

**Table 4.** Antibacterial activity of *Pseudoalteromonas* sp. against pathogenic bacteria

| Strain | Pathogen | Reference |
|---|---|---|
| *Pseudoalteromonas sp* | *V. parahaemolyticus* | [46] |
| *P. luteoviolacea* | *V. harveyi* | [47] |
| *P. flavipulchra* | *V. anguillarum, V. harveyi* | [48] |
| *P. haloplankti* | *V. ordalii* | [49] |
| | *V. anguillarum, V. alginolyticus* | |
| *P. pisicida* | *V. alginolyticus, V. cholera, V. coralliilyticus, V. parahaemolyticus, V. vulnificus* | [50] |
| *Pseudoalteromonas* | *Serratia, Marcescens, Aorantimonas* | [51] |

*Pseudoalteromonas* sp is a candidate for probiotic bacteria which was also found in isolation from the vannamei shrimp digestive tract which has the advantage of being able to degrade protein and starch [52, 53]. *Pseudoalteromonas* has antimicrobial compounds and can synergize with other bacteria so it is possible to use it for probiotics Table 4. [54]. *Pseudoalteromonas species* have been used as probiotics [55]. Bacteria used as probiotic agents must be able to survive in the digestive tract and be able to attach to the intestinal epithelium of shrimp. *Pseudoalteromonas* sp has the ability as a good antimicrobial prevention, especially for

diseases caused by Acute Hepatopancreatic Necrosis Disease (AHPND) [56] *Vibrio nigripulchritudo* [57] V*ibrio harveyi* [58]. Pigmented *Pseudoalteromonas* species, for example, P. *flavipulchra* and P. *piscicida* usually produce bioactive compounds with antibacterial, antifouling, and antibiofilm activities, whereas non-pigmented species tend to have a wider environmental tolerance than pigmented ones [59-61]. P. *flavipulchra* JG1 can synthesize antibacterial protein (PfaP) which can catalytically produce hydrogen peroxide to inhibit the growth of V. *anguillarum*.

## Conclusions

Based on the research, the highest activity index identified molecularly based on the 16s rRNA gene was obtained in as many as 6 bacterial isolates. The isolates were identified from the group *Vibrio* sp. (2) *Staphylococcus* sp. (1) and *Pseudoalteromonas* sp. (3) with an identity of 97.91% -99.91%. Strain of *Pseudoalteromonas* sp.is a bacterium that has the highest amylotlytic content and generally has antimicrobial properties against pathogens bacteria so it is a candidate for bacteria as a potential probiotic agent.

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