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Effects of the sporulation conditions on the lovastatin production by *Aspergillus terreus*

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Abstract The production of biomass and lovastatin by spore-initiated submerged fermentations of *Aspergillus terreus* ATCC 20542 was shown to depend on the age of the spores used for inoculation. Cultures started from older spores produced significantly higher titers of lovastatin. For example, the lovastatin titer increased by 52% when the spore age at inoculation rose from 9 to 16 days. The lovastatin titer for a spore age of 16 days was 186.5 ± 20.1 mg L⁻¹. The time to sporulation on surface cultures was sensitive to the light exposure history of the fungus and the spore inoculation concentration levels. A light exposure level of $140 \mu\text{E m}^{-2} \text{s}^{-1}$ and a spore concentration of $1,320$ spore cm⁻² produced the greatest extent of sporulation within about 50 h of inoculation. Sporulation was slowed in the dark and with diluted inoculants. A rigorous analysis of the data of statistically designed experiments showed the above observations to be highly reproducible.

Keywords Lovastatin · Fermentation · Sporulation · *Aspergillus terreus*

Introduction

Statins are commonly used in clinical practice to lower the blood cholesterol levels [1]. Lovastatin (C₂₄H₃₆O₅) is a natural statin produced as a secondary metabolite by various filamentous fungi including *Penicillium* sp. [3], *Monascus ruber* [4, 8] and *Aspergillus terreus* [9–11, 13]. Commercial production of lovastatin is based on *A. terreus* batch fermentation and, consequently, this

species is the focus of most research. While the effects of the medium composition and fungal morphology on lovastatin production by *A. terreus* have been studied [2, 7, 9–11, 14], there are no reports about how the various sporulation conditions and the storage conditions of the spores that will be used for inoculation might influence the biosynthesis of lovastatin. The present work examined the effects of sporulation aspects on lovastatin production in batch cultures of *A. terreus* ATCC 20542.

Response surface methodology was used for studying the effects of three factors [i.e., spore concentration at inoculation, the light intensity exposure of the fungus during sporulation, the time spent in Petri dish prior to inoculation (i.e., spore age)], on the two responses (i.e., concentrations of lovastatin and biomass) in submerged fermentations.

Materials and methods

Microorganism

The fungus used was obtained from the American Type Culture Collection, as *A. terreus* ATCC 20542. The culture was maintained on Petri dishes of PDA (potato dextrose agar). After inoculation from the original slant, the dishes were incubated at 28 °C for 5 days and subsequently stored at 5 °C. A suspension of spores was obtained by washing the Petri dish cultures with a sterile aqueous solution of 2% Tween[®] 20. The resulting suspension was centrifuged ($\sim 2,800 \times g$, 5 min) and the solids were resuspended in sterile distilled water. Spores concentration was determined spectrophotometrically at 360 nm. A standard curve was used to correlate the optical density to direct spores counts carried out with a flow cytometer (Coulter Epics XL-MCL).

Experimental design

A modified Box–Behnken design was used for sampling the factor space (age of spores or the time spent in Petri

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dish prior to inoculation in submerged fermentation; the spore inoculation level in the Petri dish; light intensity exposure of the dish). A three-level fractional design (12 runs and 3 center points) was used. The experiments were replicated so that 30 Petri dishes were initiated as precursors to submerged fermentations. Petri dishes of PDA were inoculated with a predetermined concentration of spores and placed at 28 °C in darkness, under normal illumination in the laboratory ($20 \mu\text{E m}^{-2} \text{s}^{-1}$) and under four 18 W fluorescent lamps ($140 \mu\text{E m}^{-2} \text{s}^{-1}$). Each Petri dish was photographed every 4 h to document the sporulation status. Complete sporulation was deemed to have occurred when the spores were fairly uniformly dispersed on the dish, as illustrated in Fig. 1a.

The levels of the independent variables were as follows: 1.32×10^3 , 13.2 and $0.132 \text{ spores cm}^{-2}$ for the spore concentration; 0, 20 and $140 \mu\text{E m}^{-2} \text{s}^{-1}$ for the light intensity; and 2, 9 and 16 days for the spore age. The spore concentration could also be expressed as concentration factor levels [$\log_{10}(\text{absolute concentration}/13.2)$], i.e., 2, 0 and -2, for the concentrations above, respectively. Stated this way, the light intensity factor was the only one with values that were not equally spaced when the factors were coded for response surface analyses. The response surface was fitted to normalized levels for each factor. Normalized value of a factor was calculated with the following equation:

$$X = \frac{2(F - F_{\text{mid level}})}{(F_{\text{max}} - F_{\text{min}})}, \quad (1)$$

where F was the absolute value of the factor and X the transformed or coded value. The subscripts max and min denoted maximum and minimum values. Table 1 shows the combinations of factor levels, the absolute values of the factors and the coded values used.

Once sporulated, the Petri dishes were incubated (28 °C) for 2, 9 or 16 days as shown in Table 1. After this, a suspension of spores was obtained by washing the Petri dish cultures as described above. All submerged fermentations were carried out in duplicates at 28 °C in 250 mL shake flasks filled with 50 mL of the medium [2] that contained the following components (per liter): lactose 114.26 g; soybean meal 5.41 g; KH_2PO_4 0.80 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.52 g; NaCl 0.40 g; biotin 0.04 mg; $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ 1 mg; $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ 2 mg and trace element solution 1 mL. The trace element solution contained (for 1 L of solution): $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ 100 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 50 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 50 mg and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 250 mg. The pH was adjusted to 6.5 with NaOH before sterilization. The flasks were inoculated with 500 μL of a spore suspension which had been standardized to contain 8.6×10^7 spores mL^{-1} . The inoculated flasks were held on a rotary platform shaker (150 rpm, 2.6 cm stroke) for 7 days. After this, the lovastatin titer and the biomass concentration were measured.

Analytical methods

The biomass dry weight was determined by filtering a known volume of the broth through a $0.45 \mu\text{m}$ Millipore cellulose filter, washing with sterile distilled water and freeze drying the solids.

Lovastatin was determined as its beta hydroxyacid, by high-performance liquid chromatography (HPLC) of the biomass-free filtered broth [12]. Because the fungus secretes lovastatin in the beta hydroxyacid form, the assay eliminated the conversion step to the active lactone form of the drug. Using the beta hydroxyacid form permitted rapid analysis because this form elutes earlier from a chromatography column than does the lactone form of lovastatin. Also, the beta hydroxyacid is quite stable in solution. The filtered broth containing the beta hydroxyacid form of lovastatin was diluted 10-folds with acetonitrile–water (1:1 by vol) prior to analysis [6].

Pharmaceutical grade lovastatin (lactone form) tablets (Nergadan[®] tablets; J. Uriach & Cia., S.A.) containing 40 mg lovastatin per tablet were used to prepare the standards for the HPLC analyses. Prior to use, the lactone form of lovastatin was converted to the beta hydroxyacid form by dissolving the tablets in a mixture of 0.1 N NaOH and ethanol (1:1 by vol), heating at 50 °C for 20 min and neutralizing with HCl. The resulting standard stock solution contained 400 mg lovastatin (beta hydroxyacid) per liter. The solution was held at 5 °C until needed.

High-performance liquid chromatography was performed on a Beckman Ultrasphere ODS (250 mm \times 4.6 mm I.D., 5 μm) column. The column was mounted on a Shimadzu model Lc10 liquid chromatograph equipped with a Shimadzu MX-10Av diode array detector. The eluent was a mixture of acetonitrile and 0.1% phosphoric acid (60:40 by vol). The eluent flow rate was 1.5 mL min^{-1} . The detection wavelength was 238 nm. The sample injection volume was 20 μL .

The fungal growth was examined and photographed under an inverted microscope (Leica DMIL) equipped with a CMOS camera (Evolution LC Color; Media Cybernetics Inc., Silver Spring, MD, USA) using a 400 \times magnification. Photographs were taken four times a day until sporulation was observed.

Results and discussion

The effects of light intensity and spore concentration on the time to sporulation are shown in Table 2. Any increase in either light intensity or spore concentration shortened the time to sporulation. At a given level of light, an increase in spore concentration reduced the sporulation time. Experiments carried out at light intensity increased in three levels up to $250 \mu\text{E m}^{-2} \text{s}^{-1}$ and spore concentration increased up to 1.32×10^5 spore cm^{-2} , did not further shorten the time to sporulation relative to the shortest value of 46 h in Table 2.

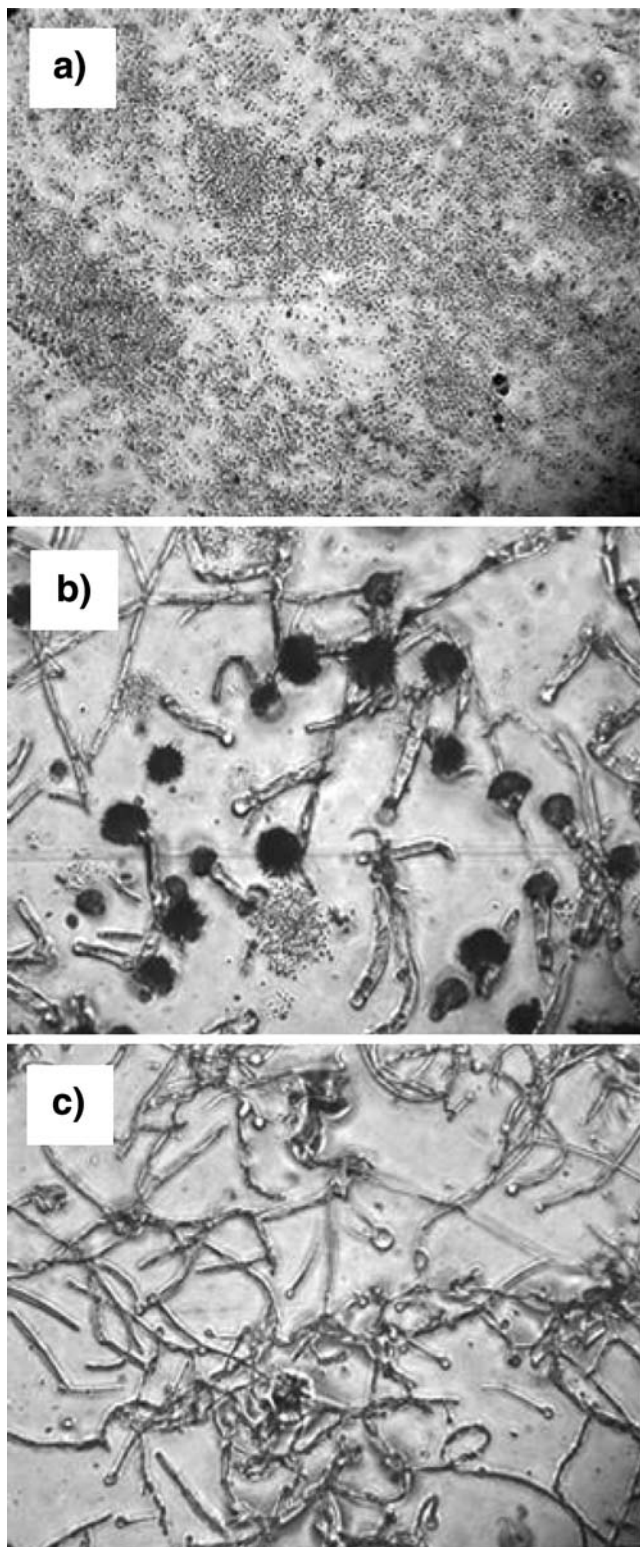


Fig. 1 Morphology of the Petri dish cultures at 50.5 h from inoculation and different light and spore titer levels: **a** 1.32×10^3 spores cm^{-2} and $140 \mu\text{E m}^{-2} \text{s}^{-1}$; **b** 1.32 spores cm^{-2} and $20 \mu\text{E m}^{-2} \text{s}^{-1}$; **c** 0.132 spores cm^{-2} and darkness

The spore concentration effect was related to a more rapid depletion of nutrients at high spore concentration and consequent earlier sporulation. Light level of up to

Table 1 Combinations of factor levels and coded values

Factors	Original levels			Coded levels		
Light intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)	0	20	140	-0.286	0	1.714
Spore concentration (spores cm^{-2})	13.2×10^{-2}	13.2×10^0	13.2×10^2	-1	0	1
Age (days)	2	9	16	-1	0	1

Table 2 Time to sporulation (h) in Petri dishes

Spore concentration (spores cm^{-2})	Light intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)		
	0	20	140
13.2×10^{-2}	90	84	74
13.2×10^0	78	71	55
13.2×10^2	71	56	46

$140 \mu\text{E m}^{-2} \text{s}^{-1}$ was an accelerator factor in spore development and had no inhibition component in the range studied. Light is known to induce both morphogenic and non-morphogenic responses during growth and development of many fungi [5]. The sporulation states attained at a given time and different initial spore concentrations and Petri dish illumination levels are shown in Fig. 1. In Fig. 1a the sporulation has occurred. In Fig. 1b the spore-containing conidiophores have not yet hatched. Figure 1c shows that the conidiophores are just beginning to form as swellings at the tips of the hyphae.

The results of the experimental design are shown in Table 3, where responses Y_1 (lovastatin titer) and Y_2 (biomass concentration) are the average values of the two replicated submerged fermentations. The table also shows the coded factor levels. The lovastatin titer ranged from 22.6 to 196.4 mg L^{-1} and the biomass concentration varied from 7.85 to 12.52 g L^{-1} .

Spore age was the main source of variation in the lovastatin titer and the biomass concentration. Spore concentration and exposure light intensity did not affect biomass concentration and lovastatin titer as much as did the spore age (Tables 4, 5).

The age of spores had opposite effects on lovastatin and biomass concentrations. Thus, an increasing spore age decreased the biomass concentration attained but raised the lovastatin titer. This behavior was associated with the fact that lovastatin is produced mainly during the stationary phase of the growth.

Further experiments carried out to test the possible influence of the genetic variability of spores of the same age produced under identical conditions of illumination revealed no significant spore-to-spore variation in biomass concentration and lovastatin titers. For example, the lovastatin titers for a spore age of 16 days (light intensity of $20 \mu\text{E m}^{-2} \text{s}^{-1}$) were 146 ± 19 and

Table 3 Lovastatin titer and biomass concentration in submerged fermentations

Block	Run	X_1 ($\mu\text{E m}^{-2} \text{s}^{-1}$)	X_2 (spores cm^{-2})	X_3 (days)	Y_1 (mg L^{-1})	Y_2 (g L^{-1})
1	1	0	0	0	116.2	9.63
1	2	-0.28	0	-1	38.2	12.52
1	3	0	1	-1	63.0	10.80
1	4	0	-1	-1	22.9	11.58
1	5	0	-1	1	171.3	7.88
1	6	0	0	0	112.5	9.08
1	7	0	1	1	158.9	8.38
1	8	1.71	1	0	68.7	10.14
1	9	1.71	-1	0	49.7	10.60
1	10	0	0	0	43.4	10.68
1	11	-0.28	0	1	162.7	7.85
1	12	-0.28	-1	0	142.9	8.21
1	13	-0.28	1	0	132.2	8.24
1	14	1.71	0	1	196.4	8.53
1	15	1.71	0	-1	119.0	11.04
2	16	0	0	0	70.7	11.50
2	17	-0.28	0	-1	38.2	12.52
2	18	0	1	-1	22.6	10.01
2	19	0	-1	-1	40.7	11.47
2	20	0	-1	1	149.0	8.27
2	21	0	0	0	91.3	9.31
2	22	0	1	1	143.4	8.61
2	23	1.71	1	0	67.6	9.56
2	24	1.71	0	0	53.9	10.20
2	25	0	0	0	40.1	11.59
2	26	-0.28	0	1	148.5	8.83
2	27	-0.28	-1	0	133.2	7.91
2	28	-0.28	1	0	149.7	8.81
2	29	1.71	0	1	147.6	8.15
2	30	1.71	0	-1	68.9	11.04

Y_1 lovastatin titer, Y_2 biomass concentration, X_1 light intensity, X_2 spore concentration, X_3 spore age

Table 4 Analysis of variance for lovastatin titer (Y_1)

Source	SSQ	<i>Df</i>	MS	<i>F</i> ratio
Age	47,822.0	1	47,822.0	43.57 ^a
Light	1.5667	1	1.5667	0.00 ^b
Concentration	132.854	1	132.854	0.12 ^b
Blocks	3,462.68	1	3,462.68	3.15 ^b
Error	27,441.3	25	1,097.65	
Total	78,860.4	29		

$R^2 = 65.2\%$

SSQ sum of squares of data, *Df* degrees of freedom, MS mean square

^a $P < 0.001$

^b $P > 0.05$

Table 5 Analysis of variance for biomass concentration (Y_2)

Source	SSQ	<i>Df</i>	MS	<i>F</i> ratio
Age	37.3688	1	37.3688	40.16 ^a
Light	0.456537	1	0.456537	0.49 ^b
Concentration	0.131611	1	0.131611	0.14 ^b
Blocks	0.244464	1	0.244464	0.26 ^b
Error	23.2651	25	0.930605	
Total	61.4665	29		

$R^2 = 62.1\%$

SSQ sum of squares of data, *Df* degrees of freedom, MS mean square

^a $P < 0.001$

^b $P > 0.05$

$186 \pm 20 \text{ mg L}^{-1}$ in cultures that had been started from spore populations and single spores, respectively. Similarly, for spores incubated in the dark to an age of 9 days, the fermentation broths produced from multiple and single spores had comparable lovastatin titers at 71 ± 11 and $106 \pm 17 \text{ mg L}^{-1}$, respectively. For the sporulation conditions of the central point, the lovastatin concentration was $71 \pm 22 \text{ mg L}^{-1}$ (multiple spores). Submerged fermentations (four replicates) initiated from different single spores were used only in assessing the effects of spore-to-spore variability on lovastatin titer. These results confirmed that increased age of spores within the tested range increased the lovastatin titers in fermentation broths produced from the spores.

Conclusions

Control of spore age at inoculation of fermentation is required for obtaining optimal production of lovastatin. Inoculation from older spores generally leads to higher titers. Titters increased by 52% when the spore age changed from 9 to 16 days.

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