Journal of General Microbiology (1979), **110**, 29–38. *Printed in Great Britain*

Production of Enzymes Degrading Plant Cell Walls and Fermentation of Cellobiose by *Ruminococcus flavefaciens* in Batch and Continuous Culture

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(*Received* 30 *May* 1978; *revised* 19 *July* 1978)

Production of enzymes degrading plant cell walls was studied using media containing cellobiose or ammonium ions (NH_4^+) as limiting nutrients. Carboxymethylcellulase (CM-cellulase), xylanase and pectin lyase were primarily cell-associated during exponential growth in batch culture but accumulated in the supernatant during the stationary phase. Activities of CM-cellulase and xylanase were higher in cellobiose-limited than in NH_4^+-limited continuous cultures, were inversely related to the growth rate and became progressively more cell-associated as the growth rate increased. The proportion of fermentation products in cellobiose-limited continuous cultures was dependent on the growth rate and the calculated cell yields per mol ATP (Y_{ATP}) varied between 11·92 and 16·39. Glutamate dehydrogenase, an ammonia-assimilating enzyme, was most active in NH_4^+-limited continuous cultures. These results are discussed in relation to the growth and metabolism of *Ruminococcus flavefaciens in vivo*.

INTRODUCTION

The plant cell wall is a major source of energy for ruminant herbivores and its degradation in the rumen is largely dependent upon the activity of three species of cellulolytic bacteria – *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Bacteroides succinogenes*. In addition to being cellulolytic, most strains of *R. flavefaciens* can also degrade hemicellulose and pectin and we have described some characteristics of the enzymes responsible for these activities in the preceding paper (Pettipher & Latham, 1979).

Rumen fluid contains enzymes active against soluble cellulose derivatives (King, 1956), but the concentration of free cellulases is generally low (Halliwell, 1957). The proportion of rumen cellulase that is associated with the bacterial cell surface may therefore be considerably higher than the 5% detected by King (1959). Evidence based on zones of clearing in cellulose agar led Hungate (1947) to conclude that *B. succinogenes* possessed a firmly cell-bound enzyme whilst those of the ruminococci were extracellular. Hungate's conclusions have not been verified experimentally and it may be significant that cellulases of several other species of bacteria from the soil or aquatic environments, such as *Cellvibrio fulvus* (Berg, 1975) and *Cytophaga* sp. (Chang & Thayer, 1977), are predominantly cell-bound.

Ruminococcus albus (Iannotti *et al.*, 1973) and several other species of rumen bacteria have been grown in continuous culture and it is apparent that their carbohydrate metabolism is greatly influenced by their rate of growth. Since the physiological status of an organism has a major effect on the activity of many anabolic and catabolic enzymes, we have examined the production and location of the cellulases, hemicellulases and pectinases produced by *R. flavefaciens* during batch culture and during continuous culture under carbon (cellobiose) and nitrogen (ammonia) limitation. The fermentation of cellobiose and

0022-1287/79/0000-8192 $02.00

the activity of the ammonia-incorporating enzyme, glutamate dehydrogenase, were also examined. A preliminary report of this work has appeared previously (Pettipher & Latham, 1978).

METHODS

Organisms and culture media. Ruminococcus flavefaciens strains 67 and 66 (Latham *et al.*, 1978) and the type strain c94 (from Dr M. P. Bryant, Department of Dairy Science, University of Illinois, U.S.A.) were cultivated in basal medium BM10 containing 0·1 % (w/v) cellobiose (Pettipher & Latham, 1979). Methods of strict anaerobic culture (Bryant, 1972) were used throughout and all cultures were incubated at 39 °C.

Carbohydrate substrates. The ability of strains c94, 66 and 67 to use various carbohydrates was tested using medium BM10 supplemented with 0·2 % (w/v) of the appropriate carbohydrate. The nature and sources of the celluloses, hemicelluloses and pectin are listed by Pettipher & Latham (1979). Growth on larchwood xylan and oak sapwood hemicellulose was assessed by measuring the disappearance of carbohydrate (Dubois *et al.*, 1956) and by the increase in total cell numbers.

Nitrogen requirements. Nitrogen requirements were determined using modified medium BM10 lacking trypticase, yeast extract and ammonium sulphate and containing 0·3 % (w/v) cellobiose. Sodium sulphate (0·09 %, w/v) was added to restore the level of sulphate and vitamins were supplied as a mixture at the following final concentrations: biotin, folic acid and vitamin B12, 50 μg l^{-1} each; *p*-aminobenzoic acid, 100 μg l^{-1}; pyridoxin.HCl, thiamin.HCl, riboflavin and thymidine, 2 mg l^{-1} each. Ammonium sulphate (0·1 %, w/v), trypticase (0·5 %, w/v) and yeast extract (0·3 %, w/v) were incorporated as nitrogen sources either singly or in combination.

Production of enzymes in batch culture. Carboxymethylcellulase (CM-cellulase), xylanase, aryl β-glucosidase and pectin lyase activities were measured during batch culture of *R. flavefaciens* strain 67 in 10 l BM10 containing 0·1 % (w/v) cellobiose as the sole energy source. Samples (50 ml) were taken immediately after inoculation and at frequent intervals until the culture reached maximum absorbance and entered stationary phase. Cells were harvested from these samples by centrifugation, resuspended in Oxoid phosphate-buffered saline (PBS) and, together with the culture supernatants, stored frozen. The enzyme activities and reducing-sugars were assayed using the methods of Pettipher & Latham (1979).

Location of CM-cellulase, xylanase and aryl β-glucosidase. Cells from late-exponential phase cultures of *R. flavefaciens* strain 67 in BM10 containing cellobiose (0·1 %, w/v) were harvested by centrifugation and resuspended in chilled PBS. A portion of the suspension was lysed at approximately 154 MPa using a French pressure cell, and the lysate was separated into soluble and particulate fractions by centrifugation at 110000*g* for 2 h. Enzyme activities were determined in the culture supernatants, suspensions of whole cells and in the soluble and particulate fractions of the cell lysate. To test for possible leakage of intracellular contents from whole cells, the supernatants obtained by centrifuging whole and lysed cell suspensions were compared for aldolase activity using a test kit (Boehringer).

Production of enzymes in continuous culture. CM-cellulase, xylanase and glutamate dehydrogenase activities were monitored during growth of *R. flavefaciens* strain 67 in continuous culture using BM10 without trypticase as the basal medium. A carbohydrate-limited medium was obtained by incorporating 0·15 % (w/v) cellobiose, and an ammonia-limited medium was obtained by reducing the concentration of ammonium sulphate to 0·01 % (w/v), adding sodium sulphate (0·08 %, w/v) and increasing the cellobiose concentration to 0·3 % (w/v). The apparatus used was described by Latham & Legakis (1976). Four to six samples (20 ml each) were collected at each dilution rate when the absorbance and cell count of the culture was stable. At least one culture volume (360 ml) of medium was allowed to pass through the growth vessel between samples. The cells, cell lysates (from treatment in a French pressure cell) and culture supernatants obtained from these samples were assayed for CM-cellulase and xylanase activities. Glutamate dehydrogenase activity was determined by measuring changes in absorbance at 340 nm after incubation of cell lysates for 10 min at 30 °C in the presence of 0·2 ml 2-oxoglutarate (0·2 M), 0·03 ml NADPH (12 mM), 0·05 ml ammonium acetate (12·8 M) and 1·57 ml imidazole buffer (0·1 M, pH 7·0).

Yield and composition of bacteria in continuous culture. Dry weight yields were obtained by drying to constant weight washed cells from 50 ml culture effluent. The protein (Lowry *et al.*, 1951) and total carbohydrate (Dubois *et al.*, 1956) contents of cells were determined from neutralized acid hydrolysates. Intracellular periodate-reactive carbohydrate was observed by transmission electron microscopy using the periodic acid–thiosemicarbazide–silver proteinate method of Thiéry (1967).

Measurement of fermentation products. Acetic acid was detected by gas–liquid chromatography (g.l.c.) after mixing 1 ml culture supernatant with an equal volume of a 50 % (w/v) aqueous suspension of Zerolit 225 (BDH) in H^+-form. One μl of the supernatant was injected on a 2 mm i.d. × 1·5 m glass column packed with Chromosorb 101 (80 to 100 mesh) held at 180 °C; injector and detector temperatures were 200 °C and N_2 (30 ml min^{-1}) was used as the carrier gas. Succinate was detected as its dimethyl ester after methylation

of 1 ml of acidified and dried culture supernatant with 1 ml methanolic HCl at room temperature for 24 h. The methyl esters were analysed by g.l.c. using glass columns packed with 20% polyethylene glycol adipate as stationary phase on Chromosorb W-AW (60 to 80 mesh) at an operating temperature of 150 °C. Ethanol, formate and lactate were estimated using the methods of Latham & Legakis (1976). Hydrogen production was estimated from a separate carbohydrate-limited continuous culture of *R. flavefaciens* strain 67 grown in medium BM10 with cellobiose (0·2%, w/v) as the limiting nutrient. The H_2 concentration in the effluent stream of CO_2 from the culture vessel was measured by g.l.c. using silica gel (80 to 100 mesh) in 6 mm i.d. × 0·45 m glass columns held at 50 °C using a Katharometer detector and N_2 (50 ml min^{-1}) as carrier gas.

Hydrogen production from sodium formate. Cells from continuous cultures were harvested anaerobically by centrifugation and resuspended in basal medium BM10 at three times their original concentration. Portions of the cell suspensions were distributed under O_2-free CO_2 to stoppered test tubes to which solid sodium formate (50 mM final concentration) was then added. After mixing, the tubes were incubated at 39 °C for 1 h and the headspace gas was analysed for H_2. Tubes of basal medium plus formate and cell suspensions without added formate acted as controls.

RESULTS

Carbohydrate and nitrogen nutrition

All strains of *R. flavefaciens* grew well on cellulose and its constituent sugars, cellobiose and glucose, but growth was poor on oak sapwood hemicellulose and xylan. Xylose, arabinose, glucose, galactose, rhamnose and mannose comprise the major carbohydrates of most plant cell wall hemicelluloses but apart from glucose, only arabinose was fermented. Among the other carbohydrates likely to be present in plant cell walls, neither pectin, starch nor their constituent sugars, galacturonic acid and maltose, were fermented. However, strains 66 and 67 were able to utilize fructose.

Ammonium ions could satisfy all the nitrogen requirements of the ruminococci and neither trypticase nor yeast extract stimulated growth significantly. Medium BM10 was therefore modified for subsequent continuous culture studies by using ammonium sulphate as the major nitrogen source, omitting trypticase and retaining yeast extract as the source of vitamins.

Production of enzymes in batch culture

Maximum cell numbers were obtained with 40% of the cellobiose remaining but utilization continued during stationary phase until only trace amounts were detectable (Fig. 1). Tests using medium BM10 containing various concentrations of cellobiose, from 0 to 6 mM, confirmed that cellobiose was the only limiting nutrient. During the early phase of exponential growth, the activities of CM-cellulase and xylanase increased in proportion to cell numbers and remained predominantly cell-associated. Both activities continued to increase during the stationary phase but increasing proportions were detected in the culture supernatant. Pectin lyase was produced in a similar manner, whereas aryl β-glucosidase was produced most rapidly during the early stages of exponential growth and reached near maximum values well before stationary phase.

Most of the aryl β-glucosidase and a large proportion of the CM-cellulase in cells harvested at late-exponential phase was intracellular (Table 1). In contrast, a large proportion of xylanase activity was associated with whole cells and considerably less with the intracellular fraction. Leakage of whole cells was slight, as less than 3% of the total aldolase activity present in lysed cells was recovered in the supernatant of whole cell suspensions. All of the cell-associated aryl β-glucosidase and more than two-thirds of the CM-cellulase were present in the soluble fraction, but more than half the xylanase activity was associated with the particulate material.

Fermentation products in batch culture

The concentrations of fermentation products increased in proportion to cell numbers during the exponential growth phase and continued to increase until cellobiose had been

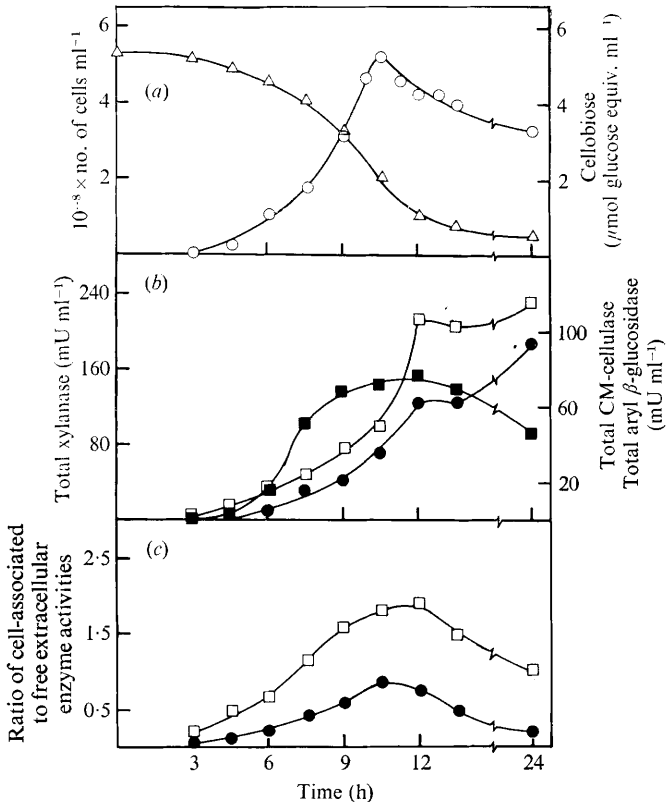

Fig. 1. Production of CM-cellulase, xylanase and aryl β-glucosidase during batch culture of *R. flavefaciens* strain 67. (*a*) Increase in total cell numbers (○) with utilization of carbohydrate (△). (*b*) Total xylanase (□), CM-cellulase (●) and aryl β-glucosidase (■) activities. (*c*) Ratio of cell-associated to culture supernatant xylanase (□) and CM-cellulase (●) activities.

Table 1. *CM-cellulase, xylanase and aryl β-glucosidase activities in whole cells, cell fractions and culture supernatant of R. flavefaciens strain 67 grown in batch culture*

	Enzyme activity (mU ml^{-1})		
Fraction	CM-cellulase	Xylanase	Aryl β-glucosidase
1. Culture supernatant	52·4	69·7	43·6
2. Whole cell suspension	28·3	82·1	47·7
3. Cell lysate	92·4	102·4	185·1
4. Intracellular*	64·1	20·3	137·4
Soluble	(70·3)†	(43·1)	(95·5)
Particulate	(29·7)	(56·9)	(4·5)

* Intracellular activity was calculated as (activity in fraction 3) − (activity in fraction 2).
† Figures in parentheses indicate percentage enzyme activities in soluble and particulate fractions after centrifuging fraction 3 at 110000*g* for 2 h.

depleted from the medium. The maximum concentrations of acetate, formate, succinate and lactate were reached after 13·5 h incubation and were 7·20, 2·94, 2·16 and 1·09 μmol ml^{-1}, respectively.

Production of enzymes in continuous culture

CM-cellulase and xylanase activities in cellobiose-limited cultures of *R. flavefaciens* strain 67 were approximately 1·7 and 2·6 times greater than in ammonia-limited cultures

Table 2. *CM-cellulase and xylanase activities in cells and culture supernatants from continuous cultures of R. flavefaciens strain* 67

Four to six separate samples taken at each dilution rate were pooled. Enzyme activity in cells was determined after lysis in a French pressure cell.

Limiting nutrient	Dilution rate (h^{-1})	CM-cellulase [mU (10^8 cells)$^{-1}$]		Xylanase [mU (10^8 cells)$^{-1}$]	
		Supernatant	Cell	Supernatant	Cell
Cellobiose	0·06	29·7 (±1·8)	25·0	45·5 (±1·1)	29·9
Cellobiose	0·10	18·4 (±1·5)	26·6	27·7 (±1·9)	42·2
Cellobiose	0·15	14·4 (±1·5)	20·1	18·8 (±1·5)	30·0
NH_4^+	0·06	10·3 (±1·8)	20·7	11·9 (±2·9)	15·5
NH_4^+	0·10	8·2 (±0·9)	19·4	10·7 (±1·3)	17·1
NH_4^+	0·15	5·5 (±0·7)	17·5	6·1 (±1·0)	13·0

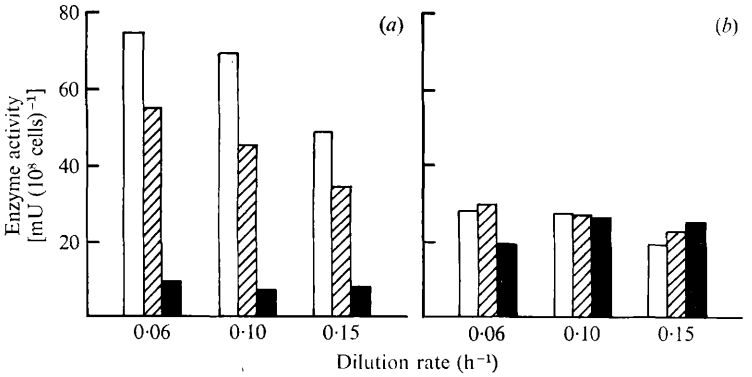

Fig. 2. Variation in total xylanase (□), total CM-cellulase (▨) and cell-associated glutamate dehydrogenase (■) activities with dilution rate and nutrient limitation during continuous culture of *R. flavefaciens* strain 67: (*a*) cellobiose-limited culture; (*b*) NH_4^+-limited culture.

but were inversely related to the growth rate (Fig. 2). The proportions of total CM-cellulase and xylanase activities that were associated with the cells increased with increasing dilution rate irrespective of the nutrient limitation (Table 2). Glutamate dehydrogenase activity was approximately three times greater in cells grown under ammonia limitation than in cells grown under cellobiose limitation and, unlike CM-cellulase, was independent of the growth rate (Fig. 2).

Cell yields and composition in continuous culture

A slight increase in cell numbers during continuous culture was observed as the dilution rate was increased (Table 3). In cellobiose-limited cultures 93% of the carbohydrate was used, but under ammonia limitation between 47 and 31% remained unfermented depending on the growth rate. Cellobiose was the only carbohydrate detected in the effluent media and analysis of the protein and carbohydrate contents of cells indicated that under ammonia limitation a substantial proportion of the carbohydrate used was retained within the cells. Most of this carbohydrate appeared to be in the form of closely packed periodate-reactive intracellular granules (Fig. 3*a*); these were less evident in cells from cellobiose-limited cultures (Fig. 3*b*).

Table 3. *Variation in cell count, hexose consumed and cell composition during continuous culture of R. flavefaciens strain 67*

Limiting nutrient	Dilution rate (h^{-1})	10^{-8} × no. of cells ml^{-1}	Hexose used [μmol (ml culture)$^{-1}$]	Dry wt [μg (ml culture)$^{-1}$]	Composition (% dry wt)	
					Protein	Carbohydrate
Cellobiose	0·06	4·92 (±0·18)	8·15	287	53·2	12·9
Cellobiose	0·10	5·54 (±0·10)	8·16	368	53·9	16·3
Cellobiose	0·15	6·32 (±0·35)	8·15	374	53·0	12·6
NH_4^+	0·06	4·80 (±0·13)	12·09	328	32·1	26·1
NH_4^+	0·10	5·49 (±0·19)	10·34	375	45·7	40·8
NH_4^+	0·15	6·38 (±0·20)	9·35	404	45·3	30·3

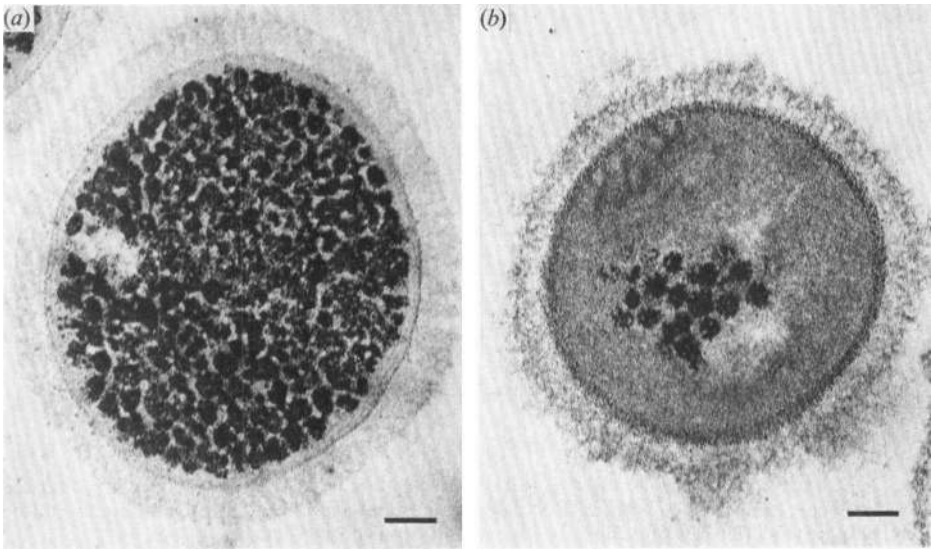

Fig. 3. Transmission electron micrographs of *R. flavefaciens* strain 67 showing intracellular granules of periodate-reactive carbohydrate. Cells were grown in continuous culture at a dilution rate of 0·06 h^{-1} under (*a*) ammonia limitation or (*b*) cellobiose limitation. Stained by the method of Thiéry (1967). Bar markers represent 0·1 μm.

Fermentation products and growth efficiency in continuous culture

Formate, and to a lesser extent succinate, accumulated with increasing growth rate in cellobiose-limited cultures, but lactate and acetate both decreased (Fig. 4). In ammonia-limited cultures the concentrations of formate and acetate were similar and remained largely unchanged as the growth rate increased but succinate decreased progressively. Variations in the concentration of lactate were large and did not appear to be related to changes in any other product. Ethanol was also present in trace amounts [less than 2·0 μmol (100 μmol hexose fermented)$^{-1}$] at all growth rates in ammonia-limited but not cellobiose-limited cultures.

As the dilution rate of cellobiose-limited cultures was increased from 0·05 to 0·24 h^{-1}, H_2 production decreased from 65·8 to 17·6 μmol (100 μmol hexose used)$^{-1}$. Cells harvested at the lower dilution rate produced 0·08 μmol H_2 (ml headspace gas)$^{-1}$ when incubated in basal medium alone and addition of sodium formate to this suspension increased the concentration of H_2 to 0·92 μmol ml^{-1}. Similarly treated cells grown at the higher dilution rate did not produce H_2 even in the presence of sodium formate.

The carbon recovery, oxidation–reduction ratio (O/R) of the fermentation products

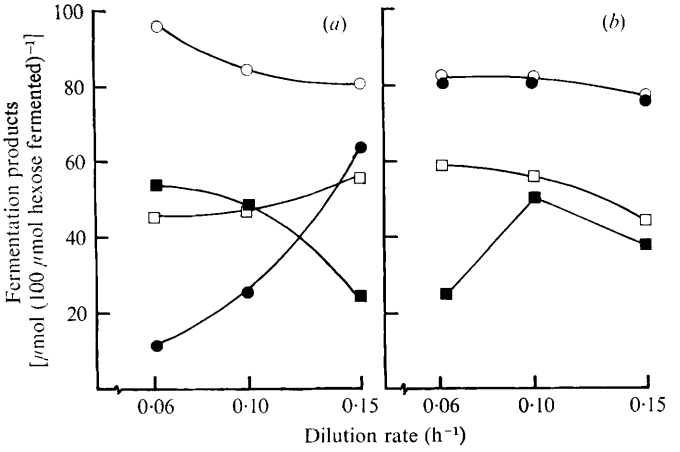

Fig. 4. Major products from the fermentation of cellobiose by *R. flavefaciens* strain 67 at different dilution rates in (*a*) cellobiose-limited and (*b*) NH_4^+-limited continuous cultures: ○, acetate; ●, formate; □, succinate; ■, lactate. Concentrations of fermentation products are expressed as μmol (100 μmol hexose fermented)$^{-1}$; the values of total hexose used were corrected for intracellular carbohydrate.

Table 4. *Carbon recoveries, oxidation–reduction (O/R) balances of fermentation products and yields of bacteria per mol ATP (Y_{ATP}) during continuous culture of R. flavefaciens strain 67*

Limiting nutrient	Dilution rate (h^{-1})	Carbon recovery* (%)	O/R balance†	ATP [mol (mol hexose fermented)$^{-1}$]	Yield [g dry wt (mol hexose consumed)$^{-1}$]	Y_{ATP}
Cellobiose	0·06	97·2	0·96	2·96	35·28	11·92
Cellobiose	0·10	90·2	0·83	2·85	45·13	15·84
Cellobiose	0·15	81·3	0·75	2·80	45·9	16·39
NH_4^+	0·06	84·6	0·81	2·83	30·59	10·81
NH_4^+	0·10	94·3	0·92	2·81	36·24	12·90
NH_4^+	0·15	81·1	0·75	2·77	43·20	15·60

* Carbon recovery was calculated as the percentage of the hexose fermented which was recovered in end-products of fermentation.

† In calculating O/R balances, net amounts of carbon dioxide and hydrogen were calculated from the following equations. CO_2 consumed or evolved = (acetate − formate) − succinate. H_2 evolved = (2 × hexose fermented) + (acetate − formate) − [lactate − (2 × succinate)].

and yield of bacteria (dry weight) per mol ATP (Y_{ATP}) were calculated from the amount of hexose fermented, the fermentation products detected and the dry weight yield (Table 4). For the purpose of calculating Y_{ATP} it was assumed that a net yield of 2 mol ATP was obtained from the conversion of glucose to pyruvate via the Embden–Meyerhof–Parnas pathway and that 1 mol ATP was synthesized during the formation of acetate from pyruvate. Since, in *R. flavefaciens*, succinate arises via the formation of oxaloacetate from phospho*enol*pyruvate (Hopgood & Walker, 1969), it is assumed that this would negate any ATP which might arise from subsequent electron transport phosphorylation associated with the reduction of fumarate to succinate (de Vries *et al.*, 1973). The Y_{ATP} calculated on this basis varied with nutrient limitation and growth rate between 10·81 and 16·39.

DISCUSSION

Enzymes produced by *R. flavefaciens* degrade plant cell walls releasing numerous mono- and disaccharides (Pettipher & Latham, 1979), not all of which can be used for growth.

The inability of strains to ferment xylose even though they can grow on xylan may be due to the lack of a specific permease required for the transport of monosaccharides into the cell (Dehority, 1967). Production of pectin-degrading enzymes (Pettipher & Latham, 1979) is of no direct nutritional advantage since, like the pectin-degrading strains of *Streptococcus bovis* (Ziolecki *et al.*, 1972), neither pectin nor its degradation products can be used for growth. Nevertheless, the possession of these enzymes may enhance the ability of *R. flavefaciens* to penetrate plant tissue and assist in revealing deep-seated fermentable carbohydrate.

Most of the CM-cellulase and xylanase is cell-associated both in batch cultures growing exponentially and in rapidly growing continuous cultures. Although these enzymes may be synthesized intracellularly, they can only be active against their macromolecular substrate, and at the same time confer a nutritional advantage to the organism, if they are located on the surface of the bacterial cell; enzymes which diffuse into the surrounding medium are of little value to the organism. The large amounts of enzymes detected free in the culture supernatant during stationary phase in batch culture or at low dilution rates in continuous culture suggested that the enzymes were escaping from the cells as a result of leakage or cell lysis rather than by active secretion. Neither CM-cellulase nor xylanase may therefore be regarded as typical extracellular enzymes and they are probably surface-bound (Pollock, 1962).

CM-cellulase and xylanase appear to be constitutive enzymes and do not perform any hydrolytic function when the organism is grown on cellobiose. The greatly enhanced CM-cellulase activity in cellobiose-limited continuous cultures suggested that synthesis of this enzyme is catabolite repressed, as previously reported for the cellulase of *R. albus* (Fusee & Leatherwood, 1972) and a cellulolytic strain of *Pseudomonas fluorescens* (Suzuki, 1975).

Most of the cellulolytic ruminococci are inefficient at utilizing amino acids, relying on ammonia as their main nitrogen source (Bryant, 1973) which, for *R. flavefaciens*, may be incorporated by glutamate dehydrogenase. Protein constitutes a small proportion of the dry weight of most grasses and is unlikely to provide a useful nitrogen source for this species. Therefore, when competing with other bacteria for rumen ammonia during growth on plant material *in vivo*, the organism may direct a significant proportion of its biosynthetic capability into synthesizing glutamate dehydrogenase as well as enzymes which degrade the plant cell wall. The observation that glutamate dehydrogenase activity increased during continuous culture under ammonia limitation supports this suggestion.

Complex interactions take place between the numerous species of rumen micro-organisms during the digestion of roughage *in vivo* (Prins & van den Vorstenbosch, 1975) and cellulolytic organisms are considered to be at, or near, the apex of a food chain which starts with the insoluble polymeric structural carbohydrates of the plant cell wall. Preliminary results suggest that free reducing-sugars do not accumulate during the growth of *R. flavefaciens* strain 67 on marble-milled filter paper, a finding previously reported for other strains of *R. flavefaciens* (Jarvis & Annison, 1967) and for *Cellvibrio fulvus* (Berg *et al.*, 1972). This raises the possibility that soluble by-products of cellulolysis by this organism are not made available for other species to ferment, as has been suggested for *Bacteroides succinogenes* (Scheifinger & Wolin, 1973). Cross-feeding of carbohydrate as a result of the activity of *R. flavefaciens* enzymes may only occur to any significant extent during the degradation of hemicellulose and pectin. Since *R. flavefaciens* does not ferment many of the carbohydrates which arise as a result of hemicellulose degradation, these carbohydrates will be available for utilization by other organisms. Moreover, any lysis of the ruminococci resulting in release of intracellular storage carbohydrate, coupled with the continuous sloughing off of the glycoprotein coat which surrounds the bacterial cell (Latham *et al.*, 1978), may provide important additional sources of carbohydrate for fermentation by non-cellulolytic species of bacteria.

Electron disposal during glycolysis was divided equally between succinate and lactate at low growth rates in cellobiose-limited cultures but became progressively biased towards

succinate as the growth rate increased. Despite the variations in growth rate and nutrient limitation, the values for Y_{ATP} were relatively constant and were similar to those reported for several species of rumen bacteria (Hungate, 1963; Hobson & Summers, 1972) and other anaerobes (Decker *et al.*, 1970).

Formate accumulated in direct proportion to cell numbers during exponential growth of *R. flavefaciens* in batch culture which was in marked contrast to results with *R. albus* (Miller & Wolin, 1973) in which formate production was a late function of growth. *Ruminococcus flavefaciens* may possess a formate hydrogen lyase, as suggested by Joyner *et al.* (1977), since formate was only a minor product at low growth rates in cellobiose-limited cultures and the addition of formate to cells from these cultures stimulated hydrogen production. While recognizing that the close similarity in the concentrations of formate and acetate arising from the fermentation of cellobiose during ammonia-limited continuous culture may be entirely fortuitous, it nevertheless seems possible that formate is derived from pyruvate by an *Escherichia coli*-type phosphoroclastic reaction. This being so, then the formate hydrogen lyase may only be active at low growth rates (since formate accumulated as the growth rate increased) and may be inactive when the organism is growing in the presence of excess carbohydrate. These tentative suggestions warrant further investigation since Miller (1975) concluded that in *R. flavefaciens* and *R. albus* formate arose from the reduction of exogenous CO_2 by hydrogen and not from the cleavage of pyruvate.

The authors thank B. E. Brooker of this Institute for performing the electron microscopy and the Agricultural Research Council for financial support (grant no. BS635) to G.L.P.

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