Isolation, Characterisation and Antagonistic Activity of Bacteria Symbionts Hardcoral *Pavona* sp. Isolated from Panjang Island, Jepara Against Infectious Multi-drug Resistant (MDR) Bacteria

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Abstract. *Pavona* sp. is highly spread over Indonesian waters including Panjang Island. Several studies showed that bacteria symbionts hardcoral were the big source of antibiotic product, but there was limited research of the bacteria symbionts with hardcoral *Pavona* sp. In this research bacteria symbionts from hardcoral *Pavona* sp. had been collected from Panjang Island, Jepara. Marine bacteria symbionts were isolated by serial dillution method, while antibacterial activity was performed by using overlay and agar block method. The total of 2 from 5 isolates were active to MDR bacteria such as *Enterobacter aerogenes* and *Acinetobacter baumanii,* the code were PHC 44/04 and PHC 44/05. Then both of them were identified by morphological and molecular DNA characterization using 16 S rRNA gene sequence. The result of 16 S rRNA identification shows PHC 44/04 has 99% similarities with *Virgibacillus salarius* strain sa-Vb 1, while PHC 44/05 shows 99% similarities with *Pseudoalteromonas flavipulchra* strain NCIMB 2033.

Keywords : MDR bacteria, Antagonistic activity, *Virgibacillus salarius, Pseudoalteromonas flavipulchra.*

1. Introduction

Multi-drug Resistant (MDR) bacteria are bacteria that acquired non-susceptibility to at least one agent in three or more antibiotic categories [1][2]. To date, the emergence of MDR bacteria has spreaded worldwide and become a major public health problem. Data from Centers for Disease Control and Prevention (CDC) showed that the minimum number of illness and deaths caused by MDR are 2,049,442 illness and 23,000 deaths per year [3]. Furthermore, the estimated data of cases and death annual number

for carbapenem-resistant *Enterobacteriaceae* are 9,300 case and 610 death; MDR *Acinetobacter* 7,300 and 500 death; Extended Spectrum Beta-Lactamases (ESBLs) 26,000 cases and 1,700 death; MDR *Pseudomonas aeruginosa* 6,700 cases and 440 death, and many more drug resistance bacteria infection cases and death [3]. Meanwhile, the case of drug resistency also occur in Asia, including Indonesia [4].

Antibiotic resistancy has been encompass any categories of antibiotics. CDC reported sixteen antibiotics resistance identified since 1940, those were Methicilin-resistant *Staphylococcus aureus* (MRSA), Penicillin-resistant *Pneumococcus* (PRP), Extended Spectrum Beta-Lactamases (ESBLs), Vancomycin-resistant *Enterococci* (VRE), and the latest is Ceftaroline-resistant *Staphylococcus* which found in 2011 [3]. Based on previous research ESBLs prevalency in Indonesia is the highest among ASEAN country, about 71.0% compared to Thailand 59.4% and *Asia Pacific Association Country* (APAC) 48,0% [5]. ESBLs *Enterobacteriaceae* resistency had covered the third generation of cephalosporin. In 2008 the percentage of ESBLs *E. aerogenes* resistant imipenem was 89.7% [6], cefoxitin resistant was 50.3% - 74.2%, ciprofloxacin resitant 51.4% and levofloxacin resistant 54.4% [7]. The resistency of the leading nosocomial agent (*Acinetobacter baumanni* and *Enterobacter aerogens*) to antibiotics such as imipenem, cefoxitin, ciprofloxacin, levofloxacin and many more are the reason to look for another new compound of antibiotics.

This condition compounded by the void development of antibiotics. Silver stated that there is a discovery void of antibiotics rather than gap since the fisrt antibiotic announce in year 1908 until 1987 [8]. The latest registered antibiotics after 1990's such as linezolid and daptomycin [3] were first found (patented) in 1978 and 1987, thus there have been no novel antibiotics discoveries since 1987[8][9]. This, lacked for the development of new novel antibiotics compound. Marine natural products have been studied as antibiotics candidat [10][11]. One of the marine natural product source is coral which have many activities as antimicrobial, antivirus, antifungal, anti-plasmodial, cytotoxic, and anti-inflamatory [12][13][14].

Pavona is a genus from family Agariciidae which commonly found in Indopacific region including Indonesia. According to previous research [15] one genus from Agariciidae which found in Panjang Island vicinity, Jepara from depth 0.5 – 7 m is *Pavona*. Although widely spread in tropical regions, the exploration of bioactive compound from *Pavona* sp is still limited. Corals which have been widely explored are Plexauridae, Clavulariidae, Xeniidae and Gorgoniidae [10]. However the main barriers for secondary metabolites from corals entering the clinical trial is the issue of availability of raw materials. The low concentration of secondary metabolites is less than 10^{-6}% by weight of the wet invertebrates [16]. It is certainly not conservative and disturb the balance of aquatic ecosystems. Therefore coral microsymbiont is the answer for the challenge. Coral symbionts bacteria have the similar compound with their host which can be source of new antibiotics compound[17]. Moreover bacteria have the characteristics of easy to breed, takes relatively shorter culture and do not require much space.

The purpose of this research were to isolate bacteria symbiont from coral *Pavona* sp, to screen antagonistic activity against human patogenic MDR bacteria, and to characterize active isolate morphologically and molecularly using PCR amplifications 16S rRNA gene sequences.

2. Materials and Methods

2.1. Sampling and isolation of symbiont bacteria

Hardcoral *Pavona* sp was collected from Panjang island vicinity, Jepara, Central Java, Indonesia by skin diving to a depth approximately 3m. During collection, *Pavona* sp was put into sterile ziplock plastic bag and placed inside the cool box.

The isolation and purification of symbionts bacteria were conducted in Tropical Marine Biotechnology Laboratory, Diponegoro University. The isolation process was conducted by serial dillution method, furthermore the purification process was carried out by streak plate method [18].

2.2. Screening of antagonistic activity

The screening of antagonistic activity was also conducted in Tropical Marine Biotechnology Laboratory, Diponegoro University. The purpose of this process was to screen the antagonistic activity between isolate and tested bacteria (MDR). The screening method against pathogenic MDR bacteria (*Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacter aerogenes,* MRSA, *Escherischia coli, Enterobacter cloacae complex, Staphylococcus haemolyticus* and *Acinetobacter baumannii*) was performed by overlay method [17]. Each 1% of the test-bacteria cultures at logarithmic phase for as much as 0,5 Mc Farland was mixed into soft agar medium which then poured on to the agar surface which was previously inoculated with isolates for seven days incubation in room temperature. The isolates which have antagonistic activity will form clear zone surround it.

The result of antagonistic activity was confirmed using agar block method [19]. Each of the isolates was cultured on the agar media for 7 days. Cylindrical pieces were cut out from each cultures. The blocks were placed on to agar medium which have been inoculated with a fix amount of test-bacteria (0,5 Mc Farland). Cultures stayed for 14 – 18 hours at 2 – 8 ^{0}C and then incubated at 37^{0}C for 24 hours. The antagonistic activity was measured in milimeter (mm) clear zone on the 24th until 48th hours. The strongest potential isolate with the greatest clear zone was characterized by morphological and molecular characterization using 16S rRNA gene sequence.

2.3. Morphological characterization

Morphological characterization was conducted by observing colony morphology and gram's staining. Observation of the colony include the colony colour, size, form, elevation and margin [18]. Gram's staining method was conducted by using gram A (crystal violet), gram B (lugol iodine), gram C (alcohol) and gram D(Safranin) [20].

2.4. DNA extraction

Chelex method with several modification was used for DNA extraction [21]. Selected colonies from the best isolate were put in to 50 – 100 µl ddH_2O and 1 ml of 0,5 % saponin in PBS 1x (stayed overnight at 4^{0}C). The incubated mixture was centrifuged at 12000 RPM for 10 minutes and discard the supernatant. As much as 1ml PBL 1x was added to the mixture and centrifuged again at 12000 RPM for 5 minutes and discard the supernatant. Then 100 µl ddH_2O and 50 µl of 20% Chelex 100 (shake well and ensure the chelex crystal mix well to the sample) were added to the final mixture. The mixture was boiled for 10 minutes and vortex once after 5 minutes. Sentrifuge the mixture with 12000 RPM for 10 minutes. The supernatan was moved in to steriled microtube and saved in -20^{0}C. The examination of DNA concentration was conducted using NanoDrop 2000 spectrophotometer (Thermo Scientific). DNA purity and concentration was calculated using 260/280 and 260/230 ratios by NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.5. PCR amplification 16S rRNA gene sequence

Primers used for PCR amplification 16S rRNA were universal primer 27 F ((5'-AGAGTTTGATCMTGGCTCAG-3) and 1492 R (5'- GGTTACCTTGTTACGACTT-3'). The PCR mixture contain GoTaq®Green Master Mix Promega (12.5 µl), primer 27 F (1 µL), primer 1492 R (1 µL), template DNA (1 µl) and ddH_2O (9.5 µl), so that the total volume was 25 µl.

PCR reaction was conducted in a MJ Mini Personal Thermal Cycler (BIO RAD) using optimation consist of initial denaturation at 95^{0}C for 3 minutes, then followed by 30 cycles of denaturation at 95^{0}C

for 1 min each cycle, annealing at 53.9^{0}C for 1 minutes, extension at 72^{0}C for 1 minutes, and followed by final extension at 72^{0}C for 7 minutes. PCR products were examined using agarose 1 % gel electrophoresis and the result was visualized by using UVIDoc HD5 (UVITEC Cambridge).

2.6. DNA sequencing

DNA template was sent to PT. Genetika Science (Jakarta, Indonesia) for 16S rRNA sequence determination. The allignment of sequences result were done using MEGA 5.05, then allignment result was inserted to the BLAST program to create homology of any closely related bacteria in the gene bank database (NCBI, http://www.ncbi.nlm.nih.gov). The result of BLAST Homology was deposite to DNA Data Bank of Japan (DDBJ, www.ddbj.nig.ac.jp) in orger to obtain the accession number.

2.7. Phylogenetic analysis

Phylogenetic tree was constructed using neighbor-joining analysis inside the program MEGA 5.05. The result of phylogenetic tree construction was examined using bootstrap method.

3. Result

3.1. Isolation and purification of symbionts bacteria

The total of symbionts bacteria from one sample *Pavona* sp. obtained 5 pure isolates with different morphology each other. Morphological characterization of each isolates was performed in table 1 as follow.

Table 1. Morphological characteristic of bacteria symbionts *Pavona* sp.

Isolate code	Colour	Size	Shape	Margin	Elevation
PHC-44/01	Pinkish white	*Pin point*	Round	Entire	Convex
PHC-44/02	White turbid	Medium	Round	Entire	Convex
PHC-44/03	Thin white turbid	Small	Round	Entire	Convex
PHC-44/04	White	Medium	Round	Entire	Convex
PHC-44/05	Orange	Medium	Round	Entire	Convex

3.2. Screening of antagonistic activity

Screening of antagonistic activity from isolates of bacteria symbionts *Pavona* sp. against MDR bacteria obtained 3 active isolates. The result of antagonistic activity screening was presented in table 2 as follow.

Table 2. Result of antagonistic activity screening from bacteria symbionts*Pavona* sp.

Isolate Code	MDR								Antagonistic Activity
	P. aerugi nosa	*K. pneum onia*	*E. aero genes*	*A. bauma nni*	MRS A	*E. coli*	*E. cloacae complex*	*S. hemoly ticus*	
PHC 44/01	-	-	-	-	-	-	-	-	Not active
PHC 44/02	+	-	+	-	-	-	-	-	Active
PHC 44/03	-	-	-	-	-	-	-	-	Not active
PHC 44/04	-	-	++	-	+++	-	-	-	Active
PHC 44/05	-	-	+	+++	+++	++	-	+	Active

Confirmation for the best isolates was conducted by using agar block method. The confirmation result was shown in table 3.

Table 3. Confirmation of antagonistic activity from isolates of bacteria symbionts *Pavona* sp.

Isolate code	***Enterobacter aerogenes* (mm)**		***Acinetobacter baumannii* (mm)**	
	Replication 1	Replication 2	Replication 1	Replication 2
PHC-44/04	29.60	30,23	-	-
PHC-44/05	-	-	15	16

3.3. Morphological characterization

The result of morphological characterization using gram staining showed that isolate PHC-44/04 was gram positive bacteria, meanwhile isolate PHC-44/05 was gram negative bacteria (fig. 1).

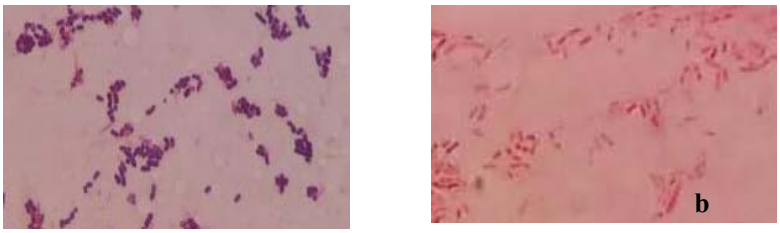

Figure 1. (a) gram staining for isolate PHC-44/04 showed gram positive bacteria, (b) gram staining for isolate PHC-44/05 showed gram negative bacteria (magnification 1000 times)

3.4. Molecular characterization and Phylogenetic analysis

The result of isolate PHC-44/04 BLAST homology showed that this bacterium was closely related to the family Bacillaceae and genus *Virgibacillus.* Meanwhile BLAST homology result for isolate PHC-44/05 showed correlation with *Pseudoalteromonas flavipulchra* as much as 99%. Phylogenetic tree showed that isolate PHC-44/04 was closely related 99% to *Virgibacillus salarius* strain Sa-Vb-1 (Fig. 2)

4. Discussion

The result of isolation obtained 5 bacteria isolate symbiont *Pavona* sp. Coral microbiota store various symbionts such as algae (dinoflagellates), viruses, bacteria and arkhaea which known as coral holobiont. Bacteria symbionts corals provide carbon, nitrogen, sulfur biogeochemical cycles and nourish also protect the coral from pathogen infection [22]. A wide variety of microbiota live in the mucus layer, tissue and skeletal calcium carbonate. The highest composition and abundant of bacteria can be found in mucus layer [23]. Mucus layer contain *mucopolysaccharida* which is nutrition for bacteria consortium. While, the amount of bacteria consortium increase it will make competition to each other. Therefore some bacteria have ability to produce secondary metabolites to maintain their existence.

The ability to inhibit the growth of MDR bacteria was different in each isolate. Isolate PHC-44/02 and PHC-44/05 inhibit in the first replication only, while isolate PHC-44/04 inhibits in the second replication. Furthermore, the antagonistic activity of isolate PHC-44/04 was confirmed by agar block method as shown in the table 3. The diameter of the inhibition zone obtained 29.60 mm and 30.23 mm. This is supported by Radjasa et al [17] which showed that the bacteria symbionts coral *Pavona* have antagonist activity against gram positive and negative bacteria.

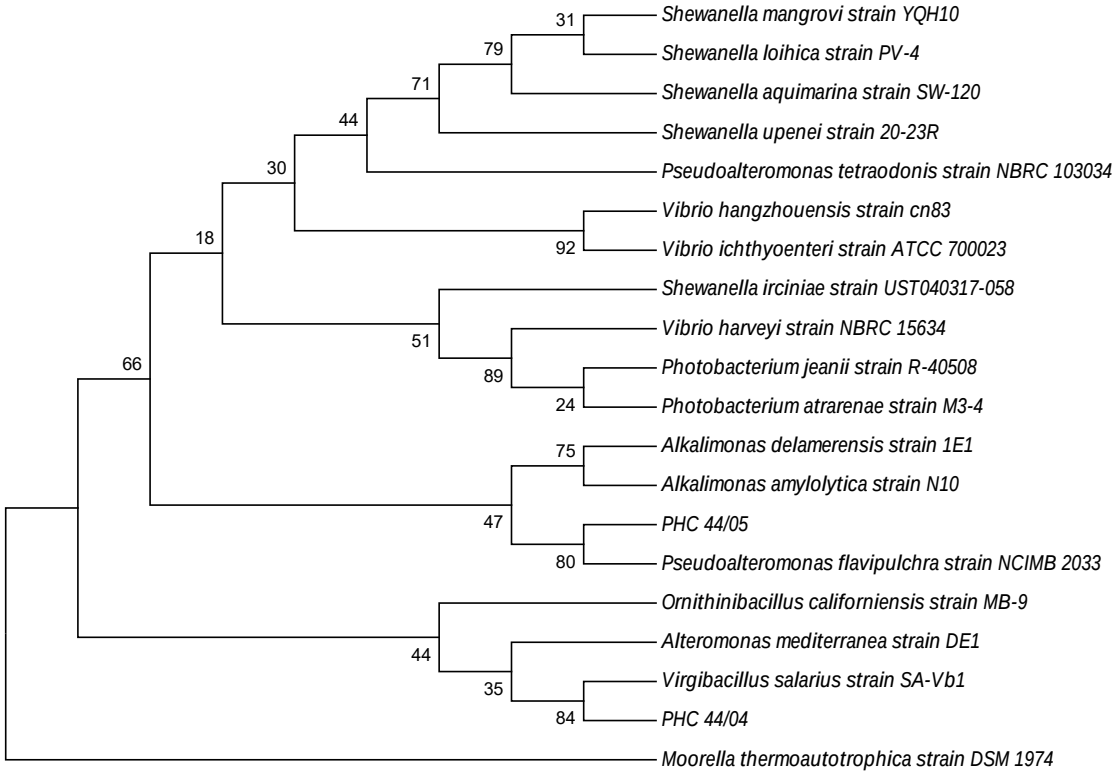

Figure 2. Phylogenetic tree from isolate PHC-44/04 and PHC-44/05

The results of microscopic identification showed that isolate PHC-44/04 stained purple (gram-positive bacteria). Meanwhile isolate PHC-44/05 showed red colour (gram negative) (fig. 1). This is due to gram-positive bacteria cell wall was thicker so it is more powerful to tie the color of the crystal violet (gram A). Thick peptidoglycan layer also makes crystal violet color does not fade when washed with gram C or alcohol. So when colored with dyes gram D (counterstain) bacteria remain purple. This mechanism was contrast with the gram negative bacteria (PHC-44/05). Moreover, microscopically isolates PHC-44/04 looks short bacillus shaped with spores inside, singly or in pairs. Those characteristics of is closely similar to the characteristics possessed by family Bacillaceae [24]. Meanwhile isolate PHC-44/05 looks bacillus shaped without spore and singly. The results of sequence homology 16S rRNA of isolate PHC-44/04 and PHC-44/05 showed similarity with *Virgibacillus salarius* strain Sa-Vb 1 with accesion number LC 190934 and *Pseudoalteromonas flavipulchra* strain NCIMB 2033 as much as 99%.

The phylogenetic tree showed that isolate PHC-44/04 is at the same clade with *Virgibacillus salarius* strain SA-Vb 1, which is halofil bacteria isolated from the Saharan Salt Lake [25]. Many strains of the genus Virgibacillus obtained from Brazilian sponge have antimicrobial activity [26]. This, suggested that many species of the genus were potential source of antimicrobial compounds. Moreover strain *Virgibacillus* sp. TS2A5 also has antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* were also found in the same waters with *Virgibacillus salarius* strain SA-Vb 1 [27]. On the other hand, isolate PHC-44/05 is in the same clade with *Pseudoalteromonas flavipulchra* strain NCIMB 2033 (fig. 2) with characteristic a marine, gram negative bacteria, lemon-yellow colour and has antibacterial activity against pathogenic bacteria [28] [29] also cytotoxic activity against sea urchin eggs [30]. This

is proven that marine bacteria symbionts have potential ability to be source of new antibiotics compound from marine environment.

5. Conclusion

Isolation and purification process result 5 pure isolate of bacteria symbionts *Pavona* sp, which are 3 or them have antagonistic activity against MDR bacteria. The strongest isolates against MDR *Enterobacter aerogenes* and *Acinetobacter baumannii* are isolate PHC-44/04 and PHC-44/05 which has 99% similarities with *Virgibacillus salarius* strain Sa-Vb 1 and *Pseudoalteromonas flavipulchra* strain NCIMB 2033.

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