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ORIGINAL ARTICLE

Isolation and Screening of Lovastatin Producing Endophytic *Aspergillus terreus* from Medicinal Plants

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ABSTRACT
Industrial scale production of lovastatin is currently being carried out by Aspergillus terreus. There is enough unexplored potential of endophytic fungi capable of producing active biocompounds of pharmaceutical importance. Hence, the present study aims to isolate and screen lovastatin producing endophytic A.terreus from apparently healthy tissues of medicinal plants. It has been found that out of 55 A. terreus isolates that were obtained from the tissues and screened for the production of lovastatin, an isolate from Hibiscus rosa-sinensis leaf produced 1.25mg/gds of lovastatin employing Solid State Fermentation (SSF) using wheat bran as substrate. The yield is on par with that produced from other endophytic sources, reported so far. The isolate was identified by partial sequencing of its 18S rRNA gene using ITS primers. Lacto phenol cotton blue stained transverse cross-section of the leaf shown colonization of the isolate within the host tissue confirming its endophytic nature. The presence of lovastatin in the fungal extract was confirmed by High Performance Liquid Chromatography (HPLC).
KEYWORDS: Endophytic fungi, Lovastatin, *Aspergillus terreus*, *Hibiscus rosa*-sinensis

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INTRODUCTION

Statins, the fungal secondary metabolites, are a class of drugs that are used in the treatment of hypercholesterolemia since they act as competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase), a rate limiting enzyme of mevolanate pathway[1]. Of late, they have also been successfully employed in the treatment of many chronic diseases such as cardiovascular diseases[2], Alzheimer's disease[3], Multiple Sclerosis[4], renal disease treatment[5], bone disorders[6] and cancer[7], to name a few. *Aspergillus terreus,* a potent lovastatin producer, is currently being used in the production of lovastatin at industrial scale. Among the other producers of the statins are *Penicillium* sp.[8], *Monascus* sp.[9], *Pleurotus* sp.[10], etc. These fungi reside in a variety of natural environments owing to their extraordinary metabolic diversity.

Endophytic fungi are the fungi that reside within the plant tissues without causing any apparent harm to the host. In spite of their enormous potential to produce diverse bio-compounds of pharmaceutical significance, they have been less explored compared to their counterparts in other habitats. In the past three decades, a great focus has been emphasized on endophytes and the metabolites they produce thereof. Lovastatin production from an endophyte of *Taxus baccata*, *Aspergillus niger* PN2, was reported by Palaniswamy et al [11].In another study, lovastatin production from *Phomopsis vexans* residing within healthy tissues of *Solanum xanthocarpum* was reported by Parthasarathy et al [12]. While there are a lot of reports on the isolation and screening of *Aspergillus terreus* from soil sources for lovastatin production, this study attempts bioprospecting of medicinal plants for the isolation and screening of high lovastatin yielding endophytic *Aspergillus terreus.*

MATERIAL AND METHODS

Sample collection: Medicinal plants namely *Ocimum tenuiflorum, Emblica officinalis, Moringa oleifera, Bacopa monnieri, Murraya koenigii, Azadirachta indica, Hibiscus rosa-sinensis, Catharanthus roseus, Withania somnifera* and *Morinda citrifolia* were collected from GKVK, Bangalore.

Isolation: The plant parts under study such as leaf, stem, root and flower (as applicable) were surface sterilized as described by Strobel [13], plated on Potato Dextrose Agar (PDA) and incubated at 25^{o}C for two weeks. Fungal hyphal tips were transferred onto fresh PDA plates and sub-cultured once in a month.

Identification: Morphological identification of the fungal isolates was carried out using lacto phenol cotton blue stain. Colonization of the fungal isolate within the host tissue was observed by making a transverse cross-section of the tissue, staining with lacto phenol cotton blue and observing under a light microscope. Partial sequencing of 18S rRNA gene was done using ITS primers [14]. The sequence was thus compared using NCBI BLAST to check the percentage of similarity with their counterparts [15].

Submerged fermentation: 50ml of cooled autoclaved soyabean medium was inoculated with three loops of culture and incubated in incubatory shaker for 7 days [16]. The experiment was done in triplicates and repeated at least twice to confirm the consistency.

Extraction of the fermented material: The fermented material was filtered and acidified (pH=3) using 1N HCl. To this, equal amount of ethyl acetate was added and kept in shaker for two hours. The organic phase was separated and allowed to dry [17]. The contents were dissolved in 1ml ethanol and stored at 4^{o}C till further analysis.

Qualitative analysis:

Thin layer Chromatography: Thin Layer Chromatography (TLC) of the extract was carried out using Toluene: Ethanol (80:20) as solvent system [16]. R_f values were calculated and compared to that of the standard drug.

Bioassay*: Saccharomyces cereveseae* spore suspension was spread on to Yeast extract Peptone Dextrose Agar (YPDA) and wells of 8mm diameter were made using a sterile borer. The fungal extract was loaded in triplicates with ethanol acting as control [18]. The zone of inhibition was measured after 18 h.

Quantitative analysis: The amount of lovastatin in the fungal extract was quantitatively estimated using alkaline hydroxylamine and ferric perchlorate reagents in acidic medium (pH 1.2±0.2). Different aliquots of fungal extract were taken in test tubes and 1ml of alkaline hydroxylamine solution was added to them and mixed well. To this, 5ml of ferric perchlorate was added and the mixture was acidified with 1ml of 2N HCl. The total volume was made up to 10ml with ethanol and incubated at room temperature for 25min. The resulting purple colored complex was read at 513nm using colorimeter [19].

Solid State Fermentation (SSF): Consistently high lovastatin yielding isolates were subjected to Solid State Fermentation using 5 g of wheat bran. 2ml of spore suspension was inoculated onto the autoclaved and cooled substrate and incubated at 28^{o}C for 11 days [11].Extraction and estimation were carried out as described previously. The experiment was done in triplicates and repeated at least twice to confirm the consistency.

High Performance Liquid Chromatography: The presence of lovastatin in the fungal extract was confirmed by High Performance Liquid Chromatography (HPLC) analyses. It was carried out using C18 column of particle size 5µm and injection volume of 20µl. Acetonitrile and water in the ratio of 60:40 (pH 3.6) acts as mobile phase with a flow rate set to 1 ml/min. Detection of compound was carried out by UV detector at 210 nm.

RESULTS AND DISCUSSION

This study documents the isolation and screening of 55 *A.terreus* isolates for the production of lovastatin. The recorded yield ranges from 6.5 mg/l to 71.5 mg/l employing submerged fermentation. Fifteen high lovastatin yielding *A. terreus* strains are given in the Table 1.

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Table 1: Yield of most potent endophytic *Aspergillus terreus* through submerged fermentation

S. No	Host	Tissue	Yield (mg/l)
1	*Bacopa monnieri*	Leaf	71.5
2	*Bacopa monnieri*	Leaf	48
3	*Hibiscus rosa-sinensis*	Flower	46.5
4	*Bacopa monnieri*	Stem	45
5	*Emblica officinalis*	Leaf	33
6	*Hibiscus rosa-sinensis*	Stem	27.5
7	*Moringa oleifera*	Stem	23
8	*Ocimum tenuiflorum*	Leaf	21.5
9	*Catharanthus roseus*	Stem	21.5
10	*Murraya koenigii*	Leaf	20
11	*Hibiscus rosa-sinensis*	Leaf	18
12	*Withania somnifera*	Root	18
13	*Murraya koenigii*	Stem	17.5
14	*Murraya koenigii*	Stem	16.5
15	*Ocimum tenuiflorum*	Stem	16.5

The most potent and consistently yielding *A.terreus* isolate from the leaf tissue of *Hibiscus rosa-sinensis,* when subjected to SSF, was found to produce a considerably high yield of 1.25mg/gds under un-optimized conditions using wheat bran as substrate. Javiel and Marimuthu [20] reported a maximum yield of 982.3 µg/gds of lovastatin from *A.terreus*. Prabhakar et al. reported a yield of 1.1 mg/gds from *A. terreus* KLVB28mu21, a mutated strain [21]. Savita et al. reported that endophytic fungi of genera *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopulariopsis, Doratomyces, Phoma* are poor producers of lovastatin as compared to their terrestrial counterparts [22]. The first report on the production of lovastatin from endophytic fungi of *Taxus baccata*, *A.niger* PN2, was given by Palaniswamy et al. [11] with a yield of 1.5 mg/gds employing solid state fermentation. Recently, Parthasarathy et al. [12] reported a yield of 550 mg/L of lovastatin from *Phomopsis vexans* residing within the healthy leaf tissues of *Solanum xanthocarpum.*

Morphologically *A. terreus* shows brownish velvety colonies. Microscopically they consist of compact biseriate conidial heads with globose shaped conidia. 18S rRNA gene sequencing result shows 97% sequence identity to its counterparts (Figure 1). The sequence was submitted to NCBI Genbank and been assigned the accession number KY780197.

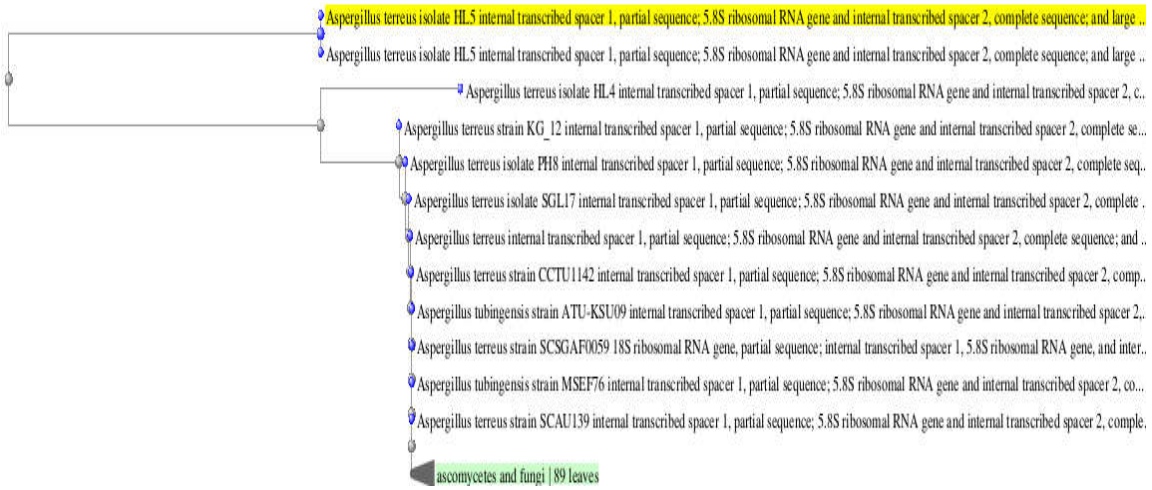

Figure 1: A portion of the phylogenetic tree generated from the BLAST search showing the sequence identity

The colonization of the endophytic isolate within the host tissue was shown by lacto phenol cotton blue stained transverse section of the leaf (Figure 2a and 2b).

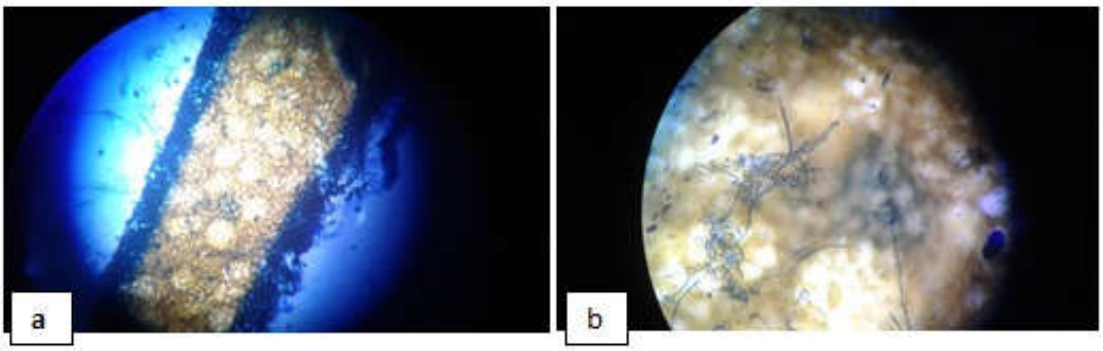

Figure 2: a) *Hibiscus rosa-sinensis* leaf cross-section showing colonization of *Aspergillus terreus* within the host tissue at 100x magnification b) *Aspergillus terreus* colonymagnified to 400x

Thin Layer Chromatography of the fungal extract confirmed the presence of lovastatin by showing similarity in the R_f values of the extract (0.70) obtained from *A.terreus* and the standard lovastatin (0.69). Yeast growth inhibition bioassay results showed zone of inhibition of diameter 1.2 cm. The presence of lovastatin in the fungal extract was further confirmed by HPLC which shows resemblance in retention time in the peaks of fungal lovastatin and standard lovastatin (Figure 3). Peaks other than that of lovastatin might be due to the presence of unidentified compounds and impurities in the sample.

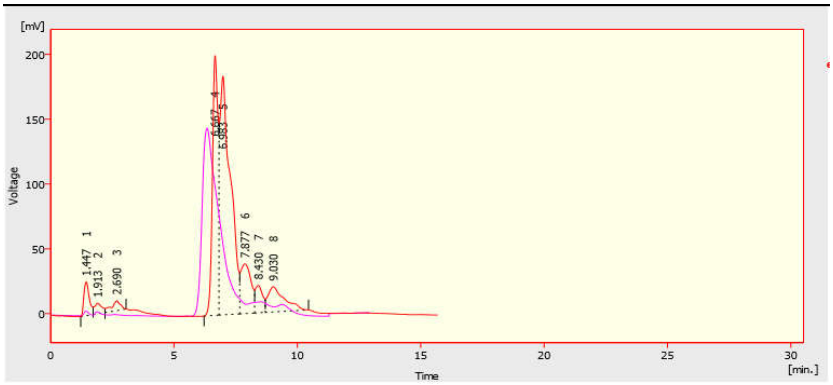

Figure 3. HPLC spectra of lovastatin produced by *A.terreus*. Retention time of fungal lovastatin 6.98 min and that of the standard lovastatin is 6.66 min

CONCLUSION

This study documented the isolation and production of lovastatin from endophytic *A.terreus,* with considerably high yield. The findings open up avenues to explore diverse microbial habitats to isolate strains with the potential to produce high yield of lovastatin. Cost-effective production of lovastatin can be achieved by optimization of production parameters and strain improvement.

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