Nutrient Leaching and End Product Accumulation in Plastic Composite Supports for L-(+)-Lactic Acid Biofilm Fermentation†

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Investigations on the leachate bioavailability, leaching rate, and lactic acid accumulation properties of plastic composite supports (PCS) were essential for large-scale or long-term lactic acid fermentation. Leachates from PCS and polypropylene discs (controls) were analyzed by the micro-Kjeldahl method; by absorbances at 260, 275, and 280 nm; and by bioassays with *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443). The amount of leached nitrogen in a 20-ml initial soaking solution had a high correlation with the soaking solution's cell density ($r = 0.87$) and absorbance at 260 nm ($r = 0.95$). Leaching rates of various PCS were evaluated by 20 20-ml simulated repeated-batch fermentations (RBF). PCS with only yeast extract as the minor agricultural ingredient had a high leaching rate and leached out 51 to 60% of the total nitrogen during the first RBF. PCS blended with dried bovine albumin, dried bovine erythrocytes, and/or soybean flour had slowed nutrient leaching (20 to 30% of the initial leached nitrogen). Hence, they could still maintain 1 g of lactic acid per liter and measurable cell density (absorbance at 620 nm, 0.4 to 0.6) at the 20th 20-ml RBF. Lactic acid accumulation properties of PCS were evaluated by soaking the supports in a 30% lactic acid solution for 72 h at 45°C. The lactic acid-soaked supports were rinsed three times and then heat treated (121°C, 15 min) in 15 ml of deionized water. The results showed that lactic acid accumulation in PCS was mainly due to absorption and had no correlation with lactic acid production or biofilm formation.

Poly(lactic acid)-degradable plastics are polyester thermoplastics with properties similar to those of acrylics and polystyrene. Some of their applications are as disposable thermoplastic products (wraps, cups, and insulators), refuse and retail bags, and agricultural mulch film, which constitutes a potential market size of 600 to 800 million lb/year (4, 12). For competition in the commodity plastics market, the development of advanced and economic fermentation processes that yield a relatively pure lactic acid solution is required. A homofermentative *Lactobacillus* sp. is the typical production organism. *Lactobacilli* are fastidious organisms, and their growth requires a specific supply of complex nutrients such as vitamins, nucleotides, amino acids, and inorganic salts (5, 11). Hence, expensive complex nutrients such as yeast extract, when added to the culture medium, significantly increase the overall fermentation cost (12).

Biofilms are a natural form of cell immobilization by which microorganisms attach to solid surfaces (2, 8). Immobilized cells are used to increase the overall cell density in the bioreactor (6, 7). We recently confirmed lactic acid biofilm fermentation by using plastic composite supports (PCS) (50% [wt/wt] polypropylene [PP] and 50% [wt/wt] agricultural products) with *Lactobacillus casei* subsp. *rhamnosus* in minimal medium (MM). The results in that study indicated that besides being a biofilm support, the PCS was also a complex-nutrient carrier (10). Hence, methods for evaluating the PCS total complex nutrients, the bioavailability of the leached complex nutrients,

the rate of nutrient leaching, and the operational time of PCS as a complex-nutrient carrier are essential for PCS blend selection.

Lactic acid is known to have antimicrobial activity (9) and caused end product inhibition of lactate dehydrogenase, the enzyme that catalyzes the formation of lactic acid from pyruvate acid (17). The PCS were shown to be porous and permeable to liquid (10). This indicates possible lactic acid accumulation inside the PCS, which in turn might impede biofilm formation and/or lactic acid fermentation.

The study reported here demonstrated that accumulation of lactic acid inside the PCS was mainly due to absorption and that it had no impact on lactic acid production or on biofilm formation. In addition, PCS containing soybean hulls, soybean flour, yeast extract, and bovine albumin behaved as slow-release carriers of complex nutrients for lactic acid biofilm fermentation in an MM environment.

MATERIALS AND METHODS

PCS. Twenty-four blends of PCS [50% [wt/wt] PP and 50% [wt/wt] agricultural materials] in duplicate were produced by high-temperature extrusion by the procedures of Ho et al. (10). The PCS in the form of extruded tubes were cut into discs (3.5 mm thick and outer diameter of 10.5 mm). PP discs (controls) of similar size were bored out of a PP sheet with cork borers. The compositions and designations of the PCS are listed in Table 1 of reference (10).

Total nitrogen in PCS. A modified standard micro-Kjeldahl method of the Association of Official Analytical Chemists (920.39C) (1) was used to determine the total nitrogen in the unground soybean hulls, unground oat hulls, soybean flour, yeast extract, dried bovine albumin, dried bovine erythrocytes (RBC), and PCS. Two grams of PCS discs (0.2 g for agricultural material) was weighed and digested with 10 ml of concentrated sulfuric acid in the presence of copper(II) selenite dihydrate (Aldrich Chemical Company, Inc., Milwaukee, Wis.) and potassium sulfate (catalysts). The liberated ammonia was distilled and collected in a receiving flask containing 10 ml of 1% boric acid. The amount of ammonia in the distillate was determined by titration with 0.1 N HCl.

Nitrogen-leaching ability of PCS. Five grams of PCS or PP (control) discs was sterilized dry (45 min at 121°C), to avoid leaching any nitrogenous compounds during sterilization (15), in a 20-ml screw-cap culture tube in duplicate. Twenty

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TABLE 1. Protein contents of agricultural ingredients determined by micro-Kjeldahl analysis

Value	% Protein (dry wt basis) ^a in:					
	Soybean hulls	Oat hulls	Soybean flour	Yeast extract	Dried bovine RBC	Dried bovine albumin
Micro-Kjeldahl method	9.8	3.4	52.1	68.8	86.8	84.7
Published	8.8 ^b	1.6–5.7 ^c	54.0 ^b	68.0 ^d	90.0 ^e	80.0 ^e

^a Percent protein = percent nitrogen × 6.25^b Adapted from table presented by Pearson (13).^c Adapted from table presented by Youngs and Brown (19).^d Adapted from product specifications of Champlain Industries Inc. (Clifton, N.J.).^e Adapted from product specifications of American Protein Corporation (Manning, Iowa).

milliliters of sterilized MM (2% glucose and mineral salts solution) was aseptically added to the culture tubes, which were then incubated in a 37°C water bath for 48 h. The initial soaking solution was aseptically transferred into sterilized screw-cap culture tubes.

The initial MM soaking solution (10 ml) was retrieved from each culture tube. The amount of nitrogen present in the solution was determined by the modified standard micro-Kjeldahl method of the Association of Official Analytical Chemists described above. The initial leached nitrogen (percent, weight/volume) from the PCS was calculated by the following formula: percent initial leached nitrogen = nitrogen in 20 ml of initial soaking solution/total nitrogen in 5 g of PCS × 100. Another sample was retrieved from the remaining initial soaking solution for measurement (with appropriate dilution) of absorbance at 260, 275, and 280 nm with a model DU 50 UV-visible spectrophotometer (Beckman Instruments Inc., Irvine, Calif.). This was aimed at evaluating the amount of leachable aromatic compounds present in the soaking solution.

Bioassays of PCS leachate. Initial soaking solutions (5 ml) from the work described in the previous section were aseptically transferred into sterilized 20-ml screw-cap culture tubes for bioassays. Each culture tube was inoculated with 0.05 mL of active *L. casei* culture (10) and then incubated in a 37°C water bath for 48 h. The lactic acid produced, glucose consumed, and cell density (absorbance at 620 nm) in the bioassayed soaking solution were determined by methods described below.

Simulated RBF studies. An assay involving simulated repeated-batch fermentations (RBF) without pH control was developed to characterize the supports' nutritional benefits. Suspended cells and PP discs were used as controls. PCS or PP discs (5 g) were sterilized dry (45 min at 121°C) in 20-ml screw-cap culture tubes. The sterilized discs were aseptically transferred into a dilution bottle containing 20 ml of sterilized MM and incubated in a 37°C water bath for 24 h. This initial soaking solution was decanted aseptically.

Each dilution bottle was then refilled with 20 ml of sterilized MM, inoculated with 0.2 ml of active *L. casei* (18-h culture), and then reincubated in a 37°C water bath for 48 h. The first RBF medium was aseptically decanted, and 80 ml of fresh sterilized MM was refilled into the dilution bottle, thus simulating an equivalent of five 20-ml RBF. The decanting and refilling processes were repeated with three consecutive transfers in 100 ml of sterilized MM, which subsequently simulated 10, 15, and 20 20-ml RBF. Decanted fermented media from each simulated RBF were evaluated for lactic acid produced, glucose consumed, and cell density (absorbance at 620 nm).

Viable biofilm population on the supports. Preliminary data on some PCS blends showed that the biofilm population in minimal medium with no pH control was similar for 20 batches of RBF. Hence, only the PCS biofilm population of the first RBF was evaluated by the stripping-sand method (10).

Analysis of bioassayed and fermented media. L-(+)-Lactic acid and D-glucose concentrations were analyzed by Waters high-performance liquid chromatography by procedures described by Ho et al. (10). Bacterial growth in lactic acid fermentation medium was monitored by measurement of the absorbance at 620 nm with a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, N.Y.).

Assay of lactic acid accumulation. Screw-cap culture tubes (in replicates of three) with weighed PCS discs were sterilized dry (45 min at 121°C) along with controls. Controls included culture tubes with no PCS discs and culture tubes containing an equivalent weight of filter papers, unground oat hulls, unground soy hulls, or PP discs. Each culture tube was filled with 20 ml of sterilized deionized water and incubated in a 45°C water bath for 72 h to hydrate the materials inside the culture tubes and to solubilize any readily leachable material from the PCS and hulls. The initial residual soaking-solution volume was measured and discarded. The difference in the soaking-solution volume was regarded as the total liquid absorbed by 5 g of dried PCS discs.

A sterilized 30% L-(+)-lactic acid solution (LAS) (30% lactic acid, 0.4% yeast extract, 2% glucose, and mineral salts solution) was dispensed into screw-cap culture tubes (15 ml for each tube) with soaked materials. The screw-cap junctions were wrapped with Parafilm (to prevent moisture loss due to evaporation), and the culture tubes were subsequently incubated in a covered water bath at 45°C for 2 weeks.

After incubation, the 30% LAS in each culture tube was decanted, and the

supports in each tube were rinsed three times in 15 ml of deionized water. The 30% LAS and the rinsed solutions from each tube were collected in a 100-ml volumetric flask. The final flask volume was made up to 100 ml with deionized water. The lactic acid concentration in the 30% LAS after treatment was regarded as the final residual lactic acid not absorbed by the materials, whereas the lactic acid concentration in the culture tube without supports or materials was treated as the initial 30% LAS lactic acid concentration.

Treated and washed materials from each tube were subsequently transferred to a 100-ml dilution bottle containing 15 ml of deionized water and were autoclaved (15 min at 121°C) in an effort to release the absorbed lactic acid. The L-(+)-lactic acid concentrations in the initial 30% LAS, the final residual 30% LAS, and the hot-water treatment solution were determined by high-performance liquid chromatography. The interstitial volume of each type of support was estimated by the following formula:

interstitial volume (ml) =

$$\frac{\text{lactic acid absorbed in the PCS (mg)}}{\text{initial 30\% LAS lactic acid concentration (mg/ml)}} \quad (1)$$

Statistical analysis. Two half sets of a five-factorial fractional design (2^{5-1}) (3) were used to evaluate the effects of different agricultural ingredients on the bioavailability of leached nutrients, the nutrient-leaching rate, and the interstitial volume of the PCS discs. The experimental design of the study followed that of Ho et al. (10). The data from each test were analyzed by analysis of variance (ANOVA) with the Statistical Analysis System package (version 6.03) (SAS Institute, Inc., 1985).

RESULTS AND DISCUSSION

PCS total nitrogen. The percentages of protein in the agricultural materials determined from the micro-Kjeldahl analysis were similar to the values obtained from the literature (13, 19) and product specification data sheets (Table 1). Soybean hulls had a greater protein content than oat hulls. Among the minor agricultural ingredients, dried bovine RBC and dried bovine albumin had greater protein contents than did yeast extract and soybean flour. The PCS total nitrogen (milligrams per gram) obtained from micro-Kjeldahl analysis matched the sum of the nitrogen contents of the agricultural ingredients ($r = 0.96$) (Fig. 1), thus indicating that most of the protein was retained in the PCS after the extrusion process.

Bioavailability of PCS leachate. The leachate from PCS discs was evaluated by measuring the soaking-solution absorbance at 260, 275, and 280 nm. The selection of these wavelengths was based on the compounds expected to be leached out from the PCS. The 260-nm wavelength corresponds to the λ_{max} of L-phenylalanine (14), riboflavin (18), and some lignin-derived aromatic compounds such as vanillic acid (16). The 275-nm wavelength corresponds to the λ_{max} of L-tyrosine (14), cobalamin (18), syringic acid, guaiacol, and catechol (16). The 280-nm wavelength corresponds to the λ_{max} of L-tryptophan, folacin (18), vanillin, and veratraldehyde (16). Hence, the absorbances at these wavelengths could indicate the concentration or presence of nutrients such as aromatic amino acids (nitrogenous compounds) and water-soluble vitamins or of the

TABLE 2. Correlation coefficients for the soaking-solution absorbances at 260, 275 and 280 nm with the amount of leached nitrogen in the soaking solution, the percentage of nitrogen leached from the PCS, and the cell density of *L. casei* in the soaking solution

Wavelength (nm)	Correlation coefficient (<i>r</i>) with:		
	Leached nitrogen in 20 ml of initial soaking solution ^a	% of initial nitrogen leached ^b	Cell density in 100 ml of initial soaking solution (<i>A</i> ₆₂₀)
260	0.95	0.82	0.86
275	0.92	0.80	0.82
280	0.90	0.76	0.81

^a Nitrogen concentration determined by micro-Kjeldahl analysis.

^b Percent initial leached nitrogen = nitrogen in initial soaking solution (mg)/total nitrogen in 5 g of PCS (mg) × 100.

Bioassays of the PCS soaking solution showed that the cell density and lactic acid concentration of the initial soaking solution had a correlation of 0.80 to 0.86 and 0.70 to 0.76, respectively, with the soaking-solution absorbance at 260, 275, and 280 nm (Table 2). This indicated that sufficient complex nutrients were being released from the PCS to support the growth of *L. casei* in MM.

For PCS with hulls alone as the agricultural ingredients (O+ and S), negligible lactic acid concentrations were detected, although an increase in cell density was observed (Fig. 3). In contrast, as shown by SAS ANOVA, yeast extract significantly increased the cell density of the soaking solution in the bioassay analysis ($P < 0.0001$). This strongly suggested that hulls alone partly met the complex-nutrient requirements of *L. casei* and that the presence of minor complex ingredients such as yeast extract was essential for the PCS to exhibit its role as a nutrient carrier. Indeed, the correlation coefficient between

FIG. 3. The cell density of *L. casei* and the amount of lactic acid it produced in the bioassay analysis were positively correlated with the absorbances (at 260 nm) of the soaking solutions (100 ml) of various PCS. O, oat hulls; S, soybean hulls; F, soybean flour; Y, yeast extract; R, dried bovine RBC; B, dried bovine albumin; +, mineral salts.

pounds were parts of the PCS leachate, whereas the amount of lignin-derived aromatic compounds in the leachate was relatively small and had no harmful effect on the growth of *L. casei*. As expected, bioassays of the soaking solution of PP discs demonstrated the absence of leached nitrogenous compounds for the bacteria to grow in the MM environment.

PCS leaching rate. Results of the initial soaking-solution micro-Kjeldahl analysis showed that high total nitrogen concentrations for the supports did not correlate with high initial leached-nitrogen percentages. As shown in Fig. 5, the initial leached-nitrogen percentage of SFYB+ was 23%, when its total nitrogen was 23 mg of nitrogen per g of support, whereas

FIG. 8. Biofilm population and lactic acid production of *L. casei* were not affected by the amount of lactic acid absorbed in the PCS. conc, concentration; O, oat hulls; S, soybean hulls; F, soybean flour; Y, yeast extract; R, dried bovine RBC; B, dried bovine albumin; +, mineral salts.

OY had 63% initial leached nitrogen when its total nitrogen was 10 mg of nitrogen per g of support. These results showed that complex nutrients in PCS with higher initial leached-nitrogen percentages depleted quickly (within one to five RBF). This in turn led to the reduction of *L. casei* growth in long-term lactic acid fermentation. PCS with lower initial leached-nitrogen percentages released their complex nutrients gradually and thus could sustain a longer operational life.

The results described above were supported by the simulated RBF analysis, which showed that although OY and OFY+ both had similar total nitrogen contents (10 and 8.4 mg/g of support, respectively), they performed quite differently at the 20th RBF (Fig. 6). The results showed that OY, with 63% initial leached nitrogen, could no longer provide nutrients for the growth of *L. casei* at the 20th simulated RBF. In contrast, OFY+, having 42% initial leached nitrogen, still sup-

ported bacterial growth, and the final lactic acid concentration in the MM was 1.18 g/liter. Indeed, SFYB+, which had the highest lactic acid concentration (1.44 g/liter) by the end of the 20th simulated RBF, possessed only 23% initial leached nitrogen.

Statistical analysis supported the observations described above and indicated that soybean hulls, soybean flour, dried bovine RBC, and dried bovine albumin slowed the release of nitrogenous compounds from the PCS. Oat hulls and yeast extract hastened ($P < 0.0001$) the leaching of nitrogenous compounds from the supports and were not recommended to be the only minor agricultural ingredients of PCS.

Absorption of lactic acid by PCS. If the accumulation of lactic acid into the PCS was mainly due to absorption, then the lactic acid concentration inside the PCS and the lactic acid concentration in the medium would be the same. Furthermore,

FIG. 9. Relationship between interstitial volume and the amount of medium absorbed by various dried PCS. supp, support; O, oat hulls; S, soybean hulls; F, soybean flour; Y, yeast extract; R, dried bovine RBC; B, dried bovine albumin; +, mineral salts.

in the case of absorption, the quantity of lactic acid accumulated inside the PCS (5 g) should be inversely proportional to the amount of residual lactic acid in the outside medium, and their sum should be equal to the initial amount of lactic acid present in 15 ml of 30% LAS. The results in Fig. 7 show that all of the PCS behaved similarly to the filter paper controls, which strongly suggested an absorption phenomenon.

The amount of lactic acid absorbed in the PCS, as indicated by the total lactic acid extracted from the PCS through high-temperature treatment, was inversely correlated ($r = -0.95$) with the amount of lactic acid left in the 30% LAS solution after 2 weeks of soaking (Fig. 7). This strongly supported the assumption that there were no specific sites in the PCS for the binding of lactic acid. The absorption of lactic acid in the

supports was due to entrapment of liquid inside the interstitial volume of the PCS, and the absorbed lactic acid could be easily retrieved by physical methods such as high-temperature treatment. This assumption was further confirmed by the fact that the amount of lactic acid accumulated in the PCS had no correlation either with the biofilm population on the supports or with the lactic acid production by *L. casei* during batch fermentation (Fig. 8).

Interstitial volume of PCS discs. The lactic acid absorbed in the PCS served as a marker to determine the PCS interstitial volume. The interstitial volume per gram of PCS calculated from equation 1 was compared with the volume of MM absorbed by 1 g of dried PCS (Fig. 9). High correlations between the two values were obtained for both the PCS with soybean

hulls ($r = 0.81$) and those with oat hulls ($r = 0.90$). This strongly suggested that the interstitial volume could be a good indicator for the porosity of PCS. From SAS ANOVA analysis, addition of soybean hulls ($P < 0.0005$), dried bovine RBC ($P < 0.0001$), and dried bovine albumin ($P < 0.0044$) increased the interstitial volume of PCS, which in turn suggested that the porosities and liquid transport capacities of these PCS were greater.

Soybean hulls outperformed oat hulls by having greater protein content and possessing the ability to increase the interstitial volume of PCS. Yeast extract was the most outstanding minor complex ingredient for supplying nitrogenous compounds in the PCS. However, because of its high leaching rate, the PCS with just yeast extract was found to be unsuitable for long-term lactic acid fermentation. Other minor agricultural ingredients, such as dried bovine RBC, dried bovine albumin, and soybean flour, were essential to ensure a gradual release of nitrogenous compounds from the PCS. The results of this study showed that there is high potential for PCS with soybean hulls, yeast extract, soybean flour, dried bovine albumin, and/or dried bovine RBC to perform as slow-releasing nutrient carriers. Finally, SFYB+ was demonstrated to have the highest lactic acid concentration and cell density in the 1st and 20th RBF, the greatest counts of viable attached cells, a relatively small contact angle (10), and a gradual release of complex nutrients. Hence, SFYB+ is recommended for long-term biofilm lactic acid fermentation studies in MM.

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