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Functional characterization of extracted lovastatin from oyster mushroom

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Oyster mushrooms, a valuable vegetarian protein source, are typically cultivated on agricultural residues such as wheat straw, rice straw, and maize cob. They are rich in diverse metabolites, contributing to their global recognition. Several countries, notably China, Japan, and India, cultivate 10-15 species of oyster mushrooms on both large and small scales for human consumption, with *Pleurotusostreatus* being particularly favored in India. India has the potential to emerge as a leading producer of pharmaceutical and nutraceutical compounds derived from these mushrooms on the global market. In our study, we conducted functional characterization using a growth inhibition bioassay. Lovastatin extracted from the mushrooms was quantified using thin-layer chromatography and colorimetry, comparing it with standard cultures. The presence of lovastatin was confirmed by thin-layer chromatography (TLC) with an Rf value of 0.88-0.93. A growth inhibition bioassay using a concentration of 0.400 µg/ml revealed that *P. ostreatus* exhibited the highest growth inhibition, followed by *P. sajor-caju*. Subsequently, the extracted lovastatin was used for expression studies targeting sterol biosynthesis genes, specifically ERG3, ERG4, ERG5, PMAIp, and UPC2. The findings indicate a negative regulatory effect on the sterol biosynthesis pathway. The present study points to the potential of oyster mushrooms not only as a nutritional resource but also as a source of bioactive compounds with pharmaceutical and nutraceutical applications, particularly in regulating sterol biosynthesis pathways.

Keywords : Ergosterol, lovastatin,*Pleurotus ostreatus*,sterol biosynthetic genes

INTRODUCTION

Oyster Mushrooms (*Pleurotus* spp.) are a good source of fiber and soluble protein, which are crucial for maintaining good health. Being easily digested (70–90%), mushroom protein is considered superior to vegetable protein. Lovastatin is thought to be an important component in lowering cholesterol, which was made by *Aspergillus terreus* mold fermentation. (Tobert *et al*., 2003). Statins are utilized for their anti-inflammatory and antibacterial properties in addition to their effects on cholesterol (Sun *et al*., 2009).

The ability to use agro-industrial waste products for sustainable bioproduction and the potential to use particular or altered microorganisms to produce new statins are two benefits of statin production by microbes. Statins also have additional biological effects, such as regression of fatty streak lesions or microvascular thelial protection function (inhibiting the early stage of diabetic microangiopathy in the context of hyperglycemia) (Arikan*et al*. 2012).

Lovastatin, a member of statin which is naturally found in various fungi that inhibit HMG-CoA reductase, decreases the immediate synthesis of mevalonate and ergosterol biosynthesis (Fig.1.). Ergosterol is central to a diverse range of cellular functions, including membrane fluidity and transportation, lipid raft formation, steroidal hormone synthesis, and oxysterol and vitamin D synthesis. However, there are still challenges to overcome such as toxicity, antifungal drug resistance, and the emergence of cross-resistance between agricultural and medical statin. This progress with the development of the

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statin drugs has partially been driven by the need for novel antifungals and anti-cholesterol in the medicine and agriculture. Expression of the genes are regulated at different stages, including: transcription, post-transcriptional modification, translation, and post-translation modification. In the present study attempts have been made for functional characterization of lovastatin extracted from oyster mushroom.

MATERIALS AND METHODS

Extraction of statin from Pleurotus species

Oyster mushroom species namely *P. florida*, *P. ostreatus* and *P. sajor-caju*, were collected from Mushroom Research Laboratory, Department of Plant Pathology, IGKV, Raipur, Chhattisgarh, India and evaluated in this research. Cultures were maintained on Potato dextrose agar (PDA) media. The lovastatin extraction process was based on previous work with some modification (Kinjal *et al*. 2023). The fruiting body collected from three different species of oyster mushrooms were shade dried, and fine powder was prepared with the help of a mixture grinder. For extraction of statin 5 ml 0.05M HCl was used for 1 gm mushroom powder in each sample, which was further kept into shaker at 180 rpm for 1 hour. Filtrate was collected by Whatman no.1 filter paper, thereafter 5 ml Dichloromethane and 4ml ethyl acetate were added and again shaken at 180rpm for 1hour. Sodium Sulphate (Na_2SO_4) was added to obtain pure form of aqueous solution. The resultant extract was used in analytical experiments.

Determination of lovastatin from the sample

Extracted lovastatin was identified by TLC performed on Silica gel with Benzene:acetone (70:30 by volume) as the mobile phase. Lovastatin upto ~10mg was extracted from *Pleurotus* species and it was dissolved in 10 ml of ethyl acetate. For the preparation of standard solution, 10 µl of standard lovastatin and equal concentration of extracted lovastatin were spotted onto silica plates. Plates were developed 3 times with mobile phase and spots were made visible using iodine crystal. Colorimetric analysis was done by following the procedure of El-din *et al*. (2010) with minor modification. Extracted statin was analyzed by preparation of the alkaline hydroxylamine reagent by adding equivalent amounts of sodium hydroxide and 12.5% hydroxylamine hydrochloride separately and mixing it. To each test tube, 1ml of the alkaline hydroxylamine reagent was added,after a minimum one-minute sufficient amount of 2.5N HCl and 5 ml of ferric perchlorate was added. The volume was increased using spectroscopic ethanol. The product with the purple-red color is measured at 513 nm.

Fungal growth inhibition bioassay

Fungal growth inhibition bioassay performed by preparing the 25 ml of PDA medium each Petri plate after solidifying poured standard statin and extracted statin from *P. ostreatus*, *P. florida* on the surface of PDA media. Sliced fungal culture was inoculated in a 25 cm diameter of Petri dish. Same experiment was repeated thrice up to 200 mcg, 400 mcg, 600 mcg and 800 mcg with standard and 400 mcg/ml of extracted statin was used to check their growth inhibition efficiency. Gene expression analysis of five selected ERG3, ERG4, ERG5, PMAIP, and UPC2 genes which are involved in ergosterol biosynthesis metabolism was carried out to understand the response of genes In these experiments, preferred medium was Potato Dextrose broth medium to analyze the performance of the genes at 400ug/ ml of concentration at mycelial stage which has been conducted at *in-vitro* condition.

RESULTS AND DISCUSSION

Biochemical analysis of lovastatin by TLC and colorimeter

Clear brown spots were observed on TLC sheet. The Rf values of lovastatin standard and test samples were calculated and presented in Table 1.

The determination of lovastatin content from different species of *Pleurotus-P. florida, P. ostrestus* and *P. sajorcaju* under different culture conditions like solid state fermentation was carried out by determining the UV spectra of lovastatin standard and differentethyl acetate extracted from the fermentation broth in 75%

Table 1 : Rf value of standard lovastatin and extracted different samples

Development stage	*P. florida*	*P. ostreatus*	*P. sajorcaju*
Mycelium	0.91	0.93	0.91
Fruiting body	0.87	0.88	0.87

Table 2 : Data representation of spectrophotometric analysis of three different *Pleurotus* species

Name of species	Mean (mg)	SD	SE	RSD
P. florida	0.552±0.02	0.012	0.012	1.983
P. ostreatus	0.463±0.04	0.040	0.023	4.347
P. sajorcaju	0.469±0.02	0.029	0.017	3.166 3.165931
	1.485	0.030641	0.018024	
			Mean	1.485±0.03
			N	3
			SD	16.6
			SE	11.7
			RSD	3.69

ethanol of *Pleurotus* species. The concentration of lovastatin was calculated spectro photometrically (two replication). The sample and the standard exhibited a peak at 513 nm in the spectrophotometer absorption (Table 2). From the results, it was deduced that three *Pleurotus* species and their stages showed a positive result for lovastatin production.Results confirmed that among the fruiting bodies of *P. florida* (0.552mg/g), *P. ostreatus* (0.463 mg/g) and *P. sajorcaju* (0.469 mg/g), *P. florida* showed the maximum amount of lovastatin in (Table2). Chen *et al.,* (2012) reported that the fruiting bodies of *P.ostreatus* from Japan, Korea, and Taiwan gave 606.5,165.3 and 216.4 mg/g lovastatin yield, respectively.

Fungal growth inhibition bioassay

The effect of lovastatin on the growth of *Pleurotus*species was evaluated using PDA medium, where statin concentrations ranged from 0 to 8 mg/ml. The results, showing growth under different statin concentrations in media, are presented in Fig. 2. In PDA media, *P. florida, P. ostreatus,* and *P. sajor-caju* isolates were strongly inhibited by a concentration of 100 mM lovastatin. *Pleurotus* species showed low sensitivity to 0.8 mg/ml lovastatin, while *P. ostreatus* showed sensitivity to 0.4 mg/ml lovastatin. As we increased the concentration of lovastatin, the growth of *Pleurotus* species stopped, but 0.2 mg/ml lovastatin appeared to be effective in promoting growth in *Pleurotus* species. Inhibition appeared to be uniform within *Pleurotus* species, as three isolates of *Pleurotus* species displayed the lovastatin sensitivity profile of *P. ostreatus.* The effects of statins on the growth of *Pleurotus* species were also examined on solid media. In the absence of statins, *Pleurotus* species displayed robust growth with production of mycelia after 4 days at room temperature. In the presence of statins, growth was sequentially substantial down at mg/ml, 10mg/mlless growth was observed (Fig.3), while lovastatin at 0.2 mg caused substantial growth induces. Although *Pleurotus* species are known to produce lovastatin as a secondary metabolite to ward off

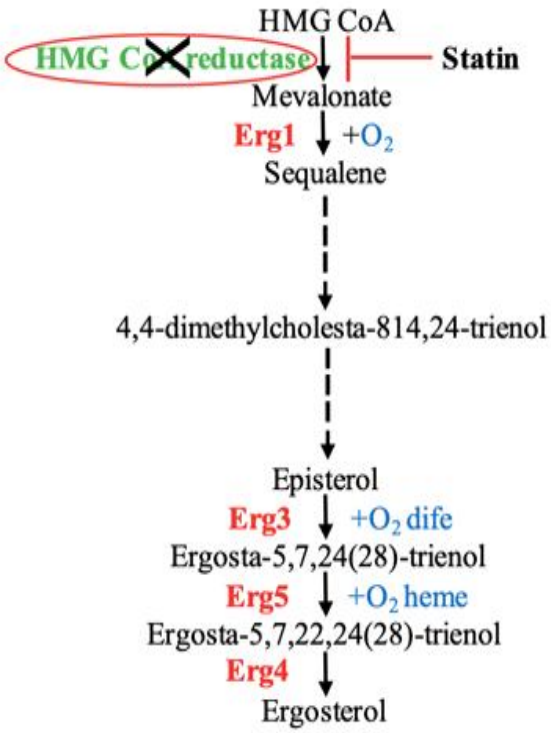

Fig.1: Ergosterol biosynthetic pathway in S. cerevisiae. Statins inhibit 3-hydroxy-3-methyl glutaryl (HMG)-CoA reductase to prevent the synthesis of mevalonate. The red color indicates downregulation Erg1, squalene epoxidase; Erg3, sterol C-5 desaturase; Erg5, sterol C-22 desaturase; Erg4, sterol C-24 reductase.

competition in their environment, high concentrations or cause a reduction in mycelial biomass

Expression of sterol pathway related genes

Several genes for expression studies were selected based on prior research. These include three key genes (ERG3, ERG4, and ERG5) directly involved in the sterol biosynthesis pathway, the transcription factor gene UPC2, and the PmalP gene, which maintains membrane fluidity. Statins are biochemical compounds that regulate ergosterol synthesis under adverse conditions such as abiotic and biotic stress. Based on these findings, we chose these five genes, along with the Actin gene, for expression analysis. When examining treated mycelia of *P. florida*, significant differences were observed for all genes compared to *P. ostreatus* and *P. sajorcaju*, as shown in Fig.4. These differences were observed across all growth stages of the oyster mushroom, including pinhead, mycelial, and fruiting body stages. Notably, the ERG5 and UPC2 genes exhibited high expression levels in the fruiting body stage, with the pinhead stage showing intermediate results. The results are given below: ERG5 and UPC2 genes showed particularly high expression levels in the fruiting body stage. Intermediate expression levels were

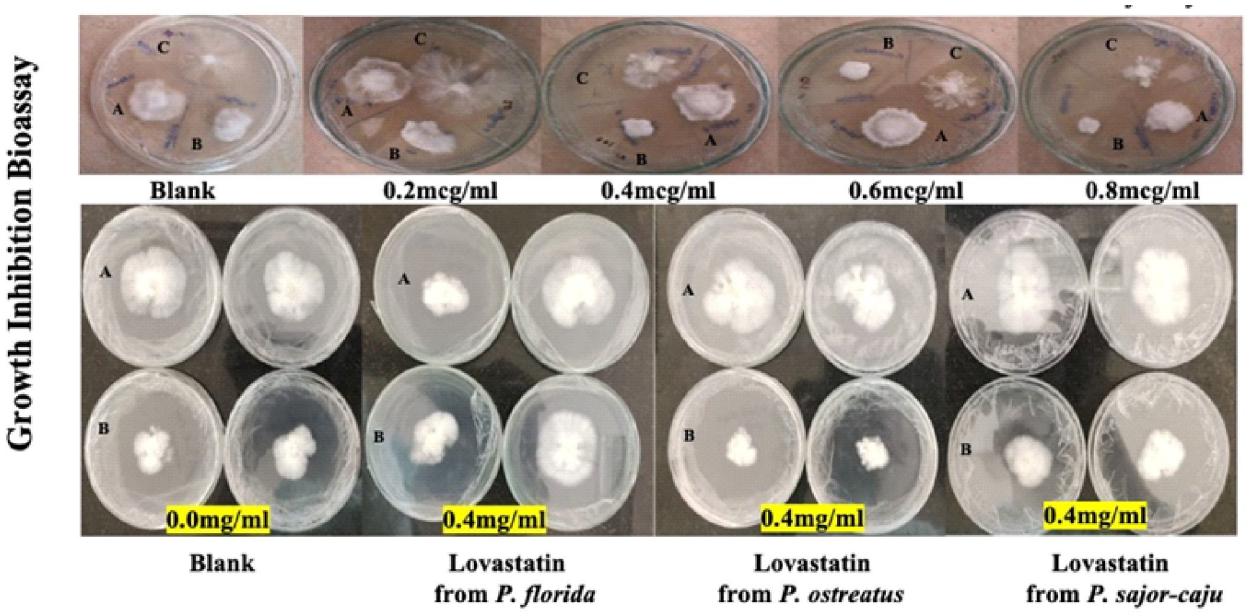

Fig 2: Mycelium Growth inhibition bioassay at various concentration of standard lovastatin and extracted lovastatin against*Pleurotus*species A. *P. florida*, B. *P. ostreatus* and *P. sajor-caju*

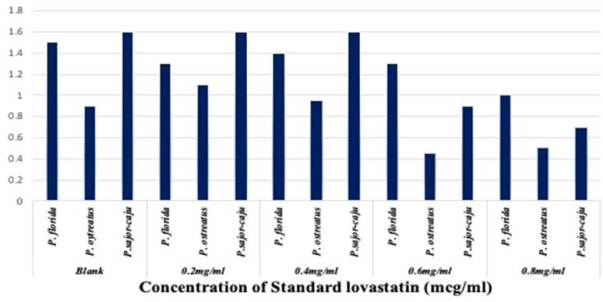

Fig 3 : Growth inhibition assay of lovastatin concentration of different *Pleurotus* species. Data represent mycelial growth in cm

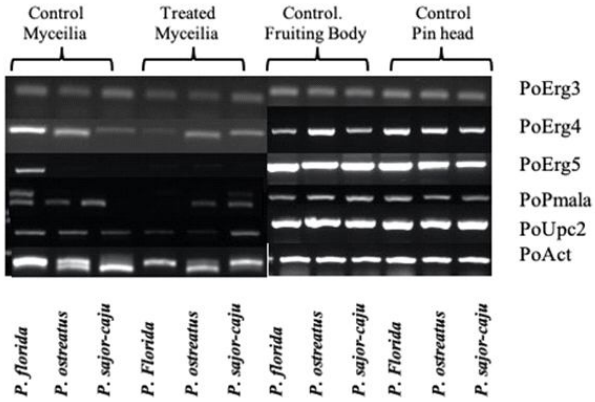

Fig 4: Gene expression analysis of different *Pleurotus* mushroom

observed in the pinhead stage. This study underscores the dynamic regulation of gene expression throughout the developmental stages of oyster mushrooms, influenced by external conditions and genetic factors.

Ergosterol is an important component of cell membrane which is regulate the membrane transportation, fluidity resulting prevention of biotic and abiotic stress it also includes in hormones and vitamins synthesis. Regulation of sterol synthesis is important to avoid accumulation of free sterols which may become irreversible effect on the cell. Such regulation of sterol levels is mainly achieved by feedback inhibition at transcriptional levels (Hughes, 2007). Lovastatin plays critical role in the accumulation of ergosterol in fungal cell membrane to inhibit the function of HMG CoA reductase and ultimately reduce the ergosterol in mevalonate biosynthesis pathway, but some other pathway is also available for synthesis of mevalonate from TCA cycle (Guerra *et al*. 2021). Upc2 belongs to the family of fungal Zn(2)-Cys(6) binuclear cluster transcription factors which is highly conserved in Ascomycetes and Basidomycetes but not found homology to the analogous mammalian sterol regulatory element binding proteins (SREBPs), that regulate cholesterol biosynthesis. *UPC2 is a* sterol regulatory gene, is upregulating ERG3 in the ergosterol biosynthetic pathway in response to ergosterol starvation (Vik*et al*. 2021). Deletion of *UPC2* has been associated with reduction of uptake of sterol across the plasma membrane (Crowley *et al.* 1998). UPC2, is a sterol regulatory gene or transcription factor, to upregulate ERG3 in the ergosterol biosynthetic pathway in response to ergosterol starvation. Mutation in *UPC2* gene showed that reduces the amount of ergosterol synthesized, even though cells are still able to synthesize the final product ergosterol because, the optimal set of sterols in the plasma membrane determines its selective permeability and contributes to the normal functioning of membrane proteins. UPC2 contributes to transcriptional upregulation of PMA1(gene encoding plasma membrane ATPase) under sterol depletion and azole stress.Further researchers observed that increased expression of PMA1 in stressed cells, in lipid rafts to maintain membrane homeostasis (Hervay*et al*. 2024).

Cholesterol-lowering activity was previously reported the in *Auricularia polytricha*, *Agaricus bisporus* and *Lentinus edodes*. In addition, Berger *et al*. (2004) observed *in vitro* and *in vivo* anti-hypercholesterolemic properties in *Pleurotus* species, such as *P. ostreatus* and *P. ferulae*. Recently, extracted lovastatin from *P. florida* demonstrated that reduced the total glycerides and cholesterol in rats fed with high cholesterol diets (Fombang *et al*. 2016). Statins bind directly to the HMGR active site and are competitive inhibitors of the enzyme with respect to HMG-CoA (Endo *et al*., 1976; 2017). All statins have structural similarity to the 3-hydroxy-3-methyl glutarate moiety of HMG-CoA and occupy the HMG-CoA binding pocket of HMGR (Istvan 2002).

CONCLUSION

Pleurotus species is an important filamentous fungus, which has been applied in the Pharma industrial production for the bio-based compound and the lipid-lowering drug lovastatin. Different extracts from four edible mushrooms species

were analyzed qualitatively and quantitatively usingthin layer chromatography and spectrophotometer to determine the presence and quantification of lovastatin. Results revealed that *Pleurotus* species produces lovastatin in the present experimental conditions and also reported a higher amoun to flovastatin produced by *P. florida* followed by *P. ostreatus* compared to other *Pleurotus* species.

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DECLARATION

Conflict of interest. Authors declare no conflict of interest.

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