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Isolation and Characterization of Extracellular Melanin Produced by *Chroogomphus rutilus* D447

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ABSTRACT

Melanins are polyphenolic or indolic dark-brown to black pigments of macromolecules and have many biological activities. In this present study, a new strain with extracellular melanin producing ability was isolated from fresh sand bones underground 40-50 cm collected from desert region at Ningxia province, China. The strain was identified to be *Chroogomphus rutilus* (*C. rutilus*) D447 by Internal Transcribed Spacer (ITS) DNA sequence. The melanin had typical synthetic melanin's physical and chemical properties including its maximum ultraviolet absorption peaks at 212 nm, strong and broad bands in region of 3.400-3.200 cm⁻¹ in infrared (IR) spectra and chemical reactions. The melanin did not dissolve in acidic or neutral water and had good stability at high temperature of 100°C for 5 h, sunlight for 40 day, ultraviolet for 240 min and conventional food additives including 5% sucrose, 5% glucose, 0.5% sodium benzoate and 1.0% potassium sorbate. This is the first report of extracellular melanin from *C. rutilus* D447 and it has potential applications in food, pharmaceutical and cosmetic industries.

Key words: *Chroogomphus rutilus*, melanin, characterization, extracellular

INTRODUCTION

Melanins are widely existed in plants, animals and microorganisms, which are of polyphenolic or indolic dark-brown to black pigments of macromolecules (Langfelder *et al.*, 2003; Butler and Day, 1998). They play important roles in organisms, such as composite compounds of cell wall structure, protection of the organisms against environmental stress from ultraviolet radiation, ionizing radiation, heavy metal ions, low or high temperature, etc. So, they have important biological activities, including free radicals scavenging ability (Zhan *et al.*, 2011; Jacobson and Tinnell, 1993; Babitskaya *et al.*, 2000a, b; Wu *et al.*, 2008; Goncalves and Pombeiro-Sponchiado, 2005; Manivasagan *et al.*, 2013), anti-radiation (Dadachova *et al.*, 2007; Ye *et al.*, 2014), antimicrobial (Manivasagan *et al.*, 2013), anti-virus (Montefiori and Zhou, 1991), antitumor (El-Obeid *et al.*, 2006), chelating heavy metal ions (Gadd and Rome, 1988), antivenin activity (Hung *et al.*, 2004) and live protecting activity (Sava *et al.*, 2003), etc. They showed a widely application prospects in food additives, pharmacology, cosmetics and other fields.

A lot of melanins have been identified from fungal sources, such as *Aspergillus nidulans* (Goncalves and Pombeiro-Sponchiado, 2005), *Azospirillum lipoferum* (Givaudan *et al.*, 1991), *Curvularia lunata* (Rizner and Wheeler, 2003), *Botrytis cinerea* (Doss *et al.*, 2003),

Exophiala pisciphila (Zhan *et al.*, 2011), *Inonotus obliquus* (Babitskaya, 2000b), *Wangiella dermatitidis* (Jacobson and Tinnell, 1993), *Alternaria alternate* (Goncalves and Pombeiro-Sponchiado, 2005), *Tuber melanosporum* Vitt. (Harki *et al.*, 1997), *Agaricus bisporus* (Stussi and Rast, 1981), *Auricularia auricula* (Zou *et al.*, 2010), *Lachnum* (Ye *et al.*, 2014) and *Hypoxyylon archeri* Berk. (Wu *et al.*, 2008), etc. In general, melanin is one part of the cell wall in fungi. Most of these fungal melanins are water-insoluble intracellular pigments. Alkaline and ultrasound-assisted extraction of melanins from fungal mycelium was of the most commonly used methods. Extracellular melanins are also founded in some fungal culture, which might be broken away from the cell wall matrix or the phenolic compounds secreted into the culture medium being slowly auto-oxidize to form melanins (Butler and Day, 1998). However, there has no information about extracellular melanin from *Chroogomphus rutilus* (*C. rutilus*). *Chroogomphus rutilus*, a species of *Gomphidiaceae* family fungus is one of the edible mushroom species. So far, a few studies on *C. rutilus* have been reported. To date, the antimicrobial, anticholinesterase, antioxidant activities and volatile compounds of the mushroom have been studied (Kalyoncu *et al.*, 2010; Yamac and Bilgili, 2006; Cayan *et al.*, 2014). In this report, an extracellular melanin producing fungi was identified to be *C. rutilus* D447 by ITS DNA gene sequence. The melanin was purified from the fungal culture and identified using chemical reaction, Fourier transform infrared and Ultraviolet-visible spectra and the stability of *C. rutilus* melanin was analyzed.

MATERIALS AND METHODS

Materials: The UNI-Q-10 column DNA extraction kit and DNA gel recycle and purification kit were purchased from Sangon Biological Engineering Technology, Shanghai, China. The DNA maker (#SM0331) was purchased from Thermo Fisher Scientific Inc. (Waltham, USA). The forward primer sequence (5-TCCGTAGGTGAACCTGCGG-3) and reverse primer sequence (5-TCCTCCGCTTATTGATATGC-3) were synthesized by Sangon Biological Engineering Technology, Shanghai, China. Potassium bromide was of spectroscopic purity and purchased from Sigma-Aldrich. Hydrogen peroxide (H₂O₂), sodium sulfite (Na₂SO₃), glucose, sucrose, sodium benzoate, potassium sorbate and citric acid were of analytical grade and purchased from China National Pharmaceutical Group Shanghai Chemical Reagents Company (Shanghai, China); All other reagents were of analytical grade.

Isolation and identification of *C. rutilus*: The *C. rutilus* strain was isolated from fresh sand bones underground 40-50 cm collected from desert region at Ninxia province, China. The strain was screened for melanin production on Czapek Dox medium and incubated for 6 days at 28°C. After incubation, the dark brown to black color area, where might be caused by melanin pigments production was noted on the medium (Fig. 1). Developments of these areas were considered for melanin production.

The strain was identified by ITS DNA sequencing. The total DNA was extracted using the UNI-Q-10 column DNA gel kit (Sangon Biological Engineering Technology, Shanghai, China) according to the instructions. The forward primer sequence (5-TCCGTAGGTGAACCTGCGG-3) and reverse primer sequence (5-TCCTCCGCTTATTGATATGC-3) were synthesized by Sangon Biological Engineering Technology, Shanghai, China. PCRs were performed with the following conditions: 35 cycles consisting of 98°C for 5 min, 95°C for 35 sec, 55°C for 35 sec and 72°C for 1 min, followed by a final extension of 4 min at 72°C. Then ITS gene sequence was sent for sequencing (Sangon Biological Engineering Technology, Shanghai, China). The sequences were submitted to GenBank (KM488533) and identified by comparison with accessible sequences in



Fig. 1: Melanin production of strain on Czapek Dox medium

GenBank with the BLAST server. Phylogenetic trees were constructed according to the Neighborjoining method using MEGA v. 5.1 software and reference sequences were obtained from the GenBank database.

Production and isolation of melanin: After slant cultured at Tryptone/Yeast Extract agar medium for 6 days at 28°C, the strain was transferred to 500 mL Erlenmeyer flasks containing 100 mL of Czapek Dox Medium. The composition of medium was as following: Sucrose 30 g; K_2HPO_4 1.0 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $NaNO_3$ 2.0 g and 1000 mL distilled water. The medium was inoculated with 10% (v/v) inoculum and mixed under 180 r/min for 54 h at 28°C. Then these culture liquid was transferred to fermentation medium with 10% inoculums and cultivated under agitation at 180 r/m for 192 h. The culture was collected and centrifuged for 20 min at 10000×g to remove the cells. The supernatant was adjusted pH to 3 with 6 mol LG^1 HCl and kept for 30 min at room temperature. Then the mixture was centrifuged for 10 min at 10000×g to obtain the crude melanin. The crude melanin was dispersed in distilled water and centrifuged again. This step was repeated until the supernatant reached to neutral pH. The precipitation was dried vacuum at 50°C and the solid was washed with petroleum ether, chloroform, ethyl acetate and alcohol, respectively. At last, the purified melanin was obtained after dried vacuum at 50°C.

Characteristics of melanin from *C. rutilus* D447: Chemical and physical stability characteristics of melanin from *C. rutilus* D447 were tested. The *C. rutilus* melanin was dissolved in different pH water and then centrifuged for 20 min at 10000 × g. Absorbance at 415 nm of the supernatant was determined to evaluate the effects of pH on solubility. The *C. rutilus* melanin was dissolved in different pH 10.0 water and heated for 1-5 h at various temperature. Then the absorbance was determined at 415 nm to evaluate the temperature stability of *C. rutilus* melanin. The melanin was dissolved in different pH 10.0 water and exposed to sunlight for 5-40 days or UV light (100 W) for 20-240 min at room temperature. Then the absorbance was determined at 415 nm to evaluate the light stability of *C. rutilus* melanin. The melanin was dissolved in different concentrations of 0-3.0% (v/v) hydrogen peroxide or 0-1.0% (w/v) sodium sulfite for 30 min at room temperature and dark place. Then the absorbance was determined at 415 nm to evaluate the oxidative stability and reducibility of *C. rutilus* melanin. The melanin was dissolved in various food

additives, including 5% (w/v) sucrose, 5% (w/v) glucose, 0.5% (w/v) benzoate sodium and 1.0% (w/v) potassium sorbate, for 24 or 48 h at room temperature and dark place. Then the absorbance was determined at 415 nm to evaluate the food additives stability of *C. rutilus* melanin.

The *C. rutilus* melanin was dissolved in pH 10.0 water to obtain 0.8 mg mL⁻¹ solution and scanned from 200 to 800 nm with UV-755B spectrophotometer to obtain Ultraviolet Visible (UV) absorption spectra (Ravishankar *et al.*, 1995). The purified *C. rutilus* melanin 2 mg was ground uniformly with 150 mg spectrometric grade KBr and pressed to a tablet. Then the infrared (IR) spectra was recorded (Paim *et al.*, 1990) on a Thermo Nicolet spectrometer (Nicolet 380 FT-IR).

Statistical analysis: The experimental results obtained were expressed as Mean±SD of triplicates.

RESULTS AND DISCUSSION

Isolation and identification of *C. rutilus* D447: Sand bones were collected from a desert region underground 40-50 cm at Ninxia province, China. The sand bones were stored in sterilized containers and transported over ice for immediately delivery to the laboratory. Total 12 strains were isolated from the sand bones, including 8 strains of bacteria, 2 strains of actinomycetes and 2 strains of fungi. The 2 strains of fungi were cultured at Czapek Dox Medium and incubated for 6 days at 28°C. There was obvious dark brown to black color rings around one of the two colonies (Fig. 1). This strain was cultured repeatedly on Czapek Dox Medium.

This isolate was tentative identified as *C. rutilus* sp. D447 based on the morphological, physiological and biochemical characteristics and it was confirmed by the ITS sequencing (GenBank KM488533) (Fig. 2). Although there are no exact similarity limits for defining specific

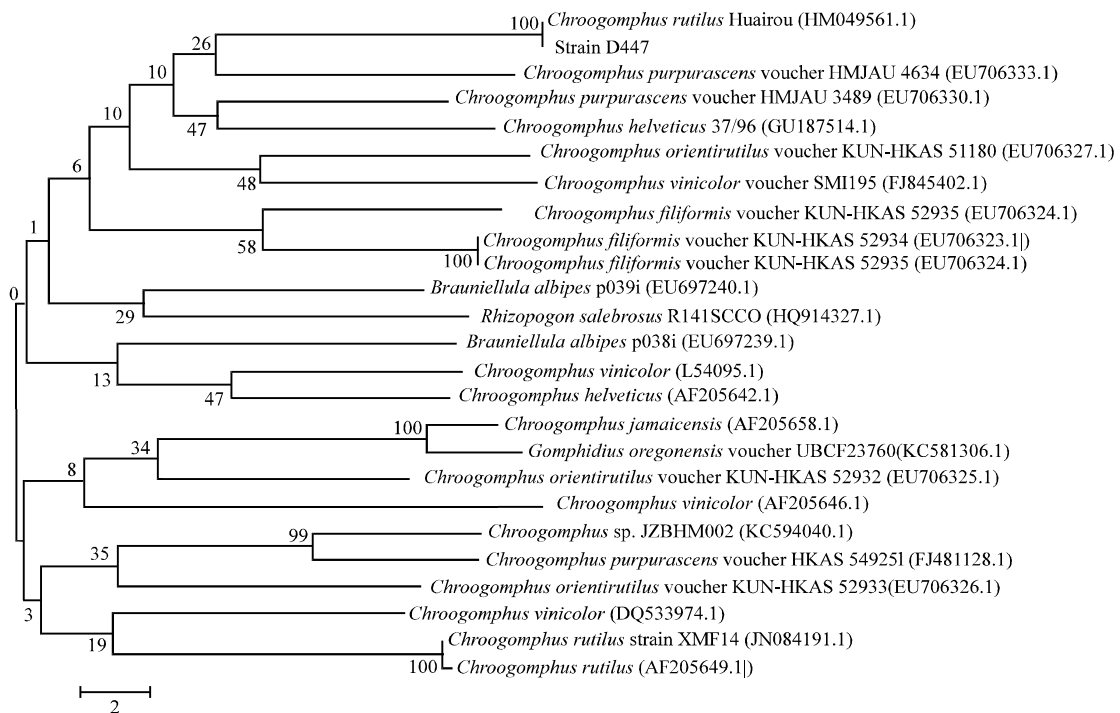


Fig. 2: Phylogenetic relationship of the ITS sequence of *C. rutilus* D447 with ITS of different *Chroogomphus*

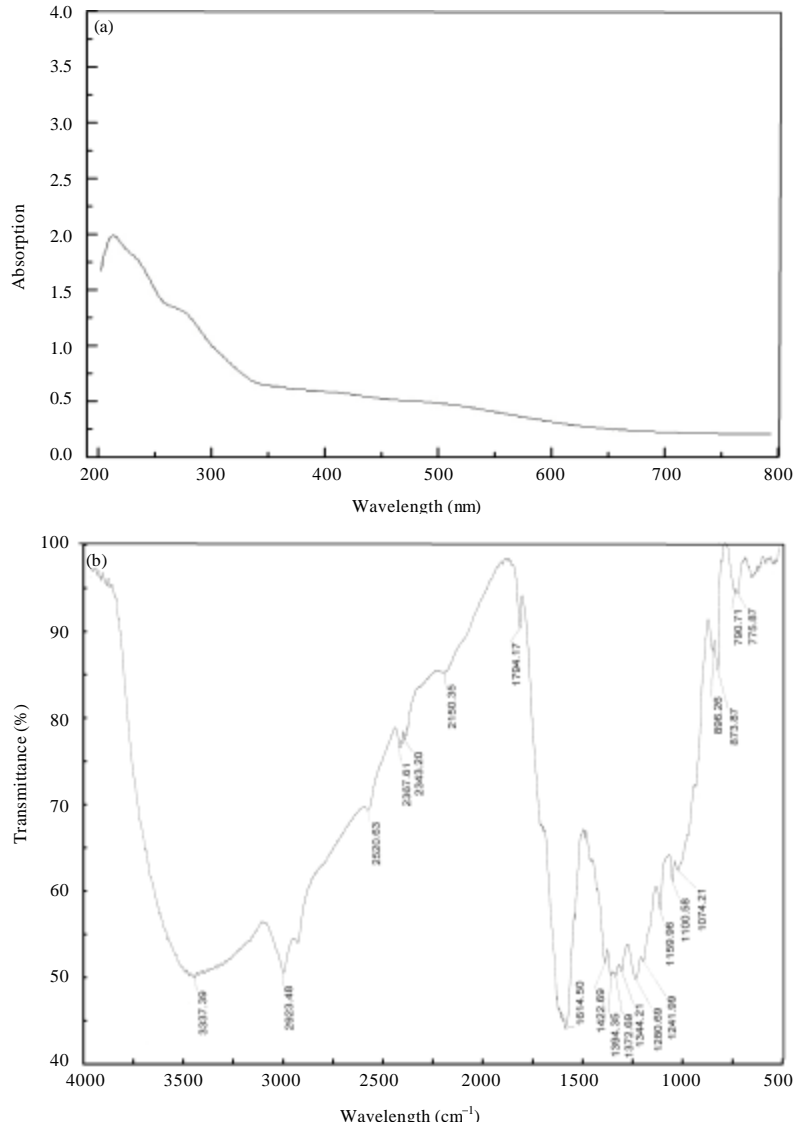


Fig. 3(a-b): Spectrum of *C. rutilus* melanin (a) Ultraviolet visible spectrum and (b) Infrared spectrum

taxa, such as genus and species, in general, species definition requires sequence similarities greater than 98%. In our study the strain showed 99% similarity to ITS gene sequences from GenBank databases and was identified as members of *Chroogomphus rutilus*.

Spectral characteristics of melanin from *C. rutilus* D447: The purified *C. rutilus* melanin had maximum absorption peaks at 212 nm (Fig. 3a), which was similar to 211 nm of *Lachnum* YM-346 melanin (Ye *et al.*, 2012). The melanins of different sources had various maximum UV-Vis absorption peaks, such as *Lachnum* 404 melanin at 210 nm (Ye *et al.*, 2014), *Actinoalloteichus* sp. MA-32 melanin at 300 nm (Manivasagan *et al.*, 2013), *Pleurotus cystidiosus* melanin at 280 nm (Selvakumar *et al.*, 2008) and *E. pisciphila* melanin at 216 nm (Zhan *et al.*, 2011), etc. The *C. rutilus* melanin showed stronger absorption in UV region and progressively less as the wavelength increased likely other fungal sources of melanin. The purified *C. rutilus* melanin did

not contain nucleic acid or protein because of no obvious absorption peak between 260-280 nm in the UV spectra.

Infrared spectra is one of the most important spectral characteristics and has been used to investigate the chemical structure of melanin, including *Lachnum* melanin, *Actinoalloteichus* melanin and *Ophiocordyceps sinensis* melanin, etc. (Manivasagan *et al.*, 2013; Ye *et al.*, 2012; Dong and Yao, 2012). The IR spectra of the purified *C. rutilus* melanin was shown in Fig. 3b, where it had strong and broad bands of OH and NH₂ stretching in region of 3.400-3.200 cm⁻¹ (Bonner and Duncan, 1962). The small pinnacle at 2923.48 cm⁻¹ might be result from oscillations of aliphatic group CH₂ (Zhan *et al.*, 2011). The *C. rutilus* melanin had another strong bands at 1614.50 cm⁻¹, which might be caused by stretching vibration of C = O of quinone neighbouring to the carboxyl group and influenced by another C = O adjacent to it (Ye *et al.*, 2012). The absorption at 1422.6-1344.21 cm⁻¹ might be due to amine groups and deformational changes in NH groups of secondary amines, CH₂ groups of aliphatic radicals, CH groups adjacent to COOH and OH groups. The 1280.69 and 1241.99 cm⁻¹ absorptions might be due to stretching vibration of carbonyl groups (Babitskaya *et al.*, 2000a). These results indicated that *C. rutilus* contained carboxyl-substituted indole quinone and methyl-substituted indole quinone structure.

Stability of *C. rutilus* D447 melanin: The *C. rutilus* melanin was dissolved in various pH water and centrifuged for 10 min. The supernatant solution was determined at 415 nm and the result was shown in Fig. 4a. The *C. rutilus* melanin was dissolved easily in strong alkaline solution, but did not dissolved in acidic or neutral water, which was of characteristics of the typical non-water-soluble melanin.

Heat processing was one of the most useful food processing technology. As food additives, they should be tolerated from heat processing. The *C. rutilus* melanin was stability at 80 or 100°C for at last 5 h and the survival rate of melanin was higher than 85%, shown in Fig. 4b. This result indicated that the *C. rutilus* melanin had good thermal stability and had broad applications of food thermal processing.

The sunlight or ultraviolet stability of *C. rutilus* was also tested in this study and the results were shown in Table 1. The *C. rutilus* melanin had good sunlight and ultraviolet stability for 40 day and 240 min, respectively. The survival rate of melanin under sunlight or ultraviolet for 40 day or 240 min was higher than 93.5 or 96.3%.

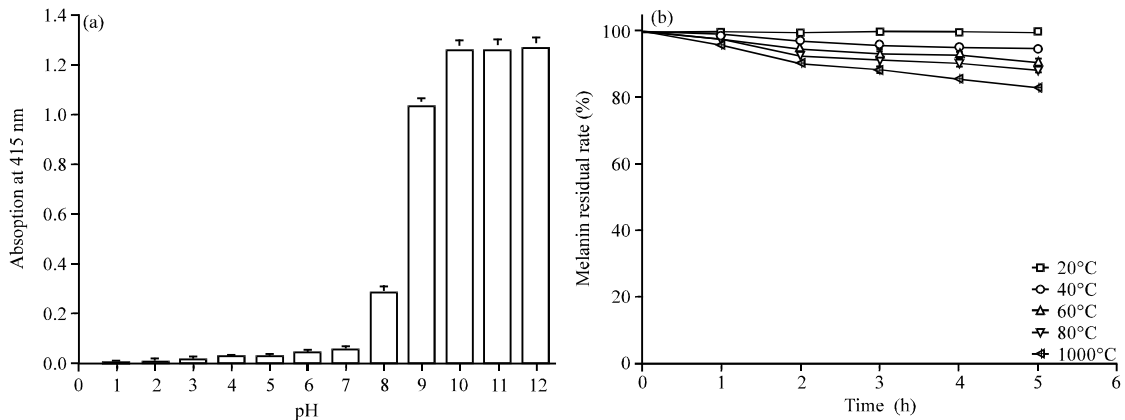


Fig. 4(a-b): pH solubility (a) and thermal stability (b) of *C. rutilus* melanin. Values reported represent Mean±SD (n = 3)

Table 1: Effects of sunlight and ultraviolet on *C. rutilus* melanin

Exposure to sunlight (days)	Melanin survival rate (%)	Exposure to ultraviolet (min)	Melanin survival rate (%)
0	100	0	100
5	101.2±1.3	20	100.6±1.2
10	100.0±1.3	40	99.5±1.2
15	99.1±1.1	60	99.2±1.0
20	98.7±1.2	120	98.5±1.1
25	98.7±1.3	180	96.8±1.3
30	97.1±0.9	240	96.3±1.3
35	96.6±1.1		
40	93.5±0.8		

Values reported represent Mean±SD (n = 3)

Table 2: Effects of hydrogen peroxide and sodium sulfite bleaching on *C. rutilus* D447 melanin

H ₂ O ₂ concentration (%)	Melanin survival rate (%)	Na ₂ SO ₃ concentration (%)	Melanin survival rate (%)
0	100	0	100
0.1	99.6±1.2	0.05	99.3±0.9
0.4	97.4±1.0	0.10	99.3±1.1
0.8	95.7±1.2	0.20	97.5±1.3
1.2	94.2±1.1	0.40	88.9±1.2
3.0	93.7±0.9	1.00	79.7±0.9

Values reported represent Mean±SD (n = 3)

Table 3: Effects of food additives on *C. rutilus* D447 melanin

Food additives	Melanin survival rate after 24 h (%)	Melanin survival rate after 48 h (%)
0	100	100
5% sucrose	100.4±1.2	100.1±1.1
5% glucose	99.8±1.1	99.8±1.0
0.5% sodium benzoate	100.3±1.1	100.0±0.8
1.0% potassium sorbate	99.4±0.9	99.1±0.8
1.0% citric acid	18.4±0.2	15.7±0.2

Values reported represent Mean±SD (n = 3)

The effects of oxidant (H₂O₂) and reduce reagent (Na₂SO₃) on *C. rutilus* melanin were shown in Table 2. Obviously, the *C. rutilus* melanin could be bleached by hydrogen peroxide or sodium sulfite, especially at high concentration, which was one of the typical characteristics of various resources melanin, such as plant melanin (Sava *et al.*, 2001), fungal melanin (Zhan *et al.*, 2011) and animal melanin (Chen *et al.*, 2008).

Sucrose, glucose, sodium benzoate, citric acid and potassium sorbate are widely used in processed food as food additives. Effects of these food additives on *C. rutilus* melanin were shown in Table 3. Obviously, most of these food additives except for citric acid did not reacted with *C. rutilus* melanin and the survival rate of melanin did not decreased for 24 or 48 h. The citric acid decreased significantly the melanin, which could be caused by pH decreasing to very low level. At low pH, the melanin would be insoluble and be precipitated. So, the *C. rutilus* melanin can be used in most of processed food except for low pH food.

CONCLUSION

In the international market, the demand of various pigments for uses of food, pharmaceutical, cosmetic and other industries, is increasing year by year. A strain was isolated from underground

sand bone with extracellular melanin producing ability and identified by ITS method to be *Chroogomphus rutilus* D447. The *C. rutilus* melanin had maximum absorption peak at 212 nm and the IR spectral characteristics showed the *C. rutilus* melanin containing carboxyl-substituted indole quinone and methyl-substituted indole quinone structure. The melanin could be decolorized by hydrogen peroxide and sodium sulfite. The melanin did not dissolve in acidic or neutral water and had good stability at high temperature, sunlight, ultraviolet and conventional food additives including sucrose, glucose, ascorbic acid, sodium benzoate and potassium sorbate. This study provided a new melanin-producing edible fungus. In future studies, we will focus on the melanin modification for improving its water solubility and biological activity including antioxidative, antimicrobial, anti-virus and other activities.

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