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Production of natural melanin by *Auricularia auricula* and study on its molecular structure

Shujing Sun [a,*], Xiaojuan Zhang [a], Shiwei Sun [c], Liaoyuan Zhang [a], Shukai Shan [a], Hu Zhu [b,c,*]

[a] College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China
[b] College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China
[c] Centre for Bioengineering and Biotechnology, China University of Petroleum (East China), Qingdao 266580, People's Republic of China

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ABSTRACT

In this study, the production and structure of melanin produced by *Auricularia auricula* have been determined and analyzed in detail. The results showed that the highest mycelial growth rate was observed in low-carbon and carbon-free medium. In low-nitrogen and nitrogen-free medium, melanin yield was very low. Deficiency of tyrosine in medium led to weak secretion of melanin. The inorganic salt could markedly influence mycelia morphology, but did not obviously impact mycelia growth rate and melanin yield. Meanwhile the condensed molecular formula ($[C_{18}(OR)_3H_7O_4N_2]_n$) and structural formula of melanin were concluded based on UV–Vis, HPLC, FTIR, NMR and elemental assay. This is an eumelanin and also a macromolecular polymer of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid. The 6 main components were phenolic hydroxyl, carboxyl, amidogen, carbonyl, methylene, methyne and sulfur. This work testified that nutritional control was very important to promote melanin production, making melanin more affordable as material in food, cosmetics and medicines.

1. Introduction

Pigments are important constituents in food additives and the demand for pigments has been gradually increasing in recent years (Eriksen, 2008). Natural pigments are considered safe, with pronounced nutritional and therapeutic benefits in comparison to synthetic pigments. Currently, the natural pigments are mostly obtained through extraction of plant materials (Kim et al., 2005; Yolmeh, Habibi Najafi, & Farhoosh, 2014). However, plant cultivation does not guarantee a standardized product, with pigment composition varying from batch to batch. Furthermore, the extraction is a very complicated and time-consuming process, which severely limits the application of natural pigments on a large scale. Microbial submerged fermentation is considered a promising alternative for the efficient production of natural pigments (Guo, Chen, et al., 2014; Guo, Rao, et al., 2014; Vinarov, Robucheva, Sidorenko, & Dirina, 2003) because it is prone to industrialization.

Melanin is one of the important natural pigments and has been widely and conventionally used in different industrial fields including food, cosmetics, pharmacology, medicines and other fields. The most interesting thing is that melanin can be used as food additives to prevent the rancidity caused by the presence of bacteria by quenching the bacterial quorum sensing (Zhu, He, & Chu, 2011). Melanins are irregular dark brown polymers that are produced in various organisms by fermentative oxidation of nitrogen containing or nitrogen-free diphenols (Aghajanyan et al., 2005). Melanins are considered strong antioxidants and have anti-virus functionality and can effectively protect living organisms from ultraviolet radiation. There are some traditional methods to obtain melanin mainly including the extraction from plant and animal materials, the chemical synthesis based on oxidation of tyrosine and its derivatives, and enzyme catalysis (Eisenman & Casadevall, 2012; Murisier & Beermann, 2006; Vinarov et al., 2003). However, these technologies have shown no potential for large-scale industrial production of melanin due to their high cost and complicated synthesis process. Because of these shortcomings

Abbreviations: A. auricula, *Auricularia auricula*; PDA, potato dextrose agar; CM, complete medium; CFM, carbon-free medium; NFM, nitrogen-free medium; TFM, tyrosine-free medium; SFM, salt-free medium; LCHNM, low-carbon and high-nitrogen medium; LNHCM, low-nitrogen and high-carbon medium; UV–Vis, ultraviolet–visible spectrum; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared spectroscopy; HPLC, high performance liquid chromatography; SEM, scanning electron microscope.

* Corresponding authors at: College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China (S. J. Sun). College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China (H. Zhu).

E-mail addresses: shjsun2004@126.com (S. Sun), zhuhufafu@gmail.com (H. Zhu).

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many researchers now explore melanin production by bacteria, mycete, or fungi, but these microorganisms often secrete many toxic secondary metabolites. The latter make the produced melanins following these routes difficult to use in food, cosmetics, pharmacology and medicines (Figueiredo-Carvalho, dos Santos, Nosanchuk, Zancope-Oliveira, & Almeida-Paes, 2014; Goncalves, Lisboa, & Pombeiro-Sponchiado, 2012; Guo, Chen, et al., 2014; Guo, Rao, et al., 2014; Wan et al., 2007). Over the last 50 years, the study of melanin structure has been attracting scientific interest and has become a key research focus in the field of natural pigments. However, the exact structure of melanin is still undefined due to its low solubility in water and most organic solvents. Melanin may exist as three different forms: eumelanins, phaeomelanins and allomelanins. Currently, the structure characterization of the first two melanins has been confirmed to a certain extent, but the structure of the third melanin has not yet been determined (Agadzhanian et al., 2011; Aghajanyan et al., 2005; Dong & Yao, 2012; Eisenman & Casadevall, 2012; Khan, Harsha, Giridhar, & Ravishankar, 2011; Stadler & Fournier, 2006; Vinarov et al., 2003; Ye et al., 2014; Zhong, Frases, Wang, Casadevall, & Stark, 2008). The main chemical monomers of eumelanin are 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) instead of benzothiazine and benzothiazole in phaeomelanin.

Auricularia auricula is a traditional Chinese edible and medicinal mushroom containing many bioactive compounds that have been reported to help in detoxification, the inhibition of bacterial quorum sensing, the lowering of blood pressure and the reduction of blood vessel fat (Ma, Xu, & Feng, 2014; Nguyen, Chen, et al., 2012; Nguyen, Wang, et al., 2012; Reza et al., 2014). *A. auricula* is an organism capable of high secretion of natural edible melanin by submerged fermentation, which can shorten and simplify the production cycle, lead to high production efficiency, and then reduce the cost. Melanin is a secondary metabolite that is generally secreted under restrictive nutrient conditions, and furthermore, melanin accumulation can be also affected by these environmental factors such as aeration and nutrition (Zhang et al., 2015). Therefore, it is important to investigate the effect of environmental conditions on the mycelial growth rate and melanin production by *A. auricula*. It is also significant for improving the melanin production, simplifying and shortening the purification process, and then increasing economic benefit. However, up to now, there are seldom reports on the investigations of the linkage between the mycelial growth rate and melanin production by *A. auricula* under nutrition stress conditions. Furthermore, it is hard to get single component of this melanin to analyze its structure.

In the present study, the growth rate and morphology of mycelia and melanin production from *A. auricula* under different nutritional conditions were determined and analyzed in detail. The chemical structure of melanin has then been systematically investigated using chromatography technologies. This specific structure and molecular formula of natural melanin is significantly different from that of melanin from *Lachnum* YM404 (Ye et al., 2014) and *Catharsius molossus* (Riley, 1997), and is rarely reported at home and abroad. In particular, chemical groups might be connected to each other through various chemical bonds.

2. Materials and methods

2.1. Strain, media, and chemicals

The *A. auricula* strain was obtained from the Fujian General Station of Technology Popularization for Edible Fungi (Fujian, China) and deposited in China Center of Industrial Culture Collection (Serial Number: 1511C0005000004283). This strain was maintained on PDA slant (potato 200 g/L, glucose 20 g/L and agar 20 g/L) at 28 °C with a periodic transfer. The stock culture was incubated on enriched PDA plate (glucose 20 g/L, yeast extract 5 g/L, peptone3 g/L, KH_2PO_4 3 g/L, $MgSO_4$ 1.5 g/L, VB_1 0.1 g/L and agar 20 g/L) at 28 °C for 7 days, and then used for culture inoculation. The following media were prepared:

Complete medium (g/L): lactose 10, yeast extract 10, tyrosine 1, $CaCl_2$ 0.1, NaCl 5;
Carbon-free medium (g/L): yeast extract 10, tyrosine 1, $CaCl_2$ 0.1, NaCl 5;
Nitrogen-free medium (g/L): lactose 10, tyrosine 1, $CaCl_2$ 0.1, NaCl 5;
Tyrosine-free medium (g/L): lactose 10, yeast extract 10, $CaCl_2$ 0.1, NaCl 5;
Salt-free medium (g/L): lactose 10, yeast extract 10, tyrosine 1;
Low-carbon and high-nitrogen medium (g/L): lactose 1, yeast extract 10, tyrosine 1, $CaCl_2$ 0.1, NaCl 5;
Low-nitrogen and high-carbon medium (g/L): lactose 10, yeast extract 1, tyrosine 1, $CaCl_2$ 0.1, NaCl 5;
Tyrosine medium (g/L): glucose 1, peptone3, $CaCl_2$ 0.1, NaCl 5, tyrosine 1, pH7.0.

All of the chemicals used in this study were of analytical grade and were purchased from Sigma (Sigma–Aldrich, China) unless mentioned otherwise.

2.2. Influence of nutritional conditions on mycelial growth rate, morphology and melanin production

The colony morphology and mycelia growth rate were studied on various agar media: CM, CFM, NFM, TFM, SFM, LCHNM and LNHCM. The mycelia of *A. auricula* grown on enriched PDA plate were transferred to solid medium by punching out one mycelial tablet (d = 8 mm) with a sterilized puncher. These plates were incubated at 28 °C for 7 days. In order to determine the melanin production, flasks (250 mL) were filled with 100 mL of CM, CFM, NFM, TFM, SFM, LCHNM and LNHCM liquid media in advance. The culture inoculation was then transferred to every flask by punching out ten mycelial tablets (d = 8 mm) with a sterilized puncher. The cultivation was conducted at 28 °C on a rotary shaker operated at 150 rpm for 8 days. In all experiments, multiple flasks at least in triplicate were run at the same time to ensure reproducibility. The Data Processing System (DPS) statistical software package (version 7.05) was employed to analyze all data and statistical significance of experimental designs.

2.3. Analysis of element composition and molecular structure of melanin

In order to explore the difference between natural melanin from *A. auricula* and other melanins, the element constitution and structure of melanin from *A. auricula* were investigated with Elemental Analyser, HPLC technology, UV–Vis spectroscopy, FTIR spectroscopy and NMR spectroscopy as below.

2.4. Analytical methods

All experiments were performed in triplicate to ensure their reproducibility, and the data presented in "Section 3" represent the mean of three independent experiments.

(1) Melanin extraction, purification and detection.

The fermentation broth of *A. auricula* was collected and squeezed through a nylon mesh to remove mycelia. The pH of