COO-2983-8
Distribution Category UC-95e

ENERGY AND PROTEIN PRODUCTION FROM PULP MILL WASTES

Annual Report
for the Period June 15, 1977-June 15, 1978

M. F. Jurgensen and J. T. Patton

Michigan Technological University
Houghton, Michigan 49931

June 15, 1978

Prepared for
THE U. S. DEPARTMENT OF ENERGY
UNDER CONTRACT E(11-1)-2983

DISCLAIMER

ABSTRACT

Studies on desugared spent sulfite liquor, DSSL, subjected to ozonation indicate that this complex organic substrate in water solution reacts readily with ozone to produce lower molecular weight organic fragments which can be metabolized by a variety of microorganisms. Ozone uptake is complete up to approximately 15 g/l and results in an increase of 35% BOD and a reduction of 16% COD. The production of BOD is pH dependent with a maximum occurring at around pH 3. Ozone is effective for removing the color bodies present in DSSL reaching a maximum of 80% at long ozonation times. Color reduction of 30% is obtained at the 15 g/l dosage.

The production of methane via fermentation of DSSL is greatly enhanced by the ozonation reaction. Methane production on raw DSSL is only 45.3 standard cc/l of DSSL. After ozonation of the DSSL during which 15 g/l of ozone are reacted the resulting product yields 1239 standard cc/l. Methane concentration in the produced gas consistently excedes 75% in contrast to 50% generally obtained on cellulosic substrates.

The hypothesis that methane is produced from acetic acid, held by several prior workers, could not be corroborated in this study. Liquor remaining in the fermenter after gas production has essentially ceased is much richer in acetic acid than ozonated DSSL. Continuous fermentation studies operated to optimize gas production produced a fermentate containing 3.96 g/l of acetic acid.

The production of protein accomplished through the growth of Torula yeast on DSSL is also enhanced by the ozonation reaction. Two variants show minimal growth on unozonated DSSL but cell densities of 5 g/l were obtained with the rough variant when this substrate has been ozonated. In contrast to the methane fermentation which showed high ozone consumption to be beneficial, the yeast

prefer very minimal ozone reaction. Maximum protein production was obtained on 10 minute ozonate in which ozone uptake is approximately 0.8 g/l.

Yeast growth was not vigorous on methane fermentate shown to be rich in acetic acid. Although the production of a possible toxicant during methane fermentation has been considered, other explanations are being sought. If the available acetic acid can be utilized for either the production of methane or protein productivity, could increse between two and three-fold.

INTRODUCTION

Lignin derivatives and other soluble organic compounds of wood are the major waste products from the pulp and paper industry. The amount of pulp mill waste effluent produced each year is enormous. For example, the sulfite pulping industry alone accounts for nearly three million tons of organic waste material annually (14). The disposal of this spent sulfite liquor (SSL) is a significant source of water pollution. Mueller and Walden (29) estimate that 2,100 gallons of spent sulfite liquor are produced for every ton of sulfite pulp manufactured.

Various techniques have been developed to dispose of this enormous quantity of waste product. Originally the bulk of this material was discharged into the streams and lakes convenient to the pulping operations (8). The rivers and bodies of water which currently receive the spent sulfite liquor are overtaxed and cannot continue to accept even the current level of discharge without detrimental environmental effects. The expansion of this segment of the pulping industry has been curtailed because of the pollution problems and the operation of many existing plants is now threatened.

Methods of treating pulping waste liquor to eliminate the pollution hazard can be generally classified as mechanical or biological. Mechanical methods generally separate the water, usually by vaporization, and dispose of the solids by burning, burying, or sales. The conventional treatment is the evaporation of the water followed by the burning of the organic solids. Besides elmininating stream pollution, this process recovers some of the

cooking chemicals and considerable energy is generated during the combustion of the solids. Nevertheless, the treatment does have its disadvantages. The burning of the organic solids creates air pollution. The evaporated water contains all the volatile organics originally present in the effluent and, thus, the condensate has an objectionably high BOD content. In addition, 20-50 percent of the energy recovered by burning the organic solids is consumed by the evaporation process which detracts from the overall thermal efficiency of the operation. Growing demand for DSSL solids for such uses as roadbinders and clay modifiers has allowed some mills to sell the residues rather than burn them.

Biological treatment seeks to avoid the expensive water removal step by enzymatic conversion of the waste effluent to a form which can be more efficiently removed. The activated sludge process, storage oxidation and aerated stabilization have been extensively studied (47). In general, these treatments remove only the low molecular weight soluble organic fractions which effectively lowers the BOD_5 of the DSSL. The high levels of lignosulfonates and sulfur are not significantly reduced (31), and the energy potential of this organic resource is sacrificed.

It is the object of this research to convert the sulfur and organics now classified as pollutants in spent sulfite liquor by means of a combination chemical-biological process into synthetic methane and proteins. The process could be self sufficient with respect to energy requirements and could make a significant contribution toward relieving the projected shortages in the energy and food supplies.

The general lack of success with biodegradation of lignosulfonates suggests that some pretreatment must be required to degrade the DSSL, or transform it into a state which could be metabolized. One such possibility is to break the lignosulfonate into smaller molecular weight fractions through ozone treatments. Stern and Gasner (46) have shown that such processing of kraft mill waste liquor did cause a shift in the molecular weight distribution of lignins to lower weight fractions. Ozonation also increased the susceptibility of the waste liquor to biological decomposition. It is likely the lignosulfonate present in DSSL would be affected in a similar manner. This technique could be used prior to a yeast fermentation to increase protein yields, or after to facilitate the removal of residual BOD and COD. The sulfur fraction of the lignosulfonate released by ozonation could be removed by stripping and/or bioreduction.

The BOD remaining after ozonation is amenable to subsequent biological treatment. The methane-producing anaerobes seem ideally suited for such a role. These organisms use fatty acids, alcohols, and carbon dioxide as substrates which are the readily available organic materials remaining in the DSSL after yeast fermentation. Methane produced from DSSL would then be used as a supplemental energy source in the processing plant.

Production of methane from organic residue by anaerobic digestion is well known. A variety of substrates ranging from activated sludge to cultured algae have been converted to methane by this process (22, 43). Considerable interest has recently developed on obtaining methane from animal wastes (12).

Methane fuel production from DSSL was earlier considered as a promising fermentation possibility due to the ease with which the gaseous product could be recovered. Calculations by Benson and Partansky (4) based on incubation studies gave a heating value of 1,430,000 BTU/ton of pulp, assuming a 25% carbon removal, and incubation at 36°C. Cultures acclimatized to DSSL by successive batch transfers or continuous fermentation were not used. Bannik and Muller (3) also found significant production of methane from DSSL, and Wiley (51) patented a process for use in sulfite pulp plants. The caloric value of the organic waste present in DSSL amounts to approximately 40 trillion BTU's annually, equivalent to 40 billion cubic feet of natural gas (33). However, the relatively low cost of other fuels at that time discouraged subsequent research on methanogenesis using pulp mill substrates. This situation would now appear to have changed.

Process Description

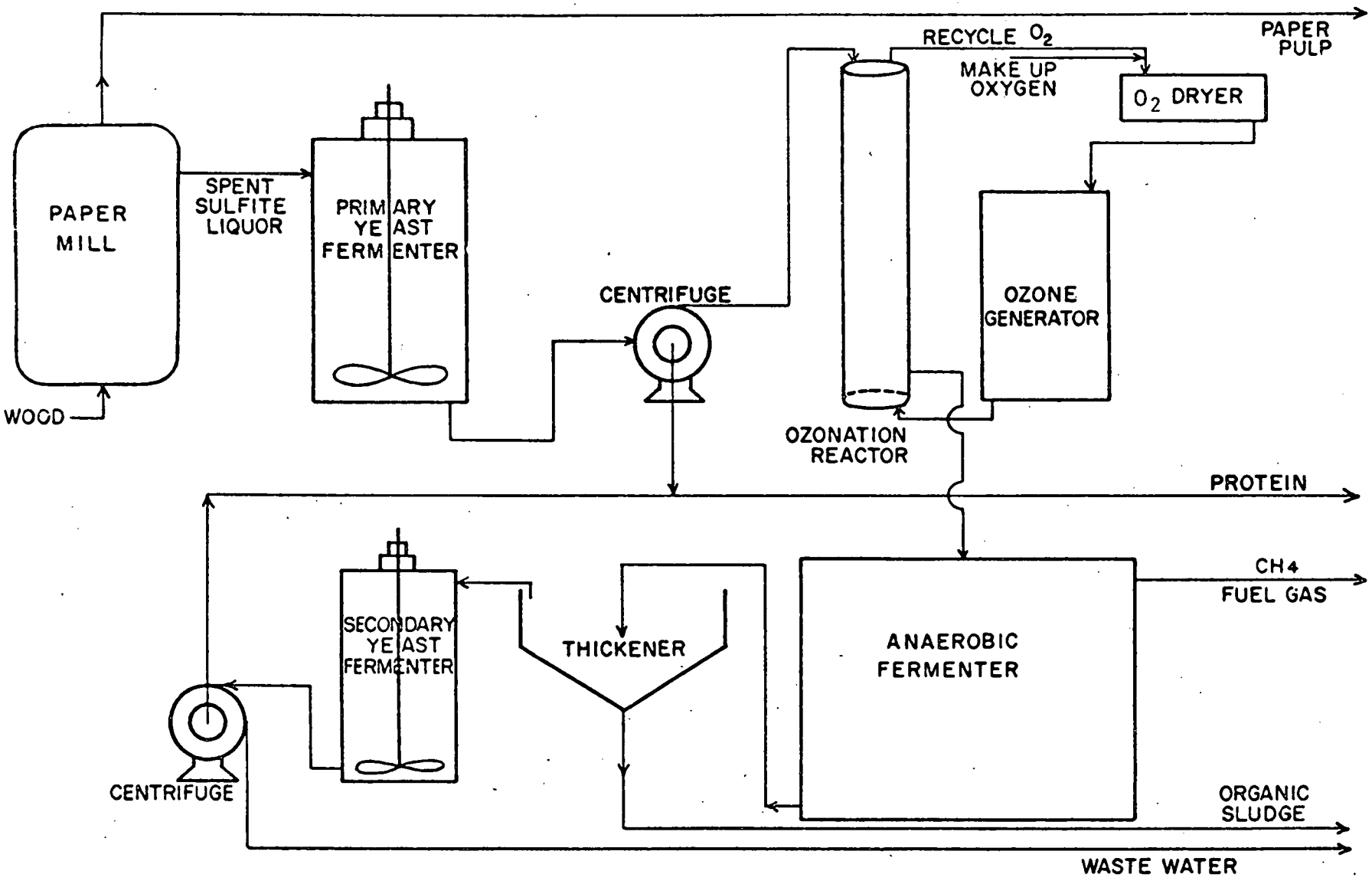
Conceptual design work has resulted in the development of a multistage process which has the potential for converting the pollutants present in sulfite pulp mill waste DSSL to useful fuels and protein. The process utilizes three fermentation stages coupled with the chemical and physical operations, ozone cracking, steam stripping, and centrifugation to synthesize and harvest useful products derived from the DSSL. This process is shown in the accompanying flow diagram, Diagram 1.

Waste liquor from the pulp mill enters the process in the primary yeast fermenter where all assimilated organics are converted to protein and CO_2.

DIAGRAM 1

PROCESS FLOW SHEET

CONVERSION OF PULP MILL WASTE TO PROTEIN AND FUEL GAS



Effluent from the primary fermenter passes through a centrifuge to harvest the protein. It then passes to the ozonator where it is treated to modify the sulfur bonds and break down the high molecular weight organics. Ozone cracked effluent is cooled and fed to the anaerobic digestor. Here a mixed bacterial culture is used to convert the remaining organics to synthetic methane. Only a few specific organics can be utilized by methane bacteria. For this reason a mixture of other organisms, largely Desulfovibrio, are needed to transform the ozonated fragments into substrate for methane synthesis.

The effluent from the anaerobic digestor may still contain unassimilated ozonated fragments which can be utilized by Torula yeast. The effluent from the methane fermenter can, therefore, be fed to secondary yeast fermenters, and then to a centrifuge for protein removal. Any BOD_5 still remaining is removed by standard treating processes such as activated sludge or soil infiltration.

During the anaerobic digestion some organic sludge, biological solids and lignin, will be produced. The effluent from the anaerobic digestor is fed to a conventional thickener for the recovery of the precipitated organics. The sludge, after dewatering, can be utilized as fuel by burning directly if no higher value alternate is available.

Staffing

At the beginning of the second year, the project was fully staffed. Two full-time laboratory technicians were employed during the summer to aid in the numerous chemical and biological tests which were routinely

conducted. In addition, Dr. Y. Lai, a lignin chemist in the Forestry Department, spent one month working on the changes in the organic fraction of SSL after ozonation.

At the beginning of September Dr. Patton left on a nine-month leave of absence to New Mexico State University. He continued to work on the project through periodic trips to Houghton and by correspondence, an arrangement that has been coordinated and approved by ERDA officials. Since Dr. Jurgensen was absent from campus from September to November 26, 1977, professional direction of the project was left to Dr. Patton, whose appointment at New Mexico State University allowed him to spend 25% of his time actively directing the research work. He was able to spend 14 days in Houghton in addition to data analysis and report preparation completed in New Mexico.

Upon his return, Dr. Jurgensen devoted full time to the project for two weeks. Continuity was maintained by Ph. D. candidate Sushil Dugar and research associate Craig Bremmon on a day-to-day basis and by periodic meetings with Drs. Beckwith and Jurgensen. Dr. Jurgensen had adequate time available during the last half of the year to bring the overall staffing for the project up to the budgeted value for the total year. Three undergraduate laboratory assistants were also employed for the second half of the year.

EQUIPMENT, MATERIALS AND METHODS

Equipment

During the first project year, all major pieces of equipment were either purchased or constructed in the University shop, and then installed. A Welsbach ozone generator, Model 1T-816, was purchased and installed as part of the equipment necessary for ozonation of the spent sulfite liquor. The ozone reaction is carried out in a 500 ml capacity 1" ID glass column 6 ft. tall. The raw DSSL is fed to the reaction column by means of a Masterflex pump. A second Masterflex pump acts as a recycle pump, moving the liquid to a mixing chamber where the pH is automatically monitored and controlled with a Radiometer automatic titrating apparatus. This equipment proved to be reliable for all experiments performed.

Fermentation apparatus and equipment required for the biological conversions and assay work were also necessary. Four continuous stirred fermenters furnished by Michigan Technological University were adapted for anaerobic studies and installed. These were also equipped with Masterflex pumps to be operable in either batch or continuous mode. Foot operated culture transfer and media preparation equipment developed at Virginia Polytechnic Institute was constructed in the chemical engineering shop and placed in operation. These elements are operating as expected, giving high quality results. A BOD incubator and associated glassware were purchased from the VWR Scientific Company and is operating satisfactorily.

Several additional pieces of equipment were necessary for extended protein synthesis experiments. An NBS Gyratory Shaker, Model G25, that contains a heater, and temperature control is used as an incubator for yeast growth. A Sorval RC2-B refigerated centrifuge was purchased for harvesting the yeast.

A Macrosonics Corporation, Model 180VF, high frequency generator was obtained from Michigan Technological University and used for sonication experiments. A 300 ml glass column reactor was constructed in the University shop to be used with the high frequency generator.

Materials

Desugared Spent Sulfite Liquor (DSSL). The waste liquor for ozone treatment was obtained from the Lake States Division of the St. Regis Paper Co., Rhinelander, Wisconsin. The effluent liquor from the pumping unit of this plant is fed to their protein production unit, where more than 90% of the wood sugars in the liquor are converted to protein by growing Torula yeast. Some of the important characteristics of the liquor are tabulated in Table 1. The actual characteristics vary depending on the type of wood used and cooking conditions employed for manufacture of pulp. The data in Table 1 represents a range for four batches obtained from the above mill over a period of one year.

Table 1

Characteristics of desugared calcium based spent sulfite liquor

Volatile solids	70 - 81 g/l
Sugars[1]	2 - 3 g/l
BOD	8250 - 10150 mg/l
COD	91500 - 109000 mg/l
Ash content	12 - 15 g/l
Calcium[1]	7 - 10 g/l
Sulfur	6 - 7 g/l
Hydrogen ion conc. (pH)	4.1

[1]From Holderby and Moggio (17).

Methane producing bacteria. Communication with leading researchers in the field of methane fermentation yielded the opinion that anaerobic bacteria required for DSSL digestion would be essentially the same from any municipal waste treatment plant. The initial culture was obtained from the anaerobic treating plant located at Brookings, South Dakota. The bacteria were acclimated for growth in DSSL by the procedure described in the Culture development section.

Yeast. The samples of Torula yeast for the yeast growth experiments were obtained from the Lake States Yeast Company, Rhinelander, Wisconsin.

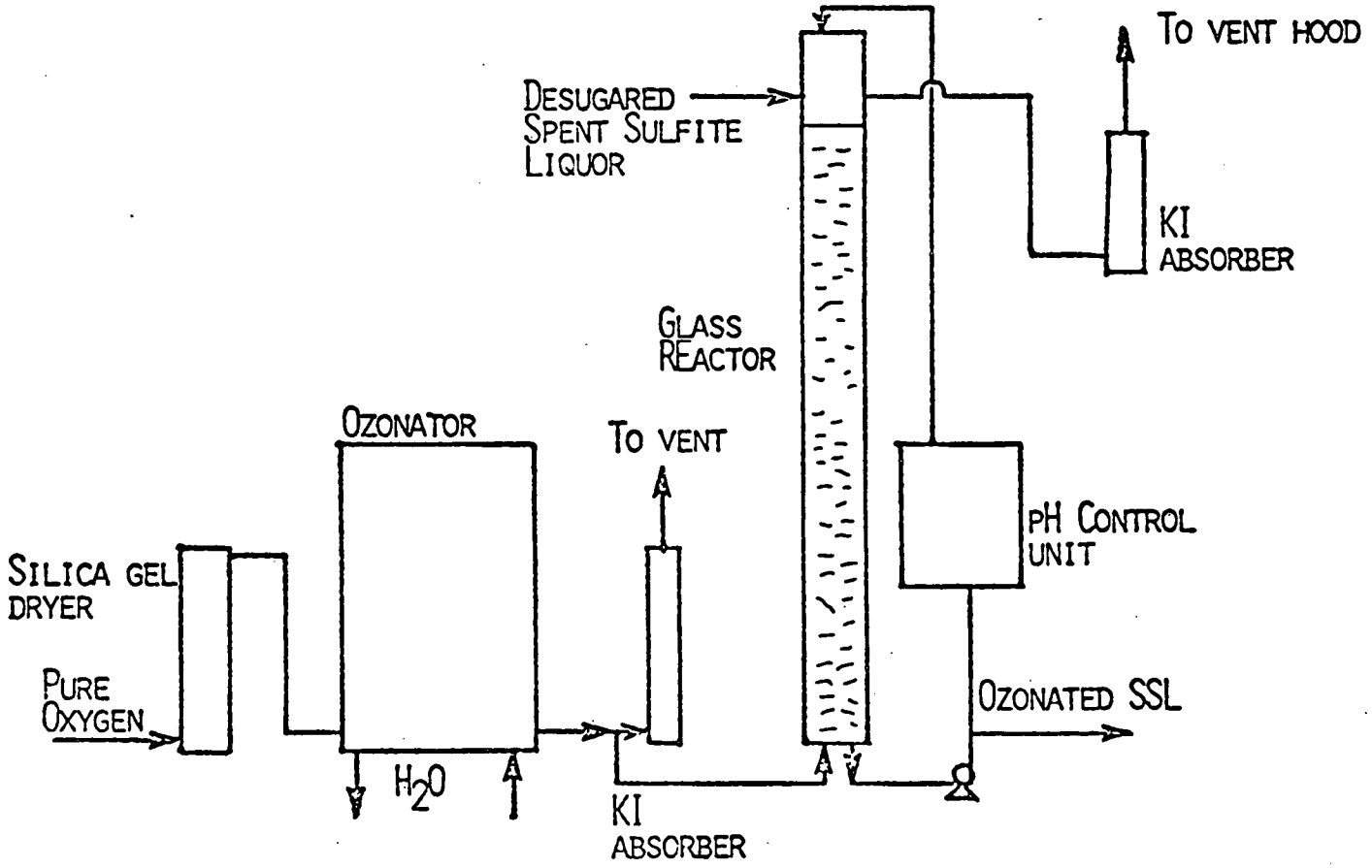
Methods

Ozonation

Ozonation is carried out in the counterflow tubular reactor described in the Equipment section, and shown in Diagram 2. All fittings for the ozonation apparatus are gas tight, and made of stainless steel, to avoid problems with corrosion. The gas stream produced by the ozonator contains

DIAGRAM 2

EXPERIMENTAL OZONATION APPARATUS



2% by weight ozone and is introduced at the bottom of the reactor. DSSL is supplied to the top of the reactor by a variable speed feed pump which could be adjusted to obtain any residence time desired.

Preliminary batch ozonation experiments indicated that pH control was essential in order to obtain reproducible results. This control was achieved by continuously circulating the liquid being ozonated through a small chamber external to the reactor which monitored and controlled the pH automatically. This system allowed the reactants to be controlled to within $\pm$0.1 pH units. Since the ozonation reaction produces organic acids and lowers pH, control was achieved by the addition of a 10% NaOH solution.

The gases leaving the reactor contain small quantities of residual ozone. The off gas is scrubbed with a 2% potassium iodide solution before the gases are exhausted through a hood. At short ozonation times only trace quantities of ozone can be detected in a reactor off gas stream. Determination of the quantity of iodide that has reacted with ozone during a given experiment provides the necessary data to calculate the extent of the ozone reaction with the lignosulfonates.

Lignosulfonates present in DSSL have a natural surfactant quality that causes undesirable foaming in the reactor. It is necessary to employ a small quantity of anti-foam, and several commercial products proved effective. Dow-Corning Antifoam A was selected for use in the bulk of the experiments.

During ozonation a small quantity of insoluble materials is produced. These solids are removed before the ozonated DSSL is used either as a medium for yeast fermentation or methane production.

Fermentation

Fermentation is carried out in continuous flow fermentation vessels, as shown in Diagram 3. Three magnetically stirred, stainless steel one liter fermenters are equipped with Masterflex pumps. Dilute (75% DSSL) liquor treated at an ozone dosage of 15 g/l is used as the only carbon source. Ammonium hydroxide (4 ml/l of 50% concentration) and potassium phosphate (0.5 g/l) are added to the media for nitrogen, potassium and phosphorus sources. Feed pH is maintained at 8.5, which controls pH in the fermenters between 7.0 and 7.5, the range most suitable for growth of methane bacteria. Since the fermenters are glass, the retention volume is monitored visually. Fermentation temperature is maintained at 35°C $\pm$ 1°C in an environmental chamber. Determinations of fermenter pH and redox potentials are performed daily using a glass combination pH electrode and a platinum redox electrode.

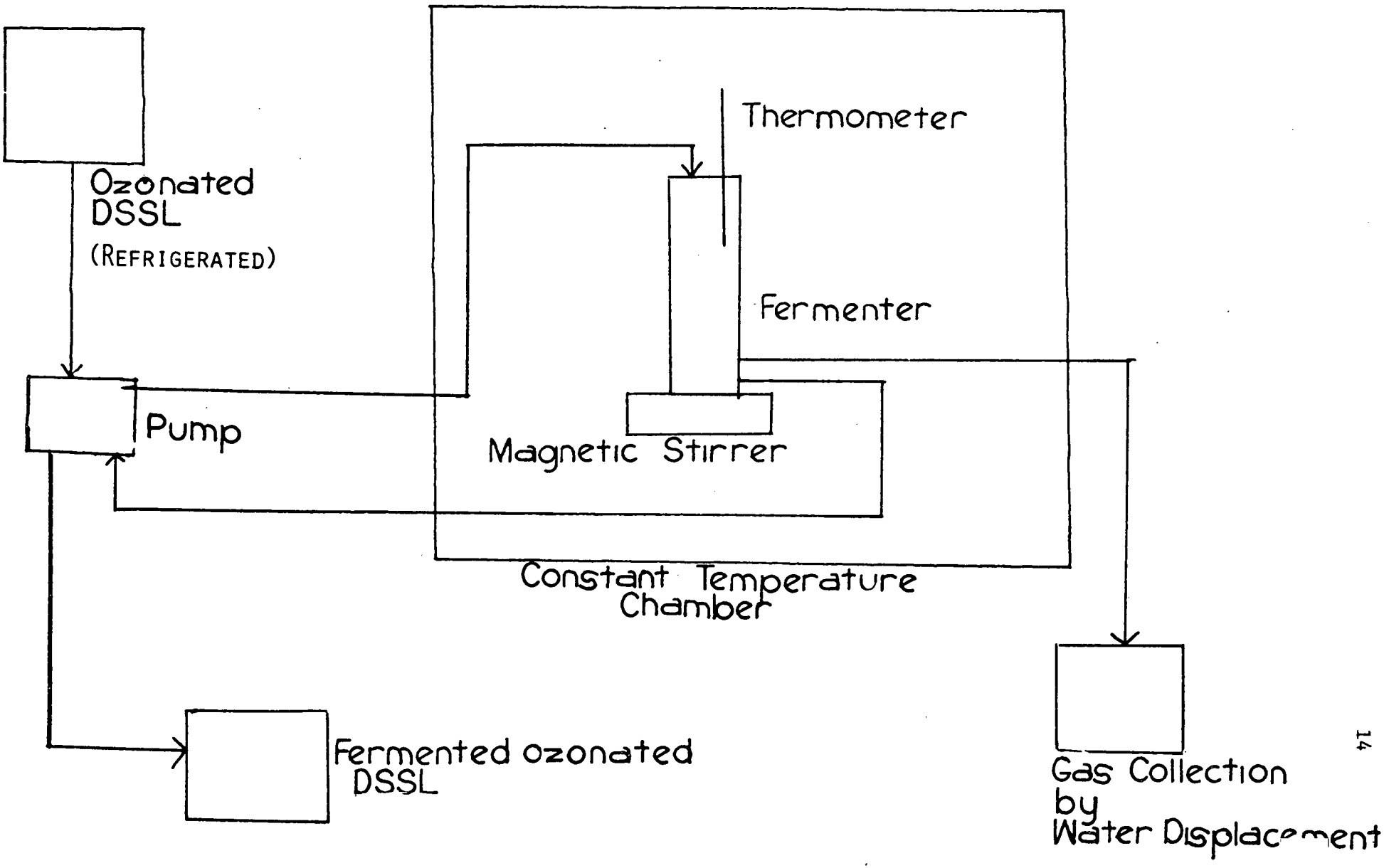
Protein Synthesis

Two variants of Torula utilus yeast were studied in batch fermentation studies. Both variants were isolated from desugared yeast plant effluent, DSSL, obtained at the Lake States Yeast Plant, Rhinelander, Wisconsin. Effluent cultures were streaked on agar plates and incubated at 35°C. After 2-3 days of incubation, one rough and one smooth variant were identified and isolated in pure culture.

Twenty flasks, 10 ozonated and 10 unozonated DSSL, were inoculated with the Torula cultures. The DSSL had been sterilized, and its pH was adjusted to 5.0. Various SSL ozonation times were used, ranging from 10 minutes to

DIAGRAM 3

EXPERIMENTAL FERMENTATION APPARATUS



2 hours. In addition, nitrogen, phosphorus, and potassium were added as supplements in the form of ammonium hydroxide (0.5 gm/l) and potassium phosphate (0.5 gm/l). The flasks were placed on a gyratory shaker, and incubated at a temperature of 33°C to provide optimum environment for yeast growth. Two uninoculated flasks (either two containing raw DSSL for the raw DSSL experiments, or two containing ozonated DSSL for the ozonated DSSL experiments) were also placed on the shaker as controls in case some chemical reaction occured which would cause precipitation.

One or two culture flasks were removed every 12 hours and analyzed for yeast production. The yeast was harvested in a Sorval RC2-B refrigerated centrifuge at 15000 RPM for 20 minutes. The liquid was decanted and the cells resuspended in distilled water and recentrifuged. This technique was repeated a second time to insure the removal of all soluble compounds.

A similar procedure was performed on the uninoculated samples to determine the quantity of solids, if any, produced by non-biological effects. The cells or solids so isolated were then dried for various periods of time at 101°C. It was found that after 1 hour of drying time a constant weight had been achieved. Yeast production was taken as the difference between the dry weight of solids obtained from yeast growth flasks minus dry weight of solids produced in the uninoculated flasks.

Effluent Analysis

Chemical oxygen demand (COD) and biochemical oxygen demand (BOD) tests are routinely performed as described in Standard Methods for the Examination of Water and Wastewater (45). An acclimated seed for the BOD_5 test is

maintained by continual aeration and daily additions of 1:50 dilutions of 3-hour ozonated DSSL-water mixture. The ozonated DSSL in the DSSL-water mixture is supplemented with 0.1 g/l K_2HPO_4, and 1 ml/l NH_4OH. One-sixth of the entire acclimated seed is discarded once a week, and replaced with raw sewage. Total sulfur was determined according to the procedure outlined by Salverson and Hogan (39).

Volatile and non-volatile fatty acid contents are analyzed by gas chromatography according to the procedure given in the Virginia Polytechnic Institute Anaerobic Manual (1). Fatty acids are identified and quantified by comparison of retention times and peak heights with those of known standards. A 6'1/4" stainless steel column packed with 15% SP-1220/1% H_3PO_4 on chromasorb WAW, a packing specifically developed by Supelco Inc. (Bellefonte, Pa.) for fermentation product evaluation, is employed for these tests. Column conditions are 120°C with a helium gas flow of 60 cc/min.

Gas production rates are recorded hourly as effluent gas bubbled into burets filled with water. Corrections are made for hydrostatic pressure, water vapor and temperature to report gas production at standard conditions. Gas produced in fermentation is analyzed by gas chromatography using a Varian Aerograph Model 180. A 6'1/8" stainless steel column packed with Porapak R runs at 40°C with a nitrogen carrier gas flow rate of 20 cc/min. and is used for all gas sample analysis.

Culture Development

Fresh, fermenting sludge was obtained from an anaerobic sludge digester in Brookings, South Dakota, and acclimated for growth in DSSL in the following manner. One hundred mls of this material was added to each of the fermenters

as inoculum, after first flushing the empty fermenter with N_2, hot copper-scrubbed to remove traces of O_2. Next a prereduced basal medium, also sparged with oxygen-free N_2, was slowly added to a total volume of 600 mls. This basal medium was used initially to develop active microbial growth and adaptation to DSSL. The basal medium was composed of $(NH_4)_2SO_4$ - 0.45 g; NaCl - 0.9 g; $MgSO_4 \cdot 7H_2O$ - 18.0 g; $CaCl_2 \cdot 2H_2O$ - 0.1 g; NH_4Cl - 0.5 g; KH_2PO_4 - 1.5 g; K_2HPO_4 - 2.2 g; Na acetate - 2.5 g; Na formate - 2.5 g; Na Lactate (60%) - 2.0 ml; methanol - 2.5 ml; ethanol - 2.5 ml; tryptone - 2.0 g; yeast extract - 2.0 g; $Na_2S \cdot 9H_2O$ - 0.5 g; cystine - 0.5 g; resazurin - 1.0 mg; trace mineral soln. (7) - 10 ml; vitamin soln. (7) - 10 ml; and distilled water to make a final volume of one liter. The final pH was adjusted to 7.2.

As soon as methane production was observed (in about 3 days), a solution of 75% basal medium and 25% DSSL ozonated for 3 hours at pH 3.0 was added and removed continuously with a 3.5 day retention time. The ratio of basal medium to ozonated DSSL was decreased in stepwise fashion from 50:50 and then to 25:75 over a one month period to acclimate the organisms to the ozonated DSSL. Eventually, the basal medium was eliminated and replaced by a medium containing 75% 3 hour ozonated DSSL, 25% distilled H_2O, 0.5 g/l K_2HPO_4, and 4.0 ml/l NH_4OH (conc.). Fermenter pH was controlled at about 7.2 by maintaining the feed pH at 8.0 with NaOH.

Bacterial Enumeration

Total anaerobic bacterial counts were made weekly from fermenter contents using the Virginia Polytechnic Institute's (VPI) modification of the Hungate roll tube technique (1). The medium used was the basal medium

previously described with the addition of 2% agar. The medium was initially gassed with copper-scrubbed N_2 during preparation, then gassed with CO_2 and H_2 at a ratio of 1:1 when samples were added to the roll tubes. This modification gave results of population counts 10 times higher than previously obtained.

Samples for bacterial counts were collected by draining a few milliliters of fermenter contents into a test tube continuously sparged by a stream of oxygen-free CO_2. The test tube was then transferred to a three-place swing-type cannula system described by VPI (1) and 9.9 ml (1 to 10) serial dilutions were made with the previously described broth. Each sample was prepared in triplicate, added to roll tubes containing warm liquid agar, mixed, rolled under cold running water and incubated at 35°C for 28 days. Total colony counts were made under a 40X binocular dissection microscope. Separate counts of black-dark brown colonies were then made to estimate the numbers of sulfur-reducing organisms.

The presence of methanogenic bacteria was initially verified by streak isolating 80 light colored colonies into separate roll tubes. After 28 days of incubation at 35°C, samples of the gas in each tube were analyzed for methane by gas chromatography. Correspondingly, 35 black-dark brown colonies were streak isolated into separate roll tubes and samples of their gas analyzed by gas chromatography. Gram stains of these isolates were performed according to the Kopeloff modification as outlined by VPI (1).

Anaerobic bacteria other than methanogens or sulfur-reducing bacteria were enumerated by plate counting using Brewer's anaerobic agar (Difco). Triplicate dilution plates were incubated at 35°C for 5 days in Torbal jars with an atmosphere of 80% N_2, 15% CO_2, and 5% H_2. This lowered the OR potential below -75 mv as indicated by a clearing of the resazurin indicator present in the agar.

Aerobic plate counts of fermenter contents were made in triplicate as described by Standard Methods for the Examination of Water and Wastewater (45) using tryptone-glucose-extract agar (Difco). Colony counts were made after 5 days of incubation at 35°C.

Concentration

For use in these experiments, 100% DSSL is defined as the liquid pulp mill waste that we received from the St. Regis Paper Co. Any concentration less than 100% (i.e. 75%) is achieved by diluting the samples with distilled water. Any concentration above 100% (i.e. 150%, 200%) is produced by evaporating some liquid from the pulp mill waste sample and thus concentrating it.

BACKGROUND

In order to discuss the achievements of the second project year, it is necessary to review the significant results of the first project year. During the first project year primary staffing was completed, and the major equipment and materials were obtained and/or installed, as described in Equipment Materials and Methods.

Preliminary data was taken to compare raw DSSL, and DSSL ozonated for 6 hours. Infra-red spectra taken for raw DSSL and 6 hour ozonated DSSL, shown in Fig. 1, suggest that significant transformation of aromatics to carboxylic acids had been caused by ozonation. The BOD_5 test results of 6 hour ozonated DSSL indicated an appreciable increase of metabolizable

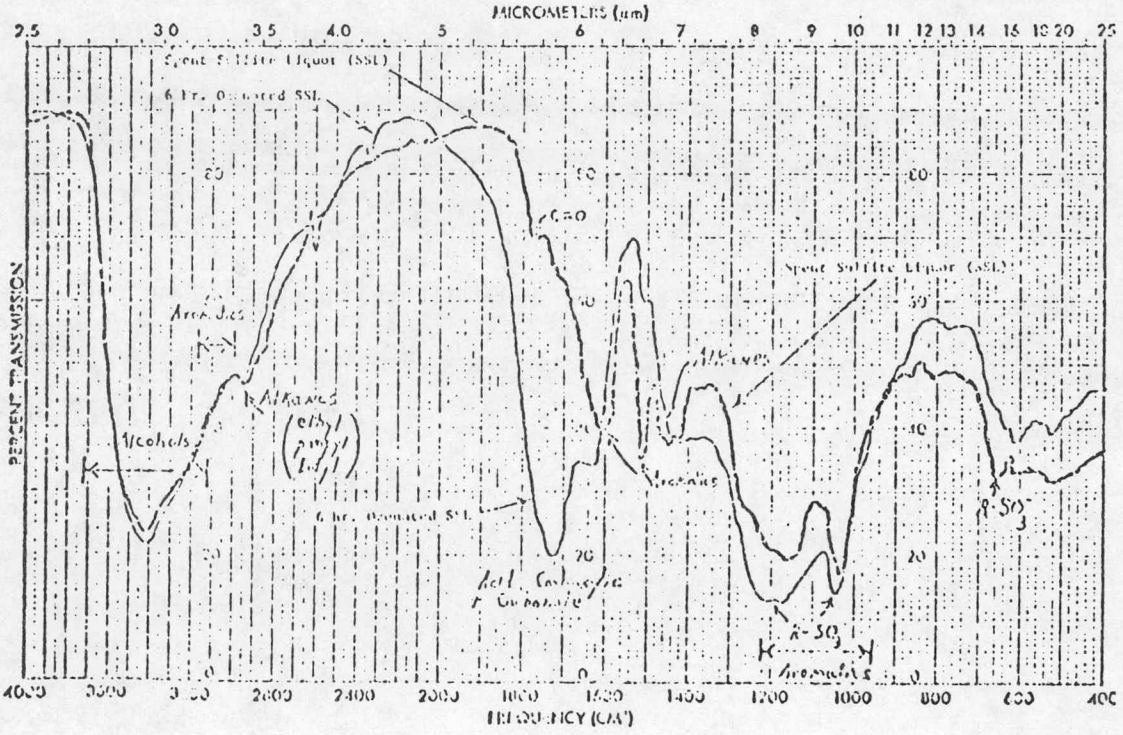


Figure 1

Infra-red Spectral Comparison between Desugared Spent Sulfite Liquor (DSSL) and 6 hr. Ozonated DSSL.

organics when ozonation was conducted at pH 3.0, while there was very little increase in BOD_5 when ozonation occured at alkaline pH. The COD of the ozonated sample was reduced the same amount, 23%, whether acid or alkaline pH's were used. Tests indicated that there was a slight decrease in the sulfur content of the sample due to ozonation.

Ozonation was conducted in continuous and batch modes of operation after completion of the construction of the packed column reactor, and results generally paralleled those obtained in exploratory flask ozonation tests. Total ozonation time was reduced because of more efficient gas-liquid contact in the column reactor. The time required for ozone to appear in the effluent gas stream varied from 3 hours at pH 3 to about 6 hours at pH 8. Ozone consumption was approximately one-half at the lower pH, and the product was more easily assimilated by the micro-organisms, consequently, all further testing was done with material ozonated at pH 3.

Micro-organisms useful for the production of methane and desulfonation were acclimated for growth in DSSL, as described in the Culture Development section. After acclimation, all reactors were run in continuous mode. The results obtained for a three week run are shown in Fig. 2. The higher curve is the reference reactor, No. 1, containing synthetic growth nutrients. The second curve is the first DSSL fermenter. Gas production from this fermenter was obtained with a residence time of about 3 days. The third curve is a fermenter running on the effluent from the second DSSL fermenter. The use of this second fermenter in series was to assess the degree of conversion obtained in the first fermenter. Very

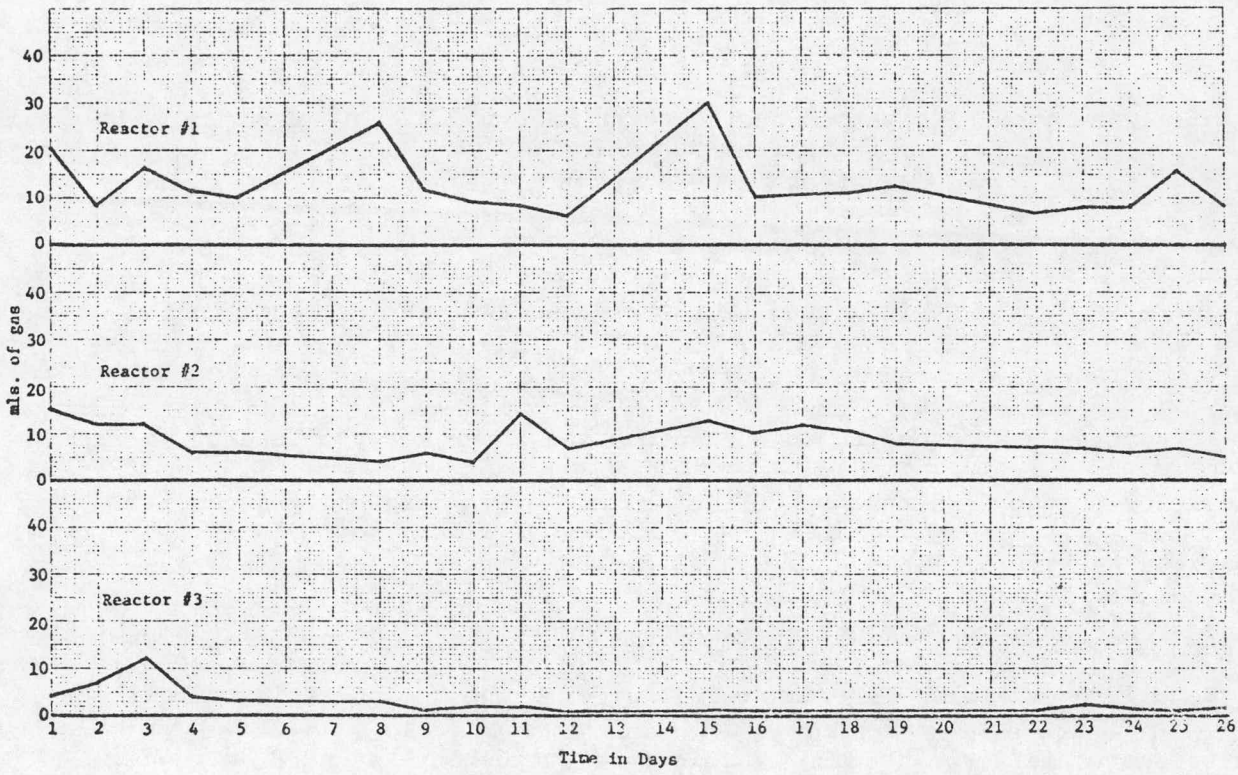


Figure 2. Gas production vs. time for three fermenters.

little methane was being produced in the third reactor, indicating almost total conversion of available substrates to methane in reactor No. 2. The gas produced from fermenters one and two is high in methane and easily supports combustion. Analysis of the gas produced using a gas chromatograph is shown in Fig. 3. Fermenters one and two produced gas having a CH_4 to CO_2 ratio of 4:1.

Preliminary protein studies showed the unozonated DSSL, would not support the growth of Torula yeast, and had a comparatively low BOD_5. Samples of the ozonated DSSL were adjusted to pH 5.0, and 0.1% dipotassium phosphate and

ammonium chloride were added to accommodate the metabolic requirements of yeast. Active yeast growth developed in a one week period.

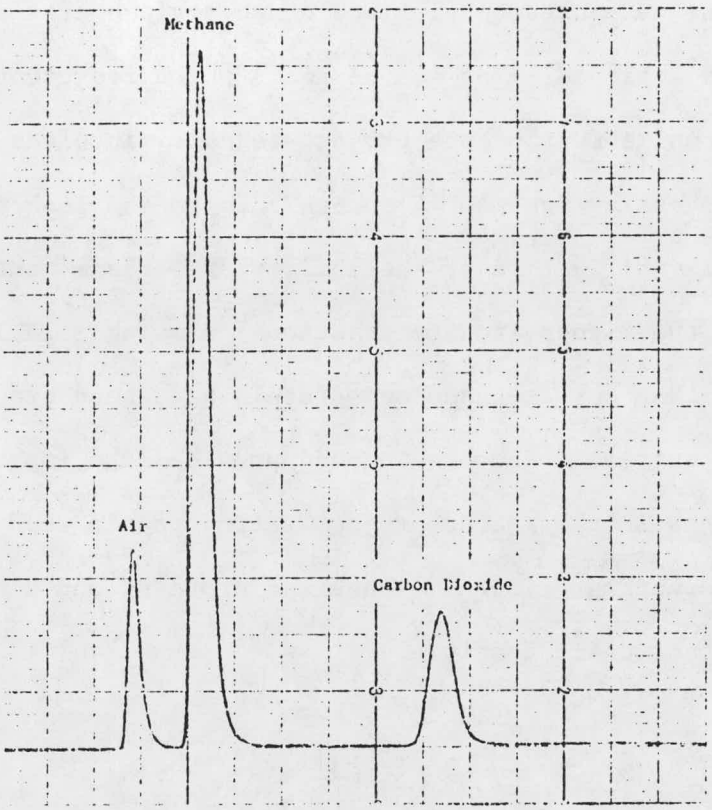


Figure 3. Gas chromatographic analysis of effluent gas from fermenter 2.

For the past 40 years, Torula yeast growth has been accomplished commercially on pulp mill waste liquor to consume the wood sugar remaining in the waste before it is disposed of. Due to changes developed in modern nomenclature, literature and microbiology journals are now calling Torula by its more specific name Candida utilus. These names are synonomous, however, and the yeast will be referred to as Torula throughout the remainder

of the paper.

The exploratory experiments conducted during the first project year served to establish two facts. Reliable operability of the many pieces of equipment which were assembled to similate bench scale operation of the conversion process was achieved. The second fact, which represents the major significant accomplishment of the project to date, involves the feasibility of the processing scheme. Although the process looked promising on paper, experimental verification of the ability of methane bacteria to convert the lignin of the lignosulfonate fractions to metabolizable organics was needed. The fact that both methane and protein have been produced in measurable yields was extremely encouraging and provided the impetus for further study. Research in the second project year hoped to find optimal conditions for the production of both methane and protein, and a means of making the process economically feasible.

RESULTS

Effect of Ozonation

Preliminary experiments studying the effect of ozonation at varying conditions of DSSL concentration, time and pH indicated the DSSL ozonated for 3 hours (15 g O_3/l) at pH of 3.0, then diluted to a 75% concentration, was a suitable medium for anaerobic fermentation and production of CH_4.

This conclusion was drawn from test results which showed that time and pH significantly affected the yield of useful substrate produced during ozonation. Reactivity of ozone with DSSL as a function of time is shown in Fig. 4 and 5. During the early stages of ozonation, the reaction

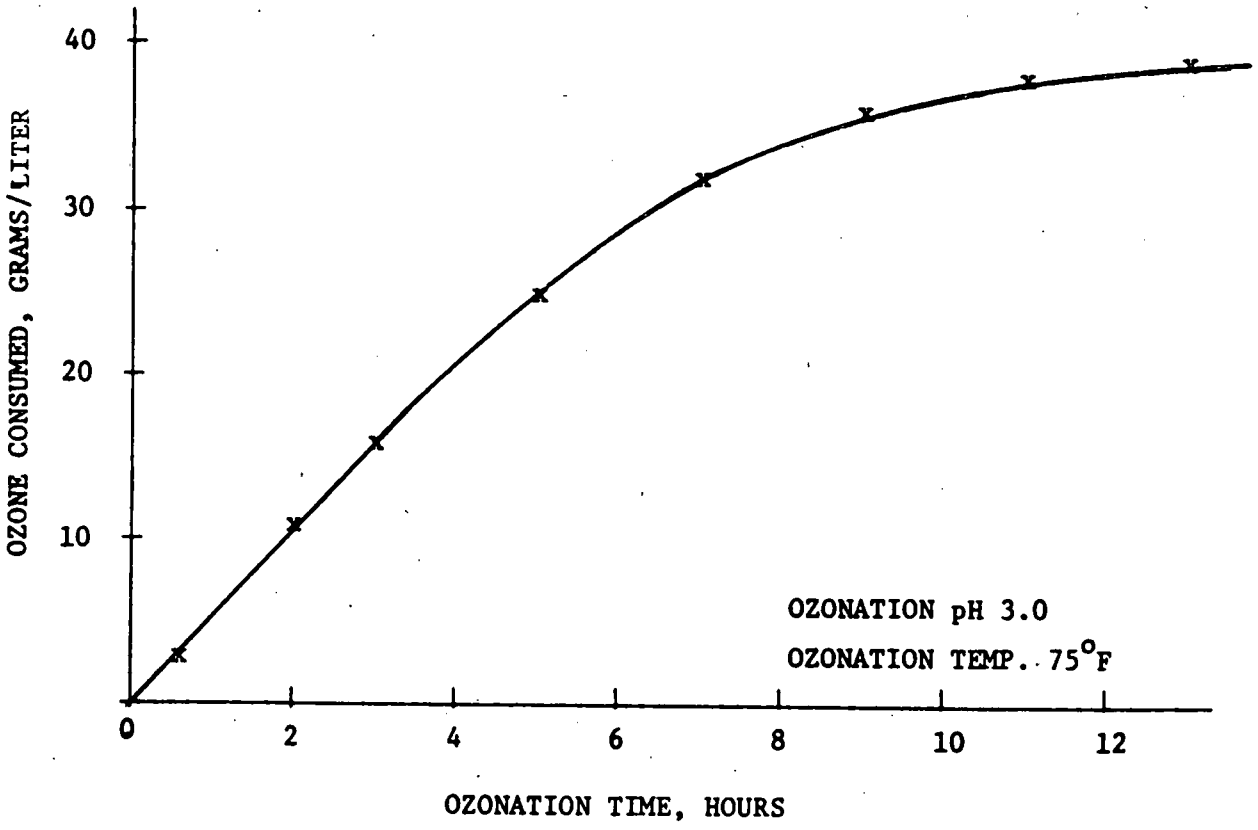


FIGURE 4. OZONE-DESUGARED SPENT SULFITE LIQUOR REACTIVITY

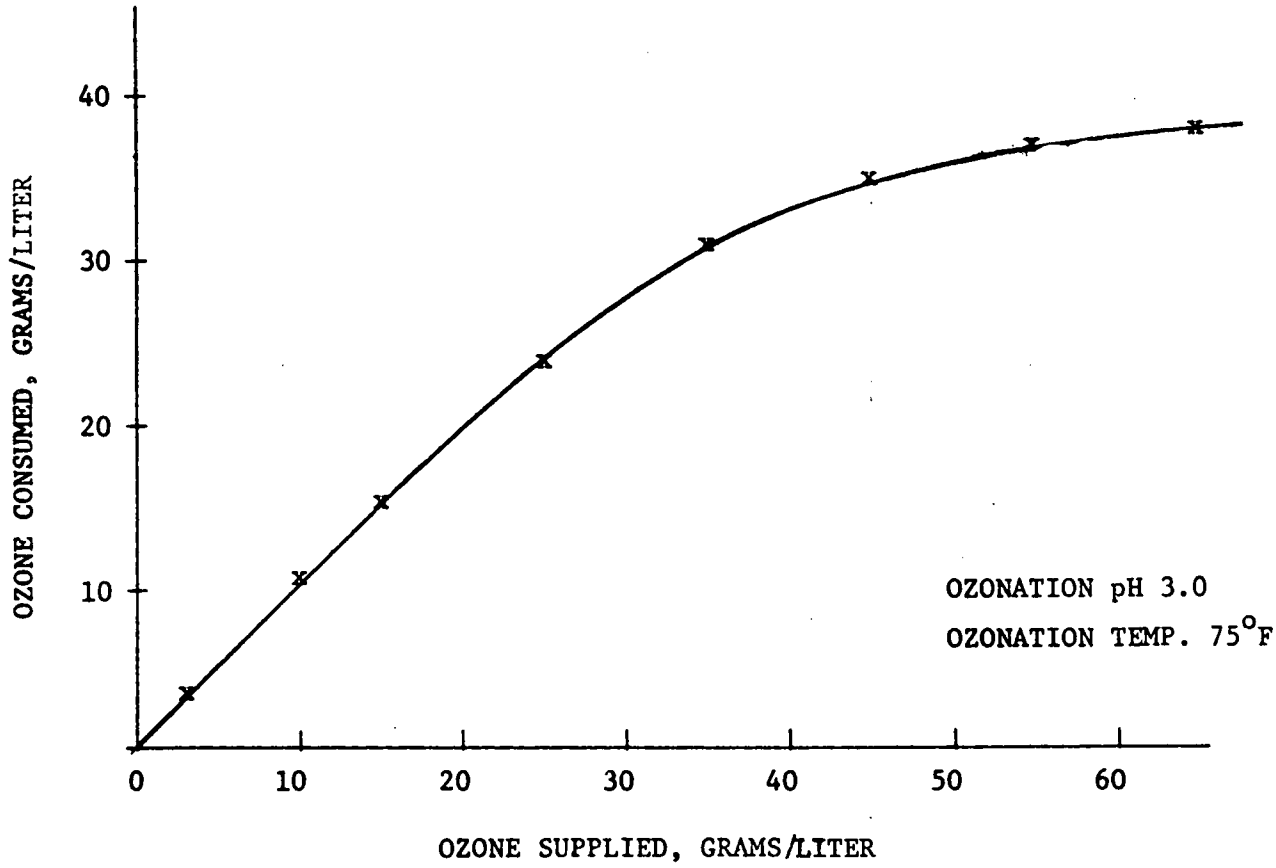


FIGURE 5. OZONE UPTAKE EFFICIENCY OF DESUGARED SPENT SULFITE LIQUOR

rate is essentially constant, and the ozone uptake efficiency is 100%. After approximately 3 hours of ozonation the organics in solution show resistance to further oxidation and the rate of reaction slows down. The ozone uptake efficiency decreases as ozone consumed approaches a value of approximately

15 g/l DSSL treated or equivalent to 3 hour ozonation time.

COD was lowered about 16% during ozonation, as shown in Fig. 6a, indicating an oxidation and breakdown of the lignosulfonate molecule. The corresponding increase in BOD_5 values (Fig. 6b) was evidence of a production of smaller metabolizable molecular fragments from the lignin molecule. Total sulfur content was lowered about 7%. Most of this was bonded to the lignin molecule since free sulfur in solution was steam stripped from the DSSL prior to yeast fermentation. This sulfur was probably oxidized from the lignin molecule to SO_2 and was removed by way of the off gas during ozonation.

The effect of pH on the production of metabolizable compounds and the concurrent reduction in COD is significant. Typical results are shown in Fig. 6c, where the effect of pH on BOD and COD for a two hour ozonation period are plotted. Although these curves tend to vary with different ozonation times, the results consistently favor low pH conditions.

Gas chromatographic analysis of ozonated DSSL indicated that significant amounts of aromatic compounds or the phenylpropane subunit of the lignin molecule were transformed to oxalic acid. Formic and acetic acid were also produced during ozonation in varying quantities, a probable cause for some of the BOD_5 increase. The gas chromatographic evidence substantiated infra red data taken during the first project year on ozonated and unozonated DSSL (see Background).

The most pronounced visual effect of ozonation on DSSL is change in color, as shown in Fig. 6d. Although initially black in color, the liquor

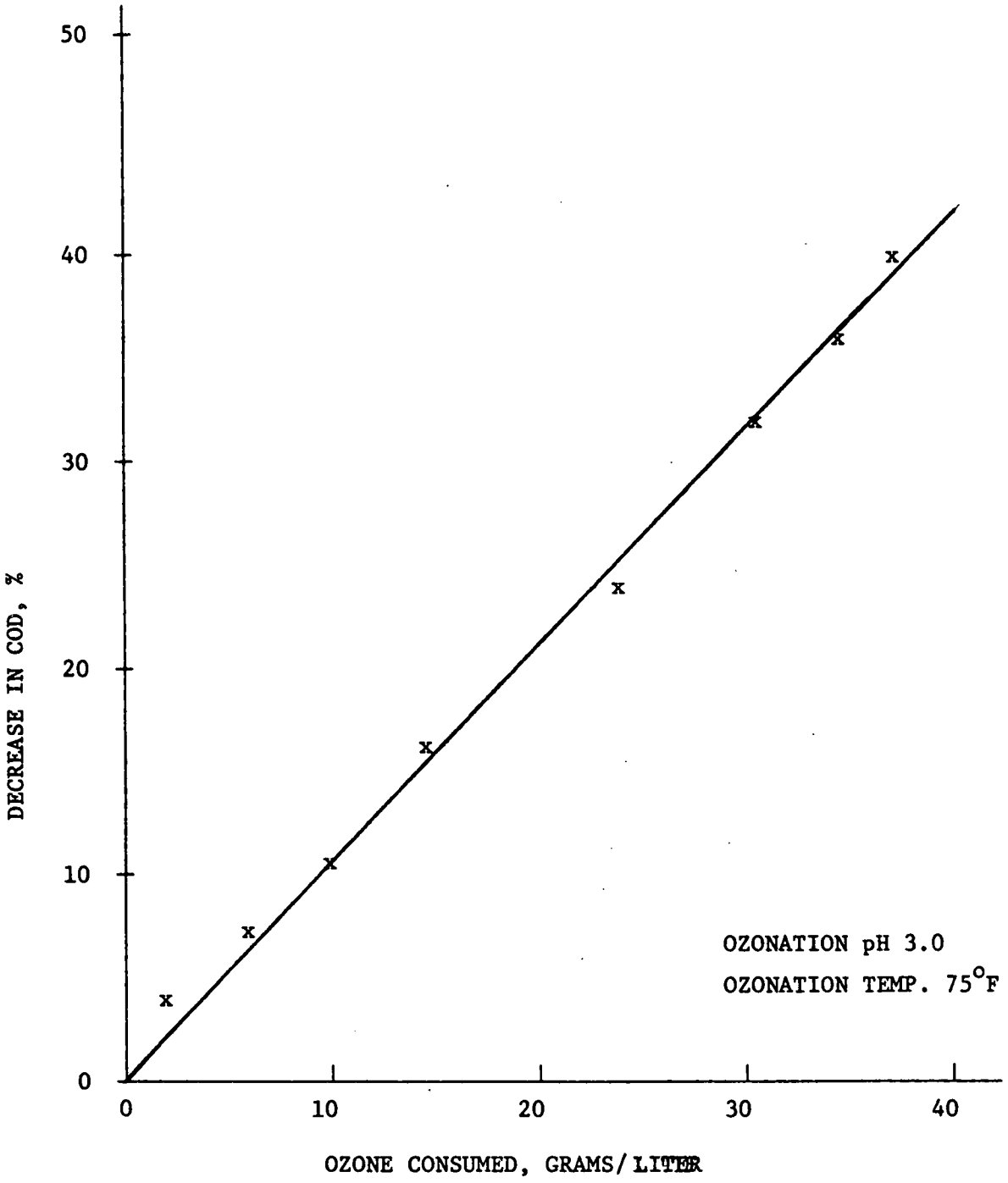


FIGURE 6a. EFFECT OF OZONATION ON CHEMICAL OXYGEN DEMAND

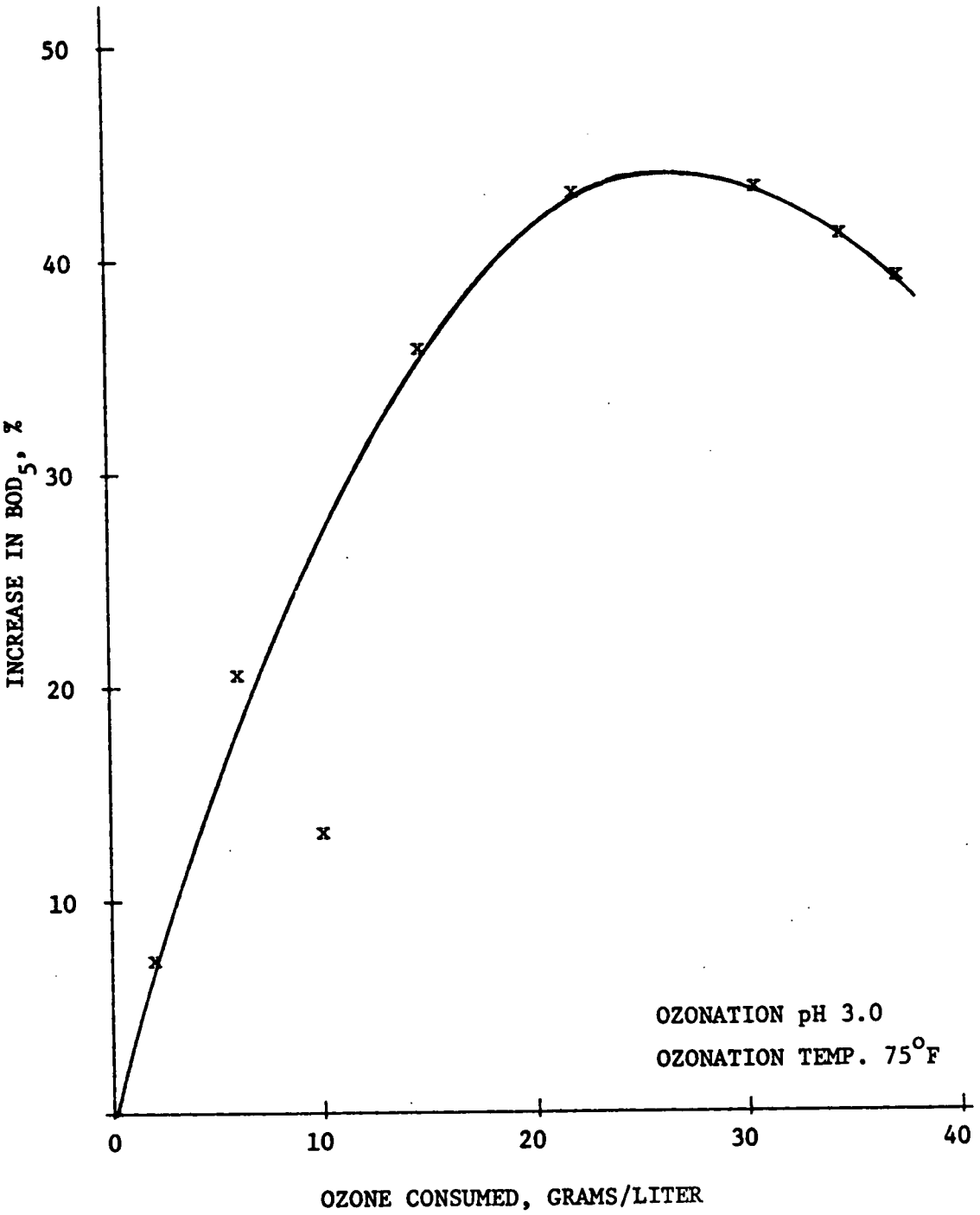


FIGURE 6b. EFFECT OF OZONATION ON BIOLOGICAL OXYGEN DEMAND

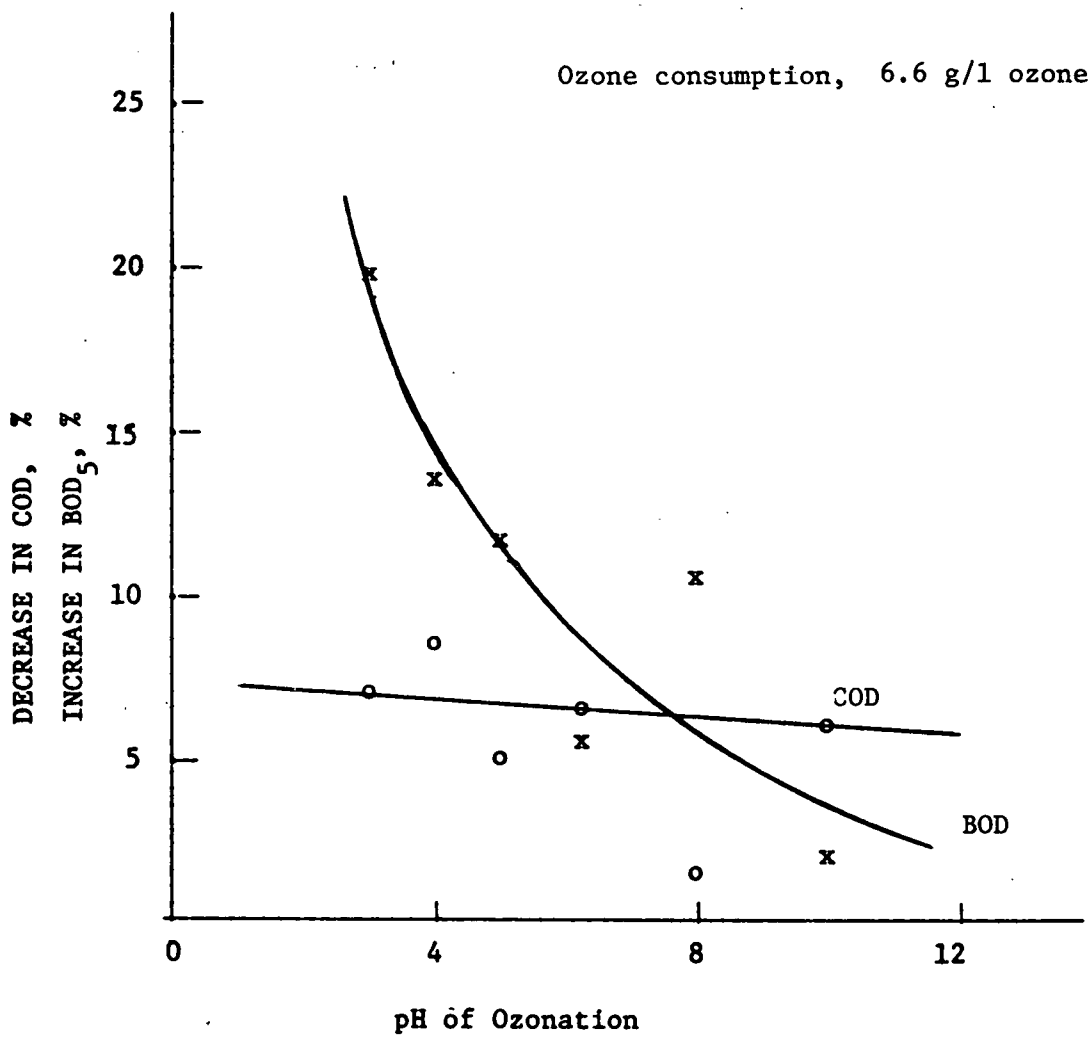


Figure 6c. Hydrogen Ion Concentration Affects Chemical and Biological Oxygen Demands During Ozonation.

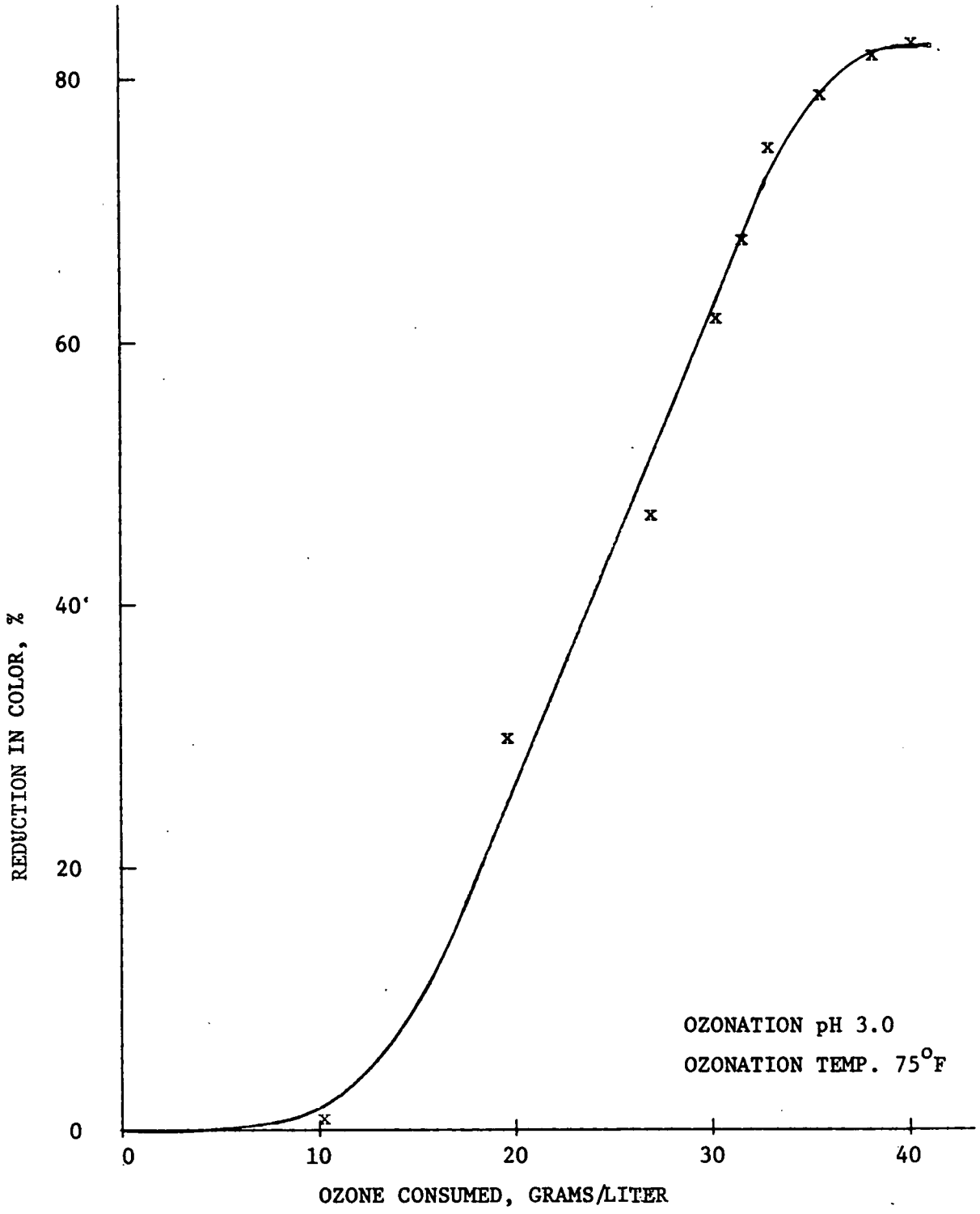


FIGURE 6d. EFFECT OF OZONATION ON COLOR OF DSSL

became orange upon addition of 15 g O_3/l and turned yellow after a 30 g O_3/l treatment. These changes in color were followed on a Spectronic 20 at 360 nm using samples diluted 1 part with 20 parts water. The reduction in color is due to destruction of the aromatic ring system along with the chromopores of lignin responsible for its color. The UV spectra, shown in Fig. 7, shows the decrease in specific absorbances in the entire 200-300 nm wavelength region.

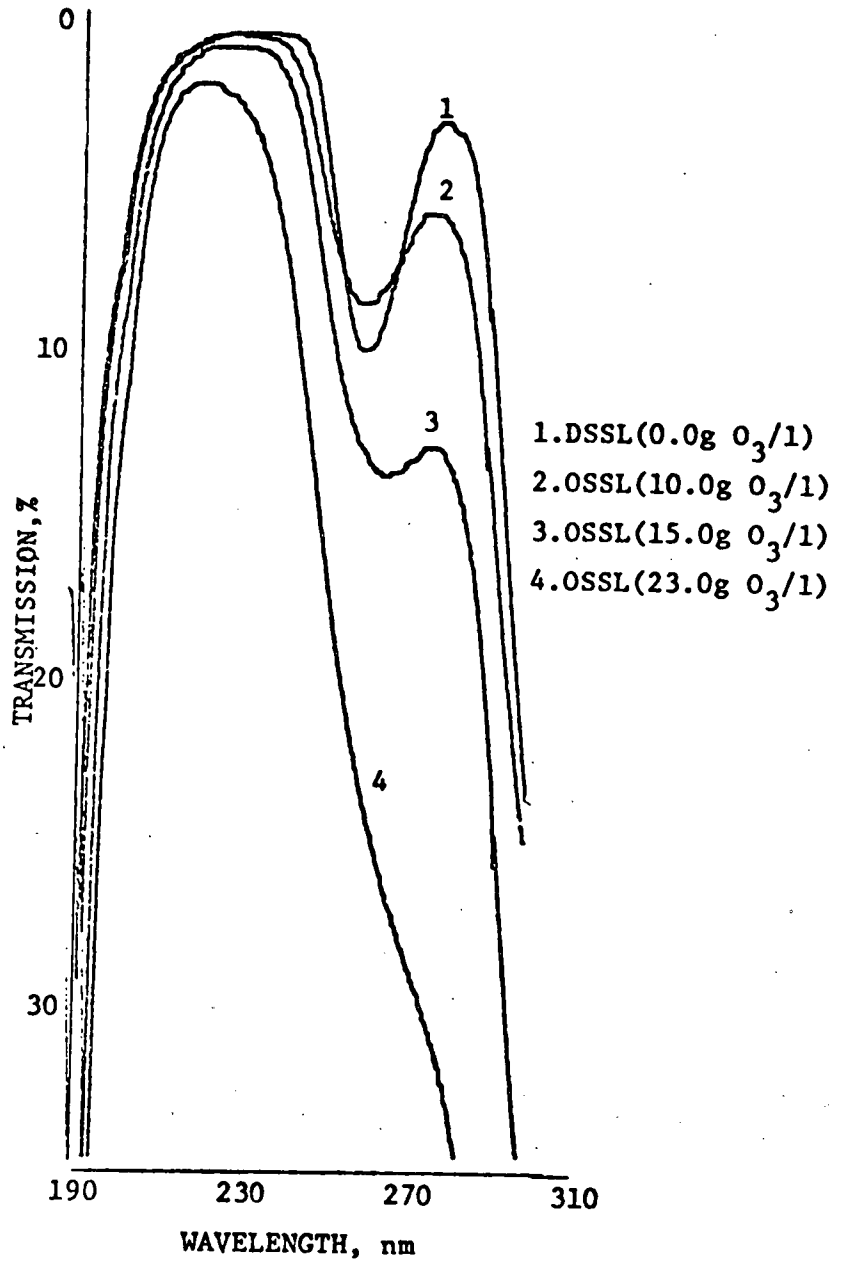


FIG. 7. UV-SPECTRA OF OZONATED LIQUOR

Methane Production

Continued studies were made with anaerobic fermenters for optimum conditions and feasibility as a method for producing methane. Early observations showed unexplained fluctuations in gas production during each 24 hour period. It was decided that these daily variations were due to uncontrollable changes in the ambient temperatures of the laboratory. This problem was eliminated by constructing a constant temperature chamber which reduced temperature fluctuations to less than $\pm$ 1°C during any given measuring period.

To determine whether ozonation was necessary to produce methane, the level of gas production which can be supported in untreated yeast plant effluent was tested by feeding one reactor a medium of 75% unozonated DSSL. The results of this test showed a general lack of metabolizable substrate in the unozonated effluent. Gas production was only a quarter of that obtained from ozonated DSSL reactors (approximately 2 mls per hour), and was composed mostly of carbon dioxide (Table 2). The results of this test established that most of the methane produced from ozonated DSSL occurs due to compounds produced during ozonation, and not from carbohydrates and cellulose remaining after fermentation with Torula. It is evident that prior yeast fermentation (discussed under Materials) has removed most of the sugars and lower weight compounds in DSSL to the point where it cannot support extensive anaerobic fermentation.

Except for preliminary testing, studies done to determine capacities of methane production were done in continuous operation. Two of the fermenters were operated under identical conditions on a substrate consisting of 3 hour ozonated DSSL (15 g O_3/l) diluted 25% (3:1) with tap

TABLE 2. Gas production from the continuous fermentation of spent sulfite liquor.[1]

Fermenter Feed	Retention Time (days)	Effluent Gas		
		Production (mls/hr)	Composition $\%CO_2$	$\%CH_4$
3 hr Ozonated DSSL[2] (15 g O_3/l)	3.3	10	21.8	78.2
3 hr. Ozonated DSSL (15 g O_3/l)	2.6	--[4]	48.6	52.4
Unozonated DSSL[3]	3.3	2	85.7	14.3

[1]All values are based on 75% concentration.

[2]Desugared yeast plant effluent ozonated at pH 3 for 3 hours.

[3]Untreated, desugared yeast plant effluent. Values from one fermenter.

[4]No steady state values obtainable. Production rates were a maximum of 20 mls/hr immediately after adjustment of retention time, then decreased slowly.

water. The results obtained from the two reactors were identical, and will be utilized as the statistical base to accurately assess future results. The working volume for the fermenters was varied from 500 to 700 mls to establish residence times between 2 and 6 days. Total gas production for these early tests averaged 8 cc/hr. This gas rate was lower than desired, but the composition averaged about 80% methane, which is exceptionally rich for synthetic gas fermentation.

Steady state testing done on the fermenters over a 3-month period maintained an average gas production of 10 ml/hr at 0°C and 1 atmosphere pressure for each of the 3 fermenters used. This is a total yield of 1700 mls per liter of feed for a 3.5 day retention time. The maximum gas production

observed at any time during fermentation was 30-40 mls/hr. The average gas production of 10 mls/hr was equivalent to 2 liters of methane per liter of undiluted ozonated DSSL, or a carbon conversion of about 5%. Gas chromatography showed the average methane concentration for the steady state period was about 75%, the balance being composed of carbon dioxide. This methane concentration is higher than that reported in the literature (50-65% methane from fermentation of agricultural and municipal wastes)(20, 21, 22). The numerical results of the tests are tabulated in Table 2. (Gas production in these tests is greater than the 2 mls/hr produced using unozonated DSSL feed.)

The average generation time for gas producing organisms appears to be about 2.3 days. When the retention time was brought below 3.0 days, gas production rates increased by about 10% at first, then rapidly dropped off. This implies a washing out of the bacterial population. When the retention time was raised to 3.5 days, a decrease in gas production was observed proportional to the increase in retention time, implying a decrease in utilizable substrate.

The ratio of CH_4 to CO_2 in the effluent gas averaged 4:1. Again referring to Table 2, it can be seen that if the retention time were lower than the apparent retention time of 3.0 days, the ratio of methane to carbon dioxide decreased markedly, to almost 50:50. This is presumably due to a decrease in the number of methanogenic bacteria as opposed to other anaerobic bacteria.

A lower ratio of CH_4 to CO_2 was also observed if a fermenter were run at a pH below the optimum of 6.8 to 7.5, or if unozonated DSSLwere used as a substrate. This range of pH along with oxidation-reduction (OR) potentials of -300 mv has been confirmed by a number of investigators (16, 36. 39).

Cappenberg reported optimum methane bacterial growth at OR potentials between -380 and -400 mv (4). In our fermenters, OR potentials fell to as low as -420 mv during continuous operation and optimum gas production. It is probable that different fermenter feed has an effect on the net OR potentials during fermentation.

Fermenter Effluent Analysis

As indicated by the data in Table 3, for the effluent stream (fermented ozonated DSSL), a cumulative drop of 32% is observed for COD values for a combined treatment of both ozonation and fermentation. Fermentation of

TABLE 3. Average values for COD, BOD, total sulfur, and fatty acids in untreated and treated SSL[1].

	Unozonated SSL	Ozonated SSL	Fermented Ozonated SSL
COD (mg/l)	70,000 (10)[2]	59,000 (10)	48,000 (10)
BOD (mg/l)	6,900 (7)	8,200 (10)	6,400 (6)
Sulfur (g/l)	4.6	4.3	3.8
Organic Acids			
Formic (mM)	-[nd]	3	2
Acetic (mM)	2	8	66
Propionic (mM)	-	-	4
Butyric (mM)	-	-	4
Oxalic (mM)	-	4	-

[1]All values are based on a 75% concentration.

[2]Numbers in parentheses indicate number of values used to determine average, all others were determined from the average of 5 replicates.

[nd]Concentrations less than 1 mM not detectable.

ozonated DSSL reduced the average COD value of the effluent by about 19%, while the average BOD value dropped 23%. There is an initial increase in BOD of about 20% after ozonation, but this substrate is used up during fermentation. When unozonated DSSL was fermented under similar conditions, COD values dropped only 7% with no noticeable change in the BOD value. A 10.5% loss in total sulfur content was observed after fermentation of ozonated DSSL. Fatty acid analysis indicated that approximately 66 mmoles per liter of acetic acid were present in the effluent along with traces of propionic and butyric acids. The oxalic acid produced during ozonation was consumed during fermentation.

Analysis of reactor performance during this period indicated that the fermentation process appeared to be substrate limited. Various nutrient supplements were added to 34 steady state runs, lasting approximately 5 days each, to determine what the limiting substrate(s) may be. During these experiments varying quantities of methyl or ethyl alcohol, vitamins, minerals, salts, and salts of low molecular weight organic acids were added (in different amounts and combinations) in an attempt to stimulate methane production. The addition of alcohol, formate, or acetate alone had no measurable effect on gas production. The addition of a mixture of methyl alcohol with vitamins and minerals stimulated gas production to 22 cc/hr, but the addition of methanol with either minerals or vitamins alone increased gas production to only 15 cc/hr. The addition of sodium lactate with or without additional vitamins and minerals also tended to stimulate production to only 15 cc/hr.

The published literature suggests the preferred substrate for methane production is acetic acid. The nutrient supplement experiments failed to show similar results. The additional of low molecular weight fatty acids had no measurable effect on fermentation. It is significant that the fatty acids are not present in unozonated DSSL at detectable quantities, but formate and acetate ions are formed after 3 hours of ozonation.

All experiments show an acetate concentration for ozonated and fermented DSSL six times larger than DSSL that has only been ozonated. This occurred in all runs which had a testing period from 3 to 5 days.

This production of 66 mM (3960 mg/l) acetic acid, as shown in Table 3, is much higher than values commonly reported by other investigators working with an aerobic digestion of organic wastes. Van Den Berg et al. (49) reported 200 mg/l acetic acid produced in the fermentation of pear wastes while Pfefferts (34) indicates a production of 880 mg/l of acetic acid from the digestion of domestic refuse. In view of the large increase in acetate after fermentation, it is not surprising that the addition of acetate produced no appreciable increase in gas production.

The fact that acetate is produced during methane fermentation suggests that the preferable processing sequence would be methane fermentation followed by yeast production. It has been previously established by other researchers that yeast can effectively utilize acetate as a growth substrate. Study was conducted to examine the feasibility of growing yeast on ozonated DSSL which has first been subjected to anaerobic digestion for the production of methane. It was anticipated that yeast production should show a signifi-

cant increase unless contaminants produced in methane fermentation compete for the limited substrate under aerobic condiitons (Protein Synthesis section).

Protein Synthesis

Continued studies were made on yeast growth. Tests were done in the manner described in the Methods section. The results of the tests are shown in Figure 8. Some yeast growth occurred in all inoculated flasks. Yeast growth on the unozonated DSSL was low, reaching a maximum concentration of 1.2 g/l after 3 days. The highest yeast growth, 5 g/l, was obtained in the DSSL ozonated for 10 minutes, while the poorest yeast development occurred in DSSL ozonated for 2 hours. This was an unexpected result and contrary to the methanation results. The maximum yield of the rough variant of Torula yeast increased approximately four times when the ozonation time was decreased from 120 minutes (2 hours) to 10 minutes, with the 10 minute treatment showing a dramatic production peak at two days and a very rapid decrease on the third day. This decrease is probably attributable to autolysis of the yeast cells. After 7 days there was very little difference in yeast growth among the various DSSL treated media. It appears that the lower yeast yields for longer DSSL ozonation treatments are related to a reduction in carbon available for yeast growth.

The data shown in Fig. 9 relate the growth of the smooth variant of Torula yeast to ozonation times. These data corroborate those obtained with the rough variant and provide additional support to the hypothesis that short ozonation times are to be preferred. Although the smooth variant apparently has the same growth rate characteristics as the rough, its ability to utilize ozone generated substrate is much less. The smooth variant also shows a

FIGURE 8. Yeast production vs. time--rough variant

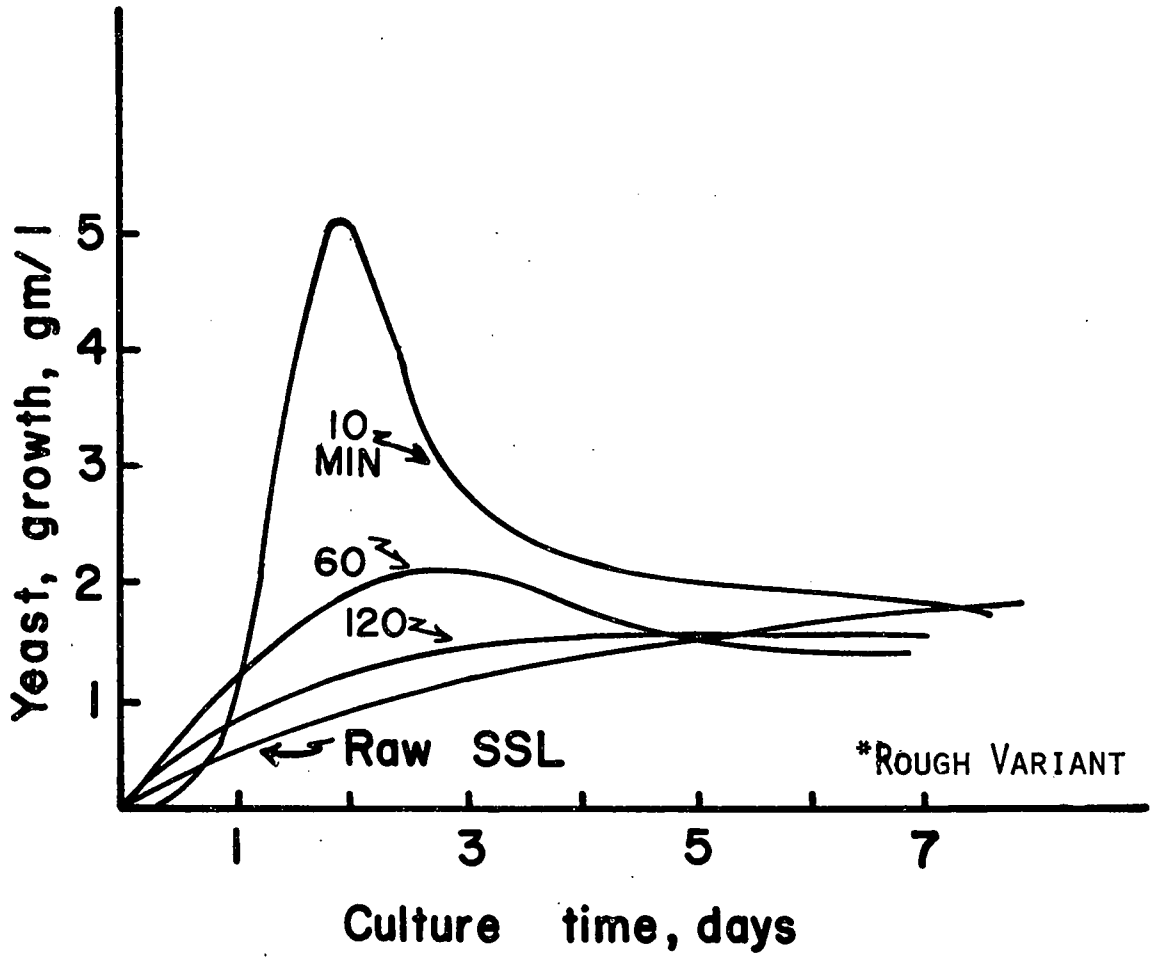
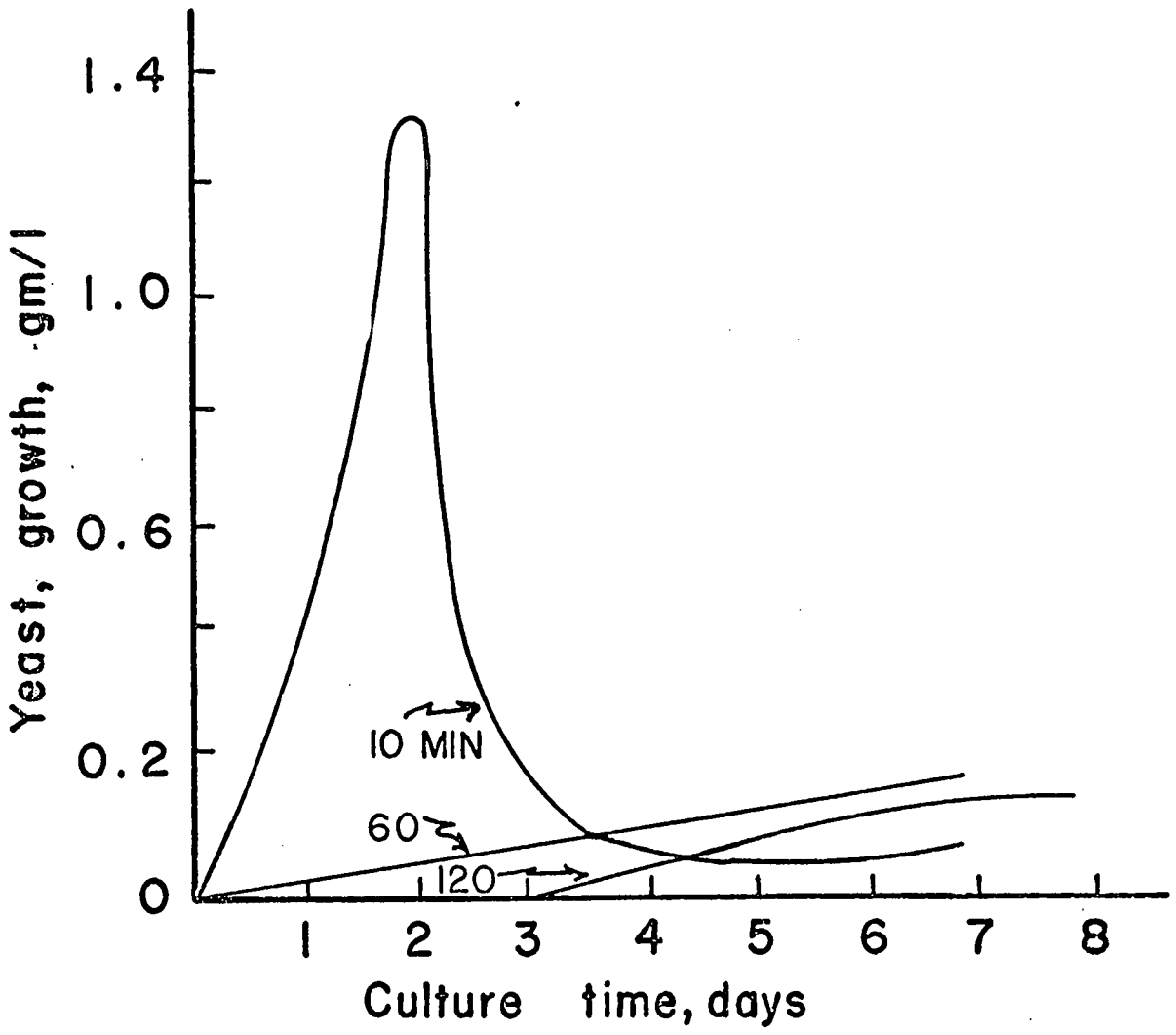


FIGURE 9. Yeast production vs. time--smooth variant



maximum production of cell mass at 10 minute ozonation time, but this production is only slightly greater than that obtained from the rough variant grown on untreated DSSL. The ability of the smooth variant to utilize substrate generated from DSSL at ozonation times greater than 10 minutes is essentially nil. Growth of the smooth variant on untreated DSSL is also negligible.

In later tests, the fermenters were changed from batch to continuous operation to confirm the yield characteristics shown in the batch tests and to confirm the suitability of the substrate for commercial operation. A fermenter similar to those previously described for the methane fermentation studies was operated on 3 hours ozonated 100% DSSL containing supplemental additions of nitrogen, phosphorus and potassium. The pH was adjusted to 4.6 and the DSSL was continuously mixed by a magnetic stirrer. Incubation temperature was 36°C. A culture of high yield rough colony variant of Torula was used as fermenter inoculum.

The yeast fermenter was run continuously for six weeks. Substrate residence times were varied from 1.5 days to 2 days during this period. Highest yeast growth was obtained at a residence time of 1.8 days, attaining an average weight yield between 2.0-2.2 g/l of SSL. This was a low yield when compared with the 4.8-5.0 g/l produced in the batch cultures, but this might be expected if the continuous flow parameters are less than optimum.

Previous analysis of effluent from various methane-producing digesters indicated appreciable production of acetate during the anaerobic fermentation of DSSL. It was speculated that significant yeast production should occur on this substrate since Torula can effectively use acetate as an energy and carbon source. However, pure culture experiments indicate that only negli-

gible yeast growth was possible on previously fermented ozonated DSSL. Since ozonation or total salt concentration are not inhibitory to this species of Torula yeast, it appears that possibly some material toxic to the yeast was produced during the fermentation process.

Other DSSL Treatment

Since the preliminary efforts to increase methane production from ozonated DSSL by the addition of growth supplements or varying reactor operating conditions failed to isolate any sufficiently effective additive, the effects of other pretreatments of DSSL prior to fermentation were examined. Previous research by Mr. Bremmon before he joined our staff indicated that sonication of sewage sludge greatly increased its biodegradability. Therefore, a study was established in which ultrasonic treatment of SSL was compared with or used in conjunction with ozonation.

All sonication treatments were performed in the same reactor having a starting volume of 300 ml of 100% yeast plant effluent. The reactor was placed in an icebath for temperature control, but substrate temperature did reach 60°C during the longer ultrasound treatments. A sound level of 41,000 hz was used in all experiments. Samples were removed from the reactor every half hour for COD and BOD determinations.

The results of these tests showed that ultrasound treatment does have an appreciable effect on the BOD and COD levels of DSSL, especially when used in conjunction with ozonation (Fig. 10). Some of these changes may have resulted from the higher substrate temperature due to sonication or to the lowering of pH to 1.5 during the combined sound and ozone treatment. It is still unclear why the combined sound-ozone treatment had such a marked effect.

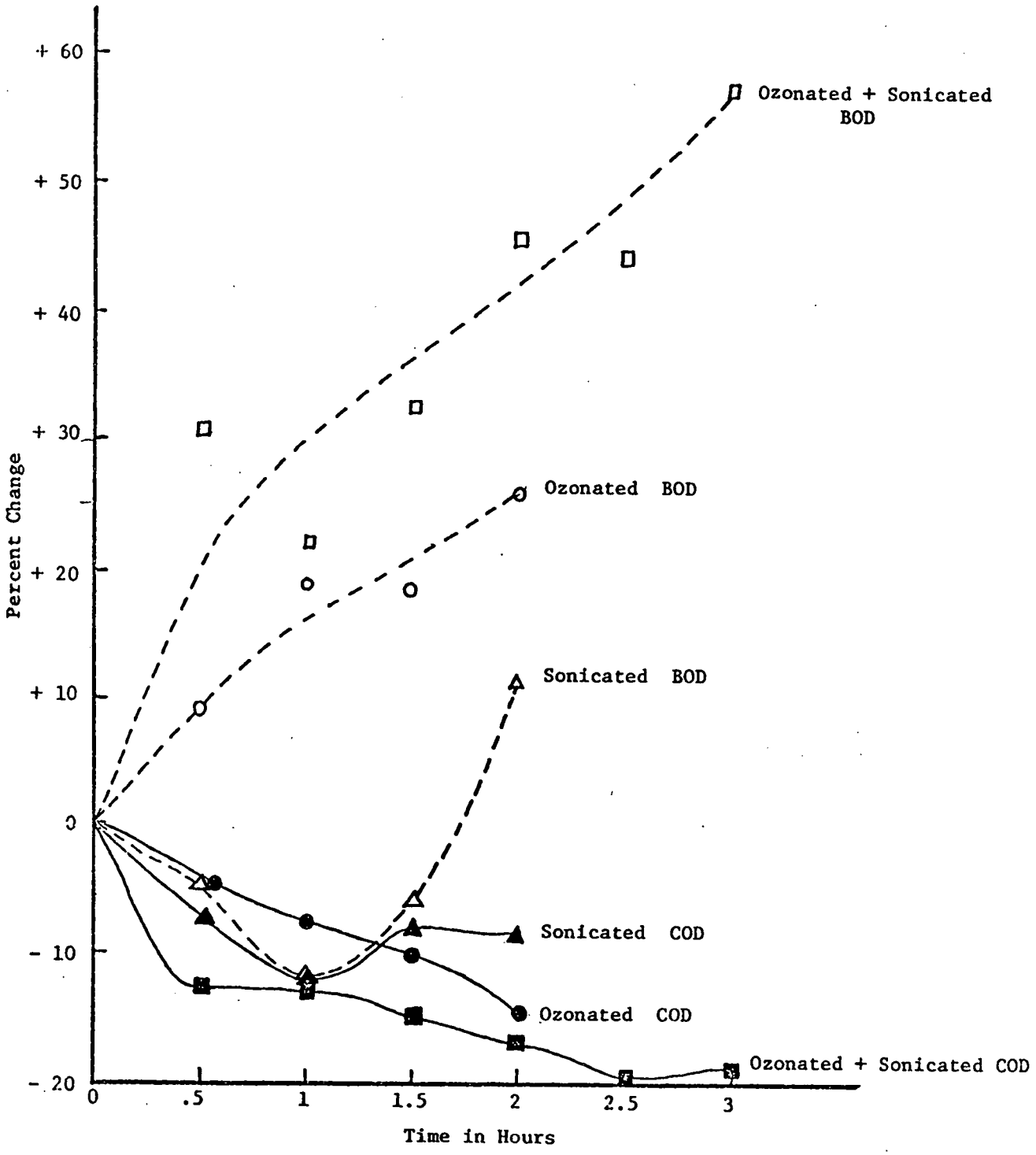


Figure 10. Changes in COD and BOD vs. time for varied treatments of DSSL with ozone and sound.

It is expected that the increased BOD levels would result in greater yeast or methane productions.

Statistical Analysis of Methane and Yeast Production

This past year we began a fractional factorial screening experiment to determine statistically the important factors in the production of both methane and yeast from DSSL. It was decided that the factors which appear most important in gas production from DSSL are: (1) concentration, (2) ozonation time, and (3) retention time. Further, we decided to measure gas production in ml of gas/ml of feed in order to reflect the actual conversion of DSSL to gas. The measurements are all taken at steady state conditions which require several weeks to achieve. After steady state is achieved, the gas production has shown little variation from day to day. Hence, at this point, it is not felt that replication of treatment levels is necessary. At this point we have measurements for 18 different treatment levels.

A preliminary regression equation was developed using the factors of concentration, retention time, and concentration-retention time interaction to predict gas production. This analysis showed concentration to be significant while retention time is not significant in the range of three to eight days. Wash out occurs at a retention time of less than three days. The concentration-retention time interaction also appears to be significant. Further tests are being run to test the significance of concentration and concentration-retention time interaction. Also further tests are being conducted to check the significance of ozonation time.

In the yeast production from DSSL, it was decided that the factors of inoculum, ozonation, and incubation time appear to be significant. Tests

were run with two different sources of inoculum to check for mutation on both 10 minute ozonated DSSL and unozonated DSSL. Also yeast growth on ozonated and unozonated DSSL which had previously had yeast growth on it but which received no new inoculum was measured. Measurements of yeast growth over various periods of time were taken and preliminary analysis shows there appears to be a significant difference in yeast production due to ozonation and there may be a significant difference due to inoculum.

Bacterial Analysis

Methane bacterial counts in the fermenters, as shown in Table 4 were derived by subtracting the counts of black-dark brown colonies from the total roll tube counts. The validity of this technique was verified when it was found that of the 80 light colored colonies randomly isolated from roll tubes, 78 were shown through gas chromatography to produce methane.

Table 4. Average bacterial enumerations for anaerobic fermenters fed 75% 3 hr. ozonated DSSL continuously for one month.

Taxa or Group	Average CFU/ml
Methane bacteria	1.7×10^9
Desulfovibrio spp. (Black-dark brown colonies)	1.7×10^9
Total anaerobic bacteria as determined by Torbal jars	5.3×10^7
Facultatively aerobic bacteria	2.4×10^7
Obligate anaerobes other than Desulfovibrio or methanogens (by deduction)	2.9×10^7

CFU = Colony forming units. Each value represents an average of 24 roll tube or plate counts.

Thirty-five of the black-dark brown colonies were also isolated and none of these were found to produce methane.

Methanogenic colonies, larger than 0.5 mm in diameter, were found to fluoresce in roll tubes when exposed to low frequency ultraviolet light. This is in accordance with the findings of Edwards and McBride (11). This fluorescence was not exhibited by any of the black-dark brown isolates. Although the fluorescence of methane bacteria has been suggested as a method of enumeration, the deduction method proved much easier and faster after it was shown through gas chromatography that nearly all the lighter colored colonies were methanogens. Enumeration of methanogens by fluorescence proved to be unreliable because colonies smaller than 0.5 mm did not emit enough light for detection.

A microscopic examination was made of 135 randomly picked light colored methanogenic colonies. Fifty-one percent of these were found to be Gram positive coccoids of short lancet shaped rods, sometimes in chains indicating the probable presence of Methanobacterium ruminantium (38, 44). Although not always the dominant species, this organism seems to be present in many forms of anaerobic digestion (22, 48).

Another 42% of the methanogens could be placed in the genus Methanobacterium on the basis of cellular morphology. These organisms, commonly present in anaerobic digestion (22, 48), represented at least two or more species of the genus Methanobacterium since various types of Gram negative rods were found. No attempt was made to identify these isolates to species level.

The remaining 7% of the 135 light colored colonies were of the genus Methanococcus. These isolates were small Gram variable to Gram negative

cocci which grew in clumps, pairs, or as single cells. This genus is also common in most anaerobic digesters (22, 48). No members of the genus Methanosarcina were observed even though they are common to anaerobic digestion (48).

Microscopic examination of the bacteria growing as black-dark brown colonies revealed all were curved to sigmoid Gram negative nonsporing rods. The production of H_2S by each of these isolates was readily detectable. These attributes indicated the probable presence of Desulfovibrio spp. (36). A culture of Desulfovibrio desulfuricans obtained from the American Type Culture Collection was comparable in microscopic and cultural examination to the 35 fermenter isolates.

Other anaerobic bacteria present in the fermenters were enumerated by dilution plates in Torbal jars. It was assumed that no methanogens would be present in these counts since the OR potential of the medium used rarely will go below -200 mv. The OR growth range for methanogens is -250 mv and below (41). Growth of Desulfovibrio also would be inhibited by lack of lactate, sulfate and other required nutrients in the medium. The absence of either methanogens or Desulfovibrio on these plates was confirmed in subsequent colony isolation. At the same time, aerobic dilution plates were made to estimate numbers of facultatively aerobic bacteria. Obligate anaerobic populations were assumed to approximate the differences between the total anaerobic counts in Torbal jars and the aerobic counts. As shown in Table 4, the facultative aerobic bacteria were 10-100 fold less numerous than either the methanogens or Desulfovibrio. Consequently, these bacteria would have had a negligible effect on the population estimates of Desulfovibrio and methanogens. However, these organisms were an active part

of the fermentation process since the fermenter feed bacterial count showed 10^4 organisms/ml of feed. Gram stains from 80 isolates from Torbal jars revealed that 39% were Gram positive rods, 19% were Gram positive cocci in chains and clusters, and 42% were Gram negative rods. Colony morphology was quite variable.

The average number of methanogens present in ozone treated SSL fermenters is high in the range most commonly encountered in the digestion of other organic wastes. Reports by various authors show a range of 10^6-10^9 methanogens/ml in sewage, domestic refuse, and animal waste digestion (11, 22, 48). The higher population of methanogens observed in ozonated DSSL fermentation as compared to those reported in the literature for sewage sludge may be due to differences in counting media or real differences due to less competition from other anaerobes in the low carbohydrate DSSL substrate. Desulfovibrio populations in fermenting DSSL are also much higher than the 3 - 5 x 10^4 counts/ml commonly found in sewage digesters (12, 29). The high content of sulfur in DSSL likely accounts for these elevated Desulfovibrio populations.

An extremely wide variety of non-methanogenic anaerobic and facultatively aerobic bacteria have been identified and enumerated in numbers exceeding 10^7 organisms/ml during anaerobic digestion (22, 48). Toerien and Hatting (48) indicated 20 different genera have been isolated and pointed out that many of these organisms were probably simply "contaminating" organisms in a resting state introduced via the digester input. But, non-methanogenic populations are considered essential to the degradation of the complex organic matter into substrates that could be used by the methanogens (48). Methanogens can utilize only H_2, CO_2, methanol, formate, and acetate as their source of carbon and energy (15, 34). It is likely that these other bacteria are supplying a large portion of the substrates used by the methanogens (36).

Since methane bacteria have been shown to use acetate as a substrate for methane production, it is surprising to find substantial amounts (66 mM/l) present in ozonated DSSL fermenter effluent. This net production of 58 mM/l (3480 mg/l) acetic acid is much higher than values commonly reported by other investigators working with anaerobic digestion of organic wastes. Van Den Berg et al. (48) reported 200 mg/l acetic acid produced in the fermentation of pear wastes while Pfeffers (34) indicates a production of 880 mg/l of acetic acid from the digestion of domestic refuse. The work by Varel et al. (50) with thermophilic methane production from cattle wastes indicated a maximum acetic acid production of 2160 mg/l. It has been shown that acetic acid can be used as a carbon and energy source for the protein producing yeast Candida utilis (37, 38). This higher acetic acid content could lead to a substantial increase in the yeast protein yield obtained from DSSL by refermentation. Over 70% of the methane produced in sewage digestion has been shown to have been derived from acetate (20, 54). Zeikus et al. hypothesized that H_2 must be present before acetate can be fermented by methanogens (54). Acetate was not shown to serve as an energy source but instead acts as an electron acceptor and is reduced to CH_4 when electrons are supplied by H_2 oxidation. The following reaction has been put forth to account for acetate utilization by methanogens (41):

$$CH_3COO^- + H^+ + 4H_2 \rightarrow 2CH_4 + 2H_2O$$

Based on this reaction, 66 mM acetate remaining in ozone treated DSSL after fermentation could stoichiometrically be converted to 3.0 l of CH_4/l. This conversion would require 6.0 l of H_2. Thus, a lack of H_2 could be limiting CH_4 production from acetate in this system. Either not enough H_2 was produced

initially or significant amounts were used in reaction unrelated to CH_4 production.

According to Postgate and other investigators (7, 34, 36), Desulfovibrio reduced sulfates and sulfites to H_2S while oxidizing lactate, pyruvate, and malate to acetic acid, CO_2 and H_2. Thus, the large population of Desulfovibrio could be responsible for producing a portion of the acetate present, along with some H_2. Bryant et al. (7) have indicated that in the presence of sufficient sulfate, the use of H_2 in the production of H_2S by Desulfovibrio is favored over the production of CH_4 via methane bacteria. Thus, the high sulfur content of DSSL and the resultant Desulfovibrio development could limit methane production by tying up H_2. Our results have shown a loss of about 0.6 g/l of sulfur during fermentation of ozone treated DSSL. In converting this sulfur to H_2S, Desulfovibrio would require 0.42 l of H_2/l of ozonated DSSL. If this H_2 were available for CH_4 synthesis from acetate, only an additional 0.21 l of CH_4/l of ozone treated DSSL could be produced. Thus, sulfur reduction by Desulfovibrio appears not to be a major inhibitor to CH_4 production in this system. Possibly other non-methanogens may be responsible for an apparent lack of H_2. Studies have shown that various H_2-utilizing non-methanogenic bacteria may participate in interspecies H_2-transfer by acting as H_2 acceptors (18, 25).

Another possible reason for the limited CH_4 production could be the relatively high salt concentration of 13 g/l in 75% DSSL. Of this total, 7-10 g/l are calcium. This concentration is not intolerable to organisms such as Candida utilis which has been economically grown in 100% DSSL for years (9). Kirsh and Sykes (12) have shown that calcium concentrations in sewage digesters could be allowed to build up to 7.0 g/l without adverse

effects on CH_4 production. However, McCarty (28) concluded that a calcium level of 8 g/l produced a definite inhibitory effect on CH_4 production in sewage sludge.

These studies have shown that most of the CH_4 produced in the fermentation of ozone-treated DSSL originates from compounds produced during ozonation and is not a result of wood sugars and other low molecular weight compounds remaining after previous yeast fermentation. In contrast, unozonated DSSL did not supply necessary substrate for comparable CH_4 production. The rate of conversion of volatile solids in ozone-treated DSSL to CH_4 is much lower than in domestic and agricultural waste digestion. Although it appears that this process is not competitive at the present, with an increasing demand and cost for energy it could be a supplemental energy to the pulping process. Acetate produced during the fermentation of ozone-treated DSSL may be used as a substrate for further fermentation by the protein-producing yeast Candida utilis.

Manuscript Preparation

It was felt by our staff that much worthwhile information had been accumulated on the ozonation of DSSL and the resultant production of methane and yeast which would be of interest to other researchers working in this field. Consequently, considerable effort was expended during this past year on preparing manuscripts for presentation to professional society meetings and for publication. These papers deal mainly with the effects of ozone treatment on DSSL and the production of methane and yeast from ozonated DSSL as shown below:

(1) Protein Synthesis from Ozonated Pulp Mill Waste: Presented at the annual AIChE meeting, Philadelphia, Pennsylvania.

(2) Bioremoval of Lignosulphonates from Pulp Mill Effluents: Submitted to the journal Process Biochemistry.

(3) Methane Production from Ozonated Pulp Mill Effluent: To be submitted to the journal Applied and Environmental Microbiology.

(4) Methane Fermentation of Pulp Mill Wastes: To be presented at the ACS Microbial and Biochemical Technology Division Meeting, Miami Beach, Florida.

(5) Ozonation of Desugared Spent Sulfite Liquor: Manuscript in preparation.

LITERATURE CITED

1. Anaerobe Laboratory Manual. 1973. 2nd ed. The Virginia Polytechnic Institute and State University Anaerobe Laboratory. Southern Printing Co., Blacksburg, Virginia.

2. Audit, M. J. 1973. Desulfovibrio Fermentation to Reduce Pulp Mill Pollution. M. S. Thesis, Michigan Technological University.

3. Bannenk, H. F. and F. M. Muller. 1952. On the utilization of waste liquors from the digestion of straw with monosulfate. Anton van Leevenhoek. 18:45-54.

4. Benson, H. K. and A. M. Partansky. 1934. The rate and extent of anaerobic decomposition of sulfite waste liquor by bacteria of sea water mud. Proc. Nat. Acad. Sci. U. S. 20:542-551.

5. Bollyky, L. J. 1973. Ozone treatment of cyanamide and planting wastes. Proc. of First International Symposium on Ozone for Water and Wastewater Treatment.

6. Bremmon, C., M. Jurgensen, and J. T. Patton. Methane production from ozonated pulp mill effluent. Applied and Environmental Microbiology. (submitted for publication).

7. Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabil. 1977. Growth of Desulfovibrio in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162-1169.

8. Canty, C., F. G. Perry, and Woodland. 1973. Pollution vs. sulfite pulp mills. Pulp Paper, 47:53-54.

9. Cappenberg, T. E. 1975. A study of mixed continuous cultures of sulfate-reducing and methane-producing bacteria. Microb. Ecol. 2:60-72.

10. Dugar, S. K., J. T. Patton, and M. Jurgensen. 1978. Ozonation improves effluent and substrate properties of desugared spent sulfite liquor. (To be submitted for publication.)

11. Edwards, T., and B. C. McBride. 1975. New methods for the isolation and identification of methanogenic bacteria. Appl. Microbiol. 29:465-469.

12. Elliott, L. F., and T. M. McCalla. 1972. The composition of the soil atmosphere beneath a beef cattle feedlot and a cropped field. Proc. Soil Sci. Soc. Amer. 36:68-70.

13. Ferry, J. G., P. H. Smith, and R. S. Wolf. 1974. Methanospirillum, a new genus of methanogenic bacteria, and characterization of Methanospirillum hungatii sp. nov. Int. J. Syst. Bacteriol. 24:465-469.

14. Gehm, H. 1973. State-of-the-art review of pulp and paper waste treatment. EPA Tech. Ser. R2-72-184.

15. Hackett, W. F., W. J. Connors, T. K. Kirk, and J. G. Zeikus. 1977. Microbial decomposition of synthetic ^{14}C - labeled lignins in nature: Lignin biodegradation in a variety of natural materials. Appl. Environ. Microbiol. 33:43-51.

16. Hauser, K. H., and R. J. Zabransky. 1975. Modification of the gas-liquid chromatography procedure and evaluation of a new column packing material for the identification of anaerobic bacteria. J. Clin. Microbiol. 2:1-7.

17. Holderby, J. M, and W. A. Moggio. 1959. The production of nutritional yeast from spent sulfite liquor. For. Prod. J. 9:21-24.

18. Iannotti, E. L., D. Karkewitz, M. J. Wolin, and M. P. Bryant. 1973. Glucose fermentation products of Ruminococcus albus in continuous culture with Vibrio succinogens: Changes caused by interspecies transfer of H_2. J. Bacteriol. 114:1231-1240.

19. Inskeep, G. C., A. N. Wiley, J. M. Holderby, L. and L. P. Hughes. 1951. Indus. & Eng. Chem. 43:1702

20. Jeris, J. S., and P. L. McCarty. 1965. The biochemistry of methane fermentation using ^{14}C-tracers. J. Water Polut. Control Fed. 37:178-192.

21. Katuscak, S., A. Hrivik, and M. Mahdalik. 1972. Ozonation of lignin IV. The course of ozonation of insoluble lignin. Paperi ja Puu. 54(12):861.

22. Kirsch, E. J., and R. M. Sykes. 1971. Anaerobic digestion in biological waste treatment. Prog. Indus. Micro. 9:155-237.

23. Kispert, R. G., S. E. Sadek, and D. L. Wise. 1975. An economic analysis of fuel gas production from solid waste. Resour. Rec. Cons. 1:95-109.

24. Le Gall, J., and J. R. Postgate. 1973. The physiology of sulfate-reducing bacteria, p. 82-133. In A. H. Rose, and D. W. Tempst (eds.), Advances in microbial physiology. Vol 10. Academic Press, N.Y.

25. Mah, F. A., D. M. Ward, L. Baresi, and T. L. Glass. 1977. Biogenesis of methane. Ann. Rev. Microbiol. 31:309-341.

26. Marshall, H. B. and A. I. Cunningham. 1956. TAPPI 39(7):171.

27. Marshall, H. B. and M. A. Johnson. 1952. TAPPI 39(1):146.

28. McCarty, P. L. 1964. Anaerobic waste treatment fundamentals. Public works 95:91-94.

29. Mueller, J. C., and C. C. Walden. 1970. Microbiological utilization of sulfite liquor. British Columbia Res. Rep. 323.

30. Nebel, C., R. D. Gottschling, and H. J. O'Neill. 1974. Ozone: A new method to remove color in secondary effluents. Pulp and Paper 142.

31. Pandila, M. M. 1973. Microorganisms associated with microbiological degradation of lignosulfonates: A review of literature. Pulp Pap. Mag. Can. 74:80-84.

32. Patton, J. T., B. Delaney, and M. Jurgensen. 1978. Protein synthesis from ozonated pulp mill waste. Paper presented at ACS meeting in Philadelphia, Penn.

33. Perry, J. H. 1950. Chemical Engineers' Handbook. McGraw-Hill, N. Y.

34. Pfeffer, J. T. 1974. Temperature effects on anaerobic fermentation of domestic refuse. Biotech. Bioeng. 19:771-787.

35. Pine, M. J., and H. A. Barker. 1954. Studies on the methane bacteria II. Fixation of atomspheric nitrogen by methanobacterium omelianskii. J. Bacteriol. 68:589-591.

36. Postgate, J. R., and L. L. Campbell. 1966. Classification of Desulfovibrio species, the nonsporulating sulfate-reducing bacteria. Bacteriol. Rev. 30:732-738.

37. Prescott, S. C., and C. G. Dunn. 1959. Industrial Microbiology. McGraw-Hill, N. Y.

38. Rose, A. H., and J. S. Harrison. 1970. The Yeasts. Vol. 3, Academic Press, N. Y.

39. Salverson, J. R., and D. Hogan. 1948. Analysis of sulfite waste liquor and lignosulfonates. Analyt. Chem. 10:909-911.

40. Sanderson, L. D. 1974. The use of anaerobic digestion to reduce the pollution content of spent sulfite liquor. M. S. Thesis, Michigan Technological University.

41. Shapton, D. A., and R. G. Board, eds. 1971. Isolation of Anaerobes. Academic Press, N. Y.

42. Sarkonen, K. V., and C. H. Ludwig, eds. Lignins Occurence, Formation Structure and Reactions. 1st ed. John Wiley Interscience, p. 471.

43. Smith, P. H., F. M. Bordeaux, and P. J. Shuba. 1970. Methogenesis in sludge. Amer. Chem. Soc. Div. Water Air Waste Chem. Gen. Paper 10:105-110.

44. Smith, P. H., and R. E. Hungate, 1958. Isolation and characterization of Methanobacterium ruminantium N. sp. J. Bacteriol. 75:713-718.

45. Standard Methods for the Examination of Water and Waste Water. 1965. 12th ed. American Public Health Association, N. Y.

46. Stern, A. M., and L. L. Ganer. 1974. Degradation of lignin by combined chemical and biological treatment. Biotech. Bioeng. 16:789-805.

47. Tempe, W. G., E. Sang, and R. L. Miller. 1973. Kraft pulping effluent treatment and reuse-state of the art. EPA Tech. Ser. R2-73-164.

48. Toerien, D. F., and W. H. J. Hattingh. 1969. Anaerobic digestion. I. The microbiology of anaerobic digestion. Water Res. 3:385-416.

49. Van Den Berg, I., C. P. Lents, R. J. Athey, and E. A. Rook. 1974. Assesment of methanogenic activity in anaerobic digestion: Apparatus and method. Biotech. Bioeng. 16:1459-1469.

50. Varel, V. H., H. R. Isaacson, and M. P. Bryant. 1977. Thermophylic methane production from cattle waste. Appl. Environ. Microbiol. 33:298-307.

51. Wiley, A. J. 1955. The microbiology of spent sulfite liquor, p. 226-254. In Microbiology of Pulp and Paper. TAPPI Monog. Ser. 15.

52. Wolf, R. S. 1971. Microbial formation of methane. Adv. Microbiol. Physiol. 6:107-146.

53. Zeikus, J. G. 1977. The biology of methanogenic bacteria. Bacteriol. Rev. 41:514-541.

54. Zeikus, J. G., P. J. Weimer, D. R. Nelson, and L. Daniels. 1975. Bacteriol. methanogenses; Acetate as a methane percursor in pure culture. Arch. Microbiol. 104:129-134.

☆U.S. GOVERNMENT PRINTING OFFICE: 1979-640-092/415