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Isolation and characterization of lovastatin producing fungi; investigating the antimicrobial and extracellular enzymatic activities

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Abstract

Lovastatin is the drug used to reduce blood cholesterol serum levels. The present study includes the isolation of new fungal species such as *Aspergillus terreus* to produce lovastatin and other key secondary metabolites that can be effectively used as antimicrobial agents. New strain was characterized by morphological and molecular identification. Lovastatin was produced by *Aspergillus terreus* by submerged fermentation, and the levels of lovastatin produced were calculated to be 405mg/l. The initial characterization of crude extract was done by yeast inhibition bioassays, thin layer chromatography and Fourier transform-Infrared spectroscopy and later confirmed by high performance chromatographic analysis. The antibacterial and antifungal activities of crude extracts were determined including the assessment of enzymatic potential of fungal *Aspergillus terreus*. The study concludes that *Aspergillus terreus* is the newly isolated fungal strain that is a potent producer of lovastatin, exhibiting various other biologically important characteristics that can be used for the exploitation in various industries.

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Introduction

Statins are the hypercholesterolemic drugs used to reduce the serum cholesterol levels in humans. Lovastatin is the first statin approved by FDA1987 USA. They act as a competitive inhibitor of HMG-CoA reductase, and enzyme involved in catalysis of the rate limiting step for the synthesis of cholesterol in humans and ergosterol in yeast. Lovastatin has been reported to be produced by various species of fungi including *Aspergillus terreus* (Subhan *et al.*, 2016).
Soil is a rich source of organic nutrients abundant in filamentous fungi. A number of extracellular products and extracts showing antimicrobial have been reported mainly from *Aspergillus* and *Penicillium* species. Since the discovery of penicillin, fungi research diverted to these microbes for the production of bioactive secondary metabolites such including antifungal, antibacterial, mycotoxins, immunosuppressant, and cholesterol-lowering agents (Petit *et al.*, 2009).

Microorganisms are being discovered for novel antibiotics that could replace the existing drug, which are non-effective against resistant microbial pathogenic strains. Such efforts subsequently led to discovery of numbers of antibiotics that can fight against pathogenic microbes. Importance of new novel antimicrobial drug and the ineffectiveness of current antibiotics are due to the re-emergence of resistant pathogens and new diseases (De Castro *et al.*, 2014).

The present study includes the isolation of lovastatin producing fungi, exploring its extra enzymatic potential and antimicrobial activities. That can generate pathways leading to novel drug design as antimicrobial agent and blood cholesterol reducing agent.

Materials and methods

Isolation of fungal isolates

Agricultural soil samples were collected from the different regions of Pakistan, targeting the wheat fields. The soil samples were taken from the depth of about 12 cm from the ground. The samples were kept in sterilized polyethylene bags and stored at 4°C till further analysis. Serial dilution plate technique was used to isolate fungal strains.

Isolation of fungal strains was carried out using sabauroud dextrose agar (SDA) medium. Fungal strains isolated were identified on the basis of their morphology by macroscopic and microscopic structures. Isolated and identified strains were maintained at fresh SDA slants and stored at 4°C.

Screening of isolates and submerged fermentation

Purified and identified fungal strains were screened for lovastatin production. Spores were diluted to 5 x 10^6 concentrations. Haemocytometer was used for spore count(Upendra *et al.*, 2013). Modified soybean meal medium (g/l) was used as screening medium; Lactose 50, Soybean meal 20, K_2HPO_4 1, $NaNO_3$ 1, $MgSO_4.7H_2O$ 0.5, Peptonized milk 10(Srividya, 2014). Each flask was inoculated by 5x 10^6 spores, containing soybean meal medium. The flasks were subjected to fermentation in shaker incubator at 30 °C and 150 rpm for 12 days.

Downstream process

At the end of 12 days fermentation, the pH of broth was lowered to 2 with 1N HCl. An equal volume of ethyl acetate was added in each flask and kept overnight for incubation at room temperature. The fermentation samples were subsequently centrifuged at 1100rpm for 10 min.

The organic layer on the top was separated, supernatant was filtered through Whatman filter paper no.1.The biomass was separated for dry biomass estimation (Upendra *et al.*, 2013).Dry cell mass was measured by filtration of cell culture/fermentation broth after centrifugation, on pre-weighed Whatman no. 1 filter paper. The biomass was dried in an oven at 65 °C for one hour. Weight of fungal biomass was calculated in mg/l. The organic phase was separated from fermentation broth by using separating funnel for each sample. EtOAc was used as an organic solvent which was concentrated using a rotary vacuum evaporator. The dried residue was dissolved in acetonitrile (ACN). The crude extracts obtained for each sample were used for qualitative and quantitative analysis (Lingappa K. *et al.*, 2004).

Yeast growth inhibition bio assay

Yeast growth inhibition bioassay was done by agar well diffusion method. *Candida albicans*ATCC90028was used as test organism. Cell suspension of *C. albicans* ATCC 90028 was prepared in normal saline and spread onto the YEPD media plates. Wells were made using a sterile borer of 8mm in diameter.100µL of fungal extracts (10 mg) was loaded into the wells. EtOAc was used as control. Plates were incubated at 30 °C for 24hrs (Lingappa K. *et al.*, 2004).

Fourier transform-infrared spectroscopy (FTIR)

FTIR measurements of the samples were performed by Tensor 27 (Bruker) FTIR-Spectrophotometer equipped with Zn Se ATR. A small amount of the crude sample was directly placed on the diamond disk. Sample was scanned for absorbance over the range from 4000 to 420 wave numbers (cm^{-1}) at a resolution of 1 cm^{-1} for each interferogram. An IR spectrum for different set of compounds was analysed.

Thin layer chromatography (TLC)

TLC Silica gel 60 F_{254} aluminium plates were used to perform thin layer chromatography. TLC plate was activated in by dry heat at 100°c for 1hr. Dichloromethane and ethyl acetate in a combination (70:30) was used as mobile phase. Sample and pure standard of lovastatin were spotted on TLC plate and allowed to dry (Samiee *et al.*, 2003). Plates were observed under UV transilluminator (254 nm). Iodine crystals were used to develop the plates. R_f value were calculated as the spots moved on the TLC plate by the following formula

$$Rf = \frac{\text{distance travelled by sample}}{\text{distance travelled by solvent}}$$

High performance chromatography (HPLC)

HPLC was carried out using reverse phase C-18 hypersil column (5 µm x 4 mm i.dx125mm length) using Agilent technologies 1220 LC. The stock standard of 500 µg/ml lovastatin was prepared. Acetonitrile was used with water in ratio of 60:40 in an isocratic system which was acidified with 0.1% orthophosphoric acid as mobile phase. The peaks were analysed at 238 nm while the flow rate was kept constant as 1.5mL/min. The injection volume was 20 µL.

Molecular identification of fungal isolate

DNA of lovastatin producing fungus was isolated using the protocol of Gardes and Burns 1993 (Gardes & Bruns, 1993). The isolate was characterized on the basis of 18 S rRNA. The primers that were used include the ITS $_1$ and ITS $_4$ as upstream and downstream primer respectively. The PCR assay was performed with 1µl of DNA template in a total reaction volume of 50 µl. The PCR reaction mixture contained 1 µl of upstream primer (10 µM) and 1µl downstream primer (10 µM),nuclease free water (22 µl) and GoTaq® green master mix, 2X (25 µl) (Promega corporation). The PCR was performed in Bio-Rad thermo cycler T100.The PCR condition include the initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72 °C for 1 min with total number of 35 cycles. The amplified PCR products were purified using Isolate II PCR and gel kit 50 preps (Bioline NSW Australia). The nucleotide blast program BLASTn ,the standard nucleotide-nucleotide basic local alignment search tool (National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA) was used to search the sequence with nucleotide homology. The sequence obtained from NCBI gene bank with maximum scores and high percentage of homology were retrieved and were aligned. Non-aligned sequences were trimmed off.

Enzyme assays

Amylase assay

Amylase production was analysed by point inoculation of fungal isolate on nutrient agar plates supplemented with 1% starch. Plates were incubated at 30°C for 24-48 hrs. Iodine crystals were used as an indicator. The culture plates were fumigated with iodine crystals. Zones of starch hydrolysis by amylase were observed around the colonies.

Cellulase assay

Cellulase production was analysed by point inoculation of fungi on agar plates supplemented with 1% carboxymethly cellulose (CMC). Plates were incubated at 30 °C for 24-48 hrs. Culture plates were flooded with Congo red dye (1mg/mL) for 10-15 min and detained with 1N normal saline for at least 30 minutes. Cleared zones of hydrolysis were observed around the colonies with cellulase activity.

Protease assay

Protease production was analysed inoculating the fungi on 1% casein agar media. Plates were incubated at 30°C for 24-48 hrs. Plates were flooded with 15 % glacial acetic acid. Opaque zone of clearance around the colonies determine the proteolytic activity.

Antifungal bioassay

Antifungal bioassays were done by disc diffusion method. Six different species of candida were used as test organisms, including *Candida albicans* ATCC 90028*, Candida lusiataniae* 634*, Candida glabrata* ATCC 90030*, Candida guilliermondii* 176*, and Candida krusei* ATCC 6258 *Candida tropicalis* ATCC 750.All the strains were obtained from School of Science, RMIT Australia. Fluconazole (100 mM) was used as a positive control. Cell suspension of yeast isolates was prepared in normal saline. YEPD was used as growth medium. Crude extract (10 µL) was loaded onto the paper disc of 5mm and allowed to dry. The dried discs were placed on the YEPD plates. Plates were incubated at 30 °C for 24hrs.

Antibacterial bioassay

Antibacterial bioassays were done by disc diffusion method. Five different gram positive and gram negative strains isolates were used as test organisms, including *Staphylococcus aureus* 344/24, *Bacillus subtilis* ATCC 633 *and E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 *and Klebsiella pneumonia* ATCC 13833. All the strains were obtained from School of Science, RMIT Australia. Ampicillin (0.2mg/ml) was used as a positive control. Cell suspension of bacterial isolates was prepared in normal saline. Nutrient agar was used as growth medium. Crude extract (10 µL) was loaded onto the paper disc of 5mm and allowed to dry. The dried discs were placed on NA media plates. Plates were incubated at 37 °C for 24hrs.

Statistical analysis

Each of the experiment was run in triplicates. Mean values with standard deviation are presented. Prism graph pad was used analyse the data.

Results and discussion

Different fungal isolates were obtained from various soils of Pakistan. 56 strains were selected from these different natural environment and agricultural soils. Pure fungal isolates were identified based on their macroscopic and microscopic features.

The values were compared with standard reference book Compendium of soil fungi (Domsch *et al*., 1980). Out of total 56 selected strains 34 (61%) were identified as member of genus *Aspergillus* and 22 (39%) were *Penicillum* spp. It shows that *Aspergillus* dominance is slightly higher than *Penicillum*in agricultural soil (Table 1).

Table 1. Isolation of fungal strain and *Aspergillus* predominance.

Sr no	Total fungal strains	*Aspergillus* sp	*Penicillum* sp
1	56	34(61%)	22 (39%)

Qualitative screening parameters

Qualitative bioassay was performed for screening of lovastatin producer. Out of 56 isolated strains only 26 strains gave positive zone of inhibition against *Candida albicans* ATCC 90028.

Further results were confirmed by HPLC, and only one strain was found to be the producer of lovastatin. The strain MF-7 gave the highest value for the crude extract as well as zone of inhibition. The production of lovastatin was independent of the biomass (Fig. 1)

Table 2. Antifungal activity analysis.

Sample and standards	*Candida tropicalis* Wm30	*Candida albicans* ATCC90028	*Candida lustani* 634	*Candida galabrata* ATCC 90030	*Candida krusie* ATCC 6258	*Candida guilliermondii 176*
Crude extracts	8±0.60	16±0.01	9±0.43	10±0.23	9±0.86	9±0.25
Lovastatin std	12±0.01	10±0.90	10±0.01	11±0.02	10±0.11	10±0.02
Control-ve	5±0.12	5±0.09	5±0.05	5±0.26	5±0.15	5±0.21
Control+ve	12±0.17	15±0.20	11±0.54	13±0.12	13±0.82	±0.09

Values (mean ± SD) are average of three samples of each fungal extract, analyzed individually in triplicate (n =1x3) *std=Standard, +ve=Positive, Negative= -ve.

Table 3. Antibacterial activity analysis.

Sample and standards	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumonia* ATCC 13833	*Staphylococcus aureus* 344/24	*Escherichia coli*ATCC 25922	*Bacillus subtilis* ATCC 633
Crude extract	8±0.82	10±0.23	9±0.65	7±0.01	18 ±0.2
Lovastatin std	10±0.01	11±0.20	10±0.12	12±0.01	15±0.3
Control-ve	5±0.18	5±0.23	5±0.07	5±0.01	5±0.01
Control+ve	15±0.01	12±0.12	13±0.82	12±0.09	13±0.65

Values (mean ± SD) are average of three samples of each fungal extract, analyzed individually in triplicate (n =1x3) *std=Standard, +ve=Positive, Negative= -ve.

Molecular identification of fungal isolate

The strain was found to be *Aspergillus terreus* (New strain) and the accession number after submission to gene bank was retrieved and allotted as KX011595 (Fig. 2.).

High performance chromatography (HPLC)

The samples were run on the HPLC. The peak of the pure standard eluted at retention time of 1.91 mins (Fig. 3B.). The peak of the sample that was found to be positive eluted with the same retention time which was found to be 1.91 min.

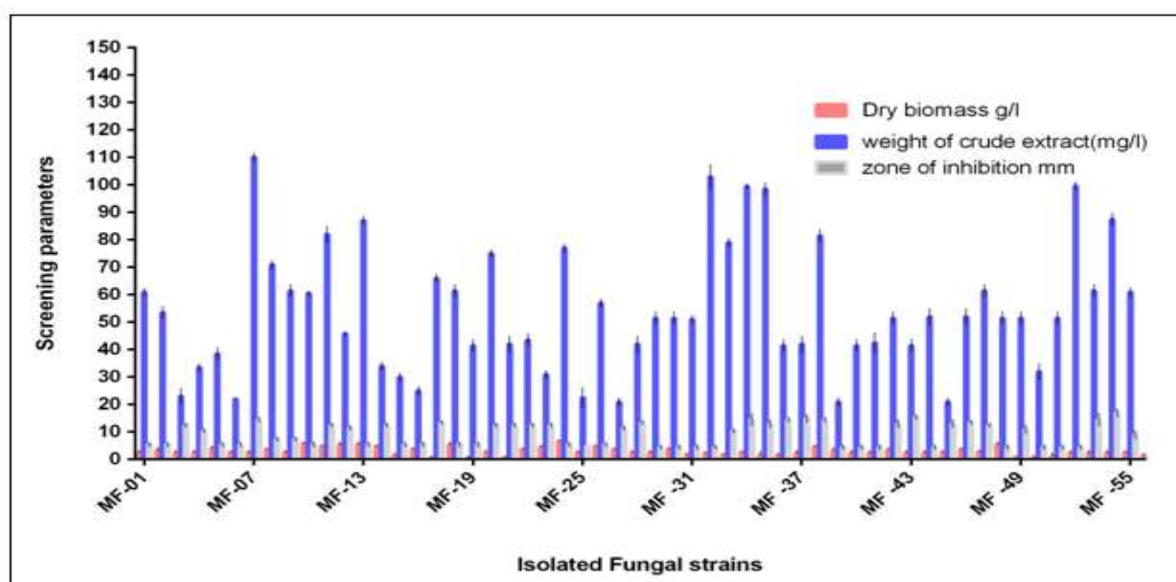

Fig. 1. Initial screening of fungal isolates on the basis of amount of biomass (g/l), crude extract (mg/l) zone of inhibition (mm).

The amount of lovastatin was quantified by drawing HPLC calibration curve at different concentrations with the standard (Fig. 3A.).

The results were further confirmed by analysis of the blank media, which gave no peak in at specified retention time (Fig. 3C.)

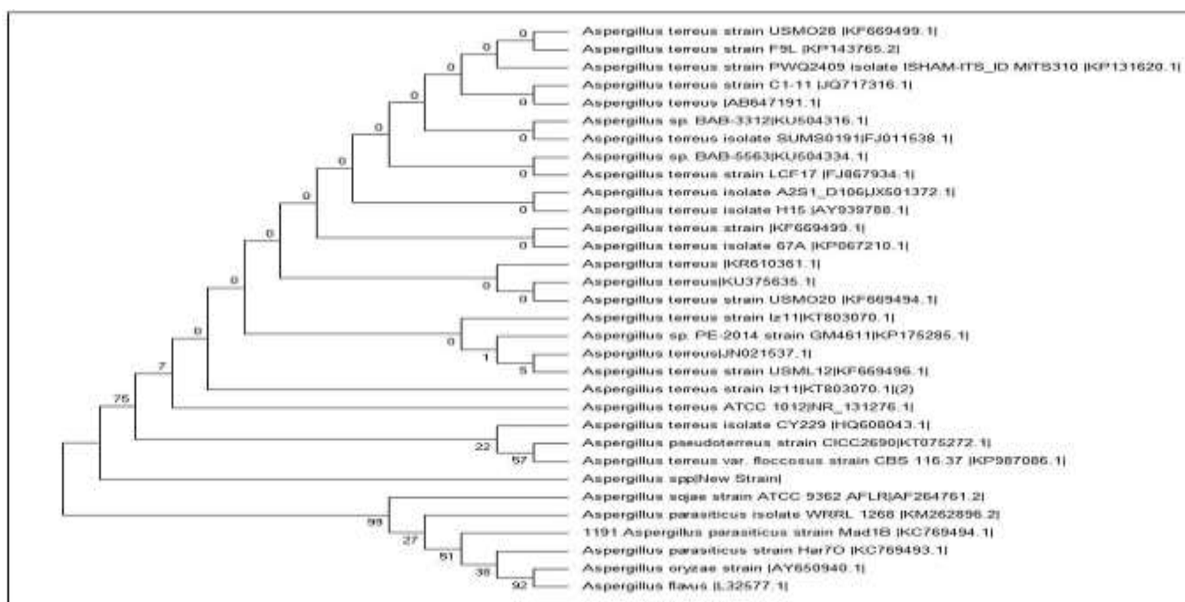

Fig. 2. Dendrogram showing relationships between 32 accessions *Aspergillus* species based on genomic sequence

Thin Layer Chromatography (TLC)

The TLC of standard was performed on silica gel plates. Dichloromethane and EtOAc in ratio of (70:30) were used as mobile phase. The spot of inoculation was marked. The distance travelled by the spot was measured and R_f value was found to be 0.3(Fig. 4.), that was similar to the R_f value of the standard pure lovastatin.

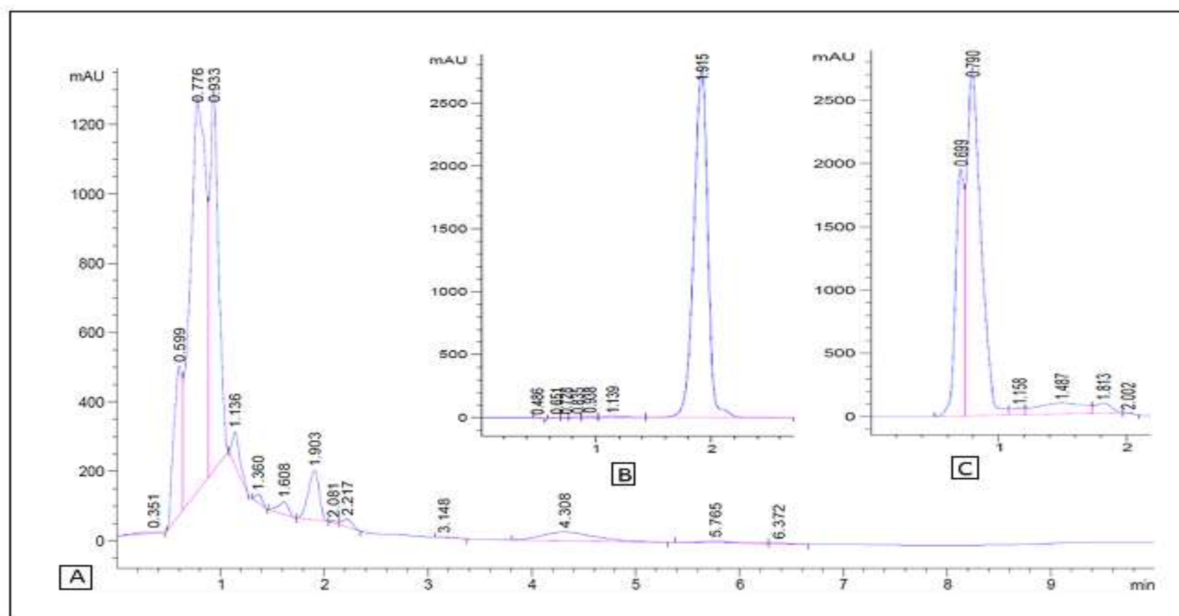

Fig. 3. HPLC chromatograms of (A) Fermented extract (B) Pure lovastatin standard; (C) Pre-culture media extract.

Fourier transform-Infrared spectroscopy (FTIR)

The FTIR results were analysed with the standard interferogram of pure lovastatin. A narow stretch at 3400-3500 describes the presence of broad hydroxyl peak (Rajeswari *et al.*, 2012).

The aliphatic and vinylic CH stretching peaks between 2900 and 3000 $cm^{-1}$and the peak at 1442.89$cm^{-1}$for the two carbonyl ester groups, and alkyne CH bend between 680–610 are the key peaks for the lovastatin compound that appeared in the crude samples as well.

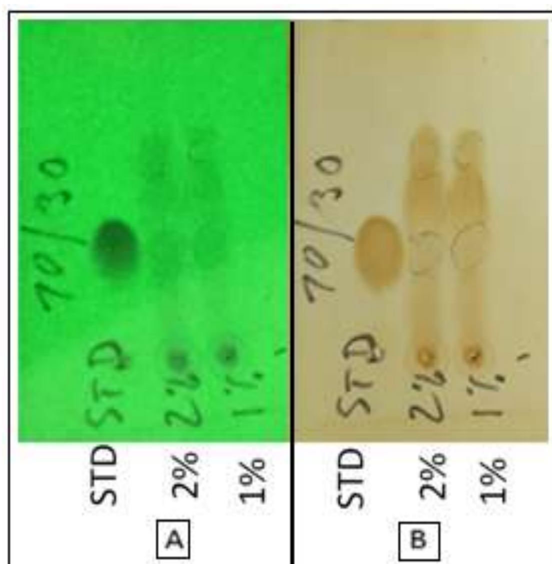

Fig. 4. TLC plate showing the two (1%, 2%) concentrations of crude extract with the pure standard (STD) (A) under short UV range (254 nm) (B) stained with Iodine crystals.

CH stretching absorptions all occur below 3000 cm^{-1}.Any band structures observed between 3150 and 3000 cm^{-1} are almost indicative of unsaturation (C=C-H) and aromatic rings. The other most important set of bands are the aromatic ring vibrations centred around 1600 and 1500 cm^{-1}, which usually appear as a pair of band structures in the FTIR of lovastatin (Fig.5a and 5b.).

Antimicrobial assays

The antifungal and antimicrobial activities were tested against various candida species, gram positive and gram negative bacterial species. Lovastatin and crude extract was active against most of the yeast isolates and some of the bacterial species as well Table 2 and Table 3. Lovastatin has been previously reported as antifungal and antibacterial agent as well (Qiao *et al.*, 2007, Thangamani *et al.*, 2015).

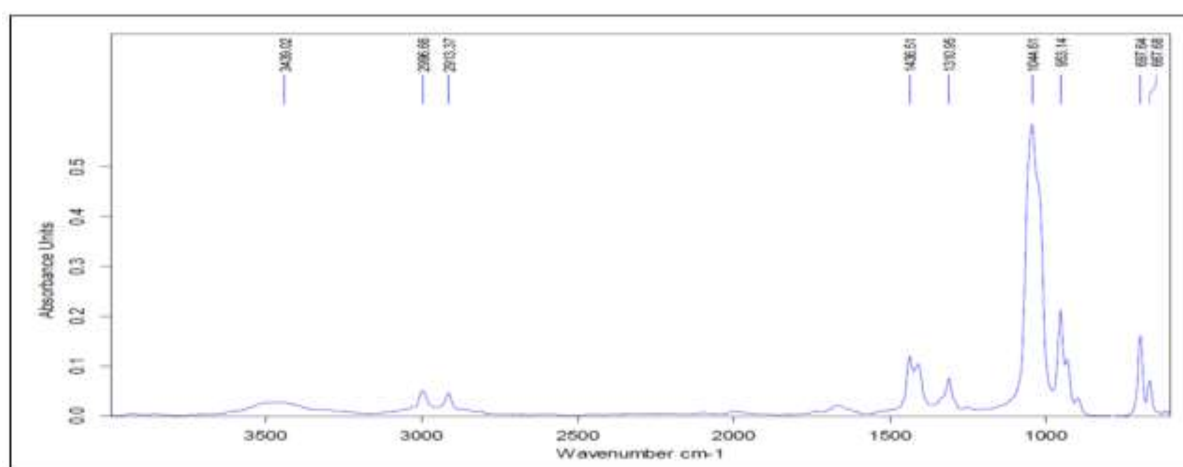

Fig. 5a. FTIR analysis of lovastatin produced by Aspergillus terreus.

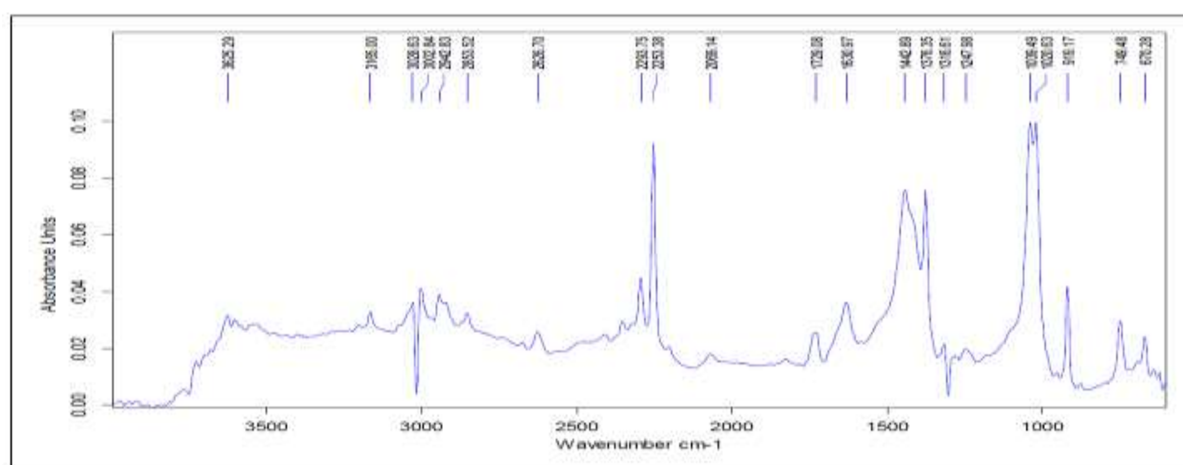

Fig. 5b. FTIR analysis of standard lovastatin.

In soil microbial life is competitive and different microbes compete for compounds necessary for their growth and survival such as carbon, nitrogen and phosphate. Successful intra and inter species competition may be ensured by production and secretion of biomolecules, which inhibits growth of other organisms by interfering with their metabolism. As a result of these competitions the microorganism produces certain antimicrobial compounds. Fungal species including entophytic fungi have a lot of potential to produce secondary metabolites, which are major source of antimicrobial compounds (Avinash *et al.,* 2015).

Enzyme assays

The isolated strain of *Aspergillus terreus* was found to be the producer for all the enzyme analysed, it was found to be the hyper producer for the amylase, but it also had the ability to produce cellulase and amylase. Cellulases and amylases are very important for various industries, they can be produced by various other sources but the demand for microbial based enzyme that is cheap, effective and highly stable is highly desirable. *Aspergillus* species have been studied and reported to produce cellulases extensively and efficiently (Flachner and Réczey, 2004).

Conclusion

The newly isolated fungi, *Aspergillus terreus* KX011595 is a potent producer of lovastatin. It exhibits various antimicrobial activities. Lovastatin can be used to treat various other diseases. The newly isolated strain has a capability to produce extracellular enzymes which finds its applications in various industries. The present study further suggests the isolation and identification of the other potential metabolite produced by these fungi, and investigation of their biological properties.

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