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Ethanol production by *Saccharomyces cerevisiae* in biofilm reactors

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Biofilms are natural forms of cell immobilization in which microorganisms attach to solid supports. At ISU, we have developed plastic composite-supports (PCS) (agricultural material (soybean hulls or oat hulls), complex nutrients, and polypropylene) which stimulate biofilm formation and which supply nutrients to the attached microorganisms. Various PCS blends were initially evaluated in repeated-batch culture-tube fermentation with *Saccharomyces cerevisiae* (ATCC 24859) in low organic nitrogen medium. The selected PCS (40% soybean hull, 5% soybean flour, 5% yeast extract-salt and 50% polypropylene) was then used in continuous and repeated-batch fermentation in various media containing lowered nitrogen content with selected PCS. During continuous fermentation, *S. cerevisiae* demonstrated two to 10 times higher ethanol production in PCS bioreactors than polypropylene-alone support (PPS) control. *S. cerevisiae* produced 30 g L^{-1} ethanol on PCS with ammonium sulfate medium in repeated batch fermentation, whereas PPS-control produced 5 g L^{-1} ethanol. Overall, increased productivity in low cost medium can be achieved beyond conventional fermentations using this novel bioreactor design.

Keywords: ethanol; fermentation; *Saccharomyces cerevisiae*; biofilm

Introduction

Ethanol, methanol, and ETBE (ethyl tertiary-butyl ether) are blended with gasoline as fuel extenders and octane enhancers. Ethanol is produced commercially by microbial batch or continuous fermentation and it is recovered by distillation. It is necessary, however, to increase productivity and ethanol concentration in the fermentation medium in order to decrease ethanol production costs, which were $1.25 per gallon in 1992 [11]. Fermentation medium costs represent about 30% of the final ethanol costs [16].

Some fermentation techniques have been developed to improve ethanol fermentation to decrease cost [3,5]. End-product inhibition can be overcome by employing a vacuum fermentor which removes ethanol continuously from the fermentation medium [5]. Another method for continuous removal of ethanol from fermentation medium is extractive fermentation, which utilizes suitable solvent and/or membrane [3]. Other commonly studied fermentor-types focus on increasing biomass concentration in the fermentor which also increases productivity and ethanol concentration [2,15,17]. Cell recycle [17], hollow fiber membrane fermentors [2], and cell immobilization [15] are being improved continuously. However, these systems have found little application commercially because of mass transfer limitation, and other limitations in long-term fermentations.

Biofilms are a natural form of cell immobilization that result from microbial attachment to solid supports [1]. Biofilms have been used in waste water treatments [14], production of vinegar by the 'quick vinegar process', mineral ore treatment [4], and lactic acid production [6,7,8–10]. Kunduru and Pometto [12,13] demonstrated the benefit of plastic composite supports (PCS) chips for enhanced ethanol production. However, PCS shape resulted in bioreactor plugging. In this paper, ethanol fermentation with different nitrogen concentration by *Saccharomyces cerevisiae* in PCS biofilm reactors containing PCS rings and disks is described. Continuous and repeated-batch ethanol fermentations with PCS-stimulated biofilm formation provided essential nutrients to *S. cerevisiae*.

Materials and methods

Microorganism and media

S. cerevisiae (ATCC 24859) was maintained in a medium containing 20 g of glucose, 6 g of yeast extract (Ardamine-Z, Champlain Industries, Clifton, NJ, USA), 0.23 g of $CaCl_2 \cdot 2H_2O$, 4 g of $(NH_4)_2SO_4$, 1 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4 per liter of deionized water and stored at 4°C. All fermentations' inocula were prepared from 24-h static culture at 30°C. For culture-tube fermentation, 5% glucose medium (5% GM) was used which contained 5% glucose, salts (as above, except ammonium sulfate), and varying the concentration of yeast extract or ammonium sulfate as nitrogen source. For continuous fermentation and repeated-batch fermentation, glucose concentration was increased to 7.5% (7.5% GM). Medium was sterilized in a 50-L B. Braun fermentor U-50 (Allentown, PA, USA) with constant agitation (220 rpm) at 121°C for 20 min. Three liters of sterile KH_2PO_4 solution were added aseptically to all culture media prior to use.

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Plastic composite-supports

Various PCS rings and discs were produced from blends of 50% agricultural materials and 50% (w/w) polypropylene (Table 1). PCS were prepared by high-temperature extrusion in a Brabender PL2000 co-rotating twin-screw extruder (Model CTSE-V, CW Brabender Instruments, South Hackensack, NJ, USA) through a tubing die assembly onto a metal rod, collected as 10–15 cm length then removed from the rod and air cooled. The barrel temperatures were 200, 220, and 200°C, the die temperature was set at 165°C, and the screw speed was 11 rpm. Polypropylene pellets (Quantum USI Division, Columbus, OH, USA), specific agricultural blends, and salts (0.46 g of $CaCl_2 \cdot 2H_2O$, 8 g of $(NH_4)_2SO_4$, 2 g of $MgSO_4 \cdot 7H_2O$ per kg) were mixed in a container and then added to the extruder hopper. The melted polypropylene was uniformly mixed with agricultural product by the co-rotating movement of the twin-screws and extruded through a pipe die with 9.5-mm i.d., and 12.7-mm o.d. for rings or with 3.2-mm i.d., and 12.7-mm o.d. for discs, and then cut into approximately 3-mm slices. Polypropylene-alone support (PPS-control) rings were hand cut out of a sheet of polypropylene by using cork bores of 7-mm i.d., and 11-mm o.d.

Culture-tube repeated-batch fermentation

The selected PCS discs (2.9 g) (Table 1), or PPS-control rings (4.8 g) with a total external surface area of 60 cm^2 were placed in 25×150-mm screw-cap culture tubes and autoclaved dry at 121°C for 30 min. After cooling, 10 ml of sterile 5% GM medium with 0.6% (w/v) yeast extract was added. The tubes were incubated at 30°C for 24 h, then aseptically decanted to remove excess particles. After adding 10 ml of fresh medium, the tubes were inoculated with 0.1 ml of a 24-h suspension culture of *S. cerevisiae*, and incubated at 30°C. Every other day, the culture medium was decanted aseptically and 10 ml of fresh sterile medium was added for seven transfers. These repeated-batch fermentations were performed in duplicate including suspension-culture fermentation with no support. At the end of the seventh transfer, medium nitrogen composition was decreased sequentially to 0.3, 0.1, and 0.05% (w/v) yeast extract, then all yeast extract was removed (ammonium sulfate medium), and finally all ammonium sulfate was removed (N-free medium) with sequential repeated-batch fermentation. The spent culture medium from each transfer was analyzed for changes in cell density, ethanol and glucose concentration. After the seventh transfer in the ammonium sulfate medium was completed, four discs were aseptically removed from each culture-tube for biofilm determination. The repeated-batch fermentations were continued by adding 7.8 ml of N-free medium to keep support and medium ratio constant. Upon completion of the seventh transfer in N-free medium, four discs were removed and analyzed for biofilm development.

Table 1 Composition of plastic composite rings and discs blends and names

PCS	Minor agricultural material(s) (w/w)	Salt
SH-SF[a]	10% soybean flour[b]	–
SH-SF-S	10% soybean flour	+
SH-YE	10% yeast extract[c]	–
SH-YE-S	10% yeast extract	+
SH-SF-YE	5% soybean flour – 5% yeast extract	–
SH-SF-YE-S	5% soybean flour – 5% yeast extract	+
SH-SF-BG	5% soybean flour – 5% bovine globulin[d]	–
SH-YE-BG	5% yeast extract – 5% bovine globulin	–
SH-SF-BA	5% soybean flour – 5% bovine albumin[d]	–
SH-YE-BA	5% yeast extract – 5% bovine albumin	–
SH-RBC	10% red blood cells	–

[a]SH: ground 20 mash and vacuum-dried soybean hulls (Cargill Soy Processing Plant, Iowa Falls, IA, USA).
[b]SF: vacuum-dried soybean flour (Archer Daniel Midland Co, Decatur, IL, USA).
[c]YE: yeast extract (Ardamine-Z, Champlain Industries, Clifton, NJ, USA).
[d]BG: dried bovine globulin; BA: dried bovine albumin (American Protein Corporation, Ames, IA, USA).

Analysis of biofilm

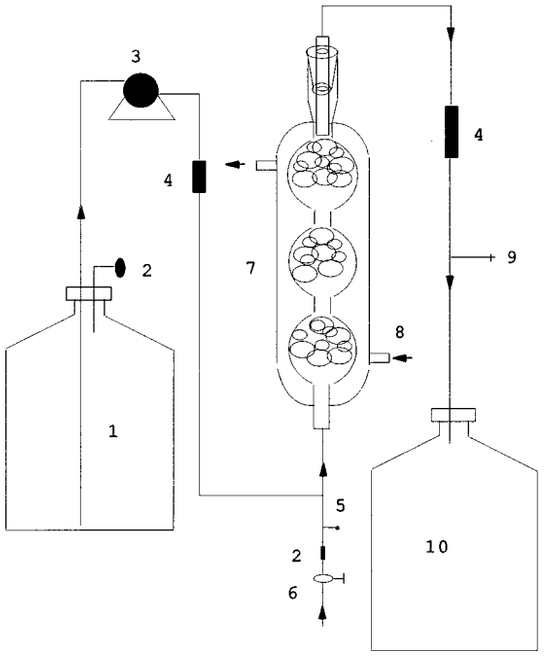
The stripping sand method [8] was used to determine the relative biofilm population on the supports. The four discs removed from each culture-tube were washed in 100 ml of sterile 0.1% (w/v) peptone water by turning the bottle upside-down 10 times, then aseptically transferred into a 25×150-mm screw-cap tube containing 5 g of sterile sand and 9 ml of sterile 0.1% (w/v) peptone water. After vortexing the tube three times in 30-s intervals, the sample was serially diluted, and 10^{-2} to 10^{-6} dilutions were spread plated onto potato-dextrose agar plates. After incubating *S. cerevisiae* plates aerobically at 30°C for 48 h, colony-forming units (CFU) were counted.

Continuous ethanol fermentation

With selected PCS (SH-SF-YE-S; see Table 1), continuous ethanol production was performed in the customized semi-packed reactor, which consisted of a 400-mm long cylindrical-bulb condenser with three bulbs used by Kunduru and Pometto [13] (Figure 1). Each bulb was packed with an equal number of selected discs and rings (6.6 g) or PPS-control rings (8.3 g) as control to give a total external surface area of 160 cm^2, and bulk volume of 60 ml. The reactor which had a working volume of 50 ml was sparged with filter-sterilized air. Feeding medium and gas entered the reactor from the bottom and exited from the top. Dilution rates varied from 0.48 to 3.84 h^{-1}. The reactor was operated at each dilution rate until steady state was achieved. Samples were collected daily and analyzed for changes in cell density, ethanol and glucose concentrations. Culture purity was frequently monitored microscopically.

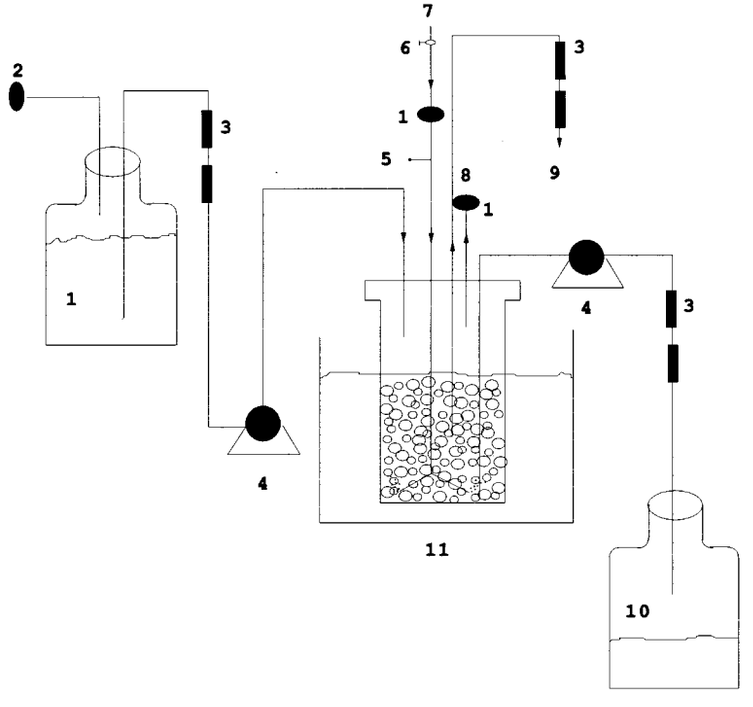
Repeated-batch ethanol fermentation

Repeated-batch fermentations were performed in customized bioreactors (300-ml Fleaker beakers) containing selected PCS. Reactors contained 36.7 g of support (725 cm^2 external surface area) which were equipped with ports for air in and out, sampling, and medium in and out (Figure 2). Reactors with all the tubing and specific supports were



1. Feed reservoir
2. Air filter
3. Pump
4. Liquid break
5. Inoculation septum
6. Manifold
7. Bioreactor
8. Water in
9. Sampling port
10. Effluent collector

Figure 1 Schematic diagram of continuous biofilm reactor.



1. Feed reservoir
2. Air filter
3. Liquid break
4. Pump
5. Inoculation septum
6. Manifold
7. Air inlet
8. Air outlet
9. Sampling port
10. Effluent collector
11. Water bath

Figure 2 Schematic diagram of repeated-batch biofilm reactor.

autoclaved at 121°C for 60 min without medium. After cooling, 120 ml of sterile medium was pumped into the reactor. The reactor was incubated at 30°C for 24 h to check sterility and to remove initial leachate from the PCS. After medium was replaced with fresh medium, each reactor was inoculated with 5 ml of 24-h yeast culture. Media in reactors were changed every 24 or 48 h with N-free medium or ammonium sulfate medium. Reactors were operated with or without air. The samples were collected daily and analyzed for glucose consumption, ethanol production, and cell density.

Analysis of culture broth

The suspended cell density in the reactors was measured by using a Bausch and Lomb Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY, USA) at 620 nm. Glucose and ethanol concentrations were determined by using a Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford, MA, USA) equipped with a Waters Model 401 refractive index detector, column heater, autosampler, and computer controller. Ethanol, glucose, and other broth ingredients were separated on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA, USA) using 0.012 N sulfuric acid as the mobile phase at a flow rate of 0.8 ml min^{-1} with a 20-μl injection volume and a 65°C column temperature.

Results and discussion

Screening various PCS blends for improved performances

In culture-tube repeated-batch fermentation, all the PCS, PPS-control, and suspension cultures in 0.6, 0.3, 0.1, and 0.05% (w/v) yeast extract media, no significant differences were observed in their performance. They all consumed 50 g L^{-1} glucose, and produced about 25 g L^{-1} ethanol. However, ethanol production decreased to 5 and 0 g L^{-1} in PPS-control culture-tubes with ammonium sulfate medium and with N-free medium, respectively, whereas all PCS culture tubes in these same media produced about 25 g L^{-1} ethanol. The improved performance of PCS was attributed to the nutrients' slow-release [9] from each support and to biofilm formation on the supports. Biofilm formation was confirmed on all support surfaces in ammonium sulfate medium as $<10^5$ CFU cm^{-2} for PPS-control, and as $>10^6$ CFU cm^{-2} for all PCS. For N-free medium, CFU cm^{-2} was slightly less, but not significantly different from that of the ammonium sulfate medium. Yeast cells located in the PCS pores were confirmed by scanning electron microscopy of the interstitial area of the PCS.

Selection of the best performing PCS was determined by plotting the average ethanol concentration of the first set of three culture-tube repeated-batch transfers to the last set of three in N-free medium (Figure 3). PCS of SH-SF-YE-S demonstrated the best performance by illustrating the highest ethanol productions in both cases. These results also suggested that a significant reduction in organic nutrients in the culture medium is possible for continuous ethanol production with these supports.

Continuous ethanol fermentation with low or nitrogen-free medium

For biofilm development, bioreactors containing PCS of SH-SF-YE-S or PPS-control were operated with 0.6% (w/v) yeast extract medium at a dilution rate of 1.92 h^{-1}. For biofilm development, bioreactors containing PCS of SH-SF-YE-S or PPS-control were operated with 0.6% (w/v) yeast extract medium at a dilution rate of 1.92 h^{-1}. Previously Kunduru and Pometto [13] reported that suspension cell culture fermentation yielded equal or lower productivities than those of PPS-control. The reason for the high dilution rate was to prevent yeast flocculation. Visible biofilm was observed after 9 days for the PCS reactors, however, flocculation was observed in the PPS-control bioreactors on days 7 to 9 (Figure 4). In the N-free medium, PCS of SH-SF-YE-S bioreactor performed better than PPS-

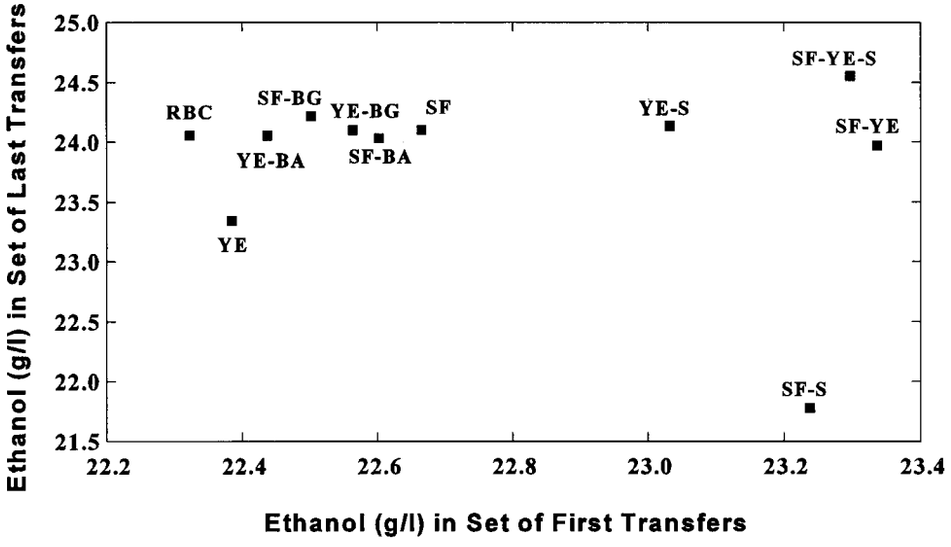


Figure 3 Comparison of various PCS's performance in culture-tube repeated-batch fermentations by *S. cerevisiae* with N-free medium. Each support had 40% soybean hulls (See Table 1). Each data point is an average of two replicates and three transfers for a total of seven transfers.

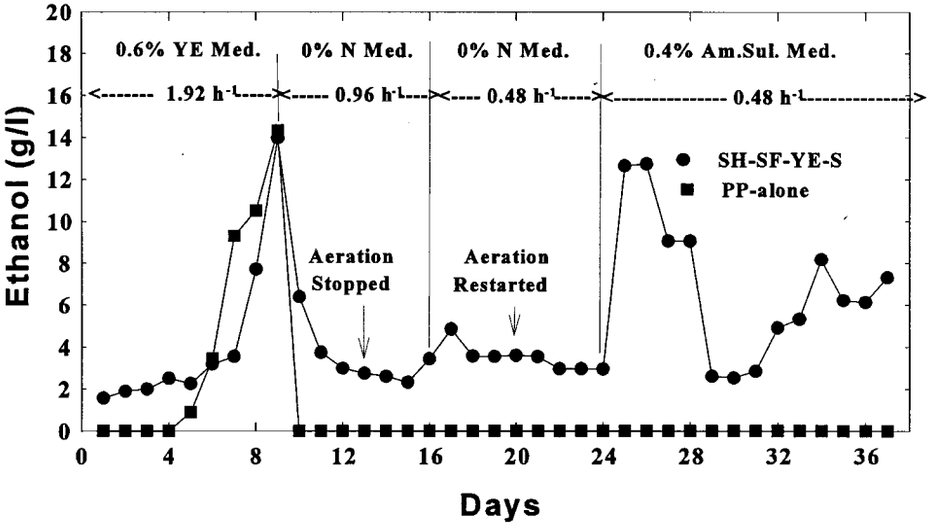


Figure 4 Continuous biofilm ethanol fermentation in various fermentation media by *S. cerevisiae* with PCS of SH-SF-YE-S and PPS-control. Each data point is an average of two replicates.

control bioreactor at the dilution rate of 0.96 h^{-1}. When the medium was changed to N-free medium, *S. cerevisiae* washed out in the PPS-control bioreactors, whereas ethanol production for PCS bioreactor was 3 g L^{-1} with productivity of 1.4 g L^{-1} h^{-1} at a dilution rate of 0.48 h^{-1} (Figure 4). When aeration was stopped, working volume in the bioreactors changed slightly due to the loss of air bubbles in the reactor, which reduced the dilution rate. Overall, ethanol production was not affected significantly with or without aeration. When the medium was changed to ammonium sulfate medium, there was no ethanol production for PPS-control support reactor, whereas an increase and a decrease in ethanol production for the PCS bioreactor were observed followed by a steady rise which most likely represents an initial response to the new medium with a possible increase in suspension cells. Ethanol production averaged 9.1 g L^{-1} for the PCS bioreactor with productivity of 4.4 g L^{-1} h^{-1} at a dilution rate of 0.48 h^{-1} which suggested a nutritional benefit to the yeast by the PCS. This increase in ethanol production where $(NH_4)_2SO_4$ was introduced into the culture medium illustrated a need for some exogenous nitrogen.

Kunduru and Pometto [13] reported productivity for *S. cerevisiae* as 76 g L^{-1} h^{-1} at a dilution rate of 2.88 h^{-1} on

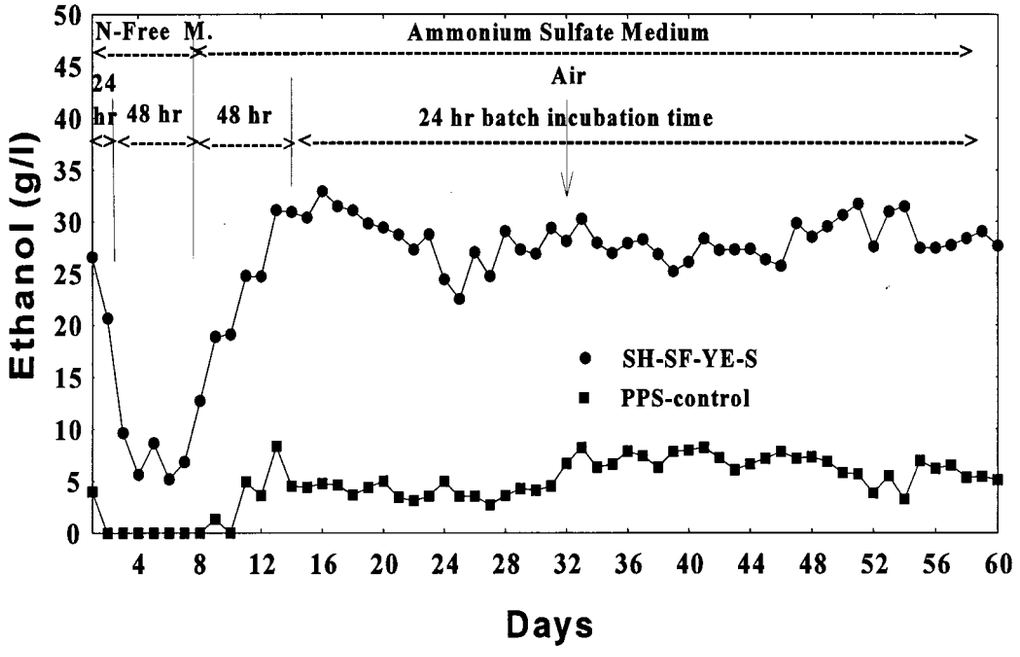


Figure 5 Repeated-batch fermentation by *S. cerevisiae* with PCS of SH-SF-YE-S and with PPS-control. Medium was changed every 24 or 48 h. Each data point is an average of two replicates.

PCS chips. However, they also reported that plugging of the reactor due to flocculation of the yeast was the biggest concern for long-term fermentation. Therefore, utilization of rings and disks in place of chips, and low nitrogen media helped to reduce flocculation and plugging of the reactor, because low nitrogen media probably encouraged the growth of yeast cells on the surface of PCS instead of growing as suspension culture which caused flocculation in long-term fermentation. Bioreactor plugging by yeast flocculation could be reduced by fluidizing the support mechanically or by increasing the medium flow rate around the support. Additionally, we had performed continuous ethanol fermentation with *Z. mobilis* in 0.1% (w/v) yeast extract medium by using PCS of 30% soybean hulls, 5% yeast extract, 5% red blood cell and salts. No differences were observed between the PCS and PPS-control bioreactors (data not shown) due to the aggressive aggregation of *Z. mobilis* which caused serious plugging problems in long term continuous fermentations.

Repeated-batch fermentation

Repeated-batch fermentations were performed initially with N-free medium for 24-h then 48-h incubations until day 7 by using PCS of SH-SF-YE-S or PPS-control supports (Figure 5). PPS-control bioreactors produced no ethanol initially, whereas PCS bioreactors produced 8 g L^{-1} ethanol. We observed a gradual increase in ethanol concentration for both PCS and PPS-control bioreactors with ethanol concentrations of 25–33 g L^{-1} and 4–9 g L^{-1} (Figure 5), respectively. No significant impact on ethanol production was observed when aeration was initiated. Overall, PCS bioreactors significantly outperformed the PPS-control bioreactors during this long-term (60 days) repeated-batch fermentation in ammonium sulfate medium with overall average ethanol production of 28.4 and 5.7 g L^{-1}, which represented a percentage yield ([produced ethanol (g L^{-1})/consumed glucose (g L^{-1})] $*$ 100) of 38.2 and 43.4%, respectively. These results confirmed the preliminary study with culture-tube repeated-batch fermentation.

Repeated-batch fermentation with *Z. mobilis* demonstrated no differences between the PCS and PPS-control bioreactor (data not shown). Again this lack of difference was attributed to this bacterium aggregating characteristics. Therefore, biofilm bioreactors are not recommended for this bacterium.

However, PCS bioreactors benefitted ethanol fermentation by *S. cerevisiae* both in continuous and repeated-batch fermentations. Utilization of low-nutrient medium is an important factor to reduce production cost. The maximum concentration was about 30 g L^{-1} which could be increased by increasing the initial glucose concentration. This research demonstrated the benefit of PCS to ethanol fermentation in long-term fermentation. Further research is needed to optimize increased ethanol concentration in the bioreactor for industrial purposes. These results for ethanol fermentation and the results of Ho *et al* [10] for lactic acid fermentation strongly indicate that this novel immobilized-cell bioreactor can be adopted for other fermentations.

Acknowledgements

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