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Microorganisms Growth in Gel Volume: Process Dynamics in Limiting Mass Transfer Conditions

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The work purpose is to find ways to effectively cultivate living microorganisms not near the outer surface, but inside the gel matrix volume by internal channels using, when the delivery of nutrients to the gel volume is carried out by a convective-diffusion mechanism. Immobilized cells development dynamics in gel under limited mass transfer conditions of nutrient across the surface was considered. The penetration depth of the nutrient from surface into the gel volume for providing a stable life of microorganisms has been estimated at 3.0 – 4.0 mm. The forming time-stable linear and branched channels possibility within the gel matrix volume has been experimentally confirmed and it usage for convective nutrient supply to the volume was tested. The qualitative nutrients diffusion regularities from the channels into the gel volume with immobilized cells are described.

1. Introduction

Currently, a new scientific direction is being formed, which is called bioprinting (Murphy and Atala, 2014). The ideas of growing human organs from stem cells no longer seems impossible and even the first successes in this direction are known (see for example, Melchels et al., 2012).
The bioprinting idea is implemented in the bio-printers. They are filled with cellular spheroids, which are applied in a certain order to a framework (scaffold) and thus form the basis for growing the organ (Rodrigues et al., 2011). However, it is not yet an organ, but rather an engineering construction that has a form of an organ. It can be called an organ when stem cells begin to grow, divide and differentiate. For this reason, the cells must be in suitable conditions to enable their immobilization. In particular, they must be provided with the necessary amount of nutrients for normal life.
Promising materials for usage in bioprinting are hydrogels (Wang et al., 2015). There is a formal analogy between the mass transfer in gels with living cells and filtration with the formation of deposits in the pores (Taran et al., 2019). Gels capillary network is able to supply living cells with nutrients and oxygen, as well as remove metabolic products. This property depends on the capillaries size and the diffusion coefficient, which decrease with increasing concentration of the dispersed phase gel (Pokusaev et al., 2015; Pokusaev et al., 2019). It was found that the diffusion rate mass transfer in gels does not exceed the maximum possible for the pure dispersion phase (Pokusaev et al., 2018). It is obvious that the transfer of nutrients through the outer boundary of the formed organ due to its large size only due to diffusion will not be able to provide living cells with the necessary amount of nutrients and oxygen.
For industrial biotechnology, the microorganisms growth problem in the gel volume is not fundamentally important, since the processes of cultivation of microorganisms in bioreactors are more convenient to carry out the deep method in the liquid phase, when there is no diffusion resistance to the mass transfer of nutrients to the cells. However, when growing tissues from stem cells in a gel, providing them with nutrients and oxygen becomes essential. In recent years, this causes an increase of interest in research related to additive methods creation (and not only) analogues of circulatory systems that provide a solution to the mass exchange problem of living cells with the environment (see, for example, Richards et al., 2017; Sasmal et al., 2018).
Further, the experimental results for determine the possibility for providing nutrition and oxygen immobilized in the gel volume of living cells directly by mass transfer through the capillary network inside the gel from the

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surface contacting with the nutrient medium will considered. Also, some results on the creation of artificial time-stable microchannels in the gel and data on modeling the nutrient medium delivery through them to living cells directly into the gel volume will presented.

2. Materials and methods

In the experiments, both pure gels based on agarose "Chemapol" and gels with the addition of yeast culture with a nutrient solution were used. The concentration of agarose in the gels is varied in the range of 0.6 – 1.5 % by weight. At such concentrations, agarose gels are optically transparent, which makes it possible to study the growth kinetics of yeast culture by non-contact optical methods, without violating the conditions of its metabolism.

Pichia polymorpha Y-314 culture was chosen as a model for experiments with living cells, since it is close to human somatic regarding the cell size (20 – 25 microns – the size of yeast cells and 25 microns – the size of human liver parenchyma hepatocytes) and life conditions. The culture is capable of glucose fermentation as the only source of carbon and grows well at temperatures from 30 to 42 °C.

To visualize the mass transfer processes and measure the diffusion rate from the channels to the volume of agarose gels in the experiments, a 1.0 % aqueous fuchsin solution was used, which is sometimes added to nutrient media. Fuchsin (hydrochloric acid rosaniline) $C_{20}H_{20}N_3Cl$ is a substance with a high molecular weight, aqueous solutions of which has a purple-red color and has a high contrast against the gel.

To study the unsteady mass transfer processes in agarose gels containing, among other things, biological cultures, an experimental setup was created that allows to register the diffusion of the substance into the samples under study (see Figure 1). For this purpose, a two-beam spectrophotometer UV-1280 manufactured by "Shimadzu" was included in the previously developed setup based on the spectrometric method. The technical capabilities of such equipment ensured the possibility for measurements in these studies by light transmission and absorption spectra at several wavelengths in the range of 190 – 1100 nm in automatic mode. In addition to the working area, a special system for measuring the position of the test sample in space was installed. For mass transfer processes registration in gel systems with artificial microchannels the working site including the photo-registering device of high resolution was used.

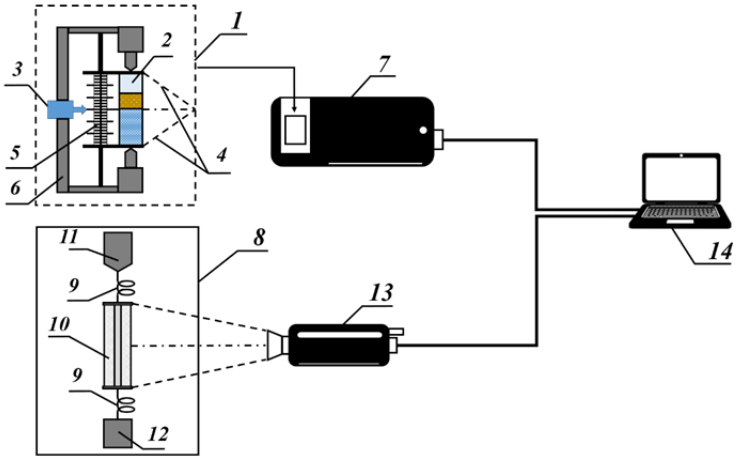

Figure 1: Scheme of the experimental setup: 1– working area 1: 2 – optical cell with gel, 3 – level indicator, 4 – working area of scanning, 5 – scale of height measurement, 6 – cell holder, 7 – Shimadzu spectrophotometer, 8 – working area 2: 9 – system of connecting channels, 10 – optical cell with gel with channels, 11 – tank with nutrient medium, 12 – collection tank, 13 – camera, 14 – computer

3. Results and discussions

3.1 Preliminary comments

Research using stem cells is a complex problem that requires the fulfillment of sterility conditions and the creation of multifactorial comfortable conditions for cell growth. The selection of suitable gels is also a difficult task related to materials science (see, for example, Lin et al., 2011). Obtaining concrete practical results will require taking into account and implementing the conditions associated with the specifics of the selected biological objects and gels. However, for the purposes of primary basic research, many exhibited qualities by different living cells and the properties of different gels have similar features. Therefore, at the first stage, it is

possible to perform experiments on simpler and more convenient objects in order to obtain results that allow to clarify the directions of research.

When performing experiments with microbiological objects, analyzing their results and creating mathematical models of mass transfer, it is necessary to take into account that the consumption rate of various nutrient medium components by yeast is different. However, the rate of consumption of each component is proportional to the concentration of cells. The accumulation of biomass of microorganisms in a periodic process of growth without any additional components has the following stages: cells adaptation period to the environment without increasing their numbers (for the using yeast culture at the optimum temperature 2 - 6 h); exponential phase, characterized by a maximum growth rate of the number of cells (4 - 8 h), stationary phase in which growth rate and death equal to (2 - 4 h) and stage of death (with the exhaustion of the nutrient medium).

The level of biomass accumulation for each strain of microorganisms is determined experimentally and depends on the initial number of seeded cells, the nutrient medium amount and temperature. The maximum rate of consumption of the nutrient medium at the carbon source, as it is the main structural component used for intracellular synthesis of all biopolymers. The rate of carbon consumption is directly proportional to the concentration of cells and is characterized by a carbon-to-biomass conversion rate.

Following the abovementioned, to describe the temporal and spatial dynamics of concentration fields for the nutrient and the number of living yeast cells in the gel, models such as population dynamics with a delay should be used (Polyanin et al., 2018). They include at least two equations. The first is a non-stationary diffusion equation describes the material balance of the nutrient taking into account its absorption by cells. The second equation is an ordinary differential equation with a delay, describing the change in the concentration of yeast over time, taking into account their reproduction by cell division. The delay is associated with the presence of cells adaptation period due to which the microorganisms growth rate at the current time will be determined by their concentration at earlier times and the duration of adaptation.

When modeling the growth of microorganisms in the gel volume under feeding through the surface, the kinetic model based on an ordinary differential equation with a delay is the most physically justified. In this case, the delay has a clear physical meaning, as the duration of the time adaptation interval of microorganisms to the environment before the beginning of division. Among the factors hindering the growth in the number of cells, consider the following: a lack of the nutrient medium during transport to the cells through diffusion, the gel mechanical resistance under increase the volume occupied by microorganisms, excretion by cells of metabolic products that inhibit their growth.

3.2 Cells reproduction kinetics under condition of diffusion limitation

The influence of mass transfer diffusion restriction on the kinetics of living cells reproduction in the volume of layered gel systems is determined. Layered samples were prepared in a spectrometric cuvette with a size of 10×10×40 mm. Initially, a lower layer of gel was formed, after stabilization of which, the next layer was applied on top. Yeast cells were seeded in the top layer of the gel, and the bottom layer remained clean. The height of the lower layer was 10 mm, and the top layer of gel with cells was 4 mm. After the formation 1.0 ml nutrient medium with small concentration was poured onto the top layer. Further, the optical permeability of the test sample was measured at a wavelength of light of 540 nm. Studies were carried out at a temperature of 30 °C.

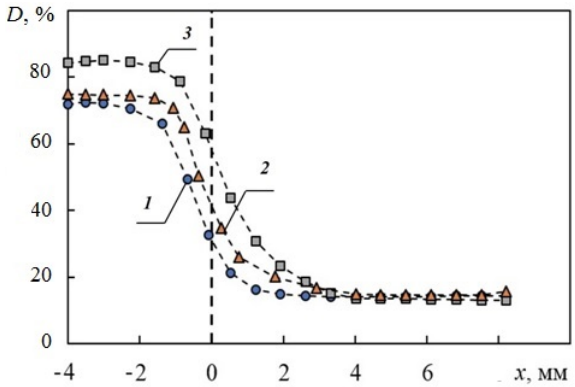

Figure 2: Dependence from depth (x, mm) at different times of the relative (to distilled water) intensity of light absorption (D, %) in 0.6 % by weight agarose gel. Notation on the horizontal x axis: 0 – the interface of pure gel and gel with cells, values with a minus - a gel layer with cells, plus - pure agarose gel. Notation of curves: 1 – 30 min after the feeding of the nutrient medium, 2 – 6 h, 3 – 24 h

Figure 2 shows the results of measurements of light absorption intensity by height of a two-layer gel sample with agarose concentration for each layer equal to 0.6 % by weight. The zero point in the height of the cell corresponds to the boundary between the interface of the pure gel and the gel with cells. Positive values of the coordinate are counted into deep the pure gel, and negative values are counted into deep the gel with cells. Dependencies are presented for different times of the process. It can be seen that in the area of the cells height $-4<x<0$ mm, the increase in the absorption intensity associated with an increase in the cells concentration in the gel is noticeable after 24 hours from the beginning of the measurement. Below the interface of the two layers of gels in the height $2<x<10$ mm, the values of the absorption intensity do not change with time.

Experiments show that within 24 hours the bottom of the cells nutrient medium does not reach, although according to calculations, based on the value of the diffusion coefficient for the gel with a weight concentration of agarose 0.6 % and in the absence of its absorption by cells, should reach. Thus, the lower gel layer with cells do not receive nutrition due to its intensive absorption by the upper layer.

The most interesting is the area near the gels layers interface in the cells height $-3<x<4$ mm. This area is transitional and an important issue is the germination of cells in the adjacent layer of gel (Pokusaev et al., 2017). As follows from curves 2 and 3, in the region of the interface between the layers of gels $-2<x<2$ mm, there is an increase in the intensity of light absorption compared to curve 1. Measurements near gel layers interface, representing a curved meniscus filled with liquid are not accurate due to increased light scattering on curved surfaces. However, the data obtained suggest that an increase in the absorption intensity after 24 h relative to the initial values is associated with an increase in the concentration of yeast cells. In this case, part of the cells in the division process passes into the lower gel layer to a depth of about 0.5 – 2.5 mm.

Results allow us to estimate the depth of localization of the cells growth zone near the surface through which the nutrient is fed, about 3.0 – 4.0 mm. Mechanism of predominant cells growth in the gel in this zone is as follows. In the case when the initial cells concentration is small and the rate of diffusion supply of the nutrient medium to the volume exceeds the rate of absorption by the cells in the adaptation state, the nutrient begins to accumulate in the gel surface layers. In this area, at times exceeding the duration of the delay, an increase in the cells concentration begins due to their division. Accordingly, the absorption of nutrients increases proportionally. With an exponential increase in the cells number, the diffusion nutrient supply into the gel through the interface does not compensate for the total nutrient absorption by cells in the gel volume. Moreover, the further away the cells are from the surface, the less they get nutrient due to its intensive absorption in the surface layers. The closer to the border, the more cells and the higher the absorption of the nutrient medium. As a result, the cells in the depth of the gel remain without nutrient, stop dividing, and most of them die. This mechanism of mass transfer leads to the fact that the cells are localized only near the surface through which the nutrient enters the gel.

3.3 Mass transfer in gel through artificial channels

To ensure the cells growth in the gel, it is necessary to introduce nutrient directly into its volume, for example, with the help of special artificial channels. In this case, the diffusion of the nutrient through the walls of the channels into the volume will provide nutrition to the cells inside the gel. To avoid internal diffusion resistance within the channel, the nutrient in them must be in motion. Thus, the artificial channels system inside the gel should functionally be similar to the blood vessels in the organs.

To solve this problem we have developed a complex system of branched channels in the agarose gel volume. Experimental modeling of the channel system was carried out as in the cells for optical measurements. As an example, in Figure 3 the photos of the obtained samples of the channel network are presented. The channels were manufactured using flexible metal cylindrical rods in gel with an agarose concentration of 1.0 % by weight. After stabilizing the gel to a temperature of 20 °C and removing the channel-forming rods from them, fuchsin was supplied to the samples through the connecting system of tubes. As can be seen in the photos, the obtained channels retain their shape and are able to perform the function of a system for transporting vessels in order to provide nutrition to the yeast culture.

In Figure 3, the capillary system of channels is shown with varying degrees of optical magnification. Some defects noted in the photos (widely colored fuchsin areas along the channels) in comparison with the whole channels were due to gel deformation at the time of channel-forming rods removal. It can be seen that a crack was formed in the channel deformed due to mechanical action, which was instantly filled with fuchsin when it was fed. At the same time, with the fuchsin arrival in the deformed region, due to the pressure created by it, an air bubble is squeezed out of it and subsequently removed from the channel with the liquid flow. It is experimentally established that channels, despite the phenomenon of syneresis in gel, remain stable over the time. It is practically confirmed that through them it is possible to pass a liquid flow of nutrient with a volumetric flow rate of several microliters per second and this does not affect the condition of the channels.

Figure 4 presents experimental results on mass transfer of fuchsin from the surface to the gel volume in the presence of artificially formed channels. In the experiment, a two-layer gel sample with an agarose concentration of 1.0 % by weight was used. In the lower layer, a single channel was formed, in the upper layer of the gel – two channels. The dimensions of the channels in both layers were the same 1 mm. The transition of the substance between the vertical channels occurs along the horizontal channel between the gel layers. Fuchsin was poured over the top layer of gel with a volume of 1.0 ml.

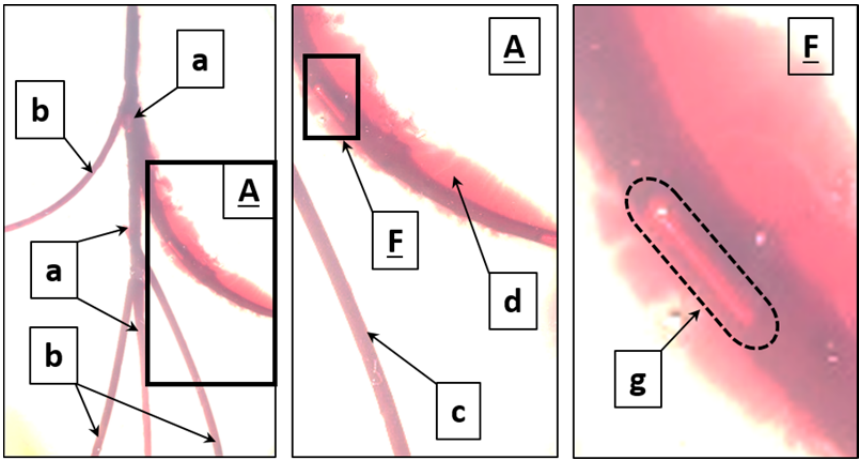

Figure 3: Fuchsin diffusion of into a gel with an agarose concentration of 1.0% by weight in the presence of a branched channels system: a – central channel, b – lateral channels, c – whole (undeformed) channel, d – deformed channel, g – air bubble in the channel

Photo 1 (in Figure 4) shows that before the fuchsin is fed, the channels are almost completely filled with dispersive moisture released from the gel. After applying fuchsin (photos 2 – 10) the process of diffusion-convective filling of fuchsin begins in the channels and gel volume. In this case, the faster mass transfer is carried out through the channels formed in the gel. This confirms the effectiveness using of the capillary channel system in additive technology for gel-based bioreactors production.
Due to the heterogeneity of the gel structure in the presence of channels in it, the task of modeling mass transfer is difficult and ambiguous. The question regarding the optimal channels structure inside the gel, which provides the necessary supply of nutrient to the cells immobilized in the gel, remains open. To find such a structure, it is necessary to develop methods for modeling mass transfer from complex shape channels (including branched) and variable cross-section, as well as to made numerical experiments with different channels configurations to determine their location and the amount needed to provide cells with nutrient.

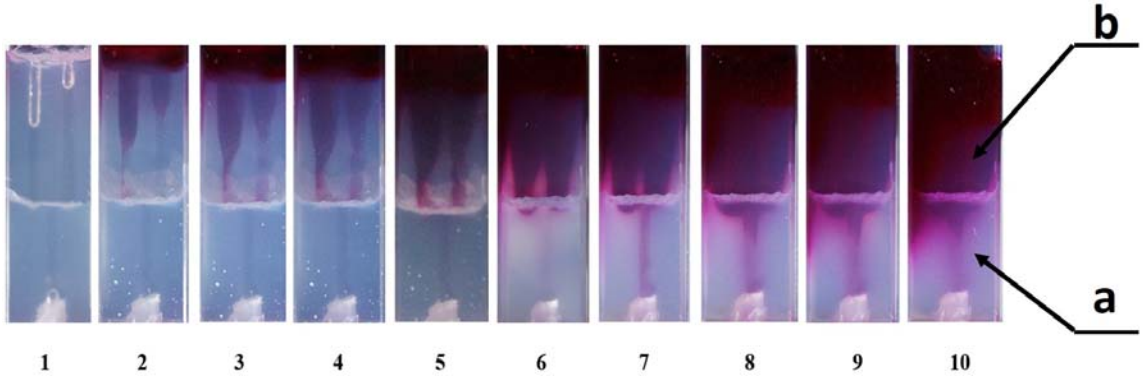

Figure 4: Time dynamics of fuchsin filling with layered agarose gel with agarose weight concentration 1.0 % and channels with a diameter of 1 mm: a – bottom gel layer; b – top gel layer; 1 – 0 min., (empty channel), 2 – 5 min., 3 – 10 min., 4 – 15 min., 5 – 30 min., 6 – 100 min., 7 – 130 min., 8 – 190 min., 9 – 230 min., 10 – 420 min.

By additive technology practically the easiest way to implement in the gel two forms of channels – linear and cross. The linear model corresponds to a capillary system penetrating the gel vertically. The advantage of

such a structure is the technical simplicity of its formation. The cross model corresponds to a system of vertical and horizontal capillaries having mutual intersections. This configuration is more difficult to implement, but the presence of intersections between the channels allows to intensify the mass transfer process and redistribute the load along the liquid flow through the entire cross-section of the gel sample.

4. Conclusions

The microorganisms growth in the gel volume is localized near the surface through which nutrient is supplied. Estimates for yeast cells growth in the agarose gel volume show that the width of such localization zone is from 3.0 to 4.0 mm, which corresponds to the depth of penetration of the nutrient into the gel under conditions absorption by cells. With an increase in the agarose concentration in the gel, the localization zone narrows.
It is established that a system of artificial channels can be created in the gel for nutrients transfer to its volume by the convective-diffusion mechanism. It is shown that channels, despite the phenomenon of syneresis in gels, remain stable over time. It is practically confirmed that through them it is possible to pass a liquid flow of nutrient with a volumetric flow rate corresponding to several microliters per second and this does not affect the condition of the channels. It is noted that defects on their surface in the form of cracks can occur for channels created by mechanical method.
Qualitative analysis shows that initially under small times, when the cells are in an adaptation state to new condition, there is a relatively rapid volume saturation of nutrient. With the beginning of the cells division stage, the nutrient concentration passes through the maximum and begins to decrease, due to increased absorption as a result of an increase in the number of cells consuming nutrition. When the number of cells after the growth stage approaches the maximum value determined by the conditions of their existence in the gel, the nutrient concentration asymptotically reaches a new stationary value.

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