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DEGRADATION OF CELLULOSIC BIOMASS AND ITS SUBSEQUENT UTILIZATION FOR THE PRODUCTION OF CHEMICAL FEEDSTOCKS

Progress Report

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DISCLAIMER

Scope of Program

This is a coordinated program to effect the microbiological degradation of cellulosic biomass and the subsequent utilization of the degradation products to produce chemical feedstocks. The microbiological aspects of cellulosic biomass degradation will focus on the use of anaerobic microorganisms which possess cellulolytic enzymes. These studies will attempt to increase the enzyme levels through genetics, mutation and strain selection. In addition, the direct use of these cellulolytic anaerobes to produce soluble products (sugars) which can then be utilized by other microorganisms to produce chemicals is also within the scope of this program. Engineering scale-up of these microbial processes is planned once the basic microbiological parameters are defined.

The second area of our major effort is devoted to the production of chemical feedstocks. In particular, three fermentations have been identified for exploration. These are: acrylic acid, acetone/butanol and acetic acid. The main efforts in these fermentations will address means for the reduction of the cost of manufacturing for these large volume chemicals.

Abstract

The degradation of cellulosic biomass continues to focus on the anaerobic thermophile Clostridium thermocellum. When grown on crystalline cellulose (MN300) in batch culture, there is an initial rapid accumulation of reducing sugars but the sugars are rapidly metabolized in later times during the fermentation. When grown on Solka floc with periodic addition of the substrate, there is a continual accumulation of reducing sugars (xylose, glucose and cellobiose) as well as ethanol and acetic acid during the entire course of the fermentation. In the presence of surfactant in the growth medium, there is an increase appearance of extracellular cellulases. A chemically defined medium is being developed for growth Cl. thermocellum in order to study the enzyme regulations. Lastly, a trinitrophenyl-carboxylmethyl cellulose substrate for determining cellulose activity appears to be a promising and rapid assay. Progress in the genetic manipulations has been cautious but promising. Preliminary evidence leads to optimistic projection on the presence of plasmids and bacteriophage in Cl. thermocellum. Mutants containing antibiotic resistant markers have been isolated which will be utilized for future investigations as to the feasibility of transformation systems in Cl. thermocellum.

The production of chemical feedstocks continues to focus on acrylic acid, acetone/butanol and acetic acid. Studies with cell free extracts of Clostridium propionicum have shown the production and accumulation of acrylic acid from lactic acid. The use of electron acceptor in cell-free systems has shown effective prevention on the reduction of acrylic acid to propironic acid. Medium development and strain selection using available acetone/butanol producing Cl. acetobutylicum have been initiated. There is every indication that these strains are capable to produce mixed solvents close to the theoretical maximum yield. An accurate and rapid method for quantifying acetic acid has been developed. This technique is being used to examine the pertinent parameters on the production of acetic acid by Clostridium thermoaceticum.

Contributors of Program

This program is under the coordination of Daniel I.C. Wang, Principal Investigator. The research and development within this program are directed by the following individuals.

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This progress report acknowledges the contribution by these individuals listed above.

TABLE OF CONTENTS

I.) Microbiology of Cellulose Degradation and Cellulase Production

A.) Enzyme Production by Clostridium Thermocellum

1.) Introduction:

In the area of cellulase production by Clostridium thermocellum efforts continue toward locating and defining the activities of the enzymes made by this organism. As reported previously, C. thermocellum produces an endoglucanase, which appears free in solution in fermentation broths and is also extractable from pellet fractions with detergent and which is active against carboxymethylcellulose. Batch fermentations have been run in which the production of the pellet associated endoglucanase has been followed by direct incubation of pellet fractions with CMC solutions normally used to assay for enzyme in soluble fractions. As pellet endoglucanase activity could be demonstrated in this manner (results herein), it was hypothesized that exoglucanase activity may be pellet associated (possibly cell bound) and that its activity may also be demonstrable by incubating pellet fractions with insoluble substrate and assaying for liberation of reducing sugars. The search for pellet (or cell) associated exoglucanase follows from the observation of rapid in vivo cellulose hydrolysis rates yet very low levels of freely soluble exoglucanase. Through demonstration of exoglucanase activity has to data been unsuccessful, we remain convinced, however, that due to the growth of the

organism on Solka floc, MN300 and Avicel celluloses the conditions can be achieved to locate and measure significant exoglucanase activity.

2.) Materials and Methods

Organism: Clostridium thermocellum obtained from the American Type Culture Collection

Growth of Organism: C. thermocellum is cultured in either anaerobic flasks or a 2 liter fermentor on the basic CM3 medium (described in our previous report 12/1/76-2/28/77) containing either MN300 cellulose or Solka floc as carbon source.

Pellet protein, residual cellulose and soluble CMCase activity are measured as described earlier.

Measurement of pellet-associated cellulase activities: Samples of fermentation broths were centrifuged (20,000xg for 15 minutes) and pellets were washed twice with distilled water. Supernatants were removed and the whole pellets were resuspended in 1 ml of sodium citrate buffer (pH 4.8) containing either carboxymethylcellulose (a 2% solution), filter paper (one 1 X 6 cm Whatman #1 filter paper strip) or MN300 powdered cellulose (50 mg). Incubation occurred at 60°C for one hour after which the samples were assayed for liberated reducing sugars using a DNS colorimeteric reagent.

3.) Results and Discussion

Figures I.A.1 and I.A.2 depict typical profiles of pellet protein, residual cellulose, pH, broth reducing

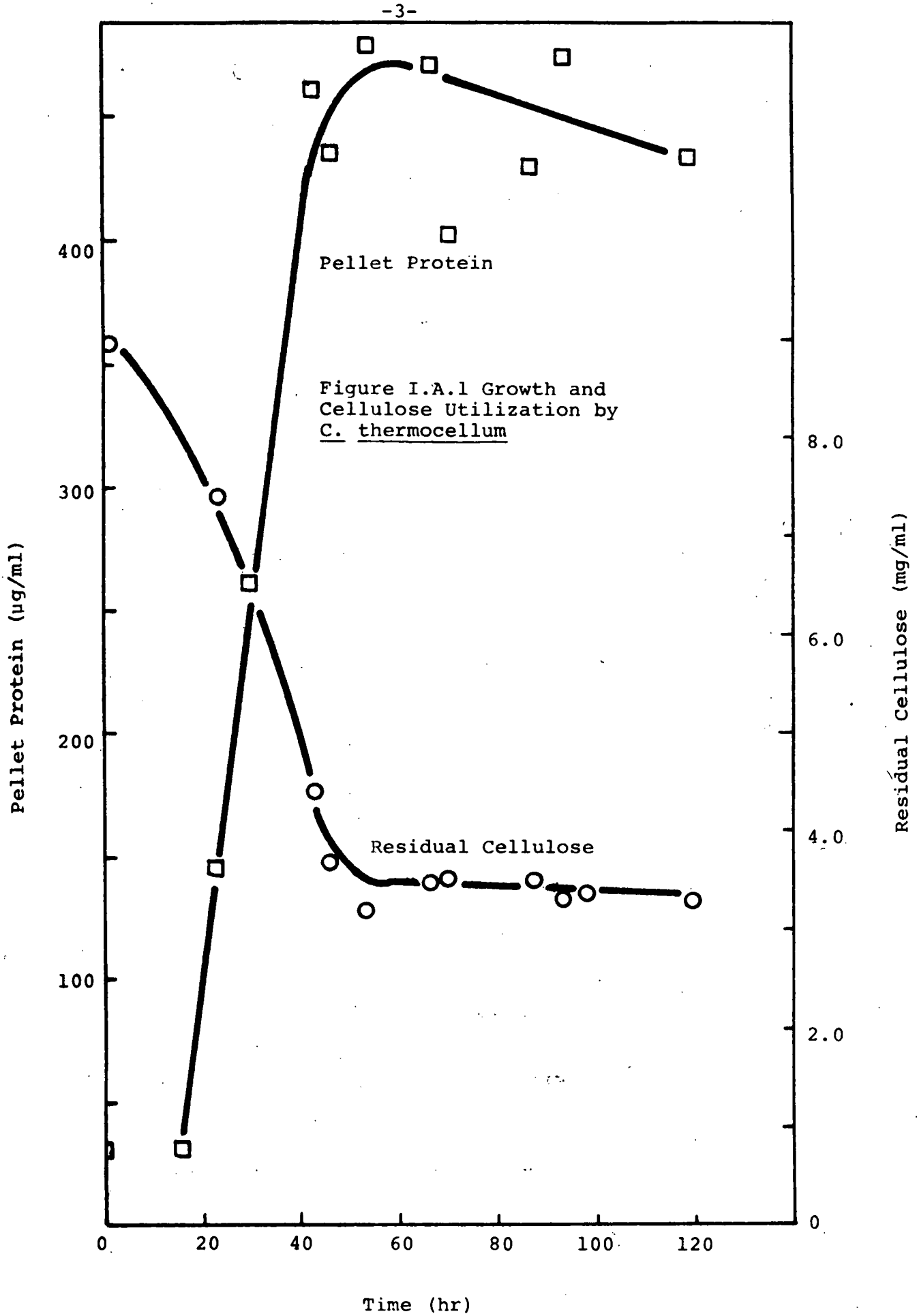


Figure I.A.1 Growth and Cellulose Utilization by C. thermocellum

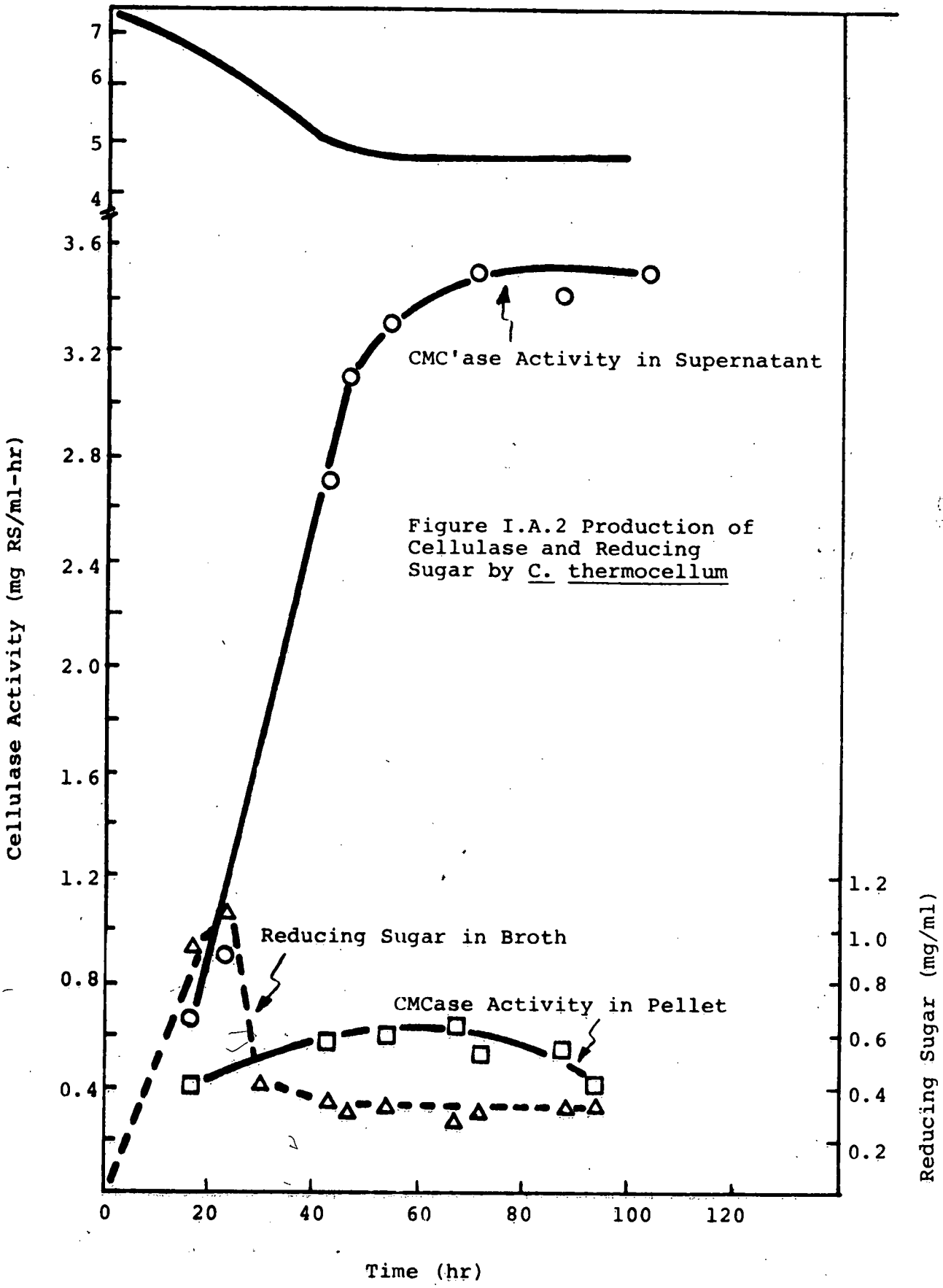


Figure I.A.2 Production of Cellulase and Reducing Sugar by C. thermocellum

sugars, supernatant CMC activities and pellet CMC'ase activities versus time for a batch fermentation run in a 2 liter fermentor (working volume, 1.5 liters) of C. thermocellum on CM3 medium with Solka floc substituted for MN300 cellulose. As is generally observed with our cultures (sparged with CO_2 and agitated at 100 rpm), broth reducing sugars accumulate for approximately 24 hours after which time they decrease sharply and remain at a low level (0.3 mg/nl) throughout the course of the fermentation.

Of the total endoglucanase (CMCase) activity present, most is free in solution at the end of the fermentation, with only ∿15% associated with the pellet fraction at maximal activity values. This amount of pellet activity is similar to the amount extractable with a citrate buffer wash but less than that extractable from pellets with Triton X100 (as reported earlier). Thus assaying for pellet activity in this manner may not account for all the measurable activity present.

When pellet fractions (whole pellets or cell pellets obtained by allowing residual cellulose to settle out) were incubated at 60°C in sodium citrate buffer (pH 4.8) with insoluble substrates, such as filter paper or MN300, no measurable exoglucanase activity was observed.

Since C. thermocellum can utilize insoluble celluloses as growth substrates, we assume that an exoglucanase of some type is produced by the organism. The assay conditions suitable for detecting endoglucanase activity may not be optimal for

the exoglucanase, especially if this latter component is not present in large quantities. There exists the possibility that the exoglucanase is not oxygen stable and this must be looked into. Alternatively, the lack of exoglucanase activity may merely be due to the low pH of the assay. Since the rate of cellulose degradation during the fermentation is highest when the pH is above that used in the assay, it follows that exoglucanase activity may become detectable when the reaction is buffered at an increased pH.

B.) Effect of Surfactants on the Growth and Cellulase Production of Clostridium thermocellum

1.) Introduction:

In our first progress report (12/1/76-2/28/77) we presented results which indicated that additional cellulase with CMCase activity could be solubilized from the pellet fraction (i.e., cells + residual cellulose) of C. thermocellum fermentation broths using a detergent, Triton X100. Based on this observation, as well as reports in the literature that cellulase production by several fungi is stimulated by surfactants in the growth medium, experiments were conducted in which C. thermocellum was cultured in the presence of Triton X100, Tween 20 and Tween 80 to determine their effect on growth and soluble cellulase production.

2.) Materials and Methods:

C. thermocellum obtained from the American Type Culture Collection was cultured in Hungate anaerobic tubes (10 ml volume) on CM3 medium containing Triton X100, Tween 20 (polyoxyethylene monolaurate) or Tween 80 (polyoxethylene monooleate) at concentrations of 0.05, 0.10, 0.15 and 0.20 per cent. Control tubes containing no surfactants were run as well. The cultures were harvested after 50 hours at 60°C at which time the following assays were performed:

a. Pellet protein was determined as described in our earlier report as a measure of cell growth.

b. The CMC assay was performed on supernatant fractions as described earlier.

c. Pellet CMCase activity was measured according to the following procedure: 2 ml of broth is centrifuged at 20,000xg for 15 minutes. Supernatant is removed and the pellet is washed twice in distilled water. One milliliter of the CMC solution (2% in sodium citrate buffer pH 4.8) is added to the pellet and incubated at 60°C for one hour. Liberated reducing sugars are measured with the DNS reagent as previously described.

3. Results and Discussion

The data for cultures containing Tween 20 and Tween 80 are summarized in Tables I.B.1 and I.B.2. No signs of growth were observed in tubes containing Triton X100. Supernatant CMCase activities were measured but due to the low values obtained, these data are omitted.

The values for pelletprotein (Table I.B.2) and CMCase activity (Table I.B.1) indicate that the surfactants inhibited growth and decreased enzyme levels when compared with the control cultures. Tween 80 appears to be less of a growth inhibitor than the Tween 20 or Triton X100.

Despite the negative effects on growth and CMCase levels, if one observes the ratio of supernatant activity to total activity, it is apparent that a greater proportion of CMCase activity is in solution in the surfactant cultures than in the controls, particularly in the case of Tween 80. This suggests that the surfactants do indeed effect solubilization of otherwise cell-associated (or solids-associated) activity.

TABLE I.B.1

Effect of Surfactant Addition on Production and Location of Cellulase by C. thermocellum

Surfactant	Concentration % (V/V)	Supn't Activity mgRS/ml-hr	Total Activity (pellet & support)	Sup activity / total activity
TWEEN 20	0.05	1.0	1.43	0.70
	0.10	0.85	1.23	0.70
	0.15	0.85	1.18	0.72
	0.20	0.80	1.04	0.77
TWEEN 80	0.05	1.5	1.92	0.78
	0.10	1.5	1.92	0.78
	0.15	1.3	1.63	0.80
	0.20	1.25	1.55	0.81
NONE (control)	0.00	1.8	2.55	0.71
	0.00	1.5	2.25	0.67

TABLE I.B.2

Effect of Surfactant Addition on Cellulase Production and Activity by *C. thermocellum*

Surfactant	Concentration % (V/V)	Pellet activity (mgRS/ml-hr)	Pellet prot (µg/ml)	Pellet act / pellet prot (µg/ml-hr/µg/ml)	Estimated DCW ** (mg/ml)	Total/DCW (mgRS/ml-hr/mg/ml)
TWEEN 20	0.05	0.43	80	5.4	0.2	7.15
	0.10	0.38	57	6.7	0.14	8.8
	0.15	0.33	40	8.3	0.10	11.8
	0.20	0.24	*			
TWEEN 80	0.05	0.42	120	3.5	0.3	6.4
	0.10	0.38	97	3.9	0.24	6.4
	0.15	0.33	95	3.5	0.24	6.8
	0.20	0.3	97	3.1	0.24	6.5
None 1		0.75	260	2.9	0.65	3.9
2		0.75	235	3.2	0.58	3.9

* not measured

** Estimated Dry Cell Weight is based on a bacterial composition of @ 40% protein

Also, the specific activities (units/mg pellet protein) and the specific total activities (units/mg dry cell weight) are higher for the surfactant cultures.

As a follow-up to this experiment, a similar experiment will be performed with lower surfactant concentrations to determine whether a level is achievable which allows growth and increased solubilization of cellulase enzymes. Another experiment in which surfactants are added to the fermentation at times after inoculation is also planned, again aimed at enhanced solubilization of cellulase.

C.) Degradation of Cellulosic Biomass to Produce Soluble Products

1.) As reported in the previous progress report (12/1/76-2/28/77) Clostridium thermocellum when grown in batch culture at 60°C on Solka floc, substantial amounts of soluble reducing sugars were detected. This appears to be a reasonable approach in the production of soluble products from cellulose which in turn can be directly used for chemical production through fermentation. In order to assess this potential in more detail, experiments have been performed using a batch fermentation with Clostridium thermocellum where the cellulose (Solka floc) was fed in a batch-wise fashion. Samples from this fermentation were then carefully analyzed to obtain a more complete picture of the products which were formed from the cellulose.

2.) Materials and Methods

The organism used in these studies was Clostridium thermocellum grown in a 1-liter Erlenmyer flask at 60°C. The medium used for this fermentation was identical to that reported in the previous progress report. The major difference being that cellulose was added periodically to the fermentation.

Various assays were performed during the course of the fermentation. Cell dry weight was determined using the optical method previously reported. The soluble reducing sugars were quantified in the following manner. A 0.2 ml sample is first filtered through a Millipore filter (0.45 μm). The filtrate is then added to 0.8 ml of citrate buffer (0.05 M, pH 4.8). To this mixture 3 ml of the dinitrosalicylic acid

(DNS) reagent was then added. The DNS reagent is composed of:

3.5 Dinitrosolicylic acid	= 1%
phenol	= 0.2%
NaOH	= 1%
Rochelle Salt	= 20%
Sodium Sulfite	=0.05%

This sample was then heated in boiling water bath for five minutes. The samples were then analyzed spectrophotometerically at 550 nm using glucose as the standard solution.

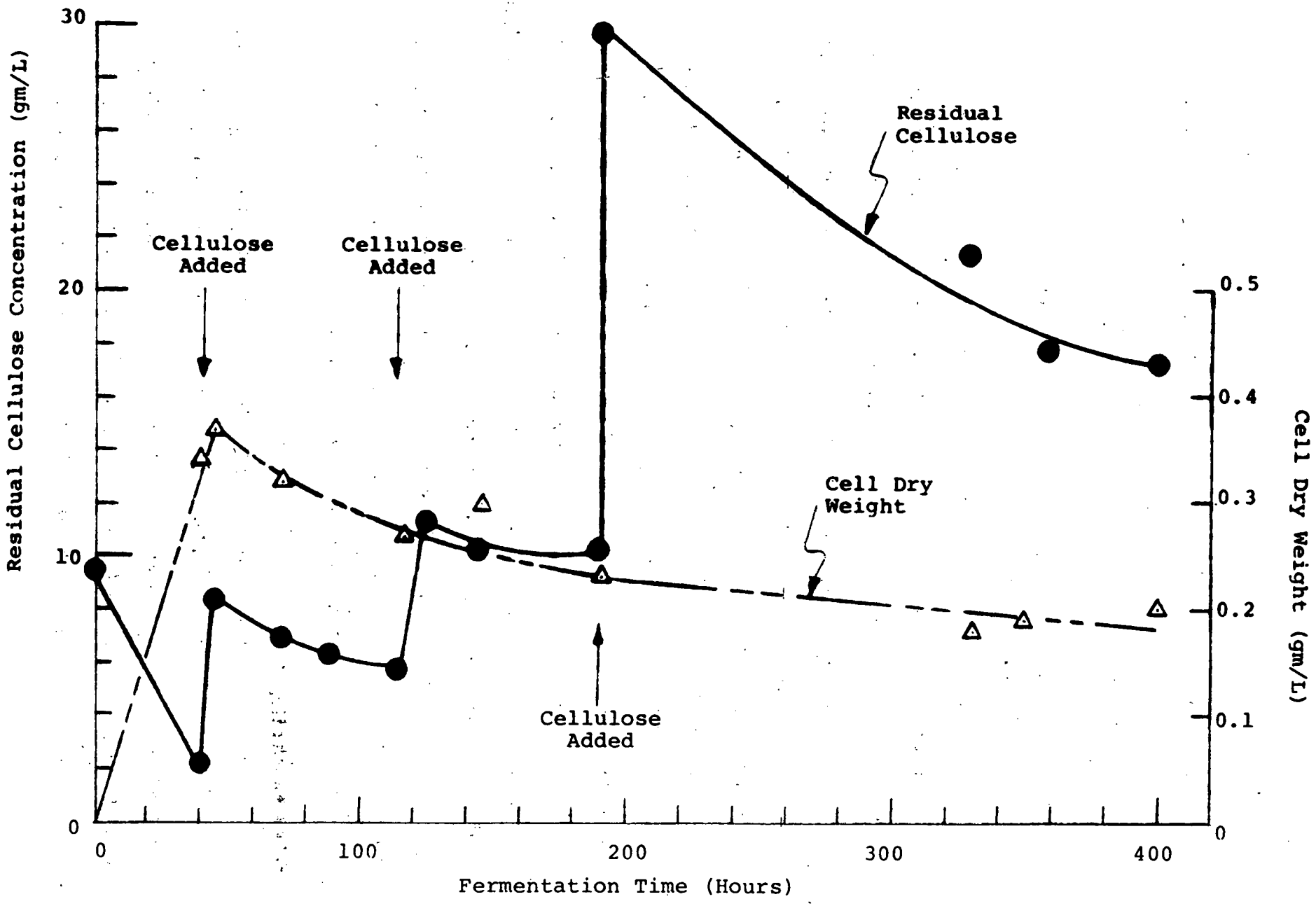
The fermentation samples were also analyzed for volatile products (ethanol and acetic acid) by gas chromatography. The column was packed with Chromosorb 101, 80-100 mesh, having a diameter of 1/8 inch and 5 feet long of stainless steel tubing. The injection temperature was 220°C and the detector temperature was 300°C. The column is first maintained at 110°C for four minutes followed by a temperature program at 20°C/min. up to 160°C. Carrier gas (nitrogen) was maintained at 20 ml/min. and methanol was used as an internal standard.

Qualitative identification of the reducing sugars was performed using silica gel plates. The solvents employed were described in the previous progress report.

3.) Results and Discussion

A batch fermentation using Clostridium thermocellum grown on Solka floc was performed over a period of 400 hours. Shown in Figure I.C.1 are the time profile of the residual cellulose and dry cell weight of this fermentation. Fresh cellulose was added at the 60th, 120th and 200th hour of fermentation as

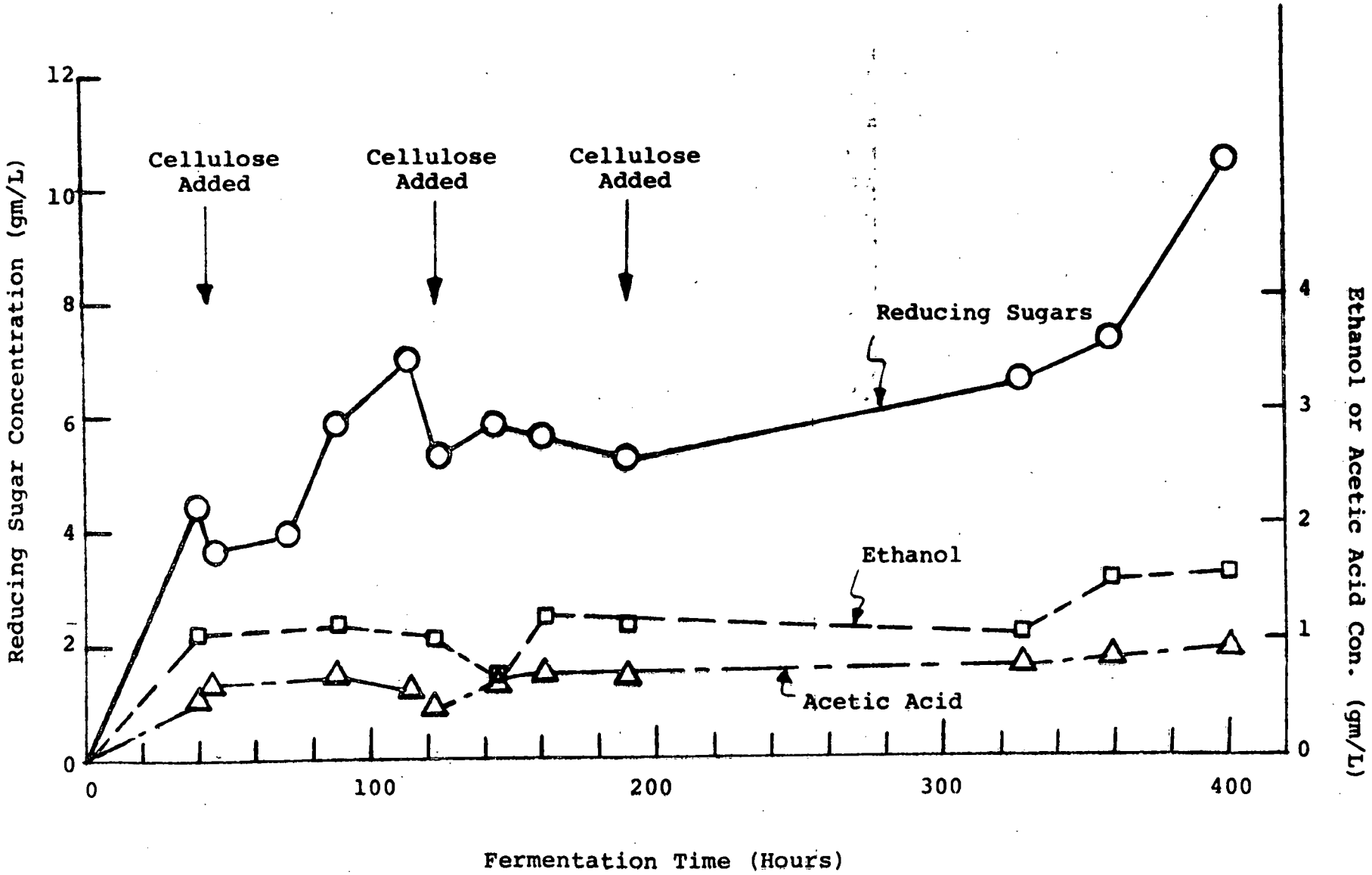
FIGURE I.C.1: Batch Fermentation of C. thermocellum with Cellulose (Solka floc) addition: Residual Cellulose and Cell Dry Weight



shown in this figure. The rate of cellulose degradation was quite rapid during the first 60 hours. During this period, cell biomass accumulates to about 0.3 gm/L. However, following the first and second additions at the 60th and 120th hour of fermentation, the rates of cellulose degradation was not as great as compared with the first 60 hours of fermentation. When the cell dry weight is examined, there is an apparent decrease following the cellulose additions. On the other hand, after the third addition of cellulose at 200th hour, the rate of cellulose degradation appears to have increased. Here again the cell dry weight indicated a slight decline.

When the products from this fermentation were analyzed, the results obtained are shown in Figure I.C.2. It is of interest to note that in addition to reducing sugars, ethanol and acetic acid both accumulate in the broth. Although the cell dry weight (Figure I.C.1) showed a decline between 60 and 120 hours, the reducing sugars showed continued increases in this same period. One should mention that the decrease in reducing sugar concentrations immediately following cellulose addition is a result of dilution of the broth. The reducing sugars continue to increase throughout the entire course of the fermentation up to the 400th hour and attain a final value of 11.8 gm/L. The results from this figure indicate that volatile products, ethanol and acetic acid, accumulate during the first 60 hours and attain their respective maximum values of about 1.5 and 1.0 gm/L respectively.

FIGURE I.C.2: Product Concentration of Batch C. thermoaceticum Fermentation with Cellulose Addition



In view of the cell dry weight relationship previously shown, there are two possible explanations on the production of soluble reducing sugars. The first of these leads one to speculate that non-growing or resting cells are capable of degrading cellulose to produce reducing sugars. This is an encouraging fact since it offers the possibility of a total cell recycle system to produce soluble products. The second explanation which could be offered and is probably more plausible being cell growth and cell lysis are occurring simultaneously. This is probably more plausible in view of the results from the previous progress report which tend to indicate that the production of soluble reducing sugars is associated with cell growth. We hope to delineate this behavior in more definitive fashion in the immediate future.

The hypothesis that cell growth and lysis is probably more reasonable can best be substantiated by examing the overall results from this fermentation shown in Table I.C.1. A total of 41.9 gm/L of cellulose was added to this fermentation of which 24.7 gm/L was degraded. However, from this amount of cellulose degraded, only 14.5 gm/L of products were recovered. Since the cell yield of C. thermocellum on cellobiose has been found to be about 0.17 gm cell/gm cellobiose, the amount of cell mass (0.2 gm/L) found at the end of the fermentation can only account for 1.2 gm/L of cellulose. This calculation shows that there is nearly 10 gm/L of cellulose unaccounted for. It is our opinion that this unaccounted for

cellulose probably was used to produce cell mass which in turn autolysed during the course of the fermentation.

As mentioned in the last progress report the reducing sugars from these fermentations are mostly glucose, xylose and cellobiose.

Table I.C.1: Product Formation by Clostridium thermocellum with Fed-Batch Fermentation of Solka Floc (T = 60°C: Fermentation cycle = 400 hours)

CELLULOSE

Cellulose Added (gm/L)	Cellulose Remaining (gm/L)	Cellulose Degraded (gm/L)
41.9	17.2	24.7

PRODUCTS FORMED

Cell Dry Weight (gm/L)	Reducing Sugar (gm/L)	Ethanol (gm/L)	Acetic Acid (gm/L)	Total Product Recovered (gm/L)
0.2	11.8	1.5	1.0	14.5

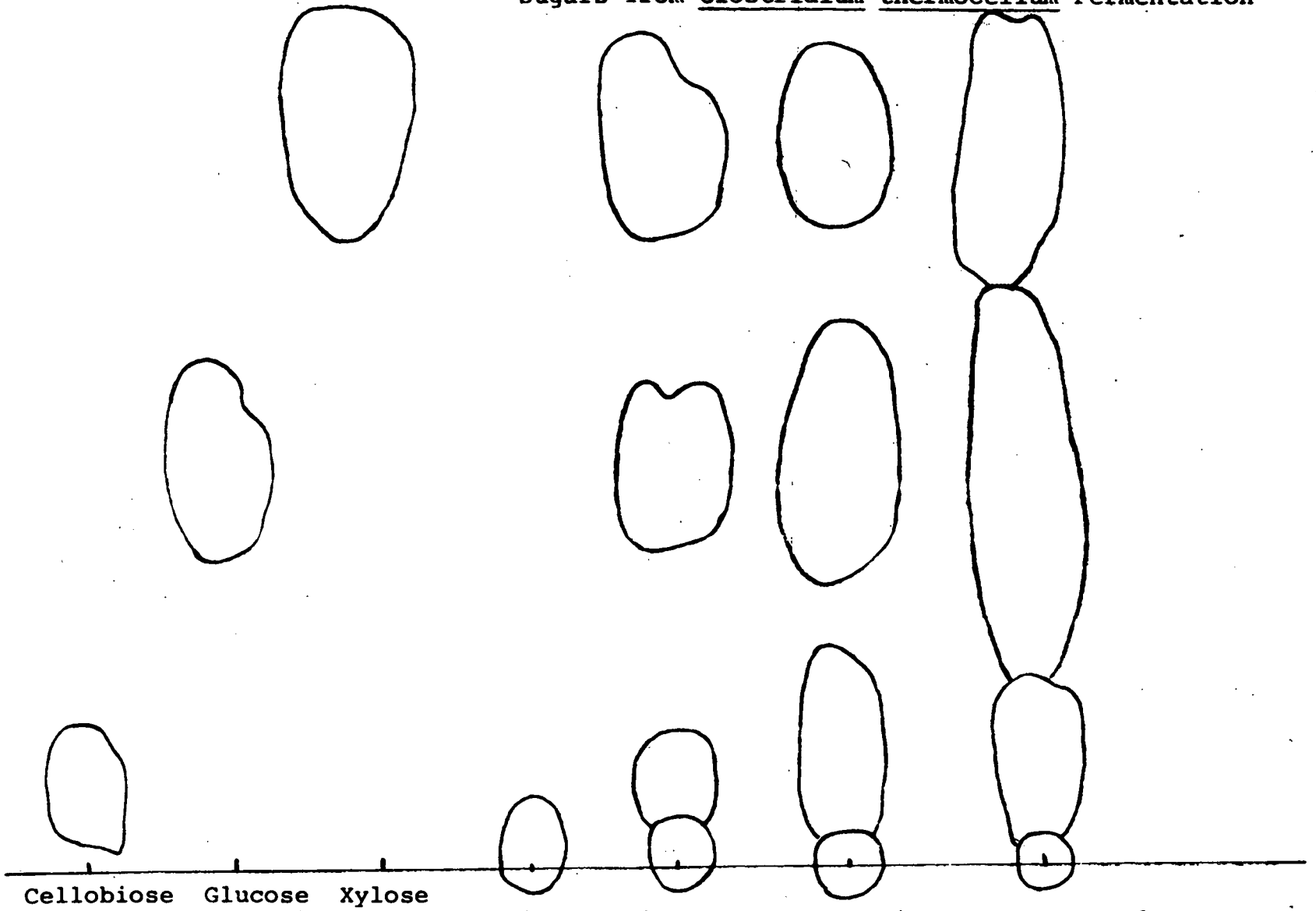
A typical silica gel chromatograph of the soluble sugars from these fermentations is shown in Figure II.C.3.

4.) Future Studies

The future studies will focus on:

- Kinetics of growth and cellulose degradation.
- Increase cell concentration to increase rate of product formation.
- Examination of real cellulosic wastes or residue: e.g. corn residue.

FIGURE I.C.3: Silica Gel Chromatograph of Reducing Sugars from Clostridium thermocellum Fermentation



Mutation and selection to increase rate of product formation.

D. Genetic Manipulations

1. Plasmid Profiles

A series of experiments are being conducted to determine if plasmids are present in C. thermocellum. C. thermocellum is grown in the following medium at 60°C for 48 hrs.

Yeast Extract (Difco)	5.6 mg/ml
Yeast Nitrogen Base without Amino Acids (Difco)	4.2 mg/ml
KH_2PO_4	1.5 mg/ml
K_2HPO_4	2.9 mg/ml
Na-thioglycollate	0.5 mg/ml
Resazurin	0.001%
Solka Floc	1%

Prior to inoculation H^3-adenine (New England Nuclear; 23 Ci/mmole) was added to a final concentration of 50 µCi/ml. At this time total acid precipitable radioactivity was measured to determine extent of isotope uptake. Representative counts are approximately 80,000 cpm/0.1 ml of culture. The cells were harvested by centrifugation, washed once in TES buffer (0.03 µ Tris-HCl, 0.005 µ EDTA and 0.05 µ NaCl) and resuspended in a 15% sucrose solution in TES buffer. The suspension was divided in three 3.25 ml aliquots. One received 100 µl of 3.0 mg/ml lysozyme solution, made up in TES buffer. One received 100 µl of 9.1% w/w Sarkosyl NL 97 made up in water. The third also received 100 µl of the

Sarkosyl solution and was then heated at 90°C for 10 min. The first two solutions were incubated at room temperature for 1 hr. The purpose of the heating at 90°C is to denature interfacing deoxyribonucleases. At this point the tube containing lysozyme received 100 µl of Sarkosyl solution and 700 µl of water, the other two tubes received 800 µl of water. Thus, three lysates were prepared: a. Lysozyme-Sarkosyl, b. Sarkosyl alone, c. Heat-Sarkosyl. The contents of each solution were ejected 10 times through a syringe fitted with a 25 G x 5/8" needle, each stroke taking from one to two seconds. This was done in order to achieve shearings of large molecular weight DNA.

Each lysis mixture (1.018 ml) was placed in a cellulose nitrate tube (5 ml). To each tube the following were added: 0.162 ml water, 1.8 ml of 1.8946 g/cm^3 CsCl solution and 20 µl of ethidium bromide (14 g/l). After mixing the tubes were layered with 2 ml of Nujol. The tubes were centrifuged in a SW 50.1 Beckman rotor at 43,000 rpm for 24 hr at 20°C. The gradients were punctured on the bottom and 2 drop fractions were collected on glass fiber filters. The filters were dried and counted for radioactivity in scintillation fluor (0.4% PPO and 0.05% dimethly-POPOP).

The results are shown in Fig I.D.1, I.D.2, and I.D. 3. Fig. I.D. 1 and I.D. 2 show no signs of satellite bands of covalently closed circular (CCC) DNA. However, in Fig. I.D.3 there is an indication that CCC DNA may be there (indicated by vertical arrows in the figure). The presence of plasmids

Fig. II.D.1 Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. Cells were lysed with sarkosyl.

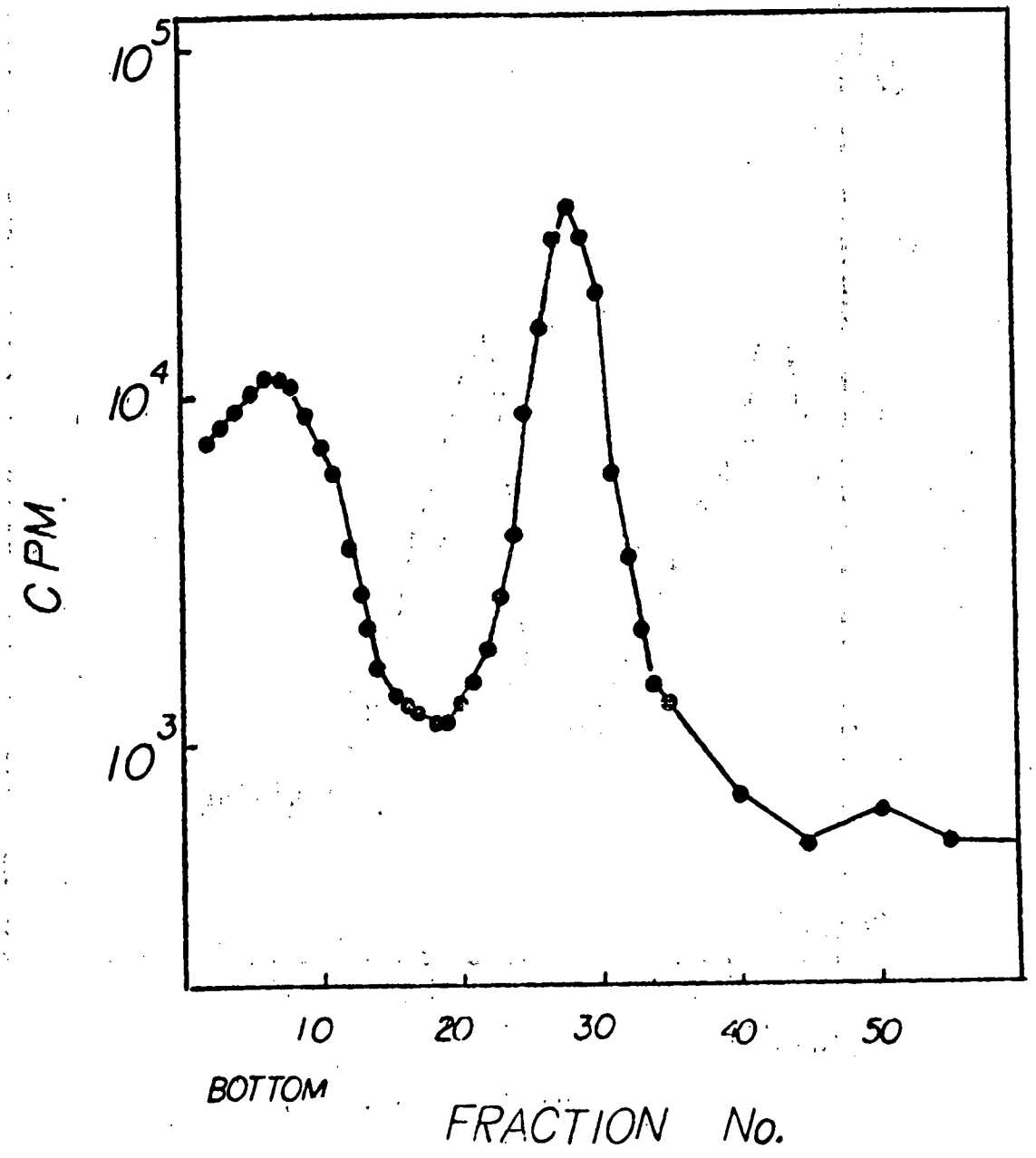


Fig.II.D.2 Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. Cells were lysed with lysozyme and Sarkosyl.

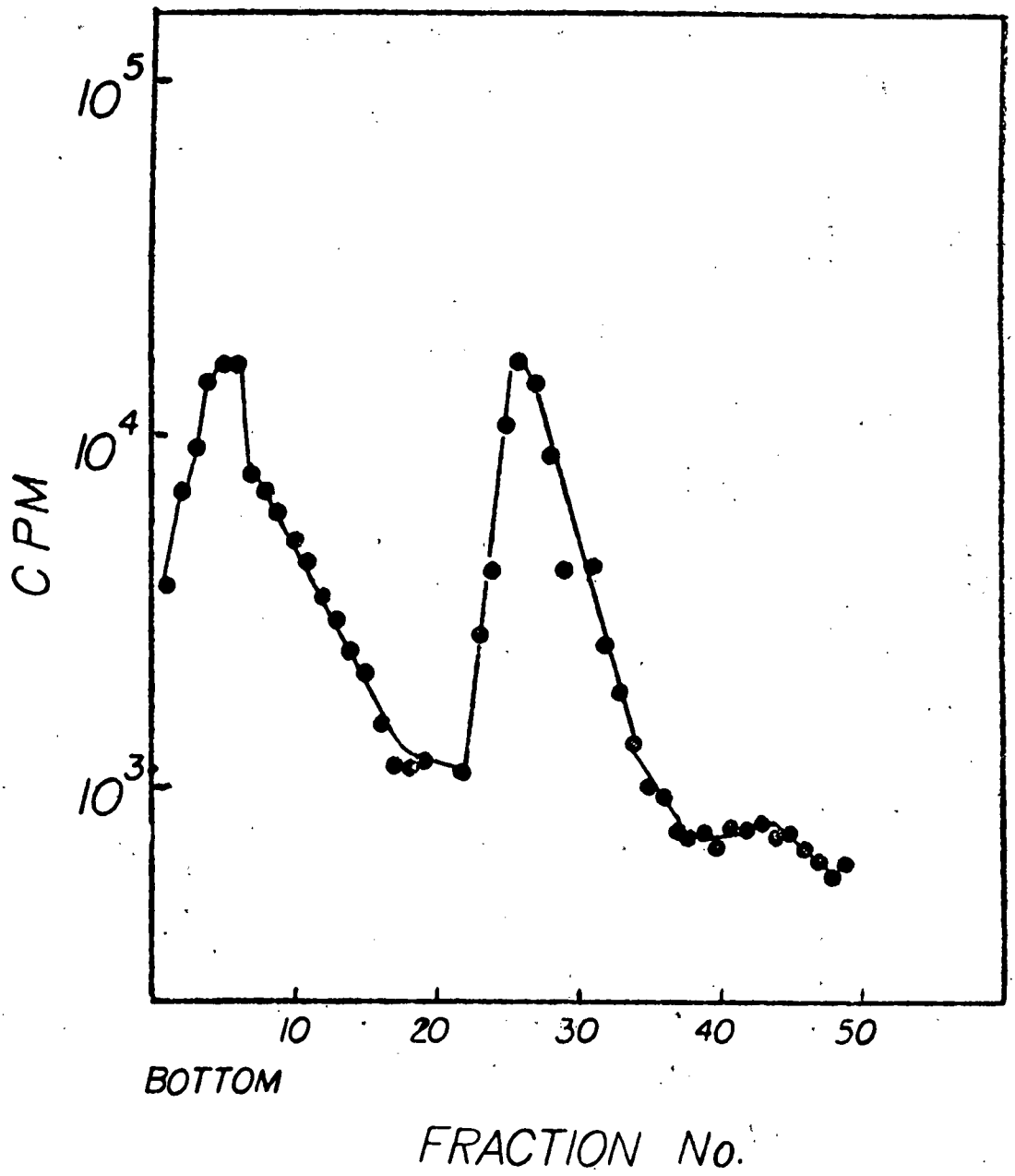
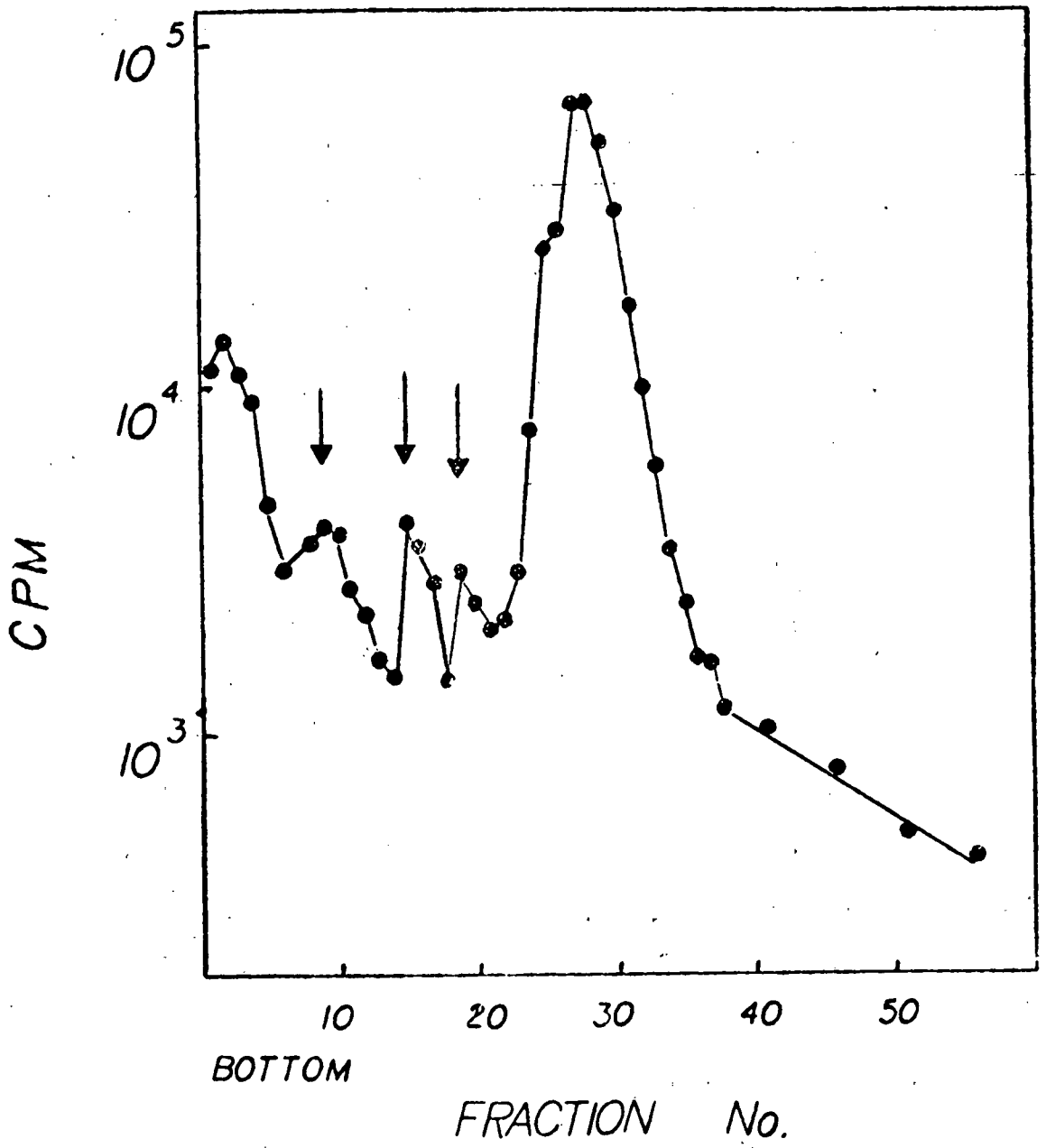


Fig.II.D.3 Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. Cells were lysed in Sarkosyl followed by exposure to 90°C for 10 min.



in the latter case may suggest that some degradation may be taking place in the unheated lysates, possibly nicking any plasmid that maybe present. The function of the heat treatment, thus, may be the inactivation of deoxyribonucleases. However, it should be noted that "suspected" plasmids in Fig. I.D. 3 may in fact represent single-stranded ends from pieces of partially denatured chromosomal DNA. In future experiments the nature of these peaks will be determined.

2. Cesium chloride Gradient Analyses

In order to determine some of the physical characteristics of C. thermocellum DNA as well as to confirm the labelling of DNA with ^{3}H-adenine, equilibrium density analyses were performed.

Cultures were grown as described in the previous section. The cells were resuspended in a solution composed of: 0.05 M Tris-HCl, 0.05 M EDTA and 0.5 mg of lysozyme per ml at pH = 8.0. The suspension was incubated at 37°C for 30 min. At this time 8.8 mg of Sarkosyl NL 97 per ml were added and the mixture further incubated at 60°C for 45 min. Finally, 28 mg of sodium lauryl sulfate per ml were added and the suspension incubated overnight at 4°C.

The lysis mixture was dialyzed against 1000 volumes of 50 mM Tris-buffer, pH 8.0 for 18 hrs at room temperature. After dialysis, lysate aliquots were placed in a mixture of CsCl ($p = 1.6977\ g/Cm^3$) and 0.54 mg Sarkosyl per ml in 5 ml cellulose nitrate tubes. Centrifugation was done at 25°C for

48 hrs at 30,000 RPM in a Beckman SA50.1 rotor. Fractions (4 drops each) were collected on 21 mm filter discs. The discs were dried, washed twice in cold 5% trichloracetic acid and once in cold acetone. After redrying, the discs were assayed for radioactivity in scintillation fluor (see previous section). Results indicated that C. thermocellum DNA has a density of 1.696 corresponding to a GC content of 37% [Schildkrant et. al. 1962, Journal of Molec. Biol. 4: 430-443; (density = 0.098) (GC mole fraction) + 1.660 = g/cm^3]. This value is close to those reported for C. thermosaccharolyticum (% GC = 36). These experiments also confirmed our ability to label C. thermocellum DNA with ^{3}H-adenine.

3. Isolation of Mutants

The following procedure was used to isolate antibiotic resistant mutants and exemplifies our mutagenesis procedure. After an unsuccessful attempt to isolate spontaneous antibiotic resistant mutants, a gamma irradiation procedure of mutagenesis was used. Prewashed CM-4 medium (see 1st Quarterly Progress Report, December 1, 1976 - March 28, 1977), with cellobiose as the carbon source, is inoculated, at a 1% level with a 48 hr culture from the same medium. When absorbance of 660 nm reaches 0.17, the culture is iced. It is then irradiated in a ^{60}Co source with 85 Krads. Survival after this dosage is approximately 0.1%. After irradiation the culture is allowed to resume growth at 60°C until absorbance reaches 0.3. At this time CM-4 (with cellobiose) agar plates containing the antibiotics are seeded with 0.1 ml of the

culture and incubated anaerobically at 60°C for 48 hrs.

Using this procedure we have been able to obtain mutants resistant to streptomycin, rifampicin and penicillin and one mutant dependent on streptomycin.

Work is now underway to examine other mutagenic agents such as U.V. light and ethylmethane sulfanate. In addition, we are also trying to isolate auyotrophic and "cellulase" deficient mutants.

The antibiotic resistant mutants are being utilized to attempt to establish a transformation system.

E. Deregulation of clostridial cellulase

1. Fermentation results

We have obtained growth, carboxymethylcellulase (CMCase) activity and trinitrophenylcellulase (TNP-Case) activity in both complex and chemically-defined media. The enzyme activities have been found in the culture supernatant and have amounted to 0.11-0.17 units/ml CMCase (u moles reducing sugar/min) and 0.09-0.13 OD units/ml TNP-Case. Attempts are now underway to determine which of the amino acids and vitamins are necessary for growth and enzyme production.

The above CMCase units are minimum figures since recent studies indicate that the liberation of reducing sugars is not linear with time and we have been running the assay for the extended period of 1 hr. It is thus possible that considerably higher levels of cellulase are actually present.

2. Yellow pigment produced by growth on cellulose

Some information has been obtained on the yellow pigment which is produced by Clostridium thermocellum when growing on cellulose but not on sugars. The pigment has been known for over 25 years but no characterization has ever been carried out on it. It binds tightly to the cellulose fibers during the fermentation. We grew C. thermocellum in complex medium 1 (See Progress Report 12-1-76 to 2-28-77) until extracellular CMCase (0.14u/ml) and TNP-Case (0.09 OD u/ml) production was about complete. After removal of the broth supernatant by centrifugation, the cells-plus-cellulose-pellet was washed with buffer (0.1N citrate buffer, pH 4.4). The washed pellet from 200ml fermentation broth was resuspended in 4ml buffer. Part of this suspension (0.5ml) was extracted with 1.5ml buffer containing 2% Triton X-100 for 10 min at room temperature. After Millipore filtration, the yellow filtrate was examined in the spectrophotometer. This revealed an absorption peak at 440nm amounting to 0.205. It will be of interest to determine whether the kinetics of pigment appearance follows those

of growth and/or cellulase production.

3. Studies on the hydrolysis of TNP-cellulose

The procedure of Huang and Tang (Anal. Biochem. 73, 369, 1976) described in the 12/1-76 - 2/28/77 progress report (pp. 42 - 44) by R. Gomez and B. Snedecor is a very useful way to assay the hydrolysis of amorphous cellulose. This assay measures the release of yellow trinitrophenyl-oligosaccharides by hydrolysis of trinitrophenyl-CMC. Further work was done on the utility of this assay using TNP-CMC supplied by R. Gomez and B. Snedecor.

a. Effect of pH on absorbance measurement.

After exposure of TNP-CMC to our crude extracellular enzyme, the reaction mixture was filtered and the pH adjusted by addition of Na_2CO_3. As shown below, the color development is not affected by pH.

pH	OD_{344}
4.7	0.48
4.9	0.49
6.0	0.49
6.9	0.49
8.5	0.48
9.3	0.46

b. Effect of enzyme concentration and reaction time on activity.

We conducted the enzyme reaction in the presence of 0.2 to 1ml of crude extracellular enzyme and measured absorbance at 30, 60 and 90 min. As shown in Fig.I.E.1, the enzyme reaction is directly proportional to time and enzyme concentration. The usual enzyme concentration used is 1ml per 3ml reaction mixture.

c. Effect of substrate concentration.

The usual substrate concentration in the assay is 20mg per 3ml reaction mixture. Reactions were conducted at 1.25, 2.5, 5, 10, 15 and 20mg TNP-CMC per 3ml. As shown in Fig.I.E. 2, the highest level did not saturate the enzyme

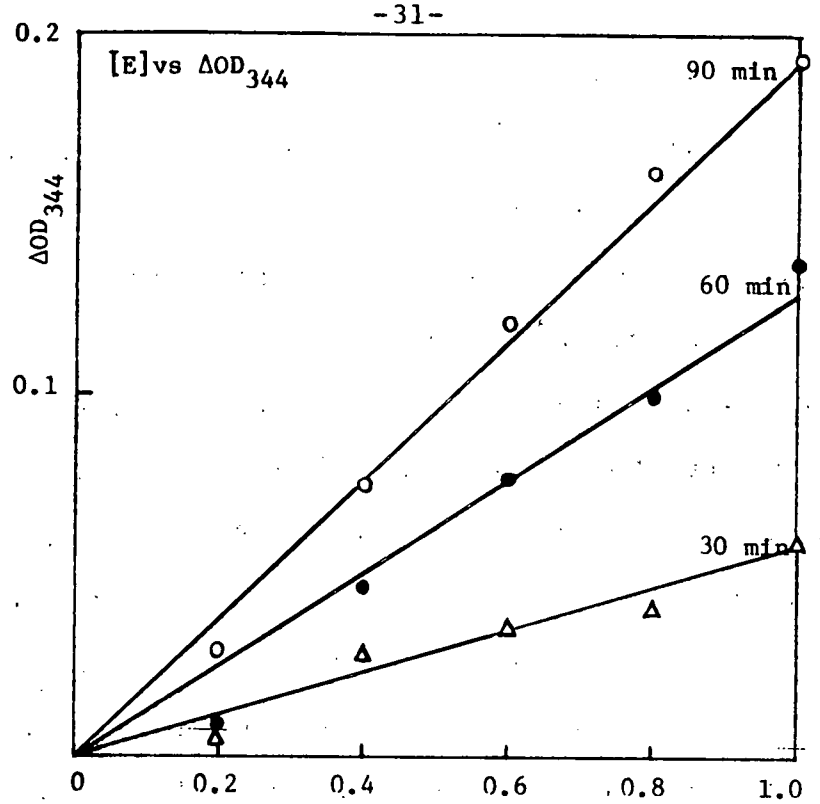


Fig.I.E.1. Effect of time and enzyme concentration on the TNP-Case reaction.

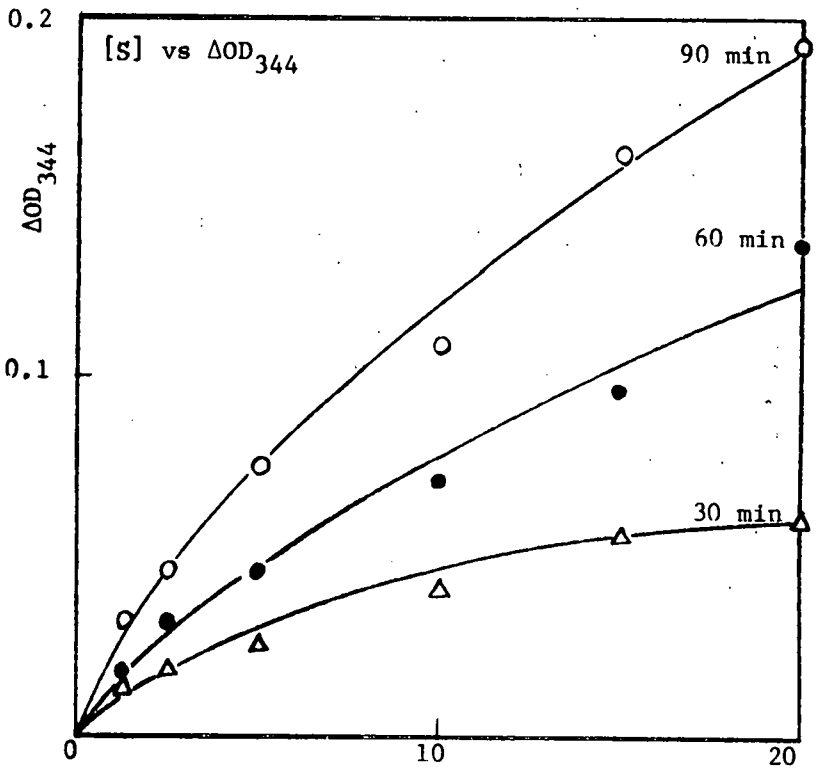


Fig.I.E.2. Effect of time and substrate concentration on the TNP-Case reaction.

indicating a high Km. The experiment was repeated using higher substrate concentrations giving the results in Fig.I.E.3 which show saturation near 40mg TNP-CMC per 3ml. A Lineweaver-Burk plot is shown in Fig.I.E.4 which is in agreement with the data of Gomez and Snedecor (Prog. Rept. 12/1/76 - 2/28/77, p. 46) i.e.,the reaction follows Michaelis-Menton kinetics. Our calculated value for Km is 3.3mg/ml which is quite high, indicating that the affinity of our extracellular enzyme to TNP-CMC is low, probably due to the large chromophore (trinitrophenol) added to the CMC. The degree of hydrolysis which we are measuring is estimated at about 1% in 120 minutes under standard conditions. This is based on the molar extinction coefficient of ε-trinitrophenyl-lysine and assuming 0.12 m moles of TNP/g TNP-CMC (Huang and Tang, 1976).

d. Effect of sugars on the reaction.

Glucose, cellobiose and xylose were added at 2mg and 20mg each per 3ml reaction mixture. As shown below, no feedback inhibition was observed. There was, in fact, a moderate stimulation of enzyme activity by the sugars.

	Relative Reaction Rate		
Additive	Expt. 1	Expt. 2	Expt. 3
None	1.00	1.00	1.00
Glucose 2mg	1.17	1.17	1.28
" 20mg	1.19	1.16	1.24
Xylose 2mg		1.16	
" 20mg		1.16	
Cellobiose 2mg		1.14	
" 20mg		1.12	

We also tested the cellulase bound to the cell-cellulose pellet for sugar inhibition after extraction of bound enzyme by the Triton X-100 treatment. No inhibition or stimulation by 20mg glucose/3ml was observed.

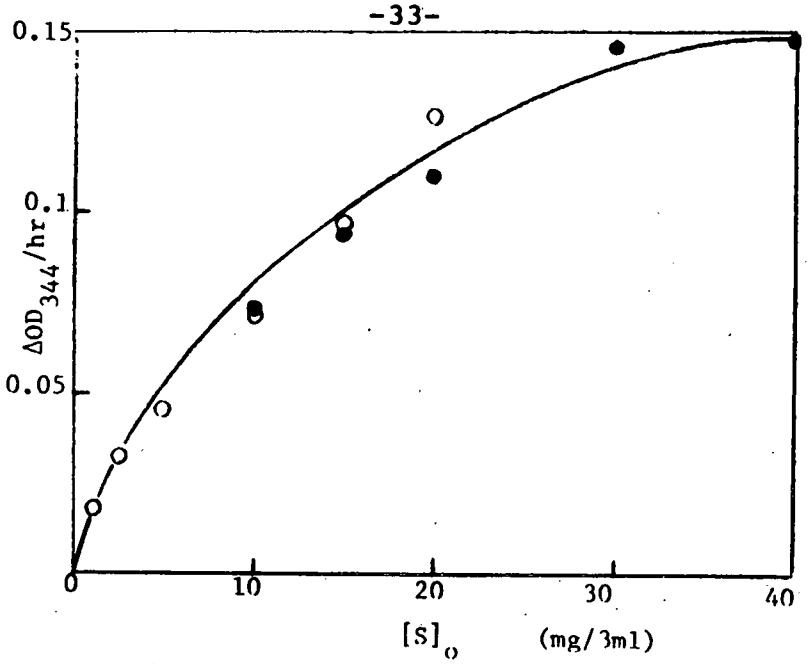


Fig.I.E.3. Initial substrate concentration vs. reaction rate.

o, Exp. Day 1 , ●, Exp. Day 5

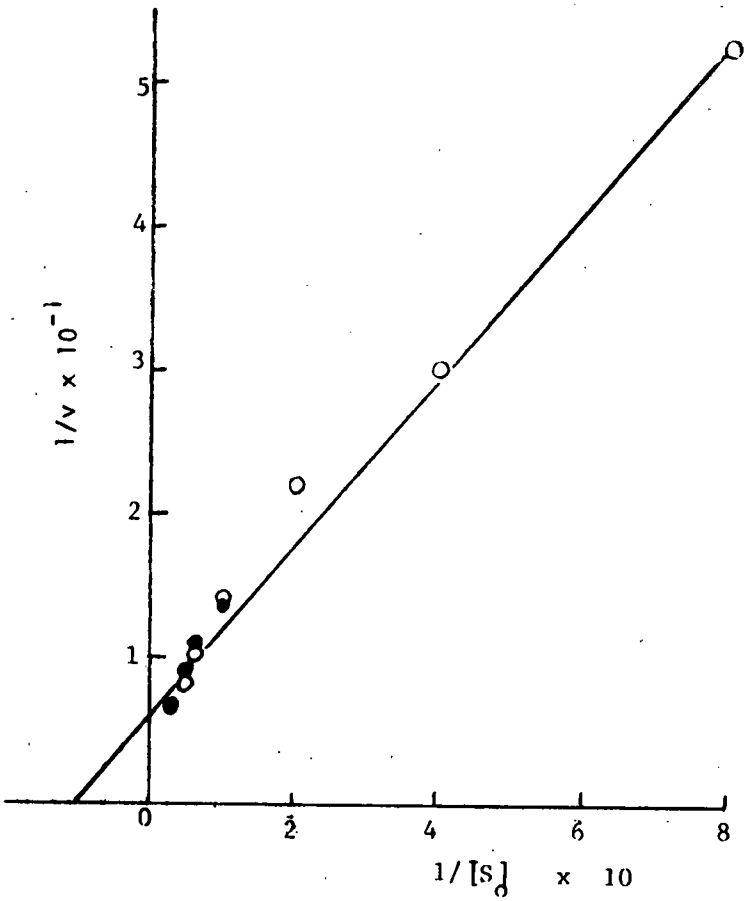


Fig.I.E.4. Lineweaver-Burk plot of cellulase reaction on TNP cellulose

S_o = mg/3ml, $v = \Delta OD_{344}/hr$

II. Production of Chemical Feedstocks

A. Production of acrylic acid by fermentation

1. Introduction

As summarized in the previous progress report, production of acrylic acid by fermentation from renewable resources is attractive because:

a. acrylic acid is a high volume chemical commodity the demand for which is predicted to approach a billion pounds per year.

b. the theoretical yield of 0.8gm acrylic/gm glucose and energy yield of 0.96 K cal recovered in acrylic acid per K cal in glucose are both high.

Consequently, fermentations where acrylic acid occurs as an intermediate are being investigated in this study. After a review of the literature the two anaerobic microorganisms chosen for study are Peptostreptococcus elsdenii and Clostridium propionicum. Background information on both of these microorganisms is to be found in the first progress report. This report summarizes a variety of studies that have been done with both microorganisms.

2. Materials and Methods

The growth media for both organisms and the anlaytical techniques for analysis of fermentation products is described in the first progress report.

Small scale fermentation studies were initiated on Peptostreptococcus elsdenii. For these studies a Bioflo fermentor (New Brunswick Scientific Co.) with a maximum working volume

of 375 ml was employed. The medium was the same used for other earlier experiments and it had initially a concentration of 10.3 gm lactate/liter as the only carbon source.

The inoculum was prepared by continuous reinoculation in tubes. 10ml of a heavy culture was transferred to the fermentor containing 350ml of medium. Nitrogen was flushed through the fermentor for the first hour and then the exit stream gas was connected to the carbon dioxide and hydrogen measuring system. Microbial growth for this type of experiment was followed by two procedures. One procedure measured the absorbance of the fermentation broth directly and in the other the fermentation broth was acidified to reduce the color indicators in the medium. The later procedure was found to be more reproducible and was used in subsequent studies.

Chemical Assays:

For these studies the Lowery procedure for protein determinations was also evaluated and found to be an acceptable technique for the measurement of protein in whole cells and in cell free extracts.

Cell free Extracts:

Cell free extracts for both organisms were prepared by the procedure of Ranhand, 1974, Applied Microbiol. 28, 66.

Inhibitors of reduction of acrylic to propionic acid:

A variety of chemicals were evaluated for their ability to prevent the reduction of acrylic acid to propionic acid by whole cells. Chemicals employed as electron acceptors were:

2,6 dichloroindophenol (Fisher). Final concentration (~0.4mM).

$K_3Fe(CN)_6$ (Final concentration 10mM)
Tetrazolium (Final concentration 10mM)
Methylene Blue (Final concentration 10mM)

Oxalic acid (final concentration 10mM) was employed as an inhibitor of lactic dehydrogenase.

3. Results:

Peptostreptococcus elsdenii fermentation kinetics in the small New Brunswick fermentor is summarized in Fig. II A 1. Also presented is the effect of adding an external electron acceptor 2,4 dichloroindophenol in the fermentor.

Immediately after the addition of 2,4 dichloroindophenol to the fermentation there was a decrease in the rate of propionate production. Bacterial growth was inhibited for approximately one half-hour. The results appear to confirm the fact that propionate production is used as one of the major pathways for maintaining the oxidation reduction balance during the fermentation. In this experiment, no acrylate was observed to accumulate during any phase of the fermentation.

Future studies with Peptostreptococcus elsdenii:

Future studies will concentrate on the development of a continuous culture system for the propagation of Peptostreptococcus elsdenii. The reasons are two fold. One is that the fermentation can be studied during continuous culture. The second is that cultivation of this organism via continuous culture will provide a source of cells with a defined physiological

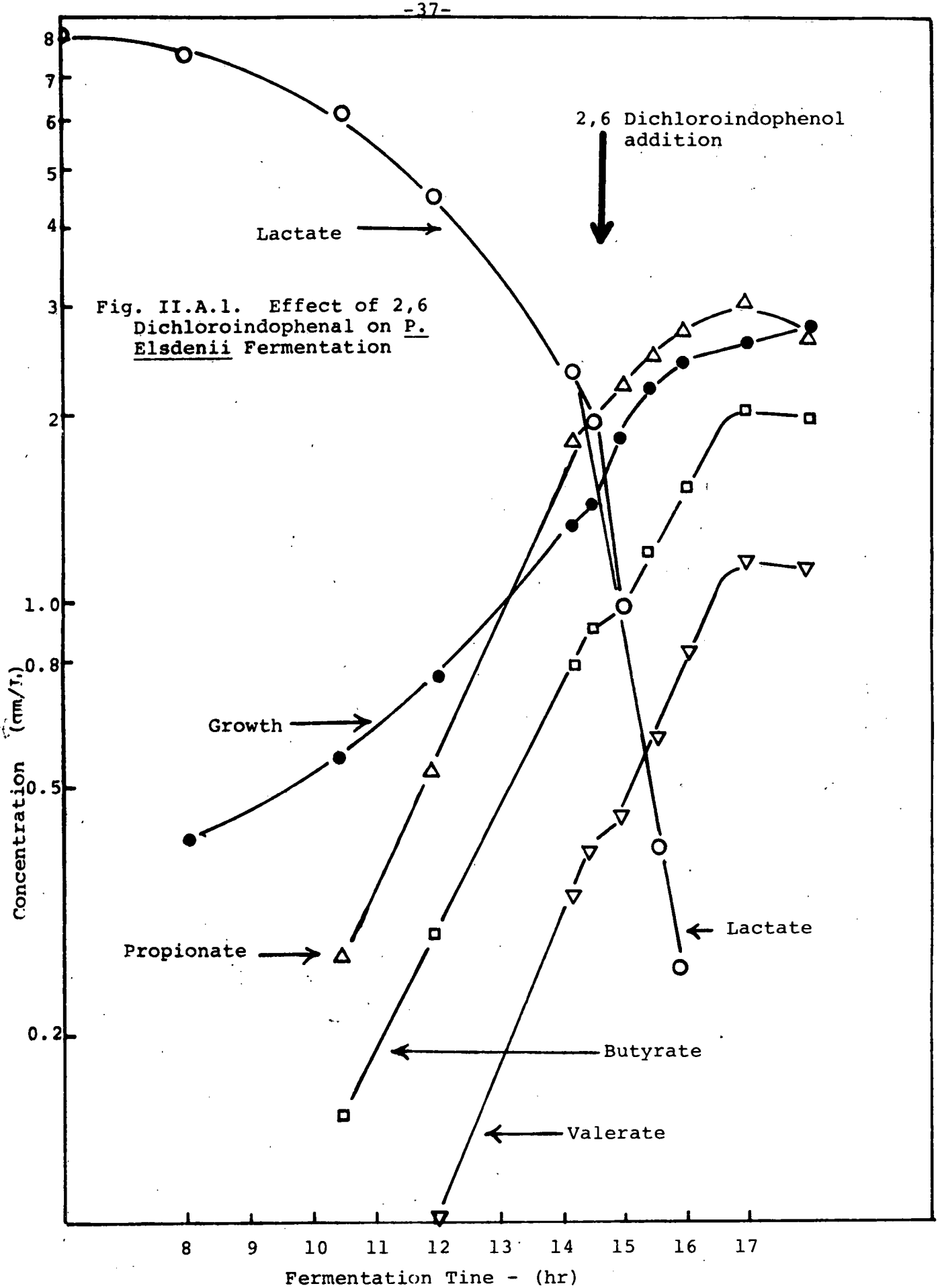


Fig. II.A.1. Effect of 2,6 Dichloroindophenal on P. Elsdenii Fermentation

state that can be used for both cell free extract and resting cell fermentations. Preliminary continuous culture experiments indicate that the fermentation and products are markedly different from those observed in batch culture.

Results with Clostridium propionicum:

A variety of experiments have been conducted; the growth medium experiments have conclusively shown that both the yeast extract and peptone are required for growth of Clostridium propionicum.

The studies with resting cells were extended and the following observations have been made:

1. Resting cell fermentations can be conducted in phosphate buffer provided that oxygen is excluded during the experiment. This observation eliminates the need for a reducing agent such as rezazurine or cysteine for subsequent studies and thus greatly simplifies the fermentation studies with resting cells.

In studies with lactate as the fermentation carbon source with resting cells no inhibitors i.e. electron acceptors were found that prevented the reduction of acrylic acid to propionic acid. This may be due to the fact that the resting cells may be impermeable to the chemicals studied.

Fermentations with cell free systems were then initiated. A cell free extract system was prepared. Crude cell free extracts were prepared as described previously and it was observed that 90% of the cells were broken. Fermentation of lactate was studied with the cell free extracts. Conversion

of lactate to propionate and acetic acid was demonstrated. Final ratios of products was 2 moles propionate to one mole acetic in accordance with previous observations with resting cells. An exciting preliminary result is that in certain cell free extracts of Cl. propionicum acrylic acid can be detected.

4. Future studies:

Now that a cell free system capable of enzymatically converting lactate to propionate and acetate with acrylic acid as an intermediate in the pathway has been demonstrated studies will be initiated that determine reaction rates and which chemicals (electron acceptors) block the reduction of acrylic acid to propionic acid.

B.) Production of Acetone/Butanol by Fermentation

1.) Introduction

The results of the economic analysis and the theoretic yield calculations of acetone and N-butanol production by fermentation were presented in the last progress report (12/1/76-2/28/77). The conclusion drawn from these results was that to reduce the manufacturing costs of the solvents, it is necessary to have a high yield of solvents and to have a microorganism more tolerant to acetone and butanol. It was found that a continuous process would be advantageous. The first step of our study was to examine some available strains of Clostridium acetobutylicum from American Type Culture Collection (ATCC) and Northern Regional Research Laboratory (NRRL). The objective was to find a strain that has a high solvent yield. This strain would then be used for future studies. At the same time, a soluble medium for growing the microorganism was developed. The soluble medium will be used for growth kinetic studies and hopefully will be easier to work with than a solid-liquid medium, such as the corn meal medium.

2.) Material and Methods.

2.1.) Microorganisms: four strains of Clostridium acetobutylicum were examined in the study. Three strains were obtained from ATCC and they were numbered as 824, 862 and 10132. The other strain B-594 was obtained from NRRL.

2.2.) Media:

2.2.1.) Corn meal medium: This medium consists of 5% corn meal which was obtained from local supermarket. Cysteine concentration of 0.05% was added to ensure a anaerobic condition in the medium. The mash was prepared by boiling the corn meal in appropriate volume of distilled water for one hour. The meal was distributed into Huntgate tubes and was sterilized at 121°C for 10 minutes. The medium was used for maintaining the cultures and was also used for the preliminary selection of the solvent production ability of the various strains.

2.2.2.) Soluble medium: The soluble medium contains the following components:

KH_2PO_4	0.75 gm
K_2HPO_4	0.75 gm
$MgSO_4$	0.20 gm
$MnSO_4 \cdot H_2O$	0.01 gm
$FeSO_4 \cdot 7H_2O$	0.01 gm
NaCl	1.00 gm
Cysteine	0.50 gm
yeast extract	5.0 gm
asparagine H_2O	2.0 gm
glucose	20.0 gm
1% resazurin solu.	1.0 ml
distilled water	1000 ml

2.3.) Inoculation procedure and growth conditions

A fermentation was started by inoculating spores of C. acetobutylicum from a corn meal mash into a fresh 5% corn meal medium. The inoculum size was 10%. This inoculated

corn meal mash was subjected to heat shock treatment by placing the tube into boiling water for 45-60 secs. The culture was allowed to grow for 24 hours at 37°C and was then used for inoculation of either the corn meal medium or the soluble medium. The size of inoculum used was 1%. The initial pH of the corn meal medium or the soluble medium was about 6.2-6.4.

2.4.) Preliminary selection of solvent producing strains.

The cultures were grown in corn meal medium in a series of Hungate tubes. The growth kinetics was followed by monitoring the pH and the product concentrations. However, due to the complexity of the corn meal medium, the substrate concentration and the cell density were not measured.

2.5.) Growth of strain ATCC 824 in soluble medium.

ATCC 824 was grown in corn meal medium. This was used for inoculation of a series of Hungate tubes containing the soluble medium. The concentrations of glucose and yeast extract were varied in one experiment. In another experiment the concentrations of asparagine and ammonium sulfate were varied. The final concentrations of cell, glucose and end products were measured.

2.6.) Methods of Analysis

2.6.1.) Cell turbidity: the cell density was measured by using a Klett-Sumerson colorimeter with a red filter.

2.6.2.) Glucose concentration: the residual glucose concentration of the fermentation broth was measured using an enzymatic kit from Calbiochem Co.

2.6.3.) End product concentrations: the products that were monitored were acetone, N-butanol, ethanol, acetic acid and butyric acid. Gas chromatograph was used for assaying these products. The column used was a 6 ft X 1/8" Telfon column packed with Chromasorb WV-AW coated with 10% AT1000. The temperature of the column was programmed from 100 C to 160 C at a rate of 20 C/min, and the final temperature was maintained for 3 minutes. The carrier gas, nitrogen, was at a flow rate of 30 ml/min. The samples analyzed was acidified and standardized by adding an acidified internal standard, n-propanol. The actual procedures were as the following:

(a) Fermentation broth was harvested from the tube and was centrifuged at 10,000 rpm for 10 minutes,

(b) Two-tenths of a millimeter of the supernatant were mixed with 0.05 ml of 25% n-propanol in diluted sulfuric acid.

(c) One ml of this mixture was used for the gas chromatograph assay.

A chromatogram of a standard solution is shown in Figure II.B.1a, and chromatogram of a fermentation sample is shown in Figure II.B.1b

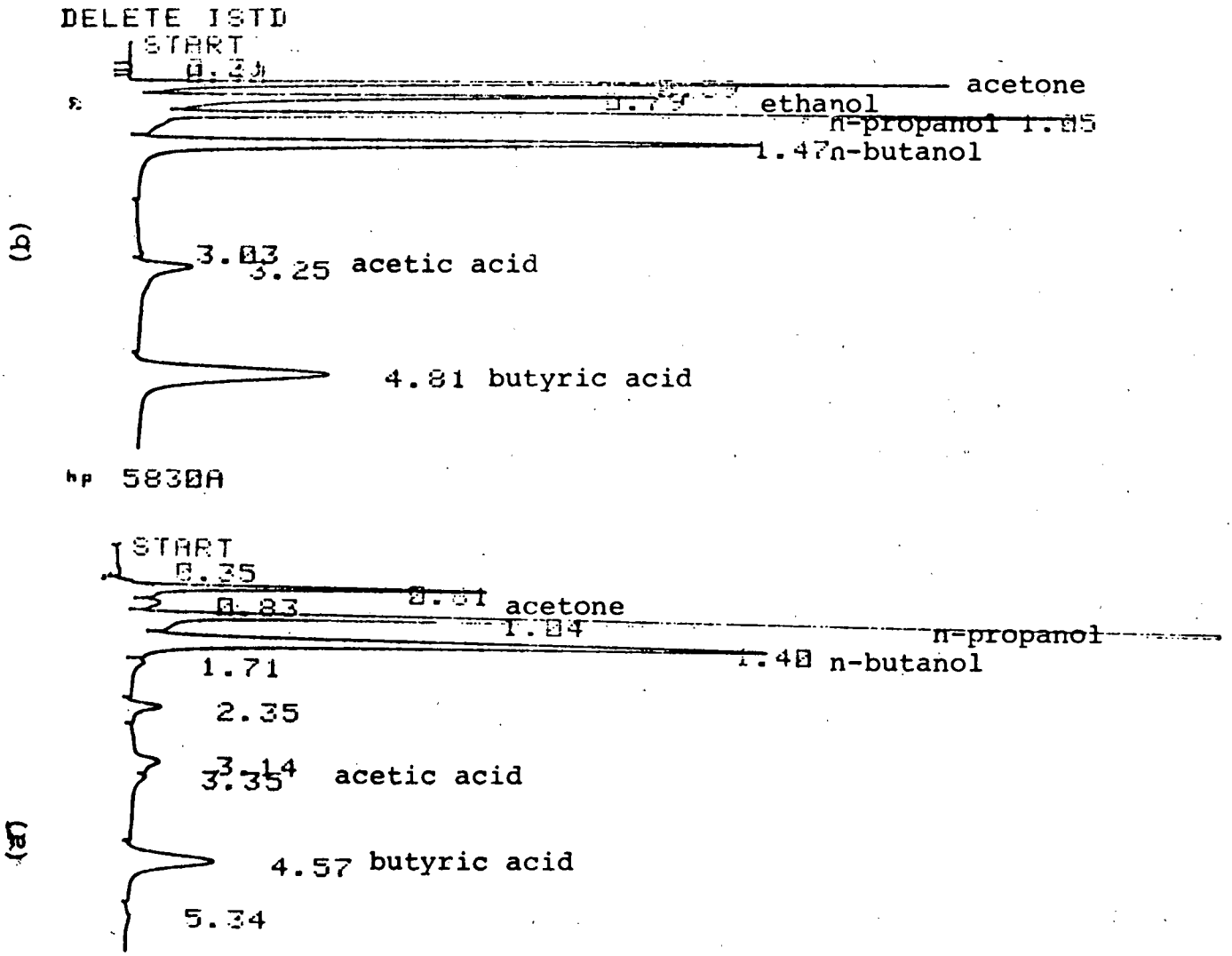
3.) Results and Discussion

3.1.) Growth of C. acetobutylicum on Corn Meal

The results of the C. acetobutylicum growing on corn meal medium for 96 hours are summarized in Table II.B.1.

A complete course of fermentation of ATCC 824 is shown in Figure II.B.2. The results of this fermentation was in-

FIGURE II.B.1: Gas Chromatography of Standard (a) and Fermentation Products (b) from C. acetobutylicum



(a)

(b)

RT	EXP RT	AREA		CAL #	AMT
0.61	0.60	177700		2	0.941
0.83	0.78	44120		3	0.228
1.04	1.04	1573009	R	1	
1.40	1.39	568800		4	1.905
3.14	3.09	116000		5	1.831
4.57	4.51	173000		6	0.811

XF: 1.2500 E+ 0 ISTD AMT: 5.0000 E+ 0

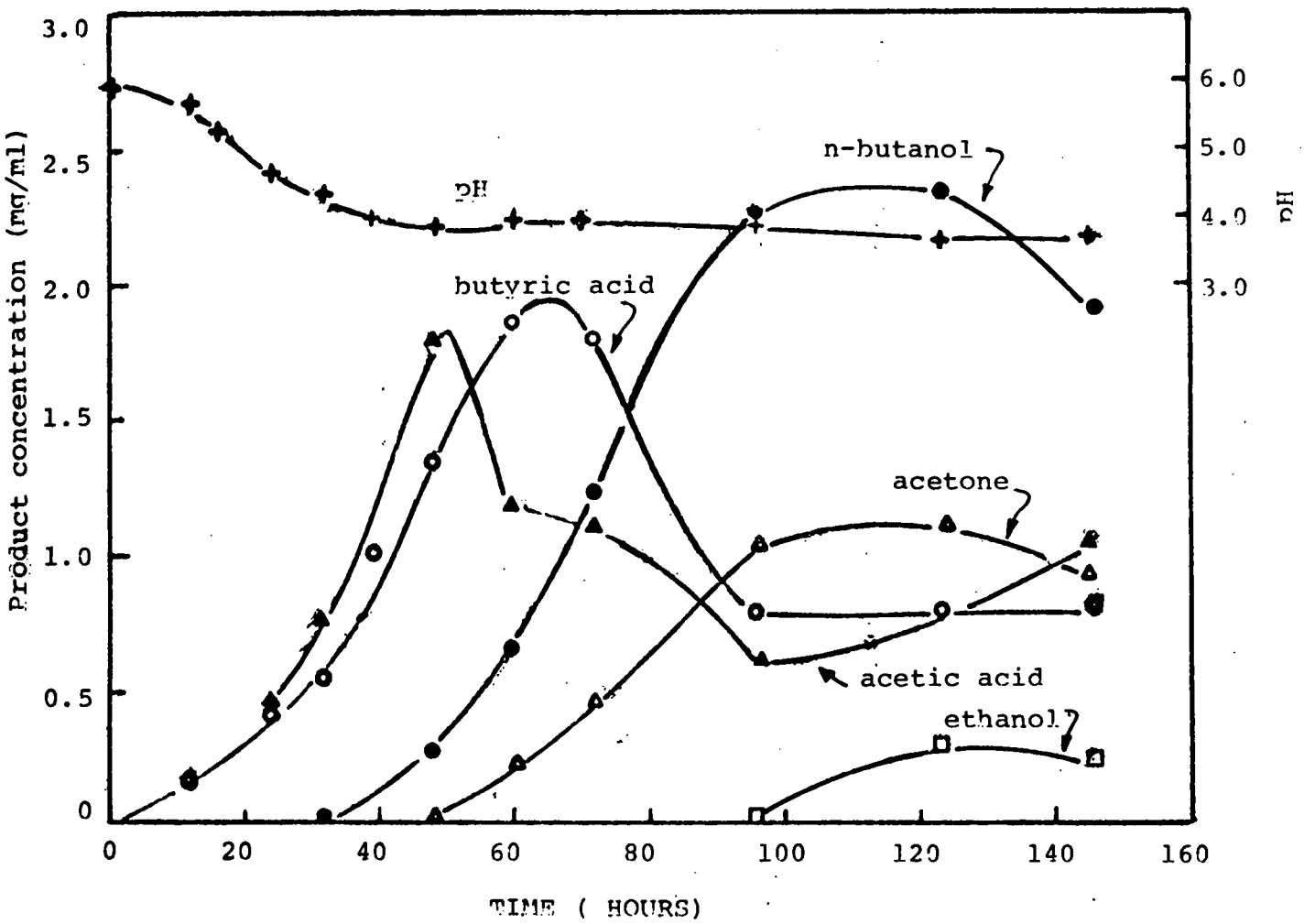
Table II.B.1

Results of C. acetobutylicium Growing on Corn Meal Medium (gms/liter)

Strain #	Final pH	Acetone	Ethanol	N-butanol	Butyric Acid	Acetic Acid
10132	4.41	0.04	0.03	-	0.34	0.31
B594	4.48	0.06	0.03	0.63	0.22	0.27
824	3.84	1.05	*	2.26	0.78	0.60
864	3.82	0.86	*	1.85	0.48	0.45

* Amount Unknown, due to error in retention time of gas chromatogram

FIGURE II.B.2: Fermentation Profile of Clostridium acetobutylicum on Corn Mash Medium



complete since the substrate concentration, the cell mass and the gaseous products were not measured. However, the general course of this fermentation agrees well with results reported by others.

From this preliminary experiment, ATCC 824 was found to produce the highest concentration of solvent from a corn meal medium. It was decided that ATCC 824 would be used for the development of soluble medium.

3.2.) Growth of C. acetobutylicum ATCC 824 on Soluble Medium.

3.2.1.) Variation of glucose and yeast extract concentrations.

The composition of the soluble medium was as described above. Three glucose concentrations were used; 10 g/l, 20 g/l and 30 g/l. The yeast extract concentration was also varied to be: 0., 0.25, 0.50, 0.75 and 1.0 g/l. The fermentation broth was analyzed after 135 hours of incubation at 37°C.

The results (Table II.B.2.) showed that the yeast extract was a requirement for growth and solvent production. However, within the range of yeast extract concentration studied, there is no distinct optimal concentration for yeast extract in the medium with respect to growth on product formation.

3.2.2.) Variation of Nitrogen Source Concentration.

C. acetobutylicum ATCC 824 was grown in the soluble medium with 20 g/l of glucose and different concentrations of asparagine and ammonium sulfate. Concentrations of asparagine ranges from 0 to 5.0 g/l, and two concentrations of ammonium sulfate were used. Duplicate tubes were used

Table II.B.2:

Results of ATCC 824 Growing in Soluble Medium with Different Concentrations of Yeast Extract and Glucose

(ALL VALUES IN GRAMS PER LITER)

Yeast Extract %	1% Glucose pH	Cell Mass	Acetone	Ethanol	Butanol	Acetic Acid	Butryic Acid	Glucose consumed
0	4.22	0.110	-	-	0.31	1.01	0.55	6.5
0.25	3.98	0.767	0.18	-	1.14	2.48	1.61	0.47
0.50	4.08	0.650	0.08	-	0.70	0.97	2.57	0.16
0.75	4.27	0.820	0.05	-	0.56	0.88	2.58	0.23
1.00	4.28	0.887	0.01	-	0.56	1.14	2.84	0.23
	2% Glucose							
0	4.16	0.113	0.04	-	-	0.06	0.50	16.48
0.25	3.93	0.713	0.17	0.13	1.13	0.82	1.63	3.69
0.50	4.01	0.680	0.07	0.12	0.63	1.04	1.71	9.42
0.75	4.15	0.880	0.10	0.18	0.49	1.48	2.72	9.80
1.00	4.26	0.860	0.09	0.19	0.60	1.85	3.16	8.18
	3% Glucose							
0	4.17	0.123	0.02	-	0.21	0.63	0.71	24.17
0.25	3.95	0.707	0.09	0.10	0.63	0.68	2.07	25.57
0.50	4.05	0.793	0.10	0.14	0.90	1.37	2.80	18.40
0.75	4.18	1.800	0.07	0.16	0.60	1.71	2.71	15.33
1.00	4.23	1.887	0.11	0.20	0.70	2.36	2.36	15.64

for this fermentation. One set of the tubes was harvested after incubation of 65 hours at 37°C and the second was harvested after 135 hours of incubation. The purpose was to make sure that the fermentation was completed at the time of harvest. The results were shown in Table II.B.3 and II.B.4. Since the medium contains yeast extract and asparagine, assumptions were made that these compounds were utilized completely. The amounts of these compounds were converted to amounts equivalent to glucose on a carbon content basis. Also, a constant cellular yield from glucose was assumed, and it was 0.15 gm dry cell/gm glucose. With these assumptions, we could calculate the amount of glucose used for product formation, and thus the product yield. The fractions of carbon recovered from the formation of the products were also calculated. The results are shown in Table II.B.5 and II.B.6. In these tables, the maximum butanol yield was calculated by converting butyric acid to equivalent butanol and adding this number to the actual butanol concentration. The sum was used for the yield calculation. Similar treatment was also performed using the acetone and acetic acid concentrations to calculate the maximum acetone yield. With the exception of a few cases, the maximum butanol and maximum acetone yields were close to the theoretical yeilds reported. The values that were higher than the theoretical yields may be explained by the variation of carbon content in the yeast extract.

Table II.B.3

Production of Solvents and Other Products by C. acetobutylicum on Soluble Medium: Effect of Asparagine and $(NH_4)SO_4$ Concentrations at 65 Hours

Asparagine conc. (g/ l)	pH	Cellmass	Acetone	Ethanol	Butanol	Acetic Acid	Butyric Acid	Residual Glucose	Glucose Consumed
	0 g/l $(NH_4)_2SO_4$	← ALL CONCENTRATIONS IN gm / liter →							
0	3.93	0.880	0.23	0.18	1.03	1.70	2.27	7.78	9.68
1.0	4.01	0.923	0.35	0.21	1.61	1.48	2.64	5.62	11.84
2.0	4.11	0.797	0.13	0.16	0.74	1.52	3.24	8.99	8.47
3.0	4.07	0.873	0.16	0.18	1.02	1.38	2.84	8.12	9.34
4.0	4.07	0.847	0.19	0.19	1.23	1.40	2.59	6.75	10.71
5.0	4.13	0.797	0.21	0.19	1.39	1.64	2.04	6.03	11.43
	1.0 g/l $(NH_4)_2SO_4$								
0	3.92	0.993	0.15	0.17	1.03	1.25	2.26	8.16	9.30
1.0	3.94	0.947	0.14	0.15	0.82	1.36	2.27	8.92	8.54
2.0	3.97	0.850	0.11	0.16	0.70	0.95	2.23	9.60	7.86
3.0	4.04	0.957	0.15	0.18	1.06	1.47	2.31	8.54	8.92
4.0	4.01	0.846	0.12	0.15	0.73	1.43	2.36	9.16	8.30
5.0	4.06	0.917	0.19	0.18	1.33	1.42	2.17	6.96	10.50
	2.0 g/l $(NH_4)_2SO_4$								
0	4.03	1.040	0.19	0.17	1.27	1.32	2.33	7.02	10.44
1.0	3.95	0.993	0.15	0.14	0.96	1.17	2.33	8.30	9.16
2.0	3.97	0.977	0.13	0.14	0.84	1.30	2.45	8.92	8.54
3.0	4.20	1.200	0.89	0.35	3.72	1.16	1.01	0.15	17.31
4.0	4.06	1.040	0.18	0.16	1.35	1.05	2.24	7.25	10.21
5.0	4.03	0.957	0.21	0.16	1.31	1.11	2.21	7.13	10.33

Table II.B.4

Production of Solvents and Other Products by C. acetobutylicium on Soluble Medium
Effect of Asparagine and $(NH_4)_2SO_4$ Concentrations at 135 Hours

Asparagine conc. (mg/ml)	pH	Cell Mass	Acetone	Ethanol	Butanol	Acetic Acid	Butyric Acid	Residual Glucose	Glucose Consumed
	0 mg/ml $(NH_4)_2SO_4$ ← ALL VALUES ARE EXPRESSED IN G/L →								
0	4.01	0.633	1.10	0.40	3.91	1.46	0.93	6.15	17.31
1.0	3.95	0.850	0.18	0.14	1.11	1.42	2.21	7.64	9.82
2.0	3.94	0.707	0.30	0.16	1.55	1.56	2.24	5.34	12.12
3.0	3.94	0.713	0.21	0.14	1.11	1.50	2.26	7.22	10.24
4.0	3.98	0.757	0.17	0.15	1.11	1.35	2.36	7.76	9.70
5.0	3.96	0.663	0.06	0.09	0.36	1.15	2.01	11.47	5.99
	1 mg/ml $(NH_4)_2SO_4$								
0	3.93	0.893	0.10	0.13	0.87	1.14	2.41	8.38	9.08
1.0	3.90	0.867	0.12	0.13	0.88	0.98	2.43	8.70	8.76
2.0	3.90	0.843	0.14	0.14	0.92	0.30	2.31	8.96	8.50
3.0	3.95	0.897	0.27	0.18	1.71	1.17	2.19	5.99	11.47
4.0	3.98	0.887	0.19	0.15	1.40	1.29	2.30	6.58	10.88
5.0	3.99	0.830	0.27	0.16	1.51	1.43	2.39	6.06	11.40
	2 mg/ml $(NH_4)_2SO_4$								
0	3.88	0.947	0.13	0.13	0.96	1.11	2.26	8.28	9.18
1.0	3.95	0.927	0.15	0.14	0.97	1.22	2.29	8.41	9.05
2.0	3.91	0.927	0.17	0.15	1.10	1.37	2.38	7.41	10.05
3.0	3.90	0.950	0.46	0.19	2.10	1.16	1.86	5.08	12.38
4.0	3.97	0.907	0.15	0.14	1.16	1.23	2.21	8.46	9.00
5.0	4.08	0.963	0.24	0.18	1.83	1.25	2.24	6.09	11.37

Table II.B.5

Calculated Product Yields from C. acetobutylicium Fermentation at 65th Hour

All yield values are expressed as gm product/gm glucose equivalent consumed

(65 hour incubation)

$(NH_4)_2 SO_4$ (gm/L)	Asparagine conc. (gm/L)	Acetone Yield	Ethanol Yield	n-Butanol Yield	Acetic Acid Yield	Butyric Acid Yield	Maximum Butanol Yield	Maximum Acetone Yield	Fraction of total Carbon from Assayed Product
0	0	0.022	0.018	0.102	0.169	0.226	0.292	0.185	0.704
	1.0	0.027	0.016	0.126	0.116	0.207	0.300	0.139	0.670
	2.0	0.009	0.011	0.051	0.106	0.225	0.240	0.111	0.526
	3.0	0.013	0.015	0.084	0.113	0.233	0.280	0.122	0.609
	4.0	0.013	0.013	0.085	0.096	0.178	0.289	0.106	0.516
	5.0	0.013	0.012	0.085	0.100	0.125	0.228	0.109	0.446
1	0	0.017	0.019	0.115	0.140	0.253	0.328	0.152	0.726
	1.0	0.015	0.016	0.088	0.147	0.245	0.294	0.157	0.660
	2.0	0.011	0.016	0.070	0.095	0.222	0.257	0.103	0.551
	3.0	0.013	0.016	0.095	0.131	0.206	0.268	0.140	0.609
	4.0	0.010	0.013	0.065	0.128	0.211	0.242	0.134	0.555
	5.0	0.013	0.012	0.091	0.097	0.148	0.215	0.107	0.485
2	0	0.019	0.017	0.130	0.134	0.238	0.330	0.149	0.724
	1.0	0.016	0.015	0.100	0.122	0.243	0.304	0.134	0.663
	2.0	0.013	0.014	0.085	0.132	0.248	0.294	0.141	0.648
	3.0	0.050	0.019	0.207	0.065	0.056	0.254	0.113	0.589
	4.0	0.014	0.013	0.106	0.082	0.176	0.254	0.093	0.535
	5.0	0.015	0.011	0.092	0.078	0.056	0.223	0.090	0.476

Table II.B.6

Calculated Product Yields from C. acetobutylicium Fermentation at 135 Hours
cell yields are expressed as gm product/gm glucose consumed

$(NH_4)_2SO_4$ conc. (gm/l)	Asparagine conc. (gm/L)	Acetic Acid	Ethanol Yield	n-Butanol Yield	Acetic Acid	Butyric Acid Yield	Maximum Butanol Yield	Maximum acetone Yield	Fraction of Total Carbon from Assayed Product
0	0	0.056	0.021	0.202	0.075	0.048	0.242	0.129	0.592
	1.0	0.016	0.013	0.099	0.127	0.197	0.375	0.142	0.653
	2.0	0.020	0.010	0.102	0.102	0.147	0.226	0.119	0.518
	3.0	0.015	0.009	0.079	0.106	0.160	0.214	0.117	0.490
	4.0	0.012	0.011	0.079	0.096	0.167	0.271	0.105	0.487
	5.0	0.005	0.008	0.030	0.097	0.170	0.173	0.099	0.397
1.0	0	0.011	0.013	0.093	0.122	0.257	0.309	0.129	0.659
	1.0	0.012	0.013	0.088	0.098	0.242	0.292	0.107	0.606
	2.0	0.013	0.013	0.086	0.121	0.215	0.267	0.130	0.593
	3.0	0.019	0.013	0.121	0.083	0.155	0.251	0.099	0.540
	4.0	0.013	0.010	0.097	0.089	0.160	0.232	0.099	0.499
	5.0	0.016	0.010	0.091	0.086	0.144	0.212	0.099	0.471
2.0	0	0.014	0.014	0.105	0.122	0.248	0.314	0.132	0.672
	1.0	0.015	0.014	0.098	0.123	0.231	0.292	0.134	0.641
	2.0	0.015	0.013	0.094	0.117	0.203	0.265	0.128	0.590
	3.0	0.031	0.013	0.143	0.079	0.127	0.250	0.107	0.555
	4.0	0.012	0.011	0.094	0.099	0.178	0.244	0.108	0.530
	5.0	0.016	0.012	0.120	0.082	0.147	0.244	0.095	0.521

From this experiment, it was difficult to conclude if there was an optimal concentration of asparagine and/or ammonium sulfate. However, we could state that under the described experimental conditions, the bottleneck on obtaining higher acetone and butanol yield is the conversion of the acids to the solvents. In a few cases, the yields of butanol and acetone were reasonably high without any special manipulation of the system. For example, the yields of acetone and n-butanol were 0.05 and 0.21 respectively when the concentrations of asparagine g/l and $(NH_4)_2SO_4$ were 3.0 and 2.0 g/l respectively. The theoretical yield for maximum acetone and butanol yields were calculated to be 0.10 and 0.27, respectively, and this assumes no other products were formed.

The results of this experiment confirm our analysis in our last progress report that the yields of the products were already close to the theoretical. This could mean that there will not be much room for manipulation for cost reduction in this area of research.

4. Future Studies

The priority of the future research are listed as the following:

- more work on the growth kinetics of the organism.
- development of a defined medium so that the yields of product can be found directly.
- search for high solvent concentration tolerant mutant
- use such mutants for continuous culture fermentation with possible cell recycle or reuseage.

C.) Production of Acetic Acid by Fermentation

1.) Introduction

The production of acetic acid by Clostridium thermoaceticum has been plagued by an analytical problem in the quantification of acetic acid in the fermentation broth.

It was felt in reviewing the available enzyme assays for acetate that none of them provided a simple sensitive procedure. The alternative scheme of gas chromatography also faces a number of problems. Acetic acid is best analyzed as an ester derivative but the high preference of acid for water versus other organic solvents make it impossible to do these extractions and subsequently form these derivates on a routine basis. The analysis of free fatty acids (FAA) in water has a number of problems of its own but overall seems to be the method of choice. The development of a quantititative method for analyzing FAA especially the lower ones has been difficult. There are two main problems: one is the molecular association of the acids in the vapor state and the other is adsorption which is generally recognized as the worst of the problems. It causes the acid to show bad tailing, irregular shaped peaks, and ghosting. A review of the problem and a suggested means to solve it was written by Cochrane (1975)*. His recommendation was that formic acid be added to the carrier stream and he demonstrated that this can overcome the adsorption problems. The reason is because formic acid creates such a polar environment and saturates

*Cochrane, G.C. (1975) A review of the Analysis Free Fatty Acid, J. of Chrom. Sci. 13: 440-446.

other adsorption sites in the column. Although he recommended the use of a liquid phase (25% NSGA on Chromosorb W) it has been our experience that the injection of large amounts of water (1-2 ml) at temperatures greater that 100°C causes considerable hydrolysis of the liquid phase. Thus we investigated the use of porous polymers as a solid phase support. Chromosorb 101 was found to work very well.

2.) Materials and Methods

The following conditions were used: A Teflon column (5 ft x 1/8" OD) contained Chromosorb 101 (mesh 80/100) was used. Conditioning was carried out at 250°C. Formic acid was incorporated into the carrier gas by passing the helium over the formic acid (once distilled 88% reagent) before entering the column.

Temperature was isothermal at 145°C, injection port temperature 200°C, detector temperature 300°C. Flow rate of carrier gas was 30 ml/min.

3.) Results and Discussion

Under these conditions we were able to get reproducabilities of $\pm$ 0.5%. The concentration of acetic acid was determined by using isopropanol as an internal standard. The ratio of the areas under the chromatographic peaks is shown in Figure II.C.1. The data show excellent linearity over the range 0.025% to 0.5% acetic acid with a standard error of 0.9%. A typical chromatograph is shown in Figure II.C.2. The isopropanol has a retention time of 2.09 min & the acetic acid a retention time of 5.74 min.

FIGURE II.C.1: Calibration of Acetic Acid through Gas Chromatography Using Formic Acid Treated Column

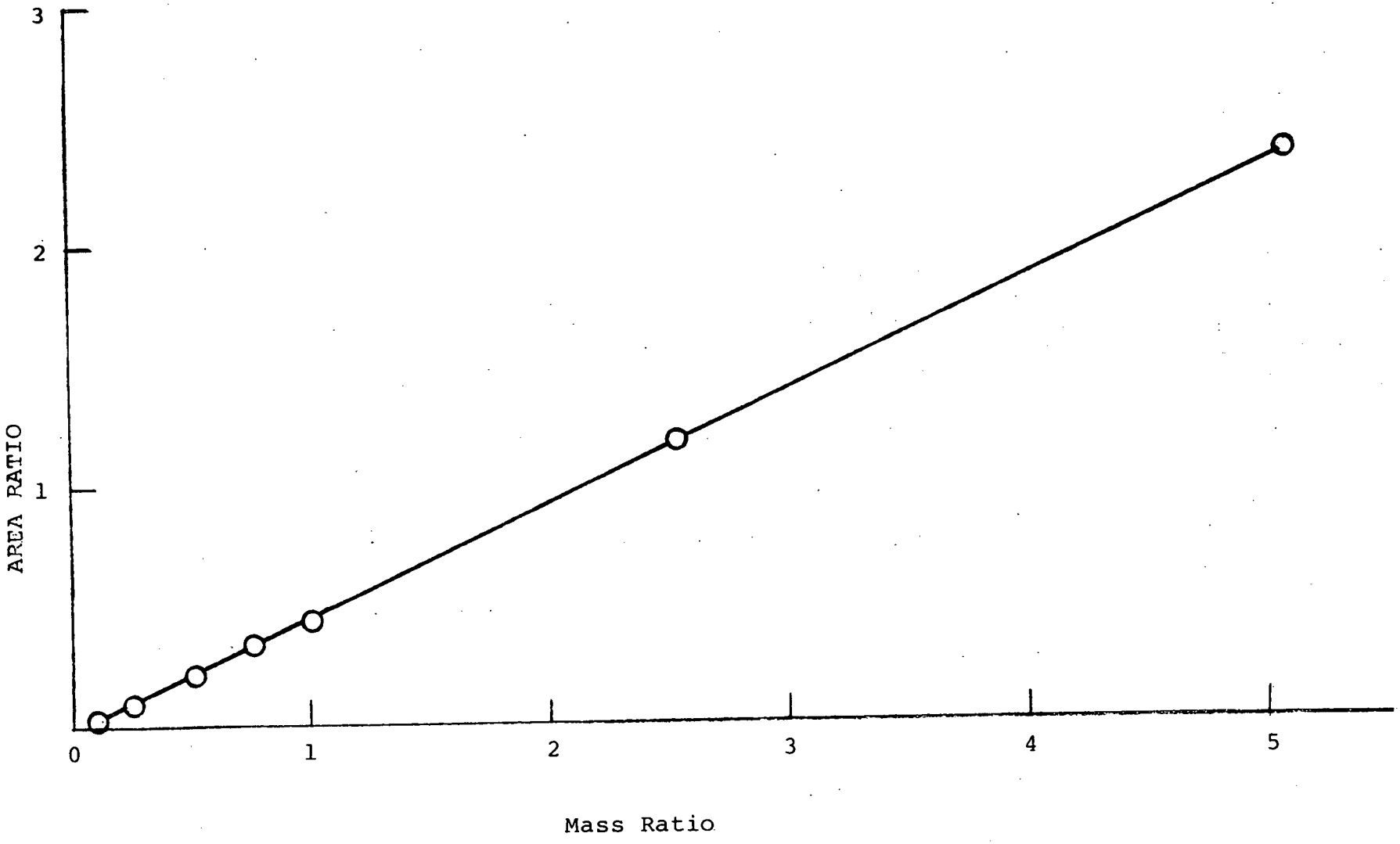
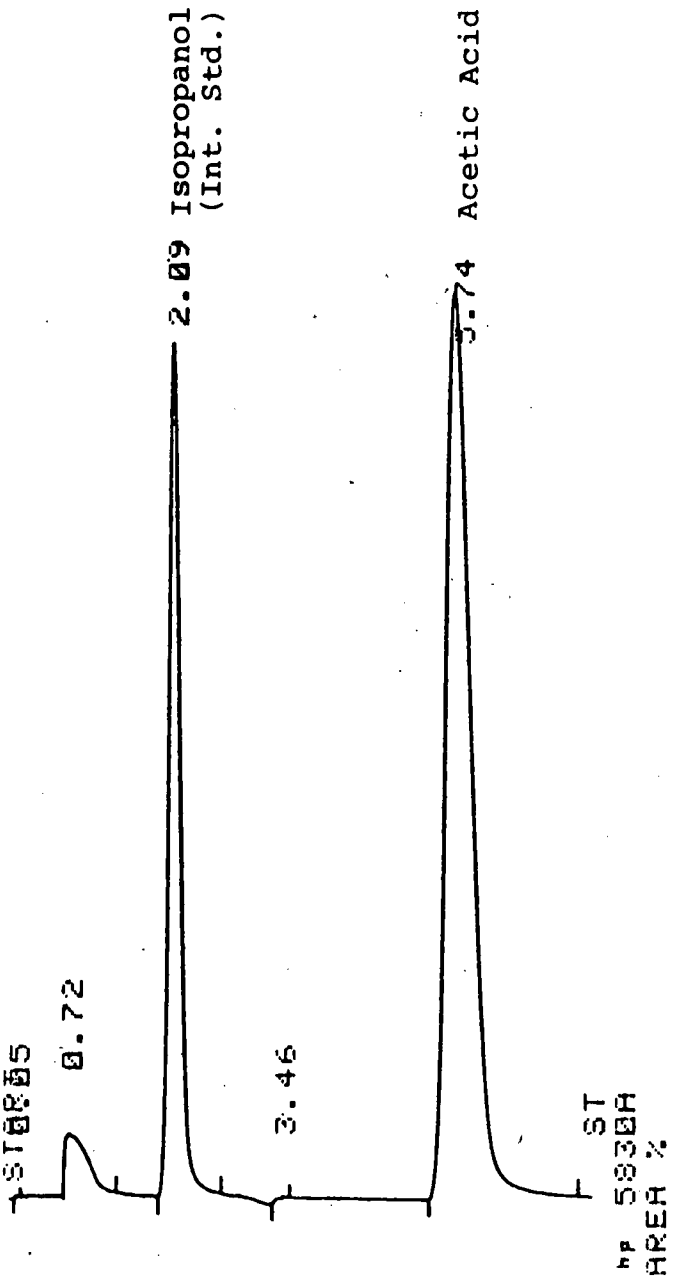


FIGURE II.C.2: Gas Chromatograph of Formic Acid Treated Column to Determining Acetic Acid Concentration



These results show that we now have an accurate and rapid method for determining acetic acid concentrations. This will now enable us to proceed with the detailed characterization of acetic acid production by C. thermoaceticum.

D. Production of Methane from Cellulose

1. Introduction

The production of methane gas from cellulosic residues by anaerobic digestion has been considered by many workers as an alternative and supplemental method for gas production. The disadvantage and limitations of existing technology for anaerobic digestion are: low rates of conversion of cellulose to methane and limited conversion of the initial cellulose to methane. With the objective of minimizing those limitations, we initially proposed to examine the kinetics of cellulose conversion to methane and test a hypothesis that the rate limiting step in the conversion is the rate of generation of reducing equivalents from glucose oxidation and the subsequent utilization of this reducing power in methane formation. This work was proposed as a limited effort (one half man year) in order to test the hypothesis and evaluate if further work should be directed towards this area. Results from the initial work on this problem are presented here.

2. Experimental Approach

Since the objective in this study is to enhance the rate of methane production from cellulose, the experimental system was an anaerobic digestor operating on cellulose as the primary carbon source. In order to examine the effect of availability of reducing equivalents on the detailed kinetics of cellulose conversion to methane, we proposed to first examine the specific rate of cellulose conversion to glucose,

glucose to fatty acids (i.e. acetate, propionate, butyrate etc) and fatty acids to CO_2 and methane. This would be done by measuring the rates of disappearance and appearance of substrates and intermediates, and by the use of radioactive materials to accurately assess not only rates but metabolic pool sizes. Such studies would be done with variable amounts of H_2 in order to measure its effect. If successful, we would propose the enhancement of reducing power, not by H_2 addition, but rather optimal control of the biological reactor.

3. Cellulose degrading mixed cultures were enriched from a mixture of 3 liter digester effluent and 1 liter raw sludge from the Nut Island Sewage Treatment Plant in Boston. The sludge was incubated anaerobically in a 4 liter flask at 38°C. After inoculation when the gas production leveled to less than 30 ml/liter/h, Solka Floc as a source of cellulose was added every second day increasing from 1.25 g/liter to 5 g/liter. If necessary, pH-adjustments were made during the first two days of operation by addition of small amounts of NaOH or H_2SO_4. After some days of operation, when no liquid was withdrawn and the pH remained constant, alkalinity was assumed to be constant.

Gas production was followed by water-displacement. The methane content of the gas was determined by absorption of the CO_2 in 20% KOH (w/v).

For determination of the isotope distribution into fermentation products derived from labeled acetate three 500 ml flasks were filled with activated sludge from one of the

cellulose fermentors. The flasks had an opening at the bottom for withdrawing samples and the stopper at the top of the flask had a gasexit. The injection of the labeled acetate solution was carried out with a syringe through the stopper. The fermentation gas was either directly led to the gas sampling trap in a small diameter tubing or prior to final collection into two CO_2-traps in sequence each of them containing 20 ml 20% KOH.

Radioactivity levels in the clarified neutral and acidified supernatant, in the cell suspension after two washings with distilled water and in the KOH from the CO_2-absorption was controlled by counting and comparing the radioactivity of KOH from each of the two traps separately. In addition the cylinders with the collected methane were stored in alkaline solution for one night, to remove residual CO_2.

The bacterial cell mass was determined gravimetrically after drying the suspension at 105°C to constant weight. The separation from the sludge particles was done by centrifugation in a Sorvall R-2B refrigerated centrifuge, equipped with a SS-34 Rotor for 10 minutes at 15,000 RPM. After the first run the supernatant was discarded and the cell- and sludge pellet was resuspended in distilled water. The supernatant after the second run was also discarded and then the clearly separated cell pellet was carefully washed off from the sludge pellet. This procedure was repeated once. Reducing sugars were determined as described previously volatile acids were determined in aquous solution by gas chromatography with a Varian 1200 GC, using 10% AT-1000 on Chromosorb W-AW

80/100 mesh (purchased from Alltech Associates, 202 Campus Drive, Arlington Heights, Ill.) as a stationary phase in a 6 ft teflon column. The jnjector temperature was set at 220°C, the column temperature was programmed from 100°C to 190°C in rates of 20°C per minute, the detector temperature was 290°C. 0.5 ul of a standard solution containing 1% of each acetic, propionic-, butyric- and valeric acid (pH 1.5) were injected as reference. Volatile solids in sludge samples were determined gravimetrically after drying at 105°C to constant weight. Total solids (ash) were determined by combustion of the dryed sludge samples at 600°C for 6 h.

4. RESULTS

a. Characteristics of a Cellulose Degrading Mixed Culture

The culture selection was carried out with digester effluent and raw sludge as described in materials and methods. After stabilization of the system, increasing amounts of cellulose up to 7.5 g/liter/day were added. With feeding rates up to 5 g/liter/day no accumulation of reduced sugars or volatile acids could be detected. Acetate was present at 0.3 to 0.7 mM. The concentration of propionate, butyrate and valerate ranged from 0.1 to 0.4 mM. The cell mass concentration increased during the first 15 days of operation about tenfold to 2.3 g/liter and then was constant at this value (Fig. II.D.1). The gas production rate increased the addition of cellulose, peaked after about 24 hr and settled down to a low value. The gas production rate at least for

FIGURE II.D.1: Gas production in a cellulose degrading digester. ● = cell mass % (g dry weight/ 100 ml sludge), o = cell mass in a parallel fermentor, ▲* = addition of 20 g Solka Floc, ▲ = addition of 30 g Solka Floc.

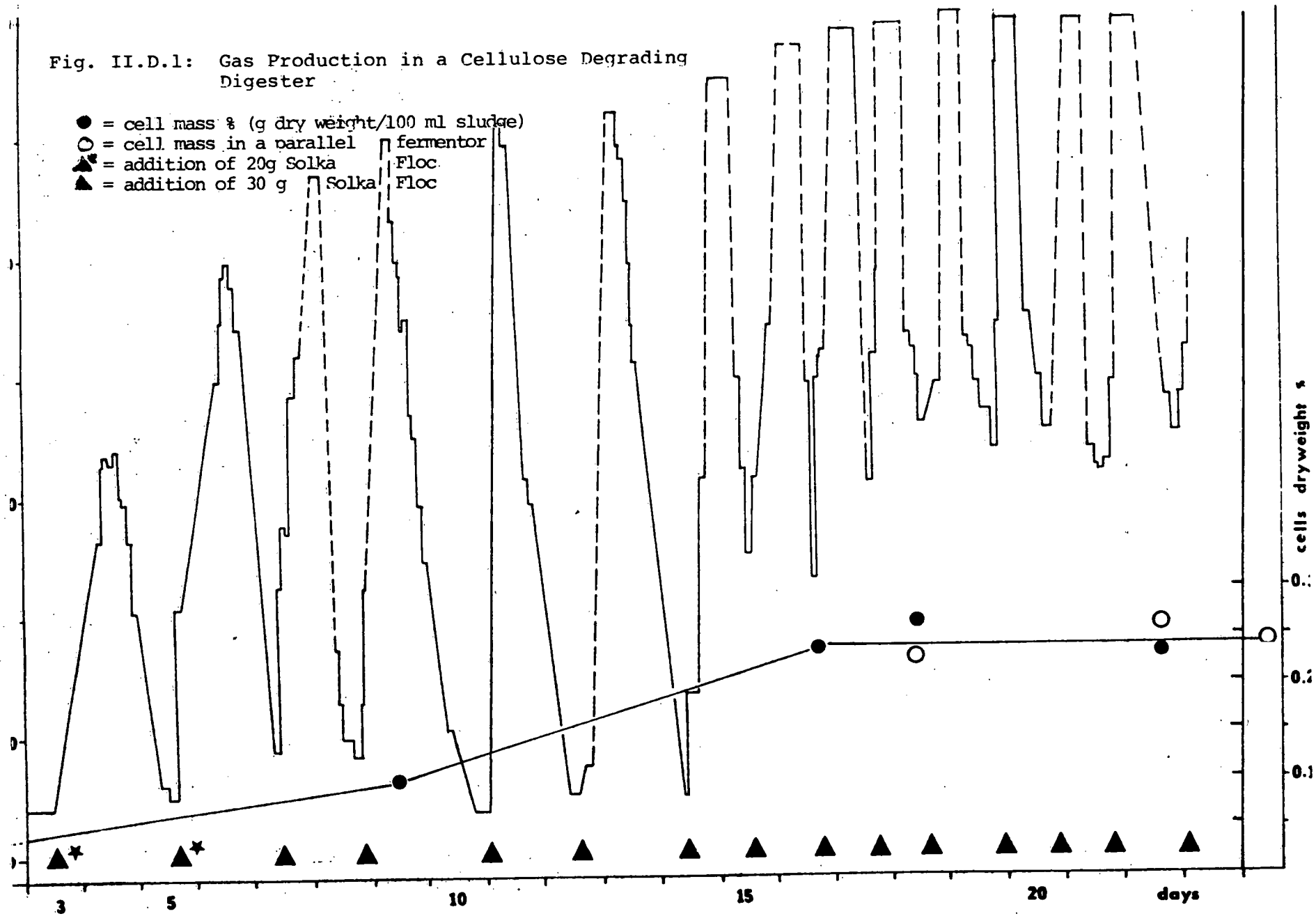


Fig. II.D.1: Gas Production in a Cellulose Degrading Digester

the first two feeding cycles after initiation were measured and are illustrated in Fig. II.D.1. Calculation of the total gas production by integration of the area under the first 2 peaks shows 27.1 liters of gas produced from 40 g of Solka Floc. For comparison the maximum theoretical possible value is 27.2 liters of gas, assuming a molecular weight of 198 for the hydrolyzed cellulose. Due to the increasing bacterial cell mass in the first two weeks of the experiment the maximum possible gas production rates are increasing with each feeding cycle. This indicates also that a balanced growth of the mixed cellulose degrading culture occurs. After two weeks of operation there is no further bacterial cell growth, death rate and growth rate are balanced. With increasing feeding rate the lowest observed gas production rates are increasing due to a saturation of the system with cellulose, as is also indicated by an increasing sludge pellet (see Table II.D.1). Continuing with higher feeding rates than 5 g/liter/day leads to overloading and finally to failure in gas production, accompanied by increases in acetate and propionate concentration to more than 1 g/liter. In the early stages of overloading, it is possible to stabilize the system again by interruption of the cellulose addition for some days carefully controlling the pH at 6.8 and then beginning cellulases feeding at low rates. However, when high acetate and propionate concentrations were present in the liquid, stabilization within two weeks could not be obtained. Therefore for further experiments fermentors were maintained at a feeding rate of 2.5 g/liter/day, this ensuring total cellulose degradation

Table II.D.1

Volatile Solids and Bacterial Dry Weight in a Cellulose Digester, Fed Increasing Amounts of Cellulose

Days after Inoculation	Dry Cell Weight g/liter	Dry Weight Sludge	Dry Weight Total g/liter
0	0.26	19.7	20
9	0.97	34.5	35.5
16	2.27	33.8	36.1
18	2.23	29.9	32.1
	2.69	37.1	39.9
22	2.25	38.3	40.5
	2.75	41.5	44.3
24	2.18	44.9	47.1
	2.38	44.9	47.3

and rapid gas production.

b. The Degradation of Acetate and Other Volatile Acids.

During cellulose degradation with our system acetate concentrations between 0.4 and 0.7 mM were measured. To increase this concentration, a fermentor fed 2.5 g cellulose/liter/day was supplemented with sodium acetate to a final concentration of 20 mM and the degradation was monitored. Fig. II.D.2 illustrates the decreasing acetate concentration, accompanied by increasing gas productivity almost immediately after addition of the acetate to the system. Half of the acid is degraded in 2-3 hr and after 17 hrs. almost all of the acetic acid is gone. A slight increase of the pH was measured, due to alkaline reaction of the sodium ions.

To determine the carbon balance from acetate and the distribution of methyl- and carboxyl groups on CO_2 and methane, 500 ml sludge from a cellulose fermentor were incubated either with nonlabeled, 1-^{14}C-labeled and 2-^{14}C-labeled acetate. After filling the sludge samples into the reaction flasks, the gas productivity was measured and compared in Fig. II.D.3) prior to acetate addition. Besides a slight loss of activity in each of the three flasks in comparison to the origin- perhaps due to oxygenation - the gas production is the same. The flasks now were inoculated either with 20 mmol of nonlabeled, 1-^{14}C-acetate and 2-^{14}C-acetate. Total gas productivity, gas production rates as well as methane and CO_2 -production were monitored and compared with gas production of sludge without added acetate. The disappearance of radioactivity from the liquid was measured. Results as shown in Fig. II.D.4 show that after

20 hrs. all the acetic acid was degraded. With carboxyl-labeled acetate a significant difference in the residual radioactivity between neutral and acidified broth was apparent, due to an increased alkalinity caused by an increase of the pH by 0.3 units and secondly due to an exchange reaction of labeled CO_2 with unlabeled CO_3^{--} and HCO_3^-. In the early stage of acetate degradation almost all of the labeled CO_2 is solubilized in the liquid, while $^{14}CO_2$ in the gas phase appears mainly in the later stage of the incubation. Overall, 43% of the carboxylgroups of acetate are released as CO_2 and 57% are reduced to methane. 2-^{14}C-acetate degradation results predominantly in the production of labeled methane, only 2% of the methylgroups are oxidized to CO_2. Radioactivity incorporated into cells was less than 0.1% of the total activity. Fig. II.D.5 shows the actual gas production and the theoretical gas production, the later calculated from data on solubilized $^{14}CO_2$ in the liquid from the tracer experiment. Subtracting the total gas productivity of the fermentor without added acetate from the gas productivity of the fermentor supplied with acetate, an amount of 880 ml total gas was produced from 20 mmoles acetate. Compared with the theoretical value of 896 ml of gas, this accounts for 98% C-recovery. The actual composition of the gas, generated from combined cellulose- and acetate degradation is 72% CH_4 and 28% CO_2, however including CO_2 causing the alkalinity, it would be 58% CH_4 and 42% CO_2. The gas composition from cellulose was 65% methane and 35% CO_2 during balanced growth. The gas composition from the degradation of acetate is 50% methane and 50% CO_2, when no external hydrogen source is available. In our experiment the

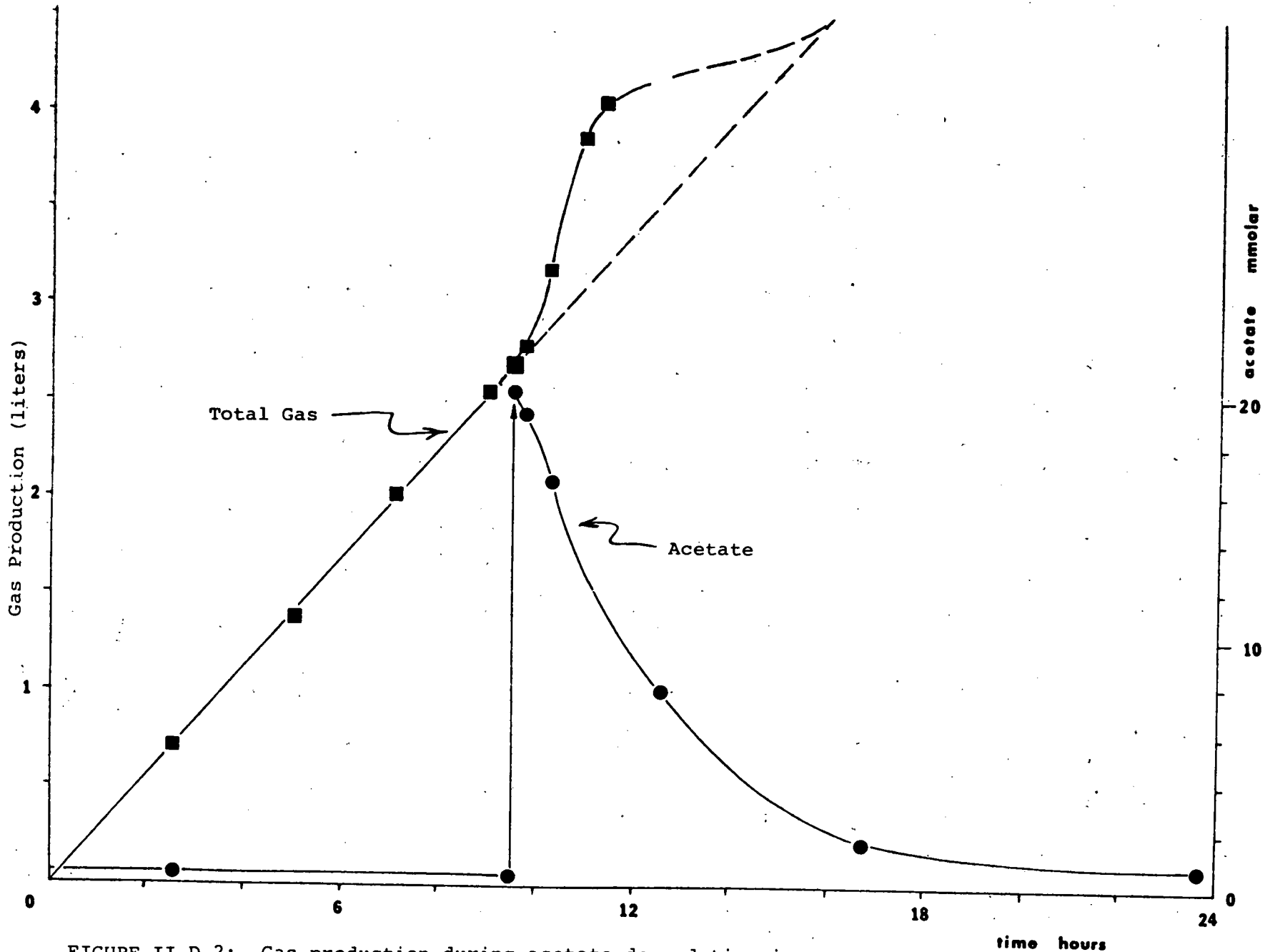


FIGURE II.D.2: Gas production during acetate degradation in a cellulose digestor.

FIGURE II.D.3: Comparison of gas production in three 500 ml-fermentors, prepared for pulse addition of acetate.

● = gas production in original digestor

o, ■, □ = gas production in fermentors, I, II, III.

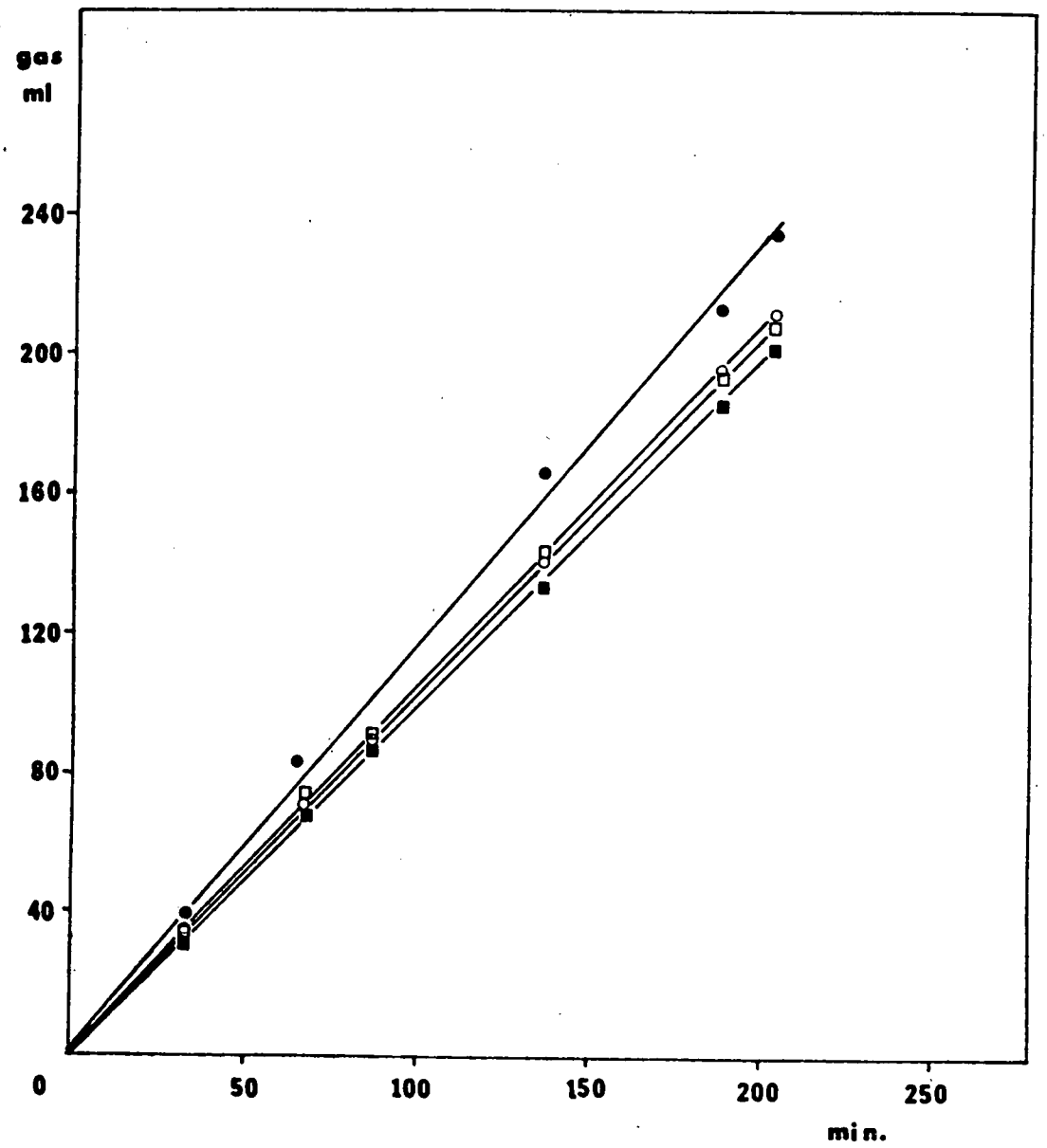


FIGURE II.D.4: Disappearance of radioactivity from acetate during combined acetate and cellulose fermentation in an anaerobic digestor.

○ = label in supernatant of centrifuged sludge samples.

● = label in acidified supernatant

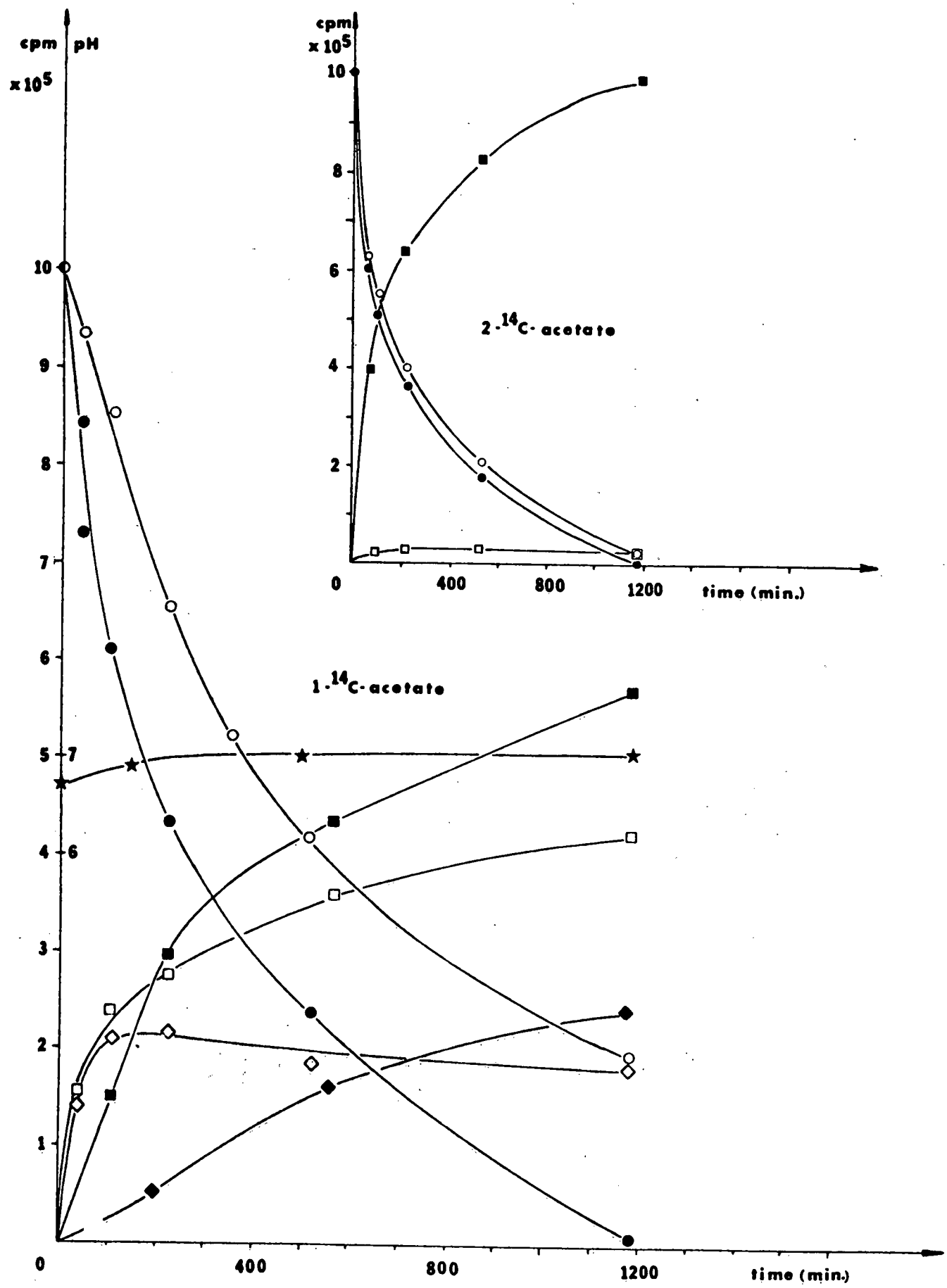
■ = labeled methane (calculated as total ^{14}C minus $^{14}CO_2$)

□ = total $^{14}CO_2$ producted

◆ = $^{14}CO_2$ obtained from KOH traps

◇ = $^{14}CO_2$ as $^{14}CO_3^-$ and $H^{14}CO_3^-$

▲ = pH


cpm x 10^5
pH
10
9
8
7
6
5
4
3
2
1
7
6
0
400
800
1200
time (min.)
1.-14C-acetate
cpm x 10^5
10
8
6
4
2
0
400
800
1200
time (min.)
2.-14C-acetate

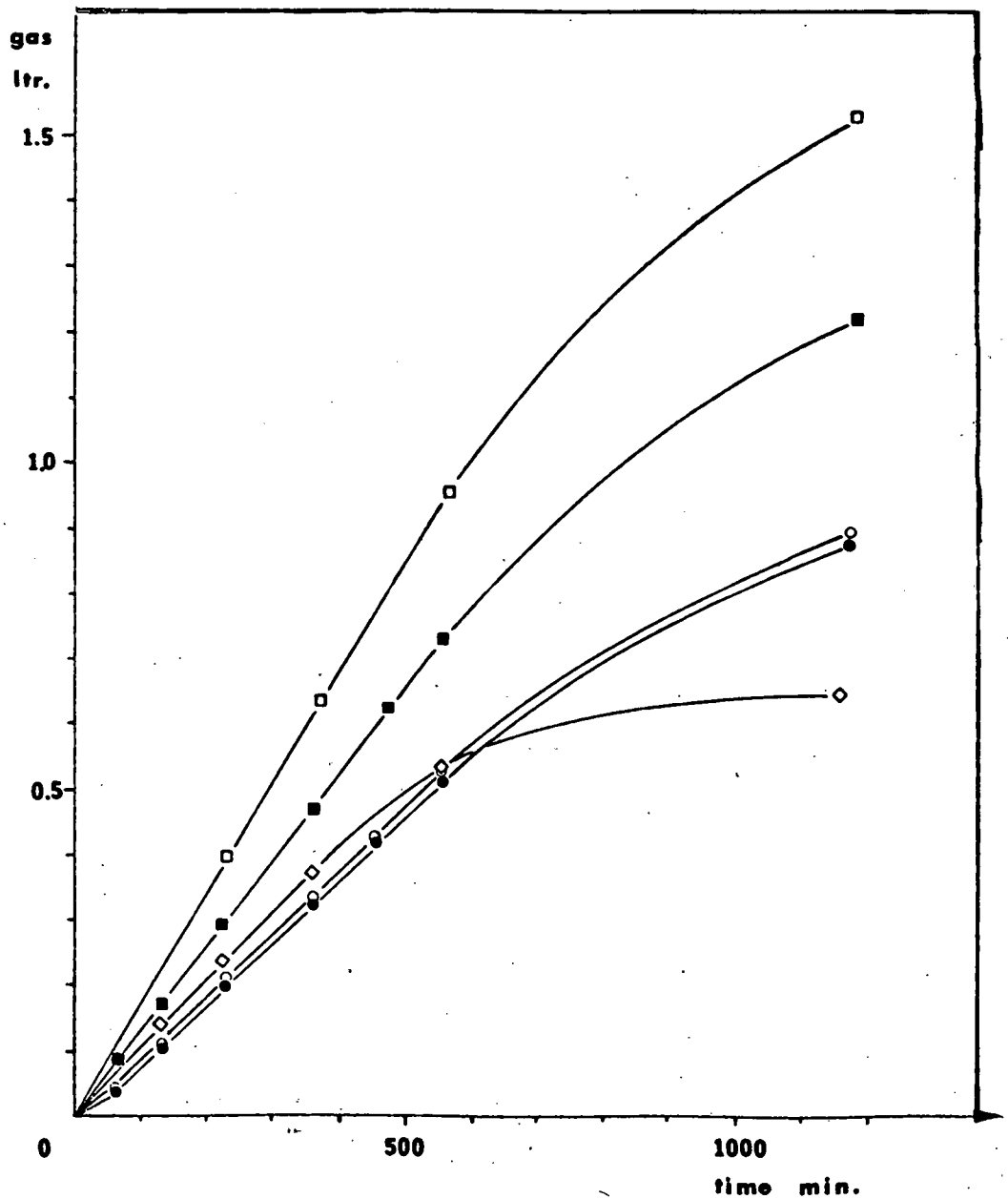


Figure II.D.5: Methane and carbondioxide Production from acetate

□ = total gas produced from acetate + cellulose

■ = total gas released from acetate + cellulose

●,○ = methane produced from acetate + cellulose

◇ = total gas produced from cellulose alone

total gas is derived from cellulose to an extent of 43% and from acetate 57%. The average gas composition was expected to be 56.5% methane and 43.5% CO_2, including the CO_2 solubilized in the broth. This total ratio would not change when hydrogen transfer to acetate occurs as it is the case in our cellulose-acetate fermentor, when the hydrogen available is only derived from acetate and cellulose. Actually this seems to be the case, because our values of the gas composition - 57% CH_4 and 43% CO_2 - are very close to the expected values.

The production rates for methane and for total gas after addition of acetate are shown in Fig. II.D.6. Total gas production rates are increasing faster than methane production rates. This indicates that at the beginning of the degradation, the ratio CH_4/CO_2 is very much shifted in the direction of CO_2 in addition to the solubilized CO_2 in the liquid. Thus, the maximum rate of gas production is reached earlier with CO_2 than with methane. In the final phase of degradation the methane content in the fermentation gas is as high as 80%. These experiments show that acetate degradation is a fast process resulting in total gasification and that no compartimentation of hydrogen takes place.

In the final experiment to a digestor fed 2.5 g/liter/day of cellulose, unlabeled volatile acids (acetate, propionate and butyrate) were added. The degradation of these acids and the gas production rates were measured. The pH was adjusted every day to 7.0. When acetate was fed instead of cellulose the degradation was faster than during feeding together with cellulose (fig. II.D.7). The higher the concentration of acetate, the more the degradation is slowed down by cellulose, accompanied

by a slight increase of the amount of propionate and butyrate. Degradation of propionate initially has a small lag-phase, but then starts rapidly, it is slowed down by cellulose and is completely stopped by butyrate addition. The propionate concentration increases a little, when the maximum capacity of the system is reached by cellulose addition. The degradation is continued when butyrate is used up and when the acetate concentration is less than 40 mM. Butyrate degradation is a very fast process with conversion rates of 2.5 mmol/liter/h at the beginning, but it is influenced by cellulose addition similar to the other acids. While butyrate is decomposed, acetate and to a minor extent propionate are enriched in the medium.

Total degradation of butyrate and propionate, added in concentrations of 40 mM to a cellulose digestor takes about 7 days, acetate degradation is much faster. The gas production rates increase with acetate and propionate addition. With butyrate addition first there is a decrease in the gas production for about 8 hr and then the rates increase again, but this may be due to cellulose addition at this point. Acetate is enriched to about 30 mM during butyrate degradation and also propionate increases, the enrichment starting, when cellulose is added at that time to the sludge. This indicates that acetate decomposition at this point is a limiting step, with acetate coming from cellulose, butyrate, possibly propionate and from external acetate sources.

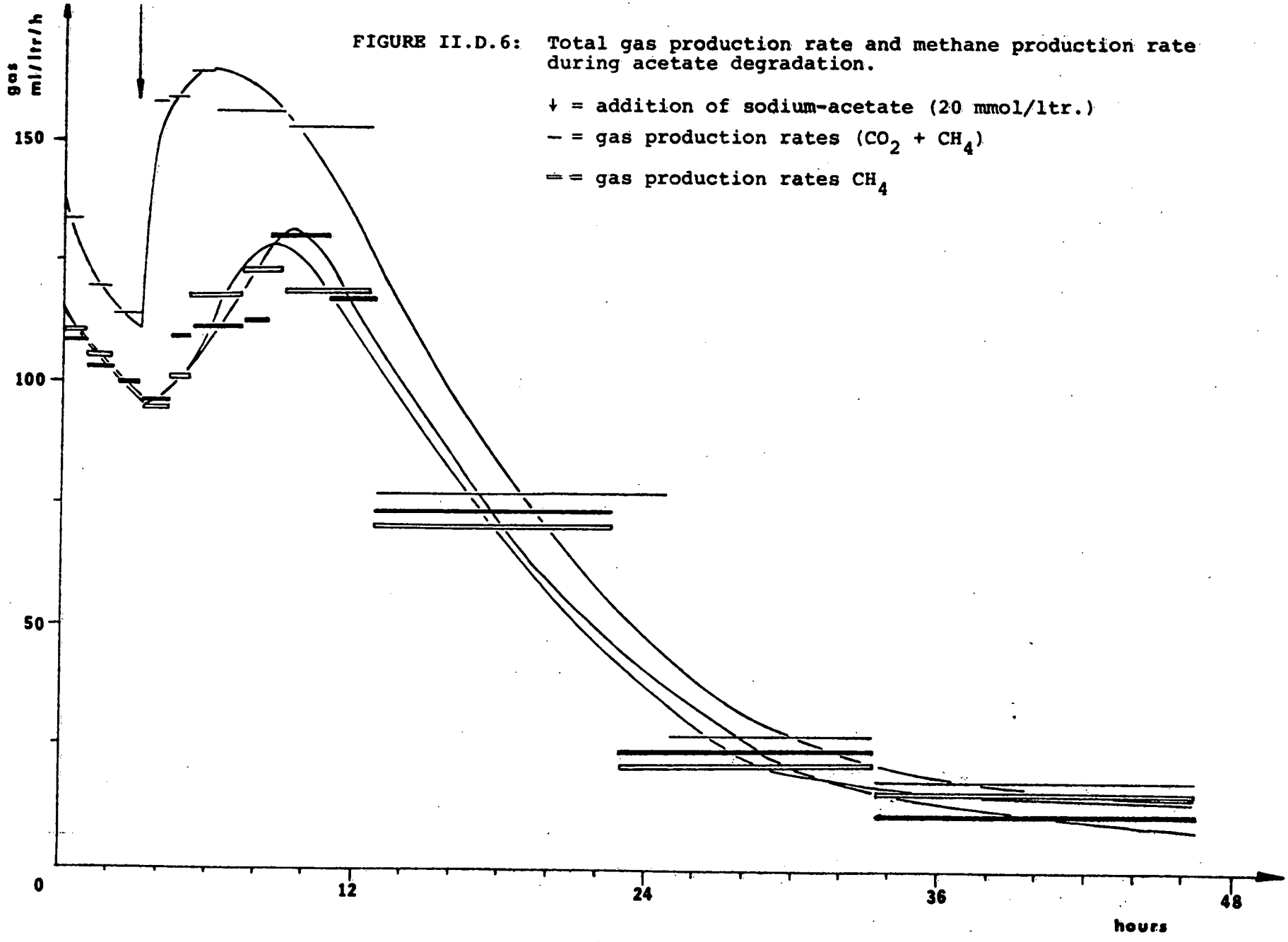


FIGURE II.D.6: Total gas production rate and methane production rate during acetate degradation.

↓ = addition of sodium-acetate (20 mmol/ltr.)

— = gas production rates (CO_2 + CH_4)

= = gas production rates CH_4

Figure II.D.7: The degradation of volatile acids and the gas productivity in a semi-continuously cellulose fermentor.

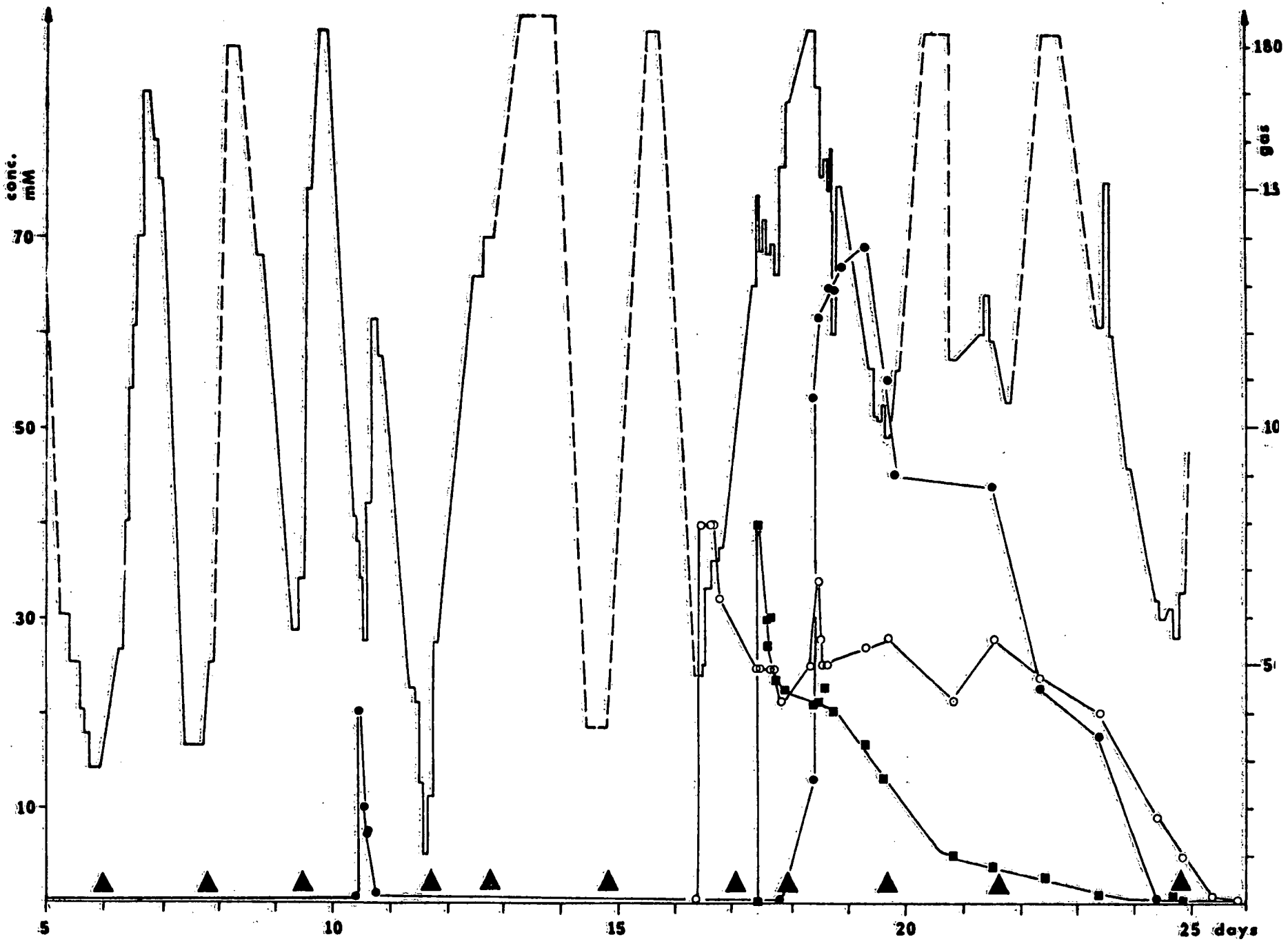
Reactor Volume: 4 liters

▲ = addition of 20 g Solka Floc

● = acetate, first peak 20 mmol/liter, second peak 40 mmol/liter added

○ = propionate, 40 mmol/liter added

□ = butyrate, 40 mmol/liter added


conc. mM
70
50
30
10
180
15
10
5
gas
5
10
15
20
25 days

5. Conclusion

A mixed culture was enriched from sewage sludge and was able to degrade cellulose and acetate rapidly and quantitatively to methane and CO_2. Pure cellulose was fermented at a maximum rate of 0.1 g/g of total cells/hour. Acetate is an intermediate of cellulose degradation and is fermented very fast in comparison to butyrate and propionate. Methane is formed from the methyl group without intermediate oxidation to CO_2 and also a great part of the methane is derived from carboxyl groups of acetate. This, however, goes at the expense of methane formation from CO_2 derived from cellulose. The total amount of methane from both substrates seems to be dependent on the amount of available hydrogen. The amount of carboxyl groups of acetate being reduced possiblely is a function of the ratio of acetate/cellulose in the broth. It may also be dependent on the strength of alkalinity.

* U.S. GOVERNMENT PRINTING OFFICE: 1977--740-306/203 Region No. 4