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ANAEROBIC BIOPROCESSING OF LOW RANK COALS

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1. EXECUTIVE SUMMARY

The most common biological processes applied to coal conversion have been focused on oxidative biosolubilization. The mechanism of such coal solubilization under aerobic conditions is probably dependent on the aromatic ring hydroxylations followed by ring scission. Such reaction results in oxygenated coal product which, in turn, makes the coal poorer fuel than the starting material. Thus, a novel approach has been made, in this project, to remove oxygen from coal by reductive decarboxylation. In Wyodak subbituminous coal the major oxygen functionality is carboxylic groups which exist predominantly as carboxylate anions strongly chelating metal cations like Ca^{2+} and forming strong macromolecular crosslinks which contribute in large measure to network polymer structure (Narayan, 1986). Removal of the carboxylic groups at ambient temperature by anaerobic organisms would unravel the macromolecular network, resulting in smaller coal macromolecules with increased H/C ratio which has better fuel value and better processing prospects. Therefore, the overall goal of this project was to find biological methods to remove carboxylic functionalities from low rank coals under ambient conditions and to assess the properties of these modified coals towards coal liquefaction. During the project period, efforts were made to establish anaerobic microbial consortia (or mixed cultures) having decarboxylating ability, decarboxylate coal with the adapted microbial consortia, isolate the organisms, and characterize the biotreated coal products. Production of CO_2 was used as the primary indicator for possible coal decarboxylation.

Rumen fluid and anaerobic waste digester sludge were chosen as prime sources for decarboxylating anaerobes. Microbial consortia which decarboxylate coal were obtained from these sources by selective enrichment methods. The cultures showing high net gas production from coal were subcultured to adapt and enrich as the desired consortia. Since two inoculum sources, i.e., rumen R2 and waste digester #1B, exhibited highest CO_2 and CH_4 production from coal, a mixture of these inocula was used to initiate the bioreactor and the subsequent enrichment studies. Microbial consortia showed higher gas production at pH 7 than at pH 6. Coal decarboxylation was examined in the presence of high initial CO_2 pressure to prove feedback inhibition, if any. Subbituminous coal seems to contain water soluble compounds which were more inhibitory than those in lignite coal. Certain microbial consortia exhibited higher gas production from coal when they were maintained in media containing no coal.

Of the four taxonomically known cultures, *Propionibacterium acidi-propionici*, *Clostridium acetobutylicum*, *Veillonella alcalescens* and *Selenomonas ruminantium*, only two, *P. acidi-propionici* and *V. alcalescens*, were selected for further examination, but were found to possess only limited coal decarboxylation activity. Except mixed cultures, 25 new isolates obtained from enriched cultures or microbial consortia also showed only limited coal decarboxylation. These results suggest that certain interactions between various organisms may play an important role in decarboxylation of coal, therefore, mixed microbial consortia and not the pure cultures need to be employed in further coal bioprocesses.

Crude enzyme study showed that the decarboxylase(s) present in the microbial consortia may be extracellular in nature. Pure enzyme study, however, showed negligible

activity on coal decarboxylation. Model polymer compounds with carboxylic groups were also used in order to better understand coal decarboxylation mechanisms.

A new configuration of batch fermentation system was successfully developed and operated to maintain anaerobic conditions over a long period and to continuously strip out CO_2 produced. The production of total CO_2 was increased with an increase in supplemental carbon source such as sodium succinate. However, it was found that quality improvement of biotreated coal (oxygen reduction, increase in H/C ratio, etc.) was not correlated with addition of supplemental carbon source. This indicates that carbon sources other than coal, after a minimal concentration, will reduce the dependence of microorganisms on $-\text{COOH}$ groups of coal for growth and energy, and make it difficult to judge the true decarboxylation from coal. Volatile fatty acids and alcohols produced by microbial consortium were almost completely consumed to produce CH_4 within 5-6 weeks, indicating conversion of some of coal monomers to these intermediates. Hydrogen gas phase (5%) increased H/C ratio of coal product probably by conservation of coal hydrogen. Inhibition of methanogenic bacteria was also effective in improvement of coal quality.

Biotreated coals were characterized using FT-IR spectroscopy, and CHN and thermogravimetric analyzers, titration of carboxyl groups and examining their solvent solubilities. Using an FT-IR spectroscopy, about 10-22% loss of carboxyl groups in coal was observed. An air leak introduced into one batch fermentor system stopped production of CO_2 . The increased carboxyl and carbonyl contents in this coal indicated that the decarboxylation of coal requires anaerobic conditions. Titration method also confirmed the removal of carboxyl groups from coal by anaerobic biotreatment. Biotreated coals from batch fermentors exhibited 1.0-6.8% increase in H/C ratio. Thermogravimetric (TG) analysis was performed to determine any impact that anaerobic bioprocessing would have on evolution of volatiles from biotreated coals. As expected, the ratio of volatile carbon to fixed carbon of biotreated coals showed up to 20% increase, suggesting that decarboxylated coal allowed the polymer chain to pull off the matrix without undergoing significant retrogressive reaction. Aromaticity of the coal was reduced and THF-solubles were greatly increased by biotreatment of coal. Liquefaction of limited biotreated coal samples did not indicate increase in liquefaction yields in comparison to untreated coal probably due to severe conditions employed during liquefaction. Interestingly, particle size of the coal was reduced during the anaerobic bioprocessing and the reduction in size was dependent upon length of exposure to the biotreatment.

2. INTRODUCTION

Coal is basically a carbonaceous material with mineral inclusions and, theoretically, microorganisms should be able to attack such a heterogeneous substance. Biotechnology can be used to bring about either a general breakdown of coal or to achieve the selective removal of particular components within its macromolecular structure. Microorganisms may attack either the carbonaceous material or the interspersed inorganic materials in coal. One route is the depolymerization of coal polymer where the breakage of various key links will serve as an aid to a subsequent liquefaction step. A second route is the reduction in oxygen content of coal making the coal more hydrogenated, thereby, improving its fuel value. Another route is to remove sulfur, nitrogen or other contaminants from the coal before combustion, thus reducing unwanted emissions.

Subbituminous and lignite coals contain high levels (about 18-20%) of oxygen. Our earlier structure reactivity studies with Wyodak subbituminous coal (Narayan, 1986) have revealed that carboxyl groups along with ether/phenolic groups (present as dioxy aromatics) are the predominant oxygen functionalities of this coal (Figure 1). For every three phenolic or phenol ether groups there are two carboxyl groups. The macromolecular structure consists of carboxylate anions (-COO^-) chelated with metal cations such as Ca^{+2} and strong hydrogen bonded crosslinks from free hydroxyl and carboxyl groups. These two significant non-covalent interactions, the chelate and hydrogen bonds, contribute to the formation of a crosslinked polymeric network structure of the coal. It has also been reported that the carboxyl groups account for an estimated two thirds of the oxygen present in this low rank coal (Sandreal and Wiltsee, 1984). Decarboxylation of coal by chemical or thermal processes occurs only at elevated temperatures. Elimination of -COOH groups is accompanied by retrogressive crosslinking reactions in coal resulting in a more intractable coal macromolecule. This is because the oligomeric chains of coal are in close proximity to each other and the carboxylate and hydrogen bonded non-covalent crosslinks are replaced by much stronger carbon-carbon covalent crosslinks.

Therefore, removal of -COOH groups under ambient conditions would result in the elimination of the carboxylate chelate and hydrogen bond crosslinks holding the macromolecular structure resulting in unraveling of the macromolecular structure. As discussed earlier, the elevated temperature conditions of chemical decarboxylation results in the creation of more cross-linked intractable coal structure. Ambient temperature decarboxylation can be achieved by using anaerobic microorganisms. Anaerobic bioprocessing of coal results in removal of oxygen by reductive decarboxylation, cleavage of ether linkages and reduction of contaminants such as sulfur via desulfurization. The process is less energy intensive since no aeration is needed and requires a simple engineering design. Contrary to this, the more common aerobic process focuses on oxidative biosolubilization of coal. The requirement of aeration during this process makes it highly energy intensive and less practical. It is well established that under aerobic conditions the mechanism of coal solubilization is probably dependent on aromatic ring hydroxylations followed by ring scission. However, even under anaerobic conditions hydroxylations by incorporation of water followed by a reductive cleavage seem to be a potential biochemical mechanism for ring scission and coal solubilization.

Carboxy and dioxy groups are major oxygen functionalities

Network polymer structure due to secondary forces - hydrogen bonding and chelate crosslinks

Figure 1. Salient Structural Features of Wyodak Subbituminous Coal (Narayan, 1989)

In this project, we studied the anaerobic biotransformations of a subbituminous coal. The key objective was to reduce the oxygen functionalities, namely carboxyl, resulting in dismantling of the macromolecular network. The resultant biotreated coal was to be examined for its liquefaction potential. Finally, the goal of the project was to understand the microbial transformations that occur during anaerobic bioprocessing of coal.

3. OBJECTIVES

The main goal of this project was to explore and establish anaerobic bioprocesses for transformations of coal to provide a good solid fuel or serve as a feed in a subsequent coal liquefaction step. The specific objectives of this project were to:

1. Develop microbial consortia using various enrichment techniques which would provide potential organisms for coal decarboxylation,
2. Develop batch bioreactor configuration for anaerobic coal decarboxylation,
3. Isolate organisms responsible for coal decarboxylation,
4. Evaluate crude and pure decarboxylase for coal decarboxylation,
5. Optimize process parameters,
6. Characterize coal products, and
7. Liquefy the decarboxylated coal

4. ACHIEVEMENTS

- ◆ Microbial consortia that can anaerobically remove oxygen from coal have been developed.
- ◆ Coal decarboxylation possible only under anaerobic condition has been shown.
- ◆ Long-term anaerobic bioreactor operation treating coal has been shown.
- ◆ Removal of oxygen from coal, using adapted anaerobic microbial consortium, in a batch system achieved.
- ◆ Biotreated coals in comparison to starting coal have been demonstrated to have a higher H/C ratio. A maximum of up to 7.0% increase in H/C ratio was achieved.
- ◆ Up to 4% oxygen content was reduced in the biotreated coal in about 6 weeks.
- ◆ Ratio between oxygen and carbon content (O/C) of the biotreated coal was reduced by about 5% in about 6 weeks.
- ◆ Thermogravimetric (TG) analysis of the biotreated coal showed increased evolution rate of volatile carbon indicating it to be a better feedstock for the liquefaction process.
- ◆ An increase in the ratio of volatile carbon to fixed carbon (VC/FC) in the batch biotreated coal was demonstrated.
- ◆ An increase in THF solubility of the biotreated coal was demonstrated.
- ◆ Several new anaerobic strains from the adapted microbial consortia that decarboxylated coal were isolated. Mixed cultures showed higher CO₂ production in comparison to pure cultures.
- ◆ Hydrogen gas phase in the batch fermentation system greatly increased H/C ratio of the biotreated coal.
- ◆ Inhibition of methanogenic bacteria in the batch fermentation increased VC/FC ratio of the biotreated coal.
- ◆ Crude enzyme study showed that coal decarboxylase seems to be mainly extracellular.
- ◆ Pure decarboxylase showed negligible coal decarboxylation activity.
- ◆ Potential biochemical mechanism occurring during anaerobic bioprocessing of coal has been speculated.

UNEXPECTED RESULTS

Reduction in the size of coal particles to ultra-fine particles was achieved during the anaerobic bioprocessing of coal. The biotreatment modified the coal so as to improve its dispersability in coal-water slurries. A U.S. patent application has been filed vide serial No. 08/016,119.

5. EXPERIMENTAL PROCEDURES

Coal

Wyodak subbituminous coal (100 mesh) was purchased from Argonne Premium Coal Sample Bank. The ultimate analysis of the coal is C=68.43, H=4.88, O=16.24, N=1.02, S=0.63, Ash=8.77 and the % moisture content=28.09.

Medium

Phosphate buffered basal (PBB) medium (Table 1) containing mineral salts, phosphate buffer, vitamins and sodium sulfide, as reducing agent, was used (Kenealy and Zeikus, 1981). Coal was added at 1.5 - 5% depending upon the experiment. Yeast extract was added at 0.05 - 0.2% and, where indicated, a supplemental carbon and energy source was added at 0.2-0.4%. The initial pH of the medium was about 7.0. The media and solutions were prepared and sterilized anaerobically as per methods described elsewhere (Zeikus, et. al., 1980).

Enrichment of Coal Decarboxylating Anaerobic Microbial Consortia

All experiments were carried out in anaerobic pressure tubes (28 ml, Bellco Glass, Inc., Vineland, NJ) and vials (158 ml, Bellco Glass, Inc., Vineland, NJ). Gases (N_2 and CO_2) and gas mixture N_2 - H_2 (95:5) were passed over heated copper fillings to remove traces of oxygen. Inocula used in this study consisted of rumen samples obtained from the dairy farms of Michigan State University, East Lansing and University of Wisconsin, Madison, and from a waste treatment site in Michigan which were stored anaerobically at 4°C until used. Media containing coal was inoculated with the mixture of the two inocula at 5%. The incubations were carried out at 37°C with shaking at 120 rpm. Culture tubes without coal were kept as control.

Batch Fermentation System

A new batch fermentation (Figure 2) configuration was developed since the first two batch fermentation systems were not suitable for long-term operation of the fermentations under strict anaerobic conditions. The new configuration consisted of inclusion of continuous N_2 gas sparge to remove produced CO_2 from the gas phase and its subsequent absorption in concentrated alkali. The N_2 gas was passed over a heated copper catalyst to remove traces of O_2 present in the N_2 gas and then bubbled through concentrated alkali to remove any contaminant CO_2 before sparging it through the fermentor. The trapped CO_2 in the alkali solution was released by acidifying the solution in a sealed tube and the evolved CO_2 in the gas phase was measured using gas chromatograph. The PBB media (about 400 ml) containing 5% coal was inoculated with a microbial consortium mixture at 5% and the fermentor was operated at 37°C and a pH of 6.9 - 7.2. Agitation was provided using a magnetic bar to minimize coal particle reduction caused by mechanical grinding as a result of metal impeller shaft. Coal samples were withdrawn at weekly interval and stored at 4°C until analyzed. Variations in the culture conditions applied to operate different batch fermentations are given in Table 2.

Figure 2. Schematic diagram of anaerobic coal decarboxylating reactor system.

Table 1. Composition of Phosphate Buffered Basal (PBB) Medium

A. Composition of mineral mixture	
Composition	Per 1.0L Medium
NaCl	0.9 g
MgCl ₂ ·6H ₂ O	0.2 g
CaCl ₂ ·2H ₂ O	0.1 g
NH ₄ Cl	1.0 g
Trace mineral solution II ^a	10 ml
Resazurin solution, 0.2%	1 ml
Distilled water to	1000 ml
a. Composition of trace mineral solution II (per L solution)	
Nitrilotriacetic acid	12.80 g
FeSO ₄ ·7H ₂ O	0.10 g
MnCl ₂ ·4H ₂ O	0.10 g
CoCl ₂ ·6H ₂ O	0.17 g
CaCl ₂ ·2H ₂ O	0.10 g
ZnCl ₂	0.10 g
CuCl ₂ ·2H ₂ O	0.02 g
H ₃ BO ₃	0.01 g
Na molybdate	0.01 g
NaCl	1.00 g
Na ₂ SeO ₃	0.017 g
NiSO ₄ ·6H ₂ O	0.026 g
SnCl ₂	0.02 g
B. Composition of vitamin solution (per 1L): dissolve in order biotin, 2 mg; folic acid, 2 mg; B ₆ (pyridoxine)-HCl, 10 mg; B ₁ (thiamine)-HCl, 5 mg; B ₂ (riboflavin), 5 mg; Nicotinic acid (niacin), 5 mg; pantothenic acid, 5 mg; B ₁₂ crystalline (cyanocobalamin), 0.1 mg; PABA (<i>p</i> -aminobenzoic acid), 5 mg; lipoic acid (thioctic), 5 mg; and distilled water to 100 ml.	
C. Composition of phosphate buffer: 15% KH ₂ PO ₄ + 29% K ₂ HPO ₄ , pH 7.0 - 7.1.	
D. PBB Medium: add vitamin solution (0.1 ml), phosphate buffer (0.1 ml), and 2.5% Na ₂ S (0.2 - 0.25 ml) as reductant, to 10 ml of mineral mixture.	

**Table 2. Summarized Culture Conditions for Batch Bioreactor Fermentations
(#1 - #18)**

Batch No.	N ₂ Sparging	Inoculum ^a	(g) per Batch Medium			Fermentation Period (days)
			Coal	Sodium Succinate	Yeast Extract	
1	No	WD1B	15 (a.r.)	0	0.2	51
2	No	R2+WD1B	20 (a.r.)	0	0.2	31
3	Yes	RW72C	20 (a.r.)	0	0.2	132
4	Yes	RW71C	20 (a.r.)	0.8	0.8	71
5	Yes	#34	20 (dry)	1.6	0.8	49
6	Yes	#34	20 (dry)	1.6	0.8	49
7	Yes	Rumen 991	20 (dry)	0	0.2	44
8	No ^b	Rumen 991	20 (dry)	0	0.2	44
12	Yes	No inoculum	20 (dry)	0	0	38
13	Yes	LC-4C3	20 (dry)	1.6	0.8 (NH ₄ Cl) ^c	57
14	Yes	LC-4C3	20 ^d (dry)	1.6	0.8 (NH ₄ Cl) ^c	57
15	Yes	No inoculum	20 ^d (dry)	1.6	0.8 (NH ₄ Cl) ^c	57
16	Yes	RW7C-10	20 (dry)	0.8	0.4	51
17 ^e	Yes	RW7C-10	20 (dry)	0.8	0.4	51
18	Yes ^b	RW7C-10	20 (dry)	0.8	0.4	51

^aWD1B, waste digester 1B; R2, rumen R2; RW72C, enriched consortium obtained from a mixed inoculum of R2 and WD1B; #34, a mixed inoculum of #3 F and #4 F subcultures (1:1); rumen 991, fresh rumen obtained on September 30, 1991 from a MSU dairy farm.

^bA mixture gas of N₂-H₂ (95:5) was sparged continuously.

^cNH₄Cl was added as nitrogen source. No yeast extract was added.

^d20 mesh Penn State coal.

^eAdd 2-bromoethanesulfonic acid (BES) at 8 mM to inhibit growth of methanogenic bacteria.

Isolation of Anaerobic Bacteria from Microbial Consortia

Prevalent anaerobic bacteria in the adapted coal decarboxylating microbial consortium were isolated using anaerobic plating techniques as described elsewhere (Jain and Zeikus, 1987). The adapted consortium was streaked on PBB medium agar plates containing 0.2% yeast extract, and 0.4% sodium succinate or 5% coal. The plates were incubated anaerobically for 1 - 5 weeks and the colonies grown on plates were picked up in the tubes containing homologous liquid medium. Isolates were subcultured and evaluated for coal decarboxylations in PBB medium with 0.2% yeast extract and 5% coal in the presence and absence of 0.4% sodium succinate.

Evaluation of Known Cultures for Coal Decarboxylation

Four well-characterized pure cultures, i.e. *Clostridium acetobutylicum*, *Propionibacterium acidi-propionici*, *Veillonella alcalescens*, and *Selenomonas ruminantium*, were examined for their coal decarboxylating ability. CO₂ production was determined during their growth in their respective media in the presence and absence of coal. All experiments were conducted in duplicate tubes. Since the media used in these studies contained very high amounts of organic nutrients, elimination or reduction of some non-essential nutrients, e.g. vitamin K and hemin for *P. acidi-propionici*, was also attempted.

Gas Chromatographic Analysis of Gases

The gas phase of tubes and vials were examined for CO₂ and CH₄ to monitor growth and decarboxylation. Gas samples were withdrawn from the headspace with a 1.0 ml glass syringe (Container Corp., Sioux City, Iowa) equipped with a gas tight mininert syringe valve (Alltech Associate, Inc., Deerfield, MI) and a 23 gauge needle. CO₂ and CH₄ gas was analyzed by injecting 0.4 ml of gas sample into a Gow-Mac series 580 Gas Chromatograph (GOW-MAC Instrument Co., Bridgewater, NJ) equipped with a thermal conductivity detector (TCD) and carbosphere column at 102°C with helium as a carrier gas. Detector temperature and helium gas flow rate were 150°C and 50 ml/min, respectively.

Chemical Characterization of Coal

The relative change in the carboxylic group content of the coal was determined by FT-IR method (Perkin-Elmer 1600 series FT-IR). Biotreated coal was collected by filtration or by centrifugation at 4000 rpm for 20 min. The coal was washed with 3N HCl for 3-4 hours to remove biomass attached to the coal followed by repeated washing with distilled water until neutral pH. Microscopic examination of washed coal did not indicate the presence of any microbial cells. The washed coal was dried under vacuum at 80°C for 24 hour. The dried coal (100 mg) was methylated using dimethylsulfate at pH 13 (Narayan, 1986). Methylated coal was centrifuged, washed several times with 1N HCl, then washed with 50 ml of hot 1N HCl, and finally with 100 ml of hot distilled water. The washed coal was dried under vacuum at room temperature. We also measured the -COOH groups in the biotreated coals by a titration method (Blom, et. al., 1957). The method involves acid washing of coal to remove cations from carboxyl groups and then adding calcium acetate to chelate calcium ions with ionized carboxylic groups. The released acetic acid was titrated with 0.02 N NaOH solution.

The elemental analysis to determine the carbon and hydrogen content was done by a CHN elemental analyzer (Perkin-Elmer model 2400). Method to prepare coal samples for elemental analyses was similar to the one used for FT-IR analysis except the methylation step. However, in later analysis acid washing process was omitted to prevent possible removal of acid soluble coal components and change of coal polymer structure by acid treatment.

Thermogravimetric (TG) analysis of the coal was conducted to provide information on the reactivity of the biotreated coal using a DuPont 9900 series TG analyzer. Sample preparation was the same as for CHN analysis. The TG analyzer was programmed as

follows: isothermal at 200°C for 5 min., rate of temperature increase at 100°C per min to 900°C, and isothermal at 900°C for 18 min. The air was manually introduced to the furnace after 8 min of reaching at 900°C.

The extent of coal biosolubilization was measured by the amount of coal product solubilized in organic solvents per unit of time (Narayan 1987; Narayan, et. al., 1987; Huang and Narayan, 198). The THF and toluene were used for extracting the coal product using the Soxhlet extraction apparatus overnight. The solvent extract was concentrated and finally dried under vacuum at 75°C for THF and 85°C for toluene.

Coal Liquefaction

Liquefaction experiments of the biotreated coal were performed by Dr. Robert Warzinsky at Pittsburgh Energy Technology Center, Pittsburgh and at Advanced Fuel Research (AFR), East Hartford, Connecticut by Dr. Mike Serio. At AFR, the liquefaction experiments were carried out at 400°C for 30 minutes with 6:1 tetrahydronaphthalene as the solvent.

6. RESULTS AND DISCUSSION

Development of Microbial Consortia

Microbial consortia which utilize coal as carbon and energy source or which decarboxylate coal while utilizing supplemental substrates as carbon and energy sources were obtained by selective enrichment methods using rumen fluid and sludge from waste treatment site as the initial source for microorganisms. Rumen fluid as prime source for decarboxylating anaerobes, was chosen firstly because cattle feed is lignocellulosic in nature (coal resembles lignin in their structural complexities) and secondly because many decarboxylating anaerobic bacteria have been isolated from it. The anaerobic sludge from the waste treatment site was selected because this facility receives a mixture of many unknown chemicals and, thus, a variety of anaerobes that may be resistant to complex chemicals are likely to be found in such a system. The enrichments were conducted in tubes, vials as well as the fermentor system. The selective pressures to develop a coal decarboxylating consortium were simple and included (i) use of coal as the sole carbon source, (ii) use of substrates such as succinate, benzoates, lactate etc. which have been reported to be decarboxylated and (iii) a mixture of coal and supplemental substrates such as succinate. In general, production of CO_2 was assumed as an indication of potential decarboxylation. Inoculum from tubes producing high CO_2 levels were subcultured into the homologous media to adapt and enrich the desired consortia.

A. Evaluation of different inoculum sources

Five different inoculum sources (3 from rumen and 2 from waste digester) were evaluated for coal decarboxylation potential in anaerobic tubes with coal (0.15 g/10 ml medium) as carbon and energy source. Vitamin solution was added as nutrient supplement, where indicated. In some cases, succinate or benzoate was added as supplemental carbon and energy source. The gas in the headspace of the tubes was examined for CO_2 and CH_4 for 5 weeks. The preliminary results summarized in Table 3 indicate that rumen inoculum R2 showed highest decarboxylation activity, as determined by CO_2 production, followed by waste digester #1B. Production of CO_2 was enhanced when the media was supplemented with vitamins indicating requirement of one or more than one vitamin by the decarboxylating microbial species. Succinate seemed a better supplemental carbon and energy substrate than benzoate. Effect of benzoate was variable but significant (Table 3). Interestingly, initial gas production from coal was low. This may suggest that coal contains some inhibitory compounds that affect the initial growth of decarboxylating organisms.

Cultures from tubes showing high decarboxylating activity were used to inoculate subsequent tubes containing supplemental carbon source with or without coal. Results of subsequent subculturing have shown establishment of microbial consortium having decarboxylating activity and its maintenance upon transfer from one tube to another (Tables 4 and 5). Important to note is the gas production by consortium #2 and #3 with succinate as supplemental carbon source and by consortium #4 in presence of benzoate (Table 4). Since rumen R2 and waste digester #1B showed higher decarboxylating activities in tubes, these were further examined, either alone or their mixture, in anaerobic vials containing 50 ml

medium with 3-5% coal. A typical time course of CO₂ and CH₄ production by the mixed consortia of rumen R2 and waste digester #1B (1:1 ratio) is presented in Figure 3.

Table 3. Gas Production from Enrichment Cultures Inoculated with Various Inoculum Sources

Inoculum Source and Conditions	14 days		35 days	
	% CO ₂	% CH ₄	% CO ₂	% CH ₄
<i>Rumen R2</i>				
Control (no coal, no vitamin)	4.20	8.51	7.52	30.43
+ Coal	4.06	4.14	7.06	26.38
+ Coal + Vitamin	4.08	11.61	8.41	36.16
+ Vitamin + Succinate	8.18	4.14	9.39	17.20
+ Coal + Vitamin + Succinate	7.92	5.58	9.72	24.82
+ Vitamin + Benzoate	4.80	4.70	7.13	14.49
+ Coal + Vitamin + Benzoate	4.98	4.42	9.11	28.78
<i>Rumen M6</i>				
Control (no coal, no vitamin)	2.16	10.15	3.19	14.12
+ Coal + Vitamin	1.36	7.48	2.51	13.63
+ Vitamin + Succinate	3.39	0.30	3.81	0.56
+ Coal + Vitamin + Succinate	3.83	0.82	4.86	10.61
+ Vitamin + Benzoate	0.89	0.31	1.30	0.53
+ Coal + Vitamin + Benzoate	0.71	0.29	1.04	0.64
<i>Rumen M8</i>				
Control (no coal, no vitamin)	0.72	0.31	0.95	1.56
+ Coal + Vitamin	0.71	0.65	1.03	0.95
+ Vitamin + Succinate	3.22	0.44	3.83	1.13
+ Coal + Vitamin + Succinate	3.63	0.68	4.42	2.35
+ Vitamin + Benzoate	1.02	0.45	1.41	1.74
+ Coal + Vitamin + Benzoate	0.78	0.36	1.19	0.58

Inoculum Source and Conditions	14 days		35 days	
	% CO ₂	% CH ₄	% CO ₂	% CH ₄
Waste Digester 1B (WD1B)				
Control (no coal, no vitamin)	1.97	7.70	2.71	13.01
+ Coal	1.26	4.74	1.67	9.41
+ Coal + Vitamin	1.79	8.82	2.91	15.66
+ Vitamin + Succinate	6.38	2.39	7.16	16.04
+ Coal + Vitamin + Succinate	4.54	8.08	5.49	14.87
+ Vitamin + Benzoate	0.39	6.07	3.88	19.46
+ Coal + Vitamin + Benzoate	1.63	4.10	2.41	10.15
Waste Digester 3B (WD3B)				
Control (no coal, no vitamin)	2.26	9.03	2.98	14.38
+ Coal	1.0	2.04	1.74	7.63
+ Coal + Vitamin	0.78	1.45	1.46	6.14
+ Vitamin + Succinate	5.94	2.31	6.33	10.80
+ Coal + Vitamin + Succinate	4.58	2.88	6.04	16.61
+ Vitamin + Benzoate	1.31	3.74	2.77	12.20
+ Coal + Vitamin + Benzoate	1.15	2.96	2.40	11.20

B. Effect of initial pH on production of CO₂ from coal in anaerobic vials

In order to help establish the microbial consortium upon transfer into new medium, it was pertinent to examine the effect of initial pH on decarboxylating activity of rumen R2 and waste digester #1B microbial consortia from tubes (Table 3). Initial media pH was adjusted to pH 6 and 7 just before inoculation. The vials were inoculated with these inocula either alone or their mixture in 1:1 ratio. The results presented in Table 6 indicate that except for #WD1B alone at pH 7, rumen R2 alone or in combination with #1B performed very well at both pH's. The mixture of consortia produced a little more gas at pH 7. This mixed consortia (RW71 and RW72) of rumen R2 and waste digester #1B was subsequently used to inoculate batch fermentor #3 and #4.

Figure 3. A typical time course of gas production from 3% coal in a vial with a mixed inoculum of rumen R2 and waste digester 1B (1:1).

Table 4. Gas Production from Subcultures of Four Microbial Consortia with Succinate or Benzoate $\pm$ Coal as Substrates

Inoculum and Conditions	14 days		35 days	
	% CO ₂	% CH ₄	% CO ₂	% CH ₄
<i>Consortium 1</i> (from 1 wk tube, R2 + Coal + Succinate)				
+ Succinate	3.47	0.45	3.40	1.07
+ Succinate + Coal	3.38	0.53	3.55	1.14
+ Benzoate	0.97	0.44	1.01	0.49
+ Benzoate + Coal	0.43	0.41	0.51	0.58
<i>Consortium 2</i> (from 1 wk tube, R2 + Coal + Benzoate)				
+ Succinate	3.17	0.30	3.18	0.33
+ Succinate + Coal	3.41	0.95	3.94	7.93
+ Benzoate	1.17	0.70	1.14	2.12
+ Benzoate + Coal	0.51	0.31	0.54	0.30
<i>Consortium 3</i> (from 1 wk tube, WD1B + Coal + Succinate)				
+ Succinate	3.34	1.69	3.21	3.29
+ Succinate + Coal	3.45	6.42	3.85	7.07
+ Benzoate	0.79	1.48	1.03	2.16
+ Benzoate + Coal	0.42	2.19	0.48	2.64
<i>Consortium 4</i> (from 1 wk tube, WD1B + Coal + Benzoate)				
+ Succinate	3.62	3.12	3.66	3.50
+ Succinate + Coal	ND	ND	3.69	2.97
+ Benzoate	0.57	1.91	0.73	2.75
+ Benzoate + Coal	0.58	3.41	1.27	7.18

Table 5. Gas Production from Subcultures of Eleven Microbial Consortia from PBB Medium Containing 1.5% Coal $\pm$ 0.2% Sodium Succinate

Inoculum and Conditions	7 days		21 days	
	% CO ₂	% CH ₄	% CO ₂	% CH ₄
<i>Consortium A</i> (from 6 wk tube, R2 + Coal)				
+ Coal + Succinate	6.57	1.16	8.08	7.11
+ Succinate	2.83	2.62	3.57	5.75
<i>Consortium B</i> (from 6 wk tube, R2 + Coal + Succinate)				
+ Coal + Succinate	7.11	3.01	7.72	5.22
+ Coal	3.77	6.04	4.23	7.61
<i>Consortium C</i> (from 6 wk tube, R2 + Coal + Benzoate)				
+ Coal + Succinate	10.1	3.35	9.96	9.30
+ Coal	3.10	5.48	3.42	7.03
<i>Consortium D</i> (from 6 wk tube, WD1B + Coal)				
+ Coal + Succinate	7.48	1.31	8.57	9.54
+ Coal	2.77	3.69	3.68	8.32
<i>Consortium E</i> (from 6 wk tube, WD1B + Coal + Succinate)				
+ Coal + Succinate	6.96	2.04	9.22	11.6
+ Coal	2.66	2.57	3.80	7.38
<i>Consortium F</i> (from 6 wk tube, WD3B + Coal + Succinate)				
+ Coal + Succinate	7.02	0.96	7.75	5.54
+ Coal	2.58	2.19	3.58	7.13
<i>Consortium G</i> (from 6 wk tube, M6 + Coal + Succinate)				
+ Coal + Succinate	3.94	3.45	7.86	6.03
+ Coal	2.14	1.74	3.48	5.29

Inoculum and Conditions	7 days		21 days	
	% CO ₂	% CH ₄	% CO ₂	% CH ₄
<i>Consortium H</i> (from 5 wk tube, Coal + Succinate; subcultured from R2 + Coal + Benzoate on day 7)				
+ Coal + Succinate	7.20	1.07	7.78	3.81
+ Succinate	2.10	1.05	3.20	5.55
<i>Consortium I</i> (from 5 wk tube, + Coal; subcultured from R@ + Coal on day 10)				
+ Coal + Succinate	6.33	1.07	6.86	4.44
+ Coal	1.72	1.69	2.83	4.10
<i>Consortium J</i> (from 5 wk tube, Coal + Succinate; subcultured from WD1B + Coal + Succinate on day 7)				
+ Coal + Succinate	5.49	3.21	6.40	6.96
+ Coal	2.32	1.66	3.12	4.82
<i>Consortium K</i> (from 5 wk tube, Coal + Benzoate; subcultured from WD3B + Coal + Succinate on day 7)				
+ Coal + Succinate	3.12	1.69	5.37	7.09
+ Coal	2.15	0.31	2.84	2.69

C. Maintenance of coal adapted microbial consortium

Significant efforts were put to maintain anaerobic microbial consortia having potential decarboxylating activity. Microbial consortia obtained from anaerobic tubes, vials or fermentors were being subcultured at about 3-4 week interval. However, it was noticed that if the consortium was maintained just on coal as carbon source, its decarboxylating activity was significantly reduced. For example, when the consortia was successively subcultured from fermentors #3 and #4 (Table 7) in tubes containing coal, consortia from fermentor #3 was inhibited and produced less gas in comparison to the consortia maintained in absence of coal. This probably indicates the possible selection of non-decarboxylators over decarboxylators either due to differences in growth rates or due to other obligate requirement for decarboxylation. Thus, microbial consortia maintained on various carbon sources were examined for coal decarboxylation (this term is used here loosely since production of CO₂

from coal is taken as an indication of its decarboxylation). For this, a new microbial consortia from rumen fluid and waste digester (RW71 and RW72) was developed in presence of coal. After it was successively transferred 3 times in absence of coal, this consortia produced a much higher net gas production from coal when it was maintained in absence of coal (Table 8). The results indicate that there was a large amount of net CO₂ and CH₄ production when this microbial consortium was maintained on various substrates as compared to when it was maintained on coal.

Table 6. Effect of pH on Gas Production from 3% Coal by Enriched Cultures

		2 days		14 days		28 days	
Inoculum*	Initial pH	% CO ₂	% CH ₄	% CO ₂	% CH ₄	% CO ₂	% CH ₄
Rumen (R2)	6	2.44	0	5.55	8.52	8.12	24.33
R2 + WD1B	6	2.69	0	4.45	8.59	6.04	19.29
R2	7	2.84	0	5.45	8.97	8.42	28.30
WD1B	7	1.52	0	2.96	4.95	3.66	9.74
R2 + WD1B	7	2.11	0	4.32	8.32	6.37	21.98

*Inoculum was added @ 5%.

Typical profiles of gas production from four microbial consortia in the presence and absence of coal are presented in Figure 4. Consortium LC exhibited highest net gas production from coal followed by consortia RW and RN. Consortium RN was not used further due to limited total gas production. Highest net gas production from coal was usually observed between 3 and 4 weeks of biotreatment period. It may be noted that consortium LC utilized lactate as carbon/energy source and NH₄Cl as nitrogen source, showing that this consortium, utilizing coal as the substrate, could be grown in a chemically defined medium.

Finally, four adapted microbial consortia were selected and maintained in anaerobic vials. These consortia, subcultured in different media, (Table 9) showed distinct coal decarboxylation profiles. Each consortium was subcultured at every 6 week interval. Coal samples decarboxylated by various microbial consortia in anaerobic vials were also analyzed for VC/FC ratio (Table 10). The results indicated that consortium RW8 was more effective than the consortium LCC5. These results are in good agreement with carbon dioxide and methane production in the headspace of the vials.

Figure 4. Gas production profiles from Wyodak coal by four adapted microbial consortia in vials

Table 7. Effect of Successive Subculturing of Microbial Consortia in Medium Containing Coal on Gas Production from Coal

Consortium*	Percent (%)		Net Gas Production from Coal (%)		
	CO ₂	CH ₄	CO ₂	CH ₄	Total
<i>1st Subculture</i>					
3F	13.82	0			
3F + Coal	13.41	5.19	-0.41	5.19	4.78
4F	14.36	0			
4F + Coal	10.24	3.05	-4.12	3.05	-1.17
<i>2nd Subculture</i>					
3F	12.62	30.83			
3F + Coal	12.24	18.39	-0.38	-12.44	-12.82
4F	10.08	19.90			
4F + Coal	11.35	19.20	1.27	-0.70	0.57
<i>3rd Subculture</i>					
3F	12.41	18.78			
3F + Coal	9.57	1.74	-2.84	-17.04	-19.88
4F	11.39	20.43			
4F + Coal	11.79	21.01	0.40	0.58	0.98

*Consortium 3F (from fermentor #3) was subcultured in medium containing 0.2% yeast extract plus 5% coal.

- Consortium 4F (from fermentor #4) was in medium containing 0.4% sodium succinate, 0.2% yeast extract plus 5% coal using coal containing cultures as respective inoculum.

- Gas production was recorded on day 14.

D. Effect of inhibition of methanogenesis and high CO₂ partial pressure on coal decarboxylation

Since the microbial consortia contains methanogens, CH₄ was always produced possibly from CO₂. It is, therefore, not simple to determine the exact amount of CO₂ produced from coal since part of CO₂ is converted to methane. 2-Bromoethanesulfonic acid (BES) has been widely used to inhibit methane production. In the experiments conducted in presence of BES, production of CH₄ was completely inhibited and CO₂ production was observed only for a week (Figure 5). This may indicate that BES also affected the decarboxylation reaction indirectly. It is also possible that the maximum coal decarboxylation takes place only in the initial fermentation period after which the coal carboxyl groups are

Figure 5. Effect of 2-Bromoethanesulfonic acid (BES) on the gas production from coal in anaerobic tubes

inaccessible to microbial action and the observed CO₂ production comes from acetate conversion to CH₄ and CO₂. Thus, inhibition of methane production will also mean no additional CO₂ production.

Table 8. Net Gas Production from Coal by Microbial Consortium (RW71C-4*) Maintained on Different Carbon Sources (on Day 13)^b

Carbon Source for Inoculum	CO ₂ %	CH ₄ %	Net CO ₂ from Coal (%)	Net CH ₄ from Coal (%)	Total Net Gas from Coal (%)
Acetate	13.09	65.55			
Acetate + Coal	14.39	66.93	1.30	1.39	2.69
Benzoate	7.22	13.99			
Benzoate + Coal	9.47	24.35	2.25	10.36	12.61
Lactate	17.82	39.16			
Lactate + Coal	19.26	42.69	1.44	3.53	4.97
Propionate	8.30	16.65			
Propionate + Coal	8.68	19.66	0.38	3.01	3.39
Succinate	12.17	31.35			
Succinate + Coal	14.73	34.14	2.56	2.79	5.35
Coal (C)	9.29	22.20			
C + Coal	5.48	13.58	-3.81	-8.62	-12.43
C + Succinate	10.09	9.10			
C + Succinate + Coal	13.58	27.32	3.49	18.22	21.70

*RW71C-4 is a fourth transfer of RW71C consortium.

^bExperimental medium includes 0.2% yeast extract and 5% coal with no other C-source. Inoculum was grown in the absence of coal for 3 days in media containing respective C-source (0.4%, except for sodium acetate (0.8%) and coal (5%)).

Again microbial consortium RW9 was subcultured in the presence of BES to confirm the above results and to characterize the biotreated coal products. The first and second subcultures of decarboxylating consortia grown on 5% coal produced 3-4% more carbon dioxide than controls without coal. TG analysis showed that the VC/FC ratio of biotreated coal was increased in comparison to control coal and that VC/FC ratio of coal with BES was slightly lower than that without BES (Table 11).

Table 9. Adapted Microbial Consortia for Coal Decarboxylation

Consortium	Origin of Inoculum	Carbon and Nitrogen Source	No. of Subculture Made
RW	Rumen (R2) + waste digester (#1B)	0.4% Sodium succinate + 0.2% yeast extract	12
#34	From #3 and #4 batch fermentor	Same as RW	8
LC	Rumen 991	0.4% Sodium lactate + 0.2% ammonium chloride	8
LCC	Rumen 991	Same as LC	8

Table 10. Thermogravimetric Analysis of Coals Biotreated in Anaerobic Vials

Sample	% Moisture	% Volatile Carbon (VC)	% Fixed Carbon (FC)	% Ash	VC/FC Ratio	% Change in VC/FC Ratio
Control Coal	28.05	32.24	33.33	6.38	0.9672	-
RW8	4.05	43.14	43.35	9.46	0.9951	2.9
LCC5	4.30	41.87	43.75	10.08	0.9570	-1.1

In order to exclude the possibility of feed back inhibition by the accumulated CO₂ on decarboxylation, experiments were set up with higher partial pressure of CO₂. The results indicate that high initial partial pressure of CO₂ did inhibit production of net CO₂ (about 16% reduction) and CH₄ (11-29% reduction) (Table 12). Further investigation may be needed to prove any feedback inhibition.

Table 11. Thermogravimetric Analysis of Coals Biotreated in Anaerobic Tubes*

Sample	% Moisture	% Volatile Carbon (VC)	% Fixed Carbon (FC)	% Ash	VC/FC Ratio	% Change in VC/FC Ratio
Control Coal	27.66	32.40	33.13	6.81	0.9779	-
RW10-C	4.89	42.81	42.46	9.84	1.0082	3.1
RW10BES-42	4.87	42.46	42.81	9.86	0.9919	1.4

*Methanogenesis was inhibited by adding 2-Bromoethanesulfonic Acid (BES) during biotreatment.

Table 12. Effect of Initial CO₂ Partial Pressure on Gas Production from Coal by Microbial Consortium (RW71-4)

Initial CO ₂ Partial Pressure (atm)	% CO ₂ ^a	% CH ₄	Total Gas (%)	Net CO ₂ Change (%)	Net CH ₄ Increase (%)
0 (Control)	16.95	34.39	51.34	-	-
0.1	14.23	30.72	44.95	-16.05	-10.67
0.3	14.26	25.06	39.32	-15.87	-29.13

^aCO₂ values were obtained by subtraction of the initial values from the final values. The experiment was conducted in anaerobic tubes for 14 days.

E. Effect of removal of water-soluble compounds on coal decarboxylation

It is known that coals have water soluble compounds which may be inhibitory to microorganisms and we observed such inhibitory effect of coal on decarboxylating organisms (Table 3). In order to examine the effect of removal of such compounds on coal decarboxylation, two low-rank coals, North Dakota lignite and Wyodak subbituminous, were used after a water wash. Unwashed coals were also used as controls. The results presented in Table 13 show higher gas production from washed coals than unwashed coals. Subbituminous coal seems to contain water soluble compounds which are more inhibitory than those present in lignite.

Table 13. Gas Production by Washed and Unwashed Coals by Anaerobic Microbial Consortium (RW71-4)

Coal Sample	After 14 Days		Difference (B - A)		Total Difference
	% CO ₂	% CH ₄	% CO ₂	% CH ₄	
<i>Lignite</i>					
Unwashed (A)	14.77	29.00	-	-	-
Washed (B)	16.11	30.32	1.34	1.32	2.66
<i>Subbituminous</i>					
Unwashed (A)	16.95	34.39	-	-	-
Washed (B)	17.13	42.58	0.18	8.19	8.37

F. Coal-Microbe Juxtaposition

Samples from the fermentor and the anaerobic tubes containing coal and microorganisms were examined for coal-microbe juxtaposition by scanning electron microscopy (SEM). Figure 6 exhibits SEM pictures showing association of bacteria (rods and cocci shape) with coal particles. These pictures could be indicative of cell bound or extracellular nature of the enzyme(s) involved in coal decarboxylation.

Decarboxylation of Coal by Known Anaerobic Microorganisms

Four pure cultures, such as *C. acetobutylicum*, *P. acidi-propionici*, *V. alcalescens*, and *S. ruminantium*, were examined for their ability to decarboxylate coal in the presence of supplemental carbon and nitrogen sources. The results presented in Table 14 indicate increased CO₂ production by *P. acidi-propionici* in growth media containing coal than without coal. *C. acetobutylicum* did not show any coal decarboxylation. Difference in CO₂ production from coal by *V. alcalescens* was moderate. *S. ruminantium* was dropped from further studies since it required CO₂ for its growth.

Table 14. Coal Decarboxylation with Known Cultures in Anaerobic Tubes Containing 5% Coal

Known Culture/ Condition	% CO ₂ in the Gas Phase		
	0 Day	5 Days	Net Change
<i>P. acidi-propionici</i>			
- Coal + Medium	0.11	13.9	
+ Coal + Medium	0.11	24.2	10.3
<i>V. alcalescens</i>			
- Coal + Medium	0.0	20.0	
+ Coal + Medium	0.0	24.3	4.3
<i>Cl. acetobutylicum</i>			
- Coal + Medium	0.08	1.06	
+ Coal + Medium	0.08	1.26	0.2

Upon further examination of *P. acidi-propionici* and *V. alcalescens* it was observed that increase in coal concentration did not increase in CO₂ production (data not reported) suggesting that decarboxylation may be limited by some unknown nutritional requirement or other factor. In order to have an idea about the mechanism of coal decarboxylation by *P. acidi-propionici*, preliminary whole cell enzyme assay system was conducted with washed and non-washed cells. Data presented in Table 15 indicate about 1.5 times increased CO₂ production in the presence of coal suggesting that the enzyme mediated decarboxylation

Figure 6. Scanning Electron Microscopic Pictures of Microbe-Coal Association
(Arrow indicates microorganism.)

reaction might occur by the direct contact of cells with coal particles. It is too preliminary an experiment to conclude whether the coal decarboxylation is due to an extracellular or cell bound enzyme. Additional experiments will be needed to understand the mechanism of coal decarboxylation, however, the scanning electron micrographs (Figure 5) do suggest the direct contact of coal and the bacterial cell, indicating a possible cell bound or extracellular type enzyme system.

Table 15. Coal Decarboxylation by Washed Whole Cells and the Supernatant of the Culture Broth of *P. acidi-propionici*

Reaction Mixture*	% CO ₂ in the Gas Phase		
	0 Hr	17 Hrs	96 Hrs
SP + C#1 (no coal)	0.38	1.12	1.33
SP + C#2 (+ coal)	0.56	1.16	1.13
SP + C#3 (heated, + coal)	0.35	0.72	0.68
SP - C#1 (no coal)	0.26	1.18	1.16
SP - C#2 (+ coal)	0.55	1.12	1.45
SP - C#3 (heated, + coal)	0.41	0.67	0.87
WC-C#1 (no coal)	0.00	0.21	0.22
WC-C#2 (no coal + vitamin)	0.08	0.25	0.22
WC-C#3 (+ coal)	0.20	0.37	0.35
WC-C#4 (+ coal + vitamin)	0.18	0.39	0.37

*Preparation of crude enzyme reaction mixture:

1. 10 ml of supernatant from culture grown with coal (SP+C)
 - SP+C#1: - coal
 - SP+C#2: + coal
 - SP+C#3: + coal with heat treated supernatant
2. 10 ml of supernatant from culture grown without coal (SP-C)
 - SP-C#1: - coal
 - SP-C#2: + coal
 - SP-C#3: + coal with heat treated supernatant
3. 10 ml of washed whole cells suspension from the culture grown without coal (WC-C)
 - WC-C#1: - coal
 - WC-C#2: - coal + vitamin
 - WC-C#3: + coal
 - WC-C#4: + coal + vitamin

Isolation of Anaerobic Bacteria and Their Decarboxylation Potential

Enrichment samples from fermentor system #3 and various microbial consortia in tubes and vials were plated onto agar plates in an anaerobic glove box. The plates were incubated anaerobically in anaerobic jars. After incubation, the anaerobic jar was opened in the glove box and well-isolated colonies were picked into PBB liquid medium. A total of about 25 isolates were obtained in the pure or mixed cultures. These isolates were stored at 4°C until use.

First seventeen isolates obtained from adapted microbial consortia were examined for their decarboxylating activity using PBB medium containing 5% coal. However, the levels of CO₂ produced were very low (< 1.0%), even in 3 weeks. Nine of the 17 isolates were further examined for decarboxylating activity using a slightly rich PBB medium containing 0.4% sodium succinate and 0.2% yeast extract (Table 16). The results indicate that the consortium R623 produced highest net CO₂ from coal. Other isolates which showed potentially good activity were A2 and C3. In addition, 8 new isolates were obtained from coal agar plates and of these, #8 which was a mixed consortium exhibited highest net CO₂ production (Table 17) when examined for coal decarboxylating activity.

Table 16. CO₂ Production from 5% Coal by Nine Selected Isolates^a

Isolate #	Net CO ₂ from Coal (%) ^b			
	Day 1	Day 3	Day 7	Day 14
A2	0.15	-0.01	1.34	1.75
B4	0.05	0.34	0.76	0.99
C3	2.08	1.37	1.62	1.68
C4	-0.87	-0.41	-0.05	0.10
D1	-0.77	-0.42	-2.09	-1.95
D3	0.80	0.99	0.29	0.28
E4	0.49	0.83	1.16	1.19
F4	-0.18	0.62	0.97	0.96
R623	0.31	0.56	5.94	6.63

^aMedium composition: 10 ml PBB medium + 0.4% sodium succinate + 0.2% yeast extract.

^bBy difference of CO₂ in the gas phase between culture containing coal and culture without coal.

Supplemental carbon sources such as benzoate, lactate and succinate were evaluated for their suitability to support growth and decarboxylating ability of these 2 potential isolates and 3 microbial consortium (Table 18). Based on the total CO₂/CH₄ production, the results show that succinate was better for the growth of consortium #8 and isolates C3 and A2 while lactate and benzoate were better for growth of consortium R623 and CON, respectively. Net

gas production, probably from coal, was less than 4% with highest gas production by consortium #8 (3.89%) and isolate C3 (2.84%). Consortium #8 was a mixed culture that contained methanogenic bacteria, thus, produces methane. When these cultures were subcultured in a medium containing sodium succinate and coal, the coal decarboxylating activity was noted to be greatly reduced. These observations were similar to the one noted for microbial consortia that were successively subcultured in the media containing coal (Table 7). Thus, it can be concluded that these pure cultures and the consortia need to be maintained in a medium without coal.

Table 17. CO₂/CH₄ Production from 5% Coal by Eight New Isolates Picked from Coal Agar Plates^a

Isolate #	Net Gas from Coal (%) ^b			
	Day 5		Day 14	
	CO ₂	CH ₄	CO ₂	CH ₄
1	1.47	0	1.67	0
2	0.67	0	1.03	0
3	1.05	0	1.61	0
4	0.08	0	0.55	0
5	-0.39	0	0.66	0
7	0.26	0	-0.39	0
8	0.53	1.13	0.78	4.62
9	2.10	0	1.59	0

^aMedium composition: 10 ml PBB medium + 0.4% sodium succinate + 0.2% yeast extract.

^bBy difference of CO₂ in the gas phase between culture containing coal and culture without coal.

Based on preliminary coal decarboxylation experiments, a total of 11 isolates with high decarboxylation activity were selected for final examination of their coal decarboxylation potential. These were subcultured in PBB medium containing 0.4% glucose and 0.2% yeast extract to get high cell density to allow substantial coal decarboxylation. Two of these isolates NHC and LC were obtained from microbial consortia enriched with fresh rumen inoculum. Among these 11 isolates, six isolates showed good growth after 3 days and the remaining took more than 5-7 days. In parallel, two known cultures, *V. alcalescens* and *P. acidi-propionici*, were also grown in their respective medium. Three day old cultures of two known and six fast growing isolates were used as the inoculum for coal decarboxylation in PBB medium containing 0.4% sodium succinate and 0.2% yeast extract. The results presented in Table 19 indicate that *V. alcalescens* exhibited highest net decarboxylation activity, followed by isolates #8 and LC. Five slow growing isolates were also compared for their coal decarboxylation ability in the same manner but these did not show much decarboxylating potential. Based on these results, *V. alcalescens* and isolate #8 were selected

as the primary candidates for enzyme (decarboxylase) study. As observed previously, total gas or net gas production from coal by known cultures or isolates was much less than that by microbial consortia, suggesting that decarboxylation of coal probably involves coordinated activities of various microorganisms in a manner similar to that is observed in anaerobic degradation of organic materials.

Table 18. Net CO₂/CH₄ Production* by Various Isolates and Mixed Culture from Coal in the Presence of Different Carbon Sources

Isolate #	% in the Gas Phase			
	Coal	Benzoate + Coal	Lactate + Coal	Succinate + Coal
After 7 Days				
8 ^b	2.15 (9.53)	0.80 (3.82)	1.07 (- 1.06)	2.56 (2.40)
R623 ^b	0.61	-0.40	0.36	0.48
C3	1.73	0.32	0.52	3.19
CON	1.17	1.18	-4.25	0.85
A2	0.24	0.24	-0.12	0.11
After 28 Days				
8 ^b	1.00 (1.78)	1.33 (4.32)	1.56 (0.16)	3.09 (0.80)
R623 ^b	2.13	-0.79	0.58	0.87
C3	2.13	-0.16	0.44	2.84
CON	0.56	0.61	-0.17	0.31
A2	0.53	0.32	0.01	0.88

() indicates value of net methane production.

*Values from respective controls (only supplemental carbon source in the absence of coal) were subtracted.

^b#8 and R623 are microbial consortia. Consortium #8 contains methanogenic bacteria.

Evaluation of Crude and Pure Enzymes for Coal Decarboxylations

1. Crude Enzyme Evaluation

(i) Cultures Grown in the Absence of Coal:

Two selected cultures, *V. alcalescens* and culture #8, were grown in the absence of coal in their respective media for crude decarboxylase study. Cell-free broth of each culture and washed cells were prepared anaerobically to conduct the enzyme study. Data presented

in Table 20 indicate that culture #8 cell-free broth showed higher CO₂ production, while *V. alcalescens* did not produce higher gas from coal than in control. On the other hand, washed cells exhibited very low enzyme activity with little increase in CO₂ in the presence of coal. The cells were washed to make sure that no loosely bound extracellular enzyme remained on the surface of the cells. These preliminary results are not conclusive, but adapted culture, i.e., culture #8, seems to have extracellular coal decarboxylating activity.

Table 19. Comparison of % Net CO₂ Production from Coal by Two Known Cultures and Six New Isolates and a Mixed Culture

Culture	On Day 1	On Day 5	On Day 10
<i>P. acidipropionici</i>	-0.07	0.22	0.19
<i>V. alcalescens</i>	-0.05	2.18	2.24
#1	0.28	0.15	0.16
#8	0.74	1.01	1.17
#9	0.13	-1.33	-0.65
NHC	-0.05	0.32	0.71
LC	0.26	0.98	1.04
MIX*	0.24	0.41	0.51

(ii) Culture Grown in the Presence of Coal

To examine the localization of enzyme(s) responsible for decarboxylation of coal, the culture #8 was grown in the presence of 5% Wyodak coal. From this, three preparations were made to examine the possible nature of the enzyme, i.e., extracellular, cell-bound, and membrane bound or intracellular. The three preparation were: cell-free broth (I), cell-free broth (II) and washed cells. The cell-free broth (I) was obtained by centrifuging the culture broth containing coal followed by filtration of the supernatant through a 0.22 μ millipore filter. Cell-free broth (II) was obtained by suspending the pellet containing cells and coal in phosphate buffer (pH 7) and shaking or sonicating it for 5 min to release cell bound enzymes(s). The mixture was centrifuged and the supernatant passed through the 0.22 μ millipore filter to obtain a cell-free broth (II) preparation. The remaining cell pellet was considered as washed cells preparation. Coal decarboxylation was examined in the presence of these three preparations in anaerobic tubes (Table 21). After an incubation for 16 hr, the gas phase analysis in comparison to control (no coal) showed net CO₂ production only in tubes containing cell-free broth (I). This observation indicated that the enzyme responsible for production of CO₂ from coal is likely to be extracellular in nature. To confirm this observation further, this experiment was repeated using only isolate #8.

Table 20. Decarboxylase Activities in Cell-free Broth and Washed Cells Prepared from *V. alcalescens* and Isolate #8

Organism	Enzyme Mixture	% CO ₂ Produced at 16 Hrs	
		Total	Net*
<i>V. alcalescens</i>	Control Coal	0.11	
	Cell-free Broth	2.49	
	Cell-free Broth + Coal	2.10	-0.50
	Washed Cells	0.07	
	Washed Cells + Coal	0.23	0.05
Isolate 8	Control Coal	0.10	
	Cell-free Broth	3.39	
	Cell-free Broth + Coal	4.54	1.04
	Washed Cells	0.0	
	Washed Cells + Coal	0.11	0.01

*Net CO₂ was calculated by subtracting control.

Table 21. Decarboxylase Activities in Cell-free Broth (I), Cell-free Broth (II) and Washed Cells Prepared from Isolate #8

Enzyme Mixture	% CO ₂ Produced at 16 Hrs	
	Total	Net*
Control Coal	0.33	
Cell-free Broth (I)	1.98	
Cell-free Broth (I) + Coal	2.86	0.55
Cell-free Broth (II)	0.51	
Cell-free Broth (II) + Coal	0.70	-0.14
Washed Cells	0.14	
Washed Cells + Coal	0.29	-0.18

*Net CO₂ was calculated by subtracting control.

Again, only supernatant (I) showed high decarboxylase activity. Any decarboxylases contained in supernatant (II) might have been very sensitive to sonification treatment and/or

be denatured after releasing from the cell which might result in no CO₂ production from coal. Therefore, supernatant(II) and intracellular decarboxylases require further investigation under well defined enzyme reaction conditions to achieve optimum enzyme activities. The presence of coal in the culture medium appears to reduce extracellular decarboxylase activity in comparison to the supernatant obtained from culture grown in the absence of coal.

2. Evaluation of Commercial Decarboxylase Enzyme

Our preliminary crude enzyme study showed that the decarboxylase(s) present in the microbial consortia that decarboxylates coal may be extracellular in nature. Therefore, attempts were made to investigate the coal decarboxylation potential of commercially available decarboxylase enzyme. First of all, phenylalanine decarboxylase was examined for coal decarboxylation. Enzymatic activity was assayed by determining carbon dioxide in the head space of the reaction vial. The final concentrations of phenylalanine, pyridoxal phosphate, coal, and enzyme in a reaction mixture were 1.6×10^{-2} M, 7×10^{-5} M, 33.3 mg/ml and 5 mg/ml, respectively. The pH was maintained at 5.5 using 0.09 M succinate buffer solution. The enzyme reaction mixture was incubated at 37°C. The data as presented in Table 22, indicate that no coal decarboxylation activity was observed in a short incubation time, whereas low activity was detected at 16 hr. It should be noted that almost all the commercially available decarboxylases are intracellular enzymes and substrate specific. Therefore, it seems unlikely that these would result in significant coal decarboxylation. However, additional decarboxylases need to be tested before any conclusions can be drawn.

**Table 22. Coal Decarboxylation Using Phenylalanine Decarboxylase
(% CO₂ in the headspace of reaction vial)**

Reaction Time	Control (enzyme only)	Enzyme + Phenylalanine	Enzyme + Coal	Net CO ₂ Production from Coal*
10 min	0.16	0.99	0.14	-0.02
30 min	0.20	2.03	0.20	0
16 hrs	4.04	8.91	4.35	0.31

*No statistical analyses were conducted.

Decarboxylation of Other Coals and Model Compounds

A. Lignite:

One of the important questions to ask is whether these microbial consortia developed with Wyodak subbituminous coal will be equally effective in removing oxygen from other coals. For this purpose, Beulah lignite was obtained from Argonne Premium Coal Sample Program. Microbial consortium RW9, which was developed and adapted on Wyodak subbituminous coal was examined for its ability to remove oxygen from lignite. Experiment

was conducted in anaerobic serum vials. Figure 7 represents the profiles of gas production from two low-rank coals that were nearly identical during the first 21 days, but after 21 days, lignite exhibited rapid increase in gas production, mainly due to methane production. On day 42, lignite produced about 0.4 atm more gas than subbituminous coal. These results have shown that the consortium RW9 is effective in removing oxygen from other low rank coals such as Beulah lignite.

B. Penn State Wyodak Coal (20 mesh)

In this project, mainly, 100 mesh Wyodak coal (Argonne Premium Coal Sample Program) was used to develop the microbial consortia and to examine their decarboxylation potential. However, to examine the utility of these microbial consortia on coals with larger particle size, a 20 mesh Wyodak coal was obtained from Penn State Coal Bank. Penn State Wyodak coal was examined for coal decarboxylation using microbial consortia #34 and LC in anaerobic vials (Figure 8). The gas production profiles from Penn State Wyodak coal was almost identical to that obtained from Wyodak coal (100 mesh; Argonne Coal Sample Program) when inoculated with the same microbial consortium. This suggests that our adapted microbial consortia decarboxylated Wyodak coals of different particle sizes.

C. Demineralized coal:

Demineralized coal obtained from Dr. Michael Serio (Advanced Fuel Research, East Hartford, CT) was examined for decarboxylation using two different microbial consortia (RW9 and LC5). Demineralized coal showed higher gas production with consortium RW9 than consortium LC5 (Figure 9). However, in comparison to control (total gas production, 0.8-1.0 atm on day 42), 60-80 % reduction in gas production was observed with demineralized coal. The pH (6.2-6.3) of culture media containing demineralized coal was lower than the pH (6.8-7.1) of untreated coal. Since demineralized coal contains high levels of free -COOH groups, these might have reduced the buffering capacity of culture medium, resulting in pH shift of the medium. Methanogens are known to be sensitive to low pH (ca. pH 6), therefore, low medium pH due to demineralized coal might have caused severe reduction in gas production, especially CH₄. Other possible explanation may be lack of some essential metal ions and trace elements in demineralized coal for growth of certain bacteria in microbial consortium.

D. Activated Carbon

Decarboxylation experiment using activated carbon was done in order to (i) find out if there is really decarboxylation of coal, (ii) to provide increased surface area, and (iii) to provide carbon for carbon dioxide formation. Accordingly, we initiated the experiment using activated carbon (100 mesh, Aldrich Chemical Cat.#: 24,2276) as blanks for coal decarboxylation in anaerobic tubes. Two microbial consortia, RW71C-5B and #34C-2, were used as the inoculum @ 5%. To PBB medium, 0.2% of sodium succinate and 0.1% of yeast extract were added in the presence or absence of coal or activated carbon. Tubes containing no coal and no activated carbon were used as controls after inoculation with the consortium. As seen in Figures 10 and 11, the gas production from tubes containing activated carbon was significantly lower than control (no coal) and the coal containing microbial consortia

Figure 7. Gas production profiles from Beulah lignite and Wyodak subbituminous coal by microbial consortium RW9

Figure 8. Gas production profiles from 20 mesh coal by two adapted microbial consortia in vials

Figure 9. Gas production profiles from medium containing 5% demineralized Wyodak coal with two different microbial consortia

Figure 10. Profiles of gas production from coal and activated carbon in PBB medium containing 0.2% sodium succinate and 0.1% yeast extract using microbial consortium RW71C-5B

Figure 11. Profiles of gas production from coal and activated carbon in PBB medium containing 0.2% sodium succinate and 0.1% yeast extract using microbial consortium #34C-2

throughout the biotreatment period. Highest net gas production with microbial consortium #34 was achieved on day 14, while with consortium RW on day 28. These results also indicate that these two consortia probably have distinct populations and, therefore, exhibit differences in the time required for decarboxylation activity. A second set of experiment using acid (3N HCl) washed activated carbon also showed similar results (Figures 12 and 13). These results show that these microbial consortia produced carbon dioxide from coal probably by decarboxylation as almost insignificant amount of carbon dioxide was produced from inert substances such as activated carbon.

E. Model Polymers

In order to better understand coal decarboxylation mechanism(s), it was necessary to use model compounds with carboxyl groups which have similar physicochemical properties to coal. Two model polymers, poly (acrylic acid) and poly (methyl methacrylate) (PMMA), were procured to examine the decarboxylation ability of the microbial consortia. Data presented in Table 23 indicate that two model polymers inhibited gas production in comparison to control with no polymer. This suggests that model polymer probably inhibited some population of the microbial consortia, especially methanogens since inhibition of CO₂ production was less than methane production in the presence of polymers. Microbial consortium RW9 was inhibited less by poly (acrylic acid) than PMMA, whereas consortium LC5 was inhibited more by poly(acrylic acid) than PMMA. This suggests that each microbial consortium probably has different microbial composition.

Table 23. Decarboxylation of Model Polymers with Two Anaerobic Microbial Consortia RW9 and LC5 for 7 Days.

Polymer	% CO ₂	% CH ₄	% Total Gas
<i>Polyacrylic Acid</i>			
Control with RW9	8.50	19.8	28.30
Polymer with RW9	7.66	7.71	15.37
Control with LC5	10.76	10.34	20.10
Polymer with LC5	2.50	0.77	3.27
<i>PMMA</i>			
Control with RW9	7.46	10.08	17.54
Polymer with RW9	4.15	1.10	5.25
Control with LC5	11.68	17.01	28.69
Polymer with LC5	8.58	14.80	23.38

These initial attempts using unmodified model polymers were not successful because the total gas production from cultures containing polymer was less than control. One of the reasons could be high initial concentration (5%) of polyacrylic acid and PMMA.

Figure 12. Profiles of gas production from coal and HCl washed activated carbon in PBB medium containing 0.2% sodium succinate and 0.1% yeast extract using microbial consortium RW71C-5B

Figure 13. Profiles of gas production from coal and HCl-washed activated carbon in PBB medium containing 0.2% sodium succinate and 0.1% yeast extract using microbial consortium #34C-2

Subsequently, in order to elucidate the coal decarboxylation mechanism(s) by anaerobic microbial consortium, PMMA was partially hydrolyzed as described elsewhere (Biermann and Narayan, 1987). Control PMMA was powdered using acetone as dissolving solvent. The partially hydrolyzed poly (methyl methacrylate), PMMA, and polyacrylic acid were decarboxylated using the consortium RW11. PMMA and its derivatives were added at 2% in anaerobic tubes. All samples were prepared in duplicate. As presented in Table 24, gas production from polymer and sodium succinate decreased in the presence of either polymer, except one condition where unhydrolyzed PMMA was used. Surprisingly, gas production from the partially hydrolyzed PMMA was greatly increased by adding 0.4% of sodium succinate. However, in the presence of 0.5 - 5% polyacrylic acid, gas production was severely decreased under all experimental conditions, indicating severe inhibition of methanogenic bacteria and decarboxylating organisms by polyacrylic acid.

Table 24. Decarboxylation of Partially Hydrolyzed Model Polymeric Compounds Using Coal Adapted Consortium RW11

Polymer Sample	% Net CO ₂	% Net CH ₄	% Total Gas from Polymer
<i>PMMA*</i>			
PMMA (0)	1.4	2.0	3.4
PMMA (0) + Succinate	-2.8	-2.5	-5.3
PMMA (24)	-0.8	-3.0	-3.8
PMMA (24) + Succinate	3.4	44.0	47.4
PMMA (48)	-0.4	-1.8	-2.2
PMMA (48) + Succinate	0.4	27.2	27.6
<i>Polyacrylate (PA)</i>			
0.05 g PA	-1.0	-21.8	-22.8
0.05 g PA + Succinate	-2.4	-29.9	-32.3
0.1 g PA	-1.4	-23.0	-24.4
0.1 g PA + Succinate	-2.4	-33.7	-36.1
0.2 g PA	-1.4	-29.6	-31.0
0.2 g PA + Succinate	-1.2	-50.9	-52.1
0.3 g PA	-0.3	-28.1	-28.4
0.3 g PA + Succinate	0.1	-41.7	-41.6
0.5 g PA	1.1	-29.0	-27.9
0.5 g PA + Succinate	-1.7	-47.3	-49.0

*Values in () followed by PMMA represent hours of partial hydrolysis of PMMA.

Coal decarboxylation by Microbial Consortium under Batch Fermentation System

A. Development of Batch Fermentation System

The initial biotreatment of coal under batch fermentation #1 was not successful either due to lack of some nutrients, significant oxygenation during long period operation without a N₂ sparge, or because of inhibition of microorganisms by some unknown factors associated with coal.

To examine if the failure of batch #1 was due to oxygenation of the system, a leak was introduced into the batch fermentation #2 when CO₂ production had begun. Figure 14 exhibits the profile of CO₂ production in batch fermentor system #2 containing 5% coal as sole carbon and energy source. The fermentor was inoculated with a mixed inoculum from rumen R2 and waste digester # 1B. As can be seen, CO₂ production was affected adversely when the air leaks were introduced. This confirmed that coal decarboxylation is possible only under anaerobic conditions. This conclusion is further supported by the FT-IR analysis of coal from fermentor system #2 (please see Table 30).

The batch fermentation configuration was, therefore, modified as illustrated in Figure 2 so as to operate it over a long period under anaerobic conditions. CO₂ produced was continuously stripped out by N₂ gas and trapped in an alkaline solution as described under experimental procedure section. The level of CO₂ produced from batch #3 remained very low (Table 25), probably due to limited biomass development since no readily available carbon and energy source was supplied. Volatile fatty acids and alcohols were produced and consumed probably to produce CH₄ (Figure 15). Since the available carbon and nitrogen sources in the fermentor system #3 were found to be limited, the batch system #4 was supplemented with 0.2% sodium succinate as supplemental carbon and 0.2% yeast extract to meet, if any, complex nutritional requirements. The purpose was to increase the biomass and the decarboxylation activity. As expected, the #4 batch fermentor system produced significant quantity of CO₂ (Table 25) and also showed high microbial population when examined microscopically. Batch fermentation system #4 and #5 (Figures 16 and 17) also showed similar overall profiles of production and conversion of acids and alcohols.

Table 25. Total CO₂ Production from Coal (Batch Fermentation System #3 and 4)

Batch No.	Coal Size (mesh)	Sodium Succinate Addition	Gas Phase	Total CO ₂ Produced (μmoles)
3	100	-	N ₂	5.1
4	100	+	N ₂	33.6

It was evident from previous experiments that periodic withdrawal of homogeneous uniform coal samples from batch reactors was difficult mainly due to insoluble nature of

Figure 14. Effect of oxygen on carbon dioxide production from 5% coal in the fermentor #2

Figure 15. Changes of volatile acid and alcohol contents in the culture broth of #3 anaerobic fermentation system

Figure 16. Changes of volatile acid and alcohol contents in the culture broth of #4 anaerobic fermentation system

Figure 17. Changes of volatile acids and alcohols in the culture broth of #5 anaerobic fermentation system.

coal, attachment of coal to glass vessel, tubings, heater, and pH probes and possible separation of coal as a result of differences in the density due to particle size. Thus, the results obtained from such samples were likely to vary and lead to erroneous conclusions. Therefore, two batch systems were set up to examine if the periodic sampling could be a cause for variations in results. Samples were to be withdrawn every week from one fermentor (#5) while another (#6) was to be operated undisturbed. At the end of about seven weeks, the results indicated that 20% more gas was produced from the undisturbed fermentor (Table 26). This difference, however, could be explained on the basis of withdrawal of about 48 ml sample of broth containing coal. Methane was detected in small amounts (< 0.01%) in the exit gas of both the fermentors. Changes in coal were studied by CHN and thermogravimetric analysis and the results are discussed under characterization of biotreated coal subhead.

Table 26. Total CO₂ Production from Coal (Batch Fermentor System #5 and 6)

Batch No.	Coal Size (mesh)	Sodium Succinate Addition	Gas Phase	Samples Withdrawn	Total CO ₂ Produced (μmoles)
5	100	+	N ₂	Yes	26.2
6	100	+	N ₂	No	32.1

2. Coal Decarboxylation using Fresh Rumen Inoculum

Fresh rumen fluid was obtained from a dairy farm of Michigan State University. Two batch fermentor systems were started using the rumen inoculum to obtain microbial consortia and to decarboxylate coal in PBB medium containing 0.05% yeast extract. Nitrogen gas was purged through #7 fermentor while a mixture of 5% hydrogen and 95% of nitrogen gas was purged through #8 fermentor to minimize possible loss of hydrogen during the decarboxylation of coal and to find any beneficial effects of hydrogen enrichment on the quality of biotreated coals. When compared to adapted inoculum in terms of total gas production, rumen fluid itself was not found to be a good inoculum for coal decarboxylation. The presence of 5% hydrogen gas in batch #8 did not stimulate the unadapted rumen bacteria and resulted in reduced carbon dioxide production in comparison to batch #7 (Table 27). This could be due to formation of methane by methanogens present in fresh rumen inoculum using carbon dioxide and hydrogen. Since the rumen fluid was found to contain 3.84% of dry matter, it is expected that elemental composition of rumen inoculum could effect the final elemental composition of biotreated coals. Therefore, we analyzed the CHN contents of dried rumen inoculum for C(41.55%), H(6.02%), and N(4.98%) which showed about 5 times higher level of nitrogen than that of coal. These results indicate that increase in final nitrogen content of the biotreated coal is mainly due to rumen inoculum because it was difficult to remove the rumen solid particles from the biotreated coal before analysis.

Table 27. Total CO₂ Production from Coal (Batch Fermentor Systems #7 and 8)

Batch No.	Coal Size (mesh)	Sodium Succinate Addition	Gas Phase	Inoculum	Total CO ₂ Produced (μmoles)
7	100	-	N ₂	Fresh rumen fluid	21.5
8	100	-	N ₂ + H ₂ (95+5)	Fresh rumen fluid	14.8

3. Coal Decarboxylation as affected by fermentor gas phase and coal particle size

Newly developed consortium LC4 was inoculated in batch #13 and #14 systems containing different coal particle size (100 mesh versus 20 mesh). The consortium LC was developed by successive enrichment of rumen inoculum on sodium lactate as carbon source and NH₄Cl as nitrogen source. Wyodak subbituminous coal (100 mesh) obtained from Argonne Premium Coal Sample Program was added in fermentor #13 whereas Wyodak coal (20 mesh) obtained from Penn State Coal Bank Program was added to fermentor #14. About 10 μmoles more carbon dioxide was produced from 20 mesh Penn State Wyodak coal than 100 mesh coal (Table 28).

Table 28. Total CO₂ Production from Coal (Batch Fermentor Systems #12 thru 15)

Batch No.	Coal Size (mesh)	Sodium Succinate Addition	Gas Phase	Inoculum	Total CO ₂ Produced (μmoles)
12	100	-	N ₂	-	0 (control)
13	100	+	N ₂	LC4	29.9
14	20	+	N ₂	LC4	39.2
15	20	+	N ₂	-	0 (control)

4. Coal Decarboxylation under Presumptive Optimum Conditions

Three new batch fermentor systems containing consortium RW were initiated under assumed optimum conditions for coal decarboxylation. The optimum conditions were presumed on the basis of previously run batch systems. Microbial consortium RW seemed to be potentially a good microbial consortium because of its stability during the maintenance/subculturing over 16 months and reproducible coal decarboxylation results. Hydrogen gas at 5% is used in fermentor #18 since our previous observations indicated that the bioprocessing of coal under hydrogen gas phase resulted in coal products with higher H/C ratio. Antimethanogenic agent (2-bromoethanesulfonic acid) was used in batch fermentor #17

to inhibit methanogenic bacteria. As presented in Figure 18, the total carbon dioxide production on day 51 was 11.2, 18.4, and 26.9 μ moles from #16, #17 and #18 batch systems, respectively. Increased amounts of carbon dioxide were obtained (Table 29) when coal was anaerobically biotreated under H_2 gas phase system (fermentor #18) than under N_2 gas phase (fermentor #16). Highest methane concentration was detected in the exit gas from fermentor #16 during the first week of biotreatment period, whereas low levels of methane were detected in the exit gas from fermentor #18. No methane gas was detected from fermentor #17, indicating complete inhibition of methanogenic bacteria by 2-bromoethanesulfonic acid.

Table 29. Total CO_2 Production from Coal (Batch Fermentor Systems #16 thru 18)

Batch No.	Coal Size (mesh)	Sodium Succinate Addition	Gas Phase	BES Addition	Total CO_2 Produced (μ moles)
16	100	+	N_2	-	11.2
17	100	+	N_2	+	18.4
18	100	+	$N_2 + H_2$ (95+5)	-	26.9

Characterization of Biotreated Coal

Biotreated as well as control (untreated) coals were characterized for loss of carboxyl groups (by FT-IR spectroscopy and titration method), elemental composition (using CHN analyzer), for evolution of volatiles (using Thermogravimetric analyzer) and for solvent solubles. The results obtained using these techniques are discussed as under:

A. Carboxyl groups

(i) FT-IR Spectroscopy

Biotreated coals from fermentors as well as tubes were washed with 3N HCl to remove biomass and then methylated for FT-IR analysis. The results for C=O as well as C-O for control coal, for coal treated in tubes using inocula from rumen R2 and waste digester #1B, and for coal treated in fermentor system #2 are presented in Table 30. Inoculum from rumen resulted in higher loss of coal carboxyl groups than the inoculum from waste digester #1B. Of the two supplemental carbon sources used, benzoate was found to be a better carbon source for coal decarboxylating organisms. However, significant amount of coal decarboxylation, as confirmed by the relative loss of carboxyl groups, was also achieved without any supplemental carbon source.

Interestingly, oxidized coal from batch fermentor system #2 exhibited a slight increase in carboxyl groups and a significant increase in carbonyl groups, confirming that the decarboxylation (loss of carboxyl groups and resultant CO_2) is possible only under anaerobic conditions.

Figure 18. Profiles of carbon dioxide production from batch fermentor systems (#16 - #18) containing 100 mesh Wyodak coal

Table 30. FT-IR Analysis of Methylated Biocoals

Sample	Wave Number Range	Peak Area	% Gain (+)/ Loss (-)
<i>C = O Region</i>			
Coal without inoculum	1782.5-1679.8	2.23×10^4	-
Coal + inoculum (R2)	1788.0-1678.8	2.31×10^4	-
Coal from #2 Fermentor (3 wk)	1783.4-1679.5	2.36×10^4	+2.2
Coal from #2 Fermentor (4 wk)	1786.0-1680.0	2.48×10^4	+7.4
Coal from Vial with WD1B	1779.2-1680.5	1.99×10^4	-10.8
Control Coal (coal + 2 x R2)*		2.39×10^4	-
Coal from Tube, + 2 x R2 (5 wk)	1779.7-1680.9	1.98×10^4	-17.2
Coal from Tube, + 2 x R2 + Succinate (5 wk)	1779.3-1679.4	2.15×10^4	-10.0
Coal from Tube, + 2 x R2 + Benzoate (5 wk)	1782.1-1680.0	1.87×10^4	-21.8
<i>C - O Region</i>			
Coal without inoculum	1389.8-1129.2	4.78×10^4	-
Coal + inoculum (R2)	1393.0-1128.2	5.26×10^4	-
Coal from #2 Fermentor (3 wk)	1391.1-1132.1	5.79×10^4	+10.1
Coal from #2 Fermentor (4 wk)	1389.5-1129.2	6.11×10^4	-16.2
Coal from Vial with WD1B	1391.2-1131.7	4.00×10^4	
Control Coal (coal + 2 x R2)*		5.74×10^4	-
Coal from Tube, + 2 x R2 (5 wk)	1392.5-1130.6	4.72×10^4	-17.8
Coal from Tube, + 2 x R2 + Succinate (5 wk)	1393.8-1132.8	5.30×10^4	-7.7
Coal from Tube, + 2 x R2 + Benzoate (5 wk)	1391.0-1130.6	4.77×10^4	-16.9

*Calculated from the result of (coal + R2)

The peak areas in FT-IR were reproducible, however, methylation reaction of coal was observed to be very sensitive to the nature of sample and reaction conditions (pH, temperature, ratio of NaOH to dimethyl sulfate) which frequently caused some problems in interpreting FT-IR data due to variations.

(ii) Titration method

The data presented in Table 31 confirmed the loss of -COOH groups in biotreated coals by a titration method (Blom, et. al., 1957). The method involved acid washing of coal to remove cations from carboxyl groups and then adding calcium acetate to chelate calcium ions with ionized carboxylic groups. The released acetic acid was titrated with 0.02 N NaOH solution. Blom, et. al., however, pointed that this method may have some shortcomings due to limited accessibility of chemical agent to coal macromolecular structure and the size effect of coal particles.

Table 31. Reduction of Carboxylic Groups from Coal by Anaerobic Biotreatment

Batch No.	% Loss of -COOH Groups
3	9.6
4	6.0
5	7.6
6	8.6
7	15.0
8	15.3

B. Hydrogen: carbon ratio

Carbon, hydrogen and nitrogen analysis of the biotreated as well as control coal samples was performed to determine the hydrogen to carbon (H/C) ratio. The results presented in Table 32 indicate that biotreated coals in the fermentor systems had an increase in H/C ratio from 1.0 to 6.8%. The relative change in H/C ratio in coal treated in vials was very less (Table 33). There was lot of CH₄ production in these tubes because of the presence of methanogens in the microbial consortia. Also CO₂ was readily available, in addition to acetate, for methane production in the closed tube systems. It is likely that coal hydrogen might have been lost in CH₄ production, since this is thermodynamically a favorable reaction:

Thus, it seems necessary that methane production be minimized so as to maximize H/C ratio in biotreated coal.

Table 32. Elemental Analysis of Biotreated Coal using CHN Analyzer

Batch System	% C	% H	% N	H/C Ratio	Increase in H/C Ratio
Control Coal (100 mesh)	68.59	5.06	1.05	0.885	-
3	68.19	5.11	0.59	0.899	1.58
4	68.72	5.12	1.42	0.894	1.02
5	69.38	5.18	1.14	0.896	1.24
6	69.48	5.36	1.30	0.925	4.52
7	68.61	5.27	1.20	0.922	4.18
8	68.31	5.28	1.24	0.928	4.86
13	64.66	4.92	1.17	0.913	3.16
Control Coal (20 mesh)	62.85	4.33	0.90	0.827	-
14	59.78	4.40	1.23	0.883	6.77
Dried Rumen Fluid	42.61	6.77	5.56	1.906	-

Table 33. Ratio Between Hydrogen and Carbon Content of the Coal Biotreated as Affected by Hydrogen

Batch System	Gas Phase	% C	% H	H/C Ratio	Increase in H/C/ Ratio
Control	-	64.94	4.86	0.898	-
#16	N ₂	61.68	4.77	0.929	3.45
#17 ^a	N ₂	60.70	4.71	0.909	1.22
#18	N ₂ :H ₂ (95:5)	61.45	4.89	0.955	6.35

^a2-Bromoethanesulfonic acid was added to inhibit methanogenesis.

It is noted that elemental composition of rumen inoculum could effect the final elemental composition of biotreated coals, since the rumen fluid was found to contain 3.84% of dry matter. Therefore, we analyzed the CHN contents of dried rumen inoculum. It showed about 5 times higher level of nitrogen than that of coal.

As seen in Table 32, mixed gas environment gave a slight higher H/C ratio than only nitrogen gas environment, suggesting possible beneficiary effect of 5% hydrogen gas on coal quality during anaerobic biotreatment. Therefore, two additional batch fermentor systems, #16 and #18, containing 5% coal, 2% sodium succinate and 0.1% yeast extract were completed under the same gas phase conditions used in #7 and #8, respectively, to verify this result and to maximize the coal decarboxylation using an adapted microbial consortium (RW71C-5A) instead of rumen inoculum.

Wyodak subbituminous coals of different particle size were decarboxylated in batch configuration systems for 56 days where microbial consortium LC was used with 0.4% sodium lactate and 0.2% ammonium chloride. 100 mesh and 20 mesh Wyodak coals were used for #13 and #14 batch system, respectively. The H/C ratio of biotreated 20 mesh Penn State Wyodak coal increased by about 6.8%. The results for fermentor #16 and #18 indicated a 3-6% increase in the H/C ratio (Table 33) confirming again increased H/C ratio in coal treated in the presence of 5% H₂ gas.

C. Evolution of volatiles

Thermogravimetric (TG) analysis was performed to determine any impact that anaerobic bioprocessing would have on evolution of volatiles from biotreated coals. One of our hypothesis (refer to our proposal) was that carboxyl groups are involved in hydrogen bonding and chelate cross-linking; and because decarboxylation occurs at only elevated temperature, the polymer chain can not escape from the matrix before undergoing retrogressive condensation reaction, i.e., there is a competition between evolution of polymer chains from the matrix and the retrogressive reaction, and the equilibrium always occurs towards retrogressive reaction. Elimination of carboxyl groups would allow the polymer chain to pull off the matrix without undergoing significant retrogressive reaction.

As presented in Table 34, eight out of eleven coal samples showed increases in VC/FC ratio by anaerobic biotreatment in the batch fermentor system. These results appear to support and confirm the hypothesis mentioned above. At this stage, we cannot confirm that the increased volatilization of the biotreated coals is due to decarboxylation or other enzymatic reaction(s). TG analysis of non-acid washed coals was also conducted. The main difference in TG analytical results between acid washed and non-acid washed coals was in the ash content. The acid treated coals showed up to 60% removal of ash content in biotreated coals.

D. Solvent Solubles

Two organic solvents, THF and toluene, were used to determine the change of solvent solubilities of coal product after biotreatment. Generally THF-soluble coal products were greatly increased by anaerobic biotreatment, while toluene-solubles were not changed to a great extent (Tables 35 and 36). The highest increase in THF solubles was absorbed in #13 batch system where the consortium LC-4C3 was inoculated in coal medium containing 1.6 g of sodium succinate and 0.8 g of ammonium chloride. Increases in toluene-solubles were observed in only two batch systems of #7 and #13. These results indicate that anaerobic

biotreatment can change the property of coal in terms of solvent solubility which would provide a better feedstock toward coal liquefaction.

Table 34. TG Analysis of Biotreated Coals in Batch Fermentor Systems

Sample	% Moisture	% Volatile Carbon (VC)	% Fixed Carbon (FC)	% Ash	VC/FC Ratio	% Change in VC/FC Ratio
Control Coal	1.14	47.18	43.53	8.15	1.084	-
#3	6.19	48.97	37.89	6.95	1.292	19.24
#4	0.16	49.69	41.07	9.08	1.210	11.62
Control Coal	0.54	46.60	43.18	9.68	1.079	-
#5	4.56	47.66	38.94	8.84	1.224	13.42
#6	4.24	45.70	41.25	8.81	1.108	2.67
#7	1.22	45.17	43.07	10.54	1.049	-2.8
#8	1.10	49.46	40.48	8.96	1.222	13.2
Control Coal	22.13	34.61	35.90	7.36	0.964	-
#13	3.81	41.49	40.96	13.74	1.013	5.1
Control Coal (20 mesh)	26.64	31.13	32.45	9.78	0.959	-
#14	4.08	39.01	40.72	16.19	0.958	-0.12
Control Coal	27.68	33.04	33.65	5.63	0.982	-
#16	5.52	41.15	41.30	12.03	0.996	1.5
#17	4.87	41.95	40.20	12.98	1.044	6.3
#18	4.99	40.28	41.33	13.40	0.975	-0.8

E. Particle Size of Biotreated Coal

We observed an unexpected reduction in the coal particle size during the anaerobic biotreatment. To further examine this phenomenon, two microbial consortia were compared in tubes with uninoculated coal (100 mesh) as control. After 6 weeks of treatment there were clear differences in the sedimentation profiles of biotreated coals and control coal. Control coal showed clear separation of coal particle from the supernatant, while biotreated coal showed very slow separation of coal particles even after 24 hr. Only a small fraction of the clear supernatant liquid was observed at the top of the culture broth in the biotreated cultures. This phenomena was more remarkably observed when 20 mesh coal (from Penn State Coal

Sample Program) was biotreated for 42 days. These results strongly suggest that there is a significant particle size reduction of coal during anaerobic bioprocessing.

Table 35. THF Extracts of Biotreated Coals from Batch Fermentation System

Batch No.	THF Solubles (%)	Increase Over Control (%)
Control	3.77	-
3	4.84	28.4
4	5.43	44.0
5	5.57	47.7
6	4.10	8.8
7	5.92	57.0
8	5.82	54.4
10	5.08	34.7
11	5.30	40.6
13	6.44	70.8
17	4.30	14.1
18	5.24	39.0

Batch fermentation systems containing 20 g of coal were also monitored for reduction of coal particle size by microbial consortium in comparison to control coal without the inoculum. To minimize the mechanical grinding effect caused by the motion of a magnetic impeller shaft a magnetic bar stirring was provided at 120 rpm for batch fermentor. Constant stream of N₂ gas was provided to ensure anaerobic condition and to trap the evolved CO₂ from the coal. Figure 19 presents the reduction of coal particles size from 100 mesh to ultrafine size. Particle size determinations were performed at DOE-PETC. The effect of slow agitating motion of a magnetic bar could minimally contribute to partial particle size reduction of control coal and biotreated coal as well. Progress reduction in coal particle size under batch fermentation system as observed under microscope is shown in Figure 20.

A patent application on coal comminution was filed with U.S. Patent office vide serial no. 08/016, 119.

Figure 19. Particle Size Distribution of 100 Mesh Control and Biotreated Coals

Figure 20. Progress Reduction of Coal Particle Size in Batch Fermentation System/Magnification (1000 X).
Biotreatment time: A = 0 hr; B = 1 week; C = 3 weeks

Table 36. Toluene Extracts of Biotreated Coals from Batch Fermentation System

Batch No.	Toluene Solubles (%)	Increase Over Control (%)
Control	1.75	-
3	1.61	-8.0
4	1.47	-16.0
5	1.52	-13.1
6	1.41	-19.4
7	1.78	+1.7
8	1.59	-9.1
10	1.58	-9.7
11	1.15	-34.3
13	1.98	+13.1
17	1.54	-12.0
18	1.33	-24.0

Material Balance

Based on the extensive analytical data, mass balance of elements of biotreated coal was conducted to evaluate the performance of coal bioprocessing by the adapted consortia. As an example, #6 batch fermentation system was chosen as presented in Table 37. The coal, on weight basis, was recovered to about 97.8% of initial weight. From the CHN analysis data, net carbon reduction was 10.53 μ moles, which was very close to actual experimental data (10.85 μ moles) of net CO₂ from coal determined by GC method. Thus, carbon recovery was 99%. However, this method requires very strict measurement of initial dry coal and final dry coal weights which may contribute to major errors to calculate mass balance of coal elements because relatively small fraction of carbon is converted to CO₂ gas and CH₄, by the microbial/enzymatic activities.

Table 37. Mass Balance of Coal Decarboxylated in the Fermentor System #6

Mass or Elemental Composition of Coal	Initial Coal (g)	Biotreated Coal (g)	Net Change	% Recovery
Coal Mass	20.0013	19.5546	-0.4467	97.8
Carbon	13.1889	13.0625	-10.53 μ moles	99.0
Hydrogen	0.92006	0.95231	+32.25 μ moles	103.5
Oxygen	3.63424	3.26757	-5.96 O ₂ μ moles	89.9

*Note: A total of 13.5 μ moles of hydrogen ion was added into the fermentor system to maintain the pH at 7.2

Coal Liquefaction

The liquefaction experiments were carried out at 400°C for 30 min with 6:1 tetrahydronaphtalene as the solvent. Table 38 shows the liquefaction yields for the untreated and the biotreated coal. The results do not show any dramatic increase in liquefaction yields although the decrease in CO₂ evolution during the liquefaction for the biotreated coal was clearly evident. The results also show a modest increase of the toluole solubles (about 7.7%) in the biotreated coal sample.

Liquefaction of the untreated and the biotreated coal samples conducted at DOE-PETC showed 88-90% yields. The liquefaction experimental conditions were too severe to differentiate the effects of the biotreatment. The fact that the coal network has been unravelled to some extent is shown by thermogravimetric analysis of the untreated and biotreated coals (Tables 31 and 32). Clearly, milder liquefaction conditions need to be employed and studied to exploit the biotreatment of the coals as an approach to enhance liquefaction yields.

Table 38. Liquefaction Yields (DAF %) of Untreated and Biotreated Coals

Coal	Toluole Solubles	Oils	Asphalt	CO ₂	CO	C ₂ H ₆	CH ₄
Untreated	37.7	23.9	13.8	3.88	0.29	0.07	0.05
Biotreated #13	40.6	25.7	14.9	0.55	0.30	0.07	0.11

Microbial transformations

During the anaerobic biotreatment of coal, the following potential biotransformation occurs:

As shown in Figure 1, decarboxylation should result in unravelling of the macromolecular network. The CO_2 production during the course of the anaerobic biotreatment seems to confirm this. The production of CO_2 could also be the result of reductive ring opening of a cyclohexanol type structure.

Proof for reduction of aromatic ring comes from the FT-IR studies of the biotreated coal (Table 39). The aromatic C-H stretching vibrations are reduced from 1.12 units to 0.7-0.9 units (about 20-35% reduction). The data also shows some reduction in peak heights of COOH and C=O in the biotreated coals in comparison to untreated coal.

During the course of the anaerobic processing of coal, methane was produced along with CO₂. The evolution of CH₄ could be explained on the following basis:

Table 39. Curve Resolved FT-IR Data of Untreated and Biotreated Coal

Coal	Free OH	Weak H-bonding ^a	Arom. py-H	COOH	C=O ^b
Untreated	0.02	1.12	1.10	0.047	0.247
Biotreated (#5)	0.04	0.81	0.72	0.042	0.208
Biotreated (#13)	0.00	0.96	0.90	0.039	0.251

^aO-dihydrox. water cluster.

^bC=O is the sum of the free and H-bonded or conjugated forms.

The required hydrogen probably comes from the coal, thus, limiting the increase in H/C ratio. An understanding of the biochemical pathways in coal biotransformations would help us to select for a specific desirable transformation. More work needs to be done in this area.

7. ACHIEVEMENTS OF MILESTONES AND REPORTING

A chart showing the completion of specific milestones is provided (Figure 21). During the contract period, monthly status reports, quarterly progress reports, annual progress reports and a final report were prepared and submitted as per the contract requirement.

U.S. DEPARTMENT OF ENERGY
MILESTONE SCHEDULE ☐ PLAN ☐ STATUS REPORTFORM APPROVED
OMB NO. 1901-1400

1. TITLE ANAEROBIC BIOPROCESSING OF LOW-RANK COALS										2. REPORTING PERIOD 09-12-90 -- 08-10-93										3. IDENTIFICATION NUMBER DE-AC-22-90PC90051																																															
4. PARTICIPANT NAME AND ADDRESS Michigan Biotechnology Institute 3900 Collins Road, P.O. Box 27609 Lansing, MI 48909										5. START DATE 09-12-90										6. COMPLETION DATE 08-10-93																																															
										7. ELEMENT CODE										8. REPORTING ELEMENT										9. DURATION										FY		FY		10. PERCENT COMPLETE																							
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11. SIGNATURE OF PARTICIPANT'S PROJECT MANAGER AND DATE										Mahesh Jain 8/5/93																																																									

9. PRESENTATIONS AND PUBLICATIONS

Presentations:

1. Jain, M. K., D. Burgdorf, and R. Narayan. 1990. Anaerobic Bioprocessing of Subbituminous Coal. Fuel Chemistry Division, ACS, August 26-31, 1990, Washington, D.C.
2. Jain, M. K., A. Lathia and R. Narayan. 1991. Decarboxylation of subbituminous coal under anaerobic conditions. Presented at the Second International Symposium on the Biological Processing of Coal. May 1-3, 1991, San Diego, California.
3. Han, O., R. Narayan, and M. K. Jain. 1992. Reductive Decarboxylation of Low-rank Coal by Mixed Microbial Cultures. Presented at the Third International Symposium on the Biological Processing of Coal. May 4-7, 1992, Clearwater Beach, Florida.
4. Narayan, R., O. Han, and M.K. Jain. 1993. Modification of Subbituminous Coal by Anaerobic Microbial Consortia. Presented at the American Chemical Society Spring Meeting, March 28-April 12, 1993, Denver, Colorado.

Publications:

1. Jain, M. K., Burgdorf, D. and Narayan, R. 1990. Anaerobic Bioprocessing of Subbituminous Coal. In: Fuel Chemistry Division Preprints, American Chemical Society, Washington, D.C., 35(3):830-838
2. Jain, M. K., Lathia, A. and R. Narayan. 1991. Decarboxylation of Subbituminous Coal under Anaerobic Conditions. Proceedings: 1991 Second International Symposium on the Biological Processing of Coal. p. 15-21, September, 1991, EPRI GS-7482.
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4. Han, O., Narayan, R. and M. K. Jain. 1992. Reductive Decarboxylation of Low-rank Coal by Mixed Microbial Cultures. Fuel (in preparation)
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10. ACKNOWLEDGMENTS

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