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Lactic Acid Production in a Mixed-Culture Biofilm Reactor†

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Novel solid supports, consisting of polypropylene blended with various agricultural materials (pp composite), were evaluated as supports for pure- and mixed-culture continuous lactic acid fermentations in biofilm reactors. *Streptomyces viridosporus* T7A (ATCC 39115) was used to form a biofilm, and *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) was used for lactic acid production. For mixed-culture fermentations, a 15-day continuous fermentation of *S. viridosporus* was performed initially to establish the biofilm. The culture medium was then inoculated with *L. casei* subsp. *rhamnosus*. For pure-culture fermentation, *L. casei* subsp. *rhamnosus* was inoculated directly into the reactors containing sterile pp composite chips. The biofilm reactors containing various pp composite chips were compared with a biofilm reactor containing pure polypropylene chips and with a reactor containing a suspension culture. Continuous fermentation was started, and each flow rate (0.06 to 1.92 ml/min) was held constant for 24 h; steady state was achieved after 10 h. Lactic acid production was determined throughout the 24-h period by high-performance liquid chromatography. Production rates that were two to five times faster than those of the suspension culture (control) were observed for the pure- and mixed-culture bioreactors. Both lactic acid production rates and lactic acid concentrations in the culture medium were consistently higher in mixed-culture than in pure-culture fermentations. Biofilm formation on the chips was detected at harvest by chip clumping and Gram staining.

Lactic acid and its derivatives have found many applications in the food and nonfood industries (6). In the nonfood area, polyesters of lactic acid can be made into degradable plastics with good tensile strength, thermoplasticity, fabricability, and biodegradability (9). Such plastics have an estimated potential yearly market of 9 billion lb (ca. 4 billion kg). Lactic acid can also be used as feedstock for the chemical and biological production of other organic acids such as propionic acids, acrylic acids, acetic acids, propylene glycol, ethanol, and acetaldehyde (9). In 1985, only 50% of the annual worldwide lactic acid production (24×10^6 to 28×10^6 kg) (15) was from renewable sources by fermentation. The rest was obtained from petroleum. The cost of lactic acid is currently $1.03/lb (50¢/kg) (10). If the price of lactic acid could be reduced, the market should expand substantially. Some methods that can be used to reduce the cost of production are microbial strain development, the design of novel bioreactors, and improved recovery processes.

Hollow-fiber, cell-recycled, artificially immobilized-cell, and biofilm reactors maintain high cell densities (4, 5, 7, 10, 12). Such reactors can generate increased volumetric productivity rates (g/liter/h). However, the use of hollow-fiber and cell-recycled fermenters is limited by high start-up costs and membrane fouling during the fermentation (10, 12). In reactors with artificially immobilized cells, production rates and yields are low because of limited diffusion rates, cell leakage, and poor cell reproduction in the beads (13). In biofilm reactors, microorganisms are immobilized by a natural attachment to solids while they continuously grow (16). Some industrial applications of biofilm reactors include biological oxidation or reduction of industrial wastes (8), Quick vinegar production (2), animal tissue culture (14), and ore treatments (2).

In this paper, the use of polypropylene chips blended with various agricultural materials as supports for biofilms was evaluated in continuous lactic acid fermentations with both pure and mixed cultures. These agricultural materials served

TABLE 1. Percent composition of pp composite supports[a]

Type of pp composite chip	% Agricultural product (wt/wt)	Minor agricultural product (5%)
Polypropylene		
Cellulose	25	
Cellulose-soy flour	20	Soy flour
Cellulose-zein	20	Zein
Corn fiber	25	
Corn fiber-soy flour	20	Soy flour
Corn fiber-zein	20	Zein
Corn starch	25	
Corn starch-soy flour	20	Soy flour
Corn starch-zein	20	Zein
Oat hull	25	
Oat hull-soy flour	20	Soy flour
Oat hull-zein	20	Zein
Soy hull	25	
Soy hull-soy flour	20	Soy flour
Soy hull-zein	20	Zein

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

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TABLE 2. Percent yield and productivity for lactic acid production by *L. casei* subsp. *rhamnosus*[a]

Flow rate (ml/min)	Results with the following chip:															
	Cellulose				Cellulose-soy flour				Cellulose-zein				Corn fiber			
	Pure		Mixed		Pure		Mixed		Pure		Mixed		Pure		Mixed	
	Yld[b]	Prod[c]	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	81.7	0.04	53.3	0.04	76.9	0.05	61.6	0.04	80.6	0.04	44.2	0.03	81.5	0.04	45.9	0.03
0.12	100	0.07	72.9	0.09	85.5	0.08	63.3	0.09	84.9	0.07	42.7	0.06	81.3	0.04	54.5	0.08
0.24	100	0.12	78.0	0.18	93.1	0.12	75.1	0.19	87.6	0.12	64.6	0.15	84.9	0.07	70.0	0.19
0.48	70.5	0.15	72.4	0.29	75.4	0.19	73.1	0.31	78.0	0.22	58.9	0.19	89.3	0.14	78.7	0.33
0.96	68.8	0.22	68.2	0.44	73.1	0.38	67.3	0.48	70.0	0.35	61.8	0.30	93.5	0.26	86.4	0.50
1.92	61.0	0.38	68.2	0.68	76.2	0.81	66.1	0.75	69.0	0.59	73.7	0.46	100	0.43	84.3	0.76

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as a carbon and nitrogen source on the support surfaces stimulating biofilm formation (4). These additives also changed the physical characteristics, making these supports more porous and absorptive for nutrients. These particular supports were selected after several other supports, like pea gravels, aluminum oxide spheres, and ceramic saddles, were screened (4). Biofilm formation on the chips was evaluated by clumping of the chips and Gram staining. The biofilm and lactic acid producers were *Streptomyces viridosporus* T7A and *Lactobacillus casei* subsp. *rhamnosus*, respectively. Increases of three- to fivefold in lactic acid production compared with the results of suspension culture fermentations were observed in selected biofilm reactors.

MATERIALS AND METHODS

Microorganisms and media. A biofilm producer, *S. viridosporus* T7A (ATCC 39115), was maintained on agar slants at 4°C for 3 to 6 weeks (11). The lactic acid bacterium *L. casei* subsp. *rhamnosus* (ATCC 11443) was maintained in MRS *Lactobacillus* broth (Difco Laboratories, Detroit, Mich.) at 4°C and subcultured every 4 weeks. For continuous fermentations, a 0.6% yeast extract medium (Difco) (pH 7) (11) and MRS *Lactobacillus* broth (Difco) (pH 6.5) were used for *S. viridosporus* T7A and *L. casei* subsp. *rhamnosus*, respectively.

Solid supports. Polypropylene composite (pp composite) chips containing 25% (wt/wt) agricultural materials were used as solid supports (Table 1). The pp composite chips were prepared by high-temperature extrusion of the polypropylene (Quantum USI Division, Rolling Meadows, Ill.) and agricultural materials (Table 1) in a Brabender PL2000 twin-screw extruder (C. W. Brabender Instruments, Inc., South Hackensack, N.J.). 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.), ground (20 mesh screen) corn fiber (Penford Products Co., Cedar Rapids, Iowa), corn starch (American Amize-Products Co., Chicago, Ill.), ground (20 mesh) oat hulls (National Oats Co., Cedar Rapids, Iowa), soybean flour (Archer Daniels Midland Company, Decatur, Ill.), ground (20 mesh) soy hulls (Iowa State University Center for Crops Utilization Research, Ames), and zein (Sigma Chemical Co.). Each agricultural material was vacuum dried for 48 h at 110°C prior to being used. Polypropylene was compounded with different levels and blends of agricultural materials, extruded as 3-mm-diameter rods, air cooled, and then cut into chips (2 to 3 mm in length). All chips compounded with protein were difficult to produce and were charred by the high temperatures employed.

Biofilm evaluations. The biofilm formed on the solid supports was evaluated by weight change, by clumping of the supports after they were dried at 70°C overnight, and by Gram staining of the pp composite chips at the end of continuous fermentations. After being dried in a flask for weight change information, supports were vigorously shaken to evaluate chip-clumping strength. In this assay, supports

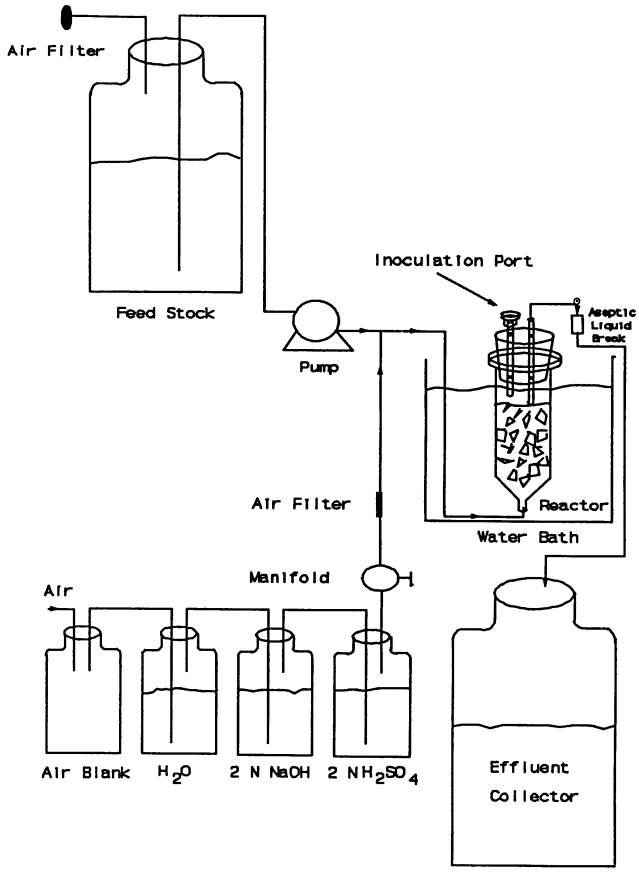

FIG. 1. Schematic diagram of biofilm reactor.

TABLE 2—*Continued*

Results with the following chip:																
Corn fiber-soy flour				Corn fiber-zein				Corn starch				Corn starch-soy flour			
Pure		Mixed		Pure		Mixe		Pure		Mixed		Pure		Mixed d	
Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
61.3	0.04	59.5	0.04	82.2	0.03	53.2	0.03	100	0.03	60.1	0.04	85.9	0.04	50.0	0.04
77.6	0.04	68.5	0.09	86.8	0.04	58.6	0.08	100	0.03	69.0	0.08	91.3	0.03	100	0.08
81.0	0.08	75.2	0.19	97.0	0.07	67.0	0.17	92.7	0.06	77.4	0.15	90.2	0.06	67.7	0.14
85.9	0.14	86.4	0.32	91.1	0.15	75.6	0.33	100	0.12	88.1	0.28	98.3	0.13	79.6	0.27
86.9	0.19	89.2	0.50	93.0	0.25	83.1	0.52	100	0.19	69.4	0.38	94.7	0.25	86.5	0.49
50.0	0.31	100	0.82	ND[d]	ND	ND	ND	100	0.26	100	0.87	100	0.39	88.0	1.04

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with good biofilm formation resisted chip separation, whereas supports with no biofilm formation separated easily. Gram stains developed on stained pp composite chips after fermentations were compared with those on uninoculated supports. For Gram staining, chips were stained by submersion, washed with alcohol-water until excess color was removed, dried at 70°C overnight, and evaluated visually for a blue color.

Continuous lactic acid fermentation. Fifty milliliters of pp composite chips was weighed and placed in a 50-ml-volume plastic syringe fitted with a silicone stopper. A 10-liter carboy containing 4 liters of specific medium for feeding was connected to a T-shaped tubing connector (Fig. 1). One arm of the T was connected by silicon tubing to the syringe at its hypodermic-needle port. The second arm of the T was connected to an air line fitted with a cotton plug to supply filter-sterilized CO_2-free air. The barrel mouth of the syringe was fitted with a silicon stopper that was penetrated by two glass connecting tubes. One tube was covered with a septum for bioreactor inoculation. The other tube was used as a medium exit line. The complete system was sterilized in an autoclave at 121°C for 1 h. After being cooled, the 50-ml reactors were placed in a water bath at 37°C. For mixed-culture fermentations, 0.6% yeast extract medium was pumped through the reactors containing sterile, dry pp composite chips. The addition of 1 ml of inoculum from a 24-h *S. viridosporus* suspension culture through the inoculum port was followed by a 24-h batch culture and 15 days of continuous fermentation with a flow rate of 0.06 ml/min at 37°C for biofilm formation. The medium was then changed to heat-sterilized MRS *Lactobacillus* broth, and each reactor was aseptically inoculated through the inoculum port with 1 ml of a 24-h *L. casei* subsp. *rhamnosus* culture which was incubated as a batch culture for 24 h at 37°C before being subjected to continuous fermentation. For pure-culture fermentation, only lactic acid bacteria were inoculated into the reactors containing pp composite chips. A reactor containing a 25-ml suspension culture was used as the control. Medium was pumped at various rates (0.06, 0.12, 0.24, 0.48, 0.96, and 1.92 ml/min). Each flow rate was held constant for 24 h. The pH, optical density (at 620 nm), percent lactic acid, and percent glucose in the effluents were analyzed every 4 or 5 h by using a pH meter, a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, N.Y.), and a Water's high-performance liquid chromatograph (HPLC) (Milford, Mass.) equipped with a Water's model 401 refractive index detector, respectively. The HPLC separation of lactic acid, glucose, and other broth constituents was achieved with a Bio-Rad Aminex HPX-87H column (300 by 7.8 mm) (Bio-Rad Chemical Division, Richmond, Calif.) with a 20-μl-volume injection loop and 0.012 N H_2SO_4 as a mobile phase at a flow rate of 0.8 ml/min.

RESULTS AND DISCUSSION

Percent yield. The percent yield is a measure of the efficiency of bioconversion of glucose to lactic acid (Table 2) and is defined as lactic acid produced (g/liter) divided by glucose consumed (g/liter). In these continuous fermentations, yields ranged from 44 (cellulose-zein; mixed culture) to 100%. For both pure- and mixed-culture fermentations, percent yield patterns were irregular, with the higher values usually correlated with the faster flow rates. Generally, percent yields were lower for mixed cultures than they were for pure cultures at the same flow rate. The biofilm former, *S. viridosporus* T7A, evidently consumed more glucose at slower flow rates, and this consumption apparently decreased at faster flow rates, resulting in the higher yields.

Productivity rates. The productivity rate (g/h) is a measure of lactic acid production per hour (Table 2). Productivity rates for several pure- and mixed-culture fermentations on pp composite supports were 2 to 3 times higher than those with the suspension culture reactor (control) at the same flow rates. The lower flow rates (0.06 and 0.12 ml/min) had productivity rates very close to those of the suspension culture, whereas the higher flow rates (0.24 to 1.92 ml/min) generally had significantly higher productivity rates for both the pure- and mixed-culture fermentations. With an estimated working volume of 25 ml, the highest volumetric productivities for all chips were about 30 g/liter/h, which agrees with results of a previous study involving *Lactobacillus helveticus* cells immobilized in calcium alginate (1).

Lactic acid production. Lactic acid concentrations were analyzed every 4 or 5 h for 24 h to determine the steady-state values. After 10 h of continuous fermentation, lactic acid concentrations were steady. Therefore, the lactic acid concentration measured at 24 h for each flow rate represented the lactic acid concentration at steady state for 14 h for each flow rate. Lactic acid levels were consistently higher for pp composite supports in both pure- and mixed-culture fermentations than for both the cell suspension culture and pure polypropylene, particularly at the three fastest flow rates (Fig. 2). Furthermore, mixed-culture fermentations with almost every support produced substantially higher levels of

TABLE 2—*Continued*

Flow rate (ml/min)	Results with the following chip:															
	Corn starch-zein				Oat hull				Oat hull-soy flour				Oat hull-zein			
	Pure		Mixed		Pure		Mixed		Pure		Mixed		Pure		Mixed	
	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	83.0	0.04	67.6	0.05	77.1	0.03	54.1	0.03	62.6	0.03	69.1	0.04	73.8	0.04	56.2	0.04
0.12	91.5	0.07	72.5	0.10	71.4	0.05	42.9	0.06	60.4	0.06	59.5	0.08	85.7	0.05	59.5	0.08
0.24	94.0	0.13	85.5	0.18	84.4	0.11	64.2	0.14	76.6	0.13	67.6	0.18	89.5	0.15	74.3	0.17
0.48	76.1	0.24	74.3	0.30	94.2	0.24	71.4	0.29	100	0.24	81.7	0.35	100	0.29	80.7	0.29
0.96	72.2	0.33	64.4	0.32	93.5	0.40	82.1	0.50	100	0.52	82.7	0.60	97.0	0.41	89.0	0.51
1.92	68.2	0.56	66.8	0.61	90.6	0.64	76.8	0.81	96.5	0.72	83.1	0.93	100	0.69	75.1	0.68

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product than did the corresponding pure-culture fermentations. These data illustrate the benefits of mixed-culture biofilm reactors for enhanced lactic acid production. Moreover, these results suggest that a higher cell density was present on each support for both pure- and mixed-culture fermentations, generating a net increase in lactic acid production.

Cell immobilization. Higher production rates in immobilized-cell cultures are the result of higher cell densities in the bioreactors (3). Compared with the starting pp composite chips, each chip Gram-stained after fermentation demonstrated an increased Gram-positive color density and a corresponding increase in clumping. These data, along with significant increases in lactic acid production when chips were used, strongly suggest that higher cell densities existed in each of the different pp composite support bioreactors. Furthermore, the benefit of the mixed-culture fermentation on cell immobilization is dramatically illustrated by comparison of the pure- and mixed-culture fermentations on pure polypropylene chips (Fig. 2). Finally, cell concentration, as determined by optical density in each reactor's effluent, indicated that suspended cells, in addition to cells contained in the biofilm, were continuously present. Both *Streptomyces* and *Lactobacillus* cells were observed in the mixed-culture effluent microscopic examination. However, all pp composite chips at harvest had weight losses of up to 15% depending on the chip type, probably because of agricultural material being leached or biodegraded.

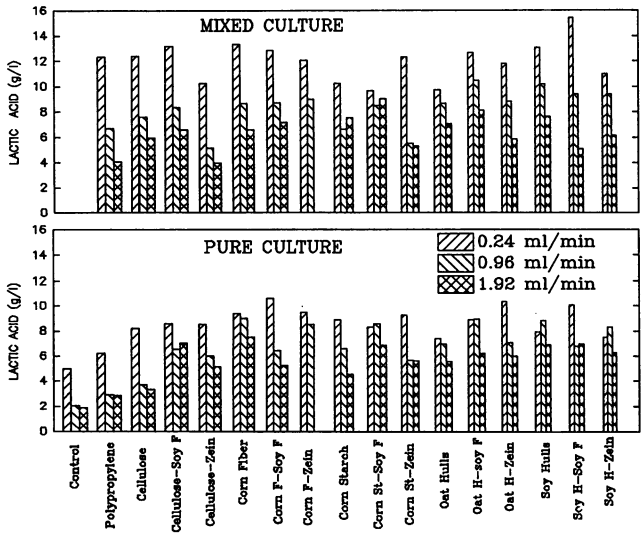

FIG. 2. Lactic acid concentrations at three flow rates on chips containing various agricultural materials compared with those at the same rates on the suspension culture and pure polypropylene chip controls. F, flour; St, starch; H, hulls.

The addition of soy flour or zein to the chips improved the retention of *L. casei* subsp. *rhamnosus* in the pure-culture reactors for cellulose and oat hulls (Table 2; Fig. 2). The addition of soy flour to pp composite chips generally enhanced lactic acid production for the mixed-culture fermentations. The composition of the chips seemed to play an important role in bioreactor performance, indicating a need to screen different microorganisms on various pp composite supports. Some of the added agricultural materials might be acting as a carbon and energy source which does not change the physical shape of the chips but might be the source of some micronutrients. However, the data suggest that certain agricultural materials have an affinity for specific bacteria, resulting in the formation of the desired biofilm. Finally, experiments involving several of the pp composite bioreactors were repeated, and every time very similar results were obtained. (Ranges for standard deviation were 0.2 to 1.0 g/liter for lactic acid and glucose concentrations in the effluents.)

Criteria for pp composite support selection. High lactic acid concentrations, production rates, and yields in the effluent, particularly at the faster flow rates, were the criteria used for choosing the best pp composite for pure- and mixed-culture reactors (Table 2 and Fig. 2). Oat hull-zein and oat hull-soy flour chips met these criteria for pure- and mixed-culture fermentations, respectively.

The biofilm-forming bacterium, *S. viridosporus* T7A, was definitely acting as a natural immobilizer of *L. casei* subsp. *rhamnosus*. Pure-culture interaction by *L. casei* subsp. *rhamnosus* with the different agricultural materials also resulted in cell immobilization and improved lactic acid production. Lignocellulosic materials (corn fiber, oat hulls, and soy hulls) generally performed better than single polymers (cellulose and starch). The productivity obtained with these biofilm reactors (30 g/liter/h) was similar to the observations with calcium alginate immobilized-cell reactors (1). Long-term studies which compare different immobilized-cell systems such as entrapped cells (calcium alginate immobilized cells) and biofilm reactors need to be done under pH-controlled conditions. Biofilm reactors have the potential of increasing production rates for many fermentations.

TABLE 2—*Continued*

Results with the following chip:																	
Soy hull				Soy hull-soy flour				Soy hull-zein				Polypropylene				Control[e] (pure)	
Pure		Mixed		Pure		Mixed		Pure		Mixed		Pure		Mixed		Yld	Prod
Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod		
77.9	0.03	58.6	0.04	83.9	0.04	63.0	0.04	78.4	0.03	69.1	0.04	100	0.02	62.2	0.04	78.3	0.03
69.6	0.06	61.7	0.09	88.9	0.07	67.9	0.08	77.1	0.06	60.6	0.09	85.0	0.04	67.8	0.09	86.0	0.05
83.5	0.11	73.3	0.19	98.6	0.14	87.5	0.22	85.5	0.11	71.4	0.16	96.1	0.09	83.4	0.17	86.8	0.07
88.3	0.27	83.5	0.33	90.6	0.27	75.8	0.28	97.8	0.24	100	0.31	96.1	0.18	90.1	0.25	100	0.06
90.5	0.51	87.2	0.59	73.6	0.39	100	0.54	95.8	0.48	88.9	0.54	100	0.20	82.6	0.38	100	0.12
93.6	0.80	89.5	0.88	69.9	0.60	68.3	0.58	100	0.72	95.2	0.71	90.8	0.33	86.1	0.47	100	0.22

[a] Percent yield and productivity were determined with pure- and mixed-culture fermentations with different pp composite chips.
[b] Percent yield (yld) was calculated as lactic acid produced (g/liter) divided by glucose consumed (g/liter) times 100%.
[c] Productivity (in g/h) is a measure of lactic acid production per hour (calculated as lactic acid produced [g/liter] times flow rate [liters/h]).
[d] ND, not determined.
[e] Control values represent the mean values of three replicate continuous fermentations for a 25-ml suspension culture of *L. casei* subsp. *rhamnosus*.

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