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ISOLATION OF SOIL BORNE FUNGI FOR PRODUCTION OF NATURAL LOVASTATIN IN SUBMERGED AND SOLID STATE FERMENTATION PROCESS

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ABSTRACT

The present research aims to select and analyse lovastatin from fungi, isolated from different soil samples. Lovastatin is competitive inhibitor of cholesterol biosynthesis by blocking 3-hydroxy, 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). Five isolates of the genus *Aspergillus* were isolated from different soil samples and *Aspergillus terreus* is an MTCC 479 strain which has been used as a standard were screened for lovastatin production in submerged fermentation for 11 days. After 11 days of fermentation, all of them showed positive results, when screened through plate assay by determining the zone of inhibition, which revealed by the fungus strain against *S. cerevisiae*. Lovastatin production was further confirmed by thin layer chromatography. It was found by colorimetric estimation that *Aspergillus sp*. (Isolate L3) to be the best producer of lovastatin upto 133.33 mg/L in submerged fermentation where as in solid state fermentation (SSF) using wheat bran as substrate highest lovastatin production by *Aspergillus sp*. (Isolate L3) was found 15.625 mg/g. High Performance liquid chromatography (HPLC) analysis found that produced lovastatin from *Aspergillus sp*. (Isolate L3) has the same retention time with the standard lovastatin i.e., 12.4 minutes. In total, we have isolated and screened 5 (L1 to L5) lovastatin producing cultures, among which Isolate L3 was found to be the best.

Key words: Lovastatin, competitive inhibitor, submerged and solid state fermentation, HPLC analysis

INTRODUCTION

Lovastatin is a hypocholesterolaemia drug approved by the Food and Drug Administration (FDA) in the year 1987 **[1]**. Lovastatin is an effective inhibitor of the enzyme hydroxymethylglutaryl coenzyme A (HMGCoA) reductase that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol and does competitive inhibition **[2]**. Cardiovascular diseases have been associated with elevated cholesterol level in blood **[3]**. Both *in vitro* and *in vivo* studies by various researchers revealed that lovastatin has potent inhibitory activity on cholesterol biosynthesis and plasma cholesterol level **[4]**. These have proven lovastatin to be effective in the therapy of patient in hypercholesterolemia condition. HMG-CoA reductase inhibition found pleiotropic effects on animal and human **[5]**. Inhibition of cellular proliferation and induction of apoptosis and necrosis in several experimental settings including that of breast cancer makes lovastatin a potential anticancer agent **[6]**. Some preclinical studies have suggested lovastatin administration in a nanobead preparation to be possibly therapeutically useful in case of repairing of fractures in humans **[7]**. There are several fungi in nature which are capable of producing lovastatin include *Aspergillus*, *Penicillium*, *Monascus*, *Paecilomyces*, *Trichoderma*, *Scopulariopsis*, *Doratomyces*, *Phoma*, *Pythium*, *Gymnoascus*, *Hypomyces* and *Pleurotus* **[8-9]**. Lovastatin is one among the statin compound commercially derived from *Aspergillus terreus* through fermentation. Lovastatin was produced by liquid surface fermentation (LSF) technique **[10]** earlier but presently submerged fermentation (SmF) techniques **[11]** are employed throughout the world for lovastatin production. Therefore, the present investigation focuses on the isolation of natural high yielding fungal strains from different places of Kalyani, Nadia, West Bengal, India for screening, fermentation, extraction and analysis of different soil fungi in producing lovastatin **[12]**.

MATERIAL AND METHODS

Sampling: Soil samples were collected from different places of Kalyani, Nadia, West Bengal, India for the isolation of lovastatin producers. Samples of soils were taken depths of 10-50 cm, after that packed into labelled sterilized polyethylene bags using a sterilized spatula, and returned to the laboratory for further examination as per described by Szakacs *et al.,* 1998.

Isolation and characterization of fungal isolates from natural samples: Isolation of desired fungal cultures was carried out using potato dextrose agar (PDA) medium containing potatoes 200 g/L (pills of it), dextrose 20g/L, agar 15g/L, by following standard microbial methods described by

Szakacs *et al.,* 1998 **[12]**, such as morphological properties (Colony colour, Shape, size, margins elevation and growth rate) and microscopic properties (conidial head, conidiophores, vesicle and conidia). Characterized and identified fungal cultures were maintained in pure culture form on PDA slants and stored at 4°C **[13]**.

Production of lovastatin by submerged fermentation: Soybean meal medium (g/L) containing Sucrose (50), Soybean meal (20), K_2HPO_4(1), $NaNO_3$(1), $MgSO_4.7H_2O$(0.5), pH 6.5 was used for lovastatin production by the selected fungi. 100 ml of Soyabean meal media in 250 ml Erlenmeyer flask was autoclaved at 121°C for 15 minutes, 3 loopful of isolates were inoculated in cooled autoclaved medium and incubated for 7 days in a rotatory shaker at 100 rpm in 22 ^{O}C temperature **[13]**.

Extraction of lovastatin from screening medium: Screening medium was filtered in order to get the culture filtrate containing lovastatin. pH of the broth was adjusted to 4.5 and then equal volume of ethyl acetate was added to the broth and kept in the rotatory shaker at 100rpm for 2 hours. Using separating funnel broth was separated from ethyl acetate (organic phase) containing lovastatin. Ethyl acetate was allowed for drying. 1ml of ethyl acetate was added to the residue. This residue was stored at 4°C and used as an extract for further analysis **[13]**.

Yeast growth inhibition bioassay: Yeast peptone dextrose agar medium was poured into the sterilized petri plates. After solidification of yeast peptone dextrose agar medium *Saccharomyces cerevisiae* (Bakers yeast) cell suspension (0.1 ml) were spread onto the sterilized medium. Then wells were made using a sterile borer of 18 mm diameter. 100µL of extract was loaded into the well. Ethylacetate was also loaded into one well as a control. Plates were incubated at room temperature for 24 h. After incubation, zone of inhibition was measured as per Kirby-Bauer test method **[13]**.

Lovastatin confirmation by thin layer chromatography: TLC plates were spotted with extracts along with the standard lovastatin. Loaded TLC plates were dipped into solvent system containing toluene and ethanol in the ratio of 80:20. These plates were exposed to - UV. lamp in order to visualize the spots and then Rf values were calculated **[13]**.

Estimation of lovastatin by colorimetric method: Stock solution of lovastatin 4mg/ml (in ethanol), was prepared along with Hydroxylamine hydrochloride 12.5% and sodium hydroxide 12.5% (in methanol). Alkaline hydroxylamine reagent was prepared by mixing hydroxylamine hydrochloride solution and sodium hydroxide solution in equal amount and filtering off the precipitate. Ferric

perchlorate stock solution was prepared by dissolving 0.8g of iron in 10ml of 70% perchloric acid. Working solution of ferric perchlorate was prepared by mixing 40ml of stock solution of ferric perchlorate and 12ml of 70% perchloric acid and the volume made up to 100ml with ethanol. In order to estimate the amount of lovastatin, 10µl of different extract was taken in a test tube, 1ml of alkaline hydroxylamine reagent was added and mixed. pH of 1.2 was maintained with the help of 2M HCl into which 5ml of ferric perchlorate reagent solution was added. The volume was made up 8 ml with ethanol. The purple red colour product was measured at 510 nm after 25 minutes **[14]**.

Lovastatin samples were analyzed using high performance liquid chromatography (HPLC). The concentrated lovastatin was diluted at a 1:1 ratio with acetonitrile and passed through a filtration assembly (0.45 µm) The samples (20 µL) were injected into the HPLC apparatus (Shimadzu LC10AT) by syringe and analyzed with a UV detector at 238 nm and Shim pack CLS-ODS (C18) column at a flow rate of 1mL/min. The mobile phase consisted of 0.1 % phosphoric acid and acetonitrile with a volume ratio of 40:60.

RESULTS AND DISCUSSION

In the present study, total 5 isolates (L1 to L5) were screened for the production of lovastatin along with the standard strains *Aspergillus terreus* MTCC 479. All fungi tested showed clear zones around the wells on bioassay. The clear zone sizes varied with a range between 1.1 to 12.2 mm. The smallest halo of 1.1 mm was exhibited by isolate L1 and highest of 12.2mm by isolate L3 **(Table 1)**.

Detection of clear zone by agar diffusion method is considered as one of the qualitative assays for lovastatin. The different clear zones indicate variations among physiological characteristics and genetic potential of the specimens **(Figure 1)**. Other influencing factor may be the ability of diffusion of lovastatin in agar and also on microbial assay method and the incubation period given for the experiment **[15]**. The fungal isolates were characterized by using standard microbiological methods which includes morphological properties such as colony colour, shape, size, margins elevation and growth rate and microscopic properties such as conidial head, conidiophores, vesicle and conidia. Characterized and identified fungal cultures were maintained in pure culture form on PDA slants and stored at 4°C. The isolates were further compared for morphological and pigmentation properties with *Aspergillus terreus* (MTCC-479) used as standard.

Lovastatin in nature is found in the form of β-hydroxy acid and lactone. The active form of lovastatin is β-hydroxy acid **[16]**. It is

suggested that the β-hydroxy acid form of lovastatin in ethyl acetate extract is having antifungal nature. Like other living beings, the cell membrane of *S. cerevisiae* has made up of lipid bilayers. Due to consist of sterol in cell wall, it is a target of antifungal activity, despite of the enzyme that is involved in the cell wall synthesis. It has been suggested that inhibition of yeast growth could be manifestation due to the contact of myosin directly with the sterol in the cell membrane, which may cause leakage of cell membrane and then loss of intracellular component. Another possibility is myosin may inhibit protein synthesis by blocking the RNA. Other mode of inhibition by myosin is on ergosterol biosynthesis which may result increase of membrane permeability and ultimately membrane damage **[17]**. At the end of 11 days of fermentation in wheat bran media isolate L3 produced the highest level of lovastatin (133.33 mg/L) by Smf and (15.625 mg/g) by SSF **(Table 2)**.

Of the 5 fungal species of 14 genera isolated from Kalyani soil samples **[9]**, nearly one-third (32%) of the strains were positive for lovastatin production. Several researchers mentioned that lovastatin production fungi are *Aspergillus oryzae*, *A.terreus*, *Doratomyces stemonitis*, *Paecilomyces variotii*, *Penicillium citrinum*, *Penicillium chrysogenum*, *Scopolariopsis brevicaulis* and *Trichoderma viride*, *Aspergillus terreus* as the best lovastatin producer (84 mg/l)[9]. *Penicillium citrinum*, *Paecilomyces variotii* and *Penicillium chrysogenum* were found to produce 61 mg/l, 56 mg/l and 35 mg/l of lovastatin, respectively **[9]**.

Although many fungal genera like *Penicillium*, *Monascus*, *Trichoderma*, *Pleurotus*, *Doratomyces*have been reported to produce lovastatin; *Aspergillus* is the most commonly used isolate for its robust nature **[18]**.

Jaivel and Marimuthu tested 10 fungal strains of seven species of five genera (isolated from natural samples) for lovastatin production by submerged fermentation process using glucose as carbon source and reported *Aspergillus terreus* (JPM3) as a best isolate producing 138.4 mg/l of lovastatin, was almost similar yielding as compared to *Aspergillus terreus* (76) reported in present study **[19]**. Sree Devi *et al*. showed various strains of *Aspergillus terreus* form soil samples, screened for lovastatin production by agar plug method and reported *Aspergillus terreus* (KSVL-SUCP-75) as highest among isolated strains yielding (360 µg/ml) in the study **[20]**. Mangunwardoyo *et al.* screened 40 selected fungal cultures from University of Indonesia culture collection (UICC) by using paper disc method, SmF process and reported *Aspergillus flavus* UICC 360 highest (85.8 mg/l) among the cultures

screened, which had shown 1.6 fold lower yield than the present reported fungal isolate *Aspergillust erreus* (L3) [**21**].

Identification of lovastatin from the highest producing isolate L3 was performed through High performance liquid chromatographic analysis. Retention time of standard lovastatin and sample was 12.42 min. and 12.443 min. respectively **(Figure 2)**.

The results confirmed that the ethyl acetate extract from *Aspergillus* sp. L3 was lovastatin. The other peaks in the sample might be due to the presence of impurities or other unidentified compounds of the sample. There different options for HPLC determination of lovastatin in fermentation broth or its extract include, (determination of the compound in open hydroxy acid form after adjustment of the pH to 7.7 **[22]** determination of lovastatin in both open hydroxy acid and lactone forms existing simultaneously **[23]**. It is possible to determine lovastatin in both forms, although when the acidified broth is extracted with ethyl acetate it exists mainly in lactone form with retention times of 12-13 minutes **[18]**. β-hydroxyacid form elutes earlier in the chromatographic column with retention time of 3.83 mins **[22]**.

Table 1: Lovastatin screening of different fungal isolates based on bioassay and TLC

Isolates	Zone of clearance (mm) by bioassay method	Rf values based on TLC (Standard Lovastatin- R_f=0.70)
L1	1.1	0.70
L2	1.9	0.77
L3	12.2	0.72
L4	10.3	0.73
L5	11.1	0.77
A.terreus MTCC 479	2.0	0.78

Figure 1: Inhibition of growth of *S.cerevisae* by lovastatin produced by isolate L3

Table 2: Yield of lovastatin produced by different fungal strainson Smf and SSF cultivation

Isolate	SmF yield(mg/l)	SSF yield(mg/g)
L1	62.5	7.29
L2	95.83	14.58
L3	133.33	15.625
L4	54.16	4.166
L5	54.16	8.33
A.terreus MTCC 479	54.16	4.166

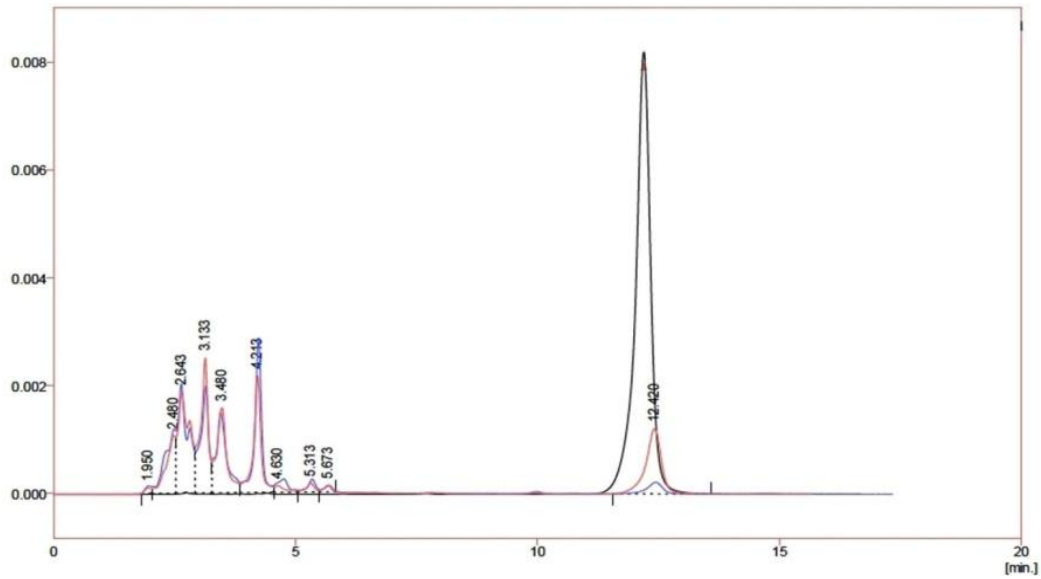

Figure 2: HPLC chromatogram of Samples and standard lovastatin (Red indicates Standard and Blue indicates sample)

CONCLUSION

The resultsconclude that the fungal isolate L3 produced comparable levels of lovastatin in both SmF and SSF conditions during screening. Further, the screening medium may be optimized to support optimal growth and production of the isolate to get a better idea of product ability of lovastatin by the screened isolates.

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