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Continuous ethanol production by *Zymomonas mobilis* and *Saccharomyces cerevisiae* in biofilm reactors

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Continuous ethanol fermentations were performed in duplicate for 60 days with *Zymomonas mobilis* ATCC 331821 or *Saccharomyces cerevisiae* ATCC 24859 in packed-bed reactors with polypropylene or plastic composite-supports. The plastic composite-supports used contained polypropylene (75%) with ground soybean-hulls (20%) and zein (5%) for *Z. mobilis*, or with ground soybean-hulls (20%) and soybean flour (5%) for *S. cerevisiae*. Maximum ethanol productivities of 536 g L^{-1} h^{-1} (39% yield) and 499 g L^{-1} h^{-1} (37% yield) were obtained with *Z. mobilis* on polypropylene and plastic composite-supports of soybean hull–zein, respectively. For *Z. mobilis*, an optimal yield of 50% was observed at a 1.92 h^{-1} dilution rate for soybean hull–zein plastic composite-supports with a productivity of 96 g L^{-1} h^{-1}, whereas with polypropylene-supports the yield was 32% and the productivity was 60 g L^{-1} h^{-1}. With a *S. cerevisiae* fermentation, the ethanol production was less, with a maximum productivity of 76 g L^{-1} h^{-1} on the plastic composite-support at a 2.88 h^{-1} dilution rate with a 45% yield. Polypropylene-support bioreactors were discontinued due to reactor plugging by the cell mass accumulation. Support shape (3-mm chips) was responsible for bioreactor plugging due to extensive biofilm development on the plastic composite-supports. With suspension-culture continuous fermentations in continuously-stirred benchtop fermentors, maximum productivities of 5 g L^{-1} h^{-1} were obtained with a yield of 24 and 26% with *S. cerevisiae* and *Z. mobilis*, respectively. Cell washout in suspension-culture continuous fermentations was observed at a 1.0 h^{-1} dilution rate. Therefore, for continuous ethanol fermentations, biofilm reactors out-performed suspension-culture reactors, with 15 to 100-fold higher productivities (g L^{-1} h^{-1}) and with higher percentage yields for *S. cerevisiae* and *Z. mobilis*, respectively. Further research is needed with these novel supports to evaluate different support shapes and medium compositions that will permit medium flow, stimulate biofilm formation, reduce fermentation costs, and produce maximum yields and productivities.

Keywords: ethanol; biofilm; plastic composite-supports; *Zymomonas; Saccharomyces*

Introduction

Ethanol can be produced by chemical synthesis from petrochemical feedstocks or by microbial fermentation from renewable plant sources. The microbial production of ethanol was an important process prior to 1940, when chemical synthesis from petrochemical feedstock became more economical. Environmental concerns and possible future depletion of petroleum reserves, however, has revived an interest in ethanol fermentation. The use of ethanol as a fuel extender for vehicles in the United States has grown to nearly 900 million gallons in 1991 [8]. In 1988, 400 million bushels of corn were utilized in USA for ethanol production, adding $1 billion to farm income [19]. Current ethanol production costs by fermentation are less than $1.25 per gallon depending upon the process used and the feedstock costs.

One approach for improved production is the use of immobilized-cell bioreactors, which retain the biocatalyst (microorganisms) in the reactor as the substrate and product migrate through. Viable cells immobilized in solid gel matrices (ie calcium alginate) as beads have been studied in packed-bed and fluidized-bed reactors [21]. However, these systems have relatively low efficiency and find limited application due to diffusional resistance of substrate or product, rapid removal of CO_2 from the reactor, and limited microbial viability for long-term production of ethanol. Improving industrial fermentation productivity requires providing for increased production rates with reduced fermentor volumes and decreased operating costs.

Biofilms represent a natural form of cell immobilization that results from microbial attachment to solid supports [3]. This paper describes the use of biofilm reactors with plastic composite-supports for enhanced ethanol production. These novel supports stimulate cell attachment and biofilm development, and act as a slow release carrier of essential nutrients. Cost of production for plastic composite-supports is $US 2–3 per pound [14]. Ethanol productivities 10–100 times greater than those in suspension culture were obtained in the biofilm reactors for *Saccharomyces cerevisiae* and *Zymomonas mobilis*, respectively. For *Z. mobilis* a maximum productivity of 536 g L^{-1} h^{-1} with 39% yield was obtained by using polypropylene-supports. This is the highest productivity reported to date.

Materials and methods

Microorganisms and media

Zymomonas mobilis ATCC 31821 was maintained in a medium containing 2% (w/v) glucose, 0.5% (w/v) yeast

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extract (Difco Laboratories, Detroit, MI, USA), 0.2% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.2% (w/v) KH_2PO_4 at 4°C and was subcultured every 2 weeks at 30°C. *Saccharomyces cerevisiae* ATCC 24859 was maintained on a medium containing 2% (w/v) glucose, 1.0% (w/v) yeast extract (Difco) and 2% (w/v) peptone at 4°C and subcultured every 4–6 weeks at 30°C.

For *Z. mobilis*, the fermention medium consisted of 0.5% (w/v) yeast extract (Ardamine Z, Champlain Industries, Clifton, NJ, USA), 0.2% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.2% (w/v) KH_2PO_4 and 10% (w/v) glucose (pH 5.8). The medium used for *S. cerevisiae* fermentations contained 0.6% (w/v) yeast extract (Ardamine Z), 0.023% (w/v) $CaCl_2 \cdot 2H_2O$, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$, 0.15% (w/v) KH_2PO_4, 0.4% (w/v) $(NH_4)_2SO_4$ and 7.5% glucose (pH 5.8). Sixty liters of fermentation medium were sterilized at 121°C for 15 min in a 72-L B Braun U50 fermentor (B Braun, Allentown, PA, USA) and transferred aseptically to pre-sterilized 50-L carboys. The phosphate salts were autoclaved separately and added to the medium aseptically after sterilization and prior to dispensing the medium into carboys.

Support materials

Polypropylene composite-chips containing agricultural materials (25% w/w) were used as solid supports. The plastic composite-supports were prepared by high-temperature extrusion as described by Kunduru and Pometto [14]. Polypropylene (Type PP-8004ZR, Quantum USI Division, Columbus, OH, USA) was compounded with 20% ground soybean-hull (Iowa State University Center for Crops Utilization Research) and 5% zein (Sigma Chemical Co, St Louis, MO, USA), or 20% ground soybean-hull and 5% soybean flour (Archer Daniel Midland Co, Decatur, IL, USA), and extruded as rods 3 mm in diameter, which were air cooled and then cut into chips 2–3 mm in length with a pelletizer.

Bioreactors

A packed-bed reactor that approximated a trickling bed was custom made for *Z. mobilis* fermentation at 30°C. The reactor consisted of a cylindrical bulb condenser 600 mm long (Corning part No. 2420–600) filled with 140 ml (65 g) of polypropylene or plastic composite-supports of soybean hull–zein with a 25-ml working volume and a flow from top to bottom (Figure 1). A constant volume of 25 ml was maintained by adjusting the height of the liquid break in the exit line. The lower and upper ends of the condenser were fitted with 3-ml syringe plungers with the top pad perforated with holes, to retain the support materials within the reactor. Liquid breaks were used both in the feed and exit lines to prevent contamination when drawing samples or switching carboys. The microorganisms were inoculated into the reactor through a rubber septum in the stopper on the liquid break in the feed inlet.

A plug-flow bioreactor was custom made for *S. cerevisiae* fermentation with continuous aeration and at 30°C. The reactor column consisted of a cylindrical bulb condenser 400 mm long (Corning part No. 2420–400) filled with the 65 ml (18.5 g) of polypropylene or plastic composite-supports of soybean hull–soybean flour with a working volume of 30 ml. The reactor was similar in construction to the one described for *Z. mobilis*, but with the feed inlet from the bottom of the reactor (Figure 2). Filter-sterilized air was supplied to the reactors for *S. cerevisiae*. Continuous fermentations in duplicate were carried out for 60 days with dilution rates of 1.92 to 15.36 h^{-1} for *Z. mobilis* and 0.18 to 5.76 h^{-1} for *S. cerevisiae*.

A continuously stirred tank reactor (CSTR) (2 L Biostat M, B Braun) with agitation at 250 rpm and a 300-ml working volume was used for suspension-culture continuous fermentation. A Y-connector was placed on the exit line with one arm of the Y connected to the effluent exit line and the other arm left open in the reactor. The height of the Y-connector was adjusted to maintain a constant volume of 300 ml in the reactor. For *Z. mobilis* suspended-culture continuous fermentation, filter-sterilized nitrogen gas was continuously supplied (160 ml min^{-1}) to maintain an anaerobic environment. For *S. cerevisiae* suspended-culture continous fermentation, filter-sterilized air was continuously supplied (160 ml min^{-1}). Liquid breaks in the feed inlet and exit line were used to prevent contamination during sampling. The fermentors were operated at 30°C with dilution rates of 0.5 h^{-1} and 1.0 h^{-1} for a week. These continuous ethanol fermentations permitted a direct comparison of biofilm reactors to suspension-culture reactor (control) by using the same medium and microorganisms.

Continuous fermentation

Each reactor (packed bed with plastic supports or suspension culture) was inoculated with 1% (vol/vol) of the specific 24-h culture and incubated in batch fermentation at 30°C for 24 h, then changed to continuous fermentation with various dilution rates. Samples were collected from the exit line at 8-h intervals and analyzed for cell density, ethanol and glucose concentration. Dilution rates were routinely confirmed by measuring the exit volumes.

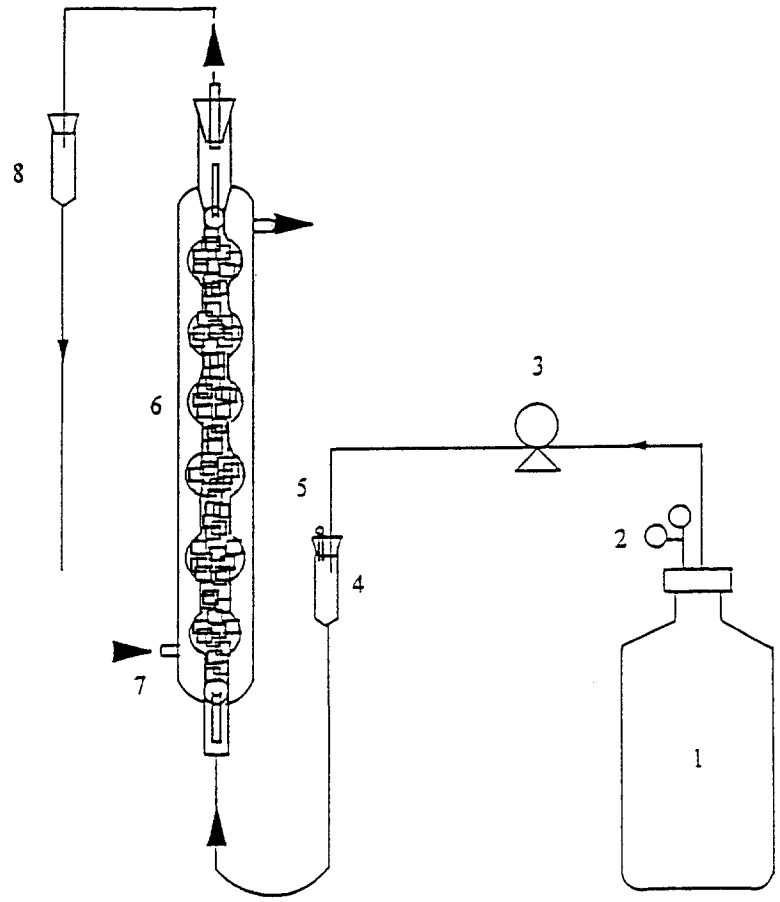
The residence time in the reactors was determined by injecting 1 ml of 1% aqueous solution of dextran blue dye (Sigma Chemical Co, St Louis, MO, USA) into the feed line of the fermentation medium. Flow rate was determined by collecting 10-ml samples until all the dye eluted from the reactor. The residence time was calculated by the fraction that had the most intense color as observed by absorbance at 540 nm.

Evaluation of the biofilm

The biofilm formed on the support material was evaluated visually by the accumulation of the cell mass on the chips and by Gram staining. Gram staining was performed on the supports after the fermentation, and the resulting color development was compared visually with the color of uninoculated Gram-stained supports [7]. Yeast cells were stained violet. Weight increase of the supports was not determined due to difficulty in removing the supports from the reactor.

Analysis of culture broth

The suspended-cell density in the reactors was measured by absorbance at 620 nm. The concentrations of glucose and ethanol were measured using a Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford,



1. Feed reservoir
2. Air filters
3. Pump
4, 8. Liquid breaks
5. Inoculation port
6. Bioreactor with supports
7. Water jacketed column

Figure 1 Schematic diagram of an upflow biofilm bioreactor used for continuous fermentation with *Z. mobilis*.

MA, USA) equipped with a Waters Model 401 refractive-index detector, column heater, autosampler and computer controller. The separation of ethanol, glucose and other broth ingredients was done on a Bio-Rad Aminex HPX-8711 column (300 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA, USA) by using 0.012 N sulfuric acid as a mobile phase at a flow rate of 0.8 ml min^{-1} with a 20-μl injection volume and a column temperature of 65°C.

Results and discussion

Continuous fermentation

For *Z. mobilis*, the reactors were operated at each dilution rate listed in Figure 3 for a week until the bioreactor achieved a dilution rate of 15.36 h^{-1} where it was operated for 5 days. It was difficult to maintain this high dilution rate because of the large consumption of medium. With a 10% glucose feed, there were 11 g L^{-1} of glucose, 34 g L^{-1} ethanol in the effluent, and a visible decrease in the biofilm. Thereafter, the fermentation was continued at a dilution rate of 7.68 h^{-1} for another 30 days.

With *S. cerevisiae*, the reactors were operated for one week at each of the dilution rates listed in Figure 4. At a dilution rate of 5.6 h^{-1}, a decrease in the visible biofilm and an overall reduction in ethanol production was observed. There were 30 g L^{-1} of glucose and 13 g L^{-1} of ethanol in the effluent with a 7.5% glucose feed. Therefore, the reactors were operated at a dilution rate of 2.8 h^{-1} for the next 30 days. The differences between replicates were within an average of 5% at each dilution rate.

Percentage yield

Percentage yield is a measure of the conversion efficiency of glucose to ethanol and is defined as ethanol produced

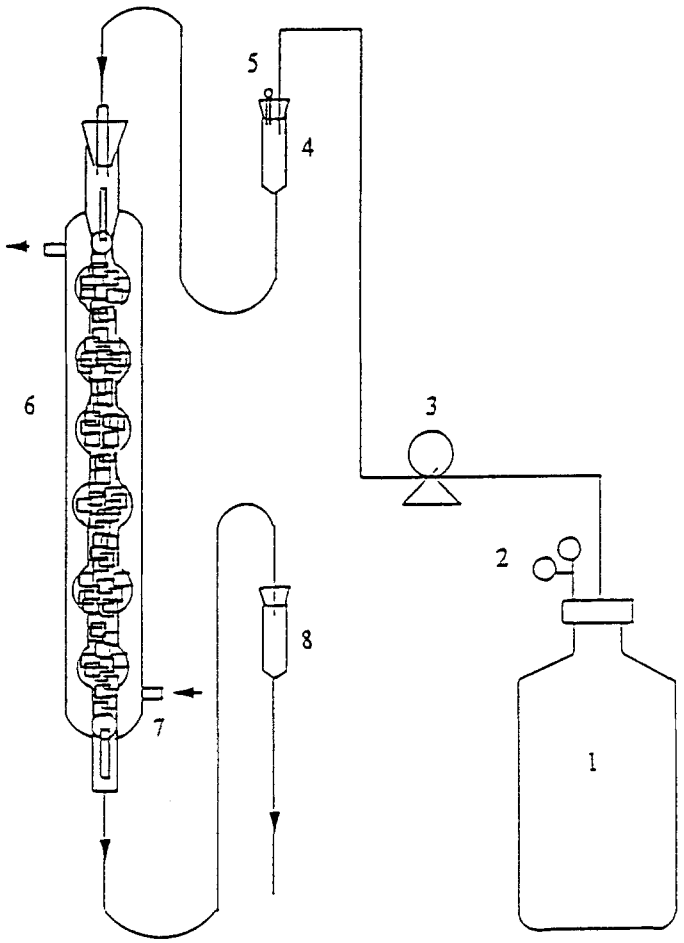


Figure 2 Schematic diagram of a downflow biofilm bioreactor used for continuous fermentation with *S. cerevisiae*.

divided by glucose consumed. The theoretical yield for ethanol production is 51% [20]. With *Z. mobilis*, the yield was 26% with suspension-culture continuous fermentation, whereas in the biofilm reactor the yields were 32–49% with polypropylene-support and 37–51% with plastic composite-supports of soybean hull–zein (Figure 3). The percentage yields for *Z. mobilis* were initially lower on plastic composite-supports during the first few days of fermentation but were comparable by the end of the first week of fermentation. With *Z. mobilis*, fermentation yields of 39 and 37% were obtained at a dilution rate of 15.36 h^{-1} with polypropylene-supports and plastic composite-supports of soybean hull–zein, respectively. Therefore, percentage yields were higher in the biofilm reactors than the suspended-culture reactors at all dilution rates evaluated even at the 15.36 h^{-1} dilution rate.

With *S. cerevisiae*, when plastic composite-supports of soybean hull–soybean flour were used, the percentage yields were 29 and 43% at dilution rates of 5.76 h^{-1} and 2.88 h^{-1}, respectively (Figure 4). A 24% yield was obtained with suspension-culture continuous fermentation of *S. cerevisiae* at a dilution rate of 0.5 h^{-1}. Therefore, at a dilution rate five-fold higher than the maximum (0.5 h^{-1}) for the suspension-culture, the percentage yields were higher in the biofilm reactors.

Ethanol productivity

Productivity (g L^{-1} h^{-1}) is a measure of ethanol production per hour (calculated as ethanol produced in g L^{-1} times the dilution rate in h^{-1}). Ethanol productivities were low in suspension-culture continuous fermentations for both *Z. mobilis* and *S. cerevisiae* at 5 g L^{-1} h^{-1}. Ethanol productivities of 76 and 40 g L^{-1} h^{-1} were obtained with *S. cerevisiae* on plastic composite-supports of soybean hull–soybean flour at dilution rates of 2.88 and 1.44 h^{-1}, respectively (Figure 4). For *S. cerevisiae* at a dilution rate of 5.76 h^{-1}, an ethanol productivity of 73 g L^{-1} h^{-1} was obtained with a 29% yield. Fermentation with *S. cerevisiae* in polypropylene-support reactors was not successful; excess cell mass plugged the reactor. This was attributed

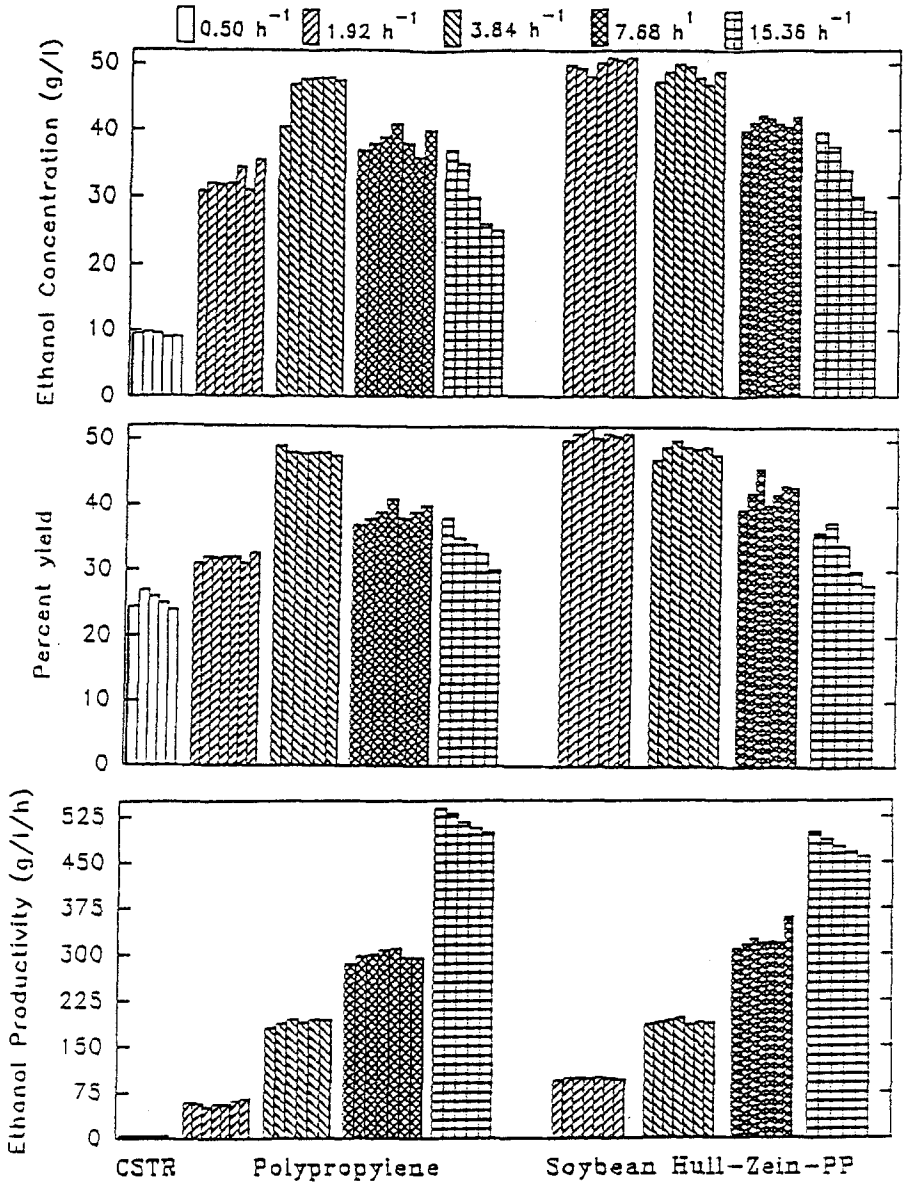


Figure 3 Ethanol concentrations, yield and productivities in downflow biofilm bioreactors used for continuous fermentation with *Z. mobilis*. Each data point represents the average value of two replicate bioreactors sampled every day. The fermentors were operated at each dilution rate for 5–7 days and increased sequentially.

to the support shape (2 to 3-mm chips) which interfered with yeast migration through the bioreactor. For *Z. mobilis* and *S. cerevisiae*, a complete washout was observed when the suspension-culture continuous fermentation was carried out at a dilution rate of 1.0 h^{-1}. For the *Z. mobilis* fermentation, a maximum productivity of 536 and 499 g L^{-1} h^{-1} was observed on polypropylene and plastic composite-supports of soybean hull–zein, respectively, at a dilution rate of 15.36 h^{-1}. These ethanol productivities are significantly higher than those reported in the literature [1,2,4–6, 9–18,21].

Benefit in ethanol production with biofilm reactors

Ethanol and glucose concentrations were analyzed from samples collected every 8 h to determine the steady-state condition. A steady-state condition was usually observed within 24 h of continuous fermentations at each of the dilution rates tested. The ethanol concentrations were consistently higher for plastic composite-support reactors for both *Z. mobilis* and *S. cerevisiae* than for suspension-culture continuous fermentation (Figures 3 and 4). The cell densities from the effluents of the bioreactor with plastic composite-supports were higher than the cell densities from the effluents of suspension-culture continuous fermentation (Figure 5). These data illustrate that the higher cell density present in the biofilm reactor (suspended-culture and immobilized-cells) corresponded to an increase in ethanol production. Furthermore, continued ethanol production with a corresponding increase in cell mass concentration in the bioreactor effluent at dilution rates above the washout for suspended-culture continuous fermentation confirmed biofilm formation. At these higher dilution rates, sus-

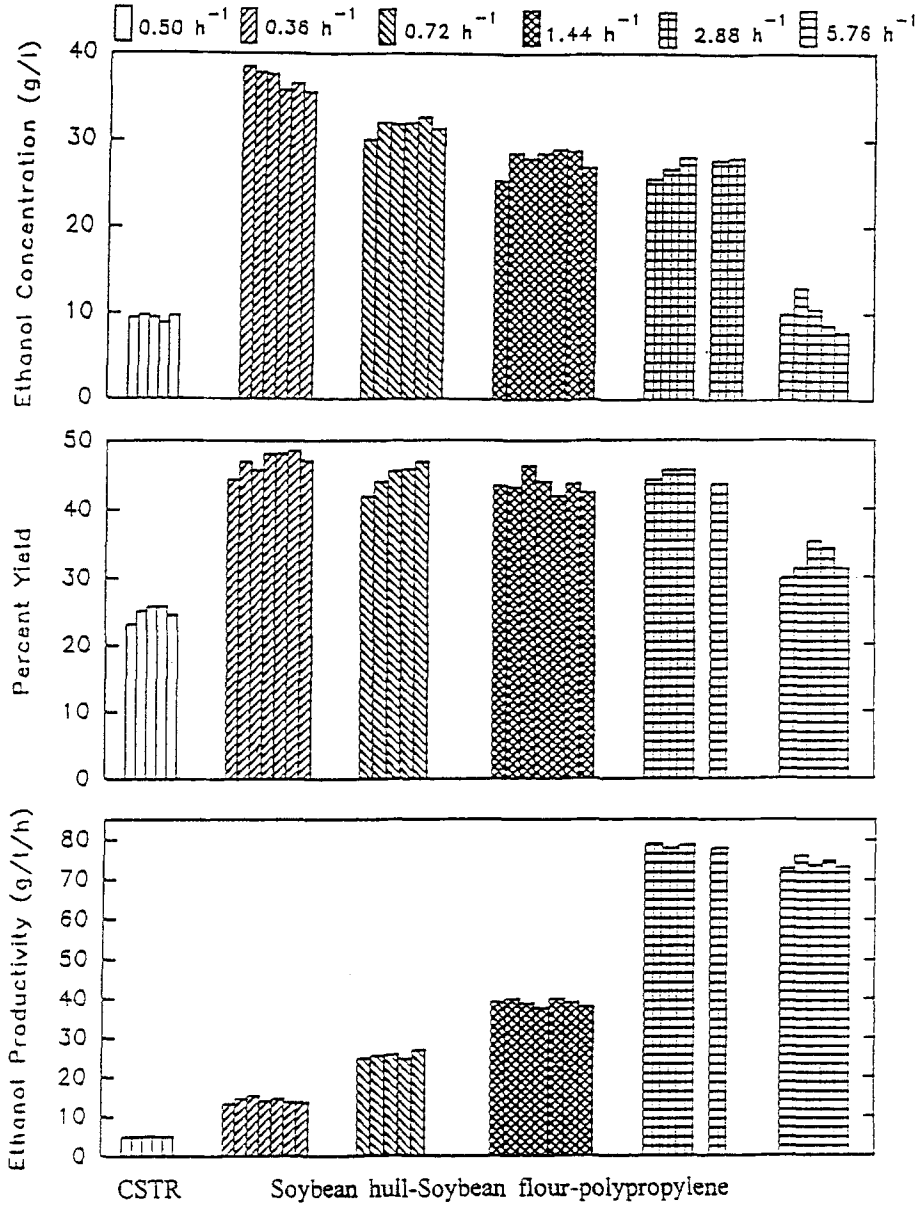


Figure 4 Ethanol concentrations, yield and productivities in upflow biofilm bioreactors used for continuous fermentation by *S. cerevisiae*. Each data point represents the average of two replicate bioreactors sampled every day. The fermentors were operated at each dilution rate for 5–7 days and increased sequentially.

pended-cell concentration increase or cell loss were linked with reduced percentage yields. In the biofilm reactors, a decrease in the visible biofilm was observed at dilution rates of 15.36 h^{-1} for *Z. mobilis* and 5.76 h^{-1} for *S. cerevisiae*. In the initial stages of the *Z. mobilis* fermentation, the ethanol concentrations were higher in the reactor with plastic composite-support of soybean hull–zein than in the reactor with polypropylene-alone supports. However, for *Z. mobilis*, within a week there was no appreciable difference in the ethanol concentrations between the plastic composite- and the polypropylene-support bioreactors. There was very little visually detectable biofilm formation on the polypropylene-supports in the initial stages of fermentation, which corresponded with the low cell densities in the effluent. However, for *Z. mobilis*, once cell mass started to accumulate due to bacterial aggregation in the trickle flow reactor, there was no difference in the performance of the two supports. The greatest ethanol concentrations (50 g L^{-1}) were obtained in the *Z. mobilis* fermentation by using the plastic composite-support of soybean hull–zein at a dilution rate of 1.92 h^{-1}. At the highest dilution rate tested (15.36 h^{-1}), the ethanol concentrations were 32 and 34 g L^{-1} in plastic composite- and polypropylene-support reactors, respectively. After 60 days of fermentation, the mean residence time was 7 min at a dilution rate of 7.60 h^{-1}. For *Z. mobilis*, the maximum ethanol concentration of 10 g L^{-1} was achieved in suspension-culture continuous fermentation with 100 g L^{-1} glucose feed and at a 0.5 h^{-1} dilution rate.

With *S. cerevisiae* the ethanol concentrations obtained were 28, 27, and 13 g L^{-1} at dilution rates of 1.44, 2.88 and 5.76 h^{-1}, respectively, on plastic composite-support reactors

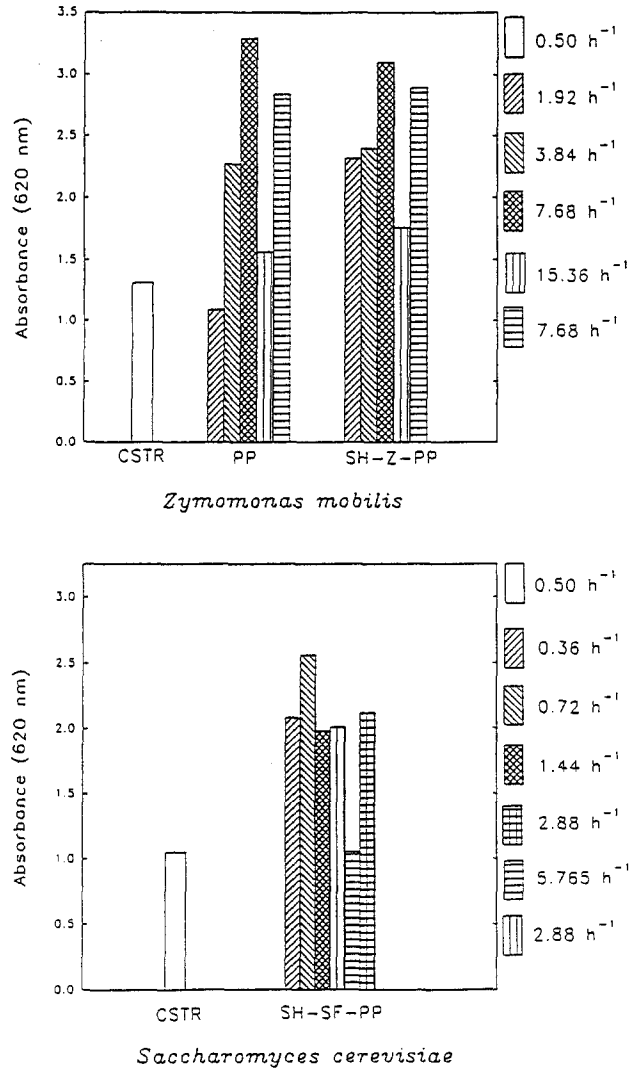


Figure 5 Absorbance (at 620 nm) of effluents from continuous fermentation reactors operated at different dilution rates. Each data point represents the average of two replicate bioreactors sampled every day.

of soybean hull–soybean flour. The mean residence times were 29 and 18 min at dilution rates of 1.44 and 2.88 h^{-1}, respectively. Even though the residence time was longer in the biofilm reactors with *S. cerevisiae*, the ethanol productivities obtained were lower than for the biofilm reactors with *Z. mobilis*. The ability to form better biofilms together with the higher glucose-uptake rates of *Z. mobilis* apparently resulted in higher productivities. For *Z. mobilis*, the mean residence time was 7 min at a dilution rate of 7.68 h^{-1}. For *S. cerevisiae*, a maximum ethanol concentration of 9.7 g L^{-1} was observed in suspension-culture continuous fermentation with 75 g L^{-1} glucose feed and a 0.5 h^{-1} dilution rate.

Support materials and bioreactor design

With *Z. mobilis* fermentations, the use of plastic composite-supports of soybean hull–zein resulted in high concentrations of ethanol and good retention of biomass in the early stages. This suggests that the soybean hull and zein in the composite-support provided some nutrients (proteins and amino acids) to *Z. mobilis* and that the plastic composite-support provided a unique surface for cell attachment. However, as this downflow fermentation progressed, the two supports (plastic composite-supports and polypropylene-alone supports) did not differ appreciably in their bioreactor effluent ethanol concentrations. We attribute this to the reactor and support shape (2 to 3-mm chips), which encouraged cell retention and which increased the back pressure and decreased the medium flow rate. Therefore, the actual flow rate was monitored and adjusted daily, to maintain the desired dilution rate.

In the *S. cerevisiae* fermentation, the polypropylene-support upflow-reactors plugged and resulted in excessive back pressure due to cell mass accumulation on the plastic support. The plugging of the bioreactor presumably was due to the flocculating characteristics of the yeast. In the preliminary studies, this phenomenon was not observed [14]. This may be because the flow rates were changed every day and the fermentation was performed for only 7 days; thus there was not enough time for the yeast to flocculate in the bioreactor. The plastic composite-support with the yeast, however, remained operational for 60 days, which

suggests that the agricultural material imparted some control of biofilm thickness. Support shape did contribute to bioreactor plugging.

Overall, the biofilm reactors for *Z. mobilis* and *S. cerevisiae* out-performed the traditional suspended-culture continuous fermentations in every aspect. However, reactor plugging significantly affects any long-term operation of this type of bioreactor. Therefore, different support shapes such as Raschig rings, or thin discs and rings need to be evaluated before scale-up of this process.

Acknowledgements

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