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Hypercholesterolemic agents by *Aspergillus terreus* in solid state and submerged fermentations

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Abstract
Hypercholesterolemic agent's production by Aspergillus terreus indigenous strains in submerged (SmF) and solid state fermentations (SSF) have been studied. To evaluate the ability to produce Hypercholesterolemic agents various cultivation media and substrates have been used. The obtained data showed good yield b Hypercholesterolemic agents *A. terreus* (1782) 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 Hypercholesterolemic agents or 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 Hypercholesterolemic agents 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* 1782 on oat bran causes the highest Hypercholesterolemic agents yield - 9.6 and 9.60 mg/g, respectively.

Keywords: Hypercholesterolemic agents, submerged fermentation, solid state fermentation, production

Introduction
Hypercholesterolemic agents 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 oxydoreductase, EC 1.1.1.34) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol (Alberts *et al*., 1980) [1]. 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) [12].
Commercial production of Hypercholesterolemic agents is conventionally performed by liquid SmF using *A. terreus* mutants (Barrios-Gonzales *et al.*, 2010) [2]. To date, there are many publications focused on studies of cultivation regimes for producing statins (Bizukojc *et al.*, 2009) [3]. 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) [6]. In the present work, production of Hypercholesterolemic agents in conventional SmF and in SSF on natural solid substrates has been studied comparatively using two domestic isolates of *Aspergillus terreus* selected from 27 strains which were isolated from saline soils and maintained in Culture Collections.

Materials and Methods
Microorganisms and inoculum preparation: *A. terreus* strains were isolated from soils of the Visakhapatnam region, Andhra Pradesh. 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 $Na_2B_4O_7 \cdot 10H_2O$, 50 mg $MnCl_2$, 50 mg $Na_2MoO_4 \cdot 5H_2O$, and 250 mg $CuSO_4 \cdot 5H_2O$ - per liter of solution (Kumar *et al.*, 2000) [8]. The flask with medium was inoculated with 3 $x10^7$ conidiospores, held on rotary shaker at 160 rpm for 2 days at 28-30 °C and then was used as inoculum.

Liquid submerged fermentation

Different glucose and lactose based Hypercholesterolemic agents 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) [11].
3. Glucose - 45, Na glutamate - 12,5, KH_2PO_4 - 5, K_2HPO_4 - 5, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H2O$ - 0,1, $ZnSO_4 \cdot 7H_2O$ - 0,2, $MgSO_4 \cdot 7H_2O$ - 0,1, trace elements - 1 ml, pH 6,5 (Hajjaj *et al*., 2001).
4. Lactose - 20, yeast extract - 8, KH_2PO_4 - 1,51, $MgSO_4 \cdot 7H2O$ - 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 Lopez *et al.*, 2003).
5. Lactose - 70, yeast extract - 8, defatted soybean meal - 0,5, polyethylene glycol 2000 - 0,5, KCl - 1, K_2HPO_4 - 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. was Hypercholesterolemic agents 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_2SO_4, concentrated to 2 ml by vacuum evaporation and used for Hypercholesterolemic agents estimation.

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, $MgSO_4$ - 0,75, $(NH_4)_2HSO_4$ - 2,3, KH_2PO_4 - 2, maltose - 5, pH -7,5. After moistening of substrate, 2,5 ml of inoculum (with spore concentration of 10^7-10^8 ml^{-1}) 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) [14].

Hypercholesterolemic agent's 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_2SO_4, concentrated to 2 ml by vacuum evaporation and used for lovastatin estimation. After SSF, Hypercholesterolemic agents 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.

Spectricrophotometric an alysis 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 (150 x 4,6 мм 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) [11]. Results and Discussion

In our work a two strains *A. terreus 4* and *A. terreus 20* were analyzed for their potential in Hypercholesteromic agents production using SmF and SSF. Because is Hypercholesteromic agents an intracellular product and mostly accumulated in mycelium, for Hypercholesteromic agents extraction we used mycelial biomass separated from cultural broth. According to Manzoni *et al.*, 83% of total Hypercholesteromic agents has been extracted from separated mycelium of *A. terreus*, only 17% from cultural filtrate, 60% of total loss at direct ext Hypercholesteromic agents raction of the whole culture (Manzoni *et al.*, 1998) [11].

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) [2, 3]. Secondary metabolism can be regulated both by nature and concentration of the carbon source, such as catabolic repression by glucose. Biosynthesis of as Hypercholesteromic agents 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 Hypercholesteromic agents 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 Hypercholesteromic agents by *A. terreus* (Casas Lopes *et al.*, 2003). Hajjaj *et al.* investigating the biosynthesis of by Hypercholesteromic agents *A. terreus* ATCC74135 have found that the use of a glucose and lactose mixture leads to a good lovastatin or Hypercholesteromic agents yield (Hajjaj *et al.*, 2001). Lai *et al.* studied the biosynthesis of hypercholesteromic agents and itaconic acid by *A. terreus* ATCC20542 and observed that HMG-CO A 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 hypercholesteromic agents production by the Hungarian strain *A. terreus* TUB F-514 was observed with the use of lactose as the carbon source and the hypercholesteromic agents titer was 400 mg/l, while the hypercholesteromic agents titer on sucrose was 40% less than on lactose (Szakacz *et al.*, 1998).

We used five different glucose- and lactose-based media. hypercholesteromic agents concentrations were estimated throughout 24 days of cultures growth. It was observed that product accumulation dependents from used media and takes place at 10-22th 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 hyper cholesteromic yield was higher in lactose-based media. It should be mentioned that their productivity is comparable with HMG Co-A yield in shake flask fermentation of *A. terreus* ATCC 20542 and *A. terreus* ATCC74135 (Bizukojc *et al.*, 2009) [3]. Data on SmF of *A. terreus 4* and *A. terreus 20* confirm that media with a slowly utilizable carbon source are preferential for hypercholesteromic agents production. During our evaluation of potential of *A. terreus* strains we also

examined the feasibility of SSF for hypercholesteromic agents 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. Hypercholesteromic agents 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 yields o Hypercholesteromic agents f 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 Hypercholesteromic agents 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).

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

A.terreus strains had nearly similar productivity which was higher than for the rest of the substrates. The yields of Hyper cholesteromic agents 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 (Comparison of data for submerged and solid-state fermentation methods for Hyper cholesteromic agents production by *A. terreus 4* and by *A. terreus 20* have demonstated 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).Wei *et al.*, 2007; Jaivel *et al.*, 2010).

Conclusions

The ability of two indigenous strains of *A. terreus* to produce Hyper cholesteromic agents 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 Hyper cholesteromic agents were 9.7 and 9.56 mg/g, for *A. terreus 4* and *A. terreus 20*, respectively.

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