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Screening and Molecular Characterization of Natural Fungal Isolates Producing Lovastatin

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Abstract

Aspergillus sp. is known to produce cholesterol reducing drug lovastatin. Limited investigations have been made on screening of high yielding strains from hitherto under-utilized natural sources. With this objective, in the present study, various fungal cultures (130 in number) were isolated from natural sources such as (soil from paddy fields, oyster mushroom beds from the fields and compost) from different regions of interiors of Karnataka and Tamil Nadu of India. All the isolates were identified through standard microbiological methods such as morphological, microscopic methods. These isolates were further cultured for the production of lovastatin through submerged fermentation process (SmF). The extract from each of the isolate was subjected to three replicates of UV spectrophotometry quantification (200-350 nm). Confirmation of the metabolite production was achieved through HPLC and Attenuated total reflectance (ATR-FTIR) chromatography using the drug lovastatin (Biocon Laboratories) as standard. Out of all, nine high lovastatin yielding fungal strains were selected by applying selection pressures. Submerged state fermentation (SmF) extract (lovastatin) of these strains also exhibited the maximum zone of inhibition (≥ 10 mm) against *Neurospora crassa* (MTCC-790) in bioassay. Fungal strains giving maximum yield of lovastatin were found to be *Aspergillus terreus* (SSM4) from oyster mushroom bed source that produced a maximum yield of lovastatin (996.6 mg/l) followed by *Aspergillus terreus* (SSM3) from compost source (900 mg/l), *Aspergillus flavus* (SSM8) from compost source (643 mg/l). High yielding *Aspergillus terreus* (SSM4) isolate was characterized by β2 tubulin gene sequencing, confirmed through phylogenetic and molecular evolutionary analyses using *MEGA* version 5 software. The present study isolated three high lovastatin yielding fungal isolates from natural samples and one of the highest yielding isolate was sequenced and deposited in the MTCC (IMTECH) Chandigarh with issued accession number *Aspergillus terreus* nhceup 11045.

Keywords: *Neurospora crassa;* Lovastatin; RP-HPLC; ATR-FTIR; β2 Tubulin gene sequencing; Phylogenetic analysis

Introduction

Lovastatin is a naturally occurring drug found in food such as red yeast rice (red rice *koji* or red fermented rice, red kojic rice, red *koji* rice, *anka*, or *ang-kak* is a bright reddish purple fermented rice, which acquires its colour from being cultivated with the mold *Monascus purpureus* and consists of lovastatin) and oyster mushroom [1]. Lovastatin (mevinolin) was the first hypocholesterolemic drug to be approved in 1987 by Food and Drug administration (FDA), USA [2]. Lovastatin is an effective competitively inhibitor of the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol [3]. Increased cholesterol levels have been associated with cardiovascular diseases (CVD) [4]. Both in vitro and in vivo studies of lovastatin in humans and animals exhibit potent inhibitory activity on cholesterol biosynthesis and plasma cholesterol level [5], thereby it is effective in the therapy of hypercholesterolemia. HMG-CoA reductase inhibition has beneficial pleiotropic effects [6]. Lovastatin have been shown to inhibit cellular proliferation and induce apoptosis and necrosis in several experimental settings including that of breast cancer, thus making them potential anticancer agents [7]. Some preclinical studies were suggested that lovastatin administered in a nanobead preparation may be therapeutically useful in hastening repair of human fractures [8]. Fermentation derived lovastatin is a precursor for simvastatin, a powerful semi-synthetic statin commercially available as Zocor™. Simvastatin is obtained via a selective enzymatic deacylation of lovastatin.

Several fungal genera including *Aspergillus terreus, Penicillium citrinum, Monascus ruber and Pleurotus* spp. have been reported to produce lovastatin [9]. FDA approved *Aspergillus terreus* as a best microorganism to produce lovastatin in 1987. *Aspergillus terreus* appears to be the most commonly used producer of this drug [10]. In earlier period, lovastatin was produced by liquid surface fermentation technique but currently submerged state fermentation (SmF) (the organism which can grow beneath the surface of the medium called as submerged fermentation process) technique [11] is employed throughout the world. 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., soils from paddy fields of Varthur, Bangalore, Karkada, Udupi Dist Karnataka, Avalapalli dam, Thatanapalli village, Hosur, Tamilnadu, compost from Karkada, Udupi District and wild oyster mushrooms from the fields of Asettipalli, Hosur Taluk, Tamilnadu and Karkada, Udupi Dist, Karnataka. The study then focused on characterization of (130 in number) isolated fungal

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strains based on morphological and microscopic properties. Then the SmF process was carried out for selected fungal isolates and confirmed the lovastatin in fermentation broth through bioassay method by using *Neurospora crassa* (MTCC-790) as a test organism followed by the UV spectroscopy in three replicates and final confirmation was achieved through RP-HPLC and Attenuated total reflectance (ATR-FTIR) chromatography using the drug lovastatin (Biocon Laboratories, Bangalore India) as standard. Total nine isolates were positive for lovastatin production. Among all, high yielding (996.6 mg/l) *Aspergillus terreus* (SSM4) isolate was characterized by β2 tubulin gene sequencing, confirmed through phylogenetic and molecular evolutionary analyses using *MEGA* version 5 software and deposited with (accession number nhceup 11045) microbial type culture collection (MTCC), Chandigarh, India.

Materials and Methods

All the chemicals and reagents used in this study were of high purity (Analytical grade, Merck and Qualigens).

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 (Table 1). Isolation of desired fungal cultures was carried out using potato dextrose agar (PDA) medium (potatoes 200g/l, dextrose 20g/l, agar 15g/l) containing oxy-tetracycline (100 ug/ml) 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 [12].

Submerged fermentation (SmF) process

The spores were collected by single spore isolation technique using 2% Tween-20 solution and diluted to 5.7×10^6 spores per milliliter. Spore counting was carried out using a hemocytometer. 7.5 ml of collected spores were added to 50 ml of seed culture medium (Composition per litre: corn steep liquor 5 g, tomato paste 40 g, oat meal 10 g, glucose 10 g, trace element stock solution 10 ml (Composition per litre: $FeSO_4.7H_2O$ 1 g, $MnSO_4.4H_2O$ 1 g, $CuCl_2.2H_2O$ 25 mg, $CaCl_2.2H_2O$ 100 mg, H_3BO_3 56 mg, $(NH_4)_6Mo_7O_{24}.2H_2O$ 19 mg, $ZnSO_4.7H_2O$ 200 mg and distilled water 1 litre (pH 6.8)). The seed cultures were incubated in a rotary shaker-incubator at 180 rpm at 28°C for 24 hours. Ten percent of the seed broth was inoculated to 50 ml of production medium and incubated in a rotary shaker-incubator at 180 rpm at 28°C for 10 days. Production media (Composition per litre: glucose 50 g, yeast extract 20 g, tomato paste 30g, oat meal 20 g, sodium acetate 10 g, ammonium sulphate 5 g, potassium dihydrogen phosphate 2 g and trace element stock solution 10 ml and distilled water 1 litre (pH 7.0) adjusted using 1 M NaOH [13]. Media optimization experiments with these species showed that glucose and glycerol are the best carbon source for lovastatin production [14].

Natural sample type	Sample code	Place	State of India
Soil	S1PT1	Thattanpalli	Tamil Nadu
	S2PK1	Karkada	Karnataka
	S3RT1	Dharmapuri	Tamil Nadu
	S4PT2	Thattanpalli	Tamil Nadu
	S1U1	Karkada	Karnataka
Oyster Mushroom	M1T1	A. settipalli	Tamil Nadu
	M2K1	Karkada	Karnataka
	M3K2	Karkada	Karnataka
	M4T2	Dharmapuri	Tamil Nadu
	M5K3	Karkada	Tamil Nadu
	M6T3	Thattanpalli	Tamil Nadu
	M7T4	A. settipalli	Tamil Nadu
Compost	COM1U1	Bramhavara	Karnataka
	COM2U2	Karkada	Karnataka

Table 1: Collected natural samples for fungal isolation.

Downstream processing of lovastatin

At the end of 10 days of fermentation, the fermentation broth was acidified to pH 3.0 with 10% 1 N HCl. Then the acidified broth was extracted with equal volume of ethyl acetate under shaking condition (180 rpm) at 70°C for 2 hrs. The fungal biomass was separated by filtration using pre-weighed Whatman No.40 filter paper. The filtrates were subsequently centrifuged at 3000 g for 10 min and the organic phase was collected. To the 1ml of organic phase 1% trifluoroacetic acid (10 ml) was added for lactonization process. Then the extract was concentrated at 80°C (without vacuum), diluted to 1 ml with acetonitrile and filtered through a 0.45 µm filter for qualitative and quantitative estimation by UV Spectrometry, high-performance liquid chromatography (HPLC) and ATR-FTIR analysis [15].

UV Spectrophotometric analysis of lovastatin

Prepared sample were analyzed qualitatively for the presence of lovastatin at different nm (210 nm-350 nm) (three replicates), subsequently lovastatin was detected and estimated at 238 nm (three replicates) using pure lovastatin (Biocon laboratories, Bangalore, India) as a standard in UV/Visible spectrophotometer. (Shimadzu, Model no UV-2450 and Software UV-probe 2.21) [16].

HPLC analysis of lovastatin

The prepared sample extracts and lovastatin standard (Biocon laboratories, Bangalore, India) was quantitatively analyzed for the presence of lovastatin in the fungal extracts. HPLC analysis was carried out at 238 nm by using reverse phased 250×4.6 mm inertsil ODS-3 C 18 column of 5 µm particle size, 20 µl loop injector, Shimadzu instrument model: Ao6SM7696M, version 5.032 software. The eluent was a mixture of acetonitrile and 0.1 per cent phosphoric acid (60:40 v/v), flow rate was 1.2 ml min^{-1} and temperature of column was at 25°C [17]. Lovastatin was quantified as its lactone form and β-hydroxy acid form, by HPLC. As the open hydroxy acid form of lovastatin is unstable, it was prepared freshly from lactone form whenever necessary. The lovastatin lactone form was suspended in 0.1M NaOH and heated at 50°C for at least one hour in a shaking water bath. Subsequently, the suspension was adjusted to pH 7.7 with 1 M HCl, filtered through 0.45 µm filters and diluted to the concentration of 5 µg/ml [18].

ATR-FTIR analysis of lovastatin

Final confirmation of lovastatin in the prepared sample was done by using FTIR/Diamond ATR, Model: FTIR-8400S, Brand Name: Shimadzu. ATR was fitted with a single bounce diamond at 45° internally reflected incident light providing a sampling area of 1 mm in diameter with a sampling depth of several microns. A small amount of the sample was directly placed on the diamond disk and liquid sample kept in liquid sample holder. Sample was scanned for absorbance over the range from 4000 to 400 wave numbers (cm^{-1}) at a resolution of 1 cm^{-1} [19].

Bio-assay of lovastatin using *Neurospora crassa* (MTCC-790)

Neurospora crassa (MTCC-790) was grown for 7-10 days on PDA slants at 28°C. Spores were harvested with sterile water. 20 µl of spore suspension of *Neurospora crassa* (MTCC-790) was transferred into a 40×40 mm size sterilized petri plate with PDA (potatoes 200 g/l, dextrose 20 g/l, agar 15 g/l). After solidification, wells were made using 8 mm diameter borer 18 and bioassay was carried out with ethyl acetate as a control and ethyl acetate extract of fungal isolates as samples [20]. Bioassay positive fungal cultures were maintained on PDA slants at 28°C.

Phylogenetic and molecular evolutionary analyses of high yield isolate

Fungal DNA was extracted with phenol-chloroform protein extraction, precipitated using ethanol and checked on 1% agarose gel for purity. PCR amplification of partial regions of β2-tubulin gene was done by using primer pairs Bt2a (5'GGTAACCAAATCGGTGCTGCTTTC 3') (forward) and Bt2b (5'ACCCTCAGTGTAGTGACCCTTGGC3') (reverse). PCR product obtained was gel purified and taken for sequencing. PCR product was sequenced with the big dye terminator cycle sequencing ready reaction kit on an ABI3730XL genetic analyzer (Applied Biosystems) instrument model/name: 3730xl/ ABI3730XL-15104-028, sequence scanner version 1.0 software [21]. Forward strand partial β 2-tubulin gene sequence (545 nt) were aligned with maximum identity score sequence *Aspergillus terreus* NRRL 255 strain (515 nt) through BLAST. Phylogenetic analyses were performed by the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbor-joining method [22]. The percentage of replicate trees (500 replicates), associated taxa clustered was done by bootstrap test [23]. The evolutionary distances compared using Jukes-cantor method [24] to infer phylogenetic tree. Evolutionary analysis was conducted using MEGA version 5 software [25].

Results

Isolation and characterization of fungal isolates from natural samples

In the present investigation, 130 fungal colonies were isolated from different natural samples (soil from paddy fields, oyster mushroom beds from fields and compost) on PDA medium. They were characterized by using standard micro biological 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 (Figure 1, Table 2). The isolates were further compared for morphological and pigmentation properties with *Aspergillus terreus* (MTCC-1782) used as standard.

Submerged fermentation process and downstream processing of lovastatin

The fungal cultures were grown under submerged fermentation conditions to screen their potential for lovastatin production. At the end of 10 days of fermentation, the final pH of the medium and fungal biomass (wet weight) was measured (Table 2) and after fermentation process organic phase contain lovastatin was extracted through downstream processing.

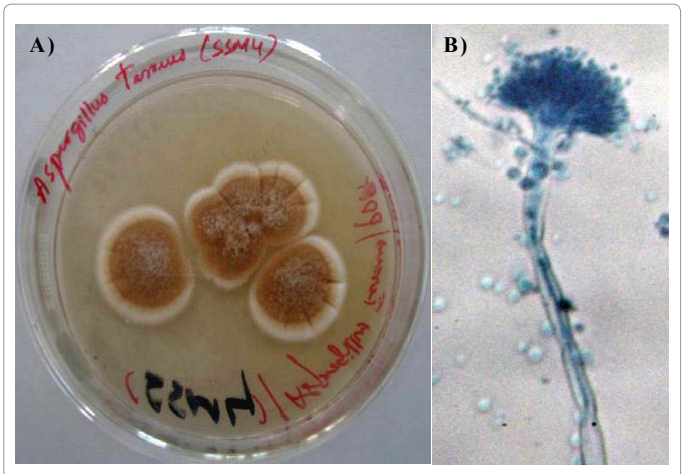

Figure 1: Characterization of *Aspergillus terreus*. A. *Aspergillus terreus* colonies on PDA plate showing cinnamon brown color; B. Biseriate conidiophores of *Aspergillus terreus* (100X).

UV spectrophotometric analysis of lovastatin smf samples

The concentration of lovastatin was calculated spectrophotometrically (three replicates). The samples and the standard exhibited a peak at 238 nm in the spectrophotometer scanning in figure 2. From the results, it was deduced that nine fungal cultures showed positive results for lovastatin production. *Aspergillus terreus* (SSM 4) yields the maximum amount of lovastatin (996.6 mg/l) followed by *Aspergillus terreus* (SSM 3) (900 mg/l), *Aspergillus flavus* (SSM 8) (643 mg/l), *Aspergillus niger* (SSM 7) (180 mg/l), *Aspergillus terreus* (SSM 6) (138.5 mg/l), *Aspergillus terreus* (SSM 1) (130.2 mg/ l), *Aspergillus terreus* (SSM 2) (128.4 mg/ l), *Aspergillus terreus* (SSM 5) (111.4 mg/ l) and *Rhizopus oryzae* (SSM9) (90 mg/l) in the screening work through SmF process.

HPLC and ATR analysis of lovastatin

HPLC analysis also confirmed quantitatively the amount of lovastatin in the fungal extract. Retention time of standard lovastatin and sample (SSM4) for β-hydroxy acid form was 9.291 and 9.477 min respectively and for lactone form, 17.225 and 17.235 respectively (Figures 3A, B and C). Qualitative analysis and quantification of lovastatin in HPLC was carried out by considering both forms of lovastatin (lactone and β-hydroxy acid form) β-hydroxy acid form elutes earlier in the chromatographic column followed by lactone form [18] and also the fermentation extract was lactonized which principally consists lovastatin in the form of lactone form.

ATR-FTIR analysis of lovastatin

The ATR-FTIR spectra of *Aspergillus terreus* MTCC-11045 smf extracts (lovastatin) were shown in figure 4. The spectrum presented characteristic peaks at 3541 cm^{-1} (alcohol O-H stretching vibration), 3003.27 cm^{-1} (olefinic C-H stretching vibration), 2335.87 cm^{-1} (methyl and methylene C-H asymmetric stretching), 1629.90 cm^{-1} (lactone and ester carbonyl stretch), 1440.87 cm^{-1}, 1375 cm^{-1} (Methyl and Methylene bending vibration), 1033.88 cm^{-1} (ester C-O-C symmetric bend), 918.15 cm^{-1} (alcohol C-OH stretch), 819.77 cm^{-1} (trisubstituted olefinic C-H) and 750.33 cm^{-1} (meta disturbed benzene-strong) confirms the presence of lovastatin in the SmF samples. Lovastatin containing the lactone ring gives characteristic peak at 1629.90 cm^{-1} [26].

Source/Place/State	Medium/dilution of sample/ sample code	Fungal isolates	Organism	Fungal biomass (g/l)	Final pH of the medium
Soil, Karkada, Karnataka	PDA/10^{-6}/S_1U_1	SSM 1	*Aspergillus terreus*	6.36	6.54
Compost, Karkada, Karnataka	PDA/10^{-3}/COM_2U_2	SSM 2	*Aspergillus terreus*	6.75	6.32
Compost, Bramhavara, Karnataka	PDA/10^{-5}/COM_1U_1	SSM 3	*Aspergillus terreus*	6.38	6.47
Oyster Mushroom beds, A. settipalli, Tamilnadu	PDA/10^{-5}/ M_7T_4	SSM 4	*Aspergillus terreus*	6.24	6.48
Compost, Karkada, Karnataka	PDA/10^{-4}/COM_2U_2	SSM 5	*Aspergillus terreus*	6.78	6.32
Oyster Mushroom beds, Karkada, Karnataka	PDA/10^{-5}/M_5K_3	SSM 6	*Aspergillus terreus*	6.42	6.35
Compost, Karkada, Karnataka	PDA/10^{-6}/COM_2U_2	SSM 7	*Aspergillus niger*	7.06	7.32
Compost, Bramhavara, Karnataka	PDA/10^{-3}/COM_1U_1	SSM 8	*Aspergillus flavus*	6.55	6.61
Oyster Mushroom beds, Karkada, Karnataka	PDA/10^{-6}/M_5K_3	SSM 9	*Rhizopus oryzae*	6.67	6.72

Table 2. Screening of fungal cultures for lovastatin production by SmF method.

Bio-assay of lovastatin using *Neurospora crassa* (MTCC-790)

The results of *Neurospora crassa* (MTCC-790) bioassay revealed that all nine isolated fungal cultures were shown zone of inhibition of growth after incubation period. *Aspergillus terreus* oyster mushroom bed (from the fields) fungal isolate (SSM 4) exhibited maximum zone of inhibition (12mm) which related to the maximum capacity to produce the drug lovastatin (Figure 5).

Phylogenetic and molecular evolutionary analyses of high yield isolate

High yielding *Aspergillus terreus* (SSM4) DNA was isolated and confirmed the purity of DNA by agarose gel electrophoresis. From isolated fungal DNA partial region of β 2-tubulin gene was amplified with the Bt2a and Bt2b primer set**.** PCR product obtained was gel purified and taken for sequencing. Forward sequence information was submitted to NCBI genbank with genbank accession number (JQ897354). *Aspergillus terreus* (SSM4) forward strand partial β 2-tubulin gene sequence (545 nt) were aligned with maximum identity score sequence *Aspergillus terreus* NRRL 255 strain (515 nt) through BLAST (Expect values 0.0 and query coverage was 94%). Out of 515 nt 512 nt (99%) were similar with 0% gaps. The evolutionary history was inferred using Neighbor-joining method (Figure 6), the optimal tree with sum of branch length=0.97315489 was shown .The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (500 replicates) were shown next to the branch. The

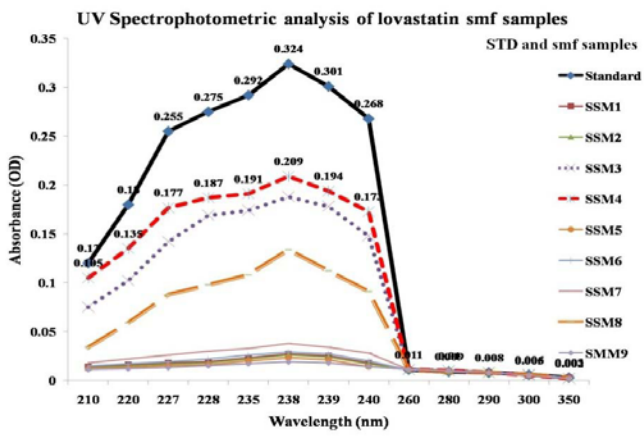

Figure 2: UV Spectrophotometric analysis of lovastatin (SmF samples).

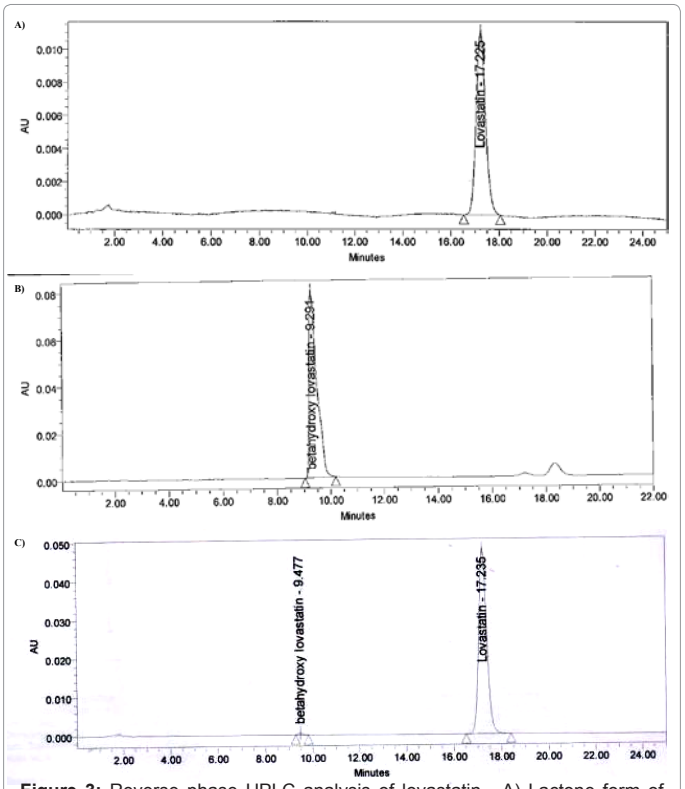

Figure 3: Reverse phase HPLC analysis of lovastatin. A) Lactone form of standard lovastatin; B) β-hydroxy acid form of standard lovastatin and C) *Aspergillus terreus* (SSM4) SmF extract.

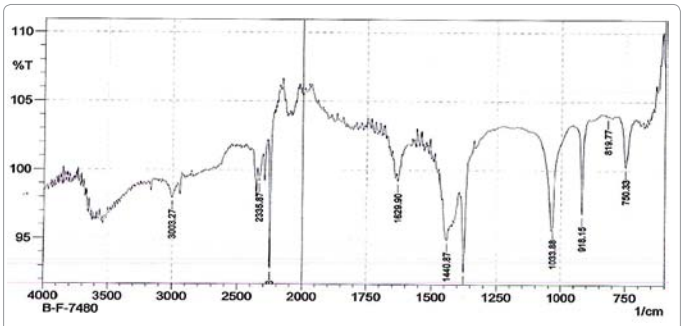

Figure 4: ATR-FTIR analysis of lovastatin (*Aspergillus terreus* (SSM4) SmF extract).

tree was drawn to scale, with branch length in the same unit as those of the evolutionary distance used to infer the phylogenetic tree. The evolutionary distance was in the units of number base substitutions per site. The analysis involved 16 nucleotide sequences. All ambiguous were removed for each sequence pair. There were a total of 595 positions in the final data set.

Discussion

In the present study, attempts were made to isolate potential, high yielding 3-hydroxy-3-methyl glutaryl CoA (HMG CoA) reductase inhibitor producing fungal strains from natural samples. Isolated fungal strains were tested by SmF process using glucose as a sole carbon source for the production of lovastatin. Nine high yielding fungal isolates were identified. Among these fungal isolates, *Aspergillus terreus* (SSM4) isolated from oyster mushroom beds (from fields) sample taken from *A. settipalli*, Tamil Nadu recorded a maximum yield of 996.6 mg/l of lovastatin. Results of bioassay using *Neurospora crassa* (MTCC790) and various analyses (U.V, HPLC and ATR-FTIR) confirmed the identity of lovastatin (with regard to standard lovastatin from Biocon laboratories, Bangalore, India). HPLC chromatogram of present study exhibited peaks for two forms of lovastatin that is β-hydroxy acid form and lactone form and confirmed with the standard lovastatin chromatogram.

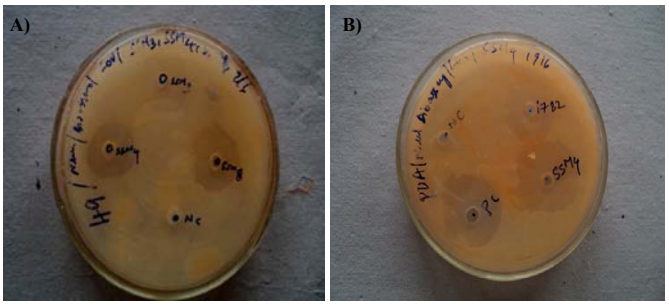

Figure 5: *Neurospora crassa* (MTCC-790) bioassay of fungal SmF extracts.

A) Zone of inhibition of SSM 3, SSM4, SSM8 and negative control (NC) ethylacetate.

B) Zone of inhibition of SSM4, MTCC-1782, positive control (PC) standard lovastatin and negative control (NC) ethylacetate.

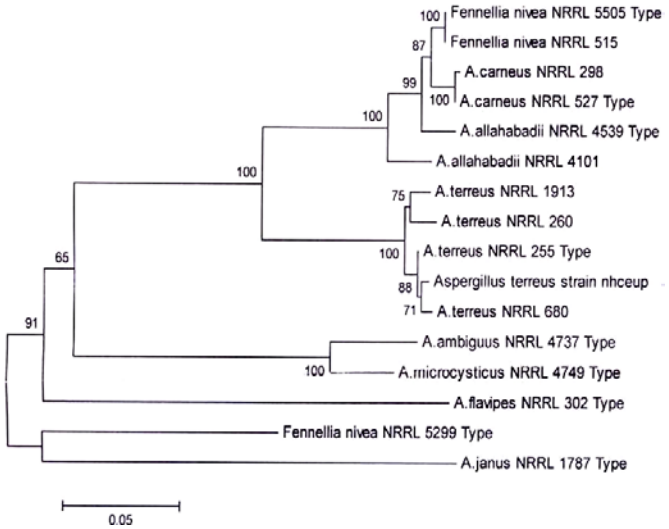

Figure 6: Phylogenetic tree of *Aspergillus terreus* strain nhceup 11045, inferred from Neighbour-Joining analysis of partial β-tubulin gene sequence.

Samiee et al. [13] screened 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 and reported *Aspergillus terreus* as a best isolate, which was yielding 17-fold less (55 mg/l) than that of present study. Jaivel and Marimuthu [27] 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 7.12 fold decreased yielding than the *Aspergillus terreus* (SSM 4) reported in present study. Sree Devi et al. [28] 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 starins, compared to 1/3 yielding (360 mg/l) of the present study. Mangunwardoyo et al. [29] 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 11 fold lower yield than the present reported fungal isolate *Aspergillus terreus* (SSM 4). Supplementing tylosin of 50 mg l^{-1} at the beginning of lovastatin biosynthesis led to the final lovastatin production of 952.7 ± 24.3 mg l^{-1} [30,31].

Considering these references as discussed above, it could be concluded that *Aspergillus terreus* MTCC 11045 (SSM4), the fungal isolate as obtained from oyster mushroom beds was found to produce highest yield (996.6 mg/ l) of lovastatin (HMG Co A reductase inhibitor). It can be also seen that the present investigation led to explore the hitherto under-utilized natural sources for fungal isolation. The design of selective media led to screening and selection of desired high lovastatin producing fungal isolates-*Aspergillus terreus*.

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We wish to express our sincere gratitude to Chairman and Principal, New Horizon College of Engineering, Bangalore for providing us with all facilities to undertake research work on "Screening and molecular characterization of natural fungal isolates producing lovastatin". We extended our sincere thanks to Biocon, Bangalore, India for their help. We also wish to express our gratitude to the officials and other staff members who rendered their help during the period of this research work. Last but not least, we wish to avail ourselves of this opportunity to express a sense of gratitude and love to friends and our beloved parents for their moral support, strength, and help and for everything.

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