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Micro-environmentally Restricted Yeast Cell Growth within Ca-alginate Microbeads*

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ABSTRACT

Micro-environmental restriction effects to yeast cell growth obtained within Ca-alginate microbeads are considered. It is complex phenomenon influenced by: (1) relaxation of expanded polymer network around the cellular clusters, (2) forces generated by cell growth inside the beads and (3) interactions between solvent, network parts and cells. The resulting effects are measured experimentally by estimating volume of microbeads and yeast cell concentration as function of time of cultivation. Comparative analysis of dynamics of cell growth and increase of microbead volume through four regimes indicates that reversible and irreversible local structural changes of Ca-alginate hydrogel induces micro-environmental restrictions to cell growth. The mechanism of restrictions includes both mechanical and electrostatic effects.

Keywords: Yeast Cells; Micro-environmental Restrictions; Ca-alginate Hydrogel Matrix; Structural Changes; Pattern of Volumetric Yeast Growth

1. Introduction

There is a growing interest in using immobilized cell systems for various applications in biotechnology, biomedicine and food technology [1-5]. The success of such applications depends on achieving suitable conditions for cell growth inside microbeads. It is based primarily on the optimization of the performance of microbead matrix. Ca-alginate hydrogel have been the most frequently used matrix for immobilization of yeast cells.

Significant attempts have been made to examine the rheological response of hydrogel matrix to stresses generated by compression, shear and tension [7-9]. However, little is known about the rheological responses of variously structured matrixes caused by cells growth. The growing cells press to the surrounding and create a new space for further cells growth inside the matrix. Such cell actions are obtained on two time scales, i.e. one is the migration time (the short-time scale) while the other is the growing time (the long-time scale).

Yeast population entrapped in Ca-alginate hydrogel matrix grew into many small cell clusters located at multiple positions in the microbeads. Interactions between clusters can be neglected [3-4]. Consequently, the number of clusters is approximately the same while the number of cells per cluster increases during cell cultivation.

Hydrogel with density in the range of 1-2 % have good mechanical behaviours [3-6] and ensure optimal nutrient transfer through the microbeads. Many authors [1-5] observed homogeneous cell distribution within 2 % and 1.5 % Ca-alginate microbeads, respectively. However, some of them [2-5] reported early suppression of cell growth within hydrogel in optimal nutrient supported systems. It should be connected with micro-environmental restrictions to cell growth.

The primary function of the alginate matrixes in biotechnology applications is to provide mechanical and biological integrity of immobilized cell population by ensuring the optimal packing state. Matrixes simultaneously transmitted mechanical signals to the cells and the developing population. As Ca^{2+} ions in bucked egg-box junction are being released by cell actions, electrostatic repulsion between the liberated chains enhances the additional swelling effects of the alginate gel. This electrostatic repulsion forces have the feedback action on cell growth [3-4].

Phenomenon of the micro-environmental restrictions generated within hydrogel includes following steps from the rheological point of view: (1) the immobilized clusters induce radial deformations of the surrounding hydrogel matrix during clusters expansion [10], (2) the radial deformations induce generation of the mechanical stress within hydrogel around the clusters (the external stress) [3,4,10,11], (3) the external stress around the clusters induces generation of the internal stress within cell clusters [11-12] and (5) the internal stress provokes the biological response of cells [2-4,13]. The biological response of cells influences metabolic activity of yeast. On that base, it is necessary to connect the rheological behaviour of hydrogel with cell growth dynamics.

Increase of the internal stress within immobilized cell clusters induces changes in cell growth dynamics [3-4]. Significant attempts have been made to examine the pattern of volume growth of yeast cell population under various nutrient supporting conditions [1,5]. The pattern of volumetric yeast growth depends on the volumetric states of single cells. The volumetric states depend on: (1) the ability to budding and (2) the cell aging [14-16]. However, little is reported about the pattern of volume growth under action of generated mechanical compres-

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sive stress within hydrogel around immobilized clusters.

The aim of this work is to estimate the micro-environmentally restricted yeast cell growth in conjunction with the structural changes of Ca-alginate hydrogel matrix.

2. Experimental Part

2.1. Imobilization and Cultivation Procedure

The 2% w/w Na-alginate solution was prepared by dissolving 10 g of sodium alginate powder (Sigma- medium viscosity) into 500 ml of distilled water. The brewer's yeast (*Saccharomyces uvarum*) was cultivated at 25 °C in sterile medium of 11% w/w extract in shake flask. Polymer/cell suspension was formed by mixing of 100 ml of Na-alginate solution with 25 ml of thick brewer's yeast suspension at room temperature. The cell suspension was forced out of the tip of the needle at constant flow rate (25.2 ml/h). The droplets were formed by the action of electrostatic forces [17].

After gelling the microbeads were placed in double distillated water to remove un-reacted material and low molecular weight byproducts. Spherical droplets were formed by extrusion of Na-alginate/yeast cell suspension into 1 % $CaCl_2$ solution. After gelling the microbeads were placed in double distillated water to remove un-reacted material and low molecular weight by-products. Microbeads with cells were cultivated in 500 ml flasks, which contained 150 ml of medium and 5 g of microbeads in each experimental group, and these were placed on orbital shaker at 115 rpm and 25 °C.

2.2. Analytical Methods

Total yeast cell concentration in the beads was estimated by using Thoma counting chamber after dissolution of the beads. The initial yeast concentration (ρ_0) in the beads was about 3 x 10^6 cells/ml. The microbeads were also sampled twice per day from the cultivation flask and cell concentration was measured in the same way.

Local cell concentration per microbead layers was calculated from the experimentally determined surface fraction of cells for various microbead cross sections. The surface fraction of cells was estimated by ultramicroton cutting the microbead. The alginate microbeads sampled for image analysis were fixed in 2.5 % glutaraldehyde, embedded in araldite, cross-sectioned by LKB III ultramicrotom and stained with hematoxylin and eosin (H&E). The images of the microbeads cross-sections (the number of sections was six for each bead) where acquired using a solid-state CCD camera (Hitachi) mounted on an inverted microscope (Nikon Diaphot), digitized by a CG-7 frame Grabber (Scion Corp., Frederick, MD) and analyzed using Image Pro Plus software. Distribution of the cells and the colonies, as well as, the surface fraction of cross sections occupied by cell colonies were determined by automatic counting and measurement of all objects darker than background and equal or larger than single cell. It was assumed that only one cell layer is visible, due to small thickness of the histological cross sections.

Diameters of the microbeads were measured with an accuracy of 10 μm using optical microscope. The average microbead diameter and standard deviations were then calculated from the measured data.

3. Resutls and Discussion

We considered the micro-environmentally restricted dynamics of yeast cell growth within Ca-alginate microbead. The nature of the restriction phenomenon should be connected with the local structural changes of the hydrogel. Cell cluster expansion induced the structural changes of the matrix and provoked the complex rheological response with additional dissipation effects.

Based on our previous experience [2-4], the dissipative effects were deeply related with the deformation of the alginate matrixes. These changes of the matrixes within interfaces around the clusters included both, the reversible deformation of domains, as well as the partial domains disintegration that caused permanent irreversible deformation. This complex process was influenced by various multi-scale interactions: the interactions between domains themselves and the interactions between chains within the domains, as well.

Consequently, we estimated both:

1) changes of the microbead volume $V_b(t)$ and

2) changes of the cell concentration per microbead ρ(t) as function of the growing time.

Changes of the microbead volume depended on: the volume changes of cell population and the volume changes of hydrogel. On one side, the volumetric state of cell population depended on the cell number and the volume of single cells. The pattern of volume growth of single cells was influenced by the budding state of the cells and the cell aging status. On the other side, the volumetric state of hydrogel matrix included the sum of local reversible and irreversible deformation contributions within the interfaces around the immobilized clusters.

Increase of the average bead volume vs. the growing time was presented in **Figure 1**.

Averaged microbead volume increased rapidly during first 3 days with the averaged rate equal to $5x10^{-4}$ ml/days. The averaged rate of increase the microbead volume decreased during next 3 days period to $6.7x10^{-5}$ ml/days. The microbead volume increased 3 times during 6 days of cultivations. The main goal of this consideration was to estimate what has been happen with the hydrogel on one side and within the immobilized cell population on the other. For additional information, we considered changes of the cell concentration as function of the growing time.

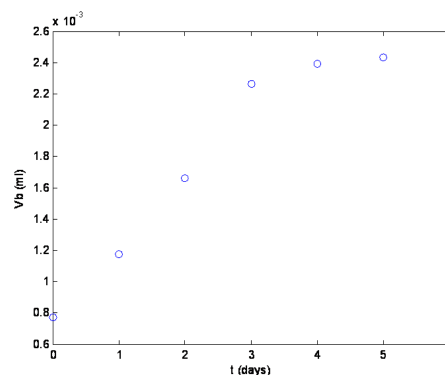

Figure 1. Increase of the averaged Ca-alginate microbead volume as function of the growing time resulted by the immobilized yeast cell growth.

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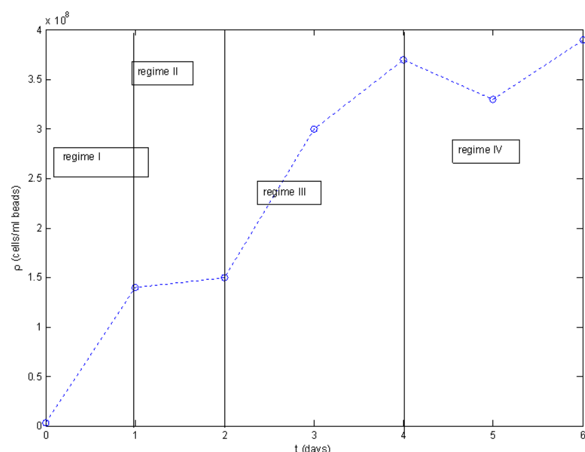

Figure 2. Yeast cell growth in Ca-alginate microbead as function of the growing time – within four regimes.

Interesting phenomenon was observed. In **Figure 2** was presented the number of cells in a bead per unit volume, ρ *vs*. the growing time, to estimate the trends of cell growth changes. For first *ca*. 1 day, the steep increase of the cell concentration up to 1.4 x 10^8 cells/ml with the averaged rate 1.37 X 10^8 cells/(ml days) was observed. It indicated good conditions for cell growth (the regime I). During next day, the rate of growth significantly decreased up to 1 x 10^7 cells/(ml days) *ca.* 13.7 times (the regime II). The cell concentration further increased to 3.7 x 10^8 cells/ml obtained at 4 days (the regime III). Then, the rate of growth slightly increased up to 6 days and the cell concentration increased to 3.9 x 10^8 cells/ml (the regime IV).

The main goal of this consideration was to estimate the micro-environmentally dynamics of cell growth. In the regime I cells had a lot of free space to grow in the swollen gel matrix. On that base, the regime I corresponded to slightly mechanically limited growth conditions. With increase of the cell number, they felt elastic resistance of the network. With further increase of cell number, the restrictions of the network became so high (the regime II). Clusters reached the critical sizes and had enough power to destroy ionic bonds. Cells were able to make available significant new free space within the network. It corresponded to increase in the cell number within the regime III. In the regime IV, cells filled free space within the network. The resistance effects within the network became high and suppressed further cell growth. The restrictions in the regime IV was caused by mechanical and electrostatic effects.

It was interesting that quit different courses induced increase of the microbead volume with the averaged rate 5x10^{-4} ml/days within first three regimes (**Figure 1**). The regimes I and III corresponded to rapid increase in the cell number. However, the regime II corresponded to the significant structural changes of hydrogel within the interfaces around the clusters. New charges were generated by breaking of the network ionic bonds in the hydrogel. The cell membranes were also partially negative charged. Such irreversible structural changes of hydrogel matrix induced additional repulsive interactions between the cells on one side and the alginate chains within the interfaces around the clusters on the other. Some authors have detected such charges [1-4]. The irreversible structural changes induced the increase of the microbead volume in the regime II. Increase of the microbead volume was suppressed within the regime 4. It was connected with the restrictions in cell growth dynamics.

4. Conclusions

The results of this study point to some important cause-consequence relationships between the matrix rheological responses and yeast cell growth within Ca-alginate microbeads. The nature of the micro-environmental restrictions to cell growth should be connected with the structural changes of hydrogel within the interfaces around the immobilized clusters. Changes within the interfaces are difficult to trace experimentally.

To estimate the complex multi-scale mechanism of cell/olymer interactions, we consider the increase of microbead volume and the increase of cell concentration as function of the growing time within four regimes. The regime I corresponds to slightly mechanically limited cell growth and local elastic deformation of hydrogel. The regime II corresponds to restricted cell growth and local plastic deformation of hydrogel. The regime III corresponds to rapid growth of cells within the additional free space within hydrogel. The regime IV corresponds to the restricted cell growth. Restriction is caused by mechanical and electrostatic effects. Based on comparative analysis we point that both mechanical and electrostatic effects contribute to micro-environmental restrictions.

This consideration should be used for optimization the performance of hydrogel matrix in order to achieve higher yeast cell concentrations.

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