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Research

Antibacterial Potential of Fungal Endophytes From Selected Seaweeds From Johor Coast

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ABSTRACT

Endophytic fungi from marine seaweed have been known to be the source of new secondary biological metabolites. The ample coast in Malaysia in particular the Johor Coast has diverse marine seaweed, home to potential marine endophytic fungi. In this light, this study aims to characterise endophytic fungi in selected seaweeds from the Johor Coast and determine the antibacterial potential. Fungal endophytes were aseptically isolated from brown seaweed, *Sargassum* sp. and green algae, *Ulva lactuca*. Macroscopic and microscopic observations were performed for characterisation as fungal genera. Sequence analysis of Internal Transcribed Spacer (ITS) suggested the five fungal isolates as *Trichoderma asperellum*, *Aspergillus aculeatus*, *Aspergillus fumigatus*, *Penicillium* sp. FKI-3389 and *Hypoxylon monticulosum*. Antibacterial activity was determined by minimum inhibition concentration assay against five human pathogenic bacteria. Only *T. asperellum*, *A. fumigatus* and *H. monticulosum* showed antibacterial potential with the latter indicating broad-spectrum antibacterial activity. As a conclusion, five endophytic fungal species were successfully determined from the brown and green seaweeds. Three of the fungal endophytes showed potential in antibacterial activity with *H. monticulosum* displayed broad spectrum activity.

Key words: Antibacterial potential, endophytic fungi, identification, seaweed

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INTRODUCTION

Bacterial resistance towards antibiotics has been a worldwide phenomenon especially in nosocomial settings (Urban-Chmiel *et al*. 2022). In light of this, alternative sources of antibiotics with different modes of action can be explored. One important environmental source for novel drugs was endophytes (Cheng *et al*., 2020). Endophytes help the host in avoiding pathogen attack through the production of antimicrobial secondary metabolites. It has been hypothesized that endophytic fungi and the host may possess similar biosynthesis pathways in producing secondary metabolites. This is due to the vertical gene transfer as suggested in the discovery of paclitaxel (taxol) from endophytic fungi *Taxomyces andreanne* (Soliman *et al*., 2013).

Seagrasses are macroalgae, autotrophic multicellular marine organisms that can be categorised into three groups: red, green and brown algae. Red algae or Rhodophyta bears pigment called chlorophyll A, phycocyanin, and phycoerythrin that provides the red colour to this distinctive species. Green algae from the Chlorophyceae class have chlorophyll a and b that provide a light green colour. Brown algae were grouped in the Phaephyceae class which contains a pigment known as fucoxanthin that displays a greenish-brown colour.

Malaysia is blessed with ample coastal with diverse marine macroalga home to potential marine fungi. Environmental endophytic fungi have been known to be a

source of new secondary biological metabolites (Saleem *et al*., 2013; Lini *et al*., 2020; El-Bondkly *et al*., 2021). Previous endophytic fungi from seagrass have been described by Sultan *et al*. (2011), Tong *et al*. (2011), Zainee *et al* (2018a; 2018b: 2019a; 2019b; 2021). This study further explores new antibacterial alternatives from seaweed's fungal endophytes.

MATERIALS AND METHODS

Seaweed collection and endophyte isolation

Brown seaweed (*Surgassum* sp.) and green seaweed (*Ulva lactuca)* were collected from a coastal area in Johor, Malaysia and prepared for endophyte isolation as described by Zainee *et al*. (2018a). Dried seaweed pieces (1 cm × 1 cm) were placed on Potato Dextrose agar (PDA) and incubated for at least seven days at 30°C.

Fungal endophyte identification

Fungal identification of pure isolated culture was examined for the following: colony morphology and colour on both surface and reverse of PDA, hyphae morphology or mycelium, and characteristics of spore-bearing structures. Morphological species characterisation was determined following the key characters described in Bridge (1985), Samson *et al.* (2006) and Visagie *et al.* (2014). PCR was employed to amplify the internal transcribed spacer (ITS) gene (Figure 1) using the following primers: ITS1F (TCC GTA GGT GAA CCT GCG G) and ITS4R (TCC TCC GCT TAT TGA GC) (Schoch *et al*., 2012).

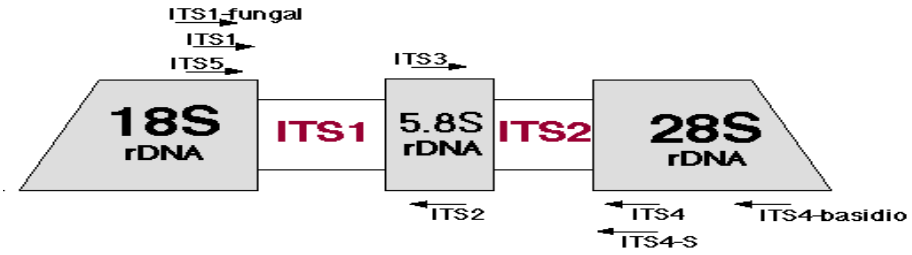


Fig. 1. Primers locations in the ITS region within the ribosomal DNA

Following sequencing of the amplified ITS products, the sequences were edited using Sequencer software version 4.0 (Gene Codes Crop, Ann Arbour, Mich., USA). Sequence alignment was conducted using Clustal W programme version 1.8 against the existing sequences data from the Genbank database (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) programme. Phylogenetic reconstruction was calculated using the Neighbour Joining (NJ) algorithm and Maximum Parsimony (MP) methods with *Schizosaccharomyces pombe* as an outgroup. The NJ analysis was run using Molecular Evolutionary Genetic Analysis (MEGA) software while the MP analysis used Phylogenetic Analysis using PAUP (PAUP) with 1000 bootstrap values respectively. Sequence analysis regarding the PCR ITS sequence size, accession number of species with the highest identity percentage and the E value was tabulated for all the isolates.

For the preparation of fungal extracts, isolates were cultured on sterile PDA for seven days at 30°C followed by growth of 3 × 6 mm mycelium in 200 mL of potato dextrose broth (PDB) at 30 ± 2°C with 100 rpm speed of agitation for 10 days. Fungal biomass was separated by filtration and metabolites in the filtrate were extracted three times with ethyl acetate (EA) in the ratio of equal volumes to broth (1:1). The Organic layer of EA was pooled together and evaporated using a rotary evaporator at 45°C. The crude extract obtained was weighed and stored at 4°C until use.

Antibacterial activity determination

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (2020). The following bacteria were tested in the assay: *Staphylococcus. aureus, Escherichia. coli, Enterococcus. faecalis, Klebsiella. pneumoniae* and *Bacillus. subtilis*. Extract concentration of stock mixture (10 mg/mL) was prepared in 1% of dimethyl sulfoxide (DMSO) followed by two times serial dilutions until 0.078 mg/mL. Test bacteria were mixed with different concentrations of extracts and incubated at 37°C for 24 hr. Bacterial viability after treatment with extracts was determined using 3-(4,5-dimethyldiazol-2-1)-2,5-diphenyl-2-H-tetrazolium-bromide (MTT) (2 mg/mL) as a dye. MIC value is equal to the lowest extract concentration that changed MTT to yellow indicative of no bacterial growth. Purple to blue colour indicates bacterial growth due to the formation of blue formazan. The assay was replicated three times.

RESULTS AND DISCUSSION

Five endophytic fungi isolates were obtained from brown seaweed, *Sargassum* sp. and green seaweed, *Ulva lactuca* (Table 1). Isolation to single colonies of endophytic fungi was a great challenge due to the close symbiotic relationship in the algae. Some of the isolates took a longer time to grow reducing the available nutrients able to be supplied by the PDA medium.

The PCR product amplified within the ITS region of the endophytic fungi isolates ranged from 500 to 700 base pairs (bp) (separation by agarose gel electrophoresis gel not shown). ITS sequence comparison is a convenient molecular tool for phylogenetic analysis and differentiating fungal populations. Genetic relationships can be evaluated for organisms at any taxonomic rank. All isolates possess similarity with the NCBI database at least 98% identity (*E*=0) (Table 2). The fungal isolates were identified as different species within the Ascomycota phylum.

Table 1. Macroscopic and microscopic descriptions of isolated endophytic fungi

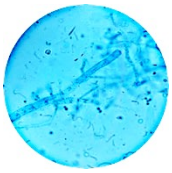
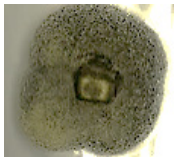
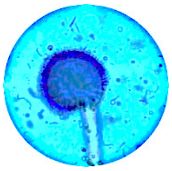
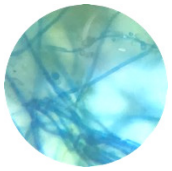
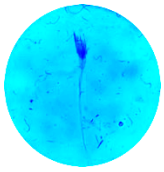
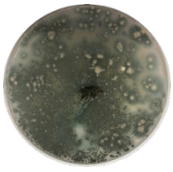
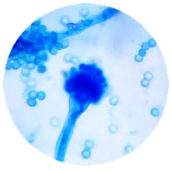
Code	Macroscopic Description	Macroscopic Observation	Microscopic Observation (1000×)	Fungal Genus Identification
A	The colony colour is dark green and the reverse is white. Its morphology displayed rough and dark green.			*Trichoderma*
B	The colony colour is black and the reverse is white—spore morphology is displayed as a black powder.			*Aspergillus*
C	The colony colour is white-yellowish and morphology showed fibrous with a waxy fibre structure.			*Hypoxylon*
D	Mycelium is bright yellow with a greenish texture.			*Penicillium*
E	The colony colour is dark turquoise and the spore morphology is dark greenish fine powder.			*Aspergillus*

Table 2. Classification of isolated endophytic fungi according to ITS sequence

Fungal Species	Sequence analysis			
	ITS Sequence size (bp)	Accession Number	*E* value	Identity percentage (%)
Trichoderma asperellum	604	MT10243.1	0.0	100
Aspergillus aculeatus	578	MN187974.1	0.0	100
Hypoxylon monticulosum	583	KJ774047.1	0.0	100
Penicillium sp. FKI-3389	603	AB455515.1	0.0	100
Aspergillus fumigatus	615	KM111293.1	0.0	100

Ascomycota phylum has been described for more than 32,000 species and it is the largest group in the fungi kingdom with variations in the biological and morphological perspectives (Webster & Weber, 2007). This phylum is known as sac fungi due to the important morphological characteristic of the sac that contains four to eight spores known as ascus. The most noticeable difference among species in this phylum is their fruiting body, spore (teleomorph) or ascospore inside its ascocarp where conidia

(anamorph) is within the conidiophore. Usually, conidia were formed at the end of the modified hyphae in a cluster or simple linkage. However, all these characteristics are dependent on growth medium, temperature, pH and moist existence.

The lowest concentration of fungal extracts required to inhibit the growth of the test bacteria was determined from the MIC values shown in Table 3. Three out of five fungi showed activity against at least one bacteria. *Penicillium* sp. FKI-3389 and *Aspergillus aculeatus* did not inhibit all test bacteria. *Trichoderma asperellum* inhibits all the tested bacteria except *E. coli*. *Aspergillus fumigatus* was able to inhibit *B. subtilis, S. aureus* and *E. faecalis*. Only *Hypoxylon monticulosum* showed the ability to inhibit all test bacteria with the lowest concentration of 1.25 mg/mL when tested against *E. coli*. Further analysis of the possible antibacterial metabolites produced by *Hypoxylon monticulosum* may provide useful knowledge in the development of future antibacterial agents.

Table 3. Minimum inhibitory concentrations (MIC) of endophytic fungi extracts against test bacteria

Endophytic fungi	Minimum inhibitory concentrations (MIC) (mg/ml)				
	B. subtilis	*S. aureus*	*E. faecalis*	*E. coli*	*K. pneumoniae*
Trichoderma asperellum	10.00 ± 0	10.00 ± 0	5.00 ± 0	NA	10.00± 0
Hypoxylon monticulosum	5.00 ± 0	10.00 ± 0	5.00 ± 0	1.25 ± 0	10.00 ± 0
Aspergillus fumigatus	5.00 ± 0	10.00 ± 0	10.00 ± 0	NA	NA
Aspergillus aculeatus	NA	NA	NA	NA	NA
Penicillium sp. FKI-3389	NA	NA	NA	NA	NA

*Average concentration ± standard deviation, *n*=3; NA: no activity

Fungal classification is numerous but a morphological observation approach is usually used. Guarro *et al*. (1999) explained that there are two different concepts regarding the technique: one is the phenotype technique that is driven by morphological characteristics. The other technique is a polythetic approach that depends on several morphological characteristics. Morphological characterisation however is not suitable for all fungi due to the nature of certain fungi that are not capable of living in a laboratory environment, different rates of sporulation, variation of life cycle and the problem of differentiating species having similar morphology (Grimm *et al*., 2005; Moore *et al*., 2005; Pang & Mitchell, 2005). Performing molecular technique is a confirmation of the morphology technique in classifying a fungus. The advantage of the molecular technique to sequence the ITS region can be attributed to the nature of DNA which is not sensitive towards environmental changes in a short period (Bridge, 1985; Visagie *et al*., 2014).

Several endophytic fungi have been isolated from *Sargassum* sp. and *U. lactuca*. *Trichoderma* was reported to be isolated from terrestrial ecology and has halotolerant capability. It has been isolated from ocean sediment regions, marine invertebrates and also algae (Thong-han *et al*., 2015). There were more than 250 reports regarding species from this genus (Bissett *et al*., 2015).

According to Wu *et al*. (2017), *Trichoderma* sp. is highly adaptive to the environment. Their growth rates are faster than other pathogens to better compete for limited spaces and nutrients. In addition, *Trichoderma* is an antagonistic pathogen that can cause mycoparasitism, a condition where the mycelium grows alongside recognized pathogen in a spiral fashion through the secretion of cell wall-degrading enzymes such as chitinases (Bailey *et al*., 2006; Sharon *et al*., 2007). Sixteen types of chitinase genes have been isolated from Wu *et al*. (2017) study of a novel endophytic fungi named *Trichoderma asperellum* GDFS1009.

Aspergillus has always been associated with a genus that caused contamination in the environment with high oxygen density (Yodsing *et al*., 2018). *Aspergillus* as marine fungi has been known to be the main contributor to secondary metabolites with oligotrophy capability. This allows it to live in a low-nutrient environment. There were around 1,300 bioactive compounds characterised by *Aspergillus* species with several biological activities including antitumor, antibacterial, anti-inflammatory and antiviral activity (Lee *et al*., 2013).

Aspergillus aculeatus and *A. fumigatus* have been isolated, identified and tested for antibacterial activity in this study. *A. fumigatus* was more active than *A. aculeatus* with the lowest MIC value of 5 mg/mL against *B. subtilis*. Previously, Xu *et al*. (2019) reported the isolation of three helvolic acid derivatives: 16-*O*-propionyl-16-*O*- deacetylhelvolic acid, 6-*O*-propionyl-6-*O*-deacetylhelvolic acid and helvolic acid from *Aspergillus fumigatus* CUGBMF17018. Antibacterial tests revealed the activity against MRSA and *S. aureus* at MIC 0.78 to 12.5 µg/mL.

Hypoxylon is a genus in the Xylaraceae family with more than 130 species identified (Sánchez-Ballesteros *et al*., 2000). Fungi from this genus have been identified as the main producer of potential bioactive metabolites and it is the largest member in the Xylaraceae family that can be found either in

the terrestrial or ocean ecology (Stadler *et al*., 2006, Stadler *et al*., 2008, Lutfia *et al*., 2021). Reports by Leman-Loubière *et al*. (2017a & b) discovered some novel compounds from sea sponges that were cytotoxic to cancer cells and had antifungal activity.

Hypoxylon monticulosum displayed the broadest spectrum antibacterial activity as it is active against all tested bacteria with the lowest MIC value at 1.25 mg/mL against *E. coli*. Cheng *et al*. (2020) have reported that the secondary metabolites of *H. monticulosum* are not very well known. This is a great opportunity for further investigation of this species that may lead towards the discovery of new metabolites, specifically antibacterial secondary metabolites. In addition, Cheng *et al*. (2020) have isolated three new compounds, hypoxyloamide, 8-methyoxynaphtalene-1,7-diol and hypoxylonol. In addition, seven newly isolated compounds from nature were reported along with 19 known compounds isolated from *H. investiens* BCRC 10F0115. Recently, it has been reported that *H. monticulosum* from marine sources has pharmacologically active compounds with antimicrobial properties including Dihydrocordoin, D-pantothenoyl-L-cysteine, caffeine and Tumonoic A acid and other biological activities (Azlan *et al*. 2024).

Penicillium sp. is in the Ascomycota class. No antibacterial activity was associated with the isolate in this study. This genus, however, is widely spread in the marine environment and received great attention due to the presence of secondary metabolites (Farha & Hatha, 2019). It was noted that 96.2% of endophytic fungi biodiversity within the swamp plants were in the Ascomycota class with *Penicillium* as the most dominant genus (Hamzah *et al*., 2018; El-Bondkly *et al*., 2021). *Penicillium* sp. from green algae, *Ulva* sp. has also been reported (Zainee *et al*., 2018a).

For future research, it is suggested that the mechanism of action of the identified antibacterial compounds, especially from *H. monticulosum* and *Aspergillus* species is further identified for new sources of therapeutic agents of antimicrobial treatment. Further investigation on other biological activities such as anti-tumour, anti-inflammatory and antiviral activities of the fungal isolates will be also interesting to determine.

CONCLUSION

Five endophytic fungi species have been isolated from brown seaweed, *Sargassum* sp. and green seaweed, *Ulva lactuca* and identified as *Trichoderma asperellum*, *Aspergillus aculeatus*, *Aspergillus fumigatus*, *Penicillium* sp. FKI-3389 and *Hypoxylon monticulosum*. *Trichoderma asperellum and Aspergillus fumigatus* have antibacterial activity while *H. monticulosum* displayed broad-spectrum activity against all tested bacteria.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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