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To cite this article: O Cristianawati *et al* 2017 *IOP Conf. Ser.: Earth Environ. Sci.* **55** 012027

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# Exploration of Fungal Association from Hard Coral against Pathogen MDR *Staphylococcus haemolyticus*

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**Abstract.** *Staphylococcus haemolyticus* are opportunistic bacteria and as the second leading cause of nosocomial infections. It is a disease causing septicemia, peritonitis, otitis, and urinary tract infections and infections of the eye. It also a phenotype resistant to multiple antibiotics commercial. There is now an urgency to find an alternative antibiotics to combat this bacteria. It has been widely reported that many bioactive marine natural products from marine invertebrate have striking similarities to metabolites of their associated microorganisms including fungi. Hard coral associated microorganisms are among of the most interesting and promising marine natural product sources, which produce with various biological activities. The proposed work focused on the discovery of bioactive compounds and also estimated the phylogenetic diversity from fungal association of hard coral against pathogen MDR *Staphylococcus haemolyticus*. A total of 32 fungal association, FHP 7 which were isolated from *Favia* sp. capable of inhibiting the growth MDR. Molecular identification based on 18S rRNA gene sequences revealed that the active fungal association belonged 100% to the members from one of the genera *Trichoderma longibrachiatum*. Accession Number LC185084.1.

**Keywords:** Fungal association, secondary metabolites, *Staphylococcus haemolyticus*, *Trichoderma longibrachiatum*

## 1. Introduction

*Staphylococcus haemolyticus* which were included in the negative staphylococcus members colonized from the human skin as an opportunistic pathogen. This bacterium as the second leading cause of nosocomial infections are isolated in conjunction with *S. epidermidis*, for example through the insertion of medical devices [1]. They are a disease causing septicemia, peritonitis, otitis, and urinary tract infections and infections of the eye [2]. *S. haemolyticus* [3], [4] that the organisms known to show resistant to multiple antibiotics commercial in a phenotype scale [1], this was reinforced their resistance to several antibiotics including heteroresistance keglukopeptida. Resistance of antibiotic become a very serious problem that caused decreasing the number of recovery, the cost increasing



rapidly for medical treatment extension and also increasing mortality of patient [5], [6], [7]. Using antibiotics against pathogenic bacteria has been increasing since the improper and uncontrolled treatment and become for Multi Drug Resistant (MDR) bacteria.

It has been widely reported many bioactive marine natural products have striking similarities with associated microorganisms from marine including fungi [8]. Marine fungal come out as an alternative solution to find potential compound from marine environment. Many potential marine-derived natural compounds were produced by marine organisms themselves or their associated microorganisms. reported that sustainability of marine organisms as the main problem of marine natural product exploration [9]. It has been almost impossible to harvest large numbers of organisms from nature without destroying the habitats.

A potential source of associated bacteria from marine invertebrates could be used for the isolation of bioactive metabolites which is collected from inhabiting the coastal waters [10]. Secondary metabolites can be found from association bacteria with soft coral because they have a similar characterization [11]. It can be possible that fungal association from hard coral can become an alternative to produce antibacterial compound which are against to pathogen MDR that fungal association from marine have potential as a natural drugs because they have an active secondary metabolites [12]. Secondary metabolites of marine fungal association are more specific than terrestrial fungal. However, the biodiversity of marine microorganisms is barely known. The biological diversity has been found in marine ecosystems which is higher than in tropical rain forests [13]. The reef invertebrates sources such as sponges, tunicates, bryozoans, soft corals and molluscs are accumulated as a large group of structurally unique natural products from the oceans. This diversity has been declared that the source of unique chemical compounds with the potential for industrial already develop as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes and agrichemicals [14].

Coral reef ecosystem in Indonesia about 85.000 km<sup>2</sup> and represented 14 % world coral reef [15]. Panjang island is located in 2 mil from west jepara beach with lasge about 19,7 ha. It was reported that Panjang island surrounded by coral reef [16]. Coral reef is a productive ecosystem which can produce a useful bioactive compound with high biodiversity in the sea [14]. It has been very well established that terrestrial microorganism are sources of valuable bioactive metabolites for more than half a century [17]. Hard coral are included as a phylum Cnidaria which has potential biomedic compound for 21% species [15]. Many bioprospeksi activity of natural compound from marine has been resulted a thousand new compound [18]. Coral is the targeted source for natural bioactive product which known have a secondary metabolites [14]. Exploration of fungal association that produced a bioactive compound has been found in marine invertebrate which have a secondary metabolites [19].

Successfully exploration of new compound was founded more than 330 compound from fungal association in 2002-2006. Some related research of bioactive compound produced by coral such as *Anthomastus bathyproctus* produced 3-Oxo sterols for cytotoxic [20], montiporyne A from *Montipora spp.* for anticancer [21] and montiporic acids from *M. digitata* as antimicrobial [22], reported that abundance and diversity of natural chemistry with some potential bioactivity can be gained into drugs discovery [18]. It is important to highlight an anti pathogen compounds that produced by marine fungi associated with invertebrates is providing the possible role an alternative to the commercial. This phenomenon has raised the importance of microbial symbionts of invertebrates as prolific sources of secondary metabolites with diverse biological activities applicable for health and industrial purposes. Therefore, it is reasonable to expect new classes of secondary metabolites are discovered which are exploration of untapped marine microbial diversity and resources will improve the rates.

## 2. Materials and Methods

### 2.1. Sampling of hard coral

Colonies of hard coral were collected from Panjang island, North Java Sea, Jepara, Central Java, Indonesia by scuba diving. Upon collection hard coral colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA).

### 2.2. Isolation of fungal symbionts from hard coral

The coral tissues were tapped on MEA medium containing chloramphenicol antibiotics, incubated at room temperature for 7 days. On the basis morphological features, colonies were randomly picked and purified by streak plates [23].

### 2.3. Inhibitory interaction tests

Antibacterial tests of hard coral-fungal symbiont against bacterial pathogen were performed by using plug method [24] and [25]. Bacterial pathogen (*S. haemolyticus*) used in this study was obtained from culture collection from Kariyadi Hospital. Culture of bacterial pathogen in the logarithmic phase was mixed with Mueller Hinton Agar (MHA) (1% v/v), which were then be poured on to the respective agar surface previously inoculated with hard coral fungal association and incubated for a day. The plates will be incubated at room temperature for 1 or 2 days. MDR activity was defined by the formation of inhibition zones around the fungal colonies.

### 2.4. PCR amplification and sequencing of 18S rRNA gene fragments

DNA extraction and amplification were also carried out at the Tropical Marine Biotechnology UPT Diponegoro University and sequencing at PT. Genetika Science. The DNA genomes from misselia fungal isolates were extracted using cheelex. The DNA concentrations were quantified by using Nanodrop. 18S rRNA fragments were generated by using universal primer ITS1 - ITS4 with base sequence ITS1: 5'- CTT GGT CAT TTA GAG GAA GTAA-3' and ITS4 : 5'- TCC TCC GCT TAT TGA TAT GC-3' [26]. These primers were used to obtain 400- 800 bp rDNA fragments for sequencing purposes. The DNA amplification was performed by Taq polimerase master mix with approximately 2 µg of total genome as a template. PCR was conducted as modification from: initial denaturation (5 min at 95<sup>0</sup>C ), 32 cycles of primer denaturation (1 min at 95<sup>0</sup>C), annealing (1 min at 56,4<sup>0</sup>C), and elongation (1 min at 72<sup>0</sup>C) followed by a final elongation step (7 min at 72<sup>0</sup>C) [27]. Purification of PCR products and subsequent sequencing analysis was conducted according to the method. The determined DNA sequence of strains were compared for homology to the BLAST database.

### 2.5. Phylogenetic analysis

All sequences were used at least 400-800 bp long. A phylogenetic tree was constructed using maximum-likelihood analysis. Only sequences of type strains was included in tree calculation. Alignment positions at which less than 50 % of sequences of the entire set of data have the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 18S rDNA. Phylogenetic analysis was performed with MEGA software packages.

## 3. Results

### 3.1. Sample collection

Samples of Hard coral were collected from Panjang Island. Based on *purposive* method, 8 different samples already collected by scuba diving. The samples was then be isolated to produce the fungal association with specific media.

### 3.2. Isolation and Purification of Hard Coral

The isolations were used for knowing and selecting of coral fungal association, which have an antibacterial potential. Fungal association were planted on Malt Extracts Agar Media (MEA). The result of fungal asosiation was about 32 isolates. The isolates were then be identified based on morfology such as colony, texture and colour [28]. Moreover, every single isolate was continued for antibacterial test to combat pathogen MDR *S. haemolyticus*.

### 3.3. Antibacterial Test

**Table 1.** Fungal Association against MDR *S. haemolyticus*

| Code   | Bacteria                           | Code   | Bacteria                           |
|--------|------------------------------------|--------|------------------------------------|
|        | <i>Staphylococcus haemolyticus</i> |        | <i>Staphylococcus haemolyticus</i> |
| FHP 1  | -                                  | FHP 34 | +                                  |
| FHP 2  | -                                  | FHP 37 | +                                  |
| FHP 3  | +                                  | FHP 6  | -                                  |
| FHP 4  | -                                  | FHP 10 | -                                  |
| FHP 5  | +                                  | FHP 15 | -                                  |
| FHP 7  | +                                  | FHP 16 | -                                  |
| FHP 8  | -                                  | FHP 17 | -                                  |
| FHP 9  | -                                  | FHP 18 | -                                  |
| FHP 11 | +                                  | FHP 19 | -                                  |
| FHP 12 | -                                  | FHP 20 | -                                  |
| FHP 13 | -                                  | FHP 24 | -                                  |
| FHP 21 | +                                  | FHP 25 | -                                  |
| FHP 30 | -                                  | FHP 29 | -                                  |
| FHP 31 | -                                  | FHP 35 | -                                  |
| FHP 32 | -                                  | FHP 36 | -                                  |
| FHP 33 | -                                  | FHP 38 | +                                  |

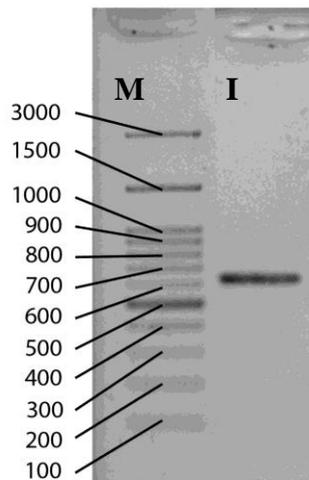
(+) success; (-) unsuccessful

Antibacterial test was showed 8 inhibition zone surrounding the plug miselia which was active combat with pathogen MDR. The growth rate of inhibition zone was observed until 9 days incubation (9x24 hours). The active isolates were signed by code FHP 3, FHP 5, FHP 7, FHP 11, FHP 21, FHP 34, FHP 37 and FHP 38. In 1x24 hours incubation, the inhibition zone was resulted 2,6 mm; 10,7 mm; 14 mm; 13 c mm; 22,3 mm; 14,6 mm; 18,7 mm dan 13,4 mm respectively. In the other hand, 9x24 hours incubation was resulted 22,3 mm; 10,7 mm; 26, 4 mm; 22,4 mm; 20,6 mm; 22 mm; 18,7 mm dan 15 mm. FHP 7 was picked up as the best isolate candidate based on growth inhibition zone.

### 3.4. Molecular identification

#### 1. Polymerase Chain Reaction (PCR)

The DNA which was amplified by using PCR 18S rRna . The PCR product was visualized by using electrophoresis and gel documentation. It was showed that FHP 7 in 610 bp long. The result of amplification was presented in Figure 1.

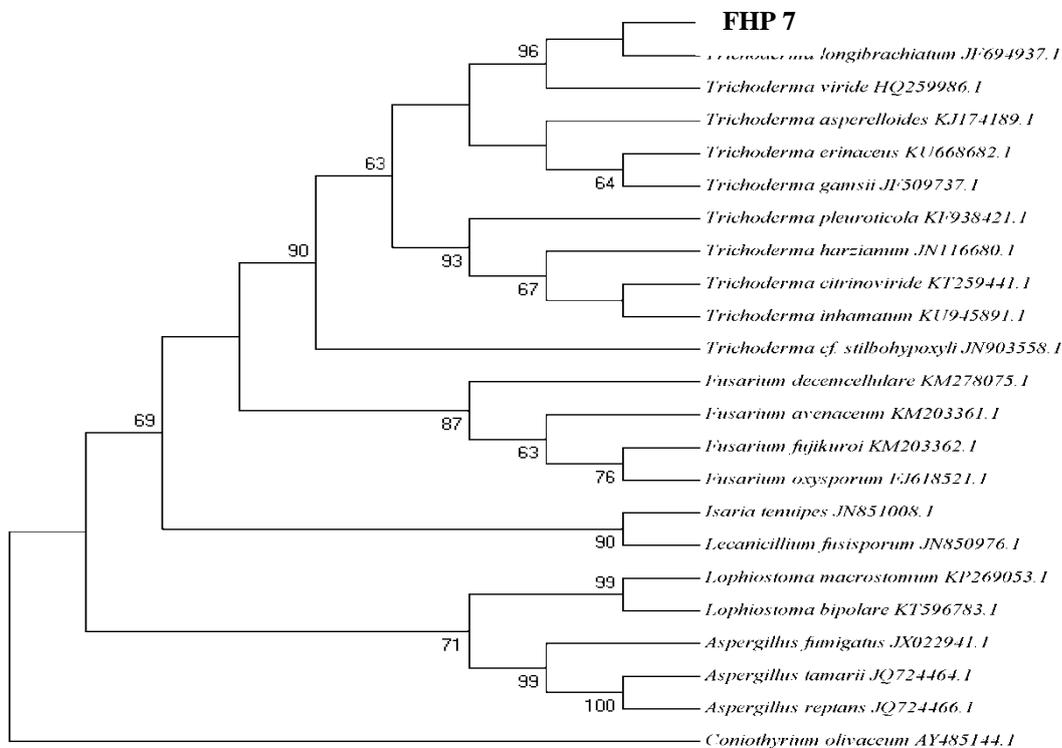


**Figure 1.** Visualization of PCR product by using electrophoresis.

where: M = *Marker Low DNA Mass Ladder*; I = FHP 7

3.5. *Phylogenetic tree*

Blast homology of FHP 7 was closely related 100 % with *Trichoderma longibrachiatum* strain JF694937.1. Analysis of phylogenetic by using Clustal W and MEGA 5 showed that phylogenetic tree of FHP 7 also closely related with genera from *Trichoderma* sp. The result of phylogenetic analysis was represented in Figure 2.



**Figure 2.** Phylogenetic tree of fungal association from hard coral

#### 4. Discussion

The samples which were collected from Panjang island was classified by using *Coral of The World* book [29] and software Coral ID [30], [31]. Genera of 8 different coral was indentified, such as Genus *Acropora* (PP-HC-16-45) (Suharsono, 2008), genus *Pachyseris* (PP-HC-16-48) [29], genus *Favia* (PP-HC-16-35 [32]. Johan (2003), Genus *Favites* (PP-HC-16-15) [29], Genus *Goniastrea* (PP-HC-16-49) [34]. Genus *Isopora* (PP-HC-16-47) [32], Genus *Pavona* (PP-HC-16-44) and Genus *Caulastrea* (PP-HC-16-43). Coral *Favia* sp. was identified through the morphology using a microscope and molecular method based on the isolate candidate which was FHP 7.

The isolation process was used specific media for fungi which was MEA (*Malt Extract Agar*). From the isolation process, it was purified 32 isolate association Fungal association that already single colony was then be identified such as colony, texture and colour [28]. The fungal association from hard coral which then be continued for antibacterial test toward pathogen *S. haemolyticus* by using plug miselia method. It was representing that 8 isolates was built an inhibition zone. Association of microorganism can produce secondary metabolites which can be used for pharmacological activity [35], [36]. Fungal association from marine invertebrate which have a bioactive compound can give the benefit for antibacteri, antifungal, citotoxic, anticancer, antioxidant, antimalaria, nematocidal, antitumor, antineoplastic, antifouling, larvasida, neurotoksik, antimycobacterial, anti HIV, antiprotozoal, antiviral, antiulcer, antiparasite, antiinflammation, and immunosuppressant [37].

FHP 7 was choosed as the best candidate of fungal association from hard coral with code PP-HC-16-35 based on the inhibition zone activity until incubation in day 9. The characteristic FHP 7 was as the bacteriocidal which is killing the bacteria toward pathogen MDR. Microscopic test by using a microcope was conducted for checking conidiophore, phialides and conidia and supported by using identification book from [38], [39]. Moreover, FHP 7 was identified as a *Trichoderma longibrachiatum* with characteristic, such as short and tight phialides, soft green conidia.

Sequencing identification of FHP 7 was used BLAST (*Basic Local Alignment Search Tool*) system that was showed that fungal association belonged 100% to the members from one of the genera *Trichoderma longibrachiatum*. The isolates can be one level species for 99-100% homology condition. [40]. Identification based on molecular identification can be used for species level analysis of fungal.

#### 5. Conclusion

A total of 32 fungal associations, FHP 7 which were isolated from *Favia* sp. capable of inhibiting the growth MDR *S. haemolyticus*. Molecular identification based on 18S rRNA gene sequences revealed that the active fungal association belonged 100% to the members from one of the genera *Trichoderma longibrachiatum*. Accession Number LC185084.1.

#### Acknowledgements

This research was partly funded by a research grant from the Ministry of Research, Technology and Higher Education, Indonesia for PMDSU (Program of Magister Leading to Doctoral Degree for Excellent Under Graduates) scholarship.

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