Biodiversity Of Endophytic Fungi from Marine Algae and Its Phylogenetic Studies

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Abstract
The selection of marine algae and surface sterilization is the critical part while working with endophytic fungus isolation. In this present investigation six green algae and three brown algae were collected from Thoonithurai, Mandapam, Rameswaram, India. Around 68 fungal endophytes were isolated from selected algae and the fungi. It was observed that colonization rate was high in *Ceratophyllum submersum* L. and low in *Ulva intestinalis* L**.** The Shannon Diversity Index were calculated was 1.77 and Shannon Equitability index was 0.99. Based on morphological identification strains were selected and further identified by 18 s r DNA ITS region sequencing analysis. The BLAST analysis result identified the closest strain from NCBI database. Out of 19 strains , six strains belonged to Ascomycota family while other endophytes were unidentified due to low sequence homology in NCBI database. The current research showed a fungal diversity among green and brown algae in Gulf of Mannar coastal range and also it act as a pioneer source of drug discovery against Multi Drug Resistance.

Keywords: Endophyte, Fungi, Marine Algae, Multi Drug Resistance, Biodiversity

Introduction

Our planet's surface comprises more than 70 % of water, which gives the way to discover novel bioactive compounds (Sayed *et al.*, 2016). Marine organisms like microalgae, macroalgae, sponges, and halophilic organisms are adapted to unique environmental factors such as salinity, pressure, low temperature, and nutrition with the help of special metabolic capabilities. This promises that a large number of novel bioactive compounds are present in marine organisms (Carroll *et al.*, 2020);(Tan *et al.*, 2001);(Lekshmi *et al.*, 2020). The term endophyte (Gr. Endon, within; Phyton, Plant) was introduced by De Bary (Ahamed & Murugan, 2019). An endophyte is defined as any micro-organism which is found within a plant that does not cause any symptomatic infection to the host (Wilson, 1995). They are an endosymbiotic group of micro-organisms that are readily isolated from any growth-promoting medium (Nisa *et al.*, 2018);(Blunt *et al.*,2013). The endosymbiotic endophytic microorganism can produce some biologically active compounds which help to prevent certain pests that are growing in the host plant. These bioactive compounds are termed secondary metabolites (Deutsch *et al.*, 2021). In the recent era, endophytes and their secondary metabolites are an unexploited source of pharmacological and industrial products and also for the production of new biological control agents (Masand *et al.*, 2015);(Kamat *et*

al., 2020). The endophytic microorganism can be bacteria, fungi, actinomyces, or viruses but fungi are found in almost all the corners of the marine habitat. Marine algae have been a ubiquitous source of marine endophytic fungi (Debbab *et al*., 2011) for the identification of novel bioactive compound research (Handayani *et al*., 2015). Marine endophytic fungi are an ecological polyphyletic group which are commonly belonging to Ascomycetes; Basidiomycetes and anamorphic fungi (Huang, 2007);(Arnold, 2007). Many studies identified novel compounds with antibiotic activity against clinical pathogens and multidrug-resistant organisms from seaweeds (Ismail *et al*., 2016) and also optimized the isolation potential of endophytes from seaweeds (Schulz *et al*., 2002). The need for development of novel antibiotics against deadly pathogenic bacteria and multi drug resistant was increased and endophytic fungal isolates from marine plant and algae possessed broad spectrum antimicrobial potential (Kamat *et al*., 2020);(*Bose et* al.,2015). This study helps to identify the isolation potential among green and brown algae and its microbial biodiversity in marine algae species collected from Thoonithurai, Mandapam, Rameshwaram. Further studies on the selected endophytes may lead to the isolation of novel bioactive compound for use in medicine.

Materials and method

Collection of algae

Fresh algal samples were collected from Thoonithurai, Mandapam, Rameshwaram, India. Brown and green algae samples were collected and differentiated in their morphological appearances and color that are transferred in to sterile plastic containers with sea water and kept in ice box during transportation to lab. The algal samples were washed to remove all debris and dirt in the external surface using sea water. The collected algae were identified and processed immediately for endophytic isolation standard protocol. After completion of sterile water wash the samples are kept in filter paper to remove excess water. The identification of macro algae was carried out in Prof. P. Jayaraman, Plant Anatomy Research Centre, Tambaram, Chennai and placed in the Herbarium for record.

Surface sterilization

The dried algal samples are rinsed with 70 % ethanol for 60 s and followed by 0.4 % sodium hypochlorite for 30 s to remove the epiphytic micro-organism from outer surface of algae. Finally, after two washes with sterile distilled water, final wash was collected in a beaker to screen for endophytic microorganisms The water washed algae was placed in the filter paper to remove excess water for 10 to 20 minutes using sterile blade and the surface samples were cut in to small segments (2.0 cm) and fine pieces were pressed in to Potato Dextrose Agar Nutrient Agar, Actinomyces agar separately, respectively. The plates are prepared using sea water and streptomyces in PDA to reduce the bacterial growth and nystatin in starch casein agar to suppress fungi isolates. Then the algal pressed plates were incubated for 7 days in dark condition at 28 $\pm$ 2 ^{0} C and AA media plates were incubated for 10 to 15 days in 37^{0} C. The colonies grown around the segments were isolated and sub cultured in slants for further studies. The pure endophyte culture are preserved in glycerol and photographed for colony morphological studies.

Molecular identification of endophytes

The isolated fungi were identified by 18 S rDNA ITS sequencing. The fungal cultures were grown in PDA slant for 7 days at 28 $\pm$ 2 ^{0} C. After incubation, the fungal mat were taken and suspended in lysis buffer and the DNA isolation were done using Expure Microbial DNA isolation kit. After DNA isolation ITS 1 (5^{1} TCC GTA GGT GAA CCT GCG G 3^{1}) and ITS 4 (5^{1} TCC TCC GCT TAT TGATAT GC 3^{1}) primers were used for DNA amplification of the fungal genome. The pure PCR product were used for Sanger Sequencing in Reginal Facility for DNA fingerprinting, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. From the raw data (ATB file), the FASTA sequence collected and the Basic Local Alignment Search Tool (BLAST) were carried out for identification of fungal species.

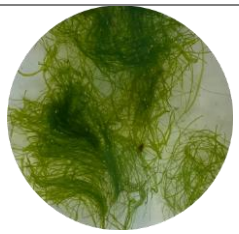
Results and discussion

Identification and authentication of algal samples

Five green algae and two brown algae were collected from Thoonithurai, Mandapam, Rameshwaram. The algal genera, species and voucher no of the algae are listed in Table 1.

Table 1 List of Identified and Authenticated algal Samples

S. No	Voucher No.	Family	Photography of Live Algae	Binomial
1	PARC/2022/4830	Gigartinaceae		*Chondrus crispus* Stackh
2	PARC/ 2022/4829	Dictyotaceae		*Padina boergesenii* Allender & Kraft
3	PARC/2022/4828	Dictyotaceae		*Padina gymnospora* (Kuetzing) Vickers

4	PARC/2022/4827	Caulerpaceae		*Caulerpa racemose* (Forsskal) J Agardh
5	PARC/2022/4826	Caulerpaceae		*Caulerpa sertularioides* (S G Gmel) M Howe
6	PARC/2022/4825	Ulvaceae		*Ulva intestinalis* L.
7	PARC/2022/4824	Ulvaceae		*Ulva lactuca* L.
8	PARC/2022/4823	Caulerpaceae		*Caulerpa taxifolia* (M Vahl)C.Agardh
9	PARC/2022/4822	Ceratophyllaceae		*Ceratophyllum submersum* L.

Isolation of endophytes

A total of 135 segments from six green algae and three brown algae were used to isolate the endophytes. Around 68 fungal endophytes from above algae were isolated shown in Figure 1. Among all the algae identified highest percentage of colonization rate (C R %) was observed in *Ceratophyllum submersum* L.while the lowest percentage was recorded in *Ulva intestinalis* L shown in Figure 2.; Table 2As per (Suryanarayanan *et al*., 2013), the colony frequency can be calculated using the formula.

$$CF\ \% = \frac{\text{No of plant segments colonized by a single fungus}}{\text{Total number of plant segments observed}} \times 100$$

Figure 1 Isolated endophytic fungal strains on PDA slant

Table 2 Colonizing frequency of endophytic fungi from different marine algae

S.No	Algae	Segment	Endophytic Fungi	CF %
1	*Chondrus crispus*	15	4	27
2	*Padina boergesenii*	15	8	53
3	*Padina gymnospora*	15	9	60
4	*Caulerpa racemose*	15	10	67
5	*Caulerpa sertularioides*	15	8	53
6	*Ulva intestinalis* L.	15	3	20
7	*Ulva lactuca* L.	15	9	60
8	*Caulerpa taxifolia*	15	6	40
9	*Ceratophyllum submersum* L.	15	11	73

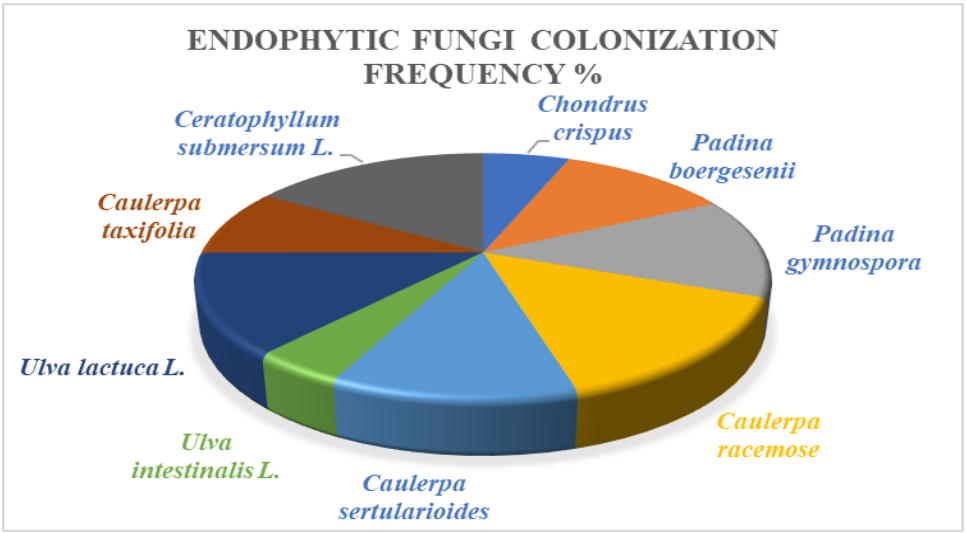


Figure 2 Endophytic fungal colonization frequency among green and brown algae

Shannon Wiener Diversity Indices (H) helps to identify the highest endophytic fungi community in the algae (Hosetti 2002) are tabulated in Table 3 and graphically represented in Figure 3. From this present investigation H index value recorded was 1.77 and it proved the high fungal diversity from the selected algal community. The H index was calculated using the formula (1)

$$H = \sum Pi(\ln(Pi)) \quad (1)$$

Here Pi= Number of individuals in the i^{th} species

S= Number of species.

using this H value Shannon equitability also identified as 0.99 using the formula E_H=H/ln (S) where H is a Shannon Wiener Diversity Indices and S is a total number of species.

Based on colonization frequency percentage, the two algae *Caulerpa racemose* and *Ceratophyllum submersum* L. were selected to determine the Sorenson's Co efficient (CC) of this fungal community using formula (2)

Sorenson's Coefficient Index (CC)

$$CC = 2C/S1 + S2 \quad (2)$$

Where, C = is the number of species the two communities have in common

S1 = is the total number of species found in community 1

S2 = is the total number of species found in community 2

The Sorenson's Coefficient index (CC) of fungal community among two algae was 0.8 which proved as High score (close to 1) (Clarito *et al*., 2020).

Table 3 Shannon Wiener Diversity Indices (H) of identified endophyte

S.No.	Fungi isolation	Frequency	Shannon Weiner Diversity Indices (H)
1	*Corynascus sepedonium*	10	-0.28
2	*Talaromyces aurantiacus*	11	-0.29
3	*Amesia atrobrunnea*	11	-0.29
4	*Microascus gracilis*	17	-0.35
5	*Curvularia platzii*	10	-0.28
6	*Chaetomium perlucidum*	9	-0.27
			H= 1.77

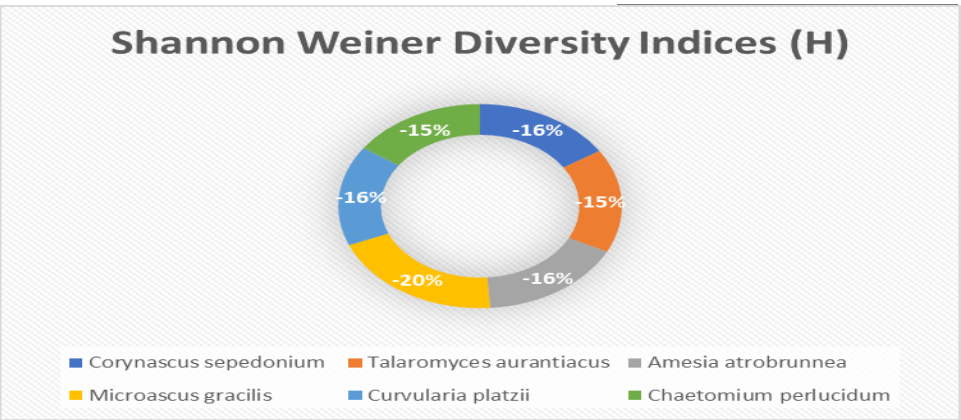


Figure 3 Diversity Indices (H) of Identified Endophyte

18s r DNA sequencing of endophytic organisms

Based on colony morphology and LCB staining the fungal colonies were identified and further authenticated by 18 s DNA Sequencing by Sanger Sequencing Analysis of ITS r DNA region. The BLAST analysis result identified the closest strain from National Centre for Biotechnological Information (NCBI) database. Out of 19 fungal endophyte, 6 fungal endophytes were identified and taxonomically identified under Ascomycota family and tabulated in Table 4 and represented in Figure 4, while the remaining endophytes are unidentified due to low sequence homology in the gene bank database. The % of identity among the closest sps were identified where 93.5 % similarity in FUG_06 which have closest sequence similarity with *Chaetomium perlucidum* . FUG_03 and FUG_04 have 100% sequence similarity with *Amesia atrobrunnea* and *Microascus gracilis* FUG_01, FUG_02, FUG_05 have 99.78%, 98.84% and 99.77% respectively which have closest sequence similarity with *Corynascus sepedonium, Talaromyces aurantiacus, Curvularia platzii* shown in Figure 5 .

Table 4 Six endophytic fungal strains identified by 18 s r DNA Sequencing

Isolates Name	Closest Relative[a]	Accession No[b]	% Identity[c]
FUG_01	*Corynascus sepedonium*	ONO59588.1	99.78
FUG_02	*Talaromyces aurantiacus*	ONO59708.1	98.84
FUG_03	*Amesia atrobrunnea*	ONO63018.1	100
FUG_04	*Microascus gracilis*	ONO63045.1	100
FUG_05	*Curvularia platzii*	ONO63065.1	99.77
FUG_06	*Chaetomium perlucidum*	ON350775.1	93.55

[a]Closest species which high % identity in BLAST Analysis, [b]NCBI Gene bank accession number in website (http://www.ncbi.nlm.nih.gov/pubmed),[c]Gen Bank accession no. of our strains deposited on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed),[d] % identiy of strain based on BLAST Analysis

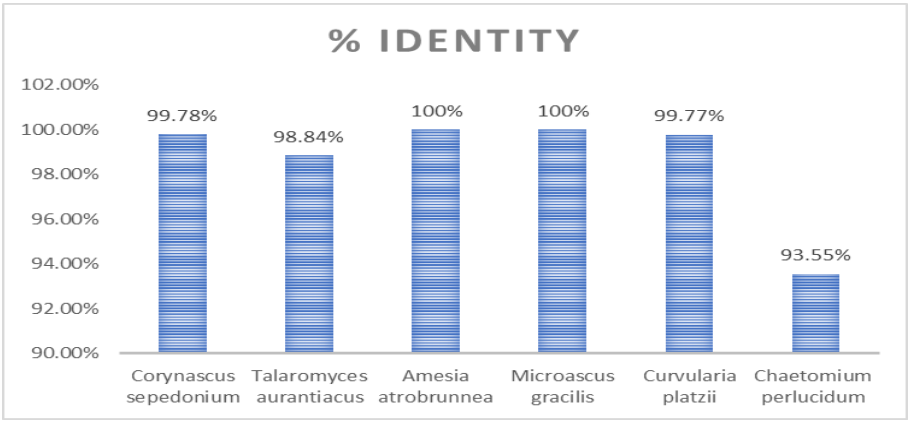


Figure 4 Closest species which high % identity in BLAST Analysis

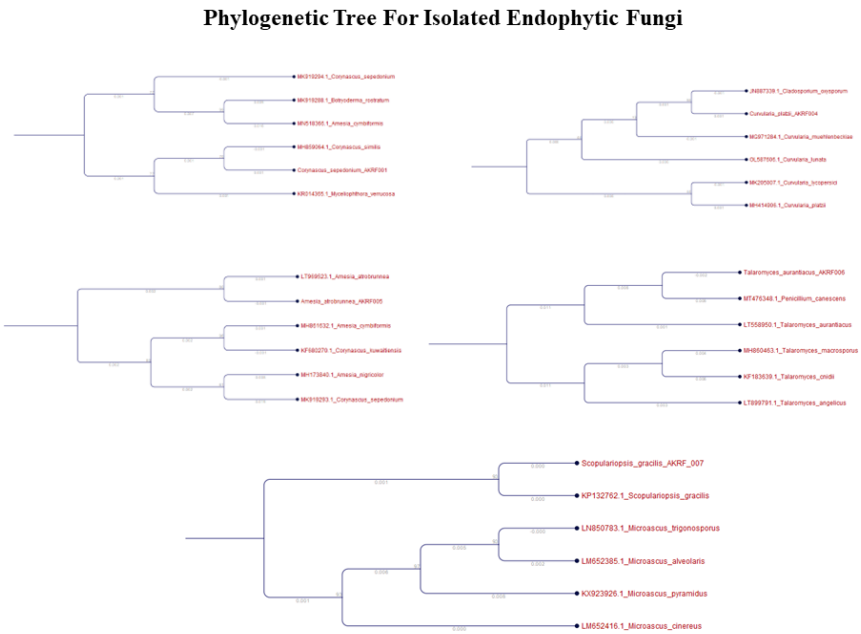


Figure 5 Phylogenetic tree of isolated endophytic fungi

Based on all the algal species investigated, *Ceratophyllum submersum* L. recorded rich fungal diversity and the organism were belonging to existing phylum Ascomycota. Most of the algal species are abundant in most common fungal genus Aspergillus. They require cellulose for their growth; however, the algal thallus contain more amount of cellulose which favored the endosymbiotic growth in marine algae (Kamat et al., 2020). Some endophytic fungi need lignin to associate with marine algae. In the case of Basidiomycota very less possibility of association in marine algae due to lack of lignin. One of the fungal isolates *Taleromycoes aurantiacus* had the ability to produce taleromycin (broad-spectrum antibiotic). Marine origin endophytic transpire as a new novel source for producing natural bioactive products which is then further used for many deadly diseases. The current research shows a fungal diversity among various green and brown algae in the Gulf of manner and also it is the pioneer source for drug discovery against multi-drug resistance.

Conclusion

Recently endophytic organism has been presented with substantial concentration which protects the host from pests, and pathogen and also helps in growth and reproduction in their host lifecycle. They have a considerable amount of bioactive which are not yet identified from most of the endophytes that endow with novel biochemical diversity. In this study, it provided treasured awareness of the biodiversity of endophytic fungi from different green and brown algae. Using 18 s r DNA sequencing, we found that both green and brown algae were rich in endophytic fungi which belong to the Ascomycota family. The Shannon Index and Sorenson's coefficient equation proved high fungal diversity among the algal species from Gulf of Mannar coastal areas. As we have promising novel bioactive compounds present in these

endophytic fungi helps to focus in the future on the extraction and purification of these bioactive compounds against anticancer assay, anti-inflammatory, and anti-microbial assays.

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Conflict interest
The authors declare that there is no conflict of interest.

Data availability statement
The datasets generated for this study can be found in the NCBI Bank, Accession numbers:
ONO59588.1; ONO59708.1; ONO63018.1; ONO63045.1; ONO63065.1; ON350775.1

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