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Ethanol Production Using Immobilized *Saccharomyces cerevisiae* in Lyophilized Cellulose Gel

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Abstract A new lyophilization technique was used for immobilization of *Saccharomyces cerevisiae* cells in hydroxyethylcellulose (HEC) gels. The suitability of the lyophilized HEC gels to serve as immobilization matrices for the yeast cells was assessed by calculating the immobilization efficiency and the cell retention in three consecutive batches, each in duration of 72 h. Throughout the repeated batch fermentation, the immobilization efficiency was almost constant with an average value of 0.92 (12–216 h). The maximum value of cell retention was 0.24 g immobilized cells/g gel. Both parameters indicated that lyophilized gels are stable and capable of retaining the immobilized yeast cells. Showing the yeast cells propagation within the polymeric matrix, the scanning electron microscope images also confirmed that the lyophilization technique for immobilization of *S. cerevisiae* cells in the HEC gels was successful. The activity of the immobilized yeast cells was demonstrated by their capacity to convert glucose to ethanol. Ethanol yield of 0.40, 0.43 and 0.30 g ethanol/g glucose corresponding to 79%, 84% and 60% of the theoretical yield was attained in the first, second and third batches, respectively. The cell leakage was less than 10% of the average concentration of the immobilized cells.

Keywords Lyophilized gels · Hydroxyethylcellulose · Immobilization · *Saccharomyces cerevisiae* · Ethanol

Introduction

Interest in biomass and bioenergy has intensified in the last few years due to the fact that the depletion of the oil reserve is much faster than previously predicted and the environmental deterioration resulting from the overconsumption of petroleum-derived

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products is threatening the sustainability of human society [1]. The negative effect of fossil fuels on the environment, especially the emission of greenhouse gases, has turned the scientist's attention to fuel alternatives. Ethanol, which is both renewable and environmentally friendly, is believed to be one of the best liquid fuel alternatives. The production of ethanol by yeast fermentation of various renewable biomass resources is continuously increasing. Hence, any improvement in the technology of ethanol production will be of huge economic importance [2]. Immobilized cell technology has been suggested as an effective means for improving ethanol fermentation [3, 4].

The selection of the proper supporting material is very important for successful performance of the immobilized cell system. Polymeric cryogels as a new family of porous materials are pointed out as carriers suitable for cell entrapment. Depending on the properties and concentrations of the precursors and on the conditions of the cryotropic gelation, cryogels of various chemical structures can be prepared [5]. Recently, we reported on immobilization of *Candida boidinii* and *Saccharomyces cerevisiae* cells in hydrogels based on UV-crosslinked poly(ethylene oxide) and hydroxyethylcellulose (HEC) [6–8]. In this study, a new lyophilization technique for immobilization of *S. cerevisiae* cells in HEC was employed and the ability of the gel matrix to retain the cells was evaluated. The activity of the immobilized yeast cells was assessed by their capacity to produce ethanol from glucose.

Materials and Methods

Yeast, Medium and Inoculum Preparation

The yeast used in this study was a commercial-grade baker's yeast, *S. cerevisiae*. The cells were grown in a medium with the following composition (per liter 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 50 g glucose. The sugar and salt solutions were autoclaved separately at 121 °C for 15 min. Initial pH was 5.

Immobilization of Yeast Cells

Cells of *S. cerevisiae* were immobilized in lyophilized gels of 2-HEC containing trimethylammoniumchloride (BBTMAC) as photoinitiator. Homogenized cell suspension, containing 10% (w/v) with respect to the polymer solution, was added under stirring at room temperature to the 2% (w/w) aqueous solution of HEC and vortexed. The resulting homogeneous solution was poured into Teflon dishes (2 cm diameter) forming about 2.5-mm-thick layer and frozen at –30 °C for 2 h. The dishes were then irradiated with UV light by Dymax 5000-EC curing equipment with 400 W metal halide flood lamp for 2 min on both sides. After immobilization procedure the gels were lyophilized. For revitalization of the cells, the gels were placed in 250-ml flasks with 100 ml nutrient medium for 20 h on a rotary shaker (100 rpm) at 30 °C. After that, the gels were used for fermentation of glucose to ethanol.

Fermentation

Fermentations were performed in 250-ml cotton-plugged shake flasks containing 100 ml of the medium and glucose as a carbon source. The flasks were placed on a rotary shaker

(100 rpm) at 30 °C for 24 h. Repeated batch fermentation was carried out in three cycles, each lasting 72 h. At the end of each cycle, the gels with immobilized cells were recovered from the flasks and immersed in fresh medium to start a new run. The fermentations were performed in duplicate.

Scanning Electron Microscopy

For scanning electron microscope, the lyophilized gels were quenched in liquid nitrogen, freeze-dried and coated with gold in a Jeol JFC-1200 fine coater. The samples were examined using the model Jeol JSM-5510.

Analytical Methods

Samples of approximately 2 ml were collected periodically for analysis. Cell concentration was estimated turbidometrically at 620 nm, after diluting the samples within the range of 0.05–0.5 units. Calibration curve was used to relate the optical density units to dry cell concentration. Samples were clarified by centrifugation (Heraus) at 2500×*g* for 15 min for products and residual sugar analysis. Supernatants were stored at –18 °C before analysis. The biomass concentration in the lyophilized gels was estimated using Kjeldahl method. Gels used for this procedure were removed from the fermentation medium, blotted with filter paper and weighed before pyrolysis. Ethanol concentration was measured by gas chromatography using Varian CP 3800 model with a capillary column WCOT fused silica (30 m×0.32 mm), equipped with flame ionization detector (FID). The injector and detector temperatures were 250 °C, and the column temperature was 200 °C. Glucose was determined by high-performance liquid chromatograph (Knauer), equipped with a refractive index detector using Aminex HPX-87H (300×7.8 mm) column (Bio-Rad) maintained at 50 °C. The sample volume was 20 μl, and 5 mM H_2SO_4 (0.6 ml/min) was used as a mobile phase. The experimental errors of the measurements were ±3%. In all figures, the size of the symbols corresponds to the experimental error.

Product (ethanol) yield was calculated as mass of the product formed per mass of the substrate used. Cell retention, R_c, was determined by dividing the mass of the entrapped cells with the mass of the matrix (gel) used for cell immobilization. The immobilization efficiency, η, represents the ratio of the concentration of the immobilized cells to the concentration of the total (immobilized plus free) cells.

Results and Discussion

Yeast cells suffer from various stresses during fermentation. One of them, coming from the yeast cell metabolism, is ethanol accumulation in the medium. Ethanol inhibits the yeast cell growth and consequently its own biosynthesis since ethanol production is tightly coupled with yeast cell growth [1]. Therefore, it has been assumed that the inhibitory effect of ethanol produced will be diminished by entrapment of *S. cerevisiae* cells into a gel since the gel acts as a protector. A lyophilized HEC gel was used as a carrier for *S. cerevisiae* cells. The HEC gels possess macroporous structure with large interconnected pores surrounded by dense walls, which impart opacity to the material. HEC is biodegradable, is inexpensive and has properties, which are conducive for large-scale production [9].

The first experiment with immobilized *S. cerevisiae* in lyophilized HEC gels was performed with initial glucose concentration of 50 g/l and 10 pieces of gels in 100 ml

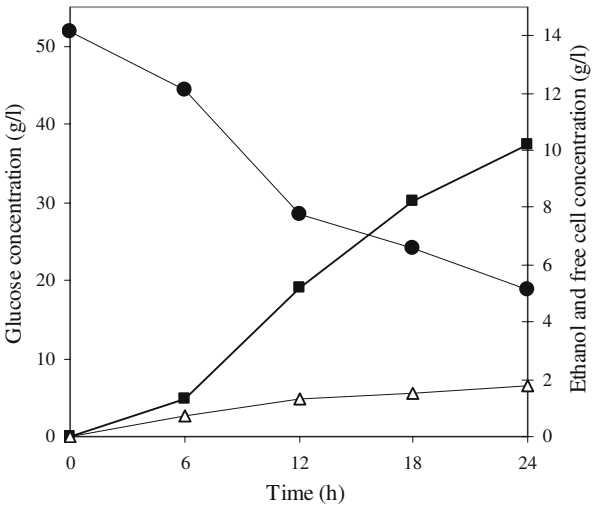

Fig. 1 Glucose (●), ethanol (■) and free cell (Δ) concentration during fermentation with immobilized *S. cerevisiae* cells

medium for 24 h. As can be seen in Fig. 1, after 24 h, immobilized *S. cerevisiae* consumed 63.7% of the glucose and produced 10.2 g ethanol/l with yield of 0.31 g ethanol/g consumed glucose. The final concentration of cells released in the medium from the lyophilized gels was 1.8 g/l.

The second experiment with the immobilized *S. cerevisiae* cells was performed with initial glucose concentration of 100 g/l and 40 lyophilized gels in 100 ml medium. To estimate the stability of the gels, three consecutive batches, each of 72 h, were performed (Figs. 2 and 3). Kinetic profiles of glucose consumption, ethanol formation and cell growth followed similar pattern (Fig. 2). Glucose was completely consumed in each of the cycles. During the first 24 h of the first batch, fast consumption of glucose coincided with fast ethanol production and cell growth in the polymer carrier. The high initial concentration of the immobilized cells (19.5 g immobilized cells/l medium) and the absence of lag phase can

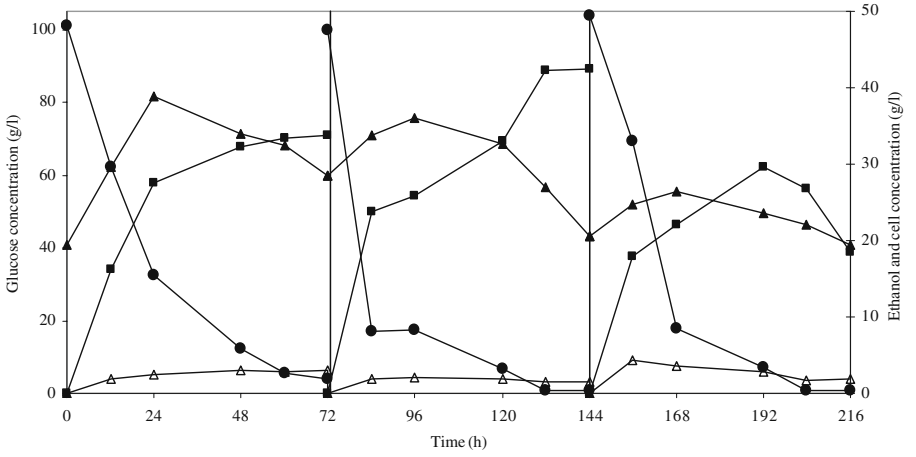

Fig. 2 Fermentation profile of immobilized *S. cerevisiae* cells during ethanol production in three batches. Concentration of (●) glucose, (■) ethanol, (▲) immobilized cells and (Δ) free cells

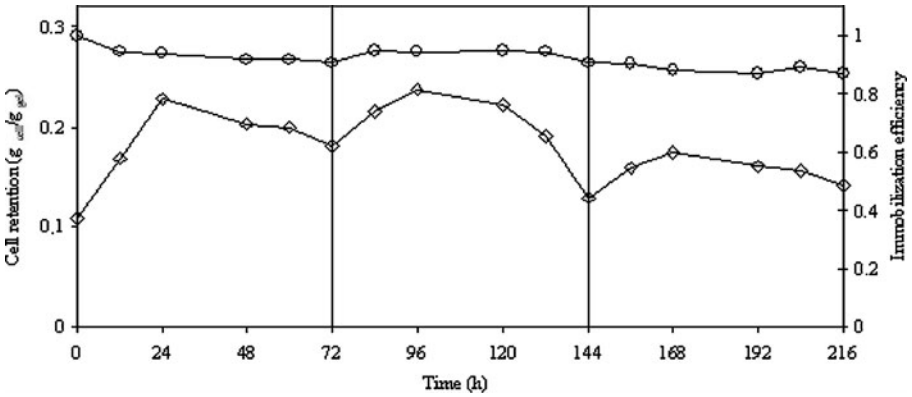

Fig. 3 Cell retention (◊) and efficiency of the immobilization (○) performed with *S. cerevisiae* cells in three batches

be explained by the revitalization of the immobilized *S. cerevisiae* cells after immobilization. The concentration of the immobilized cells (38.9 g immobilized cells/l medium) peaked at the 24th hour in the first batch. The cell free concentration gradually increased to about 3 g free cells/l medium, which was 10% of the average concentration of the immobilized cells. In the second and the third batch, the highest concentration of the immobilized cells was lower than in the first batch, reaching 36.0 and 26.5 g immobilized cells/l medium, respectively. The maximum ethanol yield was 0.40, 0.43 and 0.30 g ethanol/g glucose or 79%, 84% and 60% of the theoretical yield for the first, second and third batches. These results are similar to those reported by Sakurai et al. [10] who immobilized *Saccharomyces pastorianus* cells on three cellulose carriers (a combination of viscose and cellulose, diethylaminoethyl and trimethylammonium-2-hydroxypropyl cellulose) and used the immobilized yeast for batch ethanol production from glucose. The ethanol yield spanned from 0.44 to 0.46 g ethanol/g glucose while the cell leakage was about 12% of the immobilized cells concentration.

The changes of two important parameters that characterize the immobilization system, cell retention (R_c) and immobilization efficiency (η), were determined (Fig. 3). The amount of the entrapped cells into the gel doubled during the first 24 h reaching its maximum R_c value of 0.23 g immobilized cells/g gel in the first batch. In the second batch, the maximum R_c value was almost the same (0.24 g immobilized cells/g gel), while in the third batch it was reduced to 0.17 g immobilized cells/g gel. Throughout the repeated batch fermentation, the immobilization efficiency was almost constant with an average value of 0.92±0.03 (12–216 h) indicating that the immobilized cells were properly entrapped in the lyophilized HEC gels. From the values of the immobilization efficiency and cell retention, it was evident that the lyophilized HEC gels have very good properties for immobilization of *S. cerevisiae.* Both parameters indicated that the lyophilized gels were stable, suggesting possible use of the gels for continuous or repeated batch production of ethanol.

Santos et al. [11] reported that η and R_c values of *Candida guilliermondii* cells immobilized on an alternative support material, pretreated sugarcane bagasse, increased during the fermentation and reached maximum values of 0.51 and 0.31 g immobilized cells/g bagasse after 21 h and then sharply decreased. When sorghum bagasse without pretreatment was used for immobilization of *S. cerevisiae*, the R_c value was 0.6 g dry cell mass/g dry bagasse [12].

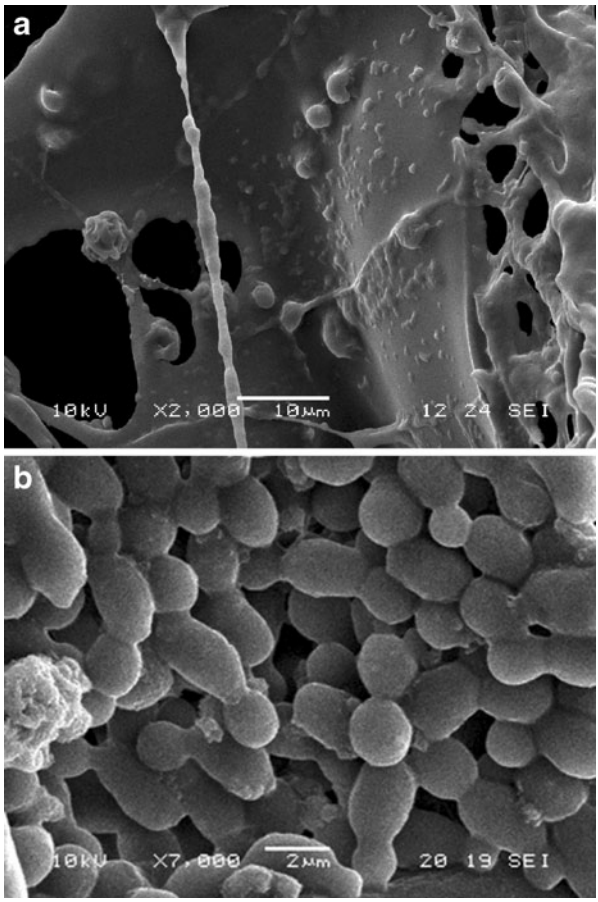

Fig. 4 Scanning electron micrograph of the HEC cryogel with immobilized *S. cerevisiae* cells (**a**) immediately after preparation and (**b**) after 72 h of fermentation

The lyophilized HEC gels were examined by scanning electron microscope and Fig. 4 shows the electron micrographs of the polymeric matrix, immediately after the immobilization of *S. cerevisiae* cells and after 72 h of fermentation. These micrographs showed that after preparation (Fig. 4a), immobilized cells were viable inside the gel and had enough space to grow in the pores of the carrier. Proliferation of the entrapped yeast cells, after 72 h of cultivation, can be clearly seen in Fig. 4b, thus confirming the suitability of the carrier and the method of immobilization. At the end of the fermentation, no visible signs of breaking the polymer walls were detected.

Conclusion

The immobilization efficiency and cell retention were used to assess the ability of lyophilized HEC gels to serve as immobilization matrices for *S. cerevisiae.* Both parameters indicated that lyophilized gels are stable and capable of retaining the immobilized yeast cells. This was also confirmed by scanning electron micrographs. The immobilized yeast cells were successfully used for ethanol production from glucose.

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