The Molecular Identification of Sea Fungus Isolates from Mangrove Litter as Antimicrobials Potential

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Abstract. Marine microorganisms, one of which is sea fungus, have the potential to produce active metabolites which have pharmacological effects. This study aims to identify pure fungus isolates from mangrove litter and tested its antimicrobial activity on bacteria Escherichia coli, Staphylococcus aureus and fungus Candida albicans. In identification result of molecular using primary Internal Transcribed Spacer, Penicillium biourgeianum species were obtained in sequences DNA CTGGGTCAC...TAT. Sea fungus isolates later are fermented using a shaker at a speed of 150 rpm for 21 days and separated between the media and mycelium. The media was extracted liquid and the mycelium was macerated using ethyl acetate. Screening test of antimicrobial activity obtains the result showing antimicrobial activity against the tested microbes. Media culture and mycelium extracts which have antimicrobial activity are then determined by the MIC and MBC values. Most of the MIC test values are 256 µg/mL, which provide strong inhibitory. MBC value gained is 512 µg/mL showing a strong bactericidal. Study result shows that sea fungus isolates produce active metabolites that mostly have antimicrobial activity against Staphylococcus aureus ATCC29737, Escherichia coli ATCC10536, and Candida albicans ATCC10231.

1. Introduction
Among the three main habitats in the biosphere, sea occupies almost 70% of the earth’s surface. Sea is a place to live for many kinds of living organisms especially microbes. Marine microbes are not only found on the sea surface, but also in the deepest part of the sea, the beach, mangrove, corals, etc. The different environment between sea and land create microorganisms which form a defense in the form of secondary metabolite with different types and potential. These secondary metabolites certainly have the effect that can be used as a natural product with certain biological activities [4].

One of the sea microorganism that has big potential to be a natural product is a fungus. Sea fungus produces a secondary metabolite which has a variety of biological activities such as antimicrobial effect [8].

Due to huge marine potential in producing products with pharmacological effect, the exploration is necessary in order to find new microorganism which produces antimicrobial compound, especially fungus. Sea fungus that has a different defense with mushrooms will produce secondary metabolite with its different activity as well. These study results are expected to become a basis for the development of new medicine in the future.
2. Material and Methods

Material used in this study is sample of fungus from mangrove litter in ecological area, distilled water, NaCl, MgSO$_4$.7H$_2$O, MgCl$_2$.6H$_2$O, KCl, CaCl$_2$.2H$_2$O, NaHCO$_3$, HCl, NaOH, physiological NaCl, yeast extract, peptone, dextrose, agar, agar media MHA (Mueller Hinton Agar), MHB (Mueller Hinton Broth), SDA media (Sabouraud Dextrose Agar), ethyl acetate, alcohol 70%, streptomycin sulphate, tetracycline HCl, chloramphenicol, ketoconazole, ammonium sulphate anhydrite, lactophenol cotton blue, Staphylococcus aureus ATCC29737, Escherichia coli ATCC10536, and Candida albicans ATCC10231. Tools used are Petri dishes, test tubes, Erlenmeyer, measuring cups, Bunsen, autoclaves, incubator, cotton, gauze, freezer, analytical balance, pH meters, baths, Ose needles, measuring pipettes, filler, micropipettes, tip, matches, beaker, disc paper, media bottle, test tube rack, label paper, mattress threads, heat-resistant paper, aluminium foil, parchment paper, Buchner funnel, rotary evaporator, micro titer plate, and UV-visible spectrophotometry.

Initial preparation begins with tools sterilization, material and making sterile artificial seawater. Later, the making of agar media for purification and sea fungus cultivation, microscopic observation along with the making of microbial test suspense [2].

2.1. Molecular Identification

DNA extraction carried out by using the method of ZR fungal or bacteria DNA miniprep kit. Afterward, the extracted DNA used as a template for the amplification stage. DNA amplification conducted using Polymerase Chain Reaction (PCR) using ITS primer. The product of PCR resulted was characterized first using DNA electrophoresis and visualized through UV transilluminator. If the characterization shows the size of the product suitable with theoretical size, then it is continued by sequencing to determine nucleotide sequences.

2.2. Fermentation and extraction

Fungal isolates were fermented in YPD sterile liquid media for 21 days using a shaker. The expected fermentation result is in the form of small mycelium in pellets and clear liquid culture. This separation result is in mycelium and liquid culture or media. Mycelium extracted through maceration technique with ethyl acetate solvent, while media extracted through liquids extraction technique. Extraction results are concentrated using rotary evaporator and dried at room temperature.

2.3. Antimicrobial activity test

Antimicrobial activity test carried out using the agar diffusion method with MHA media for bacteria and SDA for fungus. The media suspension was homogenized and then allowed to solidify and three sterile disc papers were put on the media. Extract and standard antibiotic were put in the three discs as a positive control and negative control. The standard antibiotic used is tetracycline HCl for S. aureus, chloramphenicol for E.coli, and ketoconazole for Candida albicans. Then incubation was carried out and afterward the measurement of inhibitory diameter.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The determination of MIC on Escherichia coli bacteria and Staphylococcus aureus along with Candida Albicans fungus was carried out using MHB media for bacteria and SDB for fungus with microdilution and incubation method. The smallest concentration in the well that is clear or unable to bacteria turbidity is the MIC value. The extract with growth inhibitory of the strongest tested bacteria was found in the smallest MIC value. The determination of minimum bactericidal concentration (MBC) conducted on MHA media and SDA by subculturing MIC test result on the agar media which is divided into 8 zones and incubated. The smallest concentration in the zone which is unable to
provide bacteria growth is MBC value from the extract. The extract with the strongest bactericidal was found in the smallest MBC value.

3. Result and Discussion
Preparation on YPD agar media in a liquid state was put into a sterile petri dish and put in streptomycin sulfate with a concentration of 50 mg/litre media [6]. The administration of antibiotic streptomycin sulfate aims to kill the bacteria so that they do not grow on the media. Next, the identification of microscopic carried out aseptic, when adding one to two drops of lactophenol cotton blue dye before the 70% ethanol dries to kill the living organism, lactate acid to maintain the fungus structure and cotton blue which can color chitin on the fungal cell wall. Morphological microscopic results are characterized by septate hyphae and conidial form such as Ascomycota.

Sea fungus is a eukaryote organism with more complete cell wall structure so that a very strong lysis method is necessary in order to be able to lysis the cell wall that DNA can be extracted [5]. The result of molecular biology analysis shows that the size of PCR product ± 600 pb and the identification shows Penicillium biourgeianum species with CTGGGTCAC...TAT DNA sequences.

Fermentation is a process of producing a large number of microorganism culture products. Fermentation was carried out on a shaker at a speed of 150 rpm for 21 days at room temperature. Agitation conducted in the process of fermentation in order to increase the amount of dissolved oxygen in the cultivation media [7]. Optimum fungal growth can be reached when using agitation at a speed of 150 rpm [3]. Fermentation result obtained later filtered using Buchner funnel. Filtering is done to separate the mycelium part and fermented media part. Mycelium part extracted to obtain intracellular metabolite from fungus and the media part extracted to obtain intracellular metabolite from fungus [9]. The extraction was carried out twice to gain an optimum result. The extracted result obtained was added to ammonium sulfate to attract the remaining water contained in the extract. Ethyl acetate used because it is semipolar so that it can attract semipolar fungal secondary metabolites. Next, the extract results were concentrated using rotary evaporator at 25°C temperature, 50 rpm speed, and 74 bar pressure. The concentration was carried out at room temperature so that the active metabolite compounds are not damaged by the temperature.

Based on the result of antimicrobial activity, it was found that media extract has activity against all tested microbes, yet for the mycelium extract only inhibited one type of microbe. It is because the extracellular metabolite has activity compared to its intracellular so that the nutrients needed by extra cell fluid will be fulfilled that it will influence the cell metabolism system.

Table 1. The Inhibitory Diameter of Media Extract Culture and Mycelium on the Tested Microbes

| Extract   | MIC (µg/mL) | E. coli | S. aureus | C. albicans |
|-----------|-------------|---------|-----------|-------------|
| Fungus Isolates | Media     | 256     | 256       | 256         |
|           | Mycelium   | 512     | -         | -           |

In the result of microdilution determination, the smallest MIC value obtained in the test is 256 µg/mL which is owned by the liquid media culture extract against tested microbes. A plant extract has a strong microbial activity if it has MIC value of < 500 µg/mL, media activity if it has MIC value of 600-1500 µg/mL, and weak activity if it has MIC value of > 1600 µg/mL [1]. Therefore, it can be seen that liquid media culture extract is strong against S. aureus bacteria, E. coli and C. Albicans with MIC value of 256 µg/mL each. Mycelium culture extract has media activity against E.coli bacteria with MIC value of 512 µg/mL. Minimum killing concentration is the lowest concentration showed by the absence of bacterial growth in the inoculation area.
Table 2. Minimum Inhibitory Concentration of Media Culture and Mycelium Extract against Tested Microbes

| Extract 250 µg/disk | Inhibitory Diameter (mm) |
|---------------------|-------------------------|
|                     | E. coli | S. aureus | C. albicans |
| Fungus Isolates     |         |           |             |
| Media               | 12.9    | 9.2       | 10.3        |
| Mycelium            | 10.4    | -         | -           |

The table shows that the MBC value of liquid media culture extract against tested microbes is strong in the value of 512 µg/mL and mycelium culture extract against E.coli bacteria is media. Most of the extract has MBC value at the concentration of 512 µg/mL. This shows that media culture extract has the strongest bactericidal to the three tested microbes.

Table 3. Minimum Bactericidal Concentration of Media Culture and Mycelium Extract against Tested Microbes

| Extract | MBC (µg/mL) |
|---------|-------------|
|         | E. coli | S. aureus | C. albicans |
| Fungus Isolates |         |           |             |
| Media   | 512     | 512       | 512         |
| Mycelium| 1024    | -         | -           |

4. References

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