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Continuous lactic acid fermentation using a plastic composite support biofilm reactor

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Abstract An immobilized-cell biofilm reactor was used for the continuous production of lactic acid by *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443). At Iowa State University, a unique plastic composite support (PCS) that stimulates biofilm formation has been developed. The optimized PCS blend for *Lactobacillus* contains 50% (wt/wt) agricultural products [35% (wt/wt) ground soy hulls, 5% (wt/wt) soy flour, 5% (wt/wt) yeast extract, 5% (wt/wt) dried bovine albumin, and mineral salts] and 50% (wt/wt) polypropylene (PP) produced by high-temperature extrusion. The PCS tubes have a wall thickness of 3.5 mm, outer diameter of 10.5 mm, and were cut into 10-cm lengths. Six PCS tubes, three rows of two parallel tubes, were bound in a grid fashion to the agitator shaft of a 1.2-l vessel for a New Brunswick Bioflo 3000 fermentor. PCS stimulates biofilm formation, supplies nutrients to attached and suspended cells, and increases lactic acid production. Biofilm thickness on the PCS tubes was controlled by the agitation speed. The PCS biofilm reactor and PP control reactor achieved optimal average production rates of 9.0 and 5.8 g l^{-1} h^{-1}, respectively, at 0.4 h^{-1} dilution rate and 125-rpm agitation with yields of approximately 70%.

Introduction

Lactic acid (2-hydroxypropanoic acid) exists in two optically active enantiomers, L(+) and D(–), and is widely used by the food, cosmetic, pharmaceutical, and plastics industries (Vickroy 1985). The organic acid is produced by chemical synthesis and microbial fermentation. Microbial fermentation has the advantage of being able to produce optically pure lactic acid. For fermentation processes to be competitive, however, improvements are needed to increase production rates and to maximize downstream processing. Presently, lactic acid is produced by batch fermentation because it exhibits both Type I (growth-associated) and Type II (nongrowth-associated) fermentation (Crueger and Crueger 1990). Accelerated production rates and high lactic acid concentrations have been achieved by strain development (Demirci and Pometto 1992).

To increase cell density in bioreactors, fermentation using cell immobilization by attachment and entrapment has been researched. The industrial application of cell entrapment with calcium alginate or κ-carrageenan beads is not feasible due to bead disintegration and mass transfer limitations. Attachment immobilization has been explored with several types of support materials: wood chips, porous bricks, glass (Krischke et al. 1991; Gonclaves et al. 1992; Guoqiang et al. 1992), foam (Dong et al. 1996), cotton cloth, ceramics (Gonclaves et al. 1992; Guoqiang et al. 1992), and plastic composite supports (PCS) (Ho et al. 1997a, b, c). PCS biofilm reactors have demonstrated increased lactic acid production rates, minimal lag phase, tolerance to high concentration of glucose, reduced requirement of micronutrients, and increased cell density (Ho et al. 1997a, b, c; Velázquez et al. 2001).

Continuous fermentation allows a constant flow of fresh sterile medium into a stirred tank bioreactor to eliminate the nutrient limitations that are present in batch fermentations. Microbial growth rate equals bioreactor dilution rate. Higher productivities can be achieved, but contamination and cell washout at high dilution rates for suspended cell bioreactors remain problems.

The purpose of this research was to demonstrate the effectiveness of continuous lactic acid fermentation using PCS tubes fixed to the agitator shaft for cell immobilization via biofilm reactor. Biofilm thickness was controlled by agitation.

Materials and methods

Bacterial culture preparation

Stock culture of *Lactobacillus casei* subsp. *rhamnosus* (ATTC 11443) from the American Type Culture Collection (Rockville, Md.) was maintained at 4°C in *Lactobacillus* MRS broth (Difco

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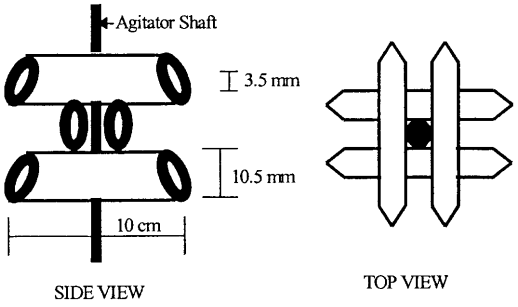


Fig. 1 Plastic composite support (PCS) and polypropylene (PP) tubes bound to *agitator shaft*

Laboratories, Detroit, Mich.). To maintain viability, monthly transfers were made into fresh medium. An active *L. casei* culture was prepared by adding 1 ml of stock solution to 100 ml of MRS and incubating as a static culture for 18 h at 37°C.

Media preparation

Lactic acid fermentation medium [40 g of glucose, 5 g of yeast extract (Ardamine Z; Champlain, Clifton, N.J.), 1 g of sodium acetate, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 7H_2O$, 0.5 g of KH_2PO_4, and 0.5 g of K_2HPO_4 per liter deionized water] was used for all experiments. Glucose, yeast extract, sodium acetate, $MgSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot 7H_2O$ were added to 86 l of water, sterilized in a B-Braun 100-D fermentor (Allentown, Pa.), with continuous agitation for 25 min at 121°C, and adjusted to pH 5.0 with sterile 3 N hydrochloric acid. Phosphate buffer solution was autoclaved separately in a 4-l carboy for 40 min at 121°C and added aseptically to other medium components. The sterilized media was aseptically transferred into two sterilized 50-l carboys equipped with a carboy filling port, a medium delivery line with a liquid break, and an air vent capped with a 0.45-µm air filter for storage.

Plastic composite support

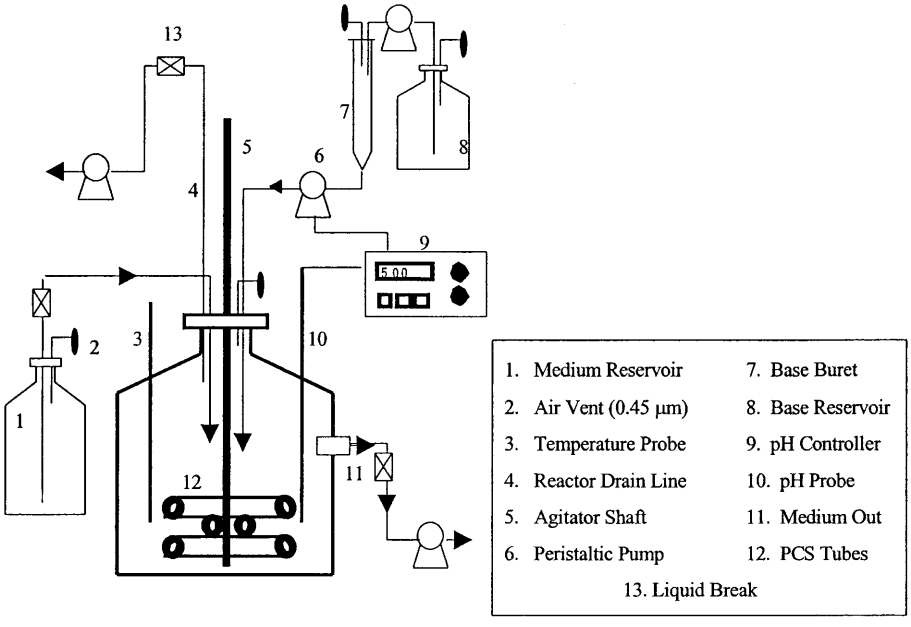
PCS tubes composed of 50% (wt/wt) polypropylene (PP), 35% (wt/wt) ground soy hull (Cargill Soy Processing Plant, Iowa Falls, Iowa), 5% (wt/wt) soy flour (Archer Daniels Midland, Decatur, Ill.), 5% (wt/wt) yeast extract, and 5% (wt/wt) dried bovine albumin (American Protein, Ames, Iowa) and mineral salts were produced according to Ho et al. (Ho et al. 1997c). These dry ingredients were mixed in a separate container prior to being poured into an extruder hopper. The twin screw co-rotating Brabender PL2000 extruder (model CTSE-V; Brabender Instruments, South Hackensack, N.J.) was operated at a rate of 11 rpm, barrel temperatures of 200, 220, and 200°C, and a die temperature of 167°C to form a continuous tube. Composite supports with a wall thickness of 3.5 mm and an outer diameter of 10.5 mm were cut into 10-cm lengths. PP tubes (control) were cut in an identical way to PCS tubes.

Continuous fermentation systems

A continuous stir tank bioreactor with PCS tubes fixed to the agitator shaft was compared with a control continuous reactor with PP tubes fixed to the agitator shaft (Fig. 1). PP and PCS tube ends were cut at an angle to allow fermentation media to flow through the inside of the tubes. Six PCS or PP tubes, three rows of two parallel tubes, were bound to the agitator shaft in a gridlike fashion. A schematic of the reactor design in a 1.2-l vessel (inside diameter of 12 cm) of a computer-controlled New Brunswick Bioflo 3000 fermentor (Edison, N.J.) is given in Fig. 2. Fermentations were controlled at 37°C and pH 5.0 with concentrated ammonium hydroxide. To determine the working volume of each reactor, water was passed through the continuous system until steady state was reached, at least five working volume exchanges. The reactor was then disassembled, drained into a graduated cylinder and the working volume determined. Agitation rates of 100 and 125 rpm did not affect working volume of reactors.

The series of steps for repeated continuous fermentation are summarized in Fig. 3. The reactor was sterilized with water for 1.25 h at 121°C. After sterilization, medium was used to dilute out water at a dilution rate of 0.6 h^{-1} overnight. The batch fermentation (1% inoculum) prior to continuous flow employed agitation of 100 rpm. Dilution rates varied from 0.1 to 1 h^{-1}. A dilution rate of 0.1 h^{-1} at 100 rpm for 5 days was needed to establish good biofilm formation on fresh PCS supports. Dilution rates were then increased. To repeat, reactors were washed with concentrated ammonium hydroxide for 2.5 h with agitation of 150 rpm, rinsed with sterile medium for 2 h with agitation of 175 rpm, and diluted with fresh sterile medium at a dilution rate of 0.6 h^{-1} overnight.

Fig. 2 Bioreactor design



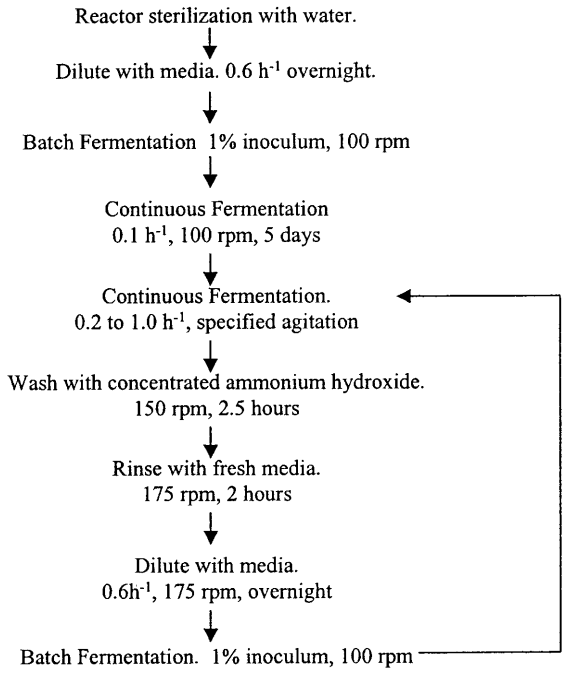


Fig. 3 Series of steps for repeated continuous lactic acid fermentations for bioreactor protocol

Long exposure at a dilution rate of 0.1 h^{-1} was not necessary for repeat fermentations. The effect of agitation (100 and 125 rpm) was also evaluated.

Sample analysis

After reaching steady state, samples were collected and analyzed for glucose consumption, lactic acid production, the presence of exopolysaccharide (EPS) and cell density. Fermentation samples were centrifuged, diluted 1:2, and filtered with a 0.45-µm filter prior to being analyzed for D-glucose and L(+)-lactic acid concentrations. Samples were analyzed using a high performance liquid chromatograph equipped with column heater, autosampler, computer controller, and a model 2410 refractive index detector (Waters, Milford, Mass.). Components were separated on a Bio-Rad Aminex HPX-87H column (300×7.8 mm) (Bio-Rad, Richmond, Calif.) with 0.12 N sulfuric acid used as the mobile phase at a flow rate of 0.8 ml/min with an injection volume of 20-µl and a column temperature of 65°C

The amount of EPS (total complex carbohydrates) present in freeze-dried fermentation medium was measured according to Ho et al. (1997c) by comparing the reducing sugar concentration determined by the Somogyi-Nelson assay with the phenol sulfuric assay (Wood and Bhat 1988). The phenol sulfuric assay hydrolyzes any polysaccharides that may be present into monosaccharides. The Somogyi-Nelson assay measures free reducing sugars prior to hydrolysis. After steady state was achieved at each dilution rate, 500-ml samples were collected. Samples were centrifuged to collect biomass and EPS, washed with deionized water, centrifuged, and freeze-dried for carbohydrate analysis.

Results and discussion

Figure 4 and Table 1 demonstrate the benefit of PCS tubes fixed to the reactor agitator shaft by increased microbial production rates, yields, and cell densities compared with a reactor with PP tubes (control).

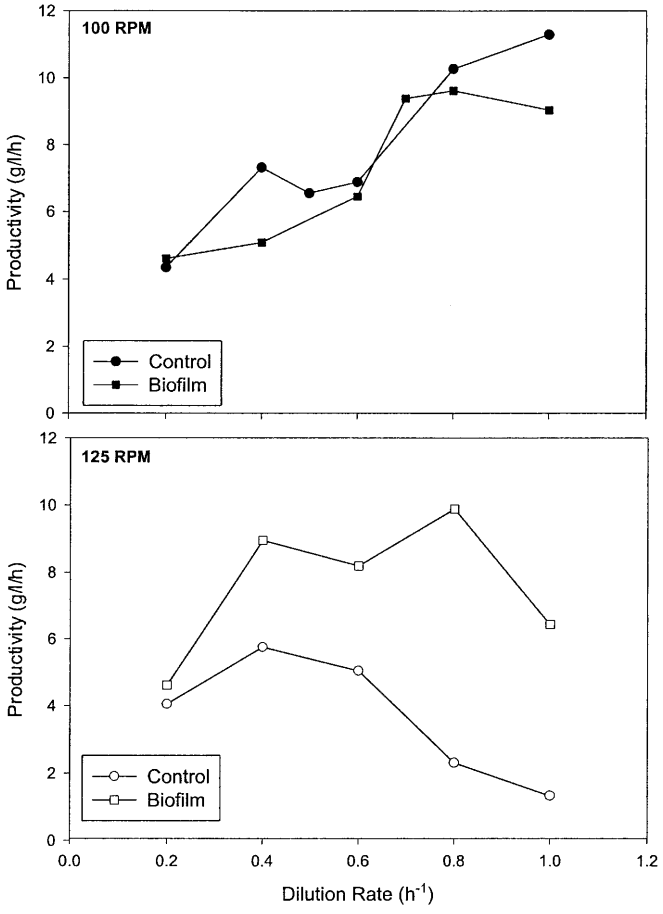


Fig. 4 Lactic acid production rates for PCS and PP control reactors. Each value is an average of repeated continuous fermentations

Agitation rates also had an effect on production rates and cell density.

At a dilution rate of 0.4 h^{-1}, the biofilm reactor demonstrated excellent productivities: 5.08 and 8.95 g l^{-1} h^{-1} with acceptable yields of 68.89 and 70.7% at agitations of 100 and 125 rpm, respectively. Furthermore, at a dilution rate of 0.6 h^{-1}, the biofilm reactor demonstrated excellent productivity, with a yield of 8.19 g l^{-1} h^{-1} and 71.68%, respectively, whereas the PP control reactor demonstrated acceptable performance at a dilution rate of 0.4 h^{-1} with a productivity rate and yield of 5.75 g l^{-1} h^{-1} and 69.55%, respectively. The PP control reactor achieved the highest average productivity of 11.29 g l^{-1} h^{-1}, at 1.0 h^{-1} dilution rate and 100 rpm agitation. The PCS bioreactor achieved the highest average productivity of 9.88 g l^{-1} h^{-1}, at 0.8 h^{-1} dilution rate and 125 rpm agitation; however, the percentage yields for the control and biofilm reactors giving these productivities were unacceptable at 52.38 and 58.61%, respectively. A typical percentage yield for *L. casei* is 70–72% (Ho et al. 1997a).

Conversion yields below 69% represent a physiological shift in the bacterium to the overproduction of EPS and reduction of lactic acid production. Since EPS is attached to supports and suspended in medium, the data

Table 1 Summary of results for control and biofilm reactors[a]

	Dilution rate h^{-1}				
	0.20	0.40	0.60	0.80	1.00
Lactic acid production (g l^{-1} h^{-1})					
Control-100 rpm	4.34	7.32	6.88	10.27	11.29
Control-125 rpm	4.05	**5.75**	5.04	2.31	1.33
Biofilm-100 rpm	**4.61**	**5.08**	6.45	9.62	9.04
Biofilm-125 rpm	**4.61**	**8.95**	**8.19**	9.88	6.43
Biofilm-125 rpm	**4.67**	6.29	5.02	4.39	7.46
% Yield[b]					
Control-100 rpm	64.50	61.39	51.35	55.88	52.38
Control-125 rpm	64.83	**69.55**	48.28	28.78	11.68
Biofilm-100 rpm	**72.12**	**68.89**	52.82	29.63	48.07
Biofilm-125 rpm	**74.73**	**70.70**	**71.68**	58.61	46.95
Biofilm-125 rpm	**70.86**	64.88	50.62	41.28	44.98
Final lactic acid concentration (g/l)					
Control-100 rpm	21.68	18.30	11.46	12.84	11.29
Control-125 rpm	20.23	**14.38**	8.39	2.88	1.33
Biofilm-100 rpm	**23.05**	**12.70**	10.75	12.03	9.04
Biofilm-125 rpm	**23.03**	**22.36**	**13.65**	12.34	6.43
Biofilm-125 rpm	**23.37**	15.73	8.38	5.49	7.46

[a] Each value is an average of repeated continuous fermentations, with the exception of biofilm reactor at 100 rpm. Biofilm fermentations are presented in the order performed with a concentrated ammonium hydroxide wash between each fermentation (see Fig. 3)
[b] Data in bold illustrate acceptable percentage yields of 68–75% for *L. casei*

Table 2 Amount of exopolysaccharide (EPS) present in suspended medium (mg/l)

	Dilution rate h^{-1}				
	0.20	0.40	0.60	0.80	1.00
Control-100 rpm	387	252	1528	175	317
Control-100 rpm	ND[a]	17	122	745	464
Control-125 rpm	ND	2955	221	297	ND
Control-125 rpm	808	138	236	391	706
Biofilm-100 rpm	1141	844	1265	2516	2021
Biofilm-125 rpm	ND	455	335	849	1993
Biofilm-125 rpm	269	273	465	1333	822

[a] Not determined; samples at this dilution rate were not collected

presented in Table 2 appear inconsistent between repeated fermentations. At a higher agitation, more EPS is sloughed off the supports and suspended in the medium; therefore, the amount of EPS reported in Table 2 is not the "absolute amount" of EPS present in the reactor. For example, under these continuous fermentation conditions, extracellular polysaccharide production maximized for the PCS bioreactor at a dilution rate of 0.8 h^{-1}, 100 rpm agitation, and 2.0 g l^{-1} h^{-1} of EPS, and for the PP control bioreactor at a dilution rate of 0.4 h^{-1}, 125 rpm agitation, and 1.2 g l^{-1} h^{-1} of EPS which produced yields of 29.63 and 69.55%, respectively. With the data being inconsistent, no correlations can be made regarding dilution rate, agitation or EPS formation. Furthermore, repeated PCS washing with concentrated ammonium hydroxide (Fig. 3) may be the cause for the reduced performance in the last 125 rpm continuous fermentation. Under normal operating conditions, a set dilution rate and agitation speed, not alkali washes, will be employed to control biofilm thickness.

There was a visible increase of suspended EPS at higher dilution rates and increased agitation. There were also visible changes in the characteristics of the freeze-dried medium. At higher dilution rates, a flaky white material surrounded the pelleted cells. This change in carbon flow by *Lactobacillus* has been observed previously. Krischke et al. (1991) reported lactic acid production rates of 10 and 13.5 g l^{-1} h^{-1} at dilution rates of 0.4 and 1.0 h^{-1}, respectively, using a fluidized bed reactor with glass fittings. They also reported reduced yields of 50% for the higher dilution rate as our data illustrate. A continuous packed bed reactor with foam glass achieved a production rate and yield of 6.2 g l^{-1} h^{-1} and 60%, respectively (Guoqiang et al. 1992); however, this packed bed reactor also made pH control difficult. Gonclaves et al. (1992) achieved a production rate of 20.1 g l^{-1} h^{-1} with a continuous packed bed made of sintered glass beads. However, the reactor could be operated for only 24 h and percentage yields were not reported.

The PCS continuous reactors in this research were operated continuously for 3 months. Repeated batch lactic acid PCS biofilm reactors have operated for over 2.5 years in our laboratory. A reactor with PCS tubes fixed to the agitator shaft provides a truly open continuous bioreactor design for an immobilized cell bioreactor. This design has advantages over packed and fluidized beds, and cell recycling bioreactors, which have problems with reduced flows and membrane fouling. Thus, this bioreactor can increase cell density, productivity, and yield for continuous lactic acid fermentations over a longer period of time. Other benefits of PCS biofilm reactors include control of biofilm thickness with agitation and excellent pH control.

The use of PCS tubes fixed to the agitator shaft of a bioreactor demonstrated a positive effect on cell density, production rates, and yields. A dilution rate of 0.4 h^{-1} and agitation of 125 rpm demonstrated the highest productivities (9.0 g l^{-1} h^{-1}) and yields (71%). Agitation had a significant effect on cell density, biofilm formation, and yields. Further research is needed to optimize and increase the final concentration of lactic acid in the fermentation media to ≥120 g/l. To achieve this desired concentration, a three- to four-reactor train may be necessary with glucose addition into each bioreactor. Furthermore, scale-up will require some geometry changes in the shape of the PCS. Currently, PCS is an extruded product. If injection-molded shapes are chosen for the best geometry (e.g., discs) then this might have an effect on biofilm development. Thus, further research is needed before commercial acceptance is achieved. Potentially, this technology has application in any fermentation designed for continuous operation such as ethanol and succinic acid production from liquified corn starch and the bioconversion of food liquid waste streams to value-added products.

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