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Hydroxyethylcellulose cryogels used for entrapment of *Saccharomyces cerevisiae* cells

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ABSTRACT

Cryogels of cellulose ether, hydroxyethylcellulose (HEC), were obtained via UV-irradiation of moderately frozen aqueous solutions in the presence of water soluble photoinitiator, (4-benzoylbenzyl) trimethylammoniumchloride (BBTMAC). The suitability of the cryogels as matrices for immobilization was studied by entrapment of *Saccharomyces cerevisiae* cells. The cryogels were characterized by measuring their rheological parameters, gel fraction yield and the degree of equilibrium swelling. The cells entrapped inside the matrix acted as pore fillers and strengthened the cryogels, while glucose incorporated into the gel had the opposite effect. The applicability of the immobilized systems was investigated by performing batch ethanol production from glucose. The operational stability of the gels was tested in batch fermentation with three cycles. The storage stability was investigated in seven repeated batches after 6 months storage of lyophilized cryogels in distilled water. The ethanol production ranged from 70% to 95% of the theoretical yield for the gels with 15% cells, and from 87% to 95% for the gels with 25% cells.

1. Introduction

Immobilization of biocatalysts first appeared in 1960s and since then it has been a widely used technique in a number of areas [1]. Immobilization of whole cells for fermentation processes has been developed to eliminate the inhibition caused by high concentration of substrate and/or product. If intact microbial cells are directly immobilized into the matrix, the removal of microorganisms from the broth can be omitted and the loss of intracellular enzyme activity can be kept to a minimum [2]. Hence, the advantages of the processes with immobilized microorganisms over the suspension cultures include the easier collection and purification of bioproducts, better stability of the biocatalyst and tolerance against high concentrations of toxic compounds [3].

In recent years, there has been a growing interest for the immobilization of microbial cells via gel entrapment. As an alternative to the chemical methods, irradiation technique has been introduced for production of gels because of its processing speed and simplicity of synthesis. Several methods have been proposed for modification of the structure of the polymer network and the size of pores, among which is the preparation of new generation of gels also known as cryogels [4]. The cryogels are highly porous polymeric materials synthesized in semi-frozen aqueous media where ice crystals act as porogen and template the continuous interconnected pores after melting. A system of large interconnected pores and high elasticity are the main characteristics of these gels. Recently, a promising type of natural polymer, hydroxyethylcellulose (HEC), which is inexpensive and nontoxic to microorganisms, has been obtained by a simple, gentle and rapid procedure with UV curing of a frozen aqueous polymer solution [5]. Hydroxyethylcellulose is usually used as thickener in food and cosmetic products, as protective colloid in polymerization processes and thickener in water-based paints. The aqueous solutions of HEC are highly viscous. To optimize the properties for the different industrial applications the HEC samples are often modified by ionic or hydrophobic groups. HEC is becoming more popular due to stringent environmental regulations, since it can be easily degraded by the cellulases [6,7].

The progressive depletion of the energetic resources, mainly based on non-renewable fuels, initiated the search for alternative fuels. Among the alternative liquid fuels, the use of bioethanol has raised a lot of interest. This is mainly due to the growing public's awareness of the problems existing on the current oil market, as well as in the environment caused by increased greenhouse emissions and global warming [8–10]. One of the most common method for ethanol production is through the fermentation. Bioethanol production is based on a proper selection of a yeast strain,

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a fermentable substrate and an appropriate fermentation system [11]. Several studies have already suggested the production of ethanol with the use of immobilized yeast in Ca-alginate beads, hydrogels and inorganic supports [12–14]. In the scientific and industrial world *Saccharomyces cerevisiae* is the most well-known microorganism for ethanol production.

In this study, for the first time, the hydroxyethylcellulose cryogel [5] was used as a matrix for entrapment of yeast cells. To our knowledge, there have been no reports on hydroxyethylcellulose being used for immobilization and culturing of *S. cerevisiae* cells. The mechanical properties of the cryogels were characterized and alcoholic batch fermentation was used to test the ability of immobilized *S. cerevisiae* to produce ethanol.

2. Experimental

2.1. Materials

Natrosol (2-hydroxyethylcellulose) (Mn = 1.3×10^6 g/mol) was obtained from Union Carbide Chemicals and Plastics Co. The (4-benzoylbenzyl) trimethylammoniumchloride (BBTMAC) purchased from Aldrich was used as a photoinitiator, without further purification. All other chemicals used were purchased from commercial sources and were of analytical grade.

2.2. Microorganism and media

Glucose medium with commercial grade baker's yeast, *S. cerevisiae*, with 29.4% dry biomass, was used for ethanol production. Prior to batch fermentation, the cells were cultured for 20 h in a nutrient medium with the following composition (per litre of distilled water): 10 g yeast extract, 2 g KH_2PO_4, 1 g NaCl, 0.2 g $CaCl_2{\cdot}2H_2O$, 1.7 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeCl_3{\cdot}6H_2O$, 2 g NH_4Cl and 20 g glucose. In the batch production of ethanol the glucose concentration was increased to 100 g/l. The sugar and salt solutions were autoclaved separately at 121 °C for 15 min. The initial pH values of both media were five.

2.3. Preparation of cryogels, without and with yeast cells

Hydroxyethylcellulose was dissolved in an appropriate amount of distilled water to obtain 2% aqueous solution at 40 °C. Then the solution was kept for 24 h at room temperature to ensure complete dissolution and homogeneity. Given amount of photoinitiator, BBTMAC, (2 wt% of the polymer mass) dissolved in 1 ml distilled water was added under stirring at room temperature. The homogeneous solution was poured into teflon dishes (20 mm diameter) forming a 2–3 mm thick layer, followed by freezing step at −30 °C for 2 h. Afterward the dishes were quickly placed in a temperature-controlled chamber and the polymer was UV irradiated in a Dimax 5000-EC UV light curing system for 2 min. The gels in the teflon dishes were left at room temperature for few minutes, turned over the other side and placed back in the freezer for additional 2 h, when they were UV irradiated again on the other side for 2 min under same conditions.

The cryogels with different glucose concentration (0.5–10% of the polymer mass) were prepared in the same way as pure hydroxyethylcellulose cryogels. Glucose was added to the polymer solution together with the photoinitiator before the freezing step.

For the formation of the immobilized biocatalysts, homogenized cell suspension, containing 2–25% (w/v) cells with respect to the polymer solution, was added under stirring at room temperature to the 2% (w/w) aqueous solution of HEC and vortexed to improve homogenization. The gelation process followed the same procedure used for preparation of pure cryogels.

2.4. Measurements of gel fraction yield and equilibrium degree of swelling

Gel fraction (GF) yield and equilibrium degree of swelling (ES) of HEC cryogel were determined gravimetrically. The gel fraction content in the dried sample was estimated by weighing the insoluble part after extraction in distilled water for 6 days at room temperature:

$$\text{Gel fraction} = \frac{\text{mass of the dried sample after extraction}}{\text{initial mass}} \times 100\ (\%) \qquad (1)$$

The equilibrium degree of swelling (ES) was determined at room temperature. Disks of dried cryogel (ϕ 14 mm) were equilibrated in distilled water for 48 h, removed from the water, blotted with filter paper and weighed. They were then dried to constant mass under vacuum and weighed again. The ES is presented as grams of swollen gel sample per gram of dried gel sample:

$$\text{Equilibrium swelling} = \frac{\text{mass of equilibrated swollen sample}}{\text{mass of dry sample}} \qquad (2)$$

2.5. Dynamic rheological measurements

Dynamic rheological measurements of the cellulose cryogels were performed on a ThermoHaake Rheostress 600 rheometer with a parallel plate sensor system (20 mm diameter) and Peltier temperature controller. Disks of the gels were extracted for 6 days in distilled water, then removed from water and blotted with filter paper before the dynamic storage (G') and loss (G'') moduli were measured in a frequency range of 0.1–10 Hz at 25 °C.

2.6. Scanning electron microscopy

For electron microscopy scanning (SEM) micrographs, samples were taken from fresh cryogels, without and with yeast cells, as well as from cultivated gels with immobilized cells. Composite cryogels were quenched in liquid nitrogen, freeze dried, and coated with gold in a Jeol JFC-1200 fine coater. At the end samples were examined by SEM using the model Jeol JSM-5510.

2.7. Fermentations

Batch fermentations with free and immobilized cells of *S. cerevisiae* in the cryogel matrices was carried out in 100 ml nutrient medium placed in 250 ml Erlenmeyer flask on a rotary shaker (100 rpm) at 28 °C. After 20 h of preculturing, the gels with immobilized cells were washed in sterile distilled water and transferred into fresh nutrient medium for ethanol fermentation, under same conditions. Forty gel pieces of about 1 ml volume were placed in each flask providing 1:2.5 v ratio of gel to medium.

For repeated batch fermentation after every run the immobilized biocatalysts were recovered from the flasks and were immersed in fresh medium to start a new batch run.

2.8. Analytical methods

In batch fermentation, approximately 2 ml samples were collected for analysis. Dry weight of yeast cells in the gels was estimated by the Kjeldahl method. Gels used for this procedure were removed from the fermentation medium, blotted with filter paper and weighed before pyrolysis. To determine the rate and the degree of cell leakage from the matrices, after the immobilization procedure, the absorbance of each sample from the fermentation

broth was measured at 620 nm using spectrophotometer, Varian, Cary 50.

Ethanol was analyzed by gas chromatography using a Varian CP 3800 with a capillary column WCOT fused silica (30 m × 0.32 mm), CP WAX 52 CB, d_i = 0.25 μm, equipped with flame ionization detector (FID) and a manual injector type 1709. The injector and detector temperatures were 250 °C, and the column temperature was 200 °C. Nitrogen was used as a carrier gas with a flow rate of 30 ml/min. Isopropanol was used as an internal standard.

Glucose concentration was determined using a reducing chemical reagent, 3,5-dinitrosalycilic acid solution following the procedure described by Miller [15].

Ethanol yield was calculated as mass of the ethanol formed per mass of the substrate consumed, while the volumetric productivity was calculated as mass of the ethanol formed per litre medium per hour.

3. Results and discussion

3.1. Characterization of HEC cryogels

Cryogels of hydroxyethylcellulose were synthesized by UV-irradiation of 2% moderately frozen aqueous solutions for 2 min at both sides in the presence of the photoinitiator (4-benzoylbenzyl) trimethylammoniumchloride (BBTMAC). Due to the cryoconcentration effect, the polymer concentration in the non-frozen liquid microphase was too high and therefore the crosslinking reactions could overcome degradation reactions during the UV curing [5].

To improve the environment of the entrapped cells the presence of some dissolved components such as salts, sugars or cryoprotectants is required. However, it is important these substances not to act as obstacles in the gelation process and not to have a negative effect on the physico-mechanical properties of the matrix [16]. Since glucose was the main substrate for the yeast cells, cryogels with different glucose concentration were also synthesized. The concentration of glucose within the gel was increased from 0.5% to 10% of the polymer mass, corresponding to concentration of 5–100 g glucose/l polymer solution, thus approaching the same concentration as the glucose in the nutrient medium. The properties of the cryogels formed are presented in Table 1 and in Fig. 1.

It is evident that the increased glucose concentration in the gel seriously affected the gel fraction and the equilibrium swelling degree in water (ES), as well as the storage modulus, G'. It is obvious that the cryogels with glucose do not posses the required mechanical strength. The addition of glucose in concentration of 100 g/l resulted in no gelation even though the system looked quite frozen. The most likely reason for no gelation was the absence of phase separation in the 10% glucose solution and correspondingly the lower local concentration of cross-linkable groups. The poor mechanical properties of the cryogels with glucose excluded them from further use for cell immobilization.

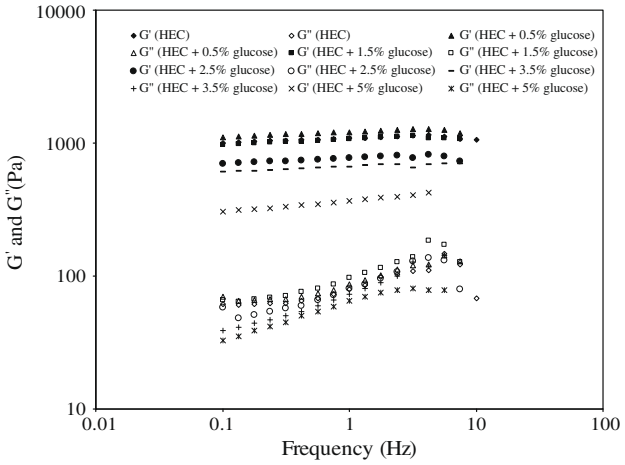

Fig. 1. Rheogram of pure 2% hydroxyethylcellulose cryogels and cryogels with different glucose concentration. Elastic (G') and storage modulus (G'') versus oscillating frequency.

Measurements of both storage (G') and loss modulus (G'') in samples of pure 2% HEC and samples supplemented with glucose (Fig. 1) depicted the changes in the mechanical spectra derived from the frequency sweeps. The curves for the cryogels with glucose corresponded to the decreased gel fraction suggesting formation of weak gels. The pure HEC cryogel, however exhibited the behavior of a true gel, with high G' values and independence of the frequency of oscillation.

To get an insight in the process of curing of cell-polymer suspensions, characterization of the cryogels with different initial cell concentration was performed. Gels with different cell concentration were synthesized in order to investigate the maximal cell load that the cryogel can retain without breaking the polymer network. The presence of entrapped cells influenced the physico-mechanical properties of the cryogels. It is important to note that immobilized biomass did not reduce the strength of the immobilized system (Table 2, Fig. 2).

Several authors [9,17,18] reported on a decrease in the mechanical properties of the gel matrices (Ca-alginate and hydrogel) as a result of the presence of microbial cells. In contrast, the HEC cryogels showed notable increase in the modulus of deformability with an increased cell upload. The entrapped cells resemble the porous fillers in their effect on the rheological properties of the HEC cryogel support. Although yeast cells are mechanically weaker and contain large amounts of liquid they were still capable of strengthening the gels as good as they were rigid mineral materials [19]. The decrease in viscosity was due to the increase in elasticity, as rheograms shown in Fig. 2. Rheological analysis carried out on cryogels with different concentration of entrapped cells showed increase in mechanical stability with the increase of cell concentration in the

Table 1
Effect of glucose concentration on the properties of cryogels prepared from 2% frozen aqueous solution of hydroxyethylcellulose.

Gel composition HEC:glucose % (w/v)	Gel composition HEC:glucose % (w/w)	Glucose concentration (g glucose/l polymer solution)	GF (%)	ES
1:0	1:0	0	83	24
1:0.5	1:0.25	5.0	77	35
1:1.5	1:0.75	15.0	54	31
1:2.5	1:1.25	25.0	38	30
1:3.5	1:1.75	35.0	30	36
1:5.0	1:2.00	50.0	20	48
1:10.0	1:4.00	100.0	–	–

Table 2
Characteristics of the HEC cryogels with different concentration of entrapped cells in respect to the polymer solution.

Gel composition HEC:cells (w/w)	Cell concentration % (w/v)	GF (%)	ES
1:1.00	2	86	28
1:2.00	5	75	25
1:4.00	10	88	21
1:6.00	15	72	18
1:10.00	25	66	23

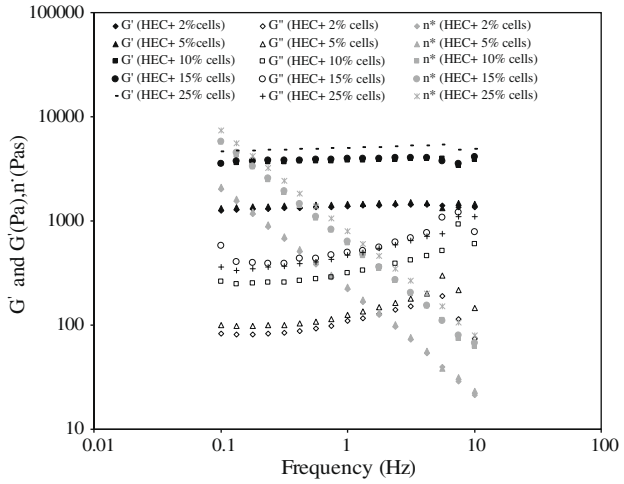

Fig. 2. Rheogram of cryogels with different cell concentration. Elastic modulus (G'), storage modulus (G'') and complex viscosity (n^*) versus oscillating frequency.

gel. Consequently, the systems with 15% and 25% cells have been chosen for further tests.

The SEM method was the method of choice for the characterization of the porous structure of the HEC cryogels. Fig. 3 shows the cross-section and the inner region of the carrier matrix with entrapped cells. The relative sizes of both the cells and the macropores can be measured. As can be seen from Fig. 3 there are many pores with size of approximately 200 μm filled with uniformly spread yeast cells yet leaving enough space for transport of the substrate and the product.

3.2. Cultivation of free and immobilized cells

Batch fermentations with freely suspended and immobilized cells were performed to investigate the benefits of the cell immobilization (Fig. 4). To compare the process parameters and to provide detailed information on glucose depletion and product formation, both experiments were carried out with the same initial glucose concentration of 100 g/l for 72 h.

The rapid glucose consumption in the first 12 h of the fermentation coincided with the maximal volumetric productivity of 1.35 g/lh and 2.28 g/lh for the system with free and immobilized cells, respectively. In this period immobilized cells produced 27.4 g/l ethanol corresponding to a product yield of 0.38 g/g (74% from the theoretical yield), which was two times more than the ethanol produced by freely suspended cells. Other authors have also reported better production rates with immobilized cells [20,21]. Ethanol is a typical primary metabolite the production of which is tightly coupled with the growth of yeast cells. When cells are immobilized, they can be protected from the inhibitory effect of the produced ethanol since the cryogel acts as a protector [22].

3.3. Operational stability of the immobilized cells

Development of stable and re-usable porous gel materials that can be dried and subsequently re-swollen is desirable. The ability

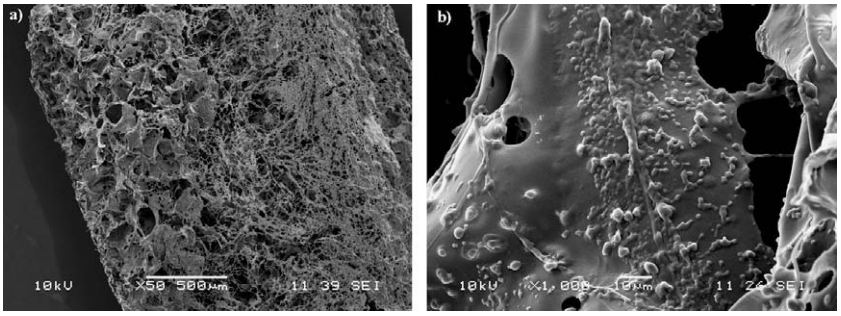

Fig. 3. SEM micrographs of 2% hydroxyethylcellulose cryogels with 15% entrapped cells to the polymer mass: (a) cross-section and (b) inner region.

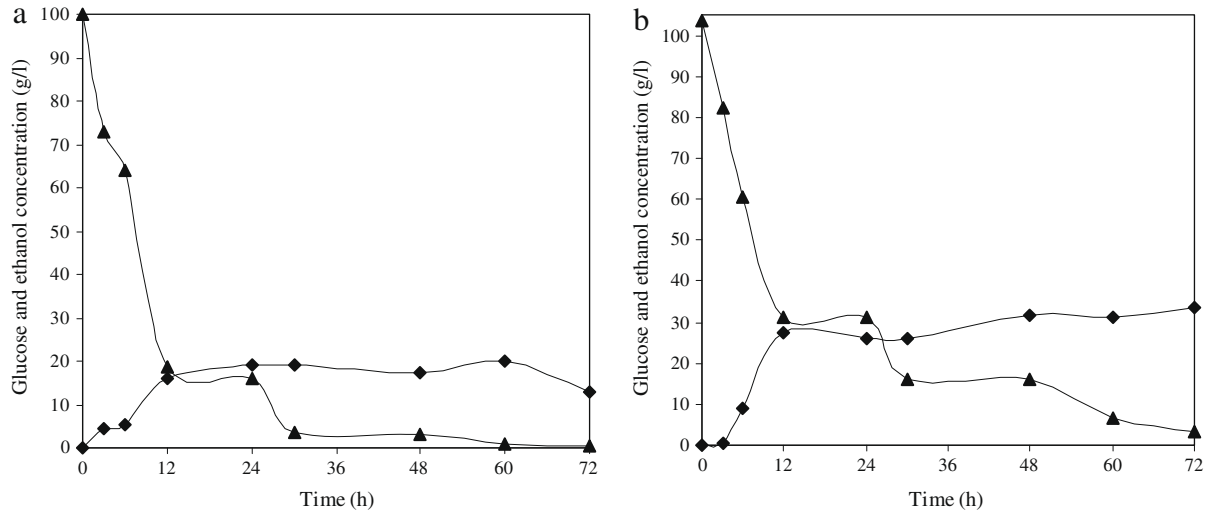

Fig. 4. Dynamics of glucose consumption (▲) and ethanol production (♦) in batch fermentation carried out with (a) freely suspended and (b) immobilized cells.

Table 3
Process parameters of ethanol production from glucose medium by *S. cerevisiae* cells immobilized in HEC gels. The initial dry biomass entrapped in the gels is 17.64 g/l, t = 48 h.

Parameters						
Batch	P	$Y_{p/s}$	$Y_{p/t}$	Q_p	$S_{residual}$	$\Delta S/S_0$
	(g/l)	(g/g)	(%)	(g/lh)	(g/l)	(%)
Cryogels						
I	37.3	0.38	74	0.77	1	99
II	37.9	0.41	80	0.79	7	93
III	24.2	0.26	51	0.49	7	93
Lyophilized cryogels						
I	34.0	0.34	67	0.71	1	99
II	23.8	0.25	50	0.49	7	93
III	22.1	0.24	46	0.46	7	93

P, ethanol concentration (g/l).
$Y_{p/s}$, measured ethanol yield (g/g).
$Y_{p/t}$, percentage of the measured ethanol yield to the theoretical ethanol yield (%).
Q_p, volumetric productivity (g/lh).
$S_{residual}$, glucose concentration at the end of fermentation (g/l).
$\Delta S/S_0$, substrate consumption (%).

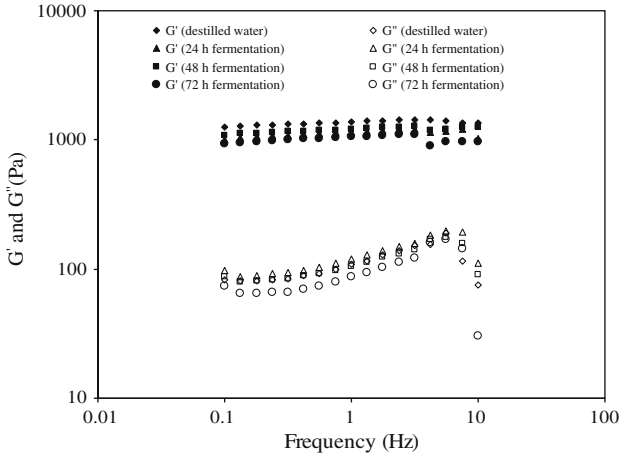

Fig. 6. Mechanical spectra of HEC cryogels with 2% cell concentration to the polymer mass after 6 days extraction in distilled water and during 3-day cultivation in glucose medium. Elastic (G′) and storage modulus (G″) versus oscillating frequency.

of drying the immobilized microbial cells simplifies their storage. Being able to survive dehydration and later on be reactivated at higher water activities in their environment is of a fundamental importance in biotechnology [23]. Accordingly, both cryogels and lyophilized cryogels with immobilized cells, were synthesized and tested. To investigate the operational stability of the immobilized cells, they were used in three repetitive runs for conversion of glucose to ethanol (Table 3). The production of ethanol at the end of the three runs carried out with cryogels and lyophilized cryogels with the same amount of initial biomass ranged from 22 to 38 g/l. The residual sugar concentrations were low in all cases (1.0–7.0 g/l) with conversions from 93% to 99%, demonstrating good efficiency of the system. Similar conversion factors of 94.4–98.8% but with ethanol productivity of about 4 g/lh were reported by Plessas et al. [24]. The highest volumetric productivities of 1.00, 1.02 and 0.88 g/lh for cryogels in the first, second and the third batch were reached after 30 h of cultivation, while the cells in lyophilized gels showed their maximal productivity of 1.10, 2.42 and 1.24 g/lh in all three batches after 12 h. The final product yields achieved by the cells immobilized in cryogels were slightly higher than the yields of cells immobilized in lyophilized gels.

To view the morphology of the cryogels with immobilized yeast cells, scanning electronic micrographs were taken after 24 and 72 h of cultivation (Fig. 5). These micrographs showed that after 24 h, immobilized cells were viable inside the gel, while after 72 h of cultivation a cell proliferation inside the gel pores was noticed and what was very important, no signs of breaking the polymer walls were detected. In favor of the stability and the suitability of the HEC cryogels for cell immobilization is the observation that the amount of cells retained in both cryogels at the end of the third run was almost 8-fold higher than the amount of free cells released in the broth.

In addition to SEM and estimation of the cell leakage, the stability of the gels was evaluated by measuring the retention of the shear storage modulus, G' on randomly taken samples during cultivation. The rheograms presented in Fig. 6 showed that there were insignificant changes in the behavior of the shear storage modulus measured on gels which were cultured in nutrient medium for 3 days compared to referent gels extracted in distilled water for 6 days at room temperature. The solid and constant line of G' values above 1000 Pa for all samples confirms the mechanical stability of the gels.

3.4. Storage stability of the immobilized cells

The storage stability of the immobilized system was investigated in a repeated batch ethanol fermentation. The lyophilized gels with immobilized cells which were used in three successive runs were stored in distilled water at 4 °C for 6 months. After this period, they were used again in seven repeated cycles, each of 48 h. The time of each run was shortened since in the previous experiments the substrate was consumed earlier than 72 h. Fig. 7 depicts the cell density, glucose concentration and product formation as a function of time.

From the first cycle, (Fig 7a) it was obvious that the cells after being stored for 6 months in distilled water, were starved and in the first 36 h had to use the substrate for growing without producing any detectable amount of ethanol. The following batches (2–5) exhibited standard behavior, which meant gradual glucose

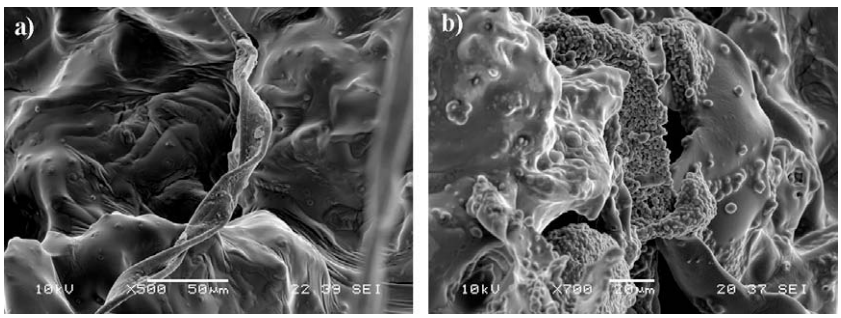

Fig. 5. SEM micrographs of immobilized yeast cells in cryogels after (a) 24 h and (b) 72 h of the fermentation.

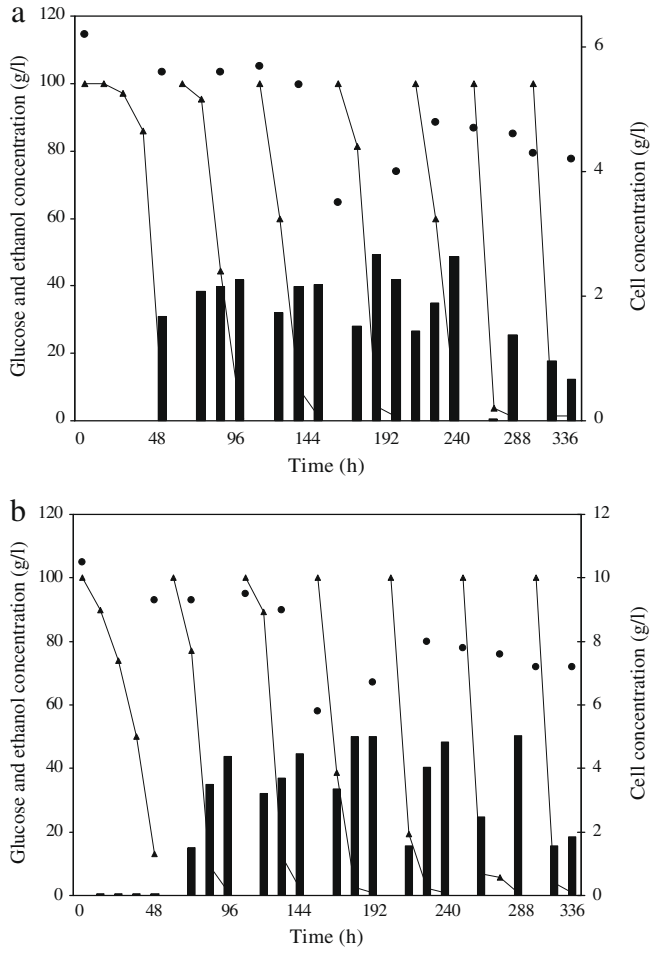

Fig. 7. Fermentation profile during ethanol production in seven consecutive runs with 17.64 g/l (a) and 29.40 g/l (b) initial biomass. Concentration of cells in lyophilized cryogels (•), concentration of glucose (▲) and ethanol (vertical bars) in the medium.

consumption and ethanol production. At the end of each cycle, glucose was completely consumed and ethanol concentration was above 40 g/l in both immobilized systems. These values of ethanol concentration corresponded to a yield from 70% to 95% of the theoretical yield for the gels with 15% cells (17.64 g_{dw}/l) and from 87% to 95% for the gels with 25% cells (29.40 g_{dw}/l). During the next two consecutive cycles, ethanol production slowly decreased leading to a production under 50% of the theoretical.

Comparison of ethanol production by different immobilized systems is presented in Fig. 8. It is evident that all immobilized systems showed similar behavior. Only the lyophilized system with 15% cells produced ethanol in the first run but after 48 h. The same system had a significant drop in the ethanol concentration at the end of the 6th and 7th batch. The two other systems started actually producing ethanol from the second run. The higher initial cell mass in cryogels (29.40 g/l) did not bring to significantly higher ethanol production. Yu et al. [21] reported on 13 repeated uses of the immobilized system in batch fermentation with constant product yield of 94% of the theoretical throughout all batches.

It is noteworthy to mention that the lyophilized cryogels despite being used (three cycles), then stored (6 months) and used again (for 14 days, seven cycles) remained intact and exhibited no signs of degradation at the end of the seventh cycle.

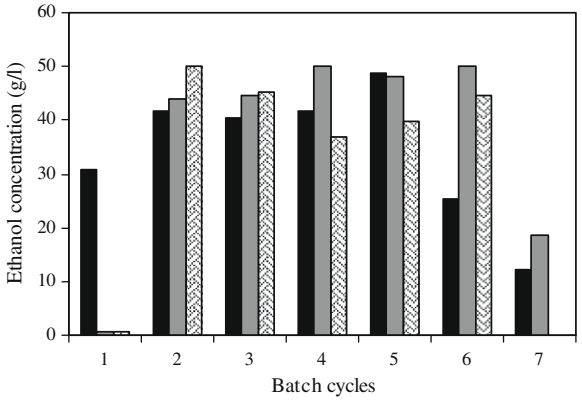

Fig. 8. Ethanol concentration during seven repeated batches with different immobilization systems. Cryogels with 17.64 g/l cell (patterned columns), lyophilized gels with 17.64 g/l (black columns) and with 29.40 g/l (gray columns) cells.

4. Conclusion

It has been shown that cryogels of hydroxyethylcellulose synthesized by UV-irradiation of moderately frozen aqueous solutions in the presence of water soluble photoinitiator are suitable matrices for cell entrapment. The performance of the immobilized systems was evaluated during repeated batch ethanol fermentation immediately after its preparation and after storage. After being stored for 6 months the cryogels were still mechanically stable with no signs of deterioration, and *S. cerevisiae* cells immobilized in the cryogels were able to produce above 40 g/l ethanol.

Acknowledgements

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References

[1] M.R. Melo-Junior, L.C. Alves, F.B. Santos, E.I.C. Beltrao, L.B. Carvalho Jr., React. Funct. Polym. 68 (2008) 315.
[2] N. Miletic, R. Rohandi, Z. Vukovic, A. Nastasovic, K. Loos, React. Funct. Polym. 69 (2009) 68.
[3] Q. Wen-tao, Y. Wei-ting, X. Yu-bing, M. Xiaojun, Biochem. Eng. J. 25 (2005) 151.
[4] A.A. El-Hadi, Process Biochem. 38 (2003) 1653.
[5] P. Petrov, E. Petrova, R. Stamenova, C.B. Tsvetanov, G. Riess, Polymer 47 (2006) 6481.
[6] S. Erkselius, O.J. Karlsson, Carbohyd. Polym. 62 (2005) 344.
[7] P. Petrov, E. Petrova, B. Tchorbanov, C.B. Tsvetanov, Polymer 48 (2007) 4943.
[8] M. Gryta, A.W. Morawski, M. Tomaszewska, Catal. Today 56 (2000) 159.
[9] T.S. Khaw, Y. Katakura, K. Ninomiya, C. Moukamnerd, A. Kondo, M. Ueda, S. Shioya, J. Biosci. Bioeng. 103 (2007) 95.
[10] J.A. Quintero, M.I. Montoya, O.J. Sánchez, O.H. Giraldo, C.A. Cardona, Energy 33 (2008) 385.
[11] A. Sheoran, B.S. Yadav, P. Nigam, D. Singh, J. Basic Microbiol. 38 (1998) 123.
[12] V. Manojlovic, J. Djonlagic, B. Obradovic, V. Nedovic, B. Bugarski, J. Chem. Technol. Biotechnol. 81 (2006) 505.
[13] R. Jovanovic-Malinovska, S.A. Amartey, S. Kuzmanova, E. Winkelhausen, M. Cvetkovska, Ch. Tsvetanov, Bull. Chem. Technol. Macedonia 25 (2006) 113.
[14] S.R. Andrietta, C. Steckelberg, M.G.S. Andrietta, Biores. Technol. 99 (2008) 3002.
[15] G.L. Miller, Anal. Chem. 31 (1959) 426.
[16] V.I. Lozinsky, F.M. Plieva, Enzyme Microb. Technol. 23 (1998) 227.
[17] V.I. Lozinsky, A.L. Zubov, E.F. Titova, Enzyme Microb. Technol. 20 (1997) 182.
[18] E. Winkelhausen, R. Jovanovic-Malinovska, S. Kuzmanova, M. Cvetkovska, Ch. Tsvetanov, World J. Microbiol. Biotechnol. 10 (2008) 2035.
[19] M. Giuliano, C. Schiraldi, C. Maresca, V. Esposito, M. De Rosa, Enzyme Microb. Technol. 32 (2003) 507.
[20] E. de Alteriis, D. Porro, V. Romano, P. Parascandola, FEMS Microbiol. Lett. 195 (2001) 245.
[21] J. Yu, X. Zhang, T. Tan, J. Biotechnol. 129 (2007) 415.
[22] F.W. Bai, W.A. Anderson, M. Moo-Young, Biotechnol. Adv. 26 (2008) 89.
[23] F.M. Plieva, A. Oknianska, E. Degerman, B. Matiasson, Biotechnol. J. 3 (2008) 1.
[24] S. Plessas, A. Bekatorou, A.A. Koutinas, M. Soupioni, I.M. Banat, R. Marchant, Biores. Technol. 98 (2007) 860.