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LOVASTATIN PRODUCTION FROM ENDOPHYTIC *MEYEROZYMA GUILLIERMONDII* ISOLATED FROM *HIBISCUS ROSA-SINENSIS*

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ABSTRACT

The current study aims to exploit endophytic fungi residing within medicinal plants as potent lovastatin producers. A total of 98 isolates from the tissues of medicinal plants were isolated and screened among which an endophytic yeast, *Meyerozyma guilliermondii,* isolated from apparently healthy leaf tissues of *Hibiscus rosa*-sinensis has been found to yield 1.65 mg/gds of lovastatin employing Solid State Fermentation (SSF) which is appreciably high as compared to that produced from other endophytic sources, reported so far. Microscopic and molecular identification techniques confirmed the endophytic nature of the isolate. HPLC and FTIR analyses evidently confirmed that the fungal metabolite was identical to the standard lovastatin.

Keywords: *Meyerozyma guilliermondii*, *Hibiscus rosa*-sinensis, Endophytic fungi, Lovastatin, FTIR

INTRODUCTION

Over the past 3 decades, the term"endophytes" has appeared more often in the fungal literature owing to their novel biochemistry and secondary metabolite production. The term was first proposed by de Bary [1] and since the point of their first isolation and culture from seeds of *Lolium temulentum*, all the plant species studied till date were found to host not less than one species of endophytes [2]. Endophytic fungi

asymptomatically colonize the apparently healthy internal tissues of almost all plant families, some being host specific, and are capable of synthesizing many overlooked biologically active compounds of pharmaceutical importance.

One of the important secondary metabolites produced by endophytic fungi is lovastatin, produced during the secondary phase of fungi growth. It was the first statin approved by FDA in 1987 as hypercholesterolemic drug that lowers cholesterol by competitively inhibiting the 3- hydroxyl-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [3]. Lovastatin has a therapeutic effect across a wide range of cardiovascular and non-cardiovascular diseases [4], osteoporosis [5], Alzheimer's disease [6], Parkinson's disease [7], multiple sclerosis [8] and renal diseases [9] to name a few. Beyond these applications, lovastatin was also found to restrict the growth of tumor cells. It has been experimentally proven that HMG-CoA reductase inhibitors inhibit cellular proliferation and induce cell deaththus making them potential anticancer agents [10].

A lot of research has been done in establishing soil fungi such as *Penicillium* sp. [11], *Monascus ruber* [12] and *Aspergillus* sp.,[3] as potential lovastatin producers whereas lovastatin as a secondary metabolite from endophytic fungi was studied and reported by Palaniswamy et al. [13] and Ramalingam et al. [14] in two different studies. This indicates that there is a great opportunity to explore new strains and target natural products from endophytic microorganisms that colonize plants in different niches and ecosystems. In the present study, an attempt has been made to screen lovastatin producing endophytic fungi from medicinal plants.

MATERIALS AND METHODS

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

Isolation of endophytic fungi: Surface sterilization of plant parts was carried out as described by Strobel et al. [15], plated on Potato Dextrose Agar (PDA) supplemented with streptomycin (150 mg/L) to prevent bacterial contamination and incubated at 25^{o}C for 2 weeks. Hyphal tips of the fungi were picked and grown on a fresh PDA plate

in pure culture and sub-cultured once in every 4 weeks.

Identification of endophytic fungi: The identification of the isolates was carried out using morphological and molecular methods. Characteristic morphological properties of the fungal cultures were observed using lacto phenol cotton blue stain. Further, transverse cross-section of leaf tissues was carried out to show the endophytic colonization within the host tissue using light microscope. Molecular identification was carried out by partially sequencing the 18S rRNA gene of the isolate using ITS primers [16]. The sequence thus obtained is presented in FASTA format and compared with that of the endophytic isolate by way of NCBI BLAST to check the percentage of similarity with the endophytic counterpart [17].

Screening of lovastatin producing endophytic fungi: Each of the fungal isolates was primarily screened for lovastatin production employing submerged fermentation (SmF). Three loopful of culture was inoculated into 50 ml of cooled autoclaved Soyabean meal medium (Composition (g/L): Sucrose-50; Soyabean meal-20; K_2HPO_4-1; $NaNO_3$-1; $MgSO_4.7H_2O$-0.5; pH 6.5) [18]. The flasks were incubated in a rotary shaker for 7 d at 100rpm. The experiment was done in triplicates and repeated at least twice to confirm the consistency.

Extraction of lovastatin: Lovastatin from fermented medium was extracted using equal amount of ethyl acetate. The system was kept in the rotary shaker at 100 rpm for 2 h and the supernatant i.e., the organic phase containing lovastatin was carefully separated and allowed to dry completely [19]. The residue was dissolved in 1ml ethanol and the extract was refrigerated at 4ºC till further analysis.

Bioassay using *Saccharomyces cerevisiae*: Yeast Peptone Dextrose Agar (YPDA) medium was poured into a sterile petriplate and after solidification, *Saccharomyces cerevisiae* cell suspension was spread onto it. Wells of 8 mm diameter were made using sterile borer and the extract was loaded in triplicates with ethanol as control. These plates were incubated at room temperature for a maximum of 18 h and then the zone of inhibition was measured [20].

Qualitative analysis by Thin Layer Chromatography (TLC): Rapid analysis of the extract was carried out by TLC using the solvent system Toluene:ethanol (80:20) [18]. Rf values were calculated and compared to that of the standard lovastatin.

Quantitative analysis by colorimetric method: Different aliquots of standard

lovastatin (4 mg/ml) ranging from 0.1 to 1 ml and the extracts of unknown concentrations were taken in test tubes. 1 ml of alkaline hydroxylamine reagent was added and mixed well. To this 5 ml of freshly prepared ferric perchlorate working standard was added and pH was adjusted to 1.2± 0.2 with 2 N HCl. The volume was made up to 10 ml with ethanol and the system was incubated at room temperature for 25 min. The maximum absorbance of the purple red color ferric chelate complex was read at 510 nm [21].

Lovastatin production by Solid State Fermentation (SSF): Consistently high lovastatin yielding strains were shortlisted based on submerged fermentation results and subjected to solid state fermentation. 5 g of wheat bran was weighed into Erlenmeyer's flask and moistened 70% with distilled water and autoclaved [13]. After cooling to room temperature, the medium was inoculated with 2 ml of spore suspension and incubated at 28ºC for 11 d. After the incubation period, extraction and estimation of lovastatin was carried out as explained previously. The experiment was done in triplicates and repeated at least twice to confirm the consistency. The extract thus obtained was store at 4°C and used for further analysis.

Confirmation of Lovastatin by HPLC: Lovastatin sample was analyzed by High performance Liquid Chromatography (HPLC) and results were compared to that of the standard drug. HPLC analysis was carried out using C18 column of particle size 5 µm, injection volume of 20 µl. The mobile phase used was Acetonitrile and water in the ratio of 60:40 and pH3.6. The flow rate was set to 1 ml/min with pressure 1300 psi. Detection was carried out by UV detector at 210 nm.

FTIR analysis: Fragment pattern of lovastatin sample was studied with the help of Fourier Transform Infrared Spectroscopy (FTIR) and compared to that of the standard drug. The analysis was carried out using Thermofisher Scientific – Nicolet 6700 Fourier Transform Infrared Spectrometer. The spectrum was captured with DTGS KBr detector; a KBr beam splitter was used for the measurement. The spectrum was scanned in the range of 4000 cm^{-1} to 400 cm^{-1}.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi: In the present study, 98 endophytic fungal isolates were obtained from surface sterilized stem, leaf and root tissues of 10 medicinal plants. Of them, the most frequently isolated genera were morphologically identified as *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Rhizopus* sp., *Mucor* sp. and *Candida* sp.

Screening of lovastatin producing endophytic fungi

Each of these isolates, when initially screened for the production of lovastatin employing submerged fermentation, gave a yield ranging between 5 mg/L to 71.5 mg/L. Of these, 12 high yielding strains are mentioned in Table 1.

Till date, lovastatin has been produced from various soil fungi namely *Penicillium sp., Monascus sp., Aspergillus sp*., etc among which *Aspergillus terreus* is the most commonly employed fungi for the production at industrial scale. Javiel and Marimuthu [22] have reported that *A.terreus* produced the maximum yield of 982.3 µg/gds using wheat bran as substrate. Lovastatin yield of 1.1 mg/gds has been reported by Prabhakar et al. using *Aspergillus terreus* KLVB28mu21 strain [23]. *Monascus purpureus* MTCC 369 produced 3.432mg/gds lovastatin using rice based medium with no other supplements [24]. Attempts have been made to exploit endophytic sources for the production of lovastatin and the first report was documented by Palaniswamy et al. [13] which reported a yield of 1.5 mg/gds employing solid state fermentation from endophytic *A.niger* PN2 residing within healthy tissues of *Taxus baccata.* In 2015, Ramalingam et al. [14] isolated and screened endophytic *Phomopsis vexans* residing within the healthy leaf tissues of *Solanum xanthocarpum* for the production of lovastatin employing submerged fermentation and quantified the amount produced to be 550 mg/L. The isolate under the current study was found to produce a notable yield of 1.65mg/gds under un-optimized conditions.

Morphological and molecular identification of *Meyerozyma guilliermondii*:

Morphologically, the culture appears as white to cream-colored smooth, flat, glossy, yeast like colonies. Lactophenol cotton blue stain of the isolate shows spherical to sub-spherical budding yeast like cells or blastoconidia with short pseudohyphae. 18S rRNAgene sequencing result shows 98% identity to the endophytic sources. The sequence was submitted to NCBI Genbank and been assigned the accession number KY780195. Lacto phenol cotton blue stained transverse section of leaf tissue shows colonization of the isolate within the host tissue (Figure 1).

Analysis of lovastatin: The Rf value of the extract (0.71) obtained from *Meyerozyma guilliermondii* was found to be same as that of the standard lovastatin (0.709). *Saccharomyces cerevisiae* plated bioassay results showed zone of inhibition of diameter

1.1 cm establishing the antifungal activity of the extract. HPLC analysis also confirmed the presence of lovastatin by showing resemblance in retention time in the peaks of fungal lovastatin (11.02 min) and standard lovastatin (10.9 min) (Figure 2). Presence of unidentified compounds and impurities in the sample resulted in the occurrence of peaks other than that of lovastatin. The FTIR spectra showed characteristic peaks at 3541.85 cm^{-1} (corresponds to hydroxyl group stretch), 2966.80 cm^{-1} (corresponds to aliphatic and vinyl C-H stretch), 1695.18 cm^{-1} (corresponds to lactone and ester carbonyl stretch) and 1215.37 cm^{-1} (corresponds to C-O stretch) [25] confirming the presence of lovastatin (Figure 3).

Table 1: Yield of lovastatin from most potent endophytic isolates

S.No	Fungal isolate	Source of the isolate	Yield of lovastatin (mg/L)
1	BS3	*Bacopa monnieri* Stem	71.5
2	BS1	*Bacopa monnieri* Stem	66.5
3	CS7	*Catharanthus roseus* Stem	65
4	AL1	*Azadirachta indica* Leaf	60
5	BL2	*Bacopa monnieri* Leaf	58
6	EL3	*Emblica officinalis* Leaf	55
7	BL1	*Bacopa monnieri* Leaf	51.5
8	OL5	*Ocimum tenuiflorum* Leaf	48
9	HF1	*Hibiscus rosa-sinensis* Flower	46.5
10	HL1	*Hibiscus rosa-sinensis* Leaf	40
11	HL5	*Hibiscus rosa-sinensis* Leaf	21.5
12	HL4	*Hibiscus rosa-sinensis* Leaf	18

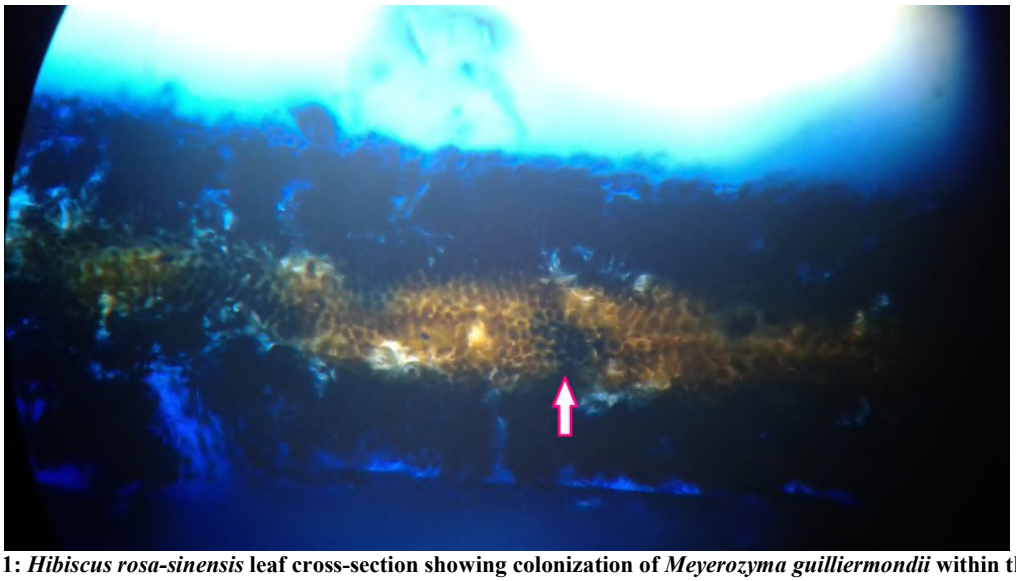

Figure 1: *Hibiscus rosa-sinensis* leaf cross-section showing colonization of *Meyerozyma guilliermondii* within the host tissue

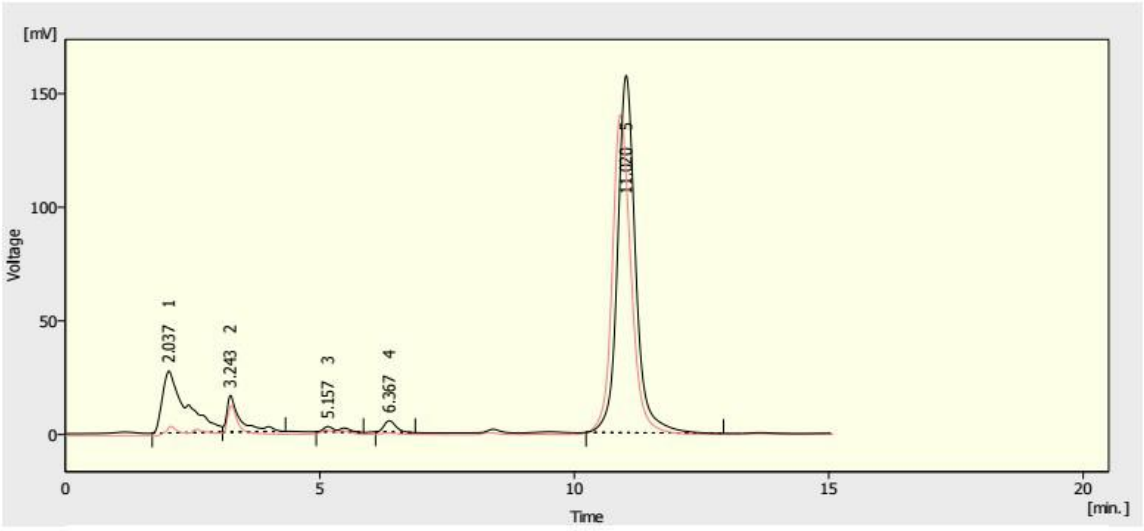

Figure 2: HPLC spectra of the fungal and standard lovastatin

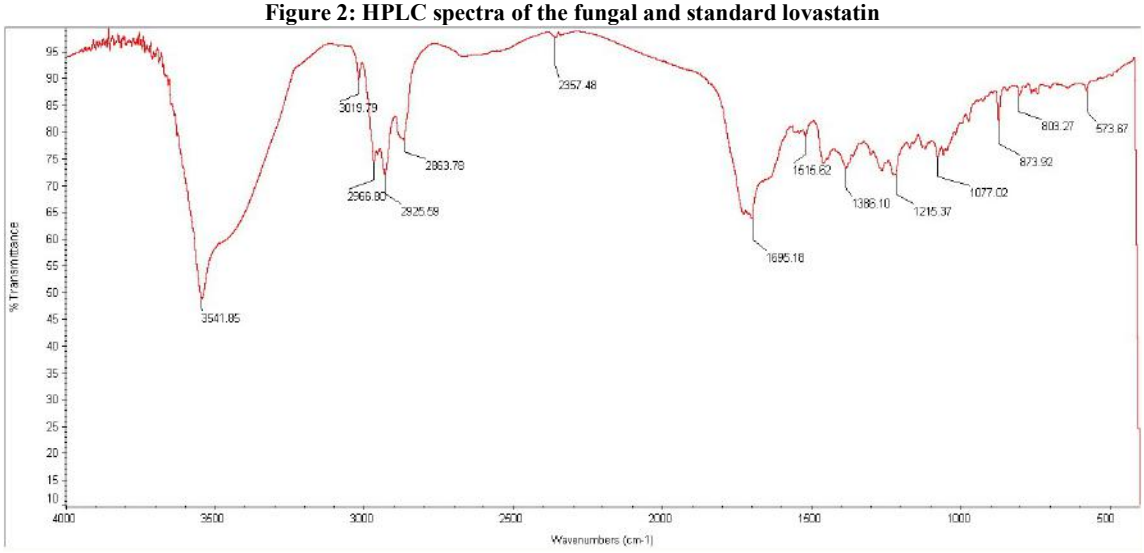

Figure 3: FTIR spectra of fungal lovastatin

CONCLUSION

This investigation, for the first time, has documented the isolation of endophytic yeast, *Meyerozyma guilliermondii,* from the leaf of *Hibiscus rosa-sinensis* and production of cholesterol lowering fungal secondary metabolite, lovastatin, from the yeast, with an appreciably high yield. This study offers scope to explore more endophytic sources with the potential to produce high yield of lovastatin. Optimization and strain improvement studies can be carried out inorder to achieve cost effective production of lovastatin which is proven to have a wide range of applications in the field of medicine for the treatment of chronic diseases that levy heavy cost burden.

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