ISOLATION AND SCREENING ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI DERIVED FROM TWO MARINE SPONGES AGAINST *ESCHERICHIA COLI*

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Abstract

Escherichia coli is known to cause several infectious diseases such as urinary tract infection and gastrointestinal disease. Marine endophytic fungi from marine sponges have potential as source of new compound against *Escherichia coli*. In the present study, two marine sponges were collected in Shwe-Thaung-Yan coastal area for the isolation of marine fungi on August, 2018. The isolation was undertaken by surface sterilization method. In this study, seven endophytic fungi were isolated from two marine sponges. Among these isolated fungi, two isolates, SF-04 and SF-05 showed antibacterial activity against *Escherichia coli*. According to fungi identification results, fungus SF-04 was identified as *Trichoderma* sp. and fungus SF-05 was identified as *Aspergillus* sp..

Keywords: antibacterial activity, *Aspergillus* sp, endophytic fungi, *Escherichia coli*, marine sponge, *Trichoderma* sp.

Introduction

Microorganisms are a rich source of structurally unique bioactive substances. Since the 1940s, over 30,000 natural products have been discovered from microorganisms, more than 10,000 of which are biologically active (Fenical, 1993). Several characteristics of microorganisms make them important sources of bioactive natural products. Many bioactive compounds, especially antibiotics, have been isolated from microbiological sources. Of the natural products that have been developed into drugs, many come from plant sources, but there have been a considerable number of important drugs harvested from microorganisms and marine sources (da Rocha, 2001). Marine derived fungi are a rich source of structurally new natural products with a wide range of biological activities (Somei and Yamada, 2005, Blunt et *al.*, 2006).

Sponges are a part of the benthic fauna and live in all areas of the marine world, from the shallow coastal seas to the deepest oceans (Levi, 1998). Most of them occur in the marine environment and only about 1% inhibits freshwater (Belarbi et *al.*, 2003). Sponges are well known to be hosts for a large community of microorganisms. Some bioactive compounds isolated from marine organisms have been shown to exhibit anticancer, antimicrobial, antifungal and other pharmacological activities (Natori et *al.*, 1994). Majority of the marine natural products have been isolated from the wide variety of marine microorganisms living in sponges' tissues (Williams et *al.*, 2007).

Escherichia coli are a common inhabitant of the gastrointestinal tract of humans and animals. They are often the most abundant facultative anaerobes in this environment. There are *E.coli* strains that are harmless commensals of the intestinal tract and others that are major pathogens of humans and animals. The pathogenic *E. coli* are divided into those strains causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites (Ketia et *al.*, 2012). Therefore, isolation and screening antibacterial activity of endophytic fungi from marine sponges were carried out in this research work.

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Materials and Methods

Sampling Site and Sponge Collection

Two marine sponge samples, *Cloina* sp. (Duchassaing and Michelotti, 1864) and *Amphimedon* sp. (Duchassaing and Michelotti, 1864) were collected at Shwe-Thaung-Yan coastal area (Lat. 17 °04' N, Long. 94° 26') nine miles up away from Chaungtha Beach of Ayeyarwady Region. Specimen collection was carried out on August, 2018. All samples of each species were collected at the depths of 0.5-3 m in the intertidal zone and gloves were worn during collection. Specimens were transferred directly to sterile plastic bags containing seawater to prevent contact of sponge tissue from air. The specimens were transported to the laboratory and processed immediately for the isolation and cultivation of fungi. Alternatively, sponge tissues were stored in refrigerator at - 80˚C for identification and future studies.

Figure 1 Map showing the location of sample collected area

Isolation of endophytic fungi

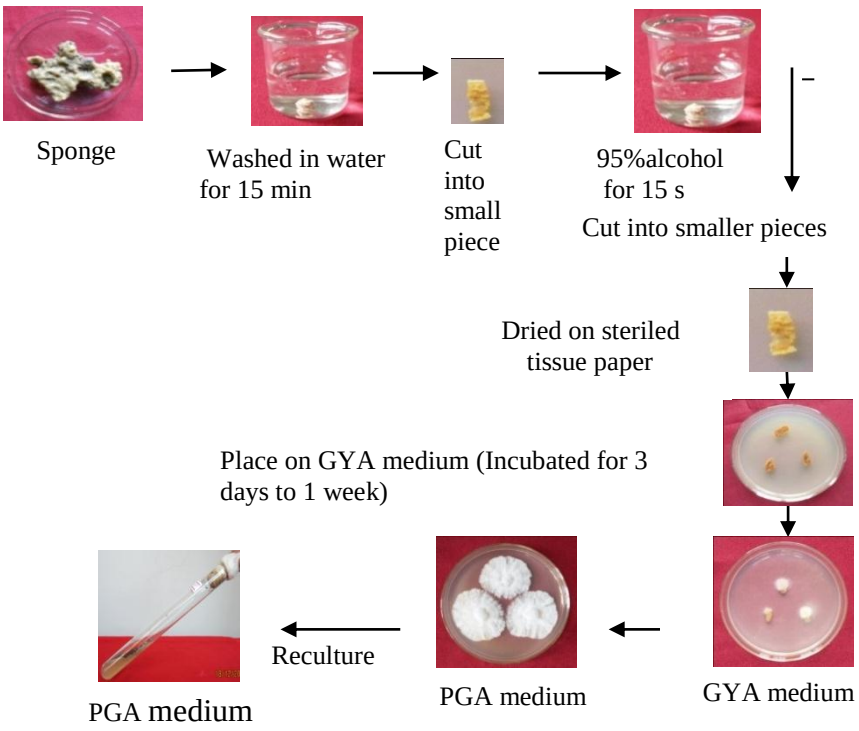
Isolation of fungi was undertaken by Surface Sterilization Method (NITE, 2004) (Figure. 2). The live sponge sample was washed with water for 15 minutes to get rid of nonspecific fungal species from seawater column on sponge surface and cut into small pieces. The cutting samples were disinfected with 95 % alcohol for 15 second and then, cut into the smaller pieces at each end and dried on the sterile tissue paper. After drying, sponge samples were plated on glucose yeast extract agar (GYA) medium containing chloramphenicol. The plates were incubated at 25˚C for 3 days to 1 week until the morphology of fungi could be distinguished. Each isolate was picked up and transferred onto the plate of potato glucose agar medium containing chloramphenicol. The resulting plates were also incubated at room

temperature for pure culture and then, maintained in the slants containing PGA medium for further studies.

The media used for the isolation of endophytic marine fungi are as follow.

GYA medium (Glucose Yeast extract Agar Medium)	
Glucose	2.0 g
Yeast extract	0.5 g
Agar	1.8 g
DW	80 ml
Seawater	20 ml
pH	6.5
(after autoclaving chloramphenicol was added to the medium.)	

PGA medium (Potato Glucose Agar Medium)	
Glucose	2.0 g
Potato	20g
Agar	1.8 g
DW	80 ml
Seawater	20 ml
pH	6.5
(after autoclaving chloramphenicol was added to the medium.)	



GYA medium = Glucose Yeast extract Agar medium

PGA medium= Potato Glucose Agar medium

Figure 2 Surface Sterilization Method for the isolation of endophytic fungi from marine sponge

Study on the Antibacterial Activities

Preliminary study for antibacterial activity against *Escherichia coli* was carried out by the paper disc diffusion assay method (NITE, 2004).

Procedure for antibacterial activity test

A cut of mycelium from seven days old culture of each plate was cultured in a conical flask containing 50 ml of seed medium and incubated at the temperature of 25˚C. After three days, 5% of seed medium was taken by sterile pipette and poured into another conical flask containing 50 ml of fermentation medium and also incubated at the temperature of 25˚C. After 7 days, a sterile paper disc (8 mm in diameter) was impregnated in the fermentation medium and dried at least for 10 hours. About 20 ml of sterilized assay medium was poured into each sterile Petri plates and added o.5 ml of liquid culture of corresponding test organisms and allowed to solidify. And then, each dried paper disc was placed in order onto the assay plate and a non-impregnated sterile paper disc was also placed on the assay plate as a control. All the plates were incubated at 25˚C for 24 hours. After 24 hours incubation, the plates were observed for the formation of clear inhibition zone around the paper disc. The clear zone was examined by measuring the diameter of the clear zone with the aid of a digital clipper.

Media used for antimicrobial activity test

Seed Medium

Glucose	2.0 g
Yeast extract	1.0 g
NaCl	0.1 g
K_2HPO_4	0.001 g
DW	80 ml
Seawater	20 ml (28 %)
pH	6.5

Fermentation Medium

Glucose	3.0 g
Glycerol	0.3 ml
Yeast extract	0.3g
Polypeptone	1.2 g
K_2HPO_4	0.001 g
$MgSO_4$	0.001 g
$CaCO_3$	0.1 g
DW	80 ml (28 %)
Seawater	20 ml
pH	6.5

Assay Medium

Glucose	1.0g
Peptone	0.3g
Agar	1.8 g
DW	80ml
Seawater	20 ml (28 %)
pH	6.5

Identification of endophytic fungi

Identification was achieved by taxonomic process set up direct comparison of specimen and by the use of keys, description and illustration. The microscopic examinations of cultures were done on PGA medium under microscope (400X) and identified according to Ando and Inaba (2004).

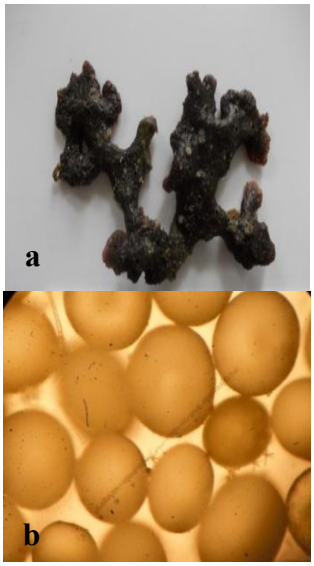
Selection of test organism

The test organism, *Escherichia coli* was obtained from the laboratory of BRBDC of Pathien University.

Results

Classified lists of sponge species collected from study area

Two species of marine sponges were collected from Shwe- Thaung- Yan coastal area. The classification system of the recorded species was followed after Khin Zar Nyo (2004).



Phylum- Porifera
Class- Demospongiae
Sub Class- Monaxonida
Order- Haplosclerida
Family- Niphatidae
Species- *Amphimedon* sp.
(Duchassaing and Michelotti, 1864)

Description - massive to repent branches (1-3cm in diameter); contains volcano-shaped oscules (2-5mm); light gray in color; soft and compressible in consistency; found on shallow reefs and seagrass beds.

Figure 3 (a) Morphology of *Amphimedon* sp. (Duchassaing and Michelotti, 1864)
(b) Micrograph of spicules (400X)

Phylum - Porifera
Class - Demospongiae
Sub-Class- Monaxonida
Order- Hadromerida
Family- Clionidae
Species - *Cliona* sp. (Duchassaing and Michelotti, 1864)

Description- thin to thick encrusting (0.2-5cm in thickness), or massive lobate; smooth; velvety surface with oscules (0.2-3cm in diameter) bearing cream colored membranes; firm and rubbery; found on the reef and seagrass environments.

Figure 4 (a) Morphology of *Cliona* sp. (Duchassaing and Michelotti, 1864)
(b) Micrograph of spicules (400X)

Isolation of Endophytic Fungi from Marine Sponges

In this study, seven endophytic fungi were isolated from two marine sponges by Surface Sterilization Method (Table 2).

In the prescent study, four fungi were isolated from marine sponge *Amphimedon* sp. and three fungI from *Cliona* sp. (Table 1).

Table 1 Isolated fungi from two sponges' species by surface sterilization method

Sample	Total Isolated Fungi	
	Total Isolated Fungi	Fungi No
Amphimedon sp.	4	**SF-01,02,03,04**
Cliona sp.	3	**SF-05,06,07**
Total Isolated Fungi	**7**	**7**

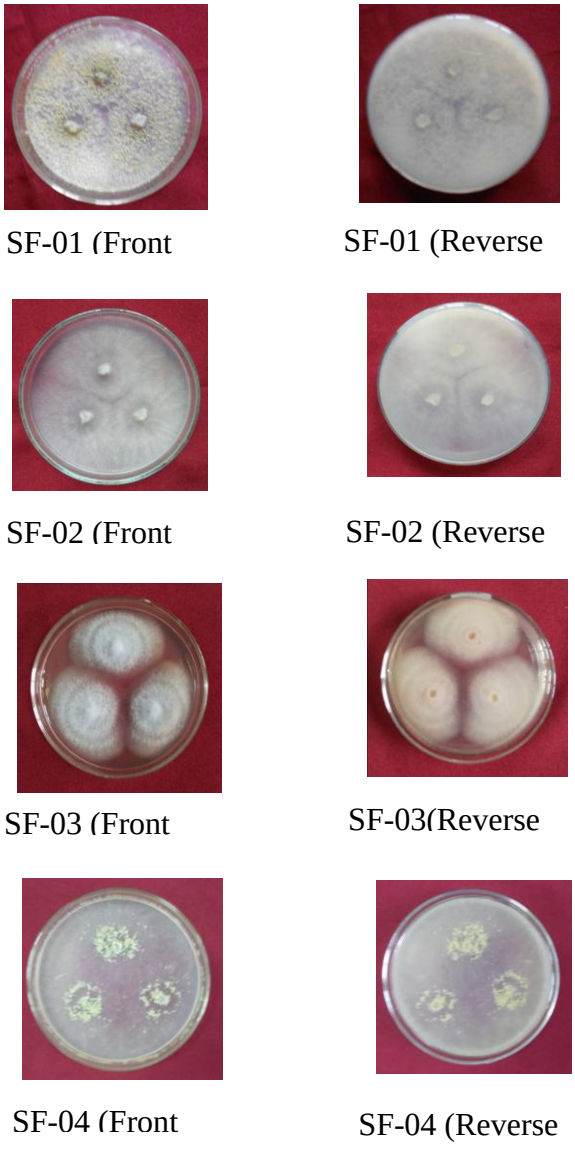
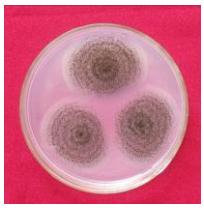


Figure 5 Morphologies of endophytic fungi isolated from *Amphimedon* sp.

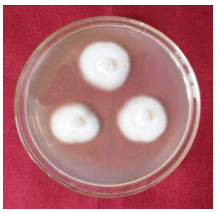


SF-05 (Front

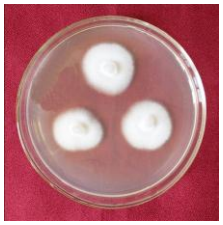
SF-05 (Reverse side)

SF-06 (Front

SF-06 (Reverse side)



SF-07 (Front



SF-07(Reverse

Figure 6 Morphologies of endophytic fungi isolated from *Cliona* sp.

Antibacterial Activity of Isolated Fungi against *Escherichia coli* by Paper Disc Diffusion Assay

Antibacterial activity was carried out by the paper disc diffusion assay method. In the present study, the fungus SF - 04 isolated from *Amphimedon* sp. and the fungus SF – 05 isolated from *Cliona* sp. were shown distinct clear zone against *Escherichia coli* and the other fungi were not shown any inhabitation zone (Table 2).

Table 2 Antibacterial Activity of Isolated Fungi against *Escherichia coli* by Paper Disc Diffusion Assay (7 days fermentation)

Fungi No.	**Inhibitory zone (mm)**
SF-01	no activity
SF-02	no activity
SF-03	no activity
SF-04	29.84
SF-05	35.48
SF-06	no activity
SF-07	no activity

Figure 7 Antibacterial Activity of Isolated Fungus SF - 04 against *Escherichia coli*

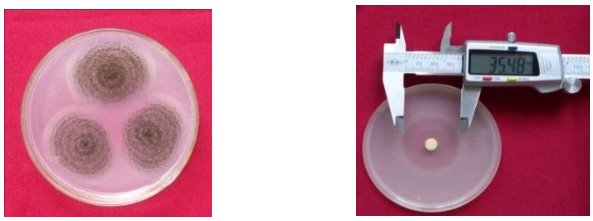


Figure 8 Antibacterial Activity of Isolated Fungus SF - 05 against *Escherichia coli*

Identification of endophytic fungus SF - 04 and SF - 05

Identification of endophytic fungui which showed the clear zone of inhabitation against *Escherichia coli* was done according to Ando and Enaba (2004). According to fungi identification results, fungus SF-4 was identified as *Trichoderma* sp. (Figure 9) and fungus SF-5 was identified as *Aspergillus* sp. (Figure 10).

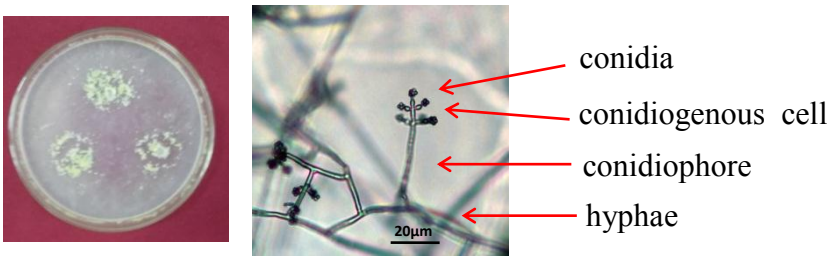


Figure 9 Morphology and photomicrograph (400 X) of fungus SF-04 (7 days old culture on PGA medium)

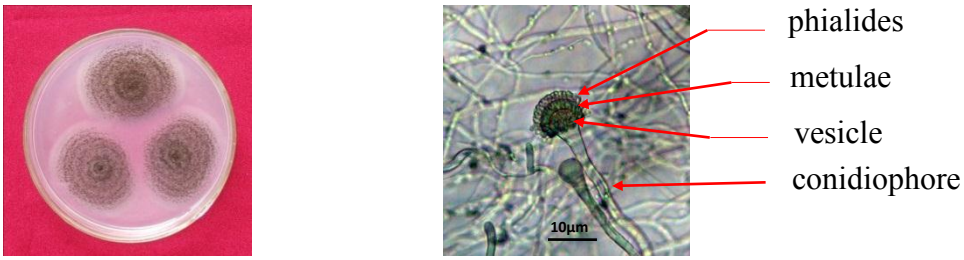


Figure 10 Morphology and photomicrograph (400 X) of fungus SF-05 (7 days old culture on PGA medium)

Identification Keys of Mitosporic Fungi (Ando and Enaba, 2004)

1. Synnemata form ---------- Synnematous fungi

1. Synnemata not form ---------- 2

2. Conidium axis curved through more than 180 ---------- Helicoconidium

2. Conidium axis not curved through more than 180 ---------- 3

3. Conidium with more than one axis; protuberances(s), other than setulae, present and more than ¼ the length of the spore body -- Stauroconidium

3. Conidium with only one axis; any protuberances, other than setuale, if present, not more than ¼ the length of the spore body ---------- 4

3. Conidial shape not above ---------- Miscellaneous fungi

4 Length/ width ratio of conidium body exceeding 15:1---------- Scolecoconidium

4. Length/ width ratio of conidium body less than 15:1---------- 5

5. Conidium with 1septa ---------- Didymoconidium

5. Conidium lacking septa (Ameroconidium) ---------- 6

6. Conidiophores not produced or not clear ---------- *Arthrinium* sp.

6. Conidiophores with or without septa, developed single and enteroblastic conidia ---------- 7

7. Conidiophores without septa, not branched and multi phialides with parallel arrangement and straight ending in a large vesicle---------- *Aspergillus* sp.

7. Conidiophores with septa, developed single and branched cluster into fascicles---------- *Trichoderma* sp.

Discussion

Marine fungi have been recognized as an important and untapped resource for novel bioactive compounds. The chemical compounds of marine microorganisms are not well known as terrestrial counterparts. However, in the last decade, several bioactive compounds have been isolated from marine fungi and bacteria. These natural compounds are new resources for the development of medically useful compounds (Donia and Haman, 2003, Anand et *al.*, 2006). Antibacterial activities among marine fungi and bacteria were a well-known phenomenon and had been demonstrated in a number of studies (Isnansetyo and Kamei, 2003, Uzair et *al.*, 2006).

In the present study, two marine sponges collected from Shwe Thaung Yan coastal area were employed for the isolation of marine endophytic fungi. Endophytic fungi were isolated by the method of Surface sterilization Method (Figure 2). In this study, seven endophytic fungi were isolated from two marine sponges (Table 1). Four fungi were isolated from *Amphimedon* sp. and three fungi were isolated from *Cliona* sp.. Easson et *al.,* 2014 mentioned the microbial diversity of *Cliona varians* to find the relationships between the microbial community patterns and sponge population structure. Antibacterial activity of all isolates against *Escherichia coli* was carried out

by paper disc diffusion assay method. In the present investigation, endophytic fungus SF – 04 inhibited on *Escherichia coli* (29.84 mm) and SF – 05 inhibited on *Escherichia coli* (35.48 mm) (Figure 7 and Figure 8). By the microscopic observations, fungus SF-04 was identified as *Trichoderma* sp. and fungus SF-05 was identified as *Aspergillus* sp. (Figure 9 and Figure 10). According to Ghisalberti and Sivasithaamparam (1991), *Trichoderma* species were well-known as antifungal producers and they have been used as biological control agent for various phytopathogenic fungi.

Conclusion

Although marine sponges have been shown the bioactive potential for various natural products, the microbial study on marine sponges are very rare. For this reason, the present study was carried out on the antibacterial activity of marine microbes associated with marine sponges in the coastal region of Myanmar. But there is still a need for the extensive study of marine microbes and their relationships to their environment.

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