Features of Biogenic Nanoparticle Formation in Agarose Gels and Their Effect on Cells Growth During Bulk Cultivation

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The following article contains critical notes about the possibility of using the observation of biogenic nanoparticles growth (OBNG) methodology to control the living cells' physiological state during their cultivation in agarose gel and its volume. The methodology's main idea is to add a low-concentrated silver nitrate solution to the immobilized cells. Cells are able to inhibit the negative effects of silver ions recovering them and forming nanoparticles are being detected by spectrometric methods. It is experimentally proved that the successful use of this methodology for cells state monitoring can only be applicable after choosing a special agarose with a low level of electroendoosmosis (EEO). This type of agarose itself does not lead to the silver nanoparticles formation. All the obtained nanoparticles are only the interaction product of silver nitrate with living cells. It was found that agarose gel layers containing silver nanoparticles prevent the migration of immobilized cells by division.

1. Introduction

Targeted cultivation of all types of cells starting from the most complex such as stem cells moving on to relatively simple like yeast cells require special devices (bioreactors). Bioreactors are meant to maintain the parameters such as temperature, pH, and osmotic pressure in certain circumstances. It is also necessary to provide growing cells with nutrition and oxygen and to get rid of the products of their metabolism. Bioreactors are designed to provide all the necessary physiological conditions for immobilized cells (Rodrigues et al., 2011). The distinction of cell growth and division factors from optimal ones leads to metabolic activity inhibition and to the growth and divisional termination (Placzek et al., 2009).

As it is considered in biotechnology, the microorganisms' cultivation is carried out in a deep way in the liquid phase (Kurtzman et al., 2011). As the result of mixing, there could not be any diffusion restrictions on mass transfer so cells have access to nutrients. The removal of cells' metabolites is also easier to carry out in the liquid environment.

Previously the observation of biogenic nanoparticles growth (OBNG) methodology was offered with the aim of monitoring the physiological state of microorganisms during their volume cultivation in a nutrient solution (Sorokin et al., 2013; Skladnev et al., 2017). The idea is that metabolically active cells are able to form nanoparticles of reduced silver (Ag°) from a solution of silver salt in a liquid environment with cells for a limited amount of time. Accordingly, the formation of nanoparticles is sharply inhibited when the physiological state of cells is weakened. Electron microscopy and optical spectrometry were used to identify nanoparticles.

Nowadays such a new scientific direction as bioprinting is being formed (Murphy and Atala, 2014). Implementation of the idea of 3D printing human organs from stem cells will provide a breakthrough in the development of regenerative medicine (Turksen, 2015). Hydrogels are promising materials where cultured cells can be mobilized for bioprinting (Wang et al., 2015; Jang et al., 2018). Gels can be used as bioinks that form the structures for cell growth (Kesti et al., 2015). The rheological properties of previously mentioned materials make it possible to form complexly configured bioreactors with immobilized cells based on layer-

by-layer stacking of variously concentrated and composed gels. The gel capillary network can be used to transport nutrients to individual cells and to remove products of cellular metabolism (Pokusaev et al., 2017b).

The problem of in-gel controlled cells growth is sophisticated and is not solved yet. One of the approaches is to create a system of artificial in-gel microchannels for uniform supply of nutrients to its volume (Pokusaev et al., 2020a), which are an analogue of the vascular circulatory system (Richards et al., 2017). In this case, the problem of controlling the cells' metabolism during their growth and division are arises. The research on the cells' reverse effect to the changes in gel properties has begun recently (Buckley et al., 2009).

The main issue of living cells' in-gel growth using bioreactors is the physiological state monitoring. Monitoring methods should be non-contact in order to prevent the impact of measurements on growing cells whenever possible. Since gel is an optically transparent media, optical methods are well suited for this purpose (Pokusaev et al., 2020b). However, the determination of changes in optical density gives approximate results taking into account that there is intensive cell division.

A simple transfer from the OBSN methodology as it was used in liquid media to the study of the physiological state of living cells in gels is not possible without pointing out their specific properties (Pokusaev et al., 2017a). For example glucose is known for contributing to the nanoparticles formation from silver salt, and this may also occur when using agarose. The resulting nanoparticles, although neutral, the gels' transport properties (Pokusaev et al., 2016). A large difference was found in the rates of nutrient diffusion in pure gels and gels with nanoparticles. As a result, their delivery to cells slows down, which results in slowing down the cell growth. For this type of system, the critical aspect is the size of nanoparticles compared to the internal scales of microchannels in the transport medium (Ozturk et al., 2010).

The area of nanoparticles contained gels' application is constantly expanding. For example, the use of nanoparticles as fillers in the production of nanocomposites from polymers has attracted much attention due to the increased demand for new materials with improved properties (Rahman and Padavettan, 2012). Gels are used in electrophoresis to separate nanoparticles by shape and size to produce calibrated nanomaterials (Hanauer et al., 2007). Recently, Rose et al. (2014) showed that strong and fast adhesion between two hydrogels can be achieved by applying of nanoparticle solution. In addition, numerous new natural and synthetic hydrogels and nanomaterials are currently being synthesized to be used in regenerative medicine and bioprinting (see, for example, Ignatieva et al., 2019; Lou-Franco et al., 2021)

This paper discusses the possibility of using the OBNG methodology to determine the physiological state of cells cultured in the gel volume. The possibility of the influence of the agarose gel composition on the intensity of nanoparticle formation is considered. The study shows that the choice of a certain type of agarose enables the formation of exclusively biogenic silver nanoparticles. In addition, it is on behalf to determine the effect of nanoparticles on the migration ability of living cells in gels as a result of their division, which may be a promising method for the formation of ordered cell structures.

2. Materials and methods

Yarrowia lipolytica yeast cells were used as a model biological object. Circumstances for cell immobilization in the gel were carried out by seeding them into a 1 mm^3 gel containing 10 % of the LB growth medium. The yeast cells' suspension was sown an agarose gel at 40 °C with a 10^7 cells/mm^3 concentration. The yeast cells seeded gel was placed into a two-layer gel structure on top of the same, but sterile gel.

The agarose's properties are known to vary depending on the manufacturer. Gels were prepared by mixing agarose with distilled water when heated to 90 °C. The mass concentration of agarose in the gel is 0.4 weight %. Two types of agarose with different levels of electroendoosmosis (EEO) are used in experiments. The first type is "Chemapol" agarose (designated as A_0), which has a relatively high level of EEO. This is important in further research, as it can be a chemical reducing agent of silver salt cations and forms a certain number of nanoparticles Ag . The second type is agarose "Serva" (designated as A_1), which has a low EEO level. It has no potential for chemical reduction of the introduced silver cations and is not able to form Ag nanoparticles independently without the presence (in the experiment) of live yeast cells immobilized in the gel. Having being prepared from both agarose A_0 and A_1, the gels demonstrated almost identical growth of yeast.

The formation of biogenic silver nanoparticles is carried out according to the OBNG methodology. A solution of silver nitrate salt $AgNO_3$ is used as a source of cations, introduced into the molten agarose gel (at a temperature of 40 °C) immediately before cooling it at a final concentrations 5 or 500 µg/ml. By the electronic microscopy it was found that even the minimal concentration of $AgNO_3$ salt (5 µg/ml) is sufficient to ensure stable silver nanoparticles formation. Both concentrations of silver nitrate are non-toxic for the selected yeast species.

The final samples of gels were formed in standard spectrometric cuvettes with a size of 10×10×40 mm. To form two-layer samples 1 ml (cm^3) of gels melted and then cooled in a water bath to 42 °C (in a liquid state), then introduced into the cuvette. After solidification of the lower layer at room temperature (~25 °C), the top

layer of gel of the same volume (1 cm^3) was similarly applied. The insertion of fresh yeast cells suspensions in the required concentration was additionally carried out in the upper gel.

A spectroscopic method is used to investigate the gels with immobilized cells and nanoparticles. To assess the time evolution of the system and control the silver nanoparticles formation, the gels' spectral density was recorded using a fiber-optic spectrometric system based on OceanOptics equipment (Pokusaev et al., 2017a). Optical fibers with a diameter of 0.2 mm are used as light guides. The main part of the experimental setup consists of a USB 2000+VIS-NIR spectrophotometer, which includes a 2048-element detector Sony ILX511. An optical cuvette contains a gel sample and is installed in an air thermostat. Incubation of cuvettes with yeast cultures immobilized in the upper layer of two-layer gel structures was carried out at a temperature of 28 °C. The system allows measurements to be made at a point of 1.0 mm^2 over the entire height of the cuvette.

3. Results and discussion

First of all, as it was recently described (Sorokin et al., 2013), the addition of silver nitrate AgNO$_3$ solution leads to the formation of nanoparticles of 25-70 nm in size within 20-30 minutes for yeast cells growing in a liquid nutrient medium. The nanoparticles formation was clearly observed using a transmission electron microscope, and recorded by spectrometry (a specific peak close to the light wavelength of 400 nm). Nanoparticles were not formed in a sterile liquid nutrient medium without living cells. The reduction of cations was stopped at the phase not exceeding clusters of silver atoms (up to 0.5-2 nm). Such clusters were detected using an electronic microscope, but they are not detected by spectroscopy.

Comparison of the yeast growth dynamics in agarose gels with a different reduction potential level should begin with a transmission spectra comparison of hydrogels with 0.4 % by weight concentration of agarose prepared from its two types A_0 and A_1 (with a reduced level of EEO). The transmission spectra for both types of agarose gels are shown in Figure 1. The spectra represent the dependence of the transmission value T (absolute units) on the wavelength λ (nm) of the transmitted light and are similar to each other, but the agarose gel A_0 is optically denser. The reason for this difference is distinction in production technology. Further special interest will represent the gel transmittance T_{600} at a 600 nm wavelength, which characterizes the increase in the living cells concentration and the change in transmittance T_{400} at a wavelength of 400 nm, which characterizes the increase in the biogenic and abiogenic nanoparticles concentration.
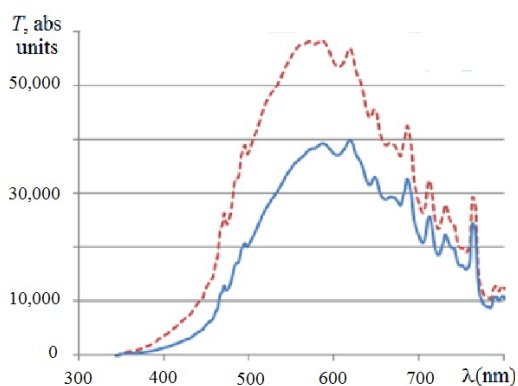

Figure 1: Hydrogels transmission spectra with the same agarose concentration of 0.4 % by weight prepared from agarose A_0 (solid line) and A_1 (dotted line)

Incubation of measuring cells with yeast cultures immobilized in the upper layer of two-layer gel structures was carried out under optimal conditions for 7 days, periodically measuring the upper and lower gels optical permeability. According to preliminary experimental measurements, the live yeast cells concentration during this time under such conditions increased by about an order of magnitude.

We should point out that it is possible to form both biogenic and abiogenic nanoparticles in gel with agarose A_0 but only biogenic ones in gel with agarose A_1. During the first two hours of the experiment visual observation revealed the yellow-brown color absence (with the silver nitrate solution added) characteristic of the forming nanoparticles for gels with agarose A_1, compared to gels with agarose A_0 (where the color already appeared after this time). The measuring results the optical transmission for upper gel samples with cells from time to time at different initial concentrations of the silver nitrate (AgNO$_3$) salt as a cations source for the formation of reduced silver Ag   nanoparticles are shown in Figure 2.

Samples observations of agarose gels various types showed that in the silver salt presence in gels with agarose A_0, a rapid formation of abiogenic Ag nanoparticles was observed, and the yeast cells presence only enhanced this color. On the contrary, when a sterile silver salt solution was applied directly in gels with agarose A_1 containing yeast cells, a gradual characteristic color development was observed in proportion to the concentration of silver cations and the yeast cells concentration.
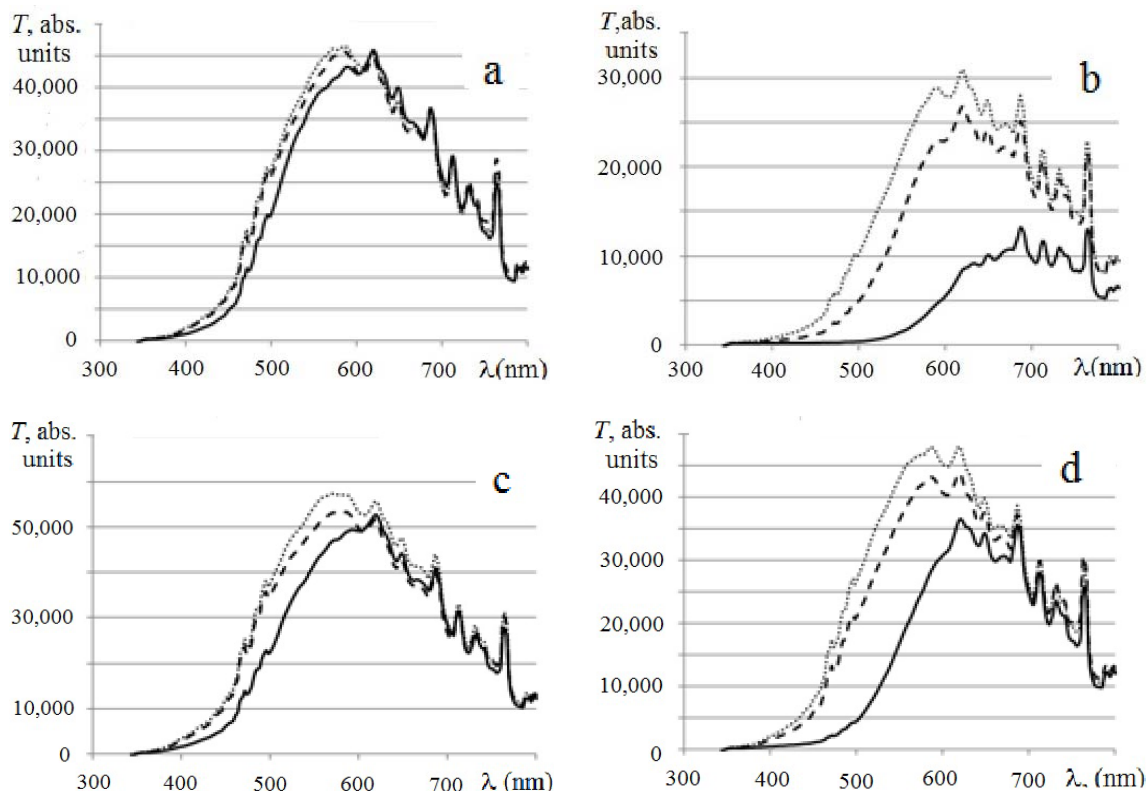

Figure 2: Transmission spectra for upper layer of bilayer hydrogels with an agarose concentration of 0.4 % by weight prepared from agarose A_0 (a and b) and A_1 (c and d) at different silver nitrate concentrations: 5 µg/ml (a and c) or 500 µg/ml (b and d). Curve designations: dotted line – two hours after preparation, dotted line – after a day, solid line - after 7 days

Figure 3 shows the results of changing the light transmission intensity of the upper layers of agarose gels using agarose of various types with an increase in the concentration of yeast under the 10^7 cells/ml initial density at different initial concentrations of the introduced silver nitrate salt. We should notice that in all cases there is an increase in the density of the immobilized cell suspension (an incomprehensible exception is the curve 1 - gel with a minimum silver nitrate addition). The most distinct increase in the yeast cells number over 7 days is observed in the silver nitrate presence with 500 µg/ml concentration (curves 2 and 6). Since the growth is more distinct in gel with agarose A_0 at high silver salt concentrations, it is likely that the rapid and efficient formation of silver nanoparticles Ag^0 protects cells from non-covered toxic Ag^+ cations by preventing their diffusion to cells.

To analyze the number of biogenic nanoparticles produced during the yeast cells cultivation, the light transmission spectra of two agarose gels types containing immobilized yeast cells, as well as different silver nitrate salt solution concentrations, were compared using spectrometry data at a light with 400 nm wave length. Figure 3 shows that within a day after the silver salt being added to gels with agarose A_0 (curves 3 and 4), the optical permeability was significantly lower than in similar gels based on agarose A_1 (curves 7 and 8) in absolute units. It was curious to find out that the light transmission in gel with agarose A1 with a maximum silver salt added with a concentration of 500 µg/ml (curve 8) and gel with agarose A_0 with a minimum of 5 µg/ml concentration (curve 3) are almost identical. It means that silver nanoparticles are formed less efficiently (only at the expense of cells) in the gel with agarose A_1 with low EEO.

The interaction of cells immobilized in agarose A_0 and silver salts under a 500 µg/ml concentration is fascinating (curves 2 and 4). The rapid formation of numerous nanoparticles, as evidenced by the minimum

light permeability level after a day of incubation - 22000 (curve 4), is also accompanied by a drop in light permeability due to the yeast culture growth in agarose A0, detected at 600 nm.

It is important to analyze the drop in light permeability for agarose gel A_1. At a high silver nitrate salt concentration, an approximately proportional decrease in light permeability is observed both due to an increase in the yeast concentration (measurements at 600 nm, curve 6) and due to the formation of nanoparticles (measurements at 400 nm, curve 8). In contrast, when a silver salt is added at a low concentration, the permeability is mainly reduced by the nanoparticles formed (measurement at 400 nm, curve 7). This result indicates that low concentrations of artificially added cation source should be used to monitor the immobilized cells physiological state by controlling from the silver nanoparticles formation.
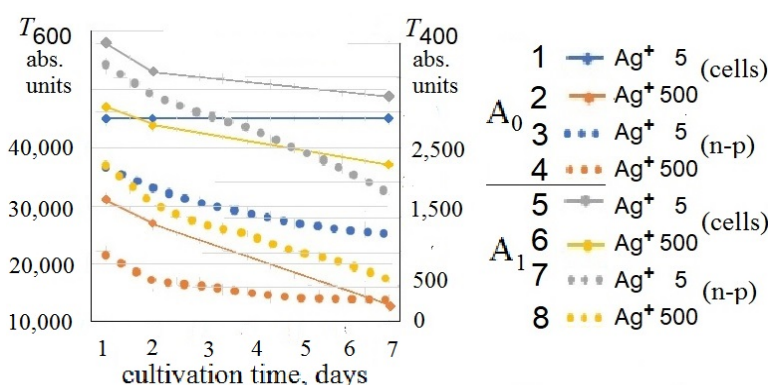

Figure 3: Transmission spectra for the upper layers of double-layer hydrogels prepared from 0.4 % by weight agarose types A_0 and A_1 containing immobilized yeast cells, as well as different concentrations of silver salt solution $AgNO_3$, as a cations source for the biogenic nanoparticles formation. Solid lines represent temporary changes in the gel layers light permeability due to the growth of the cells number (measured at a light 600 nm wavelength - left scale), point curves correspond to temporary changes in the gel light permeability caused by the biogenic Ag^0 silver nanoparticles formation (measured at a light 400 nm wavelength - right scale)

Previously, while studying the growth dynamics of yeast cells in two-layer gels systems, when both gel layers contain nutrients and only one is seeded with cells, cells germination was observed from one layer to another due to its division. Since nanoparticles change the gels properties, it can influence the dynamics of immobilized cells growth and migration. It can be assumed that yeast cells being immobilized in the volume of agarose gel cannot germinate through the gel layer containing nanoparticles. This property of gel layers with nanoparticles can be very useful in 3D bioprinting technology.

The ability of growing yeast cells to penetrate through a thin layer of gel filled with prepared Ag   silver nanoparticles was experimentally estimated for verification needs. To form such layer, a concentrated preparation of already formed (stabilized) Ag   nanoparticles (with a volume of 100 µl) was applied to the lower gel layer upper surface at the geleration time. After cooling to room temperature, the top gel layer containing fresh yeast cells was applied to the layer gel with nanoparticles.

In conclusion for all the experiments, spectrometric measurements did not show a decrease in the optical permeability over time in the lower layer under the gel layer with nanoparticles. This means that there is no yeast cells growth in the lower gel layer and this can only be if it were unable to grow through the gel layer with nanoparticles. The obtained data confirm the earlier assumption that gels layers enriched with prepared silver nanoparticles can be used to prevent the immobilized cells migration in an undesirable directions.

4. Conclusions

Adding silver nitrate concentrated below 5 µg/ml can be used to determine the cells' state cultured in the gel volume. Growing and dividing yeast cells are able to restore silver cations and form nanoparticles. It is shown that for the purpose of determining the cells' functional state by this method, it is necessary to use agarose with a low EEO level. Such agaroses themselves do not lead to the nanoparticles formation, and all the resulting nanoparticles are the product of the interaction between the silver nitrate salt and living cells.

The ability of immobilized yeast cells to overcome a thin layer of gel containing ready recovery silver nanoparticles was evaluated using the technique of multilayer gel structures. Cells germination due to division through such a layer was not detected. This method can be used to prevent spontaneous immobilized cells migration in undesirable directions.

Acknowledgments

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