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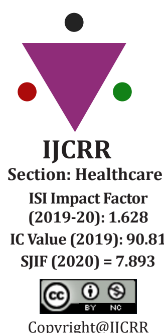

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Screening and Production of Lovastatin Producing Endophytic Fungus from *Phyllanthus reticulatus* using Oyster Mushroom Extract

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ABSTRACT

Introduction: Endophytic fungi are most commonly found in plants. They are present inside the plants and supporting plants to survive and produce many bioactive metabolites. Lovastatin is one of the secondary metabolites produced by the endophytic fungi. It is used in the treatment of high blood cholesterol and reduces the risk of cardiovascular diseases.

Objective: Screening and Production of Lovastatin Producing Endophytic Fungus from Phyllanthus reticulatus using Oyster Mushroom Extract.

Methods: In our study, we have isolated endophytic fungi from the leaves and stem of *phyllanthus reticulatus.* Biodiversity of the endophytic fungi was calculated. *Neurospora crassa* bioassay was performed to identify the best producer of lovastatin. Their Lovastatin production potential was investigated with two different mediums such as potato dextrose and the mushroom extract. After completion of the fermentation process, the broth was extracted by a simple distillation method and then analyzed for the presence of Lovastatin using UV-Visible spectrophotometer and HPLC.

Results: One of the positive and dominant endophytic fungi, *Curvularia* sp was isolated and chosen for this study. Its Lovastatin producing potential in PDA and mushroom extract medium was investigated and confirmed.

Conclusion: *Curvularia* sp was identified as a better Lovastatin producer when compared to other endophytic fungi isolated from *Phyllanthus reticulatus*. From the results of the UV and HPLC, the presence of lovastatin is confirmed.

Key Words: Endophytic fungi, Lovastatin, Bioassay, UV Spectrometry, High-performance liquid chromatography

INTRODUCTION

Endophytic fungi are the probable alternative sources for the development of novel biotechnological products like antibiotics, antimycotics, immunosuppressants and anti-cancer compounds. Such microorganisms live in the plant's internal tissue which supports the plant cells growth and metabolism. Fungal endophytes have been recognized as an excellent source for new compounds of enormous value in agriculture, industry and medicine.[1,2] They were the potential sources for the discovery of new and useful compounds or new platforms for the organic synthesis of such compounds for human benefit. Unlike organic synthetics, there is a clear need for endophytic sources of antimicrobials for their biological and chemical safety effects due to health and environmental problems.[1,3,4]

Lovastatin is one of such secondary metabolites having huge medicinal properties and also naturally produced by certain higher fungi, such as *Pleurotus ostreatus* (oyster mushroom) and closely related *Pleurotus* sp.[5] Chemical synthesis of Lovastatin strictly involves synthetic methods. It was the first statin drug which was patented[5] and approved by the United States Food and Drug Administration (FDA) in 1987.[6-9] The cost of production is too high for commercialization purposes and other commercially viable chemical processes only yield poor quality production and involve complicated production steps.[10-13] Hence, biotechnology has opened a new avenue through simple biotechnological methods to produce high quality and cost-effective Lovastatin and it is summarized in Table 1.

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Phyllanthus reticulatus belongs to the family of Euphorbiaceae, usually a dense deciduous shrub which contains lots of medicinal properties. The different parts of this plant such as leaves, dried bark, fruits, aerial parts are to treat various diseases. In the pharmacological view, it has various activities like antidiabetic, antispasmodic, hypocholesterolemic, antimicrobial, cytotoxic, hepatoprotective anti-hyperglycemic, etc.[14-19]

MATERIALS AND METHODOLOGY

Collection of Plant

Healthy leaf, stem and root of *Phyllanthus reticulates* (Poir) was collected from the Theravada regions of Sathyamangalam Taluk in a sterile ziplock bag and processed within 24 hours at the Endophytic Fungal Metabolite Research Laboratory, Department of Biotechnology, Bannari Amman Institute of Technology. [20,21]

Isolation and Identification of endophytic fungi

All the plant specimens were washed thoroughly with sterile water and endophytic fungi were isolated. [22,23] The surface sterile leaves, stem and petiole were dissected under sterilized conditions into small segments of 0.5 cm x 0.5 cm using a sterile scalpel and dried in blotting paper. They were placed equidistantly (10 segments per plate per specimen) on the freshly prepared Potato Dextrose Agar plates (PDA) amended with Chloramphenicol (50 µgmL^{-1}, Sigma Aldrich) and streptomycin sulphate (250 µgmL^{-1} Sigma Aldrich) and incubated at 25°C±1°C.[24,25]

Identification of Endophytic fungi was carried out using microscopic and morphological characteristics. Isolated pure endophytic fungal cultures were stained using lactophenol cotton blue staining method and viewed under the microscope and were shown in Figures 2,3 and 4.

Among the group of endophytic fungi isolated from the plant, best fungal isolate (Table 2) was chosen based on the highest colonization frequency and zone of inhibition in a bioassay.

Biostatistics for Species Diversity

Colonization Frequency Percentage (CF %)

The density of colonization of single endophytes species was calculated and was equal to the number of colonized segments divided by the total number of segments observed x 100.

Endophytic Infection Rate (EIR %)

Endophytic Infection Rate was determined as the number of infected plant segments divided by the total number of plant segments screened x 100.[26-30]

Screening by *Neurospora crassa* Bioassay

This screening was based upon the use of biological responses as a detection system for biologically active substances. *Neurospora crassa* (MTCC 790) was sensitive to β hydroxyl acid of Lovastatin [19] and hence, Lovastatin producing isolates create a zone of inhibition by suppressing the growth of this organism. Thus, using this principle, selected 10 endophytic fungal species were screened to identify their Lovastatin producing capabilities.[26,27]

Batch fermentation

Highly positive endophytic fungi obtained through screening was chosen for the further fermentation process. Hence, Fully grown mycelial culture (1cm x 1cm) of *Curvularia* sp from the agar plate was inoculated in 100 mL of two different media M1 and M2 (Table 3) at pH 6 in triplicates under sterile condition. All the flasks were incubated in a shaker incubator at 28°C and 180 rpm for 10 days.[6,14,16]

Extraction and confirmation of Lovastatin production

After 8 days of fermentation, the culture broth was separated by sterilized filter cloth. The culture filtrate was adjusted from pH 6 to pH 2 and was kept in a rotary shaker with an equal volume of ethyl acetate at 100 rpm for 2hrs at room temperature. After the extraction process, the broth was centrifuged at 1500 rpm for 20 min and filtered using Whatman filter paper No.1 to separate the biomass and filtrate. The filtrate was concentrated to 20 ml using rotary evaporator .[23,24,25]

The presence of Lovastatin in the fermentation broth was confirmed at the maximum absorbance by UV spectrophotometry at 238nm. Further, the sample was analyzed using HPLC with a C18 column as a stationary phase and acetonitrile and water (65:35 v/v) as mobile phase.[2]

RESULTS

Biostatistics for Species Diversity

The colonization frequency percentage of the endophytic fungal species were shown in table 4 and the endophytic fungal infection rate percentage of *Phyllanthus reticulatus* was shown in figure 5.

Screening by *Neurospora crassa* Bioassay

The zone of inhibition formed by the endophytic fungi against *Neurospora crassa* is shown in table 5. Among these, the *Curvularia* sp shows higher zones of inhibition up to 0.6cm when compared to the other organisms. Further studies were carried out using the *Curvularia* sp.

Batch fermentation

Fermentation of Curvularia was carried out in both PDB and mushroom extract medium. Every 2 days, biomass was collected, filtered using pre-weighed sterilized filter cloth and dried at 60 ˚C. Maximum OD of 0.826 in mushroom medium and 0.878 in PDB medium were observed at 238nm under UV spectrophotometer. The HPLC results of both the medium show that the peaks are closer enough to the standard lovastatin. It confirms the lovastatin production by *Curvularia* sp. The fermented biomass samples were shown in figure 6 and the HPLC results were shown in figure 7.

DISCUSSION

This study revealed that the endophytic infection rate was high in the stem segment of the selected plant and the *Curvularia* sp were more abundant in the leaves of the selected plant in terms of colonization frequency compared to other endophytic fungi isolated from the same plant.[28,29] This may be due to the environmental suitability and interaction of *Curvularia* sp within the plant. This fungus has also shown a good inhibitory effect against *Neurospora crassa* which is one of the confirmatory tests for the production of lovastatin. Thus, this dominant fungus was chosen for the investigation of lovastatin production in a shaker flask level. The production of lovastatin by *Curvularia* sp in the mushroom extract and PDB medium was confirmed by UV and the HPLC. The R_f value of lovastatin in the mushroom extract was 2.135 which is close to the hydroxyl form of standard lovastatin.[30-32]

CONCLUSION

The research findings of this study conclude that *Curvularia* sp may be used as one of the fungal isolates for the production of lovastatin in mushroom extract medium. This novel method of using these fungi for lovastatin was reported the first time and further studies may be done to enhance productivity.

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Authors contribution

MS - Batch fermentation, recovery of the product, report writing and edition

KPK - Biostatistics for Species Diversity and report writing

KR, HR and GG –Screening, report writing.

Conflict of Interest: Nil

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Table 1: Production of Lovastatin using Biotechnological Approaches

S. No	Fungus	Media components	Culture conditions	Extraction methods and yield	References
1.	*Pleurotus ostreatus*	• $CaCl_2.KH_2PO_4$ • $(NH_4)_2HPO_4$ • $MgSO_4$ • $FeCl_3$ • Malt extract • Glucose	• Culture grown aerobically • 7 days, 150 rpm • Room temperature • Submerged fermentation	• Methylene chloride and Ethyl acetate extraction • GC-MS & HPLC • Yield: 70 mg/l	Alarcon et al. 2003
2.	*Aspergillus terreus*	• 50g Glucose • 20 g Yeast Extract • 30g Tomato Paste • 20g Oat Meal • 10g Sodium Acetate	• 50ml of production medium, • pH of 7,180 rpm • 28°C for 7 days • Submerged fermentation	• EthylAcetate extraction • TLC, HPLC • Yield:55 mg/l	Samiee et al.2003
3.	*Monascus Purpureus* CCRC 31615	• 60 g glucose • 25g peptone • 5 g Corn Steep Liquor • Sodium nitrate • 5 g Ammonium chloride	• 30°C for 14 days,110 rpm • Solid State fermentation	• TLC & HPLC • Monacolin K • Yield:378 mg/kg	Su et al. 2003
4.	*A.terreus* ATCC 20542	• Lactose • Glycerol • Fructose • Yeast extract • Corn steep liquor • Soybean meal	• 28˚C and pH 5.8-6.3 and 150 rpm • Submerged fermentation	Yield: 30 mg/g	Lopez et al. 2003

Table 1: *(Continued)*

S. No	Fungus	Media components	Culture conditions	Extraction methods and yield	References
5.	*A.terreus* (ATCC 20542)	• Lactose • Yeast Extract	• 200rpm,28°c, • pH -5.5 for 10-12 days • 20% Dissolved oxygen • Stirred tank Fermenter	• Methanol • HPLC • Yield:458 mg/L	Lai et al. 2005
6.	*F. nectrioides (MH173849)*	• Glucose • Liquid Cheese Whey • Yeast extract • Histidine • Magnesium sulphate • Calcium chloride • Ferrous Sulphate • Heptahydrate • Potassium Dihydrogen Phosphate	• 28˚C and pH 6 and 180 rpm • Submerged fermentation	Yield: 1.75g100mL$^{-1.}$	Senthamarai and Kannan 2019

Table 2: List of selected endophytic fungi isolated from plant

S. No	Endophytic fungi selected	Segments
1.	*Nigrospora sphaerica*	Root
2.	*Aspergillus sp*	Stem
3.	*Penicillium* Sp	Leaf
4.	*Colletotrichum* sp	Root
5.	*Phyllosticta* sp	Leaf
6.	*Phoma* sp	Root
7.	*Cladosporium sp*	Leaf
8.	*Phyllosticta* sp	Stem
9.	*Curvularia* sp	Leaf
10.	*Fusarium* sp	Leaf

Table 3: Fermentation using two nutrient medium

S.No	Name of the media	Components
1	PDB [M1]	Potatoes infusion - 200 g Dextrose - 20g Agar agar type I -20g Final pH: 5.6 to 6
2	Mushroom extract [M2]	Oyster mushroom extract - 200 g Dextrose - 20g Agar agar type I -20g Final pH: 5.6 to 6

Table 4: Colonization frequency percentage (CF %)

S. no	Species	Colonization frequency in Percentage (CF%)		
		Leaves	Stem	Root
(a). HYPHOMYCETES				
1	*Nigrospora sphaerica*	10	-	-
2	*Aspergillus niger*	6	6	4
3	*Penicillium sp*	2	4	2
4	*Curvularia* sp	24	12	3
5	*Fusarium sp*	8	-	1
6	*Cladosporium sp*	4	4	2
(b). COELOMYCETES				
3	Colletotrichum sp	4	-	-
4	*Phyllosticta* sp	14	-	4
5	*Phoma* sp 2	10	-	-
(d). STERILE FORMS				
13	Sterile form 1	-	1	5
14	Sterile form 2	-	2	-
	Total number of Species	9	4	6
	Total number of isolates	**82**	**26**	**16**

Table 5: Zone of inhibition shown by organisms in cm

S. No	Endophytic fungi	Zone of inhibition (cm)
1.	*Nigrospora sphaerica*	0.2
2.	*Aspergillus sp*	0.3
3.	*Penicillium* Sp	0.1
4.	*Colletotrichum* sp	0
5.	*Phyllosticta* sp	0
6.	*Phoma* sp	0
7.	*Cladosporium sp*	0
8.	*Phyllosticta* sp	0.3
9.	*Curvularia* sp	0.6
10.	*Fusarium* sp	0.2

Figure 1: *Phyllanthus reticulatus*.

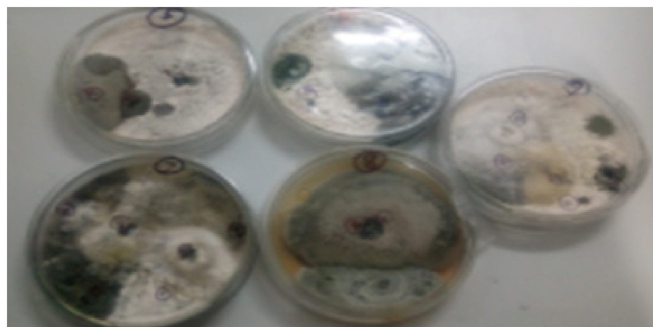

Figure 2: Isolation of Endophytic fungi.

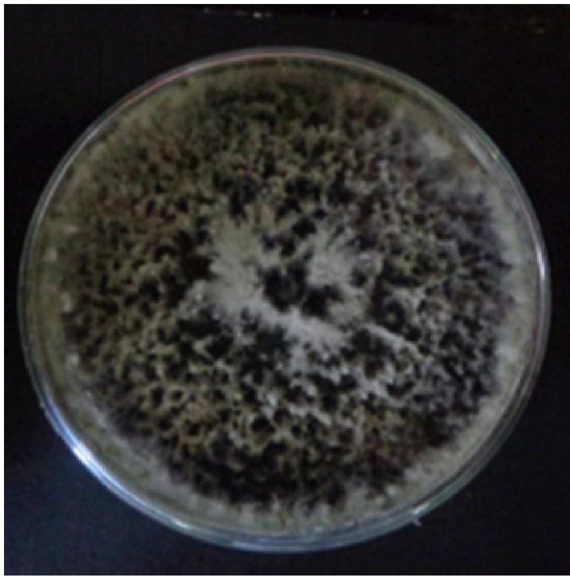

Figure 3: Plate image of Isolated *Curvularia sp*

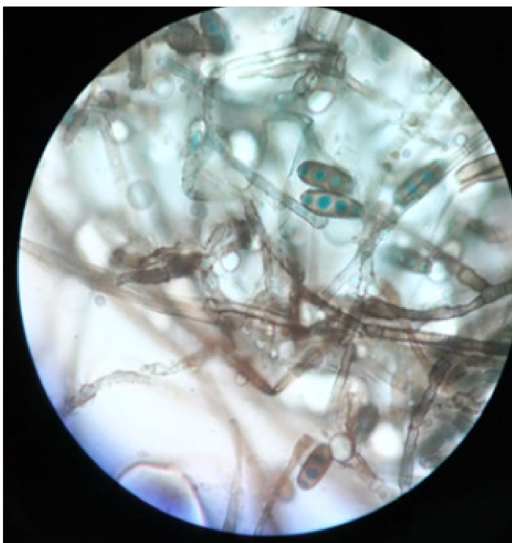

Figure 4: Microscopic image of *Curvularia s*

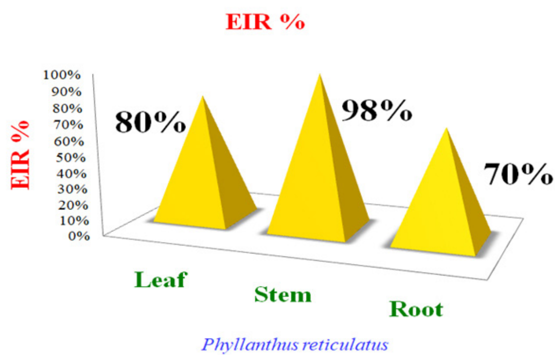

Figure 5: Endophytic infection rate of *Phyllanthus reticulatus.*

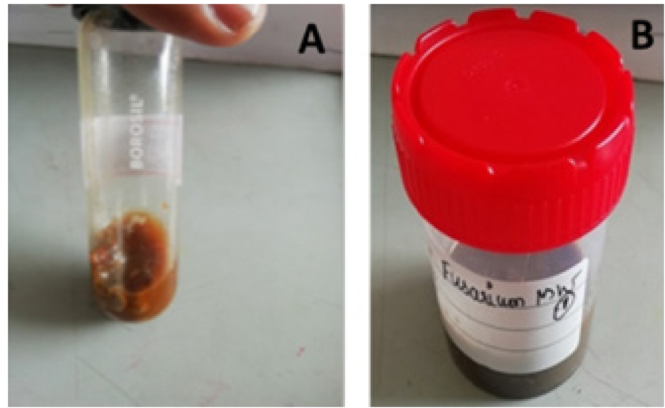

Figure 6: **A.** Biomass and B. Supernatant of fermented broth.

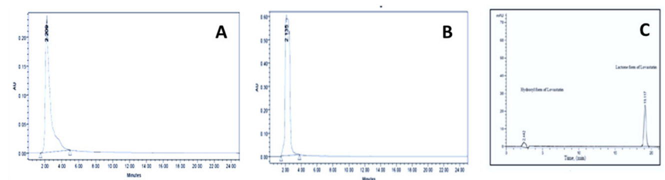

Figure 7: HPLC results of Lovastatin obtained from *Curvularia sp* in PDB and mushroom extract media. A. Lovastatin in PDB [M1]; B. Lovastatin in Mushroom Extract [M2]; C. Standard Lovastatin.