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The Use of Microporous Divinyl Benzene Copolymer for Yeast Cell Immobilization and Ethanol Production in Packed-Bed Reactor

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Abstract Microporous divinyl benzene copolymer (MDBP) was used for the first time as immobilization material for *Saccharomyces cerevisiae* ATCC 26602 cells in a bed reactor and ethanol production from glucose was studied as a model system. A very homogenous thick layer of yeast cells were seen from the scanning electron micrographs on the outer walls of biopolymer. The dried weight of the cells was found to be approximately 2 g per gram of cell supporting material. Hydrophobic nature of polymer is an important factor increasing cell adhesion on polymer pieces. The dynamic flow conditions through the biomaterial due to its microporous architecture prevented exopolysaccharide matrix formation around cells and continuous washing out of toxic metabolites and dead and degraded cells from the reactor provided less diffusional limitations and dynamic living environment to the cells. In order to see the ethanol production performance of immobilized yeast cells, a large initial concentration range of glucose between 6.7 and 300 g/l was studied at 1 ml/min in continuous packed-bed reactor. The inhibition effect of glucose with increasing initial concentration was observed at above 150 g/l, a relatively high substrate concentration. The continuous fluid flow around the microenvironment of the attached cells and mass transferring ability of cell immobilized on MDBP can help in decreasing the inhibition effect of ethanol accumulation and high substrate concentration in the vicinity of the cells.

Keywords 3-D support biomaterials · Ethanol production · Microporous divinyl benzene copolymer · Yeast immobilization

Introduction

Whole-cell immobilization involves the physical confinement of intact cells to a given region of space with the retention of catalytic activity [1]. This process frequently improves

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catalyst stability. The immobilized cells can be concentrated to higher densities within the immobilization support than is possible in normal suspension cultures, resulting in potentially high reactor productivities. Moreover, the immobilization support often can be formed into geometries that provide improved mass transfer characteristics within the microbial reactor, thus increasing the efficiency of nutrient supply and product removal throughout the cell mass [2]. Up to now, many types of support materials for yeast cell immobilization were used in reactors for ethanol production. Sodium alginate beads [1], grain residues [3], quince pieces [4], and soluble prepolymeric matrix [5] can be given as examples to those support materials.

In various cell culture applications, it has been shown that the growth and function of cells in multicellular 3-D structures is significantly different from their growth on conventional 2-D monolayer structures. Accordingly, novel in vitro culture systems that more authentically represent the cellular environment of microorganisms in nature are required for increasing our understanding of complex biological phenomena. Evidence for the functional superiority of the cells cultured in 3-D scaffolds compared to 2-D monolayer cultures has created interest in fabricating materials that can support 3-D cell growth. There are some reported researches on use of inert scaffolds fabricated from polymerized high internal phase emulsions (polyHIPE) to support 3-D cell growth in in vitro tissue culture [6] for packed-bed and pressure-driven monolith reactor applications [7]. Akay et al. [7] and Erhan et al. [8] reported a well-defined biocompatible biomaterial based on microporous divinyl benzene copolymer (MDBP) which can be used in various biotechnological applications for immobilization of bacteria. The studies focused on the bioprocess intensity which means increasing the volumetric reactivity of the biocatalyst. The bioprocess intensification was achieved by using the highly porous microarchitected cell support and its characteristic of hydrophobic surface. Microarchitecting means that the size and shapes of pores can be controlled to obtain various pore ratios of solid polymer with a repeatable process. The multicellular microstructure of the surface and inside of the final material created more convenient 3-D environment for cell growth. Because of the abovementioned microarchitecture nature of the material and surface chemistry, which gives rise to appropriate interactions between support and microbial cell membranes, it should be considered for whole-cell immobilization and for increasing mechanical strength and porosity of the matrix in packed column applications.

MDBP was previously proven to be a perfect 3-D scaffold for human, animal, and bacterial cells [3, 7, 9, 10, 11]. In this study, we used the MDBP as a microarchitected 3-D supporting material for yeast cell growth under the continuous flow conditions for the first time. The MDBP biomaterial was manufactured by an extremely easy manual process. *Saccharomyces cerevisiae* chosen as representative yeast was immobilized to MDBP scaffold. *S. cerevisiae* immobilized on MDBP was packed in a continuous column reactor and ethanol fermentation as a common biotechnological process was conducted.

Methods

Cells and Media S. cerevisiae ATCC 26602 was purchased from American Type Culture Collection. All the chemicals used in the study are Merck grade.

Preparation of Immobilized Cell Reactor The immobilized cell reactor (ICR) was made of stainless steel constructed with an internal diameter of 5 cm, 3-mm wall thickness, and 9.2-cm length, 3.5 cm of which was filled with polymeric material. Working volume of the

reactor was 180 ml. The medium was fed to the reactor from a feed tank placed beside the reactor. A peristaltic Masterflex Pump (77250-62, Cole-Parmer Instrument Company) was used to transfer feed medium from 6-l glass container. Effluent from the reactor was collected in 4-l flask. Two flow breakers were located at inlet and outlet of the reactor. The reactor was filled with 8 g MDBP with size between 3.2 and 6.3 mm and autoclaved at 121 °C for 15 min. The reactor was used in continuous mode during fermentation. Glucose and ethanol concentration was monitored at appropriate time intervals. Void volume of the reactor after being filled with the polymer was 101.5 ml.

Preculture of *S. cerevisiae* ATCC 26602 was carried out in a screw-capped bottle containing 1-l yeast basal medium with a pH of 7.0. The medium was composed of 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 10% glucose (*w*/*v*). The culture was grown at room temperature for 3 days without shaking and fed into the reactor with a flow rate of 15 ml/min for immobilization. All the experiments with immobilized cell reactor were performed at room temperature. The Russian Medium (RM) [12] with different glucose concentrations was fed into the reactor with appropriate dilution rates. Components of RM (carbon source, yeast extract (5 g/l), urea (2 g/l), buffer (2 g/l KH_2PO_4, 3 g/l K_2HPO_4) and minerals (0.2 g/l $MgCl_2.6H_2O$, 0.05 g/l $CaCl_2.2H_2O$, 0.0025 g/l $FeSO_4 \cdot 7H_2O$) were autoclaved separately and combined after cooling. The pH of the RM was adjusted to 5.5 with 1.2 M HCl. In order to prevent bacterial contamination, tetracycline was added into the medium with a concentration of 5 mg/l.

Preparation of Microporous Divinyl Benzene Copolymer MDBP was prepared by modifying the procedure described by Akay et al. [9]; 5.6 ml styrene, 2.6 ml divinylbenzene, and 1.8-ml span 80 were mixed well in a 50-ml falcon tube. Forty milliliters of 1.25% potassium peroxy disulfate solution was added into the mixture and mixed well for 5 min. The tube was incubated at 60 °C for 5 min, mixed for 3 min, and placed into the incubator again. This procedure was repeated two times. Polymer was dried for 24 h in 60 °C incubator. Dried polymer was cut into small pieces by hand and sieved through two screens.

Yeast Cell Dry Weight After fermentation, yeast cells immobilized on the polymeric material inside the reactor were dried and weighed. In order to find dry weight of the cells, weight of polymeric material placed into the reactor at the start of process was subtracted from total weight.

Microscopic Examination of Yeast Cells Polymer samples taken from the reactor were fixed in 4% glutaraldehyde solution and kept at 4 °C until examination. The polymer pieces were put into a Petri plate together with a small piece of cotton. The cotton was wetted every day in order to provide a humid environment. Polymer pieces were dried for 4 days and examined with scanning electron microscope.

Determination of Glucose and Ethanol Concentration Glucose and ethanol concentrations in the inlet and outlet were determined by high-performance liquid chromatography (Essence System, Lab Alliance, Scientific System Inc., PA, USA). One milliliter of outlet was filtered through 0.45-μm-pore-sized filter. The filtrate was analyzed by using polymer IEXH form 8-μm sugar column operated at 45 °C with 9-mM sulfuric acid running buffer at a flow rate of 1 ml/min and a refractive index detector operated at 45 °C.

Experimental Setup Continuous cultivation was conducted at two stages. For the first stage, a versatile initial concentration range of glucose between 7 and 300 g/l was fed to the

reactor to obtain the most efficient initial concentration of sugar for ethanol production at a constant flow rate after the precultivation of the packed-bed reactor. Steady-state conditions in the reactor were carried out through the precultivation. The second stage of the continuous cultivation studies, basing on the results of the first stage, was carried out to show the effect of flow rate on the reactor performance. Through this stage, the initial glucose concentration was kept constant and the reactor was operated at the flow rates of 1, 2, 3, 6, 9, and 12 ml/min.

Calculation of Studied Parameters The calculations were done according to the equations given in Table 1

Results and Discussion

Characterization of MDBP as a Biomaterial for Yeast Attachment

The biomaterial MDBP used in this study was newly tested for the immobilization of yeast cells widely applicable in immobilized form for production industry. A thick layer and a dense homogenous population of cells were observed on the surface of the polymer walls (Fig. 1b–d). Figure 1 illustrates that yeast cells are perfectly immobilized on the walls of the polymeric material. The microarchitecture of the polymer used in this study before immobilization procedure is shown in Fig. 1a. After completing continuous cultivation work, the dry weight of the cells was found to be approximately 2 g per gram of cell supporting material. This value indicates the cell attachment capacity of the MDBP.

The porosity of the polymer is about 90% and it is possible to obtain desired pore volume, pore and interconnection hole size between pores, surface chemistry, and rigidity with developed HIPE procedure. The cell supporting material was reported to be a well-defined biocompatible biomaterial in various biotechnological applications [13]. MDBP is also resistant to sterilization process and extreme conditions such as pH, temperature, toxic metabolites, etc. for any biotechnological application. In the previous study, MDBP was used for the immobilization of bacteria in a packed-bed column reactor [8]. The surface roughness of the polymer can help the cells to immobilize under the flow through conditions of the reactor. The attachment of microorganisms is based on the hydrophobic

Table 1 Equations used for calculations.

Abbreviation	Equation	Definition
V	Constant 101.5	Working volume (ml)
Q		Flow rate(ml/min)
t	V/Q	Hydraulic retention time of reactor (h)
C_{so}		Influent substrate concentration (g/l)
C_s		Effluent substrate concentration (g/l)
P		Effluent product concentration (g/l)
E (%)	$\frac{100\times(C_{so}-C_s)}{C_{so}}$	Process substrate removal efficiency (percent conversion)
r_{Su}	$\frac{-Q\times(C_{so}-C_s)}{V}$	Rate of substrate utilization (g/l per day)
Ep (%)	$\frac{100\times P}{C_{so}}$	Process ethanol production efficiency due to utilization
Pf	$\frac{Q\times P}{V}$	Ethanol production rate (g/day)

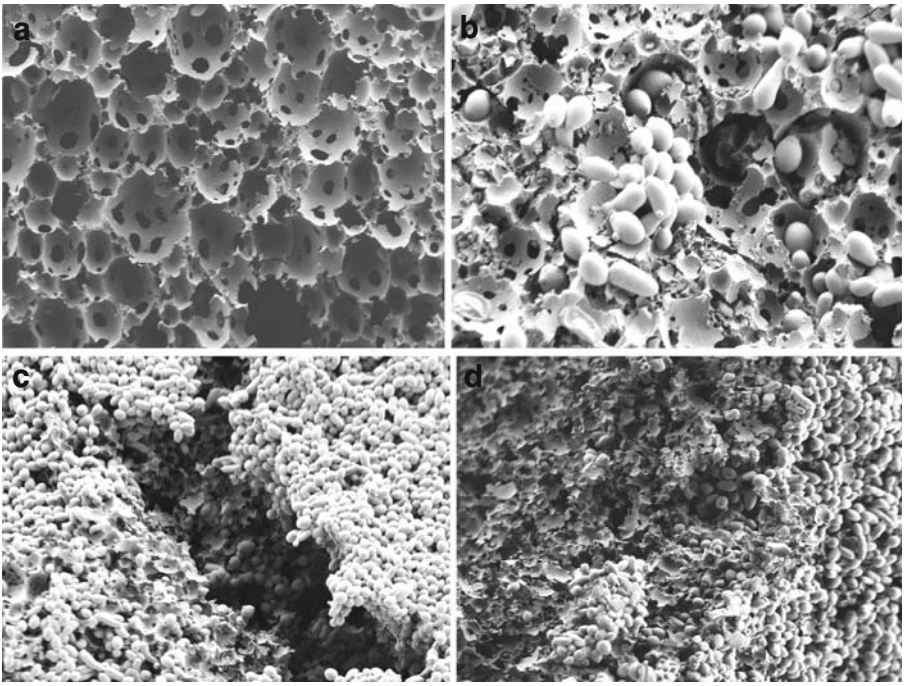

Fig. 1 Scanning electron micrographs of empty polymer (magnification ×4,000; **a**), *S. cerevisiae* immobilized on MDBP (magnification ×5,000 (**b**); magnification 2,000 (**c**)), and surface of polymer piece immobilized with cells (*right part*) and internal region not immobilized with cells (*left part*; magnification ×1,500; **d**)

interactions between cells and MDBP [8, 9]. The microarchitecture involving pores and interconnects can create more convenient 3-D living environment for cell growth. While the wall of the pores provides a large surface area to the cells, interconnects between pores help supply fresh feed and oxygen. In addition to this, degraded cell particles, dead cells that lost attachment ability, and metabolites produced during fermentation can easily be washed out from the reactor. Therefore, the immobilized biomass in the reactor can keep its freshness and dynamic living environment. On the other hand, any extracellular polysaccharide

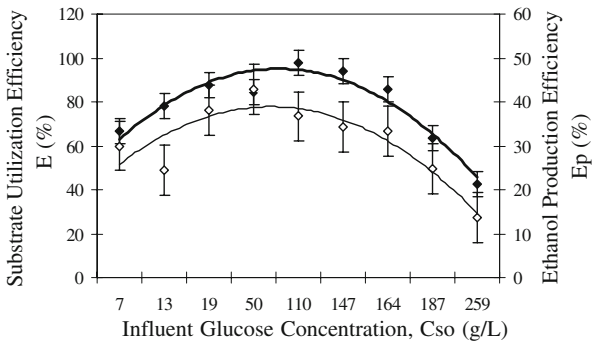

Fig. 2 The substrate utilization (*filled diamonds*, *E*) and ethanol production efficiency (*empty diamonds*, Ep) at a flow rate of 1 ml/min

matrix formation around cells was not observed from scanning electron micrographs (Fig. 1b,c). The dynamic flow conditions through the biomaterial due to microporous architecture of MDBP might inhibit extracellular matrix formation. It can be observed in Fig. 1 that immobilized cells are fresh, young, and also multiplying by the formation of small buds (Fig. 1b).

The surface chemical character of MDBP is strongly hydrophobic. The effect of surface charge of polymeric materials and hydrophobicity of yeast cell surface in attachment process was shown in several other studies. Hazen [14] and Samaranayake et al. [15] reported that cell surface hydrophobicity is involved in adherence but is not the predominant mechanism. Effect of cell surface hydrophobicity on adherence depends on microbial species. Wojciechowicz et al. [16] expressed the effect of hydrophobicity for cell adhesion by studies on *S. cerevisiae* alpha cells. Binding of alpha agglutinin, which is a cell adhesion glycoprotein, to its ligand alpha agglutinin mediates cell–cell contact during mating. Role of electrostatic interaction in adherence was also studied by Park et al. [17] and Callewaert et al. [18]. They reported that hydrophilic nature of polymers resulted in reduction in yeast cells adhesion, which agreed with our findings in this study.

MDBP was previously reported to be a well-designed inert scaffold for tissue culture studies [6] and was then used for bacterial cells as a supporting material [8]. Grifitts and Bosley [19] reported the use of another type of polyHIPE material (macroporous polystyrene-based polymer), for immobilization of bacterial cells before. Through the applications of continuous packed-bed [8] and pressure-driven monolith reactor [9], MDBP showed a perfect 3-D biomaterial character under flow conditions. Our results extremely supported and affirmed the previous findings on the use of MDBP as a 3-D cell matrix. Immobilization of *S. cerevisiae* to the MDBP was successfully performed and sustainable growth of yeast culture was achieved by means of MDBP 3-D structure mimicking natural microenvironment.

Continuous Fermentation with MDBP Immobilized *S. cerevisiae*

Continuous fermentation with *S. cerevisiae* ATCC 26602 immobilized on MDBP was investigated. The reactor was fed with RM medium with various glucose concentrations ranging between approximately 7 and 300 g/l at the flow rate of 1 ml/min. Glucose utilization and ethanol production efficiency in the reactor is presented in Fig. 2. Up to 147-g/l substrate concentration in the feed, glucose uptake, and ethanol concentration increased in the broth. The efficiency of the reactor decreased at higher substrate concentrations. Longer retention time might be necessary for sugar concentrations higher than that concentration of glucose or cells might be damaged by high pressure at higher substrate concentrations. In earlier studies, it was reported that cells might be physiologically damaged at high glucose concentrations because of osmotic pressure [20]. Najafpour et al. [21] also reported that at very high concentration of glucose the sugar conversion decreased. They also found that the maximum sugar conversion at 6-h retention time was 88.2% for 50-g/l glucose concentration in IRC with working volume of 740 ml having *S. cerevisiae* cells immobilized on sodium alginate beads. Sodium alginate beads shows a low substrate and oxygen transfer character due to its diffusional limitations. In our study, reactor efficiency reached its highest level (83.69%) when medium containing 50 g/l glucose was fed into the reactor at a flow rate of 1 ml/min which resulted in a low retention time (1.7 h) in the ICR used in our study.

The reactor was also operated at flow rates of 1, 2, 3, 6, 9, and 12 ml/min with 50 g/l glucose containing medium (Fig. 3). Shorter retention time decreased ethanol concentration in the outlet broth. The highest ethanol production rate (847 g/day) was obtained with initial

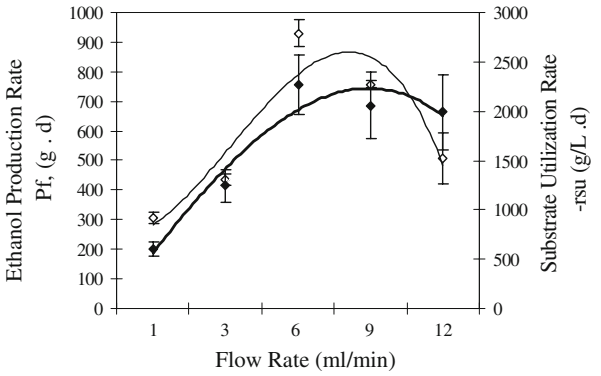

Fig. 3 Ethanol production rate (Pf; *empty diamonds*) and substrate utilization rate ($-r_{Su}$; *filled diamonds*) at different flow rates with 50 g/l glucose containing medium

glucose concentration of 50 g/l at a flow rate of 6 ml/min. For immobilized packed-bed reactors, the production rate and the substrate utilization rate increase with the increasing of flow rate at the beginning due to the diffusional limitations. Mass transfer resistance is more effective at relatively lower flow rates through and vicinity of the biofilm [8]. Therefore, higher flow rates providing necessary retention time for conducting the reaction can be preferred for higher reactor productivity.

It is well known that ethanol fermentation is inhibited by high initial glucose concentrations and accumulation of ethanol in the reactor. Figure 4 shows the relation between substrate utilization rate (r_{Su}) and initial concentration of glucose. The inhibition effect of glucose by its increasing initial concentration was observed especially at above 150 g/l, a relatively high substrate concentration (Fig. 4). Up to that concentration, substrate utilization rate increased with increasing concentration of glucose, then a sudden decrease occurred. We think that the dynamic flow conditions around the microenvironment of the attached cells and mass transferring ability of cell immobilized by MDBP can help decrease the inhibition effect of ethanol accumulation and substrate in the vicinity of the cells. However, severe decrease of substrate utilization rate could not be prevented at relatively high initial concentration of glucose (above 150 g/l).

Our results confirmed that MDBP biomaterial as a 3-D cell scaffold can successfully be used for immobilization of yeast cells in a packed-bed reactor for ethanol production. The

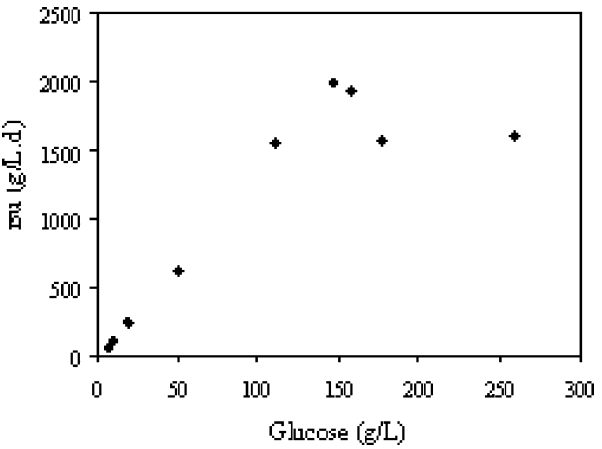

Fig. 4 The utilization rate profile of the continuous cultivation showing the substrate inhibition effect of glucose between 6.7 and 300 g/l

MDBP was occupied completely by yeast cells, forming a dense biofilm on the outer walls of the biomaterial. The biocompatible architecture of the supporting material resulted in less diffusional limitations and stable conditions for the cells due to the lack of exopolysaccharide matrix formation around cells and continuous washing out of toxic metabolites and dead and degraded cells from the reactor. Therefore, the immobilized biomass in the reactor can keep its freshness and dynamic living environment. The multicellular microporous structure of MDBP consisting of pores and interconnects can create more convenient 3-D living environment for yeast cells.

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