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ANTIMICROBIAL ACTIVITY AND BIODEGRADING ENZYMES OF ENDOPHYTIC FUNGI FROM *EUCALYPTUS*

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ABSTRACT

A total of thirty endophytic fungi were isolated from leaves and twigs of *Eucalyptus globulus* and *Eucalyptus citriodora*. Among thirty endophytic fungal isolates, four (P3MT1, P3MT2, OP4MT2 and P7ML2) are consistently producing compounds which are inhibiting *Pseudomonas aeroginosa*, *Mycobacterium smegmatis* and *Candida albicans* even after 10 generations tested under dual culture, well diffusion and disc diffusion methods. P3MT1 and OP4MT2 are inhibiting even a filamentous fungi *Penicillium chrysogenum.* The fungal isolate OP4MT2 showed highest zone of inhibition (20 mm) against *Penicillium chrysogenum* among two test fungi. The crude ethyl acetate extract of P3MT1 isolate showed highest zone of inhibition against *Candida albicans* (19 mm) by both well and disc diffusion method when compared to other fungal isolates. Another four fungal isolates (P3ML1, P6MT1, P5MT1 and P2MT1) from the same set of thirty isolates showed positive for the secretion of amylase, protease and laccase enzymes in agar plate method. Two endophytic fungal isolates (P6MT1 & P2MT1) among thirty are able to oxidize guaiacol indicating the presence of Lignin degrading enzymes. Four fungal isolates indicated presence of laccase enzymes by qualitative test were able to decolorize both methylene blue and aniline blue (synthetic dyes) in solid and liquid media. The quantitative estimation of percent decolorization of synthetic dyes by spectrophotometric method confirmed more than 90 % reduction in color is made possible by the endophytic fungi. All these fungal strains with good bioactivity are of worth studying in detail for the purification and characterization of the active compounds and enzymes.

INTRODUCTION: *Eucalyptus* genus belonging to the family of Myrtaceae grows rapidly to a gigantic size and is one among the tallest trees of the world [1]. There are about 500 species of *Eucalyptus* plants which produce essential oils [2]. *Eucalyptus* oil has antibacterial, antiviral and anti fungal components and used for the treatment of cold, influenza, rhinitis, sinusitis and other respiratory infections [3].

Almost all the plants which were studied are known to support a vast group of micro-organisms called endophytes living inside the live parts of plants [4].

Endophytic fungi are one among these endophytes living inside the plants without causing any apparent symptoms [5].

Endophytic fungi have recently gained importance due to their ability to produce bioactive metabolites, immuno-suppressants, anti-cancer compounds and biocontrol agents [6]. There are reports on endophytic fungi from *Eucalyptus* plants where they focused on density of colonization, differences in population of endophytes and sometimes production of extracellular oxidative enzymes [7]. Isolation of endophytic bacteria and fungi from *Eucalyptus globulus* has been reported and they tested for their antimicrobial activity. Some of the endophytic bacteria have shown antimicrobial activity, but none of the endophytic fungal isolates have shown antimicrobial activity [8].

The endophytic fungi are also known to produce enzymes such as lignocellulose degrading and other enzymes, including amylase, protease, tyrosinase, and laccases from medicinal plants [9]. Ligninolytic enzymes have an application in the decolorization treatment of synthetic dye other than physical and chemical methods [10]. There are very few reports on enzymes isolated from endophytic fungi from *Eucalyptus* plant. This study is aimed to isolate endophytic fungi from two *Eucalyptus* plant species and screen their ability to produce bioactive compounds and active enzymes which may have applications in pharmaceuticals, bioremediation and biofuel processing.

MATERIALS AND METHODS:

Isolation of Endophytic Fungi: Mature leaves and twigs of *Eucalyptus globulus* and *Eucalyptus citriodora* plants were collected from nearby locations of the research Institute and processed within one hour of their collection. Isolation of endophytic fungi was carried out using the protocol described by Strobel *et al.,* with slight modifications [11]. The healthy plant tissues were washed under running tap water and samples (leaves and twigs) were cut into size of an inch.

Eucalyptus citriodora samples were surface sterilized sequentially with 70 % alcohol for 90 seconds, 1% sodium hypochlorite for 90 seconds, rinsed twice with sterile water and excess water was drained from the surface of the sample by touching to the sides of the beaker before placing them on the Potato Dextrose Agar (PDA) media amended with streptomycin (200 mg/L).

The *Eucalyptus globulus* samples were surface sterilized by 70 % alcohol for 1 min, followed by 4% sodium hypochlorite for 2 min, rinsed twice with sterile water and excess water was drained and processed as above. The sterile petriplates were sealed with parafilmTM and incubated at 25°C till the fungal tips starts growing from the plant sample. Individual fungal tips started growing from the leaves or twigs were transferred to fresh PDA plates without antibiotics and allowed to grow as individual isolates. All the individual isolates after growing up to a stage stored in PDA slants at 4°C for further studies.

Antimicrobial Assay: Lyophilized bacterial and fungal cultures were purchased from Microbial Type Culture Collection (MTCC) of Institute of Microbial Technology, Chandigarh for testing antimicrobial activity. Cultures of *Pseudomonas aeroginosa* (MTCC 4676), *Mycobacterium smegmatis* (MTCC 943) and *Salmonella typhimurium* (MTCC 3232) were maintained on nutrient agar (NA) and were used for antibacterial assay. *Candida albicans* (MTCC 183) were grown on Yeast extract Peptone Dextrose Agar (YEPD) and *Penicillium chrysogenum* (MTCC 6795) were maintained on Czapek yeast extract agar (CYA) both were used as antifungal test organisms. A standard concentration of 0.5 McFarland was used for both bacterial and fungal suspension during the antimicrobial assay.

Preliminary Antimicrobial Assay: Antimicrobial activity of isolated endophytic fungi against pathogenic microbes was carried out using the method explained by Arya and Sati with slight modifications [12]. The petriplates containing respective media for the growth of bacteria and fungi were prepared and 100 µL of test organisms were spread over the surface of the agar using sterile cotton swab. Nine millimeter diameter of actively growing mycelial discs from PDA plates were cut using a sterile cork borer and placed on the surface of the agar media seeded with test bacteria or fungi.

These plates were sealed with parafilm and refrigerated at 4°C for 12 hours for complete diffusion of antimicrobial compounds from the fungal disc. Thereafter these plates were incubated at room temperature for next 12 hours for *Pseudomonas aeroginosa, Mycobacterium smegmatis, Salmonella typhimurium and Candida albicans* and 48-60 hours for

Penicillium chrysogenum and observed for the zone of inhibition. The zone of inhibition was measured in millimeter using a ruler scale. All the experiments were performed in triplicates.

Secondary Metabolite Extraction: Secondary metabolite extraction was carried out as described by Mabrouk *et al* with slight modifications [13]. Endophytic fungal isolates shown positive under preliminary screening were grown in 250 mL conical flasks containing 100 mL of potato dextrose broth (PDB) at room temperature under static condition. After 21 days of growth, mycelia were separated from the culture media by filtering through muslin cloth.

Extraction of the Filtrate: The culture filtrate was taken in a separating funnel and equal volume of ethyl acetate was added and shaken vigorously for 10 min. The miscible mixture was allowed to stand for 5 minutes and the solvent phase was collected in a flask.

Extraction of the Mycelia: The separated mycelia was washed with sterile distilled water to remove adherent media debris and then transferred to a mortar and crushed thoroughly with a pestle using ethyl acetate as solvent to obtain intracellular metabolites. The crushed material was then filtered through a muslin cloth and then taken in a separating funnel and shaken for 10 min and allowed to stand for 5 min. The crude ethyl acetate fraction from the top was collected. Ethyl acetate extract from both mycelia and filtrate were mixed and evaporated to dryness in hot air oven. The dry solid residue was dissolved in Dimethylsulfoxide (DMSO) and stored at 4 °C until used for the test.

Antimicrobial assay by Well and Disc Diffusion Method: Antimicrobial activity of secondary metabolites extracted by ethyl acetate method was determined by disc diffusion and well diffusion methods [14]. The sterile growth media plates specific for test organisms were prepared and seeded with the 100 µL of 0.5 MCF test organism. Sterile discs (Hi-media) of 6 mm diameter was impregnated with 20 µL of extract and then placed on the surface of inoculated medium. Simultaneously in the same petriplate 6 mm diameter wells were made using the sterile cork borer and 20 µL of crude extract was loaded. Similarly 20 µL of DMSO and ethyl acetate were also tested as negative controls to detect the solvent effects.

Ciprofloxacin (5 mcg disc) was used as positive control for bacteria and Flucanozole (5mg/mL) was used as a positive control for fungi. All the above tests were carried out in triplicates. The plates were incubated at 35±2°C for bacteria (*Pseudomonas aeroginosa, Mycobacterium smegmatis* and *Salmonella typhimurium*), 30±2°C for *Candida albicans* for 12-24 hours and at 28-30°C for *Penicillium chrysogenum* for 48-60 hours. The diameter of zone of inhibition after the incubation period was measured in mm using a ruler.

Extracellular Enzyme Assay: The assay for extracellular enzymes such as amylase, protease and tyrosinase was carried out with some modification to the earlier protocol [15]. Amylase activity was determined by growing fungi on glucose yeast extract peptone (GYP) agar medium with 1% soluble starch for 5 days. The fungal plates were flooded with 1% iodine containing 2% potassium iodide. The clear zone formed around the colony was considered as positive for amylase activity.

Protease assay was performed by growing the fungi on GYP agar medium amended with 0.4% gelatin adjusted pH to 6. The plates were incubated at 25±2°C for 5 days. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin precipitates with ammonium sulphate and protease digested gelatin shows clear zone around the colony.

For tyrosinase activity, fungi were grown on GYP agar medium. After incubation a mixture of 0.11% p-cresol and 0.05% glycine was added on the surface of the fungal colony. Culture plates were observed after 24 hours for the appearance of reddish brown color around the colony which indicates tyrosinase activity.

Lignin Degrading Enzyme Assay: A qualitative assay for lignin-degrading enzymes was carried out as described by Atalla *et al.,* with some modifications [16]. Six millimeter mycelial discs were cut from the edge of expanding colonies of six days old fungal cultures using the cork borer. These mycelial discs were placed on Boyd and Kohlmeyer (B&K) medium (glucose-10 g, peptone-2 g, yeast extract-1 g, agar-18 g, distilled water-1000 ml and pH-6.0) containing 4 mM guaiacol. The petriplates were then wrapped with black polythene bags and incubated at 25±2°C for 2 weeks.

Oxidation of guaiacol leads to formation of reddish brown color under and around the fungal colony. Endophytic fungi inoculated on guaiacol free media were used as control.

Laccase activity was tested by growing the fungi on GYP agar medium amended with 1-naphthol (0.005 %) (pH 6) and incubated at 25±2°C for 2 weeks by wrapping the petriplates with black polythene bags. The enzyme laccase production by the endophytic fungi will result in the oxidation of colorless 1-naphthol to a brownish-violet. The fungi grown on a 1-naphthol free media was used as control.

Decolorization of Synthetic Dyes:

- **Agar Plate Method:** The laccase positive endophytic fungi were selected for its ability to decolorize synthetic dyes such as methylene blue and aniline blue. Malt extract agar (malt extract 1% w/v, glucose 0.1% w/v and 1.5% agar) supplemented with 50 mg/L of individual dyes were used for the plate method.

  Using sterile cork borer 6 mm diameter of mycelial discs were cut from the edge of expanding colonies of seven day old fungal culture and placed on a media supplemented with dye and incubated at 25±2°C for 15 days. Plates without fungal inoculation were used as control. Observation for the rate of decolorization was made twice in a week [17].

- **Liquid Broth Method:** Decolorization of synthetic dye was carried out in broth media as described by Bonugli-Santos et *al* with slight modifications [18].

  - **First Method:** Nine millimeter fungal culture plugs were transferred to 50 ml of sterilized malt extract broth containing 50 mg/L of individual dyes (methylene blue and aniline blue) in 250 ml conical flasks and incubated at room temperature at static condition with periodic shaking for 21 days.

    Aliquots of 50 µL from the culture were taken and diluted to 1000 µl immediately after the dye addition and considered it as zero time samples. The diluted sample was centrifuged and optical density of the supernatant was measured at 665 nm for methylene blue and 600 nm for aniline blue in Perkin Elmer lambda 35 UV/VIS spectrophotometer. Every week, the same amounts of aliquots were collected and absorption maxima at 665 nm and 600 nm were recorded as above. The difference in the OD after incubation and before incubation gives the rate of dye decolorization. A control without fungal inoculation processed in the same way is considered as control. All the spectrophotometric readings were taken in triplicates and average value was calculated.

  - **Second Method**: Nine mm fungal culture plugs were transferred to 40 ml of sterilized malt extract broth and incubated at room temperature in static condition with periodic shaking. After 7 days of fungal growth 2.5 mg of individual dyes were dissolved in 10 mL (50 mg/L concentration) of sterile malt extract broth and added to the 250 ml flask containing 40 ml of fungal culture. Fifty micro liter of aliquots of cultures were taken out and diluted with distilled water to 1000 µL, centrifuged and OD at 665 nm for methylene blue and 600 nm for aniline blue measured using the spectrophotometer as explained previously.

    The day one value after adding dye was taken as initial reading and every week, sample was processed in the same way till 21 days. The percent difference in the OD indicates the rate of dye degradation. A conical flask containing the same amount of broth and dye also incubated along with this without fungi was considered as negative control. All the samples were measured in triplicates and average value was taken for calculating percent degradation of dye.

    Decolorization assay was measured in the terms of percentage decolorization using UV-Vis-Spectrophotometer.

    The percentage decolorization was calculated from the following equation [10].

% Decolorization = Initial OD-Final OD/ Initial OD x 100

RESULTS AND DISCUSSION:

Isolation of Endophytic Fungi: Isolation of endophytic fungi from surface sterilized leaves and twigs of *Eucalyptus globulus* and *Eucalyptus citriodora* yielded thirty different fungal isolates in pure form. The isolated endophytic fungi were subcultured on PDA plates for several times to negate the effects of adherent plant metabolites. One set of each fungal isolates were maintained on agar plates and slants at 4°C.

Preliminary Antimicrobial Assay: All the thirty endophytic fungal isolates were screened for antimicrobial activity by dual culture method against three bacteria, one yeast and a filamentous fungus. A total of four isolates showed inhibition against most of the tested organisms. The zone of inhibition ranged from 11 to 20 mm diameter after 12 hours of incubation for bacteria and yeast and 48 hours of incubation for a filamentous fungus. The fungal isolate OP4MT2 gave highest zone of inhibition (20 mm) against *Penicillium chrysogenum.* All the four isolates showed inhibition for *Mycobacterium smegmatis, Pseudomonas aeroginosa and Candida albicans*. None of the isolates showed inhibition against *Salmonella typhimurium,* whereas only two fungal isolates were able to inhibit *Penicillium chrysogenum*. The fungal isolates showing antibacterial and antifungal activities when compared with the control are listed in the **Table 1**. Mean diameter of zone of inhibition for triplicate values were calculated and shown along with the standard deviation. Some of the sample plates with zone of inhibition against tested microorganisms by endophytic fungi have been shown in **Fig. 1a**.

TABLE 1: ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGI TESTED WITH DIFFERENT METHODS

Endophytic fungal isolates	Microorganisms zone of inhibition in mm (mean ±SD, n=3)				
	PA	MS	ST	CA	PC
			Dual culture		
P3MT1	15.0±1.0	12.0±0.0	Nil	17.0±0.0	18.6±0.5
P3MT2	12.3±0.5	11.6±0.5	Nil	12.0±0.0	Nil
0P4MT2	12.6±0.5	13.0±0.0	Nil	16.0±1.0	20.0±0.0
P7ML2	11.0±0.0	13.3±0.5	Nil	15.0±0.0	Nil
			Well Diffusion		
P3MT1	14.3±0.6	13.3±0.6	Nil	18.6±0.6	17.6±0.6
P3MT2	10.0±0.0	11.0±0.0	Nil	15.3±0.6	Nil
0P4MT2	11.6±0.6	12.0±0.0	Nil	15.0±0.0	18.6±0.6
P7ML2	14.0±0.0	10.0±0.0	Nil	14.3±0.6	Nil
			Disc Diffusion		
P3MT1	12.6±0.6	12.0±0.0	Nil	17.3±0.6	17.3±0.6
P3MT2	09.0±0.0	09.3±0.6	Nil	13.6±0.6	Nil
0P4MT2	10.0±0.0	11.0±0.0	Nil	14.6±0.6	18.0±0.0
P7ML2	11.0±0.0	10.0±0.0	Nil	12.6±0.6	Nil
Ciprofloxacin Standard	20.0±0.0	23.0± 2.3	22.0 ±0.0	-	-
Flucanozole Standard	-	-	-	22.0±0.0	20.0±0.0

Symbols used indicates: P; plant, T; twig, L; leaf, M; Mature, **PA;** *Pseudomonas aeroginosa,* **MS;** *Mycobacterium smegmatis,* **ST;** *Salmonella typhimurium, CA***;** *Candida albicans,* **PC;** *Penicillium chrysogenum*

Endophytic fungal isolates P3MT1 and OP4MT2 are showing wide range of antimicrobial activity by inhibiting representative Gram positive and Gram negative bacteria as well as yeast and filamentous fungi. Though OP4MT2 showed highest inhibition for *Penicillium chrysogenum*, P3MT1 inhibited highest all other tested microbes. P3MT2 appears to be the least bioactive endophytic fungi among all the active isolates. While screening large number of endophytic isolates for the antimicrobial activity, dual culture method has got advantage over well diffusion and disc diffusion methods by saving lot of solvents. This will narrow down the total isolates into bioactive isolates, further these selected isolates may be used for the detailed study by growing them in large scale. Four such bioactive fungi out of thirty endophytic isolates were selected for the secondary metabolites extraction studies based on the preliminary screening in this study.

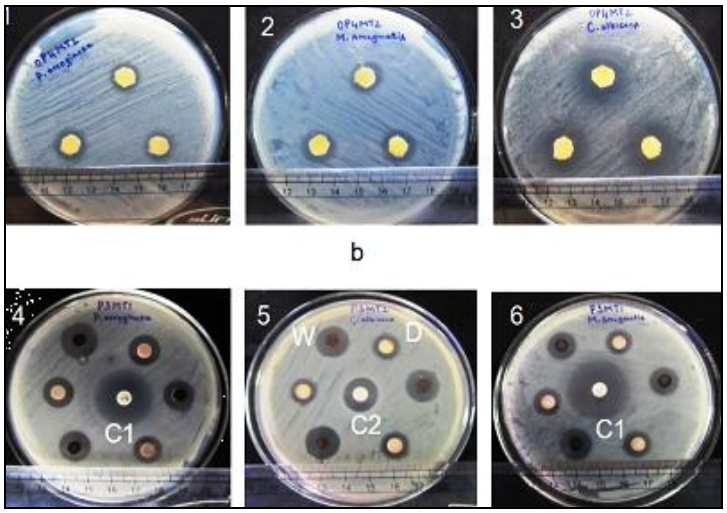

FIG. 1: INHIBITION OF PATHOGENIC BACTERIA AND FUNGI BY ENDOPHYTIC FUNGI IN DUAL CULTURE AND WELL DIFFUSION METHODS

a; Showing inhibition of *Pseudomonas aeroginosa* (1), *Mycobacterium smegmatis* (2) and *Candida albicans* (3) by OP4MT2 endophytic fungal strain in dual culture method, b; Showing inhibition of above three organisms (4, 5 & 6) by ethyl acetate crude extract of endophytic fungal strains P3MT1 and P3MT2 in well and disc diffusion method. Regular ruler is placed along the plate for the comparison. Symbols indicate: W: well, D: disc, C1-ciprofloxacin standard (positive control for bacteria) C2: Flucanozole standard (mg/ml) (positive control for fungi). DMSO and ethyl acetate extracts were used as negative control

Antimicrobial assay by Well and Disc Diffusion Method: The crude ethyl acetate extract dried and dissolved in small quantity of DMSO used for the antimicrobial assay by well diffusion and disc diffusion methods showed significant inhibition for all the four endophytic fungi previously found positive. Ciprofloxacin and Fluconazole antibiotics were used as standard for antibacterial and antifungal test respectively. Compared to the disc size of dual culture (9 mm) method, the size of the well and disc was small (6 mm) for both well diffusion and disc diffusion methods.

Though the average diameter of the inhibition zone was similar for all three types of methods, actual inhibition may be more in case of well diffusion and disc diffusion assays. The diameter of the inhibition zone in well diffusion and disc diffusion assays ranging from 10 to 19 mm and 9 to 18 mm respectively and it is comparable to the standard antibiotics. Well diffusion method is simple and better than the disc diffusion method because in the latter case sample has to be impregnated to the disc several times in small aliquots as well as sterile discs are expensive.

Where as in well diffusion you can add up to 50 uL sample at a time and incubate the plate for results immediately. The above method formulated to compare the diameter of the inhibition zones in well and disc diffusion methods. Well diffusion method showed diameter zone of inhibition little higher than disc diffusion method. These results suggest that diffusion of antimicrobial agents is much more free and higher in well diffusion method compared to disc diffusion method. These differences in inhibition zone may become crucial in dose-dependent antimicrobial studies.

Zone of inhibition by the ethyl acetate extract against three bacteria, yeast and a filamentous fungus listed in the Table 1. Petri plates showing zone of inhibition for *Pseudomonas aeroginosa* and *Candida albicans* are represented in **Fig. 1b**. Fungal isolate P3MT1 showing highest zone of inhibition against *Candida albicans* (19 mm) in both the assay methods suggest that this fungi can be a possible antibiotic source for controlling *C. albicans* infection. Similar to the preliminary screening all four endophytic fungi retained their antimicrobial activity even after growing in the submerged culture and these compounds can be extracted by a medium polar solvent like ethyl acetate.

It is evident that *Mycobacterium* has gained resistance to existing antibiotics and search for novel antibiotics has begun. Endophytic fungal isolates such as P3MT1, P3MT2, P7ML2 and OP4MT2 are producing compounds which have considerably good inhibition against *Mycobacterium smegmatis.* Inhibition of *Mycobacterium* group of organisms indicates some of these endophytic fungi may be promising candidates for the production of novel antibiotics.

Clinically latent bacteria such as *Mycobacterium smegmatis and Salmonella typhimurium* are the best examples of multiple drug resistant organisms. Developing antimicrobial agents against non-multiplying or multiplying at slow rate bacterium is a challenge in these days. Non-multiplying state of bacterium is highly resistant to multiple drugs compared to multiplying state [19].

Hence, in this study attempts were made by selecting *Mycobacterium smegmatis* as model organism to target serious pathogen *Mycobacterium tuberculosis*

and *Pseudomonas aeroginosa* and *Salmonella typhimurium* as model organisms for pathogenic species of *Pseudomonas* and *Salmonella* which have already become multidrug resistant organisms.

Fungal Enzyme Assay: Different set of four endophytic fungal isolates were able to show positive results for the enzyme assay. All the thirty endophytic fungi were screened for amylase, protease, laccase and tyrosinase enzymes, only four endophytic fungi (P3ML1, P6MT1, P5MT1 and P2MT1) were able to produce amylase, protease and laccase. None of the tested fungal isolates are producing tyrosinase enzyme in agar plate method.

Figure 2 represents the qualitative test conducted for the starch degradation by amylase (**Fig. 2a**), gelatin degradation by protease (**Fig. 2b**), guaiacol oxidation **(Fig. 2c**) and naphthol oxidation by ligninase **(Fig. 2d**). Fungi producing amylase enzymes degrade the polymer starch into oligomers or monomers.

In the qualitative analysis, starch is degraded and iodine solution will not stain that area as you can see in the **Fig. 2a** where endophytic fungi degraded 1% starch in the media.

Remaining area will be dark blue indicating the staining of starch by the iodine solution. Agar plate amended with gelatin will look opaque and protease enzyme produced by the endophytic fungi degrades gelatin to form a clear zone surrounding the fungal culture as seen in **Fig. 2b**. Enzyme assays performed on solid media detects extracellular enzymes produced and released to the media by the mycelium.

Fungal enzymes have important role in agriculture, industry and health due to their stability when compared to the enzymes produced from plants and animals [20]. There are some available evidences where endophytic fungi isolated from a mangrove plant *Rhizospora apiculata* produces extracellular enzymes [21].

In the present study, endophytic fungi producing amylase and protease needs further studies where one should quantitatively estimate the enzyme production and can take to large scale production.

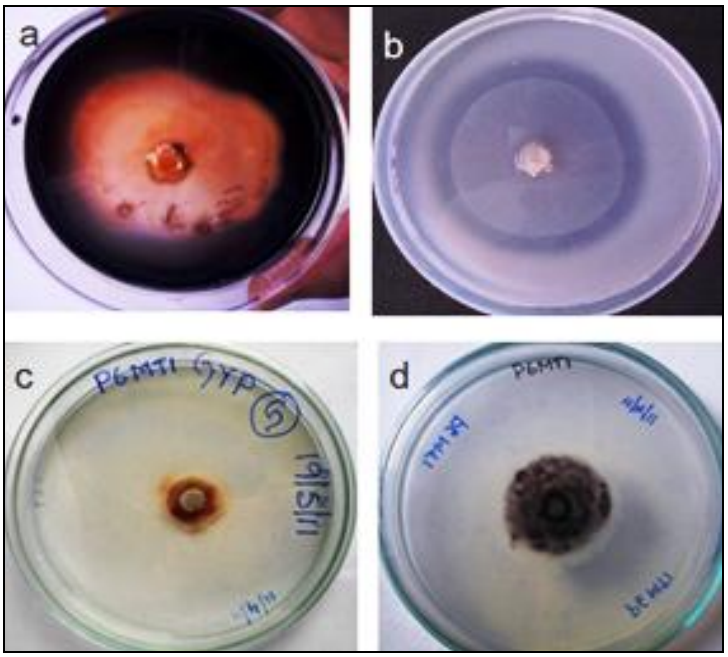

FIG. 2: QUALITATIVE ASSAY FOR AMYLASE, PROTEASE AND LIGNINASE ENZYMES FROM ENDOPHYTIC FUNGI

a; clear zone in the centre indicating degradation of starch by amylase, b; dark circle around the fungal colony showing degradation of gelatin by protease enzyme, c; reddish brown color in the center showing oxidation of guaiacol and d; brownish violet color showing naphthol oxidation by ligninase enzymes

Lignin Degrading Enzyme: Tyrosinase is known to be one of the lignin degrading enzymes and none of the endophytic isolates shown tyrosinase activity in the current study. Apart from this, there are aromatic model compounds such as Guaiacol and 1-napthol used for detecting lignin degrading enzymes. Oxidation of these compounds indicates the presence of lignin degrading enzymes [16]. Two endophytic fungi (P6MT1 & P2MT1) have shown intense reddish brown color under the fungal colony grown for two weeks in an agar plate media amended with guaiacol, which indicates the presence of lignin degrading enzymes as shown in **Fig. 2c**.

A brownish violet color was observed around the fungal colonies (P3ML1, P6MT1, P5MT1 and P2MT1) grown on agar media containing colorless 1-naphthol after four days of incubation. In the **Fig. 2d** change in the color at the center of the petriplate shows lignin degrading laccase enzymes present in the endophytic fungi are responsible for the oxidation of 1-naphthol. The ability of endophytic fungal isolates to degrade aromatic model compounds such as guaiacol and napthol shows that they can be used as the decomposers of cellulose, hemicellulose and lignin and

it also indicates that these lignin degrading enzymes can be used in the detoxification of agrochemicals and industrial effluents [16]. Since *Eucalyptus* is highly lignified, endophytic fungi are co-evolved with its host [22]. By definition, endophytes are those microbes colonizing healthy plant tissues [6]. To colonize healthy plant tissues and stay symbiotically with plants, it is expected that endophytic fungi possess and produce enzymes which can break up barriers of plant tissues and stay harmoniously.

Decolorization assay of synthetic dyes: All the four endophytic fungal isolates (P3ML1, P6MT1, P5MT1 and P2MT1) producing laccase enzymes were tested for the biodegradation of synthetic dyes in solid agar plate media. After a week of incubation both methylene blue and aniline blue were partially decolorized. Based on the color change in culture plate compared to control plate, above four fungal isolates were selected for the detailed study using spectrophotometry method.

Decolorization assay by spectrophotometry method: Decolorization of synthetic dyes using four endophytic fungi isolated from *Eucalyptus* plants were analyzed by spectrophotometric method and rate of percent degradation was plotted in a bar diagram as shown in **Fig. 3**. Percentage decolorization of methylene blue by endophytic fungal isolates P2MT1, P3ML1, P5MT1 and P6MT1 after 21 days of growth was 54.4, 60.9, 48.1 and 25.9 respectively in the first method (**Fig. 3a**).

The P3ML1 decolorized methylene blue dye highest (61%) compared to the other isolates in the study period. Whereas percentage decolorization of methylene blue by the same set of isolates was significantly high in the second decolorization method, where same amount of dye was added to the one week old fungal culture media and then grown for 21 days. In this method fungal isolates P2MT1, P3ML1, P5MT1 and P6MT1 decolorized up to 50 – 80 % within a week after the dye was added (**Fig. 3b**).

All the isolates were able to decolorize more than 95 % of synthetic dye within 21 days of exposure. The growth rate of endophytic fungi grown along with the dye was less compared to the control without dye, may be due to the effect of dye.

Similarly percent decolorization of aniline blue was tested for the same set of four endophytic fungi P2MT1, P3ML1, P5MT1 and P6MT1 employing both the methods for the same period. In case of aniline blue also, rate of decolorization was comparable to the percent decolorization of methylene blue in both methods. In this case also fungi grown in a liquid media containing 50 mg/L of aniline blue took long time to degrade the dye.

After two weeks of growth these fungi were able to decolorize up to 50 % of aniline blue. After three weeks of growth fungal isolate P5MT1 decolorized upto 85% of aniline blue (**Fig. 3c**), whereas, percentage decolorization of aniline blue added to media containing one week old endophytic fungi P2MT1, P3ML1, P5MT1 and P6MT1 decolorized up to 50 % within a week after the dye added (**Fig. 3d**). This method also achieved up to 85% of decolorization of aniline blue within two weeks and almost 100% decolorization at the end of 21 days old culture.

The rate of dye degradation between methylene blue and aniline blue by these four endophytic fungi varied within the method (**Fig. 3a and 3c**). Fungal isolates P3ML1, P5MT1 and P6MT1 could decolorize the aniline blue faster than methylene blue when they are grown along with the dye since inoculated, whereas P2MT1 showed less difference in the rate of decolorization in this method.

Fungal isolate P6MT1 grown along with the methylene blue decolorized only up to 26 % even after 21 days of incubation. This is may be due to methylene blue is inhibiting fungal growth as well as enzyme production. The same fungal isolate can degrade the methylene blue more than 50 % within a week and up to 90 % in 21 days in the second method leading to a conclusion that this fungal isolate can produces laccase enzymes even without any external influence and continues the production once it reaches to a stage.

For this reason all the fungi which are grown in pure media for a week has better capacity to decolorize the synthetic dye. Heterocyclic dyes such as methylene blue had been reported to be resistant to enzymic oxidation [17]. The present study showed more than 90% decolorization of synthetic dye is possible by endophytic fungi.

Endophytic fungi having potential ligninolytic enzymes can be used in industrial effluent treatments. This is a first kind of report where endophytic fungi showed decolorization of synthetic dyes. The results of this study also lead to a conclusion that while using fungi for the biodegradation of toxic chemicals, it is better to grow the fungi first in the downstream and then toxic effluents should be added. This may help in the faster rate of detoxification of the industrial and/or textile effluents.

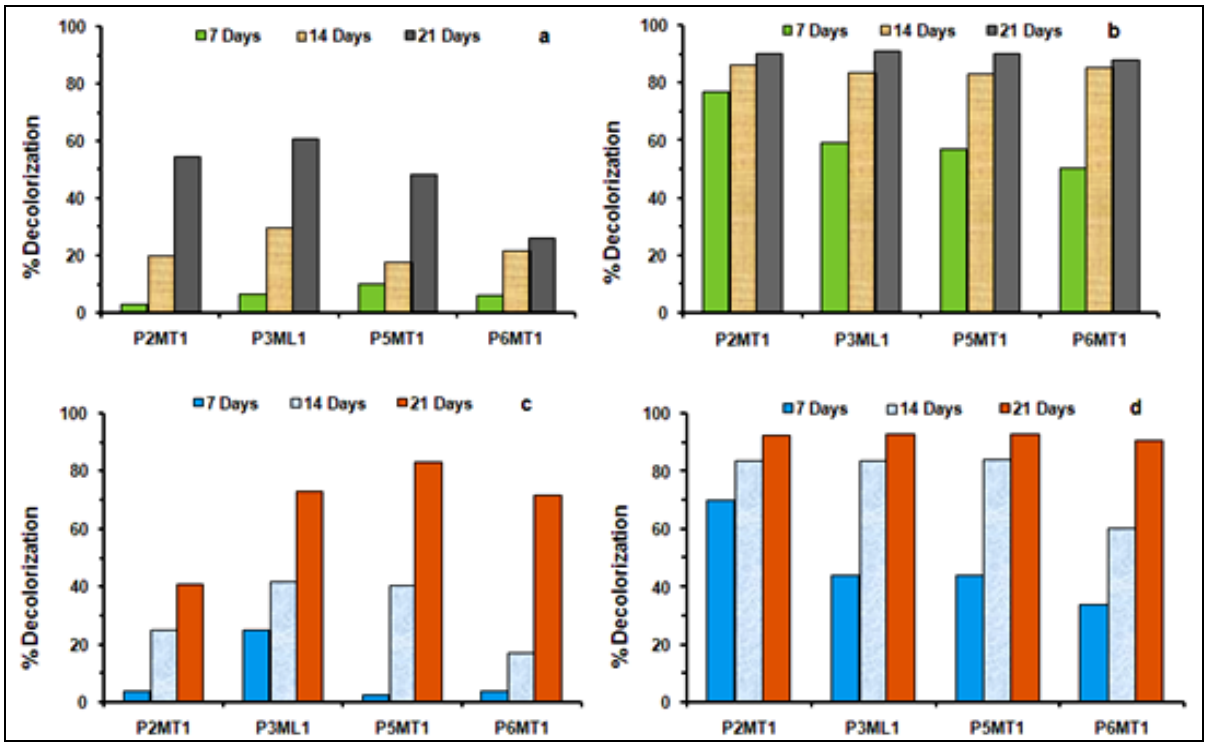

FIG.3: DECOLORIZATION OF SYNTHETIC DYES BY ENDOPHYTIC FUNGI ISOLATED FROM *EUCALYPTUS* PLANT

Percent decolorization of methylene blue drastically low when fungi grown along with dye (a) than the methylene blue added to one week old fungal culture (b), also percent decolorization of Aniline blue by the endophytic fungi is less when grown along with dye (c) than the aniline blue added to one week old fungal culture (d).

CONCLUSION: In the present study, endophytic fungi isolated from *Eucalyptus* plants have shown good antimicrobial activities and are comparable to the existing standard antibiotics. This is a promising indication for the further purification and standardization of these compounds which may have pharmaceutical and medicinal importance.

Apart from antimicrobial activity, there are endophytic fungi isolated from these plants which are able to produce pharmaceutically important enzymes; by selective modulation of these fungi might lead to large scale production of such enzymes.

The endophytic fungi which can decolorize industrial and other toxic effluents were also isolated from these plants. These indicates there may be vast varieties of economically useful endophytic fungi living inside medicinal plants which needs further exploitation for the benefit of mankind.

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