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Short communication

Immobilized cell microchannel bioreactor for evaluating fermentation characteristics of mixed substrate consumption and product formation

Tae Young Seo[a], Ki Won Eum[a], Sung Ok Han[b], Seung Wook Kim[a], Ji Hyeon Kim[c], Kwang Ho Song[a,*], Jaehoon Choe[d,**]

[a] Department of Chemical and Biological Engineering, Korea University, Seoul 136-713, Republic of Korea
[b] School of Life Science and Biotechnology, Korea University, Seoul 136-713, Republic of Korea
[c] Division of Energy and Biological Engineering, Gachon University, Gyeonggi-Do 461-701, Republic of Korea
[d] LG Chem Research Park, Daejeon 305-380, Republic of Korea

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ABSTRACT

An immobilized cell microchannel bioreactor was designed to test continuous fermentation. The fermentation set-up included a bottom hydrophilic quartz channel to immobilize cells using 0.4 wt% polyethyleneimine and a top channel designed to continuously remove metabolically generated carbon dioxide using hydrophobic polypropylene. To evaluate fermentation characteristics of immobilized cells, ethanol fermentation was carried out using *Saccharomyces cerevisiae* and *Pichia stipitis*. The immobilized cell microchannel bioreactor was used to identify long-term activity of immobilized *S. cerevisiae* cells. The continuous flow microchannel bioreactor was operated stably over a period of 1 month. The immobilized cell microchannel bioreactor was used to examine the characteristics cells that consumed mixed substrates. The concentration ratio of glucose to xylose for simultaneous utilization of hemicellulosic sugars was evaluated using the microchannel bioreactor and the results were compared with those obtained by using conventional batch fermentation with *P. stipitis*.

1. Introduction

Microchannel devices take advantage of features such as rapid heat and mass transfer rates to maximize process efficiency. These features are attractive for conducting reactions using enzymes, which can be immobilized to the device. Immobilization of cells within microchannel devices has been studied using various methods [1]. Numerous microchannel devices with immobilized enzymes have been applied for biochip technology [2,3], particularly biosensors, which take advantage of a shorter analysis time with small volumes [4]. This technique can also be applied as a genomic analysis tool using polymerase chain reactions to improve temperature controllability and reduce analysis time. Microchannel devices can also be used to examine individual cells. These devices provide the unique opportunity to observe discrete microbiological phenomena; this is not possible with traditional batch wise approaches, which show bulk-phase characteristics.

Optimization of cell culture conditions using microchannel device is faster than that using batch device [5]. Therefore, diffusion based microfluidic reactor system [6], single use microbioreactor [7], and microchemostats [8] have been developed to optimize culture conditions by integrating online measurements of dissolved oxygen and optical density. Current challenges in further development of microreactor systems include shortening experimental time and controlling variables [9].

Evaluating cell characteristics is essential for fermentation process development. However, test tubes, shake flasks, or microtiter plates are the primary devices used for testing strains to assess the fermentation processes. In this study, an immobilized cell microchannel bioreactor was operated continuously and used to evaluate cell characteristics. The loss of cell activity during ethanol production was evaluated using *Saccharomyces cerevisiae*. Further, consumption of glucose and xylose mixtures was determined and compared with that reported for batch studies [10].

2. Materials and methods

2.1. Materials

S. cerevisiae ATCC 204679 was obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA) and *Pichia stipitis* CBS 6054 was obtained from the Korea Collection of Type Culture (Daejeon, Korea). Yeast extract, peptone, glucose, and xylose were purchased from Duchefa Biochemie (Haarlem, Netherlands). Polyethyleneimine (PEI; molecular weight, 60,000), chromic acid, and hydrochloric acid were obtained from Sigma–Aldrich (St. Louis, MO, USA).

* Corresponding author. Tel.: +82 2 3290 3307; fax: +82 2 926 6102.
** Corresponding author. Tel.: +82 42 866 2393; fax: +82 42 861 2057.
E-mail addresses: khsong@korea.ac.kr (K.H. Song), jaehoon.ch@gmail.com (J. Choe).

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2.2. Analytical methods

Ethanol content was quantified using a gas chromatogram (7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and an Innowax column (30 m × 0.32 mm ID × 0.25 mm film thickness, Agilent). Oven temperature was maintained at 120 °C for 5 min. Helium gas was used as the carrier gas.

Concentrations of glucose and xylose were measured using a liquid chromatogram with a refractive index detector and an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) with 0.005 N sulfuric acid at a flow rate of 0.8 mL/min as the mobile phase. Column temperature was maintained at 55 °C. Cell concentration was determined by measuring optical density (OD) of cells in a 1-cm quartz cell at 570 nm and 600 nm for *P. stipitis* and *S. cerevisiae*, respectively, by using a UV–vis spectrophotometer. An OD of 1 was equivalent to 0.23 g/L of dry cells for *P. stipitis* and 0.275 g/L of dry cells for *S. cerevisiae*. Dry cell weight was determined by drying the washed immobilized cells at 105 °C.

Immobilized cells were examined using scanning electron microscopy (SEM, S4300, Hitachi, Tokyo, Japan). Samples were freeze-dried and fixed on an aluminum stub and coated with gold palladium at a coating thickness of 20 nm.

2.3. Cell immobilization

Cells were immobilized to the quartz channel surface using PEI [11,12]. PEI solution was prepared by dissolving 0.4 g of PEI in 1 L of distilled water; the pH was adjusted to 10 using 0.1 N NaOH. The PEI solution was then sterilized by autoclaving at 121 °C for 15 min. To prepare the immobilized cell channel, quartz plates were soaked overnight in chromic acid and dried at 30 °C for 4 h. Sterilized PEI solution was injected into the microchannel using a syringe pump (Harvard Pump 11 Plus, Harvard Apparatus, Holliston, MA, USA) and incubated for 2 h to coat the surface of the quartz channel with PEI, and then air was injected to remove excess PEI solution. Seed cultures were prepared in 500-mL flasks containing 100 mL yeast extract peptone dextrose (YPD) medium and were shaken for 18 h at 30 °C. The OD_{600} of 0.1, which correlates to 23 mg of dry weight of yeast, was taken and used for immobilization. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (pH 7.4). The cells were poured into 10 mL of 0.4 wt% sterilized PEI solution. The mixture was gently mixed on a shaker at room temperature for 2 h to coat the cells with PEI. PEI-coated cells were then centrifuged and washed 3 times with water and then resuspended in 2 mL of water. The suspension of PEI-coated cells was then injected into the quartz channel (0.26 mL) using a syringe pump and left for 2 h. The immobilized cell microchannel surface was then dried at 30 °C for 2 h by opening the top cover plate. Secure binding of cells to the quartz surface was observed using SEM after extensive washing the quartz surface under running water. The weight of loaded cell for immobilization was about 3 mg. The weight of immobilized cells was determined after drying at 90 °C for 24 h in a forced convection oven.

2.4. Continuous fermentation using immobilized cell microchannel bioreactor

The YPD medium contained 10 g/L of yeast extract, 20 g/L of peptone and varying concentrations of glucose and xylose. The continuous culture medium for *S. cerevisiae* growth contained YPD media and 30 g/L glucose. Growth of *P. stipitis* in media containing glucose and xylose in the following proportions was tested (g/L): 60/0, 50/10, 40/20, 30/30, 20/40, 10/50, and 0/60. The medium was continuously fed into the immobilized cell microchannel bioreactor using a syringe pump at a flow rate of 0.13 mL/h with a residence time of 2 h. The *S. cerevisiae* fermentation was performed in YPD media with an initial pH of 4.5 at 32 °C, whereas *P. stipitis* fermentation was conducted in YPD media at pH 6.3 and 30 °C with varying concentrations of glucose and xylose. The initial pH was adjusted using 0.1 M Tris–HCl, and the temperature was maintained in a forced convection oven. Fermentation data were collected after 5 h of continuous injection. To prevent evaporation of the collected ethanol, the outflow was cooled to 4 °C using a cooling coil. The overall experimental setup is shown in Fig. 1(a). Concentrations of glucose, xylose, and ethanol were monitored during continuous fermentation. Carbon dioxide generated during fermentation was continuously flowed through the upper channel and removed from the channel through a hole in the top plate.

2.5. Design of an immobilized cell microchannel bioreactor

A 500-μm deep and 800-μm wide flow channel was constructed from both polypropylene and quartz (50 mm × 50 mm × 3 mm) using a precision milling machine (CAT3D-M6, Datron GmbH, Mühltal, Germany). The contour length of the serpentine microchannel was 35 cm with a volume of 528 μL. The channel on the top plate composed of polypropylene was hydrophobic, while the quartz channel on the bottom was hydrophilic. Carbon dioxide gas was released through a top vent port at the end of the polypropylene channel. The product and the aqueous medium were collected through the bottom port at the end of the quartz channel.

3. Results and discussion

3.1. Immobilized cell microchannel bioreactor designed for continuous fermentation

Operating gas–liquid flow systems in micrometer-sized restricted spaces is difficult since the phase transition from liquid to gas or from gas to liquid is accompanied by a very large volume change [13]. Therefore, microstructures for complete gas–liquid separation are necessary to ensure system stability in microchannels. Gases, such as carbon dioxide, are generated through cellular metabolism during fermentation, and the gas prevented contact between the media and immobilized cells in the microchannel when both sides are constructed of quartz plates, as shown in Fig. 1(b). Flowing media through the microchannel accelerated gas production, decreasing both fermentation time and ethanol production. To efficiently separate the carbon dioxide generated during fermentation in the microchannel, the top plate was designed using hydrophobic polypropylene. The polypropylene plate was constructed to have a 110° contact angle against water and could block the inflow of hydrophilic liquid into the top channel. As shown in Fig. 1(c), the media flowed through the bottom quartz channel and the carbon dioxide flowed through the top polypropylene channel without interrupting the flow of the bottom channel. The volume of liquid in the microchannel (260 μL) was measured by determining the residence time of media injected at a constant flow rate. The media is assumed to have flowed only through the quartz microchannel since the volume of liquid measured in the microchannel was the same as the dimension of the bottom quartz microchannel.

3.2. Immobilization and performance of cells

To test the performance of immobilized cell microchannel bioreactors, ethanol fermentation was carried out using *S. cerevisiae* and *P. stipitis*. Production efficiencies of immobilized cells are represented as the change in PEI concentrations. Ethanol production decreased when PEI concentration was less than 0.4 wt%. Under the conditions used for PEI coating, no flocculation of cells into aggregates was observed. However, ethanol production decreased as PEI concentration increased to values greater than 0.4 wt%. The optimum PEI concentration for ethanol production was 0.4 wt% when *S. cerevisiae* and *P. stipitis* were immobilized.

Fermentation was performed in microchannel bioreactors by immobilizing cells 1–4 mg of cells and the resulting ethanol concentrations were measured. The greatest amount of ethanol produced was 10.74 g/L for *P. stipitis* when 3 mg of cells were immobilized on the microchannel. Ethanol production did not increase when 4 mg of cells were immobilized on the microchannel. It was assumed that further increasing the number of cells would not affect fermentation since these cells were not exposed on the surface. Therefore, 3 mg of cells is adequate for covering all surfaces of the PEI-coated microchannel. The weight of immobilized cells on the microchannel was evaluated after drying, and this value did not change over tens of hours of continuous fermentation. This suggests that not all budding cells derived from immobilized cells might have remained on the PEI-coated microchannel and that budding cells flowed out of the microchannel bioreactor. We determined the characteristics of immobilized cells independently since budding cells were separated from immobilized cells in the microchannel bioreactor.

3.3. Measurement of cell activity using immobilized cell microchannel bioreactor

S. cerevisiae was immobilized in a microchannel bioreactor and continuous fermentation was conducted for 30 days at a constant