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ISOLATION AND SCREENING OF LOVASTATIN PRODUCING FUNGI

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ABSTRACT

Lovastatin is an FDA approved blood cholesterol lowering drug and it is widely used in the treatment of hypercholesterolemia. The production of lovastatin can be carried out through the fermentation processes utilizing lovastatin producing fungi. In this study, thirty-two fungal strains were isolated from different soil samples collected from different areas of Pakistan. The isolated strains were checked under static conditions for the production of lovastatin. Among them, eight fungal strains showed the production of lovastatin in the fermentation broth. The maximum production (66 mg/mL) was shown by the strain IIB-F3. The strain was identified as *Aspergillus terreus* on the basis of its microscopic and morphological characters like texture, size, colony colour and other characteristics. This fungal strain was isolated from soil sample collected from Toli Peer, Kotli, Azad Kashmir, Pakistan. The lovastatin produced by fungi was detected by thin layer chromatography (TLC) and was quantitatively analysed through reverse phase High-Performance-liquid chromatography (HPLC) using C-18 column.

Keywords: Hypercholesterolemia, lovastatin, *Aspergillus terreus*, High-Performance-liquid chromatography (HPLC), thin layer chromatography (TLC).

INTRODUCTION

Statin was discovered for the first time in 1970s, by the Japanese microbiologist Akira Endo in the fermentation broth of a fungus *Penicillium citrinum* (Tobert, 2003). The basic reason behind its production was revealed that the microbes produced inhibitors while defending themselves against other microbes. Merck (a pharmaceutical company) isolated lovastatin first time from the fungus *Aspergillus terreus* in 1976. Lovastatin was found to be very effective for the treatment of hypercholesterolemia and others like atherosclerosis, peripheral arterial disease, sepsis, bone fracture and peripheral vascular disease (Seraman *et al*., 2010). Statins have an anti-hypersensitive effect and promote coronary collateral circulation. It also shows a favorable effect on glucose metabolism as well (Massy and Guijarro, 2001). Lovastatin is also used for treating bone disorders (Pahan, 2006) mainly bone fractures. Alzheimer's disease is also reduced by the use of statins (Hoglund *et al.*, 2004).

Two classes of statins are Natural Statins including Compactin, Lovastatin (mevacor), Simvastatin (Zocor), Pravastatin (pravachol) and Synthetic Statins including Atorvastatin (Lipitor) and Fluvastatin (Lescol). The most common statins are Lovastatin (Mevacor, Altocor), Atorvastatin (Lipitor), Pravastatin (pravachol), Fluvastatin (lescol), Simvastatin (zocor), and Rosuvastatin (Manzoni and Rollini, 2002). Lovastatin is a white crystalline powder, and it is insoluble in water. Its empirical formula is $C_{24}H_{36}O_5$ while its molecular weight is 404.55. Its IUPAC name is [(1S,3R,7R,8aS)-8-[2-[(2R,4R)-4-hydroxy-6-oxooxan-2-yl] ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl] (2S)-2methylbutanoate. It is insoluble in water but sparingly soluble in acetonitrile, ethanol and methanol. Chemical structure of lovastatin shows that it has three parts; target enzyme substrate HMG-CoA part, a hydrophobic structure which is linked covalently to the substrate. Its side groups define the solubility impact of the drugs.

In 1983, Merck initiated programs for the treatment of myocardial infarction. In 1986, it went for the approval and in 1987 the US FDA considered the clinical results and approved it on August 31, 1987 for use. The physicians started prescribing lovastatin and it showed a reduction in LDL by its dose of 80 mg daily. Its effectiveness was observed on a large scale and found that there was a decrease in 30% mortality rate (Scandinavian, 1994). No adverse side effects were noted at that time, and it was recognized worldwide as an effective drug for this purpose.

The fungal species involved in the production of lovastatin as a secondary metabolite include *Aspergillus terreus, Aspergillus niger, Monascus sp., Aspergillus flavus, Pleurotus sp, Penicillium purpurogenum, Penicillium* sp and *Trichoderma viride.* For the industrial purposes, *Monascus rubber*, *Penicillium* sp. and *Aspergillus terreus* are mostly used. However, the most famous species which is mostly used for lovastatin production is *Aspergillus terreus.* A number of studies are being carried out by using submerged fermentation for the production of lovastatin. In the process, the operating parameters are being optimized for the better yield (Manzoni *et al*., 2002). Genetically

modified organisms are also used these days for this purpose. Because of these strains, maximum yields can be obtained with minimal nutrients.

The main aim of the study was to isolate fungal strains from different soil samples to assess their potency for the production of lovastatin through fermentation. The results proved that these fungal strains have the ability to produce good quantities of lovastatin in the fermentation broth.

MATERIALS AND METHODS

Isolation of Fungi

Soil samples were collected from different regions of Pakistan including Pakpattan, Lahore, Sharakpur, Toli Peer, Sheikhupura, Swat, Phool Nagar, Kasur, Sharakpur and Multan. These samples were packed into labelled sterilized bags for the isolation of different fungi capable of producing lovastatin and further processing was done for the production, screening, and isolation of lovastatin.

Isolation of fungi was followed by serial dilution method. Soil samples were suspended in sufficient amount of sterilized water and serial dilutions of 10^{-5}, 10^{-6} and 10^{-7} were transferred on the surface of potato dextrose agar (PDA) medium supplemented with chloramphenicol (100µg/mL) to avoid bacterial growth. The plates were incubated at 30ºC for 72 hours to observe fungal growth. After incubation, those plates were selected having some fungal growth and each colony was transferred to new PDA plate for purification followed by sub-culturing onto PDA slants. The fungal strains were differentiated on the basis of different morphological characters (colours of spores such as black, green, white, brown and cottony or filamentous appearance of their colonies).

Identification of Selected Isolate

The selected fungal strain was identified by its morphological characters and most specifically by direct microscopic examination. Texture and colour of the fungal strain were observed visually. The hyphae and spores were observed under light microscope for the confirmation of the strain and identified following manual of Aspergilli (Thom and Raper, 1945). The identification was further confirmed by an expert mycologist from department of Botany, University of the Punjab, Lahore.

Fermentation Technique

Submerged fermentation was used for the production of lovastatin from the fungal isolates in shake flasks as done by Ahmed *et al.* (2013). For submerged fermentation, spore suspension was prepared by adding autoclaved distilled water (10 mL) in a fully mature slant. One mL of this spore suspension was then used to inoculate the fermentation medium. In 25 mL autoclaved potato dextrose broth, chloramphenicol (100µg/mL) was added followed by 1 mL spore suspension. The flasks were then incubated for 72 h at 30º in a shaking incubator at 120 rpm.

Extraction of Lovastatin

After completion of the fermentation, the process of extraction of lovastatin from the fermentation broth was done according to Praveen *et al.* (2015). The fungal fermentation broth was filtered using muslin cloth in order to remove mycelium. The filtrate obtained was then set to pH 3 with concentrated HCl. The extraction of lovastatin from filtrate was done by adding two equal volumes of ethyl acetate with the constant shaking of 2 h in shaking incubator which resulted in the formation of two layers after a while. Organic phase formed as a result was then collected and subjected to rotary vacuum evaporator at 45ºC under reduced pressure to remove the solvent. The dried solid residue was re-dissolved in ethyl acetate for further analysis.

For the extraction of intracellular lovastatin, mycelium was crushed by using mortar and pastel. It was then centrifuged at 6000 rpm for 15 minutes and the supernatant was subjected to the operation as stated above.

Thin Layer Chromatography (TLC)

After extraction of lovastatin, TLC was used to detect its presence by using the method developed by Haytko and Wildman, (1993). It was performed on pre-coated silica gel plate (Merck, Germany) having thickness of 1mm and an area of 20x20 cm. For the activation of silica plates, these were heated for 3 hours in a hot air oven at 45ºC. 10µL of the samples were applied to the plates along with the standard lovastatin at the base line and the plates were subjected to the solvent system which included: Solvent A-dichloromethane and Solvent B-ethylacetate in the ratio of 70:30. After the run, lovastatin was detected by exposing the plates to the Iodine fumes produced in the chromatographic chamber. The presence of lovastatin was shown by a yellow spot under UV light.

High Performance Liquid Chromatography (HPLC)

For the quantification of lovastatin, HPLC method was used as developed by Sayyad *et al.* (2007). PerkinElmer HPLC system equipped with C18 reverse phase column (SpherSIL, 5µm diameter, column size; 250x4 mm) and UV detector was used. The mobile phase consisting of acetonitrile and water in a ratio of 65:35 (pH 3.0) was used. The samples and standard were filtered by using 0.45 µm syringe filter separately before injecting. The flow rate was adjusted to 1.5mL/min. 20µL of sample was injected into column using micro syringe and absorbance was taken at 235 nm.

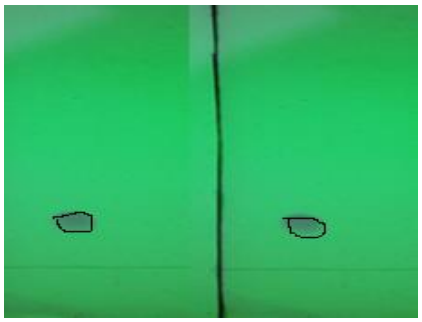
RESULTS AND DISCUSSION

Thirty-two fungal strains were isolated from soil samples collected from different areas of Pakistan and were screened for lovastatin production. Among them, eight were found positive for lovastatin production as shown in (Table 1). The presence of lovastatin was detected by using TLC which indicated that sample and standard spots both occurred at the same position as shown in (Fig. 1) whereas quantification was done by HPLC. The retention time of the standard lovastatin was 1.63 min as shown in (Fig. 2). The comparison of retention time between the standard lovastatin and the lovastatin produced by fungal strain showed the concentration of lovastatin in fermentation broth. Among eight lovastatin producing strains, highest production (66 mg/mL) was shown by the strain IIB-F3. The lovastatin was detected in the highest amount intracellularly. This particular fungal strain was isolated from the soil sample collected from Tohli Peer, Kashmir, Pakistan. The least production of lovastatin (0.22 mg/mL) was shown by the strain IIB-F2 isolated from Pakpatan.

Table 1. Screening of fungal isolates for lovastatin production using submerged fermentation

S. No	Strain code	Area of soil samples	Colony colour of fungal isolates	Amount of lovastatin (mg/ mL)	Location of Lovastatin
1	IIB-F1	Sharakpur	Light brown	7.5	Extracellular
2	IIB-F2	Pakpatan	Black	0.22	Intracellular
3	IIB-F3	Tohli Peer	Dark green	66	Intracellular
4	IIB-F4	Lahore	Olive green	25.3	Both
5	IIB-F5	Kasur	Green	2.4	Intracellular
6	IIB-F6	Sheikhupura	Dark green	9.7	Intracellular
7	IIB-F7	Swat	Greyish black	4.36	Extracellular
8	IIB-F8	Phool Nagar	Green	5.12	Intracellular
9	IIB-F9	Lahore	White	0	-
10	IIB-F10	Lahore	Black	0	-
11	IIB-F11	Kasur	Parrot green	0	-
12	IIB-F12	Swat	White	0	-
13	IIB-F13	Sharakpur	Mustard	0	-
14	IIB-F14	Pakpatan	Pale yellow	0	-
15	IIB-F15	Kasur	Yellow-green	0	-
16	IIB-F16	Phool Nagar	Pale yellow	0	-
17	IIB-F17	Lahore	Black	0	-
18	IIB-F18	Sheikhupura	Cream white	0	-
19	IIB-F19	Swat	White yellowish	0	-
20	IIB-F20	Kasur	Greyish black	0	-
21	IIB-F21	Lahore	Brown	0	-
22	IIB-F22	Sheikhupura	Brown	0	-
23	IIB-F23	Phool Nagar	Cream white	0	-
24	IIB-F24	Kasur	White	0	-

*Fermentation conditions: Incubation temperature: 30ºC; Incubation time: 72 h.



Standard **Sample (IIB-F3)**

Fig 1. TLC plate for the comparison of standard lovastatin with sample (IIB-F3) after visualization under UV. Standard and sample spots both occurred at the same position.

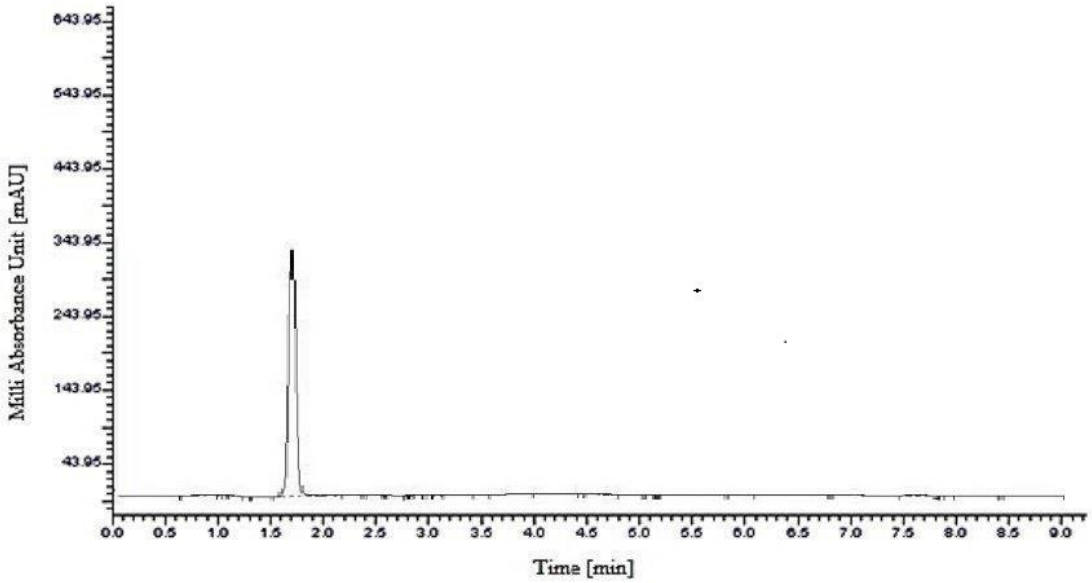


Fig. 2. HPLC Chromatogram illustrating retention time for standard lovastatin.

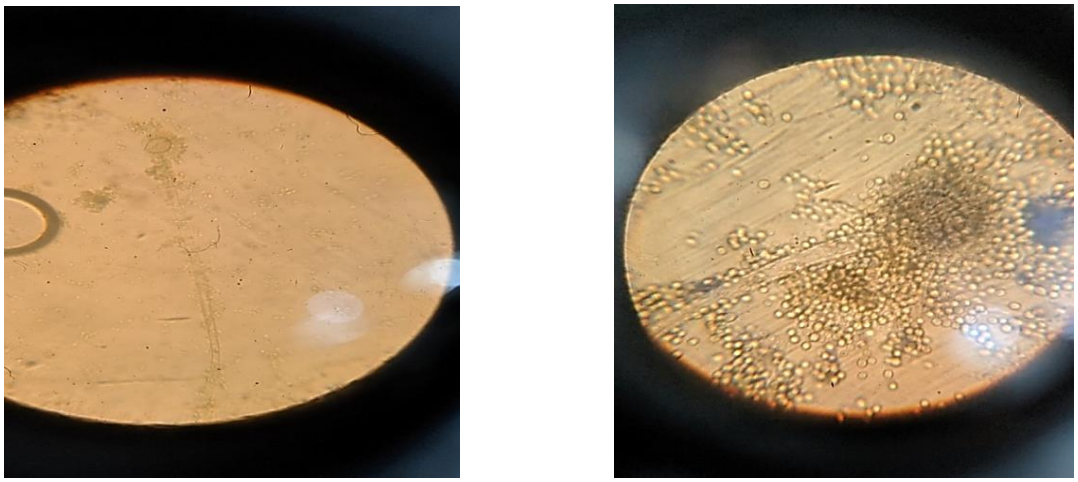


Fig. 3. Micrographs of *Aspergillus terreus* IIB-F3.

The production of lovastatin from fungi has been reported previously by Rodriguez and his colleagues (2007). The amount of lovastatin production was in accordance with the study of Arjumand *et al.* (2013) in which fermentation medium was utilized by *Aspergillus terreus* NRRL 265 for the maximum production of lovastatin which was calculated up to 212.54 mg/L lovastatin in the broth and 120.98 mg/L in the mycelium. 144 days of incubation proved to be the best for the production of lovastatin. Members belonging to the genus *Aspergillus* can be found everywhere and produce metabolites in a wide range of environmental conditions as discussed by Klich (2002). *M. paxii* AM12M; a mutant strain produced 127 mg lovastatin/L, 18 mg pravastatin/L after 16 days and 53 mg pravastatin/L after 21 days while *Aspergillus terreus* produced 230 mg lovastatin/L and 118 mg pravastatin/L after 14 days of fermentation as reported by Manzoni *et al.* (1999).

The fungal colonies were characterized morphologically and microscopically and were found to have green tufts enclosed by conidial heads emerging out of it. Conidial heads were globule and biseriate attached to philades as shown in (Fig 3). The hyphae were septate and hyaline. All these morphological characters have close similarity with *Aspergillus terreus* therefore, IIB-F3 was identified as *Aspergillus terreus*. It was further confirmed by an expert mycologist.

CONCLUSION

The present study depicts that *Aspergillus terreus* appears to be the best and promising producer of lovastatin. Further research is under progress to increase the lovastatin production by optimizing the fermentation parameters and improving the fungal strains with the help of physical and chemical mutagenesis.

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