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Bioprospecting and Detection of NRPS Gene of Sea Cucumber *Stichopus monotuberculatus* Symbiont-bacteria Against Microbial Fish Pathogens *Aeromonas hydrophila* and *Vibrio harveyi*

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Abstract. Vibriosis and septicemia are infections caused by bacteria that rise many problems on aquaculture industry. Bioprospection of marine organisms such as sea cucumber are very promising as they are widely known to contain symbiotic microorganisms having antibacterial potential. This study aimed to analyze the antibacterial potential of sea cucumber *Stichopus monotuberculatus* symbiont bacteria against *Aeromonas hydrophila* and *Vibrio harveyi*, as well as to detect its NRPS gene. The research methods used were the isolation of symbiotic bacteria in the gut of the sea cucumber *S. monotuberculatus*, screening for its antibacterial activity, identification of 16S rRNA, and detection of NRPS gene clusters. A total of 16 bacteria were isolated, where 12 isolates had the potential to inhibit the pathogen *A. hydrophila* and 7 isolates had the potential to inhibit the growth of the pathogenic *V. harveyi*. Based on the identification of 16S rRNA, the symbiotic bacteria that was able to inhibit the growth of *A. hydrophila* was *Bacillus subtilis*, whereas bacteria that inhibited the pathogen of *V. harveyi* was *Bacillus flexus*. *B. subtilis* and *B. flexus* were detected to have NRPS gene clusters with an amplicon size of about 250 bp.

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1 Introduction

Diseases in aquaculture can be caused by parasites, fungi, viruses, and pathogenic bacteria. The occurrence of disease attacks in a fish farming can result in crop failure or decreased production. The disease that can attack one of the aquatic biota, especially the shrimp group (crustacea) is Acute Hepatopancreatic Necrosis Disease (AHPND) or also known as Early Mortality Syndrome (EMS). AHPND is a disease caused by infection with the bacterium *Vibrio harveyi*. Symptoms that appear in shrimp infected with AHPND are pale and shriveled hepatopancreas, no food in the intestines and stomach, and pale yellowish shrimp body color [1]. Another disease that can be found in aquaculture, especially in freshwater fisheries, is Motile Aeromonas Septicemia (MAS). MAS is a disease caused by the pathogenic bacterium *Aeromonas hydrophila*. MAS disease is characterized by the presence of detached fish scales, bleeding from the gills, flatulence, and can cause damage to the kidneys and liver internally[2].

One of the efforts that can be done is to look for potential symbiont microorganisms that can kill pathogenic bacteria and do not damage the environment [3]. Related research in an effort to discover the potential of new bioactive compounds is called bioprospecting. Bioprospection is an abbreviation of biodiversity prospecting, a series of activities aimed at seeking and discovering the potential for new bioactive compounds through the exploration of biodiversity [4]. One of the biodiversity that has the potential to produce antibacterial and bioactive compounds is sea cucumber [5]. Sea cucumber is one of the organisms from the phylum Echinoderms and the class Holothuroidea. Sea cucumbers can be found in all coastal waters from shallow tidal areas to deeper waters [6].

Sea cucumbers also produce peptide compounds that have antibacterial activity. The main habitats of sea cucumbers are seagrass and coral. Research on the biological properties of marine invertebrates shows that most of the bioactive compounds produced by sea cucumbers [7]. The bioactive compounds produced by sea cucumbers are essential fatty acids, lectins, glycosaminoglycans (GAGs), phenolics, chondroitin sulfate, sulfated polysaccharides, cerberosidespeptides, glycoproteins, glycosphingolipids, terpenoids, glycosides, saponins, and sterols [(3)]. One of the important bioactive compounds in sea cucumber extract that has antibacterial activity is saponins (triterpene glycosides) [8].

The presence of sea cucumber symbiont bacteria provides an opportunity to use sea cucumbers as a source of bioactive compounds including antimicrobial compounds [3]. Several species of pathogenic bacteria that are often found in aquaculture are *A. hydrophylla* and *V. harveyi*. Microorganisms such as pathogenic bacteria can be inhibited by compounds from symbiont bacteria that produce antibacterial. Antibacterial effectiveness testing is a technique to measure the potential or concentration of a compound that can have an effect on microorganisms [9].

Based on the background of the problem and the potential of the sea cucumber symbiont bacteria in overcoming diseases caused by pathogenic bacteria as mentioned above, it encourages research on the potential of the sea cucumber symbiont bacteria *Stichopus monotuberculatus* as antibacterial against microbial fish pathogens along with the detection of the NRPS gene. This study aims to determine the activity of bacterial isolates associated with sea cucumber gut as antibacterial against pathogenic bacteria such as *A. hydrophylla* and *V. harveyi*.

2 Methods

2.1 Isolation of intestinal symbiont bacteria of sea cucumber *Stichopus monotuberculatus*

The method used to obtain pure isolates is the scratch method by zigzagging 1 ose of bacterial colonies on sterile MA media, then incubating for 24 hours at 30 °C. The pure isolates of the sea cucumber *S. monotuberculatus* that have been obtained were characterized for macroscopic and microscopic. Data on the macroscopic characteristics of bacterial colonies can be in the form of colony color, colony elevation, colony texture, colony shape, and colony margins. Data on the microscopic characteristics of the colonies were obtained by gram staining [10].

2.2 Antibacterial assay against *A. hydrophylla* and *V. harveyi*

Antibacterial assay was carried out by the paper disc diffusion method (Kirby-Bauer Assay). Antibacterial test was carried out by cultivating *A. hydrophylla* and *V. harveyi* bacteria on TSA media. A total of 16 bacterial symbionts in the gut of sea cucumbers were incubated on MB media and both pathogenic bacteria were grown on TSB media. The suspension of pathogenic bacteria was compared for turbidity until it was equal to the standard of mc farland 0.5 or 1.5 x 108 cells/ml culture. The suspension of the same pathogenic bacteria as McFarland was wiped using a cotton swab on the MA media, then the paper disc was affixed to the MA media. About 10 ul suspension of bacterial symbionts was dripped onto paper discs, after which they were incubated for 24 hours at 30 °C. Antibacterial activity was indicated by the formation of a clear zone around the paper disc.

2.3 16S rRNA molecular identification

The amplification of the 16S rRNA gene was carried out using the MyTaq HS Red Mix reagent. The steps taken were the Master Mix which used for 50 µL of the PCR reaction made by mixing 19 µL dH2O, 25 µL My Taq HS Red Mix reagent, 2 µL Primer 27F 10ng, and 2 µL Primer 1492R 10ng in a microtube and homogenized slowly. The Master Mix was mixed with 2 µL of DNA template (50ng/µL) in a PCR tube and homogenized. Amplification was carried out for 35 cycles with pre-denatured conditions for 3 minutes at 95°C, denaturation for 45 seconds at 95°C, annealing for 1 minute at 54°C, extension for 1 minute 30 seconds at 72°C, and final extension for 10 minutes at 72°C. The sequencing results were edited with the Bioedit application for consensus. Alignment analysis was carried out on the results of the consensus sequences and a phylogenetic tree was created using MEGA 7 software.

2.4 Detection of NRPS gene in the gut symbiotic bacteria of the sea cucumber *Stichopus monotuberculatus*

NRPS gene amplification using primers A2gamF and A3gamR. The temperature at the pre denaturation stage is 95℃ for 3 minutes. The denaturation step was carried out at a temperature of 95℃ for 1 minute. The annealing step was carried out at 69℃ for 1 minute. The extension stage was carried out at 72℃ for 2 minutes and the final extension stage was carried out at 72℃ for 10 minutes. The amplification process was carried out for 40 cycles. The finished PCR product is then subjected to an electrophoresis process. The presence of

the NRPS gene in the sample was indicated by the length of the amplicon on the agarose gel of 200-300 bp [11].

3 Results

Six teen isolates of symbiotic bacteria of sea cucumbers were selected from the results of the isolation of symbiotic bacteria and then characterized macroscopically and microscopically (Table 1). In the antibacterial screening test, there were twelve isolates that had the potential to inhibit *A. hydrophila* and seven isolates that had the potential to inhibit *V. harveyi*. The isolate with the high potential to inhibit *A. hydrophila* was BSSM-7 and the isolate with the high potential to inhibit *V. harveyi* was BSSM-2 (Figure 1 and Figure 2). The zone of inhibition produced by BSSM isolates that have antibacterial potential against *A. hydrophila* and *V. harveyi* can be seen in Table 2. The 16S rRNA gene electrophoresis results showed that the amplicon length of isolates BSSM 2 and BSSM-7 was 1500 bp (Figure 3). Based on further identification by molecular approach, BSSM-2 was identified as *Bacillus flexus* strain IFO15715 (MN accession number 326676) with 99.59% similarity and the phylogenetic tree of this isolate is shown in Figure 4. BSSM-7 was identified as *Bacillus subtilis* strain SEM 95 (Accession number MW380520) with 98.96% similarity and the phylogenetic tree of this isolate are shown in Figure 5. Detection of NRPS gene from BSSM-2 and BSSM-7 was shown through the results of agarose gel electrophoresis with an amplicon length of about 250 bp (Figure 6).

4 Discussion

Table 1. Characterization of Intestinal Symbiotic Bacteria of Sea Cucumber *S. monotuberculatus*

Isolation Code	Colony Shape	Margin	Texture	Elevation	Pigmentation	Cell Shape	Gram Staining
BSSM-1	Irregular	Irregular	Dry	Flat	Turbid White	Bacili	Negative
BSSM-2	Irregular	Irregular	Moist	Flat	Yellow	Bacili	Positive
BSSM-3	Irregular	Irregular	Moist	Umbonate	Turbid White	Bacili	Positive
BSSM-4	Irregular	Irregular	Mucoid	Umbonate	Turbid White	Bacili	Positive
BSSM-5	Irregular	Irregular	Moist	Umbonate	Yellow	Cocci	Negative
BSSM-6	Irregular	Irregular	Mucoid	Umbonate	Yellow	Cocci	Negative
BSSM-7	Irregular	Irregular	Dry	Flat	Turbid White	Bacili	Positive
BSSM-8	Irregular	Irregular	Dry	Flat	Yellow	Bacili	Positive
BSSM-9	Round	Undulate	Moist	Flat	Yellow	Bacili	Positive
BSSM-10	Round	Irregular	Mucoid	Raised	Turbid White	Bacili	Negative
BSSM-11	Irregular	Irregular	Dry	Flat	Turbid White	Bacili	Negative
BSSM-12	Irregular	Irregular	Dry	Flat	Yellow	Ovoid	Negative
BSSM-13	Round	Undulate	Moist	Raised	Turbid White	Cocci	Positive
BSSM-14	Round	Irregular	Mucoid	Raised	White	Bacili	Negative
BSSM-15	Irregular	Irregular	Dry	Flat	Yellow	Bacili	Negative
BSSM-16	Round	Irregular	Mucoid	Flat	Turbid White	Bacili	Negative

The antibacterial activity of BSSM-2 and BSSM-7 isolates can be strengthened by the detection of the NRPS gene or secondary metabolite encoding. The inhibition zone of the BSSM-2 isolate which has a high potential to inhibit the pathogen *V. harveyi* is 7.30 mm and the inhibition zone of the BSSM-7 isolate which has a high potential to inhibit the pathogen *A. hydrophila* is 7.75 mm. NRPS is a product that belongs to the secondary metabolite group with diverse properties such as antibiotics, pigments, siderophores, and toxins [12]. Microorganisms can produce secondary metabolites in their genes that function as self-

defense and resistance mechanisms. Non Ribosomal Peptide Synthetase (NRPS) is known as a secondary metabolite produced by microorganisms, including antibiotic agents [13].

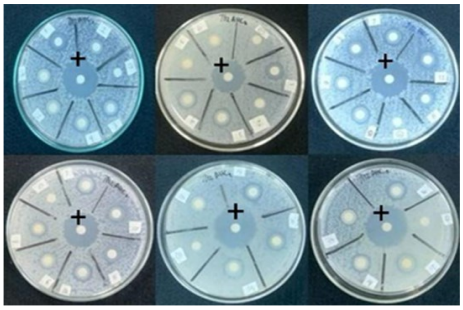

Fig. 1. Antibacterial Test of BSSM Isolates Against *A. hydrophila*

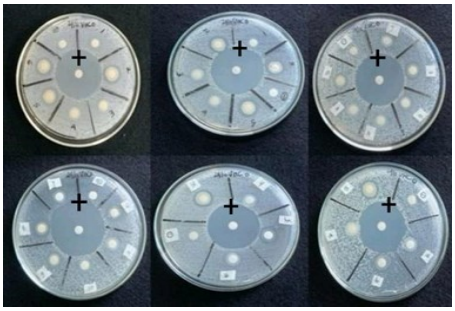

Fig. 2. Antibacterial Test of BSSM Isolates Against *V. harveyi*

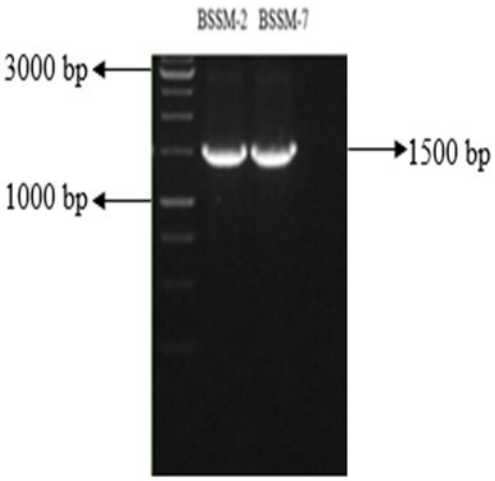

Fig. 3. 16S rRNA Gene Electrophoresis Results from Isolate BSSM-2 and Isolate BSSM-7

The results of molecular and morphological identification of BSSM-2 isolates showed similarities to the characteristics of *B. flexus*. *B. flexus* bacteria are bacteria that are grouped into gram-positive bacteria. *B. flexus* has characteristics that are rod-shaped, have yellow colonies, and flat colony elevations [14]. The results of molecular identification and macroscopic and microscopic morphological identification of the BSSM-7 isolate also showed compatibility with the bacterial species *B. subtilis*. *B. subtilis* is a gram-positive, rod-

shaped bacteria with a diameter of 0.7–0.8 m and a length of 2.0–23.0 m, having colonies with flat elevations that is turbid white [13].

Table 2. Antibacterial Activity of Symbiotic Bacteria of Intestinal Sea Cucumber against *A. hydrophila* and *V. harveyi*

Bacteria	Isolates	Inhibition Zone (mm)
A. hydrophila	BSSM-1	5,25
	BSSM-3	4,05
	BSSM-4	5,55
	BSSM-7	7,75
	BSSM-8	4,30
	BSSM-9	3,20
	BSSM-10	6,50
	BSSM-12	4,85
	BSSM-13	1,90
	BSSM-14	4,20
	BSSM-15	4,15
	BSSM-16	7,10
V.harveyi	BSSM-2	7,30
	BSSM-5	6,25
	BSSM-6	6,40
	BSSM-8	4,05
	BSSM-12	2,50
	BSSM-13	6,35
	BSSM-15	5,30

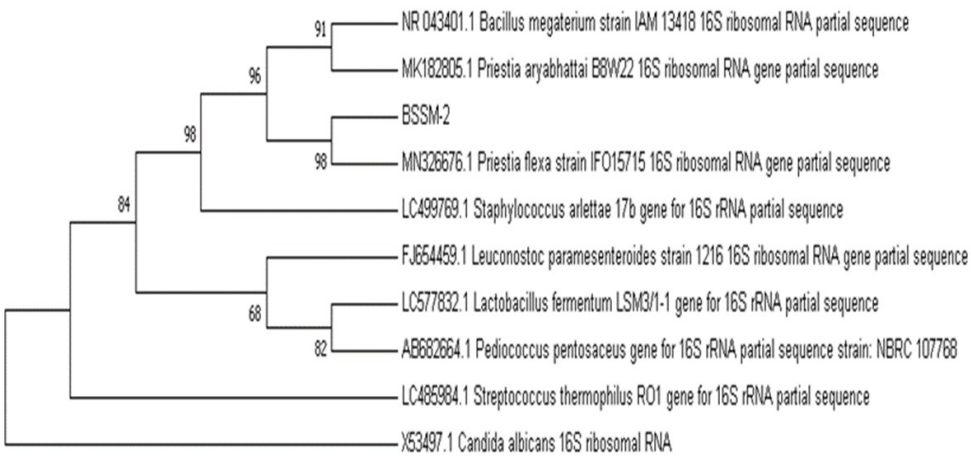

Fig. 4. Phylogenetic Tree of BSSM-2 According to 16S rRNA Gene

One of the most potent antimicrobial peptides secreted by *B. subtilis* is the biosurfactant surfactin, which exerts a strong antipathogenic effect and has diverse biological activities. B. subtilis can increase surfactin expression in a suitable cellulose environment so that it provides benefits to the host body. *B. subtilis* also produces ribosomally synthesized bacteriocins. This bacteriocin is a strong antimicrobial peptide [15]. *B. subtilis* produces a variety of antimicrobial peptides and does not produce toxins. *B. subtilis* in aquaculture has also been explored for water quality (physical, chemical, and biological), waste management

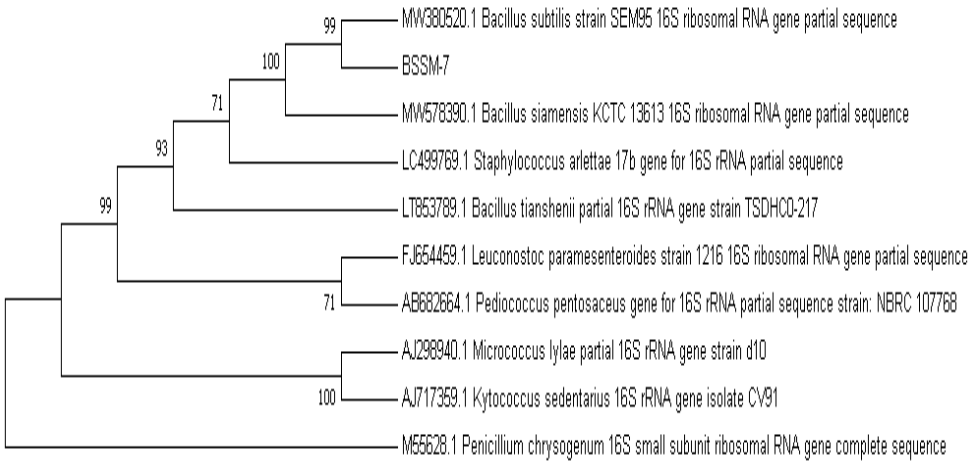

Fig. 5. Phylogenetic Tree of BSSM-7 According to 16S rRNA Gene

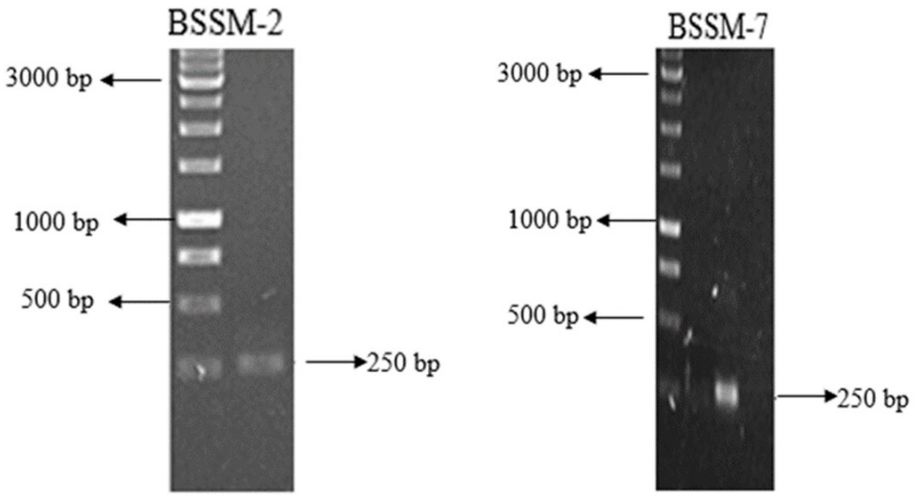

Fig. 6. NRPS Gene Detection of BSSM-2 and BSSM-7

through bioremediation, preparation of fermented food, and food quality improvement. *B. subtilis* can survive in the gastrointestinal tract because *B. subtilis* has the ability to tolerate gastric acidity, bile salts, and has the potential to adhere to intestinal epithelial cells which is considered a condition for any probiotic to provide beneficial health effects [11]. The main antimicrobial substances produced by *B. subtilis* are bacitracin, bacilin, subtilin, ericin A, bacilysocin, difficidin, lipopeptides (bacillomycin, iturin, and fengycin), and oxydifficidin. These compounds are mostly soluble, extracellular, and diffusible antimicrobial proteins or peptides that have low toxicity, high biodegradability, and are environmentally friendly [16]. *B. subtilis* has been successfully used as a probiotic, synbiotic, bioremediation or water management, and a biocontrol agent in aquaculture [15].

B. flexus produces extracellular enzymes, tolerates gastrointestinal stress and bile salts tolerance, has inhibitory activity against pathogens of the Genus Vibrio, and improves water

quality [16]. There are 15 antimicrobial bioactive metabolites that have been identified in *B. flexus*. The metabolites are isopropyl alcohol, octanoic acid, decanoic acid, dodecanoic acid, methyl tetradecanoic acid, 9-hexadecanoic acid, methyl hexadecenoic acid, heptadecanoic acid, 9,12-octadecadienoic acid methyl ester, 8,11,14-docosatrienoic acid methyl ester, octadecanoic acid, eicosanoic acid, 13-docosenoic acid, methyl docosanoic acid, and, tetracosanoic acid [17].

The results obtained from detecting the presence of the NRPS gene cluster were 250 bp. Based on the statement of Sibero*, et al.* [18], the presence of the NRPS gene cluster in the sample can be seen through the length of the amplicon on the agarose gel of 200-300 bp, then the BSSM-2 isolate and BSSM-7 isolate can be said to have an NRPS gene cluster because the size of the amplicon that was successfully electrophoresed was approximately 250 bp.

Conclusions

Sixteen isolates were isolated from the symbiont bacteria from the gut of the sea cucumber *S. monotuberculatus* from the Alang Alang Islands, Karimun, Java. There were twelve isolates that had antibacterial activity against *A. hydrophila* and seven isolate against *V. harveyi*. The best isolate against the pathogen A. hydrophila was BSSM-7 and the best isolate against the pathogen *V. harveyi* was BSSM 2. Based on 16S rRNA molecular identification, BSSM-2 had the highest sequence similarity with Bacillus flexus strain IFO15715 and BSSM-7 had the highest sequence similarity with Bacillus subtilis SEM95. BSSM-2 and BSSM-7 were identified to have NRPS gene cluster.

AB and DC would like to thanks Diponegoro University for funding this project under RPI grant number: 753-21/UN7.D2/PP/IX/2022. DW would like to thank Diponegoro University for the Postdoc Grant SK Rektor: 216/UN7.A/HK/XI/2023.

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