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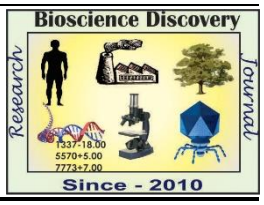

Screening of endophytic fungi isolated from *Azadirachta indica* A. Juss. for production of enzyme

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Abstract

In the present study screening of endophytic fungi for production of enzyme from medicinal plant *Azadirachta indica* A. Juss. from various regions of Aurangabad were done. Endophytic fungi were isolated from leaf with midrib, without midrib, petiole, stem. *Alternaria* sp. was dominant among isolated endophytes. Though *Colletotrichunm* exhibited lower dominance, it is isolated from all the localities. Some of the strains were screened for the production various enzymes, Some of the fungi were able to produce laccase, tyrosinase , asparinase, xylanase. *Colletotrichum truncatum* and *Alternaria sp.* were tested positive for tyrosinase production. Only *Alternaria sp.* was recorded as laccase enzyme producer.

INTRODUCTION

"Neem" which is scientifically known as *A. indica* A. Juss (Meliaceae) is one of themost effective medicinal plant in natural therapy and Ayurveda in India. Various Parts of this plant have already been reported as antibacterial (Patel and Trivedi, 1962), antiretroviral (Udeinya *et al*., 2004) antiarthritic, anti-inflammatory (Okpanyi and Ezukwk, 1981), and antiulcer (Pillai and Santhakumari, 1984). Extracts of neem also exhibited very good results against malaria (Ekanem, 1978), diabetes (Dixit *et al.*, 1986), and leukemia (Pettit *et al.*, 1983). Several reports in the recent years show that the endophytic fungi from neem produces several bioactive compounds (Li *et al.*, 2007; Wu *et al.*, 2008). According to study done by Jadhav and Pardeshi (2017) endophytic fungal extracts isolated from *Jatropha curcas* has remarkable biopesticidal activity against *Callosobruchus chinensis*.

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate disease symptoms or overt negative effects (Bacon and White, 2000). These Fungal entophytes which lives within the living tissues of higher plants without producing any apparent symptoms (Bills, 1996, Bills *et al.*,1992). Entophytes shows a protective role against insect herbivory and many are potential producers of novel antimicrobial secondary metabolites (Arnold *et al.*, 2001). Most studies have been conducted on the mere presence and identity of endophytes in the stem or leaf tissues Bettucci and Sarava, 1993; Geris *et al.*, 2003; Rodrigues and Samuels, 1999; Schweigkofler and Prillinger, 1997.

Like other organisms invading plant tissues, endophytic fungi produce extracellular hydrolases as a resistance mechanism against pathogenic invasion and to obtain nutrition from host (Sunitha *et al.*, 2013). Such enzymes include pectinases cellulases (Caldwell *et al.*, 2000), lipases (Petrini *et al.*, 1992), laccase from the endophytic fungus *Monotospora* sp. (Wang *et al.*, 2006), xylanase (Suto *et al.*, 2002), -1, 4- glucan lyase (Nielsen and OxenbØll, 1998), phosphotases (Maccheroni and Azevedo, 1998) and proteinase (Reddy and Belanger, 1996; Lindstrom and Belanger, 1994). Choi *et al.*, (2005) screened the endophytic fungi for their ability to produce

lignocellulases, amylase, cellulase, ligninase, pectinase and xylanase. According to study of Sunitha *et al.,* (2012) different thirty isolates from *Alpinia calcarata* (Roscoe) were tested for the enzymatic activity. Out of that isoltes *Cylindrocephalum* sp. gave maximum amylase activity. In another study done by Uzma *et al.,* (2016) where they isolate 112 endophytic fungi from 26 different genera and screened for enzymatic activity. Among these 112 endophytic fungi amylase activity was shown by 29% fungi, cellualse activity by 28 %, pectinase by 18% and Asparaginase by 40 % of fungi. Endophytic *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* from *Opuntiaficus indica* Mill exhinited high pectinase activity (Bezerra *et al.,* 2012).

The following research work was carried out for finding potential source of enzyme production among endophytic fungi.

MATERIALS AND METHODS

Isolation of endophytes

Five different locations were selected for sampling and were denoted as location 1, Zalta corner (Locl); location 2, the Shendra MIDC (Loc2); location 3, Osmanpura (Loc3) , location 4 , Bidkin area (Loc 4) and location 5, Khultabad (Loc 5) Leaves, stem were collected from individual plants at each location. All the samples were washed properly in running tap water for half an hour before processing. The samples were cut into small pieces. Leaves with midrib, leaves without midribs, petiole and stem samples were cut into 1.0 x 1.0 cm pieces. To eliminate epiphytic microorganisms, all the samples were initially surface treated (Petrini *et al.,* 1992). Segments of each sample were placed on potato dextrose agar (PDA). The Parafilm -sealed petri dishes were then incubated for 72 hrs. The endophytic fungi were identified according to their macroscopic and microscopic characteristics such as the morphology of fruiting structures and spore morphology. Standard taxonomic manuals were used to identify the fungal genera (Ainsworth *et al.,* (1973), Barnett and Hunter (1998). All isolated and identified endophytic fungi were assigned specific code and subcultured and cultures were kept in deep freeze.

Screening of Enzymes

Lipase

The lipase activity was assessed by growing the fungi on peptone agar medium amended with sterile Tween 20. After 4-5 days of incubation clear halos appear around the colony indicating the lipase activity (Prathyusha *et al.,* 2015).

Laccase

For screening the laccase activity GYP agar medium along with 1-naphthol (0.005%) was used. As a result of oxidation of 1-naphthol by laccase enzyme produced by endophytic fungi, a change in colour to blue takes place (Prathyusha *et al.,* 2015).

Amylase

The activity of amylase was determined by inoculating selected isolates in glucose yeast extract peptone (GYP) agar medium with 2% soluble starch. After 3-5 days incubation time, the fully formed cultures were flooded with 1% iodine in 2% potassium iodide. The clear zone in the form of halos was visualized around the fungal colony. (Prathyusha *et al.,*2015).

Cellulase

For cellulolytic activity, the isolates were grown on yeast extract peptone agar medium amended with 0.5% Nacarboxy methyl cellulose. After incubation, the plates were stained by using with 0.1% Congo red and 1M sodium chloride was used for destaining. A clear halo around the colony indicates the cellulase activity. (Prathyusha *et al.,*2015).

Tyrosinase

For tyrosinase activity, fungi were grown on GYP agar medium. After incubation a mixture of 0.11% *p*-cresol and 0.05 % glycine was overlaid on the surface of the fungal colony. Culture plates were observed after 24 hours for the appearance of red brown colour around the colony which indicated tyrosinase activity. (Maria *et.al* 2005).

Asparginase

All isolates of endophytic fungi were cultured on PDA for 7 days. The 5 mm disc of mycelium was transferred to the tested agar media. The modified agar Czapex Dox's (MCD) agar plate assay was used for the screening of asparaginase production. Control plates were MCD agar without asparagine. After incubation for five days at 30 ^{0}C, pink colour zone was observed for positive isolates (Theantana *et al.,* 2007).

Glutaminase

Glutaminase activity was observed on modified Czapek Dox medium with 2.5 % phenol red in ethanol (3ml/l).After 72 h of incubation at 26±1°C, the appearance of a pink zone around the fungal colony showed L-glutaminase activity (Gulati *et al.,* 1997).

Xylanase

For Xylanase assay XBM ($C_4 H_{12} N_2 O_6$ 5gm, Yeast Extract 0.1gm , $KH_2 PO_4$ 1gm, $CaCI_2 .2H_2O$ 0.001gm , $MgSO_4.7H_2 O$ 0.5 gm) was prepared incorporating 4 % w/v xylan and 1.6 % w/v agar. After incubation of five days flood plates with iodine stain (0.25 % w/v aqueous 12and KI) and left for 5 minutes. Stain was poured off and washed agar surfaces with distilled water. Xylan degradation around the colonies appeared as a yellow-opaque area against a blue / reddish purple colour for undegraded xylan.(Pointing 1999).

Pectinase

Pectinolytic activity was tested by growing the fungi in pectin agar medium. After 4-5days of the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl tri methyl ammonium bromide. A clear zone formed around the fungal colony indicated pectinolytic activity (Prathyusha *et al.*,2015).

Analysis of Data.

The dominant endophytes were calculated as the percentage colony frequency of a given endophyte divided by the sum of the percentage of colony frequencies of all endophytes x 100 (Kumaresan and Suryanarayanan 2002).

RESULTS AND DISCUSSION

Endophytic fungi from leaf, leaf with midrib, petiole and stem were isolated, identified and evaluated for their existence. Plants were collected from five different localities. A total 101 isolates belonging to 16 fungal taxa were obtained from 120 segments observed. Out of total endophytes isolated most of the genera are from hypomycetes.Very few from coleomycetes and five are from mycelia sterilla. *Alternaria* sp. was observed as the dominant endophytes fungus in total screened samples. Though *Colletotrichum truncatum* was showing lower dominance it is isolated from all the locations. As per present study *Penicillium* sp. showed lower dominance

(Fig.1).According to study done by Mahesh *et al.*, (2005) *Trichoderma, Penicillium* and *Pestalotiopsis* as a dominant species from neem bark collected from Mysore region.

Nine endophytic fungi of *Azadirachta indica* A. Juss. were screened for enzyme activity. Nine fungi were screened for Lipase production, out of which no any endophytes were able to produce lipase. The laccase activity was observed only in *Alternaria* by formation of clear zone around the colony. Amylase is produced by *A .flavus, Alternaria, A.sp., Cladosporium*, other fungi do not show amylase activity. Again all nine fungi were screened for cellulase production, out of which all nine endophytes were able to produce cellulase . Tyrosinase activity shown by only two endophytes i.e. *Alternaria* and *Colletotrichum truncatum*. Asperginase activity was observed only in A. *sp.2*. glutaminase and pectinase activity was not observed in any endophytes. Xylanase activity was observed in *A.flavus* and *Alternaria* by formation of clear zone around the colony (Table No.1)

Fig.1 : Dominance of endophytic fungi isolated from *Azadirachta indica*

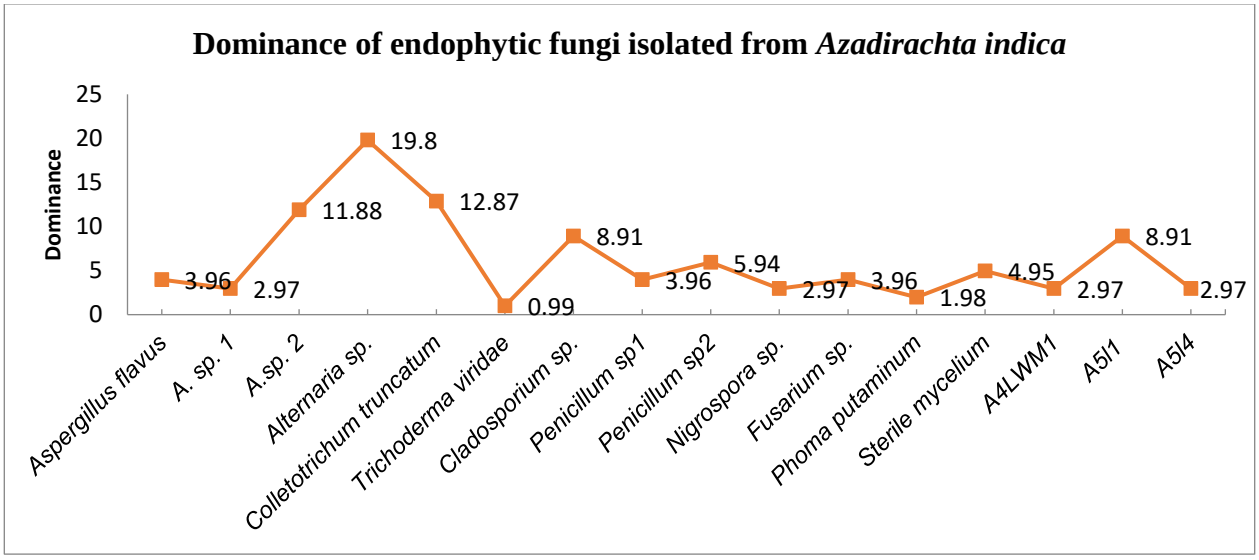

Table No.1: Detection of Enzyme from endophytes isolated from *Azadirachta indica*

Fungi	Detection of Enzyme								
	Lipase	Laccase	Amylase	Cellulase	Tyrosinase	Asperginase	Glutaminase	Xylanase	Pectinase
Aspergillus flavus	-	-	+	+	-	-	-	+	-
Alternaria	-	+	+	+	+	-	-	+	-
A.sp.1	-	-	+	+	-	-	-	-	-
Cladosporium sp.	-	-	+	+	-	-	-	-	-
Fusarium sp.	-	-	-	+	-	-	-	-	-
Colletotrichum truncatum	-	-	-	+	+	-	-	-	-
A.sp.3	-	-	-	+	-	-	-	-	-
Penicilium sp.	-	-	-	+	-	-	-	-	-
A. sp.2	-	-	-	+	-	+	-	-	-

According to studies done by Patel *et al.,* (2013) none of the endophytic fungi isolated from monocot and dicot plants able to produce lipase enzyme. In our studies also, endophytic fungi were not capable of producing lipase enzyme. In the present study endophytic fungi were not able to utilize lipids as an energy source.*Alternaria arborescence* and *Fusarium oxysporium* isolated from soil samples collected from Anamalai Hills (Tamilnadu) exhibited production of laccase enzyme (Christie and Shanmugan 2012). In another study one of the endophytic fungi *Colletotrichum gleosporide* gr. isolated from medicinal plant *Piper bettle* is found to be a laccase producer.(Sidhu *et al.,* 2014). In our study only *Alternaria* is able to produce laccase enzyme. This is the first report of endophytic *Alternaria* is producing laccase enzyme.

Most of the amylases have been produced from soil fungi such as *Aspergillus, Penicillum and Rhizopus* (Pandey *et al.,* 2000.). Very few reports are available on amylases from endophytic fungi, which are mainly explored for beneficial secondary metabolites with different bioactivity (Pimentel *et al.,* 2011.). Maria *et al*., also reported amylase production by few endophytic isolates from mangrove angiosperm *Acanthus ilicifolius* L. and mangrove fern, *Acrostichum aureum* L. In our present study four different *Aspergillus* sp. , *Alternaria* and *Cladosporium* sp. exhibited production of amylase enzyme but remaining isolates like *fusarium, colletotrichum*, another *Aspergillus* sp.and *Penicillium* sp. did not show production of amylase enzyme.All isolates are able to produce cellulase enzyme. According to studies done by Sunitha *et .al*. (2013) 32% of the endophytic fungi were able to produce cellulase enzyme . Similar result were reported by Maria *et al.,* (2005) from mangrove anigiosperm isolates. Very few references were observed about tyrosinase production from endophytic fungi, However studies done by Zaidi *et al* (2013) some of isolates from *Azadirachta indica* and *Ocimum tenuiflorum* has higher production of extracellular tyrosinase in comparison with *Calotropis gigantea* and *Lantana camara.* In the present study *Alternaia* and *Colletotrichum* were able to produce tyrosinase enzyme. This is the first time reported that endophytic isolate *Alternaria* is producing tyrosinase enzyme.

Another study done by Chandramouli and Monnanda, 2014, high level of L-asparaginase activity was observed in *Fusarium* spp. In this work only one of the Aspergillus sp. is able to produce asparaginase enzyme. Others are tested negative for the asparaginase enzyme.Wipusaree *et. al.(*2011) isolated fifty-four endophytic fungi from thai medicinal plants and examined for xylanase production. Xylanase activity was found in thirty of the isolates in primary screening by growing on solid xylan agar plates. In the present study only *A. flavus* and *Alternaria* sp.able to produce xylanase. Remaining strains were not able to produce xylanase enzyme.

None of the fungi in present study were able to produce lipase enzymes which indicate that they are not using lipids as a energy source. All are tested positive for cellulase production means they are utilizing cellulase for their survival.

Very few are producing amylase representing that all are not degrading starch, may be others are totally dependent on cellulose for living. Laccases catalyze the oxidation of a variety of phenolic compounds diamines and aromatic amines pigment formation, lignin degradation and detoxification (Solomon *et al.,*1996). As *Alternaria* sp. is producing laccase enzyme, it can be explore for further production and characterization. Tyrosinase plays major role in lignin degradation and in melanin synthesis. Endophytic *Alternaria* and *Colletotrichum* strains have potential for tyrosinase production which can be industrial source for the enzyme after detailed study. Endophytic Alternaria strain is producing most of the enzymes it can be correlate with the dominance of *Alternaria.*

In accoradnce with study done by Kamble *et al.*,(2012) *E.coli*, *Proteus* sp., *Pseudomonas* sp., *Bacillus* sp. are also source of L-asparaginase but fungi are better source of asparaginase production as they have less adverse effects than bacterial L-asparaginases (Sarquis *et al.,* 2004) . L-asparaginase is used in chemotherapy for cancerous tumors of white blood cells.In the present study only one strain of endophyte *Aspergillus* sp. demonstrated its ability to metabolize the substrate, L-asparagine. It can be further explored for industrial production of asparginase. Xylanase is one of the industrially important enzyme and it is produced by many microorganisms, such as bacteria, yeast and fungi. Endophytic *Aspergillus flavus* and *Alternaria* sp. are also potential source for the commercial production of xylanase enzyme which can be used further after optimization.

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