IMMOBILIZATION OF BREWING YEAST IN PVA/ALGINATE MICROBEADS USING ELECTROSTATIC DROPLET GENERATION

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Entrapment of cells within spheres of calcium alginate gels is widely used immobilization procedure compatible with most enzymes and living cells that can be carried out in a single step process under very mild conditions (Goosen, 1996). The biocatalyst suspension is mixed with a sodium alginate solution and the mixture is dripped into a solution containing multivalent cations (usually Ca^{2+} or Ba^{2+}). The droplets than instantaneously form gel–spheres, thus entrapping the biocatalyst in a three dimensional lattice of ionically cross–linked alginate. Droplets formed under action of gravity only are larger than 1 mm. During the last two decades new methods have been developed with a purpose of producing smaller alginate beads. The main motive was to overcome mass transfer limitations, observed in large beads, obtained by simple dropping method. One of the most successful methods was shown to be the production of alginate microbeads by electrostatic extrusion (Bugarski et al., 1993). The applied electrostatic potential accelerates the motion of charged molecules toward the droplet surface, resulting in a decrease of the surface tension and, consequently, a reduction of the droplet diameter. This method provides production of microbeads (down to 50 mm in diameter) with narrow size distributions. Another advantage of this method is potential for scale up in a multi–needle apparatus (Poncelet et al., 1999). However, alginate as an immobilization matrix has a drawback of rather low mechanical strength and susceptibility to substances, which have high affinity towards Ca^{2+} ions, such as phosphate or citrate (Smidsrod et al., 1990).

On the other hand, polyvinyl alcohol (PVA) is a synthetic, hydrophilic polymer attractive for biocatalyst immobilization because of its nontoxicity and high mechanical stability (Lozinsky et al., 1998). PVA hydrogel spatial network is formed due to hydrogen bonds between hydroxyl groups of neighboring polymer chains. PVA hydrogels are prevalently formed by multiple freezing and thawing of PVA solutions, which results in formation of a two–phase system during the thawing process and the increase of PVA concentration in the liquid phase (Ariga et al., 1987). A drawback of this technique is the exposure of cells to high temperature changes which could decrease cell activity, thus requiring the use of cryoprotectants, which, on the other hand, decrease the rigidity of gel structure. Also, beads prepared by this technique are large (usually around 3 mm) although PVA microbeads (300 mm) with immobilized anaerobic bacterium Clostridium butyricum were produced by jet cutting technique (Jahnz et al., 2001). Alternative techniques of cell or enzyme immobilization in PVA were include cross–linking by UV irradiation or with boric acid (Imai et al., 1986; Chen and Lin, 1994) and PVA–gel preparation at room temperature by means of controlled partial drying – LentiKat® technology (Jahnz et al., 2001).

A few attempts have been made to immobilize cells in PVA–alginate beads. Pattanapipitpaisal et al. (2001) immobilized *Microbacterium liquefaciens*, for chromate reduction in wastewaters, by simple dropping of a cell suspension in a PVA/alginate mixture into the $CaCl_2$ solution. The obtained beads (around 3 mm in diameter) exhibited high level of chromate removal in continuous operation without bead agglomeration or breakdown. PVA–alginate beads with immobilized *Bacillus subtilis* cells have been prepared by a "freezing–thawing" method (Szczesna–Antczak et al., 2001). The addition of alginate improved hardness of the beads, increased stability and significantly decreased cell leakage from beads.

In the present work, the possibility of production of PVA/alginate microbeads by means of electrostatic droplet extrusion was investigated. Such microbeads could be attractive for various biotechnological applications since drawbacks of both materials could be overcome, i.e. large dimensions and agglomeration of PVA particles (Bezbradica et al., 2003) and, on the other hand, low stability of alginate microbeads (Lozinsky et al., 1996). The composition of PVA/alginate blends was optimized with respect to bead diameter and microbeads with immobilized Saccharomyces cerevisiae cells were tested in repeated beer fermentations.

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Paper presented as a poster.

MATERIAL AND METHODS

Preparation of polymer yeast suspension

Brewing yeast cells (*Saccharomyces uvarum*) were initially cultivated in sterile growth medium at 25°C in shaken flasks. Cells were harvested in the early exponential phase by centrifugation. PVA powder (Sigma, St.Louis, USA) was dissolved in distilled water at a concentration of 10% mas. at 70°C. Sodium alginate powder (Protanal LF 20/40) was dissolved in distilled water at a concentration of 2% mas. at room temperature. Several blends with various volume ratios (1:1 to 1:9) of 10% PVA solution and 2% Na–alginate solution were prepared. Polymer blends were then mixed with cell suspension ($1x10^8$ cells/ml) at a ratio 4:1 resulting in polymer/cell suspensions with a final cell concentration of $2x10^7$ cells/ml.

Immobilization procedure

Spherical droplets were formed by extrusion of polymer/yeast cell suspensions through a blunt stainless steel needle using a syringe pump (Raze, Scientific Instruments, Stamford, CT) with a 5 ml plastic syringe. The cell suspension was forced out of the tip of the needle at a constant flow rate (25.2 ml/h). Electrostatic potential was formed by connecting the positive electrode of high voltage dc unit (Model 30R, Bert an Associates, Inc., New York) to the needle and by grounding the hardening bath, which was 2% $CaCl_2$ solution. Applied voltage was 7 kV, distance between tip of the needle and hardening solution was 2.5 cm, and 22–gauge needle was applied. After formation, the microbeads were stirred in the hardening solution for 60 min at 4°C. The microbeads were then transferred to olive oil and frozen at –20°C for 24 hours in order to assure formation of PVA cryogel structure. After thawing of the mixture, the microbeads were separated from the oil, washed in 0.9% NaCl solution and kept in the same solution until the use in fermentations.

Microbead size determination

A sample of 30 microbeads was taken from each experiment and diameters of microbeads were measured with an accuracy of 10 μm using a microscope. The average microbead diameter and standard deviations were then calculated from the measured data.

Fermentation studies in shaken flasks

Batch fermentations were performed in 100 ml flasks, which contained 60 ml of sterile plant wort of 11% extract and 20 g of PVA–alginate beads. The concentration of immobilized yeast cells was about $2 \cdot 10^7$ cells/ml of beads. Total of 3 fermentation experiments were performed on orbital shaker at 115 rpm and 18°C and lasted for 2 days, each.

Analytical assays

Cell concentrations and viabilities were determined following dissolution of microbeads in 0.05M Na–citrate (1g of microbeads in 5 ml of Na–citrate solution). Yeast cell concentration was estimated by using a Thoma counting chamber and cell viability was assessed by means of methylene blue staining technique. Liquid samples of fermentation medium were collected aseptically from the flasks and analyzed for specific gravity, yeast cell counts, and cell viability.

RESULTS AND DISCUSSION

Effects of polymer blend compositions on the size and of the obtained microbeads with immobilized yeast cells were investigated using mixtures of different ratios of 2% Na–alginate and 10% PVA solutions. The mean diameters (with standard errors) of beads produced by electrostatic droplet generation and subsequent freezing are presented in Figure 1.

Results indicate that the minimum bead diameter under applied conditions was 806 ± 45 mm obtained with the starting mixture composed of 80% of alginate solution and 20% of PVA solution (4:5 final mass ratio of alginate to PVA). With further increase of PVA content, bead diameter rapidly increased and the size distribution was broader. The increase of bead diameter with PVA content is in agreement with the theory of electrostatic dispersion of polymer solutions (Poncelet et al., 1999). According to this theory, the droplet diameter is reduced in the electric field due to increase of the diffusion coefficient of ionic surfactants, which is especially prominent in the case of small sodium ions (Na^+). Since PVA molecules are not charged, with the increase of PVA content in the mixture, the concentration of ionic surfactants is reduced, which results in production of larger beads. As compared to previously reported results, the obtained PVA/alginate microbeads with the composition ratio of 5:4 were of similar size as

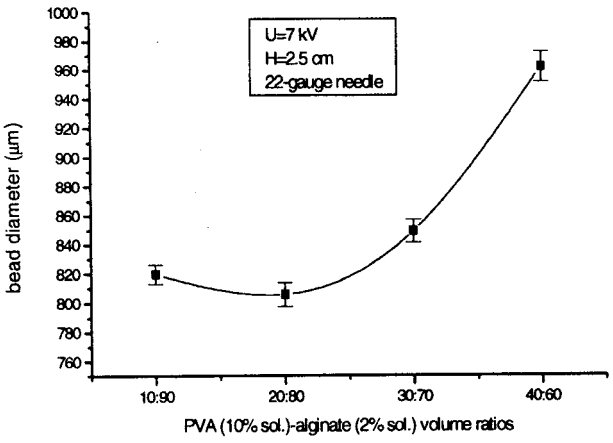

Figure 1. Effects of polymer blend composition on the size of microbeads produced by electrostatic droplet generation

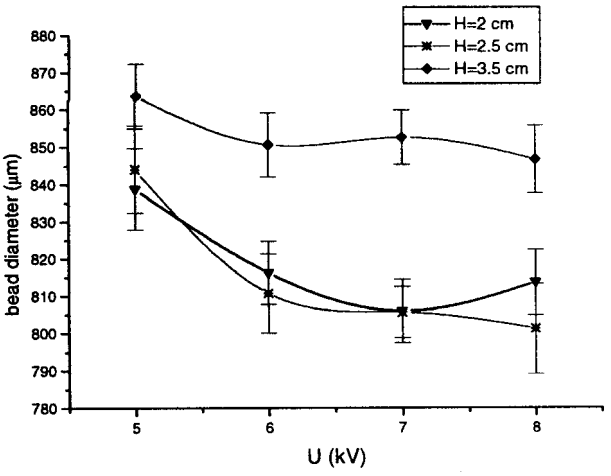

Figure 2. Effects of the applied voltage (U) and distance (H) between the needle and the hardening solution on the bead diameter

alginate microbeads produced under the same conditions of electrostatic droplet generation (Nedovic et al., 2001).

Effects of applied electrical potential and distance between the needle and the hardening solution on bead diameter are presented in Figure 2. The increase in the electrical potential above 7kV led to a slight decrease of bead diameter at significantly broader distributions. On the other hand, the decrease of the electrical potential below 6kV resulted in a significant increase in the bead diameter. The bead diameter decreased as the distance between the needle and the hardening solution was decreased from 3 to 2.5 cm. However, further decrease in the electrode distance had no additional effect on the bead diameter while a significant part of obtained particles were nonspherical.

The applied immobilization procedure had no effect on viability and activity of yeast cells immobilized in PVA/alginate microbeads at a concentration of $2 \cdot 10^7$ cells/ml. Dissolution of the PVA/alginate microbeads in 0.05 M Na–citrate following the same procedure as for dissolution of alginate microbeads required substantially longer mixing times. Additionally, microbeads obtained using electrostatic droplet generation dissolved at significantly lower rates than larger beads of the same composition obtained by simple extrusion method. These results imply more rigid structure of microbeads probably due to either change in spatial distributions of polymers in the electric field or different kinetics of PVA gelling during the freezing–thawing procedure. The activity of immobilized cells was verified in fermentation studies. Apparent attenuations of around 85% were achieved after two–day fermentations.

CONCLUSIONS

Brewing yeast cells were successfully immobilized in PVA/alginate microbeads by electrostatic droplet generation followed by freezing in oil. The composition of PVA/alginate mixture had strong influence on the diameter and size distribution of the produced microbeads. The minimum bead diameter (with narrow size distribution) was achieved when PVA:alginate mass ratio was 5:4. The optimal conditions for generation of PVA/alginate microbeads were electrical potential of 7 kV and a distance between the needle and the hardening solution of 2.5 cm. Batch fermentation studies indicated that immobilized cells remained viable and highly active. Results of this study imply that electrostatic droplet generation can be an attractive technique for cell immobilization in PVA/alginate microbeads of different compositions and properties.

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