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Optimization of a culture medium for increased mevinolin production by *Aspergillus terreus* strain

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ABSTRACT

Fungi are important sources for the production of some pharmaceutical compounds. e.g. lovastatin, mevinolin and monacolin K. These are competitive inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A reductase, the rate limiting enzyme of cholesterol synthesis pathway. Four fungal strains of *Aspergillus terreus* and one *Penicillium patulum* were tested for their potential to produce mevinolin. The fungal strains were cultivated in four different semi-synthetic media to select a fermentation medium and a fungal strain that has the ability to secret mevinolin in high yield. The fermentation followed by TLC screening. Positive results were evaluated by confirmatory HPLC. *A. terreus* J9 was the best strain for producing mevinolin with a level of 148.66 mg/L of Dox-rice medium. Cultivation a 7.5 L in fermenter, *A. terreus* J9 produced 932.15 mg/L after 96 h using Dox-rice medium at 6.5 pH. Rise in acidity or alkalinity decrease mevinolin producing ability. Ammonium sulphate in the medium as sulphur and nitrogen sources influenced greatly mevinolin production as well as incubation period. Maximum production was obtained after 36 h incubation. The maximum value of the mevinolin concentratiom (1761.6 mg/L) was attained at 400rpm after 60 h fermentation at 28 ºC. The optimized medium resulted in a significant increase of mevinolin cocentration, as compared with that obtained by the fermentation of many other *A. terreus* species.

Keywords: Aspergillus terreus, Lovastatin, Mevinolin, Monacolin K, secondary metabolite

INTRODUCTION

Mevinolin is the first compound of its kind to become available for treatment of hypercholesterolemia. Lovastatin acts by competitively inhibiting the enzyme (3-hydroxy-3-methylglutaryl coenzyme A reductase HMG-CoA) (Alberts *et al.,* 1980 and Alberts, 1988). It is active not only *in vitro* to inhibit cholesterol biosynthesis but also *in vivo* to lower plasma choesterol level in humans and animals (Kaneko *et al.,* 1978), thereby is effective in the therapy of hypercholesterolemia. Hyperchlesterolemia is a primary risk factor for the coronary artery disease, the major cause of death in many countries (Goldstein and Brown, 1984).

This fungal secondary metabolite is produced by *Aspergillus terreus*. Mevinolin was first isolated by Endo from *Monascus rubber* (Endo, 1979) and independently, by Alberts *et al.* (1980) from *A. terreus*. Since then many strains of *Monascus* (Negishi *et al.,* 1986 and Chang *et al.,* 2002) as well as a variety of other filamentous fungi including some species of *Penicillium* (Alberts, 1988), *Hypomyces, Doratomyces, Phoma, Eupenicillium, Gymnoascus, Monascus ruber, Pleurotus* and *Ttrichoderma* were found to produce lovastatin (Endo *et al.,* 1986 and Samiee *et al.,* 2003).

Behr (1998) reviewed the results obtained during the production of Monacolin k by solid state fermentation using *Monascus purpureus* strains. Fermentation process with *A. terreus* was developed to manufacture it on large scale (Buckland *et al.,* 1989). The fungus, *A. terreus*, remains the organism of choice for lovastatin production. A number of studies attempting to increase lovastatin production through more efficient processes have been documented (Alberts *et al.,* 1980 and Szakacs *et al.,* 1998). Secondary metabolism of microorganisms is parts of their normal maturation processes (Szakacs *et al.,* 1998). The design of fermentation media is critical, especially when the products are secondary metabolites. Although major improvements are generally ascribed to the development of superior strains, nutrient supplies also affect cellular productivity. Atalla *et al.* (1999) found that the commercial production of lovastatin is typically carried out using large scale of fungal fermentation. Chang *et al.* (2002) employed response surface methodology for production of mevinolin in submerged cultures by *Monascus rubber*. The optimized medium resulted in a significant increase of mevinolin yield. Lopez *et al.* (2003) found that production of lovastatin and microbial biomass by *A. terrues* (ATCC20542) were influenced by the type of the carbon and nitrogen sources used and the C:N mass ratio in the medium. The behavior of the fermentation was not affected by the method of inoculation used. Li *et al.* (2006) studied the influencing factors, such as the kind of solvents, the acidic concentration, the initial mevinolin

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concentration, and the water content as well as temperature were investigated. Two kinds of comparative reactions, hydrolysis and alcoholysis, of mevinolin in solution were studied. This detailed study on the kinetics of mevinolin transformations is valuable and meaningful for the purification, preparation, injection manufacturing, extraction, storage, etc., of mevinolin or other similar compounds. This work provides useful information for the quality control of mevinolin and mevinolin-like drugs as well.

MATERIALS AND METHODS

Fungal strains

Aspergillus terreus isolates H2, J2, J9, I2 and *Penicillium patulum* was obtained from the culture collection of the NRC, Chemistry of Microbial Products, Dept. Egypt.

Media and growth conditions

Culture media

Potato dextrose agar (PDA) and Czapek Dox agar (CDA) were used for cultivation of cultures (Haung and Ling, 1973 and Difco, 1984).

Fermentation media

Four liquid media were used as fermentation media. Czapek Dox media was modified by the addition of different agriculture wastes (150 g/L) as a sole source of carbon instead of sucrose. The modified media are: Dox-rice, Dox sayfoun and Dox-deneiba in addition to Czapek Dox. 250 mL Erlenmeyer conical flasks each containing 50 mL fermentation medium were inoculated with 1 mL spore suspension then incubated on a rotatory incubator shaken at 180 rpm for 1 week at 28 – 30 °C.

The results previously reported for mevinolin production (Atalla *et al.,* 1999 and Shahaby *et al.,* 1999) justified the investigation of laboratory large scale production of this product. This also provides knowledge of some of the essential information necessary for the fermentative production of this product on semi-industrial and industrial scale.

Fermentation was performed in a 7.5 L bench top fermenter (New Brunswick, M 1085-1003) containing 4.5 L of Dox liquid medium supplemented with 150 g/L of ground rice as the sole carbon source. After autoclaving, the sterilized medium was inoculated with a 2% of 48 h old primary seed culture (Endo *et al.,* 1979). The aeration rate was about one v/v/m (5 L/min) and stirring was maintained at 400 rpm unless mentioned otherwise at 28 °C. Samples were taken every 12 h for determining mevinolin content and pH values.

The culture filtrates were adjusted to pH 3 and extracted three times with methanol (v/v). The aqueous layer was removed and the solvent layer was concentrated under vacuum till dryness and redisolved in methanol (Atalla *et al.,*1999), to obtain on the crude extract.

Extraction, determination and purification of mevinolin

Mevinolin was extracted from the cells and the culture filtrate. The required compound was extracted from the cells according to the method adopted by Moore *et al*. (1985) and Greenspan and Yudkovittz (1985) and modified by Atalla *et al.* (1999). The crude mevinolin sample in methanol was spotted on silica gel sheets 60 F_{254} developed in an ascending manner for few hours until the solvent front was about 16 cm length. Three solvent systems were used in volume ratio as follows: dichloromethane - acetone (4/1), methanol - chloroform (10/90, v/v), and methanol - diethylether (5/95, v/v). The latter proved the most suitable, accurate and reliable solvent system for separation and identification of mevinolin. Mevinolin spot was located by their blue fluorescence on chromatograms under short and long wave UV light in comparison with the standard.

In another method, a crude sample of mevinolin obtained from the previous extraction of the fermentation medium (filtrate and mycelium) with methanol was dried and dissolved in a minimum volume of the elute ethylacetate: methylene chloride (4:6, v/v) and applied as a slurry to a silica gel column using a glass pipette. The fractions were collected in 3 mL portions with the aid of an LKB-Rac fraction collector. The fractions could be analyzed by thin layer chromatography on silica gel sheets using authentic sample as a reference and solvent system methanol/diethyl ether. The fractions were also analyzed using a UV spectrophotometer CE595 double beam digital CECIL instruments at 238 nm. The pure mevinolin fractions were pooled, concentrated to a defined volume and their purities were assured by high pressure liquid chromatography (HPLC).

Other mevinolin properties were studied such as high resolution mass spectra were obtained on a Finnigan Mat SSQ-7000 spectrometer. The infra red spectra were obtained on the Fourier Transform infrared spectrometer (FT / IR-300E) using pellet of KBr. Nuclear magnetic resonance were obtained on a Varian EM-390 MH2 NMR spectrophotometer. The compound was dissolved in dimethyl-d_6-sulphoxide (DMSO, 99.5 % deuterium).

RESULTS AND DISCUSSION

Effect of different media

The purpose of this experiment was to study the effect of different media on mevinolin production by 4 different isolates of *A. terreus* (I2, J2, J9, H2) and *P. patulum.* These isolates were isolated locally and identified at NRC lab.

The results recorded in Table 1 showed that Dox´s medium supplemented with crushed rice (150g/L) as a sole carbon source was the most suitable for mevinolin

production especially by *A. terreus* J9 which produced (148.66 mg/L) followed by *A. terreus* I2 (102.67 mg/L).

On the other hand, the addition of sayfoun to Dox´s medium, mevinolin production decreased greatly reaching 55-60% of that in Dox-rice with different fungal isolates. Dox-deneiba medium is the least suitable for mevinolin production with all fungal isolates used. The medium may contain the precursors of mevinolin biosynthesis other than the unsupplemented Dox medium. This result coincided with those obtained by Shahaby *et al.* (1999), Chang *et al.* (2002) and Samie *et al.* (2003) who found that production of lovastatin or mevacor in rice medium was superior to that of any other substrate. *A. terreus* J9 was recommended to produce mevinolin when grown on Dox-rice medium.

Table 1: Effect of different media on the production of mevinolin by fungi

Fungal Isolates	Mevinolin concentration (mg/L)			
	Rice	Sayfoun	Deneiba	Dox
A. terreus I2	102.67	63.48	57.00	29.02
A. terrues J2	80.00	66.22	57.00	45.65
A. terrues J9	148.66	82.59	59.91	38.54
A. terrues H2	82.59	48.86	29.15	11.01
P. patulum	66.22	41.28	33.36	27.85

Effect of incubation period

Production of mevinolin during different incubation periods was studied by *A. terreus* J9 using Dox-rice medium and bench top fermenter 7.5 L capacity. Following pH changes along the experiment (Table 2) indicated that maximum production was obtained at pH 6.55 (932.15 mg/L) after 96h incubation and then declined. The procedure agreed with that adopted by Chang *et al.* (2002). They employed surface methodology to increase mevinolin production in submerged cultures by *Monascus rubber.*

Table 2: Effect of different incubation periods on mevinolin production

Incubation period (h)	Concentration (mg/L)	Final pH
12	328.42	5.81
24	278.22	6.43
36	348.18	6.7
48	829.15	7.12
60	283.40	7.45
72	785.43	7.14
84	751.42	6.8
96	932.15	6.55
18	831.744	6.42
120	791.90	6.21
132	565.83	6.00
144	381.86	6.00

Effect of initial pH

The initial pH of the medium solution was adjusted at 6.5, determined every 12h and readjusted at 6.5 along the experiment. The maximum production (96.22 mg/L) was obtained after 36 h incubation (Table 3). At neutral pH, mevinolin producing ability of the fungus increased as well as incubation decreased. It was also observed that decrease or increase in pH of the fermentation solution was accompanied by decrease in production. The results agreed with those of Alberts *et al.* (1980) and Kysilka (1993) who adjusted the pH of the fermentation medium at 7.4 to produce mevinolin by *A. terreus.*

Table 3: Effect of constant pH value on mevinolin production by fungi

Incubation period (h)	pH	Concentration (mg/L)
12	6.48	4.22
24	6.47	34.33
36	6.62	96.52
48	6.09	68.34
60	6.1	66.23
72	6.82	65.75
84	7.15	54.47

Effect of some nitrogen and sulpher sources on mevinolin production

In order to determine the importance of nitrogen and sulphur compounds in the fermentation medium on mevinolin production. The addition of ammonium sulphate (3 g/L) to the medium, pH adjusted at 6.5 increased the production (105 mg/L) after 36h incubation under the optimized conditions (Table 4). The results illustrated the importance of sulphate and nitrogenous compounds for the fungus. This was in accordance with Lopez *et al.* (2003) results, they found that production of lovastatin and microbial biomass by *A. terreus* ATCC 20542 was influenced by the type of the carbon and nitrogen sources used and the C/N mass ratio in the medium.

Table 4: Effect of ammonium sulphate on mevinolin production

Incubation period (h)	pH	Concentration (mg/L)
12	6.5	26.88
24	7.22	35.95
36	6.5	105.26
48	4.6	78.7
60	5.25	65.1
72	4.5	35.95

Effect of aeration level on growth and production:

The effect of agitation speed on growth and consequently production was studied during fermentation experiments using different agitation speeds (200, 400 and 600 rpm). The aforementioned conditions were adopted also foam control. Results (Table 5) showed that 400 rpm was the

most suitable speed to obtain maximum production (1761.6 mg/L) after 60h incubation at 28-30 °C. The decrease in rpm to 200 round prolonged incubation time to 96 h to obtain maximum production 932 mg/L). On the other hand, increase of rpm to 600 round decreased production to 1165.43 mg/L after 60 h, this because high agitation speed produced fine mycelial fragments which take some time to grow to the suitable form for production. These attempts to grow and increase lovastatin production previously tried by other workers such as Szakacs *et al* (1998) and Atalla *et al.* (1999).

Table 5: Effect of the different agitation speed during fermentation

Incubation period (h)	Concentration (mg/L)		
	200 rpm	400 rpm	600 rpm
12	328.42	362.4	347.77
24	278.22	906.4	410.52
36	384.18	1269.6	480.22
48	829.15	1398.4	1036.43
60	783.40	1761.6	1165.18
72	785.43	1127.8	981.75
84	751.42	906.4	939.27
96	932.15	258.4	354.25

Effect of fermentation temperature on growth and production

The optimum temperature for fermentation process was studied during fermentation experiments using Dox-rice medium and incubation for different periods of time at temperatures range (28-32 °C) under the optimized conditions. Results (Table 6) showed that fermentation for 60 h at 28 °C was the optimal condition for mevinolin production (1761.6 mg/L). Fluctuation in temperature more or less reflected badly on fermentation production. This confirms that the design of fermentation medium is critical; especially the products are secondary metabolites (Atalla *et al.,* 1999).

Table 6: Effect of the different temperature on mevinolin production

Incubation period (h)	Concentration (mg/L)			
	26 °C	28 °C	30 °C	32 °C
12	229.89	362.4	356.16	278.21
24	194.22	906.4	815.76	328.41
36	268.92	1269.6	1369.56	384.17
48	580.4	1398.4	1358.56	829.14
60	549.8	1761.6	1585.44	832.18
72	652.5	1127.8	1015.02	785.42
84	525.99	906.4	815.76	751.41
96	311.74	258.4	335.92	383.4

CONCLUSIONS

It can be concluded that, *Aspergillus terreus* J9 was the most active strain for mevinolin production when grown on rice straw medium.
The maximum production was obtained after 36 h incubation, 400 rpm after 60 h fermentation at 28 °C.

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