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Preparation and characterization of yellow *Monascus* pigments

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ABSTRACT

Yellow *Monascus* pigments were prepared from fermentation broth after extractive fermentation in nonionic surfactant micelle aqueous solution. First, *Monascus* pigments were transferred from nonionic surfactant micelle aqueous solution into ionic liquid phase by ionic liquid based microemulsion extraction, in which the recovery yield of yellow *Monascus* pigments based on absorbance at 410 nm reached to 95.5%. Then, the yellow *Monascus* pigments in the ionic liquid phase were recovered by ionic liquid–butanol two-phase extraction, in which the recovery yield of yellow *Monascus* pigments reached to approximately 95%. Further purification of yellow *Monascus* pigments was demonstrated by removal of orange *Monascus* pigments via selective aminophilic reaction between orange *Monascus* pigments and MSG (monosodium glutamate). The results indicated that there was no aminophilic reaction between yellow *Monascus* pigments and primary amines by replacement of 4-H-pyran cycle in orange *Monascus* pigments with 2-H-pyran cycle in yellow *Monascus* pigments. This fundamental fact provides a novel strategy for removal of orange *Monascus* pigments from yellow ones. It also sheds light on why orange *Monascus* pigments but yellow ones exhibit strong cytotoxicity.

1. Introduction

Monascus species have been known to produce at least six molecular structures of pigments, which are classified into three groups based on their colors, i.e., yellow pigments (monascin ($R = C_5H_{11}$) and ankaflavin ($R = C_7H_{15}$)), orange pigments (monascorubrin ($R = C_5H_{11}$) and rubropunctatin ($R = C_7H_{15}$)) (Fig. 1), as well as red pigments (monascorubramine and rubropuntamine) [1]. Red Yeast Rice, solid state fermentation of *Monascus* sp. on rice with majority of red *Monascus* pigments as well as a few yellow ones, has a long-term history as a source of food colorant in the orient countries [2].

Orange *Monascus* pigments belong to azaphilone [3]. Azaphilones are structurally variable family of fungal polyketide metabolites processing a highly oxygenated pyranoquinone bicyclic core (known as isochromene) and a quaternary carbon center. The 4-H-pyran nucleus undergoes substitution with primary amines to form the corresponding vinylogous γ-pyridines and then changes the oxygen of pyran cycle to nitrogen, which makes the azaphilones turn red. Orange *Monascus* pigments exhibit the characteristic reaction of azaphilones, i.e., turning red in the presence of primary amines (aminophilic reaction) to produce red *Monascus* pigments as well as red *Monascus* pigment derivates with different residues depending on the kinds of primary amines [4–7]. Hajjaj et al. also reported red *Monascus* pigment derivates with glucosamine residues [8]. The non-selective aminophilic reaction between azaphilones and amino-group-containing compounds, such as amino acids, proteins, and nucleic acids, may be related to the biotoxicity as well as bioactivity of azaphilones [9,10]. Thus, the orange *Monascus* pigment rubropunctatin exhibits high cytotoxicity to human cancer cell gastric carcinoma cell BGC-823 [11] while strong teratogenic effect on check embryo as well as antibacterial activity is also recorded [12,13].

In spite of the structure similarity between orange *Monascus* pigments and yellow ones (Fig. 1), teratogenic effect of yellow *Monascus* pigments on check embryo is much less than that of orange ones [12,13]. More interestingly, the yellow *Monascus* pigment ankaflavin also shows selective cytotoxicity to cancer cell lines by an apoptosis-related mechanism while nearly no cytotoxicity on normal fibroblasts. The structural analogue yellow *Monascus* pigment monascin, however, shows no cytotoxicity to all the tested cell lines [14]. Appropriate dose of yellow *Monascus* pigments is also suggested as potential functional food colorant [3]. On the other hand, orange *Monascus* pigments are accumulated at low pH or low nitrogen concentration while red ones at nearly neutral pH or high nitrogen concentration in the submerged

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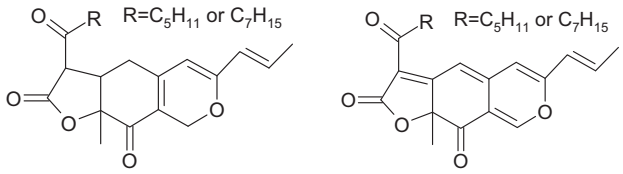

Yellow *Monascus* pigments **Orange *Monascus* pigments**

Fig. 1. Chemical structures of yellow/orange *Monascus* pigments.

culture [15]. Yellow *Monascus* pigments are the minor pigment components in the conventional *Monascus* fermentation. Thus, there is lack of basic information about yellow *Monascus* pigments, such as the aminophilic reaction between yellow *Monascus* pigments and primary amines.

In our previous work, extractive fermentation in a nonionic surfactant micelle aqueous solution had exported intracellular *Monascus* pigments into its extracellular broth, in which high fraction of yellow *Monascus* pigments was accumulated in the extracellular broth (nonionic surfactant micelle aqueous solution) [16]. The production of a large amount of yellow *Monascus* pigments by extractive fermentation provides a chance for preparation of yellow *Monascus* pigments. In the present work, extraction of *Monascus* pigments from fermentation broth by ionic liquid based microemulsion extraction and removal of orange *Monascus* pigments to achieve yellow *Monascus* pigments via selective aminophilic reaction between orange *Monascus* pigments and MSG (monosodium glutamate) as well as further purification of yellow *Monascus* pigments by crystallization were carried out. Some basic characters of yellow *Monascus* pigments, such as aminophilic reaction between yellow *Monascus* pigments and amino acids, were also evaluated.

2. Materials and methods

2.1. Extractive fermentation for production of extracellular yellow Monascus pigments

Monascus anka (China Center of Industrial Culture Collection, CICC 5013) was used in this study. Production of extracellular *Monascus* pigments by extractive fermentation in the nonionic surfactant Triton X-100 micelle aqueous solution followed the same procedure as described in our previous work [16]. Yellow *Monascus* pigments as well as orange *Monascus* pigments were accumulated in the extracellular nonionic surfactant micelle aqueous solution. The detailed information was presented in supplementary materials of Fig. S1.

2.2. Transferring of Monascus pigments by Winsor I microemulsion extraction

Transferring of *Monascus* pigments from nonionic surfactant aqueous solution to ionic liquid (IL) was conducted by ionic liquid based Winsor I microemulsion extraction [17]. The clear supernatant fermentation broth (20 ml) was mixed with 5 g (≈4 ml) IL (ionic liquid, [Bmim]PF_6) in 50 ml tube, which was subjected to Winsor I microemulsion extraction by setting up at a room temperature for phase separation for at least 1 h. The mixture was separated into two phases, where the lower one was excess IL phase and the upper one was W_m (oil-in-water microemulsion) phase (Winsor I microemulsion). The W_m phase was transferred into another tube. The excess IL phase was further washed with water for 3 times to remove the contaminated nonionic surfactant in the excess IL phase. Based on the volume and the corresponding pigments absorbance at 410 nm, the fraction of *Monascus* pigments in the excess IL phase or W_m phase was determined.

2.3. Recovery of Monascus pigments by ionic liquid–organic solvent two-phase extraction

Monascus pigments in the excess IL phase were further back-extracted by various organic solvent–IL two-phase systems, such as *tert*-butanol–IL two-phase system. Equal volume of excess IL phase and *tert*-butanol (≈15 ml) were put into 50 ml scaled tube. The mixture solution was set up at a room temperature at least 1 h for phase separation. The mixture was separated into two phases, where the lower one was IL phase and the upper one was *tert*-butanol phase. The IL phase was further extracted by equal volume of *tert*-butanol. After three times of *tert*-butanol–IL two-phase extraction, the *tert*-butanol phases were combined. The volume and the fraction of *Monascus* pigments in the combined *tert*-butanol phase were determined. Based on the volume and the corresponding pigments absorbance at 410 nm, the fraction of *Monascus* pigments in the *tert*-butanol phase was determined.

The combined *tert*-butanol phase was subjected to air stripping and slurry state of crude *Monascus* pigments was achieved. The slurry crude *Monascus* pigments were re-dissolved in cyclohexane to removal of oily materials. Crude *Monascus* pigments were achieved from clear cyclohexane phase by air stripping off solvent. The detailed information was presented in supplementary materials of Fig. S2.

2.4. Removal of orange Monascus pigments via aminophilic reaction

The major pigment components in the crude *Monascus* pigments were yellow *Monascus* pigments and orange ones. The aminophilic reaction between orange *Monascus* pigments and MSG was carried out followed a similar procedure as reported in the literatures [18]. The crude *Monascus* pigments was re-dissolved in 24 ml ethanol aqueous solution (70% (V/V), pH = 7) to prepare *Monascus* pigments ethanol aqueous solution with 46 AU (absorbance unit, multiplication of the absorbance with its dilution ratio of a sample) at 410 nm and 39 AU at 470 nm. Then excess MSG (1.5 g) was added into the *Monascus* pigment ethanol aqueous solution, which was shaken at 30 °C and 200 rpm. The residual orange *Monascus* pigments in the ethanol aqueous solution were monitored by thin layer chromatography (TLC) analysis. After the aminophilic reaction for 10 h, 190 ml cyclohexane was added into the reaction solution. The mixture solution was separated into a cyclohexane phase and an ethanol aqueous solution phase. Yellow *Monascus* pigments were partitioned into the cyclohexane phase, which was recovered by air stripping off solvent.

The yellow *Monascus* pigments were further purified by silica gel column chromatography. The yellow *Monascus* pigments in the washing solution were monitored by TLC analysis. The detailed information was presented in supplementary materials of Fig. S3. The washing solution was subjected to air stripping off solvent and yellow *Monascus* pigments had been crystallized.

2.5. Analysis methods

The concentration of pigments is usually represented by color value based on the absorbance spectrum of *Monascus* pigments at their characteristic wavelength [19]. Based on the spectrum of *Monascus* pigments, the absorbance of *Monascus* pigments at the specific wavelength was used to represent the pigment concentration, such as absorbance at 470 nm corresponding to the characteristic absorbance of orange *Monascus* pigments. The composition of *Monascus* pigments was further validated by thin layer chromatography (TLC) analysis using Silica gel 60 F_{254} TLC plate (Merck).