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Screening Antibacterial Agent from Crude Extract of Marine-Derived Fungi Associated with Soft Corals against MDR-*Staphylococcus haemolyticus*

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Abstract. Multidrug resistant *Staphylococcus haemolyticus* is a Gram-positive bacteria and member of coagulase negative staphylococci (CoNS) which has the highest level of antimicrobial resistance. This nosocomial pathogen due to skin or soft tissue infections, bacteremia, septicemia, peritonitis, otitis media, meningitis and urinary tract infections. The ability to produce enterotoxins, hemolysins, biofilm, and cytotoxins could be an important characteristic for the successful of infection. Marine-derived fungi have potency as a continuous supply of bioactive compound. The aim of this research was screening antibacterial agent from crude extracts of marine-derived fungi associated with soft corals against MDR-*S. haemolyticus.* Among 23 isolates of marine-derived fungi isolated from 7 soft corals, there were 4 isolates active against MDR-*S. haemolyticus*. The screening was conducted by using *agar plug diffusion* method. Isolate PPSC-27-A4 had the highest antibacterial activity with diameter 23±9,6 mm. The crude extract from this isolate had been confirmed to antibacterial susceptibility test and it had the highest antibacterial activity in 12.2 mm with concentration of 300µg/ml from mycelia extract. PPSC-27-A4 had been characterized in molecular, based on the sequence analysis of 18S rRNA, PPSC-27-A4 isolate was identified as *Trichoderma longibrachiatum.*

Keywords: antibacterial, associated marine-derived fungi, Multidrug resistant, nosocomial pathogen, soft coral, *Staphylococcus haemolyticus*

1. Introduction

Staphylococcus haemolyticus infection is commonly occurred in the hospital as a nosocomial infection and related to implanted medical devices [1][2]. This bacteria include in a gram positive, non-spore forming, non-motile, facultative anaerobic bacteria. The other biochemistry reactions are negative for coagulase (CoNS), DNase, ornithinine decarboxylase, phosphatase, urease and oxidase [3]. This

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nosocomial pathogen due to skin or soft tissue infections, bacteremia, septicemia, peritonitis, otitis media, meningitis and urinary tract infections [4][5]. The ability to produce enterotoxins, hemolysins, biofilm, and cytotoxins could be an important characteristic for the successful infection [6] [7]. According to [8] the biofilm formation of *S. haemolyticus* has different profile from *S. epidermidis.* 72 clinical isolates of *S. haemolyticus* have been analysed their antibiotic resistant, 89% isolates were detected resistant to all beta-lactam antibiotics encoded by *mecA* gene and 85% isolates were detected resistant to aminoglycoside encoded by [*aac(6')-Ie-aph(2")*] [8]. Not only in the hospital this pathogen was also found in the veterinary clinic. [9] mentioned that all isolates resistant to tetracycline, macrolides, and chloramphenicol. Thus, *S. haemolyticus* has the highest level of antimicrobial resistance among all CoNS species [10]. Based on this evidence, the exploration of new anti-MDR *S. haemolyticus* agent should be investigated in order to develop new therapy.
In this recent decades, [11] explained that investigation of natural product such us antibacterial agent were expanded to marine environment. One of the prospective sources for searching antibacterial agent from marine environment is soft coral [12][13]. Cladidiol is a new compound which was successfully isolated from methanolic extract of *Cladiella* sp. Its ability against several pathogens at a concentration of 50 µg/ml. Unfortunately they needed 4.5 kg dry weight [14]. A new approach to obtain a sustainable source and answer the scarcity of source is marine-derived fungi isolated from soft corals. This was supported by a statement from [15] and [16] that the microorganisms associated with macroorganisms have a symbiotic relationship with its specific host, so the fungus associated with soft corals can be used as a sustainable source of bioactive compounds because they grow faster than macroorganisms. Therefore, exploitation of soft corals could be reduced.
This research was designed to screening and characterized fungi associated with soft corals which had the crude extract potential against MDR-*S. haemolyticus.* The objective of this research may serve a pioneer of antibacterial agent with sustainable source and may provide the information to a pharmaceutical industry about marine natural product that is environmental friendly.

2. Materials and Methods

2.1. Collection of Sample

Soft corals were collected from the vicinity of Panjang Island (06°34'S and 110°37'E) using snorkeling technique. Samples were taken only 3 – 5 cm. Preservation of samples were created in a zip lock plastic bag to be stored temporarily in a cool box.

2.2. Isolation and Purification

Isolation of associated fungi was conducted using method of [17]. Fresh samples were initially sprayed with sterile sea water and cut approximately 1 cm^2. They were slashed, so that fungi on the inside of the tissue could be grown. In this research, samples were transferred as a duplo in Malt Extract Agar (MEA) added by sea water. Samples were incubated at room temperature for 3 days. Purification was conducted by separation of colony morphology (colours, shapes, and sizes).

2.3. Antibacterial Preliminary Test

MDR *S. haemolyticus* was obtained from Clinical Microbiology Laboratory of Kariadi Hospital Semarang. This test was conducted using agar plug diffusion method [18]. The associated fungi had been grown in MEA with sea water for 7-14 days. MEA which grew the associated fungi were then cut cylindrical to be transferred to the Nutrient Agar (NA) inoculated bacteria MDR *S. haemolyticus* 1x24 hours with a density of 0.5 McFarland. Both were then incubated at room temperature for 1x24 hours. Inhibition zone showed that fungi produced a potential bioactive compound against MDR *S. haemolyticus*, furthermore only the most potential isolate was extracted of its secondary metabolites.

2.4. Secondary Metabolites Extraction of Potential Isolate

Extraction was initially started with growing the associated fungi into Malt Extract Broth (MEB) with sea water for 14 days [19]. Inoculum was separated between mycelia and medium with filter paper. Medium was added with ethyl acetate (EtOAc) and mycelia were added with methanol (MeOH). Both were then separated with separatory funnel, then samples were evaporated with rotary evaporator.

2.5. Antibacterial Susceptibility Test

Crude extract from potential isolate was prepared for antibacterial susceptibility test against MDR *S. haemolyticus.* Both crude extracts from medium and mycelia were tested in various concentrations from low to high. The concentration started from 50, 100, 150, 300, and 500 µg/ml. Inoculum of MDR *S. haemolyticus* with density 0.5 McFarland was swabbed into Mueller-Hinton Agar (MHA). Crude extracts were added on blank paper disc, then incubated for 1x24 hours at room temperature. Clear zone was measured by caliper.

2.6. Molecular Identification

DNA extraction was conducted using chelex [20]. Amplification was done using PCR with primer ITS region, ITS1 as forward primer and ITS4 as reverse primer. The process of denaturation was initially at 95 °C for 3 minutes, followed by 35 cycles (denaturation at 95 °C for 1 minute, annealing at 51.4 °C for 1 minute, and extension at 72 °C for 1 minute), then post cycling in 72 °C extension for 7 minutes and set 16 °C for finishing. Furthermore, the gel electrophoresis was conducted to see the DNA bands formed and it was visualized using Geldoc. The PCR products were then sequenced and analyzed using MEGA 6.06. The sequence then compared with another sequences with the ability as antibacterial agent in phylogenetic tree using Neighbor-Joining method.

3. Results and Discussions

The infection due to MDR-*S. haemolyticus* associated with implanted medical devices has a significant impact especially in expense for hospitalized patients. Traditional antimicrobial therapy was still used as a basic control, however, this infection continue to be a challenge [7] [21]. Therefore, to minimize this incident, the discovery of bioactive compounds that inhibit MDR-*S. haemolyticus* growth is an urgent need.

3.1. Collection of Sample

There were 7 soft corals collected from vicinity of Panjang Islands, encoded with PPSC-04, PPSC-11, PPSC-16, PPSC-21, PPSC-27, PPSC-41, and PPSC-42. In this study, 7 soft corals was obtained as a host for fungi selected as a sustainable sources. They are included in *Cladiella* sp., *Lobophytum* sp., *Sinularia* sp., and *Sarcophyton* sp. Soft corals have presented the majority of potential therapeutics including anticancer agents, immunomodulators, and useful antifouling agents. They also showed significant biological activity, including antimicrobial, Ca-antagonistic and anti-inflammatory properties [22]. The previous research explained that the genus mentioned above has been studied about their microorganisms symbiont as an antimicrobial agent [23][24][25][26].

3.2. Isolation and Purification

The isolation of fungal associated with 7 soft corals obtained 23 single colony isolates. Their morphological characteristics were shown in Table 1. Among 23 isolates, 18 isolates were including in filamentous fungi or molds and 5 others were including in yeasts. [27] explained that molds grow in compact structure called mycelia and yeasts grow in a single cell colony. Previous research about isolation fungi associated with *Sinularia* sp. was successfully isolated 15 fungi using MEA [24]. [17] stated that a successful isolation depends on substrate/ media, nutrition and environment conditions such as temperature and aeration.

Table 1. The Colony Morphology of Fungi associated with Soft Coral from Panjang Island Vicinity

Isolate Code	Colour	Shape	Size	Texture	Elevation
PPSC-04-A1	White	Circle	Large	Leather	Raised
PPSC-04-A2	Cream	Circle	Small	Creamy	Flat
PPSC-04-B1	White, yellow	Circle	Medium	Leather	Raised
PPSC-11-A1	White	Circle	Large	Cotton, smooth	Raised
PPSC-11-A2	Yellow greenish	Circle	Large	Granular	Flat
PPSC-11-A3	Dark Green	Pin, Circle	Medium	Rough	Raised
PPSC-11-A4	White	Circle	Small	Smooth	Flat
PPSC-11-A5	White	Circle	Large	Cotton, slim	Raised
PPSC-11-A6	White	Circle	Large	Cotton, soft	Raised
PPSC-11-B1	White, brown	Pin, Circle	Medium	Rough	Raised
PPSC-11-B2	White yellownish	Circle	Medium	Smooth	Flat
PPSC-16-A1	Cream	Circle	Small	Smooth	Flat
PPSC-21-B1	Green dust	Circle	Medium	Velvet	Raised
PPSC-21-B2	White	Circle	Medium	Cotton, soft	Raised
PPSC-21-B3	Cream	Circle	Small	Creamy	Flat
PPSC-21-B4	Dark green	Circle	Small	Filamentous	Raised
PPSC-27-A1	Yellow, Dark green, white	Circle	Large	Powdery	Raised
PPSC-27-A2	Yellow, white, dark green, yellow greenish	Circle	Large	Powdery	Raised
PPSC-27-A3	White	Circle	Small	Smooth	Flat
PPSC-27-A4	Green, bright yellow	Circle	Large	Powdery	Raised
PPSC-27-B1	White	Circle	Large	Cotton	Raised
PPSC-41-A1	Cream	Circle	Small	Creamy	Flat
PPSC-42-A1	Cream	Circle	Small	Creamy	Flat

3.3. Antibacterial Preliminary Test

Antibacterial preliminary test was conducted to minimize time, expense, and attempt. Using agar plug method, there were 4 isolates successfully combat MDR-*S. haemolyticus*, including PPSC-11-A2, PPSC-27-A1, PPSC-27-A2, and PPSC-27-A4. This potential isolates origin from soft coral PPSC-11 and PPSC-27. According to sclerites identification [28], this soft coral was identified as *Cladiella* sp. and *Lobophytum* sp. However, only the highest antibacterial activity isolate would be extracted its bioactive compound. The antibacterial activity from 3 isolates was performed in Figure 1. The most potential isolate encoded as PPSC-27-A4 with diameter 23±9.6 mm.

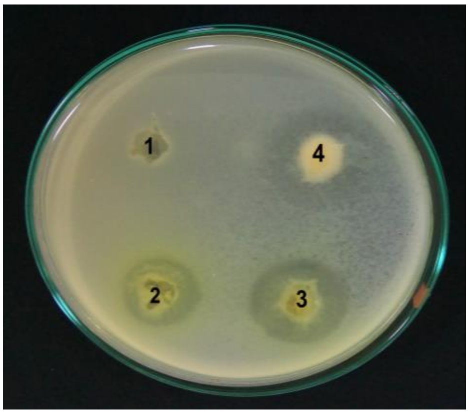

Figure 1. Antibacterial Activity from Fungi associated with Soft Coral against MDR-*S. haemolyticus*
Note: 1=PPSC-21-B4; 2=PPSC-27-A1; 3=PPSC-27-A4; 4=PPSC-27-A2

3.4. Secondary Metabolites Extraction of Potential Isolate

The purpose of secondary metabolites extraction was to find out bioactive compound from extracellular (released in medium) and intracellular (mycelia). This process was completed using polar and semi-polar solvent. [19] mentioned that organic solvent to extract bioactive compound from medium using ethyl acetate (EtOAc) and methanol (MeOH) to extract mycelia. The weight of crude extract from fermentation in 100 ml Malt Extract Broth (MEB) with sea water has been presented in Table 2. The most potential isolate encoded with PPSC-27-A4 has been extracted and tested for its antibacterial activity.

Table 2. Weight of crude extract from potential isolate encoded with PPSC-27-A4

Isolate Code	Source		Texture	Colour
	Medium	Mycelia		
PPSC-27-A4	0.0238 g	0.0395 g	Pasta	Yellow

3.5. Antibacterial Susceptibility Test

The crude extract of PPSC-27-A4 was tested for antibacterial susceptibility test in a various concentrations from low to high. Control was used to ensure the antibacterial activity from crude extract. Control positive used Tetracycline 30 µg and control negative used organic solvent. The diameter of inhibition has been shown in Table 3.

Table 3. Anti-MDR *S. haemolyticus* activity from crude extract of PPSC-27-A4

Source	Control (mm)		Concentration (mm)				
	Positive	Negative	50 µg/ml	100 µg/ml	150 µg/ml	300 µg/ml	500 µg/ml
Medium	21.5	-	10.8	11.6	11.4	11.8	11.5
Mycelia	21.5	-	11.7	10.9	-	12.2	10.3

Anti-MDR *S. haemolyticus* activity from crude extract of PPSC-27-A4 has the highest activity in 12.2 mm with concentration 300µg/ml from mycelia extract (Table 3). It means from this study, the intracellular metabolites were more effective than extracellular. Based on the literature, marine fungi majority produced hydrophobic compounds, through organic solvent extraction [29][30].

3.6. Molecular Identification

Molecular identification was used to preserve the potential isolate, thus the potential isolate could be reproduced and investigated in many cases in the future. Table 4 explained about homology analysis from sequence of PPSC-27-A4.

Table 4. Homology Analysis from Sequence of PPSC-27-A4 based on BLAST

Isolate Code	Close Relative	Homology	Acc. Number NCBI
PPSC-27-A4	*Trichoderma longibrachiatum* isolate A3S1-D3	99%	KJ767089.1

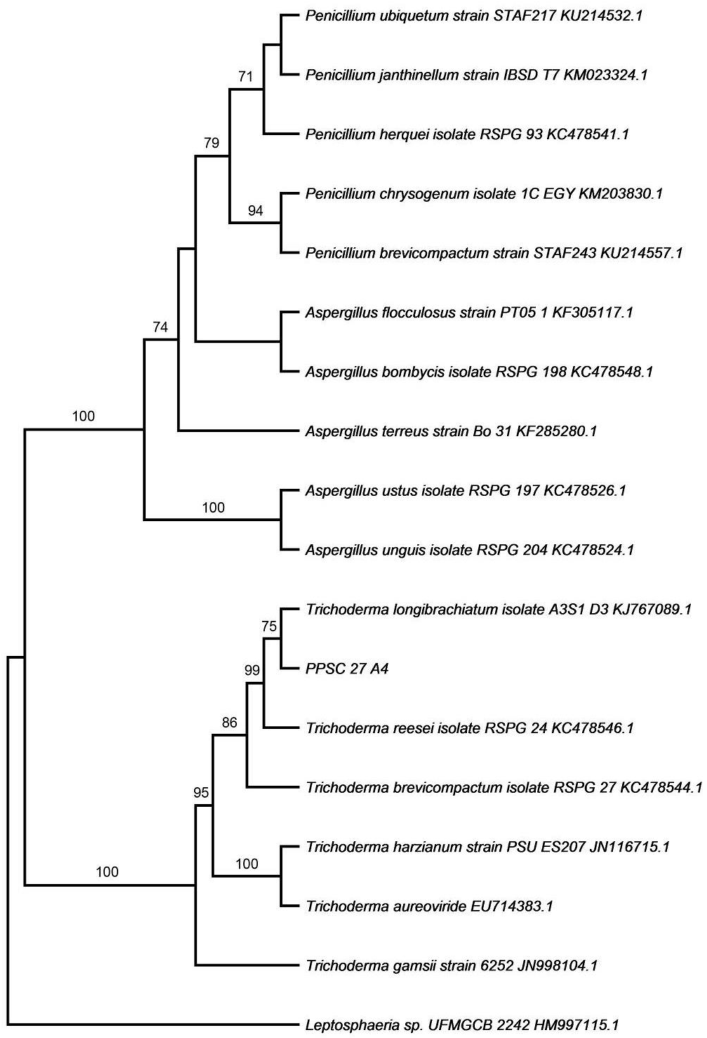

Figure 2. Phylogeny Tree based on comparison of 18S rRNA sequence using Neighbor-Joinig analysis with bootsrap 100

As mentioned by [13], the identification of potential isolate was very important, therefore, a further research is needed, so it could be reproduced. The result of sequences analysis with BLAST, this species had 99% similarity with *Trichoderma longibrachiatum* isolate A3S1-D3 (Table 4). *T. longibrachiatum* has been studied its chemical compound named ergokonin A with antifungal activity against Candida and Aspergillus species. This compound specifically could be *inhibitor of glucan synthesis in A. fumigatus* [31]. Phylogenetic tree was constructed using Neighbor-Joining to describe the relationship between this isolate and the other fungi (Fig. 2). PPSC-27-A4 was the same clade with *T. reseei* which has important metabolites in industrial products to produce second generations biofuels from cellulosic waste, that was cellulase enzyme [32].

4. Conclusion

Marine-derived fungi associated with soft corals have the ability to inhibit the growth of MDR-*S. haemolyticus*. It overcomes the supply of bioactive compound which is environmental friendly. Further research is still needed to discover chemical compound which inhibit the growth of MDR-*S. haemolyticus*.

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