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Development of technology for obtaining water-soluble bacterial melanin and determination of some of pigment properties

A.E. AGHAJANYAN[1]*, A.A. VARDANYAN[1], A.C. HOVSEPYAN[1],
A.A. HAMBARDZUMYAN[1], V. FILIPENIA[2], A.C. SAGHYAN[1]

[1] SPC "Armbiotechnology" NAS RA, Yerevan, Armenia

[2] SSI "Central Botanical Garden of the NAS of Belarus", Minsk, Republic of Belarus

Abstract

The dependence was studied of the biosynthesis of melanin on the medium pH, the intensity of aeration and the method of regulation of the levels of these parameters during the fermentation process. Based on the obtained results, a technology was developed for the production of *Bacillus thuringiensis* melanin. Optimization of the pH of the growth medium and mass-transfer coefficient regulation resulted in a rise in the melanin yield up to 13 g/l and a 20-22% increase in the efficiency of the process. The highest yield in fermentation was observed at pH 8.8-9.0. The optimal technological parameters were determined for the sorption of melanin on the ionic sorbent and its elution from the sorbent. The yield of melanin from the culture liquid at the stage of isolation and purification after drying was 78.4%. Relative intensities were determined of the EPR signal of melanin dissolved in water, depending on the dilution factor and pH, as well as the electrical conductivity of melanin.

Key words: melanin, fermentation, aeration, process optimization, sorption

Introduction

Melanins, high-molecular-weight pigments synthesized by plants, fungi, bacteria and animals, are irregular polymers of phenolic and/or indolic nature (Prota, 1992; Lyakh, 1981). Melanins are among the most widespread natural pigments which can be found in practically all living organisms. In a living cell, melanins have protective functions against UV radiation, and high and low temperatures. Melanins are used in medicine, pharmacology, cosmetics, agriculture and other areas (Borshchevskaya et al., 1999).

As inhibitors of free-radical reactions, melanins have been shown to be able to suppress the growth of malignant tumors, exert protective effects against lethal doses of ionizing radiation, inhibit the processes of lipid peroxidation and stimulate plant growth (Nosanchuk et al., 2003).

Melanins can be obtained by chemical (Pawelek et al., 1993) and microbiological syntheses (Hovsepyan et al., 2003; Robysheva et al., 2002), as well as by alkaline extraction from animal and plant tissues (Prota, 1992; Lyakh, 1981; Xin Guo et al., 2014). Due to the high cost of initial materials and the complexity of technological processes, it is difficult to perform the chemical synthesis of melanin pigments on an industrial scale. The difficulties with the isolation and purification of melanins from biological materials relate to their amorphous nature and structural features.

The main problem facing a microbiological method for obtaining melanin is that bacterial strains synthesize mainly intracellular melanin, which further complicates the processes of its isolation and purification, and therefore limits its usage. To date, there are limited data on the process of the microbial synthesis of melanins that can be found in the literature (Sansinenea et al., 2015; Sajjan et al., 2010; Gilayne et al., 2005).

The literature data on the physical and chemical features of melanin pigments mainly relate to water-inso-

* Corresponding author: SPC "Armbiotechnology" NAS RA, 14 Gyurjyan Str., Yerevan 0056, Armenia; e-mail: aghajanyanarmen@yahoo.com

luble dioxyphenylalanine melanin. Published data on the physicochemical features of water-soluble melanin are also limited (Aghajanyan et al., 2005; Aghajanyan et al., 2011).

The goal of this work was the elaboration of a technology of the production of water-soluble microbial melanin, and the determination of some physicochemical properties of the obtained pigment.

Materials and methods

The melanin-synthesizing strain *Bacillus thuringiensis subsp. galleriae* K1 was obtained from the insecticide strain *B. thuringiensis* 69-6 as a result of a chemically induced mutagenesis and multistage selection (Miller, 1972). 1-Methyl-3-nitro-1-nitrosoguanidine was used as a mutagen. The strain was stored in the Microbial Depository Center of the Institute of SPC "Armbiotechnology" of NAS RA under the MDC 11212 number. The distinguishing feature of mutants is their ability to stain the growth media (liquid and agar) to a dark-brown color, which indicates that the produced pigment is soluble (Hovsepyan et al., 2003).

The melanin-producing strain was grown on an inexpensive nutrient medium (hydrolysate of fish-paste – 1.8%, peptone – 1%, $CuSO_4$ – 0.005%, $MnCl_2$ and $ZnSO_4$ – per 0.0005%, chalk – 3% from Component Reagent Ltd., Russia).

Fermentation was performed on a nutrient medium developed in our laboratory in a "Biostat-S" fermenter with 7.0 l working capacity (Brawn, Germany). The laboratory fermenter was equipped with devices for automatic measurement and recording of technological parameters – temperature, medium pH, the content of the dissolved oxygen in the fermenter, pCO_2 and pO_2 in waste gases, and the consumption of air, as well as with devices for automatic regulation of the cultivation processes of microorganisms.

Fermentation was performed at the oxygen dissolution rate of 2.5-3.0 g $O_2 \cdot l^{-1} \cdot h^{-1}$ (1.2 volume of air to 1 volume of culture medium per minute), temperature 30-32°C, pH 7.2-8.8, overpressure of 0.03-0.04 MPa in the fermenter, and stirrer rate 700-750 rpm.

The concentration of the dissolved oxygen in the culture liquid (CL) was determined using an oxygen sensor in the fluid stream flowing out of the fermenter.

The volumetric mass transfer coefficient (K_{La}, in h^{-1}) describes the efficiency with which oxygen can be delivered to a bioreactor for a given set of operating conditions. The optimal K_{La} value of the laboratory fermenter was determined by the method of static nitrogen degassing under different modes of aeration and stirring (Kraemer et al., 2008; Vant Riet, 1979).

Monitoring of the fermentation process was performed by measuring the optical density (OD) of the solution at 315 nm in a Perkin Elmer 550S "US-VIS" (USA). The solution of the synthetic melanin (Sigma) was used as a standard.

The sorption of melanin from the CL supernatant in dynamic conditions was performed at a 0.04 cm/s linear flow rate on IA-1r anionite (Russia). Melanin elution from resins was performed using 3.5% ammonia at the eluent flow rate 0.026 cm/s.

Electron Paramagnetic Resonance (EPR) spectra were registered at 20°C with a Radiopan SE/X-2543 spectrometer (Poland) with 100 KHz frequency modulation at 9.4 GHz high frequency.

The electrical conductivity of the obtained melanin samples was determined with a device designed and assembled by us to measure substance resistivity. The device was manufactured from an organic glass (as it is not a conductor) and consisted of 3 main parts: 1) springs between which the test substance was placed, 2) clamp terminals and interconnecting wirings, and 3) a measuring voltammeter (up to 2000 MOhms). Since melanin is a hygroscopic substance, to obtain accurate results, the tablets were smeared with a colorless lacquer (Unica Super 20, Finland) and the top and the bottom were covered with foil; this eliminated the absorption of water vapors from the air.

For all experiments, the averages of 3-5 measurements are presented. Calculations were performed using Microsoft Excel. Results with $P < 0.05$ were significant.

Results and discussion

Optimization of the aeration mode in melanin fermentation

The demand for oxygen may vary during the fermentation process depending on the cultivation phase (Pirt, 1978; Ikeda, 2003). It is important to determine the optimal levels of oxygen in the culture needed for the submerged fermentation of melanin producing *B. thuringiensis* subsp. galleriae K1. In our study, the effect of the dissolution rate of oxygen and the mass-transfer coefficient on the synthesis of melanin was also examined.

The studies were conducted at a temperature range between 30-32 °C, pH = 7.2-8.8 and a dissolution rate of oxygen in the range of 1.0-4.0 g $O_2 \cdot l^{-1} \cdot h^{-1}$. The results of the experiments conducted are presented in Figure 1.

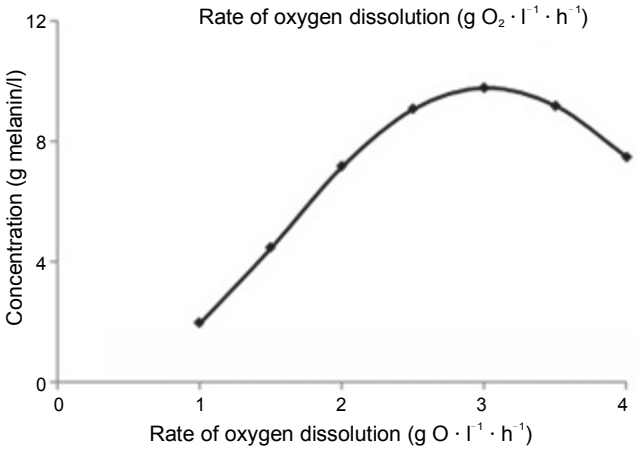

Fig. 1. The dependence of the concentration of synthesized melanin on the dissolution rate of oxygen

Our study showed that the dissolution rate of oxygen had a significant impact on the melanin biosynthesis levels. The maximum yield of melanin was observed under the oxygen dissolution rate of 2.5-3.0 g $O_2 \cdot l^{-1} m \cdot h^{-1}$ (11.5 g/l). Deviations from these values toward either a reduction or increase adversely affected the biosynthesis of melanin. Thus, the concentration of melanin in CL was reduced to 50% at the oxygen dissolution rate of 1.2-1.8 g $O_2 \cdot l^{-1} \cdot h^{-1}$. The obtained results were in correlation with the literature data (Hovsepyan et al., 2003) stating the need for relatively high oxygen dissolution rates throughout the process of melanin fermentation. However, when studying the impact of aeration on melanin biosynthesis, the need of bacterial cultures for oxygen at different stages of growth were disregarded and currently such data are not well studied in the literature.

Therefore, we examined the rate of oxygen consumption at different stages of culture growth to estimate the K_{La} values required to meet the needs of the bacterial strain producing melanin in oxygen at different stages of growth. The results are shown in Table 1.

As follows from the obtained results, the greatest consumption of oxygen was observed in the exponential growth phase (9-18 h). At the stationary growth phase (18-35 h), this consumption decreased and toward the end of the process, at the melanin biosynthesis phase (35-64 h), the oxygen consumption reached 6.8 g $O_2 \cdot l^{-1} \cdot h^{-1}$.

Table 1. Characteristics of the oxygen needs of a producer at the different phases of its growth

Parameters	Cultivation duration, hours			
	0-9	9-18	18-35	35-64
Q (g $O_2 \cdot l^{-1} \cdot h^{-1}$)	3.8	20.4	14.2	6.8
K_{La} (h^{-1})	38	200	150	70

Q – consumption of oxygen by culture

Optimization of the volumetric mass transfer coefficient

In developing stepwise K_{La} regulation, the yield of produced melanin was the controlling factor. Fermentation at which K_{La} was maintained at 150 h^{-1} throughout the whole process served as a control.

In the first series of experiments conducted according to the control K_{La} values, an acceleration of the growth of the concentration of melanin was observed; however, starting from the 35th hour of melanin biosynthesis, the rate of melanin accumulation began to slow down and this was accompanied by an increase in the duration of fermentation up to 73-74 h. Therefore, in the second series of experiments, K_{La} was maintained at an optimal level until the 35th hour and then K_{La} values were increased to 160 h^{-1}. The yield of melanin in CL was 11.5 g/l, and the duration of fermentation with the experimental K_{La} values was reduced by 5-7 h compared to the process with the control one. The results of experiments are given in Table 2.

A comparison of the parameters presented in Table 2 proves that fermentation performed according to the experimentally verified stepwise changes of the mass-transfer coefficient was more efficient than fermentation performed in control conditions.

The effect of the pH of the fermentation medium on the biosynthesis of melanin

The pH of the growth medium is one of the main factors influencing the growth of microorganisms and their physiological activity; therefore, it affects the outcome of the process of melanin biosynthesis. In this regard, it was necessary to study the effect of the pH of the medium on the melanin synthesis. The experiments were conducted in a nutrient medium that contained calcium carbonate, allowing the maintenance of the pH of the medium for a fermentation process at the same level.

As shown in Figure 2, in the control experiment in the initial hours of the culture growth the pH of the medium was 6.0-6.2, and it increased to 8.8-9.0 at the

Table 2. The main characteristics of the process of melanin fermentation at different values of the technological mode

Technological mode at different K_{La} values	The main parameters of the process				
	Specific growth rate [h^{-1}]	Specific activity of melanin formation [g/(g·h)]	Efficiency of the process [g/(l·h)]	Duration of fermentation [h]	Content of melanin in CL [g/l]
Control	0.151	0.015	0.111	72	8.0
Developed values	0.158	0.023	0.176	65	11.5
Combining of methods for regulating pH and K_{La}	–	0.045	0.216	60	13.0

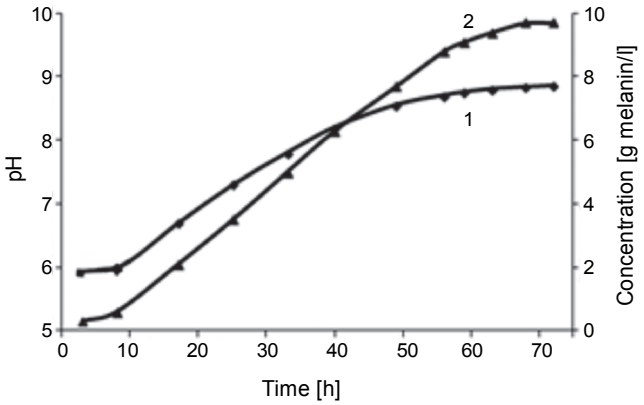

Fig. 2. The dynamics of changes in the pH (1) and accumulation of melanin (2) in the culture medium with calcium carbonate in the fermentation process

end of fermentation process. In the next series of experiments, calcium carbonate was excluded from the nutrient medium and the initial pH values of medium varied throughout the process. Since the process of melanin biosynthesis is connected with continuous alkalization of the medium, 6 M solution of hydrochloric acid was used as a titrant. To enhance the effectiveness of the fermentation, the process was divided into 3-time stages: the stage of culture growth (0-16 h); the stage of slow growth (16-22 h); and the stationary phase (22-64 h). The range of pH variation at the indicated stages was 6.0-9.0. The criteria for the selection of an optimal pH value for the phases were as follows: time of accumulation of the culture maximum titer; yield of melanin; and duration of fermentation.

At the stage of culture growth, the optimal value of the pH of the medium obtained in our experiments was 7.2-7.8. The maximum amount of biomass (titer 3-5×10^{10} cells/ml) was accumulated after 14-16 h of incubation. Deviations from the indicated pH value in both directions resulted in worsening parameters of melanin fermentation.

Our studies have also shown that the pH value in the stationary phase of cultivation (22-64 h) has a great influence on the synthesis of melanin. At this stage, pH 7.8-8.8 is optimal for melanin accumulation. Again, the deviations from the specified optimal values lead to a decrease in the yield of synthesized melanin.

Importantly, the optimal levels of the pH of the medium and the necessity of adjusting them with hydrochloric acid at various stages of culture growth were determined for the melanin fermentation process.

During melanin fermentation, when combining the pH adjustments and changes in the values of mass-transfer coefficients (Table 2), the yield of target product increased by 62.5% as compared to the control fermentation, where the constant value of K_{La} (150 h^{-1}) was maintained and pH was not regulated. Simultaneously, the duration of fermentation was reduced to 12 h.

A study of the dependence of the rate of melanin biosynthesis by *Bacillus thuringiensis* subsp. galleriae K1 on parameters such as pH and intensity of aeration, as well as the way to regulate the values of these parameters in the fermentation process, resulted in improvements in the microbiological method for obtaining melanin.

Melanin isolation and purification

To isolate melanin from CL, the sample was centrifuged at 3000 g for 20 min. To remove microsuspensions and colloidal particles, the obtained supernatant was subjected to microfiltration. To study the precipitation methods, the permeate was subjected to vacuum evaporation until melanin concentration in the evaporated solution reached 40 g/l. Studies have shown that

from the evaporated permeate solution it is impossible to quantitatively isolate melanin either by the method of precipitation with organic solvents of various nature and inorganic salts or by altering the pH of permeate (Aghajanyan et al., 2005). This is evidently connected with the nature of the protein constituting up to 30% of the melanin molecule. In our experiments, we therefore studied the possibility of isolating melanin from CL and purifying it using a sorption method (Aghajanyan et al., 2005). Our study has shown that ion-exchange IA-1r resins are the most preferable for isolating melanin from supernatant, because they have the highest sorption capacity and melanin can be easily eluted from them (Aghajanyan et al., 2005). IA-1r anionite also meets the requirements of industrial technology due to its availability, low price, high chemical stability and durability. The preferable ionic form of IA-1r resins is the Cl^--form, which provides the best sorption effect (Zhdanovich, et al., 1997). Melanin sorption on IA-1r resins (fraction – 0.5 ± 0.2 mm) is an effect of the selective interaction between the sorbate and matrix of the resin formed as a result of the polycondensation of formaldehyde, m-phenylenediamine and resorcinol (Aghajanyan et al., 2005).

Our study of the melanin sorption process in dynamic mode showed that the pH of the supernatant should be reduced to 3.7. Although in this process a part of the pigment (~20-25%) passed from the solution to the precipitate, the formed precipitate did not significantly change the effectiveness of the sorption process. The precipitate was collected on the top of the column and did not hinder the flux of the liquid. At the elution stage, the undissolved portion of melanin was dissolved in the ammonia eluate and exited the column together with the absorbed melanin. In this instance, the amount of melanin absorbed by the resins increased up to 30%.

Based on the data obtained on the IA-1r resins under dynamic mode, melanin was successfully isolated from the supernatant. First, the supernatant pH was lowered from 8.6 to 3.7. At the sorption step (before pigment breakthrough) up to 4.3 volumes of the supernatant could be passed through 1 volume of resins. The resins were then washed with demineralized water until the concentration of dry matters in the washed waters reached 0; then, melanin was eluted with 3.5% ammoniac solution. Our experiments have shown that ~97% of eluted melanin absorbed on resin was accumulated in the volume of eluate constituting 2.6 volume of the column.

The collected ammoniac eluate was subjected to a vacuum evaporation at a residual pressure of 0.01 MPa and temperature of 50-55°C and further subjected to ultrafiltration prior to drying. Afterwards, the permeate was concentrated to 250-280 g/l of melanin and the solution was dried in a stream of hot air (50-55°C). The drying resulted in the formation of a dark-brown amorphous powder with metallic luster. The yield of melanin obtained from CL after drying at the isolation stage was 78.4% of initial melanin in CL.

Our experiments have shown that at the elution stage the formed diluted fractions of eluate (ammonia concentration > 1.2%) with melanin content > 0.7 g/l can be used instead of water to prepare ammoniac eluents for the subsequent stages. With such an approach, the consumption of the ammoniac solutions used for the preparation of eluent was reduced by about 20% and the yield of melanin at the stage of its isolation from CL was increased by 2.5%. Moreover, the volume of ammoniac eluates subjected to evaporation was also reduced to 8-10%.

At subsequent stages, when isolating melanin from the supernatant, we subjected the ionite to washing until the pH reached 2.7-2.9. This led to the elimination of the resin regeneration step and a 3.5-4.0 fold reduction in demineralized water consumption.

Melanin characterization

To establish whether the obtained pigment was indeed melanin, qualitative reactions with oxidizers (H_2O_2, $KMnO_4$, $FeCl_3$) were performed. The research results showed the presence of quinoid and phenolic structures in the obtained pigment, which confirmed its melanin nature (Prota, 1992; Lyakh, 1981).

Strong reducers – glutathione and dithionite, as well as sodium borohydride, significantly decolorized the pigment, which was recovered when oxidizing conditions were created in the system. This reaction is characteristic for chemicals with quinone structures (Prota, 1992; Lyakh, 1981).

A comparison of the IR spectra of melanin extracted from natural and cultivated chaga (Kukulianskaya et al., 2002), synthetic melanin (Aghajanyan et al., 2005), and microbial melanin obtained by us (Aghajanyan et al., 2005; Aghajanyan et al., 2011) revealed similarities in respect of the main absorption bands.

The peculiarity of melanins as natural polymers containing developed systems of conjugated bonds is the

availability of unpaired electrons. All melanin molecules, without exception, have electron paramagnetic resonance (EPR) absorption as a slightly asymmetric singlet signal without hyperfine structure, with a g-factor value ranging within 2.003-2.004 (Aghajanyan et al., 2005). The paramagnetism of a natural melanin has a significant impact on its many important properties, such as electrical conductivity, chemical reactivity, antioxidant properties, and biological activity (Borshchevskaya et al., 1999).

To further evaluate the structure and properties of the obtained melanin, the EPR method was used, which enables information about paramagnetic centers to be obtained. The spectrum of a microbial melanin is a slightly asymmetric singlet without hyperfine structure with a line width between the points of the maximum incline of about 7 Gauss and a g-factor value equal to 2.003 (Aghajanyan et al., 2005). A double integration of the spectrum showed that it contained 0.21×10^{18} spin/g paramagnetic centers (Aghajanyan et al., 2005). The spectrum of the synthetic melanin was also a singlet, but the absorption line already represented a Lorentzian shape with a g-factor equal to 2.004, and a width of about 8 Gauss. The number of unpaired electrons in it was 0.37×10^{18} spin/g. The microbial melanin obtained by us was easily soluble in water, which allowed its EPR spectra to be recorded in a solution, depending on the dilution rate.

Two types of paramagnetic centers were observed in the structure of microbial melanin upon the dilution of a melanin solution to 5 mM: one was located in the conjugated chain of melanin; and the other was located on the nitrogen atom. A further dilution resulted in the disappearance of the latter paramagnetic center and a monotonic decrease of the former, which led to the conclusion that the latter center was stable, and the former was labile, as has previously been shown (Aghajanyan et al., 2005).

Moreover, the relative intensity of the EPR signal of melanin in water decreased depending on the dilution multiplicity (Fig. 3).

Thus, again, as a result of the analysis of the EPR spectra, the 2nd type of paramagnetic center in the melanin molecule was detected in a liquid phase. In the light of this discovery, the need arose to study the intensity of this signal depending on the pH. The results of the experiments are presented in Figure 4.

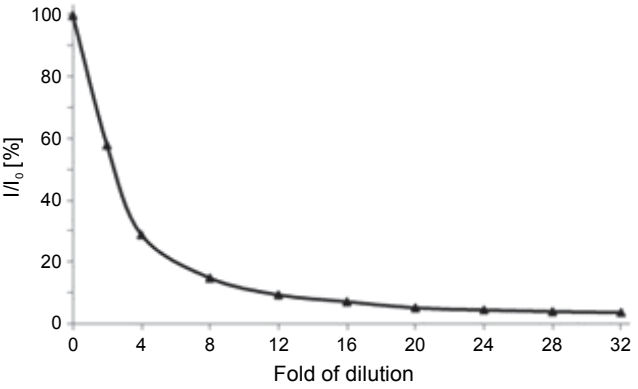

Fig. 3. The dependence of the relative intensity of the EPR signal of microbial melanin in water on the multiplicity of dilution; I – intensity of signals, I_o – maximum intensity of signals

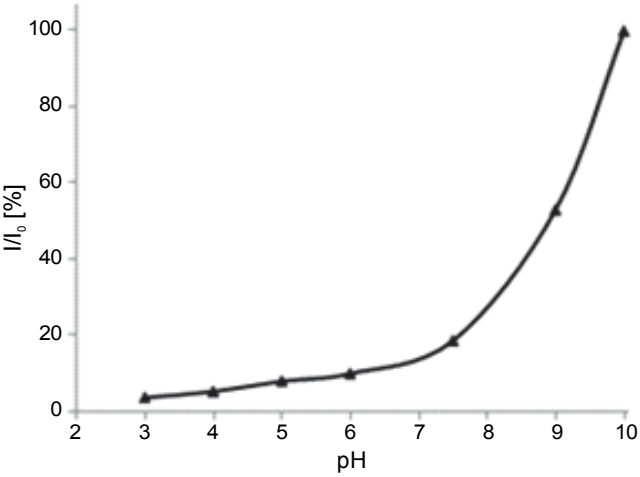

Fig. 4. The dependence of the relative intensity of the EPR signal of microbial melanin in water on the pH of melanin solution; I – intensity of signals, I_o – maximum intensity of signals

As can be seen in Figure 4, almost a linear dependence of the EPR signal intensity on the pH (in the pH range between 3 and 7.5) was observed, and at pH higher than 7.6-its sharp rise was detected. In our opinion, with the rise of pH, an evidently redistribution of the electron density occurred in a way that the concentration of unpaired electrons increased in the conjugated melanin chains. The results obtained by EPR spectroscopy contributed largely to the concepts from the early 1990s on melanins as semiconductive structures (Makordey et al., 1994).

To widen the areas of usage, the electrical conductivity of melanin isolated from CL of microbial fermentation and from black hairs of horse was studied. Three samples of melanin were selected for research: 1) a microbial melanin obtained by drying in a stream of hot air

Table 3. The calculation of melanin electrical conductivity

No.	T [m]	R [Ohm]	D [m]	S [m^2]	$\rho \cdot 10^{-8}$ [Ohm · m]	$\sigma \cdot 10^{9}$ [1/(Ohm · m)]
1	$1.45 \cdot 10^{-3}$	$170 \cdot 10^{6}$	$1.0 \cdot 10^{-2}$	$0.785 \cdot 10^{-4}$	$9.2 \cdot 10^{-2}$	$1.10 \cdot 10^{-2}$
2	$2.20 \cdot 10^{-3}$	$270 \cdot 10^{6}$	$1.0 \cdot 10^{-2}$	$0.785 \cdot 10^{-4}$	$96.0 \cdot 10^{-2}$	$1.00 \cdot 10^{-2}$
3	$0.70 \cdot 10^{-3}$	$300 \cdot 10^{6}$	$0.7 \cdot 10^{-2}$	$0.384 \cdot 10^{-4}$	$27.5 \cdot 10^{-2}$	$0.36 \cdot 10^{-2}$

T – thickness of the melanin briquette, R – resistance of the melanin briquette, D – diameter of the melanin briquette, S – surface of the melanin briquette, ρ – specific resistance of the melanin briquette, σ – specific conductivity of the melanin briquette

at 48°C; 2) a microbial melanin obtained by drying under vacuum in the presence of P_2O_5); and 3) a melanin obtained from horse hair by a standard method (Prota,1992, Lyakh,1981). Since melanin is an amorphous substance, tablets were prepared from the samples at high pressure (1.0-1.2 MPa); their diameter (D), thickness (T) and surface (S) are presented in Table 3.

The resistances of melanin samples were calculated using the following formula $\rho = RS/L$; and electrical conductivity by $\sigma = 1/\rho$. The results are given in Table 3.

As presented in Table 3, samples of the tested melanin had dielectric properties on the verge of semiconductors ($\sigma < 10^{-8}\,ohm^{-1} \cdot m^{-1}$).

Our previous studies showed that *Bacillus thuringiensis* melanin (BM) obtained by us proved to be a strong phyto-stimulator (Popov et al., 2005); its high phytostimulating effect on a commercially valuable culture of Belarus highbush blueberry (*Vaccinium corymbosum* L.) was shown. The BM induced an early initiation of the root formation phase, without the formation of a callus; it also provided growth stimulation, promoted formation of a larger number of leaves, increased the entire leaf area and improved the physiological conditions of plants (Popov et al., 2005).

Experiments conducted on albino rats with brain lesions revealed that BM promoted brain tissue regeneration, as it suppressed scar formation, improved trophicity of nervous tissues due to dilation of vessels lumens and formation of new capillaries, promoted the overall growth of nerve fibers contributing to the recovery processes after nervous tissue lesions and acted as a neuroprotector ensuring the preservation of nerve cells in the lesion areas (Petrosyan et al., 2014).

Conclusions

To carry out a complex management of the process when developing the microbiological method for obtaining melanin, technological parameters such as medium pH and K_{La} were optimized. A stepwise regulation of the technological parameters provided an increase in the yield of melanin up to 13 g/l and reduced the duration of the process.

The sorption method was efficient for the isolation of melanin from the supernatant and optimal technological parameters were determined: type of resins, their form, size of the particles of ionite, linear velocity of the liquid flow during sorption and elution, conditions of elution.

The dependence of the relative intensity of the EPR signal on the pH of the solution and the dilution factor, as well as electrical conductivity of the water-soluble melanin was determined, and this will hopefully help to enlarge the areas of its application.

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