J. Microbiol. Biotechnol. (2008), **18**(2), 322–327

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of 1,5-Dihydroxy-3-Methoxy-7-Methylanthracene-9,10-Dione by Submerged Culture of *Shiraia bambusicola*

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Received: March 22, 2007 / Accepted: May 29, 2007

1,5-Dihydroxy-3-methoxy-7-methylanthracene-9,10-dione (shiraiarin) is a kind of antitumor and antibacterial anthraquinone, and was produced for the first time from the submerged fermentation of *Shiraia bambusicola*, as confirmed by ESI-MS and NMR. The production of shiraiarin was significantly influenced when varying the carbon source, and a high amount of shiraiarin was only achieved when using lactose. The production of shiraiarin was also stimulated when using $NaNO_3$ as the nitrogen source, whereas other nitrogen sources inhibited its production. Shiraiarin was formed during the stationary phase with a pH value higher than 8. The production of shiraiarin was inhibited by sporulation.

Keywords: *Shiraia bambusicola*, shiraiarin, carbon source, nitrogen source, pH

Fungi are used in many industrial processes, such as the production of pigments [14], enzymes [3], organic acids [27], polysaccharides [35], lipids [33], and glycolipids [34]. Whereas some of these products are produced for commercial purposes, others are potentially valuable in biotechnology. It is already known that fungal secondary metabolites are extremely important for health and nutrition, giving them a tremendous economic impact [1].

Shiraia bambusicola, a fungus growing on bamboo, has been used for centuries by the Chinese to treat skin disease, and over the last few decades it has attracted much attention because of its multiple pharmacological effects, including antidepressant, antiviral, antiretroviral, and, in particular, photosensitizing activities [6, 13, 29, 37].

The submerged fermentation of *S. bambusicola* produces a red pigment named after shiraiarin, with a structure determined as 1,5-dihydroxy-3-methoxy-7-methylanthracene-9,10-dione (shiraiarin) by ESI-MS and NMR. Shiraiarin has already been separated from *Cassia italica* (a simple sparingly branched senna plant) [23], yet has not been found in *S. bambusicola* growing naturally. The structure of shiraiarin is similar to that of emodin in that both have antitumor, antibacterial, and anti-inflammatory bioactivities [19, 20, 23–25, 36]. Accordingly, using *S. bambusicola* as a new source of shiraiarin, the objective of the present study was to determine the effects of the carbon source, nitrogen source, and pH on the production of shiraiarin by a submerged culture of *S. bambusicola*.

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Materials and Methods

Fungal Material

The experiments were carried out using *S. bambusicola* grown on bamboo in southeastern China during May and July.

Culture Conditions

The basic medium contained (per liter) 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, and 0.01 g of $FeSO_4 \cdot 7H_2O$, and the initial pH was 6.83 after autoclaving.

S. bambusicola was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of the medium, and cultivated at 26°C on a reciprocal shaker at 200 rev/min for 144 h.

Preparation of Pure Shiraiarin

As the shiraiarin was secreted into the fermentation broth, it was isolated using the following procedure. The broth was filtered, and the pH of the filtrate adjusted to 3.0 by adding HCl. The mixture was then concentrated in a vacuum to one-fifth the original volume and extracted with CH_3Cl, which had been concentrated by vacuum distillation, to yield a reddish crude residue.

The crude residue was then dissolved in CH_3OH, and the pure shiraiarin prepared by chromatography under the following conditions: Kromasil C18 preparation column (250 mm×30 mm, 5 μm), mobile phase CH_3OH:H_2O (70:30), flow rate 1 ml/min,

temperature 30°C, Knauer K1900 preparation pump, Knauer K2501 detector (430 nm).

Analytical Methods

The pH value of the fermentation broth was measured using a S20 SevenEasy pH meter (Mettler Toledo), and the biomass produced in the liquid medium determined by measuring the dry weight. The cultures were harvested on filter papers, washed with 100 ml of distilled water, and dried at 80°C to a constant weight. A quantitative determination of the shiraiarin in the fermentation broth was then analyzed by HPLC, using a Knauer HPLC system (K501 pump, K2501 detector 430 nm) with Eurochrom 2000 operation software. The chromatographic conditions comprised a Kromasil C18 analytical column (250 mm× 4.6 mm, 5 μm), mobile phase of CH_3OH:H_2O (70:30), flow rate of 1 ml/min, injection of 20 μl, and temperature of 30°C.

Structure Assay

The pure shiraiarin was dissolved in $CDCl_3$ for the NMR experiments, which were carried out as described by Berger and Braun [4]. The NMR spectrum was recorded using a Varian Mercury Plus 400 MHz (Shanghai Institute of Material Medica), and a Waters Platform ZMD 4000 (Southern Yangtze University) was used to collect the electrospray ionization mass spectrometry (ESI-MS) data [8].

RESULTS

Structure Analysis

Fig. 1 shows the ESI-MS spectrum, including the *m/z* 285 in ES+ spectrum and *m/z* 283 in ES- spectrum. Thus, the molecular weight of shiraiarin should be 284. The difference of 28 (*m/z* 270 [M+1-CH_3], 242 [M+1-CH_3-CO], and 214 [M+1-CH_3-2CO] for shiraiarin) suggested

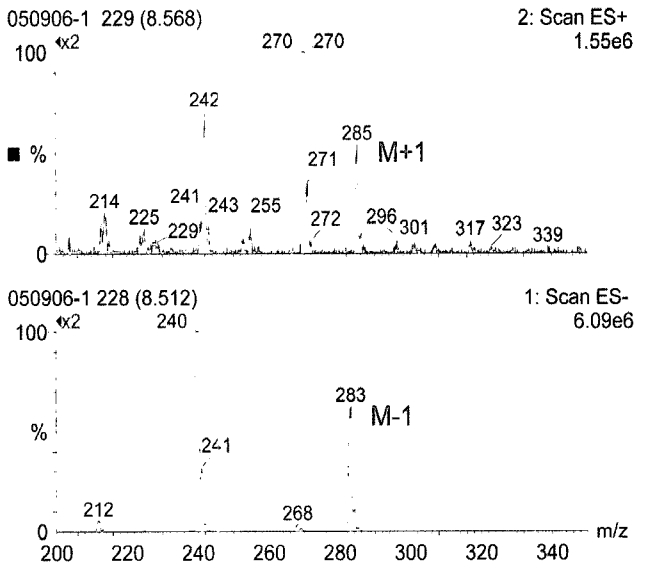

Fig. 1. Spectra of ESI-MS.
M+1, positive-ion spectrum with one adduct proton. M-1, negative-ion spectrum with one lost proton.

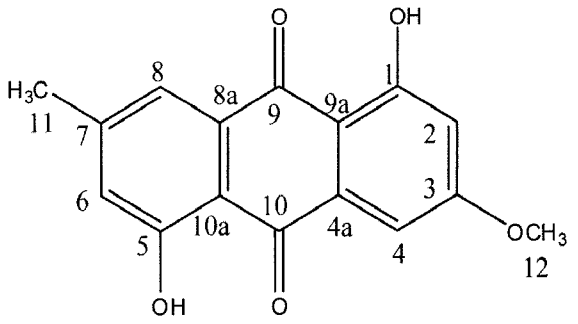

Fig. 2. Structure of the pigment isolated from fermentation broth of *S. bambusicola*.
^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.42 (s, J=2.35, 1H-2), 3.82 (3H, s, MeO-3), 7.05 (s, J=2.35, 1H-4), 6.98 (t, 1H-6), 2.38 (3H, s, Me-7), 7.45 (d, 1H-8), 12.19 (1H, s, OH-5), 12.02 (1H, s, OH-1); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 166.9 (C-1), 108.0 (C-2), 179.9 (C-3), 109.1 (C-4), 138.6 (C-4a), 163.9 (C-5), 120.4 (C-6), 147.92 (C-7), 125.4 (C-8), 117.2 (C-8a), 187.3 (C-9), 115.7 (C-9a), 186.6 (C-10), 134.9 (C-10a), 22.2 (Me-7), 56.5 (MeO-3); EMS-MS, ES+ *m/z* 285 [M+H], *m/z* 270 [M+H-CH_3], *m/z* 242 [M+H-CH_3-CO], *m/z* 214 [M+H-CH_3-2CO]; ES- *m/z* 283 (M-H); $C_{16}H_{12}O_5$, IUPAC name of metabolite is 1,5-dihydroxy-3-methoxy-7-methylanthracene-9,10-dione.

that shiraiarin has two carbonyl groups, as the difference was based on the cleavage of CO. The ESI-MS spectrum suggested a molecular formula of $C_{16}H_{12}O_5$.

The ^{1}H NMR signals of δ_H 2.38 and δ_H 3.82 indicated a chemical shift for the signals of -CH_3 and -OCH3, respectively, whereas δ_C 22.2 and δ_C 56.5 in the ^{13}C NMR spectrum indicated the signals of -CH_3 and -OCH_3, respectively. Moreover, δ_H 7.05, δ_H 6.98, and δ_H 7.45 were the signals for aromatic protons, and the other signals were attributed to two meta-couple aromatic protons (δ 6.42, J=2.35 and δ 7.05, J=2.35). Finally, δ_C 186.6 and δ_C 187.3 were the signals for the carbonyl group, which were very close, indicating that the positions of the two hydroxyl groups were not at 1 and 8. Therefore, the above results clearly confirmed that the structure of shiraiarin is 1,5-dihydroxy-3-methoxy-7-methylanthracene-9,10-dione. Fig. 2 shows the structure of shiraiarin based on the NMR and MS analyses.

Effect of Carbon Source

The ability of *S. bambusicola* to use a range of carbon sources in a defined basic medium with a $NaNO_3$ concentration of 2 g/l was investigated. Seven carbon sources (glucose, sucrose, maltose, xylose, starch, fructose, and lactose) were added to the medium to make a final concentration of 25 g/l. The growth and product formation of shiraiarin with the different saccharides are shown in Fig. 3.

S. bambusicola was able to use all the saccharides tested, yet no correlation was found between the biomass and shiraiarin when *S. bambusicola* was cultured with the different carbon sources. The best substrate for biomass production was glucose, followed by sucrose, maltose, fructose, starch, lactose, and xylose. However,

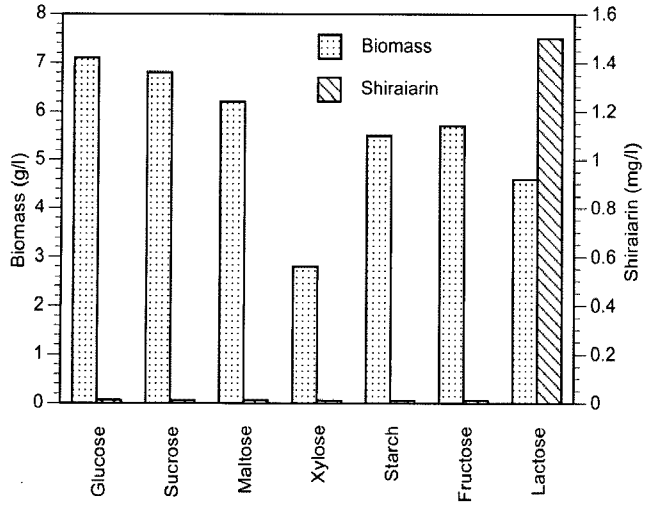

Fig. 3. Comparison of biomass and production of shiraiarin with different carbon resources.

only lactose induced shiraiarin production despite moderate cell growth.

Effect of Nitrogen Source

Different nitrogen sources (organic and mineral) were also tested (at a constant nitrogen concentration of 2 g/l, corresponding to 25 g/l of lactose). The effect on the biomass and production of shiraiarin are shown in Fig. 4, where only $NaNO_3$ stimulated the production of shiraiarin, whereas the other nitrogen sources inhibited its production.

Effect of pH

The effect of pH (with and without a buffer) on the biomass and shiraiarin is listed in Table 1, where the addition of buffers reduced the pH change during the culture of *S. bambusicola*. For the buffers tested with an initial pH of

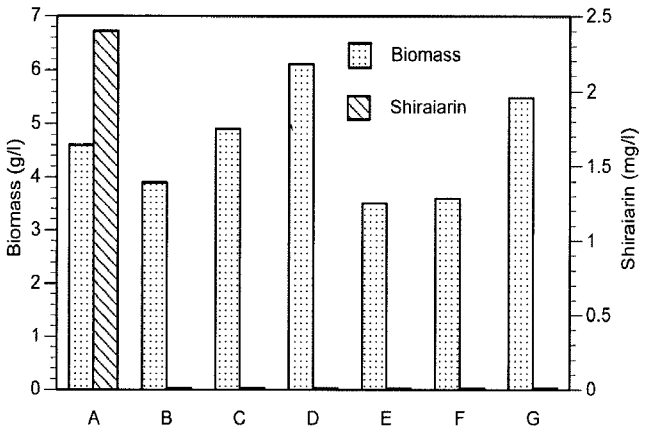

Fig. 4. Comparison of biomass and production of shiraiarin with different nitrogen resources: (A) $NaNO_3$, (B) $(NH_4)_2SO_4$, (C) wheat bran, (D) yeast extract, (E) peptone, (F) NH_4NO_3, and (G) potato extract.

Table 1. Comparison of production of shiraiarin and biomass under different pH conditions.

	pH		Shiraiarin (mg/l)	Biomass (g/l)
	Initial	End		
Controlled by buffer	5.10	5.42	0	4.6
	6.80	7.23	0	4.6
	7.46	7.87	0	4.5
	8.01	8.23	1.9	4.2
Uncontrolled	5.10	8.13	1.8	5.1
	6.83	8.41	2.5	4.6
	8.12	8.84	2.8	4.1
	9.03	9.62	2.2	3.8

Buffer: Na_2HPO_4+Critic acid; Carbon source: Lactose; Nitrogen source: $NaNO_3$.

5.10 to 7.46, *S. bambusicola* did not produce any shiraiarin. Without a buffer, the highest amount of shiraiarin was produced with an initial pH of 8.12. Therefore, the results showed that during growth on the medium containing $NaNO_3$ as the nitrogen source without a buffer, physiological basification of the cultivation medium occurred owing to the consumption of the nitrate ion and accumulation of the basic. Moreover, a high pH resulted in the production of shiraiarin, whereas *S. bambusicola* grew better under acidic conditions.

The time course of shiraiarin production by *S. bambusicola* without a buffer is shown in Fig. 5, where the biomass increased sharply after 24 to 72 h, accompanied by an increase in the pH, which allowed shiraiarin to be synthesized. Therefore, the results indicated that shiraiarin was formed during the stationary phase of cell growth when the pH was higher than 8.

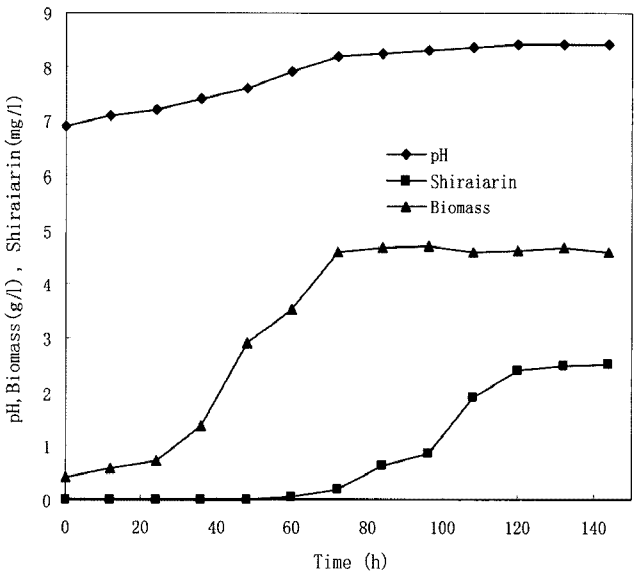

Fig. 5. Time course of shiraiarin production by *S. bambusicola* without buffer.

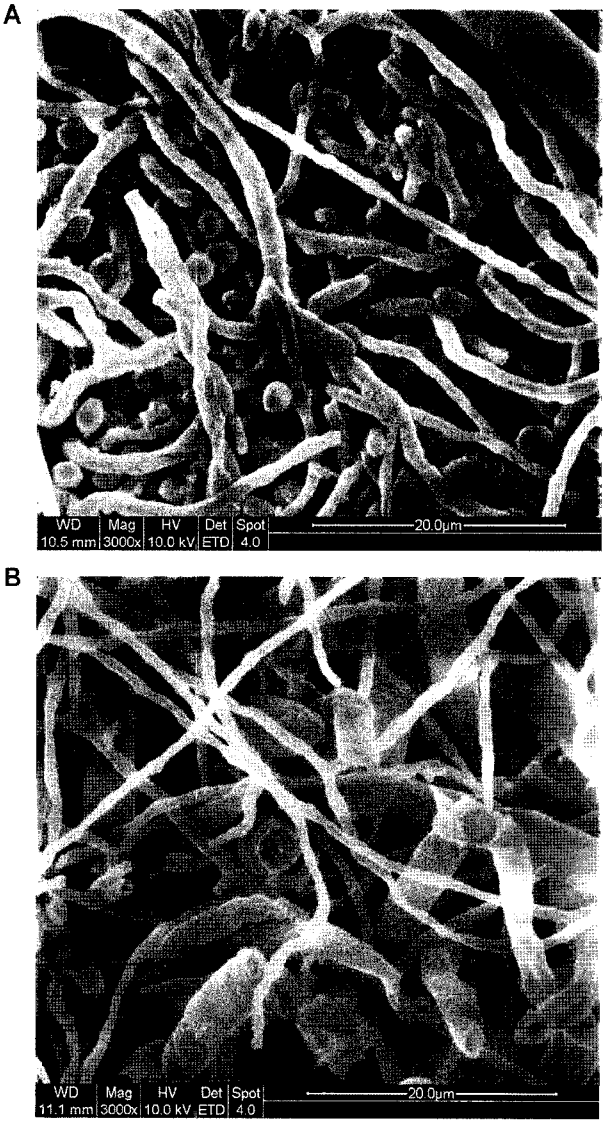

Fig. 6. Scanning electron microscopy images of mycelia taken after 120 h of cultivation: (**A**) without shiraiarin production and (**B**) with shiraiarin production.

Morphology

The morphology of *S. bambusicola* with and without shiraiarin production is shown in Fig. 6. When shiraiarin was produced, there was no sporulation and many hyphae were swollen (Fig. 6B). Thus, sporulation may inhibit the production of shiraiarin (Fig. 6A).

DISCUSSION

Fungi are of great biotechnological interest as regards the fermentative processes of secondary metabolites, which in turn are important for discovering new antibiotics. Fungi display a broad range of useful antibiotic and pharmaceutical activities, as well as less desirable immunosuppressant and toxic activities. In this study, the ability of *S. bambusicola* to produce shiraiarin was demonstrated for the first time, as shiraiarin has only previously been found in plants.

The formation of secondary metabolites is regulated by nutrients, growth rate, and enzyme induction [1]. Many fungi synthesize secondary metabolites, which are only formed in the presence of specific substrates, such as maltose [28], ethanol [21], and starch [7]. Thus, in the present study, the production of shiraiarin by *S. bambusicola* was shown to require the presence of lactose to activate the gene for the production of shiraiarin.

This is similar to the production of penicillin by *P. chrysogenum*, where lactose is required as the carbon source. The use of glucose reduces the penicillin titer drastically, with fructose, galactose, and sucrose also having a negative effect on penicillin production [5]. Thus, it would seem that *S. bambusicola* and *P. chrysogenum* have a similar gene regulation mechanism as regards the carbon source for producing shiraiarin and penicillin, respectively.

Nitrogen sources affect both primary and secondary metabolism [10], and many secondary metabolic pathways are negatively affected by nitrogen resources. In this study, only sodium nitrate induced the production of shiraiarin. There are possibly three major reasons for this: first, sodium nitrate produced physiological basification of the culture; second, as a mineral nitrogen source, NH_4^+ inhibited the production of shiraiarin (for example, idiolite production is negatively affected by NH_4^+) [9]; and third, the amino acids in the organic nitrogen sources inhibited the production of shiraiarin.

The pH regulation of gene expression in fungi is commonplace, and an analysis of pH regulatory systems has shown similarities among different fungi [12]. Generally, the medium pH not only affects the cell growth and fermentation rate, but also changes the final product yield and purity. Changing the medium pH can also induce a metabolic shift, whereas an extreme pH has been reported to be a source of water stress that can affect the structural state of DNA. Moreover, extreme acidic or alkaline conditions disrupt the hydrogen bonding in cell components, such as enzymes and lipid bilayers, having an adverse effect on cell integrity and metabolism [18, 22, 32]. Many researchers have proven that pH is an environmental factor that affects the production of second metabolites in fungi. For example, Medentsev and Akimenko [31] found that the inhibition of fungal growth as a result of increasing the medium pH to 8.0 was accompanied by the formation of dimeric naphthoquinone and aurofusarin. The key role of pH in naphthoquinone pigment synthesis can probably be attributed to the fact that at certain pHs, naphthoquinones exhibit a strong cytotoxic action against fungi [30].

In the present study, sporulation seemed to inhibit the production of shiraiarin. Nutrient supply in a fungal

culture affects the morphology and productivity [17], and secondary metabolite production in fungi is a complex process coupled with morphological development. However, although secondary metabolism is commonly associated with the sporulation processes in microorganisms [2], the present results showed the opposite, as the genes involved in sporulation and morphological changes in the cells during sporulation seemingly inhibited the shiraiarin genes. Furthermore, the enzymes involved in the development of spores also had an effect on the production of shiraiarin.

As plant resources become rare and endangered, the need for biotransforms using microorganisms is growing. Therefore, the findings of this study can be a start towards further research on the submerged culture of *S. bambusicola*.

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