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MOLECULAR CHARACTERIZATION AND MTCC SUBMISSION OF LOVASTATIN MAXIMUM YIELDING FUNGI ISOLATED FROM NATURAL SAMPLES

R S Upendra[1], Pratima Khandelwal[2], Z.R. Amiri[3]

[1]Sr. Asst. Prof, Dept. of Biotechnology, New Horizon College of Engineering, Outer Ring Road, Bellandur Post, Marathahalli, Bangalore -560103, Karnataka, India. rsupendra.nhce@gmail.com

[2]Prof & Head, Dept. of Biotechnology, New Horizon College of Engineering, Outer Ring Road, Bellandur Post, Marathahalli, Bangalore -560103, Karnataka, India
pratima2k1@gmail.com

[3]Assoc. Professor, Dept. of Food Sci. & Tech., Sari Agricultural Sciences and Natural Resources University, Sari, Iran
zramiri@gmail.com

* Corresponding Author's mailing address:
Sr. Assistant Professor, Dept. of Biotechnology,
New Horizon College of Engineering, Ring Road, Bellandur Post,
Marathahalli, Bangalore -560103, Karnataka, India
Tel: 91-80-66297777, Fax: 91-80-28440770. Email: rsupendra.nhce@gmail.com.

Abstract

Aspergillus sp. is known to produce cholesterol reducing drug-lovastatin. Lovastatin is a naturally occurring drug found in food such as red yeast rice (red rice *koji* or red fermented rice, red koji rice, red *koji* rice, *anka*, or *ang-kak*). Lovastatin (mevinolin) was the first hypocholesterolemic drug to be approved in 1987 by Food and Drug administration (FDA), USA. In the present study, three lovastatin maximum yielding wild type fungi i.e *Aspergillus terreus*- SSM4, isolated from wild oyster mushroom bed with lovastatin yield (997 µg/g dry matter), *A. terreus*- SSM3 from compost source (900 µg/g dry matter) and *A. flavus,* SSM8 from compost source (643 µg/g dry matter); obtained from the Upendra *et al.,* (2013a) were initially identified using scanning electron microscopy (SEM), further characterized at molecular level by restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing (SSM4) and 18s RNA (ITS1 - 5.8S - ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using *MEGA* version 5 software. Results confirmed the similarity between SSM3 and SSM4 isolates and they were found to be *Aspergillus terreus*, and also inferred that SSM8 is a completely different species, belongs to *Aspergillus flavus species*. Sequence information of the three isolates was submitted in NCBI GenBank with the issued accession numbers: JX419386- *Aspergillus terreus* **(**SSM3), JQ897354- *Aspergillus terreus* (SSM4), and JQ899451- *Aspergillus flavus* (SSM8). Characterized maximum lovastatin yielding wild type fungi were deposited in the MTCC IMTech- Chandigarh, with issued accession number as *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus* NHCEUPBTE 11396 (SSM8). Lovastatin in the SmF extract of *Aspergillus terreus* (SSM4) was confirmed using both ^{1}H NMR and ^{13}C NMR studies. In conclusion the presented study successfully characterized the lovastatin high yielding fungi and deposited to MTCC IMTech-Chandigarh with issued accession numbers.

Keywords: Lovastatin, RFLP, RAPD, β2 Tubulin & 18S rRNA gene sequencing, Phylogenetic analysis.

Introduction

High levels of cholesterol in the blood refer to the condition called Hypercholesterolemia also called dyslipidemia **(Durrington, 2003).** The highest % of global death today is caused by cardiovascular diseases (CVDs) occurs due to the hypercholesterolemic conditions, an estimate of 17.3 million people were died due to CVD, representing 30% of all global deaths in the year 2008 **(WHO, 2010).** An estimate of 80 % of death occurring in the low and middle income countries mainly due to CVD and affects men and women equally **(WHO, 2011).** Hypercholesterolemia can be easily treated with medications by targeting the reduction of the low density lipoprotein (LDL) cholesterol in the blood. Among the fungal polyketide metabolites, statins are in prime importance due to their anti-cholesterol nature **(Praveen and Savitha, 2012).** US Food and Drug administration approved lovastatin as the first hypo-cholesterolemic drug in the year 1987 **(Tobert, 2003).** Lovastatin competitively inhibits the enzyme HMG-CoA reductase (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34) which catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol **(Alberts *et al.*, 1980 & 1988, Upendra *et al.*, 2016).** Lovastatin found to inhibits cellular proliferation, induces apoptosis and necrosis in breast cancer **(Kalwitter *et al.*, 2010)** and also suppress the proliferation and migration of human glioblastoma cancer cells by the inhibition of Ras farnesylation mechanism **(Xia *et al.*, 2001).** Lovastatin therapy was effective in preventing creatinine clearance decline and also suppresses renal function loss in patients with kidney disease **(Buemi *et al.*, 2002).** High doses of lovastatin stimulate bone formation *in vitro* and *in vivo* and, stimulate biomechanical strength to heal fractures **(Garrett *et al.*,**

2007). Lovastatin treatment was observed to reduce the prevalence of Alzheimer's disease (AD) in patients suffering from hypercholesterolemia **(Eckert *et al*., 2005).** Lovastatin therapy improves endothelial function, modulates inflammatory responses, maintain plaque stability and prevent thrombus formation **(Palmer *et al*., 1990, Pickin *et al*., 1999).**

Aspergillus sp **(Upendra *et al*., 2013a & 2013b)** and *Monascus sp* **(Negishi *et al.*, 1986, Upendra *et al*., 2014a)** were known to produce lovastatin through polyketide biosynthatic pathway **(Lai *et al*., 2002).** Lovastatin from *Aspergillus terreus* was the first statin to be approved by FDA in 1987 for therapeutic use **(Tobert, 2003, Upendra *et al*., 2014b).** The colony morphology observations studies and sub-typing studies on the *Aspergillus terreus* species revealed that the species section was found to be more diverse in their genotype (**Balajee, 2009).** Species level identification can be achieved successfully with molecular characterization studies of β-Tubulin gene and 18s RNA (ITS1 - 5.8S - ITS2) region studies **(Henry *et al*., 2000).** Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis **(Martin and Rygiewicz, 2005).** A novel PCR-based assay (ITS1 and ITS2 regions), was developed and introduced to differentiate medically important species of *Aspergillus* not only from other opportunistic species of moulds and yeasts but also from other strains belong to the same species (**Hinrikson *et al*., 2005)**. A new species of *Aspergillus* genus, section *Terrei* was studied *using* a polyphasic approach including sequence analysis of parts of the β-tubulin, calmodulin genes and the ITS region **(Samson *et al*., 2011).** The study of molecular evolutionary process of different microorganism, comparative analysis of molecular sequence data is essential for reconstructing the species and inferring the nature and extent of selective forces shaping the evolution of genes and species **(Tamura *et al.*, 2011)**. MEGA version5 software was used for building sequence alignments and phylogenetic trees, used in evolution analysis. This software is equipped with new tools which were used in studying maximum likelihood (ML) analyses for inferring evolutionary trees, selecting best-fit substitution models (nucleotide or amino acid), inferring ancestral states and sequences (along with probabilities), and estimating evolutionary rates site-by-site **(Tamura *et al.*, 2011).** A novel method of lyophilization for the preservation of fungal strains was investigated and applied for the fungi which were found to possess high industrial value (**Bunse and Steigleder, 1991)**.

With the view of review of literature discussed, the present study aimed at identification of three lovastatin maximum yielding wild type fungi i.e *Aspergillus terreus*-SSM4, isolated from wild oyster mushroom bed with lovastatin yield (997 µg/g dry matter), *A. terreus*- SSM3 from compost source (900 µg/g dry matter) and *A. flavus,* SSM8 from compost source (643 µg/g dry matter); obtained from the Upendra *et al.,* (2013a). Study initially identified all the three lovastatin maximum yielding fungi, using scanning electron microscopy (SEM), further characterized at molecular level by Restriction fragment length polymorphism (RFLP), Random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing (SSM4) and 18s RNA (ITS1 - 5.8S - ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using *MEGA* version 5 software. Results confirmed the similarity between SSM3 and SSM4 isolates and they were found to be *Aspergillus terreus*, and also inferred that SSM8 is a completely different species, belongs to *Aspergillus flavus species*. Sequence information of the three isolates was submitted in NCBI GenBank with the issued accession numbers: JX419386-*Aspergillus terreus* **(**SSM3), JQ897354- *Aspergillus terreus* (SSM4), and JQ899451-*Aspergillus flavus* (SSM8). Characterized maximum lovastatin yielding wild type fungi were deposited in the MTCC IMTech-Chandigarh, with issued accession number as *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus* NHCEUPBTE 11396 (SSM8).

MATERIALS & METHODS

All the chemicals and reagents used in this study were of analytical Grade (Merck and Qualigens).

Analysis of lovastatin by NMR (^{1}H NMR & ^{13}C NMR) Spectroscopy

Aspergillus terreus (SSM4), SmF extract obtained from Upendra *et al*., (2013a) was analyzed by NMR (^{1}H NMR & ^{13}C NMR) spectroscopy to elucidate the srucure of lovastatin**.** The structure of lovastatin was also determined by ^{1}H NMR, ^{13}C NMR. Proton and carbon NMR measurements were performed on a Bruker Avance Ultrashield spectrometer 400.23 MHz instrument in deuterated dimethylsulfoxide and chloroform solvent ($CDCl_3$) system. Instrument was equipped with 5 mm BBO-head (^{1}H-channel, X-channel) probe with Z-gradient coils and an automatic Sample Changer B-ACS 120 (Bruker BioSpin, Rheinstetten, Germany). The data processing was performed using BRUKER X-WIN NMR 3.5 software under Microsoft Windows. All spectra were acquired at 298.0 K and the chemical shift values were reported on the δ scale relative to TMS **(Lankhorst *et al*., 1996 & Holzgrabe *et al*., 1998).**

Microorganism

The three culture of *Aspergillus species* SSM3, SSM4 and SSM8 obtained from Upendra *et al*., (2013a) were revived on the potato dextrose agar (PDA) slants and kept at 28°C in incubator for the period of seven days. After seven days of incubation, fully grown fungal cultures were stored at 4°C for further molecular characterization studies.

Scanning Electron Microscopic (SEM) confirmation of lovastatin maximum yielding fungal isolates

Selected three fungal isolates were initially confirmed by the Scanning Electron Microscopic (SEM) screening. Fungal mycelium was segmented to 0.5 to 1 cm thin sections with the blade; Segments were immersed in centrifuge tubes containing 1.5 ml of modified Karnovisk's solution (Glutaraldehyde 2.5%, 2.5% formaldehyde in cacodilato sodium buffer 0.05 M, pH 7.2, CaCl2 0.001M.) by 24 hours. Samples were than washed 3 times (each of 10 minutes) with aldehyde in 0.05 M cacodylate buffer (0.2M cacodylic acid $(CH_3)_2AsO_2H$ (MW = 138.0) 27.6 gm + ddH_2O to make 1 liter) and were immersed in tetroxide solution of 1% of osmium in 0.05 M cacodilato buffer (pH 7.2), at room temperature, in laminar flow chamber for 4 hours. Samples were washed 3 times in distilled water and were dehydrated in gradient of acetone (25, 50, 75, 90 and 100%) 10 minutes in each concentration, being repeated by 3 times in the concentration of 100%. Subsequently, the samples were submitted to CPD Balzers 030® equipment to complete dehydration. The specimens were pasted using adhesive tapes on the surface of stubs covered with aluminium and submitted to the metallization with gold using SCD

Balzers 050® equipment, in order to increase its conductivity. Finally, the specimens were observed by the scanning electron microscope LEO Evo 40®, interfaced by digital image processing software. Microscopic properties such as conidial head, conidiophores, vesicle and conidia were studied under Scanning Electron microscopy at 2000X - 3000 X magnification for all the three maximum lovastatin yielding fungal isolates **(Gonzalez and Woods, 2002).**

Molecular characterization by Restriction fragment length polymorphism (RFLP)

Fungal DNA was isolated by adopting the method as described previously by Zhao *et al*., (2001). The DNA pellet isolated was resuspended in 200 µl of TE buffer and stored at 4ºC for further analysis. Extracted Fungal DNA was subjected to electrophoresis on 1.2 % agarose gel for purity checking. PCR amplification was carried out for isolated DNA of all the three lovastatin high yielding fungal isolates of the present study. Conserved regions of 18S rRNA (ITS 1) and the 28S rRNA (ITS 4) gene (intervening 5.8S gene under the ITS 1 and ITS 2 noncoding regions) amplification was done using ITS 1 (5'TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers (118,119,167). 50 µl final volume of PCR reaction mixture **(Table-1)** was amplified following the PCR programme conditions **(Table-2)** designed for a total of 35 cycles.

Table-1. PCR reaction Mixture

Slno	Component	Volume(µl)	Concentration
1.	Triple Distilled water	36 µl	--
2.	10X assay buffer	05 µl	Tris-HCl (pH 8.4)- 200mM, KCl-500 mM $MgCl_2$-15 mM
3.	dNTP mix	02 µl	(10 mM)
4.	ITS1 primer	02 µl	50 ng/µl
5.	ITS4 Primer	02 µl	50 ng/µl
6.	Taq DNA Polymerase	01 µl	3u/µl
7.	Template DNA	02 µl	50 ng/µl

Table-2. PCR Programme Table

Segment	Step	Number cycles	Step	Temperature	Time
I		01	Initial denaturation	94ºC	5 Min
	1.		Denaturation	94 ºC	2 min
II	2.	35	Annealing	53 ºC	2 min
	3.		Extension	72 ºC	2 min
III		01	Final Extension	72 ºC	10 Min

Following amplification, 5 µl of PCR products were digested with the restriction enzyme *Hae*III, *Eco*RI and *Tag*I **(Table-3) (Munusamy *et al*., 2010).** After incubation the digested fragments were added with an aliquot of 2.5 µl of 6X gel loading dye and the fragments were separated on 1.2% agarose gel in 1X TAE buffer, stained with ethidium bromide and visualized under UV transillumination. The sizes of the digested products were determined with standard 1 kb or 100 bp molecular markers (Aristogene Laboratories Pvt. Ltd., Bangalore, India).

Table-3. Restriction digestion enzyme

Restriction enzyme	DD H_2O	Assay buffer	Template DNA	Enzyme (µl)	Final volume (µl)
EcoR I	35µl	4µl	10µl	1µl	50 µl
Hae III	35µl	4µl	10µl	1µl	50 µl
Taq I	35µl	4µl	10µl	1µl	50 µl

2.5. Molecular characterization by RAPD techniques

Random amplification was carried out for isolated DNA of all the three selected fungi using R108 random primer (5'GTA TTG CCC T-3') **(Teresa *et al.*, 2000, Raclavsky *et al.*, 2006).** 50 µl final volume of PCR reaction mixture (**Table-4**) was amplified following the PCR programme table (**Table-5**) designed for a total of 40 cycles. After amplification, the products were analyzed by the gel electrophoresis using 2% agarose gel.

Table-4. RAPD reaction Mixture

Slno	Component	Volume(µl)	Concentration
1.	Triple Distilled water	36 µl	--
2.	10X assay buffer	05 µl	Tris-HCl (pH 8.4)- 200mM, KCl-500 mM $MgCl_2$-15 mM
3.	dNTP mix	02 µl	(10 mM)
4.	Random primer	04 µl	50 ng/µl
5.	Taq DNA Polymerase	01 µl	3u/µl
6.	Template DNA	02 µl	50 ng/µl

Table-5. RAPD Programme Table

Segment	Step	Number cycles	Step	Temperature	Time
I		01	Initial denaturation	95ºC	5 Min
	1.		Denaturation	95 ºC	1 min
II	2.	35	Annealing	35 ºC	1 min
	3.		Extension	72 ºC	2 min
III		01	Final Extension	72 ºC	10 Min

Following amplification, 10 µl of amplified products were added with an aliquot of 2.5 µl of 6X gel loading dye and the amplified fragments were separated on 2% agarose gel in 1X TAE buffer, stained with ethidium bromide and visualized under UV transillumination. The sizes of the amplified products were determined with standard 1 kb or 100 bp molecular markers (Aristogene Laboratories Pvt. Ltd., Bangalore, India).

Phylogenetic and molecular evolutionary analyses selected fungal isolates

Fungal DNA was extracted with phenol-chloroform protein extraction, precipitated using ethanol and checked on 1% agarose gel for purity **(Zhao *et al.*, 2001).** PCR amplification of partial regions of β2-tubulin gene was done by using primer pairs Bt2a (5'GGT AAC CAA ATC GGT GCT GCT TTC 3') (forward) and Bt2b (5' ACC CTC AGT GTA GTG ACC CTT GGC3') (reverse) for *Aspergillus terreus* (SSM4), conserved regions of 18S rRNA amplification was done as discussed in section 2.4 **(Henry *et al.*, 2000).** PCR product obtained was gel purified and taken for sequencing **(Glass and Donaldson, 1995).** 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 **(Balajee *et al.*, 2007).** Forward strand partial β 2 tubulin gene sequence (545 nt) of *Aspergillus terreus* (SSM4) was aligned with maximum identity score sequence *Aspergillus terreus* NRRL 255 strain, forward strand 18S rRNA gene sequence of *Aspergillus terreus* (SSM3) (740 nt), *Aspergillus flavus* (SSM8) (751 nt) were aligned with maximum identity score sequence EF669586-*Aspergillus terreus* NRRL 255 strain and EF661563-*Aspergillus flavus* NRRL 4818 strain respectively through BLAST. Phylogenetic analyses of *Aspergillus terreus* SSM4 was performed using the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbor-joining method **(Saitou and Nei, 1987).** The percentage of replicate trees (500 replicates), associated taxa clustered was done by bootstrap test **(Felsenstein, 1985).** The evolutionary distances computed using Jukes-cantor method **(Jukes and cantor, 1969)** to infer phylogenetic tree. Phylogenetic analyses of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) were performed by the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbor-joining method **(Saitou and Nei, 1987).** The percentage of replicate trees (100 replicates), associated taxa clustered was done by bootstrap test **(Felsenstein, 1985).** The evolutionary distances computed using kimura 2-parameter method **(kimura, 1980)** to infer phylogenetic tree. Evolutionary analysis was conducted using MEGA version 5 software **(Tamura *et al.*, 2011).**

RESULTS & DISCUSSION

Analysis of lovastatin by NMR Spectroscopy

^{1}H-NMR Spectroscopy

^{1}H NMR (400 MHz,$CDCl_3$) determination of [*Aspergillus terreus* (SSM4)] SmF extract obtained from Upendra *et al* ., (2013a) showed resonances at δ 6.004-5.980ppm, δ 5.804-5.764 ppm, δ 5.530-5.523 ppm, δ 5.40-5.337 ppm, δ 4.632-4.579 ppm, δ 4.382-4.352 ppm (mid-field region) and δ 2.7635-2.750 ppm, δ 2.719-2.707 ppm, δ 2.641-2.627 ppm, δ 2.597-2.583 ppm, δ 2.462-2.436 ppm, δ 2.396-2.388 ppm, δ 2.281-2.275 ppm, δ 2.252-2.245 ppm, δ 2.095-2.088 ppm δ 1.998-1.937 ppm, δ 1.64-1.57 ppm, δ 1.117-1.0677 ppm, δ 0.90-0.863 ppm (aliphatic range) (Fig.1a). The ^{1}H NMR (400 MHz, $CDCl_3$) spectral prediction showed that the multiplet at δ 5.400-5.377 ppm clearly belonged to an H-atom at the hexahydronaphthalene moiety, with overlap of the signals of both atoms H6, H4 **(Fig. 1a).** The ^{1}H NMR spectral

data of lovastatin was found to be in concurrence with reported spectral data **(Holzgrabe *et al*., 1998).**

^{13}C-NMR Spectroscopy

^{13}C NMR (400 MHz, $CDCl_3$) determination of [*Aspergillus terreus* (SSM4)] SmF extract showed resonances at δ 133.04-128.33 ppm, δ 77.32-76.24 ppm, δ 41.49-11.72 ppm, **(Fig.1b).** The ^{13}C NMR (400 MHz, CDCl3) spectral prediction showed that the methylene signal at 26.81 ppm of C-3, methyl signal at 11.72 ppm of C-4" and an acetoxymethyl signal at 30.70 ppm indicating the presence of carbon-atom. The ^{13}H NMR spectral data of lovastatin was found to be in concurrence with reported spectral data **(Belwal *et al*., 2013).**

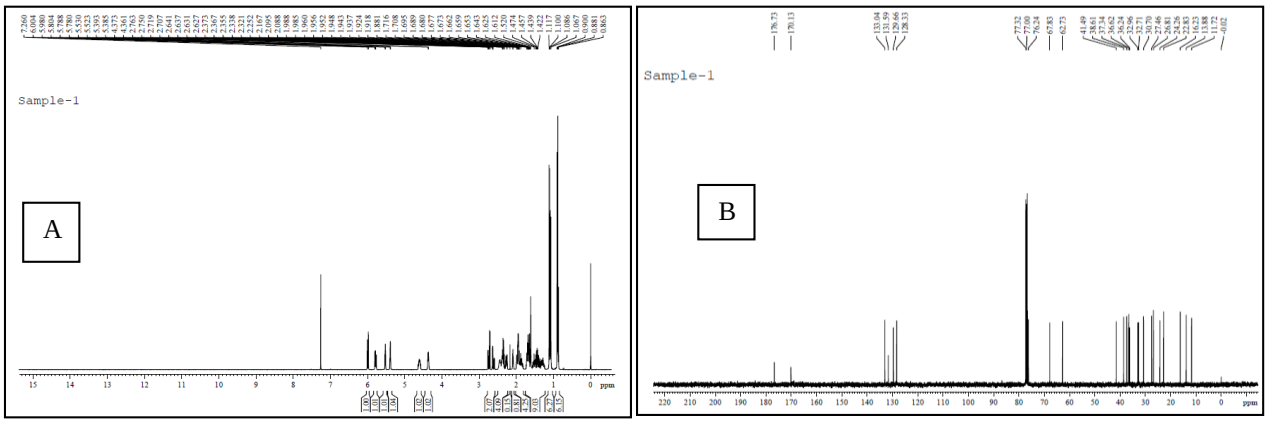

Fig.1: NMR spectrum of lovastatin (*Aspergillus terreus* (SSM4) SmF extract).
A. ^{1}H NMR spectrum of lovastatin, 400.23 MHz, solvent $CDCl_3$. B. ^{13}C NMR spectrum of lovastatin, 400.23 MHz, solvent $CDCl_3$.

Microorganism:

The fully grown cultures of *Aspergillus terreus* (SSM3), *Aspergillus terreus* (SSM4) and *Aspergillus flavus* (SSM8) on PDA plates obtained from Upendra *et al*., (2013a) were shown in **Fig.2.** *A. terreus* colonies are commonly powdery masses of cinnamon-brown spores on the upper surface and reddish-gold on the lower surface. *A. flavus* colonies are commonly powdery masses of yellow-green spores on the upper surface and reddish-gold on the lower surface.

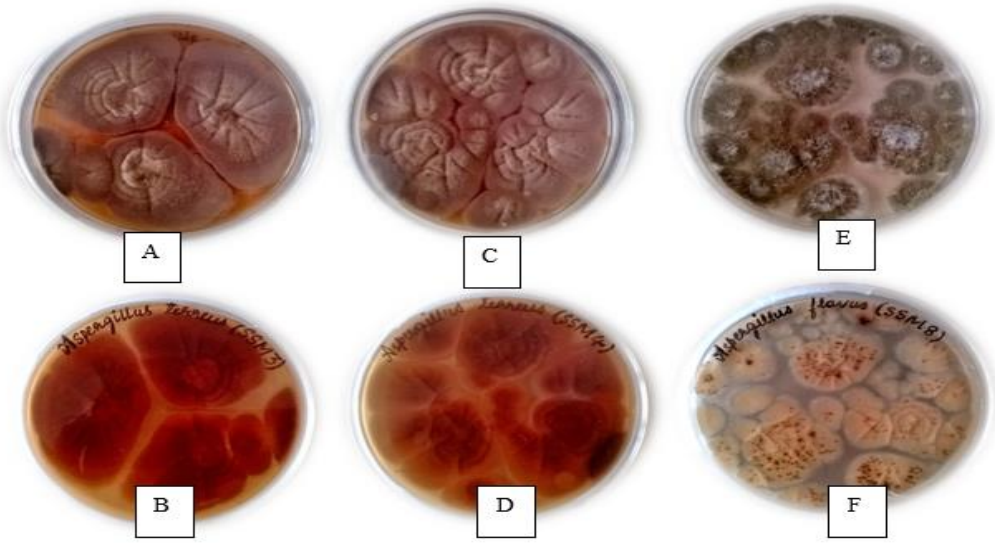

Fig 2: Selected fungal strain for molecular characterization

A. *Aspergillus terreus* (SSM3) on PDA media showing cinnamon brown on upper surface B. *Aspergillus terreus* (SSM3) showing reddish-gold on the lower surface. C. *Aspergillus terreus* (SSM4) on PDA media showing cinnamon brown on upper surface D. *Aspergillus terreus* (SSM4) showing reddish-gold on the lower surface. E. *Aspergillus flavus* (SSM8), on PDA media showing Olive to lime green color colonies upper surface. F. *Aspergillus flavus* (SSM8) showing reddish-gold on the lower surface.

Scanning Electron Microscopic (SEM) confirmation of lovastatin maximum yielding fungal isolates.

Under SEM *A. terreus* colonies were found to show characteristic properties i.e compact, biseriate, and densely columnar conidial heads (Fig 2-A, D). Smooth and hyaline conidiophores (Fig 2-B, E). The conidia are small, about 2 µm in diameter, globose-shaped, smooth-walled, and can vary from light yellow to hyaline (Fig 2-C, F). *Aspergillus flavus* hyphae are septate and hyaline (Fig 2-G). The conidiophores of are rough and colorless (Fig 2-H). Phialides are both uniseriate (arranged in one row) and biseriate. Conidia producing thick mycelial mats are often seen (Fig 2-I).

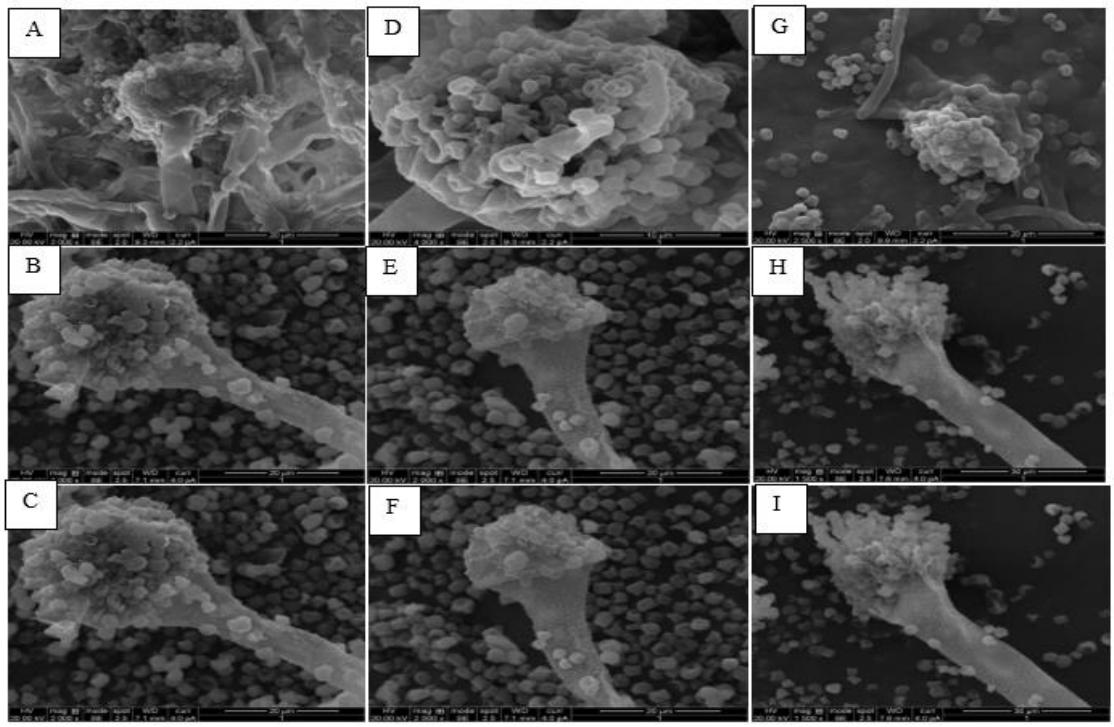

Fig 3: SEM Characterization of isolated fungal strains

A-C. Conidiophores and Conidia of *Aspergillus terreus* (SSM4). D-F. Conidiophores and Conidia of *Aspergillus terreus* (SSM3). G-I. Conidiophores and Conidia of *Aspergillus flavus* (SSM8).

Molecular characterization by Restriction fragment length polymorphism (RFLP)

The isolated and purified (1.2% agarose gel) DNA from the selected fungal cultures was shown in the Fig.4A. PCR amplification (ITS1-ITS4 primers) of genomic DNA extracted from the three selected fungi *A. terreus* (SSM3), *A. terreus* (SSM4) and *A. flavus* (SSM8) isolates with ITS1 and ITS4 primers resulted in the amplification of a product of approximately 600 bp (Fig. 4B). Upon digestion of the PCR products with the enzymes *Eco*RI, *Hae*III and *Taq*I indicated genetic variability among the isolates which varied in size of RFLP fragments and number of fragments (Fig. 4C).

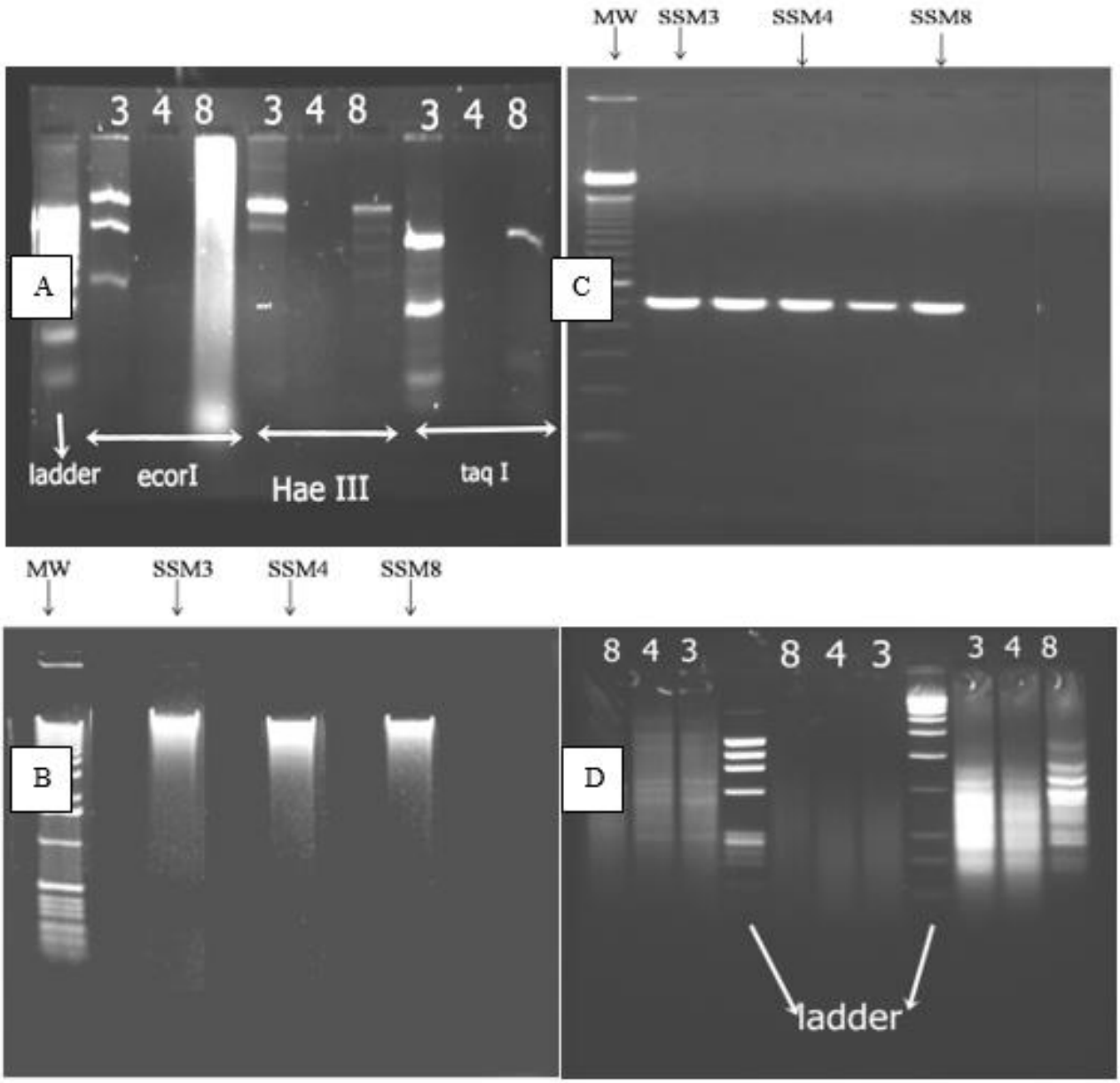

Fig. 4. Molecular characterization by RFLP and RAPD

Isolated DNA on 1.2 % agarose gel, (B) PCR amplified product (ITS region) on 2 % agarose gel, (C). Restriction patterns of the ITS regions of the ribosomal DNA of *A. terreus* (3), *A. terreus* (4), *A. flavus* (8) isolates digested with *Eco*RI, *Hae*III and *Taq*I, (D) RAPD finger printing profile of the ITS regions of the ribosomal DNA of *A. terreus* (3), *A. terreus* (4), *A. flavus* (8) isolates.

Molecular characterization by Random Amplification of Polymorphic DNA (RAPD)

RAPD-PCR amplification (random primer) product separated on 2% agarose gel of extracted genomic DNA from the three selected fungi *A. terreus* (SSM3), *A. terreus* (SSM4) and *A. flavus* (SSM8) isolates was shown Fig 4D. RAPD-PCR finger printing profiles of SSM3 and SSM4 were same and SSM8 fingerprinting profile is completely different, clearly inferring that SSM3 and SSM4 belong to same species and SSM8 belongs to the different species.

Phylogenetic and molecular evolutionary analyses of selected fungal isolates

Partial β 2 tubulin gene forward sequence information of *Aspergillus terreus* SSM4 was submitted to NCBI genbank with genbank accession number (JQ897354). Forward strand partial β 2 tubulin gene sequence (545 nt) were aligned with maximum identity score sequence *Aspergillus terreus* NRRL 255 strain through BLAST. The evolutionary history was inferred using Neighbor-joining method (Fig. 6A), 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) was shown next to the branch. The 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 (Adopted from Upendra *et al*., 2013a). 18S rRNA gene forward sequence information of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) were submitted to NCBI genbank with genbank accession numbers JX419386, JQ899451 respectively. *Aspergillus terreus* (SSM3) forward strand 18S rRNA gene sequence (740 nt) was aligned with

maximum identity score sequence EF669586-*Aspergillus terreus* NRRL 255 strain, *Aspergillus flavus* (SSM8) forward strand 18S rRNA gene sequence (751 nt) was aligned with maximum identity score sequence EF661563 *Aspergillus flavus* NRRL 4818 through BLAST. The evolutionary history was inferred using Neighbor-joining method (Fig. 6b), the optimal tree with sum of branch length = 0.10936750 was shown. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (100 replicates) was shown next to the branch. The 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 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 388 positions in the final data set.

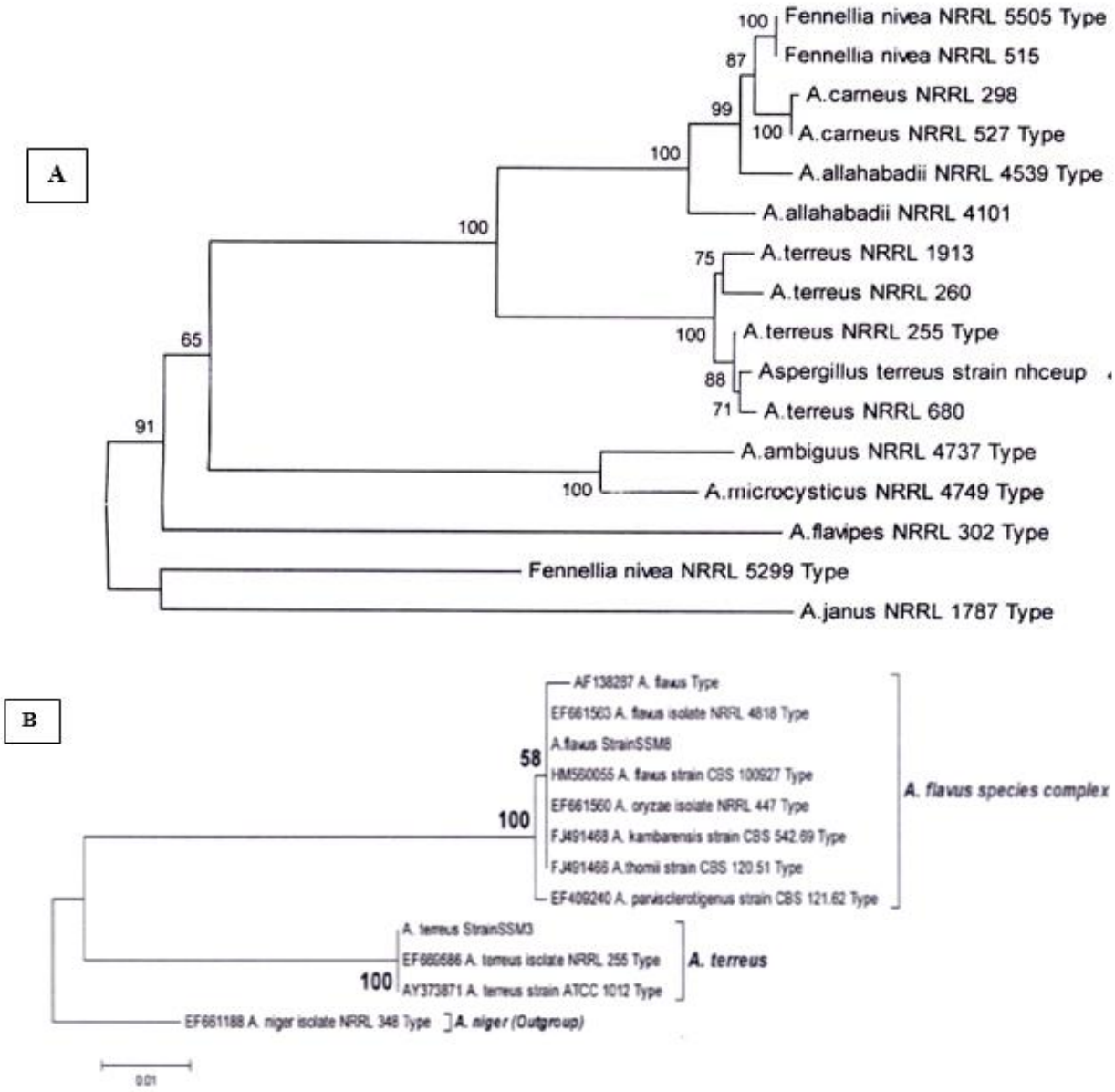

Fig 6: Phylogenetic analysis of isolated high yielding fungal strains

A. Phylogenetic tree of *Aspergillus terreus* strain nhceup (SSM4), inferred from Neighbour-Joining analysis of partial β-tubulin gene sequence (Adopted from Upendra *et al*., 2013a). B. Phylogenetic tree of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) inferred from Neighbour-Joining analysis of ITS1-ITS4 region sequencing

Deposition of lovastatin maximum yielding fungal isolates with MTCC (IMTech) Chandigarh

The maximum lovastatin yielding, molecular characterized and phylogenetically analyzed three new fungal isolates were deposited in Microbial Type Culture Collection, IMTech-Chandigarh, with issued accession number *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus NHCEUPBTE* 11396 (SSM8).

Conclusion

In the present study, attempts were made on molecular charecterization and MTCC registration of three lovastatin maximum yielding fungal cultures (obtained from the Upendra *et al.*, 2013a) isolated from natural samples. Selected fungal cultures were initially identified using scanning electron microscopy (SEM), further characterized at molecular level by Restriction fragment length polymorphism (RFLP), Random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing (SSM4) and 18s RNA

(ITS1 - 5.8S - ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using *MEGA* version 5 software. Sequence information of the three isolates was submitted in NCBI GenBank with the issued accession numbers: JX419386-*Aspergillus terreus* **(**SSM3), JQ897354-*Aspergillus terreus* (SSM4), and JQ899451-*Aspergillus flavus* (SSM8). Characterized maximum lovastatin yielding wild type fungi were deposited in the MTCC IMTech-Chandigarh, with issued accession number as *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus* NHCEUPBTE 11396 (SSM8).

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REFERENCES

- Alberts A W, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, *et al*. (1980). Mevinolin: A highly potent competitive inhibitor of hydroxymethyl glutaryl coenzyme A reductase and cholesterol lowering agent. Proceedings of the National Academy of Sciences of the United States of America, 77(7), 3957-3961.
- Alberts A W (1988). Discovery, biochemistry and biology of lovastatin. American Journal of Cardiology, 62, 10-5J.
- Balajee Arunmozhi. S. (2009): *Aspergillus terreus* complex. Medical Mycology, 47 (1), S42-S46.
- Balajee S A, Houbraken J, Verweij P E, Hong S B, Yaghuchi T, Varga J and Samson R A (2007): *Aspergillus* species identification in the clinical setting. Studies in Mycology*,* 59, 39-46.
- Belwal Chandrakant, Praveen Kumar Goyal , Anup Balte, Sandeep Kolhe, Kamlesh Chauhan, Ajay singh Rawat, Anand Vardhan (2013): Isolation, Identification, and Characterization of an Unknown Impurity in Lovastatin EP. Scientia Pharmaceutica, 8, 1-10.
- Buemi M, Senatore M, Corica F, Aloisi C, Romeo A, Cavallaro E, *et al*. (2002): Statins and progressive renal disease. Medicinal Research Reviews*,* 22, 76–84.
- Bunse T, Steigleder GK. (1991). The preservation of fungal cultures by lyophilization. Mycoses, 34(3-4), 173-6.
- Durrington, P (2003). "Dyslipidaemia". The Lancet, 362 (9385), 717–31.
- Eckert GP, Wood WG, Muller WE. (2005). Statins: Drugs for Alzheimer's disease? Journal of Neural Transmission, 112, 1057–71.
- Garrett IR, Gutierrez GE, Rossini G, Nyman J, McCluskey B, Flores A, *et al.* (2007). Locally Delivered Lovastatin Nanoparticles Enhance Fracture Healing in Rats. Journal of Orthopaedic Research, 25, 1351–57.
- Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution**,** 39:783-791.
- Glass N L and Donaldson G C (1995): Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology. 61(4), 1323.
- Gonzalez RC, Woods RE. Digital image processing. NJ: Prentice Hall, Englewood Cliffs; 2002.
- Henry T, Peter C. Iwen, Steven H. Hinrichs. (2000). Identification of *Aspergillus* Species Using Internal Transcribed Spacer Regions 1 and 2. Journal of clinical microbiology*,* 38 (4):1510-1515.
- Hinrikson, H.P., S.F. Hurst, L. De Aguirre & C.J. Morrison (2005): Molecular methods for the identification of *Aspergillus* Species. Medical Mycology; Supplement 1, 43: 129-137.
- Holzgrabe Ulrike, Bernd W.K. Diehl, Iwona Wawer (1998). NMR spectroscopy in pharmacy. Journal of Pharmaceutical and Biomedical Analysis, 17: 557–616.
- Jukes T.H & Cantor C.R. (1969). Evolution of protein molecules. (Munro H N, editor.) Mammalian protein metabolism, III. New York: Academic Press: 21-132.
- Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequence. Journal of Molecular Evolution, 16:111-120.
- Klawitter J, Touraj Shokati, Vanessa Moll, Uwe Christians, Jost Klawitter (2010). Effects of lovastatin on breast cancer cells: a proteo-metabonomic study. Breast Cancer Research, **12**, R16.
- Lai Long-Shan T. Tai-Her Tsai, and Te Chi Wang (2002). Application of Oxygen Vectors to *Aspergillus terreus* Cultivation. Journal of Bioscience and Bioengineering, 94, 453-459.
- Lankhorst P.P., M.M. Poot, M.P.A. de Lange (1996). Quantitative Determination of Lovastatin and dihydrolovastatin by Means of ^{1}H NMR Spectroscopy. Pharmacopeial Forum 22 (3), 2414–2422.
- Martin Kendall J and Paul T Rygiewicz (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology, 5, 28 doi: 10.1186/1471-2180-5-28.
- Munusamy Mohankumar, Ayyathurai Vijayasamundeeswari, Muthusamy Karthikeyan, Subramanian Mathiyazhagan, Vaikuntavasan Paranidharan, Rethinasamy Velazhahan (2010). Analysis of molecular variability among isolates of *Aspergillus flavus* by PCR-RFLP of the its regions of rDNA. Journal of plant protection research, 50 (4), 446-451.
- Negishi S, Huang ZC, Hasumi K, Murakawa S. Endo (1986): A Productivity of monacolin K (mevinolin) in the genus *Monascus*. Journal of Fermentation Engineering. 64:509–51.
- Palmer RH, Dell RB, Goodman DW. (1990). Lack of effect of lovastatin therapy on the parameters of whole-body cholesterol metabolism. Journal of Clinical Investigation, 86, 801–8.
- Pickin DM, McCabe CJ, Ramsay LE, Payne N, Haq IU, Yeo WW, *et al.* (1999), Cost effectiveness of HMG-CoA reductase inhibitor (statin) treatment related to the risk of coronary heart disease and cost of drug treatment. Heart, 82, 325–32.
- Praveen V.K and Savitha J (2012). Solid state Fermentation: An effective Method of Lovastatin Production by Fungy – A Mini review. The Open Tropical Medicine Journal, 5: 1-5.
- Raclavsky.V, J.Trtkova, V.Buchta, R.Bolehovska, M.Vackova, P.Hemal (2006). Primer 108 performs best in the RAPD strain typing of three *Aspergillus* species isolated from patients. Folia Microbiology, 51(2), 136-140.
- Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4(4), 406-25.
- Samson R.A., Peterson S.W., Frisvad J.C. and Varga J (2011). New species in *Aspergillus* section *Terrei*. Studies in Mycology, 69: 39–55.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731-2739.

- Teresa M. Diaz-Guerra, Emilia Mellado, Manuel Cuenca-Estrella, Lourdes Gaztelurrutia, Jose Ignacio Villate Navarro, and Juan l. Rodri´Guez Tudela (2000): Genetic Similarity among One *Aspergillus flavus* Strain Isolated from a Patient Who Underwent Heart Surgery and Two Environmental Strains Obtained from the Operating Room. *Journal of clinical microbiology*, Vol. 38, No. 6, p. 2419–2422.
- Tobert J A. (2003). Lovastatin and beyond: The history of the HMG CoA reductase inhibitors. Nature Review Drug Discovery, 2(7), 517–526.
- Upendra R. S., Pratima Khandelwal (2016). Physical mutagenesis based strain improvement of *Aspergillus sp.* for enhanced production of lovastatin. International Journal of Pharmacy and Pharmaceutical Science, 8 (7): 163-167.
- Upendra R.S, Pratima Khandelwal, Z.R. Amiri and Baboo M. Nair (2014b): Invited session key lecture on "Optimization of process for producing lovastatin from novel fungal isolates". Proceedings of the IV International conference on SUTPBM-2014: 28-34.
- Upendra R. S**,** Pratima Khandelwal, Z. R. Amiri, Aparna. S, Archana. C and Ashwathi. M (2014a). Isolation and Characterization of lovastatin producing food grade fungi from oriental foods. World Journal of Pharmaceutical Research, 3(10), 1404-1414.
- .
- Upendra R.S., Pratima Khandelwal, Amiri Z R, Swetha L and Mohammed Ausim S (2013b). Screening and Molecular Characterization of Natural Fungal Isolates Producing Lovastatin. Journal of Microbial and Biochemical Technology, 5(2), 025-030.
- Upendra R.S., Khandelwal P and Mohammed Ausim (2013a): A novel approach for enhancement of Lovastatin production using *Aspergillus* species. *International Journal of Agriculture and Environmental Biotechnology,* 6:779-786.
- World Health Organization, (2010). Global status report on non-communicable diseases. Geneva, World Health Organization.
- World Health Organization, (2011). Global atlas on cardiovascular disease prevention and control. Geneva, World Health Organization.
- Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD, Penn LZ (2001): Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia (Baltimore), 15, 1398–407.
- Zhao J, Fanrong Kong, Ruoyu Li, Xiaohong Wang, Zhe Wan, and Duanli Wang (2001): Identification of *Aspergillus fumigatus* and Related Species by Nested PCR Targeting Ribosomal DNA Internal Transcribed Spacer Regions. Journal of clinical microbiology, 39 (6): 2261–2266