ADVANCED STUDIES OF BIOLOGICAL INDIRECT LIQUEFACTION OF COAL

Topical Report on Task 1: Culture Identification

DOE/PC/79813--T1

DE89 010012

Published: January 1989

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Prepared for

The United States Department of Energy
Pittsburgh Energy Technology Center
Contract No. DE-AC22-88PC79813

ABSTRACT

Culture identification and characterization studies carried out at the University of Arkansas and under contract to the University of Oklahoma, Department of Botany and Microbiology, have been essentially completed. The studies indicate that the organism is indeed a new clostridial strain, to be named Clostridium ljungdahlii, strain PETC, in honor of Dr. Lars G. Ljungdahl for his work on clostridia and acetogens. C. ljungdahlii is different from other clostridial strains and similar geni in its operating conditions and choice of substrates as sole carbon and energy sources. C. ljungdahlii, strain PETC, produces ethanol as a product only at low pH levels (5-6), with acetate the primary product at higher pH levels.

ACKNOWLEDGEMENT

Financial support for this work was provided by the United States Department of Energy, Pittsburgh Energy Technology Center, on Contract No. DE-AC22-88PC79813.

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1.0 INTRODUCTION

The United States has massive reserves of coal, which represents the nations largest fossil energy source, equivalent to about 750 billion barrels of oil, or a 300 year supply at the current rate of consumption (1). Subbituminous and lignite coals account for about 60 percent of this reserve, but contain less than 40 percent fixed carbon and have an energy content of 5500-8000 Btu per pound (2).

The easiest method of utilizing coal is by direct combustion with air to recover the energy content. However, the sulfur and ash content of much of this coal is so high that environmental regulations prevent its burning, or the energy content is so low that the transportation of the coal to power plants is economically prohibitive. Nevertheless, coal will be the major source of both fuel and chemicals for the future and these problems must be resolved.

Coal may be converted to a more convenient energy source and a valuable chemical feedstock by gasification and removing undesirable components. In most gasification processes, the coal is hydrogasified by adding steam and energy at 20-70 atm and at temperatures as high as 2730°C. A gas consisting of more than 50 percent hydrogen and carbon monoxide is usually produced, having a heating value of about 450 Btu per SCF. A typical range of synthesis gas compositions from several gasification processes is shown in Table 1.1. In addition to carbon monoxide (CO) and hydrogen (H_2), small amounts of carbon dioxide (CO_2), methane (CH_4), hydrogen sulfide (H_2S), and carbonyl sulfide (COS) are also produced.

Table 1.1

Typical Composition of Synthesis Gas*

Component	Percent of Total**
Hydrogen	25-35
Carbon Monoxide	43-65
Carbon Dioxide	1-20
Methane	0- 7
Sulfur as H_2S or COS	0- 1

* average of data taken from Westfield Lurgi gasifier, Shell Kopper gasifier, and Cool Water Gasification Project, Energy Progress, (1982)

** by volume

To upgrade synthesis gas, the low-Btu gas typically undergoes a catalytic shift reaction where carbon monoxide and water are converted to carbon dioxide and hydrogen. Following purification to remove carbon dioxide and hydrogen sulfide, the hydrogen and carbon monoxide are catalytically reacted to methane and water. The water is then removed to give a gas that is 95-98 volume percent methane, with an energy content of 980-1035 Btu per SCF.

The temperatures required in most methanation processes are 300-345°C at pressures of 2.7-17 atm (3). The Exxon process employs gasification and methanation in a single step at 700°C (4,5). Most catalysts used are nickel-based; however, the Exxon process utilizes a potassium-based catalyst (4,5). Poisons for these catalysts include chlorine and sulfur (6,7). Free carbon, nitrogen, and oxygen are also potential nickel-based catalyst poisons (8).

Liquid fuels such as alcohols and petrochemicals also may be produced from coal synthesis gas by indirect coal liquefaction. An example of this is the Mobil MTG Process (9). Other processes use the coal directly to produce a

liquid fuel, which can be used to produce such derivatives as ethylene, propylene and BTX (10). These processes require elevated temperatures and pressures, often as high as 750°C and 100 atm (9).

Because high temperatures and pressures are required in the catalytic gasification and liquefaction process, losses in thermal efficiency and high energy costs occur. Thus, processes employing lower temperatures and pressures for even a portion of the overall process scheme would be advantageous.

1.1 Microbial Coal Conversion

Microorganisms may be used to convert coal to chemicals either directly or indirectly by their action on synthesis gas. Microbial processes offer certain advantages over chemical conversions. Microorganisms exist and carry out conversions at ambient temperatures and pressures, which should result in substantial energy and equipment savings. Also, yields from microbial conversions are often as high as 95 percent, since the microorganism utilizes only a small fraction of the substrate for energy and growth. Under proper conditions, microbial conversions are quite specific, generally converting a substrate into a single product, with perhaps a few by-products. These advantages are offset by slower reaction rates and special reactor considerations, such as sterility, nutrient provision, etc.

1.1.1 Direct Coal Conversion

Direct microbial conversion involves the selection of a microorganism to produce liquid and gaseous fuels by direct biological action on coal, perhaps <u>in situ</u>. Although the prospects for success are limited, there is tremendous potential for this type of coal conversion process. Indicative of the problems associated with direct microbial action on coal is the apparent toxicity of liquefied coal products and wastewater streams from the coal

conversion processes (11-15). Also, it has been extremely difficult to get active flora to flourish on the ground cover composed of coal mine spoil or tailings (16,17).

Significant attention has been devoted to the microbial conversion of the inorganic (pyrite) fraction of sulfur in coal. Microorganisms such as Thiobacillus ferrooxidans, T. thiooxidans, and Sulfobus acidocaldarius have been found to remove as much as 96 percent of the pyritic sulfur in a five-day period (18-20). Many species of fungi and a few bacterial strains have been found to degrade lignin. These include Coriolus versicolor, Sporotrichum pulverulentium, Phanerochaete chrysosporium, Aspergillus fumigatus, Metulius tremellosus, and some species of Acetiomycetes and Eubacteria (21-24).

Some lignolytic fungi have been shown to be capable of degrading lignite. Polyporous versicolor and Poria monticola have been grown on lignite agar medium. Basic salts also are required and a liquid exudate containing various chemical products was obtained (25). Also, research at the University of Mississippi has isolated fungi from a natural lignite outcrop. These isolates have been shown to utilize lignite as a sole source of organic carbon and minerals. Some of these fungi have been identified as Mucor sp., Penicillium sp., Paecilomyces sp., and Candida sp. The isolation of these organisms for direct conversion of lignite is quite promising. However, as expected, the rates of conversion are extremely slow and the development of a commercial process will be difficult.

1.1.2 Indirect Coal Conversion

A more promising biological approach, perhaps, is the indirect coal conversion of raw synthesis gas by microorganisms capable of producing alcohols, acids, aldehydes, etc. from CO, CO_2, H_2, and H_2O. Therefore, a two step process is required; conventional coal gasification, followed by

biological liquefaction. Some of these biological reactions are well-known, especially those that produce organic acids. These reactions use anaerobic organisms and give reasonable rates of reaction, while often producing a single product so that purification systems are simplified. Near ambient conditions of temperature and pressure are used so that substantial capital and energy savings are possible. A cheaper source of synthesis gas can be used, since the problem of sulfur poisoning of the catalyst can be eliminated. The sulfur tolerance of microorganisms can often be developed or sulfur can be removed biologically, if necessary.

In 1986, a culture was isolated from animal waste in the University of Arkansas laboratories that was found to be capable of producing ethanol from CO, CO_2 and H_2 in synthesis gas (26). Preliminary identification studies indicated that the bacteria was a clostridial species, and formed both acetate and ethanol as products. The stoichiometry of CO utilization was found to be (27):

$$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + CO_2 \qquad (2.1)$$

$$\Delta G^\circ = -59.9\ \text{kcal/gmole}\ CH_3CH_2OH$$

and

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \qquad (2.2)$$

$$\Delta G^\circ = -37.8\ \text{kcal/gmole}\ CH_3COOH$$

Similarly, the stoichiometry for CO_2 and H_2 utilization was found to be (27):

$$2\ CO_2 + 6\ H_2 \rightarrow CH_3CH_2OH + 3\ H_2O \qquad (2.3)$$

$$\Delta G^\circ = -23.2\ \text{kcal/gmole}\ CH_3CH_2OH$$

and

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O \qquad (2.4)$$

$$\Delta G^\circ = -18.7\ \text{kcal/gmole}\ CH_3COOH$$

When the culture was first isolated, approximately 20 moles of acetate were produced for every mole of ethanol. By continually optimizing the culture, continuous reactor studies have shown the capability of producing 3 moles of ethanol per mole of acetate. Studies are presently underway to further improve the ratio of ethanol to acetate, making use of techniques that have been successfully applied to other clostridial species (27). Successfully employed techniques include the elimination of yeast extract as a nutrient, the addition of alternate growth substrates such as cellobiose to the liquid medium and the addition of reducing agents such as benzyl and methyl viologens to the liquid medium.

1.2 Purpose

Even though it was known that the newly isolated culture is a Clostridium, it was not known whether the isolate is a new bacteriol species or an existing species now found capable of utilizing CO, CO_2 and H_2 to produce ethanol. The purpose of this report is to present the results of culture identification and characterization studies aimed of determining whether or not the culture is a new species. A subcontract has been given to Dr. Ralph Tanner, Department of Botany and Microbiology, University of Oklahoma, to carry out the bulk of this work. Dr. Tanner is very familiar with clostridia and is well-qualified to perform the necessary tasks involved in culture identification and characterization. Prior to presenting the results of the above studies, a brief review of the genus Clostridium will be given.

2.0 THE GENUS CLOSTRIDIUM (28,29)

Members of the genus Clostridium are anaerobic, spore-forming bacilli. The natural habitats of clostridia are soil and the intestinal tracts of animals and man. A few of these saprobes are human pathogens under appropriate circumstances, causing the diseases botulism, tetanus and gas gangrene. The pathogenicity of these organisms depends upon the release of powerful exotoxins or highly destructive enzymes.

2.1 Morphology and Growth

Clostridia are relatively large pleomorphic, gram-positive, rod-shaped organisms. Filamentous forms are common. All clostridial species form spores, but the conditions required for sporulation are highly variable among species. The spores are highly refractile, and are oval or spherical and usually wider than the parent cell. The spores may be central, terminal or subterminal in the cell. Most species of clostridia possess peritrichous flagella and are motile. A few species (such as C. perfringens) are encapsulated. Colony forms are variable, and hemolysis on blood agar is frequent.

Sensitivity to free oxygen is a distinguishing characteristic of the genus Clostridium. For most clostridia, oxygen is not merely growth inhibitory, but lethal. Spores, however, are not killed by oxygen. The lethal effect of free oxygen on clostridia may have several mechanisms. In many cases, it results from the formation, by the bacteria themselves, of hydrogen peroxide, which is highly toxic. Most clostridia completely lack heme proteins, and are incapable of decomposing hydrogen peroxide. However, they have a high content of flavoproteins, with some acting as oxidases in the presence of oxygen. Consequently, the exposure of cells to oxygen (air) is followed by the rapid formation and intracellular accumulation of hydrogen peroxide, by the equation:

$$\text{Substrate} + O_2 \xrightarrow{\text{flavoproteins}} \text{oxidized substrate} + H_2O_2 \qquad (2.5)$$

Not all clostridia are equally sensitive to the presence of oxygen. C. tetani, the causative agent of tetanus, requires strict anaerobic conditions. C. perfringens, on the other hand, is much less fastidious, and C. histolyticum produces small but visible colonies on aerobic blood agar plates. Clostridial spores not only survive long periods of exposure to air, but are also resistant to heat and disinfectants and germinate only under reduced conditions.

Although many species of clostridia have been named, the descriptions are for the most point fragmentary, and the taxonomy of the genus is still in an unsatisfactory state. A listing of typical species of clostridia, subdivided in terms of energy-yielding substrates and modes of energy-yielding metabolism is shown in Table 2.1.

2.2 The Butyric Acid Bacteria

Many clostridium species exemplified by the species C. butyricum, are capable of fermenting soluble sugars, and sometimes starch and pectin, to butyric acid, acetic acid, CO_2 and H_2. In some species, such as C. acetobutylicum, the fatty acids are further converted to butanol, ethanol and acetone. A starchlike polysaccharide is commmonly formed by butyric acid bacteria as a reserve material, and many of the species produce capsules. The capacity of nitrogen fixation is widespread among butyric acid bacteria.

Some species of butyric acid bacteria are proteolytic and can use amino acids as alternative substrates. In fact, butyric acid bacteria range from those which are completely nonproteolytic and depend exclusively upon

Table 2.1

Subdivisions Among the Clostridia, Primarily in Terms of Energy-Yielding Substrates and Modes of Energy-Yielding Metabolism

Substrates used	Mode of energy-yielding metabolism	Other distinctive properties	Typical species
Sugars, starch, pectin	Butyric acid (or butanol-acetone) fermentation	Many can fix nitrogen	C. butyricum, C. pastorianum, C. welchii, C. acetobutylicum
Pairs of amino acids	Strickland reaction	Proteolytic; many can also perform butyric fermentations of sugars	C. sporogenes C. tetani, C. botulinum
Single amino acids (e.g., glutamate)	Butyric or propionic acid fermentation	Nonproteolytic; often unable to attack sugars	C. tetanomorphum, C. cochlearum, C. propionici
Purines (e.g., uric acid)	Fermentation to NH_3, CO_2, acetic acid	Restricted to purines as energy sources	C. acidi-urici
Cellulose	Fermentation to acetic and succinic acids, ethanol, CO_2, H_2	Attack few or no carbohydrates other than cellulose	C. dissolvens
Sugars	Fermentation to acetic acid	-	C. thermoaceticum
Sugars	Fermentation to ethanol and CO_2	Also able to ferment a pyrimidine, orotic acid	C. oroticum
Ethanol + acetic acid	Fermentation to higher fatty acids	No other substrates fermentable	C. kluyveri
Lactic acid, some other organic acids	Anaerobic respiration, with sulfate as electron acceptor	Contain cytochromes	C. nigrificans
H_2	Anaerobic respiration with CO_2 as electron acceptor; CO_2 converted to acetic acid	No other substrates attacked	C. aceticum

fermentable carbohydrates as energy sources, to those which are strongly proteolytic and ferment sugars weakly, if at all.

2.3 Clostridia that Ferment Amino Acids

Certain clostridia, as exemplified by C. tetanomorphum, have no proteolytic ability but can obtain energy by the fermentation of single amino acids. C. tetanomorphum ferments histidine and glutamic acid. C. propionicum can ferment alanine, serine, or threonine with the formation of propionic acid as a major end-product, but is unable to attack carbohydrates.

2.4 Clostridia that Ferment Ring Compounds

Several nutritionally specialized clostridia can ferment nitrogen containing ring compounds such as purines, pyrimidines or pyridines. The best studied of these processes is the fermentation of purines by C. acidi-urici to produce acetic acid, ammonia and CO_2. C. acidi-urici is unable to use any other type of energy source.

2.5 Clostridial Fermentations of Carbohydrates

Several species of clostridia (as an example, C. dissolvens) are able to ferment cellulose to acetic acid, succinic acid, ethanol, CO_2 and H_2. C. thermoaceticum ferments soluble sugars to acetic acid. C. oroticum ferments sugars to ethanol and CO_2, and is also capable of fermenting the pyrimidine derivative orotic acid.

2.6 The Fermentation of Ethanol and Acetate

Some species of clostridia are able to use ethanol and acetate as substrates. C. kluyveri, for example, converts ethanol and acetate to higher fatty acids (mainly butyric and caproic acids).

2.7 Sulfate-Reducing Clostridia

Several clostridial species (as an example, C. nigrificans) employ the anaerobic respiration of organic compounds as a means of obtaining energy.

These organisms use sulfate as a terminal electron acceptor in a similar manner as bacteria of the genus Desulfovibrio.

2.8 Clostridia that Utilize Synthesis Gas Components

Of particular interest in this study are clostridia capable of utilizing CO, CO_2 and H_2 in synthesis gas as substrates. As will be noticed in this review, no other clostridial species was found to be capable of producing ethanol from CO or CO_2 and H_2.

2.8.1 CO Utilization

Two species of clostridia have been found to be able to utilize carbon monoxide and CO_2/H_2 as substrates. C. thermoaceticum utilizes CO to produce acetate by the equation (30):

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \qquad (2.2)$$

However, growth and CO uptake by this organism is not as rapid as with the organism Peptostreptococcus productus. Similarly, C. thermoautotrophicum was shown by Weigel in 1982 to be able to produce acetate and butyrate from CO, CO_2 and H_2. Carbon monoxide can also be converted to CO_2 and H_2 by the traditional water gas shift reaction using several clostridial species (31-35):

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad (2.6)$$

These species include C. thermoaceticum, C. formicoaceticum and C. pasteuranium.

2.8.2 CO_2/H_2 Utilization

Several clostridial species have been found to use CO_2 and H_2 as substrates. The products of these fermentations include formate, acetate and butyrate. C. purinolytricum reduces CO_2 to formate, with the formate then converted to acetate (36). C. acidi-urici synthesizes acetate from CO_2 during the fermentation of hypoxanthine (37).

As mentioned previously, C. thermoaceticum uses H_2 and CO_2 to produce acetate and C. thermoantotrophicum uses CO_2 and H_2 to produce acetate and butyrate. C. aceticum is unable to utilize CO, but also produces acetate from CO_2 and H_2 by the reaction (38, 40):

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O \qquad (2.4)$$

Small amounts of other fatty acids such as propionic, butyric and valeric acids have been reported from CO_2 and H_2 by mixed bacterial populations (40).

3.0 MATERIALS AND METHODS

3.1 Media

Several media were employed in culture isolation, culture identification and culture optimization with Clostridium sp. The media used in isolating Clostridium sp. from natural sources is shown in Table 3.1. As noted, the media was a standard basal media for strict anaerobes, containing vitamins, minerals, resazurin and yeast extract in water. The yeast extract concentration was varied in many of the experiments from the 0.1g per 100 mL concentration shown in Table 3.1. The procedure for preparing the media is shown in Appendix A.1. The media used in the pure culture experiments was as recommended by the American Type Culture Collection.

Table 3.1

Natural Source Screening Medium

	Per 100 mL of Media
Pfenning's minerals	5.0 mL
Pfenning's trace minerals	0.1 mL
B-vitamins	0.1 mL
Yeast extract	0.5 mL
Resazurin	0.1 g*
Distilled or deionized water	100 mL

* yeast extract varied considerably in the experiments

Early fermentation studies with Clostridium sp. were carried out in a basal medium containing 0.01% yeast extract, as well as B-vitamins and minerals. Yeast extract is a complex medium constituent, consisting of a wide variety of vitamins, minerals, salts and amino acids. Yeast extract has, however, been shown to affect the ratio of products produced by Clostridium sp. In addition, yeast extract is a very expensive media constituent and is

also expected to interfere with mutation/selection experiments. Experiments were thus carried out in an attempt to remove yeast extract from the medium and, in the process, develop a minimal medium for the growth of Clostridium sp. Eventually the essential amino acids for growth will be found, and an inexpensive source of these amino acids will be substituted into the medium.

The newly isolated Clostridium was first adapted to grow in a medium devoid of yeast extract following strategies similar to those employed by Savage and Drake (41) with C. thermoautotrophicum. The culture was sequentially transferred into media containing decreasing concentrations of yeast extract (0.01%, 0.0051%, 0.001%, 0%) and an enriched formulation in minerals, trace metals and B-vitamins shown in Table 3.2. To eliminate yeast extract, an enriched medium was utilized containing minerals, trace minerals, B-vitamins and an amino acid solution shown in Table 3.3. Once the culture was adapted to growth without yeast extract, all solution concentrations were halved successfully except the trace mineral solution. Next, the removal of the B-vitamins solution was attempted, but prohibited growth. However, the culture was then grown without the amino acid solution. At this point, various combinations of B-vitamins are being examined for possible elimination from the medium composition.

The medium used for culture identification studies was a basal medium containing 1 g/L yeast extract. The initial pH of the medium was 6.0, instead of 4.0 or 5.0 used in routine studies. Thus, a larger quantity of acetic acid (and less ethanol) is expected in these culture identification students, as was indeed found.

3.2 Laboratory Procedures

Detailed procedures for inoculation, gas addition and gas and liquid sampling are shown in Appendices A2-A4. These procedures were developed after

Table 3.2

Enriched Medium Composition/100 ML Medium

5.0 ml mineral solution
0.5 ml trace minerals solution
2.0 ml B-vitamin solution
0.1 ml Resazurin solution (0.1%)
80 ml distilled water

Mineral Solution	g/L
$(NH_4)_2\ SO_4$	10.0
NH_4Cl	10.0
KH_2PO_4	136.0

B-vitamins	mg/L
Biotin	20
Folic Acid	20
Pyridoxal HCl	10
Lipoic A.(Thioctica)	60
Riboflavin	50
Thiamine HCl	50
Ca-D-Pantothenate	50
Cyanocobalamin	50
P-Aminobenzoic Acid	50
Nicotinic Acid	50

Trace Mineral Solution (g/L)	
Nitrilotriacetate	1.5
$M_gSO_4 \cdot 7H_2O$	6.1
NaCl	1.0
$F_eSO_4 \cdot 7H_2O$	0.1
$CoCl_2 \cdot 6H_2O$	0.1
$CaCl_2 \cdot 2H_2O$	0.1
Zn Cl	0.1
$CuCl_2 \cdot xH_2O$	0.01
$AlK(SO_4)_2 \cdot 12H_2O$	0.01
H_3BO_3	0.01
$Na_2\ MoO_4 \cdot 2H_2O$	0.01
$NiCl_2 \cdot 6H_2O$	0.05
Na_2SeO_3	0.0005
$MnSO_4 \cdot H_2O$	0.5

Table 3.3

Amino Acid Solution Composition (16 mg/L of each)	
valine	leucine
threonine	cysteine
arginine	glutamic acid
histidine	phenylalanine
methionine	serine
lysine	asparagine
	tryptophan

several months of refinement and modification. The anaerobic techniques employed are essentially those of Hungate (42), as modified by Bryant (43) and Balch and Wolfe (44).

The development of procedures for quantitatively using gas substrates (CO, CO_2/H_2) with Clostridium sp. was necessary. An outline of the procedures for quantitative gas utilization is shown in Appendix B.

3.3 Analytical Techniques

Standard analytical techniques used on a routine basis included cell density measurements using optical density, gas analysis using gas-solid chromatography, liquid analysis using gas-solid chromatography, and reaction pH. Each of these techniques are briefly discussed in Appendix C. Other analytical techniques used in culture identification are standard microbiological techniques and are not discussed.

4.0 CULTURE ISOLATION AND CHARACTERIZATION

Bacterial isolation and characterization studies carried out at the University of Oklahoma have shown that the culture originally isolated from animal waste is a new clostridial species. Clostridium sp. is a gram-positive, motile, rod-shaped anaerobic bacterium which sporulates infrequently. The bacterium produces a homoacetic fermentation of fructose at pH 6. At high pH, it also oxidizes hydrogen and reduces CO_2 to acetic acid. The bacterium only produces ethanol at pH levels below 6.0. At higher pH levels, acetate is the only liquid phase product.

Clostridium sp. was isolated because of its ability to produce ethanol from synthesis gas. In the identification studies, the initial pH of the culture medium was 6.0. The use of a medium with a pH lower than 5.0 and/or the presence of CO as a substrate probably contributes to the production of ethanol by this microorganism. Analogous examples of switching of metabolism have been reported by Jones and Woods (45) and Lee and Zinder (46).

A list of substrates tested for growth as the sole carbon and energy source is shown in Table 4.1. As noted earlier in these studies, 1 g/L of yeast extract was added to the medium along with the substrate, and all fermentations were carried out at an initial pH of 6.0. As noted in Table 4.1 growth was obtained when H_2/CO_2, ethanol, pyruvate, xylose, arabinose and fructose were used as substrates. Clostridium sp. grew weakly in the presence of fumarate, ribose, glucose and casamino acids. In the absence of yeast extract, growth is stimulated by the addition of water-soluble vitamin solutions and/or casamino acids.

Clostridium sp. is distinguishable from related organisms by its substrate use, pH requirements, end-products of fermentation and physical characteristics. The bacteria can metabolize substrates at pH levels as low

Table 4.1

Growth of Clostridium sp on Substrates as the Sole Carbon/Energy Source

$H_2:CO_2$	+	ribose	w
CO	+	xylose	+
formate	-	arabinose	+
methanol	-	fructose	+
ethanol	+	glucose	w
pyruvate	+	galactose	-
lactate	-	mannose	-
glycerol	-	sorbitol	-
succinate	-	sucrose	-
fumarate	w	maltose	-
citrate	-	lactose	-
		starch	-
		casamino acids	w

+ positive growth
- no growth
w weak growth

as 4.0-4.2. Clostridium sp. can be distinguished from Clostridium aceticum by its pH optimum, and the utilization of glucose, formate, ribose and xylose as substrates. The bacterium can be distinguished from Clostridium barkeri by the end-products of fermentation, motility, and the utilization of glucose, lactate and xylose as substrates. Clostridium sp. is distinguishable from Clostridium formicoaceticum by the utilization of glucose, lactate, ribose and H_2/CO_2 as substrates, and is distinguished from Clostridium thermoaceticum by thermophilicity and the utilization of glucose as a substrate.

The bacterium can also be differentiated from other acetate forming species. It is distinguished from Acetobacterium sp. by spore formation and the utilization of formate, lactate and pentoses as substrates. It is distinguished from Eubacterium limosum by motility, spore formation, and the utilization of glucose, lactate, methanol, ethanol and arabinose as substrates.

4.1 Taxonomic Research in Progress/Planned

In collaboration with Carl R. Woese at the University of Illinois, a sequence analysis of the 16 S rRNA of Clostridium sp. is underway. The characterization of the gram-positive anaerobes is relatively confused (see the genus Eubacterium as an example). Characterization techniques that were standard in the past may no longer be appropriate. Researchers at the University of Oklahoma are analyzing the 16 S rRNA of approximately 60 species of gram-positive anaerobes to clarify relationships within this group.

In addition, further electron microscopy is currently underway. The mol% G+C of DNA will be determined in January 1989 and the cell wall will be characterized before June 1989. When patent clearance has been obtained, the bacterium will be deposited with the American Type Culture Collection and the Anaerobe Laboratory at Virginia Polytechnic and State University.

Photomicrographs of Clostridium sp. are shown in Figures 4.1 and 4.2. An electron photomicrograph is shown in Figure 4.3. Careful examination of the figures shows some sporulation and the tendency of the bacteria to form chains.

4.2 Bacterial Name

The bacteria has been named Clostridium ljungdahlii, strain PETC, in honor of Dr. Lars G. Ljungdahl, in recognition of his work with clostridia and acetogens.

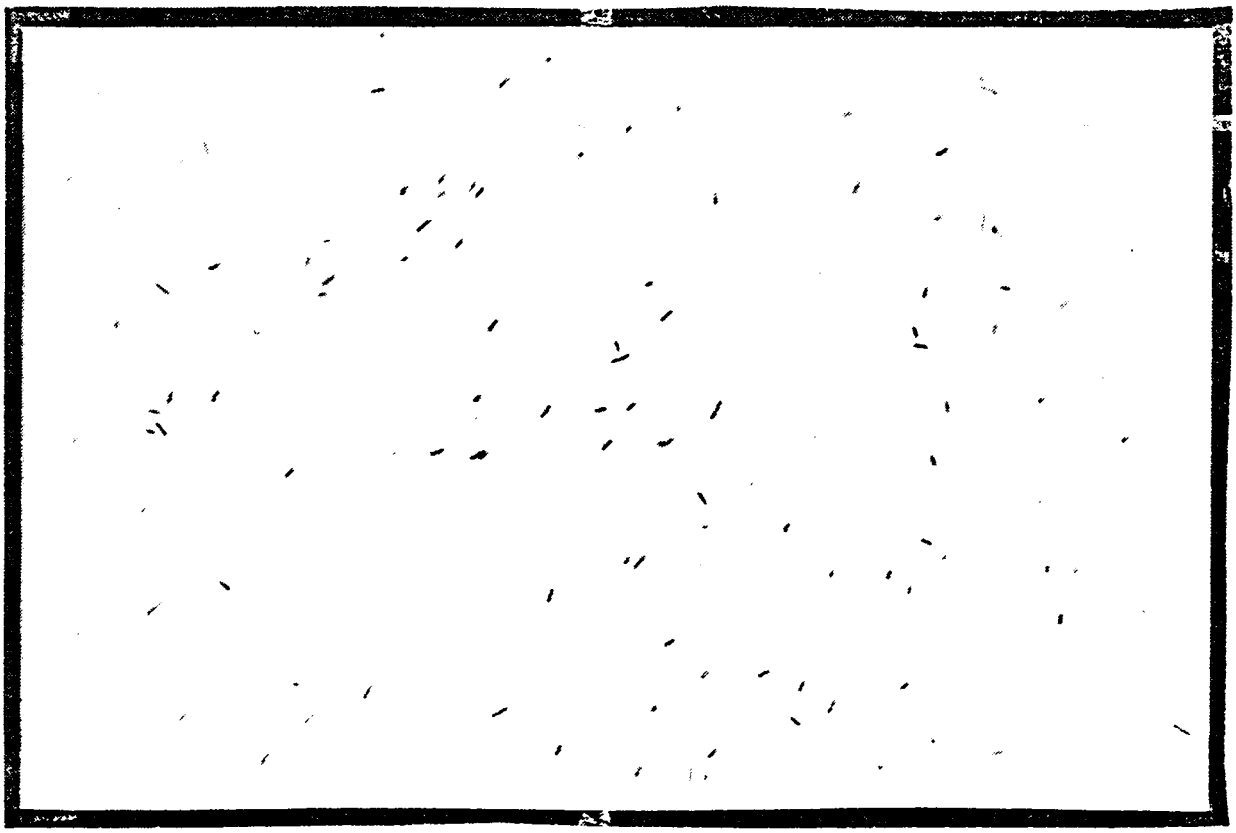

Figure 4.1. Photomicrograph of *Clostridium ljungdahlii*, strain PETC (1 μm = 0.6 mm).

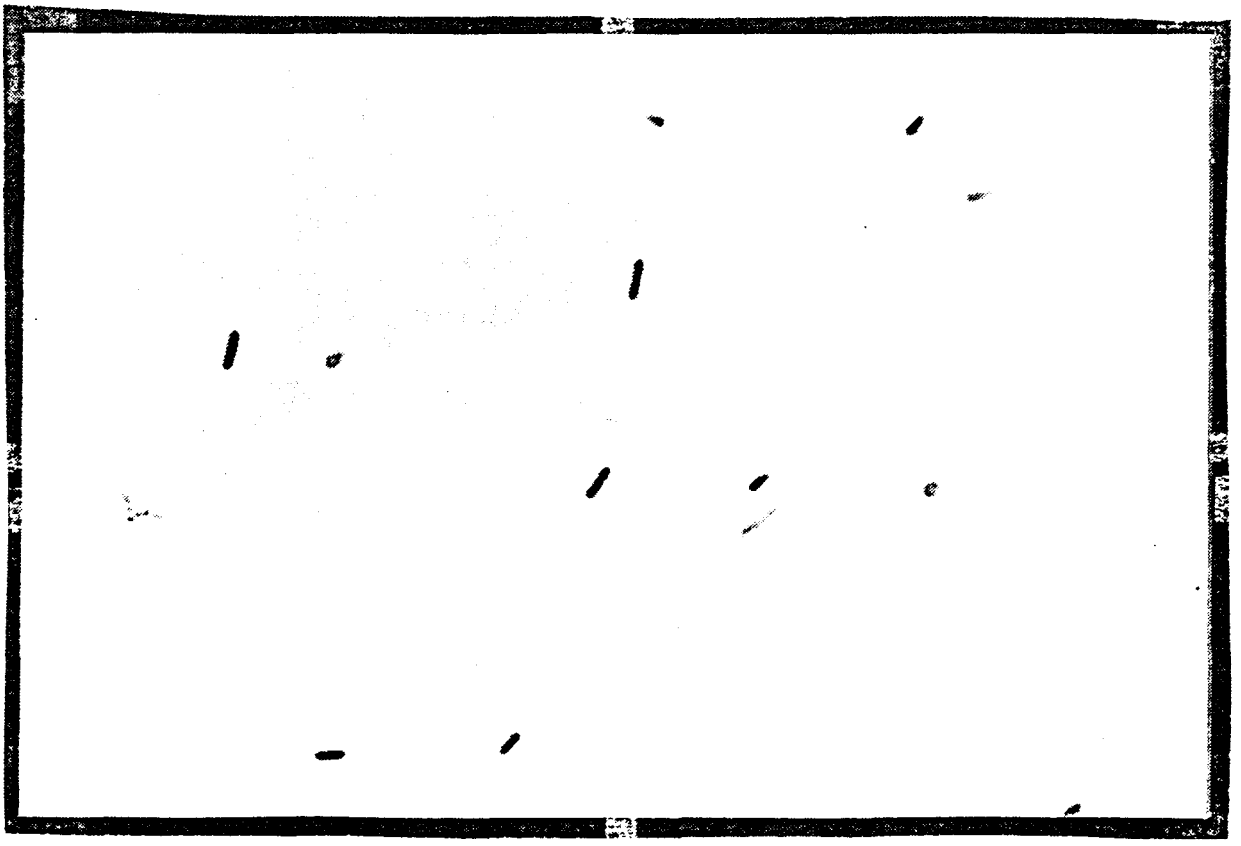

Figure 4.2. Photomicrograph of *Clostridium ljungdahlii*, strain PETC (1 μm = 1.5 mm).

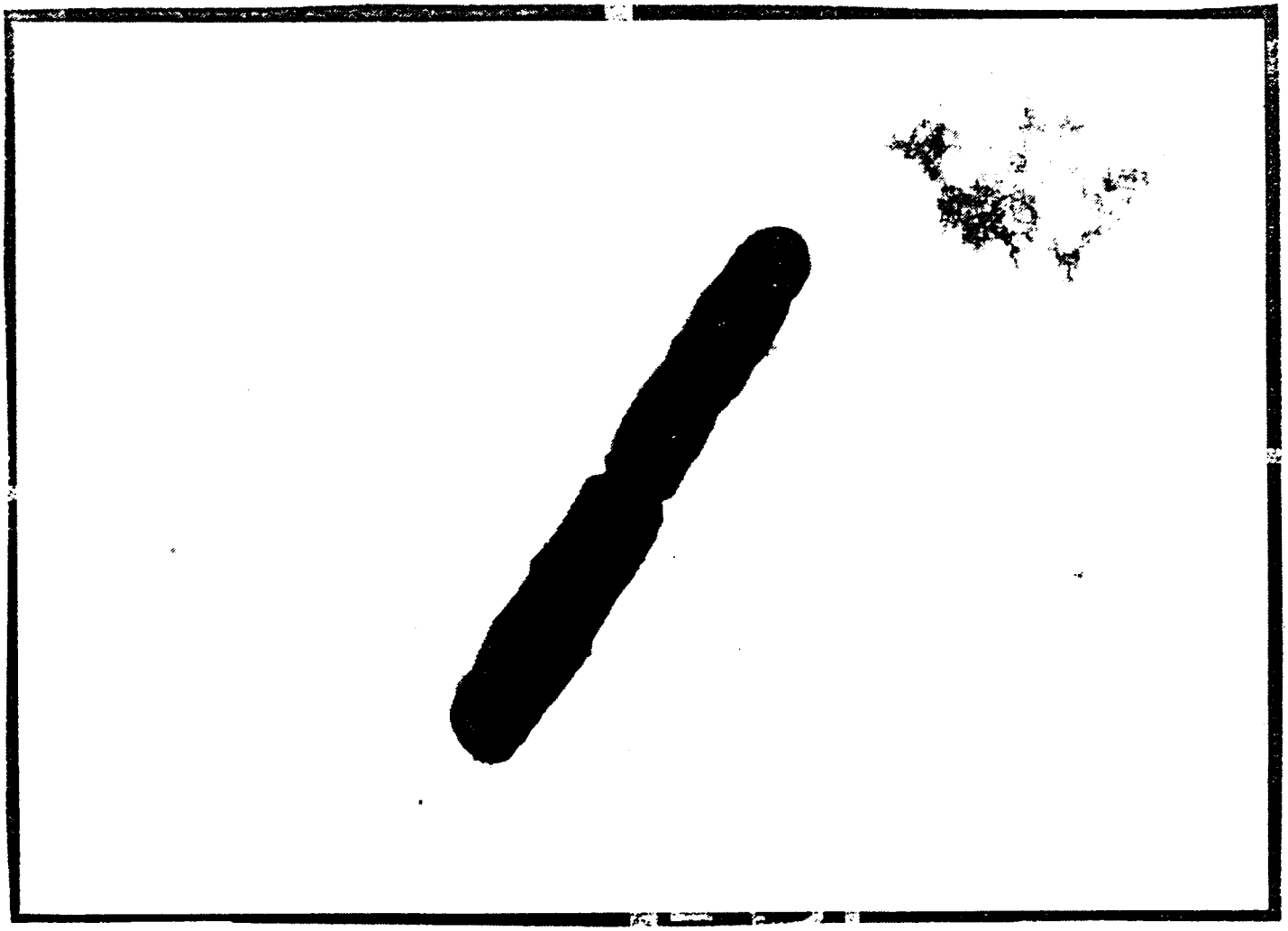

Figure 4.3. Electron micrograph of *Clostridium ljungdahlii*, strain PETC.

5.0 CONCLUSIONS

Culture identification and characterization studies carried out at the University of Arkansas and under contract to the University of Oklahoma, Department of Botany and Microbiology, have been essentially completed. The studies indicate that the organism is indeed a new clostridial strain, to be named Clostridium ljungdahlii, strain PETC, in honor of Dr. Lars G. Ljungdahl for his work on clostridia and acetogens. C. ljungdahlii is different from other clostridial strains and similar geni in its operating conditions and choice of substrates as sole carbon and energy sources. C. ljungdahlii, strain PETC, produces ethanol as a product only at low pH levels (5-6), with acetate the primary product at higher pH levels.

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7.0 APPENDICES

Appendix A
Laboratory Procedures

A.1 Procedures for Media Preparation

1. Using sterilized or disposal pipettes, mix the media constituents.

2. Test the pH of the mixture, it should be a little above what is required, since the usually drops by 0.2 units in the autoclave

   If the pH is too low,; add dropwise sterilized 10% $NaCO_3$ (sodium bicarbonate)

   If the pH is too high, add dropwise 1.0% Acetic Acid.

3. Transfer the media into a 1000 or 500 ml round bottom flask.

4. An 80% N_2, 20% CO_2 gas mixture is bubbled through the media using a 1 ml cotton plugged pipe and attached to the gas hose.

5. Allow the solution to boil for 2 minutes

6. After the solution cools, check the pH
   If the pH is correct, anaerobically transfer the solution into serum bottles and seal with a septa. (This technique will be discussed later)

A.2 Inoculation of Media with Natural Inocula

Fresh natural inocula can be added to jars of media in the anaerobic chamber. Samples must be added to the media as soon as possible after collecting the inocula, trying not to let the sample dry out. After the gas has been added (CO or synthesis gas), put the sample in the incubator. Take gas samples weekly to check the progress of the organisms in the solution. Once gas consumption has begun, transfer a portion of the inoculated media to new media, always keeping the original sample. If the original sample doesn't consume gas after a period of a month to a month and a half, it must be transferred to ensure that the organisms have not depleted the nutrients. Samples have taken from 1 to 6 months to initiate gas consumption. Thus, try not to discard the cultures to soon.

The inoculation procedure must be anaerobic or the organisms may die. The procedures for inoculation are the same as in removing liquid samples:

1. Turn on the gas that is to be used (N_2 or CO or both) and turn the gas heater on.

2. When inoculating a sample, a substance must be added to tie up any dissolved oxygen in the solution. Sodium sulfide can be used. Add 0.2 ml of NaS_2 for every 10 ml of solution. Methane inhibitors should also be added prior to inoculation. BESA or monensin in 0.1 ml quantities for every 10 ml of solution were added.

3. To add something to the solution: Take a sterile or disposable needle and syringe and flush it twice with gas by placing it in the canula. Place methanol on the bottle of NaS_2 (example) and the serum bottle, and then flame the NaS_2 bottle and insert the needle into the septa. Withdraw the exact amount of solution needed. Flame the septa of the serum bottle and put the needle in the septa. After forcing the solution into the bottle, let the plunger come up on its own. This will relieve excess pressure from the serum bottle. Withdraw the needle and dispose of it in the proper container.

4. To remove liquid samples: Flush the needle with gas and put methanol on the top of the septa. Flame the septa and insert the needle into the sample and withdraw the amount needed. Withdraw the needle and place the sample in a sampling tube. Dispose of the needle and syringe. Don't use a syringe twice due to the possibility of cross contamination. Similar procedures are used for the pure culture.

A.3. Gas Addition to Serum Bottles

1. Place methanol on top of the septa. Let it evaporate before addition.

2. Place sterile needles on the gas lines that you will be using, making sure the tubing has been autoclaved.

3. Turn the gas on and turn on the regulator by the hood. Make sure the hood fan is on. Set the regulator for the pressure that is needed. If one atmosphere is required, fill the bottle to two atmospheres, then let the pressure out with a needle. Hold the needles up in the hood and flush the gas through them for a couple of seconds. Then place the needles in the septa. Fill the bottles to pressure, then pull a vacuum. Pressure again and vacuum again. Pressure and vacuum once more. Then fill the bottles with gas once more. Remove the needles and check for leaks in the septa. Turn the gas bottle off and vent any pressure in the lines.

4. If an inert gas is needed, place the gas in a gas bag. Then, using a sterile glass syringe, take out the amount that is needed and put in the bottle. Use caution not to over pressure the syringe since they have been known to break.

   NOTE: WHEN ADDING GAS, ESPECIALLY INERTS OR TRACERS, YOU NEED TO RECORD THE BAROMETRIC PRESSURE, VOLUME OF TRACE GAS AND ROOM TEMPERATURE. This enables a mole balance to be done.

A.4 Gas and Liquid Sampling from Serum Bottles

Gas and liquid samples are to be removed using the same procedures as outlined in Appendix A.2. Be sure to shake the bottles to insure accurate readings. But make sure that liquid is not on the septa, otherwise the gas syringe may become clogged. The amount removed and the computer program used for calculation will depend on the current analysis.

Appendix B
Gas Consumption Under Quantitative Conditions

Preparation of the media was carried out anaerobically in an atmosphere of 80 percent nitrogen (N_2), 20 percent CO_2 as described by Hungate and Ljungdahl and Wiegel. The medium was then transferred to serum bottles, 157.5 mL in volume. The bottles are sealed with butyl rubber stoppers and aluminum seals. The bottles were then autoclaved at 121°C for 21 minutes. A seed culture was started from a stock culture in a bottle similar to those described above about 48 hours before the experiment was to begin. This seed culture was grown in a shaker incubator kept at 37°C and 100 rpm. Once the seed culture was ready, sodium sulfide was added to the bottle and left in the shaker incubator for about 15 minutes to allow for complete oxygen removal and temperature acclimation. Then, 10 mL of seed culture were aseptically added to each bottle. The bottles were then flushed with the gas to be employed and pressurized to the desired level (up to 3 atm maximum) with the use of adjustable check valves. A measured amount of an inert gas was then introduced with a Leur-lock syringe. In these experiments 20 cc of methane will be as inert gas. The inert gas allows determination with high accuracy the changes in total pressure inside the bottle and thus the total amount of carbon monoxide present in the gas phase.

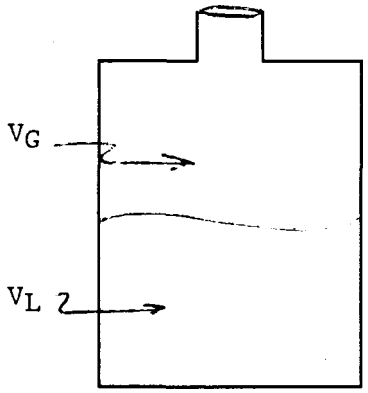
The bottles were left in the shaker incubator (100 rpm) at 37°C during the experiment. Sampling for gas composition, optical density, pH, acids and alcohols were carried out at adequate intervals. An outline of the experimental procedure is shown in Figure A.1.

The cell concentration (dry weight) may then be determined from an appropriate calibration curve of optical density as a function of dry cell weight. Acetate and ethanol concentration may be determined from a gas chromatograph calibration curve. Finally, the moles of carbon monoxide at any time may be found using the ideal gas law the gas chromatographic analysis, and the inert gas composition. The procedure is illustrated in Figure A.2.

Yields may then be determined by plotting cells or product produced as a function as the CO consumed. The yields are then found from the slopes of these plots.

Figure A.1

BATCH EXPERIMENTAL PROCEDURE FOR DETERMINING REACTION STOICHIOMETRY



V_T = 157.5 mL (total volume)

$V_L°$ = 86.5 mL (initial liquid volume)

$V_G°$ = 71.0 mL (initial gas volume)

Start-up

1. Anaerobically, fill the bottle with 75 mL of liquid medium.
2. Vent the gas phase with the desired gas mixture.
3. Sterilize the bottles in the autoclave.
4. Add 1.5 mL of Sodium Sulfide ($Na_2S.9H_2O$)(2.5%).
5. Shake for 20 minutes at 37°C.
6. Add 10 mL of seed culture.
7. Vent the gas phase with the desired gas mixture. Use variable cracking pressure check valves in the gas outlet.
8. Add 20 mL of CH_4 (inert gas) at room temperature and pressure.
9. Measure initial optical density and gas composition.
10. Place in the shaker incubator at 37°C.

Sampling

11. Sample the gas phase for gas composition (0.4 mL).
12. Sample the liquid phase for optical density, pH, ethanol and acetate (3 mL).

Figure A.2

DETERMINATION OF QUANTITATIVE INFORMATION DURING A GAS FERMENTATION

1. Cell concentration (dry weight) from OD (optical density) calibration curve.

2. Moles of carbon monoxide present in the gas phase from gas percent analysis:

   - At start-up:

$$N_{CH_4} = \frac{P_{room}\, V_G^o}{R \cdot T_{room}} \Rightarrow N_T^o = \frac{100}{(\%\ CH_4)}\, N_{CH_4}^o$$

$$N_{CO}^o = \frac{(\%CO)}{100}\, N_T^o$$

   - At each sampling time:

$$N_{CH_4}^i = N_{CH_4}^o - \sum_{j=1}^{i-1} \frac{V_{gas\ sample}}{V_G}\, N_{CH_4}^j$$

$$N_t^i = \frac{100}{(\%CH_4)}\, N_{CH_4} \Rightarrow N_{CO}^i = \frac{(\%CO)}{100}\, N_T^i$$

Thus, the moles of methane depend upon the initial amount of methane and the number of samples.

(3) Ethanol and acetate concentration from G.C. calibration curve.

NOMENCLATURE

$N^{o}_{CH_4}$ - initial moles of methane

N^{o}_{CO} - initial moles of carbon monoxide

N^{O}_{T} - initial total number of moles

$N^{i}_{CH_4}$ - moles of methane at a particular sampling time

N^{i}_{CO} - moles of carbon monoxide at a particular sampling time

N^{i}_{T} - total number of moles at a particular sampling time

P_{room} - total pressure in the room at the start of an experiment

R - universal gas constant,

$$0.0821 \frac{L \cdot atm}{gmole^{\circ} K}$$

T_{room} - room temperature

$V_{gas\ sample}$ - volume of a gas sample

V_G - volume of total gas phase

V_L - volume of liquid phase

V_T - total volume (gas and liquid)

V_G° - initial volume of gas phase

V_L° - initial volume of gas phase

Appendix C
Analytical Procedures

C.1 Bacterial Growth

Bacterial growth was determined by measurement of optical density (OD) using a Bausch and Lomb Spectronic-20 or Spectronic-21 spectrophotometers. The greater the optical density, the greater the number of cells in the culture. This procedure was necessary to monitor the viable cells in the culture. Growth of the enriched and pure culture was measured at 480 nm.

C.2 Gas Analysis

The composition of the gas phase was determined by gas-solid chromatography in a Varian 3400 gas chromatograph. Samples (0.15 mL) were injected using a Pressure-Lok syringe into a 6 ft x 1/8 in. column packed with carbosphere, 60/80 mesh, to allow the determination of H_2, N_2, Ar, CO, CH_4 and CO_2. A temperature program was required for the adequate separation of hydrogen and air components. The optimal program conditions were determined to be 30°C for 4 minutes, an increase in temperature to 125°C at 30°C/min for 5 minutes, and a final period at 200°C for 8 minutes, required to condition the column prior to analysis of the next sample and to remove any traces of water in the column. The detector and injector temperatures were both 175°C and the carrier gas used was helium at a flow rate of 40 mL/min. The total time for the analysis was approximately 20 minutes.

C.3 Liquid Analysis

Liquid analyses were also carried out by gas chromatography in the Varian 3400 gas chromatograph. Separate procedures for the analysis of alcohols and acids were required to avoid peak interference.

Alcohol analysis was performed using a 2 ft x 1/8 in column packed with Poropak QS, pretreated with NaOH. In a typical run, a 250 μl sample was mixed with 40 μl of NaOH to avoid acid peaks. The injection volume into the chromatograph was 1 μl. The oven temperature was 150°C. Both the detector and injector temperatures were 220°C and the carrier gas was helium at a flow rate of 40 μl/min. To separate methanol, ethanol, propanol and butanol, approximately 15 minutes were required.

Acid analysis was performed using a 2 ft x 1/8 in column packed with Poropak QS, previously conditioned with H_3PO_4. A 250 μl sample was mixed with 40 μl of 50% H_3PO_4. A 1 μl sample of this mixture was injected into the chromatograph for analysis. The oven temperature was 170°C. The detector and injector were both maintained at 220°C and the carrier gas was helium at a flow rate of 40 μl/min. Approximately 15-20 minutes were necessary to separate acetic, propionic and butyric acid.

To switch from alcohol to acid analysis it was necessary to change the columns, followed by conditioning at 200°C overnight.