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Enumeration of Acetogens by a Colorimetric Most-Probable-Number Assay

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Anaerobic O demethylation by acetogenic bacteria often is the first step in the mineralization of methoxylated aromatic compounds in anoxic environments. In this reaction, an ether bond is cleaved and the resulting methyl group is metabolized via the acetyl coenzyme A pathway (acetogenesis). Anaerobic O demethylation was used to assess acetogen populations. Environmental samples were diluted in anaerobic medium containing a methoxylated aromatic substrate (vanillate) and titanium(III), and acetogen titers were estimated by the most-probable-number (MPN) method. Complex formation between Ti(III) and vicinal hydroxyl groups of the aromatic products of anaerobic O demethylation results in the development of a yellow color in the medium, which can be detected by eye and monitored spectrophotometrically. High-performance liquid chromatography analysis of the yellow MPN tubes showed that they contained the product of anaerobic O demethylation of vanillate (protocatechuate). This assay was used to enumerate O-demethylating acetogen populations in environmental samples.

Acetate is a central metabolite in anoxic environments serving as a major carbon source to a variety of anaerobes (7). Acetate can be derived from the autotrophic or mixotrophic metabolism of H_2/CO_2 and/or CO by acetogens through the acetyl coenzyme A (CoA) pathway (16). The ability of acetogens to carry out anaerobic O demethylation provides them with an additional source of carbon in the form of methyl substituents derived from methoxylated compounds (8). Methoxylated substrates have been used to enrich for and to study acetogenic bacteria from a variety of anoxic environments (1, 18). The methyl groups derived from anaerobic O demethylation are channeled through the acetyl-CoA pathway to synthesize acetate, to assimilate carbon, and to conserve energy (7). Most acetogens do not further metabolize the aromatic compound, but subsequent to O demethylation, other anaerobes can modify and mineralize the aromatic ring (11, 12, 19). The proportion of acetate in anaerobic environments derived from the metabolism of methyl substituents is not known. However, acetogens are widespread in anoxic environments, and it is believed that acetogenic CO_2 fixation may account for as much as 10% of the estimated 10^{13} kg of acetate cycled globally (7).

Novel methods are needed for assessing the distribution of acetogen populations in the environment. Acetogens are difficult to distinguish in microbial communities since many other types of bacteria can utilize H_2/CO_2 or produce acetate (7), and there are no specific inhibitors of the acetyl-CoA pathway or biologically unique cofactors present in acetogens. Nevertheless, two methods have been used to enumerate acetogens in environmental samples by cultivation, one based on the detection of colonies that form acid under H_2/CO_2 (2) and the other a most-probable-number (MPN) method based on H_2/CO_2 acetogenesis (5, 6). Approaches that involve the use of molecular strategies to study acetogens in the environment have been reviewed previously (13).

A new colorimetric assay is described for the MPN enumeration of acetogens through their ability to anaerobically O demethylate methoxylated aromatic substrates. The colorimetric assay is based on the interaction of the hydroxyaromatic products of anaerobic O demethylation with titanium(III). In this study, the conversion of vanillate (4-hydroxy-3-methoxy benzoate) to protocatechuate (3,4-dihydroxybenzoate) was monitored with two environmental samples. In one of the samples, the MPN of populations enumerated by scoring for anaerobic O demethylation of vanillate was compared to the MPN of acetogens enriched on H_2/CO_2 and enumerated by acetate quantitation. With the colorimetric method, O-demethylating acetogen populations can be easily quantitated in a microtiter plate format.

(A preliminary report of this work has been presented previously [8b].)

MATERIALS AND METHODS

Sources of environmental samples. Dicamba-treated agricultural top soil from Wyoming kindly provided by Patricia Colberg and sediment collected from an orange grove drainage ditch in Manatee County, Fla., by Craig Phelps were used as inocula.

MPN medium. A modified 1019 *Acetobacterium* medium (8a) served as the medium. The medium was prepared anaerobically and contained the following (per liter): 0.1% resazurin, 1 ml; yeast extract, 0.5 g; $NaHCO_3$, 3 g; NH_4Cl, 1 g; KH_2PO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $Na_2WO_4 \cdot 2H_2O$, 4 ng; $NaSeO_3 \cdot 5H_2O$, 3 ng; $NiCl_2 \cdot 6H_2O$, 50 mg; 1.25% L-cysteine · HCl/1.25% $Na_2S \cdot 9H_2O$, 20 ml. Wolfe's mineral and vitamin solutions were added as described for ATCC 1019 medium. Added carbon sources tested, where indicated, were 2.5 mM vanillate with a headspace of H_2/CO_2 (80:20, 1 atm [1 atm = 101.29 kPa] over pressure), H_2/CO_2 (80:20, 1 atm over pressure), or N_2/CO_2 (70:30, 1 atm over pressure). All MPN tubes contained 30 mM bromoethanesulfonic acid (BESA) to inhibit growth of methanogenic bacteria. At the end of the incubation period, methane production was measured by gas chromatography as previously described (15). A Ti(III)-nitrilotriacetate (NTA) solution was prepared as described previously (14) and used at a final concentration of 0.25 mM Ti(III) with 1.0 mM NTA.

Preparation of MPN dilution tubes. One gram (wet weight) of soil or sediment was added to 9.0 ml of 1019 medium. From this first dilution (10^{-1}), serial 10-fold dilutions up to the 10^{-9} dilution were made in Balch tubes (Bellco Glass Inc., Vineland, N.J.). These dilutions were used to set up the 10^{-2} through 10^{-10} MPN tubes as follows. One milliliter from a dilution was transferred into 9 ml of 1019 medium. The 10^{-1} MPN tubes were independently prepared by adding 1.0 g of soil or sediment directly into 9.0 ml of 1019 medium. For each dilution, three or five replicates were prepared. Controls consisted of both tubes with 10^{-1} dilutions of soil or sediment that was autoclaved on three consecutive days and incubated in the presence of substrate and tubes inoculated and incubated

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without substrate. MPN tubes were incubated horizontally to enhance gas exchange. An independent subsample of the soil or sediment was used for each MPN determination.

An MPN microtiter plate format adapted from the study by Rowe et al. (17) was used to set up eight replicate dilutions in microtiter plates as follows. Two hundred microliters of a 10^{-1} dilution of soil or sediment was transferred into each well of the first column (eight wells) of a 96-well microtiter plate (Costar Corp., Cambridge, Mass.). Samples (20 μl) from each well were serially transferred into successive wells containing 180 μl of 1019 medium. Microtiter plate MPN procedures were carried out with the use of multichannel pipettors in an anaerobic chamber (Coy Laboratory Products, Glass Lake, Mich.) under an H_2/N_2 (3:97) atmosphere. Microtiter plates were incubated in an Oxoid anaerobic jar (Unipath, Ogdensburg, N.Y.), and a GasPak envelope (Becton Dickinson Microbiology Systems, Cockeysville, Md.) was used to generate H_2 and CO_2 in the gas phase. All incubations were at 30°C in the dark.

MPN computation and data analysis. The MPN program developed by Clark and Owens (3) was used to compute MPN and 95% confidence intervals. The significance of the difference between two MPN values was calculated according to the method described by Cochran (4) using the $\log_{10}$ of the titers and their corresponding standard errors (SE). Probabilities were obtained from a two-tailed z test and the level of significance used was a P value of <0.05.

Identification of O-demethylating activity in vanillate MPN tubes. MPN tubes were analyzed for O-demethylating activity after 14 days of incubation. The complex formed between Ti(III) and vicinal hydroxyl groups of hydroxylated products of anaerobic O demethylation is yellow and easily visualized. Samples (1 ml) were centrifuged to remove cells and debris, and the yellow color was monitored spectrophotometrically at 400 nm under aerobic conditions. If necessary, samples were diluted so that the absorbance would be less than 1 and thus well within the linear response range of the spectrophotometer. Simultaneously, anaerobic O demethylation activity was quantified with high-performance liquid chromatography (HPLC) by measuring loss of vanillate and appearance of protocatechuate. The clarified enrichment supernatants (25 μl) were diluted 1:40 in 0.1 N HCl and filtered before analysis on an HPLC (model SCL-10A; Shimadzu Scientific Instruments, Inc., Columbia, Md.) equipped with a Spherisorb C_{18} reversed-phase column (25 cm by 4.6 mm, 5-μm particle size; Phenomenex, Torrence, Calif.). Elution was isocratic, with a mobile phase containing 34.5% methanol, 5.4% acetonitrile, and 0.1% formic acid and a flow rate of 1 ml/min. Peaks were identified by comparing their retention times with authentic vanillate and protocatechuate standards. Eluting peaks were detected at 260 nm, and the peak area was determined with a Chromojet integrator (Spectra Physics, San Jose, Calif.).

Identification of acetogenic activity in H_2/CO_2 MPN tubes. MPN tubes were analyzed for acetate production by HPLC. After 22 days, samples were withdrawn from MPN tubes, centrifuged briefly to remove cells and debris, and diluted 1:2 in 0.01 N H_2SO_4. Samples were analyzed with the Beckman System Gold Chromatographic System (Beckman Instruments Inc., San Ramon, Calif.) equipped with a Rezex ROA Organic Acid column (30 cm by 7.8 mm, 8-μm particle size; Phenomenex, Torrance, Calif.) and autosampler (Gilson Medical Electronics, Middleton, Wis.). Elution was isocratic, with a solvent consisting of 0.005 N H_2SO_4, a flow rate of 0.5 ml/min, and a column temperature of 25°C. Peaks were detected by UV absorption at 210 nm and the peak area was measured with a Chromojet integrator. The acetate peak was identified by comparing its retention time with authentic acetate standards.

RESULTS AND DISCUSSION

Spectrophotometric and HPLC analysis of hydroxyaromatic product formation. The use of the reductant Ti(III)-NTA for a spectrophotometric assay of anaerobic O demethylation in cell extracts has been previously reported (10). In these studies, it was shown that Ti(III) does not chemically alter the aromatic acids and Ti(III) was used in reaction mixtures to monitor conversion of syringate (4-hydroxy-3,5-dimethoxybenzoate) to 5-OH vanillate (4,5-dihydroxy-3-methoxybenzoate). Ti(III) forms a yellow complex with hydroxyaromatics that have vicinal hydroxyl substituents. However, the extinction coefficients vary for Ti(III) complexes with different aromatic compounds.

The empirical relationship was examined between absorbance at 400 nm and variable concentrations of protocatechuate in the presence of 0.25 mM Ti(III). The absorbance of 1 mM protocatechuate was about 2.2, and absorbance was fairly linear in the range of 0 to 1 mM. The maximum absorbance observed was 2.5 to 3.5, and little increase in absorption was seen with 2.5 and 5 mM protocatechuate. Thus, the maximum color development is reached when the protocatechuate is approximately four times the concentration of Ti(III) and

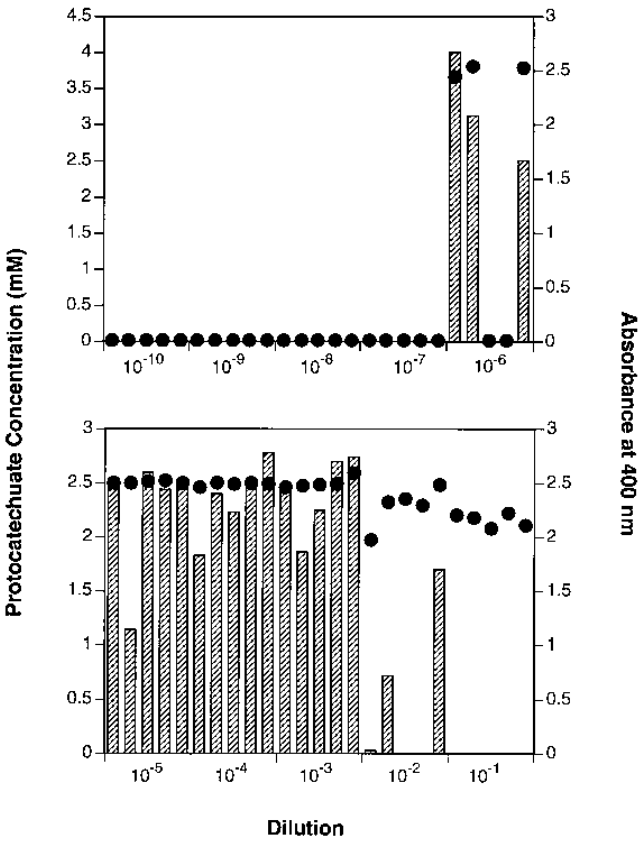

FIG. 1. Appearance of protocatechuate (▨) in vanillate MPN tubes after a 2-week incubation at 30°C. Each bar corresponds to an individual MPN tube. Five tubes per dilution were used. Absorbance (●) was measured at 400 nm. A sharp difference in absorbance indicates the presence of protocatechuate.

higher levels of protocatechuate do not result in more color. Because of the nonlinearity of absorbance at higher protocatechuate concentrations, spectrophotometric analysis was not performed to quantitate the aromatic acids but only to monitor yellow color development.

A series of MPN tubes for O-demethylating capacity was set up by using sediment from an orange grove drainage ditch. Figure 1 shows the relationship between yellow color formation, monitored visually and spectrophotometrically, and formation of protocatechuate from vanillate. The development of yellow color and appearance of the O-demethylated product, protocatechuate, are correlated, as demonstrated by the sharp difference in absorbance of the Ti(III)-protocatechuate complex. Yellow color was evident at the lowest dilutions after only 2 days of incubation. It was estimated that approximately 50 μM protocatechuate was required in MPN tubes before yellow color could be detected by eye. MPN tubes that did not turn yellow contained untransformed vanillate. Protocatechuate was the primary transformed product in MPN tubes for 10^{-3} to 10^{-6} dilutions, whereas in the higher dilutions (10^{-7} to 10^{-10}), there was no evidence of vanillate conversion to protocatechuate. By contrast, although yellow color was evident in the MPN tubes for 10^{-1} and 10^{-2} dilutions, there was substantial transformation of protocatechuate as well as vanillate, indicating that additional metabolites had been formed. Derivatives that retain vicinal hydroxyl groups will still interact with Ti(III) and form yellow complexes. The additional transformation products were not identified by HPLC but could include cat-

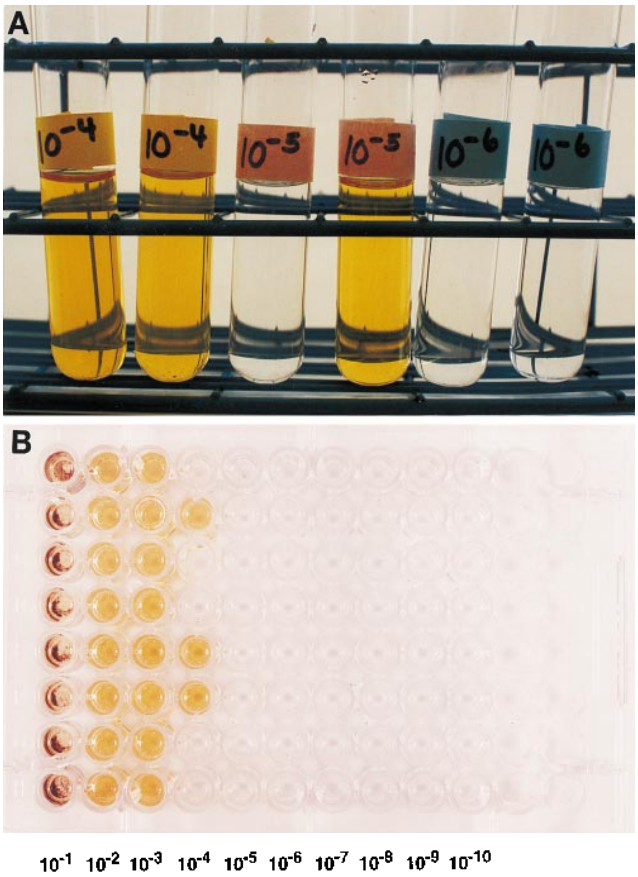

FIG. 2. Photographs showing application of colorimetric assay with three-tube MPN (A) and microtitration MPN (B) procedures. Yellow-colored tubes or wells indicate positive responses for the anaerobic O demethylation of vanillate. For the three-tube MPN procedure, only two of three MPN tubes are shown for the 10^{-4}, 10^{-5}, and 10^{-6} dilutions. For the microtitration procedure, the results for the 10^{-1} through 10^{-10} dilutions are shown.

echol, protocatechualdehyde, or 3,4-dihydroxybenzyl alcohol. Further modification and mineralization of protocatechuate may have resulted from other nonacetogenic microbial activities within the enrichment. No further change in color was observed when dilutions were left to incubate for 2 to 6 weeks. Neither autoclaved controls containing vanillate nor inoculated controls without substrate accumulated protocatechuate or developed background yellow color.

The colorimetric assay can also be used with other methoxylated aromatic substrates, including syringate (10) and ferulate (4-hydroxy-3-methoxycinnamate). However, when syringate was used instead of vanillate as the methoxylated substrate for a colorimetric three-tube MPN estimate with the sediment sample, yellow color was stably formed at higher dilutions but was only transiently formed at 10^{-1} and 10^{-2} dilutions. Loss of yellow color correlated with extensive transformation and ring cleavage of the O-demethylated products 5-OH vanillate and gallate (3,4,5-trihydroxybenzoate).

MPN of O-demethylating bacteria in two environmental samples. Two environmental samples were analyzed by the Ti(III)-based colorimetric method with microtitration and the three- or five-tube MPN procedures. Photographs of yellow color development in a three-tube MPN and a microtitration MPN procedure taken after a 2-week incubation are shown in Fig. 2A and B, respectively. MPN tubes and microtiter plate wells that were positive (yellow) and negative (clear) for anaerobic O demethylation were used to compute the MPN of viable O-demethylating acetogens present in the environmental samples (Table 1). Pairs of MPN values were examined statistically as described in Materials and Methods. For each comparison the significance of the difference between the two MPN values was calculated, and the probability (P) that such an outcome could occur by chance was determined.

TABLE 1. Microtitration and three- or five-tube MPN estimates of O-demethylating populations in soil or sediment based on the Ti(III)-colorimetric assay

Types of Sample and test	MPN/g (wet/wt)	95% Confidence interval	Log_{10} (MPN) ± SE
Soil Sample			
3-Tube MPN	1.3×10^2	2.5×10^1–4.3×10^2	2.11 ± 0.34
Microtitration[a]	4.7×10^2	2.8×10^2–7.7×10^2	2.67 ± 0.12
Sediment Sample			
3-Tube MPN	2.3×10^4	6.6×10^3–8.3×10^4	4.36 ± 0.34
5-Tube MPN	8.8×10^4	2.8×10^4–2.7×10^5	4.94 ± 0.26
Microtitration[a]	2.6×10^4	1.6×10^4–4.4×10^4	4.42 ± 0.12

[a] Microtiter plates were set up in triplicate, and the results were pooled (i.e., 24 replicates were used to compute the MPN).

For the soil sample, the MPN from the microtitration experiment was approximately fourfold higher than that from the three-tube experiment. This outcome has a P value of 0.11; thus, the observed difference is not significant at the level of $P < 0.05$. Likewise with the sediment sample, the differences between MPN values in different experiments were not significant. Specifically, the difference in MPN values between the three- and five-tube experiments has a P value of 0.17, the difference between the three-tube and microtitration MPN experiments has a P value of 0.87, and the difference between the five-tube and microtitration MPN experiments has a P value of 0.07. Hence, the data in Table 1 show that results with the colorimetric assay are consistent whether environmental samples are assayed in a microtitration or tube MPN format.

The sediment sample had an O-demethylating population 2 orders of magnitude higher than that of the soil sample, as indicated with both the three-tube and microtiter plate MPN

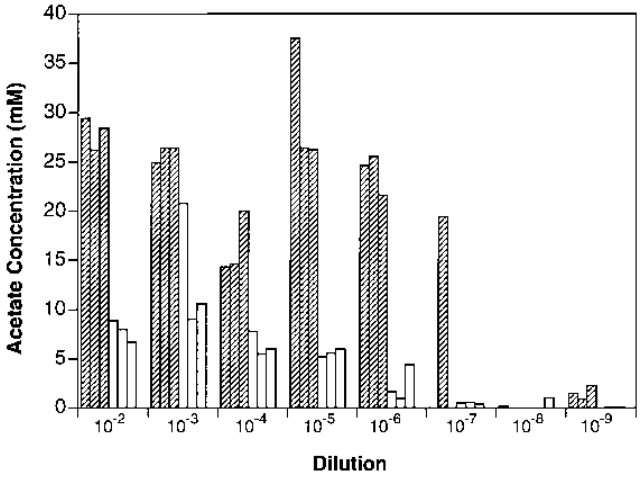

FIG. 3. Acetate production in H_2/CO_2 (▨) versus N_2/CO_2 (□) MPN tubes after a 22-day incubation. Each bar represents acetate production in an individual MPN tube.

TABLE 2. Comparison of MPN estimates of H_2/CO_2-utilizing acetogens and MPN estimates of O-demethylating bacteria in the sediment sample

Carbon source tested/ product analyzed[a]	Type of test (no. of tubes)	MPN/g (wet wt)	95% Confidence interval	Log_{10} MPN ± SE
(H_2/CO_2)/acetate	3	4.8×10^5	1.1×10^5–2.0×10^6	5.68 ± 0.34
	5	2.4×10^5	9.0×10^4–6.4×10^5	5.38 ± 0.26
Vanillate/protocatechuate	3	2.3×10^4	6.6×10^3–8.3×10^4	4.37 ± 0.34
	5	8.8×10^4	2.8×10^4–2.7×10^5	4.94 ± 0.26

[a] The positive and negative scores for acetate production in the H_2/Co_2 MPN tubes for 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions were used to compute the MPN. The positive and negative scores for protocatechuate production in the vanillate MPN tubes for 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were used to compute the MPN.

procedures. The difference in MPN values is significant and has a P value of <0.0002 with both formats, showing that the titers of O-demethylating acetogens are different in the two environmental samples.

Comparison of MPN of O-demethylating bacteria and MPN of H_2/CO_2-utilizing bacteria. The method of Doré et al. (5, 6) was used with the sediment sample to enumerate acetogens by H_2/CO_2 acetogenesis. In this method, the MPN of acetogens forming acetate from H_2/CO_2 is determined by the difference in acetate produced in H_2/CO_2 tubes compared to control tubes with N_2/CO_2 (5, 6). It was qualitatively observed that the gas-phase pressure in H_2/CO_2 tubes decreased concomitant with the formation of acetate, as expected with this method. No acetate was detected in a 10^{-1} dilution of sediment at time 0. Methane was not detected in MPN tubes, indicating the absence of methanogenic activity.

Figure 3 shows the acetate production in H_2/CO_2 or control (N_2/CO_2) MPN tubes. The 10^{-6}, 10^{-7} and 10^{-8} dilutions, which gave positive scores of 3/3, 1/3, and 0/3, respectively, for H_2-dependent acetate production, were used to compute the MPN. Table 2 shows a comparison of MPN titers of H_2/CO_2-acetogenic bacteria and O-demethylating bacteria. In both the three- and five-tube MPN experiments, H_2/CO_2 acetogenesis yielded a higher titer than that from the O demethylation of vanillate. The difference in MPN values with the three-tube format has a P value of 0.006, whereas the difference with the five-tube format has a P value of 0.31. Thus, the results of the two experiments are not in agreement since the difference between the H_2/CO_2 acetogenesis and O demethylation MPN values is not significant ($P < 0.05$) in the five-tube experiment but is significant in the three-tube experiment.

The possibility that the titer of O-demethylating bacteria was significantly lower than the H_2/CO_2 acetogenesis titer was examined subsequently by testing the three-tube MPN H_2/CO_2 tubes for O-demethylating capacity. In the anaerobic chamber, a sample was removed from each culture tube shown in Fig. 3. A 100-μl sample of culture was added to 100 μl of 1019 medium containing Ti(III) and a methoxylated substrate (vanillate, ferulate, or syringate; 2 mM final concentration). Microtiter plates were incubated in an Oxoid anaerobic jar, as described for the microtitration MPN determinations. After 14 days of incubation, which allowed adequate time for the induction of O-demethylating activity, the microtitration plates were examined for yellow color. All the H_2/CO_2 tubes positive for acetogenesis also tested positive for O-demethylating capacity with vanillate, syringate, and ferulate. Furthermore, one tube at the 10^{-7} dilution was positive for O-demethylating capacity but was negative for H_2/CO_2 acetogenesis. Thus for the sediment sample, there should have been good agreement between titers of acetogens estimated by H_2/CO_2 acetogenesis and O demethylation. The results indicate that the colorimetric MPN assay may have underestimated the titer of O-demethylating bacteria. Perhaps the culture conditions need to be further optimized. For instance, the concentration of vanillate in the medium could be somewhat toxic, and it might be appropriate to lower the concentration to 1 mM. A preincubation period in H_2/CO_2 also may be appropriate to establish the acetogen population before induction of O demethylation with a methoxylated substrate. An extension of this work will be the isolation and characterization of bacterial populations enriched with H_2/CO_2 versus vanillate to determine if the same populations arise.

We have shown the efficacy of the Ti(III)-based colorimetric assay for the enumeration of microbial populations capable of anaerobic O demethylation of vanillate and other methoxylated aromatic compounds. HPLC results confirm that the development of yellow color in MPN tubes containing Ti(III) and vanillate is indicative of anaerobic O demethylation of vanillate to protocatechuate. Although O demethylation is carried out by some sulfate reducing (12, 20) and denitrifying bacteria (21) and is also recognized as one of the earlier transformations observed during the mineralization of plant phenolic compounds by sulfidogenic, denitrifying, and methanogenic enrichments (9, 15), the MPN protocol presented in this work is tailored to the cultivation of acetogens. Insufficient levels of sulfate and nitrate are present to support the cultivation of sulfate-reducing bacteria or denitrifiers, and BESA is included in the medium to inhibit the activity of methanogens.

Two environmental samples analyzed by the colorimetric MPN method were shown to differ with respect to population titers of O-demethylating bacteria. Bacteria capable of anaerobic O demethylation were present in the soil sample although at a titer lower than that in the sediment sample. Although acetogens are obligate anaerobes, the detection of viable acetogenic populations in aerobic soil supports the findings of Wagner et al. (22) that acetogens exist within anaerobic zones of aerobic soils and that acetogenesis may contribute to carbon turnover in these environments.

In summary, the Ti(III)-based colorimetric assay is appealing because of its convenience and consistency. HPLC is not required, and a microtiter plate format is feasible. A well documented problem with the MPN method is the large sampling error associated with this technique (4). A microtiter plate format can meet this concern, since it allows for the use of a larger number of replicates and a smaller dilution ratio. The Ti(III)-based colorimetric method can be applied to the enumeration of O-demethylating acetogens. It also has potential for use in the investigation of acetogen populations associated with the animal rumen and feces and insect hindguts.

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