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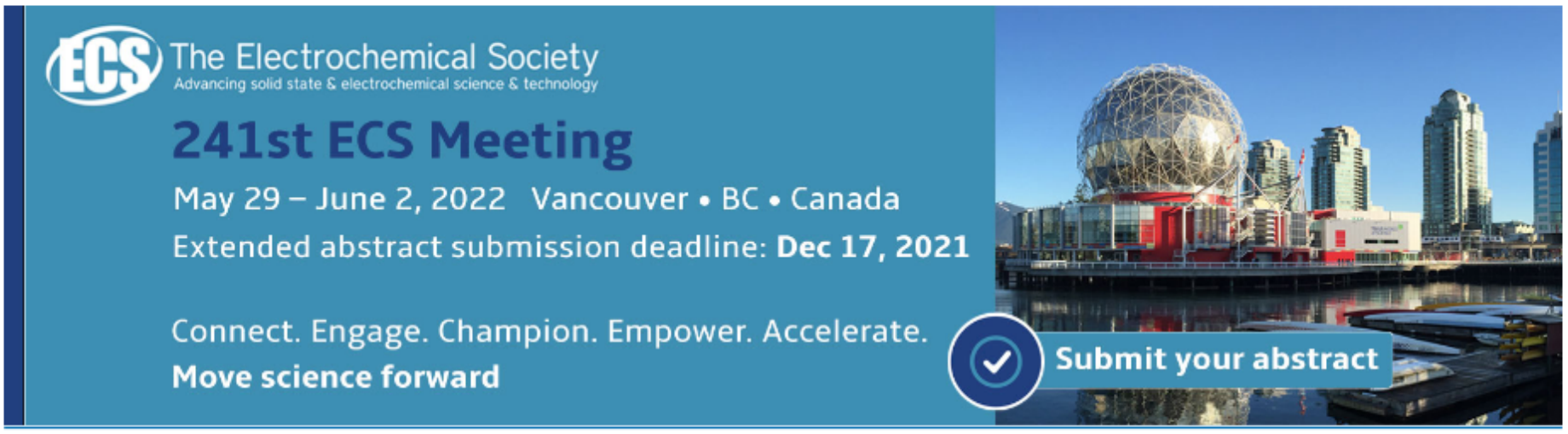

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Screening Extract EtOAc Sponge Derived Fungi Against Clinical *Staphylococcus aureus* to Obtain Sustainable Natural Product

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Abstract. The increasing resistance of pathogenic bacteria to various antibiotics worldwide has become a severe problem for medicine and human health. This study aims to determine the antibacterial activity of extract ethyl acetate (EtOAc) produced by sponge-derived fungi as an antibacterial agent. This study obtained nineteen fungal isolates from the marine sponges in Singaraja, Buleleng Bali, Indonesia. The pathogenic bacteria *Staphylococcus aureus* was obtained from patients at Abdul Moeloek Hospital, Bandar Lampung. The susceptibility test of *Staphylococcus aureus* was carried out on nine types of commercial antibiotics using the disk diffusion method. The solid-state fermentation (SSF) method on rice media carried the cultivation and co-cultivation of fungi. The bioactivity of the extract was tested against pathogenic bacteria *S. aureus*. The results of the susceptibility test to antibiotics showed *S. aureus* resistance to amoxicillin, ciprofloxacin, erythromycin. Showed that extracts A12RF, A05RF, C36RF had inhibitory activity against the growth of *S. aureus* at a concentration of 0,5mg/mL. These results indicate that co-cultivation can induce fungi to produce different secondary metabolites. This basic information is essential for further studies related to the production of fungal bioactive compounds through the co-cultivation stage in the SSF process.

1. Introduction

The tendency of pathogenic bacteria to become resistant to various types of antibiotics is a severe problem in medicine and human health. The results of a literature review related to clinical data show an increase in the resistance of pathogenic bacteria in the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Species Enterobacter*) has become a worldwide concern. As the cause of increasing disease burden, increasing mortality rate, and decreasing treatment options, ESKAPE pathogenic bacteria are a severe threat to human health. Researchers have made various efforts to overcome these problems, one of which is getting new antibiotic compounds that can inhibit ESKAPE bacteria [1].

Until now, natural products are still relied on as a source of new bioactive compounds that have antibiotic properties. Natural products ingredients have been shown to make a significant contribution in medicine, one of which is to treat diseases due to bacterial infections. However, exploring the potential of natural compounds also presents a challenge to obtain new compounds. Some of the technical challenges that are often faced include finding new sources, screening stages, isolation of active compounds, and elucidation of structures. In this study, the search for new sources focused on bioactive compounds from fungi derived from sponges [2].

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Several studies have shown that fungi are a potential source of bioactive compounds [3]. In fact, like the entophytic fungi of land plants, dereplication of bioactive compounds also often occurs in marine fungi. For overcoming one strategy is to use co-cultivation techniques with target microorganisms [4]. Until now, studies on the co-cultivation of fungi sourced from sponges are still minimal when compared to the diversity of existing marine fungi. So, the opportunity to get active compounds from sponge-derived fungi is possible.

This work focuses on the induced metabolites from the co-cultivation of marine-derived fungi with *S. aureus* under solid stated fermentation. The activity of the bioactive compounds produced by the fungus was then tested to inhibit the growth of *S. aureus,* which is known to be resistant to several types of antibiotics. According to our knowledge, the study of bioactive compounds from fungi derived from sponges is still minimal. Of course, the information obtained from this study will be very helpful in developing the potential for natural compounds in certain areas.

2. Method

2.1. Clinically Pathogenic Bacteria

The clinical pathogenic *S. aureus* used in this study was collected from Abdul Moeloek Hospital, Bandar Lampung, Indonesia. The feasibility of clinical isolates of *S. aureus* bacteria as a screening model and pathogenic bacteria were tested against several types of commercial antibiotics using the Disk diffusion method [5], according to CLSI guidelines.

2.2. Isolate Fungi

Sponge samples were obtained from coastal waters. Buleleng Bali in August 2018. Sampling was carried out randomly at five different points. Isolate fungi was deposited at the Technical Service Unit-Integrated Laboratory of the Center for Innovation and Technology Unila (UPT-LTSIT) and maintained using media malt extract (ME) and Tryptic soy agar (6.0% malt extract, 0.6% Tryptic Soy Broth (TSB), 2.0% nutrient agar (NA), and 3.8 % artificial seawater (ASW) at 30°C.

2.3. Cultivation, Co cultivation and Preparation Extract

The isolate of the fungi was cultivated in liquid media, refers to Tang *et al*. [6] with several modifications. Isolate fungi were cultured in the rice medium for 14 days at room temperature in static conditions. In the co-culture of fungi with *S. aureus*, pre-cultured *S. aureus* in complete TSB was mixed with the supernatant of the seed culture of fungi in the three Erlenmeyer flasks. The fungi growth rate was monitored during fermentation for 14 days. The cultured fungi strain was extracted using EtOAc. The filtrate obtained was concentrated using a vacuum rotary evaporator at a temperature of 95°C and a pressure of 122 mbar.

2.4. Antibacterial assay

The method of *antibacterial assay* refers to [7] Dolatabadi *et al*. (2018) with some modifications. The assay was performed using a sterile 96-well microplate. *S. aureus* was cultured on 2% (w/v) nutrient agar (NA). Inoculum (medium TSB) was adjusted to 0.5 McFarland standard turbidity (OD 0.08-0.1). The test was carried out in 3 repetitions. 0.5 mg/mL chloramphenicol solution was used as a positive control, and 0.5 mg/mL sample extract as the test compound. Incubate inoculated assay plates for 18 hours at 37°C. The optical density (OD) values were measured using a microplate reader. After that, resazurin was added and incubated for 2-8 hours, and the OD values were measured at 630 nm with an interval of 2 hours. Minimum inhibition concentration (MIC) test only on active extract.

2.5. Morphological Identification

Spores, mycelium, and sporulation structures were examined microscopically by the modified cover glass culture method [8]. Coverslip was observed under an Axio Zeiss Image A1 (100X) microscope.

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3. Result and Discussion

3.1. Antibiotic susceptibility

In this study, the pathogenic bacteria *S. aureus* obtained from patient's skin were tested against several types of antibiotics (Figure 1). Our finding showed that *S. aureus* isolates are susceptible to doxycycline hyclate (DCH), and cefadroxil (CFDX) based on the inhibition zone measurements on agar media results. *S. aureus* is resistant to the intermediate category. *S. aureus* is resistant to clindamycin (CDM), ciprofloxacin (CFX), erythromycin (ERH), lincomycin (LC), and amoxicillin (AMX). This indicates that the clinical isolate of *S. aureus* belongs to the group of ESKAPE pathogenic bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*), which are resistant to several types of antibiotics so that it can be used as a screening model for bioactive compounds.

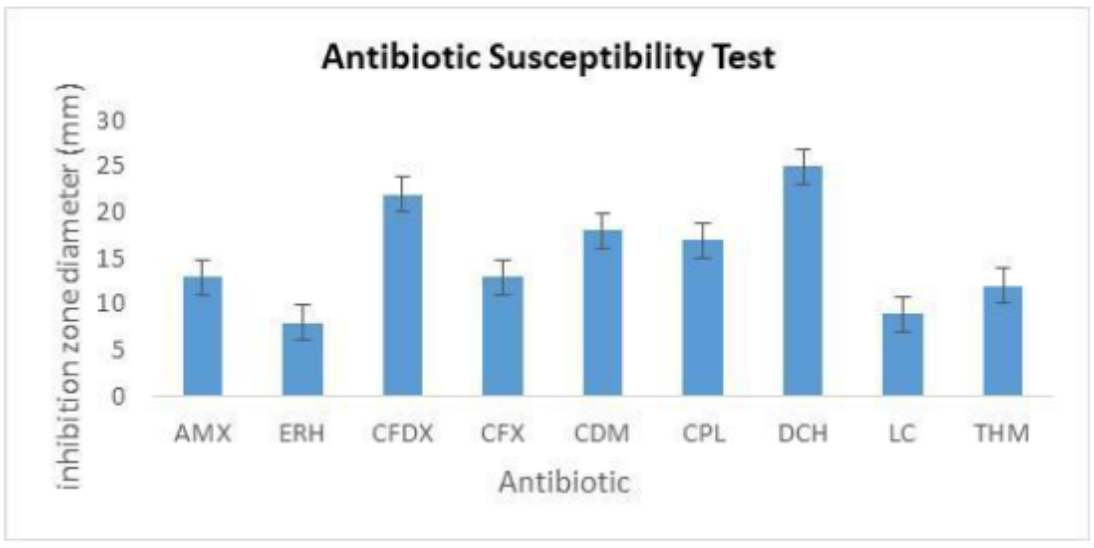

Figure 1. Antibiotic susceptibility test clinical *S. aureus*.

3.2. Isolate Fungi

Seventeen strains of isolate fungi were selected from sponges and tunicate, as shown in Table 1. The results of fungi isolation obtained information about the content of complex microorganisms contained in organisms in the ocean, such as sponges and tunicates, as has been successfully reported by Tang *et al*., 2019 obtained fungi from sponges and tunicate [9]. The study on fungi originating from the sea has a relatively small number, about 0.6% of the total number of fungi globally [10]. It is estimated that many fungi have not been isolated. Considering that fungi have a role in marine ecosystems as saprophytes, parasites, or symbionts. Furthermore, marine fungi have been reported to be present in sea fans [11], corals [12], and macroalgae [13].

3.3. Antibacterial assay

For determining the activity of the ethyl acetate extract (EtOAc) fungi that were cultured and co-cultivated using solid rice media, *S. aureus* bacteria were screened using the well plate reader 96 methods. Based on the cultivation results, isolates A02RF, A06RF, B19RF, B21RF, C30RF, and C31RF have antibacterial activity at a 0.5mg/mL concentration. Meanwhile, in co-cultivation, the same bioactivity was observed in EtOAc extracts C27RF, A05RF, D36RF, and A12RF as depicted in Table 2.

The extract of A12RF showed the highest activity compared to other extracts based on the measured OD values described in Table 2. The increase in the inhibitory power of A12RF extract on the growth of *S. aureus* can be caused by several factors, one of which is due to co-cultivation treatment. Under co-cultivation conditions, it causes competition between different microorganisms to defend themselves, thus triggering the formation of secondary metabolites through activation and transcription under stress conditions. The competition or antagonism experienced during co-cultivation is shown to lead to a significantly enhanced production of constitutively present compounds and to an accumulation of cryptic compounds that are not detected in axenic cultures of the producing strain [14]. Co-cultivation proving that this experimental approach for increasing the chemical productivity.

Table 1. Isolate Fungi

No	Sample Code	Organisms	Isolate Fungi	Color
1	18A02	Sponge	18A02RF	White
2	18A05	Sponge	18A05RF	White
3	18A06	Sponge	18A06RF	White
4	18A09	Sponge	18A09RF	White
5	18A12	Sponge	18A12RF	White-Orange
6	18A13	Sponge	18A13RF	White-Greyish
7	18B14	Sponge	18B14RF	Greyish-Black
8	18B15	Sponge	18B15RF	White
9	18B19	Sponge	18B19RF	White
10	18C20	Sponge	18C20RF	White
11	18C21	Sponge	18C21RF	White
12	18C23	Sponge	18C23RF	White
13	18C24	Sponge	18C24RF	White
14	18C27	Sponge	18C27RF	Orange-White
15	18C30	Sponge	18C30RF	White
16	18C31	Sponge	18C31RF	White
17	18D36	Tunicate	18D36RF	Brown-green
18	18E41	Sponge	18E41RF	Greyish-green
19	18E42	Sponge	18E42RF	White

Table 2. Antibacterial assay of Fungi extract against *S. aureus*

No.	Extract Code	Cultivation (OD±SD)	Co-Cultivation (OD±SD)
1.	A02RF	1.361 ± 0.019	0.599 ± 0.003
2.	A05RF	0.927 ± 0.007	1.093 ± 0.084
3.	A06RF	1.127 ± 0.017	0.620 ± 0.084
4.	A09RF	0.704 ± 0.185	0.639 ± 0.027
5.	A12RF	1.114 ± 0.008	1.059 ± 0.098
6.	A13RF	1.079 ± 0.009	0.842 ± 0.225
7.	B14RF	1.079 ± 0.076	0.572 ± 0.012
8.	B15RF	0.965 ± 0.004	0.696 ± 0.027
9.	B19RF	1.160 ± 0.211	0.581 ± 0.028
10.	C20RF	0.944 ± 0.043	0.607 ± 0.034
11.	C21RF	1.079 ± 0.012	0.534 ± 0.110
12.	C23RF	1.090 ± 0.031	0.970 ± 0.050
13.	C24RF	0.832 ± 0.041	0.492 ± 0.337
14.	C27RF	0.932 ± 0.018	1.058 ± 0.040
15.	C30RF	1.123 ± 0.019	0.966 ± 0.039
16.	C31RF	1.281 ± 0.027	0.660 ± 0.102
17.	D36RF	1.050 ± 0.034	0.888 ± 0.031
18.	E41RF	1.059 ± 0.027	0.643 ± 0.211
19.	E42RF	0.970 ± 0.326	0.650 ±0.018

3.4. Morphology selected fungi

As described in Table 2 above, EtOAc extracts from cultured fungal isolates A02RF, A06RF, B19RF, B21RF, C30RF, and C31RF had antibacterial activity. Further studies related to the morphology of each isolate are shown in Figure 2. Morphologically there are differences in the types of fungi seen from the spores produced. The fungus has spores that are shaped like flowers, round chains, and leaf-shaped. The morphology of each of these fungi can be observed and distinguished using a magnification of 100x. The observations showed that each isolate had characteristic differences in interlocking spores, mycelium, and hyphae. This shows that each isolate is a different species.

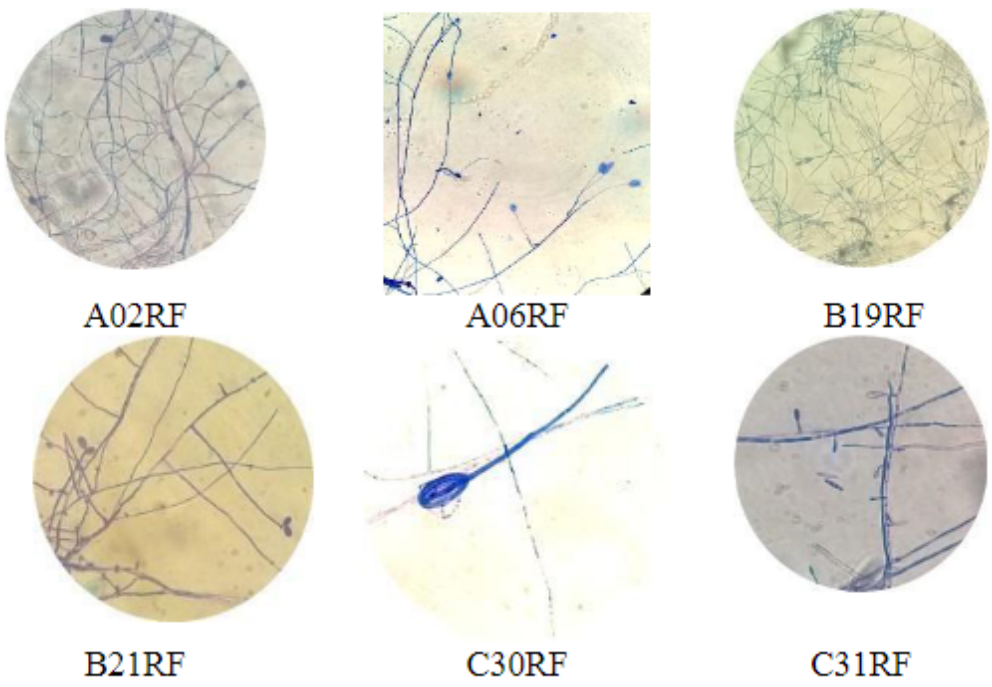

Figure 2. Morphology of fungi active Cultivation.

The same thing was observed for differences in morphological characteristics of the isolates of co-cultivated fungi, A05RF, A12RF, and C36RF. Each of these isolates showed different morphological characteristics Figure 3.

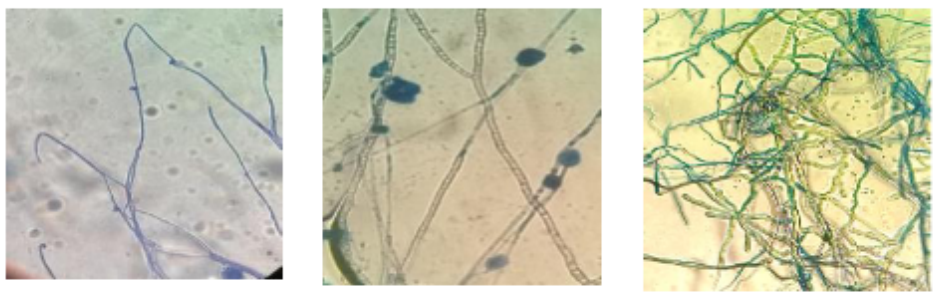

Figure 3. Morphology of fungi active Co cultivation.

The results showed that each isolate A05RF, A12RF, C36RF had different spores and hyphae forms. In detail, the characteristics for the A12RF isolate having the highest activity are as follows conidia and vesicles typically shaped like a flower, long shaped conidiophores from white to yellowish. Conidiophores are smooth-walled. Conidia are round to elliptical, hyaline to light white-yellowish, and smooth-walled. The vesicles are pretty round and 10-20 µm. This morphological feature of A12RF Marine fungal is close to the Aspergillus genus. Aspergillus is a group of Ascomycota phylum. Generally, fungi have morphological characteristics: filamentous, hyphae or septa, and are abundant in nature [15].

4. Conclusion

Based on the data obtained, it can be concluded that the isolates sponge-derived fungi were able to produce metabolites that could inhibit the growth of *S. aureus*. Co-cultivation techniques can affect the activity of the secondary metabolites produced. This information is essential as initial data for further studies related to the structural elucidation of the active compound and its mechanism of action.

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