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RESEARCH ARTICLE

Isolation and characterisation of melanin pigment from mycelial cultures of *Xylaria polymorpha* (*Ascomycota*)

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Abstract. Melanin is a heterogenic polymer of phenolic or indolic nature, possessing a broad spectrum of biological activities including radio- and photoprotective, antioxidant, chemoprotective, antiviral, antimicrobial, cytotoxic and immunostimulating activity. Based on these characteristics, natural melanin holds significant potential for applications in the fields of biomedicine, nanotechnology and materials science. Along with that, the exploration of organisms producing natural melanin remains relevant and filamentous fungi with their exceptional metabolic versatility are promising sources of these pigments. Wood-inhabiting fungi in particular are known to produce specific types of melanin as secondary metabolites. This study aimed to quantify and characterise melanin in the mycelium of various strains of *Xylaria polymorpha*, a common representative of wood-inhabiting fungi. As a result, among the ten studied strains of *X. polymorpha*, the highest melanin synthesis productivity was observed in the strain IBK 2737, reaching 180.32 ± 4.16 mg/l, while the lowest was recorded in the strain IBK 2723 at 5.17 ± 0.36 mg/l. This investigation highlights that *X. polymorpha* strains from the IBK Culture Collection show promise as a valuable source of natural melanin.

Keywords: *Ascomycota*, biomass, melanin, pigment, productivity, surface liquid cultivation, *Xylaria*

Introduction

Melanins are dark-coloured pigments, mostly brown or black, of phenolic or indolic nature, discovered among various organisms of different taxa. Like other secondary metabolites, these pigments are not essential for growth and development, but they affect the competitive ability of species and their ability to survive in certain environments (Bell, Wheeler, 1986; Menter, 2016).

Classification of melanins is based on the chemical composition of the monomer subunit structure of the pigment and is complicated due to the high heterogeneity of these pigments (Fitzpatrick, 1967). Most types of extracted melanins are resistant to chemical degradation by acids and are insoluble in most substances, which complicates their analytical characterisation. Despite the inability to use classical biochemical and biophysical methods, significant progress in understanding the structure, biosynthesis,

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localisation, function and degradation of natural melanin has been made in recent years (Rizner, Wheeker, 2003; Nosanchuk et al., 2015; Pombeiro-Sponchiado et al., 2017; Pralea et al., 2019).

Fungal melanins have attracted growing interest because of their physical and chemical properties and a broad spectrum of biological activities. Among them is defence against environmental stresses such as ultraviolet light, oxidising agents and ionising radiation (Zhdanova, Vasilevskaya, 1988; Zhdanova et al., 2000; Rosa et al., 2010). It is currently known that fungi share several pathways for the synthesis of melanin. In representatives of the *Ascomycota*, the melanin pigment is usually synthesised via the pentaketide pathway. In this pathway, polyketide synthase converts acetyl-CoA or malonyl-CoA to 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) and, after a series of reduction and dehydration reactions, the intermediates scytalone, 1,3,8-trihydroxynaphthalene, vermelone and finally 1,8-dihydroxynaphthalene (DHN) are produced. Polymerisation of DHN with the participation of the laccase enzyme leads to the formation of melanin (Thompson et al., 2000; Butler et al., 2001).

Melanin in representatives of the *Basidiomycota* is formed from phenolic precursors such as glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol. For instance, the precursor of melanin in *Agaricus bisporus* (J.E. Lange) Imbach is a metabolite of the shikimic acid pathway, γ-glutamyl-4-hydroxybenzene, which is oxidised by peroxidase or phenolase into γ-glutamyl-3,4-benzoquinone with its subsequent polymerisation (Hegnauer et al., 1985; Bell, Wheeler, 1986). Only a few fungi, for example *Cryptococcus neoformans* (San Felice) Vuill., synthesise melanin using the L-3,4-dihydroxyphenylalanine (L-DOPA) pathway, which resembles mammalian melanin biosynthesis and is the most studied type of melanin among this group of pigments (Piattelli et al., 1965; Langfelder et al., 2003). Some fungi are also characterised by the synthesis of a specific type of melanin, pyomelanin, which is formed as a result of tyrosine or phenylalanine catabolism (Turick et al., 2010). In the meantime, laboratory studies have successfully synthesised melanin using dopamine, L-3,4-dihydroxyphenylalanine (DOPA) and DHN precursors (Cao et al., 2021). However, the commercially available natural and synthetic melanins are expensive, emphasising the need for alternative sources.

Over the past decade, much research attention has been directed towards melanin pigments in fungi due to the relative simplicity of the cultivation technology and the potential for economically viable industrial-scale production, with a specific focus on lignicolous fungi. Notably, *Xylaria polymorpha* (Pers.) Grev., a common representative of wood-inhabiting fungi, has been identified in previous research as capable of producing DHN-melanin (Tudor et al., 2013). Pigment in these fungi is produced not only in response to environmental stress but also during antagonistic interactions with other fungi while colonising wood. As a secondary metabolite, it functions as both physical and chemical barriers within the wood substrate. However, there was little information on melanin production by *X. polymorpha* in culture until now.

The objective of this work was to isolate, characterise and compare the melanin productivity of natural melanin from the *X. polymorpha* strains of different origins cultivated under liquid surface conditions. Typical physicochemical characterisation, UV and Raman spectra of pigment produced by mycelial cultures were determined.

Materials and Methods

Fungal strains and cultivation conditions. The strains of *X. polymorpha* were isolated into pure culture from the entostromatal tissue of fungi collected in different regions of Ukraine. All cultures are preserved in the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine (Bisko et al., 2023). The detailed data with strain numbers in the IBK Collection, date of isolation and their origins are cited in Table 1.

The standard glucose-yeast-peptone medium (GYP, containing (g/l): glucose, 25; peptone, 3; yeast extract, 3; $MgSO_4$, 0.25; KH_2PO_4, 1; K_2HPO_4, 1) was used for the surface liquid fungal cultivation. Media were sterilised by autoclaving (20 min at 121 °C) and pH was adjusted to 6. For the experiments, mycelium was first grown in Petri dishes for 7 days at 26 ± 1 °C on a GYPA (GYP containing 21 g of agar-agar). The obtained mycelium was homogenised and sterilely inoculated in 500 ml Erlenmeyer flasks, containing 200 ml of GYP medium (10% v/v). Incubation was carried out for 30 days under surface cultivation conditions at 26 ± 1 °C, in darkness. The obtained mycelial biomass was harvested by filtration and dried at 60 °C until constant weight.

Table 1. **List of the studied strains of *Xylaria polymorpha* from the IBK Culture Collection**

Strain number	Collection site, date of isolation
2719	Ukraine, Vinnytsia Region, 49°16'13.8"N 28°26'40.1"E; 2020
2720	Ukraine, Vinnytsia Region, 49°16'13.8"N 28°26'40.1"E; 2020
2721	Ukraine, Vinnytsia Region, 49°15'31.3"N 28°26'21.0"E; 2020
2723	Ukraine, Sumy Region, Romny District, Bratske; 2020
2727	Ukraine, Vinnytsia Region, 49°15'23.9"N 28°25'56.6"E; 2020
2729	Ukraine, Kharkiv, 50°02'38.4"N 36°15'50.4"E; 2020
2736	Ukraine, Mykolaiv Region, Voznesensk District, Trykraty Forest Reserve Tract; 2020
2737	Ukraine, Mykolaiv Region, Voznesensk District, Trykraty Forest Reserve Tract; 2020
2382	Ukraine, Ivano-Frankivsk Region, Gorgany Nature Reserve; 2014
2430	Ukraine, Donetsk Region, Dronivka; 2013

Fungal melanin extraction. The pigment was extracted from the powdered mycelial biomass by treatment with hot alkali (1M NaOH at 100 °C for 2 h). After centrifugation for debris removal, the dark brown filtrate was acidified to pH 2 with concentrated HCl. The resulting black precipitate was collected by centrifugation (10000 g for 15 min). Purification of melanin involved the elimination of the carbohydrates, proteins and lipids associated with the crude residue.

Characterisation of fungal pigment. The physical and chemical properties of isolated pigment were determined in comparison with literature data and L-DOPA synthetic melanin (Sigma–Aldrich, USA) used as a standard. Diagnostic testing included solubility in organic and inorganic solvents, precipitation in 1M HCl and 1% $FeCl_3$ and reactions with oxidising agents (50% H_2O_2 and 5% $KMnO_4$ solutions).

UV–visible spectrophotometry. Melanin solutions were prepared in 1M NaOH at a concentration of 0.1 mg/ml. The UV-visible absorption spectrum of the solutions was scanned in the wavelength range of 300–900 nm with a spectrophotometer (Jenway 6850, UK) by comparing a synthetic L-DOPA melanin standard. The 1M NaOH solution was used as a reference blank.

Raman spectroscopy. Measurements were performed using a Raman spectrometer based on a single monochromator MDR–23 (LOMO), equipped with a TE–cooled CCD detector (iDus 420, Andor). The spectra were recorded in the spectral range encompassing the range of vibrations of organic molecules, 400–3500 cm^{-1}, with a spectral resolution of 2 cm^{-1}. Raman spectra were excited with a solid-state laser emitting 457 nm light. The laser power was adjusted to 1 mW, to avoid any thermal damage of the sample during the measurement.

All experiments were independently performed in triplicate and analysed with Excel statistical

Table 2. **Biomass and melanin production of the strains of *Xylaria polymorpha* from the IBK Culture Collection grown under surface liquid cultivation conditions on the GYP medium at 26 ± 1 °C**

Strain number	Biomass (g/l)	Final pH of the culture liquid*	Melanin (mg/g)	Productivity** of melanin synthesis (mg/l)
2719	13.37 ± 0.07	6.52 ± 0.09	2.33 ± 0.29	18.12 ± 2.25
2720	12.39 ± 0.03	6.90 ± 0.04	2.33 ± 0.11	17.80 ± 0.84
2721	12.63 ± 0.17	6.86 ± 0.05	2.33 ± 0.22	17.99 ± 1.65
2723	14.78 ± 0.01	6.40 ± 0.04	0.67 ± 0.04	5.17 ± 0.36
2727	11.93 ± 0.08	6.73 ± 0.02	6.00 ± 0.57	44.67 ± 4.01
2729	10.31 ± 0.05	7.23 ± 0.07	14.00 ± 0.53	103.99 ± 3.83
2736	8.74 ± 0.06	8.09 ± 0.06	15.33 ± 0.43	113.06 ± 3.32
2737	12.22 ± 0.21	7.25 ± 0.13	25.33 ± 0.57	180.32 ± 4.16
2382	10.35 ± 0.10	6.86 ± 0.13	3.00 ± 0.09	21.58 ± 0.68
2430	8.56 ± 0.08	8.12 ± 0.09	14.67 ± 0.56	96.42 ± 3.45

* The initial pH value was 6.0. ** Productivity calculated as a sum of melanin from biomass contained in 1 l of culture liquid. All results are presented as the mean of triplicate ± standard deviation.

functions using the Microsoft Office XP software. Data were recorded as means ± SD (standard deviation).

Results and Discussion

Among all strains studied, the highest pigment synthesis productivity was recorded for the strain IBK 2737 and amounted to 180.32 ± 4.16 mg/l. The lowest synthesis productivity was observed for the strain IBK 2723 with 5.17 ± 0.36 mg/l, although this strain accumulated more biomass, specifically 14.78 ± 0.01 g/l against 12.22 ± 0.21 g/l accumulated by strain IBK 2737 (Table 2).

Nevertheless, there were no significant differences in the morphology and visible pigmentation of the colonies of these particular strains, as can be seen in Fig. 1. Therefore, it is important to conduct screening of the different strains to find the producers of higher quantities of metabolites, which characteristics may also differ. Our screening revealed the challenge of predicting pigment yield based on morphological traits. Similar to exceedingly irregular in shape stromata in the natural environment, just as its name implies, mycelial colonies of *X. polymorpha* also exhibited variability in cultural morphological characteristics, especially in density and pigmentation. Colonies of *X. polymorpha* in our study were initially white, from cottony and velvety to crustose, often concentrically zonate, becoming grey or black after a week of cultivation. Another common characteristic was the production of a large number of cylindrical and mainly unbranched stromata, which were formed both during pre-cultivation on agar medium and surface liquid cultivation (Fig. 1).

Together with biomass production, at the end of the incubation period, culture liquids were measured for pH values (Table 1). The results demonstrate the increase in pH values after the cultivation process. The largest increase was observed for strains IBK 2736 and IBK 2430 with final pH values of 8.09 ± 0.06 and 8.12 ± 0.09, respectively. The increase in pH values agrees with the data on the cultivation of *X. polymorpha* under other conditions. Specifically, Tudor with co-authors (2013) when cultivating *X. polymorpha* on wood blocks reported an increase in the pH from pH 4.3 up to 5.7 for beech and from pH 4.7 up to 5.6 for sugar maple.

Identification of the isolated pigments was carried out according to the traditional scheme including a comprehensive study through chemical tests on their solubility, qualitative reactions and spectral properties. The obtained pigments presented all of the physical and chemical properties common to melanins and the experimental data within this work were found to be comparable to those reported in the literature. The results of diagnostic tests for the pigment obtained from *X. polymorpha* biomass in comparison to synthetic L-DOPA melanin are presented in Table 3.

One of the criteria for assigning pigments to melanins is their inability to dissolve in organic solvents and water combined with solubility in alkaline solutions (except for some types of melanins). The extracted pigment was soluble in NaOH, while no solubility in organic solvents (methanol, ethanol, ethyl acetate, chloroform, acetone, DMSO) was observed. Our studies also showed that 0.1 mg/ml solutions of extracted pigment in 1M NaOH were oxidised and bleached in the presence of 50% H_2O_2. With the addition of $KMnO_4$, the colour of alkaline solutions changed from brown to green followed by discolouration and precipitate formation. The addition of 1% $FeCl_3$ produced a floccular brown precipitate.

Table 3. **Physical and chemical properties of the obtained fungal pigment and synthetic L-DOPA melanin**

Characteristic	Treatment	L-DOPA melanin (standard)	Extracted pigment
Colour observation		BB	BB
Solubility in inorganic solvents	Distilled water	–	–
	1M NaOH	+	+
	1M HCl	–	–
Solubility in organic solvents	Methanol	–	–
	Absolute ethanol	–	–
	Ethyl acetate	–	–
	Chloroform	–	–
	Acetone	–	–
	DMSO	–	–
Precipitation	1% $FeCl_3$	BP	BP
	1N HCl	BP	BP
	1N H_2SO_4	BP	BP
Oxidation	50% H_2O_2	O	O
	5% $KMnO_4$	O	O

Indications in the table are as follows: – insoluble, + soluble, BB — blackish brown; BP — brown precipitate appearance; O — decolourisation.

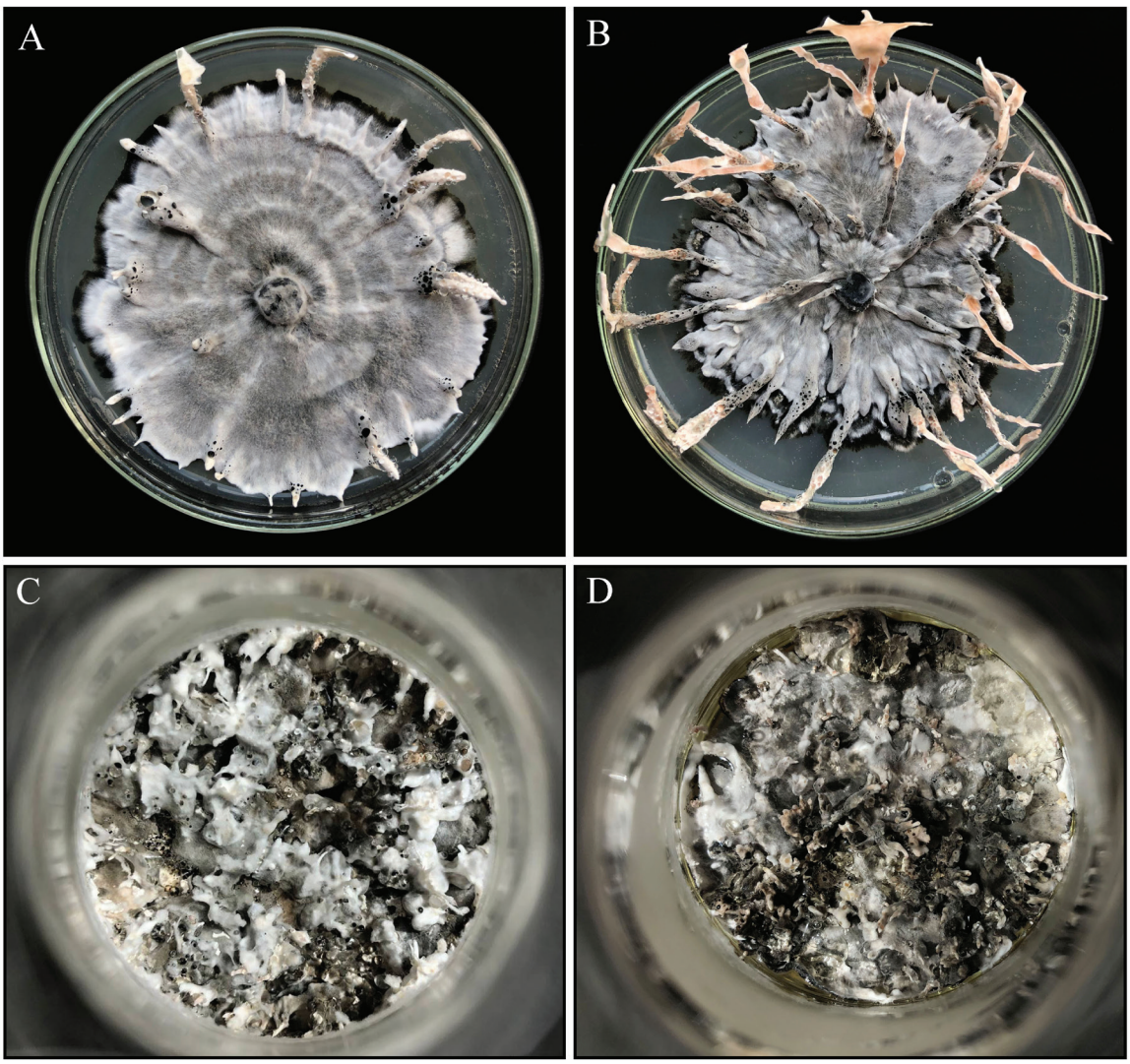

Fig. 1. Mycelial colonies of *Xylaria polymorpha*. Strains IBK 2723 (A) and IBK 2737 (B) on GYPA medium on the 20th day of cultivation. Biomass of strains IBK 2723 (C) and IBK 2737 (D) on the 30th day of liquid surface cultivation on GYP medium

UV-visible spectrophotometry of extracted pigment showed a broadband absorption of light from 300 to 900 nm, which is a typical absorption profile of most melanins including synthetic L-DOPA melanin tested here as a standard (Fig. 2). The maximum absorption was observed at 300 nm and gradually decreased with increasing wavelength. These results were similar to those of previous studies which found that the highest level of absorbance of the melanin produced by various fungi was in the UV region ranging between 200–300 nm and decreased toward the visible region (Ellis, Griffiths, 1974; Singla et al., 2021).

Raman spectroscopy showed the main peaks of extracted pigment, particularly ν_1 = 1240 cm^{-1}, ν_2 = 1310 cm^{-1} and ν_3 = 1620 cm^{-1} (Fig. 3), which are matching the set of values typically indicated characteristic melanin peaks in some fungi reported by others (De la Rosa et al., 2017; Lopusiewicz, 2018).

Lopusiewicz (2018) reported spectra of melanin from *Exidia nigricans* (With.) P. Roberts which dominated by two intense and broad peaks at about 1640 cm^{-1} and 1240 cm^{-1} (for raw melanin) and 1620 cm^{-1} and 1230 cm^{-1} (for purified melanin). The peaks can be interpreted as originating from the in-plane stretching of the aromatic rings and

the linear stretching of the C–C bonds within the rings, along with some contributions from the C–H vibrations in the methyl and methylene groups.

The spectra obtained here are also very similar to the Raman spectra for melanin from *Ochroconis lascauxensis* A. Nováková & P.M. Martin-Sanchez and *Ochroconis tshawytschae* (Doty & D.W. Slater) Kiril. & Al-Achmed, where strong bands at 1608 cm^{-1}, 1305 cm^{-1} and 1250 cm^{-1} were observed, which authors attributed to C=O, C–C stretching vibrations in aromatic compounds and to C–O stretching vibrations of hydroxyl groups, respectively (De la Rosa et al., 2017).

The actual assignment of the main melanin bands is not entirely agreed upon because, as it has been already mentioned, chemical characterisation of melanin is a complicated task given the suggestion that identical melanin structures do not exist in nature and their composition depends not only on the diverse monomeric units but also on the influence of environmental conditions during polymerisation. Nevertheless, Raman spectroscopy has proven to be a valuable tool providing insights into the principal functional groups within the melanin structure (Culka et al., 2017).

Conclusions

The productivity of melanin synthesis among the studied *X. polymorpha* strains grown under surface liquid cultivation conditions varied from 5.17 ± 0.36 to 180.32 ± 4.16 mg/l. The IBK strains 2729, 2736 and 2737 turned out to be the most productive among the studied ones. To confirm that the fungal pigment was melanin, UV and Raman spectra analyses were used. In the present study, the UV-visible absorbance spectrum of the fungal pigment produced from *X. polymorpha* showed a strong absorbance in the UV region (300 nm) and decreased toward the visible region. The Raman spectroscopic signature of fungal melanin has been detected at wave numbers ν_1 = 1240 cm^{-1} and ν_3 = 1620 cm^{-1}. Moreover, obtained pigments presented all of the physical and chemical properties common to melanins within solubility, precipitation and oxidising reaction diagnostic tests.

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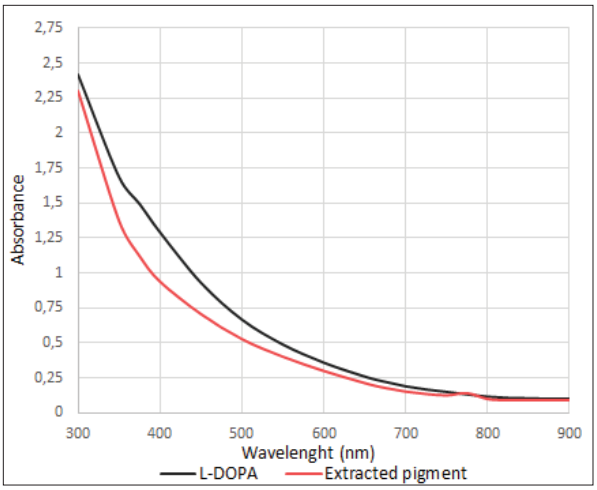

Fig. 2. UV-visible absorbance spectra of extracted melanin and synthetic L-DOPA melanin in 1M NaOH solutions (0.1 mg/ml)

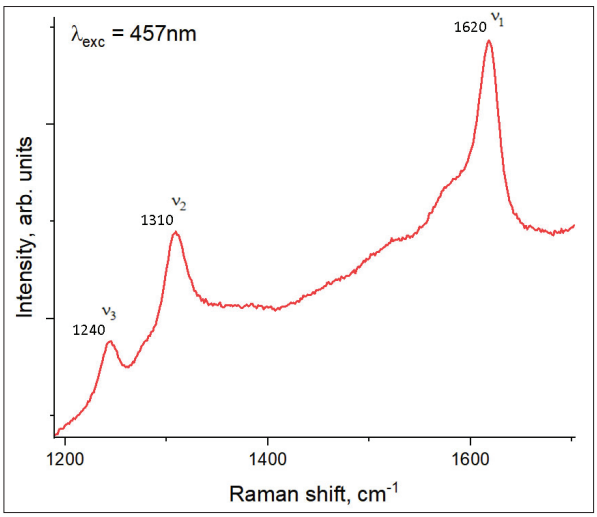

Fig. 3. Raman spectrum of the extracted melanin. A sample, recorded with λ_{exc} = 457 nm. The frequency of the main vibrational peaks is indicated

ETHICS DECLARATION

The authors declare no conflict of interest.

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Виділення та характеристика меланіну з міцеліальних культур *Xylaria polymorpha* (*Ascomycota*)

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Реферат. Меланіни — це високомолекулярні полімери фенольної або індольної природи з широким спектром біологічної активності, а саме радіо- та фотопротекторної, антиоксидантної, терморегуляторної, противірусної, антимікробної, цитотоксичної та імуностимулюючої. Зважаючи на ці та інші характеристики, меланін має значний потенціал для застосування у галузі біомедицини, нанотехнологій та матеріалознавства. Водночас, актуальним залишається пошук організмів, що продукують меланін, і гриби з їхніми винятковими метаболічними шляхами є перспективним джерелом цих пігментів. Зокрема, відомо, що ксилотрофні гриби продукують специфічні типи меланінів у процесі вторинного метаболізму. Метою цього дослідження було кількісне визначення та характеристика меланіну в міцелії різних штамів *Xylaria polymorpha*. В результаті встановлено, що серед десяти досліджених штамів *X. polymorpha* найвища продуктивність синтезу цього пігменту спостерігалась для штаму ІВК 2737 і становила 182,32 ± 4,16 мг/л, тоді як найнижча — для штаму ІВК 2723 зі значенням 5,17 ± 0,36 мг/л. Це дослідження демонструє, що штами *X. polymorpha* з Колекції культур ІВК є перспективними продуцентами меланіну.

Ключові слова: *Ascomycota*, *Xylaria*, біомаса, меланін, пігмент, поверхневе культивування, продуктивність