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Anaerobic biodegradation of methyl esters by *Acetobacterium woodii* and *Eubacterium limosum*

Shi Liu and Joseph M. Suflita

Department of Botany and Microbiology, The University of Oklahoma, Norman, Oklahoma 73019, USA

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SUMMARY

The ability of *Acetobacterium woodii* and *Eubacterium limosum* to degrade methyl esters of acetate, propionate, butyrate, and isobutyrate was examined under growing and resting-cell conditions. Both bacteria hydrolyzed the esters to the corresponding carboxylates and methanol under either condition. Methanol was further oxidized to formate under growing but not resting conditions. Unlike the metabolism of phenylmethylethers, no H_2 requirement was evident for ester biotransformation. The hydrolysis of methyl carboxylates is thermodynamically favorable under standard conditions and the mixotrophic metabolism of ester/CO_2 allowed for bacterial growth. These results suggest that the degradation of methyl carboxylates may be a heretofore unrecognized nutritional option for acetogenic bacteria.

INTRODUCTION

Methyl esters of acetate, propionate, butyrate and isobutyrate are widely used as solvents in many industrial applications [3,15,17]. These compounds also possess relatively high research octane numbers and are considered as potential biodegradable alternatives to recalcitrant gasoline oxygenates [31]. The biological formation of methyl acetate [1,13,18,28], methyl propionate [24,30] and (iso)butyrate esters [24] has been reported. However, the majority of methyl carboxylate production comes from commercial synthesis [3,35]. Understanding how these compounds are integrated into the carbon cycle is essential for assessing their environmental and health impacts. So far, there is very little information on the environmental fate of these compounds. Rakov et al. [27] reported that a *Pseudomonas* sp. hydrolyzes methyl acetate to acetate and methanol. Suflita and Mormile [31] demonstrated that several methyl carboxylates were completely mineralized by aquifer microorganisms under methanogenic conditions, presumably through the initial hydrolytic cleavage of the parent molecules. However, the reaction sequence and the identity of the requisite microorganisms were not established.

In this study, *Acetobacterium woodii* and *Eubacterium limosum* were tested for their ability to metabolize selected methyl carboxylates. These bacteria hydrolyzed all tested esters to methanol and the corresponding carboxylic acids. In the presence of CO_2, methanol was further oxidized to formate and the reducing equivalents released were coupled with CO_2 reduction to acetate. To our knowledge, this is the first demonstration of methyl ester hydrolytic activity in acetogens.

Correspondence to: J.M. Suflita, Department of Botany and Microbiology, The University of Oklahoma, Norman, Oklahoma 73019, USA.

MATERIALS AND METHODS

Bacteria and culture conditions

Stock cultures of *A. woodii* (ATCC 29683) and *E. limosum* (ATCC 8486) were grown in a defined medium [32] with 8.5 mM syringate as the sole organic carbon source under a H_2/CO_2 (8:2; 2 atm) headspace. The cultures used were obtained from single colonies grown on this medium solidified with agar (2%). Syringate-grown cultures (2%) were inoculated into the defined medium containing a methyl carboxylate (5 mM). Positive controls assessing culture viability under the incubation conditions employed 5 mM syringate as an organic carbon source. The same medium without an organic carbon source was used to measure background acetogenesis by the two inocula. All incubations were placed at 30 °C in the dark under a H_2/CO_2 (8:2; 2 atm) atmosphere.

Experiments with resting-cells

To evaluate the effect of headspace gas composition on the biodegradation of methyl esters, syringate-grown cells were harvested anaerobically [19] and suspended in 15 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.8) containing a methyl carboxylate (2 mM). Positive controls contained 2 mM syringate as an organic carbon source, while autoclaved (121 °C;

20 min) cell preparations served as negative controls. *A. woodii* and *E. limosum* cells in suspension resulted in a protein content of 1630 and 870 $\mu g\ ml^{-1}$, respectively. The headspace volume was 11 ml and was filled with either H_2/CO_2 or N_2/CO_2 gas mixtures (8:2; 2 atm). The cell suspensions were incubated in the dark at 30 °C.

Analytical techniques and chemicals

Carboxylic esters and volatile fatty acids were analyzed by gas (GC) and high-performance liquid (HPLC) chromatography, respectively [19,31]. Headspace gas pressure, culture optical density and protein concentration were measured with a transducer, a spectrophotometer [19] and the Bio-Rad DC protein assay (Bio-Rad Laboratory, Richmond, CA, USA), respectively. Teflon-lined composite rubber stoppers were used to close all incubation vessels. All methyl carboxylates tested in this study were kindly provided by the American Petroleum Institute. Other chemicals were from Aldrich Chemical Co. (Milwaukee, WI, USA) and were reagent grade.

RESULTS

Degradation of methyl carboxylates by growing cultures incubated under the H_2/CO_2 headspace

A. woodii and *E. limosum* metabolized the methyl esters of acetate, propionate, butyrate and isobutyrate to methanol and corresponding carboxylates (identified by comparing GC and HPLC retention times of the products with those of authentic standards) under conditions which allowed growth (Fig. 1). Methanol was produced and then consumed during the incubation. Other major end products were formate and acetate which were also generated in ester-unamended controls (Fig. 2). The degradation of straight chain esters was near completion within the observation period and the bacteria were able to grow at the expense of these compounds (Fig. 1). However, the transformation of methyl isobutyrate was slow and only slight growth of *E. limosum* was measured at the end of the incubation period (Fig. 1). For *A. woodii* incubations, the degradation rates of straight chain esters was positively correlated with the chain length of the acyl groups. *E. limosum* grew better under these conditions than *A. woodii* (Fig. 1) but *A. woodii* produced more acids from esters than *E. limosum* (Fig. 2). Both bacteria failed to grow appreciably in incubations that did not receive a methyl ester, even though they consumed H_2/CO_2 and produced formate and acetate (Fig. 2). The viability of the inoculum was confirmed by the typical level of growth measured in syringate-amended positive controls. Methanol was not detected in either ester-unamended or positive control incubations.

Effect of headspace gas composition on ester degradation

To test whether H_2 is required for the biotransformation of methyl esters, resting-cell suspensions were incubated under a H_2/CO_2 or N_2/CO_2 atmosphere. Based on the loss of substrate and formation of methanol (Fig. 3) and corresponding carboxylates (Fig. 4), almost identical rates of ester hydrolysis were observed for *A. woodii* cells incubated under both types of headspace. The loss of headspace gas pressure in the H_2/CO_2 incubations was much higher than that shown by the N_2/CO_2 incubations (data not shown) and large amounts of formate and acetate were produced in the former but not the latter incubations (Fig. 4). In contrast to growth experiments, near-stoichiometric amounts of methanol persisted in resting-cell suspensions under both atmospheres for longer periods of time (Fig. 3). The positive correlation between the acyl chain length of the esters and their depletion rates was more apparent in resting-cell incubations than in growing cultures. In addition, extensive degradation of methyl isobutyrate was observed. However, no acetate formation was detected in incubations amended with methyl isobutyrate (Fig. 4). Autoclaved controls incubated under both atmospheric conditions showed little or no substrate disappearance and methanol formation (Fig. 3). No formate or acetate were detected in these controls. In comparison with ester degradation, syringate transformation occurred only under the H_2/CO_2 but not the N_2/CO_2 atmosphere (Liu and Suflita, manuscript in preparation). Comparable results were also observed for *E. limosum*.

DISCUSSION

The disappearance of methyl carboxylates and the detection of stoichiometric amounts of the corresponding carboxylates in non-sterile incubations but not in autoclaved controls (Figs 1 and 3) confirmed that the transformation was microbially mediated. The depletion rates of straight chain esters were positively correlated with the length of acyl chains. At present, we do not have a biological explanation for such a phenomenon. Unlike straight chain esters, methyl isobutyrate inhibited bacterial growth (Fig. 1) and interfered with acetate production (Figs 2 and 4). The reason for such an inhibitory effect by this secondary ester is not known.

Methanol was detected as a product formed during anaerobic ester biodegradation. Previous studies with aerobic organisms also showed methanol as a product of ester hydrolysis [23,27]. The formation of methanol and the carboxylate derivatives may be achieved by O-demethylation followed by hydroxylation, or demethoxylation followed by hydrogenation. In the O-demethylation scheme, the bond rupture takes place between the ether oxygen and the alkyl carbon atom (alkyl–oxygen fission), while with demethoxylation, the bond breakage occurs between the ether oxygen and the acyl carbon atom (acyl–oxygen fission). ^{18}O labelling studies with aliphatic [5,7,26] and aryl [4,6] esters and lactone [20] in abiotic systems always showed the acyl–oxygen fission. Chemical analyses suggest that demethoxylation should be the most common mechanism among all possible alternatives, especially in alkaline conditions [12]. In consistent fashion, we propose the following generalized reaction for the initial microbial biotransformation of methyl esters: $RCOOCH_3 + OH^- \rightarrow RCOO^- + CH_3OH$.

Thus, the metabolism of methyl esters by these cells is

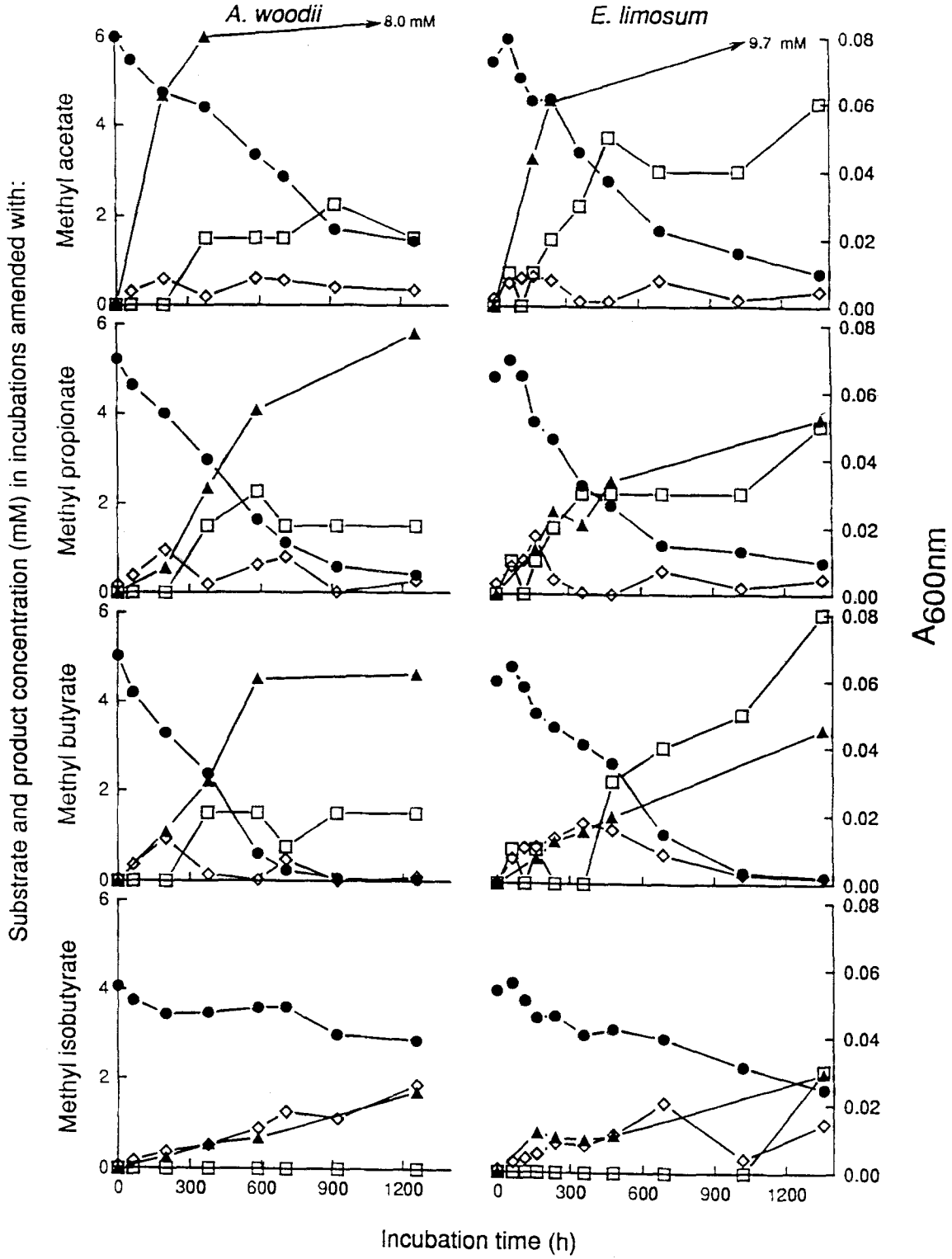

Fig. 1. Biodegradation of methyl carboxylates by *A. woodii* and *E. limosum* growing under a H_2/CO_2 headspace. Methyl carboxylates (●); Carboxylates (▲); Methanol (◇); Optical density (□).

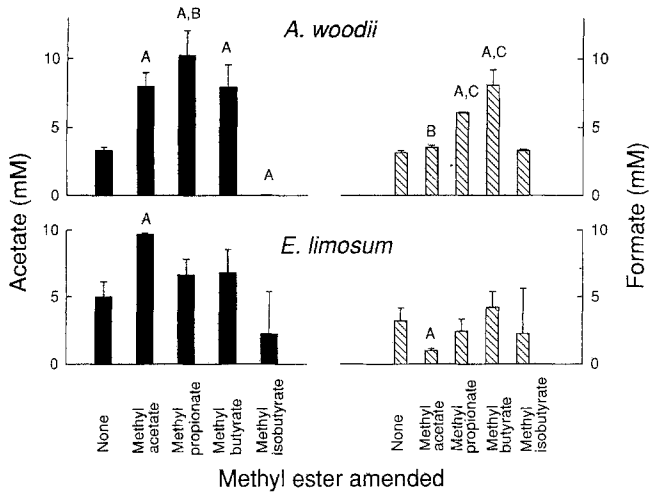

Fig. 2. Acid production measured at the end of the growth experiments. Averages and standard deviations (error bars) of duplicates. The letter A indicates that the acid produced in the ester-amended incubations was significantly ($P<0.05$) different from the ester-free incubations. The letters B and C indicate that the acid production by *A. woodii* was more than that produced by the *E. limosum* at $P<0.05$ and $P<0.1$ levels, respectively.

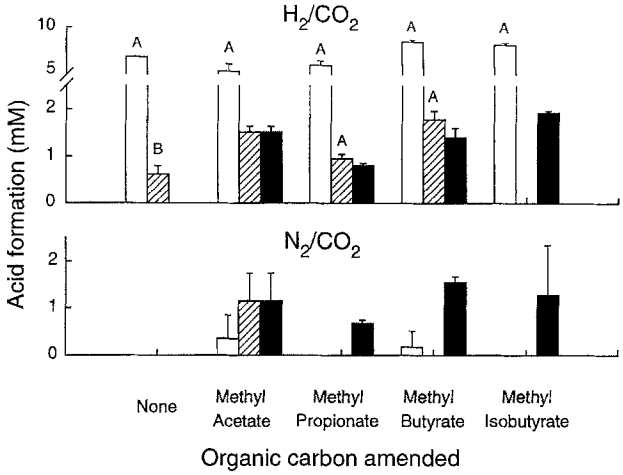

Fig. 4. Production of formate (open bars), acetate (striped bars) and the corresponding carboxylic acids (filled bars) from various methyl esters by resting-cell suspensions of *A. woodii* held under a H_2/CO_2 or a N_2/CO_2 headspace. Averages and standard deviations (error bars) of duplicates. A and B on top of the bars, respectively, indicate that the acid production levels in the H_2/CO_2 incubations was very significantly ($P<0.01$) and significantly ($P<0.05$) higher than that of the N_2/CO_2 incubations.

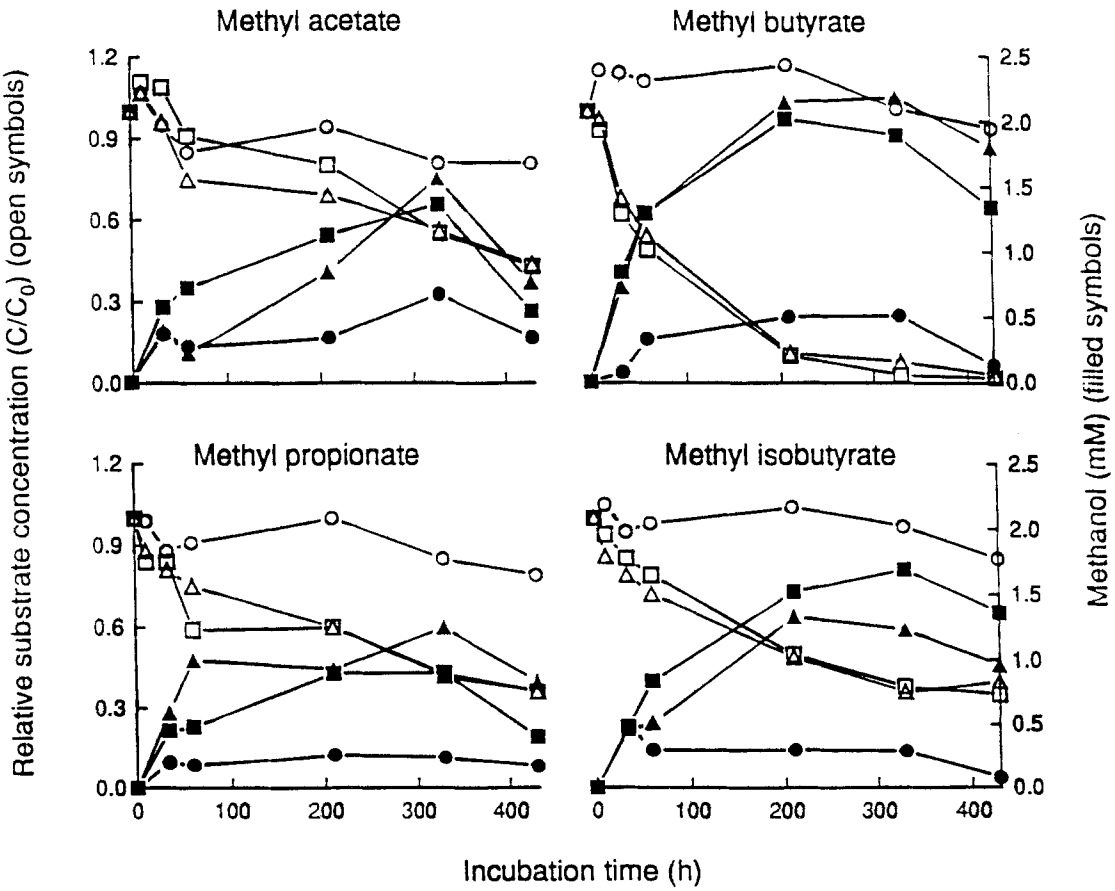

Fig. 3. Depletion of methyl carboxylates and production of methanol in resting-cell incubations of *A. woodii* under a H_2/CO_2 (□, ■) or a N_2/CO_2 (△, ▲) headspace. Autoclaved cells suspended under both atmospheric conditions (○, ●) served as the abiotic controls.

fundamentally different from the bioconversion of phenylmethylethers. With the latter, methanol is not detected as a free intermediate [2,11,14] and ^{18}O labelling studies confirmed that bond cleavage is between the oxygen atom and the methyl group [9]. In addition, the dependency for reductive activation of methyl transfer reactions during phenylmethylether metabolism has been observed [16,29] and H_2 was recently shown to supply reducing equivalents for this transformation [19, Liu and Suflita, manuscript in preparation]. However, no requirement for H_2 was evident during the biotransformation of the methyl esters (Figs 3 and 4). Thus, the initial transformations of these two classes of methoxylated compounds must proceed by different mechanisms: hydrogenolysis for the phenylmethylethers and hydrolysis for the esters.

Hydrolysis of esters is thermodynamically favorable under standard conditions (Table 1). Thus, this transformation may occur in the absence of CO_2/HCO_3^- (such a possibility was not tested in the current study). However, CO_2/HCO_3^- may impart an additional advantage to ester-hydrolyzing bacteria. CO_2/HCO_3^- can function as electron acceptor for the disposition of reducing equivalents generated during methanol oxidation (Table 1). It also helps to maintain the culture pH to favor demethoxylation. In this study, bacterial growth was observed in ester-amended H_2/CO_2 incubations (Fig. 1) but not in incubations containing just H_2/CO_2 (data not shown). In reality, microorganisms will usually encounter multiple carbon sources, especially CO_2/HCO_3^-. Mixotrophic metabolism at the expense of methyl esters and CO_2 may be a usual nutritional mode for microorganisms in environments receiving these esters. A mechanism for this metabolism is proposed (Fig. 5). The detection of formate and acetate in the ester-degrading cultures is consistent with this mechanism.

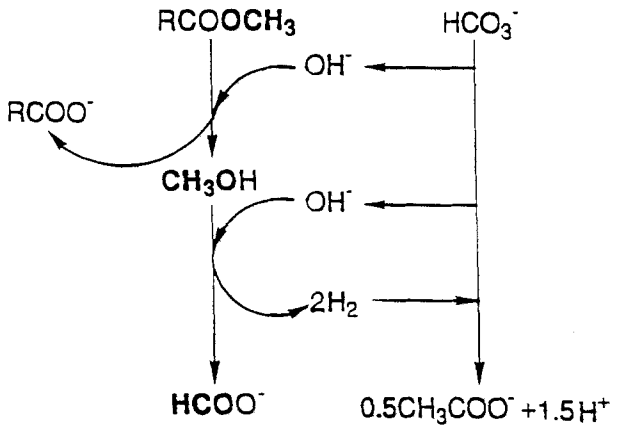

Fig. 5. A mixotrophic scheme for biodegradation of methyl carboxylates in the presence of CO_2/HCO_3^-. Reactants and products are consistent with the information in Table 1.

Methanol was consumed in growing cultures (Fig. 1) but persisted in resting-cell suspensions (Fig. 3). The failure of resting cells to metabolize methanol was also observed when this alcohol was given as the primary substrate (data not shown). Thus, the persistence of methanol in resting-cell incubations is not due to the inhibitory effect of other ester hydroxylation products. In fact, the metabolism of H_2 and CO_2 in methanol-amended suspensions (as evidenced by formate and acetate production (Fig. 4) was comparable to the ester-free controls. The lack of methanol metabolism by resting cells grown on syringate may reflect the need for induction. However, this result attests to the fact that the metabolism of methanol and phenylmethylethers is distinctly different. Alternatively, it may be that methanol metabolism

TABLE 1

Gibbs free energy available from reactions related to ester degradation[a]

Reactions	$\Delta G^{o\prime}$ kJ/reaction
A. Hydroxylation of methyl carboxylates	
$CH_3COOCH_3 + OH^- \rightarrow CH_3COO^- + CH_3OH$	−63.2
$CH_3CH_2COOCH_3 + OH^- \rightarrow CH_3CH_2COO^- + CH_3OH$	−62.8
$CH_3CH_2CH_2COOCH_3 + OH^- \rightarrow CH_3CH_2CH_2COO^- + CH_3OH$	−58.6
Summary:	AVG
$RCOOCH_3 + OH^- \rightarrow RCOO^- + CH_3OH$	−61.5
B. Other related reactions	
$CH_3OH + OH^- \rightarrow HCOO^- + 2\ H_2$	−18.5
$HCO_3^- + 2\ H_2 + 0.5\ H^+ \rightarrow 0.5\ CH_3COO^- + 2\ H_2O$	−52.3
$2H_2O \rightarrow 2\ OH^- + 2\ H^+$	80.0
Summary:	
$CH_3OH + HCO_3^- \rightarrow HCOO^- + 0.5\ CH_3COO^- + OH^- + 1.5\ H^+$	9.4
C. Mixotrophic metabolism	
$RCOOCH_3 + HCO_3^- \rightarrow RCOO^- + 0.5\ CH_3COO^- + HCOO^- + 1.5\ H^+$	−52.1[b]

[a] ΔG°_f values from Thauer et al. [33], except methyl carboxylates [34].

[b] Based on the average value of ΔG°_f for ester hydrolysis.

is tightly coupled to anabolic pathways so that the consumption of this alcohol is not evident in resting cell preparations. Similar findings have been made with CO in resting-cell suspensions of *A. woodii* [10] and *Clostridium thermoaceticum* [21,22]. Further studies are needed to clarify this issue.

The toxicity of methyl carboxylates is generally mild [3,8], except for a few reported incidences [25]. In this study, the microbial degradation of a variety of these compounds was shown and the products of this metabolism were clarified. By the nature of the products, the parent esters are likely to be environmentally compatible compounds and potentially suitable for large-scale industrial use.

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REFERENCES

1 Avedovech, R.M. Jr, M.R. McDaniel, B.T. Watson and W.E. Sandine. 1992. An evaluation of combinations of wine yeast and *Leuconostoc oenos* strains in malolactic fermentation of Chardonnay wine. Am. J. Enol. Viti. 43: 253–260.

2 Bache, R. and N. Pfennig. 1981. Selective isolation of *Acetobacterium woodii* on methoxylated aromatic acids and determination of growth yields. Arch. Microbiol. 130: 255–261.

3 Browning, E. 1965. Toxicity and Metabolism of Industrial Solvents. Elsevier, New York.

4 Bunton, C.A. and T. Hadwick. 1957. Tracer studies in ester hydrolysis. V. The kinetic form and stereochemical course of the hydrolysis of *p*-methoxydiphenylmethyl acetate. J. Chem. Soc. 3043–3047.

5 Bunton, C.A. and T. Hadwick. 1958. Tracer studies in ester hydrolysis. VI. The hydrolysis of methyl trifluoroacetate. J. Chem. Soc. 3248–3256.

6 Bunton, C.A., A.E. Comyns, J. Graham and J.R. Quayle. 1955. Preparation and hydrolysis of esters of 2,4,6-triphenylbenzoic acid. II. Mechanisms of hydrolysis. J. Chem. Soc. 3817–3824.

7 Datta, S.C., J.N.E. Day and C.K. Ingold. 1939. Mechanism of hydrolysis of carboxylic esters and of esterification of carboxylic acids. Acid hydrolysis of an ester with heavy oxygen as isotopic indicator. J. Chem. Soc. 838–840.

8 de Bruin, A. 1976. Biochemical Toxicology of Environmental Agents. Elsevier, New York.

9 DeWeerd, K.A., A. Saxena, D.P. Nagle, Jr and J.M. Suflita. 1988. Metabolism of the ^{18}O-methoxy substituent of 3-methoxybenzoic acid and other unlabeled methoxybenzoic acids by anaerobic bacteria. Appl. Environ. Microbiol. 54: 1237–1242.

10 Diekert, G., E. Schrader and W. Harderet. 1986. Energetics of CO formation and CO oxidation in cell suspensions of *Acetobacterium woodii*. Arch. Microbiol. 144: 386–392.

11 Doré, J. and M.P. Bryant. 1990. Metabolism of one-carbon compounds by the ruminal acetogen *Syntrophococcus sucromutans*. Appl. Environ. Microbiol. 56: 984–989.

12 Euranto, E.K. 1969. Esterification and ester hydrolysis. In: The Chemistry of Carboxylic Acids and Esters (Patai, S., ed.), pp. 505–588, Interscience-Publishers, New York.

13 Forney, C.F., J.P. Mattheis and R.K. Austin. 1991. Volatile compounds produced by broccoli under anaerobic conditions. J. Agric. Food Chem. 39: 2257–2259.

14 Frazer, A.C. and L.Y. Young. 1986. Anaerobic C_1 metabolism of the O-methyl-^{14}C-labeled substituent of vanillate. Appl. Environ. Microbiol. 51: 84–87.

15 Inoue, T., T. Takeuchi, N. Hisanaga, Y. Ono, M. Iwata, M. Ogata, K. Saito, H. Sakurai, I. Hara, T. Matsushita and M. Ikeda. 1983. A nationwide survey on organic solvent components in various solvent products: Part 1. Homogeneous products such as thinners, degreasers and reagents. Ind. Health 21: 175–183.

16 Kreft, J.-U. and B. Schink. 1993. Demethylation and degradation of phenylmethylethers by the sulfide-methylating homoacetogenic bacterium strain TMBS 4. Arch. Microbiol. 159: 308–315.

17 Kumai, M., A. Koizumi, K. Saito, H. Sakurai, T. Inoue, Y. Takeuchi, I. Hara, M. Ogata, T. Matsushita and M. Ikeda. 1983. A nationwide survey on organic solvent components in various solvent products: Part 2. Heterogeneous products such as paints, inks and adhesives. Ind. Health 21: 185–197.

18 Leino, M., J. Kaitaranta and H. Kallio. 1992. Comparison of changes in headspace volatiles of some coffee blends during storage. Food Chem. 43: 35–40.

19 Liu, S. and J.M. Suflita. 1993. H_2-CO_2-dependent anaerobic O-demethylation activity in subsurface sediments and by an isolated bacterium. Appl. Environ. Microbiol. 59: 1325–1331.

20 Long, F.A. and L. Friedman. 1950. Determination of the mechanism of γ-lactone hydrolysis by a mass spectrometric method. J. Am. Chem. Soc. 72: 3692–3695.

21 Martin, D.R., L.L. Lundie, R. Kellum and H.L. Drake. 1983. Carbon monoxide-dependent evolution of hydrogen by the homoacetate-fermenting bacterium *Clostridium thermoaceticum*. Curr. Microbiol. 8: 337–340.

22 Martin, D.R., A. Misra and H.L. Drake. 1985. Dissimilation of carbon monoxide to acetic acid by glucose-limited cultures of *Clostridium thermoaceticum*. Appl. Environ. Microbiol. 49: 1412–1417.

23 Mizunuma, K., T. Kawai, T. Yasugi, S.-I. Horiguchi, O. Iwami and M. Ikeda. 1992. In vitro hydrolysis of methyl acetate, a limitation in application of head-space gas chromatography in biological monitoring of exposure. Toxicol. Lett. 62: 247–253.

24 Nelson, D.R. and C.L. Fatland. 1992. Novel esters on long-chain methyl-branched alcohols in pupae of the tobacco hornworm. Insect Biochem. Mol. Biol. 22: 99–110.

25 Ogawa, Y., R. Takatsuki, T. Uema, Y. Seki, K. Hiramatsu, A. Okayama, K. Morrimoto and Z. Kaneko. 1988. Acute optic neuropathy induced by thinner sniffing: inhalation of mixed organic solvent containing methyl alcohol and methyl acetate. Ind. Health 26: 239–244.

26 Polanyi, M. and A.L. Szabo. 1934. Mechanism of hydrolysis. Alkaline saponifications of amyl acetate. Trans. Faraday Soc. 30: 508–512.

27 Rakov, D.Y., N. V. Doronina, Y.A. Trotsenko and R.M. Alieva. 1991. Growth of methylotrophic bacteria on methylacetate. Prikl. Biokhim. Mikrobiol. 27: 584–588.

28 Salinas, M.R., G. Alonso, G. Navarro and F. Pardo. 1990. Contribution to the study of the major aromatic components in the industrial processing of wine by carbonic maceration of Monastrell grapes: II. Ethyl acetate, isopentyl acetate, methyl acetate, ethyl formate and ethyl propionate. An. Bromatol. 42: 219–226.

29 Stupperich, E. and R. Konle. 1993. Corrinoid-dependent methyl transfer reactions are involved in methanol and 3,4-dimethoxybenzene metabolism by *Sporomusa ovata*. Appl. Environ. Microbiol. 59: 3110–3116.

30 Suàrez, M. and C. Duque. 1991. Volatile constituents of Lulo (*Solanum vestissimum* D.) fruit. J. Agric. Food Chem. 39: 1498–1500.

31 Suflita, J.M. and M.R. Mormile. 1993. Anaerobic biodegradation of known and potential gasoline oxygenates in the terrestrial subsurface. Environ. Sci. Technol. 27: 976–978.

32 Tanaka, K. and N. Pfennig. 1988. Fermentation of 2-methoxyethanol by *Acetobacterium malicum* sp. nov. and *pelobacter venetianus*. Arch. Microbiol. 149: 181–187.

33 Thauer, R.K., K. Jungermann and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41: 100–180.

34 Thermodynamics Research Center. 1986. TRC Thermodynamic Tables: Non-hydrocarbons. Thermodynamics Research Center, The Texas A&M University System, College Station, Texas.

35 Windholz, M. 1983. The Merck Index (10th edn), Merck & Co., Rahway, NJ.