# Screening of Lovastatin Production by Filamentous Fungi

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#### ABSTRACT

In the present study, 110 fungal strains of Persian Type Culture Collection (PTCC) including some selected strains isolated in various screening projects were tested for their potentiality to produce lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol biosynthesis. The fungal strains were cultivated in a two-stage submerged fermentation followed by screening by TLC. All positive results were evaluated by confirmatory HPLC. Nine species of four genera were found to be lovastatin producers. *Aspergillus terreus* was the best lovastatin producing strain with a level of 55 mg lovastatin per liter of screening production medium. *Iran. Biomed. J.* 7 (1): 29-33, 2003

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#### **INTRODUCTION**

ovastatin, also called Mevinolin, Monacolin K and Mevacor®, is a potent hypocholesterolemic agent first introduced by Endo [1]. This interesting polyketide is a secondary metabolite of fermentation process of various fungi such as *Monascus rubber* [1] and *Aspergillus terreus* [2].

Lovastatin and its analogs, e.g. symvastatin, are of 3-hydroxy-3-methylglutaryl-CoA inhibitors reductase (HMG-CoA reductase), a rate-limiting enzyme of cholesterol biosynthesis. These compounds, which are members of a drug family generally called "Statins", are known to exist in open hydroxy acid as well as in lactone forms (Fig. 1). The major form of lovastatin in fermentation broth is the open hydroxy acid form (Mevinolinic acid). However, it is generally in lactone form (mevinolin) when administered to the patients as drug. In vivo, the lactone form of the compound is converted to the open hydroxy acid, which is the biologically active form of the statin. The inhibitory effect of open hydroxy acid form of statins is due to their structural homology with HMG-CoA.

Several fungal genera including Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopolariopsis, Doratomyces, Phoma, Phythium, Gymnoascus, Hypomyces and Pleurotus have been reported to be able to produce lovastatin [3, 4].

In the present investigation, 110 filamentous fungi from PTCC (Persian Type Culture Collection, Tehran, Iran), including selected strains isolated during various screening projects screened for lovastatin production. The study was carried out at the Biotechnology Department of Iranian Research Organization for Science and Technology (IROST).



Fig. 1. Structural formulae of (a), lactone; (b), open hydroxy acid forms of lovastatin.

### MATERIALS AND METHODS

*Chemicals.* All chemicals used in this study were of Merck brand (Darmshtadt, Germany) unless otherwise stated.

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Organisms and fermentation condition. One hundred and ten fungi of 22 genera and 50 species from PTCC were cultured in a two-step submerged fermentation. One ml of the spore suspension (containing  $3.5-4.0 \times 10^7$  spores) of each strain was added to the seed medium according to Alberts et al. [2]. The spores were obtained following the growth of the strain on potato dextrose agar at 28°C for 14 days. The seed cultures were incubated in a rotary shaker-incubator at 180 rpm at 28°C for 24 hours. The seed medium contained of 5 g corn steep liquor, 40 g tomato paste, 10 g oatmeal, 10 g glucose, 10 ml trace element stock solution and water to 1 liter (pH 6.8). The trace element stock solution was composed of 1 g FeSO<sub>4</sub>.7H<sub>2</sub>O, 1 g MnSO<sub>4</sub>.4H<sub>2</sub>O, 25 mg CuCl<sub>2</sub>.2H<sub>2</sub>O, 100 mg CaCl<sub>2</sub>. 2H<sub>2</sub>O, 56 mg H<sub>3</sub>BO<sub>3</sub>, 19 mg (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>,2H<sub>2</sub>O, 200 mg ZnSO<sub>4</sub>.7H<sub>2</sub>O and water to 1 liter.

Ten percent of the seed broth was used as inoculum for the production medium consisting: 50 g glucose, 20 g yeast extract, 30 g tomato paste, 20 g oat meal, 10 g sodium acetate, 5 g ammonium sulfate, 2 g potassium dihydrogen phosphate, 10 ml trace element stock solution and water to 1 liter [3, 4]. The pH adjusted to 7.0 with 1 M NaOH. The 250 ml culture flasks each containing 50 ml of production medium were incubated in a rotary shaker-incubator at 180 rpm at 28°C for 7 days.

ATCC 74135, a known lovastatin producing *Aspergillus terreus*, was included in each run in order to monitor the performance of production, extraction and analysis steps.

*Extraction.* Each 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 2 hours. The samples were subsequently centrifuged at 1500  $\times$ g for 15 min and the organic phase was collected for further steps.

Thin layer chromatography (TLC). TLC was used for screening of lovastatin production. One and a half ml of each organic phase was collected from extraction step, concentrated to about 50µl using a block heater adjusted to  $45^{\circ}$ C, applied to a heat activated 20 × 20 cm Merck silica gel  $60F_{254}$  TLC plates (Art No. 1.05554). The mobile phase was dichloromethan and ethylacetate (70:30, v/v). All the plates were observed under a hand-held UV lamp (254 nm) after 3 times developing in the same mobile phase and afterward stained with iodine vapor (Fig. 2). For each TLC run, 3 lovastatin standards were applied for  $R_f$  comparison. The sensitivity of this method was at least 2.5  $\mu$ g per spot.

(A)







High performance liquid chromatography. All TLC-positive samples, were analyzed using confirmatory HPLC. One and half ml of the organic phase of each positive sample was completely evaporated and the dried residue was dissolved in 1.5 ml acetonitrile. All samples were filtered through 0.45 $\mu$ m Millex-LH (Millipor corp., Bedford, MA 01730) before injection. HPLC analysis was performed on a Knauer (Berlin, Germany) isocratic system consisting of a K-1001 pump, a K-2501 UV detector, 20  $\mu$ l sample loop injector and a 250 × 4 mm Eurospher-100 C18, 5  $\mu$ m particle size column with integrated precolumn. The mobile phase was a mixture of acetonitrile and water (60:40, v/v), which acidified

with ortho-phosphoric acid to the concentration of 0.1%. The flow-rate was maintained on 1.5 ml/min throughout the run and the detection carried out at 235 nm. The sensitivity of this assay was 1  $\mu$ g/ml.

The pure lovastatin standard in the lactone form has been generously provided by FDCL (Food and Drug Control Laboratories, Ministry of Health, Tehran) and also supplied by Sigma (catalogue no. M 2147, 98% minimum purity by HPLC). The lovastatin stock standard was prepared from the pure lactone form of the compound dissolved in acetonitrile in concentration of 500  $\mu$ g/ml and 1:10 dilution of the stock solution in the same diluent used as working standard.

As the open hydroxy acid form of lovastatin is unstable, it was prepared freshly from lactone form, whenever necessary, according to Friedrich *et al.* [5]. The pure lovastatin lactone form was suspended in 0.1 M NaOH and heated at 50°C for at least 1 hour in a shaking water bath. Subsequently, the suspension was adjusted to pH 7.7 with 1 M HCl, filtered through a 0.45  $\mu$ m filter (Millipore) and diluted to the desired concentration.

#### **RESULTS AND DISCUSSION**

The aim of this study was to find the potentiality of lovastatin production by some filamentous fungi. One hundred and ten strains of 22 genera and 50 species were screened for lovastatin production. Certain species of four genera were identified as producers of the compound (Table 1). No lovastatin detected in fermentation broth of other 18 genera. Following fungi recognized as lovastatin producers: *Aspergillus terreus* (55 mg/l), *A. parasiticus* (4.5 mg/l), *A. fischeri* (2.0 mg/l), *A. flavus* (9.0 mg/l), *A. umbrosus* (14.1 mg/l), *Penicillium funiculosom* (pinophilum)(19.3 mg/l), *Trichoderma viridae* (9.0 mg/l), *Trichoderma longibrachiatum* (1.0 mg/l), *Acremonium chrysogenum* (2.5 mg/l).

Forty-nine strains (44.5%) of the screened population showed positive TLC result, but only 31 results (28.2%) were confirmed by HPLC.

From screened strains, a *Verticillium* sp., *Beauveria bassiana, Mortierella vinacea, Paecilomyces lilacinus* showed no growth in production screening medium. Among lovastatin producers, *Aspergillus terreus* was identified as the best lovastatin producing strain (production level up to 55 mg/l). Other lovastatin producers were only able to produce the compound in a concentration less than 20 mg/l.

Gunde-Cimerman et al. [3] screened 380 fungal

strains of 50 different genera and 143 species which resulted in 43% positive TLC. Only 22% of the positive TLC results were confirmed by HPLC. They detected lovastatin production in strain of Aspergillus terreus (up to 100 mg/l) and also with Paecilomyces varioti and Pythium ultimum. They also reported lovastatin production in a concentration higher than 1 mg/l lit but not more than 4.5 mg/l, for Aspergillus flavus, A. niger, A. repens, A. versicolor, Penicillium variable, Pleospora herbarum and Trichoderma viridae. The most interesting result of their reserch was observation of lovastatin production by higher Basidiomycetus fungi, particularly Pleurotus sp., which was not mentioned in the litreture as a producer of the compound. They detected traces of lovastatin production in Agrocybe aegerita, Trametes versicolor, Agaricus bisporus and Volvariella volvacea [3].

Study of Shindia [4] was limited to 25 fungal species of 14 genera isolated from Egyptian soil as well as compost samples. The results shows that nearly one-third (32%) of the strains were positive for lovastatin production. Shindia reported lovastatin production with Aspergillus oryzae, A. terreus, Doratomyces stemonitis, Paecilomyces varioti, Penicillium citrinum, Penicillium chrysogenum, Scopolariopsis brevicaulis and Trichoderma viridae. Aspergillus terreus was the best lovastatin producer (84 mg/l) introduced in his article. The production level of Penicillium citrinum, Paecilomyces varioti and Penicillium chrysogenum were 61 mg/l, 56 mg/l and 35 mg/l, respectively. Other strains were able to produce the compound in quantities less than 10 mg/l [4].

Disregarding small modifications in fermentation conditions (such as shaking speed), this study differs from those of Shindia [4] and Gunde-Cimerman et al. [3] in which they used methanol extraction of acidified broth followed by TLC analysis. Whereas, ethyl acetate extract of acidified fermentation broth was used for TLC in our experiment. Using ethylacetate, considering good solubility of lovastatin and possibility of concentrating the organic solvent, assisted to increase the sensitivity of detection method. After methanol extraction of acidified culture media, a significant amount of lovastatin remains in open hydroxy acid form, which could not be detected using the recruited TLC system. Using ethylacetate to extract lovastatin from acidified fermentation broth were able to recover about 90% of existing lovastatin as lactone form, which can be detected by the TLC system.

**Table 1.** Genera screened for lovastatin production.

Genus	Species	Producing species
Absidia sp.	A. griseola var. Iguchi	
Acremonium sp.	Acremonium chrysogenum Acremonium sp.	A. chrysogenum
Alternaria sp.	Alternaria alternata	
Aspergillus sp.	Aspergillus terreus, A. nidulans, A. niger, A. fumigatus, A. parasiticus, A. fischeri, A. flavus, A. oryzae, A. umbrosus, A. sojae, A. foetidus, A. awamori, A. ochraceus, A. petrakii,	A. terreus, A. fischeri, A. flavus, A. umbrosus, A. parasiticus
Beauveria sp.	Beauveria bassiana	
Bipolaris sp.	Bipolaris sp.	
Blakeslea sp.	Blakeslea trispora	
Byssoclamys sp.	Byssoclamys fulva	
Chaetomium sp.	Chaetomium virescens	
Circinella sp.	Circinella muscae	
Cladosporium sp.	Cladosporium sp.	
Crysosporium sp.	Crysosporium sp.	
Cuninghamella sp.	Cuninghamella elegans	
Fusarium sp.	Fusarium fujikuroi	
<i>Mortierella</i> sp.	Mortierella vinacea	
Paecilomyces sp.	Paecilomyces lilacinus, P. varioti	
Penicillium sp.	Penicillium crysogenum, P. funiculosom (rubrum), P. funiculosum (pinophilum), P. charlesii, P. aculatum, P. purpurogenum, P. jantinellum, P. rogeforti.	P. funiculosum (pinophilum)
Phanerochaete sp.	Phanerochaete crysosporium.	
Rhizomucor sp.	Rhizomucor miehei, Rh. pusillus	
Rhizopus sp.	Rhizopus oryzae	
Trichoderma sp.	Trichoderma longibrachiatum, T. reesi, T. viridae	T. viridae, T. longibrachiatum
Verticellium sp.	Verticellium lecanii	

There are also different options for HPLC determination of lovastatin in fermentation broth or its extract: 1) determination of the compound in open hydroxy acid form after adjustment of the pH to 7.7 [5]; 2) determination of lovastatin in both open hydroxy acid and lactone forms existing

simultaneously [6]. Recruiting the presented HPLC protocol, we were able to determine lovastatin in both forms, although when the acidified broth is extracted with ethylacetate it exists mainly in lactone form (Fig. 3).



**Fig. 3.** HPLC chromatograms of lovastatin open hydroxy acid and lactone forms. (**A**), fermentation broth extract containing both open hydroxy acid and lactone forms of lovastatin; (**B**), Lovastatin standard in open hydroxy acid form; (**C**), Lovastatin standard in lactone form.



Fig. 4. Lovastatin production time-course of selected *Aspergillus terreus*.

Based on extension of the study to strain and nutritional improvement experiments carried out on the best lovastatin producing *Aspergillus terreus* strain (selected from this study) and by using different concentrations of nitrogen sources (such as Rapeseed meal or CSL) in the presence of lactose as the main carbon source, we could obtain a level of up to 400 mg lovastatin per liter of production medium (data not shown). The production timecourse study showed that about 80% of maximum lovastatin production was obtained after 7 days of incubation (Fig. 4). We recommend that the 7-day incubation duration and the applied fermentation conditions are reasonable for screening purposes. However, as the nutritional requirements of various fungi may differ, the screening medium may need to be optimized to support optimal growth and production of the population to be screened to get a better idea on potentiality and the level of production.

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