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Full Length Research Paper

Bioethanol production by immobilized *Sacharomyces cerevisiae* var. *ellipsoideus* cells

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Bioethanol can be produced by fermentation of sugars from various waste agricultural materials. Whichever system for bioethanol production is chosen, the attention must be paid to the overall economics and energy consumption. The aim of the present study was to investigate the immobilization of *Sacharomyces cerevisiae* var. *ellipsoideus* yeast cells for bioethanol production from corn meal hydrolyzates. For this purpose the biocompatible polymers such as polyvinil alcohol (PVA) and Ca–alginate were assessed. The parameters of ethanol fermentation, such as inoculum concentration in different carriers and the choice of a convenient carrier for the efficient ethanol production were studied. The maximum ethanol concentration of 10.05% (w/w) was obtained in the fermentation of corn meal hydrolyzates by 5% (v/v) of inoculum concentration of the yeast immobilized in Ca-alginate using a method of electrostatic droplet generation. The repeated batch fermentation with the yeast immobilized in Ca-alginate indicated that alginate gels degraded after the second fermentation cycle. PVA carrier exhibited better mechanical properties and stability; however lower ethanol concentrations were achieved during the fermentation.

Key words: Corn meal hydrolyzates, bioethanol, immobilization, *Sacharomyces cerevisiae* var. *ellipsoideus.*

INTRODUCTION

Ethanol is an important industrial chemical with emerging potential as a biofuel to replace fossil fuels. Ethanol can be produced by fermentation of sugars from agricultural products or waste plant materials. Whichever substrate is chosen, the attention must be paid to the overall economics and energy consumption (Demirbas, 2006).

The economic evaluation of different materials for ethanol production was thoroughly studied previously (Meo, 1984; Maiorella et al., 1984; Greg and Saddler 1995). The most commonly used ethanol producer is *Sacharomyces cerevisiae*. Efficient ethanol production requires a rapid fermentation leading to high ethanol concentrations; therefore a yeast strain must have a good specific growth rate and good specific ethanol production rate at high osmotic activities and ethanol concentration.

During batch fermentation many parameters can cause the decrease of the specific rate of yeast growth, and the inhibition can be caused either by product or substrate concentration. Ethanol production by immobilized yeast cells has been extensively investigated during last few decades. Immobilization of microbial cells showed certain technical and economical advantages over free cell system. Immobilization of cells for fermentation has been developed to eliminate inhibition caused by high concentration of substrate and product, also to enhance the productivity and yield of ethanol production (Williams and Munnecke, 1981; Sakurai et al., 2000; Kourkoutas et al., 2004; Strehaiano et al., 2006; Baptista et al., 2006).

Many support materials for cell immobilization have been reported including calcium alginate, κ-carragenan gel, polyacrylamide, γ-alumina (Nursevin et al., 2003) and orange peel (Plessas et al., 2007). According to Groboillot et al. (1994) the main advantages of the immobilization of yeast are the increase of ethanol yield and cellular stability and a decrease of process expenses

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due to the ease for cell re recovery and reutilization. Prasad and Mishra (1995) reported that other advantages of immobilized cell system over presently accepted batch or continuous fermentations with free-cells are: greater volumetric productivity as a result of higher cell density; tolerance to higher concentrations of substrate and products; lacking of inhibition; relative easiness of down-stream processing etc. However, Prasad and Mishra (1995) also concluded that in some cases, the effective-ness of immobilized cells would be lower than for a system with freely suspended cells. The main reason is because the cells deep inside a carrier particle can become inactive due either to deprivation of some essential nutrients or to accumulation of product to inhibiting concentrations.

The aim of this study was to investigate the application of alginate and PVA carriers for the immobilization of *S. cerevisiae* var. *ellipsoideus* yeast in batch ethanol fermentation. Previously, this microorganism was found to be the most superior for the fermentation of corn meal hydrolyzates among four tested yeasts: *S. cerevisiae, S. cerevisiae* var. *ellipsoideus, Saccharomyces carlsbergensis and Schizosaccharomyces pombe* (Rakin et al., 2007).

The parameters such as inoculum concentration, ethanol concentration and ethanol yield, cell density and stability of the immobilized particles in repeated batches were assessed in order to compare the convenience of the immobilization carriers for ethanol fermentation. The stability of the alginate and PVA carriers was monitored in repeated batch fermentation.

MATERIALS AND METHODS

Starch

Corn meal obtained by dry milling process was a product of corn processing factory ('RJ Corn Product', Sremska Mitrovica, Serbia). The corn meal consisted of 95% or more of particles that pass through a 1.70 mm sieve and 45% or more particles that pass through a 0.71 mm sieve, 35% or less of particles that pass through a 0.212 mm sieve. The content of the main components in the corn meal, determined by chemical analysis, was the following: starch 73.75% (w/w), proteins 6.35% (w/w), lipids 5.86% (w/w), ash 0.70% (w/w) and water 10.34% (w/w).

Enzymes and microorganisms

Termamyl SC, a heat-stable α-amylase from *Bacillus licheniformis* was used for corn meal liquefaction. The enzyme activity was 133 KNU/g (KNU, kilo novo units of α-amylases is the amount of enzyme which breaks down 5.26 g of starch per hour according to Novozyme's standard method for the determination of α-amylase). SAN Extra L, *Aspergillus niger* glucoamylase, activity 437 AGU/g (AGU is the amount of enzyme which hydrolyses 1 µmol of maltose per minute under specified conditions) was used for corn meal saccharification. The enzymes were gift from Novozymes, Den-mark. *S. cerevisiae* var. *ellipsoideus* from the collection of Depart-ment of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, Belgrade (BIB-TMFB) was used for the fermentation of hydrolyzed corn meal.

The culture was maintained on a malt agar slant. Further propagation, prior to immobilization and ethanol fermentation, was performed in 500 ml flasks in a shaking bath at 30°C for 48 h in a defined liquid media. The liquid media consisted of yeast extract (3 g/l), peptone (3.5 g/l), KH_2PO_4 (2.0 g/l), $MgSO_4 \times 7H_2O$ (1.0 g/l), $(NH_4)_2SO_4$ (1.0 g/l), glucose (10 g/l) and distilled water. The yeast suspension of a desired cell density was prepared by centrifugation of the cultured media.

Hydrolysis experiments

A 100 g of corn meal was mixed with water at the weight ratio 1:3, and 60 ppm of Ca^{2+} (as $CaCl_2$) ions was added. The mixture was then treated with enzymes in two steps, liquefaction and saccha-rification. The liquefaction was carried out at 85ºC and pH 6.0 for 1 h by adding 0.026% (v/w of starch) enzyme Termamyl SC. The liquefied mash was saccharified at 55ºC and pH 5.0 for 4 h with 0.156% (v/w of starch) enzyme SAN Extra L. The hydrolysis was performed in flasks in thermostated water bath with shaking (150 rpm), as described by Mojovic et al. (2006).

Immobilization of *S. cerevisiae var. ellipsoideus* in PVA particles

LentiKat® Liquid (80 ml) was melted at 90 to 95ºC, cooled down to a temperature of 30 to 35ºC and mixed with 20 ml of the yeast cell suspension by a magnetic stirrer. The PVA/yeast cell solution was forced out of the tip of the blunt edge needle (1 mm in diameter) by a syringe in the form of droplets on Petri dishes. Petri dishes covered with droplets were put under the sterile, laminar airflow at room temperature. Under these conditions, gellification of the droplets occurred in half an hour with a decrease of 75% of the initial mass due to water evaporation. The obtained gel particles in the form of lenses were stabilized and re-swelled in stabilizing solution (GeniaLab, Germany) for two hours. The resulting LentiKat® lenses were about 3.5 mm in diameter and 0.3 mm thick (Figure 1A) with a starting concentration of immobilized yeast cells at 1×10^7 cells/beads.

Immobilization of *S. cerevisiae var. ellipsoideus* in alginate particles

The 2% w/w Na-alginate solution was prepared by dissolving 10 g of sodium alginate powder (Sigma, medium viscosity) into 500 ml of distillated water. Polymer/cell suspension was formed by mixing of 100 ml of Na-alginate solution with 25 ml of thick yeast suspension at room temperature. Spherical microbeads were formed by extrusion of Na–alginate/yeast cell suspension through a blunt stainless steel needle using a syringe pump (Harvard Apparatures, Pump 11) with a 20 ml plastic syringe and an electrostatic droplet generator (Nisco Encapsulator, Switzerland). The cell suspension was forced out of the tip of the needle at constant flow rate (0.25 ml/min), and droplets were formed by the action of electrostatic and gravitational forces. Electrostatic potential was formed by connec-ting the positive electrode of a high voltage dc unit to the gelling bath which was 2% w/v $CaCl_2$ solution while the needle was grounded. In this way the yeast cells were entrapped in a gel matrix of Ca-alginate. After gelling the microbeads were placed in double distilled water to remove unreacted material and low molecular weight byproducts. Obtained alginate microbeads had an average diameter close to 0.8 mm (Figure 1B).

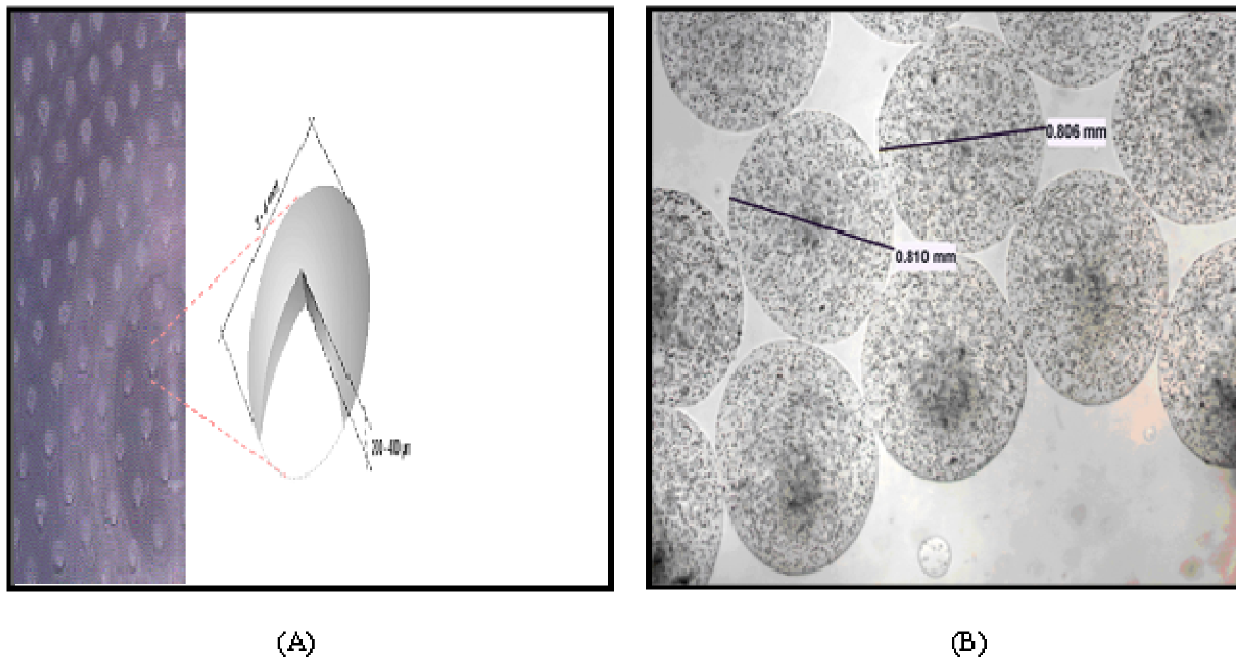

Figure 1. Microscopic images of immobilized particles. (A) Yeast cells immobilized in PVA LentiKat® lenses (3.5 mm in diameter and 0.3 mm thick); (B) Yeast cells immobilized in alginate microspheres (diameter 0.806 to 0.810 mm).

Ethanol fermentation of starch hydrolyzates

Starch hydrolyzates obtained by the two-step hydrolysis of corn starch meal were subjected to ethanol fermentation by immobilized yeasts under anaerobic conditions (pH 5.0; 30ºC; mixing rate: 100 rpm). The fermentation was performed in 500 ml flasks which contained 150 ml of medium in a thermostated water bath with shaking. The temperature profile of the process of hydrolysis and subsequent fermentation is presented in Figure 2. It was considered that the pasteurization of the substrate achieved during the enzymatic liquefaction (at 85ºC for 1 h) was a sufficient thermal treatment, and thus no additional sterilization prior to fermentation was performed. The mashes with different initial inoculum concentrations were fermented for a period up to 38 h.

Repeated batch fermentation

Five subsequent cycles of repeated batch operations with the immobilized yeast were performed using the same process conditions as in the first fermentation step, each of them lasted for 38 h.

Analytical methods

During the corn meal fermentation, the content of reducing sugars, calculated as glucose, was determined by 3,5-dinitrosalicylic acid (Miller, 1959). A standard curve was drawn by measuring the absorbance of known concentrations of glucose solutions at 570 nm. The ethanol concentration was determined based on the density of alcohol distillate at 20ºC and expressed in weight % (w/w). Indirect counting method i.e. pour plate technique was used to determine the number of viable cells. Serial dilutions of the samples were performed, and after the preset incubation time at 30ºC, colonies grown in Petri dishes were used to count the number of viable cells–colony forming units (CFU). At least three measurements were made for each condition and the data given were averages.

RESULTS AND DISCUSSION

The number of immobilized cells and ethanol concentration during the fermentation of corn meal hydrolyzates with initial glucose concentration of 176 g/l with various inoculum concentrations (2, 5 and 10%, v/v) of the yeast immobilized in alginate and PVA particles are presented in Figures 3-4.

The time profiles of the number of viable cells at 2, 5 and 10% inoculum concentrations were similar (Figure 4). The maximum ethanol concentration of 10.05 % (w/w) and the number of viable cells of 7.95×10^{8} CFU/g of beads were obtained in the fermentation with 5% of the inoculum concentration of the cells immobilized in alginate.

Results presented in Figures 3-6 indicated that the fermentation kinetics with the cells immobilized in alginate was faster than with the cells immobilized in PVA gels and in this system higher ethanol concentrations were achieved. By comparing maximum cell densities in the two assessed immobilization carriers (Figures 4 and 6) it is evident that much higher cell densities were attained in alginate beads. However, the PVA carrier can provide better mechanical properties but obviously a lower sub-

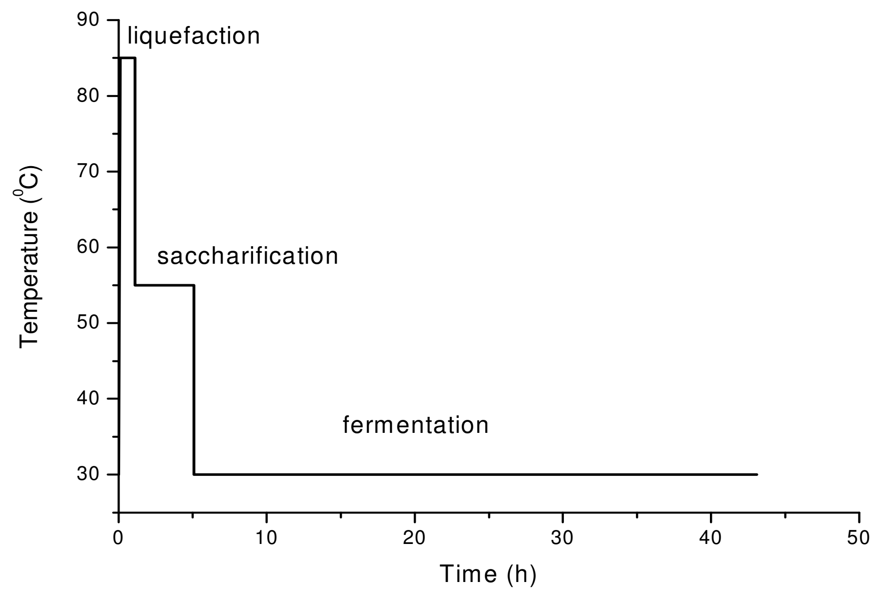

Figure 2. The temperature process profile for corn meal hydrolysis and ethanol fermentation starch hydrolyzates.

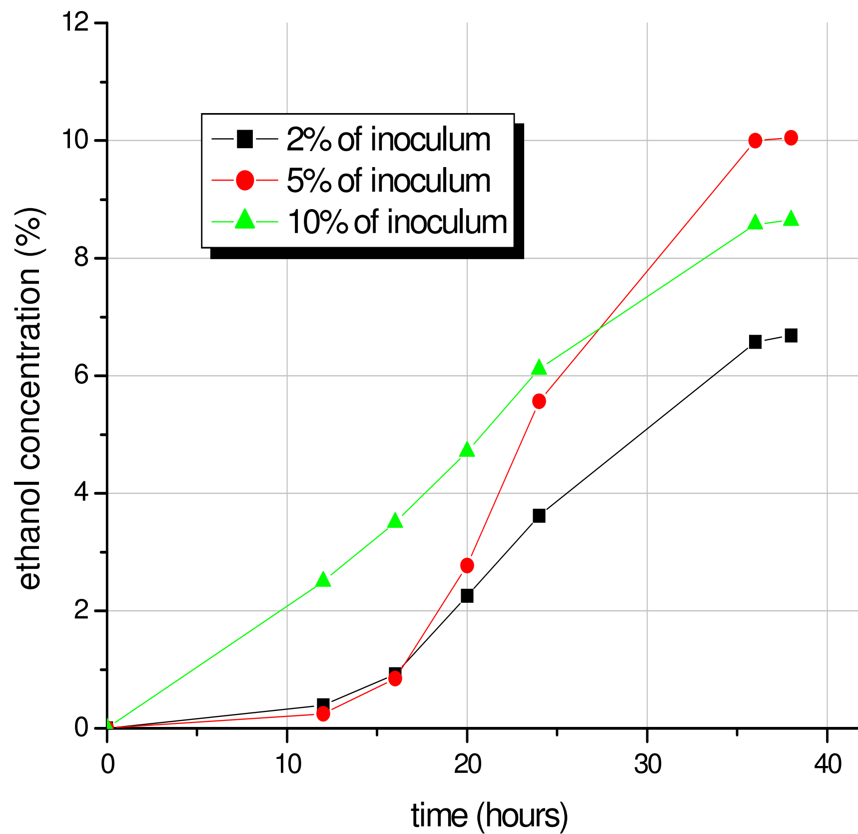

Figure 3. The ethanol concentration during the fermentation of corn meal hydrolyzates with various inoculum concentrations of *S. cerevisiae* var. *ellipsoideus* immobilized in alginate. Process conditions: pH 5.0; 30ºC; 100 rpm; initial glucose concentration 176 g/l.

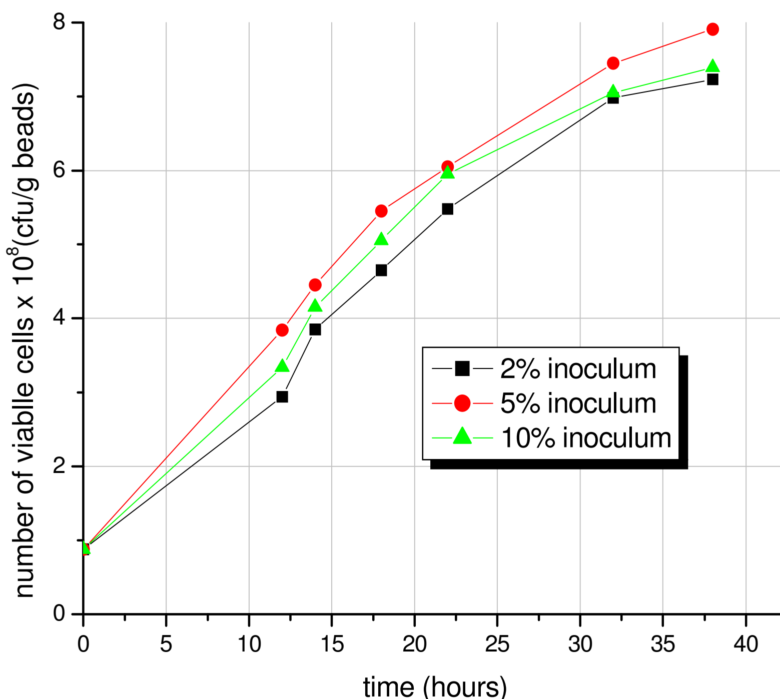

Figure 4. The number of immobilized cells during fermentation of corn meal hydrolyzates with various inoculum concentrations of *S. cerevisiae* var. *ellipsoideus* immobilized in alginate. Process conditions: pH 5.0; 30 ºC; 100 rpm; initial glucose concentration 176 g/l.

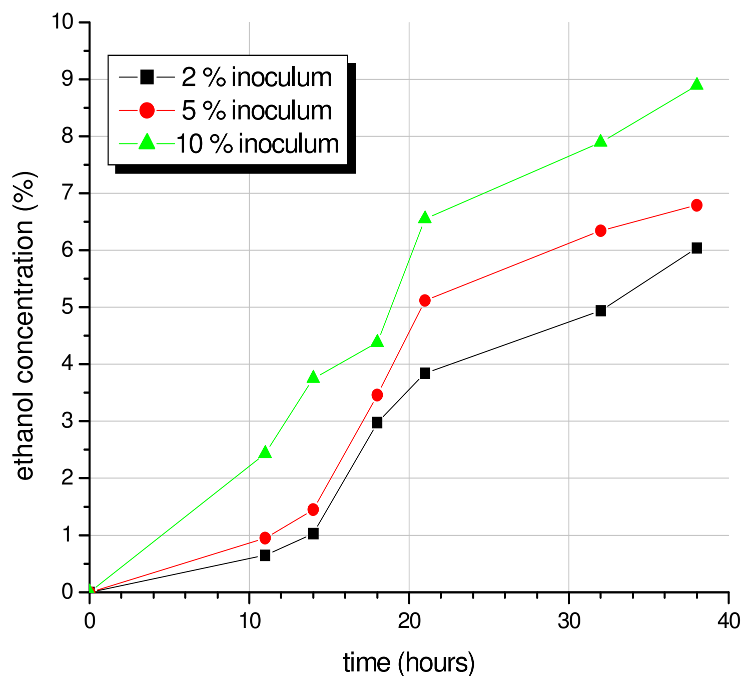

Figure 5. The ethanol concentration during the fermentation of corn meal hydrolyzates with various inoculum concentrations of *S. cerevisiae* var. *ellipsoideus* immobilized in PVA particles. Process conditions: pH 5.0; 30 ºC; 100 rpm; initial glucose concentration 176 g/l.

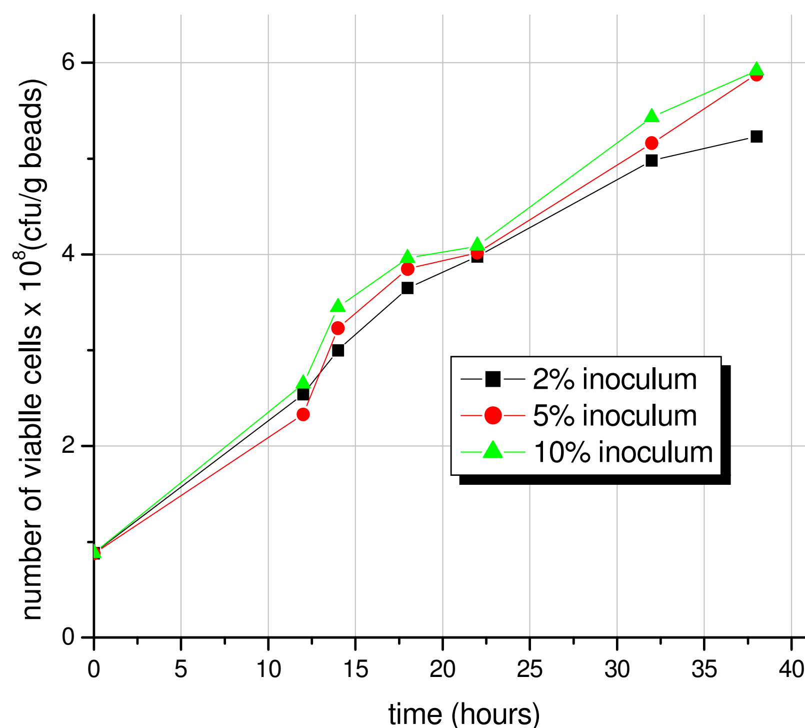

Figure 6. The number of immobilized cells during the fermentation of corn meal hydrolyzates with various inoculum concentrations of *S. cerevisiae* var. *ellipsoideus* immobilized in PVA particles. Process conditions: pH 5.0; 30 ºC; 100 rpm; initial glucose concentration 176 g/l.

Table 1. Product concentration in culture liquid after subsequent batch fermentation cycles with *S. cerevisiae* var *elipsoideus* immobilized in alginate and PVA particles.

Fermentation cycle (h)	Living cells (CFU/g beads)		Ethanol concentration (%)		Ethanol yield $Y_{P/S}$, (g/g)		% of theoretical yield	
	Alginate	PVA	Alginate	PVA	Alginate	PVA	Alginate	PVA
First, 38 h	7.95×10^{8}	5.88×10^{8}	10.05	6.95	0.568	0.394	111	77
Second, 38 h	3.35×10^{10}	6.11×10^{8}	10.10	7.20	0.574	0.409	112	80
Third, 38 h	degraded	6.05×10^{8}	-	7.01	-	0.398	-	78
Fourth, 38 h	degraded	5.98×10^{8}	-	6.97	-	0.396	-	78
Fifth, 38 h	degraded	5.90×10^{8}	-	6.97	-	0.396	-	78

Process conditions: pH 5.0; 30ºC; 100 rpm; initial glucose concentration 176 g/l; inoculum concentration 5% (v/v), initial living cells ~0.98 x10^{8} CFU/g of beads.

strate mass transport compared to alginate gel.

The production of ethanol, ethanol yield and the number of viable cells of *S. cerevieie* var *elipsoideus* immobilized in alginate and PVA in repeated batch fermentations are shown in Table 1. The number of viable cells increased significantly from first to the second cycle. The ethanol concentration and ethanol yield remained almost constant during the repeated batch fermentation with *S. cerevisiae* immobilized in alginate. This means that immobilized *S. cerevisiae var. ellipsoiddeus* in alginate gel retained its activity to produce ethanol for 3 days.

However, the third cycle of fermentation resulted in destruction of Ca-alginate beads. The destruction of alginate beads after the second cycle was due to intensive growth of cells (which reached 3.35 10^{10} CFU/g beads) and CO_2 evolution during the fermentation. It is assumed that the intensive proliferation of yeast cells inside the

matrix caused instability of Ca-alginate in acidic condition during the fermentation. Similarly, Bekers et al. (2001) investigated batch fermentation of sucrose using *Zymomonas mobilis* cells immobilized in Ca-alginate. They also reported that Ca-alginate beads disintegrated after 4-5 days. Najafpour et al. (2004) reported that the concentration of 2% w/w of alginate in beads was a suitable alginate concentration regarding the activity of beads for ethanol production in an immobilized cell reactor during 10 working days. However, higher alginate concentrations (3-6% w/w) gave more stable and rigid beads but with diffusion problems.

In contrast to alginate immobilized yeast, the cells immobilized in PVA carrier were still effective after the second fermentation cycle. The PVA particles have already been used for yeast immobilization for beer fermentation (Smogrovicova et al., 2001; Bezbradica et al., 2007). According to Bezbradica et al. (2007) the LentiKat® particles showed high mechanical and fermentative stability in beer fermentation and they can endure 30 days of operating time without significant change of cell activity, particle shape and particle size. However, our study demonstrated that the cell densities, as well as the ethanol yields were lower than those obtained in the fermentation with alginate immobilized yeast. The ethanol concentration and ethanol yield in repeated batch after the second cycle were relatively constant and slightly lower than in the second cycle. Comparing the values of all process parameters obtained in fermentation corn meal hydrolyzates with alginateand PVA immobilized yeast systems, the ethanol fermentation was faster with cells immobilized in Ca-alginate but alginate microcapsules showed lower stability in repeated batch fermentation than PVA particles.

Further benefits of utilization of immobilized *S. cerevisiae var. ellipsoideus* could be expected in continuous fermentation system, and our further research will also be focused on the improvement of the stability of alginate micro-capsules.

Conclusions

The application and the potential of alginate and PVA particles for immobilization of yeast cells during batch fermentation of ethanol from corn meal hydrolyzates was studied. The results indicated that the ethanol fermentation was faster with cells immobilized in Ca-alginate compared to the fermentation with cells immobilized in PVA gel. Under the same experimental conditions, higher cell densities and ethanol concentrations were attained in the fermentation performed by alginate immobilized yeast relative to the fermentation by PVA immobilized yeast. However, in repeated batch fermentation the particles of Calginate with immobilized yeast started to disintegrate after the second fermentation cycle. In contrast, PVA carrier provided better mechanical properties and stability but obviously a lower substrate mass transport which limited the growth within the gel and endured five cycles of repeated batch fermentation.

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