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Research Article

Production of Lovastatin by Soil Microfungi *Rhizopus oryzae*

S Rajkumar Immanuel* and P Anusha

Research and Post Graduate Department of Botany, The American College, Madurai, Tamil Nadu, India

***Corresponding Author:** S Rajkumar Immanuel, Research and Post Graduate Department of Botany, The American College, Madurai, Tamil Nadu, India.

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Abstract

Natural statins like lovastatin which is mainly produced by fungal strains act as an inhibitor of HMG-CoA reductase, an enzyme involved in the biosynthesis pathway of cholesterol. The statins decrease the level of cholesterol in blood. The aim of the study was to determine the lovastatin producing potential of soil micro fungi. In the present investigation 10 soil fungi were screened for lovastatin production using shake flask culture. The screening of potential lovastatin producing fungus was carried out using bioassay method against *Saccharomyces cerevisiae* as an indicator microorganism in the YPDA medium by measuring the zone of inhibition. The diameter of zone of inhibition ranged from 3.7 to 4.8 mm in *Rhizopus oryzae* which produced a maximum zone of inhibition. Among the lovastatin producing strains *Rhizopus oryzae* was found to be utilized maximum substrates. Genomic identification of the strain was done using 18S rDNA technique. The DNA of *Rhizopus oryzae* was extracted and purified by agarose gel electrophoresis and sequenced using the ~1.5kb 18S rRNA fragment and was amplified using the primers. Phylogenetic analysis performed by the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbor–joining method and the identity was confirmed. The fungi *Rhizopus oryzae* was screened for lovastatin production using HPLC analysis after extraction of the compound from the fermentation broth with ethyl acetate. Lovastatin quantification was carried out on extracts from the culture broth and a production level of 20.39 mg/l was recorded. Hence, from this investigation it was concluded that rapid method of determination of lovastatin can also be employed to screen lovastatin producing fungal isolates from soil and the isolate *Rhizopus oryzae* being recommended for further studies as a potent lovastatin producer.

Keywords: *Rhizopus oryzae*; Microfungi

Introduction

Lovastatin a fungal secondary metabolite, acts as one of the competitive inhibitors of the enzyme hydroxyl methyl glutaryl coenzyme A (HMG-CoA) reductase, which catalyses the conversion of HMG- CoA to mevalonate during cholesterol biosynthesis. Lovastatin is the world's widely prescribed drug to combat hypercholesterolemia and was the first statin drug which was approved by United States Food and Drug Administration in the year 1987. Lovastatin is one among the statin compound commercially derived from fungi. Fungi are important sources for the production of several pharmaceutical compounds. They produce a large variety of compounds mainly through the polyketide biosynthesis pathway. In the present study, local potential fungal lovastatin producers were isolated from various soil samples and screened for the highest activity in a shake flask system. The production was confirmed by various analytical methods.

Materials and Methods

Isolation and characterization of soil fungi

Soil samples were collected in sterile containers from various locations of Madurai, India and diluted up to 10^{-6}. The serially diluted soil samples were plated on to potato dextrose agar (PDA) supplemented with 50mg/L streptomycin to suppress the bacterial growth and inoculated at 30°C for 3-4 days until fungal growth was noted. Fungal identification was done as described by (Subramanyam, 1982).

Identification of fungus by 18S rRNA Sequencing

Fungi were grown in 50 ml Potato Dextrose Broth for 5-6 days at 28°C. The mycelia was harvested and washed with distilled water and ground with liquid nitrogen. The nucleic acid was extracted using Cetyltrimethyl ammonium bromide (cTAB) method. Polymerase chain reaction was done using universal primers ITS1 and ITS4. BLAST analysis was done with available NCBI database.

Phylogeneticand molecular evolutionary analysis

Fungal DNA was extracted with phenol chloroform protein extraction, precipitated by 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 27F (5'AGAGGTTTGATCMTGGCTCAG 3') and 1492R – (5' TACGGYTACCTTGTTACGACTT 3').

PCR product was sequenced with the big dye terminator cycle sequencing ready reaction kit on an ABI3730XL genetic analyzer. Phylogenetic tree was performed by the maximum parsimony (MP) method and molecular evolutionary relationship were inferred by using neighbour-joining method (Altschul., *et al*. 1990). Evolutionary analysis was conducted using MEGA version 5 software (Tamura., *et al*. 2004).

Lovastatin production by 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 millimeter. The seed cultures were incubated in a rotator shaker incubator at 180rpm at 28°C for 24 hours. 10% of the seed broth was inoculated to 50ml of production medium incubated in a rotator shaker incubator at 180 rpm at 28°C for 10 days. At the end of 8 days of fermentation, the fermentation broth was acidified to pH 2.0 with 10% HCL. Then the acidified broth was extracted with equal volume of ethyl acetate under shaking condition (180 rpm) 70°C for 2 hours. The filtrates were subsequently centrifuged at 3000 rpm 10 mins and the organic phase was collected. To the 1 ml 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 through a 0.45 µm for HPLC analysis.

Bioassay of lovastatin using *S. cerevisiae* was grown for 7-10 days on PDA slants at 28°C. 20 µl of spore suspension of *S. cerevisiae* was transferred into a 40 × 40 mm size sterilized petriplate with YPDA (peptone 20g, yeast extract 10g, glucose 10g, agar 15g). After solidification, discs were placed on the media and bioassay was carried out with ethyl acetate as a control and ethyl acetate extracts of fungal isolates as samples (Allarcon., *et al.* 2002). Bioassay positive fungal cultures were maintained on PDA slants at 28°C.

Results and Discussion

Isolation and characterization of Soil Fungi

Ten soil fungi were isolated from the selected soil samples (Table 1). Soil fungi such as *Pithomyces* sp, *Curvualria* sp., *Botrytis* sp, *Cladosporium* sp, *Mucor* sp, *Trichoderma* sp, *Aspergillus* sp, *Penicillium* sp, *Rhizopus oryzae, Mycocladus corimbifer* were identified (Figure2). They were characterized by using standard microbiological methods such as morphological properties (Colony color, 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).

Figure 1: Auxenic culture of Rhizopus oryzae.

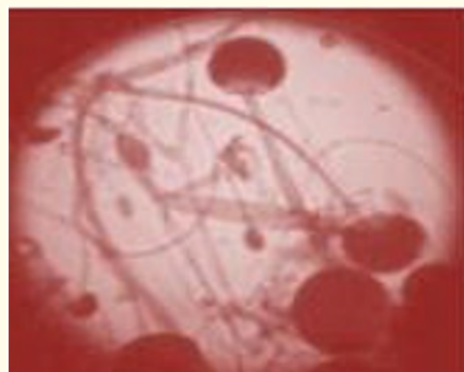


Figure 2: Microscopic view of isolated soil fungi.

S.NO	Soil type	Organisms isolated
1.	Agriculture soil	*Aspergillus sp*
2.	Agriculture soil	*Rhizopus oryzae*
3.	Black soil	*Botrytis sp*
4.	Black soil	*Cladosporium sp*
5.	Agriculture soil	*Mucor sp*
6.	Black soil	*Trichoderma sp*
7.	Black soil	*Penicillium sp*
8.	Agriculture soil	*Mycocladus corimbifer*
9.	Black soil	*Curvularia sp*
10.	Agriculture soil	*Pithomyces sp*

Table 1: List of fungi isolated from soil.

Identification of lovastatin producing fungi by 18S rRNA ITS Sequencing

The highest lovastatin yield was recorded by *R. oryzae*. when compared to other fungi, hence it was chosen for identification by molecular methods. Genomic identification of the strain was done using 18S rDNA technique. The DNA of *Rhizopus oryzae* was extracted and purified by agarose gel electrophoresis and sequenced (Figure 3). Sequenced data was aligned and analyzed for finding the closest homologs for the microbe. Phylogenetic analyses performed by the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbour-joining method and the identity was confirmed. BLAST search gave a 100% identity with that of *R. oryzae* (Figure 4). Forward sequence information was submitted to NCBI genbank.

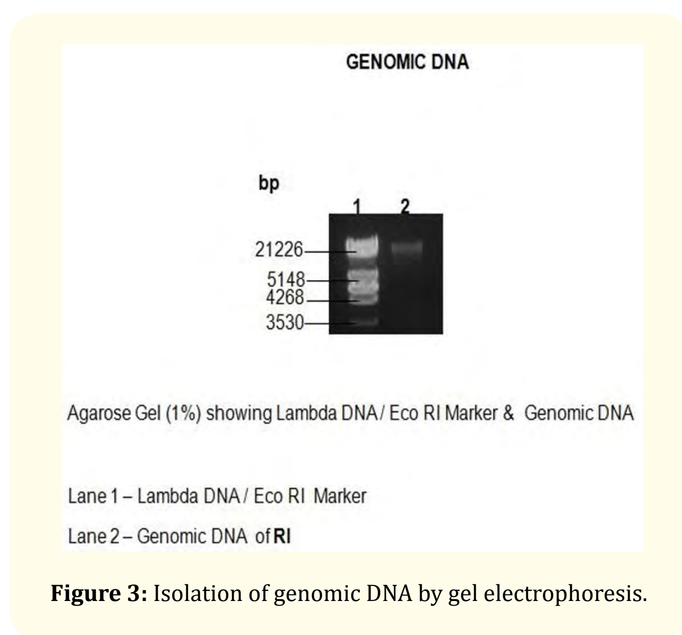


Figure 3: Isolation of genomic DNA by gel electrophoresis.

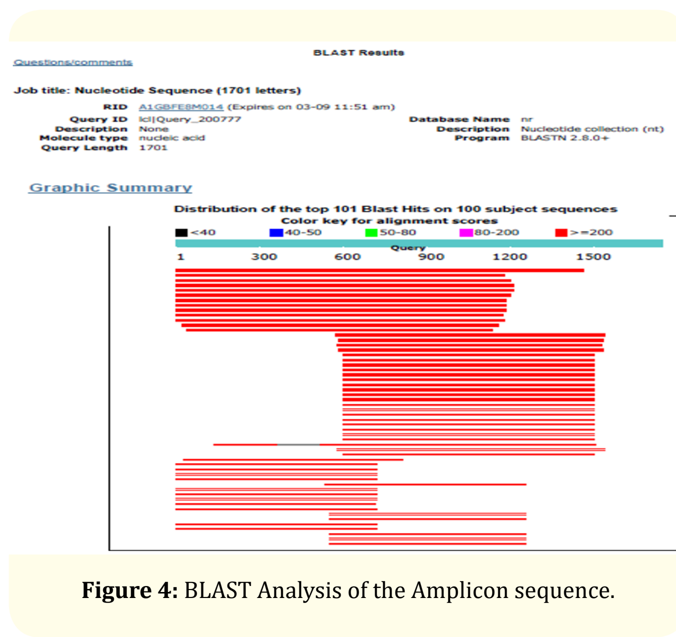


Figure 4: BLAST Analysis of the Amplicon sequence.

Phylogenetic analysis of the isolate

The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky A. and Nei M. (1992). The optimal tree with the sum of branch length = 0.36145269 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J.,1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Screening of isolate for lovastatin production by SmF

Ten strains were screened for lovastatin production using HPLC analysis after extraction of this compound from the fermentation broths with ethyl acetate. Of the screened strains only two isolates, *Mycocladus corimbifer* and *Rhizopus oryzae* were confirmed as producers of lovastatin (Table 1, Figure 5 and 6). The lovastatin standard showed a retention time of 19.1 min under the HPLC column elution conditions used (Figure 7). Lovastatin quantification was carried out on extracts from culture broths. Among positive isolates, *R. oryzae* was identified as the best lovastatin producing strain with a production level of 20.39 mg/l. The remaining lovastatin producers achieved considerably lower concentrations. The yield of lovastatin from the isolate *R. oryzae* was comparable with reported production levels in other species of Penicillium [1-7] and produced this statin at a very similar level (19.3 mg/l) to the strain selected in present study.

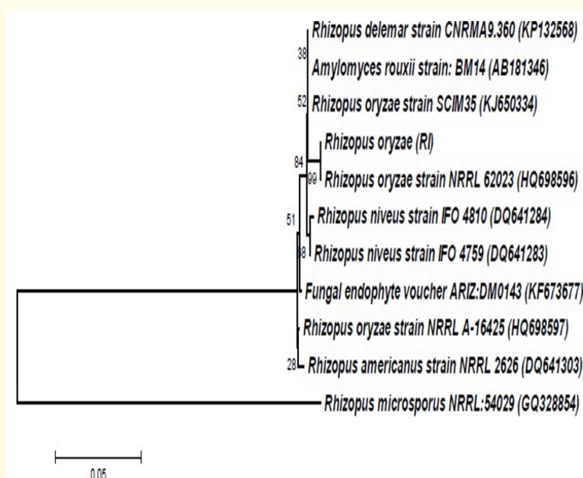
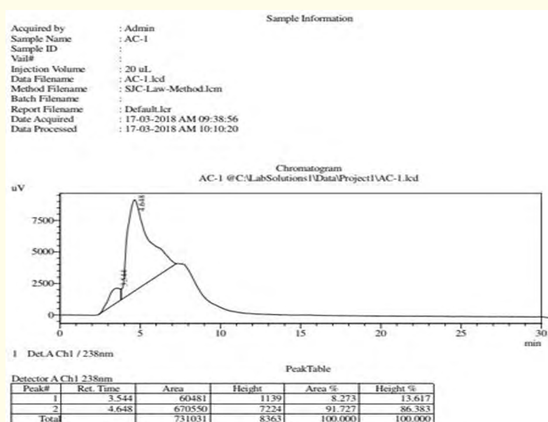


Figure 5: Molecular Phylogenic analysis of Rhizopus oryzae.

Figure 6: Extraction of statin from the isolate.



PeakTable

Detector A Ch1 238nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.544	60481	1139	8.273	13.617
2	4.648	670550	7224	91.727	86.383
Total		731031	8363	100.000	100.000

Figure 7: Elution profile of lovastatin from *Rhizopus oryzae.*

Bioassay of lovastatin using *S. cerevisiae*

The screening of potential lovastatin producing microfungi was carried out by measuring the zone of inhibition around the well, where the extracted lovastatin sample was loaded. The diameter of zone of inhibition ranged from 2 - 2.8 cm (Table 2) and *Rhizopus oryzae* produced a maximum zone of inhibition. The difference in clear zones may be due to the variation of physiology and genetic characteristics of the specimens, ability of the lovastatin to diffuse in the agar, and incubation period (VilchesFerrón., *et al.* 2005). The concentration of lovastatin was calculated using standard plot of pure lovastatin.

Estimation of Lovastatin using HPLC the preliminary production of lovastatin by *Rhizopusoryzae* was studied as batch mode under shake flask method using glucose as a sole carbon source at pH of 6.5 and temperature of 30°C, respectively. The extracts of lovastain from different isolates were subjected to HPLC analysis to confirm the amount of lovastatin produced. Chromatogram analysis indicated the peak of lovastatin standard with the retention time of 12.45 minutes (Figure 7). The extracts of the *R. oryzae* was eluted at a retention time of 12.770 minutes which was much similar to the retention time obtained from the standard. Hence, from this investigation it was concluded that rapid method of determination of lovastatin can also be employed to screen lovastatin producing fungal isolates. HPLC confirmed that the production of lovastatin in *R. oryzae* a soil fungus which has the highest potential for the production of lovastatin.

The spectrum analysis (λmax) of crude lovastatin is presented in along with standard lovastatin. Pure lovastatin has two different absorption maxima at 238 nm in HPLC, which suggests better identification of lovastatin from other compounds. The characteristics of the peaks could be due to the presence of diene. The absorption spectra of both lactone form and hydroxy acid form of lovastatin in a mixture appear similar. The quantification of lovastatin by this protocol resulted to a concentration of 0.159 mg/ml of statin. The presence of other peaks in the sample during spectrometric analysis is due to the existence of impurities or other unidentified compounds that have been extracted with the solvent [8-10].

Conclusion

Lovastatin is the competitive inhibitor of the enzyme Hydroxymethylglutaryl coenzyme A (HMGCoA) reductase that catalyzes the reduction of HMGCoA to mevalonate during biosynthesis of cholesterol. Increased cholesterol levels HDL have known to cause cardiovascular diseases (CVD). In the present study 10 soil fungi were

screened for lovastatin production using shake flask culture. Out of the 10 soil fungi isolated, two strains *Mycocladus corimbifer* and *Rhizopus oryzae* were shown their potential to produce lovastatin on the production media under submerged fermentation (SmF). The other soil isolates failed to produce lovastatin or produced lovastatin at too low concentration. Among the two lovastatin producing strains, *Rhizopus oryzae* was found to be utilize maximum substrates and lovastatin produced by the same fungi was confimed by HPLC analysis. Genomic identification of the strain was done by using 18S rRNA technique. The DNA of *Rhizopus oryzae* was extracted and purified by gel electrophoresis and sequenced using the 1.5 kb 18S rDNA fragment and was amplified by using the primers. Phylogenetic analyses performed by the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbour-joining method and the identity was confirmed. Blast search gave a 100% identity with that of *Rhizopus oryzae* (Figure 3,6). Forward sequence information was submitted to NCBI genbank. The screening of potential lovastatin producing microfungi was carried out by measuring the zone of inhibition around the well, Where the extracted lovastatin sample was loaded. The diameter of the zone of inhibition ranged from 1.1 mm to 16.6 mm. *Rhizopus oryzae* was produced a maximum zone of inhibition.

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