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Effects of Stirring and Hydrogen on Fermentation Products of *Clostridium thermocellum*

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Clostridium thermocellum **produces ethanol, acetate, H_2, and CO_2 as major fermentation products from cellulose and cellobiose. The performance of three strains of this microorganism was studied to assess the potential use in producing ethanol directly from cellulosic fiber. Depending on the bacterial strain, an ethanol/acetate product ratio from 1 to as high as 3 was observed in unstirred cultures. Vigorous stirring during growth resulted in a threefold decrease in the ethanol/acetate ratio. The H_2 content in the unstirred culture broth was three times greater than that in the stirred one. Addition of exogenous H_2 to the gas phase during growth increased the ethanol/acetate ratio much more in the stirred than in the unstirred fermentations. The addition of sufficient H_2 to the gas phase almost relieved the effect of stirring, and the ethanol/acetate ratio approached that in the unstirred condition. Addition of tritium to the gas phase of the culture resulted in the formation of tritiated water (^3H_2O), which indicates that *C. thermocellum* possesses hydrogenase(s) that catalyzes the reverse reaction. The rate of ^3H_2O formation was about three times higher in the stirred culture than in the unstirred culture. These results demonstrate that the H_2 concentration in the broth plays an important role in the product formation. The H_2 supersaturation present in the unstirred cultures is responsible for the observed effect of stirring. A hydrogen feedback control mechanism regulating the relative concentrations of reduced and oxidized electron carriers is proposed to account for the effect of hydrogen on the metabolite distribution.**

Clostridium thermocellum, a thermophilic cellulolytic bacterium, has received considerable attention for its potential application in producing ethanol directly from cellulosic fiber. This microorganism produces ethanol, acetic acid, H_2, and CO_2 as the major metabolites. Depending on the strains, ethanol/acetate ratios ranging from 1 to 1.8 have been reported (9, 20). The high level of acetate produced by these microorganisms is one of the major obstacles in the development of an economical fermentation process for ethanol production.

We isolated a strain of *C. thermocellum*, YS, that produced a significantly higher ethanol/acetate ratio than that previously reported in the literature. An ethanol/acetate ratio of about 3 was routinely achieved when the culture was grown on cellulose in serum bottles. However, the performance of this strain when cultured in a 5-liter fermentor revealed that the ethanol/acetate ratio was only about one-third of that found in the serum bottle. Results obtained from fermentors operating at several pH values (6.0 to 7.5) and analysis of the gas exiting the fermentor indicated that neither the pH nor evaporation of the ethanol could account for the observed decrease in the ethanol/acetate ratio.

Alterations of metabolite distribution have been observed in H_2-producing microorganisms (hydrogenogens) when grown in coculture with a H_2-consuming microorganism (hydrogenotroph), such as the coculture of methane fermentations (10, 11, 15). To account for such a change in the product pattern, a reduction in H_2 concentration resulting from interspecies H_2 transfer between the hydrogenogens and the hydrogenotrophs in the medium was proposed (8). Attempts to mimic the coculture by removing H_2 from monoculture fermentations have produced little success (3, 16).

In this paper, we report the remarkable effect that stirring or shaking of the culture medium has on the product pattern of *C. thermocellum*. The ethanol/acetate ratio depended on the rate of stirring. The results presented here suggest that H_2 concentration in the culture broth plays a determinative role in the fermentation product pattern.

(A portion of this work was previously reported at the 82nd Annual Meeting of the American Society for Microbiology, Atlanta, Ga., 7 to 12 March 1982.)

MATERIALS AND METHODS

Microorganisms. *C. thermocellum* YS was isolated in our laboratory from the sediment of a hot spring in Yellowstone National Park. *C. thermocellum* LQRI was obtained from J. G. Zeikus at Michigan State University. *C. thermocellum* AS-39 was obtained from A. Demain at the Massachusetts Institute of Technology. This strain is a mutant of *C. thermocellum* ATCC 27405 (American Type Culture Collection, Rockville, Md).

Medium and cultivation conditions. The CM3 medium contained the following in 1 liter of distilled water: $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 0.5 g; $MgCl_2 \cdot 6H_2O$, 1.3 g; $FeSO_4$, 1.7 mg; MOPS [3-(*n*-morpholino)propanesulfonic acid], 20.9 g; cellobiose or microcrystalline cellulose (Avicel, obtained from FMC Corp., Rockland, Maine), 20 g; cysteine hydrochloride, 1.0 g; and resazurin, 2.0 mg. The pH was adjusted to 7.3 with 3 N NaOH before sterilization. Unless indicated otherwise, 10 ml of media was anaerobically dispensed into a 30-ml serum bottle (Wheaton Industries, Millville, N.J.) under a N_2 atmosphere. The serum bottle was sealed and sterilized at 121°C for 20 min. The medium was inoculated (5% volume) with freshly grown cultures and incubated at 60°C. Unless stated otherwise, "stirring" of the cultures was achieved by laying the bottles

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TABLE 1. Effect of stirring on fermentation pattern of *C. thermocellum* strains grown on cellulose[a]

Strain	Condition	Amt of product			Ethanol/acetate ratio
		Ethanol (mM)	Acetate (mM)	Hydrogen (μmol)	
YS	Unstirred	36	12	215	3.0
	Stirred	31	32	600	0.97
AS-39	Unstirred	52	23	420	2.2
	Stirred	35	41	770	0.85
LQRI	Unstirred	28	27	490	1.04
	Stirred	20	49	900	0.41

[a] Strains were grown in 30-ml serum bottles containing 10 ml of medium.

horizontally on a rotary shaker-incubator (New Brunswick Scientific Co., Inc., Edison, N.J.) at 150 rpm. When the cultures were grown in the presence of an exogenous H_2 pressure, the H_2 was added to the gas phase of the serum bottles after inoculation.

Characterization of metabolites. H_2 was quantified by a gas chromatograph (Shimadzu, Japan) equipped with a 3-ft (1 ft = 30.48 cm), 1/8-in. (1 in. = 2.54 cm)-inside-diameter stainless steel column packed with a molecular sieve and a thermoconductivity detector. Helium was the carrier gas. Ethanol and acetate were measured with a gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) containing a 6-ft, 1/4-in.-inside-diameter glass column packed with Chromosorb 101 (150°C) and a flame ionization detector. Nitrogen was the carrier gas. Liquid samples for gas chromatographic analysis contained 0.5 N H_3PO_4 and 10 mM butanol as an internal standard. L-Lactic acid was determined by a standard enzyme assay (1).

Kinetics of ethanol and acetate production. Two sets of five serum bottles containing cellulose in CM3 medium (5 ml of medium in a 10-ml serum bottle) were inoculated with logarithmically growing cells of *C. thermocellum* YS and incubated at 60°C. One set of the serum bottles was shaken, and the other was not. At timed intervals, one serum bottle from each set was removed from the incubator and the amounts of ethanol and acetate were determined.

H_2 accumulation in the gas phase of resting cell cultures. *C. thermocellum* YS was grown on cellobiose in serum bottles (60 ml of medium in a 120-ml bottle) with vigorous stirring, harvested anaerobically, and suspended in preheated CM3 medium containing 2% cellobiose and 0.05% chloramphenicol (a protein synthesis inhibitor). Equal volumes (5 ml) of the concentrated cell suspension were injected into 10-ml serum bottles and incubated at 60°C. The medium in one set of bottles was stirred with a Teflon magnetic stirring bar, and the medium in the other set of bottles was unstirred. The amount of H_2 in the gas phase was analyzed at 30-min intervals. After 1 h of incubation, the H_2 in the gas phase was removed by flushing N_2 (30 ml/min) through the gas phase. At the end of 2 h, the amounts of H_2, ethanol, and acetate were determined.

Estimation of H_2 trapped in the culture broth. Equal volumes (20 ml) of a metabolically active culture of *C. thermocellum* YS were transferred into two sets of anaerobic serum bottles (50 ml). The bottles were incubated at 60°C with a stream of N_2 (30 ml/min) flowing through the gas phase of the bottles to remove H_2 released from the culture broth. The medium in one set of bottles was stirred, but that in the other one was not. After the cultures had been incubated for 30 min, the flow of N_2 was stopped. Immediately, 1 ml of perchloric acid was added to kill the culture. The culture medium in both sets of bottles was shaken vigorously to release into the gas phase the H_2 entrapped in the broth. The amount of H_2 released to the gas phase was determined.

Determination of ^3H_2O formation with $^3H_2$ in the gas phase. Freshly grown cells of *C. thermocellum* YS were placed into two duplicate sets of serum bottles (10 ml of medium in a 30-ml bottle) containing 2% cellobiose in CM3 medium under a N_2 gas phase. Two different amounts of cells (5 and 15% inocula) were added to each set of bottles. A 5-ml portion of $^3H_2$ gas (specific radioactivity = 2×10^7 cpm/mmol) was added to the gas phase of each set of bottles and to two serum bottles which each contained 10 ml of the CM3 medium without added cells. These two bottles were the controls for the two different culture conditions. All the serum bottles were incubated at 60°C. In one set of bottles, including one of the controls, medium was stirred at 150 rpm, and in the other set and a control medium was not stirred. A small portion of medium was removed from each bottle at 15-min intervals and centrifuged for 1 min in a microcentrifuge (Eppendorf, model 5412). A 0.1-ml portion of each supernatant was added to 10 ml of Aquasol (scintillation cocktail; New England Nuclear Corp., Boston, Mass.) and counted in a liquid scintillation counter (model LS 100 C; Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Effect of stirring on fermentation products. Tables 1 and 2 show the effect that stirring has on the metabolite distribution of different strains of *C. thermocellum* when grown on cellulose and cellobiose, respectively. In the cellulose fermentation, ethanol/acetate ratios ranging from 1 to 3 were observed in the unstirred culture. However, when the cultures were stirred, lower amounts of ethanol and higher amounts of acetate and H_2 were produced. Lactic acid was a minor product (3.5 to 5.0 mM) and was not affected. As a result of such a shift in the metabolite distribution, the ethanol/acetate ratio decreased by a factor of 3 in stirred cultures of all of the strains of *C. thermocellum* examined. Hydrogen production increased in the stirred culture proportional to production of acetate, and the hydrogen/acetate ratio remained constant. The observed effect of stirring on the metabolite distribution was not limited to the heterogeneous system in which cellulose was the substrate (Table 1). Similar results were obtained from the cultures grown on soluble cellobiose (Table 2). However, the decrease in the

TABLE 2. Effect of stirring on fermentation pattern of *C. thermocellum* strains grown on cellobiose[a]

Strain	Condition	Amt of product			Ethanol/acetate ratio
		Ethanol (mM)	Acetate (mM)	Hydrogen (μmol)	
YS	Unstirred	38	19	340	2.0
	Stirred	25	30	530	0.83
AS-39	Unstirred	47	23	400	2.0
	Stirred	36	37	650	0.96
LQRI	Unstirred	26	24	400	1.1
	Stirred	20	51	900	0.4

[a] Strains were grown in 30-ml serum bottles containing 10 ml of medium.

ethanol/acetate ratio that resulted from stirring the culture was less than that found with cellulose as the carbon source.

Kinetic profile of ethanol and acetate production. The ethanol and acetate production profiles from stirred cultures of *C. thermocellum* YS were compared to those of unstirred cultures while both cultures were growing on cellulose. Ethanol formation paralleled acetate formation for the stirred culture throughout the course of the fermentation. However, in the unstirred culture, the profiles of ethanol and acetate formation diverged as the fermentation progressed. Acetate production practically ceased after 15 h, whereas ethanol production continued at a rate comparable to that of the stirred culture for over 30 h. Consequently, the ethanol/acetate ratio gradually increased from an initial ratio of 1 to about 3 at the end of the fermentation for the unstirred culture.

H_2 accumulation in the gas phase of resting cell cultures. To demonstrate that the effect of stirring on metabolite distribution was not the result of a change in the relative enzyme concentrations, the kinetics of H_2 accumulation in the gas phase of stirred and unstirred cultures of *C. thermocellum* YS were examined, using resting cells. Chloramphenicol, a protein synthesis inhibitor, was added to the culture broth to prevent cell growth and enzyme synthesis but still permit the cells to be metabolically active. Concentrated cell suspensions (A_{600} = 10.0) were used in this experiment to ensure the detection of different metabolic patterns over a short time.

Since the stoichiometric relationship between H_2 and acetate production was unaffected by the stirring of the culture medium (H_2/acetate molar ratio of 2:1), the accumulation of H_2 in the gas phase rather than acetate was monitored during the incubation. The initial rate of H_2 accumulation in the stirred culture was three times faster than in the unstirred culture (Fig. 1). The accumulation of H_2 in the gas phase reduced the rate of H_2 evolution from the broth. However, the initial rate of the H_2 accumulation was restored after the H_2 in the gas phase was removed by a stream of N_2. After 2 h of incubation, the ethanol and acetate concentrations in the unstirred culture were 25 and 14 mM, respectively. The stirred culture had ethanol and acetate concentrations of 19 and 25 mM, respectively. The higher ethanol and lower acetate concentrations observed in the unstirred culture were consistent with previous results. However, H_2 recovered from the gas phase of the unstirred culture was insufficient to account for the amount of acetate produced. Consequently, experiments were carried out to determine the H_2 entrapped in the broth under both culture conditions.

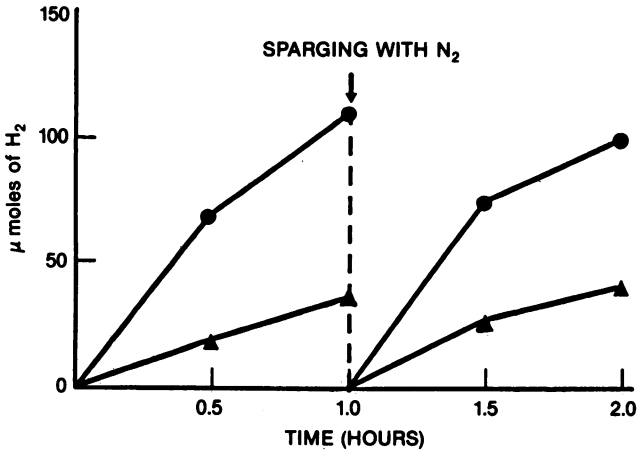

FIG. 1. Effect of stirring on H_2 production by *C. thermocellum* YS in the presence of chloramphenicol. ●, Stirred culture; ▲, unstirred culture. The gas phase was flushed with N_2 (30 ml/min) for 2 min at 1 h.

TABLE 3. Effect of stirring on ethanol/acetate ratio produced by *C. thermocellum* strains with exogenous H_2 and N_2[a]

Growth medium and strain	Ethanol/acetate ratio with 2.5 atm of:			
	Nitrogen		Hydrogen	
	Unstirred	Stirred	Unstirred	Stirred
Cellulose				
YS	3.0	0.95	3.7	2.4
AS-39	2.2	0.80	3.2	1.7
LQRI	1.1	0.40	1.2	1.1
Cellobiose				
YS	2.1	0.75	3.3	1.7
AS-39	2.0	0.95	2.6	2.0
LQRI	1.1	0.40	1.2	0.9

[a] Strains were grown in 30-ml serum bottles containing 10 ml of medium.

The amounts of H_2 entrapped in the broth (20 ml) of unstirred and stirred cultures growing on cellobiose were 31 and 10 μmol, respectively. Continuous removal of the H_2 in the gas phase by a stream of N_2 did not prevent H_2 from accumulating in the broth during growth. According to Henry's law, the amount of H_2 entrapped in the unstirred and stirred culture broth was calculated to be equivalent to equilibrium H_2 pressures in the gas phase of 2.2 and 0.72 atm (1 atm = 101.29 kPa), respectively. Despite vigorous stirring, a significant amount of the H_2 still remained in the culture broth.

Effect of exogenous H_2 on ethanol/acetate product ratios. To ascertain whether the addition of H_2 could simulate the unstirred culture condition, the ethanol/acetate ratios from three strains of *C. thermocellum* grown under increased H_2 pressure (2.5 atm) were determined. A parallel control experiment, using N_2 pressure instead of H_2 pressure, was done to evaluate the effect of pressure on the product pattern. The results shown in Table 3 indicate that the increased N_2 pressure in the gas phase did not affect the metabolite distribution, and the effect of stirring on the ethanol/acetate ratio persisted. However, the ethanol/acetate ratios were significantly increased when the cultures were grown under an exogenous H_2 pressure of 2.5 atm. The increase was much more pronounced in the stirred than in the unstirred cultures. The addition of exogenous H_2 practically offset the effect that stirring had on the ethanol/acetate ratio.

Effect of stirring rate on ethanol/acetate ratios in the presence of exogenous H_2. The relationship between the rate of stirring and the ethanol/acetate ratio was examined by growing *C. thermocellum* YS under several H_2 pressures. Figure 2 shows the ethanol/acetate ratios produced by the culture grown on cellulose (Fig. 2A) and cellobiose (Fig. 2B). In the absence of added H_2, the ethanol/acetate ratio decreased as the stirring rate increased up to 150 to 200 rpm. The effect of stirring disappeared at H_2 pressures above 1 and 2.5 atm for the cultures grown on cellulose and cellobiose, respectively. Also, the ethanol/acetate ratio increased slightly with increased stirring rate at these H_2 pressures. Larger serum bottles allowing for a greater gas/liquid volume ratio were used to reduce the increase in H_2 pressure generated by the

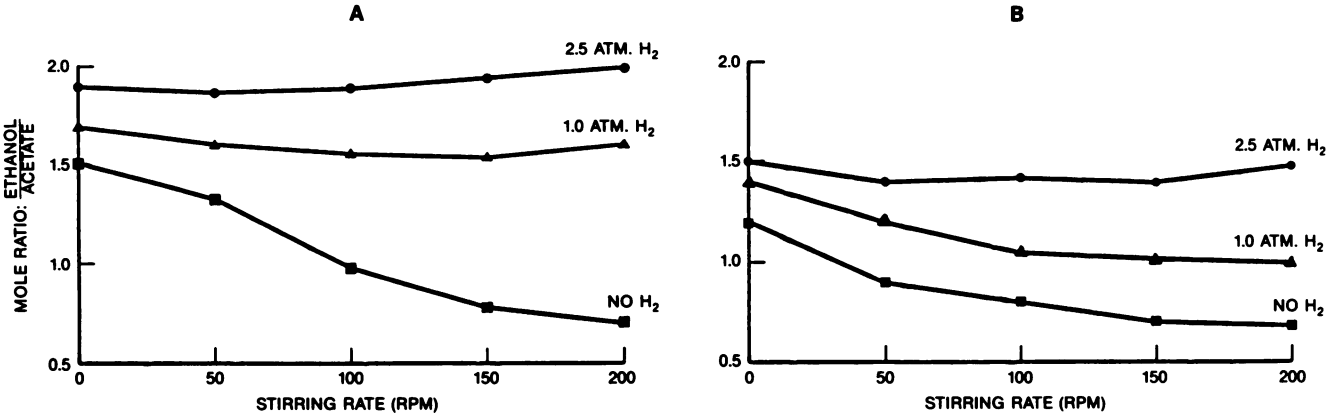

FIG. 2. Effect of stirring and added H_2 on ethanol/acetate ratio of *C. thermocellum* YS grown on cellulose (A) and cellobiose (B). Cells were cultured for 30 h in 100-ml serum bottles containing 10 ml of medium. For the "NO H_2" condition, 1 atm of N_2 replaced H_2.

bacteria during growth. As a result of this modification, the ethanol/acetate ratios obtained from the unstirred cultures were lower than those obtained from the previous experiments (Tables 1 and 2).

Rate of ^3H_2O formation from $^3H_2$ in stirred and unstirred fermentations. Tritium ($^3H_2$)-water exchange experiments were used to demonstrate the effect of stirring on the H_2-oxidation reaction by hydrogenase. Detection of the ^3H_2O in the culture broth indicated that *C. thermocellum* YS was able to oxidize H_2 to H^+ under both culture conditions. However, a much higher rate of ^3H_2O formation was observed in the stirred culture than in the unstirred one (Fig. 3). These observations indicated that the H_2 equilibration between the gas and the liquid phases was much slower in the unstirred culture than in the stirred one.

DISCUSSION

The kinetics of acetate production in the unstirred culture indicates that acetate production is inhibited by a gradual accumulation of H_2 in the culture broth. The medium becomes supersaturated with H_2 because the transfer of H_2 from the culture broth to the gas phase is impeded. Stirring the culture broth facilitates H_2 transfer to the gas phase, which relieves the inhibition of acetate formation caused by the high H_2 concentration in the medium. Consequently, lower ethanol/acetate ratios are observed. The effect that different stirring rates have on the ethanol/acetate ratio (Fig. 2) indicates that the degree of H_2 supersaturation is dependent on the rate of stirring. The impediment of H_2 transfer from the liquid to the gas phase was not overcome until the stirring rate exceeded 150 rpm.

The high degree of H_2 supersaturation that occurs in the culture broth of rapidly growing H_2-producing microorganisms had not been previously recognized at the time of this study. In fact, the H_2 produced by the microorganism was often assumed to be rapidly transferred from the medium to the gas phase to establish an equilibrium. Therefore, the H_2 concentration in the liquid phase was usually estimated from the H_2 partial pressure in the gas phase. Contrary to this assumption, the results from this study indicate that a substantial amount of the H_2 is trapped in the culture broth to form a supersaturated solution. By Henry's law, the concentration of the H_2 in the liquid phase of unstirred cultures is much higher than that estimated from the partial pressure in the gas phase when the cells are actively growing.

Existence of such a supersaturated solution has been observed in an undisturbed system. A metastable supersaturated solution of methane, oxygen, or helium up to several hundred atmospheres can exist in undisturbed water (6). A cell-free culture medium equilibrated at 8 atm of H_2 in the gas phase was shown to be stable after the H_2 in the gas phase was slowly replaced with 1 atm of N_2 (data not shown). However, stirring of the supersaturated solution resulted in the rapid evolution of H_2 to the gas phase.

The ethanol/acetate ratios obtained from cultures of *C. thermocellum* were slightly higher when grown on cellulose than on cellobiose (Tables 1 and 2). This might be explained by the fact that in the unstirred condition, the cells growing on cellulose tend to adhere to the cellulose and settle to the

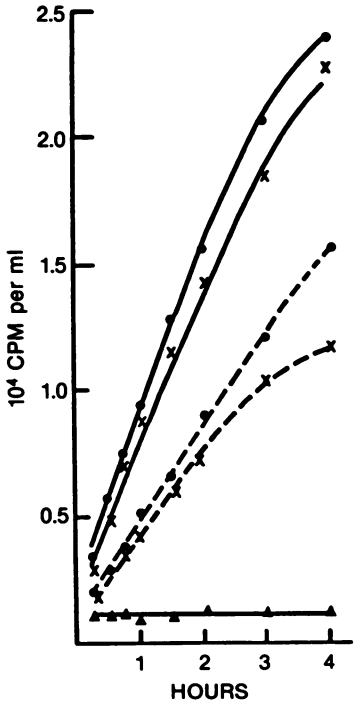

FIG. 3. Tritium ($^3H_2$)-water exchange reaction for stirred and unstirred cultures of *C. thermocellum* YS. ———, Stirred culture; – – –, unstirred culture; ×, 5% inoculum; ●, 15% inoculum; ▲, no inoculum.

bottom of the serum bottle. The H_2 produced may be trapped at the interface between the cellulose and the cell. As a result, the culture may experience a higher H_2 supersaturation when grown on cellulose than on cellobiose.

The formation of tritiated water upon the addition of $^3H_2$ to the gas phase suggests that hydrogenase(s) in *C. thermocellum* can catalyze the oxidation of H_2 to H^+. This reaction has been observed in the mesophilic strain of *Clostridium pasteurianum* (2). The relative independence of the rate of $^3H_2$ oxidation with different inoculum sizes was not expected (Fig. 3). The rate of exchange should, in principle, be proportional to the increase in cell mass in the medium. One possible explanation is that the increase in cell density proportionally increases the H_2 produced by the cells and results in a decrease in the $^3H_2$ specific activity in the medium. Such a decrease would offset the expected increase in the rate of ^3H_2O formation with the increased cell density.

The regulation of H_2 formation by *C. thermocellum* can be explained by the previously proposed mechanism of hexose metabolism (Embden-Meyerhof pathway) in which acetyl coenzyme A (acetyl-CoA) occupies a crucial branch point leading to either ethanol or acetate production (17). Stoichiometrically, 1 mol of acetyl-CoA can be converted to either 1 mol of ethanol with the consumption of 2 mol of NADH or to 1 mol of acetate and 2 mol of H_2 via the hydrogenase enzyme system. The H_2/acetate ratios of 1.8 to 1.9 observed in both stirred and unstirred cultures (Table 1 and 2) are consistent with the above mechanism. A decrease in H_2 concentration will favor H_2 formation and permit the bacterium to metabolize acetyl-CoA through the energy-efficient path leading to acetate and ATP production, resulting in lower ethanol/acetate ratios. Alternatively, increased H_2 concentration inhibits the formation of H_2 via hydrogenase. This causes more of the acetyl-CoA to be reduced to ethanol, resulting in higher ethanol/acetate ratios.

On the basis of the pathway described above, the relative amount of electrons being transferred between the two electron carriers, NADH and ferredoxin, can be estimated from the amount of ethanol and acetate produced. For example, examination of the data in Fig. 2, with respect to electron flow between NADH and ferredoxin, suggests that the electrons can flow in either direction depending on the rate of stirring and on the H_2 concentration in the medium. The increase in H_2 concentration causes the flow of electrons from the reduced ferredoxin to NAD^+ to yield NADH and vice versa. Oxidation of NADH to H_2 ($NADH + H^+ = NAD^+ + H_2$) has been considered an energetically unfavorable reaction because of a positive free energy change of 4.3 kcal/mol (1 kcal = 4,184 J) (16, 17). For this reaction to take place at pH 7.0 under standard reduction potentials, a H_2 concentration as low as 10^{-4} atm is required. Such a low H_2 pressure was thought to exist in the rumens of animals because the H_2 produced by the hydrogenogens was rapidly consumed by the hydrogenotrophs (7).

Our results indicate that a substantial fraction of the NADH can be oxidized to yield H_2 at a much higher H_2 pressure than that previously suggested (17). Similar observations have been reported for mesophilic bacteria such as *C. pasteurianum* (4), *Clostridium kluyveri* (12), and *Clostridium butyricum* (13). To account for these observations, we speculate that when the microorganism encounters a change in the H_2 concentration in the medium, a H_2 feedback control mechanism causes a change in the relative concentration of reduced and oxidized NAD and ferredoxin. Such a change causes a shift in the reduction potentials of NAD^+ and ferredoxin and allows the reaction between NADH and ferredoxin to occur. Therefore, determination of the relative amounts of NAD^+ and NADH or those of oxidized and reduced ferredoxin under various H_2 concentrations may improve our understanding of how H_2 is able to modulate the metabolite pattern.

We demonstrated that H_2 supersaturation is responsible for the higher ethanol/acetate ratios observed in unstirred compared with stirred cultures of *C. thermocellum*. Recognition of the effect of H_2 supersaturation on metabolite distribution may have an important impact on anaerobic fermentation technology (14). Significant improvements in some fermentation processes may be achieved with modifications in fermentor design and process control to maximize the retention of hydrogen in the culture medium. Recent studies on solvent fermentation by *Clostridium acetobutylicum* demonstrated that the production of solvents such as acetone and butanol can be improved by controlling the rate of agitation, as well as by maximizing the retention of H_2 in the fermentor (5, 19). Finally, the existence of the H_2 supersaturation may lead to a better understanding of the syntrophic association between hydrogen-producing and hydrogen-consuming microorganisms in anaerobic ecosystems (18).

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