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Development of Biological Coal Gasification (MicGAS Process)

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DEVELOPMENT OF BIOLOGICAL COAL GASIFICATION
(MicGAS™ Process)

ABSTRACT

ARCTECH has developed a novel process (MicGAS) for direct, anaerobic biomethanation of coals. Biomethantaion potiential of coals of different ranks (Anthracite, bitumious, sub-bitumious, and lignites of different types), by various microbial consortia, was investigated. Studies on biogasification of Texas Lignite (TxL) were conducted with a proprietary microbial consortium, Mic-1, isolated from hind guts of soil eating termites (*Zootermopsis* and *Nasutitermes* sp.) and further improved at ARCTECH.

Various microbial populations of the Mic-1 consortium carry out the multi-step MicGAS Process. First, the primary coal degraders, or hydrolytic microbes, degrade the coal to high molecular weight (MW) compounds. Then acedogens ferment the high MW compounds to low MW volatile fatty acids. The volatile fatty acids are converted to acetate by acetogens, and the methanogens complete the biomethanation by converting acetate and CO_2 to methane.

The overall focus of the research was to:

- enhance kinetics of coal biomethanation,
- reduce hydraulic retention time,
- enhance interaction between coal and microbes,
- increase solids loading,
- overcome the rate-limiting steps, and
- develop a cost effective process.

Methane production was enhanced by the addition of various co-substrates and nitrogen (organic as well as inorganic) supplements. Mass balance calculations showed that higher methane production, in the presence of carbonaceous co-substrates, was from the enhanced biogasification of TxL and not from the co-substrates. Addition of two hydrogen donors, citrate and methanol in particular, reduced the retention time to 7-8 days from the original 60 days. The citrate also appeared to sequester the metal ions from the coal that were inhibitory to microbial growth. Addition of the nitrogen amendment, Cargill 200/20, not only reduced the process cost by 116.6-fold, but also enhanced methane production significantly as compared to previously used nitrogen amendments (eg. yeast extract).

Another important parameter for cost effective coal biomethanation is coal solids loading. Preliminary economic analysis by Fluor Daniels established a target of $\geq 20\%$

solids loading for a more economical process. Texas Lignite solids loading was enhanced by 50-fold (from 0.1 to 5%), however, solids loading of ≥10% resulted in a significant decrease of methane production and an increase in carbon dioxide.

Studies at coal solids loadings of ≥10% (15% and 20%) identified the rate limiting steps as:

- the sudden drop in the initial pH of the medium after the addition of TxL,
- the accumulation of metabolic intermediates in the culture broth,
- the different pH optima of individual bacterial populations of the consortium, and
- inhibitory compounds leached from TxL into the culture medium.

Decarboxylation of low rank coals may be one of the reasons for the observed decrease in pH when the TxL was added to the culture medium. A detailed study of effects of initial pH of the nutrient (culture) medium revealed that an initial pH of 7.2 to 7.5 was required for optimal methane production. In order to overcome the effect of pH lowering after the addition of TxL, the pH of the medium was adjusted to 7.8 after adding the coal solids.

It was conjectured that the inhibitors of biomethanation at higher coal solids loading could be due to:

1) concentration of metals leaching out of coal due to degradation of coal, and
2) accumulation of by-products (eg. acetate) that could not be further metabloized under the conditions of fermentation after the coal was biodegraded.

Further investigation at ARCTECH indicated that accumulation of acetate in the medium suppressed the metabolism of the primary coal degraders, acedogens, and acetogens. In addition, the pH optimum for the methanogens (pH 8.5) is different from the other microbial populations (pH 7.5).

Therefore, in order to overcome the problems mentioned above, a dual bioreactor was used. The MicGAS Process can be carried out in a single bioreactor, however, the process was further optimized in a dual bioreactor. First, acetate was allowed to accumulate in the first upflow bioreactor (pH 7.5), and then the spent medium was transferred to a second upflow bioreactor (pH 8.5) where a methanogentic population finished the biomethanation reaction. Furthermore, studies with sub-bituminous and higher rank coals showed that biomethanation is feasible even up to 50% coal solids.

Organic and inorganic compounds of the TxL leachate were evaluated. Compounds such as phenols and metals may be responsible for growth inhibition. Washing the coal before putting it into the bioreactors helped to eliminate some of the inhibitory effects that the unwashed coal had on growth.

ARCTECH proposes to establish MicGAS as a potential remediation technology for restoration of underground gasification sites (eg. the DOE/Hoe Creek site in Gillette, Wyoming).

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1.0 Executive Summary

This report progressively summarizes the work performed by ARCTECH, Inc., from 1990 to 1995, in support of DOE/METC Contract #DE-AC21-90MC27226 (and modifications 1-11): Development of Biological Coal Gasification (MicGAS Process). The overall objective of this project was to develop a process for the direct biological conversion of low rank coals to methane with an ultimate aim to develop environmentally compatible fuel for electric power generation systems and other industrial applications.

Initial studies on the feasibility of coal biogasification were preformed under DOE contract DE-AC21-87MC23285. Additional support for developing this novel approach for coal biogasification was with Houston Lighting and Power Co. and the Electric Power Research Institute and Gas Research Institute. In these initial studies, either chemical, aerobic, or anaerobic means were used to convert the coal solids. In these same studies, microbial consortia, from various sources, were also evaluated.

Laboratory and bench-scale reactor research carried out during the 1990 to 1995 reporting period further established the feasibility of biomethanation of all ranks of coals, including different lignites (eg. Texas lignite, Neyveli, and German Brown). This biomethanation was performed by unique, anaerobic microbial consortia specifically isolated from hind guts of soil eating termites (*Zootermopsis* and *Nasutitermes* sp.) and further improved at ARCTECH. The data obtained demonstrates specificity of a particular microbial consortium to a given lignite. For example, while Mic-1 consortium produced higher methane from Texas Lignite (TxL), the Mic-4 consortium was more effective with the German Brown Coal (GBC) or Neyveli (NYL).

Development of a suitable microbial consortium was the key to the success of the Process. The Mic-1 consortium was adapted to tolerate coal loadings up to 5%; a 50-fold increase over the initial 0.01% TxL solids loading. Furthermore, studies with sub-bituminous and higher rank coals showed that biomethanation is feasible even up to 50% coal solids (ie. in the range of solid state fermentation).

A 25-fold decrease in the cost of the initial growth medium was made by replacing the nitrogen sources, Yeast Extract and Tryptic-Soy broth (YE/TSB), with Sheftone™. The addition of the Sheftone™ also eliminated the need for the costly vitamin supplements usually required by the anaerobic bacteria. Attempts to replace Sheftone™ with higher concentrations of less expensive NH_4Cl inhibited methane production. However, towards the end of the study, the Sheftone™ was replaced with a more economical nutrient medium supplement, Cargill 200/20, which decreased the cost of the initial YE/TSB-based medium by 116.6-fold.

Biomethanation of any complex substrate is a multi-step biochemical reaction performed by different populations of microbes that constitute the consortium. With

coals, the primary degraders, or hydrolytic microbes, degrade the coal to high molecular weight (MW) compounds. Then, a group of bacteria called acedogens ferment the high MW compounds to low MW volatile fatty acids. The volatile fatty acids are converted to acetate by acetogens, and the methanogens complete the biomethanation by hydrogenating the acetate and CO_2 to methane.

The chemical composition of the TxL indicates that this coal is low in hydrogen content. The effect of adding the hydrogen donors, such as citrate, lactate, formate, and methanol, to the process was studied; since the methanogentic microbial population requires hydrogen in order to hydrogenate acetate into methane. Not only did the hydrogen donors increase the TxL biomethanation, but the citrate also appeared to sequester the metal ions that were inhibitory to microbial growth from the coal. Moreover, the addition of hydrogen donors reduced the initial reaction period of 2 months to 7-11 days. Mass balance calculations showed that higher methane production was from the enhanced biogasification of TxL and because of the co-substrates alone. Data from the bench-scale bioreactor studies confirmed those obtained in the laboratory-scale reactors.

Four different bench scale bioreactor configurations, namely Rotating Biological Contactor (RBC), Upflow Fluidized Bed Reactor (UFBR), Trickle Bed Reactor (TBR), and Continuously Stirred Tank Reactor (CSTR) were evaluated for scale up studies. Preliminary results indicated highest biomethanation of TxL by the Mic-1 consortium in the CSTR, and lowest in the trickle bed reactor. However, highest methane production and process efficiency were obtained in the RBC.

Further scale-up studies indicate that the highest biomethanation at a TxL loading of up to 50% was obtained in the UFBR. Under such conditions the methane production was up to 2500 cc/day. Thus, the key accomplishments from this project are as follows:

- a five-fold enhancement of metabolic activity of termite derived Mic-1 cultures for methane production
- reduction of the fermentation time (from 60 days to 21-28 days) required for maximum methane production
- identification of the principal bacterial groups in Mic-1 consortium:
  - primary coal degraders
  - hydrocarbon degraders
  - acedogens
  - acetogens
  - methanogens.
- determining the limiting factors at coal solids loadings $\geq 10\%$ as:

- the sudden drop in the initial pH of the medium after the addition of TxL to the growth medium,
- the accumulation of metabolic intermediates in the culture broth,
- the different pH optima of individual bacterial populations of the consortium, and
- inhibitory compounds leached from TxL into the culture medium.

♦ established that agitation of cultures does not significantly enhance the methane production, however, fluidization in a UFBR does.

♦ established that co-substrates are required for coal biogasification. Nevertheless, the enhanced methane production is from theTxL and not from the co-substrates.

♦ assessed H_2S production was below 4 ppm. Thus, the biologically produced gas meets the pipleine specs for gas supply.

♦ established a formula to evaluate the efficacy and cost efficiency of co-substrates like Sheftone-T™.

♦ reduced the processing cost by 116.6-fold ($6/ton coal vs. $700/ton coal) by developing a more effective growth medium (named SNTM-CM and CNTM-CM) by replacing the YE/TSB in the original NTM medium and omitting vitamin supplements.

♦ established that among the four bench scale bioreactor configurations (RBC, UFBR, TBR, and CSTR) tested, methane production was highest in CSTR and lowest in TBR.

♦ obtained highest coal carbon conversion of up to 36% in the continuous stirred tank reactor.

♦ optimized the biomethanation process further in a dual bioreactor in order to accommodate the different pH optima of the various bacterial populations that make up the Mic-1 consortium.

2.0 Introduction

One of the most abundant fossil fuels in the USA are the low rank coals. Yet at best, this vast energy reservoir is only utilized in coal gasifiers to fuel the electric power generation plants. Not only is this method of coal utilization inefficient in terms of coal carbon converted to methane gas, it is also the major culprit for making the coal environmentally unfriendly and giving the public perception that coal is a "dirty fuel."

In recent years, under auspices of DOE funded projects, advancement in coal-conversion technologies have led to better coal carbon utilization and less emissions of air toxics. This has been achieved through the modification of coal gasifiers. Nevertheless, the capital and operating costs, together with the environmental implications associated with this method of power generation, still make it relatively cost prohibitive.

Latest studies on coal chemistry have further confirmed the heterogenous nature of coal and identified some of the high and low molecular weight lignite fractions. The postulated empirical formula[16] for the macromolecular fraction is $C_{270}H_{240}N_3S_1O_{90}$. More specifically, the macromolecular fraction is comprised of 2, and 3- ring fused aromatics of a variety of chemicals interlinked through carboxyl, etheral and different aliphatic linkages. Furthermore, the nitrogen and sulfur heterocyclics are the ones that make the coal most environmentally unfriendly in coal gasifiers as these compounds are oxidized to SO_x and NO_x[1]. A number of technologies are being developed at different DOE National labs, especially INEL, to biologically remove the SO_x and NO_x. Nevertheless, these are still in developmental stages.

Careful examination of the elemental composition of lignites indicates relative high oxygen content which makes coal more amenable to microbiological degradation. At the same time, biological processes, albeit perceived to be slow, are much more environmentally friendly because of the mild reaction conditions of temperature and pressure. This would ultimately lead to lesser, if no emission of air toxics. Seeking potential cost effective and environmentally friendly technologies for coal bioconversion is imperative due to the current awareness for a cleaner environment, stringent environmental regulations, and the quest to obtain value-added fuels. At the same time, the process by-products would result into non-fuel specialty products such as alcohols, plastics, and food additives. With this view in mind, and reviewing the history of coal economics, ARCTECH's vision for coal utilization is depicted in appendix A; this is a vision for bioprocessing coal a a high-value feed-stock.

Initial studies conducted at ACRTECH, under DOE contract DE-AC21-87MC23285, established the feasibility of coal biogasification. In these initial studies, either chemical or aerobic means were used to first convert coal into low molecular weight compounds. The coal conversion was completed via an anaerobic, biological process using microbial consortia from various sources. From these early studies, ARCTECH

established objectives and developed strategies to improve the coal bioconversion process.

Continued research conducted at ARCTECH, under the recent DOE contract, has further established the technical feasibility of biomethanation of low-rank coals by a variety of unique, anaerobic microbial consortia (eg. Mic-1, Mic-2, Mic-3, Mic-4)[6]. This direct bioconversion has been confirmed by other scientists [9, 17]. The work at ARCTECH has also demonstrated the specificity of certain anaerobic microbial consortium to a given lignite[17]. At the same time, ARCTECH developed a conceptual process design which was chosen by Flour Daniel to conduct a process economic analysis. The Fluor Daniel study[10], while acknowledging that the preliminary data showed efficient Texas lignite biogasification, also concluded that besides other factors, the following parameters must be addressed for this process to be economically viable:

1) enhanced kinetics of the coal biomethanation,
2) enhanced coal solids loading to 20%, and
3) reduce the nutrient amendment cost.

With these requirements established, ARCTECH designed studies that would further increase the relative product value of the coal bioproducts. During the period covered in this report,

1) the kinetics of methane production was enhanced by 7.5-fold,
2) coal solid loading was increased up to 50%, and
3) the cost of the medium to process the coal was decreased by 116.6-fold ($6/ton coal vs. $700/ton coal).

The market value of the coal residue remaining from the MicGAS process was also calculated. The coal residue, containing large quantities of humic acids, has a market value based on the fact that the humic acids can be made into a soil amendment product. Based on our results and calculations, the biological coal residue can contribute an additional $2000 to the value of one ton of coal.

This Final Report summarizes the experimental results obtained from laboratory and bench-scale bioreactors to further enhance the biomethanation of TxL by the Mic-1 consortium. The report also describes the kinetics of methane production as a function of physical, chemical and biological parameters.

3.0 Purpose

The overall purpose for this five year study was to evaluate and produce a cost effective and environmentally sound biomethanation process for coal substrates using cultures isolated and further adapted at ARCTECH, Inc.

4.0 Background

Increasing the use of coal, the most abundant fossil fuel source, is critical for long-term economic growth and international competitiveness of numerous U.S. industrial sectors. Sadly, utilization of this ample energy source is limited to only electric power plants. Moreover, the methods currently used to extricate the energy locked-up in coal are: 1) thermodynamically inadequate for the conversion of all of the coal-carbon into energy, and 2) environmentally 'unfriendly' due to the toxins released during the high-temperature carbonization or pyrolysis processes.

Increased awareness for the need of a cleaner, more efficient coal gasification has lead to the improvement of coal gasifiers. However, even with these modifications, a need for an improved process still exists. Numerous studies on coal composition and bioremediation of xenobiotics and other complex substrates (ie. lignin) lead to the belief that perhaps bacteria can bioconvert coal into fuel-gas and other specialty chemicals. Furthermore, bacteria could produce these products with less, if no toxins due to the low temperatures and pressures at which the bioconversions take place.

However, coal degradation is a multi-step process due to the complex structure of the coal substrate. Therefore, the biodegradation of a complex substrate usually involves numerous microbial populations (a consortium), working collectively, to metabolize various parts of the substrate.

ARCTECH has isolated and adapted microbial consortia that can efficiently bioconvert various coal substrates into methane gas (the MicGAS Process). The following describes how the biomethanation Process was developed, and how this technology has potential for: 1) restoration of underground gasification sites, and 2) increasing the value of low rank coals in terms of product manufacturing.

5.0 Methodology

5.1 General Experimental Information

All manipulations were carried out under anaerobic conditions as described by Hungate [15] and as modified by Bryant [8]. An anaerobic glove box (Coy Corporation, Ann Arbor, MI) was used for culture transfer, cell washing, and isolation of cell types from various microbial consortia. The anaerobic conditions in the glove box were maintained with an oxygen-free mixture of N_2 + H_2 (60:40). The anaerobic conditions of the culture media, reagents, stock reservoir vessels, and bioreactors were maintained by bubbling an oxygen-free mixture of N_2 + CO_2 (80:20). After preparation, the culture medium was dispensed into either pressure tubes, serum vials or Wheaton bottles. The type of vessel and working volume of medium used are summarized in Table 1. Serum vials and tubes were stoppered with rubber butyl stoppers and aluminum crimp sealed. The Wheaton bottles were stoppered with rubber butyl stoppers and screw capped.

Table 1. Quantity of medium used in different vessels

Vessel	Total capacity (mL)	Working volume (mL)
Pressure tube	27	15
60-mL serum vial	73	40
100-mL serum vial	125	50
125-mL serum vial	165	60
500-mL Wheaton bottle	700	300
1-L Wheaton bottle	1,250	500

For cultivation of pure cultures of methanogens, the medium was prepared in the same manner as above, but the head space of the serum vials or Wheaton bottles was exchanged with a mixture of H_2 + CO_2 (80:20).

Appropriate TxL concentrations (0.01% to 50%) were added prior to dispensing the culture medium into experimental vessels containing TxL. All experiments were conducted with in triplicate with the appropriate controls. Unless otherwise stated, two controls, one without nitrogen amendment, and another without TxL, but containing a nitrogen amendment, were part of each experiment. All studies were conducted with -325 mesh (44 μm) lignite (Texas or other) based on the results of previous studies at ARCTECH [26].

The evaluation of bioconversion of lignites was based on the production of total biogas, concentration (mole%) of each of the gases (CH_4, CO_2, N_2, and H_2S) of interest, volatile fatty acids (VFA), chemical oxygen demand (COD), biomass growth, bacterial morphology, pH of the culture medium, and at the end of each experimental set, the proximate composition (ash, carbon, hydrogen, moisture, oxygen and sulfur content) of the residual TxL. The data collected were analyzed to obtain kinetics for the production of methane, VFA concentration, soluble carbon, and biomass. These data were also used to calculate mass and electron balance according to Datta and Andrews[11].

5.2 Chemicals and reagents

All chemicals used were purchased from Sigma Chemical Co., VWR Scientific Co., or Fisher Scientific Co. The nitrogen amendments (e.g. agars or peptone) used for medium preparation and microbiological procedures were purchased from Difco Laboratories, MI. Sheftone T™ was ordered from Sheffields Products Co., NY. Cargill products were purchased from Cargill Protein Products, IO. Detailed composition of the metal, mineral, and vitamin solutions are listed in the appendix. Metal and mineral solutions were dispensed into 1L Wheaton bottles and autoclaved. B-vitamin solutions were filtered sterilized and stored at 4°C.

The 2.5% Na_2S reducing solution was prepared in an exhaust hood by first boiling 1L of double distilled water in a 2L flask. After boiling, 100mL of the water was removed and the remaining volume (900mL) was bubbled with N_2. When the water was cool. 25g of $Na_2S \cdot 9H_2O$ was rapidly and quantitatively transferred to the 900mL of water using the 100mL that was previously removed. When the Na_2S was completely dissolved, the final volume was adjusted to 1L, and 7-8Ml of concentrated HCl was added to adjust the pH to 9. The Na_2S solution was dispensed in 80 mL aliquots in 125-mL serum vials and N_2 was bubbled through the vials for 5-8 minutes. Then the vials were stoppered, crimp sealed and autoclaved at 121°C for 20 minutes.

5.3 Culture Media

Several culture media were used for the maintenance of anaerobic cultures and bioconversion of low rank coals. Yeast Extract + Tryptic Soy Broth (YE/TSB) solution, other nitrogen amendments, chelators, vitamins, or substrates other than coal, were always added as concentrated solutions under aseptic and anaerobic conditions. The medium was always prepared in such a manner that the medium components were not diluted by the additions. Mic cultures were grown in various media as described in Table 2. The Sheftone New Termite Medium (SNTM) and Sheftone New Termite Medium with citrate and methanol (SNTM-CM) are simply a variation of the NTM medium. Methanogens-Sludge Medium (MSM) was used for the maintenance of the methanogens, and unless otherwise indicated, the MSM was autoclaved sterilized at 121°C at 15psi. Anaerobic conditions of all media was ensured by adding 2.5% Na_2S (1mL/20mL medium) and 0.1% resauzurin. Detailed media composition is listed in the appendix.

Table 2. Culture Media used for maintenance Mic anaerobic cultures			
Medium	Organic N Source	B-Vitamin Solution	Additional Changes
NTM[a]	YE/TSB[d]	+	N/A[e]
SNTM[b]	Sheftone T™ (0.01 to 0.2%)	–	N/A
SNTM-CM[c]	Sheftone T™ (0.01 to 0.2%)	–	Sodium Citrate and Methanol

[a] New Termite Medium
[b] Sheftone New Termite Medium
[c] Sheftone New Termite Medium + 10mM Sodium Citrate and 0.5% MeOH (v/v)
[d] Yeast Extract + Tryptic Soy Broth
[e] Not Applicable

5.4 Microbial cultures

Four anaerobic, microbial consortia were used for biogasification of low rank coals. The anaerobic consortia were derived from hind gut of wood-eating termites fed on coal[25]. Mic-1 was derived from *Zootermopsis* sp. and Mic-4 from *Nasutitermes* sp. Mic-2 was developed at ARCTECH during studies performed in a rotating biological contactor. Mic-3 was also developed at ARCTECH by mixing consortia from different sources. The stock cultures were maintained frozen in 5.0% glycerol vials at -70°C. The working cultures were prepared by thawing the frozen stock cultures at room temperature and subsequent transfers under aseptic and anaerobic conditions into serum vials or Wheaton bottles containing medium and 0.1 to 1% TxL (-325 mesh). All working stock cultures were incubated at 37°C under stationary conditions for 14 to 21 days. 500-mL Wheaton bottles containing 300-400 mL NTM + 0.1% TxL were inoculated with the microbial consortia to be tested. Alternatively, in order to check the stability of consortia and avoid erroneous results due to residual TxL from inoculum(a), inoculum was prepared in bottles containing NTM without TxL. After appropriate incubation time (10 to 21 days), the cultures were aseptically harvested under anaerobic conditions, washed twice with the NTM (without YE/TSB), and resuspended in a quantity of NTM (without YE/TSB) that would give enough inoculum for the experiment to be performed. This method of inoculum preparation also provided a means of keeping the initial bacterial inoculum size constant for different treatments with in a given experiment. The bacterial numbers were spectrophotometrically quantified by measuring the absorbance of the resuspended bacterial suspension at 660 nm.

Four methanogenic cultures, *Methanobacterium formicicum, Methanospirillum hungatii, Methanosarcina* sp., and *Methanothrix* sp., were obtained from other researchers and from the University of Oregon Microbial Culture Collection. These cultures were grown and used for the acetate, formate and H_2-CO_2 conversion studies, since these methanogenic cultures are known to be present in the consortia.

5.5 Isolation of Bacterial Cultures

The consortia were diluted to 10^{-3} to 10^{-4} in sterile, buffered water. Spread plates with 15mL NTM + 2% (w/v) agar were prepared, and 0.1 mL of the dilution was spread onto the solidified medium surface (final dilution factor of culture: 10^{-4} and 10^{-5}, respectively). A 5mL NTM + 2% agar overlay with either -325 mesh TxL (0.5% w/v) or biosolubilized TxL (1.25% v/v) was melted, well mixed, and poured onto the inoculated NTM Petri plates. Control plates were prepared using overlays without coal supplements. The melted overlay was held at 45°C until use; this is the temperature at which agar will remain molten but would not adversely affect bacteria in the short time they are exposed to this temperature.

However, it was believed that plating the cultures on the medium surface did not allow adequate contact between the bacterial cells and the coal particles. Therefore, the dilution was then added directly to the coal overlay before pouring it onto the petri dish.

5.6 Microbial growth and biomass production

All microbial cultures/consortia were routinely observed by epifluorescence microscopy[4] using a phase-contrast microscope (Olympus, Model BH 2) equipped with a camera. However, total microscopic cell counts and turbidimetric measurements were not a reliable quantitative method due to adherence of microbial cells to coal particles and the physical interference of coal particles with turbidimetric measurements. Instead, microbial growth was measured as total cellular protein.

The protein analysis method (Pierce, Protein Assay #23225) was developed at ARCTECH by modifying the BCA™ to monitor the changes in microbial growth. A 1mL aliquot of the culture medium was withdrawn and either analyzed the same day or preserved by freezing the sample with 80μL of DMSO. The soluble protein in the medium was removed by centrifugation at 12,000 rpm for 10 min. The pellet containing either the cells (control) or cells + coal (experimental) was washed with physiological saline. The sample was centrifuged again to pelletize the cells and coal particles. Cellular protein was then solubilized by adding 1% (w/v) sodium dodecyl sulphate (SDS). The sample was centrifuged again at 12,000 rpm for 10 min. Solubilized protein in the supernatant was determined using the BCA™ Protein Assay. The assay protocol (having linearity in the range of 5-250 μg/mL of protein) required incubation of the reaction mixture for 30 minutes at 60°C. The change in color was recorded as absorbance at 562 nm against an appropriate blank and compared to a standard curve (bovine serum albumin 2.0 mg/mL in a 0.9% aqueous NaCl solution at

various concentrations) to determine the protein concentration.

Biomass was also measured as wet cell weight, spectrophotometrically at 660 nm (OD_{660}), and dry cell weight. The dry, cell weight was determined as follows: A 10 mL sample from each of the cultures was dispensed into a tared, dry, aluminum weigh pan. To correct for the contribution of medium components, another aliquot was centrifuged at 10,000 rpm for 30 minutes to remove bacterial cells. A 10 mL sample of the cell-free supernatant was dispensed into another tared, dry aluminum weight pan. Both samples were dried to constant weight at 110°C. The dry cell weight was calculated by deducting the weight of the centrifuged sample from the weight of the sample which was not centrifuged. From these data, the specific growth rate (μ) was calculated.

During the exponential phase, the specific growth rate, μ, of a culture can be described as:

$$\mu = (\ln x_2 - \ln x_1)/t_2-t_1$$

where: x_1 is the biomass at time t_1 and x_2 is the biomass at time t_2. Both x_2 and x_1 were measured during the exponential phase. The biomass doubling time (t_d) was determined with the following equation:

$$t_d = (\ln 2)/\mu$$

5.7 Determination of headspace gas

Gas samples from the tubes, serum vials, and Wheaton bottles were measured by a syringe for either type of reactor. Total volume of biogas from the bench-scale reactors was measured by water displacement. Samples for headspace gas analyses were obtained by directly introducing the needle of a pressure tight gas syringe into the butyl rubber stoppers on the sample containers. Prior to sampling, the pressure tight syringe was made anaerobic by flushing it with oxygen-free $N_2 + CO_2$ mixture at least three times.

Headspace gas composition was determined using gas chromatography. A GOW-MAC (Model 580) gas chromatograph (GC) fitted with a 10'x 1/8" OD stainless steel column packed with 100/120 mesh Carboseive S-II (Supelco Co.) was used to analyze the gas samples. The conditions were: thermal conductivity detector (TCD), column temperature 200°C, detector and injector temperatures 220°C, detector current 170 mA. Helium was used as a carrier gas (34 mL/min at 50 psi on the cylinder). The data were integrated by an HP integrator (Model 3396A). The calibration standard was prepared with a mixture of carbon dioxide,CO_2 (29.3%); carbon momoxide, CO (10.2%); hydrogen, H_2 (4.9%); nitrogen, N_2 (24.9%); and methane, CH_4 (30.7%). Concentration of H_2S was monitored using another gas chromatograph (Varian Model 3700) equipped with flame photometric detector (FPD).

5.8 Kinetics of Methane Production

These experiments were performed to evaluate initial biomethanation of TxL by the Mic-1, consortium. The experiments were performed in 60-mL serum vials or Wheaton bottles containing NTM and 0.01% TxL. Methane production as a function of time was evaluated.

5.9 Effect of agitation

Mic-1 consortium was grown in vials containing NTM + 0, 0.1, 1.0, 5.0, and 10% TxL. These cultures were cultivated with and without agitation (gentle mixing at 100 rpm), and each sample was monitored for methane production and chemical oxygen demand (COD). Each sample was also examined microscopically to monitor the bacterial cell populations attached to the coal particles.

5.10 Determination of chemical oxygen demand (COD)

Total soluble carbon was colorimetrically measured as chemical oxygen demand (COD) by a method available commercially from Hach Company[18]. For this study, a 1mL aliquot was centrifuged at 7000 rpm for 10 min to remove the suspended solids. An aliquot of 500 μL was added to the reaction mixture along with 1.5 mL of deionized water, incubated at 150°C for 2 hours, and the absorbance measured spectrophotometrically on a Spectronic 21 at 620 nm.

5.11 Analysis of volatile fatty acids (VFAs)

VFA were analyzed using capillary gas chromatography (Hewlett Packard 5880 A) fitted with automatic sampler according to the ASTM procedure E-260. A bonded phase, wide bore, Nukol fused silica capillary column, 15 m X 0.53 mm ID, 0.5 μm film thickness (Supelco # 2-5326) was used. The flame ionization detector (FID) was operated at 300°C. A thermal gradient program with initial oven temperature of 48°C and final temperature of 200°C was used. The initial rate was 15°C/min up to 120°C followed by 10°C/min up to 155°C and then 5°C/min to 200°C held for 20 min. The injector temperature was 205°C. Data were integrated by an HP electronic integrator. Helium was used as a carrier gas at 5 mL/min (110 psi at the pressure regulator on the cylinder). The calibration standard for VFAs was a mixture of acetic, n-butyric, iso-butyric, propionic, valeric, iso-valeric, caproic, iso-caproic, and heptanoic acids, mixed with acetone, n-butanol, methanol, ethanol and propanol. Prior to analysis, the stock solutions and samples were acidified with 50 mM phosphoric acid.

5.12 Coal analysis

Parent (original) coal and residual coal after bioprocessing were analyzed by commercial analytical laboratories.

5.13 Separation and biogasification of Texas Lignite macerals

Macerals components can be separated using various techniques such as hand-picking, sink-float, and density-gradient centrifugation[12]. The sink-float method was used in

this study to separate the three major macerals present in Texas lignite to determine which maceral fraction is more readily biodegraded to methane.

Texas lignite was demineralized using the method described by[22]. The demineralized coal was then added to a CsCl density gradient (1.6 g/mL; 1.43 g/mL; and 1.31 g/mL) in the presence of a surfactant (Brij-35), and centrifuged to separate the three maceral fractions. Macerals were washed to remove residual CsCl and surfactant before each of the three maceral types and the demineralized coal was used in biogasification experiments. Samples of demineralized lignite and each of the maceral types were submitted to Commercial Testing and Engineering for ultimate analysis.

Triplicate samples (three macerals, parent Texas lignite, and demineralized coal) at 0.1% (w/v) were added to the anaerobic NTM medium (10 ml) and were inoculated with Mic-1 consortium. The medium inoculated with the culture without coal or macerals served as controls. The samples were incubated at 37°C under static conditions. Total gas production, methane concentration, and protein concentration (biomass) were determined periodically.

5.14 Biological and chemical pre-treatment of TxL

Mic-1 was grown on TxL. After 14 days, the TxL was harvested, washed with tap water, and dried. This residual TxL was resuspended in fresh SNTM, inoculated with Mic-1 consortium and evaluated for products. In another set of experiments, TxL was extracted with tetrahydrofuran (THF) in a soxhlet. The THF soluble fraction (mobile phase, MP) and residual TxL were separated, dried, and stored. The biomethanation of THF residual, THF mobile and Mic-1 treated residual TxL was evaluated in different sets of experiments, inoculated with the Mic-1 consortium. Abiotic controls were not inoculated with Mic-1, whereas biotic control consisted of 0.2% SNTM + treated TxL + Mic-1.

5.15 Microbial resistance to inhibitors

In order to investigate the effect of inhibitory compounds leached from coal during the biogasification process at higher solids loading, 100 g of TxL were soaked for 16 hours in 1 L of 0.2% SNTM-CM (0.2% SNTM + citrate + methanol) contained in a 2-L beaker. The beaker was kept on a shaking platform (150 rpm) at room temperature. After 16 hours the contents of the beaker were filtered through Whatman filter paper #4 and the residual TxL dried at 104°C until constant weight. The leachate and residual dried TxL were used in several sets of vials as follows:

- set A ♦ Leachate + 10% untreated TxL Leachate + 5% untreated TxL, Leachate alone - as control,
- set B ♦ 0.2% SNTM-CM + 10% residual TxL(-325 mesh:micronized), 0.2% SNTM-CM + 5% residual TxL, and
- set C ♦ 0.2% SNTM-CM + 10% untreated TxL, 0.2% SNTM-CM + 5% untreated TxL, 0.2% SNTM-CM alone - as control.

5.16 Bioreactor Studies

The purpose of bioreactor (BR) studies was to generate information that could be used for preliminary economic and commercial feasibility studies on scaling-up the laboratory experiments. In all bench scale BR studies the control was NTM containing 0.2% of either YE/TSB, selected Sheffield Product, or NTM without B-vitamins solution + selected Sheffield Product. The treatments contained either 1, 5, 10, 20, or 50% TxL. After charging the culture medium (in both control and experiment reactors), all reactors were purged with oxygen-free N_2 + CO_2 (80:20) mixture. Four different basic configurations of BRs were used.

5.16.1 Rotating Biological Contactor (RBC)

A rotating biological contactor (**Figure 1**) was constructed from plexiglass and used for several experiments. The total volumetric capacity was 7.6 L and the total working volume was 6.2L. TxL (-325 mesh:micronized) was used at varying (1-10%) concentrations (w/v). Inoculum consisted of 500 mL of fresh anaerobic sewage sludge and 200 mL of Mic-1. In later experiments the inoculum was only Mic-1 or mixture of Mic-1 and Mic-4.

5.16.2 Upflow Fluidized Bed Reactor (UFBR)

The upflow fluidized bed reactor was a cylindrical column reactor with a conical bottom for more effective fluidization. The advantage of this reactor is that it provides better interaction between the microorganisms and the substrate (lignite) through pumping either the liquid culture medium, or a portion of headspace gas.

5.16.2.1 Plexiglass UFBR

In earlier studies at ARCTECH, the UFBRs constructed of plexiglass (PUFBR) were used (**Figure 2**). The total volumeteric capacity of the reactor was 700 mL, and the working volume was 550 mL. These bioreactors were used only in few experiments because of gas leaks due to improper sealing at the joints connecting various parts. Leaks of this nature made it very difficult to accurately design.

In addition, such leaks resulted in the introduction of oxygen into the anaerobic system, which inhibited methanogenesis.

5.16.2.2 Glass UFBR

In order to alleviate air leakage problems, new UFBRs were designed at ARCTECH and were constructed of glass (**Figure 3**). The advantages of using these reactors include no leakage and easy conversion from batch to continuous operation mode. The GUFBR is a glass column (H = 24" and D = 2") with ports for liquid and gas sampling. The bottom and top parts are designed in such a way that recirculation of the liquid or gas is possible. The total volumeteric capacity of these reactors was 1,250 mL, and the working volume was 800mL.

5.16.2.3 Dual UFBR

The dual UFBR is a set of interconnected "acetogenic" (ABR) and "methanogenic"

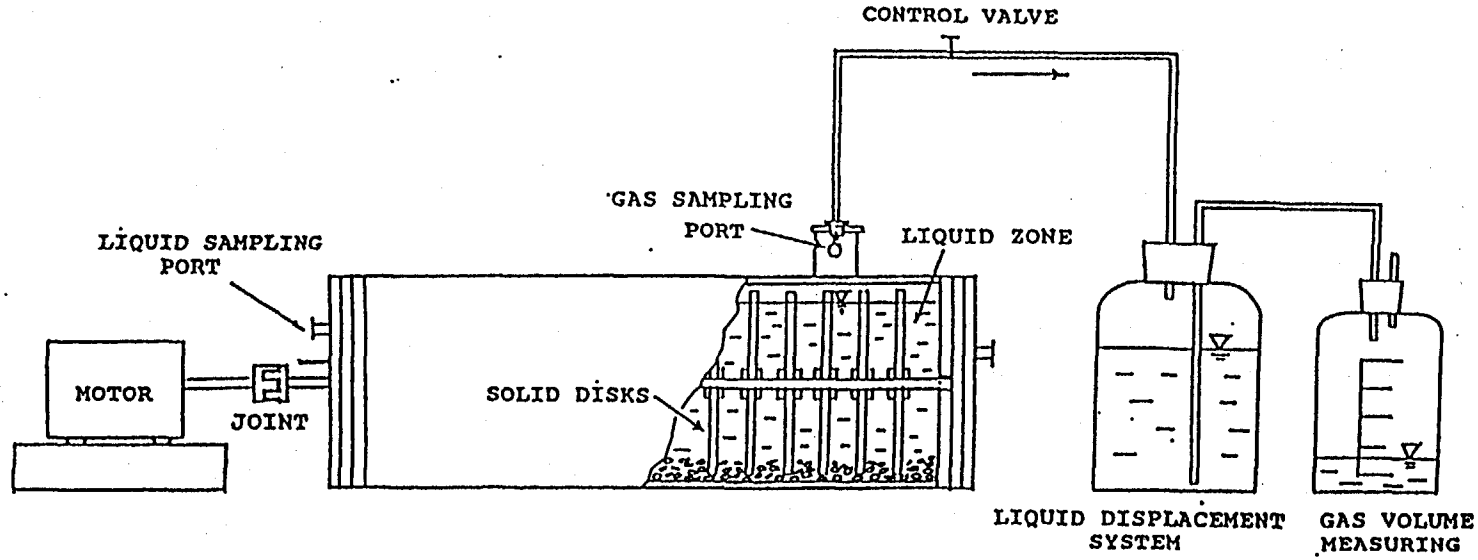


Figure 1. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Rotating Biological Contactor (RBC).

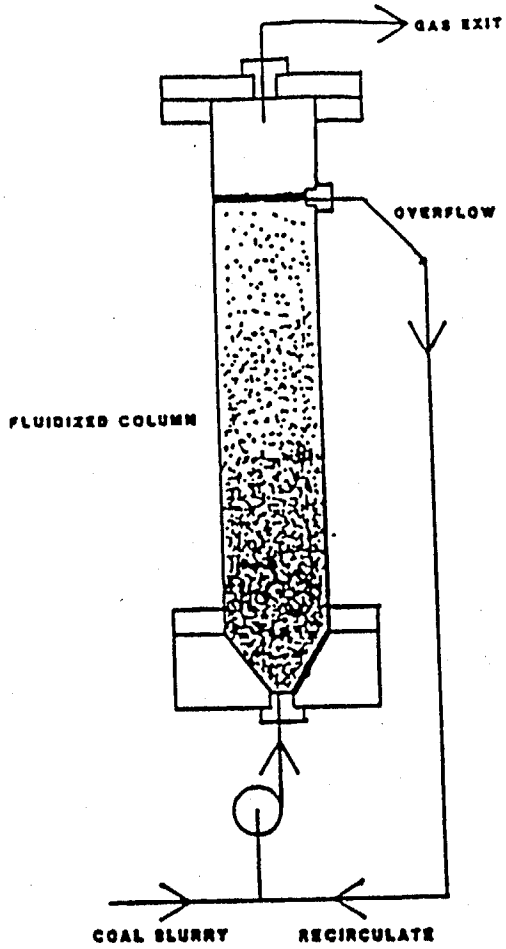


Figure 2. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Plexiglass Upflow Fluidized Bed Reactor (PUFBR).

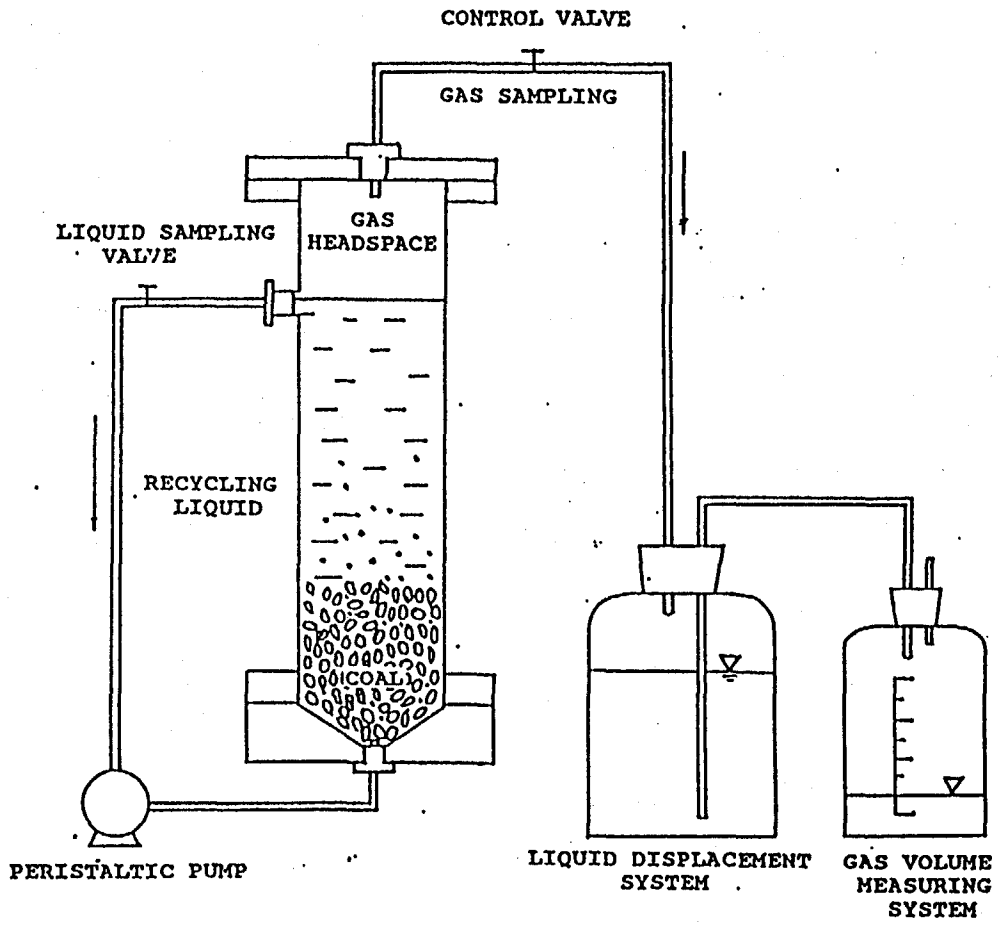


Figure 3. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Glass Upflow Fluidized Bed Reactor (UFBR).

(MBR) bioreactors. The ABR contained fermentation medium and anaerobic microorganisms, which are primary coal degraders, acedogens and acetogens (Mic-1). The MBR contained methanogens and granulated sludge consortium (GSC), specially adapted for coal bioconversion. Acedo-- and acetogens in Mic-1 consortium biodegrade TxL to acetate and lower VFAs (i.e. propionate, isobutyrate, etc.). However, accumulation of acetate in the medium can suppress the metabolism of acedo- and acetogens. Methanogens, on the other hand, convert acetate to CH_4 which allows acetogens to further convert lower VFAs to acetate. The acetate in MBRs will be consumed by methanogens for their growth and metabolism. Therefore, the hypothesis to be tested was: TxL, in the ABRs will be bioconverted into low-molecular VFAs (i.e. acetic acid) by Mic-1 consortium. At the same time, methanogens + GSC will grow and produce metabolites and some CH_4 in MBRs. After a definite period of time, the essential nutrients for Mic-1 consortium will decrease in ABR, while the concentration of acetic acid (main substrate for methanogens) will increase.This arrangement will provide better process control because of the different pH optima ranges for the two organisms. The schematic of this experiment is shown in **Figure 4.**

For the studies reported here, six GUFBR were organized into three sets (two GUFBR in each set). Each BR had a total volumeteric capacity of 1250 mL and working volume of 800 mL. All BRs contained 800 mL (final volume) of 0.2% SNTM-CM. Reactors #1, #3, and #5 (ABR) were inoculated with Mic-1 consortium (10% biomass). BRs #3 and #5 contained 5% and 10% TxL, respectively. Before addition, TxL (150 g) was pretreated with 1.5 L of water, containing 5% (v/v) methanol and 10 mM citrate. The coal-water slurry was stirred at 150 rpm for 18 hours at room temperature. Subsequently, TxL was dried at 105°C for 4 hours, cooled and used for the experiment at 5% (40 g) and 10% (80 g) solids loading. Reactors #2, #4, and #6 (MBR) were inoculated with 10% of mixed culture of methanogens + GSC. After 7 to 9 days, the medium in both ABR and MBR (in each set) was exchanged; i.e. the medium from MBR #2 (containing methanogens + GSC) was exchanged with the medium from ABR #1 (containing only Mic-1). All BRs were incubated at 35°C.

5.16.3 Trickle-Bed Reactor (TBR)

A trickle bed reactor made of plexiglass **(Figure 5)** was constructed. This reactor had a total volumeteric capacity of 2 L and working volume of 1 L. It was charged with TxL (300 g of -9 mesh, 4 g each of -325 mesh and micronized) and 700 mL NTM. The reactor was inoculated with 200 mL of sewage sludge and 100 mL of Mic-1.

5.16.4 Tank Reactors

Three types of tank reactors, continuously stirred tank, simulated anaerobic chemostat and simulated tank reactors were used.

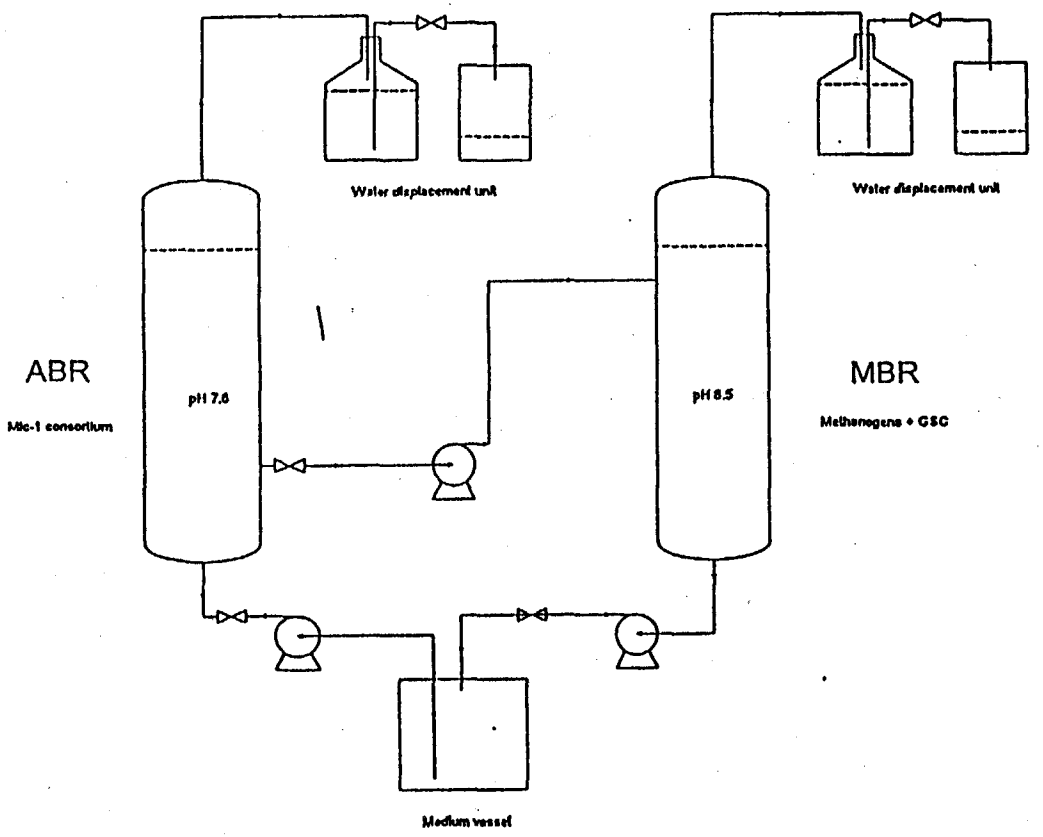


Figure 4. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Dual UFBR.

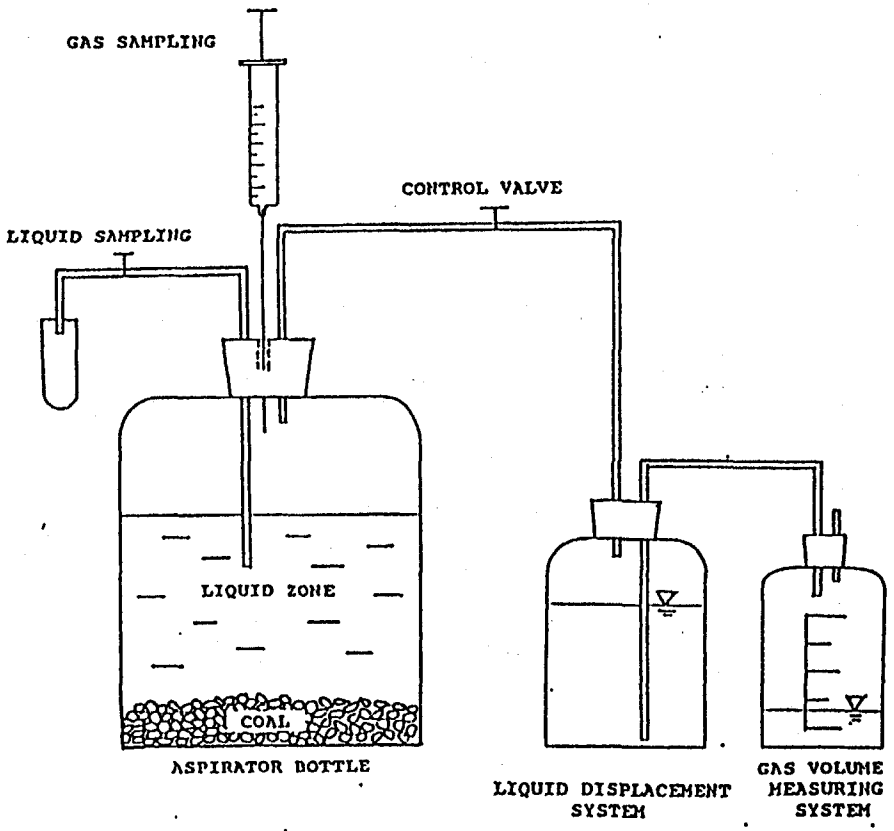


Figure 5. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Trickling Bed Reactor (TBR).

5.16.4.1 Continuously Stirred Tank Reactor (CSTR)

A Bioflo (New Brunswick Scientific Co.) fermenter with 2 L capacity vessel and stainless steel flange top (**Figure 6**) was used as a CSTR. Fermentation was carried out at agitation of 25 rpm and 37°C. One percent TxL + NTM were initially charged into the vessel and biomethanation monitored for 34 days. Periodically TxL and YE/TSB were added to the vessel so that the concentration of TxL and YE/TSB was at five and 0.2%, respectively.

5.16.4.2 Simulated Anaerobic Chemostats (SAC)

The SACs were made out of 1-L aspirator bottles that had a glass aspirator tube at the bottom (**Figure 7**). The bottles were stoppered with a two holed rubber stopper. One additional hole (port A) ran through only half of the stopper thickness. Port A was used to sample the headspace gas with a pressure tight syringe. Through one of the holes (Port B) of the stopper passed a small glass tube running into the bottle above the liquid level. This port was used for measuring total gas production by the water displacement method. Through the second hole (port C) was passed another glass tubing that ran just below the liquid level inside the aspirator bottle.

5.16.4.3 Simulated Tank Reactors (STR)

One liter aspirator bottles (**Figure 8**) simulating tank reactors were set up to monitor methane production by the indigenous sewage sludge microorganisms. The STRs contained either: sewage sludge, sewage sludge + 0.2 % SNTM, and sewage sludge + 0.2% SNTM + Tx. The STR were incubated under static and agitated conditions.

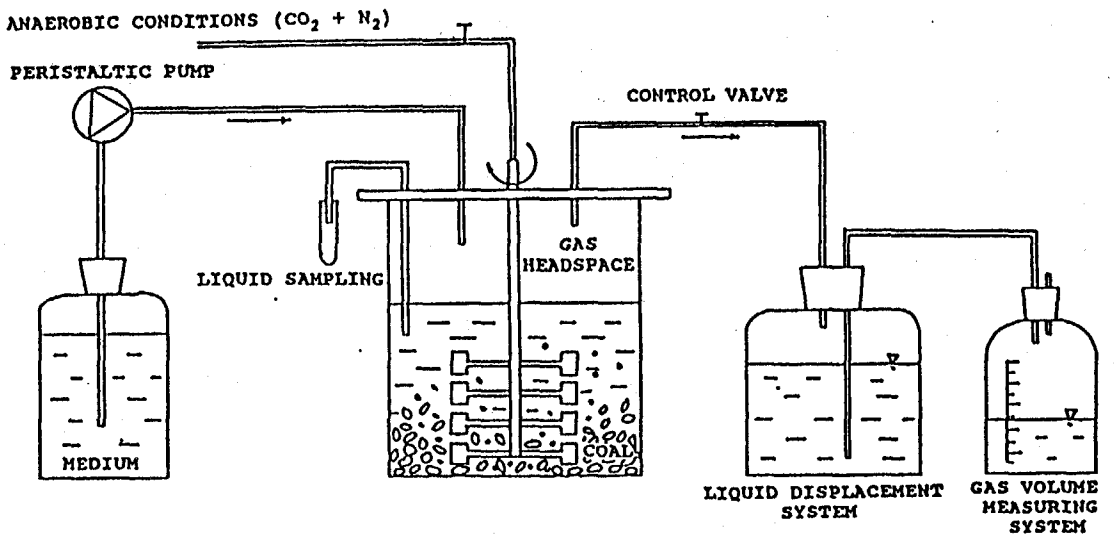


Figure 6. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Continuously Stirred Tank Reactor (CSTR).

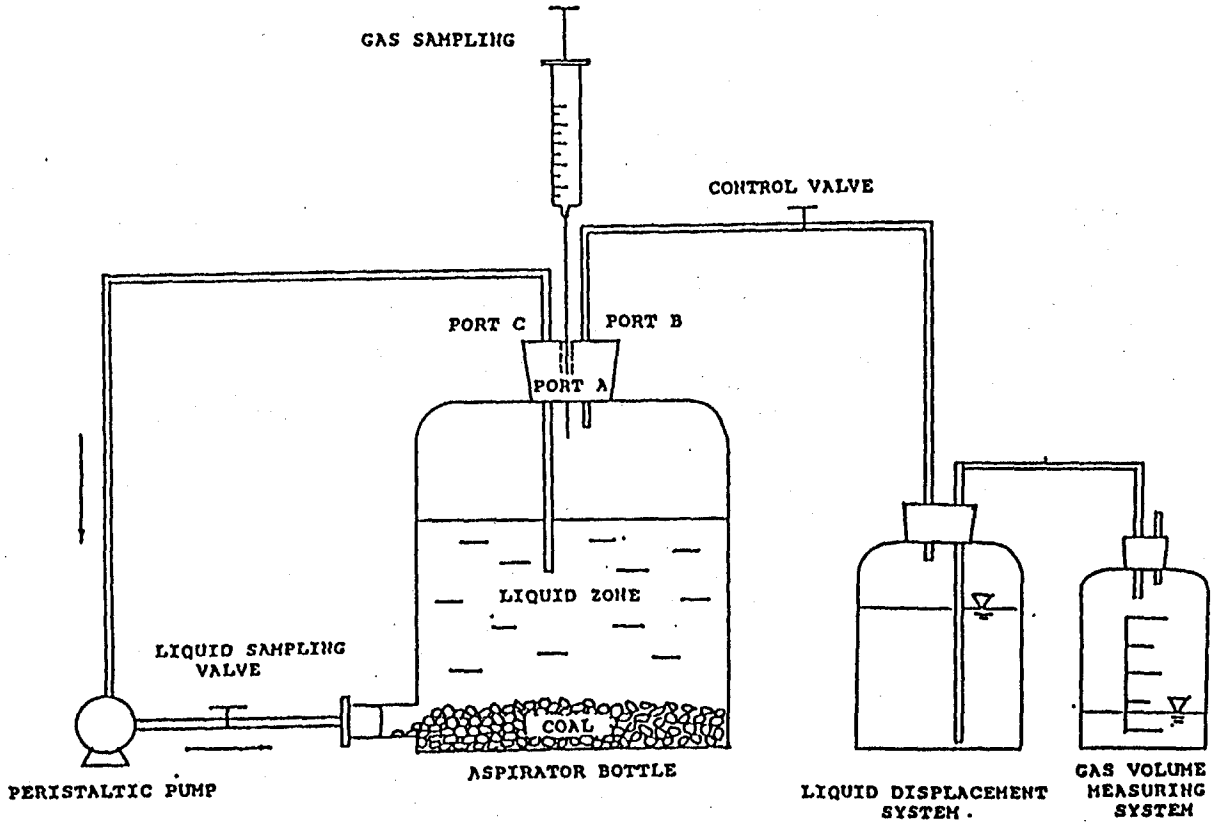


Figure 7. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Simulated Anaerobic Chemostat (SAC).

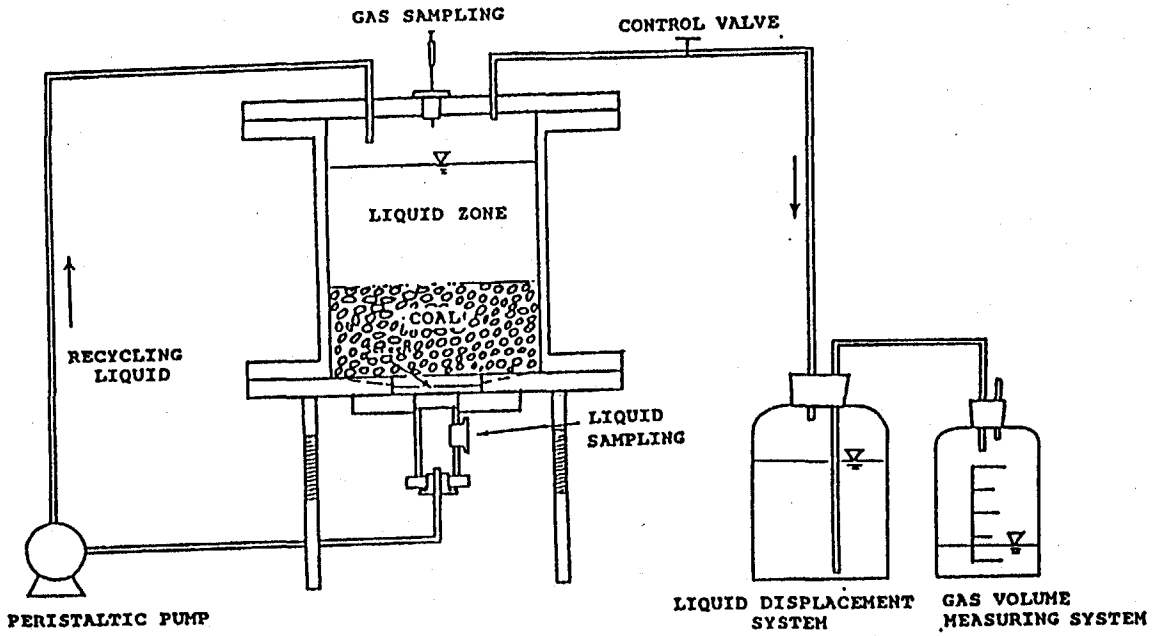


Figure 8. Experimental Set-up for Biomethanation of Texas Lignite by Mic-1 Consortium in a Simulated Tank Reactor (STR).

6.0 Results and Discussion

6.1 Kinetics of methane production

To understand the biogasification of low rank coals, several experiments were conducted to ascertain the growth characteristics of the microorganisms on coal substrates. The biogasification rates and optimal substrate concentration was also determined.

6.1.1 Adaptation of microbial consortia to coal utilization

In order to enhance and optimize the bioconversion of lignites to methane, it was essential to understand the biogasification reactions by different microorganisms. All microbial consortia (Mic-1, 2, 3, and 4) were tested on various lignites (Beulah, German brown, TxL, and Neyveli) to determine the potential biogasification process for commercial application. Most of the investigation used Mic-1 because preliminary studies showed that Mic-1, growing on TxL, appeared to be the most effective for the process. However, the original microbial consortia isolated from the termite hind gut demonstrated relatively little methane production with very long retention times. Therefore, the consortia was manipulated so that it could adapt to higher TxL loadings. This process was a step-wise procedure in which the consortium was transferred into increased concentrations of TxL.

Through adaptation, a five fold increase in methane production, from 10 to 50 mole%, was achieved **(Table 3)**. Furthermore, the methane production by the Mic-1 consortium from 1% TxL started at day 3 and reached a maximum within 21-28 days compared to approximately 2 months time in initial experiments with 0.01% TxL. The maximum rate of methane production was observed to occur between 11-14 days. These results demonstrated that the Mic-1 consortium has the ability to adapt and utilize higher TxL carbon concentrations.

Table 3. Adaptation of Mic-1 consortium

Parameter	Before Adaptation	After Adaptation
Coal Solids, %	0.01	0.1 - 1
Gas Composition, mole %	10	40 - 50
CH_4Production, cc/g coal	40	96 - 193
Time, days	60	21 - 28

Moreover, the consortium was significantly improved during the project studies. Mic-1 consortium had the potential to tolerate higher solids loading and was further enhanced to bioconvert up to 50% solids. The abilities of this consortium to tolerate high solids loading will be further discussed.

6.1.2 Growth rates and growth characteristics of Mic consortia

The growth rates of Mic-1 and Mic-3 on TxL and Beulah lignite, respectively, were determined via cellular protein. Both cultures were grown in a medium supplemented with either sodium acetate (0.5% w/v), solubilized lignite, or pulverized lignite. Solubilized and pulverized lignites were added on an equal carbon basis of 0.057% w/v. The protein concentrations in both cultures grown in the presence of lignites were higher than those with acetate. This was an indication that growth was stimulated by the presence of coal. Specific growth rates and doubling times for Mic-1 and Mic-3 are listed in **Table 4**. Knowledge of microbial growth rates was important in the process design because if the hydraulic retention time is lower than the growth rate, then microbial washout will occur. Thus, the bioreactor would be depleted of the microorganisms.

Table 4. Specific growth rates (μ) and biomass doubling times (t_d) for Mic-1 and Mic-3 growing on different lignites or acetate as a sole carbon source[a]

Consortium	Carbon source	Exponential phase, (days)	μ (days^{-1})	Doubling time, t_d (days)
Mic-1	Texas Lignite (TxL)	3-14	0.043	16.2
	Solubilized TxL	3-14	0.042	16.5
	Acetate[b]	8-18	0.061	11.4
Mic-3	Beulah lignite	9-14	0.18	3.9
	Beulah product	9-14	0.19	3.6
	Acetate[b]	8-14	0.13	5.3

[a] Protein content from medium control deducted. The numbers represent mean of three analyses.
[b] Bacterial protein from control not deducted from acetate culture

The growth characteristics of the acetate-utilizing methanogenic population(s) of the various Mic consortia were approximated by providing acetate as a sole carbon source. It was found that the acetate-utilizing methanogenic populations comprised little of the biomass in Mic-3. Biosolubilized coal product supported the growth of additional biomass (probably acetogenic microorganisms) with subsequent production of methane by methanogens. The biomass in the vials containing untreated Beulah lignite was slightly lower than that achieved with Beulah product--an indication that the consortium could be deficient in primary coal degraders (hydrolytic/fermentative).

Biomass was generally higher in the cultures grown in the presence of coal or coal product **(Figure 9)**. Therefore, bacterial growth may be stimulated by some component(s) of the coal. It was conjectured that the bacterial metabolism and growth was enhanced by the leaching of trace element(s) from coal. This hypothesis was further investigated and the results will be discussed later.

6.1.3 Characteristics of methane production

A time course acetate conversion kinetics study was conducted using a mixed bacterial culture containing predominantly *Methanothrix* sp. capable of efficiently converting acetate to methane. The *Methanothrix* culture, which were known to be present in the Mic consortia, was grown in a basal salts medium supplemented with various concentrations of acetate and YE. The culture produced methane from all concentrations of acetate, and the CH_4 production was proportional to the acetate concentration utilizedwhen using both media types. From the data obtained it was concluded that: 1) The YE concentration could be reduced to 0.05% with out negative effect on bacterial growth or methane production and 2) the growth rate was highest when acetate was at a non-limiting concentration of .1%.

There was significant difference in CH_4 production from cultures containing TxL, solubilized TxL, or those with acetate. Highest CH_4 was produced by Mic-1 grown on TxL alone **(Figure 10)**. Methane production in the cultures containing solubilized TxL was less, with a maximum of 63 cc/ g coal carbon. Despite the variability in the amount of CH_4 produced during these experiments, the data showed that maximum CH_4 production occurs between days 7 and 14. This corresponds to the time when methanogens begin active metabolism and acetate (main precursor for CH_4 production) is utilized.

Methane and acetate production from Beulah lignite and biosolubilized Beulah product by Mic-3 were also monitored during this experiment **(Figure 11)**. Nearly identical amounts of methane and acetate were produced from untreated lignite and the biosolubilized lignite product. Methane production occurred most rapidly during days 2-14, after which the rates decreased. Methane production seemed to be limited by the amount of acetate available to the methanogenic populations of the consortium.

The relatively long lag phase of the methanogenic populations has important implications for process design. During the initial coal degradation steps, when little acetate is available, significant loss of acetate-utilizing methanogens may occur. The biogasification consortium may require supplementation with methanogens after start-up or the methanogenic populations could be maintained in a separate reactor into which the coal degradation products could be continuously fed.

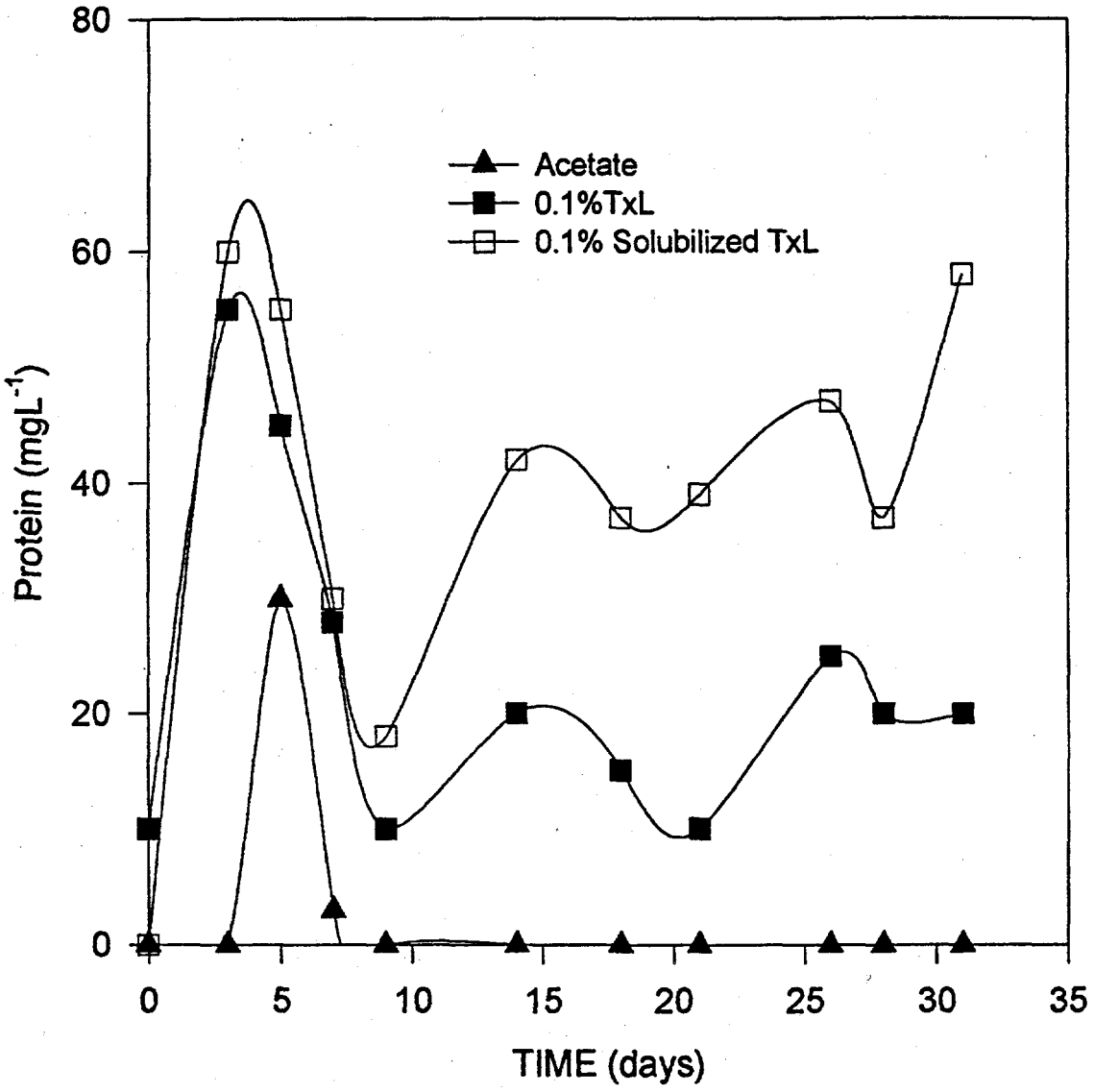


Figure 9. Growth of Mic-1 on acetate, Texas lignite or solubilized TxL as a sole carbon source.

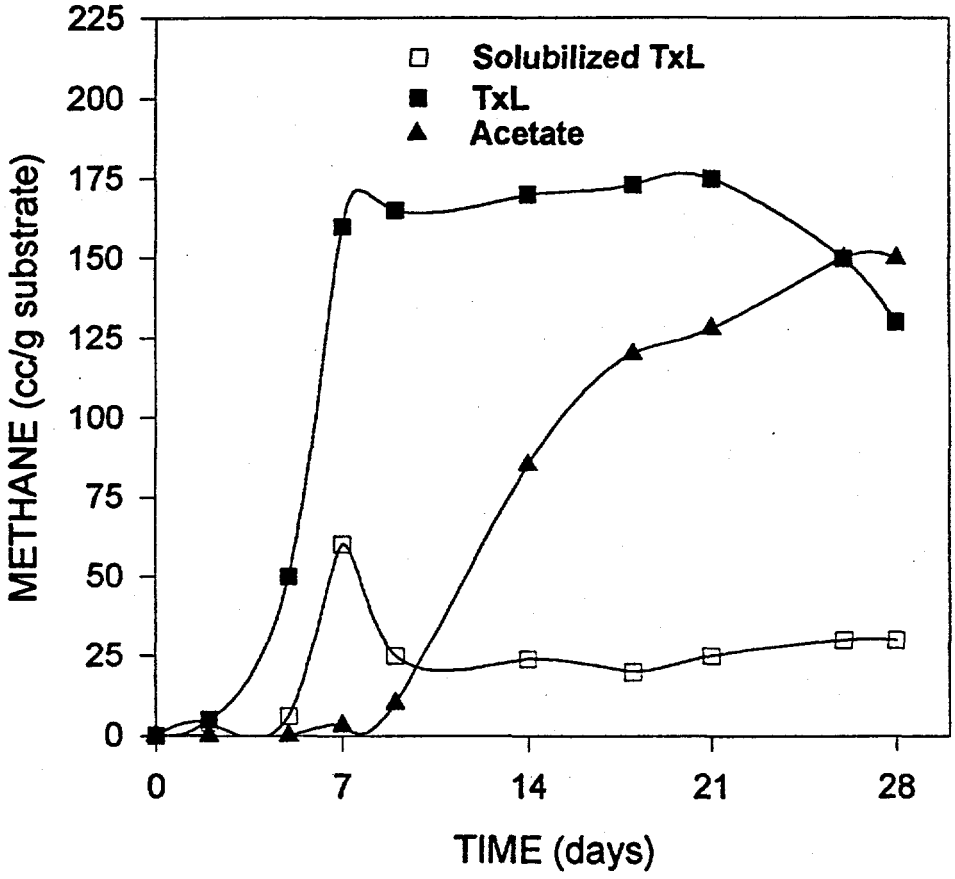


Figure 10. Methane production by Mic-1 grown on different substrates. Methane values from the coal deducted.

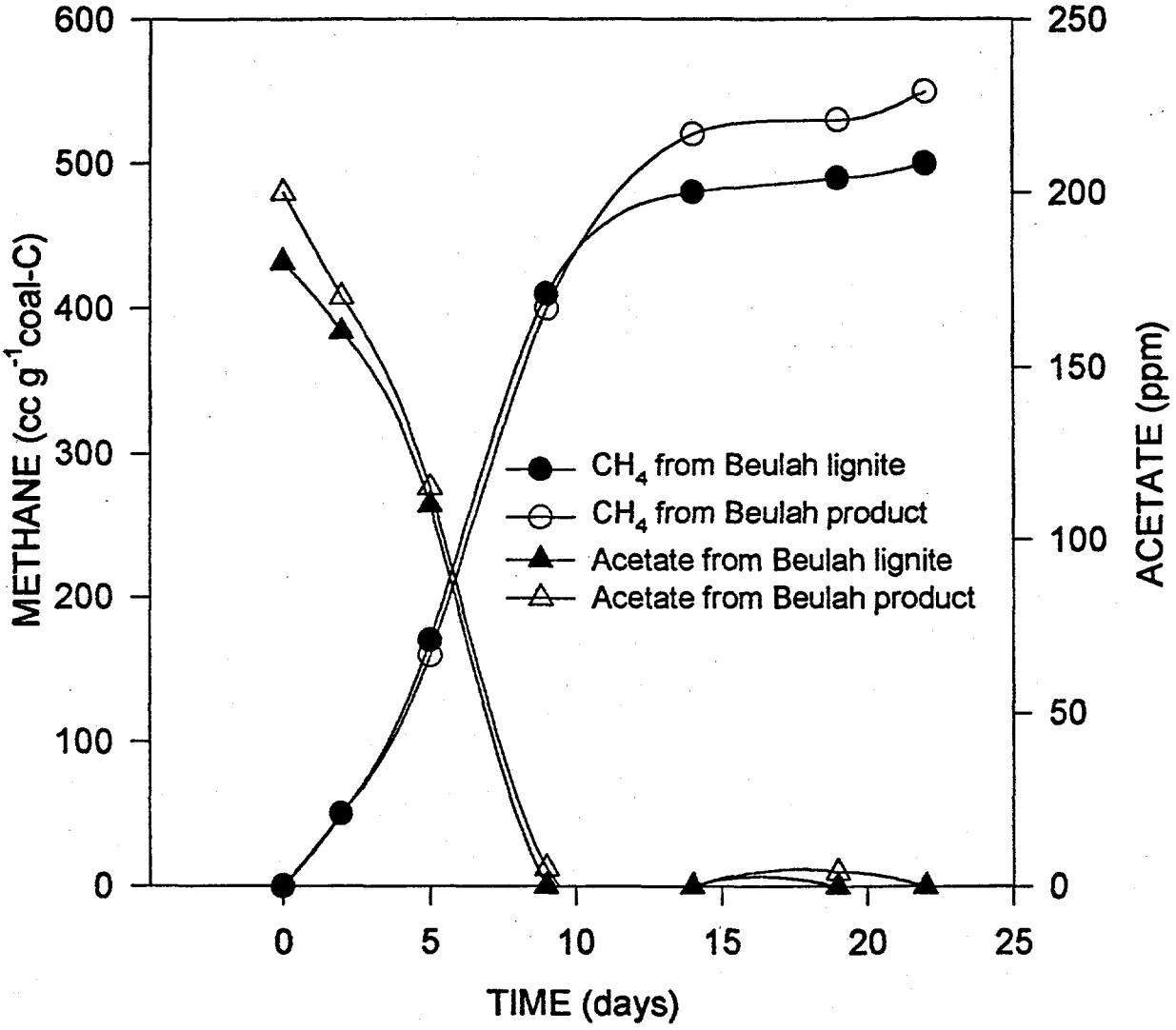


Figure 11. Biomethation of Beulah lignite and Beulah Product by Mic-3. Note the different scales of the methane and acetate.

6.1.4 Bacterial Morphology

Commonly observed morphologies constituting the Mic-1 consortium (**Table 5**) were single cells, chains, or clumps of cocci, rods (predominantly short, < 3 μm) of various lengths and thickness, curved and straight rods, both motile and non-motile rods, spore forming rods and coccobacilli. Four morphologies of methanogens were evident: *Methanococcus* sp., *Methanothrix* sp., *Methanosarcina* sp., and short rods. The short rods appeared after two weeks of incubation. As expected, the overall numbers of methanogens increased with time. Notable changes occurring in the consortium as a function of time were an increase followed by a decrease in the number of short rods, appearance of coccobacilli (possibly *Methanosarcina* sp.), and the appearance of short curved rods.

Table 5. Bacterial Morphology of Mic-1 Consortium

Predominant Morphologies	Methanogens	Major Cultural Changes
Cocci short rods (<3μm), long rods, and long filaments	*Methanococcus* sp., *Methanosarcina* sp., *Methanothrix* sp., short rods	Increase in "coccobacilli". After 2 weeks: appearance of short curved rods, increase followed by decrease in the number of short rods, increase in number of methanogens

6.2 Coal-Microbial interactions

In order to improve the CH_4 production from low rank coals, several experiments were conducted to understand how process parameters, such as agitation, substrate concentration, pH of the medium, temperature, etc., influenced the microorganisms involved in the biogasification process.

6.2.1 Coal solids loadings

Fluor Daniels performed an economic study of the MicGAS Process based on ARCTECH's laboratory scale studies on biomethanation of Texas and Beulah lignites. The Fluor Daniels study indicated a minimum solids loading of 20% lignite would be required to make the MicGAS Process economically feasible. Therefore, one of the primary goals for the biomethanation process was to increase the lignite solids loading to at least 20%.

Data on TxL biomethanation by Mic-1 consortium at solids loadings of 1% to 10% shows an inverse relationship between the TxL solids loadings and methane production (**Figure 12**). Although not encouraging, these data support previous studies at

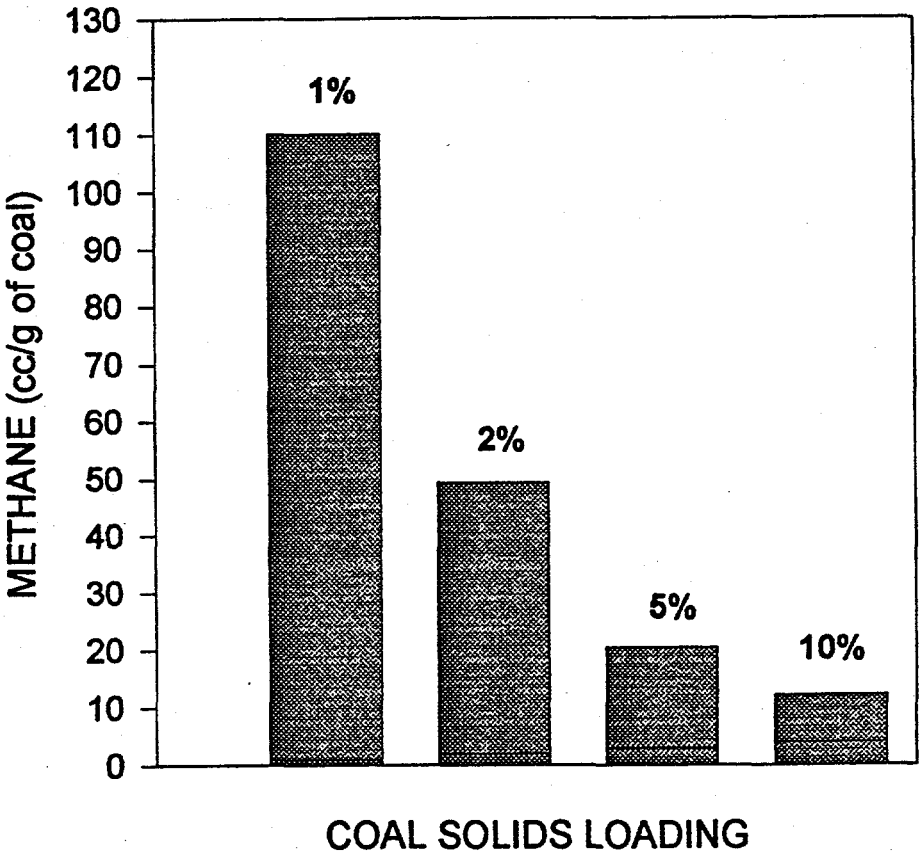


Figure 12. Methane production during biomethanetion of TxL at different solids loading by the Mic-1 consortium.

ARCTECH with Mic-2 consortium. Postulated explanation for this inhibition could be any or all of the following:

1. Inorganic materials indigenous to TxL dissolve into the culture medium, accumulate, and inhibit microbial growth and metabolism. For example, literature suggests that some TxL components such as Fe and SiO_2 [13] are inhibitory to anaerobic microbial acidogenesis [14, 20, 21].

2. Higher solids loading creates a thicker slurry that obstructs the association between the microbes and the TxL particles.

3. H_2 becomes exhausted.

4. Excessive CO_2 production changes the pH of the culture medium.

These postulations were experimentally verified during the investigation and the following was assessed.

6.2.2 Increased solids loading

The effect of coal concentrations on biogasification of TxL was further studied in vials containing 60 mL of 0.2% SNTM-CM and TxL at 5%, 10%, 15% and 20% solids loading. Mic-1 consortium was used as a 10% inoculum. Maximum CH_4 production was obtained from 5% TxL, while there was no significant difference in CH_4 production at 10%, 15% and 20% solids **(Figure 13.** At 5% solids loading, the highest CH_4 production was accompanied with increased propionate concentration during the first 14 days **(Figure 14.** A similar VFAs profile was observed in previous experiments.

The CO_2 concentration, however, significantly increased (from 500 cc to 850 cc) with increased coal solids during the experiment. Highest CO_2 production was observed in the vials containing 20% solids **(Figure 13,** and this coincides with previous observations that more CO_2 is generated at the higher solids loadings. Perhaps higher CO_2 production was due to decarboxylation of some compounds from TxL. At the same time, the reduced amount of VFAs produced during the biomethanation of TxL at higher solids loading can be explained as increased influence of inhibitory compounds released in the medium **(Fig. 14and 15).**

6.2.3 Effect of coal particle size

The effect of coal particle size on microbial growth and biomethanation was ascertained. Mic-1 was grown in 0.2% YE/TSB media containing coal samples from -28 mesh to micronized ($<10\,\mu$). No significant enhancement of CH_4 production was observed from coal particle size ranging from 595 μ to 44 μ **(Table 6).** Maximum CH_4 was obtained from vials containing micronized TxL; the micronized TxL accounted for a 47-50% increase in CH_4 production. The additional biomethanation with micronized

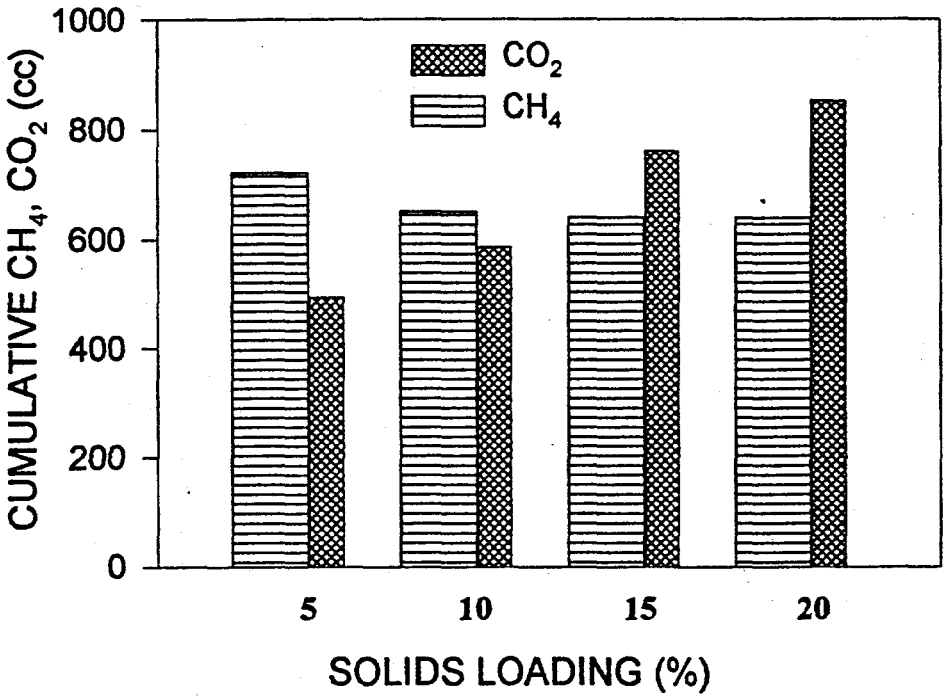


Figure 13. Cumulative CH_4 and CO_2 production during biomethanation of TxL at different solids loading in 0.2% SNTM-CM by Mic-1

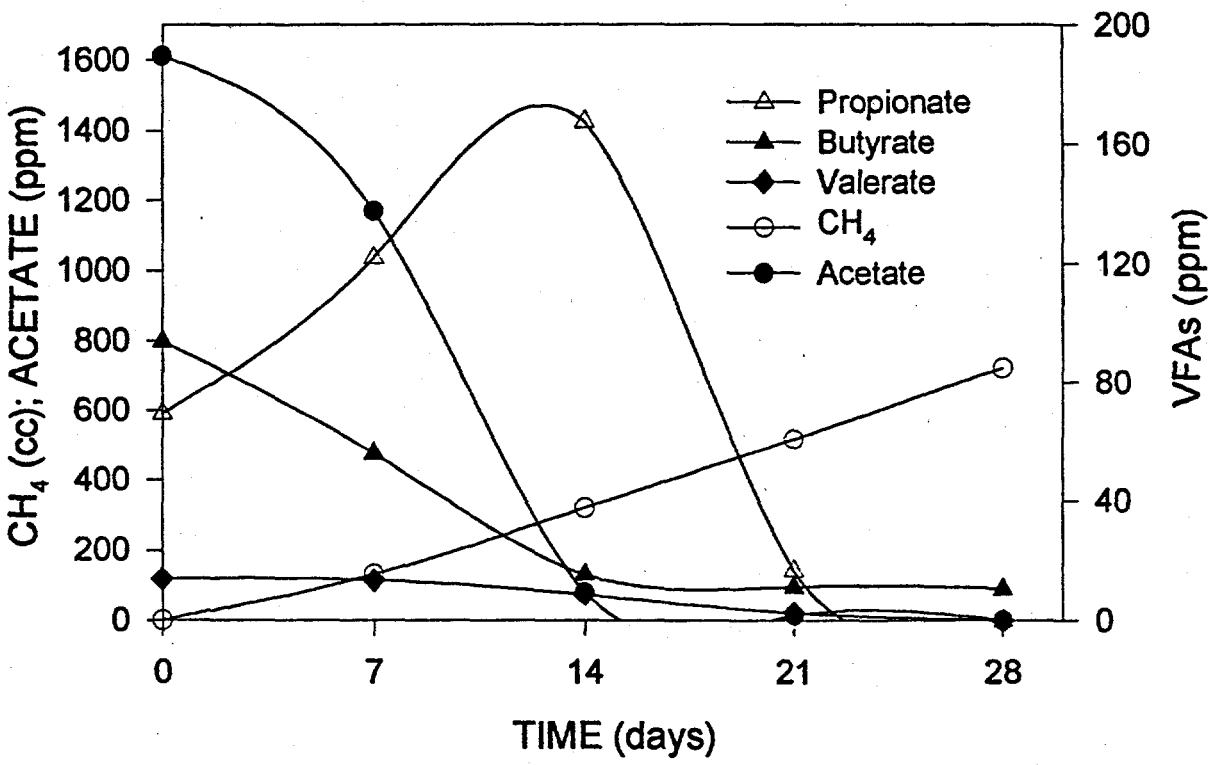


Figure 14. Effect of 5% TxL solids loading on methane and VFA production in 0.2% SNTM-CM medium.

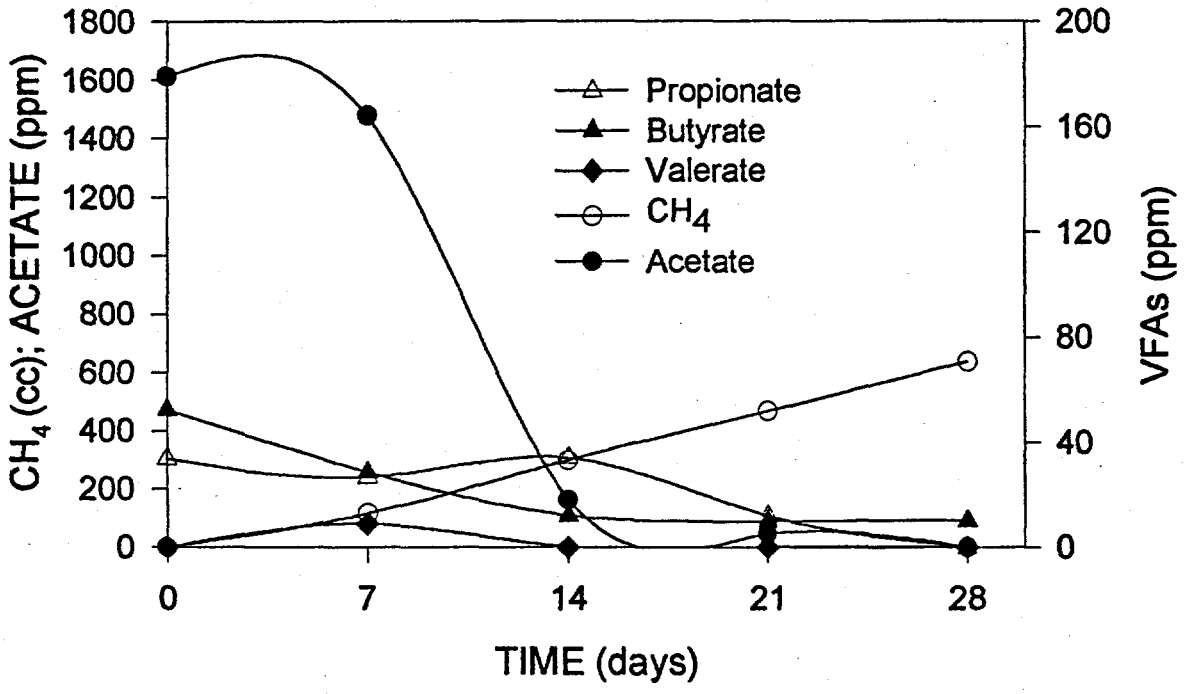


Figure 15. Effect of 20% TxL solids loading on methane and VFA production in 0.2% SNTM-CM medium.

TxL may be due to the increased surface area of substrate available to the microorganisms.

Table 6. Effect of coal particle size on biogasification of TxL by Mic-1 consortium

Particle size, mesh (μ)	CH_4/g coal, cc	CH_4/g coal-C, cc
28 (595 μ)	78	131
60 (250 μ)	85	140
200 (74 μ)	71	125
325 (44 μ)	74	130
Micronized (< 10 μ)	94	193

The effect of microbes on the coal particle size was also examined. The sizes of untreated and biologically treated TxL were compared and the data are summarized in **Table 7.** There was an overall reduction in the particle size of TxL when it was incubated with Mic-1 consortium. The least amount of -400 mesh in the treatment TxL + 0.2% SNTM + Mic-1 indicated that TxL carbon was being metabolized to CH_4. In the treatment containing additional NH_4Cl, the TxL particle size was reduced, but the coal was not being accumulated. This was indicated by lower amounts of methane produced (data not shown).

6.2.4 Effect of trace elements and coal mineral components

Studies performed at ARCTECH have indicated that the microbial biogasification process is inhibited at coal solids loadings greater than 1%. Since increasing coal solids loadings to 20% or more was a primary goal for ARCTECH's MicGAS process, it was imperative to identify which lignite component(s) cause these inhibitory effects. Several metals, including iron, manganese, magnesium and aluminum, have been found to occur in Beulah lignite at concentrations which could affect the growth of microorganisms[24]. Therefore, the effect of trace and major elements on biogasification of Beulah lignite [2] by Mic-3 was evaluated in an experiment with 0.1% Beulah lignite and several metal oxides: oxides of iron (6.2 μmoles/mL), manganese (14.1 μmoles/mL), magnesium (24.8 μmoles/mL), aluminum (9.8 μmoles/mL) and ash from Beulah lignite (1 mg/mL). Appropriate controls were prepared without metal oxides or coal ash addition. Results from the experiment evaluating methane production by Mic-3 with the various test elements are presented in **Figure 16.** Methane production was significantly lower in the cultures containing iron and magnesium oxides as compared to the control cultures. There was insignificant

Table 7. Effect of Incubation with Mic-1 consortium on the particle size of Texas Lignite

Treatment[a]	Frequency (%) of Different Particle Sizes[b] (in mesh units)				
	< 200	200	270	325	400
Untreated TxL[c]	70.97	12.18	5.00	4.76	7.08
TxL + 0.2% SNTM + Mic-1	68.86	17.10	7.50	4.42	1.86
TxL + 0.2% SNTM + Mic-1 4X NH_4Cl	58.63	13.69	6.68	3.88	17.11

[a] 0.2% SNTM medium containing 0.2% Sheftone-T™ was used with 8 g L^{-1} NH_4Cl.
[b] Pulverized TxL at 10% (w/v) Solids Loadings was used for these experiments
[c] Texas Lignite

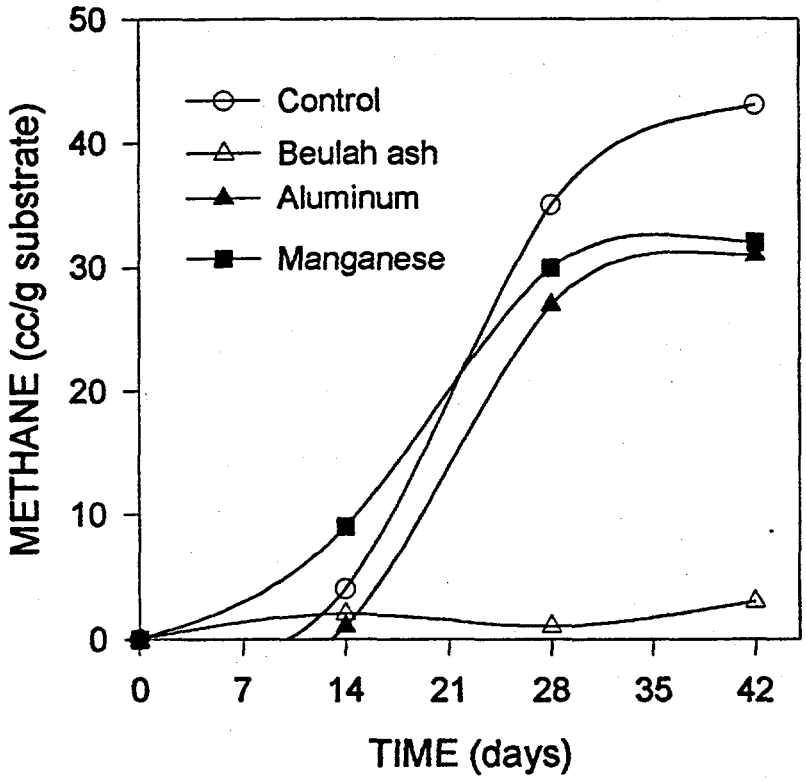


Figure 16. Effect of trace and major elements on biogasification of Beulah lignite (0.1%) by Mic-3 consortium. Methane from control deducted

difference in CH_4 production from the cultures containing manganese, aluminum oxides, and Beulah ash as compared to the controls. There was no significant difference in the cellular protein concentrations in the cultures with the various test elements.

Significantly higher levels of acetate (120-190 ppm), butyrate (20-40 ppm) and isobutyrate (20-30 ppm) were observed in Mic-3 cultures containing magnesium. This indicates that the acetate-utilizing methanogens present in the Mic-3 consortium were inhibited by magnesium. These results reaffirm the fact that anaerobic microbial acidogenesis, specially of n-butyric acid[21], a precursor of acetic acid, which is the basic substrate for methanogenesis, is inhibited by some metallic components in lignites. There were no significant differences in the VFA concentrations in the cultures containing iron, manganese, aluminum, ash, or the control cultures. In a recent publication [7], aluminum was shown to enhance methanogenesis by providing additional hydrogenesis; a process vital for methanogenesis of lignites.

These data indicated that iron and magnesium present in coal may inhibit biogasification process. This hypothesis was investigated in another experiment with TxL and Mic-1. The metals were used as salts rather than metal oxides because most metal oxides are poorly soluble in water, and therefore, controlling metal concentrations is difficult. To avoid this problem, metal salts [$FeCl_3$, $MnCl_2$, $MgCl_2$ and $AlK(SO_4)_2$] were prepared as sterile, concentrated stock solutions, and added to the medium to give a final concentration of 10 mM. However, during a preliminary trial, it was found that most of the metal salts reacted with Na_2S (used as a reducing agent in the medium) to form insoluble precipitates.

6.2.5 Biogasification of Texas Lignite macerals

Macerals are considered as the "building blocks" of coal structure. The type and concentration of maceral present in the coal is dependent upon the materials present in the swamp peat during coalification. There are 14 maceral types in all which fall into three major groups: vitrinites, liptinites (extinites), and inertinites[23]. The macerals are homogenous and exhibit unique chemical and physical properties. Of all the three groups of macerals, vitrinites are the most abundant.

Methane was produced from all three maceral types **(Figure 17)**. A maximum CH_4 was obtained from vitrinite within 20 days of incubation. Slightly less methane was produced from the extinite and liptinite maceral types over the same incubation period. The bacterial biomass also increased with time, and it is possible that the consortium members capable of using maceral breakdown products are responsible for the increase in biomass.

6.2.6 Alternate Medium Supplements

To address the economics of a coal biogasification process, less expensive medium

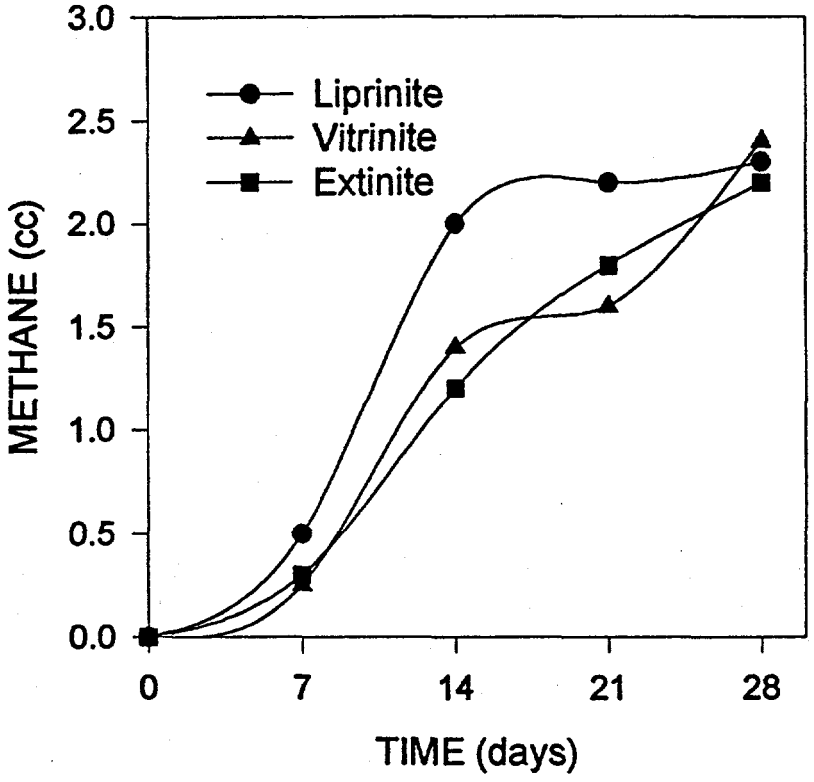


Figure 17. Biomethanation by the Mic-1 consortium from different macerals extracted from TxL.

components had to be found to replace the costly YE and/or TSB used in the NTM medium. In order to evaluate the cost effectiveness of a range of materials that could replace YE/TSB for the efficient biogasification of TxL by Mic-1 the following equation was developed...

$$\frac{(P * N)}{(M * C)} * K = \$/ ft^3$$

...where P is the price of the nutrient in dollars/pound, N is the percent concentration of the nutrient in the medium, M is the methane produced per gram of TxL, and C is the percent concentration of TxL in the medium. The constant K is a conversion factor to calculate the cubic feet of methane produced.

Using this equation, it was possible to determine the cost of the nutrient required to produce one cubic foot of methane from TxL. The YE/TSB accounted for the most of the medium cost, however, the YE/TSB was also the source for the nitrogen, trace metals, vitamins, and micronutrients required for microbial growth. The preliminary studies with various alternatives [5] to the YE/TSB indicated that Sheffield products, namely Sheftone-T™ (Sheffield Products, Norwich, N.Y.), was an economical substrate for microbial growth. In this study, Sheftone-T™ was also found to be the most economical substrate for the biomethanation of TxL by Mic-1. Hence, further studies replaced the YE/TSB in the original NTM medium and the medium was renamed SNTM. To further reduce the medium cost, the minimum concentration of Sheftone-T™ required for efficient biomethanation of TxL was also evaluated. However, the most economical and effective biomethanation was still acquired with the same 0.2% loading as the YE/TSB **(Table 8)**.

To further enhance the economics, the SNTM media was supplemented with four times (32 g/L compared to 8 g/L) the concentration of NH_4Cl. This was based on the knowledge the production costs of industrial enzymes, such as protease or lipase, can be reduced by using less expensive inorganic nitrogen constituents in the culture medium. However, additional NH_4Cl inhibited biomethanation of TxL by Mic-1 consortium **(Figure 22-B)**.

Towards the end of the MicGAS Process reporting period, still some cheaper organic nitrogen supplements were tested:

1. Gillette Foods, Inc., (NJ)
   - yeast extract 600
   - fish protein extract
   - protein extract powder 08/200

2. Pure culture products (CA)
   - boots 500

Table 8. Comparative cost of nitrogen sources for the biomethalation of TxL[a] by Mic-1

Product	Product Manufacturer	Quantity (% w/v)	Cost ($/lb)	CH_4[b]	Total Cost[c]
YE/TSB	Difco	0.20	35.45	24.2	178.82
Sheftone-T™	Sheffield	0.20	1.42	87.9	2.01
Sheftone-T™	Sheffield	0.10	1.42	36.4	2.43
Sheftone-T™	Sheffield	0.05	1.42	7.0	6.32

[a] TxL was used at 0.1% (w/v)
[b] Cumulative methane produced (cc/g TxL) after 14 days of incubation
[c] Cost (in U.S. $) of the nutrient amendment required for the production of 1 ft^3 of methane from TxL.

3. Cargill Protein products (IA)
   - soy protein products 20 PDI
   - 70 PDI
   - 90 PDI
   - soy protein products 100/90
   - soy protein products 200/20
   - soy protein products 200/70

A factional factorial method was used to test the cheapest N_2 supplement in comparison to Sheftone™. The objective was to determine which of the above products was effective in promoting significant CH_4 production cost effectively. The study indicated that Cargill 200/20 (30¢/lb) was the most effective nutrient supplement for coal biomethanation by Mic-1.

In order to further examine the medium cost reduction, Cargill 200/20 and Sheftone™, were further compared as alternative organic medium supplements. Sixty mL of NTM-CM (CM = citrate and methanol; reasons for these additions are described below) medium was placed into 125mL vials with TxL (5 and 10%), 0.2% Sheftone™ ($1.42/lb.), 0.2% Cargill 200/20 (30¢/lb), or a combination of Sheftone and Cargill (1:1 ratio).

Highest CH_4 production was obtained during biomethanation of 5% TxL in 0.2% CNTM-CM (NTM medium containing 0.2% Cargill 200/20, 10 mM citrate and 0.5% methanol). There was a slight difference in CH_4 production obtained from 10% TxL + 0.2% CNTM-CM and 10% TxL + 0.2% S+CNTM-CM. The CO_2 production was similar for all type of media used with 5% TxL and ranged from 450 cc to 500 cc **(Figure 18)**. A slight increase in CO_2 production was observed at 10% TxL **(Figure 19)**.

Data from VFA analyses showed that acetate production was a function of organic nitrogen source added in the vials containing TxL at 5% solids loading. No significant difference was observed in the vials, containing Sheftone T™ or its combination with Cargill 200/20 **(Figure 20)**. However, a significant increase in acetate and propionate production was observed in the vials containing Cargill 200/20 alone during the first seven days of the process. These results could be explained on the basis of higher C:N ratio of Cargill 200/20, which resulted in better microbial growth and enhanced biomethanation of TxL. The amount of organic nitrogen available from Cargill 200/20 was approximately 5 times higher (52.2%) than that available from the same concentration of Sheftone T™.

No significant difference in CH_4 and VFAs production was observed when 10% TxL was processed with different organic nitrogen amendments **(Figure 21)**. The results indicate that Sheftone T™ can be successfully replaced with Cargill 200/20. Therefore, the medium cost can be significantly reduced by approximately 5-fold

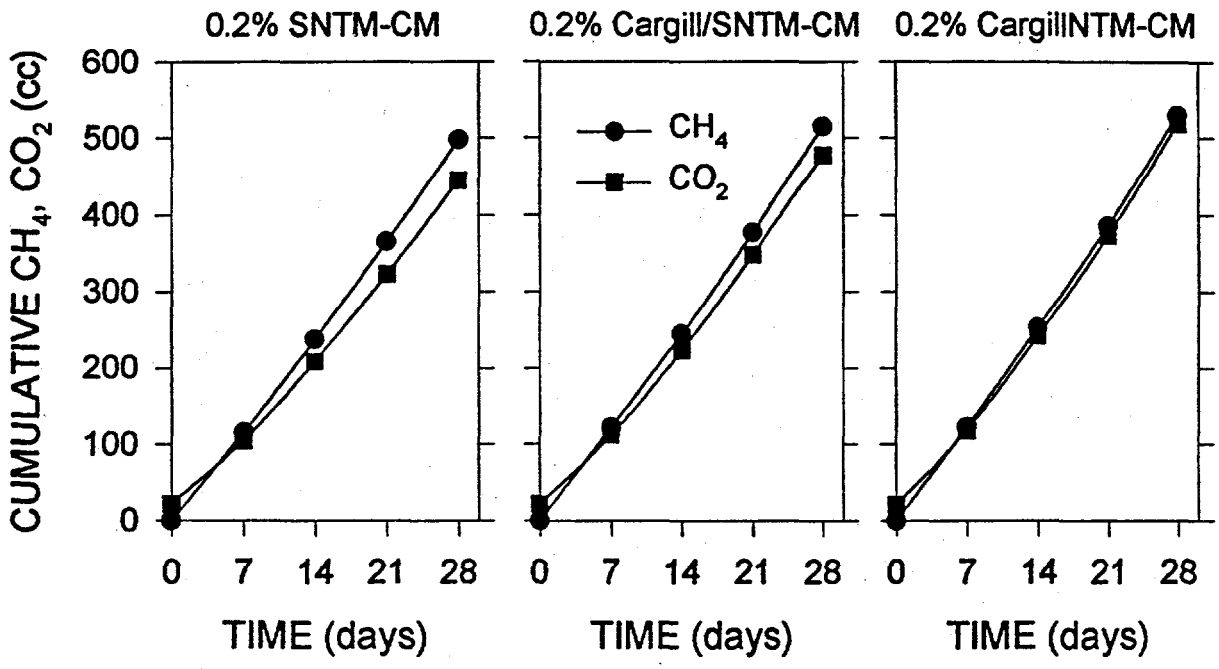


Figure 18. Effect of organic nitrogen source on CH_4 and CO_2 production during biomethanation of 5% TxL in NTM supplemented with Sheftone T™, Cargill, or a 1:1 combination.

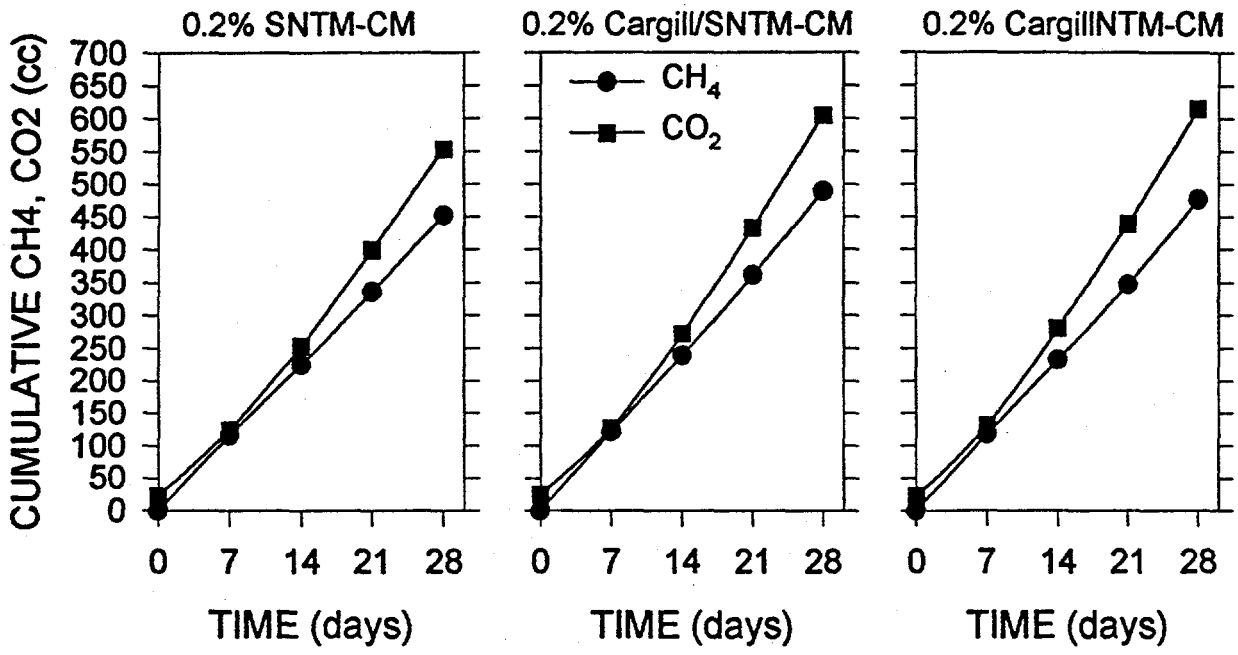


Figure 19. Effect of organic nitrogen source on CH_4 and CO_2 production during biomethanation of 10% TxL in NTM supplemented with Sheftone T™, Cargill, or a 1:1 combination.

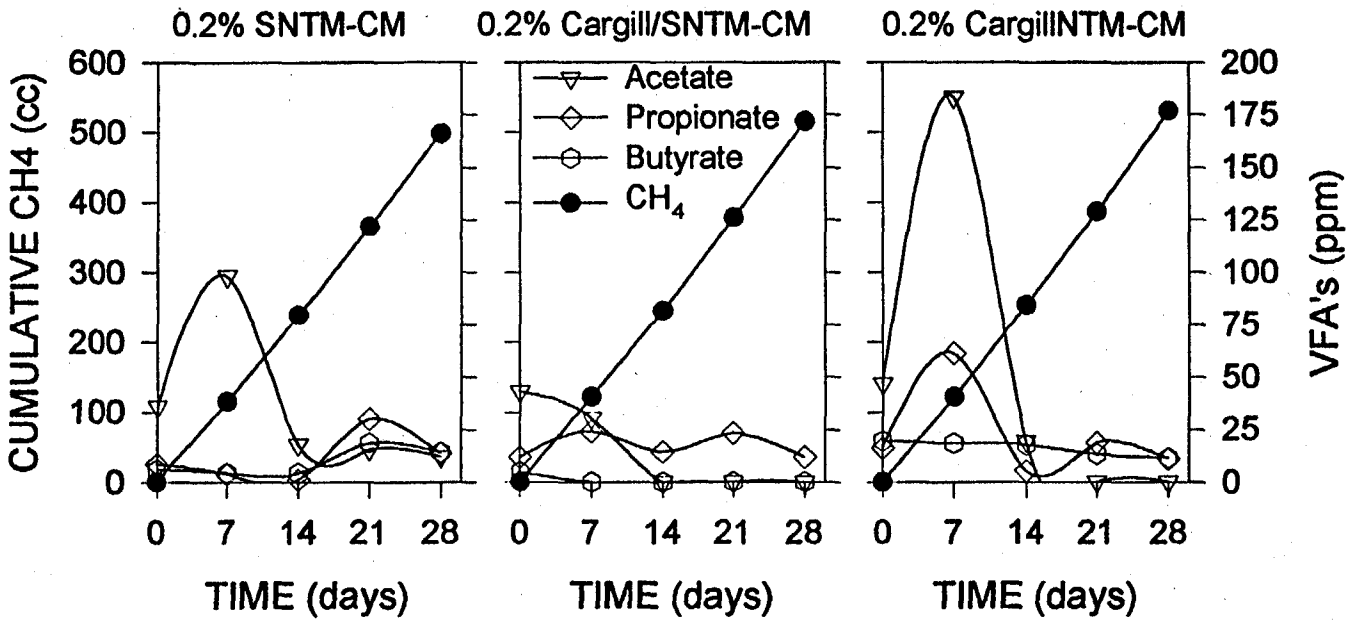


Figure 20. Effect of organic nitrogen source on CH_4 and VFA production during biomethanation of 5% TxL in NTM supplemented withSheftone T™, Cargill, or a 1:1 combination.

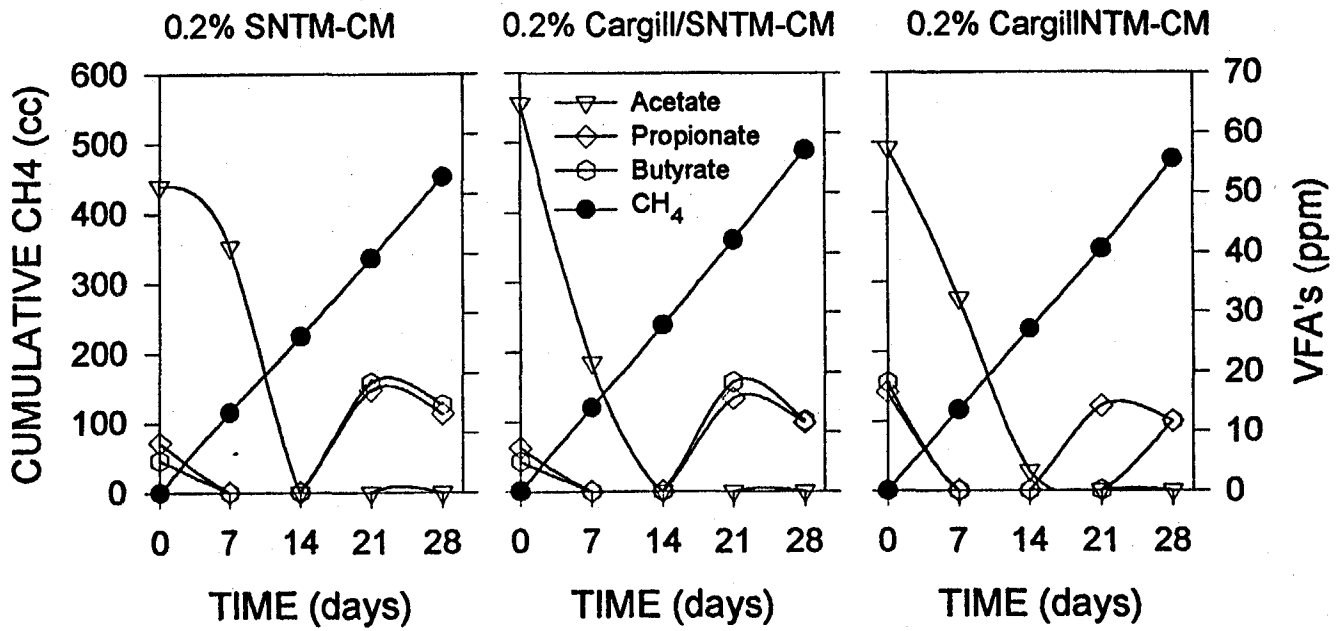


Figure 21. Effect of organic nitrogen source on CH_4 and VFA production during biomethanation of 10% TxL in NTM supplemented withSheftone T™, Cargill, or a 1:1 combination.

(Sheftone T™ - $ 1.42/lb vs. Cargill 200/20 - 30¢/lb). Overall, there was a 118-fold decrease in media cost when one compares the cost of the YE/TSB ($35.45) to the cost of Cargill 200/20.

6.2.7 Effect of B-vitamins

The NTM medium, now supplemented with the 0.2% Sheftone-T™ (SNTM), still contained the B-vitamin solution, and this addition also increased the cost of the medium. Data from Mic-1 grown in the SNTM medium, with and with out B-vitamins, showed that methane production was not affected by the absence of B-vitamin solution. Therefore, B-vitamins were not used in the process.

6.2.8 Effect of pretreatment of Texas Lignite on biomethanation

Lignites are assumed to have a micro- and a macromolecular components. Based on the results form studies at ARCTECH, especially at higher solids loadings, it is conjectured that the Mic-1 population was capable of utilizing only the micromolecular and not the macromolecular fraction of TxL. This postulation was verified by removing the micromolecular fraction either by biological or chemical treatment (*see* METHODOLOGY).

The data indicates that methane production was 1.5 fold higher in experimental vessels containing residue from biologically pretreated TxL than those containing residue from chemically (THF) pretreated TxL **(Figure 22-A)**. Biogasification and biomethanation was also inhibited in the THF soluble (mobile) fraction even though the THF was removed from the mobile fraction **(Figure 23)**. This result could be a manisfestation of incomplete removal of THF after during the pre-treatment of TxL. The most encouraging data from these experiments was that methane production was not inhibited at higher (1% vs. 5%) TxL solids loadings which were biologically pretreated **(Figure 22)**. These results are significant for numerous reasons. First of all, process economics is improved because the residual TxL can be recycled. Furthermore, this information might be a prelude towards unfolding the mechanism of biological attack on the TxL. In the absence of a detailed molecular fractionation of TxL, these data provide preliminary support to the hypothesis that the initial population of Mic-1 could not attack the macromolecular fraction of TxL, however, biological pretreatment makes the macromolecular fraction of TxL more amenable to Mic-1 consortium.

The effect of temperature treatment of substrate (lignite) was studied in vials containing NTM and TxL at 1% and 10% solids loading. One half of the vials containing TxL were autoclaved. All vials were inoculated with Mic-1 consortium. CH_4 production was monitored in both autoclaved and unautoclaved vials containing coal. Higher CH_4 was produced in the presence of 1% autoclaved TxL **(Figure 25)**. CH_4 production in unautoclaved vials were less, which indicated that during autoclaving TxL was partially solubilized and provided better conditions for microorganisms (Mic-1) to grow and bioconvert TxL into CH_4.

6.2.9 Effect of agitation

Since methane production was inhibited by solids loading greater than 1%, it was

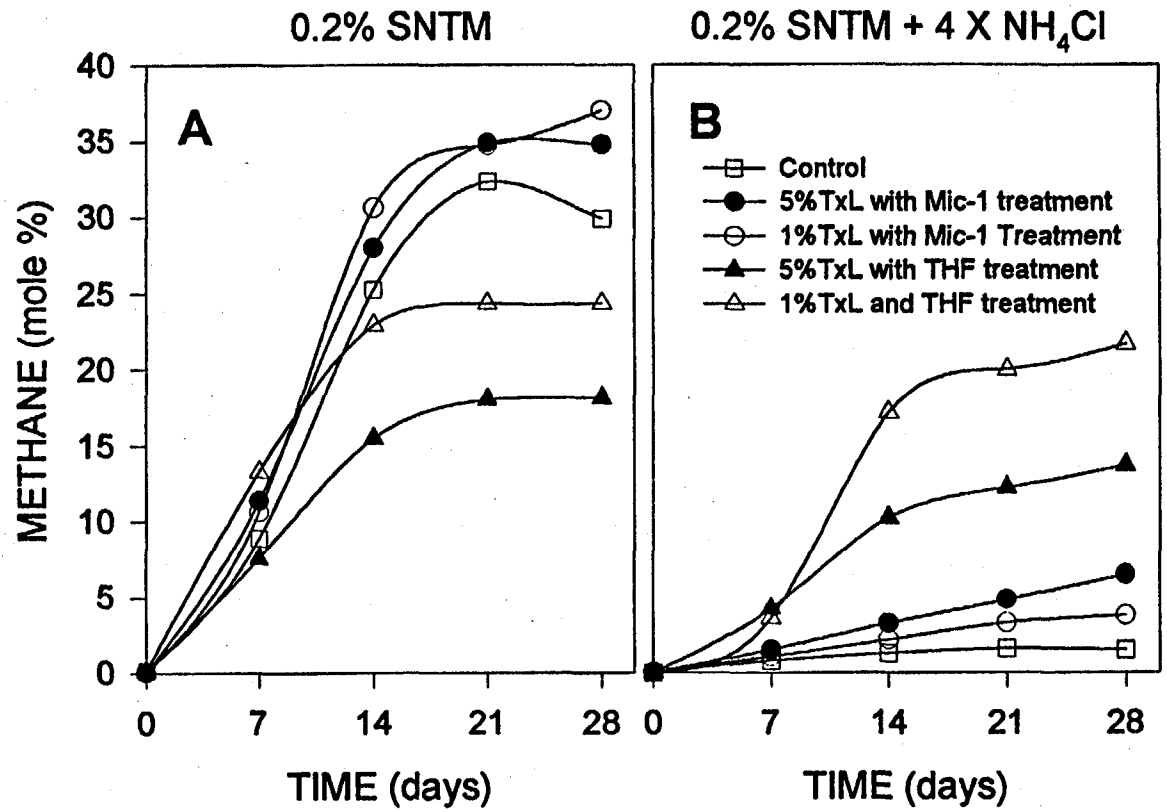


Figure 22. Methane production from biologically (Mic-1) and chemically (THF) pretreated TxL at 1% and 5% solids loading in two media

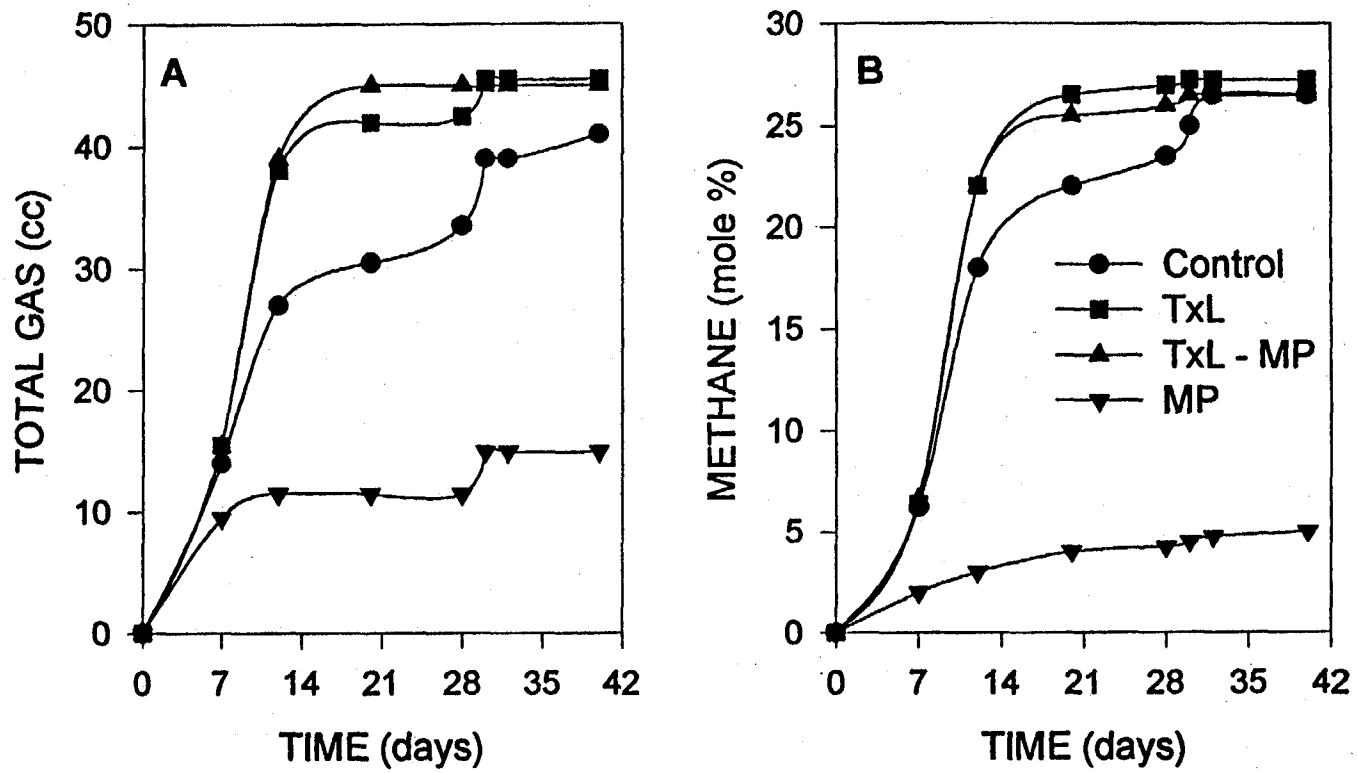


Figure 23. Total gas and methane production from chemically pretreated (THF) TxL at 0.1% solids by Mic-1 consortium. The mobile phase (MP) was extracted from TxL (TxL - MP).

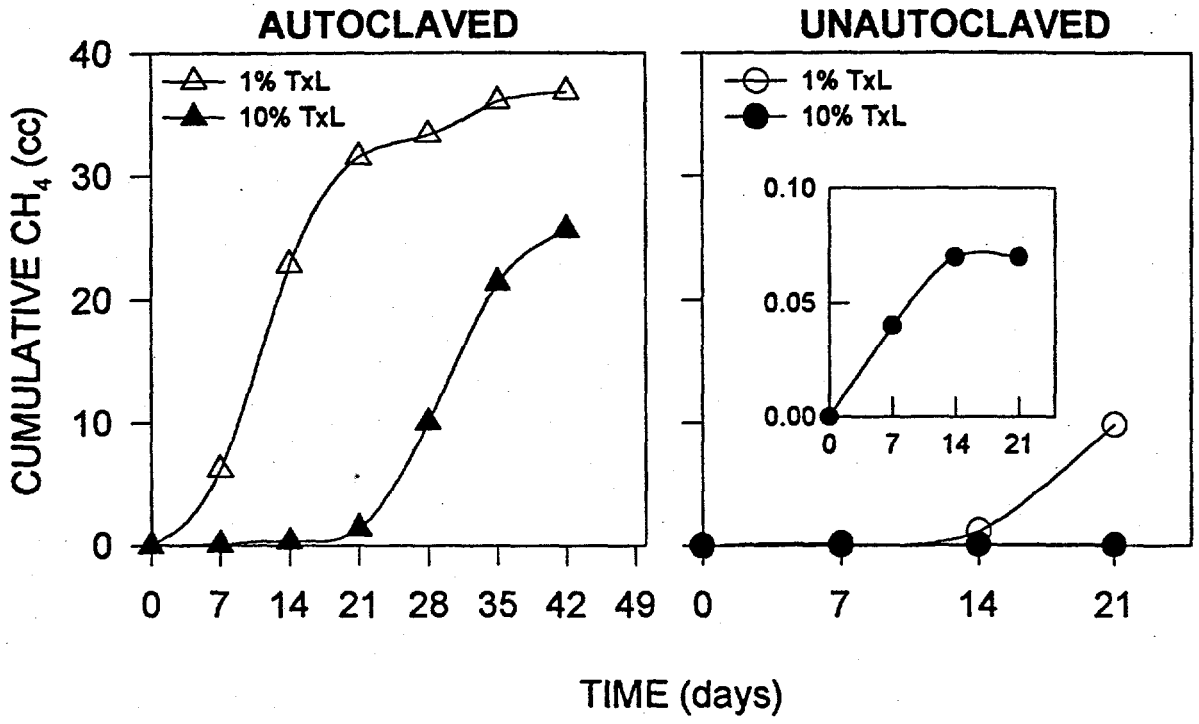


Figure 24. Effect of autoclaving on 1% and 10% TxL in NTM medium. Insert in graph shows a close-up of the methane production with 10% TxL. Note the diferences in scale on the insert

hypothesized that agitation may be required at the higher solids loading for optimal contact between the coal particles and microorganisms.

6.2.9.1 Effect of agitation on methane production

Among the three TxL solids loadings (1, 5, 10%) tested, methane production was highest in the samples containing 1% TxL and lowest at 10% TxL (**Figure 24**). Cultures growing in the 1% TxL produced approximately 80-85 cc CH_4/g TxL, whereas cultures growing in the 10% TxL produced only 3-5 cc CH_4/g TxL. However, no significant difference in actual amounts of methane production were observed between the static and agitated cultures. An interesting observation was that the static cultures reached the maximum CH_4 production approximately seven days sooner than agitated cultures. Therefore, agitation did not increase methane production at the higher TxL loadings.

6.2.9.2 Effect of agitation on COD and biomass production

The effects of agitation on the chemical oxygen demand (COD) and the biomass production are presented in **Table 9**. In all cases, the COD, which measures the total soluble carbon, decreased throughout the course of the experiment. Substrate utilization was most efficient at the 1% TxL loading. The control cultures (0% TxL) demonstrated a higher COD than the ones containing TxL. This suggested that, in contrast to the controls, the soluble carbon from TxL was utilized by the Mic-1 consortium. No significant difference in COD was seen between static and agitated samples.

Similar to the COD results, little difference was seen in the biomass production between static and agitated samples. The biomass production was greatest at the 1% TxL loading. These similarities in COD trends demonstrate that cellular growth was being supported by the TxL solubilized carbon, and TxL solids loadings greater than 1% inhibit both growth and COD. The cellular protein and COD data, coupled with the observations on methane production (**Figure 12**) by Mic-1, strongly indicate that the methanogenic population was inhibited by one or more components at higher TxL solids loadings.

6.2.10 Effects of autoclaving

Another important observation was that even before adding the Mic-1 inoculum to the culture vessels, the CO_2 concentration in the headspace gas of the cultures containing 5% and 10% TxL was significantly higher than those containing less TxL. This could be attributed to the fact that all serum bottles were autoclaved (sterilized) and more CO_2 would be released in the bottles containing the higher TxL loadings. Therefore, immediately before inoculating, all culture vessels were aseptically reflushed with the oxygen free N_2 + CO_2 (80 : 20) mixture to ensure that the headspace gas was consistent in all the treatments. After 28 days of incubation, the CO_2 concentration in the cultures grown on 5% TxL was 34 to 36 mole % as compared to the 20-25

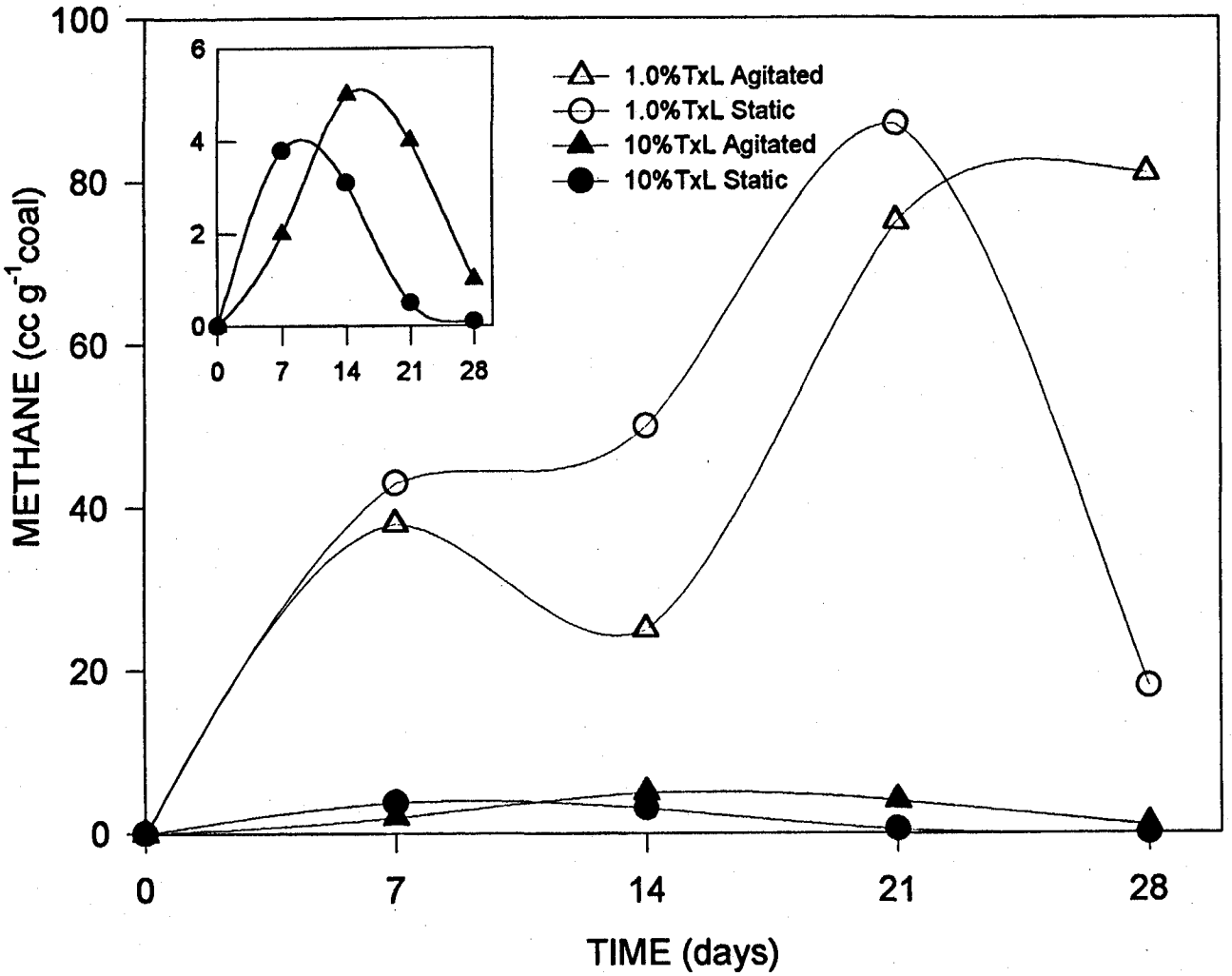


Figure 25. Biomethantaion of 1% and 10% TxL under static and agitated conditions. Insert shows close-up of the biomethanation of 10% TxL

Table 9. Total soluble carbon and biomass productionof Mic-1 Cultured in static or agitated mode at different solids loadings of Texas Lignite (TxL)

Solids Loading (%)	*Soluble Carbon (ppm)*[a] *in the Two Culture Modes*						*Biomass*[b] *in the Two Culture Modes*					
	Day 7		Day 14		Day 21		Day 7		Day 14		Day 21	
	Static	Agitated	Static	Agitated	Static	Agitated	Static	Agitated	Static	Agitated	Static	Agitated
0.0	1,156	1,100	603	733	441	499	47.7	58.6	36.7	51.7	55.3	63.1
0.1	941	866	392	454	155	216	67.8	68.5	64.2	69.1	78.8	79.0
1.0	667	717	238	281	130	138	81.2	84.4	114.1	116.1	87.2	115.1
5.0	935	993	415	369	268	259	60.8	56.1	72.9	76.1	62.0	69.4

[a]Chemical Oxygen Demand (COD) in the liquid phase. Data represents average of three replicates
[b]Total cellular protein in ($\mu g\ mL^{-1}$) in liquid phase. Data represents average of three replicates.

mole % in other culture vessels. Since the concentration of CO_2 in the headspace is vital to methanogenesis, one would expect that the higher CO_2 amounts would lead to increased CH_4 product. However, despite higher CO_2 at 5% TxL, less CH_4 was produced. This observation led to two hypotheses:

- The system was deficient in H_2 -- another essential limiting factor for biomethanation, and
- the increase in CO_2 decreased the pH of the culture medium. These factors were further investigated in a series of experiments with abiotic, autoclaved, and unautoclaved conditions.

6.2.11 Effect of Temperature

The temperature optimum for maximal growth of acetogens was determined in experiments conducted at 30°C, 35°C, and 40°C. As these cultures were derived from mesophilic environments, the expected temperature optimum for growth and activity was expected to be in this temperature range. The temperature optimum for methanogens used in this study is well known to be 37°C, and was not the subject of this study.

The COD was much higher in the autoclaved treatments containing 10% TxL **(Figure 26)**. These data clearly explain that the inhibition of biomethanation at higher solids loading is related to TxL components one of which could be CO_2. Another important indication was that the pH of culture medium needs to be buffered for efficient biomethanation of TxL, since the pH of the autoclaved media decreased drastically.

6.2.12 Effect of pH

Previous studies indicated that solids loading of $\leq$ 5% (w/v) did not significantly influence the medium pH. However, at solids loadings of $\geq$ 10%, pH of the reaction broth decreased sharply after the addition of TxL.

The mechanism of lowered pH during the addition of Texas lignite (TxL) at higher solids loading was investigated. TxL was treated with different solutions in order to modify the initial pH of TxL. Samples of pulverized TxL (20 g each) were rinsed with tap water, 0.1 M $NaHCO_3$ (pH 8.40), 0.1 M $NaHCO_3$ + 0.1 M Na_2CO_3 (pH 10.23), and 0.1 M Na_2CO_3 (pH 11.04) solutions. The TxL samples were treated for 1 minute to 16 hours with these solutions and then dried at 104°C. The pH of the reaction

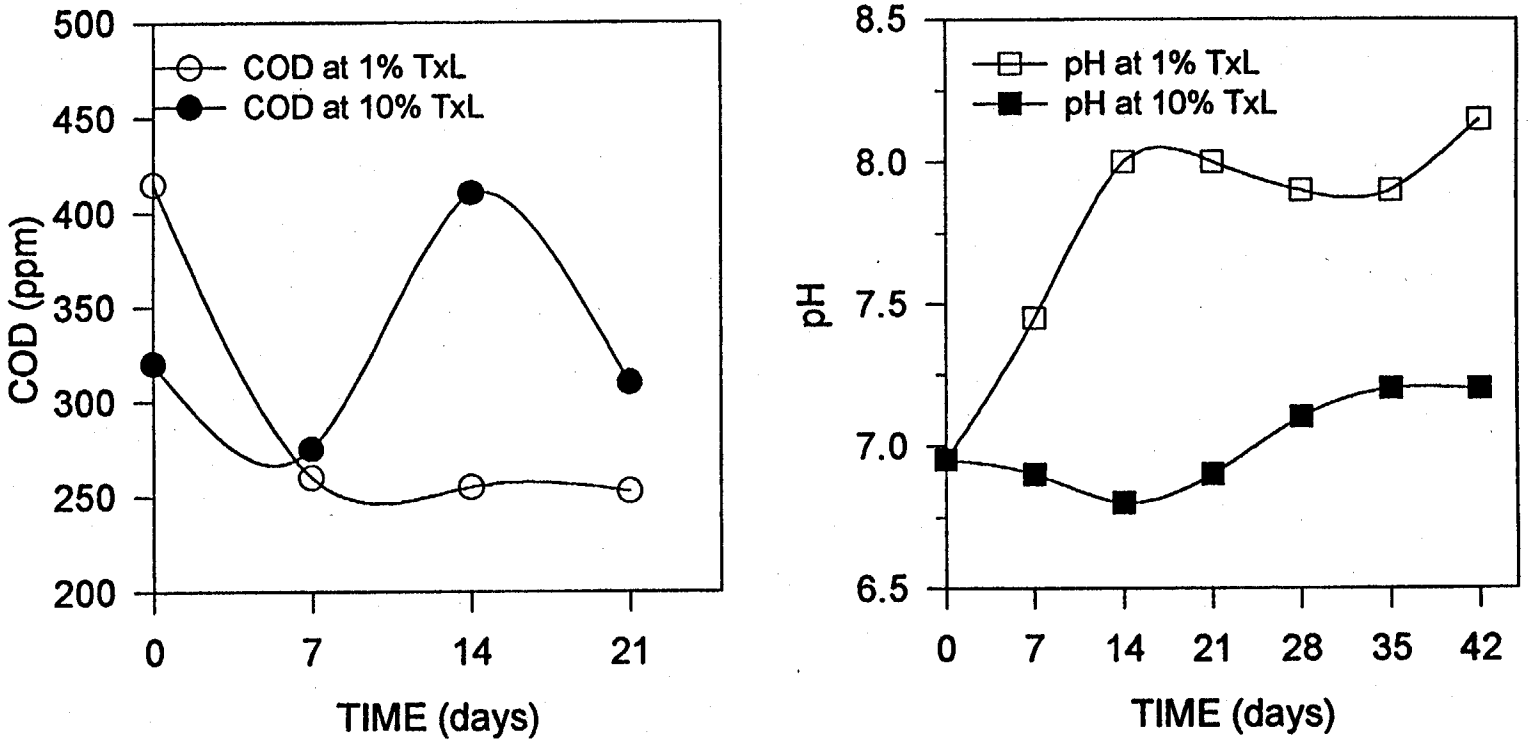


Figure 26. COD and pH of autoclaved 1% and 10% TxL in NTM medium by Mic-1.

mixture changed for each treatment (**Table 10**), and significant changes were observed in the color of the solution as well as in the washed residual TxL after drying at 104°C.

Another experiment to study the mechanism of pH lowering during biomethanation of Texas lignite (TxL) at higher solids loadings was conducted in 60-mL vials containing 40 mL of 0.2% SNTM and 10% TxL. The initial pH of the medium in both control and experimental vials was modified with 1 N HCl or 1 N NaOH to the following values: 6.5, 7.0, 7.5, 7.8 (no correction), 8.0, 8.5, and 9.0. Every seven days the control and experimental vials were monitored for total gas production, gas composition, COD, VFA, and pH changes. At ≥10% TxL solids loading, the pH of the reaction mixture decreased to 6.6 -7.6 within one hour even though the pH of the medium was initially modified to 8.5 - 9.0. This is interpreted as the effect of TxL addition. The different initial pH of the medium in both control and experimental vials did not significantly influence CH_4 production. Cumulative CH_4 production in the experimental vials containing 10% TxL at different pH values varied from 10 to 22 cc after 21 days of static cultivation (**Figure 27**). The highest CH_4 production was observed in the experimental vials without any modification of initial pH of the medium and was 53% of the CH_4 produced in the control vials. The COD measured in all experimental sets were significantly higher (1725 mg O_2/L when the initial pH of the medium was modified to 9.0) as compared to the controls (58-93 mg O_2/L, **Figure 28**). This is interpreted as higher soluble carbon in the medium released from the TxL biodegradation. When initial pH of the medium was adjusted to 9, the COD/CH_4 ratio was in excess of 440 in the experimental vials containing TxL. In contrast, the COD/CH_4 ratio for the control vials was 13-19. This indicates that correction of the initial pH of the medium can enhance biomethanation of TxL by releasing more soluble compounds in the medium and enhancing the growth of microorganisms. The changes in the pH after the initial correction were not significant and varied from 7.0 to 7.2 (**Figure 29**).

6.2.13 Effect of co-substrates

6.2.13.1 Effect of chelators, sequesters, and surfactants

Coal and some of the coal by-products, like biphenyl, are considered xenobiotics. Literature reports indicate that modifying the surfaces of xenobiotics, such as TxL, makes them more amenable to biodegradation[3]. Therefore, treatment of TxL with chelating agents and/or surfactants may improve the MicGas Process by either:

1. making the coal substrate more accessible to microbial degradation through physical means and/or,

2. chelating inhibitory products such as metals.

Table 10. Effect of different solutions on the pH of the TxL slurry during treatment of 20% Texas lignite *

Time (min)	Tap water + 20% TxL 1	0.1 M $NaHCO_3$ + 20% TxL 2	0.1 M $NaHCO_3$ + 0.1 M Na_2CO_3 + 20% TxL 3	0.1M Na_2CO_3 + 20% TxL 4
0	7.96	8.40	10.23	11.04
1	3.90	5.70	6.90	7.35
15	3.90	5.28	6.84	7.40
30	3.90	5.21	7.15	7.65
45	3.90	5.13	7.20	7.77
60	3.80	5.10	7.30	7.80
960	3.80	4.92	7.30	8.20
Observations	*Slight change in the color of solution. After drying - no change in the color of TxL*	*Color changed; partial solubilization (yellow). After drying seems more "black"*	*"Foam" formation; solubilization; dense solution with dark brown color. After drying "black" TxL*	*"Foam" formation; "dense" solution with black color. After drying looks like charcoal*

* Experiment was carried out in 300-mL flasks containing 100 ml of respective solution with TxL at 20% (w/v) solids loading. The flasks were incubated for 16 hours on a rotary shaker (150 rpm) at room temperature.

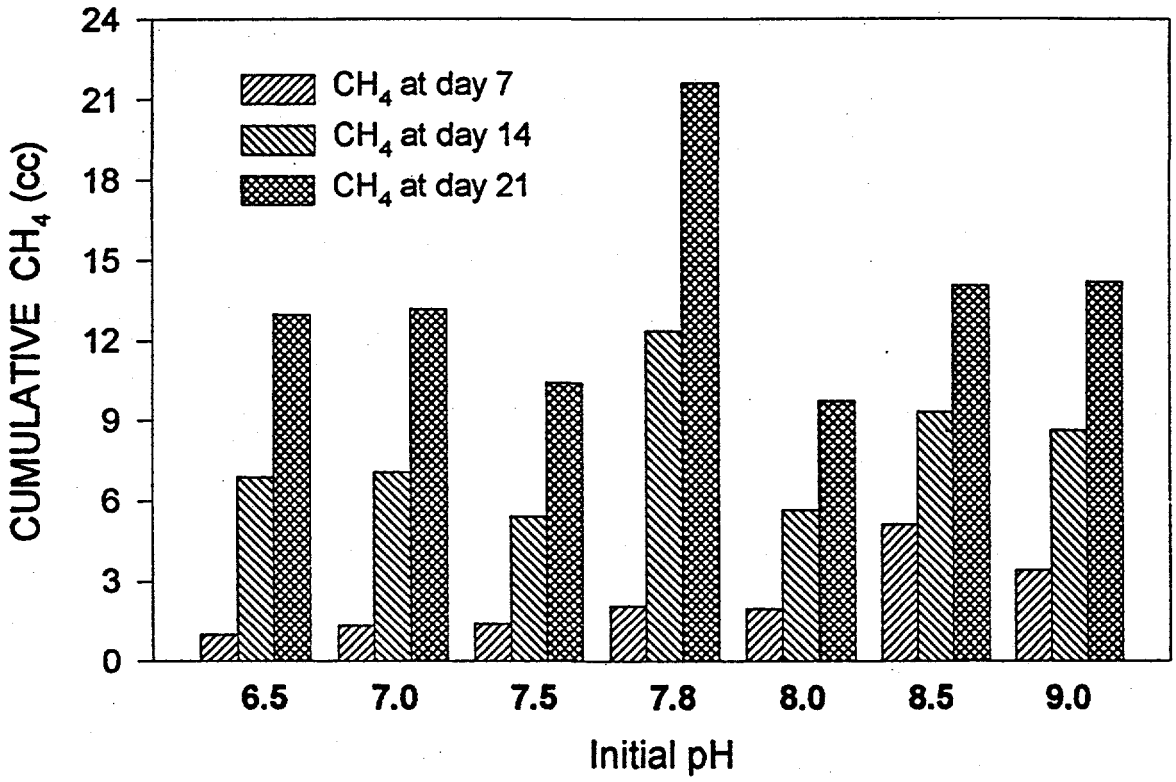


Figure 27. Effect of initial pH of the medium on methane production during biomethanation of 10% TxL in 0.2% SNTM by Mic-1 consortium

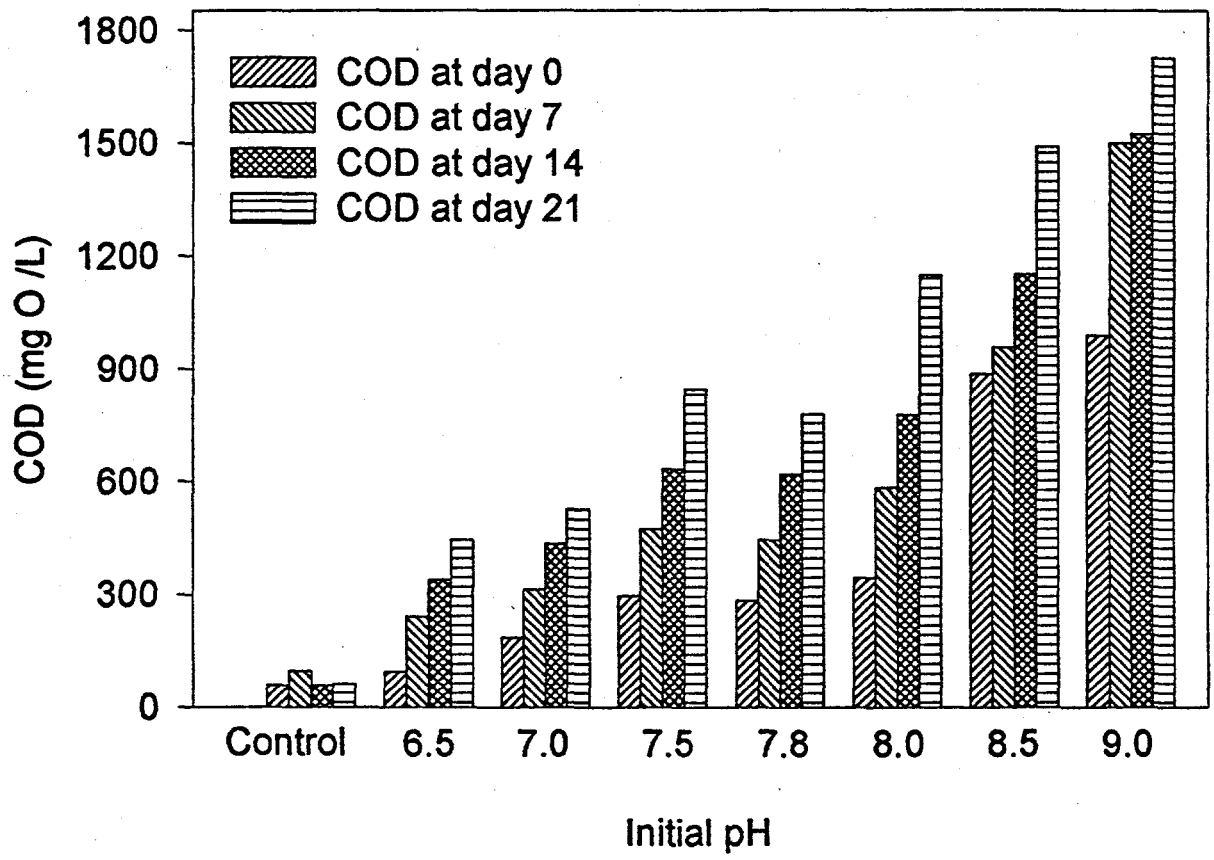


Figure 28. Effect of initial pH of the medium on COD concentrations during biomethanation of 10% TxL in 0.2% SNTM by Mic-1 consortium

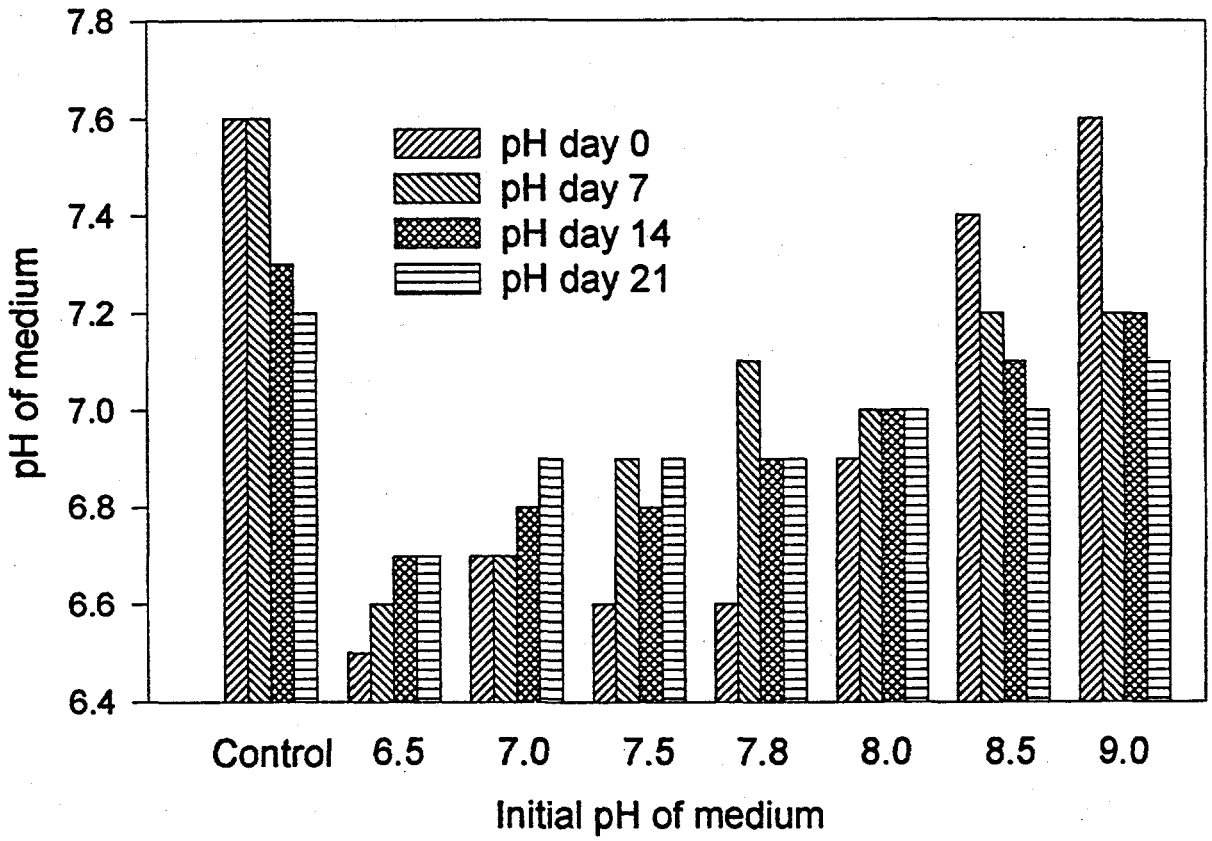


Figure 29. Changes in pH of the medium during biomethanation of 10% TxL in 0.2% SNTM with initial correction of medium pH.

These were the hypothesis which required further investigation. Unless otherwise stated, all agents were tested in NTM media with 0.1% TxL and Mic-1. Cultures were incubated for 28 days. Samples were analyzed for CH_4, CO_2, and VFAs.

6.2.13.2 Effect of chelating and sequestering agents

The sequestering/chelating agents tested were EDTA, oxalate, and citrate at 0.1, 1.0, and 10mM. The chelating affinity of a given agent is specific towards each metal. For example, EDTA has a high affinity for zinc, but a relatively small affinity for calcium. This information is useful because zinc is one of the metal ions that does inhibit anaerobic acedogenesis[21].

EDTA had little effect on biogasification. In fact, 10mM EDTA inhibited early biomethanation. This could be due to the strong chelating properties of EDTA. Cultures growing in the oxalate supplemented media only showed a slight methane production after 21 days of incubation (data not shown).

In contrast to the results from EDTA and oxalate, significantly higher methane production was observed in cultures supplemented with different concentrations of citrate, and there was a direct, positive correlation between the concentration of citrate and biomethanation **(Figures 30)**. Nevertheless, at higher (1 and 10%) TxL solids loadings, even 10mM citrate was not effective **(Figure 31)** in the NTM medium. However, addition of 1mM and 10mM citrate enhanced methane, acetate, and propionate production in the SNTM media with 1% TxL. Acetate and propionate increased and reached maximum concentrations after 7 days with 1mM citrate **(Figure 32)**. The acetate and propionate concentrations then decreased and this decrease was followed by an increase in methane. This indicates the following biochemical reaction:

propionate ----> acetate ----> methane

When the concentration of sodium citrate was increased to 10 mM, a significant increase in the concentration of acetate but not of propionate was obtained **(Figure 32)**. The profile of propionate was almost the same as in the vials that contained 1 mM citrate. At the end of the incubation, only traces of acetate were detected.

Citrate is an important mediator for the formation of acetate (main precursor for CH_4 formation) in the microbial glyoxylate cycle. At the same time, citrate also has sequestering properties for metal ions. The hypothesis to be tested was that the metals (such as Fe^{3+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, etc., present in the coal structure) are chelated/sequestered by addition of citrate, and therefore, facilitate coal carbon to CH_4 conversion by the microorganisms. The results from this experiment show that there

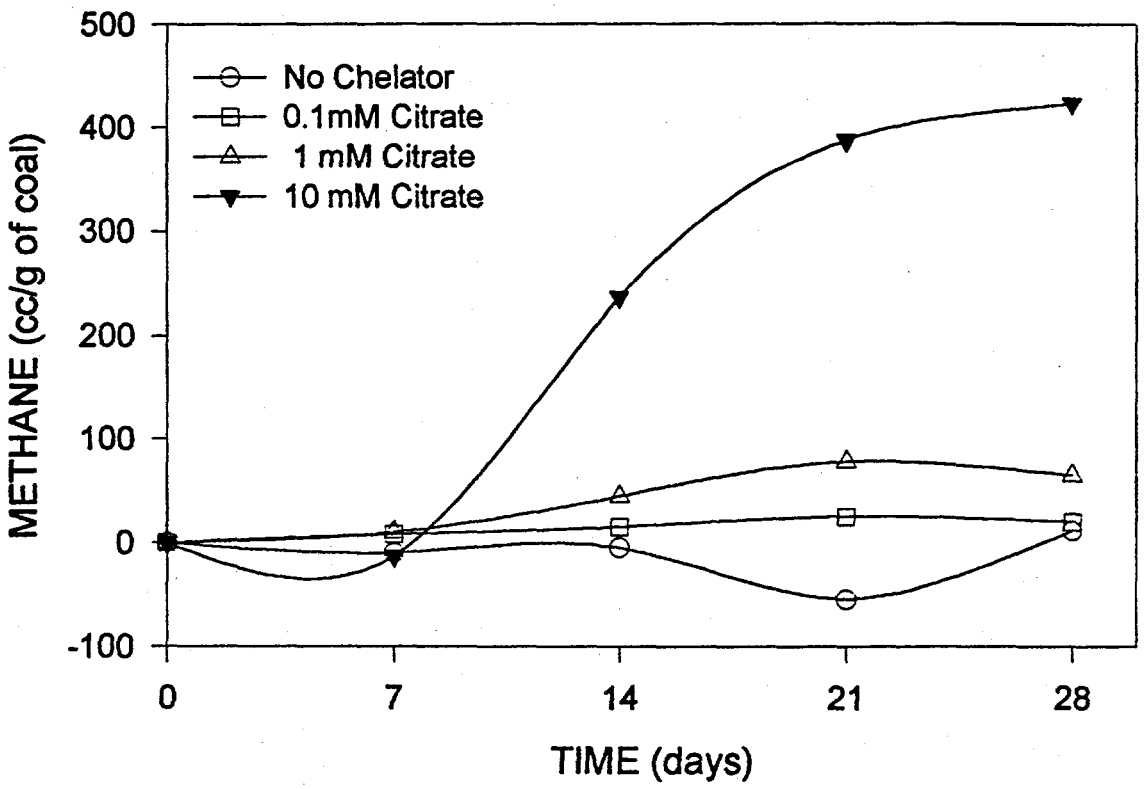


Figure 30. Effect of different citrate concentration on biomethanation of 0.1% TxL in NTM. Methane from control deducted.

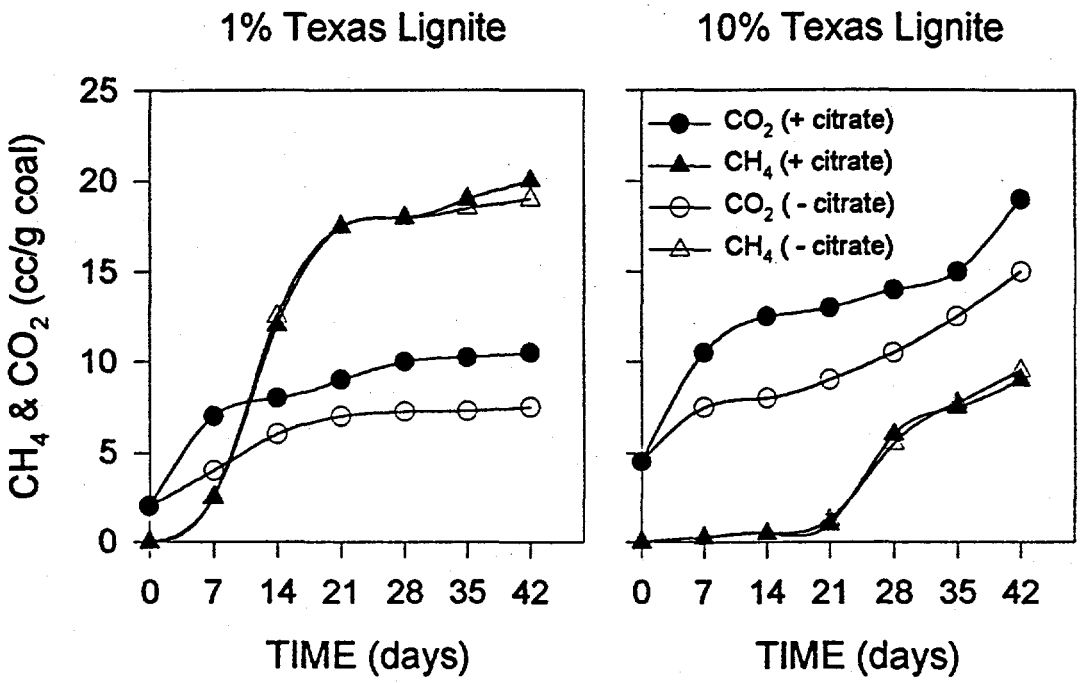


Figure 31. Effect of 10 mM citrate on CH_4 and CO_2 production during biomethanation of 1% and 10% TxL in NTM by the Mic-1 consortium.

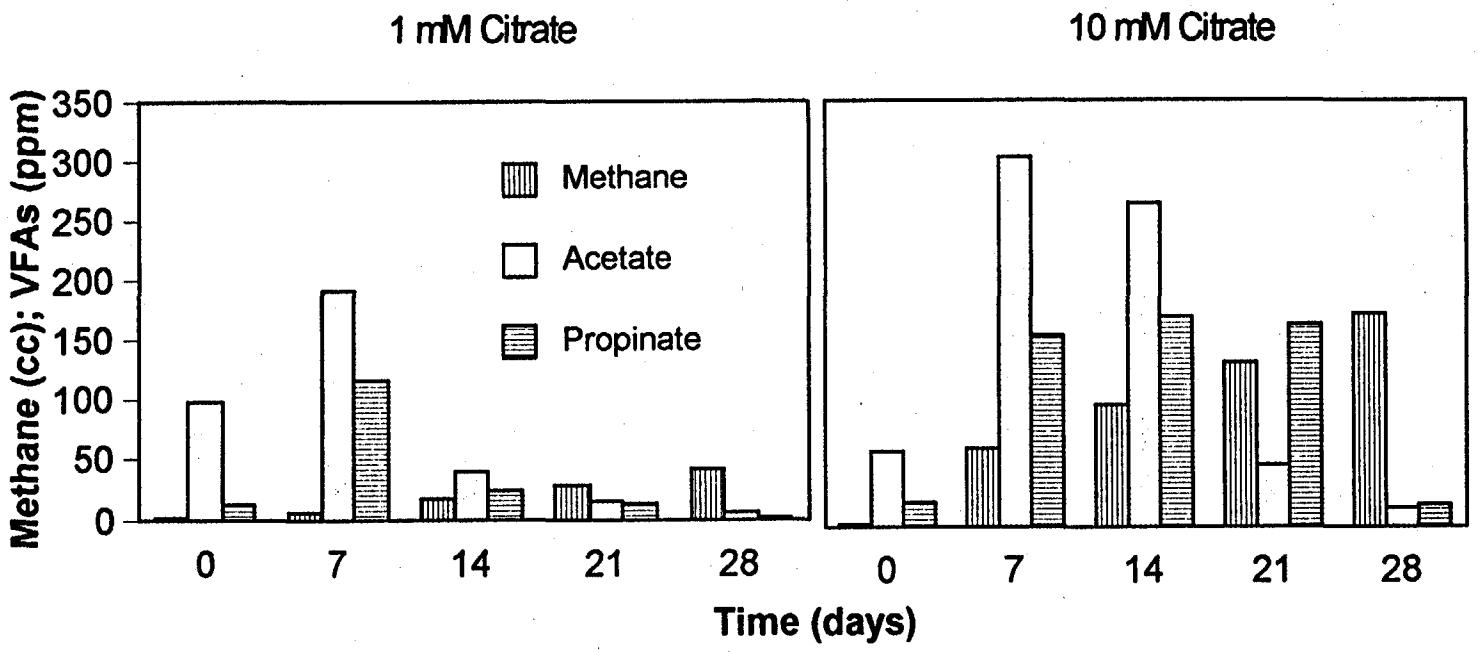


Figure 32. Effects of citrate on methane, acetate, and propinate production in 0.2% SNTM and 1%TxL

was no significant difference in the amounts of CH_4 produced in the control vials. The increased concentration of citrate (10 mM) induced more CO_2 production, compared to the rest of the control vials containing 1 mM citrate or oxalate. In the presence of 1% TxL, however, a significant increase in CH_4 and CO_2 production was observed. The highest CH_4 production was obtained in the treatment with 10 mM citrate (**Figure 33**), while the amount of CO_2 produced varied slightly (121 -132 cc for all experimental vials). This phenomenon can be explained as the leaching of the inhibitory compounds from TxL and their subsequent sequestration by the citrate, thereby allowing the bacteria to convert TxL to CH_4 more efficiently. Mass balance calculations show that CH_4 production is due to the biomethanation of TxL and not from the addition of co-substrates.

6.2.13.3 Effect of surfactants

Experiments were also conducted to study the effect of surfactants on biogasification of TxL. The surfactants tested were sodium dodecyl sulfate (SDS), Triton X-100, and Igepal CO-720, at 0.05, 0.1, and 0.2% (w/v) concentrations. Methane production was severely inhibited in all cultures supplemented with surfactants even at the lowest test concentration of 0.05%. Methane production was not observed in cultures containing either Triton X-100 or SDS. In the cultures containing Igepal CO-720, the highest methane production was 1 mole percent. The bacterial species constituting the Mic-1 consortium might have lysed at the surfactant concentrations, because SDS and Triton X-100 are used to lyse bacterial cells for nucleic acids extraction[19]. These results clearly indicate that even at the lowest concentration, surfactant amendment would not enhance biomethanation of TxL by the Mic-1 consortium.

6.2.13.4 Effect of methanol

The effect of co-substrate addition was studied in 100-mL vials containing 50 mL 0.2% SNTM and 1% TxL. Citrate, oxalate and methanol were used as co-substrates at different concentrations (10 mM, 1 mM and 0.5%, respectively). The experiment was carried out under static conditions at 37°C for 28 days. Maximum methane production was obtained from the vials that contained 0.5% methanol and 1% TxL (174 cc), while in the control vials CH_4 production was only 123 cc (**Table 11**). This can be explained by the fact that methanol is one of the simplest substrates for CH_4 formation and acts as an additional donor of protons necessary for the biomethanation process. Addition of 0.5% methanol to the experimental vials containing 1% TxL, resulted in an increase of propionate (171 ppm) concentration (**Figure 34**). The observed decrease of acetate concentration in the medium is interpreted as conversion of released acetate to CH_4 (174 cc).

6.2.13.5 Combined effect of citrate and methanol The effect of co-substrate addition on biogasification of TxL was further investigated in another experiment for evaluating

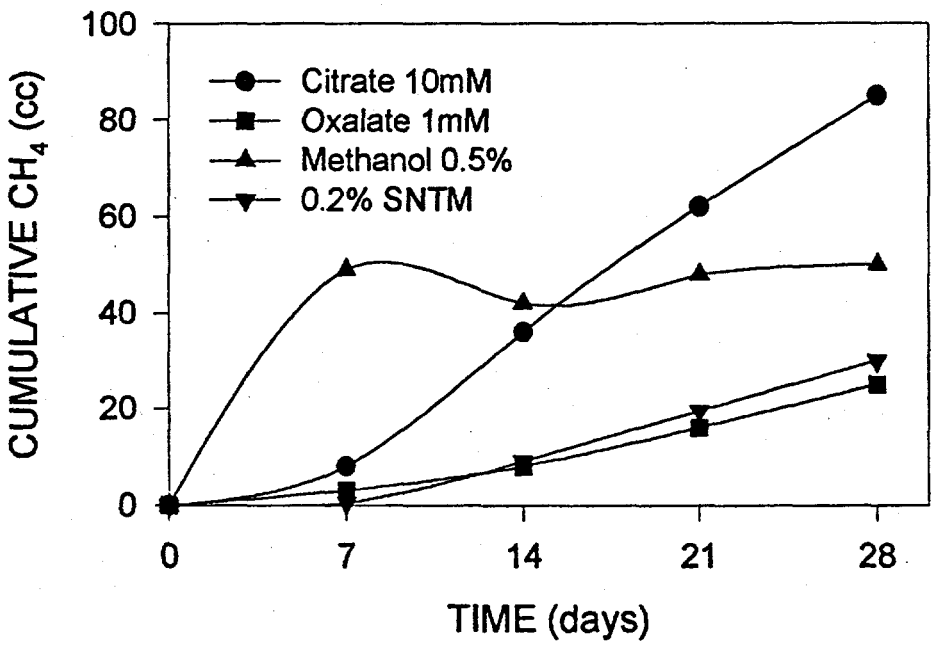


Figure 33. Effect of citrate, oxalate and methanol on CH_4 production during biomethanation of 1% TxL by Mic-1 consortium in 0.2% SNTM

Table 11. Theoretical and actual methane production from different carbon sources used as co-substrates[a]

Carbon Source	Total Carbon Available, (g)	Theoretical Production, (cc)	Actual Production, (cc)
Sheftone T (0.2%)[b]	0.044	56	5
TxL (1%)	0.314	398	7
0.2% Sheftone T + 1% TxL	0.358	455	34
Methanol (0.5%)	0.074	94	53
0.2% Sheftone T + 0.5% Methanol	0.118	150	123
0.2% Sheftone T + 0.5% Methanol + 1% TxL	0.432	549	174
Citrate (10 mM)	0.036	46	6
0.2% Sheftone T + 10 mM Citrate	0.080	102	2
0.2% Sheftone T + 10 mM Citrate + 1% TxL	0.394	500	86

[a] All calculations are based on 50 mL medium. Theoretical production - 100% of the carbon available is converted to CH_4.
[b] Based on the analysis that TxL contains approximately 60% carbon.

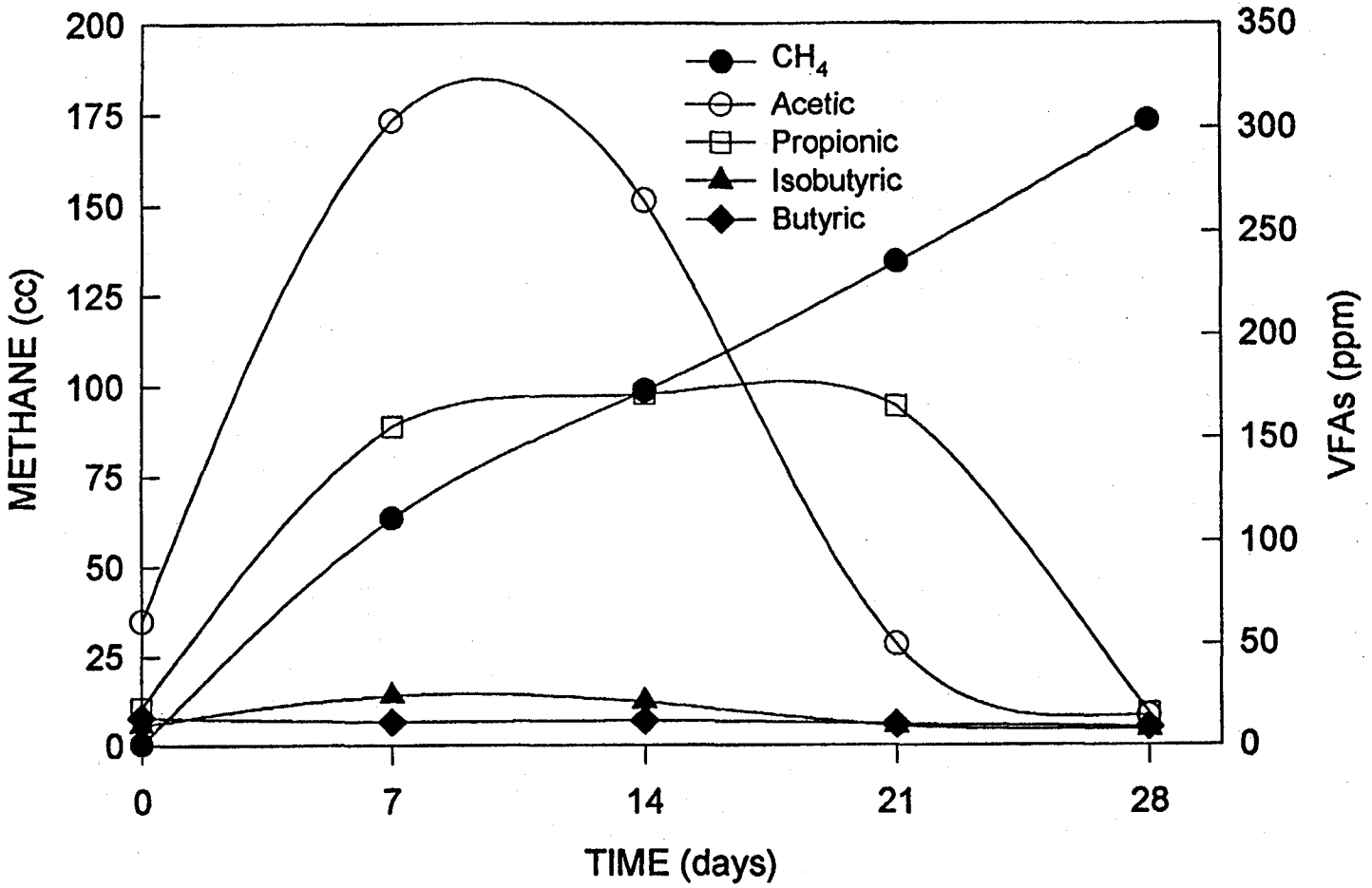


Figure 34. Effect of co-substrate addition on CH_4 production and VFA concentrations during biomethanation of 1% TxL in 0.2% SNTM supplemented with 0.5% methanol

the combined effect of citrate and methanol was conducted in 125-mL vials containing 60 mL of 0.2% SNTM or NTM, 5% TxL, and 0.5% methanol and 10 mM sodium citrate. The appropriate controls, containing SNTM and co-substrates without TxL, were also prepared. Another set of experimental and control vials was prepared with NTM. Total gas production was measured by water displacement method. Each vial was connected to a set of two vials through appropriate tubing. The first vial was filled with water, while the second vial was empty. The gas produced after a few days displaced the water in the first vial into the second vial through the connecting tubing between them. The second vial was already calibrated. The amount of water displaced from the first vial into the second vial was the excess gas produced for a defined period of time.

Highest CH_4 production was obtained in the experimental vials containing 5% TxL and 0.5% MeOH, while in the group of vials that contained 5% TxL, MeOH and citrate, CH_4 production was 400 cc (**Figure 35**). Considerably low CH_4 was obtained from the controls which did not contain TxL. The CH_4 accumulation in the vial head space started on the 3rd day of the experiment and reached up to 51-52 mole% on day 14 in the vials containing TxL + MeOH and TxL + MeOH + citrate, respectively. In the controls (groups #3 and #4), CH_4 production initiated after day 14 and slowly accumulated to 50-100 cc, however, the gas concentration was only 31 mole% and 12 mole%, respectively. Compared to the rest of the vial groups which contained organic nitrogen source, CH_4 production in groups #7 and #8 (NTM without Sheftone T™) was negligible (**Figure 35**).

These data can be explained on the basis of metabolism of methanogens where hydrogen is consumed to reduce CO_2 to CH_4 and for building biomass (cell carbon). It is known that several species of *Methanosarcina* and *Methanobacterium* can produce and consume hydrogen when grown on MeOH or acetate (Bhatnagar et al., 1987). Data from the VFA analyses indicate that in groups # 3 (SNTM + MeOH + citrate) and #4 (SNTM + MeOH) considerably high concentrations of acetate (**Figure 36**) were observed during first 14 days as a result of bacterial metabolism. The decrease in acetate concentration after day 14 was accompanied by increased production of methane and other VFAs, such as propionate, isobutyrate and isovalerate (**Figure 36**).

The reason for lesser production of CH_4 in the combinations containing 10 mM sodium citrate could be that the addition of citrate sequestered some metal ions such as Fe^{+2} and Ni^{+2} from the medium. Literature indicates that Fe^{2+} and Ni^{2+} are the prosthetic groups of the methanogenic enzymes that are responsible for the conversion of acetate to CH_4. In the experimental vials (groups #1 and #2), the addition of TxL influenced CH_4 production and VFA's concentration. Highest CH_4 was obtained in the vials containing TxL and SNTM supplemented with MeOH (group #1, **Figure 37**). The

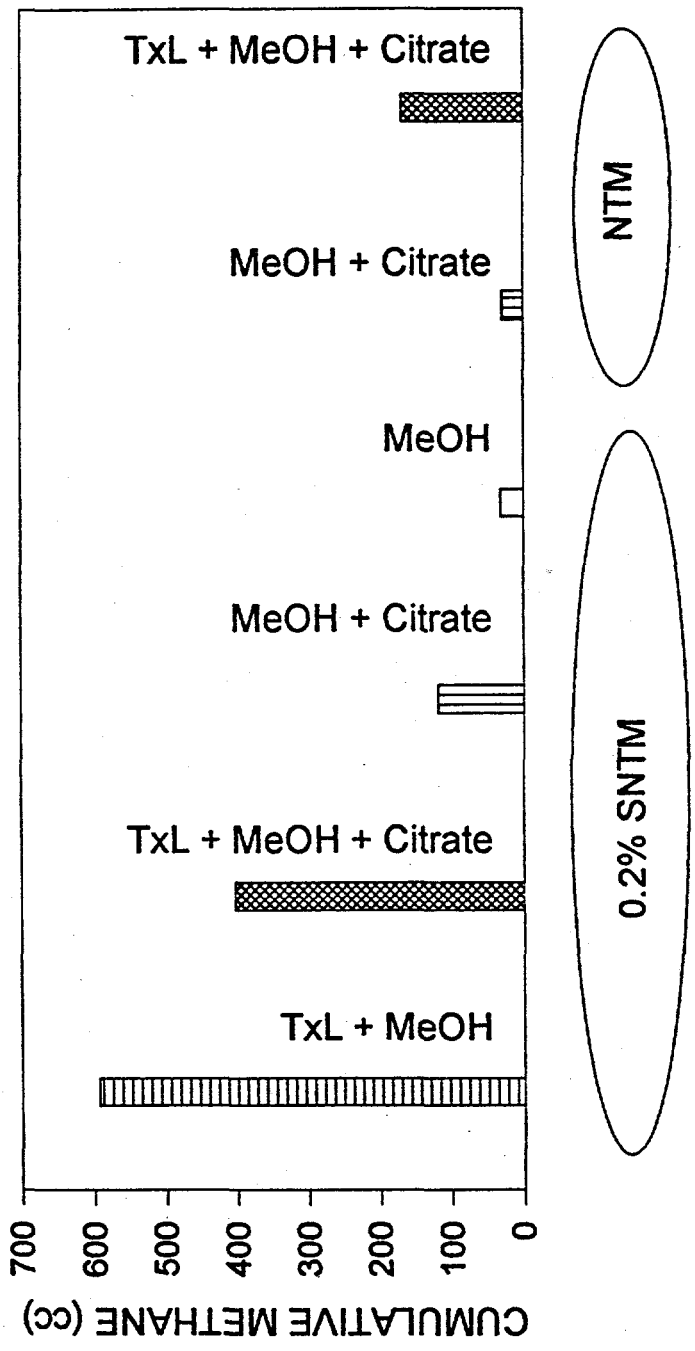


Figure 35. Effect of citrate and methanol (MeOH) on CH_4 production during biomethanation of 5% TxL in 0.2% SNTM and NTM

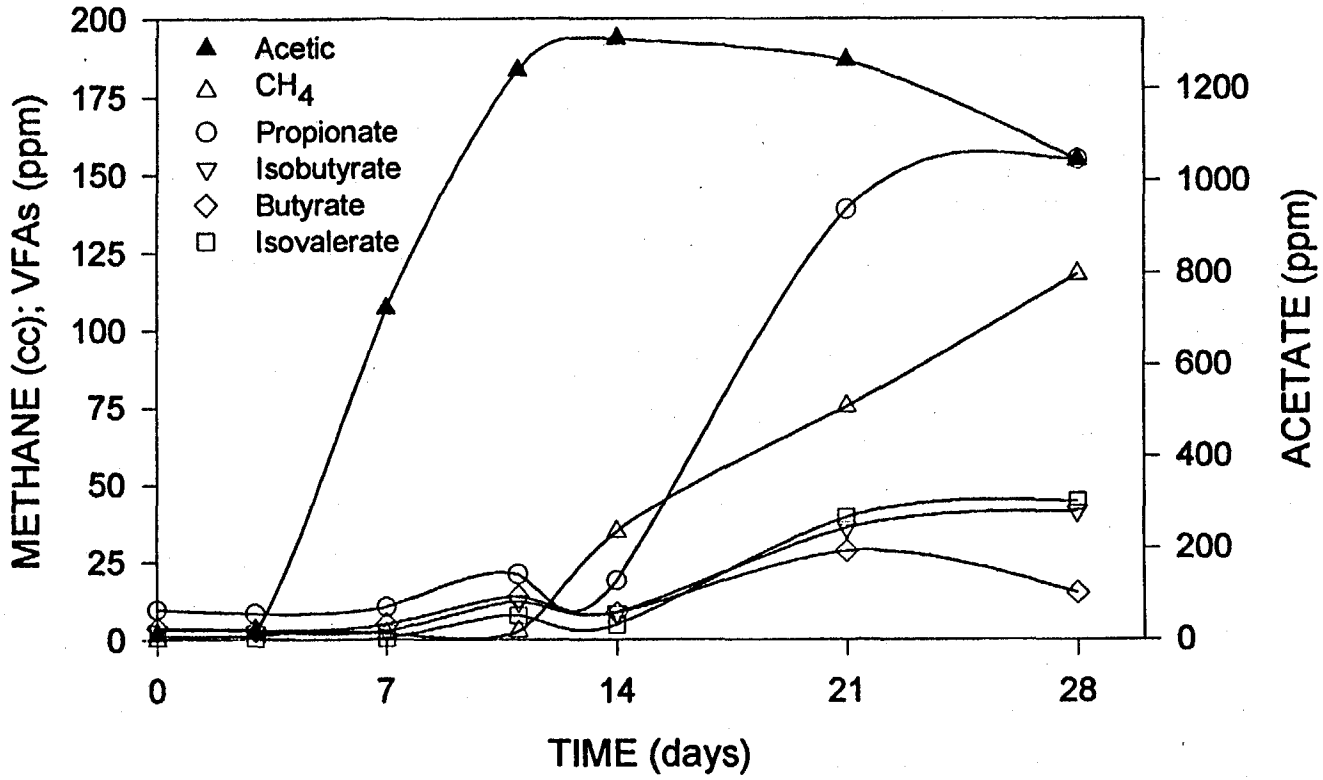


Figure 36. Effect of co-substrate (citrate and methanol) addition on CH_4 and VFA production in vials conyaining 0.2% SNTM

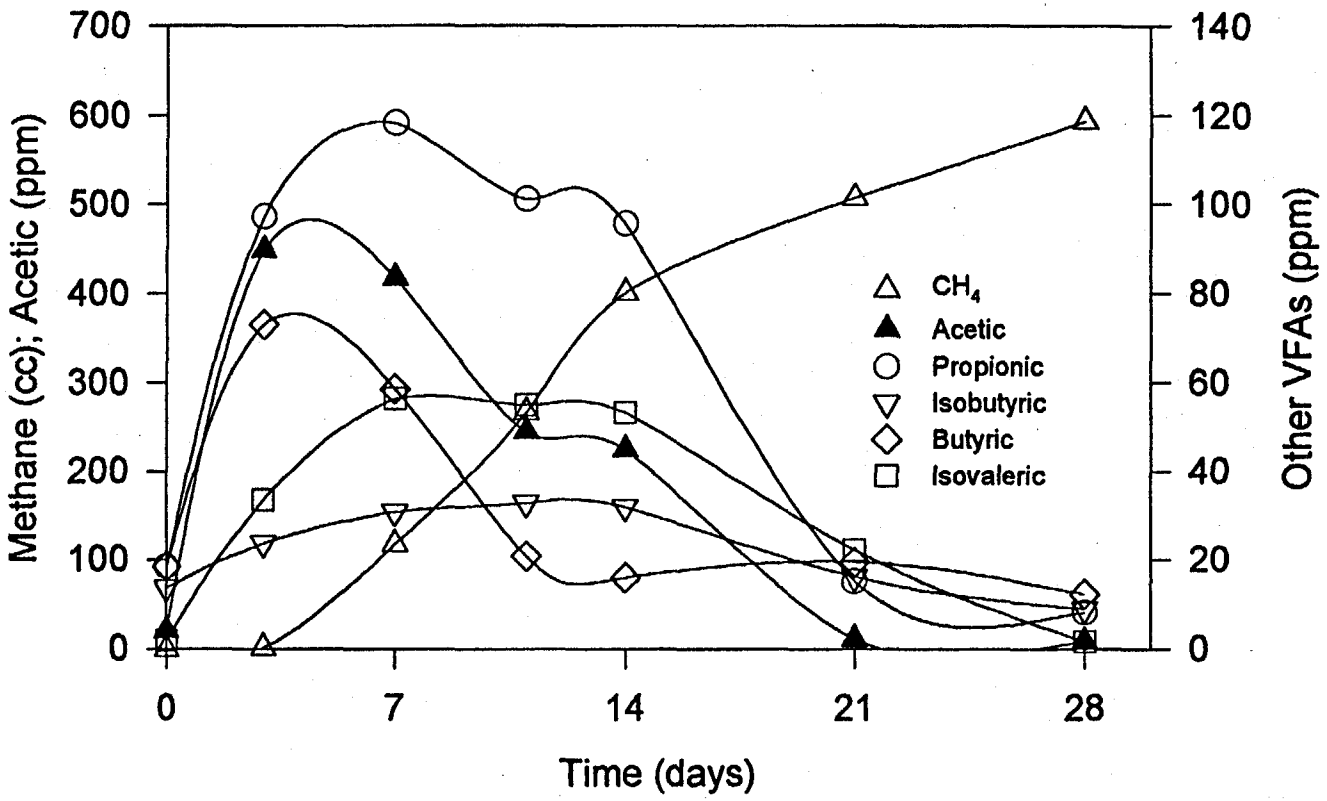


Figure 37. Effect of co-substrate (citrate) addition on CH_4 and VFA production in vials conyaining 0.2% SNTM

concentrations of acetate, propionate and butyrate in group #1 were almost 2-fold higher compared to those in group #2 at the beginning of the experiment. The data obtained support the hypothesis that citrate and methanol supplemented to 0.2% SNTM enhance biomethanation of TxL.

6.2.14 Effect of formate, lactate, and succinate

In another experiment sodium formate (CHO_2Na), L(+) lactic acid ($C_3H_3O_3$) and sodium succinate ($C_4H_4O_4Na_2.6H_2O$) were used as co-substrates at 10 mM concentration to study the effect of co-substrate addition on biomethanation of TxL. The experimental vials contained 60 mL 0.2% SNTM (or NTM) and 1% TxL. Control vials contained 60 mL of 0.2% SNTM (or NTM) only. Mic-1 consortium was used at 10% inoculum. Both control and experimental vials (in triplicate) were incubated at 37°C (static conditions). High CH_4 production (mole %) was obtained from the vials that contained 0.2% SNTM and 1% TxL supplemented with 10 mM lactate during the first 7 days of the experiment. The CH_4 production obtained from the vials that contained only lactate was 7 cc, and from the vials with TxL and lactate even lower. In the presence of organic nitrogen source (Sheftone-T™) and TxL, however, the addition of lactate enhanced the biogasification process because 87 cc of CH_4 were obtained. The addition of 10 mM formate did not show enhancement of the biogasification of TxL (66 cc). This can be explained as the inhibitory effect of formate on the growth of some constituents of the Mic-1 consortium. Still, this is a smaller amount which also affected the CH_4 production by this consortium. In the experimental vials containing 1% TxL and 10 mM succinate, a significant increase in CH_4 production was observed after the 21st day of the experiment. The presence of succinate in the vials supplemented with 1% TxL yielded 21 cc of CH_4 (4% conversion from the theoretically possible CH_4 production). In the vials with 0.2% SNTM and TxL, however, a total of 99 cc CH_4 gas was produced which increases the efficiency of TxL biogasification to 17%.

Maximum methane production was obtained in the experimental vials that contained 0.2% SNTM supplemented with 10 mM sodium citrate and 1% TxL (144 cc), while in the control vials CH_4 production was only 58 cc. The conversion efficiency was 24%. This clearly confirms that citrate is an important mediator for the formation of acetate (main precursor for CH_4 formation) in the microbial glyoxylate cycle, on the one hand, and as a sequestering agent, on the other. These results further indicate that citrate can be successfully used as co-substrate for enhancement of the TxL biogasification process. The results obtained reconfirmed our hypothesis that the metals (such as Fe^{3+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}), present in the coal structure) are chelated/sequestered by the addition of citrate. Mass balance calculations show that this increase in CH_4 production is due to the biomethanation of TxL and not because of the chemical conversion of co-substrate(s) to CH_4 **(Table 12).**

The profile of VFA concentrations in the vials containing 10 mM lactate was similar

Table 12. Theoretical and Actual Methane Production from Different Carbon Sources used as Co-substrates*

Carbon Source	Total Carbon Available(g)	Theoretical Production (cc)	Actual Production (cc)
***Sheftone* T (0.2%)**	0053	67	15
TxL (1%)	0.377	479	1
0.2% Sheftone T + 1% TxL	0.430	546	67
Lactate (10 mM)	0.022	28	7
0.2% Sheftone T + 10 mM Lactate			
1% TxL + 10 mM Lactate	0.075	95	34
0.2% Sheftone T + 10 mM Lactate + 1% TxL	0.399	507	4
	0.452	574	87
Formate (10 mM)	0.007	9	1
0.2% Sheftone T + 10 mM Formate			
1% TxL + 10 mM Formate	0.060	76	37
0.2% Sheftone T + 10 mM Formate + 1% TxL	0.384	488	1
	0.437	555	66
Succinate (10 mM)	0.029	37	1
0.2% Sheftone T + 10 mM Succinate			
1% TxL + 10 mM Succinate	0.082	104	28
0.2% Sheftone T + 10 mM Succinate + 1% TxL	0.406	516	21
	0.459	583	99
Citrate (10 mM)	0.043	55	8
0.2% Sheftone T + 10 mM Citrate			
1% TxL + 10 mM Citrate	0.096	122	58
0.2% Sheftone T + 10 mM Citrate + 1% TxL	0.420	533	4
	0.473	601	144
Methanol (0.5%)	0.074	94	53
0.2% Sheftone T + 0.5% Methanol			
0.2%	0.118	150	123
Sheftone T + 0.5% Methanol + 1% TxL	0.432	549	174

* All calculations are based on 60mL medium and 50 mL for methanol. Theoretical production = 100% of the carbon available for the conversion to CH_4

to the profile obtained from the experimental vials containing 0.5% methanol, however, CH_4 production was lower (**Figure 38**). The addition of formate did not show significant effect on CH_4 production and VFA's concentration (**Figure 39**). Nevertheless, addition of succinate to the medium resulted in considerably higher amounts of propionate after seven days of incubation. The maximum propionate production was 650 ppm on day 14 (**Figure 40**).

The effect of sodium citrate on biomethanation of TxL from the first experiment was reconfirmed. The peak in acetate concentration (1317 ppm) on day 7 was followed by a rapid conversion of this precursor to CH_4 (**Figure 41**). The VFA data confirmed the hypothesis that citrate and methanol can significantly enhance the biomethanation of TxL (**Figure 42**).

6.2.15 Microbial resistance to inhibitors

During the coal biogasification process, organic, as well as inorganic compounds, could be leached into the nutrient medium and inhibit microbial growth. Thus, the MicGAS Process would be inhibited. If inhibitory compounds do exist, then this could explain why TxL solids loading of $\geq$ 10% are inhibitory to the Process. In order to investigate the effect of inhibitory compounds possibly leached from coal during biogasification, TxL was washed with the SNTM-CM medium. Both the washed coal and the coal leachate (medium wash) was inoculated with Mic-1. Additional experimental details are described in the *METHODOLOGY*, however, the experimental design and CH_4 production from the leachate, washed (residual) coal is shown in **Table 13.**

Highest CH_4 production (600 - 630 cc) was obtained in the vials from set B containing 5% and 10% residual TxL. In comparison to the vials from sets A and C, the higher CH_4 production in the vials containing residual TxL could be explained as a result of removal of inhibitory products from coal during pretreatment of TxL with 0.2% SNTM-CM. When the leachate was used as a medium (Set A), the growth of Mic-1 was suppressed; the OD_{620} was lower compared to the controls (*data not shown*). The lower CH_4 production and suppressed growth is possibly due to increased concentration of inhibitory compounds leached from TxL. Perhaps the methanol and citrate, present in the SNTM-CM medium, cleaved and/or sequestered the inhibitory compound(s) from the coal.

The positive effect of TxL pretreatment to leach out the inhibitors can be further illustrated by comparing the ratio of CH_4:CO_2. **Figure 43** shows cumulative CH_4 and CO_2 production and their ratios. The highest ratio (1.92) for SNTM-CM can be explained as likely conversion of methanol to acetate and CH_4 by the acetogenic and methanogenic microbes in Mic-1. Even so, comparing the total CH_4 produced in all

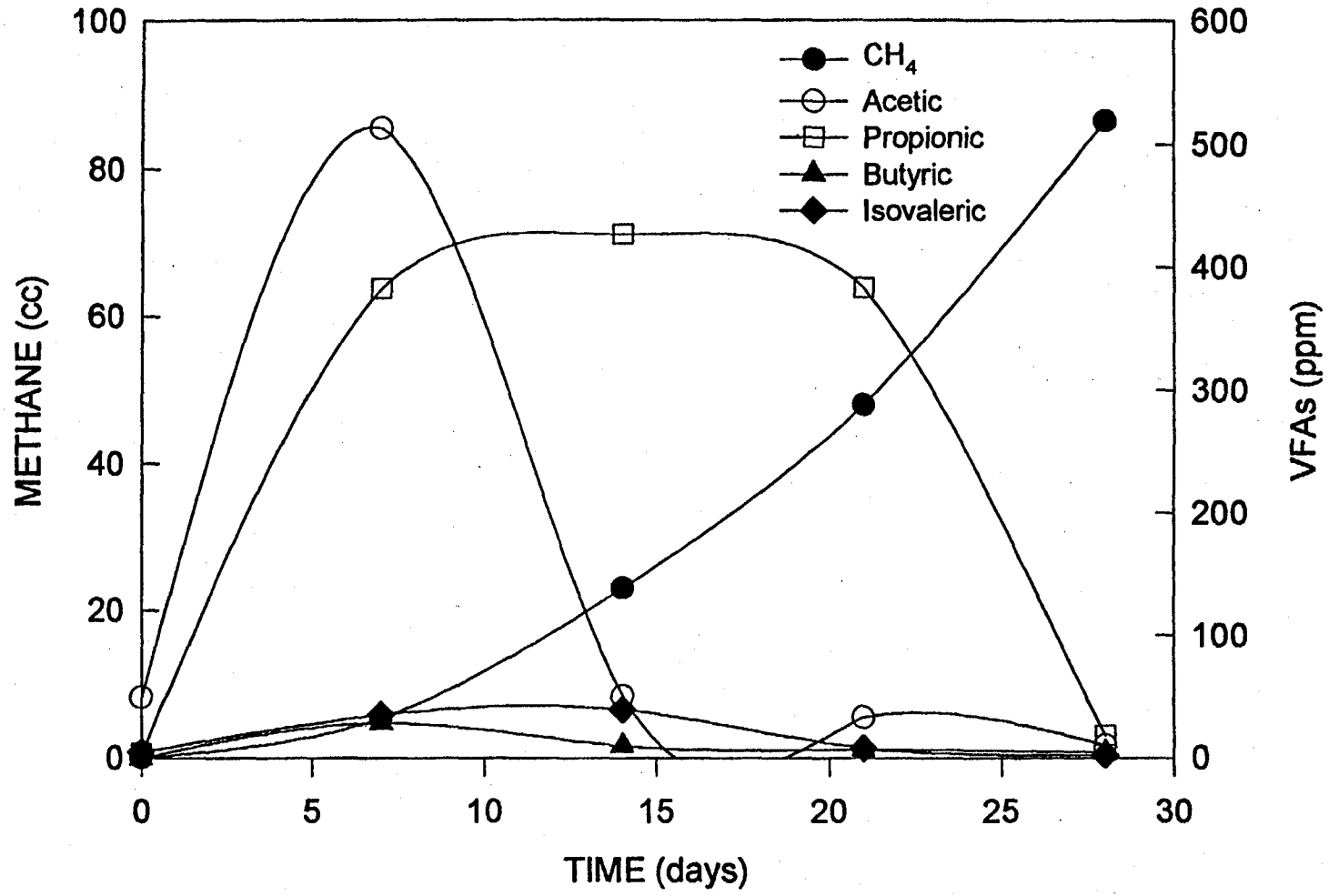


Figure 38. Effect of co-substrate addition on CH_4 and VFA production during biomethanation of 1% TxL in 0.2% SNTM supplemented with 10 mM lactate

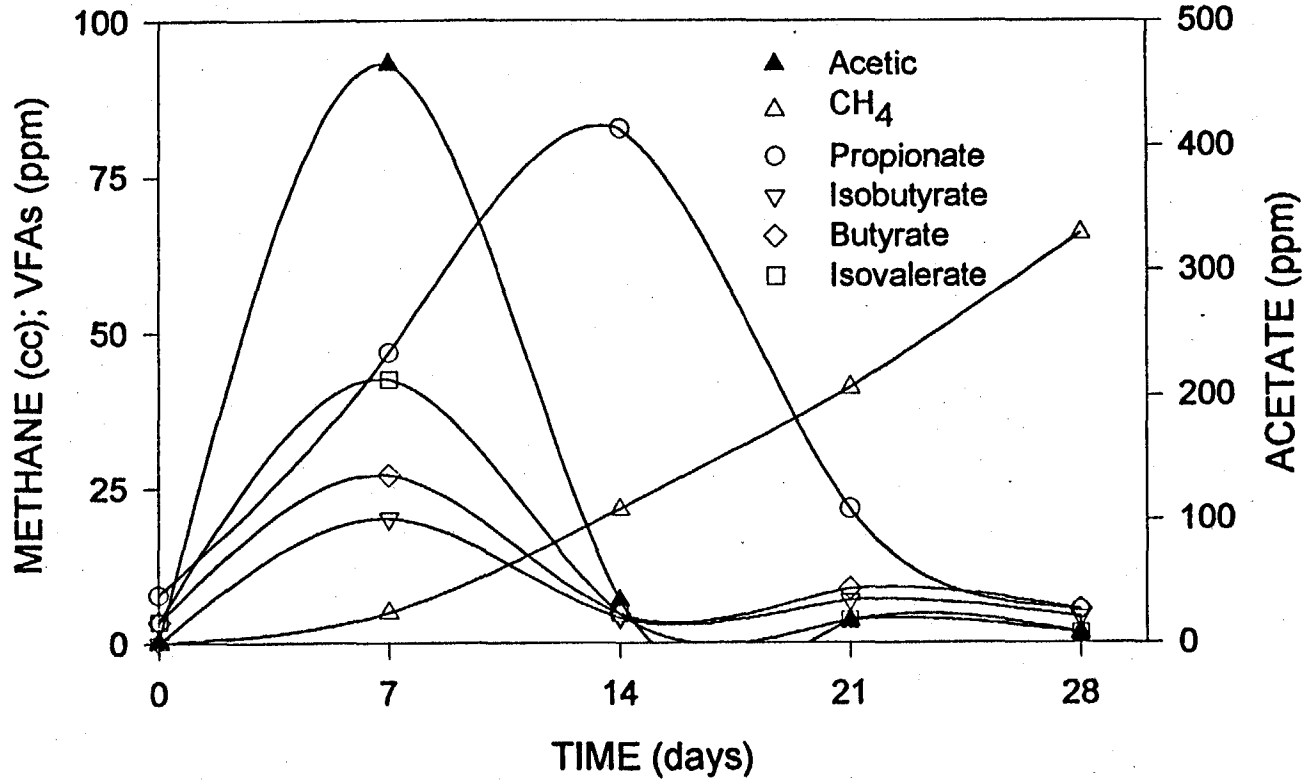


Figure 39. Effect of co-substrate addition (10mM Formate) on CH_4 and VFA production during biomethanation of 1% TxL in 0.2% SNTM supplemented with 10 mM formate

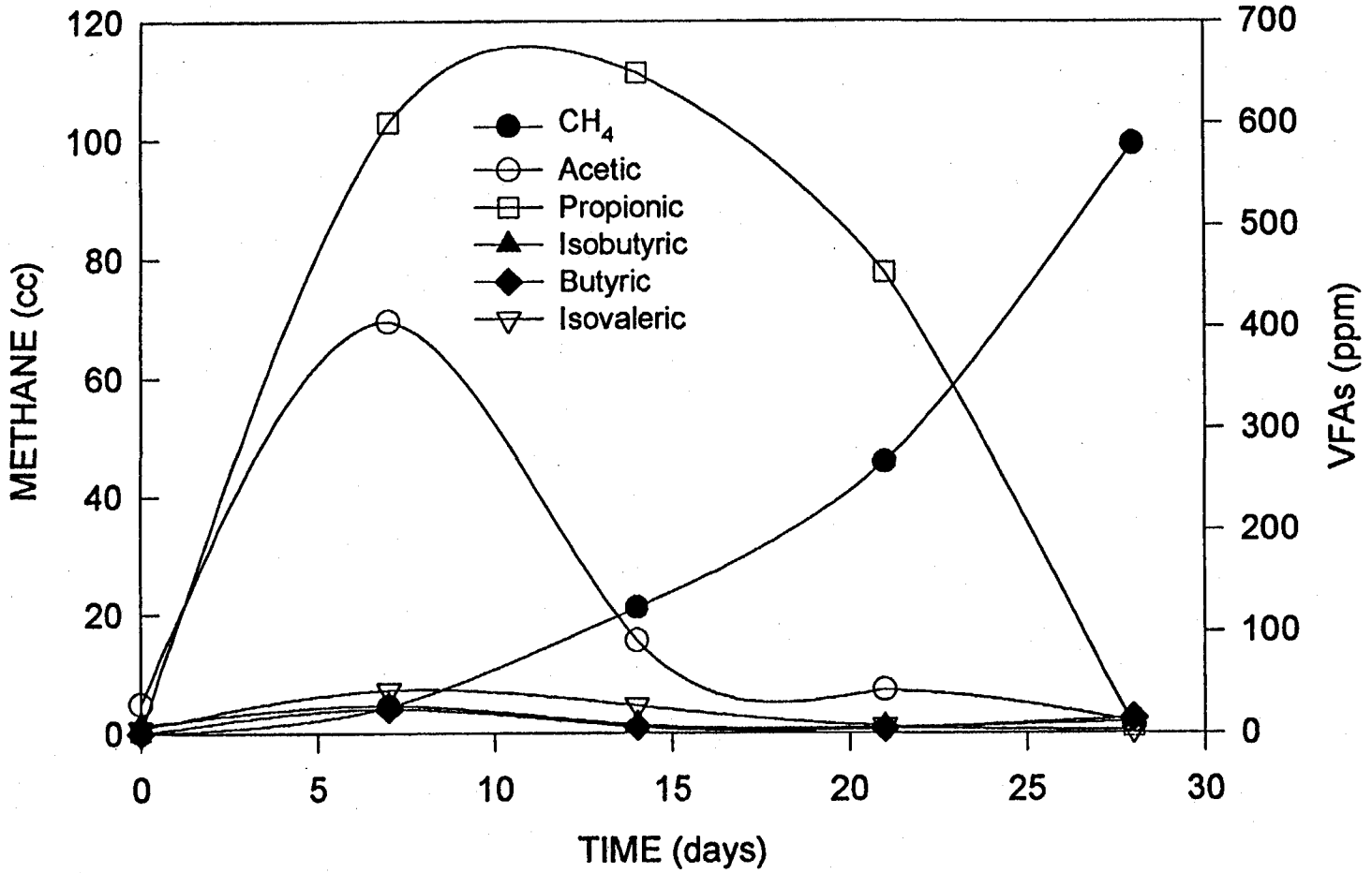


Figure 40. Effect of co-substrate addition on CH4 and VFA production during biomethanation of 1% TxL in 0.2% SNTM supplemented with 10 mM succinate.

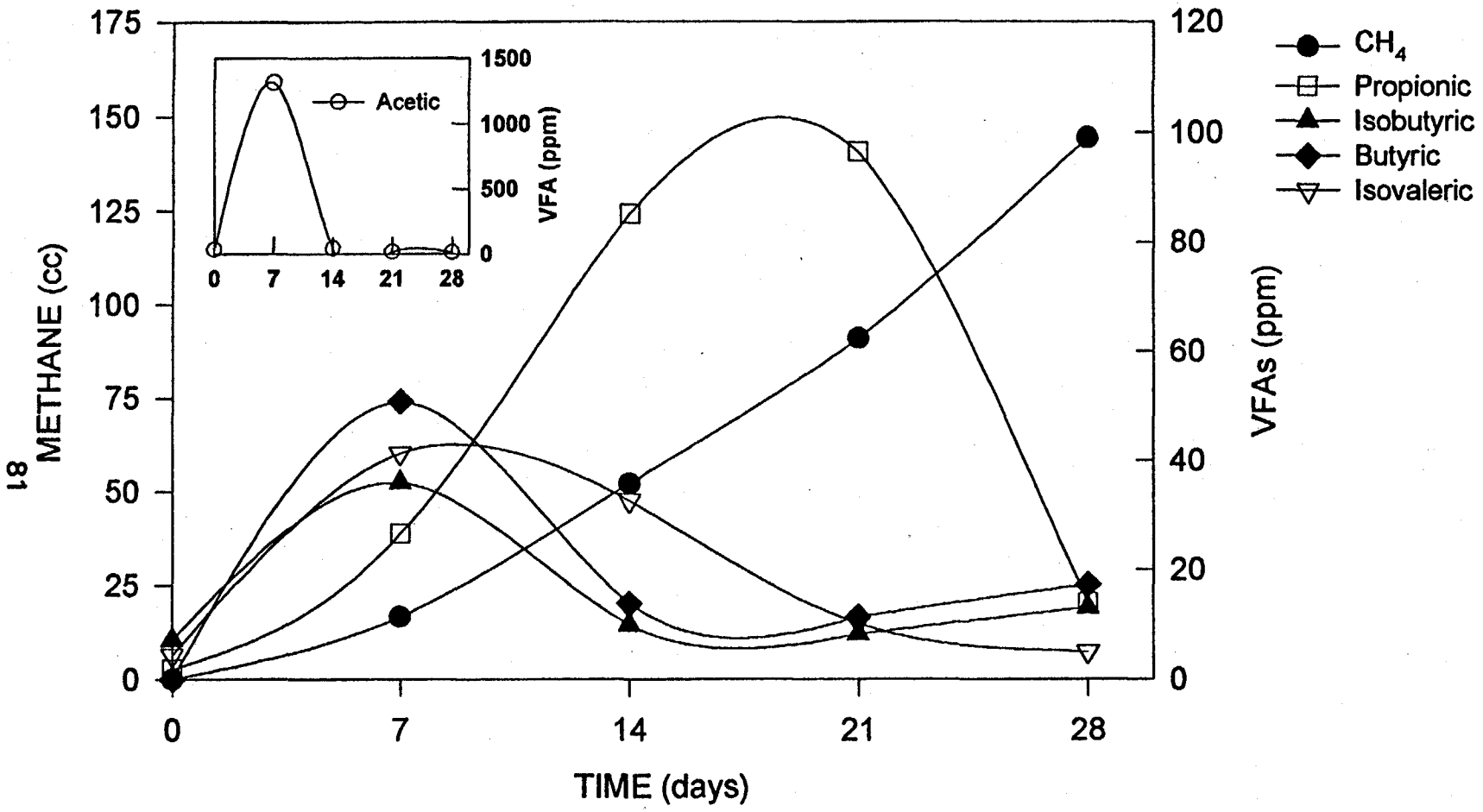


Figure 41. Effect of co-substrate addition on CH_4 and VFA production during biomethanation of 1% TxL in 0.2% SNTM supplemented with 10 mM citrate. Insert shows acetate production vs. time. Note the different scale on the insert graph.

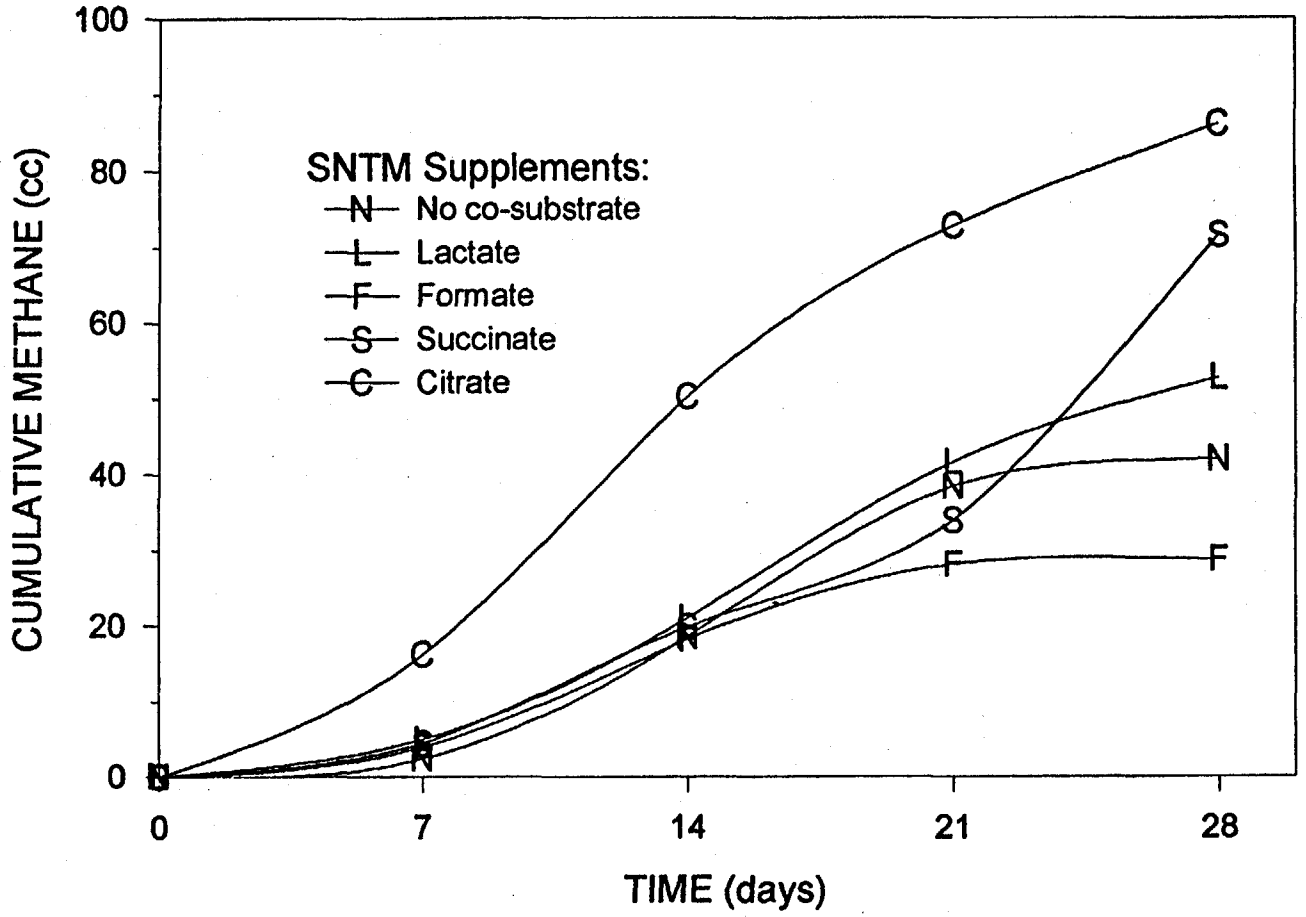


Figure 42. Effect of co-substrate addition on CH_4 production during biomethanation of 1% TxL in 0.2% SNTM. Methane from controls deducted

Table 13. Experimental set-up and range of CH_4 production by Mic-1 from various coal products in SNTM-CM[a] media

Set	Solids Loading	Additional component	Range of cumulative CH_4 production (cc)
Set A	10% untreated TxL 5% untreated TxL 0% TxL	Leachate[b] Leachate Leachate	400 - 430
Set B	10% residual TxL[c] 5% residual TxL	0.2% SNTM-CM 0. 2% SNTM-CM	600 - 630
Set C	10% untreated TxL 5% untreated TxL 0% untreated TxL	0.2% SNTM-CM 0.2% SNTM-CM 0.2% SNTM-CM	510 - 560

[a] SNTM + 10mM Sodium Citrate and 0.5% MeOH (v/v).
[b] Leachate from soaking TxL in 0.2% SNTM media. See Methodolgy for additional details.
[c] Coal washed with SNTM-CM and dried. See Methodolgy for additional details.

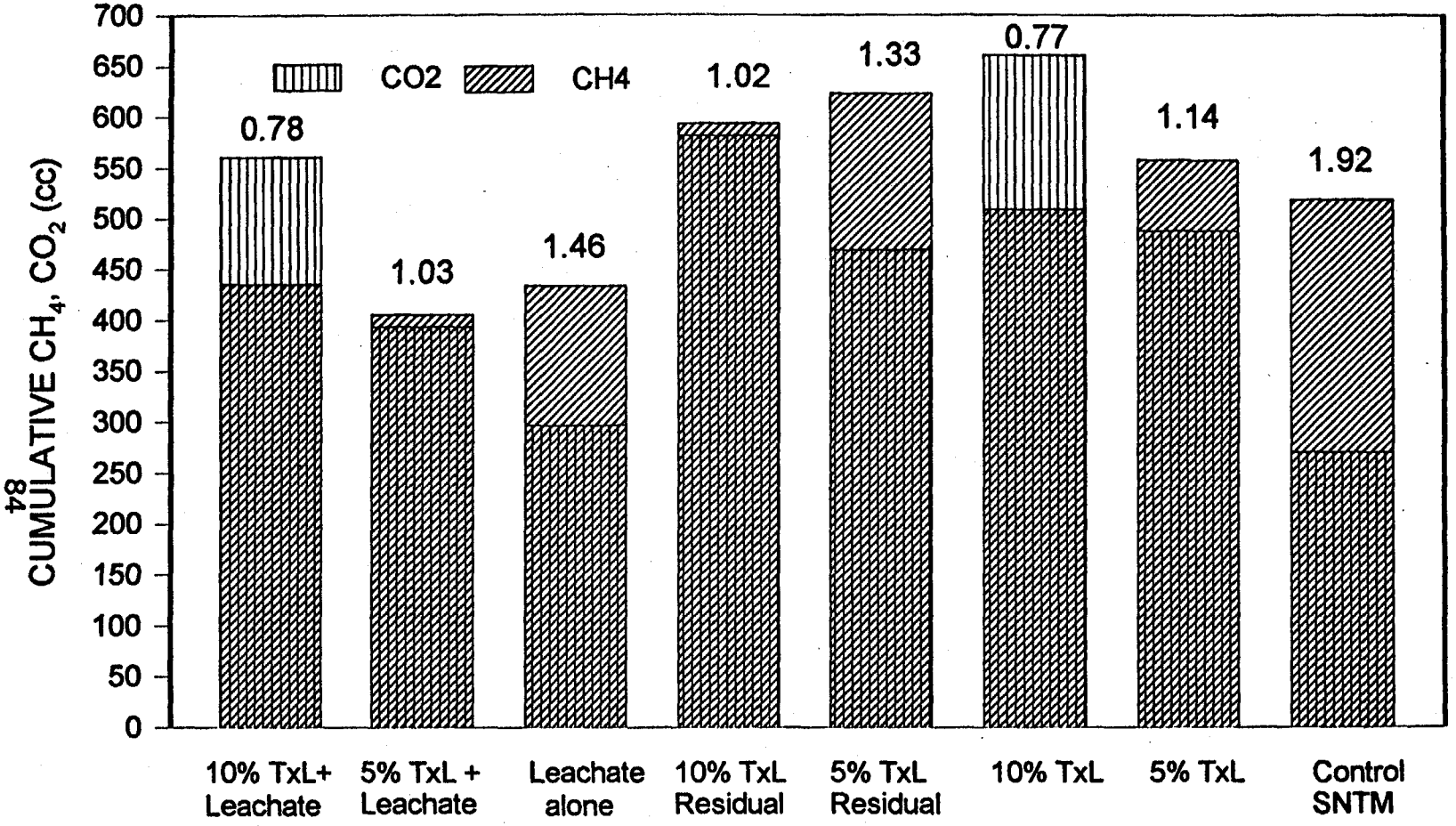


Figure 43 . Effect of coal inhibitors on CH_4 and CO_2 production during biogasification of pretreated and untreated TxL at 5% and 10% solids loading in 0.2% SNTM containing citrate and methanol (day 28). Values above bars show CH_4:CO_2 ratio.

sets, the highest CH_4 was produced when residual TxL was used as a feedstock at 5% and 10% solids loading.

The production of acetate, propionate and butyrate from 5% untreated TxL supplemented with 0.2% SNTM-CM was significantly lower compared to VFA production from 5% residual TxL (this was the TxL that had been previously pretreated with 0.2% SNTM-CM) supplemented with 0.2% SNTM-CM **(Figure 44, panel A)**. During the biomethanation of 5% residual TxL on day 7, the concentration of acetate and propionate increased up to 240 ppm and 140 ppm, respectively. When TxL was suspended in leachate only, the VFA concentrations were negligible and CH_4 production was lower **(Figure 44, panel B)**.

A similar profile for VFAs was observed at 10% TxL solids loading **(Figure 45)**. The VFA data presented here supports our hypothesis that there are compounds in coal that potentially inhibit microbial activity and because of this, higher the solids, the lower CH_4 and VFA production.

Therefore, is is conjectured that upon pretreatment with SNTM-CM, the inhibitory compounds were removed from TxL into leachate and, therefore, the growth of Mic-1 consortium and biomethanation of TxL was enhanced. Thus, an initial pretreatment with methanol and citrate is required in order to enhance the biomethanation of TxL. Preliminary data indicates that the possible inhibitory compounds are phenols or phenolic derivatives.

6.2.16 Detection of sulfur contaminant

H_2S production was measured in early experiments with TxL. The H_2S concentration was negligible in all experiments (1 to 4 ppm).

6.3 Enhancement of CH_4 production by microorganisms

To enhance the biomethanation of TxL, several isolates obtained from Mic-1 and Mic-4 consortia were evaluated.

6.3.1 Isolation of individual bacterial components of Mic consortia

Thirteen bacterial isolates were obtained from the Mic-1 and Mic-4 consortia. In order to determine whether these isolates are primary TxL degraders, they were grown in medium containing TxL. The hypothesis was that the degradation of lignite would produce an increase in soluble carbon in the liquid phase of the reaction mixture. Therefore, bacteria producing a significant increase in soluble carbon in reactions containing TxL could be considered potential primary TxL degraders. However, there

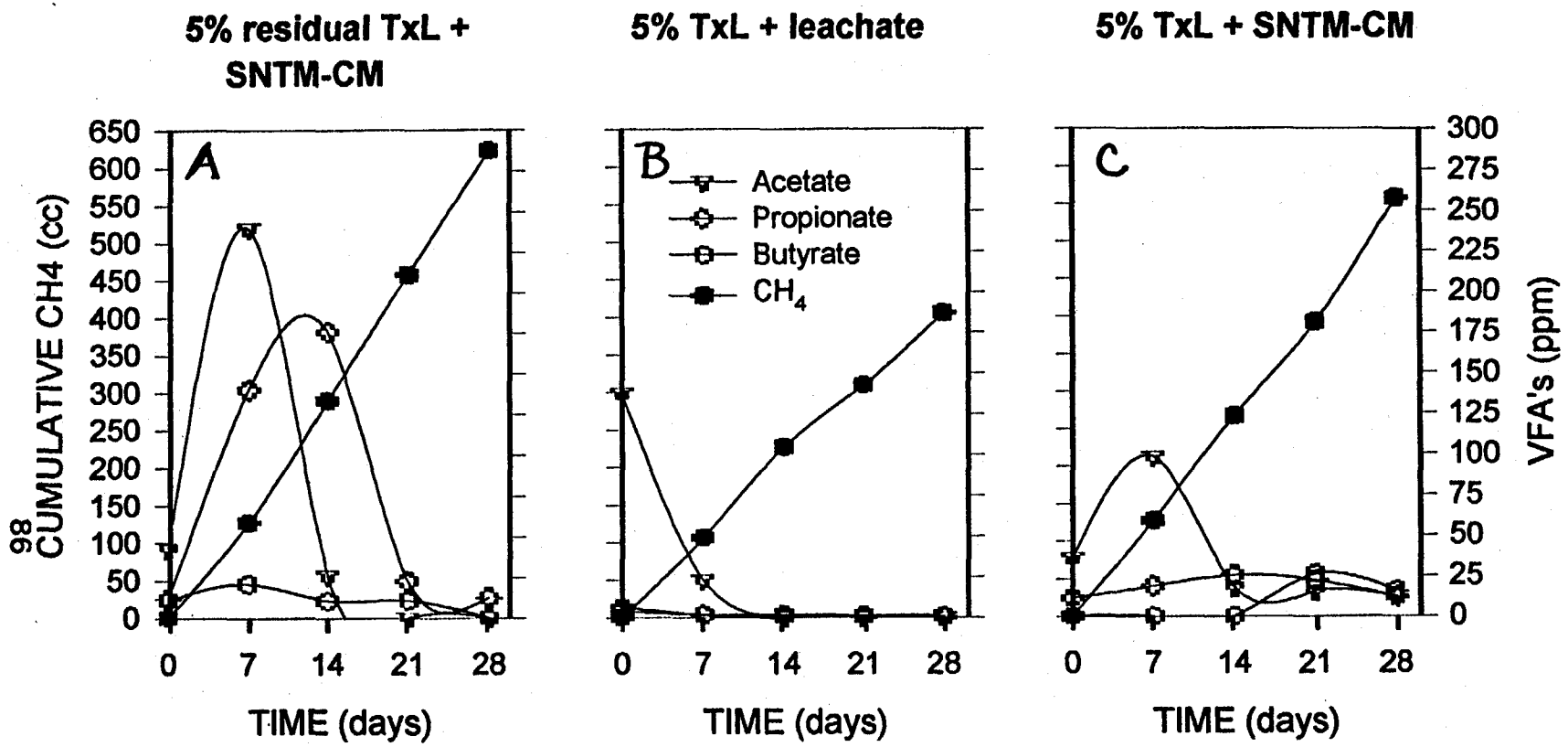


Figure 44 . Effect of coal inhibitors on methane and VFA production during biogasification of 5% TxL.

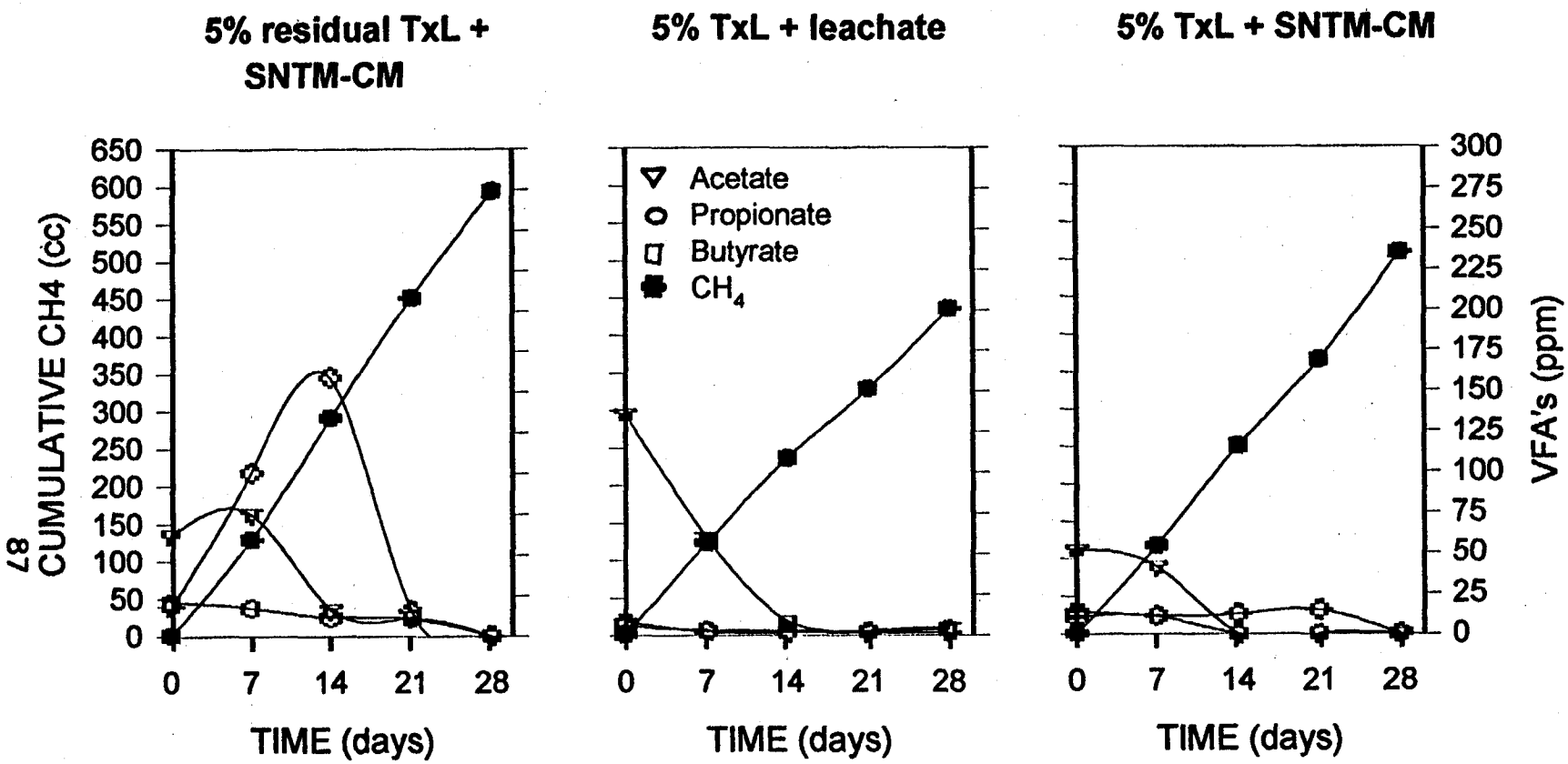


Figure 45 . Effect of coal inhibitors on methane and VFA production during biogasification of 10% TxL.

was an insignificant decrease in the levels of COD in the culture broth from these bacteria. As CH_4 and other gaseous products were not measured, it is quite possible that the bacteria utilized the soluble carbon for their metabolism for CH_4 production which could explain the reduction in COD. This possibility is supported from the data on VFA analysis **(Table 14)**. Alternatively, these data on COD can also be interperted as the net result of the synergistic metabolism of different microbial populations consisting of the Mic-1 consortium. Under that scenario, while the coal degraders might be degrading the coal into soluble carbon, manifested as an increase in COD; the metabolism of fermenters, acedogens, acetogens, and finally methanlogens feeding on that soluble carbon would result in the net insignificant change in the COD level.

Volatile fatty acid analyses **(Table 14)** indicates that each isolate (except for M4-2 and M4-8) produced acetate at a concentration greater than 100 ppm. The greatest concentration of acetate was produced by M4-1 (404 ppm), an isolate from Mic-4. The greatest concentration of acetate produced by an isolate from Mic-1 was 298 ppm, produced by M1-2. Several other VFAs were produced in smaller quantities by various isolates. The conclusion from these data is that most isolates are acedogens.

Interestingly, four isolates (M1-4, M4-2, M4-4 and M4-8) produced detectable amounts of ethanol. Certain isolates produced similar amounts of the same VFAs. Based on comparisons of the production of various VFAs, as well as other characteristics (eg. cell morphology, Gram reaction, etc.), it appears that the 13 isolates represent seven different bacterial strains. More comprehensive testing would be required to confirm this. For the purpose of this project, seven distinct bacterial strains were identified **(Table 15)**.

Several of the strains were found to be aerotolerant (able to survive oxygen exposure) rather than strict anaerobes (killed by oxygen exposure). In order to test whether the TxL biodegradation into substrates for methanogens is brought about by the aerotolerant popultion, a fresh culture of Mic-1 consortium was aerated to eliminate the strict anaerobes (ie. methanogens). This would result in an increase in soluble carbon in the liquid phase because the carbon would not be converted to methane. However, there was no increase in soluble carbon in the cultures containing TxL (data not shown). Surprisingly, methane was produced during incubation. Epifluorescence microscopy confirmed that viable methanogens were still present in the culture. Therefore, aeration did not completely oxidize the inoculum, and complete selection of aerotolerant bacteria did not occur.

During incubation, large clumps of the TxL were observed in the culture bottles with the isolates. Since the TxL was not degraded, it is possible that the bacteria modified

Table 14. Concentration of Volatile Fatty Acids and Ethanol in the Culture Broth of Individual Bacterial Isolates from Mic-1 and Mic-4 Consortia Grown on 0.1% Texas Lignite

Isolate[a]	Concentration (ppm) After 14 Days of Incubation				
	Acetic Acid	Butyric Acid	Propionic Acid	Ethanol	Other VFAs
M1-1	268	78	99	ND[b]	204
M1-2	298	ND	32	ND	149
M1-3	291	73	96	ND	211
M1-4	166	88	ND	29	179
M1-5	293	76	92	ND	213
M4-1	404	ND	55	ND	195
M4-2	58	22	ND	27	ND
M4-3	258	ND	120	ND	197
M4-4	176	84	ND	46	152
M4-5	357	ND	38	ND	173
M4-6	272	87	93	ND	215
M4-7	129	104	36	ND	ND
M4-8	64	24	ND	31	ND

[a] Isolates derived from Mic-1 are designated as M1, and those derived from Mic-4 are designated as M4

[b] ND = Non detectable levels

Table 15. Strains Designation to Isolates Obtained from Mic-1 and Mic-4 Consortia

Strain	Isolates[a]
1	M1-1, M1-3, M1-5 , M4-6
2	M1-2
3	M1-4, M4-4
4	M4-1, M4-5
5	M4-2, M4-8
6	M4-3
7	M4-7

[a] Isolates derived from Mic-1 are designated M1, and those derived from Mic-4 are M4

the TxL. To verify this hypothesis and to enhance the biomethanation, mixed sub-populations, rather than pure isolates were incubated with TxL.

The selected isolates (M1-5, M4-4, M4-5, M4-8) were used based on their capability to convert coal carbon into acetate, propionate, and butyrate. The isolates were mixed and designated as shown in **Table 16.**

The bacterial combinations did not contain any methanogens. After 28 days of static cultivation in 1%TxL, all combinations were additionally inoculated with a specific 10% (v/v) mixture of known methanogens (*Methanosarcina* sp., *Methanothrix* sp., *Methanobacterium* sp., and *Methanospirillium* sp.) in order to convert the accumulated acetate to methane. The acetate concentration in all of the vials started to decrease after the addition of methanogens. Based on the data obtained from the VFA analyses, the most promising combinations were combination D (KS14RM5K8 - 1458) and G (KS04RM4K4 - 0444). The VFA analyses supported the fact that most of the acetate was converted to CH_4 **(Figure 46)**. This confirms previous observations, based on the CH_4 production, **(Figure 47)**, that combinations D and G are the most effective for converting TxL to acetate that can hydrogenated by methanogens into CH_4.

6.3.2 Evalution of additional microbial consortia

Several experiments were conducted to study the efficiency of the granulated sludge consortium (GSC) on the biomethanation of TxL. With an aim of obtaining a more effective culture than Mic-1, TxL biomethanation by the GSC was evaluated at different inoculum concentrations (0%, 1% and 10%).

The first experiment was carried out, using 10%GSC as the inoculum, with SNTM + 1% TxL . Methane production was measured periodically in the vial headspace. After 20 days of incubation, the methane in the vial headspace was found to be $\leq$67mole%. VFA analysis showed insignificant quantities of acetic, propionic, butyric, isobutyric, valeric and isocaproic acids. Caproic acid was at 32-34 ppm concentration which was unusual.

CH_4 production was from biogasification of coal and not from the medium substrates used for growing the GSC.

The effect of two different anaerobic conditions (N_2:CO_2 and H_2:N_2) on biomethanation of Texas lignite was also studied. The hypothesis to be tested was whether H_2 in H_2:N_2 mixture can serve as an H_2 donor for CH_4 formation during biogasification of coal at 1% and 10% solids loadings. The experiment was conducted in 60-mL vials containing 0.1% SNTM + 1% or 10% TxL + 10% GSC in two sets. The medium in each set of control and experimental vials was purged with a mixture of deoxygenated H_2:N_2 (60:40) or N_2:CO_2 (80:20).

There was no significant difference in the total biogas production between the control

Table 16. Experimental Design for the Evaluation of Isolates Mixtures					
Combination/Designation		Isolates[a]			
		M1-5	M4-4	M4-5	M4-8
D	KS14RM5K8 - 1458		✓	✓	
G	KS04RM4K4 - 0444	✓	✓	✓	✓
H	KS54RM4K8 - 5448	✓	✓	✓	
I	KS15RM4K8 - 1548	✓	✓		✓
J	KS04RM5K8 - 0458		✓	✓	✓
K	KS05RM5K8 - 0558	✓		✓	✓
[a] Empty boxes designate that the isolates were not used					

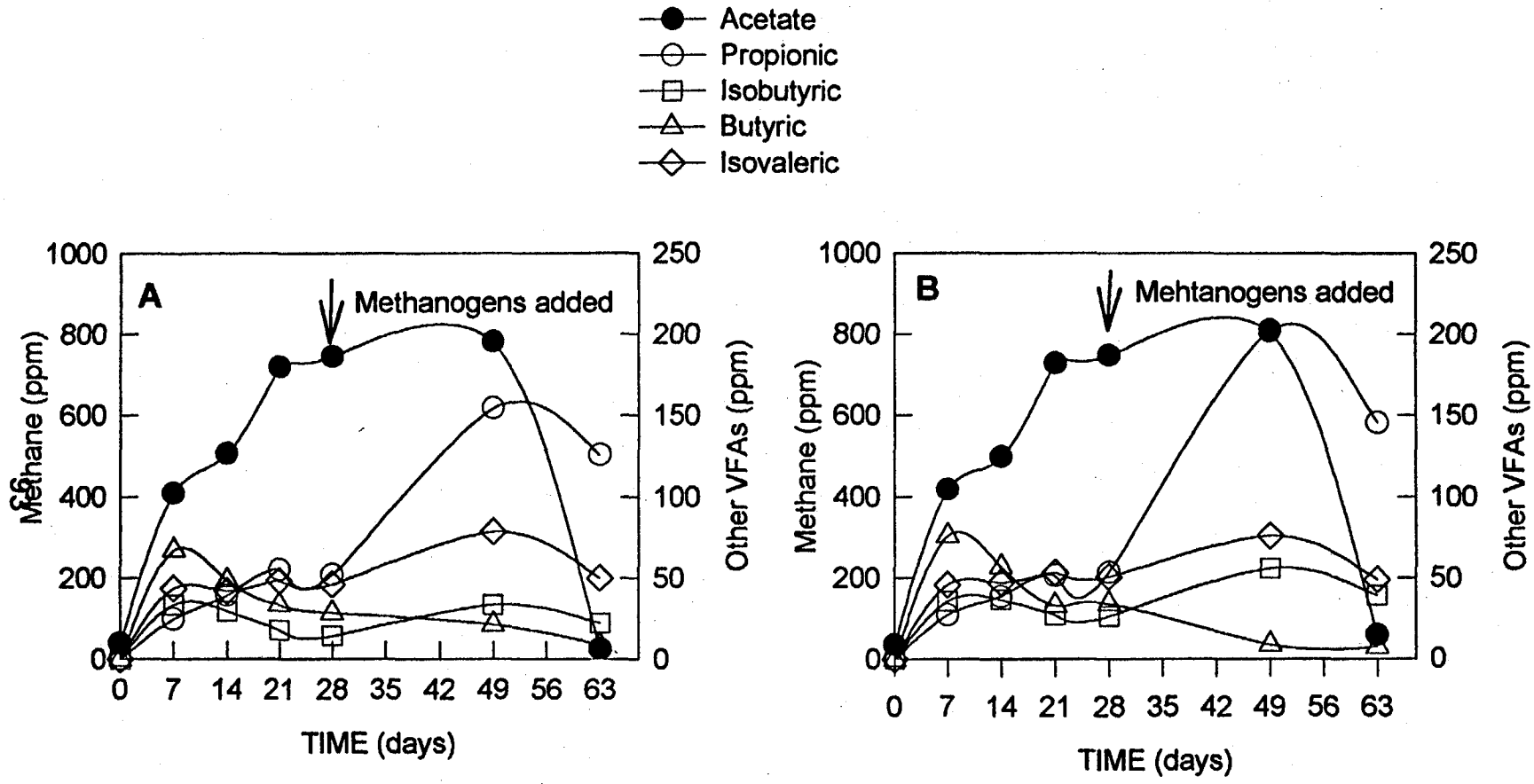


Figure 46. Time course of VFA concentrations during biomethanation of 1% TxL in 0.2% SNTM by combinations of Mic-isolates: panel A) Combination D (KS14RM5K8) and panel B) Combination G (KS04RM4K4). Methanogens were added on day 28.

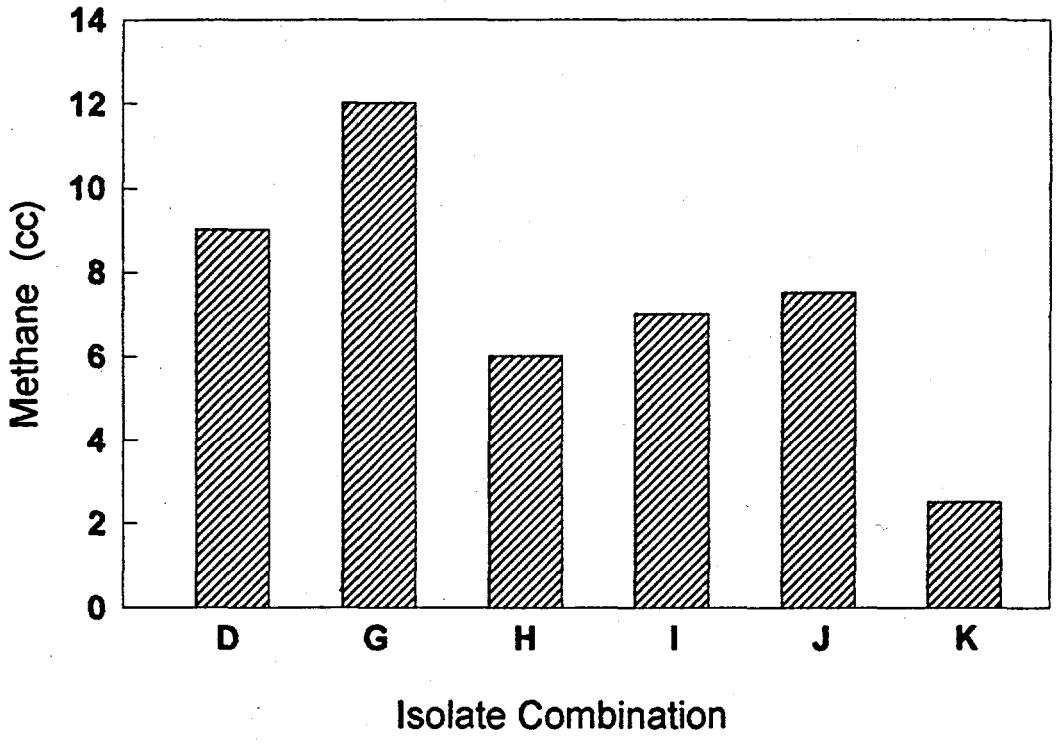


Figure 47. Methane production during biomethanation of 1% TxL in 0.2% SNTM by different combinations of Mic-isolates after the addition of methanogens on day 28.

and experimental vials. However, in the "N_2:CO_2" set total gas production was approximately 15% higher. The CH_4 production decreased with an increase in the solids loadings from 1% to 10% (Figure 48). These results confirm our previous observations on the inhibitory effect of increased solids loadings on biomethanation of TxL.

There were slight differences in the protein and COD concentrations in the experimental vials with 1% and 10% TxL. Maximum protein content was observed in the vials that contained 10% TxL and head space of N_2:CO_2. On the other hand, maximum COD concentration was not affected by the composition of head space gas mixture. The two anaerobic conditions used (H_2:N_2 and N_2:CO_2) had insignificant influence on COD (39.6 and 34.1 g O_2/L, respectively).

The concentration of VFAs varied during the course of the experiments (Figures 49, 50, 51). In both experimental sets (with 1% and 10% TxL) less propionate and isobutyrate was observed than in the control vials. Nonetheless, in all cases propionate accumulation was the highest among the VFAs monitored. This result indicates a limiting factor for overall CH_4 production from the TxL. Maximum isovalerate concentration (60.75 ppm) was obtained on day 21 in experimental vials that contained 10% GSC and 10% TxL under N_2:CO_2. Heptanoic acid was observed in the experimental vials after 10-14 days of cultivation. Maximum production of this acid (26.45 ppm) was observed on day 21 in the experimental vials containing 1% TxL. The appearance of isovaleric acid peak coincided with maximum concentration of heptanoic acid. This phenomenon was not observed in any of the previous experiments with TxL and Mic-1 consortium.

7.0 Bench scale bioreactor studies

The data presented and discussed in previous paragraphs established the technical feasibility of an anaerobic coal biomethanation process in general, and the bioconversion of TxL and other rank coals to methane and other value-added products. These data also provided sufficient information to conduct a preliminary economic evaluation of a large-scale coal bioconversion process. The economic evaluation resulted in a number of important parameters that needed to be addressed in order to develop a scale-up process. The most important among these parameters were:

a. low cost organic nitrogen supplement,
b. reduction in retention time, and
c. higher (at least >10%) coal solids loading.

A critical analysis of all data, together with recomendations from the preliminary economic analysis, indicated that prudence would be to conduct follow-up experimnets in bench-scale bioreactors. This would evaluate the scale-up potiential of the MicGAS Process at a 16-fold volume increase from the volume used in the vial studies. In these studies, the following bioreactors were evaluated:

Influence of different anaerobic conditions on CH4 production during biomethanation of 1% and 10% TxL in 0.1% SNTM by granulated sludge consortium (GSC)

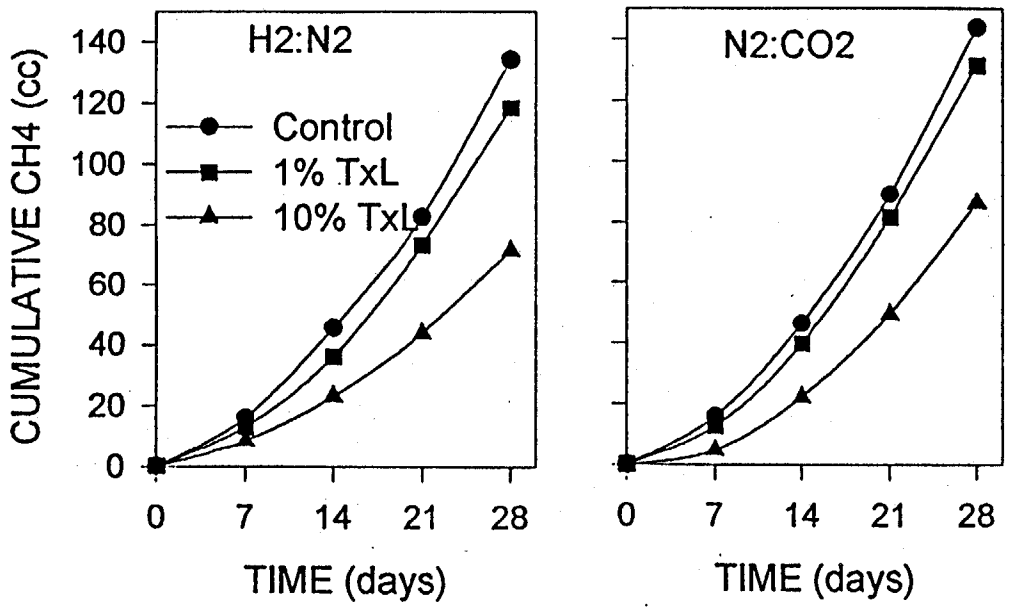


Figure 48 -- page 96

Influence of different anaerobic conditions on VFA production during biomethanation of 1% TxL in 0.1% SNTM by granulated sludge consortium (GSC)

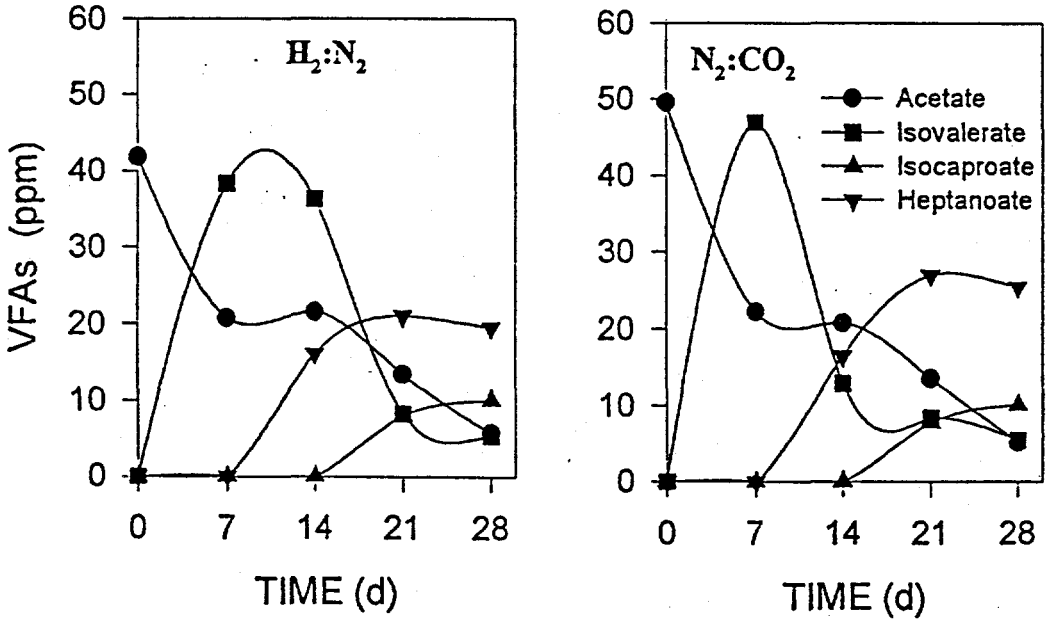


Figure 49 -- page 97

Influence of different anaerobic conditions on VFA production during biomethanation of 10% TxL in 0.1% SNTM by granulated sludge consortium (GSC)

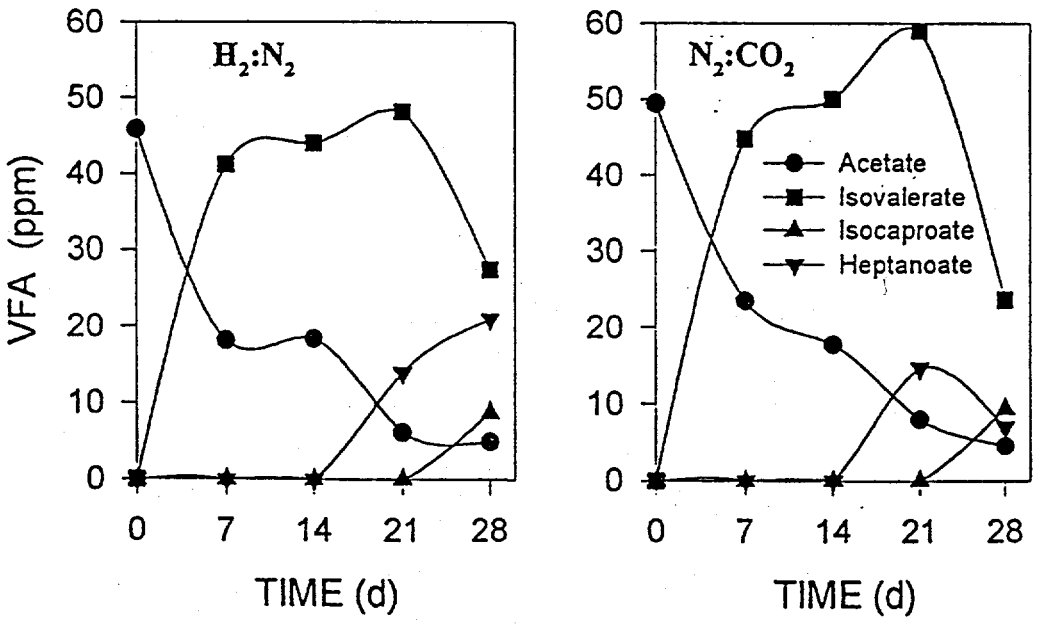
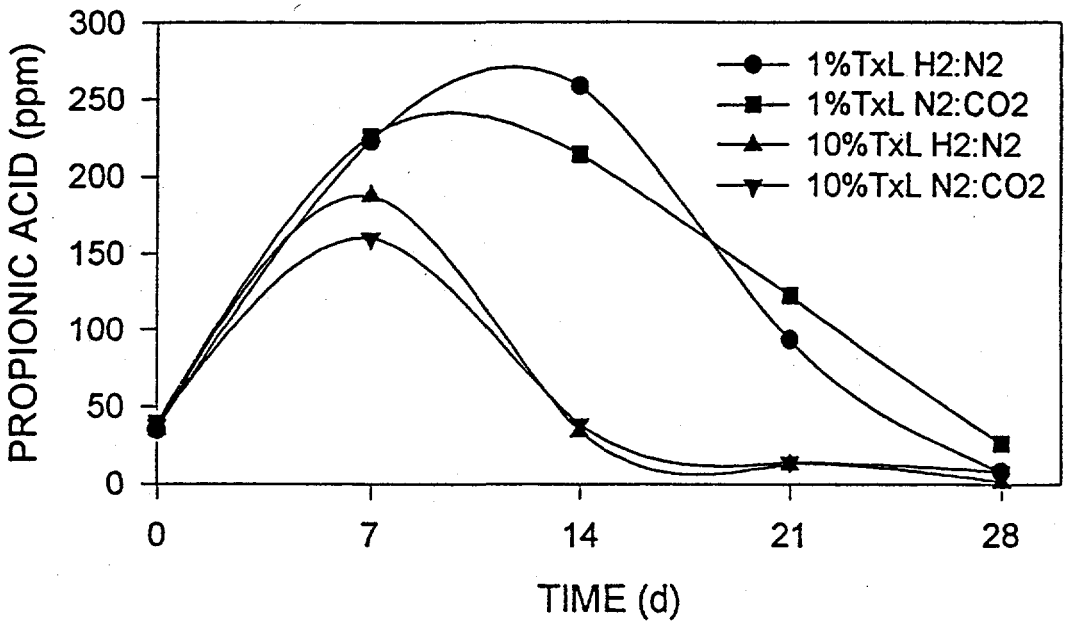


Figure 50 -- page 98

Influence of different anaerobic conditions on propionate production during biomethanation of 1% and 10% TxL in 0.1% SNTM by granulated sludge consortium (GSC)



7.1 Rotating Biological Contactor (RBC).

The maximum rate of CH_4 production began at day 4 and proceeded through day 15 (**Figure 52**). Rates of CH_4 production over this time period ranged from 496 to 603 cc CH_4/day. Control reactions produced very little methane or CO_2. Total gas production reached 11 L within 25 days. Methane concentration in the reactor headspace reached 72 mole% and CH_4 accounted for more than 60% of the total gas produced.

In a study by Fluor Daniels, it was pointed out that in order for the MicGAS Process to be commercially feasible, it is necessary to use solids loading of $\geq$20% (w/v). Biogasification of TxL at 1% and 5% solids in NTM was demonstrated using RBCs. In a latter experiment, the RBCs was charged with 10% TxL solids loadings. Initially, CH_4 production rates were high (4800 cc produced during the first 10 days). However, CH_4 production decreased over the next month (**Figure 53**). It was conjectured that the decrease in CH_4 production could be due to a loss of methanogens. To test this hypothesis, fresh inoculum (Mic-1 consortium) was added to the reactor as a source of viable methanogens and CH_4 production was monitored for another month. However, CH_4 production continued to decline. Operation of this reactor was discontinued after approximately two months.

7.2 Upflow Fluidized Bed Reactor (UFBR).

In preliminary studies conducted in plexiglass UFBR containing 1% TxL a significant increase in CH_4 production was observed (**Figure 54**). Methane concentrations of 51.96 mol% in the control and 73.21 mole% in the reactor containing 1% TxL were obtained. Higher levels of butyrate and caproate (**Figure 55**) were observed in the reactor containing 1% TxL. The acetate concentration measured in both control and experimental reactors was similar (**Figure 56**), but trace amounts of heptanoic acid were monitored in UFBR with 1% TxL. Furthermore, biomass concentration was lower in the control reactor (**Figure 56**). Another important observation was that YE/TSB addition was necessary to maintain the CH_4 production in the UFBR with 1%TxL. On the other hand, addition of YE/TSB to the control UFBR did not result in enhancement of CH_4.

In a later experiment, 5% TxL was loaded in to the glass UFBR made of glass. The headspace gas was recirculated through a peristaltic pump. Methane concentrations (48-50 mole%) were monitored in the headspace gas after 4 days of incubation. However, gas production decreased rapidly after this time. It was hypothesized that the circulated headspace gas was not able to adequately mix the large amount of TxL which was at the bottom of the reactor. This was further supported by the fact that one of the reactors containing TxL had to be discontinued because the lower circulation port became clogged with TxL. After 1 month of operation, these bioreactors were stopped.

Cumulative CH4 and CO2 production during biomethanation of 1% TxL in rotating biological contactor (RBC) by Mic-1 and sewage sludge

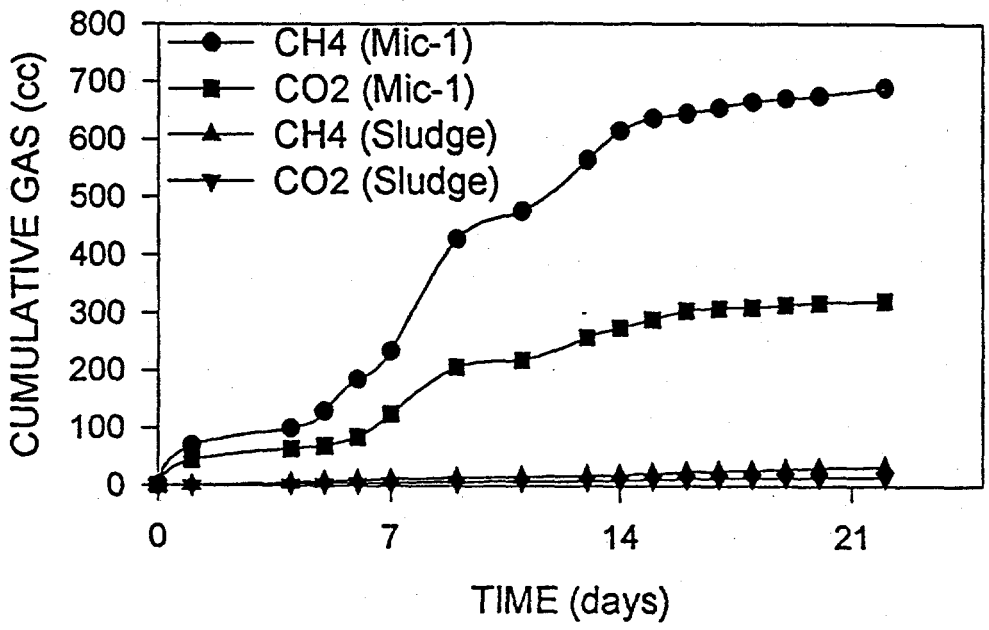


Figure 52 -- page 101

Cumulative biogas and CH4 production during biomethanation of 5% TxL in a rotating biological contactor (RBC) with 0.2% SNTM

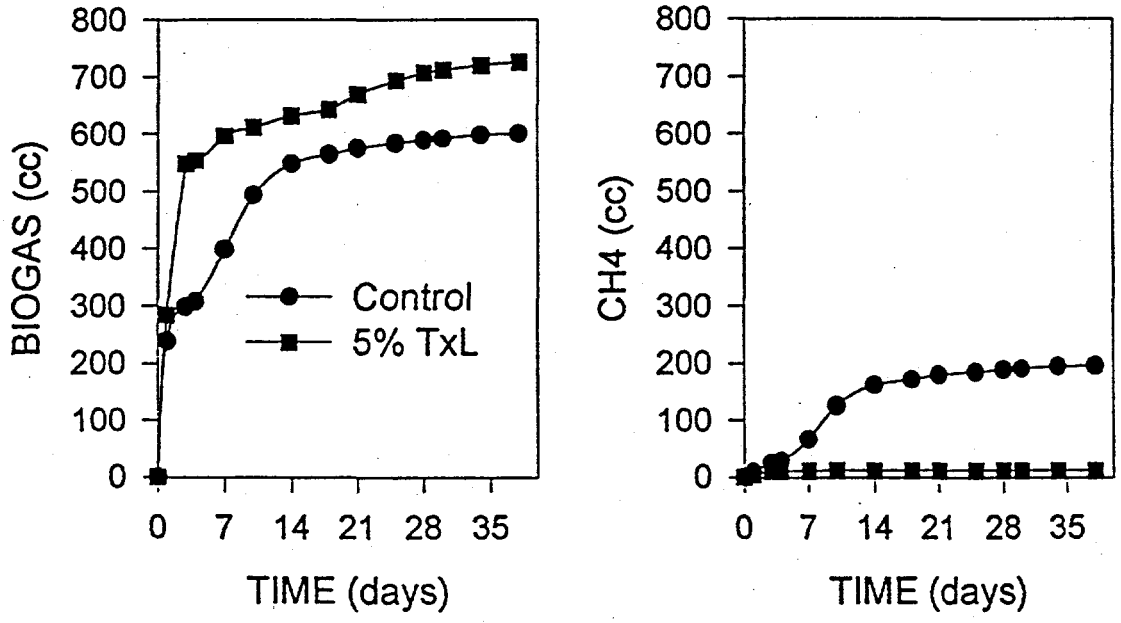


Figure 53 -- page 102

Biomethanation of 1% TxL in an upflow fluidized bed reactor (UFBR)

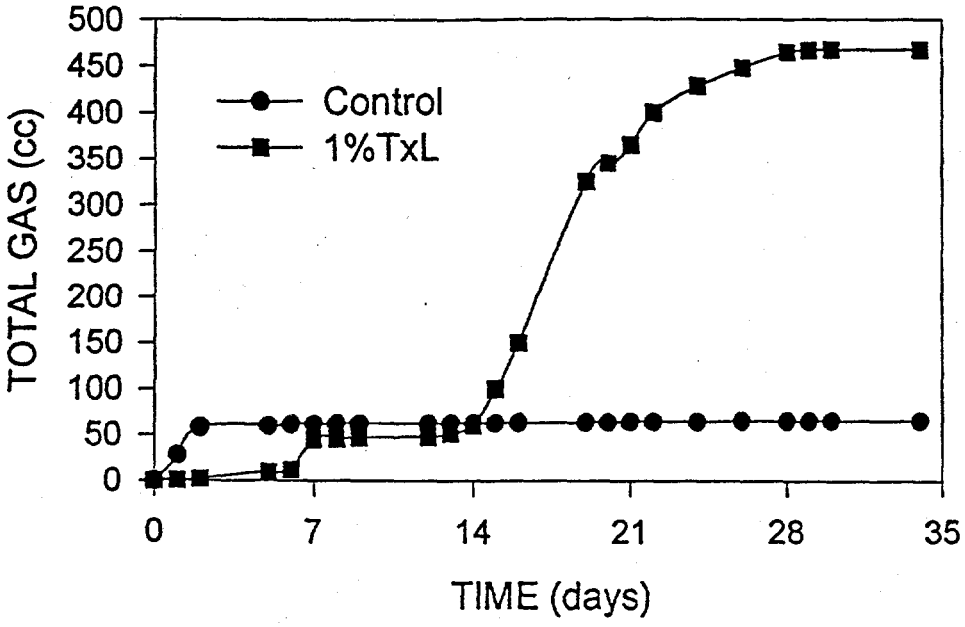
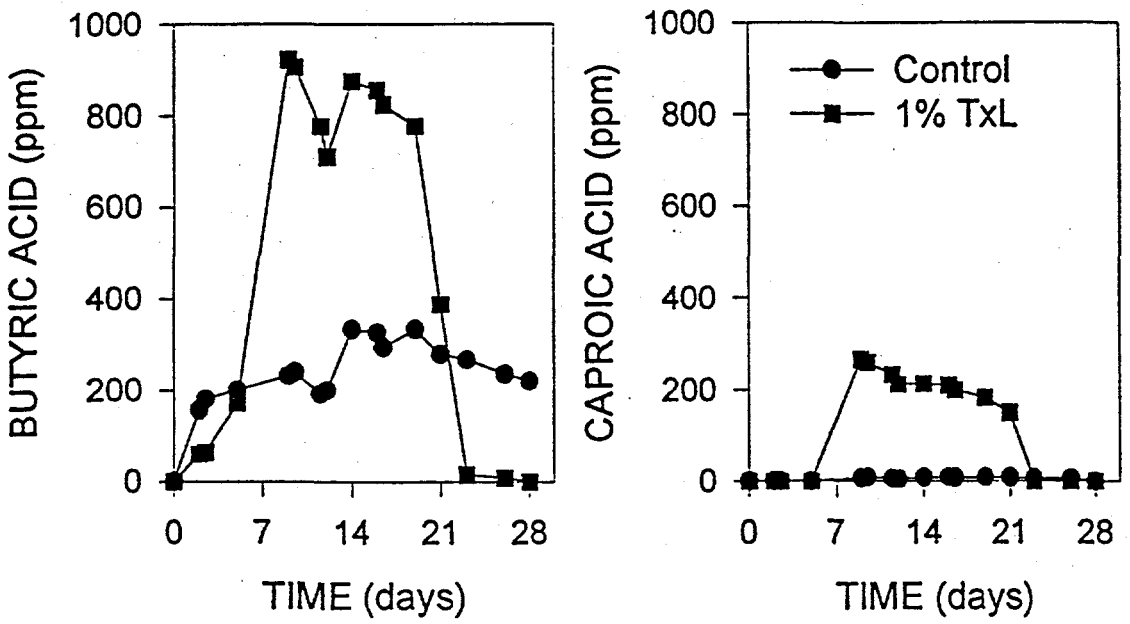
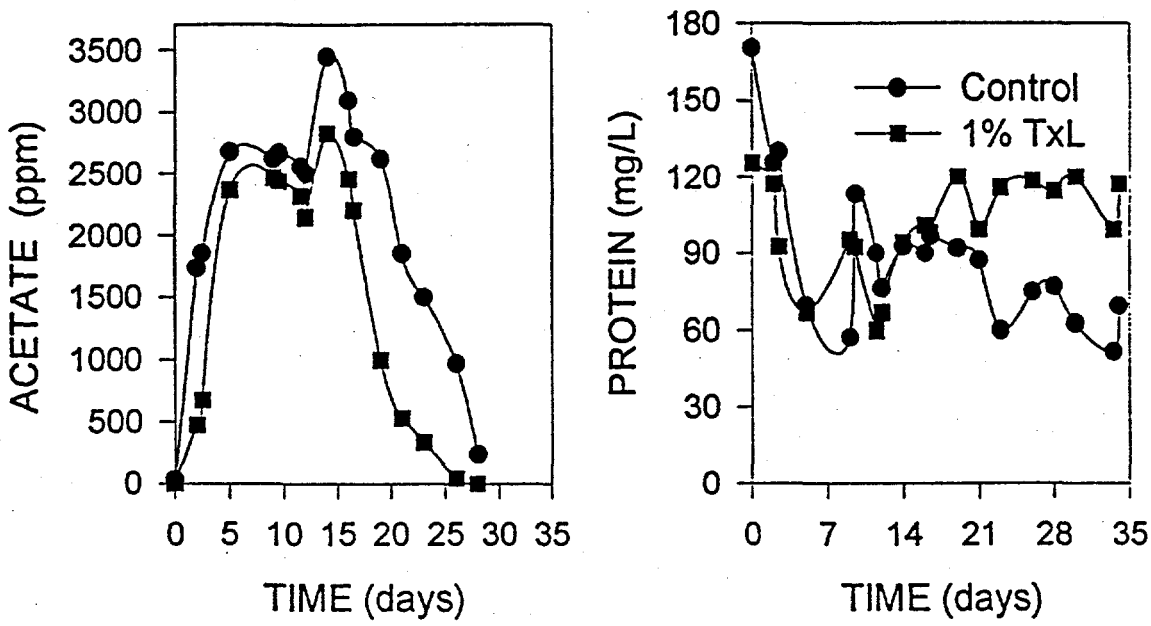


Figure 54 -- page 103

Time course of butyrate and caproate concentrations during biomethanation of 1% TxL in an UFBR



Time course of acetate and protein concentrations during biomethanation of 1% TxL in an UFBR



7.2.1 UFBR with sewege sludge

The effect of adding fresh anaerobic sewage sludge on biomethanation of TxL with and without Mic-1 was also studied. The CH_4 production was higher in UFBR containing TxL when the headspace gas was recirculated **(Figure 57)**. The data showed that for higher production of a given product, specifically developed microorganisms provide higher process efficiency than the natural microbiota. The information presented in **Figure 57** also showed that culture agitation through fluidization enhances the overall process efficiency.

7.2.2 UFBR with higher TxL solids loading

As pointed out in the economic study, the effect of higher solids loading on biomethanation of TxL in UFBR was examined. There was little difference in total gas production between the two reactors containing 5% and 10% TxL **(Table 17)**. The CH_4 concentration in the headspace was slightly higher in the reactor containing 5% TxL (18.2 mole%) than in the reactor containing 10% TxL (16.6 mole%). The CO_2 concentration was also highest in the reactor containing 10% TxL. The control (no TxL) reactor produced lower total gas, CH_4 and CO_2. The lower CH_4 production in the reactor containing 10% TxL was conjectured to be due to the lower pH (6.7) of the liquid phase, as mesophilic methanogens are generally inhibited at pHs' below 7.0.

Table 17. Biomethanation of TxL and pH in Upflow Reactors (BR) containing 0.2% SNTM after 12 Days of incubation

Treatments	Total Gas (cc)	CH_4 (mole %)	CO_2 (mole %)	pH
Control (no TxL)	122	6.6	5.8	7.6
BR 5% TxL	228	18.2	9.7	7.3
BR 10% TxL	237	16.6	16.1	6.7

7.2.3 UFBR with methanol

The UFBR were recommissioned to study the effect of methanol (CH_3OH) as hydrogen donor. The data showed that total gas and cumulative CH_4 production in the head space of reactor with CH_3OH were highest (2321 cc and 1118 cc, respectively). The peak in CH_4 production during the first 10 days **(Figure 58)** could be explained as the initial effect of addition of easy to metabolize co-substrates, like CH_3OH. Thus, in this reactor, the higher CH_4 production observed during the latter part of the experiment was due to biogasification of TxL solids loadings. This was confirmed by comparing the volumes of cumulative CH_4 produced in the reactor that did not contain CH_3OH and the one that did **(Figure 58)**. Total gas production was similar in reactors without TxL (containing only 0.2% SNTM) and the one that contained only 5% TxL in 0.2%

Influence of anaerobic sewage sludge (SS) on biomethanation TxL in an UFBR with NTM

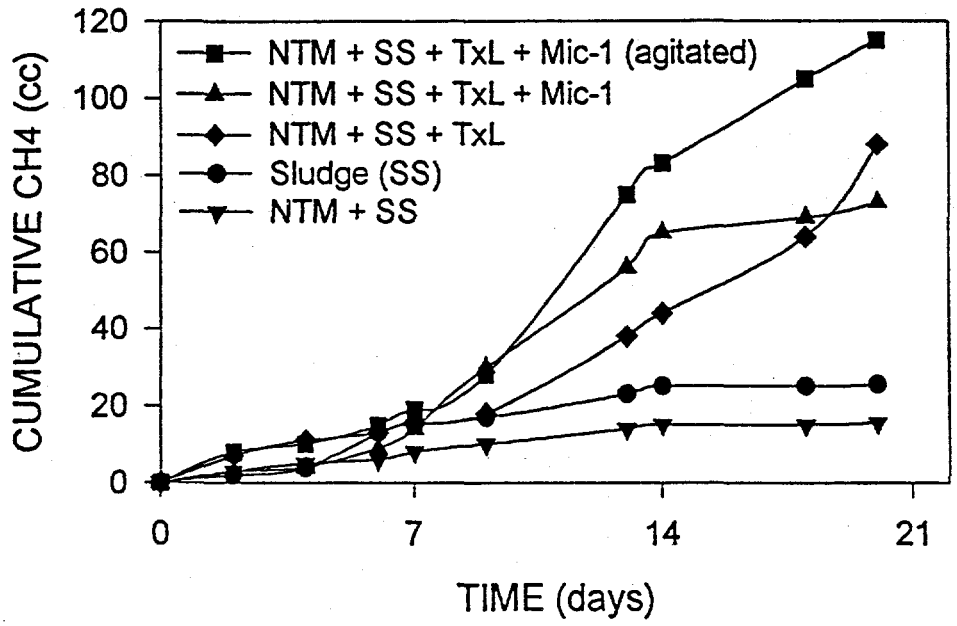
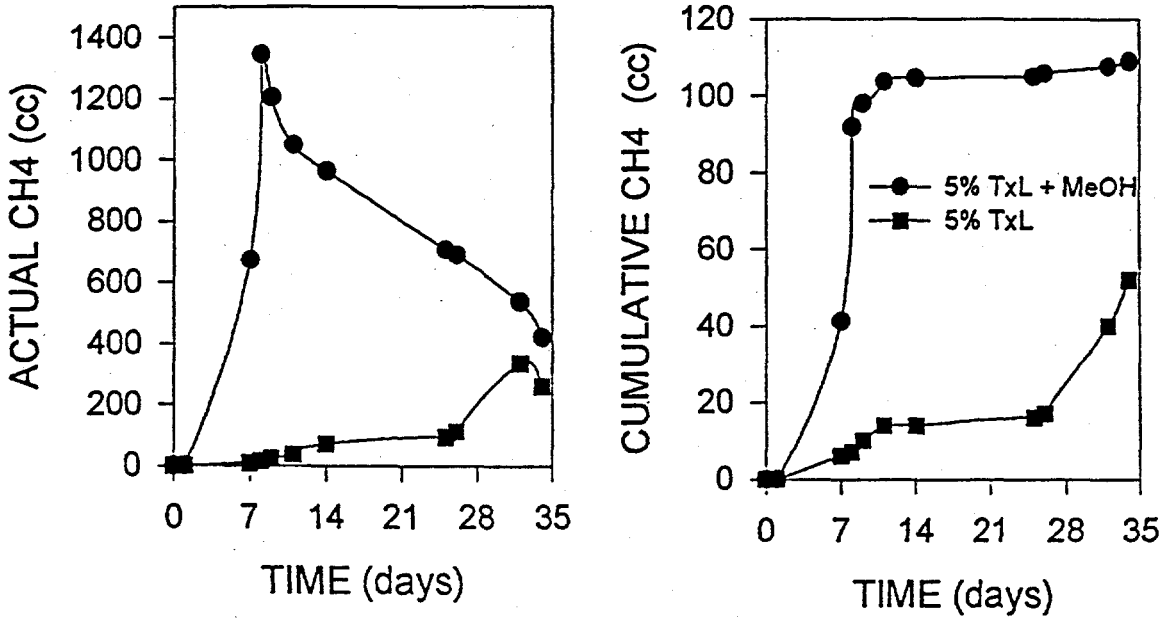


Figure 57 -- page 107

Effect of methanol (MeOH) on actual and cumulative CH4 production during biomethanation of 5% TxL in UFBRs with 0.2% SNTM + 4 X NH4Cl. Methane from controls deducted.



SNTM, but CH_4 production was observed only in the UFBR containing TxL. The net CH_4 production was highest (1344 cc) in the one containing TxL + CH_3OH. Differences in microbial growth and soluble carbon were not significant. As compared to other reactors, the addition of extra NH_4Cl to 0.2% SNTM showed significant increase in acetic, isobutyric, butyric and isovaleric acids production, but not CH_4 in the control reactor. Acetic and propionic acids were higher in the reactor with TxL + CH_3OH **(Figure 59)**, while in the one without CH_3OH, the quantity of all VFAs was lower and isobutyric and butyric acids were not observed **(Figure 60)**.

To further evaluate the effect of solids loadings on survival of methanogens, fresh inoculum (10%, v/v) containing mixture of known methanogens was added on day 25 to provide viable methanogens. Methane production (337 cc) increased only in the reactor that contained 5% TxL **(Figure 58)**.

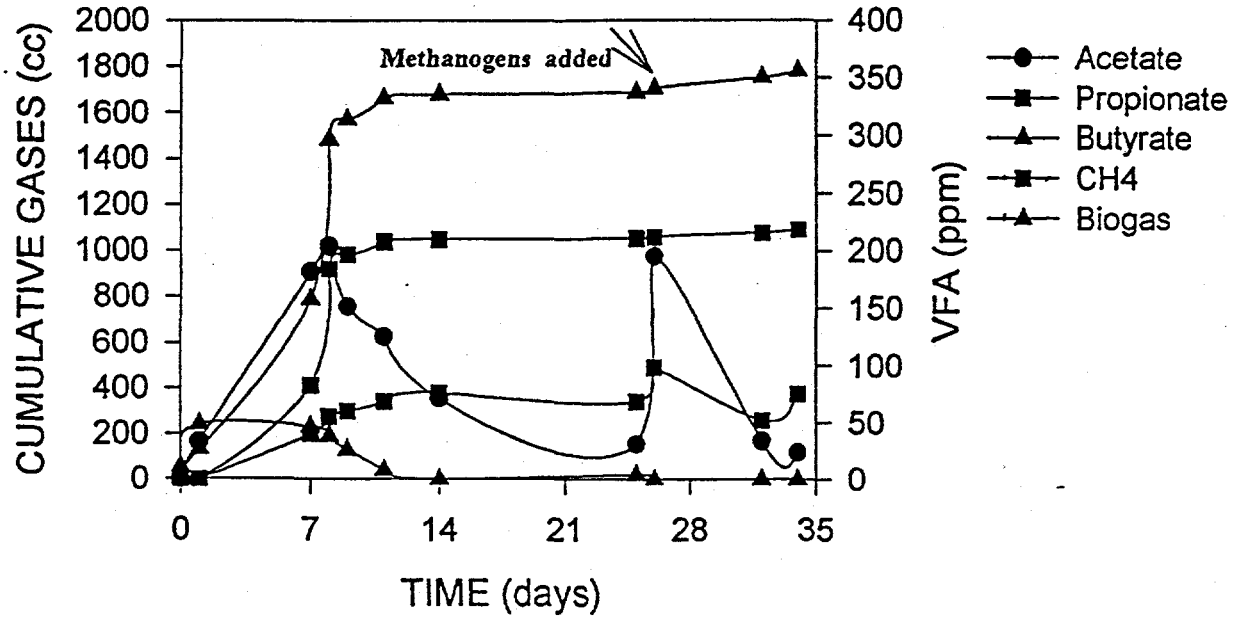
7.2.4 UFBR with citrate and methanol

The vial experiment described in preceding sections was scaled up in six upflow fluidized bioreactors to confirm the results obtained in the vials on the effect of co-substrate, surfactants, and wetting agents addition. The experimental design is presented in **Table 18**. Two media were used - SNTM and NTM. Mic-1 consortium was used as 10% inoculum (v/v) in all reactors.

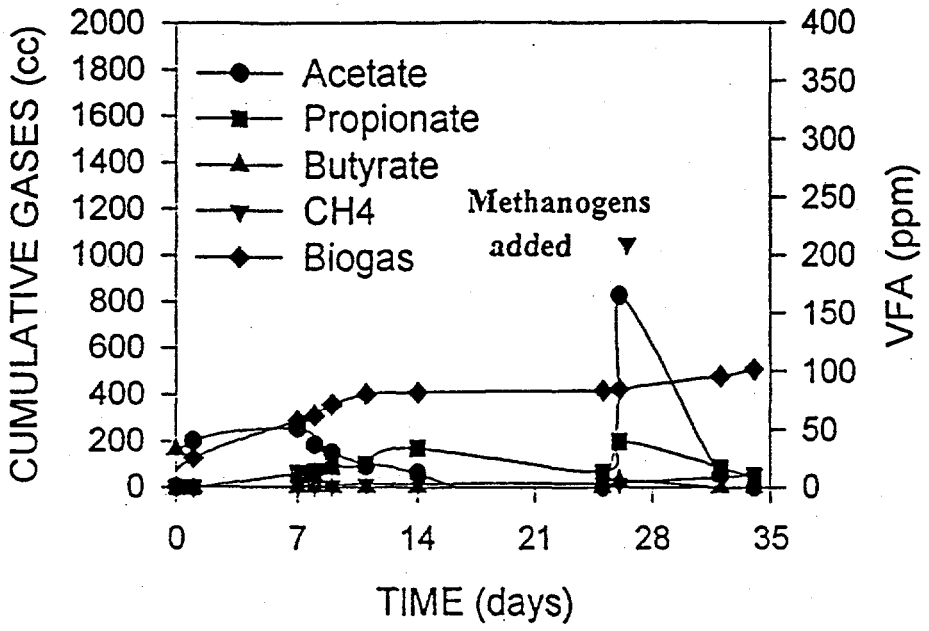
Table 18. Cumulative methane production in "6 Upflow Bioreactors with NTM & SNTM.

Reactor, #	COMPONENTS	CUMULATIVE CH_4 PRODUCTION (cc) ON DAYS			
		2	10	12	14
1	NTM + 0.5% MeOH (4 mL)	0	2	4	5
2	NTM + 5% TxL (40 g)	0	1	4	5
3	0.2% SNTM + 0.5% MeOH	0	4	8	12
4	0.2% SNTM + 5% TxL	0	8	19	30
5	0.2% SNTM + 5% TxL + 0.5% MeOH	0	128	2579	4156
6	0.2% SNTM + 5% TxL + 0.5% MeOH + 10 mM sodium citrate	0	570	3594	5227

Effect of methanol addition on biogas and VFA production during biomethanation of 5% TxL in UFBR with 0.2% SNTM + 4 X NH4Cl



Biogas and VFA production during biomethanation of 5% TxL by Mic-1 in an UFBR with 0.2% SNTM + 4 X NH4Cl



Reactor 1 was a "control" for reactor 3, reactor 2 was a "control" for reactor 4, and reactor 4 was a "control" for reactors 5 and 6. Reactors were sampled for total gas, pH, liquid and gas composition at regular time intervals.

The cumulative CH_4 and CO_2 production and some of the VFA data from bioreactors are presented in **Figures 61 to 66.** Highest CH_4 production was obtained in the bioreactor supplemented with 0.2% SNTM, 5% TxL, 0.5% methanol, and 10 mM sodium citrate **(Figure 66).** The maximum CH_4 production was found to be on day 11. The concentration of CH_4 in the total gas was up to 75 mole% **(Figure 67).** Considerably high CH_4 production with 73 mole% gas composition was also obtained in the reactor containing 0.2% SNTM, 5% TxL and 0.5% methanol. In the presence of an organic nitrogen source (Sheftone T™) the microbial activity was further enhanced and resulted in increased CH_4 production and acetate accumulation **(Figures 63 to 66),** while in its absence CH_4 and acetate accumulation were negligible **(Figures 61 and 62).** The exponential increase in cumulative CH_4 around days 11 and 12 in bioreactors #5 and #6 was accompanied by a decrease in acetate concentration **(Figure 65 and 66, respectively).** In contrast, the CH_4 production in bioreactors #3 and #4 was only 5 and 6 mole%, respectively. The acetate concentration was lower in both bioreactors **(Figures 61, 62 and 67).**

Furthermore, data from the VFA analyses showed that acetate accumulation in bioreactor #3 reached 400 ppm in two days and remained at the same level until day 10 **(Figure 63)** . In bioreactor #1, on the other hand acetate concentration reached up to the same level, but in a longer period of time (10 days) and remained in the range of 270-240 ppm **(Figure 62).** This indicates that the acetate has not been converted into CH_4. On the other hand, comparison of data from bioreactor #2 **(Figure 62)** and bioreactor #4 **(Figure 64)** shows that propionate production was higher (100 ppm) in bioreactor #4 than in bioreactor #2. There was a significant difference in acetate concentration in both bioreactors - 600 ppm on day 7 (bioreactor #4) and less than 150 ppm on day 10 (bioreactor #2). Based on these data, together with the pattern of CH_4 production in different bioreactors, it can be concluded that the presence of organic nitrogen source (Sheftone T™) enhanced both acetate and CH_4 production.

Higher concentration of acetate (approx. 600 ppm) was also observed in bioreactor #5 **(Figure 65).** The maximum acetate production was obtained in the bioreactor #6 **(Figure 66)** which contained 10 mM sodium citrate in addition to the components of bioreactor #5. The sharp decrease in acetate concentration from day 10 to day 20 indicated a rapid conversion of this precursor of methanogenesis to CH_4. On the other hand, decrease in the butyrate concentration up to day 10 and increase in the propionate concentration from day 10 **(Figure 65)** can be explained as conversion of higher volatile fatty acids (eg. butyrate) to lower ones such as acetate. In bioreactor #3 **(Figure 68)** the highest concentration among the VFA's was that of butyric acid,

EFFECT OF CO-SUBSTRATE ADDITION (0.5% METHANOL) ON CUMULATIVE CH_4 PRODUCTION AND VFAs CONCENTRATIONS IN AN UPFLOW BIOREACTOR #1 CONTAINING NTM

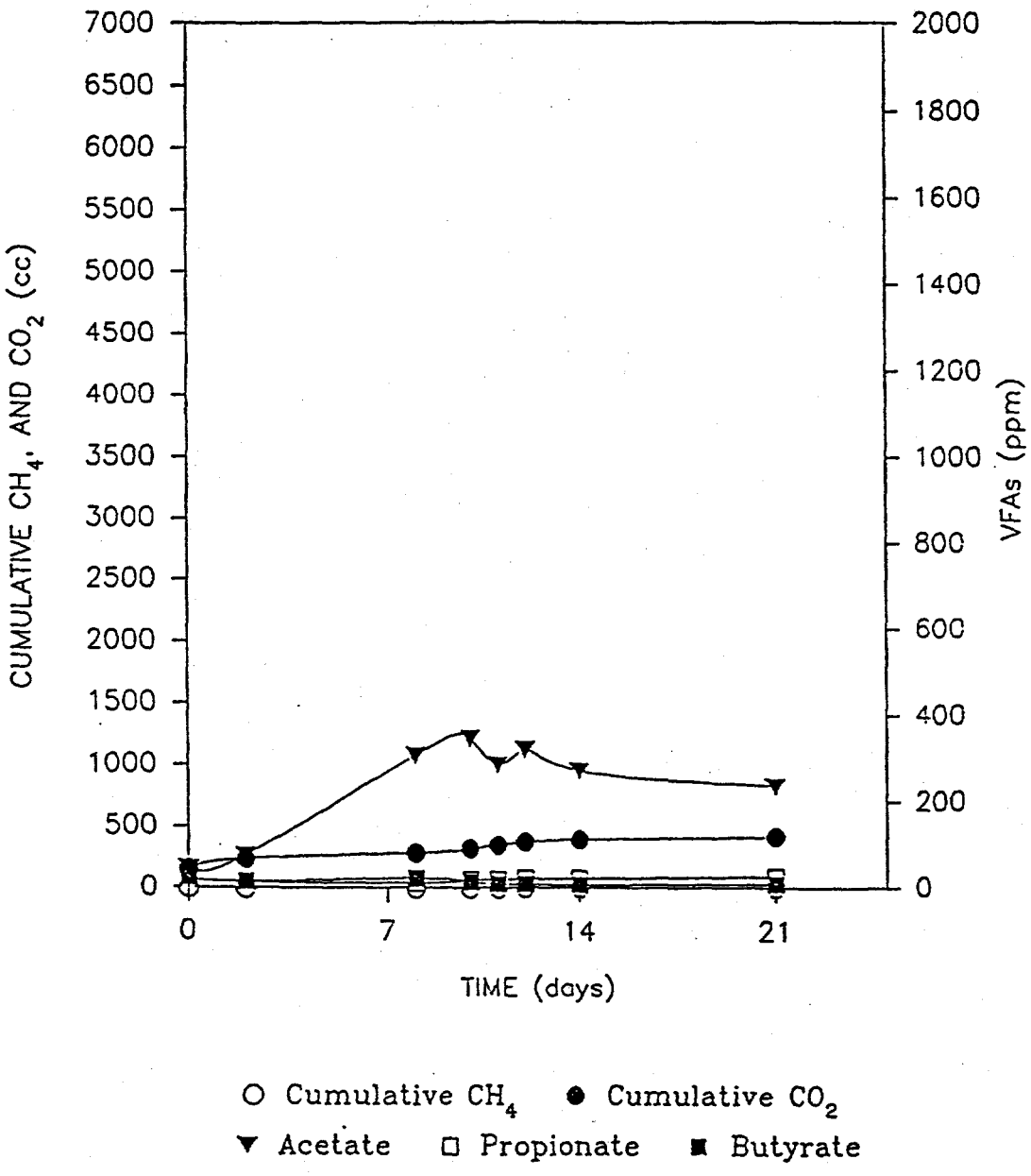


Figure 61 -- page 113

EFFECT OF CO-SUBSTRATE ADDITION – CUMULATIVE CH_4 PRODUCTION AND VFAs CONCENTRATIONS DURING BIOMETHANATION OF 5% TEXAS LIGNITE IN AN UPFLOW BIOREACTOR #2 CONTAINING NTM (no H^+-donors)

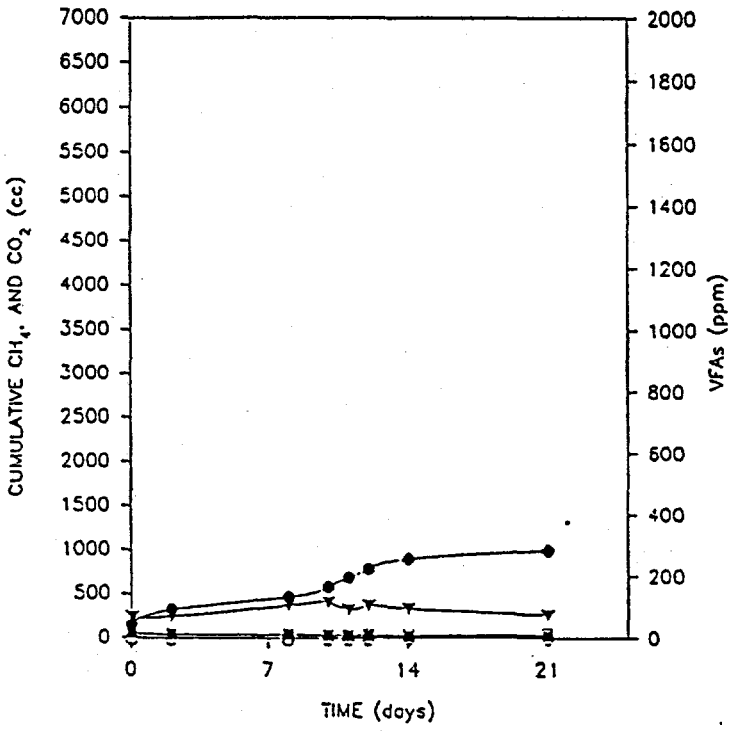


Figure 62 -- page 114

EFFECT OF CO-SUBSTRATE ADDITION (0.5% METHANOL) ON CUMULATIVE CH_4 PRODUCTION AND VFAs CONCENTRATIONS IN AN UPFLOW BIOREACTOR #3 (control - no TxL)

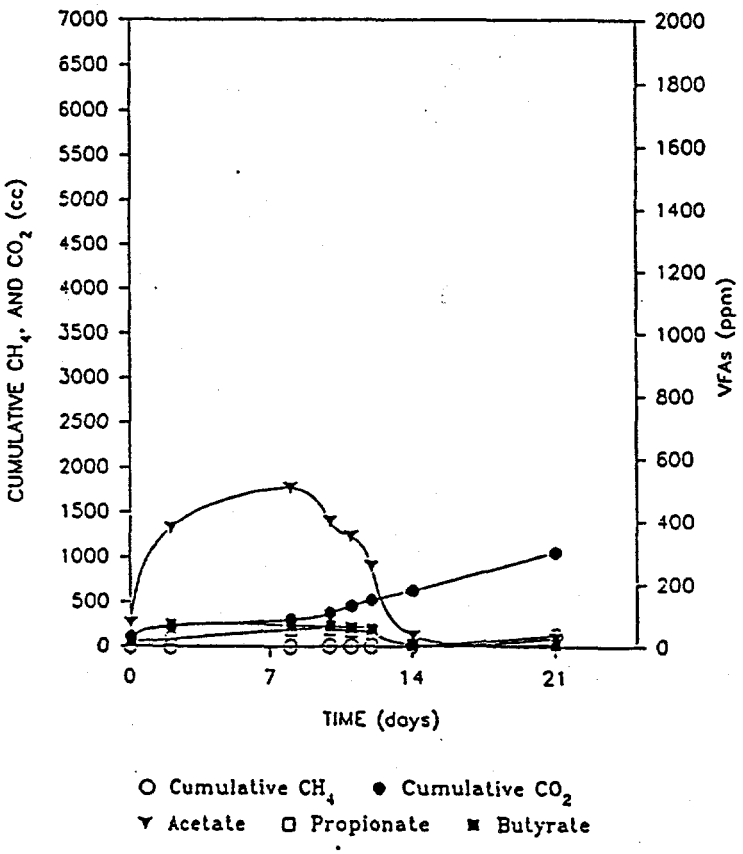


Figure 63 -- page 115

EFFECT OF CO-SUBSTRATE ADDITION – CUMULATIVE CH_4 PRODUCTION AND VFAs CONCENTRATIONS DURING BIOMETHANATION OF 5% TEXAS LIGNITE IN AN UPFLOW BIOREACTOR #4 (no H^+-donors)

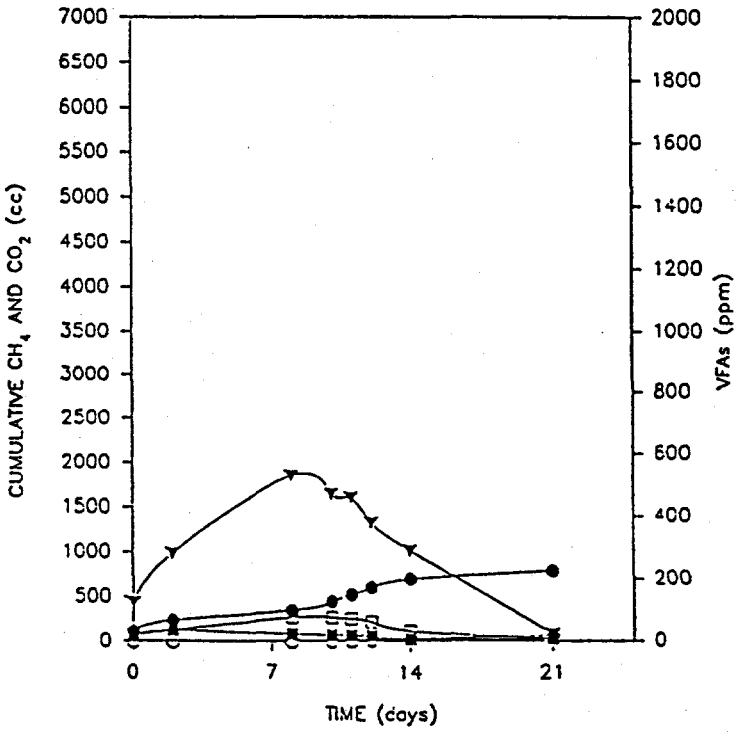


Figure 64 -- page 116

EFFECT OF CO-SUBSTRATES ADDITION (0.5% METHANOL) ON CUMULATIVE CH_4 PRODUCTION AND VFAs CONCENTRATIONS DURING BIOMETHANATION OF 5% TEXAS LIGNITE IN AN UPFLOW BIOREACTOR #5

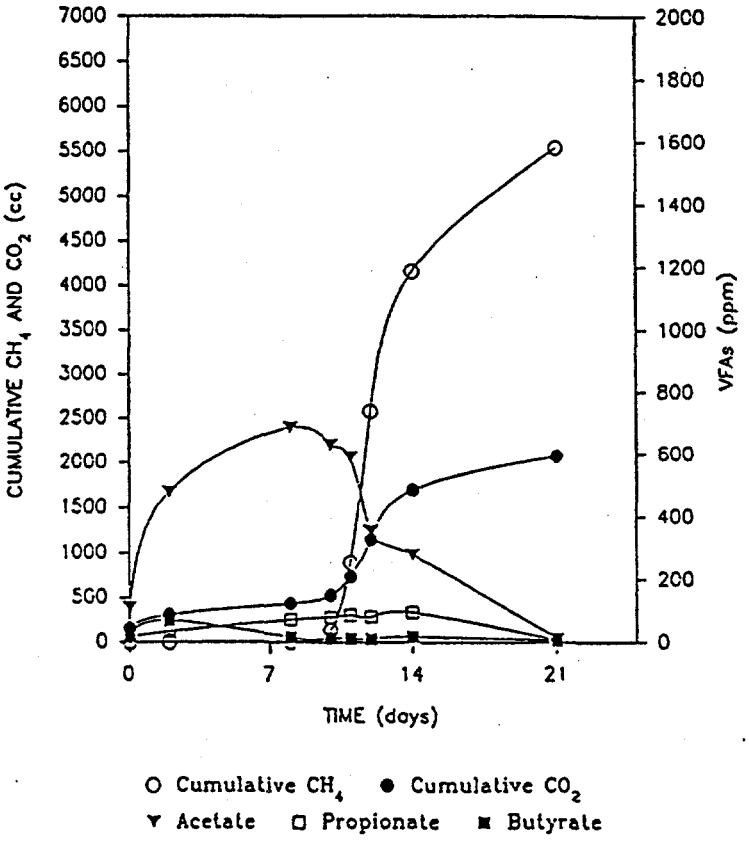
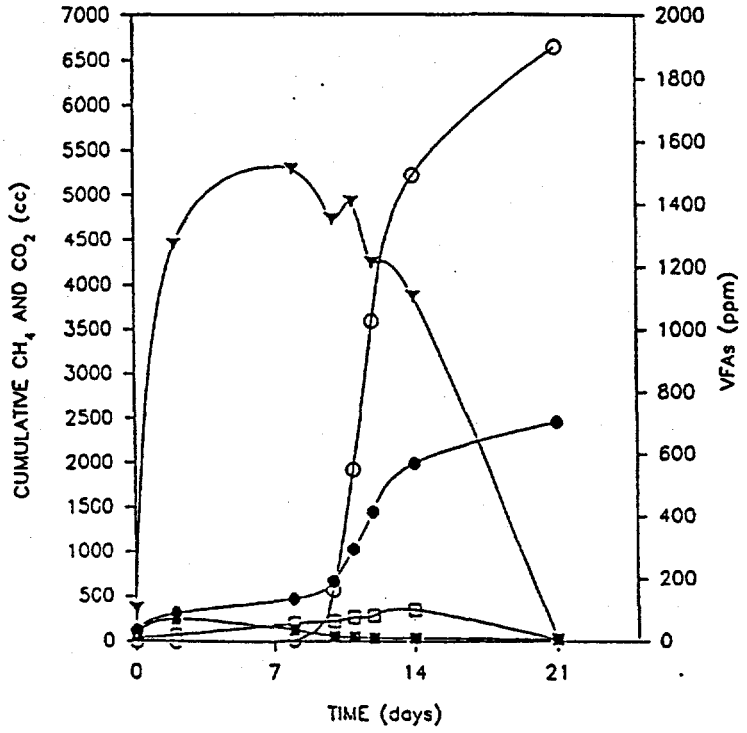
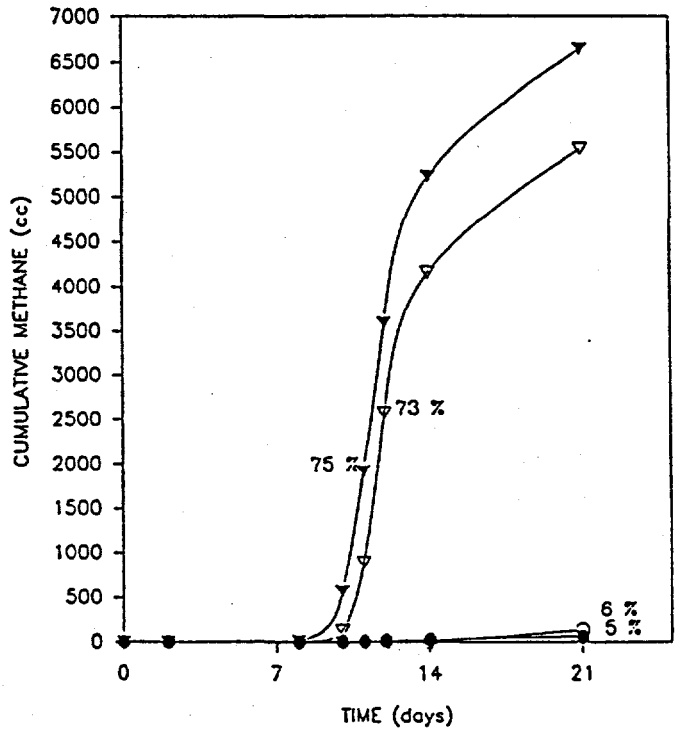


Figure 65 -- page 117

EFFECT OF CO-SUBSTRATES (0.5% METHANOL AND 10 mM CITRATE) ON CUMUALTIVE CH_4 PRODUCTION AND VFAs CONCENTRATIONS DURING BIOMETHANATION OF 5% TEXAS LIGNITE IN AN UPFLOW BIOREACTOR #6



EFFECT OF CO-SUBSTRATE ADDITION (0.5% METHANOL AND 10 mM CITRATE) ON CUMULATIVE CH_4 PRODUCTION IN UPFLOW BIOREACTORS (#3 to #6) WITH 0.2% SNTM DURING BIOMETHANATION OF 5% TEXAS LIGNITE



○ BR #3 + 0.5% MeOH
● BR #4 + 5% TxL
▽ BR #5 + 0.5% MeOH + 5% TxL
▼ BR #6 + 0.5% MeOH + 10mM Citrate + 5% TxL

* Numbers with % show the maximum mole % of CH_4 produced

Figure 67 -- page 119

TIME COURSE OF VFAs CONCENTRATION IN AN UPFLOW BIOREACTOR #3 (Control) CONTAINING 0.2% SNTM AND 0.5% METHANOL

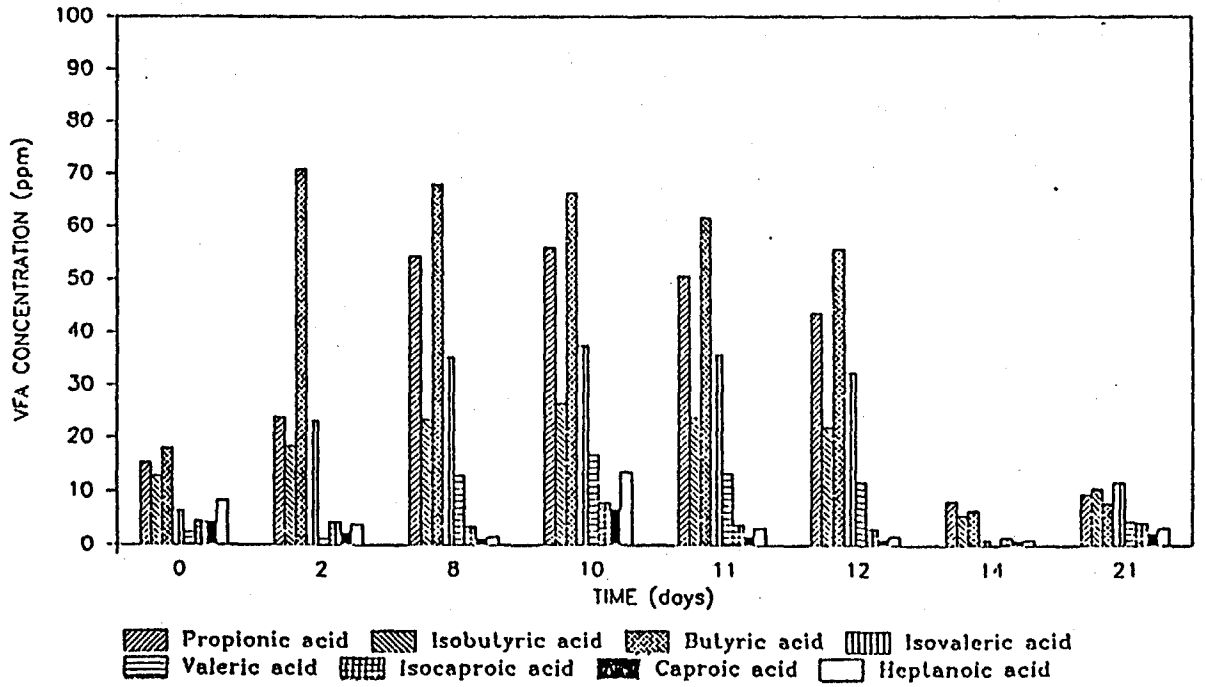


Figure 68 -- page 120

while in bioreactor #4 (Figure 69) the propionic acid concentration was significantly higher. Propionic acid concentration in bioreactor #5 and #6 (Figures 70 and 71) followed the same pattern (increased up to day 14 and decreased rapidly until day 21) as in bioreactor #4. Therefore, it is believed that in the presence of TxL, acidogens in Mic-1 consortium have better ability to degrade organic compounds to lower VFAs such as propionate (fermentation). In contrast, the higher concentration of VFA's with longer carbon chain are produced by these non methanogenic members when TxL is not present in the medium.

In order to further understand the factors affecting biogasification of TxL another experiment was conducted in upflow bioreactors. In this experiment, the effect of stepwise re-addition of methanogens, Mic-1, Sheftone T™, citrate and methanol was investigated (Figure 72) in order to enumerate the factors that have maximum influence on biomethanation. The operating conditions were kept as in the previous experiment. The bioreactors were assembled with all necessary tubing and sets of bottles for measuring the total gas produced. Initially, eight hundred mL of 0.2% SNTM-CM (0.2% SNTM with 0.5% methanol and 10 mM citrate) was used in each bioreactor. Texas lignite was added in five of the bioreactors at 5% solids loading, but not to the sixth bioreactor, which was a control. These bioreactors were inoculated with Mic-1 consortium (10%, v/v). The effect of each factor affecting biogasification process was studied as follows:

A After a definite period of operation (when all 5 bioreactors containing TxL reached "steady" state, i.e. no significant increase in CH_4 production), to 4 of them a mixture of methanogens was added (T_2). The 5th bioreactor was left as a "control" (no methanogens added), so that the effect of methanogens on CH_4 production can be compared. The rationale behind this experiment was to investigate how much is the conversion of accumulated acetate observed in the previous experiment with bioreactors in the medium during the first period of the biogasification process (T_1).

B Mic-1 consortium was added when no increase in CH_4 production was observed following the addition of methanogens. To three of the bioreactors, containing methanogens (T_2), a fresh inoculum of Mic-1 consortium (10%, v/v) was added (T_3). At this point the 4th bioreactor was left as a "control" to compare the effect of Mic-1 addition. The hypothesis to be tested was that a "fresh" Mic-1 consortium will help for more efficient biogasification of TxL through further degradation of TxL to acetate which is the substrate for methanogens. Therefore, the CH_4 production in the bioreactors (containing additional Mic-1 consortium) should increase.

TIME COURSE OF VFAs CONCENTRATION IN AN UPFLOW BIOREACTOR #4 CONTAINING 0.2% SNTM AND 5% TEXAS LIGNITE

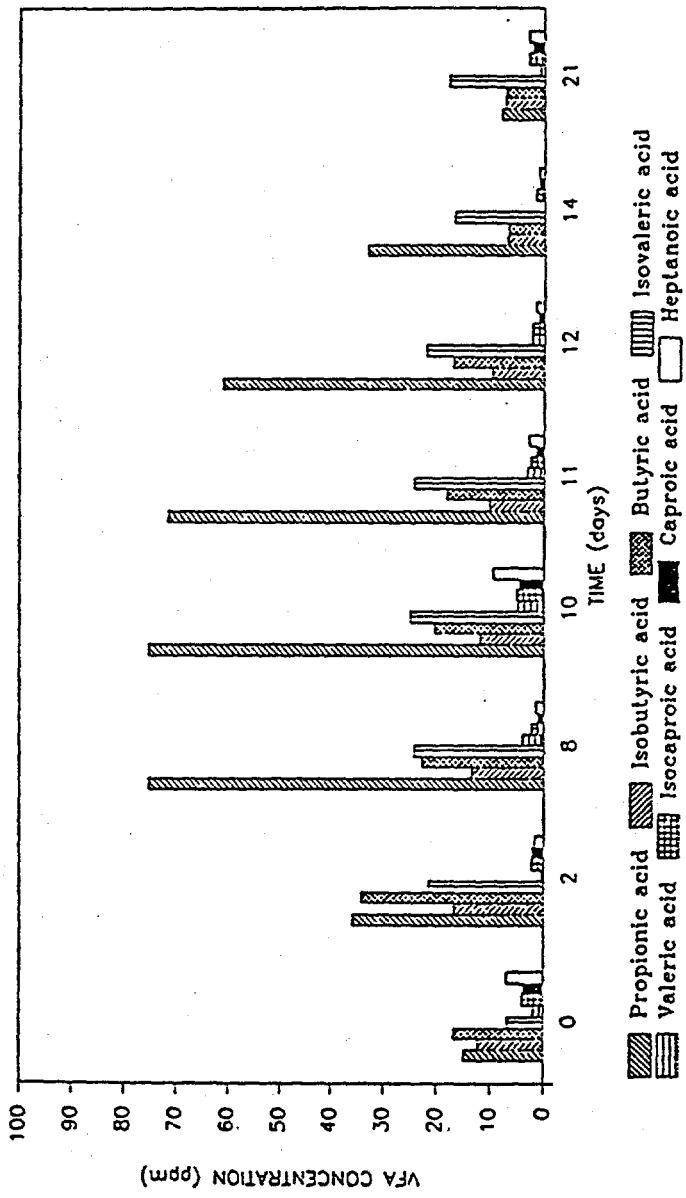


Figure 69 -- page 122

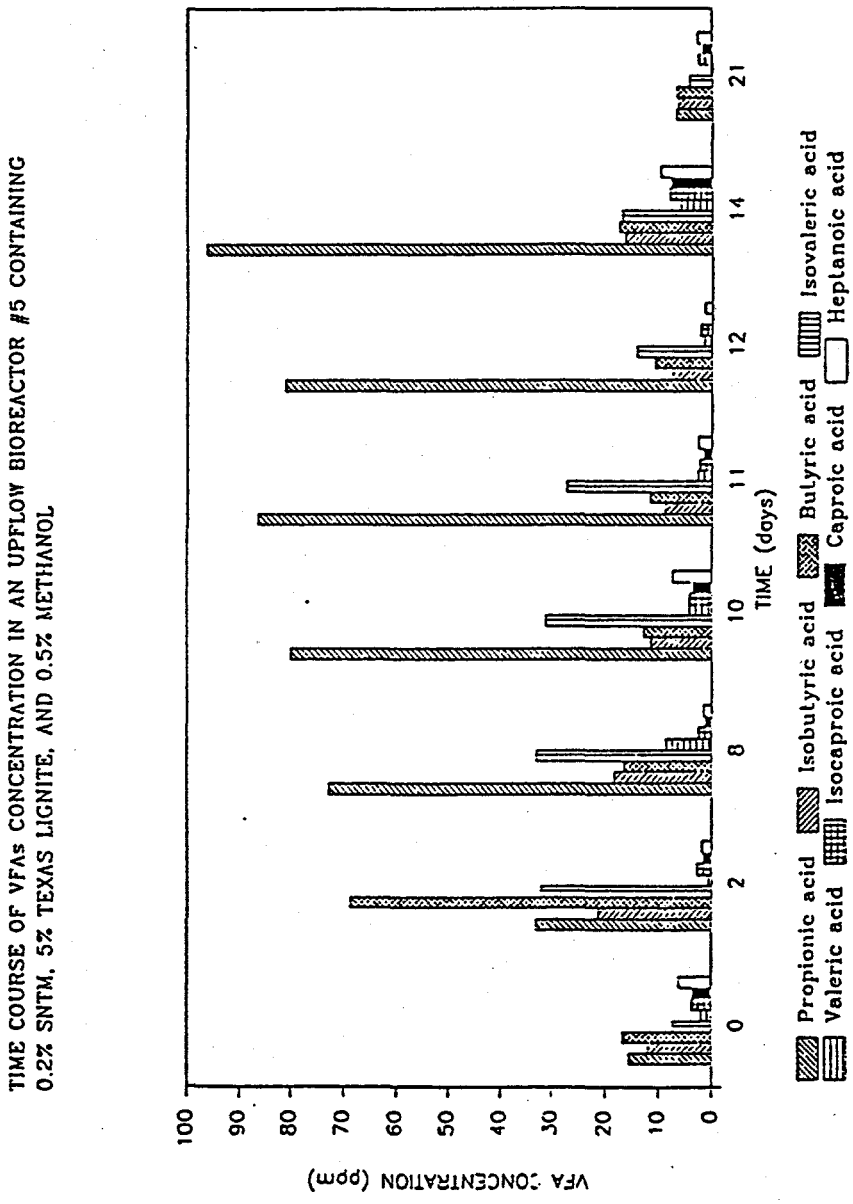


Figure 70 -- page 123

TIME COURSE OF VFAs CONCENTRATION IN AN UPFLOW BIOREACTOR #6 CONTAINING 0.2% SNTM, 5% TEXAS LIGNITE, 0.5% METHANOL, AND 10 mM CITRATE

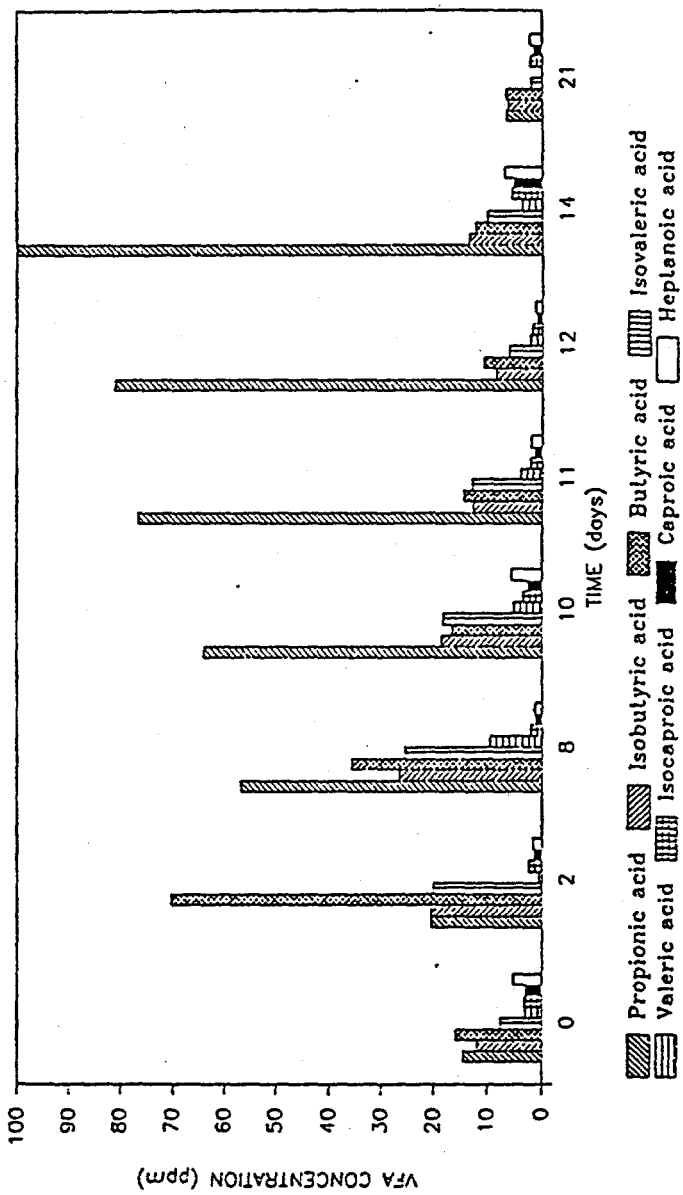


Figure 71 -- page 124

EFFECT OF SEQUENTIAL RE-ADDITION OF MEDIUM COMPONENTS ON BIOMETHANATION OF 5% TEXAS LIGNITE IN UPFLOW BIOREACTORS CONTAINING 0.2% SNTM, METHANOL AND SODIUM CITRATE

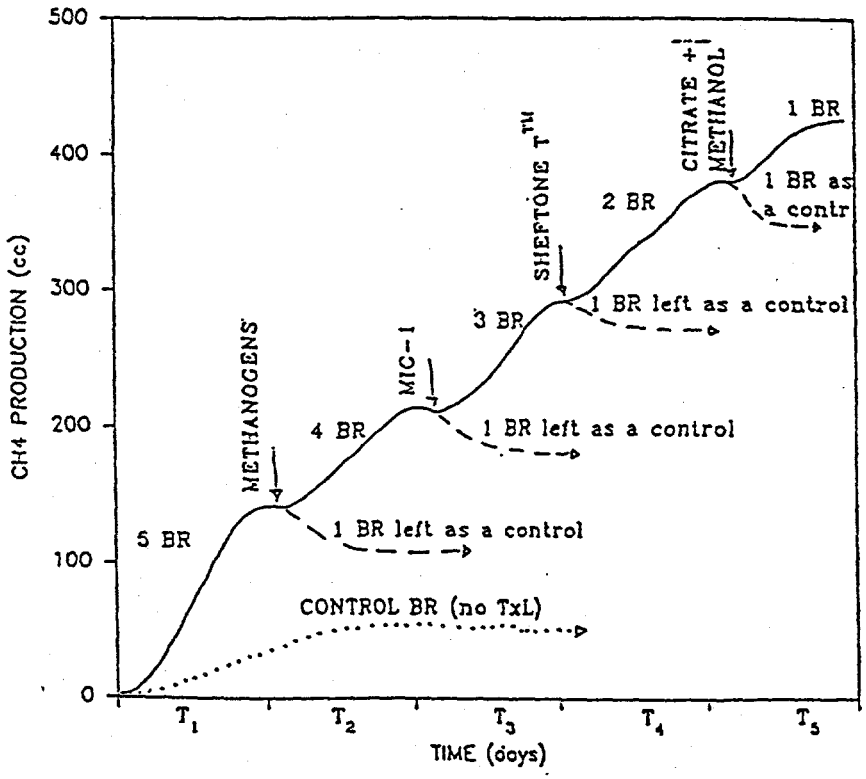


Figure 72 -- page 125

C The next hypothesis to be tested was that after the addition of Mic-1 to the medium, the major nitrogen source should be depleted which in turn will slow down the growth of all bacterial population and will result in complete slow down of the process. Therefore, addition of a new solution of Sheftone T™ (final concentration of 0.2%) will provide the necessary organic nitrogen for Mic-1 consortium (T_4). Thus, Sheftone T™ was added into two of the three remaining bioreactors, while the third bioreactor was left as a "control".

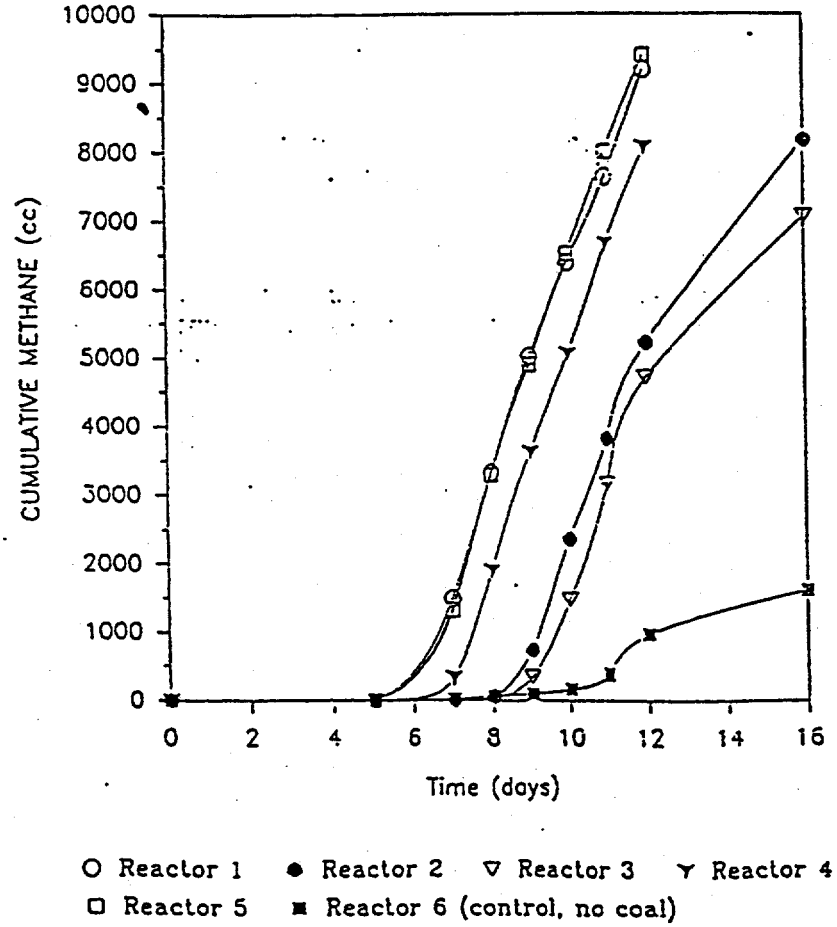
D The final "step" in this experiment was to test the effect of citrate and methanol on CH_4 production. The hypothesis to be tested was similar to that in paragraph C, i.e. until this time (T_5) citrate and methanol originally added to the medium would have been utilized and the "new" citrate and methanol will sequester more metal ions or provide additional hydrogen, respectively. Therefore, to one of the two remaining reactors, after the additions made in paragraph C, citrate (10 mM) and methanol (0.5%) were added and the other one left as control **(Figure 72)**.

The results obtained during the first 12 days of Mic-1 cultivation (T_1) show that CH_4 production in the bioreactors containing TxL ranged from 5500 to 9200 cc. Maximum CH_4 was obtained in bioreactors #1 and #5 on day 8 **(Figure 73)**. For the remaining three bioreactors the highest CH_4 production was within 9 to 11 days. This insignificant difference in the time for the maximum CH_4 production shows that biogasification process in upflow bioreactors is reproducible.

Maximum CH_4 (~18,000 cc, CH_4 produced in the control was deducted) was obtained from bioreactor #5, where all investigated factors were sequentially added during the experiment. The addition of methanogens in reactors #2, #3, #4, and #5 after the first peak of CH_4 production (9-11 days), resulted in a rapid decrease of acetate concentration and increase of CH_4. The concentration of propionate, isobutyrate, butyrate and isovalerate decreased up to 50%.

This could be explained as the "effect of product inhibition". Acidogens and acetogens in Mic-1 consortium biodegrade TxL to lower VFAs (propionate, isobutyrate, etc.) and acetate (main precursor for CH_4 production), respectively. However, after the accumulated acetate reaches a certain concentration, it suppresses the activity of acido- and acetogens. The presence of methanogens (or their addition), however, convert acetate to CH_4 which allows acetogens to further convert lower VFAs to acetate and in turn acidogens are triggered to convert coal hydrolysis products to lower VFAs.

Cumulative methane production during biomethanation of 5% TxL in fluidized upflow bioreactors supplemented with 0.2% SNTM, 0.5% methanol (4 mL) and 10 mM sodium citrate.



The addition of 10% (v/v) Mic-1 culture on day 29 to reactors #3, #4, and #5 did not show significant increase in CH_4 production. There was no significant change in VFAs concentration also. The addition of Sheftone T™ (final concentration of 0.2% w/v) to the medium, however, resulted in an increase in CH_4 production - approximately 2100 cc for 10 days **(Table 19)**. The increase in CH_4 production following the addition of Sheftone T™ in the culture medium was because thus provided organic nitrogen permitted active growth of different members (hydrolyzers, acidogens and acetogens) of Mic-1 consortium. This, in turn, resulted in overall, higher biomethanation of TxL. This result confirms our hypothesis that an additional organic nitrogen source is required during the biomethanaion process to restore the C:N ratio which is essential for the growth of bacterial population constituting Mic-1 consortium.

Table 19. Theoretical and Actual Methane Production for Each of the Major Components of the Culture Medium (0.2% SNTM-CM) without TxL*

Carbon Source	Theoretical Production (cc)[a]	Actual Production (cc)[b]	Actual Production (cc)[c]	CH_4 from TxL (cc)[c]
0.2% Sheftone T_{TM}	880	200	2100	1900
0.5% Methanol + 10 mM Citrate	2200	940	6200	5260

* All calculations are based on 800 mL of medium.

[a] Theoretical CH_4 production - 100% of the carbon available is converted to CH_4.

[b] Actual CH_4 production, calculated for 800 mL medium, based on data obtained from experiments conducted in vials containing 60 mL of 0.2% SNTM-CM.

[c] Actual CH_4 production obtained in upflow bioreactor after the addition of Sheftone T™ and citrate + methanol, respectively. CH_4 from control deducted.

Methanol (0.5% v/v) and citrate (10 mM final concentration) added to reactor #5 on day 48 considerably increased CH_4 production **(Figure 74)**. More than 6000 cc CH_4 was produced for 7-8 days after citrate and methanol were added to the medium. From the data presented in Table 1, one can see that the actual CH_4 obtained from just the citrate and methanol is ~3-fold higher than the theoretical yield and is ~7-fold higher than the actual production obtained in vials. Thus, the data clearly indicate that the amount of CH_4 produced after the addition of citrate and methanol is from

CUMUALTIVE CH_4 PRODUCTION AND VFAs CONCENTRATION DURING BIOMETHANATION OF 5% TEXAS LIGNITE IN UPFLOW BIOREACTORS CONTAINING 0.2% SNTM-CM (CH_4 from control deducted)

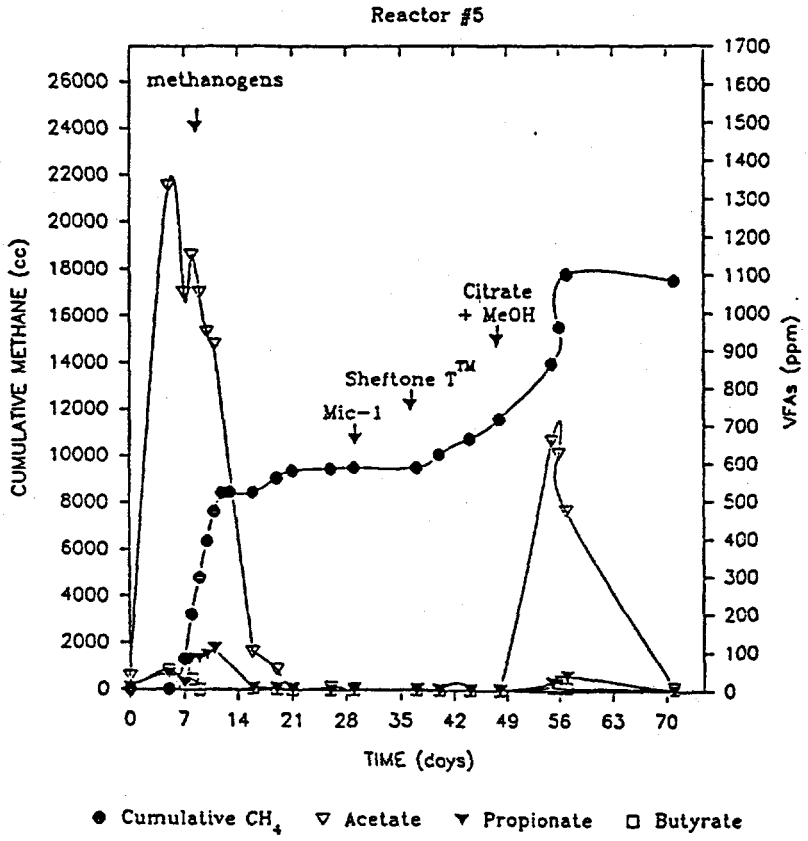


Figure 74 -- page 129

bioconversion of TxL to CH_4. Therefore, results from this experiment, conducted in upflow bioreactors, confirmed our previous observations that addition of citrate and methanol increases significantly the amount of acetate, which is subsequentially converted into CH_4 (**Figure 74**). During this period, a significant increase in VFAs concentration was also observed. The acetate concentration increased from 3 ppm to 660 ppm, propionate from 0 to 38 ppm, isobutyrate and butyrate - from 0 to 7-8 ppm (**Figure 74**). This again confirms our previous observations that addition of methanol (as precursor and wetting agent) and sodium citrate (as sequestering agent) can significantly enhance CH_4 production from TxL.

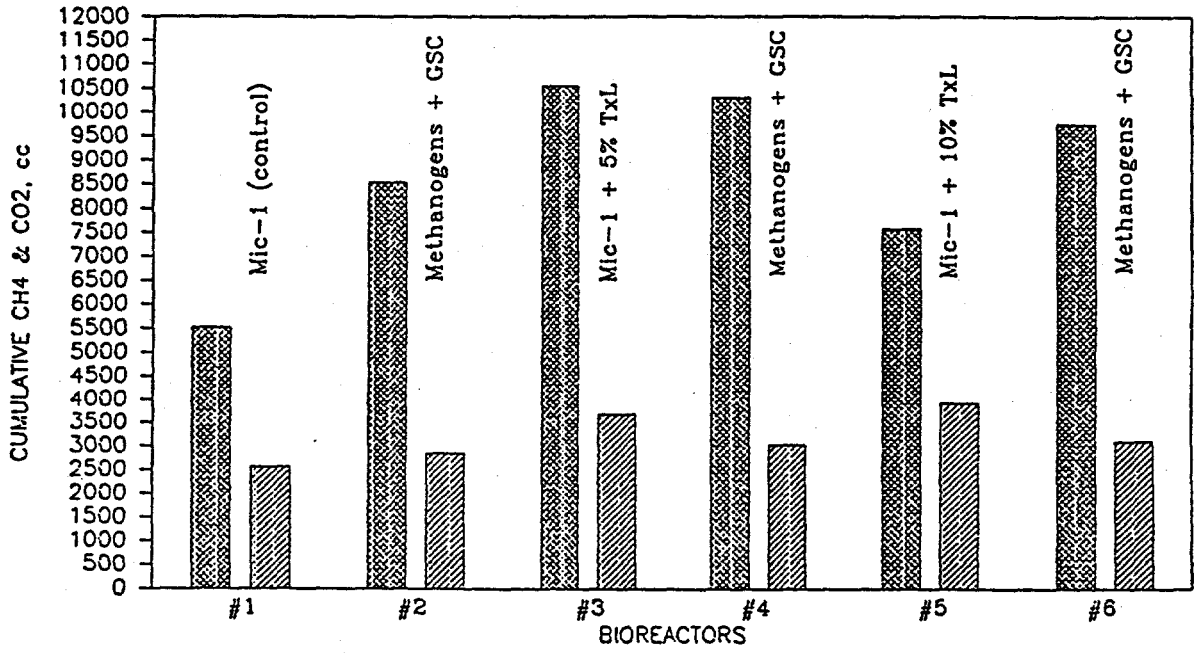
7.3 Two stage bioreactors.

Further investigation with the bioreactors indicated that the accumulation of acetate in the culture medium suppressed the metabolism of the primary coal degraders, acedogens, and acetogens of the Mic-1 consortium. In addition, the pH optimum for the methanogentic population (pH 8.5) is different from the other Mic-1 microbial populations (pH 7.5). Therefore, in order to overcome these two problems, a dual bioreactor (DBR) was designed to further optimize the efficiency of the TxL bioconversion. The DBR is a set of interconnected "acetogenic" (ABR) and "methanogenic" (MBR) bioreactors (**Figure 4**). The ABR (pH 7.5) contained fermentation medium inoculated with primary coal degraders, acedogens, and acetogens (Mic-1). The acetate from the TxL conversion was allowed to accumulate in the ABR, and then the spent medium was transferred to the second upflow bioreactor (MBR, pH 8.5) where the methanogentic population finished the biomethanation reaction. Details of this experiment is described described below.

Six upflow bioreactors were organized into three sets (two BR in each set). Each BR had a total volume of 1250 mL and working volume of 800 mL. All BRs contained 800 mL (final volume) of 0.2% SNTM-CM. Reactors #1, #3, and #5 (ABR) were inoculated with Mic-1 consortium (as 10% inoculum). In addition, BRs #3 and #5 contained 5% and 10% TxL, respectively. Before addition, TxL (150 g) was pretreated with 1.5 L of water, containing 5% (v/v) methanol and 10 mM citrate. The coal-water slurry was stirred at 150 rpm for 18 hours at room temperature. Subsequently, TxL was dried at 105°C for 4 hours, cooled and used for the experiment as 5% (40 g) and 10% (80 g) solids loading. Reactors #2, #4, and #6 (MBR) were inoculated with 10% mixed culture of methanogens + GSC. All BRs were incubated at 35°C.

Highest CH_4 production (10,560 cc) was obtained in ABR #3 containing 5% pretreated TxL after 21 days of operation, (**Figure 75**). The CH_4 production in ABR #5 (10% TxL) was lower (7600 cc). This could be explained as the effect of increased solids loading in ABR #5 and possibly the increased amount of inhibitory compounds released in the medium during bioconversion of TxL by Mic-1 despite the fact that TxL was pretreated. This result supports our previous observation and the hypothesis that

CUMULATIVE CH_4 AND CO_2 PRODUCTION IN DUAL BIOREACTORS CONTAINING 0.2% SNTM–CM AND INOCULATED WITH Mic–1 OR MIXED CULTURE OF METHANOGENS + GRANULATED SLUDGE CONSORTIUM (GSC) – day 21



Highest CH_4 production obtained in Bioreactor #3 (5% TxL)

No significant difference in CO_2 production for all bioreactors

Figure 75 -- page 131

there are compounds in coal leachates that might inhibit microbial activity, when higher coal solids loading are used and as a result the production of CH_4 and VFAs is lower.

Except for higher CO_2 production in ABR #5 (which could be because of the higher solids loading), there was no significant difference in the amount of CO_2 produced (2800 - 3000 cc) in other BRs. The data for CH_4 and CO_2 production **(Figure 75)** show that TxL (when pretreated) was converted into high amount of CH_4 (up to 75 mole %) at considerably low CO_2 concentration. Also, in the MBRs, containing only methanogens and GSC, the CH_4:CO_2 ratios were higher than CH_4:CO_2 ratios in ABRs containing only Mic-1 consortium. Thus, the dual bioreactor system would result in higher CH_4 production and provides better process conditions than the single bioreactors.

7.4 Trickle-Bed Reactor (TBR)

Within five days of operation maximum (535 cc) gas production was observed in the reactor with constant recycle. After this time the gas production was not significant **(Table 20)**. The control reactor, with no pumped recycle, produced 302 cc of gas over 5 days and continued to produce gas at low levels over 32 days with the headspace methane concentrations exceeding 56 mole%. These data indicate that pumping of the culture broth decreases biogasification efficiency and that a different pumping system must be used. It is noted, however, that biogasification does take place even at 30% TxL solids, suggesting that bioconversions at high solids loadings are possible.

7.5 Continuously Stirred Tank Reactor (CSTR)

Parameters of methane production were a little bit difficult to measure in this reactor because of the multiple inlet and outlet lines. However, biomethanation of 1% TxL was calculated based on the total headspace volume and changes in gas composition over time. Nitrogen was used as the basis in all the determinations.

The data obtained showed the highest methane production in this reactor **(Table 21)**. Nevertheless, this experiment needs further evaluation once all other parameters that are currently being evaluated have been completed.

Table 20. Methane Production and Texas Lignite Conversion During Operation of Various Bioreactor[a] Configurations

Bioreactor Type	Mode of Operation	Total Volume (L)	Head Space (L)	Total Gas Produced (L)	Total CH_4 (%)	Solids Loadings (%)	TxL Conversion (%)[c]
Rotating Biological Contactor	Batch	7.6	0.70	11.0	73.0	1	21.600
Upflow Fluidized Bed	Batch: Liquid recycle	0.7	0.08	0.007	51.9	1	0.170
	Gas Recycle	0.7	0.14	0.465	73.2	1	8.410
Trickle Bed	Batch with Liquid Recycle	0.7	0.08	0.540	24.0	30	0.054
Continuous Stirred Tank	Batch	2.0	0.85	2.2[b]	57.7	1 or 5	23.100

[a] - All the reactors were run at 1% solids loadings
[b] - Calculated value based on the total head space volume and measured N_2
[c] - According to the data presented in Table 18

Table 21. Ultimate Analysis of Texas Lignite Solids Before and After Bioconversion to Methane by Mic-1 Consortium

TxL Component	Amounts (%) Present		Change*	
	Before Biogasification	After Biogasification	Fold	%
Ash	14.0	31.8	+ 2.27	+ 127.0
Carbon	62.9	42.9	- 1.46	- 31.8
Hydrogen	4.5	4.0	- 1.13	- 11.1
Nitrogen	1.3	2.4	+ 1.85	+ 84.6
Oxygen (by difference)	16.1	18.1	+ 1.12	+ 12.4
Sulfur	1.2	0.9	+ 1.12	- 25.0

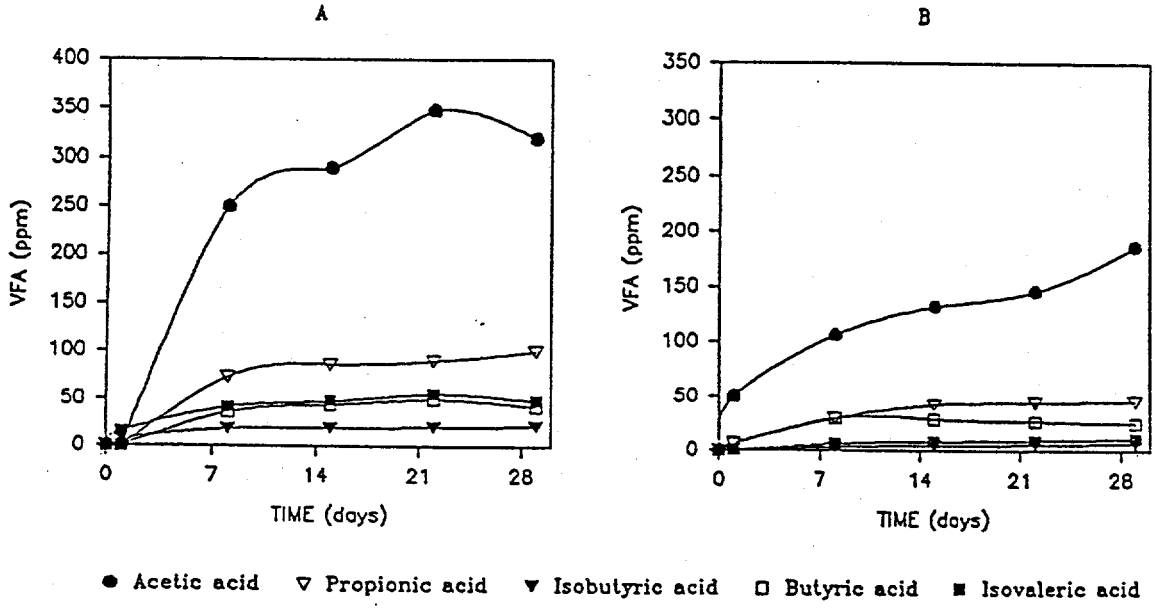
* - = decrease, + = increase

7.6 Simulated Anaerobic Chemostats (SAC)

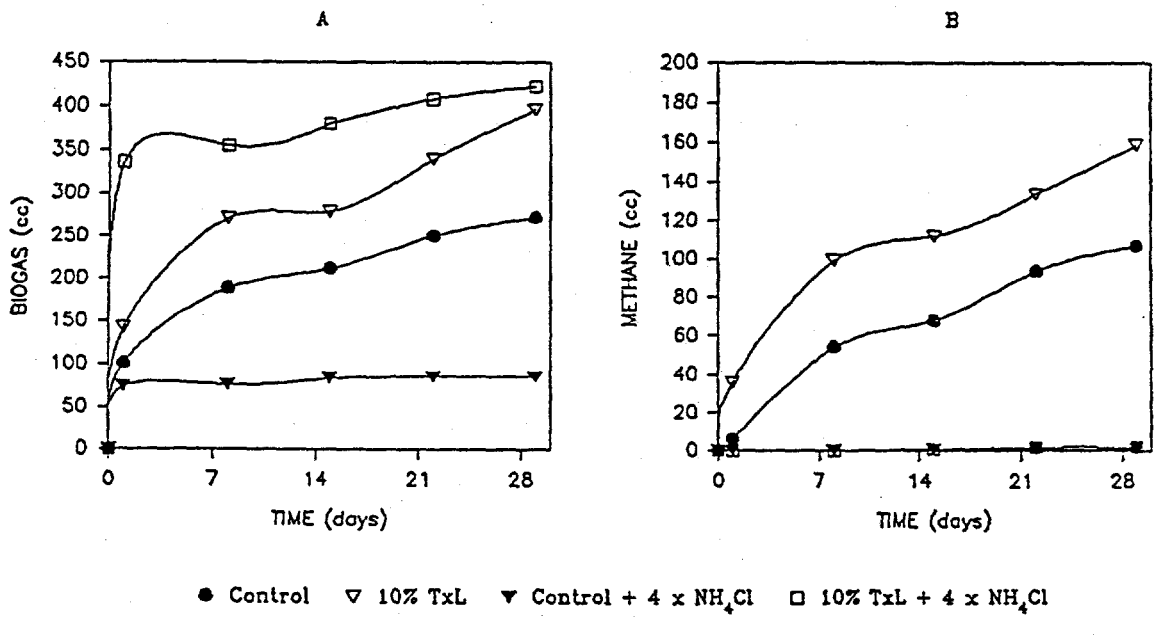
The influence of NH_4Cl on microbial prodution of VFAs and CH_4 was studied in chemostat cultures (**Figure 7**) containing 0% and 10% TxL (**Figures 76 and 77**). Gas analysis of the headspace showed high CO_2 production (513 cc) in experiments with 10% TxL and extra NH_4Cl (**Figure 76**). However, lower methane production was observed compared to the ones that did not contain TxL. This is yet another confirmation of our hypothesis that at higher TxL concentrations the CO_2 production is enhanced. The control (no TxL) reactor produced lower volume of total gas and contained low concentrations of methane and carbon dioxide (data not shown). The lower methane production in the 10% coal reactor may be due to the lower pH (6.5) of the liquid phase, as methanogens are generally inhibited by low pH. The low pH is probably due to the higher carbon dioxide concentration.

7.7 Simulated Tank Reactors (STR)

Bottle reactors, simulating tank reactors (**Figure 8**), were set up to monitor methane production from sewage sludge, 0.2% SNTM + sewage sludge and 0.2 % SNTM + sewage sludge + TxL by the indigenous sewage sludge microorganisms. These reactors were monitored for methane production when medium, sewage sludge, TxL and Mic-1 were incubated under static and agitated conditions. Data obtained from



Production of VFA in Simulated Tank Reactors with 0.2% SNTM + 4 x NH_4Cl (32 g/ L) (A) and 0.2% SNTM + 4 x NH_4Cl (32 g/ L) + 10% Texas Lignite (B).



Biogas (A) and Methane (B) Production During Biomethanation of 10% Texas Lignite in Simulated Tank Reactors with 0.2% SNTM + 4 x NH_4Cl (32 g/L).

these experiments illustrated that little methane was produced from sewage sludge or from sewage sludge + 0.2% SNTM. Significant CH_4 production took place only in presence of TxL. The maximum rate of methane production (7.4 cc CH_4/day) was observed between day 11 and day 20 when only sewage sludge microorganisms were present. The addition of Mic-1 resulted in earlier initiation of maximum methane production rates (7 days vs. 11 days) when TxL was present (data not presented). Agitation of the reaction mixture enhanced the rate of methane production from 7.4 to 8.6 cc CH_4/day. Total methane as well as total gas produced increased by 41% and 35% respectively when the reaction mixture was incubated with agitation. These data indicate the need for gentle agitation to enhance microbial/TxL contact to maximize methane production. This was previously observed in the UFBRs.

8.0 Characterization of TxL

8.1 Ultimate analysis of untreated and residual TxL

Parent (original) coal and residual coal after bioprocessing in upflow bioreactors were analyzed by commercial analytical laboratories (Little Bear Laboratories; Red Lodge, MT and Huffman Laboratories; Golden, CO). The results are shown in **Table 22.**

The data from inorganic analysis showed an insignificant change in Ca and Mg. However, the amount of Zn increased by as much as 2.8-fold. This is possibly due to the fact that the Zn was present in the medium in the form of a metal salt; during the experiment the salts were utilized by the microorganisms (Mic-1) and left as a residue on the coal surface. On the other hand, the decrease in Fe and Ni content could be explained by the fact that Fe and Ni were possibly utilized by Mic-1 consortium to synthesize enzymes which require these essential elements.

The fact that the ash content increased by almost 2% indicates that the carbon from TxL was converted into CH_4.

8.2 Evaluation of solid residue for humic acids

Samples from the bioreactor experiments were analyzed for humic acid (HA) content **(Table 23)** following a procedure developed at ARCTECH. The procedure is as follows:

> The coal sample (1 g) was treated with 10 mL of 1N KOH to solubilize TxL and extract the HA. The samples were left for 16 hours and then the supernatant (containing HA) was separated from the residual (unconverted) TxL. The pH of the supernatant was adjusted to < 2 with 1 N HCl to precipitate the HA. The precipitated HA was diluted with distilled H_2O to approximately 35 mL, centrifuged at 3500 rpm for 20 minutes, and the process of washing and centrifuging was repeated. After the second centrifugation, the pellet containing HA was washed twice with distilled H_2O and dried at 105°C until constant weight.

Table 22. Analysis of untreated and biologically treated TxL* from upflow bioreactors (BR) containing 0.2% SNTM-CM.

Sample	Major and trace elements, % of whole dry coal					Ash (%)	Moisture (%)	C (%)	H (%)	O (%)	N (%)	S (%)
	Ca	Fe	Mg	Ni,μg/g	Zn,μg/g							
Untreated TxL	0.90	2.35	0.23	93.90	5.17	17.3	4.5	57.8	3.9	24.3	1.1	1.4
BR with 5% TxL	0.94	2.10	0.28	82.00	14.30	19.5	2.6	55.8	3.8	21.4	1.5	1.4
BR with 10% TxL	0.93	2.16	0.26	86.60	11.10	19.3	2.7	55.8	3.7	21.7	1.4	1.4

* The samples were air dried at 105° for one hour prior to analysis. Results are an average of several results and were compared to NIST standards

The HA content was calculated using the following equation:

$$HA\ (\%) = W_{HA1} - W_{HA0}/W_s \times 100$$

where: W_{HA1} is the weight (grams) of centrifuge tube containing the dried HA, W_{HA0} is the weight of the centrifuge tube alone, and W_s is the weight of the sample.

Table 23. Humic acid (HA) analysis of untreated and biologically (Mic-1) treated TxL

Sample	Humic acid, %	Increase in HA content, %	Standard deviation, s	RSD, %
Untreated TxL	5.1	100	0.0025	4.967
Bioreactor with 5% TxL	8.4	165	0.0040	4.762
Bioreactor with 10% TxL	8.1	159	0.0031	3.787

The HA content increased significantly (up to 165%) after biological treatment of TxL in bioreactors containing either 5% or 10% solids loading. The results indicate that the residual coal from "MicGAS Process" can be successfully used as a feedstock for HA production.

8.3 Market value of coal residue

The approximate market value of humic acid in the coal residue was calculated. The chemical extraction, as described above, converted the biologically treated coal into HA by an average of 94.6%. Therefore, from 1 (one) ton of coal:

(2000 lbs coal) X 0.946 = 1892 *lbs* of HA / ton coal

Therefore:

$$\frac{\text{1892 lbs HA}}{\text{1 ton coal}} \times \frac{\text{gallon}}{\text{9.15 lbs}} \times \frac{\text{\$10.00}}{\text{1 gallon HA*}} = \text{\$2068/ton coal}$$

**Market value for 1 gallon of* Actosol®, *a commercial soil amendment product*

Thus, one ton of the biologically treated coal residue can contribute an additional economic value to the biogas and other chemicals (eg. VFAs) generated by the MicGAS™ Process. Additionally, only a small amounts of waste products remain because almost all (~95%) of the residue can be converted into product.

9.0 Conclusions

- A microbial consortium, Mic-1, was isolated, adapted, and further improved at ARCTECH for utilizing coals of various ranks.

- Mic-1 consortium can successfully bioconvert TxL into CH_4 and other value added products, such as ACTOSOL®, a commercial, humic acid-based, soil amendment.

- Mass balance calculations show that CH_4 production is due to the biomethanation of TxL and not from the addition of co-substrates.

- Of the four bench-scale reactors [Rotating Biological Contactor (RBC), Upflow Bioreactor (UFBR), Trickle-Bed Reactor (TBR), and Continuously Stirred Tank Reactor (CSTR)] tested, methane production was highest in the CSTR and lowest in the TBR.

- Coal biomethanation was further optimized in a dual upflow bioreactor. This design accommodated the different pH optima of the various bacterial populations that make up the Mic-1 consortium.

- Approximately 95% of the coal residue from the MicGAS™ Process can be chemically converted into humic acids. This decreases the waste products from the Process, and adds approximately $2K to the value of one ton of coal.

- Nutrient cost for the MicGAS™ Process was decreased by 116.6-fold. This was accomplished by adding the nitrogen supplement, Cargill 200/20, to replace the costly yeast extract ($6 to process 1 ton of coal vs. $700).

- The MicGAS Process should be scaled up to a 50L pilot plant and this technology may be used to restore underground gasification sites.

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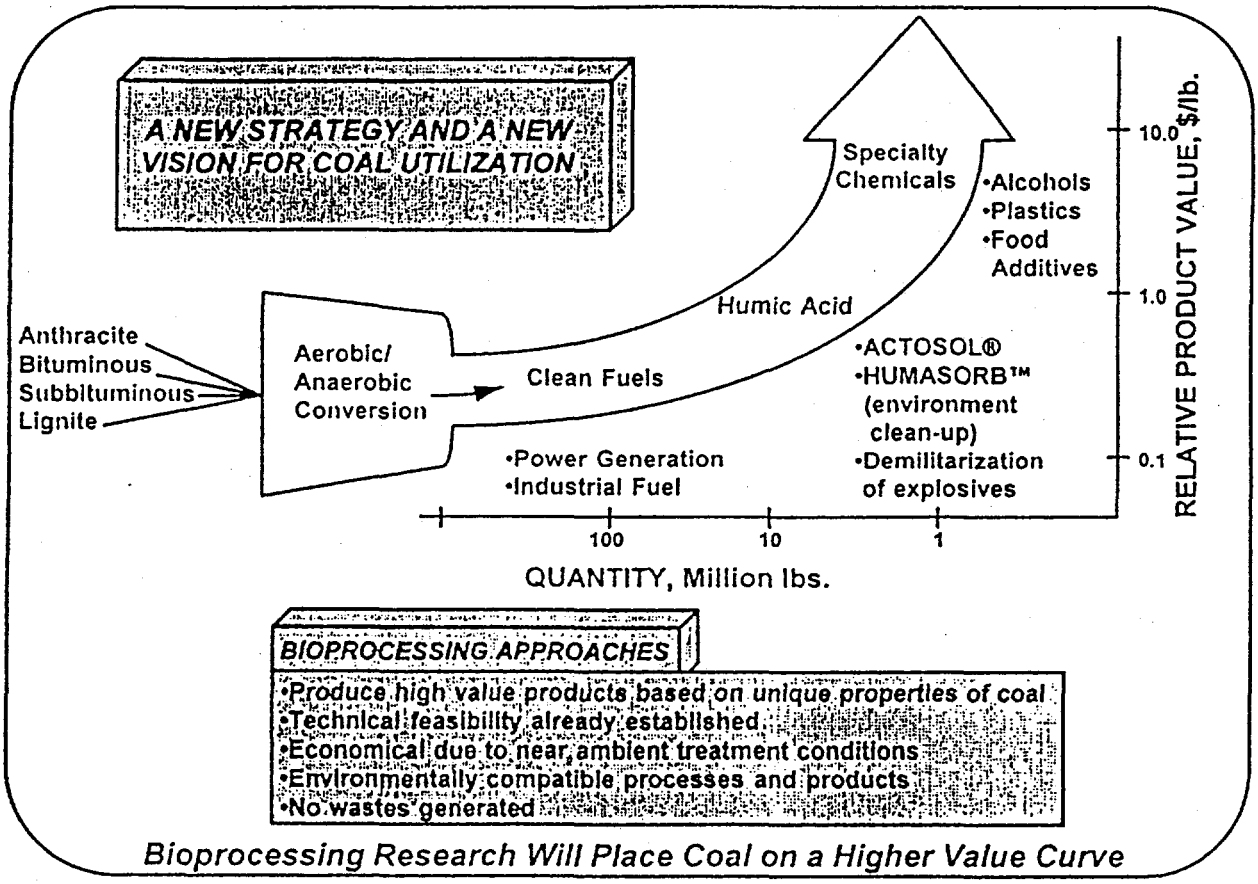
Appendicies

APPENDIX A



Bioprocessing Research Will Place Coal on a Higher Value Curve

APPENDIX B: Composition of medium of NTM and MSM Media

	NTM[a]		MSM[b]	
Component[c]	g/L	mL/L	g/L	mg/L
Yeast Extract	1.0		2.0	
Tryptic Soy Broth (TSB)	1.0		2.0	
B-Vitamins Solution I		5.0		
B-Vitamins Solution II		50.0		5.0
Pfenning's Minerals		10.0		
Pfinning's Trace Metals		1.0		
Mineral Solution I				5.0
Mineral Solution II				5.0
Mineral Solution III				10.0
Resazurin (0.1% Solution)		1.0		1.0
KCl	1.6			
$NaHCO_3$[d]	3.5		5.0	
Na_2Se_3 (0.1% Solution)[d]		1.0		1.0

[a] New Termite Medium pH = 7.0 to 7.2
[b] Methanogens-Sludge Medium pH = 7.8 to 8.0
[c] All components (except NaHCO3 and Na2SeO3) were added sequentially and only after the one added before was completely dissolved. The medium was made in water that was simultaneously boiled and purged with oxygen-free [N_2:CO_2 (80:20) for NTM and H_2:CO_2 (80:20) for MSM] mixture. Medium was then cooled in a cold water bath to ambient temperature, pH adjusted with 2N NaOH, and dispensed into 1L Wheaton bottles. The media was then purged with the appropirate oxygen-free gas mixture for at least five minutes before capping. Media was used immedately or kept at 4°C.
[d] Added with constant stirring after the medium was cooled.

APPENDIX C: Compostition of metal and mineral solutions for NTM and MSM media

	Pfenning's Minerals	Pfenning's Metals	Mineral Solution I	Mineral Solution II	Mineral Solution III
Component	Quantity (g/L)	Quantity (g/L)	Quantity (g/L)	Quantity (g/L)	Quantity (g/L)
NH_4Cl	8.00				
$CaCl_2 \bullet H_2O$	1.00	0.010	8.00		0.02
$MgCl_2 \bullet 6H_2O$	6.60		20.00		
KH_2PO_4	10.0		4.0	4	
NaCl	8.0				
H_3BO_3		0.300			0.01
$CoCl_2 \bullet H_2O$		0.200			0.15
$FeCl_2 \bullet 4H_2O$		1.500			
$MnCl_2 \bullet 4H_2O$		0.030			0.10
$Na_2MoO_4 \bullet H_2O$		0.025			0.01
$NiCl_2 \bullet 6H_2O$		0.020			0.02
$ZnSo_4 \bullet 7H_2O$		0.100			0.10
K_2HPO_4				4	
$CuCl_2 \bullet H_2O$					0.02
$FeSo4 \bullet 7H_2O$					0.01
$Na_2EDTA \bullet 2H_2O$					0.50
$NaWO_4 \bullet 2H_2O$					0.03

APPENDIX D: Composition of B-vitamin solutions for NTM and MSM media

Component	B-Vitamin Solution I Quantity (g/L)	B-Vitamin Solution II Quantity (g/L)
Biotin	20.0	2.0
Cyanocobalamin (B_{12})	100	5.0
Niacin	200	5.0
Pantothenic acid	100	
p-aminobenzoic acid	100	5.0
Pyridoxine	500	10
Riboflavin	100	5.0
Thiamine	200	5.0
Calcium panthotheate		5.0
Folic acid		2.0
Vitamin B_{12}		0.1

APPENDIX E: Cost for various YE/TSB replacements tested.

Product	Product Manufacturer	Cost ($/lb.)
YE/TSB	Difco	35.45
BHI Solids	Marcor Development Corporation	21.77
Meat Peptone	Marcor Development Corporation	8.85
Testone 900	Universal Foods	4.35
Sheftone-M™	Sheffield Products	1.72
Sheftone-T™	Sheffield Products	1.42
Cargill 200/20	Cargill	0.35