

## Lovastatin production by *Aspergillus terreus* in solid state and submerged fermentations

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### Abstract

Lovastatin production by *Aspergillus terreus* indigenous strains in submerged (SmF) and solid state fermentations (SSF) have been studied. To evaluate the ability to produce lovastatin various cultivation media and substrates have been used. The obtained data showed good lovastatin yield by *A. terreus 4* and *A. terreus 20* both in SmF and SSF. At submerged cultivation of *A. terreus 4* and *A. terreus 20* on five different glucose and lactose based media the highest titer of lovastatin has been obtained on lactose based media, namely 276 mg/l and 236 mg/l, respectively. Five various types of bran have been tested as solid substrates for production of lovastatin in SSF - wheat bran, oat bran, maize bran, rice bran and mix of wheat and peanut bran. It has been observed that fermentation of *A. terreus 4* on wheat and *A. terreus 20* on oat bran causes the highest lovastatin yield - 9.7 and 9.56 mg/g, respectively.

**Keywords:** lovastatin, submerged fermentation, solid state fermentation, production

DOI: <http://dx.doi.org/10.4314/ijest.v5i3.2>

### 1. Introduction

Lovastatin is a fungal secondary metabolite used for lowering blood cholesterol. It acts as an effective inhibitor of the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol (Alberts *et al.*, 1980). It has been shown that lovastatin very competitively inhibits the reductase which decrease serum cholesterol levels by blocking cholesterol biosynthesis.

Lovastatin has a polyketide structure and is produced as a secondary metabolite by a variety of filamentous fungi such as *Monascus (M. ruber, M. purpureus, M. pilosus, M. anka)*, *Penicillium (P. citrinum)*, *Paecilomyces viridis*, and *Aspergillus (A. terreus)* (Manzoni *et al.*, 2002).

Commercial production of lovastatin is conventionally performed by liquid SmF using *A. terreus* mutants (Barrios-Gonzales *et al.*, 2010). To date, there are many publications focused on studies of cultivation regimes for producing statins (Bizukoje *et al.*, 2009). In the last years, SSF is becoming an alternative to SmF for generating many fungal products including statins. Comparative studies have shown that solid-state fermentation has advantages over SmF such as higher and faster yield, and less water need in up-stream processing which minimizes production expense (Holker *et al.*, 2004).

In the present work, production of lovastatin in conventional SmF and in SSF on natural solid substrates has been studied comparatively using two domestic isolates of *Aspergillus terreus* selected from 30 strains which were isolated from saline soils and maintained in Culture Collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan.

## 2. Materials and Methods

**2.1 Microorganisms and inoculum preparation:** *A. terreus* strains were isolated from soils of the 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 of trace elements – 100 mg Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O, 50 mg MnCl<sub>2</sub>, 50 mg Na<sub>2</sub>MoO<sub>4</sub>·5H<sub>2</sub>O, and 250 mg CuSO<sub>4</sub>·5H<sub>2</sub>O - per liter of solution (Kumar *et al.*, 2000). The flask with medium was inoculated with 3 x10<sup>7</sup> conidiospores, held on rotary shaker at 160 rpm for 2 days at 28-30°C and then was used as inoculum.

**2.2 Liquid submerged fermentation:** Different glucose and lactose based lovastatin production media were used for SmF. 10 ml of conidiospores were inoculated in 300 ml Erlenmeyer flasks, containing 100 ml of the following media (g/l):

#1: Glucose – 10, corn steep liquor – 5, tomato paste – 40, oatmeal – 10, pH 6, (Monaghan *et al.*, 1980).

#2: Glucose – 30, glycerol – 70, peptone – 8, soybean meal – 30, pH 6,4 (Manzoni *et al.*, 1998).

#3: Glucose – 45, Na glutamate – 12,5, KH<sub>2</sub>PO<sub>4</sub> – 5, K<sub>2</sub>HPO<sub>4</sub> – 5, FeSO<sub>4</sub>·7H<sub>2</sub>O, MnSO<sub>4</sub>·4H<sub>2</sub>O – 0,1, ZnSO<sub>4</sub>·7H<sub>2</sub>O – 0,2, MgSO<sub>4</sub>·7H<sub>2</sub>O – 0,1, trace elements – 1 ml, pH 6,5 (Hajjaj *et al.*, 2001).

#4: Lactose – 20, yeast extract – 8, KH<sub>2</sub>PO<sub>4</sub> – 1,51, MgSO<sub>4</sub>·7H<sub>2</sub>O – 1,51, NaCl – 0,4, ZnSO<sub>4</sub>·7H<sub>2</sub>O – 1, Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O – 2, biotin – 0,04 mg, trace elements – 1 ml, pH 6,0 (Casas Lopez *et al.*, 2003).

#5: Lactose – 70, yeast extract – 8, defatted soybean meal – 0,5, polyethylene glycol 2000 – 0,5, KCl – 1, K<sub>2</sub>HPO<sub>4</sub> – 1, pH 6,5 (Lai *et al.*, 2005).

Fermentation was carried out at 28°C in flasks held on a rotary platform shaker at 160 rpm for 24 days. Lovastatin was extracted only from biomass after centrifugation of whole cultural suspension at 6000 rpm for 20 min. 1g of mycelium was washed with 0,05M HCl and extracted with 20 ml of acetonitrile in a rotary shaker at 160 rpm for 60 min. Extracts were dried with Na<sub>2</sub>SO<sub>4</sub>, concentrated to 2 ml by vacuum evaporation and used for lovastatin estimation.

**2.3 Solid state fermentation:** Substrates such as wheat bran, oat bran, rice bran, maize bran and mix of wheat and peanut bran were used in the solid state fermentation process. Before fermentation, substrates were ground to the size of 20 mesh. SSF was performed in 500 ml conical flasks, containing 50 g of solid substrate. The flasks were autoclaved for 40 min at 121°C, the substrate's moisture content was measured and adjusted to a level 55-65% with nutrient solution (%): glucose – 11, glycerol -16, MgSO<sub>4</sub> – 0,75, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> – 2,3, KH<sub>2</sub>PO<sub>4</sub> – 2, maltose - 5, pH -7,5. After moistening of substrate, 2,5 ml of inoculum (with spore concentration of 10<sup>7</sup>-10<sup>8</sup> ml<sup>-1</sup>) was added. The flasks were shaken evenly and incubated at 28°C for 14 days. At the end of incubation SSF substrate was dried at 100-105°C, ground using a porcelaine pestle and mortar to a fine powder and used to estimate the lovastatin content by HPLC analysis (Morovjan *et al.*, 1997).

**2.4 Lovastatin extraction:** After SmF, lovastatin was extracted from biomass after centrifugation of the whole culture suspension at 6000 rpm for 20 min. 1g of mycelium was washed using 0,05M HCL and extracted with 20 ml of acetonitrile on rotary shaker for 60 min at 160 rpm. Extracts were dried with Na<sub>2</sub>SO<sub>4</sub>, concentrated to 2 ml by vacuum evaporation and used for lovastatin estimation. After SSF, lovastatin was extracted from 1g of ground substrate using 20 ml of acetonitrile by shaking on a rotary shaker for 60 min at 160 rpm, centrifuged for 10 min at 6000 rpm, and then the supernatant was used for HPLC analysis.

**2.5 HPLC analysis of lovastatin:** Prepared extract samples, obtained both by SmF and SSF were quantitatively analyzed for the presence of lovastatin. HPLC analysis 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 1,5 ml/min and the detection wavelength 238 nm. The identity of the compound was confirmed with a commercial sample of lovastatin (Gedeon Richter) as standart (Manzoni *et al.*, 1998).

## 3. Results and Discussion

In our work a two strains *A. terreus* 4 and *A. terreus* 20 were analyzed for their potential in lovastatin production using SmF and SSF. Because lovastatin is an intracellular product and mostly accumulated in mycelium, for lovastatin extraction we used mycelial biomass separated from cultural broth. According to Manzoni *et al.*, 83% of total lovastatin has been extracted from separated mycelium of *A. terreus*, only 17% from cultural filtrate, 60% of total lovastatin loss at direct extraction of the whole culture (Manzoni *et al.*, 1998).

For liquid SmF we applied different lactose- and glucose-based media. Carbon and nitrogen sources are directly linked with the formation of biomass and metabolites, therefore these nutrients generally play a dominant role in fermentation productivity among the major culture nutrients (Barrios-Gonzales *et al.*, 2010; Bizukojc *et al.*, 2009). Secondary metabolism can be

regulated both by nature and concentration of the carbon source, such as catabolic repression by glucose.

Biosynthesis of lovastatin as secondary metabolite also has been found to depend on the carbon sources. According to many authors, a slowly utilizable carbon source is preferable for high lovastatin production. For example, Casas Lopes *et al.* testing fructose, lactose and glycerol, showed that the most slowly utilizable carbon source is lactose, and it caused the highest level of biosynthesis of lovastatin by *A. terreus* (Casas Lopes *et al.*, 2003). Hajjaj *et al.* investigating the biosynthesis of lovastatin by *A. terreus* ATCC74135 have found that the use of a glucose and lactose mixture leads to a good lovastatin yield (Hajjaj *et al.*, 2001). Lai *et al.* studied the biosynthesis of lovastatin and itaconic acid by *A. terreus* ATCC20542 and observed that lovastatin yield was almost 10 times higher on medium containing lactose than on medium containing glucose (Lai *et al.*, 2007). Szakacz *et al.* also showed that the maximum lovastatin production by the Hungarian strain *A. terreus* TUB F-514 was observed with the use of lactose as the carbon source and the lovastatin titer was 400 mg/l, while the lovastatin titer on sucrose was 40% less than on lactose (Szakacz *et al.*, 1998).

We used five different glucose- and lactose-based media. Lovastatin concentrations were estimated throughout 24 days of cultures growth. It was observed that product accumulation depends from used media and takes place at 10-22<sup>th</sup> day of growth. Results of SmF of *A. terreus* 4 and *A. terreus* 20 on glucose- and lactose-based media are presented in Table 1. As shown, in both *A. terreus* strains lovastatin yield was higher in lactose-based media. It should be mentioned that their productivity is comparable with lovastatin yield in shake flask fermentation of *A. terreus* ATCC 20542 and *A. terreus* ATCC74135 (Bizukojc *et al.*, 2009). Data on SmF of *A. terreus* 4 and *A. terreus* 20 confirm that media with a slowly utilizable carbon source are preferential for lovastatin production.

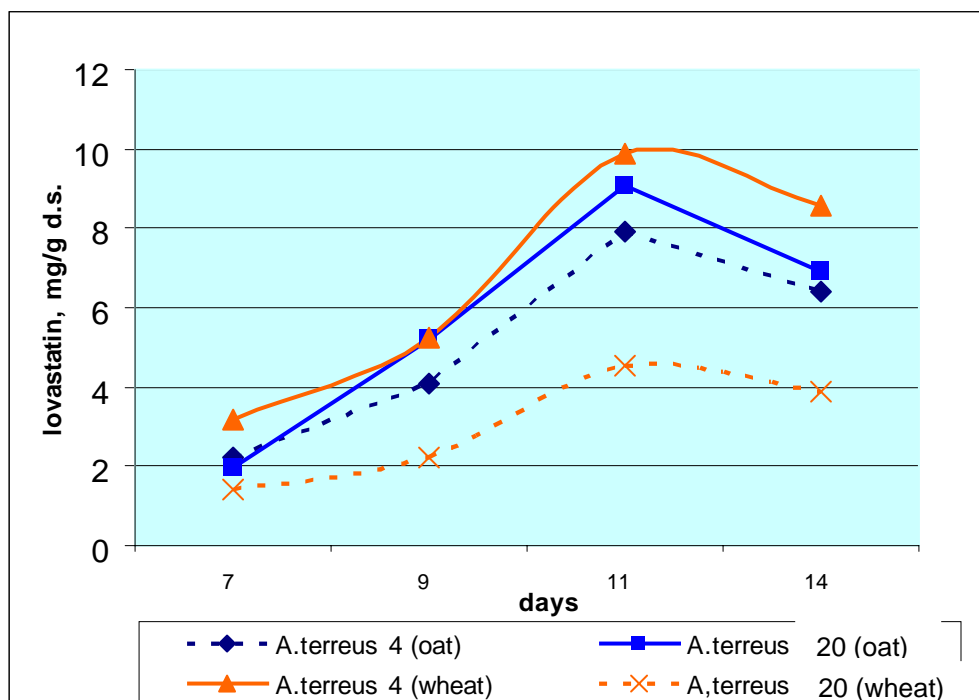
**Table 1.** Effect of carbon and nitrogen sources on lovastatin yield by *Aspergillus terreus* using submerged cultivation <sup>a)</sup>

#	Carbon source (g/l)	Nitrogen source (g/l)	Lovastatin titer (mg/l)	Time (days)	Strain
1	Glu (10)	CSL (5) TP (40) OM (10)	112	10 <sup>th</sup>	<i>A.terreus</i> 4
			121	14 <sup>th</sup>	<i>A.terreus</i> 20
2	Glu (30) Gly (70)	SM (30) PE (8)	61	16 <sup>th</sup>	<i>A.terreus</i> 4
			51	12 <sup>th</sup>	<i>A.terreus</i> 20
3	Glu (45)	Na-Glu (12,5)	137,6	10 <sup>th</sup>	<i>A.terreus</i> 4
			29,8	14 <sup>th</sup>	<i>A.terreus</i> 20
4	Lac (20)	YE (8)	136	20 <sup>th</sup>	<i>A.terreus</i> 4
			236	14 <sup>th</sup>	<i>A.terreus</i> 20
5	Lac (70 g/l)	YE (8) DSM (0,5)	276	22 <sup>th</sup>	<i>A.terreus</i> 4
			214	20 <sup>th</sup>	<i>A.terreus</i> 20

a) Glu, glucose; Gly, glycerol; Lac, lactose; CSL, corn steep liquor; TP, tomato paste; OM, oat meal; DSM, defatted soybean meal; Na-Glu, Na glutamate; YE, yeast extract; SM, soybean meal; PE, peptone.

During our evaluation of potential of *A. terreus* strains we also examined the feasibility of SSF for lovastatin production. SSF on natural solid substrates is being considered as the most common and the best option for production of microbial metabolites with use of cheap raw materials. Lovastatin production in SSF on natural solid substrates has been studied by Valera *et al.* with *Aspergillus flaviceps* (Valera *et al.*, 2005), Wei *et al.* with *Aspergillus terreus* (Wei *et al.*, 2007), Xu *et al.* with *Monascus ruber* (Xu *et al.*, 2005), who have shown lovastatin yields of 4-6 mg/g, 2,9 mg/g, 16,78 mg/g, respectively. Szakacs *et al.* used solid substrates such as wheat bran and sweet sorghum pulp and reported that SSF is superior than SmF for lovastatin production (Szakacs *et al.*, 1998).

In our experiments we used rice bran, wheat bran, oat bran, maize bran and mix of wheat and peanut bran as solid substrates grounded to the size of 20 mesh as used by Wei *et al.* (2007).



**Figure1.** Time course of lovastatin production in solid-state fermentation of *Aspergillus terreus* with oat and wheat bran

As shown in Figure1, biosynthesis of lovastatin in both strains reaches its maximum by the 11<sup>th</sup> day of growth on oat and wheat bran. The same dynamic of lovastatin accumulation we have observed at SSF on all used substrates.

**Table 2.** Effect of different solid substrates on lovastatin yield by *Aspergillus terreus* strains

Strains	Solid substrate	Lovastatin titer (mg/g)
<i>A.terreus 20</i>	Rice	2,67
	Wheat	5,54
	Oat	9,56
	Wheat + peanut (1:1)	0,4
	Maize	3,2
<i>A.terreus 4</i>	Rice	4,2
	Wheat	9,7
	Oat	8,4
	Wheat + peanut (1:1)	4,8
	Maize	4,4

The summary results presented in Table 2 show that when using oat and wheat brans as substrates both *A. terreus* strains had nearly similar productivity which was higher than for the rest of the substrates. The yields of lovastatin in extracts of *A. terreus 4* and *A. terreus 20* after SSF are comparable with ones previously reported for *Monascus ruber* (Xu et al., 2005), *A. flavipes* BICC 5174 (Valera et al., 2005), and was significantly higher than for *A. terreus 20524* (Wei et al., 2007; Jaivel et al., 2010).

Comparison of data for submerged and solid-state fermentation methods for lovastatin production by *A. terreus 4* and by *A. terreus 20* have demonstrated a clear advantage for SSF with productivity increase on solid substrates by more than 30 times (9.7 и 9.56 mg/g against 0.276 and 0.236 mg/ml, respectively).

#### 4. Conclusions

The ability of two indigenous strains of *A. terreus* to produce lovastatin in SmF and SSF have been studied. In SmF, lovastatin yield was elevated on lactose based media and reached its maximum - 276 mg/l and 236 mg/l by *A. terreus* 4 and *A. terreus* 20, respectively. In SSF with various substrates used – wheat bran, oat bran, maize bran, rice bran and mixed wheat and peanut bran - the preferred substrate was wheat and oat bran and maximum titers of lovastatin were 9.7 and 9.56 mg/g, for *A. terreus* 4 and *A. terreus* 20, respectively.

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Received February 2013

Accepted March 2013

Final acceptance in revised form March 2013