Journal of Biotechnology 212 (2015) 167–173

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Investigation of relationship between lipid and *Monascus* pigment accumulation by extractive fermentation

Bo Wang[a,b], Xuehong Zhang[c], Zhenqiang Wu[a,*], Zhilong Wang[b,*]

[a] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, PR China
[b] School of Pharmacy, and State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai 200240, PR China
[c] School of Life Science and Biotechnology, and State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai 200240, PR China

ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 19 August 2015
Accepted 20 August 2015
Available online 28 August 2015

Keywords:
Nonionic surfactant
Extractive fermentation
Microbial lipids
Pigments
Monascus anka

ABSTRACT

Fermented *Monascus* pigments have been utilized as traditional Chinese medicine and food colorant for thousands of years. Under the limited nitrogen concentration and/or low initial pH 2.5 conditions, it was observed that production of intracellular pigments and accumulation of microbial lipids (high content reaching to approximately 50% in dry cell weight) by edible *Monascus anka* exhibited a positive correlated relationship. Extractive fermentation in nonionic surfactant micelle aqueous solution selectively exported the intracellular *Monascus* pigments into its extracellular broth, in which the concentration of intracellular pigments was negligible while the extracellular one was enhanced. The extractive fermentation provides a novel strategy for shifting of the metabolic channeling from intracellular lipid accumulation to *Monascus* pigment production. High pigment concentration, i.e., approximately 40 AU of extracellular *Monascus* pigments, was achieved by extractive fermentation at a relatively high nonionic surfactant concentration 10 g/l. This phenomenon might be attributed to the nonionic surfactant micelles acting as pigment reservoirs by biomimetic of intracellular lipids.

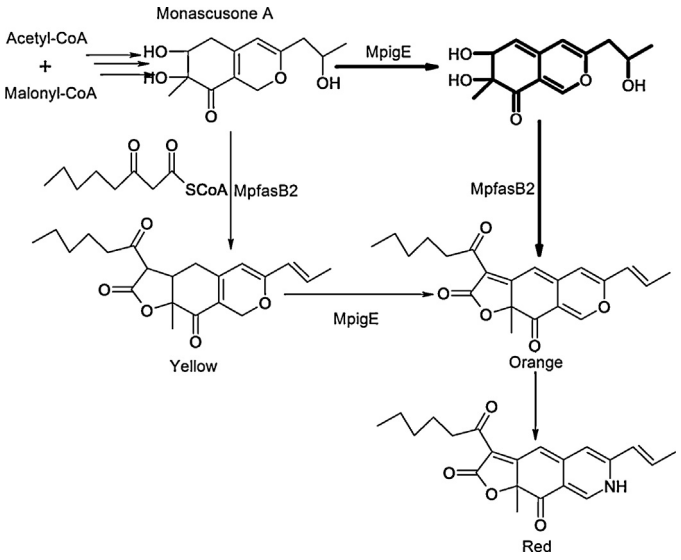
1. Introduction

The genus *Monascus* belongs to the class *Ascomycetes* and the family *Monascaceae*, which produces various secondary metabolites of polyketide structure. *Monascus* species has 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 and ankaflavin), orange pigments (monascorubrin and rubropunctation), and red pigments (monascorubramine and rubropuntamine) (Patakova, 2013). The utilization of fermented *Monascus* product as food colorant, which is known as Red Yeast Rice, has a long-term history in the orient countries (Ma et al., 2000).

Monascus pigments are fungi secondary metabolites. Their biosynthesis is generally regarded to follow a polyketide pathway. However, the detail biosynthetic pathway of *Monascus* pigments remains unclear and even controversial (Feng et al., 2012). Based on isolation and identification of the important precursor of yellow *Monascus* pigments (monascusone A), direct biosynthesis of yellow *Monascus* pigments and then further modification of the yellow *Monascus* pigments is suggested (Jongrungruangchok et al., 2004). Recently, putative gene cluster of biosynthetic *Monascus* pigments has been located and identified (Balakrishnan et al., 2013). It is found that targeted inactivation of *MpfasB2* (encoding short chain fatty acid synthase) leads to the abolishment of *Monascus* pigment biosynthesis while accumulation of monascusone A (Balakrishnan et al., 2014). More importantly, yellow *Monascus* pigments and very few of red ones are produced by *MpigE*-deleted mutant (Δ*MpigE*). On the contrary, the ability for production of orange *Monascus* pigments and red ones is recovered by *MpigE* complementation strain (*ΔMpigE::MpigE*) (Liu et al., 2014). This fact clearly indicates that yellow *Monascus* pigments can be biosynthesized independent of orange ones while aldehyde reductase (coded by *MpigE*) is the key to convert yellow *Monascus* pigments into orange ones. Thus, putative biosynthetic pathway of *Monascus* pigments can be supposed (Scheme 1), in which further chemical modification of orange *Monascus* pigments into red ones (dashing arrow) via aminophilic reaction between orange *Monascus* pigments and primary amines (Lin et al., 1992; Xiong et al., 2015a) is also involved. The corresponding precursor of orange *Monascus* pigments (bold section in Scheme 1) is also identified in *Penicillium marneffei* fermentation broth (Woo et al., 2014), which may also be the reduced product of monascusone A.

* Corresponding authors.
E-mail addresses: btzhqwu@scut.edu.cn (Z. Wu), zlwang@sjtu.edu.cn (Z. Wang).

http://dx.doi.org/10.1016/j.jbiotec.2015.08.019
0168-1656/



Scheme 1. Putative biosynthetic route of *Monascus* pigments.

The same precursor acetyl CoA is utilized for biosynthesis of both *Monascus* pigments (catalyzed by polyketide synthase) and fatty acids (catalyzed by fatty acid synthase) (Hertweck, 2009). The fatty acid composition of genus *Monascus* are highly similar. The major components are C18:1, C18:2, C16:0, C18:0, and C16:1 (Nishikaw et al., 1989). However, there is no data about the lipid content in those strains. Only one report records that *Monascus purpureus* albino strain accumulates high content of lipids while no pigment under a limited nitrogen condition (ratio of carbon to nitrogen, C/N = 80:1) (Rasheva et al., 1997), in which high content of long-chain fatty acids of C20 and C22 is observed (Juzlova et al., 1996). Till now, the metabolic regulation between lipid accumulation and *Monascus* pigment production, to the best of our knowledge, has never been previously described.

Monascus pigments are usually accumulated intracellularly. In our previous works, extractive fermentation in a nonionic surfactant micelle aqueous solution had successfully exported the intracellular *Monascus* pigments into its broth (Hu et al., 2012a,b). Thus, extracellular yellow *Monascus* pigments at low pH 2.5 (Xiong et al., 2015b) while red ones at nearly neutral pH (Xiong et al., 2015a) are accumulated during extractive fermentation. In the present work, accumulation of intracellular lipids and *Monascus* pigments was examined by submerged culture in defined media under limited nitrogen and/or low pH condition. Then, the content of intracellular pigments was perturbed by extractive fermentation in a nonionic surfactant micelle aqueous solution, which was utilized to check the relationship between microbial lipid accumulation and *Monascus* pigment production.

2. Materials and methods

2.1. Microorganism and culture

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 in a 250 ml Erlenmeyer flask with working volume 50 ml for 30 h.

The fermentation culture medium consisted of glucose 50 g, KH_2PO_4 5 g, $CaCl_2$ 0.1 g, and $FeSO_4{\cdot}7H_2O$ 0.01 g in per liter of tap water. The nitrogen source (monosodium glutamate, MSG) concentration and initial pH (adjustment with 10% (V/V) hydrochloric acid) in the fermentation medium were specified in every run. Inoculum culture broth (2 ml) was added into 250 ml Erlenmeyer flask with 50 ml of working volume. The flask was shaken at 30 °C and 200 rpm for 7 days. All experiments were triplicated.

2.2. Extractive fermentation

Extractive fermentation was carried out in a medium consisted of glucose 50 g, MSG 1 g, KH_2PO_4 5 g, $CaCl_2$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, and a specified amount of nonionic surfactant Triton X-100 in per liter of tap water with initial pH 2.5 (adjustment with 10% (V/V) hydrochloric acid). Two milliliters of inoculum culture were added into 250 ml Erlenmeyer flask with 50 ml of working volume. The flask was shaken at 30 °C and 200 rpm for 7 days. All experiments were triplicated.

2.3. Analysis methods

After fermentation culture, culture broth was normalized to 50 ml with water, in which mycelia was separated by centrifugation. After collection of mycelia, pH of the supernatant was recorded. At the same time, the supernatant (1 ml) was diluted directly with water to determine the residual glucose concentration and MSG concentration, respectively. The residual glucose concentration was determined by the standard 3, 5-dinitrosalicylic acid method (DNS). The residual MSG concentration was determined by standard ninhydrin method. In additionally, the supernatant was also used to analyze the absorbance (concentration) of extracellular pigments. The procedure was detailed in our previous work (Xiong et al., 2015a).

The mycelia after centrifugation from the culture broth were subjected to washing 3 times with equal volume of water (adjustment of pH to 2 with 10% (V/V) hydrochloric acid) and followed a similar procedure to determinate dry cell weight (DCW) by gravity (Rasheva et al., 1997) except that dry temperature was kept at 60 °C to ensure the stability of intracellular *Monascus* pigments while dry time was lasted at least overnight until constant mycelia weight reached. The dry cells were used to estimate lipid content and pigment concentration, respectively. Lipid content in DCW was determined following the standard method (Bligh and Dyer, 1959).

The intracellular pigment content in DCW was defined as the absorbance of 1 g of DCW dissolved completely in 100 ml of ethanol solution (70 %, V/V, pH 2). A certain amount of DCW (such as 0.1 g) was re-suspended in 25 ml of ethanol aqueous solution and incubated for 1 h at room temperature for extraction of intracellular pigments. Estimation of pigment concentration in the ethanol aqueous solution followed the same method as detailed in our previous work (Xiong et al., 2015a). *Monascus* pigment concentration was estimated from visible spectrum and represented as absorbance unit (AU, multiplication of the absorbance with its dilution ratio for a certain sample). The composition of *Monascus* pigments was further validated by thin layer chromatography (TLC) analysis using Silica gel 60 F_{254} TLC plate (Merck). Samples were spotted and run with chloroform/methanol/acetic acid (285:21:9) as solvent system. *Monascus* pigments were detectable at different locations under visible light: red *Monascus* pigments at origin, yellow ones at $R_f = 0.7$, and orange ones at $R_f = 0.95$.