

Potential Bacterial Symbion of Sea Urchin As a Multi-Drug Resistant (MDR) Antibacterial Agent Against *Staphylococcus aureus* and *Escherichia Coli* Bacteria

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Abstract

Staphylococcus aureus and *Escherichia coli* are pathogenic bacteria agent of many human diseases. Those bacteria infect in various levels and also been antibiotic resistants. Bacterial resistance has become a serious global problem. The purposes of this study were to isolate and identify the symbiotic bacteria of the Sea Urchin that have an antibacterial activity of the Strain Multi-Drug Resistant (MDR) against *Staphylococcus aureus* and *Escherichia coli*. Sea Urchin was taken from Panjang island, Jepara Indonesia at 2-3 m depth. The symbiotic bacteria were isolated from Sea Urchin by using dilution method and spread plate method. Phenotypic characteristics was observed on colony shape, color and texture of growing bacteria. While the streak method was used to purify bacterial symbion. The antibacterial activity test was performed using overlay method. The results showed that 3 out of 37 isolates have antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The BB.03.35 isolate was selected for molecular identification due to the largest inhibitory zone diameter. The sequence of this bacterium showed 97% homology and closely related to *Pseudoalteromonas flavipulchra*.

Key words: *Staphylococcus aureus*; *Escherichia coli*; Sea urchin; antibacterial

Introduction

Secondary metabolites are compounds that are synthesized by organisms to maintain their life from any competition with other organisms. Secondary metabolites include the substances with sophisticated and variable chemical structures, synthesized by certain variety of strains of microbial species (Sharma *et al.*, 2014). Marine organisms have evolved biochemical and physiological mechanisms that include the production of bioactive compounds for such purposes as protection, communication, and reproduction against predation, infection and competition (Kamalakkannan, 2015). Class of the animals that has the potential of secondary metabolites are Echinoderm (Aprilia *et al.*, 2012).

Sea Urchin (Echinoderm) is one of fishery products with a high economic value. This animal is excessively caught by coastal community. They even collect the eggs for food (Vimono, 2007). Spines owned by sea urchins especially in strain species *Diadema setosum* are used to move and protect itself from the attack of preys. These spines

contain poison (Abubakar *et al.*, 2012) stated that the toxin produced by sea urchins, can be utilized medicine, which has the potential to be used as an antibiotic candidate because it contains bioactive compounds. The extract of sea urchin ovary showed antimicrobial activity (Marimuthu *et al.*, 2015).

Antibiotics are the most important tools in medicine, but their efficacy is threatened by the evolution of resistance (Bhandari *et al.*, 2016). Antibiotic holds a very important role in the drug ingredients, especially in overcoming the infectious diseases in Indonesia. Antibiotics are used on diseases caused by bacteria. If a bacterium carry several resistance genes, it is called multidrug resistant (MDR) or, informally, a superbug or super bacterium (Adekunle, 2012). Continuous use of antibiotics without noticing the dosage, will accelerate the process of antibiotic resistance. Most infectious bacteria may become resistant towards some antibiotics (Walewangko *et al.*, 2015).

Infectious diseases which infect and attack humans to date are caused by pathogenic bacteria of *Staphylococcus aureus* and *Escherichia coli*.

Staphylococcus aureus bacteria can cause serious infectious diseases such as septicemia, pneumonia, endocarditis, osteomyelitis, gastroenteritis and abscesses, where its infection levels has increased over the past decade (Setiawati, 2015). *Escherichia coli* is considered to be a faecal contamination indicator in foods due to its presence in the gut, *Staphylococcus aureus* cause disease through direct invasion by toxin production and followed by tissue destruction (Onwubiko and Sadiq, 2011; Gundogan and Avci, 2014). Based on that problems, therefore, the purpose of this research is to isolate and identify the symbiotic bacteria of Sea Urchins which have the antibacterial activity of Multi Drug Resistant (MDR) strain towards the bacteria of *Staphylococcus aureus* and *Escherichia coli*.

Material and Methods

Sea Urchins samples were collected from Pulau Panjang, Jepara, April 2017. Other materials used for the analysis are the bacteria of *Staphylococcus aureus* and *Escherichia coli* are collection culture from Tropical Marine Biotechnology Laboratory, Aquadest, Zobell 2216E Media, Nutrient Agar (NA) and Nutrien Broth (NB). The tools used in the research were autoclave, incubator, laminary airflow and UV light, homogenizer, bunsen and Analytical Scales. This research was conducted at Tropical Marine Biotechnolony Laboratory, Integrated Laboratory, Diponegoro University.

Isolation of symbiotic bacteria on sea urchins

The isolation of the Sea Urchin's symbiotic bacteria was carried out using the dilution method and spread plate method. The samples of Sea Urchins were destroyed by the use of Scalpel, where its gonad and the contents of the stomach were taken out, then insert the ± 5 grams into 5 mL of sterile sea water. The sample of 100 μ L was collected from the dilution series of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and were spared into sterile petri dish which contains Zobell 2216E media which was subsequently incubated at 26°C for 2 days in Incubator. Purification of bacterial colonies was separated by inoculate loop, based on the different colors, textures and shapes of colonies on Zobell 2216E media in petri dishes (Madigan *et al.*, 2000). After the pure isolates were obtained, then it should be stored on a media to make it tilt.

Antibacterial activity

The antibacterial activity test was performed by using overlay method, and the bacterial isolates were checked for purity and maintained on nutrient

agar (Nazim *et al.*, 2014). Every one ose of Sea Urchin symbiotic bacteria are planted on Zobell 2216E marine media and incubated at room temperature for 2 days. The concentration of *S.aureus* and *E. coli* cultur was adjusted to 0,5 McFarland standards. 1mL of the test bacteria are taken and inserted into 100 mL soft agar. Soft agar that has contained test bacteria are poured into a medium that has been overgrown with the isolates of Sea Urchin symbiotic bacteria, then incubated at room temperature for 1x24 hours. The active isolates of Sea Urchin symbiotic bacteria are seen with the formation of clear zones around colony.

DNA extraction and PCR amplification

DNA extraction in bacteria begins by planting microbes in new media then it was incubated for 1x24 hours. When the microbes have grown, took it sufficiently and add 50-100 μ l aquabides and 1 ml of 0.5% saponin in PBS and then incubated overnight at 4 °C. In the next day, Used Chelex 100 for Extraction DNA from bacteria (Walsh *et al.*, 1991). DNA extract for 16S rRNA gene were amplified by PCR using Universal Primer 27 Forward (5'AGAGTTTGATCMTGGCTCAG-3') and 1492 Reverse (5'TACGGTTAACCTTGTACGACTT-3'). The PCR Mixture consisted of GoTaq® (25 μ L), Primer 27F (2 μ L), Primer 1492R (2 μ L), DNA template (2,5 μ L), and ddH₂O (18,5 μ L) (Sabdono, 2007). The PCR Program (Biorad T100) used were: denaturation at 45°C for 1 minute, anneling at 53,9°C for 1 minute, extention at 72°C for 30 second and post cycling at 72°C for 7 minute. All these stages was repeated 30 cycles.

The PCR product was analyzed by Agarose 1% gel electrophoresis and the result showed by UVI-Doc (UVITEC Cambridge). DNA sequencing was conducted at 1st Base, Singapura by Genetika Science Corporation. The sequence was inserted to BLAST search program to identify the sequences of any closely related organisms.

Results and Discussion

The symbiotic bacteria were isolated from Sea Urchin are isolated on Zobell 2216E marine media. Phenotypic characteristics was observed on colony shape, color and texture of growing bacteria. The results of morphological identification are 37 isolates of symbiotic bacteria. The data of antibacterial activity of Sea Urchin symbiotic bacteria and bacterial isolates of sea urchin symbionts was presented in Table 1 and Figure 1.

The antibacterial activity test of *Staphylococcus aureus* and *Escherichia coli* was performed using overlay method.

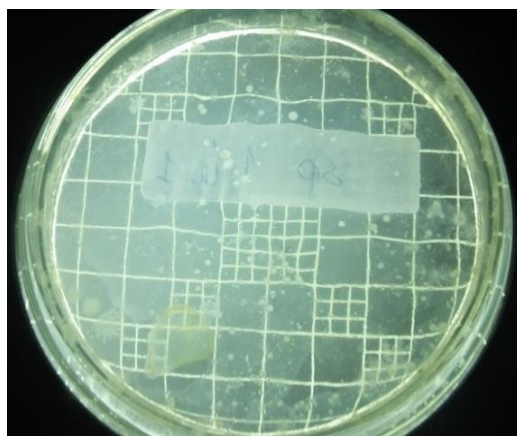


Figure 1. Colonies of Symbiotic Bacteria on Sea Urchins

Table 1. Antibacterial activity On the Symbiotic Bacteria of Sea Urchins against *Staphylococcus aureus* dan *Escherichia coli*

Isolate code	Bacterial test		Isolate code	Bacterial test	
	<i>S. aureus</i>	<i>E. coli</i>		<i>S. aureus</i>	<i>E. coli</i>
BB.01.1	-	-	BB.02.20	-	-
BB.01.2	-	-	BB.02.21	-	-
BB.01.3	-	-	BB.03.22	-	-
BB.01.4	-	-	BB.03.23	-	-
BB.01.5	-	-	BB.03.24	-	-
BB.01.6	-	-	BB.03.25	-	-
BB.01.7	-	-	BB.03.26	-	-
BB.01.8	-	-	BB.03.27	-	-
BB.01.9	-	-	BB.03.28	-	-
BB.01.10	-	-	BB.03.29	-	-
BB.01.11	-	-	BB.03.30	-	-
BB.01.12	-	-	BB.03.31	-	-
BB.02.13	-	-	BB.03.32	-	-
BB.02.14	-	-	BB.03.33	-	-
BB.02.15	+	+	BB.03.34	-	-
BB.02.16	-	-	BB.03.35	-	+
BB.02.17	-	+	BB.03.36	-	-
BB.02.18	-	-	BB.03.37	-	-
BB.02.19	-	-	BB.02.20	-	-

Description: (+) able to inhibit test bacteria; (-) Unable to inhibit test bacteria

The results in Table 1 and showed, that 3 out of 37 isolates have antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Isolates BB.02.15 has antibacterial activity against *S. aureus* and *E. coli*, while BB.02.17 and BB.03.35 isolates have antibacterial activity against *E. coli*. Antibacterial activity was indicated by the presence of clear zones which present around the isolates. Isolate BB.02.15 were able to inhibit the growth of *S. aureus* with an inhibition zone diameter of 10.4 mm and in *E. coli* bacteria, it was capable of inhibiting the growth with an diameter of 10.9 mm. While the BB.02.17 and BB.03.35 isolates were able to inhibit the growth of *E. coli* with the inhibition zone diameter of 21.3 mm and 27.2 mm, respectively.

Bacterial, protistan, and metazoan symbionts inhabit the lumen of the sea urchin digestive system – especially the intestinal region (Holland, 2013).

This is consistent with research done Abubakar *et al.* (2012) that revealed that the Sea Urchins exhibit antimicrobial activities, particularly the extracts of the guts and gonads. Gonad extract showed the high antibacterial activity against *E. coli* and *S. aureus* (Akaerina *et al.*, 2015). BB.02.15, BB.02.17 and BB.03.35 Isolates have the ability to inhibit the growth of bacteria test caused by the presence of the active compounds. The clear zone formed around the isolates indicated that there were compounds contained in the isolates. These

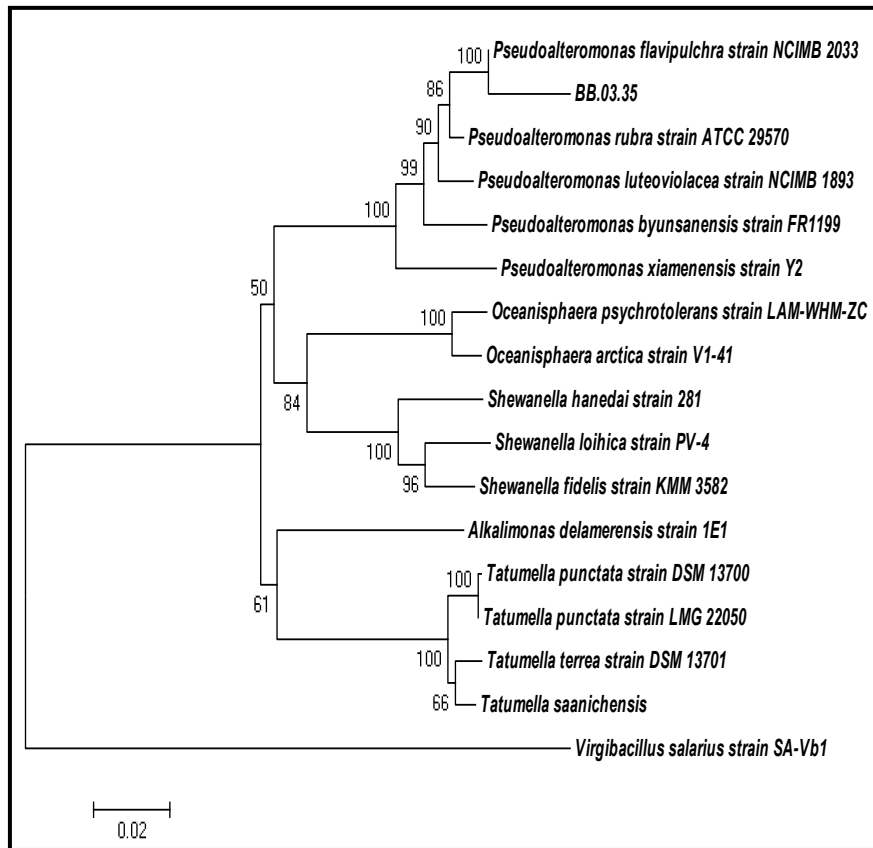


Figure 3. Phylogenetic Tree

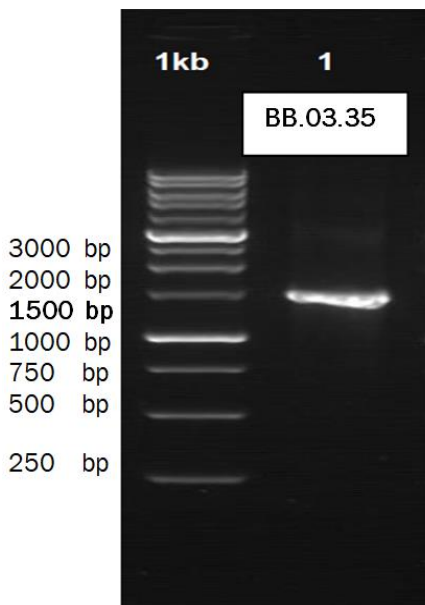


Figure 2. PCR Amplification 16S rDNA Isolate BB.03.35

compounds produced the inhibition of growth in test bacteria. With the visualization of 16S rDNA amplification was presented in Figure 2. Isolate BB.03.35 indicated a best results. Isolate BB.03.35

has a base length of 1500 bp. PCR is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Joshi and Deshpande, 2010).

Phylogenetic tree are constructed by using MEGA 6 applications. The phylogenetic tree in Figure 3 indicated the bacteria which have the closest genetic relationship with bacterial isolate BB.03.35. BLAST (Basic Local Alignment Search Tool) results showed isolate BB.03.35 have a 97% homology closely related to *Pseudoalteromonas flavipulchra*. (Offret et al., 2016) stated the genus *Pseudoalteromonas* is a Gram-negative, heterotrophic, and aerobic bacteria. *Pseudoalteromonas* strain requires a seabed for the growth and association with healthy animals as well as algae (Pelczar, et al., 2000).

Conclusion

The tree 3 out of 37 isolates have antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The BB.03.35 isolate was

selected for molecular identification due to the largest inhibitory zone diameter. The sequence of this bacterium showed 97% homology and closely related to *Pseudoalteromonas flavipulchra*. The DNA sequences generated from this study have been registered in GenBank with access numbers LC275180.

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