APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1979, p. 84-89
0099-2240/79/07-0084/06$02.00/0

Vol. 38, No. 1

# Anaerobic Biodegradation of Eleven Aromatic Compounds to Methane

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Received for publication 20 April 1979

A range of 11 simple aromatic lignin derivatives are biodegradable to methane and carbon dioxide under strict anaerobic conditions. A serum-bottle modification of the Hungate technique for growing anaerobes was used for methanogenic enrichments on vanillin, vanillic acid, ferulic acid, cinnamic acid, benzoic acid, catechol, protocatechuic acid, phenol, *p*-hydroxybenzoic acid, syringic acid, and syringaldehyde. Microbial populations acclimated to a particular aromatic substrate can be simultaneously acclimated to other selected aromatic substrates. Carbon balance measurements made on vanillic and ferulic acids indicate that the aromatic ring was cleaved and that the amount of methane produced from these substrates closely agrees with calculated stoichiometric values. These data suggest that more than half of the organic carbon of these aromatic compounds potentially can be converted to methane gas and that this type of methanogenic conversion of simple aromatics may not be uncommon.

The continuous and essential cycling of carbon in the biosphere depends upon a balance between the synthesis and degradation of organic carbon. Many aromatic hydrocarbon derivatives are industrial by-products and are considered to be not readily biodegradable. These refractory compounds, along with some naturally refractory polymers, can end up as waste material and eventually accumulate in the environment. This accumulation threatens the normal balance in the carbon cycle. There is need, therefore, to reduce the amount of refractory material in wastes which need disposal.

Lignin is a natural refractory material found in significant quantities in both domestic and agricultural wastes. It is a complex three-dimensional aromatic polymer consisting of basic phenylpropane building blocks held together by irregular carbon-carbon and diaryl-ether linkages (Fig. 1). The large molecular size, poor solubility, and complex cross-linking of lignin make it quite inaccessible to both microorganisms and enzymes.

Heat treatment under alkaline conditions has been evaluated (7) as a method for increasing the anaerobic biodegradability of lignin and other refractory material. This would reduce the amount of solids needing disposal while producing the useful by-product methane. The heat treatment of lignin is expected to partially break down its complex refractory structure and release a variety of simple aromatic compounds (7), as shown in Fig. 1.

A crucial consideration for obtaining methane from heat-treated lignin is whether simple aromatic compounds are readily biodegradable under anaerobic conditions. Several investigators (2, 4, 13) have carried out extensive studies on the aerobic degradation of aromatic compounds, yet relatively little attention has been paid to their anaerobic degradation. A variety of different anaerobic culture conditions involving both pure and mixed cultures has been used to demonstrate the decomposition of a few selected aromatic compounds during photosynthetic metabolism (3, 8), nitrate respiration (16, 18), and methane fermentation (1, 6, 9, 13).

This report presents evidence that a wide range of ligno-aromatic compounds are degradable to methane and carbon dioxide.

## MATERIALS AND METHODS

Prereduced, defined medium contained the following (per liter): resazurin, 0.001 g; $(NH_4)_2PO_4$, 0.04 g; $NH_4Cl$, 0.2 g; $MgCl_2 \cdot 6H_2O$, 1.8 g; KCl, 1.3 g; $MnCl_2 \cdot 4H_2O$, 0.02 g; $CoCl_2 \cdot 6H_2O$, 0.03 g; $H_3BO_3$, 0.0057 g; $CaCl_2 \cdot 2H_2O$, 0.0027 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0025 g; $ZnCl_2$, 0.0021 g; $FeCl_2 \cdot 4H_2O$, 0.368 g; $NaHCO_3$, 2.64 g; $Na_2S \cdot 9H_2O$, 0.5 g; and 1% (vol/vol) vitamin solution (19). The medium was buffered at pH 7.0 by a bicarbonate-$CO_2$ system with a gas atmosphere of 30% $CO_2$ and 70% $N_2$. Oxygen was removed from the medium by boiling, followed by the addition of the sodium

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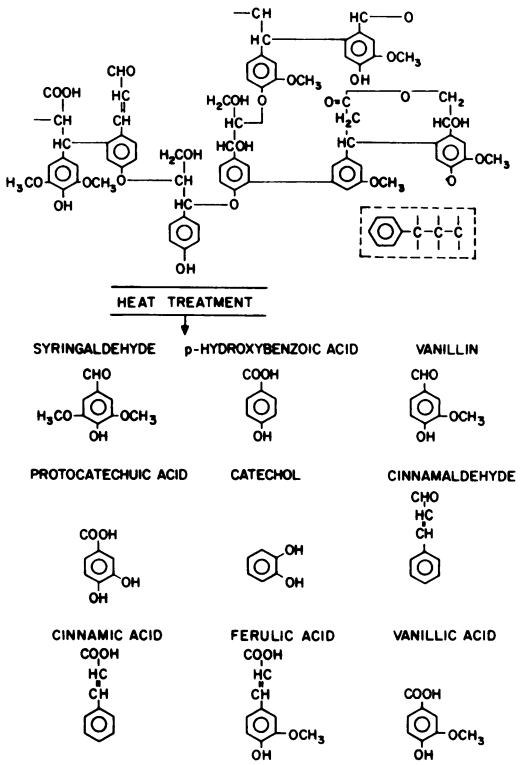


FIG. 1. *Representation of lignin and examples of the types of aromatic compounds expected to be released from heat treatment. Note the basic phenylpropane subunit which makes up the lignin molecule.*

sulfide reducing agent. Resazurin was used as an oxidation-reduction indicator. A C/N/P molar ratio of 100:15:1 was provided for aromatic substrate concentrations of 300 mg/liter used in the serum-bottle enrichments. Prereduced replacement medium, used for maintaining stock cultures, contained an aromatic substrate concentration of 10 g/liter. Additional amounts of nitrogen and phosphorus were thus required to sustain the same C/N/P ratio as in defined medium.

A serum-bottle variation of the Hungate technique for growing anaerobic bacteria was adapted from Miller and Wolin (12). Defined medium was inoculated with 10% (vol/vol) seed from a laboratory anaerobic digester fed primary settled sewage sludge on a 15-day detention time. Serum bottles (250-ml) were flushed (500 ml/min) with oxygen-free gas for 20 min before the inoculated medium was added. These cultures were incubated in the dark at 35°C.

Various types of syringes were used to maintain and monitor serum-bottle cultures. Plastic disposable syringes of different sizes were used to add and remove anaerobic culture fluid, replacement medium, or other culture-associated liquids. Syringe needles were either 18 or 20 gauge. A 1-ml gas-tight glass syringe (Hamilton no. 1001) was used to remove gas samples for analysis. The volume of gas produced by 250-ml serum-bottle cultures was measured daily by displacement of the plunger lubricated with water in a 20-ml glass syringe fitted with a 20-gauge needle. The bottle was tilted slightly while the syringe was held horizontally to minimize the effect of the plunger weight, and measurements were accurate to less than 1% error. Substrate concentration was determined by diluting a centrifuged sample and assaying it in a spectrophotometer at a known characteristic ultraviolet wavelength for each particular aromatic compound. Gas composition was determined on a Fisher-Hamilton model 29 gas partitioner. Gas production and substrate concentration were corrected for background levels by subtracting values measured in a control culture which contained no aromatic substrate.

Acclimated stock cultures were maintained in serum bottles by regular replacement of one-fifth of the culture volume with fresh, prereduced media. This replacement was made after the culture ceased converting its previous supply of substrate to gas. After the 150-ml culture was shaken for homogeneity, 30 ml was withdrawn with a syringe and either wasted or transferred as inoculum to another serum bottle. An appropriate volume of replacement medium was then added, with the balance of the 30 ml removed being replaced with defined medium.

Mass balances on the conversion of organic carbon to $CO_2$ and $CH_4$ were based upon the stoichiometry of the Buswell equation (15):

$$C_nH_aO_b + \left[n - \frac{a}{4} - \frac{b}{2}\right] H_2O \rightarrow \left[\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right] CO_2 + \left[\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right] CH_4$$

Theoretical yields of gas expected from this equation were compared with actual yields as determined by analyses of the composition of the volumes of gas produced by the serum-bottle cultures.

Cross-acclimations were set up in the following manner. Hungate tubes were flushed for 10 min with oxygen-free gas. Amounts of 7.5 ml from an acclimated stock culture were anaerobically transferred with a syringe to a set of Hungate tubes. These cultures had completed gas production before the transfer took place. Then 2.5 ml of different aromatic substrates dissolved in prereduced medium and stored anaerobically in serum bottles was added to each of the tubes. These prereduced stock solutions of aromatic substrates contained four times the usual concentration of substrate, nitrogen, and phosphorus to return the 10-ml culture to original nutrient levels. The control contained no aromatic substrate. The onset, rate, and extent of gas production in the tube containing the same substrate to which the stock culture was acclimated were compared with those in the other tubes fed with different aromatic substrates.

## RESULTS AND DISCUSSION

Initial enrichment studies indicate that the range of 11 simple aromatic compounds are degradable under strict anaerobic conditions and are summarized in Table 1. Of the many initial enrichments undertaken, most were inoculated

from a laboratory anaerobic digester fed primary settled sludge as described above. In several of the cases the initial inoculum came from a laboratory digester fed heat-treated refuse or newsprint (7) or from digester samples collected from the San Jose treatment plant. The response of the enrichments to each compound appeared quite consistent, apparently being little affected by the different sources of inocula. In general, decomposition in these enrichments was slow, first requiring an acclimation period of about 10 days to 2 weeks followed by gas production over an additional 2- to 4-week period, depending upon the compound. For example, cultures acclimated to syringic acid in 2 days, compared with 21 days for the apparently more refractory catechol. The conversion of substrate carbon to gas was also observed to differ, ranging from 63% in protocatechuic acid to 102% in syringaldehyde. Gas composition was determined as $CO_2$ and $CH_4$ with no detectable hydrogen. In most cases more than 80% of the carbon was converted to gas, clearly indicating that ring cleavage occurred under these strict anaerobic conditions. Consequently, the major portion of the substrate carbon was released as gaseous end products.

TABLE 1. *Degradation of 11 aromatic compounds in methanogenic enrichment cultures*[a]

| Substrate | Acclimation lag (days) | Period of gas production (days) | Conversion of substrate carbon to gas (%) |
|---|---|---|---|
| Vanillin ($n$ = 10) | 12 ± 1.2 | 16 ± 1.1 | 72 ± 1.4 |
| Vanillic acid ($n$ = 8) | 9 ± 1.2 | 19 ± 1.4 | 86 ± 2.8 |
| Ferulic acid ($n$ = 8) | 10 ± 0.7 | 24 ± 2.2 | 86 ± 2.8 |
| Cinnamic acid ($n$ = 3) | 13 ± 0.9 | 28 ± 1.6 | 87 ± 8.1 |
| Cinnamic acid ($n$ = 3) | 13 ± 0.9 | 28 ± 1.6 | 87 ± 8.1 |
| Benzoic acid ($n$ = 5) | 8 ± 0.5 | 18 ± 1.6 | 91 ± 7.8 |
| Catechol ($n$ = 10) | 21 ± 0.8 | 13 ± 1.1 | 67 ± 1.6 |
| Protocatechuic acid ($n$ = 5) | 13 ± 1.7 | 14 ± 1.2 | 63 ± 1.8 |
| Phenol ($n$ = 10) | 14 ± 1.2 | 15 ± 1.0 | 70 ± 3.2 |
| *p*-Hydroxybenzoic acid ($n$ = 5) | 12 ± 1.2 | 14 ± 0.9 | 80 ± 2.7 |
| Syringic acid ($n$ = 10) | 2 ± 0.5 | 15 ± 0.5 | 80 ± 1.6 |
| Syringaldehyde ($n$ = 2) | 5 ± 0.0 | 13 ± 2.8 | 102 ± 13.3 |

[a] Measurements are reported as mean ± standard error. $n$ = number of enrichments established for each substrate.

When additional substrate was fed to the same respective cultures, degradation to methane and carbon dioxide usually occurred with a significantly shortened or no lag at all. For example, Fig. 2 shows the temporal relationship of *p*-hydroxybenzoic acid degradation. Initial *p*-hydroxybenzoate enrichments required a 6-day lag, whereas subsequent utilization of additional substrate was immediate. The acclimated culture metabolized *p*-hydroxybenzoate down to undetectable levels in 3 days, compared with 17 days for the initial enrichment culture. In both cases, more than 80% of the substrate carbon was converted to gas.

To examine potential similarities in the utilization of different aromatic ring compounds, cross-acclimation studies were carried out to determine whether a culture acclimated to one substrate is simultaneously acclimated to other related substrates. To illustrate this procedure, the cumulative volumes of gas produced in four Hungate tubes containing active cultures acclimated to syringic acid are shown in Fig. 3. These results are representative of the type of responses obtained in these cross-acclimation studies. Very little gas was produced by either the control with no substrate addition or the

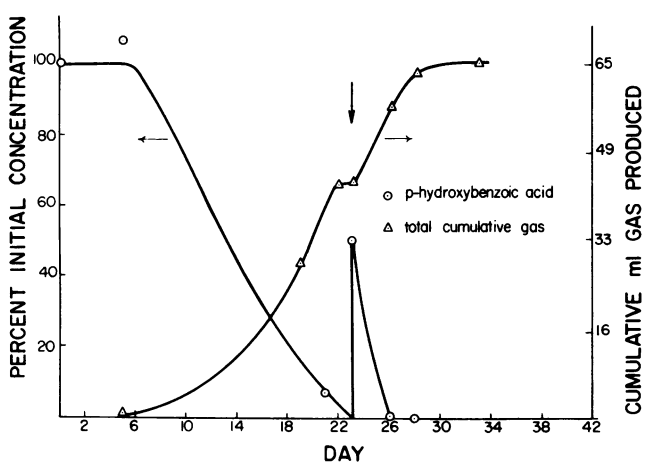


FIG. 2. *Decomposition of p-hydroxybenzoic acid with resulting production of gas. 100% initial concentration ≈ 300 mg/liter. ↓, Point in time when culture was spiked with additional substrate.*

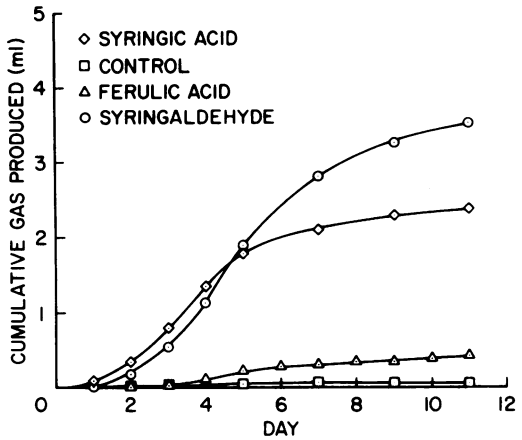


FIG. 3. *Cross-acclimation to syringic acid. All four tubes contained active syringic acid cultures. Each was fed the indicated substrate. There was a positive response to syringic acid and syringaldehyde, but not to ferulic acid.*

culture fed ferulic acid. The cultures fed syringic acid and syringaldehyde, however, produced gas immediately, indicating that the syringic acid-acclimated culture was simultaneously acclimated to syringaldehyde but not to ferulic acid. As shown in Table 1, for syringaldehyde, an unacclimated culture normally would have displayed a 5-day lag.

The results of the cross-acclimation studies are summarized in Fig. 4. Structural representation of the substrates to which individual cultures were originally acclimated are shown in the left-hand column. Substrates to which each of these cultures are simultaneously acclimated, that is, requiring no additional acclimation lag, are shown in the right-hand column. In almost all cases shown in Fig. 4, there is a high degree of structural similarity among the compounds to which each culture can readily acclimate. For example, the compounds may differ in the oxidation state of one substituent group, as in the case of the carboxyl group of syringic acid compared with syringaldehyde, or they may differ in the presence of an extra substituent group at an additional ring position, as in the case of the methoxy group of syringaldehyde compared with vanillin.

The vanillic acid-acclimated culture (Fig. 4) did not fall into this pattern because of the large number and types of compounds to which it is simultaneously acclimated. Syringaldehyde, syringic acid, and vanillin are structurally similar to vanillic acid in the oxidation state or in the presence of an additional methoxy group. On the other hand, benzoic acid, catechol, and protocatechuic acid are structurally quite different from vanillic acid in the type of substituent group at identical ring positions. For example, catechol contains a hydroxyl group at the number 3 position compared with the methoxy group of vanillic acid. In addition, it does not contain a carboxyl group.

These results suggest that the capability of a microbial population to metabolize several different aromatic structures may not be uncommon. In most cases these additional compounds are very similar in structure, differing only in the oxidation state of one substituent group or in the presence of an extra substituent group. In some cases, the compounds to which a culture is simultaneously acclimated are not similar in structure. The fact that a vanillic acid culture can readily metabolize catechol suggests that the 4-week lag initially required for catechol

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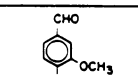
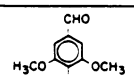
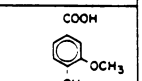
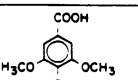
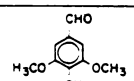
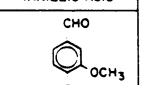
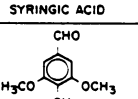
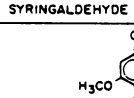
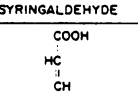
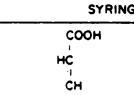
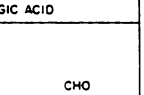
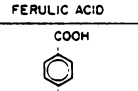
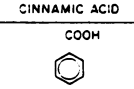
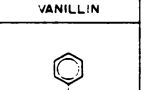
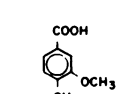
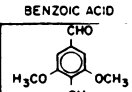
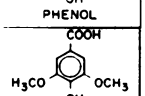
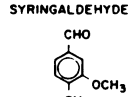
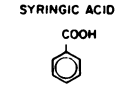
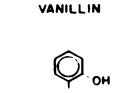
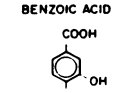
|  VANILLIN |  SYRINGALDEHYDE;  VANILLIC ACID |
|  SYRINGIC ACID |  SYRINGALDEHYDE;  VANILLIN |
|  SYRINGALDEHYDE |  SYRINGIC ACID |
|  FERULIC ACID |  CINNAMIC ACID;  VANILLIN |
|  P-HYDROXYBENZOIC ACID |  BENZOIC ACID;  PHENOL |
|  VANILLIC ACID |  SYRINGALDEHYDE;  SYRINGIC ACID<br>  VANILLIN;  BENZOIC ACID<br>  CATECHOL;  PROTOCATECHUIC ACID |

FIG. 4. *Summary of cross-acclimation results. Substrates to which cultures were simultaneously acclimated elicited immediate gas production.*

decomposition can be reduced to 2 weeks, if the initial enrichment is first made with vanillic acid before feeding with catechol. In a separate experiment, 150-ml cultures grown on 400 mg of vanillic acid per liter were fed 400 mg of catechol per liter. Immediate gas production was detected, with 85% of the catechol converted to gas over a 17-day period. For benzoate, compounds used in cross-acclimation experiments (catechol, phenol, *p*-hydroxybenzoic acid, and vanillic acid) yielded negative results, with no immediate gas production. All of the compounds are either more complex or quite different in structure from that of benzoate. Furthermore, with benzoate having been studied more extensively than the other aromatics, analysis of its anaerobic catabolism reported in the literature indicates that none of the above compounds is an intermediate formed during decomposition (5, 10, 14).

A well-acclimated stable enrichment routinely responded to additional substrate inputs by immediate gas production. Gas analysis during decomposition of selected compounds indicates that methane is produced continuously throughout the entire gas-producing period and can be seen to constitute more than half of the total gas produced (Fig. 5 and 6). For example, Fig. 5 shows the carbon balance of a vanillic acid enrichment in which 48% of the substrate carbon was converted to methane, accounting for 67% of the total gas produced. In the case of a ferulic acid culture (Fig. 6), the substrate was degraded in 4 days, whereas its conversion to methane and carbon dioxide continued during an 8-day period. Of the total gas produced, 60% was methane, accounting for 44% of the original substrate carbon.

The stoichiometry for these methanogenic conversions is illustrated as follows: vanillic acid, $C_8H_8O_4 + 4H_2O \rightarrow 4CO_2 + 4CH_4$; ferulic acid, $C_{10}H_{10}O_4 + 5.5H_2O \rightarrow 4.75CO_2 + 5.25CH_4$. These equations indicate that at least 50% of the organic carbon can be theoretically converted to methane. Based upon these stoichiometries, the experimental conversion of these compounds to methane is summarized in Table 2. The amount of methane produced from these substrates agrees closely with theoretical values (99 to 104%), whereas total gas values varied between 82 and 86% of the theoretical. Mass balances and substrate conversion of two other aromatic compounds, catechol and phenol, have been reported previously (9).

The 14 to 17% lower than theoretically predicted values for total gas can be accounted for by loss of substrate carbon to two sinks. First, approximately 5 to 10% of the carbon can be expected to end up as cell mass (17); second, formation of bicarbonate complexes such as $NaHCO_3$, $CaHCO_3^+$, and $MgHCO_3^+$ may account for the remainder. These complexes can reduce the amount of $CO_2$ reaching the gas phase up to an additional 10%, as determined by a thermodynamic equilibrium model, SOLMNEQ (11).

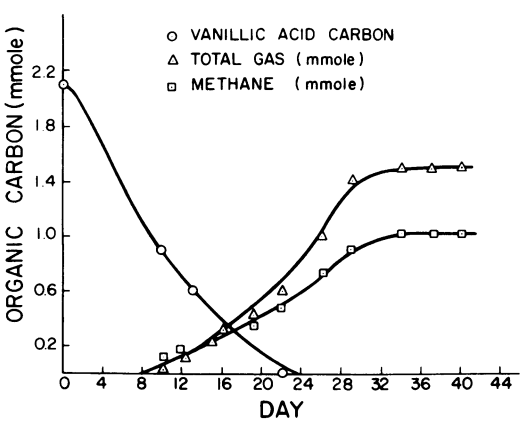



FIG. 5. *Carbon balance for a vanillic acid culture. Methane accounts for 48% of the substrate carbon converted.*

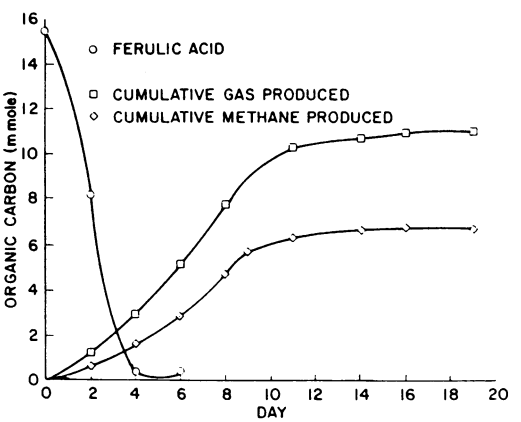



FIG. 6. *Carbon balance for a ferulic acid culture. Methane accounts for 44% of the substrate carbon converted.*

TABLE 2. *Summary of vanillic and ferulic acid gas production*[a]

| Substrate | Total gas produced (mmol) | | Methane produced (mmol) | |
|---|---|---|---|---|
| | Actual | Theoretical | Actual | Theoretical |
| Vanillic acid | 1.74 ± 0.059 ($n$ = 14) | 2.03 | 1.06 ± 0.040 ($n$ = 2) | 1.02 |
| Ferulic acid | 1.90 ± 0.022 ($n$ = 39) | 2.31 | 1.20 ± 0.028 ($n$ = 5) | 1.21 |

[a] Actual values are reported as mean ± standard error. $n$ = number of enrichments established for each substrate.

Many aromatic compounds, therefore, appear to be cleaved without the presence of molecular oxygen, under strict anaerobic conditions, and result in the gaseous end products $CO_2$ and $CH_4$. Earlier investigators had not examined as wide a range of compounds and in many cases were unsuccessful in the methane fermentation of a number of aromatics (1, 15). The two examples given here, vanillic and ferulic acids, illustrate that their degradation results in nearly stoichiometric conversion to $CO_2$ and $CH_4$ and indicate that half or more of the organic carbon in aromatic ring derivatives can be potentially converted to methane gas. Thus, it may be possible to reduce the amount of ligno-aromatic wastes needing disposal by their biological conversion to a useful product. In addition, these results suggest that aromatic hydrocarbon derivatives which find their way into highly anaerobic environments may not be refractory and can potentially be mineralized to $CO_2$ and $CH_4$.

## ACKNOWLEDGMENT

This work was supported by a grant from the Department of Energy, Fuels from Biomass Program.

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