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Production of Succinate from Glucose, Cellobiose, and Various Cellulosic Materials by the Ruminal Anaerobic Bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*

R. R. GOKARN,[1] M. A. EITEMAN,* S. A. MARTIN,[2] AND K.-E. L. ERIKSSON[3]

[1]*Department of Biological and Agricultural Engineering, Driftmier Engineering Center;* [2]*Departments of Animal and Dairy Science and Microbiology; and* [3]*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602*

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ABSTRACT

The production of organic acids by two anaerobic ruminal bacteria, *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD-1, was compared with glucose, cellobiose, microcrystalline cellulose, Walseth cellulose (acid swollen cellulose), pulped paper, and steam-exploded yellow poplar as substrates. The major end product produced by *F. succinogenes* from each of these substrates was succinate (69.5–83%), the principal secondary product was acetate (16–30.5%). Maximum succinate productivity ranged from 14.1 mg/L · h for steam-exploded yellow Poplar to 59.7 mg/L · h for pulped paper. For *R. flavefaciens*, the major end product from cellobiose, microcrystalline cellulose, and acid-swollen Walseth cellulose was acetate (39–46%), pulped paper and steam-exploded yellow poplar yielded succinate (42–54%) as the major product. Maximum succinate productivity by *R. flavefaciens* ranged from 9.21 mg/L · h for cellobiose to 43.1 mg/L · h for pulped paper. In general, much less succinate was produced at a lower maximum productivity by *R. flavefaciens* than by *F. succinogenes* under similar fermentation conditions. The maximum

*Author to whom all correspondence and reprint requests should be addressed.

succinate productivities by these two organisms are comparable to the previously reported value of 59 mg/L · h for *Anderobiospirillum succiniciproducens* grown on glucose and corn steep liquor.

Index Entries: Fermentation; succinate; *Fibrobacter succinogenes; Ruminococcus flavefaciens*; ruminal bacteria; lactate; acetate; formate; cellulose.

INTRODUCTION

Cellulosic materials are the most abundant forms of waste organic matter *(1)*. Current environmental and social issues and the realization that fossil resources are limited have stimulated interest in the conversion of renewable biomass, such as cellulosic wastes, into commodity chemicals. Research has focused mostly on the conversion of biomass to fuel ethanol *(2–4)*, but interest has grown in developing biological technologies for the production of organic acids from renewable sources *(5,6)*.

Succinic acid and its derivatives may be used as specialty chemicals for applications in foods, pharmaceuticals, and cosmetics, and as an intermediate in the production of 1,4-butanediol and tetrahydrofuran *(7–10)*. Succinic acid may be chemically manufactured from maleic anhydride via hydrogenation and hydration steps. However, microbial processes have also been considered for the production of succinic acid, for example, by using the anaerobe *Anaerobiospirillum succiniciproducens* with dextrose as substrate *(7–9,11,12)*. Direct conversion of cellulosic waste materials to succinate by anaerobic microorganisms has received limited attention, even though succinate producing cellulolytic ruminal bacteria have been identified *(13–16)*. The present study compares the direct conversion of cellulosic materials, as well as glucose and cellobiose, to succinate by two predominant ruminal cellulolytic bacteria, *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD-1.

The major fermentation end products from cellulose metabolism by *F. succinogenes* and *R. flavefaciens* are succinate, formate, and acetate *(17)*. Both organisms metabolize the glucose formed in cellulose hydrolysis via the Embden-Meyerhof-Parnas pathway and the reductive tricarboxylic acid cycle *(18,19)*. Succinate production occurs via phosphoenolpyruvate carboxykinase and fumarate reductase; acetate is produced via pyruvate kinase and acetate kinase *(18–21)*. Both organisms require carbon dioxide for the conversion of glucose to succinate; carbon dioxide is evolved during the formation of acetate *(18–21)*. These pathways are very similar to those used by *A. succiniciproducens (22)*. Even though one strain of *R. flavefaciens* has the enzymes required to produce lactate, lactate production has been previously observed only in the absence of carbon dioxide *(19)*. Neither *F. succinogenes* S85 nor *R. flavefaciens* FD1 form lactate from microcrystalline cellulose in continuous culture *(14,15)*.

MATERIALS AND METHODS

Organisms and Growth Medium

F. succinogenes S85 and *R. flavefaciens* FD-1 (provided by P. J. Weimer, USDA, U.S. Dairy Forage Research Center, Madison, WI) were cultivated in defined basal media, pH 6.5, of the following composition: $K_2HPO_4 \cdot 3H_2O$, 0.292 g/L; KH_2PO_4, 0.24 g/L; $(NH_4)_2SO_4$, 0.48 g/L; NaCl, 0.48 g/L; $MgSO_4 \cdot 7H_2O$, 0.12 g/L; $CaCl_2 \cdot 2H_2O$, 0.064 g/L; $MnCl_2 \cdot 4H_2O$, 0.1 g/L; Na_2CO_3, 4 g/L; cysteine hydrochloride, 0.6 g/L; isobutyric acid, 57.6 mg/L; isovaleric acid, 55.8 mg/L; n-valeric acid, 56.4 mg/L; 2-methyl butyric acid, 54 mg/L; resazurin, 1.0 mg/L; thiamine HCl, 0.44 mg/L; calcium D-pantothenate, 0.44 mg/L; nicotinamide, 0.44 mg/L; riboflavin, 0.44 mg/L; pyridoxine, 0.44 mg/L; biotin, 0.1 mg/L; vitamin B_{12}, 4.0 μg/L; folic acid, 2.0 μg/L; tetrahydrofolic acid, 2.0 μg/L. Neither organism catabolizes the volatile fatty acids *(23)*, but require a fraction of these acids for building cell membranes.

Fermentation Studies

Batch fermentations (500 mL liquid volume) containing 10.0 g/L of substrate were conducted in 550-mL gas-washing bottles at 39°C. Substrates included glucose, cellobiose (Sigma, St. Louis, MO), Avicel microcrystalline cellulose PH102 (FMC, Philadelphia, PA), steam-exploded yellow poplar *(Liriodendron tulipifera)* (provided by W. G. Glasser, Virginia Tech., Blacksburg, VA), and pulped paper and acid-swollen cellulose prepared by the method of Walseth *(24)*. Pulped paper (500 g) was prepared by first shredding paper (Xerox 4200, Xerox, Palo Alto, CA) in an office shredder and then pulping with 8.5 L of water for 75 min at room temperature in a laboratory pulper. Prior to its use as a substrate, pulped paper was thoroughly rinsed and then spun-dried at room temperature in a cloth drier to a 29% water content. Bacterial cultures (50 mL) grown on microcrystalline cellulose served as inocula for all substrates. Samples were anaerobically withdrawn from each gas-washing bottle using a sterile syringe and needle and stored at –20°C. For the cellobiose and glucose fermentations resazurin was not used in the medium, and growth was monitored with a spectrophotometer at 600 nm (OD). Duplicate gas-washing bottles were used for each substrate, and each washing bottle had a continuous flow of oxygen-free carbon dioxide.

Analyses

Cells and solid substrate were removed from the medium by centrifugation (13,000g, 25°C, 10 min). Acetate, formate, lactate, and succinate were measured by high-pressure liquid chromatography using a Shimadzu HIC-6A ion chromatography system with a 5 μL sample loop. The system

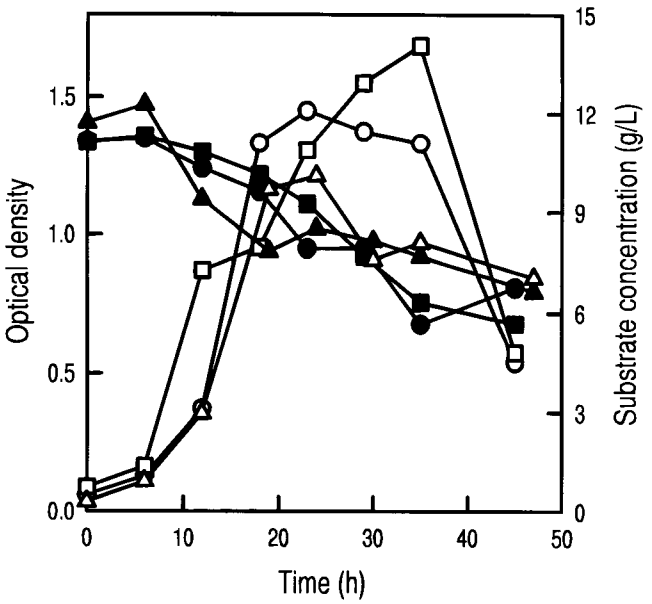

Fig. 1. Average OD (hollow symbols) and substrate concentration (filled symbols) during fermentation by *F. succinogenes* or *R. flavefaciens* on 10 g/L of soluble substrates: *F. succinogenes* on glucose (○, ●), *F. succinogenes* on cellobiose (△, ▲); *R. flavefaciens* on cellobiose (□, ■).

consisted of a model LC-6A pump, CTO-6AS oven, SCL-6B controller, and a SPD-6AV UV/visible detector using Chromatopac C-R4A software. A Coregel 64H (Interaction Chromatography, San Jose, CA) ion-exclusion column (300 × 7.8 mm ID) of 10 μm particle size and guard column were used in the analyses. Separation of fermentation products was accomplished at 47°C with 7 mN H_2SO_4 as the eluent. The UV detector operated at 210 nm, and the eluent flow rate was 0.6 mL/min. Glucose and cellobiose concentrations in the medium were determined by the DNS method *(25)*.

RESULTS AND DISCUSSION

Batch fermentation studies were conducted to compare organic acid production from simple and complex substrates. Growth of *F. succinogenes* on glucose and cellobiose and that of *R. flavefaciens* on cellobiose are shown in Fig. 1. *F. succinogenes* attained an OD of 1.0 after approx 18 h. However, the organism utilized only 40% of the glucose available in the medium. *R. flavefaciens* strain FD-1 cannot utilize glucose as an energy source *(26)*, hence, no glucose fermentations were conducted with this organism. Both organisms were able to ferment cellobiose, and a higher maximum OD was achieved with *R. flavefaciens* than with *F. succinogenes*. During cellobiose

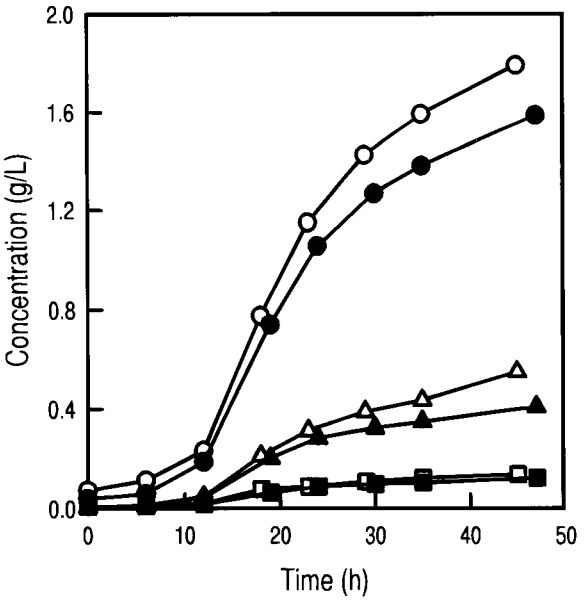

Fig. 2. Average end product concentrations from 10 g/L glucose (hollow symbols) and 10 g/L cellobiose (filled symbols) fermentations by *F. succinogenes* succinate (○, ●); acetate (△, ▲); and formate (□, ■).

fermentation, *F. succinogenes* utilized about 44% of the available substrate; *R. flavefaciens* utilized 49%. The observed cessation of substrate consumption may be caused by inhibition from a fermentation product.

The products from these fermentations by *F. succinogenes* are shown in Fig. 2. Succinate was the major fermentation product from both substrates, and the maximum productivities were 46.8 mg/L · h from glucose and 44.1 mg/L · h from cellobiose. The acetate productivities were 13.3 mg/L · h and 11.7 mg/L · h on glucose and cellobiose, respectively; the formate productivities were 3.54 mg/L · h and 3.72 mg/L · h. Lactate was not detected in these *F. succinogenes* fermentations. Fermentation of glucose results in a succinate yield of 0.38 g/g glucose and an acetate yield of 0.11 g/g glucose consumed. With cellobiose as substrate, both succinate (0.30 g/g) and acetate yields (0.08 g/g) were lower than for glucose.

Cellobiose fermentation by *R. flavefaciens* yielded acetate and formate as the major products (Fig. 3). Acetate productivity was 34.9 mg/L · h, formate productivity was 32.1 mg/L · h. *R. flavefaciens* also produced some succinate (9.2 mg/L · h) and trace lactate. The succinate production by *F. succinogenes* from cellobiose was four times greater than production by *R. flavefaciens*. The product yields by *R. flavefaciens* were 0.08 g succinate/g and 0.24 g acetate/g of cellobiose consumed.

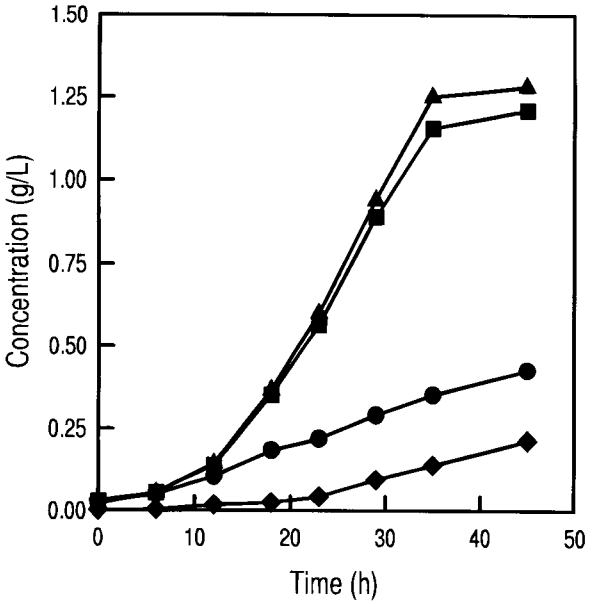

Fig. 3. Average end product concentrations from 10 g/L cellobiose fermentations by *R. flavefaciens*: acetate (▲); formate (■); succinate (●), and lactate (◆).

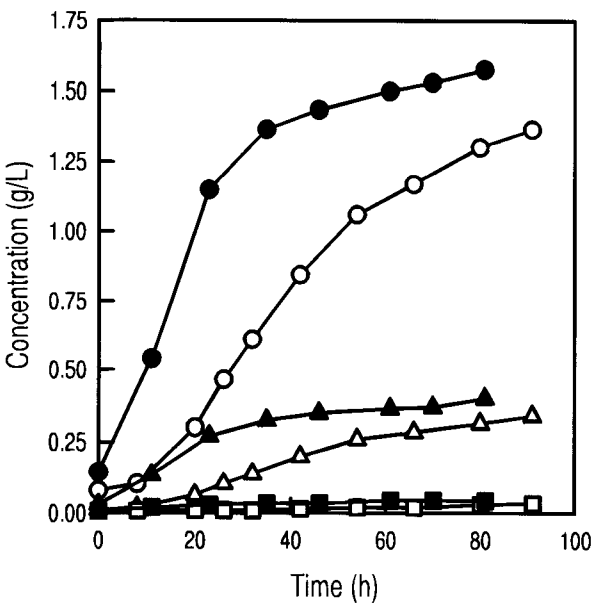

Fig. 4. Average end product concentrations from 10 g/L Walseth cellulose (filled symbols) and from 10 g/L microcrystalline cellulose (hollow symbols) fermentation by *F. succinogenes*: succinate (○, ●), acetate (△, ▲), and formate (□, ■).

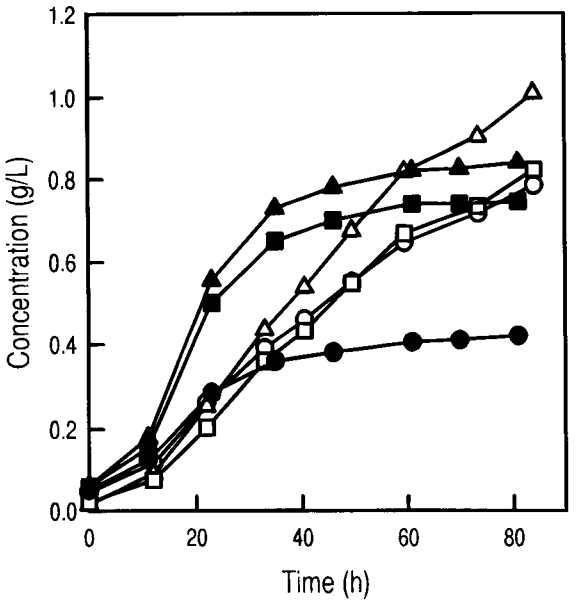

Fig. 5. Average end product concentrations from 10 g/L Walseth cellulose (filled symbols) and from 10 g/L microcrystalline cellulose (hollow symbols) fermentations by *R. flavefaciens*: acetate (△, ▲), formate (□, ■), and succinate (○, ●).

End product concentrations from the fermentations of Walseth cellulose and microcrystalline cellulose by *F. succinogenes* are shown in Fig. 4. Succinate was the major fermentation end product with maximum productivities of 34.8 mg/L · h and 18.2 mg/L · h, respectively. Acetate had maximum productivities of 8.62 mg/L · h and 4.62 mg/L · h, respectively; formate was a minor product. These organic acid production rates were lower than those obtained in the glucose and cellobiose fermentations. The succinate productivity and acetate productivity were proportionately greater for Walseth cellulose than from microcrystalline cellulose. Therefore, the succinate-to-acetate-mass ratio achieved with these substrates was similar. The increase in productivities on Walseth cellulose might be attributed to its more amorphous nature.

Figure 5 shows the results of cellulose fermentations by *R. flavefaciens*. In contrast to *F. succinogenes*, acetate was the principal end product from both substrates, with maximum productivities of 19.1 mg/L · h for Walseth cellulose and 13.5 mg/L · h for microcrystalline cellulose. Formate was produced with maximum productivities of 16.8 mg/L · h and 10.8 mg/L · h; succinate had maximum productivities of 8.85 mg/L · h and 10.2 mg/L · h, respectively. Trace lactate was also detected during the Walseth cellulose fermentation (data not shown). *R. flavefaciens* achieved approximately twice the final succinate concentration from microcrystalline cellulose as from

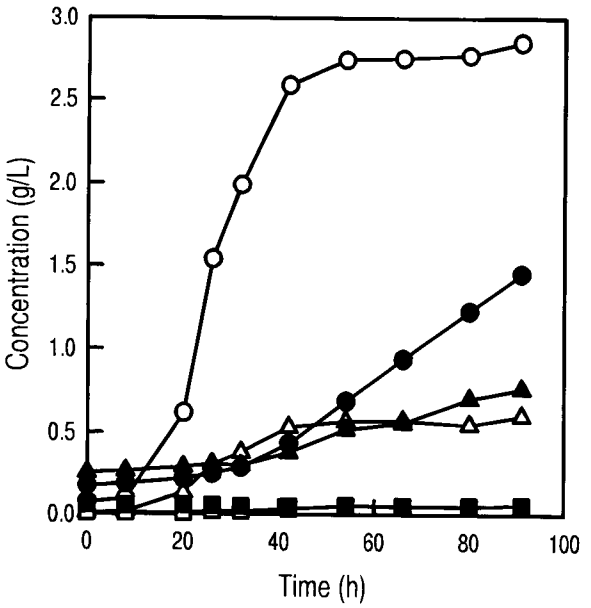

Fig. 6. Average end product concentrations from 10 g/L pulped paper (hollow symbols) and 10 g/L steamed-exploded yellow poplar (filled symbols) fermentations by *F. succinogenes*: succinate (○, ●), acetate (△, ▲), and formate (□, ■).

cellobiose, but produced less acetate and formate on the complex substrate. A striking difference occurred between the Walseth cellulose and the microcrystalline cellulose fermentations. For microcrystalline cellulose, acid production continued at the same rate through 80 h of fermentation. In contrast, the rate of acid production from Walseth cellulose was high during the first 40 h, at which time the production of each of the three acids essentially ceased. The comparatively sudden cessation of acid production in the Walseth cellulose fermentations has the appearance of end product inhibition. However, the final concentration of the three acids in the Walseth cellulose fermentations are lower than the final concentrations in either the cellobiose or microcrystalline cellulose fermentations.

The end products resulting from the fermentation of pulped paper and steam-exploded yellow poplar by *F. succinogenes* are shown in Fig. 6. For both substrates, succinate was the major product. From pulped paper, the productivities of succinate, acetate, and formate were 59.7 mg/L · h, 11.6 mg/L · h, and 0.85 mg/L · h, respectively. Succinate production from pulped paper was 3.3 times greater than from microcrystalline cellulose; acetate production from pulped paper was 2.5 times greater than from microcrystalline cellulose. Therefore, the succinate-to-acetate-mass ratio increased from 3.9 for microcrystalline cellulose to 5.2 for pulped paper. The productivity of succinate by *F. succinogenes* on pulped paper was simi-

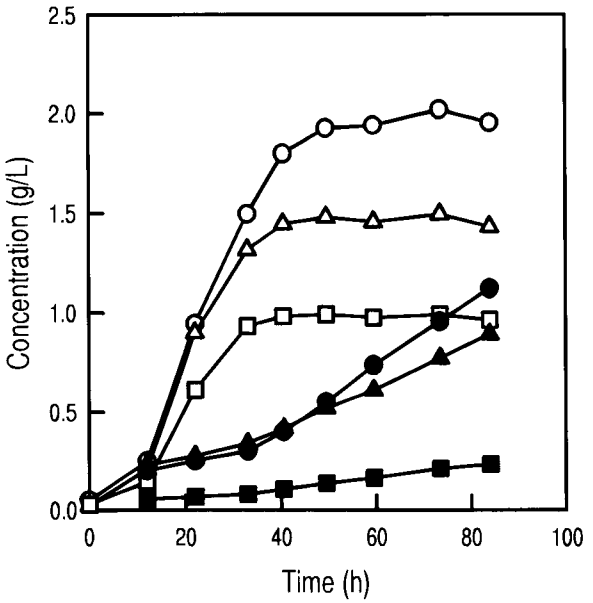

Fig. 7. Average end product concentrations from 10 g/L pulped paper (hollow symbols) and steam-exploded yellow poplar (filled symbols) fermentations by *R. flavefaciens*: succinate (○, ●), acetate (△, ▲), and formate (□, ■).

lar to the succinate productivity (59 mg/L · h) of *A. succiniciproducens* grown on glucose and corn liquor (*22*). The productivity of each of the three acids and the succinate-to-acetate mass ratio increased in the following order: microcrystalline cellulose, Walseth cellulose, and pulped paper. This order of increasing acid productivities may be associated with the decreasing crystallinity or may be caused by the increasing available surface area. In the case of steam-exploded yellow poplar, succinate (14.1 mg/L · h) and acetate (6.18 mg/L · h) production rates were lower than from pulped paper; formate was not produced. Compared with microcrystalline cellulose, acetate production was 25% greater from yellow poplar; succinate production was 20% lower. Thus, the succinate-to-acetate mass ratio averaged only 2.3 for the yellow poplar fermentation by *F. succinogenes*.

The end products from pulped paper and steam-exploded yellow poplar fermented by *R. flavefaciens* are shown in Fig. 7. In contrast to the fermentation of cellobiose, microcrystalline cellulose, and Walseth cellulose, succinate was the major product when either pulped paper or yellow poplar was the substrate. The rate of succinate production from pulped paper was 4.2 times greater than from microcrystalline cellulose; the rates of acetate and formate production were only 2.2–2.6 times greater than from microcrystalline cellulose. Therefore, the succinate-to-acetate-mass ratio increased from 0.75 for microcrystalline cellulose to 1.2 for pulped

paper. Like the Walseth cellulose fermentations (Fig. 5), the generation of these three acids was initially high, but ceased after about 40 h, even though visually significant amounts of pulped paper remained in the fermenter. In this case, however, the final concentrations of succinate (2.0 g/L) and acetate (1.5 g/L) were greater than for any other fermentation studied, so that acid inhibition might have occurred. Also, the presence of tannins in the poplar may have affected cell growth, attachment, or acid production. Greater acid production by *R. flavefaciens* from pulped paper than from microcrystalline cellulose might be similarly attributed to crystallinity or surface area. The productivities of all three acids from steam-exploded yellow poplar were lower than with pulped paper, a pattern similar to that observed with *F. succinogenes*. In contrast to *F. succinogenes*, however, *R. flavefaciens* produced less acetate and more succinate from steam-exploded yellow poplar than from microcrystalline cellulose, resulting in a succinate-to-acetate-mass ratio of 1.5, the greatest observed for all fermentations studied with this organism.

The maximum volumetric productivities for the principal organic acids from the different substrates are summarized in Table 1. For each substrate, *F. succinogenes* fermentations yielded a greater succinate mass fraction and a higher succinate productivity than the corresponding *R. flavefaciens* fermentations. For both bacteria, the greatest succinate productivity occurred with pulped paper. The highest concentration of succinate was 2.8 g/L after 40 h of pulped paper fermentation by *F. succinogenes* (Fig. 6), and 2.0 g/L after 40 h of pulped paper fermentation by *R. flavefaciens* (Fig. 7). The total acid concentrations corresponding to these fermentations were 3.4 g/L and 4.5 g/L, respectively. During fermentation of glucose or cellobiose by *F. succinogenes*, however, substrate utilization slowed when the succinate concentration had reached 1.8 g/L (and total acid concentration was 2.3 g/L). Similarly, during cellobiose fermentation by *R. flavefaciens*, substrate utilization slowed when succinate concentration reached 0.3 g/L (and total acid concentration was 3.0 g/L). These observations suggest that succinate and the total acid concentration had not caused inhibition during these soluble substrate fermentations, unless some physical or metabolic protection was afforded the organism during the solid substrate fermentations.

CONCLUSIONS

The ruminal cellulolytic bacteria *F. succinogenes* and *R. flavefaciens* produced succinate, acetate, and formate as the major end products of glucose, cellobiose, or cellulose fermentation; *R. flavefaciens* also produced trace lactate from cellobiose and Walseth cellulose. In general, *F. succinogenes* produced more succinate at a greater rate than *R. flavefaciens*. However,

Table 1
Maximum Average Volumetric Productivities and Mass Fractions of the Major Products in 0.5 L Batch Fermentations
(Standard Error from Multiple Fermentations in Parentheses)

Organism	Substrate	Volumetric Productivity (mg/L · h) Succinate		Acetate	Mass Fraction of Products Formed (g/g) Succinate:Formate:Acetate
F. succinogenes	Glucose	46.8	(1.6)	3.54 (0.24)	13.3 (0.2)	0.735:0.055:0.208
	Cellobiose	44.1	(0.3)	3.72 (0.02)	11.7 (0.5)	0.740:0.062:0.196
	Avicel	18.2	(0.1)	0.24 (0.00)	4.62 (0.00)	0.789:0.010:0.200
	Walseth cellulose	34.8	(0.0)	0.78 (0.13)	8.43 (0.14)	0.790:0.017:0.191
	Paper pulp	59.7	(1.3)	0.85 (0.06)	11.6 (0.2)	0.827:0.011:0.160
	Yellow poplar	14.1	(1.2)	0.00 (0.00)	6.18 (0.44)	0.695:0.000:0.305
R. flavefaciens	Cellobiose	9.21	(0.25)	32.1 (1.1)	34.9 (1.7)	0.120:0.421:0.458
	Avicel	10.2	(0.2)	10.8 (0.2)	13.5 (0.1)	0.295:0.314:0.390
	Walseth cellulose	8.85	(0.17)	16.8 (0.0)	19.1 (0.2)	0.197:0.375:0.426
	Paper pulp	43.1	(2.2)	23.6 (0.6)	35.1 (1.0)	0.423:0.231:0.344
	Yellow poplar	13.7	(0.0)	2.60 (0.17)	9.01 (0.12)	0.541:0.102:0.356

R. flavefaciens produced more total acid (i.e., succinate + acetate + formate). Both organisms were able to utilize steam-exploded yellow poplar as a substrate, but produced much lower rates of succinate, formate, and acetate on this substrate, compared with other cellulosic substrates.

ACKNOWLEDGMENTS

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