Immobilized Yeast Cells in Hydrogel Carriers for Bioproduction of Alcohols

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Abstract

An approach for fabrication of double-layered hydrogels systems comprising a macroporous hydrogel core with immobilized yeast cells and an outer poly(ethylene oxide) (PEO) hydrogel layer was developed. Macroporous hydrogels of various hydrophilic and temperature-responsive polymers were synthesized via both UV irradiation of moderately frozen systems and phase separation radical polymerization. Well-defined PEO outer layer were obtained by UV-induced crosslinking for extremely short time, which makes the preparation of double-layered hydrogels systems very facile and convenient procedure. Yeast cells of various concentrations can be immobilized into the macroporous core. The outer layer of PEO hydrogel encapsulates the core containing cells, thus reducing the cells leakage. The conversion of glucose to ethanol by *Saccharomyces cerevisiae* and xylitol by *Candida boidinii* was determined.

Thematic Area/Type of the project

Food, Agriculture and Biotechnology
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Keywords

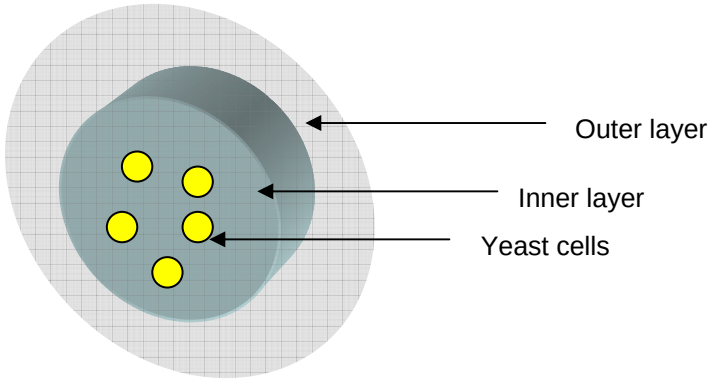
Bioethanol, Candida boidinii; cryogels, immobilization, macroporous hydrogels, Saccharomyces cerevisiae, xylitol

1. Introduction

The use of immobilized cell systems is an attractive and rapidly expanding research area because of the technical and economical advantages compared to the free cell systems. The selection of proper support matrix is very important for the successful performance of an immobilized cell system. It is desirable that the cell carrier possesses large surface area, permeability, chemical, mechanical, and thermal stability, insolubility and suitable shape. In the last few years, a new round of interest in biomass and bioenergy has been initiated. The depletion of the oil reserve is going much faster than previously predicted and the environmental deterioration resulting from the over-consumption of petroleum-derived products is threatening the sustainability of human society. Ethanol, both renewable

and environmentally friendly, is believed to be one of the best alternatives and its production is continuously increasing. For such a vast production, any improvement in its fermentation technologies will be economically important [1, 2]. Attempts are being made to bring such improvement by using immobilized yeast cells. Possessing lower energy value than that of sucrose, xylitol became more interesting for the consumers. When supplied in the diet, this naturally occurring sweetener limits the tendency to obesity, and therefore attracts individuals concerned with their body weight and health. The anticarcinogenic properties of xylitol have important commercial application. The most recent finding concerning xylitol application was reported by King et al. [3], who discovered the usefulness of xylitol as ingredient in functional foods for appetite control. Granström et al., [4] reported on the use of xylitol as a raw material for production of L-lyxose, L-xylose and L-arabinose. Xylitol is routinely produced chemically, but this process requires several purification steps, which lead to increased production cost. An alternative to the chemical process is the use of microorganisms, mainly yeasts, which directly convert xylose to xylitol. One of the strategies aiming at improving the performance of the microbial process is the use of immobilized cells. In that respect, we tried to synthesize and investigate synthetic polymer carriers for cell immobilization and to explore these systems for alcohol production, particularly ethanol and xylitol as alcohols that currently, for quite different reasons, attract attention of the society.

The project aims at preparation and investigation of double-layered hydrogels systems, where the inner hydrogel contains yeast cells and the outer layer ensures the mechanical strength and acts as barrier membrane against cells leakage. Two techniques are tested for processing porous inner polymer scaffolds: via UV irradiation of moderately frozen systems and via phase separation radical polymerization. The production of ethanol and xylitol by yeast cells immobilized in the double-layered hydrogels systems is evaluated.



2. Experimental Techniques and Methods

2.1 Formation of porous inner matrixes

2.1.1 Super-macroporous polymer hydrogels via UV irradiation (BG team)

An appropriate amount of each polymer/monomer was dissolved in distilled water under stirring to obtain homogeneous aqueous solution. Given amounts of initiator (and cross-linking agent) were added under stirring at room temperature. The resulting homogeneous solution was then kept in a freezer at minus 20 ^{o}C for 2 h. The frozen system was irradiated with full spectrum UV-vis light with

a “Dymax 5000-EC” UV curing equipment with 400 W metal halide flood lamp for 2, 5 or 10 min (irradiation dose rate = 5.7 J/cm^2.min; input power = 93 mW/cm^2).

2.1.2 Macroporous hydrogels via phase-separation method (H team)

Monomer mixtures were poured into cylindrical shape holders and filled with double processed tissue cultured water and ammonium persulfate solution. The cross-linker was adding to the solution by press-lock microsyringe, and then the solution was purged with argon for approximately 5-10 min to remove oxygen. Then, tetrametylethylenediamine was injected by microsyringe into the reaction mixture while the argon bubbled through the solution. The sample holders were kept in an oven at 50 °C for three days. After the reaction, the resulting hydrogels were cut into disks and immersed in double processed tissue cultured water to extract the unreacted materials.

2.2 Preparation of cell cultures

2.2.1 Microorganisms (FYROM team)

Saccharomyces cerevisiae, a commercial grade baker’s yeast, with 32 % dry mass and *Candida boidinii* with 29 % dry mass were used in the investigations. The yeast strain of *C. boidinii* was kindly provided by Dr. C.P. Kurtzman of the USDA, ARS Culture Collection, USA.

2.2.2 Culture media (FYROM and RS teams)

Nutrient medium used for the reincubation of the immobilized *Saccharomyces cerevisiae*, had the following composition: 0.1 %w/w KH2PO4, 1 g 0.7 %w/w MgSO4.7H2O, 0.1 %w/w (NH4)2SO4, 0.4 %w/w yeast extract and 10 %w/w glucose. The media were autoclaved at 120oC for 15 min.

Nutrient medium for inoculum preparation and fermentation with *C. boidinii* had the following composition (per liter): 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5 g casamino acids (Difco), 5 g urea and 50 g xylose.

2.3 Immobilization of yeast cells

2.3.1 Addition of yeast cells before cross-linking (BG and FYROM teams)

The polymer aqueous solutions were first mixed with yeast cells suspension to a 10% concentration (w/v) followed by addition of photoinitiator, cooling to -20 ^{o}C and irradiation with UV.

2.3.2 Soaking of yeast cell culture into already prepared cryogel (BG and FYROM teams)

Yeast cells were immobilized into the macroporous core simply by soaking. First, the polymer cryogel was prepared, freeze dried and, then, immersed in the cells suspension.

2.4 Outer layer formation (BG and FYROM teams)

Cryogels containing yeast cells were, first frozen and covered with polymer solution containing photoinitiator and crosslinking agent. Then, the samples were irradiated with full spectrum UV-vis light with a “Dymax 5000-EC” UV curing equipment with 400 W metal halide flood lamp for 2 minutes.

2.5 Batch and repeated batch fermentations (FYROM and RS teams)

Double-layered gels with immobilized *S. cerevisiae* were incubated in 100 mL nutrient medium placed in 250-mL Erlenmeyer flask on a rotary shaker at 28^{o}C. Three successive batch runs with recycling of the immobilized cells were carried out. At the end of each cycle, the fermented broth was replaced with fresh medium. Each batch lasted 3 days. Afterwards the samples were kept for 6 months in distilled water at 4 ^{o}C and tested again for ethanol production in seven successive batch runs in duration of two days.

The performance of the immobilized cells was investigated by carrying out batch and repeated batch fermentations. Immobilized cells of *C. boidinii* in the gel matrices were incubated in 100 mL nutrient medium placed in 250-mL Erlenmeyer flask on a rotary shaker at 30^{o}C. Seven successive batch runs with recycling of the immobilized cells were carried out. At the end of each cycle, the broth was unloaded, the gel with immobilized cells was washed with distilled water, and the flask was refilled with fresh medium. Each cycle (except one) lasted 7 days.

2.6 Scanning electron microscopy (BG and FYROM teams)

Micrographs of cross-sections and the interior of macroporous hydrogels were obtained by using a JEOL JSM-5510 scanning electron microscope (SEM), operating at 10 kV. The specimens were first dried, then quenched in liquid nitrogen, fixed on a glass substrate and coated with gold for 60 s.

2.7 Analytical methods (FYROM and RS teams)

Cell concentration was estimated by UV.-VIS spectrometer. Xylose, xylitol and glycerol were determined by high-performance liquid chromatograph, equipped with a refractive index detector. Ethanol was analyzed by gas chromatography using a Varian CP 3800 with a capillary column.

3. Results and Discussion

3.1 Synthesis of macroporous hydrogels (cryogels) via UV irradiation.

Biocompatible cryogels were selected to build up the core of double-layered hydrogels systems because the cryogels are macroporous materials with an open porous structure. The process of cryogel formation involves a moderate freezing of the system, a reaction of cross-linking and a subsequent thawing. In this process, most of the water is frozen and forms ice crystals while the non-freezable water and the soluble substances (polymer or monomer, photoinitiator, etc.) are accumulated into a non-frozen liquid microphase (NFLMP). The gel formation occurs in this liquid microphase and the ice crystals perform as porogens.

Macroporous hydrogels of various hydrophilic and temperature-responsive polymers were synthesized via UV irradiation of moderately frozen systems. Firstly, hydroxyethylcellulose (Figure 1) and hydroxypropylcellulose cryogels (Table 1) were prepared by UV irradiation of 1 wt.% moderately frozen systems for 2 min at an irradiation dose rate of 5.7 J/cm^{2}min using a water soluble photoinitiator, (4-benzoylbenzyl)trimethylammonium chloride, and subsequent thawing. At these experimental conditions gels of good quality and high gel-fraction yield (> 95 %) were prepared. In order to ensure regular cryostructuration, the semidilute aqueous solutions of polymer and photoinitiator were at first kept at minus 20 ^{o}C for 2 h and then irradiated with UV light. Macroporous temperature-responsive poly(N-isopropylacrylamide) and poly(N-vinylcaprolactame) hydrogels (Table 1) were synthesized via UV irradiation of 10 wt.% moderately frozen systems of the corresponding monomer, poly(ethylene glycol) diacrylate (10 wt.% to the monomer) and H_2O_2 for 10 min. H_2O_2

initiator can be successfully substituted for the benzophenone derivative thus suppressing the existence of by-products in the material.

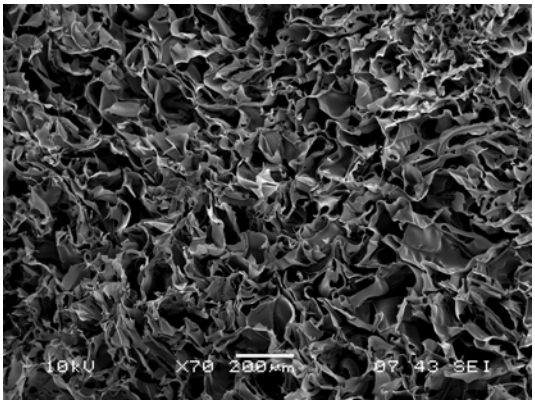


Figure 1. *SEM micrograph of hydroxyethylcellulose cryogel.*

Generally, two types of water co-exist in the polymer cryogel, water bound to the polymer via hydrogen bonds and capillary-bound water that fills the space of macropores. Since the capillary-bound water is 65 – 70 % of the total amount, the diffusion coefficient of low-molecular weight species within the cryogel is increased significantly compared to the conventional hydrogels.

Table 1. *Gel-fraction yield and degree of swelling of polymer cryogels.*

Gel	Gel-fraction [%]	Degree of swelling at 20 ^{o}C	Degree of swelling at 50 ^{o}C
Hydroxyethylcellulose	96	15	-
Hydroxypropylcellulose,	96	26	16
Poly(N-isopropylacrylamide)	93	13	5
Poly(N-vinylcaprolactame)	68	23	16

3.2 Synthesis of macroporous hydrogels via phase-separation method.

Two series of macroporous networks, as potential cell immobilizers, were synthesized from 2-hydroxyethyl methacrylate and N,N-dimethylacrylamide. The macroporous network series were successfully synthesized by phase separation polymerization in an aqueous solution. The low molecular weight and soluble hydrophilic components of the polymer networks were removed by extraction in double processed tissue cultured water. Depending on the experimental conditions hydrogels with different swelling properties can be obtained. The cross-section of the samples, analyzed by Scanning Electron Microscopy, however showed that only poly(hydroxyethyl methacrylate) hydrogels are porous (Figure 2).

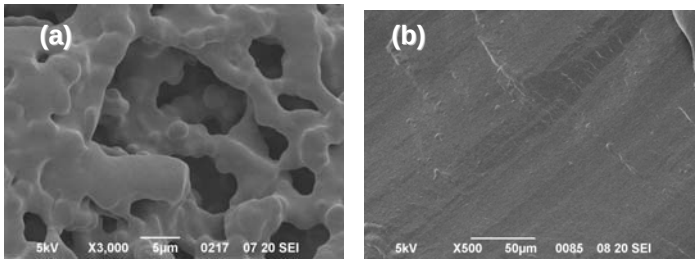


Figure 2. *Poly(hydroxyethyl methacrylate) based hydrogels (a) and polydimethylacrylamide based hydrogels b).*

In this case, poly(hydroxyethyl methacrylate) networks with high solvent volume fractions have larger pores which originate from the phase separation polymerization in an aqueous solution. The solution (water) fills the space during the polymerization and larger pores are generated. The increase of the polymer mass in the network cause smaller cavities, and the polymer matrix dominates the hydrogel system in swollen state.

3.3 Immobilization of yeast cells.

Two different approaches for immobilization of yeast cells in cryogels were tested. The first one includes mixing of the cells suspension and polymer solution followed by freezing and subsequent crosslinking. Due to the cryostructuration effect this method leads to incorporation of cells into the polymer matrix which, however, at higher cells content (> 300 wt% with respect to the polymer weight) decreases the crosslinking efficiency and, consequently, the quality of the cryogel.

The second approach is more flexible and allows immobilization of much higher amount of yeast cells into the cryogel. In this case, the polymer cryogel is prepared, freeze dried and, then, immersed in the cells suspension. As mentioned above, the cryogels are macroporous, spongy-like materials. So, by freeze drying one maintains the morphology of material and ones immersed in the suspension, the liquid fills the channels (interconnected pores) of the cryogel. This method allows immobilization of up to 2000 wt.% yeast cells with respect to the polymer weight.

For porous poly(hydroxyethyl methacrylate) samples, cell adsorption on the polymers was the only immobilization technique that could be employed. The disks were immersed in cell suspension for 2 weeks. The degree of equilibrium swelling of the sample was 3. After the sorption was over the polymer gel was slightly swollen, its consistency resembled a jelly-gum, and the color and turned white. Additionally, there were no visible signs of cell adsorption on the surface.

3.4 Synthesis of outer layer of hydrophilic hydrogel.

The outer layer has to encapsulate the macroporous gel containing yeast cells. Importantly, it has to ensure both the mechanical strength of the system and the diffusion of nutrient media and alcohols, as well as to prevent or, at least, to reduce the cells leakage. Suitable outer layer was obtained from high molecular weight poly(ethylene oxide) (HMW PEO) and crosslinking agent. It forms dense network for 2 min UV irradiation leading to well-defined outer layer. In addition, the viscosity of the semidilute PEO solution is very high, which prevents penetration into the pores of the core (in case of partial ice melting). Altering the PEO:crosslinking agent ratio one may tune the crosslinking density and the mechanical properties of the outer layer. As a rule, the increase of crosslinking agent content increases both the rigidity and density of polymer network.

3.5 Ethanol production by immobilized Saccharomyces cerevisiae

The suitability of the hydroxyethylcellulose cryogels as immobilization matrices for entrapment of cells of Saccharomyces cerevisiae was tested in a batch ethanol fermentation performed with gels containing initial cell mass concentration (dry weight) of 17.6 g/L and 29.4 g/L, which corresponds to 15 and 25%. At the beginning, the fermentation was carried out in three batches of 72 hours. Throughout the fermentation, in all batches, the substrate was totally consumed at the end of each cycle. The average volumetric productivity was 0.72g/Lh and 1.02g/Lh for the initial cell concentration of 15 and 25%, respectively. Therefore, in the further experiments, the fermentation time was shortened to 48 h. Important characteristics of the immobilized cell system are the stability of the system during the operation and its stability during the storage. The operation stability of the system

can be tested in either continuous process or by repeated batch fermentation. We opted for the latter one. The immobilized cells were stored in distilled water at 4°C for 6 months. After this period they were used in 7 repeated batches, each of 48 hours. The cell concentration inside the gels was maintained almost constant during the cycles. In course of the first cycle the cells adapted and revive their fermentative activity. The product yield was between 70-95% of the theoretical yield for the gels with 15% cells, and between 87-95% for the gels with 25% cells. It should be emphasized that the stability of the cryogels during the storage was notable. All through this time the gels kept their mechanical stability and showed no signs of degradation.

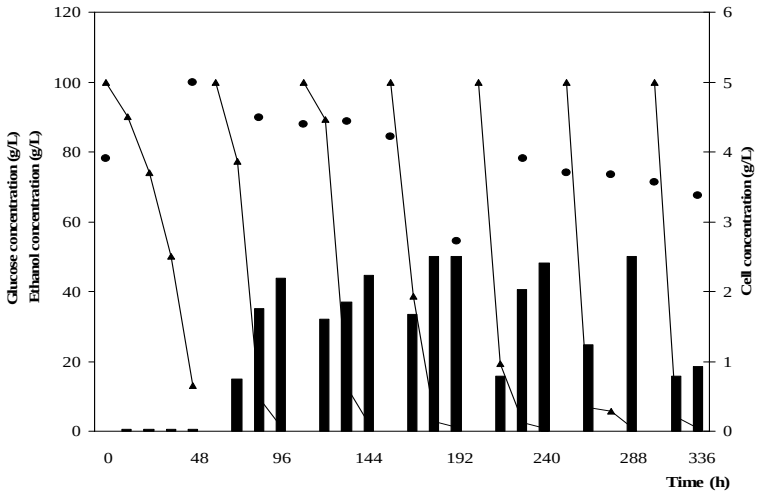


Figure 3. *Repeated batch cultivation of immobilized system with 25% cells of S. cerevisiae in hydroxyethylcellulose cryogel; biomass (●), glucose (▲) and ethanol (■)*

The performance of double-layered careers of *Saccharomyces cerevisiae* was tested at a larger scale in stirred tank bioreactor, convenient to test mechanical stability and resistance to abrasion of the biocatalyst and in fluidized bed bioreactor. The results from the fermentations studies showed that hydroxyethylcellulose cryogel coated with the layer of poly(ethylene oxide) hydrogel is a suitable matrix for yeast cell immobilization aimed for the process of glucose conversion to ethanol. After the immobilization step, yeast cells were able to propagate and kept the activity to catalyze fermentation process. Except viability of cells, another important characteristic in immobilized cell systems is the stability of the biocatalyst during long-term operation process. However, the materials were not able to withstand high shear stresses caused by non-uniform turbulent flow in a tank with rapid mechanical mixing. In case of fluidized bed reactor, there were not notable changes in a shape of gel peaces. In large scale industrial applications, mechanical stirring is envisaged, therefore, the mechanical strength of the hybrid system need to be improved.

3.6 Xylitol production by immobilized *Candida boidinii*

Preliminary tests of xylose consumption and xylitol formation during the repeated-batch culture of immobilized C. boidinii were carried out. In the first cycle 51.8% of the xylose was consumed, while in the subsequent three cycles the consumption increased up to 73.5%. This progressive improvement of the xylose consumption stopped in the fifth cycle when the consumption was lowered to 42.7%. Even though the time in the next (sixth) cycle was increased to 13 days, xylose consumption did not surpass 50%. The cell leakage of this system was minor, however, the experimental condition still has to be optimized.

4. Summary/Conclusions/Outlook, next steps

Novel double-layered polymeric hydrogels were synthesized and tested as careers of different cells. The results from the fermentations studies showed that hydroxyethylcellulose cryogel coated with thin layer of poly(ethylene oxide) hydrogel is a suitable matrix for yeast cell immobilization aimed for the process of glucose conversion to ethanol. From a technological point of view, this is a fast, convenient and effective method for design and preparation of hybrid hydrogel system. The future experiments should focus on the mechanical stability of the outer layer and the continuous fermentation for production of alcohols with double-layered hydrogels under different operating conditions.

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6. Appendices (if appropriate)

The results were published in two papers:

Winkelhausen E., Kuzmanova S., Jovanovic-Malinovska R., Cvetkovska M., Tsvetanov Ch. *Hydrogels based on u.v.-crosslinked poly(ethylene oxide)–matrices for immobilization of Candida boidinii cells for xylitol production.* World J Microbiol Biotechnol (2008)DOI10.1007/s11274-008-9707-5

Petrov, P; Petrova, E; Tsvetanov, Ch B. *UV-assisted synthesis of super-macroporous polymer hydrogels.* Polymer, doi:10.1016/j.polymer.2008.12.039

And presented at the following conferences:

Velickova E., Kuzmanova S., Winkelhausen E.,Cvetkovska M,. Tsvetanov Ch. *New immobilization technique of cell entrapment applied for ethanol production*, 4th Central European Congress on Food, Cavtat, Croatia, May 2008.

Velickova E., Petrov P., Fodor C., Manojlovic V., Nedovic V., Ivan B., Csaba, Winkelhausen E., Tsvetanov Ch. *New Carriers for cell immobilization*, “Sixth International Conference of the Chemical Societies of the South-Eastern European Countries”, Sofia, September 2008.