Extracellular Pyomelanin Pigment Production, Purification and Characterization with *Streptomyces griseus* MPPS2

Sinan BAYRAM (✉ sbayram@bayburt.edu.tr)

Bayburt Üniversitesi <https://orcid.org/0000-0002-2156-1566>

Research Article

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Abstract

Melanins are natural pigments widely distributed in nature from bacteria to humans. These complex, negatively charged, amorphous, high molecular mass natural biopolymers have many different bioactive properties such as antimicrobial, antiviral, antioxidant, liver protective effects etc. In this study, some chemical and physical properties of the extracellular pyomelanin pigment purified from *Streptomyces griseus* MPPS2 was investigated via XRD (X-Ray diffraction), FT-IR (Fourier transform infra-red) and ¹H NMR (Nuclear magnetic resonance). Additionally, the melanin pigment-producing *Streptomyces griseus* MPPS2 strain was identified at species level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and 16S rDNA sequence analysis. 16S rDNA sequence analysis result was deposited in NCBI under accession number MT825616.

Introduction

Melanins are ubiquitous natural pigments which are formed with polymerized phenolic and/or indolic compounds. These complex dark colored biopolymers are hydrophobic, negatively charged and high molecular weight. These amorphous bio-macromolecules polymerize in acidic solutions, but are insoluble in water and organic solvents. They are known to have a maximum absorbance capacity at 200-400 nm wavelengths and also, these natural pigments are characterized by its sensitivity to oxidizing agents such as H₂O₂ and its loss of color (Tarangini and Mishra, 2014; Banerjee et al. 2014).

Under adverse conditions, many microorganisms synthesize melanin pigment and this pigment protects microorganisms against external threats such as desiccation, UV radiation, temperature fluctuations, heavy metals, hydrolytic enzymes and digestion. In this way, microorganisms increase their chances of survival (Venil et al. 2013; Ye et al. 2014).

Melanin pigment has many different bioactive properties such as antioxidant, antiviral, antimicrobial, antivenin, anti-inflammatory, anti-proliferative effects. In addition to these properties, liver protective, drug carrier and UV absorbing properties of melanin pigment were also determined in different studies (El-Naggar and El-Ewasy, 2017; Zerrad et al. 2014; Teplyakova and Kosogova 2015; Sava et al. 2003; Araújo et al. 2014).

In a study performed by Dadachova and Cassadevall (2008), it was stated that the melanized fungi species found around the Chernobyl reactor responded to ionizing radiation with increased growth (Dadachova and Cassadevall 2008). These data support the radioprotective effect of the melanin pigment and show that the melanin pigment has the potential to be used as a protective agent for patients with cancer undergoing radiation therapy. After this study, in a study conducted by Schweitzer et al. (2010), melanin coated nanoparticles were used to protect the bone marrow against radiotoxicity during radioimmunotherapy. The obtained results in this study showed that melanin coated nanoparticles reduced hematological toxicity in mice treated with radioimmunotherapy. Furthermore these nanoparticles did not have a protective effect against tumor tissues (Schweitzer et al. 2010).

In another study, conducted by Ozlu et al. (2019) for breast cancer therapy, controlled release of doxorubicin from polyethylene glycol functionalized melanin nanoparticles was investigated. Results from in vitro cytotoxicity assays showed that melanin nanoparticles (MNP) and polyethylene glycol (PEG) conjugated melanin nanoparticles (PEG-MNPs) did not show any toxic effects on mouse fibroblast cells. In contrast to these results, it has been observed that doxorubicin loaded PEG-MNPs (DOX-PEG-MNP) can inhibit the proliferation of human breast cancer cells. These results support the potential for the use of melanin nanoparticles in prolonged and controlled drug release for different cancer therapeutics (Ozlu et al. 2019).

Melanin pigment has strong absorption at near-infrared region and higher photothermal conversion efficiency. Taking advantage of these properties of melanin pigment, Jiang et al. (2017) demonstrated that melanin nanoparticles has a great potential for antitumor photothermal therapy process because of its biocompatibility and biodegradability properties (Jiang et al. 2017).

In recent studies, it is seen that melanin pigment has an important potential in fields such as medical imaging, drug delivery, optoelectronics, cosmetics (Ozlu et al. 2019; Fan et al. 2014; Gogurla et al. 2020). These results provide an increasing interest in melanin pigment day by day. In this study, pyomelanin pigment production potentials of different *Streptomyces* strains were investigated and the purified extracellular pyomelanin pigment from *Streptomyces griseus* MPPS2 strain was characterized by using XRD, FT-IR and ¹H NMR and UV-Vis spectrometry. In addition to these characterization process, pyomelanin pigment producer *Streptomyces* strain identified at species level via MALDI TOF/TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometer) and 16S rDNA sequence analysis.

Materials And Methods

Determination of Pigment Producer Strains.

In order to determine pigment producer microorganisms, strains were seeded (about 1 cm²) to petri dishes containing ISP2 medium and incubated at 30 °C for 96-120 h. At the end of this incubation period, colonies that form dark colored extracellular pigment around it, were selected for pyomelanin pigment production (El-Naggar and El-Ewasy, 2017).

Identification of strains by MALDI TOF/TOF MS

Species identification of pyomelanin pigment producer *Streptomyces* strain was carried out with MALDI TOF/TOF MS (Bruker Daltonics, Autoflex Speed). For this purpose, mass signals from most abundant and conserved ribosomal protein fractions that are specific at genus, species or sub-group levels was detected. MALDI-TOF MS log (score) values range between 2.3-3.000 was interpreted as highly probable species-level identification and values range between 2.000-2.299 was interpreted as secure genus identification and probable species identification. For the extraction of ribosomal protein, formic acid method which was applied by Bizzini et al. (2010) was used and samples were prepared for MS analysis.

The samples taken from a single bacterial colony using a sterile wood applicator were transferred to the steel target plate (Ground Steel Target, Bruker Daltonics). Transferred microorganisms were overlaid with 1.0 μL of a saturated HCCA matrix (a solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile – 2.5% trifluoroacetic acid - CAS Number 28166-41-8). To allow co-crystallization, the sample was kept for drying at room temperature (Bizzini et al. 2010). Mass spectra were calibrated with the recommended BTS (Bacterial Test Standard) for MS (See Supplementary Figure 1).

DNA extraction and PCR amplification of 16S rDNA

Total genomic DNA extraction and PCR amplification process were performed with small modification based on the method previously reported by Dahal et al. (2010). Total Genomic DNA was extracted from pure cultures using the EcoSpin Bacterial Genomic DNA Kit, 50 rxn (EcoTech Biotechnology - Turkey), and stored at $-20\text{ }^{\circ}\text{C}$ until being used. During 16S rDNA gene amplification procedure, 27F (forward 5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R 1492R (reverse 5'- CGG TTA CCT TGT TAC GAC TT -3') universal primers were used. PCR reaction mixture were prepared with 2 μL extracted genomic DNA and 48 μL mastermix [10 μL 10 x PCR Buffer (100 mM Tris – HCl, 500 mM KCl, 15 mM MgCl_2 , %0,01 gelatine pH: 8,3), 2 μL dNTP mix (dATP, dGTP, dCTP, dTTP – 10 mM), 1 μL 50 μM primer 27F, 1 μL 50 μM primer 1492R, 4 μL DMSO, 6 μL (25 mM) MgCl_2 , 1 μL Taq DNA polymerase and 23 μL sterile distilled water] (Dahal et al. 2010).

Bioproduction and Purification of pyomelanin pigment

Extracellular pyomelanin pigment production and purification process was performed based on previously described by Bayram et al. (2020).

Dissolution of pyomelanin pigment and UV–VIS Spectroscopy

Purified pyomelanin pigment was dissolved in an ultrasonic water bath (Kudos) at 35 kHz with DMSO (dimethylsulfoxide). For this purpose, pyomelanin pigment was kept in ultrasonic water bath at $60\text{ }^{\circ}\text{C}$ for 30 min. Absorbance values of purified pyomelanin was recorded in the wavelength range from 200–700 nm using a UV-Vis spectrophotometer (Thermo Multiskan Go). The blank control was conducted with DMSO (Bayram et al. 2020).

X Ray diffraction analysis of purified pyomelanin

Initially, the pyomelanin sample was carefully ground in a mortar and analysis was performed by an X-ray diffractometer (Bruker - D8 Discover XRD) with Cu K α radiation ($\lambda=1.5406\text{ \AA}$) for 2θ angles ranging between 20° and 90° (Fig.1) (Mbonyirivuze et al. 2015).

FT-IR (Fourier transform-infrared) spectroscopy

Fourier transform infrared analysis was performed on a Perkin-Elmer FT-IR spectrometer and reported as wavenumbers $\nabla\text{ (cm}^{-1}\text{)}$ with band intensities indicated as s (strong), m (medium), w (weak), b (broad).

Pyomelanin particles were scanned at $4000\text{--}400\text{ cm}^{-1}$ and spectrum recorded. FT-IR spectra is shown in Fig. 2. (Vasanthabharathi et al. 2011).

^1H NMR spectroscopy

^1H NMR spectrum of the pyomelanin was obtained by Bruker NMR Spectrometer (AVANCE III - 400 MHz) at 25°C (9 Tesla) as described before by Bayram et al. (2020).

Results And Discussion

Recent studies show that melanin pigments have the potential to be used in photothermal chemotherapy applications in cancer patients and also in the controlled, prolonged drug release process (Capozzi et al. 2006; Jiang et al. 2017; Ozlu et al. 2019). These important amorphous macromolecules also have potential to be used for radio-immunotherapy to reduce hematological toxicity due to its radioprotective effect (Schweitzer et al. 2010). These properties make the melanins a much more attractive object for pharmaceutical and biomedical fields as well as scientific studies.

In this study, purified extracellular pyomelanin pigment from *Streptomyces griseus* MPPS2 strain was analyzed by X ray diffractometer (Fig. 1). Additionally, Fourier-transform infrared spectroscopy (Fig. 2), ^1H Nuclear magnetic resonance (Fig. 3) and UV-vis spectrometry (Fig. 4) spectrums interpreted by comparing it with previously published data. In addition to these analyzes, the pyomelanin pigment producer *Streptomyces* strain was characterized at the species level by 16S rDNA sequence analysis and Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry techniques. As a result of the analyzes, the pyomelanin-producing bacterium was identified as *Streptomyces griseus* at the species level and 16S rDNA sequence analysis result was deposited in NCBI under accession number MT825616.

Initially, dark colored pyomelanin pigment was scanned by using the Cu K α radiation ($\lambda=1.5406\text{ \AA}$). XRD spectrum of the pyomelanin is characterized by a broad peak centered about at 2θ . The observed 2θ values are well coinciding with the previous studies performed by different research groups (Capozzi et al. 2006; Mekala et al. 2019). Diffractograms obtained from XRD analysis is shown in fig. 1.

Secondly we used FT-IR spectroscopy to further identify the structure of the purified pyomelanin polymer. When the obtained FT-IR spectra in this study were compared with the previously performed studies, it was observed that IR spectra were highly consistent and compatible (Tarangini and Mishra, 2014; El-Naggar and El-Ewasy, 2017; Li et al. 2017). Bacterial pyomelanin pigment showed strong absorption at 3276.37 cm^{-1} and this stretching vibration band is attributed to O\H stretch. After that, a weak signal at 2918.75 cm^{-1} can be assigned as the C-H stretching band. The spectrum shows an absorption peaks at 1614.14 cm^{-1} was attributed to the NH bending. The broad infra-red absorption peak in the region between 3600 cm^{-1} and 2400 cm^{-1} attribute to the stretching vibrations of phenolic, carboxylic and aromatic amino functional groups present in the indole and pyrrole core structures. The band at around

1614.14 cm^{-1} in Fig. 2 is attributed to bending of secondary N-H group. Additionally, IR band near 1521 cm^{-1} indicates the presence of indole structure in the pyomelanin pigment. Another infra-red absorption peak about 1443 cm^{-1} seen in Figure 2 is also characteristic of melanin pigments ($\text{CH}_2\text{-CH}_3$ bending). Another characteristic IR absorption peak around 1210 cm^{-1} and this IR peak is generally attributed to the phenolic COH stretch in previously performed studies. In this study, obtained FT-IR spectra reflects the characteristic absorption peaks and band stretching vibrations of melanins. As a result of the interpretation of this data and comparison with the previous literature, we can state that the purified pigment is pyomelanin pigment. (El-Naggar and El-Ewasy, 2017; Bayram et al. 2020; Li et al. 2017).

In addition to these data, ^1H NMR analysis of the purified pyomelanin pigment was performed and spectrum of the purified pyomelanin pigment in DMSO- d_6 was measured using a 400 MHz NMR Spectrometer (Bruker AVANCE III) at 25 °C (9 T). The purified pyomelanin pigment is sparingly soluble in DMSO- d_6 . Since melanin pigment is a heterogeneous polymer, difficulties have been experienced in obtaining NMR spectra but it has been observed that NMR spectra are substantially similar to the previous literature (El-Naggar and El-Ewasy, 2017; Bayram et al. 2020). ^1H NMR spectrum of the purified pyomelanin polymer shows resonances between 6.0 and 8.0 ppm and these resonances are assigned to the indole/pyrrole repeating units (aromatic groups). The resonances observed in the 6.6 and 7.1 ppm regions confirm the presence of vinylic proton ($\text{C} = \text{C}$) in the pyomelanin pigment. In addition to these resonances, pyomelanin polymer synthesized by *Streptomyces griseus* MPPS2 shows other resonances between 0.83 and 4.23 ppm. In this region, resonance signals between 0.6-2.4 are assigned to residual protein. Finally, signals between 3.3 ppm and 4.4 are interpreted as residual solvent (DMSO and H_2O) (El-Naggar and El-Ewasy, 2017; Bayram et al. 2020).

The absorbance values obtained from UV-vis spectrophotometric measurement show that the purified pyomelanin pigment has high absorbance values in the UV region and reaches the maximum level, especially between 250-280 nm wavelengths. (Fig. 4). These obtained data overlap with previously published studies (Li et al. 2017).

In conclusion, when XRD diffractograms and FT-IR, ^1H NMR, UV-vis spectra are examined, it is observed that the pyomelanin pigment has a high similarity with the data published previously in the literature and obtained analysis results show that pyomelanin pigment highly similar to eumelanin pigment (El-Naggar and El-Ewasy, 2017; Bayram et al. 2020; Li et al. 2017).

Based on these results, it can be stated that the purified pyomelanin pigment has the potential to be used in cosmetic products due to its high level of UV absorption. In addition to these results, the chemical and physical properties of melanin pigments should be investigated more deeply and thus the structure of this enigmatic pigment should be further clarified.

Conclusion

The obtained analysis results show that the pyomelanin pigment purified from *Streptomyces griseus* MPPS2 strain has a highly similar structure to eumelanin pigment. It was observed that all obtained analysis results are compatible with the literature however, due to the heterogeneous polymer structure of pyomelanin pigments, it could not be obtained regular and clear spectra in repeated ^1H NMR analyzes. Also, in the research results published before, it is seen that the ^1H NMR spectra are not clear in the literature. In order to strengthen and support these results, it was planned to characterize melanin pigments using pyrolysis GC-MS in the future researchs.

As a result, based on these analysis results, it can be stated that purified pyomelanin pigment has the potential of use in different areas such as medicine, pharmacology and cosmetics. In addition to these properties, the amorphous semiconductor, X-ray and γ -ray absorbing properties of pyomelanin polymers should be studied in depth and their potential for use in nanocomposite and biocomposite material production should be investigated in detail.

Declarations

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Conflict of interest disclosure: The author declare no conflict of interest.

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Figures

Figure 1

X Ray diffraction spectra of the purified pyomelanin pigment.

Figure 2

FT-IR spectra of the purified pyomelanin pigment.

Figure 3

NMR spectra of the purified pyomelanin pigment.

Figure 4

UV-vis spectra of the purified pyomelanin pigment.

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