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# Production and characterization of melanin pigments derived from *Amorphotheca resinae*[§]

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**As melanin has emerged as functional pigment with cosmetic, health and food applications, the demand for the pigments is expected to increase. However, the conventional sources (e.g. mushroom, hair, and wool) of melanin production entail pigments inside the substrates which requires the costly extraction procedures, leading to inappropriate scalable production. In this study, we screened 102 of fungal isolates for their ability to produce melanin in the supernatant and selected the only *Amorphotheca resinae* as a promising candidate. In the peptone yeast extract glucose broth, *A. resinae* produced the melanin rapidly during the autolysis phase of growth, reaching up 4.5 g/L within 14 days. Structural characterization of the purified melanin from *A. resinae* was carried out by using elemental analysis, electron paramagnetic resonance, $^{13}$C solid-state nuclear magnetic resonance spectroscopy, and pyrolysis-gas chromatography-mass spectrometry in comparison with the standard melanins. The results indicate that the structural properties of *A. resinae* melanin is similar to the eumelanin which has a wide range of industrial uses. For example, the purified melanin from *A. resinae* has the potent antioxidant activities as a result of free radical scavenging assays. Consequently, *A. resinae* KUC3009 can be a promising candidate for scalable production of industrially applicable melanin.**

***Keywords***: *Amorphotheca resinae*, melanin pigments, fungal melanins

## Introduction

Melanins are natural pigments with diverse structures, and their biosynthesis involves the oxidation and polymerization of tyrosine in animals or phenolic compounds in lower organisms (d'Ischia *et al.*, 2013). Melanins can be chemically classified as four types to date; eumelanin, pheomelanin, allomelanin, and pyomelanin (Bell and Wheeler, 1986; Schmaler-Ripcke *et al.*, 2009; d'Ischia *et al.*, 2013). All melanins are known to play a major role in the coloration and protection from stress factors such as ultraviolet light and reactive oxygen species (Wang *et al.*, 2006; Brenner *et al.*, 2008; Gao *et al.*, 2011). Thus, melanins may be useful as both pigments and important bio-active compounds that can be used in cosmetic, health and food applications. Additionally, melanins exhibit unique optical and electronic properties, which researchers have used to design energy storage devices and environmental sensors (Kim *et al.*, 2013; Shillingford *et al.*, 2016). These functional properties make melanins attractive as bio-active substrates for multiple industrial applications and the demand for these pigments is expected to increase.

Isolating melanins from natural sources is typically not practical for scalable production, as only melanins originated from cephalopod ink (approximately 70% pure melanin) have been directly extracted from a natural resource (d'Ischia, 2018). In recent years, different strategies have been developed for large-scale melanin production. Artificial synthesis of melanin by the oxidation of dopamine or L-3,4-dihydroxyphenylalanine, which usually requires alkaline-pH environments, metal catalysts, and a variety of oxidants, was developed (Liu *et al.*, 2014). Extraction of melanin pigments from edible and medicinal mushrooms such as *Auricularia auricula* has been developed as well (Prados-Rosales *et al.*, 2015). However, the former can cause secondary environmental problems by anthropogenic chemicals. The latter has the problems such as slow growth of the fruiting body and laborious procedures of the extraction, which have made it difficult for the industrialized production.

Liquid cultivation of fungi may be a feasible solution for large-scale melanin production. Liquid cultivation has advantages for cost-effective and eco-friendly production because it utilizes readily available feedstocks including organic wastes. Fungi are suitable candidates of liquid cultivation for microbial cell factories as they can utilize a wide range of organic wastes and efficiently produce variable pigments such as melanins, carotene and *Monascus* pigments (Bell and Wheeler, 1986; Mapari *et al.*, 2005; Dufossé *et al.*, 2014). In case of fungal melanins, however, the pigment granules are localized to the cell wall where they are likely to be cross-linked with polysaccharides of cell wall (Eisenman and Casadevall, 2012).

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This fact leads to laborious purification process of melanins from fungal cells during which the loss of melanins occurs. Some species, however, are known to produce melanin in the liquid medium, which raises the need for isolating the fungal species capable of producing melanins outside the cells to facilitate large-scale melanin production.

In the present study, a total of 102 fungal isolates responsible for wood discoloration were evaluated for their ability to produce melanin outside the cells. Only one isolate, designated as *Amorphotheca resinae* KUC3009, was selected based on its capability to produce the black-brown pigment in the culture filtrate, making the isolate a promising source for industrial applications. Then, the profile of melanin production during fungal cultivation was measured. The purified melanin was structurally characterized by physico-chemical analyses to predict its potential industrial application. Furthermore, the antioxidant activities of the purified melanin were evaluated. These results further shed lights on providing a basis for biotechnological production of melanin pigments, using filamentous fungi.

## Materials and Methods

### Screening of fungal species for melanin production

A total of 102 fungal isolates was obtained as pure cultures from the Korea University Culture collection (KUC). Inocula for the experiments were cultured on 2% malt extract agar composed of 20 g malt extract and 15 g agar per 1 L deionized water. Peptone yeast extract glucose (PYG) broth, composed of 20 g glucose, 10 g peptone, and 5 g yeast extract per 1 L of deionized water, was used to screen for fungi capable of melanin production in the culture filtrate. Five agar plugs (5 mm diameter) of each strain were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of sterilized medium and grown on a shaker at 150 rpm at 21°C. After 14 days of cultivation, the fungal biomass in the cultures was harvested by centrifugation. Then, pH of the resulting supernatant was adjusted to 2.0 with 1 M HCl and incubated for 24 h at 21°C. Considering that the melanin is precipitated at acidic condition, the fungal strain KUC3009 which produced the largest pellet fraction of the supernatant was selected as the promising isolate.

Identification of the selected fungi with molecular characterization was conducted as follows. The internal transcribed spacer (ITS) region was selected for phylogenetic analysis of the selected fungus. Genomic DNA of the fungi was extracted using an AccuPrep Genomic DNA extraction kit (Bioneer) according to the manufacturer's protocol. The ITS region was amplified using AccuPower PCR PreMix (Bioneer) with the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTA A-3′) and LR3 (5′-CCGTGTTTCAAGACGGG-3′), as described previously (White *et al*., 1990; Gardes and Bruns, 1993). PCR was performed on a Bio-Rad MyCycler and the temperature cycles were set as follows: the initial denaturation step was performed at 95°C for 5 min, followed by 34 cycles at 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and a final 5 min extension at 72°C. DNA sequencing was carried out by Macrogen using the Sanger method with a 3730xl DNA Analyzer (Life Technologies). The sequences for ITS was deposited in GenBank under accession numbers JN-033458.1. The obtained ITS sequences was proofread and edited with the reference sequences obtained from GenBank using MEGA v.7 and multiple alignment was conducted using MAFFT 7.13 (Katoh and Standley, 2013; Kumar *et al*., 2016). If necessary, the sequence alignments were manually modified. Phylogenetic analysis was performed on ITS sequences by RAxML with the GTR + G model of evolution and 1,000 bootstrap replicates for maximum likelihood (Stamatakis, 2006).

### Growth and melanin production measurement

Fungal growth with reference to melanin production was monitored at 2-day intervals for a total incubation period of 20 days. Spores of the strain KUC3009 were collected from 10-day-old cultures on potato dextrose agar (PDA) using a surfactant (0.02% tween 80). One milliliter of spore suspension ($10^6$ spores/ml) was inoculated into each flask containing 100 ml sterilized PYG media and the cultures were agitated at 150 rpm on a rotary shaker at 27°C. At each measurement point, the biomass and cell-free culture media were obtained by centrifugation. The obtained supernatant was acidified to pH 2 with 1 M HCl and incubated for 24 h at 21°C for pellet production. The cells and the resulting precipitate were weighed after filtering the sample through 0.45-μm glass fiber filters (Whatman). All experiments were performed in at least biological triplicate to obtain standard deviations.

### Melanin preparation and purification

Melanin in the culture filtrate derived from the strain KUC-3009 was purified as follows. Cultivated broth was centrifuged and filtered through 0.45-μm glass fiber filters for biomass removal. The obtained filtrate was mixed with 1 M $NH_3{\cdot}H_2O$ in a proportion of 1:1 (v/v). After boiling in a water bath at 80°C for 2 h, the solution was adjusted to pH 2.0 with 6 M HCl and incubated without movement for 24 h at 21°C. Next, the mixture was centrifugated and the melanin precipitate was collected. The precipitate was suspended in 6 M HCl and boiled at 100°C for 4 h to hydrolyze cellular materials and nutrients associated with the melanin. The melanin was rinsed repeatedly with deionized water, re-dispersed in deionized water, and then extracted successively with chloroform, ethyl acetate, and absolute ethanol. The purified melanin was then lyophilized.

Standard samples of eumelanin, pheomelanin, and pyomelanin were prepared as follows. Melanin from *Sepia officinalis* (Sigma-Aldrich) was used as the representative of eumelanin sample. Synthetic pheomelanin was prepared by oxidation of L-DOPA and L-cysteine (Ito, 1989). L-DOPA (1.0 mM) and L-cysteine (1.5 mM) were dissolved in 100 ml of 50 mM sodium phosphate buffer, pH 6.8. The mixture was incubated at 37°C for 24 h in the presence of 5 mg mushroom tyrosinase (Sigma-Aldrich). After incubation, the mixture was acidified to pH 3 with acetic acid and incubated for 24 h at 21°C. The dark brown precipitate was collected by centrifugation and washed with 1% acetic acid, after which the melanin was lyophilized. Synthetic pyomelanin was produced by autooxidation of 10 mM homogentisic acid solution at pH 10 with constant stirring for 3 days (Schmaler-

Ripcke *et al.*, 2009). Polymerization was stopped and precipitation was started by adjusting the solution to pH 2 with 1 M HCl. After precipitation and centrifugation, the pellet was washed repeatedly with deionized water and lyophilized. As the artificial synthesis procedure of allo-melanin was not yet established, a monomer of allo-melanin, 1,8-dihydroxynaphthalene (1,8-DHN) (Sigma-Aldrich), was set as the standard.

### UV-Vis spectroscopy

The purified melanin samples were dispersed in 0.1 M NaOH. The samples were then scanned in the GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific), using a 10 mm path length quartz cuvette with 0.1 M NaOH as the reference blank. The samples were scanned without temperature equilibration at 1 nm intervals over the wavelength range from 270 to 750 nm.

### Elemental analysis

Elemental compositions (C, H, N, O, and S) of the prepared melanins were determined using a Vario-Micro Cube elemental analyzer. The combustion temperature was set to 1,150°C and the sample weight was approximately 1.8 mg. Standard samples (i.e., sulfanilic acid and benzoic acid) were first analyzed to evaluate instrument accuracy.

### Electron paramagnetic resonance (EPR)

X-band continuous-wave EPR spectra were obtained using a Bruker EMX Plus 6/1 spectrometer equipped with a dual-mode cavity (ER 4116DM). The experimental parameters were as follows: microwave frequency, 9.64 GHz; microwave power, 0.001 mW; modulation amplitude, 5 G; temperature, room temperature.

### $^{13}$C cross-polarization magic angle spinning solid state NMR ($^{13}$CP/MAS SSNMR)

Nuclear magnetic resonance (NMR) data were acquired on 400 MHz solid-state NMR spectrometer (AVANCE III HD, Bruker) operated at 100.66 MHz using a HX CP/MAS probe and 4.0-mm zirconia rotor. Spectra were obtained at 10 kHz to ensure that all rotational sidebands would fall outside of the spectral regions of interest. Typical experimental parameters were as follows: spin rate of 10 kHz; CP contact time, 2.0 msec; recycle delay, 3 sec.

### Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)

Pyrolysis was conducted at 600°C for 1 min and GC-MS analysis of the pyrolysis products was performed using an Agilent 6890N GC coupled to a quadrupole Agilent 5975i MS spectrometer. The samples were injected in split mode (10:1 ratio) at 280°C. The GC oven was held at an initial temperature of 40°C for 2 min, increased to 300°C at a rate of 6°C/min, and held at this temperature for 20 min. Separation was achieved using a 30 m × 0.25 mm (inner diameter) × 0.25 μm DB-5MS capillary column with helium 1.0 ml/min as the carrier gas. Full scan acquisitions were performed over an m/z 50–800. Mass spectral conditions were 70 eV ionization energy, 230°C source temperature, and 290°C transfer line temperature.

### Antioxidant and radical scavenging assay

The 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation-scavenging activity of the purified melanin was evaluated according to previous studies (Chen *et al.*, 2008; Wang *et al.*, 2012; Hou *et al.*, 2019). The 7 mM ABTS solution and 2.45 mM potassium persulfate were mixed and placed in darkness at room temperature for 16 h. The ABTS solution was diluted with absolute ethanol until the absorbance was 0.70 ± 0.02 at 734 nm. A series of 0.2 ml test samples were incubated with the 3 ml ABTS solution for 60 min at room temperature under dark conditions and the absorbance was measured at 734 nm ($A_1$). The ABTS solution was replaced with an equal volume of absolute ethanol and the absorbance was measured ($A_2$). The same amount of distilled water was used instead of the melanin solution and the absorbance was measured ($A_0$). The calculation of the ABTS radical scavenging rate was done by using the following equation.

$$\textit{Scavenging activity}\ (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity of the purified melanin was determined by the previously described procedure (Chen *et al.*, 2008; Hou *et al.*, 2019). First, 2 ml of different concentrations of the melanin solution and 2 ml of 0.2 mM DPPH absolute ethanol solution were taken and put into to a test tube. The samples were kept in the dark for 30 min, and the absorbance was measured at 517 nm ($A_1$). Then, the DPPH solution was replaced with an equal volume of absolute ethanol, and the absorbance was measured ($A_2$). The same amount of distilled water was used instead of the melanin solution, and the absorbance was measured ($A_0$). The ability to scavenge the DPPH radical was calculated by using the same equation described above.

## Results and Discussion

### Selection of melanin-producing fungus in culture filtrate

A total of 102 different fungal strains was examined to identify the strain with a potential for melanin production outside the cells. Most of the isolates were in the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Ophiostoma*, and their close relatives, which are generally known as melanin-producing fungi (Bell and Wheeler, 1986; Kimura and Tsuge, 1993; Pal *et al.*, 2013) (Supplementary data Table S1). However, only a single fungal strain, KUC3009 isolated from wood treated with chromated copper arsenate preservatives showed potential based on its ability to produce a black-brown culture filtrate. The species was identified as *Amorphotheca resinae* based on the phylogenetic analysis using ITS sequences (Seifert *et al.*, 2007; Bensch *et al.*, 2012) (Supplementary data Fig. S1). This group of fungi is present in various petroleum products and has also received considerable attention because of its putative involvement in the corrosion of aluminum alloys (Hendey, 1964; Parbery, 1969).

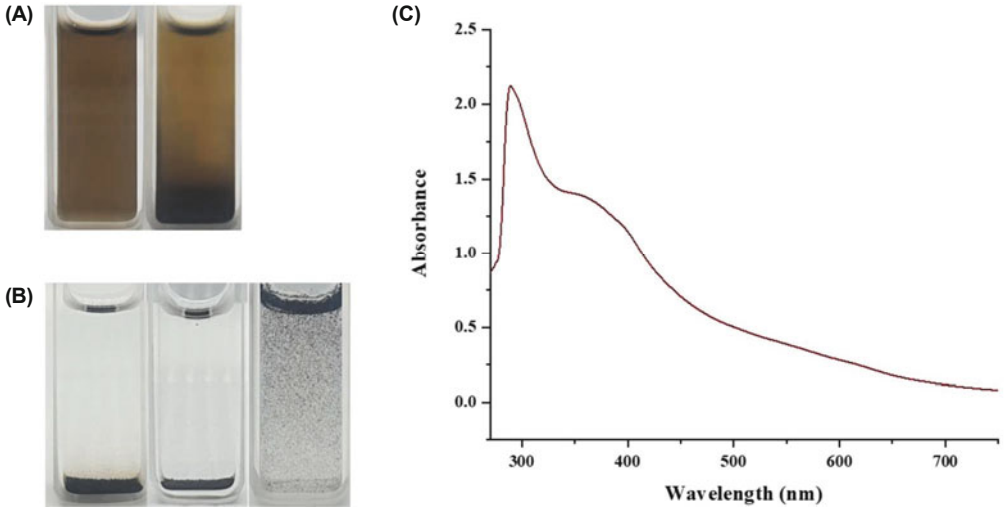


**Fig. 1. Experiments designed to confirm the black pigments as melanins (A) culture filtrate (left) precipitation of the black pigments after pH adjustment (right) (B) the pigments in ethyl alcohol (left), acetone (middle), and chloroform (right) (C) UV-Vis spectrum of the pigments in the range between 270–750 nm.**

However, few studies have evaluated melanin production in the culture filtrate by *A. resinae* up to date (Gadd and de Rome, 1988).

Melanin pigments displayed common features such as precipitation in acidic solutions, insolubility in various solvents and increasing absorbance towards the higher energies (Tran *et al*., 2006; Riesz, 2007; d'Ischia *et al*., 2013). Accordingly, we conducted the pH adjustment of culture filtrate, solubility tests, and UV-Vis measurements to confirm the pigment in the culture filtrate as melanins. As shown in Fig. 1A, the pigment was precipitated as the pH of culture filtrate of *A. resinae* was adjusted to 2.0. Then, the lyophilized precipitates showed excellent insolubility in water and various organic solvents such as absolute ethanol, acetone, and chloroform as depicted in Fig. 1B. In addition, the dispersed pigments in 0.1 M NaOH showed broadband absorbance which increased monotonically towards the higher energies as shown in Fig. 1C, probably due to structural heterogeneity of melanins (Tran *et al*., 2006). Consequently, we determined the black-brown pigment in the culture filtrate as melanins and selected the *A. resinae* as the promising isolate for scalable melanin production.

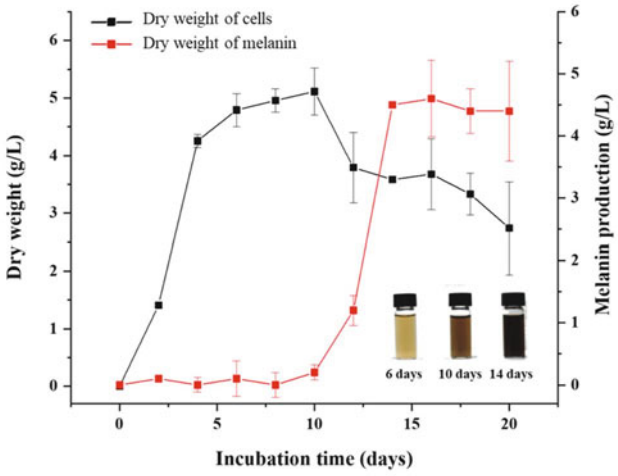


**Fig. 2. Cell growth and melanins production by *A. resinae* KUC3009.** Dry cell weight and the melanin content in the culture filtrate were measured during 20 days of cultivation at 2-day intervals.

## Growth and melanin production by *A. resinae* KUC3009

Melanin production in the culture filtrate and fungal growth of *A. resinae* KUC3009 were examined during the 20 days of cultivation in PYG culture media as shown in Fig. 2. The fungal growth profile exhibited classical growth curve of filamentous fungi and the melanin production appeared to exhibit the similar characteristic kinetics as common secondary metabolite production (Vrabl *et al*., 2019). The fungal biomass sharply increased until 4 days and then entered a stationary phase. Then, the declining phase began after 10 days at which the fungal biomass decreased slowly. At the same time, the rapid synthesis of melanin outside the cells began and reached a maximum value of approximately 4.5 g/L in 14 days, causing considerable darkening of the culture filtrates.

Two mechanisms for melanin formation outside the cells have been proposed (Bell and Wheeler, 1986). The first is external secretion of phenol oxidases such as laccases and tyrosinases, which oxidize phenolic compounds of various origins. The second is secretion of phenolic compounds into the external environment where they are autoxidized under environmental conditions or oxidized by enzymes later released from the fungus during autolysis. The biosynthetic characteristics of *A. resinae* KUC3009 observed in this study may be attributable to the latter possibility because the melanin production in the culture filtrate increased during the declining phase. These characteristics of melanin production were analogous to those of two ascomycetes, *Aureobasidium pullulans* and *Aspergillus nidulans*, in a way that the melanin formation increased after termination of the exponential growth phase (Rowley and Pirt, 1972; Gadd, 1980). However, the exact mechanisms of melanin formation should be further investigated.

The yield and productivity of the fungal melanins in the culture filtrate was comparable to the previous ones. *Gliocephalotrichum simplex* MTCC 5489 can produce 4.6 g/L of

**Table 1.** Elemental composition of melanins

| Types of melanin | Elemental composition (%)[a] | | | | |
|---|---|---|---|---|---|
| | C | H | N | O | S |
| *Sepia* melanin | 32.6 (0.8) | 3.2 (0.4) | 7.1 (2.1) | 36.0 (0.3) | 0.2 (0.0) |
| Synthetic pheomelanin | 45.9 (0.7) | 3.7 (0.4) | 9.0 (0.4) | 31.1 (1.2) | 9.2 (0.9) |
| Synthetic pyomelanin | 52.0 (2.3) | 3.2 (0.1) | 0.5 (0.6) | 41.0 (1.4) | 0.1 (0.1) |
| 1,8-DHN | 75.1 (0.7) | 4.9 (0.0) | 0.5 (0.6) | 20.4 (1.3) | 0.2 (0.1) |
| *A. resinae* KUC3009 melanin | 52.7 (0.4) | 4.6 (0.1) | 6.9 (0.1) | 34.2 (0.9) | 0.8 (0.3) |

[a] Values represent the average of three replicates and ones in parentheses are the standard deviations.

fungal melanins in the culture filtrate through optimization process (Jalmi *et al.*, 2012). *Armillaria cepistipes* Empa 655 approximately can produce 27.98 g/L of fungal melanins in the culture filtrate within 161 days of cultivation (Ribera *et al.*, 2018). The melanin yield of *A. resinae* is similar to that of *G. simplex* and lower than that of the strain Empa 655. However, it should be noted that the current melanin productivity for *A. resinae* is approximately 0.32 g/L/day which is higher than 0.17 g/L/day of the strain Empa 655 since it takes shorter time for *A. resinae* to produce melanins. The incubation period of *G. simplex* in the optimized culture condition was not exactly indicated, so clear productivity comparison between *A. resinae* and *G. simplex* was impossible. Considering that the optimization process of culture condition of *A. resinae* has not yet been completed, the fungus can be a promising isolate for renewable source of melanin production into the liquid medium, paving the way for melanins to be produced in a scalable manner.

### Structural characterization of the purified melanin from *A. resinae* KUC3009

Despite the intensive studies, melanins, which are generally regarded as complex and amorphous polymer, are not fully understood. The four types of melanins (eumelanin, pheomelanin, allo-melanin, and pyomelanin) are known as the pigments responsible for fungal dark coloration (Bell and Wheeler, 1986; Schmaler-Ripcke *et al.*, 2009). These melanins have different chemical structures and elemental compositions, as each is derived from different precursors. Both eumelanin and pheomelanin are derived from a common precursor, dopaquinone, formed by oxidation of L-tyrosine, while the latter requires L-cysteine for their biosynthesis (d'Ischia *et al.*, 2013). Eumelanin typically includes repeat pyrroles and indole units with 6–9% nitrogen such as 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid (Pezzella *et al.*, 1997). Pheomelanin differs from eumelanin in that the oligomer structure includes benzothiazine and benzothiazole units. In contrast, allo-melanin and pyomelanin are formed by the polymerization of non-nitrogenous phenolic compounds, 1,8-DHN and homogenistic acid, respectively. Given the difference of the precursors among melanins, elemental analysis can provide useful information for identifying specific types of uncharacterized melanins.

Table 1 shows the elemental composition of the purified melanin from *A. resinae* KUC3009 compared to those of the standard melanins. The purified melanin obtained from *A. resinae* KUC3009 showed similar characteristics as melanin biosynthesized from *Sepia officinalis*, which is a representative example of a well-characterized eumelanin. A nitrogen content close to 7% was clearly detected in the purified melanin, whereas the sulfur contents were limited to only 1%. Additionally, the empirical formula of C:N:H of the purified melanin (8.9:1:9.3) was most similar to that of *Sepia* melanin (5.4:1:6.3) and other previously reported eumelanins (Magarelli *et al.*, 2010; d'Ischia *et al.*, 2013), except for the high proportions of carbon and hydrogen. These results suggest that the type of melanin obtained from *A. resinae* KUC3009 can be classified as eumelanin-like pigment; further detailed

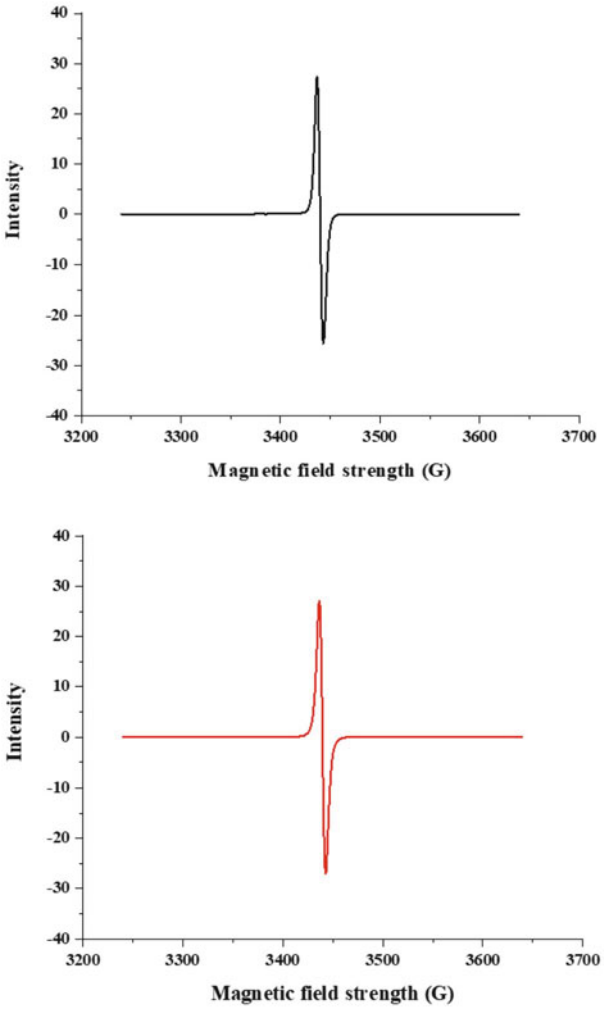


**Fig. 3.** Electron paramagnetic resonance of melanin produced by *A. resinae* KUC3009 (top) and *S. officinalis* (bottom)

structural characterization was conducted for comparison with *Sepia* melanin.

Eumelanin possesses a unique free radical signature attributable to indolequinone species present in the pigment, which can be detected by EPR (Sealy *et al*., 1982). Figure 3 shows the EPR spectra of melanin from *S. officinalis* and *A. resinae* KUC3009 measured at approximately 9.64 GHz. The X-band EPR spectra of both *Sepia* melanin and *A. resinae* KUC3009 melanin revealed only a single band with 5.5 and 6 G wide. Furthermore, the g-factor values of both *Sepia* melanin and *A. resinae* KUC3009 melanin were 2.00401 and 2.00386, which were close to 2.004. At the X-band (microwave frequency approximately 9 GHz) at which standard EPR spectrometers are operated, the EPR spectra of eumelanin showed a single and slightly asymmetric line 4–6 G wide with a g-factor close to 2.004 (Sealy *et al*., 1982; d'Ischia *et al*., 2013). These results suggest that fungal melanin obtained from *A. resinae* KUC3009 possesses an indolequinone species as observed in *Sepia* melanin.

$^{13}$C CP/MAS SSNMR was conducted to verify the organic functional groups related to indolequinones species present in the purified melanin of *A. resinae* KUC3009. Figure 4 shows the $^{13}$C NMR spectra for melanin obtained from *A. resinae* KUC3009 and *S. officinalis* as a comparison. The NMR spectra of *A. resinae* KUC3009 melanin showed similar resonances, displaying three characteristic spectra regions; these data were consistent with those previously reported for eumelanin (Duff *et al*., 1988). Three characteristic spectral regions were identified: 10–90 ppm, aliphatic carbons, most likely due to proteinaceous material; 90–160 ppm, aromatic carbons, including indole or pyrrole type carbons within the polymer; and 160–225 ppm, carbonyl carbon atoms from amides, carboxylates, and quinones, which may be associated with the melanin polymer and proteinaceous material (Adhyaru *et al*., 2003).

For the broad aromatic spectral envelope centered at about 128–130 ppm, such band is the result of overlaps of resonances from slightly dissimilar aromatic indole-based constituents (Prados-Rosales *et al*., 2015). However, the spectra also indicated a remarkable difference between the two melanin in intensity from the alkyl chains ($[CH_2]_n$, 30 ppm). The prominence of alkyl chains from NMR signals also agreed with the elemental analysis result indicating larger percentages of carbon and hydrogen (Table 1). Large proportions of alkyl chains found in *A. resinae* KUC3009 melanin may be derived from the strong bond with nutritional materials which contains a lot of carbon sources. For reference, this is analogous to the previous studies of melanin biosynthesis which raised the possibility of tight association between cellular materials and melanins in *Cryptococcus neoformans*

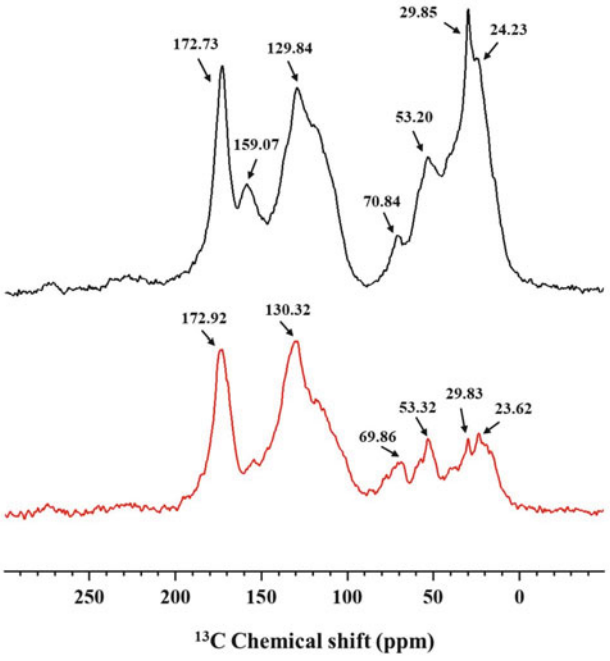


**Fig. 4. 400 MHz $^{13}$C NMR spectra of *A. resinae* melanin (top) and *Sepia* melanin (bottom), measured at 10 kHz MAS using the quantitatively reliable multiple cross-polarization magic-angle spinning technique.**

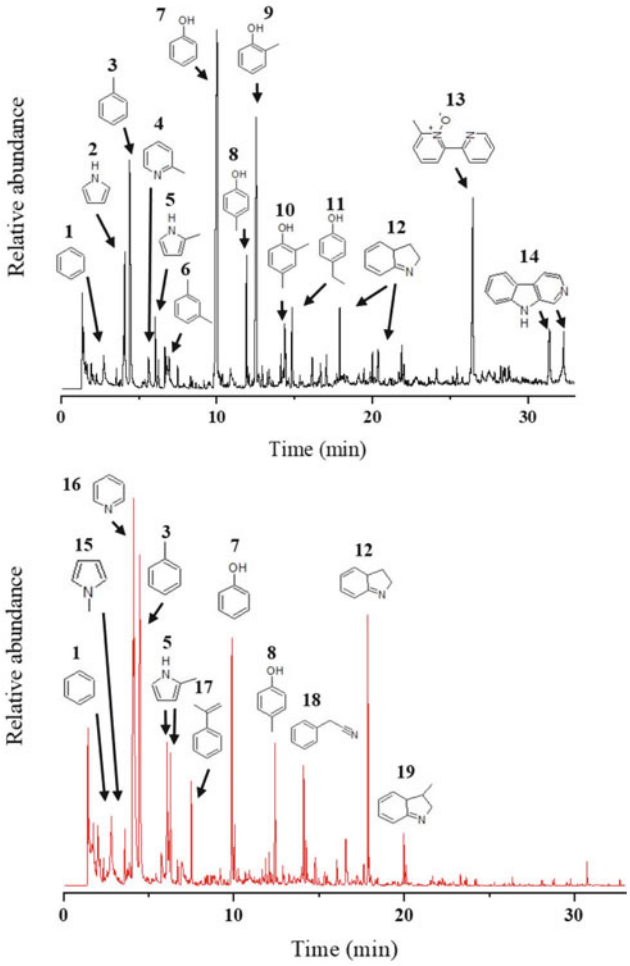


**Fig. 5. Ion chromatograms following pyrolysis at 600°C for 1 min of melanin from *A. resinae* KUC3009 (top) and *S. officinalis* (bottom).** The numbers indicate major pyrolysis products of melanin: 1, benzene; 2, pyrrole; 3, toluene; 4, 2-methylpyridine; 5, 2-methyl-pyrrole; 6, m-xylene; 7, phenol; 8, 4-methyl-phenol; 9, phenol; 10, 2,4-dimethyl-phenol; 11, 4-ethyl-phenol; 12, indole; 13, 6-methyl-2,2′-bipyridine 1-oxide; 14, 9H-pyrido[3,4-*b*]indole; 15, 1-methyl pyrrole; 16, pyridine; 17, styrene; 18, benzenepropanenitrile; 19, 3-methyl-indole.

(Chatterjee *et al*., 2012).

The chemical fingerprint of the degradation products obtained by Py-GC-MS can verify the former results of structural characterization, particularly for an amorphous polymer such as melanin (Dworzański, 1983; Glass *et al*., 2012). The profiles of compounds formed during thermal degradation of melanin from *A. resinae* KUC3009 and *S. officinalis* melanin are shown in Fig. 5. The distribution of the pyrolysis products from *A. resinae* KUC3009 melanin showed common characteristics with the standard eumelanin. Various aromatic compounds such as phenol, styrene, pyridines, and their alkylated homologs were detected, mainly accounting for the degradation products of melanin. Furthermore, the presence of indoles and pyrroles or their alkylated homologs from *A. resinae* KUC3009 melanin was confirmed. These degradation products are typically observed in various eumelanin sources including *Sepia* melanin, supporting the results of EPR and NMR analysis (Dworzański, 1983; Stępień *et al*., 2009; Glass *et al*., 2012).

### Antioxidant activity of the purified melanin from *A. resinae* KUC3009

It is widely reported that reactive oxygen species and excessive free radicals cause oxidative chain reactions that damage biomolecules such as proteins and DNA, which cause a wide variety of diseases and aging (Amens, 1983). Consequently, discovering new antioxidants and their applications are becoming more significant. As a number of fungal secondary metabolites are known to play a role in scavenging radicals, we evaluated the antioxidant activity of the purified melanin to expand the availability of the pigment for food and biomedical applications.

The ABTS and DPPH radical cations are the commonly used organic radicals to determine the antioxidant activity of single compounds and other complex mixtures. Accordingly, we used ABTS and DPPH assays to evaluate the antioxidant activity of the melanin. Figure 6 shows the results of ABTS and DPPH scavenging activities of the *A.resinae* KUC3009 melanin with the four levels of concentration. As shown in Fig. 6, the radical scavenging rates showed high dependency on the concentration of melanin derived from *A. resinae* KUC3009. Then, the $IC_{50}$ values of antioxidant activities for ABTS and DPPH were determined as 1.47 and 0.44 mg/ml, respectively. These values were higher than those of melanins from edible and medicinal mushroom such as *Auricularia auricula* (Wu *et al*., 2018; Liu *et al*., 2019). The above results indicate that the purified melanin has the potent ability to scavenge ABTS and DPPH cation radicals. Even though the scavenging assay gave important information about antioxidant activity, both *in vitro* and *in vivo* studies should be conducted in the future.

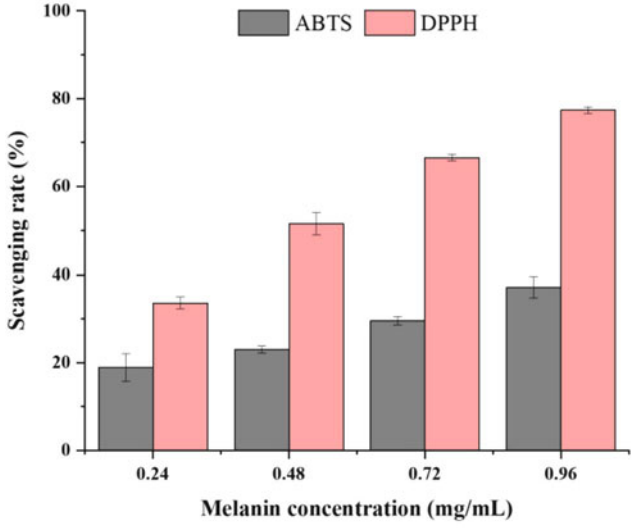


**Fig. 6. Antioxidant activities of the purified melanin from *A. resinae* KUC-3009.**

## Conclusion

In summary, we selected the fungus *A. resinae* KUC3009 which produce the melanin pigment in the culture filtrate. This strain produced the melanin pigment with a maximum yield of 4.5 g/L within 14 days. Physico-chemical analyses indicated that the purified melanin can be classified as a eumelanin-like pigment with multiple alkyl chains. Unlike other types of melanins, the chemical properties of eumelanin like pigments of hair and *Sepia* ink have been widely examined, suggesting that eumelanin can be applied as a bio-based material (Brenner *et al*., 2008; Kim *et al*., 2013; Wang *et al*., 2016). Furthermore, as the purified melanin possess the antioxidant activities, making the availability of food and medical application. Therefore, *A. resinae* KUC3009 can be a promising candidate for melanin production, and the purified melanin sufficiently possess the potential as a bio-based material.

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