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ORIGINAL PAPER

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Production of red pigments by the insect pathogenic fungus *Cordyceps unilateralis* BCC 1869

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Abstract Production of red pigments (naphthoquinones) by the insect pathogenic fungus *Cordyceps unilateralis* BCC 1869 was investigated in this study. Cultivation conditions, including temperature, intitial pH of medium, and aeration, were optimised to improve the yield of total naphthoquinones in shake-flask culture of *C. unilateralis*. The highest yield of total naphthoquinones (3 g L^{-1}) was obtained from a 28-day culture grown in potato dextrose broth with an initial pH of 7.0, at 28°C with shaking-induced aeration at 200 rpm. An extraction process for isolation of the targeted naphthoquinone, 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (3,5,8-TMON), from a culture of *C. unilateralis*, was also developed. The yield of 3,5,8-TMON obtained was about 1.2 g L^{-1} or 40% of total naphthoquinones. The stability of 3,5,8-TMON was very high, even upon exposure to strong sunlight (70,000 lx), high temperature up to 200°C, and acid and alkali solutions at concentrations of 0.1 M

Keywords Naphthoquinones · *Cordyceps unilateralis* · Insect pathogenic fungi · Red pigment production · Optimisation

Introduction

The utilisation of natural pigments in foodstuff, cosmetic and pharmaceutical manufacturing processes has been increasing in recent years. This is due to concern about the harmful effects of synthetic pigments and their industrial by-products on humans and the environment [7]. Natural pigments can be obtained from two major sources, plants [16, 19] and microorganisms [3, 4, 10, 17, 22, 24]. The latter are of great interest owing to the stability of the pigments produced [21], and the availability of cultivation technology [11, 20].

Cordyceps unilateralis BCC 1869, an insect pathogenic fungus, was found to produce six extracellular red naphthoquinones [12], namely, erythrostominone, deoxyerythrostominone, 4-*O*-methyl erythrostominone, epierythrostominol, deoxyerythrostominol, and 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (3,5,8-TMON) (Fig. 1). Some of these were previously isolated from the fungus *Gnomonia erythrostoma* [4]. These red naphthoquinones have been found to possess broad spectrum antibacterial [4, 5] and mild antimalarial [12] activities. Interestingly, the chemical structures of the naphthoquinones produced by *C. unilateralis* are similar to those of the commercial red pigments shikonin and alkanin, the red metabolites of the plant roots of *Alkanna tinctoria* [19] and *Lithospermum erythrorhizon*, respectively [16]. Shikonin and alkanin have been widely used in pharmaceuticals as wound-healing agents, anti-microbial substances [13, 18, 19, 23], and colourants for cosmetics, fabrics and foods.

Our attention was drawn to the naphthoquinones produced by *C. unilateralis* because of their deep blood-red colour and lower cytotoxic properties, particularly of 3,5,8-TMON [12], suggesting a potential role as colourants for cosmetics, fabrics and food. For commercial application, optimisation of fermentation conditions in order to produce competitive yields from *C. unilateralis* is necessary, and is the subject of this paper. We also report a simple method for the extraction of 3,5,8-TMON.

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Materials and methods

Microorganism and inoculum preparation

Cordyceps unilateralis BCC 1869 was collected from the Khao Luang National Park, Thailand and identified by

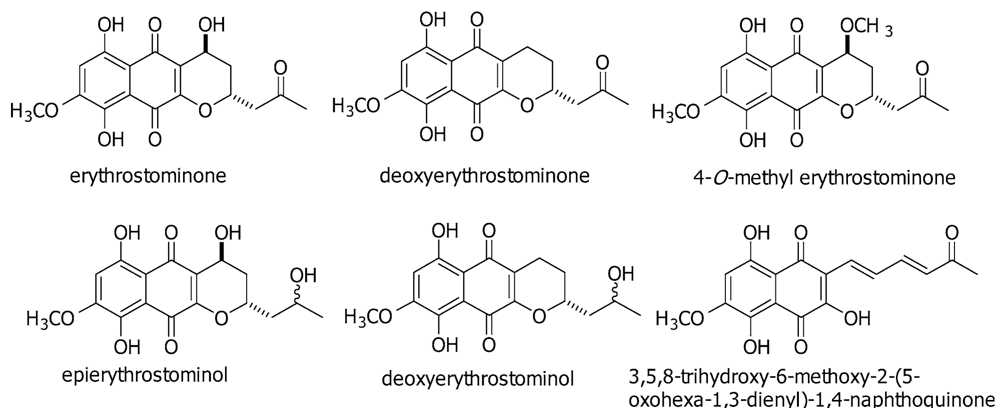

Fig. 1 Structures of six derivatives of naphthoquinones produced by *Cordyceps unilateralis* BCC 1869 [12]

N. Leslie Hywel-Jones of the Mycology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The culture was deposited in the BIOTEC Culture Collection.

The culture used throughout the experiment was maintained on potato dextrose agar (PDA: Difco, Detroit, Mich.) slants at 22°C. For inoculum preparation, the fungus was initially grown at 22°C on a PDA plate for 10 days. A 0.7-cm^2 plug from the outer zone of the colony was punched with a sterile cutter and transferred to 25 ml potato dextrose broth (PDB) medium in a 250 ml flask, and grown at 22°C under basal (static) conditions [8] or on a rotary shaker at 200 rpm for 7 days.

Optimisation of culture conditions

The culture conditions examined were temperature, pH, and aeration. Experiments were conducted in shake flasks and the fungus grown under various temperatures (22, 25, 28, 30, and 35°C), different initial pH ranging from 5 to 8 (pH 6 to 8 adjusted with 1 M NaOH) or controlled pH at 5 by phosphate buffer, and shaking at 200 rpm or static conditions. Fungal growth and naphthoquinone production were monitored weekly, and all experiments performed in duplicate.

Analytical methods

For measurement of growth and total naphthoquinones, fungal cultures were filtered through filter paper (Whatman No. 1), the pellet freeze-dried, and weighed. Naphthoquinones in the filtrate were quantified by a colorimetric method determining absorbance at 500 nm using a double beam spectrophotometer (UV-Vis, Varian, Palo Alto, Calif.). Total naphthoquinones was calculated by the equation, $A = \varepsilon\ L\text{conc}^n$, where A is absorbance at 500 nm, L is length of cell (1 cm), ε is average molar absorptivity of total naphthoquinones (6,456 L mol^{-1} cm^{-2}), and conc^n is concentration (mol L^{-1}) of pigments.

To determine the distribution of naphthoquinones in the medium, the filtrate was extracted twice with an equal volume of ethyl acetate, and the combined organic layer concentrated under reduced pressure to obtain a red crude extract. This extract (20 μL) was subjected to HPLC on a reversed phase C_{18} column (Novapak, 8×100 mm) equipped with a Waters 600 pump connected with a Waters 2487 photodiode array detector. MeCN:H_2O (50:50, v/v) and 0.5% formic acid was used as eluent with a constant flow rate of 1 mL min^{-1}, and fractions monitored by UV absorption at 254 nm. Each naphthoquinone was identified and quantified by comparing the retention time and peak area with that of authentic naphthoquinones isolated from the culture broth of *C. unilateralis* [12].

Extraction process for the naphthoquinone 3,5,8-TMON

The extraction process was modified based on the information that erythrostominone could be chemically transformed to 3,5,8-TMON by heating it under acidic conditions [12]. One litre of culture grown under optimised conditions was filtered to separate the mycelium, the broth acidified to pH 4 with 2 M HCl, and concentrated to 200 ml in a rotary evaporator. The concentrated culture broth was heated at 100°C for 1 h, cooled down, and the pH adjusted to 7 with 1 M KOH. The neutral broth was kept at 4°C for 24 h in order to precipitate 3,5,8-TMON, and the precipitate was finally separated from the broth by centrifugation at 12,000 *g*. The red precipitate was washed sequentially with water and ethanol, dried under vacuum, and the yield determined. Purity of 3,5,8-TMON was determined by nuclear magnetic resonance (NMR) analysis. Dry powder of 3,5,8-TMON was dissolved in deuterated dimethyl sulfoxide (DMSO- d_6), and subjected to ^{1}H NMR spectrophotometry (Bruker DRX 400 NMR spectrometer, Bremen, Germany), operating at 400 MHz for protons. The spectra of 3,5,8-TMON was identified based on data previously reported by Kittakoop et al. [12]:

3,5,8-TMON; UV λ_{max} (MeOH) nm (log ε): 236 (3.97), 280 (3.94), 331 (4.05), 402 (4.12); IR V_{max} (KBr) cm^{-1} : 3,498 (OH), 1,716 C=O), 1,667, 1,583, 1,477, 1,289, 1,220, 855, 811, 736; EIMS *m/z* (rel. int.): 330 (100) $[M]^+$, 315 (32), 287 (86), 273 (35), 245 (60), 244 (52), 121 (25); HR-EIMS *m/z*: 330.0743 $C_{17}H_{14}O_7$ $[M]^+$, requires 330.0739); ^{1}H NMR (400 MHz; DMSO-d_6): 13.71 (1H, s, phenolic OH), 12.26 (1H, s, phenolic OH), 7.62 (1H, dd, J = 15.5 and 15.4 Hz, H-2′), 7.42 (1H, dd, J = 15.5 and 15.4 Hz, H-3′), 7.24 (1H, d, J = 15.5 Hz, H-1′), 6.83 (1H, s, H-7), 6.21 (1H, d, J = 15.5 Hz, H-4′), 3.93 (3H, s, 6-OMe), 2.26 (3H, s, H-6′).

Stability tests for 3,5,8-TMON

3,5,8-TMON powder was subjected to heat, light and acid/alkali stability. Heat stability of 3,5,8-TMON was carried out by means of thermal gravity analysis using simultaneous TGA-DTA (STA 449C, Netzsch, Germany), heating from 30°C to 850°C with a scanning rate of 20°C/min, under air atmosphere. Stability to light of 3,5,8-TMON was evaluated by exposure to sunlight (maximum intensity about 70,000 lx) for 12 h, and determined by NMR analysis. Stability of 3,5,8-TMON under acid and alkali conditions was independently performed for 24 h in 0.1 M HCl and 0.1 M NaOH solutions. Changes in 3,5,8-TMON were examined by ^{1}H NMR analysis.

Results and discussion

Growth and naphthoquinone production under basal conditions

Figure 2 shows growth and naphthoquinone production of *C. unilateralis* grown under basal conditions. Maximal biomass was at day 35, and the growth rate estimated at 0.099 day^{-1} . Production of naphthoquinones showed a typical profile of a secondary metabolite, and was not associated with growth [6, 15]. Maximum yield of naphthoquinones (2 g L^{-1}) was observed at around day 50 or at its almost dead phase of growth. The specific production of total naphthoquinones under these conditions was about 350 mg g-$cell^{-1}$.

Effect of temperature

Temperature had little effect on the growth of the fungus, with maximum yield at 22°C and growth completely inhibited at 35°C (Fig. 3). However, naphthoquinone production was affected by temperature; with an increase in yield from 22°C to 30°C. The highest yield (1.8 g l^{-1}) and specific production (304 mg g-$cell^{-1}$) of naphthoquinones were detected at 28°C. This is similar to naphthoquinone production by *Fusarium solani* [17] and the red pigment/citrinin produced by *Monascus ruber* [9]. The optimum temperature for pigment production by these fungi was at 30–32°C, with an optimal temperature for growth of 26 °C. These correspond with secondary metabolite production in other microorganisms, in that the optimum temperature is generally higher than that for growth [1, 14].

Effect of pH

The pH of the culture medium has been reported to play a key role in naphthoquinone synthesis [2, 3, 10, 15]. Our results demonstrated that biomass and naphthoquinone production was slightly affected by the initial pH of the medium (Fig. 4a). The highest biomass and naphthoquinone production was observed when the initial pH of the culture medium was set at 7. The yield and specific

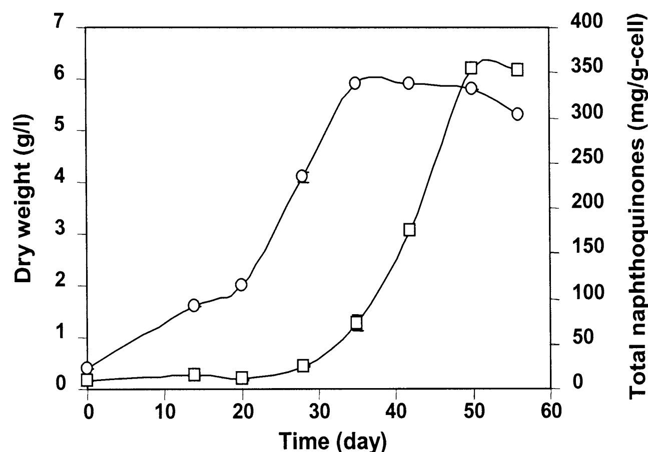

Fig. 2 Time course of growth (*circles*) and naphthoquinone production (*squares*) by *C. unilateralis* BCC 1869 under basal conditions

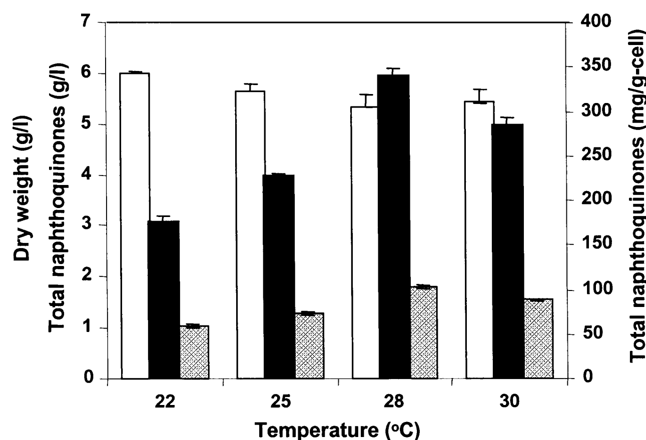

Fig. 3 Biomass and naphthoquinone productions of *C. unilateralis* BCC 1869 grown at the temperatures indicated for 40 days (where the maximal biomass was obtained). *Open bars* Dry weight (g L^{-1}), *black bars* naphthoquinone specific production (mg g-$cell^{-1}$), *shaded bars* total naphthoquinones (mg L^{-1})

production of naphthoquinones increased to 2.5 g L^{-1} and 424 mg g-cell^{-1}, respectively.

Changes in the pH of the medium were also monitored throughout the cultivation process. In all cases the medium became acidic (pH 5–6) during biomass production, increasing to about pH 7 when naphthoquinones were produced, suggesting the involvement of pH in naphthoquinone synthesis. To investigate the effect of pH on naphthoquinone synthesis, an experiment was undertaken with a buffer to maintain the pH of the medium at 5. Biomass production was enhanced when controlled at pH 5, whereas the level of naphthoquinones was dramatically reduced (Fig. 4b). These results indicate that production of naphthoquinones by *C. unilateralis* is influenced and regulated by the pH of the medium, with the best yield obtained at pH 7.

Effect of aeration

Aeration, by shaking at 200 rpm, significantly reduced cultivation time, and enhanced the yield of naphthoquinones (Fig. 5). Biomass production rapidly increased and reached stationary phase within 7 days, while it took 21 days when the cultures were grown under static conditions. The growth rate of shaken cultures was about 0.231 day^{-1} . Maximum naphthoquinone production was attained at day 28, while cultures incubated under static conditions reached their maximal production at day 50. Moreover, the yield of total naphthoquinones of the fungus grown under shaken conditions (with optimal temperature and initial pH) was enhanced up to 3 g L^{-1}, corresponding to a specific production of 605 mg g-cell^{-1} (Fig. 5).

Change of pH in shaken cultures of *C. unilateralis* was also monitored. Two pH regimes were observed during the fermentation process (Fig. 5); the pH of the medium was lower than 7 during biomass production, while naphthoquinones accumulated when fungal growth decreased and the pH increased to neutral.

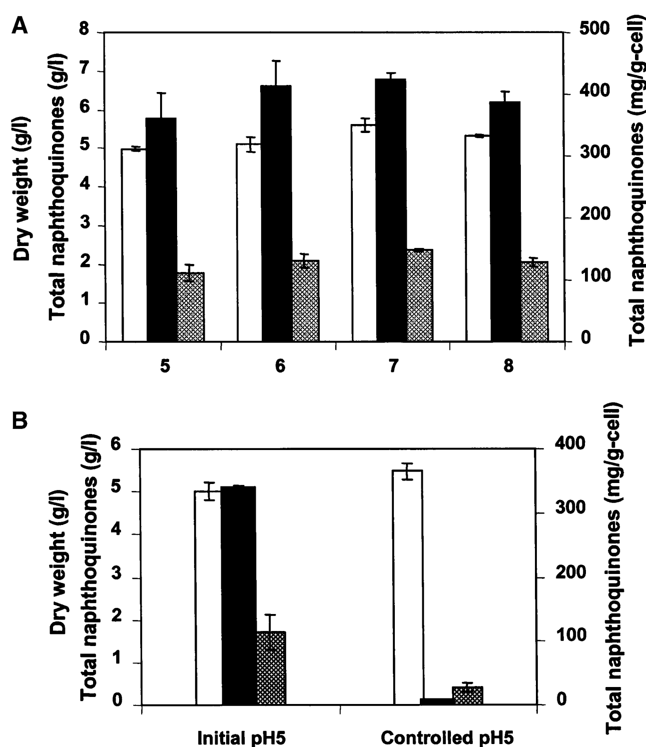

Fig. 4 Biomass and naphthoquinone production of *C. unilateralis* BCC 1869 grown at 28°C for 40 days under various initial pH values (**a**) and controlled pH (**b**). *Open bars* Dry weight (g L^{-1}), *black bars* naphthoquinone specific production (mg g-cell^{-1}), *shaded bars* total naphthoquinones (mg L^{-1})

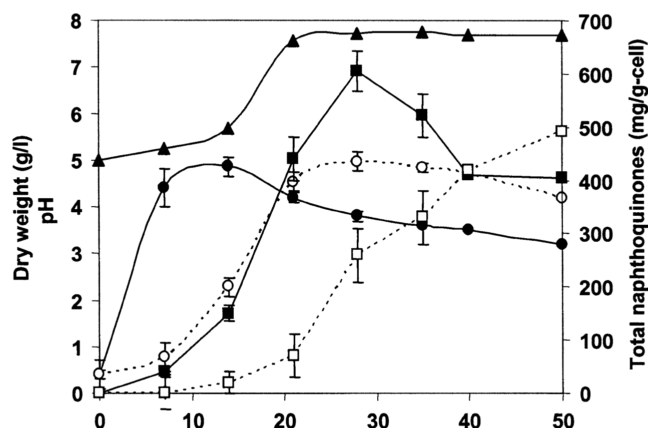

Fig. 5 Time profiles of growth and naphthoquinone specific production of *C. unilateralis* BCC 1869 grown at 28°C, initial pH 7, under static or shaken conditions. *Open symbols* Static cultures, *filled symbols* shaken cultures. *Circles* Dry weight (g L^{-1}), *squares* specific naphthoquinone production (mg g-cell^{-1}), *filled triangles* pH

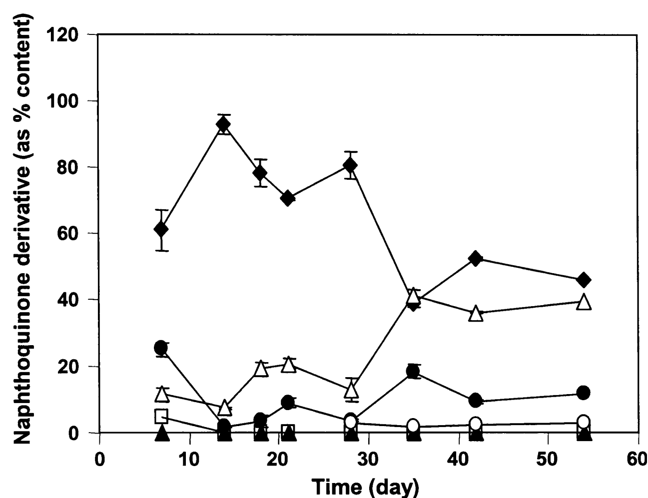

Fig. 6 Naphthoquinone distribution in *C. unilateralis* BCC 1869 during a 60-day fermentation of a culture incubated at 28°C, initial pH 7 and shaking at 200 rpm. *Filled diamonds* Erythrostominone, *filled triangles* 4-*O*-methyl erythrostominone, *open squares* deoxyerythrostominone, *open triangles* epierythrostominol, *open circles* deoxyerythrostominol, *open circles* 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (3,5,8-TMON)

Composition of naphthoquinone derivatives during the fermentation process

As mentioned previously, of the six naphthoquinones produced by *C. unilateralis*, 3,5,8-TMON is the most desirable due to its lack of cytotoxicity [12]. We therefore examined production of 3,5,8-TMON as well as other naphthoquinones during the course of fermentation at 28°C, initial pH 7 and shaken at 200 rpm (optimised culture conditions), and the results are shown in Fig. 6. Throughout the fermentation, the major derivative was erythrostominone, with the highest level of 90% at day 14, thereafter declining with time of incubation. The level of 4-*O*-methyl erythrostominone was very low and almost undetectable throughout. Deoxyerythrostominone was detected only at an early stage of growth, with a level of 7%. The levels of epierythrostominol and deoxyerythrostominol increased with time, and their maximal levels were about 40% and 15%, respectively. 3,5,8-TMON was detected at day 28, and was constant at 5% throughout the fermentation period.

Extraction yield and stability of 3,5,8-TMON

Since the proportion of 3,5,8-TMON in cultures of *C. unilateralis* was rather low, we developed a simple method to improve the yield of this compound. By employing this technique, naphthoquinones of a 28-day old culture of *C. unilateralis* grown under optimised conditions were chemically transformed to 3,5,8-TMON. The 3,5,8-TMON powder was poorly soluble in water and organic solvents. It was easily precipitated and separated from the culture broth. The yield of 3,5,8-TMON was about 1.2 g L^{-1} or 40% of total naphthoquinones.

^{1}H NMR spectra of 3,5,8-TMON after exposure to strong sunlight, and acid/alkali conditions were unchanged when compared with a control (data not shown). Thermal stability of 3,5,8-TMON demonstrated that this compound remained intact up to 200°C, and partially decomposed (20%) at 300°C. It was gradually decomposed with increased temperature, and completely degraded at 700°C. These findings indicate that 3,5,8-TMON is extremely stable even upon exposure to sunlight and high temperatures up to 200°C, and is also resistant to acid and alkali conditions.

Conclusion

By optimisation of physical conditions for submerged culture of *C. unilateralis* BCC 1869, a high yield of naphthoquinones (3 g L^{-1}) was achieved, and the developed extraction process provided a yield of 3,5,8-TMON of up to 1.2 g L^{-1}. This red pigment was very stable in sunlight, high temperature and acid/alkali conditions, suggesting a good potential for commercial utilisation of *C. unilateralis* BCC 1869 as an alternative source for red pigment production.

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