Diversity and bioactive profile of endophytic mycoflora in mangroves

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Abstract

The present study deals with the mangrove associated endophytic fungi and their bioactivity profile. The fungal endophytes were isolated from the leaves of three mangrove species (*Aegiceras corniculatum, Lumnitzera racemosa* and *Ceriops tagal*) and identified by morphological and molecular methods (Internal Transcribed Spacer and β-tubulin (Ben A) sequencing). The isolates mainly belonged to Ascomycota (95%) and the Basidiomycota comprised only 5%. They come under 5 classes, 11 orders, 16 genera and 25 species. Under Ascomycota, four classes, viz., Sordariomycetes (44.5%), Eurotiomycetes (32.46%), Dothideomycetes (17.28%) and Saccharomycetes (1.83%) comprising 10 orders were recorded. Under Basidiomycota a single class viz., Agaricomycetes (3.93%) comprising 1 order (Agaricales) was observed. *Colletotrichum* (30.2%) was the dominant genus followed by *Aspergillus* (17.8%), *Penicillium* (14.1%), *Phyllosticta* (9.1%) etc. *Penicillium citrinum* was the dominant fungus (22%) in *A. corniculatum*; *Colletotrichum siamense* (31%) in *L. racemosa* and *Aspergillus sydowii* (35%) in *C. tagal* (Fig. 3). Among the isolates, the Colonization frequency (CF%) was maximum for *Colletotrichum siamense* (18%) followed by *Phyllosticta capitalensis* (11.6%). *Penicillium citrinum* was found in all the three plant species. Isolates from *Ceriops tagal* were found to exhibit higher antibacterial as well as hydrolytic enzyme production potential. *Aspergillus montevidensis* EF 30, *Cladosporium oxysporum* EF 316, *Colletotrichum siamense* EF 272, *Penicillium chrysogenum* EF 362, *Colletotrichum fruticola* EF 22, *Ascotricha chartarum* EF 374, *Aspergillus sydowii* EF 239, *Talaromyces purpurogenus* EF 313, *Meyerozyma caribbica* EF 347 and *Schizophyllum commune* EF 309 were segregated as potential strains for hydrolytic enzyme production. *A. aculeatus* EF 10, *T. purpurogenus* EF 313, *C. oxysporum* EF 316, *C. fruticola* EF 74, *P. capitalensis* EF 187, *A. sydowi* EF 226 and *P. chrysogenum* EF 363 showed notable antimicrobial property. *T purprogenus* EF 313 endowed with pigment (red) production has potential for application in dyeing industry. During the current study, 25 different species of endophytic fungal species could be recovered from three host mangrove plants and some of them were having high bioactive potential for possible commercial applications with respect to bioremediation and as antimicrobials in medicine and aquaculture.

Introduction

Endophytic fungi are a group of organisms that live inside the plant tissues without causing any harm to the host (Schulz, 2006; Hyde, 2008). They mainly belong to ascomycetes group, whereas basidiomycetes, deuteromycetes and oomycetes are rarely found. They play a key role in the physiological processes of the host plants i.e., improving nutrient and water uptake, nitrogen fixation, abiotic and biotic stress tolerance and protecting the host from attack by microbial pathogens, animals and nematodes (Selim, 2012). The interaction between the host and the endophytes modulates the metabolic process of the endophytes resulting in the production of molecules having bioactive properties. Endophytic fungi are increasingly been recognized as a rich source of enzymes and other bioactive metabolites (Aly, 2010; Strobel, 2003; Porras-Alfaro, 2011; Deshmukh, 2015; Ratnaweera. 2017). These enzymes play an important role in getting protection from pathogens, obtaining nutrients from host and in mineralization.

It can be used in several areas, including food processing, leather industry, detergents, pharmaceuticals and medical therapy (Alberto, 2016; Lobo, 2005; Martinho, 2019).

Mangroves are halophytic plants confined to tropical and subtropical coastlines. These ecosystems are considered as a transition zone between marine and terrestrial habitat with unique properties i.e., high salinity, tidal flooding, and anaerobic soil and sludge (Wang, 2014). These distinctive ecosystems support the growth of microorganisms having particular ecological, morphological, biological and physiological adaptations. So, the endophytic fungi from mangroves activate certain metabolic pathway and produce biomolecules (alkaloids, phenolics, saponins, flavonoids, terpenoids, steroids etc.) to survive within the unique ecosystem (Salini, 2015). The diversity and bioactivity profile of endophytes depend highly on the environment where it lives. Traditionally, mangrove species are widely used in folklore medicine for the treatment of dyspepsia, hepatitis, asthma, diabetes and stomach problems (Bandaranayake, 1998). Generally, plants having ethnobotanical history are mostly recommended for the study of endophytic fungi. The black mangrove, *Aegiceras corniculatum*, belongs to the myrsinaceae family and is distributed in coastal and estuarine areas of India. In ancient time, the people used it for the treatment for rheumatism, arthritis, inflammation, asthma, diabetes etc. An anticancer compound, aegicoroside A was isolated from the leaves of *A. corniculatum* (Vinh, 2017). *Lumnitzera racemosa*, belonging to Combretaceae family is a small tree found in the Asian coast. Fluid obtained from incisions made in the stems have been applied externally for the treatment of herpes infections and itches. Early reports showed that the extracts of this plant possessed antibacterial (D'Souza, 2010), antifungal, antihypertensive (Lin,1993), protein tyrosine phosphatase 1B (PTP1B) inhibitory, hepatoprotective, and antioxidant activities (Ravikumar, 2011). *Ceriops tagal* belongs to the family of Rhizophoreaceae and is predominant in tropical and sub-tropical areas. The ethno-medicinal uses of the barks, leaves and roots of this plant include the treatment of hemorrhage, diabetes, malaria and ulcers (Tiwari, 2008). Endophytes possibly get the genes responsible for the bioactive property of the host plants as reported in case of Taxol, the multi- billion dollar drug from the Yew tree which was later detected in an endophytic fungus associated with the tree and thereby the isolation and identification of endophytes for the exploration of bioactive compounds are very important. Therefore, three mangrove plants having ethnobotanical history were selected for the present study.

Materials And Methods

Sample collection

Three different mangrove species, *Aegicerus corniculatum, Lumnitzera racemosa* (9°07'13.8"N 76°28'40.5"E) and *Ceriops tagal* (8°56'10.4"N 76°33'08.2"E) from Kollam (India) were selected for the study. Leaves from 10 healthy plants of each species were randomly collected and kept in polythene bags, transported to laboratory and stored at 4 °C in a Refrigerator. Samples were processed within 24hrs of collection.

Isolation of Endophytic fungi

Fungal endophytes were isolated from the plant samples by the modified method of Kumaresan (2001). The leaves were thoroughly washed under running tap water to remove dirt and dust. Then the leaves were surface sterilized by dipping in 70% ethanol for 1min, followed by Sodium hypochlorite (4% v/v) for 2 min. Surface sterilized samples were rinsed twice for 1 min in sterile water and the water on the surface was removed with blotting paper. The leaves were cut into small segments (about 0.5 cm^2) under aseptic conditions in a Laminar flow Chamber. To evaluate the effectiveness of the disinfection process, imprints of the leaf samples were taken on Potato Dextrose Agar medium and the presence/absence of microbial growth was observed after incubation. Absence of growth indicated effective surface sterilization. Leaf segments from each plant species (1080, 300 and 900 from *A. corniculatum*, *L. racemosa* and *C. tagal* respectively) were placed in Petri dishes containing Potato Dextrose Agar (PDA) medium amended with 150 mg/l chloramphenicol. The number of leaf segments to be used was fixed so as to get a minimum of 100 fungal isolates from each plant species. These petri dishes were sealed, incubated for four weeks at 28 ± 2 °C and examined periodically. Fungal growth from the leaf segments were recorded and the colonies were isolated into PDA slants, purified and 3 day old cultures in potato dextrose broth were stocked at -80 °C.

Identification of endophytic fungi based on morphological and molecular characteristics

Morphological examination

The isolates were spot inoculated on PDA plates and incubated for one week at 28 ± 2 °C. Morphological features such as colony characteristics, growth pattern and colony colour (front and reverse) were noted. Micromorphology was recorded using Scotch tape method (Yang, 2007). For this, the sticky side of a clear cellophane tape was first applied to the surface of a colony and then the tape was placed sticky side down on a drop of Lactophenol cotton blue placed on a clean glass slide. The spores and hyphae were examined under Inverted Research Microscope (Zeiss, Axio Observer 3, Germany) . The isolates could be classified up to genera level based on their spore morphology (Barnett, 1987; Ellis, 1988).

Molecular characterization

Genomic DNA isolation

Fungal genomic DNA was extracted by salting out method following Miller (1988). Pure cultures were grown in PD broth under static condition for 24 – 48h. Mycelia were homogenized in 500 µl Solution 1 (50 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 2% SDS). Added 0.5µl of Proteinase K (20 mg/ml), vortexed and incubated for 2 hours at 55 °C in a water bath. Then the lysed cell suspension was chilled on ice for 10 minutes and added 250µl of NaCl (6M) and inverted several times to mix the lysate. The lysate was again chilled on ice for 5 minutes and centrifuged for 15 minutes at 8000rpm. Transferred the

clear supernatant into fresh vial and added twice the volume absolute Isopropanol. Then incubated the mixture at 4 °C in a Refrigerator for 12- 18hours to precipitate the DNA. The DNA was pelletized by centrifugation at 11000 rpm for 15 minutes and the pellet was washed with 500µl of 70% ethanol (3 times)followed by 95% ice cold ethanol (twice) and dried under vacuum, The pellet was dissolved in 30µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored in -20 °C Freezer.

Amplification of ITS region

The internal transcribed spacer (ITS) region of the fungal rDNA was amplified using the primer pairs ITS1 (5'-CGTAGGTGAACCTGCGG-3') and ITS4 (5'- CTCCGCTTATTGATATGC-3') (White, 1990). The polymerase chain reaction was performed in a final volume of 25µl; the reaction mixtures contained 1X standard Taq buffer (10 mM Tris –HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM each primer, 1U Taq DNA polymerase (Fermentas, Inc.) and 1-2 µL of DNA template (10-100 ng). The Thermocycler programme consisted of initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 45 seconds and extension at 72 °C for 1 minute and a final elongation step at 72 ^{o}C for 10 minutes. The PCR amplicons were separated by electrophoresis through 1% (w/v) agarose gel and visualised under ultraviolet light after staining with ethidium bromide. The resultant PCR products were used for Amplified Ribosomal DNA Restriction Analysis (ARDRA).

Amplified ribosomal DNA restriction analysis (ARDRA)

The ITS amplicons were digested using three restriction enzymes i.e., *Hinf* 1, *Sdu* 1 (Fermentas, France), and *Cfo* 1 (Sigma, USA) in separate reactions. The 20µl reaction mixtures contained 8 µL PCR product, 2 µL buffer (50 mM NaCl, 10 mM Tris- HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol), 8 µL milli Q and 2 µL (5 units) of each restriction enzyme. The reaction mixture was incubated at 37 °C for 16 hours. Enzyme inactivation was performed by heating the reaction mixture at 65 °C for 15 minutes. The digestion products were separated by 2% (w/v) agarose gel electrophoresis and recorded using a Gel documentation system (Syngene, G Box). Dendrogram was constructed based on the ARDRA profile of different fungal isolates and a representative strain from each cluster selected for sequencing. In cases where the fungal isolates which showed same percentage similarity with the ITS region of more than one species, amplification of ß-tubulin (Ben A) was performed. The Ben A gene was amplified by the same Thermocycler programme of ITS except the annealing temperature, 55 °C with the primer pairs Bt2a and Bt2b. The PCR amplicons were sequenced at Scigenome, Kochi. The nucleotide sequence of ITS and Ben A were compared with the reference sequences using BLASTn against GenBank database at NCBI. The sequences were submitted in GenBank (NCBI).

Screening for Antibacterial activity

Screening for antibacterial activity of the crude extract of endophytic fungi was performed by the Kirby-Bauer disk diffusion method. The assay was done against 12 bacterial pathogens viz., *Escherichia coli* (MTCC 1610), *Edwardsiella tarda* (MTCC 2400), *Pseudomonas aeruginosa* (MTCC 741), *Bacillus cereus*

(MTCC 1272), *Staphylococcus aureu*s (MTCC 3061), *Aeromonas hydrophila* (MTCC 1739), *Vibrio cholerae* (MTCC 3906), *Vibrio parahaemolyticus* (MTCC 451), *Vibrio alginolyticus* (MTCC 4439), *Vibrio harveyi* (BCCM 4044), *Vibrio proteolyticus* (BCCM 3772) and *Vibrio fluvialis* (MCCB 130). Crude extract of fungal culture was impregnated on sterile filter paper disks (6mm) and placed on the surface of Muller Hinton agar plate (Muller Hinton broth, 2.1g; NaCl, 0.5 g (15 g for vibrios); agar, 20 g; distilled water, 1 L, pH 7) already seeded with the test microorganisms. The plates were incubated at 37 ± 2 °C/28 ± 2 °C and the zone of inhibition was noted.

Screening of hydrolytic enzyme production

Extracellular enzyme production was tested by spot inoculation of the fungal isolates on various nutrient agar media plates supplemented with the corresponding substrates. After incubation at 28±2 °C for 5-7 days, the plates were flooded with reagents and observation was recorded. The details of the media and reagents used and the appearance for a positive reaction are given in Table 1.

Results

Endophytic fungi from mangroves

A total of 382 endophytic fungal isolates could be obtained from the leaves of the three mangrove species. Isolation frequency was found to be significantly high in the host plant *L. racemosa* (58%) followed by *C. tagal* (11.7%) and *A. corniculatum* (9.4%) (Fig 1). The colony morphology of fungal mycelium in potato dextrose agar (PDA) varied from powdery, cottony, and velvety to leathery and glabrous nature. Surface topology was mainly flat, rugose, umbonate and verrucose type. Both pigmented and non-pigmented fungal isolates were obtained. Twenty-five fungal species, including 2 yeast species could be found (Table ST1, supporting information). Out of the 382 isolates, 96% belonged to the phyla Ascomycota and 4% to Basidiomycota. Under Ascomycota, four classes, viz., Sordariomycetes (44.5%), Eurotiomycetes (32.46%), Dothideomycetes (17.28%) and Saccharomycetes (1.83%) comprising 10 orders i.e., Eurotiales (32.46%), Glomerellales (0.26%), Botryosphaeriales (9.16%), Hypocreales (36.65%), Pleosporales (6.02%), Diporthales (5.5%), Sacchraomycetales (1.83%), Capnodiales (1.83%), Sordariales (0.52%) and Xylariales (0.26%) were recorded. Under Basidiomycota a single class viz., Agaricomycetes (3.93%) and order Agaricales (3.93%) were observed (Fig. 2). The isolates belonged to sixteen genera i.e., *Alternaria, Ascotricha, Aspergillus, Candida, Chaetomium, Cladosporium, Colletotrichum, Diaporthe, Escovopsis, Fusarium, Meyerozyma, Neurospora, Penicillium, Phyllosticta*, *Schizophyllum* and *Talaromyces*. Most abundant genus was *Colletotrichum* (30.2%) followed by *Aspergillus* (17.8%), *Penicillium* (14.1%) and *Phyllosticta* (9.1%). The most abundant species was *Colletotrichum siamense* (19.37%), *Penicillium citrinum* (12.04%), *Aspergillus sydowii* (9.69), *Phyllosticta capitalensis* (9.16%) and *Colletotrichum fruticola* (7.85%). *Penicillium citrinum* was the dominant species (22%) in *A. corniculatum*; *Colletotrichum siamense* (31%) in *L. racemosa* and *Aspergillus sydowii* (35%) in *Ceriops tagal* (Fig.3). Colonization frequency (CF%) was maximum for *Colletotrichum siamense* (18%) followed by *Phyllosticta capitalensis* (11.6%) and *Colletotrichum fruticola*

(9.6%) (Table 2). *Penicillium citrinum* was found in all the three plants and species such as *A. chartarum, E.weberi* and *N. intermedia* were unique to *C. tagal*, *L. racemosa* and *A. corniculatam* respectively and were absent in other mangrove species. *S. commune* was the only Basidiomycota found as endophyte.

Phylogenetic analysis of endophytic fungi deliver insight into the evolutionary relations of the endophytic fungal isolates (Fig. 4). In *A. corniculatum,* the largest clade, Eurotiomycetes comprised of three genera *Aspergillus, Talaromyces* and *Penicillium*. Sordariomycetes clade consisted of 5 genera: *Chaetomium, Colletotrichum, Diaporthe, Fusarium* and *Neurospora*. *Schizophyllum commune* (EF 309), the only Basidiomycota obtained in our study, formed a separate clade. Two yeast isolates viz., *Candida orthopsilosis* (EF 367) and *Meyerozyme caribbica* (EF 380) formed the two subclades of Saccharomycetes. In *L. racemosa*, Sordariomycetes formed the main clade, consisting of four genera: *Colletotrichum, Diaporthe, Escovopsis* and *Fusarium*. Eurotiomycetes formed two sub clades, consisting of two isolates EF 99 and EF 162 that grouped with *Penicillium* and *Aspergillus* respectively. In *C. tagal* also Eurotiomycetes is the main clade comprising three genera: *Aspergillus, Talaromyces* and *Penicillium.* Sordariomycetes clade included *Ascotricha* and *Colletotrichum*. Two yeast genera (*Candida* and *Meyerozyma*) and the isolate belonging to basidiomycota formed separate clade.

Antibacterial property

In general, 11% of the total endophytic fungi (382 Nos), showed antibacterial activity; 15.6% of the isolates from *A. corniculatum*, 10.3 % from *C. tagal* and 8.0% from *L. racemosa* (Fig. 5, Table ST2). About 15% of the isolates inhibited *S. aureus* and *B. cereus*,14% inhibited *V. cholera*, 6% *A. hydrophila* and 6% *V. harveyi*. *A. aculeatus* EF 10 from *A. corniculatum* and *P. citrinum* EF 360 from *C. tagal* inhibited six bacterial pathogens. *A. aculeatus* EF 10*, P. chrysogenum* EF 363*, P. citrinum* EF 360*, P. capitalensis* EF 187*, T. purpureogenus* EF 313*, C. fruticola* EF 74*,* and *E. weberi* EF 67 inhibited the growth of both Gram - positive and Gram-negative bacteria. *A. alternata* EF 26 and *C. oxysporum* EF 316 inhibited only the Gram-positive bacteria whereas *A. sydowii* (EF 226) only Gram-negative bacteria.

Hydrolytic Enzyme production

Most of the isolates were positive for amylase, protease and lipase. Asparaginase, glutaminase, pectinase and cellulase production by the isolates were also notable. However, very few isolates were positive for laccase, ligninase and tyrosinase. None of the isolates exhibited chitinase activity. From the study, it is evident that, *Aspergillus montevidensis* EF 30*, Cladosporium oxysporum* EF 316*, Ascotricha chartarum* EF 374 *Aspergillus sydowii* EF 239, *Colletotrichum fruticola* EF 22, *Colletotrichum siamense* EF 272, *Meyerozyma caribbica* EF 347 *Penicillium chrysogenum* EF 362, *Talaromyces purpurogenus* EF 313 and *Schizophyllum commune* EF 309 were remarkable source of the hydrolytic enzymes (Table ST3, Supporting information). Isolates from *Ceriops tagal* exhibited maximum enzyme production potential followed by *Aegiceras corniculatum* and *Lumnitzera racemosa* (Fig. 6).

Discussion

The endophytic assemblage of a plant depends on the host (Petrini, 1981; Elamo, 1999) as well as environmental factors such as altitude, humidity, density of canopy (Petrini,1981) and precipitation (Suryanarayanan,1998; Rajagopal, 2000). In the present study, the colonization frequency varied with the host species which was consistent with the previous report that endophytic community composition is influenced by the host species (Suryanarayanan, 1998; Kumaresan, 2001, Li, 2016;). The foliar endophytic fungal community of the three hosts (*A. corniculatum*, *L. racemosa* and *C. tagal*) showed remarkable difference from the previous reports (Kumaresan, 2001; Xing, 2011) indicating that the geographical location/environmental factors of the sampling site also affect the community composition of endophytes. In the present study, phylogenetic analysis based on the ITS and Ben A sequencing confirmed 25 species distributed in 16 genera, belonging to phylum Ascomycota and Basidiomycota. Mangrove endophytic fungi commonly belong to phylum Ascomycota (Xing, 2011, Hamzah; 2018) and the same observation was made in our study also.

In the present study, Genus *Colletotrichum* was the most frequent fungal endophyte obtained from the mangrove plants. This genus is already reported as widely distributed in different mangrove species (Kumaresan, 2001; de souza, 2013; Rajamani, 2018). Besides this genus, *Aspergillus* and *Penicillium* were also found predominant. *Aspergillus* was the dominant endophyte isolated from the roots of *Rhizophora apiculata, Rhizophora mucronata* and *Bruguiera gymnorrhiza* Andaman and Nicobar Islands (Thorati; 2016). *Penicillium* spp. have a wide host range as foliar endophytes and have been reported from diverse mangrove species (Kumaresan, 2001, Xing, 2011).

At species level, each mangrove harboured diverse fungal species as endophytes while only one or two species were dominant in each host. In the current study, the dominant endophyte of each plant species was different, and its abundance were restricted to that particular host and less frequently obtained from other hosts. This is in agreement with the previous studies (Kumaresan, 2001, Rajamani, 2008). The endophytic composition revealed that some of the species are common to more than one host i.e., *A. alternata, A. aculeatus, P. citrinum* and *P. chrysogenum*; also reported by some of the earlier workers (Xing, 2011, Devi, 2012, Hamzah, 2018).

Alternaria alternata is a cosmopolitan species, commonly isolated from a wide range of plants as both endophytes and pathogens (DeMers, 2022). In the present study, the occurrence of this species in *A. corniculatum* and *L. racemosa* was 13% and 6% respectively. Previously, this species was reported as an endophyte from *A. corniculatum* and *Avicennia marina* (Li, 2016). This species is the causative agent of leaf spot disease in several plants (Chen, 2018; Aung. 2020).

Only a single isolate of *Ascotricha chartarum* and *Chaetomium globosum* could be isolated from the host *C. tagal* and *A. corniculatum*. *A. chartarum* had been identified previously only from the bark of *Rhizophora mucronata* (Ananda, 2002). The occurrence of the genus in the environmental mycobiota is comparatively lower than others (Khan, 2018). *Chaetomium globosum* is saprophytic fungus belonging to the mould family chaetomiaceae. This species is the causative agent of onychomycosis or superficial

infections (Wang, 2016). Formerly, the species was isolated from *Avicennia marina* (Kumaresan, 2001) and *Ceriops tagal* (Li, 2021) as an endophyte.

Aspergillus aculeatus is a cadmium resistant fungus reported to improve turf quality, chlorophyll content, and accelerating the plant growth and photosynthesis of Bermuda grass (Xie, 2014). It also enhanced the salt stress tolerance of plants by producing Indole-3-acetic acid and siderophores (Li, 2017). In the present study, this species could be recovered from the two hosts i.e., *A. corniculatum* (5%) and *L. racemosa* (2%). This species was reported as an endophyte from the Ecuador mangrove forest and *Rhizophora stylosa* (Xing, 2011). *Aspergillus flavus* is a saprophytic soil fungus, that act as an opportunistic pathogen in preharvest and postharvest seed crops by producing carcinogenic secondary metabolite, aflatoxin (Klich, 2007). Our study revealed that *A. flavus* is the second most abundant endophytic fungi of *C. tagal*. Wide distribution of this species among different mangrove species has been reported in a previous study (Ravindran, 2012). A single isolate of *Aspergillus montevidensis* recovered from *A. corniculatum*, which was previously reported as a clinical pathogen, could be isolated from different body sites, from superficial to deep tissue infections (Siqueira, 2017). There are no earlier reports on the endophytic nature of this species. *Aspergillus sydowiii* is a common saprotrophic soil fungus and the soil strains are harmless whereas some marine strains cause aspergillosis in coral sea fans (Gorgonia ventalina) (Ein-Gil, 2009; Soler-Hurtado, 2016). In the agriculture field, *A. sydowiii* facilitate the phosphate solubilization and thereby increasing the phosphorus uptake of plants (Baron, 2018). It was the most predominant endophytic fungi obtained from *C. tagal* in our study. Prior studies showed that this species is commonly isolated as an endophyte from both terrestrial and marine hosts (Teuscher, 2006; Bhattacharya, 2018; Handayani, 2018; Wang, 2021).

Penicillium chrysogenum is widely studied for secondary metabolite production and regulation processes (Fierro, 2022). This is used for the commercial production of β-lactam antibiotic (Guzman-chavez, 2018). In the current study, the number of isolates of *P. chrysogenum* were comparatively low. Earlier studies have reported this species as an endophyte in different mangrove plants (Devi, 2012, Huang, 2016, Rahaman, 2020). *Penicillium citrinum* is a filamentous fungus, commonly distributed world-wide and isolated from different sources such as soil, plants and indoor environments (Houbraken, 2010) and is a source of citrinin, a potent mycotoxin. *P. citrinum* is the dominant endophytic species found in *A. corniculatum* and *C. tagal* in this study. *Penicillium georgiens* is a soil fungus causing infections in *Allium cepa* (Yee, 2014). There is no literature reported till now regarding the endophytic nature of this species. *Talaromyces purpureogenus* (a teleomorph of the genus *Penicillium*), a red pigment producing filamentous fungus was isolated from both *A. corniculatum* and *C. tagal*. This species was reported as an endophyte from several terrestrial plants (Hu, 2019; Feng, 2020; Sharma, 2022) as well as sea weeds (Kumari, 2018). Our study is the first report of endophytic *T. purpureogenus* from mangroves.

Phyllosticta capitalensis has been repeatedly isolated worldwide from healthy plant tissues and is reported as a minor plant pathogen (Wikee, 2013). In the present study, this species was only recovered from *L. racemosa* and it was the second most dominant species in that host plant. Reports showed that

this species was the dominant endophyte of different mangrove species in Andaman Island (Rajamani, 2018).

Genus, *Colletotrichum* is primarily described as the causative agent of diseases in various plant species (Cannon, 2012; de Silva, 2019; Khodadadi, 2019, Chung, 2020). *C. asianum, C. fruticola, C. gloeosporoides* and *C. siamense* were reported to be associated with the anthracnose disease in mango (Li, 2019; Tovar-pedraza, 2020). *Colletotrichum* as a dominant fungal endophyte has been reported in other mangroves such as *Acanthus ebracteatus* and *Xylocarpus granatum* (Rajamani, 2018).

Schizophyllum commune, is the only basidiomycota obtained in our study. The species was isolated from both *A. corniculatum* and *L. racemosa*. It is the most commonly found fungi and could be isolated from all continents except Antartica (Ohm, 2010). Studies showed that it can cause infections in humans and plants but mainly lives as a saprobic fungus causing white rot (Premamalini, 2011; Lahbib, 2016; Tian, 2018; Itoh, 2021, Galvez, 2021). Previously, this species has been reported as an endophyte from *Avicennia officinalis* (Joel, 2012).

Neurospora intermedia is the only mould belonging to the genus *Neurospora* which is used in the food production. It is used to produce the Indonesian dish “oncom merah”. The species is a rich source of the yellow pigment carotenoids. The biomass of the fungi enriched with nutrients such as essential amino acids and omega-3 and 6 fatty acids (Gmoser, 2018). In the current study, this species could be isolated only from *A. corniculatum*. Few reports were available on the endophytic nature of *N. intermedia*. This species having bioremediation potential was reported from sugar cane previously (Wang, 2017).

Fusarium species is gaining much attention due to its plant pathogenicity, causing infections in almost all economically important plants and thereby affecting the agriculture industry (Dongzhen, 2020). Moreover, some of the species act as an opportunistic human pathogen causing damages in the cornea, nail and skin (Gupta, 2000). In the present study*, F. incarnatum* also could be recovered, which was formerly isolated from *A. corniculatum* (Li, 2008).Two yeast species, *Candida orthopsilosis* and *Meyerozyme caribbica* were isolated from *A. corniculatum* and *C. tagal* respectively. Formerly, both these yeast species were recovered as endophytes from the fruits of Camu-Camu (Matos, 2021) and its fermentation capability was also evaluated.

Diaporthe commonly found as endophyte/ plant pathogen are distributed in a wide host range and has been recorded as one of the most predominant mangrove endophytic fungi (Zong-shan, 2008; Chen, 2009). A study evaluating the species diversity of the culturable endophytic fungal community of Brazilian mangrove forest indicated that this genus was found in all sampling sites, in both the seasons, in all plant parts and in all plant species selected (De Souza, 2013). In the present study, this genus was obtained only from the two hosts; *A. corniculatum* and *L. racemosa.*

Escovopsis are reported only from the fungus gardens of attine ants. These anamorphic fungi are potentially virulent parasite of the attine ants and fungal cultivars (Reynolds, 2004). *Cladosporium* species are cosmopolitan in distribution in plants as endophytes and in foods, textiles and other organic

matter as saprobes (Bensch, 2012). It also acts as a pathogenic agent for plants, animals and humans. *C. oxysporum* was isolated earlier from the root of *Avicennia officinalis* by direct plating and damp chamber incubation (Ananda, 2002).

Antibacterial activity

Endophytic microflora serves as a rich source of novel natural compounds with a wide spectrum of biologically active agents. A significant number of fungal endophytes having antimicrobial activity were reported from mangroves in the past decades. Both crude and ethyl acetate extract of endophytic fungi from different mangrove species showed broad range of antibacterial activity (Buatong, 2011; Onn, 2016; Nia, 2017). Previously, an α-glucosidase inhibitor, altenusin derivative, was purified and characterized from the mangrove endophyte, *Alternaria alternata* (Liu, 2016). Secondary metabolites produced by this endophytic species isolated from different plants possessed antimicrobial, antioxidant, antibiofilm, anti-quorum sensing as well as antitumour activity (Fernandes, 2009; Rashmi, 2018, Chandra, 2021). Ethyl acetate extract of endophytic *Cladosporium oxysporum,* significantly inhibited the activity of bacteria (Sugijanto, 2016). Secondary metabolites from *Phyllosticta capitalensis*, an endophyte of *Brugiera sexangula* exhibited antibacterial and antifungal activity (Xu, 2021). Endophytic *Colletotrichum* spp. from different plant species possessed antimicrobial activity against various pathogens (Du, 2020; An, 2020). Our study revealed that, endophytic species of *P. citrinum, P. chrysogenum* and *A. aculeatus* possessed broad spectrum activity against both Gram positive and Gram -negative bacteria. *Penicillium chrysogenum* produces a broad range of secondary metabolites such as roquefortines, fungisporin (a cyclic hydrophobic tetrapeptide), siderophores, penitric acid, ω-hydroxyemodin, chrysogenin, chrysogine, sesquiterpene PR-toxin and sorbicillinoids (Guzman-chavez, 2018). An antibacterial compound, 3,1'-didehydro-3[2" (3‴,3‴- dimethyl-prop-2-enyl)-3"-indolylmethylene]-6-methyl pipera-zine-2,5-dione purified from the mangrove endophyte *P. chrysogenum* showed significant inhibitory activity against *Vibrio cholera* (Devi, 2012). Austinol and Penicimarin G and H, two antibacterial compounds from endophytic *P. citrinum* isolated from *Brugiera sexangula* (He, 2017), exhibited activity against both Gram positive and Gram negative bacteria. Secalonic acid derivative from endophytic *A. aculeatus* induced apoptosis in triple negative breast cancer cells by reactive oxygen species formation (Farooq, 2020). *A. sydowiii* from both marine and terrestrial hosts possessed antimicrobial, antioxidant and cytotoxic activity (Handayani, 2018; Bhattacharya, 2018; El-sayed, 2022). The fungal genus *Aspergillus* from different mangrove hosts is well known as a potential source of various bioactive molecules having antibacterial property (Handayani, 2017; Nurunnabi, 2020; Ramirez, 2020). Antibacterial and anticancer activity of the silver nanoparticles synthesized using endophytic *T. purpureogenus* are reported earlier (Hu, 2019; Sharma, 2022).

Enzymatic activity

Endophytic fungi are rich source of extracellular enzymes (Bezerra, 2012) that are suited to industrial application because of its low-cost production and reliability. Fungal enzymes are gaining importance because of its stability at high temperature and extreme conditions than the plant and animal derived

enzymes. These enzymes can be widely used in manufacturing of food, beverages, confectioneries, textile and leather. Amylase is an industrially important enzyme that can function at diverse pH and temperature and may be employed in the hydrolysis of polysaccharides (Liu, 2008). Proteases represent two-thirds of global industrial enzyme requirements. They are widely used in pharmaceutics, food industries and also in industrial bioremediation purposes. Lipases are multipurpose biocatalyst and had been widely used in several industries such as biodiesel, food, leather, textile, detergents and pharmaceuticals (Chandra 2020). Microbial lipases are very dynamic and proficient because they do not need cofactors and able to act on a large group of substrates (Toghueo, 2017). In the current study, more than 85% of the isolates produced all these three enzymes which is in agreement with the previous reports of endophytic fungi from two mangrove species (Raviraja, 2005). Notable enzyme production (amylase, lipase and protease) could be observed for endophytic fungal species from medicinal plants also (Alberto 2016, Toghueo, 2017).

Asparaginase, an amidohydrolase which cleave the aminoacid asparagine into aspartic acid and ammonium is one of the major chemotherapeutic agents routinely used in the treatement of Acute Lymphoblastic Leukemia (Moubasher, 2022). Another important amidohydrolase from microbial source, the Glutaminase which hydrolyse glutamine to glutamic acid and ammonia. The enzyme has gained much attention in the recent years due to its potential application in the cancer treatment, flavour enhancing agent as well as anti-retroviral agent (Abbas, 2016). In the present study, nearly 64% of the tested isolates were able to produce these two enzymes. Purification, characterization and antineoplastic behaviour of L-asparaginase from different endophytic fungi were studied (Manasa, 2014; Hatamazadeh, 2020; Moubasher, 2022). *Trichoderma* sp., a potent producer of l-asparaginase was isolated from *Sonneratia alba* (Prihanto, 2019). But the reports on enzyme, glutaminase from the endophytes is limited.

Cellulase, the third most industrially important enzyme with a wide range of applications in industries such as textile, paper, food and feed, biofuel, detergent and also in waste management. Cellulase activity was detected in 53% of the isolates. Previously, 27.67% of endophytic fungal isolates from the medicinal plant of Western Ghats, showed cellulolytic activity (Uzma, 2016). In another study, all the isolates from *Acanthus ilicifolis* and *Acrostichum aureum* exhibited cellulase activity (Maria, 2005). The endophytic fungi of Brazilian mangrove forest also exhibited cellulolytic activity (Martinho, 2019). Pectinases play a key role in the phytopathologic processes, plant-microbe interaction and decomposition of dead plant material. Pectic enzyme may be acidic or alkaline depending upon their source of origin. Acidic pectinases were synthesized by eukaryotic organisms while alkaline pectinases were derived from prokaryotes (Kubra, 2018). Pectinase were extensively applied in food, agricultural and environmental sectors. Pectinolytic activity was observed in 65% of our endophytic isolates. Endophytic fungi (57%) from Thai orchid showed high pectinase activity (Sopalun, 2020). Maximum pectinase activity was observed in *Talaromyces* sp. from *Calophyllum inophyllum* (Sunitha, 2017).

Tyrosinases are a group of binuclear copper enzymes and play a major role in the synthesis of tannin, lignin, flavonoids and regulate the redox potential during plant respiration and wound healing (Nawaz, 2017). This enzyme is useful in pharmaceutical, food bioprocessing and environmental applications. In

our study, 24% of the tested isolates displayed tyrosinase activity. More than 50% of endophytic fungal isolates from medicinal plants were producers of tyrosinase (Zaidi, 2013). Laccases are copper containing oxidoreductase enzyme, widely distributed in higher plants, bacteria, fungi and insects (Shraddha, 2011). The broad substrate specificity of laccase makes it highly applicable for various industries such as paper, textile, food and bioremediation (Singh, 2020). First report of laccase producing endophytic fungi were obtained from *Cyanodon dactylon* (Wang, 2006). In this study, among the total isolates screened, 22% were positive, which is lower than that of other enzymes. This is in agreement with the findings of Toghueo, 2017, where only 33% of the isolates were producers of laccase. Endophytic fungi *Fusarium* sp. of Brazilian mangrove forest was found to possess laccase activity (Martinho, 2019). Ligninase has broad range of applications especially in the degradation and detoxification of lignocellulosic waste (Kumar, 2020). In the present study, 23% of the fungal isolates showed ligninolytic activity. Earlier studies on enzyme activity of mangrove endophytic fungi indicated that 60% of the isolates were positive for ligninase (Martinho, 2019). Moreover, ligninase producing capability of endophytic fungi from plants such as *Dillenia indica* (Prasher, 2021) and *Brucea javanica* (Choi, 2005) were also reported.

DNase or deoxyribonuclease are enzymes that non-specifically cleaves DNA to release 5'-phosphorylated di, tri and oligonucleotide products. The removal of extracellular DNA is crucial for reducing the inflammatory response and maintaining homeostasis. DNase has a wide range of applications in biomedicine i.e., for the treatment of cystic fibrosis and chronic obstructive pulmonary diseases (Laukova, 2020). Among the total isolates screened, 44% showed DNase activity. Extracellular DNase activity of *Cryptococcus* sp. (Sanchez, 2010) and an inducible DNase from a thermophilic fungus were also reported earlier (Landry, 2014). Chitinases are glycosyl hydrolases, that can be applied in the preparation of pharmaceutically important chitooligosaccharides, single cell protein production, sea food waste management and control of mosquitoes and nematodes (Kuddus, 2014). There are no endophytic fungal isolates having chitinolytic activity obtained in our study. Foliar endophytic fungi from Pichavaram mangrove forest were potent producers of chitinase (Rajulu, 2011). A salt- tolerant chitinase was purified from *Talaromyces sitipitatus*, an endophytic fungus of *Avicennia marina* (Paranetharan, 2018).

Conclusion

The study provides insight into the diversity of foliar endophytic mycoflora of three mangrove species viz., *Aegiceras corniculatum, Lumnitzera racemosa* and *Ceriops tagal* from Kerala coast. A total of 382 isolates belonging to 25 species could be recovered from the leaf tissues of the three mangroves. Eleven percent of these isolates exhibited antibacterial activity. Hydrolytic enzyme production potential was also significant especially with reference to cellulase, asparaginase, glutaminase and pectinase. Metabolites originated from fungal endophytes hold promise to be further developed as a potent agent in therapeutics and industries. It can be concluded that endophytic mycoflora of the leaf tissues of mangroves of Kerala coast, could be a promising source of antibacterial agent and industrially important enzymes with commercial and biotechnological applications.

Declarations

Ethics approval : Work does not involve any experiments that needs ethical approval

Availability of data and material: *The datasets generated during and/or analysed during the current study are available at GenBank (NCBI).*

Conflicts of interest/Competing interests: The authors declare that they have no known competing financial interests or personal conflicts that could have appeared to influence the work reported in this paper.

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Authors' contributions: RP and RMR *conceptualized the idea and designed the work plan. Sample collection and experimental work were performed by RMR. Data analysis was done by RMR, ASM, DK, and MS. The first draft of the manuscript was written by RMR and all authors corrected and commented on previous versions of the manuscript. All authors read and approved the final manuscript.* RP supervised the work.

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Tables

Table 1 Media and reagents used for testing hydrolytic enzyme production by the endophytic fungi

Hydrolytic Enzymes tested	Medium (g /L Sea water of 25ppt)	Reagents used	Appearance
Amylase	Glucose yeast extract peptone (GYP) agar (Glucose 10.0 g; yeast extract 0.1 g; peptone 0.5 g;.,agar 20 g) supplemeted with starch 2%	Lugol's iodine (1% iodine and 2% Potassium iodide in Distilled water)	Clear zone
Lipase	GYP agar supplemented with tributyrin 1%	Nil	Clear zone
Protease	GYP agar + gelatin 2%	15% Mercuric chloride solution (HCl, 20 ml; Distilled water, 80ml)	Clear zone
Cellulase	GYP agar + Carboxy methyl cellulose 1%;	0.2% aqueous Congo red followed by destaining with 1M NaCl	Clear zone
Chitinase	GYP agar + colloidal chitin 5%;	Nil	Clear zone
Tyrosinase	GYPagar tyrosine 0.5%; agar 20 g)	Nil	Clear zone with brownish periphery
Laccase	napthol 0.05%; agar 20 g)	Nil	Reddish brown
Asparaginase	Glucose 2g; KH_2PO_4 1.52g; KCl 0.52g; $MgCl_2$ 0.52g; $FeSO_4.7H_2O$ 0.01g; phenol red 0.009%; L-Asparagine 1%; agar 20g	Nil	Pink colouration around the colony
Glutaminase	Glucose 2g; KH_2PO_4 1.52g; KCl 0.52g; $MgCl_2$ 0.52g; $FeSO_4.7H_2O$ 0.01g; phenol red 0.009%; L-Glutamine 1%; agar 20g	Nil	Pink colouration around the colony
Ligninase	Crawford's agar Methylene blue 0.2%; agar 20g)	Nil	Clear zone
Pectinase	Yeast extract agar (Yeast extract 10g; pectin 1%; agar 20g	Lugol's iodine solution (1% iodine and 2% potassium iodide in Distilled water)	Clear zone
DNase	DNase agar (Peptone 5g; Beef extract 3g; DNA sodium salt 2g; agar 20g)	1N HCl	Clear zone

Table 2: Colonization frequency of endophytic fungi from the three mangroves

Species	*A. corniculatum*	*C. tagal*	*L. racemosa*
Alternaria alternata	1.2	-	3.3
Ascotricha chartarum	-	0.1	-
Aspergillus aculeatus	0.3	-	1.6
Aspergillus flavus	-	2	-
Aspergillus montevidensis	0.1	-	-
Aspergillus sydowiii	-	4	-
Candida orthopsilosis	0.1	0.4	-
Chaetomium globosum	0.1	-	-
Cladosporium oxysporum	0.5	-	-
Colletotrichum asianum	0.4	-	-
Colletotrichum fruticola	-	-	9.6
Colletotrichum gloeosporides	-	0.7	-
Colletotrichum siamense	2	-	18
Diaporthaceae sp	0.5	-	5
Diaporthe hongkongensis	0.5	-	-
Escovopsis weberi	-	-	8
Fusarium incarnatum	0.4	-	0.3
Meyerozyme caribbica	0.1	0.1	-
Neurospora intermedia	0.2	-	-
Penicillium chrysogenum	0.1	0.3	-
Penicillium citrinum	2.3	3	0.6
Penicillium georgiens	0.1	-	-
Phyllosticta capitalensis	-	-	11.6
Schizophyllum commune	0.4	1	-
Talaromyces purpureogenus	0.1	0.1	-

Figures

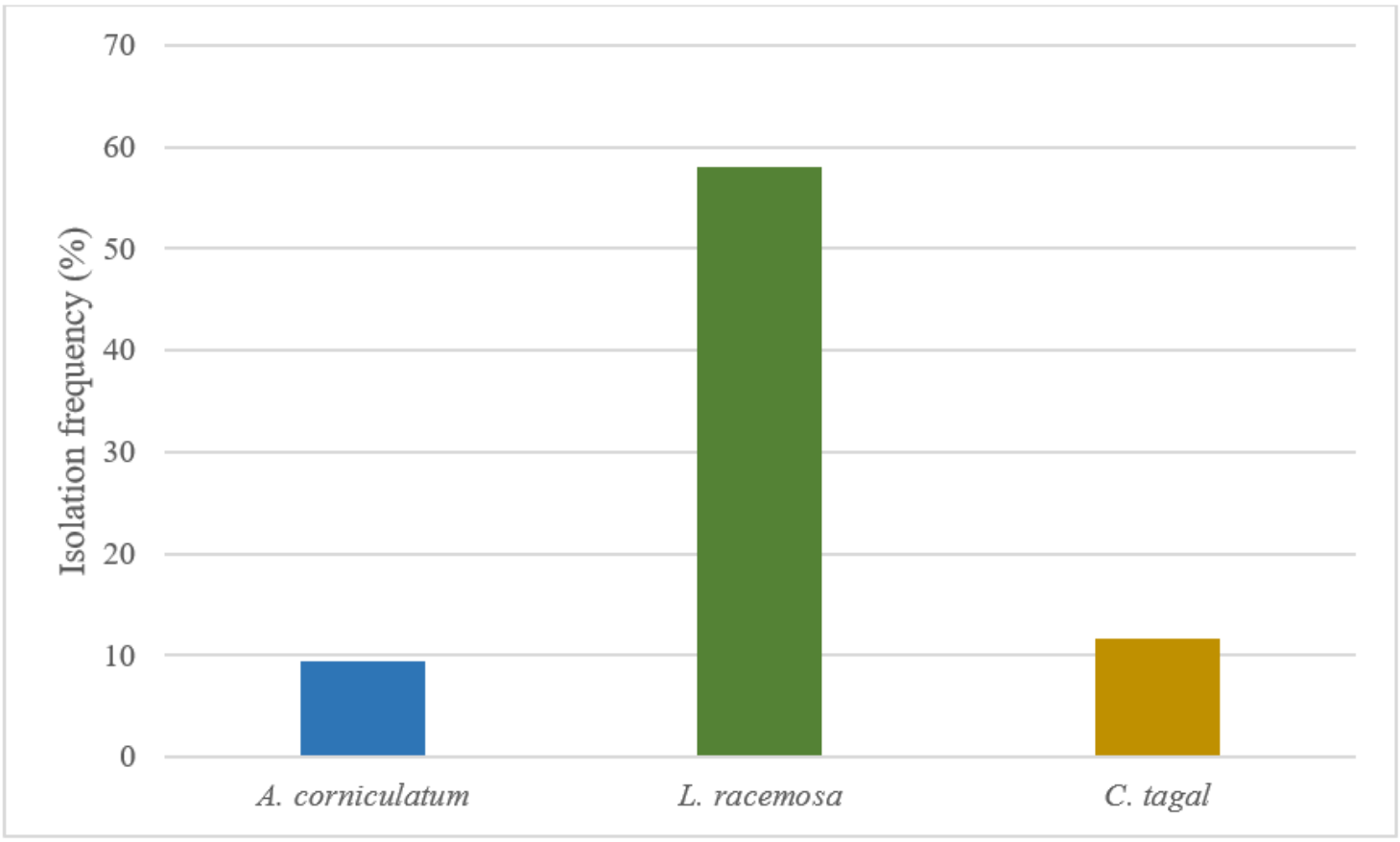


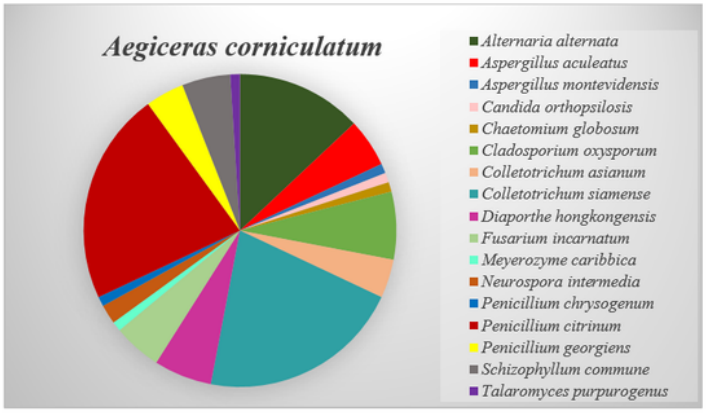
Figure 1

Isolation frequency of fungal endophyte from three mangroves

Figure 2

Taxonomic classification of endophytic fungi from mangroves a) *A. corniculatum*, b) *L. racemosa* and c) *C. tagal*.

a



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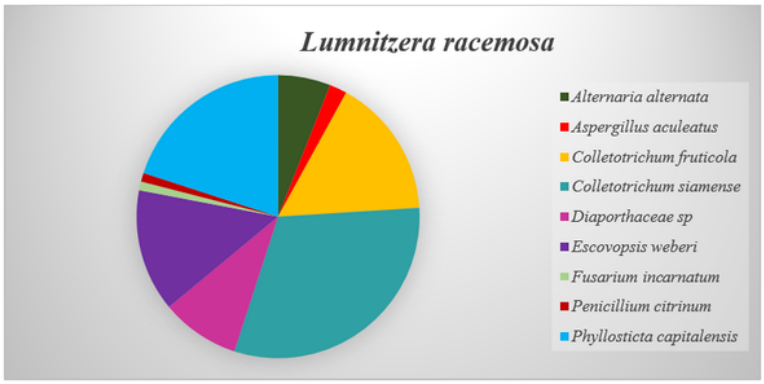

c

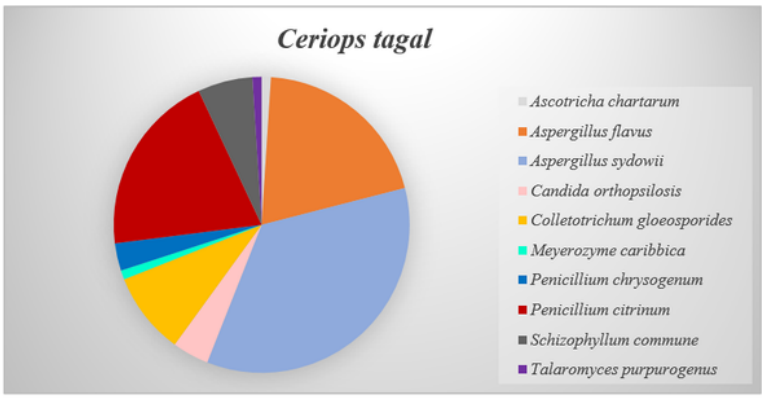

Figure 3

Species composition of endophytic fungi from mangroves a) *A. corniculatum*, b) *L. racemosa* and c) *C. tagal*.

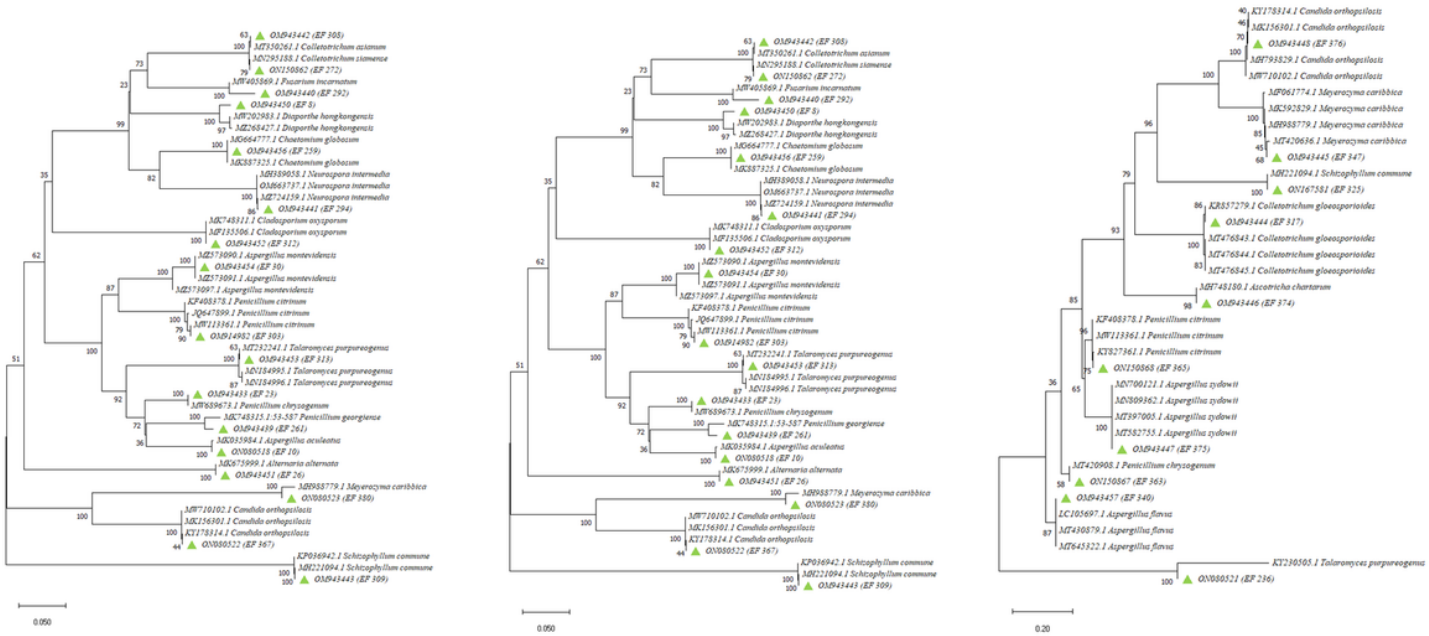

Figure 4

Neighbour joining tree based on ITS-rDNA sequences showing the grouping among endophytic fungi obtained from a) *A. corniculatum*, b) *L. racemosa* and c) *C. tagal*

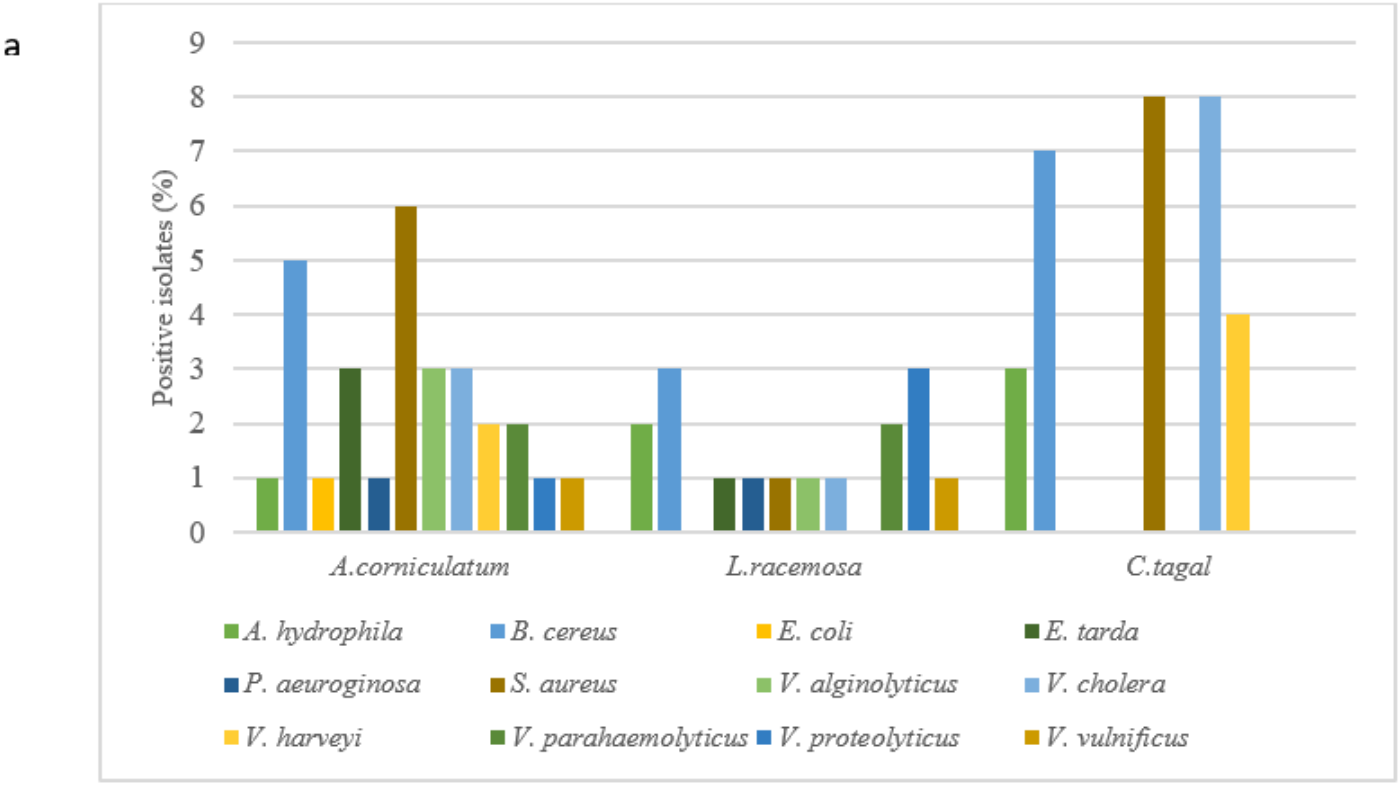

Figure 5

Antibacterial activity profile of the endophytic fungi from mangrove plants

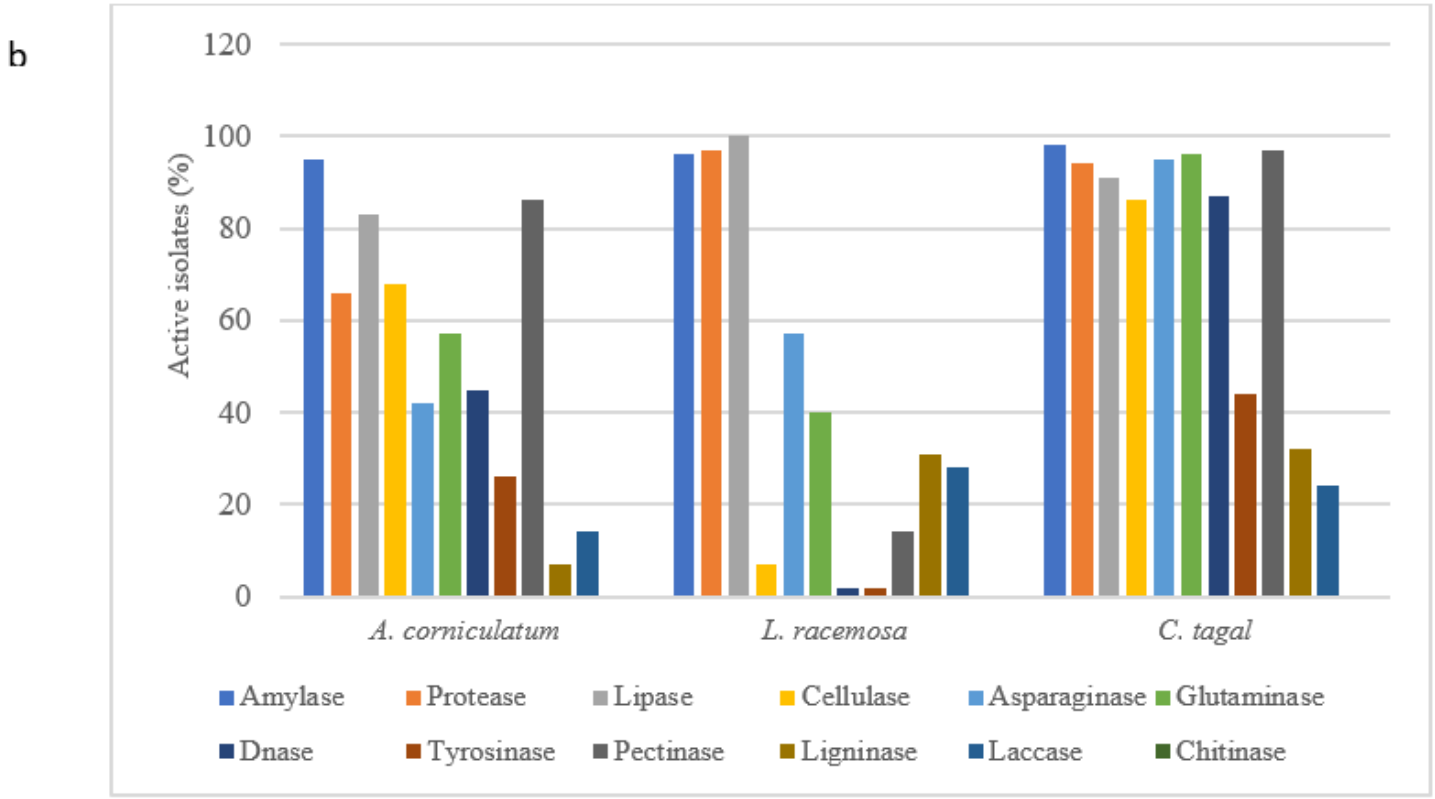

Figure 6

Hydrolytic enzyme production by endophytic fungi from mangrove plants

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableST1.docx
- TableST2.docx
- TableST3.docx