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Utilization of Microbially Solubilized Coal

Preliminary Studies on Anaerobic Conversion

Scientific Note

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ABSTRACT

The solubilization of low-ranked coals by fungi, such as *Paecilomyces*, in defined submerged culture systems has been demonstrated. Current efforts focus on the conversion of the aerobically-solubilized coal into less oxidized products. Anaerobic methanogenic consortia have been developed that can remain active and viable in the presence of the aqueous coal product or vanillin, a coal model compound. The results suggest that a methanogenic consortium was able to produce methane and carbon dioxide from the product of coal biosolubilization by *Paecilomyces* as a sole carbon source. Work continues on the development of cultures able to convert the aqueous coal product and its various fractions into methane or fuel alcohols.

Index Entries: Coal solubilization; coal; methanogenesis; biogasification; coal gasification.

INTRODUCTION

The development of biological processes for fossil energy utilization has received increasing attention in recent years. Since the first report of microbial solubilization of lowranked coals by white-rot fungi in 1982 (*1*), efforts have focused largely on identifying other organisms capable of catalyzing this transformation (*2–6*) and elucidating the underlying chem-

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istry (*4,5,7–9*). As a result, the composition of the biosolubilized coal product and its potential uses have not been fully examined.

It has been established that treatment of low-ranked coals with aerobic coal-solubilizing microorganisms results in the production of high molecular weight, polar, heterogeneous material with a relatively high oxygen content (*2,4*). This material may represent intermediates in the process of microbial coal breakdown. It may be argued that aerobic solubilization would result eventually in the production of low molecular weight oxidized coal "monomers," i.e., substituted aromatic acids or aldehydes, or phenols. Evidence for the production of compounds of this type is described in an accompanying paper (*10*). These compounds themselves may have minimal value as fuel. However, oxidized aromatic compounds have been shown elsewhere to be degraded under anaerobic conditions (*11,12*) with the production of methane (*13,14*). Methanogenesis from these compounds, moreover, suggests a metabolic flux through fatty acids and potentially through fuel alcohols (*15*). Thus, the potential exists for converting low-ranked coal to more conventional fuels—methane or fuel alcohols—via two separate, sequential microbial processes.

The work described here is the result of tests carried out to determine the feasibility of a two-step biological conversion of low-ranked coal to methane. The proposed process requires an initial solubilization of coal under aerobic conditions. The product of that activity provides a feed stream for a subsequent anaerobic transformation. The current work focused on the development of anaerobic cultures capable of utilizing this material as a sole carbon source. The overall goal was demonstration of methanogenesis from a biosolubilized coal.

METHODS

Preparation of Biosolubilized Coal

Organism and Culture Conditions

The fungus, *Paecilomyces* TLi, isolated from coal in this laboratory (*2*) was used for coal solubilization experiments. Biosolubilized coal product for subsequent anaerobic processing was routinely prepared from *Paecilomyces* cultures grown in defined Minimal I medium containing 0.1% maltose, under previously described culture conditions(*7*). Wyodak subbituminous coal pretreated with nitric acid (*16*) was the substrate used in all experiments. The spent medium from cultures containing coal gave a positive result in a spectroscopic assay (*7*) for solubilized coal. This procedure solubilized ~10% of the added coal for a estimated final concentation of 2.5 g of solubilized coal/L. This solution was measured by dry weight (and confirmed by total density) to have 5.6 g/L nonvolatiles, suggesting the presence of residual salts. The spent medium was stored frozen until use.

Acclimation Experiments

Organisms

The anaerobic biotransformations were carried out by an uncharacterized microbial consortium isolated from a municipal anaerobic sludge digester.

Media and Cultivation

The medium for the initial methanogenic acclimation experiments consisted of the following (per liter): yeast extract, 1.5 g (later 0.75 g); KH_2PO_4, 2.0 g; urea, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2$, 0.3 g; $(NH_4)_2SO_4$, 1.4 g; NH_4Cl, 6.0 g; micronutrient solution, 3.0 mL; and iron solution, 3.0 mL. NaOH was used to adjust to pH 7. The micronutrient solution consisted of (per liter): H_3BO_3, 0.1 g; $A1K(SO_4)_2 \cdot 12H_2O$, 0.1 g; $CuSO_4 \cdot 5H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 40.0 g; $ZnSO_4 \cdot 7H_2O$, 1.0 g; $MnSO_4 \cdot H_2O$, 5.0 g; and $NiCl_2 \cdot 6H_2O$, 40.5 g. The iron solution contained 100 g/L iron chelate. To this either 0.5 g/L vanillin or 37 mL solubilized coal product plus 1 g/L methanol was added.

Duplicate cultures were grown from each transfer. Active cultures from two of the previous flasks were transferred every 3 to 4 d with 150 mL of media being placed in a 250 mL Erlenmeyer flask. Approximately 15 mL inoculum was used in each transfer. A two-hole rubber stopper with 0.25 in OD glass tubing was used to seal the flask. Viton tubing was attached to the glass tubing, and the flasks were sparged with nitrogen for 20 min. Transfers of cultures to fresh medium were done with a pipet that had been sparged with nitrogen for 5 min. All transfers were made with nitrogen flowing through the flasks. After the transfer was complete, paraffin was used to seal the stopper. The inlet tubing was clamped off, and the exit tubing was inserted in an inverted graduated cylinder filled with water in order to measure gas production. The flasks were gently shaken while incubated in a water bath at 33°C.

Analysis

Gas composition of the flask headspace was determined by withdrawal of a 1 mL sample through the Viton tubing with a gas-tight syringe and analysis with a Poropack Q column on a Bendix Portable Ambient Temperature Gas Chromatograph (GC) with N_2 carrier gas. Total organic carbon (TOC) analysis was performed on some grown cultures and media using a Beckman model 915A TOC with a model 865 IR analyzer and determined by the difference of total and inorganic carbons to within ± 5 mg/L. Methanol was measured by GC on Chromosorb 101 in a Varian 3700 with FID with He carrier gas. Aromatics, such as vanillin, were measured on a HP-5890 Capillary Gas Chromatograph (cGC) with either a DB-Wax or DB-5 15-m Megabore column. All samples were filtered prior to analysis with a 0.2 μm filter to remove all cell and particulate matter.

Methane Production from Solubilized Coal

Organisms

An uncharacterized microbial consortium was isolated from a municipal anaerobic sludge digester, as above, and the anaerobic sediment of a coal fly ash settling pond. *Clostridia acetobutylicum* ATCC #824 was used in one experiment as a possible supplemental acetogenic culture.

Media and Cultivation

In order to demonstrate the production of methane from biosolubilized coal, a defined medium was developed. This medium was modified from a prereduced defined medium described by Healy and Young (*13*) The vitamin solution was added at a concentration of 0.5 mL/L and consisted of (per liter): biotin, 2.0 mg; folic acid, 2.0 mg; thiamine HCl, 5.0 mg; riboflavin, 5.0 mg; pyridoxine HCl, 10.0 mg; cyanocobalamine, 0.1 mg; nicotinic acid, 5.0 mg; DL-calcium pantothenate, 5.0 mg; thioctic acid, 5.0 mg; and *p*-aminobenzoic acid, 5.0 mg. This vitamin solution contained essentially all of the same nutrients, in equal or greater concentrations, as the one used by Healy and Young (*13*). Resazurin was used as the redox indicator and sulfite as the reducing agent.

Nitrogen was the oxygen free gas. Later, this was modified with 9% CO_2 and 3% H_2. The gas was bubbled through a solution of Titanium III citrate to strip residual oxygen. The medium was boiled in a stoppered flask with gas flowing. After the indicator was reduced, the media was cooled with gas flowing. Then, 0.5 g/L $Na_2S{\cdot}9H_2O$, 0.5 mL/L vitamin solution, and either 0.5 g/L vanillin or 0.2 g/L of the coal solution were added to the medium. It was not sterilized except by the above procedure. The medium was distributed at 150 mL/250 mL flasks, and the transfer performed, as above, with 15 mL of inoculum. However, this protocol diluted the cultures more than they apparently grew under these strict conditions (i.e., washout); therefore, the transfer protocol was modified to retain biomass by allowing the cultures to settle and replacing the clear supernatant with fresh prereduced media. Since the culture apparently grew as a film on the inorganic preciptates present, this technique appeared relatively effective and simple at retaining the biomass while replacing more than 80% of the supernatant. The flasks were modified to allow gas samples to be removed through a teflon septum in a glass T-tube. Owing to the small volumes of gas produced, the gas was measured by positive displacement with a frictionless syringe (*13*). The gas samples were analyzed by the same GC techniques as above.

RESULTS

Acclimation Experiments

Anaerobic cultures grown on methanol in undefined medium containing yeast extract plus vanillin initially showed lower average gas produc-

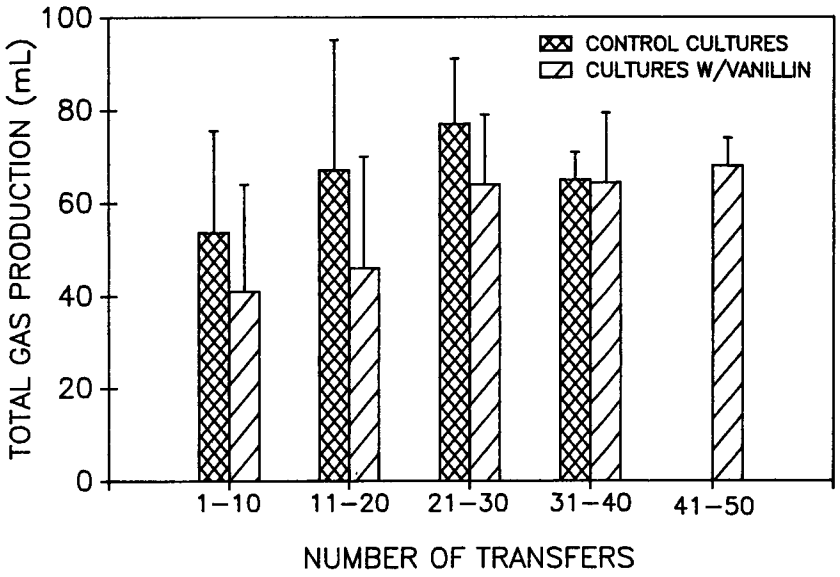

Fig. 1. Acclimation of methanogens grown on methanol (1 g/L) to the presence of vanillin, a coal model compound. Vanillin was added to 0.5 g/L. Transfers were made every 3–4 d. Biogas includes methane and CO_2.

tion than the controls without vanillin (Fig. 1). Although this difference fell within the limits of experimental error, the possibility of toxicity or inhibition owing to vanillin could not be ruled out. For this reason, cultures were allowed to acclimate to growth in the presence of vanillin. After a number of transfers, the gas production stabilized to that of the control culture. GC analysis showed that essentially all of the added methanol was consumed in 4 d in both the control and vanillin cultures. This observation indicates that, despite some initial decrease in production, vanillin at this concentration is not inhibitory to the methanogens.

We were also interested in the fate of the vanillin in these cultures, especially after a long number of successive transfers. Capillary gas chromatography was used to test for the disappearance of the 0.5 g/L of vanillin added. Samples of the 3- to 4-d-old cultures were tested and had between 10 and 100 mg/L vanillin. They also had a peak identifiable as acetate. The controls without the inoculum showed no change in the vanillin concentration. These findings suggest that the vanillin is being at least partially degraded under these experimental conditions. However, vanillin is apparently not converted to gas during the 3–4 d between transfers since a measurable increase in gas production was not observed. This finding is consistent with those of Healy and Young (*13*), who reported that gas production from vanillin by anaerobic sludge in a minimal medium occurs (after an initial 12-d lag) over a 16-d period with 72% conversion of substrate carbon to gas (CO_2 and CH_4). In those experiments, over half the

gas produced appeared after the vanillin was no longer detectable. Methanol was probably the most significant source of methane in the work described here. However, TOC analysis suggests that carbon was also used from the yeast extract and urea. The control and vanillin cultures exhibited a similar decrease in carbon content that cannot be accounted for by the utilization of methanol alone. It is clear that when other carbon sources are present, low levels of additional gas production from vanillin (or any other organic, such as the solubilized coal) would be masked. For this reason, subsequent experiments with solubilized coal were performed in minimal medium.

A parallel set of experiments was performed in which the vanillin was replaced by solubilized coal product. Similar rates and yields of total gas and methane production from methanol were observed over 20 successive transfers (data not shown). This result indicates that the level of coal product added was not inhibitory to the anaerobic cultures used here and, in particular, was not toxic to methanogens.

Methane Production from Solubilized Coal

Figure 2 shows the evolution of gas, including methane, with the unfractionated solubilized coal as the sole carbon source. Gas yields are much lower in these experiments than in Fig. 1, because these cultures were grown in minimal medium with a complex carbon source. The culture was transferred from a previous enrichment culture in the defined medium. Other identical trials showed lesser, but appreciable production of total gas (1–7 mL) and methane. An increase in the CO_2 concentration was recorded in all trials. Sterile technique was not used owing to the stringent conditions. Uninoculated controls showed no growth or gas production.

The defined medium with the 37 mL solubilized coal/L as the sole carbon source was measured to have 68 mg/L of organic carbon from TOC analysis. Complete conversion to CO_2 and CH_4 at 30°C would yield about 21 mL of gas. The solubilized coal substrate used here had a carbon content of approximately 54% (*10*). Based on the amount of solubilized coal added to cultures, total anaerobic conversion of this coal-derived material was predicted to yield 16 mL of gas. The reason for the difference between the values for carbon content is not clear. Here, a maximal gas production of 9 mL was achieved. Methane was about 25% of this total gas. The final supernatant was measured to have 15 mg/L of organic carbon.

This incubation was very slow, exhibiting a 25-d lag and not completing production till almost 100 d had passed. Methane production slowed dramatically after 60 d. This slowdown may be owing to exhaustion of the easily metabolizable organics or an increase in the redox level of the media. An increase in the redox potential was evident by the observation that the medium had a slight pink tint at the end of the run. The transfer

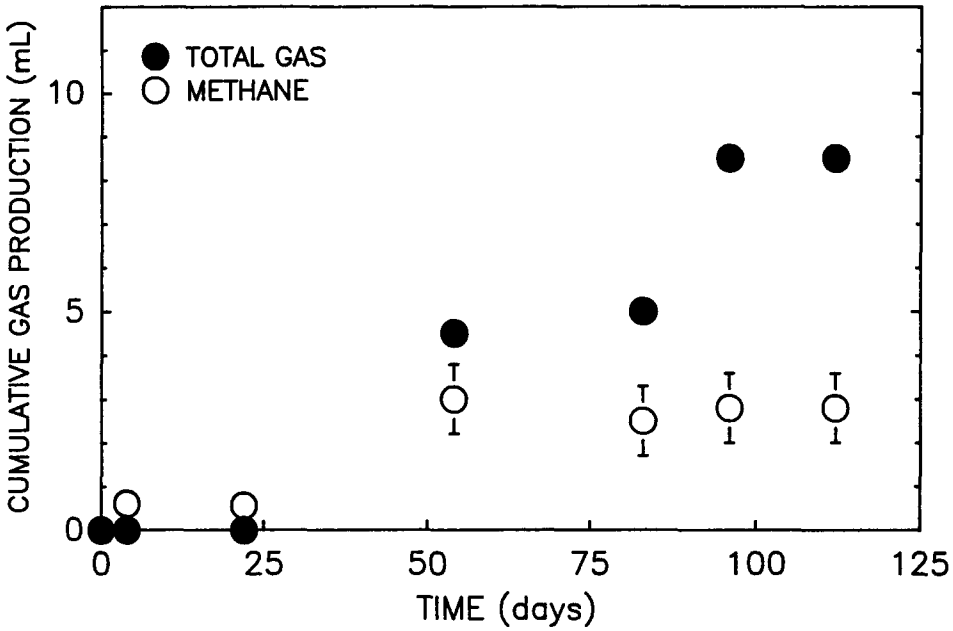

Fig. 2. Cumulative biogas production from an anaerobic culture grown on biosolubilized coal. The error bars on the methane values are estimates of the combined measurement errors, including those of the GC and volume of the flask headspace.

of this culture at 112 d to fresh medium has shown continued gas and methane production to this date.

During a previous trial, the viability of the methanogens was tested by removing a portion of a culture growing for 25 d on coal as a sole carbon source and placing it in a medium with methanol and acetate. Within 4 d, methane and CO_2 were produced, indicating that the methanogens remained active despite slow conversion rates. This test was repeated at the end of this incubation. Methane and CO_2 were produced from the methanol and acetate, further indicating the viability of the methanogens.

It may be possible to increase the methane production by supplementing the anaerobic consortium with microorganisms potentially able to degrade aromatics to low molecular weight compounds (such as acetate or methanol) metabolizable by the methanogens. This concept was tested in a preliminary manner by supplementing the consortia grown on the solubilized coal product with *Clostridium acetobutylicum* ATCC #824 grown in minimal medium containing glucose. The *Clostridium* was added to triplicate to sealed 125 mL bottles prepared with the prereduced media containing the solubilized coal liquid and the consortium, as above. Controls were prepared as above, but without the *Clostridium.* After 45 d of stationary incubation, the headspace gas was analyzed. The bottles with

the *Clostridium* had methane concentrations of 8±2%, vs 3±1% in the controls. These results must be confirmed since part of the gas production may be owing to residual carbon (i.e., glucose) in the *Clostridium* inoculum.

DISCUSSION

Our preliminary findings suggest that the conversion of low-ranked coal to more readily utilizable fuel is possible via a two-step microbial process. Aerobic treatment of subbituminous coal, described in an accompanying paper (*10*), generates a mixture of oxidized water-soluble organic compounds, including a low molecular weight fraction that is presumably aromatic. The present work demonstrates the further anaerobic conversion of this solubilized coal. Methanogenic consortia were shown to be active in the presence of the unfractionated biosolubilized coal product or vanillin, an oxidized, low molecular weight coal model compound. A methogenic consortium was apparently able to produce methane and carbon dioxide from the product of fungal coal biosolubilization as a sole carbon source.

The precise nature of the substrate for anaerobic gas production is not known. The solubilized coal product used here was produced by fungal action over the course of a 14-d incubation. Growth and solubilization took place within an inorganic salts medium (*17*) containing 0.1% maltose. Experimental evidence suggest the fungus consumes the maltose substrate within 2–3 d (unpublished results). The likelihood that residual maltose is carried over into the anaerobic conversion, therefore, is minimal. Excreted fungal metabolites are possible sources of organic carbon; however, none was detected by HPLC analysis. The experimental procedure followed in the anaerobic experiments described here, including more than three transfers in defined minimal medium, suggests that there is not sufficient residual carbon in transfer inocula for gas producton. Autolysis of the biomass is possible, but unlikely to be sustained after successive transfers. Indeed, controls incubated with fresh anaerobic sludge in minimal medium with no carbon source produced some gas initially, but none in the second or following transfers. The vitamins as a carbon source are present in negligible quantities; total conversion of the vitamins might yield less than 5 μL of gas.

Biosolubilized coal has been shown elsewhere to be a suitable substrate for microbial metabolism (*10,18*). This knowledge, coupled with the presence of low molecular weight and/or oxidized materials in the solubilized coal substrate (*10*), plus the demonstrated ability of anaerobic cultures to metabolize a low molecular weight oxidized aromatic compound to methane, suggests that solubilized coal is indeed a substrate for methanogenesis.

The methanogenic activity measured here decreased over time (perhaps owing to a change in redox potential), but the methanogens remained

viable and able to consume typical methanogenic substrates, such as acetate and methanol. This observation, coupled with the apparent resumption of methanogenic activity in the presence of acetate, indicates that the activity of the methanogens is not limiting in this overall process. The depolymerization and further degradation of the solubilized coal polymers may be a key factor in methanogenesis.

These preliminary results encourage continued research in this area. Effort is needed in developing the methanogenic and degradative anaerobic cultures in order to improve the yields, rates and consistency of the bioconversions. This goal may be achieved by adding potential aromatic-degrading organisms to the consortium. There may also be ways of modifying the aerobic solubilization step to increase depolymerization.

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