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Screening For the Production of Lovastatin by Different *Aspergillus* Species.

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ABSTRACT

Nineteen *Aspergillus* isolates was obtained from different localities including 5 isolates from air, 7 isolates from soil cultivated with *Phanera variegate*, 4 isolates from soil cultivated with *Plumeria alba* and 3 isoates from non cultivated soil. The 19 isolates belonging to 9 species as follows 4 isolates of *A. flavus*, 3 isolates of *A. ochraceus*, 3 isolates of *A. versicolor*, 2 isolates of *A. niger*, 2 isolates of *A. terreus* and one isolate of each of the following *A. fumigatus*, *A. nidulans*, *A. sydowii*, *A. tamari* and *A. ustus*. The dry biomass was varied with the *Aspergillus* spp. and also with the type of samples. The highest dry biomass was obtained from the 2nd sample type which was soil cultivated with *Phanera variegate* where *A. ochraceus* (AS34), followed by *A. ochraceus* (A10) and *A. versicolor* (S53) with values 29.1, 28.8 and 28.6 g/l, respectively. The highest lovastatin produced *Aspergillus* was *A. terreus* (S57), followed by *A. fumigates* (S32), *A. nidulans* (S33), *A. ochraceus* (AS34), *A. versicolor* (S52NB) and *A. flavus* (96C); where the amount of lovastatin was 6.03, 1.53, 0.95, 0.75, 0.73 and 0.73 mg/l, respectively. Also, the results showed that there was *Aspergillus* spp., which first recorded in lovastatin production such as *A. tamari*, *A. utus*, *A. nidulans*, *A. sydowii*, *A. ochracaus.* Also, the highest two *Aspergillus* spp. in production of lovastatin were obtained from air. The final pH was increased in all *Aspergillus* spp. in general except in *A. niger* (38 and S58) and *A. nidulans* (S33) with values 2.7, 4.0 and 5.5.

Keywords: *Aspergillus*, lovastatin, HPLC, dry biomass

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INTRODUCTION

Lovastatin (also known as mevinolin or monakolin k) is one of group of drugs known as statins that was prescribed for decades to control cholesterol; they are competitive inhibitors of HMG-COA reductase enzyme in the biosynthetic pathway of chlosterol [1]. HMG-CoA reductase inhibitors decrease hepatic cholesterol production, which in turn leads to increased LDL receptor turnover, enhanced hepatic LDL-cholesterol uptake, and ultimately decreased plasma LDL-cholesterol level [2]. Numerous multicentered trials have demonstrated the efficacy of HMG-CoA reductase inhibitors in reducing mortality and morbidity in both primary [3] and secondary prevention [4] of coronary artery disease. Furthermore, it was also discovered that their use to be associated with long-term reduction in cerebrovascular events particularly after an initial coronary event [5,6].

These agents were also shown to have pleiotropic cardiovascular and antiatherosclerotic effects, including reversal of endothelial dysfunction, inhibition of monocyte recruitment, antioxidant activity, down-regulation of angiotensin II receptors, immunomodulation, reduction in inflammatory response, plaque stabilization, reduction in ventricular arrhythmias, and decrease in thrombogenicity [7-9]. Indeed, recent clinical studies have shown that treatment with HMG-CoA reductase inhibitors in acute coronary syndrome decreases short-term recurrent ischemia [10], and similarly after a transient ischemic attack they may suppress recurrences [11].

However, increasing clinical evidence suggests that statins, independent of their effects on serum cholesterol levels, have recently been shown to have promising anticancer activity and a potential role in the prevention and treatment of cancer. Specifically, statins have been shown to exert several beneficial antineo-plastic properties, including decreased tumor growth, angiogenesis, and repression of tumor metastasis and induction of apoptosis by inhibiting the mevalonate (MVA) pathway [12-14]. Lovastatin, recently been reported to decrease the incidence of melanoma in lovastatin-treated patients. Lovastatin may enhance the effectiveness of chemotherapeutic agents in the treatment of malignant melanomas [12,15]. Lovastatin inhibited the growth of gastric cancer cells [16] and pancreatic cancer [17], induces apoptosis of ovarian cancer cells [15] and in colon cancer cells [18]. However, if lovastatin could be administered intravenously or into tumor-feeding arteries and high lovastatin concentration maintained, it will achieve new avenues for cancer treatment would be opened [18].

Lovastatin was isolated for the first time from *Monascus ruber* [19], it was also isolated from numerous fungal strains of *M. purpureus* and *M. anka* [20], *M.* paxi [21], *Aspergillus oryzae*, *A. terreus*, *A. fischeri*, *A. flavus*, *A. niger*, *A. umbrosus*, *A. parasiticus*, *A. versicolor*, *Penicellium funiculosum*, *P. citrinum*, *P. chrysogonum*, *P. expansum*, *P. janthinellum*, *P. spinulosum*, *P. variabile*, *Trichoderma viridae*, *T. longibrachiatum*, *Accremonium Chrysogenum*, *Cylinderocarpon radicicola* [22,23], *A. flavipes* [24], *P. purpurogenum* [25].

In this study we tried to isolate and screen *Aspergilllus* isolates from the Egyptian soil and air for production of lovastatin.

MATERIAL AND METHODS

Soil samples

Soil samples were collected from different localities in Giza governorate, some of soil samples were collected from fields cultivated with *plumeria alba* and *Phanera variegate* (Table 1). The soil samples were air dried and grinded to fine particles and ready to use.

Table 1: Localities and type of collected samples used for isolation of *Aspergilli*

No.	Type of sample	Locality
1	Air	Giza
2	Soil cultivated with *Phanera variegate*	Faculty of Science, Cairo University garden (Giza)
3	Soil cultivated with *Plumeria alba*	Faculty of Agriculture, Cairo University garden (Giza)
4	Non cultivated soil	Faisal Road (Giza)

Isolation and purification of Aspergillus species

Nineteen isolates of *Aspergillus* have been collected from different regions in Egypt by using soil dilution method and direct isolation method and from air in botany department. They are isolated on Potato dextrose agar (PDA) and Czapek yeast extract Agar media. These pure cultures are persevered on slants of Czapek dox agar and kept at 4ºC as a stock.

Screening of the different fungal strains for the production of lovastatin

The different fungal isolates were screened for the production of lovastatin according to the following: Stock fungal strains were grown on czapek Dox with yeast extract agar at 28°C for 7 days and spores were harvested with a sterile solution of spore buffer (2% Tween 20 (v/v) and 0.85% NaCl (w/v)), Screening of lovastatin was carried out using a two-stage process consisting of growth (seed) and production step. For the growth step, different fungal strains were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium. Flasks of the growth or the seed medium were inoculated with 500 µl of spore suspension and incubated at 30°C in a rotary shaking incubator at 250 rpm with for 2 days. Then 20 ml of mycelia were used to inoculate 200 ml of production medium, and then the flasks were incubated at 250 rpm and 27°C for 10 days. The medium for growth and production stages was composed of composed of (g/l): glucose, 45; peptone, 24; yeast extract, 2.5; and poly ethylene glycol 6000, 2.5. The pH was adjusted to 6.8 before autoclaving. Three replicates were prepared for each fungal strain. Lovastatin concentrations and mycelial dry biomass were measured; also final pH of the production media was measured.

Extraction of lovastatin

Culture filtrate was acidified to pH 3 with 10% HCl and extracted with equal volume of ethyl acetate four times. The combined extracts were dried by using (Na_2SO_4) and concentrated by using rotary evaporator to a final volume of 4 ml.

HPLC measurement of lovastatin

The concentrated extracts were analyzed qualitatively and quantitatively for lovastatin by using HPLC (Young-Lin, South Korea) using C18 Column equilibrated at 25°C. Samples were analyzed using an isocratic method, with the mobile phase consisting of HPLC-grade acetonitrile and aqueous 0.1% H_3PO_4 in the ratio of 40:60 (v/v). The flow rate was 1.5 ml/ min, the injection volume of the samples was 20 µl, using UV detector and the peak detection was at 235 nm. Standard lovastatin was prepared by dissolving 3 mg of lovastatin in 1.25 ml acetonitrile, Lovastatin eluted in approximately 5.9 min under these conditions.

Identification of isolated Aspergillus species

Macroscopic and Microscopic examination

Aspergillus spp. were identified using morphological characteristics as possible with the aid of the books [26,27,28] using macro-morphological crieteria (colony color, colony size at different temperatures, colony reverse color) and micro-morphological criteria (color and shape of conidial head, length and color of conidiophores, size of conidia , presence or absence of hülle cells and their shapes, presence or absence of ascomata and ascospore, shapes of vesicles in *Aspergillus* (globose, clavate, subclavate), conidiophores have or do not have septum). Media used for identification: Malt Extract Agar (MEA) and Czapek Agar (CZ).

RESULTS AND DISCUSSION

The main goal of screening was to find new producers of the lovastatin .ethylacetate extract of the fermentation broths of nineteen *Aspergillu*s isolates were examined by using HPLC as described above. The nutritional and cultural conditions employed in this study were appropriate for screening lovastatin production by the isolates of different *Aspergillus* spp.; under these experimental conditions eighteen strains were identified as producers of the compound. No lovastatin detected in fermentation broth of only one strain A10 a strain of *Aspergillus ochraceus*.

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From results represented in Table 2 the 19 *Aspergillus* isolates was obtained 5 isolates from air, 7 isolates from soil cultivated with *Phanera variegate*, 4 isolates from soil cultivated with *Plumeria alba* and 3 isoates from non-cultivated soil. The 19 isolates belonging to 9 species as follows 4 isolates of *A. flavus*, 3 isolates of *A. ochraceus*, 3 isolates of *A. versicolor*, 2 isolates of *A. niger*, 2 isolates of *A. terreus* and one isolate of each of the following *A. fumigatus*, *A. nidulans*, *A. sydowii*, *A. tamari* and *A. ustus*.

Table 2: Identification of the *Aspergillus* spp. isolated from different localities and different types of sample

Symbol	Type of sample	Species
19	Air	*Aspergillus flavus*
96C		*Aspergillus flavus*
S32		*Aspergillus fumigatus*
S57		*Aspergillus terreus*
S52NB		*Aspergillus versicolor*
S59	Soil cultivated with *Phanera variegate*	*Aspergillus flavus*
69		*Aspergillus flavus*
S58		*Aspergillus niger*
38		*Aspergillus niger*
AS34		*Aspergillus ochraceus*
A10		*Aspergillus ochraceus*
S53		*Aspergillus versicolor*
A22	Soil cultivated with *Plumeria alba*	*Aspergillus ochraceus*
75R		*Aspergillus sydowii*
75C		*Aspergillus terreus*
AO		*Aspergillus ustus*
S51	Non cultivated soil	*Aspergillus tamari*
S33		*Aspergillus nidulans*
S52B		*Aspergillus versicolor*

The dry biomass was varied with the *Aspergillus* spp. and also with the type of samples. The data in Table 3 showed that the highest dry biomass was obtained from the 2nd sample type which was soil cultivated with *Phanera variegate* where *A. ochraceus* (AS34), followed by *A. ochraceus* (A10) and *A. versicolor* (S53) with values 29.1, 28.8 and 28.6 g/l, respectively. On the other hand the lowest dry biomass was obtained by *A. ochraceus* (A22) in the 3rd sample type (soil cultivated with *Plumeria alba* with value 11.6 g/l.

Table 3: Mycelial dry weight (±SD) of different *Aspergillus* spp. after 10 days in relation the sample type

Species	Symbol	Dry biomass (g)	Type of sample
A. flavus	19	16.0±0.46	Air
A. flavus	96C	20.1±0.23	
A. fumigatus	S32	23.9±0.69	
A. terreus	S57	20.7±0.40	
A. versicolor	S52NB	16.7±0.44	
A. flavus	S59	18.2±0.46	Soil cultivated with *Phanera variegate*
A. flavus	69	25.2±0.26	
A. niger	S58	22.0±0.46	
A. niger	38	22.5±0.35	
A. ochraceus	AS34	29.1±0.25	
A. ochraceus	A10	28.8±0.46	
A. versicolor	S53	28.6±0.46	
A. ochraceus	A22	11.6±0.42	Soil cultivated with *Plumeria alba*
A. sydowii	75R	25.6±0.31	
A. terreus	75C	15.2±0.31	
A. ustus	AO	22.8±0.36	
A. tamari	S51	16.0±0.26	Non cultivated soil
A. nidulans	S33	21.9±0.25	
A. versicolor	S52B	20.3±0.2	

The data in Table 4 revealed that the highest lovastatin produced *Aspergillus* was *A. terreus* (S57), followed by *A. fumigates* (S32), *A. nidulans* (S33), *A. ochraceus* (AS34), *A. versicolor* (S52NB) and *A. flavus* (96C); where the amount of lovastatin was 6.03, 1.53, 0.95, 0.75, 0.73 and 0.73 mg/l, respectively. Also, the results showed that there was *Aspergillus* spp., which first recorded in lovastatin production such as *A. tamari*, *A. utus*, *A. nidulans*, *A. sydowii*, *A. ochracaus.* From this results we noticed that the highest two *Aspergillus* spp. in production of lovastatin were obtained from air. The final was increased in all *Aspergillus* spp. in general except in *A. niger* (38 and S58) and *A. nidulans* (S33) with values 2.7, 4.0 and 5.5.

Table 4: Final pH of the production medium and the amount of lovastatin production after 10 days and the initial pH was adjusted at 6.8

Species	Symbol	Final pH	Lovastatin	Type of sample
A. flavus	19	7.0	0.31±0.03	Air
A. flavus	96C	7.0	0.73±0.03	
A. fumigatus	S32	7.4	1.53±0.21	
A. terreus	S57	7.6	6.03±0.42	
A. versicolor	S52NB	8.0	0.73±0.05	
A. flavus	S59	7.1	0.12±0.03	Soil cultivated with *Phanera variegate*
A. flavus	69	7.0	0.55±0.03	
A. niger	S58	4.0	0.21±0.01	
A. niger	38	2.7	0.17±0.01	
A. ochraceus	AS34	7.5	0.75±0.02	
A. ochraceus	A10	7.4	0.00±0.00	
A. versicolor	S53	7.1	0.34±0.01	
A. ochraceus	A22	7.5	0.48±0.02	Soil cultivated with *Plumeria alba*
A. sydowii	75R	7.5	0.37±0.01	
A. terreus	75C	6.6	0.20±0.01	
A. ustus	AO	7.7	0.26±0.01	
A. tamari	S51	8.4	0.35±0.01	Non cultivated soil
A. nidulans	S33	5.5	0.95±0.04	
A. versicolor	S52B	7.3	0.25±0.01	

A. versicolor, *A. flavus*, *A. terreus* and *A. niger* have been described by other investigators to have the ability to produce lovastatin, *A. terreus* [22,23], *A. versicolor* [23], *A. flavus* [22,23,25,30] and *A. niger* [23,25]; but the following species did not mentioned before in literature *A. tamari*, *A. utus*, *A. nidulans*, *A. sydowii*, *A. ochraceus* that are promising new lovastatin producers.

The advantage of this study was no need media with a complicated composition containing a lot of ingredients for screening; the media used with simple composition with natural ingredients for screening further experimentation in order to enhance and optimize the production of lovastatin especially for species that produce it with concentration less than 1 mg/l as this low concentration of lovastatin may be due to experimental conditions. Also it can be work on genes for increasing lovastatin production, as the presence of lovstatin in their extracts mean the presence of the gene cluster responsible for production of lovastatin in the nucleic acid of these isolates.

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