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Growth of a Strictly Anaerobic Bacterium on Furfural (2-Furaldehyde)

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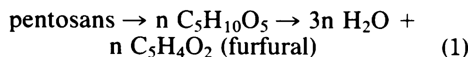
A strictly anaerobic bacterium was isolated from a continuous fermentor culture which converted the organic constituents of sulfite evaporator condensate to methane and carbon dioxide. Furfural is one of the major components of this condensate. This furfural isolate could degrade furfural as the sole source of carbon and energy in a defined mineral-vitamin-sulfate medium. Acetic acid was the major fermentation product. This organism could also use ethanol, lactate, pyruvate, or fumarate and contained cytochrome c_3 and desulfoviridin. Except for furfural degradation, the characteristics of the furfural isolate were remarkably similar to those of the sulfate reducer *Desulfovibrio gigas*. The furfural isolate has been tentatively identified as *Desulfovibrio* sp. strain F-1.

Furfural (2-furaldehyde) occurs naturally in some essential oils. It is prepared industrially by treating pentosans contained in straws or brans with hot sulfuric acid, which hydrolyzes the pentosans and then dehydrates the resulting pentoses (19, 25) as follows:



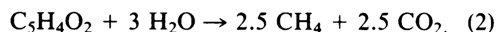
$$\text{pentosans} \rightarrow n\ C_5H_{10}O_5 \rightarrow 3n\ H_2O + n\ C_5H_4O_2\ \text{(furfural)} \quad (1)$$

Thus, furfural is also formed during the concentration of certain aqueous wastes containing pentosans; in paper mills which use the bisulfite pulping process, sulfite evaporator condensate (SEC) is a by-product of concentrating sulfite-spent liquor (5, 19). SEC may contain (per liter) up to 100 mmol of methanol, 400 mmol of acetic acid, 40 mmol of sulfite, and 30 mmol of furfural (7). Furfural is also formed during heat treatment of municipal wastes (16).

Furfural is regarded as toxic. It is used as a germicide (25), and, at concentrations found in SEC, furfural inhibits growth and fermentation of glucose by yeast (1, 3, 21).

Only a few microbial transformations of furfural are known (12). Yeast reduces this compound to 2-hydroxymethylfuran, and *Acetobacter ascendes* dismutes the aldehyde to the alcohol and acid. McCarty and co-workers (16) report that furfural at concentrations of 3 mmol/liter may be converted to methane by acclimatized anaerobic sludge cultures.

Recent investigations have shown that furfural at concentrations up to 30 mmol/liter along with the other organic materials in SEC may be very efficiently converted to methane by continuous fermentation (5, 7) as follows:



$$C_5H_4O_2 + 3\ H_2O \rightarrow 2.5\ CH_4 + 2.5\ CO_2 \quad (2)$$

In a previous publication (7), we reported briefly on the isolation of two of the major organisms from these cultures: a methanogenic bacterium (*Methanosarcina* sp.), which converted acetic acid and methanol to methane, and a sulfate reducer (*Desulfovibrio* sp.), which reduced sulfate (or sulfite) at the expense of ethanol. The objective of this study was to isolate the organism responsible for the primary attack on furfural. To our knowledge, this is the first report on a strictly anaerobic bacterium which will grow on furfural as the sole source of carbon and energy.

MATERIALS AND METHODS

Isolation and growth of organisms. The anaerobic culture technique of Hungate (10) as modified for use with serum tubes (2) and serum bottles (17) was used throughout the course of this work, except where otherwise indicated. All flasks were sealed with black butyl rubber stoppers (Bellco Glass, Inc.). A gas phase of 80% N_2–20% CO_2 (vol/vol) and an incubation temperature of 37°C was used. The furfural isolate was enriched and isolated from a 10-liter digester treating SEC at 37°C (7) on M1 medium modified from that of Brune and co-workers (7). After autoclaving, furfural was added to the tubes as indicated. The composition of M1 medium was (per liter): resazurin, 1 mg; NH_4Cl, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; NaCl, 0.2 g; $Na_2HPO_4 \cdot 2H_2O$, 1 g; $NaH_2PO_4 \cdot 2H_2O$, 0.8 g; minerals-1, 3 ml; minerals-2, 13 μl; Na_2S, 1.25 mmol; $Na_2S_2O_4$, 0.23 mmol; $(NH_4)_2SO_4$, 1 mmol. Minerals-1 contained (per liter): $MnCl_2 \cdot 4H_2O$, 475 mg; $CoCl_2 \cdot 6H_2O$, 170 mg; $CuSO_4 \cdot 5H_2O$, 50 mg;

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$ZnSO_4 \cdot 7H_2O$, 180 mg; $AlK(SO_4)_2 \cdot 12H_2O$, 18 mg; H_3BO_3, 10 mg. Minerals-2 contained (per milliliter): $Na_2SeO_3 \cdot 5H_2O$, 2 mg; $Na_2WO_4 \cdot 2H_2O$, 2 mg; $Na_2MoO_4 \cdot 2H_2O$, 2 mg. The pH was adjusted to 7.4 with $KHCO_3$ before 5 ml of medium was dispensed into roll tubes or 50 ml was dispensed into 100-ml serum bottles. For isolation, 2% agar (Difco Laboratories) was added. Stock cultures were kept in test tubes sealed with pyrogallol plugs in medium M2, which contained ethanol-sulfate-$CaCO_3$-soft agar as described elsewhere (20). Cultures were transferred once every 4 weeks.

For growth curves, medium M3 was used. It was a modification of the ethanol-sulfate-bicarbonate medium 1 of Schoberth (20). M3 medium contained (per liter): resazurin, 1 mg; NH_4Cl, 0.5 g; 1 M potassium phosphate buffer (pH 7.0), 1 ml; trace minerals and vitamins (26), 10 ml each; $FeSO_4 \cdot 7H_2O$, 20 mg; Na_2SO_4, 4 g; $NaHCO_3$, 2 g; $KHCO_3$, 1.7 g; sodium thioglycolate, 0.5 g; $Na_2S \cdot 9H_2O$, 0.1 g; sodium dithionite, 40 mg. The pH was 7.2 to 7.4.

Analytical procedures. Fatty acids and furfural were assayed by gas chromatography (Hewlett-Packard 5840A) with a flame ionization detector. A glass column (2 m by 2 mm) with 0.3% Carbowax 20M–1% H_3PO_4 on Carbopack C, 60 to 80 mesh (Werner Guenther-Analysentechnik), was used. The oven temperature was 160°C. Samples to be assayed were separated from suspended bacteria by centrifugation, diluted to a final concentration of 2 to 10 mmol of acetic acid per liter, and adjusted to about pH 2.0 by addition of HCl before injection (1 μl). Each determination was repeated six times. The detection limit was less than 1 mmol of acetic acid per liter. C_1 to C_4 alcohols, 2-furancarboxylic acid, and furfuryl alcohol were assayed on a Porapak QS column, using similar conditions. Concentrations of furfural were routinely checked with a spectrophotometer (Zeiss, DM4) at 276 nm. This method allowed the detection of less than 1 μmol/liter. Lactate and pyruvate were measured by using commercially available enzyme kits (Boehringer Mannheim Corp.). Protein was measured by the method of Bradford (6). The optical density of cell suspensions was measured in culture tubes with a Spectronic 88 colorimeter at 660 nm (path length, about 1.5 cm).

Phosphate buffer extracts of ethanol-grown cells were analyzed for cytochromes by absorbance spectrometry (14), using a Zeiss DM4 spectrophotometer. Desulfoviridin was assayed by fluorescence spectrometry (14); the supernatant of X-press-treated cell extracts was cleared by filtration through cellulose nitrate paper that was made alkaline (14), and spectra were taken with an Aminco SPF-500 fluorescence spectrometer (exciting wave length, 365 nm). H_2S formation was qualitatively checked by blackening of lead acetate paper.

RESULTS

Isolation of the furfural isolate. In the continuous fermentation of SEC, the steady-state concentration of furfural is below its detection limit of 1 μmol/liter (7). Further experiments under batch conditions showed that initial concentrations of furfural in liquid medium above 10 mmol/liter inhibited bacterial growth and degradation of furfural. Predominant organisms in cultures with furfural concentrations below 10 mmol/liter were motile, large vibroid to spirilloid rods. Initial attempts to isolate bacterial colonies on agar roll tubes with medium M1 and furfural failed since we were not able to set up a concentration of furfural high enough to allow detectable growth of colonies but also low enough not to inhibit the bacteria. Therefore, the anaerobic agar shake technique (4) was modified for repeated additions of small amounts of furfural so as to keep a steady concentration in the vicinity of growing colonies below an inhibitory level; anaerobic culture tubes (18 by 142 mm, Bellco Glass, Inc., no. 2946) closed by butyl stoppers (size no. 2 [Bellco]) contained 5 ml each of molten agar medium M1 (M1A). The tubes were inoculated with 0.1 ml of serially diluted furfural enrichment cultures. After the tubed, inoculated agar medium M1 had been solidified at room temperature, 1 ml of 10-mmol/liter furfural dissolved in sterile oxygen-free water was injected into each tube. The tubes were incubated at 37°C in an upright position (Fig. 1). Once per week, the spent furfural solutions were withdrawn and replaced by fresh solutions. Thus, a furfural gradient could develop from the top to the bottom in the agar columns. After 6 to 8 weeks, beige disk-shaped colonies of about 1 mm in diameter had developed in some tubes (Fig. 1).

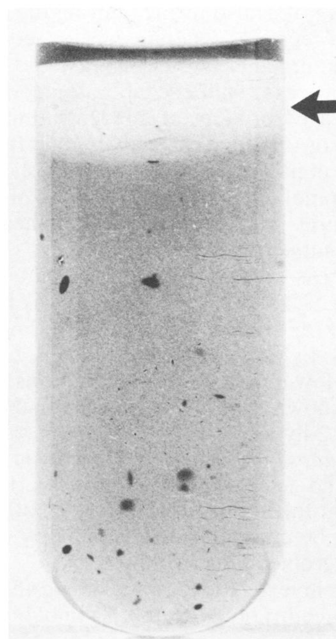


FIG. 1. Colonies of furfural-degrading anaerobes in an agar shake tube. Arrow shows dilute solution of furfural.

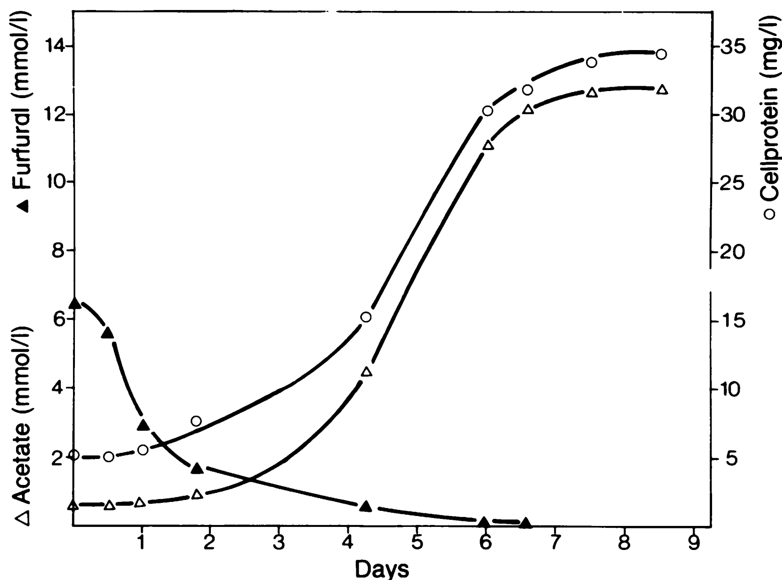


FIG. 2. Formation of acetic acid and cells (protein) in furfural-containing medium by the furfural-degrading organism at 37°C.

The agar columns were blown out of these tubes into sterile petri dishes, using sterile cotton-plugged Pasteur pipettes flushed by a gentle stream of argon. Colonies were picked, suspended in fresh medium M1, diluted in agar medium M1, incubated, and fed with furfural as described above.

Repeating this procedure twice resulted in colonies which consisted entirely of actively motile vibroid rods. They could be subcultured in liquid medium M1 containing 5 mmol of furfural per liter. Purity of the culture was checked by microscopic observation and by dilutions into AC medium (Difco). This medium allowed growth of possible contaminants only.

Characteristics of the furfural isolate. Growth of these organisms on medium M1 with furfural was very poor. Medium M3, which had been used by us to isolate sulfate reducers from SEC fermentors (7), gave better results. Figure 2 shows a typical growth curve of the furfural isolate on medium M3 supplemented with 6.2 mmol of furfural per liter. Furfural was completely metabolized within 7 days, accompanied by increases of acetic acid and cells (measured as cell protein). One can calculate from Fig. 2 that 1.89 mol of acetic acid and 4.55 g of cell protein were formed per mol of furfural degraded. The cell yield was 9.1 g/mol, assuming a cell protein content of 50%. In other experiments, yields of acetic acid varied somewhat between 1.61 and 1.9 mol/mol of furfural degraded. The lag between degradation of furfural and increases in cells and acetate suggests that an intermediate was produced before acetate production commenced. However, we could not detect furfuryl alcohol, 2-furoic acid, propionic acid, or butyric acid.

Cells of the furfural isolate were vibroid rods which became actively motile only after a few days, when most of the furfural had disappeared. The gram-negative cells were about 0.8 by 3 μm and were motile by means of a single polar flagellum (Fig. 3). These characteristics bear close resemblance to the description of the sulfate reducer *Desulfovibrio gigas* SMS-1 (DSM 496), which uses fumarate, pyruvate, lactate, or ethanol in the presence of sulfate (20). These compounds also supported growth of the furfural isolate (Table 1). For stock cultures, the furfural isolate was grown in medium M2 and transferred monthly.

Since growth did not occur in ethanol, furfural, or lactate media exposed to oxygen (resazurine turned pink), we conclude that this bacterium was strictly anaerobic. The cells contained cytochrome c_3 and desulfoviridin as indicated by absorption and fluorescence spectra of cell extracts (14, 20); absorption peaks of sodium dithionite-reduced extracts were at 420, 524, 552 (cytochrome c_3), and 630 nm (desulfoviridin); peaks of air-oxidized extracts were at 408 (cytochrome c_3) and 630 nm (desulfoviridin); and at an exciting wavelength of 365 nm, alkaline cell extracts showed red fluorescence (peak at 606 nm), which is typical for desulfoviridin. Cytochrome c_3 and desulfoviridin are electron carriers typical for sulfate-reducing bacteria (18).

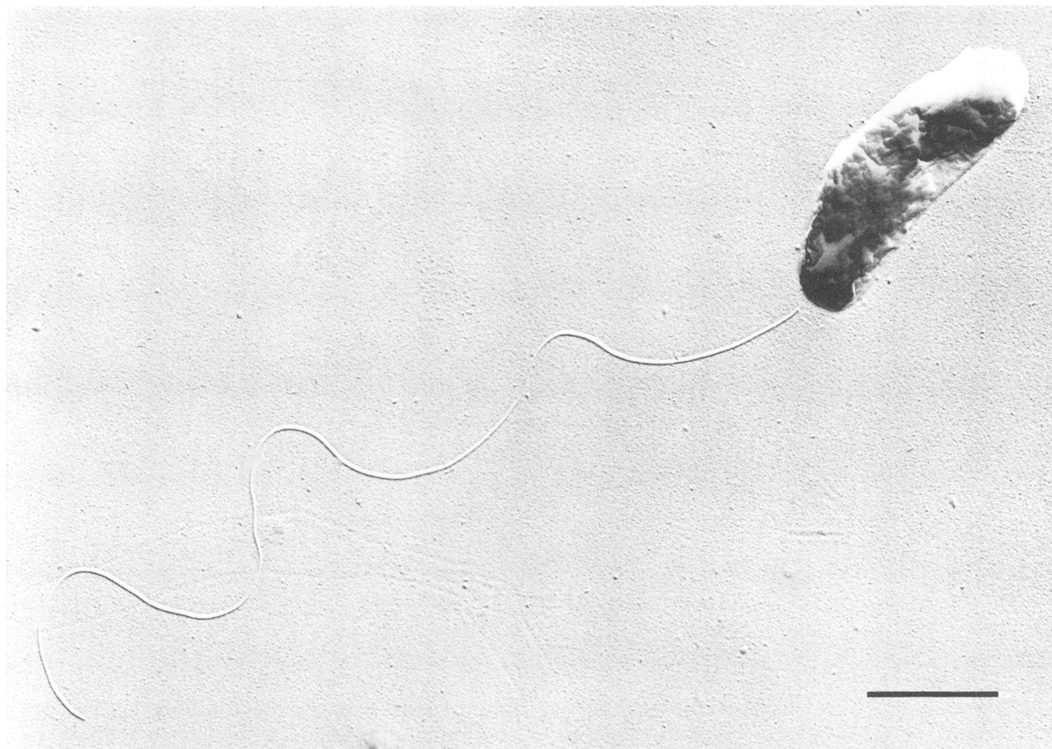


FIG. 3. Electron micrograph of the furfural-degrading bacterium showing a polar flagellum. Platinum-carbon shadowed preparation. Bar represents 1 μm.

In view of these characteristics, the furfural isolate has been tentatively identified as *Desulfovibrio* sp. strain F-1. A culture has been deposited with the German Culture Collection (DSM, Göttingen, FRG). The DSM accession number is 2590.

DISCUSSION

In the continuous fermentation of SEC, furfural at concentrations of 5 to 30 nmol/liter in the feed is degraded very efficiently (7); neither furfural nor its dissimilation products 2-furfuryl alcohol and 2-furoic acid can be detected in the fermentor. It has also been shown that furfural is nearly quantitatively converted to methane as in formula 2 by this mixed culture under batch conditions and that about 2 mmol of acetic acid appears as an intermediate per 1 mmol of furfural converted (7). This suggested that an organism might be isolated from this culture which would degrade furfural to acetic acid.

To our knowledge, the present work is the first report that a strictly anaerobic bacterium can use the heteroaromatic aldehyde furfural (regarded as toxic; 1, 3, 21, 25) as the sole carbon source for biosynthesis and for gaining cellular energy. Examples for the degradation of other heteroaromates containing nitrogen are the fermentation of nicotinic acid by *Clostridium barkeri* (24), the fermentation of purines and pyrimidines by certain clostridia (9), and the

TABLE 1. Organic substrates tested for support of growth of the furfural isolate[a]

Substrate tested	Final absorbance of culture (at 660 nm)
Ethanol	0.96
Pyruvate (sodium)	0.80
DL-Lactate (sodium)	0.70
Fumarate (sodium)	0.68
Blank	0.06

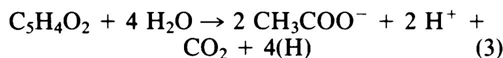
[a] Sulfate-containing medium M3 was supplemented with 1% carbon source, inoculated with 10% furfural-grown culture (cells were actively motile), incubated at 37°C, and checked each day for absorbance and motility. Final absorbance was reached after 7 days. Cells in the ethanol through fumarate cultures were actively motile and showed H_2S formation. Compounds tested which did not serve as substrates for the growth of the furfural isolate (absorbance < 0.18, no motile cells) were formate, acetate, propionate, butyrate, malate, methanol, glycerol, mannitol, and lactose.

degradation of cyanuric acid and atrazine by a novel facultative anaerobe (11).

Furfural, which contains oxygen, adds a novel compound to this list. By applying the method of substrate gradient-shake cultures as used in this study, one might be able to isolate anaerobes able to attack still other inhibitory substrates, which have been previously considered nonutilizable for growth under strictly anaerobic conditions.

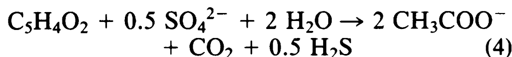
Acetic acid was the major product of the conversion of furfural (Fig. 2). Ethanol, fumarate, lactate, or pyruvate could replace furfural as a carbon substrate. Although H_2S formation from sulfate was detected qualitatively (blackening of lead acetate paper; Table 1), this has yet to be made quantitative. Cells of the furfural isolate were vibroid rods (Fig. 3), actively motile, possessed polar flagella, and contained cytochrome c_3 and desulfoviridin. According to these characteristics, the furfural isolate was tentatively identified as *Desulfovibrio* sp. strain F-1. The large cell shape of *Desulfovibrio* sp. strain F-1 suggested a certain relationship with *D. gigas* (13, 20). *D. gigas* SMS-1 (DSM 469) could also be grown on medium M3 supplemented with 6 to 9 mmol of furfural per liter. The yield of acetic acid was 1.6 to 1.8 mmol/mmol of furfural. Whether this characteristic may be found in other sulfate-reducing bacteria merits further investigation.

The conversion of furfural to acetic acid in the ratio of nearly 2 (Fig. 2) is as follows:



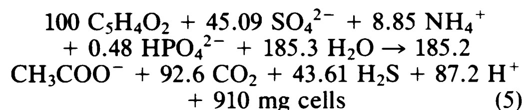
$$C_5H_4O_2 + 4\ H_2O \rightarrow 2\ CH_3COO^- + 2\ H^+ + CO_2 + 4(H) \quad (3)$$

Including sulfate reduction, the formula is as follows:



$$C_5H_4O_2 + 0.5\ SO_4^{2-} + 2\ H_2O \rightarrow 2\ CH_3COO^- + CO_2 + 0.5\ H_2S \quad (4)$$

Table 2 shows that formula (3) or (4) could adequately describe the conversion of furfural to acetic acid and cells; the amount of acetate found was in excellent agreement with the value calculated from cell yield. A complete approximate fermentation formula (in millimoles) for this pathway as calculated from Table 2 could be as follows:



$$100\ C_5H_4O_2 + 45.09\ SO_4^{2-} + 8.85\ NH_4^+ + 0.48\ HPO_4^{2-} + 185.3\ H_2O \rightarrow 185.2\ CH_3COO^- + 92.6\ CO_2 + 43.61\ H_2S + 87.2\ H^+ + 910\ \text{mg cells} \quad (5)$$

(An average formula for cells of *Desulfovibrio vulgaris*, $CH_{1.64}O_{0.32}N_{0.24}S_{0.04}P_{0.013}$ (22), was used for this calculation.).

TABLE 2. Products formed per 100 mmol of furfural decomposed by *Desulfovibrio* sp. strain F-1

Product	Amt found	Amt calculated
Acetate	189 mmol	185.2 mmol[a]
Cells	910 mg[b]	910 mg
CO_2	ND[c]	92.6 mmol[a]
C recovery	83%	100%

[a] Calculated from the cell yield shown, assuming that cell carbon was derived from furfural and using a value for the elemental cellular composition of *D. vulgaris* (22).

[b] The yield coefficient was 9.1 g/mol.

[c] ND, Not determined.

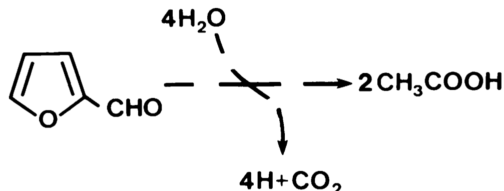


FIG. 4. Conversion of furfural to acetic acid by *Desulfovibrio* sp. strain F-1.

The conversion of furfural to acetic acid (Fig. 4) by sulfate-reducing bacteria or other strict anaerobes has not been reported previously. The initial metabolic steps could be similar to those in the degradation of furoic acid by a *Bacillus* spp. (8), which initiate the sequence by hydroxylation of the *O*-heteroatomic nucleus. An analogous hydroxylation of an *N*-heteroaromatic compound (nicotinic acid) is known to occur in *C. barkeri* (24). It will be necessary to find intermediates to elucidate the pathway of furfural degradation in *Desulfovibrio* sp. strain F-1. Although we could not detect compounds other than acetic acid in the experiments shown in Fig. 2 and Table 2, we do not preclude that in addition, small amounts of other substances such as ethanol, pyruvate, butanol, or hydrogen (15, 22, 23) may have been produced when the yield of acetic acid was somewhat lower than 1.9 mmol/mmol of furfural.

With the isolation of *Desulfovibrio* sp. strain F-1, all major organisms necessary for anaerobic treatment of SEC are now characterized. Apart from this applied aspect concerning waste purification, the present paper may also contribute to extending research on the degradation of toxic chemicals by anaerobic bacteria.

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LITERATURE CITED

1. **Azhar, A. F., M. K. Bery, A. R. Colcord, R. S. Roberts, and G. V. Corbitt.** 1981. Factors affecting alcohol fermen-

tation of wood acid hydrolysate. Biotechnol. Bioeng. Symp. **11**:293–300.

2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanolsulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
3. **Banerjee, N., R. Bhatnagar, and L. Viswanathan.** 1981. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Biotechnol. **11**:226–228.
4. **Barker, H. A.** 1936. Studies upon the methane-producing bacteria. Arch. Microbiol. **7**:420–438.
5. **Benjamin, M. M., J. F. Ferguson, and M. E. Buggins.** 1982. Treatment of sulfite evaporator condensate with an anaerobic reactor. Tech. Assoc. Pulp Pap. Ind. **65**:96–102.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Brune, G., S. M. Schoberth, and H. Sahm.** 1982. Anaerobic treatment of an industrial wastewater containing acetic acid, furfural and sulphite. Process Biochem. **17**:20–35.
8. **Dagley, S.** 1978. Pathways for the utilization of organic growth substrates, p. 305–388. *In* I. C. Gunsalus, L. N. Ornston, J. R. Sokatsch (ed.), The bacteria, vol. 4B. Academic Press, Inc., New York.
9. **Gottschalk, G.** 1979. Bacterial metabolism. Springer, New York.
10. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes. Methods Enzymol. **3B**:117–132.
11. **Jessee, J. A., R. E. Benoit, A. C. Hendricks, G. C. Allen, and J. L. Neal.** 1983. Anaerobic degradation of cyanuric acid, cysteine, and atrazine by a facultative anaerobic bacterium. Appl. Environ. Microbiol. **45**:97–102.
12. **Kieslich, K.** 1976. Microbial transformation of nonsteroid cyclic compounds. Thieme, Stuttgart.
13. **Le Gall, J.** 1963. A new species of *Desulfovibrio*. J. Bacteriol. **86**:1120.
14. **Le Gall, J., G. Mazza, and M. Dragoni.** 1965. Le cytochrome c_3 de *Desulfovibrio gigas*. Biochim. Biophys. Acta **99**:385–387.
15. **Lewis, A. J., and J. D. A. Miller.** 1975. Keto acid metabolism in *Desulfovibrio*. J. Gen. Microbiol. **90**:286–292.
16. **McCarty, P. L., L. Y. Young, D. C. Stuckey, and J. B. Healy, Jr.** 1977. Heat treatment for increasing methane yields from organic material, p. 179–199. *In* H. G. Schlegel and J. Barnea (ed.), Microbial energy conversion. Pergamon Press, Oxford.
17. **Miller, T. L., and M. J. Wolin.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27**:985–987.
18. **Postgate, J. R.** 1979. The sulphate-reducing bacteria. Cambridge University Press, Cambridge.
19. **Prager, B., P. Jacobsen, and F. Richter.** 1933. Beilsteins Handbuch der organischen Chemie, vol. 17, 4th ed., p. 272. Springer, Berlin.
20. **Schoberth, S.** 1973. A new strain of *Desulfovibrio gigas* isolated from a sewage plant. Arch. Microbiol. **92**:365–368.
21. **Soboleva, G. A., V. I. Golubkov, and A. M. Vitrinskaya.** 1973. Effect of furfural on the cytochrome system of yeasts. Mikrobiologiya **42**:441–444.
22. **Traore, A. S., C. E. Hatchikian, J.-P. Belaich, and J. Le Gall.** 1981. Microcalorimetric studies of sulfate-reducing bacteria: energetics of *Desulfovibrio vulgaris* growth. J. Bacteriol. **145**:191–199.
23. **Traore, A. S., C. E. Hatchikian, J. Le Gall, and J.-P. Belaich.** 1982. Microcalorimetric study of the growth of sulfate-reducing bacteria: comparison of the growth parameters of some *Desulfovibrio* species. J. Bacteriol. **149**:606–611.
24. **Tsai, L., and E. R. Stadtman.** 1971. Anaerobic degradation of nicotinic acid. Methods Enzymol. **8B**:233–249.
25. **Windholz, M., Budavari, L. Y. Stroumtsos, and M. N. Fertig (ed.).** 1976. The Merck Index, 9th ed. Merck and Co., Rahway, N.J.
26. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.