BIOLOGICAL PREPARATION TECHNOLOGY

Yeast *Debaryomyces hansenii* within ORMOSIL Shells as a Heterogeneous Biocatalyst

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Received November 11, 2016; in final form, December 26, 2016

Abstract—The relevant sol-gel encapsulation methods to design ORMOSIL (organically modified silica) protective shells around *Debaryomyces hansenii* yeast cells have been studied. Encapsulation is based on spontaneous “cell-in-shell” 3D structures. The physiological activity of the sol-gel encapsulated cells was determined by measurement of the oxidative activity of a BOD biosensor containing ORMOSIL-encapsulated cells. It was shown that the shells around the microbial cells did not prevent substrate diffusion and had excellent protection from extreme environmental factors (heavy metal ions, high pH, and UV radiation). In addition, it was found that the BOD biosensor surpasses some other known biosensors in their basic characteristics, including sensitivity, stability, and reproducibility. These results are important for the development of innovative methods of cell encapsulation and the creation of biocatalysts used in biotechnology and ecology.

Keywords: biosensor, biochemical oxygen demand, heterogeneous biocatalyst, yeast, sol-gel, encapsulated cells, artificial spore, cell in shell, organically modified silica (ORMOSIL), *Debaryomyces hansenii*

DOI: 10.1134/S0003683818070062

INTRODUCTION

Immobilized microorganisms offer an efficient approach to the intensification and increased economic attractiveness of current biotechnological processes. They lead to more relevant technical decisions as compared with cell suspensions. Immobilized cells retain their vitality and high concentration in restricted space over a long time period, which is a competitive advantage for their use in industry and ecological biotechnology [1, 2].

Microencapsulation is a contemporary method of microorganism immobilization based on structures consisting of cells encircled by semipermeable shells (cell-in-shell structures). These artificial coats protect cells from the influence of physical and chemical stress factors. Therefore, these types of cell-in-shell structures are called ‘artificial spores’ because of their similarity to natural endospore, living [3]. Cell-in-shell biohybrid can be a stable biocatalyst with a substantial innovation potential [4].

There are unicellular organisms with natural protective shells, for example, diatoms. Diatoms can create silica coats and, thus, form an exoskeleton to produce mechanically strong structures. These structures protect cells from adverse environmental factors, including other microorganisms [5]. Diatoms encouraged researchers to create cells encapsulated within mineral shells based on silicon compounds, silica gels (SiO₂), or organomodified silica gels (ORMOSILS, ormosil). Such shells can retain water without significant swelling, as well as they are chemically and biologically inactive and mechanically strong. Organomodified silica gels are more flexible as compared with SiO₂ gels. Their porosity can be controlled, which is important for a comfortable cell environment and high velocity of substance fluxes [6].

Sol-gel methods are usually used to obtain the cell-in-shell biohybrids. These methods do not require expensive equipment, and they are efficient and ecologically pure. Moreover, the main advantage of these methods is the use of mild conditions for sol-gel reactions, which is important for the immobilization of living cells [7]. Silicon allc oxides (for example, alkoxysilane and alkyl-alkoxy silane), which are hydrolyzed with subsequent condensation to create particles of sol, and then gel, can be precursors for sol-gel synthe-

Abbreviations: BOD—biological oxygen demand, GGM—glucose—glutamate mixture, CFU—colony-forming unit, MTES—methyltriethoxysilane, PEG—polyethylene glycol 3000, SEM—scanning electron microscopy, TEOS—tetraethoxysilane, UV—ultraviolet, BSE—back scattered electrons, SE—secondary electrons.

sis. The silica gel structure depends on a variety of factors: the structure of precursors (alkoxysilanes and alkyl-alkoxy silanes with nonhydrolyzed Si-C bond); their concentrations and ratios; the pH level and catalyst; and the presence of other constituents—organic solvents, amphiphilic polymers, and even cells.

In the presence of cells, the silane precursors normally form monolithic gels with immobilized microbial cells [8–10]. Specific technologies are required to obtain the shell around the individual cell. These technologies include, for example, the gas-phase conditions for sol-gel synthesis or the formation of a catalytic matrix from polyelectrolyte on the cell surface or lipid layer for the directed formation of nanostructured silica near the cell surface [3].

In the earlier work, we proposed simple and mild conditions for sol-gel synthesis that favor the spontaneous formation of ormosil shells around yeast cells [11]. In that study, we showed the dynamics of the formation of sol particles and fractal gel structure on the surface of yeast cells. Also, the direct participation of yeast in the spontaneous formation of 3D-structure of the cell-in-shell biohybrids was demonstrated [12]. It should be noted that in the process of the sol-gel synthesis, when alkoxysilanes undergo hydrolysis and polycondensation, alcohol is released and can be toxic for living cells. Unlike the oleaginous yeasts *Cryptococcus curvatus* VKM Y-3288, the methylotrophic yeasts *Pichia angusta* VKM Y-2559 consume alcohols as substrates and retain their activity after encapsulation under these conditions. This allowed the use of their encapsulated form to develop a biosensitive system for methanol detection [12] and to create a biofilter for the recovery of methanol wastewater [13]. At the same time, the acetic acid bacteria *Gluconobacter oxydans* cannot be encapsulated under the same conditions, which suggests that other microorganisms cannot always form protective shells on the cell surface and save their vitality.

Due to their halo-, osmo-, and cryotolerance, as well as their ability to oxidize a broad range of substrates, including a number of xenobiotics, the yeasts *Debaryomyces hansenii* VKM Y-2482 are very promising in environmental protection and biotechnology [14]. *D. hansenii* with ORMOSIL protective shells should be advantageous in biocatalysis. Moreover, new microbe species involved in studies of the sol-gel processes, which are important in the creation of ormosil shells around living cells, can contribute to the further development of methods for elaboration of efficient and stable heterogenic biocatalysts based on the cell-in shell 3D-structure.

The goal of this study is to find the protective function of ormosil shells for *Debaryomyces hansenii* yeasts, as well as the other important biocatalytic characteristics of encapsulated yeasts as a constituent of a BOD-biosensor based on an oxygen electrode.

EXPERIMENTAL CONDITIONS

Microorganism Cultivation

The cultivation of the yeast *Debaryomyces hansenii* VKM Y-2482 was carried out in a rich mineral medium (liquid glucose-peptone medium): 10 g/L glucose (Sigma, United States), 5 g/L peptone (Sigma), 0.5 g/L yeast extract (Sigma). Cells were incubated with aeration at 29°C for 18–20 h in 750 mL vessels. Next, the biomass was centrifuged (4000 g) at room temperature for 10 min. The sediment was washed three times with a 20 mM phosphate buffer (pH 6.8; Sigma). Each wash was followed by 5 min of centrifugation. The biomass was weighed and stored in microtubes at 4°C.

Estimation of Cellular Physiological Activity

Cellular physiological activity was estimated according to changes in the respiration activity with a BOD sensor at the addition of substrate. For this purpose, a 7×10^{-4} mL cell suspension was laid on a glass filter Whatman GF/A (Sigma) and dried for 15 min. The ready heterogeneous biocatalyst was brought to the surface of an oxygen electrode connected to a EXPERT-001-4.0.1 thermooximeter (Econiks-Expert, Russia) and washed just before its use in phosphate buffer for 5 min.

Biomaterial Immobilization and the Formation of a Recognizing Element (Microbial Electrode)

A cell suspension (0.005 mL; $1.3 \pm 0.1 \times 10^9$ CFU/mL) was added to 20% PEG solution in phosphate buffer (0.022 mL; 33 mM; pH 6.8) and mixed for 5 min. Furthermore, 0.015 mL TEOS (Sigma) and 0.085 mL MTES (Sigma) were added, and the mixture was mixed again for 5 min. After that, 0.005 mL of catalyst (the 0.2 M the sodium fluoride solution) was added and mixed for 15 min. An aliquot of 0.007 mL was laid on a porous glass filter Whatman GF/A (Sigma) and dried for 15 min. Finally, a heterogeneous catalyst was placed on the surface of the oxygen electrode. The modified microbial electrode was washed with phosphate buffer for 5 min just before the usage.

Biosensor Measurements

Measurements of soluble oxygen were done using an EXPERT-001-4.0.1 pH-meter/ion meter/BOD thermo-oximeter (Econix-Expert Ltd., Russia) coupled to the modified microbial electrode.

The buffer solution (20 mM phosphate buffer; pH 6.8) and a sample were placed in a 5-mL cuvette. The components were mixed with a magnetic mixer (200 rpm/min). The samples were introduced with automatic variable volume micropipettes (Biotech): 200–1000, 20–200, and 0.5–10 μ L. A mixture of

Fig. 1. SEM of encapsulated (a) and nonencapsulated (b) in sol-gel matrix *D. hansenii* VKM Y-2482 yeast cells.

glucose and glutamic acid (GGA) in a 1 : 1 weight ratio was used as the standard accepted in Russia and other countries for BOD₅ (five days BOD). According to the regulatory documents [15], it equals 205 mg/L and corresponds to a solution of 150 mg/L glucose and 150 mg/L glutamic acid (GGA concentration; BOD₅ = 0.68).

Scanning Electron Microscopy

To prepare samples of *D. hansenii* cells, a drop of cell suspension ($2.0 \pm 0.2 \times 10^8$ CFU/mL) was laid on the surface of conducting scotch (JEOL, Japan) and incubated for 5 min. The drop was then dried with filter paper, and the surface was washed several times with water to remove unbound cells. Next, the scotch with the sample was fixed on the surface of the sample holder, and a platinum-carbon mixture was sprayed with a JEE-4X vacuum-spraying device (JEOL, Japan). SEM analysis was performed with a JSM-6510 LV (JEOL) scanning microscope at low vacuum mode (30 Pa) to register backward reflected electrons (BREs) and at high vacuum mode to register secondary electrons (SEs).

Effect of Heavy Metal Ions on the Respiration of Immobilized Microorganisms

Salts of different heavy metals (see below) were added to buffer solution at the maximal permissible concentrations (MPCs) for water sources or at concentrations exceeding 10-, 25-, 50-, and 100-fold.

Effect of pH on the Respiration of Immobilized Microorganisms

McElveen buffers (Sigma) with different pH values (2–12) were placed in cuvettes with subsequent addition of samples.

BOD₅ Measurement

Standard dilution was used as a reference method for BOD₅ measurements. Analysis was carried out as described [15]. Measurements of soluble oxygen were done using an EXPERT-001-4.0.1.

RESULTS AND DISCUSSION

3D structure of Encapsulated Yeasts

TEOS (15 vol %), which is completely hydrolyzed, was used as a silane precursor for the synthesis of organomodified silica gels. MTES (85 vol %), which has a nonhydrolyzed Si-C bond, was also used for this purpose. The hydrolysis rate restricts the entire process at a neutral pH under conditions of basic catalysis (NaF catalyst), which leads to microparticle formation and a porous gel structure.

There are spheres with a rough surface. They represent individual cells covered with organosilica shells, and these encapsulated cells with the sol-gel matrix formed the entire structure together (Fig. 1a).

The spontaneous formation of similar structures was observed earlier in the case of the methylotrophic yeasts *Pichia angusta* BKM Y-2559 and the oleaginous yeasts *Cryptococcus curvatus* VKM Y-3288 [12].

So, the formation of the ‘cell-in-shell’ structure of biohybrids in the conditions of sol-gel synthesis does not depend on the yeast type. Each yeast cell initiates of ormosil capsule formation. In other words, different yeast species can participate in the formation of the similar structures of the cell-in-shell biohybrids. This means that functional characteristics of the yeast cell surface seem to be the principle factor in the self-organization of the biohybrid 3D architecture.

Respiratory activity is one of physiological characteristics of encapsulated microorganisms. This characteristic can be determined with a thermooximeter. Decrease of oxygen content within the electrode space

Fig. 2. Effect of heavy metal ions on respiratory activity of suspended (a) and encapsulated (b) *D. hansenii* VKM Y-2482 yeast cells. BOD values from the Russian normative documents were used.

is determined by cellular respiratory activity. This approach is used to design BOD biosensors with GGA as a substrate.

Wastewater can contain toxic substances that affect microorganisms. To determine the protective function of the ormosil shells, the respiration of encapsulated cells was registered in the presence of stress factors.

ORMOSIL Shell Protects Yeast Cells from the Toxic Effects of Heavy Metal Ions

The role of the ormosil shell in protection against heavy metal ions was studied based on the respiratory activity of encapsulated and nonencapsulated microorganisms during GGA oxidation in the presence of the ions (Fig. 2).

The biosensor response (encapsulated yeast) remained unchanged independently of the presence or

absence of metal ions (Fig. 2b). The respiratory activity of the encapsulated yeast decreased by only 20%, while the MPC was increased by 100 times. Conversely, nonencapsulated yeasts decreased their respiratory activity by several times (Fig. 2a). These results are reliable in that the organosilica shell around the yeast cell represents the protective function.

Role of the ORMOSIL Shell in the Protection of Yeast Cells from Extreme Environmental pH Levels

The encapsulated yeast *D. hansenii* is a promising biocatalyst to be used in ecobiotechnology. Industrial wastewater can have very high acidic or alkaline conditions; therefore, it is important to determine whether ormosil shells are capable of cell protection under these extreme conditions (Fig. 3).

Fig. 3. Dependence of respiratory activity on pH in encapsulated and suspended cells of *D. hansenii* VKM Y-2482 measured with the BOD-biosensor.

The highest cellular respiratory activity is in the range 5–9, which is consistent with the yeast optimum [16]. The respiration of unprotected cells is completely inhibited at low pH, while encapsulated cells retain 50% of their respiratory activity at a pH of 8.0, even at a pH of 2.0. Similar results were observed in an alka-

line conditions. It should be noted that encapsulated cells completely recover their activity when the buffer pH is returned to the medium values. Conversely, the activity of unprotected cells was suppressed irreversibly. This effect can be explained by stable hydrate envelope around the cells. Under these conditions, protonation of water molecules is insufficient to inhibit the physiological activity, even at pH 2.0.

Role of the ORMOSIL Shell in the Protection of Yeast Cells from UV radiation

The materials based on SiO₂ are known to be impermeable for short and medium wave range of UV radiation. UV radiation is commonly used in microbiology, biotechnology, and medicine for equipment sterilization; therefore, it is important to know how efficiently ormosil shells protect living cells under these conditions. The characteristics of a BOD biosensor with biosensitive elements based on encapsulated and nonencapsulated cells were studied after UV irradiation ($\lambda = 254$ nm) for 5 h. The respiratory activity of the encapsulated yeasts was largely unchanged, while the nonencapsulated cells died.

The results indicate that ORMOSIL is universal in *D. hansenii* protection from a variety of adverse environmental factors. In particular, UV radiation can be used to clean biocatalysts of microbial concomitant.

Fig. 4. Substrates oxidized by ORMOSIL-encapsulated *D. hansenii* VKM Y-2482. The biosensor signals are represented in the percentage of the response to the ethanol addition.

Fig. 5. Calibration curve of the BOD biosensor.

Fig. 6. Correlation of biosensor BOD measurements with those carried out by the standard method (see experimental section).

Encapsulated *D. hansenii* Cells Capable of Oxidation of a Variety of Substrates

One of the most important characteristics of biocatalyst use in ecobiotechnology is the ability to use a broad range of organic compounds. Therefore, it was important to study whether an ormosil shell can pre-

vent the influx of different substrates and their oxidation (Fig. 4).

Figure 4 shows that encapsulated *D. hansenii* can oxidize alcohols, saccharides, amino acids, and organic acids. *D. hansenii* yeast can also metabolize aromatic and surface-active compounds. Since these substances are constituents of industrial and public waste, the biocatalyst in question is of significant interest for its use in ecobiotechnology.

It should be noted that, according to our results, an ormosil shell did not prevent diffusion of the oxidized substrates from the environment to the cells.

Properties of BOD Biosensor Based on Encapsulated Yeasts

The dependence of the BOD biosensor reply on the GGA content is hyperbolic; it follows the kinetics of enzymatic microbial oxidation of the substrates (Fig. 5).

The characteristics of the BOD biosensor were compared with properties of other biosensors (see Table 1). The *D. hansenii*-based biosensor had a ten-fold higher sensitivity (higher sensitivity coefficient) than the biosensor based on the same yeast immobilized in polyvinyl alcohol hydrogel [17]. The stability of that mediator biosensor was almost three times lower than that for our device [18]. Thus, *D. hansenii*-encapsulated cells can serve as the basis of a BOD biosensor characterized by high sensitivity and stability.

To evaluate the biosensor and to compare this method with other procedures, eight samples of river and well water, as well as waste and snowmelt water from the Tula Region, were used. Statistical analysis of the BOD results showed that two samplings obtained by different methods were similar in their reproducibility (Fig. 6).

BOD values obtained with the biosensor based on encapsulated yeasts and those obtained by the standard method were similar in their error limits. This suggests that the biosensor based on ORMOSIL-encapsulated *D. hansenii* is promising for the monitoring of wastewater.

In this study, ORMOSIL-encapsulated microorganisms were obtained under the mild conditions of the sol-gel synthesis from tetraethoxysilane and meth-

Table 1. Comparison of the BOD biosensor based on *D. hansenii* VKM Y-2482 with other known biosensors

Characteristics	Biosensor based on immobilized yeasts		
	in silicium-organic sol-gel matrix	in polyvinyl alcohol film [4]	on graphite-paste electrode [2]
Transducer type	Oxygen electrode	Oxygen electrode	Mediator biosensor
Sensitivity coefficient	$0.0480 \pm 0.0001 \text{ min}^{-1}$	$0.0045 \pm 0.0003 \text{ min}^{-1}$	$0.40 \pm 0.01 \text{ nA L/mg O}_2$
Range of measurement, mg/L	0.5–5	0.7–206.7	375–1400
Stability, days	40	30	15
Relative standard deviation, %	6.7	4.2	4

yltriethoxysilane in the presence of polyethylene glycol at the pH values which were close to neutral pH. Under these conditions, ormosil shells directly formed around the *D. hansenii* VKM Y-2482 cells, which were viable even in the presence of releasing alcohol. The coats around the living cells represent a universal protective function, which is promising for the development of stable heterogeneous biocatalysts based on the encapsulated yeasts. *D. hansenii* VKM Y-2482 demonstrated high oxidation activity in the case of many substrates under extreme conditions, which also suggests that this strain shows significant potential for ecobiotechnology and biotechnology.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research and the Administration of the Tula Region (project no. 16-43-710183) and by Tula State University within the framework of the scientific project no. 8709.

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Translated by A. Boutanaev