



**United  
Financial  
Group**

2022



## **Development of manufacturing technology "Alginar".**

### **September 2020 (initiation of work)**

Characteristics of the natural strain 365 *Nadsoniellanigra* var. *hesuelica*.

The natural strain 365, characterized by coal-black pigmentation, was isolated by E.L. Ruban in 1957 from a sample taken by I.P. Ruban on the Antarctic island of Heswell during the 2nd Antarctic Expedition (1956-1957).

The black pigment (melanin) of the Antarctic black yeast *Nadsoniellanigra* var. *hesuelica*. Its significance for pharmaceuticals was noted in the materials of the UN University, Tokyo, when discussing the International Program for the Search and Practical Application of Biologically Active Substances Synthesized by Antarctic Organisms, in particular, microorganisms.

Strain 365 was described in detail and named *Nadsoniellanigra* var. *hesuelica*, after the name of the island where it was found. In November 1969, postgraduate work with the melano-pigment *Nadsoniellanigra* var. *hesuelica* 365, an Antarctic black yeast, was completed. Its results are presented in the dissertation of S.P. Lyakh "Melanopigment of the Antarctic black yeast *Nadsoniellanigra* var. *hesuelica* and its physiological function" (Moscow, 1970, 578 p.), in 17 publications on the topic and in the monograph by S.P. Lyakh and E.L. Ruban "Microbial melanins", Moscow, Science. Publication of two subsequent monographs directly related to melanin-antarctic topics (S.P. Lyakh "Adaptation of microorganisms to low temperatures", 1976, Moscow, Science and S.P. Lyakh "Microbial melaninogenesis and its functions", 1982, Moscow, Science), reviews on the characteristics of the microflora of Antarctica and experimental articles on the same subject are the results of almost 15 years of work.

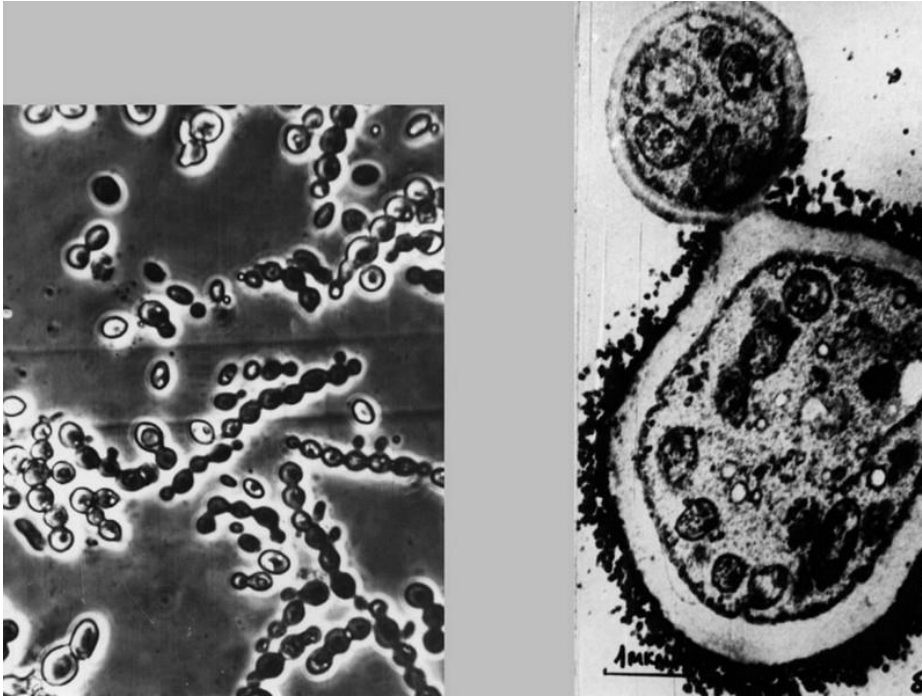


Image 1. Luminous microscopy culture cells strain *Nadsoniellanigra* var. *hesuelica* 365.

**December 2020**

Primary spectral study of Astromelanin.

EPR spectrum

From an article in 1969 Ruban-Lyakh: the concentration of paramagnetic centers in the pigment ranges from  $0.3$  to  $1.6 \cdot 10^{19}$  spin / g (essentially a quantitative assessment of antioxidant properties). In this sample No. 3 -  $1.8 \cdot 10^{14}$  spins / mg - i.e.  $1.8 \cdot 10^{17}$  spin/g, which is slightly lower than above. For comparison, sample No. 2 - enomelanin isolated from grape skins in 1986 - an experimental batch - the order of concentration of paramagnetic centers approximately coincides.

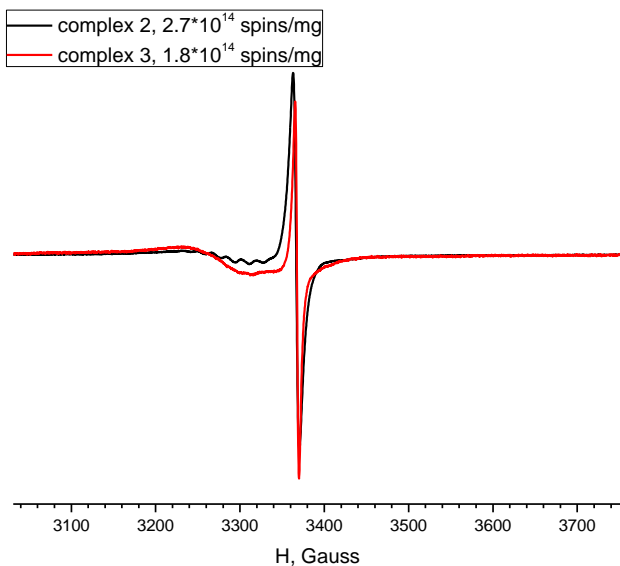


Image 2. EPR spectrum of astromelanin and enomelanin samples

An analysis of the NMR spectra of nuclear magnetic resonance showed that there are quite a few aromatic structures in this sample, which is surprising, since in modern literature, aromatic conjugated structures are always present in various melanins.

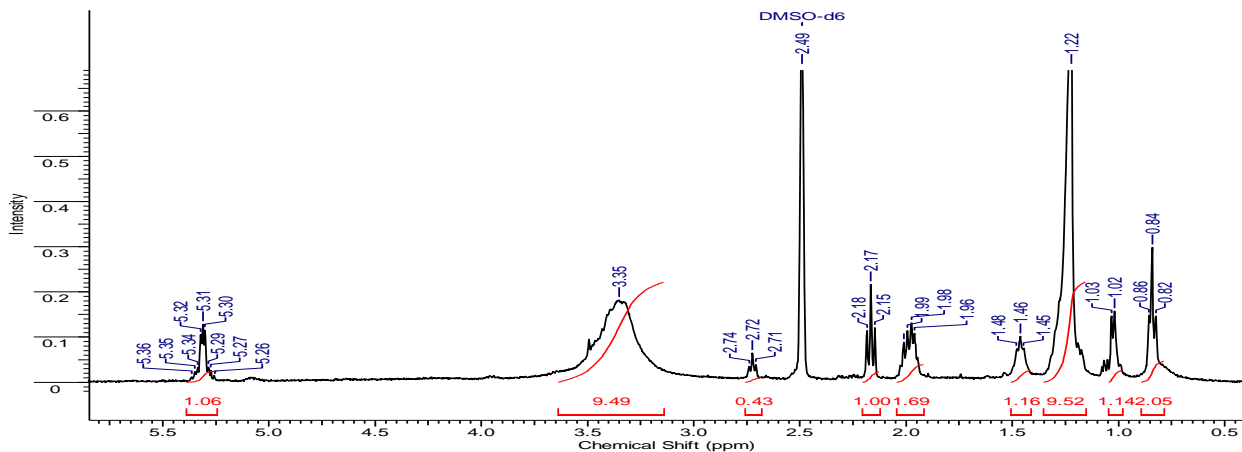
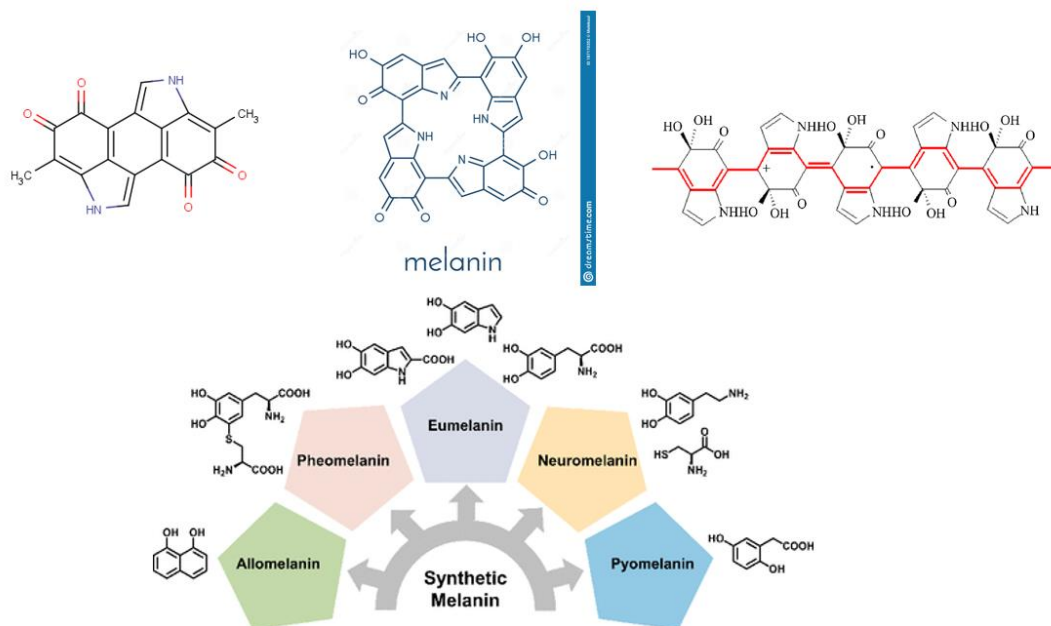


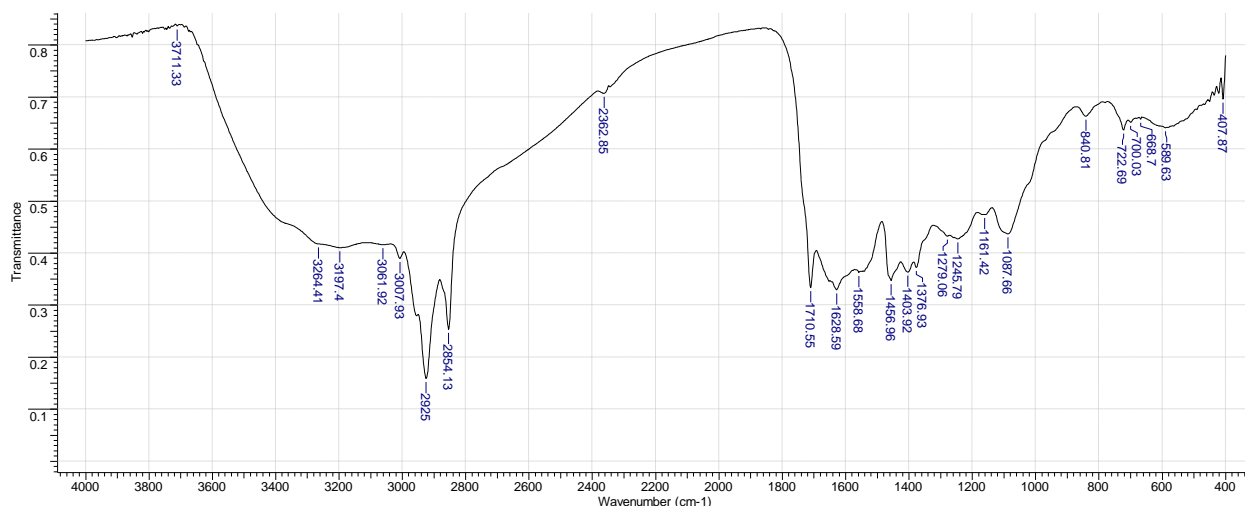
Image 3. NMR-1H spectrum of astromelanin in DMSO - D<sub>6</sub>



Rice. 4. Proposed structures of various melanins

### IR spectrum

In the region of  $3600-3100\text{ cm}^{-1}$  there is a wide intense band corresponding to stretching vibrations of OH groups linked by inter- and intramolecular hydrogen bonds. Stretching vibrations of C-H in methylene groups are manifested by intense absorption with maxima in the range of  $2850-2960\text{ cm}^{-1}$ . Absorption bands of medium intensity with peaks in the region of  $1710-1540\text{ cm}^{-1}$  correspond to the stretching vibrations of carbonyl groups (and possibly N - H). In the region of  $1460-1370\text{ cm}^{-1}$ , bands of skeletal vibrations of O-H groups are observed. The ether bonds of the =COC groups (as well as C - N) appear in the region of  $1280-1160\text{ cm}^{-1}$ . There is also an absorption of medium intensity with a peak at  $1087\text{ cm}^{-1}$  associated with the participation of the C-O-H group in skeletal vibrations.



Rice. 5. IR spectrum of astramelanin in a KBr tablet

## February 2021

Due to the fact that unmodified melanin is a water-insoluble polymer, rapid screening of samples for a qualitative assessment of the performance of commercially available melanins, as well as fractions released during processing of fungal strains, is difficult. Therefore, samples of commercial melanins were stirred in dimethyl sulfoxide at room temperature for 48 hours, and the resulting solution was examined using EPR. EPR experiments were carried out at the boiling temperature of liquid nitrogen.

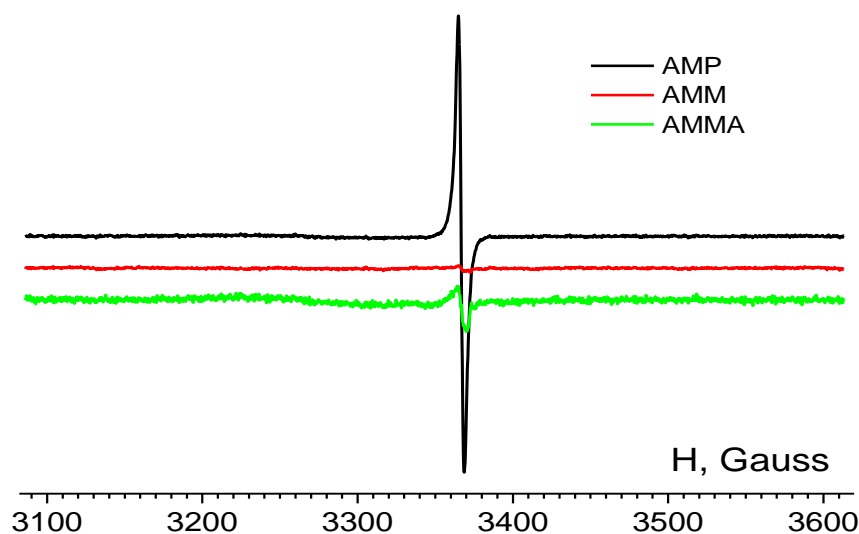


Image 6. Comparison of melanins dissolved in DMSO

**AMP  $4 \cdot 10^{14}$  spin / mg**

AMM  $1 \cdot 10^{13}$  spin / mg

AMMA  $4 \cdot 10^{13}$  spin / mg

The dimethyl sulfoxide-soluble melanin fraction of sample No. 1 turned out to be an order of magnitude better than sample No. 2. Thus, it is possible to predict the best anti-cancer activity in melanin AMP.

## March 2021

A strain of Antarctic yeast *Nadsoniella nigra* var. *hesuelica* and practical work has begun on the cultivation of raw materials for production. The necessary equipment for the work was purchased.

**April 2021**

Spectral testing of various commercially available melanins revealed their inconsistency with the declared structures and quality. It was customary to take control of every stage of melanin production.

It is planned to work out a method for isolating melanin from cells. This is the most important thing, because often, even with a slight deviation from the methodology, a product is obtained contaminated with inactive impurities and up to 80%.

While working on the melanin extraction technique, each stage will be controlled by spectral methods until the required product purity is achieved.

**June 2021**

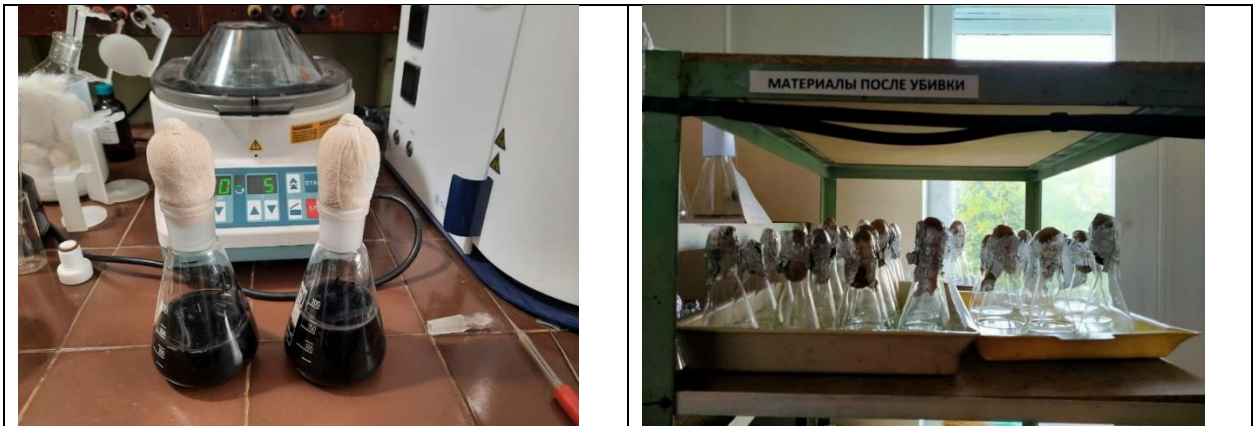


Image 7. The first cultivated strain of *Nadsoniella nigra* var. *hesuelica*



Image 8. *Nadsoniella nigra* var. *hesuelica* growing with different components of nutrient substrates.



Dilution of the strain in a liquid medium on various aqueous substrates was tested. The optimal substrate for the rapid and stable growth of the strain was found. Cells of the strain were isolated from aqueous substrates and prepared for extraction of melanin. The conditions for keeping the best reproduction and accumulation of melanin in the strain on a solid nutrient medium were selected.

## July 2021

The literature on methods for isolating melanin from various plants and fungi has been studied in detail.

Laboratory equipment was selected and purchased for breeding strains of melanin-producing fungi in the required quantities, to vary the conditions for optimal growth and maximum pigment yield.

Started cultivation of two different strains under normal conditions.



Image 9. The first large-scale experiment on the cultivation of *Nadsoniella nigra* var. *Hesuelica* in a liquid nutrient medium.

The total volume of culture grown in the laboratory was - 20l

## July 2021

The choice of methods for separating the strain from the cultivation medium was made. The extraction method must be selected with minimal losses under mild conditions, to reduce cell destruction and melanin leaching into the solution.

This is a very important stage, the results of which determine the quality and purity of the finished product.



Image 10. Specialist mycologist Ph.D. while studying *Nadsoniella nigra* var. *hesuelica* .

## October 2021

We independently isolated the first batch of Melanin from our own raw materials of black yeast *Nadsoniella Nigra*. We selected the necessary parameters for the isolation of melanin from the finished yeast mass, which we grew for 3 months, since there are several methods of isolation.



An EPR study was conducted to determine the antioxidant activity of various melanin images, including our own.

Our sample showed the highest antioxidant activity, which is comparable to the data from the works of S.P. Lyakh

A comparative study of precipitates and solutions of commercially available melanins and melanins isolated according to the original method from the *Nadsoniella* strain was carried out. Nigra (VKM F -2137) by EPR method.

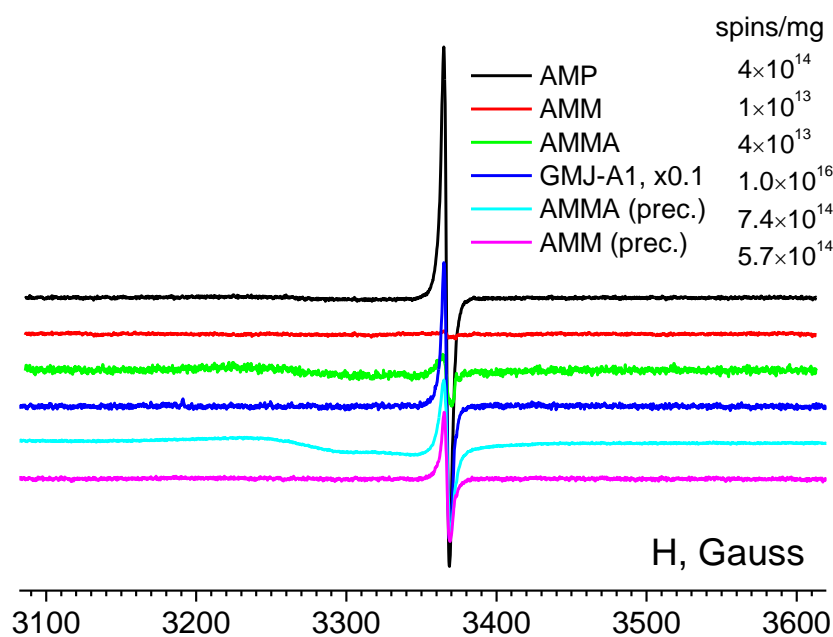


Image 11. Study of the EPR of soluble and insoluble melanins

As can be seen from the diagram, the number of spins per 1 mg of a substance is at least an order of magnitude higher in insoluble precipitates ( AMMMA and AMM ( prec ) -  $10^{-14}$  versus  $10^{-13}$  spin / mg in DMSO solution.

Melanin GMJ - A 1 isolated by the original method from the *Nadsoniella* fungus strain Nigra turned out to be the most active and contains 2 orders of magnitude more radicals- $10^{-16}$  spin / mg . For convenience, in the diagram, the data for this melanin was multiplied by 0.1 so as not to go beyond the graphs. In terms of 1 g of melanin, the number of spins is  $10^{-19}$  , which is quite enough to provide high antioxidant activity.

## **October 2021**

Over the past two months, we have obtained 10 liters of *Nadsoniella* suspension from cultivated strains. *Nigra* and 10 liters of suspension of *Exophiala Alcalophila*. These strains have proven to be good producers of animal melanin. By long-term selection of a method for isolating the corresponding strains from the biomass, 17 g of dry *Nadsoniella* cells were obtained. *Nigra* and 32 g of *Exophiala Alcalophila* cells. According to our original methods for isolating melanin from dry cells, 1.5 g of purified primary melanin (*Nadsoniella Nigra*) and 2.5 g of melanin from cells of *Exophiala Alcalophila*.

The more than high antioxidant activity of these strains was also confirmed by the EPR method. ( $10^{* 19}$  spins per gram). This figure is the highest to date of all known strains of fungi.

We examined these samples and established the possible structure of melanins by solid-phase NMR spectroscopy. It should be noted that our data correlate with literature sources. Moreover, water-soluble forms of melanins have been prepared for further research.

## **November 2021**

A technology for obtaining a water-soluble form of melanin has been developed.

Melanin derived from a yeast structure has been tested.

Studies were carried out on the antioxidant activity of melanin samples isolated by various methods (EPR). This type of activity indirectly indicates the oncological resistance of the samples: the higher the antioxidant activity, the higher the toxicity of this compound in relation to oncological cells. As a result of the study, it was demonstrated that the own melanin isolated by our group of biochemists has one of the highest activity rates among other samples.

Several types of melanin from various raw materials are in the works, including black Antarctic pigment.

## **June 2022**

The technology for extracting melanin from raw materials has been improved. The output is 150% more of the finished substance from the same volume of raw materials, which reduces the cost of production.

## **September 2022**

Getting ready for small scale industrial production

