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SOME PROPERTIES OF MELANIN PRODUCED BY *Azotobacter chroococcum* AND ITS POSSIBLE APPLICATION IN BIOTECHNOLOGY

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The strain of bacteria *Azotobacter chroococcum*, producing and excreting melanin, was isolated. Melanin excretion was observed only on Ashby medium with benzoic acid, but not on the medium with mannitol as carbon source. Some properties of bacterial melanin (absorption spectrum, reduction of permanganates to manganates, rate of bleaching by hydrogen peroxide, co-precipitation with calcium ions) and conditions of its production (dependence on copper ion concentration) were described. A scheme of isolation and purification of bacterial melanin was proposed. It was found that partially purified melanin accelerates growth of tomato, rape, radish and lettuce seedlings.

Additionally, it was shown that crystals are formed on the surface of solid growth medium, in the bulk and on the surface of bacterial streak. Putative link between melanogenesis and crystal formation, as well as practical application of melanin production by bacteria *Azotobacter chroococcum* are discussed.

Key words: *Azotobacter chroococcum*, melanin, crystal, calcification.

Melanins, black or brown pigments, are biopolymers formed with such precursors as 5,6-dihydroxyindole, 8,10-dihydroxynaphtalene, catechol, homogentisic acid [1–4]. Melanins have the defined physical and chemical properties. They reduce many compounds, including silver ions, ferricyanide, potassium permanganate [5, 6]; have specific light absorption spectrum and are precipitated by strong acids; can function as antioxidants [2, 3, 6, 7], electron shuttles or terminal acceptors [8, 9], metal cation chelators [2, 7, 10, 11], etc.

Production of melanins is characteristic for many microorganisms, mainly for soil and marine bacteria [2], and also for some parasitic bacteria and fungi. In particular, among soil bacteria, melanins are produced by actinomycetes (some *Streptomyces* species [12, 13]), species of *Azotobacter* [4, 14–16] and *Azospirillum* [17], and some strains of *Rhizobium* [18, 19]. Among marine bacteria melanins are produced by *Shewanella algae* [8, 9], *Shewanella colwelliana* [1, 20], *Marinomonas mediterranea* [21]; among pathogenic bacteria they are *Burkholderia cepacia* [22], *Klebsiella pneumoniae* [18], *Legionella pneumophila* [23], *Mycobacterium leprae* [24], *Proteus mirabilis* [25], etc. In some pathogenic bacteria, for instance *Vibrio cholerae*, melanogenesis may be induced by different stresses [26].

Functions of melanins in microorganisms are partially discovered. Melanins are considered to defend parasitic bacteria and fungi against reactive oxygen species formed by immune cells [2, 7, 22], while fungal or bacterial spores — against injury from ultraviolet irradiation [27]. Some marine bacteria may use melanin as electron carrier for generation of membrane potential and solubilization of insoluble metal salts [9]. Despite that, significance of melanins for marine and soil microorganisms has not been sufficiently cleared up. Nowadays, melanin production is under intensive investigation due to its unusual properties and broad spectrum of practical application, particularly in cosmetics and pharmacology [3, 28–30]. Semiconducting properties of melanin are well-known [31]. Antitumor properties of allomelanins were recently shown [32, 33].

Investigation of microbial melanins gives many perspectives for biotechnology and medicine. It was found that bacterial-derived melanins have the same properties as animal ones; hence, these melanins may have similar application. For example, bacterial melanin was shown to be effective defense for fibroblast cultures against ultraviolet radiation and may be promising for use in photoprotective creams [3, 28].

In medical aspect, melanogenic microorganisms are a very suitable model system to study an impact of different factors on melanin production, let alone the fact that melanins play important defensive role in pathogenic bacteria [2, 22]. As it was mentioned above, this defense is mainly connected with melanin capability to neutralize reactive oxygen species. However, it was found that melanins may also attenuate bactericidal action of antibiotics [34].

The aim of current research was to investigate conditions for melanin production by *Azotobacter chroococcum* and some physicochemical properties of this pigment. Contribution of melanin to crystal formation by *A. chroococcum* was also shown. Detailed analysis of literature showed that the last observation may not be accidental, as melanization is often associated with calcium salt crystallization in living organisms.

Materials and methods

Bacterial cultures were grown on modified solid Ashby medium, containing 2.1 g/l benzoic acid, 0.4 g/l K_2HPO_4, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.3 g/l NaCl, 2.5 mg/l $FeSO_4 \cdot 7H_2O$, 2.5 mg/l $Na_2MoO_4 \cdot 2H_2O$, 2.0 mg/l $CuSO_4 \cdot 5H_2O$, 2 g/l $CaCl_2 \cdot 2H_2O$ and 18 g/l agar; pH of the medium was routinely adjusted to 7.6. In some experiments, carbon sources like mannitol, glucose, fructose, sucrose and starch were used instead of benzoic acid. These carbohydrates were added into growth medium at concentration 20 g/l.

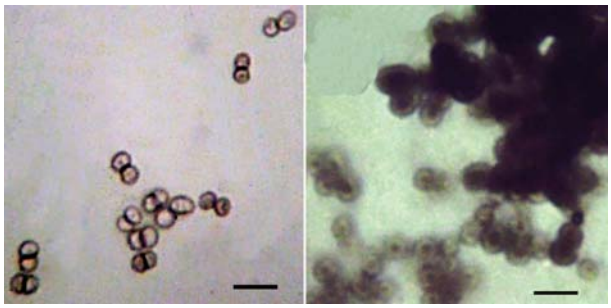
All photomicrographs were made by a USB-camera HB-35 for microphotography under magnifications ×120, ×600 and ×1350 (oil immersion objective).

For analysis of copper effect on pigment production, bacterial cultures were grown in slopped microvials in 1.5 ml of the liquid Ashby medium with benzoic acid without additional aeration. Under control conditions, medium did not contain copper salts. Experimental cultures contained $CuSO_4$ in range of 1–100 μM. After 5 days of cultivation, the cultures were centrifuged at 5000 g and pigment production was measured spectrophotometrically with the use of Spekol 211 (Carl Zeiss Jena, Germany) at wavelength 400 nm against sample with growth medium. Absorption spectrum of the pigment measured using spectrophotometer SF-46 (LOMO, USSR).

Seeds of lettuce (*Lactuca sativa* L.), tomato (*Solanum lycopersicon* Mill.), rape (*Brassica napus* var. *oleifera* DC) and radish (*Raphanus sativus* var. *radicula* Pers.) were used for testing pigment effect on plant growth. In control, 32 seeds were sown on the Petri plates with distilled water solidified with agar. After 7 days of growth, the length of the seedlings was measured. Experimental plates were prepared in the same way, but partially purified pigment was resuspended in the agar in concentration 1 mg/ml.

Results and discussion

The strain of *Azotobacter chroococcum*, used in this study, was isolated from podzol soil near Ivano-Frankivsk city (district «Pasichna») by the «single-soil-grains» method [14]. Bacteria of the isolated strain were Gram-negative, which formed whitish, slimy streaks that turned brown with age on Ashby medium with mannitol. The cells in aged cultures were 3–5 μm in diameter and occurred in pairs (Fig. 1). Capsules were observed around the cells. Bacteria were capable to grow on glucose, fructose, sucrose, starch and mannitol as carbon sources.



A *B*

Fig. 1. **Cells of *Azotobacter chroococcum* associated in pairs (*A*) and with a capsule visible around cells in pigmented nutrient broth (*B*). Oil immersion with magnification ×1350 (size bar = 15 μm)**

It is known that cultures of *A. chroococcum* become dark with age due to accumulation of black pigment [15]. The black pigment is thought to be a catechol-type melanin, or allomelanin [4]. Its synthesis does not require the presence of precursor molecules (e.g., tyrosine, dihydroxyphenylalanine, catechol, etc.) in the culture medium. Some authors suggest that such precursors are formed as by-products in aged cultures [16]. Melanin, formed in aged cultures of *A. chroococcum*, usually does not diffuse into the medium, but stays in the cells [15]. The same was observed for some other nitrogen-fixing bacteria. For instance, pigment floccules were deposited on

the medium surface only after month of cultivation of *Azospirillum brasilense* ATCC 29145 in the nitrate-based broth [17].

In our case, pigment was produced within approximately five days of cultivation, staining the bulk of the liquid medium or forming the dark halos around bacterial streaks on the solid medium (Fig. 2). However, such excretion and enhanced production of the melanin were observed only on Ashby medium with benzoic acid, but not with mannitol as a carbon source. Pigmentation did not develop under streaks that may indicate a need in air access for melanin production and excretion.

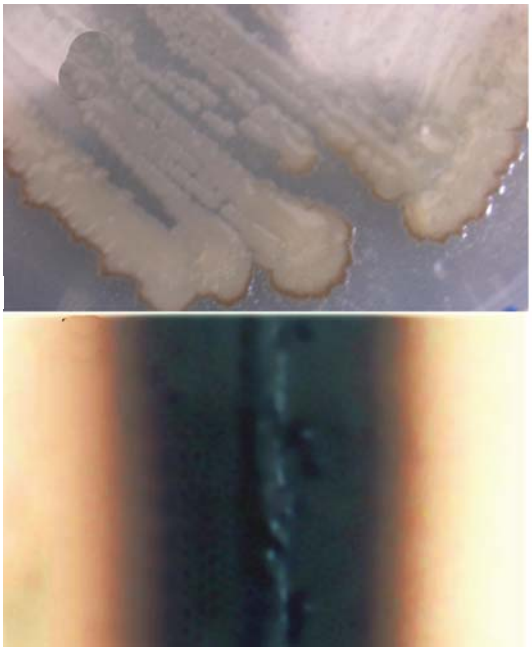


Fig. 2. ***Azotobacter chroococcum*** **streaks on Ashby agar with mannitol (*A*) and benzoic acid (*B*)**

Similar excretion of the pigment was also observed on a medium with L-tyrosine inoculated with a recombinant *Escherichia coli* strain, which expressed a tyrosinase [34], and also in marine bacteria *Shewanella algae* BrY [9] and *Shewanella colwelliana* D [20]. We have not found reports on enhancement of melanogenesis in *A. chroococcum* by benzoic acid. At the same time, the benzoic acid could suppress the production of melanin in some cases [4].

Benzoic acid metabolism in bacteria leads to catechol formation [35–38]. At the next stage, benzene ring of catechol is cleft with yield of *cis,cis*-muconic acid. Further biochemical transformations of the latter metabolite give products that enter tricarboxylic acid cycle [38]. It suggests that this metabolism may be stopped at the level of catechol, which is then oxidized by catechol oxidase, forming melanoid compound. Some authors suppose that polymerization of catechol may occur due to action of hydrogen peroxide, formed by flavin dehydrogenases [16, 39], or other reactive oxygen species [4].

Earlier, the stimulation of the pigment synthesis in *A. chroococcum* by copper ions was reported [4, 16]. We also found that the pigment synthesis was enhanced significantly at certain concentrations of copper sulphate in the growth medium (Fig. 3).

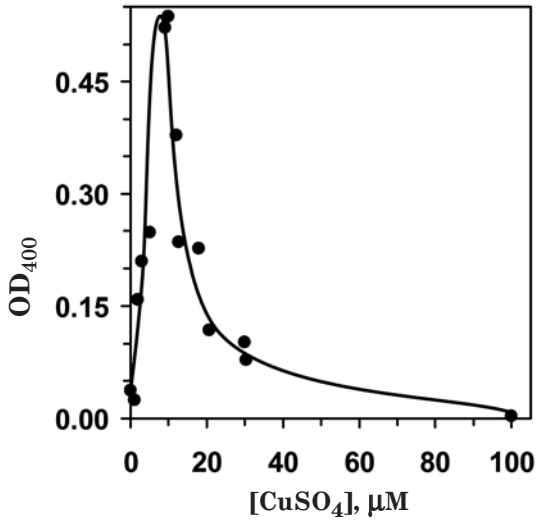


Fig. 3. **The effect of $CuSO_4$ on the diffusible pigment production by *Azotobacter chroococcum***

Cupric sulphate in concentration of 10 μM promoted maximum production of the melanin, while higher concentrations suppressed this process. High concentrations of copper sulphate may suppress bacterial growth and decrease pigment yield. These facts suggest that enzyme from polyphenol oxidase family may participate in melanin production by *A. chroococcum* bacteria. In fact, these enzymes contain copper ions in their active site. However, copper ions may also be involved in pigment formation by reactive oxygen species, because they can interact with hydrogen peroxide, giving hydroxyl radical [7].

The pigment, produced by *A. chroococcum*, had some properties, common for melanins. In particular, it was dissolved in alkali and insoluble in other solvents; coprecipitated with calcium ions. Light absorption spectrum (Fig. 4) of the pigment was typical for melanins [4, 34]. The spectrum had a small shoulder at wavelength 260 nm and no peaks. Bacterial melanin was bleached by hydrogen peroxide and reduced permanganates to

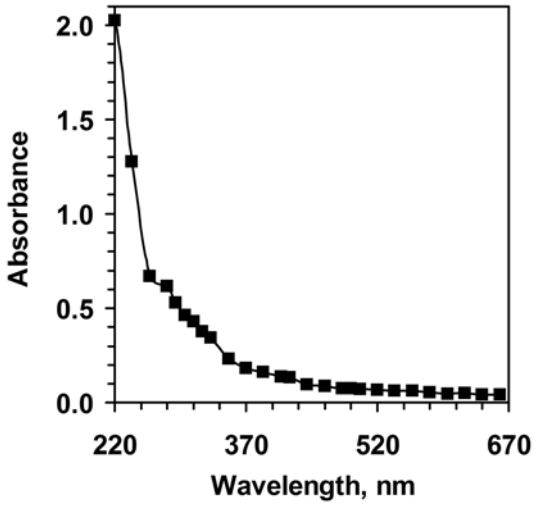


Fig. 4. **Absorption spectrum of the partially purified diffusible melanin from *Azotobacter chroococcum***

manganates. Hydrogen peroxide (5%) bleached the pigment relatively slowly: optical density of the pigment alkaline solution at wavelength 400 nm was dropped near 0.2 units per hour (Fig. 5). 0.01 N $KMnO_4$ solution changed color from pink to green under titration by the alkaline melanin solution (OD_{400} = 0.5). This indicates the formation of manganate ion. Alkalization of 0.01 N $KMnO_4$ solution did not cause color change.

Yet one noticeable feature of the pigment was its potential capability to co-precipitate with calcium hydroxide. When 30% NaOH was added to the pigmented cell-free broth, the formation of amorphous black floccules, easily separated by centrifugation, was observed. Supernatant was colorless after precipitation of the floccules. Formation of the floccules also occurred after addition of $CaCl_2$ to the alkaline solution of the partially purified melanin.

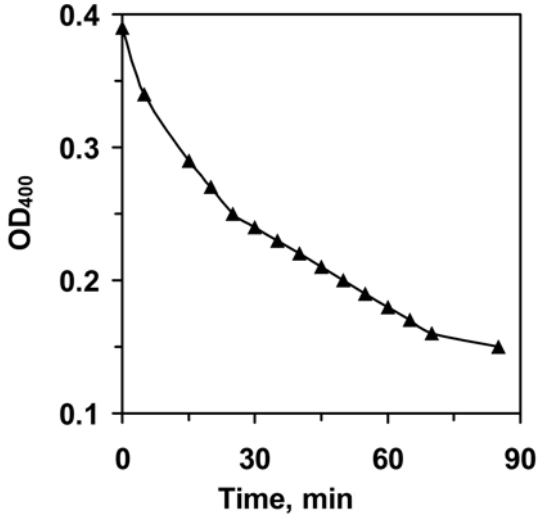


Fig. 5. **Bleaching rate of the diffusible melanin from *Azotobacter chroococcum***

These observations suggest that the floccules are the complex of melanin with $Ca(OH)_2$ formed under alkalization of the medium containing calcium ions. Calcium binding capacity is not a distinguishable property of the melanin from *A. chroococcum* because it was also described for many types of melanins [10, 11]. In our work this capacity laid in the basis of partial pigment purification method (Fig. 6): addition of 30% NaOH solution to cultural medium precipitated complex of the melanin with calcium ions; at the next step, calcium ions were washed out by hydrochloric acid, which simultaneously precipitated melanin and solubilized calcium to $CaCl_2$. After washing and settlement, precipitated melanin was dried. Dried preparation was dissolved in 30% NaOH solution.

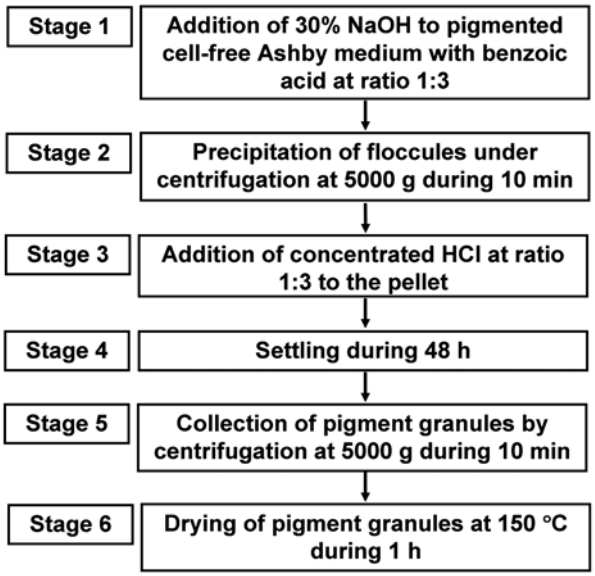


Fig. 6. **Scheme of partial purification of bacterial melanin**

Melanin co-precipitation with calcium ions may underlie the next observation — crystal production and concentration of the crystals especially within pigmented zones of the agar.

Microscopic analysis showed the presence of various size crystals which were located in the black halos around bacterial streaks (Fig. 7, *A–F*). The crystals were rare or absent at all in non-pigmented zones of solid medium (Fig. 7, *B*). At the border between dark and light zones of the agar, crystal size decreased (Fig. 7, *C*). Crystals were seen on the surface of bacterial

streaks (Fig 7, *D*) and within the streak bulk (Fig. 7, *E*), as well as in zones contacting the slime of the bacterial streaks (Fig. 7, *F*).

Up to the present, many authors indicated that some soil and marine bacteria are able to produce calcite, aragonite, struvite and other calcium minerals on different media [40–43]. It is suggested that crystal formation may play an important role in calcium homeostasis of these bacteria [44]. It was shown that bacterial calcite production occurs in alkaline environment abundant with calcium ions [42, 44]. Similar conditions were in the current study. We did not determine the type of crystals formed by the bacteria in our investigation. Nevertheless, the data of other authors, growth conditions, and shape of the crystals suggest the latter are likely a form of calcium carbonate. In particular, crystal shape in most cases was similar to rhombohedral twin lamellae (Fig. 7), what is attributable for calcite. It is not excluded, that the presence of paramagnet compound (i.e. melanin) in the medium promotes formation of calcium salt crystals. An ability of paramagnets to promote crystal formation, especially calcification, is partially confirmed by recent data [45].

Thus, our research showed a simultaneous combination of two well-documented and independent phenomena of melanin and crystal production by soil bacteria *A. chroococcum*. Simultaneous melanin and crystal production has not previously been described for microbial system. However, similar situation is observed in some cases of ochronosis (alkaptonuria), which is accompanied with calcium pyrophosphate dihydrate formation [46]. In ochronotic patients, polymerized black-coloured melanin-like homogentisic acid deposes and accumulates in different tissues, what leads to their calcification [47]. Also, melanization and calcification occur simultaneously in odontogenic cysts [48]. Some examples are also known for healthy animal tissues. In particular, horns of some species of rhinoceros have melanized bands, which contain high concentrations of calcium salts [49]. Increased calcium levels were also found in humeri of heavily spotted individuals of barn owl [50].

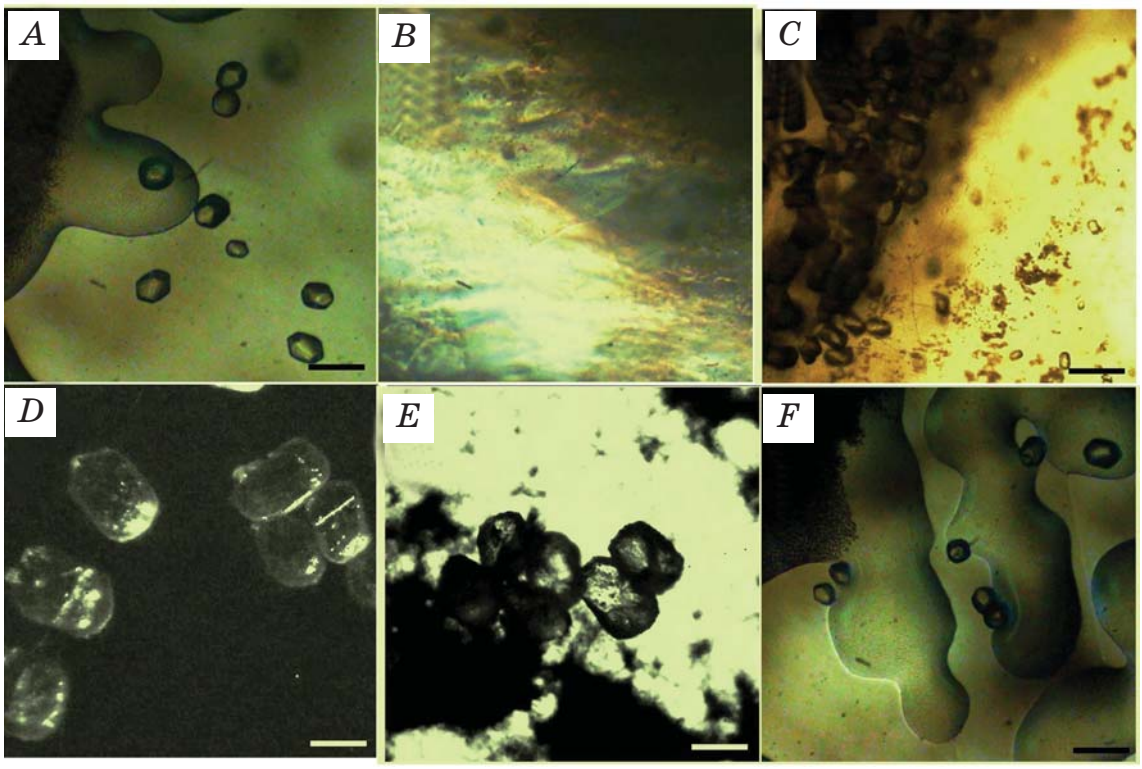


Fig. 7. **Crystals: on solid Ashby medium with benzoic acid (*A*), in non-pigmented zones (*B*), in transition zones between dark and light zone (*C*), upon the surface of streaks of bacteria (*D*), into bulk of the streak (*E*), in zones contact with slime of the streaks (*F*). For figures *A, C, F* the bar = 170 μm, for figures *E, D* the bar = 35 μm**

It is well known that melanins, especially plant allomelanins, are closely related to soil humic substances [2, 51]. In turn, humic substances can promote plant growth. We checked the last property for the melanin from *A. chroococcum*. Indeed, partially purified bacterial melanin increased the growth of lettuce (*Lactuca sativa* L.), tomato (*Solanum lycopersicon* Mill.), rape (*Brassica napus* var. *oleifera* DC) and radish (*Raphanus sativus* var. *radicula* Pers.) when added into distilled water solidified with agar in concentration 1 mg/ml. 7-day seedlings were 1.4–1.8-fold longer after germination on solidifed distilled water with melanin, than in control. Maximum effect was observed for tomato shoots, while minimum

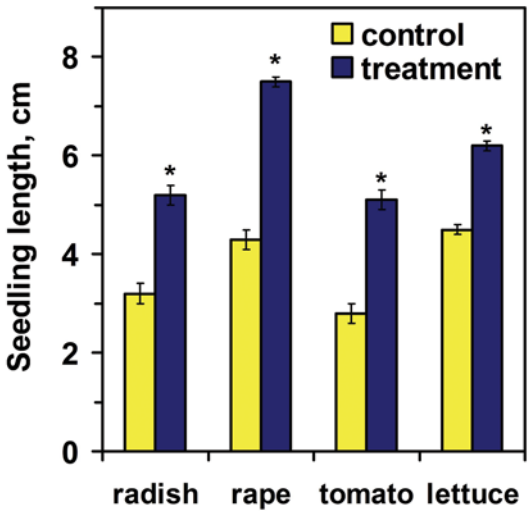


Fig. 8. **The effect of the *A. chroococcum* melanin on the plant shoots growth. Asterisk indicates significant difference by Student's t-test with $P < 0.05$ ($n = 63–83$) from the mean for control group**

one was for lettuce (Fig. 8).

Promotion of plant growth by humic acids is referred to their capability to absorb micronutrients and enhance their availability to plants [52]. However, medium for seed germination in our investigation did not contain any nutrients. Hence, the observed effect is difficult to explain by micronutrient-absorbing capacity of the melanin. Alternatively, melanins can act as plant hormones, what was described for humic substances [53], or improve water-holding capacity of a substrate [54].

Today, an ecological role of melanin for some marine bacteria seems clear. In particular, in *Shewanella algae* this role consists in transfer of electrons to ferric iron [8]. This allows release ferrous iron from insoluble salts and hydroxides. At the same time, the significance of melanization for many soil bacteria and fungi remains poorly understood. Here, we have presented the observation of an unusual melanin production type by the nitrogen-fixing bacterium *A. chroococcum*. In our case, both production and excretion of melanins occurred. Inductor of melanin production and excretion was found to be benzoic acid as a sole carbon source in the growth medium. In addition, melanin production was associated with formation of the crystals found into and onto bacterial streaks, in the slime and within pigmented zones of surrounding solid medium. Taken together, our data could help to elucidate of an ecological role of melanins produced by non-parasitic soil bacteria and a general role for melanization processes in nature.

Investigation of melanin production by *A. chroococcum* bacteria is important also for biotechnology and molecular biology. In the first case, we obtain a very suitable system for production of melanin in significant amounts with use of relatively cheap growth substrates. Melanin itself may have a very wide spectrum of practical application. For example, in 1974 it was shown that melanins are prospective amorphous semiconductors [31]. In addition, some works report ability of melanins to slow down human immunodeficiency virus propagation [29, 30], and their anti-tumor properties [32, 33]. In our work it was shown that melanins can accelerate plant growth at early stages of development. Application of melanins in molecular biology is possible in the case, if synthesis of the pigment is regulated by single gene or operon. Then this gene (or operon) may serve as a suitable marker for plasmid transfer from one organism to another. This marker system is a good alternative for those markers as β-galactosidase or proteins, which respond for resistance to antibiotics [13]. In particular, in bacterial studies heterological expression of L-tyrosinase is successfully used for the present [12, 34, 55]. In these studies, strain, bearing L-tyrosinase gene, was able to produce melanin on medium with L-tyrosine. After all, isolated strain of *A. chroococcum* may be as indicator for the presence of benzoic acid and its salts in different substrates, because of excretion of synthesized melanin occurs only in presence of benzoic acid.

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НЕКОТОРЫЕ СВОЙСТВА МЕЛАНИНА, ПРОДУЦИРОВАННОГО *Azotobacter chroococcum*, И ЕГО ВОЗМОЖНОЕ ПРИМЕНЕНИЕ В БИОТЕХНОЛОГИИ

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Выделен штамм бактерий *Azotobacter chroococcum*, продуцирующих и экскретирующих меланин. Экскреция меланина наблюдалась только на среде Эшби с бензойной кислотой и отсутствовала на среде с маннитом в качестве источника углерода. Описаны некоторые свойства бактериального меланина (спектр поглощения, восстановление перманганатов до манганатов, скорость обесцвечивания под действием пероксида водорода, соосаждение с ионами кальция). Предложена схема выделения и очистки бактериального меланина. Установлено, что частично очищенный меланин в 1,4–1,8 раза ускоряет рост проростков томата, репы, редиса и салата-латука. Кроме того, было показано образование кристаллов на поверхности агаризованной питательной среды, в толще и на поверхности бактериальных штрихов. Обсуждается возможная связь между меланогенезом и образованием кристаллов, а также практическое применение продуцирования меланина бактериями *Azotobacter chroococcum*.

Ключевые слова: *Azotobacter chroococcum*, меланизация, кристаллы, кальцификация.

ДЕЯКІ ВЛАСТИВОСТІ МЕЛАНІНУ, ПРОДУКОВАНОГО *Azotobacter chroococcum*, ТА ЙОГО МОЖЛИВЕ ЗАСТОСУВАННЯ У БІОТЕХНОЛОГІЇ

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Виділено штам бактерій *Azotobacter chroococcum*, які продукують і екскретують меланін. Екскрецію меланіну спостерігали тільки на середовищі Ешбі з бензойною кислотою, а на середовищі з манітолом як джерелом вуглецю вона була відсутня. Описано деякі властивості бактеріального меланіну (спектр поглинання, відновлення перманганатів до манганатів, швидкість знебарвлення пероксидом водню, співосадження з іонами кальцію). Запропоновано схему виділення та очищення бактеріального меланіну. Встановлено, що частково очищений меланін в 1,4–1,8 раза пришвидшує ріст проростків рослин томату, ріпаку, редису та салату посівного. У зонах агаризованого середовища, забарвлених меланіном, було виявлено кристали. Показано, що кристали утворюються на поверхні агаризованого живильного середовища, у товщі та на поверхні бактеріальних штрихів. Обговорюється можливий зв'язок між меланогенезом і утворенням кристалів, а також практичне застосування продукування меланіну бактеріями *Azotobacter chroococcum*.

Ключові слова: *Azotobacter chroococcum*, меланізація, кристали, кальцифікація.