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*

Keywords: Lovastatin, Screening, Fungal strains, *Aspergillus terreus*

INTRODUCTION

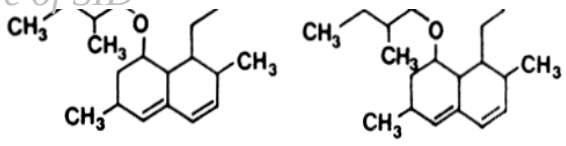
Lovastatin, also called Mevinolin, Monacolin K and Mevacor®, is a potent hypocholes-terolemic agent first introduced by Endo [1]. This interesting polyketide is a secondary metabolite of fermentation process of various fungi such as *Monascus rubber* [1] and *Aspergillus terreus* [2].

Lovastatin and its analogs, e.g. symvastatin, are inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase **(**HMG-CoA reductase), a rate-limiting enzyme of cholesterol biosynthesis. These com-pounds, 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*, *Tricho-derma*, *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.

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 × 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 $FeSO_4.7H_2O$, 1 g $MnSO_4.4H_2O$, 25 mg $CuCl_2.2H_2O$, 100 mg $CaCl_2.2H_2O$, 56 mg H_3BO_3, 19 mg $(NH_4)_6Mo_7O_{24}.2H_2O$, 200 mg $ZnSO_4.7H_2O$ 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 ×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 50 µl using a block heater adjusted to 45°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 µg per spot.

(A)

Fig. 2. Picture of TLC plate. **(A)**, after staining with iodine vapor; **(B)**, after staining with iodine vapor an enhancing under UV light. Lanes 4, 8 & 12, lovastatin standards .

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 µm 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 µl sample loop injector

and a 250 × 4 mm Eurospher-100 C18 5 µm particle size column with integrated pre-column. 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 µg/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 µg/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 µm 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., *Beau-veria 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 con-centration 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 chryso-genum*, *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*, *Paecilo-myces 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 concen-trating 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*	
Mortierella 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 time-course 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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