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Silica sol-gel encapsulated methylotrophic yeast as filling of biofilters for the removal of methanol from industrial wastewater

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ABSTRACT

This research suggests the use of new hybrid biomaterials based on methylotrophic yeast cells covered by an alkyl-modified silica shell as biocatalysts. The hybrid biomaterials are produced by sol-gel chemistry from silane precursors. The shell protects microbial cells from harmful effects of acidic environment. Potential use of the hybrid biomaterials based on methylotrophic yeast *Ogataea polymorpha* VKM Y-2559 encapsulated into alkyl-modified silica matrix for biofilters is represented for the first time. Organo-silica shells covering yeast cells effectively protect them from exposure to harmful factors, including extreme values of pH. The biofilter based on the organic silica matrix encapsulated in the methylotrophic yeast *Ogataea polymorpha* BKM Y-2559 has an oxidizing power of 3 times more than the capacity of the aeration tanks used at the chemical plants during methyl alcohol production. This may lead to the development of new and effective industrial wastewater treatment technologies.

1. Introduction

Wastewater from methanol plants is considered to be extra acidic, the pollutants include methanol, other lower and higher alcohols and formic acid [1]. At the wastewater treatment facilities methanol can be oxidized by saprophytic microflora of activated sludge. Methanol polluted wastewater of BOD_{total} = 516 mg/l and COD = 639 ml O_2/l can be cleaned up to BOD_{total} = 10.5 mg/l using aeration tanks; the efficiency of waste removal equals 95%, and the productivity can reach 1000 g/(m^3 day) [2]. However, the wastewater that is passed through activated sludge into aeration tanks should be diluted or purified up to 30 mg/dm^3 (10 mmol/dm^3), because higher concentration of methyl alcohol can even inhibit microflora of activated sludge. Microorganisms are resistant to high concentrations of lower alcohols can be used on biofilters for preliminary wastewater cleaning. The methylotrophic yeast *Ogataea polymorpha* VKM Y-2559 (*Hansenula polymorpha, Pichia angusta*) is one of methanol-degrading microbial cells which have an efficient enzymes for methanol oxidation [3,4].

One of the modern approaches to the design of heterogeneous packing blend containing microbial cells is to create a protective shell around each of the cells. Many innovative accomplishments in the field of biomaterials are inspired by nature. For example, in diatoms one of the most important components of the cell wall is silica, which creates a glass-like protective shell around the cell [5]. This nature-created approach served as a model for the development of technologies for live cells encapsulation into silica matrix [6–13]. The creation of living hybrid materials based on the silica encapsulated cells is a new trend in biotechnology [6,9,12]. Silica shells have protected microbial cells against mechanical, thermal and biological effects [13]. The sol-gel method used for development of silica based materials does not require expensive equipment, it is economical and safe for the environment [14].

The initial immobilization of the microorganisms into the sol-gel matrix was accomplished using *Saccharomyces cerevisiae* yeast cells, which is involved in the alcohol fermentation processes [11]. Dickson and Ely [12] proved that in a sol-gel process the shells are formed around cyanobacteria. The silica shells are sufficiently porous to allow relatively rapid diffusion and transport of metabolites and waste products. This is an important aspect of using such biocatalysts. Based on bacteria *Pseudomonas* sp. *P2* immobilized into silica sol-gel matrix, an optical biosensor to analyze commercial mixtures of *p*-chlorbenzenes has been developed [15]. We have shown that it is possible to provide a controlled formation of alkyl-modified silica shells around methylotrophic yeast *Ogataea polymorpha* VKM Y-2559 [16,17]. They are formed spontaneously and it is likely that surface-based groups of cells act as the centers of silica sol-gel matrix formation. The silica shells efficiently protect

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yeast cells from the effects of heavy metal ions and UV radiation [16].

This is the first report studying how the silica shell around the microbial cell affects biodegradation of methyl alcohol by *Ogataea polymorpha* VKM Y-2559 and establishes a foundation for future studies of C1-compounds biodegradation for industrial application.

2. Method description

2.1. Microorganism strain

The methylotrophic yeast *Ogataea polymorpha* VKM Y-2559 were received from the National Collection of the Institute o Biochemstry and Physiology of Microorganisms (Pushchino, Russia).

2.2. Yeast cultivation

The cultivation of the yeast *Ogataea polymorpha* VKM Y-2559 was carried out in a nutrient medium of the following composition: $(NH_4)_2SO_4$—2.5 g/dm^3, $MgSO_4$—0.2 g/dm^3, $KH_2PO_4{\cdot}3H_2O$—0.7 g/dm^3, $NaH_2PO_4{\cdot}2H_2O$—3.0 g/dm^3, yeast extract—0.5 g/dm^3, glycerol—8.3 cm^3 and trace elements—$MnSO_4$—0.0012 g/dm^3; $CoCl_2{\cdot}6H_2O$—0.0003 g/dm^3; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$—0.0002 g/dm^3; $CaCl_2{\cdot}2H_2O$—0.0015 g/dm^3; $FeSO_4{\cdot}7H_2O$—0.01 g/dm^3; EDTA—0.001 g/dm^3. Microorganism cultures were grown in Erlenmeyer flasks of 750 cm^3 (150 cm^3 medium volume) at 28 °C and aerating in a shaker at 190 rev/min. The inoculum was added in the amount of 1.5% by volume of the medium to a final concentration of ~10^6 CFU/cm^3. The biomass was placed in a flask containing 200 cm^3 of phosphate-free medium and induced with methyl alcohol (2 cm^3). Induced biomass was centrifuged at 5000 rev/min (1700g) for 15 min, the pellet was washed with phosphate buffer solution (20 mmol/dm^3, pH 7.6). The yeast biomass was stored in test-tubes at +4 °C.

2.3. Yeast encapsulation

The silane precursors used were tetraethoxysilane (TEOS, Sigma-Aldrich) and methyltriethoxysilane (MTES, Sigma-Aldrich). A mixture of silane precursors with hydrophobic additives MTES content from 85% (vol.) with respect to the total amount of the silane precursor was used. The porogen polyethylene glycol 3000 (PEG) and the catalyst, NaF, pH 7.6, were used. A suspension of yeast cells of volume 0.25 cm^3 ($1.3 \pm 0.1 \times 10^9$ CFU/cm^3) in phosphate buffer solution (20 mmol/dm^3 pH 7.6) was added to 0.1 cm^3 of a 20% solution of polyethylene glycol 3000 (PEG) (Ferak Berlin) in phosphate buffer solution and stirred for 3 min (Elmi CM-70M07), then a mixture of tetraethoxysilane (TEOS) (Sigma) and methyltriethoxysilane (MTES) (Sigma) of volume 0.5 cm^3 was added and mixed again for 3 min. A mixture of silane precursors with hydrophobic additives MTES content of 85% vol. with respect to the total amount of the silane precursor s was used. Then, 0.025 cm^3 of the catalyst solution of 0.2 mol/dm^3 NaF was added and stirred for 15 min; 0.005 cm^3 of the amount was withdrawn, spread on a porous glass filter (Whatman GF/A, Sigma) and dried for 15 min at 20 °C. A piece of glass fiber filter with immobilized cells (3 × 3 mm) was washed prior to use with a phosphate buffer solution for 5 min, then placed on a surface of Clark oxygen electrode and fixed with a nylon mesh.

2.4. Immobilization of yeast cells on fiber glass filter

The microbial biomass was washed with phosphate buffer solution (20 mmol/dm^3, pH 7,6) and centrifuged (2000 rev/min, 10 min). The buffer solution was added to the residue with the resultant biomass content of 150 mg/cm^3 ($1.3 \pm 0.1 \times 10^9$ CFU/cm^3). The microorganism suspension (0.003 cm^3) was applied on a porous fiber glass filter (Whatman GF/A, Sigma) and air dried for 15 min at 20 °C. A piece of glass fiber filter with immobilized cells (3 × 3 mm) was placed on the surface of Clark oxygen electrode and fixed with a nylon mesh.

2.5. Scanning electron microscopy (SEM)

Samples of the yeast cell *Ogataea polymorpha* for scanning electron microscopy (SEM) were first fixed in 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer pH 7.2 for 1 h at 4 °C and then post-fixed in 2% solution of osmium tetraoxide in the same buffer for 4 h at 20 °C. They were further processed and dried in a freeze drying device JFD- 320 (JEOL, Japan) according to the manual instruction. Further, the samples of the cells and the silica sol-gel matrix were applied to the surface of the metal plating and were coated with platinum-carbon mixture in a vacuum sputtering equipment JEE-4X (JEOL, Japan). Electron microscopic analysis of the samples was performed on a scanning electron microscope JSM-6510 LV (JEOL, Japan).

2.6. The effect of pH on respiratory activity of encapsulated microorganisms

Electrochemical measurements were done using an EXPERT-001-4.0.1 pH-meter/ion meter/BOD thermo-oximeter (Econix-Expert Ltd., Russia) coupled to a per- sonal computer operated by specialized software EXP2PR (Econix-Expert Ltd., Russia), which enabled recording and processing of sensor signals. The measured parameter (biosensor response) was the maximum rate of oxygen concentration change at the addition of substrates (mg/dm^3 min). The sensors were Clark oxygen electrodes (Econix-Expert Ltd., Russia) with immobilized microbial cells. The solution was mixed by a magnetic mixer (200 rpm). A phosphate-citrate buffer solution with a pH range from 2 to 12 was used.

2.7. Preparation of the heterogeneous biocatalyst for filling column type biofilters

Glass beads (diameter 3.3 ± 0.3 mm) were used as carriers for filling of the column. To activate the glass surface the beads were immersed into 0.1 M solution of HCl for 24 h before use. The modified beads was transferred in the column, washed with buffer (pH = 7.6). The methyl and ethyl alcohol in the eluate was monitored by gas chromatography.

2.8. Gas chromatography

The methyl and ethyl alcohol content was measured on a chromatograph “Crystal 5000.2” (Chromatec, Russia) using a flame ionization detector and a capillary column DB-FFAP (50 m × 0.32 mm 0.50 μm) (Agilent, USA). Analysis conditions were as follows: the column oven temperature 70 °C, the evaporator temperature 200 °C, detector temperature 2–50 °C, flow rate of carrier gas (helium) 0.10 dm^3/h.

3. Results and discussion

3.1. Organo-silica shells formation around yeast cells

Fig. 1 shows a 3D structure of the heterogeneous biocatalyst based on immobilized methylotrophic yeast *Ogataea polymorpha* VKM Y-2559 in a methyl-modified silica matrix derived from silane precursors MTES and TEOS in a ratio of 85/15 by sol-gel method