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Bioactive Metabolite from *Aphyllophorales sp.* an Endophytic Fungus Isolated from *Breguiera Cylindrica*

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Abstract

The significance of natural products in the drug discovery and development has been reported extensively. The natural products from mangrove fungi, which are known to be a rich source of bioactive metabolites, are of biotechnological interest. As most of these fungi grow in a unique and extreme habitat, they certainly will play a crucial role in meeting the demand for screening novel bioactive compounds. This is the first report on isolation of basidiomycete endophytic fungus, *Aphyllophorales* sp. (JQ34006) from mangrove plants, *Breguiera cylindrica.* Among the 44 strains isolated, the ethyl acetate extract of *Aphyllophorales* sp. exhibited pronounced activity in preliminary antimicrobial screening. The isolated bioactive compound showed an Rf value of 1.5 in Thin layer chromatograpy (TLC) analysis. In high performance liquid chromatography (HPLC) a prominent peak was detected at retention time 2.314 minutes. The FT-IR analysis showed the presence of a benzene ring in the compound represented by characteristic functional group vibrations. The isolate exhibited protease activity. The results indicated that further investigations are still needed to discover other potential of this isolate.

Keywords: Antimicrobial, basidiomycota, mangrove, electrophoresis

Endophytic fungi are defined as fungi residing the living plant tissue without causing any apparent diseases or injuries to the host plant. To date, many studies have elucidated the ubiquity of the endophytic fungi in plants and the mutualistic relationship between the fungal endophytes and the host plant. It was also documented that fungal endophytes could exert beneficial activities on host plants such as favorable, growth-promoting effects, increasing host fitness and contributing to effective host defense against pathogens, herbivores, or abiotic stress. Endophytes have a powerful ability to transform complex compounds. The potential for biotransformation and a set of specific enzymes could allow them to survive and reproduce (Zikmundova *et al.,* 2002). In addition, experiments have confirmed that, after they have coexisted for a long time, endophytes can produce effective bioactive substances from their host plants *in vitro*, especially when active ingredients are extracted from the host plants subject to a variety of constraints. It is worth noting that fungal endophytes also have the ability to utilize various organic compounds such as carbon sources, which enables them to play an important role in the degradation of structural components, plant leaf litter and wood (Osono and Takeda, 2001), and that some fungi with marked decomposing

abilities are associated with ligninolytic activity (Urairuj *et al.,* 2003). In addition, it has been shown that they have the potential to decompose environmental pollutants and to improve the soil microenvironment (Xiao *et al.,* 2010). Nowadays, fungal endophytes have been recognized as relatively unexplored and potential sources of bioactive secondary metabolites for exploitation in medicine, agriculture, and industry. Furthermore, antimicrobial substances that could be produced by endophytic fungi have drawn even greater attention. This gives rise to people's interest in screening endophytic fungi for discovery of novel metabolites.

Mangroves are coastal wetland forests established at the intertidal zones of estuaries, backwaters, deltas, creeks, lagoons, marshes and mudflats of tropical and subtropical latitudes. Among the marine ecosystems, mangroves constitute the second most important ecosystem in productivity and sustained tertiary yield after coral reefs (Qasim and Wafar, 1990). Mangrove plants have morphologically and physiologically adapted to habitats with high salinity, tidal inundation, high wind velocity, high temperature and anaerobic clayey soils. These forests are of great ecological importance, social significance and economic value. A mangrove forest is considered a dynamic ecotone (or transition zone) between terrestrial and marine habitats (Gopal and Chauhan, 2006).

Scientists have realized that mangrove fungi are important to mangrove helping them for adapting to the extreme environment and suggested that they are promising sources for screening new products (Strobel *et al.,* 2004). With the development of new molecular targets, there is an increasing demand for novel molecular diversity for screening. Undoubtedly, the natural products from mangrove fungi, which are known to be a rich source of bioactive metabolites that are of biotechnological interest certainly will play a crucial role in meeting the demand for screening novel compounds.

The wide variety of endophytes that exist within the world's mangrove plants have not been fully investigated. The unique habitats of endophytes make them more purposeful and selective in biological conversion and degradation. The aim of this study was to find out novel bioactive metabolite from endophytic fungi from mangrove plants. A novel unreported endophytic aphyllophorales species was isolated from the leaf of *Breguiera cylindrica* and the ethyl acetate extract of its culture showed pronounced antimicrobial activity.

Materials and methods

Sample collection

Healthy leaves from two mangrove species *Breguiera cylindrica* and *Rhizophora candelaria* were selected for the study. Healthy leaf samples were collected from Kattukandam at Ayiramthengu, Fisheries Resource Management society, Kollam dist, Kerala. Samples were transported to the laboratory as quickly as possible following collection and processed within 24 hours. A vigorous prewashing of plant material was performed under running tap water to reduce the number of surface micro flora and to remove soil or dust particles.

Isolation of endophytic fungi

The isolation of endophytic fungi from *Breguiera cylindrica* and Rhizophora *candelaria* was carried out as described by Strobel *et al.,* (1996) with minor modifications. Sample fragments were successively surface sterilized by immersion in 70% ethanol for 1 min, 5.25% sodium hypochlorite solution for 5 min, 70% ethanol for 30 s and sterile distilled water for 3 to 5 s. The cut surfaces of the segments were placed on Petri dishes containing potato dextrose agar (PDA) (oxoid) supplemented with chloramphenicol (50 µg/ml, Merck) and streptomycin sulphate (250 µg/ml, Sigma) to suppress bacterial growth. The inoculated plates were incubated at 28°C until the outgrowth of endophytic fungi was discerned. Isolates were then transferred to PDA plates free of antibiotics for purification.

Extraction and isolation of crude ethyl acetate extracts from Endophytic fungi

Each of the pure cultures was cultivated on PDA at 28°C for 7 to 14 days. Three pieces (0.5 × 0.5 cm2) of mycelia agar plugs were inoculated into 500 ml Erlenmeyer flasks containing 40 ml potato dextrose broth (PDB) and incubated at room temperature for four weeks under stationary conditions. The broth culture was filtered to separate the filtrate and mycelia. The filtrate was extracted three times by shaking with an equal volume of ethyl acetate (EtOAc). The culture broths were pooled and dried in a rotary evaporator (Buchi, Switzerland). The extract residue was dissolved in di methyl sulfoxide (DMSO) and stored at 4°C as stock solution for antimicrobial bioassay.

Antibacterial assay

The crude ethyl acetate extracts of the seven endophytic

fungi were tested for their antibacterial activity against *Proteus, Klebsilla, Escherichia coli, Pseudomonas aeruginosa*. Antibacterial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Pre-warmed Mueller-Hinton agar (MHA) (oxoid) plates were seeded with 10^6 CFU suspension of test bacteria. Endophytic extracts dissolved in DMSO (1 mg/ml) were pipette onto sterile paper discs (6 mm diameter, oxoid) and placed onto the surface of inoculated agar plates. Plates were incubated at 37°C for 24 h. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. DMSO was used as negative control.

Screening and identification of potential isolates

The efficient isolate from the above experiment was selected and used for further studies *i.e.* to elucidate biocontrol mechanisms

Morphological identification

The selected efficient isolate was identified by observing the colony morphology *viz* colour, texture, colony pigmentation on front and reverse positions of the PDA plate and microscopic identification was done by lacto phenol cotton blue staining.

Molecular identification

Fungal genomic DNA of the selected isolate was extracted using alkali lysis method. Its quality was evaluated on 0.8% Agarose Gel. A single band of high-molecular weight DNA was observed. D1/D2 region of LSU (Large subunit 28S rDNA) gene was amplified by PCR from the above isolated genomic DNA. A single discrete band was observed when resolved on Agarose Gel.

Using the primers DR - 5'-GGTCCGTGTTTCAAGACGG-3' and

DF- 5'-ACCCGCTGAACTTAAGC-3' the fungal DNA was amplified. The PCR amplicon was purified by column purification. DNA sequencing was carried out with PCR amplicon. The D1/D2 region of LSU (Large subunit 28S rDNA) gene sequence was used to carry out BLAST with the database of NCBI GenBank.

Test for enzyme activity of the mangrove endophytic fungi

Amylase assay

Amylase activity was assessed by growing the fungi on glucose yeast extract peptone (GYP) agar medium (glucose. 1 gm; yeast extract 0.1 gm; peptone 0.5gm; agar 16 gm; distilled water 1000 ml; pH 6) with 2% soluble starch. After incubation the plates were flooded with 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was considered positive for amylase activity (Maria *et al.,* 2005).

Protease assay

Protease assay was performed by growing the fungi on the GYP agar medium amended with 0.4 gelatin (gelatin 8 gm in 100 ml distilled water, sterilized separately and mixed with sterile GYP agar medium) adjusted the pH to 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin precipitated with ammonium sulphate and digested area around the colony was clear (Maria *et al.,* 2005).

Thin layer chromatography

The ethyl acetate extract of fungal culture filtrate was loaded on TLC plates and developed using chloroform: methanol (9:1). The dried plates were exposed to iodine vapour and the Rf value was calculated.

Fourier Transform –Infra Red (FT-IR) spectroscopy

The culture filtrate extract was analysed using FT-IR using KBr thin film technique. The range of measurement is from 4000 to 667.

High Performance Liquid Chromatography (HPLC)

The component was detected with SPD20 UV-VIS detector (254nm) data acquisition and processing was done by LC solution system (Shimadzu). Bioactive compound was analyzed by reverse phase liquid E-18 column. The mobile phase water/acetonitrile (72:28 v/v) flow rate was set at 0.8 ml/min and the UV detector was set at a wave length of 290 nm. **(**Ashish Baldi *et al.,* 2008) Data acquisition and processing were performed using LC solution system (Shimadzu).

Results

A total of 44 fungal isolates were obtained from the two selected mangrove plants *Breguiera cylindrica and*

Rhizophora candelaria. Out of 44 isolates seven endophytic organisms which showed consistent morphology and colony characters obtained from the learves of the manqrove species were selected from the leaves of the mangrove species *Breguiera cylindrica* and Rhizophora *candelaria* were selated for further know is using Potato dextrose Agar and Yeast Extract Glucose Agar medium.

From among the 44 isolates, ethyl extracts of the seven isolates showed inhibitory activity toward the test organisms *Proteus* sp., *Pseudomonas* sp., *Klebsilla* sp. and *E. coli.* Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts on Muller Hinton Agar. Each of endophyte displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 0.3 to 1.8 mm (Table 1). Replicates are maintained for each set of experiments.

Table 1: Antimicrobial activity of the crude ethyl acetate extracts of seven endophytic fungi.

Isolate	Zone of inhibition (mm)*			
	Protease sp.	*Pseudomonas* sp.	*Klebsilla* sp.	*E. coli*
PRL21	0.8	0.7	0.7	0
PRL32	0.6	0.3	0	0
YBL21	0.6	0	0.4	0.7
YBL22	0.8	0.8	0.6	0.6
PBL11	0.8	0	0.6	0
YRL32	1.3	0.6	0.8	0
PBL12	1.8	0.7	0.8	0.7

* Values are average of three replicates.

Stronger antibacterial activity against selected bacteria was displayed by isolate PBL12. The inhibition zones against *Proteus* sp., *Pseudomonas* sp., *Klebsilla* sp. and *E. coli.* was 1.8, 0.7, o.8 and o.7 mm, respectively. Therefore PBL12 selected for morphological and molecular identification.

In potato dextrose agar the isolate PBL12 formed white puffy colony. These colonies in the Potato Dextrose broth showed the production of red pigment after 4 to 5 days of incubation. In microscopic examination thread like cylindrical interconnected network of hyphae were observed. The isolate was identified as a member of the *Aphyllophrales* sp.

The **Aphyllophorales** is an obsolete order of fungi in the Phylum Basidiomycota, class Hymenomycetes. The order is entirely artificial, bringing together a miscellany of species now grouped among the clavarioid fungi, corticoid fungi, cyphelloid fungi, hydnoid fungi, and poroid fungi. classification systems based on cladistic analysis of DNA sequences.

The molecualr identification of the isolate PBL 12 was done. Based on the D1/D2 Region- PCR amplification, sequencing and BLAST analysis it was found that the fungal culture PBL12 showed 99% similarity with Aphyllophorale s sp. (Accession No: DQ327656.1) and 98% similarity with *Cerrena consors* (Accession No: FJ821518.1) (Figure 1). The sequences of the selected isolate was deposited in the NCBI gene bank and got the accession number **JQ34006.**

In the test for enzyme activity of the isolated endophytic fungi, the isolate PBL12 (JQ34006) showed negative result in amylase assay and positive result in protease assay.

In TLC analysis the distance travelled by the solvent measured as 18 and the distance travelled by the compound measured as 12. Rf value was calculated as 1.5. FT-IR spectra analysis showed the characteristic stretching frequency at 1150-1120 cm^{-1} due to normal alkanes, vibration involving in phase C-C-C stretch mixed with in plane CH_3 rock absorb weakly at this stretching frequency. The X- CH_3 rock vibration deforms the HCX bond angle and like the CH_3 symmetry deformation, is sensitive to the electro negativity of the X substituent. Approximate CH_3 rock frequency of S-CH_3 about 1030-950 cm^{-1}. In aryl-CH_3 groups a band involving CH_3 rock is usually found in stretching frequency 1070-1010 cm^{-1}. In the range 1275 ± 25 cm^{-1} the CH_2 wag vibration bands are intensified in CH_2-Cl .,1642-1562 cm^{-1} frequency stretches have been studied having 1,4 benzo and naphtha quinine dizzied group., The presence of benzene ring in the compound strongly support the band stretches 1073, 1011, 1125 and 1076 cm^{-1} in the FTIR result. The stretch 1073 mono substituted benzene are seen and in 1011 ring bending vibration of vibration of benzene ring ,1125 ± 14 cm^{-1} ortho substituted benzene and in 1076 cm^{-1} meta substituted benzene are seen. For the control and PBL12 spectrum the stretches 1440-1400 cm^{-1} stretches indicate the HO-CH secondary alcohol (Figure 2a and 2b)

In HPLC analysis of the extracted bioactive compound was obtained are major peak at retention time (RT) 2.314 minutes was obtained (Figure 3). The peak area was found to be 1091482 cm^2

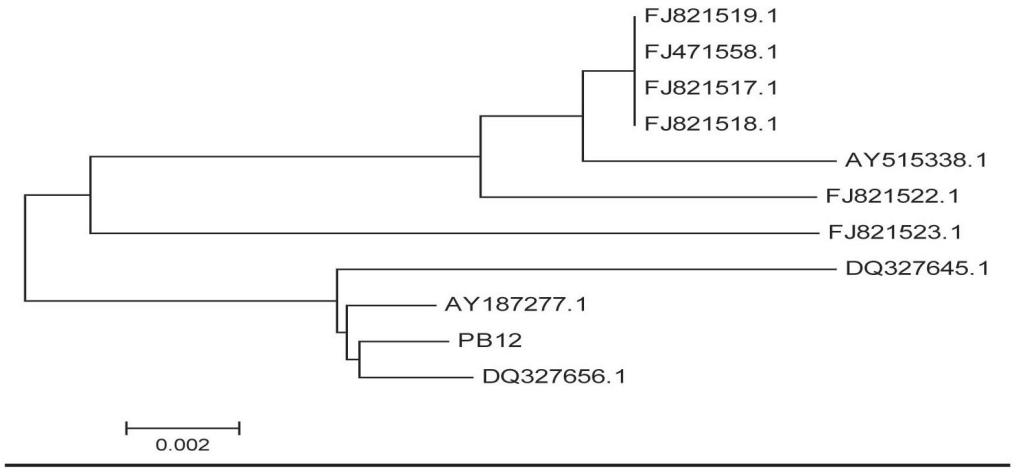

Fig. 1: Phylogenetic tree showing relationship of PBL12
PBL12 culture shows maximum similarity with DQ32765.1*(*Aphyllophorales sp. DIS 296a

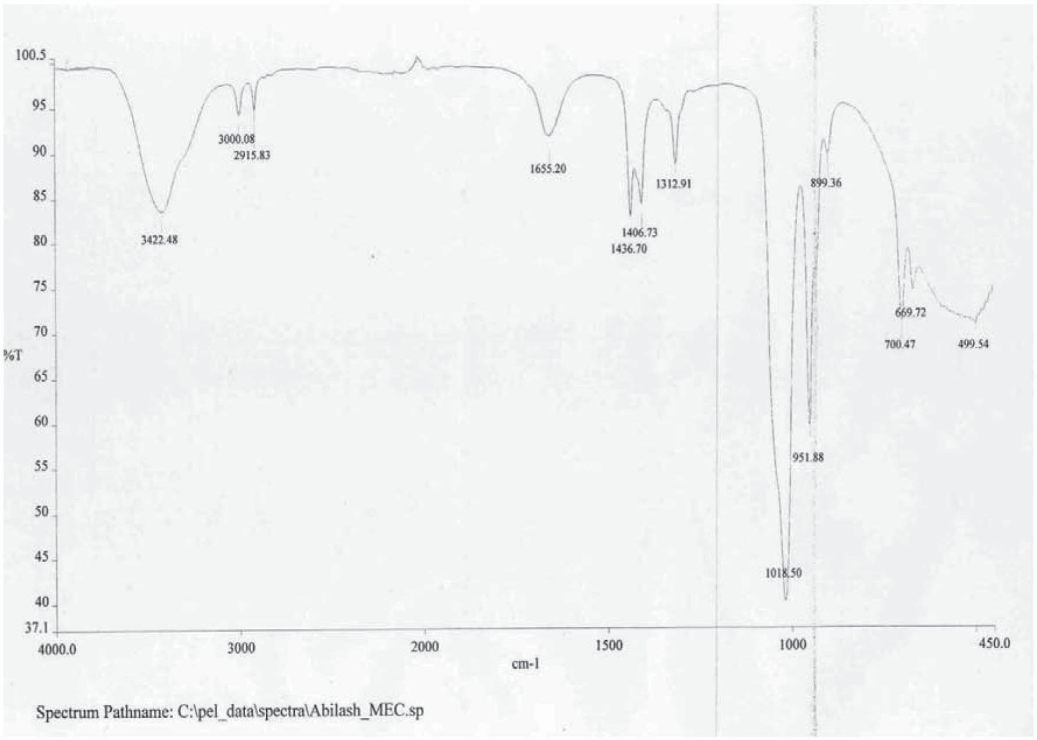

Fig. 2a: FT-IR spectrum of control

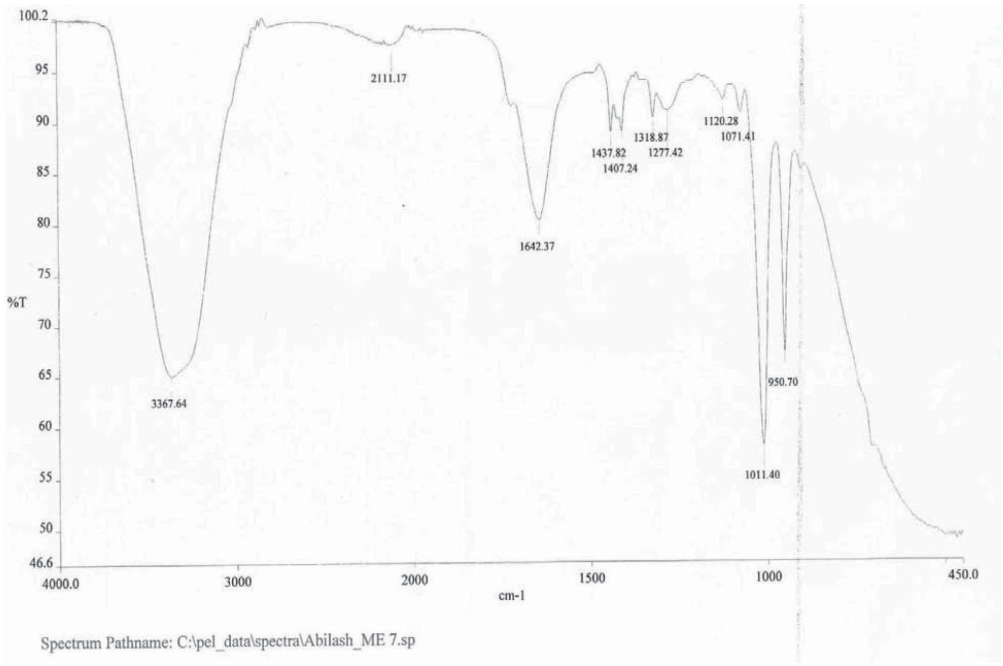

Figure 2b: FT-IR spectrum of PBL12

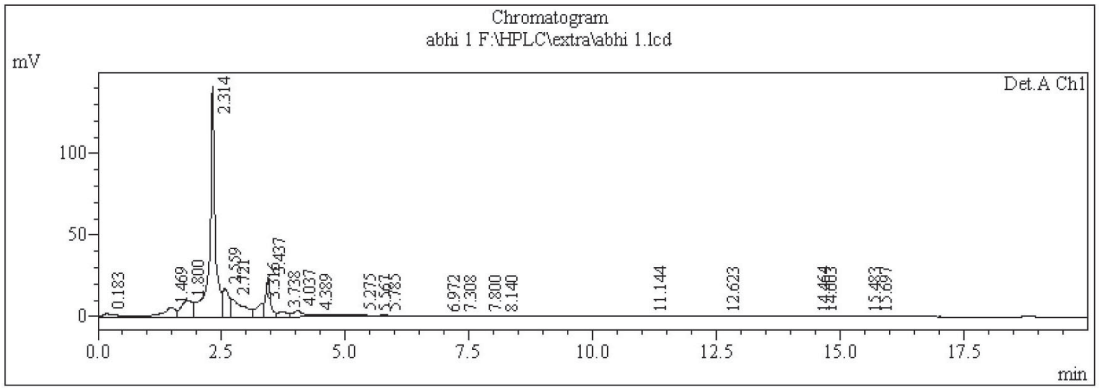

Figure 3: HPLC analysis of the bioactive compound from PBL 12

Discussion

The Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host (Shiomi *et al.*, 2006). Fungi have been widely investigated as a source of bioactive compounds. An excellent example of this is the anticancer drug, taxol, which had been previously supposed to occur only in the plants (Strobel and Daisy, 2003).

The endophytic fungi, the latent phytopathogen or mutalistic symbiont present in inter or intracellular in the normal tissue of host plants, have proved to be a promising source of secondary metabolites with normal structures and of strong bioactivities (Tan and Zou, 2001). However only few plants have been studied for their endophytic biodiversity and their potential to produce bioactive compound. Endophytes are now considered as an outstanding source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments (Strobel *et al.*, 2004).

Mangrove endophytic fungi are of enormous scientific interest, for two major reasons. First, they constitute the second largest part of the earth's marine fungi. Second, mangrove fungi often possess unique structures, metabolic

pathways, reproductive systems, and sensory and defense mechanisms because they have adapted to extreme environments, so the mangrove fungi represent a source of unique genetic information.

This work was initiated on the basis of the hypothesis that endophytic fungi produce bioactive metabolite(s) which play an important role to provide protection to their host against infectious agents and environmental factors. Secondly, mangrove plants are known to have pharmaceutical properties, and their associated endophytes could be capable of producing similar substances. The rational of the proposed work is that after its successful completion, identification of novel bioactive metabolites could lead to the development of novel pharmaceutical agents against human diseases.

During the isolation the diversity of endophytic population was found higher in *Breguiera cylindrica* than *Rhizophora candelaria.* Although several studies as fungi associated with mangrove detritus have been published, there are few studies as the endophytic fungi of mangrove plants and most of them are confined to foliar endophytes.

Endophytes have already been reported as being producers of antimicrobial compounds. In a similar study a total of 187 strains of endophytic belonging to 136 species also been tested as antimicrobial assay (Pelaez *et al.,* 1998). The production of antimicrobial substance was detected in 45 strains. In this study difference were observed among isolates from the same species with respect to their ability to produce antimicrobial metabolite. Of these endophyte Aphyllophorales sp PBL12 was significantly inhibitory to all the tested organisms. Its strong and wide spread antimicrobial activity suggests that the strain PBL12 possessed the potential of producing antimicrobial metabolites.

The identification of fungi usually has been based on the characteristic of their macroscopic and microscopic morphology. However, molecular analysis of fungal rDNA provides a powerful technique for assessing fungal diversity. The use of molecular tools aided in rapid identification of cultured fungi to the genus and strain level. In this study, fungal molecular identification of the most active isolate PBL12, based on 18S ribosome RNA sequence analysis revealed that isolate PBL12was identified as *Aphyllophorales* (JQ34006).

Fungal enzymes are gaining importance in agriculture, industry and human health, as they are often more stable (at high temperature and extreme pH) than the enzymes derived from plants and animals. Fungal enzymes are used in manufacturing food, beverages, confectioneries, textile and leather and help simplifying the processing of raw materials. Wood-inhabiting marine fungi serve as a potential source of exoenzymes (Rohrmann and Molitoris, 1992; Raghukumar *et al*., 1994). Kumaresan and Suryanarayanan (2002) studied the extracellular enzyme production by the foliar endophytic fungi of *Rhizophora apiculata* and demonstrated their involvement in litter degradation after senescence. Protease activity was seen in the selected endophyte of current study.

TLC was used for analytical separations of larger quantities of materials. Because sample mass loading capacity is proportional to thickness, thicker layers are used (>250µm up to 1mm). The thickness of the plate, polarity and choice of solvent are important factors in the separation of alkaloids. On exposure to iodine vapour a yellow colour spot developed at Rf value 1.5cm.

One of the most commonly used techniques for structure analysis by IR spectroscopy is FTIR attenuated total reflectance (ATR) spectroscopy (Tuleva *et al.,* 2002). FT-IR is a measurement technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. It is used to identify unknown materials present in a specimen. In the FT-IR spectra of the extracted bioactive compounds the presence of benzene ring in the compound supported the band stretches 1073 cm^{-1},1011 cm^{-1},1125 cm^{-1} and 1076 cm^{-1}.

HPLC is a form of column chromatography used frequently in analytical chemistry. High performance liquid chromatography (HPLC) is not only appropriate for the complete separation of different bioactive compounds; but can also be coupled with various detection devices (UV, MS, evaporative light scattering detection, ELSD) for identification and quantification of metabolites. In this case HPLC coupled with UV was used for the analysis.

Fungi have proven themselves as valuable sources of natural products for agriculture as well as biomedical development for over a half century (Biabani and Laatsch, 1998). As fungi thrive in competitive environments, it is hypothesised that their metabolic compatibility has been strongly influenced by natural selection. Bioactive product discovery depends on the knowledge of habitats where fungi are abundant and the strength of culture collection. Studies on mangrove endophytic fungi were initiated recently and it has been realised that mangrove plants harbor an extremely

diverse endophytic fungal flora (Suryanarayanan and Kumaresan, 2000; Ananda and Sridhar, 2002). Screening this fungal resource for novel metabolites and enzymes and their application are major goals of current research to accomplish environment-friendly technological development.

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