APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1987, p. 1232–1235
0099-2240/87/061232-04$02.00/0

Vol. 53, No. 6

Altered Electron Flow in Continuous Cultures of *Clostridium acetobutylicum* Induced by Viologen Dyes

GOVIND RAO AND R. MUTHARASAN*

Department of Chemical Engineering, Drexel University, Philadelphia, Pennsylvania 19104

Received 22 December 1986/Accepted 9 March 1987

The physiological response of *Clostridium acetobutylicum* to methyl and benzyl viologen was investigated. Viologen dyes at low concentrations (at levels of parts per million [micrograms per milliliter]) caused significant metabolic shifts. Altered electron flow appeared to direct carbon flow from acid to alcohol production accompanied by decreased hydrogen evolution. Reducing equivalents normally released as free hydrogen were directed toward formation of NADH which, in turn, resulted in increased alcohol production. In addition, it was shown that solvent production can take place at pH 6.3. Contrary to previous reports, butanol production appears to be independent of high levels of acetate-butyrate and glucose.

Clostridium acetobutylicum, a saccharolytic strict anaerobe, is great interest because of its ability to ferment a wide variety of substrates to the commercially useful products acetone, butanol, and ethanol (20, 30). Fermentation by this organism is characterized by two distinct phases. In the first phase, growth is rapid with copious hydrogen evolution and acetic and butyric acid production. As a result, the pH decreases to a characteristically low value (typically, about 4.5). This is followed by a second, slower growth phase with decreased hydrogen evolution and solvent formation, with partial uptake of the acids that were formed during the first growth phase. The two phases are termed acidogenic and solventogenic, respectively. Like most saccharolytic strict anaerobes, hydrogen evolution is the means by which this organism rids itself of excess electrons (9, 30).

Metabolism. The metabolic pathway of *C. acetobutylicum* is shown in Fig. 1. Glucose is fermented via the EMP pathway to pyruvate. Pyruvate oxidation to acetyl coenzyme A requires obligatory ferredoxin (Fd) reduction. Reduced Fd (FdH_2) is oxidized by hydrogenase, which regenerates Fd and releases electrons as molecular hydrogen. In addition to formation by pyruvate oxidation, FdH_2 may be formed by NADH oxidation. This permits the regeneration of NAD^+. This reaction is controlled by the activity of NAD-linked Fd oxidoreductase (15, 24). The reaction is reversible, and depending on culture conditions, either FdH_2 or NADH may be formed. It should be noted that the reverse reaction (NADH formation from FdH_2) is inhibited by NADH and proceeds only on rapid removal of the NADH that is formed (24). The mechanism of NADH removal in vivo is alcohol formation (Fig. 1). It is this mechanism that we have sought to use to redirect carbon flow toward the alcohols.

There has been considerable interest recently on the production of solvents by the use of *C. acetobutylicum*. These include several investigations related to metabolic characterization of the factors governing solvent production. It has been suggested that the levels of undissociated butyric and acetic acids in the extracellular environment regulate the onset of solventogenesis (19, 22). Results of recent studies (8, 26) point to intracellular levels of butyric acid as the governing factor for the switch to take place. Results of another study (23) indicated that high glucose levels are necessary. Other investigators have found low pH (typically, less than 5) to be a requirement for the onset of solventogenesis (8, 12, 22, 25). Various studies have been performed on nutrient limitations (2, 4, 7, 23). Intracellular enzyme activities have been measured in both acidogenic and solventogenic phases (3). Factors such as hydrogen partial pressure and agitation rates have been found to influence solvent production (6, 27, 28). The ability of the organism to sporulate has been reported to influence solvent-producing capability over several generations (7, 14, 17). Recently, intracellular pH and membrane potential in the two phases have been measured (8, 11, 12, 26). Solventogenesis appears to be acompanied by a decrease in transmembrane potential and an increase in the transmembrane pH gradient. Regulation of electron flow pathways in *C. acetobutylicum* can also be influenced by carbon monoxide sparging (5, 16, 21). Carbon monoxide inhibits clostridial hydrogenase causing transfer of electrons from FdH_2 to NAD (mediated by NAD: Fd oxidoreductase), resulting in the increased availability of NADH. Because the solvent-producing steps (for example, butanol from butyrate and ethanol from acetaldehyde) require NADH, a larger carbon flow to alcohols is observed at the expense of the acids. From the forgoing discussion it can be concluded that electron flow dictates the direction of carbon flow in this organism.

In this study we examined the physiological effects of the artificial electron carriers methyl and benzyl viologen in glucose-limited continuous cultures of *C. acetobutylicum*.

MATERIALS AND METHODS

C. acetobutylicum ATCC 824 was maintained at 4°C in reinforced clostridial medium (Oxoid Ltd., Basingstoke, England). Initially, a growth medium containing KH_2PO_4, K_2HPO_4, $MgSO_4$, $MnSO_4$, and peptone at 0.5, 0.5, 0.2, 0.01, and 5 g/liter of distilled water, respectively, was used at a dilution rate of 0.023 h^{-1}. We found, however, that this medium was unable to support growth at a dilution rate of 0.2 h^{-1}, and so a medium containing $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $MgSO_4$, $FeSO_4$, $MnSO_4$, NaCl, and yeast extract at 3.0, 0.8, 0.8, 0.2, 0.01, 0.01, 0.02, and 4 g/liter of distilled water, respectively, was used.

The dilution rates and glucose concentrations (along with other parameters) that were employed are summarized in

* Corresponding author.

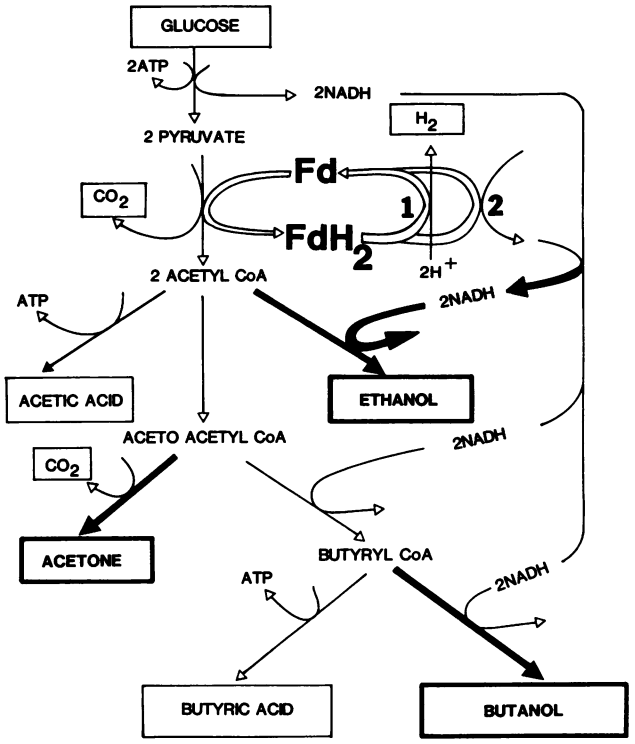

FIG. 1. Metabolic pathway of *C. acetobutylicum*. The following enzymes are shown: hydrogenase (1), NAD-linked Fd oxidoreductase (2). CoA, Coenzyme A.

Table 1. Glucose was autoclaved separately from the rest of the medium components. Autoclaving was done at 121°C for 20 min. Steady state was determined to have been reached when hydrogen, glucose, and optical density measurements were seen to be within 5% over three residence times. This was reconfirmed later with liquid product analysis. The viologen dyes were added in the oxidized state (colorless) to the culture after steady state was reached, at the indicated time, as a pulse (3 ml of a 1-g/liter solution that was sterilized by passing it through a filter [pore size, 0.22 μm]). This resulted in an initial viologen concentration of 6 mg/liter (6 ppm) in the fermenter. Subsequently, the viologens were continuously diluted by the incoming feed.

Fermentation was carried out in a 500-ml working volume in 1-liter fermenters (The VirTis Co., Inc., Gardiner, N.Y.). pH was measured and logged continuously on a computer (Apple II). The same computer also controlled pH by automatically adding 4 N NaOH. The culture broth was sparged with oxygen-free nitrogen prior to and after inoculation to ensure anoxic conditions. Sparging was maintained throughout all the experiments. All tubing that was used was made of Viton (Du Pont Co., Wilmington, Del.) to eliminate oxygen entry. The inoculum was 10% by volume and was in the exponential growth phase. Incubation and growth were at 37°C. Samples were centrifuged at 10,000 × *g* for 5 min,

TABLE 1. Experimental conditions

Parameter	pH	Feed glucose (g/liter)	Dilution rate (h^{-1})
Expt 1	6.4	8.5	0.023
Expt 2	6.3	11.8	0.228

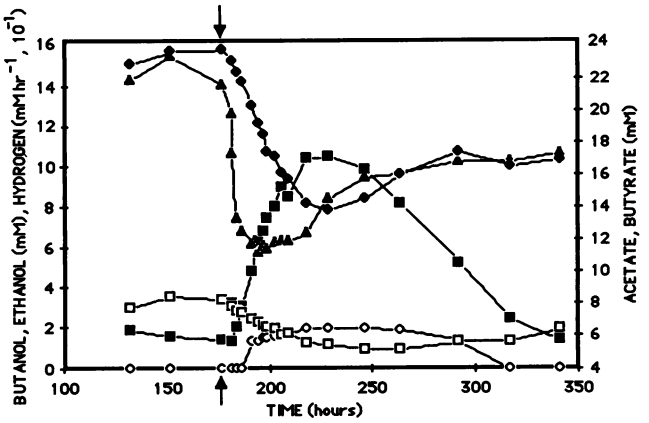

FIG. 2. Transient response of the culture to a 6-mg/liter pulse of methyl viologen. Arrows denote the point of addition. The following products are shown: ethanol (○), butanol (■), acetate (□), butyrate (◆), and hydrogen evolution rate (▲). D = 0.023 h^{-1}.

and the supernatant liquids were stored at 4°C until they could be analyzed.

Hydrogen evolution was measured on line with a gas chromatograph (model 8 AIT; Shimadzu) equipped with a column (6 ft [183 cm]; Chromosorb 102) and a TC detector. The column temperature was 100°C, and the detector temperature was 110°C. Liquid products were acidified and analyzed on a gas chromatograph (model 9A; Shimadzu) equipped with a column (6 ft [183 cm]; AT-1600 3% T-Port A; Alltech Associates, Inc., Deerfield, Ill.) column and a flame ionization detector. The column temperature was held at 100°C for 3 min, followed by a ramp of 10°C/min to 160°C, where it was held until the last component (butyric acid) eluted. Injector and detector temperatures were 200°C. Glucose was measured on a glucose analyzer (model 27; Yellow Springs Instrument Co., Yellow Springs, Ohio). Optical density was determined at 540 nm on a spectrophotometer (UV-160; Shimadzu).

RESULTS AND DISCUSSION

Several viologen-perturbed steady-state chemostat experiments were conducted to establish the reproducibility of culture response. Of these, the results of two continuous culture experiments are shown in Fig. 2 and 3. As soon as the viologen was added, a change in color of the broth was observed (blue for the methyl viologen experiment and violet for the benzyl viologen experiment), indicating that the viologen underwent reduction. The sharp decreases in hydrogen and acetic and butyric acids were simultaneously accompanied by increases in butanol and ethanol. Acetone formation was not observed at any time in either of the experiments. The glucose uptake rate during the transient response did not change, and glucose remained at 0.01 g/liter at all times in both experiments. It is to be noted that butanol production was essentially zero, both before the introduction and after the washout of the viologen(s). The maximum viologen concentration was present at the time of its addition (Fig. 2 and 3). In both cases the cultures appeared to return to their initial steady states after the viologens washed out of the reactor. The qualitative response of the organism to a pulse injection of viologen was identical. The fact that the two experiments were carred out at growth rates an order of magnitude apart, in different media, and exposed to two

different viologens indicates that the results are generic and independent of the growth rate.

It has been reported that a pH of less than 5 is a prerequisite for solventogenesis and that solvent-forming enzyme activities are 10- to 70-fold higher in solventogenic cells (3, 22). In our experiments we observed that the cells entered solventogenesis almost immediately in response to the addition of viologen. The rapidity of the system response to the viologen disturbance suggests that the enzymes of butanol and ethanol production can be induced in acidogenic cells. It is therefore reasonable to conclude that the response to viologen addition results in very rapid changes in the carbon flow pathway. These results are similar to those obtained by sparging continuous cultures of *C. acetobutylicum* with carbon monoxide. In contrast to viologen addition, total inhibition of hydrogenase appears to take place and glucose uptake decreases during the transient period of carbon monoxide sparging (21). As mentioned earlier, our results were obtained under conditions of glucose limitation at all times in both experiments. Another important feature is that the carbon monoxide sparging experiments were performed at solventogenic pHs (4.5 and below), while the results reported here were obtained at acidogenic pHs (6.3 and above).

Because the metabolic shift due to viologen addition was extremely rapid and glucose limitation was maintained, it is possible to make the approximation that the culture is in a quasi-steady state during the transient response. It is then possible to calculate butanol productivity as a function of viologen concentration for each of the experiments. Butanol productivity q_B, is calculated as $q_B = (1/x)(DB + dB/dt)$, where B is butanol concentration, x is the cell concentration, D is the dilution rate, and dB/dt is the rate of change in butanol concentration in the chemostat. The viologen concentration is calculated as $V = V_0 e^{-tD}$, where V and V_0 are the instantaneous and the initial viologen concentrations, respectively, and t is the time period following viologen introduction into the chemostat. Butanol productivity, according to the equations provided above, is plotted as a function of viologen concentration in Fig. 4. The higher (initial) viologen concentrations were omitted due to the kinetics of dilution of products from the chemostat. There was a linear relationship in both cases, suggesting that there is a direct dependence of butanol formation rate on viologen concentration. Because butanol formation requires the reduction of equivalents in the form of NADH, it can be concluded that the addition of viologen raises the NADH throughput in the alcohol-dependent pathways. There are two distinct mechanisms by which this may take place. One possibility is that specific NADH levels increase during solventogenesis and the other is that there is an increase in the NADH-NAD turnover rate. Results of in vitro studies have shown that NADH strongly inhibits NADH formation from reduced Fd (24). This supports the higher turnover rate hypothesis.

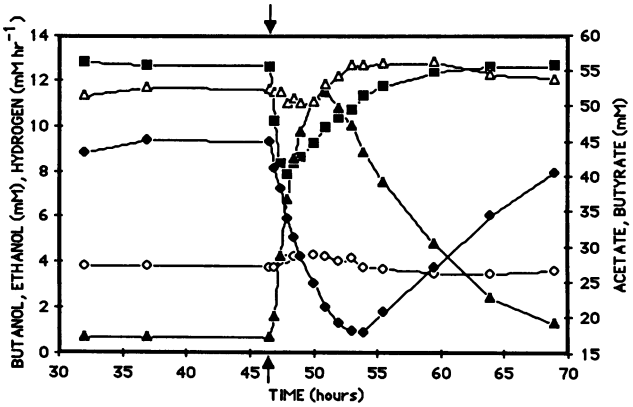

FIG. 3. Transient response of the culture to a 6-mg/liter pulse of benzyl viologen. Arrows denote the point of addition. The following products are shown: ethanol (◇), butanol (▲), acetate (◆), butyrate (△), and hydrogen evolution rate (■). $D = 0.228\ h^{-1}$.

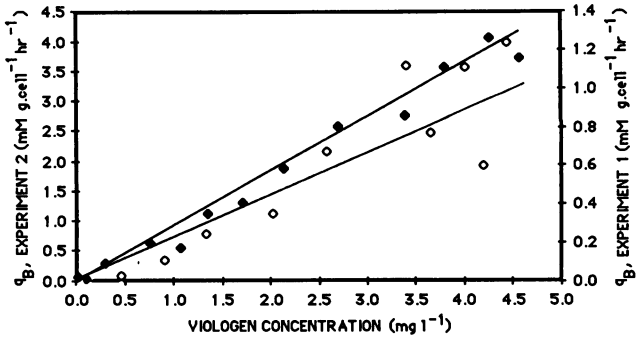

FIG. 4. Relationship between butanol productivity of the chemostat and viologen concentration in the fermentor during the transient results from experiment 1 (◇) and experiment 2 (◆).

As stated above, the change in broth color that was observed indicates that viologen reduction takes place in the fermenter. Because this reduction requires mediation by hydrogenase, FdH_2 would have to compete with viologen molecules for active sites on hydrogenase (1, 29). The result, therefore, would be a competitive blocking of FdH_2 oxidation via hydrogenase. However, FdH_2 oxidation must take place to regenerate Fd for pyruvate oxidation to proceed. Therefore, the alternative mechanism of FdH_2 oxidation, that is, NAD^+ reduction via ferredoxin reductase is activated. This reaction, however, is inhibited by NADH itself and can proceed only if the NADH that is formed is oxidized. This is realized in the form of alcohol production by the cell, resulting in NADH consumption and NAD^+ regeneration.

Other thermodynamically feasible reactions leading to increased NADH throughput that may be taking place are direct reduction of either NAD^+ or Fd by the reduced viologen. In the former case there would be direct NADH formation. In the latter case, NADH formation would take place via FdH_2. It has been observed that the viologens can substitute for Fd in vitro and directly donate electrons to NAD^+ (1, 18).

The effects of viologen addition observed in vivo in our experiments are probably due to a combination of the factors described above. The similarity of viologen response and results of carbon monoxide sparging experiments, however, suggests that the predominant mode of action in this study was probably due to competitive inhibition of hydrogenase.

The results presented in the discussion above are also consistent with previous experimental observations that show decreased hydrogen evolution due to hydrogenase inhibition during solventogensis. Hydrogenase is inhibited either by carbon monoxide sparging or increased hydrogen partial pressure (6, 21, 27, 28). Accordingly, it can be stated as a general inference that any mechanism that decreases hydrogenase activity favors increased alcohol production.

Indirect validation of this conclusion can be made from results of recent studies in which a decrease in membrane potential and an increase in transmembrane pH gradient prior to solventogenesis have been shown (8, 11, 13, 26). Hydrogenase is membrane bound and has several redox-sensitive iron-sulfur groups in its active sites (1). It is posible that as the membrane pH gradient increases, hydrogenase activity is reduced, thereby inducing solventogenesis. An explanation for the observation that the addition of butyrate and acetate to the medium at neutral pH causes solvent formation can also be explained by the fact that weak acids are known to be uncouplers (10, 13). Their addition affects the redox environment of hydrogenase and probably results in its decreased activity. In the present context, the viologens may also be affecting the redox environment of the hydrogenase complex.

It has been shown that alcohol formation is possible at pH values greater than 6.3 in *C. acetobutylicum*. The addition of viologen dyes has been shown to direct metabolic flow in *C. acetobutylicum*. It is believed that these observations hold true for a wide class of anaerobes that are dependent on hydrogenase for disposal of excess reducing equivalents. Furthermore, the macroscopic observation of increased solvent formation is directly linked to decreased hydrogenase activity. The addition of viologen has been shown to be a useful method for manipulating metabolic pathways in *C. acetobutylicum*.

LITERATURE CITED

1. **Adams, M. W. W., L. F. Mortenson, and J. S. Chen.** 1981. Hydrogenase. Biochim. Biophys. Acta **594**:105–176.
2. **Andersch, W., H. Bahl, and G. Gottschalk.** 1982. Acetone butanol production by *Clostridium acetobutylicum* in an ammonium limited chemostat at low pH values. Biotechnol. Lett. **4**:29–32.
3. **Andersch, W., H. Bahl, and G. Gottschalk.** 1983. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. Eur. J. Appl. Microbiol. Biotechnol. **18**:327–332.
4. **Bahl, H., M. Gottwald, A. Kuhn, V. Rale, W. Andersch, and G. Gottschalk.** 1986. Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **52**:169–172.
5. **Datta, R., and J. G. Zeikus.** 1985. Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. Appl. Environ. Microbiol. **49**:522–529.
6. **Doremus, M. G., J. C. Linden, and A. R. Moreira.** 1985. Agitation and pressure effects on acetone-butanol fermentation. Biotechnol. Bioeng. **27**:852–860.
7. **Gottschal, J. C., and J. G. Morris.** 1981. Non production of acetone and butanol by *Clostridium acetobutylicum* during glucose and ammonium limitation of continuous culture. Biotechnol. Lett. **3**:525–530.
8. **Gottwald, M., and G. Gottschalk.** 1985. The internal pH of *Clostridium acetobutylicum* and its effects on the shift from acid to solvent fermentation. Arch. Microbiol. **143**:42–46.
9. **Gray, C. T., and H. Gest.** 1965. Biological formation of molecular hydrogen. Science **148**:186–192.
10. **Holt, R. A., G. M. Stephens, and J. G. Morris.** 1984. Production of solvents by *Clostridium acetobutylicum* cultures maintained at neutral pH. Appl. Environ. Microbiol. **48**:1166–1170.
11. **Huang, L., C. W. Forsberg, and L. N. Gibbins.** 1986. Influence of extenal pH and fermentation products on *Clostridium acetobutylicum* intracellular pH and cellular distribution of fermentation products. Appl. Environ. Microbiol. **51**:1230–1234.
12. **Huang, L., L. N. Gibbins, and C. W. Forsberg.** 1985. Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acetogenic and solventogenic conditions. Appl. Environ. Microbiol. **50**:1043–1047.
13. **Huesemann, M., and E. T. Papoutsakis.** 1986. Effect of acetoacetate, butyrate and uncoupling ionophores on growth and product formation of *Clostridium acetobutylicum*. Biotechnol. Lett. **8**:37–42.
14. **Jones, D. T., A. V. Westhuizen, S. Long, R. Allcock, S. J. Reid, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43**:1434–1439.
15. **Jungermann, K., R. K. Thauer, G. Leimenstoll, and K. Decker.** 1973. Function of reduced pyridine nucleotide ferredoxin oxidoreductases in saccharolytic clostridia. Biochim. Biophys. Acta **305**:268–280.
16. **Kim, B. H., P. Bellows, R. Datta, and J. G. Zeikus.** 1984. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol. **48**:764–770.
17. **Long, S., D. T. Jones, and D. R. Woods.** 1984. The relationship between sporulation and solvent production in *Clostridium acetobutylicum* P262. Biotechnol. Lett. **6**:529–534.
18. **Maeda, H., and S. Kajiwara.** 1985. Malic acid production by an electrochemical reduction system combined with the use of diaphorase and methylviologen. Biotechnol. Bioeng. **27**:596–602.
19. **Martin, J. R., H. Petitdemange, J. Ballongue, and R. Gay.** 1983. Effects of acetic and butyric acids on solvents production by *Clostridium acetobutylicum*. Biotechnol. Lett. **5**:89–94.
20. **McNeil, B., and B. Kristiansen.** 1986. The acetone butanol fermentation. Adv. Appl. Microbiol. **31**:61–92.
21. **Meyer, C. L., J. W. Roos, and E. T. Papoutsakis.** 1986. Carbon monoxide gassing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **24**:159–167.
22. **Monot, F., J. M. Engasser, and H. Petitdemange.** 1983. Regulation of acetone butanol production in batch and continuous cultures of *Clostridium acetobutylicum*. Biotechnol. Bioeng. Symp. **13**:207–216.
23. **Monot, F., J. Martin, H. Petitdemange, and R. Gay.** 1982. Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. Appl. Environ. Microbiol. **44**:1318–1324.
24. **Petitdemange, H., C. Cherrier, G. Raval, and R. Gay.** 1976. Regulation of the NADH and NADPH-ferredoxin oxidoreductases in clostridia of the butyric group. Biochim. Biophys. Acta **421**:334–347.
25. **Roos, J. W., J. K. McLaughlin, and E. T. Papoutsakis.** 1984. The effect of pH on nitrogen supply, cell lysis and solvent production in fermentations of *Clostridium acetobutylicum*. Biotechnol. Bioeng. **27**:681–694.
26. **Terraciano, J. S., and E. R. Kashket.** 1986. Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **52**:86–91.
27. **Yerushalmi, L., and B. Volesky.** 1985. Importance of agitation in acetone-butanol fermentation. Biotechnol. Bioeng. **27**:1297–1305.
28. **Yerushalmi, L., B. Volesky, and T. Szczesny.** 1985. Effect of increased hydrogen partial pressure on the acetone butanol fermentation by *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **22**:103–107.
29. **Yu, L., and M. J. Wolin.** 1969. Hydrogenase measurement with photochemically reduced methylviologen. J. Bacteriol. **98**:51–55.
30. **Zeikus, J. G.** 1980. Chemical and fuel production by anaerobic bacteria. Annu. Rev. Microbiol. **34**:423–464.