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Antibacterial Activity of Freshwater Sponge *Oncosclera asiatica* Against *Escherichia coli* and *Staphylococcus aureus*

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Abstract

Freshwater sponges are animals from the *Porifera phylum* that live in freshwater. The sponge used is *Oncosclera asiatica* was taken from Kali Porong, East Java. Seventeen isolates of bacteria have been obtained from isolation. Antibacterial potential testing was performed by *paper disc inhibition* assay using *Escherichia coli and Staphylococcus aureus* as pathogenic bacteria and amoxicillin as a positive control. The antibacterial activity test showed that four isolates have the potential activity. The isolates with the highest inhibition zones were identified using a 16S rRNA.The results of BLAST showed isolate number 2 was *Pseudomonas moraviensis* with 99.51% similarity. The phylogenetic tree analysis was build using the MEGA X program. The results of the phylogenetic tree analysis showed that *P.moraviensis* had a bootstrap value of 100% with a genetic distance value of 0.001. *P. moraviensis* isolates screened for the presence of Non-Ribosomal Peptide Synthetase (NRPS) gene by *A2gamF* and *A3gamR* primers. The amplification result from NRPS gene showed positive meaning that *P.moraviensis* genome contained NRPS gene.

Keywords: *Oncosclera asiatica, antibacterial activity, Pseudomonas moraviensis, NRPS gene, bacterial symbiont*

INTRODUCTION

Indonesia is a country with abundant water resources. One of them is freshwater resources. Fresh water includes rivers, lakes, and estuaries. In freshwater waters, there are also several types of sponges. These sponges are known as freshwater sponges. Sponges are the most primitive multicellular animals that live in various types of waters ranging from fresh, brackish and marine. They live at the bottom of the water and usually attach themselves to hard substrates such as rock or coral and compete with other adhering organisms for space and food (Cheng et al., 2008). Apart from being a food source, symbiotic microorganisms from sponges use the porous body of the sponge as a host for shelter and protection. This can be seen from the association between sponges and bacteria. Bacteria can contribute to host defense by excreting antibiotics and other bioactive substances (Proksch *et al.* 2002; Rajendran, 2016). This creates an opportunity for bioprospecting sponge symbiont bacteria, where the benefits of these symbiotic bacteria need to be explored in order to obtain new biological products. The term antibiotic comes from the word 'antibiosis' which literally means 'against life'. In the past, antibiotics were considered as organic compounds produced by one microorganism that were toxic to other microorganisms (Rajendran, 2016). As a result of this idea, antibiotics were originally defined broadly as substances, produced by a single microorganism or biological origin. Some antibiotics are able to kill other bacteria completely, some antibiotics only able to inhibit their growth. Those that kill bacteria are called bactericidal while those that inhibit the growth of bacteria are called bacteriostatic. Although antibiotics generally refer to antibacterials, antibiotic compounds are distinguished as antibacterial, antifungal, and antiviral to reflect the group of inhibited microorganisms (Etebu, 2016). In this research, the sponge used was *Oncosclera asiatica* (unpublished taxonomic data) from Institute Technology Surabaya. Since the symbiont sponges from the fresh water still underexplore when it compared with the marine sponges. Thus,

This study aims to determine the diversity and antibacterial activity of symbiotic bacteria from the sponge *Oncosclera asiatica* as a source of new antibiotics.

MATERIALS AND METHODS

FRESH WATER SPONGE SYMBION BACTERIA ISOLATION

The sponges samples were isolated from Porong River located about 40 km south of Surabaya the province of East Java, Indonesia. The river is one of the canals from the main streams of Brantas River in Mojokerto City, which flows eastwards toward Madura Strait. The sponge sample washed with sterile distilled water and then vortexed and weighed one gram and then grounded using a mortar. After it was fine, put the sample into a falcon bottle and then dissolved in nine ml of sterile distilled water. Dilutions of 10^{-1} to 10^{-6} were carried out by taking one ml from the first dilution and then putting it into a tube containing 9 ml of diluent (sterile distilled water). dilution was carried out up to 6 times. The bacteria present in the sponge samples were inoculated on NA solid media by the pour plate method. The isolateswere taken as much as 1 ml to be inoculated on NA solid media in a petri dish aseptically then incubated at 37ºC in an incubator for 24 hours (Marzuki et al 2014).

ANTIBACTERIAL ACTIVITY TEST OF FRESHWATER SPONGE SYMBIONT BACTERIA

The pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus* were cultivated into 5 ml of NB liquid media and incubated 18 hours at room temperature with agitation 200 rpm until OD1. As much as 1ml of both pathogen with the dilution factor 10^{-1}-10^{-4} put into 9ml sterile distilled water. As much as 1 ml *E.coli* and *S. aureus* put into a petri dish containing NA media. Furthermore, the pathogenic bacteria that have been poured into a petri dish, flattened using a spreader and then allowed to dry. The next step is to put a paper disc sterile into a petri dish. Each paper disc was dripped with 20 µl of liquid culture of bacterial isolate and positive control (amoxicillin). Then incubated for 48 hours. After incubation, the isolates were observed which showed a clear zone.

DNA ISOLATION

Genomic DNA isolation of the sponge symbiont bacteria *O. cf asiatica* was performed by centrifugation of 1 ml of the culture overnight for 2 minutes at 13000-16000 rpm. The supernatant was discarded. For gram-positive bacteria, there is a specific treatment by adding lysozyme to the pellet, then incubated at 37℃ for 30-60 minutes, followed by centrifugation for 2 minutes at 13000-16000 rpm. In the stage of cells lysis, it was done by pouring 600µl of Nuclei Lysis Solution and slowly homogenized with a micropipette. After being homogeneous, it was incubated for 5 minutes at 80℃ and then cooled at room temperature. RNAse Solution mix was added as much as 3µl, and incubated for 15-60 minutes at 37℃. In the protein precipitation stage, 200µl of protein precipitation solution was added, homogenized with a vortex, and incubated in the freezer for 5 minutes. Then followed by centrifugation at 13000-16000 rpm for 3 min. Furthermore, it enters the stage of precipitation and DNA rehydration. At this stage, the supernatant was transferred to a sterile microtube which already contained 600 µl of isopropanol. Then it was centrifuged and the supernatant was discarded. As much as 600 µl of 70% ethanol was added and centrifuged at 13000-16000 rpm for 2 min. The ethanol was discarded and the wet microtubes were dried for 10-15 minutes at room temperature. The DNA pellet was then rehydrated by adding the Rehydration solution and incubated at 4ºC overnight (Promega 2020).

BACTERIAL DNA AMPLIFICATION

After checking the DNA purity using nanodrops, amplification plasmid DNA of freshwater sponge bacteria was carried out by the PCR process using a thermocycler with universal primers 27F (forward) and 1492R (reverse). The PCR process performed out in a 50 µl reaction, consisting of 12.5 l MyTaq PCR Kit (Thermo Scientific 76 l ddH2O,), 8 l of forward and reverse primers, and with 2 µl of DNA template. Initial denaturation was carried out at 95ºC for 3 minutes, followed by 35 cycles consisting of denaturation at 95ºC for 45 seconds, annealing at 54ºC for 1 minute. The reaction was terminated with final elongation at 72ºC for 10 minutes. PCR analysis was performed by electrophoresis technique using 1% agarose gel. The results of the electrophoresis were seen using an electrophoresis doc gel.

16S rRNA GENE SEQUENCING

The DNA sequencing process was performed by sending samples of 50 µl bacterial DNA with concentration 100 ng/µl to *Genetica Science*. Then the results of the DNA sequencing

were analyzed using *BLAST* on the website *(http://www.ncbi.nlm.nig.gov)* to match the species data in the *gene bank* to find the bacterial species.

NONRIBOSOMAL PEPTIDE SYNTHETASE

DNA amplification by PCR process using a thermocycler with primers A3F(GCSTACSYSATSTACACSTCSGG) - A7R (SASGTCVCCSGTSCGGTAS). The PCR process was performed in a 50 µl reaction, consisting of 12.5 l MyTaq PCR Kit (Thermo Scientific 76 l ddH2O,), 8 µl of forward and reverse primers, and with 2 µll of DNA template. Initial denaturation was carried out at 95ºC for 5 minutes, followed by 35 cycles consisting of denaturation at 95ºC for 30 seconds, annealing at 51ºC for 1 minute. The reaction was terminated with final elongation at 72ºC for 10 minutes. PCR analysis was performed by electrophoresis using 1% agarose gel and 100 bp DNA marker Electrophoresis results can be seen with gel electrophoresis doc.

PHYLOGENETIC TREE ANALYSIS

Nucleotide sequences alignment performed by ClustalW software. Phylogenetic trees analysis were constructed by the neighbour joining method in Molecular Evolutionary Genetics Analysis (MEGAX) software. Bootstrap values after maximum-likelihood analysis are indicated in the nodes and branches.

RESULT AND DISCUSSION

FRESH WATER SPONGE SYMBION BACTERIA ISOLATION

The Freshwater sponge *O. asiatica* obtained from Porong River, East Java was isolated for the bacterial symbiont. Frommacroscopic and microscopic identification, it showed the diversity of colony morphology The observation of macroscopic identification including: colony shape, margin, elevation, texture, color, gram properties, and cell shape. Microscopic identification includes cell morphology and gram properties.

Tabel 1. Macroscopic and Microscopic Identification of Fresh water sponges symbiont

No	Macroscopic morphological	Microscopic description
1	Shape : irregular Margin: Irregular Elevation : Flat Texture: Dry Color : white	Ovoid Gram negative
2	Shape : Rhizoid Margin: Rhizoid Elevation : Flat Texture: Dry Color : white	Streptobasil Gram negative
3	Shape : Round Margin: Smooth, entire Elevation : Convex Texture: Mucoid Color : white	Streptobasil Gram positive
4	Shape : Round Margin: Smooth, entire Elevation : Umbonate Texture: Mucoid Color : white	Basil Gram negative
5	Shape : Round Margin: Smooth, entire Elevation : Convex Texture: Moist Color : white	Basil Gram negative
6	Shape : Round Margin: Smooth, entire Elevation : Flat, raised margin Texture: Moist Color : white	Basil Gram positive
7	Shape : Round Margin: Smooth, entire Elevation : Raised, spreading edge Texture: Moist Color : white	Ovoid Gram negative
8	Shape : Round Margin: Smooth, entire Elevation : Raised, spreading edge Texture: Moist Color : white	Basil Gram negative
9	Shape : Round Margin: Smooth, entire Elevation : Raised, spreading edge Texture: Moist Color: white	Basil Gram negative
10	Shape : Round Margin: Smooth, entire Elevation : Raised, spreading edge	Basil Gram negative

	Texture: Moist Color : white	
11	Shape : Round Margin: Smooth, entire Elevation : Convex Texture: Moist Color : white	Coccus Gram positive
12	Shape : Round Margin: Smooth, entire Elevation : Convex Texture: Moist Color : white	Basil Gram negative
13	Shape : Round Margin: Smooth, entire Elevation : Convex Texture : Moist Color : white	Streptococcus Gram negative
14	Shape : Filamentous Margin: Filamentous Elevation : Flat, raised margin Texture : Dry Color : white	Basil Gram negative
15	Shape : Round Margin: Smooth, entire Elevation : Convex Texture: Mucoid Color : white	Basil Gram positive
16	Shape : Round Margin: Smooth, entire Elevation : Flat Texture: Mucoid Color : white	Basil Gram positive
17	Shape : Round Margin: Smooth, entire Elevation : Convex Textur: Moist Color : white	Basil Gram negative

Based on the results of the isolation of freshwater sponge symbiont bacteria, 17 (seventeen) different colonies were obtained. The identification of isolates were done by macroscopic and microscopic observation.

Based on the results of macroscopic observations, it was found that all colonies of symbiotic bacteria of the freshwater sponge *O. asiatica* were white with colony shapes generally *round, filamentous, irregular*, and *rhizoid*. Margins are *irregular, rhizoid, filamentous,* and *smooth entire*. The elevation is *flat, convex, umbonate, flat raised margin, raised, spreading edge*. Freshwater sponge symbiont bacteria *Oncosclera asiatica* has *a dry, mucoid,texture* and *moist* (Table 1) According to Bai (2010), In conventional microbiology, scientists have developed ways to identify and classify different bacterial species based on colony morphology which are generally described in terms of qualitative measures of height, shape, edge, surface, and pigmentation.

Based on microscopic observations in table 1, it was found that the symbiotic bacteria of the freshwater sponge *O. asiatica* in the twelve isolates had gram-negative properties, while five isolates were gram-positive bacteria where the cell wall structure of gram-positive bacteria was more complex than that of gram-negative bacteria. Freshwater sponge symbiont bacteria *O. asiatica* havecell forms *ovoid, bacilli, coccus, streptobacilli, and streptococcus.* According to Amaleena (2014) Christian H. Gram in 1884 found the Gram stain classification remains an important and useful technique today. This technique classifies bacteria as Gram positive or negative based on their morphology and differential staining properties. Gram-negative bacteria will be red in color, this is because the cell walls of gram-negative bacteria are thinner so they cannot withstand crystal violet complexes when given an alcohol solution. So that gram-negative bacteria will take on a red color when given safranin.

ANTIBACTERIAL ACTIVITY TEST OF FRESHWATER SPONGE SYMBIONT BACTERIA

The antibacterial activity test of the freshwater sponge symbiont *O. asiatica* was doneagainst the pathogenic bacteria *E. coli and S. aureus*. The result showed in Table 2.

Table 2. Bacterial Activity Test Freshwater sponge symbiont

Isolate	*E. coli* (mm)	*S. aureus* (mm)
2	1.0	1.3
4	(negative)	0.8
14	0.6	2.9

Based on the observations, only isolate 2, isolate 4, and isolate 14 appeared to have a clear zone. In the pathogen *E.coli*, isolate 2 had a clear zone width of 1.0 cm, isolate 14 had a clear zone width of 0.6 cm. In the pathogen *S aureus*, isolate 2 had a clear zone width of 1.3 cm, isolate 4 had a clear zone width of 0.8 cm, isolate 14 had a clear zone width of 2.9 cm. The clear zone/inhibition zone indicated that the isolate of the freshwater

sponge symbiont *O. asiatica* released secondary metabolites. However , it could also indicate competition for food , so that the isolates of the freshwater sponge symbiont *O.asiatica* inhibited the growth of *E. coli* bacteria and *S. aureus*. However, the inhibition zone was not as large as the positive control. This is in accordance with the opinion of Maida (2019). The zone of inhibition is indicated by the presence of a *clear zone* and is used as a determinant of the level of bacterial resistance to antibiotics. The larger the diameter of the clear zone formed, the more inhibited the growth of bacteria.

However, in a follow-up test using the supernatant, the result of the test was negative (no inhibition zone appeared). This is because the sample bacteria contained intracellular secondary metabolites. According to (Pelczar and Chan, 2008), Antibacterial is a compound that is used to inhibit the growth of bacteria. Antibacterial is present in bacteria in the form of secondary metabolites (extracellular and intracellular). The mechanism of inhibition of bacterial growth by antibacterial compounds is generally performed by damaging protein synthesis, changing membrane permeability, disrupting cell walls and inhibiting enzyme work (Pelczar and Chan, 2008). Compounds that play a role in damaging cell walls include phenols, flavonoids, and alkaloids. These antibacterial compounds have the potential to inhibit the growth of pathogenic bacteria *S. aureus* and *E. coli*. Based on the results, isolate number 2 had the largest inhibition zone, so isolate number 2 would be used for phylogenetic analysis and detection of NRPS genes.

MOLECULAR IDENTIFICATION OF SPONGE SYMBIONT BACTERIA *Oncosclera cf asiatica*

Molecular identification of the tested bacteria was initiated by sequencing the 16rRNA gene by isolating the genomic DNA of isolate number 2 of the sponge symbiont *O.asiatica*. Isolate number 2 was chosen because it has the best inhibition zone compared to isolate 4 and isolate 14. DNA isolation begins with cell lysis, then DNA extraction, then checks the level of DNA purity (purification). according to ahmed et al (2014), the isolation of bacterial genomic DNA generally includes three stages, namely cell wall release, genomic DNA extraction, and DNA purification using nanodrops. The result of the concentration of genomic DNA that was successfully extracted in isolate 2 was 325.6ng/µl as shown in table 3.

Table 3. nanodrop results of the sponge symbiont *O.asiatica*

Sample ID	NucleicAcid Concentration	Unit	A260	A280	260/280
SS-2	325.6	ng/µl	6.512	3.104	2.10

Based on the nanodrop results in Table 3, the DNA sample has a purity value 2.10 where that value already has pure DNA. This is in accordance with Ghatak (2013), the absorbance quotient (OD260/OD280) provides an estimate of DNA purity. The quotient value of the absorbance ratio of 2.0 was considered good (pure DNA). A ratio below 1.8 indicates protein contamination, while a ratio above 2.1 indicates RNA contamination.

The results in the DNA isolation stage have good purity values. Then proceed to amplify DNA using the PCR (*Polymerase Chain Reaction*) process and electrophoresis to see the success of the PCR process. The PCR process goes through 3 stages, denaturation, *annealing*, and elongation. This is in accordance with the opinion of Powledge (2004) that there are three basic steps in PCR. In the first step, the genetic material must be denatured, that is, the helical strands must be removed and separated by heating to 90-96°C. The second step is hybridization, in which the primer binds to its complementary base on a single strand of DNA. The third is DNA synthesis by polymerase. Starting from the primers, polymerases can read template DNA strands and match them to complementary nucleotides very quickly. The result is that two new helices replace the first helix. Then after amplification, the DNA is electrophoresed to determine whether the DNA has a *smear* or not.

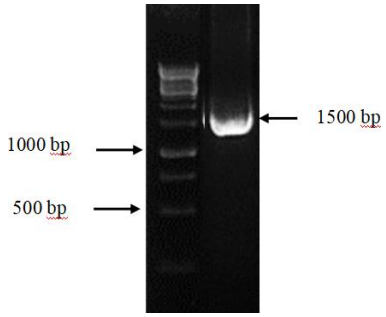

Figure 1. Results of 16S rRNA Gene electrophoresis

Based on the results in Figure 1. It can be seen that the results of DNA isolation have good bands. This is indicated by intact DNA bands, no tails and no smears so that they are clearly visible. This is appropriate with Syafaruddin (2011), PCR amplification results show that the amplified DNA produces good DNA bands where the DNA bands are very clear, thick and bright. The result of base calculation after comparison with DNA ladder is 1000-1500bp. After electrophoresis, the DNA samples were sequenced and then *BLAST*. The results are *BLAST* presented in Table 4.

Table 4. BLAST results of the sponge symbiont *O.*.

Description	Max Score	Total Score	Query Cover	E-Value	Ident	Accession
*P*seudomonas moraviensis strain 3N 16S ribosomal RNA gene, partial sequence	2591	2591	99%	0.0	99.51 %	KJ528878.1

The results of *BLAST* analysis in Table 4 showed that the isolate code number 2 of the sponge symbiont *O.*is *Pseudomonas moraviensis* with a similarity of 99.51%. and *E-value* 0.0. the smaller the value of *E.value*, the better the alignment. This is in accordance with Claverie and Notredame (2001). Based on the results obtained, the description of each species has 99% identity. The limit value is said to have similar structure and folds based on sequence similarity if the identity value is at least 25% and has an E-value of 0.0. A score above 50 indicates aresult *BLASTn* reliable.

In the molecular analysis, a phylogenetic tree is also included to make it clearer in seeing the relationship between species. According to Patwardhan (2014) Phylogeny is an abstract phenomenon and cannot be observed directly. It is something that happened in the past and must be reconstructed using the available evidence. By studying a phylogenetic tree, it is possible to obtain a quick overall picture of a given species and its relationship to other phylogenetically close species. The results of molecular phylogenetic analysis can be represented in a diagram in the form of a phylogenetic tree.

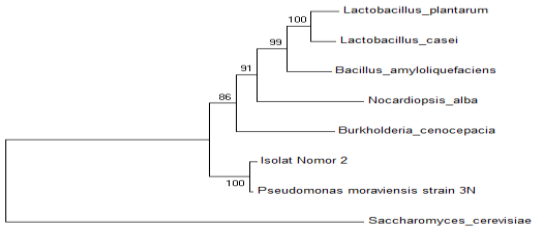

Figure 2. Neighbour-joining phylogenetic trees obtained from nucleotide sequences from *Oncosclera c.f. asiatica* symbiont bacteria showing the relationships between strains number 2 with a similarity of 99.51% with *Pseudomonas moraviensis*. The percentage at nodes refers to significant bootstrap values of 1000 replicates. The evolutionary distances were computed using the maximum composite likelihood method in MEGAX.

Based on the results of the phylogenetic tree analysis in Figure 2, the bootstrap value of the isolate code number 2 of the sponge symbiont *O. asiatica* has a value of 98%. This is in accordance with Hillis and Bull (1993) that the results of the phylogenetic tree analysis with bootstrap values above the scale (>70%) are closely related, the results from bootstrap values below the scale (<30%) are incorrect results. Based on the results and statements above, that the reconstruction of the phylogenetic tree is correct and reliable. Based on the results in Figure 2. it can be seen that *Saccharomyces cerevisiae* has the most distant kinship relationship, so it can be concluded that the selection of *outgroup* and *ingroup* is correct.

NonRibosomal Peptide Synthetase

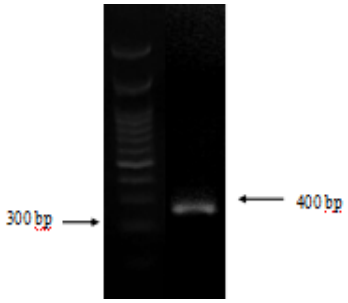

Figure 3. *NonRibosomal Peptide Synthetase*

Based on the results in Figure 2, isolate number 2 of the sponge symbiont bacteriua *O.asiatica* contained the nrps gene because it has a PCR product of 300-400bp. This is in accordance with Sulistyani (2013), NRPS is a gene that encodes secondary metabolite compounds which are peptide derivatives. However, not all microorganisms have the NRPS gene. The NRPS PCR results are said to be positive by showing that based on the electrophoresis results, all NRPS gene PCR products have a size of 300-400 bp.

CONCLUSION

There were 17 isolates of symbiont bacteria from freshwater sponge *O.asiatica* The symbiont of the sponge *Oncosclera c.f asiatica* that had antibacterial potential against *E. coli* and *S. aureus* were 3 isolates, namely isolate 2, isolate 4, and isolate 14, with isolate 2 having the best antibacterial activity. Isolate 2 which was identified molecularly showed the highest sequence relationship with the species *Pseudomonas moraviensis strain 3N.* Isolate 2 had the NRPS gene that encodes antibacterial compounds.

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