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Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production

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Abstract Novel plastic supports consisting of polypropylene blended with oat hulls/soybean flour or oat hulls/zein were evaluated as supports for mixed- and pure-culture, repeated-batch, lactic acid fermentations in biofilm reactors. *Streptomyces viridosporus* T7A (ATCC 39115) was used to form a biofilm for mixed-culture fermentations, and *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) was used for L-lactic acid production. The pure- and mixed-culture biofilm reactors were operated as repeated-batch fermentors with pH controlled at 5 for more than 2 months in which each reactor's medium was changed every 3 days for 24 batches. The plastic-composite supports performed better than polypropylene-alone supports. Significantly ($P<0.05$) higher concentrations of lactic acid were produced by the mixed- and pure-culture biofilm bioreactors with corresponding plastic-composite supports (55 g/l and 60 g/l respectively) than with polypropylene-alone supports (48 g/l for both mixed and pure culture). However, the percentage yields, maximum productivity, glucose consumption rates, and growth rates (based on the mass of suspended cells only) were not significantly different between reactors. Maximum lactic acid concentration was consistently greater for the plastic-composite support biofilm reactors. In the suspension culture at pH 5 without plastic supports, maximum lactic acid concentration at days 3 and 5 was 48 g/l and 60 g/l, respectively. These results confirm that the use of plastic-composite supports is recommended for pure-culture lactic acid production in long-term repeated-batch fermentation, and that cell immobilization was occurring.

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Introduction

Lactic acid is the smallest natural molecule to exhibit optical activity. It exists in two isomeric forms, D(–)-lactic acid and L(+)-lactic acid (Vick Roy et al. 1983). Lactic acid can be produced chemically from coal, petroleum, and natural gas, and biologically from the bioconversion of carbohydrates, agricultural and industrial wastes, and plant biomass (Vick Roy et al. 1983). It is used by the food industry and as a chemical feedstock for the production of other organic acids, polypropylyeneglycol, ethanol, acetylaldehyde, and degradable plastics. Polylactic acid (Lipinsky and Sinclair 1986) is a degradable plastic scheduled to go into commercial production in 1996 by Cargill.

In immobilized-cell bioreactors, the biological catalysts (microbial cells) are fixed into a matrix (artificial or natural), whereas the substrate and products, respectively, are constantly entering and leaving in the mobile phase. Accelerated microbial production rates and high concentrations of lactic acid can be achieved by strain development (Demirci and Pometto 1992) or by increased cell density in the bioreactor. Cell immobilization is one method used to increase the cell concentration in the bioreactor and to increase lactic acid production. In a continuous fermentation, Stenroos et al. (1982) immobilized *Lactobacillus delbrueckii* in calcium alginate, which produced 12 g/l lactic acid at a rate of 0.2 g l^{-1} h^{-1}. Tuli et al. (1985) immobilized *Lactobacillus casei* in polyacrylamide gels and obtained a maximum lactic acid concentration of 31 g/l with a 0.64 g l^{-1} h^{-1} production rate. Audet et al. (1988) used κ-carrageenan enriched with locust bean gum to immobilize *Streptococcus thermophilus, Lactobacillus bulgaricus,* or *Streptococcus lactis.* Some workers, however, have reported shrinkage and decreased strength of calcium alginate beads during lactic acid fermentation because calcium ions were displaced by lactate ions (Eikmeier and Rehm 1987; Roy et al. 1987). Lactic acid production is a type-I fermentation (Crueger and Crueger 1990) or growth-associated fermentation, which also

causes gel disruption. Batch-type fermentation is commonly used to produce lactic acid in industry. In batch fermentation, Lacroix et al. (1990) immobilized growing cells of *L. casei* in κ-carrageenan/locust bean gum for batch fermentation. They observed softening of the gel matrix after 17 h fermentation, prohibiting reuse of immobilized cells as inoculum in subsequent batch fermentation.

Biofilms are a natural form of cell immobilization. Biofilms consist of the natural attachment of microorganisms to an inert support (Characklis 1990), and are an essential component of the trickling filters of many waste-water treatment facilities (Characklis 1990). They have also been used for the production of acetic acid (Quick vinegar production), ethanol, and polysaccharides, and for metal ore leaching (Bryers 1990). Filamentous microorganisms like molds and actinomycetes will naturally stick to solid surfaces. There are also non-filamentous bacteria that will produce an extracellular polysaccharide, which acts as a natural glue immobilizing the parent cell and other microorganisms. This natural form of cell immobilization has the disadvantage of cell sloughing and little control of immobilized-cell thickness.

Demirci et al. (1993a, b) illustrated enhanced lactic acid production via pure- and mixed-culture biofilm continuous fermentations that used some novel plastic composite supports (patent pending). These supports consisted of a 25% (w/w) blend of agricultural material with micronutrients in polypropylene. In short-term (7-day) studies, pure- and mixed-culture continuous fermentations produced 30 g l^{-1} h^{-1} and 35 g l^{-1} h^{-1}, respectively. In this research, we examined the benefits of these plastic-composite supports in long-term repeated-batch fermentation. Significant increase in maximum lactic acid concentration was achieved for 3-day, repeated-batch fermentations when plastic-composite supports were used for pure- and mixed-culture biofilm reactors.

Materials and methods

Microorganisms and media

The lactic acid bacteria *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) were maintained in Lactobacillus MRS medium (Difco Laboratories, Detroit, Mich.) at 4° C and subcultured every 4 weeks (Demirci et al. 1993a). The biofilm forming *Streptomyces viridosporus* T7A (ATCC 39115) was maintained on yeast extract/malt extract/agar slants at 4° C for 3–6 weeks (Pometto and Crawford 1986).

Solid supports

The plastic-composite supports (2–3 × 3 mm) were prepared by high-temperature extrusion of polypropylene (Quantum USI Division, Cincinnati, Ohio) and agricultural materials in a Brabender PL2000 twin-screw extruder (C. W. Brabender Instruments Inc., South Hackensack, N.J.) as described by Demirci et al. (1993a). Any free materials associated with the plastic-composite supports were removed by sifting before use. The supports evaluated were polypropylene chips (bulk density: 0.51 g/ml) for a control, polypropylene composite chips (bulk density: 0.28 g/ml) containing 20% (w/w) ground (20 mesh) oat hulls (Ralston Purina, National Oats Co., Cedar Rapids, Iowa) and 5% (w/w) soybean flour (Archer Daniels Midland Company, Decatur, Ill.) for mixed-culture fermentations, and polypropylene composite chips (bulk density: 0.32 g/ml) 20% (w/w) containing ground oat hulls and 5% (w/w) zein (Sigma Chemical Co., St. Louis, Mo.) for pure-culture fermentations. On the basis of the previous studies of Demirci et al. (1993a) these plastic-composite supports were selected for mixed- and pure-culture fermentations. Polypropylene-alone supports (control) were also extruded and pelletized by using the same procedure.

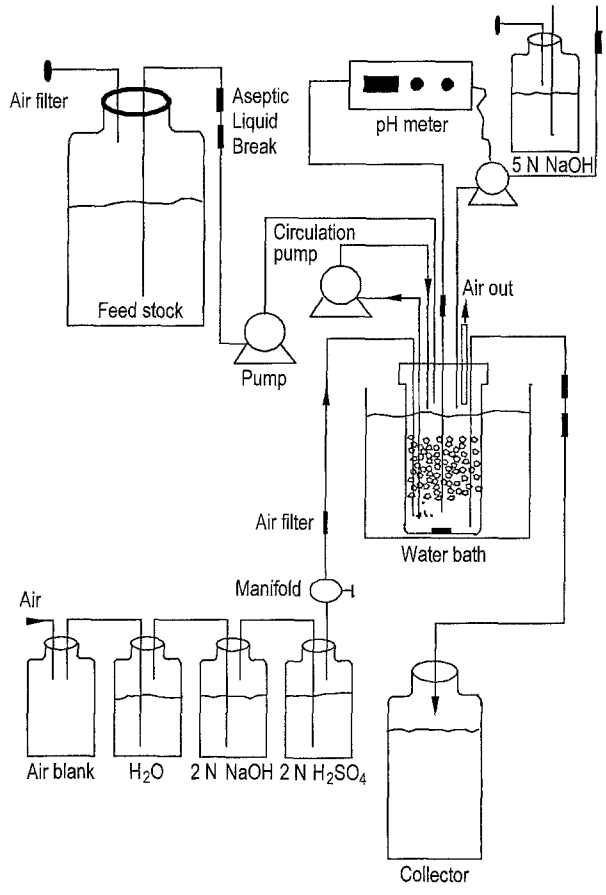

Fig. 1 Schematic diagram of batch reactor

Design and preparation of reactors for repeated-batch fermentation

Four custom-fitted 1800-ml Fleaker beakers (Corning Glass Works, Corning, NY) were constructed with circulation loop, base addition, aeration, sampling port, medium-feeding lines, pH probe, and magnetic stirring bar for mixing in each reactor (Fig. 1). The unit was operated in a 37° C water bath. In Fleaker beakers, 500 ml polypropylene or specific plastic-composite supports were heat-sterilized dry at 121° C for 75 min. The customized tops with various ports, tubing, and pH electrode were sterilized in a separate Fleaker beaker with water at 121° C for 20 min. In 20-l carboys each fitted with air filter and medium outlet feedline, 18-l culture medium was heat-sterilized at 121° C for 3.5 hours. After sterilization, each bioreactor was aseptically connected.

Repeated batch fermentations

Mixed culture

For mixed-culture fermentations, 500 ml 0.6% (w/w) yeast extract (Difco Laboratories, Detroit, Mich.) medium in nitrogen-free mineral salts solution [5.03 g Na_2HPO_4, 1.98 g KH_2PO_4, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.05 g $CaCl_2 \cdot 2H_2O$, plus 1 ml trace-element solution (Pridham and Gottlieb 1948) per l deionized H_2O; pH 7.1–7.2] was aseptically pumped into the reactors, which contained sterile polypropylene-alone (control) or oat hulls/soy flour plastic-composite supports. A 10-ml inoculum from a 24-h *S. viridosporus* suspension culture was added through the inoculum port. The reactors were continuously aerated with sterile CO_2–free air. The medium in the reactors was continuously recirculated from the bottom to top by a circulation pump at 75 ml/min. Every 3 days the medium from both reactors was drained and replaced with fresh medium for a total of 15 days. Culture growth and purity were checked by Gram staining and microscopic examination during the fermentation. The medium was then changed to 500 ml heat-sterilized lactic acid fermentation (LAF) medium [80 g glucose, 10 g yeast extract (Difco Laboratories), 0.6 g $MgSO_4 \cdot 7H_2O$, 0.03 g $MnSO_4 \cdot H_2O$, 1.0 g sodium acetate, 0.5 g K_2HPO_4, and 0.5 g KH_2PO_4 per l deionized water, pH adjusted to 5.0] and aseptically inoculated with 10 ml 24-h culture of *L. casei*. Mixed-culture reactors were continuously aerated with CO_2–free air throughout the study.

Pure-culture

For pure-culture reactors, 500-ml LAF medium was pumped into the reactors containing sterile polypropylene-alone (control), or oat hulls/zein plastic-composite supports. A reactor containing no supports (suspension culture) with a working volume of 500-ml was also prepared as another control. A 10-ml 24-h culture of *L. casei* was aseptically inoculated into each reactor. Pure-culture reactors were purged daily for 30 min with sterile CO_2 at 500 ml/min.

For reactors with and without supports, after 3 or 5 days incubation, respectively, the LAF medium was drained, and fresh LAF medium was pumped into each reactor. Batch fermentations with supports were repeated 24 times for a total of 72 days. For the suspension culture without support, batch fermentations were repeated 7 times for a total of 35 days. The pH in each reactor was maintained at pH 5 by addition of 5 M NaOH. Samples were taken twice a day for glucose, lactic acid, and cell density (absorbance 620 nm) measurements.

Sample analysis

Absorbance (620 nm), percentage lactic acid, and percentage glucose in the samples were analyzed by using a Spectronic 20 spectrophotometer (Milton Roy Co, Rochester, NY) and a Water's high-performance liquid chromatograph (HPLC) (Milford, Mass.), both equipped with Water's model 401 refractive-index detector. The HPLC separation of lactic acid, glucose, and other broth constituents was achieved on a BioRad Aminex HPX-87H column (300×7.8 mm; BioRad Chemical Division, Richmond, Calif.) by using a 20-μl injection loop and 0.006 M H_2SO_4 as a mobile phase at a flow rate of 0.8 ml/min at 65° C.

Maximum productivity (dp/dt), glucose consumption ($-ds/dt$), and growth rate (dx/dt; suspended cells only) were determined for each repeated-batch fermentation by linear regression calculation ($r^2 > 0.9$). Percentage yield was calculated by dividing the maximum lactic acid concentration (g/l) produced by the total glucose consumed (starting glucose minus residual glucose at medium change).

Statistical analysis

A one-way analysis of variance with Dunnett's method for comparison was used to determine significant differences ($P < 0.05$) between solid supports and different culture combinations by using the Sigma Stat software (Jandel Scientific, Corte Madera, Calif).

Results

All the plastic supports were submerged in the medium (Fig. 1). Continuous pH control was maintained in the lower medium zone. The external circulating pump maintained a steady flow of medium and free cells through the floating support. However, data fluctuations between repeated batches were evident (Fig. 2). This was probably the result of intermittent improper pH adjustment caused by occasional interruption in medium mixing when the magnetic stir bar made contact with the submerged tubing in the reactor. This improper mixing resulted in an overadjustment of pH by excess alkali addition. Furthermore, biofilm formation on the supports was most conspicuous for the plastic composite supports, but was also evident on the polypropylene-alone supports for both the mixed- and

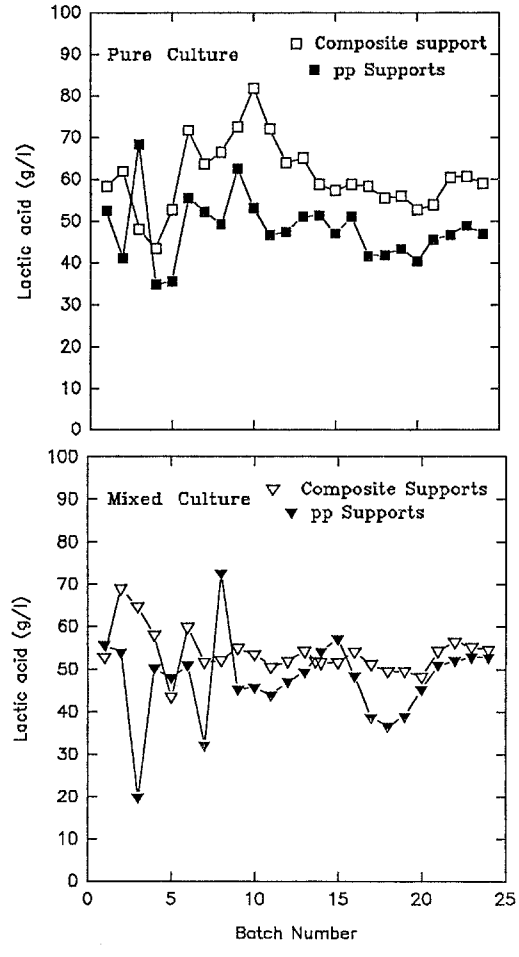

Fig. 2 L-Lactic acid production for 24 repeat-batch fermentations with a 3-day incubation for each batch. *pp* Polypropylene

Table 1 Physiological differences between the mixed- and pure-culture biofilm reactors

Description	Mixed culture		Pure culture		
	Oat hulls/soy flour[a]	Polypropylene[a]	Oat hulls/zein[a]	Polypropylene[a]	No supports[b]
Maximum lactic acid concentration (g/l)	55*,**±5.2	48*,**±9.9	60*±8.4	48*,**±7.6	60±11.2
Final glucose remained (g/l)[c]	8.1±6.1	20.6±10.2	14.8±14.6	20.6±15.5	25.2±20.6
Percentage yield[d]	76**±7.4	79**±8.18	77**±8.6	77**±10.6	87±5.7
Maximum productivity (dp/dt) ($g\,l^{-1}\,h^{-1}$)	0.75±0.18	0.69±0.22	0.81±0.19	0.70±0.22	0.65±0.22
Maximum glucose consumption rate ($-ds/dt$) ($g\,l^{-1}\,h^{-1}$)	1.0±0.23	0.91±0.34	1.04±0.33	0.84±0.29	0.90±0.40
Maximum growth rate (dx/dt) [g (dry weight) $l^{-1}\,h^{-1}$][e]	0.06**±0.049	0.09±0.059	0.03**±0.06	0.03**±0.060	0.15±0.12

* Significant difference ($P<0.05$) when compared within cultures and between composite supports (mixed versus pure culture)
** Significant differences ($P<0.05$) when compared with the suspension-culture results
[a] Mean of 24 repeat-batch fermentations with 3-day incubations each with standard deviations
[b] Mean of 7 repeat-batch fermentations as a suspension culture with 5-day incubations each with standard deviations. The mean lactic acid concentration at day 3 was 48 g/l (±15)
[c] Initial glucose concentration was 80 g/l
[d] Percentage yield was calculated dividing the maximum lactic acid concentration (g/l) by glucose consumed (g/l)
[e] Represents suspended cells in the effluent only

pure-cultures. Support clumping was evident in all reactors, which resulted in medium channelling and reduced substrate-to-cell contact. Therefore, mechanical stirring is recommended for mixing in future experiments, or an accelerated gas flow might better fluidize the supports.

For plastic-composite supports, maximum growth-rate values, which represent suspended cells and not immobilized cells, were low for the 3-day biofilm reactors (0.03–0.06 g l^{-1} h^{-1}) and were significantly higher for the 5-day suspension culture (0.15 g l^{-1} h^{-1}) (Table 1). Despite this reduced concentration of suspended cells and shorter fermentation time, lactic-acid production was the same or slightly lower for the biofilm reactors. This difference in suspended-cell growth rate confirms that a large portion of the bacterial cell-mass was naturally immobilized on the solid support especially on the plastic-composite supports.

In the suspension culture at pH 5 without plastic supports, the maximum lactic acid concentration at day 5 was 60 g/l (Table 1). On day 3, however, the average lactic acid concentration was 48 g/l (±15), which was similar to the value with the polypropylene-alone supports for the pure- and mixed-culture biofilm reactors. Furthermore, this 3-day mean value was significantly different ($P<0.01$) from that for the pure-culture bioreactors with the oat hulls/zein supports (60 g/l), confirming enhanced activity for the biofilm reactor. On the other hand, the yield for suspended cells is higher at 87% than those for biofilm reactors at 76%–79%. This difference might be directly related to the higher maximum productivities for the biofilm reactors of 0.69–0.81 g l^{-1} h^{-1} compared to the suspended cultures of 0.65 g l^{-1} h^{-1}.

Discussion

The pure- and mixed-culture biofilm reactors were run as repeated-batch fermentations for more than 2 months during which each reactor's medium was changed every 3 days for a total 24 times. The only operational difference between pure- and mixed-culture systems was aeration. For the mixed-culture bioreactor, continuous aeration was required because of the biofilm-forming *Streptomyces* requirement for O_2. From our previous study (Demirci et al. 1993a), lactic acid production by *L. casei* was not affected by aeration. *L. casei* is considered to be indifferent to oxygen but prefers an environment with a reduced oxygen tension. Furthermore, in the mixed-culture bioreactor, lactic acid bacteria potentially can locate in the *S. viridosporus* biofilm in an area with a reduced oxygen tension (Characklis 1990). For the pure-culture systems, CO_2 purging of the reactors kept the oxygen tension low and reduced support clumping. Gas bubbles in all reactors maintained each one in a semi-fluidized state. The suspension culture also was purged daily for 30 min with sterile CO_2 gas at 500 ml/min.

As predicted from our first study (Demirci et al. 1993a), plastic-composite supports performed better than polypropylene-alone supports. In fact, significantly ($P<0.05$) higher concentrations of lactic acid were produced by the mixed- and pure-culture bioreactors with plastic-composite supports than with polypropylene alone (Table 1). Goncalves et al. (1992) suggested that surface charge and pore size did not affect *Lactobacillus rhamnosus* attachment to different inert supports at low dilutions rates (0.3–0.48 h^{-1}). Agricultural material blended with the polypropylene probably stimulated biofilm formation on the support surface by serving as a carbon and/or nitrogen source, by pres-

enting a favorable surface energy, and/or by increasing the absorption of microorganisms to the solid supports. The percentage yields, maximum productivity, glucose consumption rates, and growth rates (based on the mass of suspended cells only) were not significantly different between reactors. However, productivities were greatest for the bioreactors with plastic-composite supports, illustrating a slightly improved rate for lactic acid production. *Streptomyces* and *Lactobacillus* cells, were also observed in their corresponding spent culture media for all 24 repeated-batch fermentations *via* Gram staining with microscopic examination. *Streptomyces* were always present in very low cell numbers.

In this study, the pure-culture bioreactor produced significantly ($p<0.05$) more lactic acid than did the mixed culture (Table 1). In our initial study (Demirci et al. 1993a), however, the mixed-culture biofilm reactors with plastic-composite supports consistently produced higher concentrations of lactic acid than did the pure-culture biofilm reactors. The purpose of the mixed-culture bioreactor was that the biofilm former would provide a matrix on the solid supports that would stimulate the immobilization of the lactic acid bacteria. This different observation could be due to the long-term nature of this current study (72 days) compared to the short-term nature of the initial study (7 days). Furthermore, the initial study was a continuous fermentation producing a maximum of 16 g/l (mixed culture), whereas this current study was a repeated-batch fermentation (3-day incubation) producing a maximum mean lactic acid concentration of 60 g/l (pure culture) and 55 g/l (mixed culture) with 14.8 g/l and 8.1 g/l residual glucose in the medium respectively (Table 1). No significant difference was observed in percentage yield.

These results suggest that the plastic-composite supports for pure-culture lactic acid production in repeated-batch fermentation can be repeatedly used for long-term fermentations. Similar concentrations of lactic acid were achieved in a 3-day pure-culture biofilm fermentation with plastic-composite supports and in a 5-day suspension-culture fermentation. This shortened fermentation time can significantly reduce production costs. Finally, all indications are that the cells are being naturally immobilized on these novel materials. The actual microbial attachment to these novel supports still needs to be characterized.

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