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Evaluation of plastic composite-supports for enhanced ethanol production in biofilm reactors

M Reddy Kunduru and AL Pometto III

Department of Food Science and Human Nutrition, Center for Crops Utilization Research, 2312 Food Science Building, Iowa State University, Ames, Iowa 50011, USA

Biofilms are a natural form of cell immobilization that result from microbial attachment to solid supports. Biofilm reactors with polypropylene composite-supports containing up to 25% (w/w) of various agricultural materials (corn hulls, cellulose, oat hulls, soybean hulls or starch) and nutrients (soybean flour or zein) were used for ethanol production. Pure cultures of *Zymomonas mobilis*, ATCC 31821 or *Saccharomyces cerevisiae* ATCC 24859 and mixed cultures with either of these ethanol-producing microorganisms and the biofilm-forming *Streptomyces viridosporus* T7A ATCC 39115 were evaluated. An ethanol productivity of 374 g L^{-1} h^{-1} (44% yield) was obtained on polypropylene composite-supports of soybean hull–zein–polypropylene by using *Z. mobilis*, whereas mixed-culture fermentations with *S. viridosporus* resulted in ethanol productivity of 147.5 g L^{-1} h^{-1} when polypropylene composite-supports of corn starch–soybean flour were used. With *S. cerevisiae*, maximum productivity of 40 g L^{-1} h^{-1} (47% yield) was obtained on polypropylene composite-supports of soybean hull–soybean flour, whereas mixed-culture fermentation with *S. viridosporus* resulted in ethanol productivity of 190 g L^{-1} h^{-1} (35% yield) when polypropylene composite-supports of oat hull–polypropylene were used. The maximum productivities obtained without supports (suspension culture) were 124 g L^{-1} h^{-1} and 5 g L^{-1} h^{-1} with *Z. mobilis* and *S. cerevisiae*, respectively. Therefore, for *Z. mobilis* and *S. cerevisiae*, ethanol productivities in biofilm fermentations were three- and eight-fold higher than suspension culture fermentations, respectively. Biofilm formation on the chips was detected by weight change and Gram staining of the support material at the end of the fermentation. The ethanol production rate and concentrations were consistently greater in biofilm reactors than in suspension cultures.

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

Introduction

Ethanol is a primary alcohol that can be produced by chemical synthesis from petrochemical feedstocks and by microbial fermentation of renewable plant products. Ethanol is used as a motor fuel additive, most commonly in a blend with gasoline known as gasohol. The chemical industry uses ethanol as a feedstock and as a solvent [1]. Ethanol is considered appropriate as a turbine fuel for peak load electric utilities requirements [29]. In the US, more than half of the denatured alcohol is sold as a solvent for nitrocellulose coatings, shellacs, inks, hydraulic fluids, liquid detergents, soaps, deodorants, perfumes, antiseptics and lotion. Undenatured ethanol is used by the cosmetic, pharmaceutical and food industries in the production of vitamins, flavors and essences, mouthwashes, blood products and fortified wines [13, 15], and as a growth substrate for single cell protein production [8].

Ethanol production costs by fermentation were less than $1.25 per gallon in 1992, depending on the process used and feedstock costs [13]. Raw materials cost up to 70% of the final price [23]. Conventionally, ethanol is produced by batch and continuous fermentations. To improve fermentation, several techniques have been investigated. These include vacuum fermentors [9], packed-bed tower fermentors [16], cell recycling [26], hollow fiber membrane reactors with recycling [6] and immobilization of cells [4, 7]. Various immobilization procedures such as covalent coupling, adsorption onto solid inert carriers, and entrapment in semi-permeable inert supports such as hydrogels, fibers and membranes were used. Supports such as κ-carrageenan gels [14], calcium alginate [17], ion exchange resins [19], vermiculite [3] and γ-alumina [18] have been used for cell immobilization. Viable cells immobilized in solid gel matrices (ie calcium alginate) as beads have been studied in packed-bed [31] and fluidized bed reactors [24, 28]. However, these systems have relatively low efficiency, and find limited applications due to the diffusional resistance of substrate or product, rapid removal of CO_2 from the reactor, and decreased microbial viability for long-term production of ethanol. Improving industrial fermentation productivity requires development of increased production rates with reduced fermentor volumes and decreased operating costs.

Biofilms are a natural form of cell immobilization that result from microbial attachment to solid supports [5]. Biofilms have been used in waste water treatment plants [20], for production of vinegar by the 'quick vinegar process', mineral ore treatment [8] and lactic acid production [11]. This paper describes the use of biofilm reactors that use plastic composite-supports for enhanced ethanol production. Our goal was to identify the plastic composite-support and culture combination best for long-term and for

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Correspondence: AL Pometto III, Department of Food Science and Human Nutrition, Center for Crops Utilization Research, 2312 Food Science Building, Iowa State University, Ames, Iowa 50011, USA
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more detailed studies. A three- and eight-fold increase in ethanol productivity was obtained in biofilm reactors containing plastic composite-supports with *Z. mobilis* or *S. cerevisiae* as ethanol producer, respectively. Fermentations were performed with *Z. mobilis* or *S. cerevisiae* as the ethanol producers in pure culture and with either of the ethanol producers and *S. viridosporus* as the biofilm-former in mixed-culture fermentations.

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 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 Laboratories) and 2% (w/v) peptone (Difco Laboratories) at 4°C and subcultured every 4–6 weeks at 30°C. Biofilm-former *Streptomyces viridosporus* T7A ATCC 39115 was maintained on 0.6% yeast extract agar slants at 4°C for 3–6 weeks at 37°C [21].

For continuous fermentation the medium for *Z. mobilis* had 0.5% (w/v) yeast extract (Difco), 0.2% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.2% (w/v) KH_2PO_4. Glucose concentrations of 10 and 12% (w/v) were used for pure- and mixed-culture fermentation, respectively. The medium used for yeast fermentations contained 0.6% (w/v) yeast extract (Difco), 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 and 0.4% (w/v) $(NH_4)_2SO_4$. The glucose concentration was 7.5% and 10% with pure- and mixed-culture fermentations, respectively. Phosphate salts were autoclaved separately and then added to the medium aseptically before fermentation was started.

Table 1 Composition of polypropylene composite-supports[a]

PP-composite chip	Major agricultural production (%)	Minor agricultural product (5%)
Polypropylene	–	–
Cellulose	25	–
Cellulose–soy flour	20	Soy flour
Cellulose–zein	20	Zein
Corn hull	25	–
Corn hull–soy flour	20	Soy flour
Corn hull–zein	20	Zein
Corn starch	25	–
Corn starch–soy flour	20	Soy flour
Corn starch–zein	20	Zein
Oat hulls	25	–
Oat hulls–soy flour	20	Soy flour
Oat hulls–zein	20	Zein
Soy hulls	25	–
Soy hulls–soy flour	20	Soy flour
Soy hulls–zein	20	Zein

[a]Seventy-five percent of each chip consisted of polypropylene.

Support materials

Plastic composite-supports containing agricultural materials (25% w/w) were used as solid supports (Table 1). The plastic composite-supports were prepared by high-temperature extrusion of the polypropylene (Type PP-8004ZR, Quantum USI Division, Columbus, OH, USA) and agricultural materials in a Brabender PL2000 with a counter-rotating twin-screw compounding extruder (CW Brabender Instruments, South Hackensack, NJ, USA) using the method of Demirci *et al* [11]. The barrel temperatures were 200, 210 and 220°C, the die temperature was 220°C and the screw speed was 20 rpm. The agricultural products used were cellulose (Sigma Chemical Co, St Louis, MO, USA), corn starch (American Maize-Products Co, Cedar Rapids, IA, USA), ground (20 mesh) oat hulls (National Oats Co, Cedar Rapids, IA, USA), soybean flour (Archer Daniel Midland Co, Decatur, IL, USA), ground (20 mesh) corn hulls (Penford Products Co, Cedar Rapids, IA, USA), and zein (Sigma). Each agricultural material was vacuum dried for 48 h at 110°C prior to extrusion. Polypropylene pellets and specific agricultural blends were mixed for several minutes in a container and then added to the extruder hopper. Polypropylene was compounded with different levels and blends of agricultural materials. The melted polypropylene was uniformly mixed with agricultural product by the counter-rotating movement of the twin screws and extruded as 3-mm diameter rods, air cooled and then cut into chips 2–

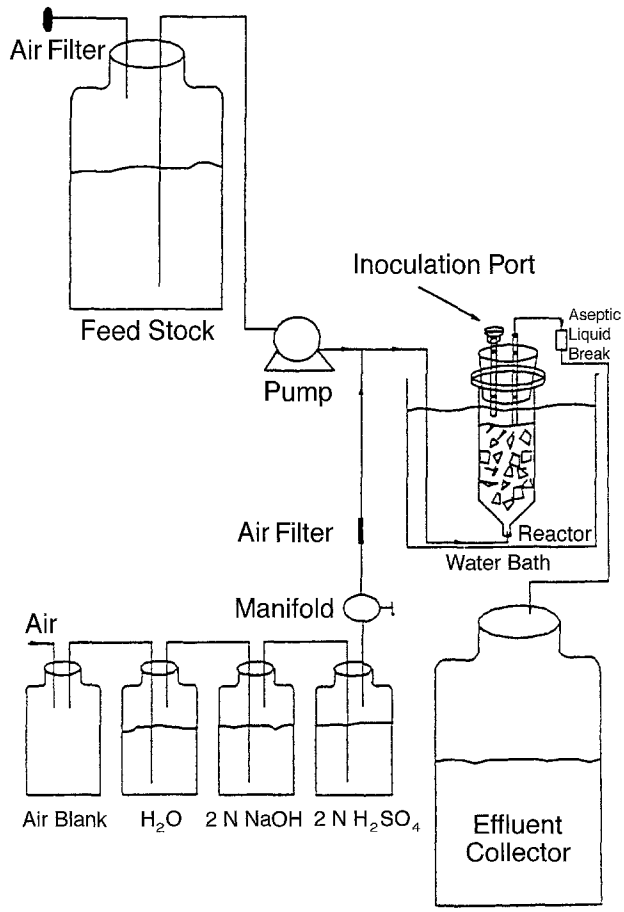

Figure 1 Schematic diagram of the experimental setup of biofilm reactor [11].

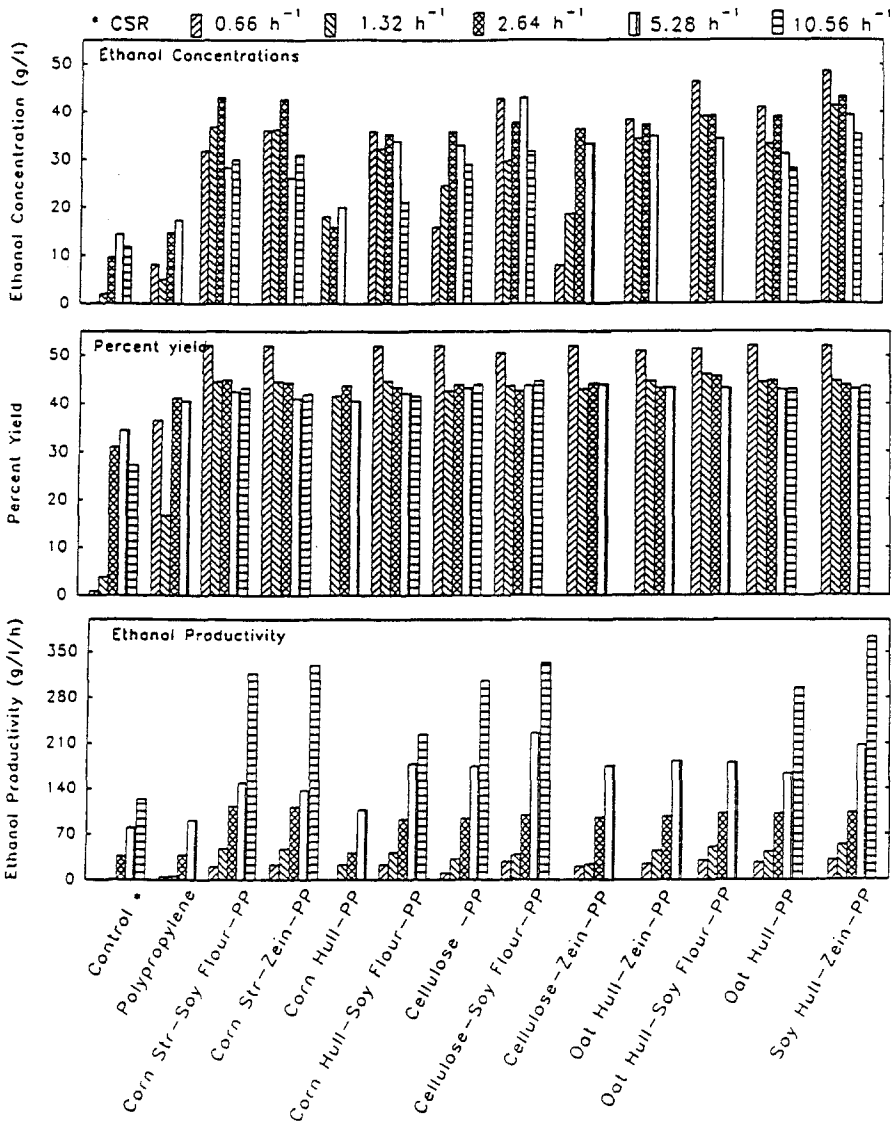

Figure 2 Ethanol concentrations, yield and productivity in pure-culture fermentation with *Z. mobilis* using 10% glucose feed at increasing dilution rates under anaerobic conditions.

3 mm in length with a pelletizer. Polypropylene extruded with protein-containing agricultural material was difficult to extrude and was charred by the high temperatures employed.

Evaluation of biofilm

The biofilm formed on each support material was evaluated at the end of each continuous fermentation by weight change, by clumping after drying at 70°C overnight, and by Gram staining the chips. After washing and drying each support in a flask, the final weight of each support was determined, then the supports were shaken vigorously to evaluate chip-clumping strength [12]. Supports with appreciable biofilm resisted separation, whereas supports within any biofilm formation separated easily. Gram staining was performed on supports after the fermentation, and the resulting color development was compared visually with the color of uninoculated Gram-stained supports.

Continuous ethanol fermentation

Fermentation was carried out in 60-ml plastic syringes with an estimated working volumes of 20 ml using the method of Demirci *et al* [11] (Figure 1). A 9-L carboy containing 4.5–6 L of sterile medium was fed into the syringe at its needle port. A T-connector in the feed line was used to supply filter-sterilized air for yeast and streptomycetes or nitrogen for other bacteria. The wide mouth of the syringe was fitted with a silicone stopper that contained two glass tubes. One port was covered with a septum and used for inoculation, and the other was used as an exit line. The system contained liquid breaks in the feed and exit lines to prevent contamination of the medium reservoir and the reactors during sampling or during changing of the medium for the mixed-culture fermentations. The syringe was filled with 50 ml (average weight of 18.65 g and density of 0.373 g ml^{-1}) of a plastic composite-support and was clamped tightly with the silicone stopper at the wide mouth

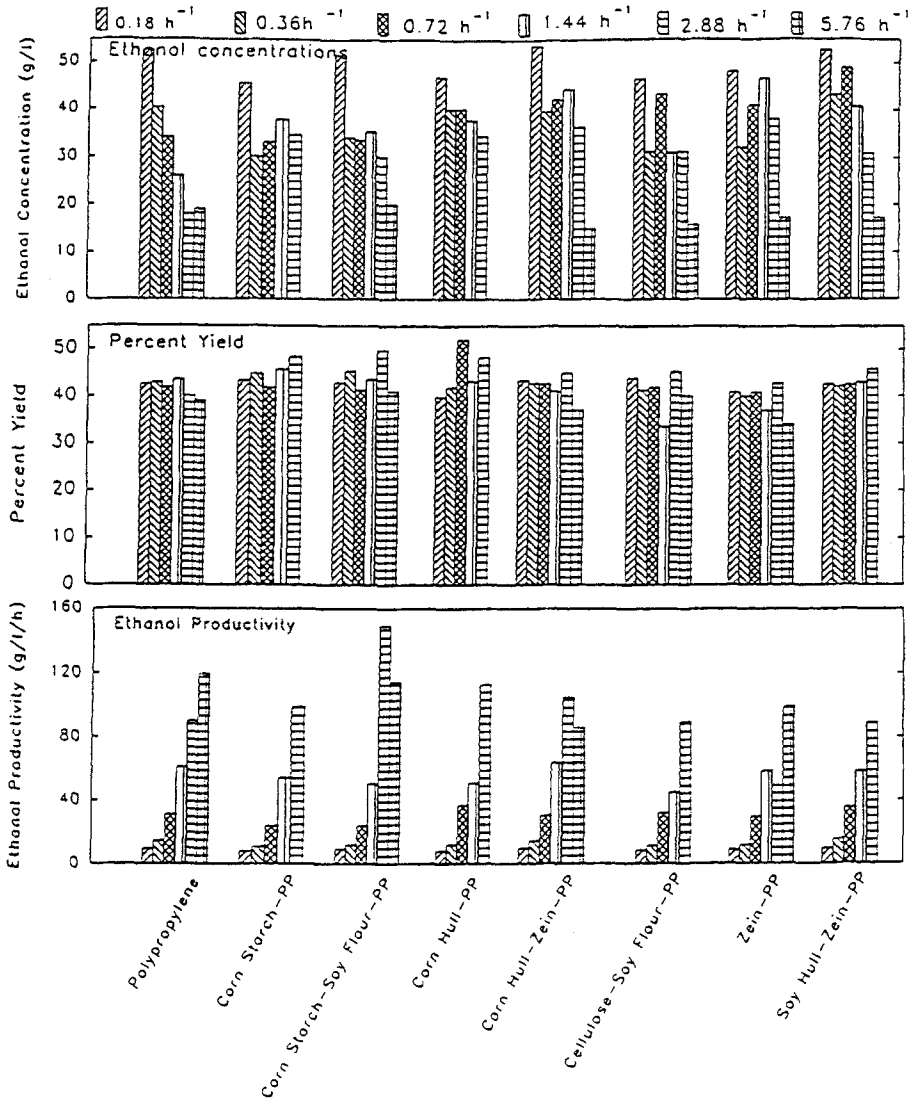

Figure 3 Ethanol concentrations, yield and productivity in mixed-culture fermentation with *Z. mobilis* and *S. viridosporus* with 12.5% glucose feed at increasing dilution rates under aerobic conditions.

end, then sterilized by autoclaving it at 121°C for 30 min. Specific culture medium was sterilized by autoclaving it at 121°C for 85 min and was then aseptically connected to each reactor. For mixed-culture fermentation, the reactors were inoculated with 1 ml of *S. viridosporus* spore suspension ($\sim 1.0 \times 10^9$ spores ml^{-1}). Each reactor was incubated in batch fermentation at 37°C for 24 h and then changed to continuous fermentation at a dilution rate of 0.18 h^{-1} for 10 days to develop a biofilm. The medium was switched, and the reactors were inoculated with 1 ml of the ethanol-producing bacterium or yeast and incubated at 30°C. A 24-h batch fermentation was followed by a continuous fermentation at various dilution rates (0.08, 0.36, 0.72, 1.44, 2.88, 5.76, 0.48, 0.96, 1.92, 3.84, 7.68, 0.66, 1.32, 2.64, 5.28, 10.56 h^{-1}). The fermentation was anaerobic when *Z. mobilis* was the ethanol producer [29]. Each dilution rate was maintained for 24 h, and samples were collected at 5 to 6-h intervals. The control reactor contained polypropylene supports in pure- and mixed-culture fermentations and no supports in pure-culture fermentations. Reactors without supports, with a working volume of 20 ml, each contained a magnetic stir-bar to prevent the culture from settling at the bottom for the *Z. mobilis* fermentation. Each continuously stirred reactor (CSR) was placed on a magnetic stir plate, and was suspended in a 30°C water bath.

Analysis of culture broth

The suspended cell density in the reactors was measured by absorbance at 620 nm. Percentages of glucose and ethanol were measured 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. 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) 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.

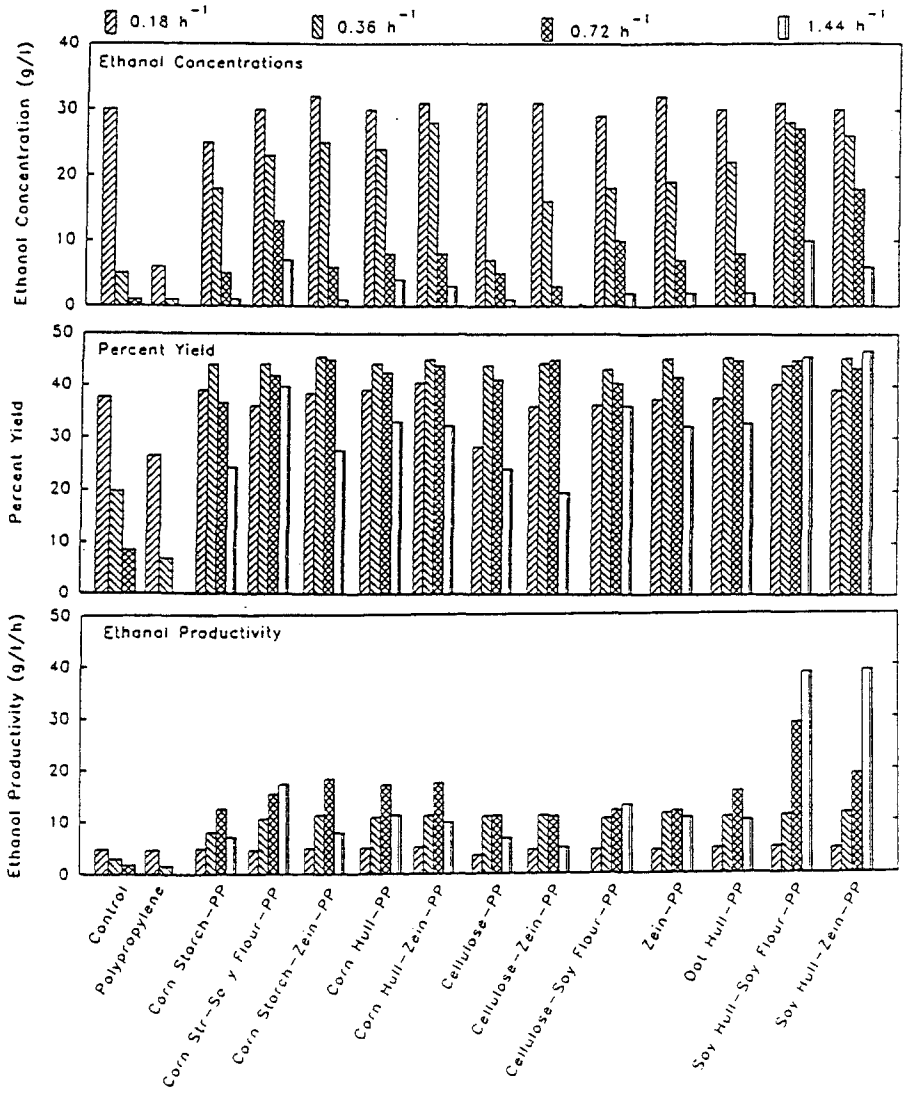

Figure 4 Ethanol concentration, yield and productivity in pure-culture fermentation with *S. cerevisiae* using 7.5% glucose feed at increasing dilution rates under aerobic conditions.

Results and discussion

Percentage yield

The percentage yield is a measure of the conversion efficiency of glucose to ethanol and is defined as ethanol produced divided by glucose consumed times 100. Theoretical yield for ethanol production is 51% [30]. The percentage yield for pure cultures of *Z. mobilis* ranged from 36 to 52% (Figure 2). Generally, the percentage yields were lower for mixed-culture fermentations (Figure 3) than for pure-culture fermentations at the same dilution rates, suggesting that the biofilm-former *S. viridosporus* utilized some of the glucose for cell maintenance and growth. There was no appreciable difference in the percentage yields among the various composite-supports tested. For *Z. mobilis* pure-culture fermentations the yields were consistently greater with the plastic composite-supports than the yields obtained from the controls with polypropylene alone or with suspension-culture fermentations at all the dilution rates tested. With the *Z. mobilis* mixed-culture reactors, there was no appreciable difference in the yields among the polypropylene alone and composite-supports. Biofilm formation by *S. viridosporus* on the polypropylene supports most likely helped to retain *Z. mobilis* in the bioreactor. A similar pattern was observed for pure- and mixed-culture fermentations with *S. cerevisiae* (Figures 4 and 5). The percentage yield for fermentation with pure- and mixed-culture of *S. cerevisiae* was much lower than that obtained with *Z. mobilis*, with ranges from 8 to 47% for pure-culture fermentation and from 15 to 38% with mixed-culture fermentation.

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 h^{-1}). In suspension-culture fermentations, ethanol productivity was very low for both *Z. mobilis* and *S. cerevisiae* (Figures 2 and 4). For *S. cerevisiae* with pure polypropylene supports, the productivity improved in mixed-culture fermentations. In plastic composite-support reactors, productivities were generally 4–9 times higher in pure-culture fermentations of *Z. mobilis* compared with

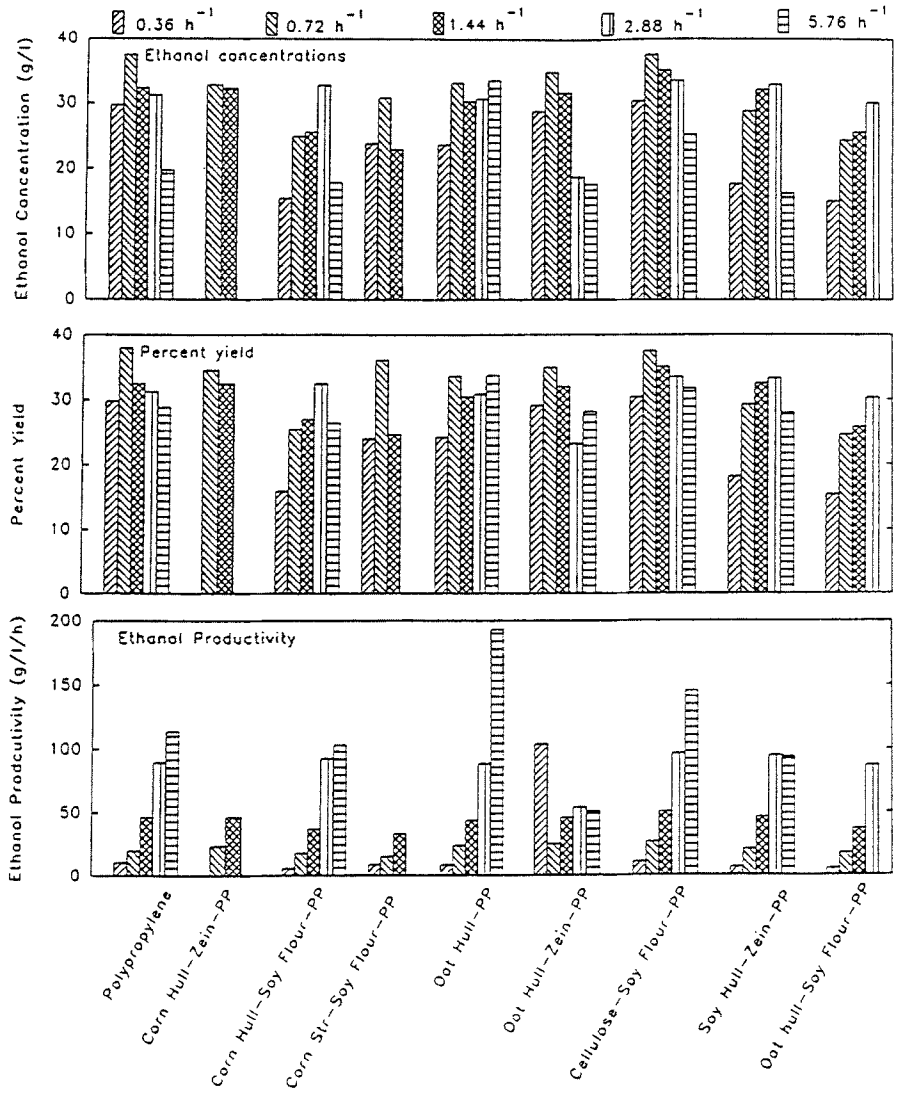

Figure 5 Ethanol concentrations, yield and productivity in mixed-culture fermentation with *S. cerevisiae* and *S. viridosporus* using 10% glucose feed at increasing dilution rates under aerobic conditions.

pure-culture fermentations of *S. cerevisiae*. Cell wash-out was not observed in any of the plastic composite-support reactors with *Z. mobilis* fermentation, even at the highest dilution rate of 10.56 h^{-1} (Figures 2 and 3). In plastic composite-support reactors, the productivities were much lower in mixed-culture fermentations of *Z. mobilis*, which could be caused by the continuous supply of air needed for the aerobic *S. viridosporus* to grow. Cell growth rate and ethanol productivity for *Z. mobilis* have been reported to decrease with increasing oxygen supply; ethanol productivity is more sensitive than the growth rate to oxygen supply [27].

In plastic composite-support reactors with *S. cerevisiae*, the productivities were higher in mixed-culture fermentations. Use of the plastic composite-support reactors with *S. cerevisiae* resulted in higher productivities than the suspension cultures or pure-polypropylene support reactors in both pure- and mixed-culture fermentations. For *Z. mobilis*, the highest productivity of 364 g L^{-1} h^{-1} was obtained in pure-culture fermentation with soybean hull–zein–polypropylene composite-supports (Figure 2), and a productivity of 149.5 g L^{-1} h^{-1} was obtained on corn starch–soybean flour–polypropylene composite material with mixed-culture fermentations (Figure 3). For *S. cerevisiae* plastic composite-support reactors, the highest productivity obtained with pure cultures was 40 g L^{-1} h^{-1} on composite supports of soybean hull–soybean flour–polypropylene (Figure 4). For *S. cerevisiae* plastic composite-support reactors, the highest productivity obtained with mixed-culture fermentation was 190 g L^{-1} h^{-1} on oat hull–polypropylene and 150 g L^{-1} h^{-1} on oat hull–soybean flour–polypropylene (Figure 5). These productivities are significantly higher than those reported in the literature (Table 2).

Table 2 Summary of immobilized-cell ethanol fermentations

Microorganism	Substrate	Max EtOH conc/productivity	Type of reactor or special technique	Ref
Saccharomyces cerevisiae	Glucose	190 g L^{-1} h^{-1}	Mixed-culture biofilm reactor with oat hull–PP as support material	This study
Saccharomyces cerevisiae	Glucose	40 g L^{-1} h^{-1}	Biofilm bioreactor with soy hull–soy flour–PP as support material	This study
Saccharomyces cerevisiae	Sugar cane juice	135 g L^{-1} in 8 h	Removal of toxic end products by high alcohols and activated carbon	[31]
Saccharomyces cerevisiae	Sugar cane water	42–53 g L^{-1}	Simultaneous extraction and fermentation	[26]
Saccharomyces cerevisiae	Glucose	18 g L^{-1} h^{-1}	Rice staw packed bed reactor	[10]
Saccharomyces uvarum	Non aseptic cane molasses	6.2 g L^{-1} h^{-1}	CSTR with five-stage system for substrate recirculation	[6]
Zymomonas mobilis	Glucose	13 g L^{-1} h^{-1}	Batch vertical rotating immobilized cell reactor	[2]
Zymomonas mobilis	Glucose	63 g L^{-1} h^{-1}	Continuous vertical rotating immobilized cell reactor	[2]
Zymomonas mobilis	Glucose	42–46 g L^{-1} h^{-1}	Cell reactor with trickle flow operation and sponge as packing	[22]
Zymomonas mobilis	Sucrose	92 g L^{-1} h^{-1}	Sugar conversion efficiency of 60% with 10% sucrose feed	[25]
Zymomonas mobilis	Glucose	364 g L^{-1} h^{-1}	Biofilm reactor with soy hull–zein–pp as support	This study
Zymomonas mobilis	Glucose	149.4 g L^{-1} h^{-1}	Mixed-culture biofilm reactor with corn starch–soy flour–pp as support	This study

Ethanol production

Ethanol and glucose concentrations for each dilution rate were analyzed from samples collected every 5–6 h over a 24-h period to determine the steady-state condition. Typically, a steady-state condition was observed after the first 10 h of continuous fermentation at each dilution rate tested. For both microorganisms, the ethanol concentrations were consistently higher for plastic composite-support reactors in both pure- and mixed-culture fermentations than for cell-suspension cultures or reactors containing pure polypropylene as support material. For *Z. mobilis* from soybean hull–zein, polypropylene alone and suspension culture reactors, the cell densities in the effluents of the composite supports with *Z. mobilis* showed an absorbance (620 nm) of 1.17, 0.18 and 0.10, respectively. This high cell density in the continuous fermentation effluent with plastic composite-supports indicates enhanced cell growth and a nutritional benefit to the bacteria from the support.

The agricultural material blends in the plastic composite-support reactors provide some essential nutrients to the microorganism and/or they provide a surface for cell attachment promoting biofilm development. Cell attachment was confirmed by the intense color of the Gram-stained harvested supports. There was also a 10–15% increase in the plastic composite-support's weight at harvest indicating cell attachment. Support materials from bioreactors illustrating the highest productivity for both microorganisms also demonstrated excellent clumping, weight gain and retention of color on Gram staining. Further long-term studies are needed to evaluate the performance of these composite-support materials for use in continuous fermentations.

Support materials benefits

Commercial production of plastic composite-supports is estimated to cost $US 2–3 per pound. In pure-culture fermentations with *Z. mobilis* the reactors containing plastic composite-supports of soybean hull–zein, corn starch–soybean flour, and cellulose–soybean flour yielded high concentrations of ethanol and good biomass retention. These results indicate that the nutrients (amino acids) supplied by soybean flour and zein in the composite-supports can provide a better environment for biofilm development. In mixed-culture fermentations with *Z. mobilis*, the reactors containing plastic composite-supports of oat hull–zein, and soybean hull–soybean flour resulted in better ethanol productivities. In pure-culture fermentation with *S. cerevisiae* plastic composite-supports of soybean hull–soybean flour, corn hull–zein, and soybean hull–zein performed better, whereas plastic composite-supports of oat hull–zein, and oat hull–soybean flour had a better performance in mixed-culture fermentation. In this study with the various plastic composite-supports and culture combinations, good yields and ethanol concentrations were not obtained for every material. Only the plastic composite-supports that performed better than the polypropylene-alone support will be investigated further.

These data suggest that the agricultural product in the composite-supports improved ethanol fermentation by stimulating biofilm formation, and by providing additional nutrients, thereby improving the rate of ethanol production. The presence of soybean hulls with soybean flour or zein resulted in good biofilm formation and in improved productivities with *Z. mobilis* and *S. cerevisiae* in pure-culture fermentation. Plastic composite-supports of cellulose with soybean flour or zein did not yield the same results. These results suggest that the presence of lignocellulosic material, which contain cellulose, hemicellulose, lignin and protein, was required for biofilm formation.

Z. mobilis is the preferred organism for use in biofilm culture reactors because of its productivity and its cell-aggregation characteristics. Mixed-culture fermentations with *Z. mobilis* did not improve ethanol productivity, but did reduce yields. Therefore, mixed-culture fermentations with *Z. mobilis* and *S. viridosporus* are not recommended

for use in biofilm reactors for ethanol production. However, mixed-culture fermentations may be considered with *S. cerevisiae* to obtain higher productivities but with greatly decreased yields. The type of plastic composite-support material used in a bioreactor depends on the microorganism(s) used, as demonstrated by the performance of different cultures employed in this research. Overall, these results indicate that a substantial gain in ethanol productivities can be achieved with biofilm bioreactors. Further research is needed to characterize the mechanisms by which plastic composite-support reactors can benefit ethanol fermentation by stimulating cell attachment and biofilm formation (cell immobilization), by performing as a slow release carrier for nutrients, and by enhancing suspended cell density in the bioreactors.

Acknowledgements

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