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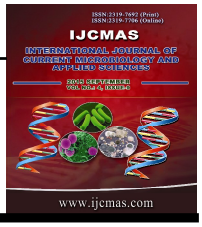

Original Research Article

Effect of the Principal Nutrients on Simvastatin Production by Wild Strain *Aspergillus terreus* 20 in Submerged Fermentation

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Simvastatin, *Aspergillus terreus*, Statins production

A B S T R A C T

The present study had been shown the ability of productivity increase of simvastatin direct synthesis by indigenous strain *Aspergillus terreus* 20 by optimization of cultivation medium. The highest simvastatin yield has been observed on media where the ratio of carbon source to nitrogen source corresponded to 10 and 14, and simvastatin titer was 84.5 and 120.0 mg/l, respectively.

Introduction

Simvastatin, a semi-synthetic lovastatin derivative, is a known hypocholesterolemic agent that is widely used due to the lack of undesirable side effects and high absorbability in the stomach. It is also reported that simvastatin prevents and reduces the risk of Alzheimer's disease (AD), slowing down the synthesis of Ab42, β-amyloid peptide associated with AD (Tan and Sinkai, 2007). In this regard, the search for new technologies of simvastatin production is demanding increasing attention.

For the industrial production of simvastatin it is commonly used semi-synthetic process, involving chemical modification of the lovastatin side chain. It is time-consuming multi-step chemical process that occurs in very severe conditions (Manzoni *et al.*, 1998; Manzoni and Rollini, 2002). Although the difference in molecular structure between the simvastatin and lovastatin resides only 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, nevertheless production of simvastatin by direct fermentation is considered impossible because DMB is not normally synthesized by the lovastatin producers, in particular by *Aspergillus terreus* (Morovjan *et al.*, 1997).

Recently, much attention is given to the possibility of biocatalytic method for production of simvastatin. Thus, using the different approaches it has been obtained the strain *A. terreus* with hybrid polyketide synthetase, capable in vivo to synthesize 2,2-dimethylbutyrate. The transformed

strain can produce simvastatin instead of lovastatin by the direct fermentation (Van den Berg *et al.,* 2007; Xie and Tang, 2007).

In previous studies we have identified for the first time that local strains *A. terreus*, producing lovastatin, possess the ability to accumulate simvastatin in the culture as final product of fermentation (Gulyamova *et al.,* 2014; Nasmetova *et al.,* 2013).

This paper presents data showing the possibility of increasing the efficiency of the direct synthesis of simvastatin in the wild strain *A. terreus 20* by the optimization of medium composition.

Materials and Methods

Microorganisms and inoculum preparation

A. terreus 20 was isolated from soils of Navoi region, Uzbekistan. Isolates were maintained on potato dextrose agar at 5°C. Conidiospores were harvested 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.

Liquid submerged fermentation: 10 ml of 5-days inoculum were transferred into 500 ml flasks containing 100 ml medium (according to table 1). Fermentation was carried out at 28°C in flasks held on a rotary platform shaker at 160 rpm for 24 days.

Statins extraction: Extracts were obtained 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 and concentrated to 2 ml by vacuum evaporation.

HPLC analysis

HPLC was carried out in a reverse phase Zorbax Eclipse XDB C-18 (3,0x100 mm) column. The mobile phase consisted of acetonitrile and water (70:30 by volume) containing 0.1 % phosphoric acid. Pharmaceutical-grade simvastatin (Gedeon Richter, Poland) was used to prepare the standards for HPLC analysis. Experiments were repeated three times.

Result and Discussion

Previous studies on the statin-synthesizing activity in the *A. terreus* indigenous strains showed that the strain *A. terreus 20* is able to synthesize kindred to lovastatin substance corresponding, by retention time, to the simvastatin standard used for HPLC analysis. Thus a relatively high yield of simvastatin was observed at lactose based media, namely 1300 μg/l. The presence of simvastatin in extracts of strains' biomass was also proved by mass-spectrometry analysis and thin-layer chromatography (Nasmetova *et al.,* 2013). Fact of discovery of simvastatin implies the existence in culture the mechanism of DMB synthesis necessary for the formation of the side chain of simvastatin (Tan and Sinkai, 2007).

It is known that carbon and nitrogen sources play a dominant role in fermentation productivity since these nutrients are directly linked with the formation of biomass and metabolites (Barrios-Gonzales and Miranda, 2010; Bizukojc and Ledakowicz, 2009; Hajjaj *et al.,* 2001).

Therefore, to study the potential of the strain *A. terreus 20* in elevating the simvastatin yield, in our experiments we used media with different concentrations of the primary carbon source – lactose, and nitrogen source - yeast extract as described previously by Szakacs *et al.* (1998).

A. terreus 20 grew for 24 days on media containing lactose (3.0%, 5.0% and 7.0%), yeast extract (0.3% and 0.6%) and $NaNO_3$ (0.1% and 0.2%). Simvastatin concentrations were estimated within the dynamics of culture growth. According to many authors, the media with use of excess of carbon source but with a limited source of nitrogen leads to a highest level of productivity (Casas Lopez *et al.*, 2003). Thus, Lai *et al.* investigating the biosynthesis of lovastatin by *A. terreus* ATCC20542 in submerged fermentation with use of optimized conditions succeeded in elevating the titer of lovastatin in two or three times (Lai *et al.*, 2007).

Table.1 Scheme of the optimization of cultivation conditions *A. terreus 20* with regard to carbon and nitrogen source[a]

Medium composition (%)	**Medium #1**	**Medium #2**	**Medium #3**	**Medium #4**
Lactose	3.0%	5.0%	7.0%	7.0%
Yeast extract	0.6%	0.6%	0.6%	0.3%
$NaNO_3$	0.1%	0.1%	0.1%	0.2%
Simvastatin titer (mg/l)	9.45	9.66	84.5	120.0

Note: [a] In addition to the components in table 1, the media contained the following (%): $MgSO_4 \cdot 7H_2O$ – 0.05; NaCl – 0.05; $MnSO_4$ – 0.16; $ZnSO_4 \cdot 7H_2O$ – 0.34; $CoCl_2 \cdot 6H_2O$ – 0.2

Figure.1 Dynamics of simvastatin accumulation by *A. terreus* 20 in media #3 and #4

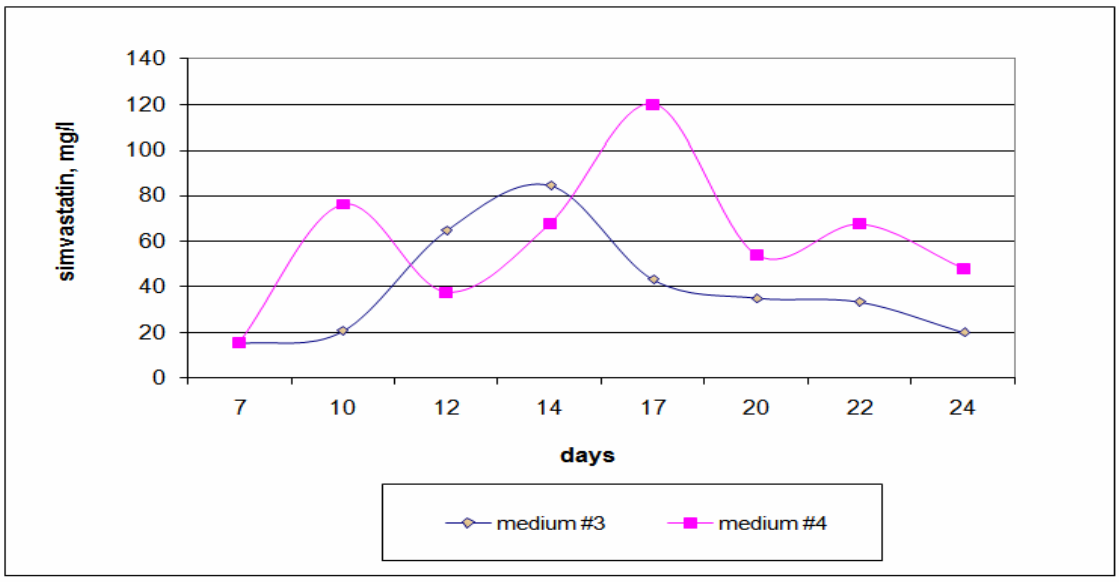
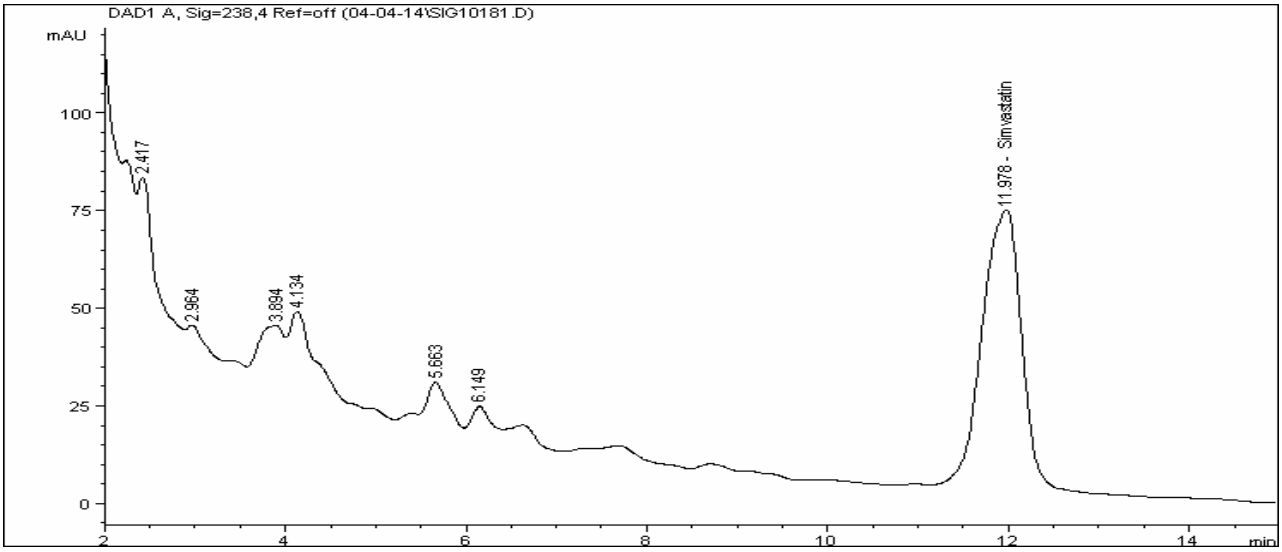


Figure.2 HPLC-chromatogram of extract *A. terreus 20*



Indeed, we have also found that the increase of carbon / nitrogen ratio leads to an elevated simvastatin titer. The data presented in table 1 show that the highest simvastatin yield was observed in media #3 and #4 where the ratio of carbon source to nitrogen source corresponded to 10 and 14, and simvastatin titer was 84.5 and 120.0 mg/l, respectively.

As shown in figure 1, accumulation of simvastatin within the dynamics of growth *A.terreus* 20 reaches its maximum by the 14th day of growth in medium #3, while three peaks were observed – by the 10th, 17th and 22nd day of culture growth in medium #4.

Figure 2 presents the HPLC profile of the extract from biomass of *A. terreus* 20 on 17th day of culture growth in medium #4. The peak corresponding by the retention time to the standard of simvastatin has been observed (Fig. 2). It should be noted that the initial titer of simvastatin in *A. terreus* 20, when cultivated in a medium as described previously by Casas Lopez *et al.* (2003), was 1.29 mg/l, which is 100 times lower than when strain was cultivated in the optimized medium #4. Thus, indigenous strain *A. terreus* 20 has a high potential for the production of simvastatin, and this can be observed in the selected cultivation media.

The above results suggest that the indigenous strain *A. terreus* 20 has a high potential for statins production, since apart from lovastatin this strain has biochemical mechanism for regulating simvastatin biosynthesis that, by-turn, is largely dependent on the composition of cultivation medium. According to information currently available to us, it is the first report on simvastatin synthesis as a product of direct fermentation in *A. terreus*.

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