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Composition of statins produced by indigenous strain of *Aspergillus terreus*

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Abstract

Composition of statins produced by *Aspergillus terreus 20* indigenous strain in submerged fermentation (SmF) has been studied. Identification of statin compounds in fungal extracts by LC-MS-MS analysis revealed 6 polyketide metabolites: lovastatin (LV) in lactone, acid and methyl ester forms, pravastatin (PV), monacolin L (ML) and simvastatin (SV). For the first time it has been revealed the ability producing simvastatin by *A. terreus* by direct fermentation.

Keywords: Aspergillus terreus, statins, composition, HPLC, LC-MS-MS analysis, mass spectra.

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1. Introduction

Statins are fungal secondary metabolites which specifically inhibit HMG-CoA reductase on early rate-limiting step in cholesterol biosynthesis (Alberts *et al.*, 1980). Statins reduce total cholesterol and low-density lipoprotein levels and therefore have been used as cholesterol-lowering drugs (Seenivasan *et al.*, 2008; Endo, 2004). Natural statins are lovastatin, compactin and pravastatin. The most profound producers of natural statins are *Aspergillus terreus*, *Monascus ruber* and *Penicillium citrinum* (Barrios-Gonzales *et al.*, 2010; Manzoni *et al.*, 2002). Simvastatin, the second leading statin in the market, is a lovastatin's semisynthetic derivative. The difference in molecular structure between these two polyketides resides in the C-8 carbon position of the side chain where lovastatin carries a 2-methylbutyrate moiety, while simvastatin - a 2,2-dimethylbutyrate (DMB) moiety. Because DMB is not normally produced by *A. terreus* this fungus is considered not to be able to synthesize simvastatin (Barrios-Gonzales *et al.,* 2010, Seenivasan *et al.,* 2008). To date commercially simvastatin is being obtained by direct alkylation of lovastatin.

Recently, we reported about good lovastatin production by indigenous strains *A. terreus 4* and *A. terreus 20* both in SmF and solid state fermentation (SSF) (Gulyamova *et al.*, 2013). This paper based on current study presents the data indicating the composition of statin metabolites produced by *A. terreus 20* in submerged fermentation.

2. Materials and Methods

2.1 Microorganisms and inoculum preparation: A. terreus 20 was isolated from soils of Navoi region, Uzbekistan. Isolates were grown on Czapek-Dox agar slants at 28°C until complete sporulation. Conidiospores were harvested from slants with 5 ml of sterile solution of 0,85% NaCl, 0,2% Tween 80 and transferred into 250 ml Erlenmeyer flasks containing 50 ml medium (g/l): 10 g glucose, 10 g oat meal, 10 g corn steep liquor, 0,2 g polyethylene glycol, and 10 ml trace elements – 100 mg $Na_2B_4O_7 \cdot 10H_2O$, 50 mg $MnCl_2$, 50 mg $Na_2MoO_4 \cdot 5H_2O$, 250 mg $CuSO_4 \cdot 5H_2O$- per liter of solution. The flask with medium was inoculated with $3x10^7$ conidiospores, held on rotary shaker at 160 rpm for 2 days at 28-30°C and then was used as inoculum (Kumar *et al.*, 2000).

*2.2 Submerged fermentation:*10 ml of conidiospores were inoculated in 300 ml Erlenmeyer flasks, containing 100 ml of following media (g/l): lactose – 20; yeast extract – 4; KH_2PO_4 – 1,51; NaCl – 0,4; $ZnSO_4 \cdot 7H_2O$ – 1; $Fe(NO_3) \cdot 9H_2O$ – 2; biotin – 0,04 мг, trace elements – 1 ml, pH 6,0 (Casas *et al.*, 2003). Fermentation was carried out at 28°C in flasks held on a rotary platform shaker at 160 rpm for 24 days.

2.3 Statin extraction: Statins were extracted from biomass after centrifugation of the whole culture suspension at 6000 rpm for 20 min. 1g of mycelium was washed by 0,05M HCl and extracted with 20 ml of acetonitrile on rotary shaker for 60 min at 160 rpm. Extracts were dried with $Na_2SO_{4,,}$ concentrated to 2 ml by vacuum evaporation and used for analysis.

2.4. LC-MS-MS analysis: Mass spectra of extracts were taken on Q-TOF LC-MS Agilent Technologies 6520B instrument under following conditions: ion source ESI+, positive ion electrospray method, drying gas flow rate 5 l/min, drying gas temperature 300°C, ion acceleration voltage on skimmer 35V, fragmentor 175V, MS range 150 – 1000 m/z, targeted MS-MS 50 – 1000 m/z, collision energy – 30, 40, 50, 65. Samples were injected on Zorbax SB C18 column, 3 µm, 150x0,5 mm (Agilent Technologies 1200) with mobile phase: A - 0,1% formic acid, B – acetonitrile + 0,1 % formic acid. Elution on Agilent Technologies 1260 Cap Pump at 15µl/min: 5 min - 60%, 15-20 min – 90%, 25 min – 60% of mobile phase B. There were conducted 3 replications.

2.5. LC-MS-MS analysis of statins: HPLC was carried out in a reverse phase Zorbax Eclipse XDB C-18 (150x4,6 mm i.d., 5 µm) column. The mobile phase consisted of acetonitrile and water (60 : 40 by volume) containing 0,1 % phosphoric acid. The sample injection volume was 20 µl, the eluent flow rate was 1.5 ml/min and the detection wavelength 238 nm. Pharmaceutical-grade lovastatin (Gedeon Richter) and simvastatin (Ivex Pharmaceuticals) tablets were used to prepare the standards for HPLC analysis (Morovjan *et al*., 1997). There were conducted 3 replications.

3. Results and Discussion

In previous studies it was established that *A. terreus 20* indigenous strain produces sufficiently high amount of lovastatin both in SmF and SSF (Gulyamova et al., 2013) but composition of statins was not studied. The presence of lovastatin in *A. terreus 20* extracts was analyzed by HPLC (Figure1).

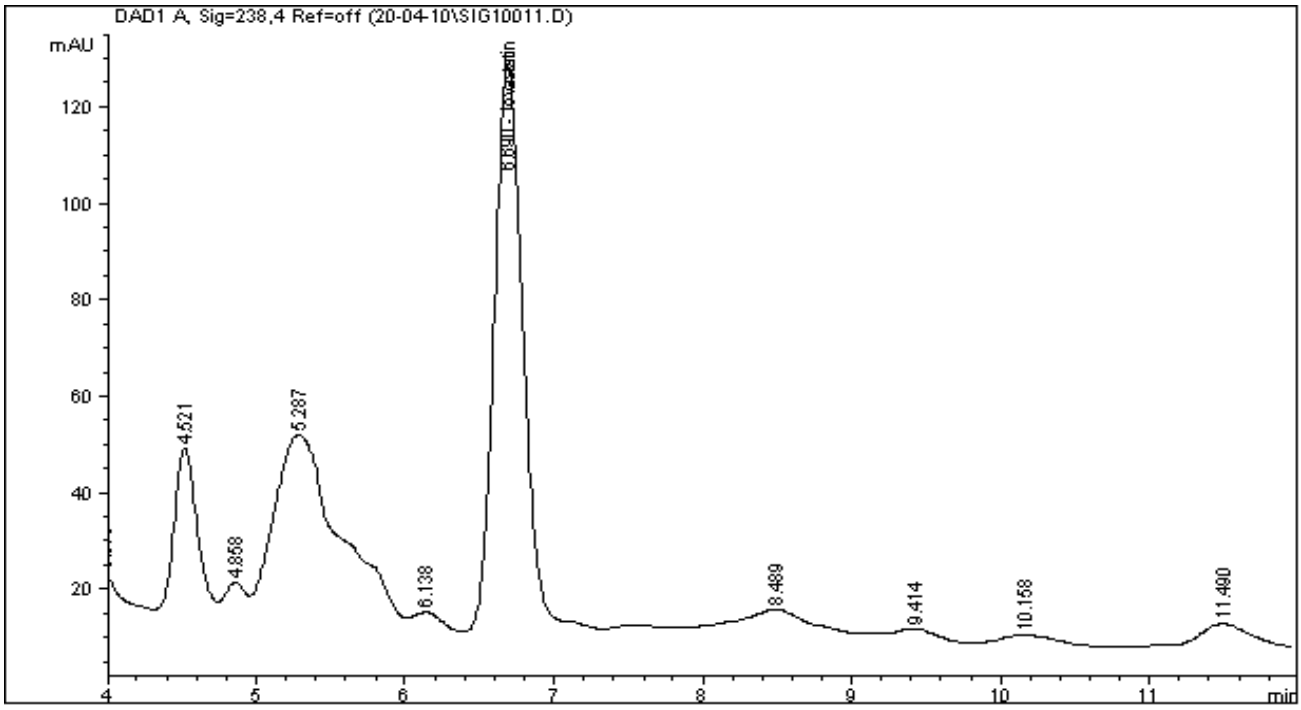

Figure 1. HPLC-chromatogram of extract of *A. terreus 20*

In this study, the identification of statin metabolites in *A. terreus* acetonitrile extracts was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic conditions on Q-TOF LC-MS Agilent Technologies 6520B instrument. To avoid or limit the interference from background, the multiple reactions monitoring analysis mode was used instead of single ion monitoring (e.g., MS-MS instead of MS). TIC-chromatogram of *A. terreus 20* extracts

presented in Figure 2. As opposed to HPLC-chromatogram a number of high intensity compounds were observed in hydrophobic region of TIC- chromatogram.

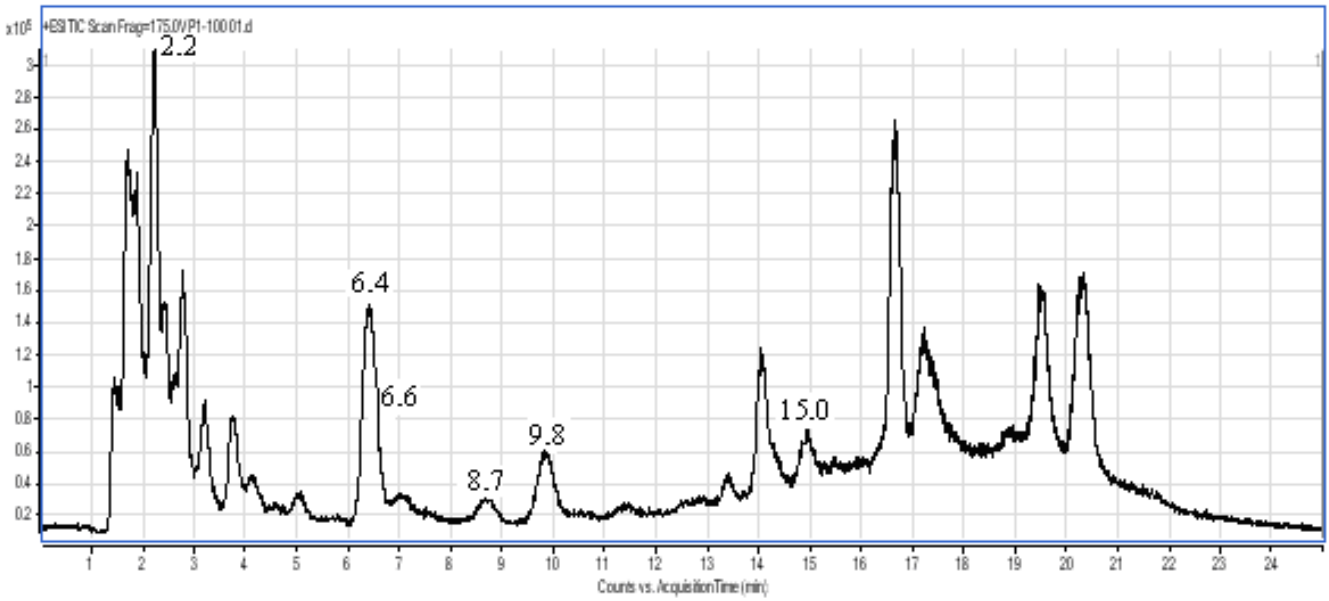

Figure 2. TIC–chromatogram of extract of *A.terreus-20.*
The peaks from left to right are ordered PV (2.2 min), LV (6.4 min), LA (6.6 min), SV (8.7 min), ML (9.8 min), LM (15.0 min)

For identification of each compound Targeted MS-MS was used. According to data shown in Figures 3 and 4 MS-MS spectra of acetonitrile extracts of *A.terreus 20* indicates the presence of compounds with molecular mass of lovastatin and simvastatin ($(M+H)^+$ 405,2205 m/z and $(M+H)^+$ 419,2362 m/z, respectively) as lactones.

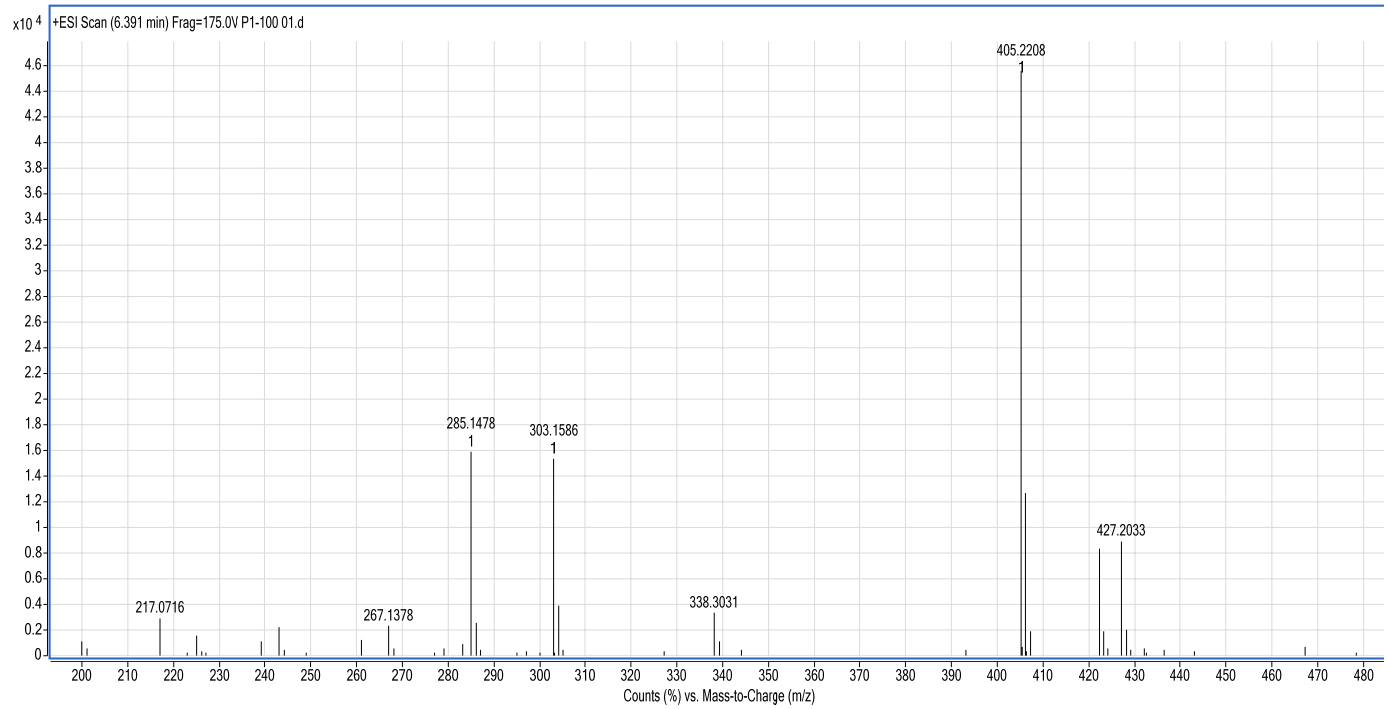

Figure 3. MS spectra of lovastatin $(M+H)^+$ 405,2205 m/z

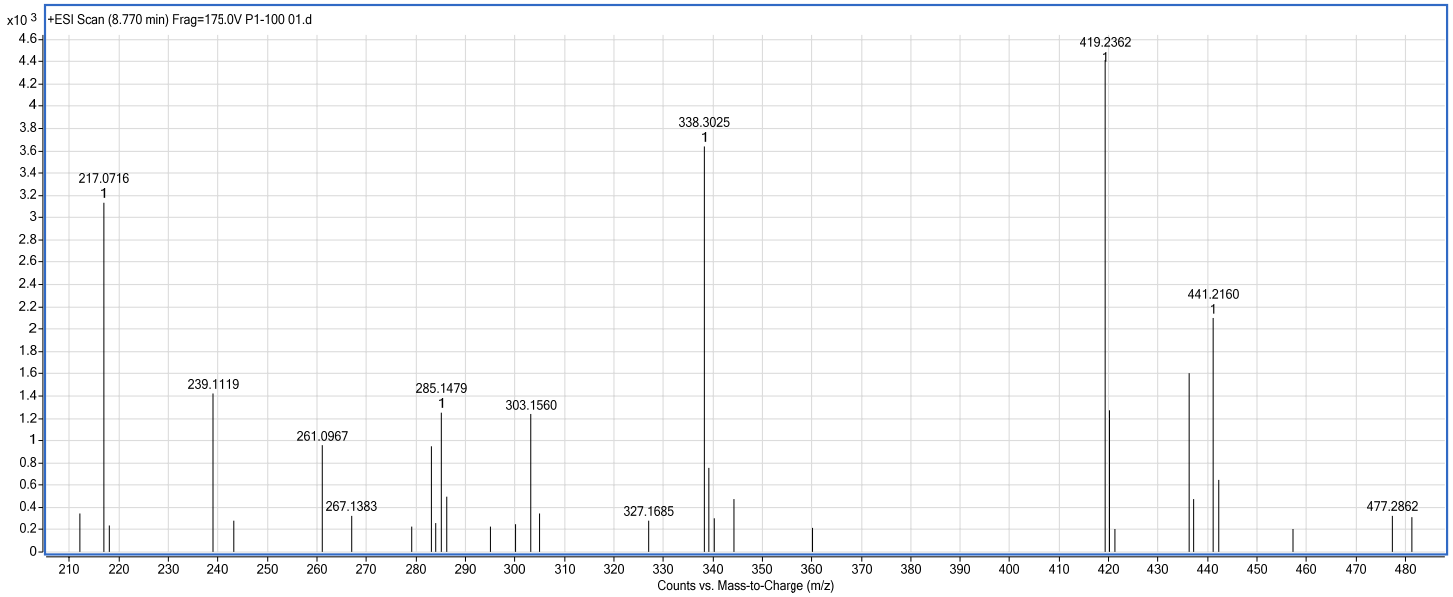

Figure 4. MS spectra of simvastatin $(M+H)^+$ 419,2362 m/z

Experimental results reveal that lovastatin was presented in three different forms: as lovastatin lactone $(M+H)^+$405,2205 m/z, lovastatin hydroxyacid ($(M+H)^+$ 422,2469 m/z) and lovastatin methyl ester ($(M+H)^+$ 436.3890 m/z) . There are also derivatives in the samples, appropriate to pravastatin ($(M+H)^+$ 425,1159 m/z) and monacolin L ($(M+H)^+$ 305,1173 m/z) (Table 1).

Table 1. MS data of statins in *A. terreus 20* extracts

Compound	**Molecular ion (M^+)**	**Retention time (min)**
Pravastatin (PV)	425,1159 m/z	2,2
Lovastatin (LV)	405,2205 m/z	6,4
Lovastatin acid (LA)	422,2469 m/z	6,6
Simvastatin (SV)	419,2362 m/z	8,7
Monacolin L (ML)	305,1173 m/z	9,8
Lovastatin methyl ester (LM)	436,3890 m/z	15,0

Manzoni *et al*. reported that some *A. terreus* strains can produce appreciable yields of pravastatin as well as lovastatin (Manzoni *et al.,* 1998). But determination of SV in extracts was quite unexpected because of normally *A. terreus* is considered not to be able to produce this derivative due lack of DMB endogenous synthesis (Barrios-Gonzales *et al.*, 2010, Seenivasan *et al.*, 2008). Moreover, for the last years advances in the biochemistry and genetics of lovastatin have allowed the development new biotechnological processes for obtaining of SV. One of the biotechnological approaches for its production would be the enzymatic synthesis of SV from monacolin J (MCJ) with acyltransferase LovD (Xie *et al.*, 2007). Using combinatorial biocatalytic approach it has been engineered *A. terreus* strain with hybrid polyketide synthase enable to synthesize DMB in vivo as side chain of SV. Transformed strain of *A. terreus* can produce SV instead of LV by direct fermentation (Van den Berg *et al.*, 2007). Hereby, as opposed to previous studies of *Aspergillus* strains indigenous *A. terreus 20* obviously is able to synthesize DMB promoting SV production as final product of fermentation.

4. Conclusions

Our results demonstrate that composition of statins producing by *A. terreus 20* includes 6 polyketide metabolites: LV in lactone, acid and methyl ester forms, PV, ML and SV. Obtained data indicates the existence in *A. terreus 20* biochemical mechanism for biotransformation which allowed formation and accumulation of SV in the culture as a final fermentation product. Although the particular pathway of SV production has not been studied yet, detection of this statin presumes ability of *A. terreus 20* to synthesize DMB as side chain precursor. On the basis of obtained data we conclude that indigenous strain *A. terreus 20* could be an alternative to the biotransformation process cited in the literature (Barrios-Gonzales *et al.*, 2010).

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