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THE "GREEN" SYNTHESIS OF GOLD NANOPARTICLES BY THE YEAST *HANSENULA POLYMORPHA*

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Metallic nanoparticles (NPs) are important objects of nanotechnology due to their potential utilization in industry and medicine. Metallic NPs can be obtained through different physics-chemical methods as well as through "green" synthesis. In this research, the thermotolerant yeast *Hansenula polymorpha* was used as a biological tool for the "green" synthesis of gold NPs (AuNPs) from tetrachloroaurate (TCA). The processes of TCA reduction with yeast culture and extracellular formation of the stabile bioAuNPs were studied. The methods of transmission electron microscopy, scanning electron microscopy, fluorescent microscopy, UV-visible absorption spectroscopy were used to characterize the final products. The influence of various parameters, namely, time and concentration of TCA on the generation of AuNPs, was investigated. Our findings might be eventually interesting for fundamental and applied biological sciences, in particular, for the study of molecular mechanisms of cell protection from stress, caused by exposure to toxic compounds, as well as for the development of the novel effective and economical methods for obtaining new nanomaterials.

Keywords: Nanomaterials, bionanoparticles of gold, "green" synthesis, yeast *Hansenula polymorpha*.

Recent advances in nanotechnology have enabled the exploration of nanomaterials for diverse applications. Metallic nanoparticles (NPs) play a significant role in nanotechnology due to their potential utilization in industry and medicine [15]. Metallic NPs can be synthesized through different methods, including "green" reduction of correspondent ions [21]. Among the variety of nanomaterials, gold nanoparticles (AuNPs) are of considerable interest due to their versatility and potential use in chemistry, biology, and medicine. AuNPs possess numerous advantages, such as low cytotoxicity, facile modification of their surfaces, straightforward synthetic processes and biocompatibility [2, 5]. After cellular uptake, NPs can act as tiny, precise and powerful heaters (thermal scalpels) to kill cancer [3, 20], and they are capable of inducing apoptosis in B-chronic lymphocytic leukemia [13]. Such novel properties make metal NPs an ideal nanomaterial for promising applications in biological analysis and imaging, drug delivery, environmental monitoring, industrial catalysis, and electronic devices [6].

Due to their toxicity, NPs of noble metals have perspectives for pharmacology. However, despite their increasing impact on the market, many relevant issues are still open. These include the molecular mechanisms governing the NPs-cell interactions, the physico-chemical parameters underlying their toxicity to different types of cells, the lack of standardized methods and materials, and the uncertainty in the definition of general strategies to develop smart antibacterial drugs and devices based on NPs [12, 19].

The biosynthesis of NPs using microorganisms as emerging bionanotechnology has received considerable attention due to a growing need in environmentally friendly technologies [23]. Furthermore, the green synthesis of metallic NPs offer better manipulation, stabilization, and control over the crystal growth due to slower kinetics [17-18]. Many plants, polysaccharides, bacteria,

fungi, yeasts, DNA, RNA, proteins, and polypeptides are known to produce nanostructured mineral crystals and metallic NPs with the properties similar to chemically synthesized materials, while exercising strict control over size, shape and composition of the particles. Biological agents secrete a great deal of enzymes that bring about enzymatic reduction of metallic ions [4, 11, 16, 22].

The exact mechanism for the NPs "green" synthesis has not yet been elucidated in detail [10, 14].

In our recent experiments [24], different types of mono- and bi-metallic NPs of noble metals were synthesized by chemical methods and characterized.

In the current manuscript, we describe the methods of AuNPs biosynthesis using yeast *H. polymorpha* as a reducing and protecting agent, as well as the characteristics of resulting bioAuNPs (further — bioNPs). We propose our conception of the extracellular "green" synthesis and stabilization of bioNPs which is based on these results and earlier obtained data.

Materials and methods

Tetrachloroauric acid trihydrate (TCA), sodium hydroxide, inorganic salts, chloroform and Butvar solution B-98 were purchased from Sigma-Aldrich, USA. All buffers and standard solutions were prepared using the water purified by the Milli-Q system (Millipore).

The size and structure of NPs were studied with Atomic Force Microscopy (AFM), Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and UV-Visible spectroscopy as described earlier [24].

As a tool for the "green" synthesis of AuNPs the recombinant strain of the yeast *H. polymorpha* NCYC495-pGAP1-HsARG1 (*leu2car1 Sc:LEU2*) was used. The cells were cultivated as described in detail in our previous paper [23].

The biosynthesis of AuNPs by cell culture was performed according to the following procedure: after reaching the exponential growth phase, the cells were supplemented with TCA to the final concentration of 0.5 – 1 mM (control cells without TCA addition) and incubated up to 5 days. Each experiment was performed in triplicate and repeated at least three times.

The process of TCA reduction to Au^0 was monitored daily by sampling an aliquot of the tested sample and recording its absorption spectrum as well as TM, AFM and SEM.

Statistic treatment of measurement's results and the level of correlation between the values of the results obtained by different analytical methods will be calculated by Origin 8.0 and Microsoft Excel.

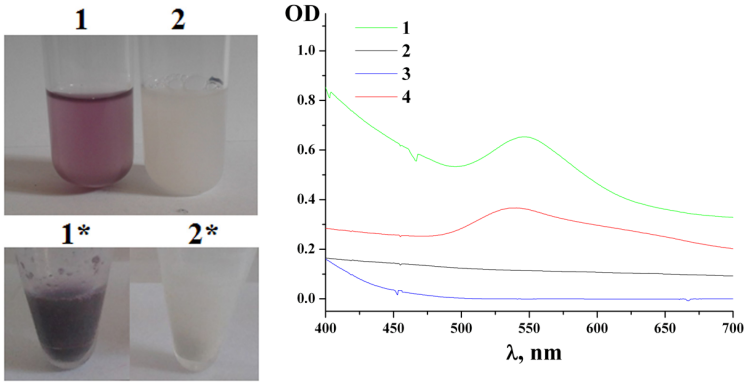
Results and discussion

The first brief characteristics of the process of TCA reduction and bioNPs generation were obtained by visual observation and UV-Vis absorption spectra (UVAS) (Fig. 1). The colour of the cell's suspension changed to dark red in comparison with that of the control one (Fig. 1 a, 1-2). The spectrum of the dark red cultural liquid (CL) - supernatant after cells removal has a peak of plasmon resonance at 540 nm (Fig. 1, b, 1), which is specific for AuNPs [1]. At the same time, the color of precipitated cells also changed (Fig. 1, a, 1*) – as a result of AuNPs accumulation inside the cells and formation of NPs-modified cells (further – cell-NPs).

To get the quick estimation of the sizes of metallic particles synthesized and to study their composition, SEM microscopy and XRM analysis were performed (Fig. 2). Fig. 2, a-b proves the generation of a large amount of individual spherical NPs in the cell culture, cultivated for 2 days in the presence of 1 mM TCA. XRM analysis of these NPs based on SEM data (indicated by the arrow in Fig. 2, b) proves the appearance outside the cells of Au^0 with characteristic peaks Kα at 2.1 keV (Fig. 2, c).

The more complex Au^0-containing structures on the surface of the cells, clusters with the size of 2 to 2.5 μm, are demonstrated in Fig. 2, d-f. The sizes of these clusters ranged from 50 to 100 μm. XRM analysis of these NPs based on SEM data (indicated by the arrow in Fig. 2, e) proves the appearance of Au^0 (Fig. 2, g). The cluster was reported to consist of glutatione, nitrate reductase and other proteins involved in bioreduction and stabilization of the formed bioNPs [9, 11].

Thus, the SEM and XRM results demonstrate the generation of AuNPs outside the cells in cell culture, incubated with TCA under growth conditions.



a b

Fig. 1. BioNPs formation: *a* – images of the cells suspensions (*1, 2*) and of the pellets of the corresponding cells (*1*, 2**); *b* – UVAS of CL (*1, 2*) and control solutions (*3, 4*). The cells were cultivated in the presence of 1 mM TCA (*1*) and without its addition (*2*); water solutions of initial 1 mM TCA (*3*) and the chemically synthesized AuNPs (*4*)

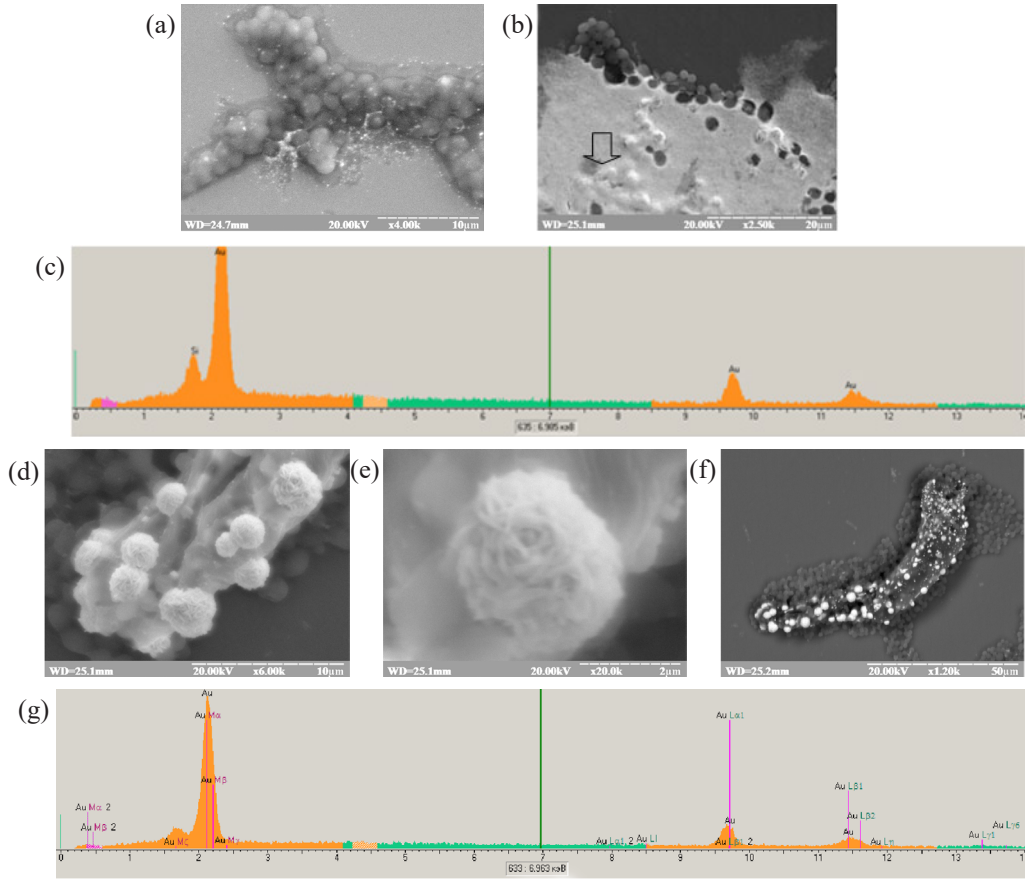


Fig. 2. Characteristics of bioAuNPs, synthesized "*in vivo*"(cultivated for 2 days in the presence of 1 mM TCA): SEM micrographs *(a, b, d-f)* and X-ray-spectral characteristics (c, *g*).

The exact size of the extracellular bioNP was estimated by the AFM method: the values of the average diameter were in the range of 20 – 40 nm (data not shown).

We assume that AuNPs synthesis takes place outside the cells due to TCA reduction with organic metabolites of yeast culture followed by bioNPs formation and stabilization. Then bioNPs penetrate through the cell wall and accumulate inside the cell. The results of TEM analysis have confirmed this suggestion (Fig. 3, a – b). It is worth mentioning that bioNPs, being in a rather high concentration (1 mM) after the penetration into a cell, cause the loss of cells-NPs ability to grow in agar medium (Fig. 3).

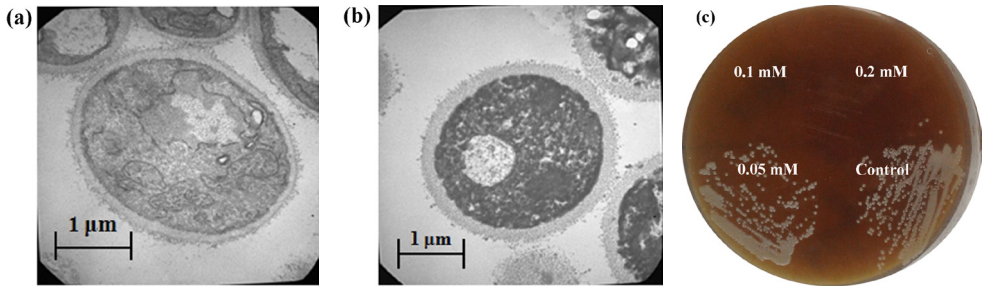


Fig. 3. Characteristics of the cells-NPs (b, c) in comparison with the control cells (*a, c*): Cells-NPs as well as conterol cells were cultivated during 4 days in the presence of 1 mM TCA (b) and 0.05 mM TCA, then aliquots of resulted cells were analysed by TEM (a, b) and growth ability (c)

In our previous research we have demonstrated that chemically synthesized NPs of noble metals [24], being in minimal concentration, proved to be non-toxic for the cell: under 3-day cultivation with 0.06 mM NPs, the yeast cells did not significantly affect either the dynamics of growth or enzyme (arginase) activity in cell free-extracts. We have demonstrated that the NPs uptake by cells was dependent on the concentration of NPs, exposure time and particles sizes. According to SEM- and TEM studies, AuNPs were shown to accumulate inside the cell [24].

It is worth mentioning that our conception of the extracellular formation of bioNPs is based on our previous results. It was discovered that the yeast *Pichia guilliermondii* is able to reduce chromate extracellularly with the formation of stable soluble Cr(III or V)-biocomplexes - bioNPs of Cr_2O_3 [7-9].

Fig. 4 (a-d) demonstrates the generation of the spherical clusters formed during bioreduction. According to the data of X-Ray spectral microanalysis (Fig. 4, e-f), these clusters consist of organic molecules which enveloped the AuNPs. The sizes of the clusters ranged from 6 to 10 µm.

It should be mentioned that the images in Fig. 4, c and d are identical, differing only in the shooting mode on SEM device: in Fig. 4, d micrographs are presented in the "compo" mode. This mode allows differentiating the nature of components: all organic atoms do not shine, although inorganic (AuNPs) ones glow brightly. These results were confirmed by the X-Ray spectral analysis (Fig. 4, e, f). The XRM data of AuNPs (which are glowing brightly in Fig. 4, d) have proved the formation of Au^0-containig compounds (Fig. 4, e). The dark grey color of the spherical cluster presented in Fig. 4, c corresponds to the molecules which contain atoms of C, O *etc* (Fig. 2, e).

Thus, the yeast *Hansenula polymorpha* was shown to be a promising tool for the development of a simple cost-effective method to obtain gold bioNPs by reducing toxic TCA.

We have demonstrated the extracellular generation of bioAuNPs of different sizes (20-40 nm) and shapes by the yeast *H. polymorpha*. The optimal conditions (concentration of inorganic ions and incubation time) required for the AuNPs formation were chosen. The nanosize and structure of NPs were proved using scanning, atomic force and transmission microscopy. The advantage of the "green" approach consists in the rapid growth of yeasts with a high-yield generation of AuNPs outside the cell, providing for the economic efficiency of the method.

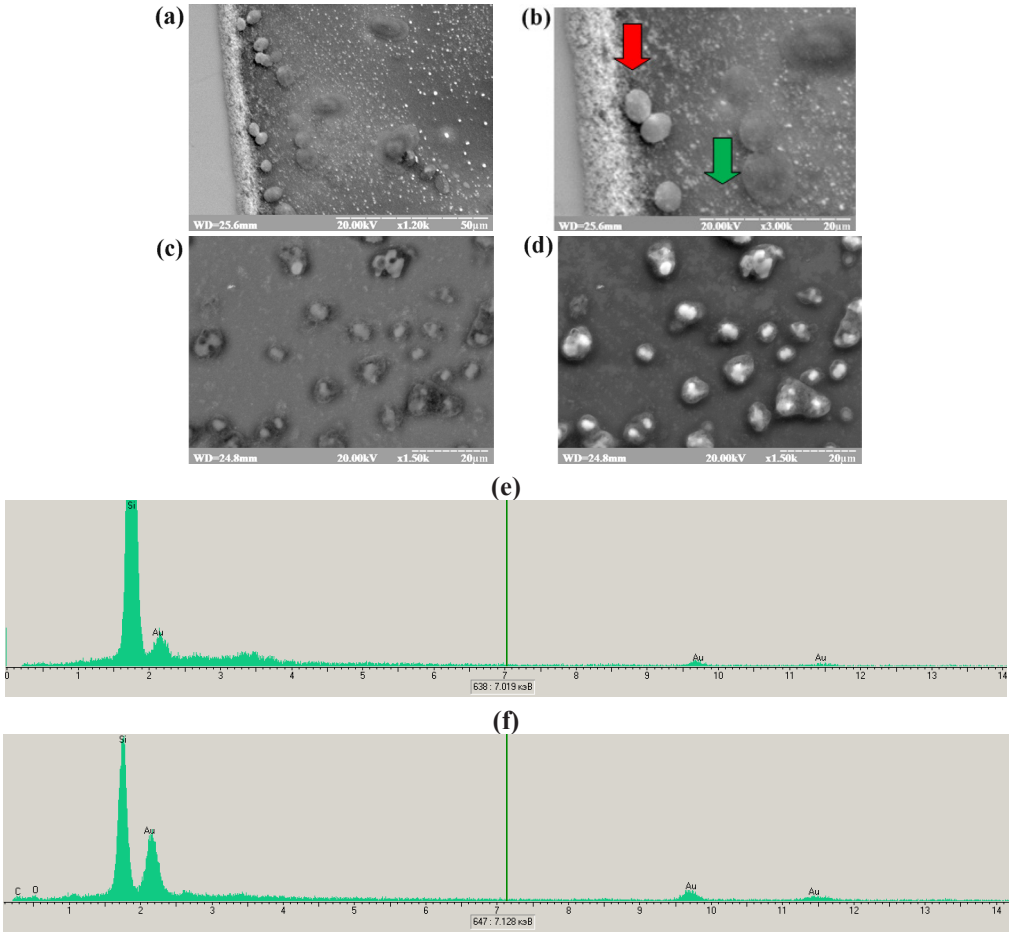


Fig. 5. SEM images (a – d) of Au-clusters formed extra-cellulary after cells growth in the presence of TCA during 4 days. X-ray-spectral characteristics (e, f) of the samples, indicated by red and green arrows, respectively

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"ЗЕЛЕНИЙ" СИНТЕЗ НАНОЧАСТИНОК ЗОЛОТА ДРІЖДЖАМИ *HANSENULA POLYMORPHA*

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Наночастинки металів (НЧ) є важливими об'єктами нанотехнологій завдяки перспективам їхнього застосування у промисловості й медицині. Металеві НЧ можуть бути отримані різними фізико-хімічними методами, а також «зеленим» синтезом. У даному дослідженні термотолерантні дріжджі *Hansenula polymorpha* було використано як біологічний інструмент для «зеленого» синтезу наночастинок золота (АuНЧ) з тетрахлораурату (ТХА). Було досліджено процеси редукції ТХА дріжджовою культурою та формування позаклітинних стабільних біоАuНЧ. Характеристику кінцевого продукту було здійснено із застосуванням методів трансмісійної електронної мікроскопії, скануючої електронної мікроскопії, флуоресцентної мікроскопії, УФ-видимої адсорбційної спектроскопії. Було вивчено вплив різних параметрів, зокрема, часу та концентрації ТХА, на формування АuНЧ. Наші результати можуть бути цікавими для фундаментальної та прикладної біологічної науки, особливо для досліджень молекулярних механізмів захисту клітини від стресу, спричиненого токсичною сполукою, а також для розробки нових ефективних і економічно вигідних методів одержання нових наноматеріалів.

Ключові слова: наноматеріали, біонаночастинки золота, "зелений" синтез, дріжджі *Hansenula polymorpha.*

"ЗЕЛЕНЫЙ" СИНТЕЗ НАНОЧАСТИЦ ЗОЛОТА ДРОЖЖАМИ *HANSENULA POLYMORPHA*

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Наночастицы металлов (НЧ) являются важными объектами нанотехнологий благодаря перспективам их использования в промышленности и медицине. НЧ металлов можно получать различными физико-химическими методами, а также «зеленым» синтезом. В данном исследовании термотолерантные дрожжи *Hansenula polymorpha* были использованы как биологический инструмент для «зеленого» синтеза наночастиц золота (АuНЧ) из тетрахлораурата (ТХА). Были изучены процессы редукции ТХА дрожжевой культурой и формирование внеклеточных стабильных биоАuНЧ. Конечный продукт характеризовали, используя методы трансмиссионной электронной микроскопии, сканирующей электронной микроскопии, флуоресцентной микроскопии, УФ-видимой адсорбционной спектроскопии. Было изучено влияние различных параметров, в том числе времени и концентрации ТХА, на формирование АuНЧ. Наши результаты могут быть интересны для фундаментальной и прикладной биологичной науки, особенно для исследований молекулярных механизмов защиты клетки от стресса, вызванного токсическим соединением, а также для разработки эффективных и экономически выгодных методов получения новых наноматериалов.

Ключевые слова: наноматериалы, бионаночастицы золота, "зеленый" синтез, дрожжи *Hansenula polymorpha.*