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Coupled aminophilic reaction and directed metabolic channeling to red *Monascus* pigments by extractive fermentation in nonionic surfactant micelle aqueous solution

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ABSTRACT

Orange *Monascus* pigments are biosynthesized during *Monascus* fermentation and the orange *Monascus* pigments are further modified into red ones by the aminophilic reaction between orange *Monascus* pigments (azaphilones) and primary amines (various amino acids). The aminophilic reaction involves reagent incompatibility due to the presence of water-insoluble orange *Monascus* pigments and water-soluble amino acids. In the present work, intracellular orange *Monascus* pigments were extracted into the extracellular broth by extractive fermentation in a nonionic surfactant micelle aqueous solution. The transfer of orange *Monascus* pigments from cell interior into its extracellular broth also provided chance for micellar catalysis of the aminophilic reaction between orange *Monascus* pigments and MSG (monosodium glutamate). Combining intracellular biosynthesis of orange *Monascus* pigments and chemical modification of orange *Monascus* pigments catalyzed by micellar catalysis in the extracellular broth by extractive fermentation, the metabolic channel was directed to production of red *Monascus* pigments. High concentration of red *Monascus* pigments (free of teratogenic orange ones) of 35 OD at 510 nm was achieved in the extracellular broth.

1. Introduction

The genus *Monascus* belongs to the class *Ascomycetes* and the family *Monascaceae*, which produces various secondary metabolites of polyketide structure. *Monascus* sp produces at least six molecular structures of pigments, which are classified into three groups based on their colors, i.e., yellow *Monascus* pigments (monascin and ankaflavin), orange *Monascus* pigments (monascorubrin and rubropunctatin), and red *Monascus* pigments (monascorubramine and rubropuntamine). Red Yeast Rice is the product of solid-fermentation of *Monascus* sp on rice, which has a long-term history as food colorant in the orient countries. The major pigments of Red Yeast Rice are red *Monascus* pigments as well as a few yellow ones [1].

Azaphilone is a class of fungal metabolites with diverse biological activities [2,3]. *Monascus* pigments belong to azaphilones [4,5]. It is believed that orange *Monascus* pigments are biosynthesized during *Monascus* fermentation while red ones are resulted from the aminophilic reaction between orange *Monascus* pigments (azaphilones) and amino group-containing compound [6]. There are many reports about production of water-soluble *Monascus* pigments by extraction of *Monascus* pigments from mycelia and then modification with amino acids [7–9] or glucosamine [10] via aminophilic reaction. Many of biological activities, such as anti-microbe [11,12], cytotoxicity [13], anticancer [14], anti-obesity [15], and anti-inflammation [16–18], may be related to the aminophilic reaction between azaphilones and amino group-containing compounds. However, the diverse amino group-containing compounds, such as amino acids, proteins, and nucleic acids, lead to non-selective aminophilic reaction. Orange *Monascus* pigments also exhibit significant teratogenic effect on chick embryo while the corresponding derivates of red *Monascus* pigments with amino acid residues have much less of toxicity [19,20]. Contrast to the close concern to citrinin toxicity in *Monascus* pigments [21], such as the forbidding sale of *Monascus* pigments contaminated with citrinin in European Union [22], both academy and public pay little attention to the toxicity of orange *Monascus* pigments.

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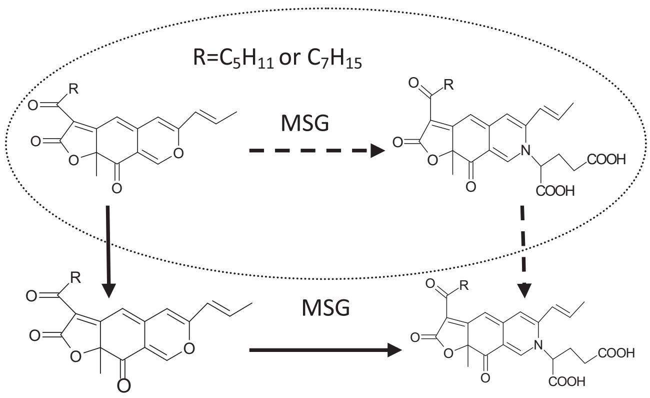

Fig. 1. Schematic representation of aminophilic reaction during microbial fermentation. Aminophilic reaction between orange *Monascus* pigments and MSG can be occurred in cell interior during conventional submerged culture, in which the water-soluble red *Monascus* pigment derivates with MSG residue are secreted into its culture medium (dotted arrows). Aminophilic reaction between orange *Monascus* pigments and MSG is catalyzed by micellar catalysis in extracellular micelle aqueous solution during extractive fermentation, in which water-insoluble orange *Monascus* pigments are exported into its extracellular culture medium by extractive fermentation (solid arrows).

The aminophilic reaction between orange *Monascus* pigments and hydrophilic amino acids (such as monosodium glutamate (MSG)) is limited by reagent incompatibility due to the lipophilic nature of orange *Monascus* pigments while the solubility of hydrophilic MSG in aqueous solution. There are various ways to solve the problem of reactant incompatibility. An obvious approach is selection of solvent or combined solvent capable of dissolving both lipophilic and hydrophilic species, such as selection of 50% methanol aqueous solution as the aminophilic reaction (between orange *Monascus* pigments and MSG) medium [23]. Alternatively, this kind of reaction can also be carried out in a liquid-liquid two-phase system consisting of two immiscible solvents containing phase transfer reagents (such as quaternary ammonium), which is known as phase transfer catalysis [24]. Surfactant micelle aqueous solution is able to solubilize various species with a very broad polar spectrum, which is an alternative medium to overcome incompatibility problem and then accelerate reaction rate by compartmentalization and concentration of reactants (known as micellar catalysis) [25,26]. The large interface area of micelles can even be utilized as a template to induce regioselectivity [27].

Orange *Monascus* pigments are produced intracellularly during conventional submerged culture. The chemical modification of orange *Monascus* pigments into red ones by aminophilic reaction between orange *Monascus* pigments and primary amines occurs intracellularly (dotted arrows of Fig. 1). The aminophilic reaction in cell interior is limited by the intracellular aqueous environment. The assimilation of amino acids is affected by the concentration of amino acids as well as pH in the culture medium [28]. Furthermore, amino acids can be utilized as nitrogen source for microbial growth and metabolism. Very high nitrogen concentration leads to microbial growth while accumulation of very few of the secondary metabolite *Monascus* pigments [29]. Thus, the composition of *Monascus* pigments depends largely upon culture medium, including carbon and nitrogen source as well as pH [30,31]. It is also observed that color value of extracellular water-soluble red *Monascus* pigments increases with the increase of MSG concentration in spite of maintaining relatively high concentration of other intracellular *Monascus* pigments. Further increase of MSG concentration leads to accumulation of biomass [32,33]. In our previous work, extractive fermentation of intracellular *Monascus* pigments was carried out successfully in the nonionic surfactant Triton X-100 micelle aqueous solution, in which microbial growth maintains very well while the intracellular *Monascus* pigments are exported into its extracellular culture medium [34]. This novel fermentation strategy provides the chance for enhancing the aminophilic reaction of orange *Monascus* pigments in extracellular culture medium (solid arrows of Fig. 1), such as micellar catalysis in the extracellular nonionic surfactant micelle aqueous solution, regulation of pH, and adjustment of MSG concentration. It is anticipatable that the extracellular aminophilic reaction should enhance the synthesis of red *Monascus* pigment derivates with MSG residue.

The present work aims at export of orange *Monascus* pigments from cell interior into extracellular broth by extractive fermentation in nonionic surfactant micelle aqueous solution, in which chemical modification of the orange *Monascus* pigments by micellar catalysis in the extracellular micelle aqueous solution is integrated into one process. Then, production of high concentration of red *Monascus* pigment derivates with MSG residue by this integrated bioprocess was carried out.

2. Materials and methods

2.1. Microorganism and fermentation

Monascus anka (China Center of Industrial Culture Collection, CICC 5013) was used in this study. The strain was maintained on potato dextrose agar (PDA) medium (potato 200 g, glucose 20 g, and agar 15–20 g, per liter of tap water) at 4 °C.

The medium composition of inoculum culture was glucose 20 g, yeast extract 3 g, peptone 10 g, KH_2PO_4 4 g, KCl 0.5 g, and $FeSO_4{\cdot}7H_2O$ 0.01 g (per liter of tap water). Inoculum culture was conducted at 30 °C and 200 rpm for 30 h in a 250 ml Erlenmeyer flask with 50 ml of working volume.

The fermentation culture medium consisted of glucose (or cornmeal) 50 g, KH_2PO_4 5 g, $CaCl_2$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, and a specific amount of MSG, per liter of tap water. The initial pH was adjusted to 5 with 10% (V/V) hydrochloric acid. For extractive fermentation in nonionic surfactant micelle aqueous solution, 50 g/l Triton X-100 was added into the fermentation culture medium. One milliliter of inoculum culture was added into 100 ml Erlenmeyer flask containing 25 ml of the fermentation culture medium. The flask was shaken at 30 °C and 200 rpm for 7–8 days. Well-mixed fermentation broth was withdrawn at a certain time point for pigment concentration analysis. All experiments were triplicated.

2.2. Aminophilic reaction of orange Monascus pigments

Orange *Monascus* pigments with a few of yellow ones were produced by *Monascus* fermentation in an aqueous medium using $(NH_4)_2SO_4$ as nitrogen source and glucose as carbon source with an initial pH 2.5, which was detailed in our previous work [28]. After submerged culture, fermentation broth was centrifuged to collect mycelia. The intracellular *Monascus* pigments in mycelia were extracted using 70% ethanol aqueous solution (pH = 2) as extractant. The extract was subjected to filtration and crude *Monascus* pigments were achieved by vacuum evaporation of the filtrate. The major pigments in the crude *Monascus* pigments were orange *Monascus* pigments with a few of yellow ones, which was dissolved in 70% ethanol aqueous solution (pH = 2). The ethanol aqueous solution with concentrated pigments (approximately 50 absorbance units at 470 nm, absorbance unit is the multiplication of the absorbance with its dilution ratio of a sample) was used for the following aminophilic reaction.

Aminophilic reaction between crude orange *Monascus* pigments and MSG was carried out in different solutions, such as 5 g/100 ml Triton X-100 micelle aqueous solution and 70% ethanol aqueous solution with a specific MSG concentration. Reaction solution was adjusted to a certain initial pH with 10% (V/V) hydrochloric acid