Journal of PharmaSciTech
ISSN: 2231 3788 (Print)
ISSN: 2321 4376 (Online)
Research Article

Purification and Characterization of Lovastatin from *Aspergillus terreus (JX081272.1)*

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Abstract

Attempt has been taken for identification and characterization of lovastatin producing strain from soil sample. Ten different samples were collected from of Subarnarekha River bank, India. Sample was diluted and pure colony was screened. The potential lovastatin producing strain was identified and characterized by microscopic and molecular techniques. 18S rDNA technique was applied for molecular characterization and the sample was identified as *Aspergillus terreus* having gene bank accession number is *JX081272.1.*

Keywords: identification, characterization, 18sr-DNA technique, Gene bank, *Aspergillus terreus*

Introduction

Lovastatin, a cholesterol-lowering drug is an important secondary metabolite from fungi through the polyketide pathway.[1] *Aspergillus terreus* is a fungus (mold) commonly used in industry to produce important organic acids, such as itaconic acid and *cis-aconitic* acid. The fungi, *Aspergillus terreus* is widely used for commercial lovastatin production. As a competitive inhibitor of 3-hydroxymethylglutaryl-CoA reductase, lovastatin has been used in medicine to lower the level of the endogenous cholesterol in a human organism. Keeping in view of the demand, usefulness and cost effectiveness of the lovastatin in the industry, it is very important to focus on their overall production. The considerable research works have been carried out using *Aspergillus terreus* for the production of lovastatin.

A. terreus has a worldwide distribution but more frequently occurs in tropical and subtropical areas. It is a telluric fungus contributing to the decomposition of organic matter because of its cellulolytic, lipolytic and amylolytic activities. *Aspergillus terreus* belongs to the group A. flavipes.[2] With a worldwide distribution, it is the most commonly isolated species from cultivation soils but it also occurs in non-cultivated soils.[3] It is very frequently found under tropical and subtropical climates as a contaminant in food storage sites. *A. terreus* may cause opportunistic infection in people with deficient immune systems. It is refractory to Amphotericin B therapy.[4]

Recent phylogenetic studies have shown that Aspergillus section Terrei includes the species A. terreus sensu stricto, *Aspergillus carneus, Aspergillus niveus, Aspergillus alabamensis, and A. terreus var. aureus.*[5] These species are morphologically indistinguishable, and can only be identified with molecular techniques. In addition, molecular identification helps us to better understand to identify and characterize.[6]

In this study we collected the soil sample from Subarnarekha River Bank Jharkhand, India. The strain which is produced lovastatin was isolated and characterized.

Materials and Methods

Chemicals and Analysis

The chemicals used were of analytical grade commercially available in India. The software package, MEGA-4 software was used for Molecular Evolutionary Genetics Analysis.

Microorganism

The microorganism used in the present study was isolated from the soil sample of Subarnarekha River, India. Subarnarekha River (also called Swarnarekha River) flows through the Indian states of Jharkhand (23°18′N 85°11′E), West Bengal and Orissa. The Subarnarekha River passes through areas with extensive mining belt of copper and uranium ores. However, there are some food (Bakery and Diary) industries also set up near the bank of the Subarnarekha River. As a result of the unplanned effluent discharges activities of the mining and other industries, the river is getting polluted. Therefore, the soil near the Subarnarekha River bank is enriched with resourceful microbial flora for isolation of potent microbial strain. These strains may sustain with the bakery and diary industries' waste rich in oil and fat.

Isolation and screening of lovastatin producing strains

Isolation study was carried out from ten soil sample which was collected from different places. The soil was diluted with saline

water and cultured for 7 days at 28^{0}C in Potato Dextrose Agar (PDA) media amended with chloramphenicol 150 mg/L to decrease the amount of bacterial contamination. After seven days, fungi were observed, and individual hyphal tips of the various fungi were removed and placed on a new PDA medium, and incubated at 28°C for at least 7days.[7] To screen the lovastatin producing potent fungi strain, lovastatin production was carried out using isolated fungal strains through submerged fermentation. In the fungal fermentation, a two-stage technique was applied, which included seed (for cell growth) and production (for lovastatin). For seed culture, the Erlenmyer flasks (250 ml) containing 30 ml medium were inoculated with 108 spores per ml of isolated fungi and then incubated at 28°C in a rotary shaker at 150 rpm. The spore suspension of various fungi was prepared separately by adding 5 mL of saline solution to hyphal tips of the various fungi which was scraped from PDA plate and were shaken vigorously for 1 min. The seed medium used was composed of (g/L, w/v): Lactose, 12; yeast extract (YE)-5; soybean meal (SM)-5; glucose-15; CaCO3-1.5 and a trace element solution, 10 mg/L (pH7.5).[8]After 24h in seed, the most prominently grown mycelia were transferred to the production stage.

Identification and characterization lovastatin producing strain

Microscopical identification of the fungal isolate with maximum lovastatin producing strain was carried out. Genomic identification of the strain was done using 18S rDNA technique. Genomic DNA was isolated from the pure culture pellet.[9] The ~1.5 kb 18S rDNA fragment was amplified using the primers; 27f (50-AGA GTT TGA TCC TGG CTC AG-30) as forward and 1492r (50-TAC GGT TAC CTT GTT ACG ACT T-30) as reverse primer.[10] Sequence data was aligned and analyzed for finding the closest homologs for the microbe.

Purification of lovastatin

The fermented broth was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was discarded and fermentation broth was adjusted to pH 3.0 by concentrated HCl followed by addition of equal volume of ethyl acetate to the whole fermentation broth. Extraction was carried out on a rotary shaker at 180 rpm at ambient temperature for 2h. The samples were subsequently centrifuged at 1500× g for 15 min and the organic phase was collected. The organic phase was completely evaporated and the dried residue was used for for HPLC analysis and characterization purpose.[11]

Characterization of purified lovastatin

HPLC analysis of lovastatin

The dried residue was dissolved in 1.5 ml acetonitrile. The samples were filtered through 0.22μm filter paper. The sample was qualitatively and quantitatively analyzed using HPLC. For the analysis of sample, HPLC system (Waters ™ 600) equipped with UV/Visible Detector was used. Chromatographic separation of lovastatin was performed on a C_{18} hypersil column (4.6mm×250 mm; 5 μm particle size; Waters, USA). Mobile phase used was acetonitrile and 0.1% phosphoric acid (60:40, v/v), at a flow rate of 1 ml/min. Temperature of the column oven was maintained at 30°C. Sample (20μl) was injected and analyzed at 236 nm using UV-Visible detector.[11]

Results and Discussion

Isolation and identification of phenol-degrading strains

The results microscopical study has been presented in the Fig. 1. 18S rDNA sequence analysis identified the strain as *Aspergillus terreus*.[4] 18S rDNA gene sequencing was submitted in gene bank and gene bank accession number is JX081272.1.

A BLAST search of all of the sequences was performed to identify the isolates. In order to investigate the presence of cryptic species, we performed a phylogenetic study (Fig. 2).[4] The neighbour-joining method was used to construct the phylogenetic tree. The data were first analyzed by use of the Tamura-Nei parameter distance calculation model with gamma-distributed substitution rates, and the neighbour-joining tree was constructed with MEGA version 4. A bootstrap analysis with 1000 replications was performed to determine the support for each clade. Reference sequences retrieved from GenBank were included.[4]

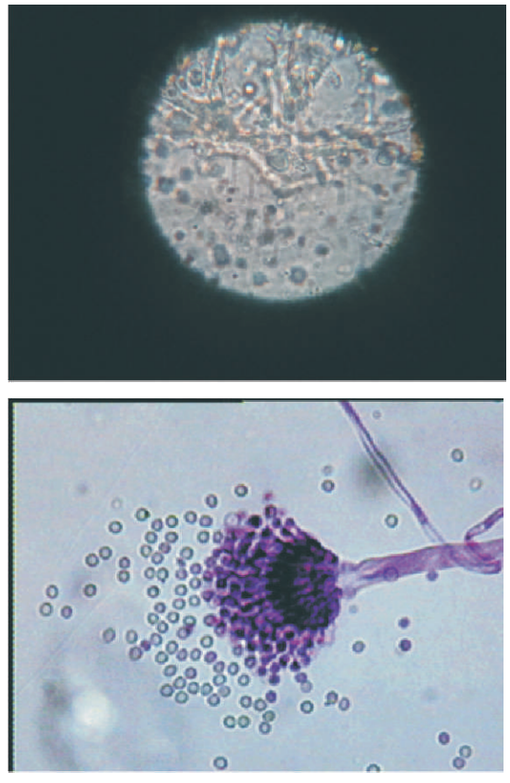

Fig. 1: Microscopic analysis of lovastatin producing strain

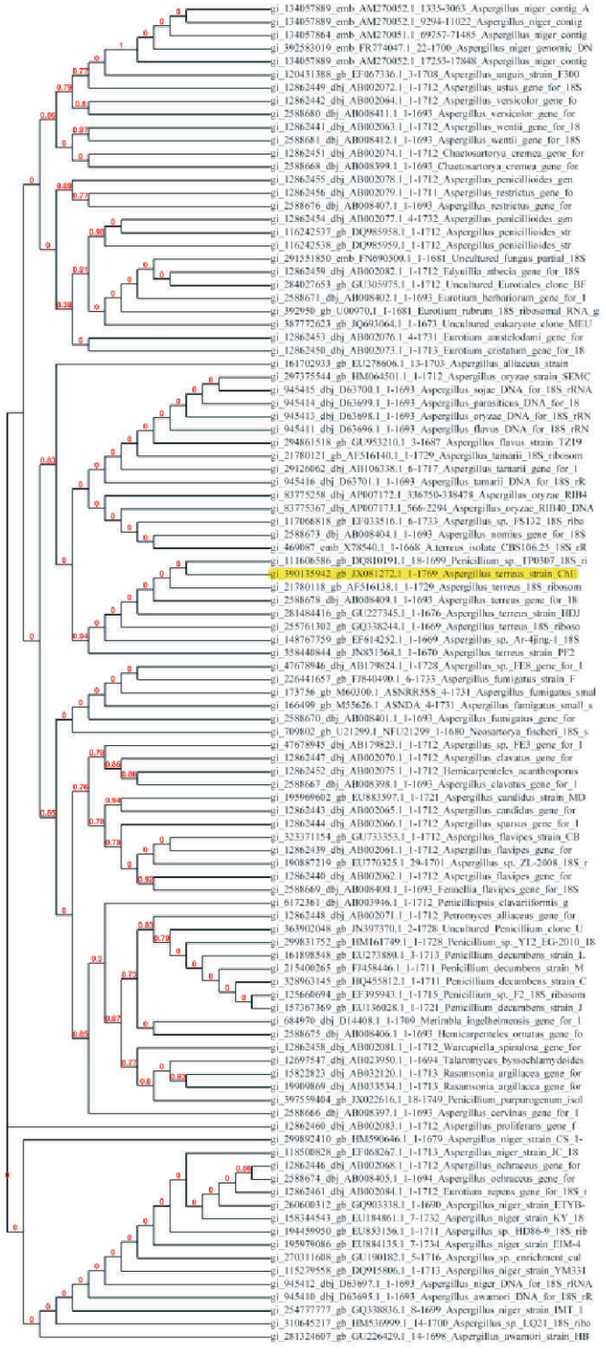

Fig. 2: Phylogenic analysis of isolated sample

Purification and characterization

The fermented broth was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was discarded and fermentation broth was adjusted to pH3.0 by concentrated HCl followed by the addition of equal volume of ethyl acetate to the whole fermentation broth. Extraction was carried out on a rotary shaker at 180 rpm at ambient temperature for 2h. The samples were subsequently centrifuged at 1500× g for 15 min and the organic phase was collected. The organic phase was completely evaporated and the dried residue was used for HPLC analysis and characterization purpose.

HPLC analysis of lovastatin

The dried residue was dissolved in 1.5 ml acetonitrile. The samples were filtered through 0.22μm filter paper. The sample was qualitatively and quantitatively analyzed using HPLC. The HPLC chromatogram of lovastatin (standard) and purified sample are shown in Fig. 3a-b. From the chromatogram it was evident that the retention time of our purified sample is 7.529 min (Fig. 3b) which resemble with retention time (7.534) of standard lovastatin (Fig. 3a). The chromatogram represented that the sample is lovastatin having 95.60% purity.

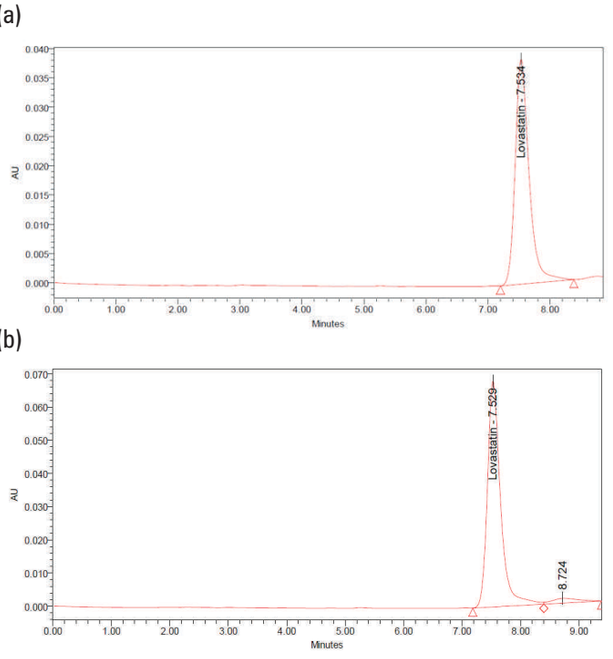

Fig. 3: HPLC chromatogram of (a) standard lovastatin; and (b) sample

Conclusion

Microscopic and molecular characterization of isolated strain which produced lovastatin was successfully carried out to identify the fungal strain. Microscopic and Molecular (18 sr DNA) technique was applied to identify the fungal strain and the strain was Aspergillus terreus having the gene bank accession number is JX081272.1

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