THE EFFECTS OF PH AND FERMENTATION MEDIA ON THE ANTIBACTERIAL ACTIVITY OF SECONDARY METABOLITE PRODUCING FROM MARINE DERIVED FUNGI*

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Abstract

The seagrass, *Enhalus acoroides*, was collected from Pho Htaung Gyaing, Shwe-Thaung-Yan Sub Township on June, 2019 for the isolation of marine derived fungi. The isolation was undertaken by Surface Sterilization Method. Six endophytic fungi were isolated and their antibacterial activities were tested against three test organisms. In the present study, three isolates showed the antibacterial activity against *Escherichia coli* and among them, fungus FE- 05 showed the highest activity on *Escherichia coli*. Therefore, fungus FE - 05 was selected for the investigation of the effects of pH and fermentation media on the antibacterial activity against *Escherichia coli*. In this study, the maximum antibacterial activity of secondary metabolite produced from fungus FE- 05 was observed in Potato Yeast Extract Medium under pH 6.5. According to identification result, the selected fungus FE - 05 was identified as *Cladosporium* sp.

Keywords: antibacterial activity, *Cladosporium* sp., endophytic fungi, *Escherichia coli*, seagrass

Introduction

Various researchers stated that endophytic fungi were a good source of bioactive natural products. Most of the investigations on endophytic fungi have been isolated from terrestrial plants (Naik et *al.*, 2008, Andrade- Linare et *al.*, 2011). However, bioactive natural compounds produced from endophytic fungi of marine plants including seagrass species have been rarely studied (Sakayaroj *et al.*, 2010).

Seagrasses are a relatively small group of flowering plants and distributed all along the three Coastal Regions of Myanmar, namely the Rakhine Coastal Region, the Ayeyarwady Delta and the Gulf of Mottama Coastal Region and the Tanintharyi Coastal Region. In Myanmar, twelve species of seagrasses were recorded (Soe-Htun et *al*., 2009).

Seagrasses played important roles in marine ecosystem. They served in stabilizing soil particles, reducing wave energy and providing a large shelter for a variety of marine animals (Hori et *al*., 2009). Some species of seagrasses were used as traditional medicine such as malaria and skin disaeases in India (Kumar et *al*., 2008).

Escherichia coli are the common facultative anaerobes inhabit the gastrointestinal tract of humans and animals (Ketia et *al.*, 2012). Most of *E. coli* strains are harmless but other strains can cause diseases such as watery diarrhea, bloody diarrhea and urinary tract infections (Nataro and Kaper, 1998).

Since microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites (Supaphon et *al*., 2014). For this reason, the objectives of the present research were to isolate and screen the endophytic fungi for antibacterial activities and to observe the effects of pH and fermentation media on the antibacterial activities of secondary metabolites produced from endophytic marine derived fungi.

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Materials and Methods

Sampling Site and Samples Collection

Figure 1 Map showing the location of sample collected area

The whole plant samples of *Enhalus acoroides* (Linnaeus f.) Royle were collected at Pho Htaung Gyaing, Shwe-Thaung-Yan Sub Township (Lat. 17 ˚10' 46" N, Long. 94˚ 28' 13" E) about thirteen miles up away from Chaungtha Beach of Ayeyarwady Region. Specimen collection was carried out on June, 2019. All specimens were collected in the intertidal zone and gloves were worn during collection. Specimens were transferred directly to sterile plastic bags containing seawater to prevent contact of plant tissue from air. The specimens were transported to the laboratory of Marine Science Department and processed immediately for the isolation and cultivation of fungi. Alternatively, some specimens were stored in refrigerator at - 80˚C for identification and future studies.

Isolation of endophytic fungi

The isolation was carried out by Surface Sterilization Method (NITE, 2004). Seagrass samples including leaves and rhizomes were washed thoroughly by running tap water. The plant parts (leaves and rhizomes) were surface- sterilized in 70 % ethanol for 3 minutes and rinsed into sterile water. The samples were dried on sterilized paper. The dried samples were then cut at the edge and then, placed onto the Glucose Yeast Extract Agar (GYA) medium (NITE, 2004) containing o.3g /ml of Penicillin G. Plates were incubated at room temperature for 3-7 days until the outgrowths of endophytic fungi were observed. The fungi were subcultured to produce pure culture on Low Carbon Agar (LCA) medium (NITE, 2004) and then stored in the slant for further investigation such as the screening of antibacterial activity.

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
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5
(after autoclaving Penicillin G was added to the medium.)	

Low Carbon Agar (LCA) Medium (NITE, 2004)	
Glucose	1.0 g
Sucrose	0.2 g
K_2HPO_4	0.1 g
$MgSO_4.7H2O$	0.05 g
KNO_3	0.1 g
KCL	0.05 g
Agar	1.8 g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5
(after autoclaving Penicillin G was added to the medium.)	

Study on the Antibacterial Activities

Preliminary studies for antibacterial activities against three tests were carried out by the paper disc diffusion assay method (NITE, 2004). The three test organisms, *Staphylococcus aureus*, *Pseudomonas fluorescence* and *Escherichia coli* were obtained from the laboratory of BRBDC of Pathien University.

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 150 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. 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. All assays were carried out in triplicate.

Media used for antimicrobial activity test

Seed Medium	
Glucose	2.0 g
Yeast extract	1.0 g
NaCl	0.1 g
K_2HPO_4	0.001g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5

Fermentation Medium (Potato Glucose Yeast Extract Medium)	
Potato	20g
Glucose	2.0 g
Yeast extract	0.5g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5

Assay Medium	
Glucose	1.0g
Peptone	0.3g
Agar	1.8 g
Distilled Water	100ml
pH	6.5

Study on the effect of fermentation medium for the antibacterial activity of secondary metabolite

To investigate the effect of fermentation medium, the antibacterial activity of secondary metabolite produced from the selected isolated fungus was studied in four fermentation broths (Endo and Inaba, 2004) namely, Glucose Yeast Extract Medium, Potato Glucose Yeast Extract Medium, Glucose Malt Extract Medium and Glucose Malt Extract Peptone Medium. Four kinds of fermentation medium are as follows.

I. Glucose Yeast Extract Medium	
Glucose	2.0 g
Yeast extract	0.5g
$CaCO_3$	0.1 g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5

II. Potato Glucose Yeast Extract Medium	
Potato	20g
Glucose	2.0 g
Yeast extract	0.5g
$CaCO_3$	0.1 g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5

III. Glucose Malt Extract Medium	
Glucose	2.0 g
Malt extract	0.5g
$CaCO_3$	0.1 g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5

III. Glucose Malt Extract Peptone Medium	
Glucose	2.0 g
Malt extract	0.5g
Peptone	0.5g
$CaCO_3$	0.1 g
Distilled Water	80 ml
Seawater	20 ml (30 ‰)
pH	6.5

In this study, 100 ml of each broth was taken in 250 ml conical flasks. These flasks were autoclaved at 121°C, for 1 hour. After autoclaving, each flask was inoculated with five mm disk of the fungus inoculum grown on PDA medium. The inoculated flasks were incubated at 25°C for 7 days under stationary condition. The broth was filtered through sterilized Whatman filter paper No.1 and the culture filtrates were then tested for antibacterial activity against test pathogens by using paper disc diffusion assay.

Study on the effect of pH on the antibacterial activity of secondary metabolite

The optimization of pH of the fermentation media on the antibacterial activity of secondary metabolite was done by carrying out the fermentation study at five different pH values 5.0, 5.5, 6.0, 6.5 and 7.0 (Furtado *et al*., 2005). For each pH value, 100 ml of Potato Glucose Yeast Extract Medium (adjusted to desired pH by using either 1N NaOH or 0.1 N HCI) was taken in 250 ml conical flasks. These flasks were autoclaved at 121°C for 1hour. Three replicates were used for each pH values. A cut of mycelium (five mm diameter) from seven days old colony of selected fungus was added as an inoculum in each flask. The inoculated flasks were incubated at 25°C for 7 days under stationary condition. The filtration was done through sterilized Whatman filter paper

No. 1 and various filtrates were tested for antibacterial activity against the test pathogens by using paper disc diffusion assay.

Identification of endophytic fungi

Identification was achieved by means of observation on macroscopic features and detail microscopic characteristics of colonies. The microscopic examinations of selected fungus was done on MEA medium under microscope (Olympus, CX 41) in Marine Science Department and identified according to Ando and Inaba (2004).

Results

Classification of seagrass species collected from study area

The seagrass species was collected from Pho Htaung Gyaing, Shwe- Thaung- Yan Sub-township. The classification of the recorded seagrass species was referenced according to Soe-Htun et *al.* (2009).

Phylum- Tracheophyta
Class- Magnoliopsida
Order- Alismatales
Family- Hydrocharitaceae
Genus - Enhalus
Species- *E. acoroides*
(Linnaeus f.) Royle, 1839.

Description – Plant erect; the rhizome thick, about 1-2cm in diameter with tough black fibers; shoots pronounced at the node, with 3-6 leaves; leaf blades flat and linear, 70-180 cm long, 0.8-2.0 cm wide, with 35-55 nerves and ribs at the margin, apex obtuse, base narrow without lingual, margin slightly serrulate in young leaves.

Figure 2 Habit of *Enhalus acoroides* (Linnaeus f.) Royle.

Isolation of Marine Derived Endophytic Fungi from *Enhalus acoroides*

In the prescent study, six endophytic fungi were isolated from the seagrass, *Enhalus acoroides* by Surface Sterilization Method (Table 1). Two fungi were isolated from rhizome of the collected seagrass plant and four fungi from leaf (Table 1, Figure 3).

Table 1 Isolated fungi from *Enhalus acoroides* by surface sterilization method

Sample	Part use	Isolated fungi	Fungi No.
Enhalus acoroides	Rhizome	2	FE-01,02
	Leaf	4	FE- 03,04,05,06
Total isolated fungi		6	6


FE-01 (Front side)


FE-01 (Reverse side)


FE-02 (Front side)


FE-02 (Reverse side)


FE -03 (Front side)


FE -03(Reverse side)


FE -04 (Front side)


FE -04 (Reverse side)


FE -05 (Front side)


FE -05 (Reverse side)


FE -06 (Front side)


FE -06 (Reverse side)

Figure 3 Morphologies of endophytic fungi isolated from *Enhalus acroides*

Antibacterial Activities of Isolated Fungi against Three Test Organisms by Paper Disc Diffusion Assay

Antibacterial activities of six isolated fungi were carried out by the paper disc diffusion assay method against three test organisms, namely *Staphylococcus aureus*, *Pseudomonas fluorescence* and *Escherichia coli*. In the present study, the three fungi (one fungus isolated from rhizome and two fungi from leaf) showed distinct clear zone against only *Escherichia coli*. All of isolated endophytic fungi did not show any activities on the other test organisms, *Staphylococcus aureus* and *Pseudomonas fluorescence* (Table 2).

Table 2 Antibacterial Activity of Six Isolated Fungi against Three Test Organisms by Paper Disc Diffusion Assay (7 days fermentation)

Fungi No.	Test Organisms		
	Staphylococcus aureus	***Pseudomonas fluorescence***	***Escherichia coli***
FE-01	no activity	no activity	23.52 ±0.33 mm
FE -02	no activity	no activity	no activity
FE -03	no activity	no activity	no activity
FE -04	no activity	no activity	24.25 ±0.35 mm
FE -05	no activity	no activity	27.75 ±0.43 mm
FE -06	no activity	no activity	no activity

± standard deviation (SD)

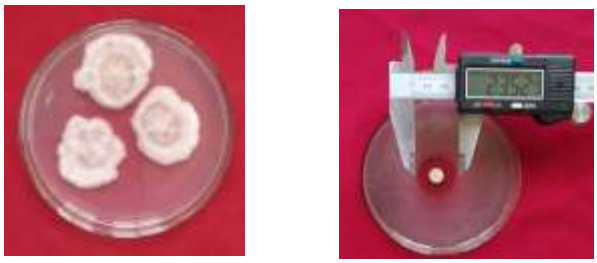


Figure 4 Antibacterial Activity of Isolated Fungus FE-01against *Escherichia coli*

Figure 5 Antibacterial Activity of Isolated Fungus FE-04 against *Escherichia coli*

Figure 6 Antibacterial Activity of Isolated Fungus FE-05 against *Escherichia coli*

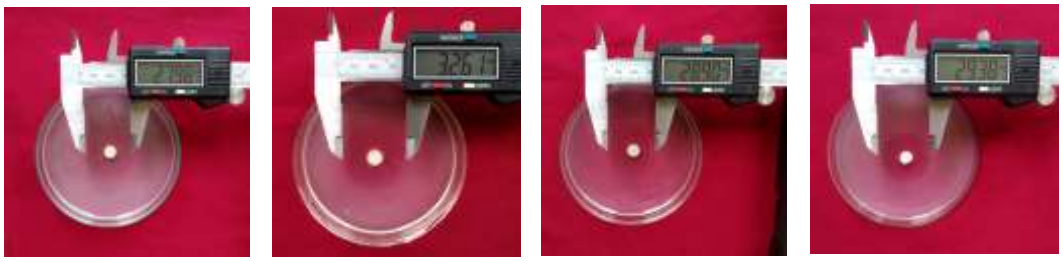
Effect of Fermentation Medium on the Antibacterial Activity of Secondary Metabolite

To investigate the effect of fermentation medium, the antibacterial activity of secondary metabolite produced from the selected isolated fungus FE- 05 was studied in four fermentation broths (Endo and Inaba, 2004). In this study, Potato Glucose Yeast Extract Medium showed the maximum inhabitation zone of 32.61 ±0.35 mm among the rest of other fermentation media (Table 3, Figure 7).

Table 3 Antibacterial Activity of Secondary Metabolite Produced from Selected Fungus FE – 05 on the Four Kinds of Fermentation Medium

Fermentation medium	Inhibitation Zone (mm)
I. Glucose Yeast Extract Medium	27.98 ±0.34 mm
II. Potato Glucose Yeast Extract Medium	32.61 ±0.35 mm
III. Glucose Malt Extract Medium	28.90 ±0.35 mm
IV. Glucose Malt Extract Peptone Medium	29.38 ±0.35 mm

± standard deviation (SD)



(I) (II) (III) (IV)

Figure7 Antibacterial Activity of Secondary Metabolite Produced from Selected Fungus FE – 05 on the Four Kinds of Fermentation Medium

Effect of pH on the Antibacterial Activity of Secondary Metabolite

The optimization of pH of the fermentation media on the antibacterial activity of secondary metabolite was done by carrying out the fermentation study at five different pH values 5.0, 5.5, 6.0, 6.5 and 7.0. In this study, maximum inhabitation zone of 32.33 ±0.27 mm was observed at pH value of 6.5 (Table 4, Figure 8).

Table 4 Antibacterial Activity of Secondary Metabolite Produced from Selected Fungus FE – 05 on Five Different Kinds of pH Value

pH Value	Inhibitation Zone (mm)
pH 5.0	27.42 ±0.38 mm
pH 5.5	29.55 ±0.35 mm
pH 6.0	32.26 ±0.34 mm
pH 6.5	32.33 ±0.27 mm
pH 7.0	29.84 ±0.37 mm

± standard deviation (SD)

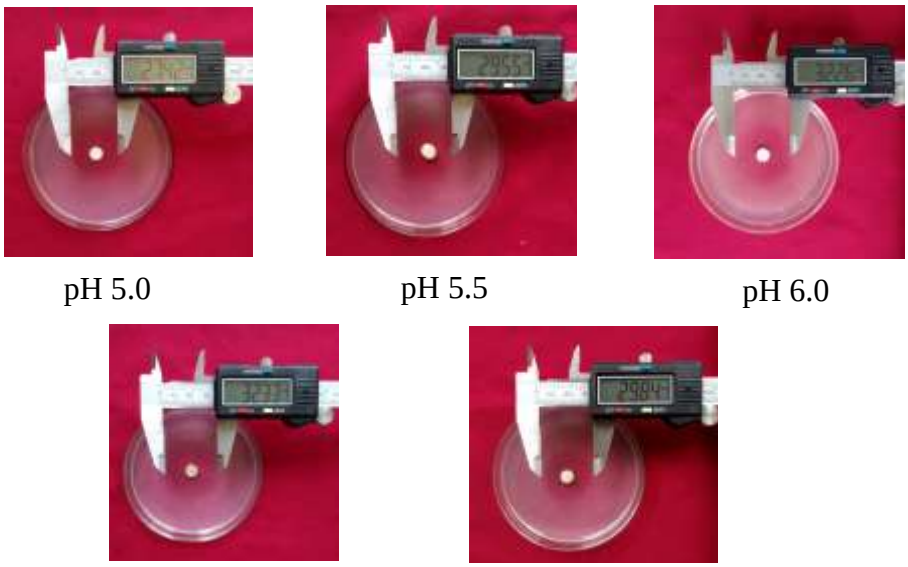


Figure 8 Antibacterial Activity of Secondary Metabolite Produced from Selected Fungus FE – 05 on Five Different Kinds of pH Value

Identification of endophytic fungus FE - 05

Identification of selected endophytic fungus which showed the clear zone of inhabitation against *Escherichia coli* was done according to Ando and Inaba (2004).

Macroscopic Features of endophytic fungus FE - 05

Colonies are slow growth. Texture is velvety to powdery. The color is olivaceous green to olivaceous brown.

Microscopic Features of endophytic fungus FE - 05

Colonies produce septate hyphae. Hyphae, conidiophores and conidia are pigmented. Conidiophores are distinct from vegetative hyphae, erect and straight, mostly unbranched. Conidia are produced in branched acropetal chains, consist of one to two-celled, and have a distinct hilum. Conidia are close to the conidiophore where the chain branched and forming the "shield-shaped" appearance. According to these external morphology and microscopic results, fungus FE-05 was identified as *Cladosporium* sp. (Figure 9).

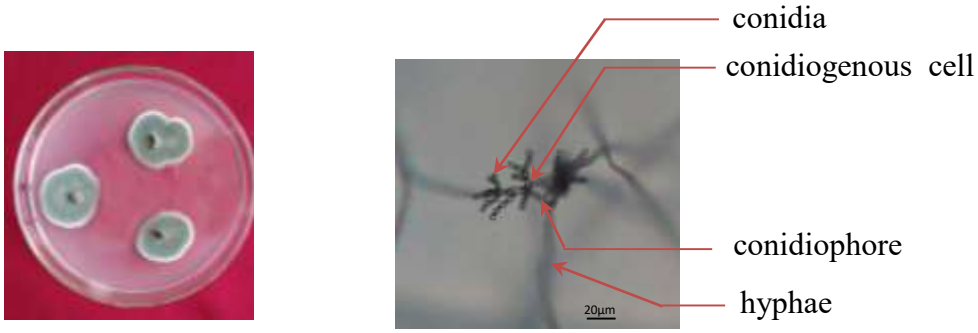


Figure 9 Morphology and photomicrograph (800 X) of fungus FE-05

Discussion

Marine derived fungi were known as the important colonizers of a large variety of organic substrates including sponges, corals, mollusks, seagrasses, seaweeds and other invertebrates. They also act as endophytes, saprobes, parasites and pathogens in the marine ecosystem (Loque et *al*., 2010). In the recent years, several bioactive compounds have been isolated from various marine microbes. These bioactive natural compounds represent the new resources for the development of medically useful compounds (Anand et *al.*, 2006).

In the present study, one of the seagrass species, *Enhalus acoroides* collected from Shwe-Thaung-Yan coastal area were employed for the isolation of marine endophytic fungi. In this study, only six endophytic fungi were isolated from *Enhalus acoroides*, two fungi from rhizome and four from leaf (Table 1). Antibacterial activities of all isolates against three test organisms were carried out by paper disc diffusion assay method. In the present investigation, endophytic fungus FE-05 showed the maximum inhabitation zone against *Escherichia coli* (Table 2 and Figure 6). All of isolated fungi did not show any antibacterial activity on the other test organisms, *Staphylococcus aureus* and *Pseudomonas fluorescence*. However, Than Than Aye (2019) described that five endophytic fungi isolated from three seagrasses species have been shown the antibacterial activity on *Pseudomonas fluorescence*.

In this research work, four fermentation broths were used in the investigation of effects of fermentation medium and it is found that Potato Glucose Yeast Medium showed the maximum inhabitation zone. Moreover, the effects of pH on the fermentation medium were also studied with five different pH values. In this research work, the maximum inhabitation zone was observed at pH value of 6.5. This result is closely related with the environmental condition of intertidal zone in which seagrass species grown very well. By the microscopic observations, fungus FE-05 was identified as *Cladosporium* sp. (Figure 9).

Conclusion

Although seagrass plants have shown the bioactive potential for various natural products, the microbial studies on seagrass species are very rare. For this reason, the present study was carried out on the screening of antibacterial activity of marine microbes associated with seagrass plants in the coastal region of Myanmar. But there is still a need for the extensive study of other environmental parameters such as temperature, salinity, alkalinity, etc. and these parameters greatly influence on the growth and metabolic production of marine microbes.

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