
Production potential of water-soluble *Monascus* red pigment by a newly isolated *Penicillium* sp.

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A new isolate from medicinal plant endophytes was identified as *Penicillium* sp. (HKUCC 8070). A water-soluble red pigment was produced by this strain in potato-dextrose broth, malt-extract broth and a chemically defined medium containing glutamate as a nitrogen source. The red pigment produced was identified as heat-stable, polyketide *Monascus* red pigment. The highest yield of the red pigment was 1107 mg l⁻¹ obtained from the culture of *Penicillium* sp. (HKUCC 8070) grown on the malt-extract medium.

Key words: biotechnology, endophytes, fungal pigments,

Introduction

Natural colorants are considered to be safer than synthetic ones, and their applications in foods, cosmetics and pharmaceuticals are growing rapidly (Lauro, 1991). There are a number of natural pigments, but only a few are available in sufficient quantities for industrial production. Production of pigments from microorganisms is advantageous over other sources because microorganisms can grow rapidly which may lead to a high productivity of the product (Kim *et al.*, 1999). *Monascus* species are widely used as a microbial source for natural red pigment production (Kim *et al.*, 1999). *Monascus* red pigments are of polyketide origin and are used commercially in the orient as non-toxic colorants for colouring rice wine, "Koji", soybean, cheese and red meat (Hajjai *et al.*, 1999). The red pigments have attracted worldwide commercial interest (Júzlová *et al.*, 1996; Spears, 1988), but little information is available on the production of this pigment by other microbial sources. It has been reported that *Penicillium* may be the potential candidate to produce polyketide structure compounds (Júzlová *et al.*, 1996). In the course of the

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screening for new red pigment producing strains, we found a new isolate, *Penicillium* sp. (HKUCC 8070), which could produce a bright red pigment while grown on potato-dextrose agar. The red pigment was subsequently identified as Monascus red pigment. In this paper, we report the taxonomy of this new isolate and the production potential of this water-soluble Monascus red pigment by this organism.

Material and methods

Chemicals and media

Monascus pigment standard was purchased from Wako Chemical Ltd. (Tokyo, Japan). Potato-dextrose agar, corn-meal agar, potato-dextrose broth and malt extract were purchased from Difco Laboratories (Detroit, MI, USA). All other chemicals and organic solvents used in this study were purchased from Sigma Co. (St. Louis, MO, USA). A chemically defined medium was also used, which contained (per litre), 10 g glucose, 5 g monosodium glutamate (MSG), 5 g K₂HPO₄, 5 g KH₂PO₄, 0.1 g CaCl₂, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g ZnSO₄·7H₂O, and 0.03 g MnSO₄·H₂O. The initial pH of the media was adjusted to 6.5 with phosphoric acid prior to sterilization at 121 °C for 20 minutes. The pH of the culture was maintained by the phosphate buffer during cultivation (Hajjai *et al.*, 2000).

Isolation and identification

Leaves from *Polygonum multiflorum* were collected from Tai Po Kau Country Park, Hong Kong and brought back to the laboratory in sterile plastic bags. The samples were surface sterilized in 75% ethanol→ 5% sodium hypochloride→ 75% ethanol at 2-minute intervals, and then inoculated on to the potato-dextrose agar medium (Brown *et al.*, 1998). A few days later, single spores that grew out were isolated. The morphology of the specimen was observed under a microscopy (Olympus, Japan). For cryoscanning electron microscopy (Leica, Cambridge SEM 440), a single colony grew on the potato-dextrose agar was cut out with the medium and critical point dried mounted on aluminium stubs using carbon cement. Preparation steps of the material followed the methods described by Ho *et al.* (1999).

Cultivation

The stock culture was kept on the potato-dextrose agar in a Petri-dish at 23°C and subcultured every three months. A spore suspension of 10^8 spores ml^{-1} was prepared by washing the 10-day old culture with sterile distilled water supplemented with 0.1% Tween 80. Erlenmeyer flasks (250 ml), each containing 100-ml liquid medium were inoculated with 5% of the spore suspension and incubated at 25°C in an orbital shaker at 180 rpm for 14 days.

Determination of colony radial growth rate

A 0.05 ml spore suspension (10^8 spores ml^{-1}) was inoculated into the centre of the Petri- dish and cultivated in the dark at 25°C for 14 days. Measurements of colony diameter were made at 24-hour intervals as described by Trinci (1969).

Determination of cell dry weight concentration

One-hundred ml of 14-day old fermentation broth was filtered through a pre-weighed Whatmann No. 1 filter paper and the mycelium pellet was washed twice with distilled water. The collected mycelia and filter paper were freeze-dried to constant weight.

Viscosity measurements

At the end of cultivation, fermentation broth was harvested by centrifugation at 3000 g. The viscosity of the culture broth was determined at 25°C using Brookfield viscometer (model DV-II, Stoughton, MA) with a UL adaptor at rotational speeds of 10 rpm.

Isolation of red pigment

One-hundred ml fermentation broth was centrifuged at 3000 g to remove the mycelium, spore and other non-soluble particles. The red supernatant solution was vacuum concentrated on a rotary vacuum evaporator (Büchi rotary system, Switzerland) and extracted with water saturated n-butanol for several times till the lower layer was free of red colour. The organic upper layer was vacuum dried and then mixed with 10 ml chloroform. The dark orange-colour chloroform layer was discarded and the undissolved red residual was dried under nitrogen gas before being dissolved in 5 ml n-butanol. The n-

butanol solution was filtered through a 0.45 µm Millipore® filter (Millipore, USA) and then directly injected into the HPLC machine for the determination of red pigment. For intra-cellular red pigment, the freeze dried mycelia was extracted with 95% methanol several times until the debris of the mycelia was colourless. The extract was collected and vacuum dried as the steps described above.

HPLC analysis

Red pigments were analysed by HPLC (Waters, Milford, MA, USA) equipped with two 510 pumps and a spectrophotometer detector. The extract solution was analysed by using a Bondapak C₁₈ column (300 × 3.9 mm I.D., 10 µm, Waters) at 25°C as described as Lin *et al.* (1992).

Results and Discussion

Taxonomy

The *Penicillium* sp. was isolated on three occasions as an endophyte of *Polygonum multiflorum*. The isolation of *Penicillium* sp. as endophytes is not uncommon (e.g. Suryanarayanan *et al.*, 2000, Romero *et al.*, 2001) and their particular isolation was striking in that which produced a bright red pigment on potato-dextrose agar used. For the identification of the fungus, corn meal agar and the potato-dextrose agar were used. This strain grew rapidly to form a grey covert pale green-blue colony with a diameter of 70 mm after incubation for 14 days at 25°C in darkness on both agars. The reverse of the colonies was dark yellow-brown on corn meal agar and red-brown on potato-dextrose agar. Dark red water-soluble pigment was produced only on potato-dextrose agar. Morphological observations were made under a microscope and a scanning electron microscope (Cambridge SEM 360). The strain grown on potato-dextrose agar at 25°C for 3 days and produced conidia. Conidia which were mainly monoverticillate formed in long dry conidial chains, formed on flask-shaped phialides, terminating with an indistinct neck. The conidia were globose to subglobose, 1-2 µm in diameter and with a rough surface (Figs 1a,b). From the above characteristics, strain HKUCC 8070 was identified as *Penicillium* sp.

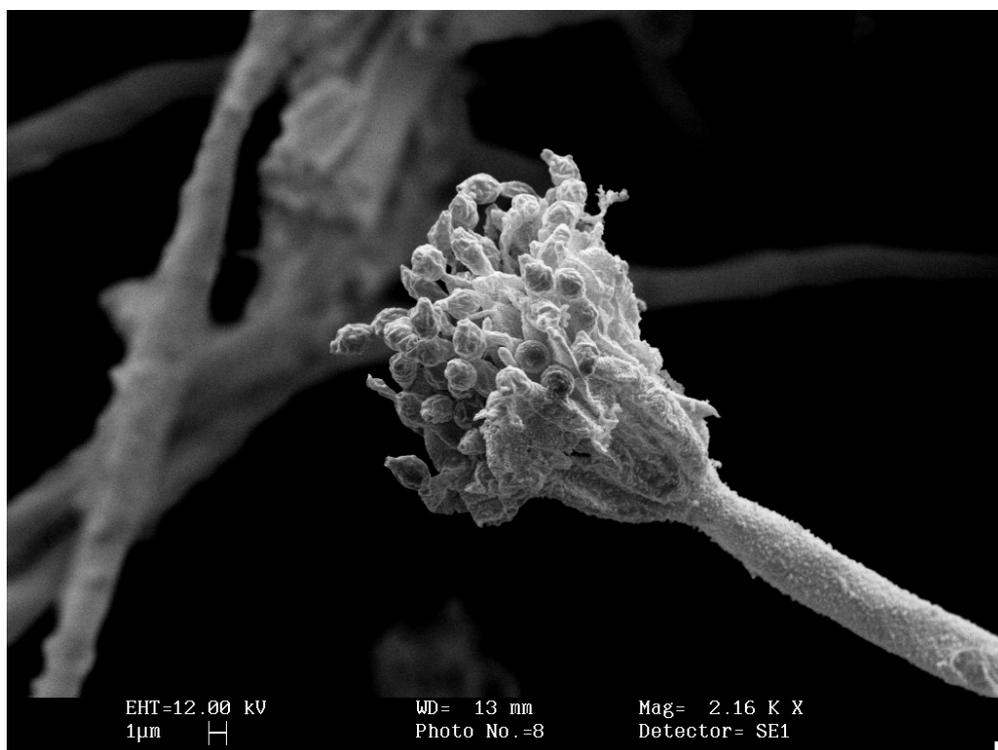


Fig. 1a. Scanning electron micrograph of penicillia of strain HKUCC 8070 on potato-dextrose agar. Bar = 10 μ m.

Growth characteristics

In this study three different media were selected to identify and investigate the red pigment secretion property. The newly isolated *Penicillium* sp. can grow and produce red pigment in both potato-dextrose broth and malt extract broth (8 g of malt extract powder dissolved in 100 ml distilled water). It was found that the viscosity of the fermentation broth was relatively high while using potato-dextrose broth and malt-extract broth. At the same time, to decrease the broth viscosity, a chemically defined medium was also selected. The kinetic growth parameters and viscosity of the fermentation broth of the *Penicillium* sp. grown in the three media are shown in Table 1. The producing morphology of *Penicillium* sp. in submerged culture was dispersed filaments in potato-dextrose broth and malt extract broth, while cultured in the chemically defined medium small pellets were found. Pelleted suspensions of fungal cells are generally not viscous and usually only deviate from Newtonian behaviour at

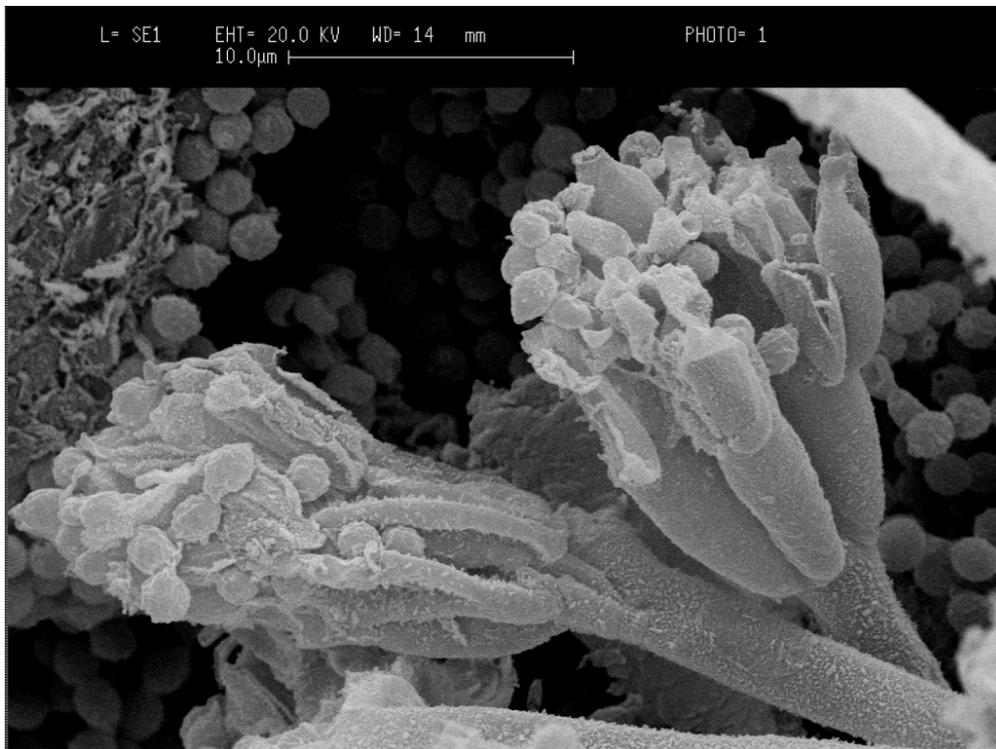


Fig. 1b. Scanning electron micrograph of penicillia of strain HKUCC 8070 on potato-dextrose agar. Bar = 10 μm .

high biomass concentrations (Gibbs *et al.*, 2000). In the chemically defined medium, pellet formation of *Penicillium* sp. led to decreased viscosity of the fermentation broth (0.83 cp as compared to 13.03 cp and 9.83 cp in the potato-dextrose broth and malt-extract broth, respectively). As a result, the O_2 transfer rate was improved, which in turn stimulated biomass production. The cell dry weight concentration of the chemically defined medium (4.69 g l^{-1}) was higher than those from the potato-dextrose broth (4.27 g l^{-1}) and malt-extract broth (3.99 g l^{-1}). The specific growth rate in the submerged cultures of the three media however, did not show apparent differences. No relationship was observed between pellet formation and specific growth rate of *Penicillium* sp. in this investigation. The kinetics of colony growth are identical with those of unbranched hyphae (Trinci, 1969). In this study, the colony radial growth rate of *Penicillium* sp. was the highest (4.80 mm day^{-1}) in the chemically defined

Table 1. Kinetic parameters of growth and Monascus pigment production of *Penicillium* sp (HKUCC 8070) grown on different media*

Parameters	Potato dextrose broth	Malt extract broth	Chemically defined medium
Specific growth rate, μ (h^{-1})	0.043 \pm 0.004	0.045 \pm 0.001	0.044 \pm 0.002
Cell dry weight concentration (g l^{-1})	4.27 \pm 0.06	3.99 \pm 0.15	4.69 \pm 0.25
Colony radial growth rate (mm day^{-1})	2.78 \pm 1.05	2.93 \pm 0.08	4.80 \pm 0.03
Viscosity of fermentation broth (cp)	13.03 \pm 0.66	9.83 \pm 0.88	0.83 \pm 0.05
Yield of pigment (mg l^{-1})	904	1107	479

*Data are expressed as mean \pm standard deviation of 3 replicates. medium as reflected by a higher rate of increase in the hyphal length on this medium (Caldwell and Trinci, 1973).

Identification of the red pigment secreted from different media

The identification of the red pigments extracted from the potato-dextrose broth, malt extract broth and the chemically defined medium was achieved by comparing retention times and absorbance spectra against authentic Monascus red pigment. Authentic Monascus pigment standard was dissolved in n-butanol before HPLC analysis to minimize the effect of solvent on HPLC profile. The typical chromatograms and absorbance spectra obtained from the standard solution and the tested samples are shown in Figs 2 and 3. As shown in Fig. 2, the retention time of the tested pigments from the potato-dextrose broth, malt extract broth and the chemically defined medium was 5.54 minutes, 5.56 and 5.68 minutes, respectively which was identical to that of the authentic standard (5.65 min). The absorption spectra of the red pigments extracted from the potato-dextrose broth, the malt extract broth and the chemically defined medium were almost the same as the authentic Monascus pigment (Fig. 3). These results indicated that the red pigments produced by *Penicillium* sp. in the potato-dextrose, malt extract broth and the chemically defined medium are the same as the authentic Monascus pigment.

The calibration curves of the peak-area (A) ratio against the concentration (C, mg ml^{-1}) for the authentic Monascus pigment ($A = 2E+07C - 251045$, $r^2 = 0.9995$) give a linear response over the tested range of concentrations (Fig. 4). The detection limit is 0.5 mg l^{-1} . The relative standard deviations of replicate injections ($n = 3$) for this compound was 1.44%. The recovery was determined to be 92.7% by the standard addition method in order

Fig. 2. Typical chromatograms of the *Monascus* pigment standard and the red pigments extracted from the culture of *Penicillium* sp. (HKUCC 8070) grown on the malt extract broth, potato-dextrose broth and the chemically defined medium. a. *Monascus* pigment standard; b. red pigment extracted from the culture with malt-extract medium; c. red pigment extracted from the culture with potato-dextrose broth; d. red pigment extracted from the culture with the chemically defined medium.

to demonstrate the accuracy of the analysis. The contents of the red pigment secreted from the mycelia on the potato-dextrose broth, the malt extract broth and the chemically defined medium were calculated by comparison to the calibration curve of the authentic monascus pigment. The concentration of red Monascus pigment secreted in the culture with the potato-dextrose broth, malt extract broth and the chemically defined medium was 0.05 g l^{-1} , 0.11 g l^{-1} and 0.01 g l^{-1} , respectively. The red pigment produced and secreted in the culture of the chemically defined medium was lower than the others. It has been reported that metabolite production by some fungi was reduced if grown as pellets which was due to the transportation limitation of the nutrients (Wang and Webb, 1995). In the chemically defined medium, the cell hyphae formed tight compact pellets, which might limit the diffusion of nutrients and oxygen. The red pigment extracted from the mycelia grown in the potato-dextrose broth, malt extract broth and the chemically defined medium were of 20%, 25% and 10% of cell dry weight, respectively. In *Monascus* strains, the pigments were produced mainly in the cell-bound state and the intra-concentration of pigments was higher than that secreted into the fermentation broth (Lin *et al.*, 1992). In this study, the red pigment content of the mycelia grown on all media was higher than that obtained from the fermentation broth. The Monascus red pigment can excrete in the medium after reacting with an NH_2 unit of amino acids, and glutamate is a rich source of this NH_2 group (Pastrana *et al.*, 1994). In this study, monosodium glutamate was the sole nitrogen source in the chemically defined medium and the results are in agreement with the previous report (Lin and Demain, 1991). In malt-extract broth, there are more free amino acids than in potato-dextrose broth. Consequently, more red pigment was excreted in the malt-extract medium on reacting with these amino acids. In the chemically defined medium, the pigment production was the lowest among all three media tested. It has also been reported that maximum red pigment production might occur when conidiation was reduced (Shepherd, 1977). On the other hand, the lower pigment production might also be due to the improper carbon source used and composition of micronutrients (Chen and Johns, 1993).

Fig. 3. Absorption spectra of Monascus pigment standard and red pigments extracted from the culture of *Penicillium* sp. (HKUCC 8070) grown on the potato dextrose broth, malt extract broth and chemically defined medium. a. Monascus pigment standard. b. red pigment extracted from culture with the malt- extract medium c. red pigment extracted from the culture with the potato-dextrose broth d. red pigment extracted from the culture with the chemically synthetic medium.

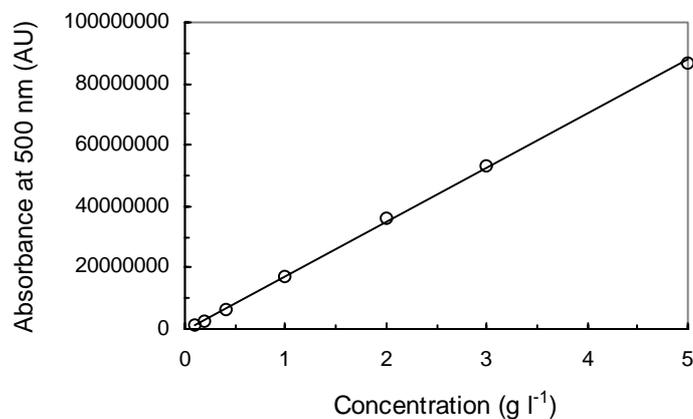


Fig. 4. Calibration curve for the determination of Monascus red pigment by HPLC analysis.

The results indicated that the newly isolated *Penicillium* sp. had the ability to synthesize glutamate based polyketide pigment. It has been reported that tetraketide was the precursor for both citrinin (an anti-bacteria mycotoxin) and red pigments production during the growth of *Monascus ruber*. A branch point existed at the tetraketide level which led to two different synthetic pathways, namely, tetraketide → penaketide → hexaketide → red pigment, and tetraketide → intermediate → citrinin. The different enzymatic reaction at the tetraketide level would account for a different production of pigments from a hexaketide (Hajjai *et al.*, 1999). The polyketide pathway is the major route for the formation of secondary metabolites in filamentous fungi (Chandler *et al.*, 1992). Further research is needed in order to characterize the enzymes that catalyse the reactions at this branch point to facilitate the development of proper strategies for highly selective production of the water-soluble red pigment.

Physical-chemical properties the tested pigments

A comparison of some properties of the red pigment extracted from the potato-dextrose broth and the chemically defined medium, and the authentic *Monascus* pigment was made. The three pigments were all heat-resistant, and could be autoclaved. The sensitivity of the pigment to light was also investigated. The aqueous butanol pigment solutions were placed in a Pyrex test tube and located 10 cm from the top of the fluorescence light tube (300

Lux) for 48 hours. HPLC analysis of the solutions showed all the pigments were light sensitive, and a loss of 29%, 47% and 69% was observed for pigments from the chemically defined medium, the potato-dextrose broth and the authentic *Monascus* pigment, respectively. In contrast, all of the pigments were stable over the pH range of 2~10. All of these properties indicate that the red pigment obtained from the new isolate of *Penicillium* sp. has great potential for food and beverage industries.

Acknowledgements

The authors would like to acknowledge the staff in the electron microscopy unit, Queen's Mary Hospital, Hong Kong for SEM observation. Helen Leung and Megan Yap are thanked for their technical assistance.

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(Received 1 January 2005; accepted 30 March 2005)