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Response Surface Analysis of the Effects of pH and Dilution Rate on *Ruminococcus flavefaciens* FD-1 in Cellulose-Fed Continuous Culture

Y. SHI[1] AND P. J. WEIMER[1,2]*

Department of Bacteriology, University of Wisconsin-Madison,[1] and U.S. Dairy Forage Research Center, Agricultural Research Service, United States Department of Agriculture,[2] Madison, Wisconsin 53706

Received 6 April 1992/Accepted 19 May 1992

The ruminal cellulolytic bacterium *Ruminococcus flavefaciens* FD-1 was grown in cellulose-fed continuous culture with 20 different combinations of pH and dilution rate (*D*); the combinations were selected according to the physiological pH range of the organism (6.0 to 7.1) and growth rate of the organism on cellulose (0.017 to 0.10 h^{-1}). A response surface analysis was used to characterize the effects of pH and *D* on the extent of cellulose consumption, growth yield, soluble sugar concentration, and yields of fermentation products. The response surfaces indicate that pH and *D* coordinately affect cellulose digestion and growth yield in this organism. As expected, the net cellulose consumption increased with increasing *D* while the fraction of added cellulose that was utilized decreased with increasing *D*. The effect of changes in pH within the physiological range on cellulose consumption was smaller than that of changes in *D*. Cellulose degradation was less sensitive to low pH than to high pH. At low *D*s (longer retention times), cellulose degradation did not follow first-order kinetics. This decreased rate of cellulose digestion was not due to poor mixing, limitation by other medium components, or preferential utilization of the more amorphous fraction of the cellulose. The cell yield increased from 0.13 to 0.18 mg of cells per mg of cellulose with increasing *D*s from 0.02 to 0.06 h^{-1} and decreased when the pH was shifted from the optimum of 6.5 to 6.8. The effect of pH on cell yield increased with increasing *D*. The reduced cell yield at low pH appears to be due to both an increase in maintenance energy requirements and a decrease in true growth yield.

Microbial fermentation in the rumen is an essential process in the conversion of feed materials to the volatile fatty acids and microbial cell protein that are used by the ruminant animal for growth and milk production. The ruminal habitat is characterized by a flux of its contents and by a variety of growth rates and metabolic activities of individual microbial species under environmental conditions that vary over time. Among the more than 60 species of ruminal bacteria (18), four predominant cellulolytic species play an important role in digesting plant fibers, which compose the bulk of the diet of forage-fed ruminants. Of the many environmental factors affecting the cellulolytic process, rumen pH and dilution rate (*D*) are two of the most important (2, 3, 5, 6).

Numerous studies have suggested that the metabolism and survival of cellulolytic bacteria are strongly dependent on rumen pH. The optimum pH for growth of the most important species of ruminal cellulolytic bacteria is ~6.5 (30). Studies with individual species of ruminal cellulolytic bacteria have indicated that most have very narrow pH ranges for growth (26). Similarly, both the rate and extent of cellulose degradation by mixed ruminal microflora or pure cultures of ruminal cellulolytic bacteria have been reported to be inhibited at pHs of ≤6.3 (8, 29). Reduced cellulose digestion may be due to the reduced prevalence (25) or activity (8) of cellulolytic species.

Continuous culture studies with both pure and mixed cultures of ruminal bacteria have revealed that *D* is positively related to the efficiency of microbial growth (9, 12, 28). Although the dilution rate in the animal (i.e., the rate of passage) can be increased by cooling the animal or increasing the intake of certain salts, this often causes an increased rumen pH and a decrease in dry matter digestibility (13, 17).

Hoover et al. (9) evaluated the independent effect of and relationship between liquid *D* and pH on the growth yield and fermentation patterns of mixed ruminal microflora in continuous cultures at four different pHs (4.5, 5.5, 6.5, and 7.5) for each of four different dilution rates (0.04, 0.08, 0.12, and 0.16 h^{-1}). However, many of these pH and *D* combinations are beyond the reported pH tolerance range of ruminal cellulolytic bacteria and the dilution rate of ruminal contents. It is not clear how the cellulolytic process and microbial growth are affected by pH and *D* combinations within the physiological growth range of individual cellulolytic species.

The recent development of continuous-culture devices for growth of microorganisms on insoluble substrates (14, 19, 32) has afforded an opportunity to obtain quantitative data on the fermentation of specific fiber components. In this report we use one such device, combined with response surface analysis of the data, to evaluate the effects of pH and *D* on cultures of *Ruminococcus flavefaciens*, a predominant ruminal cellulolytic bacterium. The response surfaces provide quantitative information on how cellulose consumption, growth yield, and product formation by this organism are affected by simultaneous changes in its normal physiological range of pH and possible rumen dilution rates.

MATERIALS AND METHODS

Organism and growth medium. *R. flavefaciens* FD-1 was grown in a modified Dehority medium (27). The modifica-

* Corresponding author.

tions included (i) removal of casein hydrolysate and cellobiose, (ii) addition of clarified rumen fluid (2.5%, vol/vol); and (iii) addition of 4.8 g of Sigmacell 20 microcrystalline cellulose (sieved to particle size of <45 μm; Sigma, St. Louis, Mo.) per liter of medium.

Continuous culture. The continuous culture system described previously (32) was modified by including a pH control system. All experiments were performed in a 940-ml-working-volume fermentor at 39°C. The pH was maintained by automatic addition of 2 N H_2SO_4 or 2 N NaOH via a Cole-Parmer model 5652 pH controller. The medium slurry in the reservoir was homogenized by stirring and diffusive gas sparging with CO_2 and was delivered to the culture vessel by CO_2 segmentation with a peristaltic pump. Microbial contamination was monitored regularly by microscopic observation. Washout points were defined as the D and pH combinations at which steady-state conditions could not be achieved. A washout was detectable by a dramatic increase in reactor cellulose concentration and by microscopic examination, which revealed that attachment of cells to cellulose particles decreased to near zero.

Analyses. All samples were taken at 6- to 20-h intervals. For each combination of pH and D tested, the data were averaged from 5 to 13 samples collected over a 2- to 7-day period at the system steady state, when the residual cellulose concentration and cell yield were basically constant. Under these conditions, the microbial growth rate (μ) was equal to D (23). The cellulose concentration in the reactor or medium reservoir was determined from ~20-ml samples (weighed to 0.001 g) by a modified neutral detergent method (31). Paired 1.5-ml samples were microcentrifuged for 5 min at 12,000 × g to prepare culture supernatants. The soluble and reducing sugars in supernatants were measured by the anthrone (15) and dinitrosalicylic acid (16) methods, respectively. Fermentation acids in culture supernatants were analyzed by high-performance liquid chromatography as described previously (32). The three major fermentation acids were separated at retention times of 10.7 min (succinic acid), 12.3 min (formic acid), and 13.3 min (acetic acid) and were well resolved from lactic acid (11.8 min), which was not produced.

The cell mass was estimated from the nitrogen content of cell pellets, which was measured with a Carlo Erba NA1500 nitrogen analyzer (32). Molar cell yields were calculated by assuming that the biomass formula for the organic component of the biomass was $C_5H_7O_2N$ and that cells contained 90% organic material (19). To confirm the validity of this formula for this organism, strain FD-1 was grown in triplicate batch cultures on cellobiose. The cell pellets were washed twice with deionized water and dried overnight at 105°C. The average measured carbon/nitrogen ratio of these dried samples was 5.01 ± 0.12.

Recovery of cellulose for crystallinity measurements was complicated by the strong adherence of cells to cellulose and by the necessity of keeping the cellulose wet enough to prevent recrystallization. A 540-ml effluent (from a culture operated at a D of 0.019 h^{-1} at pH 6.59) was amended with 10.9 ml of 5% methylcellulose (15 cP) and stirred overnight at 6°C. The suspension was centrifuged at 400 × g for 10 min, the pellet was respended to the original volume with distilled water, and the suspension was centrifuged again. The resulting pellet was resuspended in deionized water and vacuum filtered through a 3-μm-pore-size polycarbonate membrane with small water washings. The wet cake was resuspended in ~15 ml of deionized water, dispensed into 12 microcentrifuge tubes, and then pulse-centrifuged for 3 s. Pellets were washed six times with 1 ml of deionized water. The final combined pellet was resuspended in 30 ml of deionized water. The resulting cellulose slurry was stirred vigorously while 1,500-μl aliquots were transferred to 16 Pyrex tubes (18 by 150 mm). The relative crystallinity index was determined from the kinetics of hydrolysis of this cellulose in hot 6 N HCl. HCl (1,490 μl of a 12.1 N solution) was added to each tube. The tubes were sealed with plastic caps and placed in a boiling-water bath. Residual cellulose was determined gravimetrically for a series of hydrolysis time points within the range of 0 to 6.2 h after vacuum filtration through 25-mm 3-μm-pore-size polycarbonate membranes and drying at 105°C (33). Since *R. flavefaciens* was not completely detached by the methylcellulose treatment and the cellulose slurry used to measure the relative crystallinity index still contained a significant population of attached cells, a correction was included for microbial cell mass in the remaining material (0.32 to 3.83%, determined by nitrogen analysis as described above). The natural logarithm of the corrected percent cellulose remaining was plotted versus time within the range of 2 to 6.2 h; this plot directly yields the relative crystallinity index (anti-ln of y intercept at time zero). The data were compared to those obtained for the original SC20 substrate hydrolyzed in an identical manner.

Statistical model. A response surface analysis (1) was used to examine the influence of pH and dilution rate on the following fermentation parameters: fractional cellulose consumption (i.e., the proportion of added cellulose that was consumed); cell yield; soluble sugar concentration; molar yields of acetate, succinate, and formate; and the ratio of products (e.g., the molar ratio of acetate to acetate plus succinate). The region of study was a pH range of 5.8 to 7.1 and a D range of 0.017 to 0.12 h^{-1}. Regression equations were calculated from the observed data of 20 steady-state continuous-culture experiments by using a modification of the model of Farrand et al. (4), parameter = $\alpha + \beta D + \gamma P + \delta D^2 + \varepsilon P^2 + \zeta DP + \eta D^2P + \theta DP^2 + \omega$, where α, β. . . . η, θ are regression coefficients, D is the dilution rate (per hour), P is the pH, and ω is a random error. By using the General Linear Model of the SAS program (26a), the above equation was fitted to the observed data to produce the predictive equations. The simultaneous effects of D and pH on each fermentation parameter were visualized by using the CONTOUR subroutine to generate the response surfaces within the D range of 0.015 to 0.06 h^{-1} (the normal range of rumen D values) and within the pH range of 6.0 to 7.0 (the approximate growth range of the bacterium).

RESULTS

Growth range of the organism. Figure 1 shows the domains of D and pH in which steady-state growth was obtained for *R. flavefaciens* FD-1 with microcrystalline cellulose as the energy source. Within the normal range of rumen dilution rate (0.015 to 0.06 h^{-1}), steady-state growth was obtained within the pH range of 6.0 to 7.1. At pH values of ~6.5, growth was obtained at D values as high as 0.10 h^{-1}. Microscopic examination of cultures revealed that cellulose colonization by bacterial cells was essentially complete at the steady state in all runs within the D range of 0.015 to 0.06 h^{-1}. Unattached cells were present at higher density under conditions of low D.

Cellulose consumption. Figure 2A and Table 1 show the interaction between pH and D on the percentage of cellulose consumption. The statistical model gave excellent fit of the observed data ($P < 0.001$, $r^2 = 0.836$; i.e., 84% of the variation in cellulose consumption was due to pH and D

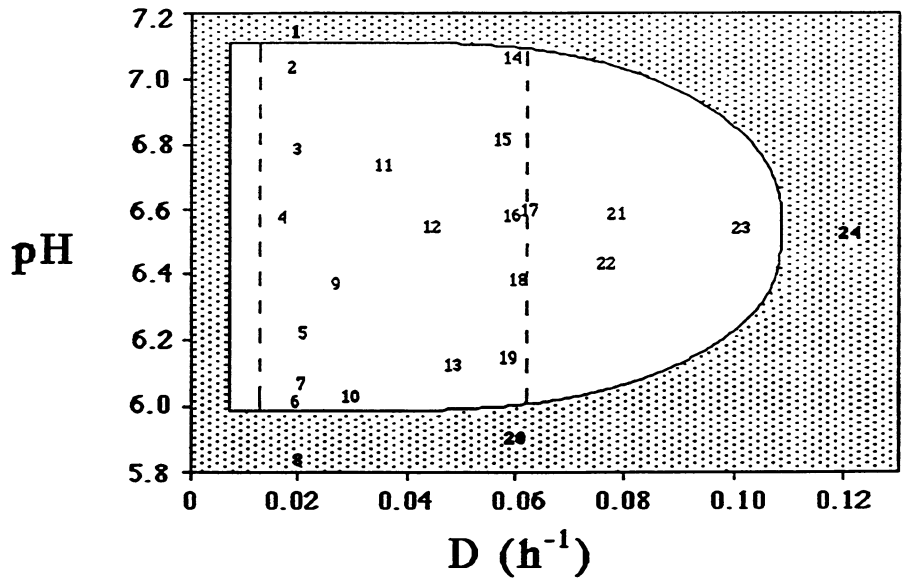

FIG. 1. Summary of the combinations of D and pH used for continuous-culture experiments. Numbers correspond to the individual runs in Table 1. The stippled region approximates the D and pH combinations with which steady-state growth was not obtained. The box enclosed by the dashed lines represent the pH-D domains included within the contour maps in Fig. 2.

alone). The highest percentage of cellulose consumption was obtained and also predicted at a lower D (≤ 0.03 h^{-1}) and an intermediate pH (ca. 6.2 to 6.7). Although the total range in fractional cellulose consumption was relatively small (0.68 to 0.80), it is clear that more complete consumption of added cellulose occurred at lower Ds (longer retention times).

Data from steady-state runs within the optimal pH range showed a nearly linear increase in the total amount of cellulose consumed as D increased (Fig. 3A), whereas the total amount of cellulose consumed showed little change with changing pH at a D of 0.02 or 0.06 h^{-1} (Fig. 3B). The results suggest that the total amount of cellulose consumption was affected by D much more strongly than by pH. These data for *R. flavefaciens* are in accord with those of Hoover et al. (9), who reported that increased D has a major effect on increasing fiber digestion by mixed ruminal microflora at the more physiological pH levels.

Using the formula derived from the statistical model and its calculated coefficients (Table 2), we calculated a series of predicted values of fractional cellulose consumption at reasonable selected combinations of pH and D. For these calculations, the concentration of cellulose in the reservoir (C_o) was set to 4,800 mg/liter, and the predicted cellulose concentration in the reactor (C) was calculated as $C_o - [C_o \times$ (predicted fractional cellulose consumption)]. Plots of these predicted C_o/C values versus retention time ($1/D$) were nonlinear (Fig. 4), suggesting that cellulose digestion does not follow first-order kinetics with respect to cellulose at low D. The relationship between C_o/C and t_R for a given pH was well described by a second-order polynomial equation (Table 3). Cellulose digestion at a D of 0.019 h^{-1} at pH 6.59 was not improved by doubling the concentration of ruminal fluid in the culture medium.

Cellulose crystallinity measurements showed that the relative crystallinity index of reactor cellulose (D = 0.019, pH 6.59) was 89.5, compared with 86.2 for the original SC20 substrate, indicating that residual cellulose was only slightly enriched in its crystalline content during the fermentation, even at long retention times.

Cell growth yield. The observed cell yield data fit the statistical model very well ($P < 0.001$, $r^2 = 0.814$). Maximum cell yield was observed and predicted at high Ds and within a pH range of 6.5 to 6.8 (Fig. 2B and 5). Both pH and D had strong effects on the cell yield. However, the steep cell yield contours at high Ds and low pHs (Fig. 2B) suggested that the effect of pH on cell yield increased with increasing D.

Using the formula derived from the statistical model and its calculated coefficients, we calculated predicted cell yield values at various combinations of pH and D. By calculating Y at a constant pH for a series of different D values, we generated a Pirt plot ($1/Y$ versus $1/D$), the slope of which equals the maintenance coefficient m and the y intercept of which equals $1/Y_g$ (Y_g is the true growth yield) (Table 3) (23). Repetition of this process for other pH values revealed that both the maintenance requirement (0.063 g of cellulose per mg of cell per h) and true growth yield (0.30 mg of cells per g of cellulose consumed) are basically constant within the observed optimum pH for growth (6.4 to 6.6). Both the true growth yield and the predicted cell yield decreased when the pH shifted from this optimum range. However, Pirt plots were nonlinear at pHs of ≥ 6.8.

Residual soluble sugar. The response surfaces for soluble sugar concentration are shown in Fig. 2C. Since the concentrations of soluble sugar at all combinations of pH and D were relatively low (<0.5 mM), the total changes were quite small. Furthermore, the statistical model did not fit the residual sugar concentration data ($P < 0.10$, $r^2 = 0.570$) as well as it fit the cellulose consumption and cell yield data. Like fractional cellulose consumption values, soluble sugar concentrations were higher at low Ds. Parallel assays with the dinitrosalicylic acid reagent (for reducing sugars; data not shown) and the anthrone reagent (for soluble sugars) gave similar results, suggesting that almost all of the soluble sugar is glucose (i.e., cellobiose and higher cellodextrins are present only at very low concentrations). The increased sugar concentration at low Ds precluded estimation of the K_s for the soluble sugars and is in accord with similar observations for *Ruminococcus albus* (19).

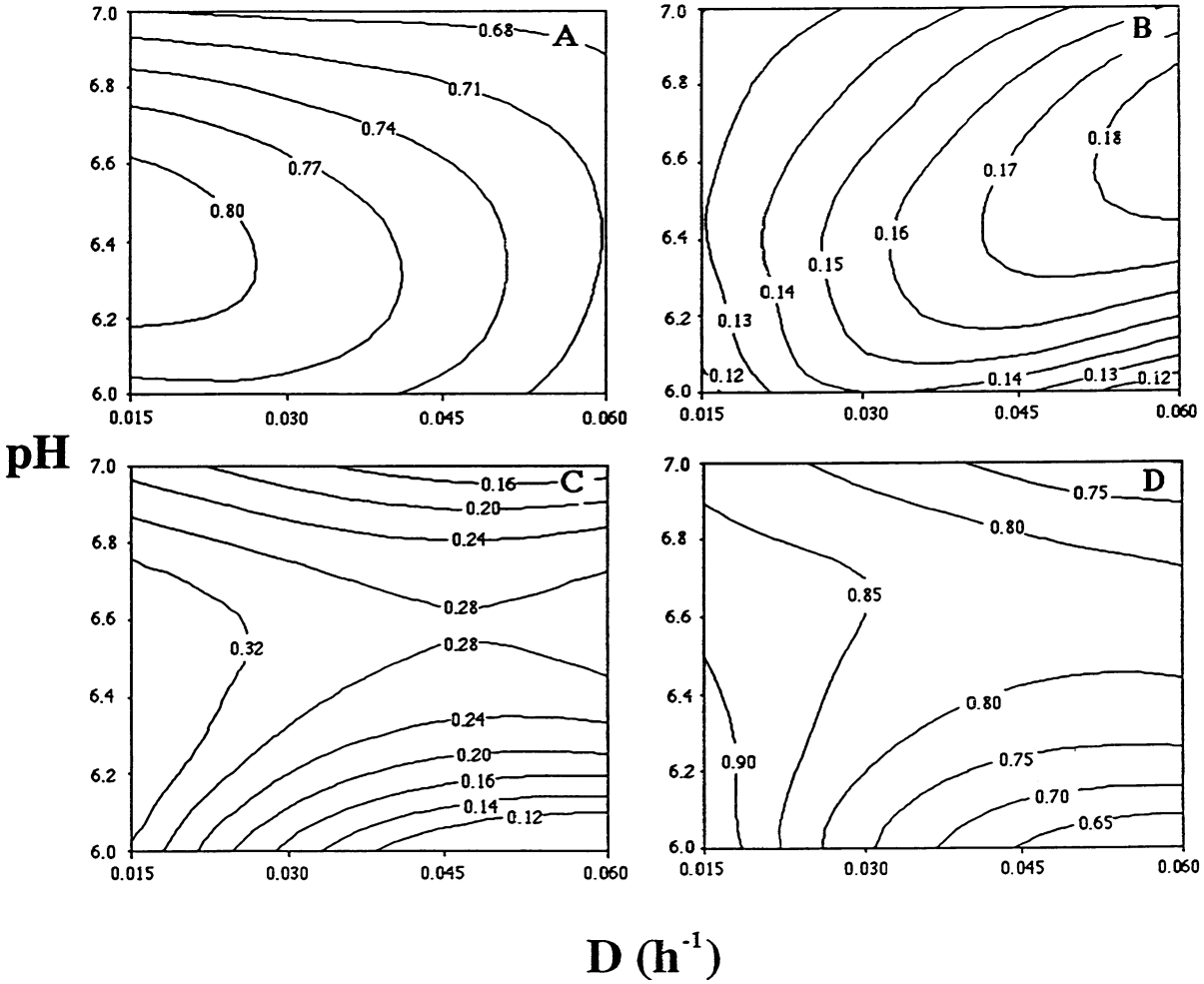

FIG. 2. Response surfaces for the *R. flavefaciens* fermentation in cellulose-fed continuous culture. (A) Fraction of added cellulose consumed. (B) Cell yield (grams of cells per gram of cellulose consumed). (C) Residual soluble sugar concentration (millimolar). (D) Acetate yield (moles of acetate produced per mole of anhydroglucose consumed). See Table 2 for statistical parameters and the text for a discussion of goodness of fit.

Fermentation product yields and ratios. Fermentation products included acetic, formic, and succinic acids but not ethanol, lactic acid, or C_3 to C_6 volatile fatty acids. Hydrogen was probably produced (32), but it was not quantitated.

Acetate was the most predominant fermentation product with all of the combinations of pH and D tested. The predicted equation for acetate yield gave only a moderate fit to the observed data ($P < 0.04$, $r^2 = 0.641$). A change in acetate yield from 0.65 to 0.90 mol/mol of anhydroglucose with varied pH and D combinations is shown in Fig. 2D. More acetate was produced at lower D and pH values, roughly parallel to the fractional cellulose consumption and soluble sugar concentration. Little change in the acetate yield was observed with changing pH at a D of ≤ 0.03 h^{-1}; however, the effect of pH on acetate yield clearly increased with increasing D.

Succinate and formate, the other major products, together accounted for 55 to 70 mol% of the total acid produced. The amount of succinate was higher than that of formate under most experimental conditions. The statistical model did not successfully predict the molar yield of succinate ($P < 0.62$, $r^2 = 0.311$) or formate ($P < 0.26$, $r^2 = 0.464$), suggesting that these yields are primarily dependent on factors other than pH and D alone.

DISCUSSION

In general, the concentrations of soluble sugars were low at all values of pH and D, suggesting that cellulose depolymerization to soluble oligosaccharides is the rate-limiting step in cellulose fermentation, as has been reported for *R. albus* (19). The accumulation of 0.1 to 0.5 mM glucose but very little cellobiose at all experimental conditions is in agreement with the reported inability of this strain to grow on glucose and its relatively high affinity for cellobiose (7).

It was quite surprising that plots of predicted C_o/C versus retention time (Fig. 4) revealed a departure from first-order kinetics for cellulose consumption at low Ds. This deviation is not due to inaccurate prediction by the statistical model, because a plot of observed C_o/C values versus t_R from runs at similar pH values (runs 4, 9, 12, 16, 21, and 23) was also nonlinear. Since t_R and C_o are independent variables, the departure must be due to a high value for C, perhaps resulting from (i) poor mixing, (ii) differential rates of hydrol-

TABLE 1. Summary of cellulose-fed continuous cultures of *R. flavefaciens* FD-1 at steady state with various combinations of pH and *D*

Run no.[a]	*D* (h^{-1})	pH	n[b]	Amt of cellulose (mg/liter/h)		Fraction of cellulose consumed	Amt of cells produced[c] (mg/liter/h)	Cell yield[d]	Concn (mM) consumed (−) or produced							% C recovery[e]	Molar yield			Molar ratio[f]	
				Added	Consumed				Cellulose[g]	Cells[h]	Soluble sugar	Acetate	Succinate	Formate	CO_2[i]		Acetate	Succinate	Formate	a/a+s	s/s+f
1	0.020	7.19	0																		
2	0.019	7.05	5	87.65	59.05	0.674	6.43	0.109	−19.19	2.69	0.21	16.72	11.05	4.15	1.52	84.9	0.871	0.576	0.216	0.602	0.727
3	0.020	6.80	6	92.10	63.82	0.693	7.70	0.121	−19.70	3.07	0.23	16.15	11.63	5.87	−1.35	84.9	0.820	0.590	0.297	0.581	0.665
4	0.017	6.59	9	77.71	67.42	0.868	8.91	0.132	−24.48	4.17	0.43	21.42	16.58	7.98	−3.14	93.7	0.875	0.677	0.326	0.564	0.675
5	0.020	6.26	8	92.20	74.14	0.804	8.06	0.109	−22.87	3.21	0.22	22.30	17.69	11.79	−7.18	99.6	0.975	0.774	0.516	0.558	0.600
6	0.019	6.02	7	88.79	68.72	0.774	8.21	0.119	−22.33	3.44	0.37	19.78	11.55	9.13	−0.90	84.7	0.886	0.517	0.409	0.631	0.559
7	0.020	6.06	10	94.98	69.72	0.735	7.18	0.103	−21.52	2.86	0.24	22.21	17.25	11.52	−6.56	103.7	0.986	0.802	0.535	0.563	0.600
8	0.020	5.85	0																		
9	0.027	6.38	9	134.73	107.65	0.799	15.44	0.143	−24.61	4.56	0.49	23.09	11.82	16.49	−5.22	88.7	0.938	0.480	0.670	0.661	0.418
10	0.029	6.03	8	135.40	108.34	0.802	18.01	0.166	−20.92	4.95	0.33	16.76	10.91	10.11	−4.26	82.4	0.801	0.522	0.483	0.600	0.519
11	0.035	6.75	7	153.48	113.93	0.742	19.99	0.176	−20.09	4.55	0.33	15.39	11.41	11.66	−7.68	88.0	0.766	0.568	0.580	0.574	0.495
12	0.044	6.56	11	217.98	166.85	0.766	25.92	0.155	−23.41	4.69	0.29	18.56	13.42	7.84	−2.70	86.5	0.793	0.573	0.335	0.580	0.631
13	0.048	6.14	10	220.99	166.42	0.753	26.16	0.159	−21.40	4.34	0.03	14.80	9.69	9.58	−4.47	78.8	0.692	0.453	0.448	0.592	0.503
14	0.059	7.08	7	281.31	184.85	0.657	27.55	0.149	−19.34	3.72	0.12	12.49	11.76	10.28	−9.55	80.8	0.646	0.608	0.532	0.515	0.534
15	0.059	6.83	6	281.31	193.40	0.688	34.28	0.177	−20.23	4.63	0.13	17.12	13.85	13.83	−10.56	96.1	0.846	0.685	0.684	0.553	0.500
16	0.059	6.59	9	269.69	190.39	0.706	39.00	0.205	−19.92	5.27	0.31	17.66	12.49	9.61	−4.44	99.3	0.887	0.627	0.475	0.586	0.565
17	0.061	6.60	7	287.80	190.38	0.662	35.32	0.186	−19.26	4.61	0.30	16.84	10.27	10.25	−3.68	92.1	0.874	0.533	0.532	0.621	0.501
18	0.060	6.40	7	277.48	201.84	0.715	31.80	0.160	−20.77	4.23	0.12	14.56	12.32	13.73	−11.49	84.0	0.701	0.593	0.661	0.542	0.473
19	0.059	6.16	8	276.65	185.56	0.671	25.37	0.137	−19.42	3.43	0.18	14.18	11.84	10.88	−8.55	83.8	0.730	0.610	0.560	0.545	0.521
20	0.059	5.94	0																		
21	0.076	6.46	5	371.72	259.08	0.697	49.78	0.192	−21.04	5.22	0.45	18.44	10.01	9.47	−1.64	91.9	0.876	0.476	0.450	0.642	0.514
22	0.078	6.60	13	366.02	217.00	0.646	41.11	0.189	−17.17	4.20	0.43	12.13	6.85	7.26	−1.98	78.6	0.706	0.400	0.423	0.639	0.486
23	0.101	6.56	11	490.56	268.26	0.536	55.05	0.205	−16.40	4.35	0.52	15.41	10.05	8.08	−2.72	102.8	0.940	0.613	0.493	0.605	0.554
24	0.120	6.56	0																		

[a] For indexing purposes only. The chronological order of the runs was 17, 12, 16, 4, 22, 23, 24, 21, 9, 5, 8, 7, 19, 20, 18, 15, 14, 3, 2, 1, 6, 10, 13, 11. The data from runs 4, 9, 12, 16, 21, and 23 were reported previously (32).

[b] n, number of samples analyzed at steady state. An *n* value of 0 indicates a washout point.

[c] Calculated from measured milligrams of N per liter and an assumed cell formula $C_5H_7O_2N$ (organic materials are assumed to compose 90% of the cell mass) with the formula, milligrams of cells/liter/hour = (milligrams of N/liter)(113 × *D*)/(0.9 × 14).

[d] Milligrams of cells produced per milligram of cellulose consumed.

[e] Calculated as [2(mM acetate) + 4(mM succinate) + (mM formate) + 6(mM soluble sugar as observed) + 5(mM cells)]/[6(mM anhydroglucose consumed) + (mM CO_2)].

[f] a/a+s, acetate/acetate + succinate ratio; s/s+f, succinate/succinate + formate ratio.

[g] Millimolar cellulose consumed (as anhydroglucose) = [(milligrams of cellulose per liter in the reservoir) − (milligrams of cellulose per liter in the reactor)] × (*D*/162) (162 represents the molecular weight of the anhydroglucose monomer).

[h] Millimolar cells was calculated as (mg of cells/liter/h)/(*D* × 125.6), where 125.6 is the formula weight of cells (equal to 113 mg of $C_5H_7O_2N$ divided by 0.9 mg of $C_5H_7O_2N$ per mg of cells). (See reference 19.)

[i] CO_2 was calculated according to the following assumptions: (i) 1 mol of CO_2 was produced per mol of acetate produced and (ii) 1 mol of CO_2 was consumed per mol of succinate or formate produced. Thus, the data comprise values for acetate, succinate, and formate. A negative value indicates net CO_2 consumption.

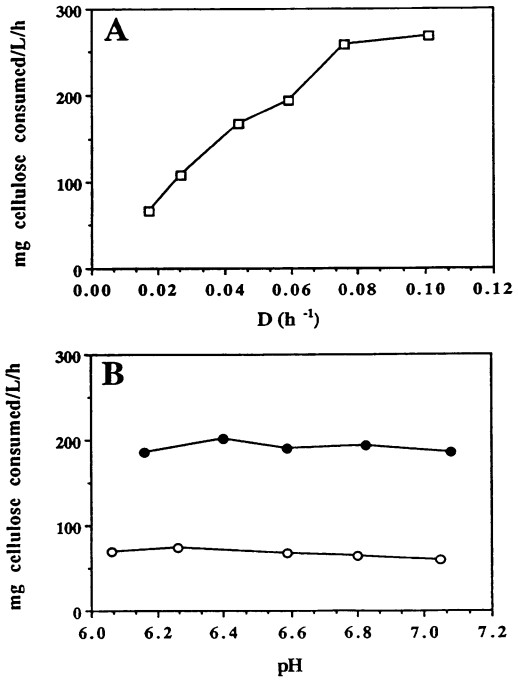

FIG. 3. Observed volumetric rates of cellulose consumption in cellulose-limited chemostats of *R. flavefaciens* FD-1. (A) Effect of *D* at pHs near the optimum (6.38 through 6.59) for cellulose consumption (runs 4, 9, 12, 16, 21, and 23). (B) Effect of pH at two different dilution rates: ○, ~0.02 h^{-1} (runs 2, 3, 4, 5, and 7); ●, ~0.06 h^{-1} (runs 14, 15, 16, 18, and 19).

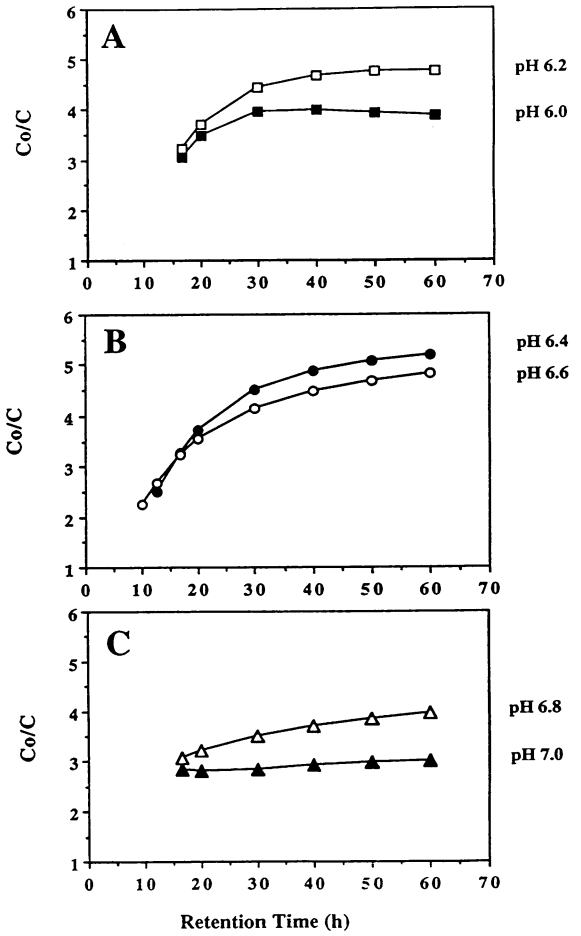

FIG. 4. Predicted ratio of reservoir cellulose concentration to reactor cellulose concentration at different values of pH and retention time (1/*D*).

ysis of amorphous and crystalline components of cellulose, (iii) limitation for nutrients in the medium other than cellulose, or (iv) changes in regulation of energy metabolism at low growth rates.

Poor mixing would cause a continuous artificial elevation of *C*. This possibility can be excluded, since (i) steady-state values of *C* were readily obtained (i.e., cellulose did not accumulate in the reactor), (ii) the measured values of *C* in the reactor and effluent were essentially identical, and (iii) the yield of soluble fermentation products (which migrate with the fluid phase) per unit of cellulose (which migrates with the solid phase) consumed was independent of t_R.

The relative crystallinity index of reactor cellulose (cultured at a longer t_R and the optimum pH) and reservoir cellulose were similar, suggesting that the amorphous and crystalline components were degraded at similar rates and that the decrease in the cellulose digestion rate at long t_Rs is not due to an accumulation of a more slowly hydrolyzed form of cellulose.

TABLE 2. Regression coefficients and statistical summary for parameters of the cellulose-limited fermentation of *R. flavefaciens* FD-1[a]

Coefficient	Fraction of cellulose consumed	Cell yield (mg/liter/h)	Yield (mmol) of:			Soluble sugar concn (mM)	Ratio[b]	
			Acetate	Succinate	Formate		a/a+s	s/s+f
α	−20.288	0.479	14.941	−14.098	−23.547	−3.431	12.146	11.253
β	300.805	−94.541	−804.942	217.619	378.108	−629.507	−384.046	−188.597
γ	−6.485	−0.241	−3.708	5.422	8.088	1.614	−3.712	−3.317
δ	−681.425	−798.895	2021.465	3985.015	1857.217	1131.180	−988.881	779.409
ε	−0.498	0.028	0.241	−0.480	−0.678	−0.154	0.297	0.261
ζ	−86.140	38.231	215.990	−120.300	−144.820	171.920	129.454	50.510
η	101.864	119.600	−299.396	−599.940	−290.995	−157.509	150.228	−111.822
θ	6.113	−3.570	−14.375	13.075	13.435	−11.798	−10.787	−3.459
r^2	0.836	0.814	0.641	0.312	0.467	0.570	0.428	0.589
P	0.001	0.001	0.043	0.618	0.255	0.102	0.336	0.082

[a] See the text for model equation.
[b] a/a+s, acetate/(acetate + succinate) ratio; s/s+f, succinate/(succinate + formate) ratio.

TABLE 3. Predicted C_o/C, true growth yields, and maintenance coefficients at various pH values

pH	C_o/C polynomial formula[a]	r^{2a}	$Y_g{}^b$	m^b	r^{2b}
6.00	$1.1891 + 0.1069t_R - 0.00133t_R^2$	0.964	0.268	0.070	1.000
6.20	$0.8840 + 0.1772t_R - 0.00201t_R^2$	0.989	0.279	0.064	0.999
6.40	$0.9976 + 0.1683t_R - 0.00175t_R^2$	0.996	0.297	0.063	0.992
6.60	$1.5837 + 0.1189t_R - 0.00114\,t_R^2$	0.998	0.301	0.063	0.960
6.80	$2.3067 + 0.0537\,t_R - 0.00046t_R^2$	0.999	0.278	0.059	0.893
7.00	$2.8661 + 0.0049\,t_R + 0.00014t_R^2$	0.946	0.234	0.051	0.737

[a] Polynomial equations were calculated from data of predicted C_o/C. r^2, regression coefficient.

[b] Y_g is the predicted true growth yield (grams of cells per gram of cellulose consumed), and m is the predicted maintenance coefficient (grams of cellulose consumed per hour per gram of cells). The data were calculated from Pirt plots obtained by solving the predicted growth yield equation (see the text and Table 2) at the indicated pHs and at dilution rates of 0.060, 0.050, 0.033, 0.025, 0.020, and 0.017 h^{-1} (equivalent to retention times of 16.67, 20, 30, 40, 50, and 60 h, respectively). r^2, linear regression coefficient.

The plots of C_o/C versus t_R showed that cellulose digestion followed first-order kinetics when t_R was less than 30 h (Fig. 4). Under these conditions, the concentration of cellulose (rather than other medium components) limited the rate of cellulose digestion. It is unlikely that nutrients other than cellulose limited cellulose digestion at longer t_Rs, because the media used throughout all our experiments were identical, except that the ruminal fluids varied both within and among runs because samples were collected on different dates. Nevertheless, systematic variation in C_o/C, rather than the random variation expected from random changes in ruminal fluid composition, was observed. Moreover, doubling the ruminal fluid at a longer t_R ($D = 0.019\ h^{-1}$, pH 6.59) did not increase cellulose utilization.

Carbohydrate metabolism in several ruminal bacteria has been shown to be influenced by growth rate (11, 21, 22). Pettipher and Latham (22) have reported that with a decreasing dilution rate, cellobiose-limited continuous culture of *R. flavefaciens* displayed increased amounts of carboxymethylcellulase and xylanase in culture supernatants. These workers suggested that these enzymes were escaping from cells as a result of leakage or cell lysis rather than by active secretion. Cellulases only function when in contact with their macromolecular substrates. For *R. flavefaciens* growing on insoluble cellulose as the sole energy source, cellulolytic enzymes that diffuse into the aqueous phase of the culture are of little value in hydrolyzing cellulose, which is completely covered with bacterial cells and their extensive glycocalyx. If the proportion of cell-associated cellulase decreases with decreasing growth rate, cellulose digestion at a low D (low growth rate) may be limited not by the concentration of cellulose but by the amount of enzyme at the cellulose surface.

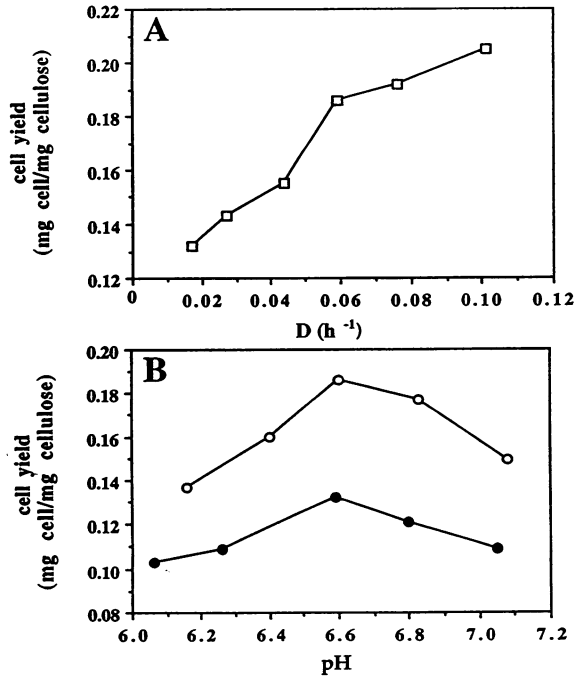

FIG. 5. (A) Effect of D on the observed cell yield of *R. flavefaciens* FD-1 at pHs near the optimum (6.38 to 6.59) (runs 4, 9, 12, 16, 21, and 23). (B) Effect of pH on observed cell yield: ○, D ~0.02 h^{-1}, pH 7.05 to 6.06 (runs 2, 3, 4, 5, and 7); ●, D ~0.06 h^{-1}, pH 7.08 to 6.16 (runs 14, 15, 16, 18, and 19).

The decreased rate of cellulose digestion that we observed at low Ds may also be due to reduced cellulase synthesis. The predicted proportion of cellulose consumption devoted to maintenance, calculable as mY/D, increased with decreasing growth rate. At pH 6.6, the proportion increased from 26% (t_R = 16.67 h) to 55% (t_R = 60 h) (Table 4). This dramatic increase at low growth rates may lead to a change of physiological status of organism and trigger regulatory changes (e.g., reduced synthesis of cellulolytic enzymes) that result in a decreased rate of cellulose digestion. However, hypotheses regarding the locations of cellulases and their potential regulation by growth rate are difficult to test, since enzyme activities within the glycocalyx are not readily measurable.

Our data indicate that *R. flavefaciens* FD-1 has a higher growth yield and lower maintenance requirement than those reported for *R. albus* (20). Within the optimum pH range for growth (6.4 to 6.6), the predicted true growth yields and maintenance requirements for *R. flavefaciens* are essentially

TABLE 4. Fraction of cellulose consumed for maintenance (m) at different combinations of retention time and pH

Retention time (h)	Fraction of cellulose used for m[a] at pH:		
	6.2	6.6	7.0
16.67	0.216	0.255	0.190
20.00	0.260	0.278	0.193
30.00	0.315	0.346	0.237
40.00	0.416	0.415	0.300
50.00	0.470	0.483	0.372
60.00	0.520	0.551	0.447

[a] Calculated as mY/D.

constant. The reduction in observed and predicted cell yields at nonoptimal pHs may be caused by (i) changes in cell composition (which would cause errors in our estimate of cell mass), (ii) changes in maintenance energy requirements, or (iii) shifts in fermentation pathways causing variable overall ATP yields (24). Since growth of these cultures is limited by the rate of cellulose hydrolysis, appreciable carbohydrate storage is unlikely. Thus, cell composition would not be expected to vary to the extent required to produce the large observed change in cell yield (Table 1 and Fig. 5).

Reduced cell yields at lower pHs appear to be due to a combination of increased maintenance energy requirements and decreased true growth yields (i.e., reduced ATP yields during catabolism). The former probably reflects the increased energy required to maintain intracellular pH at stronger transmembrane proton gradients, whereas the latter is most likely due to shifts in catabolic pathways. Another strain of *R. flavefaciens* (strain C) is known to synthesize ATP via phosphoenolpyruvate carboxykinase during succinate formation and via pyruvate kinase and acetate kinase during acetate formation (10). It is not known whether any strain of *R. flavefaciens* can also synthesize ATP in the reduction of fumarate to succinate. If such were not the case, then synthesis of succinate and acetate from C_3 intermediates would proceed with a different ATP yield. However, assignment of growth yield shifts in *R. flavefaciens* FD-1 to changes in end product ratios will require a more complete understanding of the catabolic pathways of this strain.

Reduced cell yields were also observed and predicted at pH values of ≥6.8. Because the Pirt plots of predicted $1/Y$ versus t_R were nonlinear, the resulting predicted values of Y_g and m are uncertain and the relative contributions of these two components to cell yields cannot be assessed.

In general, most of the fermentation parameters that were predicted well by the response surface equations showed relatively little change with pH at low Ds (<0.03 h^{-1}). However, at higher Ds, these parameters generally showed strong sensitivity to pH in both directions away from the pH optimum. The extensive cellulose fermentation at near pH 6.0 by our cultures of *R. flavefaciens* suggests that ruminal cellulose digestion may not be as pH sensitive as has been suggested by other pure culture studies (26), although clearly the organism does not grow at pH values below 5.9 (Fig. 1). Although cellulose digestion by a single species of ruminal cellulolytic bacterium in continuous culture cannot be directly extrapolated to the degradation of forage fiber by the entire microbial population in a discontinuously fed rumen, these pure-culture data might provide a general model for the cellulolytic bacterial growth in the rumen, if the cellulose fermentation characteristics of *R. flavefaciens* FD-1 are similar to those of other ruminal cellulolytic bacteria.

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