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The bacteria *Bacillus* sp. Potential Probiotics in the Digestive Tract of Silver Pompano *Trachinotus blochii*

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ABSTRAK

Isolation of *Bacillus* sp. the potential for probiotics in the digestive tract of Silver Pompano has never been carried out. Therefore it is necessary to research the isolation and selection of *Bacillus* sp. potential probiotics in the digestive tract of Silver Pompano to increase growth and feed efficiency. Bacteria were isolated from the digestive tract of Silver Pompano to ensure that the isolates obtained were from *Bacillus* sp. then gram staining and spore staining were performed on the isolates obtained. After that, a Proteolytic, Lipolytic, Amylolytic Activity test was carried out, then a Resistance Test to Stomach Acid and Bile Salts, as well as a bacterial growth test for 24 hours. There are six isolates of *Bacillus* sp., which have the potential for probiotics in Silver Pompano's digestive tract, which has the ability of proteolytic, lipolytic, and amylolytic enzyme activity and is resistant to acid and alkaline conditions.

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Bakteri *Bacillus* sp. Berpotensi Probiotik dalam Saluran Pencernaan Ikan Bawal Bintang *Trachinotus blochii*

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ABSTRACT

Isolasi *Bacillus* sp. yang berpotensi probiotik pada saluran pencernaan ikan bawal bintang belum pernah dilakukan. Oleh karena itu perlu dilakukan penelitian isolasi dan seleksi *Bacillus* sp. probiotik potensial dalam saluran pencernaan ikan bawal bintang untuk meningkatkan pertumbuhan dan efisiensi pakan ikan. Bakteri diisolasi dari saluran pencernaan ikan bawal bintang untuk memastikan bahwa isolat yang diperoleh berasal dari *Bacillus* sp. kemudian dilakukan pewarnaan gram dan pewarnaan spora pada isolat yang diperoleh. Setelah itu dilakukan uji aktivitas proteolitik, lipolitik, amilolitik, kemudian uji resistensi asam lambung dan garam empedu, serta uji pertumbuhan bakteri selama 24 jam. Terdapat enam isolat *Bacillus* sp. yang berpotensi sebagai probiotik pada saluran pencernaan ikan bawal bintang, yaitu memiliki kemampuan aktivitas enzim proteolitik, lipolitik, dan amilolitik serta tahan terhadap kondisi asam dan basa.

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INTRODUCTION

Silver pompano is one of the potential aquaculture commodities and has economic value. In silver pompano aquaculture, there are several problems, one of which is feed; high feed prices can cost up to 70% of the total production

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costs. To overcome this problem, it is necessary to take steps to increase feed efficiency in aquaculture. Improving feed efficiency can be done by giving probiotics.

Probiotics are food additives in the form of live microorganism cells that have a beneficial effect on the host animal that consumes them through balancing the microorganism flora in the digestive tract, thus helping the digestive system and supporting probiotic bacteria in the digestive tract, especially in the large intestine. (Irianto, 2007). Probiotic bacteria can be used as growth promoters to increase feed efficiency by improving enzyme activity and feed digestibility (Akhter *et al.*, 2015); Widanarni *et al*., 2015; Suprayudi *et al.* 2016). To increase the success of utilization by the host, it is possible to isolate probiotic bacteria from the same species (Widanarni *et al*., 2015).

One of the probiotic species that is often used in aquaculture is *Bacillus* sp. The bacteria *Bacillus* sp. can produce enzymes capable of metabolizing large amounts of fat, protein, and carbohydrates that can effectively increase the activity of digestive enzymes (Han et al., 2015; Liu et al., 2017). These bacteria are also known to have the ability to produce spores that are resistant to chemical compounds and physical agents (Slepecky and Hemphill 2006).

The bacteria *Bacillus* sp. ND2 increased the daily growth rate significantly in catfish and had a better feed conversion ratio than the control treatment (Sumartini *et al* . 2018). The bacteria *Bacillus subtilis* BS3, *Bacillus amyloliquefaciens* BS4, and *Bacillus cereus* BS6 isolated from the digestive tract of sunu grouper were able to improve the growth performance of sunu grouper (Astuti 2018). Isolation of *Bacillus* sp. potential for probiotics has never been done. Therefore it is necessary to research the isolation and selection of *Bacillus* sp. potential probiotics in the digestive tract of Silver Pompano to increase growth and feed efficiency.

MATERIALS AND METHODS

Isolation and Purification of Probiotic Candidate Bacteria

Bacteria are isolated from the digestive tract of Pompano. The intestine obtained was crushed aseptically, and every 1 gram was dissolved with sterile *phosphate-buffered saline* solution (PBS; NaCl 0.8 g, KCl 0.02 g, Na2HPO4 0.15 g, KH2PO 0.02 g, distilled water 100 mL). Subsequently, serial dilutions of the samples obtained were carried out from a concentration of 10^{-1} to 10^{-3}. The diluted sample was then incubated at 80ºC for 10-15 minutes (Slepecky and Hemphill 2006). The suspension was then inoculated on *complete seawater* (SWC; *bacto peptone* 1.25 g, *yeast extract* 0.25 g, *glycerol* 3 mL, seawater 750 mL, distilled water 250 mL, *bacto agar* 15 g) and incubated for 24 hours at temperature 29ºC. The bacterial isolates were then separated based on the colonies that repeatedly grew to obtain pure bacterial cultures. To ensure that the isolates obtained were from *Bacillus* sp., then gram staining and spore staining were performed on the isolates obtained.

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Gram stain

Bacterial preparations were made by dripping one drop of distilled water on the object glass. The colonies were taken using an ose needle, then smeared into the distilled water on the object-glass. Bacterial smear preparations were homogenized and spread over the slide. The object glass were then fixed on a Bunsen until dry. Fixation is done not directly over the flame but given a distance so that the bacterial cells are not damaged. The staining procedure for bacterial smear preparations was started by dripping 2-3 drops of Gram A solution (crystal violet dye), left for 1 minute, then washed with running water and air-dried. In the second stage, 2 drops of Gram B solution (potassium iodide/KI solution) were added, left for 1 minute, then washed with running water and air-dried. Then 2 drops of Gram C solution (acetone-alcohol solution) were added, left for 30 seconds, then washed with running water and air-dried. Finally, 2 drops of Gram D solution (safranin dye) were added, left for 30 seconds, then passed with running water and air-dried. The stained bacterial preparations were observed using a microscope with a magnification of 1000 times.

Spore Coloring

The bacteria observed were fixed with distilled water, then the bacterial smear was flooded with malachite green and put on a stove for 2-3 minutes. The dye is inhibited from evaporation and boiling. Thereafter, the preparations were cooled, rinsed in distilled water and dried. Safranin was added to the test, waited for 30 seconds, and rinsed off with distilled and dried water. The preparations were observed under microscopy with a magnification of 1000 times.

Selection of Probiotic Bacteria

A series of tests were carried out on the selection of probiotic bacteria in the gastrointestinal tract of Pompano. The bacterial selection stage to obtain probiotic candidates was carried out following the procedure performed by Widanarni *et al*. (2015) and includes the following:

Proteolytic, Lipolytic and Amylolytic Activity Test

This test aims to measure the ability of each isolate to produce protease, lipase and amylase enzymes. Probiotic bacteria candidates were cultured at 29ºC for 24 hours on media with the addition of 2% skim milk for proteolytic assay, 2% starch for amylolytic assay, and 2% olive oil for lipolytic assay. The protease activity was indicated by a clear zone around the isolates grown on agar media enriched with skim milk. Amylase activity was shown by the colour around the hermit becoming bright yellow on media supplemented with carbohydrates after being flooded with 1% KI solution. And the results of lipase activity were indicated by the presence of bright green colour in the isolates grown in media enriched with fat sources after being flooded with saturated CuSO4 solution.

Resistance Test to Acid and Alkaline Conditions

The resistance of gastric acid and alkaline conditions was used to test bacteria's ability to survive in the stomach, which has a low acidity (pH), and the digestive tract, which has an alkaline pH. A total of 1 mL of probiotic candidate

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bacterial suspension was inoculated into a test tube containing 9 mL of sterile SWC broth media with pH 2.5 (the addition of HCl decreased pH) and pH 7.5. (pH was increased by the addition of NaOH). At 29oC, the suspension was incubated. Using the spread plate method, observations were made at 2, 4, 6, and 8 hours after inoculation by counting the number of colonies that grew. The difference in colony growth between treatment and control indicated resistance to gastric acid and bile salts (media with pH 7). The smaller the difference, the more resistant the bacteria obtained are to gastric acid and bile salts.

Bacterial Growth Test

This experiment was carried out to determine the growth curve of bacteria in order to obtain the exponential phase. 1 mL of the bacterial isolate was cultured in 9 mL of liquid SWC media and incubated in a water bath shaker for 24 hours at 29°C. Bacterial growth was monitored every 2 hours by measuring the optical density (OD) with a spectrophotometer at 620 nm. The density value can be used to calculate the bacteria's growth curve, allowing you to determine the best time to harvest bacteria.

RESULTS

Isolation and Purification of Probiotic Candidate Bacteria

Bacteria isolated from the digestive tract of Pompano obtained 15 bacterial colonies. Then the results of observations under a microscope there are 13 rod-shaped bacteria and three cocci-shaped bacteria. The results of gram staining were four gram-negative isolates and 11 gram-positive isolates. In the results of the spore staining, seven isolates could absorb malachite green and eight isolates that could not absorb malachite green. From these results, further for the enzyme test were bacteria that had the form of bacilli, gram-positive, and had spores, there were six isolates, namely isolates A, B, C, I, M, and O. The results of observation of shape, gram test, and spore test presented in Table 1.

Table 1. Observation of Shape, Gram Test, and Spore Test

Isolate	Form	Gram	Spore Test
A	Basil	Positive	Spores
B	Basil	Positive	Spores
C	Basil	Positive	Spores
D	Basil	Positive	Non Spore
E	Cocci	Negative	Non Spore
F	Basil	Positive	Non Spore
G	Basil	Positive	Non Spore
H	Basil	Positive	Non Spore
I	Basil	Positive	Spores
J	Cocci	Positive	Non Spore
K	Cocci	Negative	Non Spore
L	Basil	Negative	Non Spore
M	Basil	Positive	Spores
N	Basil	Negative	Non Spore
O	Basil	Positive	Spores

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Enzyme Test

The purpose of the enzyme test was to measure the ability of each isolate to produce protease, lipase and amylase enzymes. Isolates A, B, C, I, M, and O could produce protease, lipase, and amylase enzymes. Enzyme test results are presented in Table 2.

Table 2. Enzyme Test Results

Isolate	Protease	Lipase	Amylase
A	+	+	+
B	+	+	+
C	+	+	+
I	+	+	+
M	+	+	+
O	+	+	+

Resistance Test to Acid and Alcaline Conditions

The resistance of bacterial isolates to acid changes in the digestive tract is presented in the following figure 1.

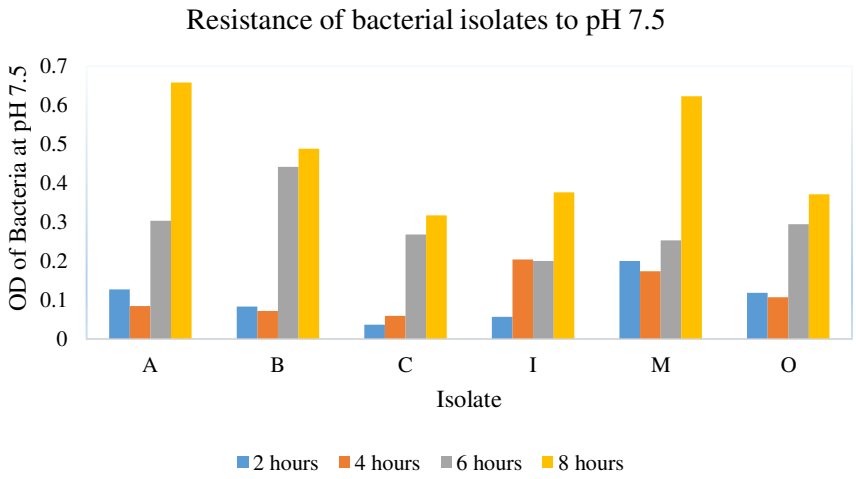

Figure 1. Resistance of bacterial isolates to pH 7.5 at various observation periods

The resistance of bacterial isolates to alcaline changes in the digestive tract is presented in the following figure 2.

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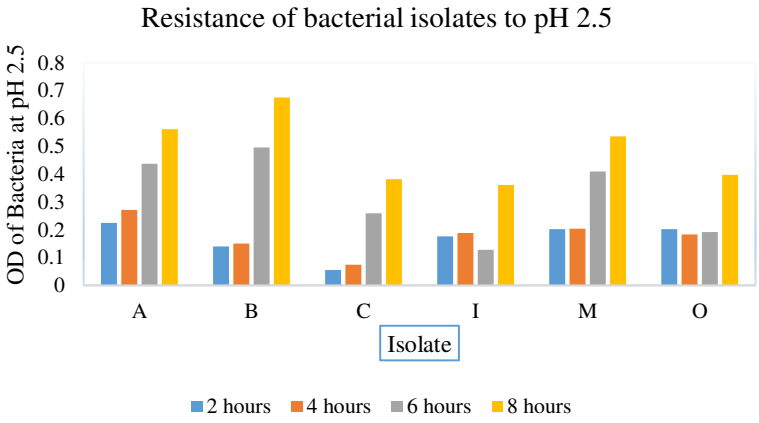
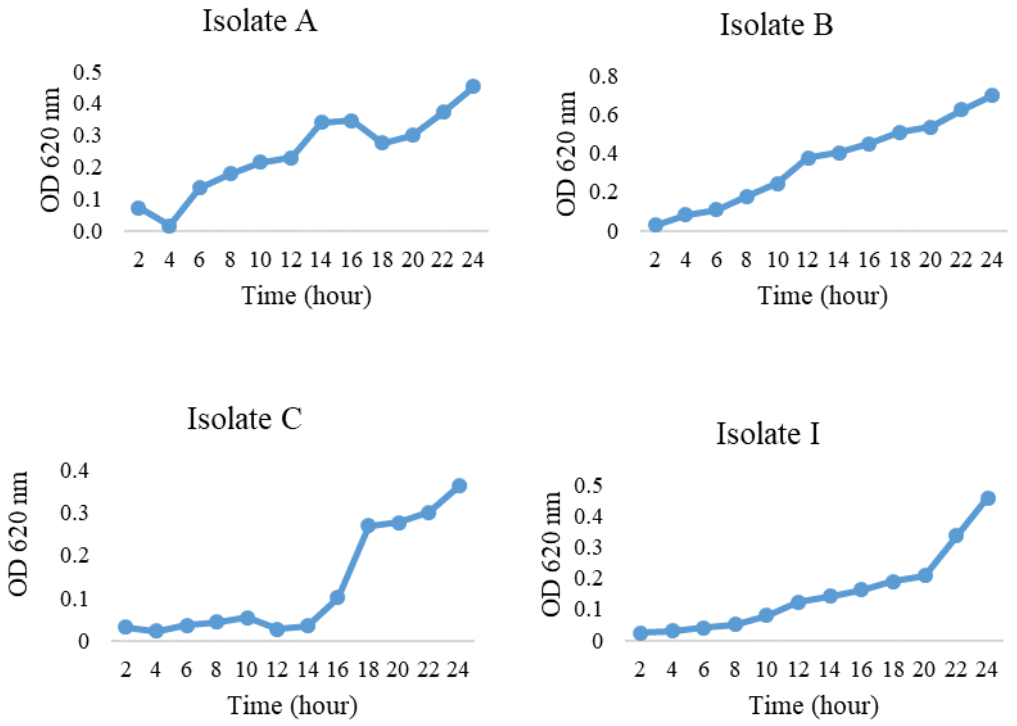


Figure 2. Resistance of bacterial isolates to pH 2.5 at various observation periods

Bacterial Growth

Growth curve of bacteria are shown in Figure 3. The time obtained is used as a reference for harvesting bacteria. The highest bacterial growth in isolates A, B, C, I, M, and O was at 24 hours.



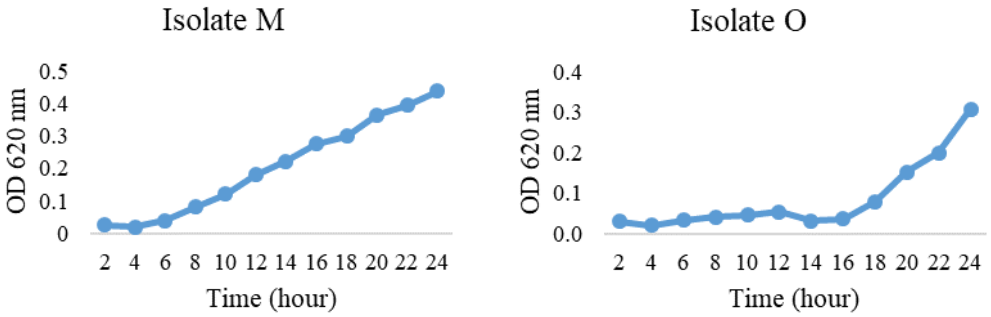


Figure 3. Growth curve of bacteria isolate

DISCUSSION

Isolation of bacteria from the gastrointestinal tract of silver pompano produced 15 bacterial isolates that were able to survive after 15 minutes of heat treatment at 80°C. Heat treatment aims to obtain spore-forming bacteria of the species Bacillus (Slepecky and Hemphill, 2006). From the gram staining results, it is known that 10 of the 15 bacteria obtained from the isolation are gram-positive, rod-shaped bacteria. The results of spore staining on rod-shaped bacteria showed that 6 out of 10 bacteria obtained could produce endospores, which indicated that the bacteria belonged to the *Bacillus* sp. *Bacillus* sp. capable of producing spherical, oval or cylindrical endospores. Endospore formation is very common in this type of bacteria as an environmental survivorship strategy (Toldar, 2009). *Bacillus* produces spores that are more resistant to disinfectants, drying, and heat than their vegetative cells so that they stay alive for a long time (Kuebutornyea *et al.*, 2019).

Further examination of probiotic candidates for these eight bacteria showed that the eight bacterial isolates were capable of producing protease, lipase and amylase enzymes. Extracellular enzymes produced by candidate probiotic bacteria will help enzymes in the digestive tract of the host to hydrolyze nutrients in the feed, and as a result, the process of digestion and absorption in the digestive tract of fish will take place more easily (Afrilasari *et al.*, 2016).

Resistance to acidic and alkaline conditions is an essential criterion for probiotics to survive in the host gastrointestinal tract (Amraii *et al.*, 2014). The probiotic candidate bacteria obtained were able to survive in both acidic and alkaline conditions because the isolates were isolated from the digestive tract of fish, so they had adapted to the very high pH fluctuations. Bacteria that are resistant to acidic and alkaline pH conditions can live in the digestive tract where these bacteria can help the process of food absorption and can increase fish growth. The digestive tract of fish has a harsh environment because it consists of digestive enzymes, pH variations, and bile salts. To select candidate isolates of probiotic bacteria, it is important to pay attention to pH tolerance. In addition, changes in pH ranging from 1 to 7.8 in the digestive tract of fish occurred during pepsin activity, and pH values higher than 7.8 occurred during lipid activity. The potential of bacterial isolates to tolerate low pH is important in selecting

probiotics. The condition of pH 2 was able to affect the survival rate of probiotics, while bile salts were also able to affect the mortality of probiotics (El-Saadony *et al.*, 2021).

Bacteria have 4 growth phases, namely: lag phase, exponential phase, stationary phase and death phase. The lag (adaptive) phase for isolates A and B was at 0-4 hours, isolate C at 0-14 hours, isolate I at 0-8 hours, isolate M at 0-6 hours, while isolate O was at 0-16 hours. In the adaptive phase, there is slow growth influenced by bacterial activity in the process of adjusting to environmental conditions such as pH, temperature, and nutrition conditions (Mardanela, 2016). Cumulatively, the lag phase is an adaptive response to stress and damage, bacteria have a shorter time to adapt, depending on their tolerance to stress (Bertrand, 2019). The exponential phase is a phase of increasing microbial activity that changes shape and also increases the number of cells to the maximum speed so that a curve is obtained in an exponential phase. The exponential phase for isolates A and B was at 4-24 hours, isolate C at 14-24 hours, isolate I at 8-24 hours, isolate M at 6-24 hours, while isolate O was at 16-24 hours. The bacterial growth curve in this study aims to determine the logarithmic phase to determine the harvest time. The number of cells in the logarithmic phase is the largest number of other phases.

CONCLUSION

There are six isolates of *Bacillus* sp., which has the potential for probiotics in the digestive tract of silver pompano, which can produce protease, lipase, and amylase enzymes and survive in acidic and alkaline conditions.

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