Antimicrobial activity of fungi isolated from the marine sponges collected from Sekotong Beach Lombok, Indonesia

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Abstract. The search for new antibiotics from a variety of sources that have the potential to produce bioactive metabolite compounds is essential to solve the increasing problem of microbial resistance. Recently, one of the most widely studied for new antimicrobial sources is marine derived fungi. The aims of this study were to isolate endophytic fungi of marine sponges collected from Sekotong Beach Lombok, Indonesia and determine their antimicrobial activity. Fungi isolation were conducted in yeast extract, peptone, and dextrose (YPD) agar medium and each pure fungi was fermented for 21 days in YPD broth medium. Culture media and mycelium were separated by filtration method. The culture media was extracted by liquid liquid extraction and mycelium was extracted by maceration method using ethyl acetate. The dried extracts then tested for their antibacterial activity against Escherichia coli ATCC8739, Staphylococcus aureus ATCC6538, and Candida albicans ATCC 10231 using paper disk diffusion method. Extracts which showed antimicrobial activities on 250 ug/disk content were determined their minimum inhibitory concentration (MIC) and minimum bactericidal or Fungicidal concentration (MBC or MFC). The results of this research were obtained 12 fungi isolates that were coded as FSKT1 until FSKT12. The lowest MIC and MBC value against E. coli was showed by mycelium extract of FSKT10 with concentration of 128 and 256 ug/mL, respectively. The lowest MIC and MBC value against S. aureus was showed by medium extract of FSKT9 with concentration of 64 ug/mL and against C. albicans with concentration of 128 and 512 ug/mL, respectively. The antimicrobial activity observed in this research indicates that the endophytic fungi of the marine sponge could be considered as new sources of antibiotics.

Key words: antimicrobial, endophytic fungi, marine-sponge, MIC, MBC, MFC

1. Introduction
The Search for new antibiotics from various sources that have the potential to produce bioactive metabolites is an important thing to overcome the resistance of antibiotic. The potential source should be easy to obtain, renewable, and environmentally friendly [1]. Natural products such as derivatives of prokaryotic bacteria, eukaryotic microorganisms, plants and various animals are still the main sources of molecular discovery new antibiotics. As for microbial products and plant products are occupying the top rank as a source of discovery of new antimicrobial compounds until now [2].

The geographical position of the Indonesian archipelago is very strategic because it is the centre of maritime traffic intercontinental. Total sea area of Indonesian covering 5.9 million km² Indonesia as a tropical country, rich for biological source, and expressed as a high level of biodiversity. Indonesia has around 2000 species of fish from 7000 species in the world. In addition, the Indo Pacific waters, which
is mostly located in Indonesian waters, is a centre of diversity world coral reefs, with more than 400 species [3]. Therefore, Indonesia as a maritime country, it has enormous sea potential. In this case, by utilizing the available potential, research on the discovery of antimicrobial compounds can be developed. One of the ways is to conduct research on endophytic microbes that live in various marine biota and mangrove ecosystem.

Endophytic microbes, especially endophytic fungi, are known to be very useful as a source of bioactive secondary metabolites production [4]. Endophytic fungi are fungi that grow on healthy organisms in various tissues [5]. The endophytic fungi have been reported produce bioactive secondary metabolite that have been proven acts as an antimicrobial. Endophytic fungi can be found in various marine organisms like sea sponges and mangroves.

The purpose of this study was to obtain fungal isolates from sponges collected from Lombok Island which produce bioactive secondary metabolites as antimicrobial agent.

2. Materials and Method

2.1. Sample Sponges
The sea sponge samples were collected from Lombok Island by Research Center for Oceanography, Indonesian Institutes of Sciences, Jakarta. Samples were put into sterile zip plastic bag and directly stored in the cool box to be taken to laboratory in Bandung. Storage of samples was carried out at in the freezer before the samples were used.

2.2. Isolation of Endophytic Fungi.
Fungal isolation was carried out under aseptic conditions. Sample surfaces was washed by using sterile artificial sea water three times [6] and soaked with 70% ethanol (v / v) for 30 seconds. Samples were then dried and cut about 1 cm x 1 cm using a sterile knife. The open parts of samples were inoculated on the surface of the isolation media on the petri dish. The Petri dishes were incubated at 25°C for about 7 days until the growth of fungi were observed. If more than one type of fungus growth on the petri dish, each colony was transferred to the new media. The transfer process to a new media was repeated until get the pure colony [7].

2.3. Identification of Macroscopic and Microscopic
The Macroscopic characteristics of fungi are observed visually by looking at the shape of the colonies or hyphae, the colour of the surface and the back of the colony, and the texture of colony. The microscopic characteristics observed the characteristics of spores or conidia and hyphae. The fungi specimen was prepare for microscopic observation. The object glass was cleaned with 70% ethanol then a few drops of 0.9% NaCl were placed on the slide. The pure fungi isolate was taken using a circular Ose needle and scratched on the drop of 0.9% NaCl. The dye of lactophenol cotton blue was added 1-2 drops. Then covered with a glass cover object and observed under a microscope [8].

2.4. Screening of antimicrobial activity
Screening of antimicrobial activity was carried out on pure fungal collection. Pure fungal strain was small-scale fermented in YPG liquid medium for 3 weeks. The fermentation results were screened to separate media and mycelium, then each was extracted with ethyl acetate. Water content was removed from the obtained extract by the addition of anhydrous ammonium sulphate. The extract was then concentrated using a rotary evaporator.

Screening of activity test was performed by mixing a suspension of bacteria or fungi with MHA medium (Mueller Hinton Agar). Dry extract of known mass was dissolved in methanol and dropped on a paper disc that had been placed on an agar medium. After incubation, the presence or absence of inhibition produced by the extract was observed. The extract which had an inhibition was tested for microdilution. The medium and suspension of bacteria or fungi by the same amount were inserted into the micro plate well. Extract concentration series to be tested was created, i.e 1024 - µg. After an
incubation period, the smallest concentration that gave inhibition (MIC) against the growth of bacteria or fungi was determined and the value of minimum bactericidal concentration (MBC) was subsequently determined.

3. Results and Discussion

The isolation process was carried out in aseptic conditions. The used of artificial sea water in the isolation and growth media were conducted to get marine environment [9]. Based on their macroscopic and microscopic characteristic, from the isolation process was produced 12 pure fungi strain. The strain names were coded by FSKT1-FSKT12.

The cultivation of pure fungi were carried out by fermentation to produce secondary metabolites intra and extracellular. The extract of media culture and biomass were tested for antimicrobial activity. Secondary metabolites are known to be useful as competitive weapons to protect against bacterial and fungal threats [10]. Fermentation was carried out in YPD liquid medium with medium speed agitation at 150 rpm for 21 days. Fermentation done by giving agitation (movement) can produce more dissolved oxygen which is important in the production of secondary metabolites [11]. The results of this fermentation were liquid as media culture (YPD liquid media) and solids (biomass) which showed variations in pellet size formed and changed the colour of the media.

Extraction of culture media and biomass were carried out to isolate secondary metabolites. In general, the bioactive secondary metabolites were semi polar compounds. Therefore, ethylacetate was used as a solvent to extract the secondary metabolites.

Screening of extracts for antimicrobial activity was conducted against *Staphylococcus aureus*, *Eschericia coli* and *Candida albicans* by using agar disk diffuse method. The result of this experiment is shown in Table 1.

### Table 1. Result of Antimicrobial Activity Screening

| Sample | Extract Type | *E. coli* | *S. aureus* | *C. albicans* |
|--------|--------------|-----------|-------------|---------------|
| FSKT1  | Media        | -         | 7.0         | -             |
|        | Mycelium     |           | 6.9         | -             |
| FSKT2  | Media        | 9.3       | 7.5         | 11.5          |
|        | Mycelium     | -         | 7.2         | -             |
| FSKT3  | Media        | -         | 7.5         | -             |
|        | Mycelium     | -         | 7.6         | -             |
| FSKT4  | Media        | -         | 7.4         | -             |
|        | Mycelium     | -         | 6.7         | -             |
| FSKT5  | Media        | -         | 6.4         | -             |
|        | Mycelium     | -         | 7.0         | -             |
| FSKT6  | Media        | -         | 6.5         | -             |
|        | Mycelium     | -         | -           | -             |
| FSKT7  | Media        | -         | 7.3         | -             |
|        | Mycelium     | -         | -           | -             |
| FSKT8  | Media        | -         | 7.7         | -             |
|        | Mycelium     | -         | -           | -             |
| FSKT9  | Media        | 7.5       | 8.1         | 8.85          |
|        | Mycelium     | -         | -           | -             |
| FSKT10 | Media        | -         | 9.0         | -             |
|        | Mycelium     | 7.6       | 8.2         | -             |
| FSKT11 | Media        | -         | 8.2         | -             |
|        | Mycelium     | -         | 7.0         | -             |
| FSKT12 | Media        | -         | -           | -             |
|        | Mycelium     | -         | 8.4         | -             |

**Note.** - : no inhibition zone; zone inhibition of positive control, tetracycline: $30.0 \pm 2.5 (E. coli)$, $30.5 \pm 1.2 (S. aureus)$; ketokenazole 15.4 $\pm 1.7 (C. albicans)$; zone inhibition of negative control, methanol: -
Most of the extracts of fungi samples inhibit the growth of *S. aureus*. There were four samples showed antimicrobial activity against *E. coli* i.e. mycelium extract of FSKT1 and FSKT10 as well as extract of media of FSKT2 and FSKT9. The extracts which active against all the test microbes were shown by two samples extracts. They were the extract of culture media of FSKT2 and FSKT 9.

The extracts that showed antimicrobial activity were further assay to determine the minimum inhibition concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) based on CLSI method. Determination of MIC was done by using microdillution method. The smallest concentration that provides a clear solution on the microplate well was stated as MIC. The results of the MIC were used as a reference to determine MBC/ MFC. The wells that provide MIC and higher concentration were inoculated into agar media that contained the test microbes. The smallest concentration that showed no microbial growth on agar media was stated as the MBC/MFC. The result of MBC and MFC is shown in Table 2.

The lowest MIC and MBC value against *E. coli* was showed by mycelium extract of FSKT10 with concentration of 128 and 256 μg/mL, respectively. The lowest MIC and MBC value against *S. aureus* was showed by medium extract of FSKT9 with concentration of 64 μg/mL, respectively and against *C. albicans* with concentration of 128 and 512 μg/mL, respectively. Based on MIC and MBC determination, the strength of antimicrobial agents could be classified as bactericidal/fungicidal or bacteriostatic/fungistatic. The ratio MBC to MIC (MIC index value) of antimicrobial agents less than 2 classified as bactericidal [12]. Therefore, there were several extract have bactericidal activity such as extract media of FSKT2, FSKT 9, and extract mycelium of FSKT10 against *E. coli*, the extract media of FSKT2-11 and the extract mycelium of FSKT2, 4, 5, 10,11 against *S. aureus*. Fungicidal activity was shown by the extract media of FSKT2 against *C. albicans*.

Table 2. Results of MBC and MFC

| Sample  | Extract Type | MIC (μg/mL) | MBC/MFC (μg/mL) |
|---------|--------------|-------------|-----------------|
|         |              | *E. coli*   | *S. aureus*     | *C. albicans* | *E. coli* | *S. aureus* | *C. albicans* |
| FSKT1   | Media        | -           | 2048            | -             | > 2048    | -           | -             |
|         | Mycelium     | 2048        | -               | > 2048        | -         | -           | -             |
| FSKT2   | Media        | -           | 512             | 512           | 1024      | 512         | 1024          |
|         | Mycelium     | -           | 64              | -             | 2048      | -           | -             |
| FSKT3   | Media        | -           | 2048            | 1024          | 1024      | 1024        | -             |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT4   | Media        | -           | 2048            | 1024          | 1024      | 1024        | -             |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT5   | Media        | -           | 2048            | 2048          | 2048      | 2048        | -             |
|         | Mycelium     | -           | 2048            | > 2048        | 2048      | 2048        | -             |
| FSKT6   | Media        | -           | 2048            | 2048          | 2048      | 2048        | -             |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT7   | Media        | -           | 128             | -             | 128       | -           | -             |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT8   | Media        | -           | 128             | -             | 256       | -           | -             |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT9   | Media        | 256         | 64              | 128           | 512       | 64          | 512           |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT10  | Media        | 256         | -               | -             | 512       | -           | -             |
|         | Mycelium     | 128         | 128             | 256           | 256       | -           | -             |
| FSKT11  | Media        | -           | 128             | -             | 256       | -           | -             |
|         | Mycelium     | -           | -               | -             | -         | -           | -             |
| FSKT12  | Media        | -           | 256             | -             | 512       | -           | -             |

Note: - : not conducted
4. Conclusion
As a result of the isolation of fungi from sea sponges were obtained 12 of fungi strain, namely FSKT 1-12. The lowest MIC and MBC value against E. coli was showed by mycelium extract of FSKT10 with concentration of 128 and 256 ug/mL, respectively. The lowest MIC and MBC value against S. aureus was showed by medium extract of FSKT9 with concentration of 64 ug/mL and against C. albicans with concentration of 128 and 512 ug/mL, respectively. The antimicrobial activity observed in this research indicates that the endophytic fungi of the marine sponge could be considered as new sources of antibiotics.

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