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Evaluation of Tyrosinase Producing Endophytic Fungi from *Calotropis gigantea, Azadirachta indica, Ocimum tenuiflorum* and *Lantana camara*

Kamal Uddin Zaidi[1*], Abin Mani[1], Ayesha S. Ali[2] and Sharique A. Ali[2]

[1]Molecular Biotechnology Laboratory, Centre for Scientific Research and Development, People's University, Bhopal M.P 462037, India.
[2]Department of Biotechnology, Saifia College of Science Bhopal-462001, India.

Authors' contributions

The work described in this article was conducted and designed by author KUZ which was guided and support of authors ASA and SAA. The development of article, organizing of analysis and the literature work was done by authors AM and KUZ. All authors had read and approved the draft of this final manuscript.

Research Article

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ABSTRACT

Aims: The present study focuses on evaluation of tyrosinase producing endophytic fungi from *Calotropis gigantea, Azadirachta indica, Ocimum tenuiflorum* and *Lantana camara.*
Place and Duration of the Study: The present study was conducted at centre for Scientific Research and Development People's Group Bhanpur, Bhopal India during January 2011 to December 2012.
Methodology: The endophytic fungi were isolated from leaves, root and stem of *Calotropis gigantea, Azadirchta Indica, Ocimum tenuifloram* and *Lantana camara* and cultured on malt extract agar. Isolates were evaluated for tyrosinase production qualitatively and quantitatively on modified Czapex Dox's agar.
Results: Out of fifty isolates, twenty seven isolates showed tyrosinase production in agar plate assay. It was observed that endophytes isolated from *Azadirachta indica* and *Ocimum tenuiflorum* has higher production of extracellular tyrosinase in comparison with *Calotropis gigantea* and *Lantana camara.*
Conclusion: Phylum basidiomycete is considered to be the prominent source of

**Corresponding author: Email: zaidi.kamal92@gmail.com;*

tyrosinase. This finding indicates that the endophytic fungi are also a promising source and are more suitable for large scale productions.

Keywords: Endophytic fungi; tyrosinase; Calotropis gigantean; Azadirachta indica; Ocimum tenuiflorum; Lantana camara.

1. INTRODUCTION

Tyrosinase (E.C. 1.14.18.1) is known for the production of melanin, which is distinguished for the hydroxylation of a monophenol and conversion of o-diphenol to o-quinone. It also performs a variety of functions in an organism ranging from pigmentation to defense to sclerotization [1]. This enzyme is essential for pigmentation, important factors in wound healing and primary immune response. It has a great applicability in environmental, pharmaceuticals, cosmetic and food industries. Tyrosinase enzyme is ubiquitous in nature and is isolated from different plant, animal, fungal and bacterial species. Intracellular Fungal tyrosinases were firstly characterized from the edible mushroom *Agaricus bisporus* [2-4]. Selinheim et al. [5] firstly reported extracellular fungal tyrosinase from a filamentous fungus. In the search of better and efficient sources, the present study was under taken to explore the viability of endophytes for the production of Tyrosinase enzyme, since it plays vital role in the decomposition of plant parts. Endophytes are known for its anti bacterial, antitumour, immunomodulatory, anti inflammatory and antiviral activities despite this, to the best of our knowledge, there are no studies indicating endophytes for tyrosinase production. The present study focused on extracellular tyrosinase from endophytic fungi of *Calotropis gigantea, Azadirchta Indica, Ocimum tenuifloram* and *Lantana camara,* which are some of the prominent herbal medicines.

2. MATERIALS AND METHODS

2.1 Plant Materials

Leaves, Root and Stem of *Calotropis gigantea, Azadirchta indica, Ocimum tenuifloram* and *Lantana camara* were collected from Bhopal region in India.

2.2 Isolation and Culturing of Endophytic Fungi

Young matured leaves, stems and roots were harvested from the plants; washed free of dirt and mopped dried. The plant materials were transported from the collection site in ice; labeled and sealed in poly bags. The specimens were washed in running tap water followed by sterile distilled water wash and decontaminated with 70% ethanol for 1 min. 3.0% sodium hypochlorite for 4 min, 70% ethanol for 1 min. and then rinsed with sterile distilled water for 3 times. 3 mm discs were cut from the leaves using a sterile hole-punch and 1 mm high segments were cut from the stems and root [6]. Each tissue parts were cultured in malt extract agar plates of pH 6.0 [malt extract (20 g/l), rose bengal (0.033 g/l), chloramphenicol (50 mg/l), agar (15 g/l)] and was incubated at 28ºC until fungal mycelia develops [7]. The hyphal tips were cut and transferred on to potato dextrose agar (PDA) of pH 6.0 (Potato infusion 200 g/l Dextrose 20 g/l Agar 20 g/l). Half strength PDA was used for sub culturing and stock culturing.

2.3 Identification of Fungi using Cotton Blue Staining

Identification was based on colony characteristics, hyphal morphology and spores formation of the fungal cultures. The mycelia of 2-3 mm from the colony edge were stained with lactophenol cotton blue and cover slip was placed without pressure. The preparation was observed under low and high magnification.

2.4 Qualitative Analysis of Tyrosinase

All isolated endophytic fungi were cultured on PDA for 7days and a 5 mm disc of mycelium was transferred to modified Czapex Dox's agar, pH 6.0 (Tested agar media) of Saxena and Sinha 1981 [8] (glucose 2.0 g/l, L- tyrosine 10.0 g/l, KH2PO4 1.52 g/l, KCl 0.52 g/l, MgSO4.7H2O 0.52 g/l, CuNO3.3H2O 0.001 g/l, ZnSO4.7H2O 0.001 g/l, FeSO4.7H2O 0.001 g/l) and control test agar media without tyrosine. All the plates were incubated at 28ºC and formation of reddish brown zone radius and colony diameters were recorded after an incubation period of five days.

2.5 Quantitative Analysis of Tyrosinase

Dopachrome formation was determined by the method of Dastager 2006 [9] with some minor modification 10 ml of Czapex Dox's broth medium of pH 6.0 was inoculated with one loop full of spores and subjected to stationary stage at 28ºC for seven days. Tyrosinase activities were estimated by mixing 2 ml of the culture filtrate and 1 ml of 0.4% substrate solution L-dopa (HIMEDIA) and incubating at 37ºC for 30 min for 5 min. Red coloration indicated the dopachrome formation and was read spectrophotmetrically at 475 nm. The difference in absorbance is proportional to the enzyme concentration. The catalytic conversion of 1µmol of substrate to product in 1 minute with 1.35 changes of absorbance is equal to 1 unit.

3. RESULTS

3.1 Isolation of Endophytic Fungi

From the four medicinal plants under study 50 endophytic fungi were recovered. The isolation is illustrated in the Table 1.

Table 1. Number of endophytic fungi isolated from four medicinal plant species on malt extract agar at 30ºC

Medicinal plants species	No of entophytic fungi			
	Stem	Root	Leaf	Total
Calotropis gigantea	3	4	5	12
Azadirachta indica	8	3	4	15
Ocimum tenuiflorum	3	5	4	12
Lantana camara	6	3	2	11

3.2 Identification of Isolates

The pure endophytic cultures isolated were identified up to the genus level. Hyphal morphology of isolates and characteristics of the spores were observed using lactophenol

cotton blue stain under the light microscope. The cultural and morphological identification of the isolates are given in Table 2.

Table 2. Identification of endophytic fungi isolated from plants under study

Isolate no.	Cultural characteristics	Morphological characteristics	Species
1,5,11,13,14,28 ,39,29,30,31,34 ,35,36,37	White to green colour, white aerial growth, velvety,	Mycelium septate, branched, conidiophore arising from a foot cell, vesicle racket shape, primary and secondary sterigmata present on ¾ portions, conidia born on sterigmata tip.	*Aspergillus* spp
8,16,18,28,32,3 3,38, 42	Rapid growing, flat, filamentous, and velvety, woolly, or cottony in texture. The colonies are initially white and become blue green, gray green, olive gray, yellow or pinkish in time.	Septate hyaline hyphae (1.5 to 5 µm in diameter), simple or branched conidiophores, metulae, phialides, and conidia are observed. Metulae are secondary branches that form on conidiophores. The metulae carry the flask-shaped phialides.	*Penicillium* spp.
6,7,10,12,15,17 ,20,24,48	Greenish black colony, reverse color brown, mycelium white, growth velvety.	Mycelium septate, branched, conidia longitudinally septate transverse present in airopetal manner, conidia pear shaped, conidia mycelium brown in color.	*Alternaria* spp.
2,3,4,26	White colony, aerial growth white, Reverse colour white, growth cottony.	Mycelium septate, branched, conidia borne on sporodochium, sickle shape conidia, mycelium light in color	*Fusarium* spp.
9,41,43,46,47,4 9	Colonies are yellow to dark yellowish-green, consisting of a dense felt of conidiophores or mature vesicles bearing phialides over their entire surface.	Aspergillus hyphae are septate and show dichotomous, 45°angle branching. Larger hyphae may resemble hyphae of the Zygomycetes.	*Aspergillus flavus*
19,21,22,23,25, 27,40,44,45,50	Yellowish black, aerial growth white, Reverse colour white, growth velvety	Mycelium septate, branched, vesicle round shape, primary and secondary sterigmata present, conidia present on conidiophores above vesicle	*Aspergillus nidulans*

3.3 Qualitative Analysis of Tyrosinase

Out of 50 isolates grown in the tested agar media only 27 isolates has shown tyrosinase activity. The formation of brownish red zone by the fungal colonies (Fig. 1) indicates tyrosinase production. Among the 27 tyrosinase producing isolates; 19 isolates had brownish red zone around the colonies indicating extracellular enzyme production and 8 isolates had intracellular enzyme production, showing brownish red zone within the colonies. Isolate TYR-26 from *Azadirachta indica*, TYR-32 and TYR-38 from *Ocimum tenuiflorum* showed the largest zone among the studies samples. These three isolates were the non sporulating fungi.

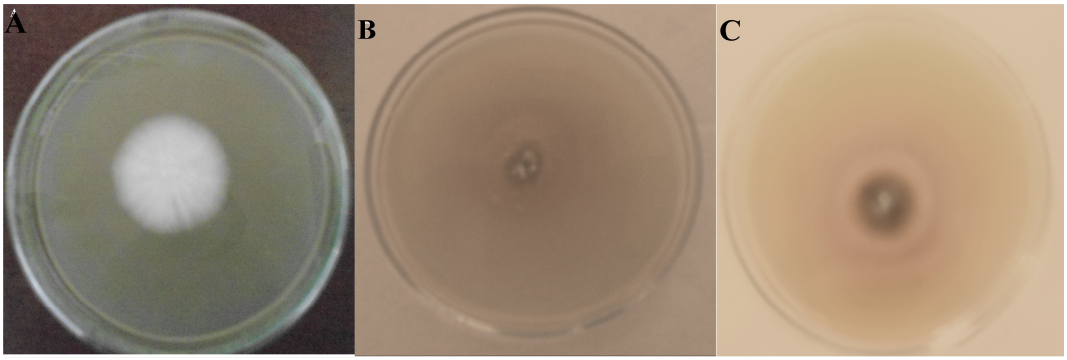

Fig. 1. Representative tyrosinase endophytic fungi on MCD agar. (A) Control plate without tyrosine, (B) brown zone around the isolate showing extracellular tyrosinase production, (C) brown zone within the isolate indicating intracellular tyrosinase production

3.4 Quantitative Analysis of Tyrosinase

The 19 extracellular tyrosinase producing fungal isolates were analyzed for dopachrome formation subjected by spectophotometrically at 475 nm. The dopachrome formation of 19 isolates revealed tyrosinase activity between 0.05 to 3.2 U/ml as shown in Table 3. It was observed that isolates TYR-26, TYR-32 and TYR-38 were having higher tyrosinase activities of 2.8, 3.2 and 2.43 U/ml respectively, which is similar to the tested agar media.

Table 3. Colony diameters and tyrosinase activities of nineteen extracellular tyrosinase producing isolates

Medicinal plants	No of isolates	Tyrosinase activity U/ml(L-DOPA)	Colony diameter (cm)
Calotropis gigantea	TYR-1	0.52	1.2 ± 0.1
	TYR-5	0.054	1.3 ± 0.1
	TYR-8	1.55	2.3 ± 0.1
	TYR-11	0.37	1.4 ± 0.1
Azadirachta indica	TYR-13	0.24	3.8 ± 0.1
	TYR-14	0.32	2.1± 0.1
	TYR-16	1.6	1.2 ± 0.1
	TYR-18	1.2	1.1 ± 0.1
	TYR-20	0.38	2.3 ± 0.1
	TYR-24	0.53	4.2 ± 0.1
	TYR-26	2.8	4.8 ± 0.1
Ocimum tenuiflorum	TYR-28	0.32	3.9 ± 0.1
	TYR-32	3.2	2.9 ± 0.1
	TYR-33	0.84	3.3 ± 0.1
	TYR-35	0.21	1.2 ± 0.1
	TYR-38	2.43	3.4 ± 0.1
Lantana camara	TYR-39	0.24	4.3 ± 0.1
	TYR-42	0.89	1.6 ± 0.1
	TYR-48	0.52	1.9 ± 0.1

4. DISCUSSION

Selinheim et al. [5] first reported extracellular tyrosinase from a filamentous fungus, Trichoderma reesei coded by tyrosinase gene tyr2 [10]. Phylogenetic study on the whole tyrosinase protein sequences, showed that fungal tyrosisnases are clustered in basidomycetes, ascomycetes and deuteromycetes [11]. The present study revealed the presence of tyrosinase production of endophytic fungi in *Calotropis gigantea*, *Azadirachta indica, Ocimum tenuiflorum* and *Lantana Camara.* The study conducted by Sun et al. [12] indicated the association of endophytic fungi in the decomposition of plant materials having lignin degrading enzymes of endophytic. Tyrosinase is one of the lignin degrading enzymes and endophytic isolated from *Calotropis gigantea*, *Azadirachta indica*, *Ocimum tenuiflorum* and *Lantana camara* shown tyrosinase activity reveals the association characteristic and recycling system of natural ecosystems, whereas the tyrosinase inhibitory property of these plants enable they vegetate growth. Osvaldo et al. [13] also reported the production of tyrosinase in free living mycelium in ripen fruit bodies. The tyrosinase production from different sources viz., Trifolium pretense [14], Crocus sativus [15], *Agaricus bisporus* [16], *Pycnoporus sanguineus* [17] and *Aeromonas media* [18], reveals high variability of tyrosinase producing fungal sources.It was observed that endophytes isolated from *Azadirachta indica* and *Ocimum tenuiflorum* has the highest extracellular tyrosinase production as compared to *Calotropis gigantea* and *Lantana camara*. This study signifies the potency of endophytic fungi for the tyrosinase production, which has a higher industrial application due to the flexibility of fungi in fermentation process and product recovery as compared to the other higher organisms that are used in tyrosinase production.

5. CONCLUSION

The present study exposed other promising tyrosinase producing fungal sources which will be a great help to the industrial production of this enzyme, which also points the scope for exploring newer floras for more valuable, economical and flexible natural sources. More over this study also showcases the value of association for energy utilization in the ecosystem. More studied has to be carried out to characterize therapeutic potentiality of these medicinal plants with its endophytic flora and their therapeutic associations.

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COMPETING INTERESTS

It is ensured that there is no conflict of interest regarding the research work and to any of the data that are provided in the manuscript. This manuscript has not been previously published elsewhere and is not currently submitted to any other journals and will not be submitted elsewhere before a decision is obtained from this journal.

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