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Bioprospecting of Lovastatin Producing Fungi Isolated from Soil Samples

Prakash Chaynika and Shivakumar Srividya*
Department of Microbiology, Centre for PG studies, 18/3, 9th Main, Jayanagar 3rd Block, Jain university, Bangalore-560011, INDIA

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Abstract

The work attempts to select and analyse lovastatin from isolated molds of different soil fungi. Lovastatin an inhibitor of 3-hydroxy, 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) enzyme, is a competitive inhibitor of cholesterol biosynthesis. Of the 15 isolates of genus Aspergillus isolated from different soil samples, Aspergillus terreus MTCC 479 strains screened for lovastatin production in submerged fermentation after 7 days of fermentation, all of them showed positive when screened through bioassay method by the zone of inhibition exhibited by the fungus against S.cerevisae. Lovastatin production was further confirmed through the laboratory analytical technique TLC. Colorimetric estimation identified Aspergillus sp. no.76 to be the best producer of lovastatin with a level of 137.5 mg/L. by submerged fermentation. SSF using wheat bran also supported highest lovastatin production by Aspergillus sp. no.76 (18.75 mg/g). High Performance chromatography (HPLC) analysis confirmed that lovastatin from Aspergillus sp. no. 76 has the same retention time with the standard (12.4 minutes).

Key words, Bioassay, fungi, lovastatin, submerged and solid state fermentation, , TLC, HPLC analysis,

Introduction

Lovastatin (mevinolin), the first hypocholesterolemic drug was approved in 1987 by Food and Drug administration (FDA), USA[1]. Lovastatin is an effective competitive inhibitor of the enzyme hydroxymethylglutaryl coenzyme A (HMGCoA) reductase (mevalonate, NADP1 oxidoreductase, EC 1.1.1.34) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol[2]. Increased cholesterol levels have known to be associated with cardiovascular diseases (CVD)[3]. Potent inhibitory activity on cholesterol biosynthesis and plasma cholesterol level by lovastatin in humans and animals as shown by both *in vitro* and *in vivo* studies [4], have proven lovastatin to be effective in the therapy of hypercholesterolemia. HMG-CoA reductase inhibition has beneficial pleiotropic effects[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 hastening repair of human fractures[7]. Lovastatin, produced by microbial fermentation, is a precursor for simvastatin, a powerful semi-synthetic statin commercially available as Zocor TM, which otherwise is obtained *via* a selective enzymatic deacylation of lovastatin.

Fungi capable of producing lovastatin include *Aspergillus*, *Penicillium*, *Monascus*, *Paecilomyces*, *Trichoderma*, *Scopolariopsis*, *Doratomyces*, *Phoma*, *Phythium*, *Gymnoascus*, *Hypomyces* and *Pleurotus*[8,9].

Lovastatin is one among the statin compound commercially derived from *Aspergillus terreus* through fermentation. Though lovastatin was produced by liquid surface fermentation (LSF) technique earlier[10] currently submerged fermentation (SmF) techniques[11] are employed throughout the world. Extremely low yield, extensive downstreaming processing and the consequent high capital and operating cost of lovastatin production has led to several attempts based on solid state fermentation (SSF) in an attempt to minimize the production expenses[12].

Therefore, in this present investigation, the focus was on isolation of natural high yielding fungal strains from different hitherto un-attempted sources from India *viz.,* Karnataka, Kerala, Tamilnadu and Jharkhand. Further we describe the screening, fermentation, extraction and analysis of different soil fungi in producting lovastatin. Bioprospecting selected microorganisms from different samples will contribute in identifying a potential strain for industrial production of lovastatin.

Material and Methods

Sampling: Soil samples were collected from different places of Karnataka, Kerala, Tamilnadu and Jharkhand for the isolation of lovastatin producers.

Isolation and characterization of fungal isolates from natural samples: Natural samples were collected from different regions of Bangalore and nearby regions in Karnataka and Tamil Nadu, India. Isolation of desired fungal cultures was carried out using potato dextrose agar (PDA) medium containing potatoes 200g/l, dextrose 20g/l, agar 15g/l, by

following standard microbial methods 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].

Isolation and characterisation of soil fungal isolates: Potato dextrose agar media was used for the isolation of fungus. Genus of the isolates were confirmed by the microscopic observation using lacto phenol cotton blue stain.

Production of lovastatin by submerged fermentation: Soyabean meal medium (g/l) containing Sucrose (50), Soyabean 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 100rpm[13].

Extraction of lovastatin from screening medium: Screening medium was filtered using filter paper in order to get the culture filterate containing lovastatin. pH of the broth was adjusted to 2 then equal volume of ethylacetate was added in the broth and it was kept in the rotatory shaker at 100rpm for 2 hours. Using separating funnel broth was separated from ethylacetate(organic phase) containing lovastatin. Ethylacetate was allowed for drying. 1ml of ethylacetate was added in the residue. This residue was used as an extract for further analysis[13].

Yeast growth inhibition bioassay: Yeast peptone dextrose agar medium was poured into the sterilized petriplates. 0.1ml of After solidification of yeast peptone dextrose agar medium *Saccharomyces cerevisiae* (Bakers yeast) cell suspension were spreaded onto the sterilized medium wells were made using a sterile borer of 18mm 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 24hours. After incubation zone of inhibition was measured[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 hand U.V. lamp in order to visualize the spots. Rf values were calculated[13].

Estimation of lovastatin by colorimetric method, Stock solution of lovastatin 4mg/ml (in ethanol), Hydroxylamine hydrochloride 12.5% and sodium hydroxide 12.5% (in methanol) were prepared. Alkalinehydroxylamine 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 10mlof 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 upto 100ml with ethanol.

In order to estimate the amount of lovastatin, 10µl of different extract was taken in a test tube, 1ml of alkalinehydroxylamine 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 with ethanol. The purple red colour product was measured at 510nm after 25minutes[14].

Results and Discussion

In the present study, total 15 isolates were screened for the production of lovastatin along with the standard strains *Aspergillus terreus* MTCC 479. All fungi tested (100%) showed clear zones around the wells on bioassay. The clear zone sizes varied with a range between 1.3 to 12.2 mm. The smallest halo of 1.3 mm was exhibited by isolate no.57 and highest of 12.2mm by isolate 76 (table 1, figure-1). Detection of clear zone by bioassay method is considered to be a qualitative assay for lovastatin. The different clear zones were produced due to the variations in physiology and genetic characteristics of the specimens. Other influencing factor could be the ability of the lovastatin to diffuse throught the thickness of the agar, total microbial assay and the incubation period[15].

Figure-1
Inhibition of growth of *S.cerevisae* by lovastatin produced by isolate 76

The fungal isolates were characterized by using standard microbiological methods 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. The isolates were further compared for

morphological and pigmentation properties with *Aspergillus terreus* (MTCC-479) used as standard.

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)
12	6.5	0.54
11	5.6	0.77
76	12.2	0.7
18	10.3	0.7
64	5.6	0.77
A.terreus MTCC 479	2	0.54
69	3.3	0.54
72	5.6	0.77
48	2.5	0.4
17	4.6	0.72
13	1.3	0.63
68	1.3	0.72
57	1.3	0.66
4	1.6	0.66
16	1.6	0.54

Lovastatin in nature is found in the form of β-hydroxy-acid and lactone. The first one is an active form[16]. The β-hydroxy-acid form of lovastatin in ethylacetate extract is an antifungal. The cell membrane of *S.cerevisiae* has lipid bilayers. The compostion of the cell wall consists of sterol which is a target of antifungal activity despite of the enzyme that is involved in the cell wall synthesis. The mechanism of antiyeast inhibition could be attributed to, i. mycosin contact directly with the sterol in the cell membrane causing leak of cell membrane and the loss of intracellular component, ii. mycosin attached with RNA and inhibition of protein synthesis iii. mycosin inhibited ergosterol synthesis causing increase of membrane permeability and membrane damage[17].

Isolates showing zone clearance size above 1.6 mm by bioassay method were subjected to submerged and solid state fermentation for lovastatin production.

The Rf values of the fungal extracts obtained in TLC (using toluene and ethanol in the ratio of 80,20) were also found to be similar to that of the standard drug (0.7) (table 2). TLC confirmation was followed by further estimation of lovastatin at 238 nm. Both sample and the standard exhibited λmax at 238nm.

Quantitative analysis of lovastatin production by the different selected fungal isolates ranged between 16.66 mg/L to 133.33 mg/L by Smf and between 4.166 to 15.625 mg/g by SSF using wheat bran, respectively, at the end of 11 days of fermentation. *Aspergillus* sp. no. 76 produced the highest level of lovastatin (133.33 mg/L) by Smf and (15.625 mg/g) by SSF.

Table-2
Yield of lovastatin produced by different fungal strains on Smf and SSF cultivation

Isolate No.	SmF yield(mg/l)	SSF yield(mg/g)
76	133.33	15.625
18	95.83	14.58
12	62.5	7.29
72	54.16	4.166
11	54.16	8.33
64	54.16	4.166
17	41.66	5.20
69	37.5	11.45
48	29.16	13.54
479	16.66	8.33

Of the 25 fungal species of 14 genera isolated from Egyptian soil as well as compost samples[9], nearly one-third (32%) of the strains were positive for lovastatin production. Lovastatin production was reported with *Aspergillus oryzae*, *A.terreus*, *Doratomyces stemonitis*, *Paecilomyces varioti*, *Penicillium citrinum*, *Penicillium chrysogenum*, *Scopolariopsis brevicaulis* and *Trichoderma viridae*, with *Aspergillus terreus* as the best lovastatin producer (84 mg/l)[9]. *Penicillium citrinum*, *Paecilomyces varioti* 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*, *Do- ratomyces* have been reported to produce lovastatin; *Aspergillus* is the most commonly used isolate for its robust nature[18]. Screening of 110 fungal strains of nine species of four genera from Persian type culture collection (PTCC) including some selected strains from various screening projects by two stage fermentation process reported *Aspergillus terreus* as the best isolate, which yielded 2.5-fold less (55 mg/l) lovastatin than that obtained in the present study.

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. isolated various starins 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 *Aspergillus terreus* (SSM 4) [21].

Identification of lovastatin from the highest producing isolate no. 76 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 ethylacetate extract from *Aspergillus* sp. no. 76 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, 1) determination of the compound in open hydroxy acid form after adjustment of the pH to 7.7[22]; 2) 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 ethylacetate 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].

Conclusion

Considering these references as discussed above, it could be concluded that the fungal isolate no.76 selected in this study produced comparable levels of lovastatin in both SmF and SSF conditions during screening. It can be also seen that the present investigation led to explore the hitherto under-utilized natural sources for fungal isolation. Further, the screening medium may be optimized to support optimal growth and production of the isolate to get a better idea on potentiality and the level of lovastatin production by this isolate.

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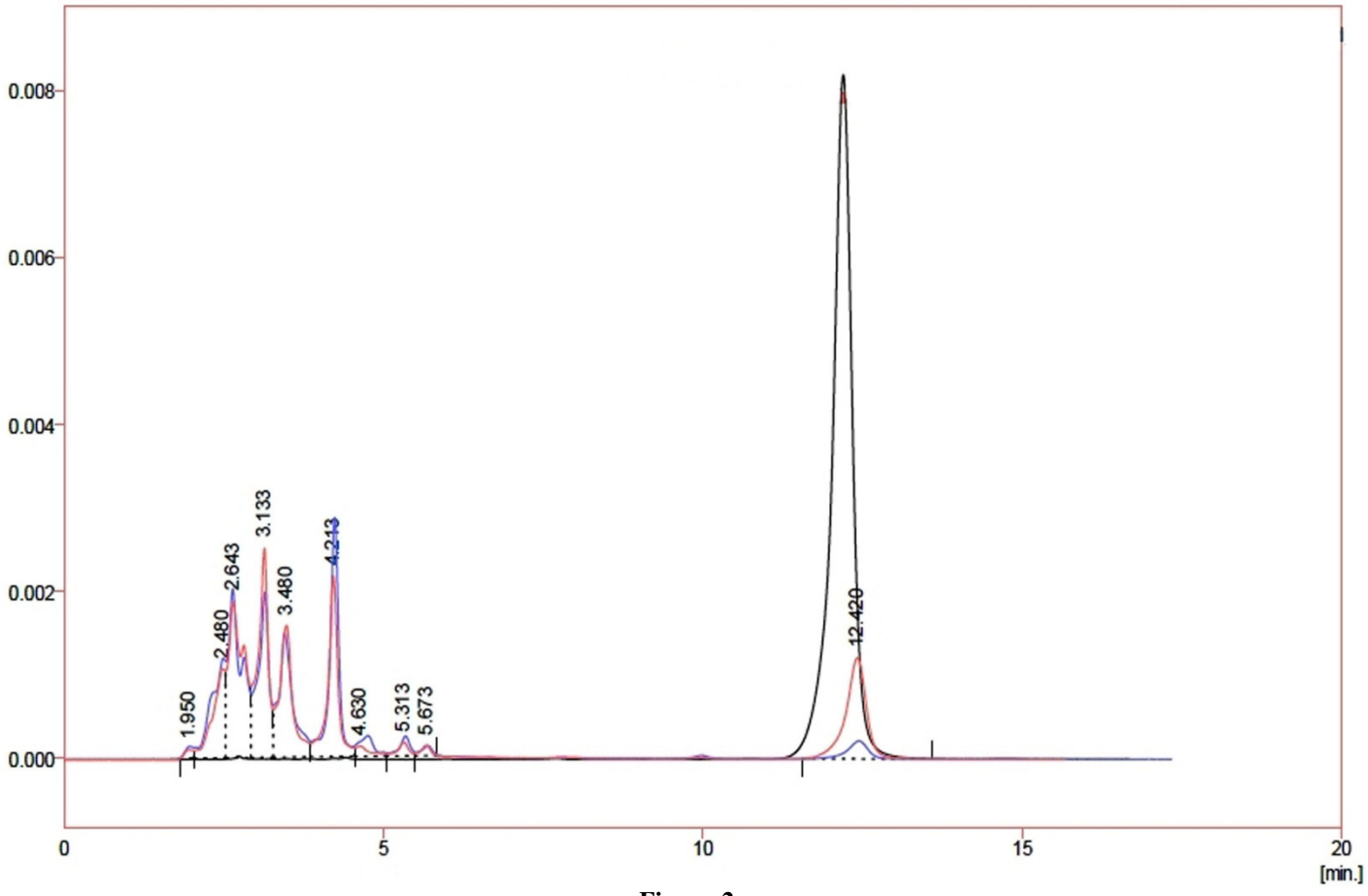

Figure-2
HPLC chromatogram of Samples and standard lovastatin

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