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MASTER

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

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Prepared for

THE U.S. DEPARTMENT OF ENERGY
CONTRACT NO. EG-77-S-02-4198

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

Report for First Quarter

February 1, 1978 through April 31, 1978

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SCOPE OF PROGRAM

This is a coordinated program to effect the microbiological degradation of cellulosic biomasses and 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 conversion from cellulosic biomasses to liquid fuel (ethanol) and/or soluble sugars by the cellulolytic, anaerobic organism is also within the scope of this program. Process and engineering scale-up, along with economic analyses, will be performed throughout the course of the program.

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 utilization of biomass will require the effective degradation of cellulose and hemicellulose. For this reason, studies on the xylanase activity of Clostridium thermocellum and other thermophilic anaerobes have been continued. It has been shown that the xylanase and CMCase activities of C. thermocellum during fermentation are nearly identical. Furthermore, extracellular enzymes from this organism exhibit equal xylanase and cellulase activities regardless of the type of cellulosic biomass used for growth. For example, when grown on corn stover containing cellulose and hemicellulose, or Avicel or cotton lacking hemicellulose, identical kinetics of cellulase and xylanase accumulations were observed. These results suggest that the enzyme complex is non-specific with respect to its hydrolytic actions on the substrate. However, it should be mentioned that even though C. thermocellum is capable of degrading xylans, it is not able to metabolize pentose or its oligomers. To effectively use all of the degradation products from biomass, especially xylans, we have continued our studies on an unidentified thermophilic and anaerobic (HG-2) organism that can metabolize xylose. This strain is also capable of producing extracellular xylanase as well as being environmentally compatible with C. thermocellum. Lastly, a variant of C. thermocellum has been isolated which is able to metabolize glucose. More important, this variant produces extracellular-cellulases (CMCase and filter paper activity) when it is cultivated on glucose.

The enzyme complex from C. thermocellum continues to show resistance to feedback inhibition in every assay system tested. No inhibitory effect on Avicel hydrolysis by 50 mg/l of glucose, cellobiose, or xylose was observed. Enzyme activity as measured by the hydrolysis of Remazol Brilliant Blue-bound MN300 cellulose was shown to be unaffected by 300 g/l of glucose or xylose and 100 g/l of cellobiose. A buffer system has been developed which will aid the problem of pH control in tube and shake flask cultures. It was found that MOPS buffer at 0.1 M will suitably maintain neutral pH to allow maximal cell growth and production of cellulases. Lastly, a new hyper-producing mutant has been isolated from our previous mutant AS-39. This new mutant, designated as AS-39-22, showed an increase of 40-50% in Avicel-hydrolyzing ability as well as TNP-CMCase production.

A number of different biomasses has been examined with respect to their degradation characteristics by C. thermocellum. These include thermally exploded poplar, corn cob granules, corn stover and sugar cane bagasse. C. thermocellum has been found

to be capable of degrading all of these materials, but the amount of reducing sugars accumulated was found inversely related to particle size. To increase biomass surface area, as well as biomass concentration, a packed-bed cellulose fermentor has been constructed and tested. It is possible to load up to 160 g/% of cellulose in such a reactor. C. thermocellum was shown to be adsorbed onto the bed and degrade cellulose producing reducing sugars while remained adsorbed on the cellulose bed.

The production of ethanol directly from cellulosic biomass by C. thermocellum continues to be a major effort in this program. Through serial adaptation and transfer, an isolate (S-4) has been obtained which can tolerate ethanol up to 5% (V/V). This isolate still retains its cellulolytic activity equal to the parent. Batch culture studies using cellobiose showed the production of ethanol up to 1% (V/V) with no observable inhibition. In addition, this isolate was also shown to be active in the degradation of corn stover. To further increase the tolerance of C. thermocellum to ethanol, the effect of temperature has been examined. Temperatures ranging from 45 to 65°C have been tested. An increase to ethanol tolerance was observed when a sub-optimal growth temperature of 50°C was tested. On the other hand, the resistance to acetate was found to be independent of the growth temperature.

A method for generating protoplasts from growing cultures of C. thermocellum has been developed. Maximum stabilization of the protoplasts was obtained with 20% sucrose. However, this concentration of sucrose is inhibitory to growth; thus, it precludes its use as a stabilizer of viable L-forms. Alternatively, polyethylene glycol shows promise as a stabilizer. Work on the plasmids of C. thermocellum continues to concentrate in their isolation and characterization. To date, the plasmids exhibit an anomalous sedimentation behavior in sucrose gradients. The data indicate that this is due to aggregation of the plasmids.

Studies on acrylic acid production have continued actively. Acrylic acid production by Clostridium propionicum has been further studied in order to optimize the oxidation of propionic and to acrylic acid. Optimal concentrations of resting cells and the effect of propionic acid concentration have been determined. The amount of acrylate produced has been found to increase with increasing propionate concentration, as well as with increasing cell concentration. Acrylate production is also stimulated by the presence of lactate in the reaction mixture.

Studies have been conducted on the aerobic oxidation of propionic acid to acrylic acid by resting cells and cell-free extracts prepared from Escherichia coli. Although a variety of experimental conditions have been employed, no acrylic acid was detected. Similar experiments with lactic acid also failed.

Production of lactic acid has been investigated this quarter by employing mixed cultures of Clostridium thermocellum and a thermophilic lactic acid producing bacterium. Preliminary results are very encouraging. A mixed culture fermentation lasting 140 hours was successful. It was found that lactic acid concentration in the fermentor was 5.0 gm/l at 15 hrs after inoculation of the mixed culture. Reducing sugars increased to a level of 2.5 gm/l during the same time period. From the 60th to the 120th hours of the rate of lactic acid accumulation remained constant, but at a low rate (0.05 gm/l-hr), the results demonstrate direct conversion of cellulose to lactic acid via mixed culture fermentation.

The acetone-butanol fermentation has focused on means of increasing butanol tolerance by Clostridium acetobutylicum. The effect of temperature on inoculum preparation and growth has been hypothesized to influence solvent tolerance. Inoculum and growth temperatures ranging from 30 to 45°C were tested in various combinations. It was found that when an inoculum temperature of 30°C and followed by a growth temperature of 30°C, 50% increase in the butanol tolerance can be achieved.

Acetic acid production by Clostridium thermoaceticum has continued to isolate product tolerant strains. A new isolate (S-3) has been obtained which can tolerate up to 60 g/l of sodium acetate. Detailed studies have also shown that using pH control (7.0) the acetate ion is the causative agent in the inhibition of growth. Additional results have confirmed that non-growing cells of C. thermoaceticum can produce acetic acid. These confirmation studies were performed using an antibiotic rifamcin which is able to totally inhibit growth, but the organism shows continued acetic acid production. In addition, quantitative examination of the viable cell population has also been performed and also shows the production of acetic acid in the absence of growth. Cell recovery studies have also been initiated to examine cell recycle fermentations. An inexpensive flocculant, Bentonite, has shown to be effective at 1 g/l to enhance the sedimentation rate of C. thermoaceticum by 3700-fold.

DISTRIBUTION LIST

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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. The percentage efforts of the Principal and Co-Principal Investigators on this project are shown along with the names of the different participants.

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This progress report acknowledges the contributions by the individuals listed above, as well as the research assistants, post doctoral research associates and technical assistants associated with this project.

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I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. ENZYME PRODUCTION BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

The economic feasibility of a microbial process for biomass utilization is largely dependent upon the price of the raw material to be hydrolyzed and further converted to products. If extensively pretreated, purified celluloses are required, the process immediately becomes less desirable compared to a process based on the use of a cheap cellulosic material. This substrate constraint has prompted the examination of C. thermocellum's behavior on materials such as corn stover. Since a significant portion of this and other "realistic" substrates is hemicellulose, or xylan, a need for information concerning the utilization and enzymatic hydrolysis of xylan by C. thermocellum arose.

Initial studies in this vein were reported last period (Dec. 1, 1977-Feb. 28, 1978). The results indicated that xylan itself was unsuitable as growth substrate, yet the extracellular enzymes produced during fermentation on Solka floc (a material which is 10% xylan) could hydrolyze xylan as well as soluble CMC. Indeed, the kinetic profiles of extracellular xylanase and CMCase accumulation were nearly identical, suggesting the possibility that the same enzyme is responsible for both activities. That xylanase and cellulase are the same enzyme, however, was not proven conclusively.

Follow-up studies to the initial work have been performed. C. thermocellum has been cultured on corn stover and the presence of xylanase has been demonstrated. The organism was also cultured on avicel and cotton, substrates which contain no hemicellulose; nonetheless, xylanase was measured. A fermentation with a mixture of Solka floc and xylan as substrate was run to determine if xylan would be utilized under these conditions, compared with its poor use when cultured on xylan alone. Sugars accumulating during this fermentation as well as corn stover fermentations were subjected to HPLC analysis to determine the products of simultaneous cellulase and xylanase hydrolysis.

2. Materials and Methods

a. Culture Conditions

C. thermocellum, ATCC 27405, was cultured in CM3 medium, as described previously, with a variety of materials as growth substrates. When the organism was cultured on avicel or cotton, it had previously undergone three successive transfers, in flasks, in CM3/avicel medium. This would effectively dilute any hemicellulose that may have remained in the original stock culture tube (CM3/Solka floc) by a factor of approximately 1×10^6 . Two different types of corn stover were used as substrates. The difference was the size of the particles, finely powdered or coarsely ground in a Wiley mill.

b. HPLC Analysis

The system is the same as described in a previous progress report (Sept. 1, 1977 - Nov. 30, 1977) with the following changes: The column used is a Whatman Partisil 10-PAC analytical carbohydrate column preceded in series by a guard column containing HC-Pellosil, a silica pellicular packing material. The solvent used is an 80:20 mixture of acetonitrile and 0.0025M KH_2PO_4 (pH 5.0 with KOH) respectively. Mixed bed ion exchange resins were no longer employed for sample clean-up. Solka floc samples were ultra filtered as described previously. Samples from corn stover cultures were passed through Pasteur pipettes containing Waters Bondapak Corasil/AX and Bondapak Porasil/C18 to remove lignins and organic acids.

Other Methods

All enzyme assays and the reducing sugar assay have been described in a previous progress report.

3. Results and Discussion

The final values of xylanase and CMCase activities, and their ratios, for flask cultures of C. thermocellum on Solka floc, corn stover, avicel and cotton, are presented in Table I.A.1.

TABLE I.A.1

<u>Substrate</u>	<u>*Xylanase Mg/ml·hr</u>	<u>*CMCase mg/ml·hr</u>	<u>Xylanase CMCase</u>
Solka floc	7.0	6.75	1.04
Corn Stover	1.95	1.9	1.03
Avicel	3.0	3.2	0.94
Cotton	4.8	5.0	0.96

*The variation in the values among the substrates reflects the time when the fermentations were ended as well as C. thermocellum's ability to grow and produce enzymes on the particular substrate.

The kinetic profiles of xylanase and CMCase during a fermentation on Solka floc were presented in our last report and were shown to be practically identical. This is true of the xylanase and CMCase profiles for avicel and corn stovers as well. (Only a final sample of the cotton culture was taken due to sampling difficulties; thus the kinetic pattern was not followed). Thus, not only are the final ratios of xylanase to CMCase approximately equal to 1 (or more importantly, constant), but also the ratios of the two activities throughout the fermentations. Because of the similarity of the kinetics of both kinds of enzyme production regardless of the substrate tested, together with the presence of xylanase in avicel and cotton cultures (substrates unlikely to induce an enzyme specific for xylan), a plausible explanation is that the same nonspecific enzyme is responsible for both cellulose and xylan hydrolysis. The significance of this inference is the following: if xylanase and CMCase activities are indeed effected by the same enzyme, then any genetic improvements in cellulase activity of C. thermocellum should be accompanied by a concomitant improvement in xylanase activity. Thus, rather than having to "hit" two gene loci for hyper-producing mutants, a single mutation may be all that is necessary to cause increases in both activities. Such a mutant would therefore have improved capabilities for degrading both the hemicellulose and cellulose present in natural substrates.

When C. thermocellum was grown in batch, no pH control, on 0.5% Solka floc plus 0.5% xylan, both the cellulose and xylan were degraded as can be seen by the residual solids curve in Figure I.A.1. Reducing sugars, measured both as xylose and as glucose, accumulated to approximately 5g/l. The maximum level of pellet protein reached, 500 µg/ml, which is roughly equivalent to 1 g/l of cells, seems too high for the amount of carbohydrate consumed. It is thought that the unusually high value of pellet protein can be attributed to interference by xylan in the Lowry protein assay. If the Lowry values are correct, then the yield of cells on the mixed substrate is around 20% which is higher than the usually observed 15% on cellulose. In Figure I.A.2, the kinetic profiles of extracellular xylanase and CMCase are shown. As in the case of the other substrates tested, the profiles and final levels reached are very similar.

In Figure I.A.1, we saw that both cellulose (Solka floc) and xylan had to have been degraded since 1.0 - 1.5 g/l of solids remained at the end of the fermentation from the initial amount of 10 g/l total. However, in order to determine whether any of the xylan had actually been utilized for cell growth (as opposed to simply accumulating in the broth) under these conditions, it was necessary to analyze the accumulated sugars by HPLC. Such an analysis would ideally indicate whether certain sugars were preferentially consumed while

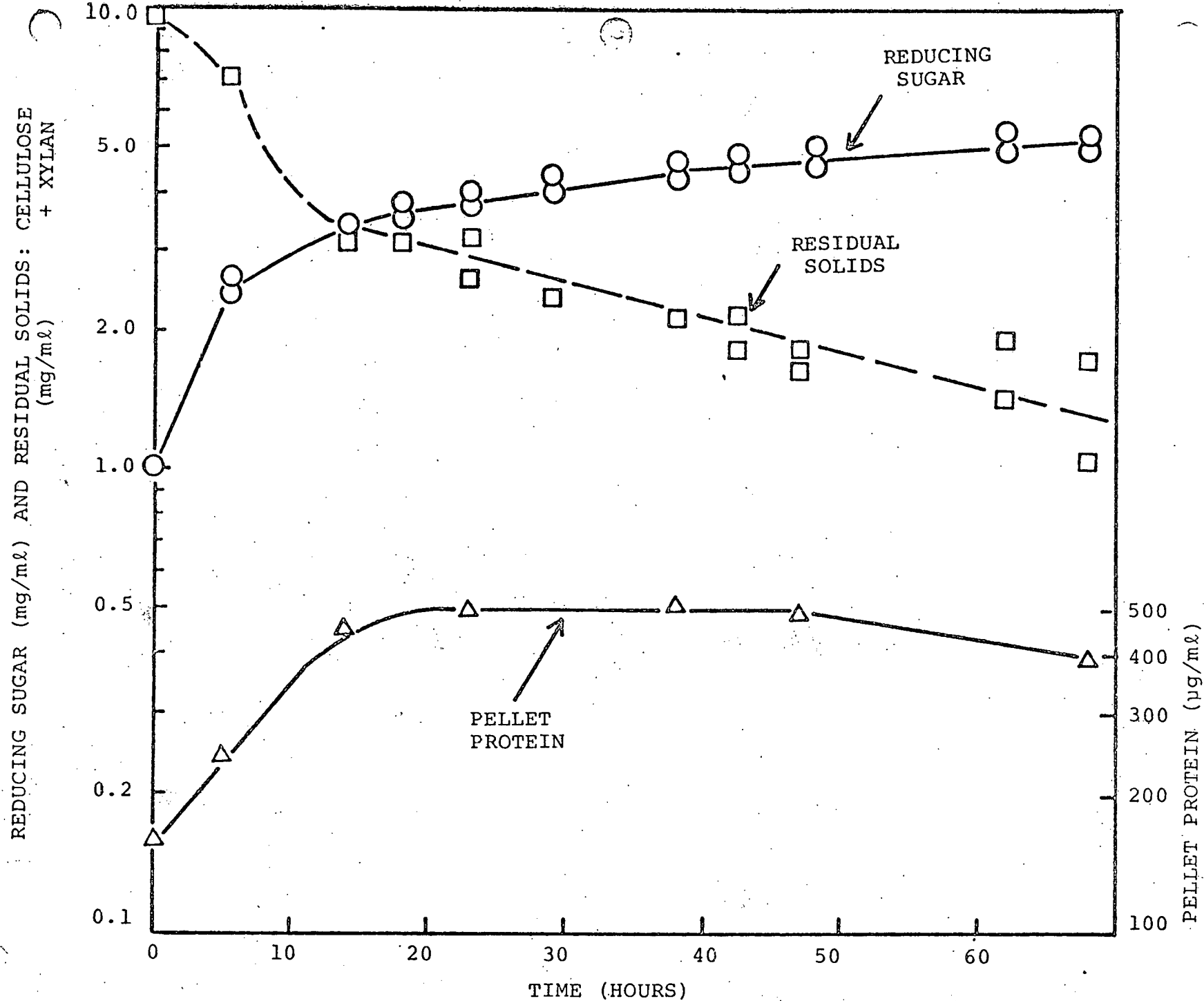
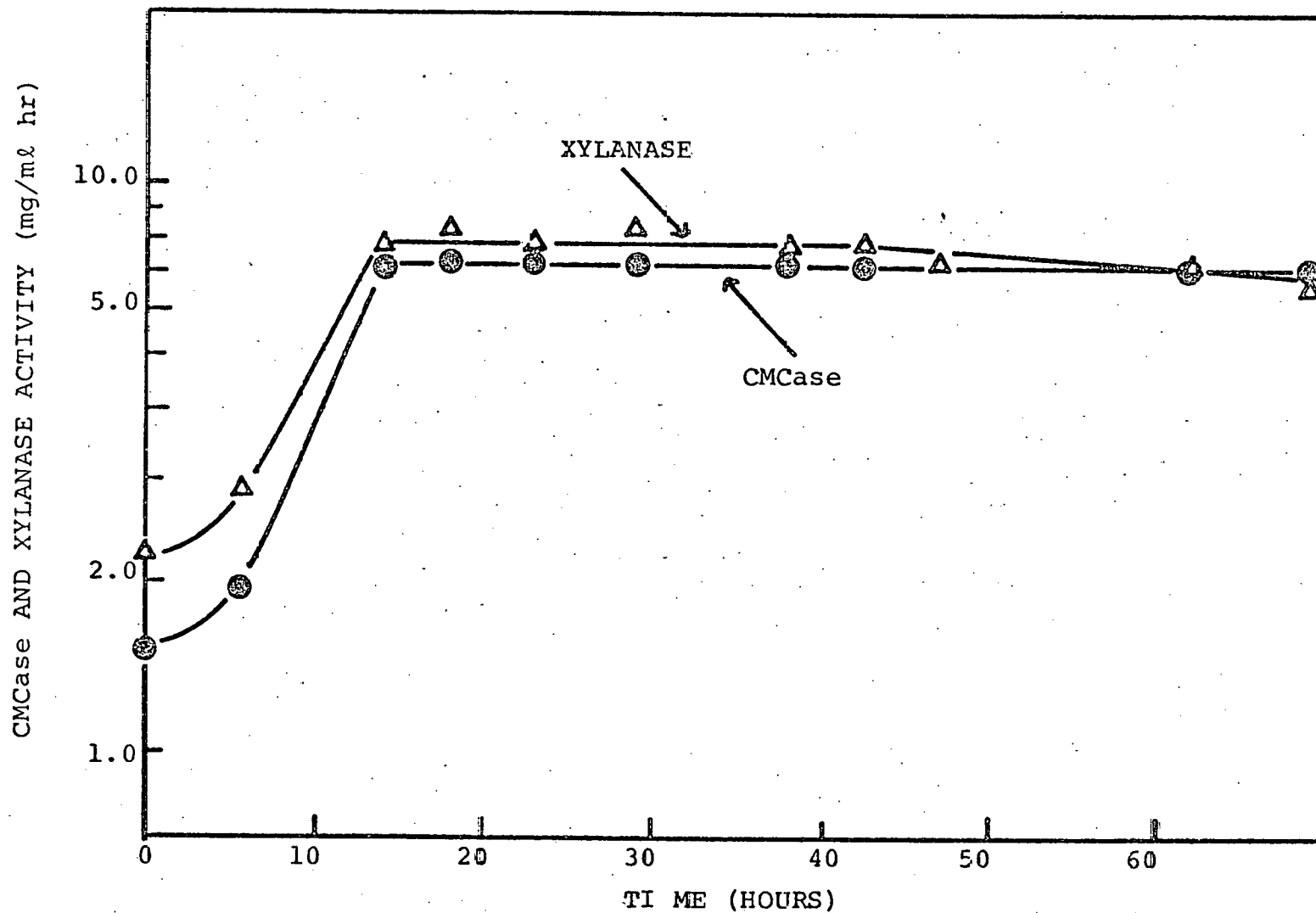


FIGURE I.A.1: KINETICS OF BATCH FERMENTATION OF *C. THERMOCELLUM* ON CM 3 MEDIUM CONTAINING 0.5% SOLKA FLOC + 0.5% XYLAN

FIGURE I.A.2: CMCase AND XYLANASE ACTIVITY PROFILES FROM FERMENTATION OF C. THERMOCELLUM ON 0.5% SOLKA FLOC PLUS 0.5% XYLAN



others that were also hydrolyzed accumulated. Unfortunately, this analysis is complicated by the fact that glucose and xylobiose do not resolve. This can be seen in Figures I.A.3 and I.A.4. Figure I.A.3 is a chromatogram of a standard mixture of xylose, glucose and cellobiose. Glucose has a retention time of ~11.4 minutes. Figure I.A. 4 is a chromatogram of a xylobiose sample obtained from the Finnish Sugar Co. It is claimed to be 90% xylobiose, with 5% contamination by glucose and 5% by some trisaccharide. If the major peak in this chromatogram (peak #2) is indeed xylobiose, then it is obvious that its retention time is so similar to that of glucose that the two would be indistinguishable as a mixture. There is the possibility that peak #2 is glucose and peak #3 is xylobiose. Until a purer preparation of xylobiose can be obtained, it is impossible to make any qualitative or quantitative assessment of glucose and xylobiose accumulated in fermentations on substrates containing significant fractions of cellulose and nemicellulose. Figures I.A.5 and I.A.6 are chromatograms of broth samples from the mixed substrate fermentation and a fermentation on powdered corn stover, respectively. In Figure I.A.5, it is obvious that in this 48 hour sample the major amount of material present appears to be glucose or xylobiose or perhaps both. Significant amounts of xylose and cellobiose accumulate as well and the kinetic pattern of these two sugars is shown in I.A. 7. It should be noted that at time

FIGURE I.A.3: HPLC CHROMATOGRAM OF STANDARD SUGARS: XYLOSE, GLUCOSE AND CELLOBIOSE, EACH AT 1.0 g/l

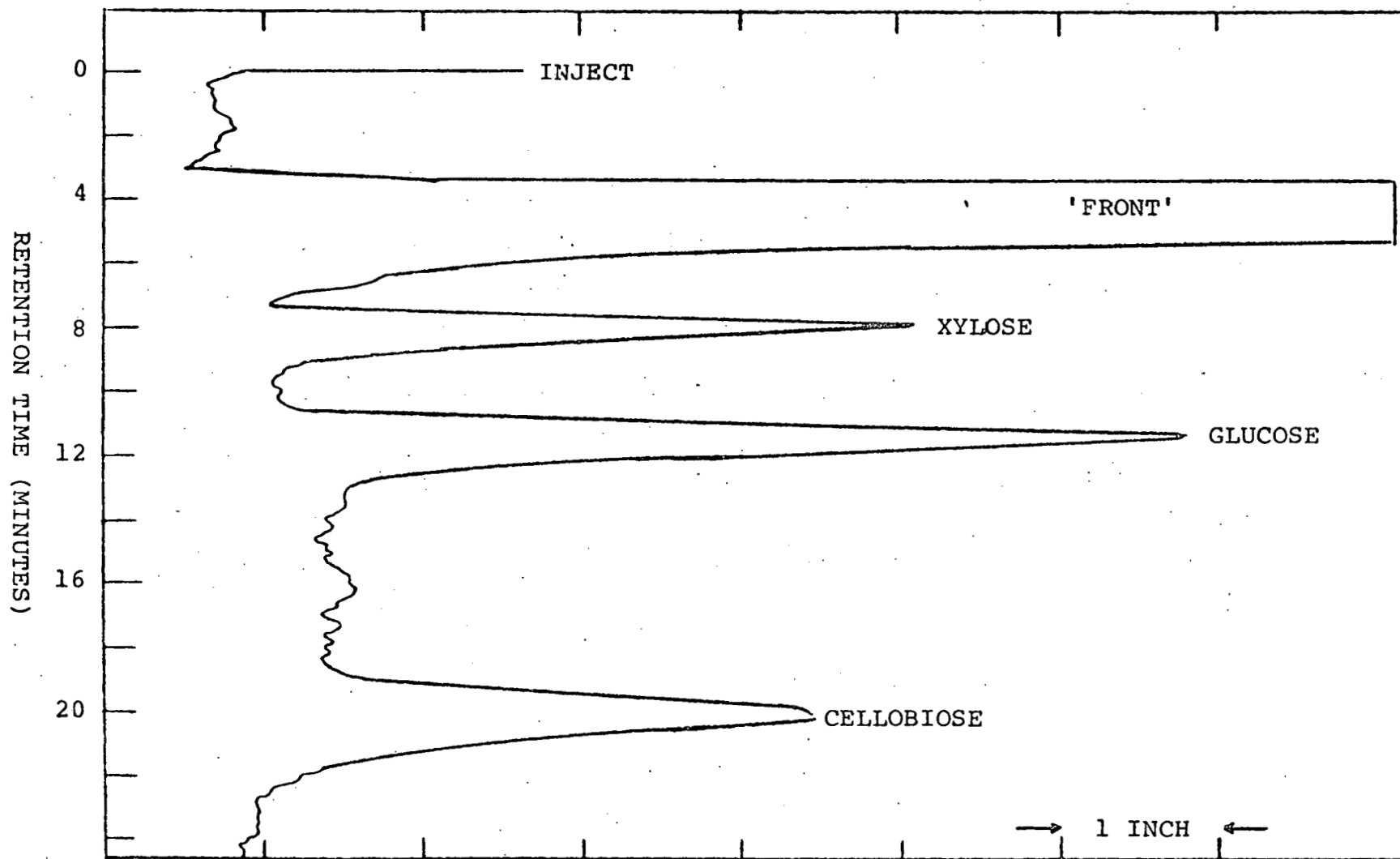
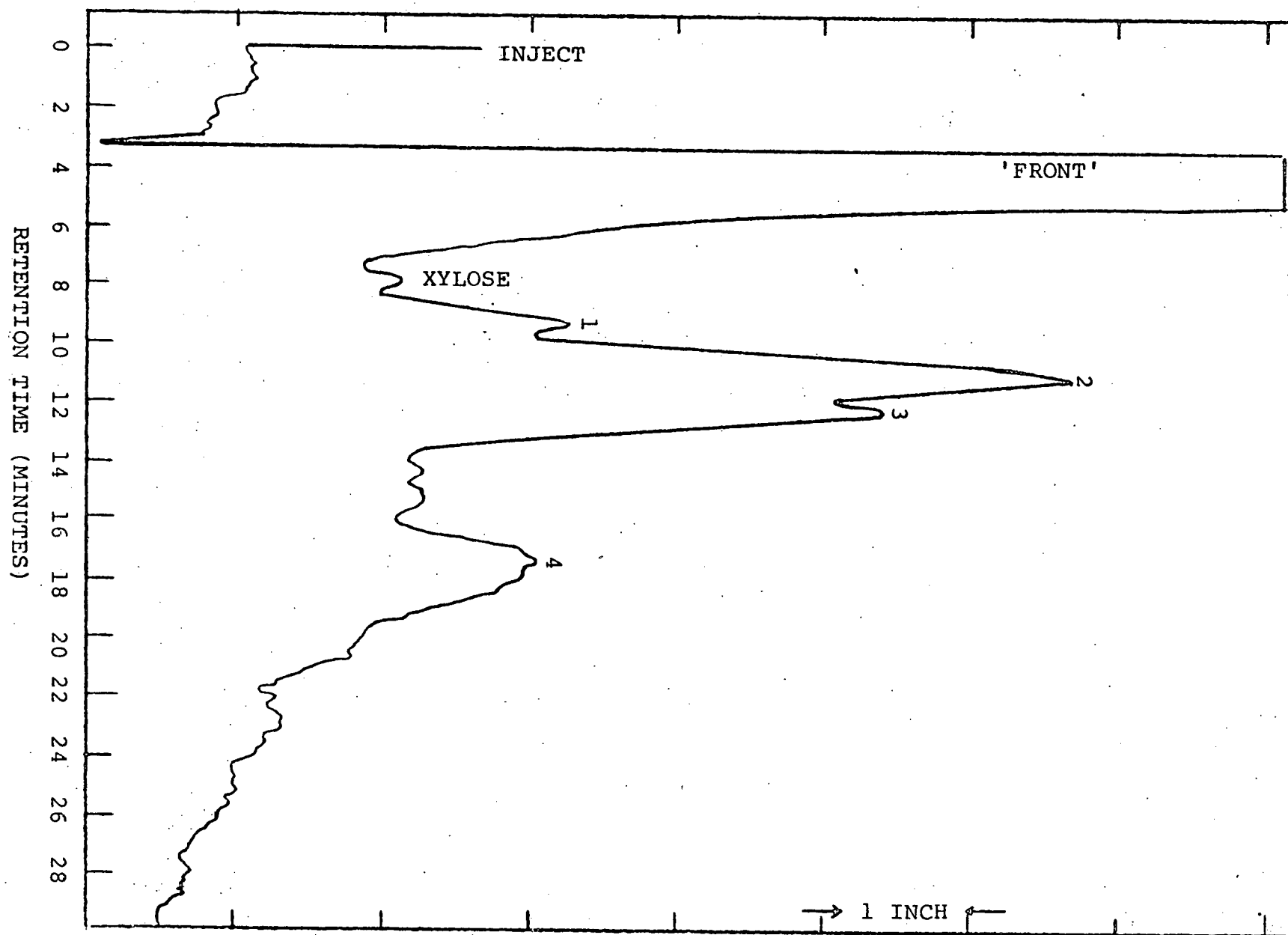


FIGURE I.A.4: HPLC CHROMATOGRAM OF "XYLOBIOSE" SAMPLE



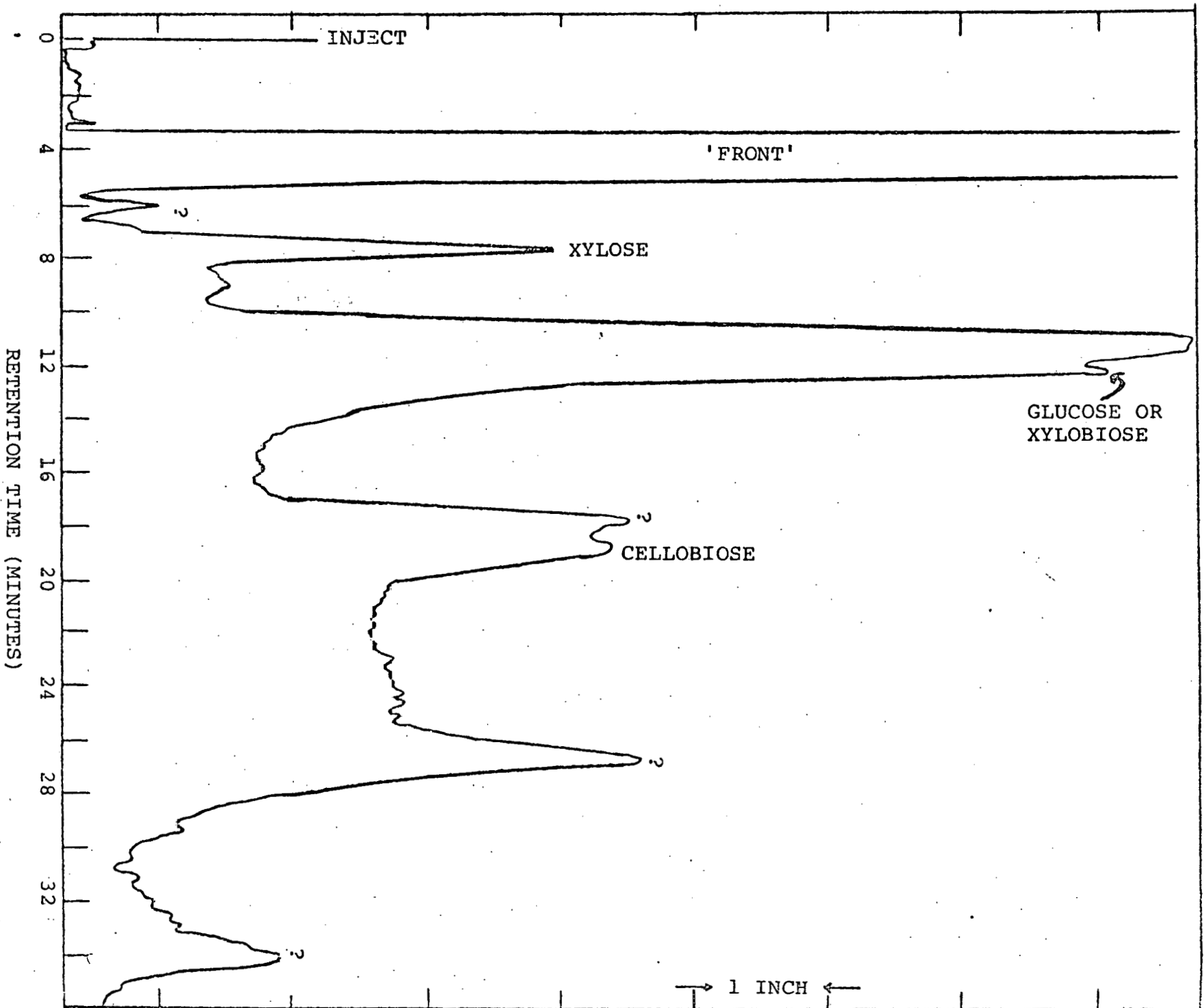


FIGURE I.A.5: HPIC CHROMATOGRAM OF BROTH SUPERNATANT FROM
FERMENTATION OF C. THERMOCELLUM ON SOLKA
FLOC AND XYLAN

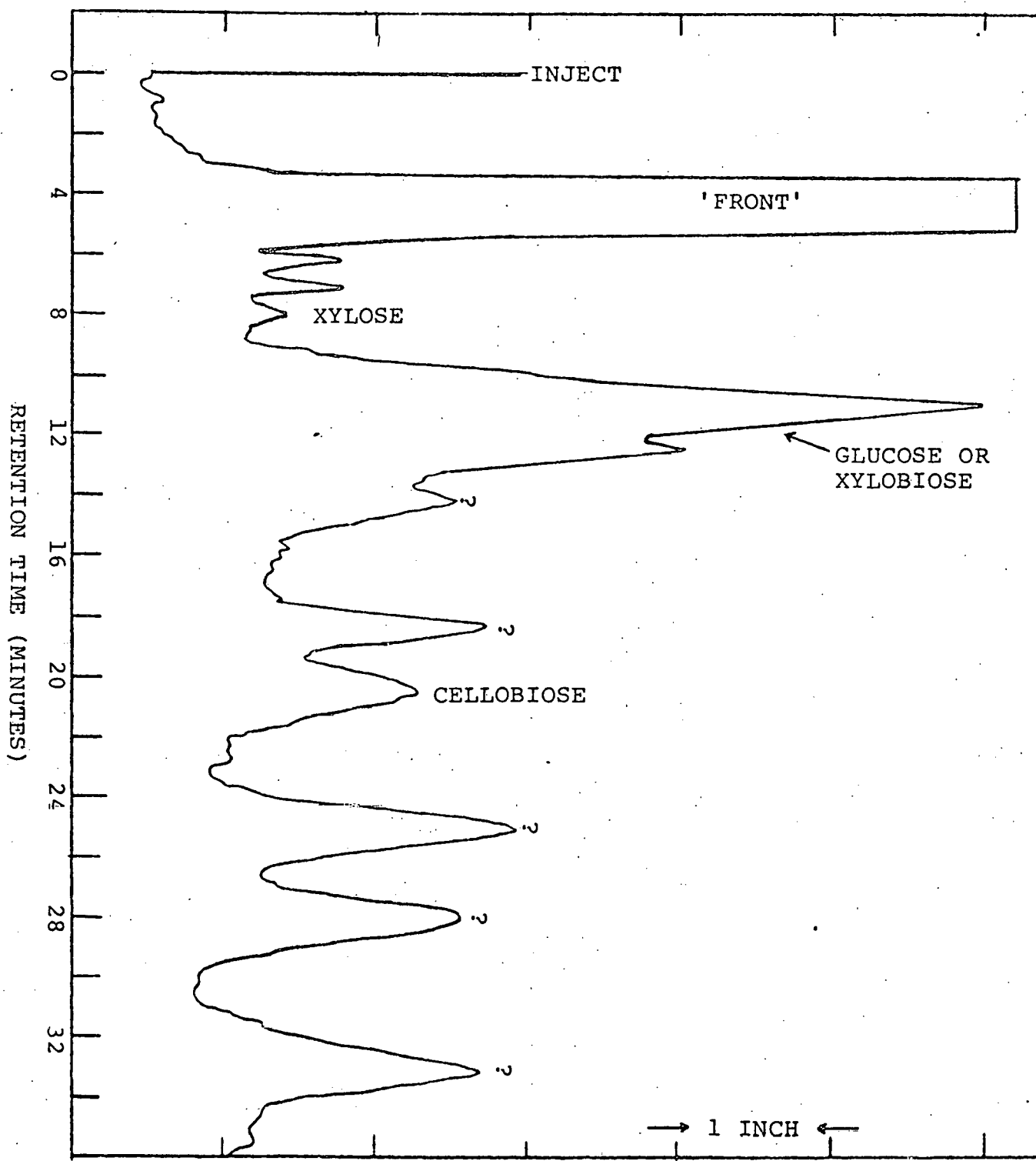
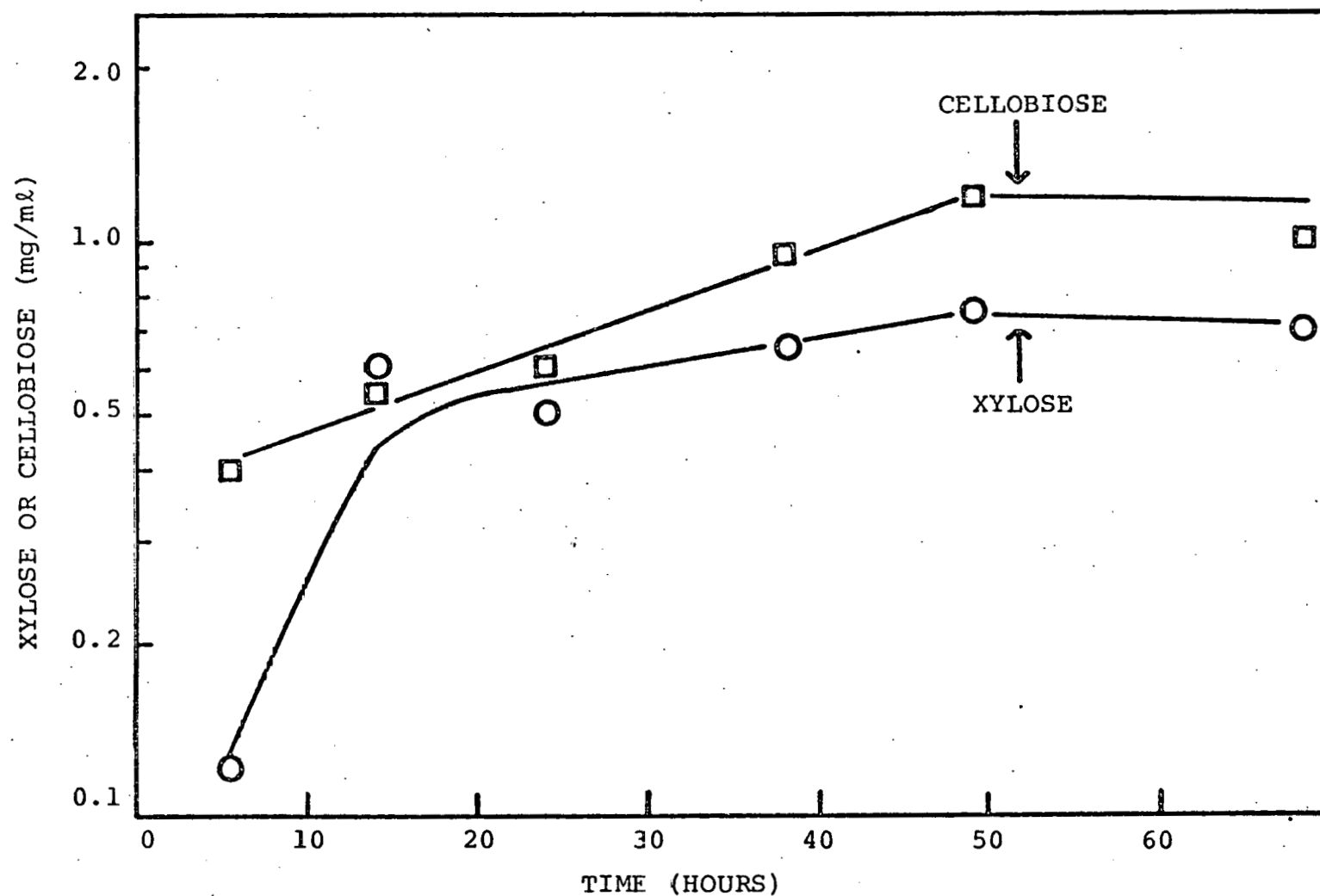


FIGURE I.A.6: HPLC CHROMATOGRAM OF BROTH SUPERNATANT FROM FERMENTATION OF C.THERMOCELLUM ON CORN STOVER

FIGURE I.A.7: PROFILE OF XYLOSE AND CELLOBIOSE ACCUMULATION,
AS ANALYZED BY HPLC, FROM FERMENTATION OF
C.THERMOCELLUM ON 0.5% SOLKA FLOC + 0.5% XYLAN



zero, no xylose or cellobiose were present.

Figure I.A.6 is a chromatogram of a 68 hr. sample from a fermentation on corn stover. Again the major material appears in the glucose-xylobiose region. Some cellobiose (~ 0.5 g/l) and a small amount of xylose (~ 0.2 g/l) are present. It is obvious that there are several unidentified peaks (which had not been seen when the mixed bed ion exchange resins were used for precleaning samples). It is likely that some of these compounds contribute to the total reducing sugar value and thus partially explain the discrepancy between the earlier HPLC analysis and total reducing sugars measured. None of the three peaks beyond cellobiose is cellotriose, as the latter has a retention time of ~ 38 minutes. These 3 peaks are present early in the fermentation and remain at relatively the same height in all samples analyzed. They are present in the broths of Solka floc cultures as well.

Until glucose and xylobiose can be resolved, little else can be said except that one or the other sugar, or possibly both are the major hydrolysis products of substrates like corn stover. As glucose is the major hydrolysis product from purer celluloses, it is speculated that it is a major product from this natural substrate as well.

B. Degradation of Cellulosic Biomass to Produce Soluble Reducing Sugars by Clostridium thermocellum (the Packed-Bed Fermentor)

1. Introduction

In any actual process for the production of reducing sugars and other useful products from biomass, a necessary requirement will be the ability to operate or feed cellulose at high concentrations. Concentrated cellulose slurries are highly viscous, difficult to pump and difficult to stir in conventional agitated fermentors. However, the mechanical properties of cellulose could be effectively exploited in a packed-bed fermentor where the cellulose forms the stationary phase. In this fashion, a high concentration of cellulose can be maintained and overcoming the previous problems. In this segment of the report, we will present our initial findings in the use of a packed-bed cellulose fermentor for the degradation of biomass to produce reducing sugars.

2. Materials and Methods

a. Organism

Clostridium thermocellum ATCC 27405 was used through this study.

b. Culture Conditions

The organism was grown on CM3 medium (Progress Report COO-4198-1). The Ca and Mg concentrations were reduced for this study to one-half of that previously reported to prevent precipitate formation. Inoculum was prepared by growing the organism on cellobiose (10 gm/l) in CM3 medium for 24 hours.

c. Description of Packed-Bed Fermentor

The packed-bed fermentor was made of glass containing two main parts as shown in Figure I.B.1. The packed-bed portion is a glass cylinder having 300 ml total volume. The upper portion of this fermentor contains a glass bulb which was used to separate the gas evolved during fermentation. In addition, in the upper section of the packed-bed, glass wool was packed to act as a retaining medium for the cellulose. Medium can be pumped to the packed-bed through an extended glass tube into the bottom of the bed. Liquid flows upward through the bed into the gas-liquid separator. The entire bed was submerged into a constant temperature water bath at 60°C. Although not shown in Figure I.B.1, it is also possible to circulate the fermentation broth through the packed-bed in an external loop. In this external loop, samples can be removed and pH can be controlled through base addition.

d. Analytical Techniques

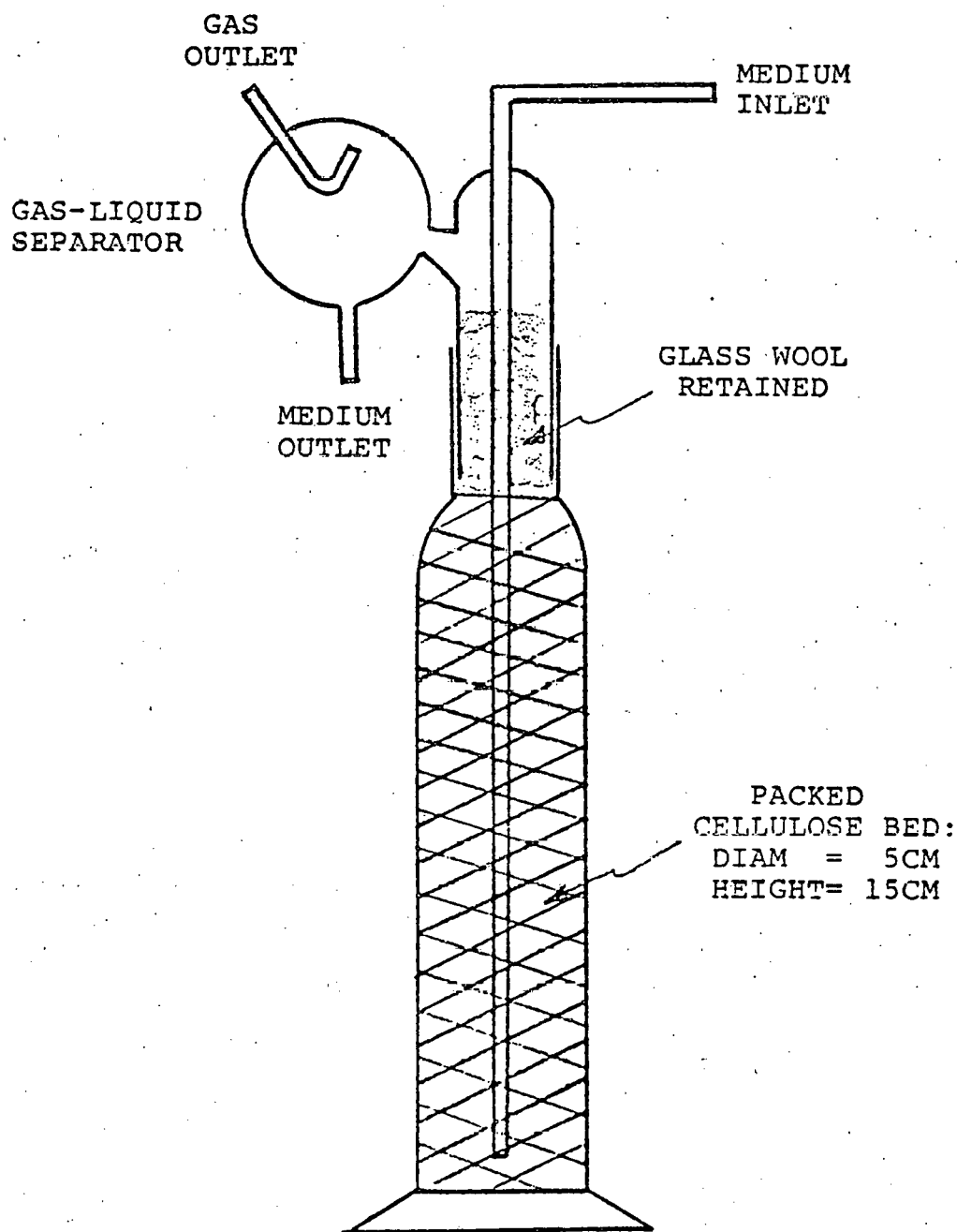
The analytical techniques used to monitor the fermentations have all been reported in our previous reports. These include reducing sugars by DNS, cell growth by turbidity (Klett-Summerson), acetic acid and ethanol by gas chromatography and CMCase by DNS reagent.

3. Results and Discussion

a. Fluid Dynamic Characterization of Packed Bed

It was recognized at the onset of our packed-bed fermentor studies that liquid flow through the cellulose

FIGURE I.B.1: DIAGRAM OF PACKED-BED FERMENTOR (SCALE 1:2)

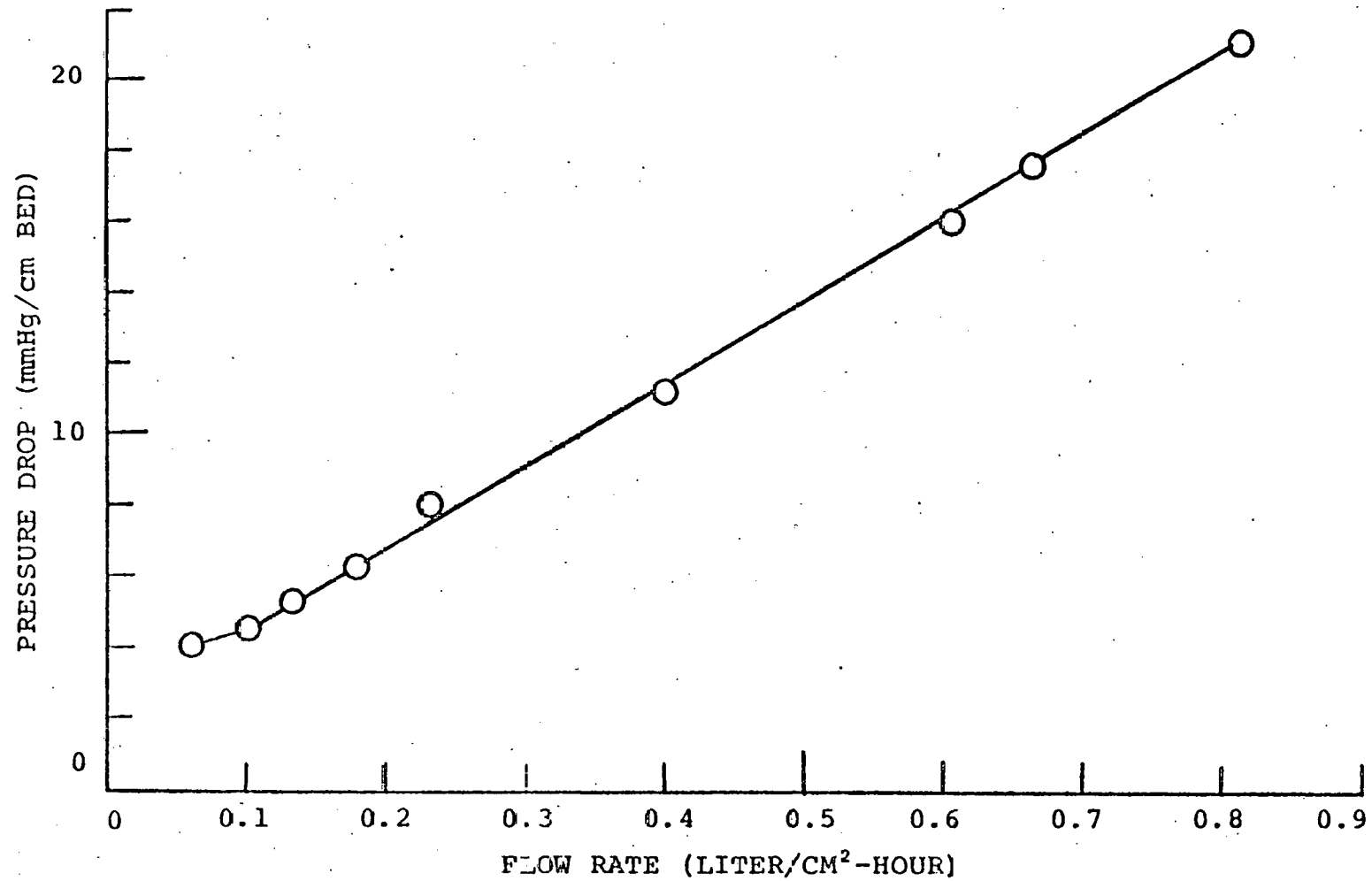


will be required. This requirement arises from the need for gas removal due to metabolism, to achieve continuous operations, and to operate with liquid recirculation for environmental control. In order to estimate the rate of liquid flow which can be achieved, pressure drop studies in the packed-bed using Solka floc were performed. The bed was loaded with 167 grams of cellulose per liter which has a total cross sectional area of 48 centimeters. Water was then circulated through and the pressure across the bed was then measured. The results from these studies are shown in Figure I.B.2. It is seen that a linear relationship between the flow rate and pressure drop is obtained. Furthermore, reasonably high flow rates can be achieved at modest pressure drops. For example, for the packed-bed fermentor as shown in Figure I.B.1, it is possible to circulate a liquid flow of 38 liters/hr. ($0.8 \text{ l/cm}^2\text{-hr}$) at a total pressure drop of 300 mm Hg ($20 \text{ mm Hg/cm} \cdot 15 \text{ cm}$). This moderate pressure drop can be easily attained in our laboratory system and the liquid flow appears to be reasonable at this time to achieve the necessary objectives.

b. Continuous Packed-Bed Fermentation Without Liquid Recirculation

The first trial in examining the performances of the packed-bed cellulose fermentor was attempted in a continuous mode of operation. Cellulose (Solka floc) was packed into the fermentor at a concentration of 50 gm/liter. The

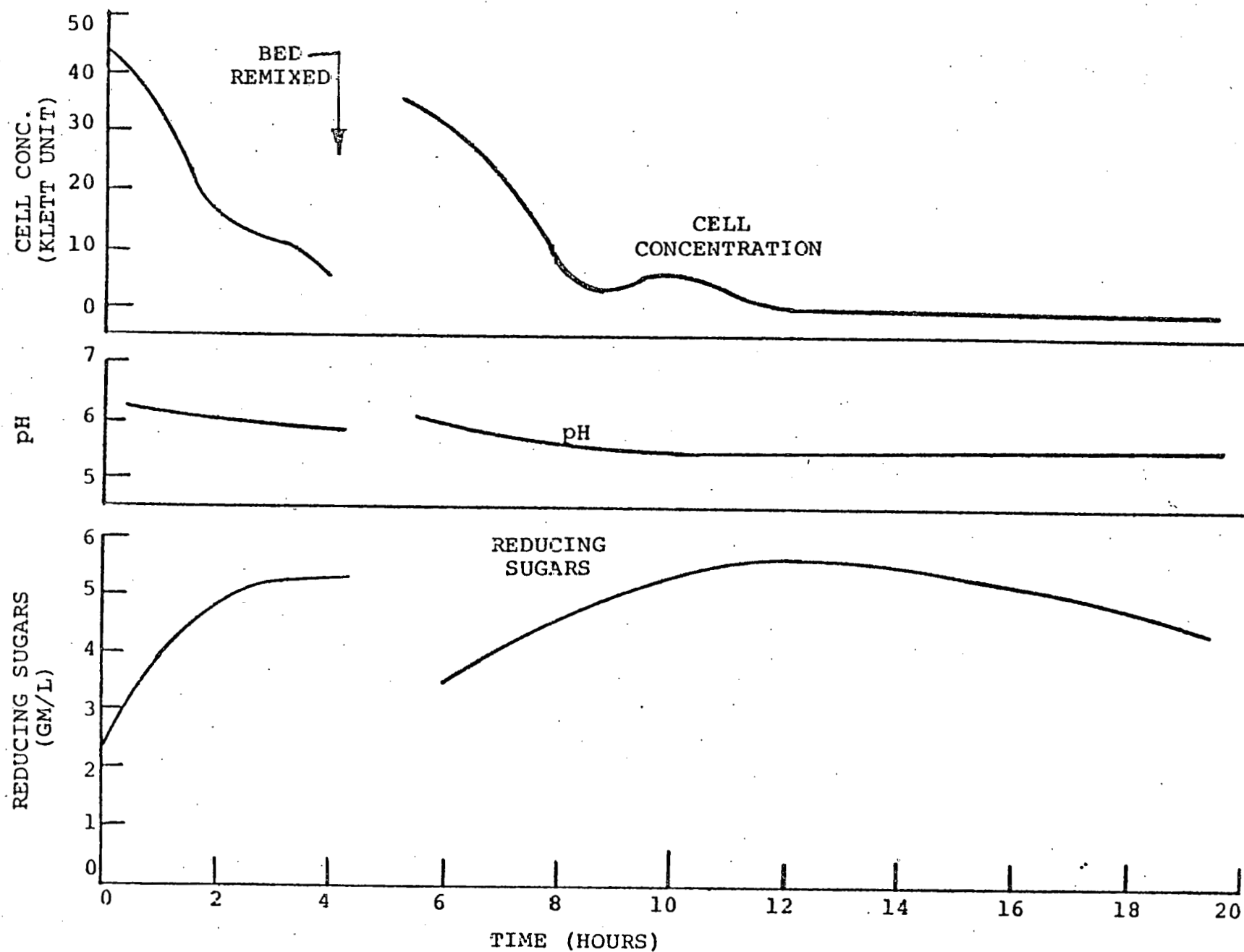
FIGURE I.B.2: PRESSURE DROP OF PACKED-BED FERMENTOR
(CELLULOSE LOADING = 167 GM/LITER)



medium was CM3 and its composition previously presented (COO-4198-1). The start-up procedure consisted of the following. Approximately 200 ml of inoculum was pumped to the packed-bed displacing about 60% of the liquid initially in the fermentor. Medium feed and removal at 21 ml/hr, equivalent to a dilution rate of 0.07 hr^{-1} , was initiated 5 hours after inoculation. During the first 20 hours, there was little microbial action as observed by the physical appearance of the cellulose bed and the lack of gas formation. However, after 20 hours, considerable activities began to be expressed by the microorganisms. The results during this period of fermentation are shown in Figure I.B.3.

It can be seen from this Figure that the reducing sugar concentration began to increase from 2 gm/l to 5.5 gm/l. This was accompanied by a decrease in the pH indicating that acidic products (acetic) are being formed. It is also interesting to note that the cell concentration (Klett units) also began to decrease during this period. It is difficult to interpret this data, but the following explanation seems plausible. In the first 20 hours, a fraction of the inoculated cells was biologically inactive and this fraction was being continuously washed-out from the fermentor. However, the active organisms were able to adsorb onto the cellulose producing more cells which also are able to adhere onto the substrate. The net result being the formation of the end products, reducing sugars, acetic acid and ethanol.

FIGURE I.B.3: DEGRADATION OF CELLULOSE (SOLKA FLOC) BY *C. THERMOCELLUM* IN CONTINUOUS PACKED-BED FERMENTOR (DILUTION RATE = 0.07 HR^{-1})



During this active growth phase (first 4 hours of Figure I.B.3), we noted large amounts of gas formation as well as a shrinkage of the bed. Due to the utilization of the cellulose, it became necessary to remix and repack the bed at about the 4th hour as shown in Figure I.B.3. This repacking procedure is the reason for the discontinuity of the results shown in this Figure. Again, a transient period of behavior resulted and finally attaining a steady-state operation as shown from this Figure. During this steady-state period, the exit cell concentration was quite low (less than 10 Klett units corresponding to about 0.03 gm/liter) and the reducing sugar concentration was constant at about 5.5 gm/liter. At the end of the operation (20 hours), the packed-bed was disassembled and approximately 57% of the cellulose had been hydrolysed and about 0.85 gm/liter of cells was attached to the undegraded biomass.

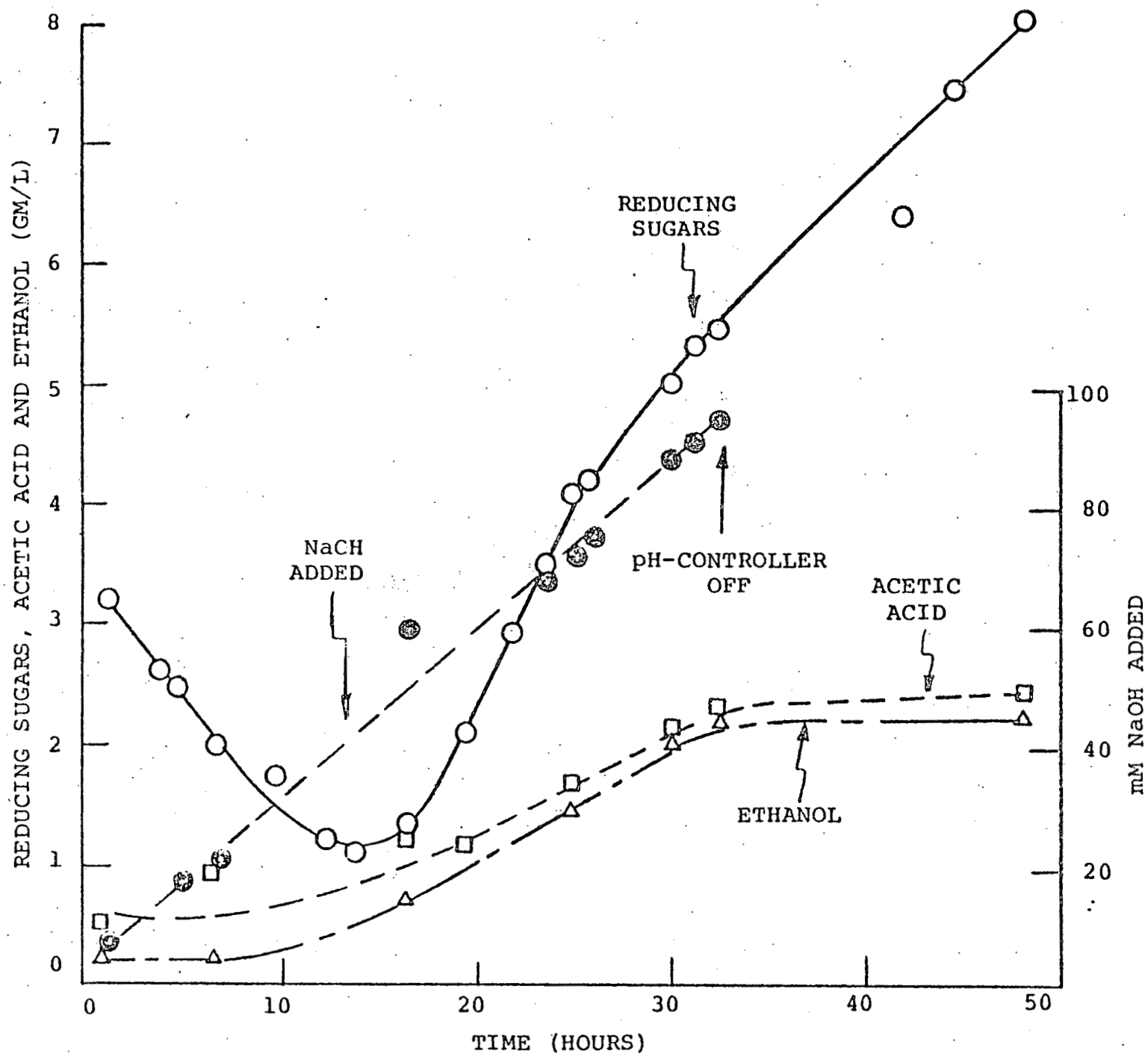
c. Batch Packed-Bed Fermentation with Liquid Recirculation

Results from the preliminary study on the continuous operation of the packed-bed fermentor suggested that data analysis and interpretation are difficult due to the transient behaviors. It was therefore decided that the packed-bed fermentor be examined in a batch-mode where the medium is recirculated through the bed by an external loop. The bed (300 ml) was packed with Solka floc (35 gm) equivalent to a

concentration of 110 gm/liter. The recirculation loop was a vessel one liter in volume where pH (6.1) and temperature (60°C) were both controlled. The liquid circulation rate through the bed was maintained at a rate of 1.6 liter/hour. Four hundred milliliter of a 24-hour old inoculum grown on cellobiose was used. The results from this study are shown in Figure I.B.4.

Since the inoculum used was a healthy culture, immediate gas evolution was noticed. In addition, the medium exiting from the packed-up was totally clear containing no cells. The initial decrease in the reducing sugar was the result of metabolism of the organism on the residual cellobiose from the inoculum. Upon exhausting of the cellobiose in the first 12 hours, the organism began to produce reducing sugars, acetic acid and ethanol from the cellulose. It should be noted that, throughout the course of the fermentation, we did not observe significant amounts of cells exiting from the packed bed. During the subsequent 20 hours (12 to 32 hours), product formation was maximum. Unfortunately, at this time, the pH-controller malfunctioned and therefore the pH decreased immediately to about 5.6. This was accompanied by the cessation of acetic acid and ethanol formation, but reducing sugars continued to accumulate. The addition of NaOH during the portion when the pH controller operated properly, indicated a linear rate. A final reducing sugars concentration of 8 gm/liter was found at the end of the fermentation.

FIGURE I.B.4: BATCH OPERATED PACKED-BED FERMENTATION WITH pH CONTROL



The results from this fermentation are summarized in Table I.B.1. There are several interesting observations which should be noted. The degree of cellulose degradation (67%) was quite comparable to earlier studies using Solka floc. More important, the total cells which were adsorbed onto the cellulose bed were found to be nearly 14 gm/liter. This is extremely high as compared to stirred tank fermentations where cell concentrations are typically 1.0 to 2.0 gm/liter. This result indicates that the packed-bed served as an excellent collector where inherent cell recycle can be achieved. These results are extremely encouraging since they show that the use of packed-bed fermentors could be a solution in attaining high substrate and product concentrations.

4. Future Work

The future studies on this segment of the research include:

- Detail studies to obtain kinetic parameters of packed-bed fermentor.
- Examine the degradation of corn stover in packed-bed fermentor.
- Continuous operation of packed-bed fermentor with capability of cellulose addition.
- Attain high cell mass leading to high productivity and high product concentrations.

TABLE I.B.1: SUMMARY OF RESULTS FROM BATCH PACKED-BED FERMENTATION BY C. THERMOCELLUM USING SOLKA FLOC

Cellulose concentration in packed-bed	110	gm/liter
Cellulose concentration calculated over total volume (including recirculation reservoir)	38	gm/liter
Cellulose degraded	67	%
Total time	48	hours
Final cell concentration in packed-bed	14	gm/liter
% cell in solution	10	%
Final reducing sugars concentration	8	gm/liter
Final ethanol concentration	2.2	gm/liter
Final acetic acid concentration	2.4	gm/liter
Final CMCase activity	36	mg/ml-Hr

C. Production of Ethanol from Cellulose by Clostridium Thermocellum

1. Isolation and Characterization of New Ethanol Tolerant Strain (S-4) of C. Thermocellum

a. Introduction

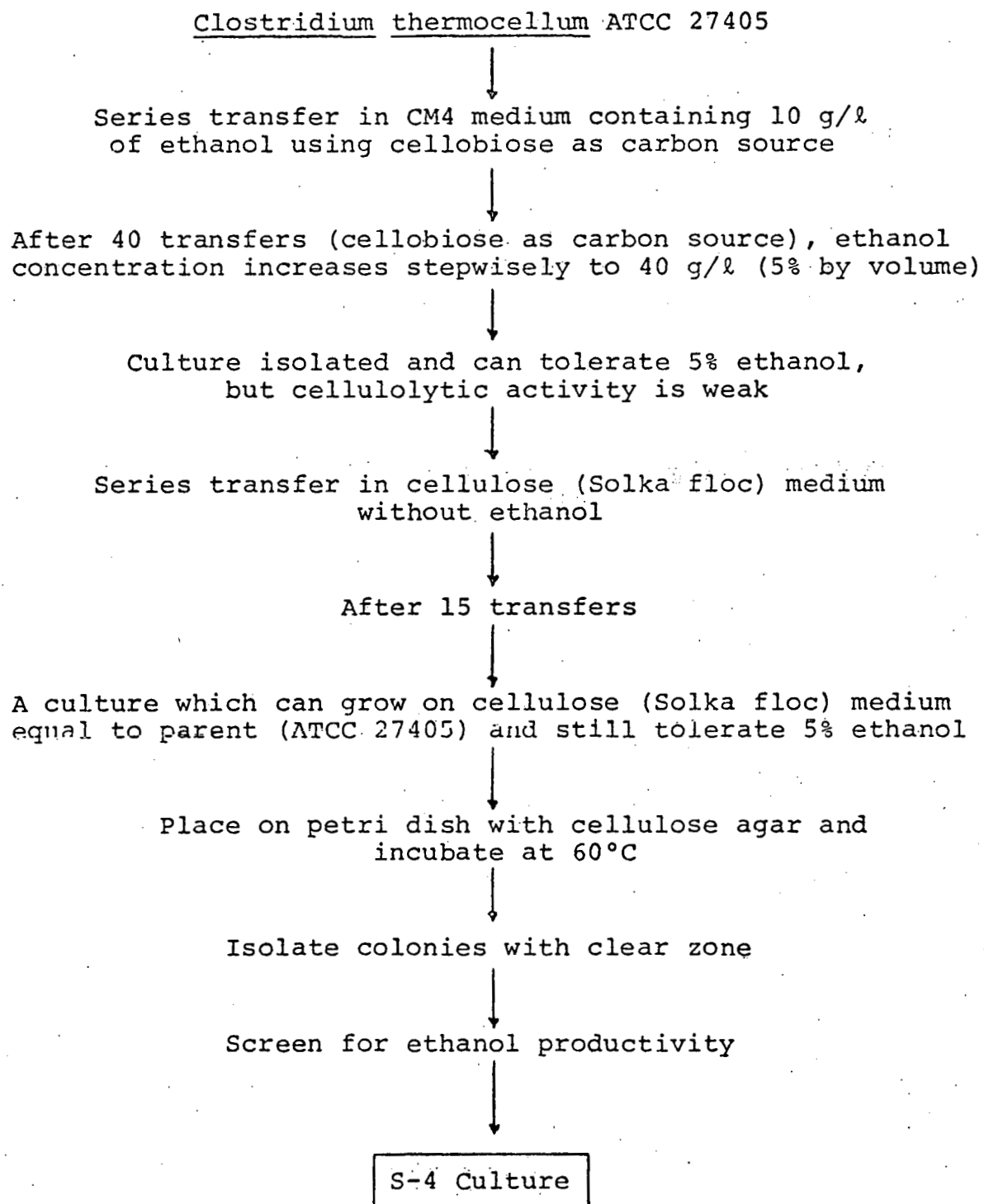
In our last Progress Report (COO-4198-5), we reported that at 3% of ethanol or acetic acid causes nearly total inhibition of growth for C. thermocellum. To produce ethanol from biomass economically, the concentration of ethanol must be increased. In this period of our contract research, we have therefore devoted a significant effort in the isolation of an ethanol tolerant strain of C. thermocellum. The characterizations of this new strain will be presented in this report. In addition, some preliminary results of this new strain on its degradation capabilities on corn stover will also be presented.

b. Materials and Methods

The microorganism used throughout this study was Clostridium thermocellum. The parent strain, designated as ATCC 27405, was originally obtained from American Type Culture Collection, Rockville, Maryland. The new strain isolated for ethanol tolerance is designated as "S-4". The medium used in the studies is CM4 whose composition has already been reported.

An adaptation technique was used in the isolation of the ethanol tolerant strain. The procedure employed in our adaptation and selection program is shown in Figure I.C.1.1. The parent, ATCC 27405, was first serially transferred in CM4

FIGURE I.C.1.1: ADAPTATION AND ISOLATION PROCEDURE FOR ETHANOL TOLERANT STRAIN OF CLOSTRIDIUM THERMOCELLUM



medium using cellobiose as the carbon source and containing 10 g/l of ethanol. The parent was then transferred in liquid medium (cellobiose as substrate) containing stepwise increase of ethanol up to 40 g/l (5% V/V). A total of 40 transfers were performed during this adaptation process to higher concentrations of ethanol. A strain which can tolerate 5% V/V of ethanol was isolated, but was found to have weak cellulolytic activity when grown on Solka floc. This strain was then passed through 15 transfers in the absence of ethanol onto medium containing Solka floc as the carbon source. The ability of this strain to grow on Solka floc equal to the parent, but tolerant to 5% (V/V) ethanol were the criteria used. Single colony isolation was then performed using solid medium containing Solka floc. This isolate is designated as "S-4" and subsequently tested for its properties.

All of the analytical procedures have already been reported and will not be repeated. The only exception is the determination of ethanol through gas chromatography. This was achieved using a Teflon column (5 ft X 1/8" OD) containing Chromosorb 101. Samples were acidified using 1 drop of 5N HCl for each milliliter of fermentation broth. The column temperature was programmed from 120°C to 160°C at a rate of 20°C/minute. The initial temperature was maintained for 2.5 minutes and the final temperature maintained for 3 minutes in order to remove other organic acids from the

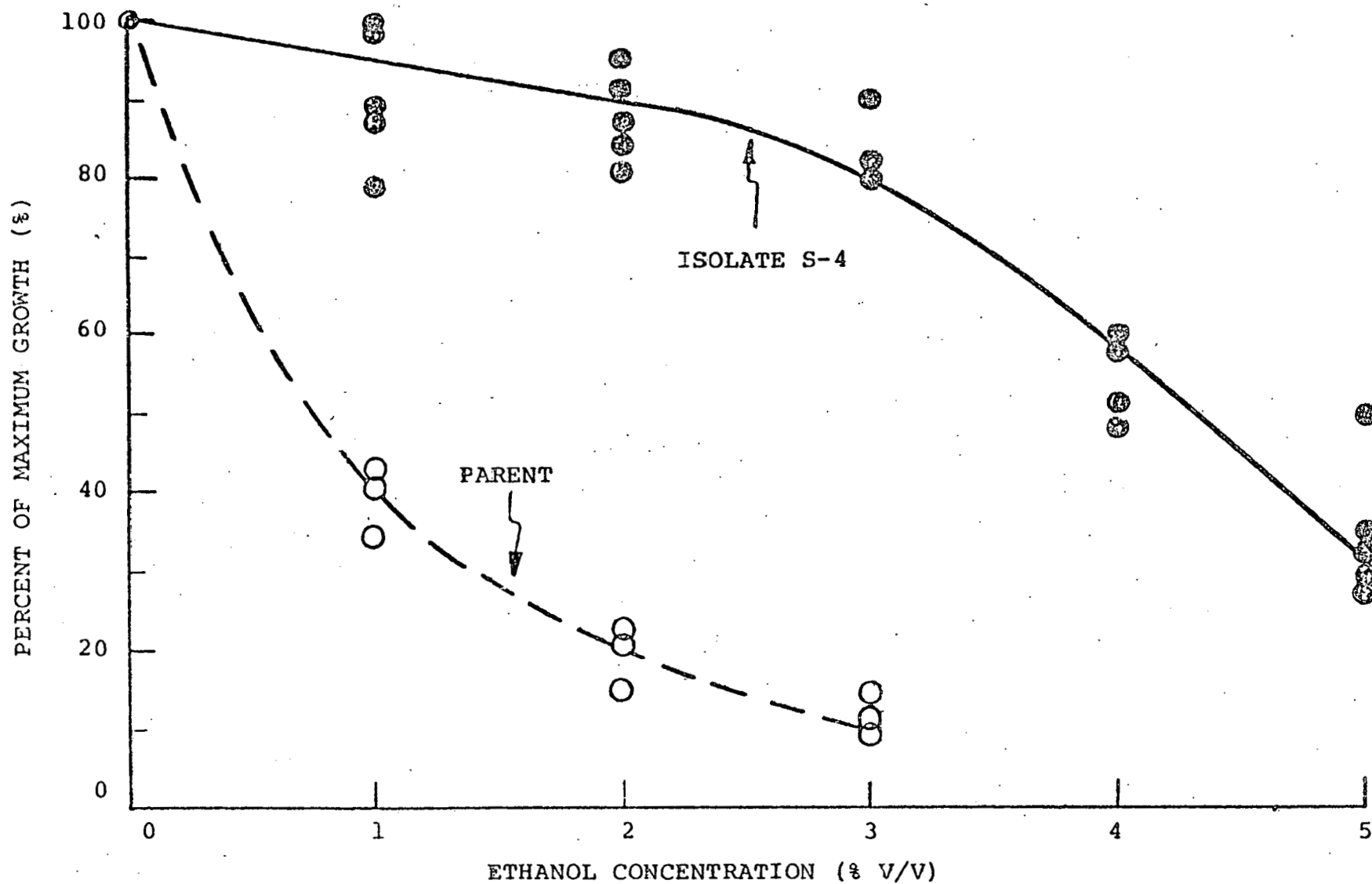
column. Nitrogen was used as a carrier gas at a flow rate of 60 ml/min.

The corn stovers used in our studies were kindly furnished by Professor George T. Tsao, School of Chemical Engineering, Purdue University, West Lafayette, IN. The original corn stover contained cobs, stalks, and leaves in sizes up to 6 inches in length. It therefore necessitated some degree of size reduction. This was accomplished through ball-milling, kindly performed by the U. S. Army Natick Laboratories, Natick, MA (Drs. M. Mandel and F. Allen).

c. Results and Discussion

Using the adaptation and isolation procedures previously outlined, a strain of Clostridium thermocellum, designated as "S-4" was found to tolerate ethanol up to 5% V/V. This isolate was then tested for its growth capabilities in Hungate tubes containing cellobiose as the carbon source. The isolate was inoculated into medium containing ethanol up to 5% (V/V) and growth was measured as a percent of maximum (0% ethanol) after 40 to 50 hours of incubation. The results from these studies are shown in Figure I.C.1.2. The tolerance of the parent strain to ethanol is also shown in this Figure. It can be seen that the new isolate (S-4) exhibits a much improved tolerance to ethanol, especially between 2 to 3% V/V. For example, at 2% ethanol, the percent of maximum growth for S-4 and the parent were respectively 90% and 20%. It should be noted

FIGURE I.C.1.2: COMPARISON OF ETHANOL TOLERANCE ON GROWTH FOR PARENT (ATCC 27405)
AND ISOLATE S-4 OF CLOSTRIDIUM THERMOACETICUM

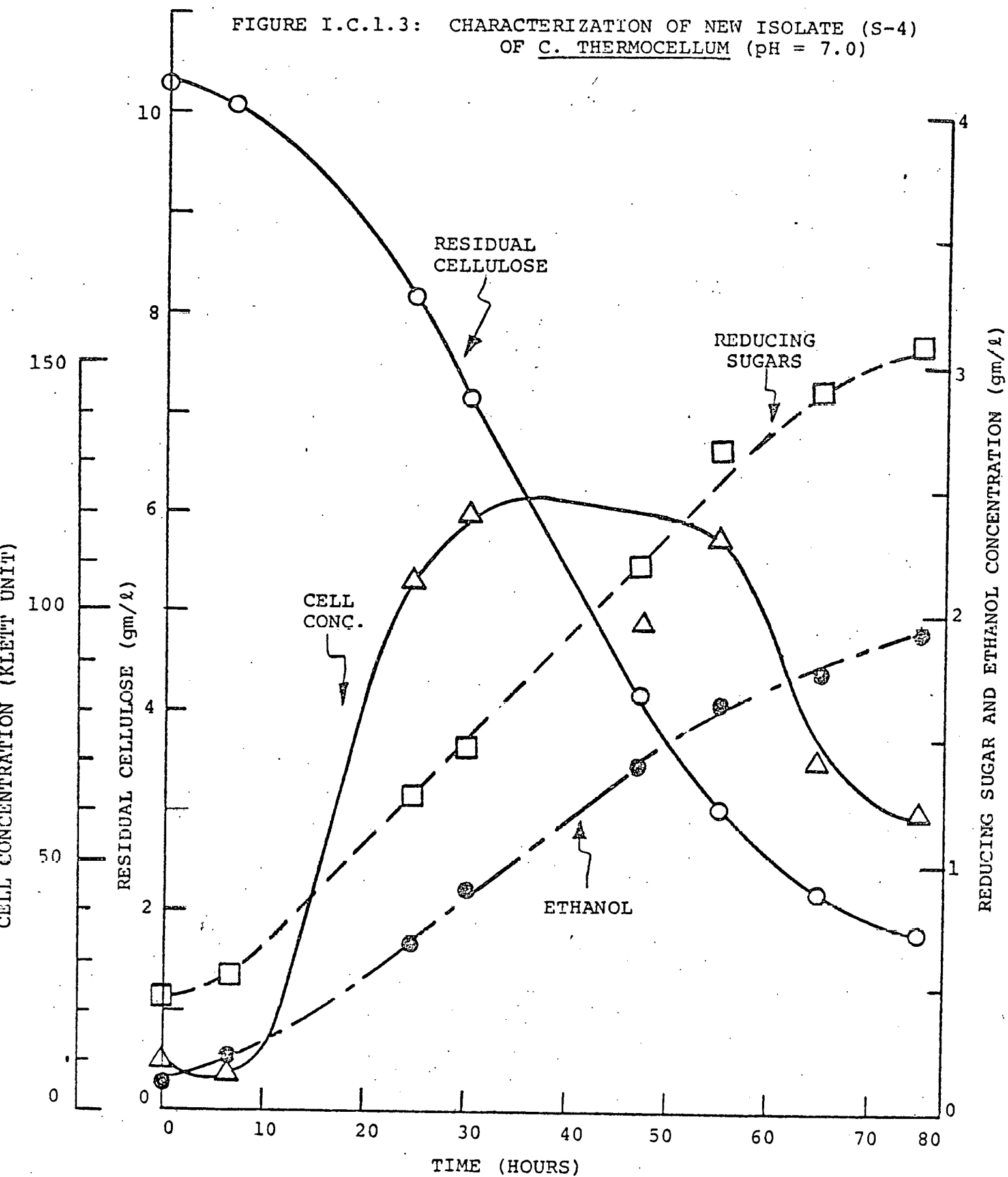


that the new strain is still inhibited by 5% ethanol to the extent of about 70%.

Since our adaptation and selection method employed cellobiose as the carbon source, the quantitative ability of the new strain to degrade cellulose is not known. To test strain S-4's cellulolytic capabilities, a fermentation study was performed using Solka floc as the substrate and the results are shown in Figure I.C.1.3. From an initial cellulose concentration of 10.5 g/l, over 80% of the substrate was degraded. This degree of degradation is quite similar to the parent strain of Clostridium thermocellum. It is seen from the result that the final reducing sugars obtained was 3.1 g/l and ethanol was 2.0 g/l. Although acetic acid production was not measured, it is reasonable to assume that its behavior will be similar to those found in the past (i.e., equal to the amount of ethanol). We conclude therefore the new isolate (S-4) is able to degrade cellulose at efficiencies similar to the parent. It is interesting to note that if we assume that acetic acid formation was equal to that of ethanol, nearly 7 g/l of products (reducing sugars, ethanol and acetic) can be derived from 8.5 g/l of cellulose degraded.

Having now the knowledge that the new isolate is able to degrade cellulose, we next asked the question whether this strain is able to degrade cellulose in the presence of ethanol. Experiments were therefore performed where the parent and

FIGURE I.C.1.3: CHARACTERIZATION OF NEW ISOLATE (S-4)
OF C. THERMOCELLUM (pH = 7.0)



S-4 were first cultivated on Solka floc as the carbon source for two days and in the absence of ethanol. These cultures served as the inoculum for subsequent testing. 24 Hungate tubes were prepared for each strain where the initial ethanol concentration was increased from 0 to 5% (V/V) in 1% increments. These tubes were then inoculated and incubated at 60°C without pH control for different periods of time. The residual sugar concentration and the reducing sugar formation were then measured during incubation. The results from these studies are shown in Tables I.C.1.1 through I.C.1.6 for ethanol concentrations of 0, 1, 2, 3, 4 and 5% (V/V), respectively. In general, the results showed that at all concentrations of ethanol tested, the new strain (S-4) is able to degrade cellulose and produce reducing sugars at a greater rate than the parent.

Quantitatively, the results are in agreement with the ethanol sensitivity results which were shown previously in Figure I.C.1.2. As mentioned earlier, the most dramatic range of increased tolerance by the new strain (S-4) is between 2 to 3% ethanol. To illustrate this behavior, the results from Table I.C.1.3 where the ethanol concentration was 2% (V/V) are graphically illustrated in Figures I.C.1.4 and I.C.1.5. It is seen from Figure I.C.1.4 that the degree of cellulose degradation at 2% ethanol for the new strain (S-4) is significantly improved over that of the parent. The accumulation of reducing sugars (Figure I.C.1.5) similarly show improvement of the new

TABLE I.C.1.1: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 0% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	7.1	5.6	1.8	2.4
4	4.4	3.8	4.1	4.2
7	1.9	1.5	6.8	6.4
10	0.9	0.6	7.8	7.3

TABLE I.C.1.2: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 1% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	8.6	6.0	1.6	2.2
4	8.0	4.3	2.7	4.0
7	6.2	2.1	3.4	6.1
10	2.5	1.0	6.7	7.0

TABLE I.C.1.3: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR FORMATION BY PARENT AND STRAIN S-4 OF CLOSTRIDIUM THERMOCELLUM.

(ETHANOL CONCENTRATION = 2% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	8.9	6.6	1.7	2.3
4	8.2	5.1	2.5	4.0
7	7.1	2.5	3.5	6.0
10	6.1	1.5	4.0	6.7

TABLE I.C.1.4: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 3% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	9.1	7.5	1.7	2.2
4	8.4	6.1	2.2	3.5
7	7.2	3.9	3.0	5.2
10	6.3	2.9	3.7	5.9

TABLE I.C.1.5: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 4% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	9.2	7.8	1.3	2.1
4	8.6	7.6	2.0	3.1
7	7.6	4.5	2.7	4.2
10	6.8	4.3	3.2	5.1

TABLE I.C.1.6: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION OF PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 5% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	9.3	8.7	1.2	1.8
4	9.0	8.1	1.8	2.5
7	8.1	7.0	2.3	3.3
10	7.3	6.3	2.7	3.7

FIGURE I.C.1.4: COMPARISON OF CELLULOSE (SOLKA FLOC) DEGRADATION
BY PARENT AND ISOLATE S-4 OF C. THERMOCELLUM

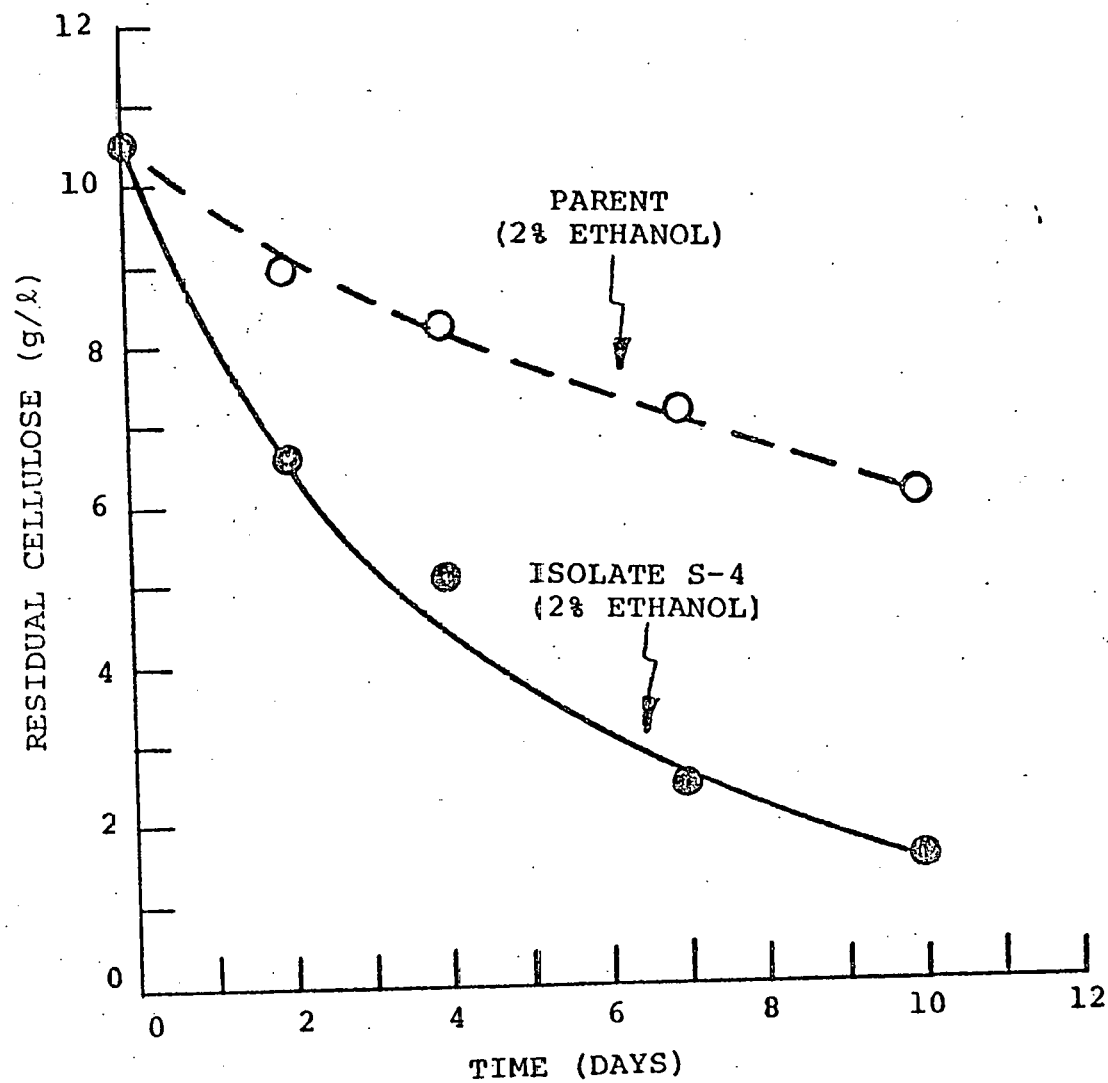
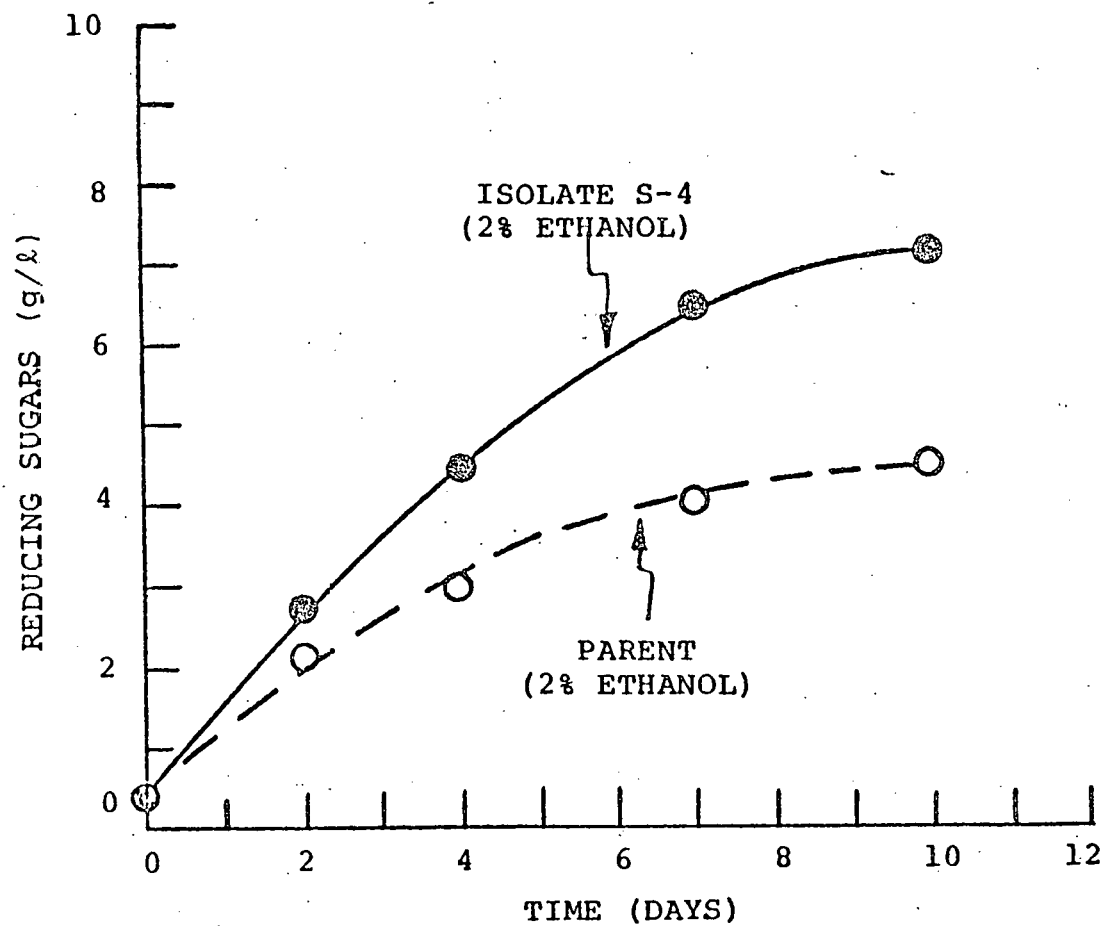


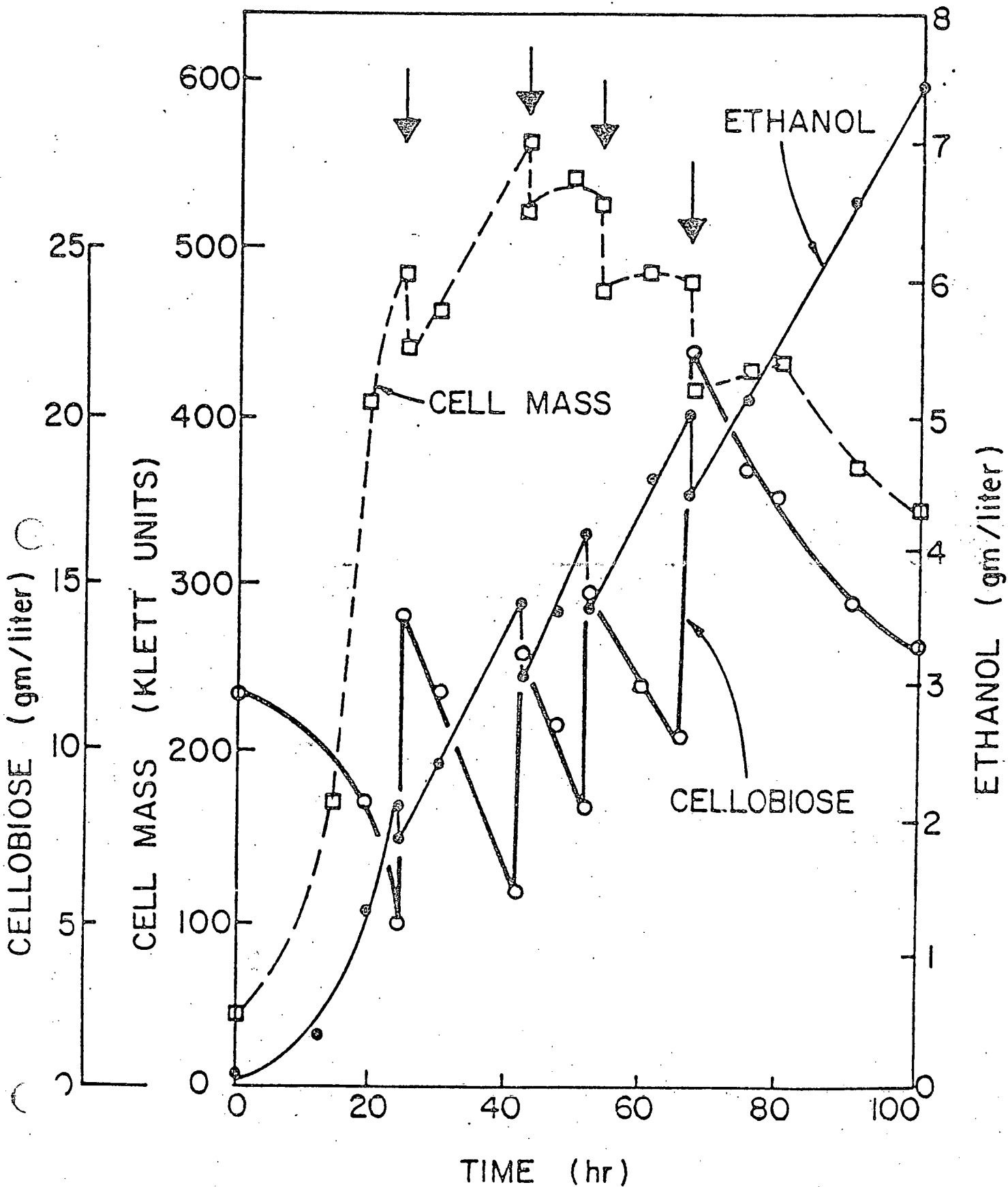
FIGURE I.C.1.5: COMPARISON OF REDUCING SUGAR PRODUCTION FROM CELLULOSE (SOLKA FLOC) BY PARENT AND ISOLATE S-4 OF C. THERMOCELLUM



isolate as compared to the parent. These results confirm that the new strain is able to degrade cellulose at higher concentrations of ethanol at rates greater than the parent. It also reinforces our selection technique for isolating useful strains for product formation. We feel we now have available a simple and expedient selection method for a future isolation program to obtain organisms with the desired properties.

At this point, it still remained to be answered whether the new ethanol tolerant strain is able to produce ethanol at concentrations higher than the parent. Unfortunately, we have the additional problem when high concentration of ethanol is desired. This being our inability to maintain or feed high concentration of insoluble biomass to our fermentations. For example, in our previous Report (COO-4198-5, Figure I.C.3, page 32), we resorted to batch-feeding of cellulose in order to obtain high substrate concentrations. In this previous study, about 4 g/l of ethanol and acetic acid each was obtained when a total cellulose concentration of 45 g/l was used through feedings. Even then, at these product concentrations, there was still 15 g/l of undegraded cellulose. In order to test the new strain as to its capability in producing ethanol, we decided to employ a simpler system in seeking this answer. This is the use of a soluble substrate, cellobiose, fed periodically to the fermentation. We feel that the results from such a substrate are translatable in view of our other findings. The results from this experiment are shown in Figure I.C.1.6.

FIGURE I.C.1.6: PRODUCTION OF ETHANOL BY NEW ISO-LATE (S-4) OF C. THERMOCELLUM



As seen from this Figure, the initial (up to 20 hours) rate of cell growth (Klett unit) was quite rapid and exhibited typical exponential behavior. The initial ethanol formation also was exponential. Ethanol production continues in a linear fashion even though the cell growth decreased. Although acetic acid production was not measured, one would expect its concentration to be about equal to that of ethanol. A final concentration of 7.5 g/l of ethanol was obtained even though, at this time, no decrease in its rate has been observed. However, due to the dilution effect when nutrients were added, the corrected equivalent concentration of ethanol was actually 9.1 g/l. If we also assume that 9.1 g/l of acetic acid was also produced, a very impressive conversion yield had been achieved as shown below. In this experiment, a total of 45 g/l of cellobiose was added and 13 g/l remained at the end. Therefore, the sum of the product yield (ethanol = 9.1, acetic acid = 9.1, cellobiose utilized = 32) was calculated to be 0.57 grams of product per gram of substrate. These results encourage us to continue our efforts in the direct conversion of cellulosic biomass to useful products.

The final series of experiments were performed to determine the degradation capability of the new strain (S-4) on corn stovers which were recently furnished by Professor G. T. Tsao. As mentioned in the Materials and Methods Section, the only pretreatment was ball-milling of the corn stover.

Unfortunately, the dark brown color of the corn stover suspension and the suspended particles prevented us from measuring cell growth during fermentation. The fermentation was inoculated from a seed culture grown on Solka floc. pH was manually controlled at a value of about 7.0, fermentation temperature was 60°C and the initial corn stover concentration was 36 g/l. The results are shown in Figure I.C.1.7. The formation of reducing sugars (open circles), ethanol (open triangles) and acetic acid (closed squares) showed an initial rapid rate. This was followed by a decreased rate of product formation, but constant over the entire fermentation cycle. At the end of the fermentation, 8 g/l of reducing sugars accumulated and 2.2 g/l of ethanol and acetic acid each also formed. It should be mentioned that the organism did not undergo any previous adaptation to this new biomass (corn stover). We believe that serial transfer and adaptation studies should enable us to increase the degradation rate. It is sufficient to state, on the first transfer, that this organism is able to impressively metabolize a totally new substrate possessing radically different physical and chemical properties.

The degree of biomass conversion by Clostridium thermocellum is likewise quite impressive and interesting. Shown in Table I.C.1.7 are the compositions of corn stover, wheat straw and rice straw as reported by Dr. C. R. Wilke. It is seen that the sum of the carbohydrates in corn stover is only

FIGURE I.C.1.7: DEGRADATION OF CELLULOSE (CORN STOVER) BY
CLOSTRIDIUM THERMOCELLUM, STRAIN S-4

(INITIAL CORN STOVER = 36 g/l)

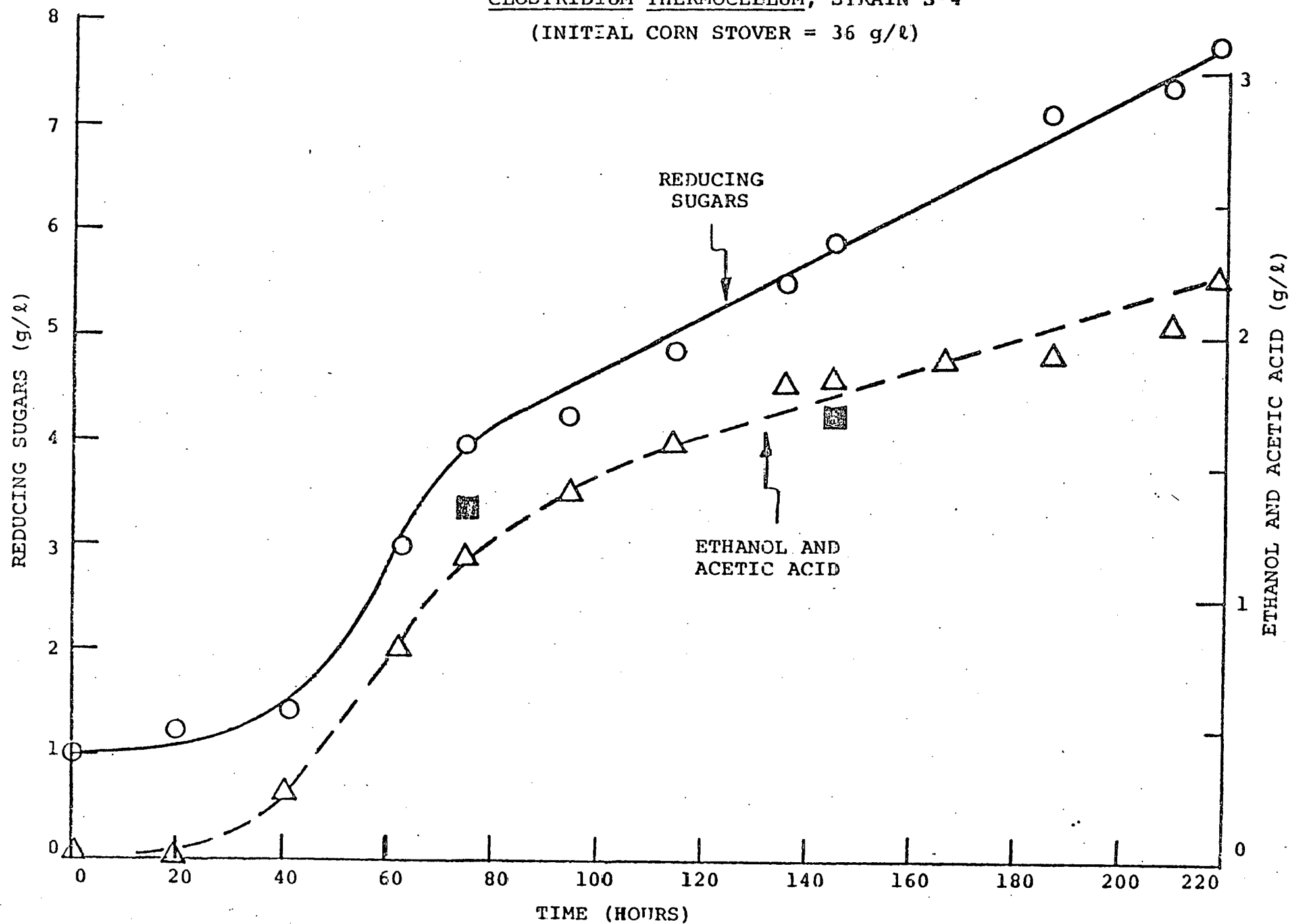


TABLE I.C.1.7: COMPOSITION OF DIFFERENT BIOMASSES *

	<u>Corn Stover</u> (%)	<u>Wheat Straw</u> (%)	<u>Rice Straw</u> (%)
Glucan	35.1	32.9	36.9
Mannan	0.25	0.72	1.6
Galactan	0.75	2.16	0.4
Xylan	13.0	16.9	13.0
Arabinan	2.8	2.11	4.0
Σ Carbohydrate	51.9	54.8	55.9
Lignin	15.1	14.5	9.9
Ash	4.3	9.6	12.4
Aceotropic benzene- alcohol extractives	5.5	7.2	4.4
Protein	4	3	-
Unidentified	18	8	10
Σ	98.8	97.1	92.7

* Data from Dr. Charles R. Wilke, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720.

51.9%. In our fermentation using corn stover, an initial value of 36 g/l was employed. This is equivalent to a total carbohydrate concentration of 18.7 g/l. From this total, Clostridium thermocellum was able to produce 8 g/l of sugars and 2.2 g/l of ethanol and 2.2 g/l of acetic acid. This is equivalent to a conversion yield of 0.66 grams of product per grams of carbohydrate. We believe this yield is quite excellent and shows further encouragement for our project objectives.

d. Future Work

The future studies on this segment of the project include:

- Further increase the tolerance of C. thermo-
cellum to ethanol.
- Incorporate the packed-bed fermentor to
achieve high product concentrations.
- Increase the yield of ethanol from biomass
by preventing the formation of acetic acid: this can be achieved
through mutation and the use of inhibitors.
- Examine other biomasses such as bagasse,
straw, Poplar, etc.

2. Growth Inhibition of C. thermocellum by Fermentation Products

a. Introduction

These studies were designed to determine the inhibition of C. thermocellum by fermentation products, as well as the effect temperature has on this inhibition.

b. Results and Discussion

1. Growth of C. thermocellum at Different Temperatures

Figure I.C.2.1 shows the growth curves of C. thermocellum within the range 45°C to 65°C. This range includes both suboptimal and superoptimal temperatures. The culture is capable of maintaining balanced growth at all temperatures. Figure I.C.2.2 indicates that the optimum temperature is around 58°C and shows that the activation energy for growth is 23 Kcal/mole. The growth rates represented in Figure I.C.2.2 are average values of several experiments.

2. Growth Inhibition of C. thermocellum by Ethanol at 60°C Experimental Protocol

An exponentially growing culture of C. thermocellum is challenged with different concentrations of ethanol by injection of 1 ml of the proper dilutions. At intervals, the optical density at 660 is measured and the inhibition index calculated according to the definition:

$$\text{Inhibition Index (II)} = 1 - \frac{A_x^t - A_x^{t_0}}{A_o^t - A_o^{t_0}}$$

where A_x^t = Adsorbance at time t, in the presence of X grs/l of ethanol

A_o^t = idem, at zero time

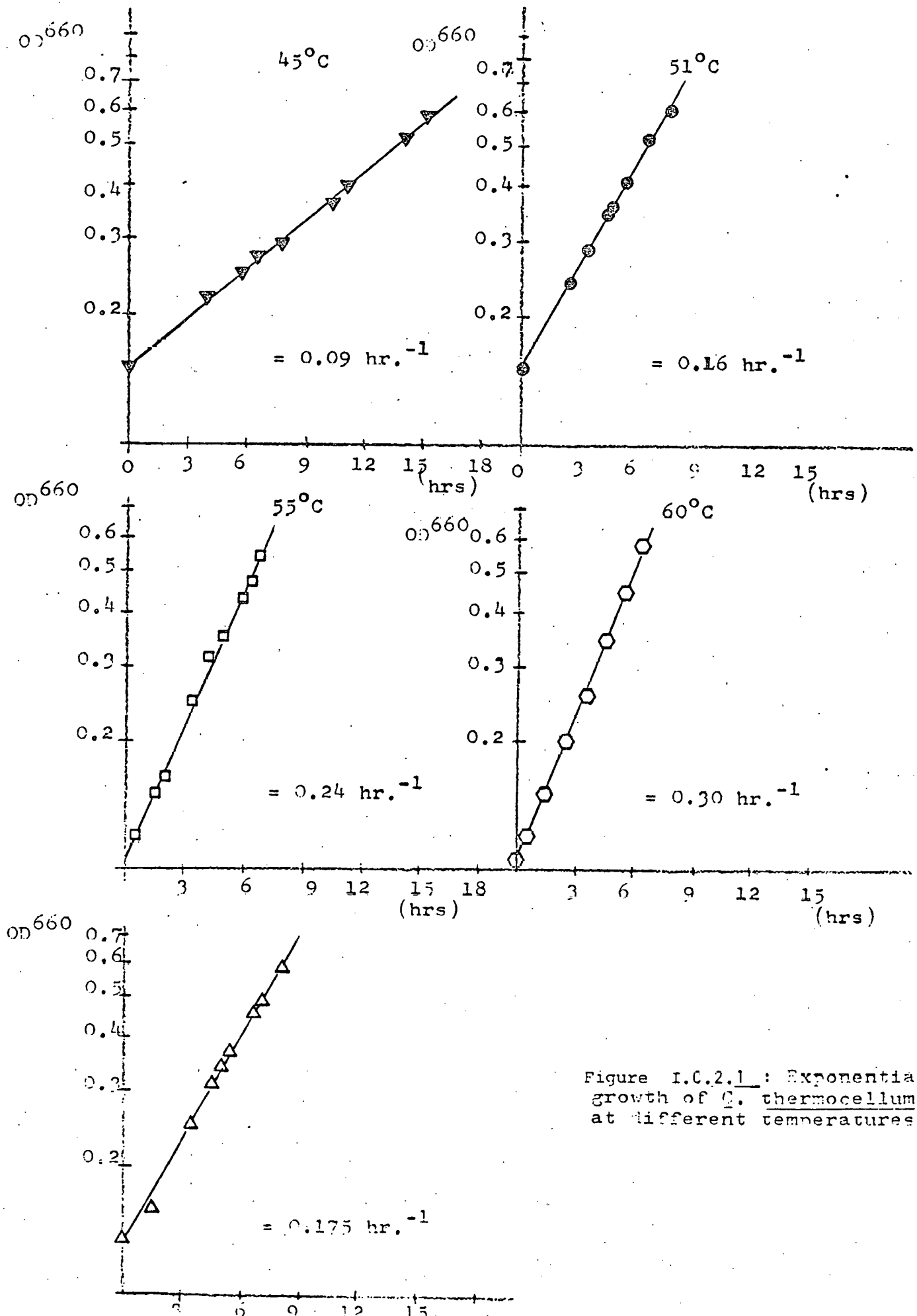


Figure I.C.2.1 : Exponential growth of *C. thermocellum* at different temperatures.

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A_O^t = Adsorbance at time x, in the absence of ethanol

$A_O^{t_0}$ = idem, at zero time

The inhibition index termed "cell yield" is the value for $t = 20$ hrs after addition.

Figure I.C.2.3. shows the results of a typical experiment. Figure I.C.2.4. shows the time dependence of the inhibition index. In order to compare results of different experiments, the II are calculated after one generation time of the control culture.

3. Growth Inhibition of *C. thermocellum* by Acetate and Ethanol at Different Temperatures

Using the same experimental protocol previously described, *C. thermocellum* cultures grown at different temperatures for 3 to 4 generations were also challenged with ethanol or with sodium acetate (pH 7.4) concentrations of 0, 2, 5, 8, 10, 13, 15, 18, 20 and 25 grs/lt; the inhibition indexes at each temperature were calculated as defined previously and the concentrations for 50% inhibition and for 25% inhibition calculated by interpolation in the corresponding II vs. ethanol concentration curve.

Figure I.C.2.5. summarized the results. While the acetate response is independent of the temperature of growth, the inhibitory effect of ethanol is clearly a function of that temperature. The maximum resistance to ethanol is attained at about 50°C in the suboptimal range. At both extreme temperatures the reduction in ethanol resistance is obvious.

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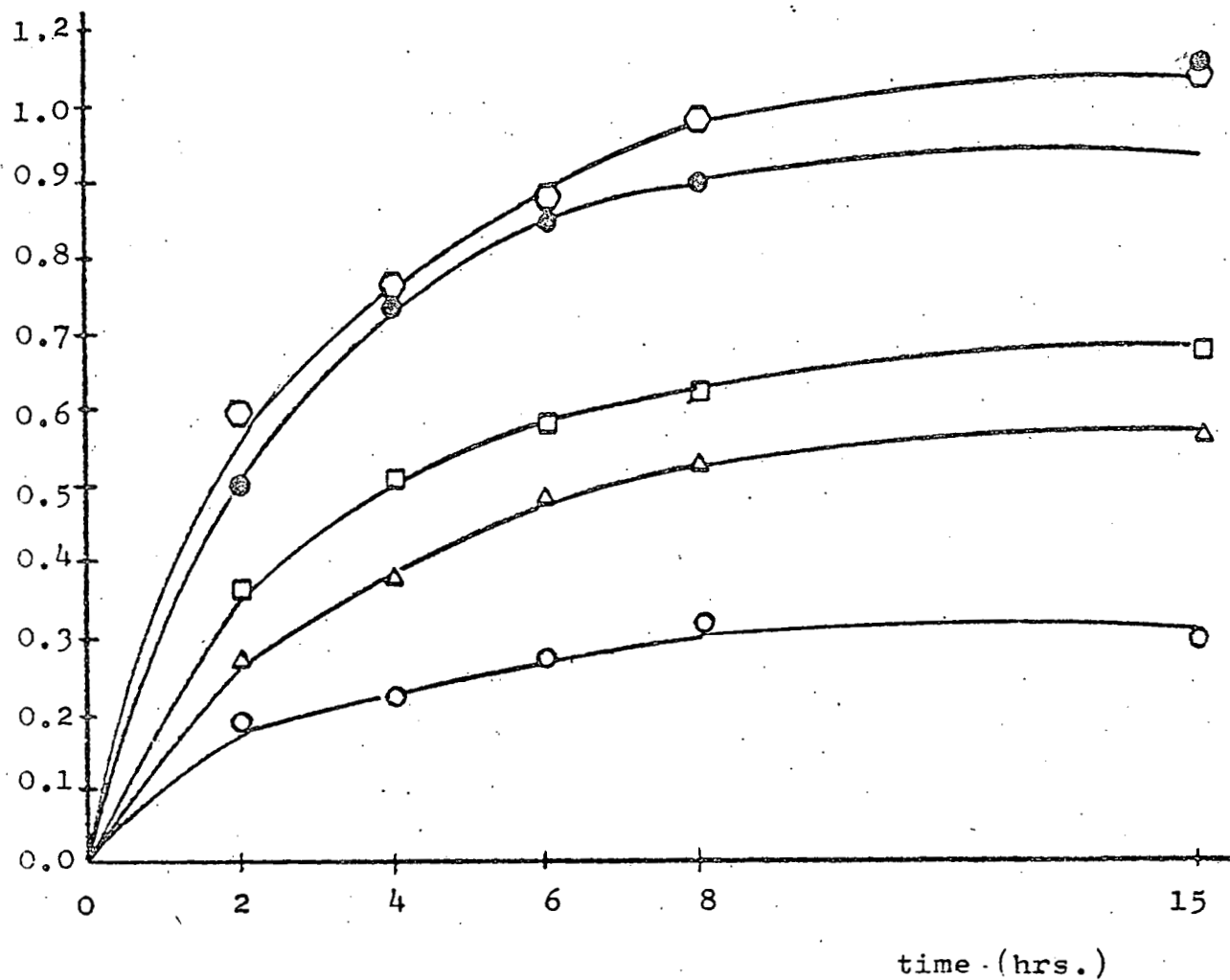


Figure I.C.2.4 : Time dependence of the inhibition index in ethanol.

- 5 grs/lt
- △ 10 grs/lt
- 15 grs/lt
- ⊗ 20 grs/lt
- ◇ 25 grs/lt

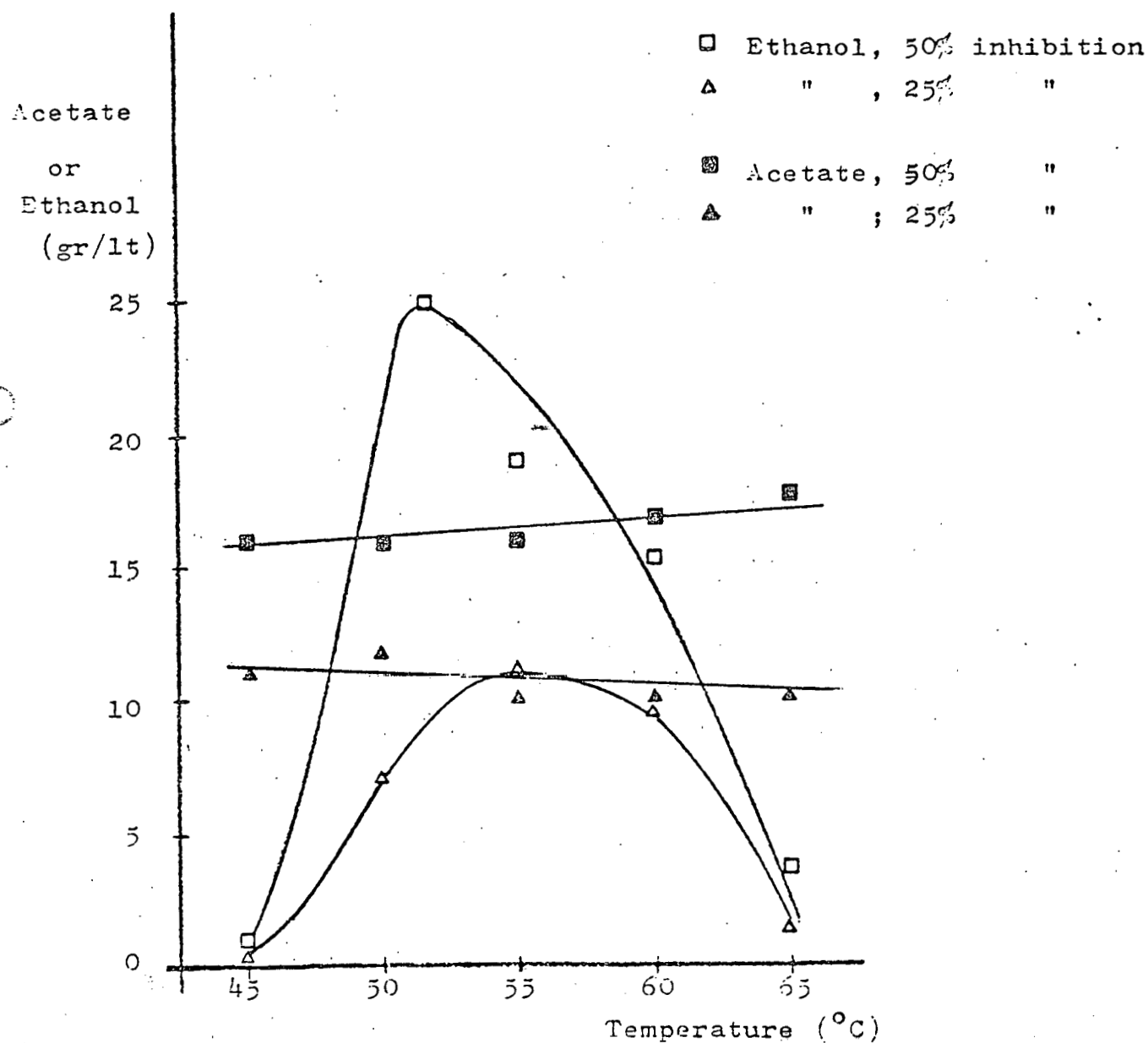


Figure I.C.2.3 : Ethanol and acetate concentrations for 50% and 25% inhibitions at different temperatures.

c. Future Work

- To investigate the effect of cultural nutrition on the dependence that ethanol toxicity has on temperature.

- The inhibition exerted by other fermentation products of C. thermocellum, as well as related compounds is currently under investigation

Once this baseline data is available, we will study the lipid composition of cell membranes, as well as the possibilities of its alteration by environmental manipulations, with the specific aim of increasing the resistance to these fermentation products.

D. Degradation of Different Cellulosic Biomasses by *Clostridium thermocellum*

1. Introduction

The importance of raw material costs in the overall process of cellulose degradation stresses the need for cheap and abundantly available biomass sources. The natural choice for preliminary investigation falls on materials that are wastes or by-products of the large existing agricultural industry. The choice of substrates was also influenced by consideration of the physical nature of the materials and their suitability for continuous feeding.

In this context, studies on degradation of thermally exploded lignocellulose poplar, corn cob granules, corn stover and sugar cane bagasse by *C. thermocellum* were performed. Special consideration was given to the effect of particle size distribution on the production yields. The economic aspect of particle size reduction was also analyzed.

2. Materials and Methods

a. Poplar

The thermally exploded lignocellulose poplar used as main substrate was obtained from the Pulp and Paper Research Institute of Canada (for details, see page 15 of previous Progress Report COO-4198-5). Fermentations were run at 60°C in anaerobic shake flasks containing 300 ml of CM 3 medium. A 3% inoculum of *C. thermocellum* grown on Solka floc (SW 40,

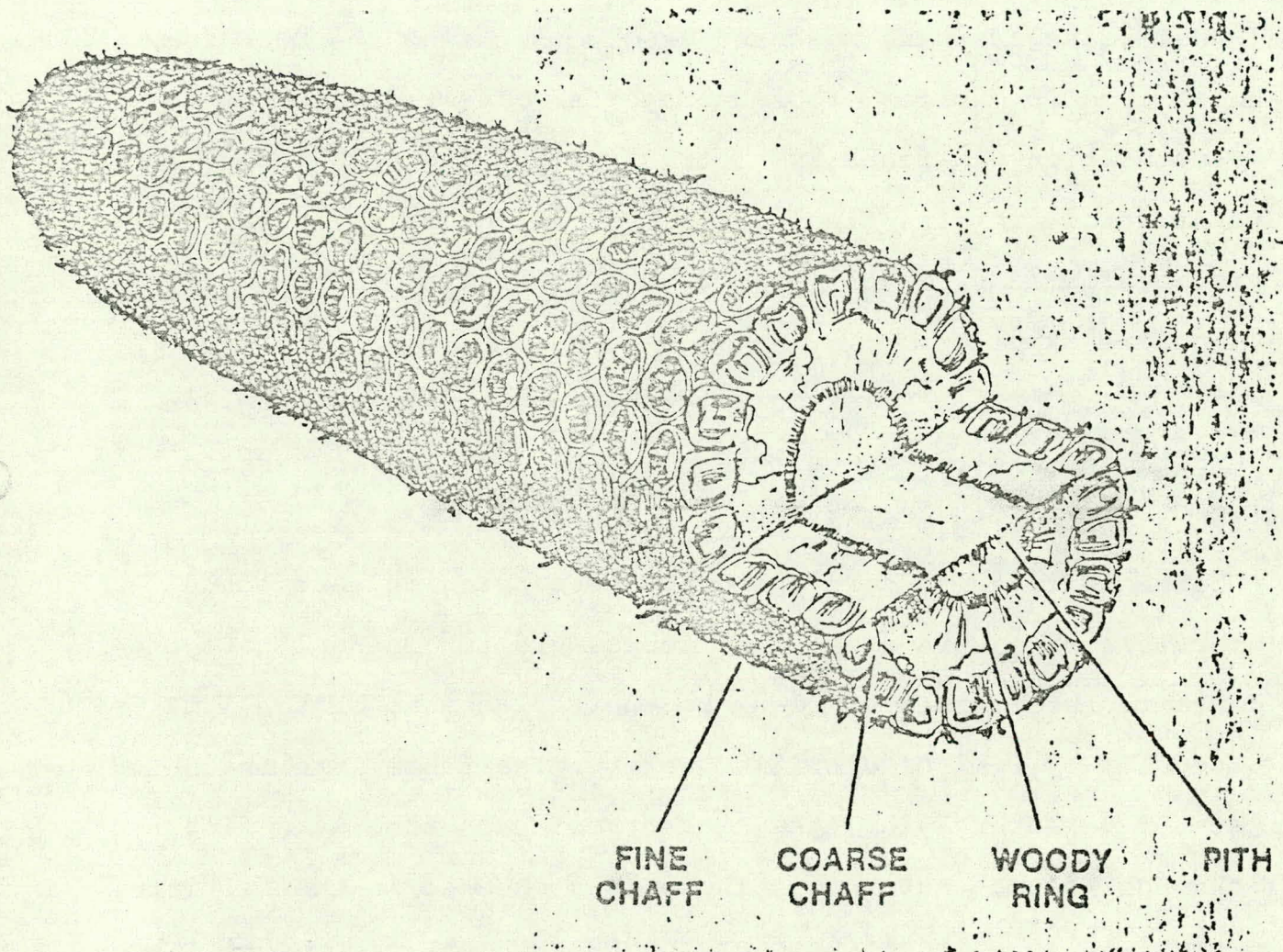
Brown Co.) was used. The effect of the pretreatment of the substrate was studied by using as substrates the original poplar, an alkaline washed poplar, an ethanol washed poplar and a Solka floc control. The alkaline wash consisted of weighing wet poplar equivalent to 10 g of dry solids, adding 100 ml 1N NaOH and centrifuging at 3,000 xg for 10 minutes. The precipitate was suspended in 100 ml distilled water and recentrifuged. The final precipitate was resuspended in 100 ml distilled water. Ten ml samples were taken in triplicate and filtered through Whatman Paper #1 for determination of loss of solids. The remaining slurry was used for preparation of the medium. The ethanol wash was performed identically using 95° % ethanol.

b. Corn Cobs

The corn cobs granules were obtained from The Andersons, Cob Division, P.O. Box 119, Maumee, OH 43537. The term GRIT-O'COBS is the trademark for material from the woody rings of the corn cob. The term LITE-R-COBS is the trademark for the combination of fine chaff, coarse chaff and pith (see Figure I.D.1). The samples are designated by numbers that refer to their particle size. Hence, GRIT-O'COBS 814 contains particles that went through screen 8 and were retained by screen 14 (U. S. standard screens). LITE-R-COBS -30 contains all particles that could go through screen 30.

Experiments were run at 60°C in anaerobic shake flasks containing 300 ml of CM 3 medium. Corn cob granules

FIGURE I.D.1: NOMENCLATURE CONCERNING CORN COBS



(From the publication "Primary Technical Properties
of Grit-O'Cobs ®, The Andersons, Cob Division)

of five different particle sizes were used as main substrates. A 3% inoculum of C. thermocellum grown in Solka floc was utilized. Samples were periodically withdrawn over the 90-hour time course of the fermentation and the pH profile was followed. Reducing sugars were measured as glucose using the DNS assay described in earlier progress reports. The reducing sugars accumulation was found by subtracting the initial glucose concentration from the final one. Residual cellulose assays were not performed since the size of the particles did not allow accurate sampling.

To overcome the problem of sampling, an experiment was run by culturing C. thermocellum in Hungate tubes containing 9 ml of CM 3 medium and 1 ml of inoculum. Fourteen different corn cob granules varying in source and particle size were used. The tubes were run in duplicate and a third tube served as blank by the replacement of the 1 ml inoculum by 1 ml of distilled water. The tubes were incubated at 60°C. After 75 hr, the contents of each tube were filtered through Whatman Paper #1. The pH was checked in the filtrate, which was then centrifuged at 20,000 x g for 15 minutes. The degraded substrate was expressed as 100 minus the percentage of undegraded corn stover in the sample to undegraded corn stover in uninoculated blank. A reducing sugars DNS assay was performed in the supernatant. The reducing sugar accumulation is expressed after correction of the reducing sugar concentration in the blanks.

c. Corn Stover and Sugar Cane Bagasse

A Wiley milled corn stover sample was obtained from Professor George Tsao, School of Chemical Engineering, Purdue University, West Lafayette, Indiana. A hammer milled sugar cane bagasse sample was obtained from Ander-Cane Inc., 1310 Carbio Center, Naples, Florida 33942. Both samples had a moisture content of 6% (dry basis).

An experiment was conducted in Hungate tubes containing 9 ml of CM 3 medium and 1 ml of C. thermo-cellum inoculum grown in Solka floc on 1 ml of distilled water (uninoculated control). The time course of the 60°C fermentation was followed by examining sets of tubes along 72 hr. Each set was composed of triplicates of inoculated corn stover and bagasse tubes, plus one inoculated Solka floc tube. Duplicates of uninoculated corn stover and bagasse tubes plus one inoculated Solka floc tube were also part of the sample set as controls.

Samples were filtered through Whatman Paper #1 for determination of residual substrate. The pH was checked in the filtrate, which was then centrifuged at 20,000 x g for 15 minutes. A reducing sugar DNS assay was performed on the supernatant.

Another experiment was performed to determine the particle size distribution of the corn stover and sugar cane bagasse used. Ten grams of sample were manually sifted

for 3 minutes through a set of Tyler standard screens; the mass fraction of total sample retained by each screen was determined gravimetrically.

3. Results and Discussion

a. Poplar

The washing procedures used for pretreatment of the poplar caused considerable losses of solids as can be seen from Table I.D.1.

TABLE I.D.1: EXPLODED POPLAR LOSSES DURING PRETREATMENT

Pretreatment of Poplar	% Solids Lost
Alkaline Wash	59
Ethanol Wash	48

No significant growth was detected in the flasks containing treated or untreated poplar while the Solka floc control sustained normal growth as indicated by color change and pH drop.

b. Corn Cobs

The reducing sugars accumulation and the final pH of broths obtained in shake flask experiments using different size corn cob granules are shown in Table I.D.2.

TABLE I.D.2: PERFORMANCE OF CORN GRANULES AS A CARBON SOURCE IN C. THERMOCELLUM FERMENTATIONS (SHAKE FLASK)

Carbon-Source	Reducing Sugars Accumulation (mg/ml)	Final pH
GRIT-O'COBS 814	1.34	6.55
GRIT-O'COBS 1420	1.50	6.50
GRIT-O'COBS 4060	2.93	6.30
LITE-R-COBS 1030	2.44	6.45
LITE-R-COBS -30	1.97	6.40
SOLKA FLOC	0.78	6.35

Unfortunately, a contamination has probably occurred in the Solka floc control flask and the accumulation of sugars is quite low. The highest sugar accumulation is obtained with GRIT-O'COBS particles of dimensions between 297 and 429 μm . Since the particles are not spherical, but rather needlelike, the dimensions found in the screen analysis refer to the second major dimension (in this case, the thickness of the particle).

Table I.D.3 shows the results obtained in Hungate tubes for the 14 types of corn cob granules. The final pH in uninoculated tubes was 7.2, while in the inoculated tubes it was in the range of 6.0 - 6.2. This data are plotted in

TABLE I.D.3: PERFORMANCE OF CORN COB GRANULES AS C-SOURCE IN
C. THERMOCELLUM FERMENTATIONS (HUNGATE TUBES)

C-Source	Sample	% Substrate Consumption	Reducing Sugars Production (mg/ml)
GRIT-O'COBS 814	a	8	-
	b	11	-
	1014		
	a	33	-
	b	35	1.38
	1020		
	a	38	1.58
	b	35	1.38
	1420		
	a	-	2.10
	b	34	1.58
	1440A		
	a	37	1.70
	b	39	1.53
	1440B		
	a	45	1.80
	b	43	1.60
	1440C		
	a	43	2.05
	b	40	1.78
	2040		
	a	45	2.15
	b	46	2.00
	4060		
	a	55	2.38
	b	57	2.35
	-20		
	a	49	2.58
	b	50	2.13
	-40		
	a	61	2.70
	b	62	2.75
	-60		
	a	64	2.88
	b	66	2.95
LITE-R-COBS 1030	a	44	1.85
	b	45	1.68
	-30		
	a	54	2.25
	b	55	2.30

Figure I.D.2 where substrate consumption and reducing sugar accumulation are shown as a function of particle size. The data from GRIT-O'COBS fits reasonably well a straight line and yields the following correlations:

$$\begin{aligned} X &= 1.62 - 1.86 \log D \\ \text{and} \\ Y &= 32.0 - 37.5 \log D \end{aligned}$$

where: X = reducing sugar accumulation (g/l)

Y = % corn cob consumed

D = particle size (mm)

The data from LITE-R-COBS seems to follow the same trend, but the number of samples is insufficient for any conclusion.

c. Corn Stover and Bagasse

Figures I.D.3 and I.D.4 show the time profile of the fermentation of bagasse corn stover and Solka floc. The spread of the data can be explained in terms of the difficulty in reproducing exactly the same conditions in individual Hungate tubes as opposed to shake flasks, where samples are always withdrawn from the same broth. The corn stover showed higher substrate utilization and higher reducing sugar accumulation although the final values (around 7.6 g/l residual substrate and 1 g/l reducing sugars) are relatively low as compared to the Solka floc control.

The differential screen analysis of the corn stover and bagasse samples is shown in Table I.D.4, where

FIGURE I.D.2: EFFECT OF PARTICLE SIZE OF CORN COB GRANULES (GRIT-O'COBS) ON
SUBSTRATE CONSUMPTION AND REDUCING SUGARS ACCUMULATION BY
CLOSTRIDIUM THERMOCELLUM

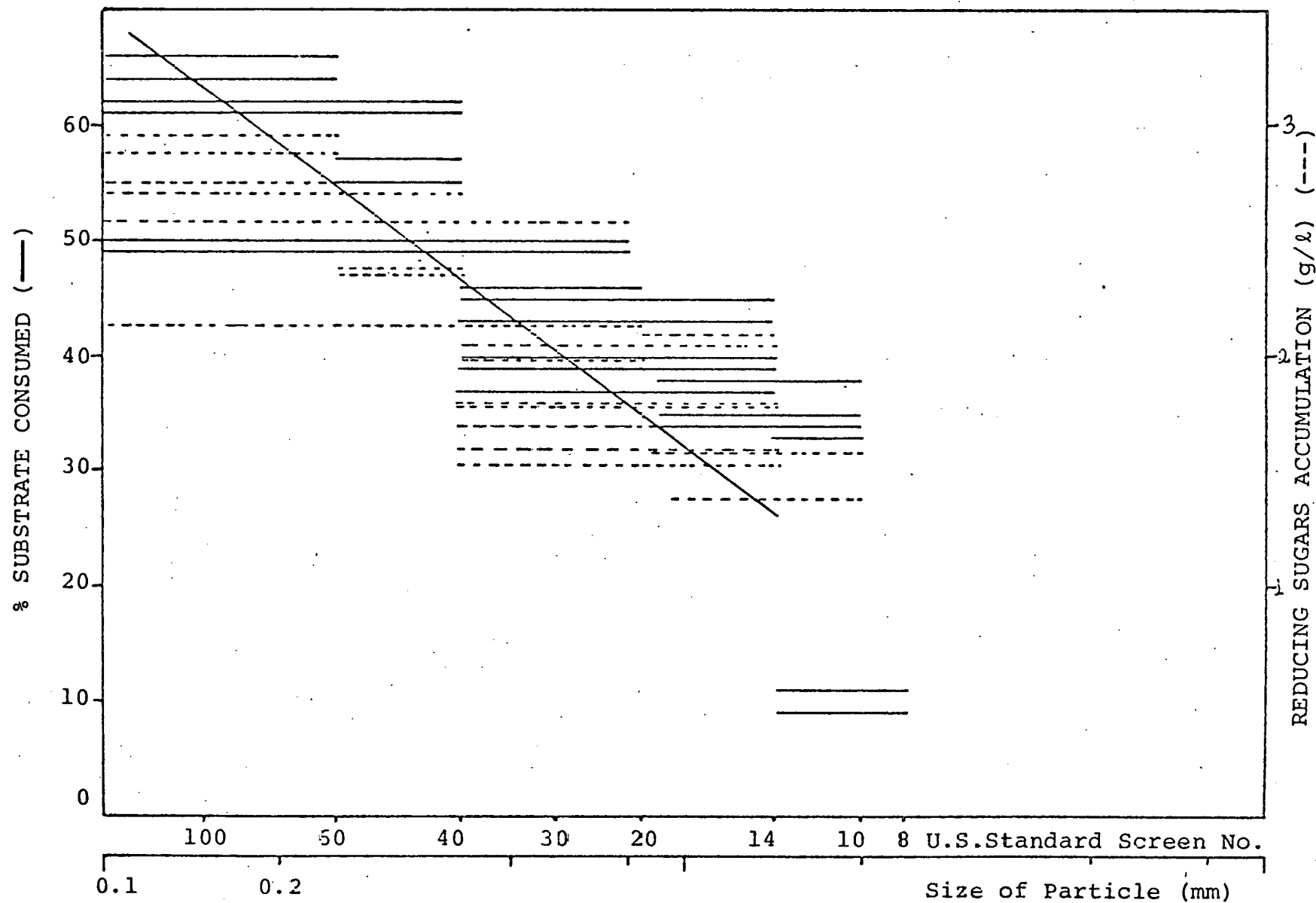
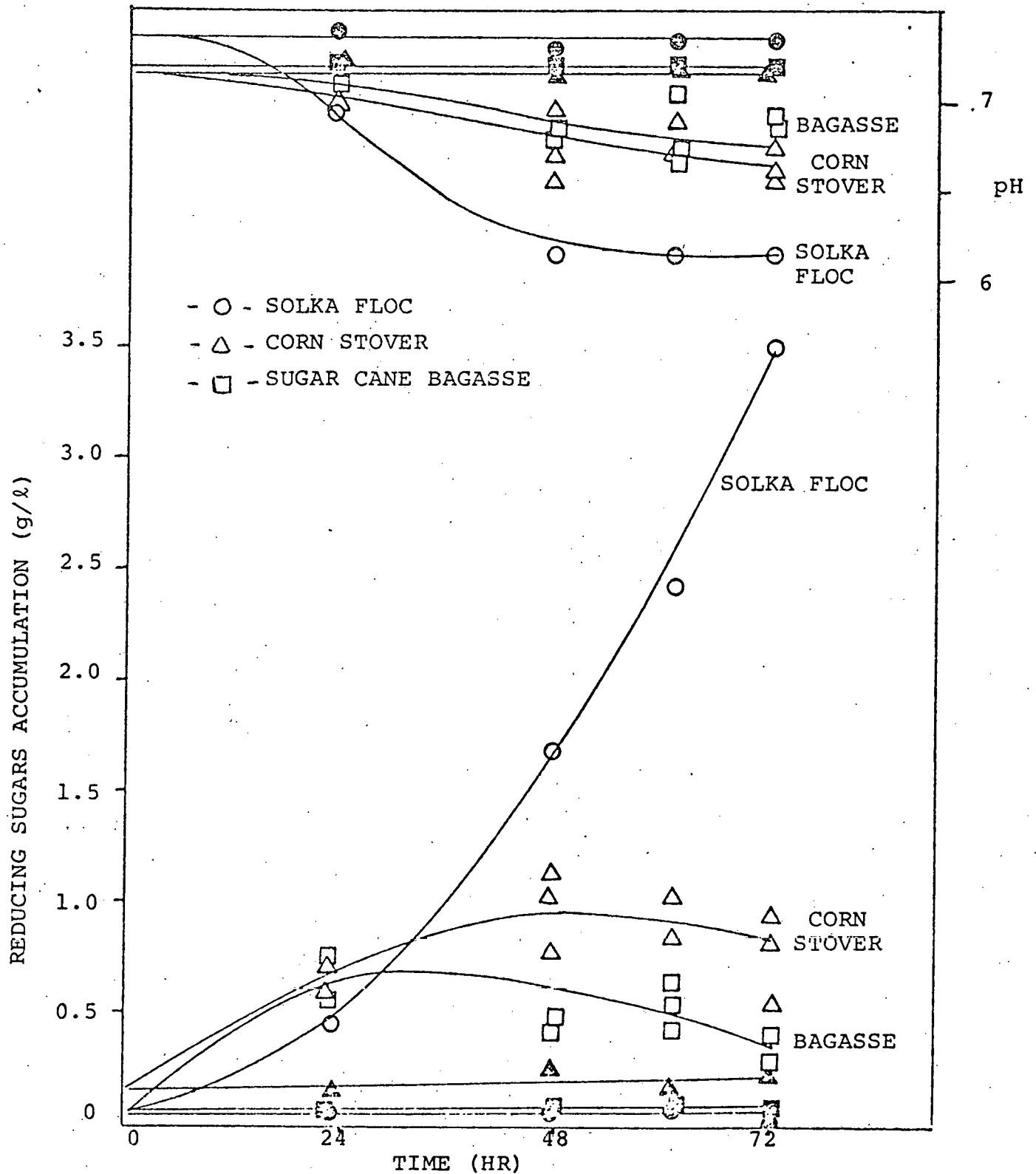


FIGURE I.D.3: REDUCING SUGARS ACCUMULATION AND pH DURING GROWTH OF *C. THERMOCELLUM* IN CM3 MEDIUM WITH CORN STOVER OR SUGAR CANE BAGASSE AS MAIN C-SOURCE (OPEN SYMBOLS REFER TO INOCULATED MEDIUM; CLOSED SYMBOLS REFER TO UNINOCULATED MEDIUM)



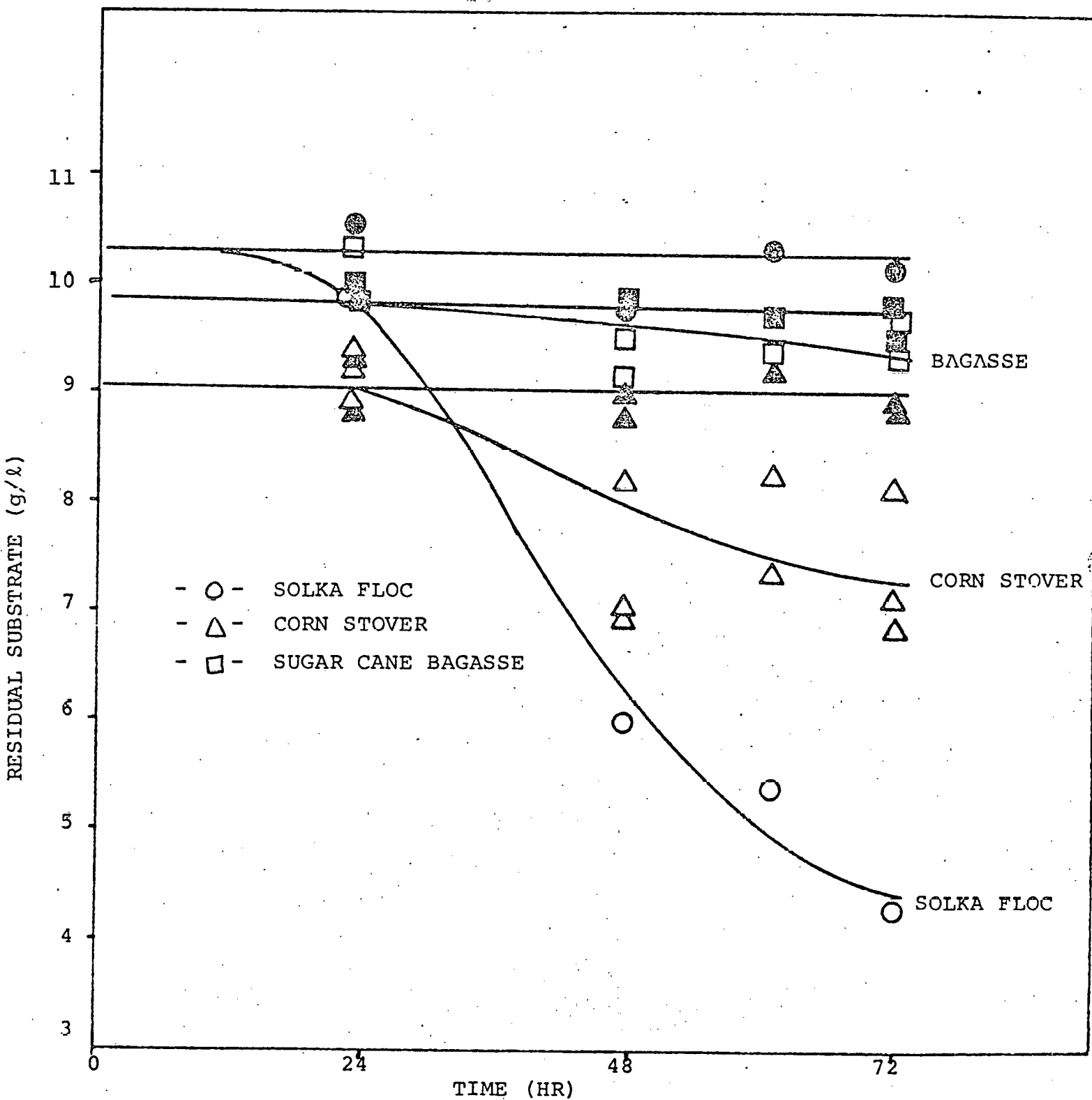


FIGURE I.D.4: RESIDUAL SUBSTRATE DURING GROWTH OF C. THERMOCELLUM IN CM3 MEDIUM WITH CORN STOVER OR SUGAR CANE BAGASSE AS MAIN C-SOURCE

TABLE I.D.4: DIFFERENTIAL SCREEN ANALYSIS OF CORN STOVER AND SUGAR CANE BAGASSE

MESH passed/retained	$D_{p,n}$ (mm)	$\Delta\phi_n$				$\bar{D}_n$ (mm)
		Corn Stover		Sugar Cane Bagasse		
		Sample 1	Sample 2	Sample 1	Sample 2	
4/ 6	3.327	0.0042	0.0000	0.0058	0.0185	4.013
6/ 8	2.362	0.0083	0.0058	0.0132	0.0140	2.845
8/ 10	1.651	0.0423	0.0666	0.0762	0.0779	2.007
10/ 14	1.168	0.1803	0.1949	0.1733	0.1784	1.410
14/ 20	0.833	0.3075	0.2639	0.2700	0.2704	1.001
20/ 28	0.589	0.1844	0.2006	0.2151	0.2224	0.711
28/ 35	0.417	0.1319	0.1347	0.1443	0.1411	0.503
35/100	0.147	0.1270	0.1077	0.0669	0.0664	0.282
parr		0.0142	0.0260	0.0353	0.0108	0.074
$A_w(\times 10^2 \text{ cm}^2/\text{g})$		3.3	2.5	2.5	2.1	

$D_{p,n}$ is the second largest major dimension of particles retained by screen n (i.e., the mesh opening of screen n), $\Delta\phi_n$ is the mass fraction of total sample that is retained by screen n having passed through screen $n-1$ and $\bar{D}_n$ is the arithmetic mean of $D_{p,n}$ and $D_{p,n-1}$. The specific area of the samples was calculated according to the equation: (McCabe & Smith, Unit Operations of Chemical Engineering, McGraw Hill, 1976):

$$A_w = \frac{6}{\psi_s \rho_p} \sum_{n=1}^{n_T} \frac{\Delta\phi_n}{\bar{D}_n}$$

where: n_T = number of screens

A_w = total surface of one unit mass of sample

ρ_p = particle density

ψ_s = sphericity of particle

The particle density was estimated by water volume displacement and was found to be 0.42 g/ml for corn stover and 0.59 g/ml for bagasse. The sphericity was assumed to be 0.7 as suggested by McCabe & Smith for crushed materials. The calculations show that the corn stover sample had a higher specific area than the sugar cane bagasse.

d. Discussion

The use of exploded poplar as a substrate in cellulose fermentations would have the advantage of the flowability

of a slurry for the purpose of feeding continuous reactors. However, the results obtained are not encouraging since even if the growth kinetics could be improved using the pretreated poplar the losses during the washes seem to be uneconomical.

Corn cob granules appear to be a good substrate, but its use in fermentation processes would have to compete with other end-uses such as ingredients in fertilizers and animal feed.

Some plausible hypothesis to explain the dependency of sugar accumulation and substrate utilization on particle size of corn cob granules are the unfolding of tertiary and quaternary structures of cellulose and the greater specific surface area for microbial attack in smaller particles. The utilization of finer corn cob granules seems to be quite promising. Their flowing characteristics will apparently fit well in a solids feeding system for continuous fermentation processes. Evidently, the use of finer granules involves an energy intensive step of particle size reduction. The power required for crushing and grinding from large feed can be estimated by Bond law (McCabe & Smith):

$$\frac{P}{m} = \frac{K_b}{D_p}$$

where P is the power required by the grinder, m is the feed rate, D_p is the diameter of the particles produced and K_b is

a constant that depends on the type of machine and on the material being crushed.

According to this theory, a reduction in final particle size from 1 mm to 0.5 mm would require an increase of power consumption by 40%. On the other hand, according to the correlations obtained for corn cob granules, such reduction would cause an increase of 35% in the reducing sugar accumulation and in the substrate consumption. As described earlier, the raw materials represent a major portion of the costs obtained by cellulose fermentation; recent economic estimates made in our laboratory place the raw materials in the range of 50-60% of the product costs. Therefore, the increase in power required for size reduction is largely compensated by the increase in yield of product from total substrate.

The same rationale might apply to corn stover and sugar cane bagasse so the performance of these substrates in a finely divided form will be investigated. According to assays performed in the Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, the cellulose content of corn stover is around 35%. Accordingly, the substrate utilization level of 25% obtained for corn stover in these preliminary studies (with no particle size selection) is quite encouraging since the maximum level would be around 35%. In regards to the sugar cane bagasse used, its specific area is rather low (around $2 \times 10^2 \text{ cm}^2/\text{g}$) so this might partially explain the low

sugar accumulation and substrate consumption. An improvement is expected from particle size selection.

4. Future Work

- Determine the influence of particle size of corn stover and sugar cane bagasse in the fermentation pattern of cellulose degradation.

- Study by electron microscopy the spatial arrangement of substrate particles and microbial cells during the growth of C. thermocellum in order to improve growth conditions.

- Complete construction and set-up of continuous culture system using a solids feeder in order to perform preliminary runs using various natural substrates.

E. Genetic Manipulations

This phase of the project has continued to emphasize the development of gene transfer systems in C. thermocellum. Specifically, we have concentrated in characterizing the techniques for protoplast induction and stabilization, towards the goal of achieving cell fusion, as well as the development of procedures for plasmid isolation.

1. Protoplast induction and stabilization in C. thermocellum

We have developed two different methods for the formation of protoplasts from C. thermocellum. One involves the use of non-growing washed cells while in the second we induce protoplast formation in actively growing cells. The first method using resting cells was described in previous reports. The main problem of this method was the variability in cell viability in different experiments which ranged between 0.1-20%. Mg^{++} and Ca^{++} concentrations did not affect the viability (Table I.E.1.1.) while the viability was decreased as the temperature of protoplast induction was increased (Table I.E.1.2.). In the second method to induce protoplast formation from growing cells, the viability of the cells was not affected. This is probably due to the fact that this method does not involve any aerobic manipulations and the protoplast inducers and stabilizers are added anaerobically into the growing culture.

Table I.E.1.1. Viability of cells at different Ca^{++} and Mg^{++} concentrations

CaCl_2 mM	-	20	40	60	-	-	-	-
MgCl_2	-	-	-	-	-	5	12.5	25
% viability	10	17	6.6	2.5	5	4	7	4

Table I.E.1.2. Viability of cells at different temperatures

viability %	0°C 20	25°C 15	37°C 0.25	44°C 0.1

a. Protoplast induction in actively growing cultures

C. thermocellum cultures were grown in CM4-cellobiose medium, inoculated into CM4-cellobiose containing 5% sucrose and grown to $OD_{660} = 0.3$ at 60° . Lysozyme (0.5 and 100ug/ml) was added at this point and further incubated at 60° , after 3 hr incubation the samples from the cultures were observed and counted in the microscope, OD_{660} was followed throughout the incubation period. No cells were observed in any of the lysozyme treated cultures, some protoplasts were seen and a large quantity of cell debris could be seen. When higher concentrations of lysozyme were used (500 and 1000 ug/ml) the protoplasts aggregated and the OD_{660} increased with incubation time due to the accumulation of a white precipitate (presumably denatured lysozyme).

Penicillin (100, 500 and 2500 u/ml) was added as protoplast inducer with or without lysozyme. The only detectable effect of penicillin was growth inhibition. When added in addition to lysozyme, penicillin did not affect the results. In all the cultures where lysozyme was present the OD_{660} went down drastically after 5-10 min. incubation at 60° and a large quantity of cell debris was observed under the microscope. The results indicated that 50 ug/ml of lysozyme, without penicillin, was adequate to induce protoplasts in growing cells.

b. Protoplast stabilization

In order to stabilize the induced protoplasts, cultures were grown in CM4-cellobiose medium to the exponential phase. The cultures were then incubated for 30 min. at 45° and various stabilizers were added at this time before the addition of

lysozyme. Protoplast stabilization was detected by checking the drop in OD₆₆₀ and by microscopic observation.

c. Effect of temperature on protoplast stabilization

C. thermocellum cultures were grown to the exponential phase at 60°C in CM4-cellobiose, then transferred for 30 min. at 45°C. Sucrose was added to 0, 5 and 10% final concentration and 50ug/ml lysozyme were added and the cultures incubated 10 more min. at 45°C. Finally the cultures were transferred to different temperatures and their OD₆₆₀ followed. Fig. I.E.1.1. indicates that in the absence of sucrose an increase in temperature from 50 to 60°C has a stabilizing effect in O.D. The same stabilization obtained at 60°C without sucrose can be seen at 50°C when the cultures contain 10% sucrose. The behavior of the cultures in 5% sucrose is similar to that in the absence of sucrose. Therefore, at this point we chose 60°C and 5% sucrose as our standard conditions. This is due to the fact that C. thermocellum grows optimally at around 60°C and 5% sucrose has little or no effect on growth kinetics. However, it should be noted that in this experiment, even optimal conditions showed a 60% loss in O.D.

d. Effect of cultural age on protoplast stabilization

Cultures were grown to different stages of growth (O.D.'s ranging from 0.1 to 0.8), one-half of all the cultures were brought up to 5% sucrose and all were transferred to 45°C for 30 min. At this point lysozyme at a final concentration of

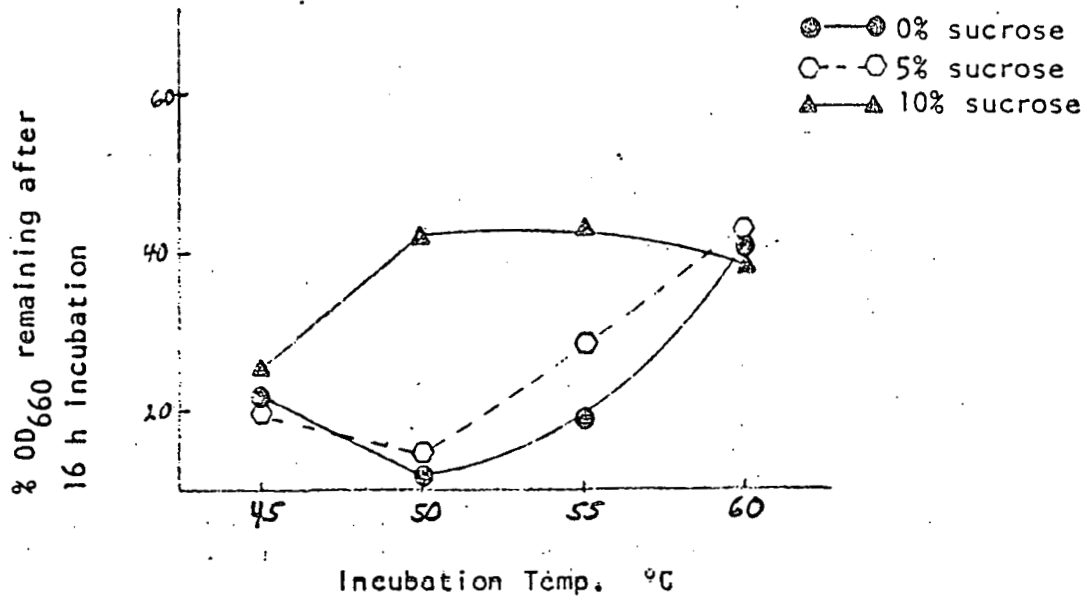


Fig. I.E.1.1. Effect of incubation temperature on the fermentation of protoplasts in actively growing cells.

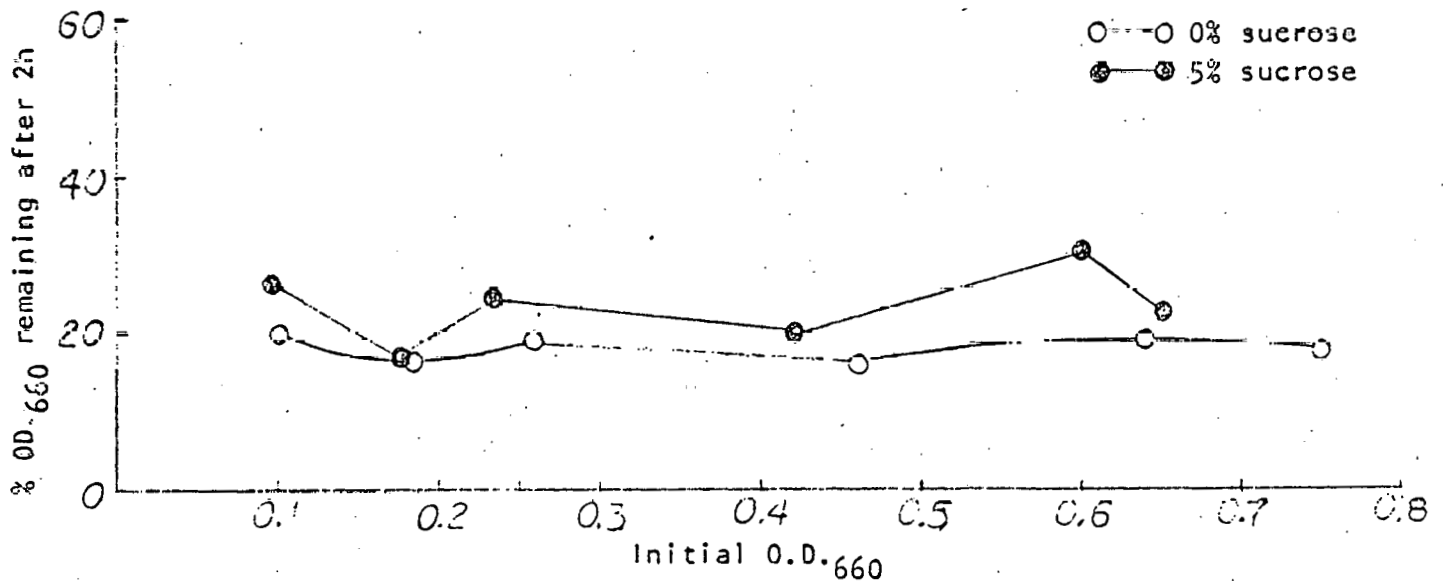


Fig. I.E.1.2. Effect of growth phase on protoplast induction and stabilization

50ug/ml was added, incubation was continued at 45°C for 10 min. and at 60°C for 2 hr. The results shown in Fig. I.E.1.2. indicate that culture age has no effect on protoplast stability whether 5% sucrose is present or not. In this experiment, approximately an 80% reduction in O.D. was observed.

e. Stabilization of protoplasts as function of NaCl concentration and 5 or 10% sucrose

Cultures were grown as above and NaCl and sucrose were added as stabilizers before addition of 100ug/ml lysozyme. Fig. I.E.1.3. shows that NaCl has only a marginal stabilizing effect above that seen with 5% sucrose.

f. Stabilization by high sucrose concentrations

Cultures were grown in CM4-cellobiose to the exponential phase and transferred to 45°C for 30 min., sucrose to different final concentrations was then added followed by 50ug/ml lysozyme. Fig. I.E.1.4. shows the results obtained when the incubation was carried on at 45°C or when the cultures were transferred to 60°C after 10 min. incubation at 45°C. In Fig. I.E.4.A. it can be seen that after 45 min. incubation at 60° or 45°, 15-30% sucrose stabilizes the induced protoplasts. However, after 20 hr. incubation (Fig. I.E.1.4.A.) at 45° or 60° at sucrose concentrations of 25 and 30% the protoplast lyse while at 15-20% sucrose the majority of protoplast are still present.

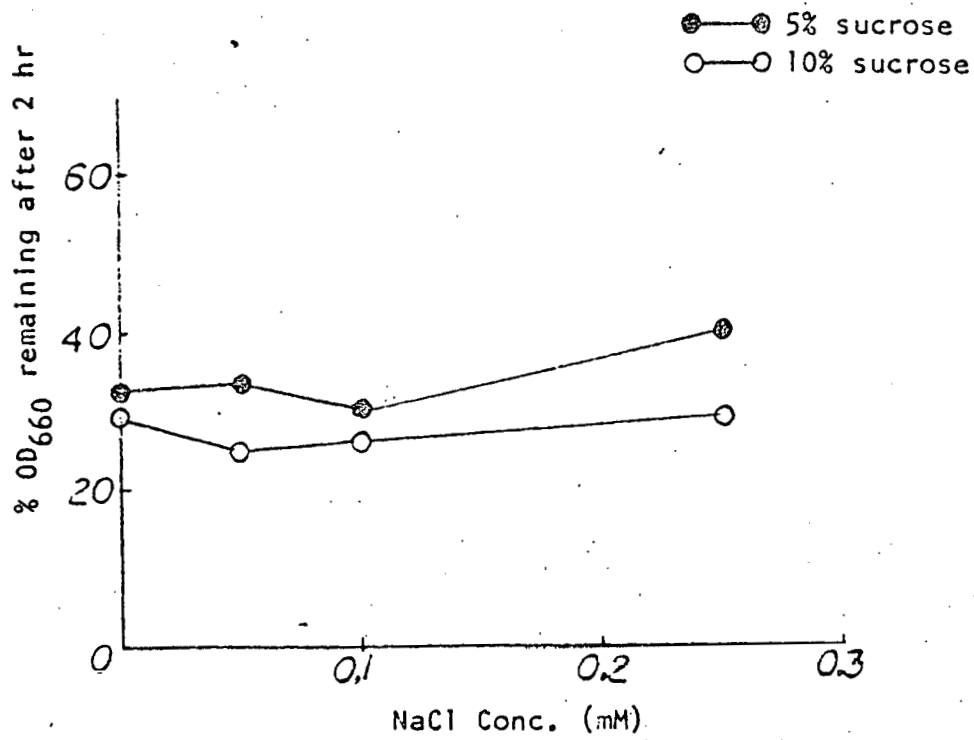


Fig. I.E.1.3. Effect of NaCl concentration on the stability of protoplasts

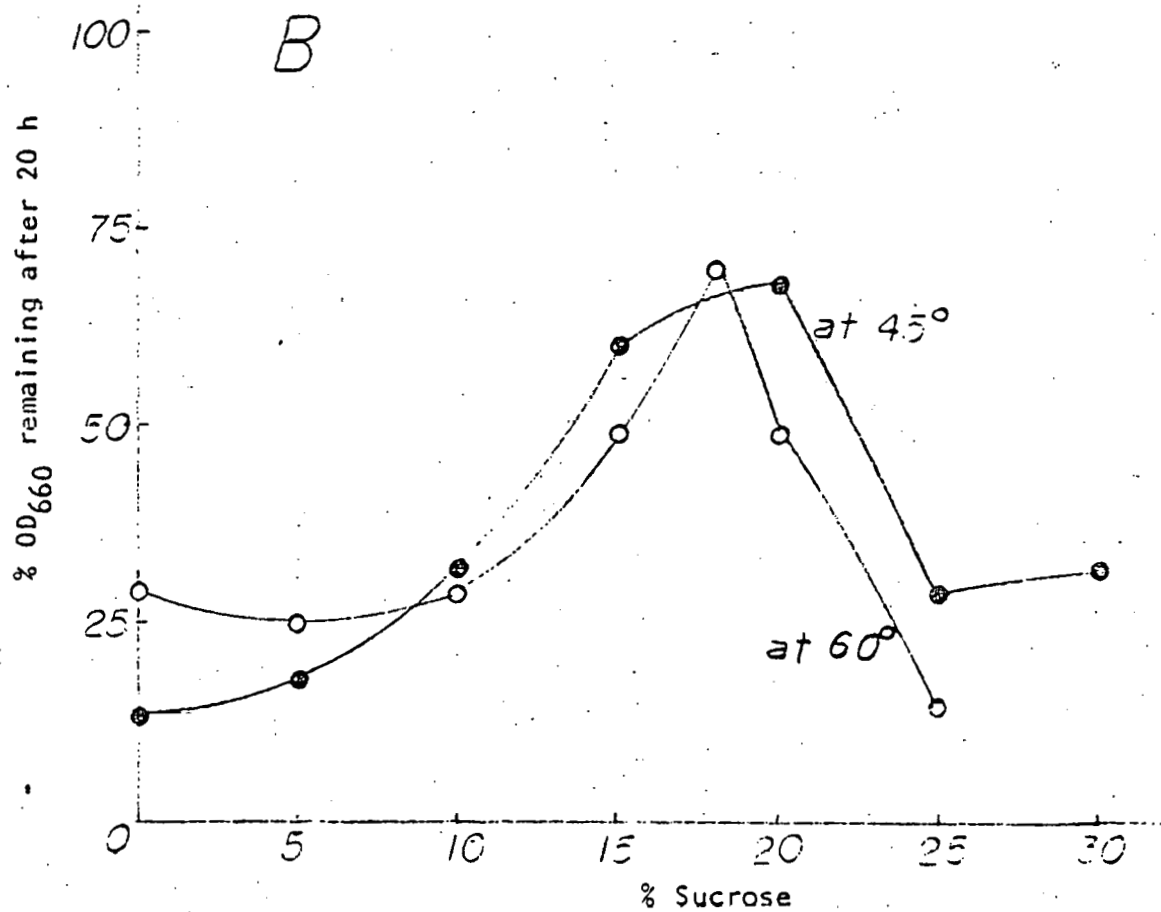
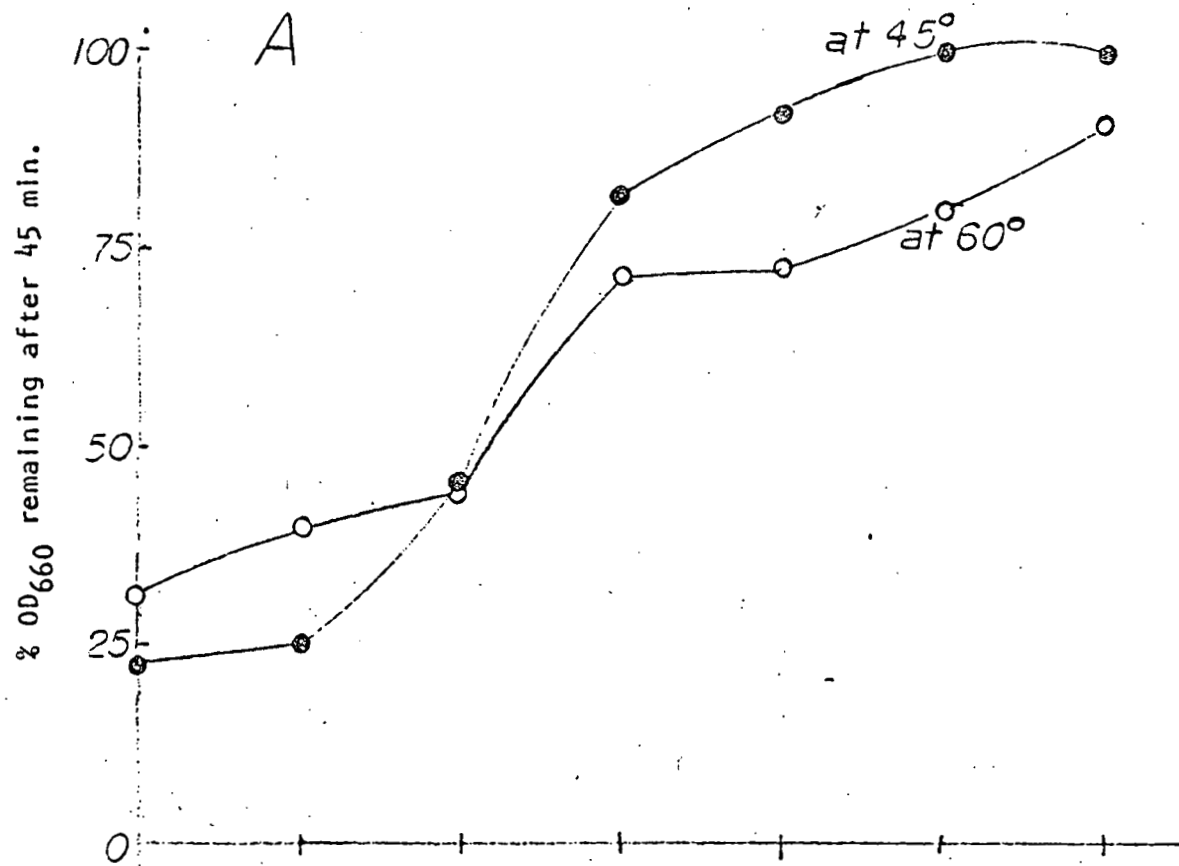


Fig. I.E.1.4. Effect of sucrose concentration on protoplast stabilization after 45 min. (A) and 20 h (B) incubation at 45°C and 60°C.

g. Stabilization by cellobiose, mannitol and polyethylene glycol

Protoplasts were induced in the presence of either cellobiose, mannitol or polyethylene glycol at different concentrations as stabilizers (added before lysozyme). Fig. I.E.1.5. shows that polyethylene glycol and cellobiose are better stabilizers than sucrose at concentrations of 10% but not at lower concentrations, while mannitol is not a good stabilizer even at 10% concentration.

h. Conclusions and Future Work

The method of inducing protoplasts in actively growing cells seems to have several advantages over the method of protoplast induction of resting cells that was described in earlier reports. Protoplasts from actively growing cells are readily formed with low lysozyme concentrations (as low as 0.5ug/ml) leaving no detectable cells and forming mostly free protoplasts without visible cell wall attached. On the other hand, protoplasts induced from resting cells require high lysozyme concentration (4ug/ml) for their formation and appear mostly with cell wall remnants attached to them. The greatest advantage in using growing cells to induce protoplasts is that the cells do not have to be exposed to aerobic manipulations and therefore losses in cell viability are avoided.

Protoplasts induced from growing cells are well stabilized by the presence of 18% sucrose (up to 70% OD₆₆₀ remained). Ten percent polyethylene glycol and 10% cellobiose are also fair stabilizers. However, 10% sucrose inhibits growth by 50%,

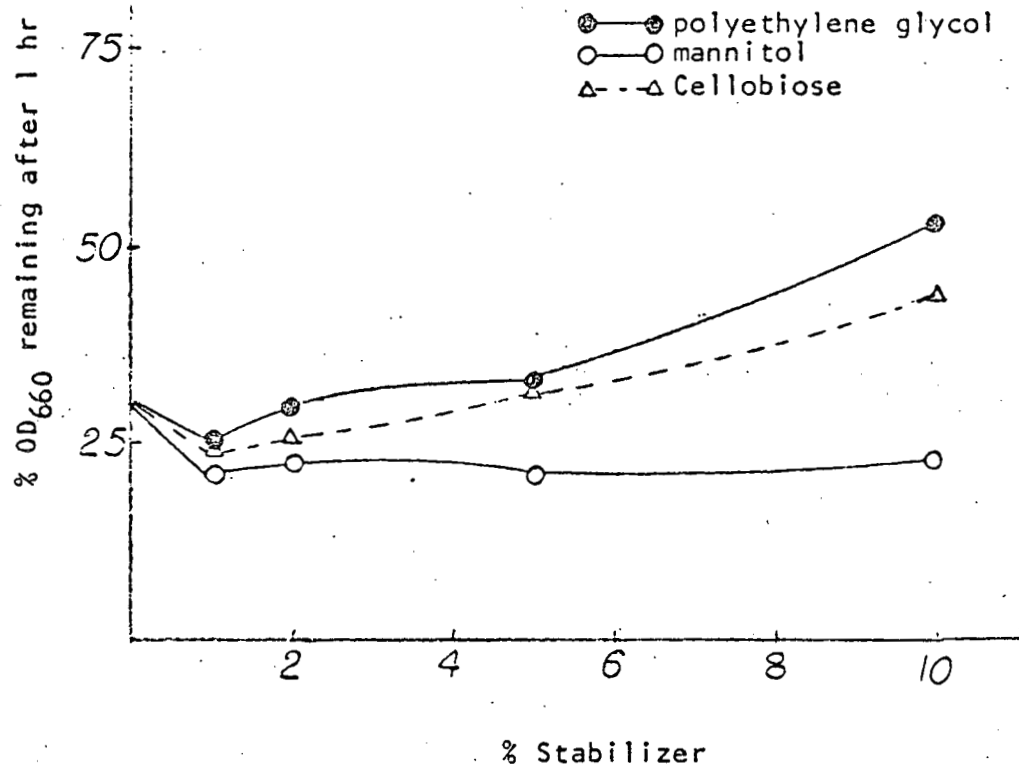


Fig. I.E.1.5. Effect of Polyethylene glycol, Mannitol and Cellobiose on protoplast stabilization

therefore 18% sucrose would not be suitable for protoplast regeneration. Ten percent cellobiose inhibits growth by 70% and 10% polyethylene glycol does so by 35%. Therefore, polyethylene glycol might be a useful stabilizer for regeneration experiments since it is a good stabilizer and is a weak growth inhibitor.

We are currently trying to obtain regenerated cells from the protoplasts induced from actively growing cells and those will be used in future experiments as means for gene transfer by fusion of different strains.

2. Isolation of plasmids from C. thermocellum

Work on the bulk isolation of the plasmid DNA and characterization of the initially isolated complex is continuing. As reported previously, the sedimentation behavior of the isolated material suggested the formation of large aggregates of some sort during isolation. Since alkaline sedimentation had resulted in the partial destruction of the aggregation, sedimentation at a series of intermediate pH values was carried out in an attempt to isolate a homogenous DNA species, as well as to gain information about the aggregate.

Material recovered from an ethidium bromide-caesium chloride gradient was dialyzed and applied to 5-20% sucrose gradients of specified pH. The results at pH 7.6, 9.2 and 9.7 are shown in Figures I.E.2.1., I.E.2.2. and I.E.2.3. It can be seen that over this pH range, no change in sedimentation behavior occurs. The result at pH 10.3 is shown in Figure I.E.2.4. It can be

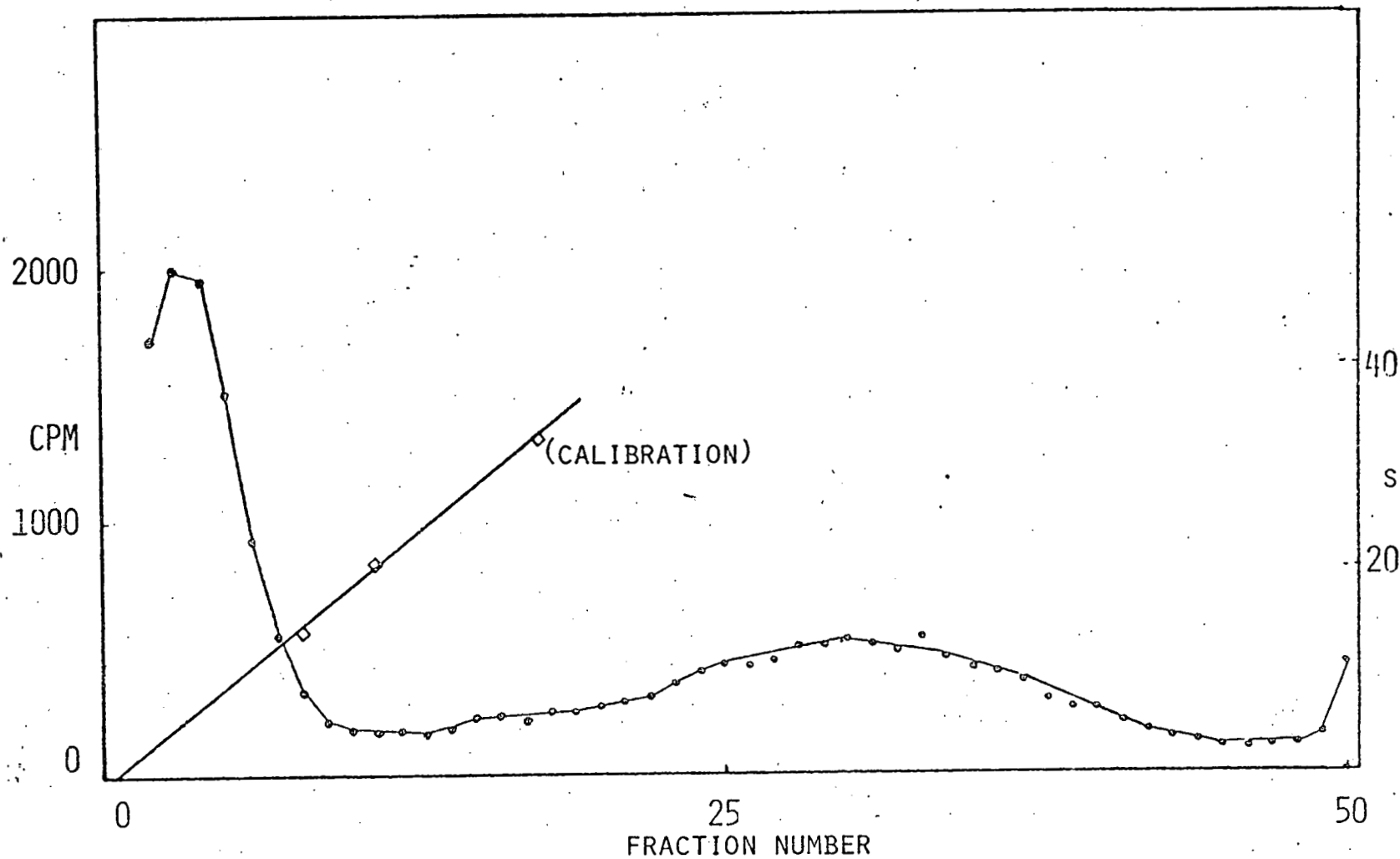


Figure I.E.2.1. Sedimentation velocity analyses of material recovered from CsCl-ethidium bromide gradient satellite peak. Five to 20% sucrose, 20°C, $\omega^2 t = 1.02 \times 10^{11}$ rad²/sec, pH 7.6

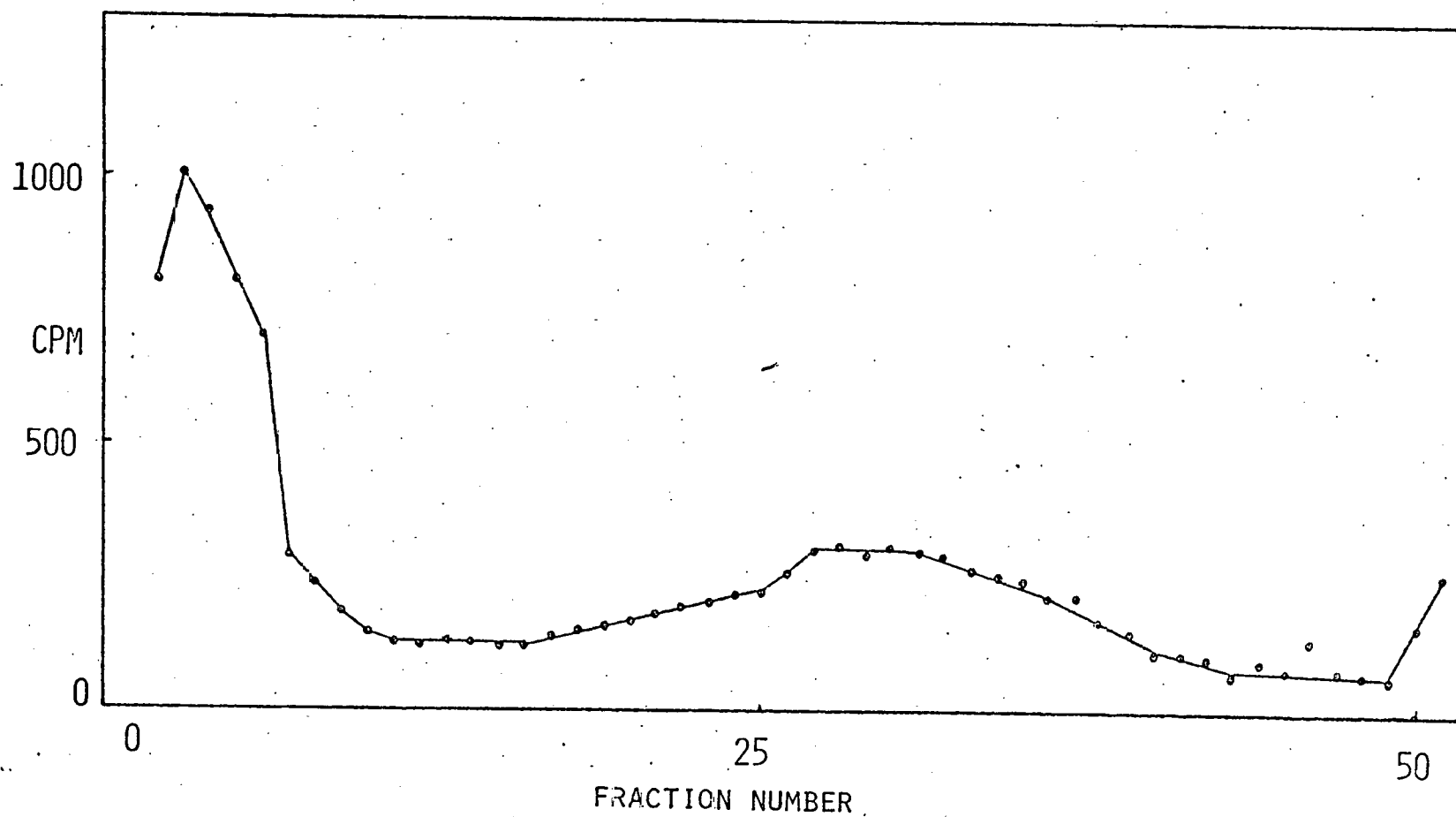


Figure I.E.2.2. Sedimentation of same material as in Fig. I.E.2.1., pH 9.2.

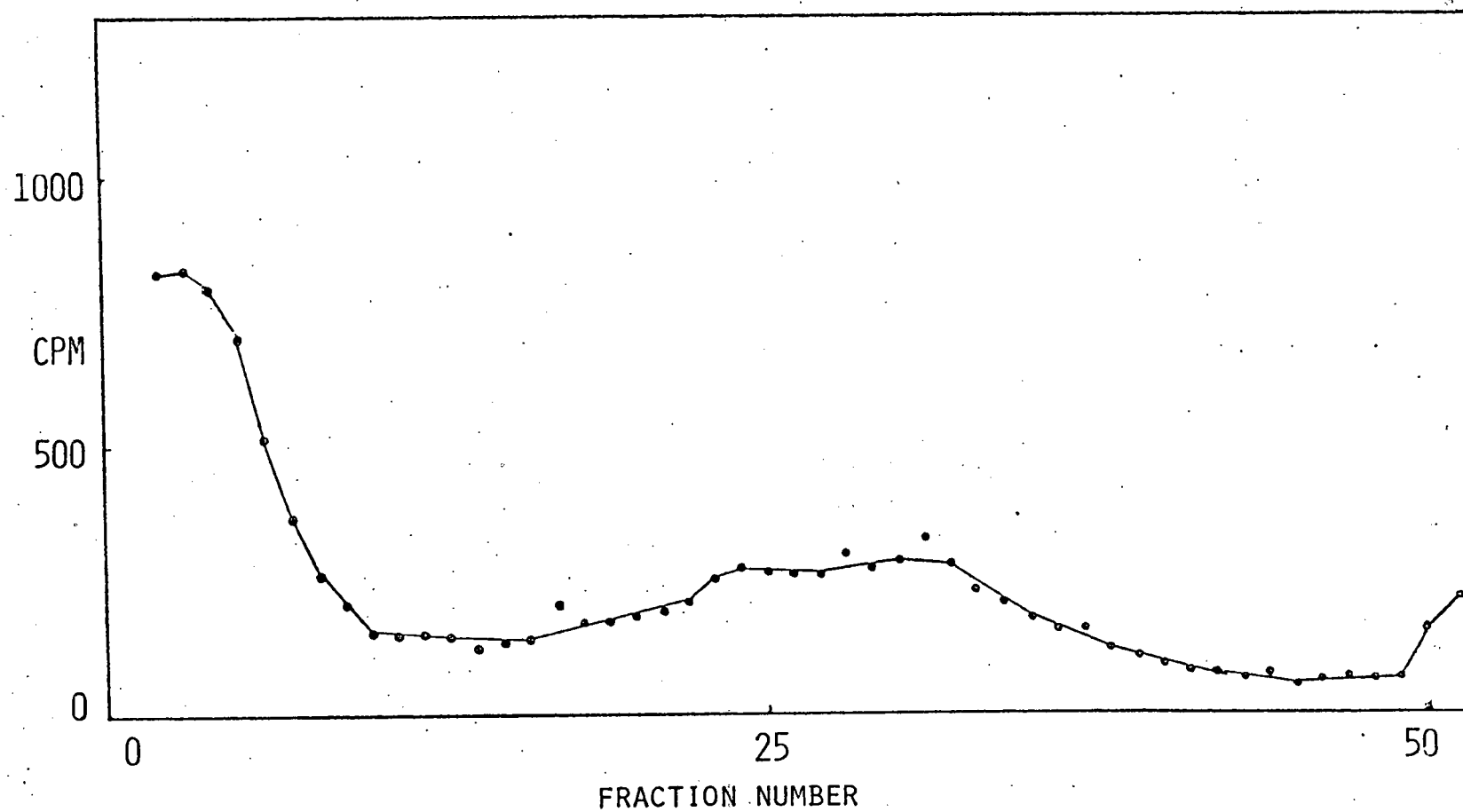


Figure I.E.2.3. Sedimentation of same material as in Fig. I.E.2.1., pH 9.7.

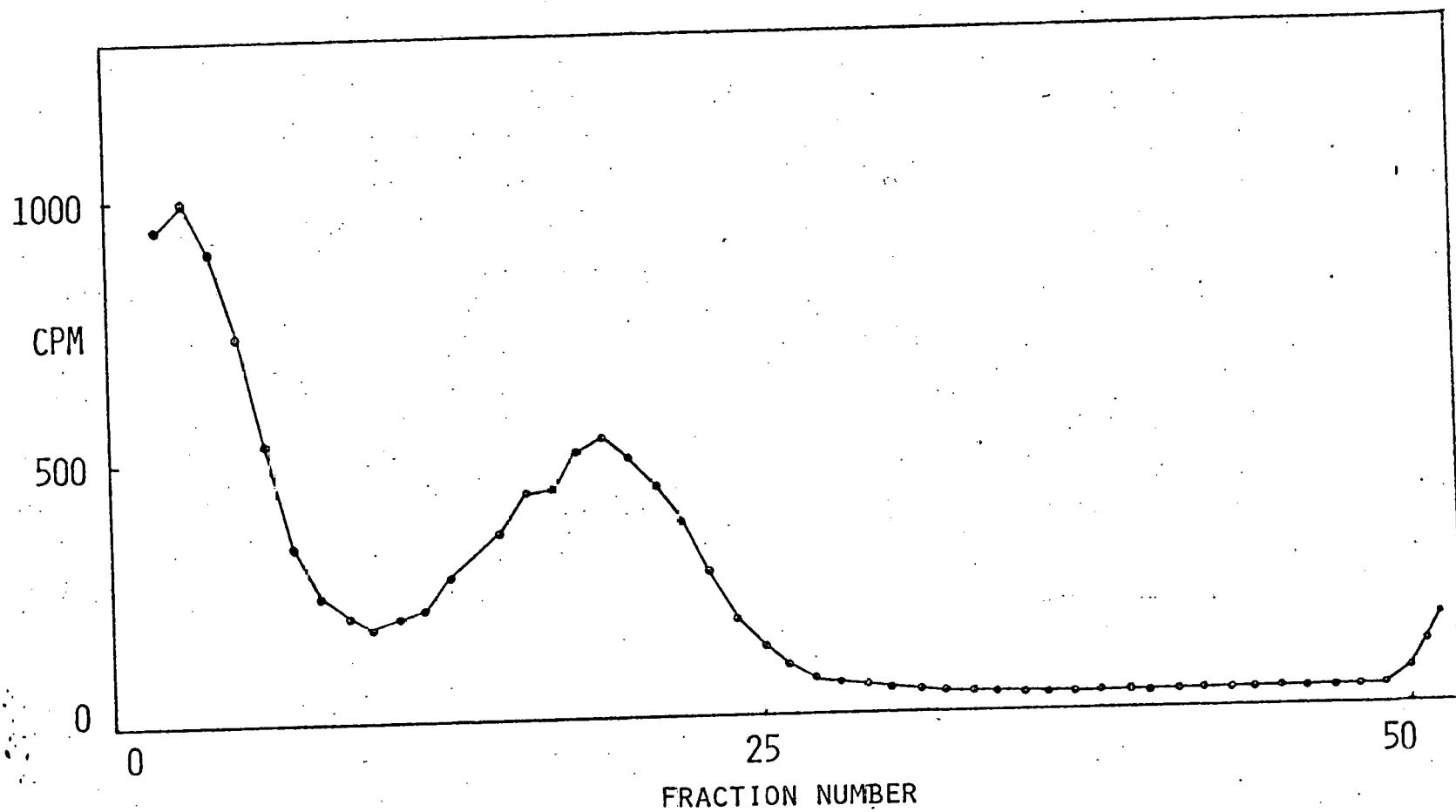


Figure I.E.2.4. Sedimentation of same material as in Fig. I.E.2.1., pH 10.3.

seen that the material sediments more slowly and with a narrower distribution, but it is still broader than a homogenous DNA peak would be.

Sedimentation at pH 11.3 is shown in Figure I.E.2.5. This results in a further reduction in peak width and velocity, and sedimentation at pH 12.5, shown in Figure I.E.2.6., reduces these further still.

A composite of these results is shown in Figure I.E.2.7. for easy comparison. The behavior shown is consistent with the random breakage of an aggregate, and nonhomogenous peaks always result.

Since sedimentation at pH 10 has been reported in some cases to remove material responsible for the alkaline degradation of plasmid DNA, alkaline sedimentation of material which had been sedimented at pH 10.3 was carried out.

Figures I.E.2.8., I.E.2.9., and I.E.2.10. show the results when material from a pH 10.3 sucrose gradient was recovered and sedimented at pH 7.6, 10.3 and 12.5. The first shows that the effect of the pH treatment is not reversible, the second is a control, and the third shows that the result of sedimentation at pH 12.5 is the same, whether or not it is preceded by treatment at pH 10.3.

This alkaline gradient was also centrifuged for a longer period to determine whether more than one peak could be resolved. The results for material with pretreatment and without are shown in Figures I.E.2.11. and I.E.2.12. No separate peaks are resolved.

To determine the cause of this and to attempt another method

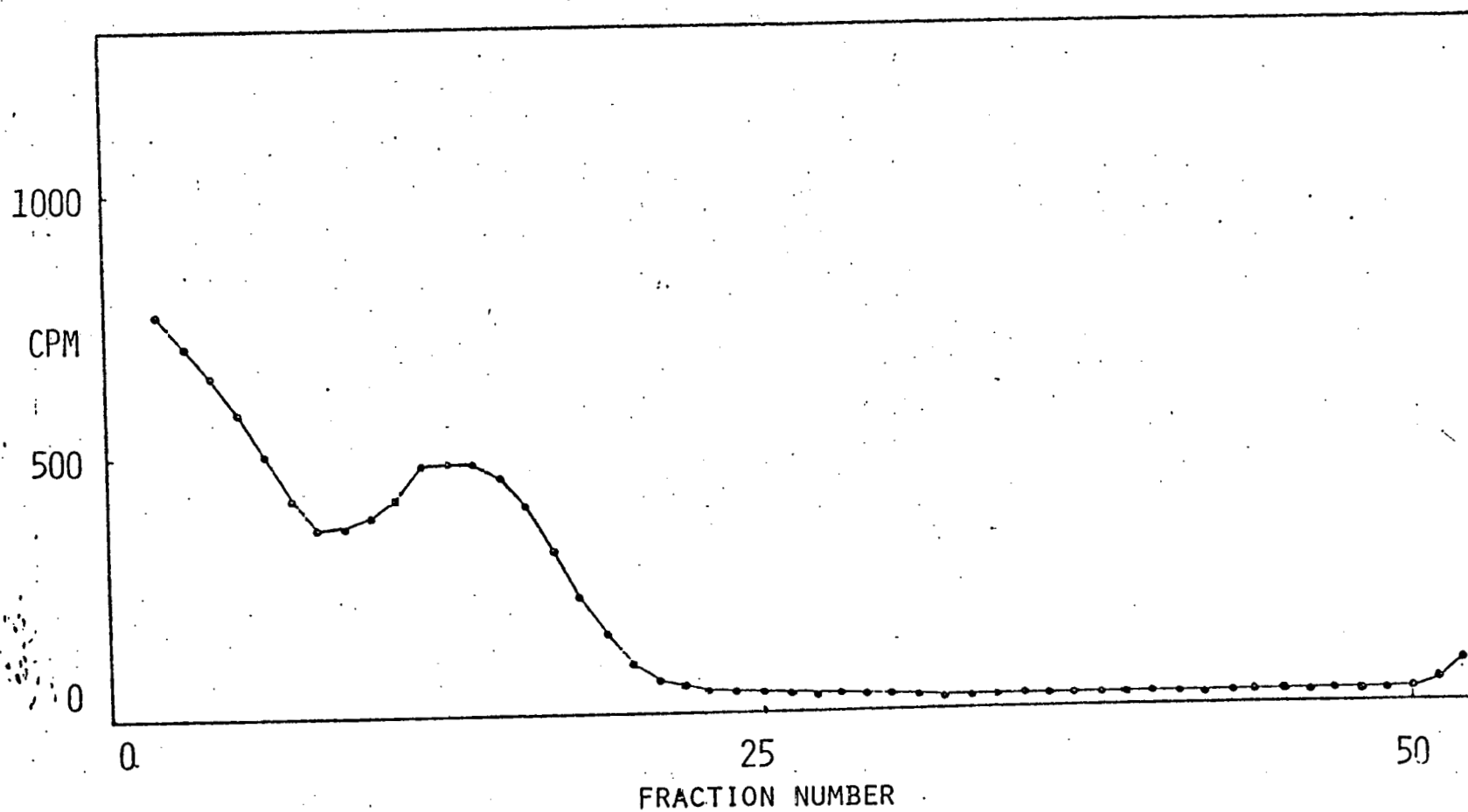


Figure I.E.2.5. Sedimentation of same material as in Fig. I.E.2.1., pH 11.3.

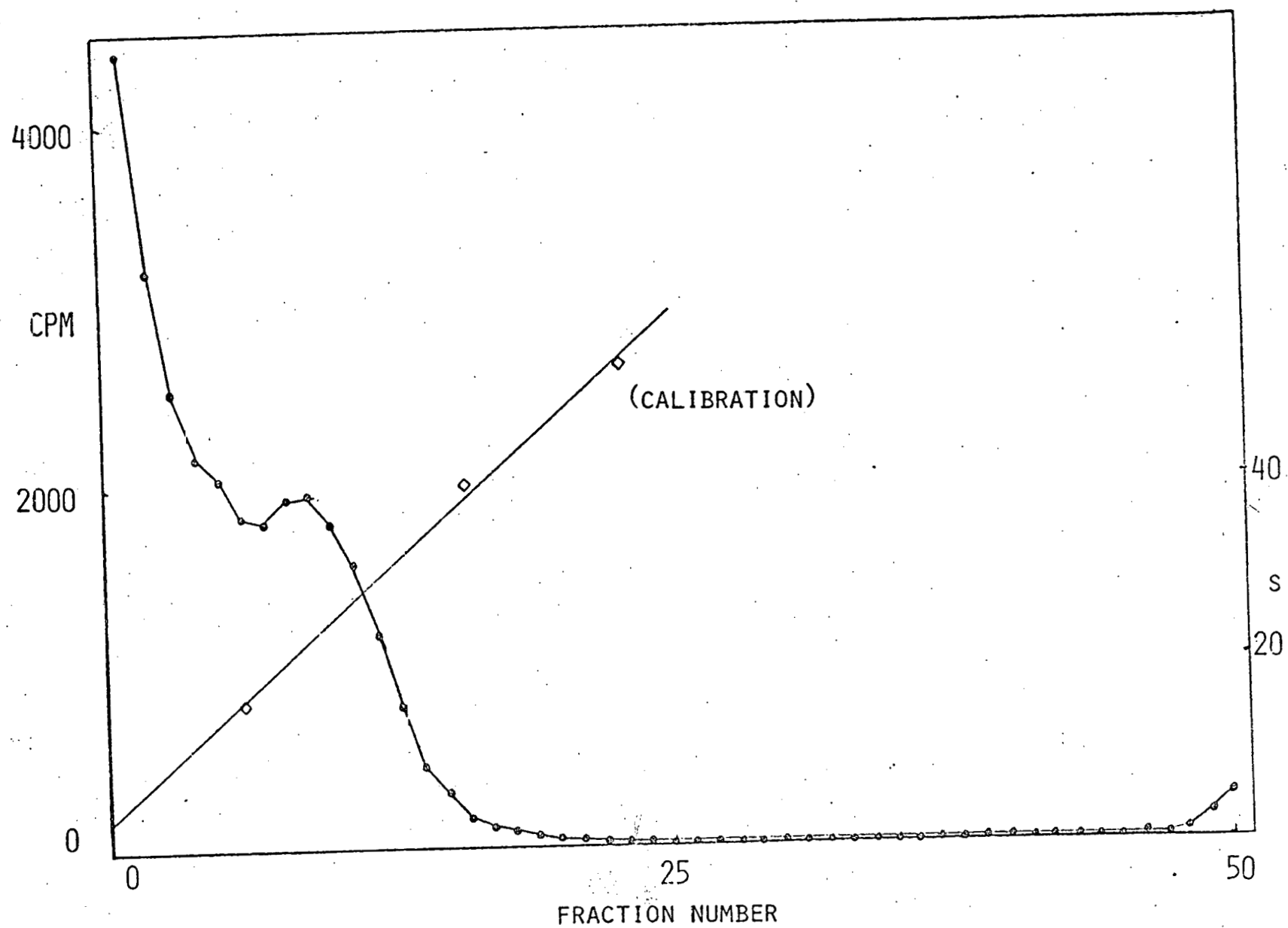


Figure I.E.2.6. Sedimentation of same material as in Fig. I.E.2.1., pH 12.5.

Figure I.E.2.7. Composite of Figures I.E.2.1. through I.E.2.6.

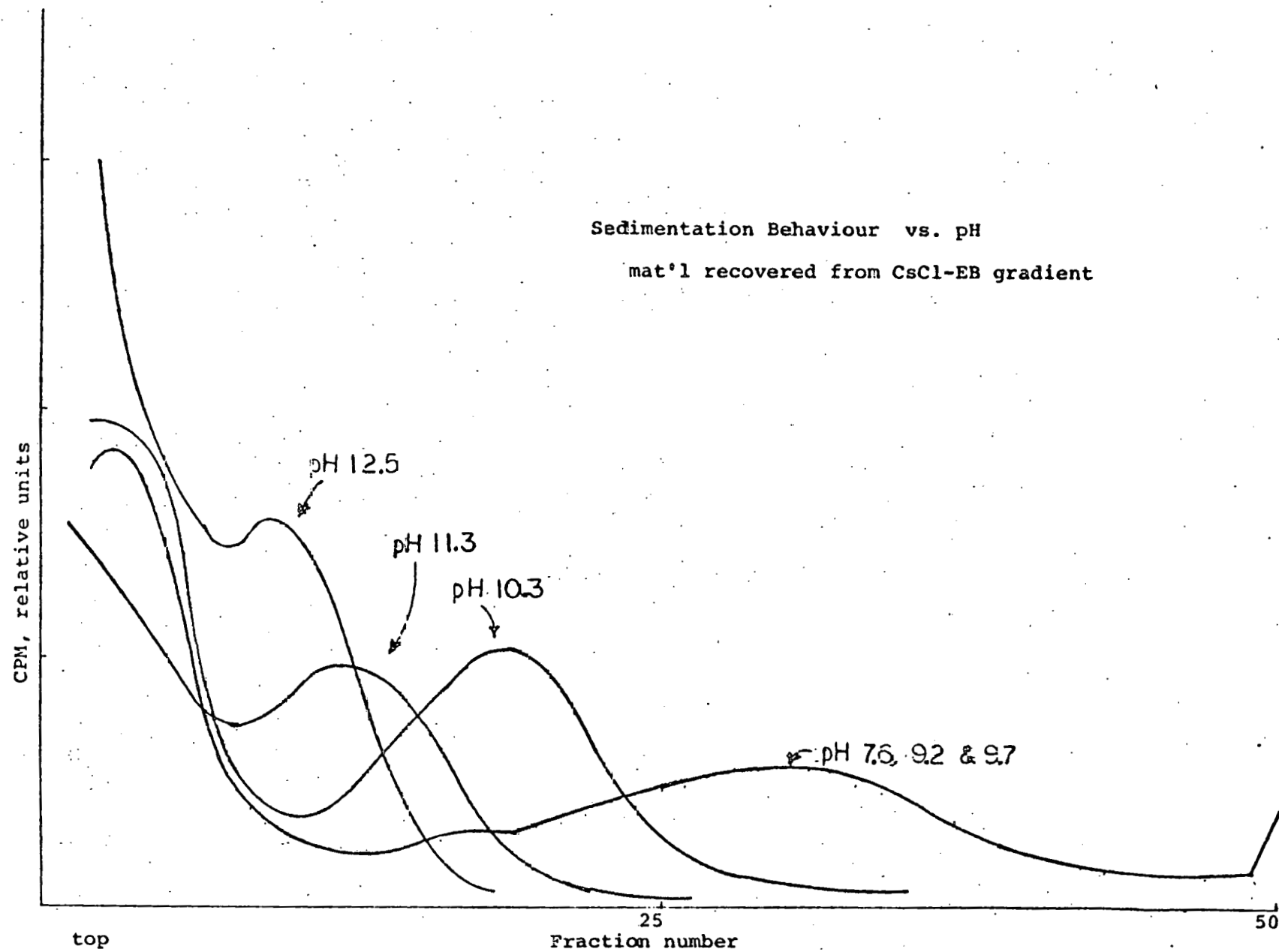


Figure I.E.2.8.

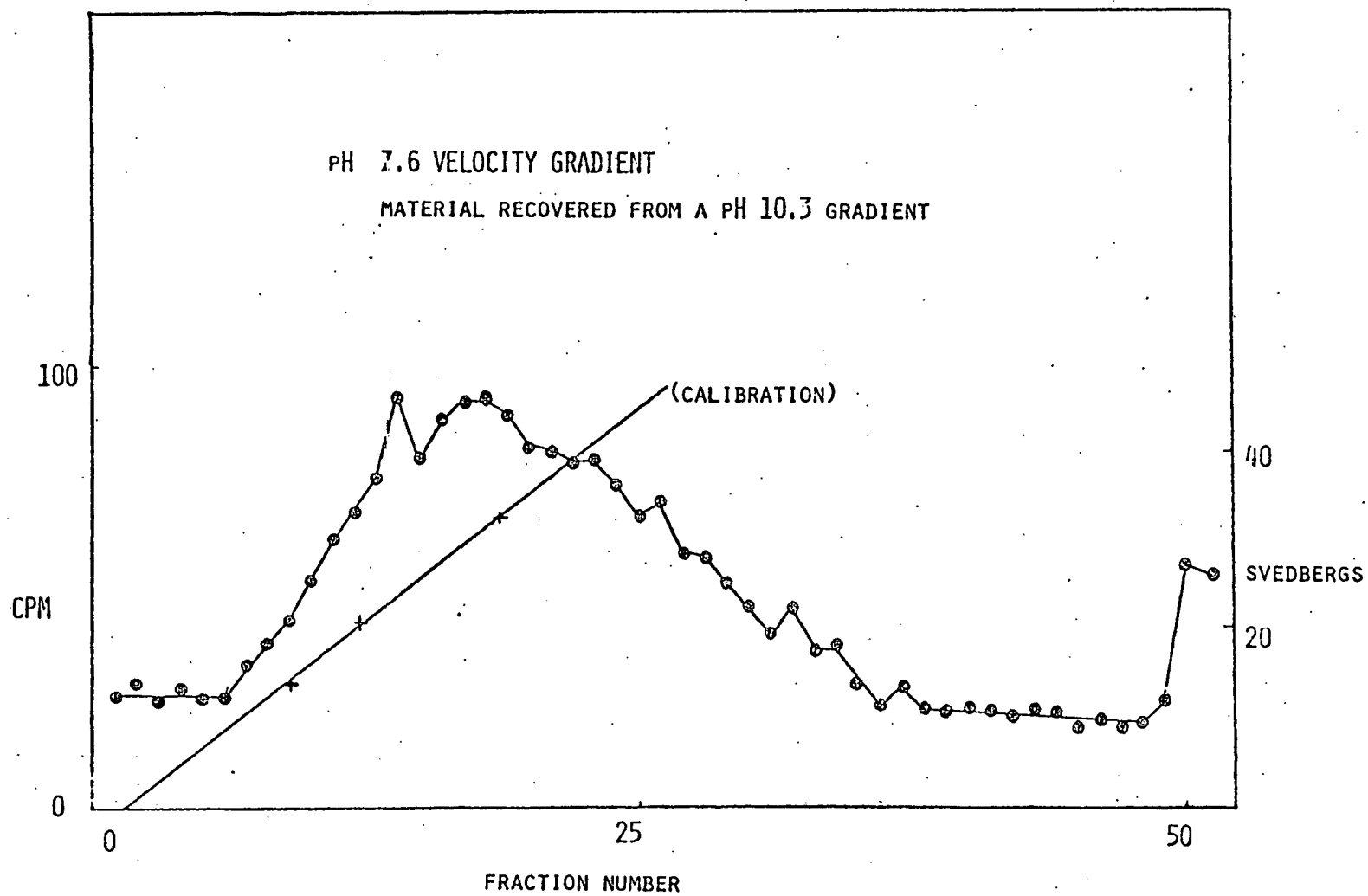


Figure I.E.2.9.

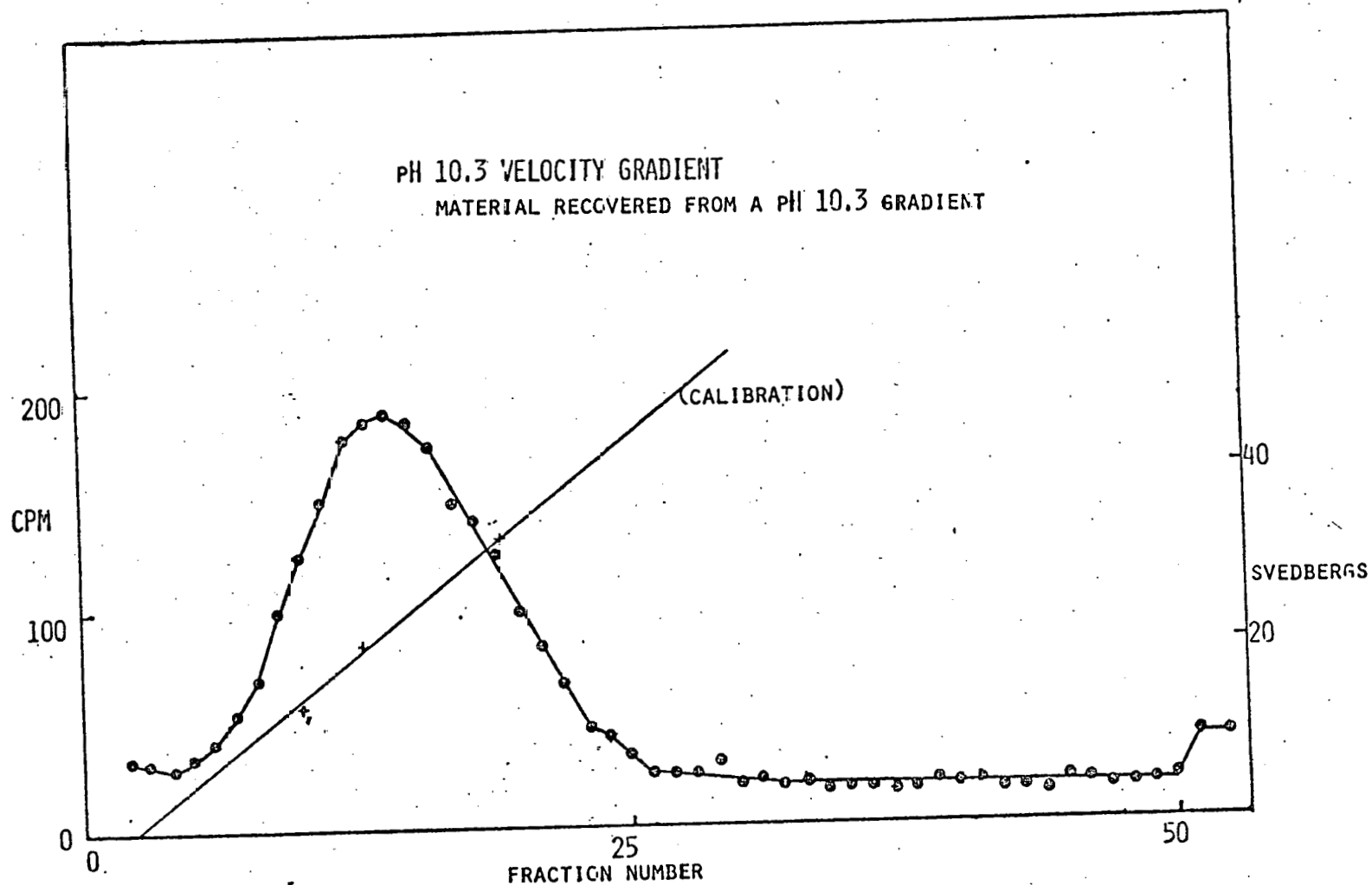
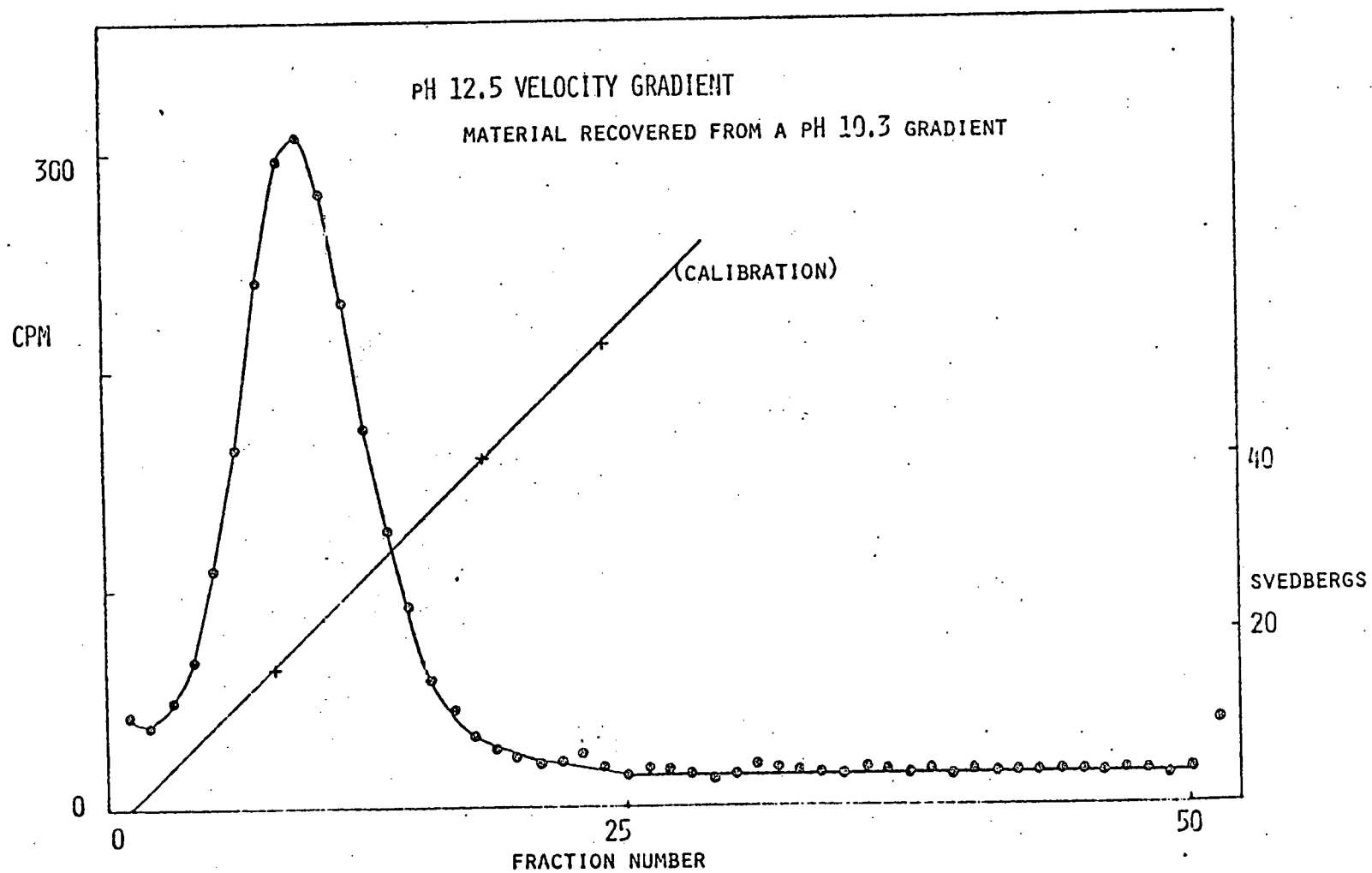


Figure I.E.2.10.



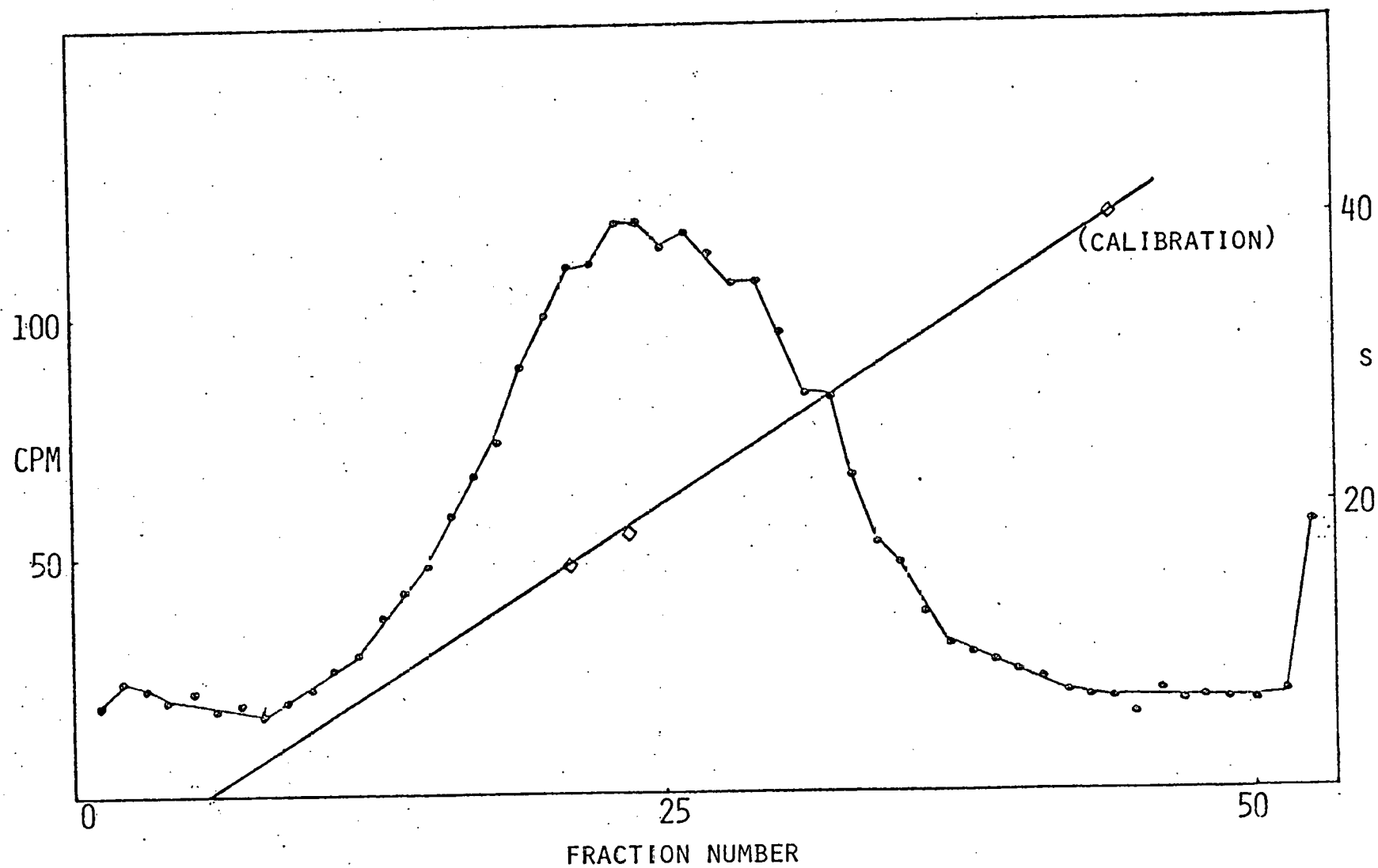


Figure I.E.2.11. Extended pH 12.5 sedimentation of same material as in Figures I.E.2.8. through I.E.2.10.

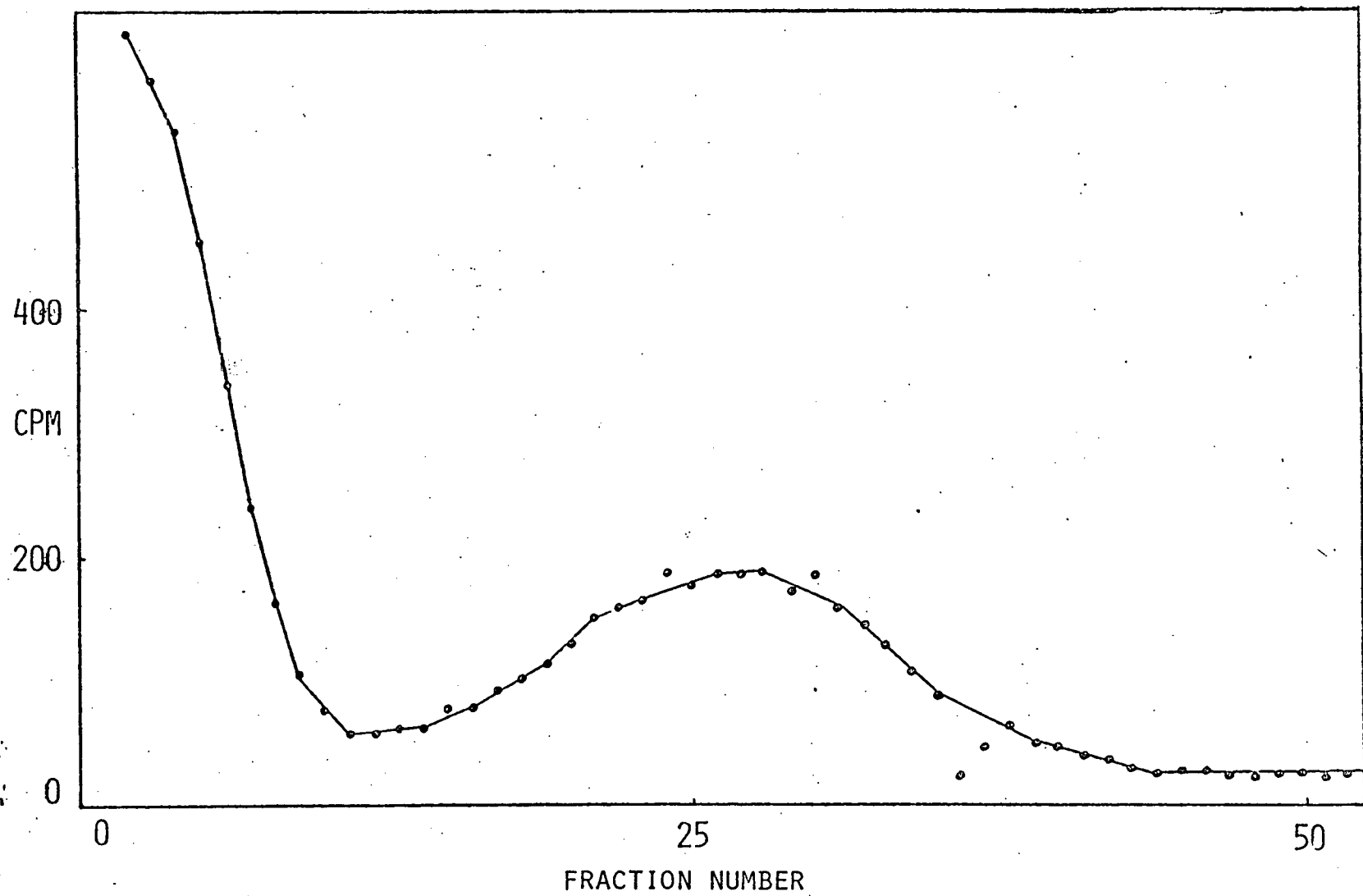


Figure I.E.2.12. Extended pH 12.5 sedimentation of same material as in Figure I.E.2.1.

of purification, material isolated from a cesium chloride-ethidium bromide gradient was subjected to different enzyme digestions followed by neutral sucrose sedimentation.

A positive control for the presence of DNA is shown in Figure I.E.2.13, when the material was treated with pancreatic DNAase. Protease K and ribonuclease, as shown in Figures I.E.2.14. and I.E.2.15. had no effect. S_1 nuclease, Figure I.E.2.16, which is specific for single stranded nucleic acid resulted in the destruction of the complex, suggesting that denatured DNA and denatured regions of the plasmid DNA are responsible for the aggregation.

In the next quarter work we will continue to attempt to elucidate the nature of the plasmides in C. thermocellum. For this purpose polyacrylamide gel electrophoresis will be employed. In addition, we are re-examining the possibility of transformation for gene transfer in C. thermocellum. A search for infecting particles (bacteriophages) from natural sources as well as from other strains of C. thermocellum has been initiated.

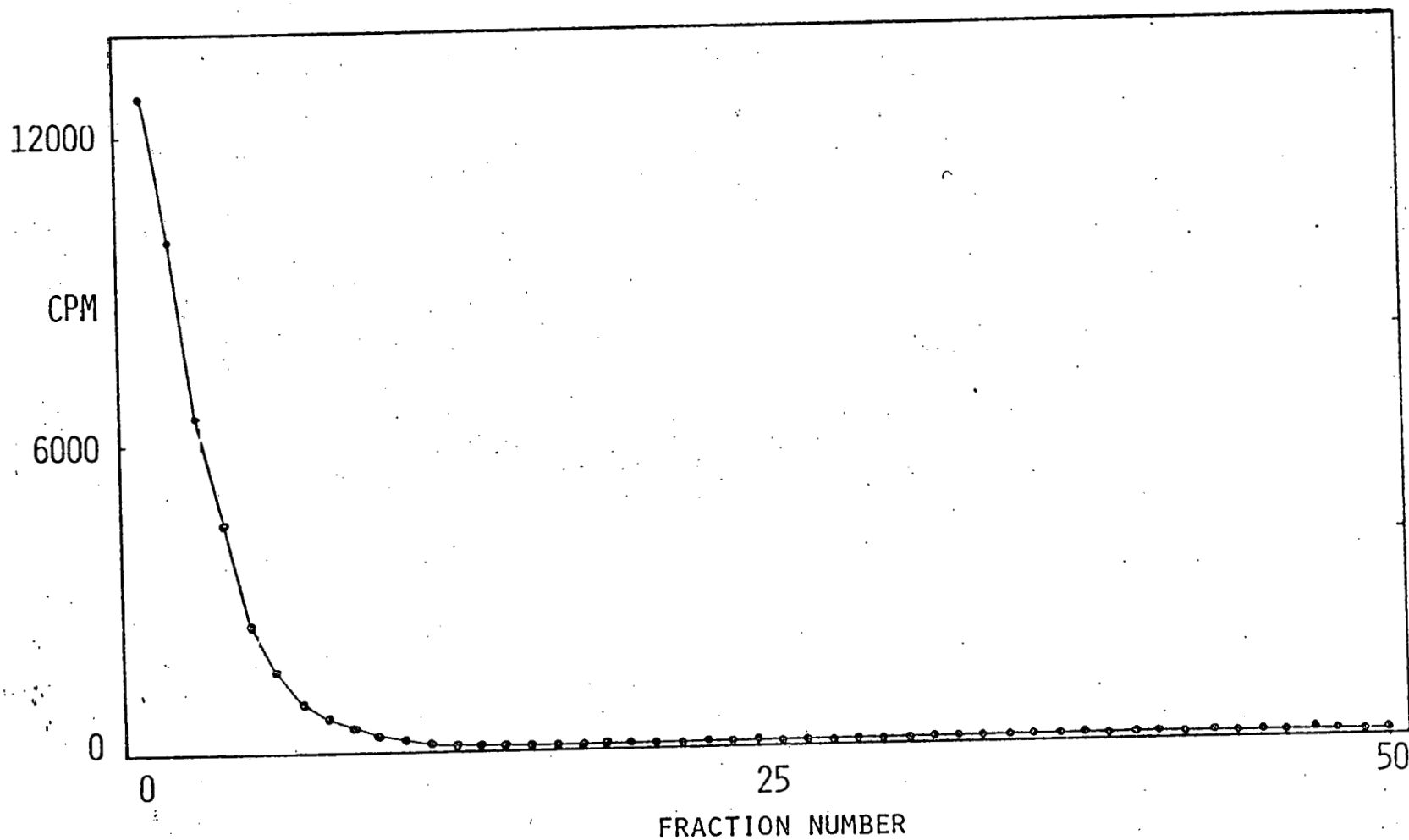


Figure I.E.2.13. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with pancreatic DNAase. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11}$ rad²/sec.

Figure I.E.2.14. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with protease K. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11} \text{ rad}^2/\text{sec}$.

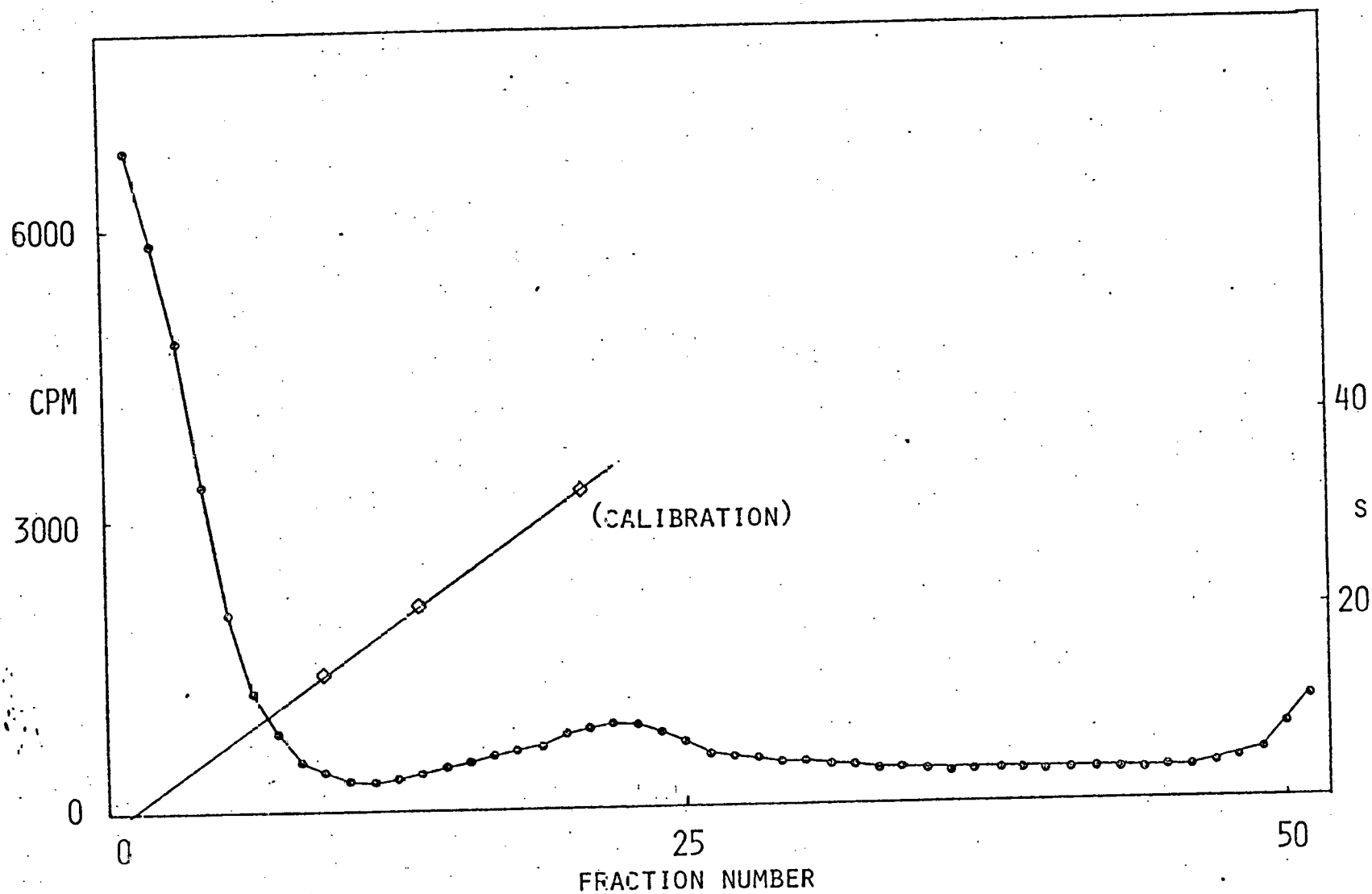


Figure I.E.2.15. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with RNAase. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11}$ rad²/sec.

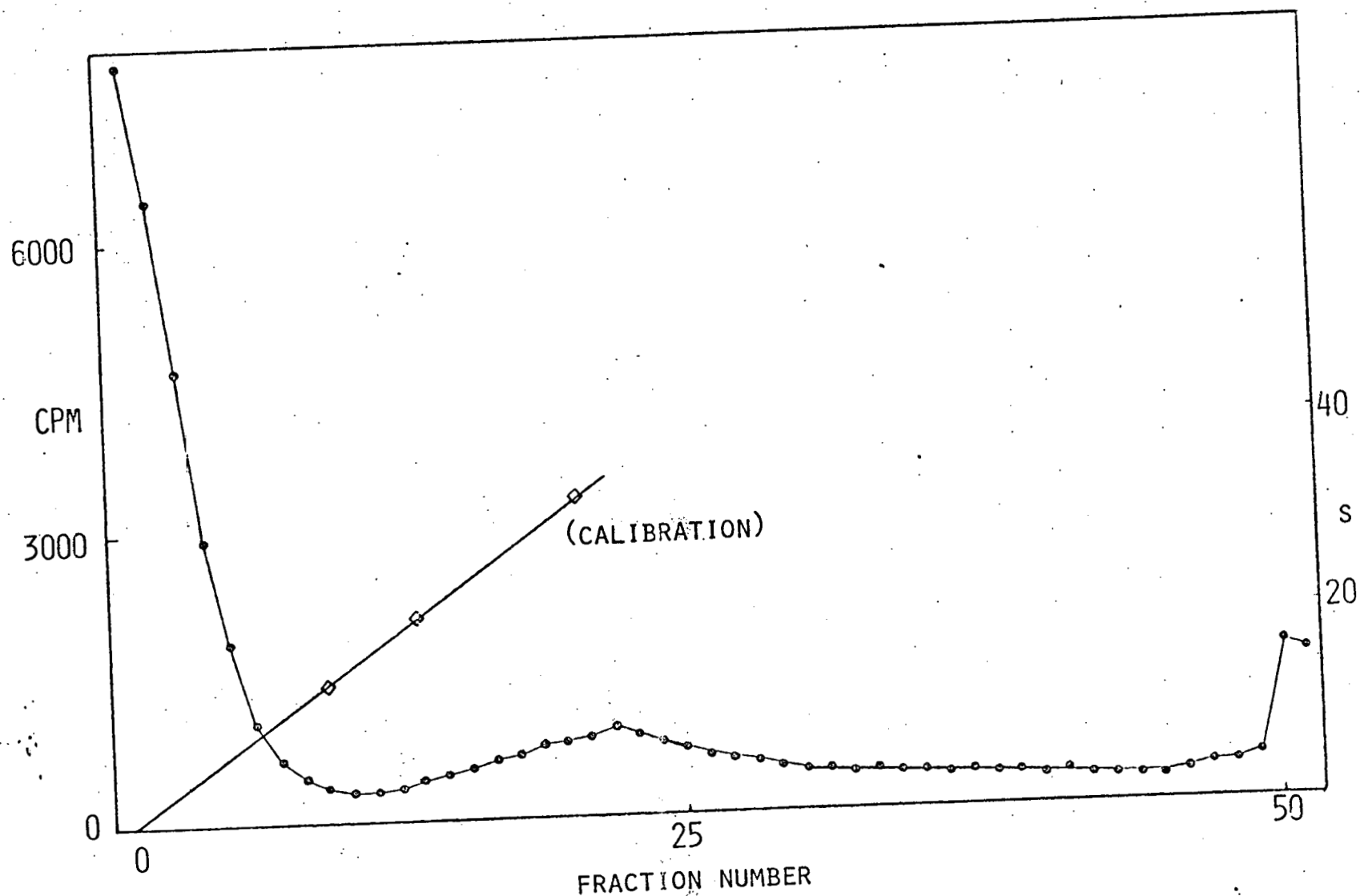
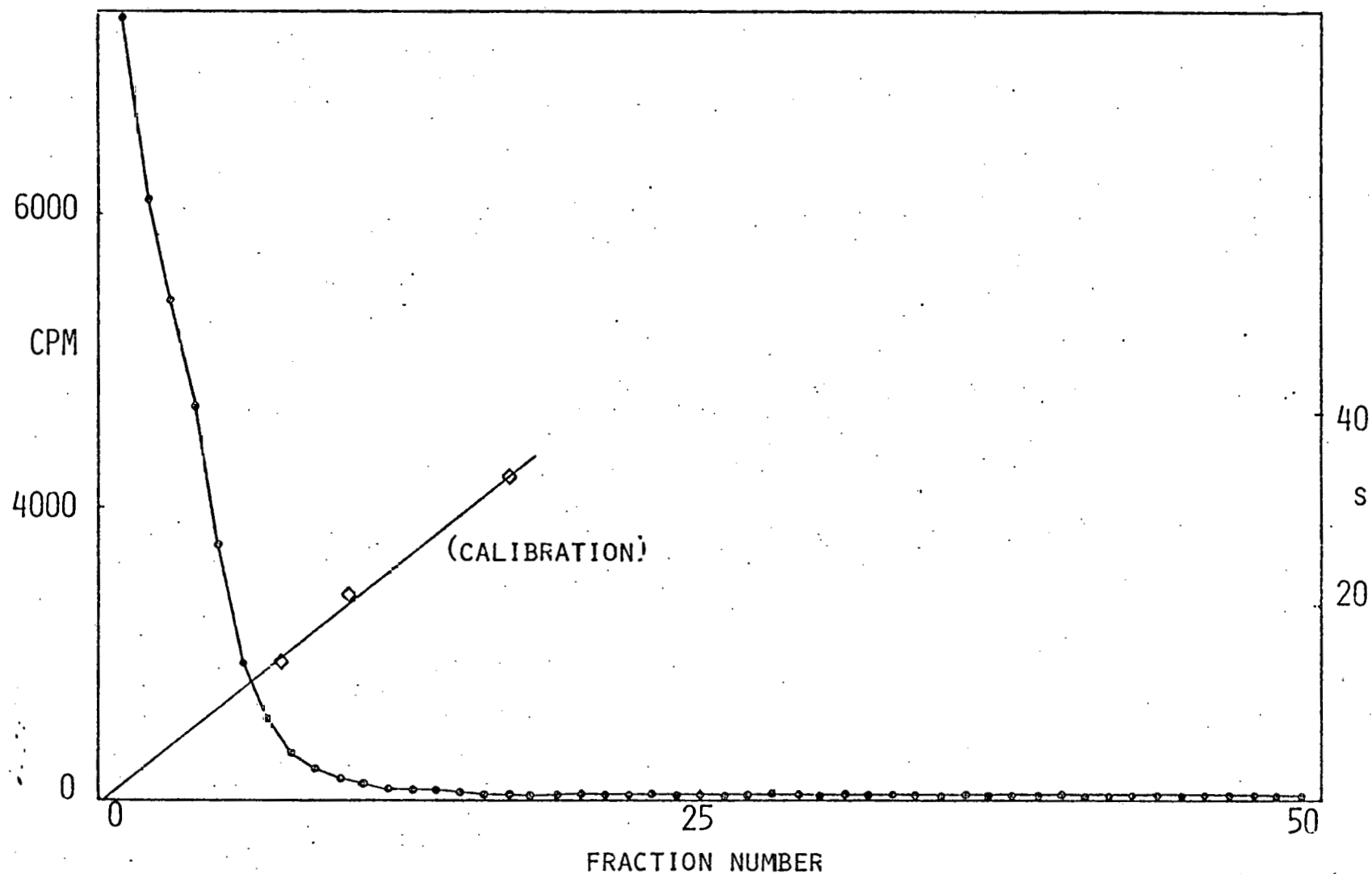


Figure I.E.2.16. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with S_1 nuclease. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11} \text{ rad}^2/\text{sec}$.



F. Deregulation of Clostridial Cellulases

1. Resistance of *C. thermocellum* Cellulase to Sugars

In two previous Progress Reports (8-31-77, 11-31-77), we showed that the addition of 300g of glucose, xylose or cellobiose per liter of enzyme reaction mixture did not inhibit TNP-CMCase activity. To examine the effect of these sugars on the hydrolysis of a more crystalline substrate, a final concentration of 0.05 g/l of each sugar was added to the enzyme solution and Avicel hydrolysis was measured. Again, the sugars did not inhibit enzyme activity.

To examine the effect of high concentrations of sugars on the hydrolysis of a crystalline cellulose, we used an assay involving chromogen release from Remazol Brilliant Blue-bound MN300 cellulose (RBB-MN300). Because the activity of *C. thermocellum* cellulase measured by this assay was low, a concentrated enzyme solution was prepared by ethanol precipitation. The reaction mixture consisted of 1 ml of a 10-fold concentrated enzyme solution, 2 ml of 0.1 M citrate buffer pH 4.5, 15 mg RBB-MN300 and various concentrations of sugars. After incubation at 60°C for 20 hours, the increase in the optical density of the supernatant was measured at 595 nm. As shown in Figure I.F.1., 300 g/l glucose or xylose and 100 g/l cellobiose did not inhibit enzyme activity.

The results of the RBB-MN300 assay agree with those of Avicel hydrolysis and TNP-CMCase activity. From these data,

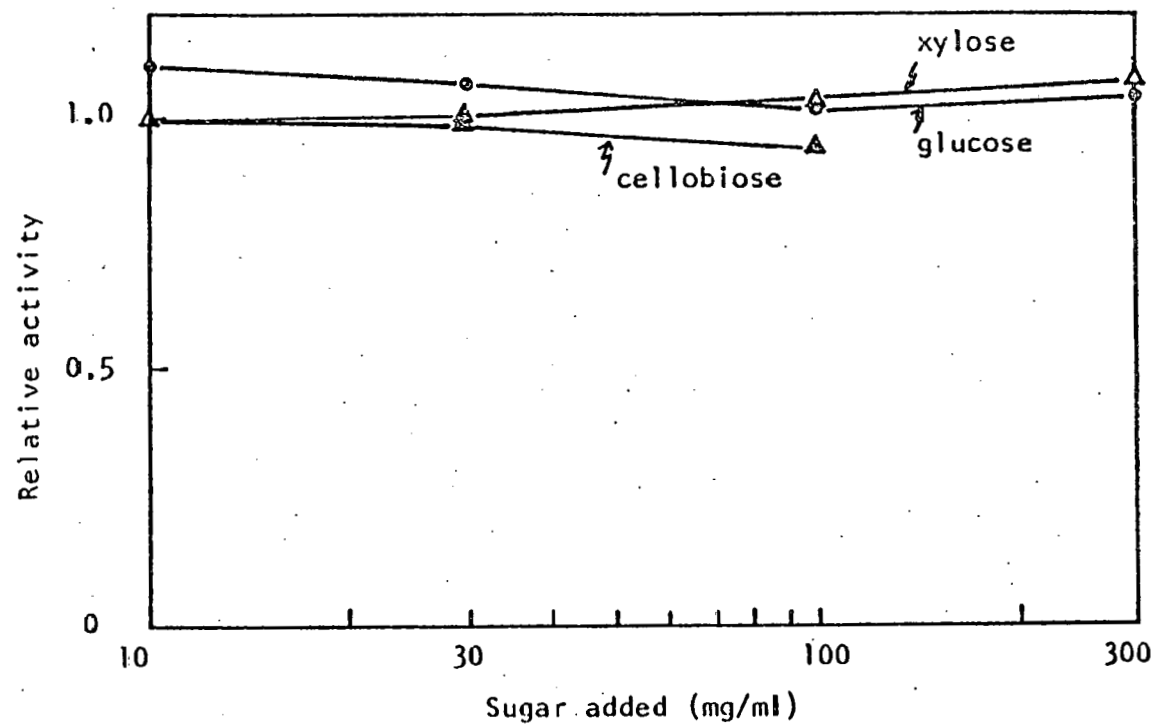


Figure I.F.1. Effect of sugars on NBB-MN300 cellulose hydrolyzing activity. Activity without added sugars is 1.0. Activity in the presence of sugars was expressed relative to this value.

we can conclude that, unlike most cellulases, the C. thermo-cellum enzyme is resistant to end product inhibition.

2. pH Maintenance During Flask Cellulase Fermentation

Preliminary experiments showed that neither phosphate buffer nor Tris.HCl buffer was an acceptable buffer for the growth medium. Morpholinopropane-sulfonic acid buffer (MOPS) did prove to be useful. Figure I.F.2. shows the effect of MOPS concentration in CM3 cellobiose medium on pH maintenance, growth, and TNP-CMCase production. In all cases, the initial pH was adjusted to 7.0. From our results, we decided that 0.1M MOPS was a suitable concentration for growth plus enzyme production.

Figure I.F.3. shows the effect of initial pH on the time course of growth and TNP-CMCase production in CM3 cellobiose medium containing 0.1M MOPS. In Figure I.F.4., the data of Figure I.F.3. on growth and TNP-CMCase production for the first 21 hours are replotted against the average pH during that time. We conclude that the optimum pH for growth and production is 6.8. However, it should be noted that the cultures begun slightly below and slightly above pH 7.0 (which showed the highest cellulase titers) showed significant cell lysis. Whether such high titers are the result of lysis or whether even higher enzyme levels would result if lysis were prevented is a question for future work. We doubt that the lysis is caused by carbon or nitrogen limitation but it could be due to formation of a toxic by-product such as ethanol and/or acetic acid.

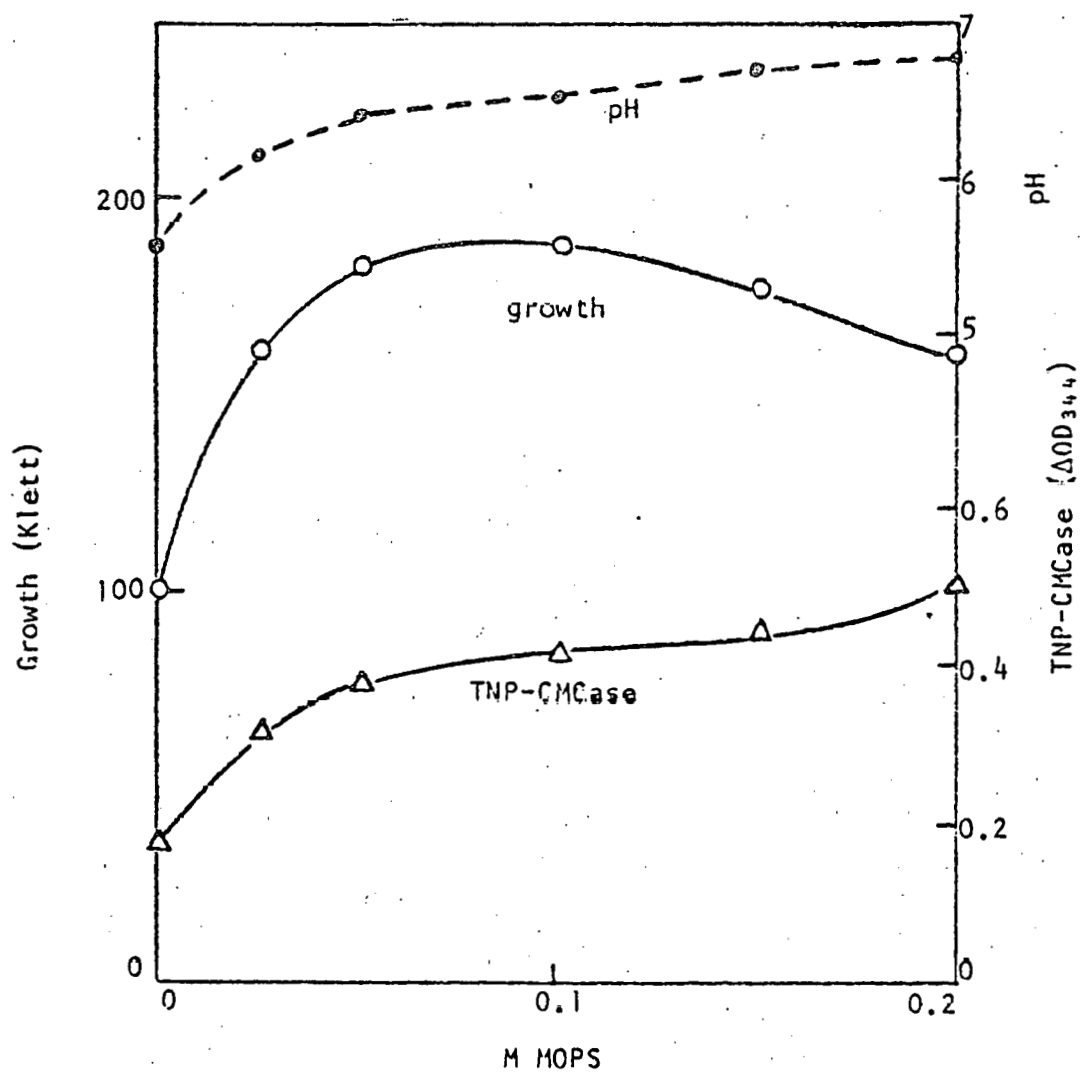


Figure I.F.2. Effect of concentration of MOPS on pH maintenance, growth, and TNP-CMCase production.

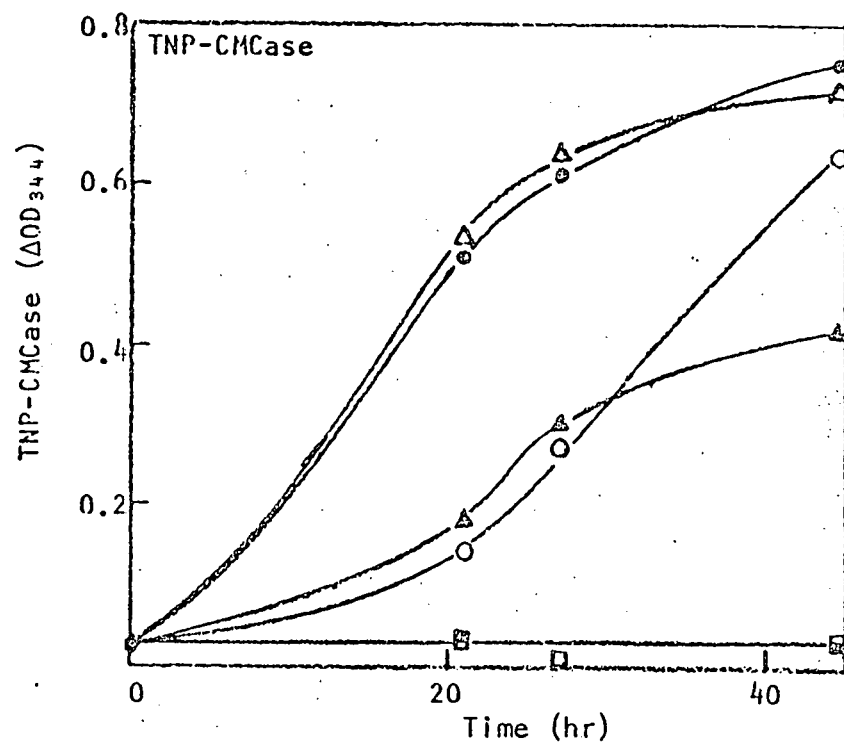
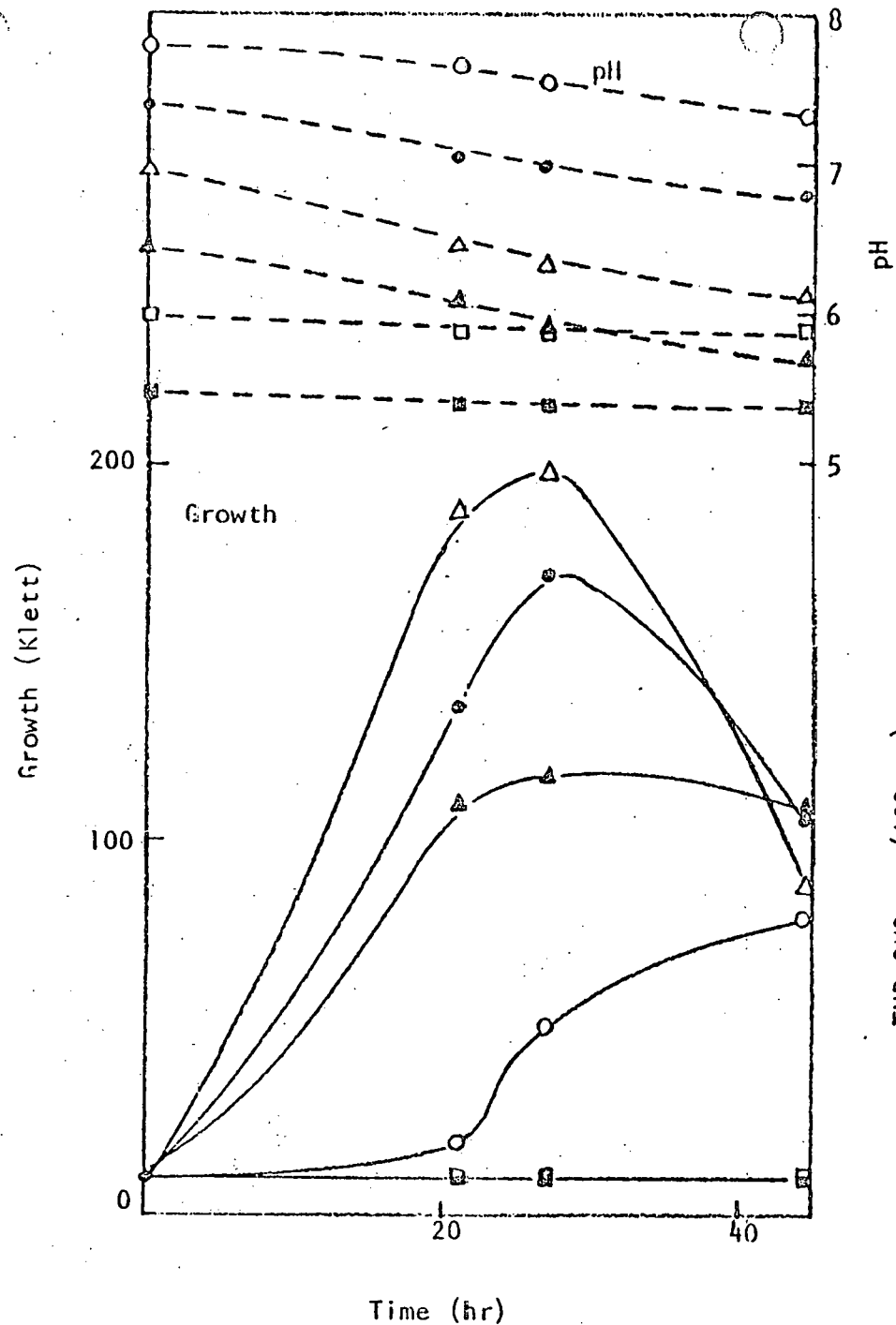


Figure I.F.3. Effect of pH on growth and TNP-CMCase production. In the presence of 0.1M MOPS, initial pH was adjusted as shown by each symbol.

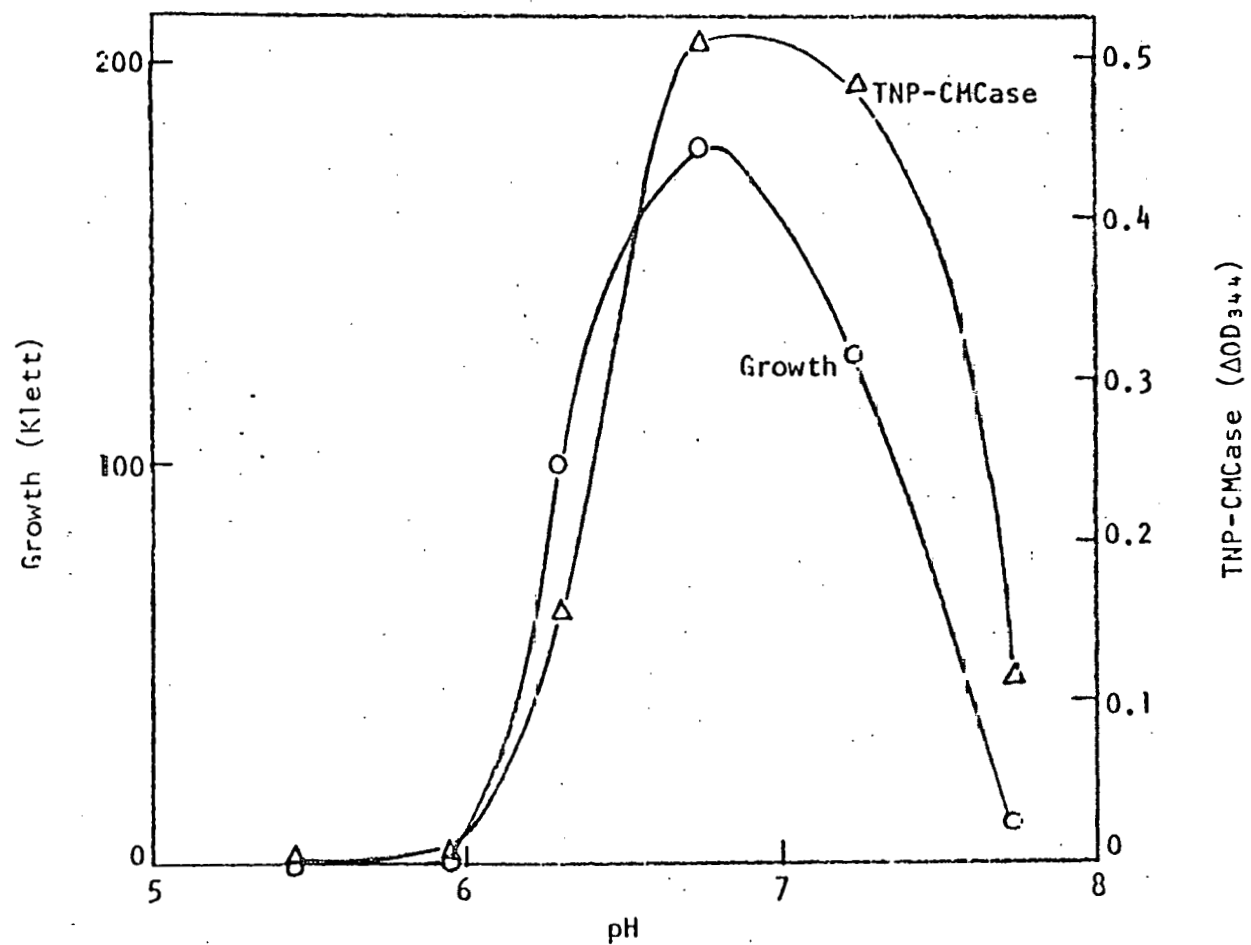


Figure I.F.4. pH vs. growth and TNP-CMCase production. Growth and TNP-CMCase production between 0 to 21 hr in Figure I.F.3. were plotted against average value of pH in the same period.

3. Determination of Intracellular TNP-CMCase Activity

Examination of C. thermocellum for intracellular TNP-CMCase activity was done. Mutant AS-39 or parent cells grown on cellobiose for 24 hours were ruptured by three methods: (1) grinding with quartz sand, (2) ultrasonication and (3) lysozyme treatment. The cell extracts were tested for TNP-CMCase activity. As shown in Table I.F.1., intracellular TNP-CMCase activity represents less than 5% of the total TNP-CMCase activity.

4. Chloramphenicol Inhibition of Growth and Cellulase Production

To determine if the presence of cellulase activity in the extracellular broth is the result of de novo protein synthesis, chloramphenicol, an inhibitor of protein synthesis, was used. A 10% inoculum of AS-39 cells was added to Hungate tubes containing CM3 cellobiose medium (0.5%) and various amounts of chloramphenicol. Growth was measured after 24 hrs incubation at 60°C. Figure I.F.5 shows the effect of chloramphenicol on growth; the minimum inhibitory concentration was 20 mg/l.

The effect of chloramphenicol on TNP-CMCase production was studied in replacement culture. AS-39 cells grown on CM3 cellobiose medium for 24 hrs were centrifuged, washed once with fresh medium, and resuspended in the same medium either with or without chloramphenicol. As a control, washed cells were also resuspended in medium with or without chloramphenicol

Table I.F.1. Ratio of intracellular and extracellular TNP-CMCase

Method of disintegration of cells	Intracellular/extracellular	
	Parent	AS-39
grinding with quartz sand*	-	0.034
Ultrasonication**	-	0.042
Lysozyme treatment***	0.0023	0.014

*30 min grinding in 0.02M citrate buffer pH 4.5

**1 min sonication. By increasing the period more than 1 min, enzyme activity in extract decreased.

***4.2mg/ml lysozyme in 0.01M Tris-HCl buffer, pH 7.2, 45 C, 3 hr. Decrease of turbidity of reaction mixture stopped after 3 hr incubation.

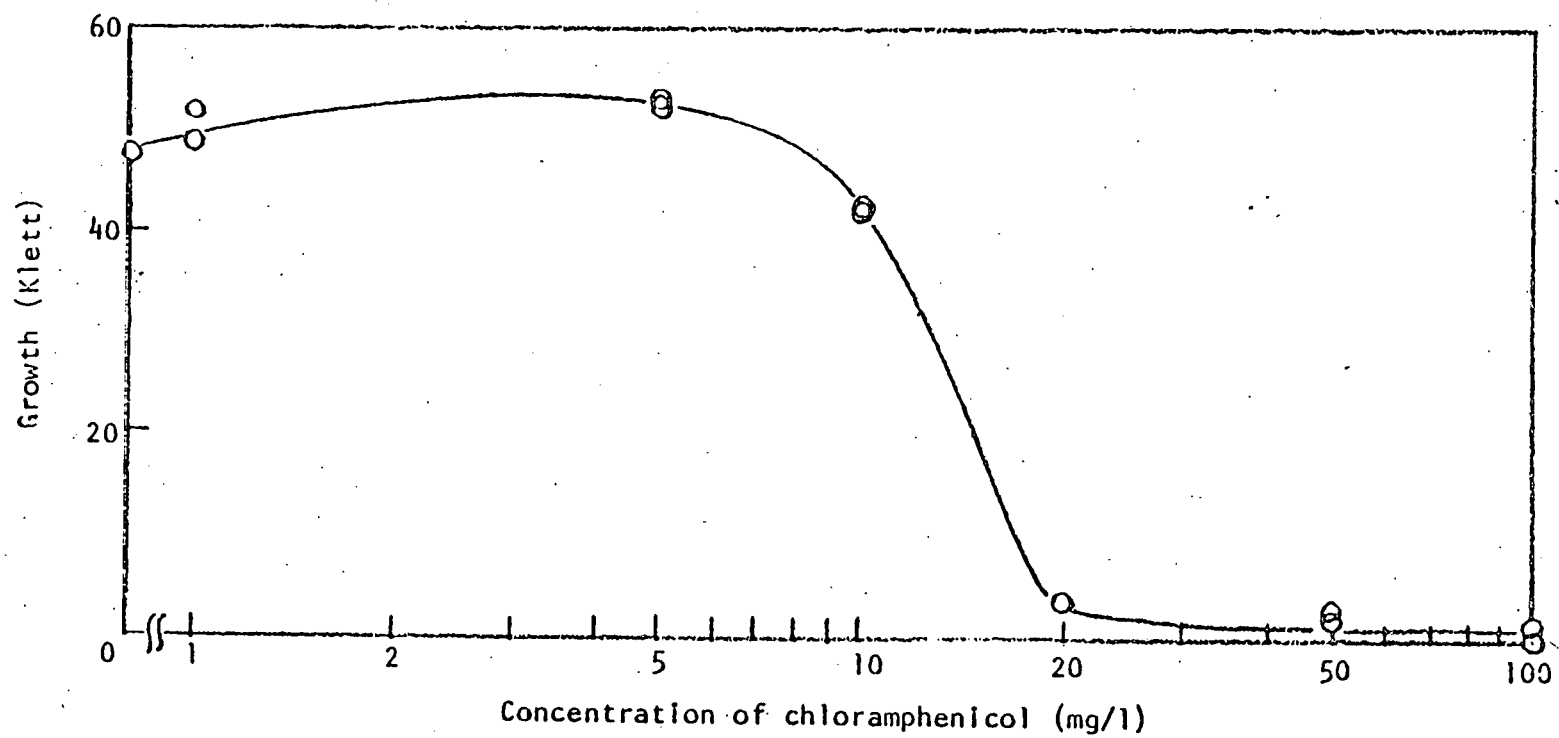


Figure I.F.5. Effect of chloramphenicol on growth.

Table I.F.2 Effect of chloramphenicol on TNP-CMCCase production in a replacement culture

Time (hr)	Addition		Klett	pH	TNP-CMCCase ($\Delta OD_{344}/\text{ml culture}$)	
	CB*	CM**			Extracellular	Intracellular***
0			78	6.8	0.014	0.001
	-	-	73	6.8	0.011	0.001
22	+	-	167	5.7	0.128	0.007
	-	+	73	6.8	0.020	0.001
	+	+	93	6.5	0.027	0.002

*cellobiose 1%, **chloramphenicol 100 mg/l

***Washed cells with 10mM Tris-HCl buffer, pH = 7.2 were incubated with 4.2 mg/ml lysozyme for 3 hr at 45 C in the same buffer, and then TNP-CMCCase activity in supernatant was measured. Activity was expressed per ml culture broth.

but without cellobiose. After 22 hours of incubation at 60°C, growth and extracellular and intracellular TNP-CMCase activities were measured (Table I.F.2). Again, the intracellular TNP-CMCase activity was quite low. The cellobiose-free control showed no change in optical density, pH, or TNP-CMCase activity both with and without chloramphenicol. Addition of 100 mg chloramphenicol/l to the cellobiose containing culture strongly inhibited both growth and the increase in extracellular TNP-CMCase activity. These results suggest that the presence of C. thermocellum cellulase in the extracellular medium is due to de novo protein synthesis and the enzyme(s) is excreted after protein synthesis.

5. Isolation of Hyperproducing Cellulase Mutants by U.V. Irradiation

In the previous report, we reported the isolation of mutant AS-39 from UV-irradiated cells of C. thermocellum ATCC 27405. This mutant produces twice the cellulase activity of the parent according to TNP-CMCase and Avicel hydrolysis assays.

Mutant AS-39 was further exposed to UV irradiation with the intention of mutating it to higher levels of cellulase overproduction. Since no new mutant was found among 30,000 colonies screened, the isolation procedure was modified as follows. The UV irradiated cells were mixed with molten CM3 agar medium containing 1% Avicel as the sole carbon source and then

poured into Petri plates. Colony formation efficiency within the agar was much better than that on the surface. Thus, the number of colonies that appeared agreed with the number we expected from microscopic counts. The probable reason is that, during inoculation and incubation, the cells within the agar are exposed to less oxygen than those on the surface.

Colonies exhibiting large clear zones were taken up into a sterile Pasteur pipet and blown by nitrogen into Hungate tubes containing CM3 medium with 0.5% Avicel as the sole carbon source. After incubation for 2 to 4 days at 60°C, tubes with actively growing cultures were measured for an increase in reducing sugars. Such a selection method should allow the isolation of cultures able to hydrolyze crystalline cellulose.

From about 350,000 survivors of mutagenesis, we tested 90 colonies in tube culture. Mutant AS-39-22 showed 50% more Avicel hydrolyzing ability than its parent AS-39 the first time tested. When this culture was transferred to CM3 Avicel and CM3 cellobiose media, it had 45% and 41% more TNP-CMCase activity than the parent, respectively.

6. Mutation and Isolation of a Cellulase Hyperproducer Using Chemical Mutagenesis

Further mutation of AS-39 has been attempted using two well known mutagens, N-methyl-N'-Nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS). Two procedures were tested. In the first, log phase AS-39 cells, washed and resuspended in pH 7.5 phosphate buffer, were exposed to 3% EMS

(final concentration). Survivors were plated on the surface of CM3 Avicel (2-3%) with cellobiose (0.2%) plates. After incubation at 60°C, plates were examined for large clear zones of cellulose hydrolysis. We did not isolate any overproducers by this method.

In the second procedure, log phase AS-39 cells were delivered into tubes containing molten CM3 Avicel (1-2%) with cellobiose (0.2%) agar and the agar was poured into plates. Filter paper discs (6.35 mm diam.) dipped in 5%-100% EMS (in methanol) were placed in the center of the plate's surface. NTG was also tested in this way with concentrations ranging from 1.25 to 50 µg per disc. The plates were incubated at 60°C. During this time, the material on the disc diffused into the agar forming a concentration gradient. Thus far, about 3,000 colonies from the disc technique have been examined. One has shown an increase of 20% in TNP-CMCase activity in liquid culture as compared to its parent AS-39. We plan to continue the chemical mutagenesis program with these and other modified procedures which may prove successful. The EMS has been more useful up to this point since it does show inhibition around the disk. For some unknown reason, NTG does not cause growth inhibition.

7. Studies on Xylan and Xylanase

In further attempts to find a carbon source which does not lead to cellulase production, xylan was tested as

major carbon source. Xylanase, TNP-CMCase and CMCase activities were determined using ethanol-precipitated fermentation broth. The values were compared to those of CM3-cellobiose and CM3 MN300 fermentations. Xylanase activity was measured by adding 0.6 ml of the ethanol-precipitated sample to 3 ml of a 2% xylan suspension in 0.05M citrate buffer, pH 4.5. The reaction mixture was incubated at 60°C for 1 h, centrifuged to remove xylan and 1.2 ml of the supernatant was assayed for reducing sugars (dinitrosalicylic acid method). To determine the background level of reducing sugars, 0.2 ml of the ethanol-precipitated sample was incubated in 1.0 ml of the citrate buffer without xylan under the conditions described. Xylanase activity is expressed as mg of reducing sugars produced/ml supernatant/h. Xylan alone in citrate buffer incubated as above does not release any detectable reducing sugars.

Table I.F.3 shows that the amount of the various enzyme activities detected in MN300 cellulose or xylan fermentations is low compared to the amounts detected in the CM3 cellobiose culture. This result could be explained if the enzymes were formed on MN300 or xylan, but enzyme adsorption to the polymers occurred. This possibility was tested. Into a capped vial, we added 2.5 ml of enzyme solution, 2.5 ml of 0.05M citrate buffer pH 4.5, and 0.5 g of polymer; these were incubated at 60°C for the times specified. Duplicate vials were used for each data point. The vials were removed from the

Table I }.

Effect of different carbon sources on growth and enzymatic activities of C. thermocellum ATCC 27405

Fermentation Time (hr)	Carbon Source 1% (w/v)	Dry cell weight* mg/ml	TNP-CMCase ΔOD_{344} /ml.hr	CMCase mg sugar/ml.hr	Xylanase mg sugar/ml.hr
0	Cellobiose	0.027	0.014	0.416	0.562
12.5		0.465	0.101	2.160	1.479
41.0		0.435	0.116	2.208	1.562
64.0		0.420	0.105	2.000	1.354
0	MN 300	0.114	0.003	0.083	0.120
12.5		0.435	0.006	0.375	0.229
41.0		0.450	0.019	0.316	0.262
64.0		0.450	0.037	0.475	0.591
0	Xylan		N.T.**	0.129	0.125
12.5			0.021	0.238	0.216
41.0			0.016	0.396	0.408
64.0			0.021	0.404	0.429
0	None***	0.024	0.012	0.088	0.116
12.5		0.057	0.018	0.258	0.229
41.0		0.036	0.023	0.316	0.200
64.0		0.036	0.024	0.266	0.196

*Calculated from Klett values. Could not be done with xylan cultures due to poor settling of polymer.

**Not tested

***Except for yeast extract.

incubator, put on ice, and their contents were centrifuged at 7500 x g for 8 minutes to separate the polymer. The percentage of the original TNP-CMCase activity remaining in the supernatant was measured. As seen in Figure I.F.6, starch, xylan and MN300 cellulose immediately adsorbed 30%, 50%, 78% of the TNP-CMCase activity respectively. If the data of Table I.F.3 are corrected for enzyme adsorbance to the polymer, enzyme production on xylan and MN300 is still considerably lower than production on cellobiose. The results suggest that xylanase and cellulase are constitutive enzymes in C. thermocellum.

Though growth of C. thermocellum on some insoluble substrates, such as cellulose MN300, can be determined by Klett measurement after 2 hr settling of the substrate by gravity, this method cannot be used to measure growth on xylan because the particle size is quite heterogeneous and some particles are small and do not settle out. Therefore, we attempted to use protein determination to measure growth. Two ml of whole broth was centrifuged at 7,500 x g, washed three times with 0.85% NaCl and subjected to the Lowry protein assay. We observed that readings of background levels (i.e., zero time) of protein in the xylan and MN300 fermentations were so high that it was not possible to measure growth with this method. The data are shown in Table I.F.4. We suspected that the polymers somehow interfered in the assay. To determine whether the alkaline treatment in the procedure was responsible, xylan and

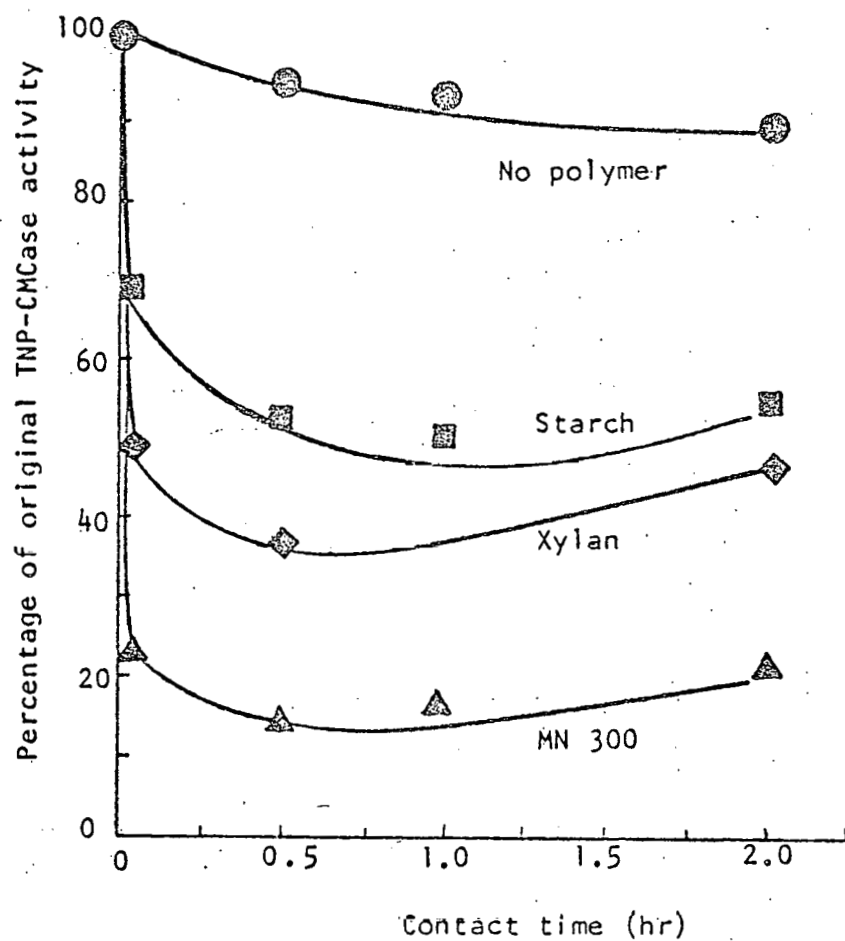


Figure I.F.6. Adsorption of TNP-CMCase activity to sugar polymers at 60°C. 0.05g polymer was added to 2.5ml enzyme solution and 2.5ml 0.05M citrate buffer, pH 4.5.

Table I.F.4. Protein determinations on whole broths by the Lowry method

Fermentation time (hr)	Cellobiose broth		MN300 broth		Xylan broth	
	OD ₅₅₀	protein μg/ml	OD ₅₅₀	protein μg/ml	OD ₅₅₀	protein μg/ml
0	0.006	0	0.526	510	0.276	275
20	0.117	110	0.602	590	0.341	338
44	0.156	152	0.596	584	0.365	360
64	0.165	160	0.628	614	0.314	310

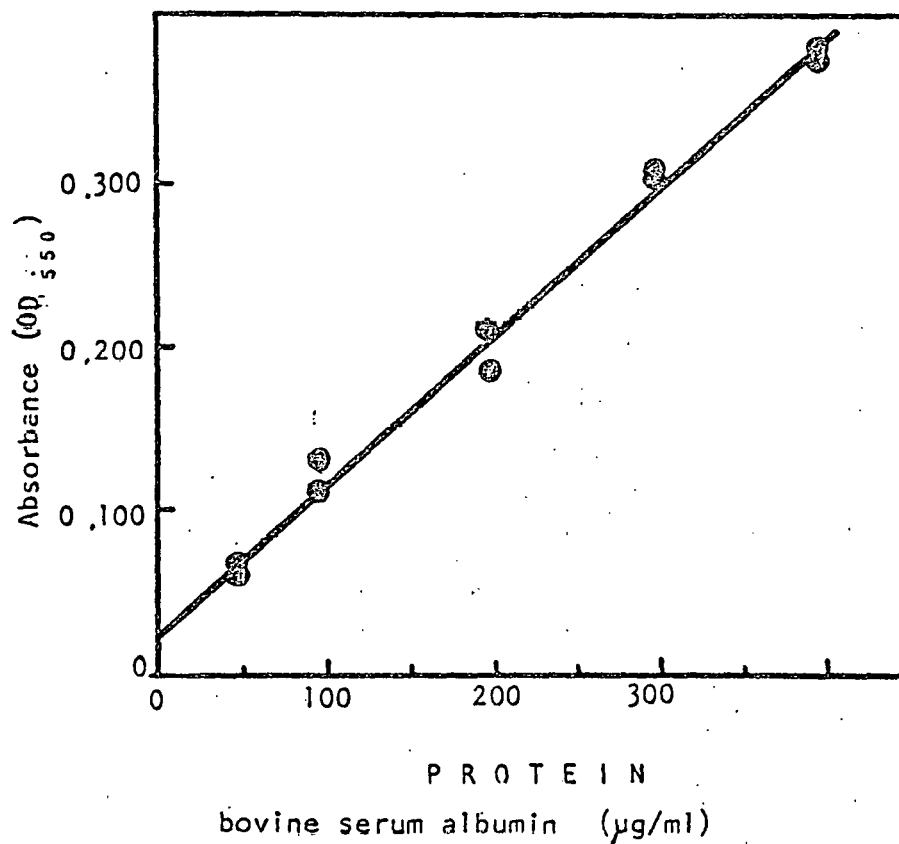
MN300 cellulose themselves were subjected to the Lowry procedure with and without the alkaline hydrolysis step. The results in Table I.F.5 show that both 10 mg/ml xylan and MN300 release a large amount of absorbance equivalent to 450 and 400 µg/ml protein. The absorbances found with only water treatment were much lower indicating that alkaline treatment liberated material interfering with the Lowry protein assay.

Auxanographic plate tests were also done to ascertain whether growth occurs on xylan. CM3 medium less carbohydrate was seeded with C. thermocellum and poured into petri dishes. After solidification, dry xylan was added with a spatula to one portion of the plate. After 5 d incubation, we looked for a growth zone-around the xylan spot, but failed to see clear-cut growth. On other plates in which the xylan was distributed through the agar, we noted its hydrolysis by the heavy inoculum added to the plate.

We thus have failed to see any clear-cut indication of growth on xylan in our experiments. However, growth on cellobiose or MN300 does lead to xylanase production.

8. Effect of Nitrogen Source on Growth and Enzyme Production

Table I.F.6 lists the fourteen compounds that were evaluated for their effects on growth and enzyme production. All were tested at a concentration of 0.27g of nitrogen per liter. Five of them which showed either high volumetric enzyme titers or high specific enzyme values were selected for



	Absorbance (OD ₅₅₀)	
	Polymer treated for 15 min at 100°C	
	(with 0.1N NaOH)	(with water)
XYLAN	0.225, 0.223	0.061, 0.069
MN 300	0.174, 0.199	0.019, 0.006

Table I.F.5. Interference in the Lowry method for protein estimation by alkaline treatment of both cellulose and xylan. Samples contained 10mg/ml of polymer. After heat treatment the hydrolysate was diluted 1:2.

Table I.F.6. Nitrogen Sources Tested for Growth

Ammonium sulfate	Glutamate
Ammonium nitrate	Aspartate
Ammonium chloride	Lysine
Sodium nitrate	Arginine
Potassium nitrate	Leucine
Urea	Isoleucine
	Valine
	Casamino acids

further analysis. They were casamino acids, urea, valine, ammonium nitrate, and ammonium sulfate. Figure I.F.7 shows growth and enzyme production for these five nitrogen sources over a nitrogen concentration of 0.02 g/l to 0.5 g/l. Ammonium salts were the best nitrogen source for growth, but urea appeared best for TNP-CMCase production. Further examination of urea's stimulatory effect at higher concentrations was conducted. Concentrations of urea nitrogen from 0.02 g/l to 2 g/l were tested on CM3 cellobiose medium less $(\text{NH}_4)\text{SO}_4$ containing the usual 0.2% yeast extract or a higher (0.6%) yeast extract concentration. Both filter and heat-sterilized urea were examined. The results in Figure I.F.8 show that urea nitrogen concentrations higher than 0.5 g/l have no stimulatory effect when the medium contains the normal level (0.2%) of yeast extract. However, when yeast extract is increased to 0.6% (which in itself increases enzyme production), urea increases production until 1.0 g/l urea nitrogen is reached. Filter-sterilized urea behaved just like autoclaved urea.

The reason we tested yeast extract at higher levels in the above urea experiment is that we had just observed a slight stimulatory effect of raising its concentration from the usual 0.2% to 0.6% in CM3-cellobiose medium containing $(\text{NH}_4)_2\text{SO}_4$. In Figure I.F.9, it can be seen also that yeast extract can serve as sole nitrogen source, but that further supplementation with $(\text{NH}_4)_2\text{SO}_4$ stimulates growth and enzyme formation.

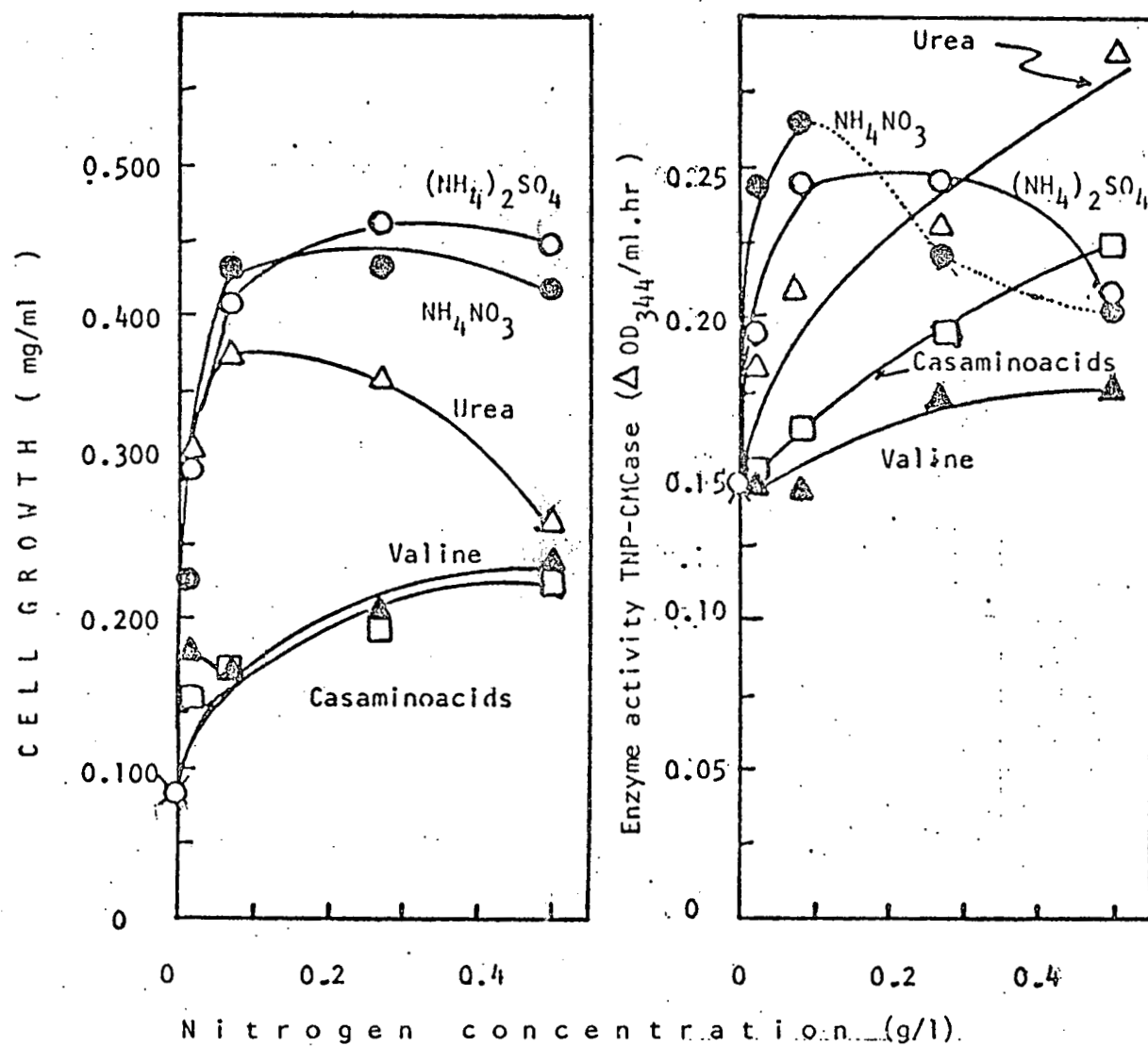


Fig. I.F.7. Growth and enzyme production using various nitrogen sources.

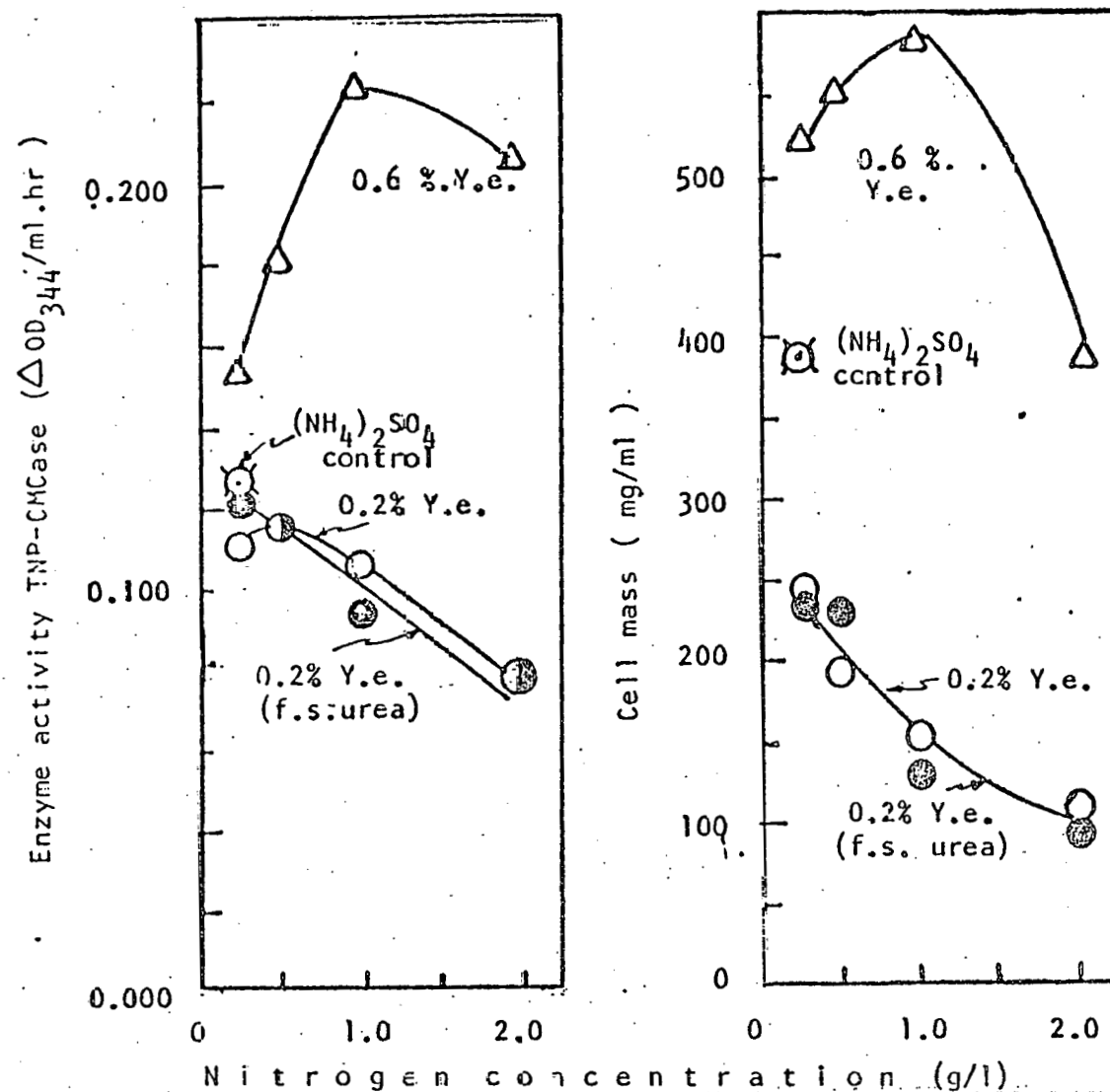


Figure I.F.8. Effect of urea at different concentrations in CM-3 celloblouse medium (0.2% Y.e.) and in the same medium with yeast extract increased to 0.6%. f.s. urea = filter-sterilized urea; otherwise the urea was autoclaved.

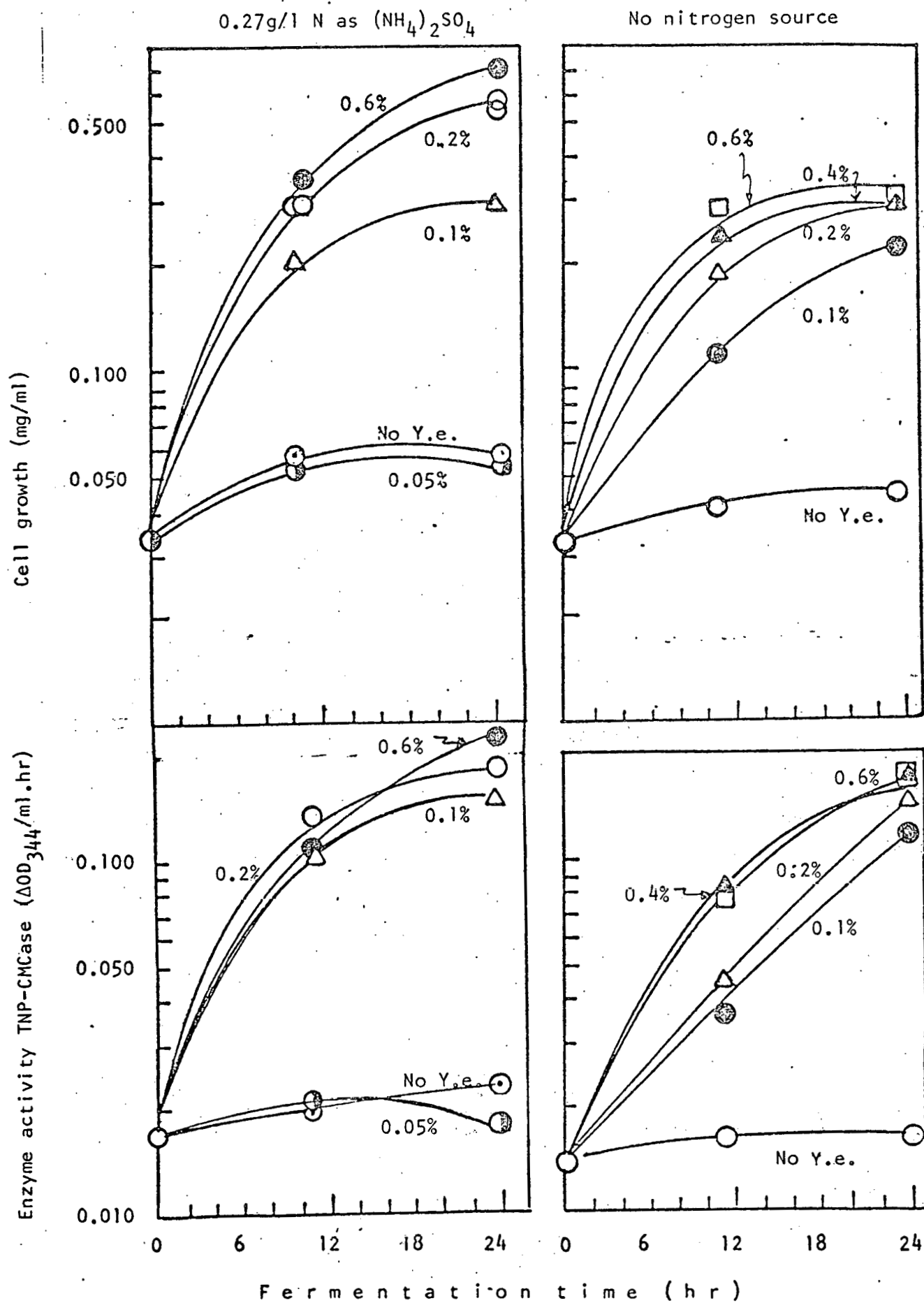


Fig. I.F.9. Growth and enzyme production at different yeast extract concentrations in the presence and absence of $(\text{NH}_4)_2\text{SO}_4$. Symbols on the left and right do not correspond.

We conclude that the nitrogen source combination best for volumetric TNP-CMCase production is 1 g/l of urea nitrogen (i.e., 2.14 g/l urea) plus 6 g/l yeast extract.

9. Discrepancy between M.I.T. and Wisconsin Reports

Ng, Weimer and Zeikus (Arch. Microbiol. 114, 1, 1977) of the University of Wisconsin have reported that C. thermocellum LQ8 produces cellulase on cellulose, but not on cellobiose. We have always found cellobiose to support extensive cellulase production by C. thermocellum ATCC 27405. Inoculum size was varied from 1% to 10% (v/v). In all cases, TNP-CMCase was made. Strain LQ8 produced titers of 0.17 to 0.25 $\Delta OD_{344}/\text{ml} \cdot \text{hr}$ whereas the values for ATCC 27405 were 0.18 to 0.21. Ethanol-precipitated supernatants of both strains also showed CMCase activity; the potencies were similar. At a discussion with Zeikus at the recent ASM Meeting in Las Vegas, he agreed that ATCC 27405 produces cellulase on cellobiose.

G. Evaluation of Clostridium thermocellum Strains for Xylanase and Cellulase Activities

1. Introduction

In the last Progress Report (COO-4198-5), we observed that all strains of C. thermocellum tested by us had the ability to degrade xylan. However, only the HG isolates were able to grow on xylan as the sole carbon source as measured by increases in OD⁶⁶⁰ and production of ethanol.

To quickly assay the degradation reactions, we have spotted representative C. thermocellum strains on agar plates with different carbon sources. Experiments to determine the specific cellulase and xylanase activities have also been performed.

2. Materials and Methods

a. All experiments were performed in test tube cultures with CM-4 medium containing 6 gr/liter of carbon source. Agar plates contained the same carbon source concentration.

b. Reducing sugars were determined using the DNS assay as described in earlier progress reports.

c. ATCC-2705 and LQ8 strains of C. thermocellum growing on different carbon sources were evaluated for filter paper activity (FPase) CMCase activity and xylanase activity.

Activities were measured following precipitation and concentration of extracellular proteins. Ethanol at 40% (V/V) was chosen to precipitate the proteins. Ethanol was

added to culture supernatants and the samples were maintained overnight in an ice bath. After precipitation, the samples were centrifuged, supernatants discarded and pellet resuspended in citrate buffer at different concentrations to determine enzyme activities.

Xylanase activity was measured by adding 3 ml of 2% xylan in citrate buffer (0.05 M pH - 4.8) to 0.2 ml of sample in duplicate tubes. For each sample, a control tube was included which received no xylan (this serves as a reference for background of reducing sugars in sample) and another tube which received xylan, but no sample. All samples and control tubes were incubated for 1 hour at 60°C. After the incubation, the tubes containing xylan were centrifuged at 12,000 rpm for 10 min and 1.2 ml of the supernatants were transferred to tubes for DNS assay.

CMCase and FPase were performed as described previously.

3. Results and Discussion

We had previously obtained preliminary evidence that all strains of C. thermocellum tested by us had the ability to degrade xylan. To confirm this observation, agar plates with CM4 and different carbon sources were inoculated with ATCC-27405, LQ8 and HG-2 strains. Four aliquots of culture supernatants, 20 µl each, were spotted on each plate.

As may be seen in Table I.G.1, ATCC and LQ8 strains previously grown in either Solka floc or cellobiose were able to degrade xylan. HG-2 strain previously grown in xylan, xylose or cellobiose is also able to hydrolyze xylan.

Two additional observations can be made from these data. One is the fact that HG-2 does not produce zones of clearing in Solka floc plates as expected since they are not able to grow on cellulose as the sole carbon source. Another is that ATCC and LQ8 strains when spotted on CM4-xylose agar plates showed no growth. Plates containing yeast extract and no other carbon source always supported some growth. Thus, it may be supposed that growth of HG-2 on Solka floc was due to the presence of yeast extract. In addition, it appears that xylose may be inhibitory to ATCC and LQ8 strains.

Since it was clear that all strains tested degrade xylan, we attempted to correlate growth with hydrolysis of xylan and the accumulation of reducing sugars.

Table I.G.2 shows that only HG-2 causes increases in optical density of the medium, indicating cell growth, while the medium inoculated with LQ8 strains shows a decrease in optical density, indicating xylan degradation. The optical density of these tubes was followed for 4 more days and the O.D. did not increase.

Another interesting observation was that HG-2 did not accumulate reducing sugars while growing in xylan. Confirmation of this result was obtained by paper chromatography

TABLE I.G.1: GROWTH OF DIFFERENT STRAINS ON VARIOUS CARBON SOURCES

<u>C. thermocellum</u> <u>Strains Grown In:</u>	<u>Xylan</u>	<u>Solka</u> <u>floc</u>	<u>Xylose</u>	<u>Yeast</u> <u>Extract</u>	<u>MN300</u>	<u>Microcrystalline</u> <u>Avicel</u>
ATCC-cellulose SF	GR-L	GR-L	(—)	GR	GR-L	GR-L
ATCC-cb	GR-L	GR-L	(—)	GR	GR-L	GR-L
LQ8-cellulose SF	GR-L	GR-L	(—)	GR	GR-L	GR-L
LQ8-cb	GR-L	GR-L	(—)	GR	GR-L	GR-L
HG-xylan	GR-L	GR-L	GR	GR	GR	GR
HG-xylose	GR-L	GR	GR	GR	GR	GR
HG-cm	GR-L	GR	GR	GR	GR	GR

GR = Growth on surface

(—) = No visible growth

(L) = Clearing zone indicative of substrate hydrolysis

Cb = cellobiose

TABLE I.G.2: GROWTH AND ACCUMULATION OF REDUCING SUGARS BY
C. THERMOCELLUM ISOLATES GROWING IN XYLAN

OD⁶⁶⁰

<u>Strain</u>	<u>0 hours</u>	<u>15 hours</u>	<u>25 hours</u>	<u>40 hours</u>	<u>63 hours</u>
HG-2	0.34	0.80	0.84	0.87	0.92
ATCC	0.34	0.28	0.24	0.20	0.17
LQ8	0.38	0.30	0.32	0.27	0.22

Reducing sugars: as mg xylose/ml broth

	<u>0 hours</u>	<u>15 hours</u>	<u>25 hours</u>	<u>40 hours</u>	<u>63 hours</u>
HG-2	.05	0.05	0.1	0.1	0.05
ATCC	.05	2.15	2.4	2.5	2.7
LQ8	.05	1.85	2.3	2.3	2.7

of the fermenting broth using n-butanol/acetic acid/water at < 2:1 as solvent and aniline-hydrogen phtalate as staining mixture. HG-2 strain did not produce any stainable spot of reducing sugars.

A study was conducted to determine the influence that carbon sources have on the different enzymatic activities. ATCC and LQ8 were grown on different carbon sources and as can be seen in Table I.G.3 it is clear that all activities tested were always present as long as growth occurs. It is interesting that the ATCC strain will grow in glucose and produce all extracellular enzymes. This is in contract to what has been previously reported for C. thermocellum. It should be noted, however, that growth in glucose exhibited a longer lag than in cellobiose. This may be indicative of either a phenotypic adaption to glucose or the selection of mutants with the capacity to utilize glucose.

4. Future Work

- Determine the ability of HG-2 strain to produce FPase, CMCase and xylanase. This may serve to better define strain HG-2 physiologically and to obtain more information about the mechanisms by which these enzymes are produced.

- Determine if the ability of C. thermocellum to grow on glucose as the sole carbon source is a stable mutation or a phenotypic adaption.

TABLE I.G.3: EFFECT OF CARBON SOURCE ON THE EXPRESSION OF EXTRA-CELLULAR ENZYMATIC ACTIVITIES IN C. THERMOCELLUM

Strain	Activity	Solka Floc	xylan ²	Cellobiose	None	Glucose	MN300
ATCC	FPase	0.010	0.014	0.042	0.002	0.030	0.005
	CMCase	1.5	0.75	4.35	0.025	1.42	1.82
	xylanase	1.2	0.57	3.45	0.020	1.25	1.05
LQ8	FPase	0.018	0.004	0.018	NM	ND	ND
	CMCase	2.5	0.27	2.05	NM	ND	ND
	xylanase	3.1	0.38	2.75	NM	ND	ND

Activity was determined at the end of the exponential phase of growth.

FPase and CMCase activity are expressed as $\frac{\text{mg Glucose}}{\text{ml-HR}}$

xylanase activity is expressed as: $\frac{\text{mg xylose}}{\text{ml-HR}}$

ND = not done

II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. Production of Acrylic Acid by Fermentation

1. Introduction

Because of the high value (~77¢/kg) and potentially high yield from glucose (~0.8 g/g), acrylic acid remains attractive as a component of an integrated process for ethanol production. Production of acrylic acid as a portable and overall process to produce ethanol could greatly improve the economic aspects of ethanol production from biomass.

During this progress report period, efforts have been directed to production of acrylic acid employing an anaerobic microorganism, Clostridium propionicum, and a facultative microorganism, Escherichia coli. In addition to research on the production of acrylic acid, research is also continuing vigorously on the production of lactic acid which potentially is an important precursor to acrylic acid.

2. Fermentation Studies with Clostridium propionicum

a. Introduction

The Progress Report (COO-4198-5) covering the period from December 1, 1977 through February 28, 1978, summarized the exciting and significant finding that acrylic acid could be produced by resting cells preparations derived from C. propionicum. These findings were also reported at the 18th Annual Meeting of the American Society for Microbiology, held May 14 - 19, 1978. The paper entitled "Production of Acrylic

Acid by Clostridium propionicum" was co-authored by M. Akedo, R. Torregrossa, C. L. Cooney and A. J. Sinskey and presented by Mr. M. Akedo. Research conducted this quarter on the production of acrylic acid by C. propionicum has had two objectives. The first objective is to investigate acrylic acid production kinetics from propionate and/or lactate. The second objective is aimed at increasing the yield of acrylic acid from the oxidation of propionate by resting cell suspensions of C. propionicum.

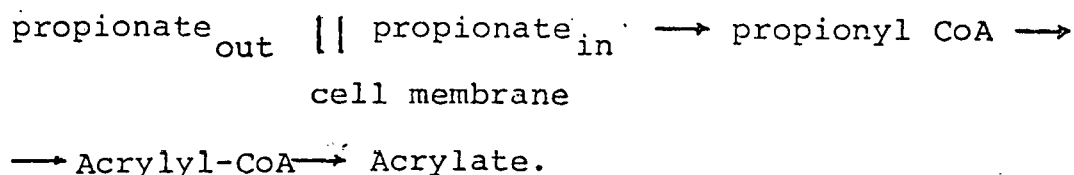
b. Materials and Methods

Procedures for conducting the basic experiments and analyses of products have been described previously (Progress Report COO-4198-5, Period December 1, 1977 - February 28, 1978). Cellular protein was measured using the modified Biuret procedure of Strickland (J. Microbiol. (1954) 5:698).

c. Results and Discussion

1. Effect of Propionate Concentration

The amount of acrylic acid produced by resting cell suspensions was found to be dependent on propionate concentration up to a concentration of about 200 mM (Figure II.A.1). The oxidation of propionate to acrylate is believed to require a minimum of four (4) steps:



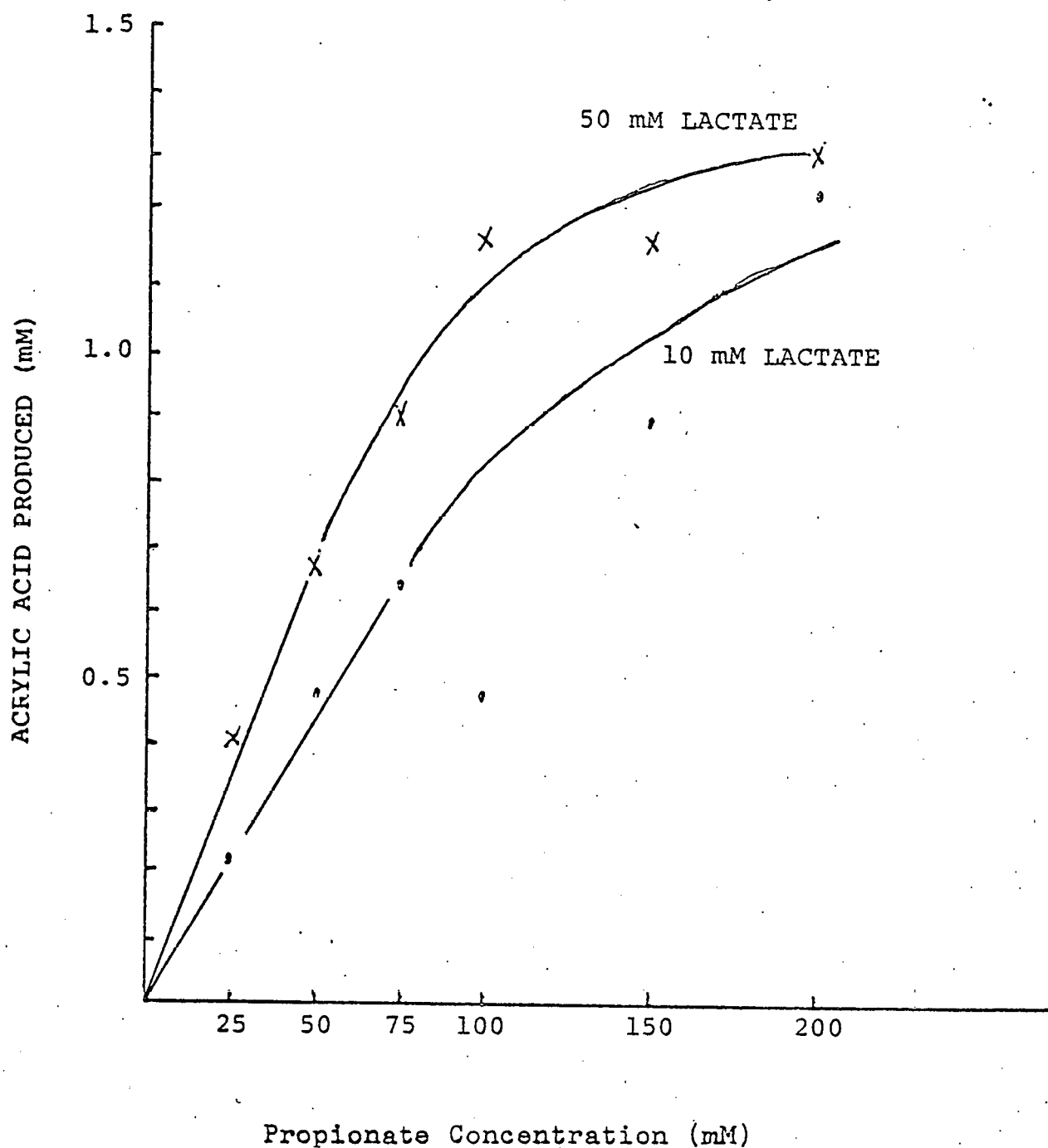


Figure II.A.1: Dependence of acrylic acid production on the concentration of propionate and lactate. Incubation mixtures contained triethanolamine buffer (0.05M, pH 7.5), cell suspension (0.05g wet cell weight/ml) and indicated concentrations of propionate and lactate. Incubation was at 37° for 4 hrs.

The large amount of propionate required for saturation may indicate that transport of propionate into the cell is the rate limiting step in the overall conversion.

2. Effect of Lactate and Oxygen

Previous findings have demonstrated that lactate addition to a resting cell preparation oxidizing propionate to acrylic acid appears to stimulate acrylic acid production (Progress Report COO-4198-5, December 1, 1977 - February 28, 1978). To date, lactate alone has not been used as a starting substrate even though we have demonstrated that resting cells of C. propionicum can metabolize lactate producing propionate and acetate and that propionate can be converted to acrylate in the presence of oxygen. Therefore, experiments were designed in which lactate is first converted to propionate under anaerobic conditions and the resulting propionate may then be oxidized under aerobic conditions to acrylic acid.

Five different environmental conditions and combinations were therefore tested to explore the above approach. They were:

- (1) anaerobic (Figure II.A.2)
- (2) aerobic (Figure II.A.3)
- (3) anaerobic → aerobic (Figure II.A.4)
- (4) Anaerobic → aerobic → anaerobic (Figure II.A.5)
- (5) aerobic → anaerobic (Figure II.A.6)

FIGURE II.A.2: ANAEROBIC FERMENTATION OF LACTATE
BY RESTING CELLS

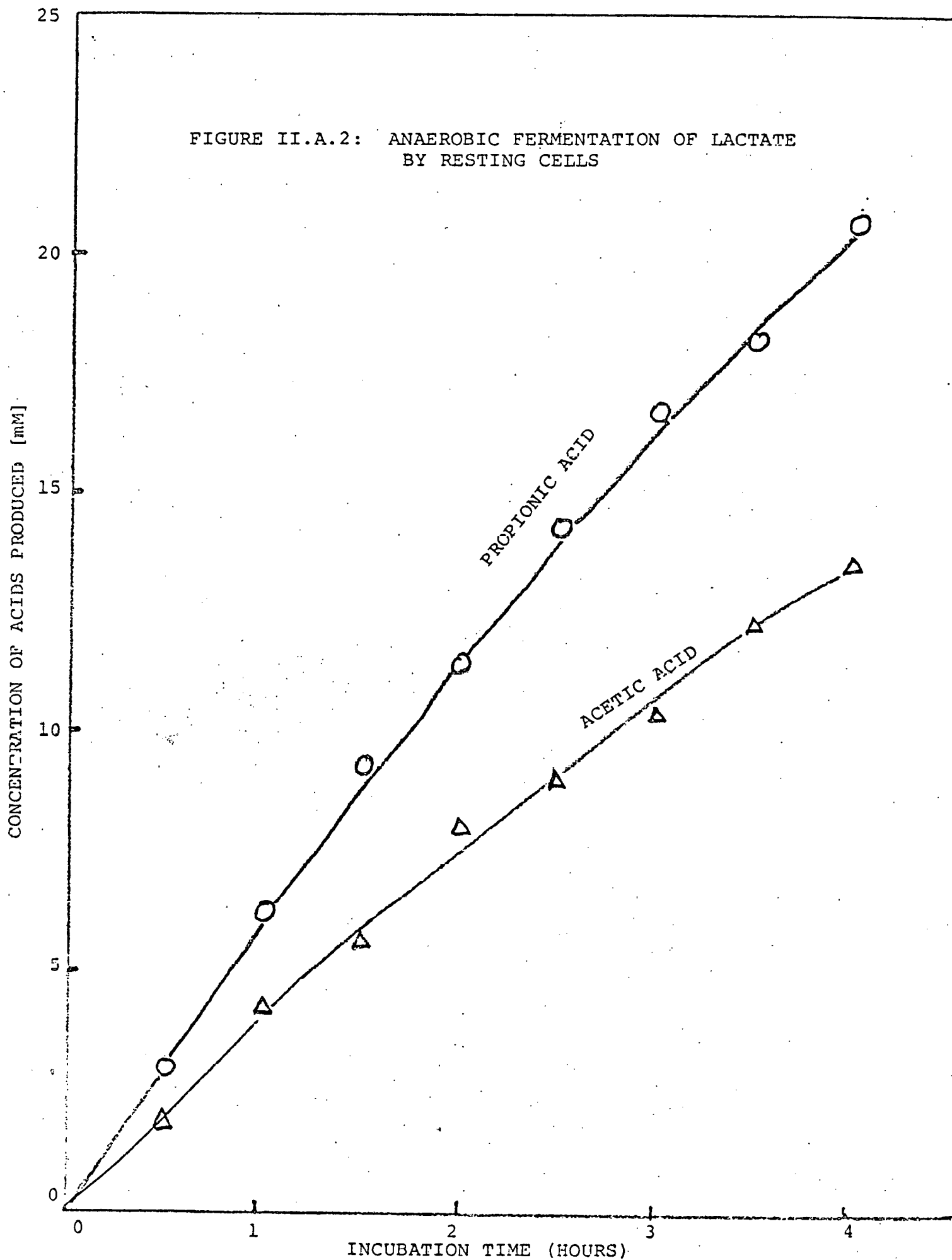


FIGURE II.A.3: AEROBIC FERMENTATION OF LACTATE BY RESTING CELLS

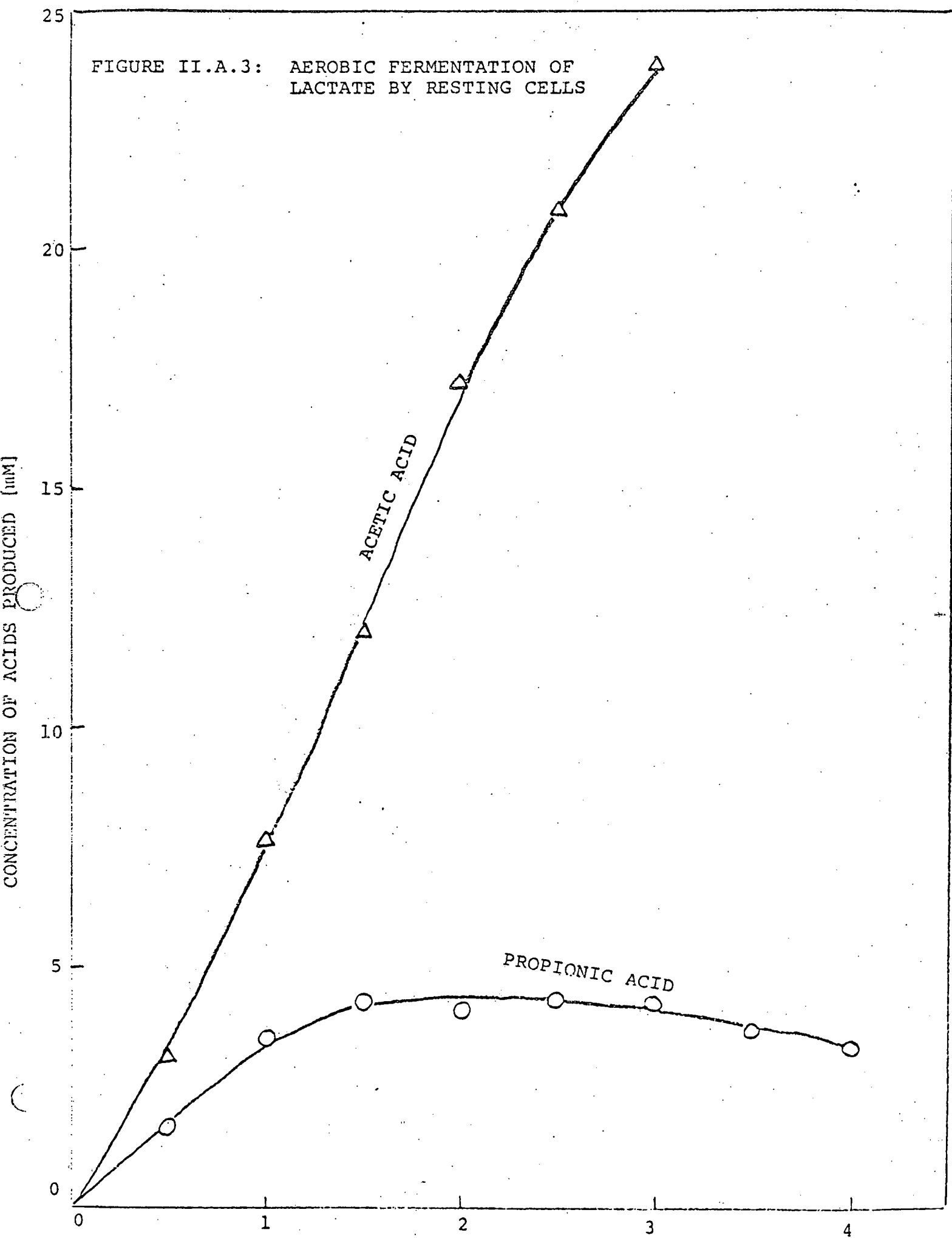


FIGURE II.A.4: LACTATE FERMENTATION UNDER THE INFLUENCE OF ENVIRONMENT

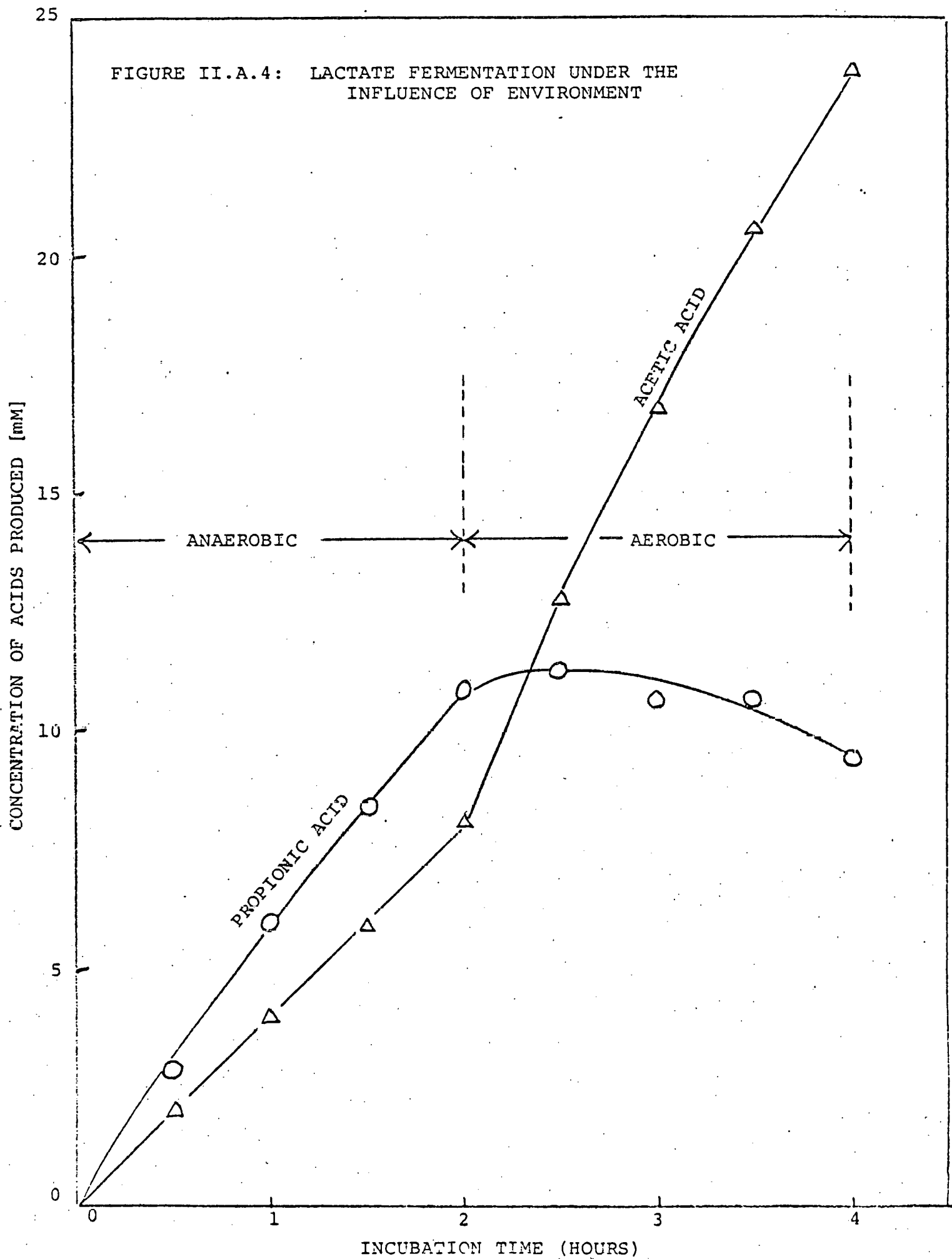


FIGURE II.A.5: LACTATE FERMENTATION UNDER THE INFLUENCE OF ENVIRONMENT

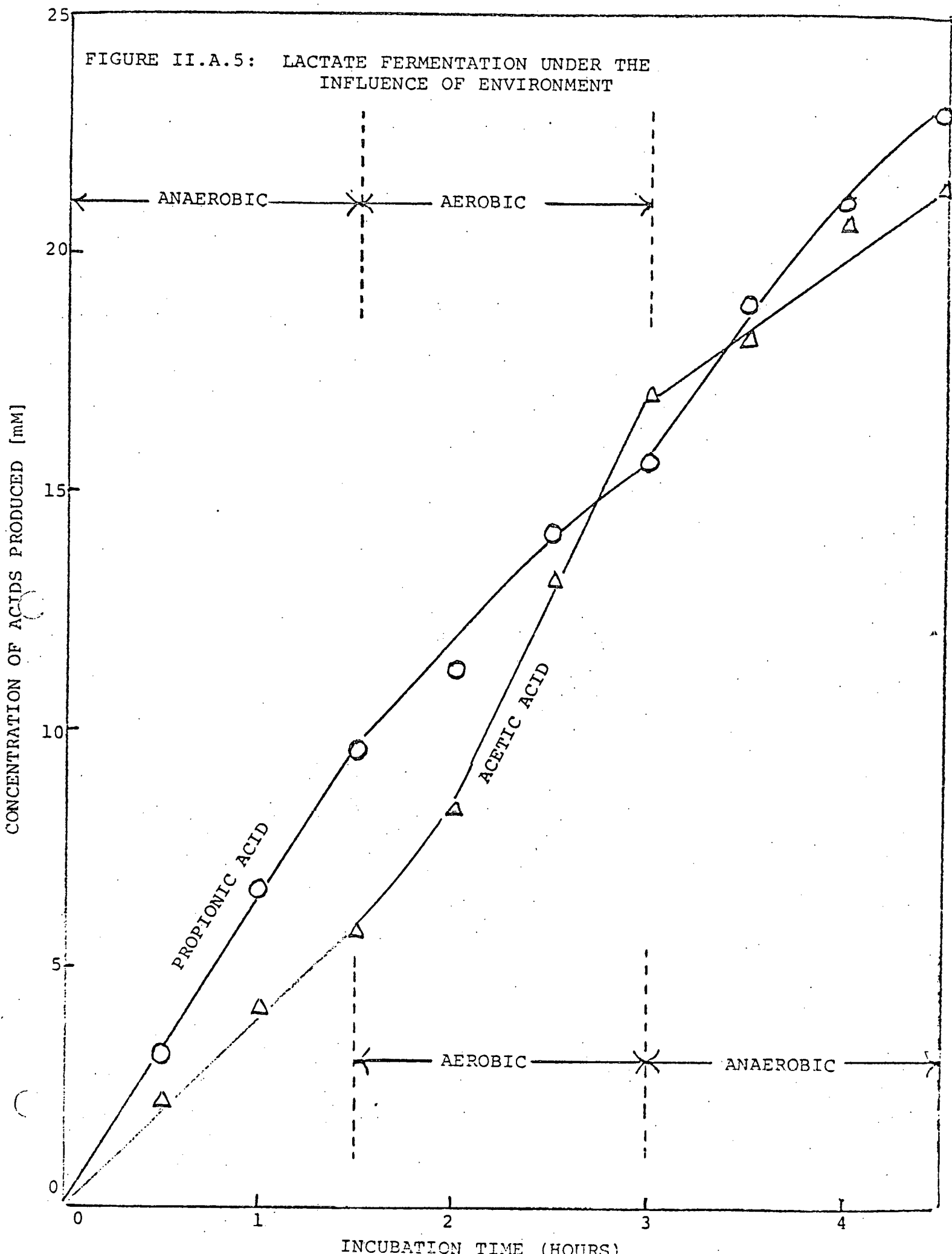
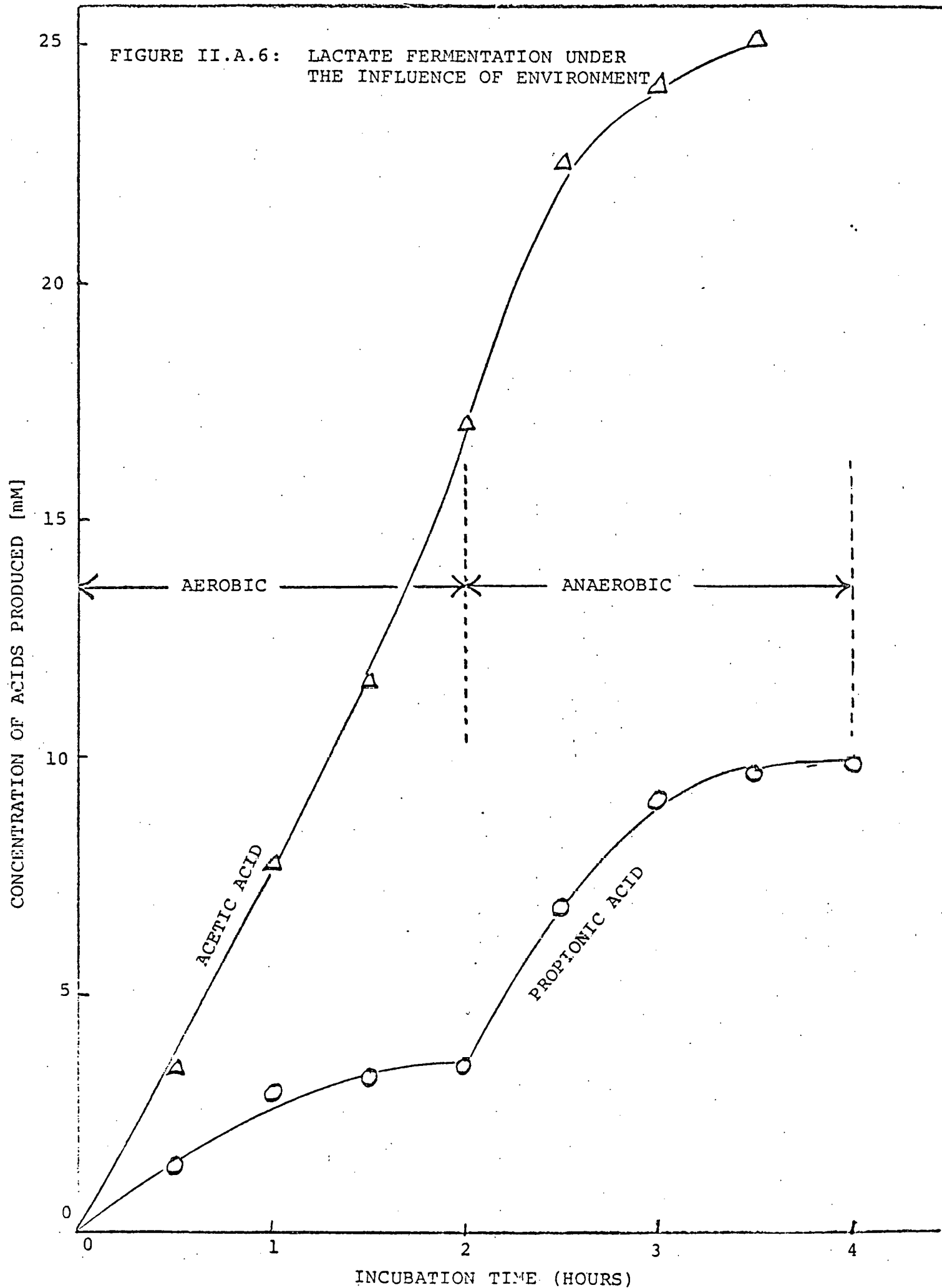


FIGURE II.A.6: LACTATE FERMENTATION UNDER THE INFLUENCE OF ENVIRONMENT



Each figure summarizes the results. In the presence of oxygen, propionate production was significantly decreased and the rate of propionate production also decreased. On the other hand, acetate production was stimulated in the presence of oxygen and the rate of acetate production increased. The fact that oxygen can act as an electron acceptor and thus alter the oxidation-reduction balance in the fermentation of lactate is clearly demonstrated in Figure II.A.5). Unfortunately, no acrylic acid accumulation was detected under these conditions even though other experiments demonstrated that lactate in the incubation mixture appears to stimulate acrylic acid production when exogenous propionic acid is the substrate. Therefore, it is highly unlikely that the stimulatory effect of lactate is due to lactate being converted to acrylic acid. A possible explanation of these observations is that the oxidation of lactate to pyruvate and eventually to acetate increases the cellular pool of acetyl-CoA. The excess acetyl-CoA is then readily available to activate propionic acid to propionyl-CoA and the propionyl-CoA is then oxidized. Thus, the lactate becomes a source of acetyl-CoA. The CoA moiety is then transferred to propionate via CoA transferase. When acrylic acid is released, the CoA moiety may be recycled. Acyl-CoA transferase has been extensively characterized in Peptostreptococcus (Megasphaera) elsdenii (Tung, K.K. and W.A. Wood, J. Bacteriol. (1975) 124:1462). Its characterization in C. propionicum remains to be conducted.

3. Optimization of Propionate Oxidation

Efforts that are concerned with the optimization of propionate oxidation reactor conditions are addressing the following variables:

- (a) concentration of propionate
- (b) concentration of lactate
- (c) cell concentration
- (d) pH
- (e) ionic strength
- (f) growth phase
- (g) oxygen tension

To date, studies have been undertaken on the first three variables--concentrations of lactate, propionate and cell mass. The propionate concentration was varied from (0 mM to 200 mM) while lactate concentration was controlled at 10 mM or 50 mM. Again, it is observed that acrylic acid production is stimulated in the presence of lactate. Increasing amounts of propionate also resulted in a higher amount of acrylate (Figure II.A.1). As cell concentration is increased, acrylic acid production also is increased (Figure II.A.7).

d. Future Studies with C. propionicum

Efforts will continue in the optimization of acrylic acid production from propionate. In addition, the ability to grow large quantities of cells and store them will be investigated. The ability to grow large quantities of cells

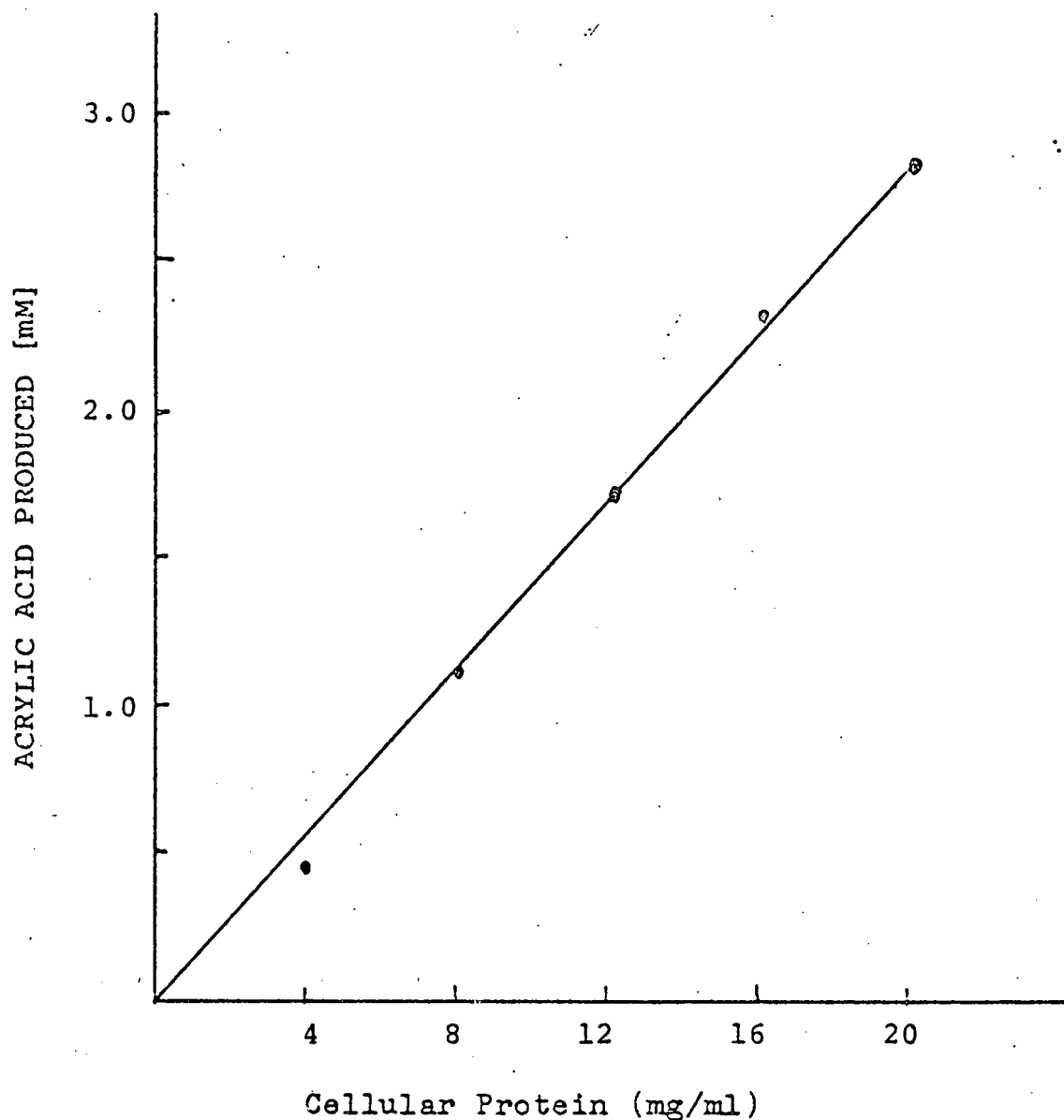


Figure II.A.7: Acrylic acid production as a function of cell concentration. Reaction mixtures contained triethanolamine buffer (0.05M, pH7.5), sodium propionate (0.2M) and varying amounts of cell suspension. Incubation was at 37° for 2 hrs with shaking in an aerobic atmosphere. Each point represents the average of duplicate reactions.

and store them will greatly reduce the time required for performing experiments. Preliminary experiments of a qualitative nature indicate that cells stored at -20°C retain the ability to produce acrylate.

3. Aerobic Oxidation of Propionic Acid to Acrylic Acid by *Escherichia coli*

a. Introduction

In previous progress reports, we outlined a research approach with aerobic organisms having the objective of accumulating acrylic acid via oxidation of propionic acid employing *Escherichia coli*. Our objective during this quarter was to resolve the feasibility of using *E. coli* ATCC 9637-2 for the aerobic bioconversion and accumulation of acrylate from propionate.

The research has concentrated on the isolation of the mutant as outlined in earlier reports. In addition, investigations on propionate oxidation by resting cell suspensions have been conducted. Preliminary experiments with cell-free extracts have been initiated. In the previous report, it was assumed that propionyl CoA could be carbonylated to succinyl CoA via methyl malonyl CoA. However, now in the light of the fact that *E. coli* cannot synthesize the cofactor vitamin B_{12} , this would be an unlikely pathway. Data presented on the metabolism of two, three and four carbon acids can, however, be interpreted on the basis of conversion of pyruvate to phospho(enol) pyruvate and its subsequent carboxylation to oxalacetate. The requirement for

C4 compounds could thus be satisfied by this pathway. Phospho(enol) pyruvate is known to inhibit the activity of isocitrate lyase, and this would explain the low levels of the enzyme under conditions which the cellular yield of propionate equalled that of succinate.

Inspection of our methods for the isolation of several mutants has revealed areas where selection could be made more specific. Some new techniques have been used for this purpose.

b. Materials and Methods

Culture conditions and measurement of growth have been described in earlier reports, as have been procedures for chromatographic assays and for mutagenesis.

1. Non-Volatile F.A. Assay

The gas chromatographic assay for methyl esters of non-volatile fatty acids was modified. The oven temperature was raised from 120°C to 135°.

2. Resting Cell Suspensions

20 ml of an M9 + 0.2% glucose starter culture, inoculated from a propionate slant and grown for 24 h was added to 1 liter of M9 + 0.2% propionate in a 4-liter flask. The cells were harvested in the exponential phase after 36 h. Cells were resuspended in 0.1 M phosphate buffer (pH 7.0) containing other M9 constituents, minus a nitrogen source, to an approximate concentration of 1 gm wet cell wt. per 50 ml medium. 0.2%

propionate was added unless stated otherwise. 1 ml samples were dispensed in 13 x 100 mm tubes and shaken by a 'wrist-action' shaker at 37°C. The reaction was terminated by addition of 0.1 ml 50% H₂SO₄. Samples were extracted with ether and analyzed as usual.

3. Cell-Free Extracts

Cells were grown as described in Section 2. Cells (3 gms wet cell weight) suspended in 10 ml 0.1 M tris buffer (pH 7.4) containing 1 mM dithiothreitol were disrupted by sonication for 90 secs., in 15 sec. pulses followed by 1 min. intervals for cooling in ice. Suspensions were centrifuged at 15K x g for 30 min. at 4°C. The supernatant was dispensed in 1 ml quantities to prevent repeated thawing of the samples and stored at -20°C.

The standard reaction mixture in 0.5 ml consisted of 100 µl cell free extract (containing approximately 20 mg protein/ml) and 400 µl 0.1 M Tris buffer (pH 7.4) containing 0.5 mM NAD. The reduction of NAD was followed spectrophotometrically at 340 nm using a Gilford recording spectrophotometer.

As a precaution against possible action of NADH oxidase, the samples were flushed with nitrogen.

c. Results and Discussion

1. In the previous progress report, we identified aspects of our selection and enrichment procedure that required closer inspection.

(a) Enrichment of the mutants by penicillin G treatment of exponentially growing acetate positive cells resulted in enrichment of only 0.005%. Also, the growing cells were often found to be penicillin resistant. A hundred fold greater enrichment was necessary. The presence of 2 mg/ml ampicillin, either alone or with penicillin G resulted in a consistent 10^5 -fold decrease in viable cells during enrichment for 480 minutes.

(b) The method for selecting isocitrate lyase mutants was also reviewed. We had been selecting these mutants on differential media plates containing 0.01% glycerol and 0.1 or 0.2% acetate. Data on mutants actually isolated by this system has been presented earlier. Stable ace-A mutants were not found. It became clear that ace A mutants plated on such media could not be distinguished from wild type cells on the basis of colony size. Mutants lacking isocitrate lyase could conceivably metabolize the roughly 1 mM glycerol and synthesize sufficient TCA intermediates to metabolize acetate normally. Our inability to isolate the mutant could result from this. The following alternatives, besides replica plating have been studied.

(1) Enriched organisms were first grown on 0.1% acetate plates for 48 to 72 hours following which the plates were sprayed with roughly 0.1 ml of a 10% solution of glycerol (corresponding to .05% in the agar). New colonies

appearing after further incubation would have the ability to utilize glycerol without being able to use acetate.

(2) This method could be further improved by spraying glyoxylate instead of glycerol and hence selecting for ace A mutants directly. To overcome possible acetate depletion in the agar, acetate is mixed with glyoxylate. Newly growing colonies would only be ace A. We have attempted the first method. Roughly 300 plates were screened without obtaining an ace A mutant. Both screening of a larger population and the second method will be undertaken.

2. Resting cell suspensions of the wild type organism were investigated. The data are presented in Tables II.A.1 and II.A.2. Propionate oxidation was almost completely inhibited in the presence of lactate. Oxamate and oxalate did not interfere significantly. Acrylate reduced the rate of oxidation some 55%. Neither acetate nor any other product of propionate metabolism was observed by the GC assay for volatile fatty acids used to assay for acrylate.

Acrylate was not metabolized by the cells when present with propionate. Propionate could not be metabolized under anaerobic conditions. The resting cell suspension experiment was repeated using lactate as a substrate (Table II.A.3). The analogs and inhibitors did not have a marked effect on the rate of lactate metabolism. Oddly, 2 peaks one at retention time of 2.33 min and another appearing as a shoulder on the internal standard (methyl oxylate) were observed to increase during

TABLE II.A.1: OXIDATION OF PROPIONATE BY RESTING CELL
SUSPENSIONS OF E. COLI

Substrate	Conc. %	Residual Propionate Conc. (gms/l)			
		Time (min)			
		0	120	300	540
Propionate	.2	2.0	1.61	0.45	0
	.4	4.0	2.67	1.75	1.25
	.6	6.0	-	4.77	3.87
	.8	8.0	6.29	5.84	4.34
Propionate + Glyoxylate	.2+	2.0	1.49	.710	0
Propionate Anaerobically	.2	2.0	1.83	1.97	2.26

TABLE II.A.2: OXIDATION OF PROPIONATE BY RESTING CELL SUSPENSIONS OF E. COLI IN THE PRESENCE OF SUBSTRATE ANALOGS AND ENZYME INHIBITORS

	Substrate	Concentration of propionate unoxidized time in mins				
		0	30	90	150	225
1	propionate 0.2% —	2.0	1.83	1.09	1.02	0.63
2	+ (NH ₄) ₂ SO ₄ 0.1%	2.0	1.99	1.05	0.55	0.47
3	+ HCO ₃ ⁻ 0.1%	2.0	-	1.04		
4	+ Lactate 0.2%	2.0	2.0	1.97	1.98	1.79
5	+ B hydroxy-propionate 0.1%	2.0	1.72	1.66	1.22	0.85
6	+ propionate 0.1%	2.0	-	1.76	2.01	1.78
7	+ acrylamide 0.1%	2.0	1.72	1.36	1.28	0.90
8	+ acrylate 0.1%	2.0	1.85	1.78	1.57	1.25
9	+ cyclopropane carboxylic acid 0.1%	2.0	1.84	1.72	0.92	1.05
10	+ oxalate 0.1%	2.0	1.66	1.62	1.19	0.85
11	+ oxamate 0.1%	2.0	-	1.63	1.30	1.14

TABLE II.A.3: OXIDATION OF LACTATE BY RESTING CELL SUSPENSIONS

Substrate		Metabolized Lactate gms/l			
		time (min)			
		0	120	300	540
1	Lactate 0.5%	5.0	3.44	1.84	0.98
2	+ Propionate 0.2%	5.0	3.42	2.27	1.52
3	+ oxamate 0.2%	5.0	3.72	2.12	1.35
4	+ acrylate 0.1%	5.0	3.07	2.48	2.15
5	+ arsenite	5.0	3.36	2.54	1.72

the time course (Figure II.A.8). The peaks were not free carboxylic acids, nor pyruvate or β hydroxy propionate. Although they appear at RT's where unmethylated propionate and acrylate appear, it is not clear how they could escape methylation. We are attempting to identify these products of lactate metabolism.

3. Cell free extracts are being studied for their ability to oxidise propionate in the presence of ATP, NAD, and CoA or Acetyl CoA. Very high endogenous rates of NAD reduction interfere with analysis. Dialysed extracts are being prepared.

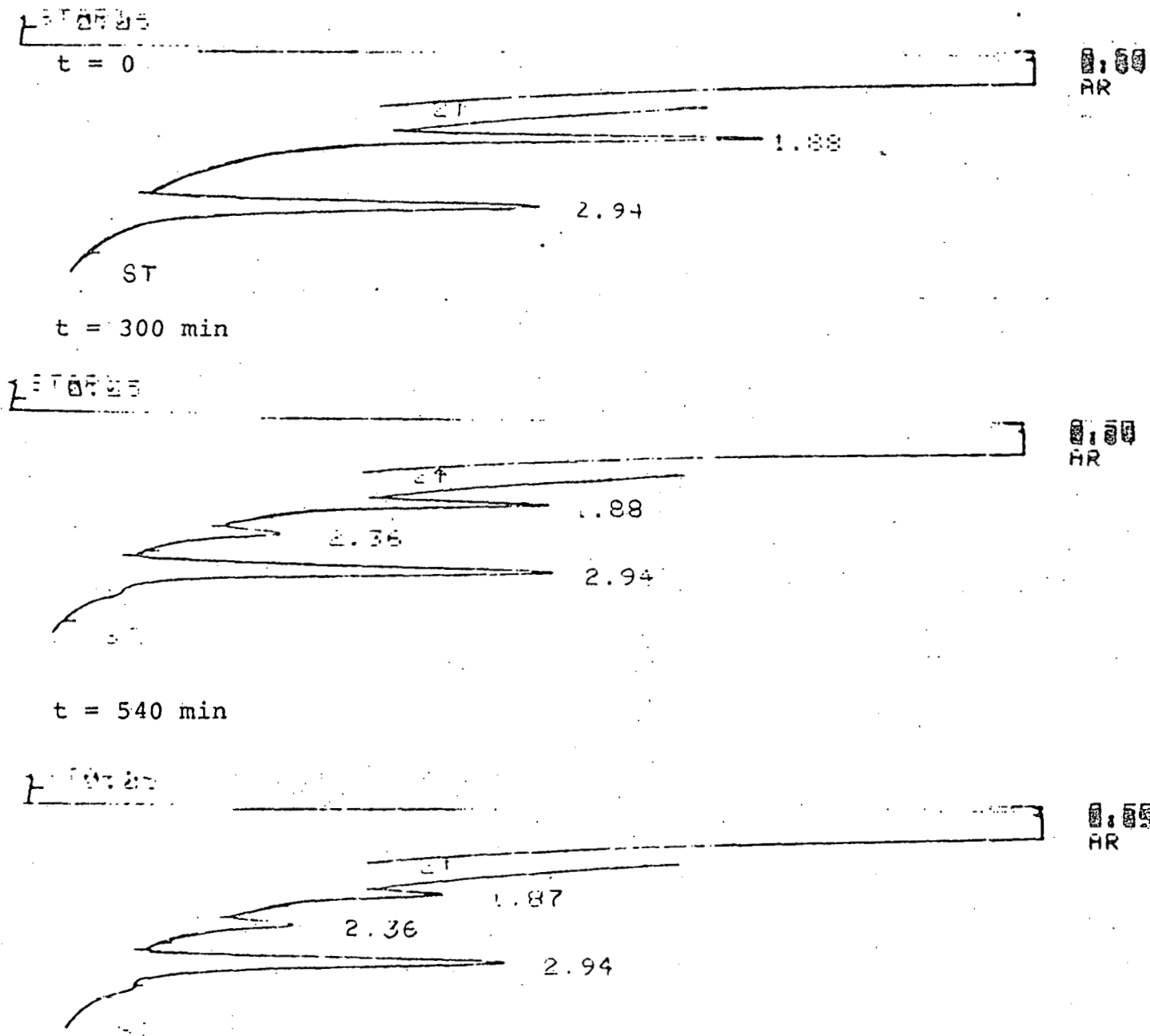
d. Future Work

- Cell free extracts capable of oxidising propionate in the presence of ATP, CoA and NAD will be prepared. They will be used to aid in the identification of an acrylate intermediate and in characterizing mutants deficient in propionate metabolism.

- Identify the products of lactate metabolism by resting cell suspensions.

- Isolate propionate negative, lactate positive mutants, irrespective of their ability to metabolize acetate as the sole carbon source.

FIGURE II.A.8: LACTATE UPTAKE AND APPEARANCE OF UNKNOWN PEAK AT RT 2.36 AND SHOULDER ON PEAK RT 2.94



RT = 1.87 = Lactic Acid - methyl ester

RT = 2.94 = Internal Standard Oxalic Acid - methyl ester

4. Production of Lactic Acid

a. Introduction

In this section of the report, production of lactic acid, which is a key intermediate in the acrylic acid conversion pathway, is being reported on.

Since our final objective is the conversion of cellulose into useful chemicals, it is especially important to evaluate the integration of lactic acid production with the degradation of cellulose.

Basically, the approach is to grow C. thermocellum with the lactic acid producer, "Thermolactic bacterium", as a mixed culture. Cellulose is to be the only added carbon source. A commensalistic type of interaction is expected to take place, where C. thermocellum growing on cellulose will provide enough soluble sugars to support growth of Thermolactic bacterium and, therefore, result in accumulation of lactic acid.

This report covers the results on the characterization of "Thermolactic bacterium" isolated and investigated as a lactic acid producer at 60°C. This bacterium was tested as a mixed culture with C. thermocellum for lactic acid accumulation using Solka floc as the only carbon source. Data of physiology of Lactobacillus planterium is also presented.

b. Materials and Methods

1. Microorganisms

Lactobacillus plantarum NRRL-B-477 is a homo-fermentative lactobacilli, when grown on Glucose. It accumulates

lactic acid plus acetic acid when grown on Xylose. The optimal growth temperature is 37°C. "Thermolactic bacterium" is a homo-fermentative, thermophilic lactic acid producer obtained from DuPont Corp. Its optimal temperature for growth is 60°C. Clostridium thermocellum ATCC-27405 (see Report COO-4198-1, pages 2-3).

2. Growth Medium

The medium utilized for growth and production of lactic acid has been described in previous progress report (COO-4198-2, March - May, 1977, pages 52-53). This medium was used for the cultivation of L. plantarum and "Thermolactic bacterium".

Preservation of "Thermolactic bacterium" was done in the same medium; however, the glucose concentration was 2.5 gm/l, and the buffer capacity of the medium was doubled.

3. Inoculum Preparation

The standard procedure for inoculum preparation was followed (see Report COO-4198-4, pages 108-109). Handling and growth of C. thermocellum was done following the methodology and growth conditions described in previous reports (see Report COO-4198-1, pages 2-3).

4. Fermentor

A 5 liter Magnaform bench scale (New Brunswick Co.) Fermentor was used at 3 liter working volume. pH and temperature were controlled at constant values.

5. Assays

During the fermentation, kinetics and concentrations of the following parameters were determined.

- (a) cell concentration
- (b) lactic acid
- (c) acetic acid
- (d) reducing sugars
- (e) ethanol

In all cases, we used the standard methodology described in previous reports for each parameter.

c. Results and Discussion

1. Conversion of Pentose Sugars into Lactic Acid by *L. plantarum*

In order to improve reducing sugar utilization during cellulose degradation, we investigated removal of pentoses by using *L. plantarum* NRRL-B473. The initial characterization of the strain was done with respect to carbon sources utilization. The results obtained are shown in Figure II.A.9. It was found that *L. plantarum* can utilize glucose, cellobiose and xylose as carbon sources with accumulation of lactic acid. However, for the case where xylose is metabolized, there is accumulation of acetic acid together with lactic acid. This is due to the fact that the cell splits 5 carbon sugars into 3 and 2 carbon products. The typical numbers in this experiment are shown in Table II.A.4. Conversion values of 0.9 gm lactic

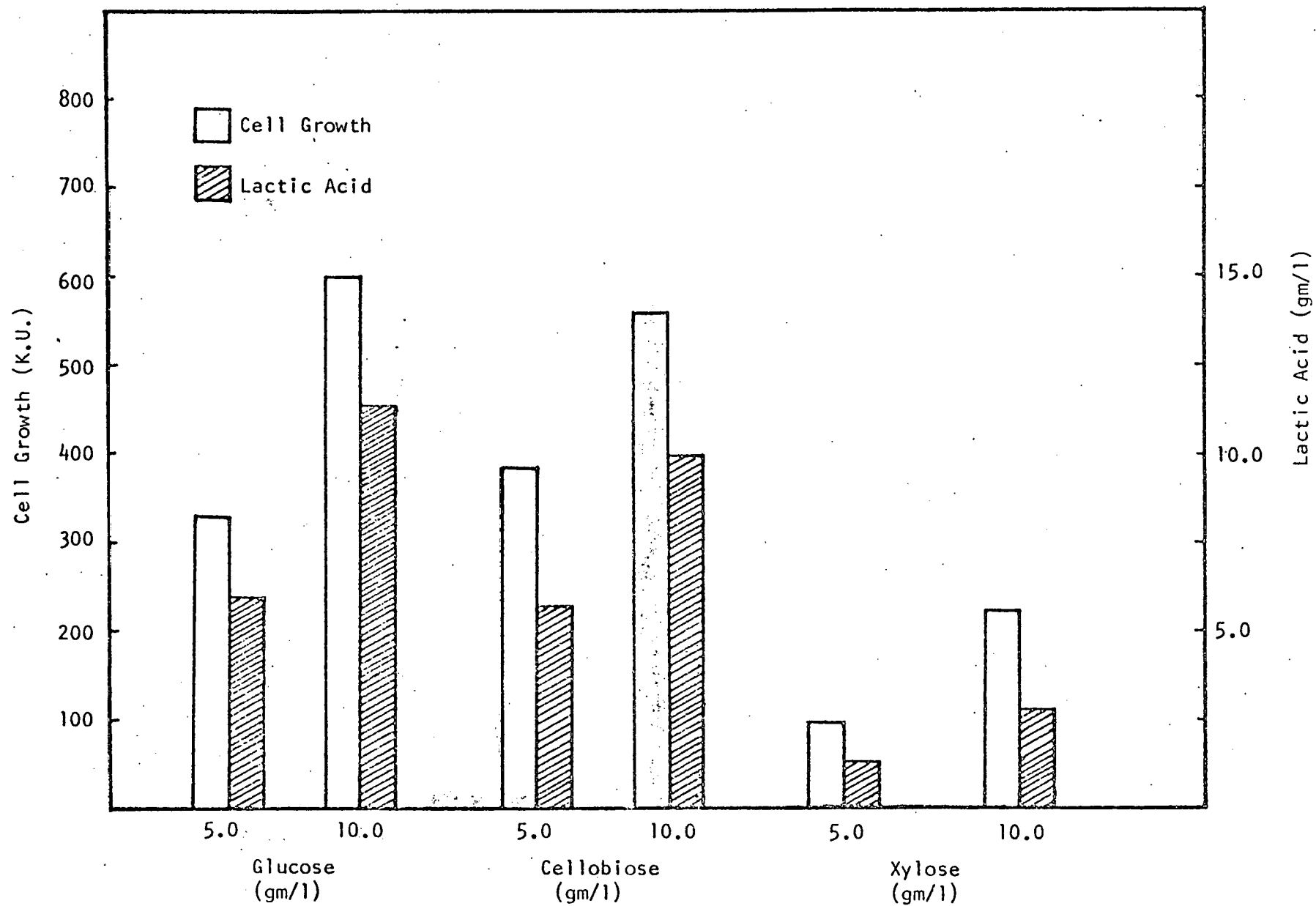


FIGURE II.A.9: CARBON SOURCES METABOLIZED BY LACTOBACILLUS PLANTARUM NRRL-B473 UNDER NO pH CONTROL

TABLE II.A.4: CARBON SOURCE UTILIZATION BY
L. PLANTARUM NRRL-B473

Carbon Source	Conc (gm/l)	Cell Conc (gm/l)	Lactic Acid (gm/l)	Acetic Acid (gm/l)	Conversion (gm Lactic/gm Sugar)
Glucose	5	0.83	4.9	-	0.98
	10	1.30	8.7	-	0.92
Cellobiose	5	1.05	4.9	-	0.98
	10	2.41	7.6	-	0.89
Xylose	5	0.55	2.5	1.9	0.38
	10	1.03	5.0	3.8	0.38

acid/gm sugar consumed were obtained for both glucose and cellobiose. However, only 50% of xylose was converted to lactic acid.

Figure II.A.10 shows the kinetics for xylose metabolism by L. plantarum under controlled pH. This experiment was done to evaluate the kinetic constants of the fermentation, and also to quantify the capacity of the microorganism to accumulate lactic acid under optimal conditions. A specific growth rate value of 0.21 hr^{-1} was found for the logarithmic phase. The complete set of data analysis is shown in Table II.A.5.

2. Production of Lactic Acid at High Temperatures (60°C) by A Thermophilic Bacteria, "Thermolactic bacterium"

One of the limitations to design of the original mixed culture system was meeting the need for a thermophilic bacteria which were able to grow and produce lactic acid at 60°C. Such bacteria was found and then characterized with respect to carbon sources utilized. The results obtained are shown in Figure II.A.11. "Thermolactic bacterium" was able to grow on glucose and fructose as carbon sources, with the consequent accumulation of lactic acid. It appears "T. bacterium" was able to grow on xylose, but further experiments are required to confirm this result.

The results shown in Figure II.A.12 demonstrate the performance of "T. bacterium" under pH controlled conditions. The kinetics of the experiment showed that it is

FIGURE II.A.10: KINETICS OF GROWTH OF LACTOBACILLUS
PLANTARUM NRRL-B-443 ON XYLOSE
pH CONSTANT = 6.0; TEMP. = 37°C

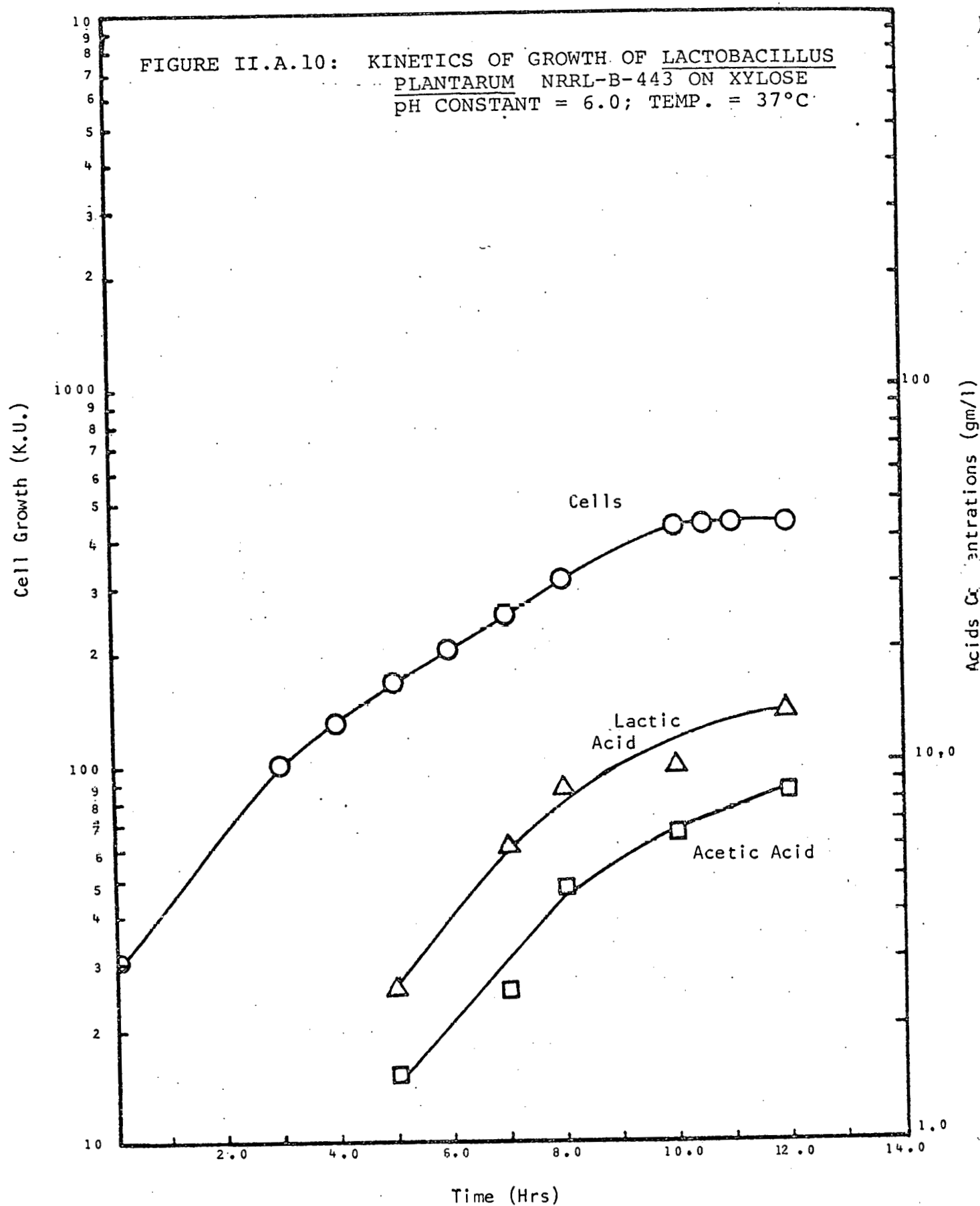


TABLE II.A.5: KINETICS OF GROWTH AND PRODUCT ACCUMULATION OF
LACTOBACILLUS PLANTARUM NRRL-B473 ON XYLOSE

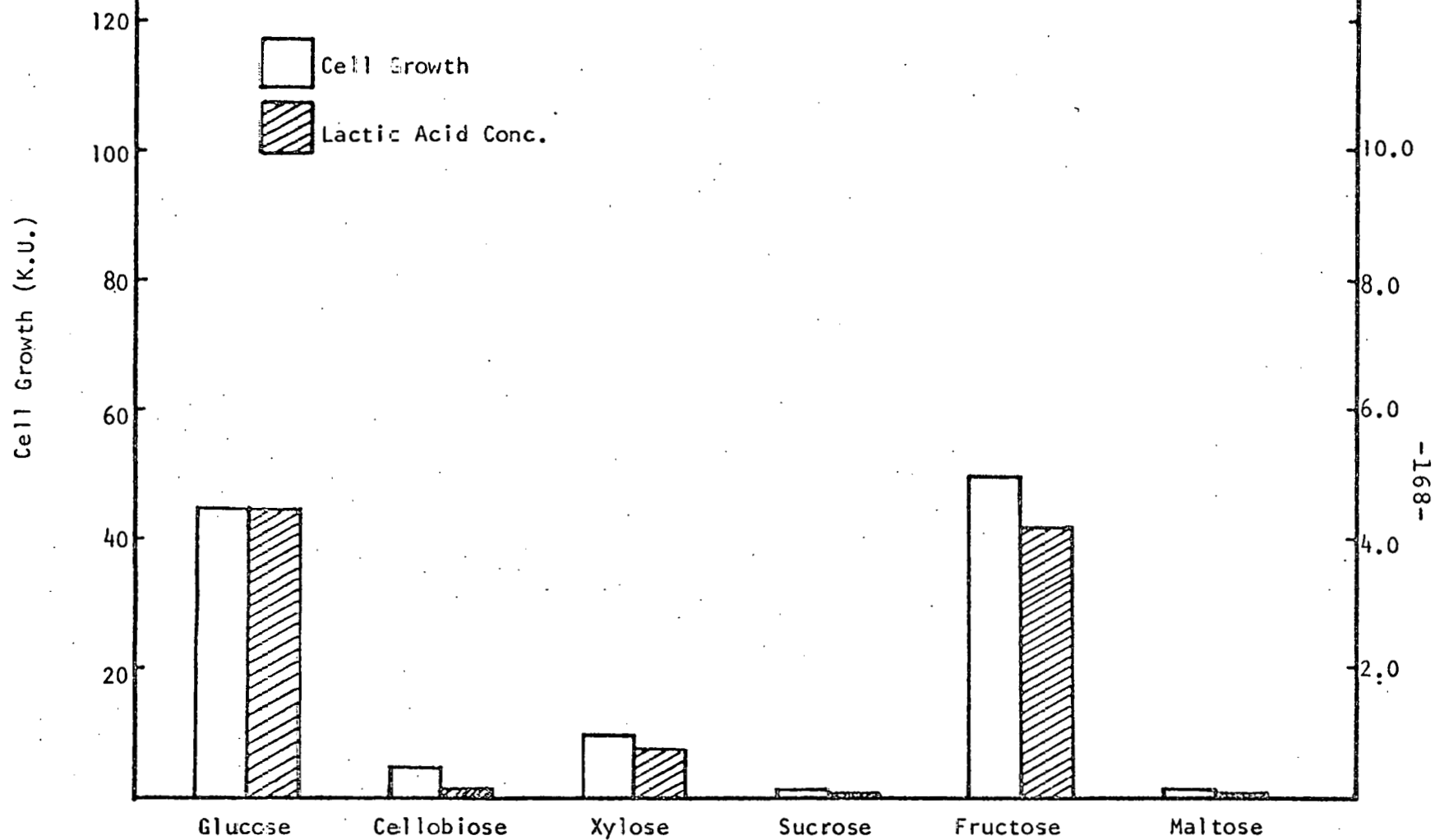
Sample N°	Age (Hrs)	Cell Conc (gm/ℓ)	Lactic Conc (gm/ℓ)	Acetic Acid (gm/ℓ)	Specific Growth Rate (1/Hr)	Productivity ($\frac{\text{gm lactic ac}}{\text{g/ℓ}}$)	Conversion ($\frac{\text{gm lactic ac}}{\text{gm xylose}}$)
1	3	0.307					
2	4	0.40	1.8	1.0	0.26		0.56
3	6	0.61	3.9	2.1	0.21	1.05	0.59
4	8	0.93	7.0	4.6	0.21	1.55	0.58
5	10	1.29	9.9	6.5	0.16	1.45	0.53
6	12	1.29	13.1	8.3	0.0	1.60	0.62

pH Controlled at: 6.0

Temperature : 37°C

Initial Sugar : 25 gm/ℓ

FIGURE II.A.11: CARBON SOURCES FOR THERMOLACTIC BACTERIA



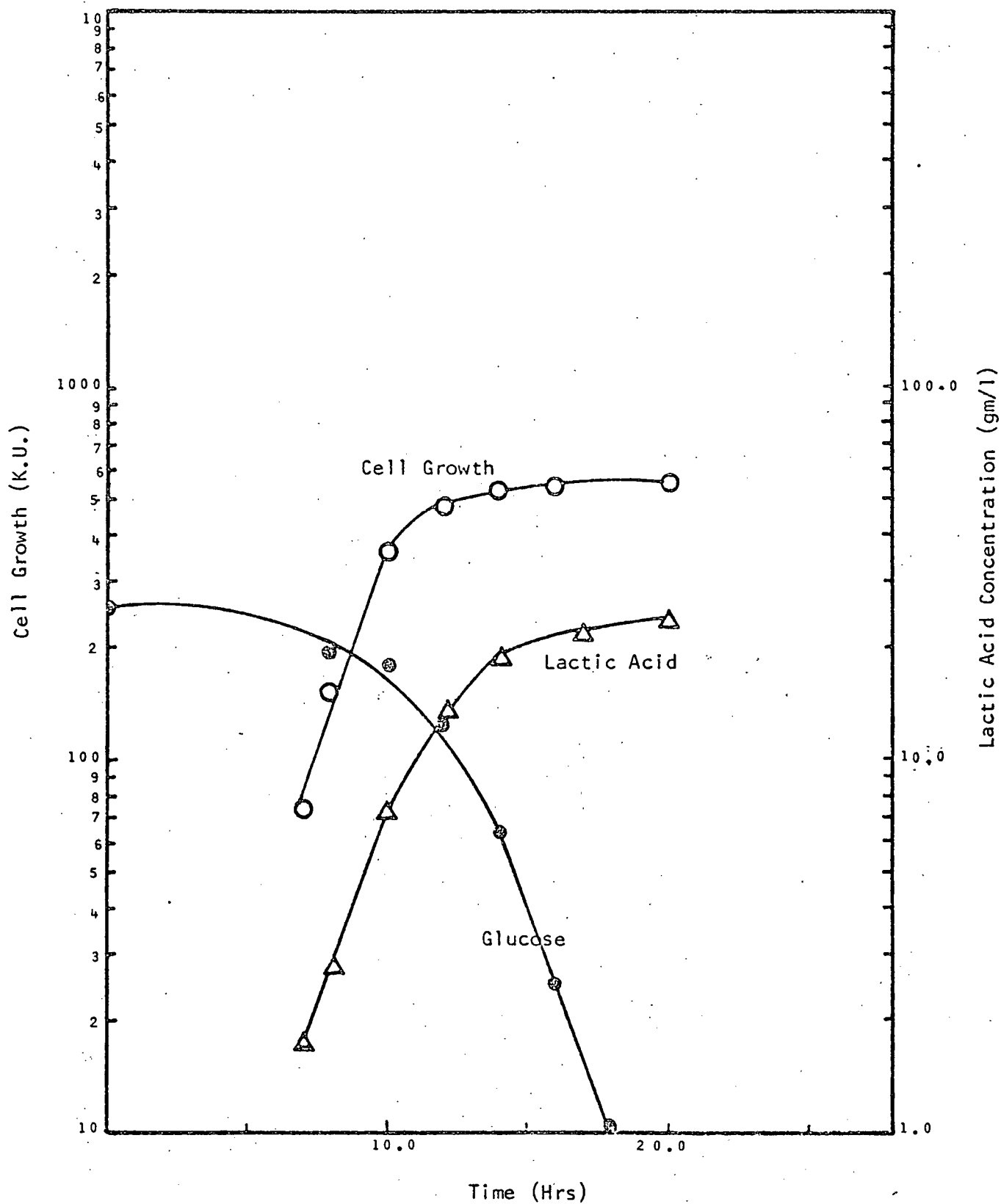


FIGURE II.A.12: KINETICS OF GROWTH AND PRODUCT ACCUMULATION FOR THERMOLACTIC BACTERIUM (CRD) ON GLUCOSE
 pH CONTROLLED = 6.5
 TEMPERATURE = 60°C

able to metabolize glucose and, with the production of lactic acid, the specific growth rate was shown to be a very strong function of the lactic acid concentration. The specific growth rate decays very rapidly with increasing concentrations of lactic acid. Typical conversion values of 0.8 - 0.9 gm lactic acid/gm glucose consumed were found (Table II.A.6).

These results demonstrate the capacity of T. bacterium to accumulate l-lactic acid at 60°C when growing on glucose, and therefore provide a basis for its utilization in mixed culture experiments with C. thermocellum.

3. Lactic Acid Accumulation in a Mixed Culture Population

A mixed culture population of C. thermocellum and T. bacterium using Solka floc as the only carbon source was conducted. The results of such an experiment are shown in Figure II.A.13. This figure represents the time profile for growth and product accumulation, for the mixed culture experiment. The culture was started with C. thermocellum only until the 42nd hour, then "T. bacterium" was inoculated. Lactic acid and reducing sugar were followed during the fermentation. It was found that lactic acid accumulated up to 5.0 gm/l after 15 hrs during the mixed culture fermentation. Reducing sugars increased up to 2.5 gm/l during the same period of time. From the 60th to 120th hours, the rate of lactic acid accumulation was constant at a very low value (0.05 gm/l-hr). Reducing

FIGURE II.A.6: KINETICS OF GROWTH AND PRODUCT ACCUMULATION OF THERMOLACTIC BACTERIUM

Sample	Age	Cell Growth	Residual Glucose	Lactic Ac	Sp Growth Rate	Productivity <u>gm Lactic Ac.</u>	Conversion <u>gm Lactic Ac.</u>
N	(Hrs)	(gm/l)	(gm/l)	(gm/l)	(l/Hr)	1-Hr	mg Glucose
1	8	0.46	19.34	2.79	0.62		
2	10	1.01	17.29	7.16	0.35	2.31	0.71
3	12	1.23	9.10	13.26	0.17	2.96	0.92
4	14	1.63	6.46	18.10	0.07	2.41	0.83
5	16	1.63	3.0	19.6	0.03	1.50	0.89
6	18	1.69	1.5	21.6	0.03	2.0	0.91
7	20	1.66	0.0	23.7	0.0	2.0	0.94

pH controlled at 6.5

Temperature 50 C

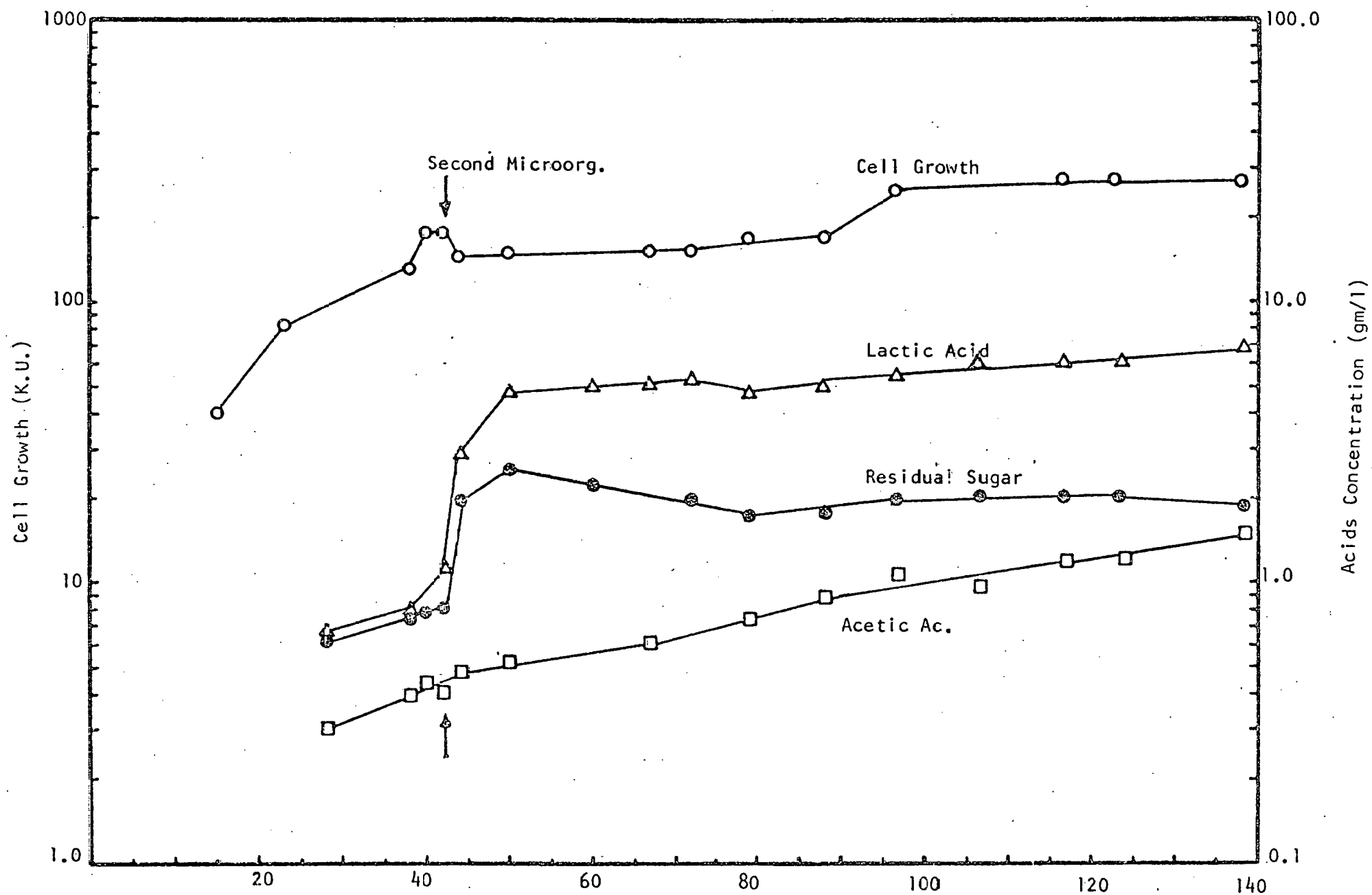


FIGURE II.A.13: KINETICS OF PRODUCT ACCUMULATION FOR A MIXED CULTURE POPULATION OF C. THERMOCELLUM AND T. BACTERIUM

sugar concentration remained constant below 2.0 gm/l. Acetic acid and ethanol accumulated in the normal way (up to 1.0 gm/l). The results in Table II.A.7 show that after 60 hrs, 75% of the cellulose was consumed, and 58% of it went into lactic acid during the fermentation. Further information on this experiment is given in Table II.A.7 and Figure II.A.14.

Although there is still much room for improvement, the results obtained so far are encouraging and demonstrate the feasibility of a mixed culture system for the production of lactic acid directly from cellulose.

4. Future Experiments

Future experiments will concentrate on the biochemical engineering aspects of the mixed fermentation. Optimization studies conducted in the future should lead to a methodology and process for the direct conversion of cellulose to lactic acid and other useful products.

TABLE II.A.7: KINETICS OF PRODUCT ACCUMULATION FOR A MIXED POPULATION OF
C. THERMOCELLUM AND T. BACTERIUM

Sample (N°)	Age (Hrs)	Cell Growth (K.V.)	Lactic Acid (gm/l)	Acetic Acid (gm/l)	Ethanol (gm/l)	Red. Sugars (gm/l)	Res. Cellulose (gm/l)
1	40	175	0.93	0.41	0.40	0.76	6.93
2	42	180	1.13	0.45	0.44	0.79	6.52
3	44	145	1.60	0.48	0.43	1.0	5.90
4	46	148	2.30	0.49	0.45	1.35	4.85
5	48	149	3.2	0.51	0.48	1.81	3.9
6	50	150	4.75	0.55	0.55	2.50	2.56
7	55	150	4.80	0.57	0.59	2.30	2.61
8	60	150	4.90	0.58	0.65	2.1	2.23

pH CONTROLLED AT: 6.8
 TEMPERATURE : 60°C

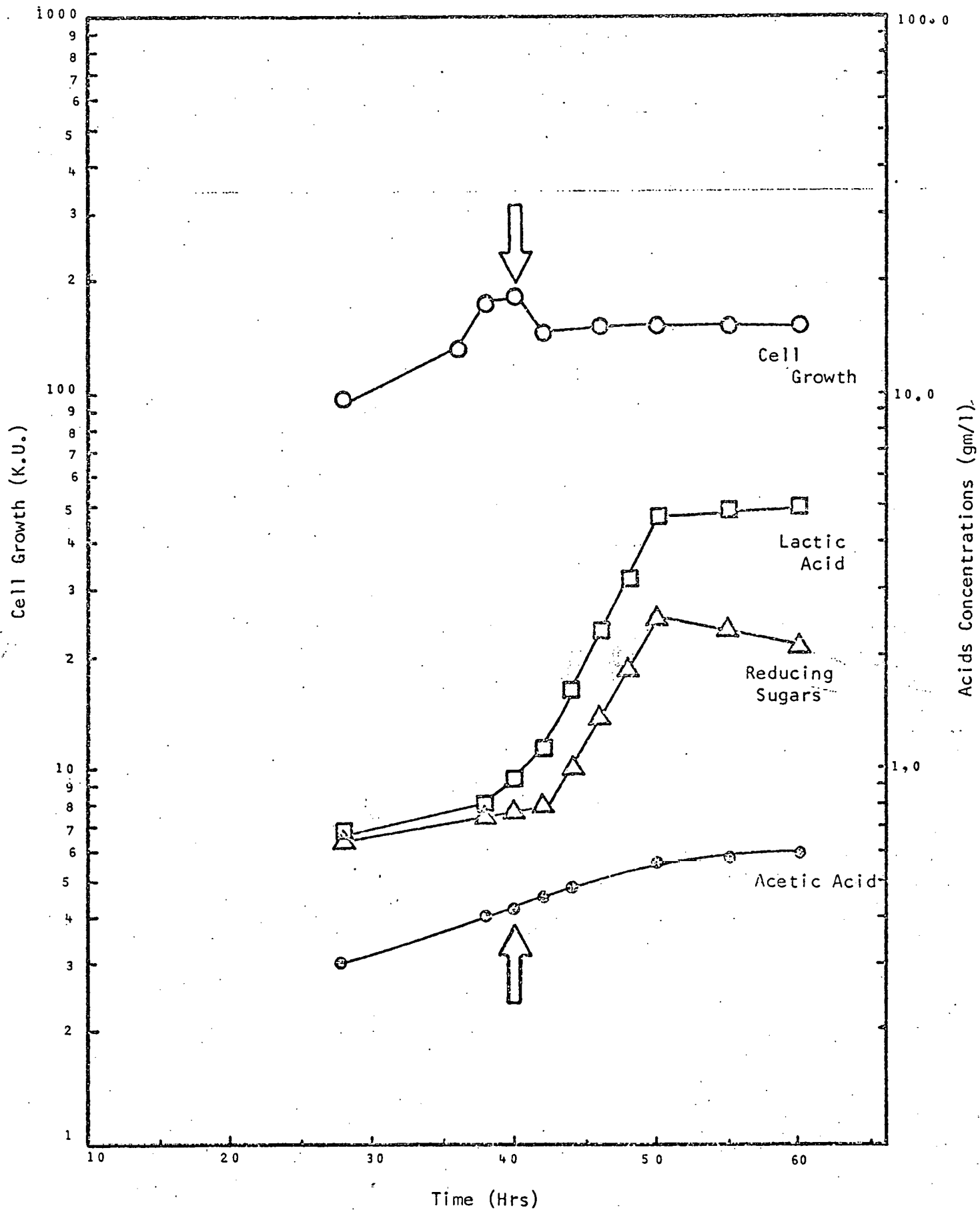


FIGURE II.A.14: KINETICS OF PRODUCT ACCUMULATION FOR MIXED POPULATION OF C. THERMOCELLUM AND T. BACTERIA

B. Production of Acetone and Butanol by Fermentation

1. Introduction

One of the problems encountered in the acetone/butanol fermentation is the low volumetric productivity of the mixed solvent (g/l-hr). This is caused mainly by the toxicity of n-butanol on Clostridium acetobutylicum resulting in a low final concentration of the mixed solvents. The concentration of n-butanol that causes total growth inhibition for C. acetobutylicum was determined to be 17 g/l (Progress Report COO-4198-4). The toxic mode of action of n-butanol towards C. acetobutylicum has not been established. However, based on previous reports on the toxicity of n-butanol towards other organisms and the physical properties of n-butanol as an organic solvent, n-butanol probably disrupts the structure of the membrane by partitioning into the lipid bi-layer.

It has been well established that the fatty acid composition of the bacterial membrane affects the permeability, transport properties and fragility of bacteria, such as E. coli (Haest et al., 1972; Akamatsu, 1974). Techniques for manipulating the composition of the membrane fatty acids have been reported and have been applied to both yeast and bacteria. The synthesis of fatty acid is either inhibited by using an antibiotic, cerulenin (Omura, 1976), or impaired by mutation (Silbert, 1975). Then, extraneous fatty acids were fed to these microorganisms to obtain membranes with a different fatty

acid profile. However, these techniques are quite involved, and therefore we decided to approach this problem in a different manner.

The membrane of living organisms has to be maintained in a liquid-crystalline structure to carry out the essential functions. Since saturated fatty acids usually have higher melting points than unsaturated fatty acids, cells growing at higher temperatures could have higher content of saturated fatty acids. This, in turn, could impart liquid-crystalline structures to the membrane.

Thus, we hypothesized that C. acetobutylicum growing at temperatures other than 37°C might have a significant change in the fatty acid content in the membrane. Such change might possibly cause a different resistance to n-butanol.

2. Materials and Methods

a. Inoculum Preparations

A culture of C. acetobutylicum ATCC 824 started from spores was allowed to grow in an anaerobic flask containing 250 ml soluble medium at 37°C. The culture was used to inoculate another 5 anaerobic flasks, each containing 250 ml soluble medium. These flasks were incubated at five different temperatures: 25, 30, 37, 45 and 50°C. From the mid-log phases of growth, these cultures were then used for the inhibition study.

b. Growth Inhibition Study

Since only cultures incubated at 30, 37, and 45°C were able to grow, only these three temperatures were studied. Nine series of Hungate tubes were used where each series consisted of tubes containing 5 ml soluble medium with n-butanol ranging from 0 to 25 g/l. These tubes were inoculated with 5 ml of appropriate inoculum and incubated at one of the three temperatures. The experimental design of these studies is tabulated in Table II.B.1. The turbidities of these tubes were followed by using a Turner Spectrophotometer set at 660 nm. For cultures that showed growth, at least one doubling of cell mass was monitored.

3. Results and Discussion

The growth rates of each culture at the different temperatures and different concentration of n-butanol were calculated from the turbidity measurements. The results are presented in terms of the growth inhibition index which is defined as:

$$\text{Growth Inhibition Index} = 1 - \mu/\mu_0 \quad (1)$$

where μ = specific growth rate in presence of n-butanol
(hr⁻¹)

μ_0 = specific growth rate without n-butanol added
(hr⁻¹)

The results from this inhibition study when the growth temperature was at 37 and 45°C are shown in Figure

TABLE II.B.1: EXPERIMENTAL DESIGN ON THE EFFECT OF N-BUTANOL
ON GROWTH OF C. ACETOBUTYLICUM AT DIFFERENT
TEMPERATURES

Inoculum Temperature (°C)	Growth Temperature (°C)		
	30	37	45
30	A	D	G
37	B	E	H
45	C	F	I

NOTE: Each letter represents a series of 10 tubes.

II.B.1 and Figure II.B.2 respectively. The effects of n-butanol on these cultures are similar and confirmed the results we previously reported (Progress Report COO-4198-4). The onset of butanol inhibition on growth is at about 10 g/l. Fifty percent inhibition on growth occurs at about 14 g/l. Total inhibition of growth was observed at 17 g/l. When the growth temperature was at 30°C, very different results were obtained. These results are shown in Figure II.B.3. The cultures originated from inoculum grown at 37 or 45°C (not shown) responded similarly as the others (Figure II.B.1 and II.B.2). However, the culture that was started from inoculum grown at 30°C has a higher resistance to n-butanol. The 50% inhibition concentration of n-butanol is 21 g/l. This is about 1.5 times of the concentrations obtained at the other temperatures. Total inhibition of growth occurs when butanol concentration reaches 25 g/l.

This study does not, however, confirm the hypothesis initially set forth. However, a marked difference of resistance to butanol for the microorganisms was observed when the growth temperature was varied. The possibility of increasing the tolerance of C. acetobutylicum towards n-butanol by lowering the temperature was revealed. This would be helpful in process design to optimize the volumetric productivity of mixed solvent.

4. Future Work

- Continue study of operating conditions for higher productivities.

FIGURE II.B.1. EFFECTS OF N-BUTANOL ON GROWTH OF C. ACETO-
BUTYLICUM ATCC 824 AT 37°C

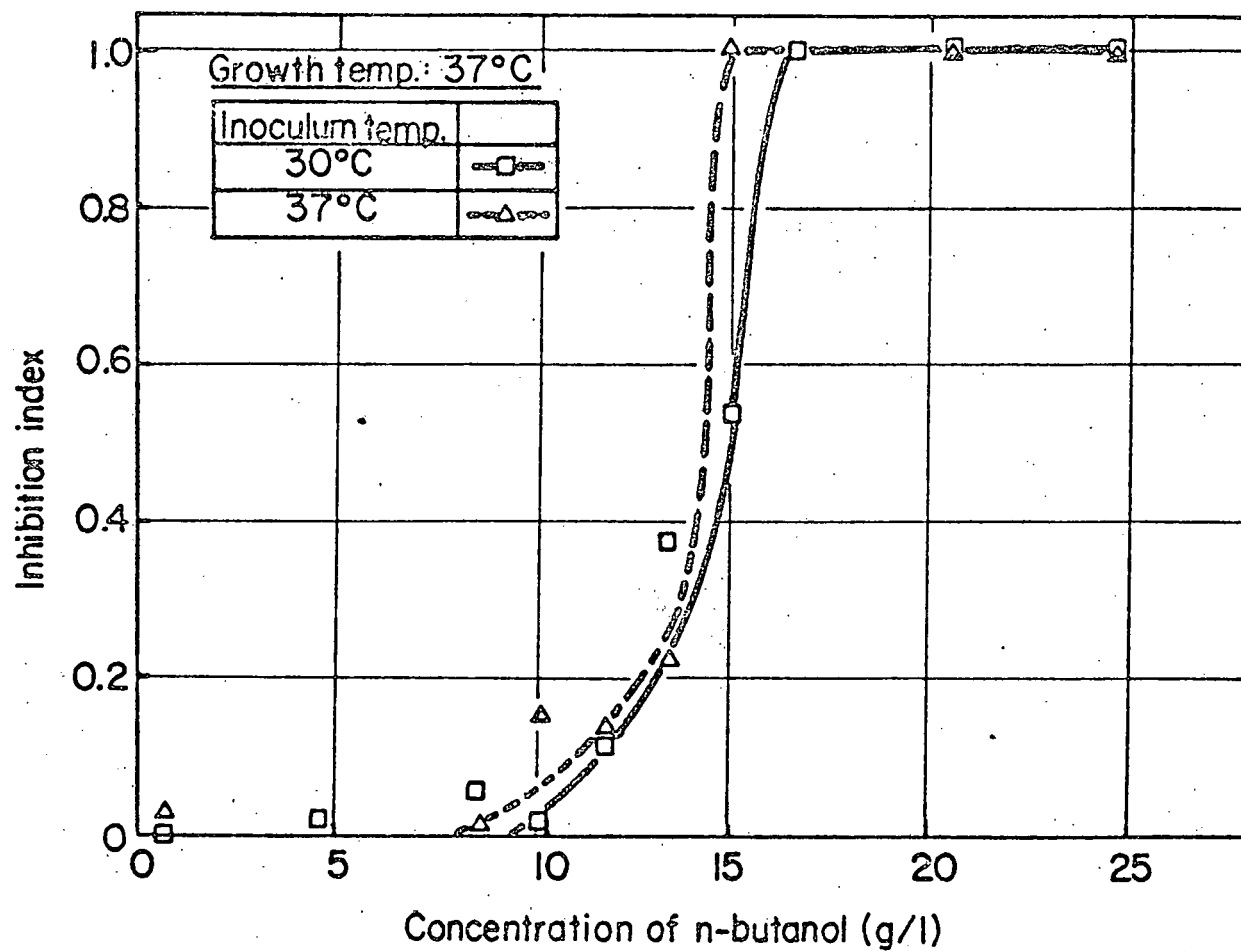


FIGURE II.B.2: EFFECTS OF N-BUTANOL ON GROWTH OF C. ACETO-
BUTYLICUM ATCC 824 GROWING AT 45°C

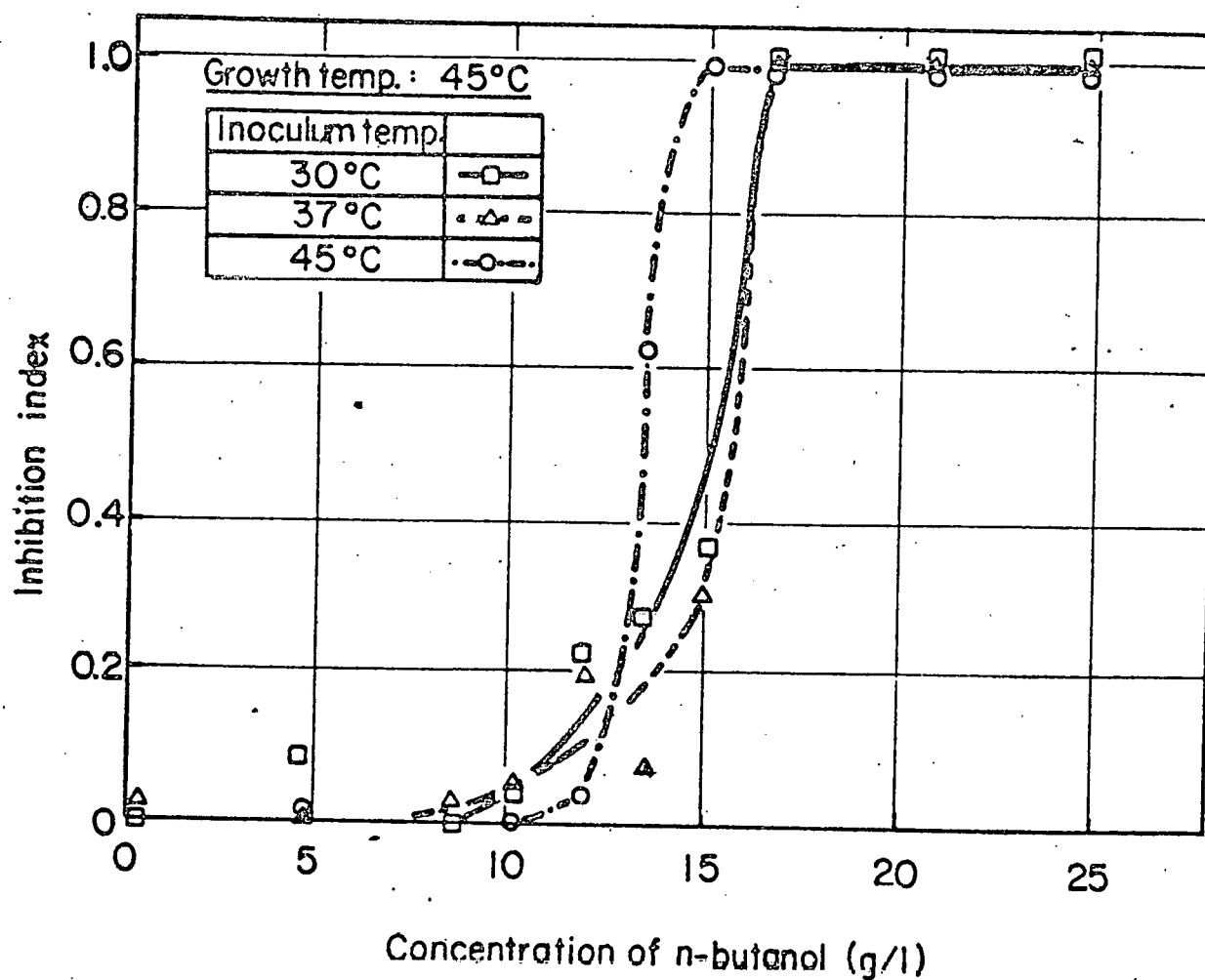
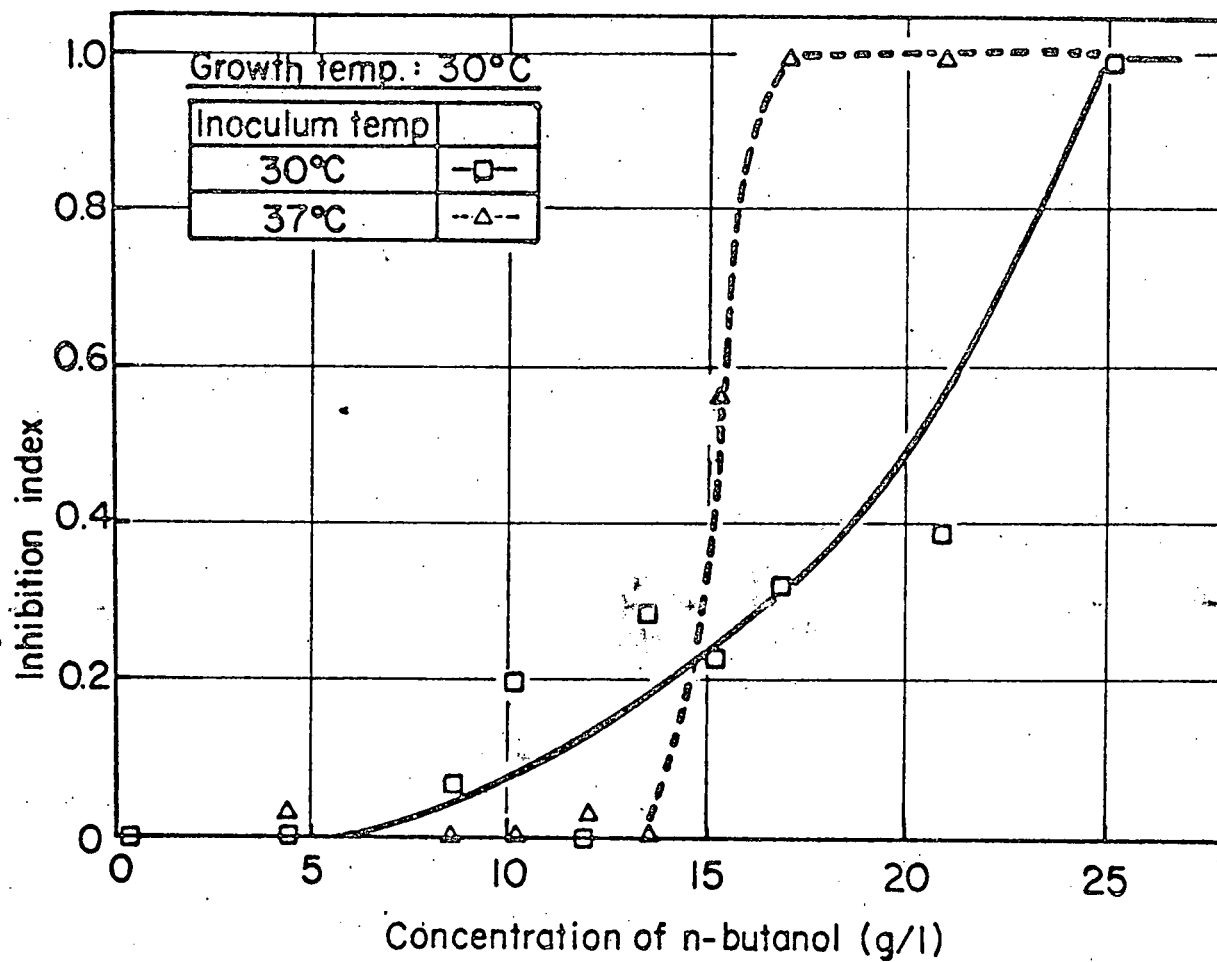


FIGURE II.B.3: EFFECTS OF N-BUTANOL ON GROWTH OF C. ACETO-
BUTYLICUM ATCC 824 GROWING AT 30°C



- ⊙ Continue study of activities of resting cells.
- ⊙ In situ extraction using resting cells or immunized cells.

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C. Production of Acetic Acid by Fermentation

1. Introduction

In a previous Progress Report (COO-4198-4), we had reported the isolation of a strain (S-2) of Clostridium thermoaceticum which was able to tolerate higher concentrations of sodium acetate than the parent. This isolate has undergone further adaptation and selection to increase its tolerance to acetate. Some of the properties of this new isolate (S-3) will be presented in this report.

In the last Progress Report (COO-4198-5), the kinetic analysis on cell growth and acetic acid production suggested both growth and non-growth associated product formation are possible. The possibility of using non-growing cells for acetic acid production might be quite important in the development of an industrial process. In view of the previous findings, a number of supplemental studies have been performed to directly assess whether non-growing cells can indeed produce acetic acid. The results from these studies will be helpful in the conceptualization as to the type of fermentation system most appropriate for acetic acid production.

There is little doubt that the best desirable method in the production of acetic acid will be a continuous process. It is not certain, however, at this time the exact type of fermentation system which might be used. Some possible considerations include: continuous culture with and without

cell recycle, resting cells in packed columns or immobilized, or in situ product removal fermentors. We have therefore initiated studies in all of these areas in order to assess the problems which might be encountered in each of these systems. These studies, along with those others, should allow us to conclude the most viable production scheme in the design of a process.

2. Materials and Methods

Most of the analytical methods used in our studies have already been presented in previous progress reports and, therefore, will not be repeated. The only new experimental method which has not been reported is the studies on cell flocculation. These experiments were performed using cells from exponential phase of growth in shake flasks and mixed with different concentrations of the flocculant (Bentonite, mineral hydrocolloid, American colloids Co., Skokie, Illinois). The cell suspension containing Bentonite was mixed for about one minute and then transferred to the sedimentation column. The sedimentation column was made of a 2.5 cm diameter glass tube and 30.5 cm in length. Anadnoted divisions on the tube allowed the measurement of the sedimented cell-liquid interface for calculating the sedimentation rates. Samples from the top and bottom in the column were removed after 30 minutes of sedimentation for measuring the respective cell concentrations.

3. Results and Discussion

a. Properties of New Acetate Tolerant Strain (S-3)

A newly isolated strain (S-3) of Clostridium thermoaceticum has been under examination with respect to its acetate tolerance properties. Shown in Figure II.C.1 is the relationship between sodium acetate concentration to the specific growth rate of this isolate. The new strain is able to tolerate acetate up to 60 gm/liter before total growth inhibition results. In comparison to the parent and the previous isolate (S-2), total inhibition resulted at sodium acetate concentrations of 43 and 50 gm/liter, respectively. These results again confirm that the use of the adaptation technique offers an expedient method for strain selection.

The question was also asked as to which ion (cation or anion) exerts the toxic effect on the growth of C. thermoaceticum. Although it has been assumed that the anion (acetate) is the causative agent, this has never been tested in a definitive fashion. A series of experiments were therefore performed and the results shown in Table I.C.1. Sodium acetate alone and in combination with sodium chloride was tested to examine their effect on the growth of C. thermocellum (S-3). It is seen from Table II.C.1 that, as the sodium acetate was increased from 40 to 60 gm/liter, the specific growth rate decreased from 0.038 hr^{-1} to zero. However, when sodium acetate was tested in combination with sodium chloride, the results

FIGURE II.C.1: RELATIONSHIP FOR NEW STRAIN (S-3) OF
CLOSTRIDIUM THERMOACETICUM BETWEEN
SODIUM ACETATE CONCENTRATION AND
GROWTH

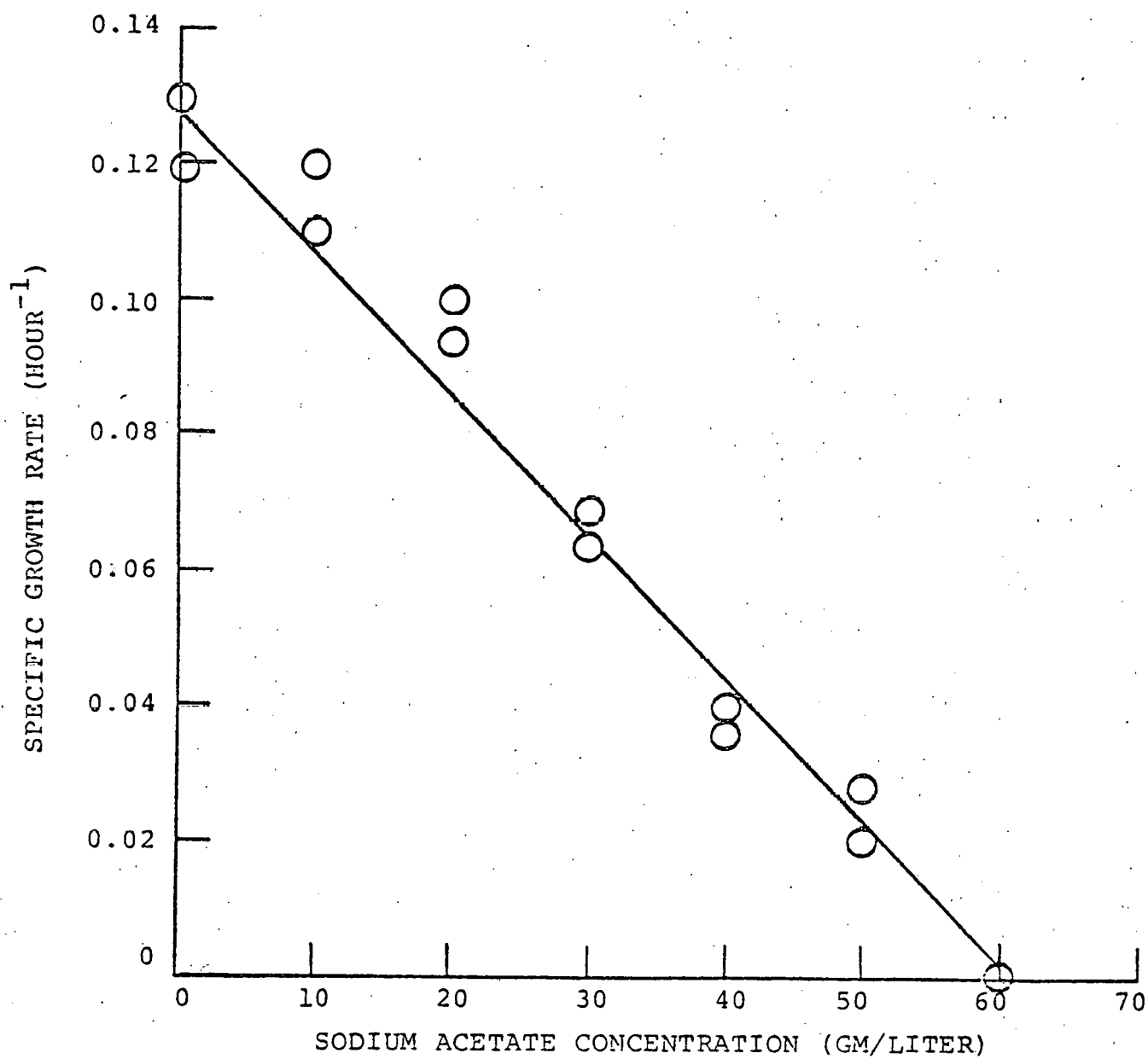


TABLE II.C.1: EFFECT OF SODIUM AND ACETATE ION CONCENTRATIONS
ON THE GROWTH OF CLOSTRIDIUM THERMOACETICUM

Sodium Acetate (gm/l)	Sodium Chloride (gm/l)	Sodium Ion (gm/l)	Acetate Ion (gm/l)	Specific Growth Rate (Hour ⁻¹)
40	0	11.2	28.8	0.038
50	0	14.0	36.0	0.025
60	0	16.8	43.0	0
40	10	15.1	28.8	0.032
30	10	13.3	21.6	0.055

showed that the increase in the concentration of the sodium ion did influence the specific growth rate, but to a much lesser extent than the acetate ion. For example, at a constant acetate concentration of 28.8 gm/liter, when the sodium ion was increased from 11.2 gm/liter (NaAC = 40 gm/liter) to 15.1 gm/liter (NaAC = 40 and NaCl = 10 gm/liter), a small decrease in the specific growth rate was observed (0.038 to 0.032 hr^{-1}). A linear interpretation of the data at 54 gm/liter (between 50 and 60 gm/liter) of sodium acetate was made which is equivalent to a sodium ion concentration of 15.1 gm/liter and acetate concentration of 38.9 gm/liter. At this condition, the interpolated specific growth rate was calculated to be 0.015 hour^{-1} . On the other hand, at the same sodium ion concentration (15.1 gm/l) but lower acetate concentration (28.8 gm/l), a significantly higher specific growth rate (0.032 hr^{-1}) was obtained. These results show that the acetate ion is the main causative agent in exerting a toxic effect to the growth of C. thermoaceticum.

b. Production of Acetic Acid by Non-Growing Cells

Since all of our previous results have indicated the possibility of non-growing cells to produce acetic acid, a number of independent experiments were performed to address this question more definitively. To test the capability of non-growing cells to produce acetic acid, the following experiments were performed. Three shake flasks were inoculated

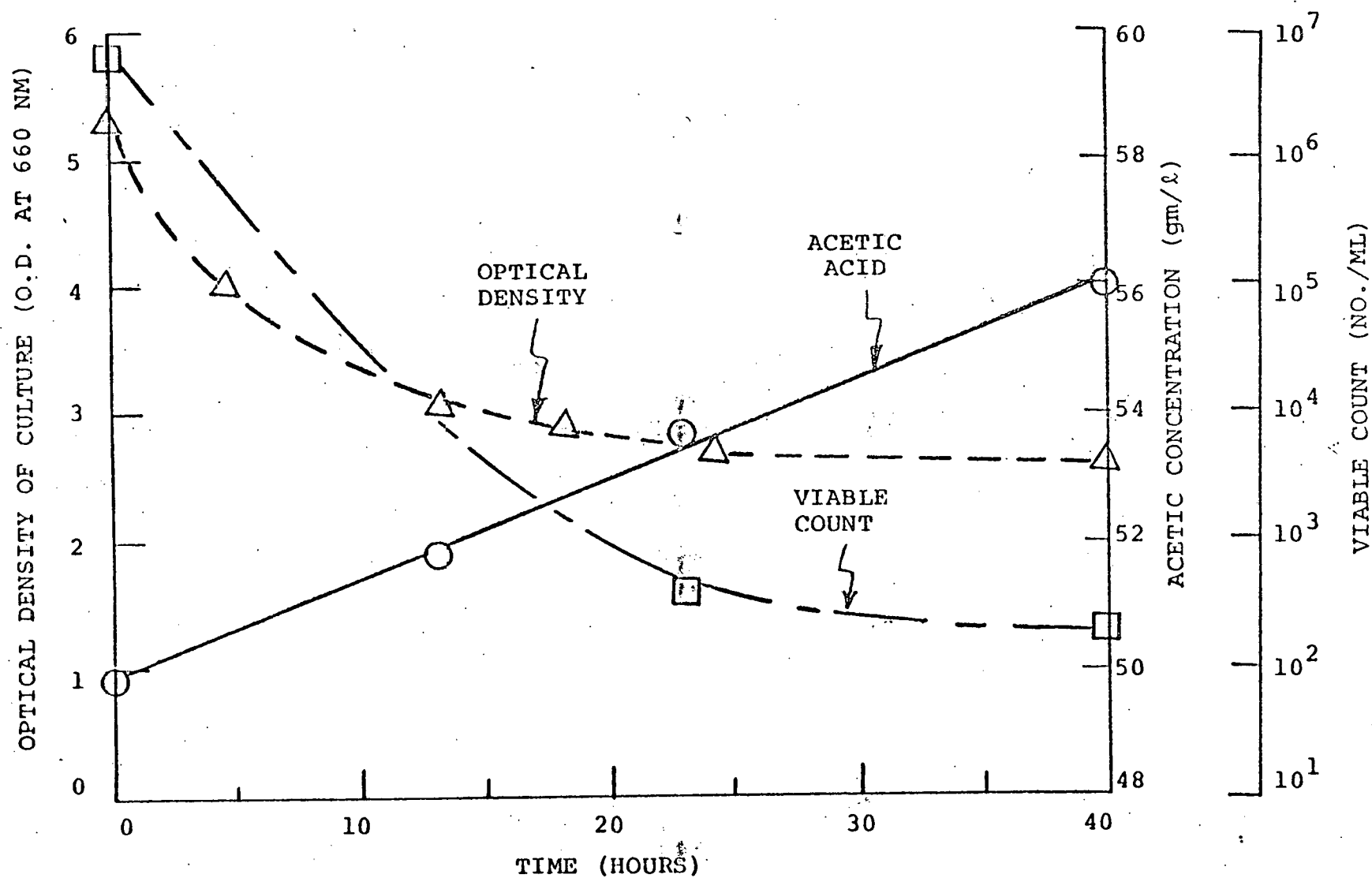
and allowed to grow and produce acetic acid until cessation of both occurred. To one of the cultures, additional glucose and bicarbonate buffer were added and the second, with no addition, served as control. Since the antibiotic rifamcin has been found to totally inhibit growth, 5 mg/liter of this antibiotic was also added to the third culture along with glucose and bicarbonate buffer. All three flasks were then allowed to incubate at 60°C for 36 additional hours. The results from this study are shown in Table II.C.2. It is seen that, when glucose and bicarbonate were added, in the absence and presence of rifamcin, acetic acid continues to be produced. Since the culture containing the antibiotic cannot possibly achieve further growth, we conclude that resting cells are indeed capable of producing acetic acid.

In order to further elucidate this phenomenon, a different type of experiment was also performed. Concentrated suspension of C. thermoaceticum was first prepared through centrifugation. This concentrated suspension was then inoculated into the complete medium, but containing 10 gm/liter of glucose and 50 gm/liter of acetic acid (actually added equivalent to 70 gm/liter sodium acetate). The culture was then incubated at 60°C and the acetic acid, optical density and viable cell count were determined. The results are shown in Figure II.C.2. Acetic acid was observed to continue to be produced from 50 to 56 gm/liter. However, the optical density of the

TABLE II.C.2: THE EXAMINATION OF NON-GROWING CELLS OF C. THERMOACETICUM ON THEIR ABILITY TO PRODUCE ACETIC ACID (INCUBATION AT 59°C FOR 36 HOURS)

Description	Initial Acetic Acid (gm/l)	Final Acetic Acid (gm/l)
Control (no glucose)	15	15
Glucose + Bicarbonate	15	20
Glucose + Bicarbonate + 5 mg/l Rifamcin	15	22

FIGURE II.C.2: ACETIC ACID PRODUCTION BY CLOSTRIDIUM THERMOACETICUM AT HIGH PRODUCT CONCENTRATION: BEHAVIOR OF CELL LYSIS AND VIABILITY



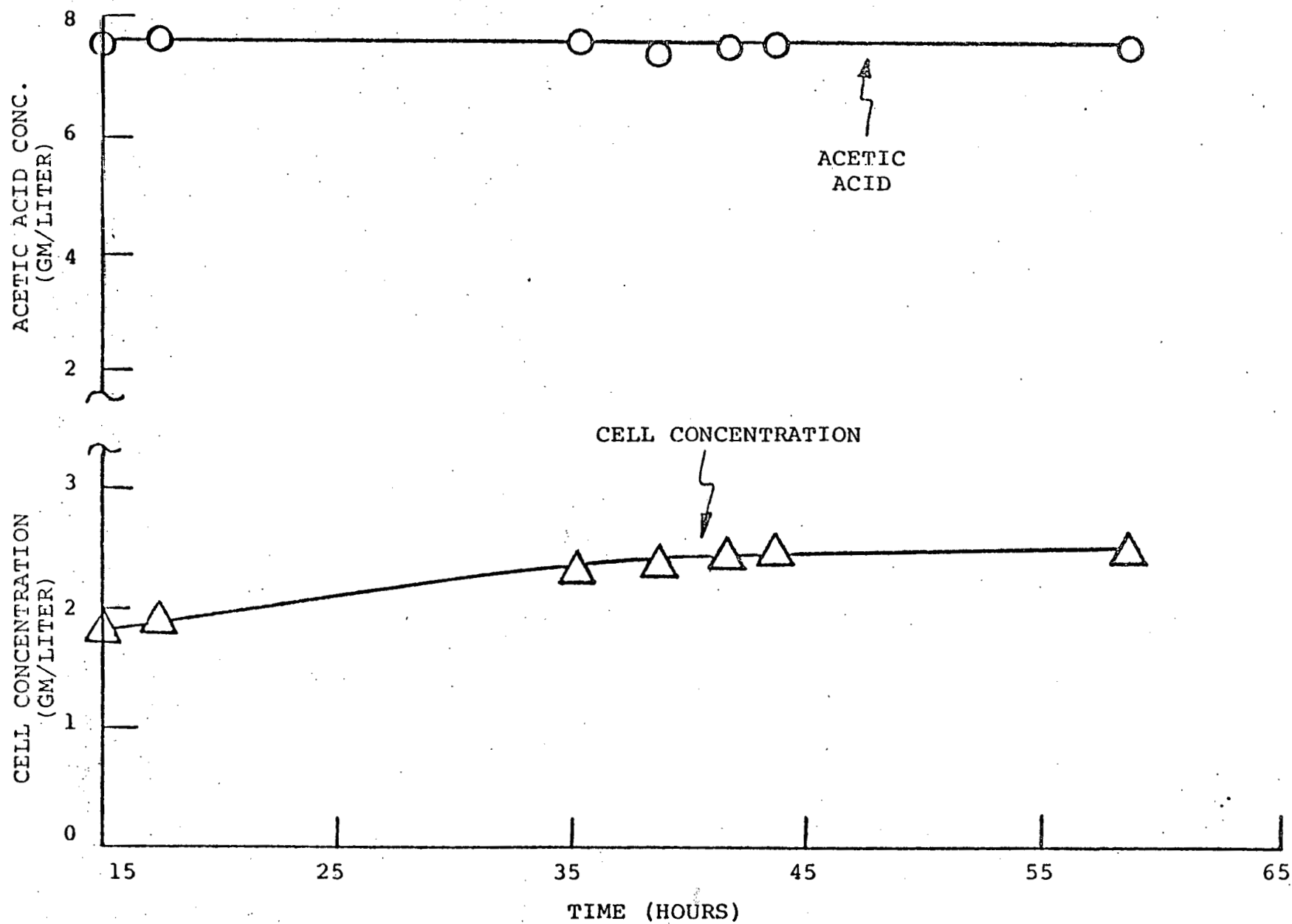
culture showed a continual decrease to approximately 50% of the initial value. The decrease in optical density would indicate cell lysis. The viable cell count during the same period decreased from about 5.8×10^6 per ml to 2×10^2 per ml. One can reasonably conclude that, during this period of acetic acid production, no net cell growth had occurred. These results further substantiate the ability of resting cells to produce acetic acid and encourage our efforts in further examining this concept for product formation.

c. Continuous Culture, Cell and Product Recovery

It is envisioned that ultimately a continuous process will be employed for the production of acetic acid. So far, all our studies have been performed using batch cultivation of Clostridium thermoaceticum. Therefore, we have initiated continuous culture studies to compare these results with our previous findings. Although only one continuous culture experiment has been completed, the results will be presented since they serve as a useful guide for future experimental programs.

The continuous culture study was performed in a 1-liter total volume fermentor using pH control (7.2), CO_2 overlay and 60°C . An inlet glucose concentration of 10 gm/liter and a dilution rate of 0.07 hour^{-1} were maintained. The results from 40 hours of steady-state operation are shown in Figure II.C.3. It is seen that the acetic acid concentration was 7.5 gm/liter and the cell concentration was 2.5 gm/liter. The

FIGURE II.C.3: CONTINUOUS CULTURE OF CLOSTRIDIUM THERMOACETICUM
($T = 60^{\circ}\text{C}$, $\text{pH} = 7.2$, $D = 0.07 \text{ Hr}^{-1}$)

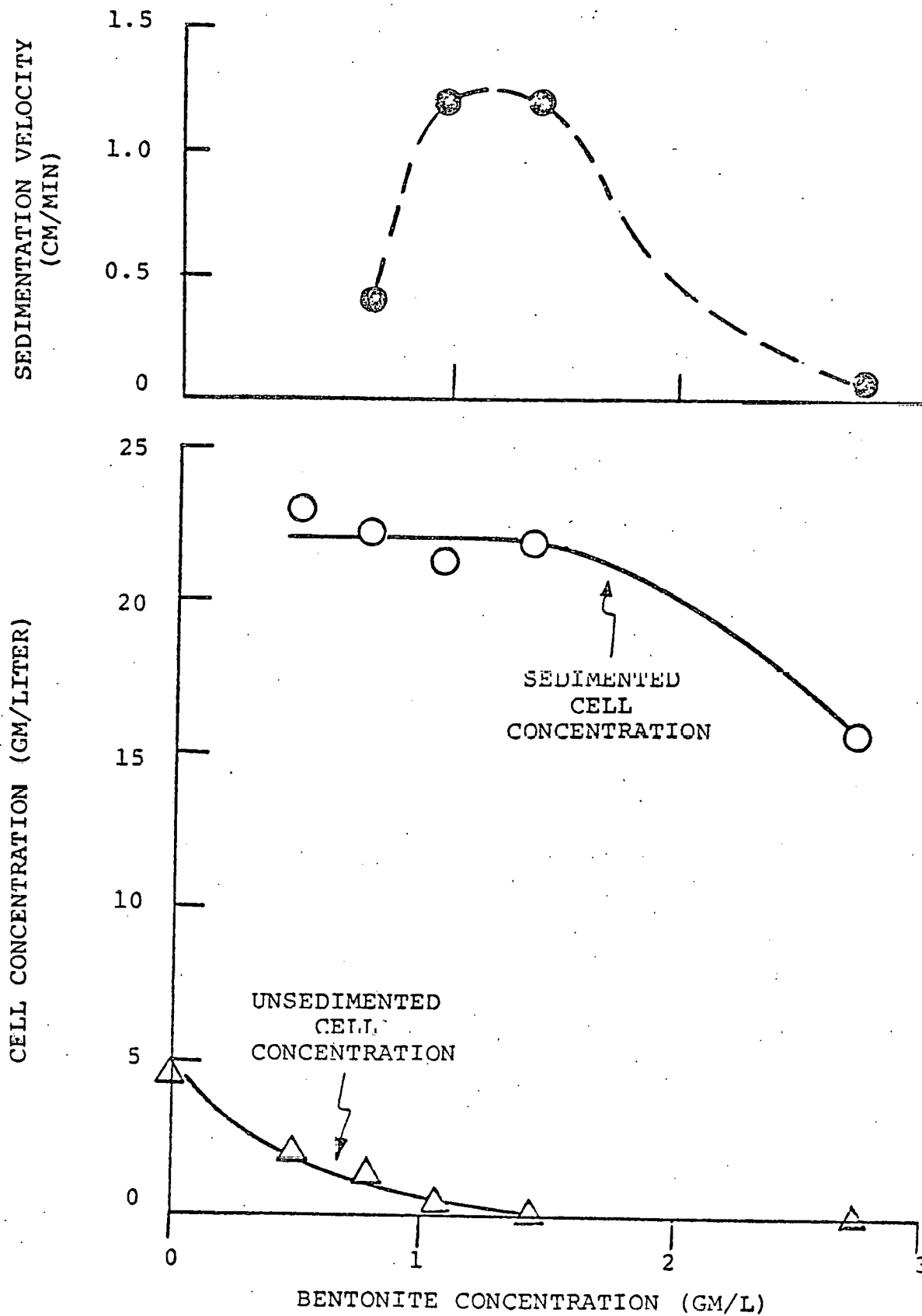


volumetric productivity and specific product formation were calculated to be respectively 0.53 gram acetic per liter per hour and 0.21 gm acetic acid per gram of cell per hour. The specific product formation is slightly lower than that found from batch culture studies. For example, from our previous Progress Report (COO-4198-5, Figure II.C.5) at the same specific growth rate of 0.07 hr^{-1} , the specific product formation rate was found to be 0.28 gm acetic/gm cell-hr. Our explanation for the differences is the following. The data from the batch culture studies were obtained when the acetic acid concentration (15 gm/l) was higher than that in the continuous culture (7.5 gm/l). Since the production of acetic acid is both growth and non-growth associated, one would expect that more energy will be required to maintain the intracellular integrity as the external acetic acid concentration is increased. This increase in the energy utilization, often called "cell maintenance", is achieved through the degradation of glucose to acetic acid where ATP is derived. We therefore hypothesize that, due to a higher acetic acid, more energy was expended corresponding to a higher non-growth associated acid formation. It is the difference in this non-growth associated acetic acid formation rate which reflected the lower specific product formation of our continuous culture study. We therefore predict that the specific product formation rate will be increased when the acetic acid concentration is increased.

It is also envisioned that the continuous process for acetic acid production will use either cell recycle or immobilized resting cells. In both cases, the recovery of cells in an economical way will be required. Since Clostridium thermoaceticum physically is quite small (about one micron in diameter), conventional cell recovery methods (e.g. centrifugation or filtration) will probably be impractical. We have therefore initiated studies on finding flocculating agents which will enhance our cell recovery technique. Our experiments have focused on the use of a mineral hydrocolloid, Bentonite, to flocculate C. thermoaceticum. This agent is selected in view of its low cost (about 2¢/lb) and its known effectiveness to flocculate microorganisms.

The results of our flocculation experiments are shown in Figure II.C.4. Bentonite concentrations up to 3 gm/liter were tested. At approximately 1 to 1.5 gm/liter of Bentonite, the sedimented cell concentration was 22 gm/liter from an initial cell concentration of 5 gm/liter. The supernatants at these flocculant concentrations were essentially void of cells and our calculations show a 99% recovery efficiency. The sedimentation velocity of the flocculated cells at 1 to 1.5 gm/liter of Bentonite was found to be 1.2 cm/minute. The sedimentation velocity of C. thermoaceticum in the absence of flocculation was calculated to be 0.00033 cm/min. These results show a sedimentation enhancement factor of 3700 fold when Bentonite

FIGURE II.C.4: FLOCCULATION OF CLOSTRIDIUM THERMOACETICUM
USING BENTONITE (INITIAL CELL CONC.= 5 GM/L)



is used. We conclude, therefore, from these impressive sedimentation rates that Bentonite will be an excellent agent for the recovery of C. thermoaceticum. We have also examined the potential adverse effect of Bentonite on the growth and acetic acid production by C. thermoaceticum. Bentonite concentrations of 0.5 to 1.0 gm/liter were tested and showed no effect on growth and product formation. We might add that the cells grown in the presence of Bentonite exhibited the same flocculation characteristics upon removal from the fermentor.

The last segment of the past research efforts was devoted to in situ product recovery. Although we have a modest effort in isolating acetate tolerant strains, there does exist a more attractive alternate. This being the production of the free acid and the in situ removal of the product leading to a more economic process. We have focused our efforts on the use of liquid ion exchangers.

We envision that a liquid ion exchanger in the hydrocarbon phase could be incorporated and thus allowing the extraction of acetate and changing the partition coefficient so that the hydrocarbon phase would be favored. From a process point of view, the following is envisioned. Acetic acid is extracted into the hydrocarbon phase with the ion exchanger. The hydrocarbon phase is removed and the acetic acid is recovered by cracking the ionic bond between the acid and the exchanger with heat. For this purpose, a tertiary amine would be required

as the ion exchanger so that the formation of the amide with acetic acid is avoided. The ion exchanger is recombined with hydrocarbon and added back to the fermentor.

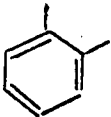
The use of such a system requires compatibility with the organism used to produce acetic acid and that the ionic bond can be broken without excessive amounts of heat.

In the first phase of experimentation, a suitable hydrocarbon phase was selected by evaluating its effect on the growth of C. thermoaceticum. The solvents tested are shown in Table II.C.3. Two things are apparent. Only the long chain alkanes are suitable and the cyclic compounds are better suited than their straight-chained counterparts.

Tridodecylamine (laurylamine) used as the ion exchanger in the first experiments was found to be particularly effective at dissolving the cell walls. Detergency was expected to decrease with the length of the hydrocarbon chain. However, some compromise must be reached because the small chain triamines are very water soluble. Conceivably, trihexylamine or tricyclohexylamine would be suitable for this purpose. As neither of these compounds were available for use, particularly the latter, the compound tribenzylamine was examined. Here, cells in contact with tribenzylamine in either decane or hexadecane were found to grow well.

The next phase involved determining the temperature at which the ionic bond between acetic acid and the

TABLE II.C.3: EFFECT OF ORGANIC SOLVENTS ON THE CELL GROWTH OF C. THERMOCELLUM

<u>Solvent</u>		<u>Cell Growth</u>
Hexane		-
Ligroin	C ₅ -C ₈	-
Octane		-
Cyclooctane		+
Decane		+
Hexadecene		++
o-Xylene		-

amine would thermally crack, to allow the recovery of the acid. With the triamines, the pKa is 10.5. Therefore, tridodecylamine was used for this experiment. The acetic acid was recovered between a temperature of 170°C to 185°C. Subsequent experimentation with pKa reductions to 9.5, in this case quinoline, the acetic acid was recovered at a reduced temperature of 155°C to 170°C.

Although quinoline itself is not a useful compound for the recovery of acetic acid, derivatives of pyridine might be useful since the pKa of pyridine is about 5.0. One potentially useful compound would be 2,4,6 tri-n-hexylpyridine. Furthermore, the compound tricyclohexylamine may also be a useful compound because its extremely hindered steric configuration would show a much lower pKa than the trialkylamines.

4. Future Work

- Evaluation of kinetics using continuous culture.
- Design gravitational sedimentation unit to be used with continuous culture with cell recycle.
- Studies with resting cells, determination of their stability and an examination of the use of polyacrylamine gel entrapment to increase that stability.
- Acetate removal using ion-exchange.

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

Report for Second Quarter

May 1, 1978 through July 31, 1978

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THE U.S. DEPARTMENT OF ENERGY
CONTRACT NO. EG-77-S-02-4198

SCOPE OF PROGRAM

This is a coordinated program to effect the microbiological degradation of cellulosic biomasses and 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 conversion from cellulosic biomasses to liquid fuel (ethanol) and/or soluble sugars by the cellulolytic, anaerobic organism is also within the scope of this program. Process and engineering scale-up, along with economic analyses, will be performed throughout the course of the program.

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

Studies concerning the 'cellobiase', or glucohydrolytic, properties of Clostridium thermocellum have been started in order to determine if the accumulating cellulose degradation end products can be enhanced for glucose (with a subsequent decrease in cellobiose and larger cellodextrins). In non-pH-controlled cellobiose fermentations, glucose accumulation is a non-growth associated event occurring when the pH of the medium is low. When the pH of the medium is controlled, however, no glucose accumulation occurs. Glucose accumulation is both growth and non-growth associated in non-pH-controlled cellulose fermentations. An implication of these preliminary results is that the cells, or the enzyme(s) responsible for converting cellobiose to glucose, may be manipulated environmentally to increase the final yield of glucose from cellulose.

The packed-bed cellulose fermentor continues to show promise using Clostridium thermocellum. Through the use of nitrogen balances, cell growth can now be determined indirectly. It has been found that C. thermocellum adsorbs tightly onto cellulose and in the adsorbed state continues to degrade cellulose and produce soluble sugars, ethanol and acetic acid. Typical degradation of cellulose in the packed-bed fermentor ranges from 60 to 70%.

Detailed characterization of the new strain S-4 of Clostridium thermocellum which has a higher tolerance to ethanol (5% V/V) has been performed. It has been definitely shown that this strain (S-4) is able to metabolize glucose, fructose, cellobiose and cellulose. It is interesting to note that the new isolate (S-4) produces significantly higher ratio of ethanol to acetic acid. Carbon and oxidation-reduction balances have lead us to conclude this strain is deficient in the hydrogenase and thus can produce the high ratio of ethanol to acetic acid. Studies using untreated and alkaline pretreated corn stover show that C. thermocellum has higher cellulolytic degradation rates when the substrate has undergone pretreatment.

A second strain of C. thermocellum (C9) resistant to ethanol has been isolated. This strain, in addition to being resistant to ethanol, is also resistant to n-propanol, n-butanol, butyrate, propionate, lactate and β -hydroxybutyrate, indicating a fundamental change in the response of this strain to a wide variety of chemicals. Studies also confirmed the fact that C9 is a stable mutant and not the result of a phenotypic adaptation.

Preliminary studies have indicated that the regeneration of C. thermocellum protoplasts is possible. Using 5% polyethylene glycol as an osmotic stabilizer, we were able to obtain regeneration of more than 20% of the original number of protoplasts. Research for plaque-forming bacteriophages has been initiated.

Approximately 50% higher cellulase production was observed in GS medium which is a modification of CM-3 medium in which MOPS buffer is used, yeast extract is increased and urea replaces $(\text{NH}_4)_2\text{SO}_4$. In both media, mutant AS-39 produced twice as much enzyme as the parental C. thermocellum ATCC 27405. At the high (0.6%) yeast extract concentration, glucose and fructose (but not mannose or mannitol) were utilized. Cellulase was also produced. Both growth and volumetric cellulase production was about one-half as great on glucose and fructose than on cellobiose. Addition of cysteine to the TNP-CMC assay system increased activity by 65%. Mutagenesis studies have continued.

The assay conditions of cellulases from C. thermocellum have been investigated. Filter paper activity and CMCase activity have been optimized in terms of reaction kinetics and temperature.

The evaluation of different substrates for use by C. thermocellum was pursued. The preliminary studies with paper mill primary sludge as substrate showed no significant growth of C. thermocellum, but the results with cotton shearings are encouraging, with a reducing sugar accumulation over 4 g/l under non-pH-controlled conditions. A major effort was also devoted to the construction and set-up of a continuous culture system using a solids feeder.

Studies on acrylic acid production have continued to proceed vigorously. Two major accomplishments have resulted from research efforts put forth this quarter. The first is that the highest concentration of acrylic acid to date -- 1.2 g acrylic acid/liter -- has been obtained with resting cell preparations of Clostridium propionicum. The addition of lactate to the reactor plus the use of methylene blue as an electron resulted in the high concentration of acrylic acid. The second significant development is that cells of C. propionicum have been immobilized and shown to retain 50% of their biological activity for synthesis of acrylic acid. Because of the extremely encouraging results obtained with C. propionicum the research with E. coli on acrylic acid production is to be terminated. Studies on the direct production of lactate, a precursor to acrylate, from cellulose have proceeded well.

Using mixed cultures of C. thermocellum and a recently isolated thermophilic homo-lactate forming organism, the direct conversion route is being explored.

Resting cell fermentations using Clostridium acetobutylicum have been performed at 30 and 37°C in hope of increasing the butanol concentration. At both temperatures, the final butanol concentration achieved was about 12 g/l. Resting cells during conversion from glucose to the mixed solvent indicate cell lysis. However, the product profiles using resting cells are quite similar to the growing cell fermentations. Conversion efficiencies from glucose to products are also similar to the growing and resting cell fermentations.

The high acetate tolerant strain (S-3) of Clostridium thermoaceticum has undergone further characterizations. This strain is able to produce sodium acetate up to about 70 g/l (i.e. ~50 g/l equivalent acetic acid) at pH of 7 and at 60°C. Resting cells of C. thermoaceticum has definitely demonstrated its non-growth associated mode of acetate formation. Conversion of glucose to acetic acid by resting cells ranges from 0.81 to 0.92 g acetic/g glucose. However, the specific acetate formation rate (g acetic/g cell-hr) of the resting cells is universally proportional to the sodium acetate concentration. At the highest sodium acetate concentration tested (70 g/l), the resting cells are unable to produce acetic acid.

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6

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. The percentage efforts of the Principal and Co-Principal Investigators on this project are shown along with the names of the different participants.

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This progress report acknowledges the contributions by the individuals listed above, as well as the research assistants, post doctoral research associates, and technical assistants associated with this project.

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I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. ENZYME PRODUCTION BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

It has been demonstrated that Clostridium thermocellum (ATCC 27405) is capable of degrading cellulosic materials to hexose (and pentose) mono- and disaccharides, and that these end products accumulate in the broth of non-pH-controlled fermentations. In terms of their general utility as substrates for other microorganisms in subsequent fermentations, the hexose monosaccharides, principally glucose, stand out as the preferred hydrolytic end-products of cellulose degradation. Therefore, a detailed assessment of the β -glucosidic (cellobiose splitting) and glucohydrolytic (cleaving of glucose units from the ends of cellulose chains) properties of C. thermocellum has been started. An objective of this study is to determine the conditions or means by which the conversion of accumulated cellobiose (or celloextrins) to glucose can be enhanced, thereby increasing the final level of accumulated glucose.

The putative model for cellulose hydrolysis by fungal cellulolytic systems (suggested by other investigators) features a complex of extracellular cellulases which degrade cellulose to cellobiose through exo- and endoglucanase activities. The cellobiose then becomes the substrate for a β -glucosidase, also extracellular, which cleaves this disaccharide

into glucose units. Whether such a scheme is applicable for the clostridial cellulase system is presently under investigation.

One component of the clostridial system which is known to be different than that of fungi is its "cellobiase". It is claimed that the cellobiose splitting enzyme of C. thermocellum is an intracellular cellobiose phosphorylase (Alexander, J.K., Methods in Enz., 28, 944, 1970). If this is the only enzyme responsible for the liberation of glucose, then the accumulation of glucose in the medium would be precluded, i.e., the appearance of glucose would remain an intracellular event for intact cells. Yet, during non-pH-controlled fermentations on cellulose, glucose does indeed accumulate.

This observation has prompted several questions: Is the cellobiose phosphorylase truly intracellular? Is cellobiose the only substrate from which glucose can be released or is there a cellulase capable of cleaving glucose units from longer cellodextrins? Does glucose accumulate in cellobiose fermentations? To begin answering these questions, glucose accumulation in fermentations of C. thermocellum on cellulose and cellobiose was measured and a kinetic analysis of the data has been performed.

2. Materials and Methods

a. Growth of Organism

C. thermocellum was cultured in CM3 medium containing 0.5% yeast extract and 1.0% Solka floc or cellobiose.

In one experiment, 10 g/l of sodium bicarbonate was added to the medium (which was reduced by bubbling CO₂). The greater buffering capacity maintains the pH of the medium closer to neutrality.

b. Glucose Measurement

For cellobiose-grown cultures, glucose was measured by the glucostat assay (Worthington Biochemicals), a method based on the use of glucose oxidase specific for glucose. For the cellulose-grown culture, glucose accumulation was followed by HPLC so that cellobiose accumulation could be measured as well. The HPLC methodology has been described in previous progress reports.

c. Cellobiose Measurement

Residual cellobiose in cellobiose-grown cultures was measured by the DNS assay for reducing sugars, using cellobiose to construct the standard curve. When glucose is also present in the broth, the values for residual cellobiose will appear higher, but can be corrected by glucose analysis.

3. Results

In Figures I.A.1 and I.A.2, the kinetics of cell growth (Klett units), glucose accumulation, and substrate utilization for C. thermocellum grown on CM3/cellobiose medium are presented. Figure I.A.1 is a composite of data from two fermentations A & B, and an uninoculated control, which were

FIGURE I.A.1: KINETICS OF *C. THERMOCELLUM* GROWN ON CM3/CELLOBIOSE MEDIUM (FERMENTATIONS A AND B)

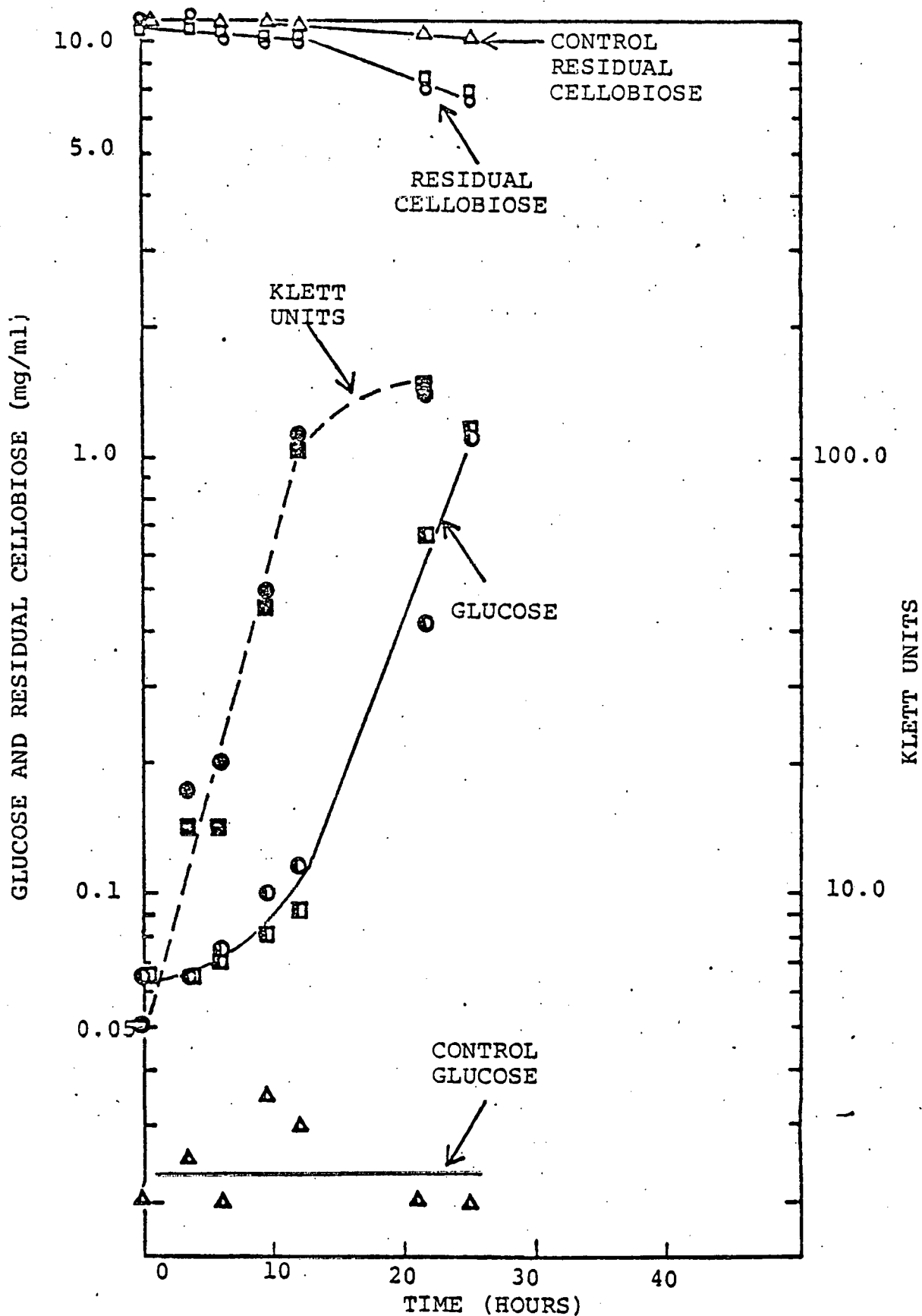
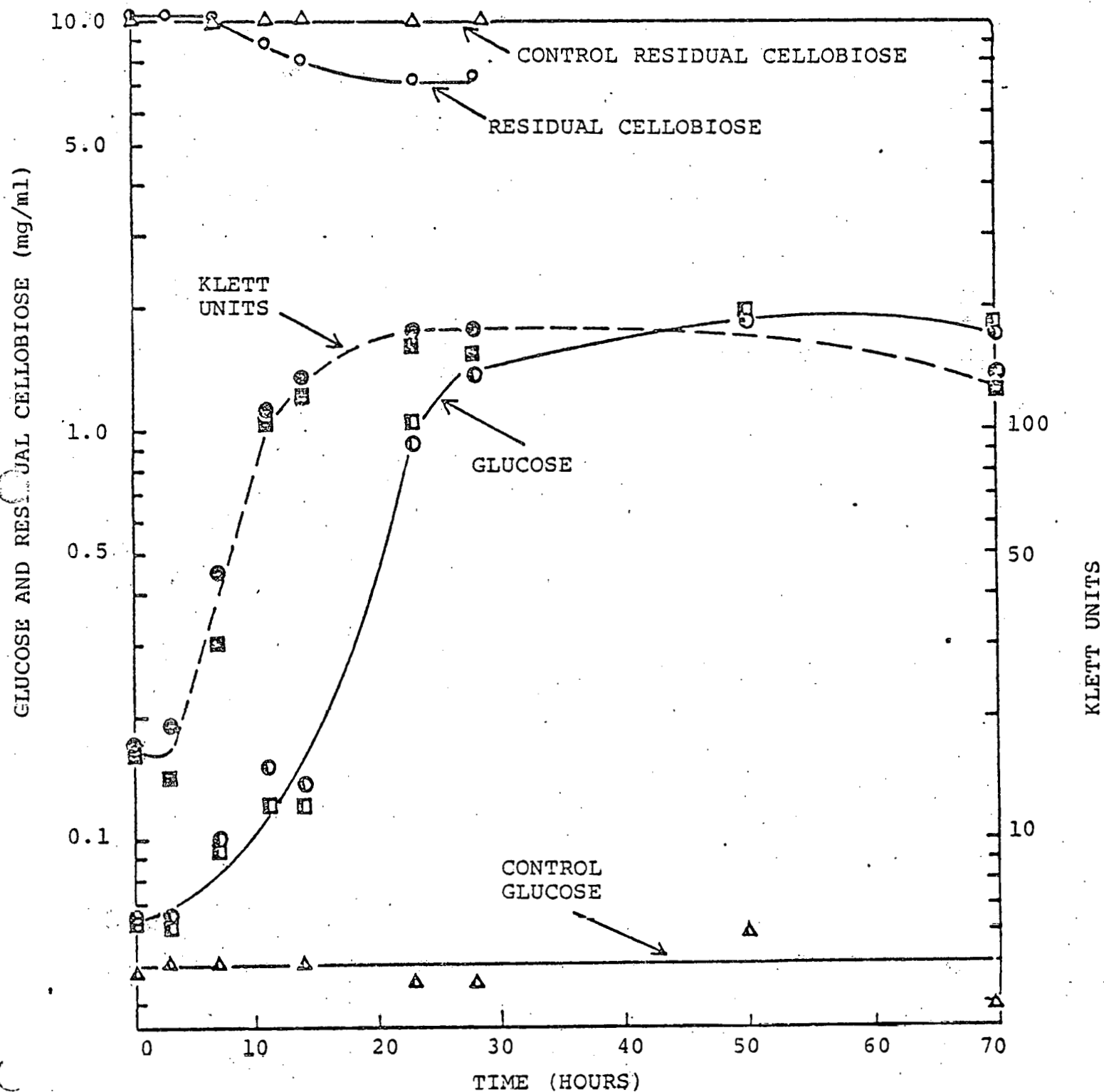


FIGURE I.A.2: KINETICS OF *C. THERMOCELLUM* GROWN ON CM3/CELLOBIOSE MEDIUM (FERMENTATIONS D AND E)



terminated after 25 hours of incubation at 60°C. Similarly, Figure I.A.2 is a composite of data from fermentations D & E, and their uninoculated control, which were run under the same conditions, but were allowed to incubate for 70 hours. In these non-pH-controlled fermentations, very little cellobiose is consumed, with 7 g/l remaining at the end of the fermentation. (Due to the presence of glucose, however, ~6 g/l of residual cellobiose would be a more accurate figure.) Glucose has accumulated to 1 g/l after ~25 hrs and at 50 hours, as seen in Figure I.A.2, the glucose concentration is approximately two grams per liter. It is apparent from these figures that the rise in glucose occurs most rapidly after culture turbidity measurements have begun to taper off. This will be discussed in more detail below.

Figure I.A.3 depicts the kinetics of two cellobiose fermentations (G and H) to which 10 g/l of sodium bicarbonate was added to buffer more closely to pH 7.0 throughout the fermentation (see Figure I.A.7 for pH profile). The results are different from those in Figures I.A.1 and I.A.2. Only 1 g/l of residual cellobiose remained at the end of the fermentation. Very little glucose accumulated (~0.04 g/l at 25 hrs) and it eventually disappeared.

Finally, the kinetics of various fermentation parameters from a cellulose-grown culture are shown in Figures I.A.4 and I.A.5. In Figure I.A.5, total reducing sugars were

FIGURE I.A.3: KINETICS OF *C. THERMOCELLUM* GROWN ON CM3/CELLOBIOSE MEDIUM, CONTAINING 10 g/l SODIUM BICARBONATE (FERMENTATIONS G AND H)

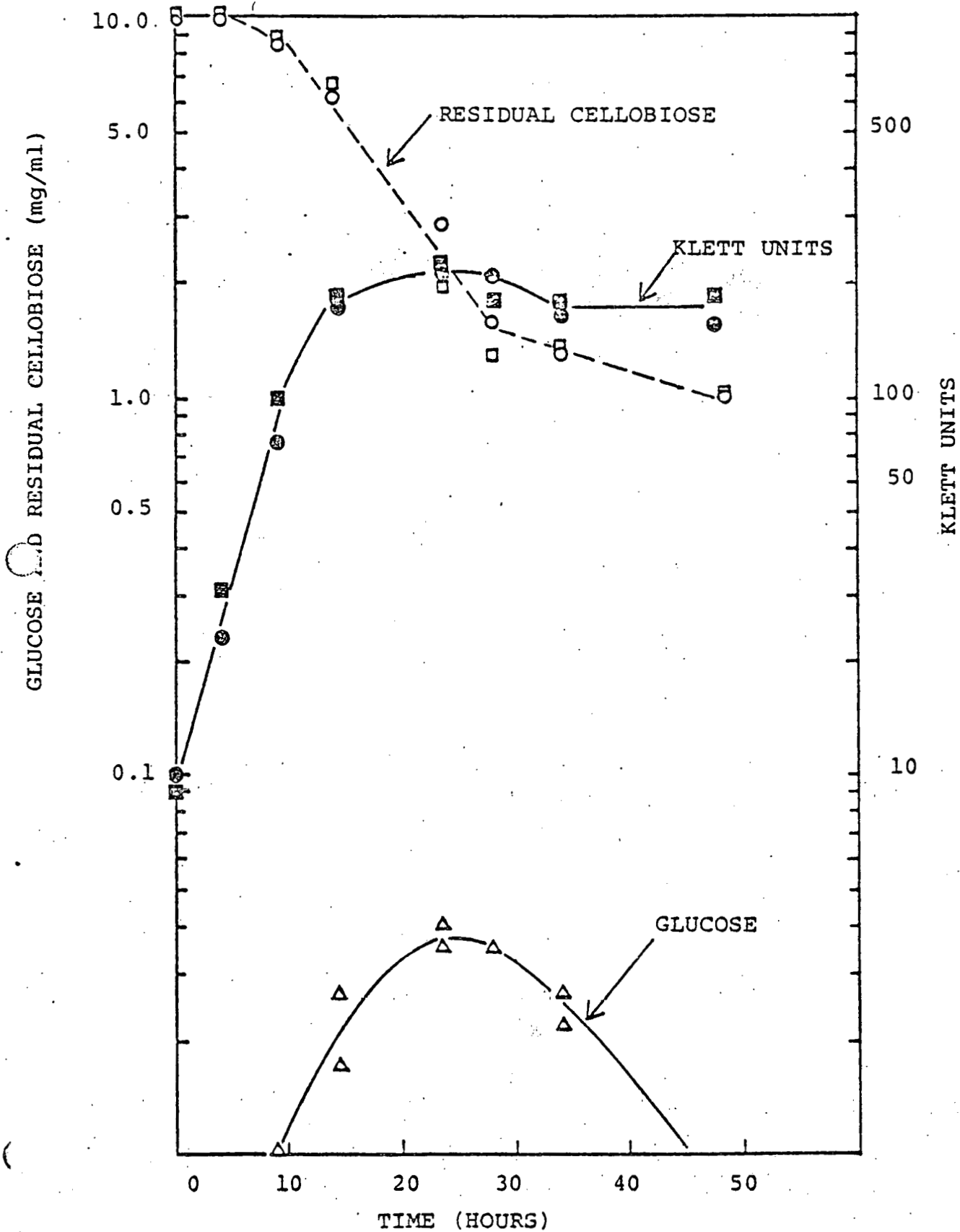


FIGURE I.A.4: KINETICS OF C. THERMOCELLUM GROWN ON CM3/
SOLKA FLOC MEDIUM (CELLULOSE FERMENTATION)

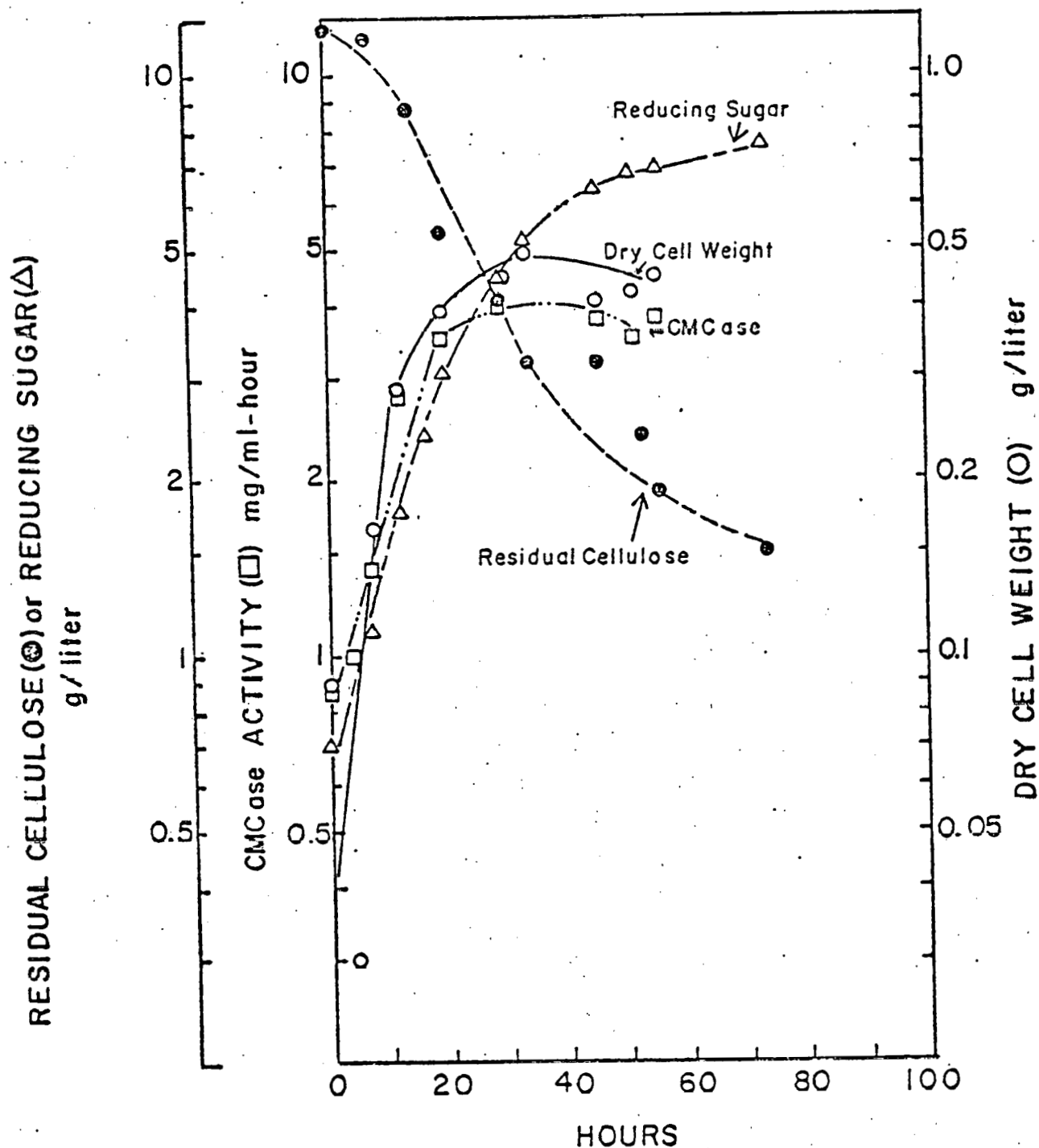
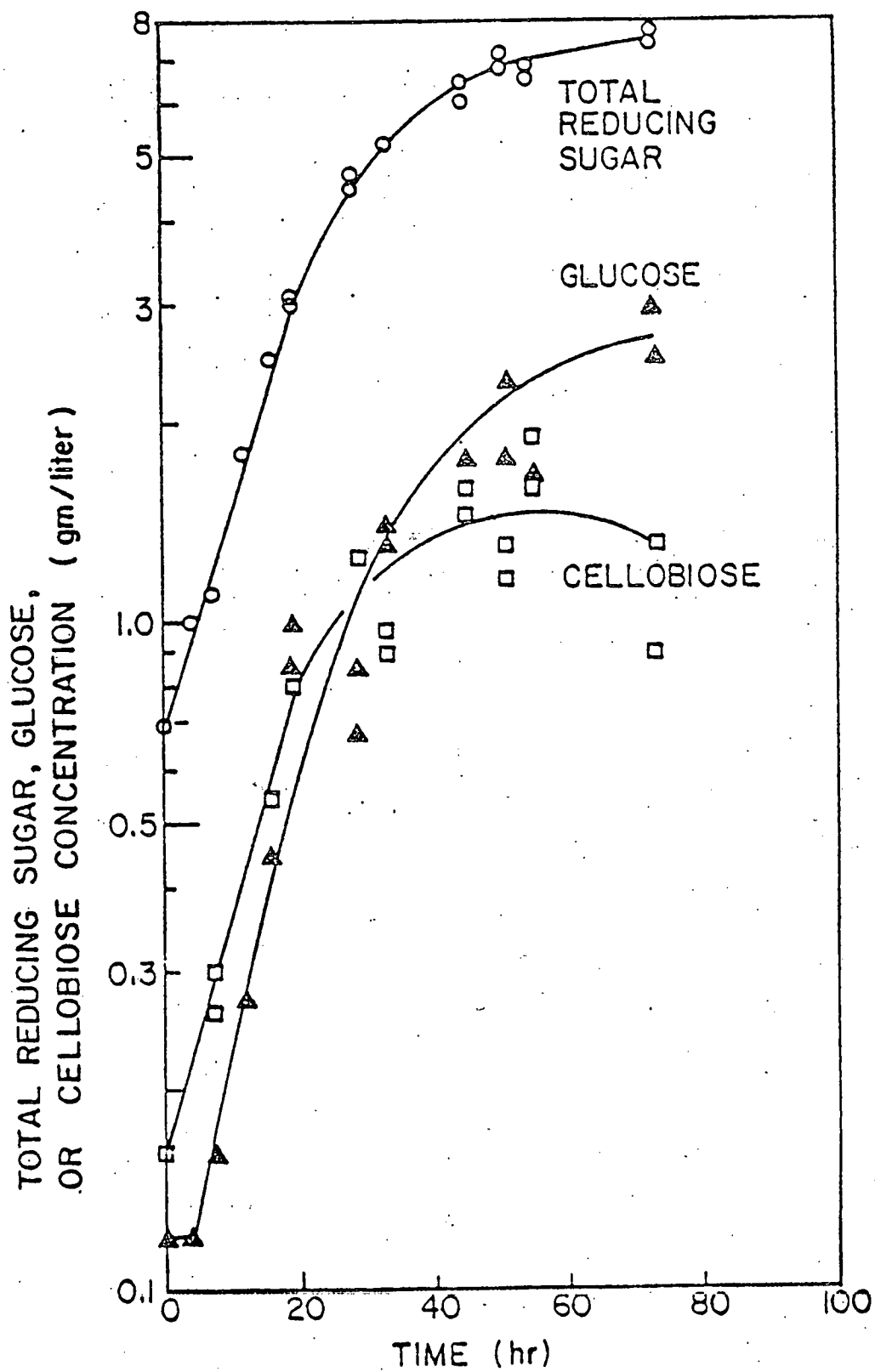


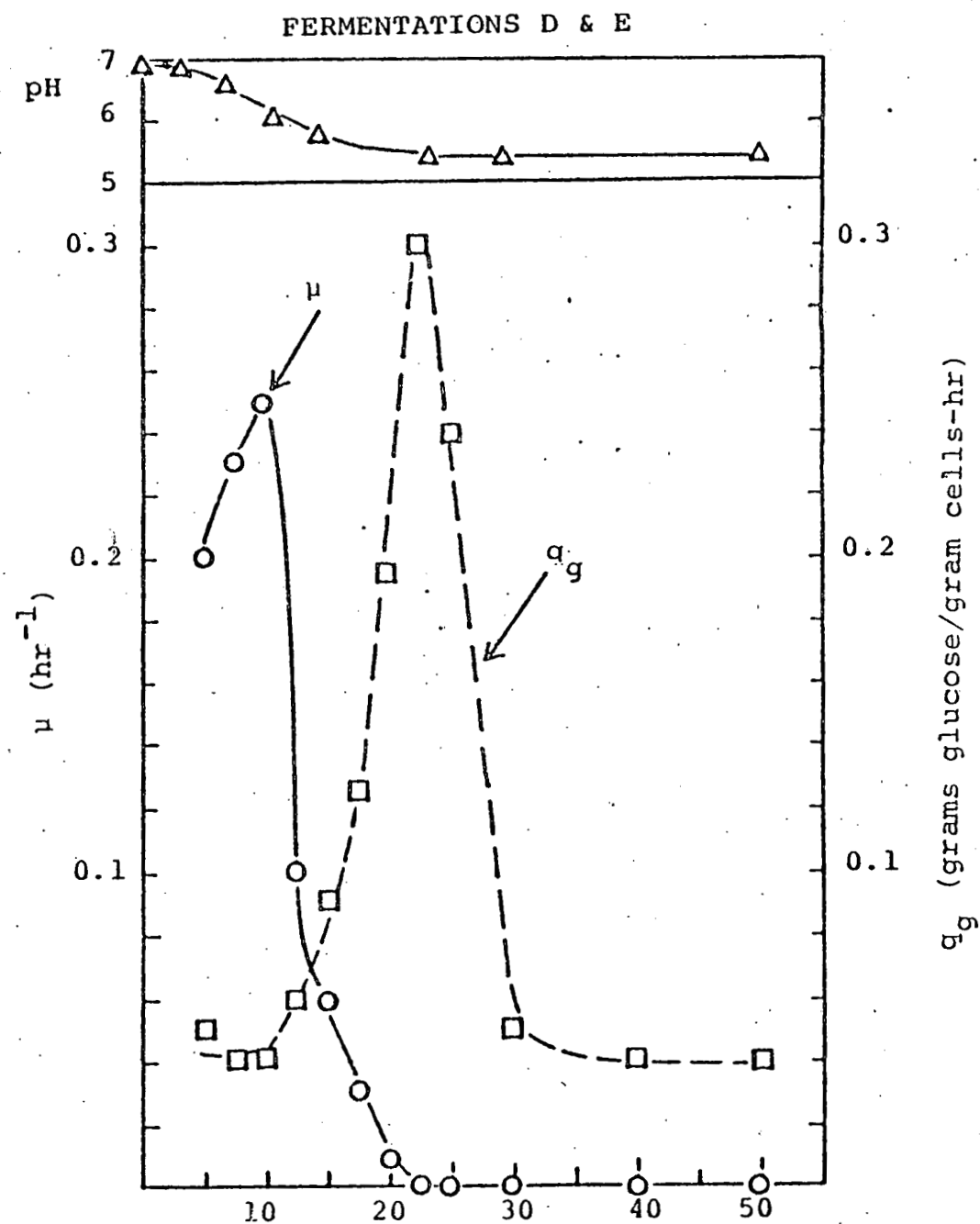
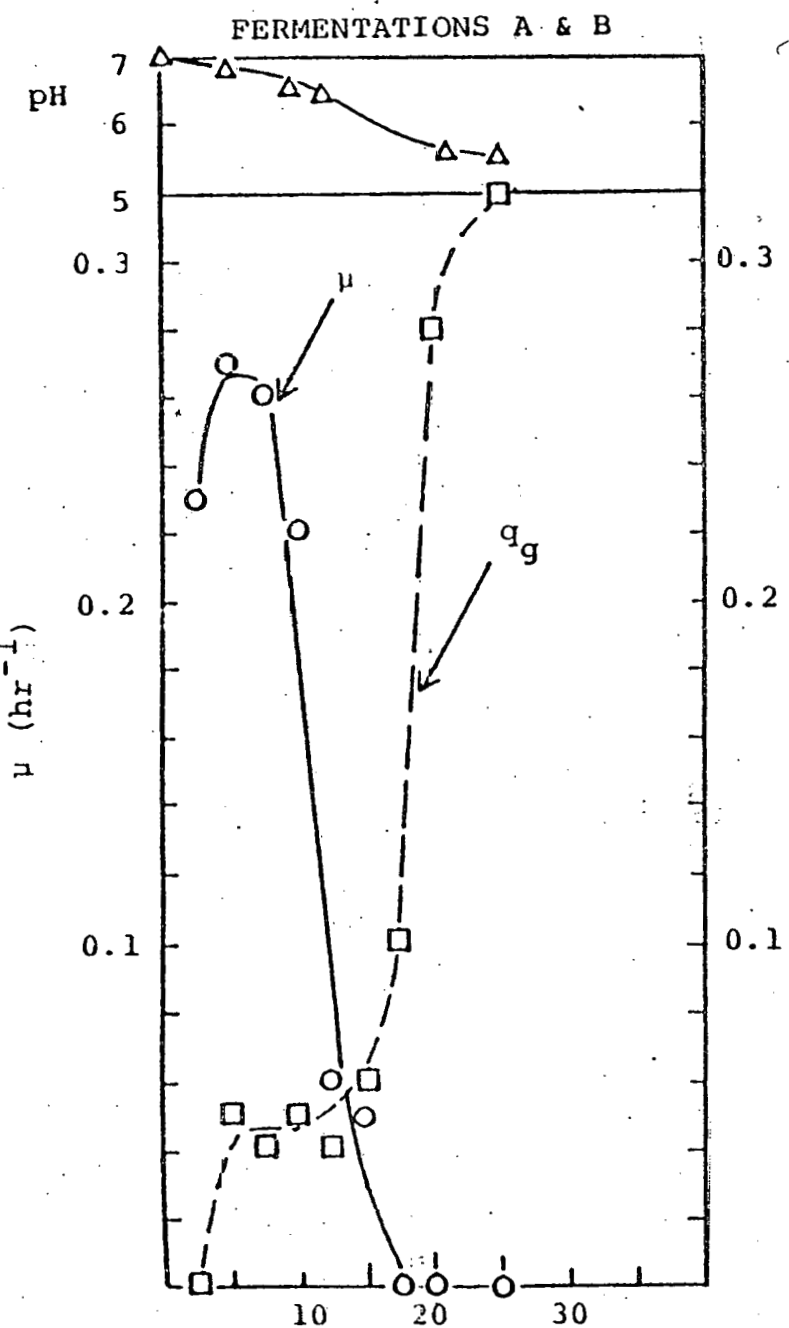
FIGURE I.A.5: SUGAR ACCUMULATION DURING CELLULOSE FERMENTATION BY C. THERMOCELLUM



determined by the DNS assay while the curves for glucose and cellobiose accumulation were generated from HPLC data. What is most noteworthy about Figure I.A.4 are the changes that have occurred in the kinetic behavior of C. thermocellum since we began working with it a year ago (see Figure I.A.9 in Progress Report COO-4198-1). Maximum growth rates and volumetric rates of cellulose degradation have more than doubled, fermentation times are much shorter, and a significant amount of cellulose is degraded to reducing sugars even after the active growth phase has been completed. As shown in Figure I.A.5, glucose and cellobiose are the major sugars that accumulate. Their accumulation begins early in the fermentation, with glucose concentrations eventually exceeding the cellobiose concentration.

The data from the curves in Figures I.A.1-5 were taken and were subjected to the analysis presented in Figures I.A.6 and I.A.7. In Figure I.A.6, the specific growth rate (μ , hr^{-1}) and the specific glucose accumulation rate (q_g , gm glucose per gm cells per hour) for the cellobiose fermentations A, B, D and E, are plotted versus time during the fermentation. (Dry cell weight was calculated from the observed conversion, 100 Klett units = 0.3 g/l cells.) Also shown are the combined pH profiles from the fermentations. It is readily apparent that the maximum specific growth rate and the maximum specific glucose accumulation rate occur at distinctly different times

FIGURE I.A.6: SPECIFIC GROWTH RATE, SPECIFIC GLUCOSE ACCUMULATION RATE AND pH PROFILES VS. TIME FOR FERMENTATIONS A, B, D AND E

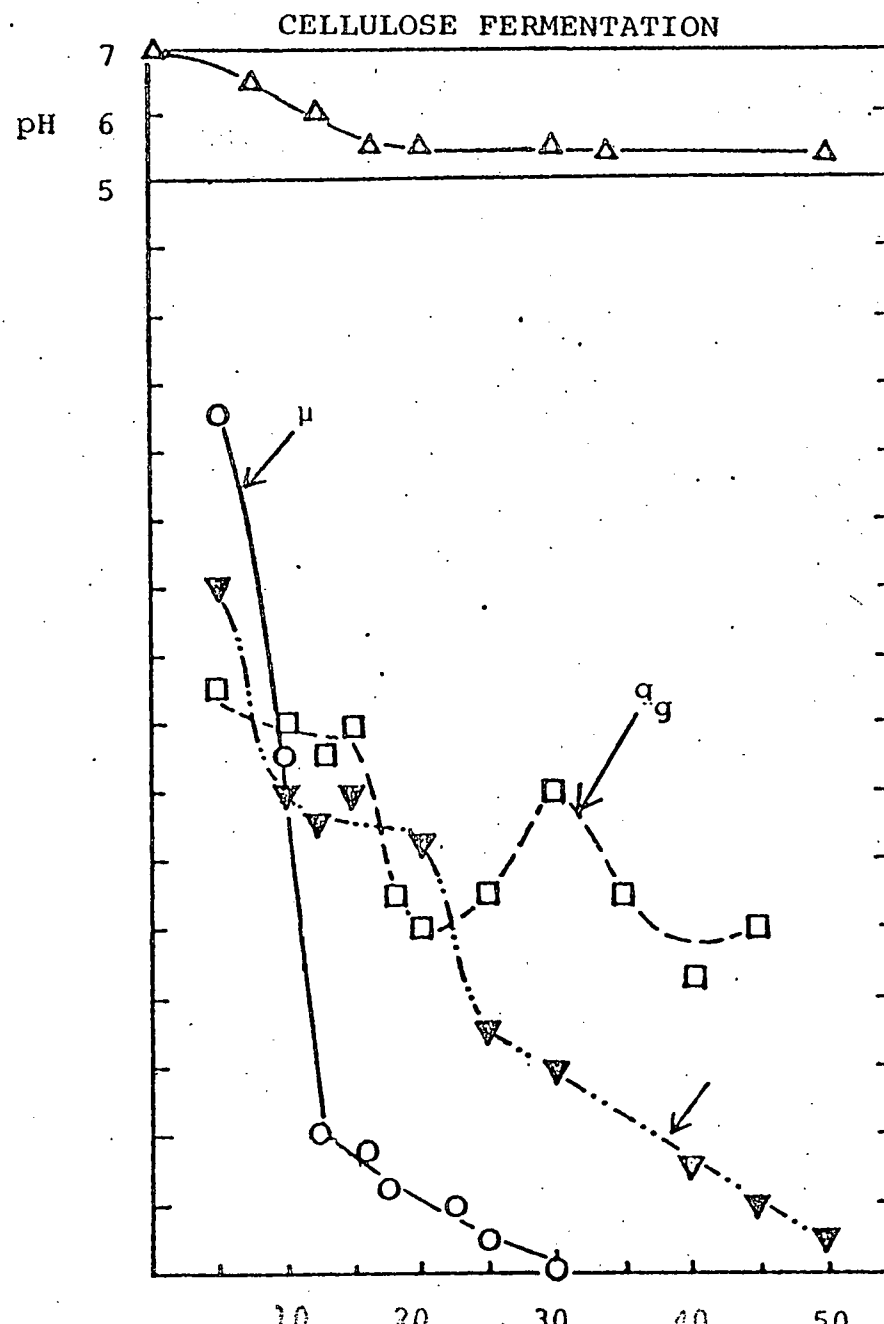
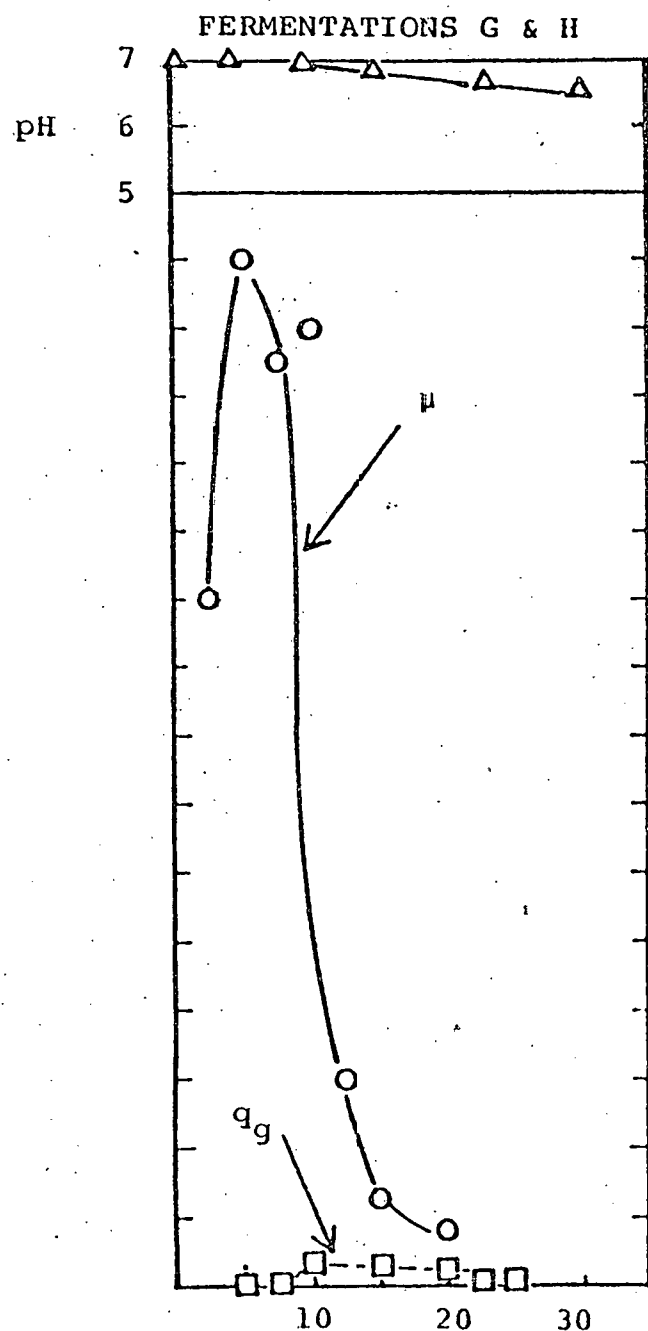


during the fermentation. Whereas the maximum specific growth rate occurs between the 5th and 10th hours, the specific glucose accumulation rate reaches a maximum value between the 20th and 25th hours, a time when the pH has dropped to its minimum value of 5.4. The accumulation of glucose in these cellobiose-grown cultures thus appears to be a non-growth associated event.

In Figure I.A.7, the situation is strikingly different. Here, the plots of μ and q_g vs. time for the fermentation on cellobiose with added bicarbonate and μ , q_g and q_c (gm cellobiose per gm cells per hour) vs. time for the cellulose fermentation are shown. In the more buffered (pH ~7) fermentation there is virtually no glucose accumulation. In the cellulose fermentation, glucose accumulation appears to be both growth and non-growth associated, and even seems to go through two distinct phases, i.e. before and after 20 hrs. The maximum specific glucose accumulation rate for the cellulose fermentation is lower (by roughly 1/2) than observed in the cellobiose (with no bicarbonate) fermentation although the rate is more consistent throughout the cellulose fermentation and still remained at 0.1 g glucose/g cells-hr when the culture was terminated. The specific cellobiose accumulation rate is maximum when μ is highest and declines thereafter. It continues to decline as q_g goes through its second peak.

Before proceeding with the discussion of these results, it might also be mentioned that samples from the cellobiose

FIGURE I.A.7: SPECIFIC GROWTH RATE, SPECIFIC GLUCOSE AND CELLOBIOSE ACCUMULATION RATE, AND pH PROFILES VS. TIME FOR FERMENTATIONS G, H, AND CELLULOSE FERMENTATION



fermentation were run on the HPLC to see if any cellodextrins larger than cellobiose were present (since C. thermocellum is claimed to possess a cellodextrin phosphorylase with synthetic capabilities (Alexander, J.K., Methods in Enz. 28, 944, 1970)). No cellodextrins could be detected, but this may be as much a reflection of the insensitivity of the refractometer (less than 0.25 g/l of cellotriose, etc., would not be detected) as it is an indication of the actual absence of such compounds.

4. Discussion

These results raise several questions:

- a. If the cellobiose splitting enzyme is intracellular, why does glucose accumulate extracellularly in non-pH-controlled fermentations?
- b. Why doesn't glucose accumulate in cellobiose fermentations in which the pH remains neutral?
- c. In cellulose cultures, why is there both growth- and non-growth related accumulation of glucose?

The physical integrity of the cell or cell surface under non-pH- and pH-controlled conditions may perhaps provide an answer to the first two questions. One plausible explanation is that the acid conditions of the non-pH-controlled cultures are responsible for cell lysis; thus the intracellular enzyme becomes released at the end of the fermentation (when the pH drops) and is free to cleave cellobiose to glucose extracellularly (until it is finally denatured causing the glucose

accumulation rate to drop). However, the culture turbidity measurements do not indicate significant autolysis, although growth and lysis could have been occurring simultaneously. An alternative explanation is that the cellobiose phosphorylase, rather than being intracellular, is membrane or cell-wall bound. The acidic conditions may affect the cell surface in such a way that, even though lysis does not occur, the cell is more "leaky" or permeable. Thus, glucose (and/or cellobiose) which should have been transported into the cell remains or leaks outside the cell and accumulates. (This presumes that a membrane-bound cellobiose phosphorylase may play a role in sugar transport.) Another possibility is that an extracellular cellobiase is produced that is highly pH dependent (around pH 5.5), but not very stable.

When the pH remains around neutrality, on the other hand, the cells or cell membrane may remain intact. Thus, the intracellular cellobiose phosphorylase or the products of a membrane-bound cellobiose phosphorylase would no longer leak out of the cell. Consequently, we would see no glucose accumulation. Alternatively, the neutral pH may favor growth and cellobiose consumption for other reasons, resulting in a lack of cellobiose as substrate for the cellobiase at the time during the fermentation its activity is usually observed.

That glucose accumulation appears to be growth-associated (as well as non-growth associated) during cellulose fermentation

may be explained in the following way. The glucose accumulating prior to 20 hours (the time when little glucose is observed in cellobiose cultures) may be cleaved from a substrate other than cellobiose. In other words, it is likely that the extracellular cellulase is cleaving glucose units off larger cellodextrins (either by a random or specific exoglucohydrolytic action). These larger cellodextrin substrates were not available in the cellobiose-grown cultures and thus we did not observe a growth related glucose accumulation. The glucose accumulation after 20 hours probably results from further cellodextrin hydrolysis and also cellobiose hydrolysis. Indeed the peak in q_g at 30 hours is similar to the observed peak for q_g in the cellobiose fermentation. Another bit of evidence to argue in favor of conversion of cellobiose to glucose at this time is the declining rate in q_c while glucose continues to accumulate.

The above explanations for glucose accumulation by C. thermocellum are only speculative and remain to be proven. Future work will include the following: experiments to determine whether an intentional disruption of the cell or damage to the cell surface will increase the conversion of cellobiose to glucose, as well as experiments in which the extracellular protein in cellobiose cultures is measured to determine whether there is an increase at the time of maximal glucose accumulation. This should help to clarify whether autolysis or a release of protein is occurring.

B. Degradation of Cellulosic Biomass to Produce Soluble Reducing Sugars by C. thermocellum.

1. Packed-Bed Fermentor

a. Introduction

In any industrial process for the production of useful products from biomass, the ability to operate at high cellulose concentration is quite essential. Concentrated cellulose slurries are highly viscous, difficult to pump and difficult to mix in conventional agitated fermentors.

In the previous progress report (COO-4198-6) we have demonstrated the feasibility of using a packed-bed fermentor in which the cellulose forms the stationary phase. In this manner, high concentration of cellulose can be maintained without resorting to mechanically feeding during the course of fermentation. The results indicated that the packed-bed serves as an excellent collector where inherent cell recycle can be achieved. However, cell accumulation could not be measured during the course of fermentation without destructively terminating the fermentation.

In the present report we have used nitrogen balances as an indirect and non-destructive method for the measurement of cell mass. Studies in a stirred tank fermentor have shown that cell mass can be estimated from the nitrogen - Kjeldahl analysis of supernatants. Consequently, cell mass accumulation in a packed-bed fermentation could be determined.

b. Materials and Methods

The organism used, culture conditions and the analytical techniques were described in a previous report.

Modified packed bed fermentor

The modified packed-bed fermentor was similar to the packed bed fermentor described in a previous report. The reactor is composed of two sections and the lower part was a glass cylinder (450 ml volume), which has a capacity to hold 50 g of dry cellulose (solka floc). A glass tube inlet was located symmetrically at the bottom of this cylinder. The upper part and the lower part were connected through a ground glass joint (60/71). In the upper part, the outlets for broth and gases were located. A stainless steel screen (mesh 12) was used to retain the cellulose tightly packed in the lower part of the fermentor.

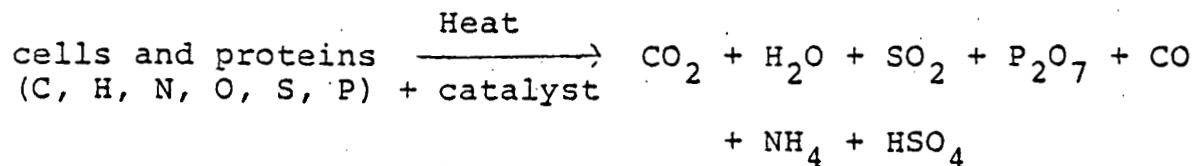
Spectrophotometric determination of total nitrogen

The determination of total nitrogen was performed on micro-Kjeldahl digests of sample as described by Kjeldahl (1883) and Munro (1969).

The organic matter in the sample is oxidized by heating with refluxing sulfuric acid and added catalysts to speed up oxidation.

Kjeldahl, J. Z. Anal. Chem. 1883, 22, 366.

Mammalian Protein Metabolism, Ed. H.N. Munro, Academic Press 1969, Vol. III, Ch. 30.



The ammonia in the digest is then reacted with sodium hypochlorite to form chloramine, which is then reacted with phenol to form indophenol, a blue product which is measured spectrophotometrically. The intensity of the color is proportional to the quantity of ammonia in the digest.

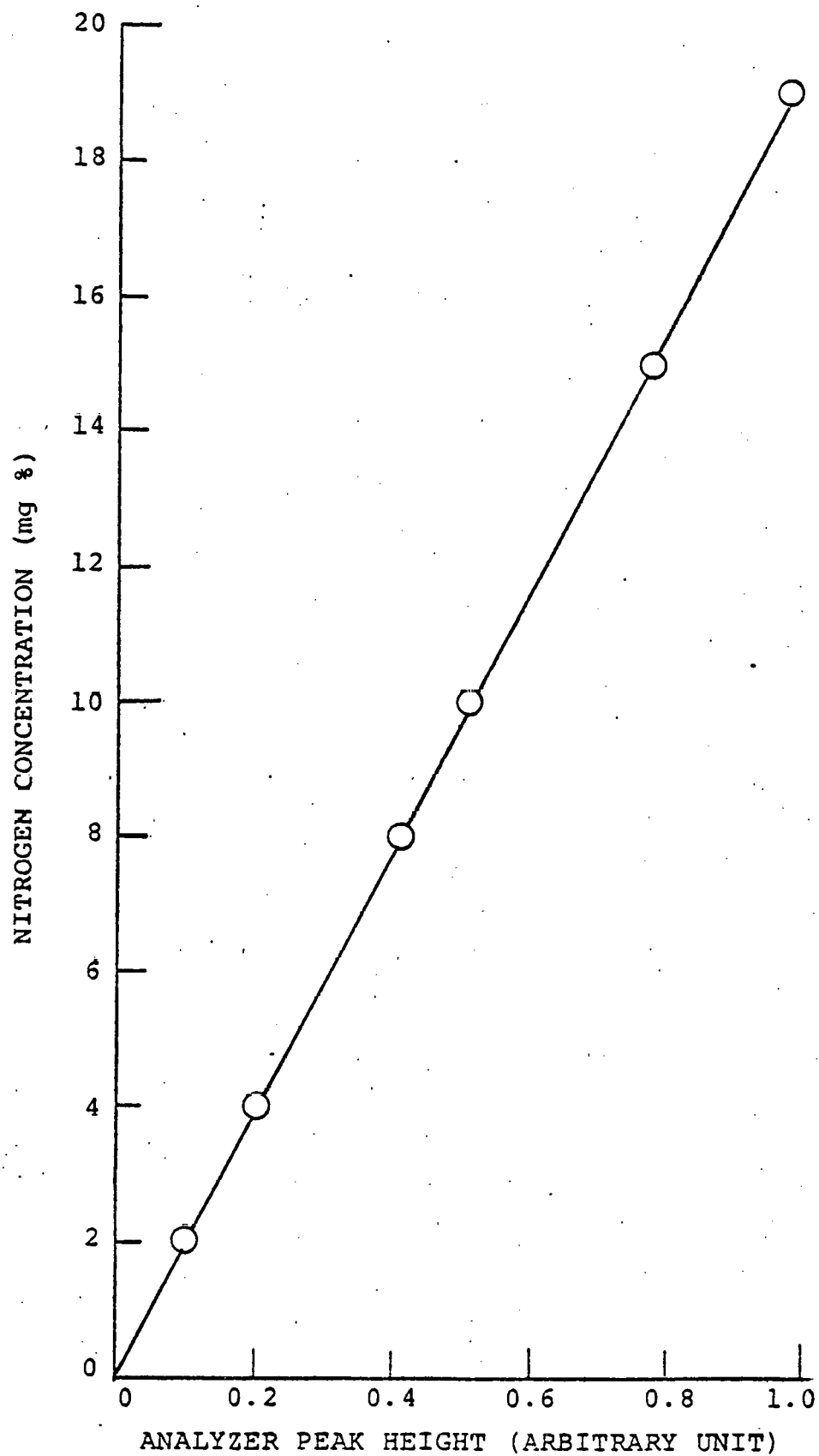
The calibration curve for the color reaction is presented in Figure I.B.1.1. The reaction is automatically processed on an enzyme analyzer (Technicon). The recorder readout is correlated to nitrogen content in mg per cent. The calibration is linear in the range of 0.5 mg% to 20 mg% nitrogen using $(\text{NH}_4)_2\text{SO}_4$ as standard.

c. Results and Discussion

Comparison of methods for the estimation of cell mass in a stirred-tank fermentation of cellulose.

Previous results have indicated that the estimation of cell mass by Klett measurements of the filtered broth could be misleading. It was observed that in some packed-bed fermentations that the concentration of cells in the circulation loop fell to zero; yet the cells seemed to grow continuously on the packed cellulose. These cells produced gas, reducing sugars and acid. In stirred tank fermentations, when

FIGURE I.B.1.1: CALIBRATION CURVE OF NITROGEN DETERMINATION



the culture approached the stationary phase, Klett measurements of filtered broth declined sharply, which could be interpreted as a rapid cell lysis.

It was postulated that when cells approach stationary phase they become firmly attached to the cellulose and are easily retained on the cellulose during filtration prior to the optical density measurements. To obtain a quantitative picture as to the fate of the cells during fermentation a careful examination of the cell mass accumulation was performed. The following question was asked: Is the decrease in Klett readings due to rapid lysis of cells or due to a change in the binding of cells to cellulose? Another important issue is the cell accumulation during fermentation in a packed-bed cellulose fermentor. The only direct method for measuring cell mass is to separate the cellulose from the cells. This requires the termination of the fermentation and cannot be monitored in a continuous fashion during fermentation. The alternative is an indirect method for the determination of cell mass. Nitrogen balance seems a plausible method. Nitrogen is present in the medium in the yeast extract and as ammonia. It is converted to cell mass and soluble proteins. Nitrogen is not converted into gaseous products and therefore appears to be a reliable approach to obtain an overall balance. The reliability of nitrogen balance for determination of cells mass could be

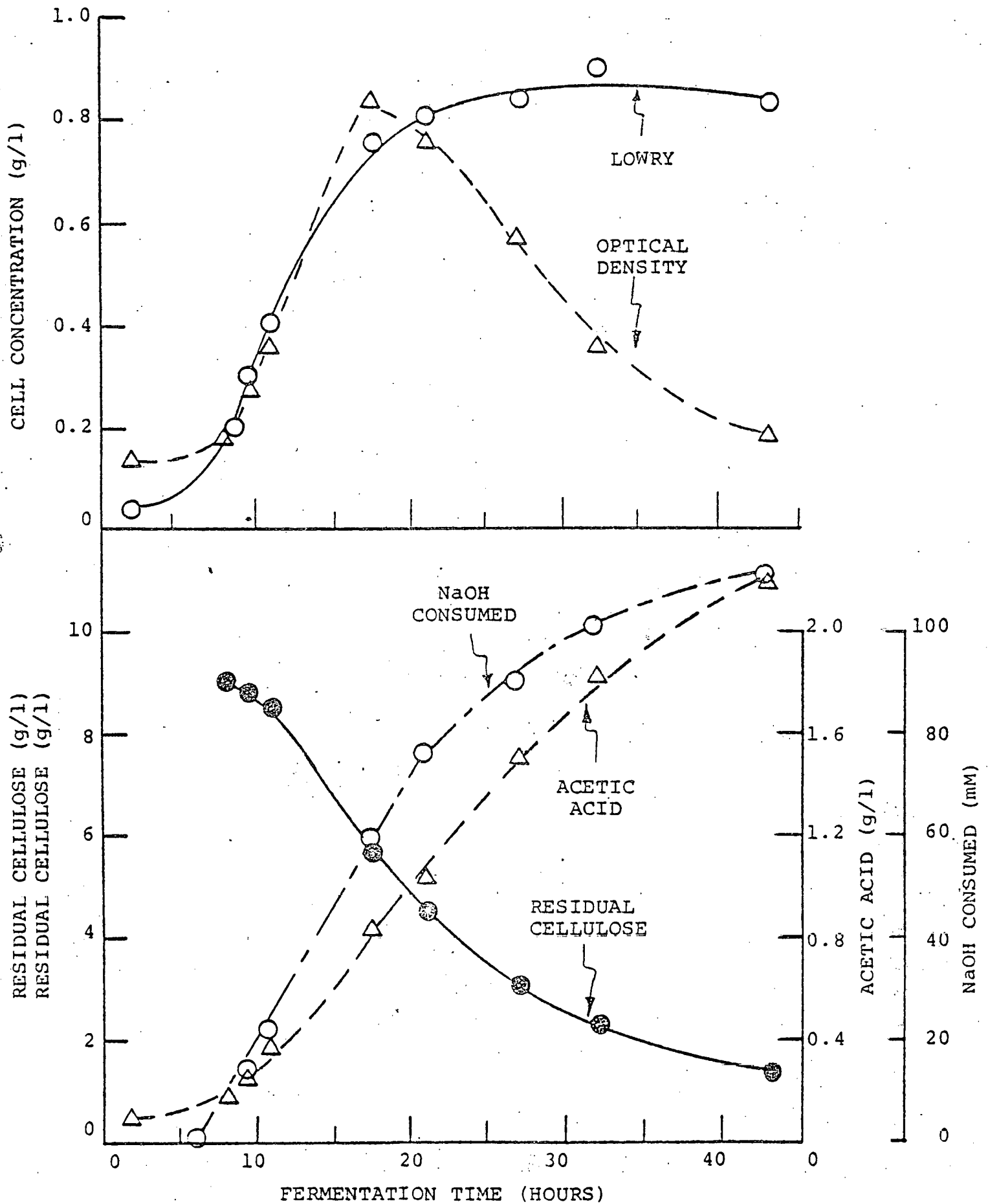
tested in a stirred tank fermentation where removal of homogeneous samples is possible.

Fermentation was carried out in a 5 liter fermentor having a working volume of 3.5 liter. The medium used was CM3 with 9 g/l finely ground Solka-floc type FCC. Throughout the fermentation the pH was controlled at 6.8 by the addition of NaOH. Samples were removed and analysed as follows. Supernatants were obtained by centrifuging the broths for 3 minutes at 10,000 g. Supernatants were used for determination of total nitrogen by Kjeldahl, reducing sugars by DNS and acetic acid concentration by gas chromatography. Precipitates from the above were washed with equal volume of phosphate buffer-saline and collected again by centrifugation. The washing was repeated. The washed pellets were used for total nitrogen and for Lowry protein assay. The Lowry assay required the destruction of cellulose material by boiling for 15 minutes in 0.2 m NaOH.

In Figure I.B.1.2 the results of the fermentation are presented. About 40 hours after inoculation initiation of growth occurs. There is a rapid decrease in the cellulose concentration. Within 43 hours, 7.7 g of cellulose are utilized representing 85% degradation.

In the first 10 - 15 hours there is a rapid production of acetic acid, reducing sugars (not shown), and consumption of NaOH. Cell mass calculated from optical

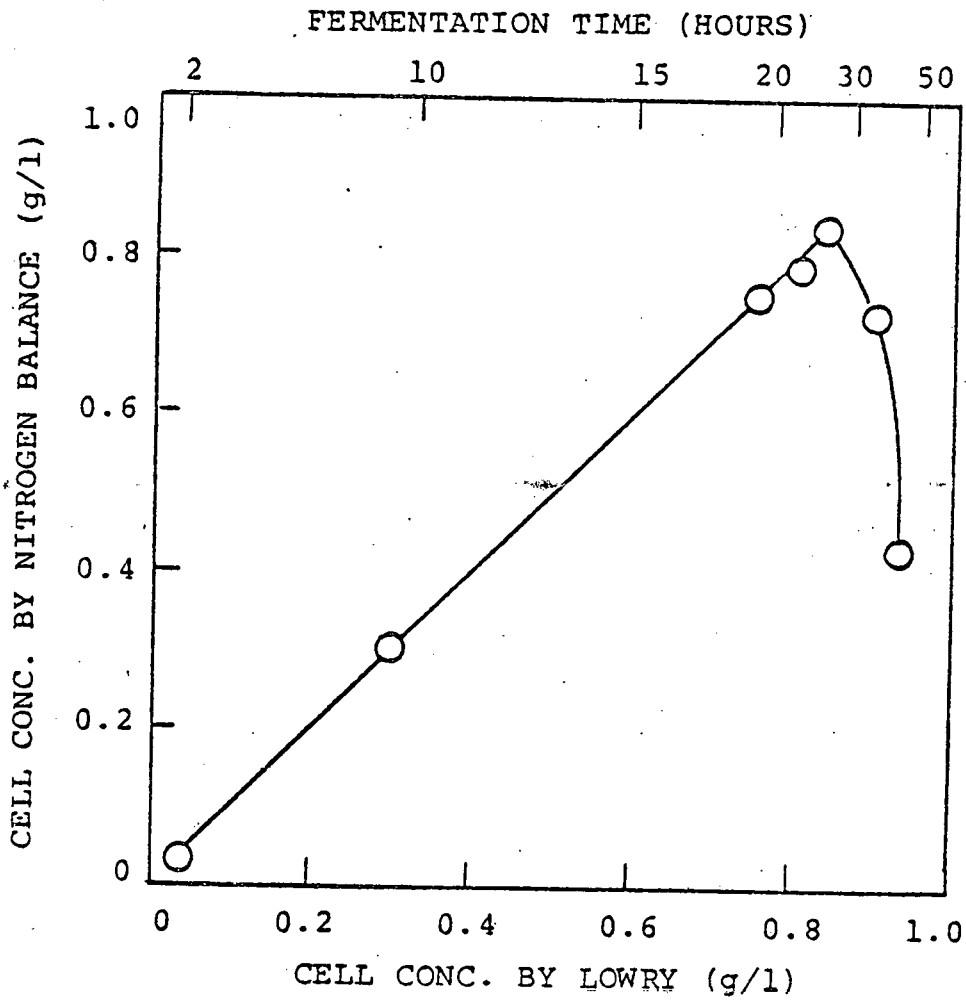
FIGURE I.B.1.2: FERMENTATION PROFILE OF *C. THERMOCELLUM* GROWN ON SOLKA FLOC IN AGITATED FERMENTOR



density (Klett) readings as well as Lowry protein of the precipitates increased in a parallel fashion up to 17 hours. As the fermentation progresses, the two techniques for monitoring cell growth deviate markedly. Cell mass by protein analysis of the precipitate increases until the 25th hour of fermentation and then remains essentially constant until the end of the fermentation. On the other hand the Klett readings decrease rapidly and after 43 hours they are only marginally higher than at the beginning of fermentation. These results are interpreted as being that C. thermocellum undergoes critical changes during the course of the fermentation. As the culture approaches the stationary phase, cells become strongly attached to the residual cellulose fibers. Cellular activity of the adsorbed cell is still evident since cellulose continues to be degraded and products are produced. The level of cellular mass in the system remains essentially constant for a long period of time (20 hours), with no net growth of the cells nor cell lysis as indicated by Klett measurements. We further conclude that optical measurements of cell growth could be quite misleading.

The correlation between dry cell weight calculated from Lowry protein assay and calculated by nitrogen balance is presented in Figure I.B.1.3. The protein content of the bacterium have been determined twice on a cellobiose grown culture and was found to be 60%. Nitrogen content of C. thermocellum was estimated at 12%.

FIGURE I.B.1.3: COMPARISON OF CELL CONCENTRATION DETERMINATION
BY DIFFERENT METHODS OF C. THERMOCELLUM
GROWN ON SOLKA FLOC



The results are in good agreement until the 27th hour of fermentation. During later times there is a marked decrease in the values obtained by nitrogen balance. The values presented in Figure I.B.1.3 were calculated from the decrease of nitrogen in the medium, during the course of fermentation. The discrepancy after the 27th hour could be explained in view of the results presented in Table I.B.1.1.

Table I.B.1.1

Nitrogen Kjeldahl Analysis on
Supernatants and on Precipitates

<u>Fermentation</u> <u>Time (hours)</u>	<u>Dry Cell Weight</u> <u>(g/l) Calculated</u> <u>from Supern.</u>	<u>Dry Cell Weight</u> <u>(g/l) Measured ac-</u> <u>tually on perc.</u>	<u>% Deviation</u>
21	0.783	0.792	1.1%
27	0.842	0.825	2.0%
44	0.425	0.675	59%

We have observed that towards the end of fermentation there is excretion of proteins into the medium (due to lysis of cells or excretion of enzymes). Those proteins present a source of nitrogen that interferes with the calculation of cell mass. These proteins can be precipitated with 6% TCA with an improvement of the correlation for later times in the fermentation. It is concluded that nitrogen-balance is a reliable indirect method for the determination of cell mass accumulation: provided soluble proteins could be removed especially towards the end of fermentation.

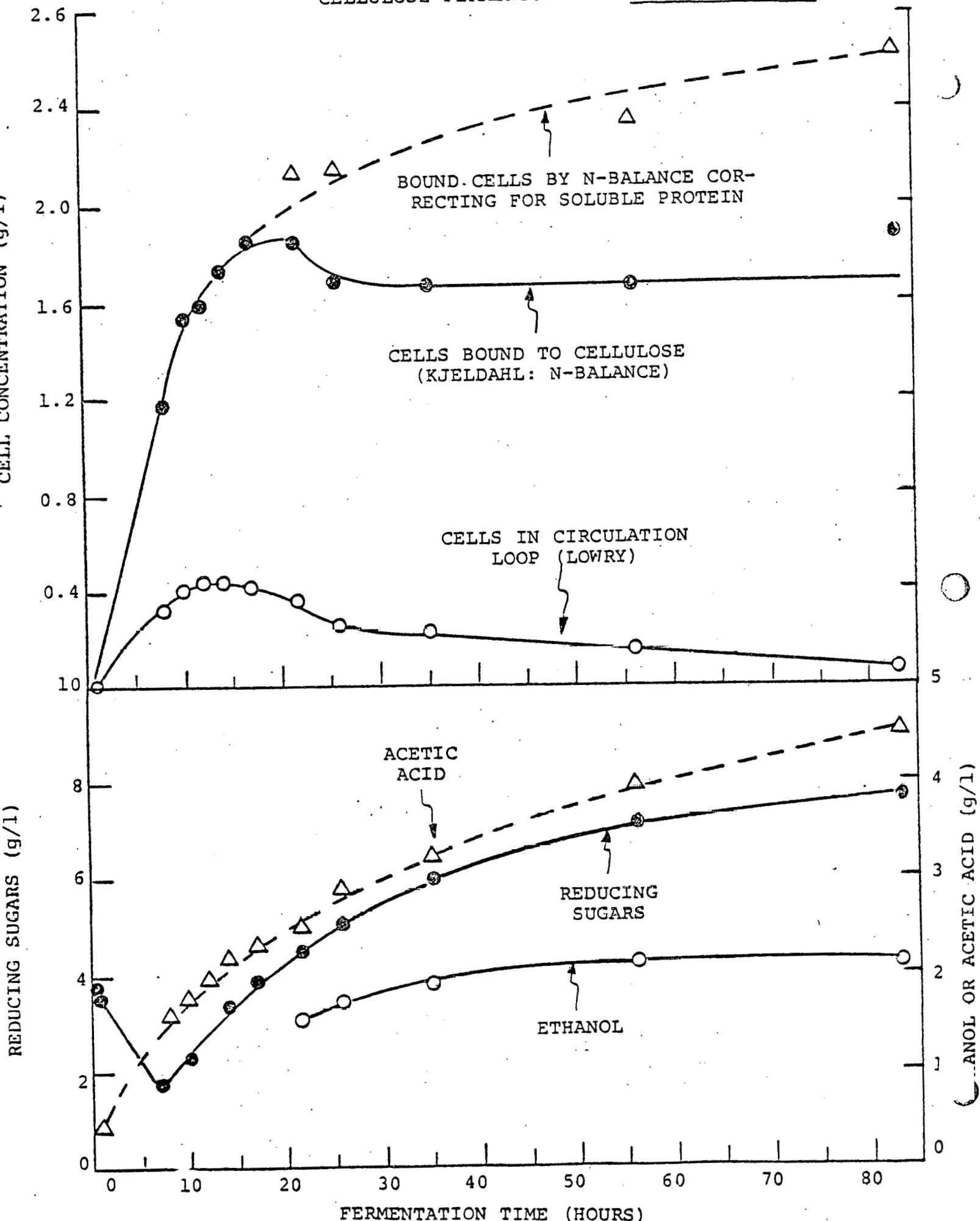
Cell mass accumulation in a packed-bed cellulose fermentor.

The relationships among cells, proteins and the cellulose matrix were partially revealed during a packed-bed fermentation with C. thermocellum. The packed-bed consisted of 50 g of solka-floc. The concentration of solka-floc when calculated over the entire system, including the volume of the circulating loop, was 40 g/l. Inoculum consisted of a 20 hour culture of C. thermocellum grown on cellobiose and the inoculum volume was 45% of the total. About 0.16 g of cells were inoculated. pH was controlled at 6.8 through the addition of NaOH.

The time course of fermentation is presented on the lower part of Figure I.B.1.4. Rapid accumulation of reducing sugars, acetic acid and ethanol occurred up to the 20th hour, followed by a much lower rate until the 83rd hour. At the end of the fermentation products accumulation were: 8 g/l reducing sugars, 4.5 g/l acetic acid and 2,3 g/l ethanol. This represents a 61% of the cellulose degradation.

Detailed characterization of the cell mass accumulation were performed using the following techniques. Direct measurement of cells free in the circulation loop were made. Samples drawn from the circulation loop were precipitated at 10,000 g and cell mass was determined by the Lowry protein assay. Cell mass was also determined through indirect measurement of bound cells by the decrease of total nitrogen

FIGURE I.B.1.4: KINETICS OF FERMENTATION OF PACKED-BED CELLULOSE FERMENTOR WITH C. THERMOCELLUM



of a cell free supernatant (Kjeldahl). Direct measurement of TCA precipitable proteins was also performed of samples taken from the Circulation loop. The cell free supernatant was treated with equal volume of 20% TCA. The protein in the precipitate was measured by Lowry assay. Cell accumulation plus protein accumulation on the residual cellulose was verified on the 83rd hour when the fermentation was stopped. The residual cellulose was drained and washed with water and 1N-NaOH. The protein content of the combined washings was determined.

The results in the upper part of Figure I.B.1.4 depict an initial rapid increase in cell mass (17th hour), followed by disappearance of cells in suspension from the circulation loop. After 20 hours of fermentation there is sufficient soluble protein to interfere with the nitrogen balance. Using the Kjeldahl nitrogen method mentioned above a strange pattern was obtained (see Figure I.B.1.4, upper part, middle line). This technique does not differentiate between nitrogen from cellular material or nitrogen in excreted proteins. However, TCA precipitable protein was measured and it enabled us to calculate cell mass accumulation on the cellulose according to the following equation:

$$\left\{ [N]_{t=0} - [N]_{t_1} + [\text{TCA - protein}] \times 0.16 \right\} \times \frac{1}{0.12} = \text{d.c.w g/l}$$

N = nitrogen concent. in mg/ml at the beginning of fermentation

t = 0 and at t₁

[TCA - protein] = concentration of protein precipitated mg/ml

The upper curve in Figure I.B.1.4 summarizes these calculated values. It is seen that cell mass continues to increase at a very slow rate approaching stationary phase after 40 hours. The decrease of cell mass in the circulation loop is a further proof for the enhanced binding of cells to the cellulose. These results clearly show that the use of optical density measurements for determining cell growth is inadequate. The use of a more tedious but reliable method, nitrogen balance, is far superior for accurately estimating cell growth on cellulosic substrates.

d. Future Work

The future studies in the packed-bed fermentor include:

- Examination of kinetics of growth and product formation at high cellulosic biomass concentrations (e.g. > 100 g/l)
- Investigate other biomass such as corn stover and poplar in the packed-bed fermentor.
- Increase productivity of the packed-bed fermentor through higher loading of cell mass onto support.

C. Production of Ethanol from Cellulose by Clostridium thermocellum

1. Characterization of New Ethanol Tolerant Strain (S-4)

a. Introduction

In the previous Progress Report (COO-4198-6), we reported an ethanol tolerant strain (S-4) isolated from Clostridium thermocellum ATCC 27405. This strain can tolerate up to 5% ethanol (V/V) and can also degrade cellulose at high ethanol concentrations. In this quarter, we have continued to examine the properties of strain S-4 to include studies on its metabolism and kinetics of ethanol production.

In addition, experiments using corn stover as carbon source and soybean meal as nitrogen source were also performed. The results from these experiments will be presented.

b. Materials and Methods

The microorganism used throughout this study was strain S-4. The basic medium used for growth was CM4 and its composition has already been reported. Slight modifications were made in some cases. These changes will be specified along with the presentations of the results.

The determination of formic acid was achieved through gas chromatography using two Teflon coated columns (8 ft x 1/8" OD) containing Chromosorb 101. Samples were acidified using 0.2 ml of 5 N HCl for each milliliter of fermentation

broth. Temperature was isothermal at 170°C; injection port temperature 225°C, thermal conductivity detector temperature 275°C, flow rate of carrier gas (N₂) at 70 ml/min for both columns.

For the lactic acid assay, two methods were used. The procedure using lactate dehydrogenase was the same as Sigma Technical Bulletin No. 826-UV. The other method was colorimetric determination, using P-hydroxydiphenol and sulfuric acid, developed by S.B. Barker and William H. Summerson (J. Biol. Chem., 138:535 (1941)). The former can measure only L-lactic acid, the latter can measure both L and D forms of lactic acid.

All other analytical procedures have already been reported and will not be repeated.

c. Results and Discussion

In order to obtain more detail properties of the new strain, S-4, various carbon sources were tested in CM4 medium. Strain S-4 can grow on the monosaccharides D-glucose and D-fructose; it also fermented the disaccharide cellobiose as well as cellulosa. All pentoses tested failed to support the growth of S-4 which include xylose, arabinose, ribose and xylan. These results were similar to that reported by Alexander et al. (J. Bacteriol., 105:220 (1971); ibid., 105:226 (1971)), but slightly different from Alexander's strain 651 in that D-mannitol cannot support the growth of strain S-4.

In order to make sure that the growth of S-4 in D-glucose and D-fructose is not due to contaminants, we have performed a series of transfers in CM4 medium using glucose or fructose as the carbon source. If the growth is due to the contaminants, it was reasoned that S-4 would be diluted out after several transfers. After 6 transfers or more, we then performed plate count on cellulose agar medium, as well as growth of the culture on cellobiose as the control. All cultures grew on glucose, fructose and cellobiose.

In order to make certain that the growth is not due to the utilization of yeast extract (5 g/l), we performed studies using these carbon sources in shake flask. This allows us to measure the substrate consumption, as well as products formation. The results are shown in Figures I.C.1.1 through I.C.1.4. In all cases, the fermentation profiles and product ratios are quite similar. These studies conclusively show that S-4 is able to utilize glucose, fructose, cellobiose and cellulose.

It is very interesting to note that the ethanol production is much greater than acetic acid production. Our data, reported in Progress Report COO-4198-5, showed that Clostridium thermocellum ATCC 27405 will produce ethanol and acetic acid at an approximate ratio of 1:1. However, it can be seen from Figures I.C.1.1 through I.C.1.4 that S-4 no longer behaves in this fashion.

FIGURE I.C.1.1: FERMENTATION PROFILE OF GLUCOSE BY
C. THERMOCELLUM (S-4)

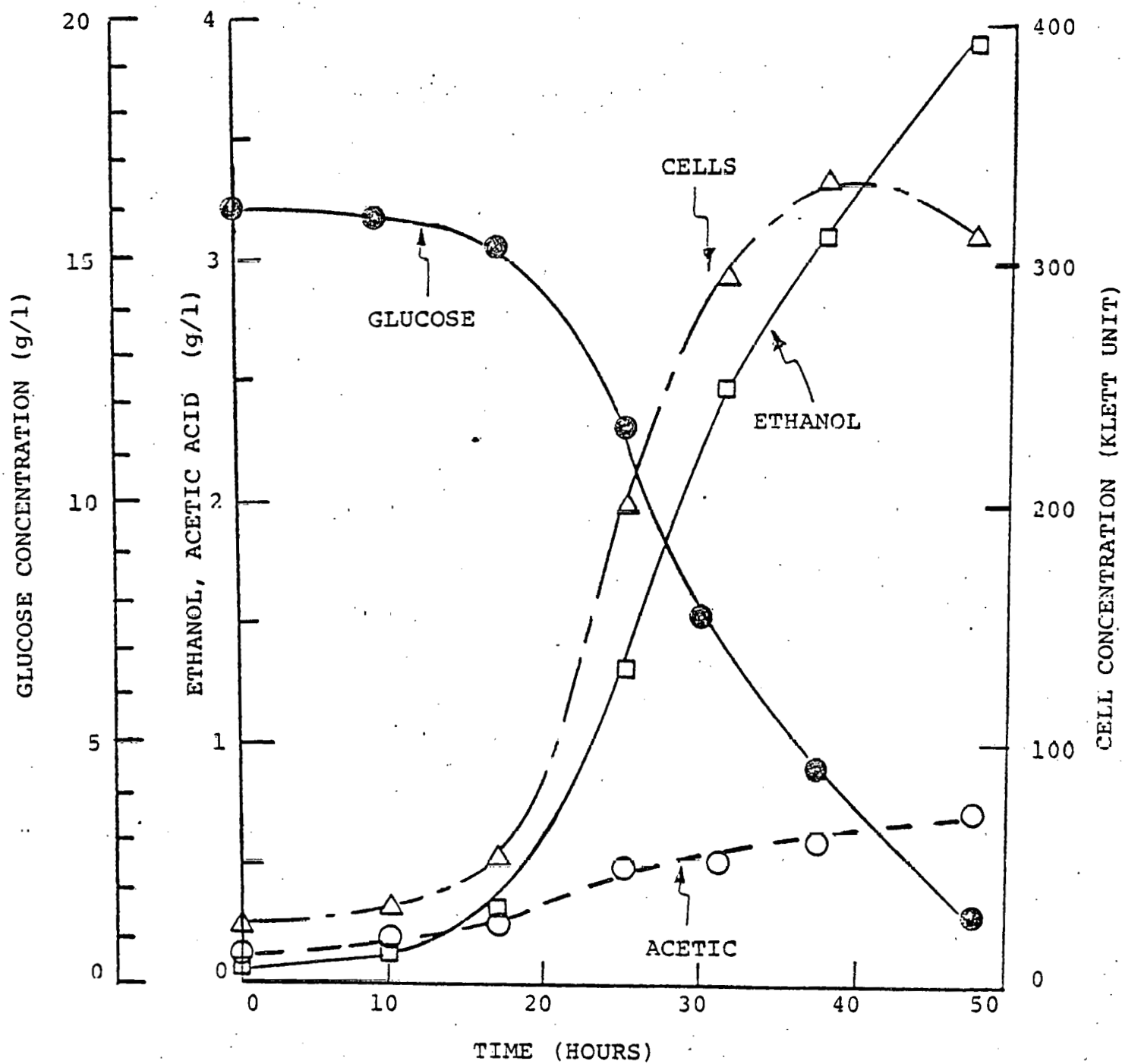


FIGURE I.C.1.2: FERMENTATION PROFILE OF FRUCTOSE
BY C. THERMOCELLUM (S-4)

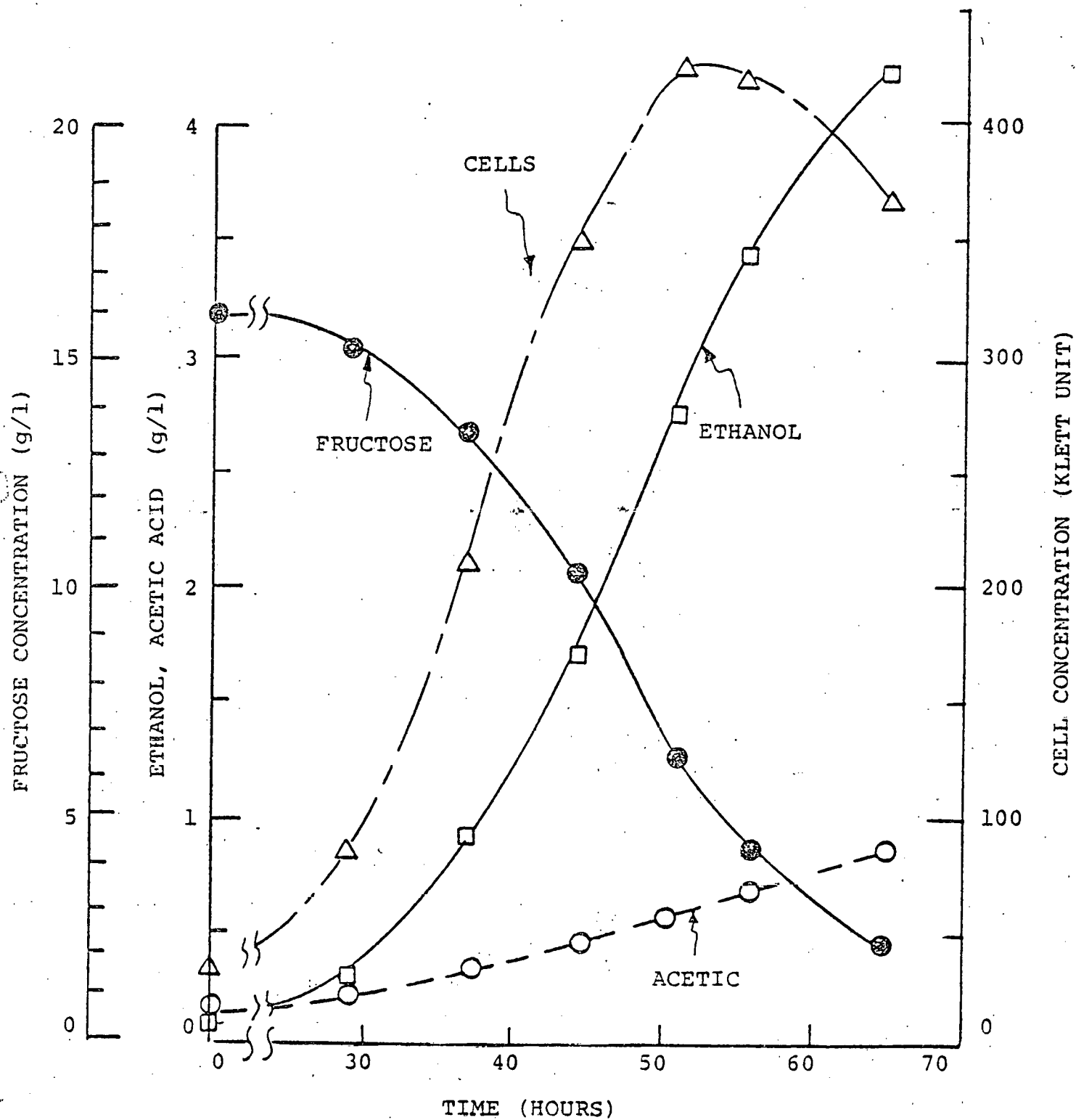


FIGURE I.C.1.3: FERMENTATION PROFILE OF CELLOBIOSE
BY C. THERMOCELLUM (S-4)

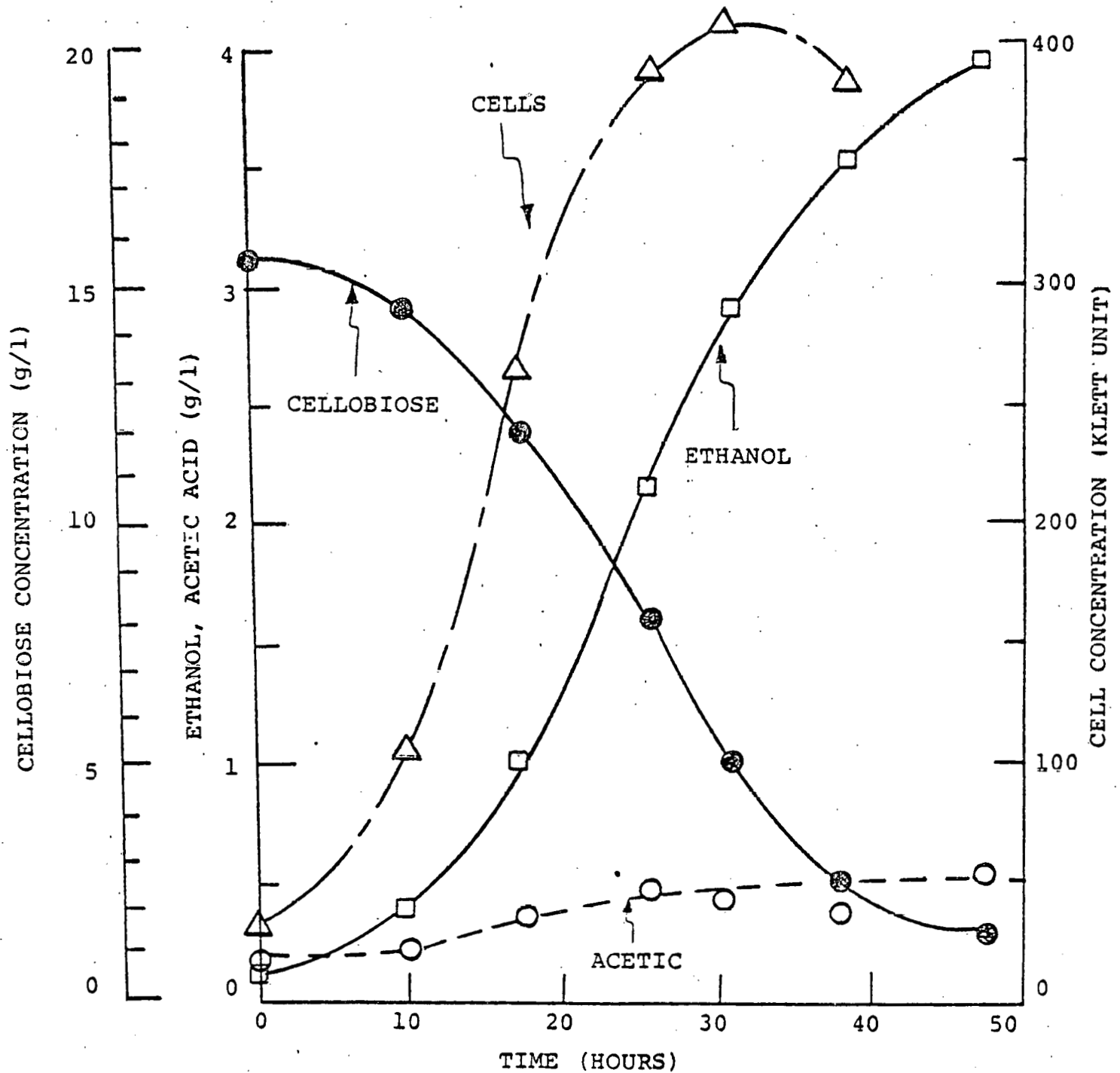
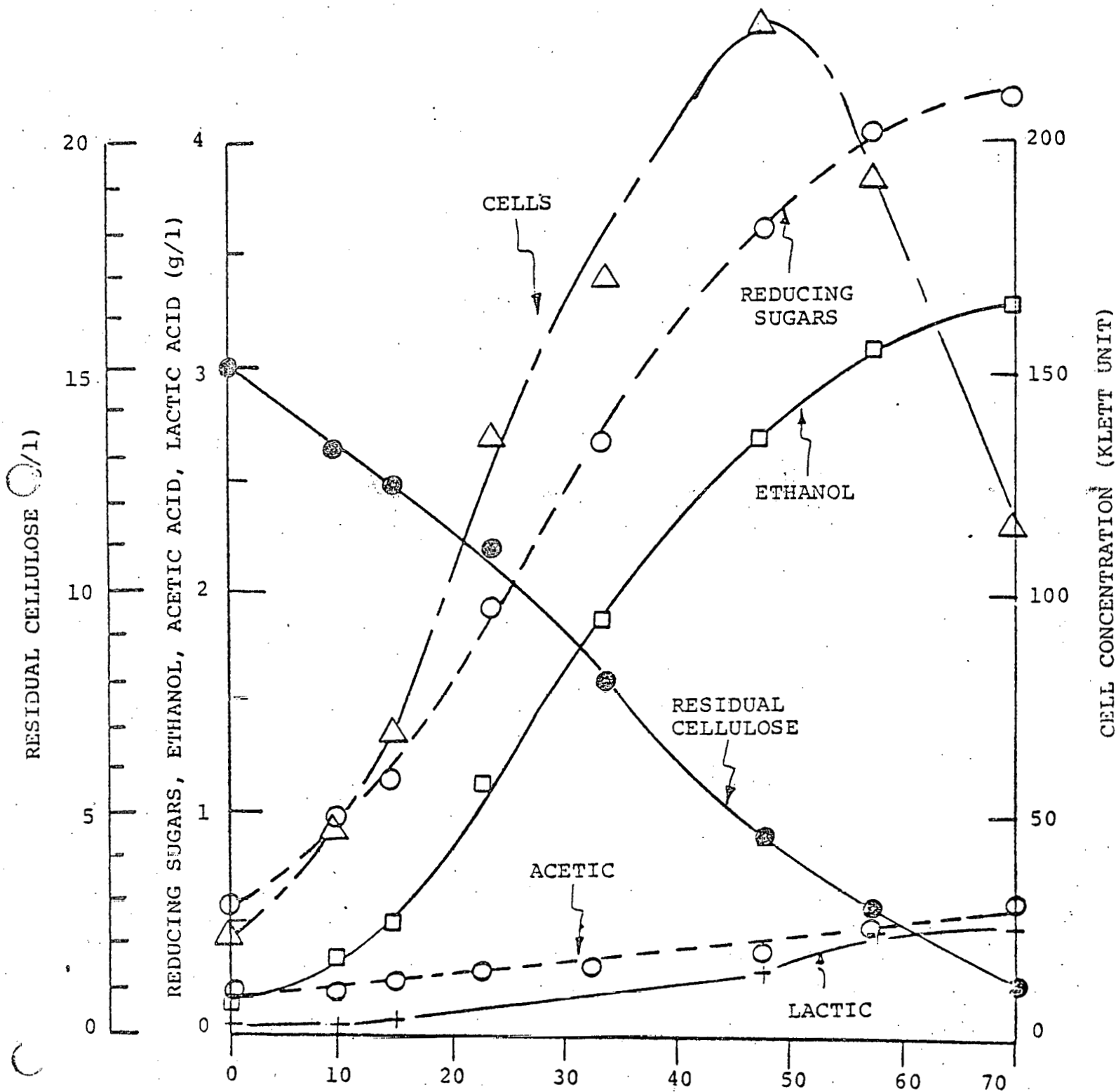
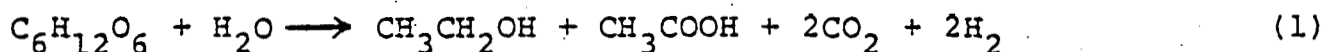


FIGURE I.C.1.4: FERMENTATION PROFILE OF CELLULOSE (SOLKA FLOC) BY C. THERMOCELLUM (S-4)



In view of the observations that strain S-4 produces significantly higher concentrations of ethanol as compared to acetic acid, it was felt that a more detailed analysis of the results is warranted. It should also be mentioned that the fact that strain S-4 is capable of producing ethanol in preference to acetic acid is an extremely desirable property in achieving the direct production of fuel (ethanol) from biomass. To perform our analysis as to the reason why S-4 is able to produce a higher ratio of ethanol to acetic acid, the biochemical degradation pathway by clostridia is shown in a simplified manner in Figure I.C.1.5. In this figure, the pathway of reducing power generation and H₂ formation in clostridia are summarized. Under normal conditions, only one mole of ethanol will be produced from each mole of glucose fermented, as well as the production of one mole of acetic acid, two moles of CO₂ and two moles of H₂. The fermentation can be represented by the following equation (1).



In order to examine the behaviors when strain S-4 was used to degrade cellulose, the results from Figure I.C.1.4 were used to obtain carbon as well as oxidation-reduction balances. The results from these analyses are shown in Table I.C.1.1. The carbon recovery was found to be 90.4%.

FIGURE I.C.1.5: FUNCTION OF FERREDOXIN IN THE METABOLISM OF SACCHAROLYTIC CLOSTRIDIA.

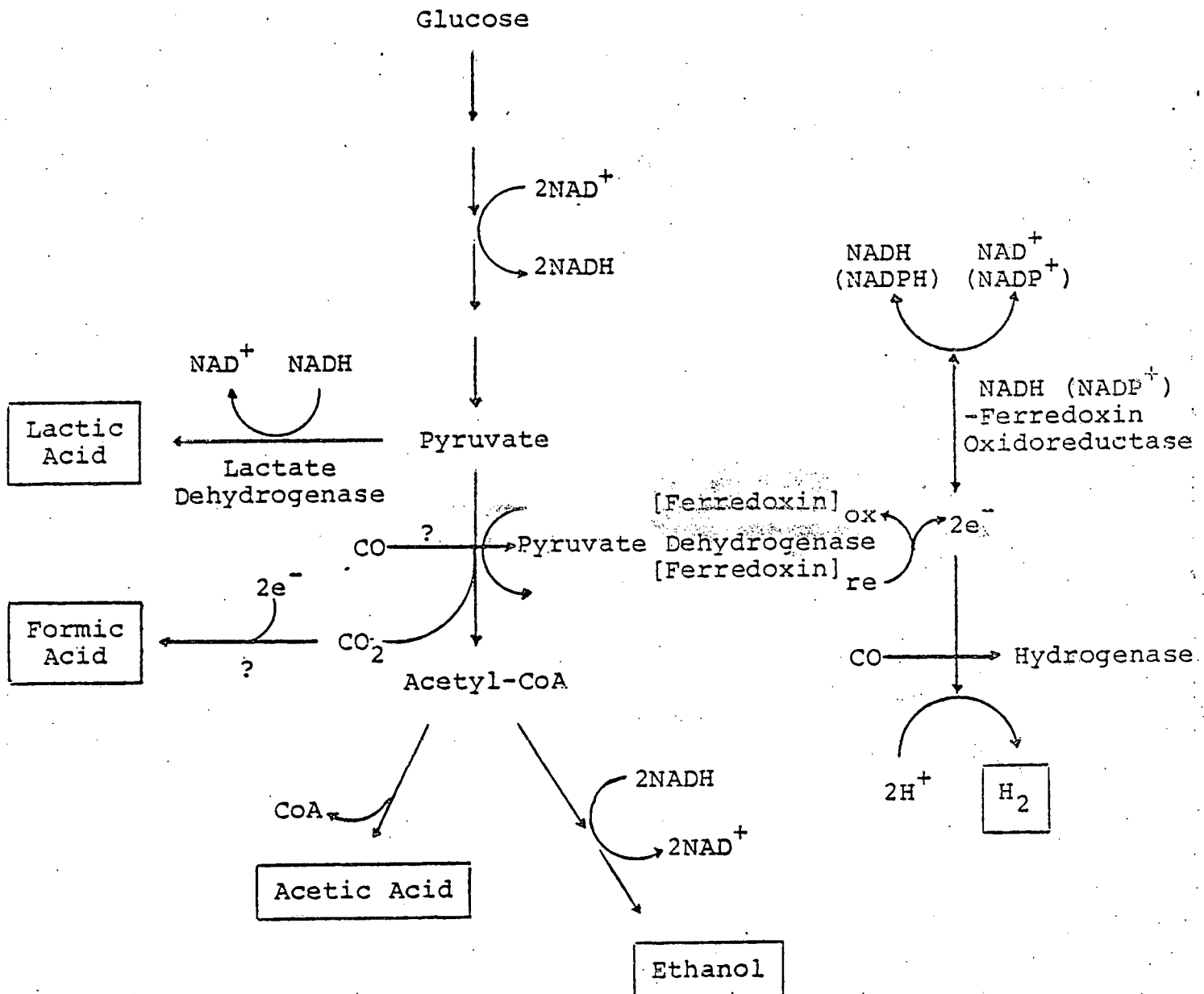


TABLE I.C.1.1: CARBON AND OXIDATION-REDUCTION BALANCES FOR FERMENTATION
OF CELLULOSE BY CLOSTRIDIUM THERMOCELLUM S-4

Products	Amount		g carbon/l	Unit O/R value per mole	Overall O/R Value	
	g/l	mM			Case I	Case II
Reducing Sugar ¹	5.22	29	2.083	0	0	0
Acetic Acid	0.42	7	0.163	0	0	0
Lactic Acid	0.45	5	0.18	0	0	0
Ethanol	3.25	68.8	1.651	-2	-137.6	-137.6
CO ₂ ²	3.34	75.8	0.910	+2	+151.6	+151.6
H ₂ ³	(0.15)	(75.8	-	-1	- 75.8	0
Cell Mass	0.8	-	0.4 ⁴	-	-	-
Total Carbon Recovery			5.397	0	+151.6 - 213.4	+151.6 - 137.6
Cellulose Degraded	13.57	83.8	5.971			
Total Carbon Recovery in Percent of Theoretical Value			$\frac{5.397}{5.971} = 90.4\%$			
Oxidation- Reduction Index					$\frac{151.6}{213.4} = 0.71$	$\frac{151.6}{137.6} = 1.10$

¹

² The amount of reducing sugar was detected after acid hydrolysis (5% HCl, boil for 1 hour).
Calculated CO₂ evolution: Assume 1 mole of CO₂ produced for each mole of Ethanol or Acetic

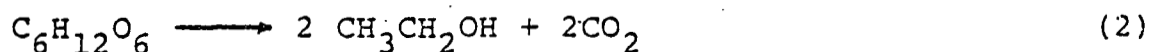
³ Acid produced.

Calculated H₂ evolution: Case I : Assume 1 mole of H₂ produced for each mole of CO₂ evolution.

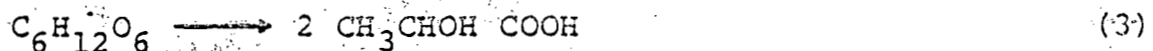
⁴ Case II: Assume no H₂ produced at all.

Assume 50% carbon in the cell dry weight.

Two cases for the oxidation-reduction balances were performed. It can be seen from Table I.C.1.1 that, in the case of strain S-4, in order to balance the oxidation-reduction reactions, there must be very little or no H_2 formation. This would mean that almost all the reducing power generated by pyruvate dehydrogenase reaction was not used to convert the proton to hydrogen gas. Instead, it is postulated that the proton was used to produce NADH, and thus increasing the production of ethanol or lactic acid. Thus, the fermentation by S-4 can be represented by the sum of equations (2) and (3).



or



Simultaneous to the studies shown in Figure I.C.1.1 through I.C.1.4, an alternate approach to increase the production of ethanol preferentially to acetic acid had been proposed. In examining the pathway of degradation (Figure I.C.1.5), it was postulated that if the formation of hydrogen can be inhibited, higher amounts of ethanol should result. Since carbon monoxide is a known inhibitor of metallo enzymes, it was reasoned that by introducing this gas, the hydrogenase activity should be depressed. As a result of this inhibition, it was hoped that the accumulation of reduced

ferredoxin will effect the reduction of NAD^+ to NADH and thus increase ethanol production.

Carbon monoxide atmosphere was introduced into an exponentially growing flask culture of S-4, using Solka floc as carbon source. The results are shown in Figure I.C.1.6. After carbon monoxide was introduced, the production of ethanol and acetic acid decreased, but the production of lactic acid increased. Since pyruvate dehydrogenase also contain non-heme iron atom, it is very possible that this enzyme was also inhibited by carbon monoxide. We conclude that the metabolic pathway is inhibited by carbon monoxide toward the direction of lactic acid production. Table I.C.1.2 shows the carbon balance and oxidation-reduction balances from this experiment. Here again the analysis showed that hydrogen formation is probably also suppressed through the action of carbon monoxide.

Thus far, all of the studies with strain S-4 have been performed in shake flasks which does not allow the control of pH. We were interested to observe the behavior of this strain when the pH was controlled. Therefore, a 7 liter agitated fermentor containing 5 liter of medium controlled at a pH of 7.0 was used. The results are shown in Figure I.C.1.7. Since our previous results have shown that this new strain is able to metabolize glucose, this fermentation was performed using this as the carbon source.

FIGURE I.C.1.6: FERMENTATION PROFILE OF CELLULOSE (SOLKA FLOC) BY *C. THERMOCELLUM* UNDER CARBON MONOXIDE ATMOSPHERE

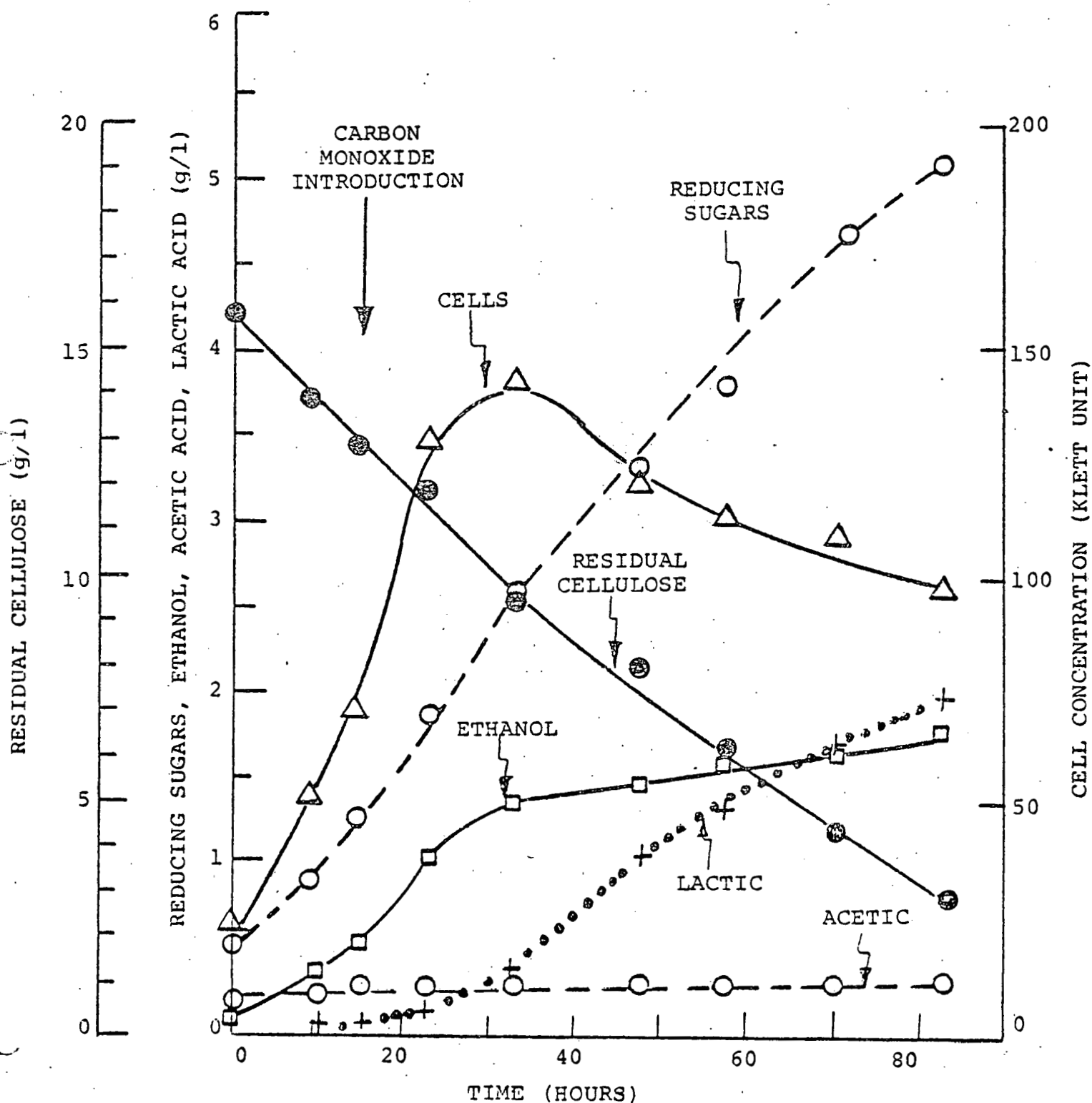


TABLE I.C.1.2: CARBON AND OXIDATION-REDUCTION BALANCES FOR FERMENTATION OF CELLULOSE BY CLOSTRIDIUM THERMOCELLUM S-4 UNDER CO ATMOSPHERE

Products	Amount		g carbon/l	Unit O/R value per mole	Over-all O/R Value	
	g/l	mM			Case I	Case II
Reducing Sugar*	6.89	38.3	2.756	0	0	0
Acetic Acid	0.089	1.5	0.036	0	0	0
Lactic Acid	1.87	20.8	0.75	0	0	0
Ethanol	1.62	35.2	0.845	-2	-70.4	-70.4
CO *	1.615	36.7	0.44	+2	+73.4	+73.4
H *	(0.073)	(36.7)	-	-1	-36.7	0
Cell Mass	0.5	-	0.25*	-	-	-
Total Carbon Recovery			5.077		+ 73.4 - 107.0	+73.4 - 70.4
Cellulose Degraded	12.84	79.3	5.650			
Total Carbon Recovery in % of Theoretical Value			$\frac{5.077}{5.650} = 89.9\%$			
Oxidation-Reduction Index					$\frac{73.4}{107.1} = 0.69$	$\frac{73.4}{70.4} = 1.04$

* See footnotes of Table I.C.1.1.

FIGURE I.C.1.7:- CONTROLLED pH (7.0) FERMENTATION BY CLOSTRIDIUM THERMOCELLUM USING GLUCOSE

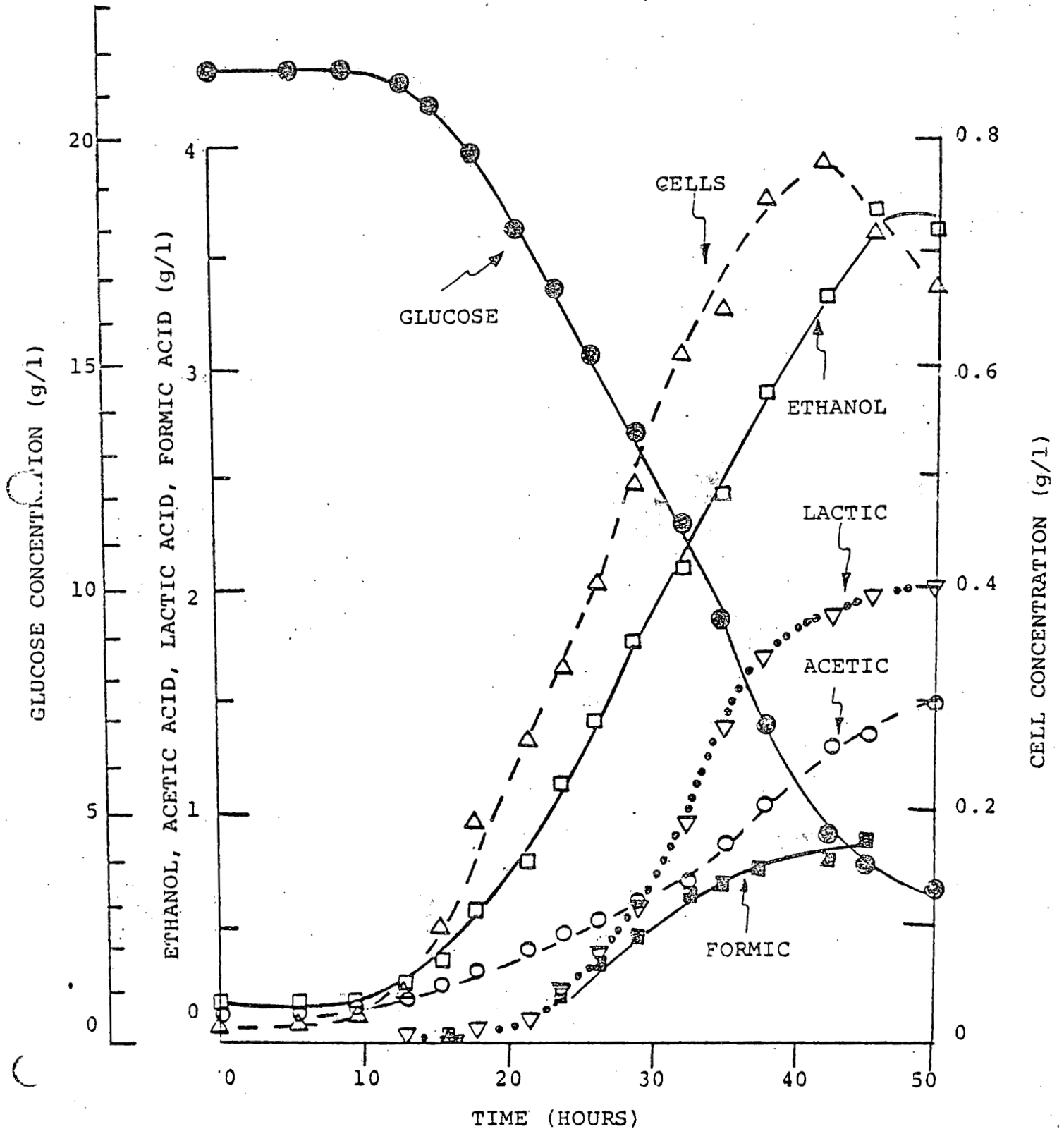
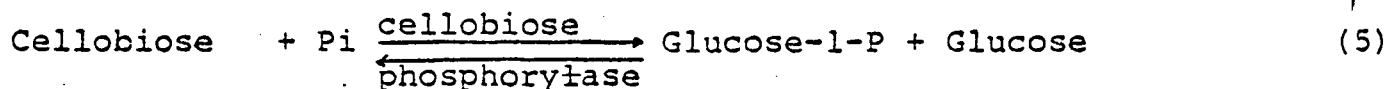
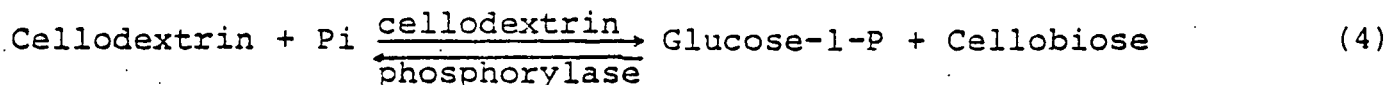


Figure I.C.1.7 showed the product profiles for this fermentation were similar to the previous results. The production of reduced products (i.e. ethanol and lactic acid) is significantly greater than oxidized product (acetic acid). The lactic acid measured by lactate dehydrogenase method and p-hydroxydiphenyl-sulfuric acid method gave about the same amount of lactic acid indicating that the lactic acid produced by strain S-4 is L-lactic.

We also analyzed other possible products, such as butyric acid, valeric acid, propionic acid, acetone, isopropanol, butanol, 2,3-butanediol, acetoin or diacetyl, but none was found. We also examined the sugars that remained in the fermentation broth by paper chromatography. It was found that cellobiose and perhaps some oligosaccharides accumulated during the fermentation. Figure I.C.1.8 shows the result of the paper chromatography and it is seen that cellobiose accumulated during fermentation.

The formation of cellobiose and cellodextrin is possible according to Alexander et al. (J. Biol. Chem., 243, 2899 (1968); ibid., 244, 457 (1969)), since Clostridium thermo-
cellum will produce cellobiose phosphorylase and cellodextrin phosphorylase which catalyzes the following reactions:



Both enzymes are reversible, and in vitro, cellobiose phosphorylase is favored in the direction of phosphorolysis, but cello-dextrin phosphorylase is favored in the direction of synthesis. It is very possible that, under high glucose concentration, cellobiose and cello-dextrin were synthesized from glucose by these enzymatic reactions.

The kinetics of growth and ethanol production from Figure I.C.1.7 were carefully analyzed in order to reveal the manner in which ethanol formation occurs. If the production of ethanol can be achieved through growth and non-growth associated modes, the following mathematical model can be formulated.

$$q_p = \alpha \mu + \beta$$

where, q_p = specific productivity of ethanol
(g ethanol/g cell-hr)

μ = specific growth rate
(g cell/g cell-hr)

α = growth associated product formation constant
(g ethanol/g cell)

β = non-growth associated product formation constant
(g ethanol/g cell-hr)

If we plot q_p versus μ , the slope of this plot should give the growth associated product formation constant α , and the intercept at $\mu = 0$ should give the non-growth associated product formation constant β . The data from Figure I.C.1.7 were

analyzed in this fashion and shown graphically in Figure I.C.1.9. The maximum values of μ and q_p from Figure I.C.1.7 are 0.31 (g cell/g cell-hr) and 0.70 (g ethanol/g cell-hr) respectively. The calculated value of α and β from Figure I.C.1.9 are 1.8 (g ethanol/g cell) and 0.14 (g ethanol/g cell-hr) respectively; indicating that the ethanol production is produced through both growth and non-growth associated modes.

In the previous Progress Report, we presented results about the degradation capability of strain S-4 on corn stover. Additional experiments were done during this quarter. Figure I.C.1.10 showed the effect of corn stover concentration on the production rates of ethanol, acetic acid and reducing sugar. The production rate was directly proportional to corn stover concentration. Data for higher concentration (above 4% corn stover) could not be obtained because it is not possible to operate in shake flasks at this corn stover concentration.

In order to increase the production rate, we also attempted corn stover pretreatment. The results were shown in Figure I.C.1.11. NaOH was first added to the corn stover medium to obtain a pH of 11. The corn stover medium was then sterilized and neutralized. No washing procedure was performed.

It can be seen from the results in Figure I.C.1.11 that alkaline pretreatment of corn stover can enhance the microbial activity during fermentation. The plot on the

FIGURE I.C.1.8: PAPER CHROMATOGRAM OF REDUCING SUGARS FROM
GLUCOSE DURING FERMENTATION BY CLOSTRIDIUM
THERMOCELLUM (pH = 7.0):

SOLVENT: n-Butanol:Acetic Acid:H₂O
4:2:1

STAINING REAGENT: Aniline Hydrogen Phthalate
(10%)

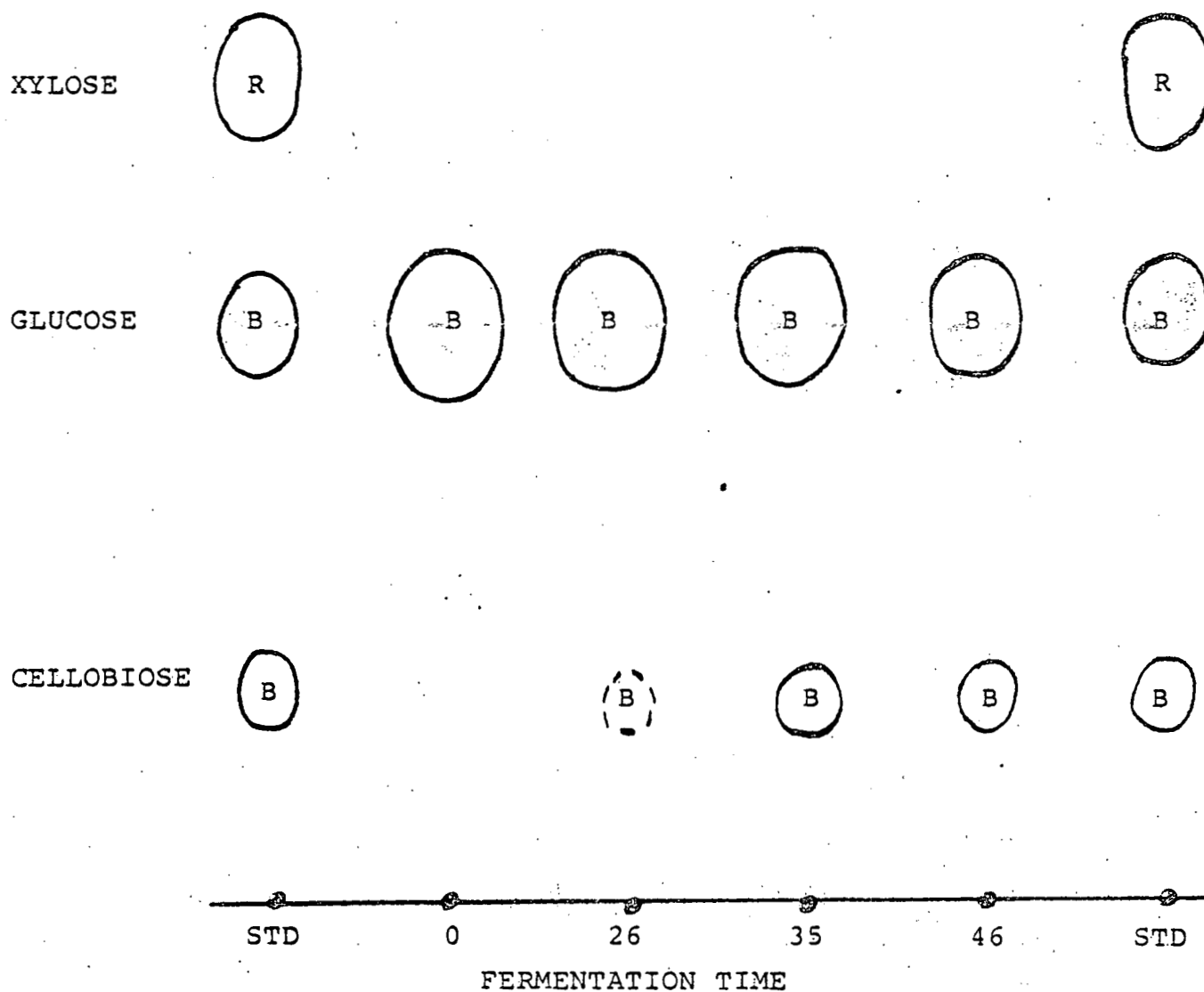


FIGURE I.C.1.9: KINETIC RELATIONSHIP OF GROWTH AND PRODUCT FORMATION BY *C. THERMOCELLUM* (S-4) GROWN ON GLUCOSE

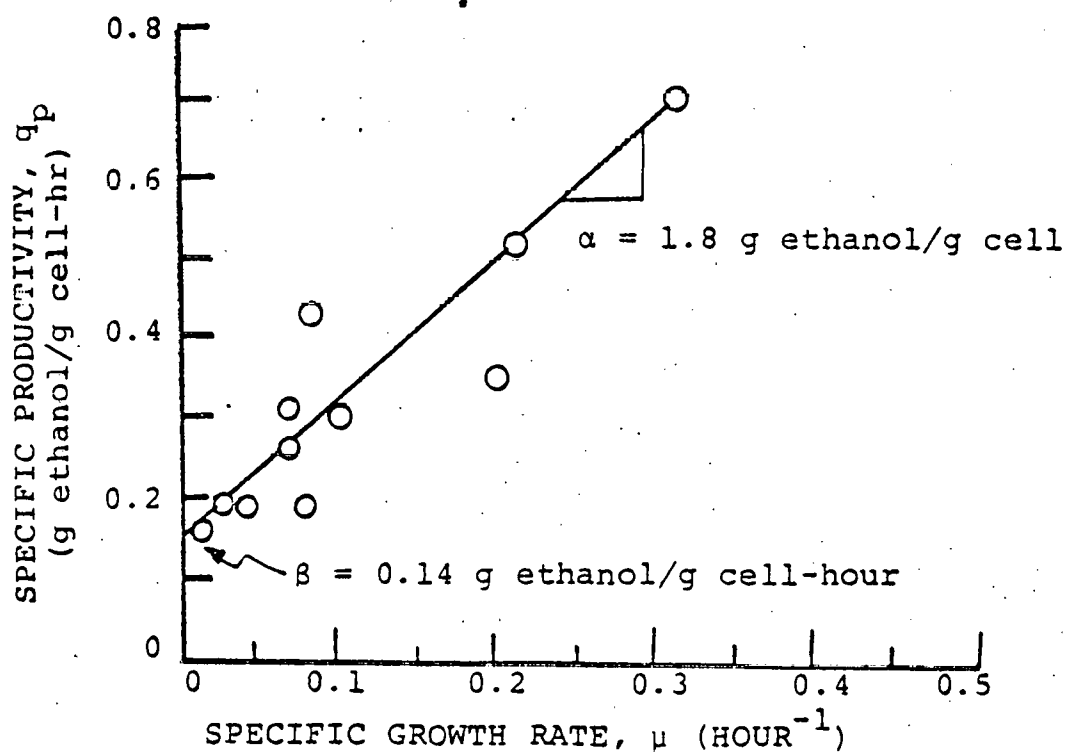
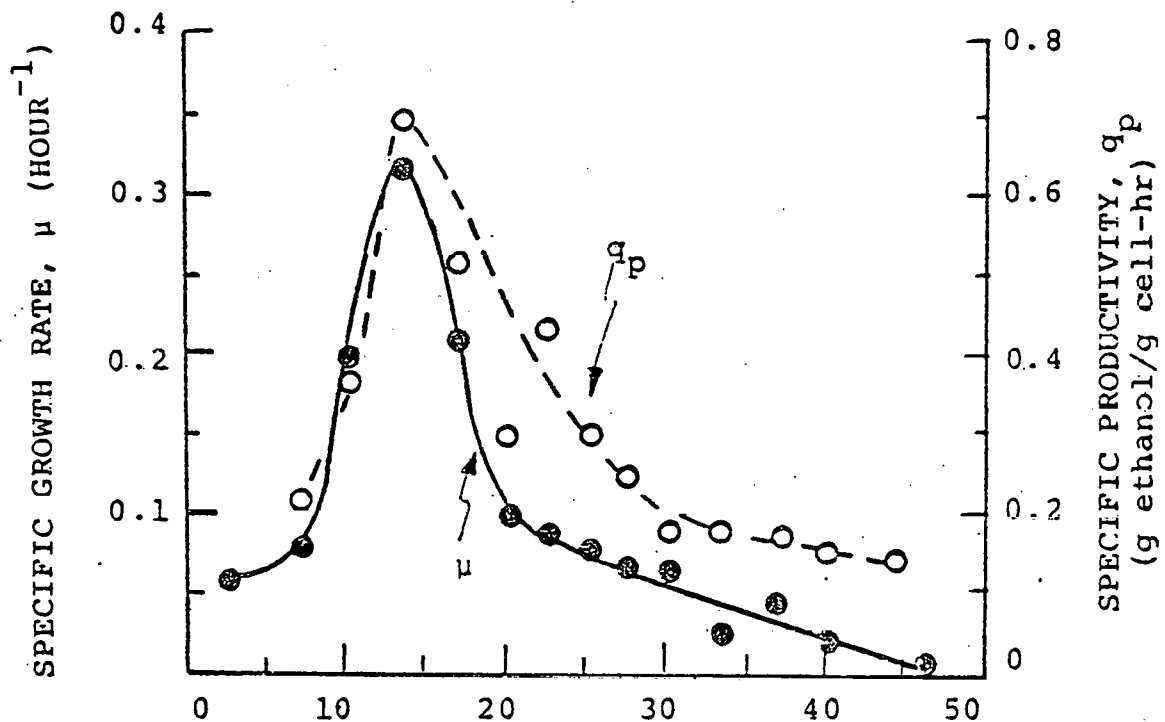


FIGURE I.C.1.10: EFFECT OF INITIAL CORN STOVER CONCENTRATION ON VOLUMETRIC PRODUCTIVITY OF PRODUCTS BY CLOSTRIDIUM THERMOCELLUM (S-4)

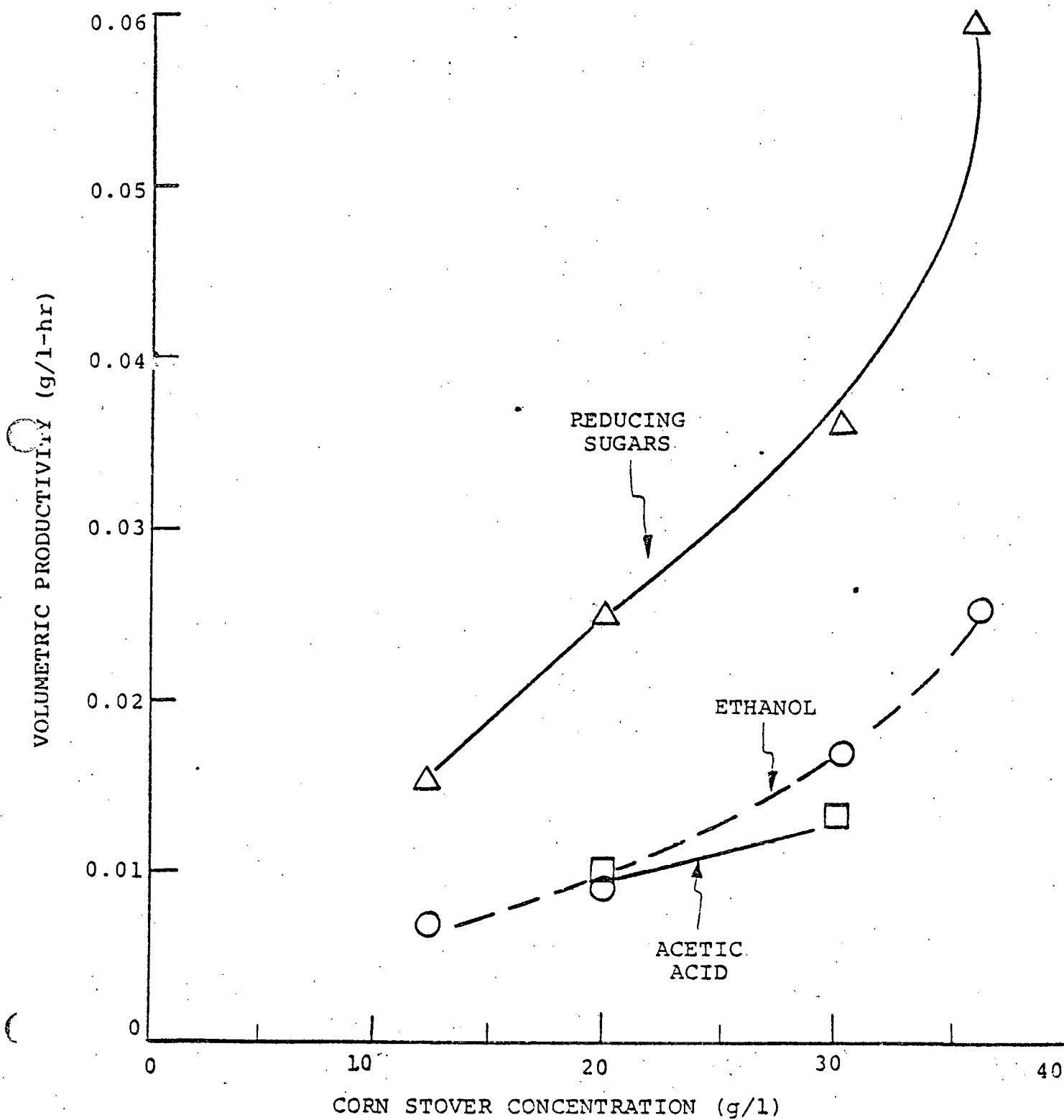
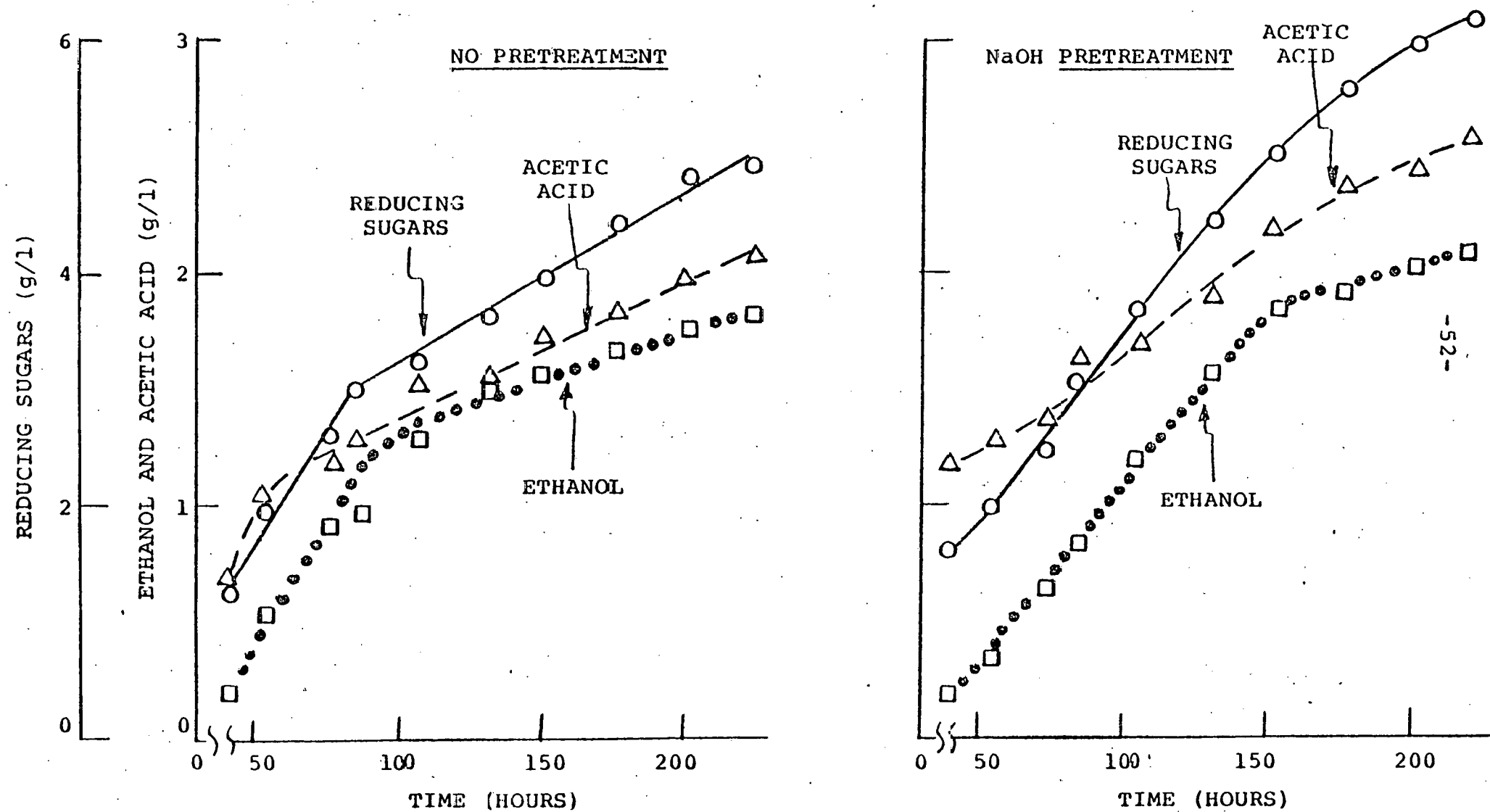


FIGURE I.C.1.11: PRODUCT FORMATION FROM CORN STOVER BY *CLOSTRIDIUM THERMOCELLUM* (S-4)
WITH NO PRETREATMENT AND NaOH PRETREATMENT (CORN STOVER = 30 g/l)



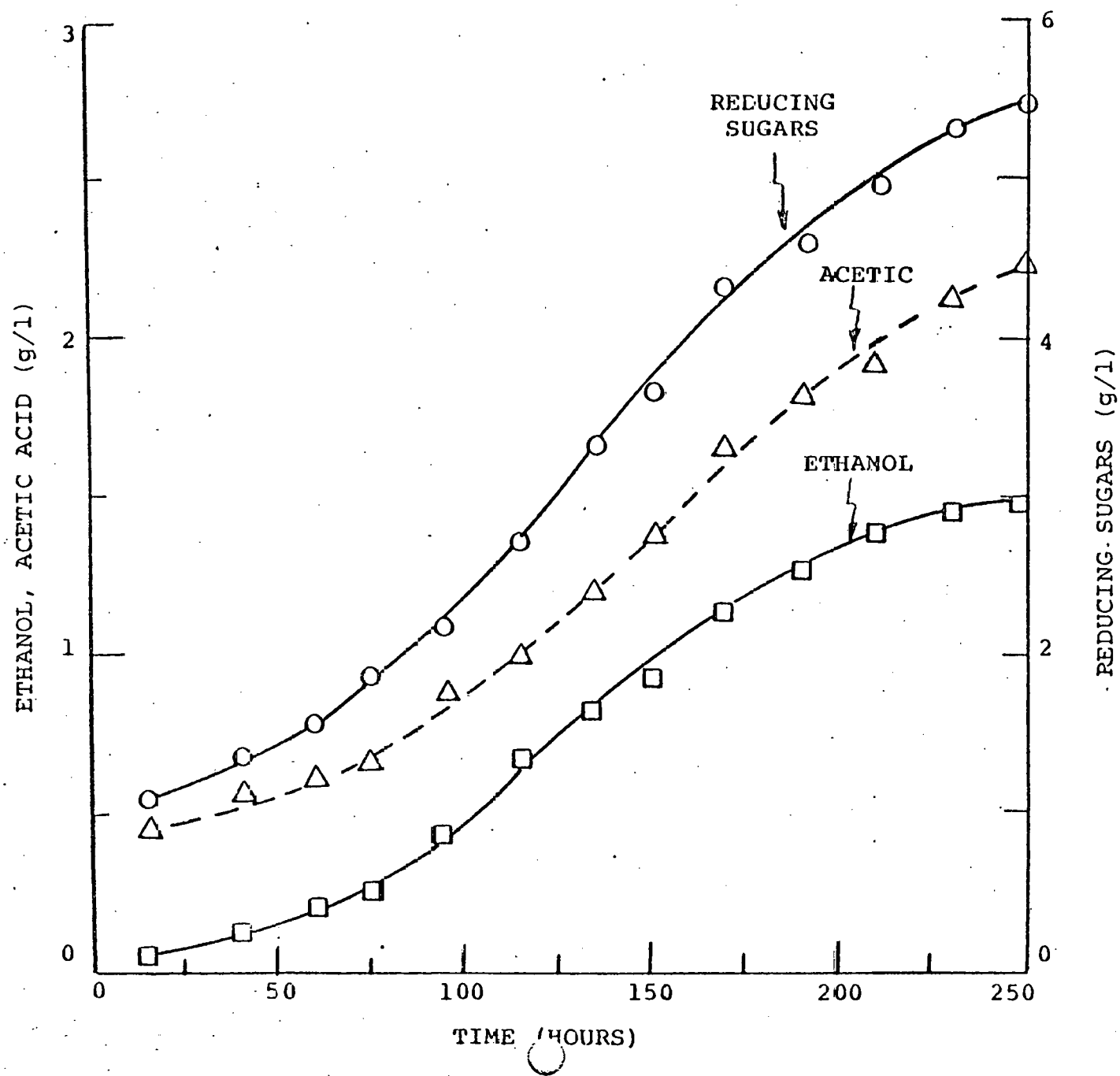
left in the Figure represents no pretreatment and on the right represent NaOH pretreatment. The rates of product formation (ethanol, acetic acid and reducing sugars) are all higher when the stover was pretreated. Further studies with corn stover along with pretreatment will be performed.

Lastly, an experiment using soybean meal as nitrogen source was carried out in order to eliminate the expensive yeast extract from the medium. The results are shown in Figure I.C.1.12. (No pretreatment was performed.) These results are encouraging since they show that the use of cheaper nitrogen source, soybean meal, can be used to produce useful products.

d. Future Work

- Increase the yield of ethanol from biomass by developing the best environmental conditions.
- Incorporate the packed-bed fermentor to achieve high production rate and high product concentration.
- Examine other biomasses such as bagasse, straws, wood, waste pulp, etc.
- Further selection studies to increase ethanol tolerance.
- Attempts to elucidate the mechanisms leading to preferential formation of ethanol to eliminate other fermentation products which will ultimately result in the direct production of liquid fuel (ethanol) from biomass.

FIGURE I.C.1.12: FERMENTATION PROFILE OF CORN STOVER (30 g/l) BY CLOSTRIDIUM THERMOCELLUM (S-4) USING SOYBEAN MEAL (10 g/l)



2. Studies on Wild-Type *C. thermocellum* and Isolate C9

In these studies, a new ethanol-resistant *C. thermocellum* strain, C-9, was isolated and characterized. The inhibition of this strain and wild-type *C. thermocellum* by fermentation products is further investigated. In addition, the issue of glucose utilization is taken up.

a. Isolation of an ethanol-resistant strain of *Clostridium thermocellum*

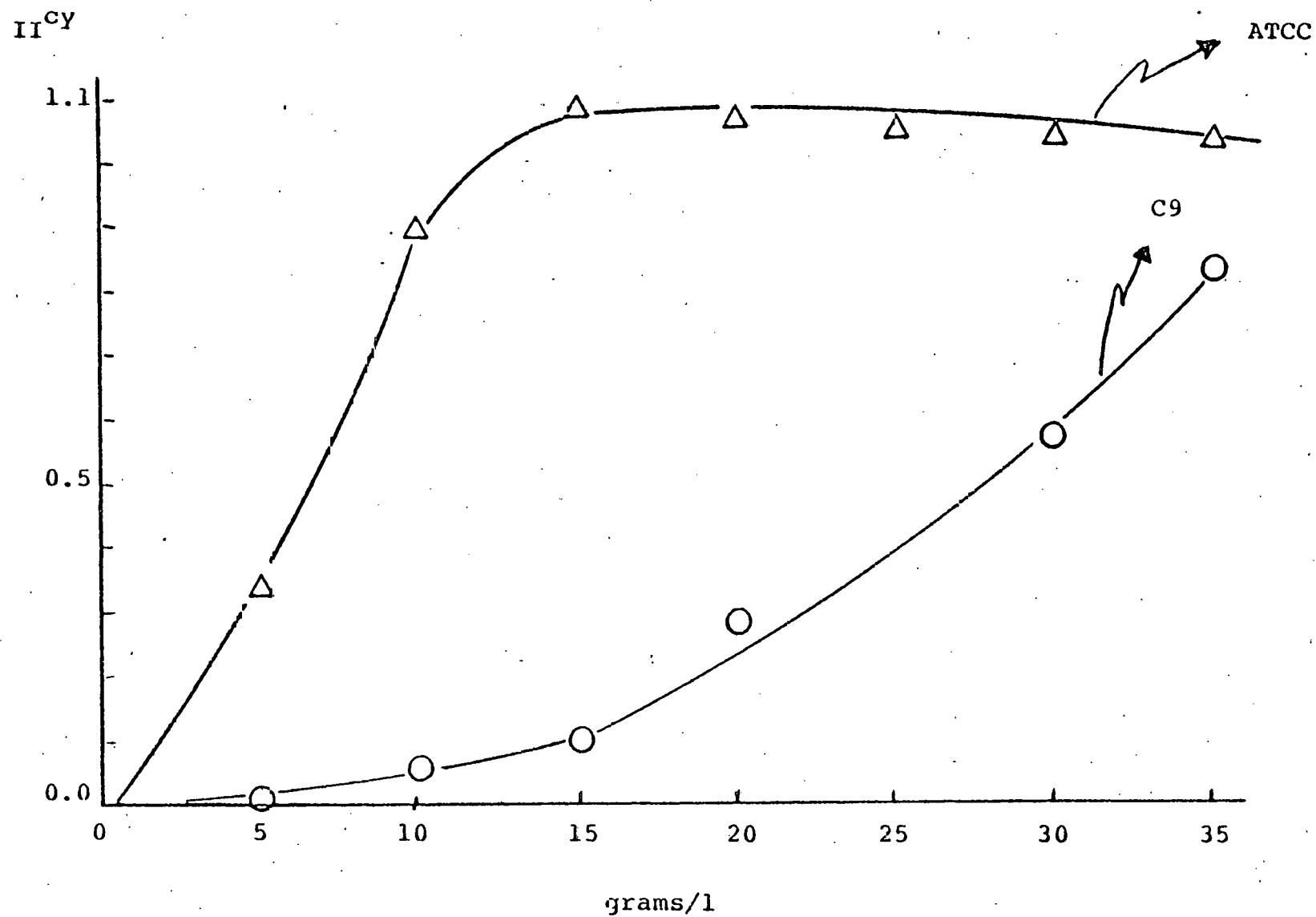
i. enrichment procedure:

An ethanol resistant strain (50% inhibition at 25 grs/lt) has been obtained after nine sequential transfers of *C. thermocellum* in CM4 broths containing increasing amounts of ethanol, using α -cellulose (sigma) as C-source. The criteria for the selection of more resistant cultures has been the II^{120} (inhibition index measured after 120 hours of growth at 60°C); those cultures that showed a lower II^{120} at a given ethanol concentration were transferred into broths with higher ethanol content. After nine sequential transfers the strain named C9 was obtained. An inhibition index experiment was performed as described in our last progress report section (I.C.2), and the results are shown in Figure I.C.2.1.

ii. confirmation procedure:

From the control tube of this experiment (0 grs/lt of ethanol), C9 cells were diluted in 0.1% peptone-water + 0.05% sodium thioglycollate and plated on CM4-Cb without ethanol; well isolated single colonies were inoculated into CM4-Cb and

Figure I.C.2.1 Growth Inhibition by Ethanol. Plot of Inhibition Index (Cell Yield) vs. Ethanol Concentration in Grams/L.



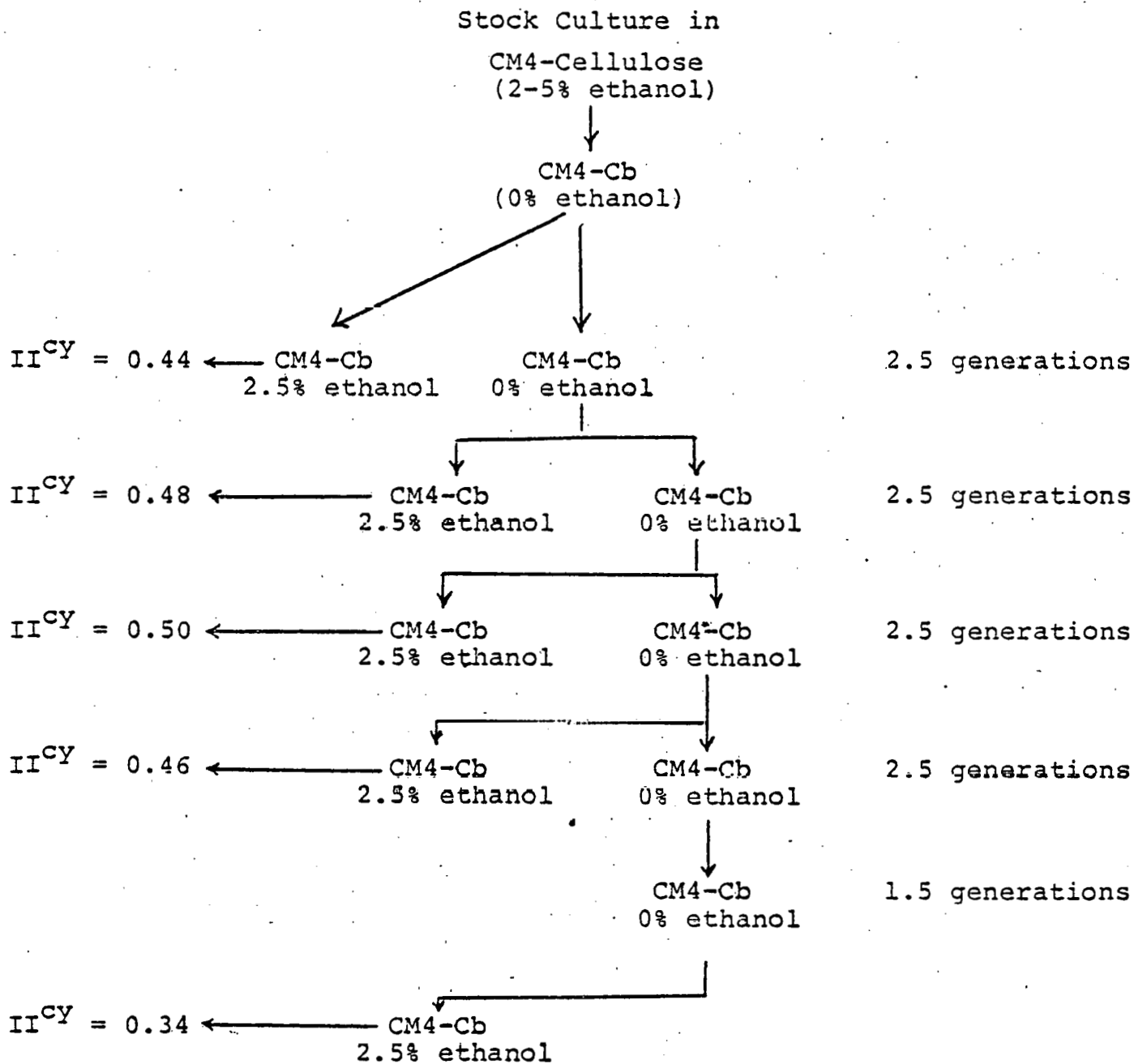
CM4 - cellulose (Solka-floc) broths in the absence of ethanol. After 48 hrs of growth at 60°C in CM4-cellulose, stock tubes were prepared containing 25 grs/lt of ethanol. To ascertain whether the resistance of ethanol of C. thermocellum C9 was due to a mutational change or if it was the phenotypic expression of a conditioned culture, the following confirmation procedure - in the absence of a selective pressure - was carried out (see Figure I.C.2.2).

After a prolonged cultivation in the absence of ethanol, the C9 strain retains its ability to grow in 25 grs/lt - ethanol containing broths, with an II (cell yeild) of about 50%. Our conclusion was therefore, that C. thermocellum C9 is an ethanol-resistant mutant.

iii. other characteristics of C. thermocellum C9
and sugar fermenting abilities

C. thermocellum C9 exhibits the same fermentation pattern as the wild type strain. In CM4 medium (containing 5 grs/lt of yeast extract) both strains are able to ferment cellobiose, D-fructose, D-glucose and D-sorbitol, as well as cellulose; C9, however, has a reduced ability to produce the yellow pigment during cellulose fermentation. Neither of the strains, on the other hand, was able to ferment, after 72 hrs at 60°C, D- and L-arabinose, D-arabitol, D- and L-fucose, D-galactose, D- and L-lyxose, D-mannose, L-rhamnose, D-ribose, L-sorbose, D-tagatose, D-xylose, lactose, manitol, melibiose, maltose, raffinose, stachyose or threalose.

Figure I.C.2.2 Confirmation Procedure



Note: II^{CY} (inhibition index measured for cell yield).

b. Growth rate

C. thermocellum C9 grows better on CM4-cellobiose medium at 46°C (generation time of 6.24 hrs) than the wild type strain (Generation time 7.45 hrs). This situation is reversed at control temperatures (around 60°C) at which the generation times were 3.0 hrs for the wild type and 3.9 hrs for the C9 strain.

c. Enzymatic activities

C9 and wild type strains were assayed for filter paper-ase, xylanase and carboxymethyl cellulose activities, by the ethanol precipitation technique, as previously described.

The results are shown below.

	<u>mg R.S</u> <u>ml.hr: mg.prot.</u>		<u>Ratio</u>
	<u>ATCC</u>	<u>C9</u>	<u>C9/ATCC</u>
Filter paper-ase	0.48	0.96	2
Xylanase	37.1	59.1	1.6
CMC-ase	55.1	57.7	1.05

The protein concentration of the reaction mixture was assayed by the Bio-Rad Protein assay system using the standard curve for Bio-Rad Protein Standard. The C9 strain excretes an increased level of filter paper-ase activity. This result is not surprising since it has been selected in α -cellulose, which has a 67% cristallinity index (Zeikus et al., Arch. Micro. 114:1-7, 1977).

d. Growth inhibition of C. thermocellum C9 and wild type strains by fermentation and related products

Three groups of compounds, alkanols (ethanol, propanol and butanol), short-chain fatty acids (acetate, propionate and butyrate-sodium salts, pH 7.4) and hydroxyacids (lactate and β -hydroxy-butyrate-sodium salts, pH 7.4), were used to challenge both strains during balanced growth at 50°C. The experimental protocol was at that described in our last progress report, (Section I.C.2). The results of this experiment are shown in Figures I.C.2.3 and .4.

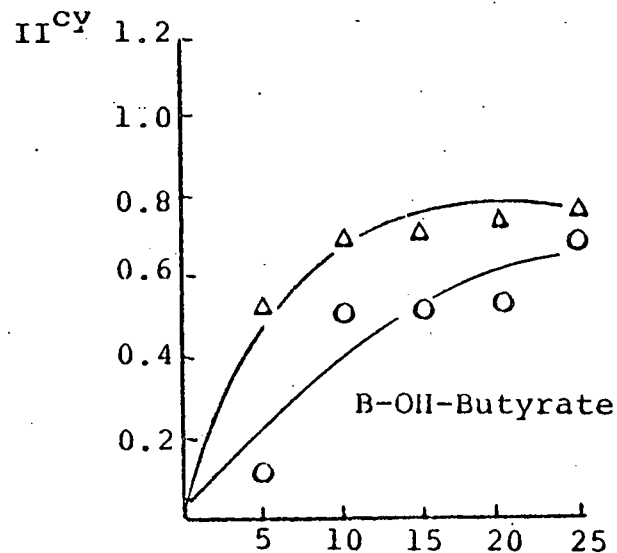
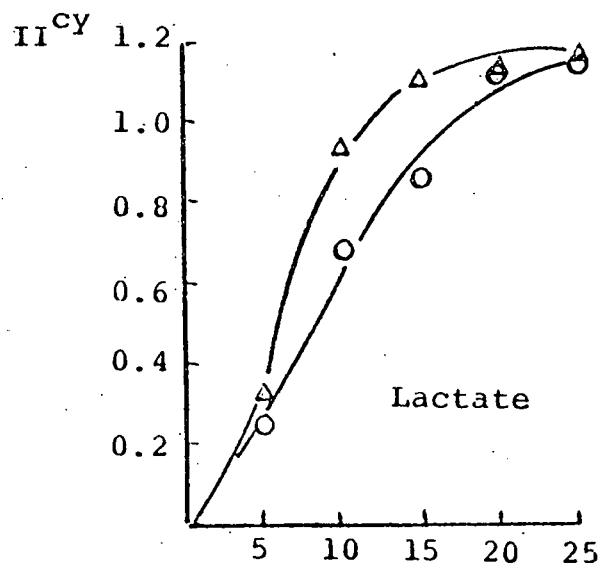
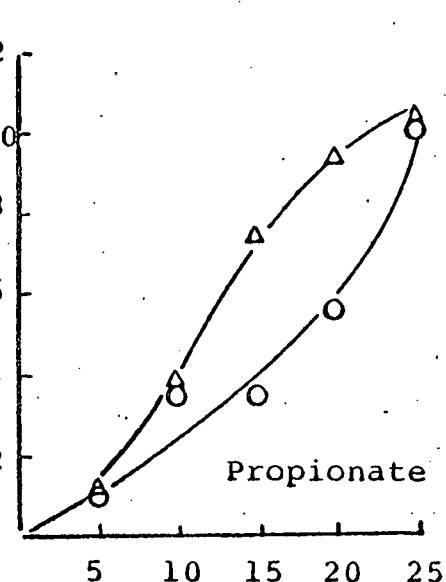
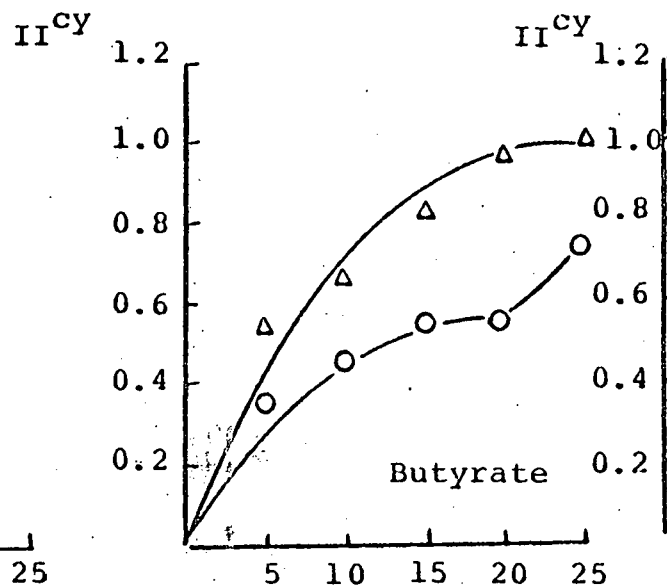
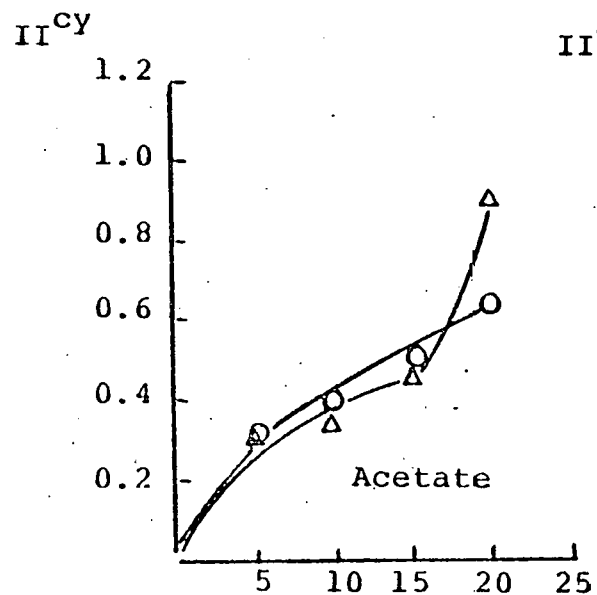
The ethanol-resistant strain C9 has increased resistance against all the compounds tested, except for acetate, against which both C9 and wild type strains show a similar response.

These data show that alcohols are more inhibitory than the sodium salts of the short chain fatty acid-salts tested, and that the larger the chain, the higher the growth inhibition promoted by the compound, except for the hydroxyacid-salts group.

e. Growth of C. thermocellum in glucose as C-source
i. introduction

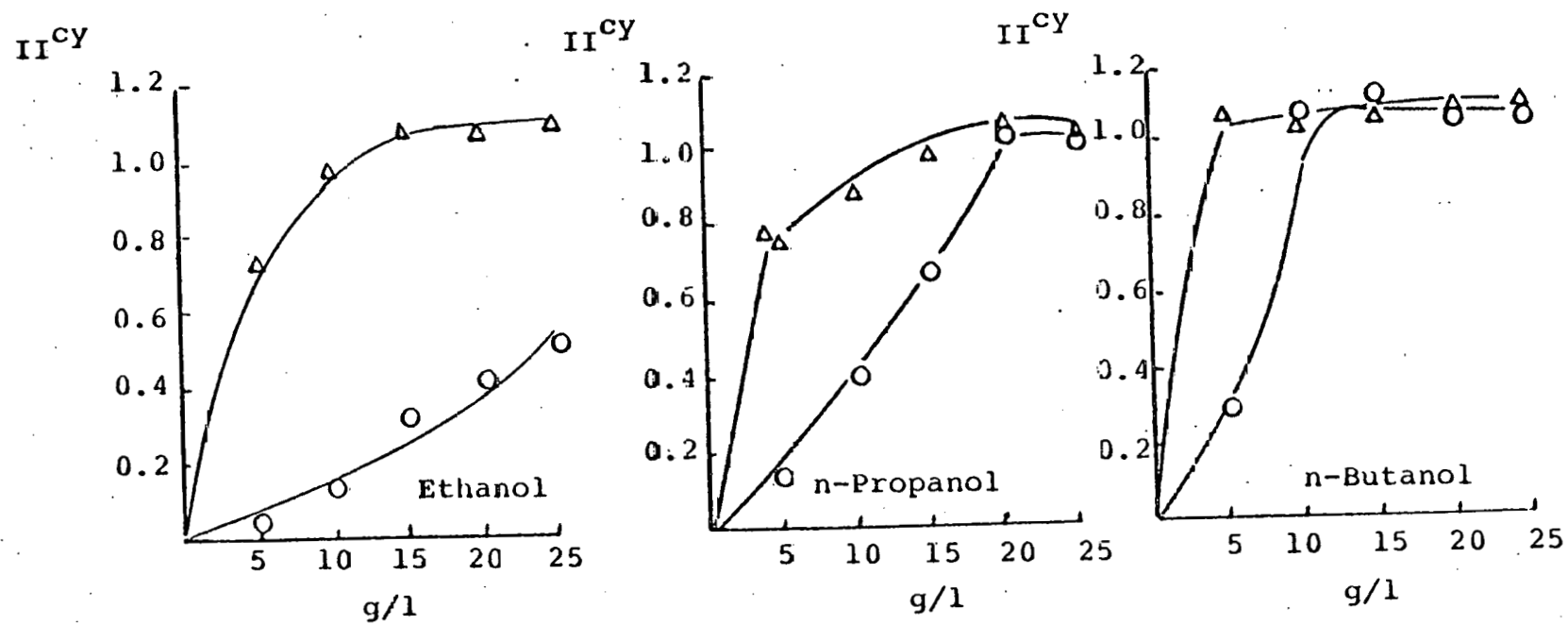
There has been much controversy about this point in the literature since the early isolation of C. thermocellum by R.H. McBee in 1954 (J. Bacterial, 67:505-506, 1954), who stated the absence of growth in glucose. In 1971, N.J. Patni and J.K. Alexander (J. Bacterial, 105:220-225, 1971), reported

Figure I.C.2.3 Growth Inhibition by Fatty Acids Salts. Plot of Inhibition Index (Cell Yield) vs. Salt Concentration in Grams/L.



△ C9
○ ATCC (w.t.)

Figure I.C.2.4 Growth Inhibition By Alcohols. Plot of Inhibition Index (Cell Yield) vs. Alcohol Concentration in Grams/L.



Δ C9
 $\circ$ ATCC (w.t.)

that limiting glucokinase - an inducible enzyme - was responsible for that lack of growth. Glucokinase was fully synthesized when higher amounts of yeast extract were present in the media. In spite of this, T.K. Ng, P.J. Weimer and F.G. Zeikus (Arch. Microbiol. 114:1-7, 1977), reported the absence of growth in glucose and attribute this occasional occurrence to a contamination problem.

Our results suggest that induction is necessary for growth of C. thermocellum to occur in a glucose-yeast-extract containing medium.

ii. induction is required for growth in glucose

A long lag has been observed before growth takes place (4 to 6 days) when C. thermocellum grown in CM4-cellobiose is inoculated into CM4-glucose broth. The question we asked was, is this lag due to a phenotypic lag or to the appearance of an occasional mutant, or contaminant, which developed in CM4-glucose broths. To answer this question we performed an evaluation of the growth in CM4-glucose by the Most Probable Number technique (MPN). The results are shown in the following table and indicate that growth in glucose occurs after a phenotypic lag and that all bacteria in the population have the capacity to grow on glucose.

<u>Dilution</u>	<u>Cellobiose</u>	<u>Glucose</u>
	<u>Growth at 24-48hrs.</u>	<u>Growth at 80-100hrs.</u>
10 ⁻¹	+ + +	+ + +
10 ⁻²	+ + +	+ + +
10 ⁻³	+ + +	+ + +
10 ⁻⁴	+ + +	+ + +
10 ⁻⁵	+ + +	+ + +
10 ⁻⁶	+ + +	+ + +
10 ⁻⁷	+ + +	+ + +
10 ⁻⁸	+ + +	+ + +
10 ⁻⁹	+ + +	+ + +

Future Work:

- . To characterize the resistance of C9 strain at different temperatures.
- . To determine differences in lipid composition between C9 and wild type strains
- . To determine what factors are necessary for C. thermocellum to grow in glucose.

D. Genetic Manipulations

The first priority of this portion of the project continues to be the development of gene transfer systems in C. thermocellum. Development of techniques for using protoplasts to this end continues, and is concerned with achieving reproducible regeneration of normal cells from C. thermocellum protoplasts. In addition, experiments have been initiated which are aimed at achieving the transformation of these protoplasts, using the techniques at their current state of development.

1. Protoplast regeneration

The best conditions for protoplast induction and stabilization were found to be when actively growing cultures of Clostridium thermocellum are treated with lysozyme, and polyethylene glycol (PEG) is used as stabilizer (last progress report).

We attempted to use the above conditions to obtain regeneration of the induced protoplasts. We had found that 10% PEG would be a very good stabilizer while not being inhibitory for cell growth. Since no regeneration could be observed in liquid cultures, agar or soft agar media was used. 10% PEG inhibited the solidification of CM4 agar or soft agar and we therefore used 5% PEG. This is still a good stabilizer. We used the method of soft agar overlayed on hard agar plates. Cells were grown in CM4 medium to an OD₆₆₀ of 0.3, transferred to 45° for thirty minutes, brought to 5% PEG and then 50 ug/ml lysozyme was added and the culture was incubated for a further fifteen minutes at 45° and 45 minutes at 60°. (A control culture was not treated with lysozyme.) At this stage no cells could be observed (under the microscope) from the treated culture

and only protoplasts and cell debris could be seen. The protoplasts and the control cells were centrifuged and washed twice with CM4 containing 5% PEG, and then diluted in peptone water with or without 5% PEG, 0.5 ml of the diluted suspension was added to soft agar (2.5 ml) and overlayed over the agar plates. The agar and soft agar used to plate PEG-containing dilutions also contained 5% PEG. Plating was carried out in an anaerobic glove box. The plates were incubated anaerobically for 48 hours at 60°.

The results obtained are shown in Table I.D.1.1. colonies were formed only from the protoplasts plated in the presence of 5% PEG while without PEG no colonies appeared. It seems to be a regeneration of more than 20% when the number of colonies on the 5% PEG plates is compared to the number of protoplasts counted in a Pretroff-Hauser counting chamber, although the counting of protoplasts under the microscope is not very accurate.

This procedure is not yet reliably reproducible, so work will be directed at improving the reliability of the regeneration technique.

The ability to reliably regenerate protoplasts will allow us to test several different gene transfer methods. Among these, the two which will receive the most attention are PEG-facilitated transformation of protoplasts and PEG-mediated protoplast fusion.

2. Transformation/Transfection

The collection of suitably marked DNA's with which to test the transformability or transfectability of C. thermocellum is in progress and protoplast transformation experiments have been initiated.

Table I.D.1.1 Regeneration of C. Thermocellum Protoplasts

	<u>Lsozyme Treated culture</u>		<u>Control Culture</u>	
	<u>-PEG</u>	<u>+5%PEG</u>	<u>-PEG</u>	<u>+5%PEG</u>
plate count, $\frac{\text{cfu}}{\text{mg}}$	0	3×10^7	2×10^8	1.2×10^8
microscope count cells or protoplasts/ml	-	1.4×10^8 (protoplasts)	-	1.2×10^8 (cells)

a. Search for Plaque-Formers:

One such marked DNA is plaque-forming phage DNA. Accordingly, a method for isolating plaque formers in C. thermocellum was adopted. A likely inoculum is shaken with an equal volume of 0.1 M MgSO_4 . This is coarsely filtered and then membrane-filtered through a 0.45 micron filter. The filtrate is added to a young culture of C. thermocellum for enrichment. When the culture has grown, a filtrate of the broth is mixed at various dilutions with growing cells and plated using a soft agar overlay. Activated sludge from the Boston works and compost from a local source have both been tested without success. This search will continue, using the effluents of anaerobic, thermophilic digestors as source.

b. Protoplast Transformation Trials

A first attempt has been made to transform lysozyme-treated cells using chromosomal DNA from antibiotic resistant mutants of C. thermocellum already in hand. The results of this are not yet available.

Future work will include attempts to transform these lysozyme-treated cells with genetically marked covalently closed circular DNA obtained from other organisms. Some C. perfringens resistance factors recently obtained from another investigator will get early attention.

3. Plasmid Isolation

Work aimed at elucidating the nature and behavior of plasmids in C. thermocellum continues, at a lowered priority.

The method of Hansen and Olsen, J. Bacteriol. 135:227-238 (1978), was used for the first attempt to isolate and characterize these plasmids using agarose gel electrophoresis. No plasmid band was detected. This work will be repeated using other lysis methods.

E. Deregulation of Clostridial Cellulase

1. Introduction

In our earlier report (3/1/78 to 5/31/78), while studying the effect of nitrogen source on growth and enzyme production, we showed that yeast extract concentration is an important factor in the growth medium as it can serve as sole nitrogen source. It was also shown that an increase in yeast extract concentration from 0.2% to 0.6% results in an increase in enzyme production. Our studies on the maintenance of pH during the flask cellulase fermentation have shown that morpholinopropane-sulfonic acid (MOPS) buffer at 0.1M concentration is a suitable buffer for growth and enzyme production in shake flasks.

In this report, we shall present our results on growth and enzyme production in the "GS" medium containing higher yeast extract concentration (0.6%), urea (as the nitrogen source) and MOPS as the buffer in comparison to the CM-3 medium.

Growth and production of cellulase is also studied by replacing cellobiose with various other sugars such as glucose, fructose, mannose and mannitol at the new higher yeast extract concentration. The purpose is to check whether any of these sugars are utilized by the organism for growth and production of cellulase when yeast extract is raised to 0.6%, as claimed in some literature reports using other strains of C. thermocellum.

We also report on the stimulatory effect of a thiol reducing agent (cysteine-HCl) on the TNP-CMCase assay.

In previous progress reports, we reported on the isolation of a cellulase-overproducing mutant of C. thermocellum ATCC 27405,

designated as AS-39, after UV radiation. Further mutation experiments with mutant AS-39 using UV radiation has not resulted in the isolation of higher cellulase over-producers, and so other methods of chemical mutagenesis using EMS and NTG are being employed.

2. Materials and Methods

a. Organism:

C. thermocellum ATCC 27405 and the mutant obtained from this strain (mutant AS-39) were used in this study.

b. Anaerobic Methods:

The anaerobic culture technique of Hungate as modified by Bryant was used throughout the course of this work. Anaerobic culture tubes contained 10 ml of medium. Flask cultures were grown in 500 ml Erlenmeyer flasks that contained 200 ml of medium. All flasks were sealed with neoprene stoppers.

c. Cultural Conditions:

The organisms were grown in CM-3 medium and/or the new GS-medium. In GS medium, which is modified from CM-3 medium, ammonium sulfate is replaced by urea (2.14 g/l) and yeast extract is used at a higher (0.6%) concentration. In addition, GS medium contains 0.1 M MOPS buffer. The initial pH of GS medium is adjusted to 6.8. The cysteine-HCl and the sugars are sterilized separately and are added at the time of inoculation under constant vigorous gassing with N₂ (See Table I.E.1.).

Stock cultures of the organisms were maintained through weekly transfers of 1 ml culture into fresh medium. All cultures were incubated at 60°C without shaking.

Table I.E.1.

Composition of CM-3 and GS media

Ingredients	CM-3	GS
KH_2PO_4	1.5 g	1.5 g
K_2HPO_4	2.9 g	2.9 g
$(\text{NH}_4)_2\text{SO}_4$	1.3 g	-
Urea	-	2.14 g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1.0 g	1.0 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.15 g	0.15 g
$\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$	1.25 mg	1.25 mg
cysteine-HCl	1.0 g	1.0 g
yeast extract	2.0 g	6.0 g
Resazurin	2.0 mg	2.0 mg
Cellobiose	10.0 g	10.0 g
MOPS	-	20.93 g
Final volume	1 liter	1 liter
pH	7.0	6.8

Growth was measured as absorbance using the red filter in the Klett instrument.

d. Analysis of Cellulolytic Activity:

In the supernatant assay, cultures were centrifuged at 10,000 x g for 10 minutes and 1 ml of this broth supernatant was added to 2 ml of a 1% suspension of TNP-CMC in 0.1 M citrate-buffer (pH 4.5). A zero-time sample was millipore-filtered immediately. A control was run in which the broth culture was substituted by buffer. The tubes were incubated at 60°C for 1 hour and millipore filtered. Absorbance was read at 344 nm, the increase in absorbance between 0 and 60 min was calculated, and the control value was subtracted from the experimental value. The TNP-CMC activity is represented as absorbance units/hr/ml enzyme.

e. Mutagenesis

We have been working with a plate mutation system using ethyl methanesulfonate (EMS) in 5-100% concentration (in methanol) added to 6.35 mm filter paper discs. The disk was placed on CM3 agar medium with 2% avicel and 0.2% cellobiose seeded with a suspension of log phase cells of AS-39. We have developed this method further according to the following procedure. Sandwich plates are prepared by using 10 ml of CM3 agar medium without cellobiose or avicel for the top and bottom layers. The middle layer consists of 15 ml of CM3 agar medium with a reduced amount of cellobiose (0.05%) from that previously used, 2% avicel and a suspension of log phase cells of mutant AS-39. Filter paper discs (6.35 mm diam.) are dipped in a 100% solution of EMS and are placed on the center surface of the top agar layer.

Plates are then incubated at 60°C for 7 days. Promising (zone-size) colonies are removed by a sterile Pasteur pipet and ejected into Hungate tubes containing CM3 medium (0.5% cellobiose) under N₂. These are then incubated at 60°C for 40 hours, and TNP-CMCase levels are assayed. Identically prepared plates using CM3 medium (at pH 6.5 and the normal pH 7.0), and replacing EMS-filter paper discs with 2-5 mg of crystals of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) have also been tried.

3. Results and Discussion:

Approximately 50% higher cellulase production was observed in the new GS medium, i.e., when 0.1 M MOPS buffer was used in the medium along with 0.6% yeast extract and 0.21% urea. The increase in enzyme production was observed with both parent and mutant. Figure I.E.1. shows the growth and I.E.2. the cellulase production by the parent and the mutant AS-39 in CM-3 medium and the modified GS medium throughout the growth cycle. The AS-39 mutant has repeatedly shown twice the TNP-CMCase activity compared to the parent strain when CM-3 medium was used. A similar superiority is seen in the GS medium.

Another important question is the utilization of glucose and other carbohydrates by this organism. C. thermocellum has been described as a thermophilic anaerobe that ferments cellu-

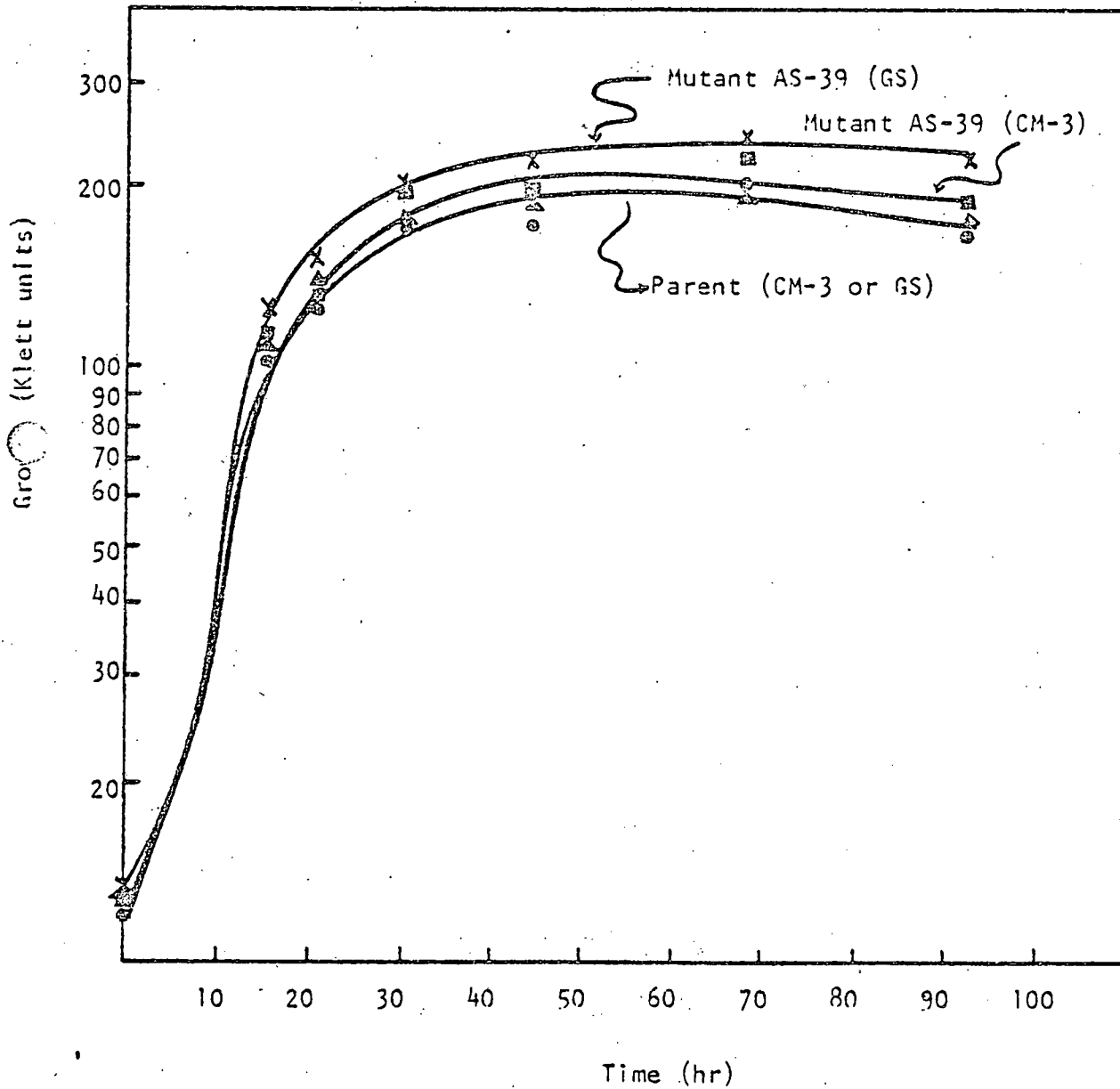


Fig. I.E.1. Growth of *C. thermocellum* ATCC 27405 (parent) and mutant AS-39 in CM-3 and GS media.

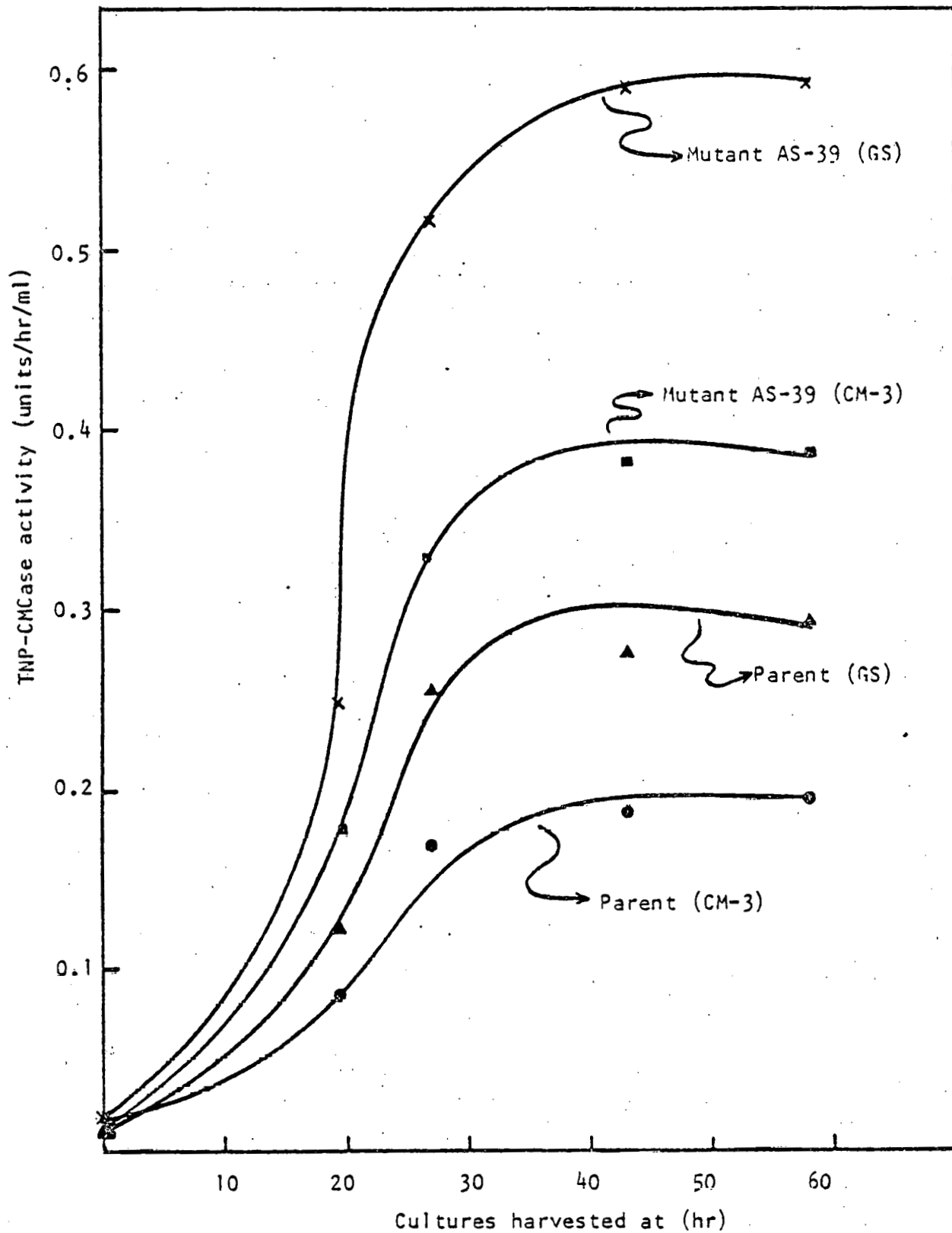


Fig. I.E.2. TNP-CMCase production by strains ATCC 27405 and mutant AS-39 in CM-3 and GS media.

lose, hemicellulose and cellobiose but is unable to ferment glucose, fructose, mannose, mannitol and a number of other carbohydrates at low (0.05 - 0.1%) yeast extract concentrations.

Figure I.E.3. shows the growth kinetics of the mutant AS-39 using different carbon sources in the GS medium. It can be seen that significant growth of this organism occurs on glucose when yeast extract concentration of the medium is elevated.

Similar results were observed when fructose was used as the main carbon source in the GS medium. Though the growth of the organism was a little less compared to glucose supplemented medium, the TNP-CMCase activation of the culture was the same as that of glucose-grown cultures. In the medium containing 0.2% yeast extract some growth on glucose and fructose can also be seen. At either low or elevated concentrations of yeast extract, growth was not observed when mannose and mannitol were used as the major source of carbon.

It was interesting to observe that the organism not only grows on glucose and fructose under the elevated yeast concentrations but also produces cellulase. Table I.E.2 shows the enzyme activity obtained from different sugars. About 50% as much cellulase activity was observed from the cultures grown on glucose and fructose compared to the cultures grown on cellobiose.

In this study, we have thus confirmed the findings obtained by others (with other strains) that C. thermocellum ferments glucose and fructose when a high yeast extract concentration is included in the medium. Under these conditions, it also produces a significant amount of cellulase. Our data fail to confirm the

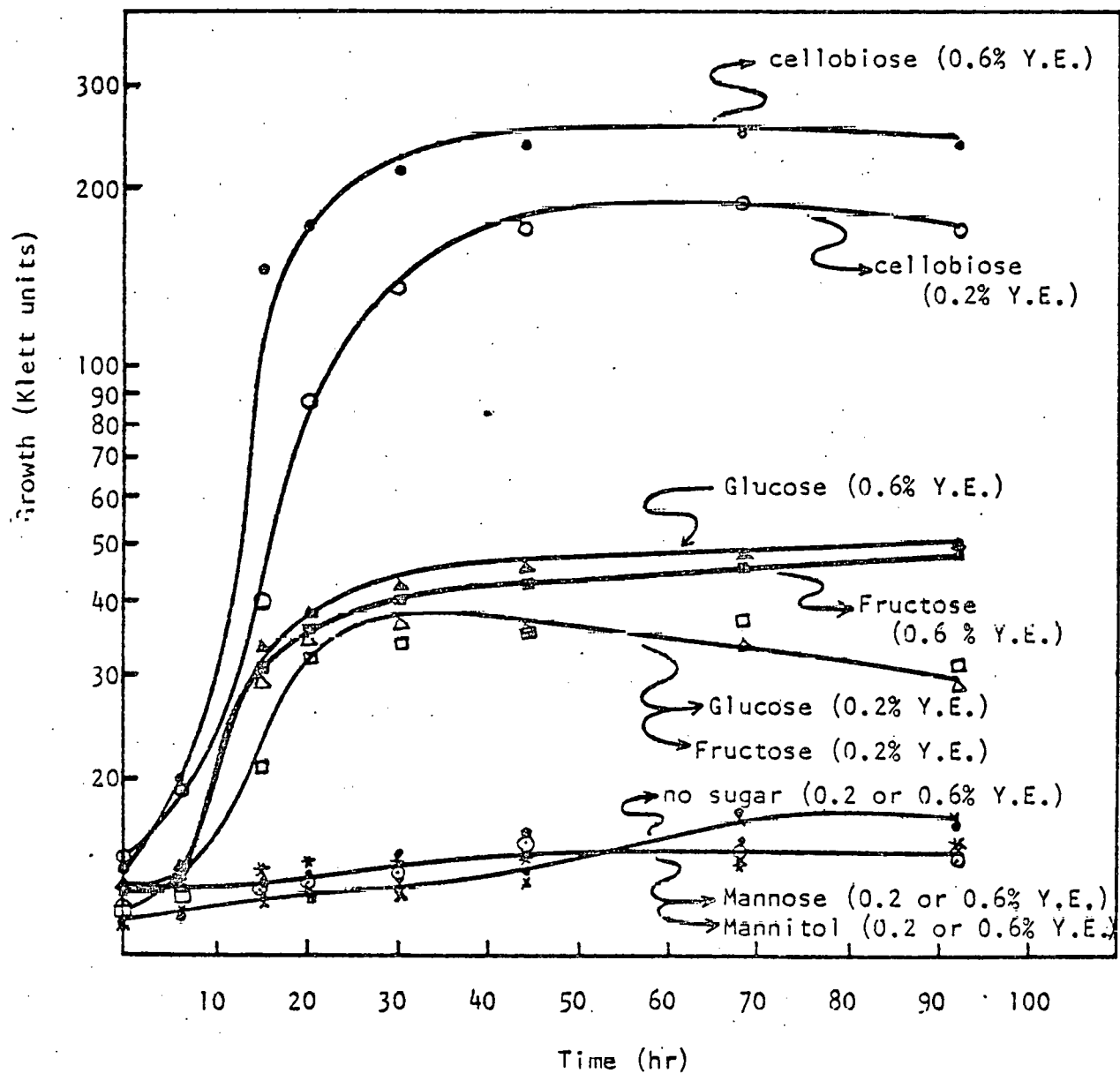


Fig. I.E.3. Growth of *C. thermocellum* AS-39 on carbon sources at two levels of yeast extract.

Table I.E.2.

TNP-CMCase activity of cultures grown on cellobiose, glucose and fructose as the carbon source in GS medium containing different amounts of yeast extract

	TNP-CMCase Activity					
Carbon Source	Cellobiose (1%)		Glucose (1%)		Fructose (1%)	
Yeast extract (%)	0.2	0.6	0.2	0.6	0.2	0.6
Cultures harvested at						
48 hr	0.245	0.297	0.150	0.140	0.108	0.121
72 hr	0.284	0.281	0.114	0.107	0.125	0.125
96 hr	0.282	0.280	0.140	0.157	0.114	0.135

reported ability of C. thermocellum to grow on mannose and mannitol.

The growth media, both CM-3 and the modified GS medium, contain cysteine-HCl, a strong reducing agent. It was of interest to study the effects of cysteine-HCl on enzyme activity in vitro. Table I.E.3. shows the effects of various concentrations of cysteine-HCl on cellulase activity. It was noted that cysteine at a concentration as low as 20µg/ml stimulated TNP-CMCase activity by 45%. Maximum stimulation (65%) was observed when 1mg of cysteine was included per ml of reaction mixture. This is the same concentration of cysteine which is used in the fermentation medium (1 gm/liter).

After establishing the optimum concentration of cysteine-HCl for maximum stimulation of TNP-CMCase activity, a time curve of the enzyme assay with this amount of cysteine-HCl was studied (Figure I.E.4). A linear relationship between the TNP-CMCase activity and the incubation time (at 60°C) was observed up to 60 minutes in the presence of the cysteine-HCl in the reaction mixture.

In the plate mutagenesis procedure, during incubation, the mutagen diffuses from the disk down and outwards into the agar medium forming a concentration gradient and a zone of no growth (100% kill or inhibition). Colonies growing at the edge of the zone are smaller, with smaller Avicel hydrolysis zones, while colonies further out are bigger with correspondingly larger hydrolysis zones. Furthermore, the colonies are generally more uniform in size and appearance than those from plates prepared in the previous way. The sandwich plate method eliminates spreading

Table I.E.3.

Effect of cysteine-HCl on TNP-cellulase activity in vitro

Cysteine-HCl added to the reaction mixture* (mg/ml)	TNP-CMCase activity units/hr/ml	% increase
0 (control)	0.293	control
0.1	0.443	51
0.5	0.456	55
1.0	0.484	65
1.5	0.448	53
2.0	0.470	60

*cysteine-HCl was added before the incubation at 60°C for 1 hr.

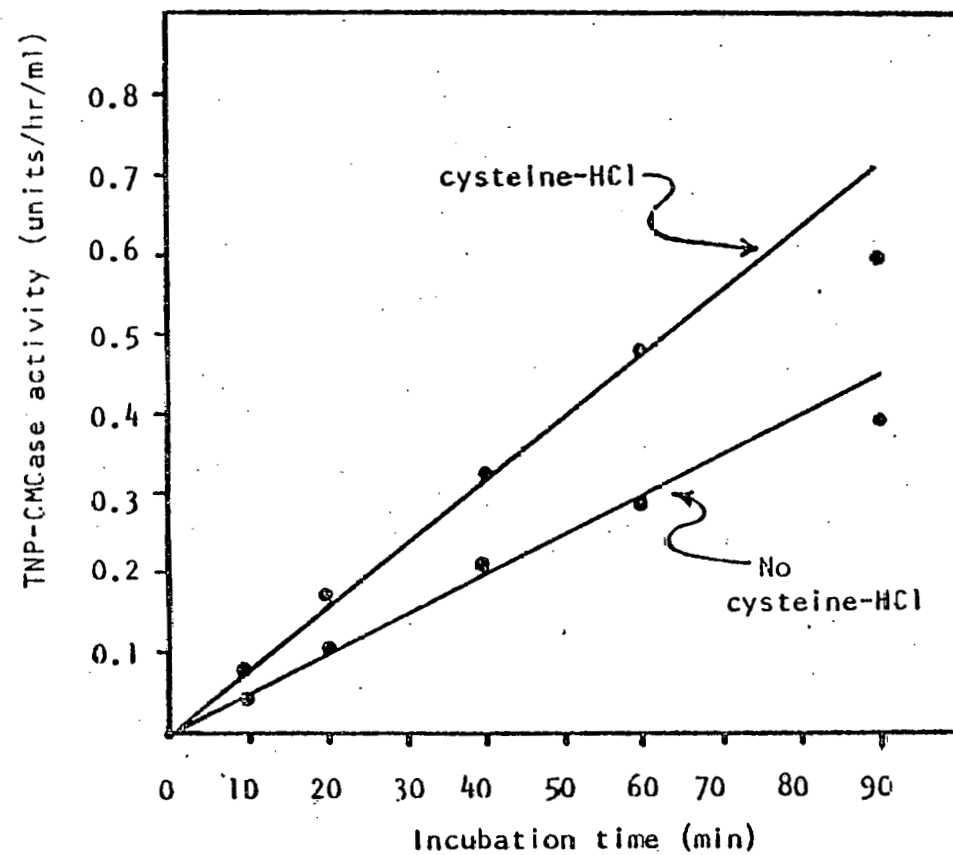


Fig. I.E.4. Effect of 1 mg/ml cysteine-HCl on TNP-CMCase activity

colonies at the air/agar and agar/plastic interfaces. Use of 2-5 mg of NTG crystals produces a smaller zone of no growth than does EMS, while in previous experiments NTG concentration of 100-200 µg/6.35 mm filter paper disc showed no inhibitory effect. There is about a 50% inhibition of cell growth on control plates at pH 6.5 (at which NTG is more stable) than at the usual pH 7.0.

Thus far, over 65,000 colonies from sandwich plates treated with EMS have been screened using mutant AS-39 as the parent culture. One possible mutant, CM-17-3, on first assay showed a 100% increase in TNP-CMCase activity, and this is being tested further. From sandwich plates treated with NTG, about 6,000 colonies have been screened with none so far indicating higher cellulase enzyme levels than the parent AS-39.

4. Future Work

Future studies on this segment of the research will include:

- . a study of possible glucose repression of cellulase synthesis during growth on cellulose and cellobiose at high yeast extract concentrations;
- . a study of the ability of glucose to support cellulase production after repeated transfers in glucose medium in the absence of cellulose and cellobiose;
- . a study of the ability of vitamins to replace the low (0.1%) yeast extract requirement of growth on cellobiose;
- . a study of the nature of the factor in yeast extract allowing growth on glucose;
- . a study of the effect of surface active agents on cellulase production.

- . Chemical mutagenesis in liquid cultures in anaerobic flasks and Hungate tubes, using different concentrations of EMS and NTG for varying times of exposure. Mutagen concentration/exposure time values giving 50% and greater inhibition of growth will be tested and survivors will be plated out on higher Avicel concentrations (3-5%) and with less cellobiose (0.01% or 0%). In addition, mutagenized suspensions of cells or possible cellulase overproducing mutants will be subjected to serial transfer into fresh Avicel medium over several weeks, in hope of selecting for the fastest grower and producer of cellulase.

F. Other Enzyme Studies on C. thermocellum

1. Optimization of Cellulase Assay Conditions

a. Introduction

Purification studies of the enzyme(s) responsible for cellulolytic activity in C. thermocellum require an implicit faith in the cellulase assay procedure. Prior to further attempts at purification, a series of experiments designed to optimize this assay were conducted.

b. Materials and Methods

All experiments were carried out on 48-hr cultures of C. thermocellum ATCC, grown at 60°C from a 10% inoculum of stationary phase cells. The growth medium in each case was CM-4 with 5% cellobiose as carbon source.

After incubation cells were harvested by chilling at 0°C for 15 min then centrifuged at 12,000 R.P.M. for 15 min in a cold Beckman JA-14 Rotor. The supernatant was decanted, then spun again. The clarified broth was made 40% in ethanol and incubated at 4°C for 24 hr, followed by centrifugation at 12,000 R.P.M. for 15 min. The pellet was resuspended in 1/10 the original broth volume of 0.05 M Na citrate, pH 4.8, and stored frozen at -16°C. This ethanol-precipitated enzyme preparation was used in all subsequent assays.

c. Standard Assay Procedures

Exoglucanase as filter paper activity was measured by incubation of 0.5 ml of enzyme with a 1 x 2 cm strip of Whatman #1 paper at 60°C for 60 min. Blanks for this reaction include citrate buffer, containing the substrate only, and

enzyme containing no substrate. Activity was measured against a blank containing buffer only. After incubation, the reaction was stopped by addition of 1.0 ml DNS reagent, then boiled, and the absorbance measured at 550 nm. Liberation of reducing sugars was expressed in units of m moles glucose liberated per hour. Endoglucanase as CMC activity was measured by incubation of 0.05 ml enzyme with 1.0 ml 2.0% CMC in citrate buffer, with addition of 1.15 ml buffer to a final volume of 1.2 ml. The reaction is incubated at 60°C for 60 min, then stopped by addition of 3.0 ml DNS reagent. The samples are quantitated as above.

Both assays are linear to 60 min, as illustrated in Figures I.F.1, 2. These and all subsequent assays were conducted in replicate tubes.

d. Kinetic Studies

Cellulase activity with respect to time was measured as a function of enzyme concentration as follows: various amounts of an enzyme preparation were assayed under standard conditions, the total reaction volume maintained at 0.5 and 1.2 ml for the filter paper and CMC assays, respectively by the addition of buffer. The time course of release of reducing sugars is illustrated in Figures I.F.3 and I.F.4. Initial rates are proportional to protein concentration (Figure I.F.5). These data are presented in Tables I.F.1 and I.F.2.

e. Temperature Studies

Activity was assessed as a function of temperature by incubation of the standard reaction mixture for 60 minutes

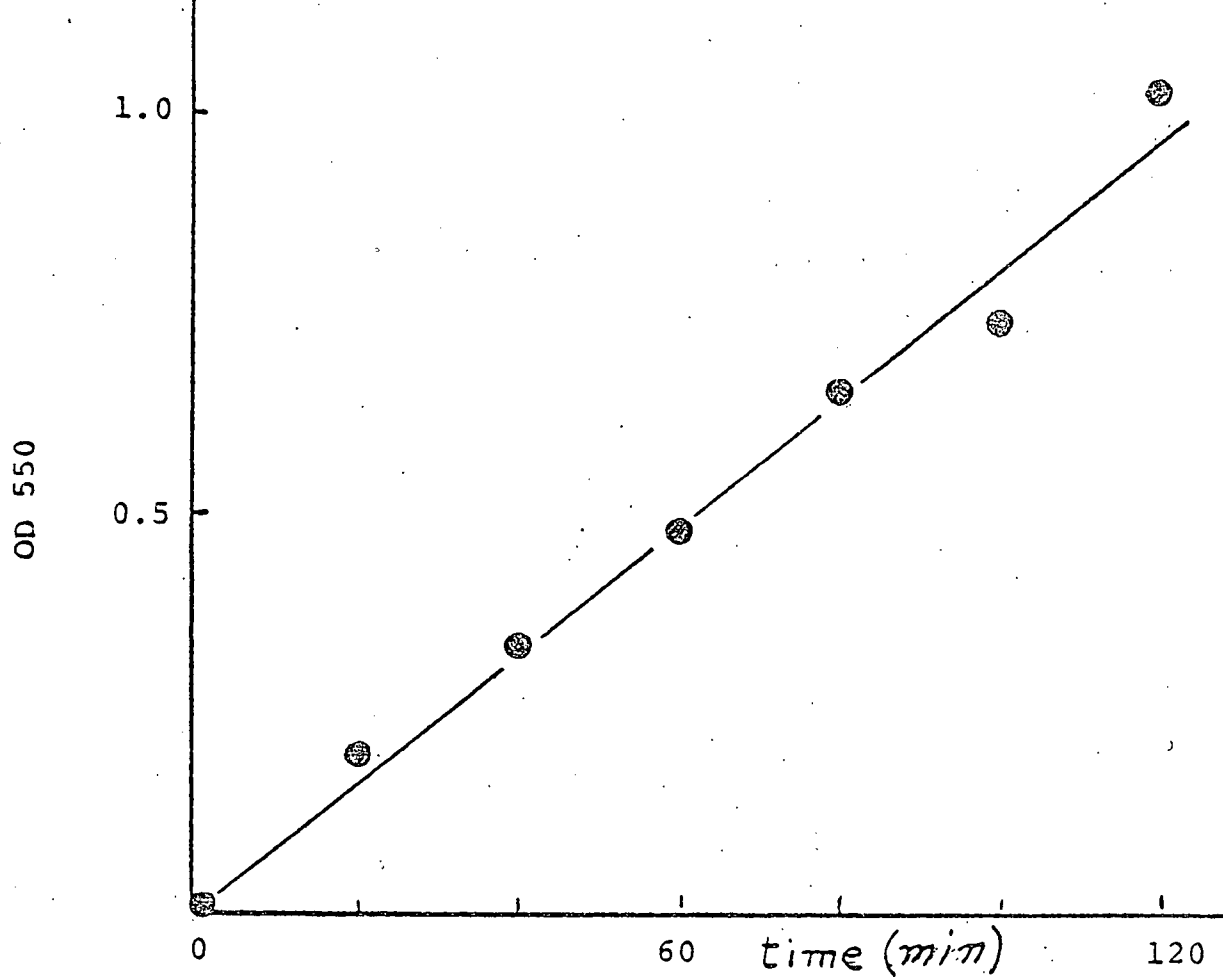


Figure I.F.2 Standard CMC Assay

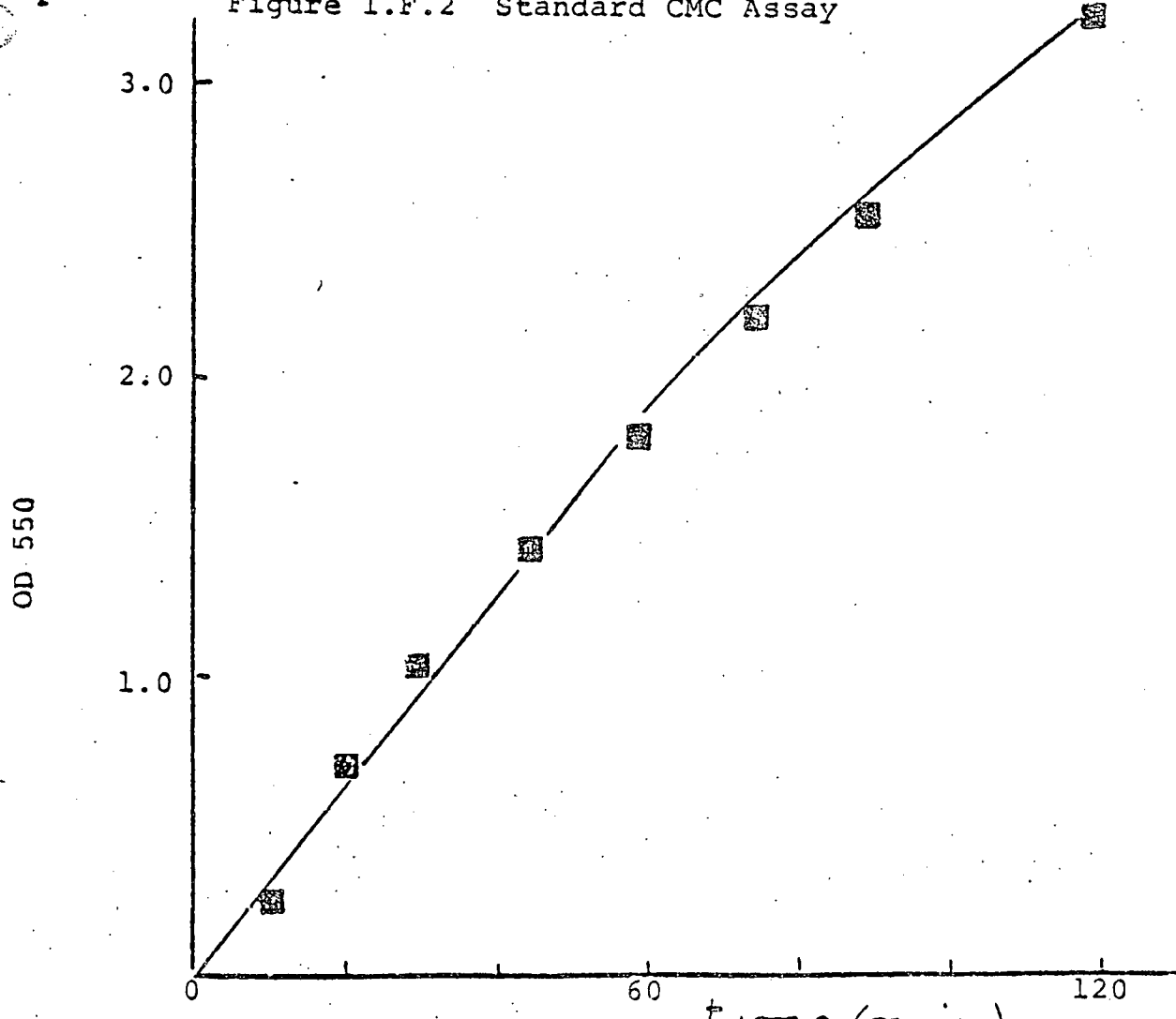


Figure I.F.3 Filter Paper Activity vs. Enzyme Concentration Over Time

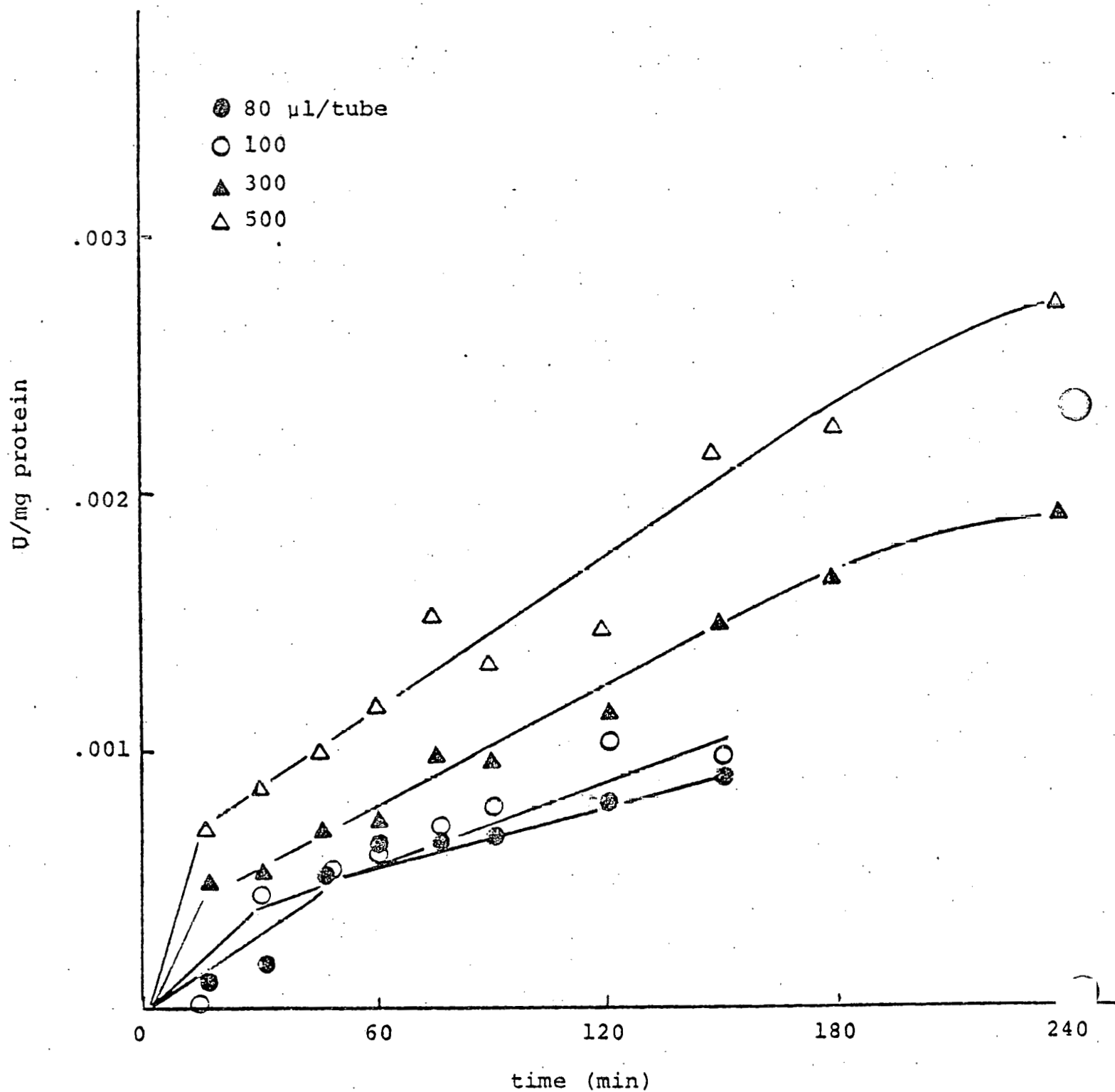


Figure I.F.4 CMCase Activity vs. Enzyme Concentration Over Time

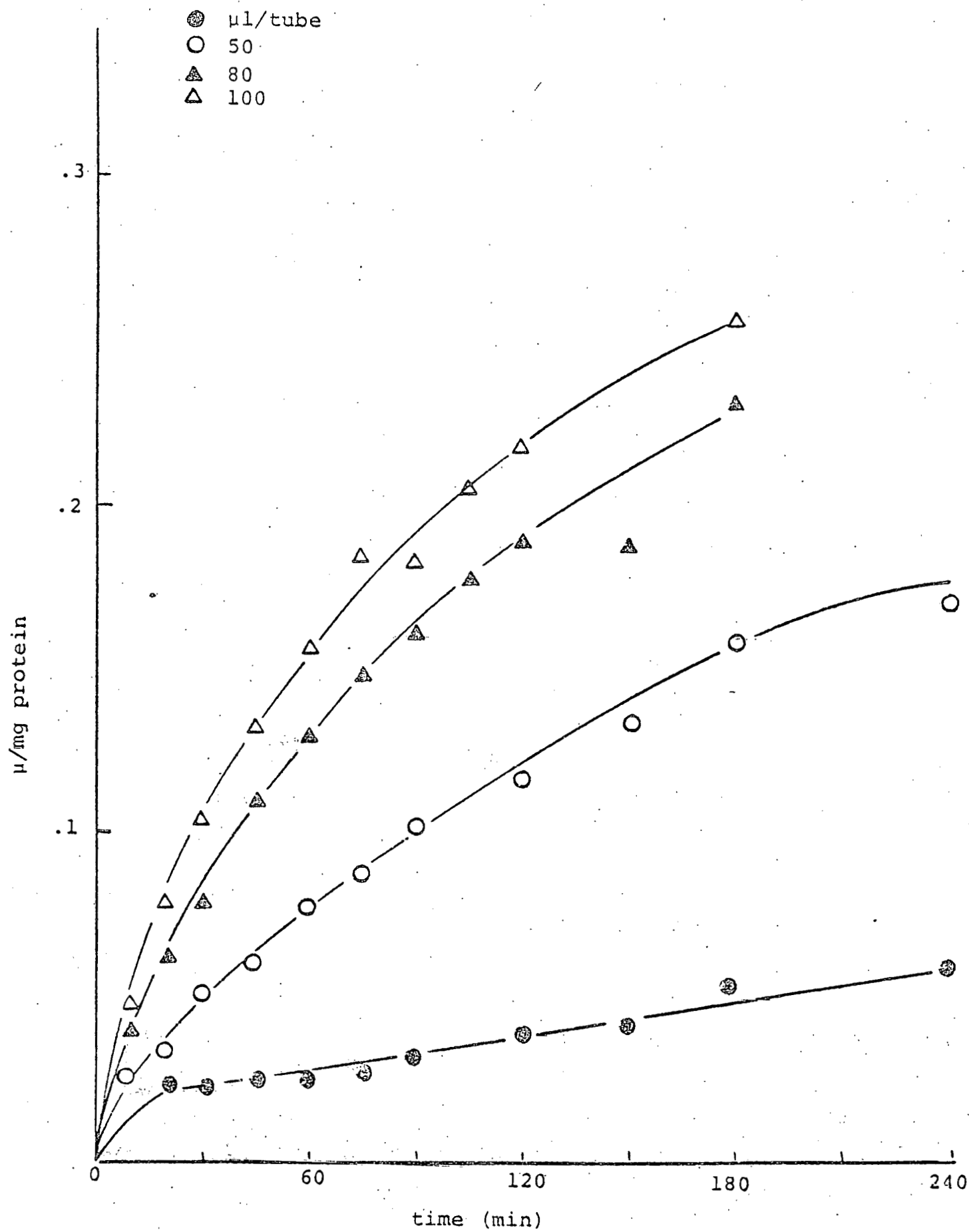


Figure I.F.5 Reaction Rate vs. Enzyme Concentration

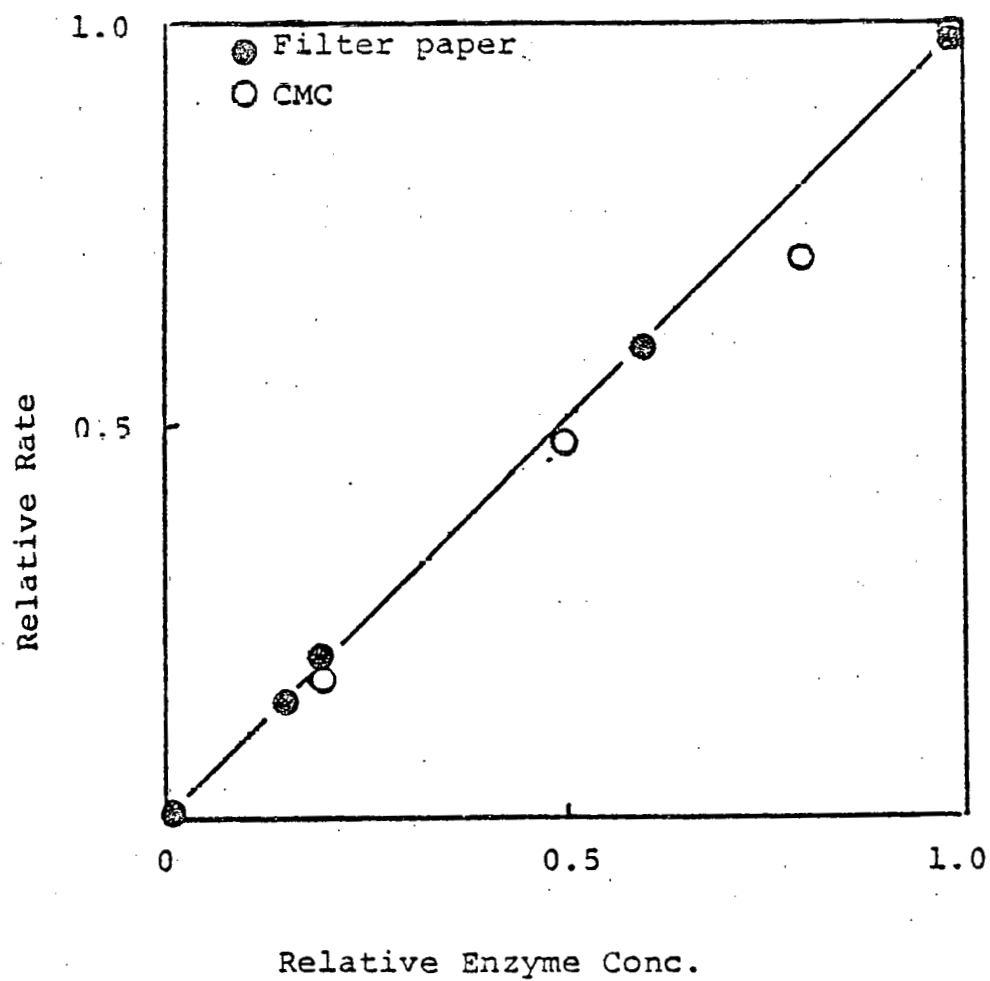


Table I.F.1. Filter Paper Activity vs. Enzyme Concentration

<u>Vol. Enzyme (ml)</u>	<u>Initial Rate (V/ml)</u>	<u>Relative Volume</u>	<u>Relative Activity</u>
0.080	0.00035	0.16	0.152
0.10	0.00047	0.20	0.204
0.30	0.00137	0.60	0.443
0.50	0.00230	1.0	1.00

Table I.F.2 CMC Activity vs. Enzyme Concentration

<u>Vol. Enzyme (ml)</u>	<u>Initial Rate (V/ml)</u>	<u>Relative Volume</u>	<u>Relative Activity</u>
0.020	0.0188	0.20	0.178
0.050	0.0500	0.50	0.473
0.080	0.0744	0.80	0.704
0.10	0.1056	1.0	1.00

at temperatures ranging from 50°C to 80°C in 5° increments. The results as plotted in Figure I.F.6 and I.F.7 indicate a temperature optimum of 65-70°C for both filter paper and CMC degradation.

f. pH Studies

Preliminary data indicate optimum for both reactions at some pH above the buffering range of sodium citrate (3.8-6.2). Optimization will require development of a suitable buffering system.

g. Future Experiments

Separation of endo- and exoglucanase activities with subsequent purification of the latter, will continue via traditional enzymological methods. Simultaneously, the search continues for an endoglucanase mutant, ie. one with no activity toward CMC. This strain should yield exoglucanase activity unmasked by the larger quantities of endoglucanase synthesized by the wild-type cell.

Figure I.F.6 Filter Paper Activity vs. T.

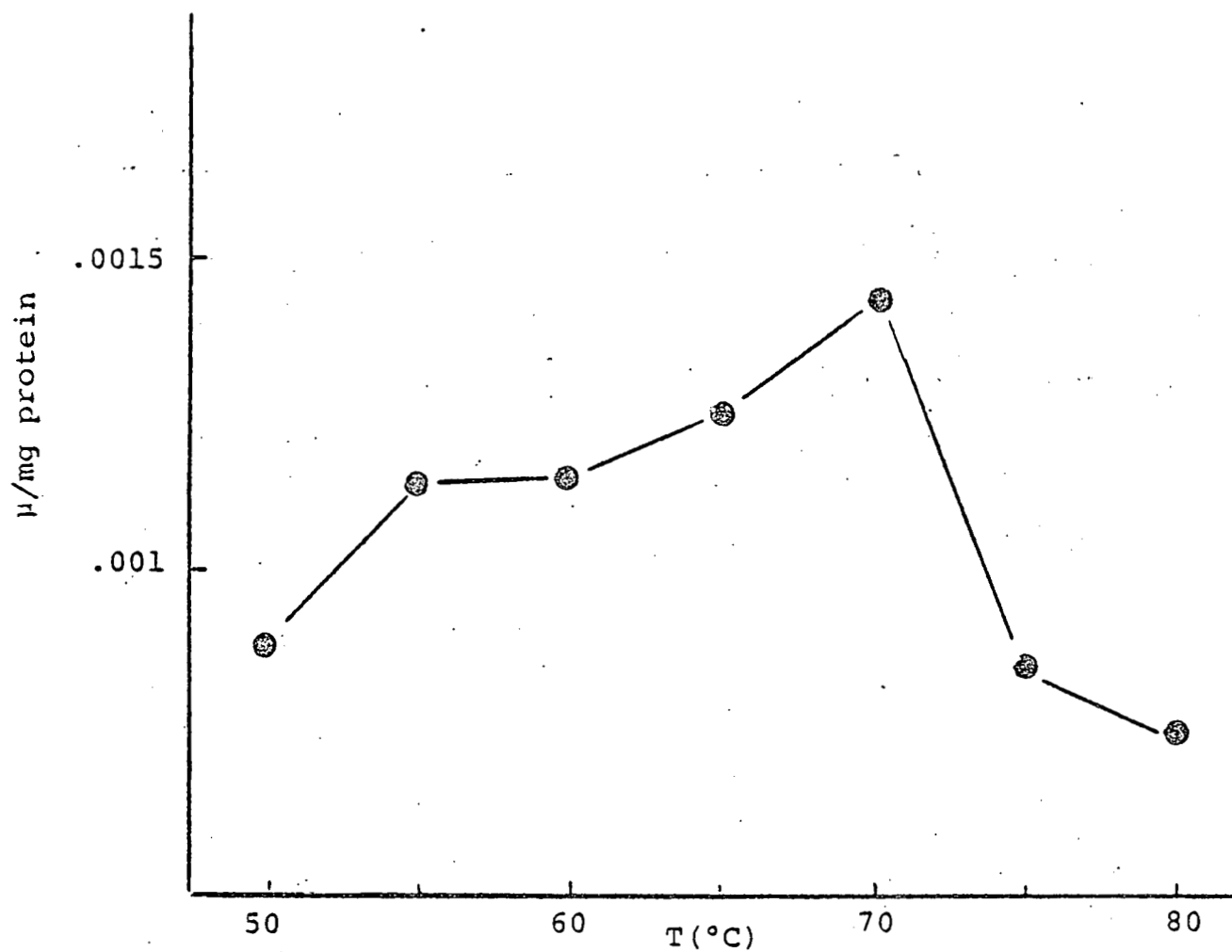
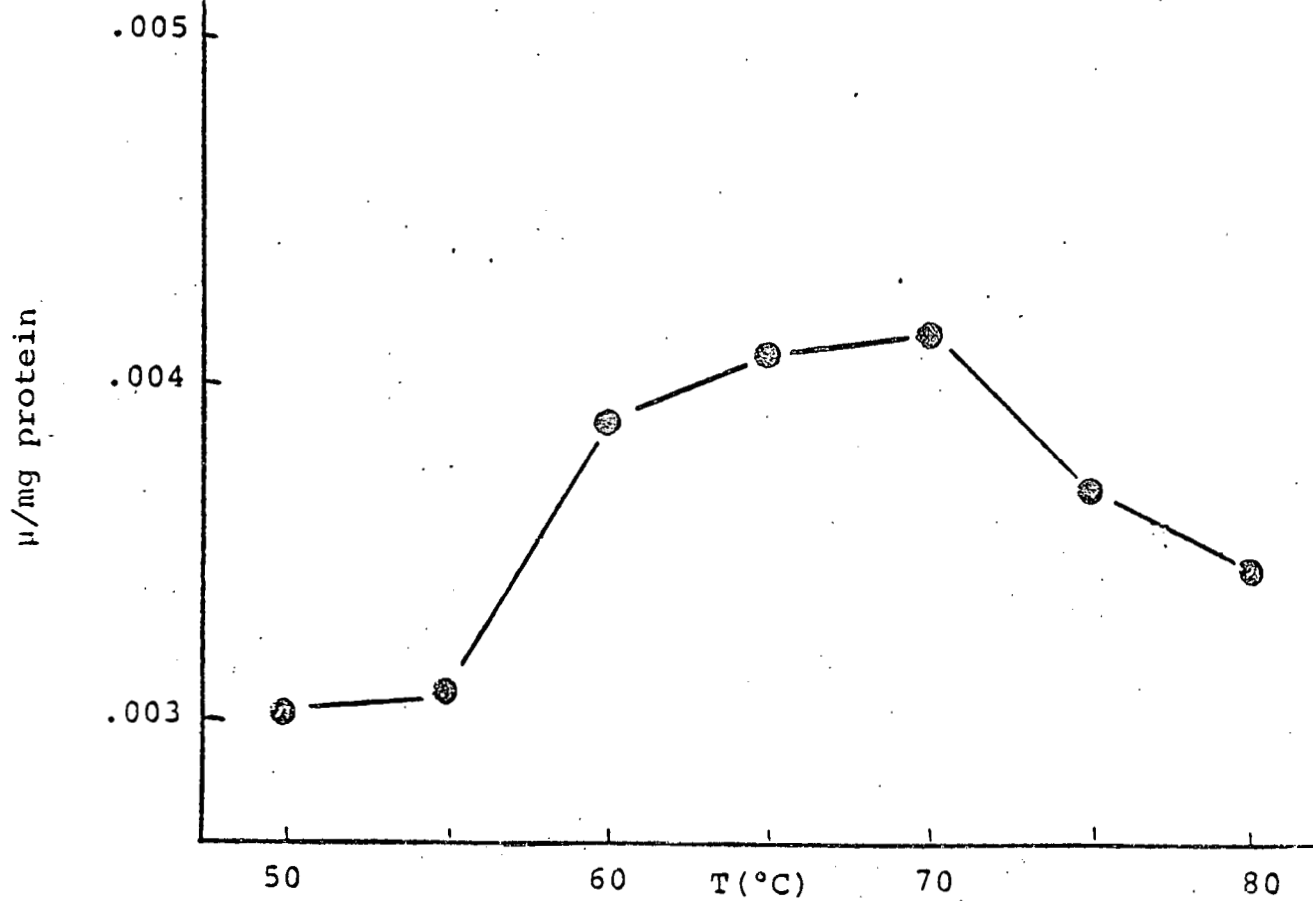


Figure I.F.1.7 CMCase Activity vs. T.



2. Evaluation of C. thermocellum Strains for Xylanase and Cellulase Activities

a. Introduction

We have previously reported enzymatic activities of C. thermocellum strains ATCC-27405 and LQ8 growing on different carbon sources. Isolate HG-2 has also been tested. HG-2 seems to have the three activities, although in contrast with the activities in ATCC-27405 and LQ8, in this organism the activities seem to be induced by the presence of xylan. At this point we are able to detect filter paper, carboxymethyl-cellulose, and xylanase activities on the culture supernatants of ATCC-27405, LQ8 and HG-2.

Preliminary evidence indicates that xylose inhibits the growth of C. thermocellum strain ATCC 27405 on cellobiose.

To check the efficiency of ethanol precipitation for the recovery of enzymatic activities, Diaflo ultrafiltration membranes from Amicon Corp. were employed. PM-10 membranes seem to yield the highest specific enzymatic activities and an acceptable flow rate, so it will be employed from now on in the characterization of the cellulase and xylanase enzymes.

SDS and native preparative polyacrylamide gel electrophoresis have been done on culture supernatants of C. thermocellum strains. Using this technique, it is possible to see that the patterns of extracellular proteins are alike for the ATCC-27405 and LQ8 strains when these are grown on cellobiose, while the pattern obtained from isolate HG-2 is different.

This suggests that this isolate is a different species.

Electrophoresis will also serve to monitor enzyme recovery during the course of their purification by gel filtration and ion exchange column chromatography.

b. Materials and Methods

1. Experiments were either performed in test tube cultures or 300 ml culture flasks with CM-4 medium containing 6 gr/liter of carbohydrate.

2. ATCC-27405 and HG-2 strains were evaluated for filter paper activity (FPase) CMC activity (CMCase) and xylanase activity (XLase). Activities were measured following the concentration of extracellular proteins by precipitation or ultrafiltration. Ethanol at 40% (V/V) was chosen to precipitate the proteins. Ultrafiltration was performed with Diaflo membranes from Amicon Corp. Ethanol was added to culture supernatants and the samples were maintained overnight in an ice bath. After precipitation, the samples were centrifuged, the supernatants were discarded, and the pellet was suspended in 0.05M citrate buffer pH 4.8 to determine enzyme activities. Ultrafiltration was performed through an Amicon Model 52 ultrafiltration cell under a constant N_2 pressure of 45 lb/in². Ultrafiltration membranes used were: Amicon XM-100A, XM-50, PM-30 and PM-10 (The numbers refer to the molecular weight cut-off, in thousands).

3. Protein measurements: Because of the interference of reducing sugars in the Lowry assay, the Bio-Rad protein assay was employed. The Bio-Rad assay is a dye-binding assay based

on the differential change of dye in response to various concentrations of protein (Bradford, 1976). 0.2 ml of sample and 0.2 ml of a blank (citrate buffer) were pipetted into separate test tubes. 5 ml dye reagent was added and the absorbance was read at 595 nm. The protein concentration was determined from a standard curve.

4. Sodium dodecylsulfate polyacrylamide (9%) gel electrophoresis was carried out as described by Laemki (1970). Native polyacrylamide (9%) gel electrophoresis was an adaptation of the former method in which 10% SDS and 2-mercaptoethanol were eliminated from the gels and sample buffer. Gels were stained with 0.25% Coomassie Blue in 50% methanol, 7% acetic acid and diffusion destained in 5% methanol and 7% acetic acid. Gels were preserved by drying under vacuum. Ten-fold concentrated samples containing 0.45mg/ml of protein were diluted in half with sample buffer (final concentration 20% glycerol, 20% SDS, and 2.5% 2-mercaptoethanol), and from this mixture a 40 ml sample used for electrophoresis. Electrophoresis was done at 40V, constant voltage for about 11 hours.

c. Results and Discussion

The enzymatic activities of HG-2 were assayed during its growth on a variety of cellulosic and non-cellulosic carbohydrates. Experiments were done with CM-4 medium and the results were obtained at the end of the exponential phase of growth. Enzymes were ethanol precipitated.

As can be seen from Table I.F.2.1 enzymatic activities,

Table I.F.2.1 Effect of Carbon Source on the Expression of Extracellular Enzymatic Activities in HG2 Isolate

<u>Stain</u>	<u>Activity</u>	<u>Solka Floc</u>	<u>MN300</u>	<u>Xylan</u>	<u>Cellobiose</u>	<u>Y.E.</u>	<u>Glucose</u>	<u>Xylose</u>
HG2	FPase	NM	NM	0.004	0.002	NM	NM	0.004
	CMCase	0.05	0.045	0.32	0.085	NM	0.035	0.050
	Xylanase	0.04	0.035	1.45	0.005	NM	0.015	0.035

* NM: No Measurable activity at supernatant concentration 1/10

* FPase and CMCase expressed as $\frac{\text{mg Glucose}}{\text{ml-HR}}$

* Xylanase as : $\frac{\text{mg xylose}}{\text{ml-HR}}$

although low, are present in every carbon source tested. This is consistent with the hypothesis that the weak growth of HG-2 observed on Solka floc and MN-300 is supported by the hemi-cellulose present in these. But because activities increase when the culture is growing on xylan compared with those obtained when the culture is growing on xylose, cellobiose or glucose, an experiment was performed to determine the rate of development of enzymatic activities over time.

From Table I.F.2.2 it can be inferred that the HG-2 isolate, when grown on xylose develops very little enzymatic activity compared with that obtained when grown on xylan. This observation could be accounted for if these enzyme(s) are induced in the presence of xylan.

In the last progress report, it was reported that no colonies of strains ATCC-27405 or LQ8 arise when plated on CM4-xylose. A possible explanation is that xylose could be a non-metabolizable substrate as well as an inhibitory substance. To test these hypotheses an experiment was performed in which ATCC-27405 was grown on a CM-4-cellobiose medium (6 gr/liter) with the addition of variable quantities of xylose. When 6 gr/liter of xylose were added an inhibition index of 0.29 was observed (Table I.F.2.3). The experiment is not conclusive as the osmotic pressure of the medium due to the high concentration of substrate can explain this inhibition. Alternate explanations could be that substrate competition for the active site of the transport enzyme occurs or that xylose exerts its inhibition intracellularly.

Table I.F.2.2 Rate of Development of Enzymatic Activities Over Time in Strain HG2 Growing on Xylan and Xylose

Strain	Activities	7hrs.	12hrs.	18hrs.	24hrs.
HG2	FPase	NM	NM	NM	NM
Xylose	CMCase	0.012	0.020	0.025	0.075
	Xylanase	0.010	0.020	0.030	0.080

Strain	Activities	12hrs.	24hrs.	48hrs.	72hrs.
HG2	FPase	NM	0.004	0.001	NM
Xylan	CMCase	0.06	0.22	0.40	0.17
	Xylanase	0.27	1.05	1.50	0.50

Table I.F.2.3 Inhibition Index of Different Concentrations of Xylose on ATCC-27405 Forming on CM-4 Cellobiose at 6 grs/Liter.

<u>Substrate</u>	<u>Inhibition Index</u>
Control Cm-4-Cb	0
CM-4-Cb + 1 gr/L xylose	— 0.05
CM-4-Cb + 2 gr/L xylose	— 0.08
CM-4-Cb + 3 gr/L xylose	— 0.16
CM-4-Cb + 4 gr/L xylose	— 0.18
CM-4-Cb + 5 gr/L xylose	— 0.29
CM-4-Cb + 6 gr/L xylose	— 0.29
Control Cm-4-xylose only	— >>1 (no growth)

* Values obtained at the end of logarithmic rate of growth

* Inhibition index as:

$$1 - \frac{\text{Absorbance OD}^{660} \text{ sample with variable quantity of xylose}}{\text{Absorbance OD}^{660} \text{ control (CM4-Cb alone)}}$$

* When inhibition index is 0 = no inhibition
>>1 = complete inhibition

At this point one doubt, however, arises: does ethanol precipitation in some way alter the enzyme(s) with an accompanying loss of activity? It was thought that ultrafiltration of culture made through non-cellulosic filters (Diaflo filters from Amicon Corp.) would provide a control to check the recovery of activities. Differences in activities would mean that ethanol could produce some damage to the enzyme(s) involved in the degradation of cellulose and xylan. Ultrafiltration might also be useful for separating the enzymes of interest from each other on the basis of molecular weight. However as seen in Table I.F.2.4 to I.F.2.7 this method was unsuccessful. Consistently the highest activities for every activity tested were obtained with the PM-10 filters. XM-50 and PM-30 membranes did not yield adequate recoveries perhaps some quantity of enzyme remained trapped on the membrane. Activities were also retained by XM-100A filters, which indicate that the enzyme(s) which we are looking for have a molecular weight of more than 100,000.

SDS and native preparative electrophoresis have also been done. It was evident that in native gels patterns of extracellular proteins are largely the same in C. thermocellum strains ATCC-27405 and LQ8 grown on cellobiose, however banding patterns for the HG2 isolate growing on xylose or xylan are quite different. This could mean that HG2 and ATCC-27405 are very different microorganisms. These patterns will also be useful in identifying the peaks from gel filtration and ion-exchange chromatography.

Table I.F.2.4 Recovery of Activities for C. thermocellum ATCC-27405
Growing on Cellobiose

Method of Concentration	Protein	FPase	CMCase	XLase	Specific Activities for	
					CMCase	XLase
Ethanol	0.46	0.020	1.55	1.25	3.37	2.71
XM-50	0.48	0.046	2.35	1.50	4.90	3.12
PM-30	0.42	0.038	2.10	1.20	5.00	2.85
PM-10	0.46	0.046	2.70	1.55	5.87	3.36

Table I.F.2.5 Recovery of Activitiy From HG2 Growing on Xylan

Method of Concentration	Protein	FPase	CMCase	Xlase	Specific Activities for	
					CMCase	Xlase
Ethanol	0.39	NM	0.42	1.35	1.08	3.46
XN-50	0.81	0.010	0.025	2.20	1.23	2.71
PM-30	0.64	NM	0.80	1.75	1.25	2.73
PM-10	0.64	0.005	0.87	2.30	1.36	3.59

* Protein: as mg/ml, liquid supernatant concentrated 1/10

* FPase, CMCase, Xlase as $\frac{\text{mg glucose or xylose}}{\text{ml-HR}}$

* Specific activities as : $\frac{\text{mg glucose or xylose}}{\text{ml-P}} / \text{mg/ml protein.}$

Table I.F.2.6 Recovery of Activity from ATCC-27405 Growing on Cellobiose

Method of Concentration	Protein	FPase	CMCase	XLase	Specific Activities for	
					CMCase	XLase
Ethanol	0.68	0.036	3.5	2.01	5.14	2.95
XM-100A	0.53	0.044	2.95	1.85	5.56	3.49
PM-10	0.73	0.040	4.0	2.80	5.47	3.83

Table I.F.2.7 Recovery of Activities From HG2 Growing on Xylose

Method of Concentration	Protein	FPase	CMCase	XLase	Specific Activities for	
					CMCase	XLase
Ethanol	0.22	NM	0.035	0.055	0.15	0.25
XM-100A	0.20	NM	0.12	0.25	0.60	1.25
PM-10	0.33	0.008	0.34	0.35	1.03	1.06

d. Future Work

We are able to detect enzymatic activities on the supernatant of ATCC-27405 and HG2 cultures. How many enzymes are responsible for these activities? Gel filtration and ion exchange chromatography will be employed to separate these activities.

If C. thermocellum strains must utilize substrates with a high content of hemicellulose to grow, the phenomenon of non-utilization, and perhaps xylose inhibition should be studied.

G. EVALUATION OF SUBSTRATES FOR USE BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

The screening of different cellulosic biomasses with respect to degradation by C. thermocellum was continued. The basic properties of the substrates considered to be desirable for the process are abundant availabilities, low cost and suitability for continuous feeding.

Earlier work with agricultural residues (see Progress Report COO-4198-6) had suggested corn stover to be a better substrate than sugar cane bagasse. It was then speculated that this better performance could be explained by the 50% higher specific surface area (cm^2/g) of the corn stover sample used. To check the validity of this hypothesis, C. thermocellum was now grown on these two substrates using defined particle sizes ranging from 20 to 100 mesh.

One of the problems often mentioned in the literature concerning the degradation of cellulosic substrates is their fairly high content of hemi-cellulose and lignin. Most materials that are agricultural wastes or by-products contain 20-50% cellulose and 50-80% hemicellulose and lignin. A common way to overcome this problem is to effect an alkaline pretreatment of the substrate in order to remove most of the hemicellulose and some of the lignin. This preliminary step in a commercial process would considerably raise the total production cost. The idea of utilizing a by-product of a process

that inherently included a favorable pretreatment was considered to be ideal. Since most wood pulping processes for paper production involve an alkaline treatment and an Na_2S treatment (to increase the rate of delignification), the use of paper mill primary sludge as a C-source for C. thermocellum was investigated.

Another substrate tested was cotton shearings, which are by-products of the textile industry. Shearing is a finishing operation in which uneven threads are mechanically cut or trimmed from the face of a fabric resulting in extremely short fibers which have presently no end-use in the textile industry.

Besides this search for new substrates, a major effort was devoted to the construction and set-up of a continuous culture system using a solids feeder. The objective of such a system would be to obtain process data that would be later utilized in process design and cost analysis. •

It has been shown previously that for some materials the substrate particle size has a major impact on cellulose degradation and reducing sugar accumulation. Yet a detailed picture of the spatial arrangement of substrate particles and microbial cells during the growth of C. thermocellum is still not available. In order to obtain such knowledge, this work dealt also with the observation of the growth of this organism using Scanning Electron Microscopy (SEM).

2. Material and Methods

a. New Substrates

The hammer milled corn stover and the Wiley milled sugar cane bagasse samples used were part of the same lot previously employed (see Progress Report COO-4198-6). Samples of the two materials were sifted for 2 minutes through a set of Mesh Tyler series screens and the fractions in the range of 20-28 (589-833 μ), 28-35 (417-489 μ) and 35-100 (147-417 μ) mesh were used as C-sources for C. thermocellum. The experiment was conducted in Hungate tubes containing 1 ml of Solka floc inoculum plus CM3 medium up to a final volume of 10 ml. Tubes were prepared in duplicate for each substrate/particle size combination. Controls consisted of both Solka floc tubes and uninoculated tubes. The time course of the 60°C fermentation was followed by examining sets of tubes after 25 and 72 h.

The cultures were filtered through Whatman Paper No. 1 for determination of residual solids. The pH was measured in the filtrate which was then centrifuged at 20,000 x g for 15 minutes. A reducing sugar DNS assay was performed on the supernatant. This same procedure was followed in later reducing sugar determinations.

Another experiment was performed to test the possibility of using paper mill primary sludge as a substrate. The sample was obtained from Crown Zellerbach (Central Research

Caruas, Washington 98607). The moisture content of the sample was determined to be 80% on a weight basis. The pH of a 10% suspension is 8.0. According to the information provided by the manufacturer, 50% of the total solids is cellulose from kraft, magnesite and mechanical pulping processes, while ash represent 31%. The experiment was run at 60°C in anaerobic shake flasks containing 300 ml of CM3 medium with 1% dry sludge. A Solka floc flask was used as a control. A 3% inoculum of C. thermocellum grown on cellobiose was utilized. The reducing sugar production was determined by the DNS assay. The residual solids pattern was not followed since the nature of the substrate made sampling inaccurate.

This experiment was repeated using washed sludge as a substrate. The wash was done by centrifuging a 5% sludge slurry at 3,500 x g for 15 minutes and resuspending the precipitate in distilled water. Duplicate 10 ml samples taken before and after the wash were dried to assess eventual solid losses. Conditions were the same as in previous experiment except that sludge concentration in CM3 medium was 0.8% solids.

Samples of bleached and green dyed cotton shearings were obtained from West Point Pepperell (West Point, Georgia 31833). The experiment was conducted at 60°C in anaerobic shake flasks containing 300 ml of CM3 medium (1% shearings). A Solka floc flask was again used as a control. A 3% inoculum of C. thermocellum grown on cellobiose was utilized. Reducing

sugar production was followed by the DNS assay. Again, it was not possible to assay for residual solids due to the nature of the substrate.

b. Continuous Culture Set-Up

The fundamental problem with the continuous culture set-up for cellulose degradation is the feeding of the insoluble cellulosic substrates. The approach chosen here was the use of a solids feeder connected to the fermentation vessel by means of a funnel attached to a 59 cm long duct. Both funnel and duct were made out of glass in order to minimize accumulation of particles on the walls. Several designs of ducts were tried. The most successful design (see Figure I.G.1) is based on the same principle as a Venturi flowmeter. A flow of nitrogen is forced through the constricted length of tubing and carries the substrate particles. The set-up was tested with corn cob granules (GRIT'O-COBS-60, The Andersons, Cob Division, P.O. Box 119, Maumee, NH 43537) of maximum diameter 300 μ .

c. Scanning Electron Microscopy

C. thermocellum was grown at 60°C in anaerobic shake flasks containing 300 ml CM3 medium with 1% ball milled corn stover (Dow Chemical Co., Midland, Michigan). A 10 ml (3%) inoculum grown on Solka floc was used; 10 ml sterile distilled water were added to the uninoculated control. At 43 h, these broths were used to inoculate (3%) media in similar flasks. When the fermentation in these second transfer flasks

reached 45 h, 2 ml aliquots were centrifuged at 13,000 x g for 10 minutes and the precipitate was resuspended in distilled water and recentrifuged. The dehydration of the precipitate was done by the method of Kurtzman et al. (Appl. Microbiol. 28: 708-712) which consists of resuspending the precipitate in progressively higher concentrations of ethanol (10, 30, 50, 95 and 100% ethanol were used). The final suspension was air dried in a desiccator for 24 h prior to gold coating. The samples were examined with a Cambridge Stereoscan Mark II SEM at magnifications ranging from 800 to 8000.

To verify whether this sample preparation procedure was washing the cells off the substrate particles, culture broths of C. thermocellum grown on ball-milled corn stover and Solka floc were examined under the light microscope after each step of the procedure in both supernatant and precipitates. A wet mount technique was used to prevent the rapid evaporation of ethanol from the microscope slides at the final stages of the dehydration process.

3. Results and Discussion

a. New Substrates

The residual sugar accumulation profile for corn stover and sugar cane bagasse of defined particle sizes is shown in Figure I.G.2. The final pH in the Hungate tubes was 6.3 for the corn stover, 6.8 for the bagasse and 6.2 for the Solka floc control. When analyzing these figures, it should

be kept in mind that experiments performed in Hungate tubes usually show less intense cellulose degradation and sugar production compared to shake flask or fermentor performance. From Figure I.G.2, it can be seen that for this particle size range no definite correlation can be found between sugar accumulation and particle size. One can also conclude that for the same particle size or for the same specific area (taking into account the difference in densities) corn stover is still a better substrate than sugar cane bagasse. The results obtained with this hammer milled corn stover are around 60-80% of the reducing sugar production obtained previously for corn cob granules of similar size, but the lower cost of stover could compensate for the lower production yields.

No growth was detected when the paper mill primary sludge was used as a substrate while the Solka floc control reached 80% substrate consumption and a reducing sugar concentration around 5 g/l at a final pH of 5.6. This fact was rather surprising since it was hoped that the alkaline pretreatment would have left an easily degradable cellulosic material. Khan and Trottier (Appl. Env. Microbiol. 35: 1027-1034) have reported that sulfides are toxic to many anaerobes at fairly low concentrations so one seemingly plausible explanation for the inability of growth of C. thermocellum was that the sulfide used in the pulp treatment was toxic to this organism.

Hence, a water wash pretreatment was conducted with the objective of solubilizing the sulfide salts. The

solubility of Na_2S at 10°C , for instance, is 15.4 g/100 g so the treatment employed should have been effective in reducing sulfide salts. No significant weight losses were detected as a result of the washing procedure, but still no growth was observed along 15 days of incubation.

The results obtained with the cotton shearings are much more encouraging. The reducing sugar production is shown in Figure I.G.3. For both substrates (bleached and dyed shearings), the reducing sugar accumulation is higher than 4 g/l. An interesting feature of these substrates is the long lag phase for both bleached shearings (around 5 days) and dyed shearings (around 7 days). A lag phase of 8-9 days had been previously observed in this laboratory for C. thermocellum grown on surgical cotton. A final pH of 5.8 was observed for both types of cotton shearing while Solka floc had a final pH of 5.6. When the culture containing dyed shearings entered log phase, the medium became reddish and the longer lag phase observed for this substrate may be related to the green dye or products generated from it. This experiment will be repeated using inoculum grown on cotton shearings to shorten the lag phase. This fermentation process might be of interest to the textile industry since the color variations and the short staple length of these fibers have so far prevented their use in many suggested end-uses including the viscose process for rayon.

b. Continuous Culture Set-Up

The main problem found in the equipment set-up has been the connection between the feeder and the fermentation vessel. At room temperature, the use of a plain vertical glass tube is effective but, at 60°C, moisture condensation occurs onto the walls of the lower portion of the glass tube, causing accumulation of particles and finally clogging the tube.

The Venturi principle used in the design shown in Figure I.G.1 has proved to be quite successful. Since the flow of N_2 is constant, the gas reaches a higher velocity at the constricted length of tubing, which creates a depression zone. This allows the flow of particles into the fermentor. The length of constricted tubing upstream from the funnel connection is around 20 times larger than the tubing diameter; this was designed in order to assure laminar flow at the funnel connection.

At room temperature, this apparatus was shown to be able to deliver corn cob granules at rates above 0.50 g/min with a precision of a hundredth of a gram. The performance of this set up remains to be tested during longer periods of time at 60°C which will be needed for continuous culture.

c. Scanning Electron Microscopy

The examination of the corn stover cultures with SEM did not provide any conclusive evidence about the morphology of substrate particles. It was not possible to

delineate the presence of C. thermocellum cells, nor the type of colonization occurring. Both inoculated and uninoculated samples indicated the presence of fairly large structures which appeared to be crystals of salt precipitate. For future work, the salts added to the medium shall be filter sterilized to avoid precipitate formation during autoclaving.

The idea of checking the sample preparation technique by light microscopy could not be done successfully with corn stover cultures since the shade formed at the edge of the particles made impossible the observation of cell attachment. Such a check was done with Solka floc grown cultures. It was found that, during ethanol dehydration, although the supernatants showed a very small cell concentration (indicating that centrifugation procedure was appropriate), the resuspended precipitates showed the major portion of the cells in a free form. When final samples corresponding to resuspension in pure ethanol were examined, most cells appeared in solution, but some still seemed to be attached to the Solka floc particles. Other techniques for sample preparation for SEM shall be tried, possibly by the use of a liquid nitrogen freezing process.

4. Future Work

- Operation of the continuous culture system using a solids feeder to obtain process data for cellulose degradation by C. thermocellum.

- Further study of performance of cotton shearings as substrate for C. thermocellum.

- Study of colonization pattern of C. thermocellum on substrate particle by means of SEM.

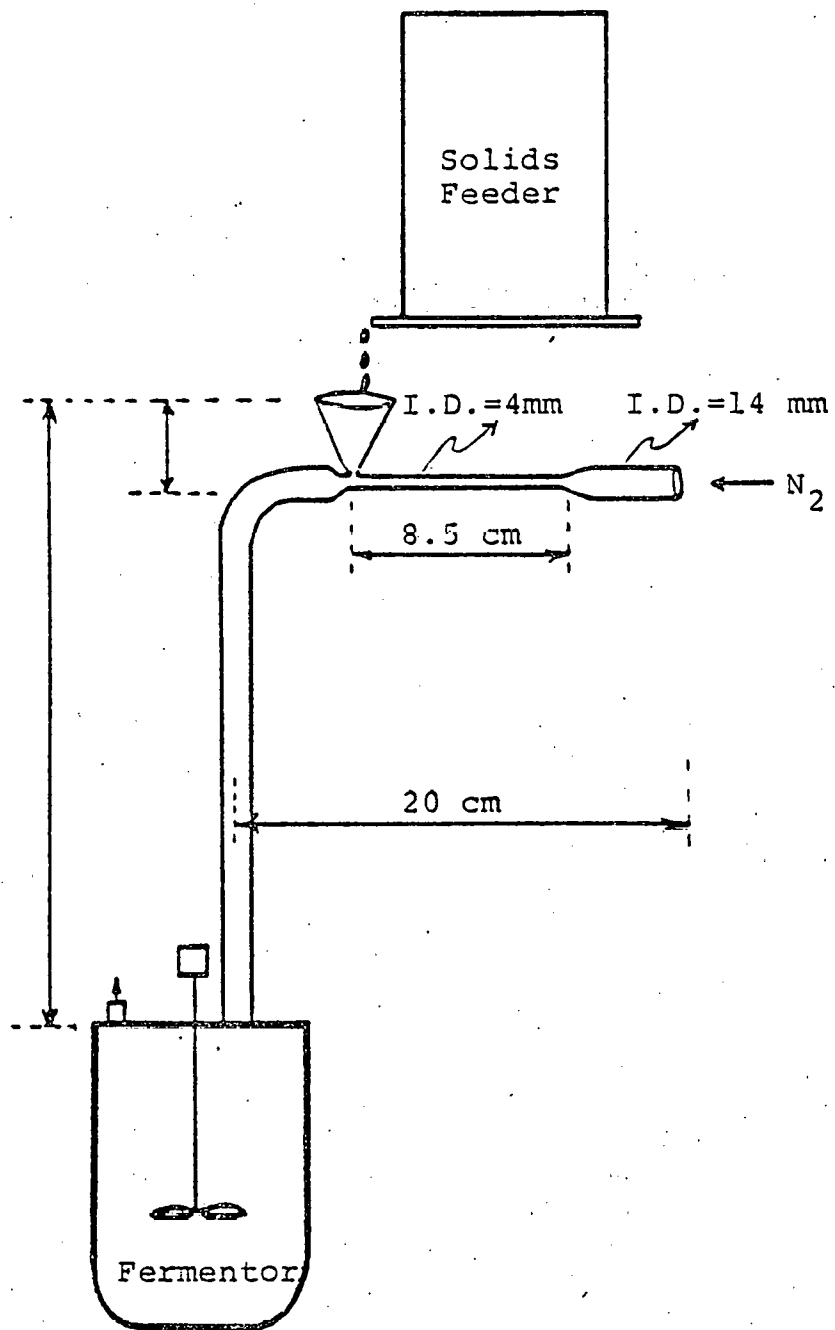


FIGURE I.G.1: DESIGN OF SOLIDS FEEDING APPARATUS FOR CONTINUOUS CULTURE SET-UP

FIGURE I.G.2: REDUCING SUGAR ACCUMULATION PROFILE FOR C.THERMOCELLUM GROWN ON CORN STOVER AND SUGAR CANE BAGASSE OF DEFINED PARTICLE SIZE IN THE RANGE OF 20 TO 100 MESH

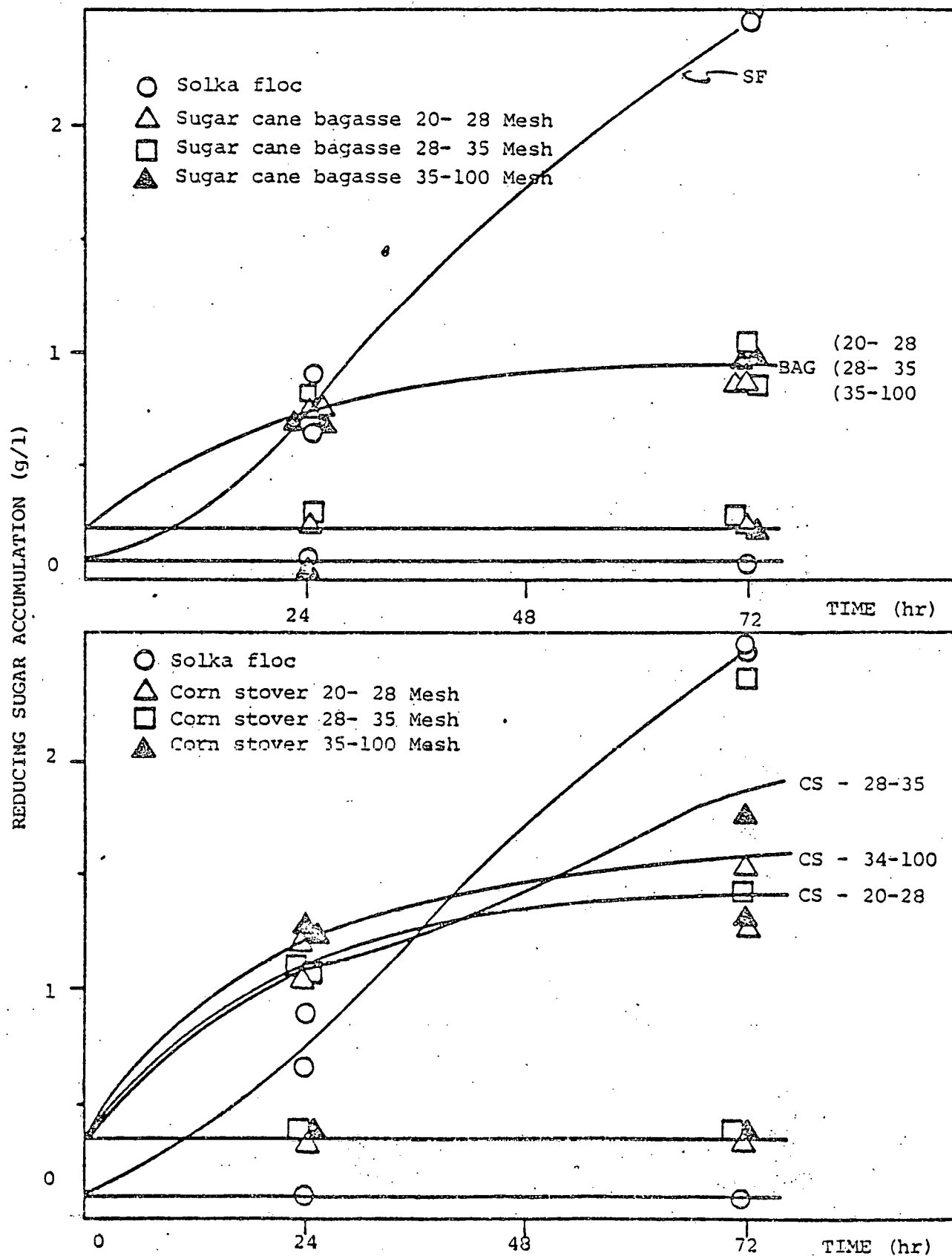
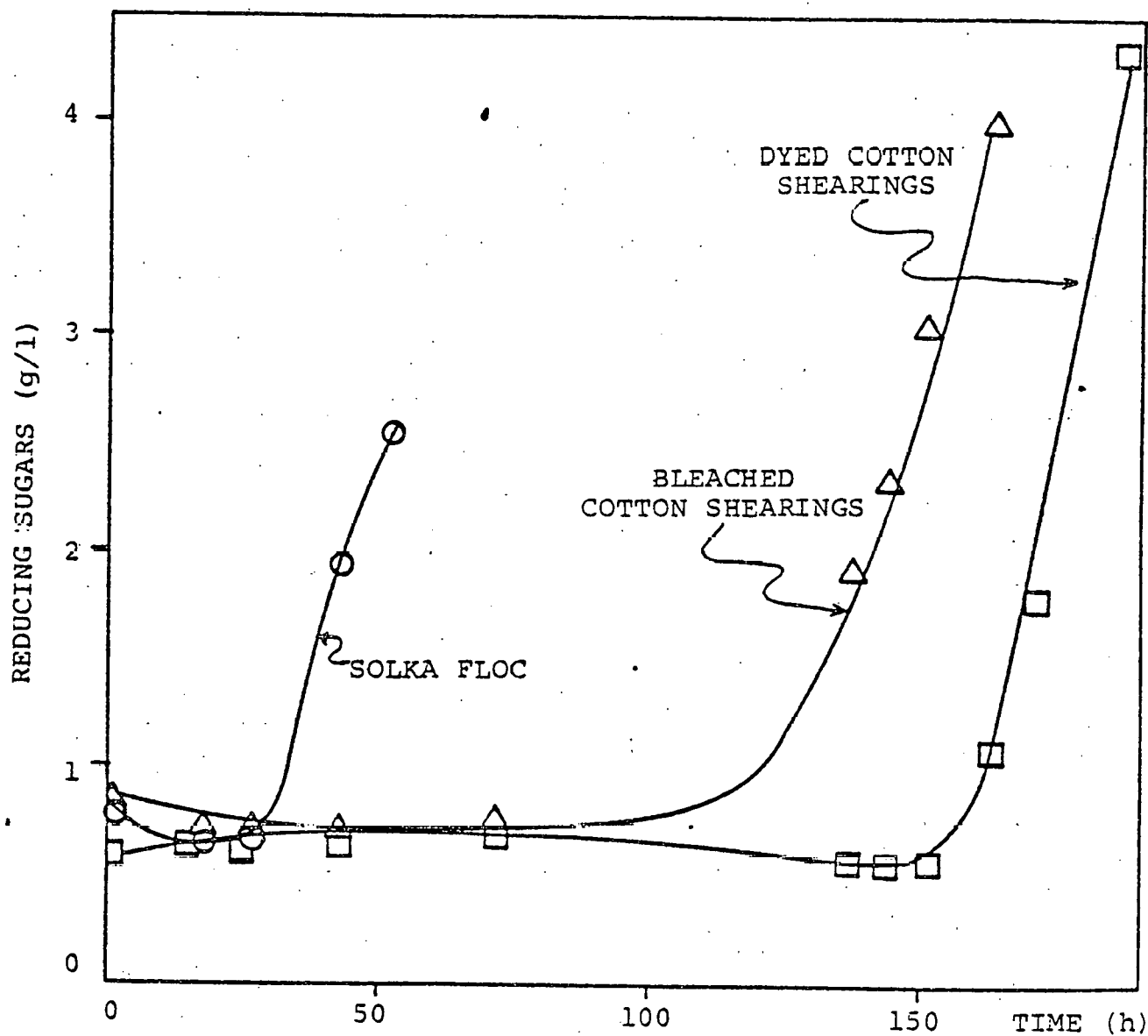


FIGURE I.G.3: REDUCING SUGAR ACCUMULATION FOR CULTURE OF C. THERMOCELLUM GROWN ON BLEACHED AND DYED COTTON SHEARINGS AND ON SOLKA FLOC



II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. Production of Acrylic Acid by Fermentation

1. Introduction

During the course of this project, a microbial process for the production of acrylic acid has been developed. This represents the first microbial production of this commodity chemical. Production of acrylic acid from cellulosic biomass as part of an integrated process for fuels and chemicals production could greatly enhance the economics of ethanol production because of the high value (e.g. 35-40¢/lb.) for acrylic acid.

In the most recent report period, we have achieved a concentration of 1.2 g acrylic acid/liter and have demonstrated the stimulating effect of lactate and methylene blue on its formation. In addition, cells of Clostridium propionicum have been immobilized and shown to retain a major portion of their biological activity.

At this point, we have decided to cease our efforts on the development of a process for conversion of propionate to acrylate with E. coli and focus only on C. propionicum.

Studies on the direct production of lactate, a precursor to acrylate, from cellulose have proceeded well. Using mixed cultures of C. thermocellum and a recently isolated thermophilic, homo-lactate forming organism, the direct

conversion route has been explored. Efforts to improve this step towards a complete process for acrylate are continuing.

2. Acrylic Acid Production by Clostridium propionicum

a. Introduction

Continuing efforts have been made to optimize the reaction conditions for the production of acrylate from propionate with C. propionicum. There are several important variables that need to be optimized. These include:

- (1.) propionate concentration
- (2.) lactate concentration
- (3.) methylene blue concentration
- (4.) hydrogen ion concentration (pH)
- (5.) temperature
- (6.) phase of growth
- (7.) oxygen availability
- (8.) cell concentration

In this report, results from an optimization of some of the above variables for the maximum production of acrylate from propionate is presented. Since our last progress report it should be noted that when both lactate and methylene blue are present, as much as 17 mM (1.2 g/l) acrylate is produced from propionate.

We have also pursued the use of immobilized whole cells for acrylate production. Results to date are quite successful in the use of this technique.

b. Materials and Methods

Clostridium propionicum was grown in complex medium containing peptone and yeast extracts (see previous progress reports) with α -alanine as the carbon source. Cells were harvested at late exponential or early stationary phase and washed with 0.03% Na_2S solution. Washed cells were suspended in 50 mM triethanolamine-HCl (10 mM, pH 7.5) buffer. These suspended cells were used for experiments. All experiments were carried out in a test tube (13 x 100mm) with magnetic stirring at 37°C. Cell concentrations were 0.1 g wet weight cells/ ml of reaction mixture (approximately 20 mg/ml dry weight). 200 μl of reaction mixture were withdrawn and 40 μl of 50% H_2SO_4 was added to stop the reaction. Volatile acids were extracted with ether (200 μl) and assayed by gas chromatography with valeric acid as an internal standard.

Cell Immobilization

Cell suspensions were prepared as previously described for resting cell experiments except that the washed cell pellet was suspended in triethanolamine buffer (10 mM, pH 7.5) containing 1mM MgSO_4 (TEA-Mg buffer). To 9 ml of cell suspension (2 g wet cell weight) were added 1.5 g acrylamide and 0.080 g N,N'-methylene-bis-acrylamide with stirring under a nitrogen atmosphere at 40°C. Then 0.25 ml 10% ammonium persulfate solution was added to the mixture followed by 0.5 ml of a 10% tetramethylethylen-

diamine (TEMED) solution. The mixture polymerized after about 5 minutes at 4°C. The gel was then homogenized in 50 ml TEA-Mg buffer for 90 sec in a Waring blender maintained at 4°C. The gel particles were washed three times with 100 ml TEA-Mg buffer. With this procedure the concentration of cells was approximately 0.2 g/g gel. The efficiency of entrapment was 85-90% as judged by measuring the amount of protein in the combined gel washings.

The gel suspension was assayed for acrylic acid production in tubes in a manner identical to the resting cell assay procedure (200 mM propionate, 50 mM TEA buffer, pH 7.5, aerobic). In addition, the use of an immobilized cell column reactor was investigated. A substrate mixture containing 0.2 M sodium propionate, 0.025 M sodium (acetate, 0.1 M triethanolamine buffer (pH 8.0), 0.2% methylene blue and 0.001 M MgSO_4 was pumped at a rate of 7.5 ml/hr through a column (2.5 x 7 cm) of gel particles (10 g packed gel weight). Effluent samples were assayed for acrylic acid.

c. Results and Discussion

(1) Effect of Lactate Concentration

In the previous report, the stimulatory effect of lactate on the production of acrylate from propionate was shown. We have now determined the optimum concentration of lactate. It was observed that low concentration of lactate increased the final yield of acrylate,

but high concentration of lactate did not. It is important to note that the initial rate of acrylate production was decreased as lactate concentration increased (Figures II. A.2.1 and 2). After 8 hour incubation the reaction went to completion. Using an 8 hour incubation the final yield of acrylate from proprionate was investigated as a function of proprionate and lactate concentration. The results are shown in Figures II.A.2, 3 and 4. Increasing amounts of propionate resulted in increased amount of acrylate. Lactate had its maximum effect at a concentration of 20 mM. These same results are replotted in Figure II.A.2.4. In an additional experiment shown in Figure II.A.2.5. the optimum occurred at 30 mM lactate.

(2.) Effect of pH

Three buffer systems were used to investigate the effect of pH in the range from 4.0 to 10.5. 25 mM of lactate was also added to the reaction mixture to stimulate acrylate production. The optimum pH value was 8.0 - 8.5 as seen in Figure II.A.2.6.

(3.) Effect of Methylene Blue

Methylene blue is an electron acceptor; on reduction, the color changes from blue to colorless. The reduced compound is readily reoxidized in air. We investigated the effect of methylene blue on acrylic acid production under both anaerobic and aerobic conditions.

FIGURE II.A.2.1: EFFECT OF LACTATE ON THE PRODUCTION OF ACRYLATE FROM PROPIONATE

- Reaction mixture (total volume 2 ml) contained:

triethanolamine-HCl buffer (pH 7.5)	50 mM
propionate	200 mM
lactate	0-5 mM
- Incubated at 37°C under air atmosphere with mechanical agitation
- Samples (200 μ l) were taken at each time interval and transferred to a tube containing 40 μ l of 50% H₂SO₄

—○— 0 mM lactate
—●— 10 mM lactate
—△— 20 mM lactate
—▲— 50 mM lactate

Figure 11.A.2.1.

ACRYLIC ACID PRODUCED (MM)

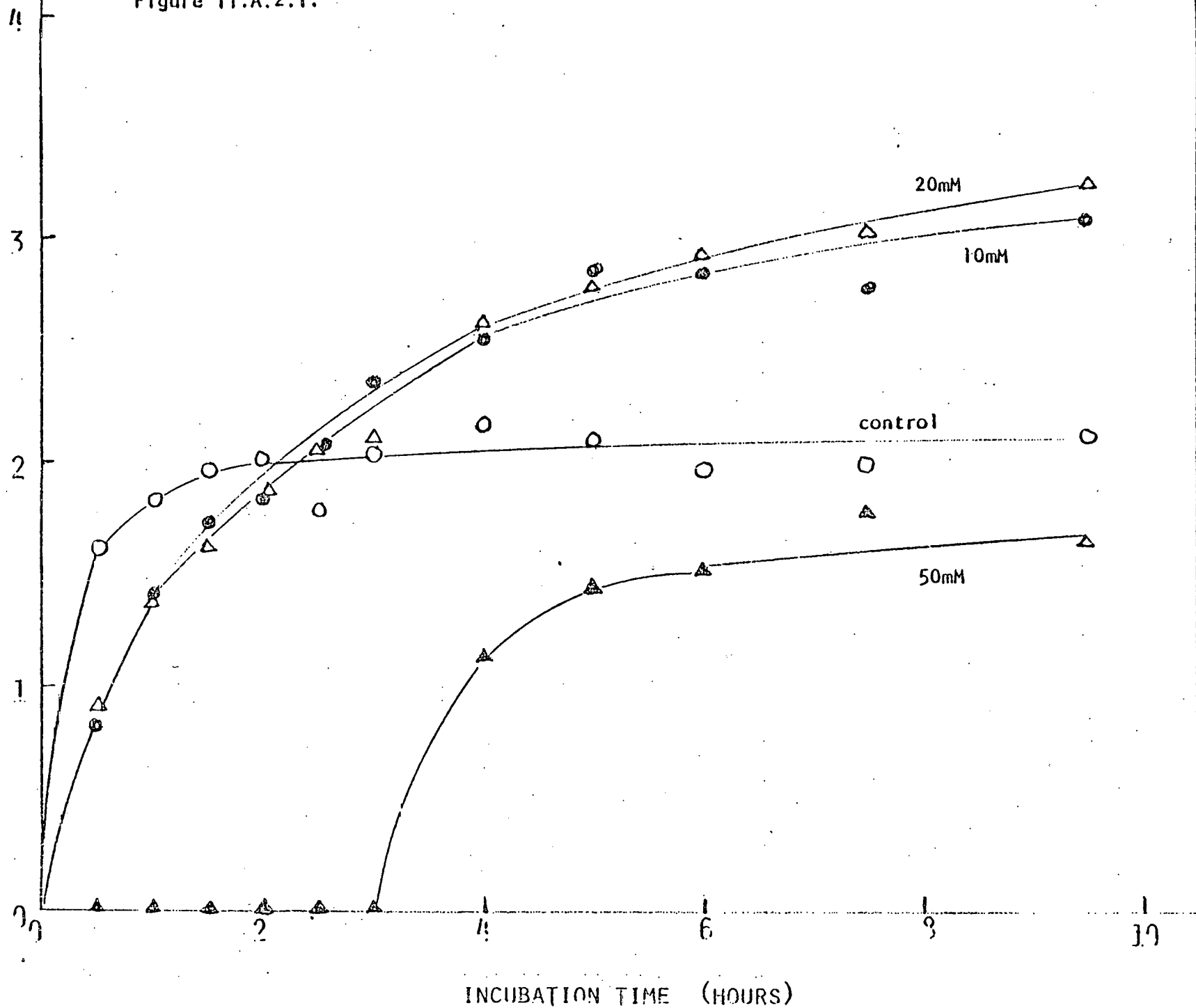


FIGURE II.A.2.2.: EFFECT OF LACTATE ON THE PRODUCTION OF
ACRYLATE FROM PROPIONATE

- Reaction mixture (total volume 2 ml) contained:

triethanolamine-HCl buffer (pH 7.5)	50 mM
propionate	200 mM
lactate	0 ~ 100 mM
- Incubated at 37°C under air atmosphere with mechanical agitation
- Samples (200 μ l) were taken at each time interval and transferred to a tube containing 40 μ l of 50% H₂SO₄

—○—	0 mM lactate (control)
—△—	10 mM lactate
—●—	20 mM lactate
—□—	30 mM lactate
—△—	50 mM lactate
—■—	100 mM lactate

Figure 11.A.2.2.

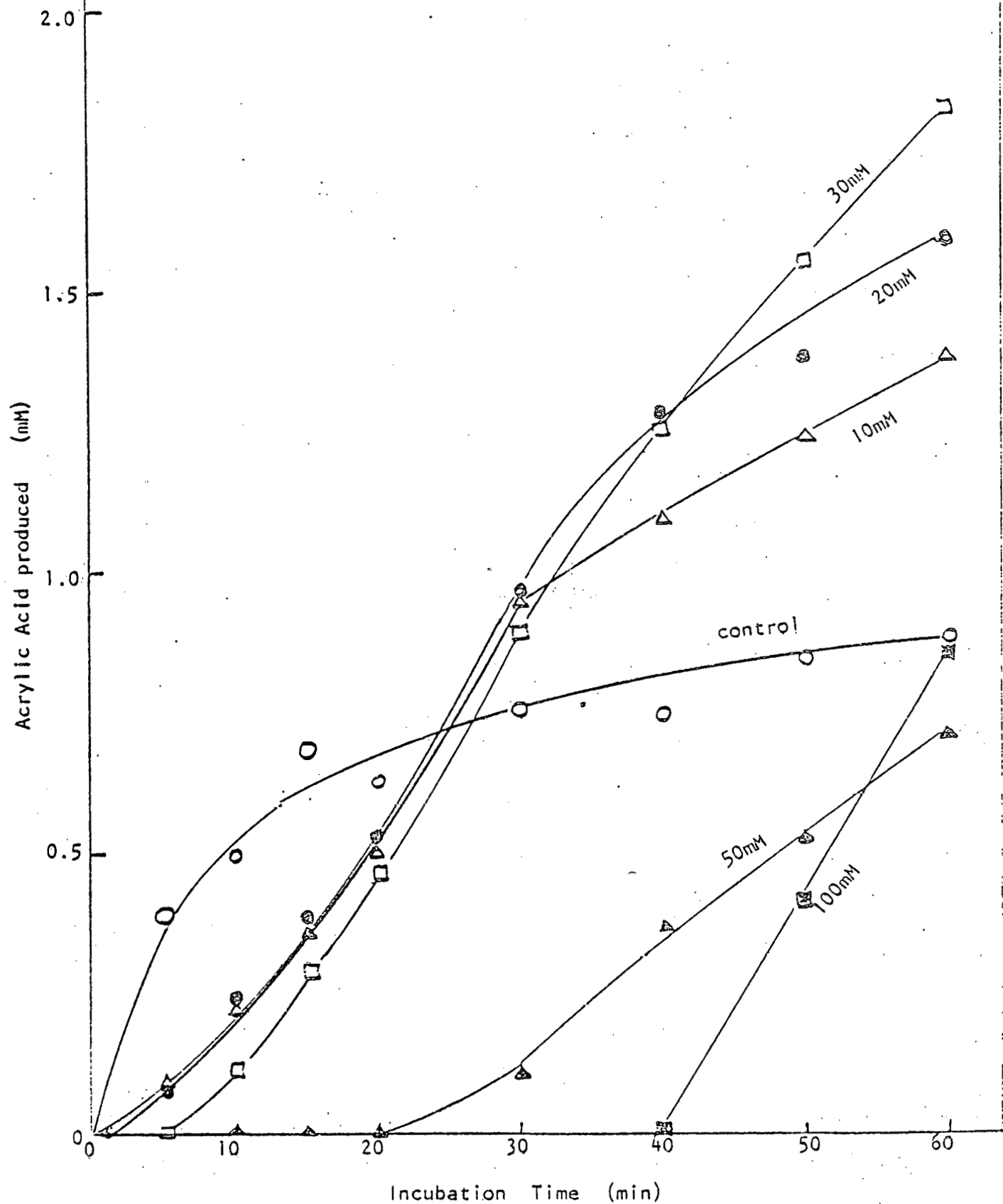


FIGURE II.A.2.3: EFFECT OF LACTATE CONTRATION ON THE PRODUCTION OF ACRYLATE AS A FUNCTION OF PROPIONATE CONCENTRATION

- Reaction mixture (total volume 200 μ l) contained:
 - triethanolamine-HCl buffer (pH 7.5) 50 mM
 - propionate 0 ~ 200 mM
 - lactate 0 ~ 100 mM
 - Incubated at 37°C for 8 hours under air atmosphere with mechanical agitation
 - reactions were stopped with the addition of 40 μ l H₂SO₄ (50%)
-
- 0 mM propionate
 - 25 mM "
 - 50 mM "
 - ▲— 75 mM "
 - △— 100 mM "
 - 150 mM "
 - 200 mM "

Figure 11.A.2.3

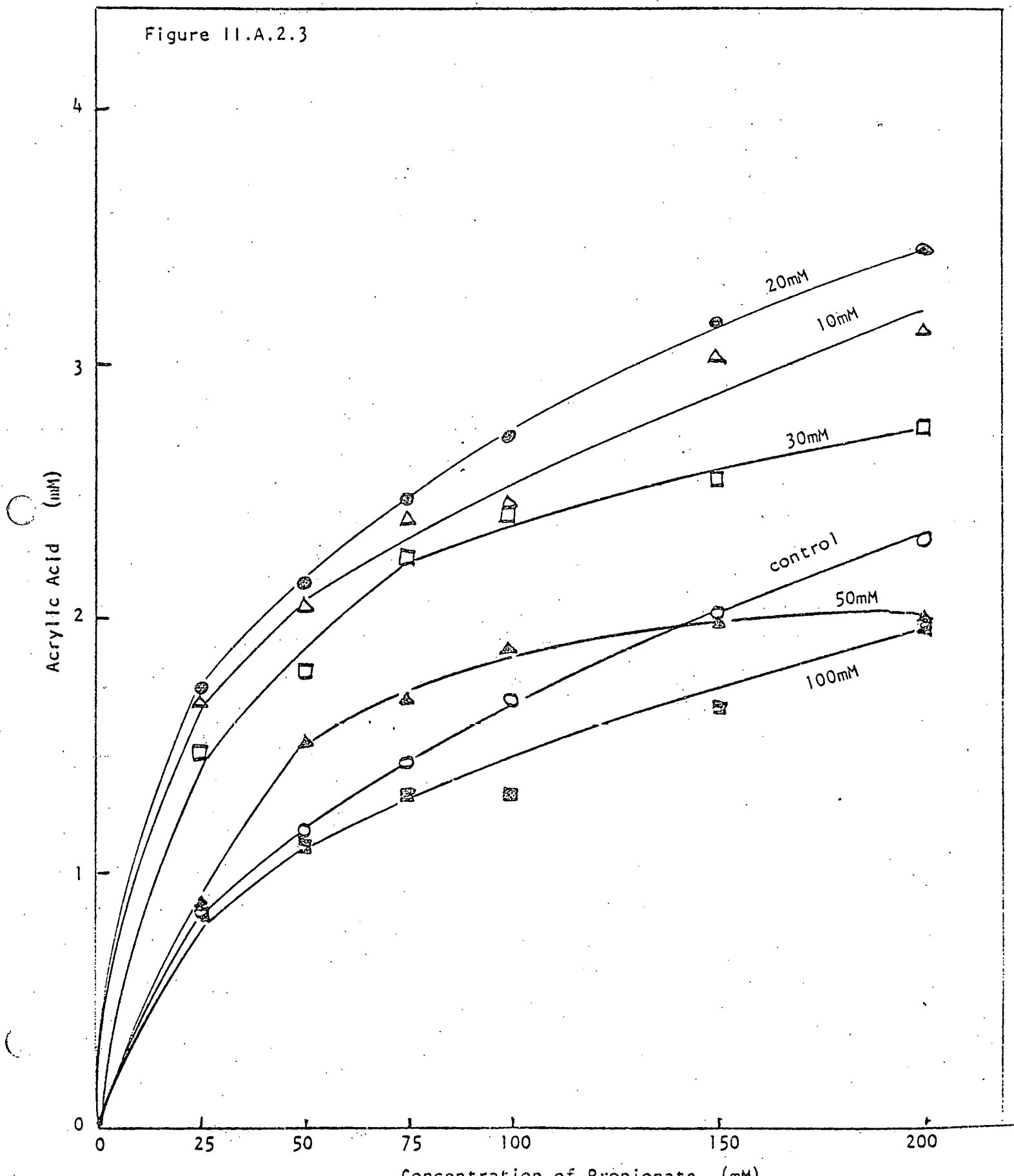


FIGURE II.A.2.4: EFFECT OF PROPIONATE CONCENTRATION ON THE PRODUCTION OF ACRYLATE AS A FUNCTION OF LACTATE CONCENTRATION

- Reaction mixture (total volume 200 μ l) contained:

triethanolamine-HCl buffer (pH 7.5)	50 mM
propionate	0 ~ 200 mM
lactate	0 ~ 100 mM
- Incubated at 37°C for 8 hours under air atmosphere with mechanical agitation
- Reactions were stopped with the addition of 40 μ l H₂SO₄ (50%)
 - 0 mM lactate
 - △— 10 mM lactate
 - 20 mM lactate
 - 30 mM lactate
 - ▲— 50 mM lactate
 - 100 mM lactate

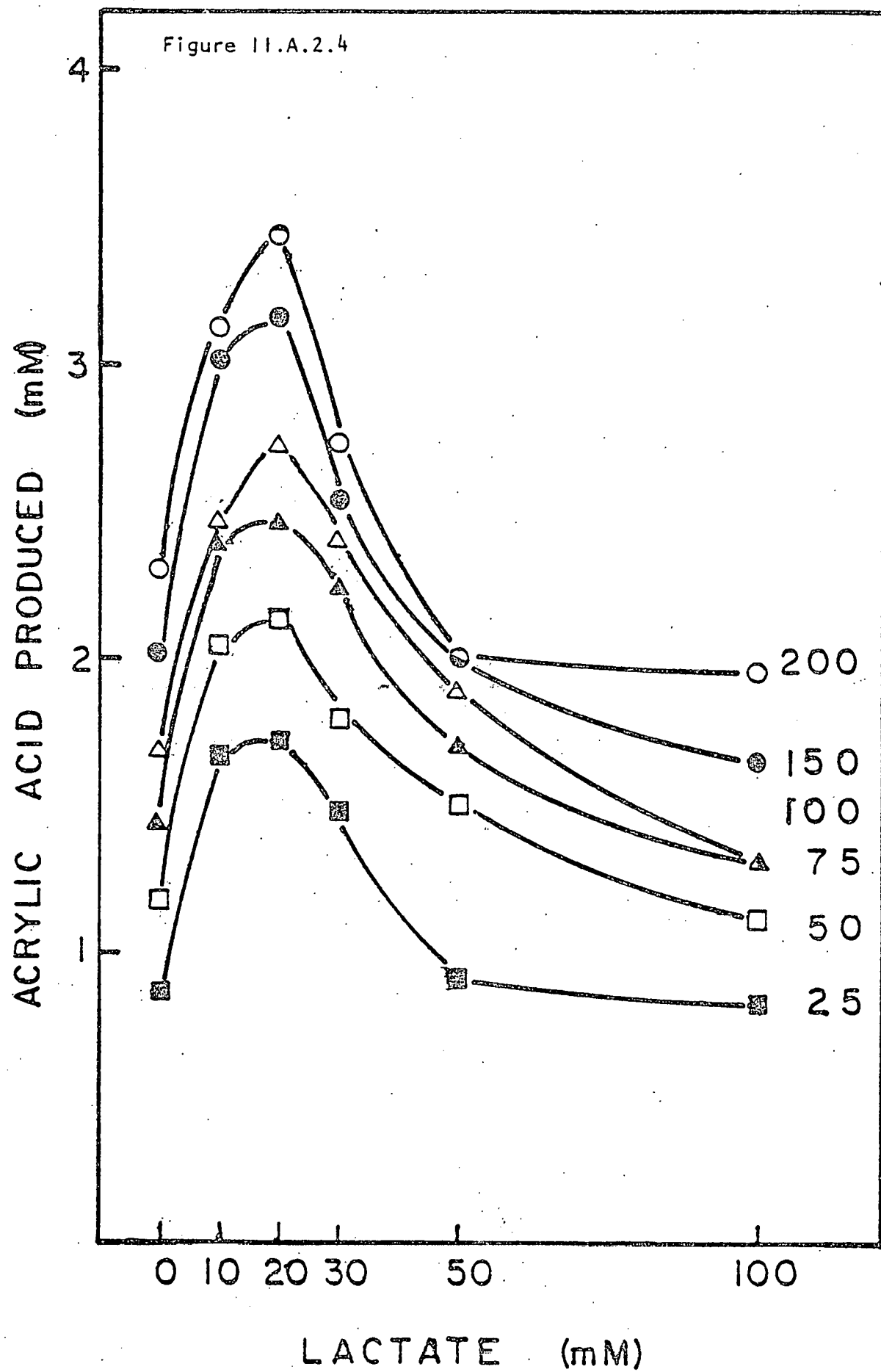


FIGURE II.A.2.5: EFFECT OF LACTATE ON THE PRODUCTION OF
ACRYLATE FROM PROPIONATE

- o Reaction mixture (total volume 2 ml) contained:

triethanolamine-HCl buffer (pH 7.5)	50 mM
propionate	200 mM
lactate	0 ~ 100 mM
- o Incubated at 37°C for 8 hours under air atmosphere with mechanical agitation
- o Samples (200 µl) were drawn after 8 hour incubation and placed in a tube containing 40 µl of 50% H₂SO₄

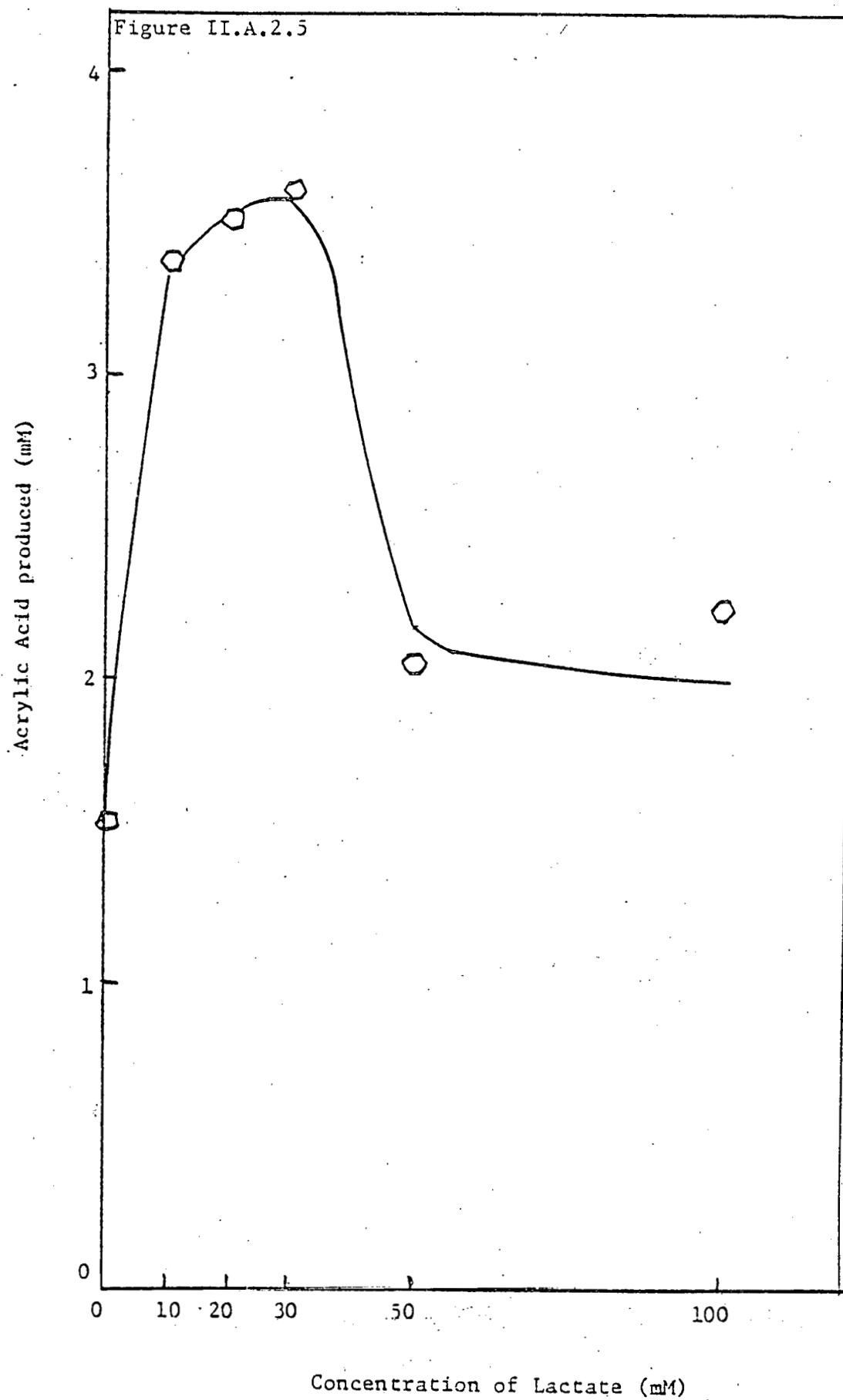


FIGURE II.A.2.6: EFFECT OF pH ON THE OXIDATION OF PROPIONATE TO ACRYLATE

- o Reaction mixture (total volume 200 μ l) contained:

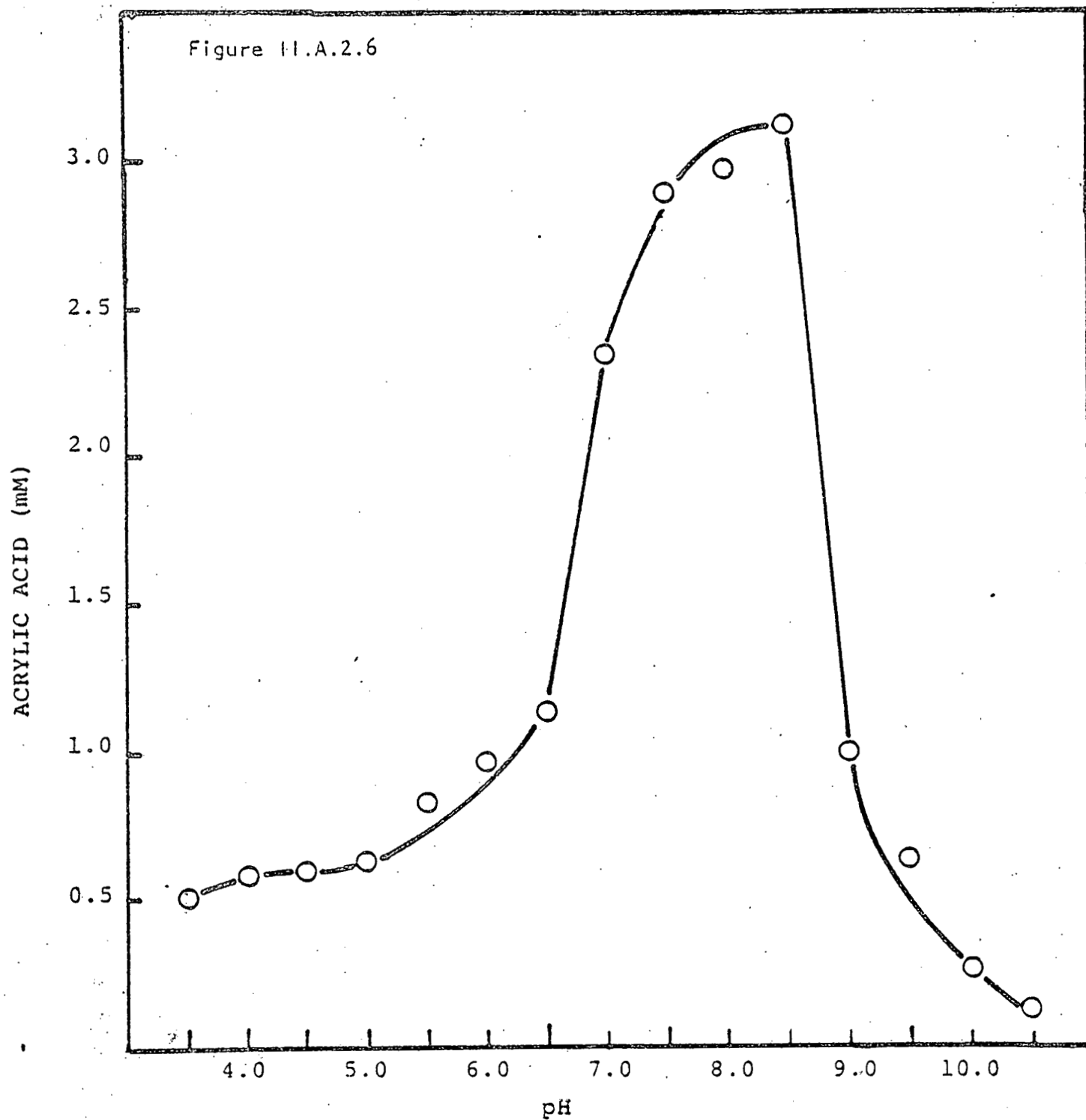
pH buffer*	50 mM
propionate	200 mM
lactate	25 mM
- o Incubated at 37°C under air atmosphere with mechanical agitation for 8 hours
- o Reactions were stopped with the addition of 40 μ l H_2SO_4 (50%) after 8 hour incubation

* The following buffer systems were used:

{ citric acid - Na_2HPO_4 for pH 4.0 ~ 6.5
triethanolamine hydrochloride - NaOH for pH 7.0 ~ 8.5
sodium carbonate (Na_2CO_3) - sodium bicarbonate ($NaHCO_3$)
for pH 9.0 ~ 10.5

EFFECT OF pH ON THE OXIDATION OF PROPIONATE TO ACRYLATE

Figure H.A.2.6



Under anaerobic condition (Figure II.A.2.7), there was no acrylate production without methylene blue. Increasing the amount of methylene blue (up to 0.2%) resulted in an increase in the initial rate of acrylate production and in a high final acrylate concentration. However, once the methylene blue is reduced (as observed by the color change), the acrylate was readily metabolized and disappeared.

Under aerobic condition as shown in Figure II.A.2.8 the initial rate of acrylate production was increased as methylene blue increased, but the final amount of acrylate was not as great as these control when 0.1% or less methylene blue was used.

(4.) Combined effect of lactate and methylene blue

In the above experiments, the effect of lactate or methylene blue was investigated independently. Here the production of acrylate was examined in the presence of both lactate and methylene blue. It was observed that acrylate production was greatly stimulated only when both lactate and methylene blue were present in the reaction mixture as seen in Figure II.A.2.9. If we removed either of these, no accelerated production of acrylate was observed.

The presence of air (or oxygen) was found to be essential for the stable accumulation of acrylate (Figure II.A.2.10).

FIGURE II.A.2.7: EFFECT OF METHYLENE BLUE ON THE OXIDATION OF PROPIONATE TO ACRYLATE UNDER ANAEROBIC CONDITION

- Reaction mixture (total 2 ml) contained:

triethanolamine-HCl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0 ~0.2%

- Incubated at 37°C under N₂ atmosphere with mechanical agitation

- Samples (200 µl) were taken at each time interval and reaction was stopped with 40 µl of 50% H₂SO₄

—○— 0 % methylene blue (control) ..
—□— 0.05 % methylene blue
—△— 0.1 % methylene blue
—●— 0.2 % methylene blue
—▲— 0.2 % methylene blue (Under aerobic condition)

arrow (↓) indicates the color change of methylene blue from blue to colorless

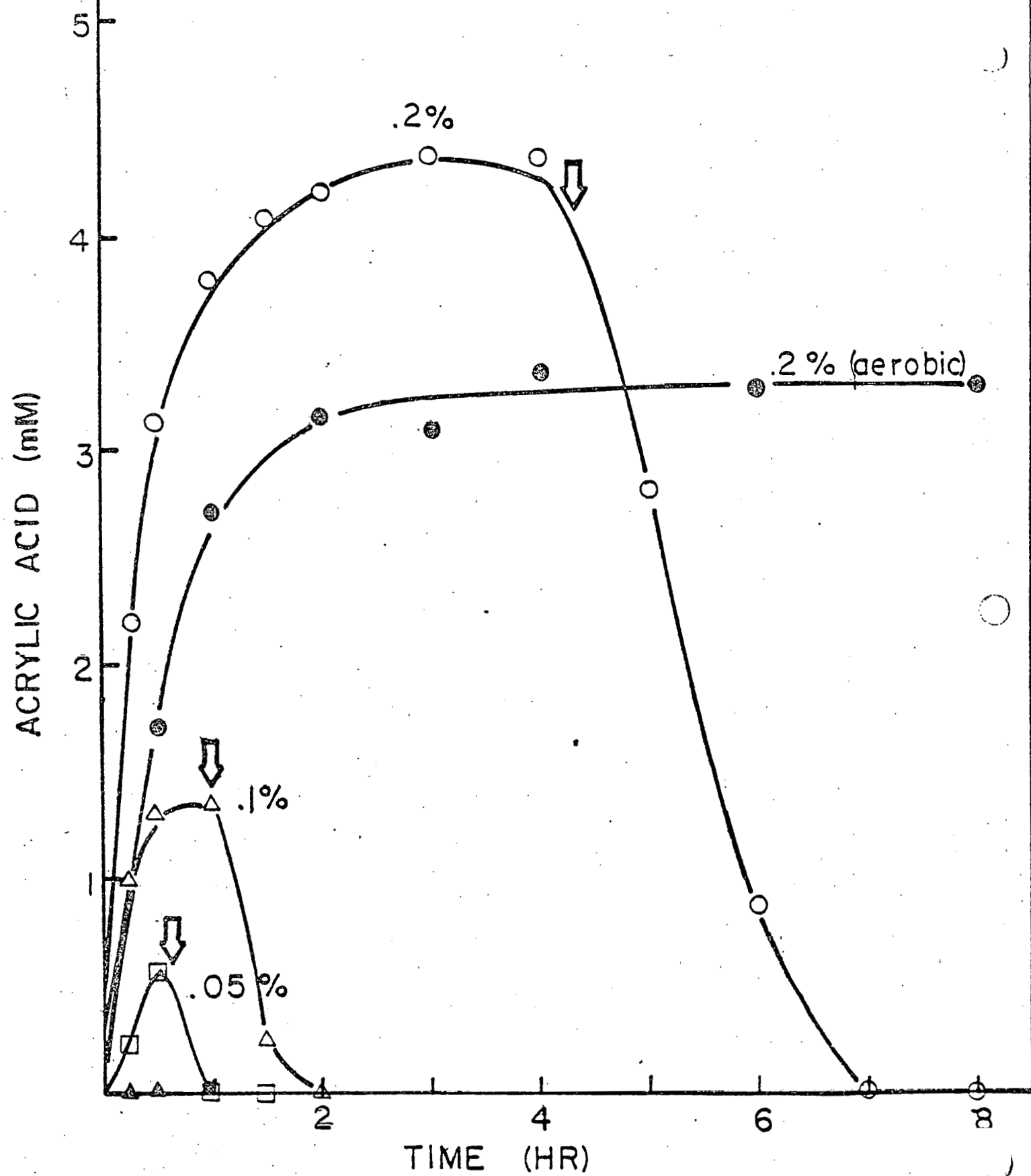


FIGURE II.A.2.8: EFFECT OF METHYLENE BLUE ON THE OXIDATION OF PROPIONATE TO ACRYLATE UNDER AEROBIC CONDITION

- Reaction mixture (total 2 ml) contained:

triethanolamine-HCl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0 ~ 0.2 %
- Incubated at 37°C under air atmosphere with mechanical agitation
- Samples (200 µl) were drawn at each time interval and transferred to a tube containing 40 µl of 50% H₂SO₄
 - 0 % methylene blue (control)
 - 0.05 % methylene blue
 - △— 0.1 % methylene blue
 - 0.2 % methylene blue

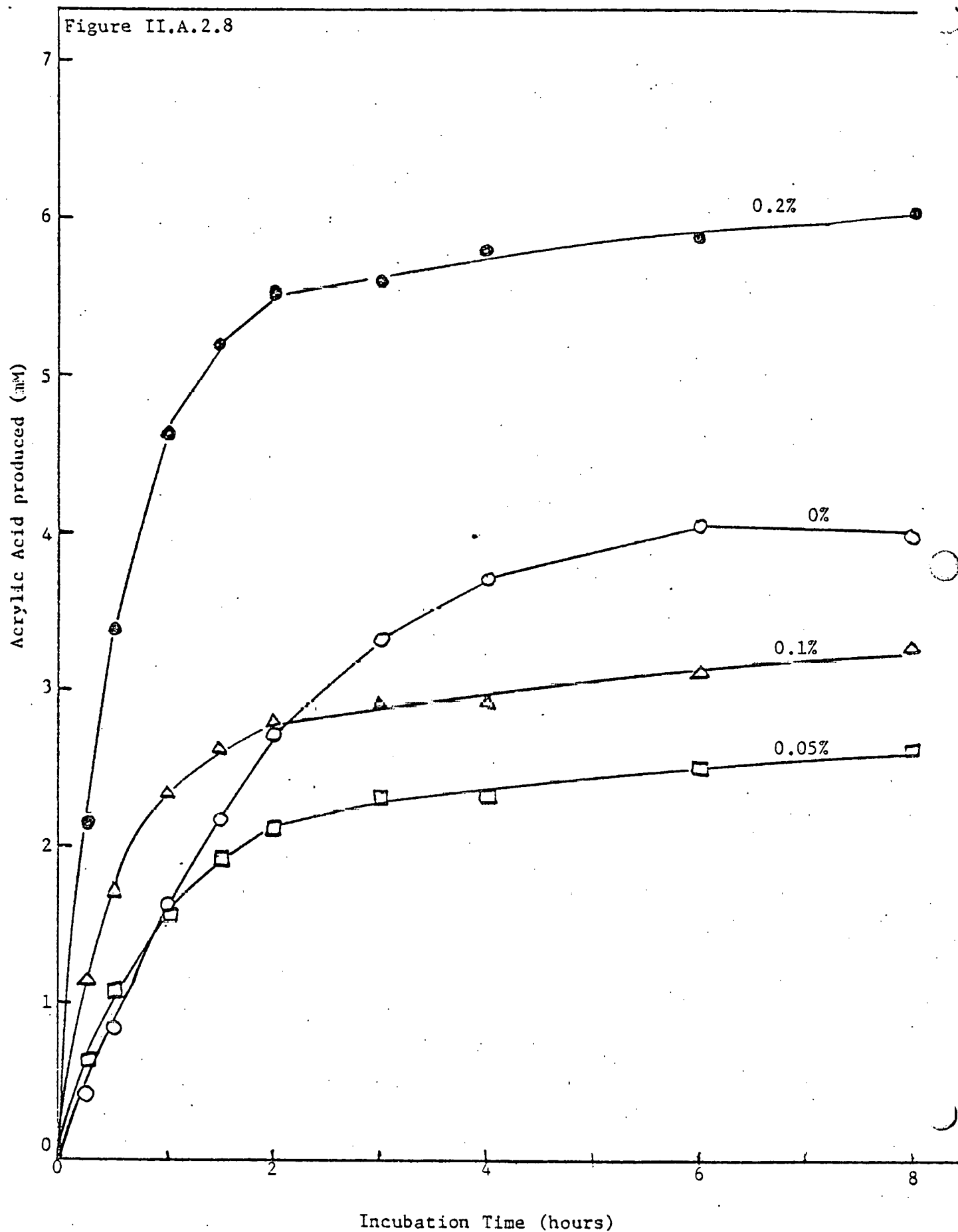


FIGURE II.A.2.9: PRODUCTION OF ACRYLIC ACID FROM PROPIONATE
IN THE PRESENCE OF METHYLENE BLUE AND LACTATE

- Reaction mixture (total volume 2 ml) contained:

triethanolamine-HCl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
lactate	25 mM

- Incubated at 37°C under air atmosphere with mechanical agitation

- Samples (200 μ l) were taken at each time interval and transferred to a tube containing 40 μ l of 50% H_2SO_4

- propionate + methylene blue + lactate
- propionate + methylene blue (- lactate)
- △— propionate (- methylene blue) + lactate
- propionate (- methylene blue) (- lactate)
- ▲— (- propionate) + methylene blue + lactate

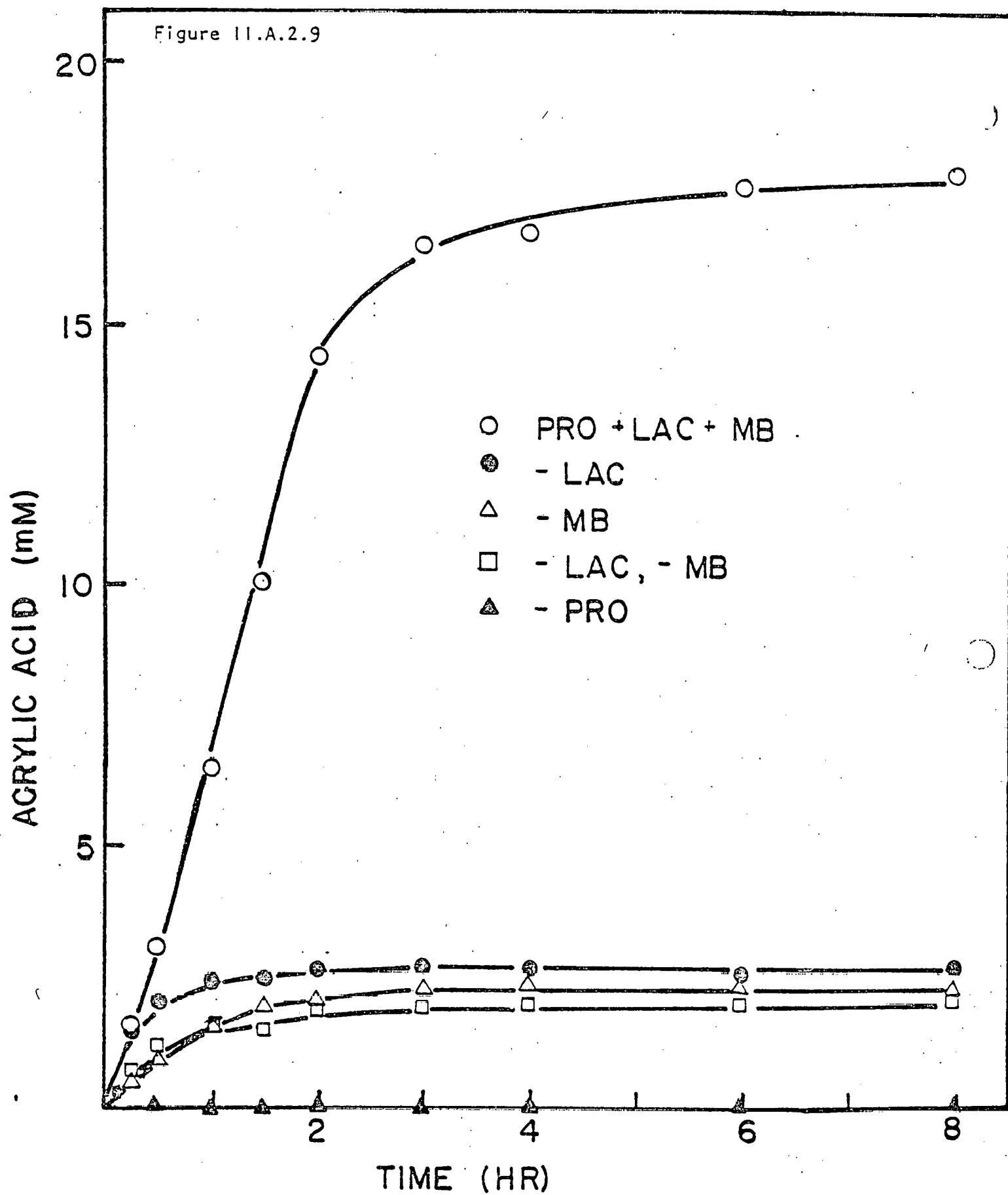


FIGURE II.A.2.10: EFFECT OF AIR (OR OXYGEN) ON THE PRODUCTION OF ACRYLATE FROM PROPIONATE

- Reaction mixture (total 2 ml) contained:

triethanolamine-HCl buffer (pH 8.5)	50 mM
propionate	200 mM
lactate	25 mM
methylene blue	0.2 %

- Incubated at 37°C under either anaerobic or aerobic condition with mechanical agitation
- Samples (200 µl) were taken at each time interval and transferred to a tube containing 40 µl of 50% H^2SO_4 in order to stop reactions

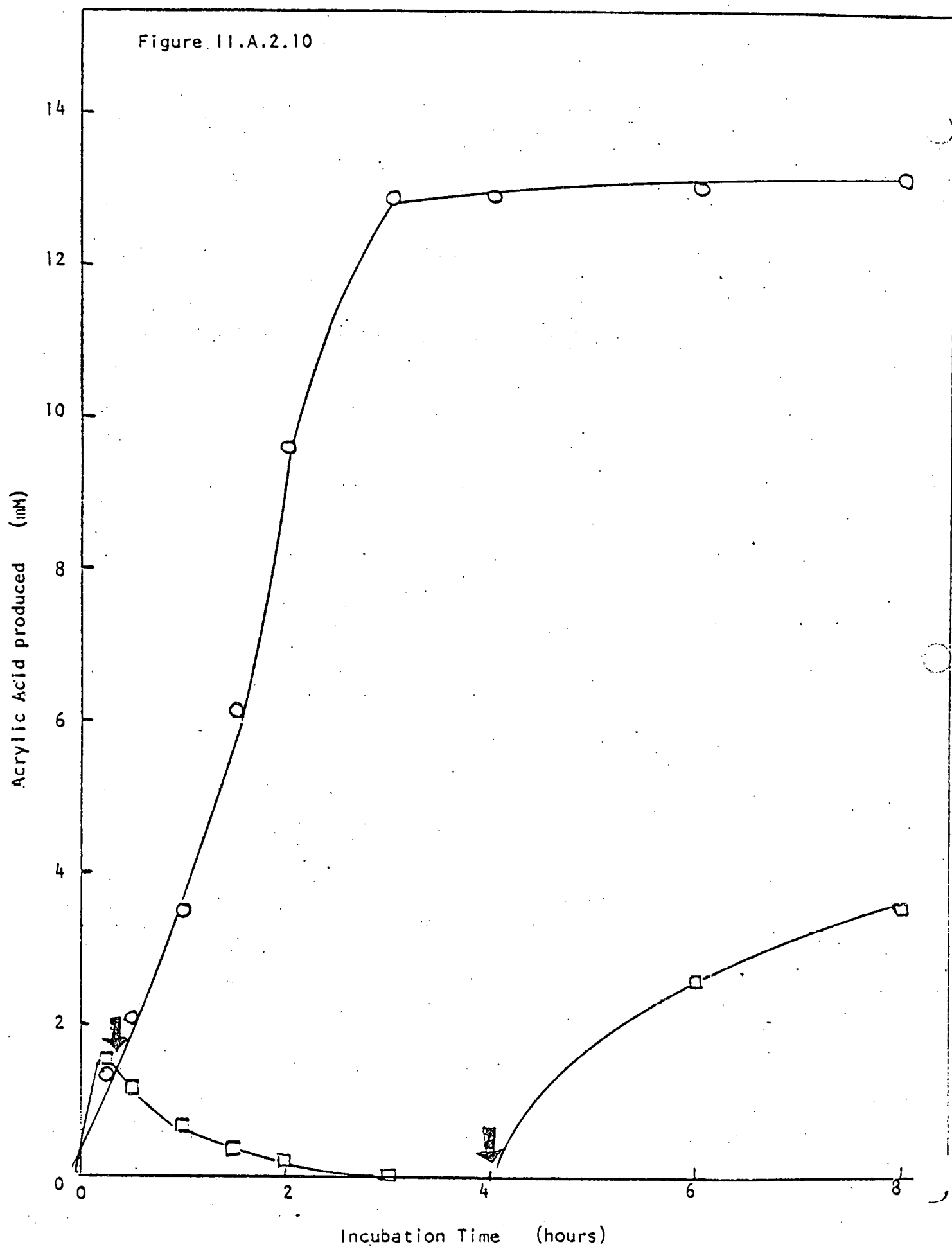
—○— under aerobic condition

—□— under anaerobic condition

↓ indicates the color change of methylene blue from blue to colorless

↓ indicates the atmosphere shift from anaerobic to aerobic condition. At this moment, methylene blue turned back to blue color

Figure 11.A.2.10



(5.) Cell Immobilization

Since the production of acrylic acid by resting cell preparations of C. propionicum is in effect a bioconversion process rather than a fermentation process, an investigation into the use of immobilized whole cells has been initiated. Because the oxidation of propionate to acrylate requires prior activation of propionate to propionyl-coenzyme A (CoA), the use of the purified, immobilized enzymes is not practical. However, as with immobilized enzymes, the fixation of whole cells on or within an inert support offers several advantages over the use of free cells as in a conventional fermentation process. These include the facilitated removal and reuse of cells, the possibility of eliminating product inhibition or consumption of product by the use of novel column reactor designs which allow the manipulation of reaction conditions and swift removal of products, the operation of the process in a continuous fashion and the potential stabilization of enzyme activity. In this section we report preliminary experiments on the immobilization of C. propionicum cells by entrapment in polyacrylamide gel. The immobilized cell preparation retained good activity for the production of acrylic acid.

Preliminary results based on tube reactions in which approximately equal amounts of free and immobilized cells were compared, indicate that polyacrylamide gel-entrapped C. propionicum cells retain about 50% of the

activity of free cells with respect to acrylic acid production (Table II.A.2.1). This result is encouraging for a "first attempt" and is comparable to many immobilized cell systems. There is, however, room for improvement by optimizing the many parameters involved in immobilization. Also, as noted above, immobilization appears to result in somewhat increased stability of cells during storage.

In a separate experiment, polymerization was allowed to proceed in a glass petri dish such that a thin layer of gel-entrapped cells was formed. After washing the surface, 5 ml of substrate solution was added and the dish incubated statically at room temperature for 48 hours. At that time, 10.06 mM acrylic acid was observed in the clear overlayer. Incubation of a suspension of free cells of approximately the same cell concentration for 48 hours resulted in 9.42 mM acrylic acid.

An investigation of the use of an immobilized cell column reactor has just been initiated. To date technical problems with equipment and reactor design have prevented an extended continuous flow experiment. However, one run has been performed in which a substrate-buffer mixture (50 ml) was pumped through a column of gel particles in a recycle loop. After 18 hours (about 3 cycles), the gel column was washed by pumping TEA-Mg buffer without substrates for 18 hours (without recycle). This was followed by pumping fresh substrate-buffer mixture (50 ml)

TABLE II.A.2.1: COMPARISON OF ACRYLIC ACID PRODUCTION IN
FREE AND POLYACRYLAMIDE GEL-ENTRAPPED CELLS*

	ACRYLIC ACID (mMoles/l-hr)		
Experiment	Free Cells	Immobilized Cells	%
A	2.68	1.15	43
B	2.07	1.02	49
C	2.48	1.36	55

* Reaction mixtures contained 200 mM sodium propionate, 50 mM TEA buffer (pH 7.5) and approximately 20 mg/ml dry cell weight. Incubation was for 2 hours at 37°C.

in recycle again for 18 hours. At the end of the first 18 hour period the acrylic acid concentration was 1.26 mM, and at the end of the second 18 hour reaction the concentration was 1.09 mM. There was no acrylic acid detectable at the end of the buffer "wash". Although the amount of acrylic acid produced was small, these results do indicate that a continuous-flow immobilized cell column reactor may be useful in determining certain characteristics of immobilized cells such as stability and also indicate the potential for continuous production of acrylic acid.

(6.) Storage Stability

The ability to store cell suspensions with minimal loss of activity would eliminate the time consuming process of growing and harvesting cells prior to every set of experiments. Several storage conditions were investigated with the aim of achieving maximum retention of activity. A fresh cell suspension of known activity was divided into three portions. All three portions were stored for 48 hours under a nitrogen atmosphere in 10 mM triethanolamine buffer (pH 7.5). All assays were performed in the standard manner with propionate as the substrate. One portion was stored at 4°C, another at -20°C and the final portion at -20°C in the presence of 10% glycerol and 10 mM cysteine. In addition, cells immobilized in polyacrylamide gel (see section on Cell Immobilization)

were stored at -4°C for 48 hours and their activity compared to that of the freshly immobilized cells. The results are shown in Table II.A.2.2. Freezing of cells, even in the presence of glycerol results in extensive loss of activity. The results do indicate that a cell suspension stored at 4°C could be used for 1 or 2 days. Other storage methods should be investigated for longer storage periods. These methods could include lyophilization and other drying methods as well as investigation of other cryoprotective agents such as DMSO (dimethylsulfoxide). The increased stability of immobilized cells is encouraging and should be pursued.

(7.) Nutrient Requirements of Clostridium propionate.

Clostridium propionicum grows to an optical density of about 0.50 in yeast-extract, peptone broth. It is desired, however, to create a defined medium which supports similar growth. Experiments were done to determine amino acid and vitamin requirements.

Through a process of elimination of the twenty amino acids, we determined that methionine is a requirement, tyrosine suppresses growth when added with methionine, tryptophan and possibly serine have stimulatory effects when added with methionine.

Since C. propionicum can grow on yeast-extract, peptone broth which does not contain glycine,

TABLE II.A.2.2: STABILITY OF PROPIONATE-OXIDIZING ACTIVITY
(ACRYLIC ACID PRODUCTION) OF CELL SUSPENSIONS
STORED UNDER VARIOUS CONDITIONS

CONDITION OF STORAGE*	PER CENT OF ORIGINAL ACTIVITY
4°C	75
-20°C	50.5
-20°C/10% glycerol/10 mM cysteine	55
Polyacrylamide gel-entrapped cells	90

* See text for details

aspartate, glutamate, or proline, we eliminated these as requirements. For the rest of the experiments, we prepared a stock medium containing the same vitamins and minerals as in the complex medium. Various combinations of amino acids were added to Hungate tubes and relative growth was compared by optical density to negative (no AA's) and positive (all AA's) controls. The buffer medium to which the amino acids were added contained:

	Final conc.	Use
Yeast Nitrogen Base (YNB)	6.7 g/l	Vitamins & minerals
α -alanine	8.0 g/l	C-source
Resazurin		Oxygen indicator
Cysteine	0.3 g/l	Reducing agent
Amino acid stock solution	0.05 g/l	

The procedure followed for these studies was to prepare a culture from the seed and incubate at 37° for 24 hours. The medium at double strength was adjusted to pH 7.0. The amino acid solutions at double strength were also adjusted to pH 7.0. Into each Hungate tube was added: 5.0 ml α -ala-YNB stock (2 x strength), 0.5 ml each amino acid (20 x) and 4.5 ml H₂O to give a final volume of 10 ml. The tubes were flushed with nitrogen, autoclaved, and flushed with nitrogen again. Then 0.1 ml of KH₂PO₄/K₂HPO₄ buffer was added to the tubes and they were inoculated with 3 ml of seed culture and incubated at 37°C.

The 17 amino acids were separated into 6 groups according to their chemical structure. The sulfur group (#4), containing cysteine, methionine, and cystine was required since it was present in all tubes with most growth and was absent from tubes with no growth. The "amide group" containing asparagine and glutamine could not support growth, and showed no stimulatory effects. The necessity of the other 4 groups was not clear but they appeared to be stimulatory.

To determine which of the remaining 15 amino acids were required, tubes with combinations of amino acids eliminating one amino acid per tube were examined. There was no growth without methionine or tryptophan, so these appeared to be requirements. Valine and tyrosine were possibly stimulatory since there was growth, but less than with all the amino acids.

Growth was examined with combinations of these 4 amino acids to determine if each is a requirement or a stimulatory. Methionine is required - there is no growth when it is absent. Tryptophan is stimulatory when added to methionine, but cannot support growth alone. Valine is non-essential. Tyrosine suppresses growth with methionine present.

To check the stimulatory effect of each of the four amino acids, they were added to YNB and methionine. Our controls were amino acids, methionine alone

and all amino acids. Cysteine and phenylalanine had the same growth as methionine alone and therefore were not stimulatory. Serine was perhaps stimulatory. Tryptophan had the same growth as all amino acids and was definitely stimulatory with methionine.

The maximum growth on yeast extract and peptone was OD ~ 0.50 and the maximum growth on YNB + α - alanine + amino acid solutions and cysteine was OD ~ 0.20. Therefore, something was lacking from the second medium. Either the amino acid solutions were not sufficiently supportive of growth or α -alanine was a poor carbon source. We tested growth on YNB and vitamin-free casamino acids to see if the amino acid solutions were deficient. Next we added NH_4HCO_3 to the α -ala-YNB medium as a source of CO_2 . Growth was the same on vitamin-free casamino acids as on our amino acid solutions and growth on vitamin-free casamino acids was the same with or without bicarbonate. So, we conclude that the amino acid solutions are sufficient and that bicarbonate has no effect on growth.

To specify the vitamins required by C. propionicum we made a vitamin-medium containing the nutrients of the complex medium and to it we added vitamins and compared growth to negative (no vitamins) and positive (all vitamins) controls. The composition of Vitamin-Free Medium was

	<u>Final conc.</u>	<u>Reason</u>
Na ₂ HPO ₄	7 g/l	Buffer
KH ₂ PO ₄	3 g/l	Buffer
NH ₄ Cl	1.0 g/l	N for amino acid biosynthesis
MgSO ₄ · 7H ₂ O	0.25 g/l	Mineral
FeSO ₄ · 7H ₂ O	.01 g/l	Mineral
CaSO ₄ · 2H ₂ O	2.5 ml/l	Mineral
α-alanine	8.0 g/l	C-source
Cysteine	0.3 g/l	Reducing agent
Resazurin	1.0 ml of 0.2% solution	O ₂ indicator
Vitamin-free casamino acids	10 g/l	Amino acid

The vitamin solutions were:

	<u>(20 x)</u>
Biotin	.01 g/100 ml
Ca Pantathenate	.4 g/100 ml
Folic Acid	.02 g/100 ml
p-Aminobenzoic Acid	.01 g/100 ml
Pyridoxine HCl	.4 g/100 ml
Riboflavin	.4 g/100 ml
Thiamine HCl	.4 g/100 ml

Our first experiment was to eliminate one vitamin per tube and compare growth by optical density. Because of the color of the vitamins and some mineral precipitate, the background was too great to clearly detect growth

differences. However, possibly folic acid may be required.

We next tried agar plates with each plate lacking one vitamin. Again the difference in the color of the plates and the rough agar made precise comparison of growth difficult. This work will be continued.

d. Future Work

• Complete the optimization of acrylate production with cell suspension of C. propionicum by examining the effects of temperature, growth phase, and cell concentration.

• Examine in more detail the role of lactate in stimulating acrylate production for propionate.

• Complete the nutritional study on vitamin requirements.

• Determine conditions for immobilization of C. propionicum cells in polyacrylamide gel which yield optimum specific activity and stability of acrylic acid production.

(a) Examine effect of the concentration of acrylamide monomer and the degree of cross-linking.

(b) Examine effect of cell concentration, temperature, pH, ionic strength, divalent cations.

• Perform a prolonged continuous-flow immobilized cell column run to determine the half-life for acrylic acid production.

- Examine various parameters for the continuous production of acrylic acid such as flow rate, substrate concentrations, etc.

3. Aerobic Oxidation of Propionic Acid to Acrylic Acid by Escherichia Coli

a. Introduction

This report summarizes results of attempts to produce acrylic acid from propionic acid using E. coli ATCC 9637-2.

Results obtained by others with a pseudomonas enzyme extract demonstrated the hydration of acrylyl-pantetheine to lactyl-pantetheine. Radiorespirometric analysis of labelled propionate oxidation by E. coli strengthened the suggestion that propionate was metabolised via acrylyl-CoA to lactate. Further work by various investigators with (Monerella buoffi, A. glaucus, P. digitatum, P. aenerginosa, amongst) other microorganisms supported this hypothesis.

(1) Choice of the Organism

Characteristics desirable for a suitable organism were (a) One in which acrylyl-CoA had been strongly suggested as an intermediate of propionate oxidation, (b) in which propionate could be metabolized solely via acrylyl-CoA, maximizing the conversion yield (c) and in which efficient mutation could be attempted.

E. coli w (ATCC 9637-2) was selected.

It metabolized propionate via lactate and α -hydroxy glutarate. C_4 compound synthesis, essential for growth on propionate could be achieved via lactate by the glvoxylate cycle,

phospho (enol) pyruvate carboxylase or via α -hydroxy glutamate. An ace^A (isocitrate lyase) mutation in this organism would block α -hydroxyglutarate synthesis owing to lack of glyoxlate synthesis. Hence, all the propionate could be metabolized solely through the lactate pathway. Such mutants in E. coli ω have been reported to grow on propionate comparably with the wild type cells. Ace^A mutants in strains K-12 and E-26 are unable to grow on propionate, since they obtain C_4 compounds, unlike strain ω , primarily from α hydroxyglutarate.

(2) Line of Approach

Central to this project was the objective to create appropriate mutations resulting in acrylate accumulation from propionate oxidation. Two enzyme lesions were aimed for (a) an isocitrate lyase mutation and (b) a second mutation that would result in the phenotype propionate negative - lactate positive. Resting cell suspensions of the above mutant would have to be used to effect the bio-conversion.

b. Materials and Methods

The analytical procedures and conditions for growth, mutagenesis, enrichment and selection have been given in earlier progress reports.

c. Results and Discussion

(1) Growth Kinetics

Growth kinetic experiments were performed on a range of carbon sources at 0.2% concentration and in

M9 medium. Acrylate and isobutarate did not support growth. However, the cells could grow on glucose and propionate in the presence of 0.1% acrylate. Growth on propionate was preceded by a long lag of between 48 and 72 hours when inoculated from a slant or stationary phase culture. This lag could be reduced significantly, to 12 hours, by addition of a sparking quantity (0.5 mM) glucose, to propionate. Diauxic growth was observed. The doubling time of E. coli w on propionate was 510 minutes. HCO_3 added in equimolar concentration to propionate decreased the doubling time. The presence of 0.1% acrylate increased the doubling time on both glucose and propionate, the former from 90 minutes to 360 minutes.

(2) To study propionate metabolism in these cells, their maximum cell concentrations were compared when grown on equimolar concentrations of succinate, propionate and acetate (refer to previous two progress reports). At concentrations below 20 mM the maximum cell growth on propionate closely followed that on succinate, suggesting a common metabolism following propionate carboxylation. At concentrations above 20 mM however, the trend reversed and propionate growth levels were intermediate between the two. The cell yield dropped continuously, from 1.25 times that on succinate and acetate to an equal value. An increase in isocitrate lyase levels with a decrease in yield suggested a shift from phospho enol

pyruvate carboxylase mediated carboxylation to glyoxylate cycle mediated C_4 compound production.

The results obtained were not by themselves conclusive evidence for a shift in metabolism, but confirmed results obtained elsewhere.

(3) Resting Cell Suspensions

Wild type resting cell suspensions were studied for their ability to oxidize propionate. Potential chemical inhibitors of the enzymes involved were used (progress report March to May 31, 1978). No volatile fatty acids could be detected in ether extracts of the cell suspension despite propionate uptake. Propionate uptake was less in the presence of lactate, propionic acid, acrylate, cyclopropane carboxylate and oxamate. However, acrylate accumulation could not be detected.

With lactate as substrate a major peak appeared, that could be oxalacetate. The presence of acrylate with lactate inhibited the formation of oxalacetate, and lactate uptake. Arsenite did not cause pyruvate accumulation, despite lactate uptake. Under these conditions of growth, lactate was possibly not metabolized significantly by pyruvate dehydrogenase, but instead via phospho enol pyruvate carboxylase. The role of α hydroxy glutarate synthase pathway could not be determined.

(4) Cell Free Extracts

Both from the point of view as a method to characterize propionate negative mutants and to identify acrylyl-CoA as an intermediate in propionate oxidation, it was necessary to standardize a cell free system capable of oxidizing propionate.

Oxidation was followed spectrophotometrically by monitoring NAD^+ reduction. Undialysed samples had a very high rate of endogenous reduction of NAD^+ and dialysed samples were inactive even in the presence of 0.5 mM NAD^+ , 0.4 mM CoA, 0.5 mM propionate, 1 mM ATP-Mg and 4 mg protein/ml sample at pH 7.0.

The samples, however, retained lactate dehydrogenase, succinate dehydrogenase and isocitrate lyase activity. Nitrogen sparging of samples to inactivate NADH oxidase did not influence the observed rates.

(5) Mutation

The rational for this approach has been discussed previously. Acetate negative, propionate negative-lactate positive cells were isolated. However, from amongst the acetate negative mutants a stable ace^A mutant could not be isolated. From amongst propionate negative - lactate positive mutants, not one was able to oxidize propionate when it was added to lactate grown, resting cell suspensions.

The problem appeared to be the inability to achieve satisfactory enrichment, permitting the screening of a large enough population of potential mutants.

The protocol for mutation with EMS has been discussed in the progress report for Dec. 1 to Feb. 28, 1978. Roughly, a 70% inactivation of viable cells was obtained. A control for mutagenesis was performed by enriching the cells in trypticase soy broth and comparing the number of auxotrophic cells unable to grow on glucose synthetic medium. 10-20% of the cells recovered on trypticase soy were unable to grow on glucose-M9, indicating the extent of mutagenesis.

Enrichment of mutagenised cells was carried out by growing cells to the stationary phase in glucose. A 15% suspension of these cells in acetate synthetic medium was allowed to double once before being treated with penicillin A or ampicillin. Antibiotic enrichment was performed both in hypertonic medium and in normal medium. The latter method was also modified. Every half generation time the cells were centrifuged, washed and resuspended. This was done to prevent cross feeding of mutants by lysing cells.

Maximum enrichment of acetate negative cells was obtained using 500 µg/ml ampicillin in normal medium. 1×10^{-5} cells survived the treatment. This corresponded to a turbidity decrease of 60-70%. 80% of this decrease occurred within 1 doubling period, while the

remainder occurred over a total of 3-4 generations. Surviving cells were tested for antibiotic resistance and were found sensitive. Surviving cells were selected as described in the progress report for March to May 31, 1978.

The acetate negative cells were assayed for the isocitrate lyase lesion as previously described. The metabolic phenotype was determined by replica plating on lactate, propionate, EMB, isobuturate, acrylate, glucose, acetate and glyoxylate.

In the absence of an ace^A mutant, a propionate negative - lactate positive mutant was searched for, directly from the wild type cells. This was despite the fact that any propionate negative cell obtained would be either a double mutant with a lesion in both branches after propionyl CoA, or before propionyl CoA.

The difficulty faced was again one of enrichment. Ampicillin resulted in survival of 1×10^{-3} to 1×10^{-4} cells. To increase the enrichment, cells were washed after centrifugation, recovered in glucose and re-enriched with ampicillin. However, glucose recovered cells had a lag of 72-96 hours. Mutants isolated that were propionate negative - lactate positive were unable to oxidize propionate. The lesion was probably in the activation step.

d. Conclusions

As a consequence of the lack of success for acrylate production with E. coli it has been decided to terminate this approach to acrylate production. While the results of the studies continue to be consistent with the postulated pathway with acrylate as an intermediate, the alternative metabolic paths available point to the difficulty in this approach. Future studies will therefore focus on acrylate production from Clostridium.

4. Microbial Degradation of Cellulose and Accumulation of Lactic Acid

a. Introduction

The main objective of this portion of the study is the bioconversion of crude cellulosic materials into lactic acid which will be used as a key intermediate for acrylic acid production. A system of two microorganisms was selected to accomplish this objective. In the first stage, C. thermocellum should degrade cellulose with the accumulation of reducing sugars that are used by a thermolactate bacterium which accumulates lactic acid as final product. The characterization of the second microorganism in the system has to be done to define its properties as a single culture and to apply this information to study the interactions in a mixed culture. This report deals with the study of thermolactic bacterium with the immediate objective of identification of the strain. We expect that this information will

simplify the analysis of the mixed culture interactions. The results of some mixed culture studies are also presented. These mixed culture experiments confirmed the earlier results where no negative interactions were found, while each microorganism was able to grow on a different substrate. All the results obtained so far are encouraging with respect to the possibilities of a mixed culture system for the bioconversion of cellulose to lactic acid.

b. Materials and Methods

(1) Microorganisms:

- (a) Clostridium thermocellum ATCC-27405

See report COO-4198-1. Page 2-3.

- (b) "Thermolactic bacterium" (trivial name:

A. Homofermentative, thermophilic and facultative microorganism, Gram(+) and non-sporulating.

(2) Culture media

The media for growth of C. thermocellum and "T. bacterium" have been described previously (see reports COO-4198-1, page 2-3; and COO-4198-6, respectively).

For the growth of the mixed culture population a combined medium was used and is described as follows:

TABLE II.A.4.1: MIXED MEDIUM COMPOSITION (MIXME)

INGREDIENT	CONCENTRATION (gm/l)
Glucose	Variable
Cellobiose	Variable
Yeast extract	5.0
Trypticase	5.0
Tryptone	1.5
Cystxetine	0.1
Thioglycollate	0.5
KH_2PO_4	3.0
K_2HPO_4	4.4
$(\text{NH}_4)_2\text{SO}_4$	1.3
MgCl_2	1.0
CaCl_2	0.15
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1.25 mg/l
$(\text{NH}_4)_3$ Citrate	1.0
Tween 80	0.5 ml
Resazurin (1% solution)	1.0 ml
Antifoam FG10	0.2 ml
Salt Solution	2.5 ml
H_2O to 1.0 liter	

Salt Solution: gm/l

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	11.5
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.68
$\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$	2.4
H_2O	100.0 ml.

(3) Inoculum preparation

The standard procedure for inoculum preparation was followed (see report COO-4198-4, pages 108-109). Handling and growth of C. thermocellum was done following the methodology and growth conditions described in previous reports (see report COO-4198, pages 2-3).

(4) Fermentor

A 5.0 liter Magnaferm bench scale (New Brunswick Scientific) fermentor was used with 2.0 liter working volume. pH and temperature were controlled at constant values noted in the text.

(5) Assays

During the fermentation, the following kinetic parameters were followed: cell concentration, lactic acid, acetic acid, ethanol, reducing sugars and glucose. In all cases, we used the standard methodology described in previous reports.

c. Results and Discussion

(1) Growth of T. bacterium in the presence of oxygen .

In order to study the metabolic properties of 'T. bacterium', its resistance to oxygen was evaluated. Mixed medium was used with 2.0 gm/l glucose. Samples were treated differently with respect to oxygen availability. The results are shown in Table II.A.4.2. Oxygen is seen to have a stimulatory effect on growth. The ability to grow with oxygen is confirmed by the catalase test.

(2) Temperature range for growth of "T. bacterium".

This experiment was designed to evaluate the temperature range for growth of "T. bacterium" using mixed medium. The results are shown in Figure II.A.4.1. Cell growth is observed at 30°C but not at 25°C; therefore, cell growth is seen at 60°C but not at 65°C; therefore the maximum growth temperature is somewhere between 60-65°C. With respect to optimum temperature no conclusion can be drawn since it is apparent that it will be between 45 and 60°C and data are not yet available.

(3) Adaptation of "T. bacterium" for growth on xylose.

This experiment was performed to adapt "T. bacterium" to grow on xylose as a carbon source. The importance of these results is evident from an examination of the composition of reducing sugars produced from cellulose degradation: xylose, cellobiose and glucose. The experimental procedure was as described in Figure II.A.4.2. From a typical batch fermentation of "T. bacterium" on glucose, four samples were removed during logarithmic growth. Each sample was transferred to a series of 3 tubes containing: xylose (3.0 gm/l), xylose-glucose (3.0-0.1 gm/l) and medium with no sugar added. Samples were incubated for 12 hours and the best grown sample was transferred to tubes with the same medium and again incubated. The transfer operation was repeated 6 to 7 times. The results of those

TABLE II.A.4.2: EFFECT OF OXYGEN ON THE GROWTH OF
"T. BACTERIUM"

<u>Sample</u>	<u>Incubation Conditions</u>	<u>Growth⁽¹⁾ (K.U)</u>	<u>pH</u>	<u>Lactic Acid (gm/l)</u>	<u>Catalase⁽²⁾ (Units/ml)</u>
1	Anaerobic	108	4.68	2.5	550
2	Microaerophilic	130	4.70	2.6	500
3	Aerobic*	140	4.60	2.7	620

Notes:

* Tubes with cotton plugs to allow gas exchange

(1) Incubation time 16 hours.

(2) Method: Beers & Sizer (1952)

1 unit = 1 micromole of H_2O_2 per minute of $25^\circ C$

serial transfers are shown in Figure II.A.4.3. It can be seen that "T. bacterium" was able to grow on xylose although the optical density was not increased markedly after a series of 6 transfers.

(4) Fermentation products of "T. bacterium" on xylose.

In previous batch culture experiments, the fermentation products from xylose were studied. The results are shown in Table II.A.4.3. After 24 hours about 50% of the sugar was converted to lactic acid as the major product, as well as some ethanol and acetic acid; these products were in low concentration. No carbon dioxide was formed during the fermentation.

(5) Adaptation of "T. bacterium" for growth on Cellobiose.

Since cellobiose is a major product of cellulose degradation, it is important to develop the ability of "T. bacterium" to grow on cellobiose. The experimental procedure followed was the same as the one used for xylose adaptation, described in Figure II.A.4.2. Results from transferring the culture into a series of tubes and continuing the serial transfer every 12 hours are shown in Figure IIA.4. There was a continuing improvement after each transfer until eventually the optical density was maintained constant after each transfer at which point the culture was considered to be adapted to grow on cellobiose.

TABLE II.A.4.3: FERMENTATION PRODUCTS FROM "T. BACTERIUM"
GROWING ON XYLOSE AS CARBON SOURCE

<u>Sample No</u>	<u>Age (Hrs)</u>	<u>Cell Growth (gm/l)</u>	<u>Lactic Acid (gm/l)</u>	<u>Acetic Acid (gm/l)</u>	<u>Ethanol (gm/l)</u>	<u>Res. Xylose (gm/l)</u>
1	23	0.30	9.3	0.75	0.82	8.2
2	38	1.60	16.4	0.95	0.92	1.5

NOTES:

- (1) Mixed Medium with 20 gm/l xylose.
- (2) 40 hrs fermentation

TABLE II.A.4.4: KINETICS OF SUGAR UTILIZATION (GLUCOSE AND CELLOBIOSE) BY "T. BACTERIUM"

<u>Sample No</u>	<u>Age (Hrs)</u>	<u>Glucose (gm/l)</u>	<u>Red. Sugars (gm/l)</u>	<u>Cellobiose (gm/l)</u>	<u>Lactic Acid (gm/l)</u>
1	0	10.0	20.5	10.5	0.69
2	2	8.2	18.7	10.5	1.3
3	4	7.45	17.3	9.85	2.3
4	5	2.40	13.1	10.1	4.4
5	6	0.56	8.9	8.34	9.6
6	7	-	5.7	5.70	12.6
7	8	-	3.3	3.3	16.1
8	9	-	0.91	0.91	18.4

(6) Mixed substrate utilization by "T. bacterium".

The composition of mixed medium includes both glucose and cellobiose as carbon sources because it was assumed that C. thermocellum can grow on cellobiose preferentially and "T. bacterium" can grow on glucose. However, since "T. bacterium" can utilize both sugars, an experiment was designed to evaluate the preferential utilization of sugars by "T. bacterium" in mixed medium. A batch fermentation was designed and the kinetics of sugar utilization was followed. The results of this experiment can be observed in Table II.A.4.4 and also Figure II.A.4.5. It can be seen that glucose was used preferentially with respect to cellobiose under the conditions studied.

(7) Mixed culture growth on soluble sugars.

This experiment was designed to evaluate the potential interactions of the mixed population of C. thermocellum and "T. bacterium" when growing together on a mixture of sugars. The experiment was designed as a batch culture system with glucose and cellobiose as carbon sources. The results obtained are shown in Figure IIA.4.6. The experiment was started as a batch culture of C. thermocellum. After 10 hours "T. bacterium" was inoculated to start the mixed culture experiment. Total cell growth, lactic acid, ethanol and acetic acid productions were followed as criteria for

growth of the two strains. The results obtained showed that lactic acid accumulated up to 10.0 gm/l. This demonstrated the growth of "T. bacterium". Acetic acid and ethanol accumulated in a pattern similar to a normal single culture of C. thermocellum. From these results, we can conclude that the growth of a mixed population is possible where each microorganism is able to grow independently with no apparent negative interactions under the experimental conditions studied.

d. Future Work

- Future experiments will focus on the degradation of crude cellulosic materials such as corn stovers using the mixed culture population.

- In order to better understand the system, specific measurements for cell growth and substrate concentration in the broth are required. Therefore, analytical techniques will be developed to measure glucose, cellobiose, xylose, C. thermocellum and "T. bacterium" concentrations, as well as all the normal fermentation products.

FIGURE II.A.4.1: TEMPERATURE RANGE FOR GROWTH OF "T. BACTERIUM"

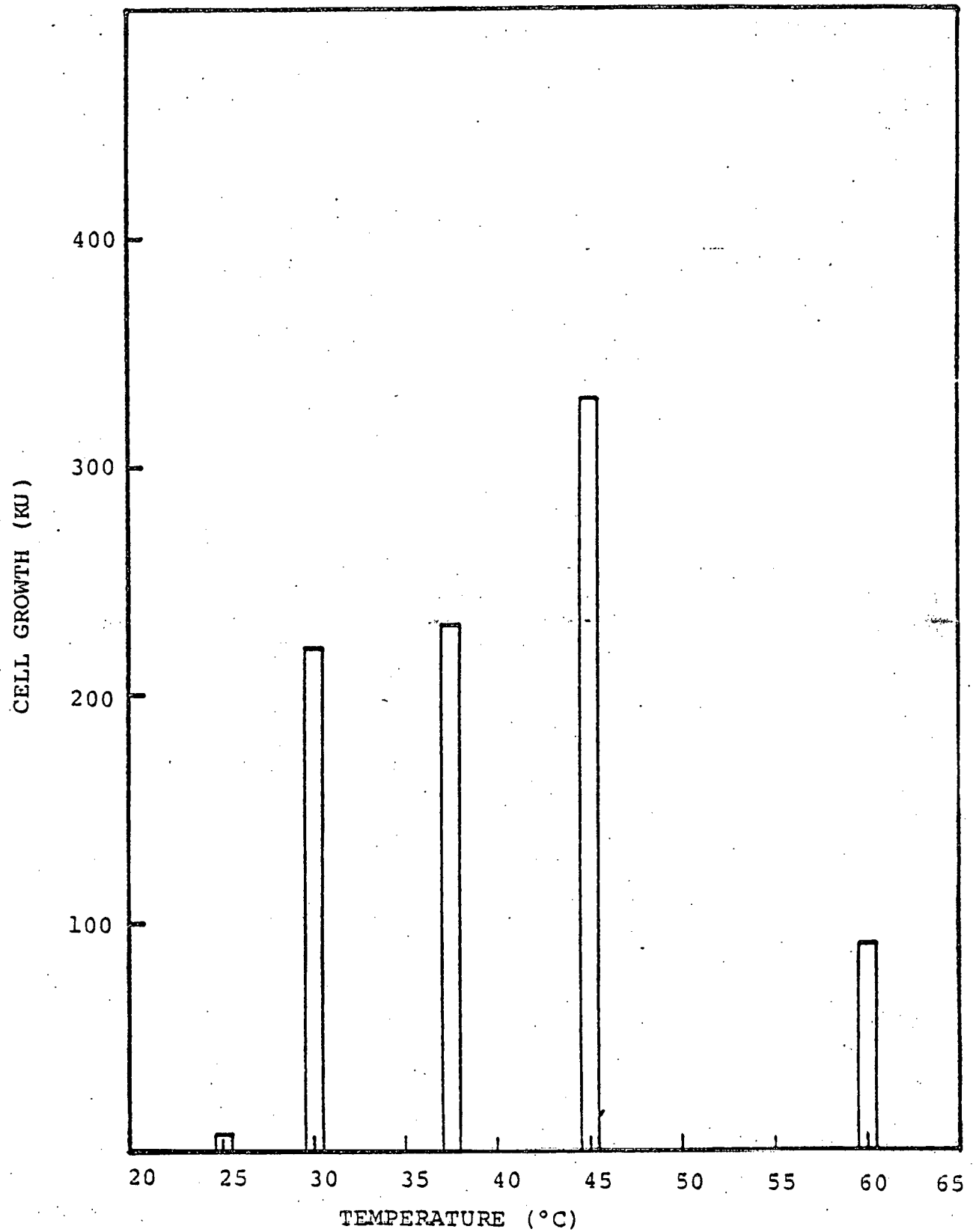


FIGURE II.A.4.2: SCHEMATIC PROCEDURE FOR ADAPTATION OF
"T. BACTERIUM" TO GROW ON XYLOSE

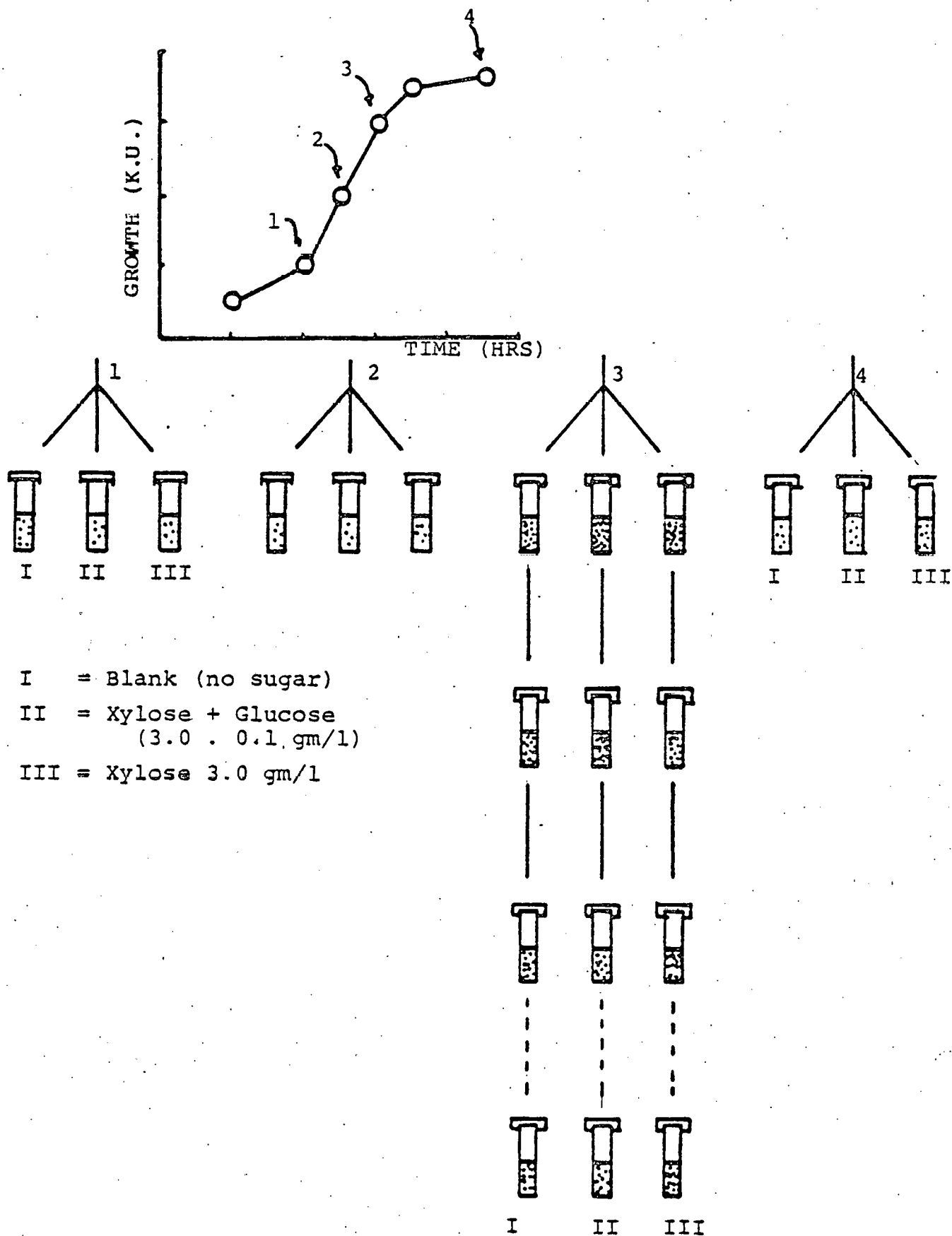


FIGURE II.A.4.3: ADAPTATION OF "T. BACTERIUM" TO GROWTH ON XYLOSE AT 60°C.
SERIAL TRANSFERS WERE DONE EVERY 12 HRS.

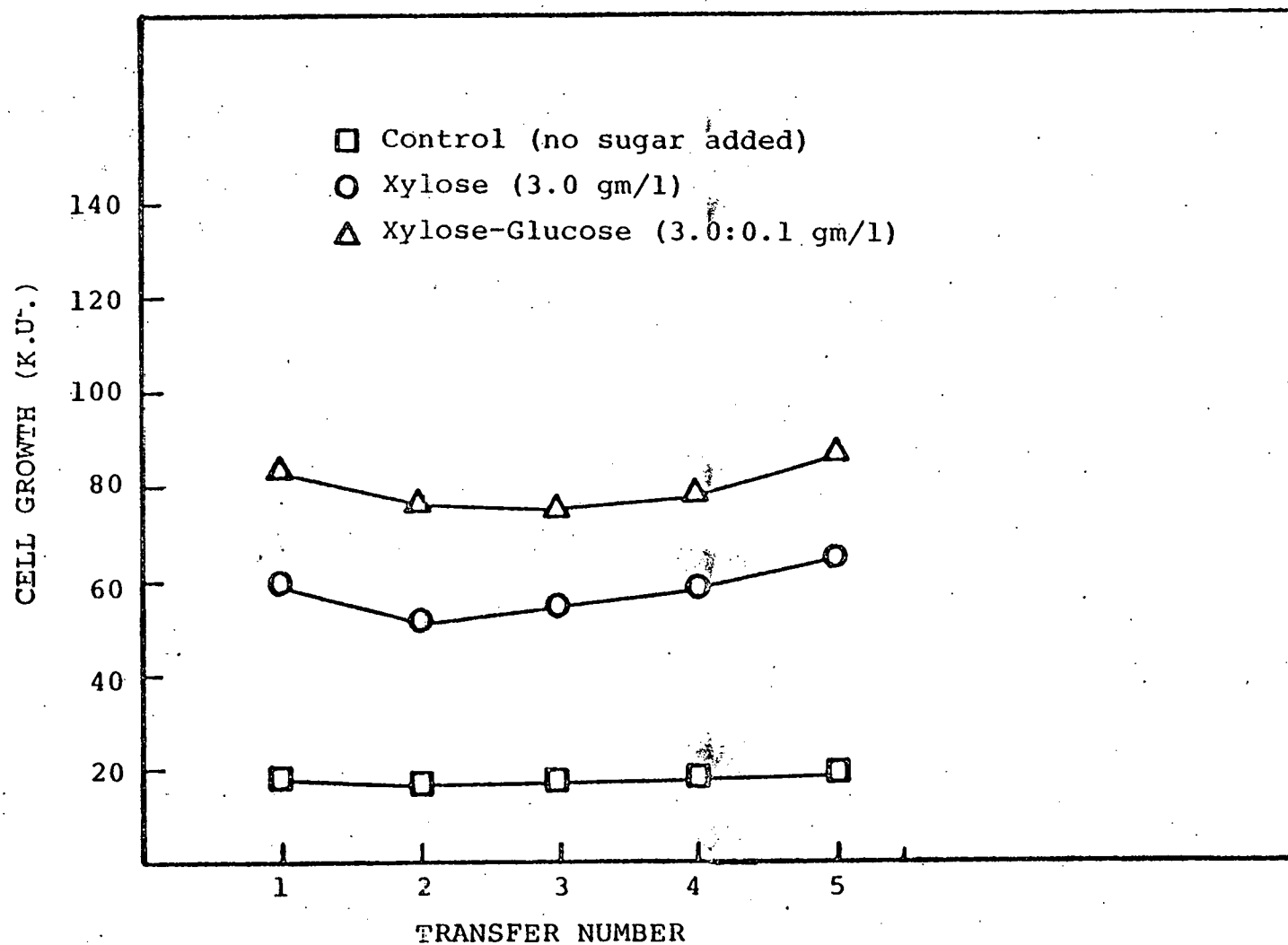


FIGURE II.A.4.4: ADAPTATION OF "T. BACTERIUM" TAKEN FROM LOGARITMIC PHASE OF GROWTH ON CELLOBIOSE

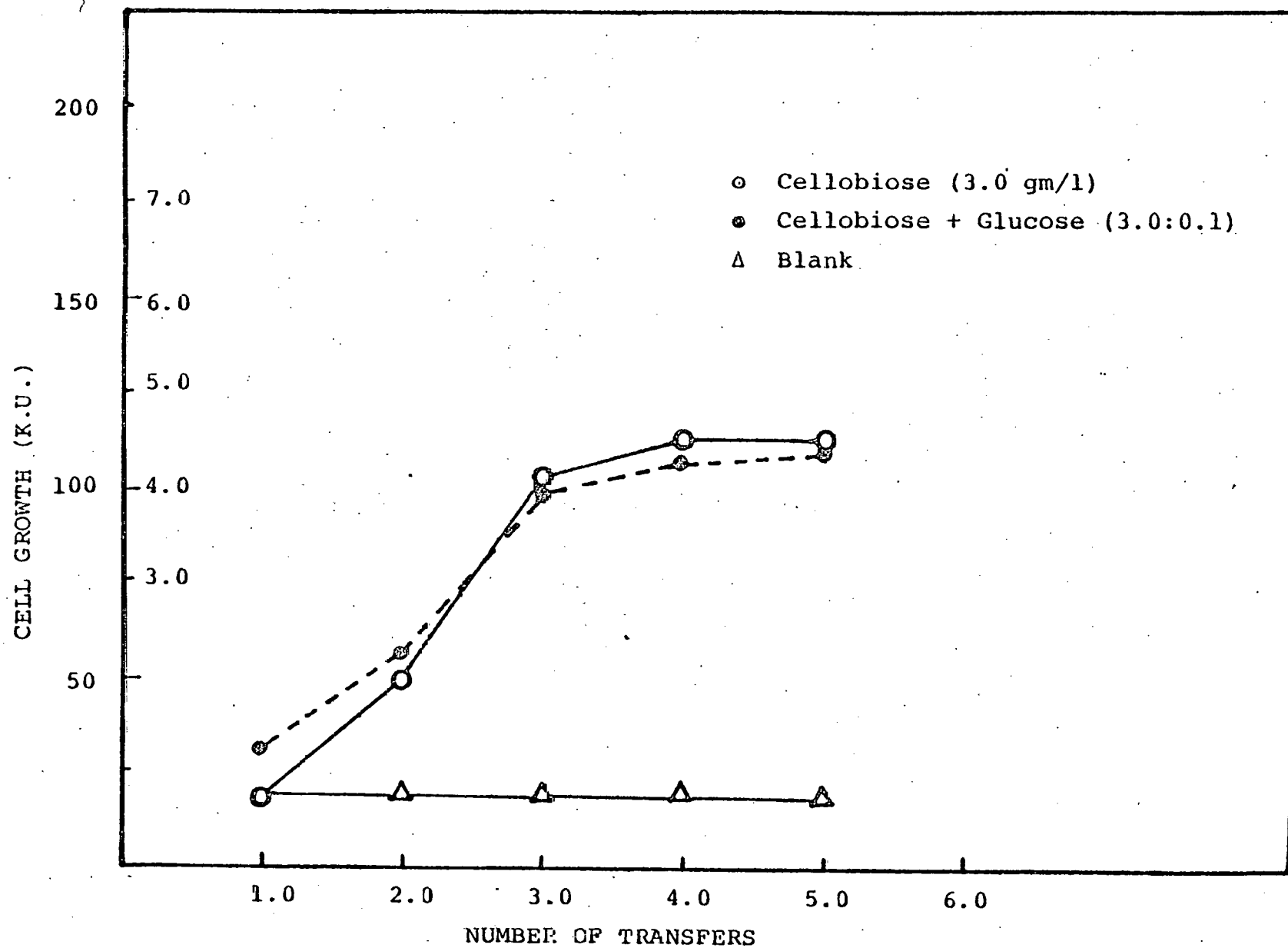


FIGURE II.A.4.5: MIXED SUBSTRATE UTILIZATION BY "T. BACTERIUM"
(GLUCOSE-CELLOBIOSE) IN BATCH CULTURE AT 60°C

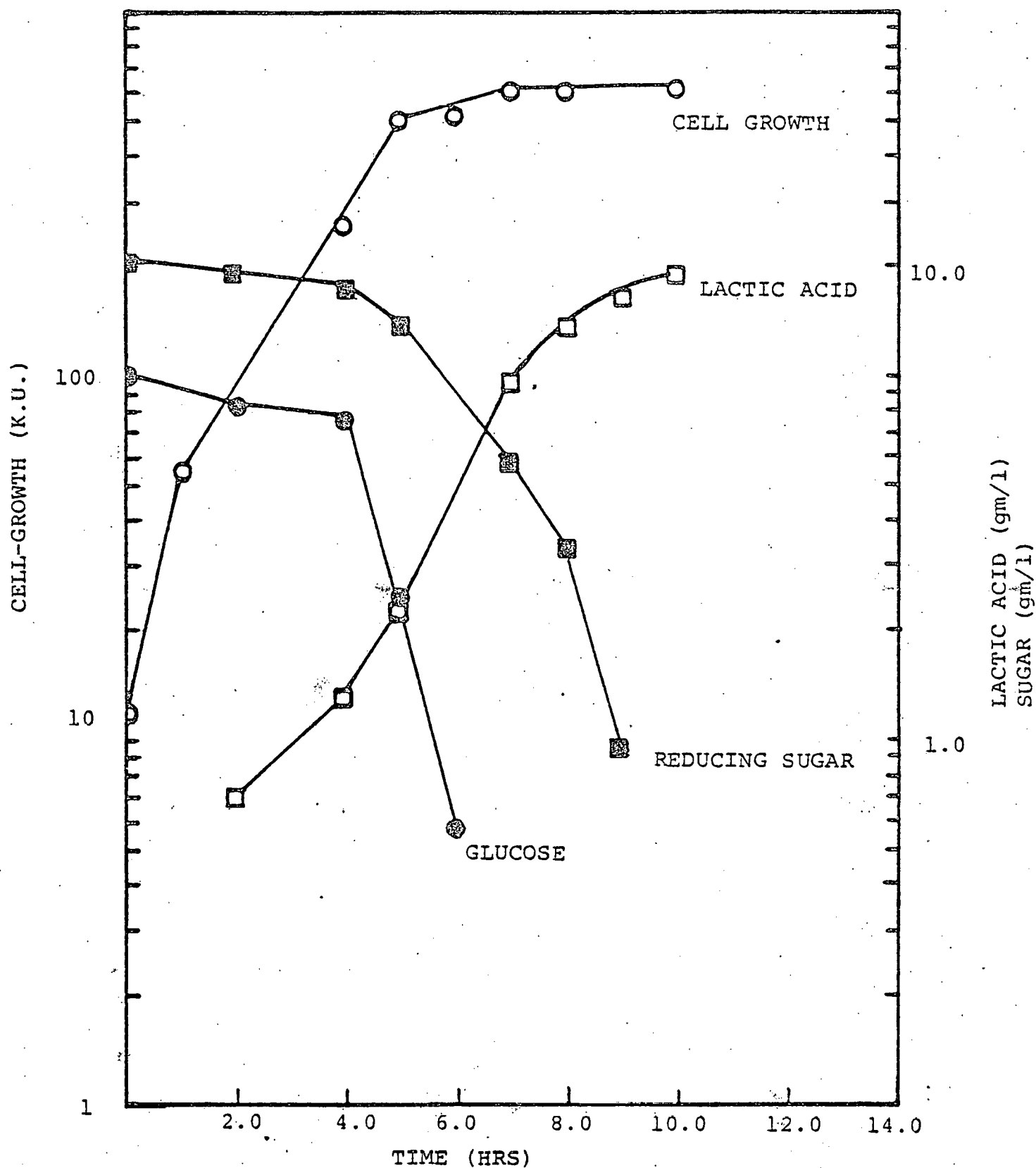
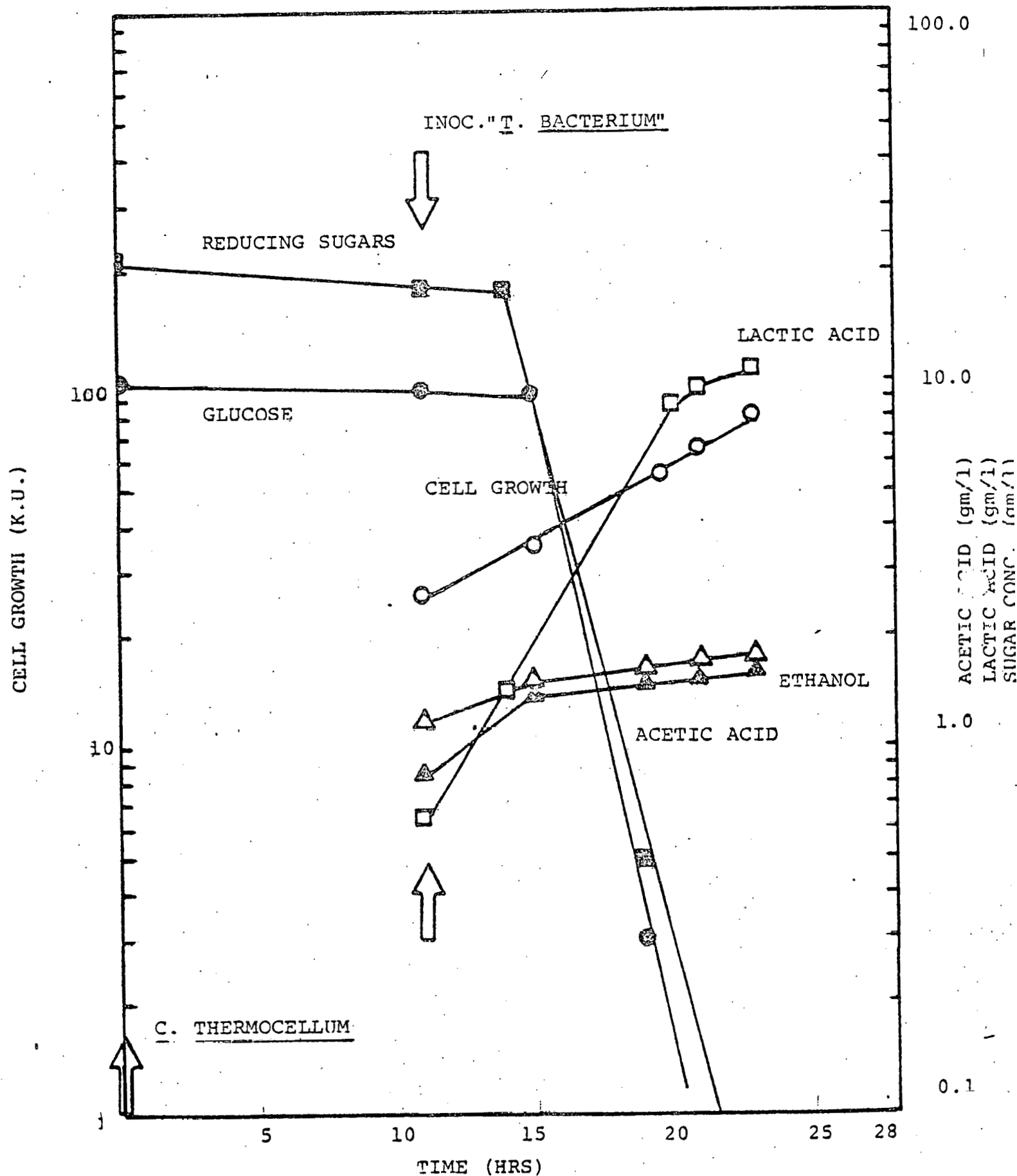


FIGURE II.A.4.6: MIXED CULTURE OF C. THERMOCELLUM AND "T. BACTERIUM" ON GLUCOSE AND CELLOBIOS E IN BATCH CULTURE



B. Production of Acetone and n-Butanol by Fermentation

1. Introduction

It has been shown previously that washed C. acetobutylicum cells harvested from a batch fermentation retained the capabilities to ferment glucose to produce acetone and n-butanol. The concentration of n-butanol obtained was 6 g/l and the acetone concentration was 3 g/l (Progress Report COO-4198-5). These concentrations were lower than those obtained in a growing cell fermentation of C. acetobutylicum. Also, in the last Progress Report (COO-4198-6), we reported the resistance of C. acetobutylicum towards n-butanol on growth temperature. It was shown that C. acetobutylicum had a higher resistance to n-butanol when grown at 30°C as compared to 37°C. In this report, we will present results of a detail study of C. acetobutylicum grown at 30°C and to compare the performances of resting cells at 30°C and 37°C.

2. Materials and Methods

a. pH Controlled Fermentation

The pH controlled fermentations were carried out in a 5 liter fermentor with 3 liter working volume. The procedures for the fermentations at 30°C (Run 13) and 37°C (Run 12) were identical and were described in Progress Report COO-4198-4.

b. Resting Cell Experiments

Two temperatures were used for the resting cells experiment, 30°C and 37°C. These experiments were

numbered as Run R2 (37°C) and Run R3 (30°C). The procedures for both experiments were the same. The cells for Run R2 (37°C) were harvested at the 22nd hour of fermentation, Run 12, which were grown at 37°C. The cells for Run R3 (30°C) were harvested at the 28th hour of fermentation, Run 13, which were grown at 30°C. The harvesting times reflected the maximum cell densities of the respective fermentations. In both of the resting cell experiments, the pH was controlled above 5.0 by titration with 2.0 N NaOH.

The cell harvest was achieved by placing 1.5 liters of fermentation broth on ice at the specified time. The cells were centrifuged at 3000 x g for 15 mins. at 4°C. The cell pellets were washed once with 900 ml of the following solution:

	<u>g/l</u>
KH ₂ PO ₄	0.75
K ₂ HPO ₄	0.75
NaCl	1.0
Cysteine	0.5
Glucose	10.0

The purpose of the wash was to remove the excess yeast extract from the fermentation broth. NaCl was added to the washing solution to maintain the tonicity of the solution. The cells were then resuspended into 500 ml of cell free reaction mixture which contained the following ingredients:

	<u>g/l</u>
KH_2PO_4	0.75
K_2HPO_4	0.75
MgSO_4	0.20
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
NaCl	1.00
Cysteine	0.50
Glucose	100.00

This mixture was essentially the same as the original fermentation medium minus the yeast extract and asparagine .

These reactions were carried out in a 1-liter fermentor agitated by a magnetic stirrer. The gaseous atmosphere of the fermentor was maintained anaerobic using nitrogen. Samples were taken at different intervals of time. After centrifugation to separate the cells, the supernatants were frozen until analyses for substrate and products.

3. Results and Discussion

a. pH Controlled Fermentations

The results of the batch fermentation performed at 37°C (Run No. 12) were similar to those of the previous runs reported. The results of the batch fermentation performed at 30°C (Run No. 13) are shown in Figure II.B.1. Since the operating temperature was lowered, the rate of the fermentation was expected to be lower. The final concentrations

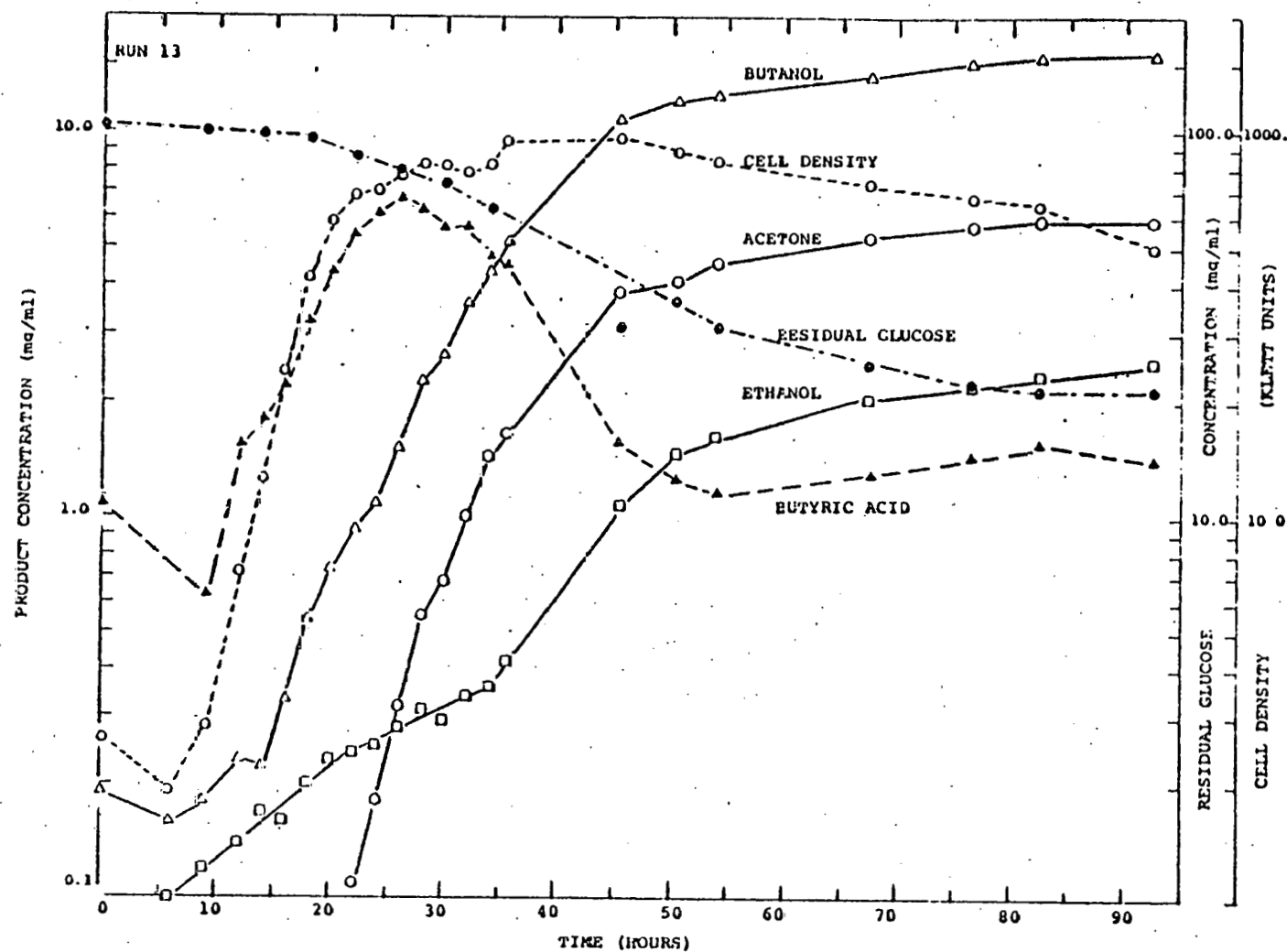


FIGURE II.B.1: pH CONTROLLED FERMENTATION OF *C. ACETOBUTYLICUM* ATCC 824 GROWING IN SOLUBLE MEDIUM WITH GLUCOSE AT 30°C (RUN NO. 13)

of n-butanol and acetone reached 15 and 6 g/l respectively. The concentration of n-butanol was slightly higher than that obtained at 37°C (12 g/l). This value, however, is considerably lower than what was expected (21 g/l) based on the inhibitory studies reported in last progress report. The product yields at 30 and 37°C are listed in Table II.B.1. From this table, we can conclude that a fermentation temperature of 30°C did not contribute significantly to increase the product yield or final concentration improvements.

b. Resting Cell Experiments

The results of the resting cell experiment using cells grown at 37°C are shown in Figure II.B.2. The incubation temperature of the resting cells was also 37°C. The pattern of the products using resting cells was similar to a normal growing cell fermentation. There was a lag time of about 10 hours before the production of butyric acid was detected. The production of acetone and n-butanol began at about the 20th hour and continued until their concentrations reached 6 g/l and 12 g/l respectively after 40 hours of reaction. However, during the course of the experiment, the optical density as an indicator of cell concentration decreased steadily. However, the optical density decrease at about the 20th hour ceased and up to the 35th hour one observes an increase in optical density. This may have been caused by the "cryptic growth" of the culture and also appears to reflect the product formation during the same period of time.

TABLE II.B.1: A SUMMARY OF THE RESULTS OF FERMENTATION AT 37°C (RUN NO. 12) AND 30°C (RUN NO. 13)

Growth Temperature	30°C (No. 13)	37°C (No. 12)
Length of time to achieve max. solvent conc. (hr).	~80	~25
Max. growth rate (hr^{-1})	0.30	0.53
Final n-butanol conc. g/l	15.70	8.90
Final acetone conc. g/l	5.95	3.30
Total glucose consumed g/l	81.0	50.3
Yield of n-butanol g/g	0.19	0.18
Yield of acetone g/g	0.07	0.07
Productivity of mixed solvent (butanol, acetone, ethanol) g/l-hr.	0.29	0.50

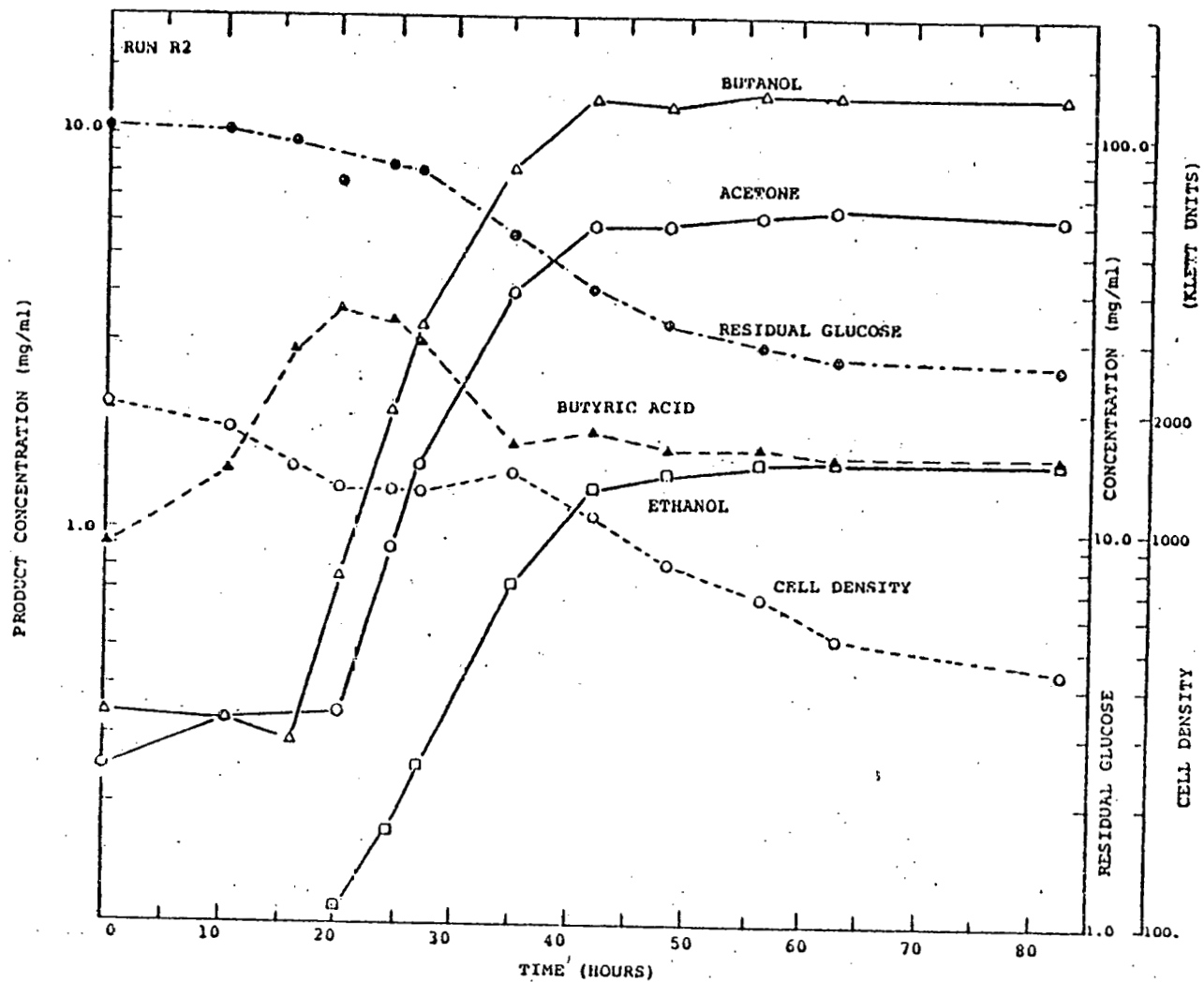


FIGURE II.B.2: RESTING CELL EXPERIMENT OF *C. ACETOBUTYLICUM* ATCC 824 AT 37°C
(CELLS WERE OBTAINED FROM FERMENTATION RUN NO. 12)

The results of the resting cell experiment performed at 30°C (Run R3) are shown in Figure II.B.3. The cells were also harvested from a 30°C fermentation. There was no lag period as seen from the accumulations of butyric acid, butanol, acetone and ethanol, which occurred immediately. The concentration of butyric acid leveled off at about the 20th hour. The final concentration of n-butanol and acetone were 12.5 g/l and 5.8 g/l respectively at the end of the experiment after 56 hours of reaction. The final concentration of ethanol was 1.4 g/l.

The cell density decrease of the resting cells was also observed in Run R3 (Figure II.B.4). However, the decrease was steady and when plotted in a semi-log fashion indicating a first order decay (see Figure II.B.4). The re-growth phase in Run R2 became quite obvious as seen in the figure. The half-life at the 30°C was about 43 hours and the half-life at the 37°C was about 27 hours: the re-growth period was not taken into account at 37°C.

The product yields from Run R2 and Run R3 are summarized in Table II.B.2. When these results were compared to the results in Table II.B.1, we found that they were very similar.

4. Future Work

- Define the factors that affect the stabilities of the resting cells.

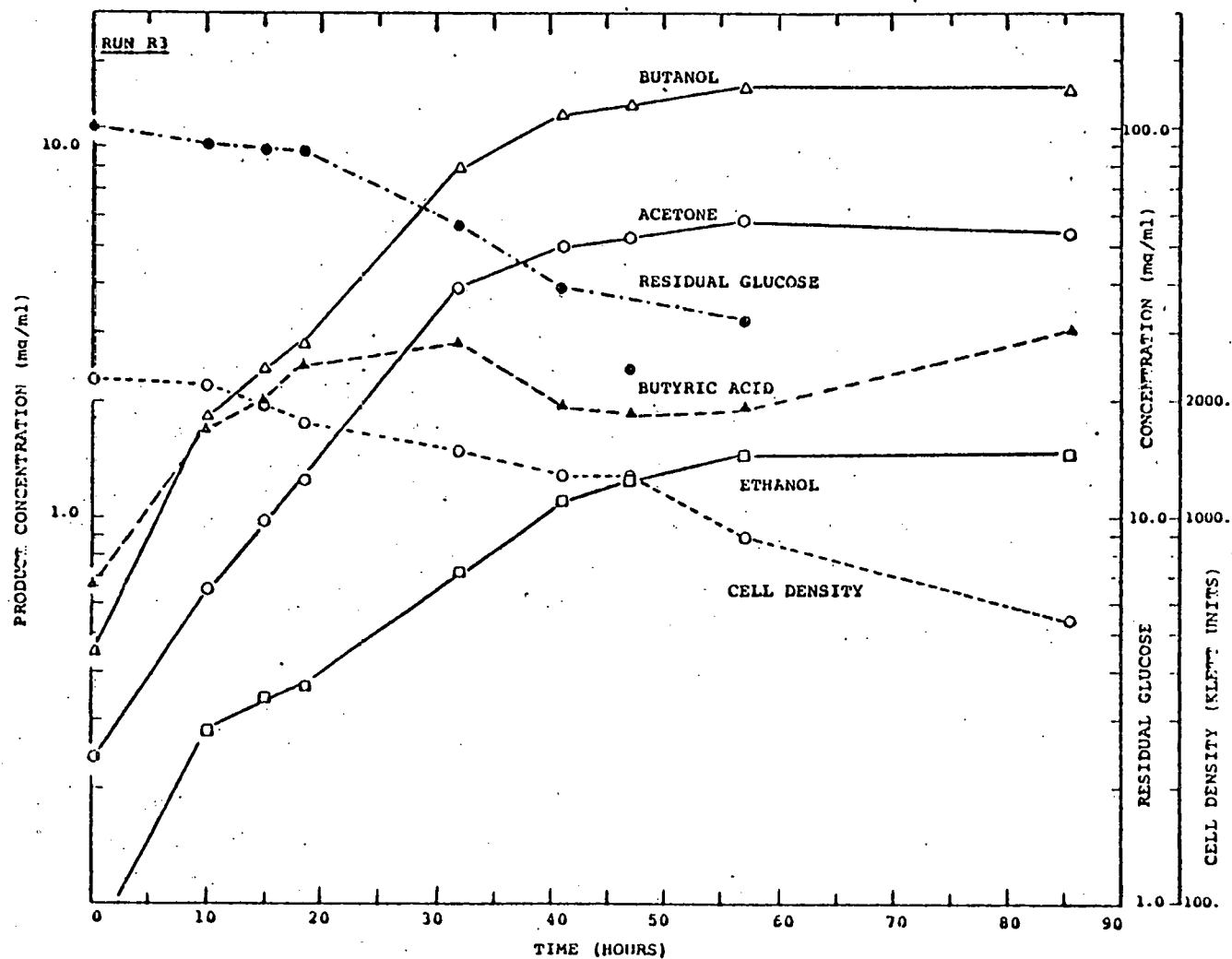


FIGURE II.B.3: RESTING CELL EXPERIMENT OF *C. ACETOBUTYLICUM* ATCC 824 AT 30°C
(CELLS WERE OBTAINED FROM FERMENTATION RUN NO. 13)

FIGURE II.B.4: THE DECAY OF THE CELL DENSITIES IN THE RESTING CELL EXPERIMENTS (RUN NO. R2 AND RUN NO. R3)

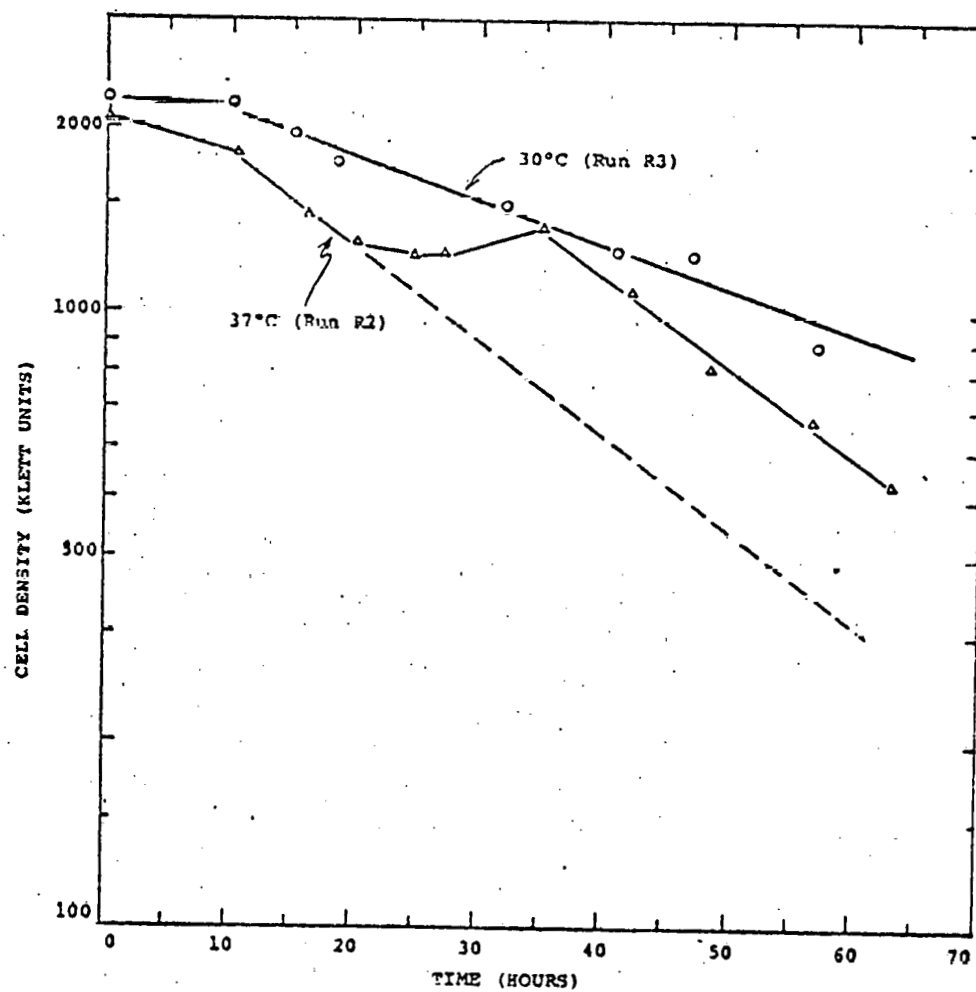


TABLE II.B.2: SUMMARY OF RESULTS USING RESTING CELLS
OF C. ACETOBUTYLICUM

Reaction Temperature	30°C (R2)	37°C (R3)
Fermentation time (hr.)	57.	56.
Final butanol conc. (g/l)	12.79	12.52
Final acetone conc. (g/l)	5.86	6.24
Final ethanol conc. (g/l)	1.44	1.48
Total glucose consumed (g/l)	69.5	76.5
Yield of n-butanol (g/g)	0.18	0.16
Yield of acetone (g/g)	0.08	0.08
Yield of ethanol (g/g)	0.02	0.02
Productivity of mixed solvent (g/l-hr)	0.35	0.34

- Stabilization of the resting cells through environmental manipulation.
- Stabilization of the resting cells by immobilization.
- Study of the kinetics of the immobilized cells for further process design and development.

C. Production of Acetic Acid By Fermentation

1. Introduction

In the last progress report (COO-4198-6), we reported a new strain of Clostridium thermoaceticum (S3) that can grow on higher acetate concentrations. The properties of S3 is further studied using pH-controlled fermentations.

It was also shown that non-growing cells can produce acetic acid. Additional studies on non-growth associated acetic acid production will be reported.

2. Materials and Methods

The fermentation was performed in 5 l. New Brunswick fermentor with working volume of 3.5 l. The temperature was controlled at 59.5° C and pH controlled at 7.0 using NaOH and with CO₂ overlay. The other analytical methods have already been presented in the previous reports.

For the resting cell experiments, cells were harvested from the fermentor, centrifuged, washed twice with phosphate buffer and resuspended into glucose-mineral salt medium with different concentrations of sodium acetate. The composition of glucose-mineral salt medium is shown in Table II.C.1.

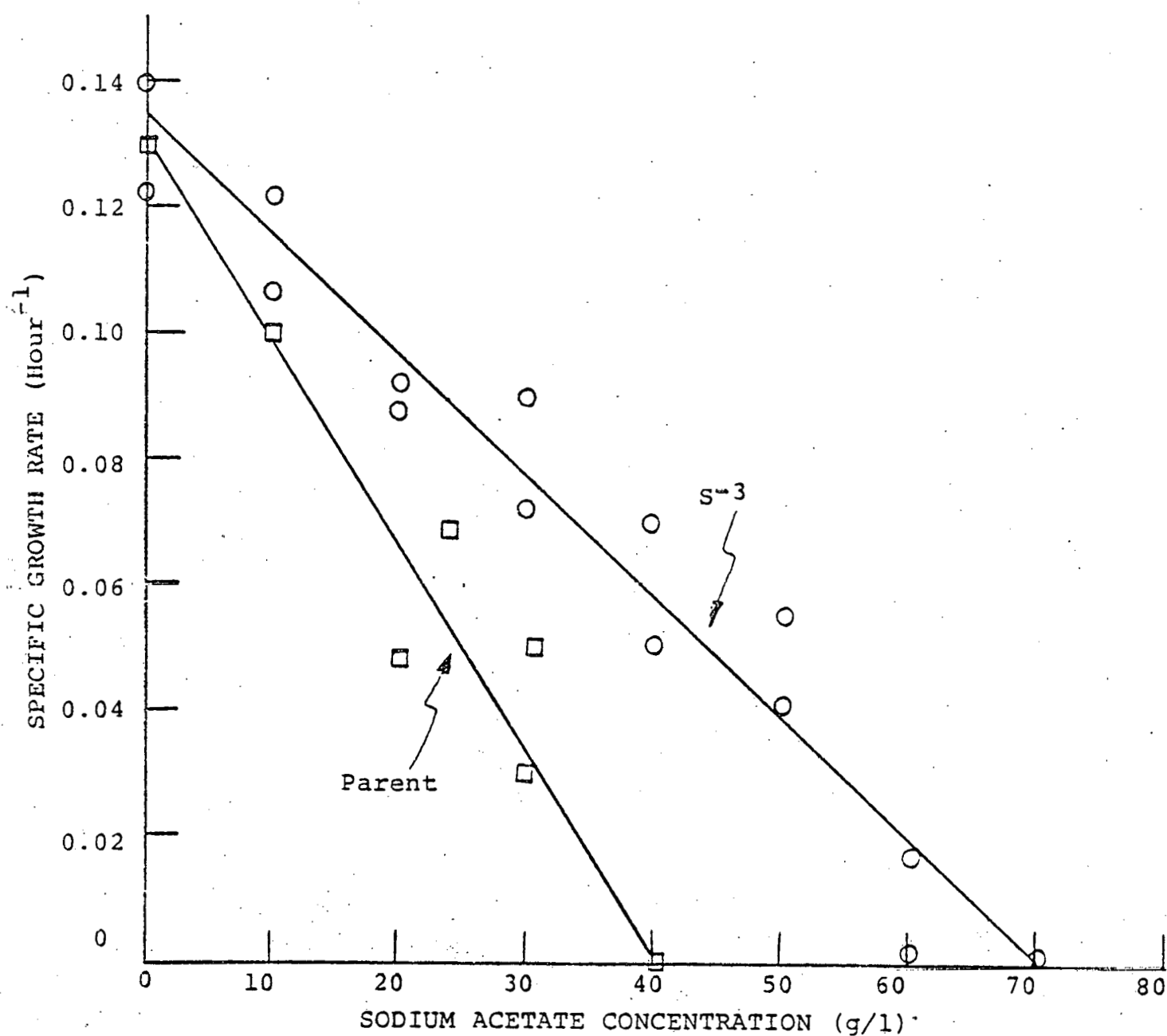
3. Results and Discussions

The relationship of the specific growth rate of the new strain, S-3, and the parent at different sodium acetate concentrations is shown in Figure II.C.1. Total inhibition of growth of S3 occurs at 70 g/l of sodium acetate (51 g/l of

TABLE II.C.1: COMPOSITION OF GLUCOSE-MINERAL SALTS MEDIUM

Glucose	18	g/l
Sodium bicarbonate	16.8	g/l
Potassium phosphate (microbasic)	5.1	g/l
Potassium phosphate (dibasic)	7.0	g/l
Sodium thioglycollate	0.5	g/l
Magnesium sulfate	0.25	g/l
Cobalt nitrate	.029	g/l
Calcium chloride	.016	g/l
Sodium molybdate	.12	g/l

FIGURE II.C.1: COMPARISON OF NEW ISOLATE (S-3) WITH PARENT STRAIN OF CLOSTRIDIUM THERMOACETICUM WITH RESPECT TO GROWTH AT DIFFERENT SODIUM ACETATE CONCENTRATIONS

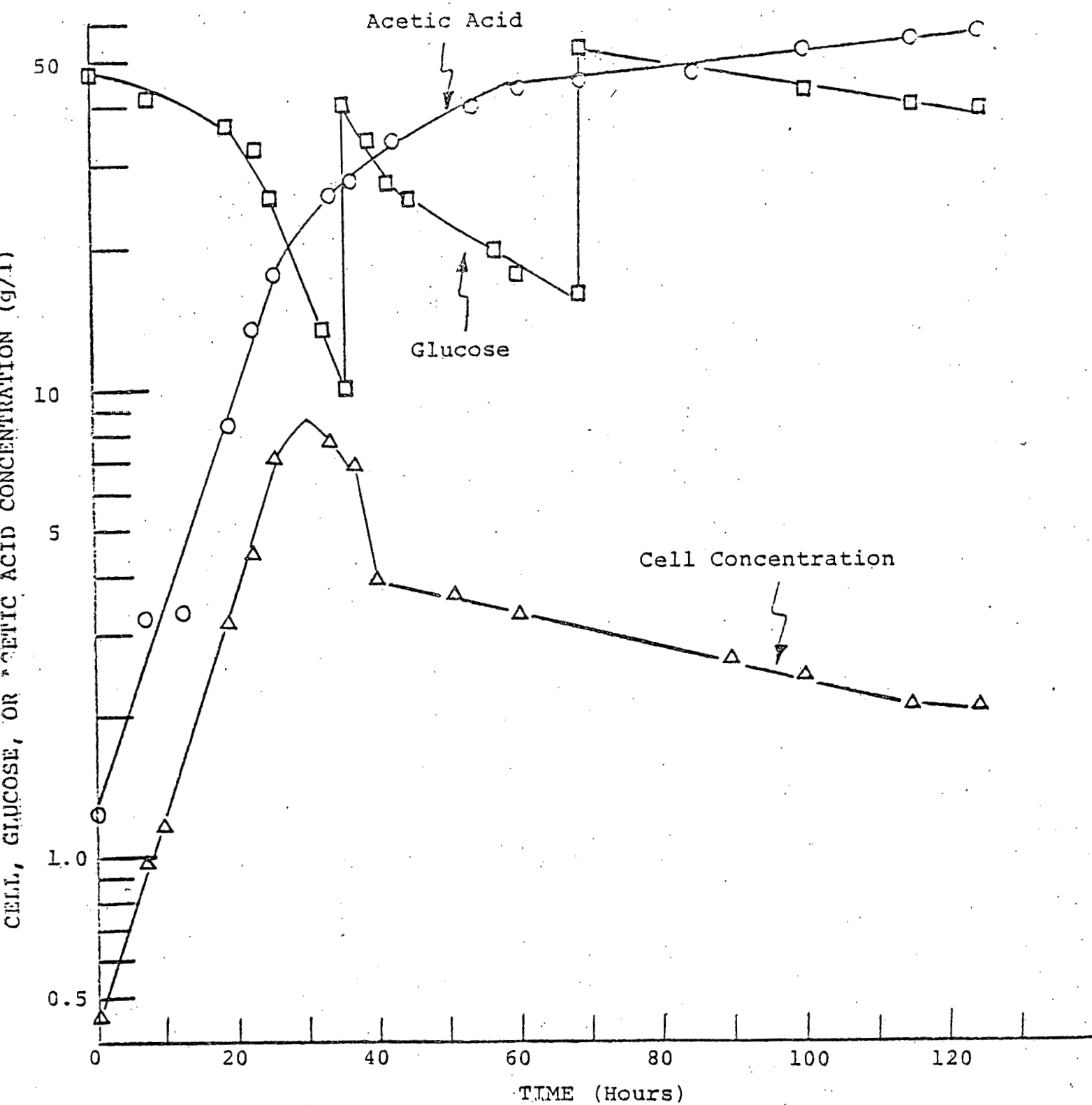


acetic acid) as compared to 40 g/l of sodium acetate (30 g/l of acetic acid) for the present.

The fermentation profile of S3 under pH control at 7.0 is shown in Figure II.C.2. The patterns of cell growth and acetic acid production are similar to those reported earlier (COO-4198-4). Cell mass, measured by optical density, increased exponentially for the first 30 hours, and decreased from then on to the end of the fermentation. This decrease of cell mass is thought to be caused by lysis of cells due to high ionic strength. Acetic acid increased exponentially for the first 50 hours, and reached 40 g/l, then the production rate decreased and reached 56 g/l by 125 hours. A summary of the results from Figure II.C.2 is shown in Table II.C.2. A total of 108.2 g/l of glucose was added with 38.6 g/l remained after 125 hours. The conversion yield (gm acetic acid per gm glucose) is 0.81 which is in agreement with the previous results.

TABLE II.C.2: SUMMARY OF pH-CONTROLLED FERMENTATION OF S3

Total glucose added	108.2 g/l
Total yeast extract	8.5 g/l
Total tryptone	8.5 g/l
Total glucose remained	38.6 g/l
Total acetic acid formed	56.0 g/l
Conversion yield	0.81 (g. acetic acid/g. glucose)



• FIGURE II.C.2: FERMENTATION PROFILE OF CLOSTRIDIUM THERMOACETICUM
S-3 (pH = 7.0)

Upon a more detailed examination of the data, the relationship of volumetric productivity (gm acetic acid/l-hr) and specific productivity (gm acetic acid/ gm cell/ h.), versus acetic acid concentration is shown in Figure II.C.3. The volumetric productivity is highest (1.4 g/l/h) when acetic acid concentration is between 12-22 g/l, corresponding to the period when cell mass is highest at about 6 - 7 g/l. The specific productivity decreases with increasing acetic acid concentration. This is in agreement with the observation that specific growth rate decreases with increasing acetic acid concentration. If we assume that acetic acid is produced following the mathematical model proposed in an earlier progress report (COO-4198-4):

$$q_p = \alpha \mu + \beta$$

q_p = specific productivity

α = growth associated acetate formation constant

μ = specific growth rate

β = non-growth associated acetate formation
constant

Since α and β are constants, decrease of μ (specific growth rate) will also cause a decrease of q_p (specific productivity).

Using this model, a plot of specific growth rate (μ) versus specific productivity (q_p) is shown in Figure II.C.4. The growth associated acetate formation constant (α) is 1.4 gm acetate/ gm cell and the non-growth associated constant (β) is 0.1 gm acetate/ gm cell-hr.

FIGURE II.C.3: RELATIONSHIPS OF VOLUMETRIC AND SPECIFIC PRODUCTIVITIES OF C. THERMOACETICUM TO ACETIC ACID CONCENTRATION

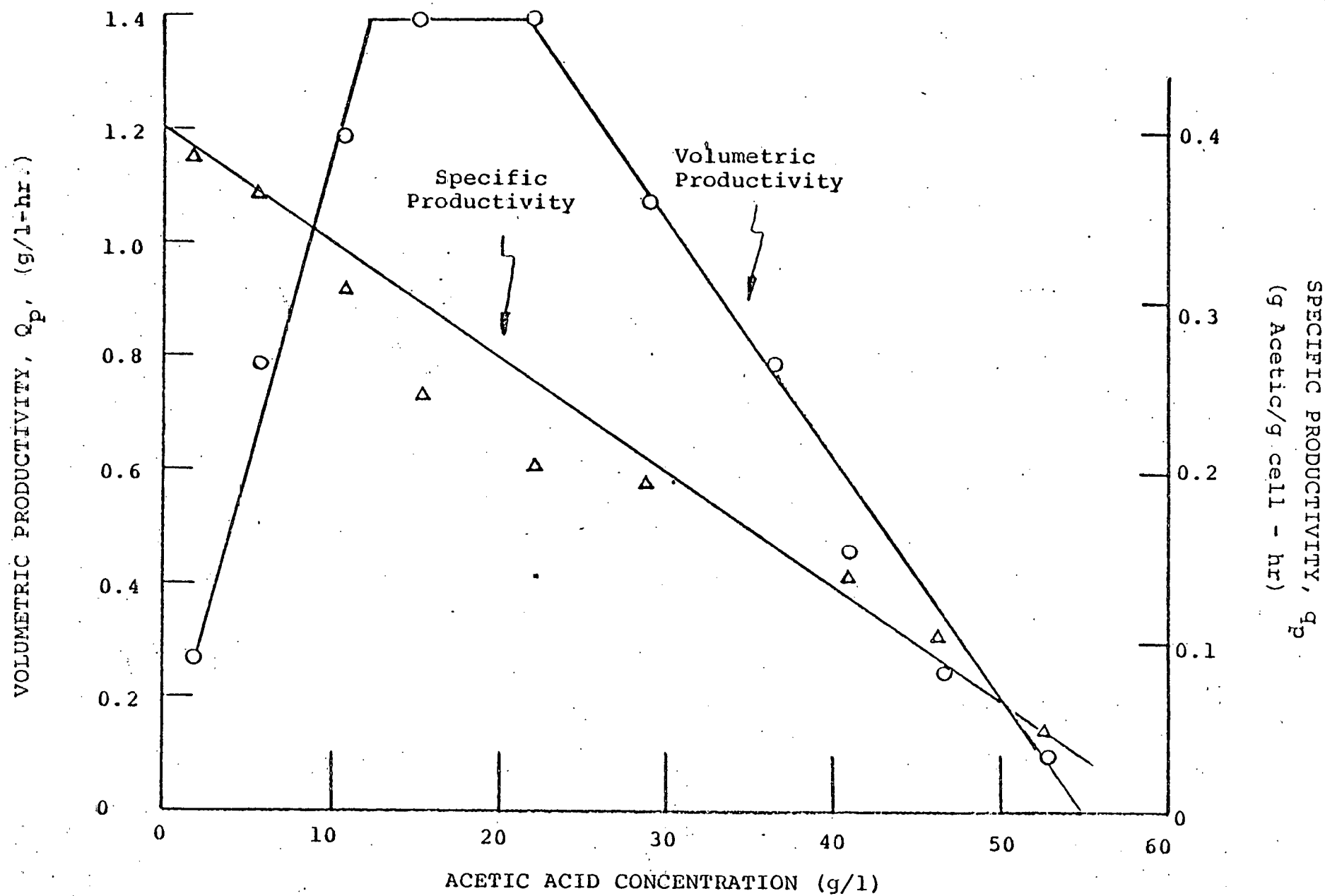
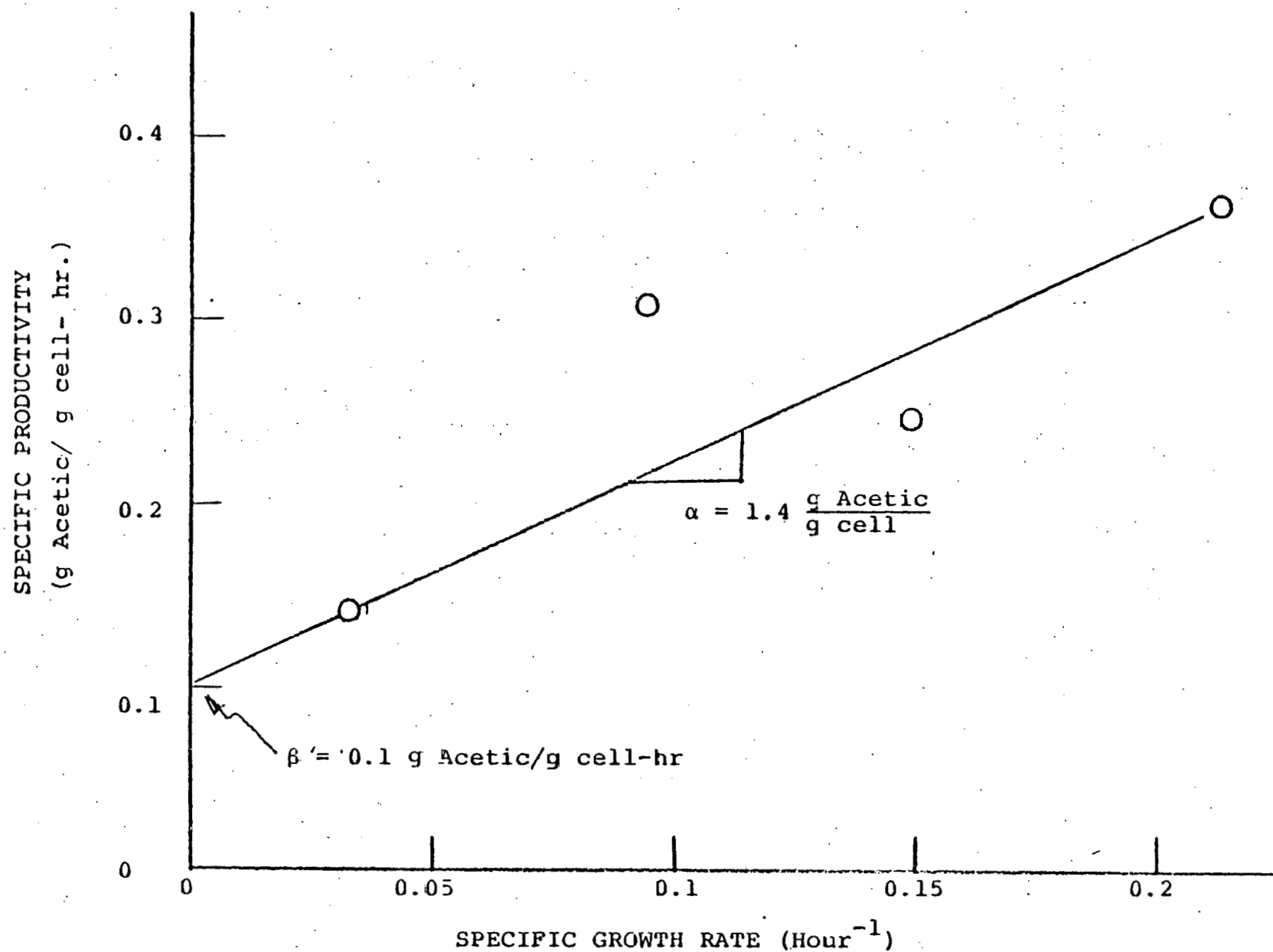


FIGURE II.C.4: RELATIONSHIP BETWEEN SPECIFIC GROWTH RATE
TO SPECIFIC PRODUCTIVITY



A typical fermentation profile of non-growing cells of Clostridium thermoaceticum is shown in Figure II.C.5. The typical conversion yield of acetic acid from glucose is between .85 - .92. The rate of acetic acid production is highest at the beginning, and levels off with time. The production rate is 0.52 g/l/h for the first 10 hours, 0.29 g/l/h for the second 10 hours, and only 0.09 g/l/h from 20th to 40th hours. This decrease of production rate could be due to (1) instability of cells or (2) inhibition effect due to low pH.

The effect of different concentrations of sodium acetate (converted to equivalents of acetic acid) on acetic acid production of non-growing cells is shown in Figure II.C.6 and Figure II.C.7. Rate of acid formation is determined by the rate of glucose consumed. A noticeable inhibition effect of acid production is observed with increasing acetate concentration. However, during the experiment massive lysis of cells was observed for all cultures when sodium acetate was added. However, in the calculation of specific rate of non-growth associated acetate production, the cell concentration used was the initial cell concentration, without taking into account the effect of lysis. It is therefore possible that the decrease of acid production rate could be due to the indirect effect of cell lysis.

FIGURE II.C.5: PRODUCTION OF ACETIC ACID BY C. THERMOACETICUM USING RESTING CELLS

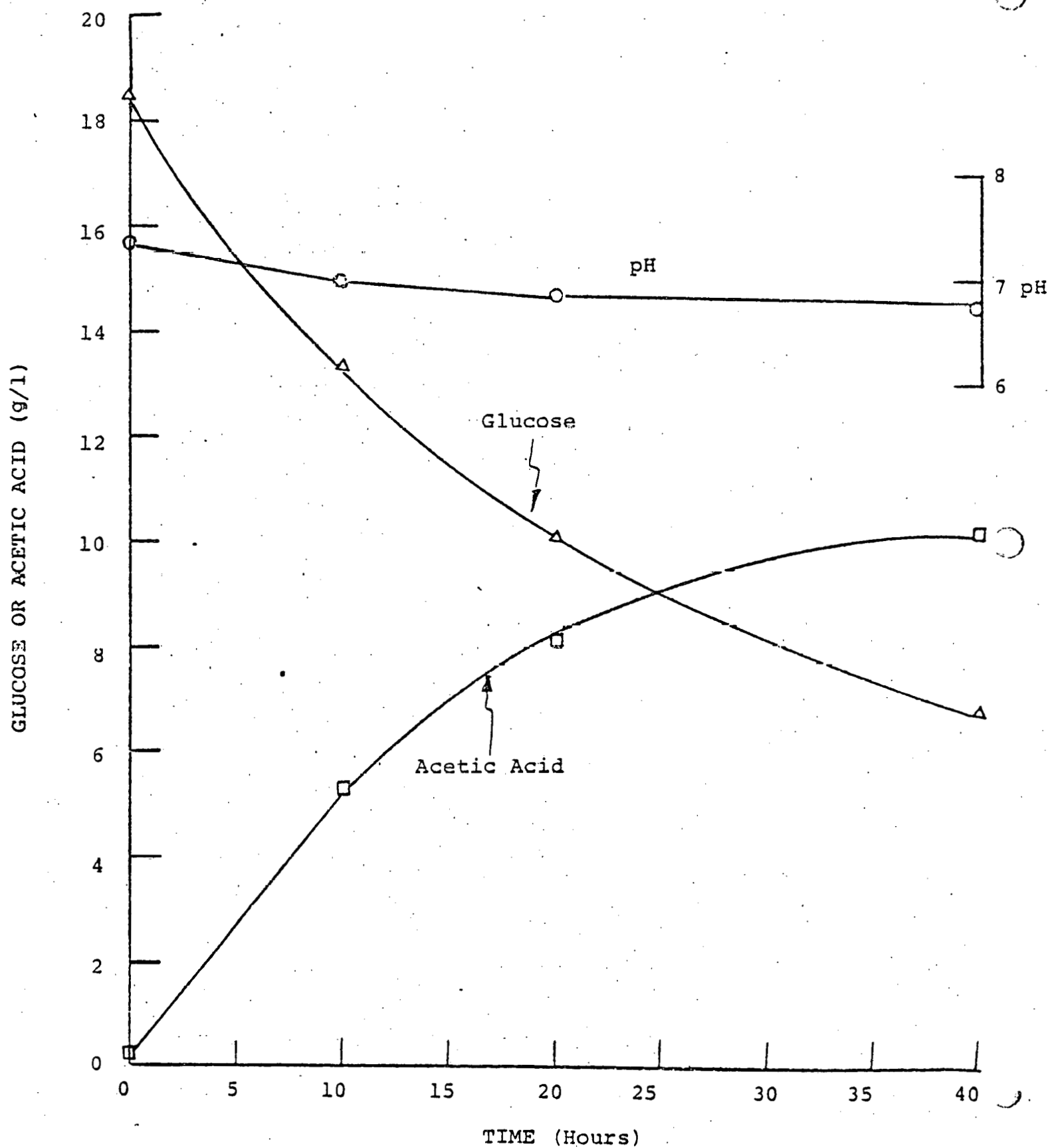


FIGURE II.C.6: KINETICS OF GLUCOSE UTILIZATION BY RESTING CELLS OF *C. THERMOACETICUM* AT DIFFERENT ACETIC ACID CONCENTRATIONS

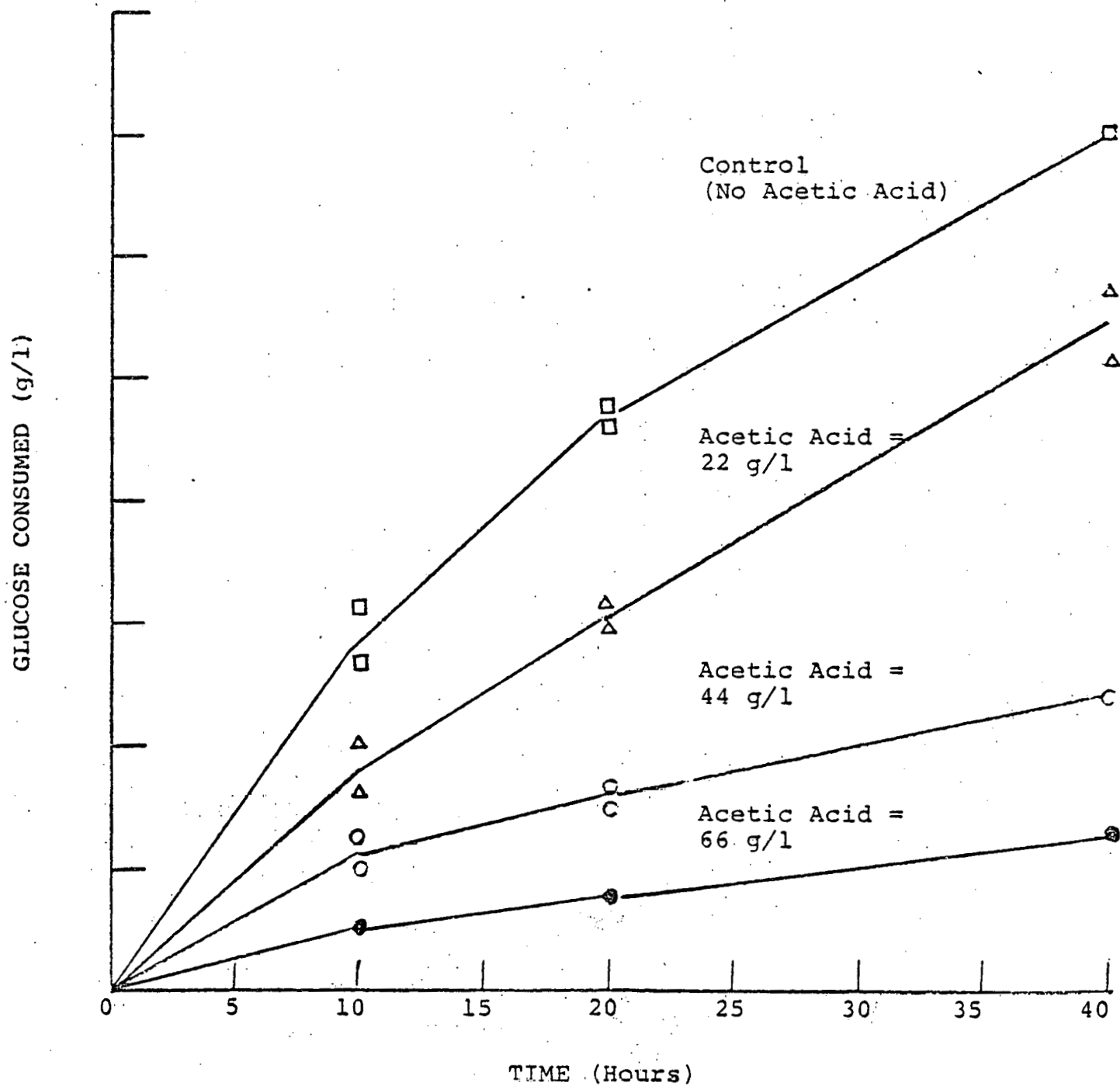
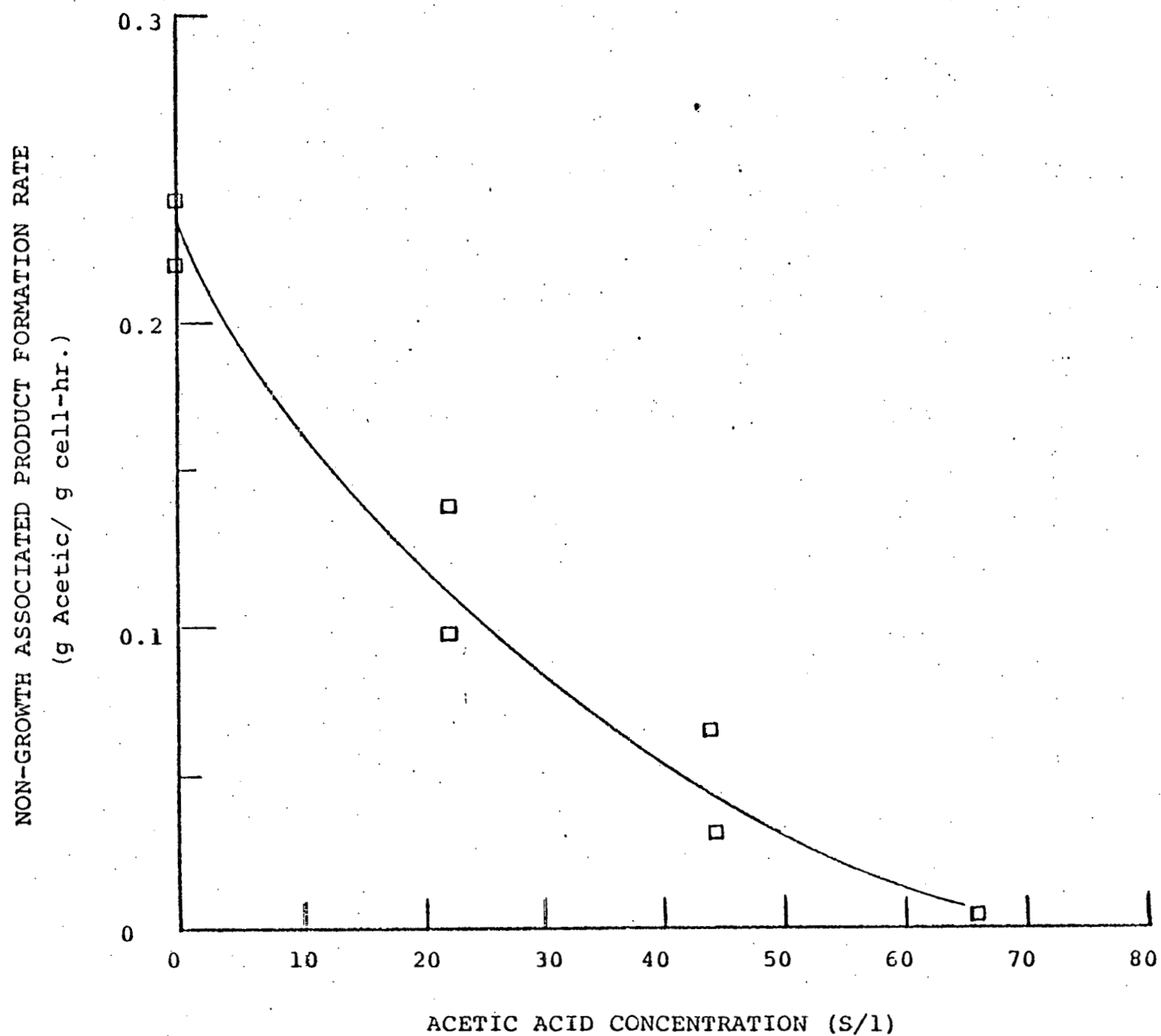


FIGURE II.C.7: RELATIONSHIP BETWEEN ACETIC ACID CONCENTRATION AND NON-GROWTH ASSOCIATED PRODUCT FORMATION RATE USING RESTING CELLS OF C. THERMOACETICUM



4. Future Work

- Further selection of strains that can tolerate higher acetate concentration.
- Determine the cause of lysis, and find the the appropriate means to eliminate it.
- The use of immobilized whole cells to increase productivity and to increase stability.
- Design high productivity bioreactors to effect product formation.

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

Report for Third Quarter

August 1, 1978 through October 31, 1978

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Prepared for

THE U.S. DEPARTMENT OF ENERGY
CONTRACT NO. EG-77-S-02-4198

SCOPE OF PROGRAM

This is a coordinated program to effect the microbiological degradation of cellulosic biomasses and 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 conversion from cellulosic biomasses to liquid fuel (ethanol) and/or soluble sugars by the cellulolytic, anaerobic organism is also within the scope of this program. Process and engineering scale-up, along with economic analyses, will be performed throughout the course of the program.

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 accumulation of fermentable sugars during the fermentation of Clostridium thermocellum on cellulose is an important and unique ability of our anaerobic process for biomass utilization. We have continued to study and to elucidate the factors responsible for this accumulation of reducing sugars. Previous studies have shown glucose to be a major product accumulated during fermentation on cellulose by C. thermocellum. We have examined the glucose accumulation more closely using an enzymatic assay to allow us to differentiate between glucose and xylobiose accumulation; HPLC analysis will not separate these two sugars. The results have shown that glucose accumulation is entirely non-growth associated, suggesting that xylobiose may accumulate during growth. To elucidate the reason for glucose accumulation, cellobio-hydrolase activity has been measured during growth and stationary phases of C. thermocellum, with negative results showing an absence of such enzyme activity. At this point, the reason for glucose accumulation remains to be identified.

Characterizations on the growth and production of ethanol and other products by Clostridium thermocellum (S-4) have continued. It was found that for C. thermocellum the specific productivity (g ethanol/g cell-hr.) for ethanol is both growth and non-growth associated. The specific ethanol productivity is directly related and increases linearly with the specific growth rate. However, the specific productivity of lactic acid formation is inversely related to increasing specific growth rate. Carbon and oxidation-reduction balances again indicate that the new isolate S-4 is deficient in the hydrogenase enzyme. This behavior again shows that a high ratio of ethanol to acetic acid (14:1) production can be achieved. Lastly, nutritional studies have shown that iron is a critical component leading to increased rates of ethanol production by C. thermocellum.

Controlled fed-batch fermentations have been performed with C. thermocellum using glucose as the carbon source. These experiments were performed to determine the highest concentration of ethanol attainable. Unfortunately, the maximum ethanol accumulated was only 8.5 g/l. Analysis of the residual glucose shows the formation of cellobiose. Detailed studies will be performed to seek answers to this problem.

A number of different biomasses has been tested with strain S-4 of C. thermocellum. These include exploded poplar, sugar cane bagasse, corn cobs, sweet gum, rice straw and wheat straw. Rice and wheat straws were shown to be most readily degraded by C. thermocellum. Calculations with rice straws showed a degradation efficiency of 0.84 gm product/gm wheat straw degraded.

To effect a more complete utilization of biomass, the use of HG-2, an anaerobic thermophile, to produce ethanol from xylose has been studied. HG-2 was found to grow rapidly (doubling time = 2 hours) on xylose and can produce ethanol and acetic acid as catabolic by-products. Adaptation and selection studies on HG-2 have been initiated. An isolate designated as HG-3 has been obtained which has a higher tolerance for ethanol.

Mixed culture studies have been initiated using corn stover as the carbon source. Clostridium thermocellum and HG-2 have been cultivated as a mixed microbial system. Results indicate that the reducing sugars produced through the cellulolytic action of C. thermocellum on corn stover can be effectively metabolized by HG-2 to produce ethanol and acetic acid.

Our attempts to isolate plaque forming units derived from C. thermocellum have continued. None of the strains examined so far contained mitomycin or U.V. inducible prophages. A qualitative examination of the sensitivity of each of the C. thermocellum strains on hand, to a variety of metal salts and antibiotics, revealed differences in sensitivity which could be used as genetic markers. Attempts to achieve inter-species transfer of antibiotic resistance from C. perfringens to C. thermocellum have not been successful.

Efforts in the nutritional and environmental manipulations, as well as deregulation studies on Clostridium thermocellum, have continued. Clostridium thermocellum grew and produced cellulase on cellobiose, on glucose and on a glucose-cellobiose mixture, indicating that cellulase production in this organism is not subject to glucose repression. Glucose utilization was extensive but growth on this hexose was characterized by a long lag phase. For growth on glucose, a higher concentration of yeast extract was required than for growth on cellobiose. The utilization of carbohydrates by C. thermocellum ATCC 27405 was further examined in detail. The maximum cell concentration was obtained on fructose (.45 g/l) followed by cellobiose (.44 g/l) and glucose (0.18 g/l). However, maximum cell yield (g cells/g initial sugar) was obtained in cellobiose (.21) followed by

glucose (.19) and fructose (.07). When the ATCC 27405 was conditioned for growth in glucose and then transferred to cellobiose, glucose and fructose, the same trends as with cells conditioned for growth in cellobiose were obtained. However, maximum cell concentrations and cell yields were highest in all conditions when the cells were conditioned for growth in glucose. The differences in maximum attainable cell concentrations and cell yields are believed to be due to differences in the way these sugars are transported. Yeast extract could be replaced by eight vitamins plus casein hydrolysate, but not by the vitamins alone. By mutagenesis of C. thermocellum AS-39, we have isolated two mutants which produce 3 times as much cellulase as AS-39.

Purification of the cellulase enzyme from C. thermocellum has been initiated. Ammonium sulfate precipitation followed by gel filtration of C. thermocellum extracellular protein has yielded purification factors of 3.7-fold for filter paper activity and 2.0-fold for CMCase activity. Elution profiles from Biogel A-5m indicate a population of heterogeneous molecular weights (10,000 to 5,000,000 daltons) for both filter paper and CMCase activities.

Considerable progress has been made in the optimization of conditions for conversion of propionic acid to acrylic acid by free and immobilized cells of Clostridium propionicum. The optimal conditions for propionate dehydrogenation are: initial propionate concentration 200 mM, methylene blue concentration 0.2%, lactate or α -alanine concentration 20-25 mM, pH 8.5, temperature 32°C with cells harvested from the late exponential growth phase. Using these conditions with free cells of C. propionicum, we have achieved concentrations of acrylic acid in excess of 40 mM. Studying the immobilization of whole cells, we have shown that it is possible to prepare polyacrylamide gels containing C. propionicum that retain 70-80% of their biological activity. The use of immobilized cells is of particular importance for the development of reactor systems for production of acrylic acid. Our ultimate goal is to derive acrylic acid from cellulosic biomass. A first step in this conversion is the utilization of a mixed culture of C. thermocellum to produce sugar from cellulose and a thermophilic, homo-lactate organism converting the sugars to lactic acid. This second organism has been identified as Bacillus coagulans and work is currently in progress to define its behavior in mixed cultures with C. thermocellum.

Detailed studies using resting cells of Clostridium acetobutylicum in the presence and absence of penicillin G have been performed. It has been shown that in the presence of

penicillin G, C. acetobutylicum is unable to metabolize glucose to produce mixed solvents (acetone or butanol). Therefore, redirections will be needed to achieve high productivity solvent fermentations for this system. In situ extractive fermentation appears to be one such solution. Using corn oil as the extractant, total n-butanol in excess of 15 g/l has been achieved. This concentration of butanol has never been achieved in conventional fermentations. Search for additional extractants is now in progress.

The production of acetic acid by resting cells of Clostridium thermoaceticum has focused on the reason leading to cell instability. "Cell lysis" was shown to occur in the presence, as well as the absence, of chloramphenicol. These results indicate lytic phenomenon alone does not explain the loss of the organism's ability to produce acetic acid. Studies will continue to determine the reason and seek answers to increase cell stability. Xylose has been examined as the carbon source for growth and acetic acid formation by C. thermoaceticum. Product yield of about 0.8 gm acetic/gm xylose was obtained.

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6

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. The percentage efforts of the Principal and Co-Principal Investigators on this project are shown along with the names of the different participants.

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This progress report acknowledges the contributions by the individuals listed above, as well as the research assistants, post doctoral research associates, and technical assistants associated with this project.

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I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. ENZYME PRODUCTION BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

As a continuation of the work described in the last progress report (COO-4198-7) concerning the conversion of cellulose to glucose by Clostridium thermocellum ATCC 27405, studies have been extended to further assess the β -glucosidase, or cellobiase, activity produced by C. thermocellum and to determine the kinetics of glucose accumulation during fermentations on cellulose. Since glucose is an important intermediate in the overall scheme of cellulose degradation and subsequent fermentative production of chemical feedstocks, information about the mechanism or enzymes involved in its production must be established so that we may find ways to enhance the final proportion of glucose among the other soluble cellulose hydrolysis end products and so that we may more rationally design mixed-culture fermentations with C. thermocellum and other anaerobic thermophiles (e.g., C. thermoaceticum or Bacillus coagulans) which utilize the glucose (released from cellulose by C. thermocellum) as a growth substrate.

Experiments were performed to measure whether extracellular 'cellobiase' is produced by C. thermocellum,

during fermentation on cellobiose, and to determine whether an intracellular 'cellobiase' may be released at the end of cellobiose fermentations as a result of cell lysis. In addition, the kinetics of glucose accumulation during cellulose fermentations was followed using the enzymatic Glucostat assay to compare the results with those previously obtained by HPLC analysis.

2. Materials and Methods

a. Growth of Organism

C. thermocellum was cultured in 500 ml flasks containing 300 ml of CM3 medium with 0.5% yeast extract. The carbon sources were either solka floc cellulose (SW40) or cellobiose at a concentration of 1.0%. No pH control was used.

b. Glucose Measurement

For both the cellobiose-grown and solka floc-grown cultures, glucose was measured enzymatically by the Glucostat assay (Worthington Biochemicals).

c. Protein Measurement

Extracellular protein production by C. thermocellum was measured with the Bio-Rad Protein Assay, a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. Bovine serum albumin (Sigma) was used to prepare standards.

d. Cellobiase Assay

The extracellular protein in the supernatant of the broth from a cellobiose fermentation was precipitated at 4°C in 40% ethanol overnight. The precipitate was resuspended in 0.05 M sodium citrate buffer, pH 5.4, at one half the original volume of the broth to effect a two fold concentration. A substrate solution containing cellobiose was prepared in the following way. In citrate-phosphate buffer, pH 5.4, (0.1 M citric acid: 0.2 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$: H_2O , 1:1:2) cellobiose was dissolved to a final concentration of 10 mg/ml (1.0%) and cysteine was added as reducing agent to a final concentration of 2 mg/ml (0.2%). (The pH was chosen as pH 5.4 to match the pH of the medium during fermentation when the specific rate of glucose accumulation is maximal. A citrate-phosphate buffer was selected to allow for a possible phosphorolytic cleavage of cellobiose.) One milliliter of the enzyme solution was added to one milliliter of the substrate (cellobiose) solution and the mixture was incubated, in the presence of air, at 60°C. After one hour, the DNS assay for reducing sugars was performed on the enzyme-substrate mixtures. The absorbance at 550 nm was compared to that of a zero time unincubated control and also to a control containing the substrate solution mixed only with buffer. Any increase in absorbance of the test samples over the controls would indicate a liberation of glucose from cellobiose.

3. Results

Because Clostridium thermocellum is known to possess an intracellular cellobiose phosphorylase and since glucose accumulation has been shown to be primarily a non-growth associated event during cellobiose fermentations, one plausible explanation for glucose accumulation was that autolysis occurred during the organism's stationary phase, releasing an intracellular enzyme that would catalyze the hydrolysis of cellobiose to glucose extracellularly. However, turbidity measurements of cell density did not indicate any significant cell lysis, nor did measurements of extracellular protein accumulation during the fermentation of C. thermocellum on cellobiose suggest that cell lysis had taken place. The results from two fermentations on CM3/cellobiose are shown in Figure I.A.1., where extracellular protein production, culture turbidity (Klett units) and glucose accumulation are plotted against time. For both fermentations it is seen that when culture turbidity reaches a plateau so does the extracellular protein level. There appears to be no stationary phase release of protein to correspond with the accumulating glucose. Assuming that no intracellular cellobiose phosphorylase was escaping, another plausible explanation for the glucose accumulation was that the organism did produce an extracellular cellobiase which functioned when the medium pH dropped from

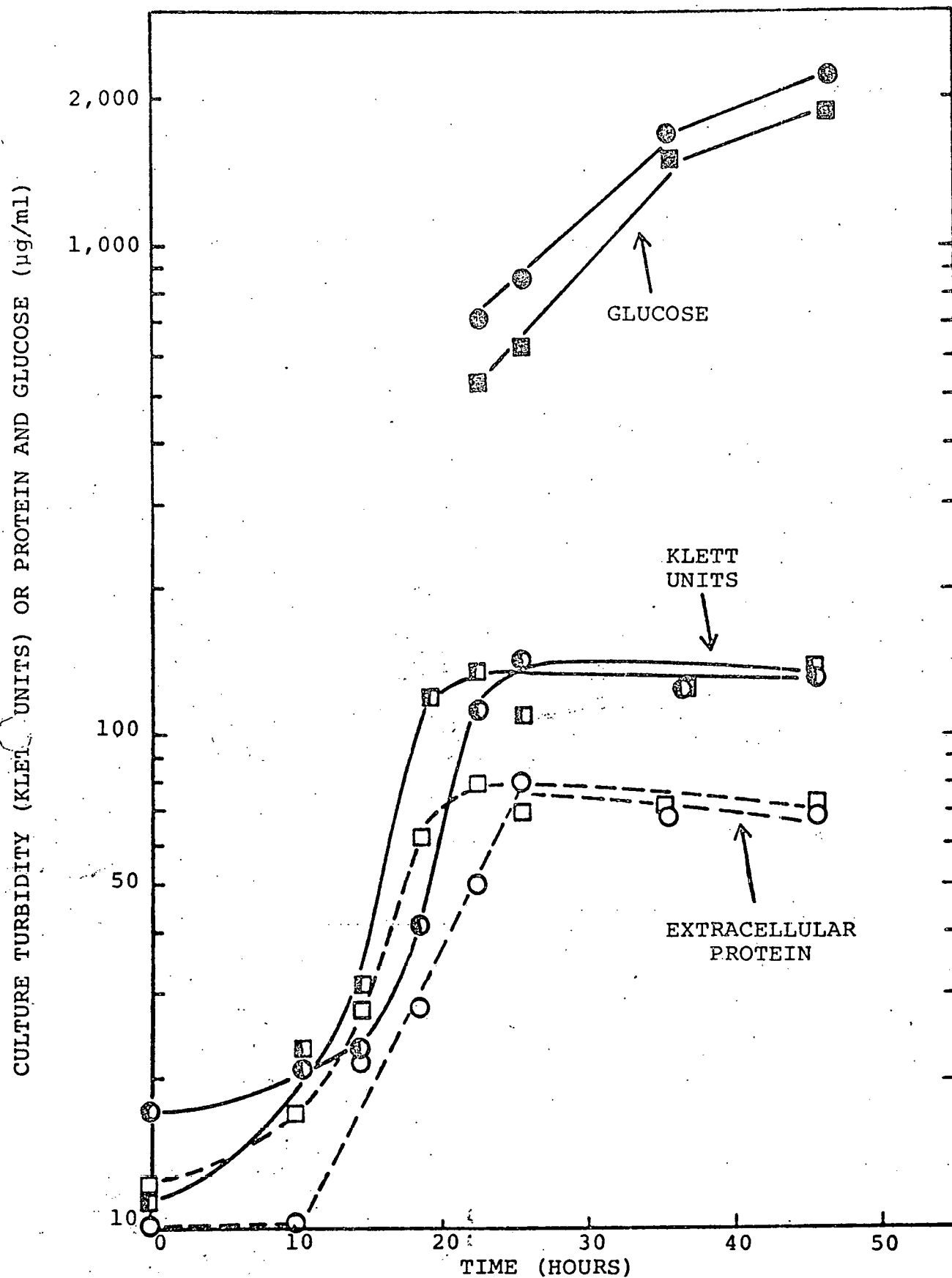


FIGURE I.A.1: KINETICS OF GROWTH, GLUCOSE ACCUMULATION AND EXTRACELLULAR PROTEIN PRODUCTION DURING FERMENTATION OF C. THERMOCELLUM ATCC 27405 ON CM3/CELLOBIOSE

pH 7.0 to pH 5.4, the final pH of the broth. However, when extracellular cellobiase was assayed according to the procedure described above, no activity could be measured as shown in Table I.A.1. In this table, the absorbances at 550 nm, after the DNS assay for reducing sugars, are presented for broth samples taken from a cellobiose fermentation at 20 and 40 hours (times when the pH of the medium was within the range of 5.4-5.6), as well as the zero time point control and the substrate (no enzyme added) control. The absorbance values correspond to the standard cellobiose concentration of 5 mg/ml which is the concentration of substrate used in this assay.

TABLE I.A.1.

Sample Time (Hrs.)	pH	Abs. 550 nm		
		Substrate Solution Cellobiose	Zero Time Control	Incubated at 60°C for 1 hr.
20 a	5.6	1.29	1.28	1.27
b		1.31	1.26	1.27
40 a	5.4	1.29	1.29	1.31
b		1.31	1.33	1.20

In the previous progress report (COO-4198-7), a kinetic analysis of the specific growth rate and specific glucose and cellobiose accumulation rates (determined by HPLC analysis) during a fermentation of C. thermocellum on Solka floc

was presented as shown in Figure I.A.2. It was hypothesized that the growth-associated glucose accumulation, which had not been observed during cellobiose fermentations, may have been the result of an exoglucosidolytic activity which cleaved glucose units from the ends of larger cellodextrin chains, substrates that would not be present in a cellobiose medium. However, since solka floc contains up to 10% hemicellulose or xylan, and since we have encountered difficulties with the separation of xylobiose from glucose on our HPLC analytical column, it may have been that the 'glucose' which we observed to accumulate in a growth-associated manner during solka floc fermentations was really xylobiose, and that actual glucose accumulation occurred as a non-growth associated event, similar to that of cellobiose fermentations. As a first experiment to check the HPLC measured glucose, C. thermocellum was again grown on CM3/solka floc medium, but glucose accumulation was measured with the enzymatic glucostat assay. Figure I.A.3 depicts the kinetics of cell mass, reducing sugar and glucose accumulation during a fermentation without pH-control. When the enzyme assay is used, it can be seen that glucose does not accumulate until after dry cell weight measurements indicate that growth has leveled off. This separation of glucose accumulation from growth is more clearly shown in Figure I.A.4 where the specific growth rate, μ , and the specific glucose accumulation rate, q_g , (data from Figure

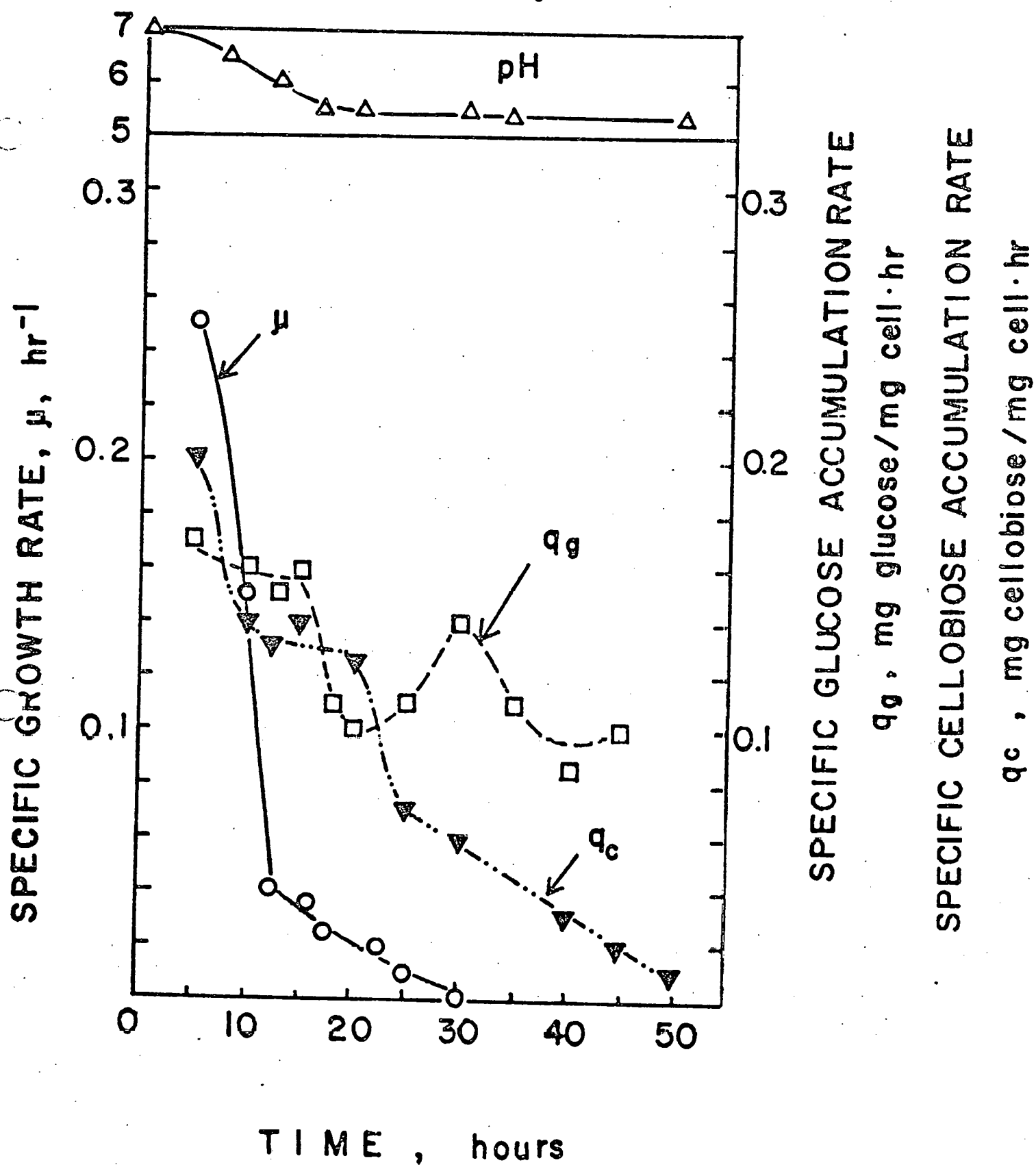


FIGURE I.A.2: SPECIFIC GROWTH RATE, SPECIFIC GLUCOSE AND CELLOBIOSE ACCUMULATION RATES, AND pH PROFILE VS TIME FOR FERMENTATION OF *C. THERMOCELLUM* ATCC 27405 ON CM3/SOLKA FLOC (HPLC DATA ANALYZED TO OBTAIN q_g , q_c)

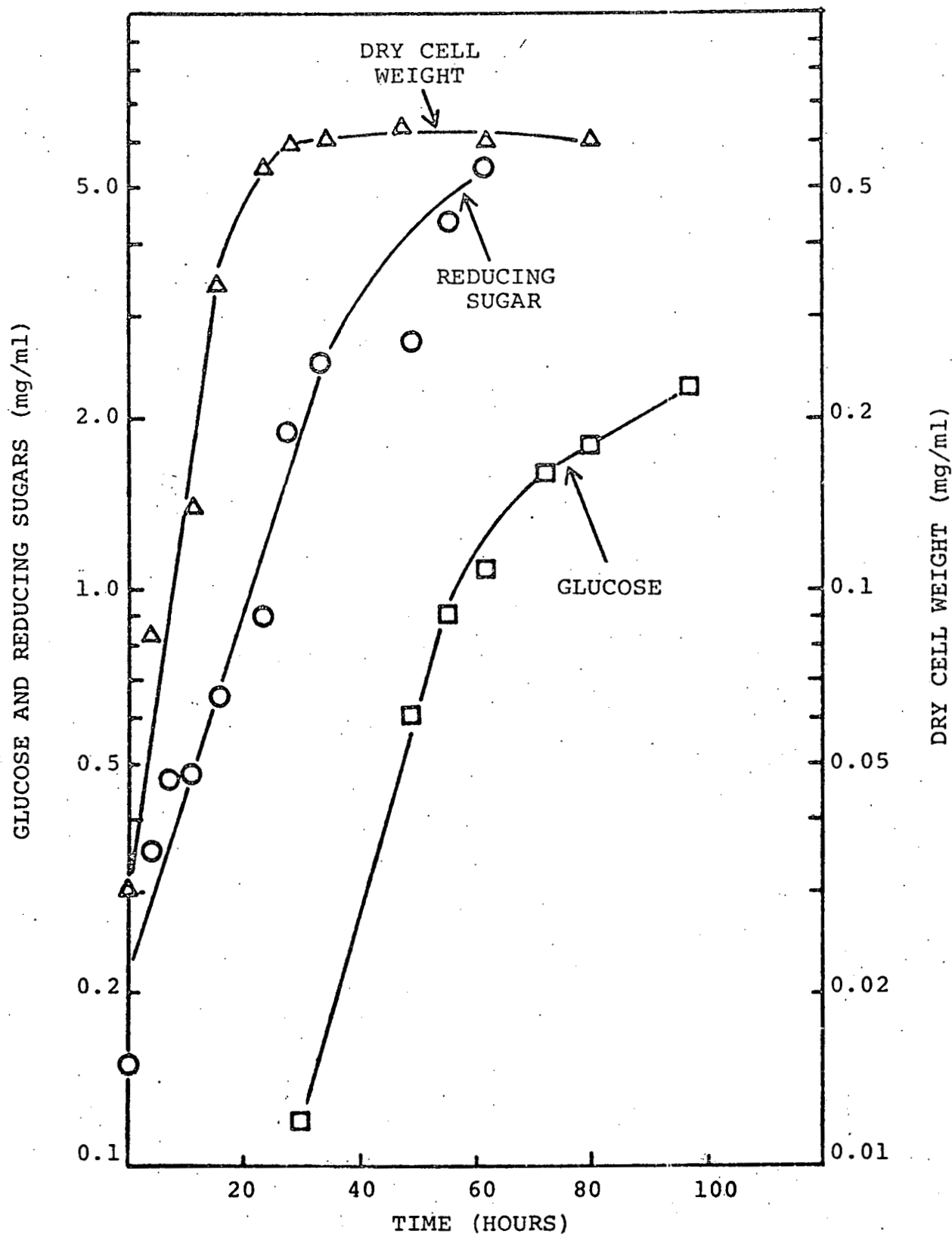


FIGURE I.A.3: KINETICS OF GROWTH, REDUCING SUGAR ACCUMULATION AND GLUCOSE ACCUMULATION (BY GLUCOSTAT METHOD) DURING FERMENTATION OF *C. THERMOCELLUM* ATCC 27405 ON CM3/SOLKA FLOC

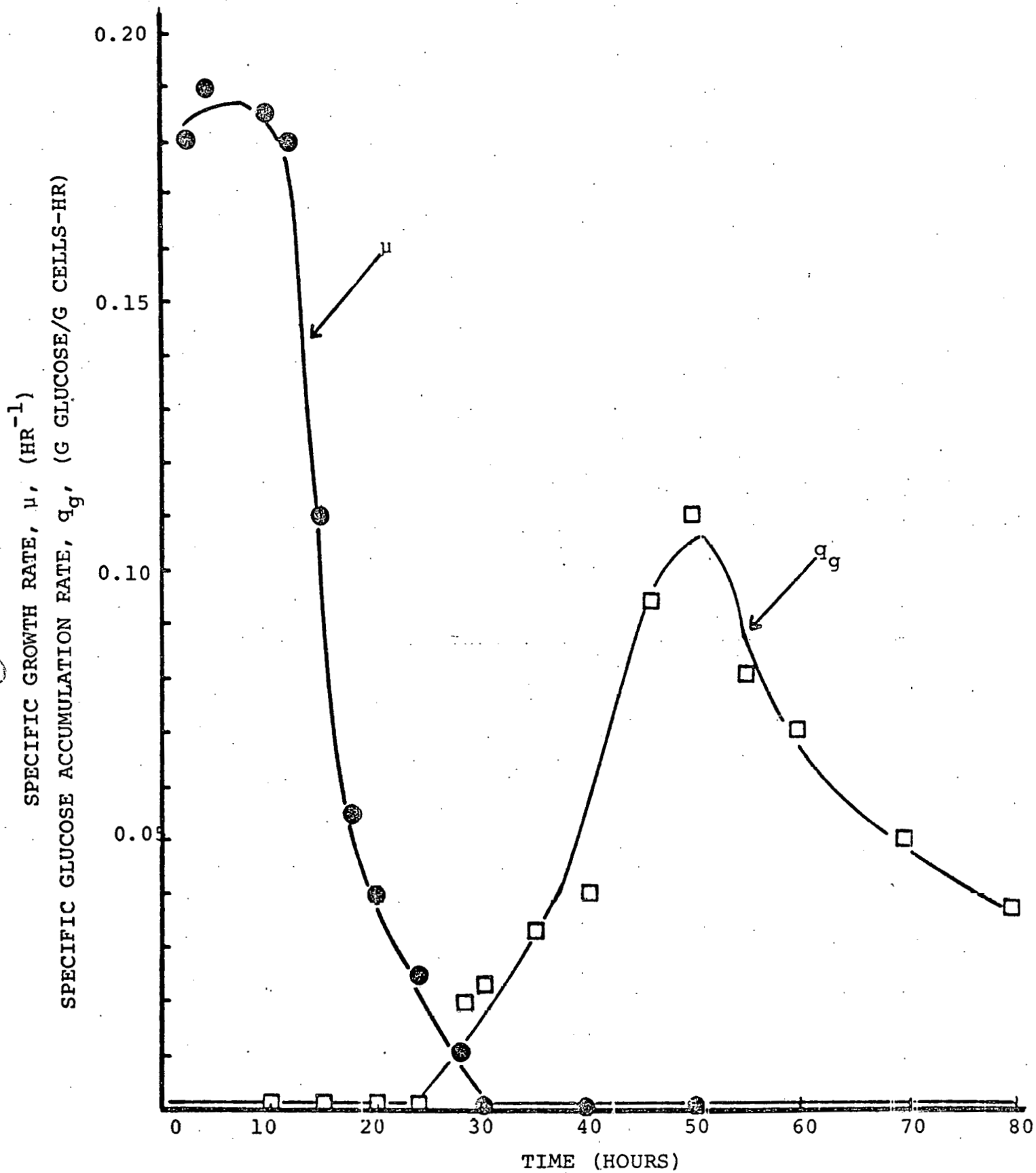


FIGURE I.A.4: SPECIFIC GROWTH RATE AND SPECIFIC GLUCOSE ACCUMULATION RATE VS. TIME FOR FERMENTATION OF *C. THERMOCELLUM* ATCC 27405 ON CM3/SOLKA FLOC (GLUCOSTAT DATA USED TO OBTAIN q_g)

I.A.3) have been plotted as a function of time. As in the cellobiose fermentations, glucose accumulation during a solka floc fermentation, measured by the glucostat assay, appears to be a non-growth associated event, occurring when the pH of the medium has dropped to 5.4.

4. Discussion

Measurements of culture turbidity and extracellular protein production during fermentations of C. thermocellum ATCC 27405 on cellobiose failed to positively show the occurrence of autolysis which might account for the non-growth associated glucose accumulation via the release of the intracellular cellobiose phosphorylase into the medium where it could act extracellularly. Similarly, an assay to measure extracellular cellobiase production did not indicate the presence of such an enzyme in the fermentation broth. There are several explanations why glucose accumulation occurs during cellobiose fermentations even though an extracellular cellobiase activity has not been measured, be it an extracellularly produced enzyme or a released intracellular enzyme. An obvious explanation is that the conditions of the cellobiase assay were unsuitable for measuring the appropriate enzyme activity. For instance, anaerobiosis may have been necessary. Two alternative possibilities are that a cell-surface-bound enzyme is responsible for the degradation of cellobiose to glucose, or that

the intracellular cellobiose phosphorylase is responsible for the cleavage and that the resulting glucose-1-phosphate is retained within the cell for further catabolism, while the other glucose unit is excreted into the medium. These possibilities will be looked into for future work.

The discrepancy between HPLC and glucostat data for glucose accumulation during solka floc fermentations may be explained, as mentioned in the Results section, if accumulating xylobiose is being artifactually detected as glucose during the active growth phase of the organism. This hypothesis will be tested by collecting the 'xylobiose-glucose' fractions (from samples taken during the active growth phase) eluting from the HPLC column and subjecting them to paper chromatography to determine whether both sugars are present. HPLC will be further used to study the kinetics of cellulose degradation, as a means of measuring whether there is a random or specific time sequence in the breakdown of cellulosic materials to cellodextrins and then to disaccharides and finally to the monosaccharides, glucose and xylose, which are useful substrates for subsequent fermentations.

B. PRODUCTION OF ETHANOL FROM CELLULOSIC BIOMASS BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

In our last progress report (COO-4198-7), we reported that the new strain of Clostridium thermocellum, S-4, produces a significantly higher ratio of ethanol to acetic acid. Carbon and oxidation-reduction balances have led us to conclude that this strain is deficient in the hydrogenase and thus can produce the high ratio of ethanol to acetic acid. The kinetics of ethanol production were examined and found that it is produced through both growth and non-growth associated modes. In this quarter, we have continued to examine the kinetics of production of other products. In addition, the degradation capabilities of C. thermocellum, strain S-4, on various cellulosic biomasses were also examined. The results from these experiments will be presented.

2. Materials and Methods

The microorganism used through this study was C. thermocellum, strain S-4. The basic medium used for growth was CM4. Modifications were made in some cases and these changes will be specified along with the presentations of the results.

The rice straw and wheat straw used were obtained from Professor Charles R. Wilke, Lawrence Berkeley Laboratory, University of California, Berkeley, California. Rice straw is

the crop of Fall, 1975, in Butte county; wheat straw is the crop of Fall, 1975, in Yolo county. Both are 0.25 - 0.35 mm fraction of 2 mm Wiley milled. The sweet gum tree sawdust was obtained from the University of Georgia. The sources of other biomasses have already been reported.

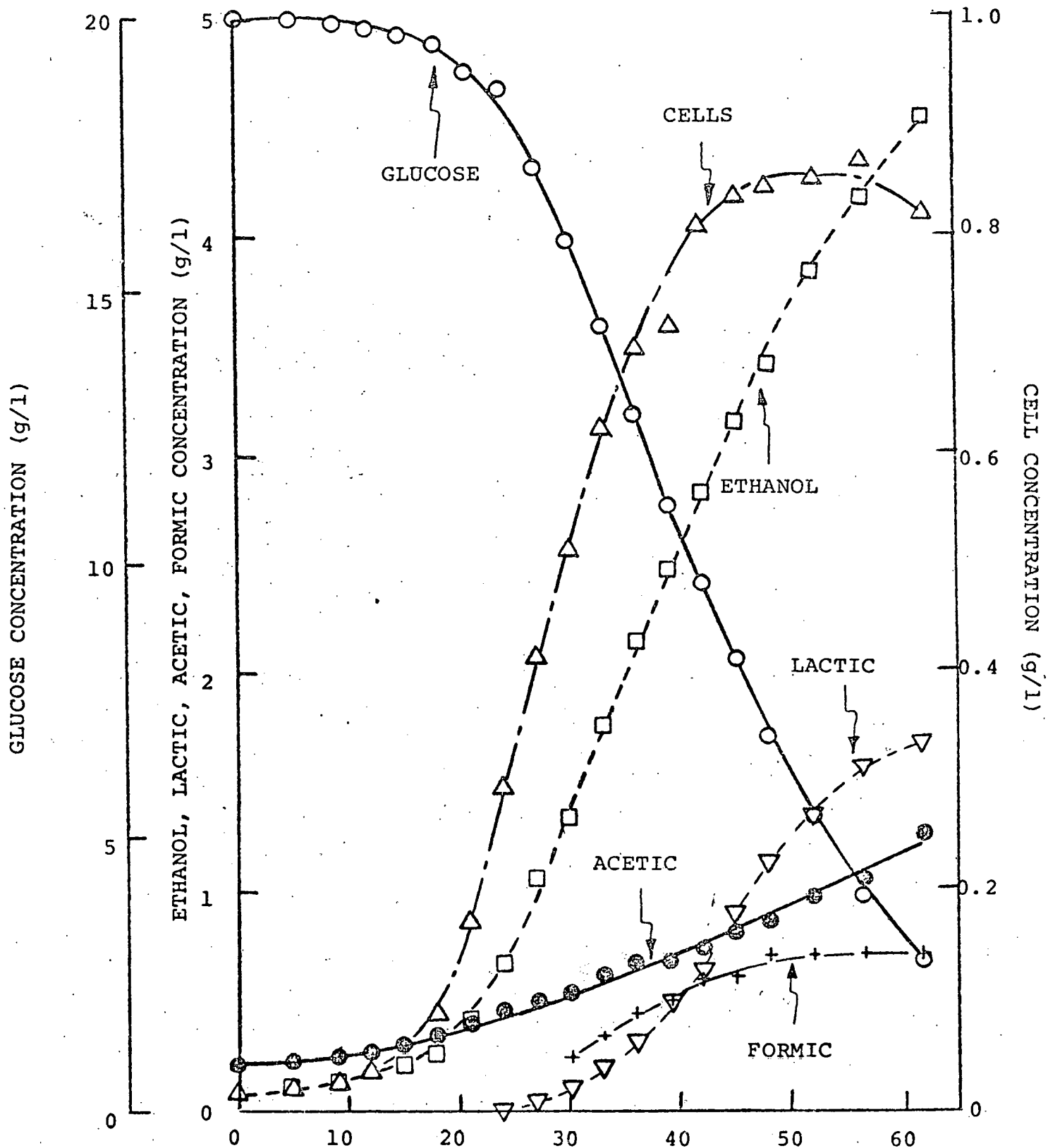
All the analytical procedures have already been reported and will not be reported.

3. Results and Discussion

In order to study the kinetics of products formation and the effects of environmental conditions, several fermentations were performed in a 7-liter fermentor with a 5-liter working capacity. Figure I.B.1 shows a typical fermentation profile with pH controlled at 6.5; initial yeast extract concentration was 3 g/l and glucose concentration was 2%. The production of reduced products (i.e., ethanol and lactic acid) is significantly greater than oxidized product (acetic acid) and is similar to the previous results. The kinetics of growth and ethanol production can be achieved through growth and non-growth associated modes and the mathematical proposed in an earlier progress report (COO-4198-7) was as followed:

$$q_p = \alpha \mu + \beta$$

FIGURE I.B.1: FERMENTATION PROFILE OF Clostridium thermo-
cellum (S-4) WITH pH CONTROL (6.5) IN LOW
IRON CONCENTRATION MEDIUM



where q_p = specific productivity, gm product/gm cell-Hr.
 μ = specific growth rate, Hr⁻¹
 α = growth associated product formation constant,
gm product/gm cell
 β = non-growth associated product formation constant,
gm product/gm cell-Hr

A plot of specific growth rate (μ) versus specific productivity (q_p) is shown in Figure I.B.2. The growth associated ethanol formation constant is 1.5 (g ethanol/g cell).

The production of lactic acid was analyzed in the similar manner and the results also shown in Figure I.B.2. In contrast to the positively growth associated mode of ethanol formation, lactic acid production shows a negatively growth associated mode. The specific productivity of lactic acid has an inverse relationship with the specific growth rate. These results indicated that higher growth rate will increase the productivity of ethanol and decrease the productivity of lactic acid, thus increasing the yield of ethanol production.

Because both pyruvate-ferredoxin oxidoreductase and ferredoxin are non-heme iron protein, it was expected that iron might play an important role in the production of ethanol. Therefore, the effect of iron concentration on the kinetic parameters was examined.

Figure I.B.3 shows the fermentation profile with pH controlled at 6.5, FeSO₄·7H₂O concentration was 0.01 g/l:

FIGURE I.B.2: KINETIC RELATIONSHIPS OF GROWTH AND PRODUCT FORMATION BY C. thermocellum (S-4) AT LOW IRON CONCENTRATION

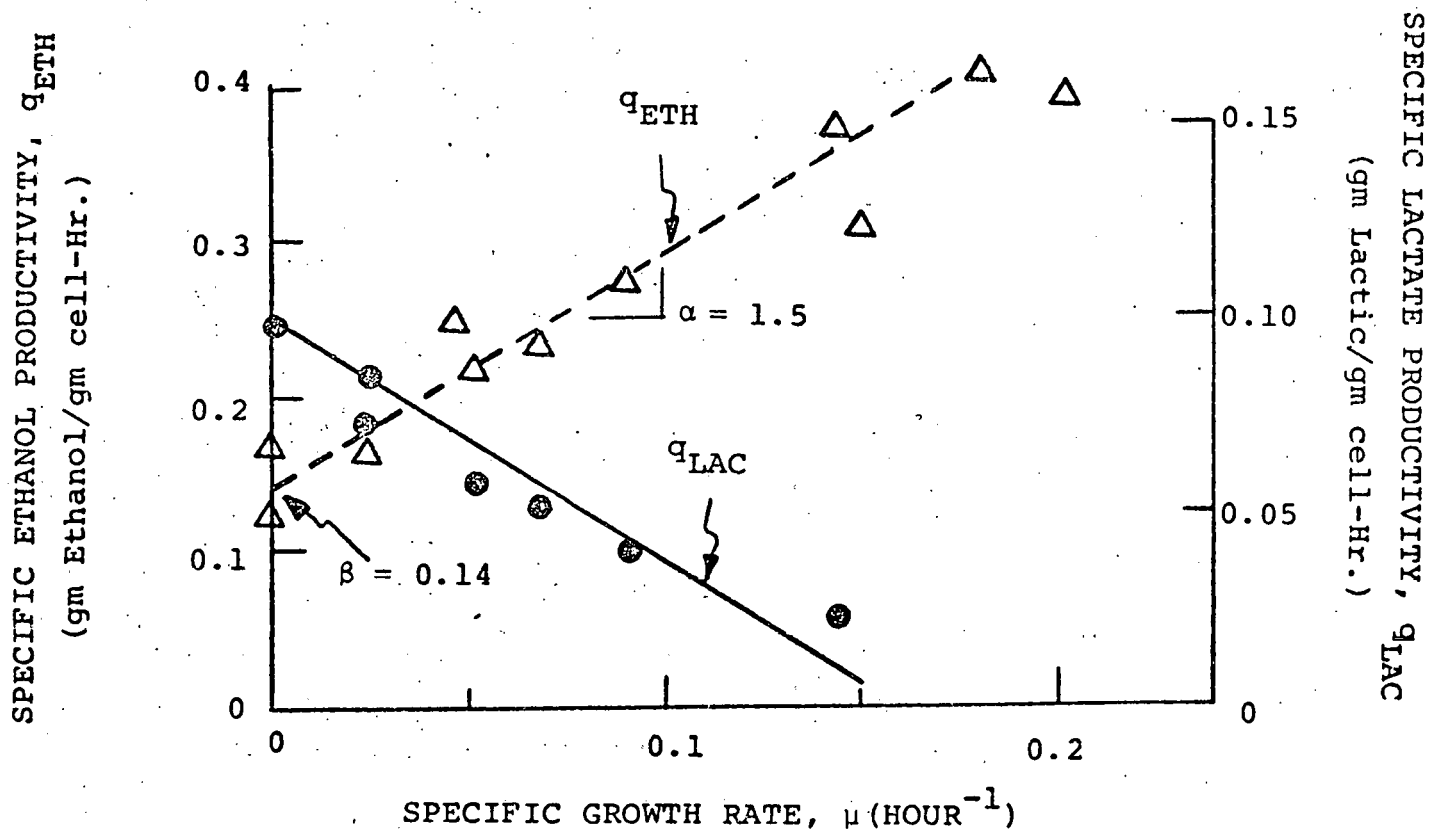
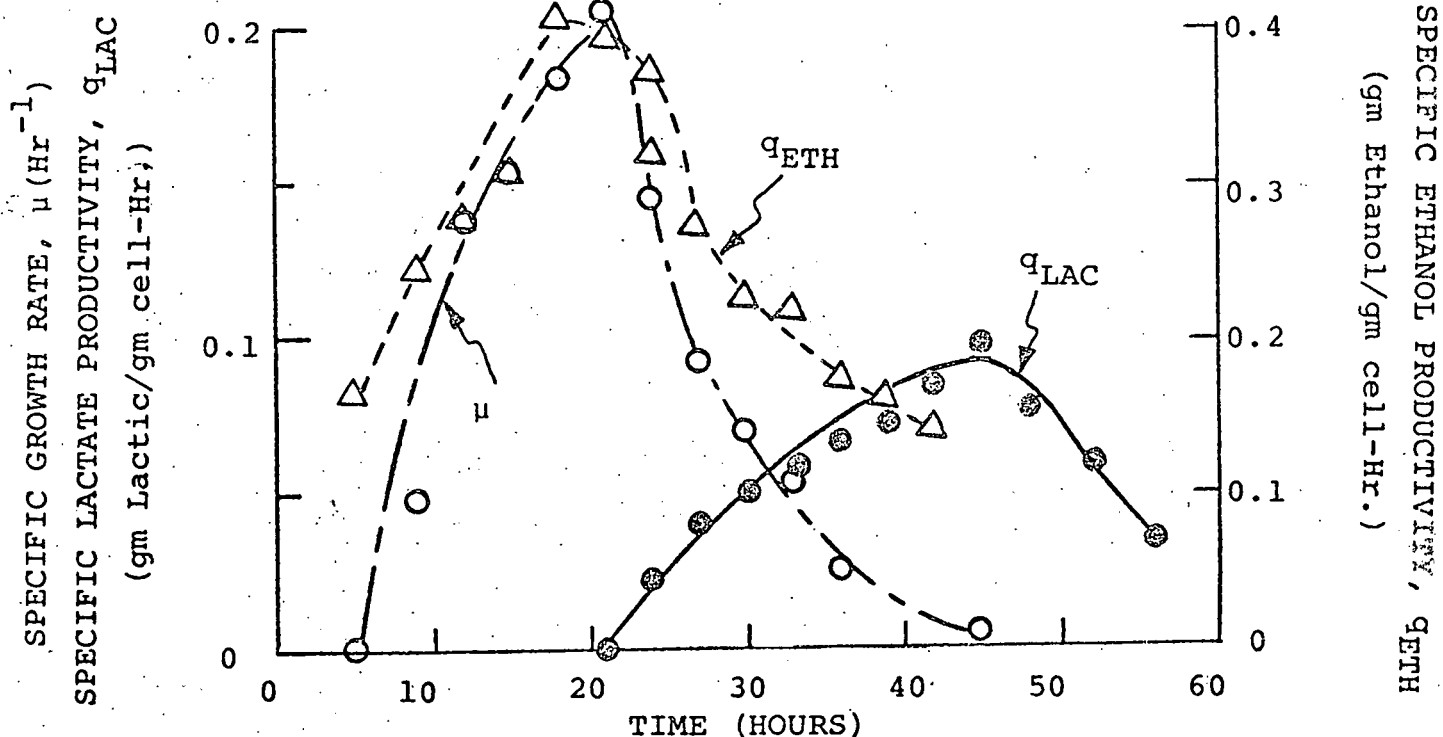
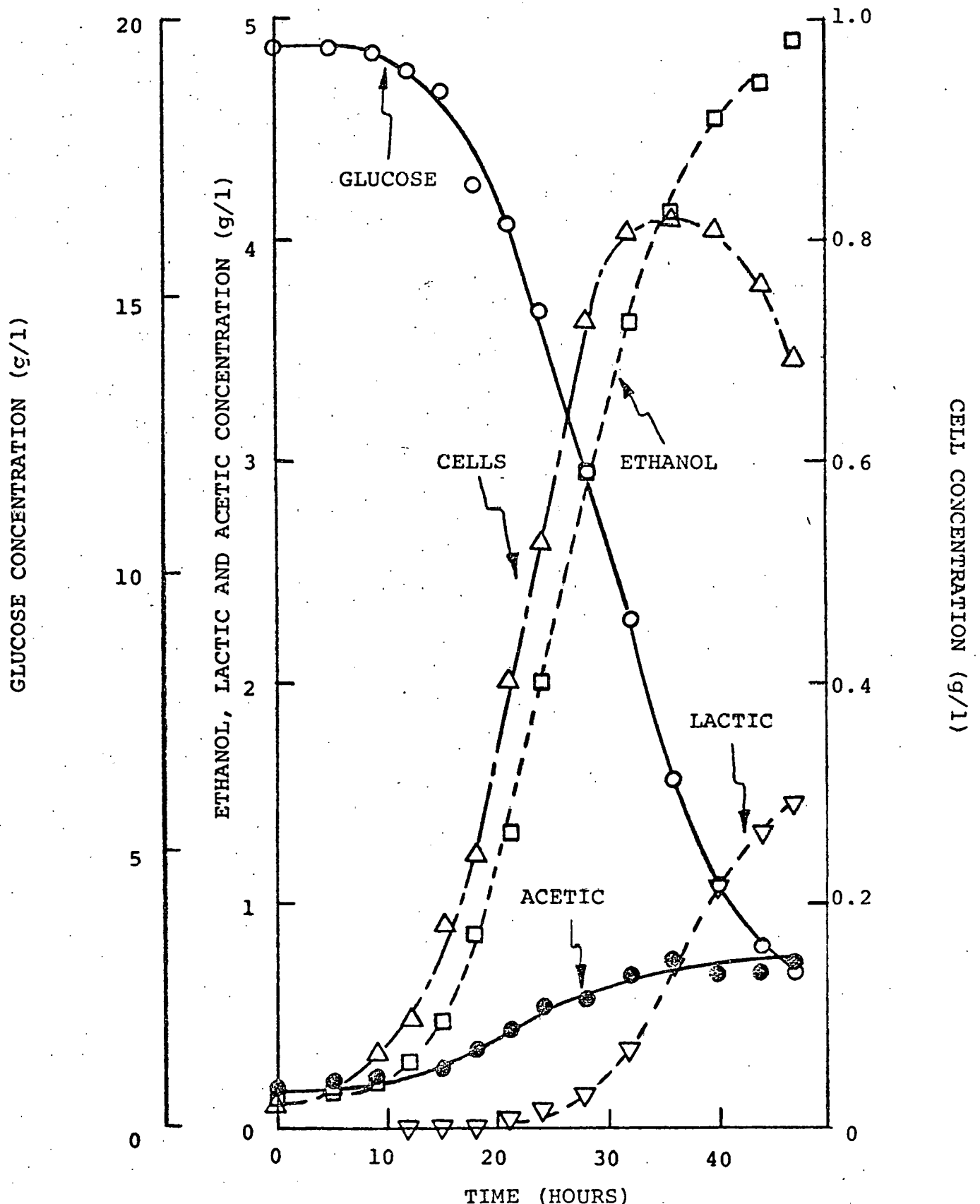


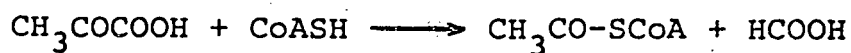
FIGURE I.B.3: FERMENTATION PROFILE OF Clostridium thermo-
cellum (S-4) WITH pH CONTROL (6.5) IN HIGH
IRON CONCENTRATION MEDIUM



eight times higher than that of CM4 medium. Other conditions are the same as the other experiments. The data from Figure I.B.3 were analyzed in the same way as described previously, and shown graphically in Figure I.B.4.

For ethanol production, the calculated value of α and β from Figure I.B.4 are 2.6 (g ethanol/g cell) and 0.14 (g ethanol/g cell-hr) respectively. It can be seen that growth associated constant α increased significantly as iron concentration increased while non-growth associated constant β remained the same. Again, lactic acid production shows a negatively growth associated mode.

Another important effect of iron concentration was the formation of formic acid. Formic acid, as shown in Figure I.B.5, is the key intermediate of one-carbon unit synthesis in clostridia. Formate accumulated when low iron concentration (1.25 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$) was used and disappeared when iron concentration was increased to 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$. There are at least 4 different mechanisms for formate production. The mechanism in Clostridium butyricum and Clostridium butylicum which formate is generated from pyruvate by pyruvate formate lyase according to the following reaction:



This is the most reasonable one to explain the observed phenomenon.

FIGURE I.B.4: KINETIC RELATIONSHIPS OF GROWTH AND PRODUCT FORMATION BY C. thermocellum (S-4) AT HIGH IRON CONCENTRATION

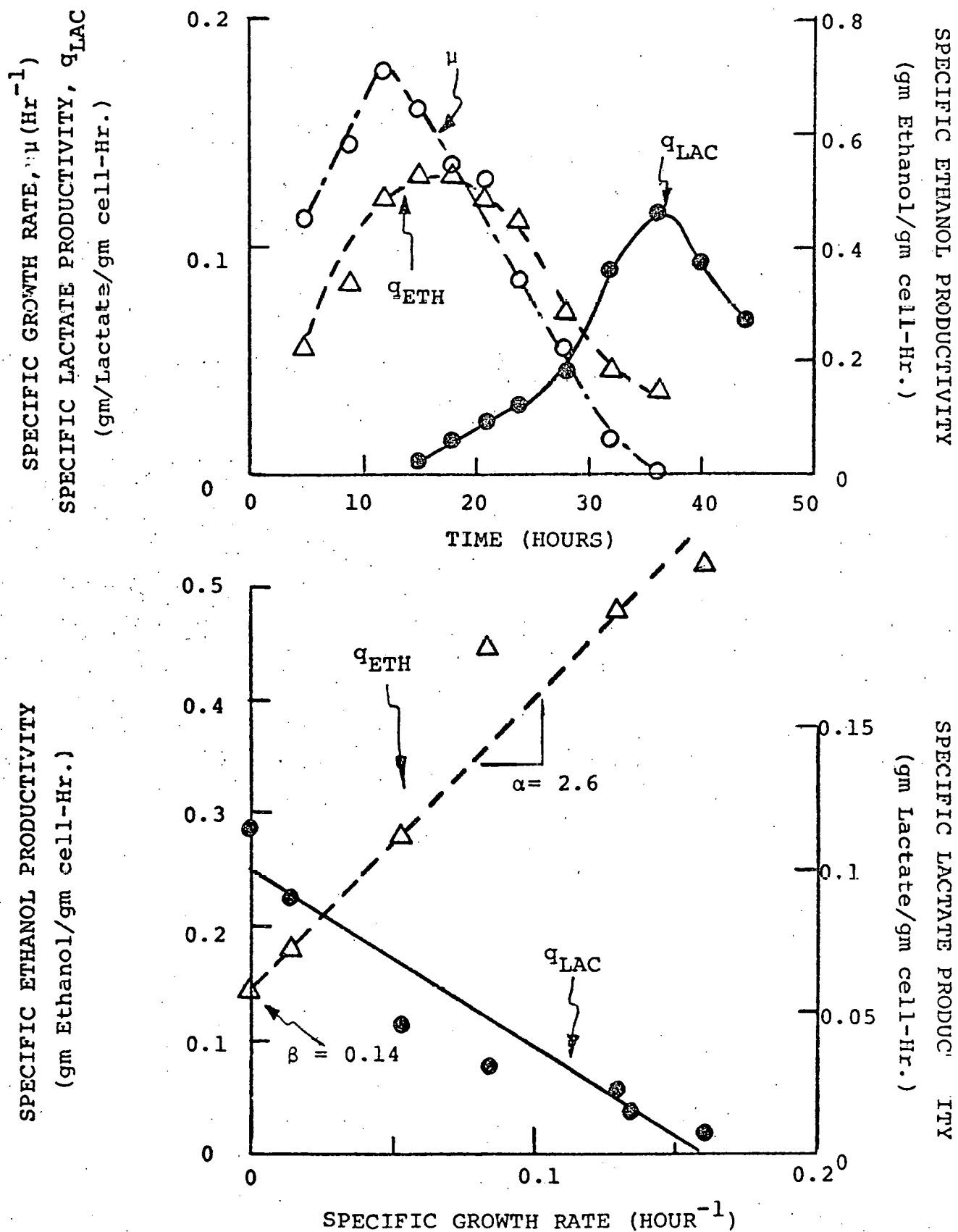
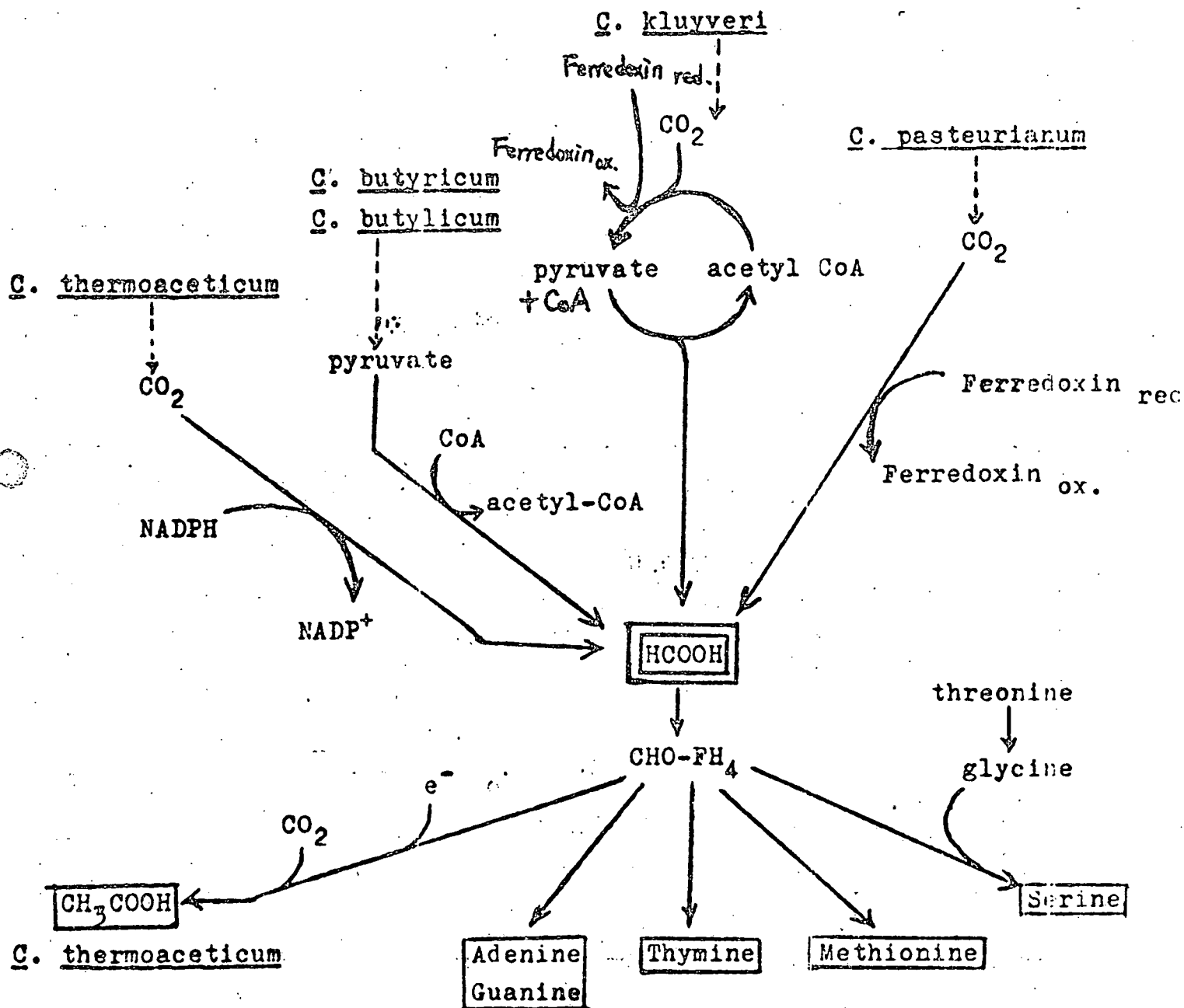


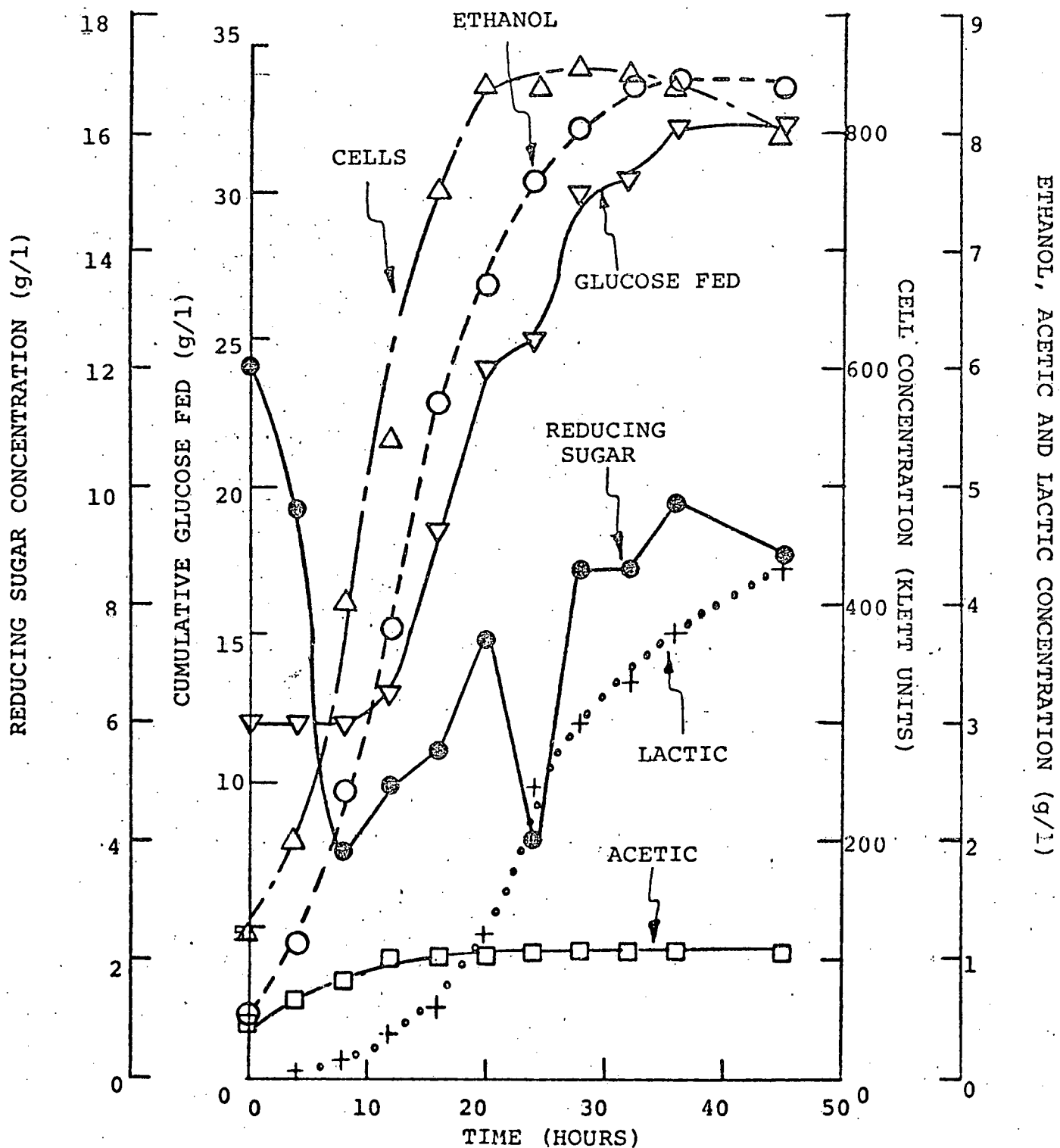
FIGURE I.B.5: PATHWAY OF ONE-CARBON UNIT SYNTHESIS IN CLOSTRIDIA



It is well known that flavodoxin, which has a similar low potential, can replace ferredoxin in certain reactions and are synthesized by many bacteria, especially in media low in iron concentration. It is reasonable to explain the phenomenon of formic acid accumulation in the same way that C. thermocellum can synthesize pyruvate formate lyase replacing pyruvate ferredoxin oxidoreductase to produce acetyl CoA in medium low in iron concentration. In this fashion, C. thermocellum is able to maintain the production of acetyl CoA from pyruvate at a certain level and leading to the accumulation of formic acid. If this is true, then it can be concluded that CM3 and CM4 medium are deficient in iron.

At this point, it still remained to be answered what is the highest ethanol concentration the strain S-4 can produce. A fed batch fermentation was used in seeking this answer. The concentration of glucose was maintained between 5-10 g/l and pH was controlled at 6.5. The results from this experiment are shown in Figure I.B.6. As seen from this figure, the initial rate of cell growth and ethanol production was quite rapid up to 20 hours. The acetic acid production occurred only in the very beginning then stopped while the lactic acid production started only when the growth rate decreased. A final concentration of 8.4 g/l of ethanol, 4.3 g/l of lactic acid and 1.0 g/l of acetic acid were obtained. A total of 32.3 g/l of glucose and 8.4 g/l of yeast extract was added and 8.8

FIGURE I.B.6: FERMENTATION PROFILE OF FED BATCH SYSTEM
WITH C. thermocellum (S-4) AT pH 6.5



g/l of reducing sugar remained after 45 hours fermentation. However, from the qualitative analysis of the reducing sugar by using paper chromatography, it was found that a large portion of the reducing sugar was cellobiose (the concentration was about one third of that of glucose). Moreover, from the HPLC analysis, it was revealed that about 2 g/l of cellobiose was accumulated. Although higher oligosaccharides could not be demonstrated due to the sensitivity of these methods, it was believed that some oligosaccharides also accumulated during the fermentation by the enzymatic reaction of cellobiose phosphorylase and cellodextrin phosphorylase.

Due to the presence of oligosaccharides, the reducing sugar concentration was underestimated. Therefore, acid hydrolysis (5% HCl, boiled for one and half hours) of the fermentation broth was performed in order to know the actual sugar concentration. It was found that the final sugar concentration increased from 8.8 g/l to 9.8 g/l. The actual reducing sugar concentration was then used to calculate the carbon and oxidation reduction balance and ethanol yield of this fermentation.

Table I.B.1 shows the carbon and oxidation-reduction (O/R) balance from this experiment. Here again, the analysis of the O/R balance showed that there must be very little or no hydrogen formation during the fermentation.

TABLE I.B.1: CARBON AND OXIDATION-REDUCTION BALANCE FOR
FERMENTATION OF GLUCOSE BY STRAIN S-4
(C. THERMOCELLUM)

PRODUCTS	AMOUNT		g carbon/l	UNIT O/R VALUE per MOLE	OVERALL O/R VALUE	
	g/l	mM			CASE I*	CASE II*
Ethanol	8.4	182.6	4.38	-2	-365.2	-365.2
Lactic acid	4.3	47.8	1.72	0	0	0
Acetic acid	0.6	10	0.24	0	0	0
CO ₂ *	8.47	192.6	2.31	+2	+385.2	+385.2
H ₂ *	0.385	(192.6)	-	-1	-192.6	0
Cell mass*	2.5	-	1.25	-	-	-
Total carbon recovery			9.90		+385.2 -557.8	+385.2 -365.2
Glucose fermented	22.5	125	9.0	0		
Yeast extract consumed (30%)**	2.52	-	1.08	0		
Total carbon recovery in percent of theoretical value	$\frac{9.9}{9.0 + 1.08} = 98.2\%$					
Oxidation- Reduction Index					$\frac{385.2}{557.8} = 0.69$	$\frac{385.2}{365.2} = 1.05$

* See previous progress report.

** Assume 30% of yeast extract was consumed. 40% of consumed yeast extract was carbon.

The yield of ethanol was calculated to be 37% of fermented sugar. Compared to 45% in yeast alcohol fermentation, those results are very encouraging since it is now almost possible to compete with yeast alcohol fermentation as far as ethanol yield is concerned.

In this fed batch experiment, the fermentation stopped when a total of about 14 g/l of products were accumulated. It is believed that other nutrients were in excess at the end of the fermentation and the concentration of products is not high enough to inhibit the fermentation. Therefore, there are some unknown reasons contributing to the cessation of the fermentation. This might be due to sporulation or it may also be due to capsule formation (i.e. further polymerization of cello-dextrin). Lastly, clostridia has been reported to be able to produce bacteriocin and this should be investigated in order to seek answers to this problem.

In the overall process of cellulose degradation, cheap and abundantly available biomasses are required. Therefore, other potential biomasses including rice straw, wheat straw, corn cob, sugar cane bagasse, sweet gum tree sawdust and exploded poplar were examined. These experiments were performed using 25 g/l of biomass, 3 g/l of NaOH. After sterilization, CO₂ was sparged until pH is 7.0, 10 g/l of soybean meal was then added and 10% of seed culture was then inoculated. The results are summarized in Figure I.B.7. It can be seen from

FIGURE I.B.7: FERMENTATION PROFILE OF VARIOUS CELLULOSIC BIOMASS BY C. THERMOCELLUM (S-4)

(A) EXPLODED POPLAR

(B) SUGAR CANE BAGASSE

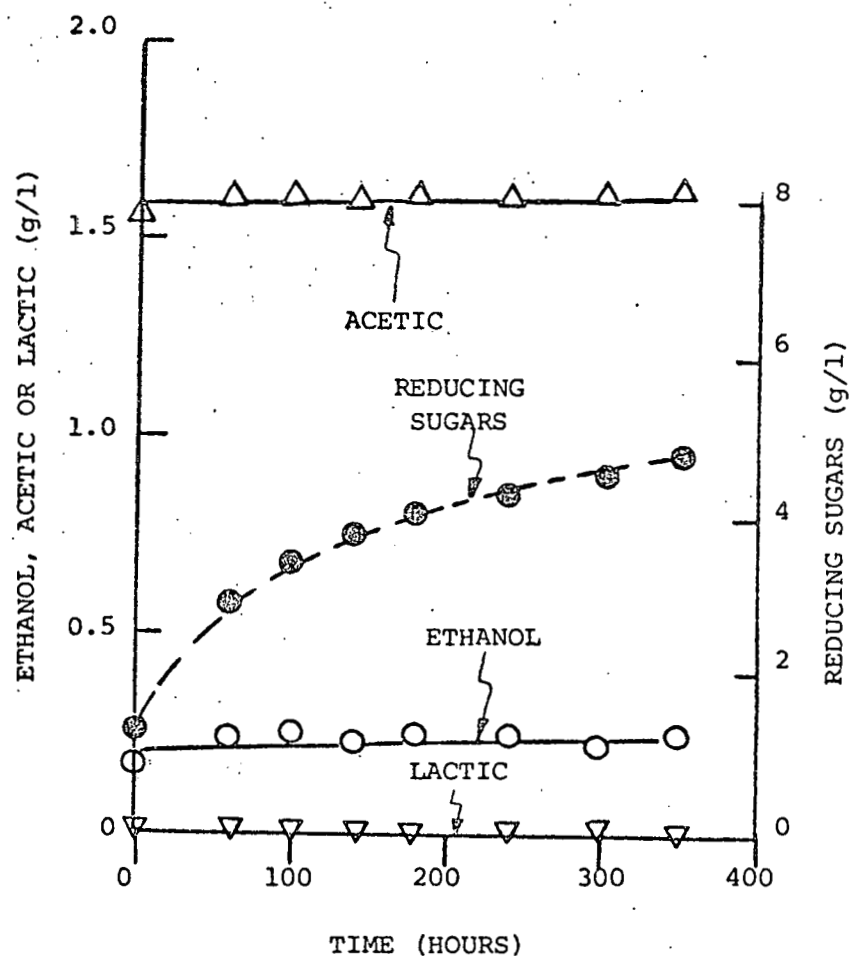
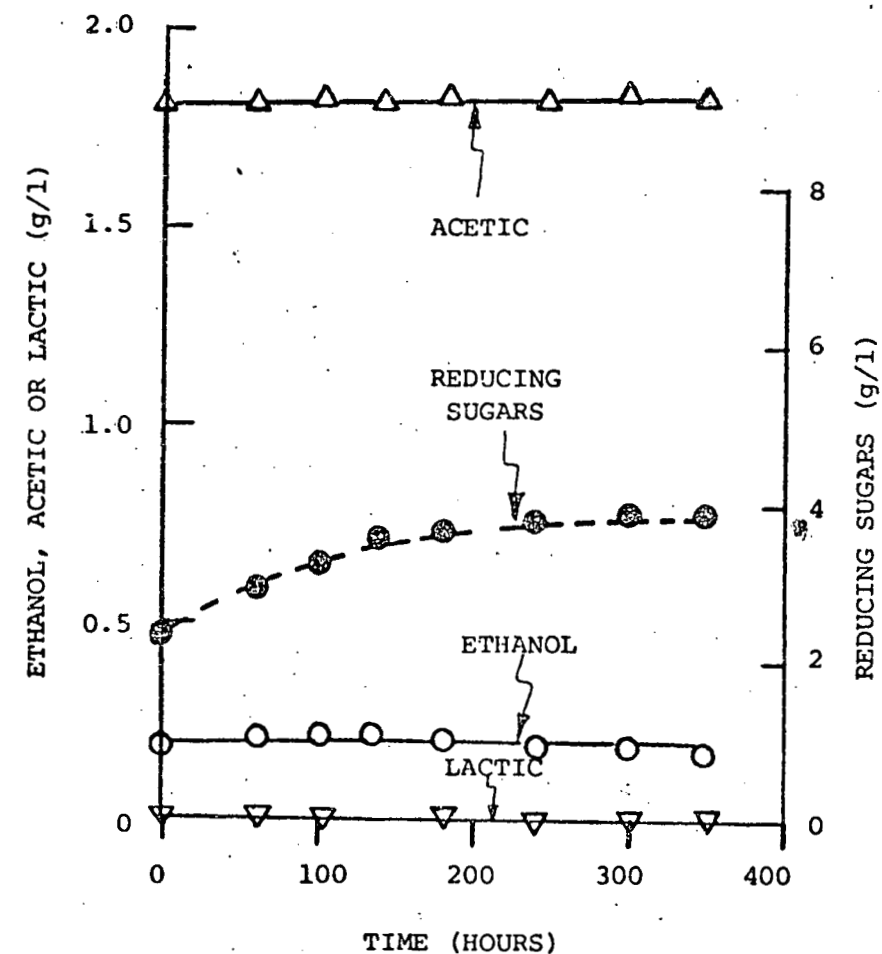
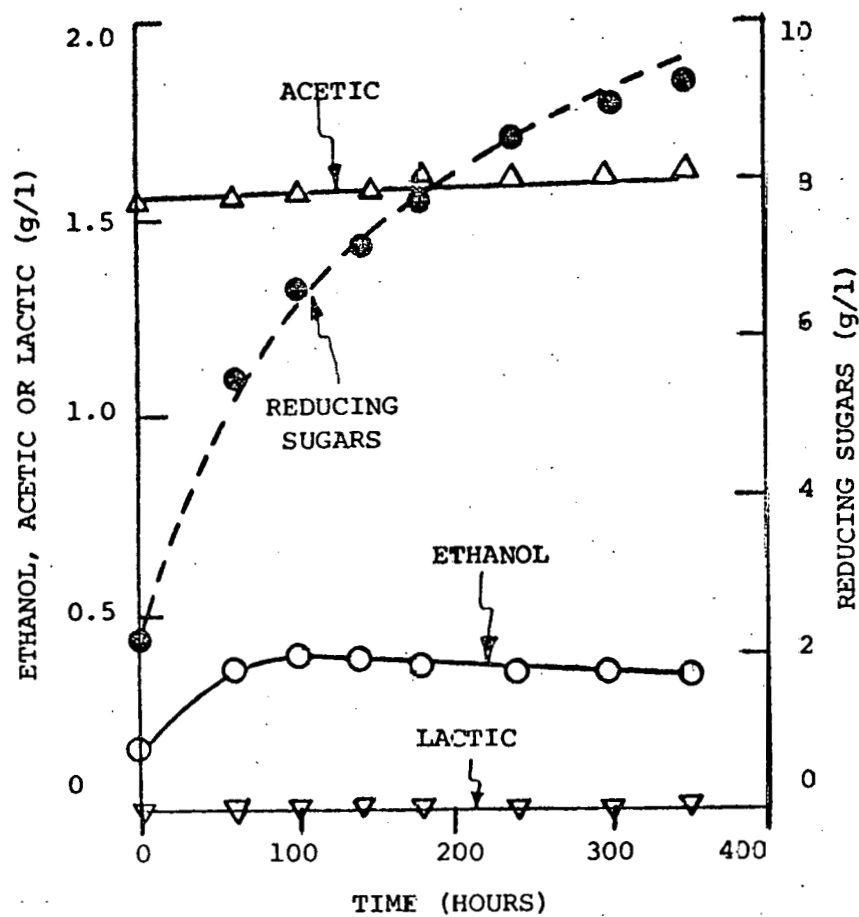


FIGURE I.B.7: CONTINUED

(C) CORN COBS



(D) SWEET GUM

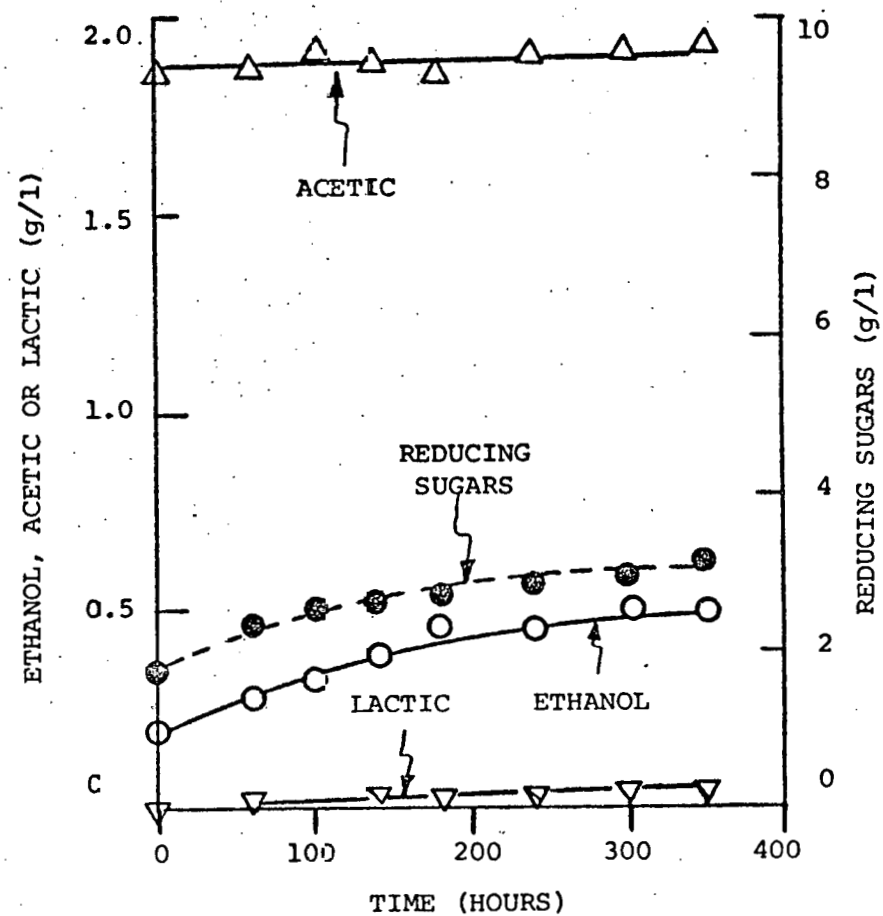
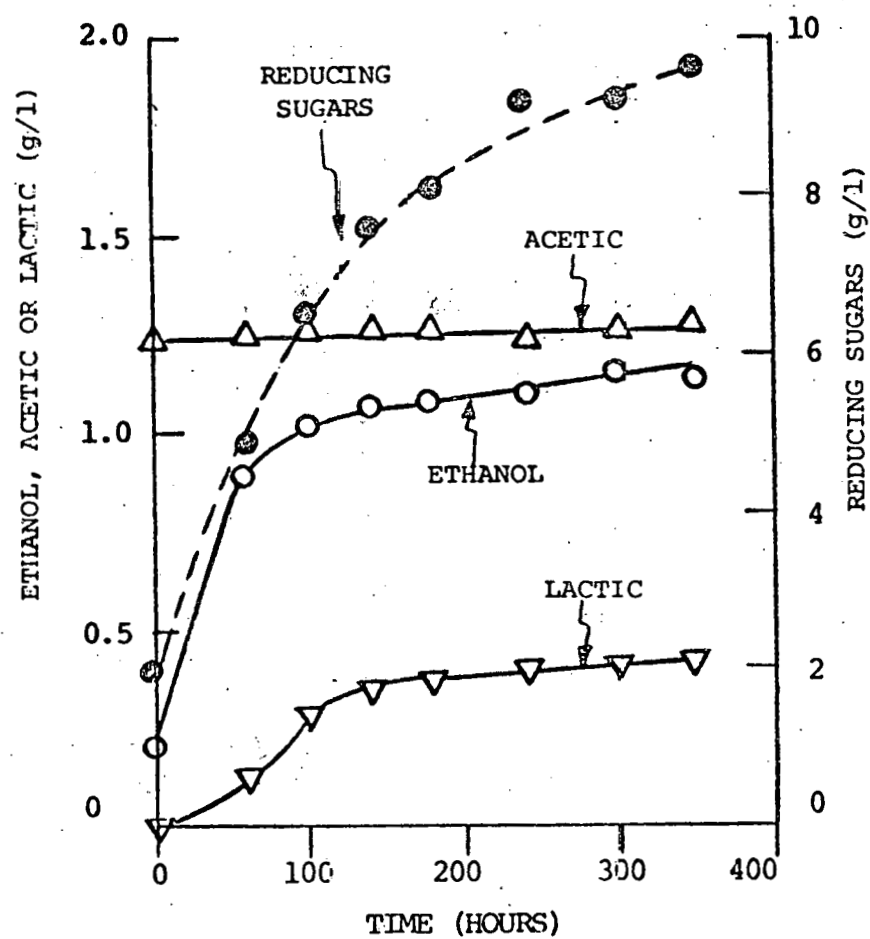
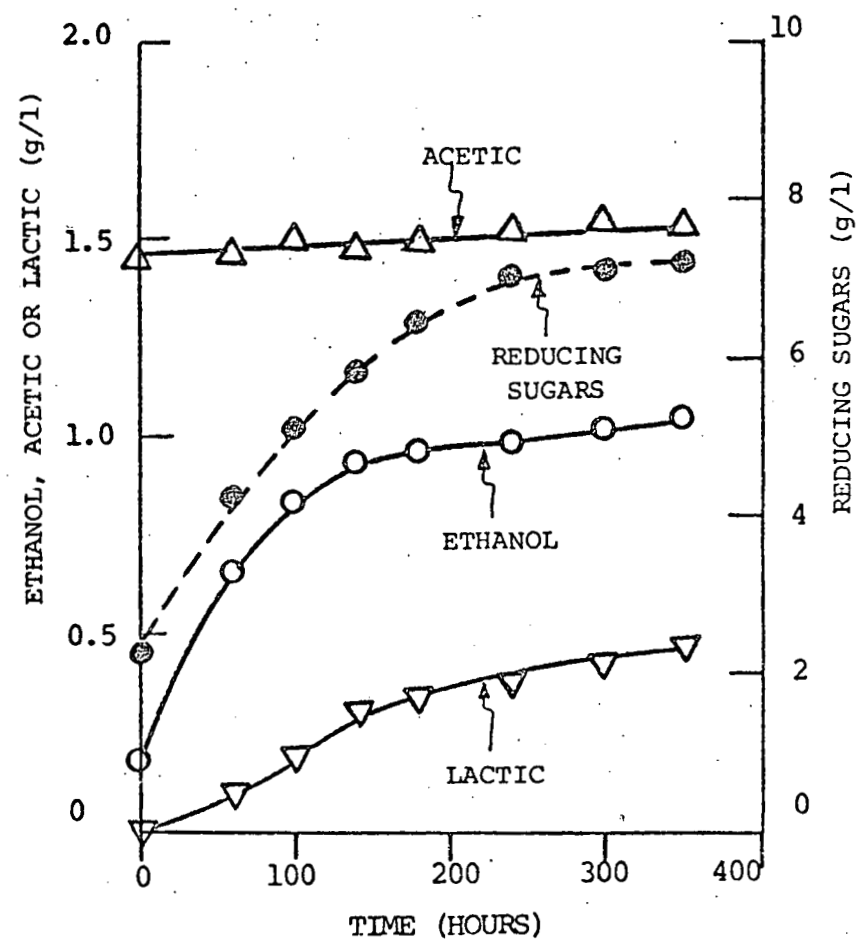


FIGURE I.B.7: CONTINUED

(E) RICE STRAW



(F) WHEAT STRAW



these results that straws are the best substrates among these biomasses tested. Since the production of ethanol is growth associated, one might use this index for growth of the organism. If one follows this rationale, we conclude that strain S-4 cannot metabolize sugar cane bagasse and exploded poplar. This may be due to the high lignin content or other toxic substance in these materials. Similarly, sweet gum tree sawdust and corn cob support only poor growth of strain S-4. The particle size of sweet gum tree sawdust is very large, up to 5 mm or more, and thus it can be explained by the limiting of surface area. The corn cob used in this experiment contains very fine particles and was not limited by surface area but by inhibitory effect of toxic substance, maybe lignin. The rapid accumulation of reducing sugar may be due to the extracellular cellulase reaction.

The best biomass, rice straw, produced 1.17 g/l of ethanol, 0.42 g/l of lactic acid and 9.6 g/l of reducing sugars. After acid hydrolysis, the actual concentration of reducing sugars was found to be 16.5 g/l. This represents a total of 18.1 g/l of products were accumulated. If one assumes that the carbohydrate contents of 70% and 40% for rice straw and soybean meal, respectively, this is equivalent to a total carbohydrate concentration of 21.5 g/l in the medium. From this total, strain S-4 is able to produce 18.1 g/l of products. This is equivalent to a conversion yield of 0.84 gram

of products per gram of carbohydrate. This yield is quite excellent and shows further encouragement for the project objectives.

4. Future Work

- Continue the packed-bed fermentor to achieve high production rate and high product concentration.
- Investigate the reason why fermentation stopped when only 14 g/l of products accumulated. Examine whether sporulation or polymerization of sugars have anything to do with the inhibition, also the possibility of bacteriocin production.
- Further efforts to increase ethanol tolerance.
- Mutation to direct catabolism only to ethanol.

C. ETHANOL PRODUCTION USING XYLOSE BY HG-2 AND CORN STOVER DEGRADATION BY MIXED CULTURE OF C. THERMOCELLUM AND HG-2

1. Introduction

One of the problems associated with the use of Clostridium thermocellum to produce ethanol from biomass is its inability to metabolize the degradation products arising from hemicellulose. In order to effect a more complete utilization of biomass, we have initiated studies on the metabolism of xylose using an anaerobic and thermophilic organism designated as "HG-2." This microorganism was isolated by personnel in our laboratory, but has not yet been identified. Some of the interesting properties of isolate HG-2 include its ability to metabolize pentose and hexoses at anaerobic and thermophilic conditions. These latter traits are particularly significant with respect to our overall objective of effective biomass utilization. Specifically, it offers us a potentially compatible mixed microbial system where the cellulolytic activities of Clostridium thermocellum are employed to produce ethanol and reducing sugars (xylose) from the cellulosic biomass and the metabolic activities of HG-2 to produce ethanol from hemicellulose degradation products. Therefore, during the past contract period, we have also initiated mixed culture studies using these two organisms to examine the feasibility for such a biomass utilization scheme.

2. Materials and Methods

Pure culture studies using HG-2 were performed in anaerobic shake flasks at 60°C. In these studies, either xylose or glucose was used as the carbon source. Substrates utilization were measured using the DNS method for reducing sugars which has been reported in previous progress reports. Adaptation studies to select higher ethanol tolerant strains of HG-2 were also performed. The protocol for adaptation and selection was identical to that reported in progress report COO-4198-6. Analytical methods such as ethanol and acetic acid concentration determinations were performed through gas chromatography as reported in other progress reports.

Mixed culture studies were performed using the procedure outlined below.

A 10% inoculum of C. thermocellum (S-4) was used in each of two anaerobic flasks containing 300 ml of medium. The medium contained corn stover (25 g/l), soy flour (10 g/l) and salts as in the CM-3 medium. Sodium bicarbonate buffer (12 g/l) was used to maintain the pH at 7.0 and sodium thio-glycolate was used to maintain anaerobiosis. After 24 hrs. of growth at 60°C, one flask was re-inoculated with an additional 10% inoculum of HG-2. The second flask served as a control for C. thermocellum alone. Samples were periodically removed and assayed for ethanol, acetic acid, and reducing sugars as described in previous progress reports.

3. Results and Discussion

a. Studies on HG-2 in Pure Culture

Growth and products formation by HG-2 using glucose as the carbon source are shown in Figure I.C.1. Mass doubling time of this organism on glucose was calculated to be about 2 hours (specific growth rate = 0.35 hr^{-1}). As can be seen from Figure I.C.1 that both acetic acid and ethanol are produced and appear to be associated with growth. Final acetic acid and ethanol are produced and appear to be associated with growth. Final acetic acid and ethanol concentrations were respectively 2.8 and 1.5 g/l. In view of this ratio of acetic acid to ethanol, we conclude that other catabolic product(s) must also be produced. Unfortunately, we did not perform analysis on these other products. One could postulate, for example, lactic acid which is at the same oxidation-reduction state as ethanol being one of the other major products of catabolism.

When xylose was used as the carbon source for HG-2, results of growth and products formation obtained are shown in Figure I.C.2. Here again, the mass doubling time was calculated to be about 2 hours (specific growth rate = 0.35 hour^{-1}). It is interesting to note that the results in this figure indicate that the final ethanol and acetic acid concentrations were nearly the same. Specifically, a final ethanol

FIGURE I.C.1: GROWTH AND PRODUCT FORMATION
BY HG-2 ON GLUCOSE

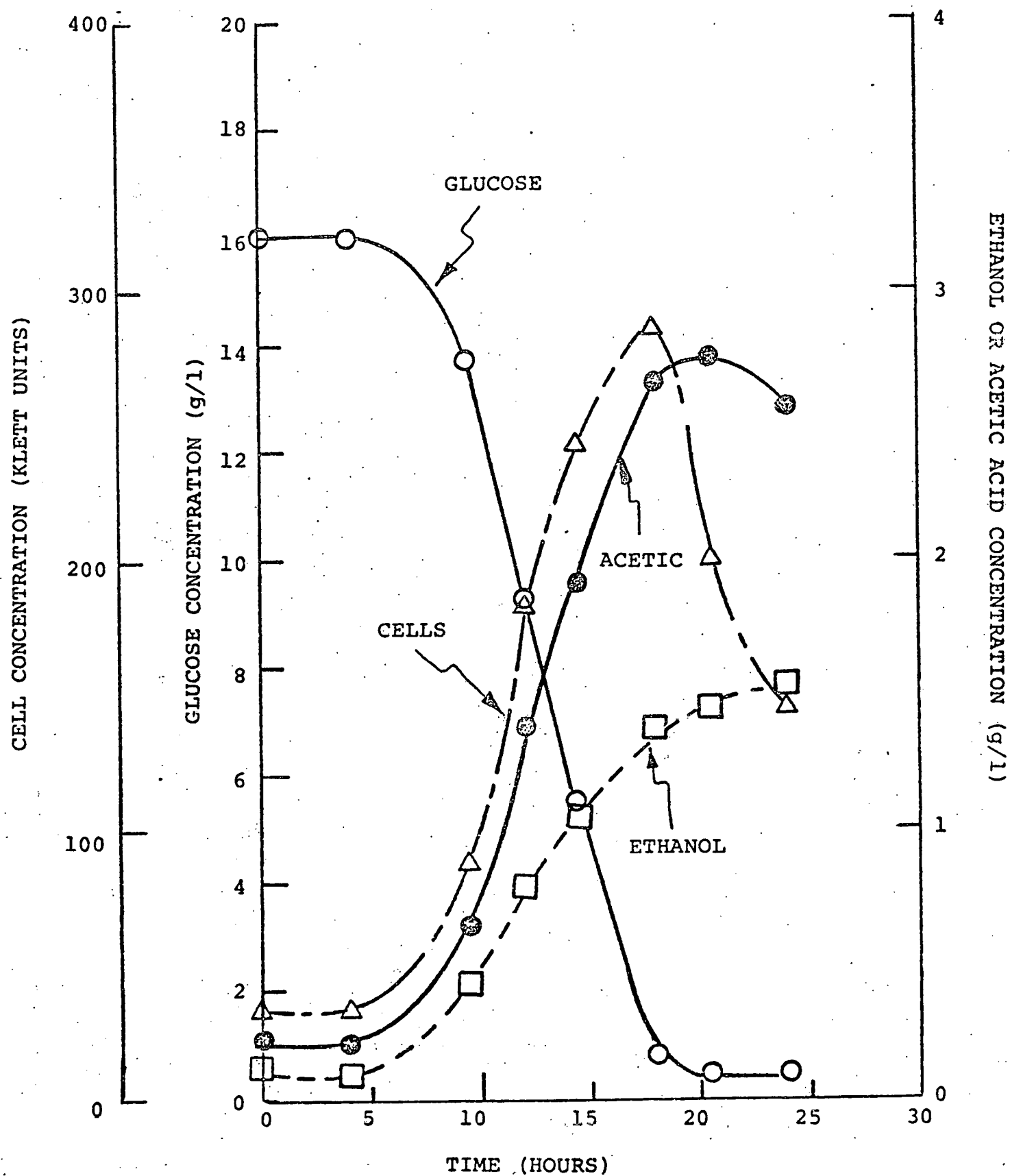
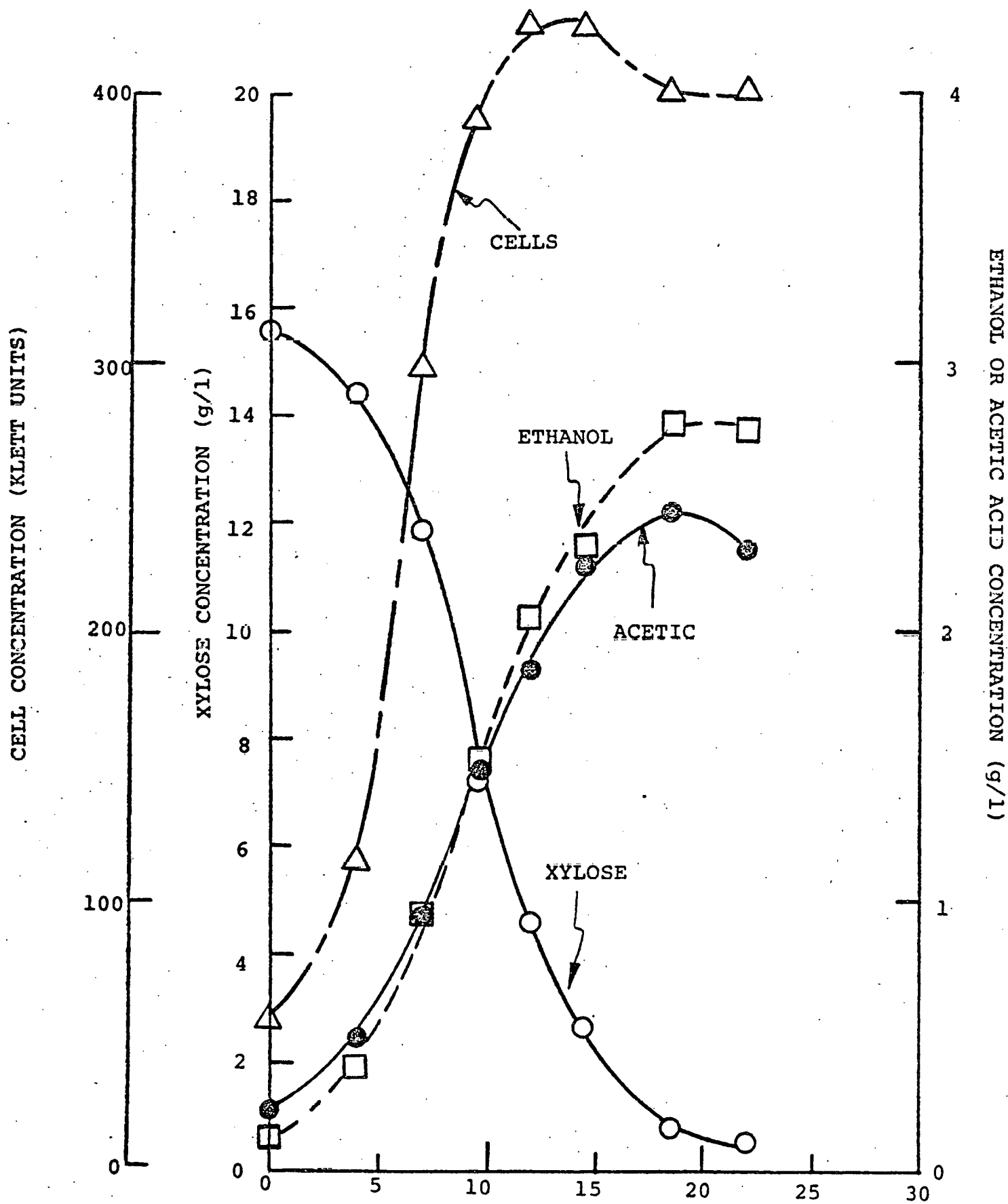


FIGURE I.C.2: GROWTH AND PRODUCT FORMATION
BY HG-2 ON XYLOSE



concentration of 2.8 g/l and acetic acid concentration of 2.5 g/l were attained.

In view of our previous experiences with Clostridium thermocellum with respect to its tolerance to ethanol, we decided to embark immediately to a selection program with HG-2 to increase its tolerance to end products. It was rationalized that we should attempt to isolate higher product tolerant strains of HG-2 before attempting to perform more detailed characterizations on this organism. Therefore, an adaptation and selection program was initiated to isolate higher ethanol tolerant strains of HG-2. The results from these studies are shown in Figure I.C.3. It can be seen that the parental strain of HG-2 exhibits a 50% growth inhibition at an ethanol concentration of 1.8% (V/V). After adaptation and six transfers in higher alcohol concentrations, an isolate designated as HG-3 was obtained. This new strain now exhibits a 50% growth inhibition at an ethanol concentration of 2.6% (V/V). We might add that this selection procedure was achieved over a period of three weeks. These results reinforce us as well as being encouraging in demonstrations on rapid capabilities in the isolating mutants with the desirable properties.

During the selection studies with HG-2, the tolerance of this organism to acetate was also examined. These results are shown in Figure I.C.4. It is interesting to note that the parental strain of HG-2 exhibited a higher

FIGURE I.C.3: ETHANOL TOLERANCE OF PARENT
(HG-2) AND ISOLATE HG-3

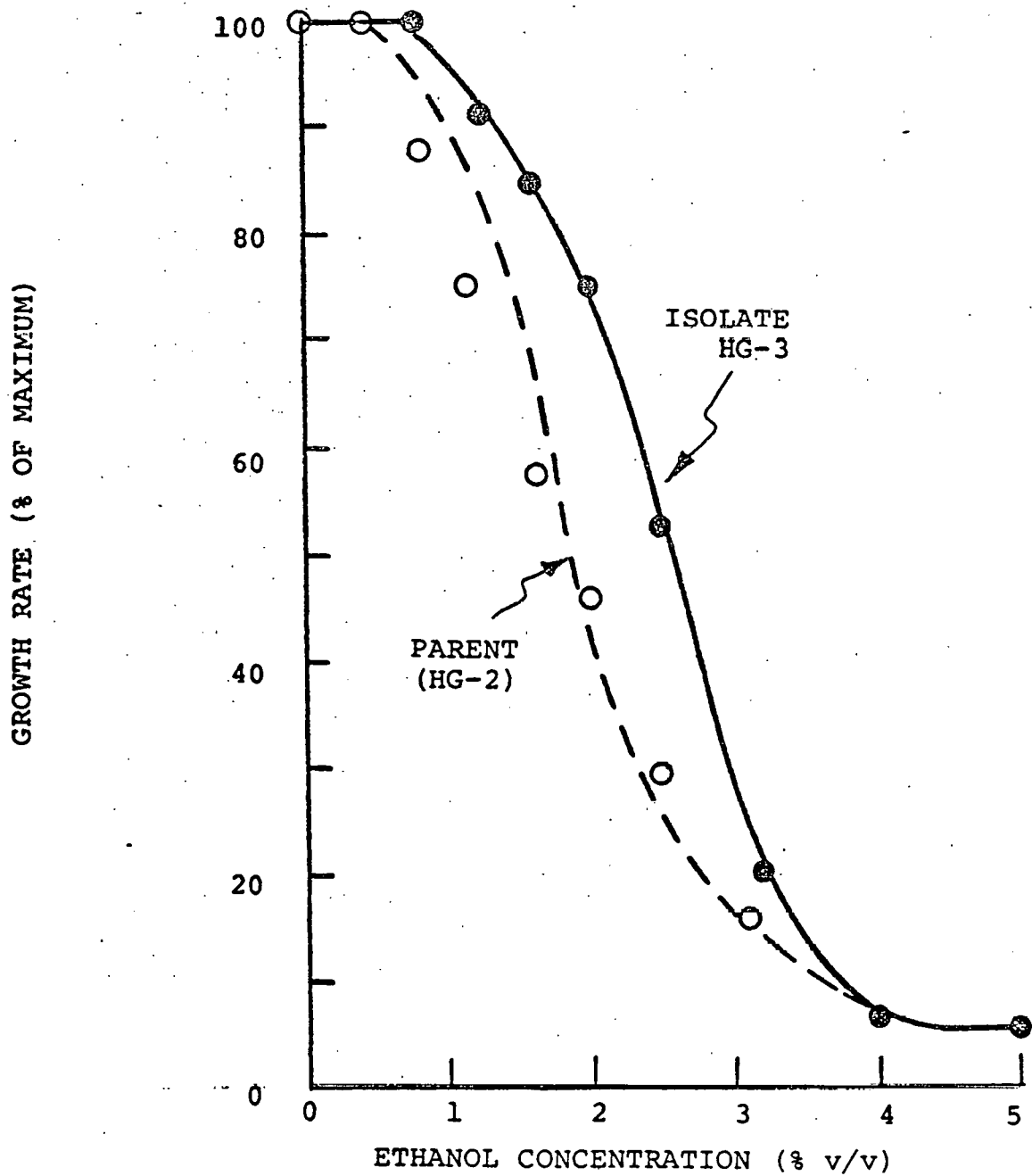
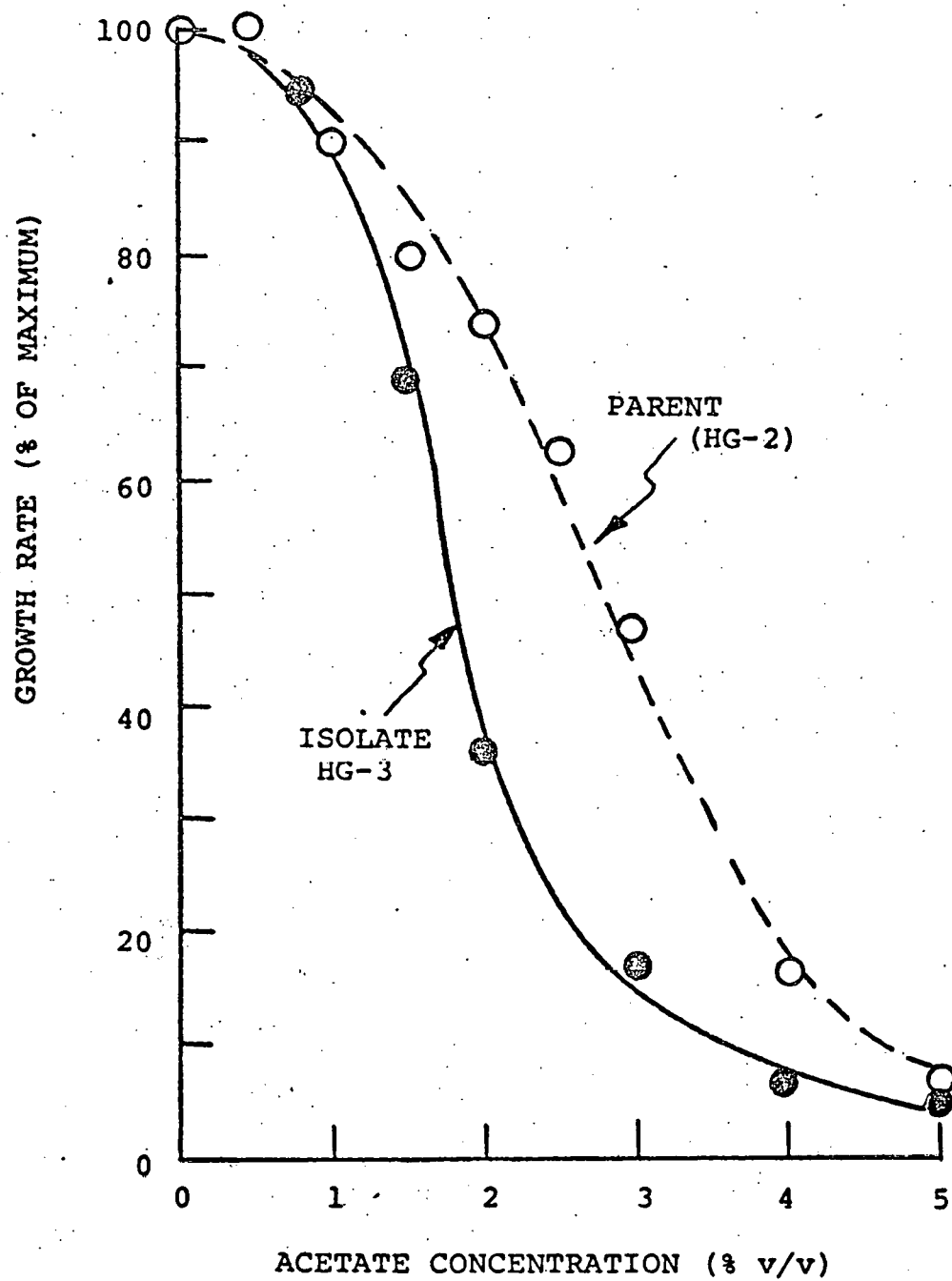


FIGURE I.C.4: ACETATE TOLERANCE OF PARENT
(HG-2) AND ISOLATE HG-3



tolerance to acetate than the new isolate HG-3. For example, 50% growth inhibition of the parental strain occurred at an acetate concentration of 2.9% (V/V), whereas the new isolate HG-3 exhibited the same growth inhibition at 1.8% (V/V) of acetate. At this moment, we do not have a logical explanation as to this observation. Further studies will be required to delineate the implication of such a behavior.

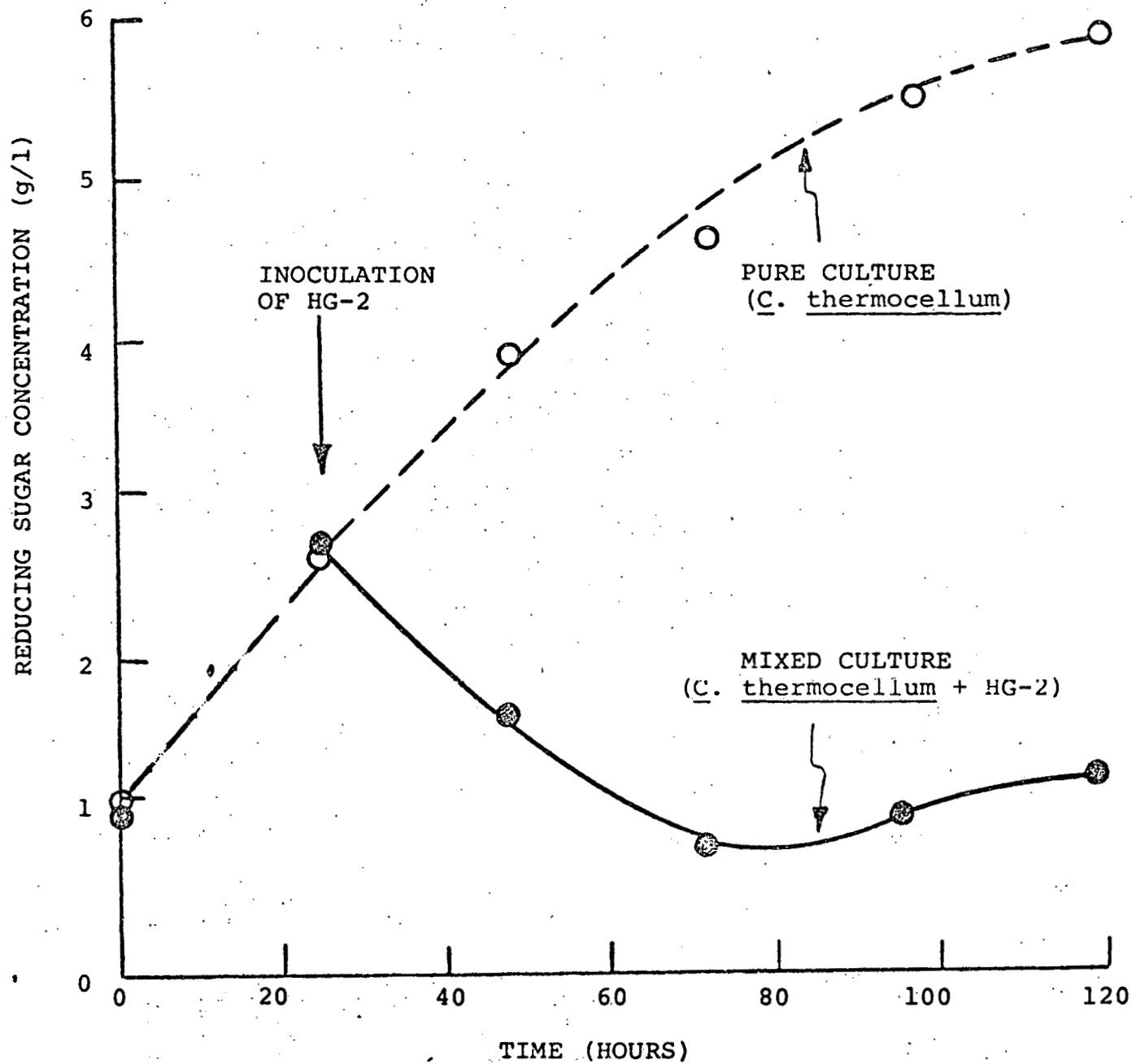
b. Studies on Corn Stover Degradation by Mixed Culture of Clostridium thermocellum and HG-2

Studies using mixed culture of C. thermocellum and HG-2 were performed to obtain preliminary indications as to their environmental compatibilities. It is our objective to pursue this approach in more detail should positive results be found. These preliminary findings will now be presented.

As mentioned in the Materials and Methods section in this report, Clostridium thermocellum (S-4) was allowed to grow in anaerobic shake flasks using corn stover as the carbon source. After 24 hours of growth, HG-2 was then inoculated. The behaviors of the pure culture (C. thermocellum) and mixed culture are shown in Figure I.C.5, I.C.6 and I.C.7.

In Figure I.C.5, the fermentation time profiles with respect to the formation of reducing sugars in pure and mixed culture are shown. It can be seen that the production of soluble reducing sugars by pure culture of C.

FIGURE I.C.5: REDUCING SUGAR ACCUMULATION FROM CORN STOVER BY PURE CULTURE OF C. thermocellum AND MIXED CULTURE OF C. thermocellum and HG-2



thermocellum continues to increase throughout the course of the fermentation. On the other hand, when HG-2 was inoculated, one observes a decrease in the reducing sugars as fermentation progresses. At the termination of the study, the residual reducing sugar in the mixed culture system was less than 1 g/l. These results indicate that the two organisms can maintain their biological activities in a compatible fashion.

During the course of these fermentations, concentrations of acetic acid and ethanol were also determined. Shown in Figure I.C.6 is the time profile of acetic acid formation for the pure and mixed culture systems. It can be seen that for C. thermocellum in pure culture, acetic acid formation reached a maximum value of 1 g/l at about 50 hours. However, for the mixed culture, the rate of acetic acid formation was significantly greater than that found for the pure culture. Furthermore, production of acetic acid continued up to 70 hours of fermentation attaining a final concentration of 3 g/l.

The behaviors on the production of ethanol by the pure and mixed culture are similar to that found for acetic acid. When C. thermocellum was grown as a pure culture, ethanol formation continues up to 100 hours of fermentation. When a mixed culture was used, the rate of ethanol formation as well as the final concentration attained were

FIGURE I.C.6: ACETIC ACID PRODUCTION FROM CORN STOVER BY
PURE CULTURE OF C. thermocellum AND MIXED
CULTURE OF C. thermocellum AND HG-2

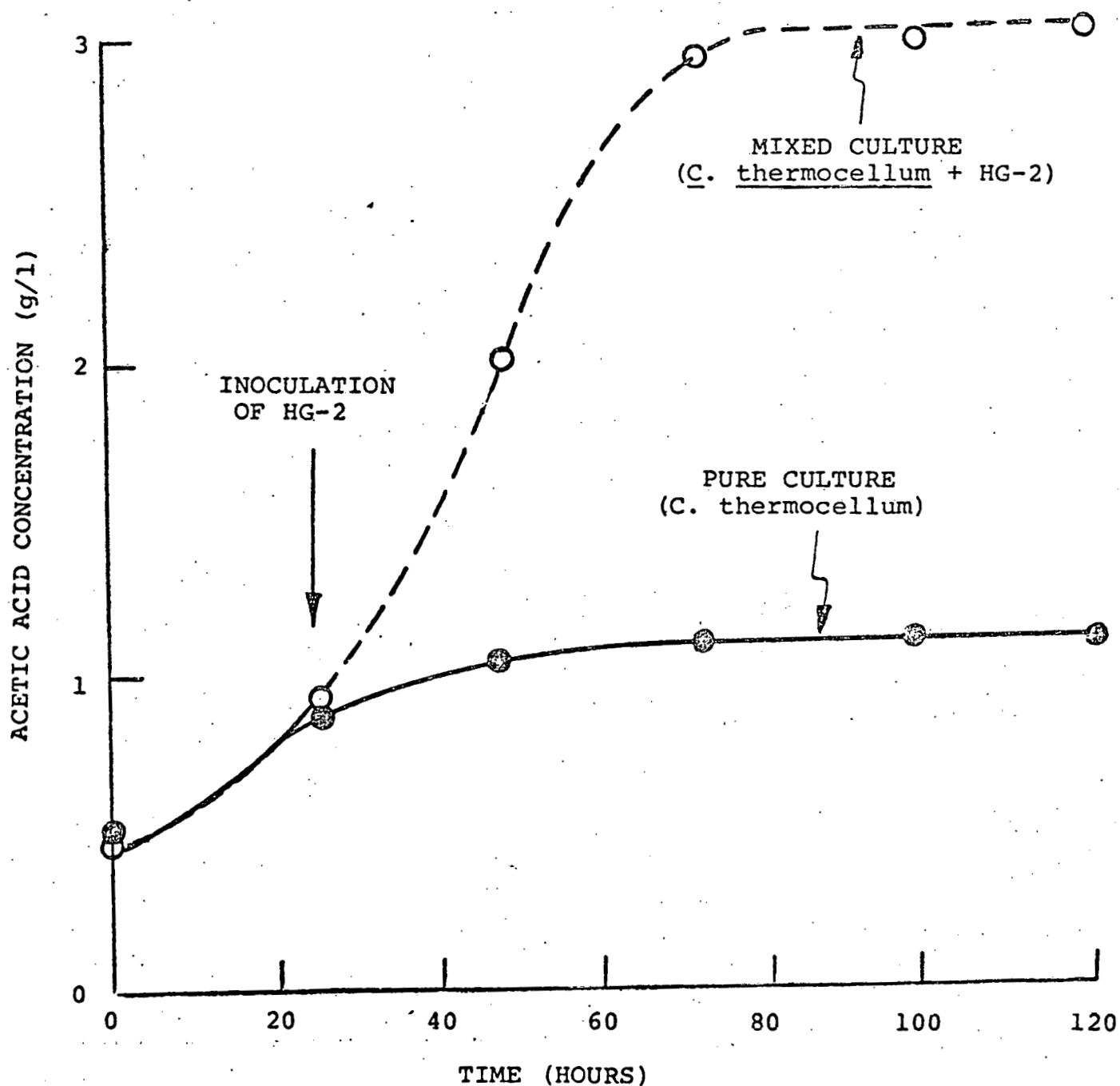
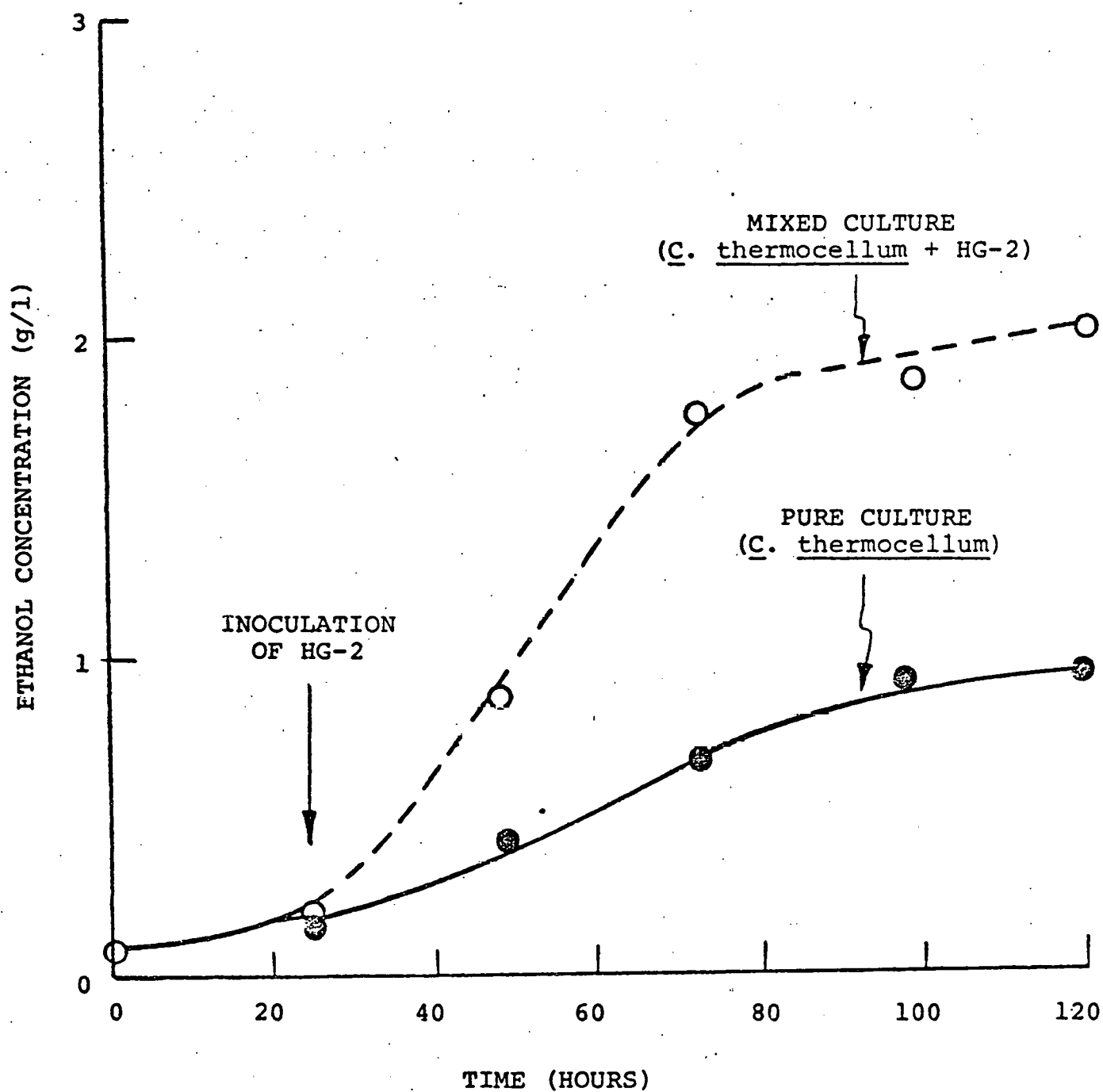


FIGURE I.C.7: ETHANOL PRODUCTION FROM CORN STOVER BY
PURE CULTURE OF C. thermocellum AND MIXED
CULTURE OF C. thermocellum AND HG-2



both significantly greater than that found in the pure culture system. In view of the results from the reducing sugar time profiles (Figure I.C.5), we conclude that HG-2 can actively metabolize the degradation products from corn stover as a result of the cellulolytic activities of C. thermocellum. These results are encouraging since they indicate no abnormal behaviors when a mixed culture of C. thermocellum and HG-2 are cultivated using a cellulosic biomass.

4. Future Work

Our future studies in this area consist of:

- Further adaptation, mutation and selection of HG-3 to increase its ethanol tolerance.
- A mutation program to effect direct catabolism of HG-3 to produce ethanol as the only product.
- Further exploration using mixed cultures to increase rate and product concentration.
- Determine product yields of HG-2 and mixed culture of C. thermocellum and HG-2.

D. GENETIC MANIPULATIONS

1. Search for Phages

More attempts were made to isolate plaque-forming units to test the transfectability of C. thermocellum, as described in the last progress report (No. 7). The filtrates used as inocula were obtained from two sources. One came from a digester operated by the Meat Animal Research Center, Nebraska. This digester is fed with cattle manure and operates at 55°C. The other sample came from a thermophilic digester in Illinois which utilizes cellulosic agricultural waste. Neither of these yielded plaque formers.

The possibility that a plaque former could be obtained from a lysogenic strain of C. thermocellum was examined also. Filtered supernatants from cultures of C. thermocellum obtained from different sources were cross-checked for plaque-forming ability against each of the strains by an agar spot test. Filtrates from cultures which had been disrupted with chloroform, as well as from cultures which had not, were used. No plaques were observed.

A test was made to determine if any of the strains could be induced to lyse by agents commonly known to induce lysogens in other bacteria. In one experiment a range of mitomycin C concentrations increasing by two-fold steps, between 1/16 to 16 µg/ml was added to growing cultures of each of the C. thermocellum strains. The optical density of these was followed until growth stopped (Figure I.D.1.1).

Figure I.D.1.1. Treatment of growing cultures of *C. thermocellum* strains with mitomycin C.

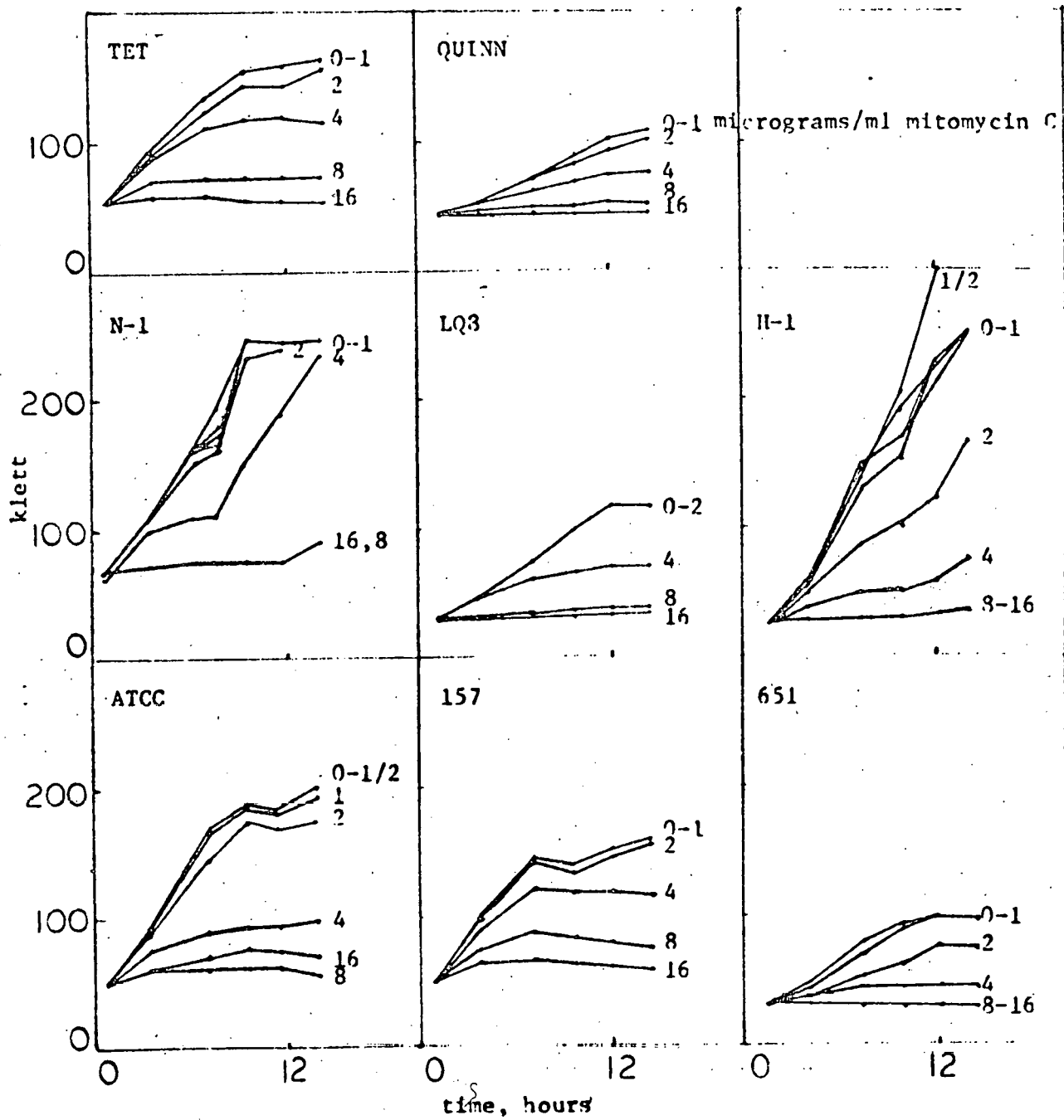
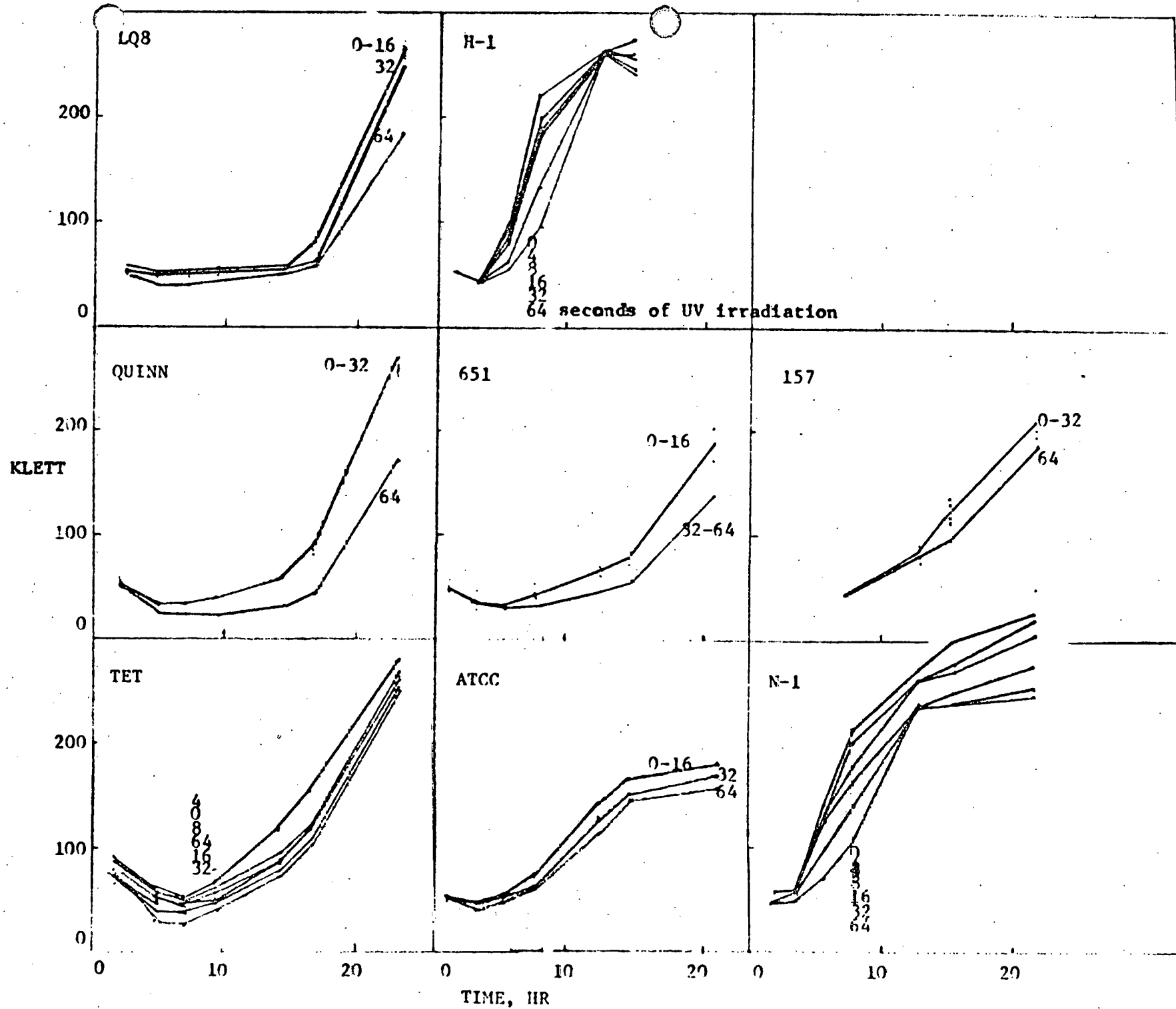


Figure I.D.1.2. Effect of U.V. radiation on the subsequent growth of C. thermocellum strains



In no case was lysis of the culture induced.

The same experiment was carried out using ultraviolet light to irradiate washed cells. The cells were kept in the dark after irradiation and used to inoculate tubes of fresh mediums. No dose given to any of the strains induced lysis (Figure I.D.1.2).

To determine if phage-induced lysis was occurring without gross cultural lysis, the supernatants of mitomycin-treated (4 µg/ml) and untreated C. thermocellum cultures were examined by electron microscopy. The cultures were cleared by low-speed centrifugation and then the supernatants were pelleted and washed by high-speed centrifugation. The pellets were suspended in a small volume of water and negatively stained for examination. While phage-like particles (70 nm icosahedrons) were observed, as has been observed in other occasions, no increase in their numbers resulted from mitomycin C treatment of the cultures.

2. Sensitivity to Antibiotics and Metal Salts

A qualitative examination of the sensitivity of each of the C. thermocellum strains on hand, to 65 metal salts and antibiotics, was made by placing impregnated discs on overlays of seeded agar. After incubation, the presence or absence of an inhibitory zone was noted. Qualitative differences in sensitivity, which could be used as markers or could be extra chromosomally determined are shown in Table I.D.2.1.

NATURAL RESISTANCE OF C. THERMOCELLUM

COMPOUND	ZONES OF INHIBITION (MM)					
	651	QUINN	TET	157	ATCC	
SODIUM SILICATE	0	0	0	0	0	
VANADYL SULFATE	9.9	9.6	8.3	10.8	8.5	
ZINC CHLORIDE	8.1	7.4	8.2	0	0	X
SODIUM ARSENATE	N.D.	0	17	0	16.5	X
ANTIMONY POTASSIUM TARTRATE	44	48	38.2	46.6	43	
LEAD NITRATE	0	0	0	0	0	
BERYLLIUM NITRATE	0	0	0	0	0	
FERROUS CHLORIDE	10.3	8	11.2	8.7	10	
FERRIC CHLORIDE	10.2	10.6	10.1	10.3	10.3	
COBALT CHLORIDE	11.2	0	0	0	0	X
NICKEL CHLORIDE	7.6	0	0	0	0	X
CUPROUS CHLORIDE	9.6	9	9.2	9.2	14.4	
CUPRIC CHLORIDE	13.7	14.2	13.6	14.1	8.6	
THALLIUM ACETATE	15.2	14.2	18	13.4	0	X
SODIUM TUNGSTATE	7.2	N.D.	8.4	N.D.	0	X
SULFANILAMIDE	0	0	0	8.4	0	X
KANAMYCIN	0	0	0	25.2	0	X
TRIMETHOPRIM	0	0	0	0	14	X
CERULENIN	0	0	0	0	38	X

ANOTHER 30 CPDS TESTED WITHOUT SIGNIFICANT DIFFERENCES.

3. Mating

Resistance factor transfer by mating on plates has been reported to occur between strains of C. perfringens (Brefort et al., Plasmid 1:52-66, 1977). The possibility that such a resistance could be transferred from a C. perfringens strain (CP600) used by Brefort and co-workers to any of our strains of C. thermocellum by inter-species mating was examined. Mating was carried out on plates at 52°C. Selection for anti-biotic resistance in C. thermocellum was done at 60°C. No evidence of resistance factor transfer to any C. thermocellum strain was obtained.

4. Future Work

- Effect of temperature on phage recovery from C. thermocellum.
- Initiation of construction of cloning vectors.

E. DEREGULATION OF CLOSTRIDIAL CELLULASE

1. Introduction

In our previous progress report (COO-4198-7), we presented several interesting results that required further investigation. One was that Clostridium thermocellum can grow and produce cellulase on glucose if elevated levels of yeast extract are used in the medium. In this report, we shall present the results of experiments in which a range of yeast extract concentrations were tested in both cellobiose and glucose containing media and growth and cellulase production were measured.

We have further studied growth and enzyme production on glucose. Glucose consumption, growth and cellulase production have been measured in media containing glucose, cellobiose, and mixtures of the two. Our objective is to determine if glucose plays a regulatory role in cellulase production.

Our attempts to devise a chemically defined medium will be described. Various substitutions for yeast extract were tested in cellobiose medium. These include mixtures of vitamins and amino acids and a protein hydrolysate.

Our cellulase-overproducing mutant, AS-39, described in earlier reports, was subjected to further mutation by physical and chemical agents. Two promising second generation mutants have been isolated and characterized.

2. Materials and Methods

a. Organism

C. thermocellum ATCC 27405 and its mutant AS-39 were used in this study.

b. Anaerobic Methods

See progress report COO-4198-7, page 71.

c. Cultural Conditions

The organisms were grown and maintained on GS medium which is described in progress report COO-4198-7, page 71. In the nutritional studies, yeast extract in the GS medium was replaced by various mixtures of vitamins, amino acids, and a protein hydrolysate. Growth was measured as absorbance using a red filter in a Klett instrument.

d. Analysis of Cellulolytic Activity

Cultures were centrifuged at 7850 x g for 10 minutes; cellulolytic activities of the supernatants were determined as follows:

1. TNP-CMCase Activity

One ml of supernatant is added to 2 ml of 1% TNP-CMC suspension in 0.1 M citrate buffer (pH 4.5). Zero-

time activity is determined by Millipore-filtering immediately after the addition of the supernatant. A control is also run in which the supernatant is replaced by 1 ml of the citrate buffer. After thorough mixing, the experimental tubes are incubated at 60°C for 1 hr. The contents are then Millipore filtered; absorbance of the filtrate is measured at 344 nm. The value at zero-time is subtracted from that at 60 minutes. Activity is expressed in units which are defined as follows:

$$1 \text{ unit} = 0.01 \text{ O.D.}_{344} \text{ increase/min.}$$

2. Avicel Hydrolyzing Activity

One ml of supernatant is added to 2 ml of 1% Avicel (Type RC-591, FMC Corp.) suspension in 0.1 M Na-citrate buffer (pH 4.5). Zero-time activity is determined by Millipore-filtering immediately after the addition of the supernatant. A control with 1 ml of buffer instead of supernatant is also run. After thorough mixing, the tubes are incubated in a boiling-water bath for 5 minutes, then cooled immediately; absorbance at 550 nm is read. The increase in absorbance from 0 to 2 hours is measured. The amount of reducing sugar liberated as glucose is read off a glucose standard curve. A unit of Avicel hydrolyzing activity is defined as follows:

$$1 \text{ unit} = 1 \text{ } \mu\text{mole glucose/min.}$$

3. Filter Paper Activity

Filter paper activity was determined using a modified method (Montenecourt et al., Biotechnol. Bioeng., XX, 297, 1978) which employs filter paper discs as the substrate. One ml of supernatant is added to an 18 mm test tube containing 0.5 ml of 0.05 M Na-citrate buffer (pH 4.8). The disc, which has a 1/2 inch diameter, weight of 46-48 mg and is 95% α -cellulose, is placed at the bottom of the tube; after vortexing, the tubes are incubated at 50°C for 1 hour. After incubation, 3 ml of DNS-reagent is added; the tubes are then incubated in boiling water for 5 minutes. At that point, 16 ml of distilled water is added to the tubes and absorbance at 550 nm is read. A zero-time sample is prepared in which the DNS-reagent is added immediately after the filter paper disc and boiled. A control tube without a filter paper disc is also run to correct for any reducing sugars present in the supernatant. The increase in absorbance between zero-time and 1 hour is determined. The amount of reducing sugar liberated as glucose is read off a glucose-standard curve prepared by the DNS-reagent method. A unit of filter paper hydrolyzing activity is described as follows:

1 unit = 1 μ mole glucose/min.

DNS-Reagent Method

To prepare the glucose standard curve, three ml of DNS-reagent is added to 1 ml of glucose solutions whose concentrations range from 0.1 - 1.0 mg/ml. The tubes are incubated in boiling water for 5 minutes. After cooling, absorbance at 550 nm is read.

3. Results and Discussion

A number of experiments were done to study the possibility of glucose repression of cellulase production in C. thermocellum AS-39. We used GS medium described in our previous progress report, replacing or varying the cellobiose content where indicated. The organism grew and produced cellulase in medium containing 1% glucose after a lag phase of 50 h (Figure I.E.1). Increasing the glucose or cellobiose concentration to 2% did not stimulate either growth or cellulase production. There was no inhibition of growth or cellulase production when 1% glucose was added to medium containing 1% cellobiose (Table I.E.1). When the specific production of cellulase was calculated, there was no significant difference between that produced on cellobiose, glucose or cellobiose plus glucose. It thus appears that glucose repression of cellulase production does not exist in this organism. These findings are important because the production of most cellulases is repressed

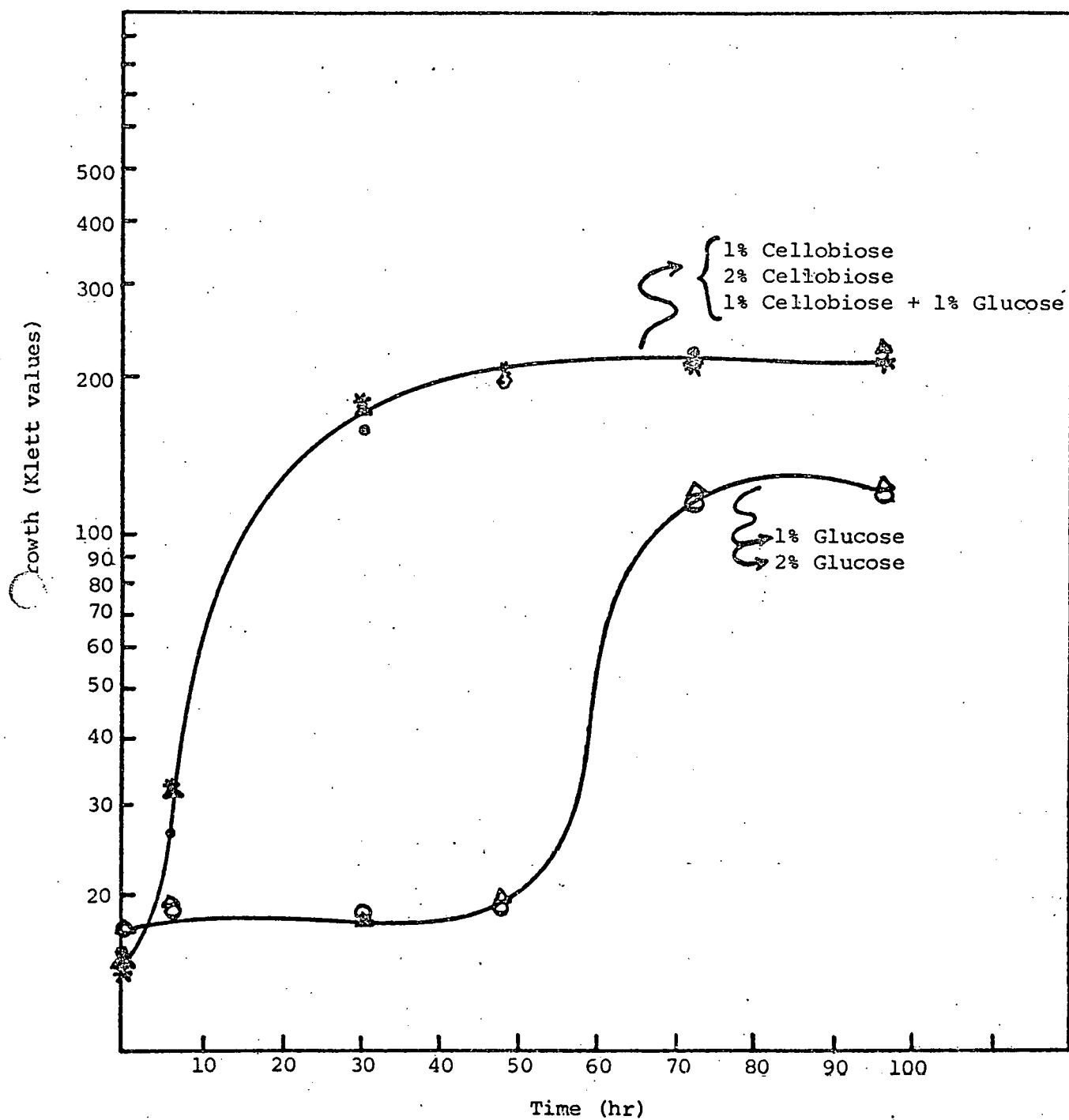


Fig. I.E.1. Growth of *Clostridium thermocellum* mutant AS-39 on cellobiose, glucose and mixtures of the two.

Table I.E.1. LACK OF GLUCOSE REPRESSION OF CELLULASE¹ IN AS-39

CELLOBIOSE (g/l)	GLUCOSE (g/l)	RESIDUAL SUGAR ² (g/l)	ENZYME PRODUCTION (units/ml sup.)	GROWTH (Klett units)	SPECIFIC ENZYME PRODUCTION [(u/ml·Ku) x 10 ³]
10	0	3.8	1.12	221	5.1
0	10	0.8	0.61	139	4.4
10	10	3.7	0.98	217	4.5
20	0	5.0	1.11	217	5.1
0	20	5.2	0.53	128	4.1

1) TNP-CMCase assay at 168 hr; 1 unit = increase in OD₃₄₄ of 0.01/min.

2) Reducing sugar as glucose at 200 hrs.

3) Maximum Klett values up to 168 hrs.

by the products of the enzymatic reaction. Such repression by glucose, for example, limits cellulase production in Trichoderma reesei.

Carbon source utilization was also studied in this experiment; as can be seen in Table I.E.1, 92% and 75% of the glucose was utilized in the 1% and 2% glucose media respectively. In the case of cellobiose media, reducing sugar utilization was 62% at 1% cellobiose and 75% at 2% cellobiose. In medium containing both glucose and cellobiose, each at 1%, there were no signs of inhibition of sugar utilization; approximately 80% utilization was observed.

We next examined the effect of various concentrations of yeast extract on glucose and cellobiose fermentations. Figure I.E.2 shows that C. thermocellum AS-39 failed to grow on cellobiose when the yeast extract concentration was 0.05%. Twenty and 40% maximum growth was observed when 0.1% and 0.2% yeast extract, respectively, were used. In the case of glucose, the organism failed to grow when 0.05% and 0.1% yeast extract were used. About 70% of maximum growth was observed when 0.2% yeast extract was used. Thus, our results show that the organism requires more yeast extract to grow on glucose than on cellobiose.

The effect of yeast extract concentration on cellulase production is shown in Table I.E.2. The highest volumetric titers were observed at the highest (0.6%) yeast extract

Fig. I.E.2. Growth of Clostridium thermocellum mutant AS-39 on cellobiose and glucose using different concentrations of yeast extract.

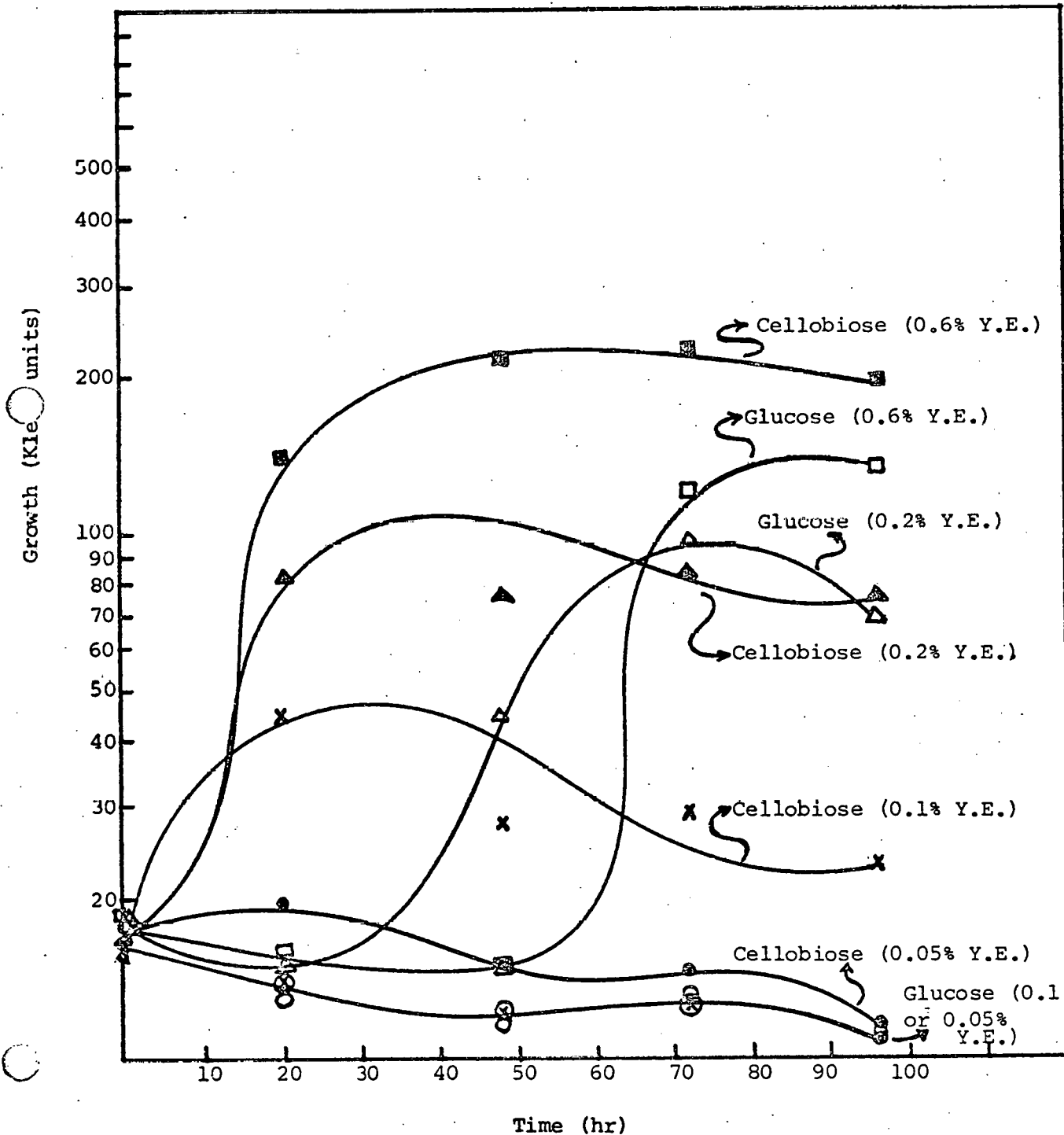


Table I.E.2. TNP-CMCase¹ PRODUCTION BY AS-39 IN CELLOBIOSE AND GLUCOSE

YEAST EXTRACT %	CELLOBIOSE (1%)			GLUCOSE (1%)		
	ENZYME ² PRODUCTION (units/ml sup.)	GROWTH ³ (KLETT UNITS)	SPECIFIC ENZYME PROD. (units/ml·Ku) x 10 ³	ENZYME ² PRODUCTION (units/ml sup.)	GROWTH ³ (KLETT UNITS)	SPECIFIC ENZYME PROD. [(u/ml·ku) x 10 ³
0	0	12	-	0	13	-
0.05	0	20	-	0	13	-
0.1	0.48	45	10.7	0	18	-
0.2	0.87	85	10.3	0.55	98	5.6
0.6	1.74	224	7.8	1.21	120	10.0

1) 1 unit = increase in OD₃₄₄ of 0.01/min.

2) Assays done after 72 hrs of growth.

3) Maximum Klett value up to 72 hrs.

concentrations in the cases of both glucose and cellobiose. Although volumetric titers were higher on cellobiose than on glucose, specific cellulase titers were similar.

To develop a chemically-defined medium, we have continued our nutritional studies testing various defined mixtures as yeast extract replacements. Other authors such as McBee, Quinn, and Alexander have individually reported that C. thermocellum can be grown successfully on yeast extract-free medium supplemented with vitamins alone or together with amino acids. We have tested these yeast extract replacements but have failed to obtain growth after successive transfers of the culture.

The results presented in Table I.E.3 show that, in our hands, McBee's mixture of 5 vitamins failed to support growth in the absence of yeast extract. Furthermore, neither Difco Vitamin-free Yeast Base (glucose NH_4 , 3 amino acids, no vitamins) nor Difco Yeast Nitrogen Base without Amino Acids (NH_4 , 9 vitamins), no amino acids) replaced yeast extract (Table I.E.4). Poor growth was observed with Quinn's mixture of amino acids and vitamins. The only supplement which gave fair growth was Alexander's mixture No. 2 which contains eight vitamins and no amino acids. Since Alexander has found a simpler mixture of 5 vitamins (Alexander's mixture No. 1) to support growth (personal communication), we used the 120 hr. growth in Alexander's mixture No. 2 to inoculate the experiment described in Table I.E.5.

TABLE I.E.3. Effect of replacement of yeast extract by McBee's vitamin mixture on growth of C. thermocellum AS-39

Supplement	Growth (Klett values)* at hour				
	24	48	72	96	120
None	0	0	0	1	0
Yeast extract	174	218	238	242	228
McBee Vitamin-mixture**	8	8	8	6	4

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

** Thiamine-HCl, 200; riboflavin, 200; calcium pantothenate, 200; pyridoxine, 200; biotin, 2µg/liter.

TABLE I.E.4. Effect of yeast extract replacements on the growth of C. thermocellum AS-39

Supplement***	Growth (Klett values)* at hour				
	24	48	72	96	120
None	11	11	12	7	7
Yeast extract	132	218	218	211	206
Difco Vitamin-free yeast-base	0	0	1	0	0
Difco Yeast-nitrogen base w/o amino acids	6	5	5	3	0
Quinn's mixture****	5	30	39	38	37
Alexander's mixture, No. 2*****	3	48	58	57	71**

* Initial absorbance of the culture medium was subtraced from the Klett values obtained at different times.

** Used as an inoculum for the next experiment (Table I.E.5).

*** All concentrations of vitamins are expressed as µg/liter and the concentrations of amino acids are expressed as mg/liter.

**** Quinn's mixture: Thiamine-HCl (200); riboflavin (200); calcium pantothenate (200); pyridoxine (200); biotin (2); folic acid (8); PABA (4); DL-phenylalanine (80); L-tyrosine (50); L-tryptophan (70); L-cystine (60) L-cysteine (70); and L-methionine (70).

***** Alexander's mixture No. 2: Pyridoxine (1000); niacin (1000); riboflavin (1000); biotin (10); folate (10); thiamine (1000); pantothenate (1000); and B₁₂ (10).

TABLE I.E.5. Effect of replacement of yeast extract by Alexander's vitamin mixture on the growth of C. thermocellum.

Supplement	Growth (Klett values)* at hour:				
	24	48	72	96	120
Yeast extract	214	245	235	220	218
Alexander's mixture No. 1***	26	37	40	59	67**

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

** Used as an inoculum for the next two experiments (Tables I.E.6 and I.E.7).

*** Pyridoxine, niacin and riboflavin (1 mg/liter) and biotin and folate at 10 µg/liter.

In Alexander's simple vitamin mixture No. 1, we obtained a low degree of growth (Table I.E.5). Again, the 120 hour growth was used as inoculum for the next experiment. Here we attempted to obtain growth again in Alexander's mixture No. 1, but in addition, we eliminated one vitamin at a time. Table I.E.6 shows that Alexander's mixture No. 1 failed to support growth as did all mixtures containing only 4 vitamins. Thus, sustained growth through multiple passages is not supported by Alexander's mixture of 5 vitamins.

We next returned to Alexander's mixture (No. 2) of eight vitamins which had given a small amount of growth in the experiment of Table I.E.4. As seen in Table I.E.7, we failed to obtain growth in this mixture. On the other hand, when 0.5% casein hydrolysate was added to the vitamins, considerable growth was observed.

In summary, the simplest medium which has supported growth of strain AS-39 is Alexander's mixture No. 1 of eight vitamins plus casein hydrolysate. Further work is underway to confirm this finding and simplify this mixture.

The cellulase-overproducing mutant, AS-39, was subjected to further mutation by ethylmethane sulfonate, nitrosoguanidine, and UV-radiation. Two promising candidates for increased cellulase production were isolated and characterized; they were designated AM-5 and CM-17-3.

TABLE I.E.6: Effects of replacement of yeast extract by Alexander's vitamin mixture on the growth of C. thermocellum.

Yeast Ex- tract	Supplement					Growth (Klett values)* at hour				
	Pyri- dox- ine	Nia- cin	Ribo- flavin	Bio- tin	Fo- late	24	48	72	96	120
-	-	-	-	-	-	0	0	0	0	0
+	-	-	-	-	-	162	171	247	241	235
-	-	+	+	+	+	0	0	0	0	0
-	+	-	+	+	+	0	0	0	0	1
-	+	+	-	+	+	1	0	1	0	2
-	+	+	+	-	+	2	0	0	0	1
-	+	+	+	+	+	3	2	0	1	3

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

TABLE I.E.7: Effect of replacement of yeast extract by Alexander's vitamin mixture and casein hydrolysate on the growth of C. thermocellum.

Supplement			Growth (Klett values*) at hour:			
Yeast extract	Alexander's mixture No. 2	Vitamin-free acid hydrolysate of casein	24	48	72	96
+	-	-	238	238	230	196
-	+	-	4	5	2	3
-	+	5 g/liter	9	68	115	137
-	+	10 g/liter	30	69	119	135
-	+	17.5 g/liter	9	65	116	137

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

** All vitamins were used at the concentrations mentioned in Table I.E.4.

AM-5 was obtained in the following manner. C. thermocellum AS-39 cells grown on CM-3 medium were UV-irradiated for 60 seconds (99% kill) and plated on CM-3 agar containing 2% Avicel and 0.5% cellobiose. After incubation for 8-12 days, 17 colonies showing large clear zones were transferred to Hungate tubes containing 10 ml of CM-3 medium with 0.5% cellobiose. After incubation for 48 hours, growth and TNP-CMCase activity were measured.

CM-17-3 was obtained by EMS mutagenesis followed by the screening of 65,000 colonies on agar plates containing 2% Avicel and 0.05% cellobiose. Thirty colonies were selected for tests in tubes on the basis of clear zone diameters. Of these, CM-17-3 proved to be the best cellulase producer.

Table I.E.8 shows that both CM-17-3 and AM-5 exhibit about 3 times as much TNP-CMCase activity as their parent, AS-39. The table also shows an increase in Avicel and filter paper hydrolyzing activity over AS-39.

4. Future Work

Future studies on this segment of research will include:

- Studying the ability of glucose to support cellulase production after repeated transfers in glucose medium in the absence of cellulose and cellobiose.

Table I.E.8.

CELLULASE PRODUCTION BY MUTANT CULTURES

CULTURE	GENERATION	TNP-CMC		AVICEL		FILTER PAPER	
		u/ml. sup. ¹	specific activity ²	u/ml. sup. ³	specific activity ²	u/ml. sup. ³	specific activity ²
ATCC 27405	1	0.22	1.1	0.011	0.055	0.012	0.060
AS-39	2	0.61	2.2	0.022	0.080	0.018	0.066
CM-17-3	3	1.6	6.1	0.035	0.14	0.031	0.12
AM-5	3	1.6	5.9	0.041	0.15	0.035	0.13

1) 1 unit = ΔOD of 0.01 per minute

2) [units/ml·Ku] $\times 10^3$

3) 1 unit = 1 μ -mole reducing sugars as glucose per minute

- Continued testing of various vitamins and amino acids to replace the yeast extract requirement.

- Continued mutagenesis and selection for cellulase-hyperproducing mutants.

F. PURIFICATION OF CELLULASE

1. Introduction

As a preface to genetic manipulations and reactor design, efforts are underway to isolate and purify the enzyme(s) responsible for cellulolytic activity in C. thermocellum.

2. Materials and Methods

a. Preparation of Crude Enzyme

A crude extract of clostridial cellulase was prepared as follows:

Cultures of C. thermocellum ATCC 27405 were grown in CM-4 containing 5% cellobiose. Incubation was at 60°C without shaking. After 48 hr, cells were chilled for 15 min and harvested by centrifugation at 18,000 xg for 15 min. The supernatant was decanted and centrifuged again. This clarified broth was filtered through an Amicon Pm-10 filter at ambient temperature. The filter cake was resuspended in 1/10 the original broth volume in 0.05 M sodium citrate pH 5.7, and stored frozen at -16°C. An enzyme so prepared is stable for at least two months.

b. Ammonium Sulfate Precipitation

The enzyme preparation was further concentrated by ammonium sulfate precipitation. The extract was made 80% saturated by addition of a cold, saturated solution, and incubated at 0°C for 2 hr. It was then spun at 18,000 xg for 15 min. The supernatant was discarded. The pellet was resuspended in 1.5 ml of citrate buffer.

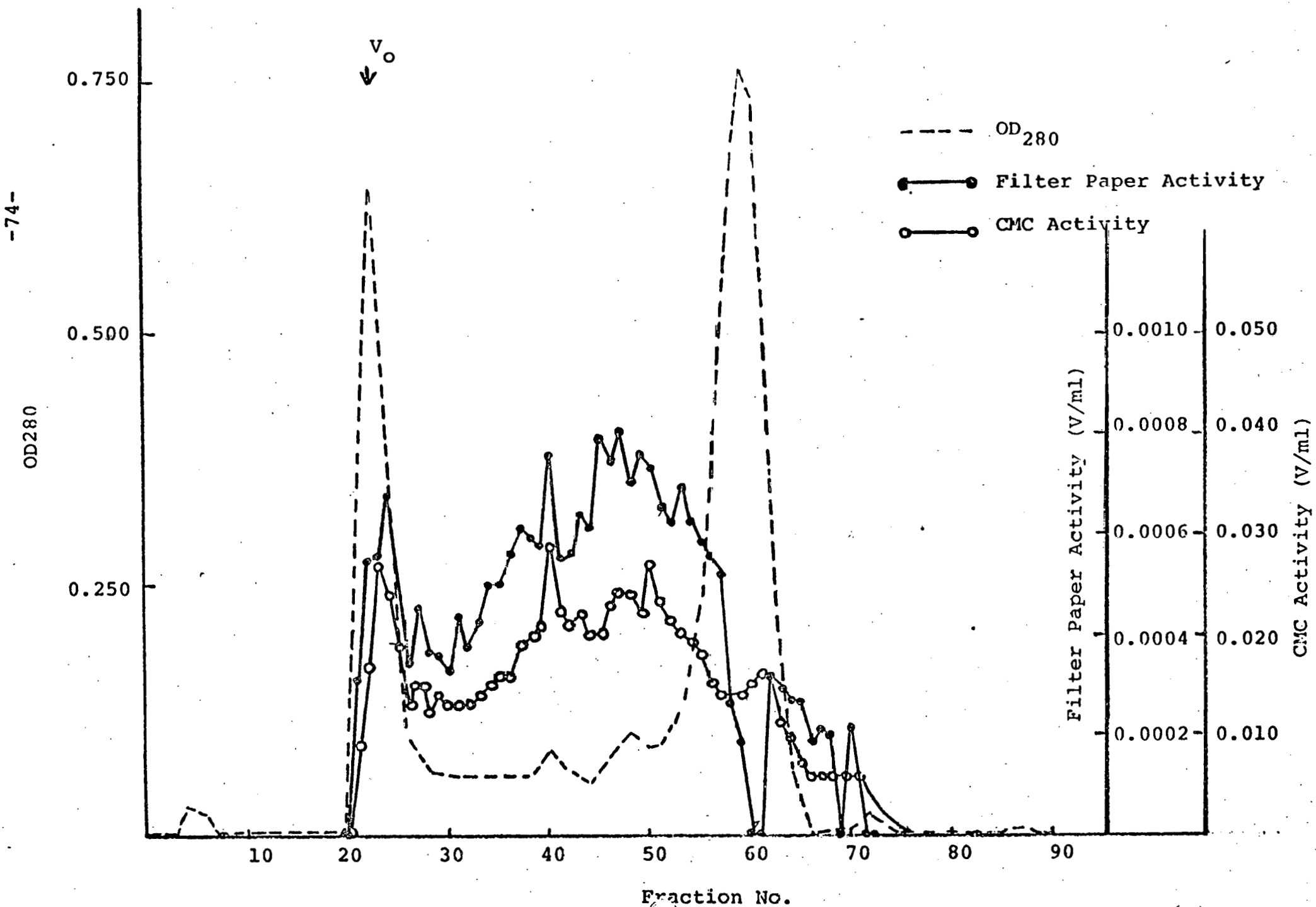
c. Gel Filtration

The ammonium sulfate precipitate was applied to a 2.5 x 35 cm column packed with Biogel A-5m, 100-200 mesh, at ambient temperature. This agarose gel is a molecular sieve of 5×10^6 exclusion limit; its effective operating range is $1 \times 10^4 - 5 \times 10^6$ daltons. The column has been previously equilibrated with 0.05 M Na citrate pH 5.7. The flow rate was maintained at 25 ml/hr. Fractions of 2.3 ml were collected. Absorbance at 280 nm, as well as filter paper and CMC activity in the column fractions, were monitored. Cellulase activity was measured as described previously (Progress Report 6/1 - 8/31/78, p. 85-86). Results are presented in Figure I.F.1.

. 3. Results and Discussion

No single discrete peak of either enzyme protein or enzyme activity was observed. Instead, there exists a large, heterogeneous population of enzyme complexes which ranges in size from approximately 10,000 to 5 million, or more, molecular weight. Activity profiles for CMC and filter paper are approximately identical, with some variation in the lower molecular weight region (higher fraction numbers). Activities overlap, but do not coincide with the large peak of OD280. These profiles indicate the presence of enzyme aggregates of a monomer of approximately 10,000 daltons. This preparation is substantially contaminated with nucleic acids, which comprise a large proportion of the major protein peak.

Figure I.F.1. Elution profiles of protein and cellulase activity through Biogel A-5m



Three separate enzyme pools, including fractions 20-34, 35-59, and 60-70, were pooled and reanalyzed for protein and nucleic acid content by the Warburg-Christian method, and for filter paper and CMC activity. Results are presented in the form of purification tables (Tables I.F.1 - I.F.3). It is evident that ammonium sulfate precipitation, followed by this chromatographic step provided some purification of the enzyme(s). It is however, of note that endo-and exo-glucanase activities, as represented by CMC and filter paper activity, respectively, are not concentrated to the same degree.

4. Future Work

Pooled fractions from this column will next be subjected to affinity chromatography on a CMC-Agarose column. Ideally, purification will proceed to near homogeneity, permitting further characterization studies. In the event of incomplete purification, other traditional enzymological approaches will be considered.

Table I.F.1 Protein and Nucleic Acid Analysis of Fractions

<u>Fraction</u>	<u>Procedure</u>	<u>Volume</u> (mg)	<u>Protein</u> (mg/ml)	<u>Total Protein</u> (mg)	<u>Protein Recovery</u> (%)	<u>Nucleic Acid Content</u> (%)
I	Crude Broth	344	NA*			NA*
II	Ultra-Filtration	34.4	0.54	19.	100	NA*
IV	Gel Filtration					
20-34		34.4	0.110	3.8	20.	5.5
35-59		57.5	0.077	4.4	23.	15.
60-70		25.3	0.038	0.96	5.0	37.

Protein and nucleic acid content as estimated by the Warburg and Christian Method.

*NA = Not assayed.

Table I.F.2 Purification of Filter Paper Activity

<u>Fraction</u>	<u>Procedure</u>	<u>Volume</u> (ml)	<u>Protein</u> (mg/ml)	<u>Activity</u> (U/ml)*	<u>Total Activity</u> (U)	<u>Specific Activity</u> (U/mg)	<u>Recovery</u> (%)	<u>Purification</u> (X)
I	Crude Broth	344	NA**	≤ 0.0005	-	-	-	1
II	Ultra-Filtration	34.4	0.54	0.00118	0.041	0.0022	100	1
IV	Gel Filtration							
20-34		34.5	0.110	0.00038	0.013	0.0035	32.	1.6
35-59		57.5	0.077	0.00056	0.032	0.0073	78.	3.3
60-70		25.3	0.038	0.00031	0.0078	0.0082	19.	3.7

* 1 Unit = Amount of enzyme producing 1 mMole glucose/hr at 60°C.

** NA = Not assayed.

Table I.F.3. Purification of CMC Activity

<u>Fraction</u>	<u>Procedure</u>	<u>Volume</u> (ml)	<u>Protein</u> (mg/ml)	<u>Activity</u> (U/ml)	<u>Total</u> <u>Activity</u> (U)	<u>Specific</u> <u>Activity</u> (U/mg)	<u>Recovery</u> (%)	<u>Purification</u> (X)
I	Crude Broth	344	NA*	≤0.005				1
II	Ultra- Filtration	34.4	0.54	0.0836	2.9	0.15		1
IV	Gel Filtration							
20-34		34.5	0.110	0.0120	0.41	0.11	14.	.73
35-59		57.5	0.077	0.0228	1.3	0.30	45.	2.0
60-70		25.3	0.038	0.0098	0.25	0.26	8.6	1.7

G. UTILIZATION OF CARBOHYDRATES BY C. THERMOCELLUM

1. Ability to Utilize Glucose

In order to establish if growth in glucose of C. thermocellum is due to the selection of pre-existing mutants having this ability or due to the whole population being able to do so, the following experiment was carried out. C. thermocellum grown to mid-exponential phase in CM-4 medium with cellobiose (6g/l) as the carbon source was submitted to a three-tube Most Probable Number (MPN) analysis. The MPN count was obtained in CM-4 with cellobiose (6g/l) as the carbon source and in CM-4 with glucose (6g/l) as the carbon source. The results are shown in Table I.G.1.1. The counts obtained in glucose were slightly lower than those obtained in cellobiose. These results demonstrated that a large proportion (>10%) of the cellobiose-grown culture is able to grow in glucose. This frequency is much too large for it to be due to spontaneous mutation. Thus, we conclude that wild type C. thermocellum ATCC 27405 is able to grow on glucose as the sole carbon source. This conclusion has to be viewed in light of the fact that our current strain is a single colony isolate from cellulose plates.

2. Effect of Carbohydrate Concentration on Cell Concentration And Yields

The effect of initial carbohydrate concentration on maximum cell concentration and apparent cell yields was determined. Apparent cell yield is defined as grams of cells per grams of initial carbohydrate concentration.

Table I.G.1.1 Three-tube MPN counts of C. thermocellum ATCC
27405 obtained in glucose and cellobiose.
Inoculum was grown in cellobiose.

<u>Sugar</u>	<u>MPN Count (cells/ml)</u>		
	¹ <u>Minimum</u>	<u>Mean</u>	¹ <u>Maximum</u>
Cellobiose	7.0×10^8	4.3×10^9	2.1×10^{10}
Glucose	1.5×10^8	9.3×10^8	3.8×10^9

¹Minima and maxima are the extremes of the 95%
condifence interval.

Stationary cultures grown in cellobiose (6g/l) for approximately 10 generations were transferred to CM-4 medium containing cellobiose, glucose or fructose at various concentrations. Optical density at 660 nm was followed until the maximum O.D was reached. This O.D value was converted to g cells dry weight/l from previous constructed standard curves, and plotted against the initial carbohydrate concentration (Fig. I.G.2.1). The results indicate that C. thermocellum under conditions where carbon is not limiting, is able to reach a higher cell concentration when grown in cellobiose than when grown in glucose at equivalent sugar concentrations. In addition, growth in fructose is not observed until the initial carbohydrate concentration is 6 g/l. At higher concentrations of fructose the final cell concentration continues to increase until it reaches a level equivalent to that obtained in cellobiose.

The same experiment was repeated with cultures which were grown on glucose prior to the experiment for at least 10 generations. The results are shown in Figure I.G.2.2. This time, although the maximum cell concentration in glucose was still lower than in cellobiose under conditions where carbon is not limiting, the maximum cell concentration in glucose was higher than that obtained with cellobiose-grown cells (Fig. I.G.2.1). Growth in fructose was similar for both cellobiose and glucose grown cells. However, in the latter, growth was observed at a lower fructose concentration. Table I.G.2.1 summarizes the results obtained in Figures I.G.2.1 and

Figure 1.G.2.1 Effect of carbohydrate concentration on maximum cell concentrations of cellobiose-grown cells of ATCC 27405.

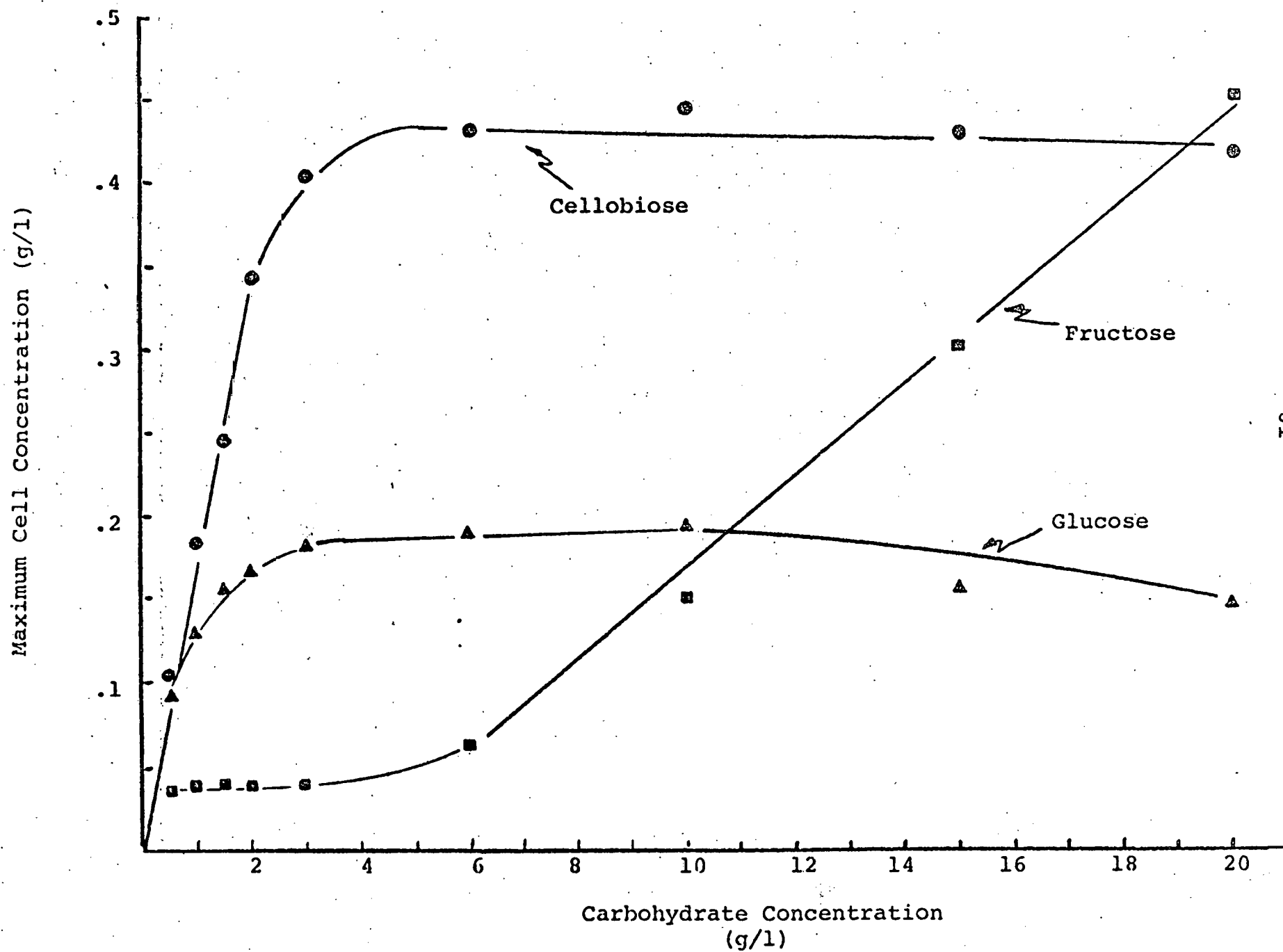


Figure I.G.2.2. Effect of carbohydrate concentration on maximum cell concentrations of glucose-grown cells of TCC 27405.

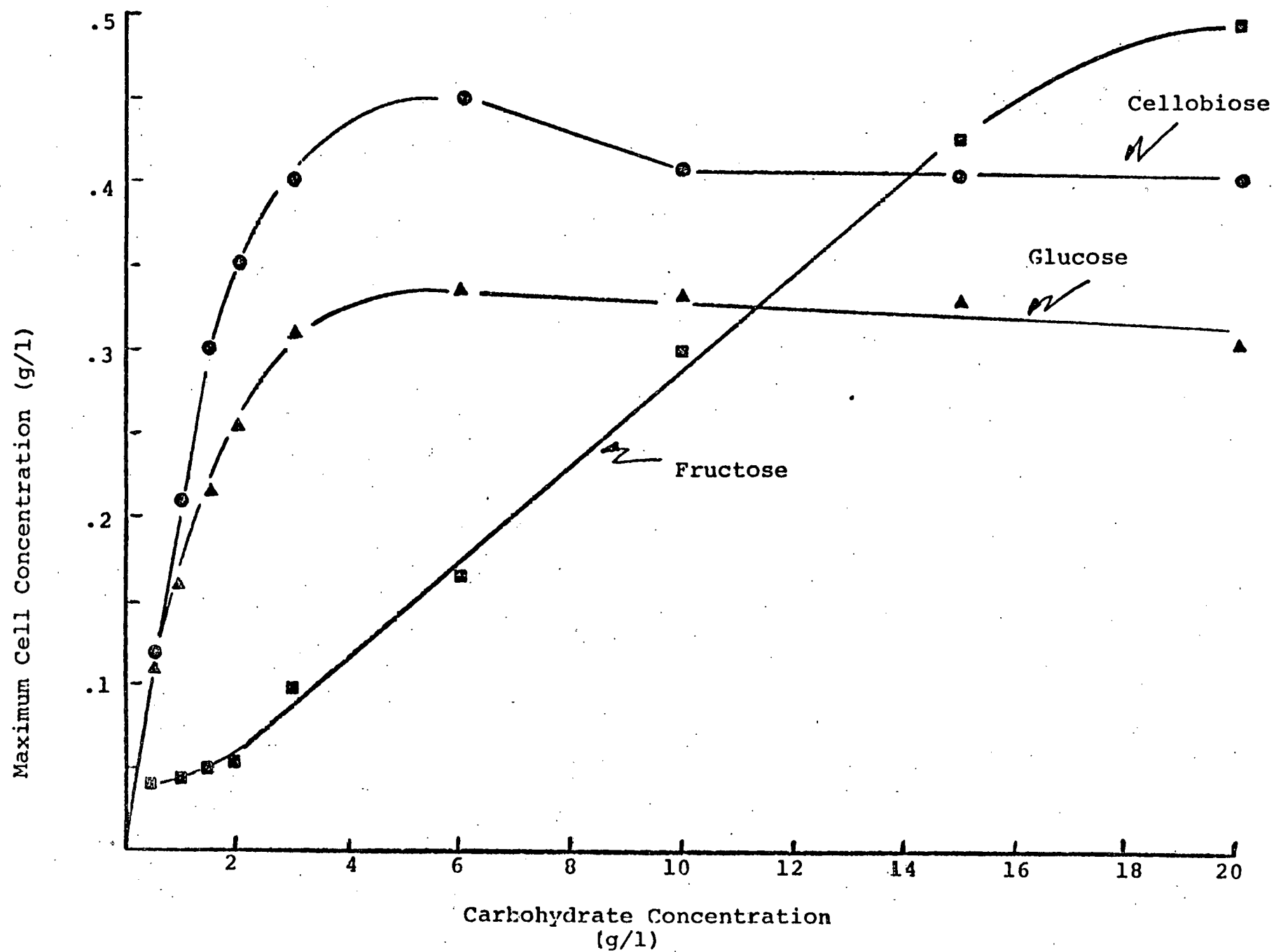


Table I.G.2.1. Apparent cell yields¹ obtained for C. thermocellum ATCC 27405 grown in various carbon sources.

Inocula were grown either in cellobiose or glucose at 6 g/l and then transferred to glucose, cellobiose or fructose at various concentrations.

<u>Inoculum</u>	<u>g Sugar</u>	<u>Cell Yields¹</u>		
	<u>1</u>	<u>Cb</u>	<u>Glu</u>	<u>Fru</u>
Cellobiose Grown	0.5	0.210	0.190	0.070
	1.0	0.185	0.130	0.040
	1.5	0.160	0.103	0.027
	2.0	0.170	0.080	0.018
	6.0	0.072	0.031	0.011
	20.0	0.021	0.008	0.023
Glucose Grown	0.5	.240	.230	.080
	1.0	.210	.160	.045
	1.5	.200	.143	.033
	2.0	.175	.128	.028
	6.0	.075	.056	.028
	20.0	.021	.015	.025

¹ Cell yields are expressed as g of cells (dry weight) per g of initial sugar.

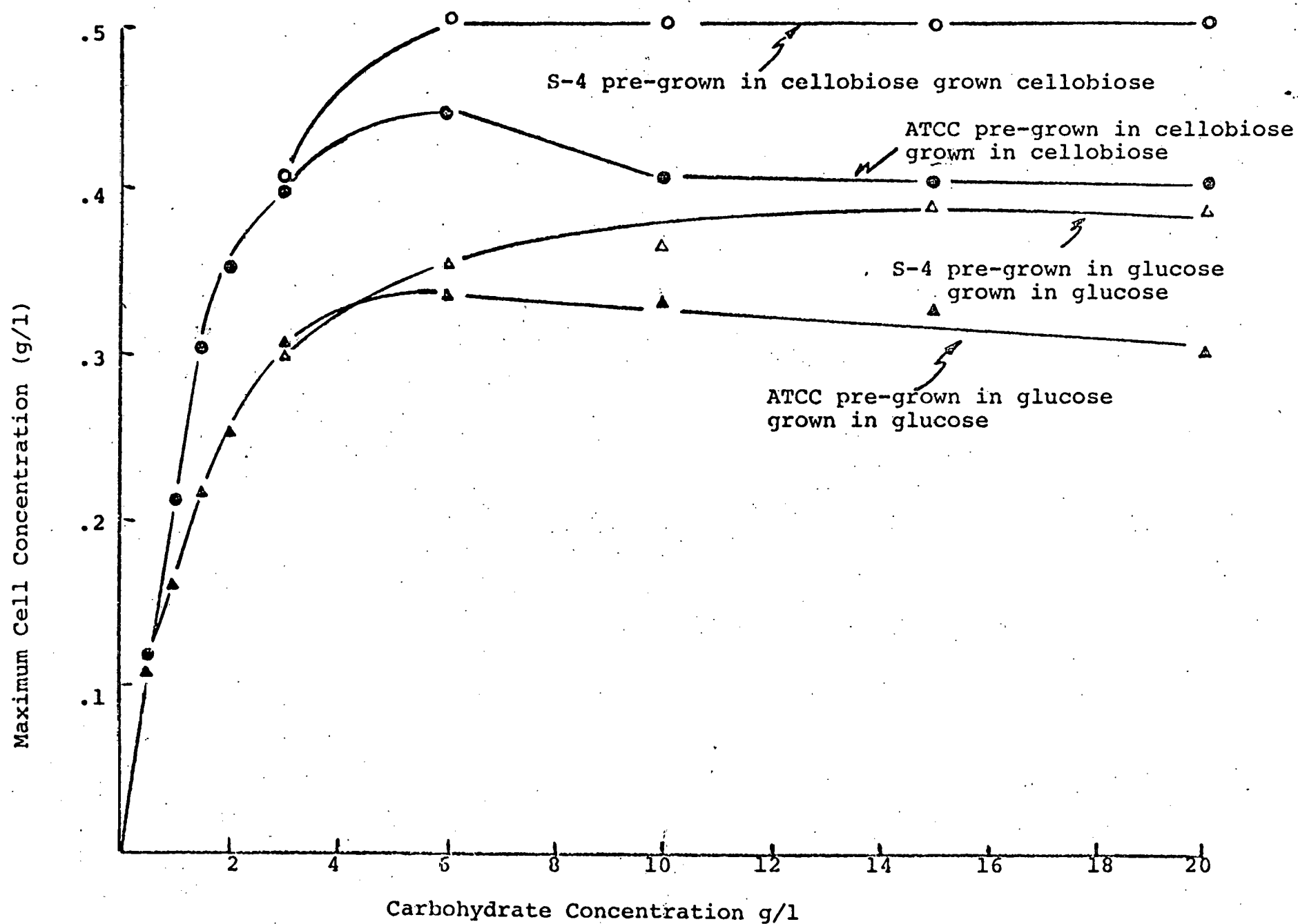
The ethanol resistant strain (S-4) was compared to ATCC 27405 (Figure I.G.2.3). The results demonstrated that S-4 can reach higher cell concentrations in both glucose and cellobiose. However, as with ATCC 27405, with strain S-4 the maximum attainable cell concentration in cellobiose is greater than that obtained in glucose.

There are essentially two reasons why apparent cell yields may change depending on carbon source, one is dependent on the manner in which the sugars are transported into the cell and the other is dependent on final catabolic products obtained. The data obtained so far does not permit us to differentiate between these two alternatives. However, in view of the large differences obtained and previously conducted end product analyses it is difficult to build a hypothesis based on differences in catabolic products. At this time we would like to propose that the differences observed are due to differences in the way in which C. thermocellum transports its sugars.

3. Effect of Yeast Extract on Carbohydrate Utilization

The current literature dealing with the utilization of glucose by C. thermocellum alludes to the fact that this microorganism will only grow in glucose at high yeast extract concentrations. We have tested this claim with our strain. C. thermocellum ATCC 27405 was grown in CM-4 medium containing cellobiose or glucose at 6 g/l and varying concentrations of yeast extract. Optical density was followed until a maximum

Figure I.G.2.3 Comparison of ATCC 27405 and S-4 according to their growth on cellobiose and glucose



was obtained. The results obtained are shown in Figure I.G.3.1. From this graph ratios of maximum cell concentration in cellobiose to glucose, at various yeast extract concentrations were calculated and are shown in Table I.G.3.1. The results indicate that whenever growth in cellobiose occurred it also was seen in glucose. However, it appears that yeast extract does have a stimulatory effect on the growth of C. thermocellum on glucose compared to cellobiose.

4. Future Work

- To determine true cell yields (g cells/g of carbohydrate utilized) of C. thermocellum grown in glucose, cellobiose and fructose.
- To determine the dependence of the distribution of fermentation end products on type of substrate.
- To initiate studies to investigate the mode of transport of glucose, fructose and cellobiose by C. thermocellum.

Figure I.G.3.1 Effect of yeast extract concentration on the growth of C. thermocellum ATCC 27405.

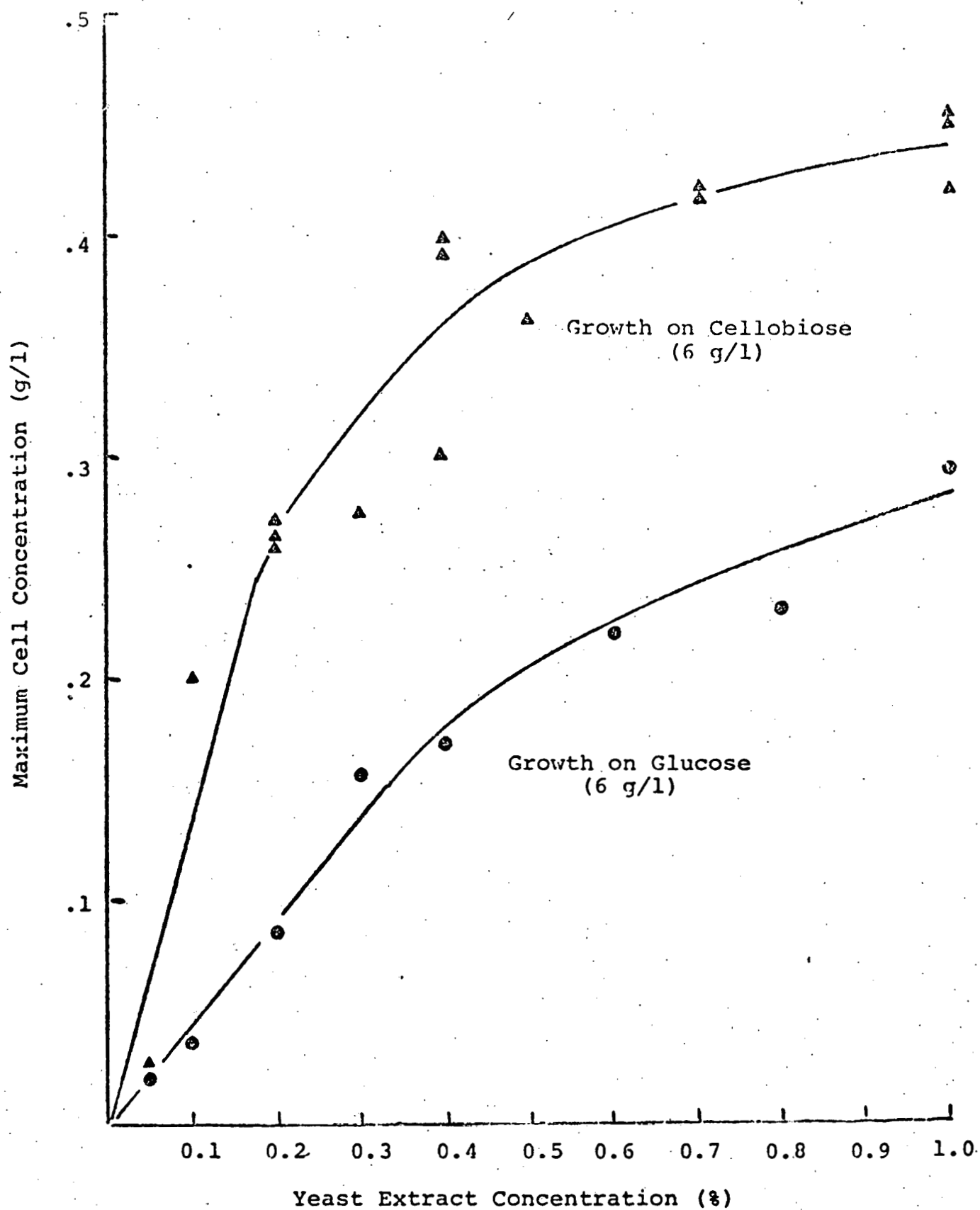


Table I.G.3.1. Ratios of maximum cell concentrations obtained in cellobiose (6 g/l) and glucose (6 g/l) at various yeast extract concentrations.

<u>[YE]%</u>	<u>Cb/Glu</u>
0.05	3.25
0.1	3.00
0.2	3.18
0.3	2.40
0.4	2.03
0.5	1.88
0.6	1.79
0.7	1.71
0.8	1.63
0.9	1.59
1.0	1.53

II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. PRODUCTION OF ACRYLIC ACID BY FERMENTATION

1. Introduction

Four aspects of acrylic acid production were investigated during this recent period of research. They were: (1) determination of the optimum conditions for conversion of propionic acid to acrylic acid with cell suspensions of Clostridium propionicum; (2) examination of the role of α -alanine and lactate on acrylic acid accumulation from propionic acid; (3) examination of cell immobilization procedures for production of acrylic acid; and (4) conversion of cellulose to lactic acid as an intermediate in acrylate production.

2. Materials and Methods

Experimental procedures for work with Clostridium propionicum were the same as previously reported. Modifications in procedures are described in figure legends or tables. The following is an update of the methods used in pure and mixed culture studies devoted to lactate production from cellulose:

a. Microorganisms

1. Clostridium thermocellum ATCC 27405 and Bacillus coagulans, a thermophilic, facultative homolactic bacterium.

b. Culture Media

The basic medium used for growth and inoculum preparation of B. coagulans was the MIXED MEDIUM described previously (COO-4198-6).

For sporulation experiments, done for identification purposes, the media used were:

1. Duncan and Strong modified medium.

<u>Ingredient</u>	<u>Concentration (gm/l)</u>
Yeast Extract	4.0
Trypticase	15.0
Soluble Starch	10.0
Sodium thioglycollate	1.0
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	10.0
Agar	20.0

2. Bacto-Tam agar (modified)

<u>Ingredient</u>	<u>Concentration (gm/l)</u>
Yeast Extract	5.0
Trypticase	5.0
Dextrose	2.0
K_2HPO_4	0.5
MnSO_4	0.2
Agar	20.0

3. Mixed-Medium agar

The composition of this medium has been previously described (see page 166, report COO-4198-7).

c. Biochemical Tests

For identification purposes, we used commercial kits with micro tests for metabolic products. These kits are manufactured by BBL Products under the trademarks of BBL MINITEK:

1. Procedure for differentiation of Enterobacteria
2. Procedure for differentiation of Anaerobes

3. Results

a. Determination of the Optimum Conditions for the Conversion of Propionic Acid as Acrylic Acid with Cell Suspensions of Clostridium propionicum

Parameters examined for their influence on the oxidation or dehydrogenation of propionic acid to acrylic acid included: temperature, pH, methylene blue concentration, and growth phase of cells used in the experimental system.

1. Effect of Methylene Blue

Determination of the role of methylene blue represents a continuation of experiments reported on in the last progress report. The reaction mixture was contained in a total volume of 2 ml, 50 mM triethanol-amine HCl buffer (pH 8.5), 200 mM propionate, 20 mM lactate and concentrations of methylene

blue that varied from 0 to 0.5%. Samples were incubated at 37°C in air with mechanical agitation. 200 μ l samples were collected at the times indicated in Figure II.A.1 and analyzed for acrylic acid as described in previous progress reports. Highest concentrations of acrylic acid were detected in the samples containing 0.2% methylene blue. However, initial rate of production was maximal at 0.4% methylene blue (Figure II.A.2).

2. Effect of Temperature

Samples containing standardized reaction mixture, 0.2% methylene blue and 20 mM α -alanine (experiments to be described later show that 20 mM α -alanine is optimal) were incubated at different temperatures. The cell concentration corresponded to 2.8 mg protein/ml of reaction volume.

The samples were incubated at temperatures between 10° and 60°C and assayed for acrylic acid after 4 hr of incubation. As seen in Figure II.A.3, the optimal temperature for dehydrogenation of propionate to acrylate is between 30 to 35°C. The experiment was repeated with a higher cell concentration (5.19 mg protein/ml), and the results indicated that 32°C is the optimal temperature (Figure II.A.4). Note that, under these conditions, the final concentration of acrylic acid was 41 mM. The resulting specific productivity is 2.0 mM acrylic acid/mg protein/ml per hr.

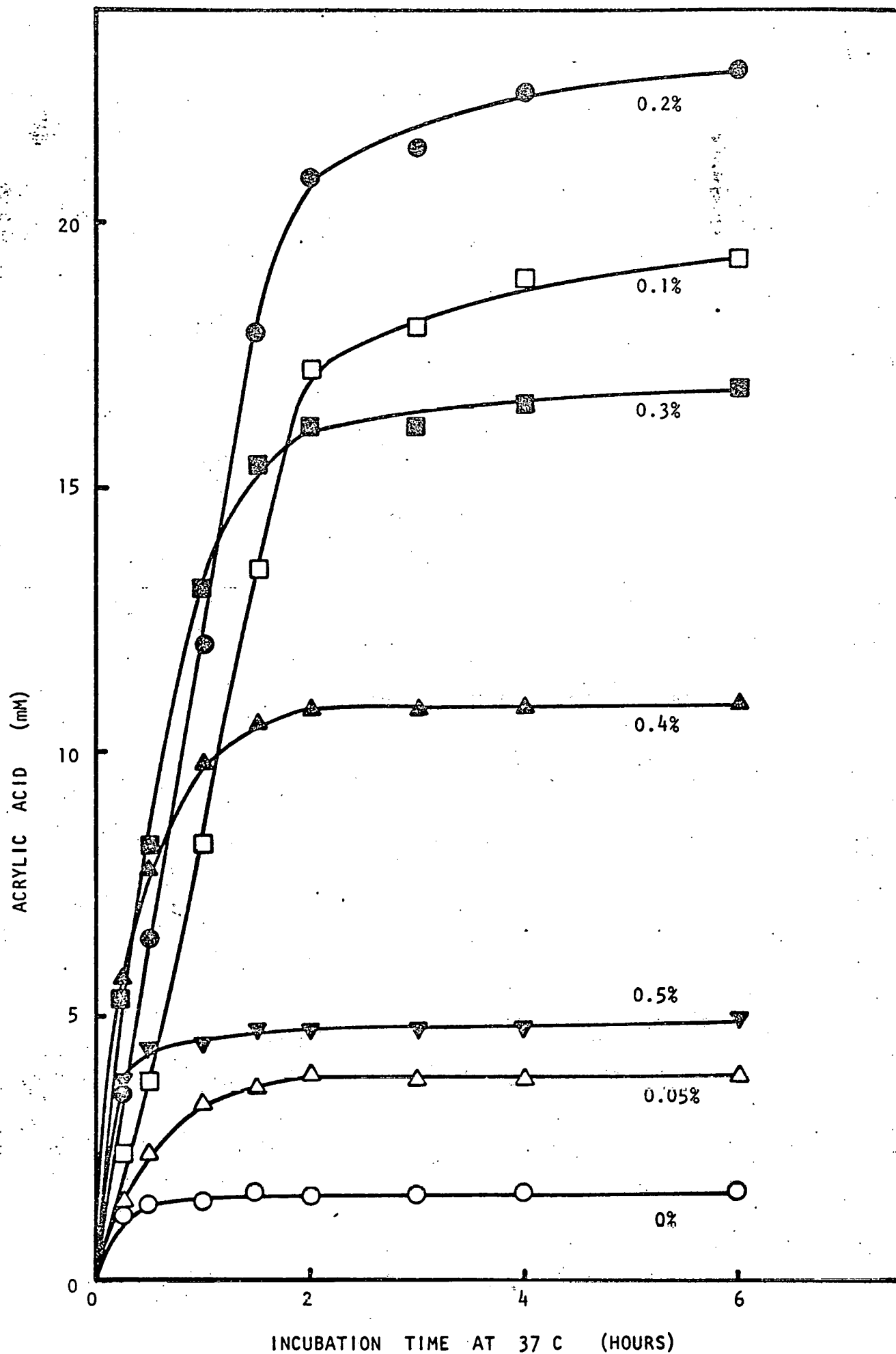


FIGURE II.A.1: EFFECT OF METHYLENE BLUE CONCENTRATION ON ACRYLIC ACID PRODUCTION

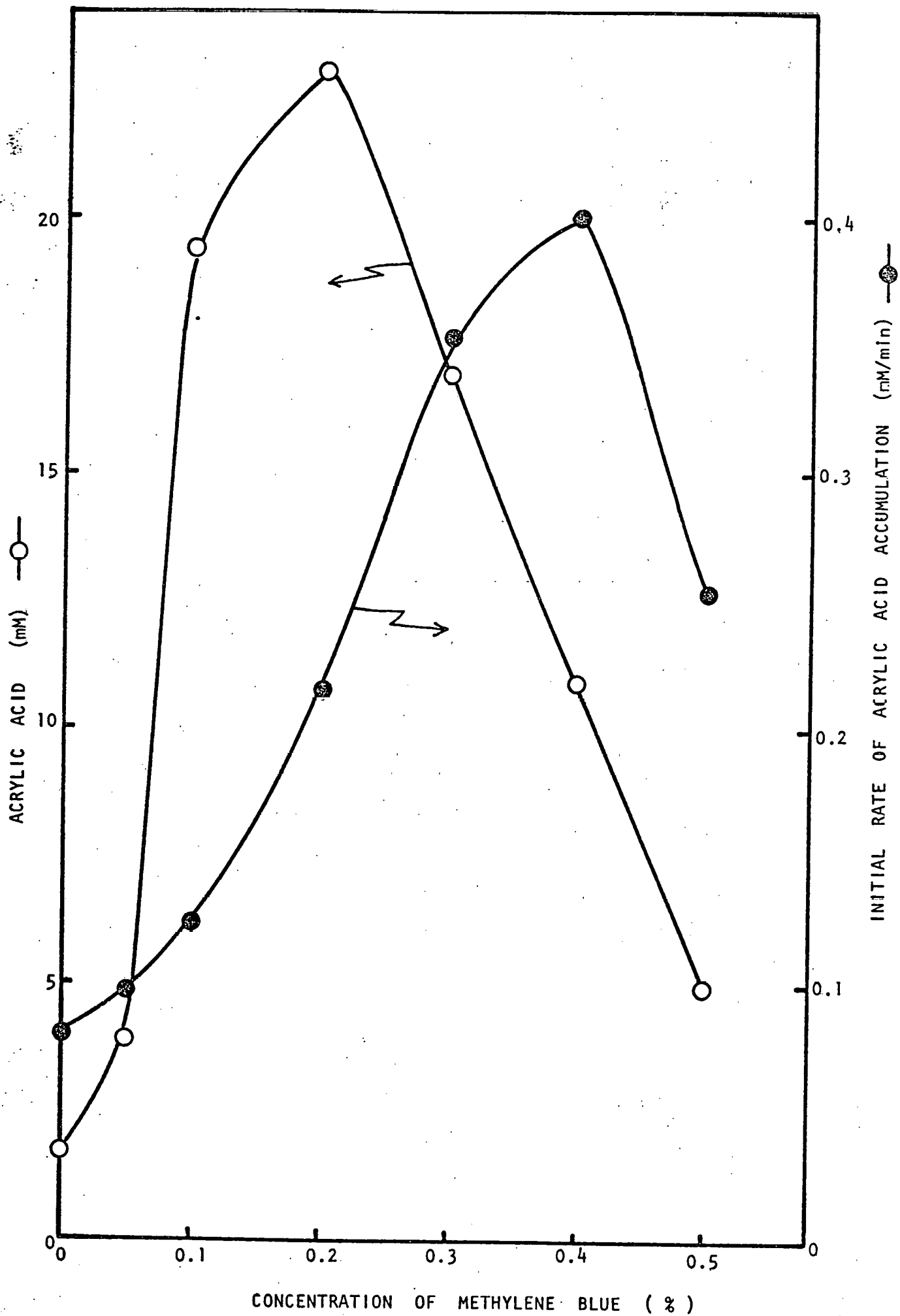


FIGURE II.A.2: EFFECT OF METHYLENE BLUE ON ACRYLIC ACID PRODUCTION

FIGURE II.A.3: EFFECT OF TEMPERATURE ON THE CONVERSION
OF PROPIONIC ACID TO ACRYLIC ACID

- Reaction Mixture (total volume 500 μ l) contained:

triethanolamine-Hcl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
α -alanine	20 mM
cellular protein	2.87 mg/ml

- Incubated under air atmosphere with mechanical agitation for 4 hours in either water bath or constant temperature room.

FIGURE II.A.3

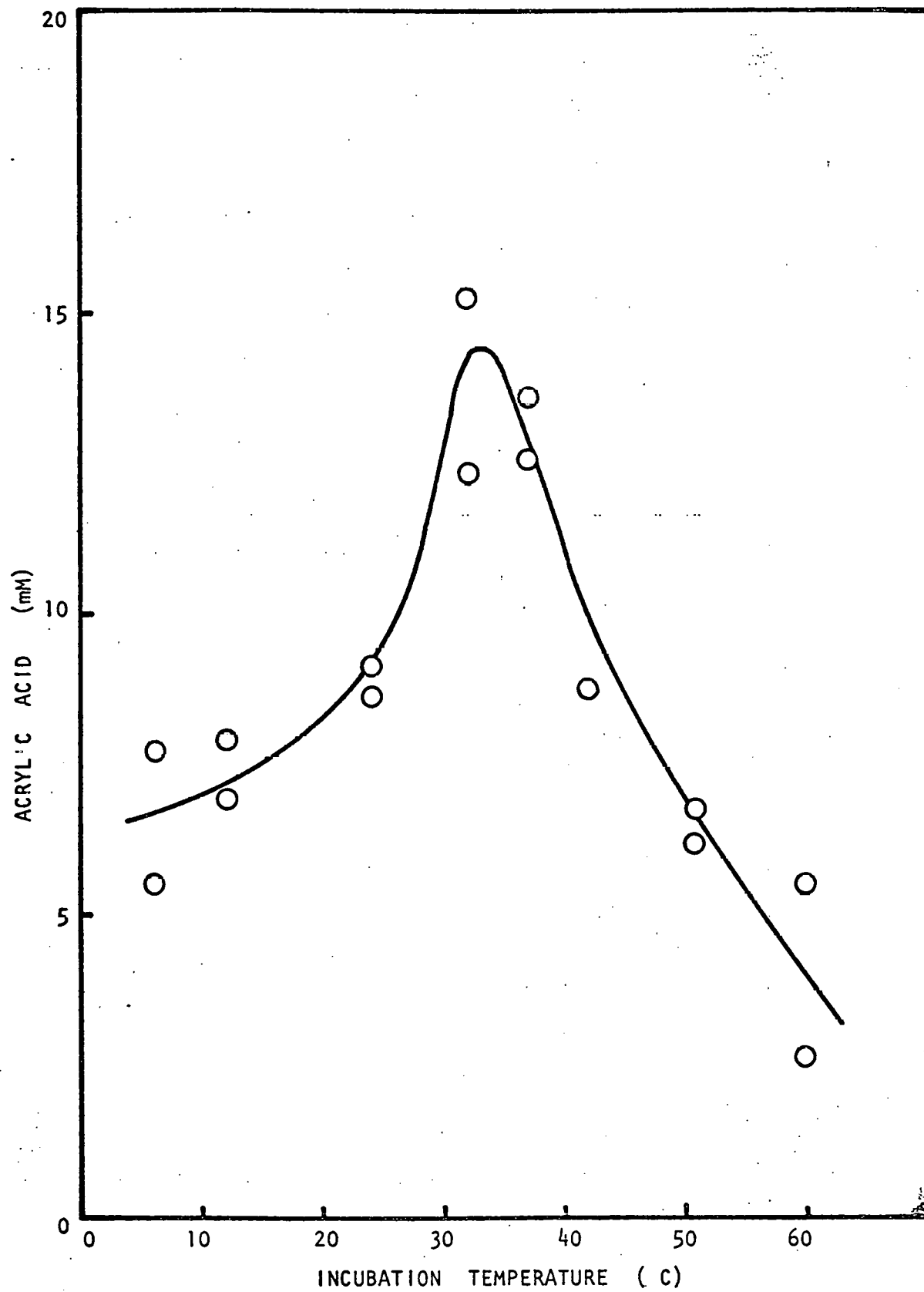


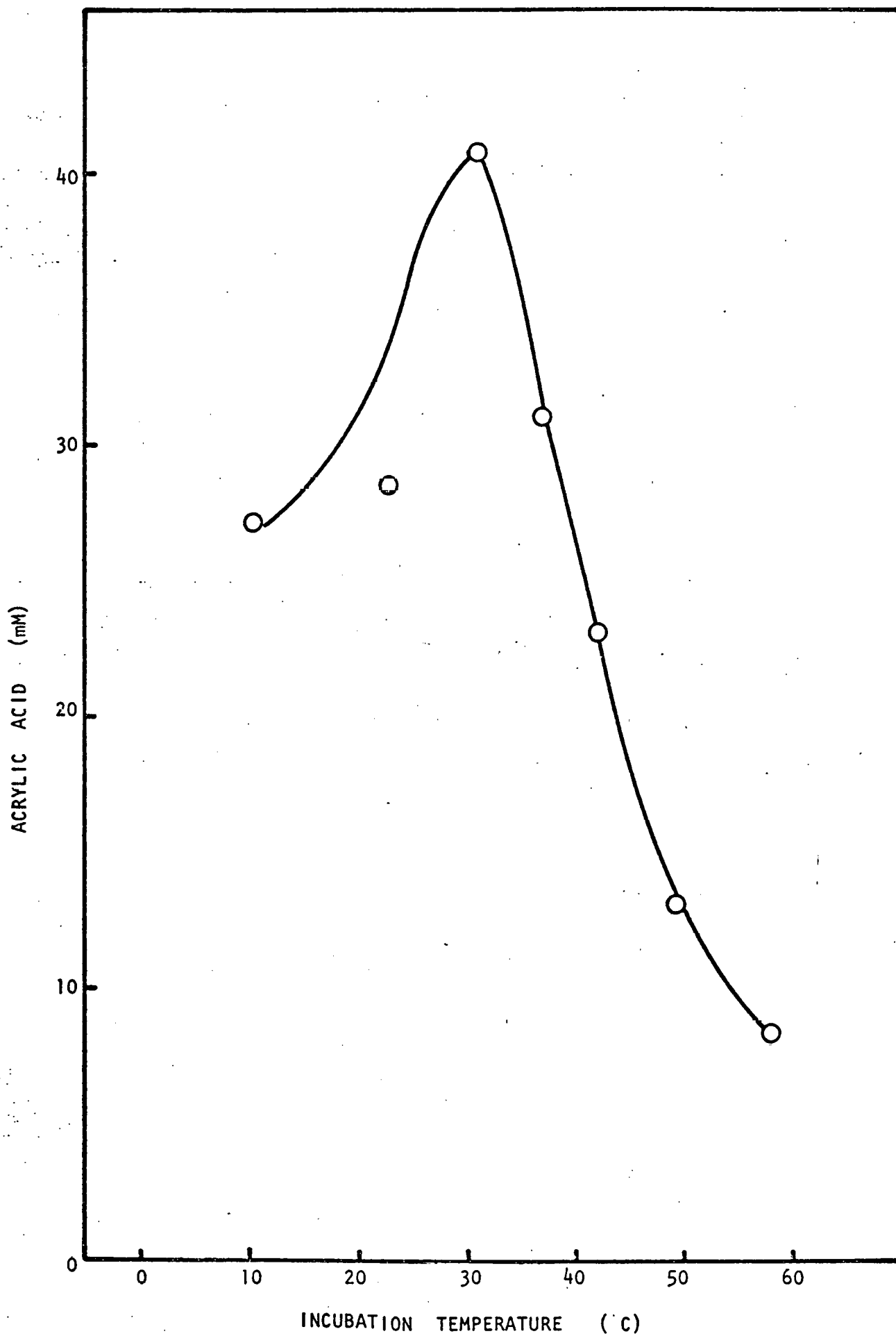
FIGURE II.A.4: EFFECT OF INCUBATION TEMPERATURE ON THE CONVERSION OF PROPIONIC ACID TO ACRYLIC ACID

- Reaction mixture (total 500 μ l) contained:

triethanolamine-HCl buffer	50 mM
propionate	200 mM
methylene blue	0.2 %
α -alanine	20 mM
cellular proteins	5.19 mg/ml

- Incubated under air atmosphere with mechanical agitation for 4 hours in either water bath or constant temperature room.

-98-
FIGURE II.A.4



Since all other previous experiments were conducted at 37°C, it can be assumed that the efficacy of the system was underestimated. For example, the final concentration of acrylic acid was 30% greater at 32°C (41 mM) than at 37°C (31 mM).

3. Conversion of Propionic Acid to Acrylic Acid as A Function of Cell Concentration

The effect of cell concentration on final level of acrylic acid from 200 mM propionate was determined at 37°C; protein concentrations were used as a measure of cell concentration. As summarized in Figure II.A.5, the final concentration of acrylic acid was proportional to cell concentration. It also appears from Figure II.A.5 that cell concentration is the limiting reactant in the system presently being characterized.

4. Effect of Growth Phase

Clostridium propionicum cells were harvested at different times during the growth phase. Results are summarized in Figure II.A.6 and Figure II.A.7. This data confirms earlier observations that cells harvested shortly before the onset of the stationary phase produced the most acrylate from propionate.

The optimal conditions for propionate dehydrogenation to acrylate appear to be as follows:

FIGURE II.A.5: CONVERSION OF PROPIONIC ACID TO ACRYLIC ACID
AS A FUNCTION OF CELL CONCENTRATION

- Reaction mixture (total 500 μ l) contained:

triethanolamine-Hcl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
α -alanine	20 mM

- Incubated at 37°C under air atmosphere with mechanical agitation for 4 hours.
- Cell concentrations were expressed as total cellular proteins per ml. Proteins were assayed by modified Lowry method with Bovine Serum Albumin (BSA) as standard.

FIGURE II.A.5

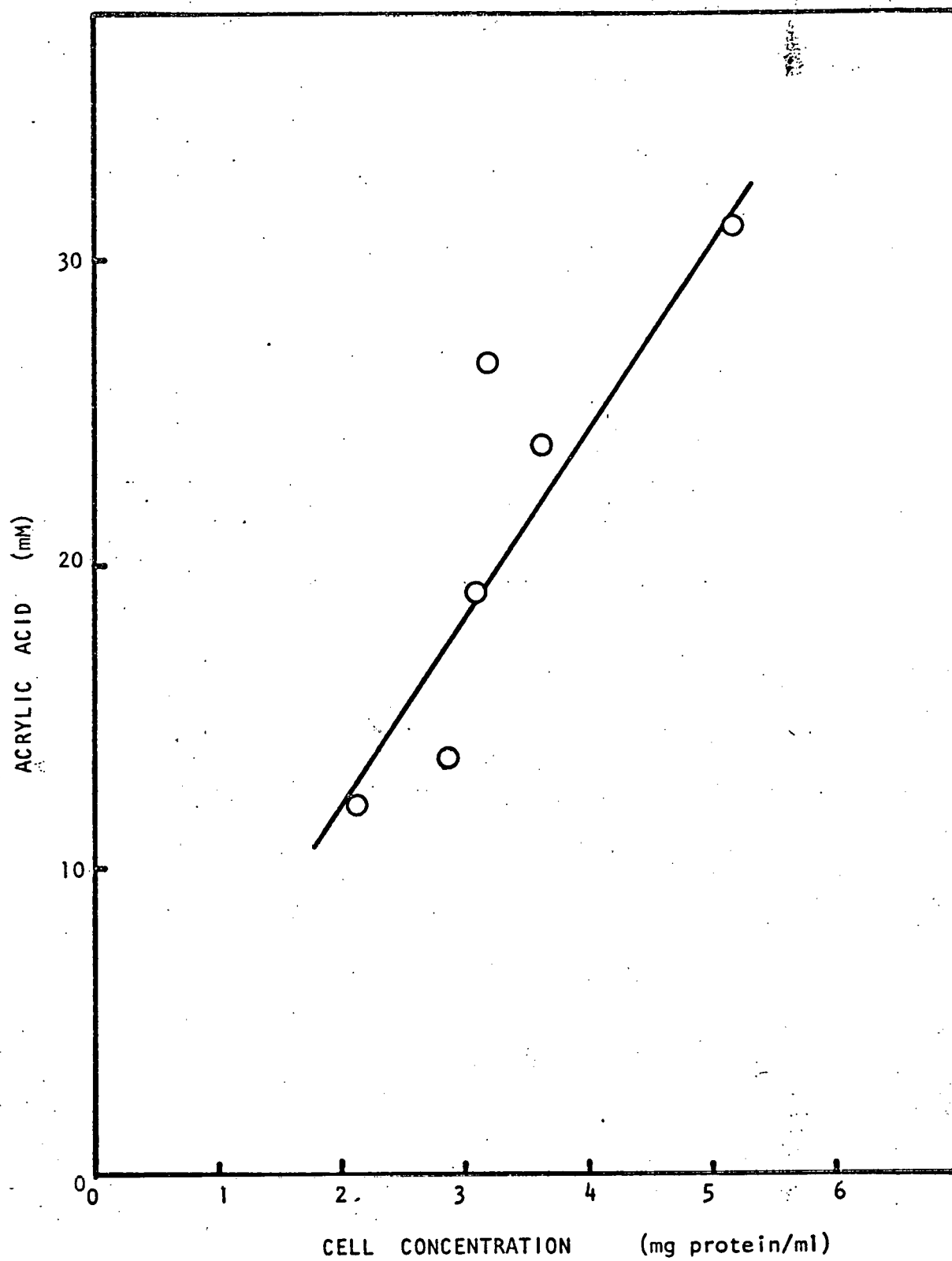


FIGURE II.A.6: ACRYLIC ACID PRODUCTION AS A FUNCTION OF GROWTH PHASE OF CELLS

- Optical density of growing cells measured in Klett units.
- ▲—▲ Wet weight per liter of cells.
- Acrylic acid produced at 4 hours after initiation of bioconversion using cells harvested at various growth phases.

The medium was inoculated with a 5% (V/V) inoculum of an actively growing pure culture.

FIGURE II.A.6

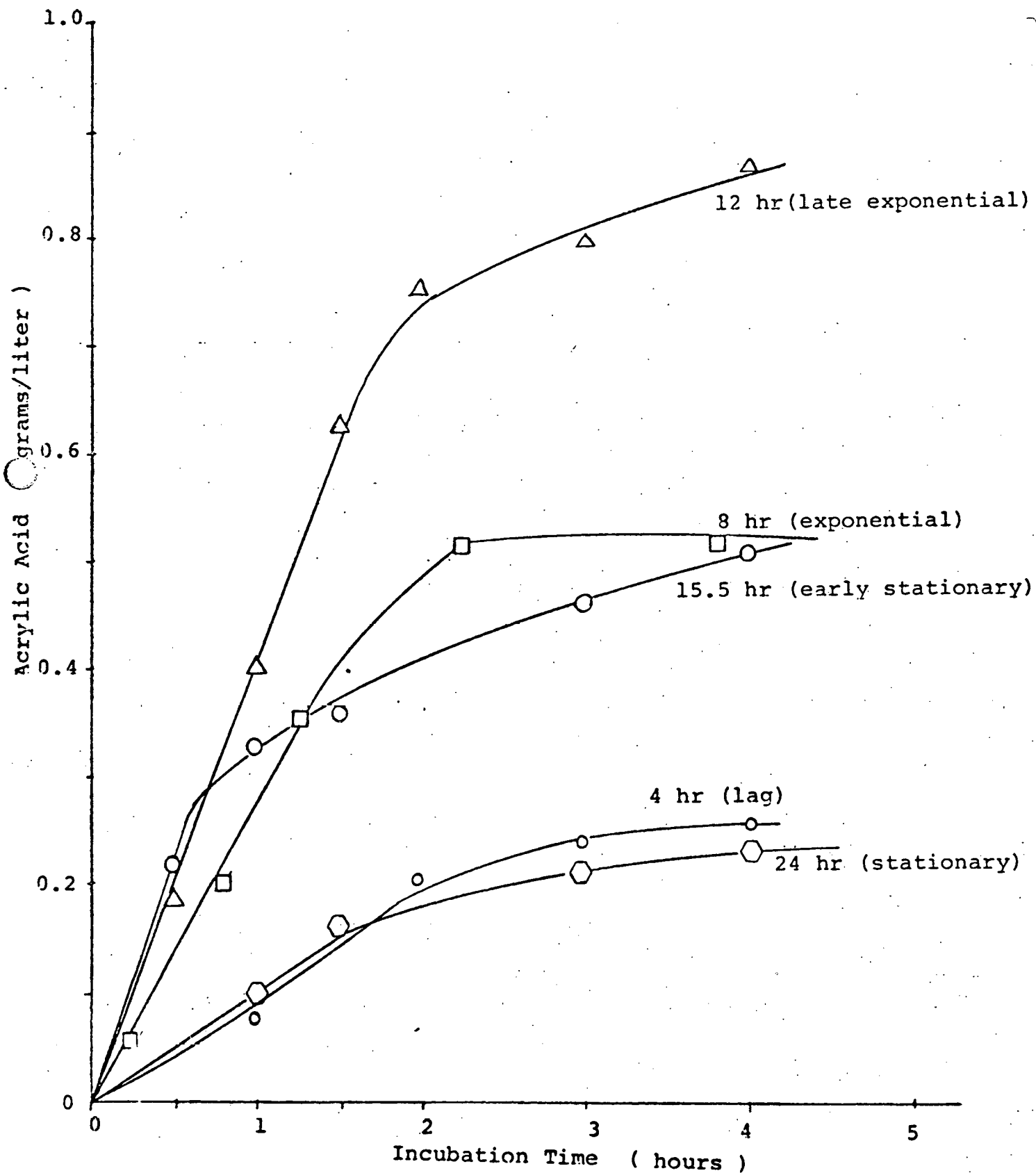
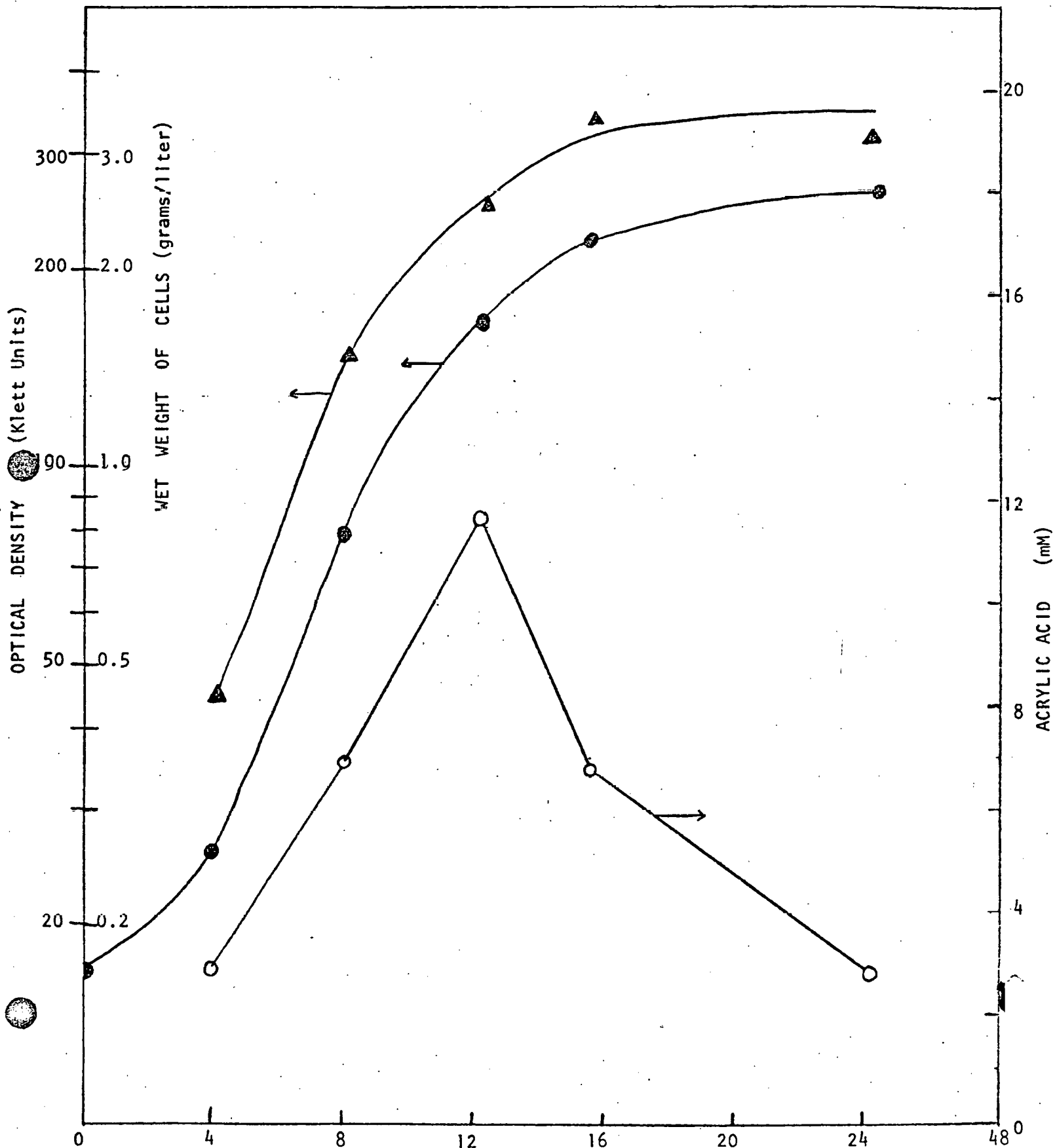


FIGURE II.A.7: EFFECT OF THE HARVEST TIME ON ACRYLATE PRODUCTION. RESULTS ARE TAKEN FROM FIGURE II.A.6.



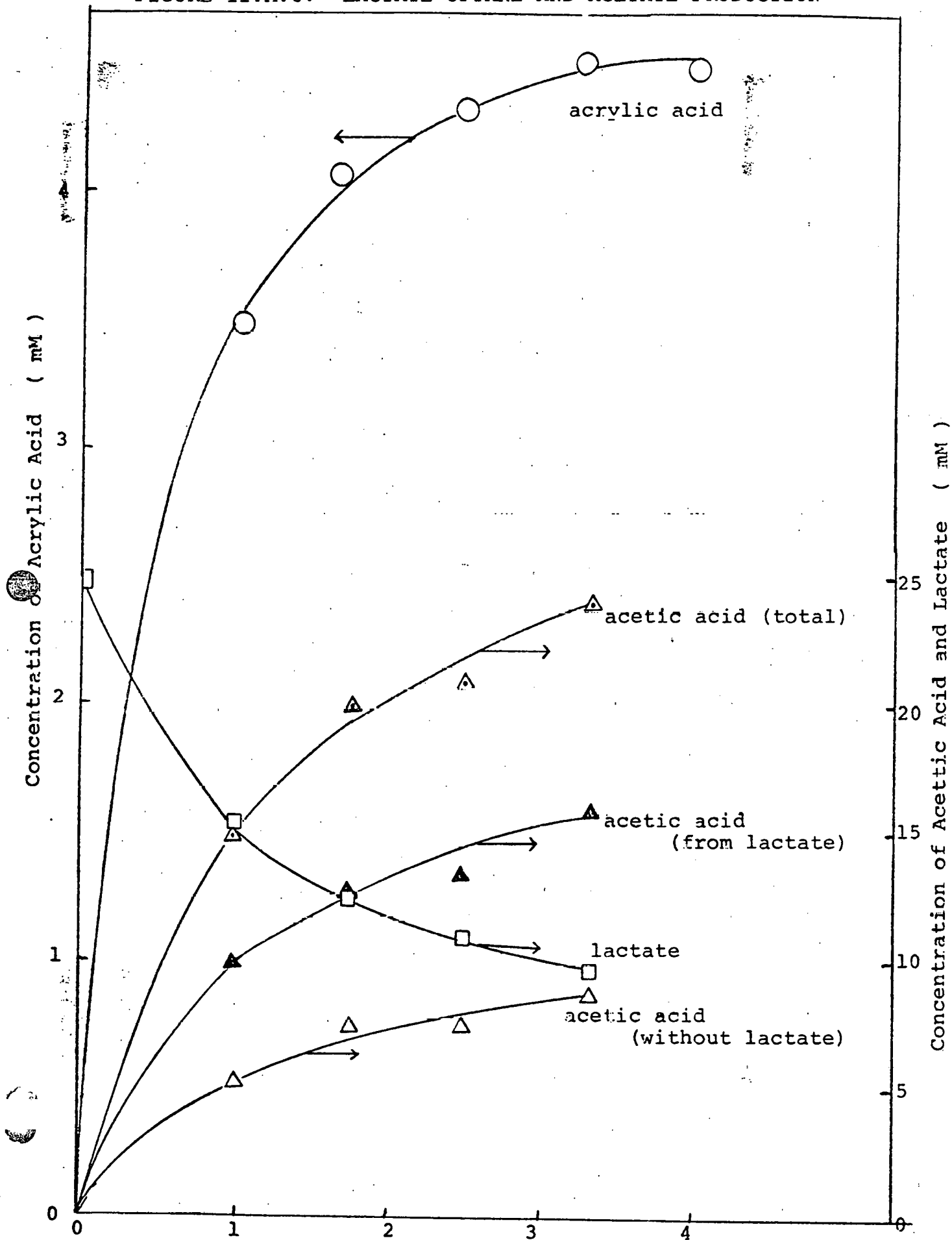
propionate concentration	200 mM
Methylene blue	0.2 %
lactate or α -alanine	20-25 mM
pH	8.5
temperature	32°C
growth phase	late exponential
cell concentration (measured by protein)	>5 mg/ml cellular protein

These optimization experiments have resulted in the use of standardized conditions which lead to reproducible results.

b. Effect of Biochemical Intermediates on Acrylic Acid Accumulation from Propionic Acid

As previously described, the addition of lactate to the reaction mixture greatly stimulated the conversion of propionate to acrylate. A standard reaction mixture (200 mM propionate, 0.2% methylene blue with or without 25 mM lactate in 50 mM Tris buffer, pH 8, and containing 20% net weight of cell suspension) was used at 37°C. The reaction was followed as a function of time. As summarized in Figure II.A.8, it was observed that the lactate is converted entirely to acetic acid. Thus, it is hypothesized that lactate is stimulating the dehydrogenation reaction by generating acetyl CoA. In view of this possibility, intermediates other than lactate were evaluated for

FIGURE II.A.8: LACTATE UPTAKE AND ACETATE PRODUCTION



their ability to stimulate dehydrogenation. As summarized in Table II.A.1, α -alanine and pyruvate have a stimulating effect identical to lactate, as expected. β -alanine and β -hydroxy propionate were ineffective as stimulants. Additional experiments (data not shown) indicated that β -alanine and β -hydroxy propionate were taken up by the cells and metabolized. However, this did not affect the amount of acrylic acid produced.

c. Immobilization of Clostridium propionicum for Production of Acrylic Acid

Immobilized whole cells may have advantages over free resting cell suspensions of C. propionicum, including facilitation of separation of the "biocatalysts" in stirred tank reactors, greater mechanical support and packaging properties necessary for a tubular flow reactor and increased stabilization of catalytic activity. During the course of this progress report period, two different organic immobilizing systems were studied and some preliminary results were obtained.

1. Polyacrylamide Gels (PAG)

Encouraging preliminary results with this system were reported in the previous progress report. Since then, crosslinking of the gel, loading of cells on the polymer, rates of acrylate production in presence of any of the components of the reaction mixture have been investigated.

TABLE II.A.1: EFFECT OF INTERMEDIATES ON THE CONVERSION OF
PROPIONIC ACID TO ACRYLIC ACID

Intermediates	m moles acrylic acid/g cellular protein	
	α -alanine grown	β -alanine grown
none	0.91	0.15
α -alanine	4.34	3.25
lactate	2.24	0.49
pyruvate	2.47	0.65
β -alanine	0.84	0.23
β -OH-propionate	0.90	0.17

- o Reaction mixture (total volume 500 μ l) contained:

triethanolamine-HCl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
intermediate compound	20 mM

- o Incubated at 37°C under air atmosphere with mechanical agitation for 4 hours.
- o Amount of cellular proteins were measured by modified Lowry method.

i. Toxicity of Polymerization Reactants

With PAG gels system, TEA-Mg buffer was replaced by Tris pH 8.0 and initial experiments evaluated the toxicity of the various components of the reaction (Figure II.A.9). N, N, N', N'-tetramethylethylenediamine (TEMED) and N,N'-methylene bis (acrylamide) (Bis), had essentially no effect on the cell system. On the other hand, ammonium persulfate caused a slight reduction in rate and reduced the final amount of acrylate produced by 30%. Acrylamide was the most toxic decreasing the rate of production. Final level of acrylate was the same as that observed for persulfate added to the system. The important finding, however, is that a significant fraction of cellular activity remains after treatment with any of the reagents and the chemicals required for immobilizing cells will not inactivate cellular activity.

ii. Effect of Degree of Cross-linking on Cellular Activity

Percent cross-linking is defined as the percent of cross-linking agent added with the monomer. A 1% cross-linked gel was rubbery, elastic and difficult to homogenize and retained only 50% catalytic activity (Figure II.A.10). 15% cross-linked gels were brittle and easy to homogenize. Though Figure II.A.10 shows a maximum activity at 2.5% cross-linking, repeated experiments indicate that except for 1% cross-

FIGURE II.A.9: TOXICITY OF POLYACRYLAMIDE GEL COMPONENTS ON
ACRYLIC ACID PRODUCTION

●—● Control bioconversion, without gel components. Contained 0.2 gm Na-propionate, 50 mM tris buffer pH 8.0, 25 mM Na-lactate, 0.2% methylene blue. Agitated under air atmosphere.

All other runs contained above components in the reaction mixture, plus below mentioned gel component.

Δ—Δ Plus potassium persulfate, 2.5 mg/ml (100 μl of 2.5% fresh solution).

∇—∇ Plus "BIS" (N, N'-methylene-bis acrylamide) 8 mg/ml

□—□ Plus 5 μl TEMED (N, N, N', N'-Tetramethyl-ethylene-diamine).

○—○ Plus acrylamide monomer 150 mg/ml.

FIGURE II.A.9

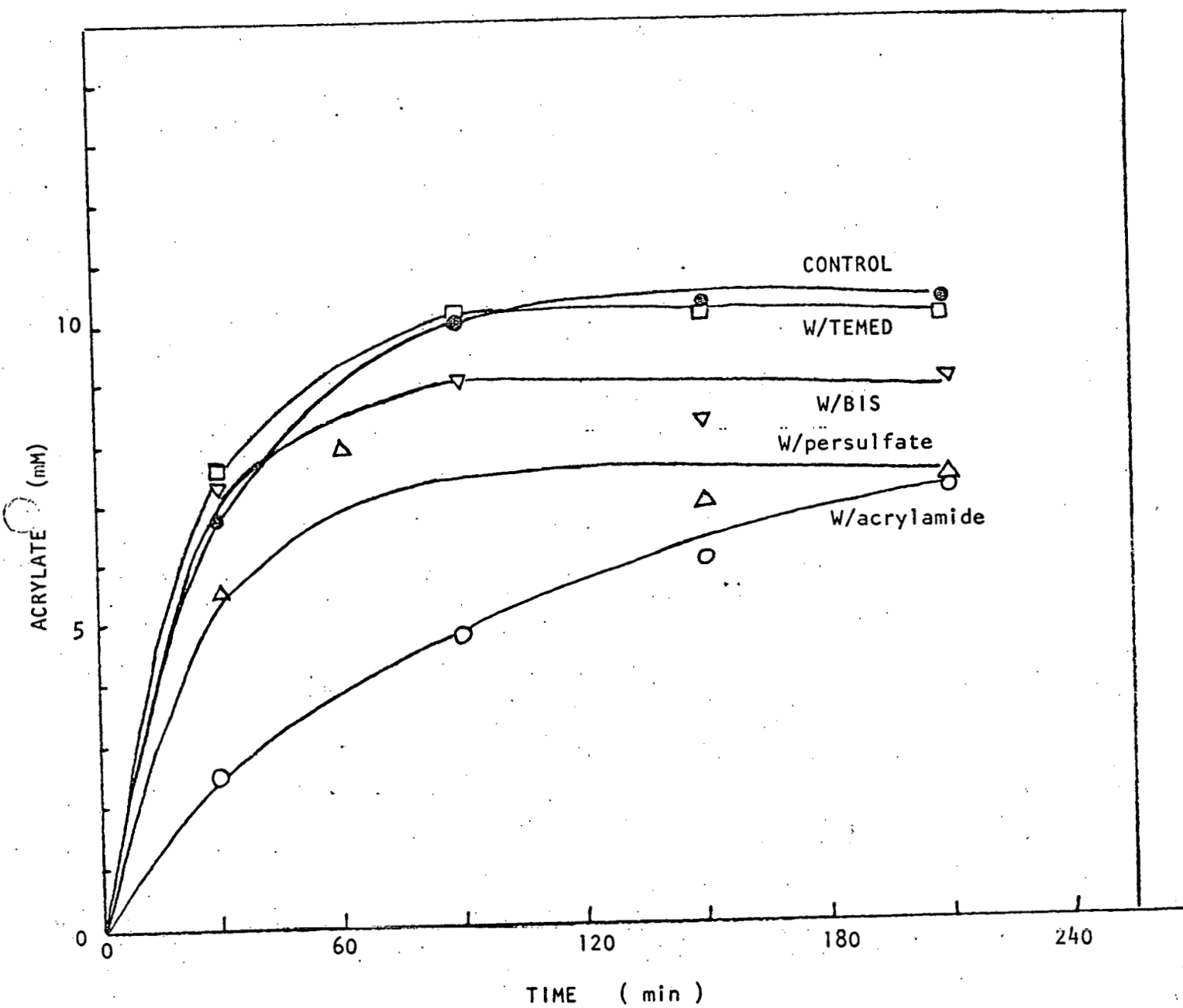
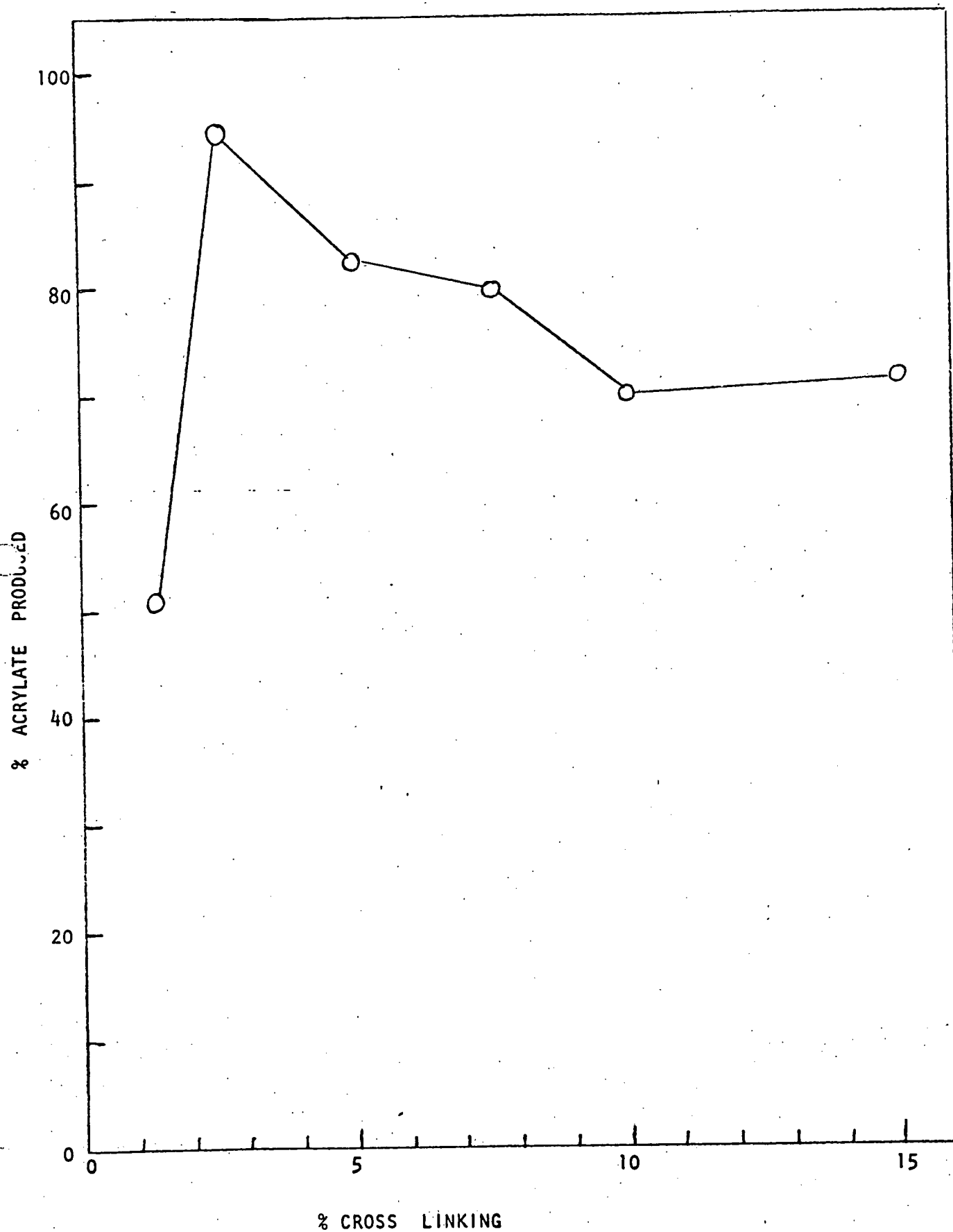


FIGURE II.A.10: EFFECT OF PERCENTAGE CROSSLINKING OF THE
GEL ON ACRYLIC ACID PRODUCTION

o—o Represents acrylic acid produced after 3.5 hrs.
of bioconversion using standard conditions for
bioconversion, as a percentage of acrylic acid
produced by a control reaction using immobilized
resting cells.

FIGURE II.A.10



linking which is consistently less active, gels between 2.5% and 15% cross-linking typically retain 70-80% biological activity.

iii. Cell Loading of the Gels

Cell concentrations ranging from 500 mg/ml net wt to 50 mg net wt/ml resulted in a wide range of gel activities. With 50 mg net wt/ml, no activity was detected. With higher cell concentrations, acrylic acid could be detected. It was found that a cell concentration of 400 mg net wt/ml had 13% of activity of 200 mg net wt/ml of control cells.

2. Polyacrylamide-CO-N-acrylory Succinimide Gels (PAN)

PAN gels were prepared according to the method of Whitesides et al. (J. Am. Chem. Soc. 100, 302, 1978).

PAN, a water soluble polymer was dissolved in 4 ml (4-(2-hydroxyethyl)-1-piperazeneethanesulfonic acid) (HEPES) buffer 300 mM, pH 7.5 succinimide buffer. The solution is stirred and allowed to polymerize after the addition of 50 μ l 500 mM dithiothreitol, 400 μ l triethyltetramine, (TET), and 0.5 ml cell suspension containing variable amounts of cells (typically 300 gms wet wt/liter) with continuous stirring at 25°C. The resulting gel is crushed in a mortar and

worked five times with a total of 100 ml TRIS buffer, 10 mM, pH 8.0. The washings are normally analyzed for protein. Results obtained with this type of gel will be presented in the next progress report.

d. Microbial Degradation of Cellulose for The Production of Lactic Acid

Continuing with efforts to convert cellulosic materials into lactic acid by means of mixed cultures, we conclude with the final characterization and identification of the second microorganism of the system. This lactic acid producer is a thermophilic, homolactic facultative bacterium. The physical and biochemical characteristics show that it belongs to the genera of Bacilli and its metabolic properties are very close to the species coagulans. Therefore, we believe it to be a strain of Bacillus coagulans.

1. Growth in Defined Medium

It is desirable to grow B. coagulans on a chemically defined medium, to study its metabolic performance and its behavior in a mixed culture. The results obtained on such a medium are shown in Table II.A.2 compared to growth on the normal, complex medium. The growth was poor and future work will focus on the need for vitamins and amino acids.

TABLE II.A.2: GROWTH OF B. COAGULANS ON A CHEMICALLY
DEFINED MEDIUM

	<u>AGE</u> <u>(HRS)</u>	<u>pH</u>	<u>GROWTH</u> <u>(K.U.)</u>	<u>LACTIC ACID</u> <u>(gm/l)</u>
Control	24	4.58	225	2.3
Defined Medium	24	6.1	45	0.6

2. Classification

Table II.A.3 presents a summary of the physical and biochemical characteristics of B. coagulans. By comparing these properties to those reported for gram positive bacilli in Bergey's manual, we found that our micro-organism fits very close the description of Bacillus coagulans. The key characteristics considered for its classification were: It is a rod, gram(+), spore forming catalase positive, homolactic, and facultative with no gas production.

Sporulation of B. coagulans is low in frequency and difficult to observe. Three different agra media were tested before spores were detected. The results are shown in Table II.A.4. Mix-media agar plates under aerobic conditions gave the positive results with respect to sporulation. Poor growth and no sporulation were found when cells were incubated under anaerobic conditions. Sporulation was difficult to see under the microscope, but was easily detected by the heat

TABLE II.A.3: SUMMARY OF THE CHARACTERISTICS OF B. COAGULANS

MORPHOLOGY

Form	Rods, slightly curved
Size	0.5 - 0.7 μ m width and 3 - 5 μ m length
Arrangement	Single, mostly pairs and short chains
Motility	Non-motile when grown on liquid or agar medium
Staining	Young cells are gram(+), gradually gram(-) with age
Spore Formation	Hard to sporulate. No sporulation in liquid medium. Spores observed in Agar plates low frequency. Hard to notice on the microscope. Detected only by heat test (80°C/10 min).

CULTURAL CHARACTERISTICS

Liquid Agar	Fast growth in 15 hrs in microaerophilic or anaerobic conditions.
Liquid Medium	Long lag (12 hrs) when inoculum entered stationary phase. Log phase inoculum grow without a lag with specific rates up to 0.7 l/hr.
Agar Plates	No growth under anaerobic conditions by streaking technique. Excellent growth under aerobic conditions, colonies were round, white, opaque, 2-3 mm.

BIOCHEMICAL CHARACTERISTICS

Gas Formation	No gas is produced
Oxygen Relation	Anaerobic facultative
Temperature Relationship	Maximum growth between 45-55°C. Grows between 30-63°C at slower rates on the extremes.
Fermentation Products	Homolactic fermentation when growing on hexoses. Acetic acid and ethanol are also produced when growing on pentoses.
Catalase	Positive
Indol	Negative

TABLE II.A.3 (Contd)

BIOCHEMICAL CHARACTERISTICS (Contd)

Nitrate Reductase	Negative
H ₂ S	Negative
Esculine	Positive
Grows on	Glucose, Fructose, Arabinose, Xylose, Salicin, Rhamose
Low Growth	Lactose, maltose, mannitol
No Growth	Glycerol, Inositol, starch, sucrose, Citrate malonate, Citrate
Lysine, Phenyl- alamine, or- nithine	Not decomposed
ONPG	No β -galactosidase produced
Urea	Urease negative
Azide	No growth at 0.2 gm/l

BIOCHEMICAL TESTS

These tests were done by using the MINITEK kits from BBL Products. These are microtests on filter paper circles, which detect the appearance of a metabolic product, upon growth of the microorganism. Inoculum was prepared by suspending cells obtained from colonies on agar plates. The results were analyzed after 24 hrs at 60°C.

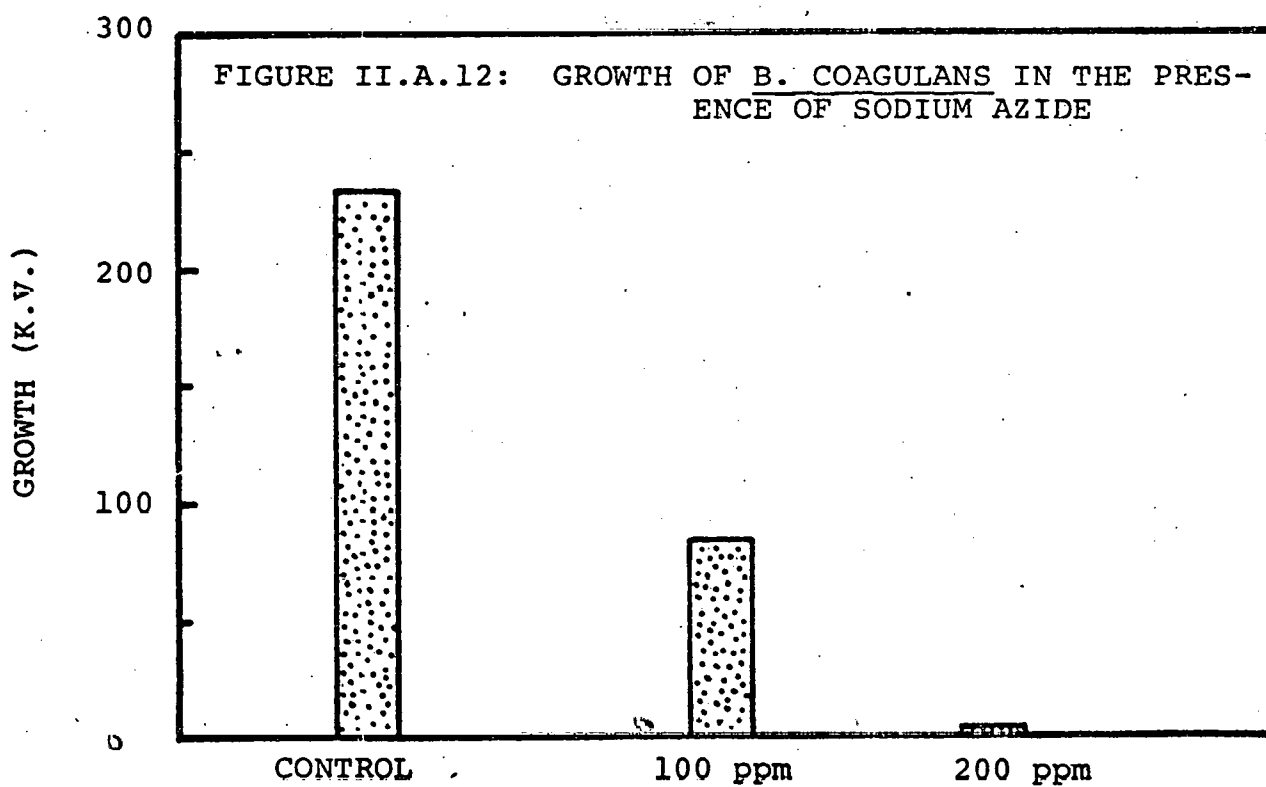
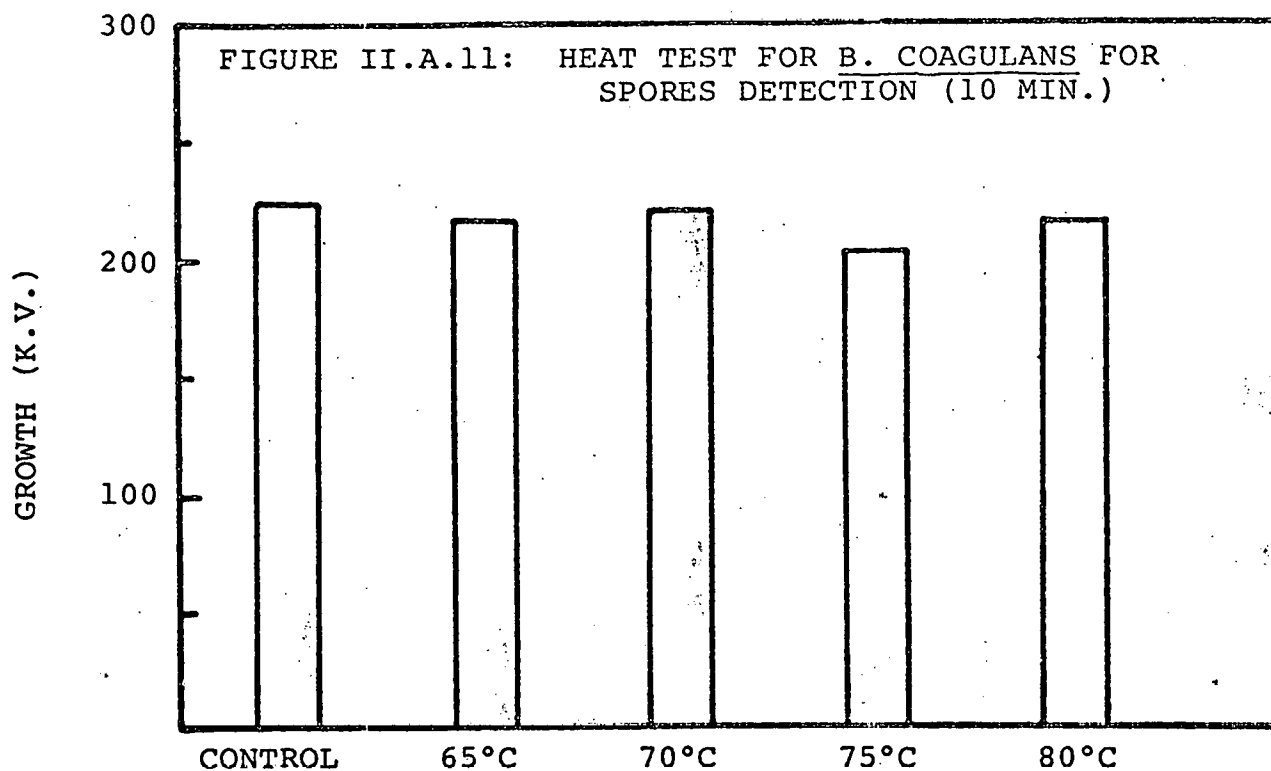
TABLE II.A.4: SPORULATION OF BACILLUS COAGULANS

MEDIUM	CONDITION	AGE (HRS)	GROWTH	SPORES
Duncan	Aerobic	72	(-)	-
	Anaerobic	72	(-)	-
Bacto-Tam	Aerobic	72	(+)	(-)
	Anaerobic	72	(-)	-
Mix-Medium	Aerobic	72	(++)	(+)
	Anaerobic	72	(<u>+</u>)	(-)

test which consisted of heating a cell suspension from agar plates at different temperatures for 10 minutes and then incubating for 24 hrs at 60°C. The results obtained are shown in Figure II.A.11. Cells were able to grow as well as a non-heated control when heated at 80°C which is conclusive of the presence of spores. Growth in the presence of sodium azide, in a test designed to detect the presence of cytochromes or hemo-proteins involved in the energy metabolism of the microorganism. Some growth (Figure II.A.12) was observed in mix-medium after 48 hrs when 100 ppm of sodium azide were present. No growth was observed at 200 ppm, which is conclusive of the sensitivity of the microorganism to sodium azide, and the involvement of hemo-proteins in its metabolism, probably in the mechanism of energy generation.

4. Future Work

- The immobilized cell preparations of C. propionicum will be investigated further to seek optimum reactor concentration and improvement in stability.
- Biochemical experiments to be conducted include
 - i. further refinement of C. propionicum nutrient requirements
 - ii. development of mutagenising conditions for preparation of strain improvement program
 - iii. initiate cell-free experiments to further elucidate role of lactate or α -alanine in stimulating the conversion of propionate to acrylate



- We will study the nutritional requirements of C. thermocellum and B. coagulans in an attempt to obtain a chemically defined medium which support rough growth of both microorganisms in mixed cultures.

- Experimental mixed population will be studied on solid substrate looking at the rates of cellulose degradation and the accumulation of lactic acid.

B. PRODUCTION OF ACETONE AND N-BUTANOL BY FERMENTATION

1. Introduction

In the two earlier progress reports (COO-4198-5 and COO-4198-6), we have reported that washed cells of Clostridium acetobutylicum were able to utilize glucose for the production of acetone and n-butanol. It was concluded then that such activities were results of the "resting cells." In this report, we performed experimental studies using "resting cells" in the presence of penicillin G to determine whether this type of cell preparation is truly able to produce solvents in the absence of growth. In addition, the study of using corn oil as an extracting agent for n-butanol in a batch fermentation was also performed.

2. Materials and Methods

a. Sensitivity of C. acetobutylicum towards Penicillin G

C. acetobutylicum was grown at various concentrations of penicillin G ranging from 0.1 to 10 units/ml. A soluble medium containing 20 g/l glucose was used. The final growths of the cultures were determined through optical density measurements after 48 hours of incubation at 37°C. The results were compared to a control where no penicillin G was added.

b. Resting Cell Experiment of C. acetobutylicum
in Presence of 100 units/ml Penicillin G

The procedures and apparatus used were described in the progress report designated as COO-4198-6. The only difference in this experiment was that penicillin G was added to the suspending medium. The final concentration of penicillin G was 100 units/ml.

c. Resting Cell Experiment using Cells Prepared
under Strict Anaerobic Conditions

The cells harvested from a fermentation were subjected to similar treatment as in the above experiments. However, these experiments were performed to ensure that the cells were not exposed to oxygen. The washing solution and suspending medium in these experiments were degassed before use. All the washing and suspensions were prepared inside an anaerobic hood containing $N_2:CO_2$ (95:5). The centrifugations were performed using air-tight bottles.

The cell pellets after washing were resuspended in medium and distributed to the following tubes. Five milliliters of the cell suspension were added to 5 ml medium. The final cell concentration was about 12 g/l. Types of incubation medium are shown below.

<u>Tube</u>	<u>Medium</u>
I	Phosphate, 10mM; cysteine, 0.5 g/l; pH, 6.7
II	Phosphate, 10mM; cysteine, 0.5 g/l; glucose, 20 g/l; pH, 6.7
III	Phosphate, 10mM; cysteine, 0.5 g/l; glucose, 20 g/l; yeast extract, 1 g/l; pH, 6.7
IA	Same as I, and penicillin G, 100 units/ml
IIA	Same as II, and penicillin G, 100 units/ml
IIIA	Same as III, and penicillin G, 100 units/ml

d. Extractive Fermentation with Corn Oil

A 5-liter fermentor was used in this experiment. The description of the fermentation system was reported earlier (COO-4198-4). The total working volume of the fermentor was 3 liters. Equal volume of a solubles medium containing 50 g/l of glucose was added to corn oil. The soluble medium and corn oil were sterilized separately. Temperature of the fermentation was controlled at 37°C, and the pH was controlled at above 5.0 by addition of 2N NaOH. Agitation of the fermentor was maintained at about 500 rpm. The high agitation rate was used to ensure an efficient contact between the two phases as well as providing means to withdraw equal volumes of the two phases during fermentation.

The samples taken at different time intervals were allowed to settle into two separate phases on ice for

about 10 minutes. From the aqueous phase, the cell density was determined by optical density measurement. The supernatants of the aqueous samples after centrifugation were frozen for subsequent substrate and product analyses. The oil phase was also extracted with equal volume of distilled water for n-butanol concentration determination. This serves as a double check on the measurement of the concentration of n-butanol in the oil phase. Cell dried weight of the samples could not be obtained due to the interference caused by the corn oil.

3. Results and Discussion

a. Sensitivity of C. acetobutylicum towards Penicillin G

The inhibition of growth by penicillin on C. acetobutylicum is shown in Figure II.B.1. The growth of C. acetobutylicum was inhibited by penicillin G at all concentrations above 1 unit/ml.

b. Resting Cell Experiment in Presence of 100 Units/ml of Penicillin G

The resting cell suspension in the presence of 100 units/ml penicillin G had no activity. Glucose was not metabolized during the 50 hours period and no product formation was observed.

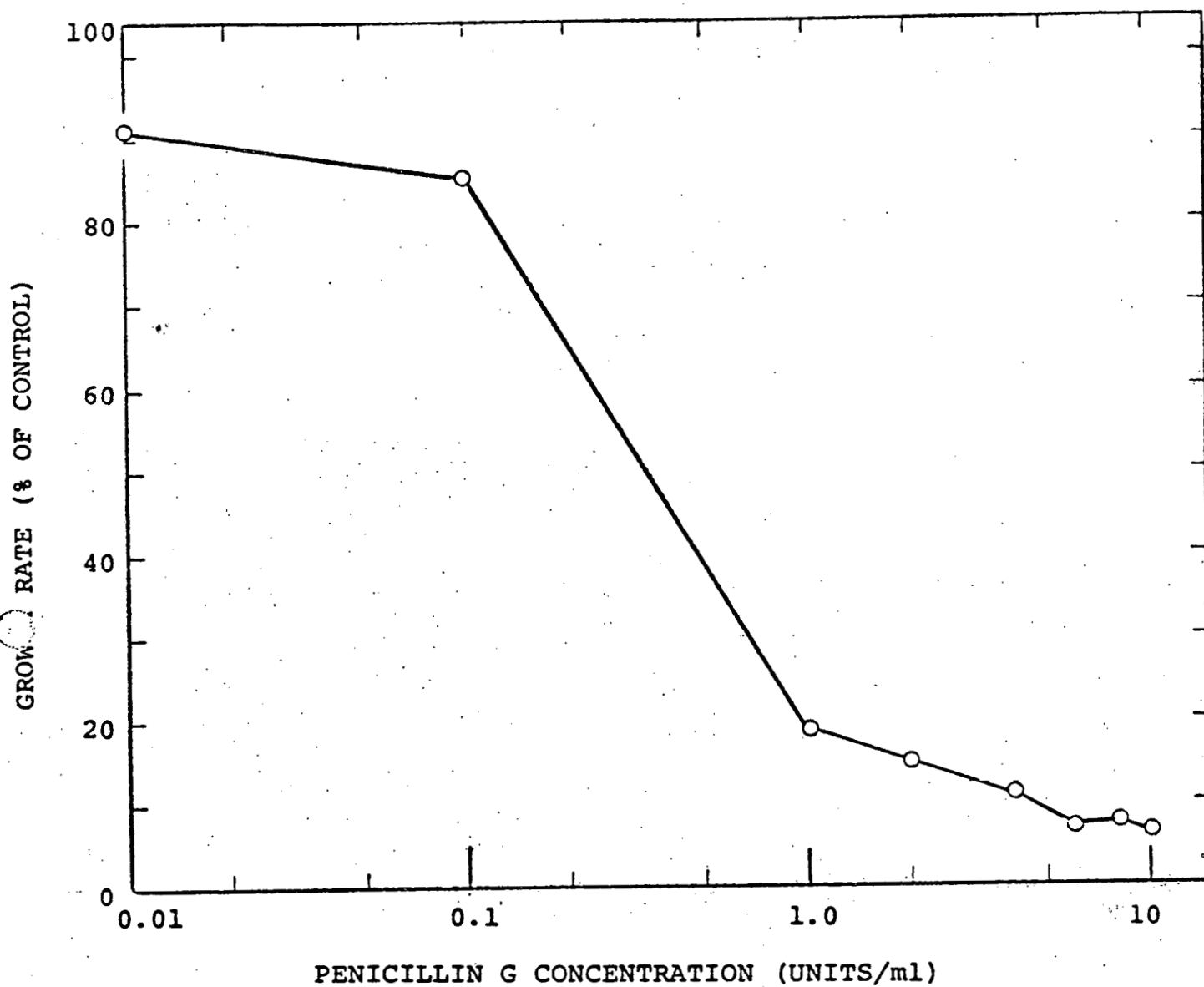


FIGURE II.B.1: EFFECT OF PENICILLIN G ON THE GROWTH OF
Clostridium acetobutylicum (ATCC 824)

The mode of action of penicillin is the inhibition of enzymes relating to cell wall synthesis in gram positive microorganisms. In the presence of penicillin G, there is no new cell synthesized. Assuming that penicillin has no effect on cells that were intact, these results suggested that "resting cells" are not able to produce solvents.

c. Resting Cell Experiment using Cells Prepared under Strict Anaerobic Conditions

The results from the previous experiment suggests that either the resting cells do not have the capabilities to produce the solvents, or the resting cells are damaged during the preparation stage. One of the most possible antagonizing factors is oxygen which could inhibit the growth of this organism. However, the results of the resting cell experiment performed using the greatest care to exclude oxygen exposure to the cells suggested that oxygen is not the inactivation factor. These results are shown in Table II.B.1. Again, in the absence of penicillin G, significant formation of products were observed, and in the presence of penicillin G no product accumulation was noted.

From these results, we conclude that the microorganisms that are not growing are incapable of producing acetone and n-butanol. The reported activities in earlier experiments are probably due to the regrowth of the cells due to

TABLE II.B.1: RESTING CELL EXPERIMENT. CELLS PREPARED IN ABSENCE OF OXYGEN. EXPERIMENT CARRIED OUT IN HUNGATE TUBES. CELL CONCENTRATION OF EACH TUBE WAS ABOUT 12 g/l d.c.w.

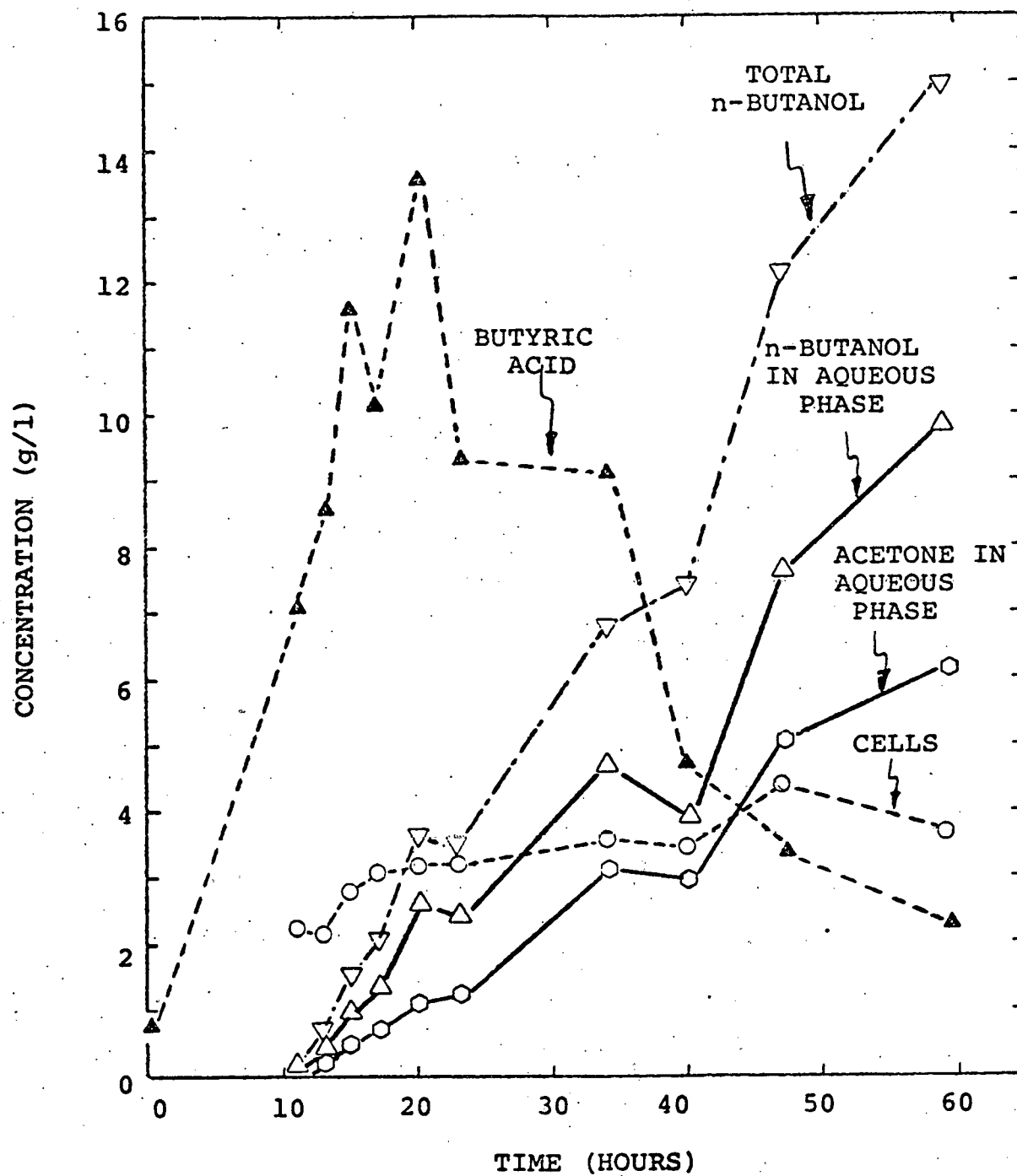
Medium	Penicillin Conc. (unit/ml)	Product Conc. (g/l) after 48 Hrs.			
		acetone	ethanol	n-butanol	butyric acid
I	0	0.03	0.05	0.22	0.32
II	0	0.08	0.07	0.34	1.00
III	0	0.26	0.12	0.98	1.01
IA	100	0.03	0.05	0.06	0.32
IIA	100	0.03	0.04	0.07	0.43
IIIA	100	0.04	0.07	0.05	0.26

endogenous metabolism. This is also supported by the observation of a slight increase of cell density in one of the resting cell experiments in report COO-4198-6.

d. Extractive Fermentation with Corn Oil

The results of this batch fermentation using in situ extraction are shown in Figure II.B.2. The general pattern of the fermentation are similar to the batch fermentations reported in the earlier reports. The butyric acid in the aqueous phase increased up to a concentration of 8 g/l before acetone and n-butanol began to appear. The final concentration of n-butanol in the aqueous phase was 10 g/l, and the acetone concentration was about 6 g/l. The n-butanol in the oil phase was about 5 g/l at the end of the fermentation. Thus, a total concentration of 15 g/l n-butanol was produced. The concentration of acetone in the oil phase was not determined. However, using a distribution coefficient of 0.3, the total acetone concentration was calculated to be 7.8 g/l. The fermentation ended due to the depletion of glucose which was about 2 g/l. The total glucose consumption was 93 g/l. (About 50 g/l of glucose was added to the fermentor at the 15th hour.) Therefore, the solvent yield from glucose was 0.24. This value is comparable to values obtained in other fermentations. However, compared to a conventional batch fermentation, the extractive fermentation takes about 2.5 times as much time to

FIGURE II.B.2: TIME PROFILE OF in situ EXTRACTIVE
FERMENTATION (CORN OIL) USING
Clostridium acetobutylicum



consume 100 g of glucose (60 hours vs 25 hours). Nonetheless, these results demonstrate the feasibility of increasing the final product concentration by extraction of n-butanol into an inert phase. Thus, if an extracting agent with a higher distribution coefficient for n-butanol is used, the final concentration can be increased even further. These results are quite encouraging in the use of in situ extraction to overcome product toxicity.

4. Future Work

- Extend study of extractive fermentation by selection of better extracting agent.
- Initiate study of high-cell-density packed column reactor for production of solvent.

C. PRODUCTION OF ACETIC ACID BY FERMENTATION

1. Introduction

In the previous progress reports, it has been shown that the production of acetic acid by Clostridium thermoaceticum is through both growth associated and non-growth associated modes. The production of acetic acid using resting cells was reported in progress report COO-4198-7. It was observed in growing and in resting cell experiments, cell lysis occurred and could be one of the factors causing the decrease of acetic acid production. Further studies were carried out to determine the cause of cell lysis, and also its effect on the production of acetic acid by resting cells.

Since xylose is one of the major reducing sugars produced by degradation of cellulosic biomass, preliminary studies were performed to determine the ability of C. thermoaceticum to use xylose as carbon source for acetic acid production.

2. Materials and Methods

The resting cell experiment was carried out in a 1-liter fermentor with a working volume of 750 ml. Anaerobic environment was maintained by passing gaseous CO₂ through the broth. The temperature was controlled at 60°C and pH was controlled at 7.0 by using 5N NaOH.

The experiments for xylose utilization were carried out in hungate tubes, using the complex medium as reported earlier, except that glucose was replaced by xylose.

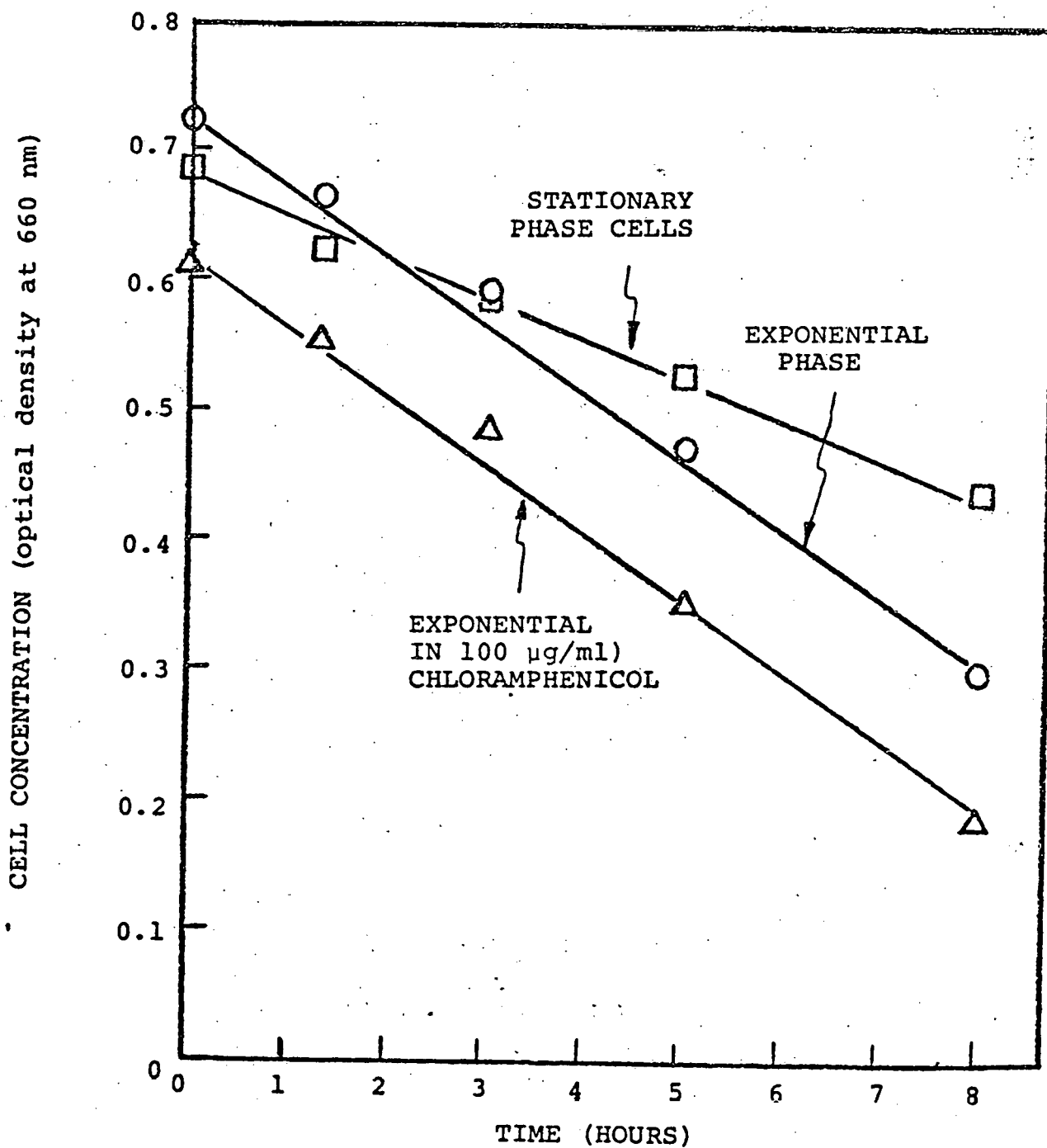
The other analytical methods have already been presented in the previous progress reports.

3. Results and Discussion

In the fermentation of Clostridium thermoaceticum under pH control, cell lysis was observed at high acetate concentration. Similarly, cell lysis was observed in the resting cell experiments. One possible explanation is the involvement of autolytic enzymes, similar to systems found widely in various gram-positive and gram-negative bacteria, such as in Bacillus licheniformis (Forsberg & Rogers, 1974, J. Bacteriol. 118, 358), or in Streptococcus faecalis (Cornett, Redman & Shockman, 1977, J. Bacteriol. 133, 631).

When cells are harvested, washed and resuspended into buffer of relatively high ionic strength (e.g. 0.3 M sodium phosphate, pH 7.1), rapid decrease of optical density was observed. This decrease of optical density is interpreted as cell lysis. Exponentially grown cells have a significantly higher rate of cell lysis than stationary grown cells, as shown in Figure II.C.1. If an autolytic system is causing the cell lysis, one method to verify such a phenomenon is to see whether this decrease of OD would be stopped with the addition of

FIGURE II.C.1: DECREASE OF OPTICAL DENSITY OF Clostridium thermoaceticum IN PHOSPHATE BUFFER



chloramphenicol. Chloramphenicol at concentrations between 50-200 $\mu\text{g/ml}$ was shown to inhibit cell growth, and was used for this experiment. However, no significant effect on the lysis rate was observed as shown in Figure II.C.1. More efforts will be spent to study this "apparent" cell lysis behavior.

The production of acetic acid by resting cells under pH control is shown in Figure II.C.2. The kinetic profile is similar to the results presented in a previous report. The production rate is highest at the beginning and decreases with time. The cell mass, measured optically, also decreases steadily during the experiment from an initial value of 1.8 g/l to the final value of 0.7 g/l. The rate of decrease of acetic acid fermentation, however, is faster than the rate of decrease of cell mass. These results that the decrease of acetic acid production by resting cells is probably due to a combination of cell lysis and cell instability.

The production of acetic acid and cell mass at different concentrations of xylose is shown in Figure II.C.3. The conversion yield was found to be 0.76 gm acetate/gm xylose consumed, and the cell yield is about 0.21 gm cell/gm xylose consumed. The specific growth rate is $.14 \text{ hr}^{-1}$. These results indicate that C. thermoaceticum can use xylose efficiently for acetic acid production with high conversion yield.

FIGURE II.C.2: PRODUCTION OF ACETIC ACID BY RESTING
CELLS OF C. thermoaceticum AT pH 7.0

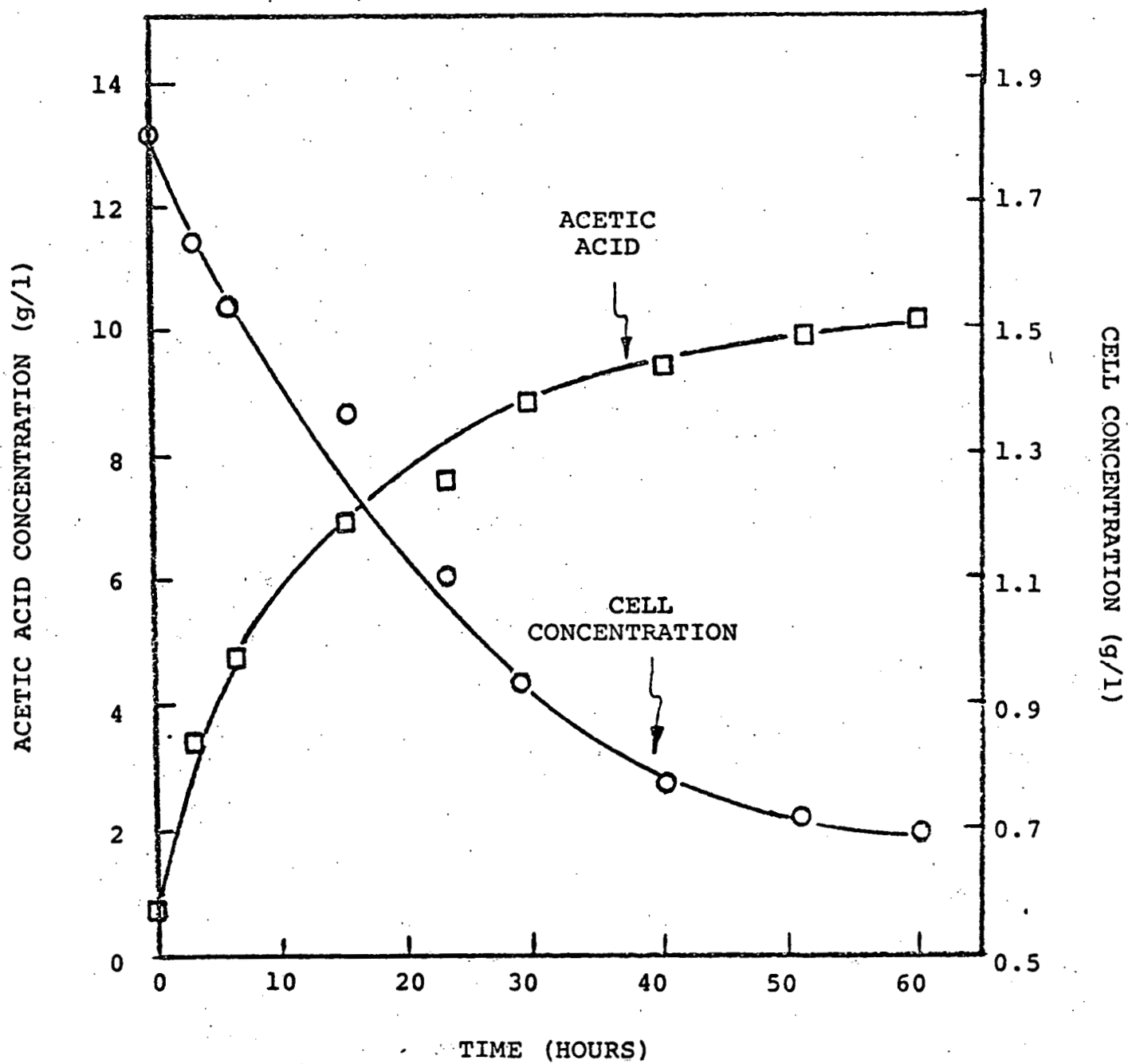
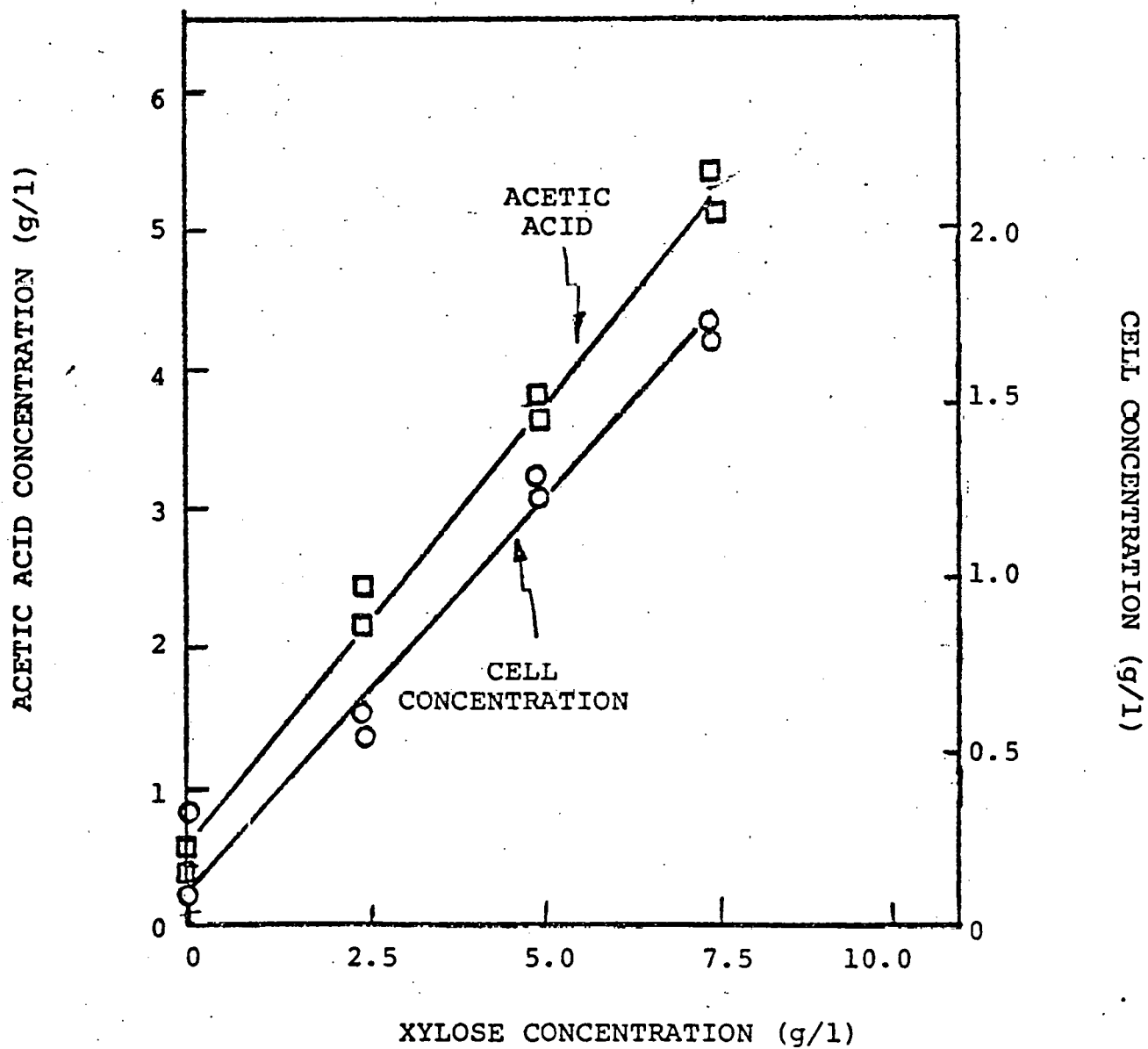


FIGURE II.C.3: PRODUCTION OF ACETIC ACID BY Clostridium thermoaceticum ON XYLOSE



4. Future Work

- Continue to study the causes of cell lysis, and find the appropriate means to eliminate it.
- Cell immobilization to increase stability and productivity of resting cells.
- Design of high productivity bioreactor.

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

Report for Fourth Quarter

November 1, 1978 through January 31, 1979

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THE U.S. DEPARTMENT OF ENERGY
CONTRACT NO. EG-77-S-02-4198

SCOPE OF PROGRAM

This is a coordinated program to effect the microbiological degradation of cellulosic biomasses and 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 conversion from cellulosic biomasses to liquid fuel (ethanol) and/or soluble sugars by the cellulolytic, anaerobic organism is also within the scope of this program. Process and engineering scale-up, along with economic analyses, will be performed throughout the course of the program.

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 materials by Clostridium thermocellum has focused on nutritional requirement and mutation and selection for high cellulase producers. At high yeast extract concentration (6 g/l), C. thermocellum uses glucose as carbon source for growth and cellulase production. During 5 sequential transfers, cellulase production remained constant.

We are obtaining growth with the 8 vitamin mix of Alexander replacing yeast extract. The two vitamins which appear essential are pyridoxine and biotin. Sequential transfer experiments have proven that only these two vitamins are required for growth.

Superiority of our first generation mutant, AS-39, has been confirmed by Zeikus and Lamed at U. Wisconsin. We found this culture to be 1 fold improved over ATCC 27405 in endo- and exo- β -glucanase production. The Wisconsin workers only examined the endo- β -glucanase levels and found a 4-fold increase. They have our 2nd generation mutants, but U. of Wisconsin has not yet examined them.

We have isolated a spontaneous 3rd generation mutant by rapid sequential passage through Avicel tubes. The new culture (JMT-11) is about 40% improved over its parent (CM-17-3).

Addition of cysteine at 5 mg/ml to the Avicel assay system increases the activity by about 40% while addition of this thiol reducing agent at 1 mg/ml to the filter paper assay system results in about a 25% increase in activity.

In the area of genetic manipulation, experiments have been initiated to clone C. thermocellum DNA in E. coli. Total DNA from C. thermocellum has been digested with restriction enzymes. A method for the quantitation of restriction is being developed.

The enzymatic activity of cellulase from Clostridium thermocellum has been evaluated and compared using several standard assays for cellulase activity. While the volumetric rates of cellulose hydrolysis are in appearance low (0.06 IU/ml), but when calculated on a specific basis these values are 0.2 IU/mg of protein and 0.086 IU/mg of cell mass when utilizing the filter paper assay. In the case of CMC

hydrolysis the specific activities are 5.3 IU/mg of protein and 2.3 IU/mg of cell mass. These values are for the activities of the wild type C. thermocellum and are within approximately a 5-fold range of cellulase activity expressed by Trichoderma reesii 9414. It is thus becoming clearer that the anaerobic bacteria produce a set of very active cellulase enzymes.

In the analysis of soluble sugar production from hydrolysis of cellulosic biomass, it was difficult to distinguish glucose from xylobiose in HPLC analysis. It is known that when C. thermocellum is grown with pH control little or no glucose accumulates in the growth medium as shown by enzymatic methods for detection of glucose. Under such conditions, using Solka floc (containing a fraction of xylan), as the cellulose sources, reducing sugars including xylobiose were shown to accumulate. Furthermore, the accumulation of xylobiose occurs throughout much of the active portion of the fermentation. These results confirm the occurrence of this disaccharide during the hydrolysis of cellulose in C. thermocellum fermentations.

The production of a liquid fuel, ethanol, using Clostridium thermocellum has focused on means to increase the tolerance of this organism to ethanol, increasing the rate of product formation and directing the catabolism to achieve high ethanol concentration with respect to other products. Through the adaptation and selection procedure using progressively higher ethanol concentrations, two strains (S-6 and S-B) from C. thermocellum (S-4) have been isolated. Both of these new strains have shown higher tolerance to ethanol than strain S-4. Experiments using strain S-6 have shown total degradation of Solka floc (initially at 35 g/l) can be achieved. Products from degradation are cell mass (~1.3 g/l), ethanol (8.3 g/l), reducing sugars (6 g/l) and acetic acid (1 g/l). When S-6 was tested using 35 g/l of corn stover, similar product profiles were obtained: cells (1.5 g/l), ethanol (4 g/l), reducing sugars (5.2 g/l) and acetic acid (1 g/l).

When the new strain S-B was examined, a number of unexpected behaviors resulted. It was found that this strain, in addition to being able to grow on glucose, cellobiose and Solka floc, has the ability to metabolize xylose. This behavior led us to suspect the new isolate is a stable mixed culture of Clostridium thermocellum and Clostridium thermosaccharolyticum. When strain S-B was cultivated on Solka floc, total degradation (initial Solka floc at 35 g/l) resulted. The products of degradation were cell mass (1.3 g/l),

ethanol (15 g/l), acetic acid (5 g/l) and no reducing sugars. Using 35 g/l of corn stover, 70% degradation resulted leading to the formation of cell mass (1.5 g/l), ethanol (4.2 g/l), acetic acid (2.3 g/l) and again no reducing sugars.

Attempts to increase the rate of cellulose degradation using Clostridium thermocellum have been performed in preparation for our future studies with the packed-bed fermentor. It was rationalized that through pre-concentration through centrifugation of the cells, a higher inoculum concentration can be attained and thus leading to higher degradation and product formation rates. This concept was tested and using corn stover as the substrate. Although the initial volumetric rate was increased through the use of concentrated cell inoculum, the increase was not proportional to what was to be expected. We believe that the redox potential may have been the cause for this abnormal behavior. Studies will be continued to delineate the reason for the unexpected poor results.

In order to increase the production of ethanol in preference to other catabolites, environmental manipulation studies have been performed. The direction of catabolism by C. thermocellum when grown on cellobiose was examined in the presence of lactate and acetate. It was shown in high levels of lactate and/or acetate initially placed into the medium will cause an increase in the level of ethanol production.

Continued studies have been performed to evaluate alternative substrates for Clostridium thermocellum. A re-evaluation of the use of cotton shearings, a by-product of the textile industry, has shown that adaption of C. thermocellum greatly shortens the lag time in the fermentation and yields up to 0.4 gms producing sugars per gm of cotton shearings fed. Studies with corn cob granules (particle diameter of 0.25 mm) have shown that it is possible to achieve 40% degradation of a 40 gm per liter suspension in 100 hrs with accumulation of 7.5 gms per liter of sugar. This is an overall yield of 0.25 grams producing sugar per gm of corn cob granules. These results further indicate the versatility of Clostridium thermocellum in using available cellulosic materials.

To achieve the effectively utilization of hemicelluloses in biomass, studies on the utilization of xylose have continued. The previous xylose utilizing organism designated as HG-2 has been identified to be Clostridium thermosaccharolyticum. Studies with a higher ethanol tolerant strain (HG-3)

have been conducted. The new strain (HG-3) shows a higher ethanol to acetic acid production ratio than HG-28 (parent) when grown on xylose, anaerobically and at 60°C. C. thermosaccharolyticum has a diverse metabolic activities towards many different carbohydrates. Lower pH during fermentation (pH 6.0 or 6.5) favors the production of ethanol over acetic acid and lactic acid. Fed-batch fermentation using xylose have shown excellent conversion efficiency by this organism. From a total of 57.89 g/l of xylose consumed, 14.5 g/l of ethanol, 15.3 g/l of lactic acid, 10 g/l of acetic acid and 2.3 g/l of cell mass resulted which corresponds to a conversion yield of 0.688 g product/g xylose. Mixed culture studies using C. thermocellum and C. thermosaccharolyticum have also been performed. Results show a compatible mixed fermentation can be achieved leading to the formation of the desired catabolites.

Efforts have been initiated to evaluate a process design using a mixed culture of Clostridium thermocellum and C. thermosaccharolyticum to directly convert hammer milled corn stover to ethanol in a single stage fermentation. The design is being done for 25 x 10⁶ gallons of 95% ethanol per year. This design is based on our best available experimental data show to provide an estimate of capital and operating costs for such a plant.

The production of other chemicals through fermentation continues to focus on acrylic acid, acetone/butanol, acetic acid and lactic acid. Optimization of acrylic acid production from propionic acid by Clostridium propionicum has demonstrated the ability to accumulate 40 mM of acrylic acid corresponding to 0.55 gm/l of acrylic acid per mg of cell protein present at an initial rate of 3.8 mmol acrylic acid per liter per hour. A major bottleneck at this point is the apparent instability of the bioconversion system for acrylic acid. The effect of oxygen and acrylate on system stability was examined and it was shown that oxygen was primarily responsible for the cessation of acrylic acid production. Furthermore, it was shown that the activity of acrylate synthesis is quite stable under anaerobic incubation. Closer examination of alternative electron acceptors suggests possible improvements in acrylic acid production. Acrylic acid was shown to have a mixed inhibition of acrylate synthesis in that both the K_m and the maximum rate of reaction decreased with increasing acrylic acid concentration. An interesting observation has been made in the anoxic incubation of C. propionicum with acrylic acid followed by oxygen mediated dehydrogenation of propionate to acrylate. In such a reaction system, a new and unidentified compound has been

observed. Efforts are proceeding to identify this reaction product. In the future, an increased effort will focus on the use of alternative electronic acceptors, the role of lactate in stimulation of acrylate production and the stabilization of the reaction system.

Realizing that the desired substrate for acrylic acid production is lactic acid, work has continued on the direct fermentation of cellulose to lactic acid with mixed cultures of Clostridium thermocellum and Bacillus coagulans. In a fed batch fermentation at pH 6.8 and 60°C, 69% of a total of 40 gm/l of cellulose was degraded to form primarily lactic acid with some ethanol and acetic acid. The amount of lactic acid formed (7 gm/l) far exceeds that amount that C. thermocellum would produce if grown on cellulose by itself. There were little or no residual sugars and it is felt that the B. coagulans utilizes both residual hexoses and pentoses. The feasibility of producing lactic acid directly from cellulose has been shown and future work will focus more on the problems of acrylic acid production rather than lactic acid formation.

The acetone/butanol production using Clostridium acetobutylicum has focused on means to increase the tolerance of this organism to butanol. Mutation, adaptation and selection for tolerant strains have not been successful. However, using in situ extractive fermentation has yielded favorable results. Corn oil has shown to be an effective extractant having no adverse effect on the biological activity of this organism. Through the use of batch extractive, in situ fermentations, butanol concentration calculated on the aqueous phase of about 20 g/l has been achieved. It should be mentioned that the previous maximum butanol concentration attainable without in situ extraction is only 12 g/l.

The production of acetic acid by Clostridium thermoaceticum has focused on the use of immobilized whole cells. It is hoped through immobilization that higher cell stability can be achieved and thus attaining higher productivity. Favorable results have been obtained when C. thermoaceticum are immobilized onto an ion exchange resin, Ambergard XE-352. These immobilized cells have been shown capable of producing acetic acid. Future studies are planned to increase the loading onto the resin, as well as to determine the operational stability of such a system.

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This progress report acknowledges the contributions by the individuals listed above, as well as the research assistants, post doctoral research associates, and technical assistants associated with this project.

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I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. Enzyme Production by Clostridium thermocellum

1. Introduction

Comparison of the cellulolytic activity of C. thermocellum's enzymes with those of other cellulase producing organisms often leaves the erroneous impression that the thermophilic anaerobe's ability to hydrolyze cellulosic materials is several orders of magnitude lower than that of the best mutant fungal strains of T. reesei. The misinterpretation results from the conventional expression of cellulase activity as a volumetric titer, "units/ml", the amount of product liberated from an assayed substrate per time per volume of fermentation broth. For a more meaningful and equitable comparison, however, expression of enzyme activities on a specific basis is necessary. For this reason, the maximum cellulase activities on the widely used substrates, carboxymethyl cellulose and filter paper, are presented based on the amount of extracellular protein and cell mass produced during a fermentation on cellulose of the wild type Clostridium thermocellum ATCC 27405. This presentation also serves the purpose of updating the specific enzyme activities appearing in earlier progress reports which

were measured prior to the now standard practice of implementing pH control during fermentation, an environmental measure which has significantly enhanced volumetric cellulase titers.

2. Materials and Methods

Enzyme activity: The measurement of cellulase activity on Sigma carboxymethyl cellulose and Whatman No. 1 filter paper has been described in an earlier progress report (COO-4198-1). Residual solids were removed from the fermentation broth by centrifugation prior to the cellulase assays.

Culture conditions: Clostridium thermocellum ATCC 27405 was grown in CM3 medium, containing 10 grams per liter MN300 cellulose and 5 grams per liter yeast extract, in a two liter fermentor. Sodium hydroxide (5N) was added automatically to maintain the pH of the medium at pH 7.0.

Protein measurements: Extracellular protein was measured with the Bio-Rad Protein Assay described in Progress Report COO-4198-8. Pellet protein was measured by the Lowry protein assay (see Progress Report COO-4198-1) to obtain an estimate of dry cell mass based on a 50% by weight cellular protein composition.

3. Results

The volumetric and specific enzyme activities of C. thermocellum's cellulase on CMC and filter paper are presented in Table I.A.1. One International Unit is equal to one micromole of glucose equivalent per minute.

TABLE I.A.1

<u>Activity</u>	<u>Substrate Assayed</u>	
	<u>Filter Paper</u>	<u>CMC</u>
Volumetric	0.65 mg RS*/ml-hr 0.06 IU**/ml	17.5 mg RS*/ml-hr. 1.60 IU/ml
Specific		
a) Based on extra-cellular protein	0.2 IU/mg protein	5.3 IU/mg protein
b) Based on dry cell weight	0.086 IU/mg cell mass	2.3 IU/mg cell mass

* RS: reducing sugar (glucose equivalent), measured by DNS assay

** IU: International Unit

4. Discussion

C. thermocellum's volumetric activity on filter paper of 0.06 International Units per milliliter of broth is considerably lower than that of the best T. reeseii mutants

which produce 9 to 14 International Units/ml. However, if C. thermocellum's cellulase activity is compared with that of T. reeseii 9414 on a specific basis, one finds that the difference between the bacterial and fungal activities is not so great. For instance, it has been reported that when T. reeseii is grown on 4% pretreated cotton, 9 IU/ml of cellulase and 9 mg/ml of extracellular protein are produced. Though direct measurements of cell mass are not made during the fungal fermentation, it is assumed that approximately 15 to 20 grams per liter of dry cell mass are produced. Thus, the fungal specific activities are estimated to be 1.0 IU/mg extracellular protein and 0.45 to 0.60 IU/mg cell mass. It appears that the cellulase of the wild type strain of Clostridium thermocellum is only five-fold less on the basis of extracellular protein and five to seven-fold less on the basis of cell mass than that of the mutant fungal strain. In light of the recent isolations of enhanced cellulase producing mutants of which C. thermocellum ATCC 27405 was the parental strain, the gap between the fungal and bacterial specific cellulase activities is even smaller. Furthermore, if C. thermocellum's ability to degrade cellulose is compared with that of the fungi based on the volumetric rate of cellulose degradation during fermentation, one finds that C. thermocellum with sustained maximal rates of 0.8-0.9 gm cellulose (solka floc) degraded per liter per hour surpasses the slow growing fungi.

B. Production of Soluble Sugars from Cellulosic Biomass
by Clostridium thermocellum

1. Introduction

As discussed in the last progress report (COO-4198-8), in the course of studying glucose accumulation during non-pH-controlled fermentations of C. thermocellum ATCC 27405 on solka floc cellulose, we have observed a discrepancy between HPLC and Glucostat measurements of glucose. By HPLC analysis, glucose accumulation during fermentation appears to be both a growth and non-growth-associated event, whereas the enzymatic Glucostat assay indicated that glucose accumulation occurs in a non-growth-associated mode only. It was hypothesized that the discrepancy was due to a growth-associated accumulation of xylobiose, derived from the hemicellulose fraction of solka floc. Xylobiose, the disaccharide of xylose, would artifactually be detected as glucose since the resolution of these two sugars on the HPLC analytical column presently used is poor. To test this hypothesis, C. thermocellum was grown on solka floc under pH-controlled conditions and samples from the fermentation were subjected to HPLC analysis. As had been shown previously, with pH control, no glucose accumulates in the medium. Thus, these conditions would allow us to selectively follow the kinetics of xylobiose accumulation by HPLC analysis. That no glucose accumulation occurred was monitored with the Glucostat assay.

2. Materials and Methods

Culture conditions: *Clostridium thermocellum*

ATCC 27405 was grown in a 14-liter Chemaptec fermentor (working volume 10 liters) on CM3 medium containing 10 grams per liter of solka floc BW40 and 5 grams per liter yeast extract. The pH of the medium was controlled automatically by the addition of 5N sodium hydroxide.

Measurement of accumulating sugars: Total re-

ducing sugar accumulation was measured by the DNS assay. Glucose was measured with the Glucostat (Worthington Biochemicals) assay. Xylobiose accumulation was followed by HPLC analysis as described in Progress Report COO-4198-6.

3. Results and Discussion

Xylobiose could not be measured quantitatively, as the amount of xylobiose standard available was sufficient for identification of the chromatographic peak but not sufficient for the construction of a xylobiose standard curve by which to quantitate the sugar. Nevertheless, the accumulation of xylobiose, reflected by the increasing height of the peak corresponding to this sugar, was followed. The results are shown in Figure I.B.1, where xylobiose peak heights for the samples analyzed by HPLC are plotted as a function of fermentation time. Reducing sugar accumulation

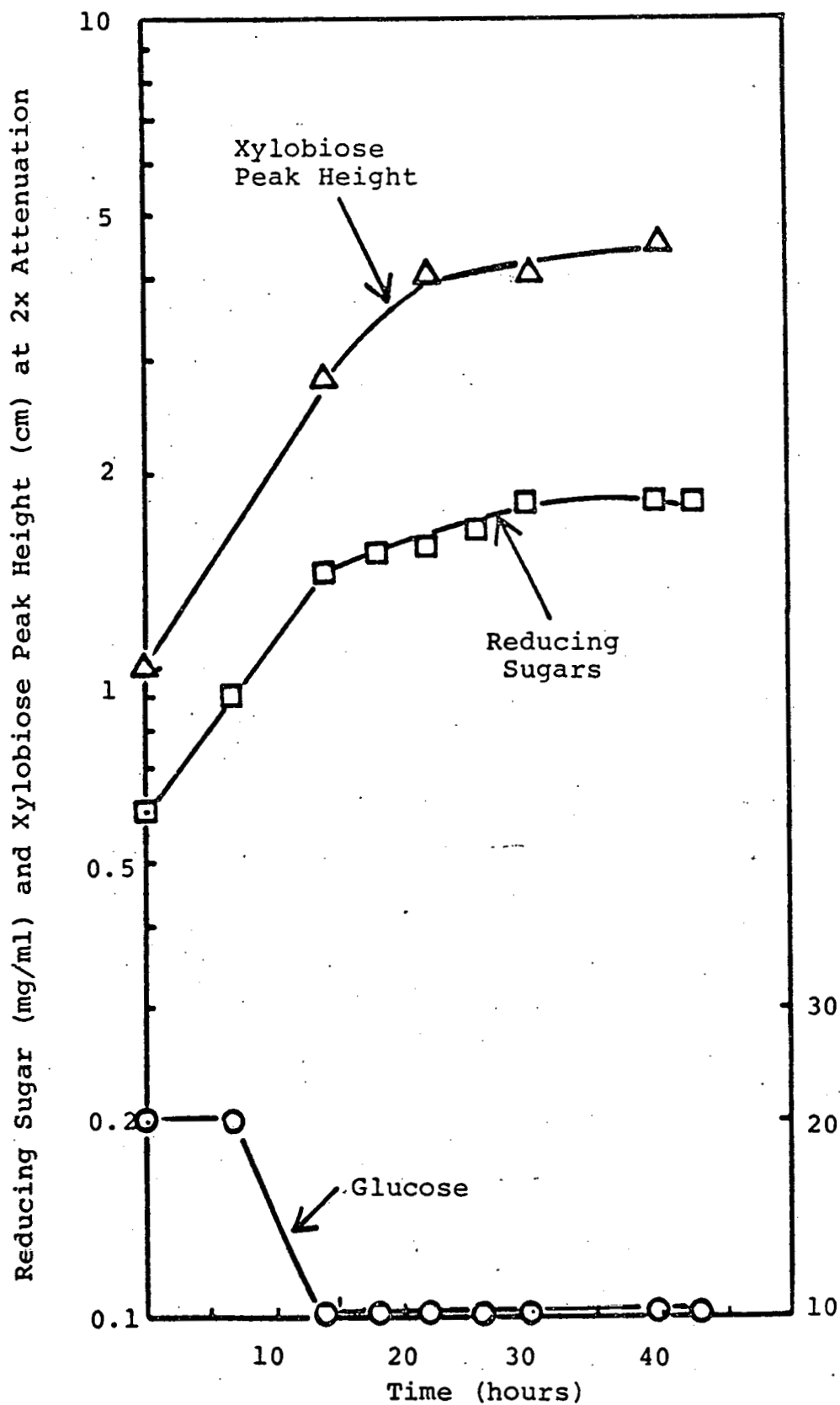


FIGURE I.B.1: Kinetics of Soluble Sugar Production During Fermentation of *C. thermocellum* ATCC 27405 on Solka Floc Cellulose

and glucose are plotted as well. It can be seen that 20 $\mu\text{g/ml}$ of glucose was present at the beginning of the fermentation, but by 15 hours the concentration had dropped to 10 $\mu\text{g/ml}$ where it remained throughout the rest of the fermentation. Xylobiose, on the other hand, appears to accumulate throughout the fermentation and follows a kinetic pattern similar to that of total reducing sugars which reached a final concentration of 1.8 grams per liter. It is plausible that the major portion of the reducing sugars measured by DNS is xylobiose, since no glucose or cellobiose accumulated in this fermentation.

For this fermentation, no assessment was made for cell mass accumulation. However, since it is known that reducing sugar accumulation is a growth associated event early in the fermentation and since the increasing heights of the xylobiose chromatographic peaks display a kinetic pattern like that of total sugars, a growth-associated accumulation of xylobiose is strongly suggested. This would then explain the observed discrepancy between HPLC and Gluco-stat measurements of glucose. Early in the fermentation of solka floc under non-pH-controlled conditions, mostly cellobiose and xylobiose appear in the medium. It is not until after the growth rate of the bacteria have declined substantially that the onset of glucose accumulation begins.

It should be recalled that in previous HPLC analyses, before the discovery of the resolution problem with

glucose and xylobiose, it was impossible to close the material balance between the total reducing sugars measured by DNS and the sum of the individual sugars (xylose, glucose and cellobiose) quantitated by HPLC analysis. It is quite likely that the amount of xylobiose which accumulates is sufficient to close the material balance. Efforts will continue to achieve the separation of glucose and xylobiose so that the two sugars may be quantitated accurately and the closure of the material balance completed.

C. EFFECT OF C. THERMOCELLUM FERMENTATION PRODUCTS
ON GROWTH AND PRODUCT ACCUMULATION

1. Growth Inhibition

Since the last progress report, we have performed an extensive investigation on the effect that acetate, propionate, butyrate, lactate, β -hydroxybutyrate, ethanol, propanol and butanol, exert on the growth rate of C. thermocellum ATCC 27405 and its ethanol resistant derivative C. thermocellum C9. In particular we have studied the growth of both strains in the presence of several concentrations of those organic compounds (range 0 to 30 grs/lit) at different growth temperatures (from 47°C to 64°C). The data are still in its processing period and will be used to calculate useful operational parameters such as rate inhibition index and other classical parameters currently utilized to describe the inhibition of biochemical processes. The results will be summarized in our next progress report.

2. Product Accumulation

a. Introduction

From the group of inhibitors we have characterized we have selected the three of them which accumulate in the broth during normal fermentation (namely, acetate, ethanol and lactate). We have investigated the effect that the addition of concentrations producing rate inhibition indexes below 0.5 have in the production of ethanol and acetate.

b. Materials and Methods

C. thermocellum ATCC 27405 was grown in CM4

medium, with cellobiose as C-source, in the presence of 0 to 8 grs/lit of acetate, lactate and ethanol. O.D.⁶⁶⁰ measurements were taken at hour intervals; at selected points in the growth curve, 0.5 ml aliquots were withdrawn to quantitate the amount of ethanol present in the medium. These aliquots were diluted (1:2) with 2.5N HCl and injected in a gas chromatograph, Hewlett-Packard 28756A, using a 6 ft. column packed with Chromosorb 101. Quantitation was performed by the internal standard method using isopropanol (1 gr/lit) as reference compound. The oven temperature was 135°C and the carrier gas flow rate was 60 ml/min. One µl of concentrated formic acid was injected before each sample injection to elute residual acetate.

c. Results

Figures I.C.1, I.C.2, I.C.3 and I.C.4 show the growth and product accumulation on CM-4-cellobiose medium and on CM-4-cellobiose medium amended with 8g acetate/l, 2.5g lactate/l and 4g ethanol/l, respectively.

Figures I.C.5.A, I.C.5.B and I.C.5.C show the levels of ethanol and acetate reached after 48 hr. of growth in CM4-cellobiose in the presence of acetate, lactate and ethanol. The quantitation of large amounts of acetic acid in our analytical system is not accurate enough, therefore we do not show acetate levels when the culture was grown in the presence of acetate.

d. Discussion

Our data show that both lactate and acetate stimulate

Figure I.C.1. Growth kinetics and product accumulation in CM-4-cellobiose.
No inhibitors added.

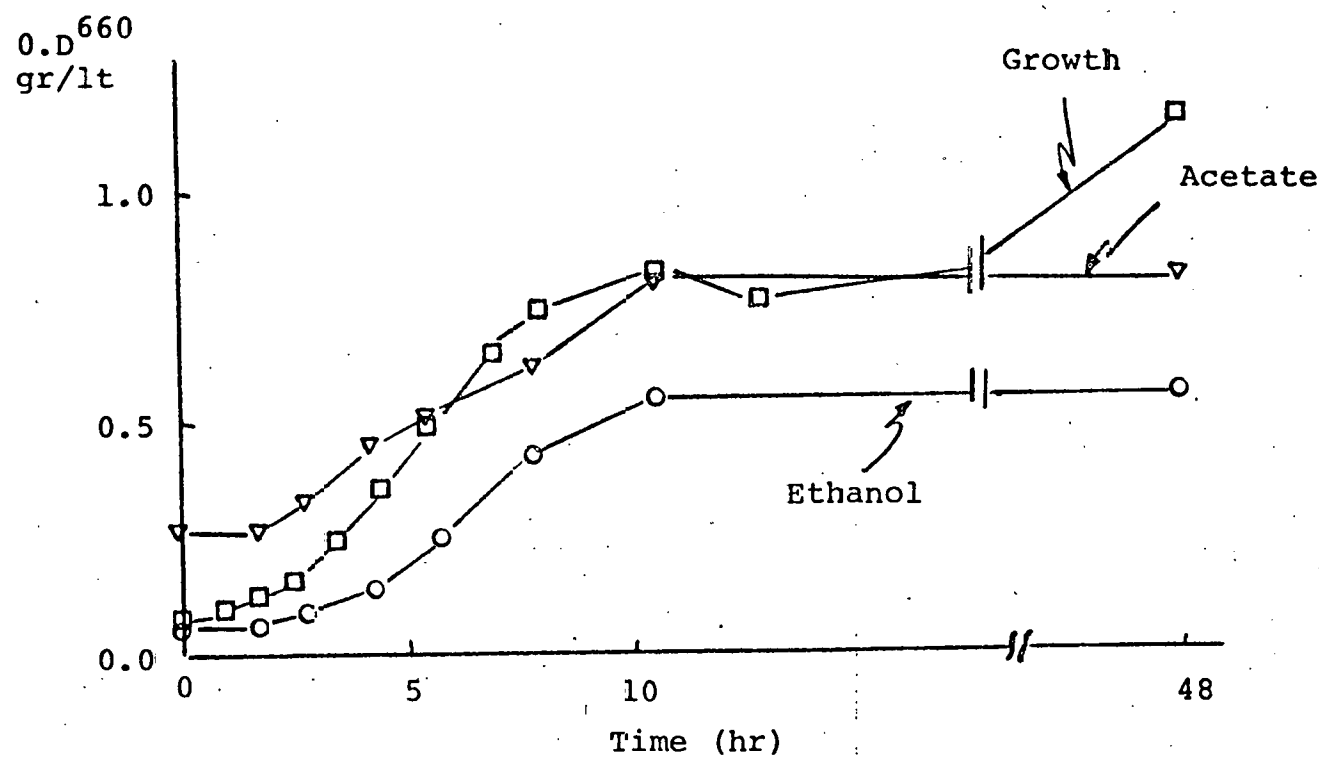


Figure I.C.2. Growth kinetics and product accumulation in CM4-cellobiose. Acetate (8g/l) were added at zero time.

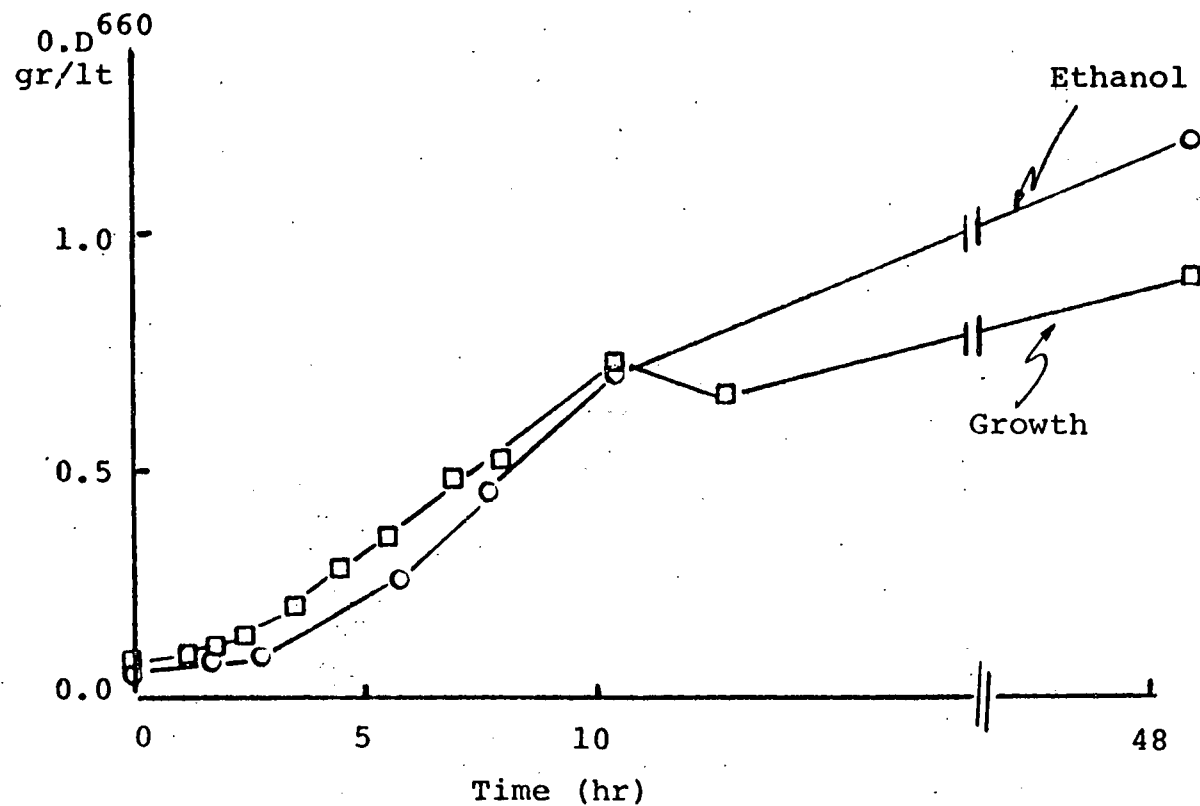


Figure I.C.3. Growth kinetics and product accumulation in CM4-cellobiose.
Lactate (2.5 g/l) was added at zero time.

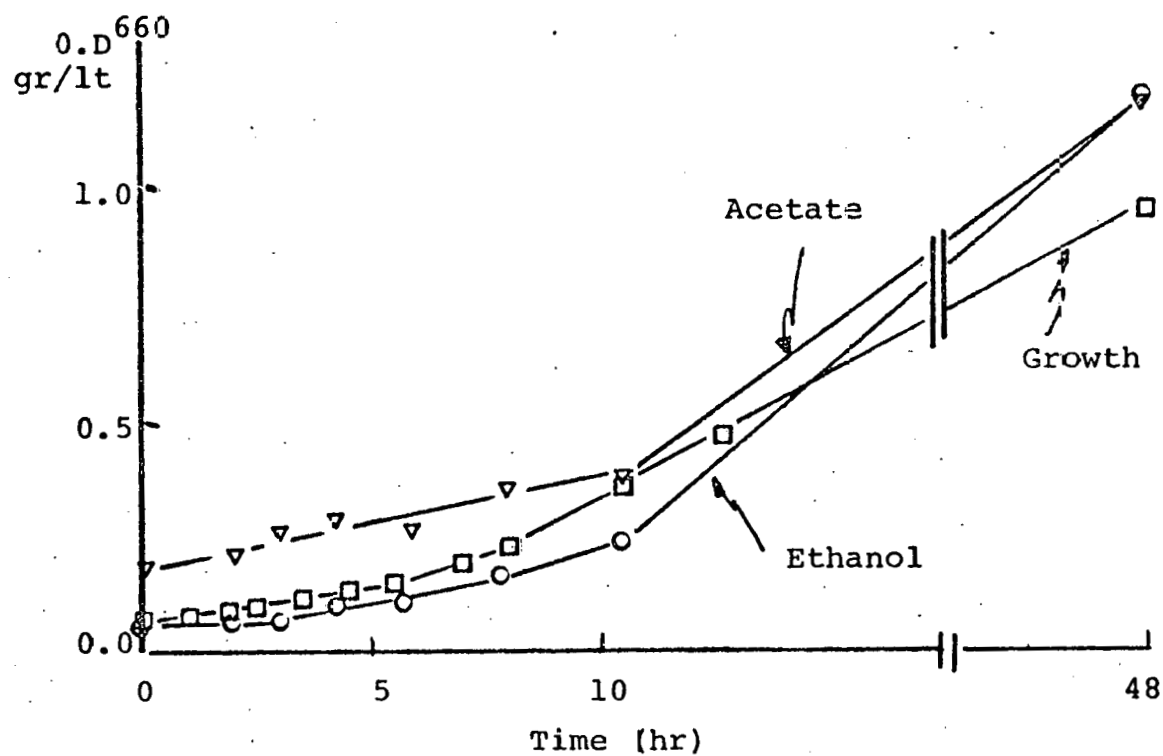
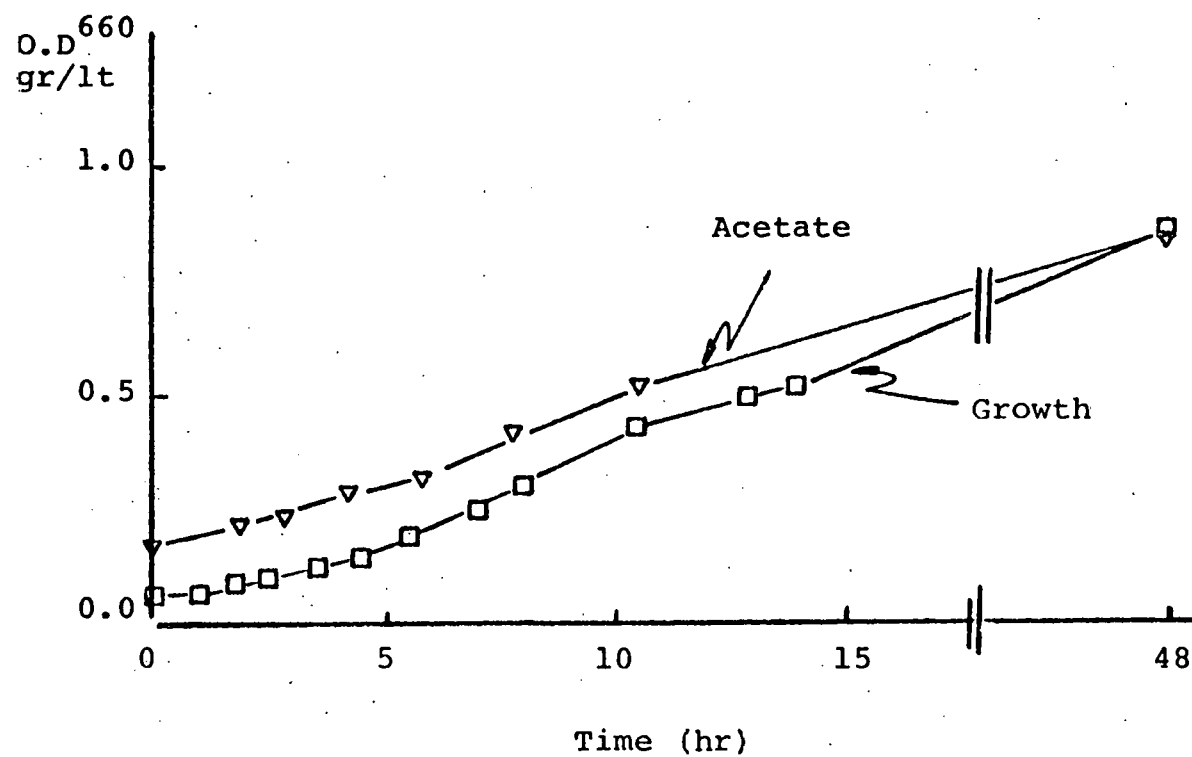


Figure I.C.4. Growth kinetics and product accumulation in CM4-cellobiose. Ethanol (4g/l) was added at zero time.



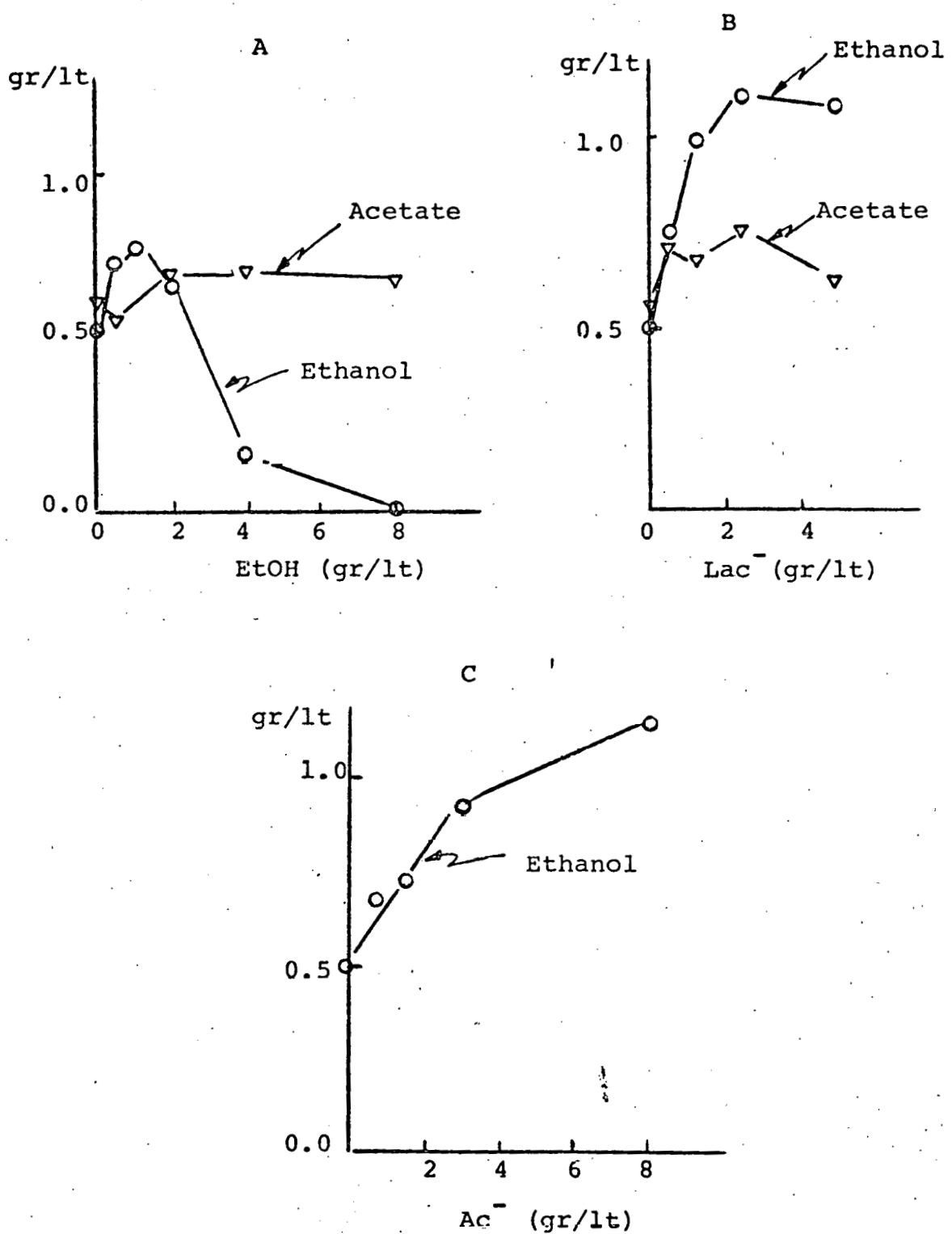
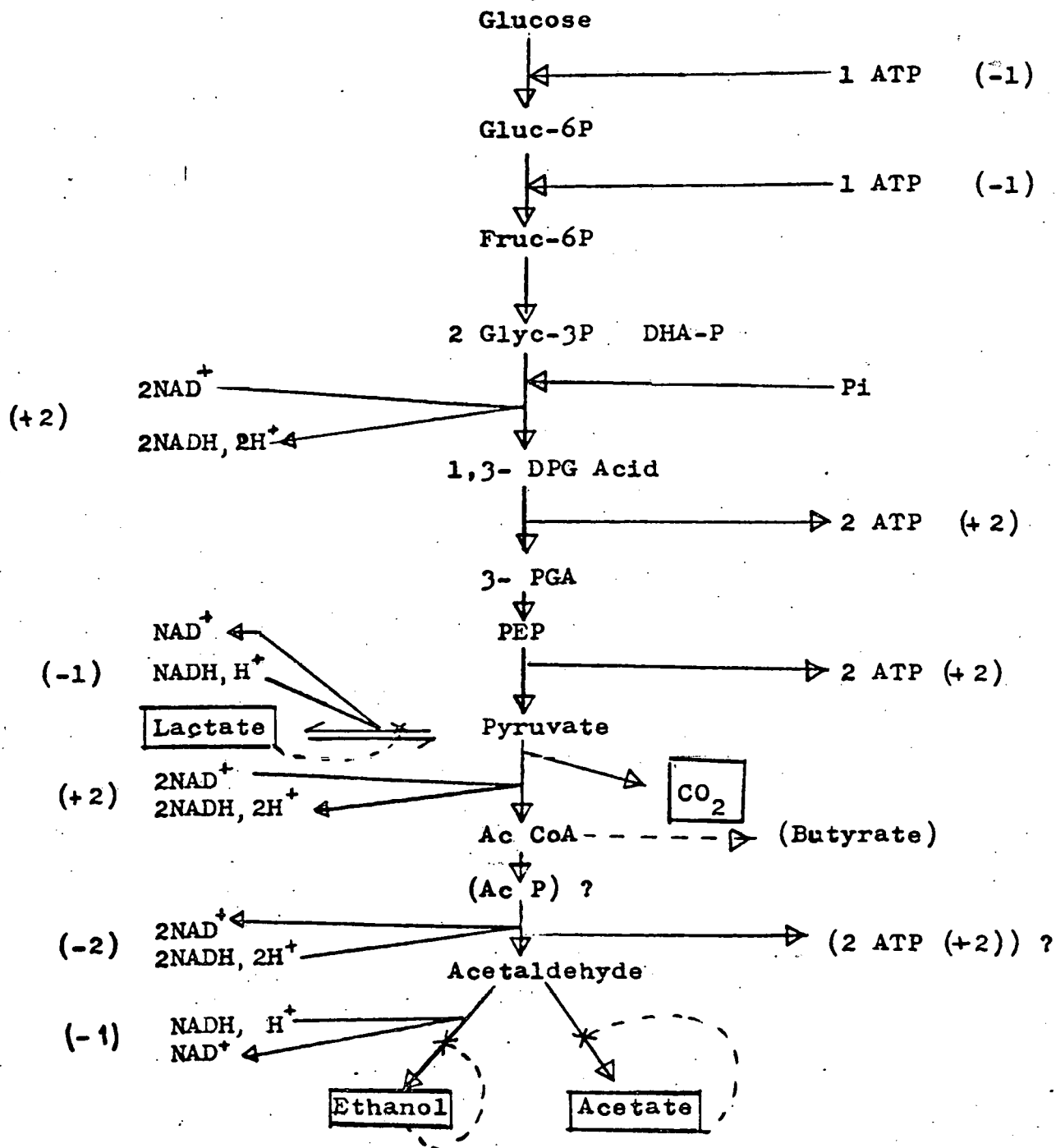


Figure I.C.5. Product formation after 48 hr. in CM-4-cellobio
 A. Effect of initial ethanol concentration
 B. Effect of initial lactate concentration
 C. Effect of initial acetate concentration

Figure I.C.6. Proposed pathway for glucose catabolism by C. thermocellum. Dotted lines represent feedback or repression.

Moles of ATP /mole of glucose



the production of ethanol in C. thermocellum ATCC 27405, while ethanol has the opposite effect. The increased level obtained in this experiment is double of that presented by the control at no inhibitor concentration. Preliminary results on acetate accumulation suggest that acetate may inhibit its own synthesis. The fact that lactate stimulates the production of ethanol indicates that lactate blocks its own synthesis so that the reduced piridine nucleotides must be oxidized by ethanol synthesis (see Figure I.C.6).

e. Future Work

- i. To characterize the behavior of the ethanol resistant strain C. thermocellum C9.
- ii. To evaluate lactic acid production in C. thermocellum ATCC and in C9 strains.

D. GENETIC MANIPULATIONS

1. Identification of HG-2

In order to identify HG-2 in preparation for its use as a host and as a source of cloned DNA, a battery of tests were performed. These tests unambiguously identified HG-2 as Clostridium thermosaccharolyticum, and can be summarized as follows:

Test Results in Agreement with Species
Description for C. thermosaccharolyticum
(Bergey's Manual, 8th ed)

Carbohydrates utilized:

- Arabinose
- Cellobiose
- Glucose
- Maltose
- Mannose
- Salicin
- Starch
- Sucrose
- Xylose

Carbohydrates not utilized:

- Mannitol
- Glycerol

- Melizitose
- Sorbitol
- Rhamnose
- Cellulose

Other Tests:

- Produces gas from carbohydrates
- Gelatin not hydrolysed
- Terminal spores
- Motile
- Long, thin sporulating anaerobe. During sporulation, longer, thinner, granulated forms are present in accordance with Pheil and Ordal (Appl. Microbiol. 15:893-898, 1967).
- Urease negative
- Catalase negative
- Gram negative reaction
- Produces acetic and butyric acids
- Indole not produced from tryptophan
- Not aerotolerant

Exception to agreement:

- Lactose not utilized by HG-2.

2. Lysozyme Treatment of C. thermosaccharolyticum HG-2

To test lysozyme treatment as a method of making protoplasts from HG-2, growing cells were suspended in lysozyme-containing P medium. This medium is modified from that given by Hopwood et al. (Nature 268:171-174, 1977) and is composed as follows:

Sucrose	103 g/l
K ₂ SO ₄	0.25 g/l
MgCl ₂ ·6H ₂ O	2.03 g/l
Cysteine·H ₂ O	0.5 g/l
KH ₂ PO ₄	0.05 g/l
Tris Base	3.03 g/l
CaCl ₂ ·2H ₂ O	1.84 g/l
MnSO ₄	5 μM
ZnSO ₄	5 μM
CuSO ₄	1 μM
CoCl ₂	1 μM
FeSO ₄	1 μM
NaMoO ₄	1 μM

The pH of KH₂PO₄ and Tris base was adjusted to 7.5 with HCl and this solution was autoclaved separately. CaCl₂·2H₂O was also autoclaved separately. Lysozyme concentrations up to 800 μg/ml were used but only rare protoplasts were produced. In the course of this experiment, the fact that this culture excretes large amounts of slime was noted. Chemical and/or genetic means

of removing the slime are being sought. The growth of HG-2 in the presence of sucrose is shown in Figure I.D.1.

3. Cloning

Preliminary work on the cloning of C. thermocellum DNA in E. coli has been performed. The necessary Memorandum of Understanding and Agreement has been approved and placed on file with the MIT Institutional Biohazards Committee and a P-2 laboratory has been established.

Total DNA from C. thermocellum has been prepared and analyzed by sucrose gradient centrifugation to ascertain that it contains a large amount of high molecular weight DNA. Digestion of this DNA with a variety of commercial restriction enzymes has been carried out and monitored using horizontal agarose gel electrophoresis. A method for the quantitative determination of the extent of digestion is being developed for the preparation of a defined partial digest for use in cloning.

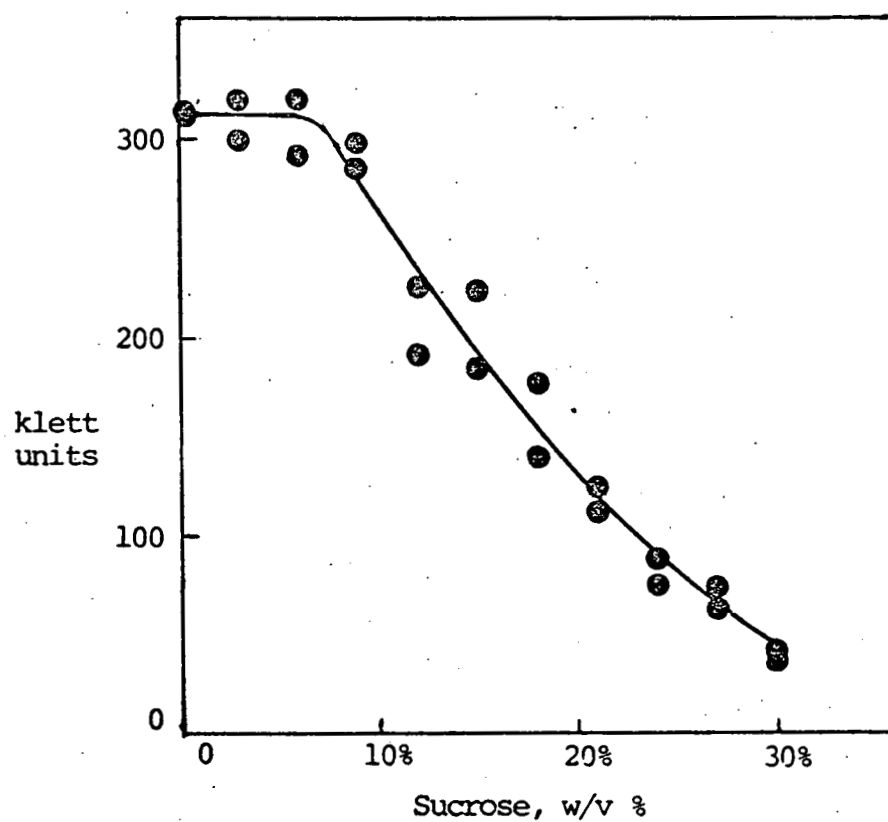
Equipment for the manipulation of large numbers of clones has been constructed. This equipment will also be used for replica-testing of mutagenized C. thermocellum cells.

4. Future Work

a. Mutants of C. thermosaccharolyticum HG-2 which do not excrete slime are being sought.

b. C. thermosaccharolyticum HG-2 will be treated with combinations of penicillin, lysozyme and hyaluronidase to effect protoplasting.

Figure I.D.1. Final cell densities of C. thermosaccharolyticum HG-2 in fluid thioglycollate medium containing sucrose.



c. Cloning of DNA from C. thermocellum in E. coli will be carried out.

E. MICROBIOLOGICAL STUDIES ON CLOSTRIDIUM THERMOCELLUM AND ITS CELLULASE PRODUCTION

1. Introduction

In our earlier report (COO-4198-7), we presented results showing growth and production of cellulase in glucose medium containing a high (0.6%) yeast extract concentration. It was considered important to determine the ability of glucose to support growth and cellulase production during repeated transfers in the absence of cellulose and cellobiose. These results are presented in this progress report.

We have continued our attempts to devise a chemically-defined minimal growth medium.

The stimulatory effect of a thiol-reducing agent (cysteine-HCl) on trinitrophenyl-carboxymethylcellulose (TNP-CMC) hydrolysis was shown earlier (Progress Report COO-4198-7). In this report, we present studies on the effect of this reducing agent on Avicel hydrolysis and filter paper hydrolysis activities.

Our continuous attempts to obtain improved cellulase-producing mutants have resulted in the successful isolation of a new (third generation) cellulase-hyperproducing mutant, JMT-11 and results of our mutational experiments are presented in this report.

2. Materials and Methods

a. Organism

C. thermocellum ATCC 27405 and its mutants AS-39, CM173 and JMT-11 were used in this study.

b. Anaerobic Methods

See Progress Report COO-4198-7, page 71.

c. Cultural Conditions

The organisms were grown and maintained on GS medium which is described in Progress Report COO-4198-7, page 71. In the nutritional studies, yeast extract in the GS medium was replaced by various mixtures of vitamins, amino acids and a protein hydrolysate. Growth was measured as absorbance using the red filter in a Klett-Summerson colorimeter.

d. Analysis of Cellulolytic Activity

TNP-CMCase, Avicel-hydrolyzing and filter paper-hydrolyzing activities were assayed as described in Progress Report COO-4198-8, page 53-55.

3. Results and Discussion

Glucose utilization: A series of experiments were performed to study the ability of C. thermocellum to utilize glucose as sole source of carbon for growth and the production of cellulase. The results presented in Table I.E.1 indicate that C. thermocellum CM-17-3 grows on glucose and produces cellulase during 5 sequential transfers. It thus appears that glucose repression of cellulase production does not exist in this organism. These findings are especially important because the production of cellulases by all other microorganisms is repressed by glucose, the product of the enzymatic reaction, thus limiting their use as cellulase producers.

TABLE I.E.1

EFFECTS OF SEQUENTIAL TRANSFER OF C. THERMOCELLUM CULTURE
CM-17-3 IN GLUCOSE--GS MEDIUM ON GROWTH AND TNP-CMCASE PRODUCTION

Transfer No.*	Growth (Δ Klett units)			TNP-CMCase (Units/ml.)	
	24h.	48h.	72h.	48h.	72h.
1	80	126	134	1.60	1.38
2	119	135	140	1.29	1.30
3	106	160	172	1.40	1.43
4	112	156	168	1.38	1.45
5	103	139	163	1.37	1.33

*Transfer to the next flask of the series was made at 48h.

Growth and production on corn stover and sorbitol:

Various commercially available types of CMC, as well as sorbitol and corn stover were compared to cellobiose, Avicel and MN-300 cellulose for their ability to support growth and enzyme production by C. thermocellum (Table I.E.2). It was observed that none of the 6 CMC types studied could support growth. Sorbitol also failed to support growth. Corn stover supported growth and about 10% extracellular TNP-CMCase production compared to cellobiose grown cultures, while Avicel and MN-300 supported good growth and about 50% of normal extracellular enzyme production. It should be realized that in the case of corn stover, Avicel and MN-300, a considerable proportion of cells and enzyme are adsorbed to the cellulose particles.

Development of chemically-defined medium: In order to develop a chemically defined medium, we have continued our nutritional studies. As shown in previous reports, casein-hydrolysate plus eight vitamins could replace yeast-extract for growth. In Table I.E.3, it can be seen that this was confirmed. However, the table also shows that the vitamin mix alone, as well as the casein digest alone, supports growth. The latter observation is probably due to contamination of the enzyme-hydrolyzed casein with vitamins since the amino acid mix was inactive by itself. The conclusion that only vitamins were necessary was confirmed in second and third transfer experiments using the "vitamins only" flask from the previous experiment as inoculum. In both of these transfer experiments, growth took place on vitamins alone, casein-hydrolysate alone and on the mixture of the two.

TABLE I.E.2

GROWTH OF C. THERMOCELLUM CM-17-3 AND ENZYME PRODUCTION
ON VARIOUS COMMERCIAL CARBON SOURCES

Carbon Source	Growth (Δ Klett units)					TNP-CMCase (Units/ml.) at 48h.
	24h	48h	72h	96h	120h	
Cellobiose	123	174	184	178	161	1.97
Sorbitol	12	9	11	8	7	0.0
Corn Stover	55	59	62	65	65	0.20
CMC 41	17	14	18	21	14	0.0
CMC 7H4	16	15	14	17	15	0.0
CMC 9M31	18	19	19	17	19	0.0
CMC 12M31	11	6	5	9	9	0.0
CMC 7H	26	13	7	10	14	0.0
CMC M 14045	Incubation at 60°C Results in Formation of Very Viscous Thick Slurry-like Substance					0.0
MN-300*	160	225	345	320	280	0.86
Avicel*	180	240	360	305	250	0.84

*Exact determination of growth is difficult in these flasks due to the presence of very fine particles of cellulose.

TABLE I.E.3
EFFECTS OF REPLACEMENT OF YEAST EXTRACT BY AN AMINO ACID MIXTURE,
VITAMIN MIXTURE AND/OR CASEIN HYDROLYSATE ON THE GROWTH OF C. THERMO-
CELLUM CM-17-3

Supplement to Medium Less Yeast Extract	Growth (Δ Klett Units)				
	24h	48h	72h	96h	120h
Yeast Extract	150	172	194	176	158
8 Vitamins + Casein Hydrolysate (ENZ)	89	104	116	98	90
Casein Hydrolysate (ENZ)	0	75	61	45	27
Amino Acid Mix**	8	0	0	0	3
8 Vitamins	0	88	95	71	52*

*Used to inoculate the next experiment.

**Approximating the composition of casein hydrolysate.

Our next task was to find out the essential vitamin(s) required for growth. In this series of experiments, one vitamin was omitted at a time and the effect of each deletion on growth was studied.

It appeared from the first experiment (see Table I.E.4) that only pyridoxine is required for growth.

However, it was immediately clear from the results of experiment 2 that biotin is also a requirement (Table I.E.4). In this experiment, individual flasks were inoculated from the corresponding flask of experiment 1 with the exception of the "no pyridoxine" flask which was inoculated with the "8 vitamin" inoculum. There was total absence of growth when biotin was not included in the medium. In the first experiment, growth in the absence of biotin probably resulted because of biotin carry-over from the "8-vitamin" inoculum.

This requirement for pyridoxine plus biotin was further supported by the results of our next experiment (Table I.E.5). Growth did not occur when a biotin-depleted inoculum was used to inoculate a flask containing all vitamins except biotin. Similarly, a pyridoxine-depleted inoculum did not grow in the absence of pyridoxine. The effect of pyridoxine concentration is also shown in Table I.E.5. Although the scatter in the data is bad, there is a suggestion that 15X pyridoxine (15ug/ml) might be optimal although 1X (1ug/ml) is certainly sufficient for growth.

TABLE I.E.4

EFFECTS OF DELETION OF INDIVIDUAL VITAMINS ON
GROWTH OF CLOSTRIDIUM THERMOCELLUM CM-17-3

Supplement	Growth (Δ Klett units)			
	Expt. 1		Expt. 2	
	24h	48h	24h	48h
Yeast Extract	100	163	106	173
8 Vitamins	43	48	25	117
8 Vitamins less Pyridoxine	0	0	2	4
8 Vitamins less niacin	35	45	24	113
8 Vitamins less riboflavin	37	46	25	112
8 Vitamins less biotin	37	42	2	0
8 Vitamins less folate	34	42	23	125
8 Vitamins less thiamine	28	37	17	129
8 Vitamins less pantothenate	36	44	25	118
8 Vitamins less B ₁₂	37	45	44	120

TABLE I.E.5
EFFECT OF BIOTIN AND PYRIDOXINE ON GROWTH OF C. THERMOCELLUM
CM-17-3

Inoculum	Supplement	Growth (Δ Klett)		
		24h	48h	72h
Biotin-Depleted	Yeast Extract	84	164	157
	8 Vitamins	52	113	92
	8 Vitamins less Biotin	0	12	6
Pyridoxine-Depleted	8 Vit. less Pyridoxine	0	1	3
	8 Vit.; 1X* pyridoxine	29	72	83
	8 Vit., 2X	27	78	106
	8 Vit.; 5X	23	64	109
	8 Vit.; 7X	23	67	121
	8 Vit.; 10X	25	60	61(?)
	8 Vit.; 15X	22	50	147

*1X Pyridoxine = 1 μ g/ml

A similar type of experiment was performed in which pyridoxine concentration was studied in the presence of biotin as the only other vitamin in the medium (Table I.E.6). It again appeared that an increase in pyridoxine level to 15X increased growth to its maximum. However, more important than the effect on growth is the effect on enzyme formation which indicates 1X pyridoxine to be optimal in the presence of 1X biotin. In fact, 65% as much enzyme was produced in the presence of 1X pyridoxine and biotin as was observed with yeast extract despite the fact that growth was only 27% as much.

It was considered important to prove that the two vitamins could support the growth of sequentially transferred cultures. As shown in Table I.E.7, this is the case. Enzyme production has not yet been determined in this series of experiments.

From these experiments, it can be concluded that the two vitamins, pyridoxine and biotin, are capable of replacing yeast extract for growth of C. thermocellum and that a chemically-defined medium is now available. However, the optimum level of biotin must still be established.

Effect of cysteine in assay mixture: Media used to grow C. thermocellum contain cysteine-HCl, a strong reducing agent. In our earlier report (COO-4198-7), we showed that 1mg/ml cysteine added to a TNP-CMC assay mixture stimulates TNP-CMC activity by 65%. We thus studied the effect of

TABLE I.E.6

EFFECT OF PYRIDOXINE CONCENTRATION ON GROWTH OF *C. THERMOCELLUM*
and CM-17-3 ENZYME PRODUCTION IN BIOTIN-CONTAINING MEDIUM

Inoculum	Supplement	Growth (Δ Klett)			TNP-CMCase (units/ml)
		24h	48h	72h	72h
Biotin-depleted	1X Biotin	2	6	2	0
	1X Pyridoxine	2	0	2	0
	1X Biotin + 1X Pyridoxine	9	42	52	1.73
8 Vitamins	Yeast Extract	123	193	189	2.61
	1X Biotin + 2X Pyridoxine	2	43	66	1.39
	" " + 5X "	8	79	74	1.08
	" " + 7X "	5	78	68	1.30
	" " + 10X "	3	59	89	1.06
	" " + 15X "	5	56	108	1.41
	" " +100X "	7	57	90	1.15

*1X Pyridoxine = 1μg/ml; 1X biotin = 10ng/ml

TABLE I.E.7
EFFECTS OF SEQUENTIAL TRANSFER OF THE CULTURE IN MEDIUM CONTAINING 1X PYRIDOXINE AND BIOTIN ON GROWTH OF
C. THERMOCELLUM CM-17-3

Transfer No.	Growth (Δ Klett)		
	24h	48h	72h
1	2	43	66
2	12	22	48
3	32	58	-
4	39	84	-
5	42	89	

*In each case, the previous transfer was used as inoculum.

cysteine-HCl on Avicel hydrolyzing activity and filter paper activity.

It can be seen (Table I.E.8) that cysteine-HCl stimulated Avicel hydrolyzing activity by about 40% at 5mg/ml.

Similarly, filter paper activity was stimulated by about 25% when 1mg/ml cysteine-HCl was used.

These results in addition to our earlier reported results indicate that the inclusion of a strong thiol-reducing reagent, cysteine-HCl, in the reaction mixture allows an increased rate of cellulose hydrolysis.

Isolation of a new hyper-producing strain: A cellulase-overproducing strain JMT-11 has been isolated by sequential passage of the C. thermocellum CM-17-3 culture through the GS medium containing Avicel as the main source of carbon. The tubes containing this medium were initially inoculated with the CM-17-3 culture. After seven days of incubation, a small portion of cells containing the characteristic yellow pigment from this tube was transferred to another tube. After 48 hours, cells were transferred to a third tube and the same procedure was repeated several times. After eleven such transfers, the population was tested for TNP-CMCase production after transfer to a tube containing cellobiose medium. After 48 hours of growth, the culture supernatant was assayed for cellulolytic activities. Data shown in Table I.E.9 indicated about a 40% improvement in TNP-CMCase titer in the first tube test and about 90% in the second.

TABLE I.E.8
EFFECT OF CYSTEINE-HCL ON AVICEL HYDROLYZING AND FILTER PAPER
ACTIVITY IN VITRO

Cysteine-HCl Added to the Reaction Mixture (mg/ml)	Avicel* Hydrolyzing Activity Units/ml.	Filter Paper** Activity Units/ml.
0.0	0.085	0.030
0.1	0.084	0.028
0.5	0.083	0.032
1.0	0.090	0.038
1.5	0.083	0.037
2.0	0.104	0.038
5.0	0.118	0.035
7.5	0.111	0.035
10.0	0.116	0.038
15.0	0.109	0.037

*Avicel Type PH 105 was used

**Filter paper discs were used in this assay as described by Montencourt et al., Biotechnol. Bioeng. 20, 297 (1978). To convert to international FPase units, the above values should be multiplied by 2.8.

TABLE I.E.9

COMPARISON OF CELLULOLYTIC ACTIVITY OF PARENT CM-17-3
AND NEW SPONTANEOUS MUTANT (JMT-11)

Type fermentation	Major Carbon Source	Cellulase (48h)			
		TNP-CMCase (μ /ml)		Avicel (u/ml)	
		CM-17-3	JMT-11	CM-17-3	JMT-11
Single Tubes	cellobiose	1.00	1.38	ND*	ND
Triplicate Tubes	cellobiose	0.68	1.29	ND	ND
Single Flasks	cellobiose	2.16	3.10	0.045	0.064
	Avicel	1.01	1.55	0.022	0.026

*ND = not done

Enzyme production by JMT-11 was also studied in flasks containing cellobiose or Avicel as major carbon source (Table I.E.9). Our results indicate that JMT-11 produces about 40% more TNP-CMCase than its parent (CM-17-3) when grown in cellobiose and about 50% more when Avicel was used as carbon source for growth. Production of Avicelase was improved in the mutant by about 40% when growing on cellobiose and about 20% when growing on Avicel.

In Table I.E.10 is shown the ability of supernatants from 4 cultures to hydrolyze various types of Avicel after growth on cellobiose. It can be seen that JMT-11 is about 4-5 times as active as the original ATCC culture. Also of interest is the different activities observed on different types of Avicel, all from FMC Corporation. It appears that hydrolysis of type PH 105 (the only one that does not contain CMC) is fastest. We however do not know the influence of particle size on hydrolysis rate.

It is thus apparent that JMT-11 is a better cellulase producer than the CM-17-3 culture and henceforth this 3rd generation mutant will be used for further studies.

We have also received confirmatory data of superiority of our first generation mutant (AS-39) from Zeikus and Lamed (Univ. Wisconsin). We found this culture to be 1-fold improved over ATCC 27405 in endo- and exo- β -glucanase production. The Wisconsin researchers have only examined the endo- β -glucanase

TABLE I.E.10

HYDROLYSIS OF VARIOUS AVICEL TYPES BY SUPERNATANTS OF OUR STRAIN-SELECTION PROGRAM

Culture	Avicelase activity (units/ml)		
	Avicel RC591	Avicel RC501	Avicel PH105
ATCC 27405	0.011	0.021	0.028
↓			
AS-39	0.020	0.044	0.058
↓			
CM-17-3	0.033	0.065	0.095
↓			
JMT-11	0.047	0.088	0.124

levels and found a 4-fold increase. They have our 2nd generation mutants but have not yet examined them.

Additional strain development work: EMS-Agar/plate mutagenesis procedures reported in previous progress reports have been continued. Using mutant CM-17-3 as the parent, 17,000 colonies have been screened, and 17 were selected for TNP-CMCase activity, with none indicating higher activity than CM-17-3. This method is being continued with the elimination of cellobiose from the GS-Avicel agar medium. Also, serial transfer of JMT-11 in GS-Avicel tubes is being continued. In addition, a new method of selection for spontaneous mutants (using JMT-11 as the parent) is presently being conducted. Using Hungate tubes containing GS medium with filter paper as the major carbon source, cells are transferred each day to a new tube. This is done when the outer surface of the filter paper contains a very thin layer of cells and yellow pigment. Vortexing the tube releases the cells from the filter paper into the liquid medium, from which a small portion is transferred to a new tube. To date, eighteen such transfers have been made, and samples will now be transferred to GS-cellobiose medium for testing production of cellulase. In addition, these serial transfers using avicel and filter paper will be intermittently subjected to EMS mutagenesis and further selection.

4. Future Work

Future studies will include:

Completion of nutritional experiments.

Study of the degradation of corn stover by soluble enzyme from each of our mutants. The aim is to determine whether increased enzyme activity as measured on artificial substrates is relevant to the hydrolysis of natural substrates.

Continued mutagenesis and selection of cellulase-overproducing mutants.

F. CARBOHYDRATE UTILIZATION OF C. THERMOCELLUM

1. Introduction

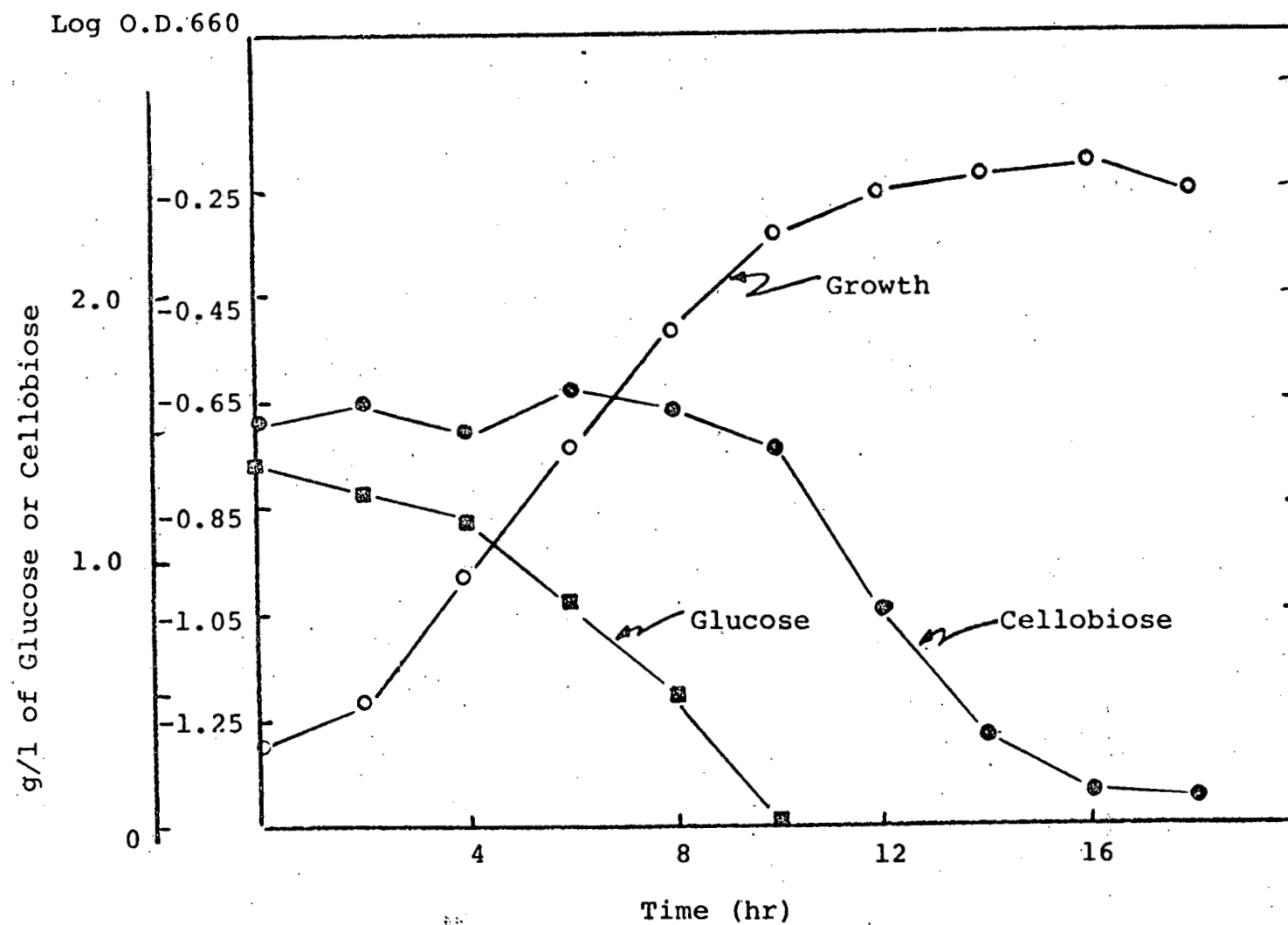
C. thermocellum has been previously reported to accumulate reducing sugars during growth in solka floc or corn stover. This accumulation may be at least partly due to a differential preference of the microorganism towards growth in the different sugars generated from cellulose hydrolysis. During the past quarter we have investigated the growth and utilization kinetics of C. thermocellum growing in mixtures of glucose and cellobiose.

2. Materials and Methods

C. thermocellum ATCC 27405 adapted for growth on glucose or cellobiose was used. Adaptation was achieved by growing the culture at 60°C for at least 20 generations in the respective carbohydrates (6 g/l). The cultures were inoculated into CM-4 medium containing approximately 1.5g of cellobiose/l and 1.5g of glucose/l. Growth at 60°C was followed by optical density at 660nm. The levels of glucose in the medium were determined through the colorimetric assay of glucose oxidation with glucose oxidase. The levels of cellobiose were obtained from the difference between total reducing sugars and glucose. All values were corrected for the levels of non-utilizable reducing sugars present in CM-4 medium.

Figure I.F.1. shows the results obtained when the culture has been adapted to growth in glucose. It is evident

Figure I.F.1. Growth and sugar utilization of C. thermocellum adapted to growth in glucose.



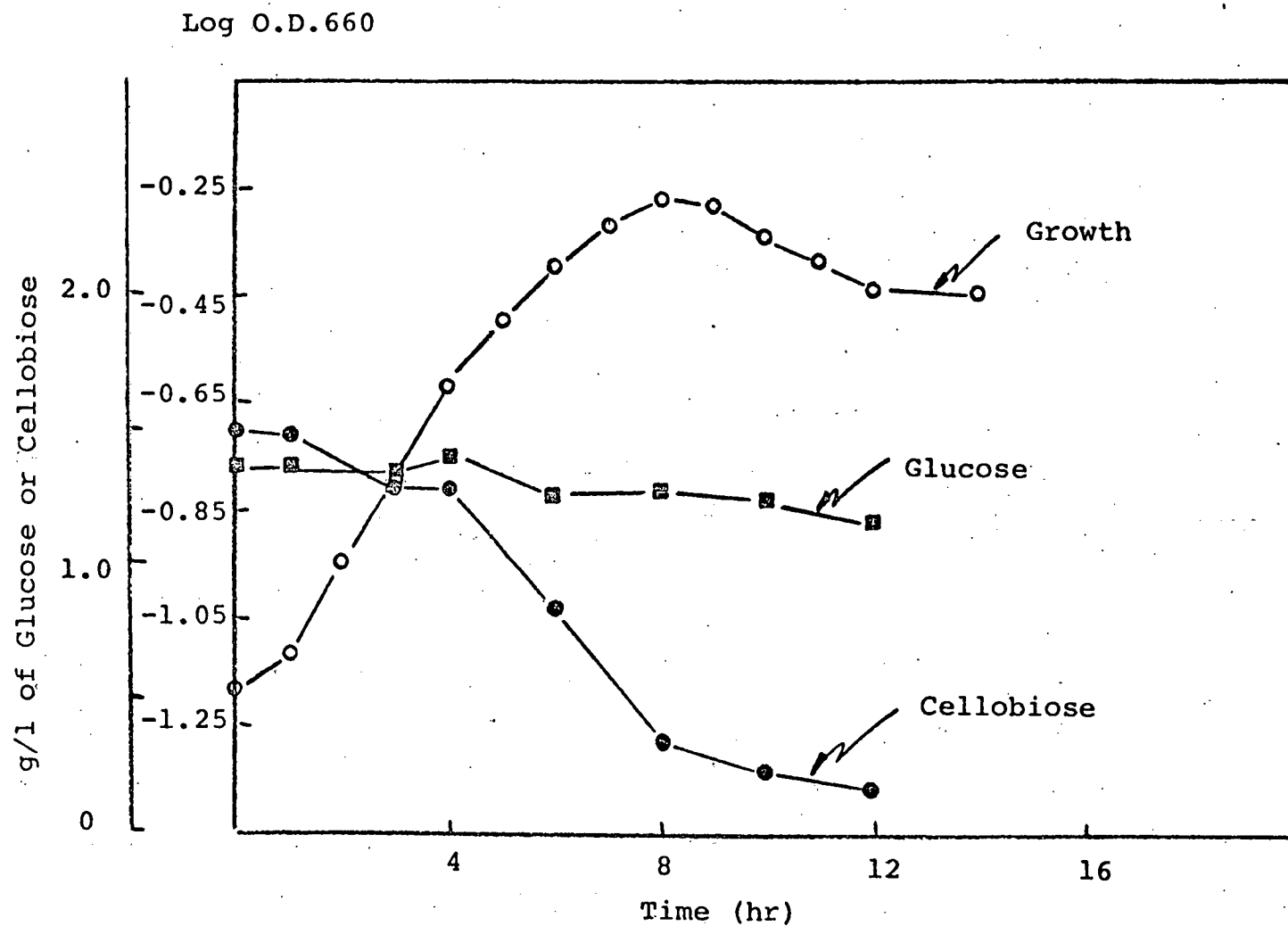
from the data that these glucose-adapted cultures used glucose first and cellobiose was not utilized until the glucose was exhausted from the medium.

In contrast, when the culture was first adapted for growth in cellobiose and then exposed to a mixture of glucose and cellobiose (Figure I.F.2.), the preferred carbon source was cellobiose. In fact, this culture did not consume any detectable glucose during the first 8 hr. of fermentation. At the end of 12 hrs. 200 mg/l of glucose has been utilized. At the end of 72 hrs. 1g of glucose/l had been utilized. This utilization occurred without any detectable increases in cell mass. It should be pointed out, however, that our glucose assay does not distinguish between partial or complete catabolism of glucose.

The lack of glucose utilization in the presence of cellobiose cannot be interpreted as repression by the latter on the former. This is due to the fact that control experiments in which glucose was the only added carbon source demonstrated that glucose utilization was delayed for 16 hrs. after inoculation. Thus, one cannot distinguish between cellobiose repression of glucose utilization and a long lag time for glucose utilization independent of the presence of cellobiose.

Once cellobiose is hydrolyzed, the catabolic pathways of a cellobiose and glucose are indistinguishable, thus the effects we have observed are probably due to the mechanisms of glucose uptake and cellobiose uptake and hydrolysis.

Figure I.F.2. Growth and sugar utilization of C. thermocellum adapted to growth in cellobiose.



3. Future Work

We will continue to investigate the utilization of glucose and cellobiose by C. thermocellum, in particular the mechanisms of transport of these sugars.

G. PRODUCTION OF LIQUID FUEL, ETHANOL

1. Production of Ethanol by Clostridium thermocellum

a. Introduction

The production of ethanol by Clostridium thermocellum using cellulosic biomasses has continued to focus on increasing the tolerance of this organism and on increasing the product concentration. A selection program to increase the tolerance of C. thermocellum (S-4) has yielded a number of different strains. These mutants have been tested in different cellulose to evaluate their degradation and product formation capabilities. The results from these studies will be presented in this section.

b. Materials and Methods

The adaptation and selection of ethanol tolerant mutants were performed using strain S-4 (see COO-4198-7, -8) of Clostridium thermocellum. The medium used for fermentation studies as well as the analytical procedures have been reported previously. The adaptation and selection protocol for ethanol tolerant strain is shown in Table I.G.1.1. The original organism for the adaptation and selection program was C. thermocellum, strain S-4. In the presence of 1% V/V ethanol, this strain was transferred during its growth in the exponential

TABLE I.G.1.1: PROTOCOL OF ADAPTATION AND SELECTION OF
ETHANOL TOLERANCE STRAIN OF C. thermocellum

1. Clostridium thermocellum (S-4) in presence of 1% V/V ethanol:
10% Inoculum, transferred from glucose → Solka floc →
cellobiose (repeated once)

2. Criterion used to increase ethanol concentration:
cell concentration attained in minimal time to a value
of 0.4 O.D. unit

3. Ethanol concentration increased by 0.2% V/V:
10% Inoculum transferred from glucose → Solka floc →
cellobiose: repeated at constant ethanol concentration from
glucose → Solka floc → cellobiose



4. Total number of transfers to reach 8% V/V ethanol = 65

5. Test for growth at various ethanol concentrations:
strains S-6 and S-B.

phase from glucose to Solka floc to cellobiose using a 10% inoculum. At this initial ethanol concentration (1% V/V) this passage from one carbon source to another is repeated once again. The reason for the passage into the different carbon sources, particularly Solka floc, is to ensure that the isolate can grow in the presence of ethanol and still possess its cellulolytic degradation capability.

The criterion which was used to increase the concentration of ethanol is a combination of cell density attained in a given length of time. In our procedure we have used a cell concentration when the optical density reaches 0.4 O.D. unit (~ 0.5 g/L of cell). Our experiences have shown in this region and range of cell concentration, the growth of the organism is usually in the exponential phase. In addition to the attainment of a given cell density, we have selected to use the time required to reach this desired optical density as a variable. This is to say that the minimal time to reach 0.4 O.D. unit, which varies from transfer to transfer, is a second criteria of our selection procedure. In this fashion we are able to select those isolates which have rapid rates of growth on the different carbon sources.

This procedure is repeated through increasing the ethanol concentration by 0.2% V/V. At the new but higher ethanol concentration, the entire procedure is again

repeated. At each increased ethanol concentration the sequential transfer from glucose to Solka floc to cellobiose is performed twice. A total of 65 repeated transfers were performed using this adaptation and selection procedure. After these 65 transfers are completed, the final ethanol concentration reached was 8% (V/V). The isolates were then tested systematically with respect to its growth and tolerance to ethanol.

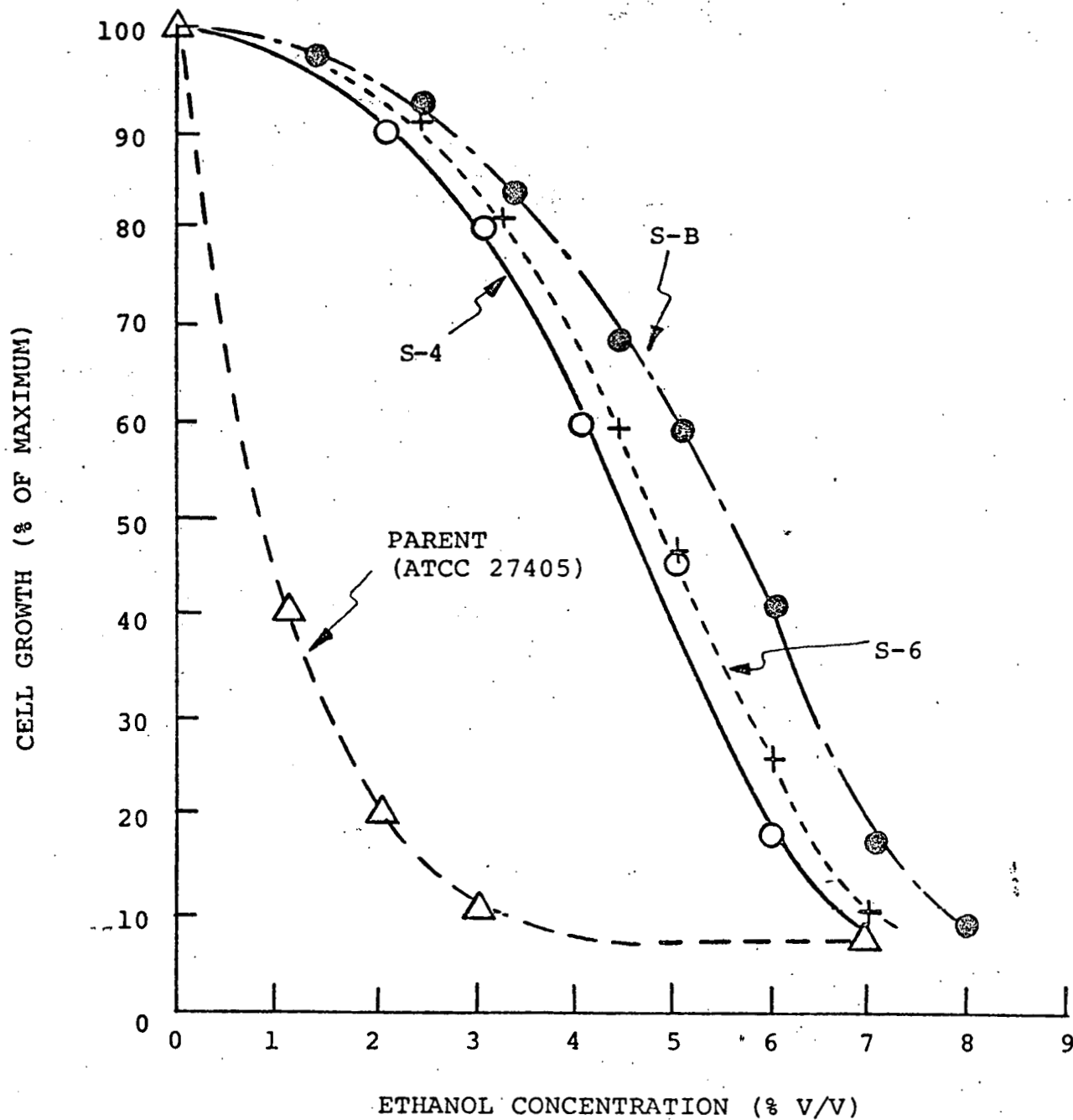
c. Results and Discussion

Through the adaptation and selection procedure two new strains designated as C. thermocellum S-6 and S-B were isolated. The ability of these isolates to grow in the presence of different ethanol concentrations is shown in Figure I.G.1.1. The tolerance of the parent (ATCC27405) and the earlier isolate S-4 to ethanol is also shown in this figure. It can be seen that strain S-6 has slightly higher tolerance to ethanol than S-4. Strain S-B shows further improvement towards ethanol tolerance than isolate S-6.

Strain S-6 was then examined with respect to its rate of growth on various carbon sources. In these experiments four carbon sources were tested: glucose, cellobiose, Solka floc and xylose. The fermentations were performed at 60°C in anaerobic flasks using manual pH

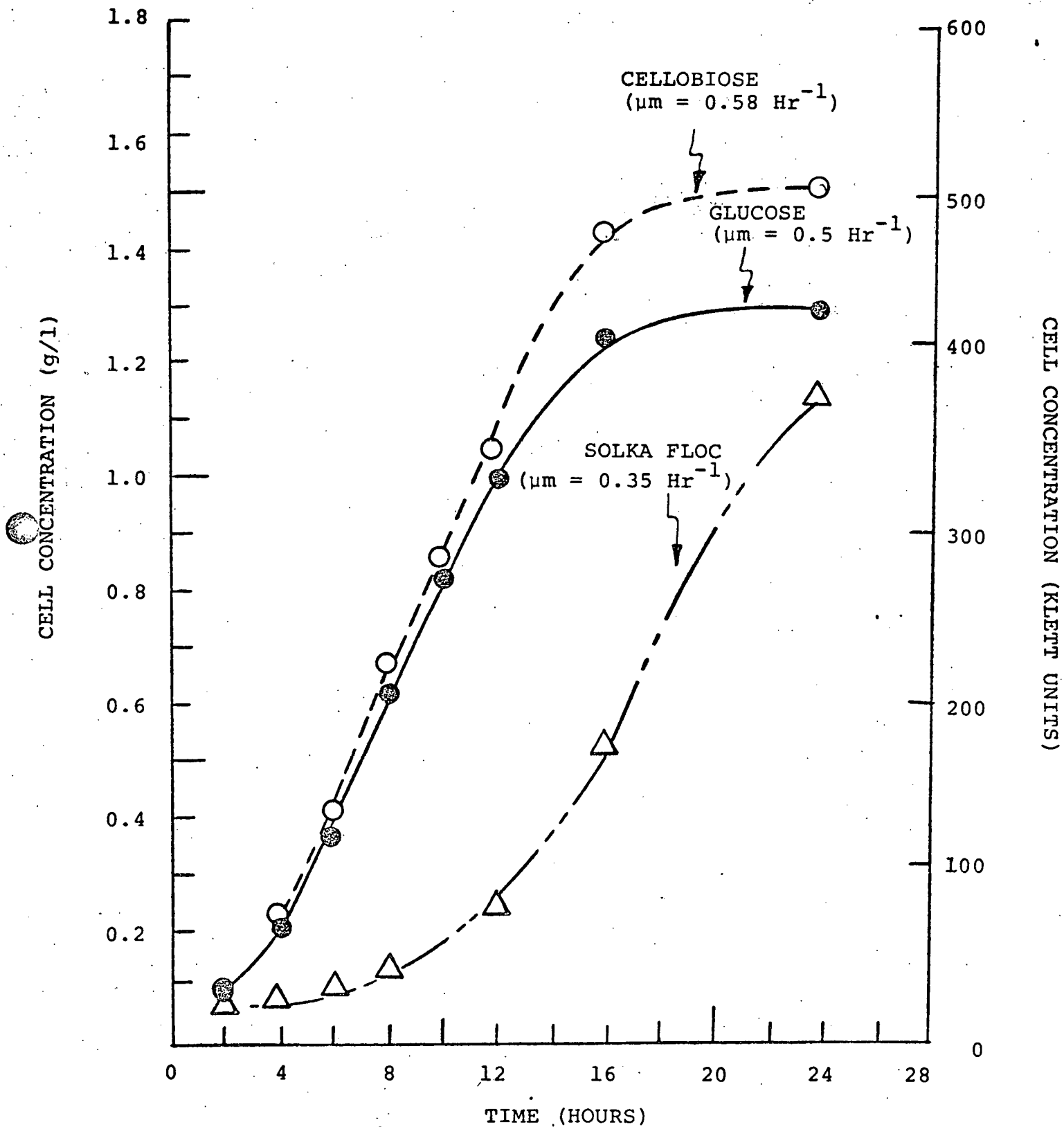
FIGURE I.G.1.1:

COMPARISON OF ETHANOL TOLERANCE FOR DIFFERENT
STRAINS OF CLOSTRIDIUM THERMOCELLUM:
(ATCC-27045 → S-4 → S-6 → S-B)



control. The latter was achieved by the addition of NaOH periodically (e.g., every 2, or 4, or 8 hours) to bring the pH back to about 7.0. The results of these growth studies on the different carbon sources are shown in Figure I.G.1.2. It was found that strain S-6 was not able to utilize xylose. This is expected since C. thermocellum is known not to be able to metabolize this sugar. It can be seen from Figure I.G.1.2 that excellent growth of S-6 was obtained on soluble carbohydrates such as glucose and cellobiose having respective maximum specific growth rates of 0.5 hour^{-1} and 0.58 hour^{-1} . Furthermore, excellent rate of growth was also found on Solka floc for strain S-6. The maximum specific growth rate was calculated to be 0.35 hour^{-1} . It should be mentioned that through our selection procedure the maximum specific growth rates of the new isolates on the different carbon sources have all increased significantly. For example, through the course of our contract the maximum specific growth rate of C. thermocellum on Solka floc has been continually increasing. Our first progress report (COO-4198-1) presented a maximum specific growth rate for this organism (parent ATCC27405) of approximately 0.07 hour^{-1} . At the present time the newest isolate S-6 exhibits a maximum specific growth rate of 0.35 hour^{-1} . It is not totally surprising to have attained the higher growth rates for our new isolates.

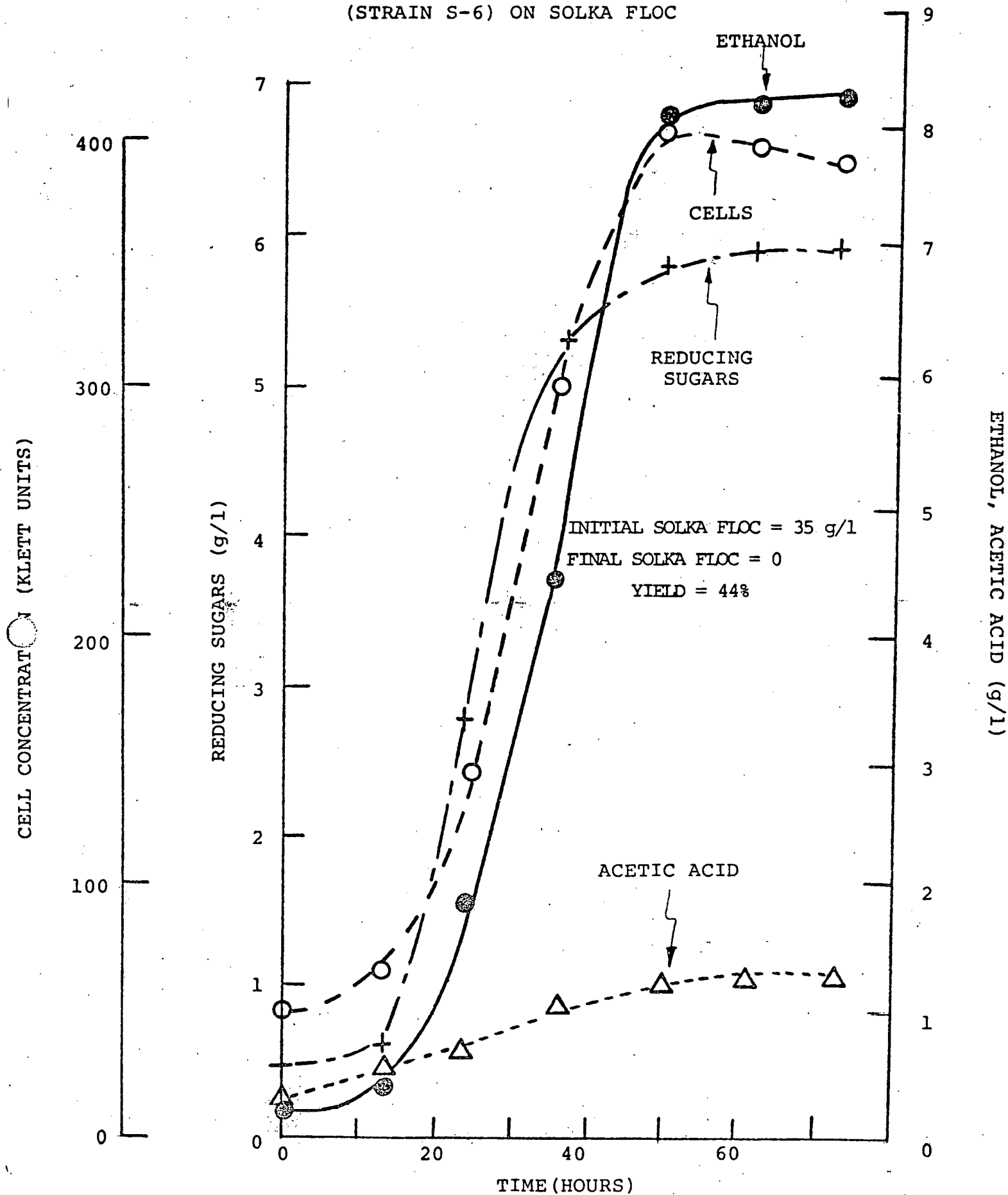
FIGURE I.G.1.2:
GROWTH OF C. THERMOCELLUM (STRAIN S-6)
ON VARIOUS CARBON SOURCES
(XYLOSE, NO GROWTH)



This is because in the course of our selection procedure which is designed primarily to increase the organism's tolerance to ethanol there has also been a secondary selection pressure which is its increased ability to grow on the different carbon sources. For example, if one examines the selection protocol shown in Table I.G.1.1, it is seen that the minimal time to achieve growth up to a given cell density.

The ability of the new strain, S-6, to degrade Solka floc leading to the formation of different catabolites was then examined using anaerobic shake flasks. All experiments were conducted at 60°C using manual and periodic pH control at about a value of 7.0. The results are shown in Figure I.G.1.3. The initial Solka floc concentration was 35 g/l which is the maximum which can be introduced into a liquid medium. Above a Solka floc concentration of 35 g/l a viscous gel results. It can be seen from Figure I.G.1.3 that the cell growth is quite excellent as well as the product formation. A final ethanol concentration of 8.2 g/l and reducing sugar concentration of 6 g/l resulted. Similar to the original properties of C. thermocellum, isolate S-4, the net formation of acetic acid was quite small amounting to about 1 g/l. These results again indicate that ratio of ethanol to acetic acid production is in the region of 8:1.

FIGURE I.G.1.3:
 FERMENTATION PROFILE OF CLOSTRIDIUM THERMOCELLUM
 (STRAIN S-6) ON SOLKA FLOC



From an initial concentration of 35 g/l of Solka floc, no residual cellulose was found through gravimetric analysis at the termination of the fermentation. When the overall yield of products (ethanol, reducing sugars and acetic acid) is calculated, it was found the 0.44 g. of product/g. of Solka floc was obtained. These results are quite exciting since they show total utilization of cellulose accompanied by a reasonable high yield.

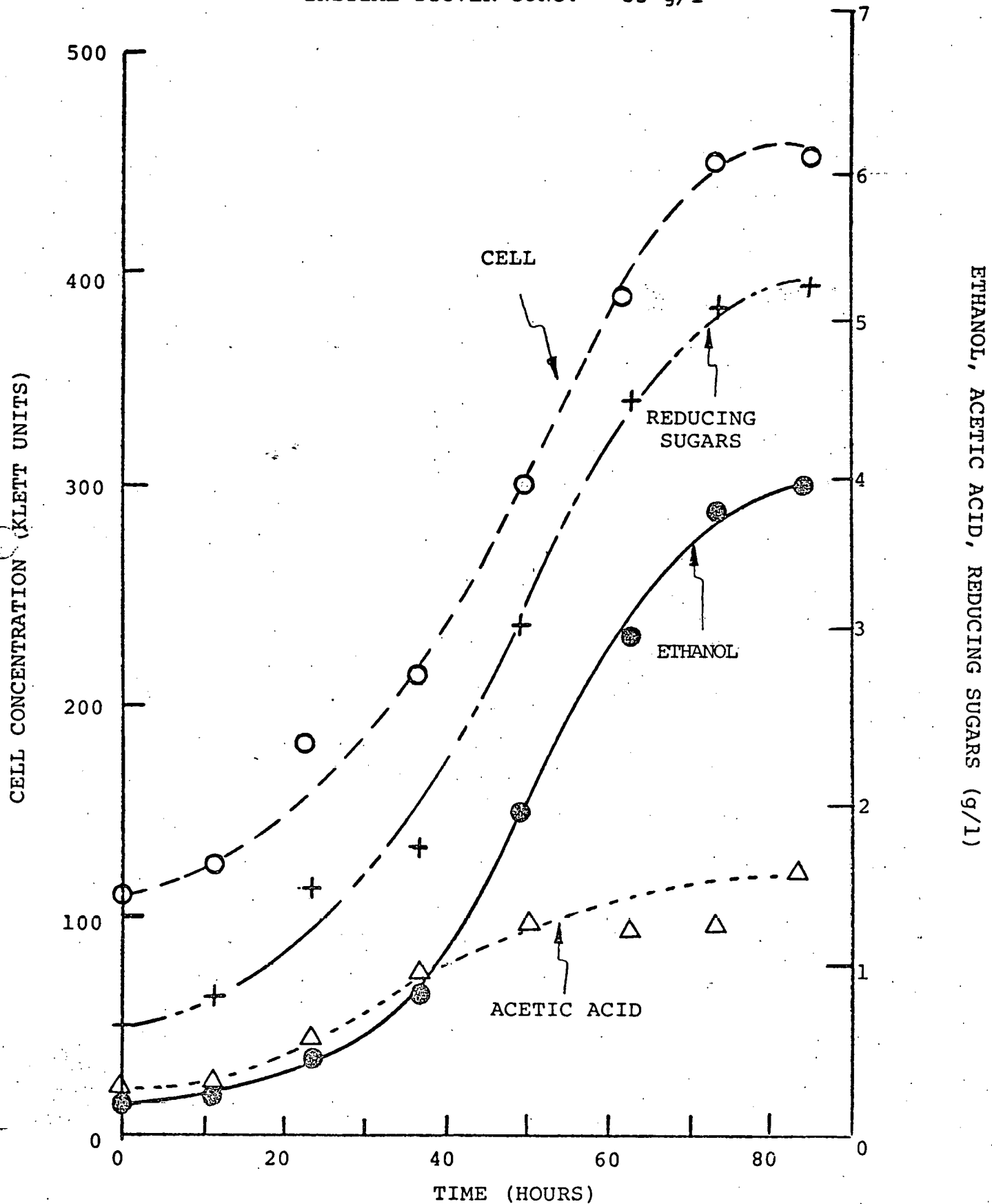
The same type of anaerobic shake flask experiments using isolate S-6 was then performed using corn stover. The initial concentration of corn stover was also 35 g/l which represents the upper limit in which a solid substrate can be placed into a liquid medium. The results from this study are shown in Figure I.G.1.4. The degradation products from corn stover by S-6 are quite similar when compared to Solka floc. However, the rate and final concentration of these products formed are lower when corn stover is used as the substrate. For example, the net formation of reducing sugars, ethanol and acetic acid are respectively 5 g/l, 4 g/l and 1 g/l. Unfortunately the final corn stover concentration was not determined but a qualitative estimate yields a new value of about 50% degradation. These results are nevertheless extremely encouraging to lead us to continue our program for the continual improvement of this organism on its degradation capability on corn stover.

FIGURE I.G.1.4:

FERMENTATION PROFILE OF CLOSTRIDIUM THERMOCELLUM

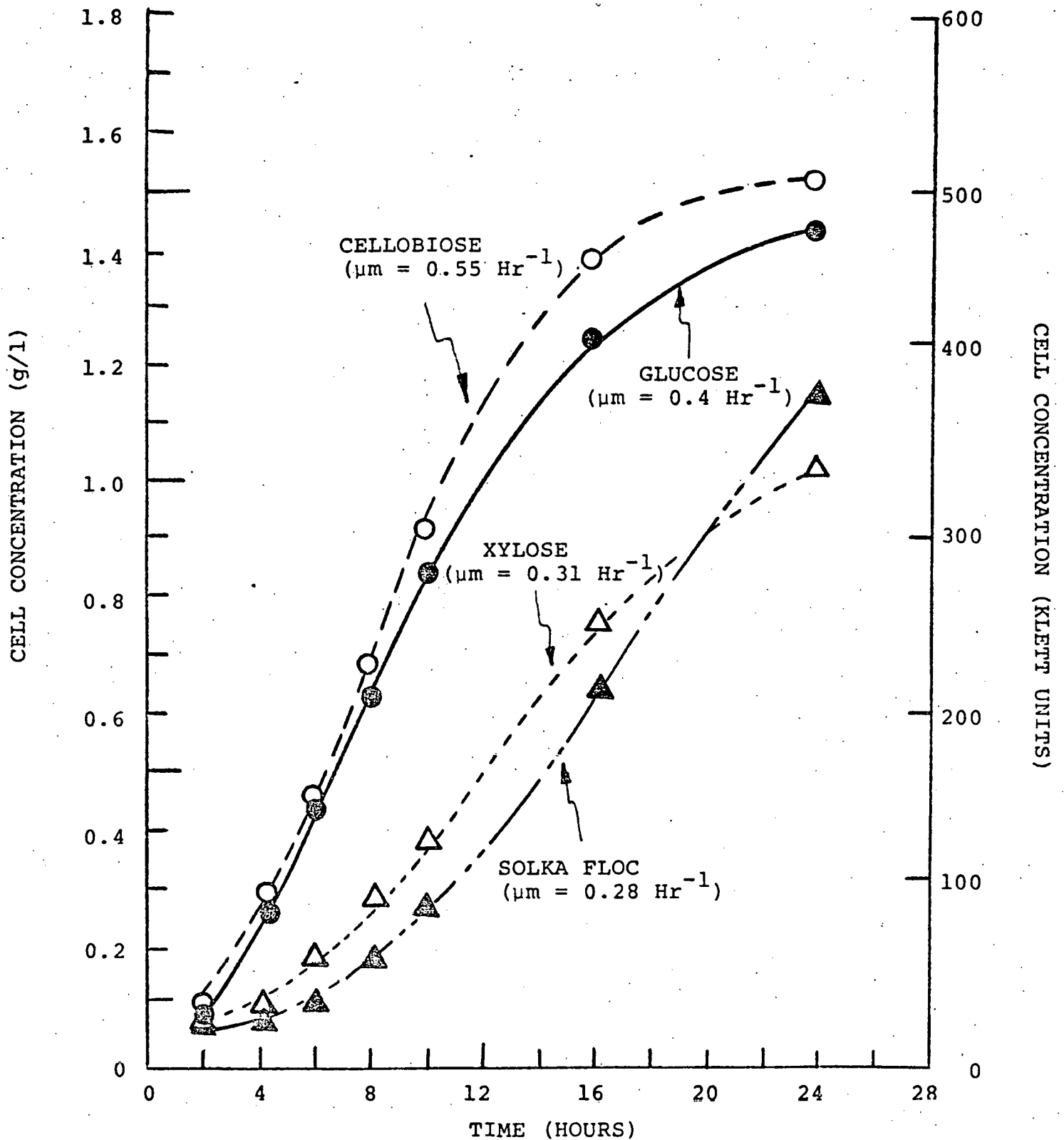
(STRAIN S-6) USING CORN STOVER

INITIAL STOVER CONC. = 35 g/l



As mentioned in the earlier part of our discussion (Figure I.G.1.1) a second strain designated as S-B has also been isolated. This strain was shown to have even higher tolerance to ethanol than S-6. The ability of this new isolate, S-B, to grow on different carbon sources was also performed. The growth of S-B on cellobiose, glucose, xylose and Solka floc is shown in Figure I.G.1.5. It can be seen that the maximum specific growth rate of strain S-B on cellobiose and glucose are quite excellent at respective values of 0.55 hour^{-1} and 0.4 hour^{-1} . These growth rates are similar to strain S-6. When this new strain S-B was grown on Solka floc, again a high maximum specific growth rate of 0.28 hour^{-1} was obtained. What is most striking is the ability of the new isolate (S-B) to grow on xylose and having a maximum specific growth rate of 0.31 hour^{-1} . The ability of this isolate to metabolize xylose leads us to suspect that a mixed culture may have been obtained. This is quite conceivable since within the same laboratory we have been actively studying the xylose utilizer Clostridium thermosaccharolyticum. This organism sporulates readily and is extremely stable to heat during sterilization. It should be mentioned, however, that if S-B is a mixed culture, it is an extremely stable system. For example, upon repeated transfer (10 times) into medium containing xylose as the only carbon source, subsequent transfer onto Solka

FIGURE I.G.1.5:
GROWTH OF "C. THERMOCELLUM" (STRAIN S-B)
ON VARIOUS CARBON SOURCES



floc yields immediate growth. From a technical and process point of view there is no reason why such a mixed culture can not be capitalized on to effectively degrade cellulose and to product the desired products.

This new strain S-B was then tested using manual pH controlled fermentations in shake flasks and Solka floc as the carbon source. The initial concentration of Solka floc was again 35 g/l and the results are shown in Figure I.G.1.6. It can be seen that the fermentation products using strain S-B are quite different as compared to S-6, the most dramatic difference being the total lack of reducing sugar formation. The primary catabolites found were ethanol (final concentration 15 g/l) and acetic acid (final concentration 5.5 g/l). In view of these findings, one would suspect that the new isolate S-13 is a mixed culture of Clostridium thermocellum and Clostridium thermosaccharolyticum. This is quite logical since the former is able to degrade the cellulose producing ethanol, acetic acid and reducing sugars. The latter organism can then utilize the soluble sugars to produce ethanol and acetic acid. In view of the ratio of ethanol to acetic acid found (~ 3:1) the hypothesis of a mixed culture is further reinforced. It should be noted, however, that from 35 g/l of Solka floc no cellulose was found at the end of the fermentation. This represents an overall product yield (ethanol + acetic) of 0.6 g of product/ g. Solka floc which is quite excellent.

FIGURE I.G.1.6:

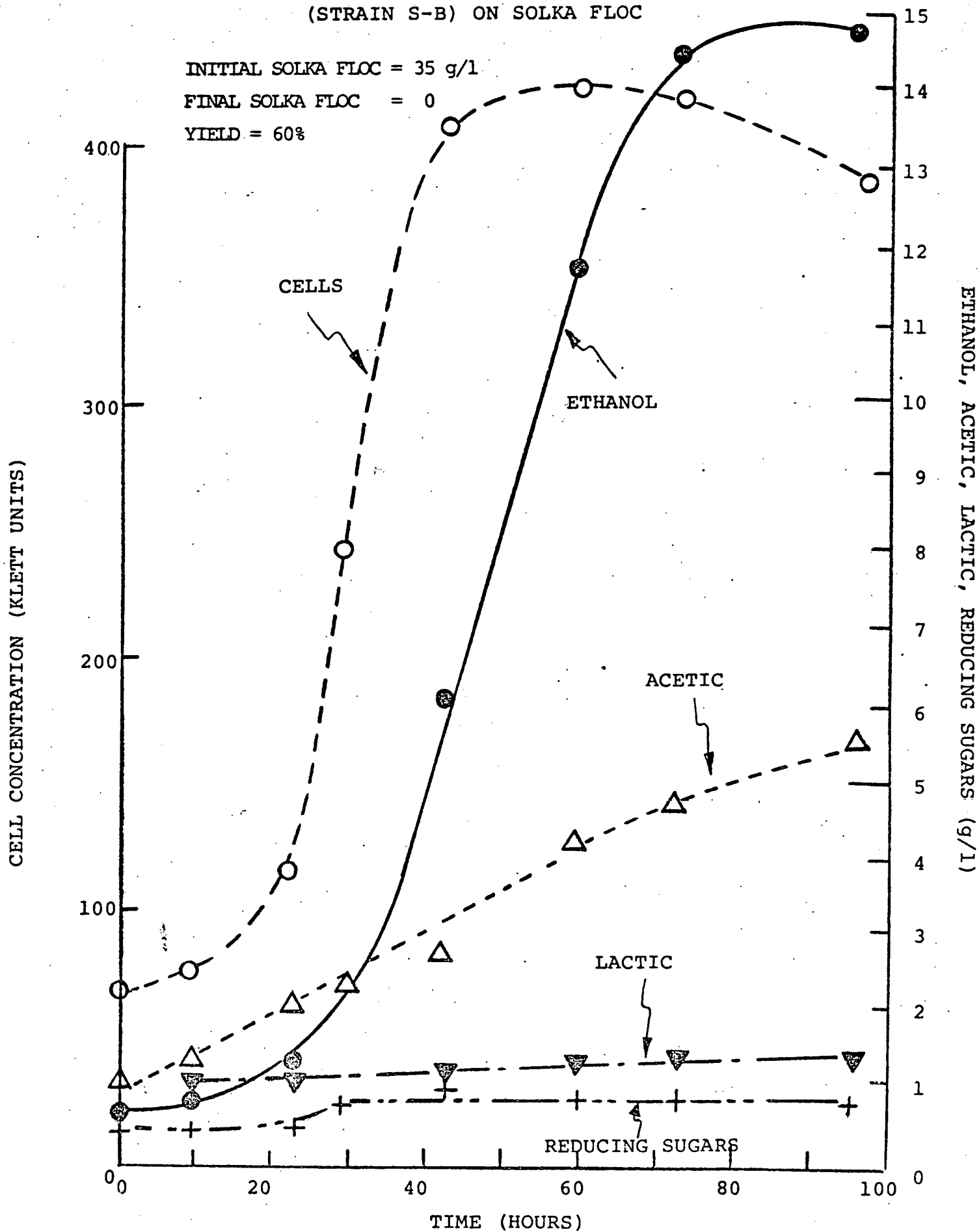
FERMENTATION PROFILE OF "CLOSTRIDIUM THERMOCELLUM"

(STRAIN S-B) ON SOLKA FLOC

INITIAL SOLKA FLOC = 35 g/l

FINAL SOLKA FLOC = 0

YIELD = 60%



The last series of experiments were performed using strain S-B and corn stover as the substrate. Again, an initial concentration of 35 g/l of stover was employed. The results are shown in Figure I.G.1.7. The fermentation product profiles when corn stover is used are similar to those found for Solka floc. Again, very little reducing sugars are found and the primary catabolites were ethanol (4.2 g/l) and acetic acid (2.3 g/l). In these experiments the final concentration of corn stover was measured gravimetrically. From an initial value of 35 g/l, the final corn stover concentration was determined to be 10 g/l. This represents about a 70% degradation of stover. Calculated overall yield of product from stover was 0.26 g of product/g stover degraded. The qualitative and quantitative nature of the undegraded corn stover was, however, not performed. These results are again encouraging, even though we realize that a mixed culture may have been isolated, to lead us to future process improvement studies.

d. Future Studies

The future studies include:

- Characterization of strain S-B.
- Continue mutation, adaptation and selection on ethanol tolerance.
- Mutation and selection to achieve directed catabolism to single product (ethanol).

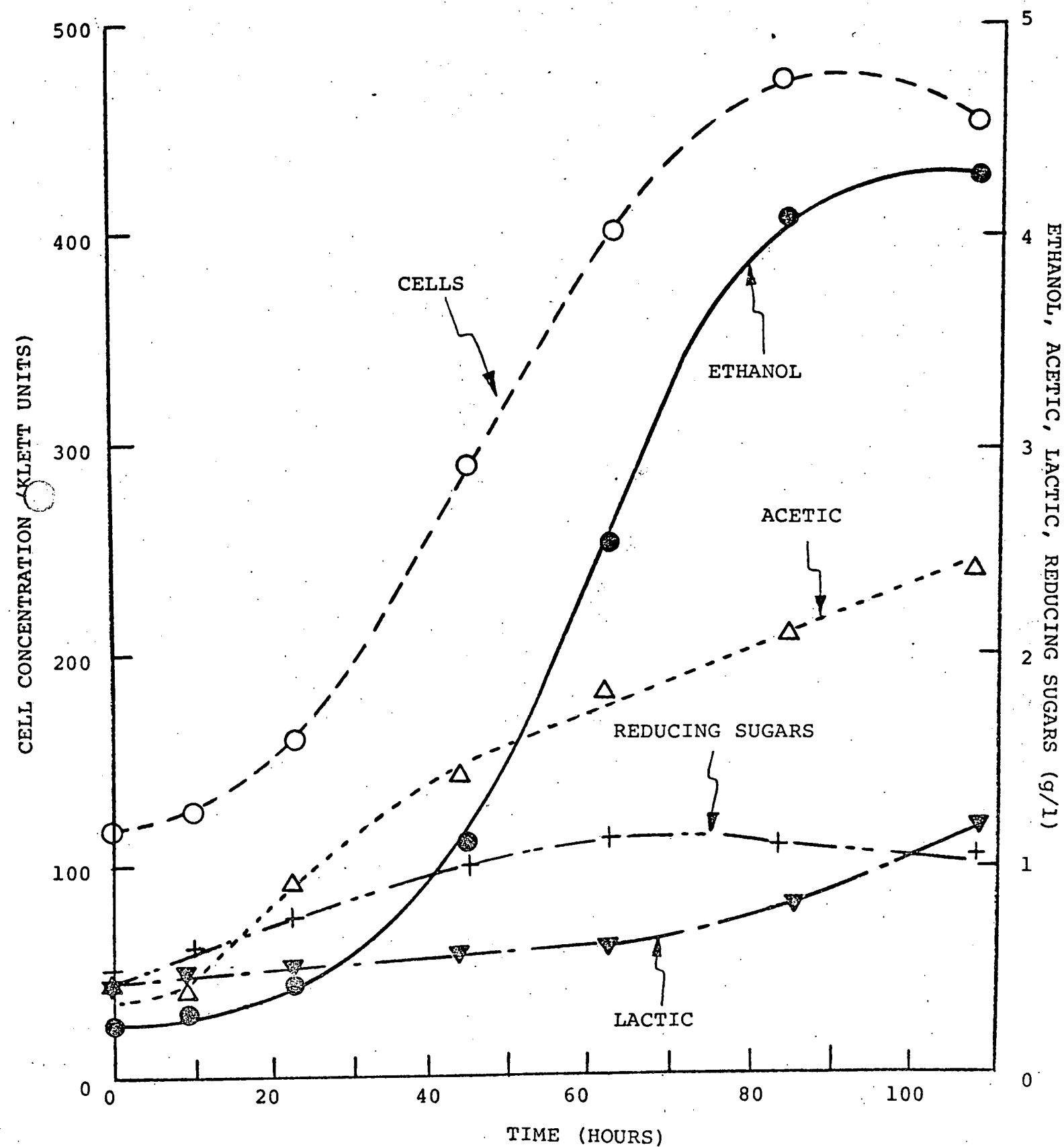
FIGURE I.G.1.7:

FERMENTATION PROFILE OF "CLOSTRIDIUM THERMOCELLUM"

(STRAIN S-B) USING CORN STOVER

INITIAL STOVER = 35 g/l

FINAL STOVER = 10 g/l



- Increased loading of cellulose concentration using packed-bed fermentor.
- Examination of different biomasses.

2. Ethanol Production by Clostridium thermosaccharolyticum Using Xylose

a. Introduction

Hemicellulose, a major component of natural biomass, must be effectively utilized if an economic process of ethanol production is to be realized. Although our previous results have shown that Clostridium thermocellum is able to degrade hemicellulose in biomass to soluble monomer and oligomers (xylose, xylobiose, etc.) unfortunately this organism is not able to metabolize five carbon sugars. We have therefore devoted a substantial effort to effectively utilize xylose and in particular the production of ethanol.

In our last progress report (COO-4198-8) we had reported the isolation of an organism designated as HG-2 which is able to metabolize xylose. Since then we have identified this isolate to be Clostridium thermosaccharolyticum. This organism possesses many properties which are totally compatible in the event a mixed culture fermentation with Clostridium thermocellum is desired. For example, C. thermosaccharolyticum is an anaerobe with thermophilic properties (temperature optimum $\sim 60^{\circ}\text{C}$). In this period of the contract we have performed a number of experiments to further characterize this organism using xylose as the substrate. The results from these studies will be presented in this section.

b. Materials and Methods

The organism used throughout the program is Clostridium thermosaccharolyticum. The original strain had the designation HG-2. Through an adaptation and selection program, a higher ethanol tolerant strain HG-3 has been isolated.

The composition of the basic medium (CM) for our studies is shown in Table I.G.2.1. Xylose at 15 g/l or when varied for specific reasons was obtained from Sigma Chemical Company (X-2000), St. Louis, Missouri. Fermentations were conducted in Hungate tubes (10 ml of medium), anaerobic shake flasks (200 ml medium in 500 ml flasks), or 7-liter agitated New Brunswick fermentors (4 liter of medium). Medium sterilization was achieved at 121°C for 30 minutes and the xylose was sterilized separately. Analytical methods for the different products have been reported in previous progress reports.

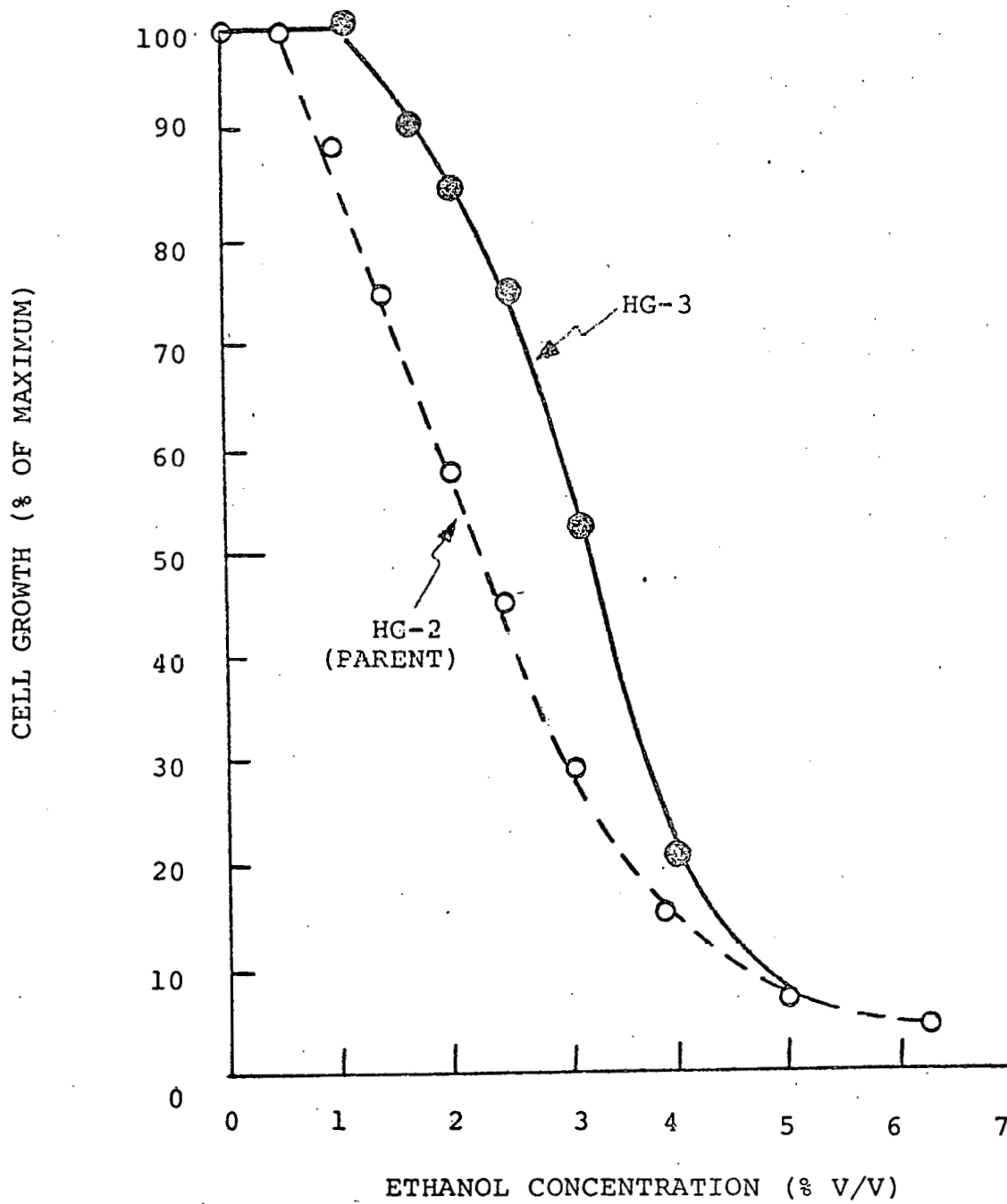
c. Results and Discussion

The growth tolerance of Clostridium saccharolyticum towards ethanol for the parent (HG-2) and strain HG-3 is shown in Figure I.G.2.1. The reason for repeating this figure, although it was already presented in progress report COO-4198-8, is because of an error in the previous report. The ethanol concentration had been reported in % W/V and

TABLE I.G.2.1: COMPOSITION OF CM MEDIUM FOR STUDIES
WITH CLOSTRIDIUM THERMOSACCHAROLYTICUM

Xylose or other carbon sources	15	g/l or variables
Yeast extract	5	g/l
KH_2PO_4	1.5	g/l
Na_2HPO_4	3.0	g/l
Urea	1.0	g/l
$\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$	1.0	g/l
CaCl_2	0.15	g/l
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1.25	mg/l
Na-thioglycolate	0.5	g/l
0.2% Resazurin solution	1	ml/l
pH	7.0	

FIGURE I.G.2.1:
COMPARISON OF ETHANOL TOLERANCE FOR
DIFFERENT STRAINS OF CLOSTRIDIUM THERMOSACCHAROLYTICUM



should be V/V. It can be seen that a slight improvement towards ethanol tolerance resulted from the new strain HG-3.

The growth and product during fermentation using xylose for strain HG-2 are shown in Figure I.G.2.2. Ethanol and acetic acid are produced at about equal concentrations (about 2.4 g/l). The behavior of new strain HG-3 on xylose is shown in Figure I.G.2.3. It can be seen that this higher ethanol tolerant strain favors the production of ethanol (3.1 g/l) as compared to acetic acid (1.8 g/l). In both instances (strain HG-2 and HG-3) total utilization of xylose, initially at 15 g/l, resulted. The conversion efficiency based on ethanol and acetic acid on xylose is quite low and therefore leads us to suspect that other catabolites may have been formed. This behavior will be examined in more detail at a later time in this section.

In order to have a more detailed characterization on the metabolism of Clostridium thermosaccharolyticum (strain HG-3), different carbon sources were therefore examined. The results are shown in Table I.G.2.2. It can be seen that C. thermosaccharolyticum possesses a diverse metabolic activity on many carbon sources. Excellent growth as seen by the maximum specific growth rates attained on the different carbohydrates. With the exception of lactose and cellobiose, it can be stated that this organism is equally effective with respect to growth on the other carbon sources tested. The ethanol and acetic acid production on

FIGURE I.G.2.2: GROWTH AND PRODUCT FORMATION OF STRAIN HG-2
(*C. THERMOSACCHAROLYTICUM*) ON XYLOSE

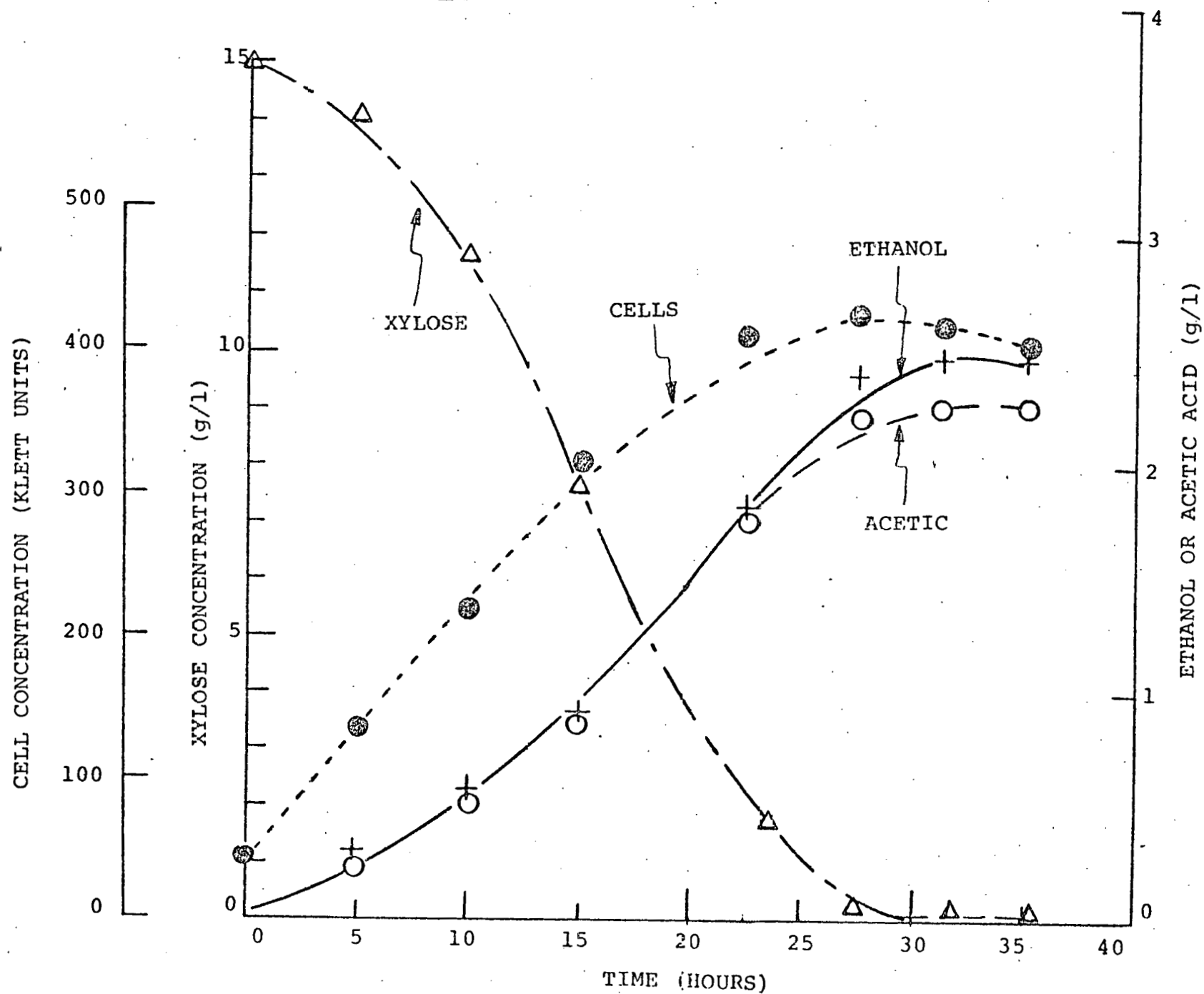
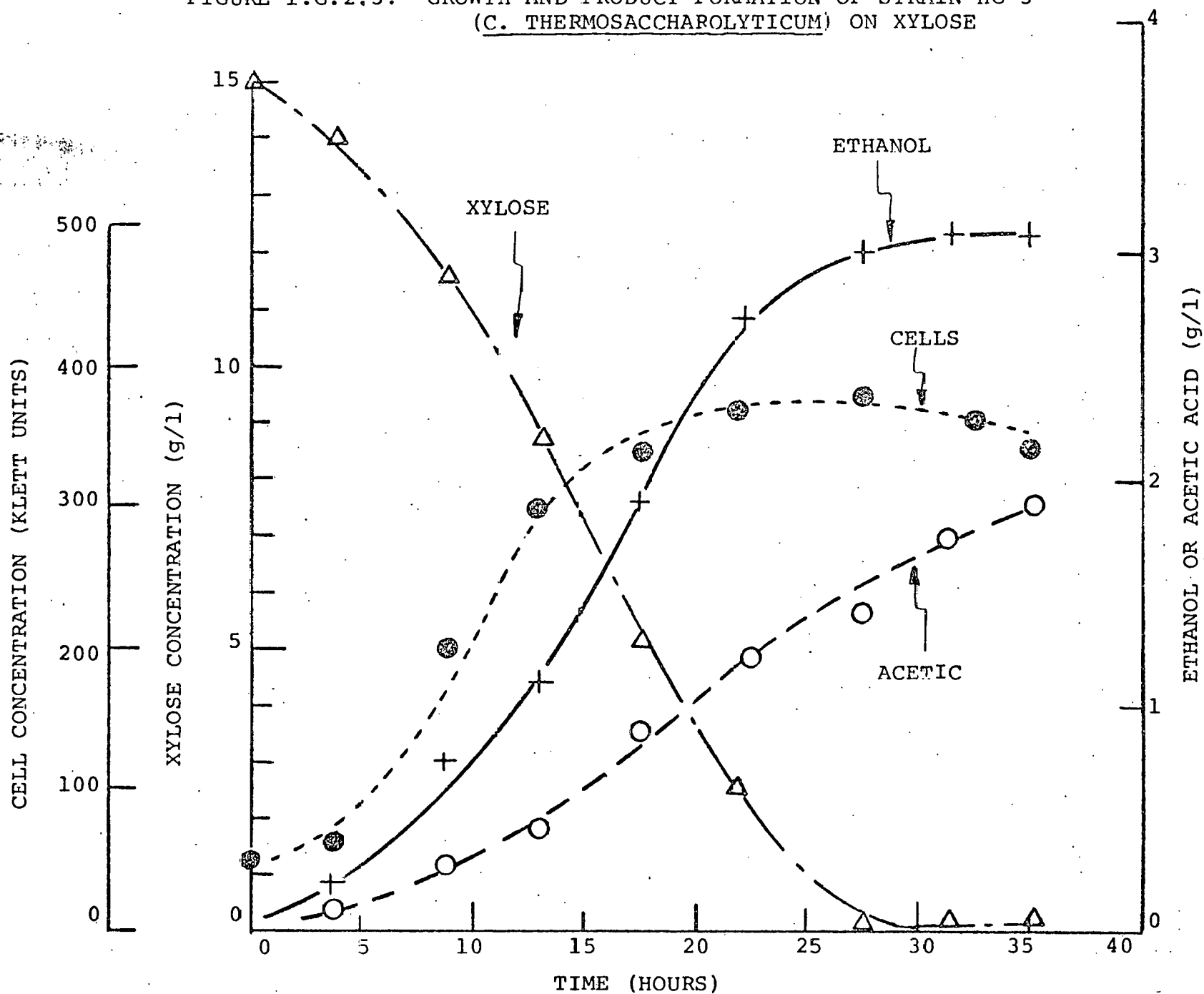


TABLE I.G.2.2: BEHAVIOR OF CLOSTRIDIUM THERMOSACCHAROLYTICUM
(STRAIN HG-3) ON DIFFERENT CARBON SOURCES*

<u>Carbon Source</u>	<u>Maximum Specific Growth Rate (hr⁻¹)</u>	<u>Cell Conc. (g/l)</u>	<u>Ethanol (g/l)</u>	<u>Acetic (g/l)</u>	<u>Ratio of ethanol/acetic acid</u>
Xylose	0.25	1.08	3.6	1.7	2.12
Glucose	0.25	0.87	1.4	1.9	0.74
Fructose	0.25	0.72	2.34	1.42	1.67
Cellobiose	0.15	0.76	1.80	2.32	0.77
Sucrose	0.25	0.63	3.7	1.7	2.18
Lactose	0.07	0.24	0.88	0.68	1.30
Galactose	0.25	0.63	1.80	1.04	1.73
Maltose	0.24	0.98	2.60	1.5	1.73
Mannose	0.22	0.55	1.58	2.16	0.73

* Urea as nitrogen sources.

FIGURE I.G.2.3: GROWTH AND PRODUCT FORMATION OF STRAIN HG-3
(C. THERMOSACCHAROLYTICUM) ON XYLOSE



the different substrates were also measured. It is interesting to note that the highest ethanol to acetic acid ratio was achieved when xylose was used as the substrate. This is very encouraging since our main objective in the use of this organism is to effectively utilize this pentose sugar.

The effect of pH on the rate of growth and product concentration and distribution was also examined. The rationale in these studies was to see whether lower pH (thus higher H^+ ion concentration) might be an environmental variable which could be used to favor the production of ethanol. It was reasoned that at a lower pH the production of acetic acid might be inhibited due to difficulty in transport by this organism and thus leading to a higher ethanol to acetic acid ratio. The pH was adjusted manually and periodically using a bicarbonate buffer. Temperature of growth was $60^{\circ}C$ and all assays for the products were performed after 35 hours of growth. The results are shown in Table I.G.2.3. It can be seen that between the pH value of 6.5 to 7.4, growth of the organism was quite similar having equal specific growth rates and final cell concentrations. However, at the lower pH value of 6.5, higher concentration of ethanol was obtained as well as having a more favorable ethanol to acetic acid ratio. At pH of 6.0, lower rate of growth was obtained but the ethanol to acetic acid ratio was higher than at higher pH. Above a pH of 7.8,

TABLE I.G.2.3: EFFECT OF pH ON THE GROWTH AND PRODUCT FORMATION BY C. THERMOSACCHAROLYTICUM ON XYLOSE

pH	Maximum Specific Growth Rate (Hr ⁻¹)	Final Cell Conc. (g/l)	Final Ethanol Conc. (g/l)	Final Acetic Acid Conc. (g/l)	Ethanol to Acetic Ratio
6.0	0.15	0.96	3.65	1.7	2.15
6.5	0.23	1.08	3.8	1.7	2.24
7.0	0.25	1.14	3.1	1.8	1.74
7.4	0.21	0.96	2.3	1.6	1.48
7.8	No growth	-	-	-	-

no growth was observed. These results suggest that a more favorable product ratio can be obtained by maintaining a lower pH during fermentation.

In the CM medium as shown in Table I.G.2.1, yeast extract at 5 g/l had been used. In order to examine the effect of yeast extract concentration on the product formation, concentrations ranging from 1 to 5 g/l were tested. The results are shown in Table I.G.2.4. It can be seen that the final cell concentration (35 hours) increases with increasing yeast extract concentration but not in a linear fashion. For example, at 3 g/l of yeast extract, comparable results were obtained when compared to 5 g/l of yeast extract. It therefore appears that the yeast extract concentration can be reduced without significant loss in cell growth or product formation. Future studies will examine other nitrogen sources, particularly inorganic forms, on the growth of this organism.

Thus far all of the experiments using strain HG-3 have employed an initial xylose concentration of 15 g/l. Furthermore, the cessation of ethanol and acetic acid production has been shown to occur when all of the xylose has been consumed. In order to see what is the highest product concentrations attainable as well as delineating in more detail the quantitative nature of the different products, a batch-fed xylose fermentation in 7-liter agitated fermentor using strain HG-3 was performed. The xylose concentration in the

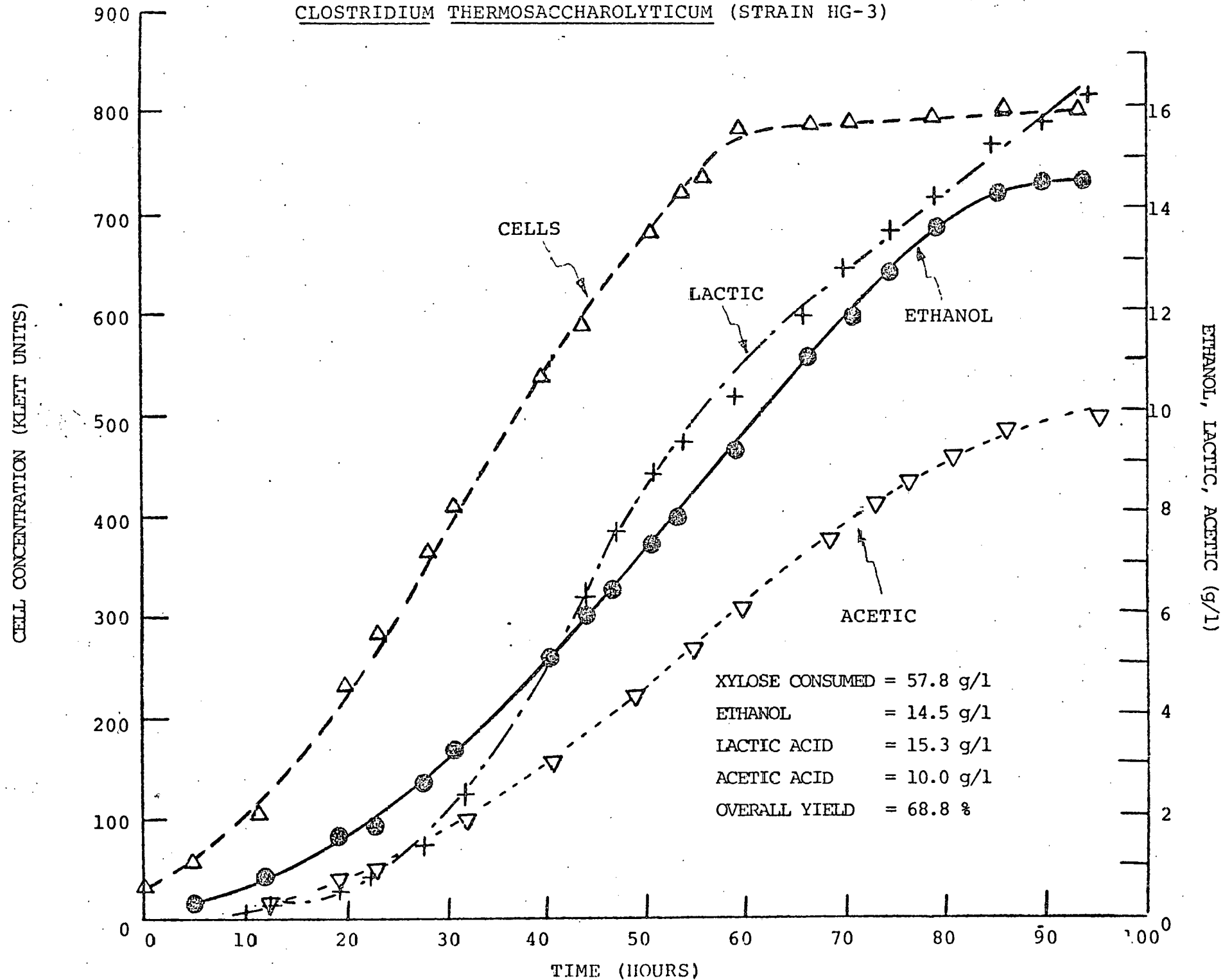
TABLE I.G.2.4: EFFECT OF YEAST EXTRACT CONCENTRATION ON THE GROWTH AND PRODUCT FORMATION USING C. THERMO-SACCHAROLYTICUM (HG-3) AND XYLOSE

Yeast Extract Concentration (g/l)	Final Cell Conc. (g/l)	Final Ethanol Conc. (g/l)	Final Acetic Acid Conc. (g/l)
1	0.58	2.3	1.4
2	0.80	2.9	1.4
3	0.95	3.4	1.7
4	1.05	3.4	1.7
5	1.10	3.5	1.7

medium was maintained between 3 to 9 g/l through batch addition and the pH was controlled at 6.5. The results from this study are shown in Figure I.G.2.4. A total of 67.8 g/l of xylose was consumed along with the feeding of 14 g/l of yeast extract. At the end of the fermentation, 14.5 g/l of ethanol, 15.3 g/l of lactic and 10 g/l of acetic acid resulted. The overall product yield (ethanol, lactic and acetic) was calculated to be 0.688 g product/g xylose. One should note that the sum of the product concentrations was 39.8 g/l! The results from this fermentation also offered the reason as to why low yield of product when only ethanol and acetic acid were considered. This is because lactic acid is a major catabolite during metabolism on xylose. The final cell concentration as measured optically was 800 Klett units which corresponds to 2.34 g/l of cells. Since 39.8 g/l of products were formed, the calculated specific biosynthetic capability of this organism was 17 g product/g cell. This is an extremely efficient organism with respect to its specific ability to convert xylose to product.

The results from the fed-batch fermentation (Figure I.G.2.4) were further analyzed with respect to the kinetic parameters of the different products. This was done in order to see whether specific growth rate might be used as a control variable to effect a more favorable distribution of the desired product, ethanol. The kinetic model for analysis

FIGURE I.G.2.4: FERMENTATION PROFILE USING FED-BATCH XYLOSE OF CLOSTRIDIUM THERMOSACCHAROLYTICUM (STRAIN HG-3)



was the combination of growth and non-growth associated mode for product formation and can be mathematically expressed as:

$$q_p = \alpha \mu + \beta$$

q_p = specific production formation rate
(g product/g cell-hr)

α = growth associated constant
(g product/g cell)

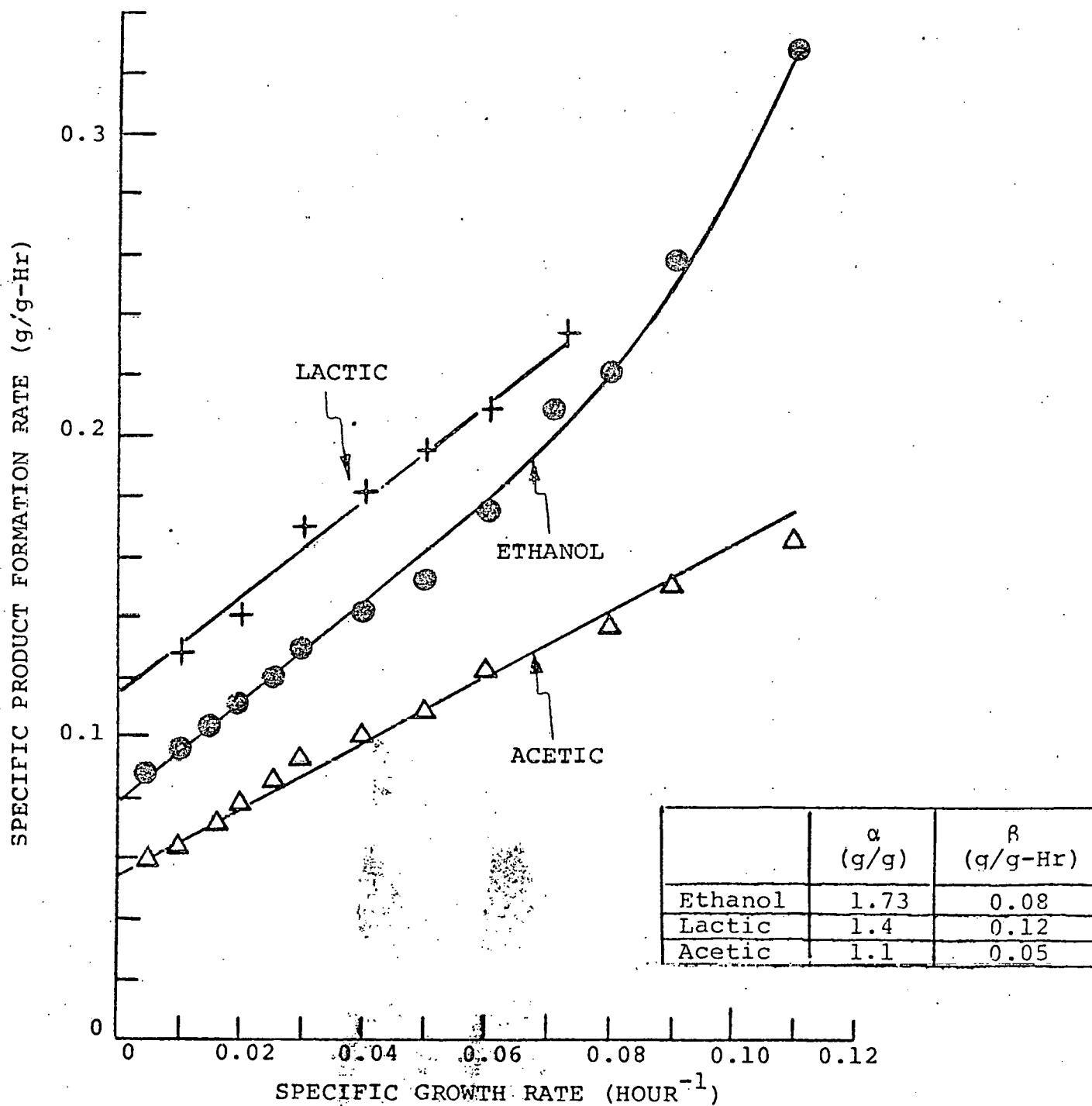
μ = specific growth rate (Hr^{-1})

β = non-growth associated constant
(g product/g cell-hr)

Using the data in Figure I.G.2.4, the specific product formation rates for ethanol, lactic acid and acetic acid versus specific growth rate are shown in Figure I.G.2.5. It can be seen that all of the products are formed through both growth and non-growth associated modes. The calculated constants for the different products are also shown on this Figure. From a comparison on the magnitudes of the different constant, we conclude that manipulation of the specific growth rate cannot be used effectively to favor one product to another. This is because the growth and non-growth associated product formation constants are quite comparable for one catabolite to another. It therefore appears that other techniques will have to be developed in order to achieve a more favorable ethanol fermentation from xylose.

The effect of ferrous (Fe^{+2}) ion in favor of ethanol over lactic acid production by Clostridium

FIGURE I.G.2.5: PRODUCT FORMATION CONSTANT FOR C. THERMO-SACCHAROLYTICUM (HG-3) IN BATCH-FED XYLOSE FERMENTATION



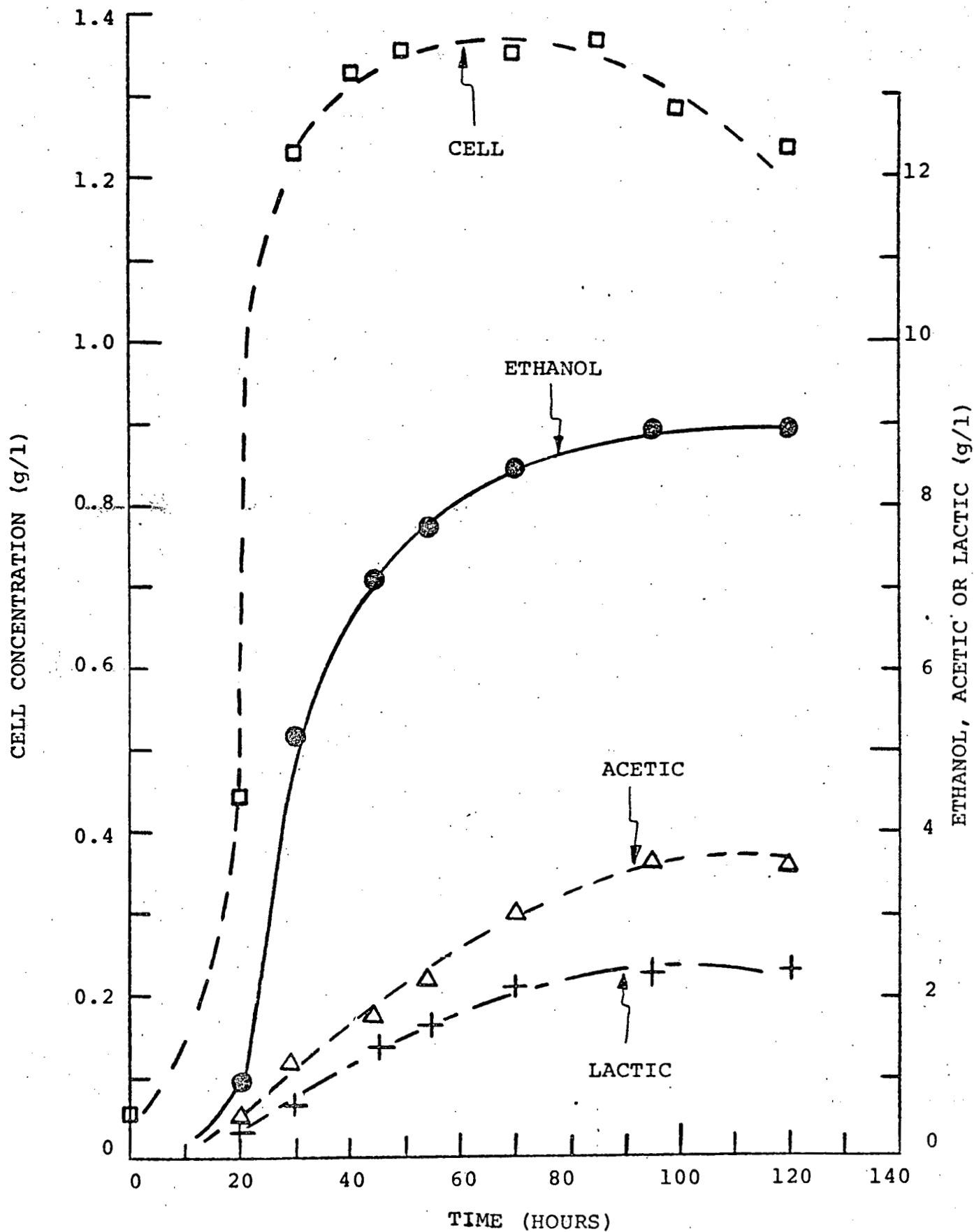
thermocellum has been shown in our previous Progress Report (COO-4198-8). In order to see whether by increasing the Fe^{+2} concentration to accomplish the same task, experiments were also performed using Clostridium thermosaccharolyticum. The results from these studies are shown in Table I.G.2.5. It can be seen that as the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ concentration was increased from 1.25 mg/l (CM medium) to 5.0 mg/l, there is a more favorable ratio of ethanol to lactic acid. However, further increases in the Fe^{+2} ion concentration beyond 5 mg/l does not appear to improve product distribution. It is recommended, however, that the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ concentration be increased in the CM medium for future studies.

A series of experiments using pure culture of Clostridium thermocellum (S-4) and mixed culture with the addition of Clostridium thermosaccharolyticum (HG-3) on Solka floc were performed at various $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations. These experiments were conducted to assess the compatibility of these two organisms as well as to gain insight as to the product distributions and concentrations. A typical fermentation profile where C. thermosaccharolyticum was inoculated after ten hours of growth is shown in Figure I.G.2.6. It can be seen that extremely high specific growth rate resulted when a mixed population is used on a cellulosic material such as Solka floc. The product concentrations and their distributions from these studies are shown in Table

TABLE I.G.2.5: EFFECT OF Fe^{+2} CONCENTRATION ON THE PRODUCT CONCENTRATION AND DISTRIBUTION DURING FERM-ENTATION ON XYLOSE BY C. THERMOSACCHAROLYTICUM (HG-3)

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/l)	Cell Conc (g/l)	Ethanol (g/l)	Lactic Acid (g/l)	Acetic Acid (g/l)
1.25	1.05	3.6	3.6	1.7
5.0	1.60	4.4	2.2	1.8
10	1.65	4.4	2.2	1.8
20	1.65	4.2	2.4	1.6

FIGURE I.G.2.6: MIXED CULTURE OF *C. THERMOCELLUM* AND *C. THERMOCACCHAROLYTICUM* GROWN ON SOLKA FLOC



I.G.2.6. It can be seen that extremely high specific growth rate resulted when a mixed population is used on a cellulosic material such as Solka floc.

The product concentrations and their distributions from these studies are shown in Table I.G.2.6. At high Fe^{+2} concentration, as would be expected, a more favorable distribution of ethanol over the other catabolites was obtained. These results are extremely encouraging since they show a total compatibility of the two organisms as well as the favorable product distribution ratio.

d. Future Work

Future studies with Clostridium thermo-
saccharolyticum include:

- Further adaptation and selection to increase its tolerance to ethanol.
- Mutation program to effect direct catabolism to a single product, ethanol.
- Detailed studies with mixed cultures with Clostridium thermocellum.
- Nutritional studies to decrease nutrient costs.

TABLE I.G.2.6: PURE CULTURE AND MIXED CULTURE OF CLOSTRIDIUM THERMOCELLUM (S-4) AND CLOSTRIDIUM THERMOSACCHAROLYTICUM (HG-3) STUDIES USING SOLKA FLOC (33 g/l)

Type of System	FeSO ₄ .7H ₂ O Conc	Final Cell Conc	Ethanol Conc.	Lactic Conc	Acetic Conc
	(mg/l)	(g/l)	(g/l)	(g/l)	(g/l)
<u>C. thermo-</u> <u>cellum</u>	5.0	0.9	7.3	3.8	2.7
<u>C. thermo-</u> <u>cellum</u> + <u>C. thermo-</u> <u>saccharolyticum</u>	5.0	0.95	8.0	4.0	3.0
<u>C. thermo-</u> <u>cellum</u> + <u>C. thermo-</u> <u>saccharolyticum</u>	12.5	1.35	9.0	2.3	3.7

3. Growth and Productivity of C. Thermocellum on Corn Stover Using Concentrated Inoculum

a. Introduction

The volumetric productivity of ethanol observed on natural cellulosic biomasses is lower than that on soluble sugars or refined cellulose such as Solka floc. Since the volumetric productivity ($\frac{dP}{dt}$) is related to the amount of cells (X) present and their growth rate (μ) ($\frac{dP}{dt} = \alpha\mu X + bX$), it appears likely that the total cell mass when grown on corn stover might be lower leading to lower productivity. A hypothesis that can be experimentally verified is the effect of independently varying the total cell concentration on the rates of corn stovers degradation and the appearance of soluble products (e.g. reducing sugars, ethanol, and acetic acid). The typical 10% inoculum from mid-log phase cells only represents about 0.1 gms/liter of cells. In the following experiment, we describe the use of cell concentration to achieve much higher initial inoculum cell loadings and their effect on the metabolism of cellobiose and corn stovers.

b. Materials and Methods

The higher ethanol producing variant of Clostridium thermocellum (ATCC 27405) S-4 was transferred in log phase to 3 anaerobic flasks (10% V/V) containing 450 ml

of CM 3/cellobiose medium. At approximately 250 Klett in optical density, the flasks were moved into an anaerobic glove box, pooled, and distributed to six 250 ml centrifuge bottles. The tightly capped bottles were centrifuged at 4°C and 10,000 x g for 20 minutes to precipitate the cells; and returned to the anaerobic chamber. The resazurin indicator dye remained yellow throughout the harvesting indicating that a sufficiently low redox potential had been maintained. These cells were subsequently used to inoculate three fresh shake flasks. The flasks contained 250 ml medium with 15 g/l of cellobiose/CM 3 medium. One centrifuge bottle served as inoculum for 1 flask, 2 for a second, and 3 in the third. The inoculum concentration corresponded in these flasks contained respectively 0.75, 1.13 and 1.35 gm of cells per liter. The flasks were incubated at 60°C in the glove box, and sampled every 1.5 hours. Measurement of optical density, ethanol, acetic acid, reducing sugars and pH were performed as previously described.

After 7 hours of incubation, the flasks were pooled and redistributed to 6 sterile centrifuge bottles and harvested as before. These six tubes were used to inoculate three fresh flasks containing 300 ml of CM3 medium with 27 grams per liter corn stover. As before, the cells were distributed 1/6, 1/3 and 1/2 to each flask. Samples were taken every 24 hrs and reducing sugars, ethanol, and acetate measured.

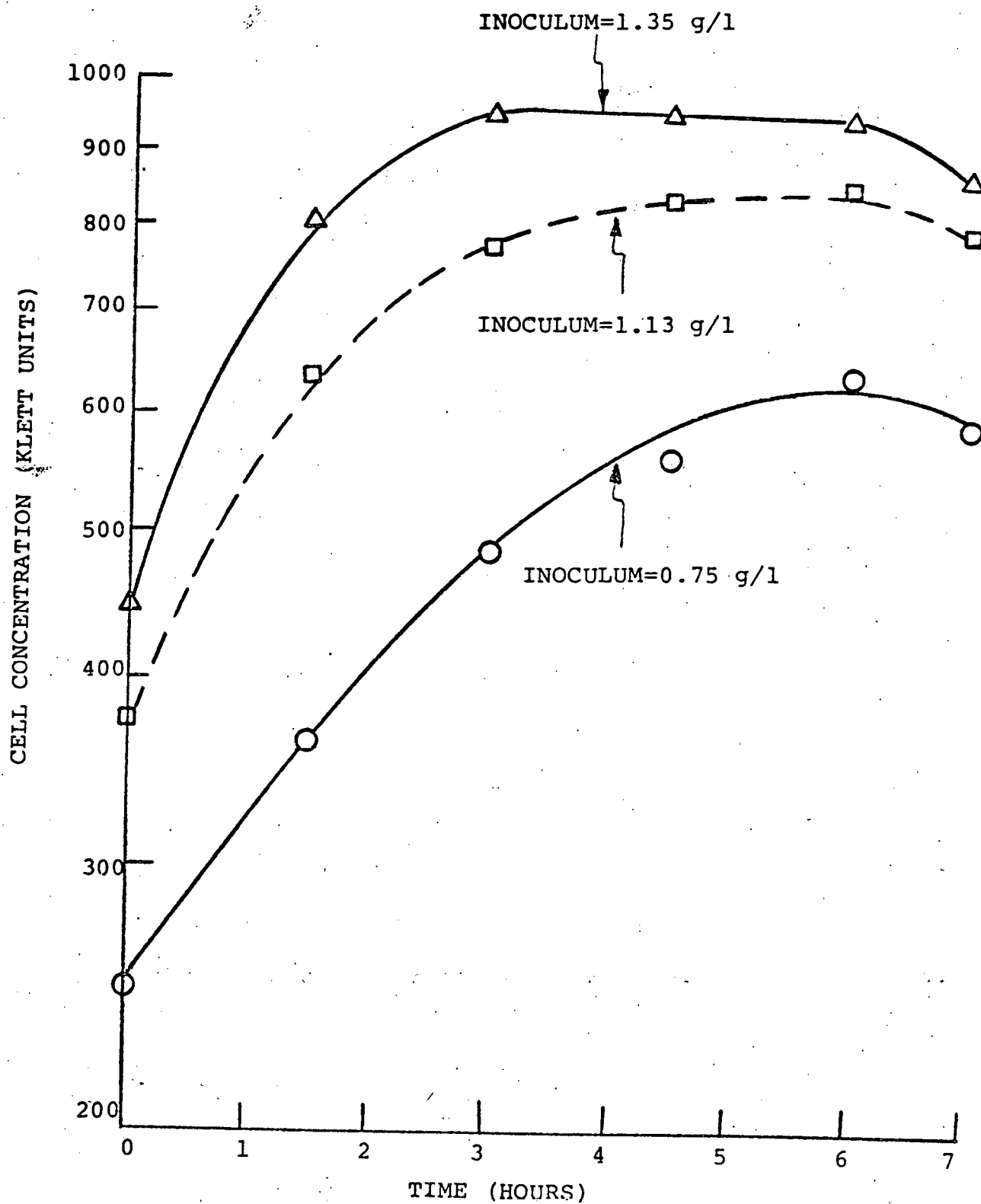
c. Results and Discussion

i. Concentrated Cells on Cellobiose

Cell growth on cellobiose after concentration by centrifugation proceeded immediately with no discernable lag phase (Figure I.G.3.1). An initial growth rate of 0.39 hr^{-1} for the dense inoculum (1.35 g/l), 0.36 hr^{-1} (1.13 g/l), and 0.25 hr^{-1} (0.75 g/l) for the less concentrated cells were observed. This result would not be expected from uniformly exponentially growing cells which should have identical maximum growth rates. One plausible explanation for this effect could be the presence of a short lag phase of these cells whose duration increased with decreasing inoculum size. This lag phase could be brought about by differences in the redox potentials. Many anaerobes will grow satisfactorily at redox potential $-200 \text{ mV } E_h$; this is approximately the level at which thioglycolate or cysteine can maintain. However, many strict anaerobic species must reduce further the redox potential to as low as -400 mV before they can achieve significant growth. If cells are able to reduce their redox environment, it is then reasonable to state that a denser inoculum could reduce the medium faster and thus achieve its maximum growth rate sooner.

The growth rates of all these cultures also decrease rapidly after 2 hours of incubation first. This

FIGURE I.G.3.1: GROWTH OF C. THERMOCELLUM (S-4) ON CELLOBIOSE
USING CONCENTRATED CELL INOCULUM



effect is probably attributed to the increasing pH drop observed with increasing cell density (Figure I.G.3.2).

The initial volumetric acetate production using the concentrated cell inoculum varies from 0.093, 0.080 and 0.053 gms of acetic/liter hour for the 1.35, 1.13, and 0.75 gm/l inoculum densities. Respectively, (Figure I.G.3.3) the initial specific acetate productivities were found to be 0.068, 0.071, and 0.071 gms acetate/gram cell hour for the decreasing concentrations of inoculum. This close agreement reveals that initial specific acetate productivity is not a function of cell concentration. Due to the variations in the pH profile associated with cell growth at different cell densities, little can be concluded from the final values of acetate accumulation.

The initial volumetric rates of ethanol production for the 3 cultures were 0.25, 0.12, and 0.013 gms ethanol per liter hour. (Figure I.G.3.4.) Normalizing the cell mass, this is equivalent to a specific productivity of 0.14, 0.08, and 0.01 gms ethanol/gm cell hour for decreasing inoculum sizes, respectively. Part of the reasons for this divergence in initial specific productivity may be the effect of different redox potentials. The equilibrium of the NAD/NADH coupled reaction between acetaldehyde and ethanol is influenced by the local redox potential. For example, the two half reactions:

FIGURE I.G.3.2: pH PROFILE OF C. THERMOCELLUM (S-4) GROWN ON CELLOBIOSE USING CONCENTRATED CELL INOCULUM

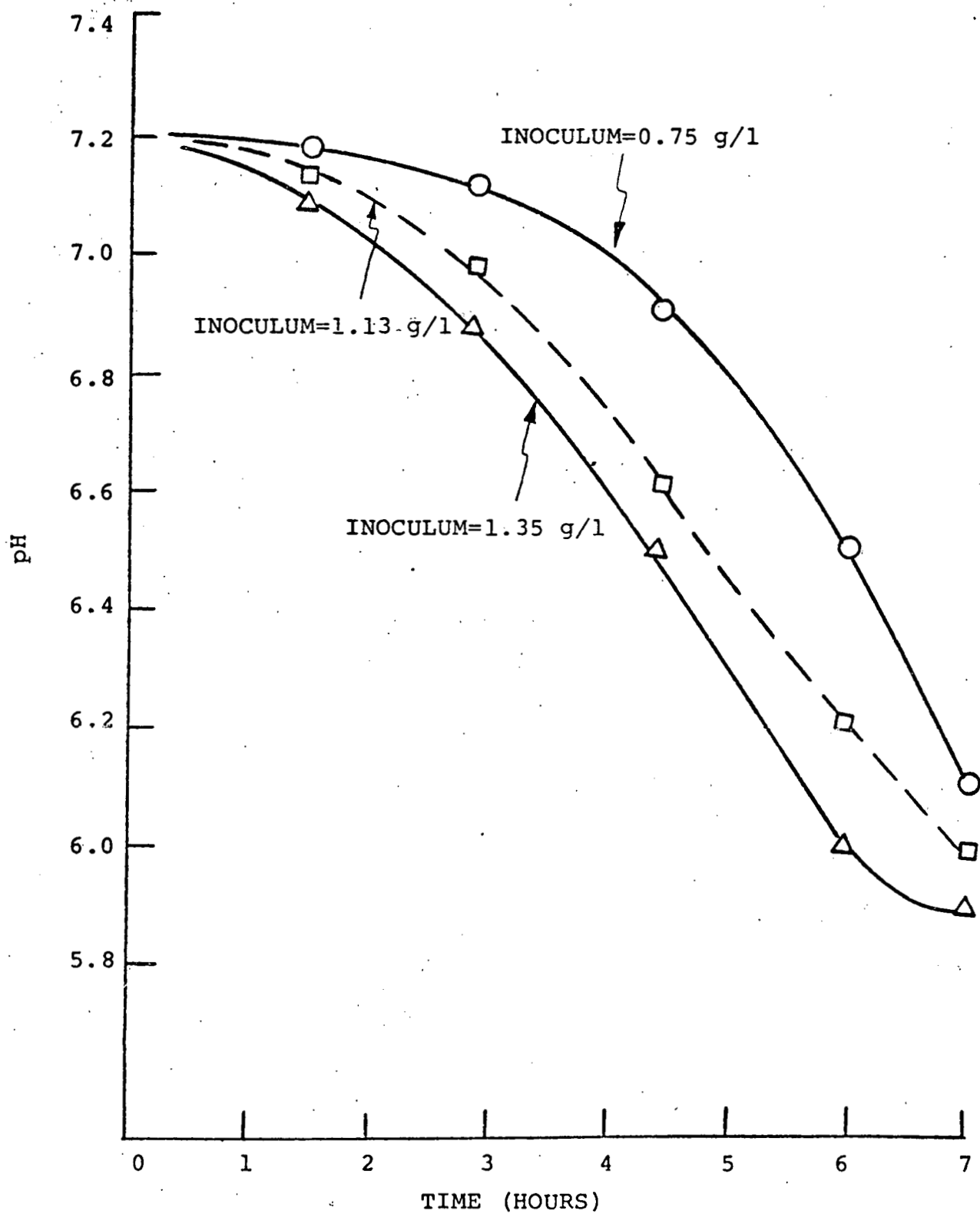


FIGURE I.G.3.3: ACETIC ACID PRODUCTION BY C. THERMOCELLUM ON CELLOBIOSE USING CONCENTRATED CELL INOCULUM

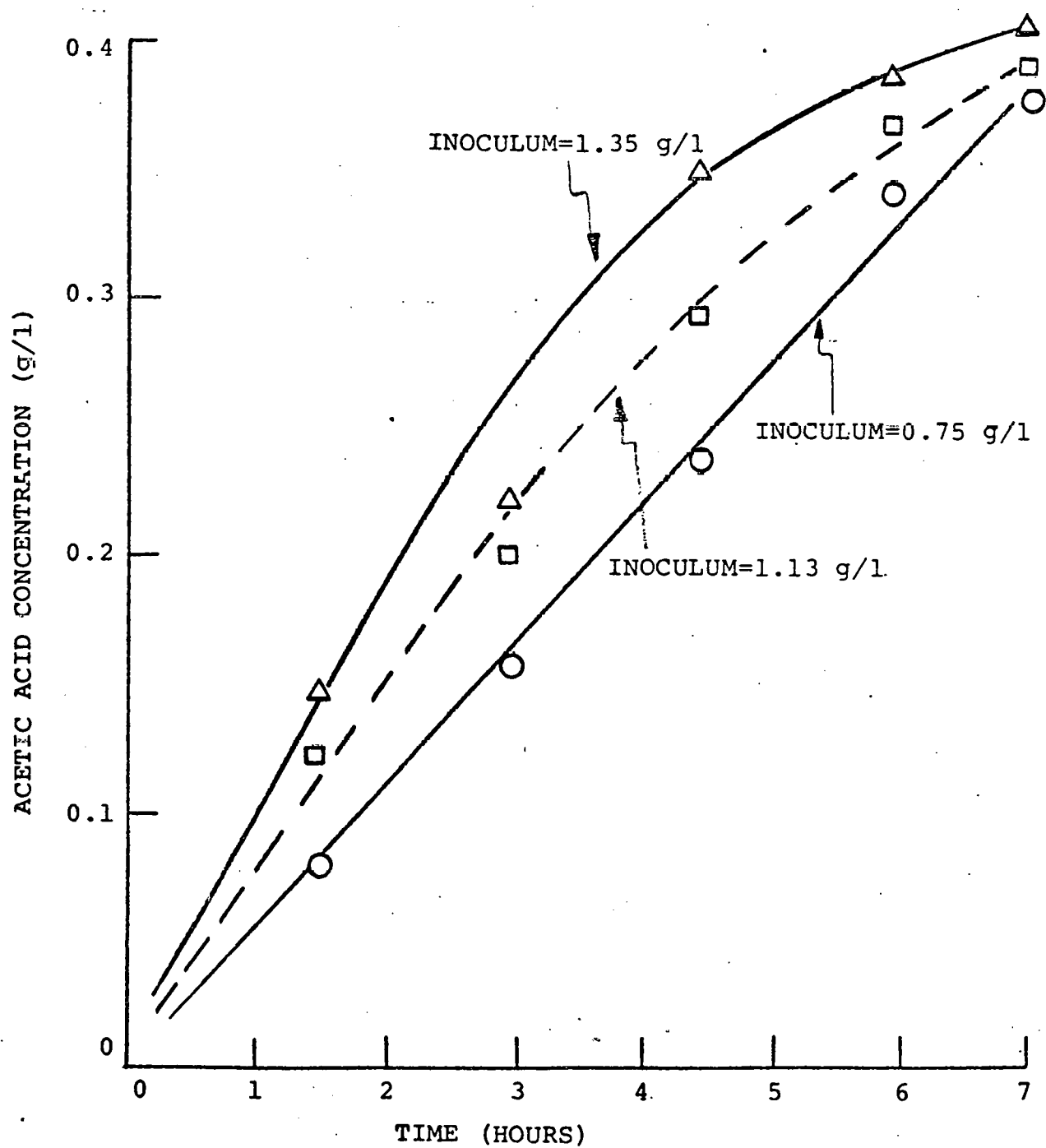
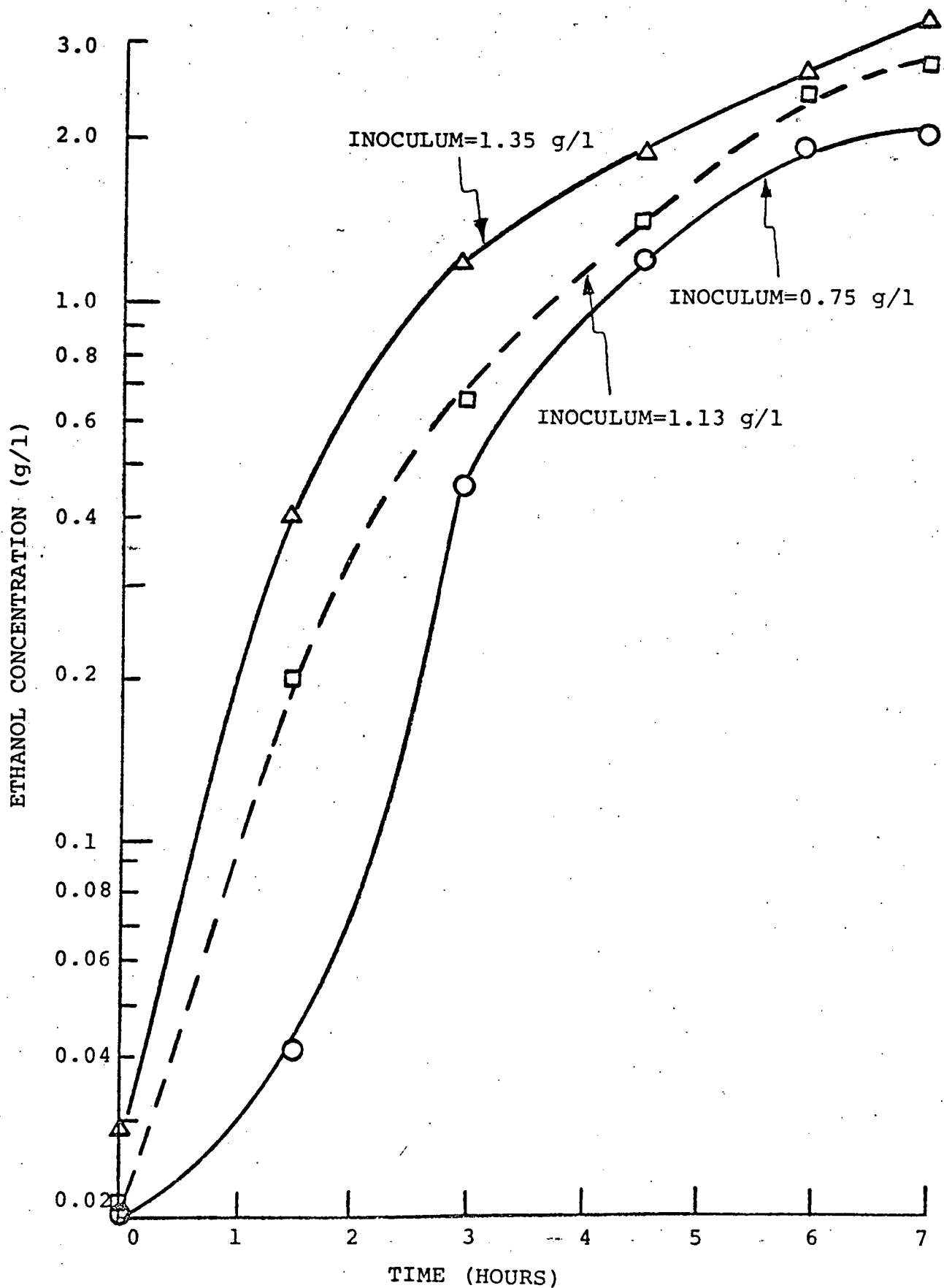
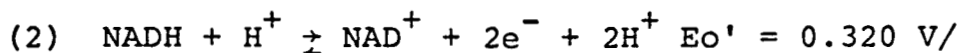
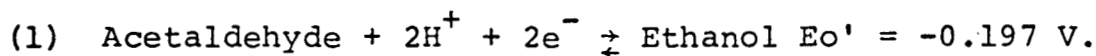
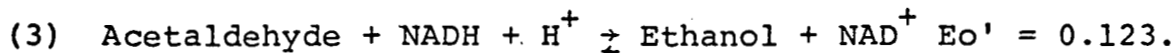


FIGURE I.G.3.4: PRODUCTION OF ETHANOL BY C. THERMOCELLUM (S-4) ON CELLOBIOSE USING CONCENTRATED CELL INOCULUM





Sum to give:



The standard potentials at 25°C and pH 7 also sum to give the combined E_o' value for the couple.

The Nernst equation for a 2 electron transfer then predicts:

$$(4) \quad E_h = E_o' + .03 \log \frac{e^- \text{ acceptor}}{e^- \text{ donor}}$$

and

$$(5) \quad E_h = 0.197 + 0.03 \log \frac{\text{Acetaldehyde}}{\text{Ethanol}} + 0.320 - 0.03 \log \frac{\text{NAD}^+}{\text{NADH}}$$

or

$$(6) \quad E_h = 0.123 + 0.03 \log \frac{(\text{Acetaldehyde}) (\text{NADH})}{(\text{Ethanol}) (\text{NAD}^+)}$$

Thus, as the E_h of the medium decreases the equilibrium constant ($\log K$) also decreases. Assuming $[\text{NADH}]/[\text{NAD}^+] = 1.0$,

E_h [Ethanol]/[Acetaldehyde] at equilibrium

-0.1	0.17
-0.2	368:1
-0.4	2×10^9 :1

Although this is only one representative reaction from the pathways of C. thermocellum, it is clear that the redox environment may contribute to product distribution and concentration.

ii. Concentrated Biomass on Corn Stover

The inoculation of corn stover with 1.2, 2.4 or 3.7 gms/l cells resulted in a volumetric production of reducing sugars as depicted in Figure I.G.3.5 of 0.031, 0.038 and 0.042 grams/liter hour, respectively. This volumetric rate does not compare favorably with the previously observed value of 0.043 g/liter hour using a 10% inoculum of S-4 at this same concentration of corn stovers. This difference in rates of reducing sugar production can be partly explained if one assumes that of the cellulase enzymes are extracellular. Therefore, a 10% inoculum with no pre-concentration contains a significant quantity of cellulase, whereas the supernatant free cell pellets (concentrated inoculum) may contain little or no extracellular activity. In Figure I.G.3.6, the initial volumetric productivity plotted against the inoculum size show the rapidly "saturating" effect of increasing inoculum concentration.

The production of ethanol on corn stover are shown in Figure I.G.3.7 using different sizes of inoculum. The volumetric productivities were nearly identical over the 3 fold range of cell concentration increasing from 0.026, 0.028,

FIGURE I.G.3.5: PRODUCTION OF REDUCING SUGARS BY C. THERMOCELLUM (S-4) ON CORN STOVER USING CONCENTRATED CELL INOCULUM

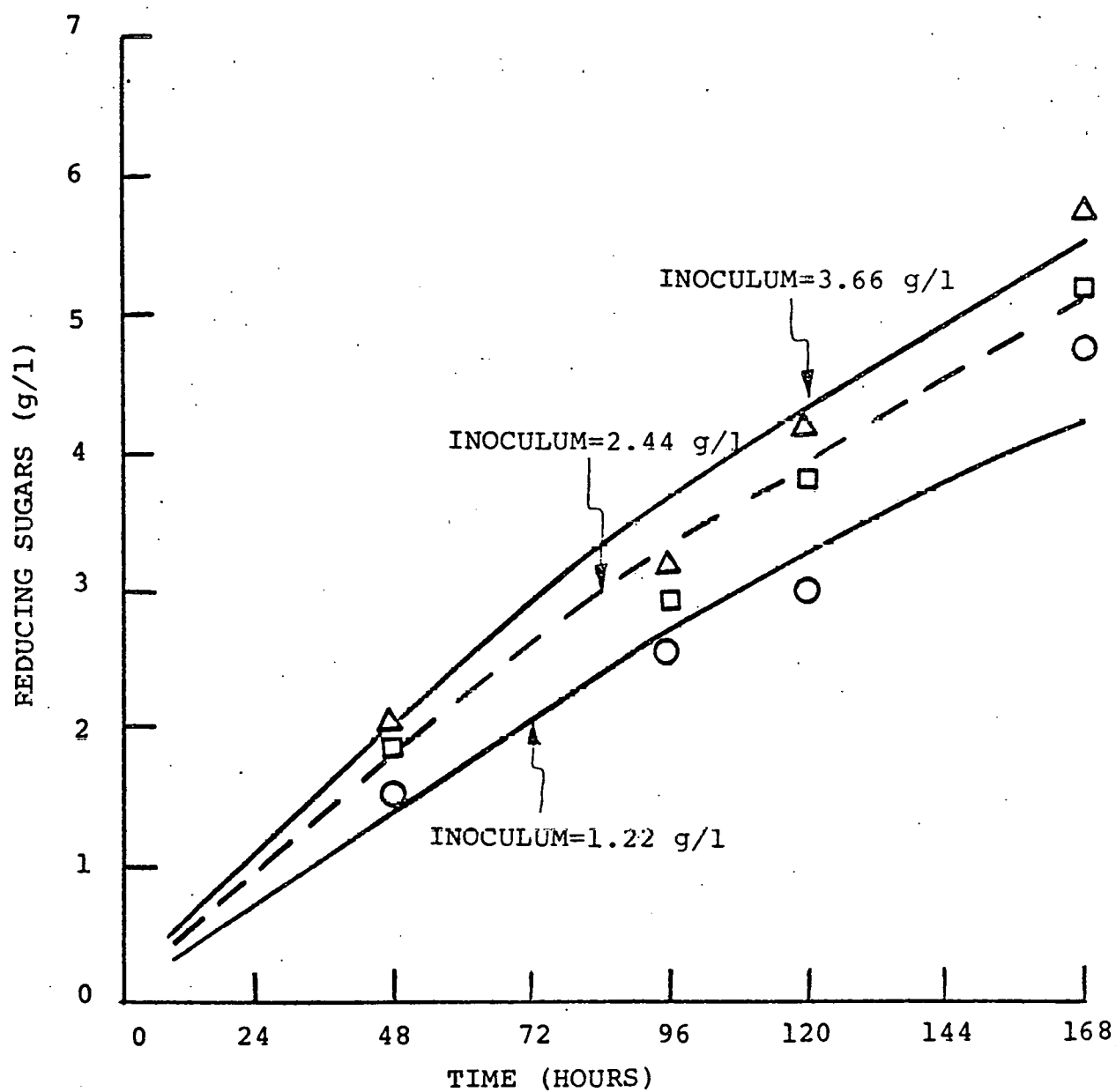


FIGURE I.G.3.6: EFFECT OF INOCULUM CONCENTRATION ON VOLUMETRIC REDUCING SUGAR PRODUCTIVITY BY C. THERMOCELLUM (S-4) USING CORN STOVER

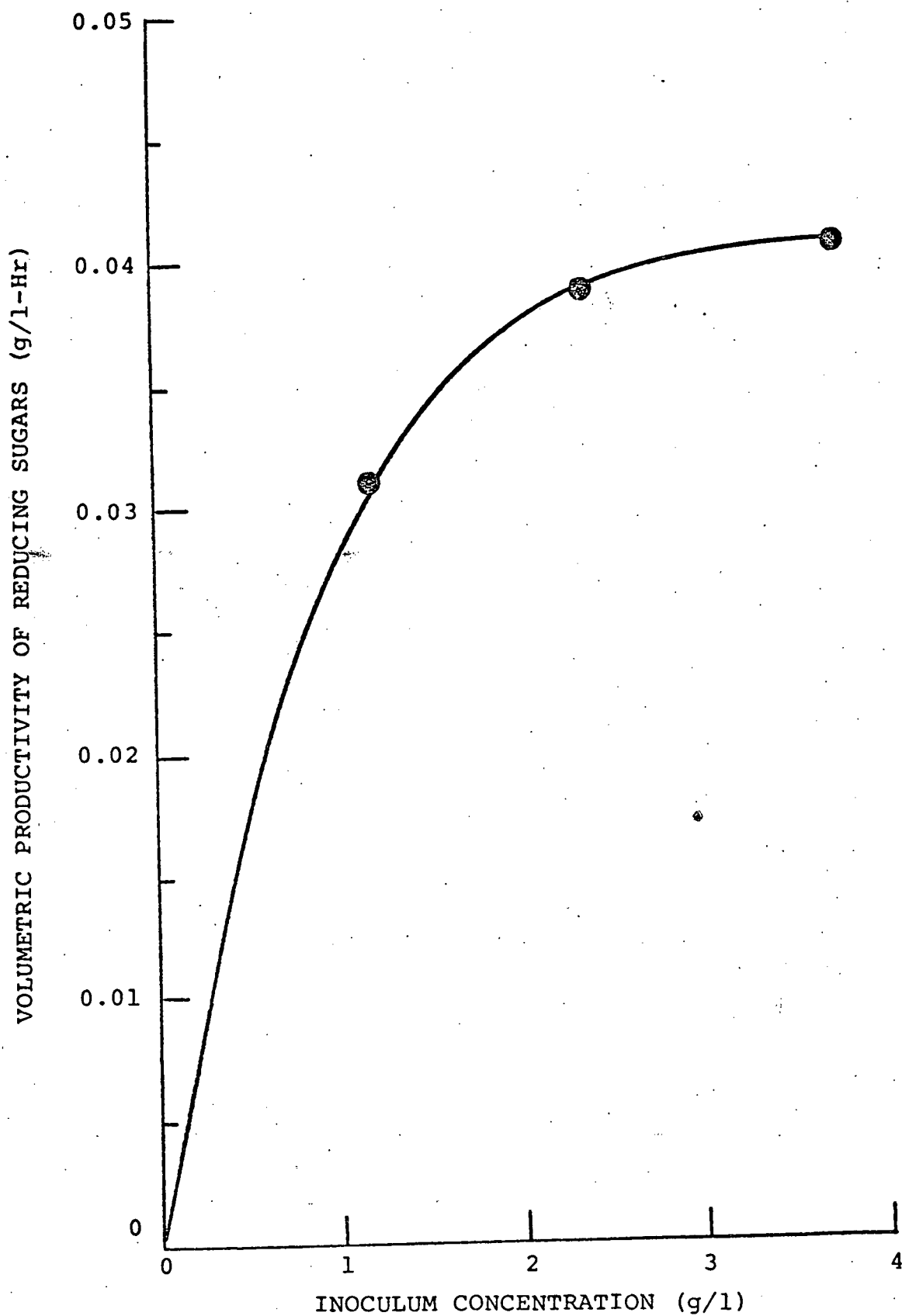
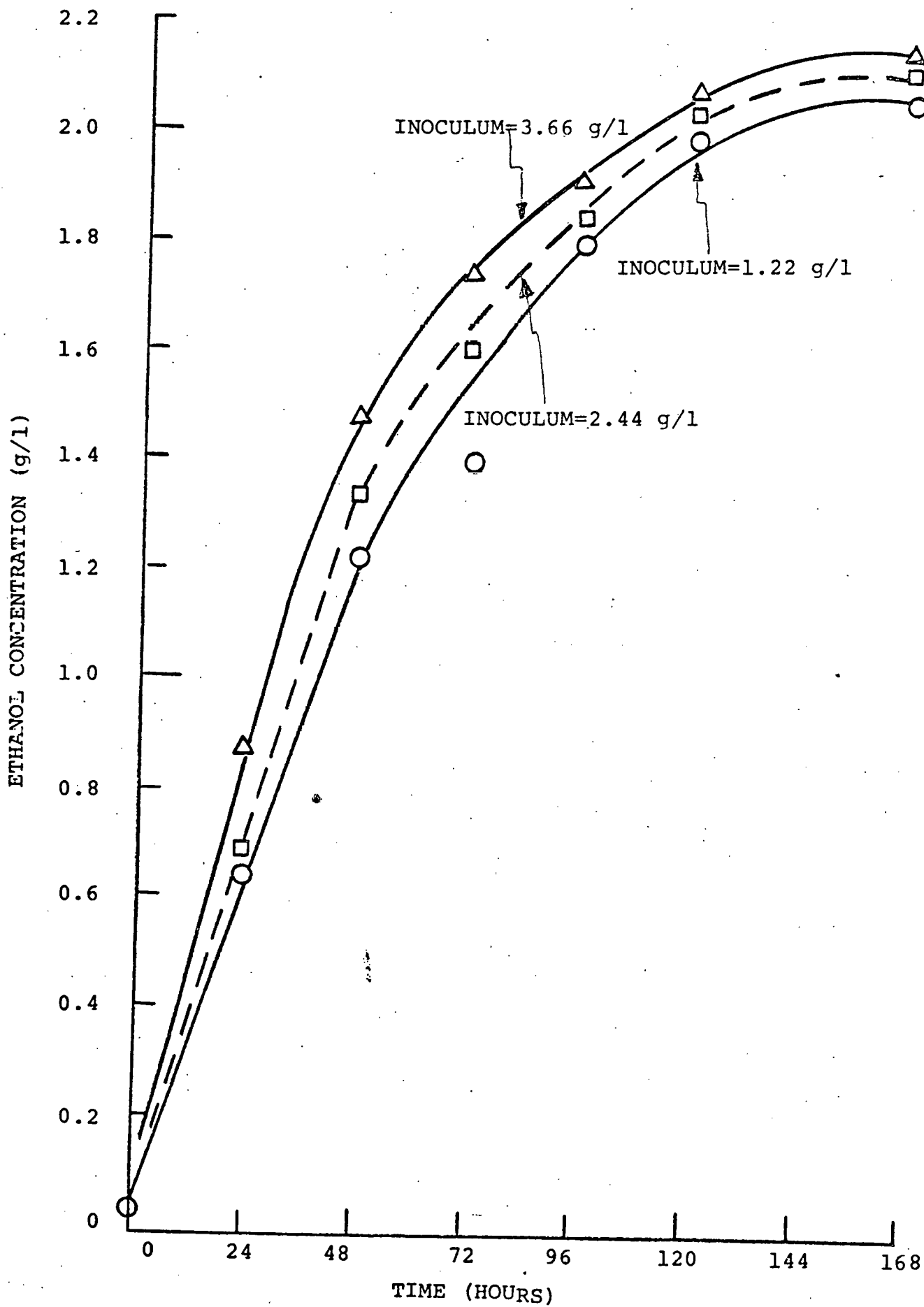


FIGURE I.G.3.7: ETHANOL PRODUCTION BY *C. THERMOCELLUM* (S-4) ON CORN STOVER USING CONCENTRATED CELL INOCULUM



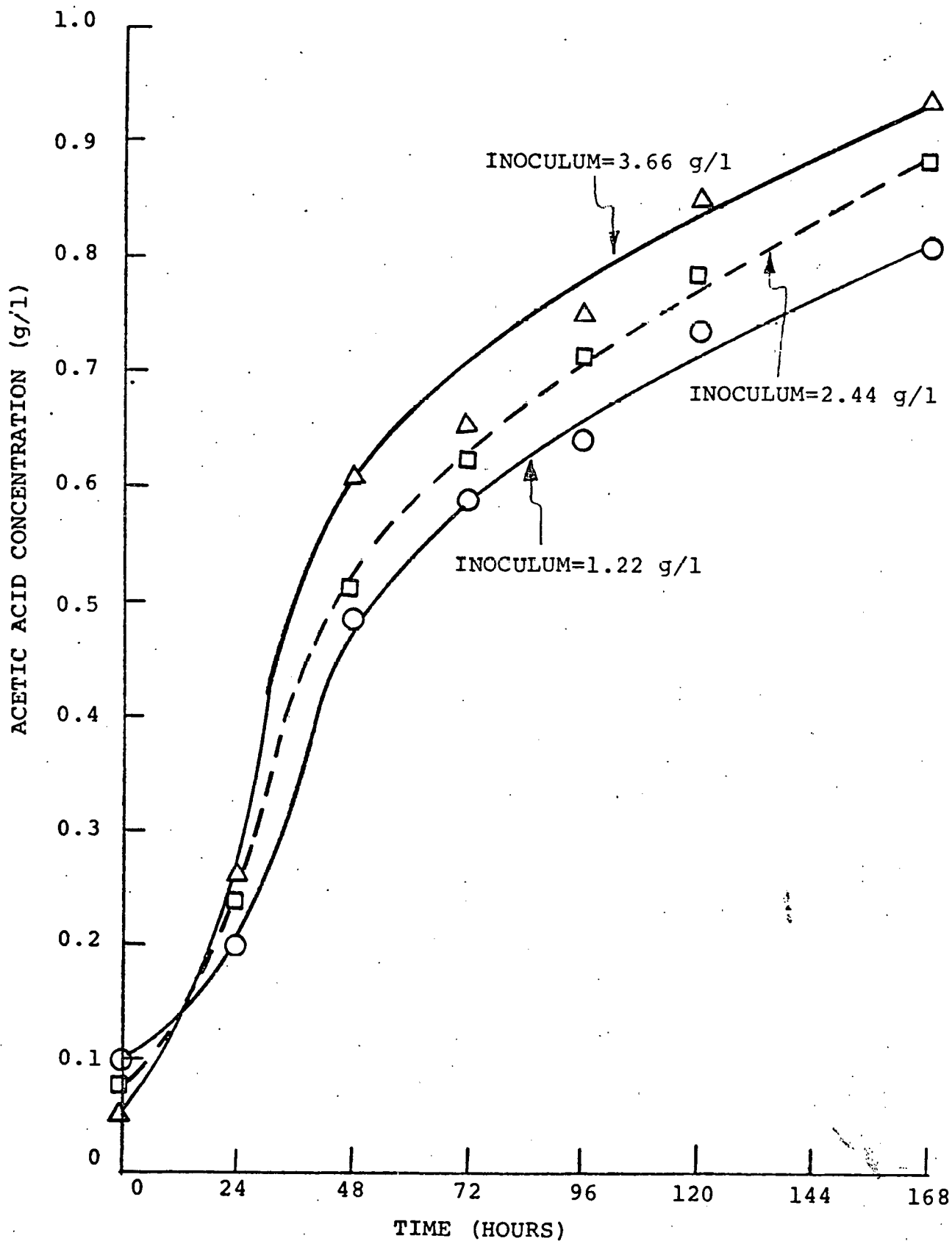
to 0.30 grams of ethanol/1 hour. These similar volumetric productivities of ethanol are not surprising in view of the negligible differences in rates of reducing sugar production which presumably act as the rate limiting control for the whole process. These rates, however, compare favorably with the maximum volumetric rate of ethanol production of 0.015 gms ETOH/1 hr previously observed with a 10% inoculum of S-4 on 27 grams/1 corn stover (COO-4198-8). Finally, the volumetric productivity of acetate by concentrated cells on corn stover (Figure I.G.3.8) increases with increasing cell inoculum density, but the specific productivity also decreases from 0.01 g/gm cell hr to 0.004 g/gm cell/hr and 0.0027 gm/gm cell hr. Thus reconfirming the limitation of cell growth on corn stover at higher cell inoculum density.

d. Future Work

- Future work will focus on the effect of increasing substrate concentration on the kinetics of corn stover degradation. These high concentrations will be achieved with the packed bed type bioreactor.

- The effect of redox potential on the "lag" time and distribution of products such as ethanol, acetate, and lactate will be examined.

FIGURE I.G.3.8: ACETIC ACID PRODUCTION BY C. THERMOCELLUM (S-4) ON CORN STOVER USING CONCENTRATED CELL INOCULUM



4. Process Problems in Cellulose Utilization by C. Thermocellum

a. Introduction

The search for natural cellulosic biomasses for degradation by Clostridium thermocellum was continued. Preliminary experiments with cotton shearings (a by-product of the textile industry, see Progress Report COO-4198-7) had been done to investigate their potential use as a substrate in C. thermocellum fermentations. Those results had been encouraging, but indicated a fairly long lag phase (5 and 7 days for bleached and dyed cotton shearings, respectively). Hence, the experiment was repeated using seed culture grown on cotton shearings to check its effect in shortening the lag phase of the organism.

Work was also continued on another natural substrate, corn cob granules. Since previous experiments had involved a fairly low substrate concentration (1%) a higher concentration (3%) was now employed.

In addition to the work with new crude substrates, a major effort was devoted to a preliminary process design for an ethanol plant using corn stover as the raw material. The process is based on a continuous culture system using a mixed culture of Clostridium thermocellum strain S-4 and C. thermosaccharolyticum. The long-range objective of

such a study is fourfold: (1) to determine the overall stability and technical feasibility of such a system; (2) to identify the need for particular microbiological data not yet available; (3) to identify process steps of higher cost sensitivity that deserve technological improvement; (4) to investigate the economical viability of the system from its cost analysis.

b. Material and Methods

i. New Substrates

In order to investigate the effect of the nature of the seed culture on the length of the lag phase of C. thermocellum ATCC 27405 cultures growing on cotton shearings, this organism was grown at 60°C in Hungate tubes containing 9 ml of CM3 medium with 1% bleached or green dyed cotton shearings. Experiments were also done in anaerobic shake flasks containing 300 ml of the same medium. Samples of the shearings had been obtained from West Point Pepperell (West Point, Georgia 31833). Sequential transfers were made using a 10% (1 ml) inoculum in Hungate tubes and a 3% (10 ml) inoculum in shake flasks.

C. thermocellum ATCC 27405 was also grown at 60°C in anaerobic shake flasks containing 300 ml of CM3 medium with 3% GRIT-O'COBS -60 (The Andersons, Maumee, Ohio 43537) and 0.5% yeast extract. A 3% inoculum grown on Solka floc was utilized. Reducing sugars were assayed by the

DNS method with glucose standards. For determination of residual solids, 5 ml samples of broth were filtered through a Whatman paper #1 and dried at 70°C for 24 hr.

ii. Process Design

The process design was based on an ethanol-from-corn-stover plant with a yearly production of 2.5×10^7 gal of 95% ethanol (1.6×10^8 lb ethanol). The composition of corn stover was assumed to be 50% cellulose, 30% hemicellulose, 15% lignin and 5% ash (the latter was determined experimentally by ignition in a muffle furnace at 560°C for 5 hr).

The organisms selected for the mixed culture continuous fermentation, C. thermocellum S-4 and C. thermosaccharolyticum, are both thermophilic anaerobes that have shown to be compatible in preliminary experiments in this laboratory (Progress Report COO-4198-8). The design involves a continuous single step fermentation with pH controlled at 6.5 where cellulose and hemicellulose are broken down by C. thermocellum S-4 to ethanol, lactic acid and pentoses; the pentoses serve as a substrate for C. thermosaccharolyticum in the production of ethanol, lactic acid and acetic acid. As reported earlier, the tolerance to ethanol of the 2 organisms has been increased through serial adaptation in ethanol, resulting in 50% inhibition of maximum growth at 33 g/l for C. thermocellum S-4 and at 25 g/l for C. thermosaccharolyticum. These values were

used to assess the limitations imposed by ethanol tolerance on the optimization of the process.

c. Results

i. New Substrates

The sequential transfer of C. thermocellum cultures grown on cotton shearings showed that in Hungate tubes by the third transfer the fermentation was essentially completed after 3 days for bleached shearings and after 4 days for dyed shearings, with the final pH around 5.7 - 5.8. In shake flasks by the second transfer the fermentation was completed after 80 hr for both bleached and dyed shearings, with a final pH around 5.6 - 5.7. This represents a 50% reduction in fermentation time as compared to previous experiments with seed grown on Solka floc. The rates of reducing sugar formation had been previously shown to be similar to that of Solka floc with a yield around 0.4 g reducing sugar/g cotton shearing fed. The very limited supply of substrate did not allow for more detailed studies, but these results suggest that with the adaptation of the culture to the new substrate the kinetics of the fermentation are encouraging in the light of commercial processes. The more classical agricultural biomasses such as corn stover involve a certain labor and/or equipment cost for collection and transportation to the plant for subsequent utilization as a substrate. Since the cotton shearings are a by-product produced "in situ"

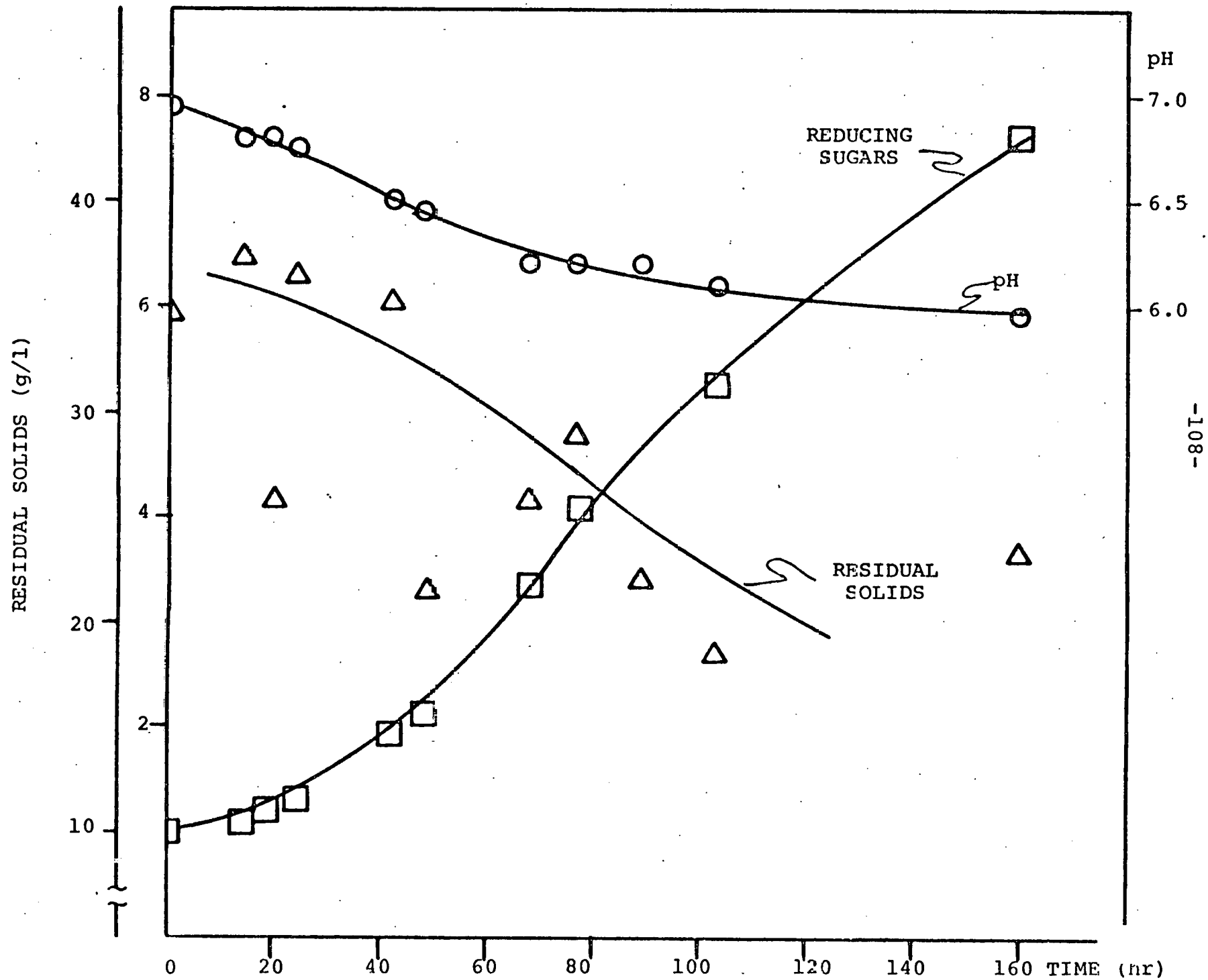
at the textile plant, the possibility of having an adjacent fermentation system would represent considerable savings on the cost of the input to the process.

The reducing sugar accumulation and the residual solids and pH profiles of C. thermocellum grown on 3% corn cob granules (particle diameter = 0.25 mm) are shown in Figure I.G.4.1. The level of reducing sugar reaches over 7.5 g/l, which represents an overall yield of 0.25 g reducing sugar/g corn cob granules fed. Hence, the yield obtained from 3% substrate is slightly lower than that previously obtained for 1% substrate (0.29 g/g) and described in Progress Report COO-4198-6. The larger scatter in the residual solids data reflects the difficulty of pipetting homogeneous samples of the broth slurry; the data suggests that approximately 40% of the original substrate was consumed.

ii. Process Design

A schematic flowsheet of the process is shown in Figure I.G.4.2. The process involves storage and hammer milling of the corn stover, preparation of the culture medium, continuous sterilization of the medium, mixed culture fermentation for the production of ethanol in a one-pass system with no cell recycle, filtration of the broth through a continuous rotary drum filter for removal of cells and undegraded substrate, distillation of the effluent for production of fuel grade ethanol and final storage of ethanol.

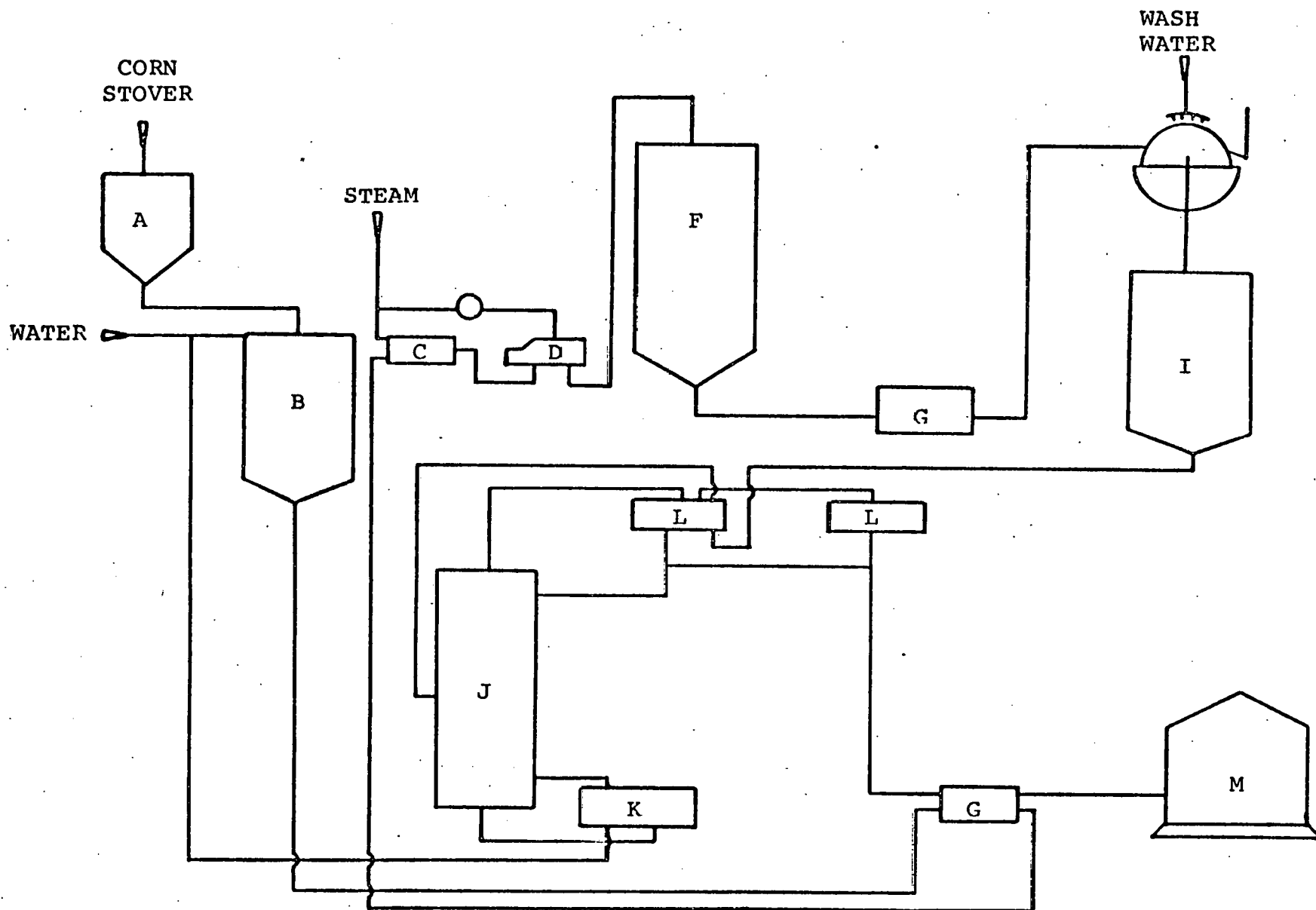
FIGURE I.G.4.1: KINETICS OF THERMOCELLUM ATCC 27405 CULTURE GROWING ON CM3 MEDIUM WITH 3% CORN COB GRANULES



Legend to

FIGURE I.G.4.2: FLOWSHEET FOR ETHANOL PRODUCTION FROM CORN STOVER

- A - Mill
- B - Mixing Tank
- C - Sterilizer
- D - Flash Evaporator/Heat Exchanger
- E - Compressor
- F - Fermentor
- G - Heat Exchanger
- H - Drum Filter
- I - Hold-Up Tank
- J - Distillation Column
- K - Reboiler
- L - Condensor
- M - Storage Tank



A model was developed taking into account:

(a) the mass balances for cells, substrate and products for each culture; (b) the ethanol tolerance limitations of each culture; (c) the dependence of specific production rates of each product on the specific growth rate of the organisms; (d) the stoichiometric relations between substrates and products (to obtain theoretical yields). This model, which will be presented in detail in the next Progress Report, indicates that for the present data the overall yield of the process is about 0.25 g ethanol/g corn stover fed.

d. Future Work

- To implement on a computer the mixed culture model for production of ethanol from corn stover in order to facilitate the optimization of the system and future incorporation of new data.

- To perform a cost analysis of the process to determine total capital investment requirement and ethanol unit cost.

II. Production of Chemical Feedstocks

A. Production of acrylic acid by fermentation

1. Introduction

In the last progress report COO-4198-8 additional results were presented that demonstrate the conversion of sodium propionate to sodium acrylate by resting cells of Clostridium propionicum. Optimization of the many parameters affecting bioconversion have resulted in a maximum product accumulation of approximately 40mM acrylic acid, corresponding to roughly 0.55g/l acrylic acid per mg cellular protein at an initial rate of 3.8 mmoles acrylic acid/l-hr.

Two major objectives remain outstanding: (a) the complete conversion of propionate to acrylate, and (b) the utilization of lactate as the substrate for acrylate production. One obstacle or bottleneck that is preventing achievement of the former objective is the fact that acrylate synthesis ceases after 180 minutes of the initiation of bioconversion. Thus, the short life of the biocatalyst is partially responsible for the low conversion yields observed to date.

In this report, results are presented that contribute to our understanding the reasons that the catalyst is short-lived under the optimized bioconversion conditions as defined in progress report COO-4198-8. Suggestions for future work are made on the basis of these findings.

2. Materials and Methods

Clostridium propionicum ATCC 27405 was grown, harvested and used to convert sodium propionate to sodium acrylate as described in progress reports COO-4198-7 and COO-4198-8. Any modification to the system is specifically mentioned with the data presented.

3. Results

a. Effects of Atmospheric Air on the Bioconversion of Propionic Acid to Acrylic Acid by C. propionicum

C. propionicum is an anaerobic organism which under standard handling techniques often is exposed to oxygen. C. propionicum has been shown to grow readily when returned to anoxic atmospheres. Thus oxygen in "limited" quantities has not been found to be bactericidal even though it may in fact be bacteriostatic. The role of oxygen exposure upon handling and the subsequent production of acrylic acid has not been determined.

To determine whether oxygen exposure affects acrylate production, cells were exposed to air by agitating them vigorously for 180 minutes prior to the addition of propionate substrate plus other bioconversion components and then examined for acrylate production. In the experiment cells were incubated under standard conditions of 200mM Na propionate, 53mM methylene blue, 25mM Na lactate, 50mM Tris buffer, pH 8.0 before and after a 180 min exposure to air at 32°C. The standard reaction mixture is to be referred to in subsequent sections as "propionate reaction mixture" or "PRM". Figure II.A.1 indicates that the cells exposed to air

Figure II.A.1

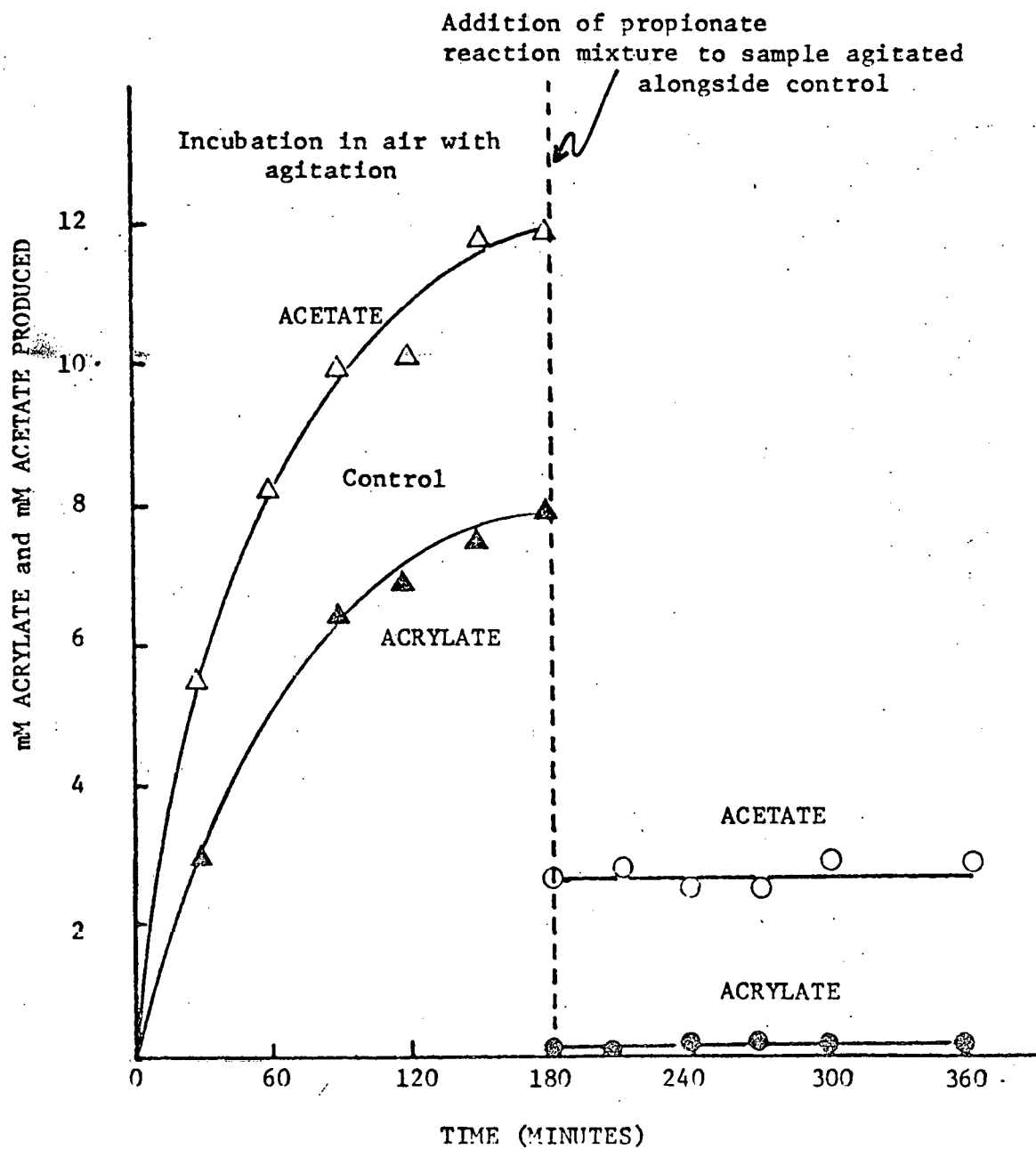
Effect of prior exposure to air on *C. propionicum* ability to form acrylic acid.

Products (Δ - Δ) Acrylate and (Δ - Δ) Acetate of control bio-conversion from 0 to 180 mins, performed under standard conditions of 200mM Na propionate, 53mM methylene blue, 25mM Na lactate, 50mM TRIS buffer pH 8.0. This substrate, plus other components for bioconversion will be referred to as 'propionate reaction mixture' or "PRM". The bioconversion was performed using 1000ug samples in 13 x 100 mm glass test tubes, with agitation in air atmosphere at 32°C. Samples were withdrawn and assayed for volatile fatty acids eluting from the column before valerate.

To determine loss of activity on exposure to air, a cell suspension in 50mM TRIS buffer pH 8 was agitated in air alongside the control. No PRM component was added; no samples were withdrawn. At 180 min the 'PRM' was added. The final concentration of all components of the bioconversion mixture equalled the control. No correction was made however for endogenous metabolite accumulation. Samples were withdrawn and (products, ($\bullet$ - $\bullet$) acrylate, and ($\circ$ - $\circ$) acetate) assayed at indicated times between 180 and 360 minutes.

Fig. II.A.1

LOSS OF CELLULAR ABILITY TO PRODUCE ACRYLIC ACID
AFTER EXPOSURE TO AIR



for 180 minutes cannot produce acrylate or acetate from propionate and 25mM lactate present in the PRM mixture. To ensure that the observed loss in biological activity was in fact a result of exposure to air and not some other ill-defined factors, an additional control experiment was conducted in which C. propionicum cell suspension was "preincubated" anoxically in an N₂ atmosphere for 180 minutes prior to addition of propionate and other bioconversion components. The results in Figure II.A.2 indicate that cellular activity is maintained during the preincubation period. Thus exposure to air atmosphere is then implicated as being at least partially responsible for the short life of acrylate producing system.

Similar experiments were performed with C. propionicum cell suspensions "preincubated" in the presence of methylene blue as the electron acceptor instead of oxygen. A decrease in biological activity due to prior exposure to methylene blue is also deduced from the data in Figure II.A.3. This data also suggests toxicity or loss in biological activity is due to the presence of an exogenous electron acceptor.

These experiments provide several directions for new research. For example:

- i. The conversion of Na propionate to Na acrylate should be done under anoxic conditions with a non-toxic electron acceptor.

- ii. The importance of lactate during anoxic bioconversion is also in question. The rapid uptake of 25mM lactate

Figure II.A.2

Maintenance of cellular activity in an anoxic N₂ atmosphere

- (Δ-Δ) acrylate produced and Δ-Δ produced in CONTROL run, similar to that present in Fig. II.A.1.
- (●-●) acrylate and (0-0) acetate produced by cells that were allowed to incubate 180 min in an anoxic environment prior to bioconversion in air atmosphere. Between 0 and 180 min, the cell suspension was incubated anoxically in a nitrogen atmosphere. No 'PRM' component was added, neither were samples withdrawn. At 180 min, the 'PRM' was added, such that the final concentration of all components equalled that of the control. No allowance was made for anoxic endogenous metabolic product accumulation, though propionate, acetate and acrylate are now known to accumulate endogenously during anoxic incubation (Fig. II.A.9). The entire bioconversion was then agitated under aerobic atmosphere and samples withdrawn periodically and assayed as usual.

Fig. II.A.2

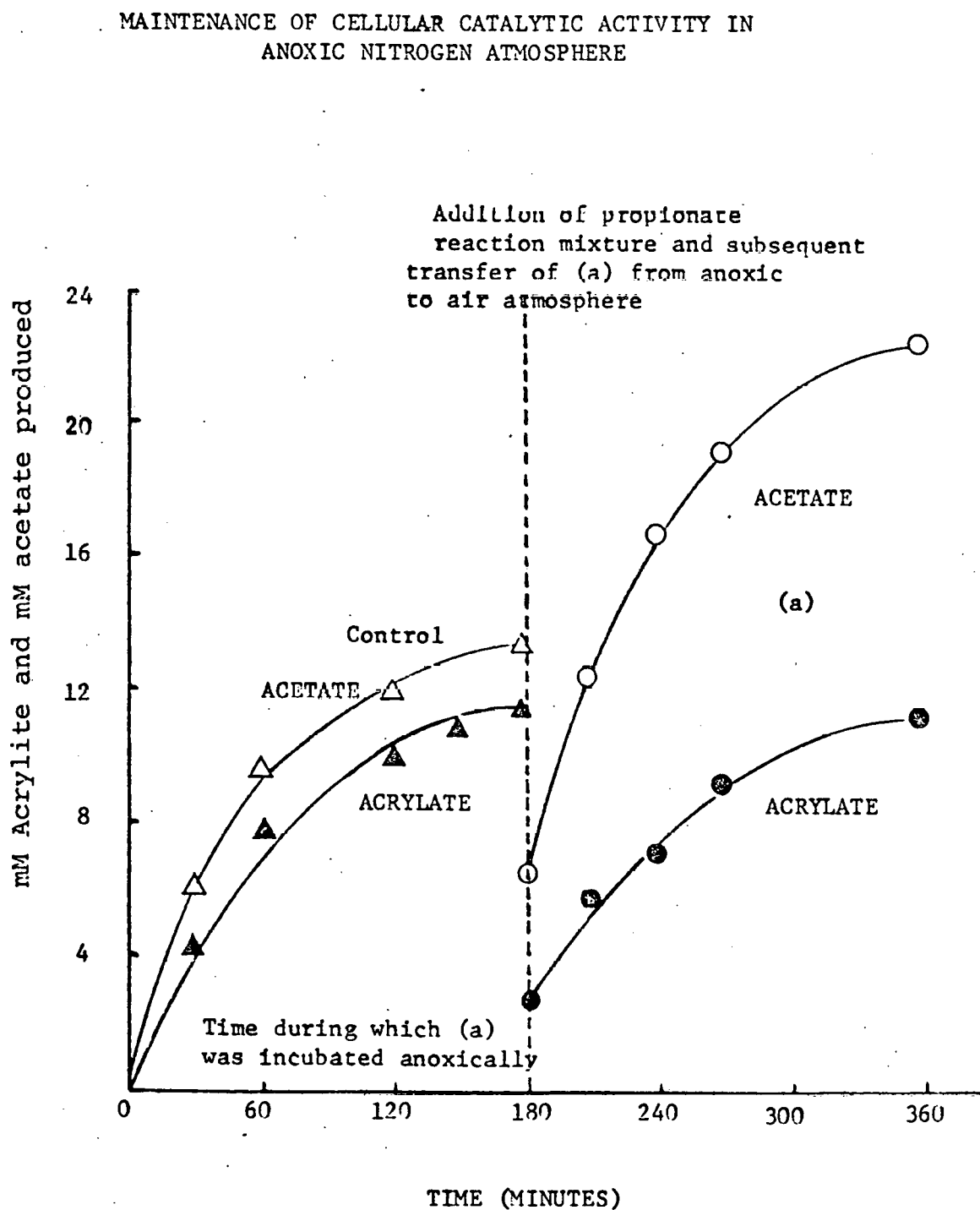


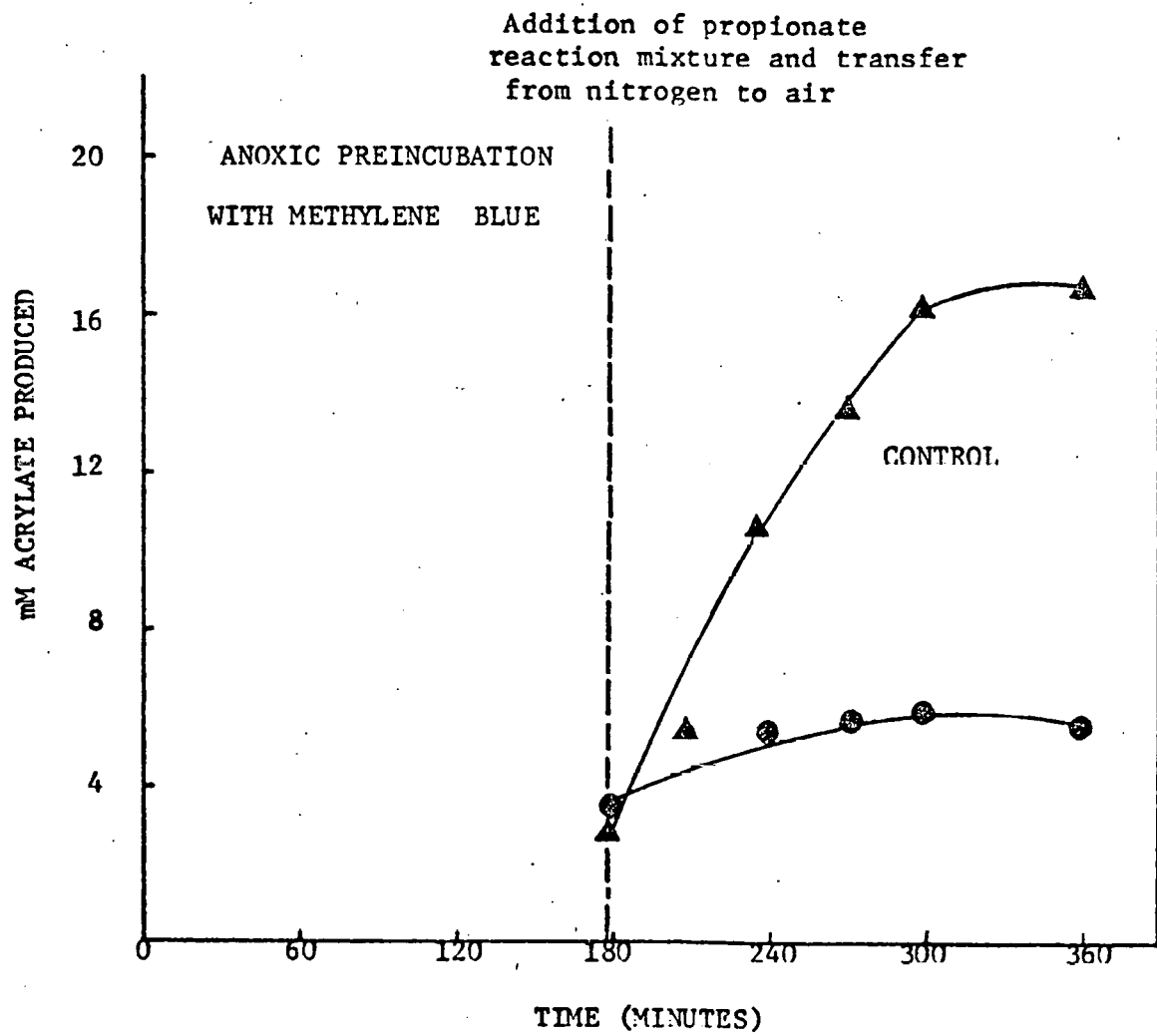
Fig. II.A.3

Effect of preincubation with methylene blue on *C. propionicum* cellular activity

- Acrylic acid produced by *C. propionicum* cell suspension using optimal bioconversion techniques after initial incubation with methylene blue. A resting cell suspension in 50mM TRIS buffer pH 8.0 was incubated anoxically between 0 and 180 minutes with 53mM methylene blue. No samples were taken during this period. at 180 min, "PRM" was added to arrive at optimal final concentrations (no correction was made for endogenous product accumulation during "preincubation"), and the bioconversion was performed in air atmosphere between 180 and 360 minutes.
- ▲-▲ Control: A cell suspension was treated as above with the exception that no methylene blue was added during the first 180 minutes.

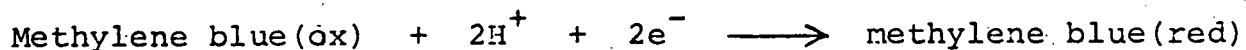
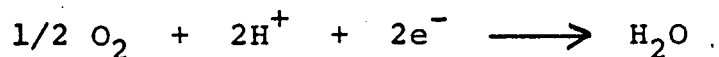
Fig. II.A.3

Effect of preincubation with methylene blue on C. propionicum



to nearly 25mM acetate and 50mM NADH is itself enough to reduce the 53mM methylene blue added. Lactate linked activation and the bioconversion could possibly be carried out in two separate stages; the first stage could be carried out without the presence of an electron acceptor. Support for this possibility is presented later in this progress report.

iii. Another aspect to be considered is the control of dissolved oxygen in the system. To oxidize 53mM methylene blue, approximately 26mM molecular oxygen is required since



Under existing conditions it is estimated that roughly 0.2mM dissolved oxygen exists in the reaction mixture. Limiting the dissolved oxygen further, so as to permit continuous regeneration of the reduced methylene blue only, may be feasible and thus one may increase the stability of the system.

iv. Regeneration of inactivated cells and reusing them remains a definite possibility. Figure II.A.4 in this report and Figure II.A.2.10 in progress report #7 show preliminary data of what happens when cells are transferred from air to nitrogen atmosphere after having produced acrylic acid in air atmosphere. Figure II.A.4 shows that cells transferred from air to nitrogen after 90 minutes of exposure to air with bioconversion taking place can reduce acrylate, while those exposed to 180 minutes of air during bioconversion cannot.

Fig. II.A.4

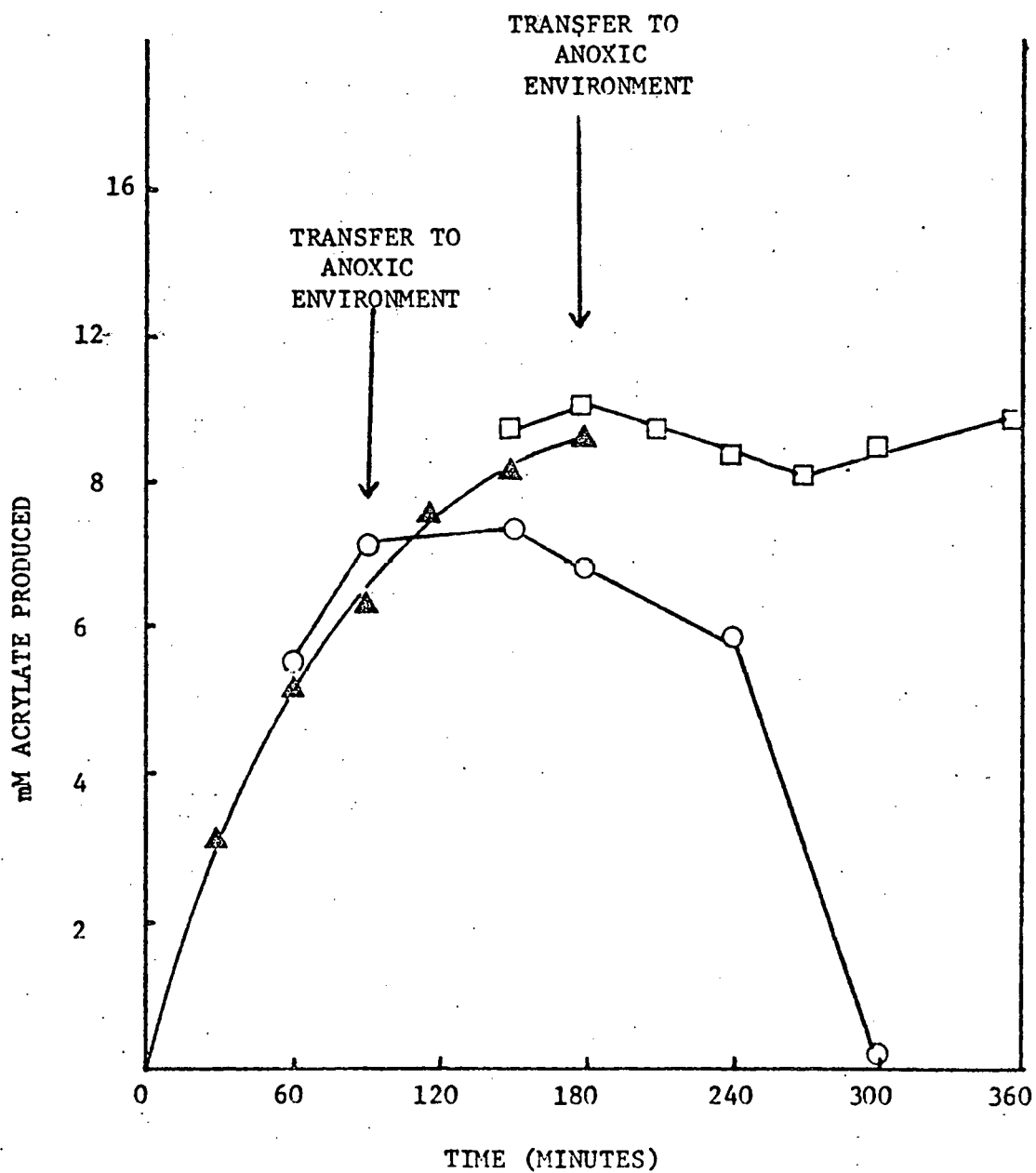
Inactivation of C. propionicum observed as the inability to reduce acrylate

▲-▲ Control: acrylate produced using standard optimized conditions enumerated in Fig. II.A.1 legend.

o-o and ■-■ acrylate in samples run parallel to the above control. Two samples, indicated by open circles and open squares were transferred from air to nitrogen atmosphere, one at 90 minutes and the other at 180 minutes, indicated by arrows. Acrylate uptake was followed.

Fig. II.A.4

INACTIVATION OF CELLS IN AIR RESULTS IN THE
INABILITY TO REDUCE ACRYLATE



Data from progress report COO-4198-7 shows that cells capable of reducing acrylate, on being returned again to aerobic conditions produce acrylate at a slower rate than fresh cells. The site of inactivation may be a specific enzyme. Also the viability of some cells may be lost. Viable counts are being obtained as MPN taken as a function of exposure to dissolved oxygen and time. Activity of cells as a function of dissolved oxygen and time is also to be studied to establish a causal relationship. Regeneration possibilities will be studied in greater depth in the next research period.

v. The studies on preincubation with methylene blue have provided some additional insight into the role of the electron acceptors. As summarized below methylene blue with a E'_O of 11mV is not the best electron acceptor for the half reaction in which reduction of acrylic occurs with an E'_O of roughly 185mV.

<u>Half Reaction</u>	<u>E'_O at pH 7.0</u>
$1/2 O_2 + 2H^+ + 2e^- \longrightarrow H_2O$	$E'_O = 816mV$
$2,6\text{Dichlorophenolindophenol} + 2H^+ + 2e^- \longrightarrow 2,6, - \text{DCPP (RED)}$	$E'_O = 220mV$
$\text{Acrylyl CoA} + 2H^+ + 2e^- \longrightarrow \text{Propionyl CoA}$	$E'_O = 185mV$
$\text{Methylene blue}_{(ox)} + 2H^+ + 2e^- \longrightarrow \text{Methylene Blue}_{(RED)}$	$E'_O = 11mV$

By calculating equilibrium constants using the Nernst equation derivative $[E'_O = \frac{2.3RT}{nF} \log K'^{eq}]$ it is evident that much lower concentrations of a primary electron acceptor like 2,6-DCPP could

replace methylene blue with the same ability to accept electrons. The use of other primary and final electron acceptors will be looked into during the next research period.

vi. In addition, bioconversion studies with cell free systems will be investigated. Initially investigations into oxygen lability will be determined. Development of a cell free system is critical for understanding the kinetics of acrylic acid production.

b. Role of Acrylate in inhibiting Acrylic Acid Formation: Product Inhibition.

In attempting to determine the cause for the short life of the system, it was thought that the product itself, acrylic acid, might inhibit further bioconversion. Figure II.A.5 represents data on the rate of acrylate production vs. substrate (propionate) concentration as a function of time in the presence of various concentrations of added acrylate. Michaelis-Menten type saturation kinetics were not observed over the range of propionate concentrations studied (50 to 300mM). A double reciprocal Lineweaver-Burk plot of the data, Figure II.A.6 does give some additional insight into the possible mode of inhibition. A word of caution! It should however be noted that this kinetic data was obtained with resting cell suspensions and not with cell free extracts. More confidence and meaning will result from kinetic experiments that are in fact obtained with cell free extracts. Moreover, two additional simplifications have been employed in presenting the data. The first is a unireactant model which has been employed for the study of a bisubstrate system and the second

Fig. II.A.5

PLOT: RATE OF ACRYLATE PRODUCTION VS. SUBSTRATE CONCENTRATION (PROPIONATE) AS A FUNCTION OF INHIBITOR CONCENTRATION (ACRYLATE), ADDED TO THE REACTION MIXTURE AT TIME) 0 MIN.. RATE WAS CALCULATED IN THE REGION BETWEEN 30 AND 120 MIN.

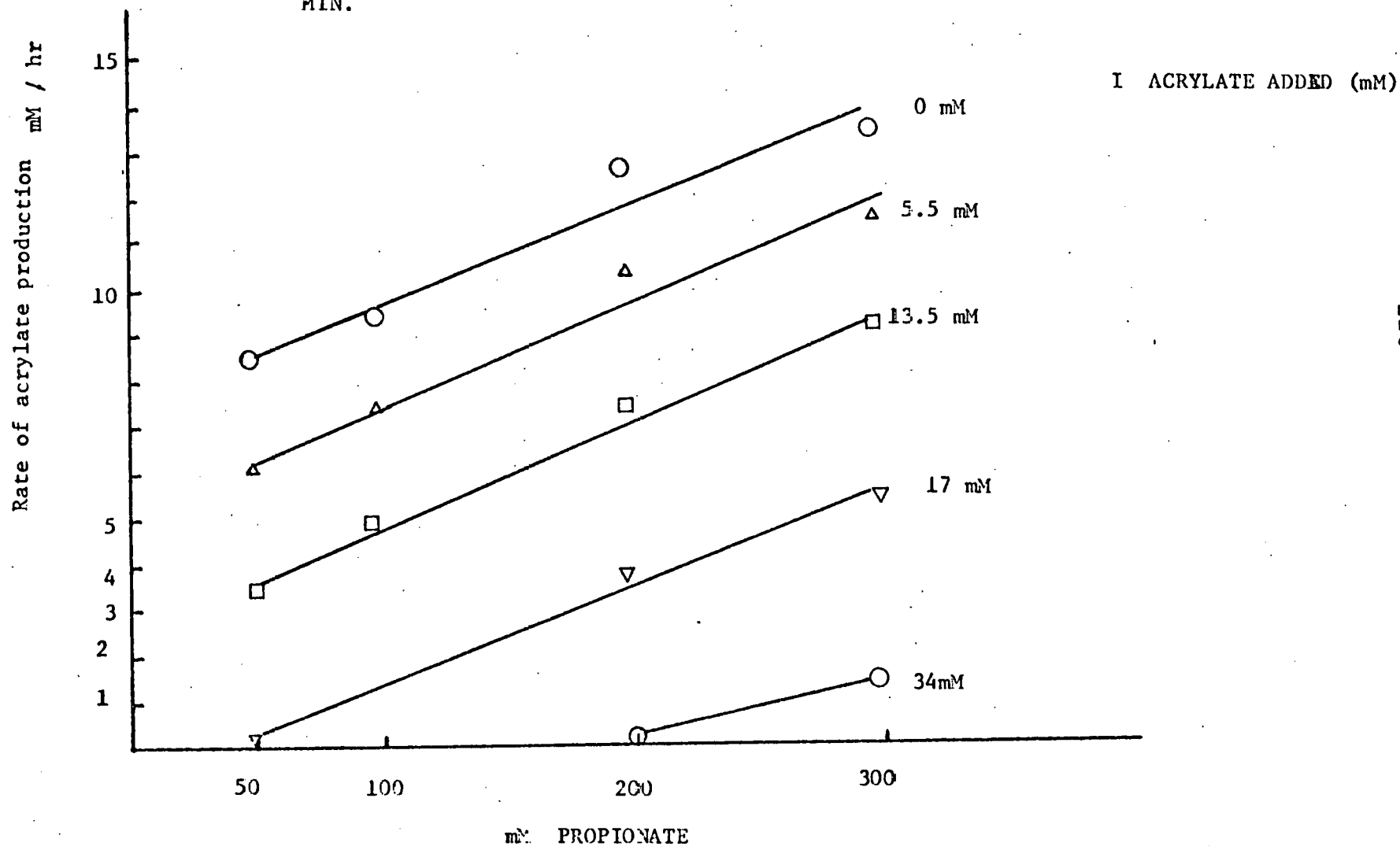
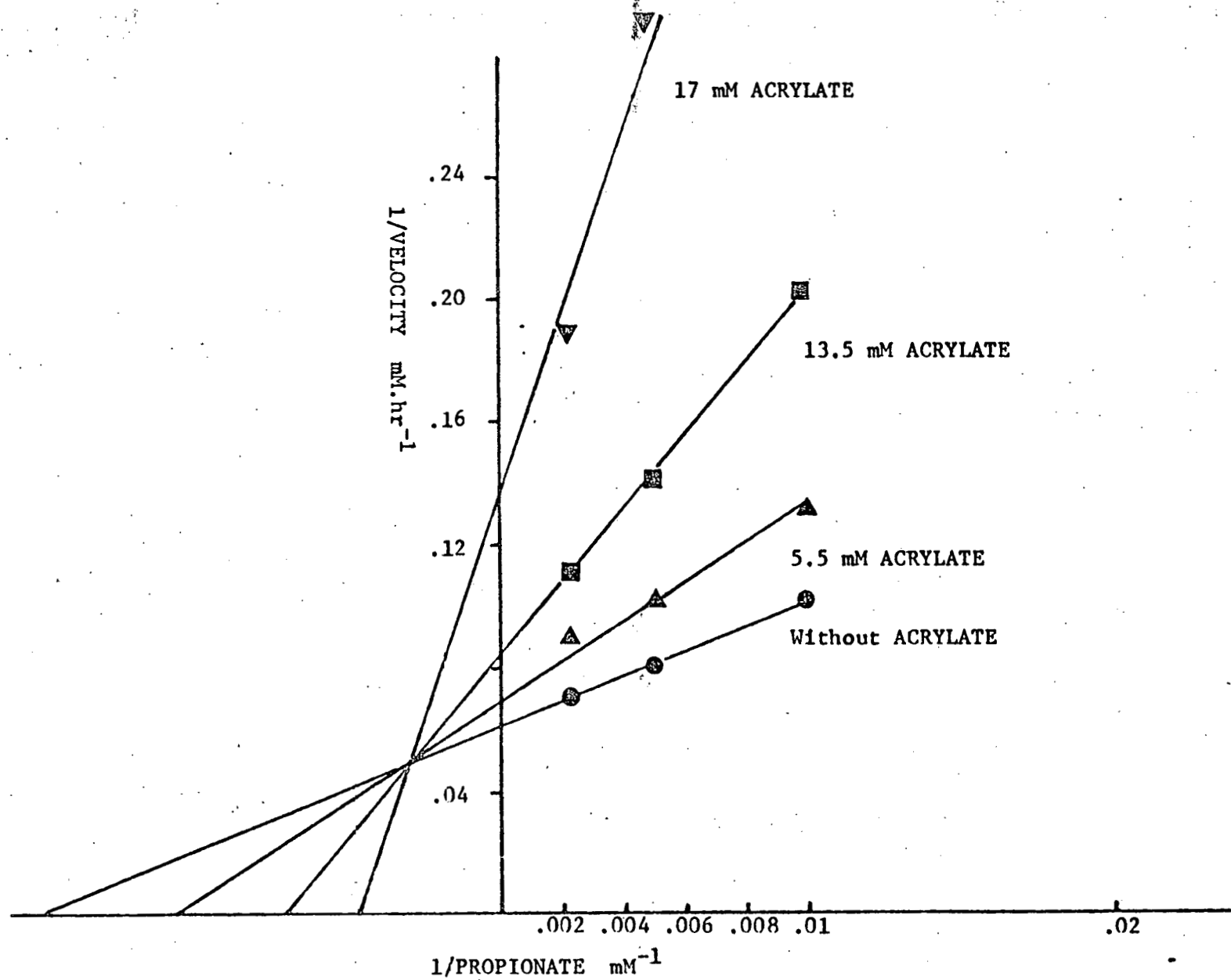


Fig. II.A.6

DOUBLE RECIPROCAL PLOT (LINEWEAVER-BURK) OF THE RATE OF ACRYLATE PRODUCTION VS. VARYING SUBSTRATE CONCENTRATIONS (PROPIONATE) AS A FUNCTION OF INHIBITOR (ACRYLATE) CONCENTRATION



is that the rate limiting step of this multienzyme system is taken to be the rate of propionyl CoA to acrylyl CoA bioconversion which is measured as the rate of acrylate accumulation. The latter factor makes calculations of K_m and V_{max} and K_i less meaningful in the traditional sense of enzyme kinetics.

The data however proposes a linear mixed type inhibition consisting of a change in both K_m and V_{max} of the system with increasing inhibitor concentration. Since in this instance, product and inhibitor are identical, the product once formed would change the dissociation constant of the reaction from K by a factor α to αK . This implies that acrylate and propionate would bind at 2 separate sites, an improbability in our system. More likely is the occurrence of simple competitive inhibition. The change in V_{max} is possibly an artifact of the complicated nature of studying enzyme kinetics in whole cells. Competitive inhibition would be easy to understand since the K_m of the enzyme for acrylyl CoA is likely to be much lower than that for propionyl CoA.

Though inhibition of the bioconversion under air atmosphere by acrylate is clearly observed, not much information is known regarding the site of inhibition, or the role of oxygen in inhibition by acrylate. Results in Figure II.A.8 show that the site is unlikely to be the acrylate metabolizing system itself. Influence of the inhibitor (acrylate) on acetate production and lactate uptake needs to be determined and will be done in future experiments.

c. The Optimum Propionate Concentration

In the process of studying inhibition kinetics, data concerning acrylate production as a function of propionate concentration was obtained. Surprisingly a substrate concentration up to 800mM did not saturate the system. (Figure II.A.7). Both the rate and final amount of product continue to increase. The data is presented with the intention of showing that 200mM propionate is not optimal in the present system. However, the cell batches in this study were only capable of producing a molar yield of $0.03 \frac{\text{moles acrylate}}{\text{moles propionate}}$ corresponding to a total of 7mM propionate against a maximum yield of 0.18 corresponding to 36mM acrylate from propionate that has been obtained in other studies. Hence though in this cell batch acrylate production increases linearly, it is doubtful that the same would occur at higher product yields with acrylate inhibition intervening. Yields and rates of product formation will be plotted vs substrate concentration with the results from optimum conversion yield studies.

d. Anaerobic Systems

i. Acrylate Inhibition

To determine whether the enzymes of anaerobic metabolism of acrylate were inhibited by acrylate itself, concentrations between 0 and 108mM acrylate were added to the cell suspensions at time 0 minutes. Rapid metabolism of the inhibitor resulted, such that up to 108mM acrylate was metabolized within 45 minutes (Figure II.A.8). The products formed were propionate and acetate occurring in the ratio 3:2, a deviation from the ratio from the normal ratio of 2:1 expected on the basis of electron

Fig. II.A.7

ACRYLATE PRODUCED AS A FUNCTION OF SUBSTRATE (PROPIONATE) CONCENTRATION

The bioconversion of propionate to acrylate was conducted using optimised conditions. Only the substrate concentration was altered. The reported concentration of acrylate produced corresponds to the amount produced after 180 mins. The reported values are relative to the amount produced using 200mM propionate. The closed and open circles are results from separate runs.

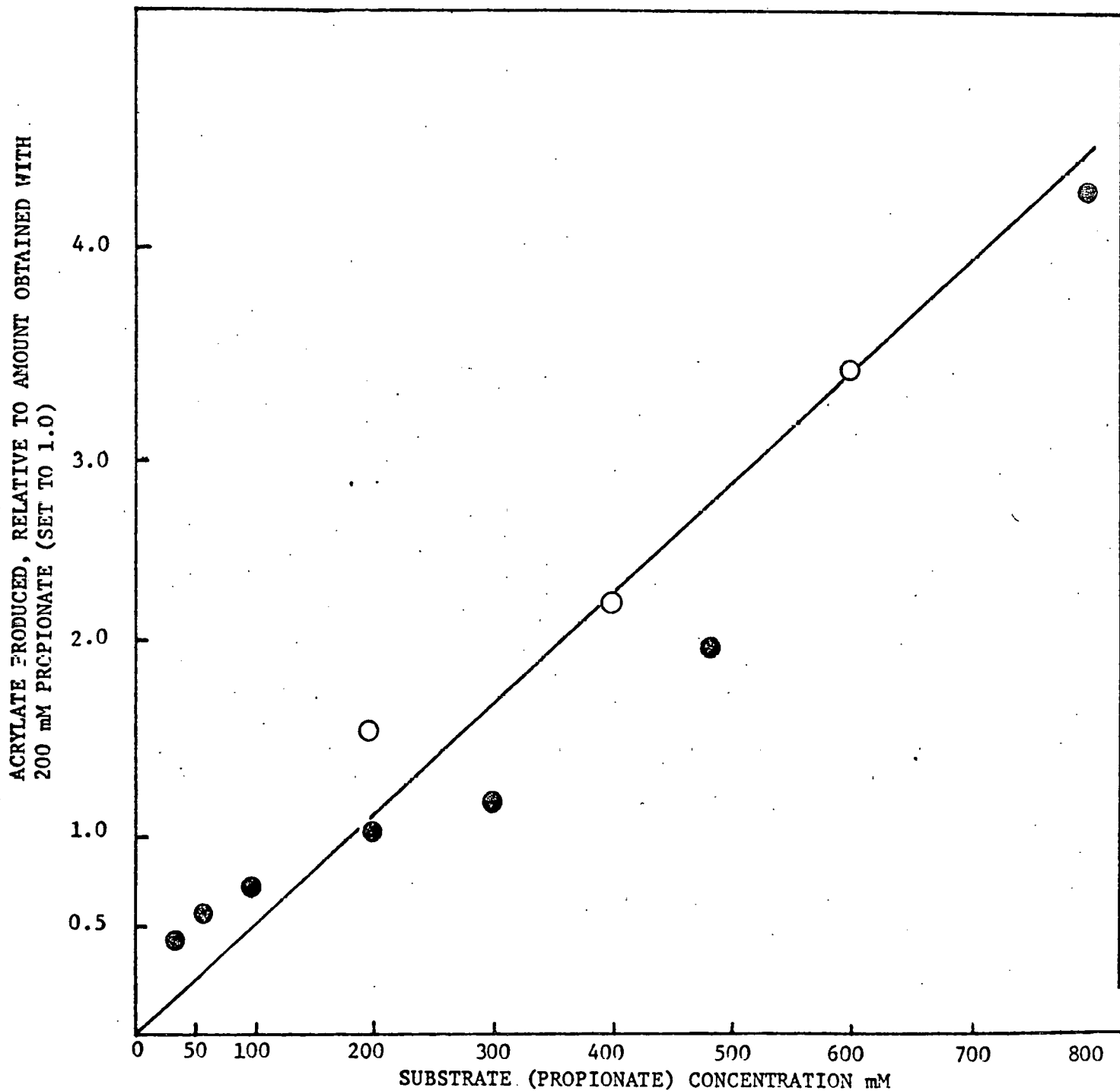


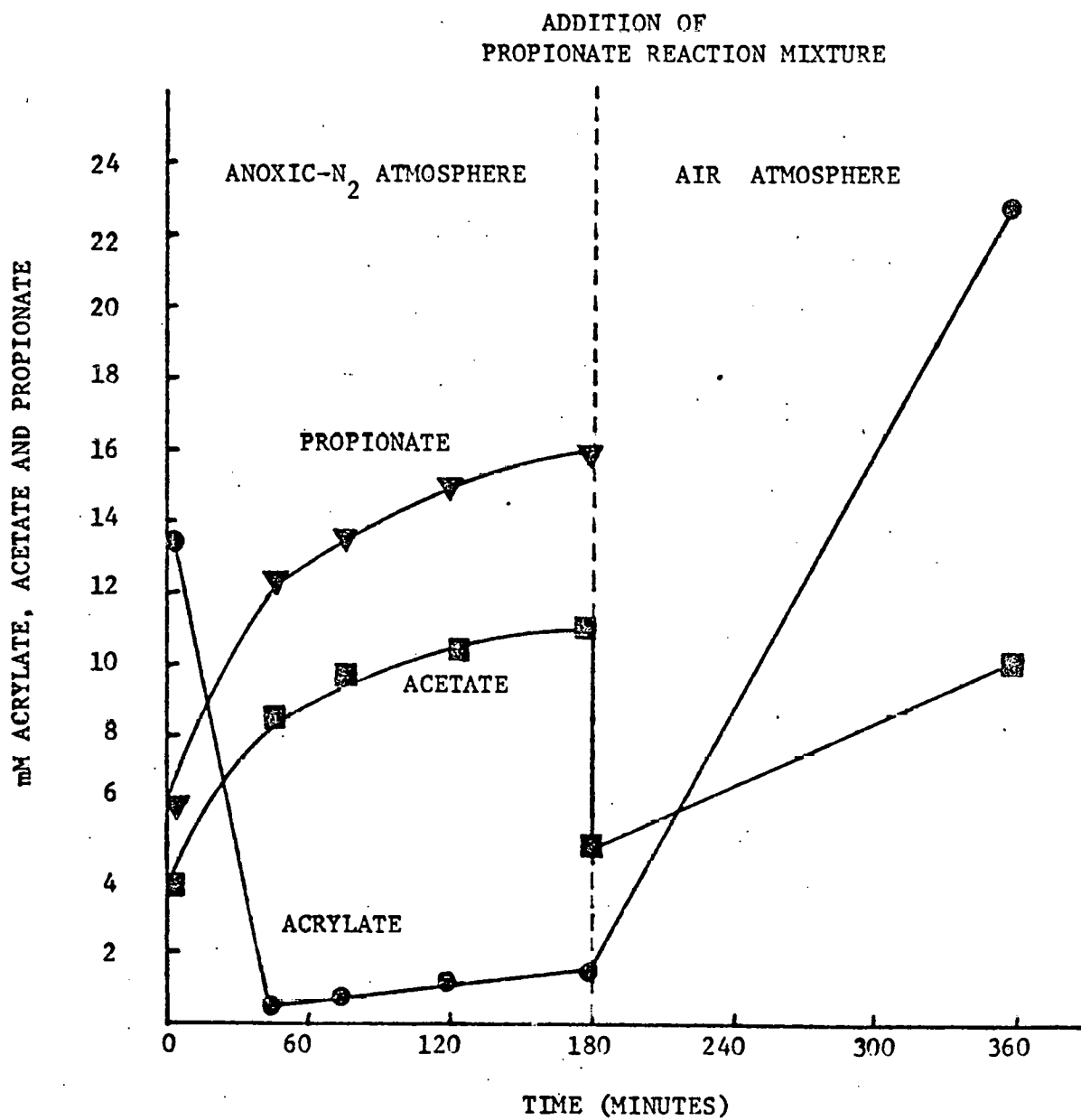
Figure II.A.8

Anoxic uptake of Acrylate and subsequent activation of acrylate production

(●-●) Acrylate, (■-■) acetate and (▼-▼) propionate produced by a cell suspension in 50mM TRIS buffer pH 8.0, incubated anoxically in nitrogen atmosphere at 32°C. Na-acrylate was added and its uptake followed by assaying samples at indicated times. After 180 minutes of anaerobic metabolism, propionate reaction mixture was added to the cells such that the final concentration of all components were optimal. the cells were then agitated in air for 180 min. Acrylate concentrations at time 0 minutes were varied from 0 to 108mM in different runs. This run represents the addition of 13.5mM acrylate.

Fig. II.A.8

ANOXIC UPTAKE OF ACRYLATE AND SUBSEQUENT ACTIVATION
OF ACRYLATE PRODUCTION



balancing between acetate and propionate. An additional product has been observed as a peak on the gas chromatograms and it occurs between propionate and acrylate. The new peak increases in amount with the time of anaerobic metabolism with (a) acrylate or (b) both acrylate and lactate together as substrates and even (c) as a result of endogenous metabolism. The identification of this compound by GC-MS techniques is being pursued, following which detailed acrylate uptake kinetics will be studied. The apparent absence of acrylate inhibition to anoxically suspended cells opens up possibilities of increasing product yield by conducting the bioconversion anoxically.

ii. Anaerobic Endogenous Metabolism

The cell suspension in buffer only began to accumulate propionate and acetate in the ratio of 2.5:1 (Figure II.A.9). Two surprising observations were made; the accumulation of a compound corresponding to acrylate and another corresponding to the unknown mentioned above. Acrylate accumulation under anoxic conditions is a contradiction, but as can be seen, it occurred also in Figure II.A.8. The endogenous acrylate production at various initial concentrations of "inhibitor" (acrylate), both with and without lactate are compiled in Figure II.A.11. (In Figure II.A.9, the concentrations of the unidentified compound are not in mM units. They are arbitrary units, represented as such only to show its increasing accumulation.)

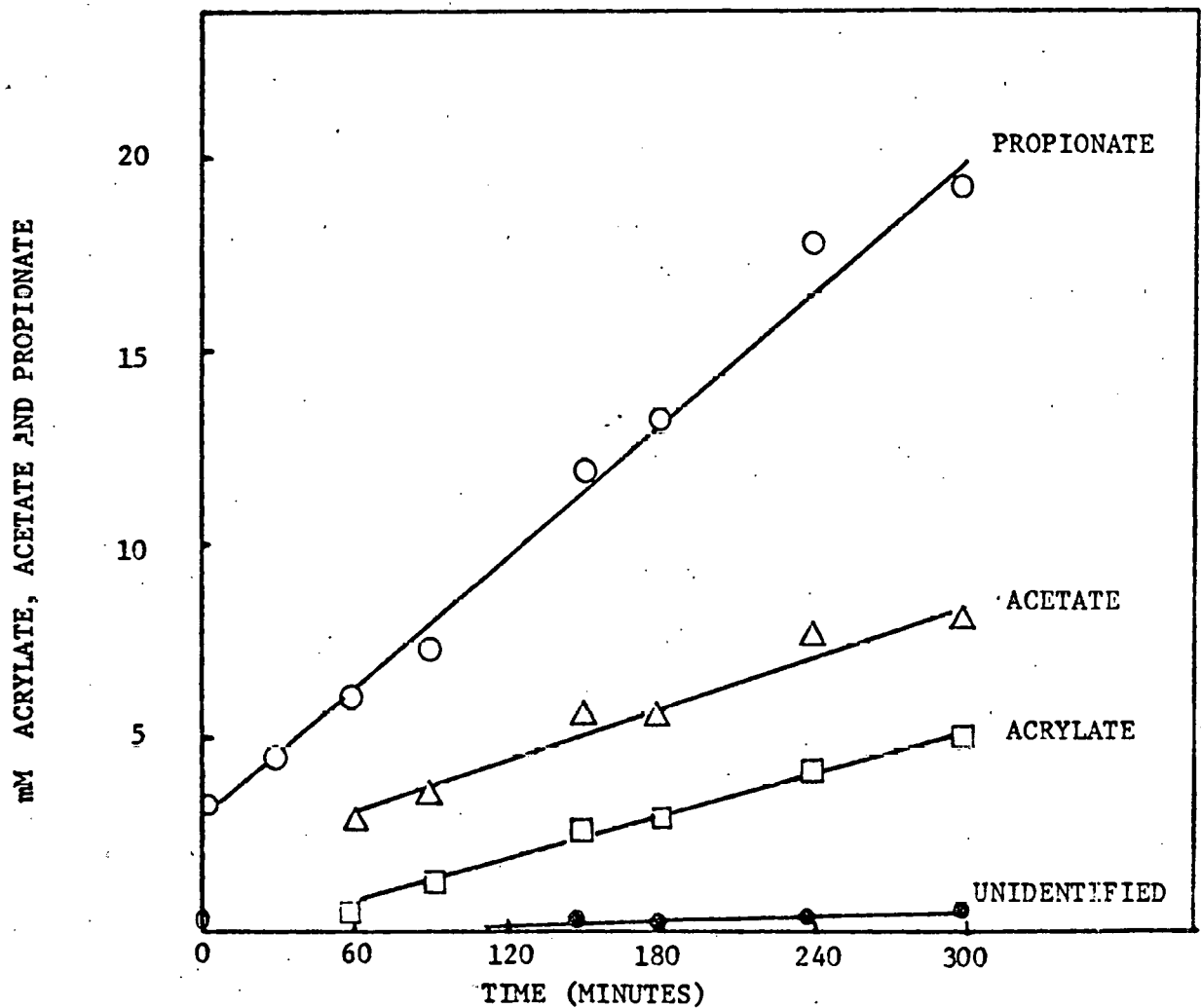
iii. Anaerobic Uptake of Lactate and Acrylate

Figure II.A.9 shows results similar to those of acrylate uptake represented in Figure II.A.8. No endogenous acrylate accumulation occurred. With lactate, the unidentified peak

Fig. II.A.9

ENDOGENOUS ANAEROBIC METABOLISM

Clostridium propionicum cells were harvested, pooled and washed X3 in 50 mM TRIS buffer pH 8.0 with 0.03% Na₂S and resuspended in 50mM TRIS pH 8.0. The suspension was incubated anoxically at 32 C in a N₂ hood. At indicated times, samples were withdrawn, acidified and assayed.



accumulated to a maximum level, while propionate and acrylate maintained the 3:2 ratio. A similar ratio as a result of anaerobic uptake of lactate has been observed before in Figure II.A.4 of progress report COO-4198-6.

iv. Increase in Bioconversion Yield by "Preincubation" of Cells

Both Figures II.A.8 and II.A.10 are divided into 2 parts, an anaerobic metabolism followed by addition of propionate, methylene blue, lactate and oxygen (the standard aerobic bioconversion). A definite increase in acrylate production is observed during bioconversion in air atmosphere after the cells have been preincubated in acrylate or lactate or both. Figure II.A.11 represents the compiled data of acrylated produced after preincubation with acrylate at concentrations ranging from 0 to 108 mM. A three fold increase is obtained in the presence of 300mM lactate even without acrylate.

A possible explanation is that incubation of the cells anoxically with lactate or acrylate may induce enzyme synthesis of those enzymes directly concerned with the acrylate metabolic pathway. Although, the addition of protein synthesis inhibitor like chloramphenicol would resolve the question, the study of initial acrylic acid production rates of cells preincubated with lactate and cells without preincubation shows that preincubated cells have an initial acrylate production rate that is 400% greater than previously reported.

A two stage bioconversion appears appropriate. Activation with an optimum lactate concentration without electron acceptor, followed by a second stage of anoxic or aerobic bio-

Figure II.A.10

Anaerobic uptake of acrylate in the presence of lactate and subsequent activation of acrylate production

(Δ - Δ) propionate, ($\square$ - $\square$) acetate and (0-0) acrylate produced exactly as in Fig. II.A.8, with the exception that 300mM lactate was added in addition to acrylate. Acrylate concentrations ranging from 0 to 180mM were used in different runs. This run represents addition of 4g/l acrylate at time 0 minutes.

Fig. II.A.10

ANAEROBIC UPTAKE OF ACRYLATE IN THE PRESENCE OF LACTATE
AND SUBSEQUENT ACTIVATION OF ACRYLATE PRODUCTION.

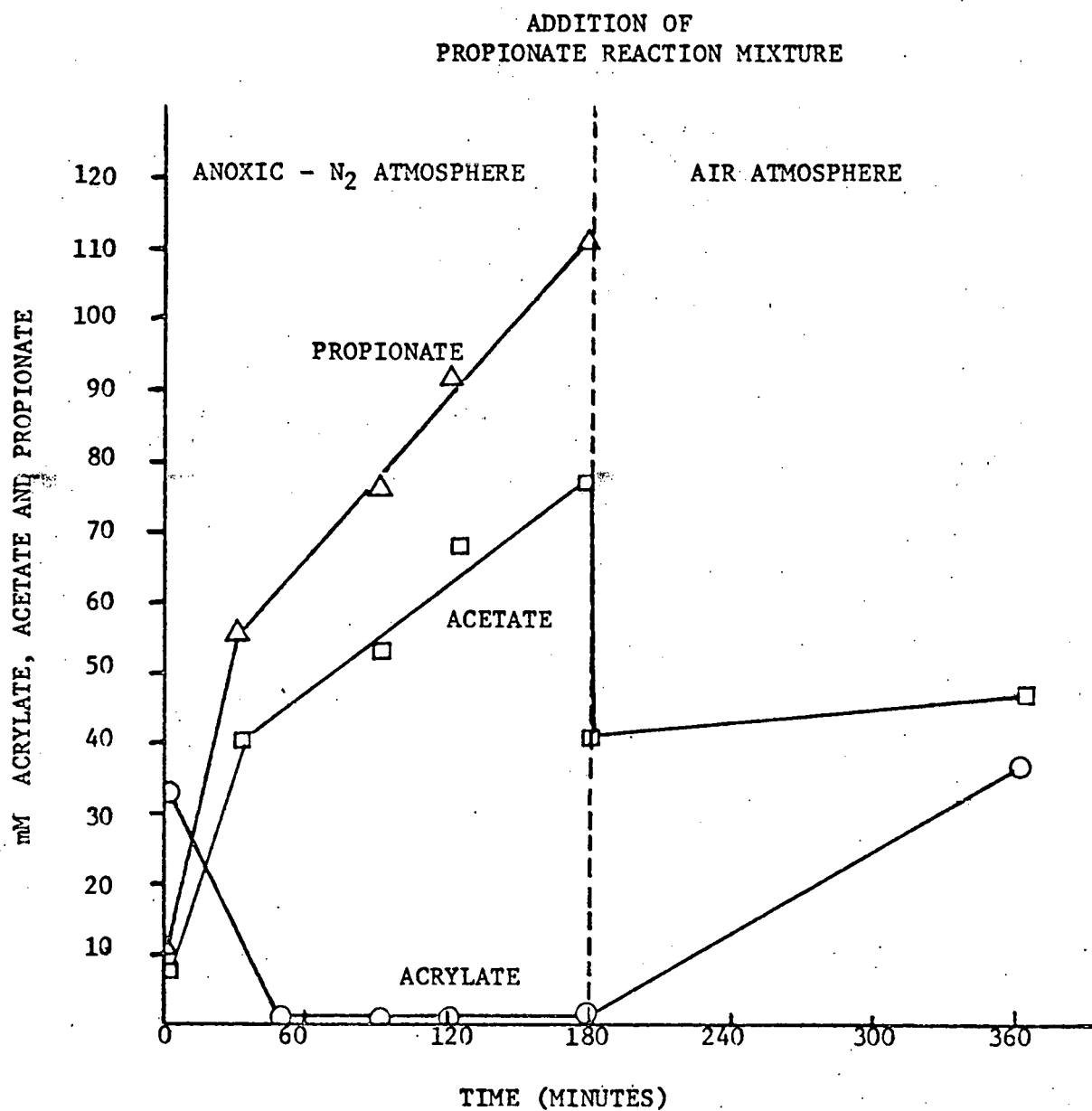


Figure II.A.11

Acrylate produced from cells preincubated anoxically at different concentrations of acrylate, with and without lactate

The results of acrylate produced by the two experiments involving anoxic anaerobes uptake (0 to 108mM) with and without lactate (300mM) followed by aerobic bioconversion is compiled in this graph.

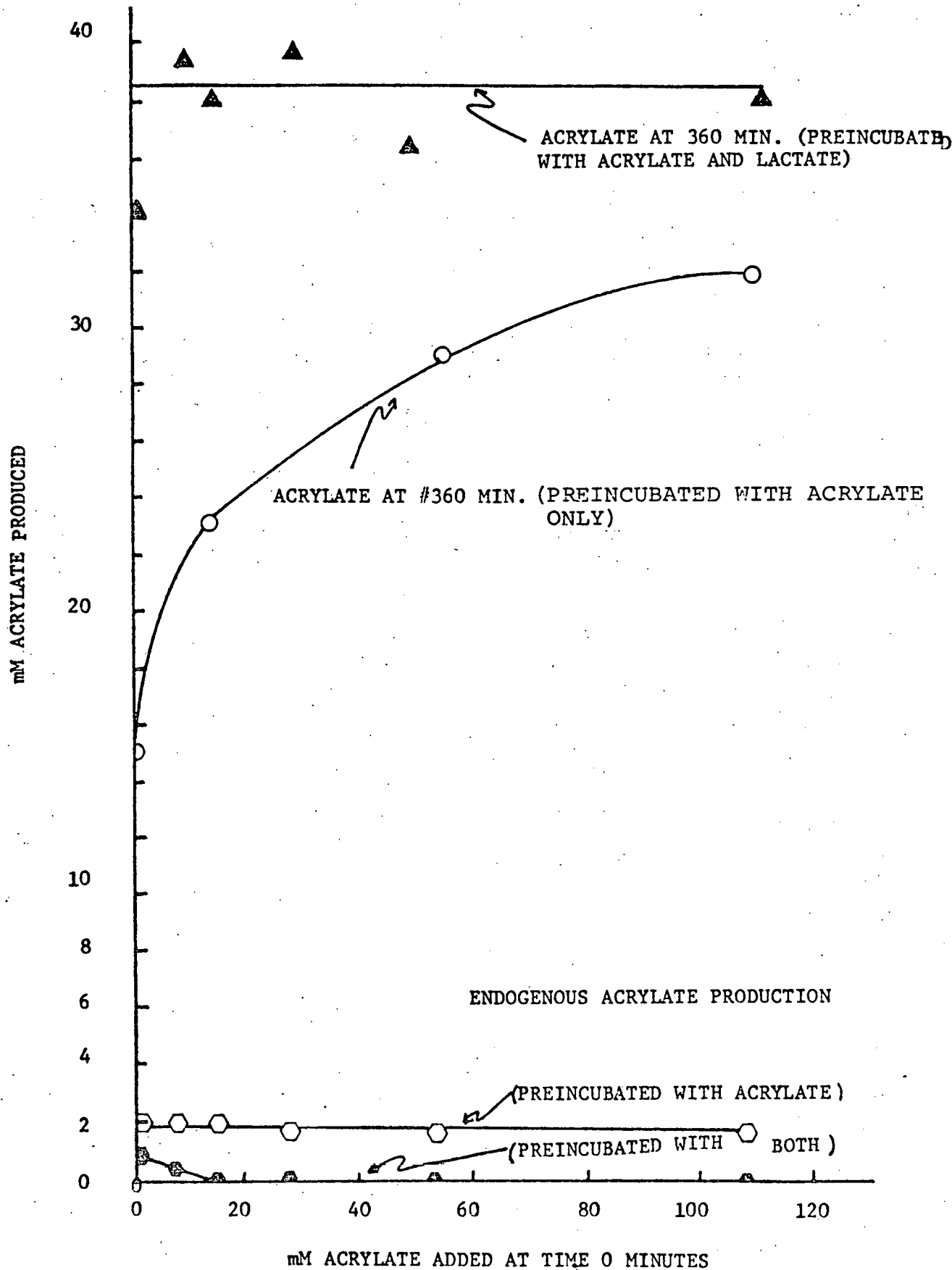
(▲-▲) Acrylate produced in air atmosphere using standard bioconversion conditions after preincubation in lactate 300mM and the indicated amount of acrylate (0-108 mM).

(○-○) Acrylate produced in an air atmosphere using standard bioconversion conditions in acrylate at the various concentrations (0-108mM).

Endogenous acrylate production are levels of acrylate produced at the end of the anoxic preincubation by what is thought to be endogenous metabolism of the cells.

Fig. II.A.11

ACRYLATE PRODUCED FROM CELLS PREINCUBATED ANOXICALLY AS
A FUNCTION OF ACRYLATE ADDED AT TIME 0 MINUTES, WITH AND
WITHOUT 300 mM LACTATE



conversion of propionate to acrylate in presence of an electron acceptor. Experiments in this area are in progress.

e. Future Studies

Many of the future studies proposed have been summarized in each of the individual sections. Briefly, recapitulating the following aspects will be addressed in future studies.

- Alternative electron acceptors will be tested.
- Role of lactate will be further studied.
- Regeneration of the biocatalyst will be examined.
- Cell-free extracts will be prepared and kinetic studies conducted with them.
- The unknown intermediate or product will be identified.

B. PRODUCTION OF ACETONE AND BUTANOL BY FERMENTATION

1. Introduction

The major problem of the fermentation process to produce acetone and butanol by C. acetobutylicum has been identified to be the inhibitory effect of n-butanol. This end product of the fermentation inhibits the growth of C. acetobutylicum at a concentration about 13 to 15 g/l. Therefore, research efforts have been directed to overcome the problem of n-butanol inhibition. The approach to this problem can be divided into two areas: (1) Selection of butanol resistant strains of C. acetobutylicum, and (2) in situ removal of n-butanol by liquid-liquid extraction. The selection of butanol resistant strains was attempted by adapting C. acetobutylicum to grow in the presence of high concentration of n-butanol. In situ removal of n-butanol was performed in batch fermentations using corn oil as the extracting agent. In this report, results from studies are presented and discussed.

2. Materials and Methods

a. Selection of n-butanol resistant strain of C. acetobutylicum

Culture of C. acetobutylicum originated from spores stored in corn mash medium was used to inoculate a

a soluble medium containing 12 g/l of n-butanol. The soluble medium contains glucose, yeast extract, and salts. The composition of this medium was reported earlier. (COO-4198-2). After 24 hours of incubation, the culture was transferred using 10% inoculum, into fresh medium containing 12 g/l of n-butanol. This transfer was performed repeatedly. After every 5 transfers, the tolerance of the culture towards n-butanol was tested. The test was conducted by using 1% inoculum of a 12 hour culture to inoculate tubes containing the soluble medium. The concentration of n-butanol in these tubes ranged from 0 to 20 g/l. Final growth of the culture in each tube was measured through absorbance after 24 hours of incubation at 37°C.

b. In situ extractive fermentation

The fermentation system and the procedures for sampling and assays were described in the previous report (COO-4198-7). The medium used was reported in the earlier reports (COO-4198-2), and the initial glucose and yeast extract concentrations are 100 g/l and 10 g/l respectively. The purpose of this fermentation is to examine the effects of the corn oil on the fermentation kinetics and product conversion yields. In this fermentation, we also want to examine the feasibility of extending the production of butanol and acetone by replacing the oil phase at selected time intervals. The

replacement of oil at the proper time during fermentation can maintain the concentration of n-butanol below the inhibitory level. The replacement of corn oil in the fermentation was accomplished by interrupting the agitation of the fermentor to effect phase separation within the fermentor. The corn oil was then withdrawn from the fermentor and fresh corn oil was added. At the same time, glucose and yeast extract were added. The timing of the oil replacement was determined by monitoring the aqueous n-butanol concentration closely through gas-chromatography of the aqueous samples. However, due to the time involvement in sample preparation, there was a lag time of about one hour before the exact concentration of n-butanol was known.

3. Results and Discussions

a. Selection of N-butanol Resistant Strain

After in excess of 20 transfers of the culture in presence of 12 g/l of n-butanol, there was no obvious increase in the resistance of the organism towards n-butanol. The transfer is still being carried on. However, it appears that new procedures for the selection have to be used. This is because the mechanism of mutation of a microorganism to be able to resist the inhibitory effects of its own product could be complicated and involves more than, for example, a single mutation. Therefore, the procedure used may not be able to select such strains due to the rare occurrence of the desired mutant.

Thus, in order to increase the chances of selecting a butanol resistant strain, the use of mutagenic agents to induce mutation will be used. Also, reports by some Russian workers (Jerusalimskij, 1958; Abdullaeva, 1959) suggested that the use of continuous culture has enable them to adapt C. acetobutylicum to grow in the presence of up to 2.5% of n-butanol. Therefore, continuous culture for the selection of n-butanol resistant strains will be studied.

b. In situ Extractive Fermentation

The results of the extractive fermentation are shown in Figure II.B.1. and II.B.2. The concentrations of the products, butanol, butyric and acetone, and glucose in the aqueous phase are shown in Figure II.B.1. The vertical arrows on the top of the figure denote the replacements of corn oil. The patterns of the fermentation before the first oil replacement followed closely to those of a batch fermentation without extraction. The accumulation of butyric acid accompanied the cell growth. The productions of acetone and butanol started after the concentration of butyric acid accumulated over 5 g/l. The cell density at the first oil replacement was about 3.5 g/l.

After the first oil replacement, the cell concentration increased steadily to about 5 g/l at the 30th hour. However, there was no significant amount of butyric acid accumulated during this period. The butanol concentrations in the aqueous

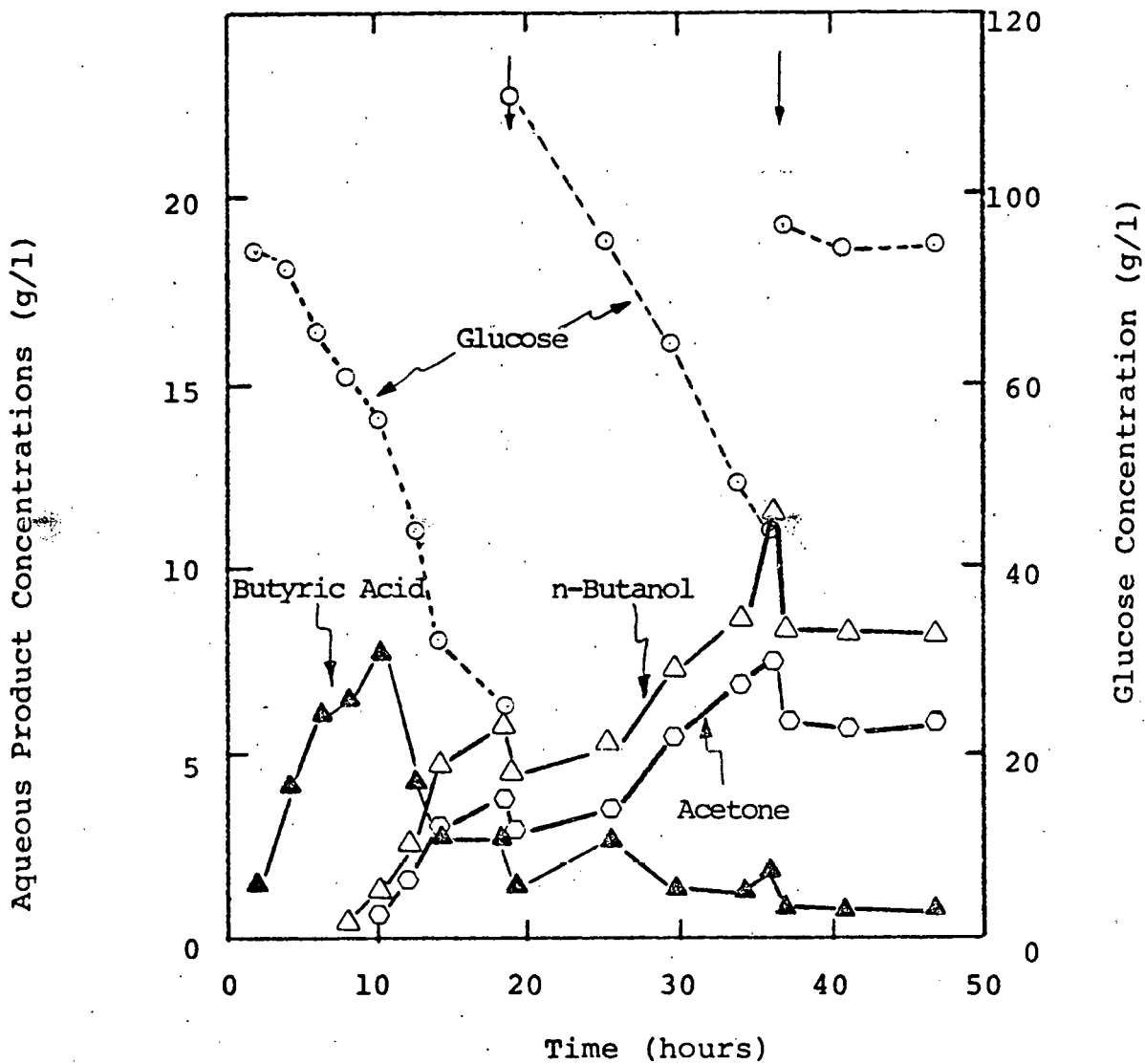


FIGURE II.B.1: In situ Extractive Fermentation of *C. acetobutylicum* using corn oil as the extractive agent. Concentrations of products and substrate in aqueous solutions are shown.

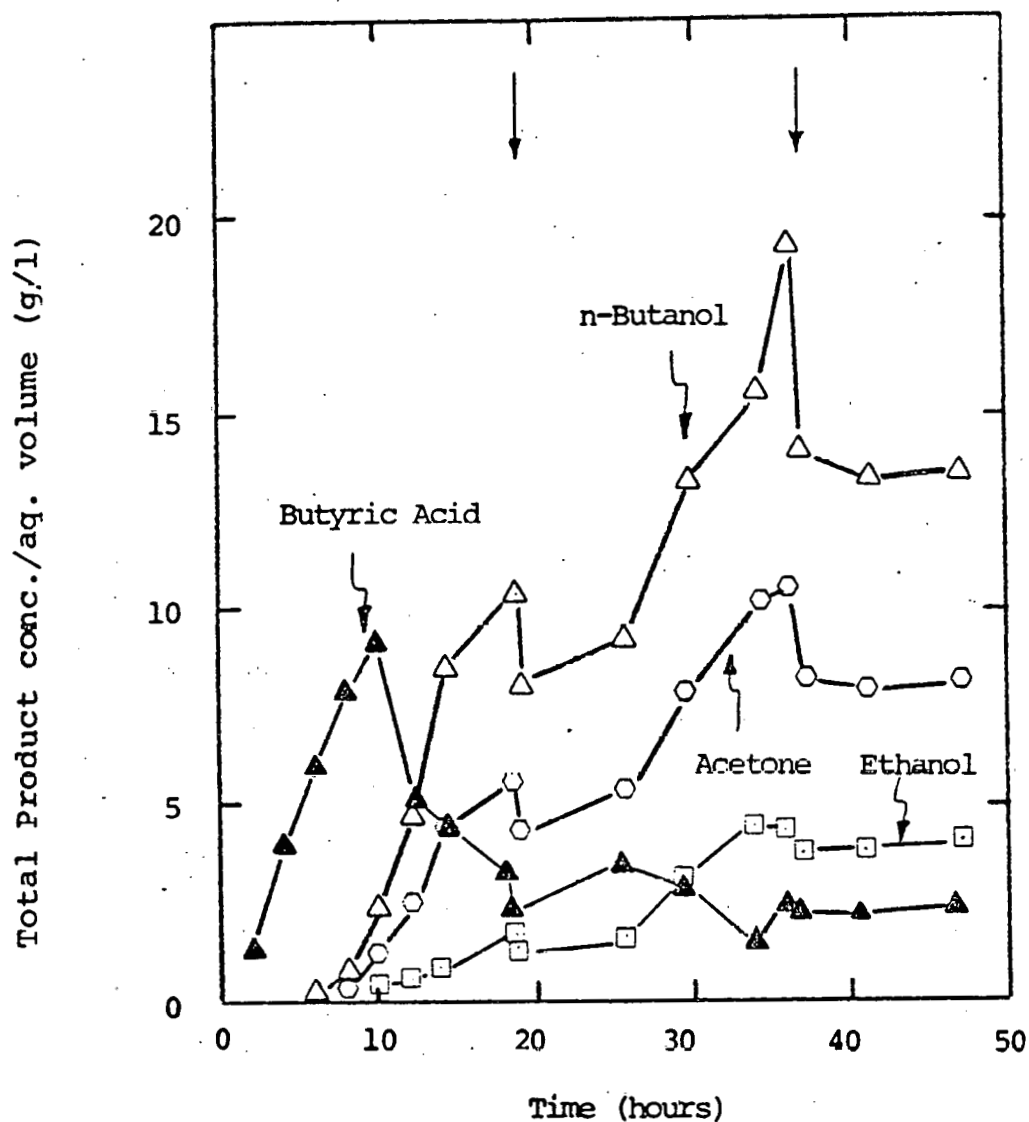


FIGURE II.B.2: Results of extractive fermentation of *C. acetobutylicum* using corn oil as extracting agent. Total product concentrations per aqueous volume are shown.

phase reached 12 g/l at the 36th hour. The concentration of acetone was about 7 g/l at the same time.

The total amounts of products in the fermentor were calculated by summing the amounts of the products in the aqueous and the oil phases. These values when divided by the aqueous volume represent the concentrations of the equivalent product concentration in the fermentor if the oil phase was absent. These results are shown in Figure II.B.2. The highest concentration of butanol based on the aqueous volume was 19 g/l. This means that by using an extraction phase, the average concentration of butanol per unit aqueous volume can be extended beyond the inhibitory level.

The results of this extractive fermentation are summarized in Table II.B.1. In this table, the results from a batch fermentation without oil extractions are shown. The productivities of the products in the extractive fermentation were calculated by using the values at the 36th hour, and are expressed as g/l (aqueous) - hour. The productivities and the conversion yields are similar in both cases which indicate that there is minimal effect of the corn oil on the biological activities of C. acetobutylicum.

However, there was a rapid loss of biological activities of the culture after the second oil replacement. The exact reasons for this loss are not known and will be subjected to further studies.

TABLE II.B.1: Summary of Results of Batch Fermentations of
C. acetobutylicum With and Without Oil Extraction

	Total Conc. of Prod. Per Aqueous Volume (g/l)		Product Yields From Glucose (g/g)		Total Solvent Vol. Productivity (g/l-hr)
	Acetone	Butanol	Acetone	Butanol	
Batch Fermentation	6.9	13.2	0.09	0.18	0.84
Extractive Batch Fermentation	10.6	19.6	0.10	0.18	0.84

From this study, it is concluded that the presence of corn oil does not effect the productivities and conversion yields of the fermentation, and the average toxic product concentration could be increase in a fermentor by use of an extracting agent.

4. Future Work

- Selection of n-butanol resistant strain by - mutation and continuous culture.
- Further examination of extractive fermentation to include the cause, of the ceasation of the reported fermentation and the design of continuous in situ extraction fermentation.
- Studies of high cell density continuous fermentation for the acetone-butanol process.

References cited:

Jerusalimskij, N.D. 1958. A study of the process of development of microorganisms by the continuous flow and exchange of media method. Continuous cultivation of microorganisms, a symposium. Prague.

Abdullaeva, G.V. 1959. Thesis at Institute of Microbiology AN SSSR. As cited in Microbiology (English Translation of Microbiologist). 1959, 28, 596.

C. Production of Acetic Acid by Fermentation

1. Introduction

In the previous progress reports, it has been shown that two of the factors for the decrease of acetic acid production by Clostridium thermoaceticum may be attributed to cell instability and cell lysis. One of the common procedures used to increase cell stability is the immobilization of whole cells. Other advantages of cell immobilization include the facilitated recovery and reuse of cells, reduction of raw material cost, and the possible development of a continuous process through novel reactor design.

Two methods of cell immobilization have been examined: (1) entrapment of cells in polyacrylamide gel and (2) adsorption of cells onto anionic resin, Ambergard XE 352. In this section, preliminary results from these experiments are reported.

2. Materials and Methods

The procedure for polyacrylamide gel entrapment was described in Progress Report COO-4198-7 and is summarized as follows. Cells were harvested at exponential phase of growth and were washed twice with 0.1 M phosphate buffer. To 10 ml of cell suspension (2.5 gm wet cell weight), acrylamide monomer (1.9 gm), N-N'-methylene-bis-acrylamide (100 mg), 5% tetramethyl-ethylene-diamine (1.25 ml), and 2.5% potassium per sulfate (1.25 ml) were

added with stirring under anaerobic atmosphere at 4°C. The mixture polymerized in about 5 minutes. The preparation was then washed three times with phosphate buffer, and the amount of cells washed away was determined by measuring the protein content in the washings.

The physical and chemical properties of Ambergard XE 352 resin (obtained from Rohm and Haas) is shown in Table II.C.1. It was chosen because of its high porosity, its strongly basic functionality, and its operation in packed column at relatively high flow rate without experiencing noticeable pressure drop (at 55°C, pressure drop is at 1.5 psi/ft with flow rate of 9 gal/min/ft²). Loading of cells onto the resin was achieved by adding 1 gm of resin into 20 ml of freshly harvested cell suspension and mixed for 30 min. The amount of cells adsorbed was determined by the difference of cell concentrations before and after adsorption.

The medium composition and other analytical methods have already been described in the earlier progress reports.

3. Results and Discussions

Preliminary experiment of immobilization by polyacrylamide gel was performed in test tubes. Acetate production of free and immobilized cells using glucose buffer and yeast extract medium was determined and shown in Table II.C.2. The activity was expressed in terms of specific acetate formation (gm acetate/gm cell-hr) for the first 15 hrs.

TABLE II.C.1

PHYSICAL AND CHEMICAL CHARACTERISTICS OF AMBERGARD XE-352

Physical Form	Uniform, Spherical Particles
Moisture Content	70 - 80%
Density	1.04 gm/cc
Porosity (cc pore/gm dry resin)	1.0 - 1.6
Pore Diameter Mean Range	7 microns 2.3 - 23 microns
Surface Area	3 - 6 M ² /gm
pH Limitations	5 - 9
Maximum Temperature	77°C
Screen Grading	20 - 50 Mesh

TABLE II.C.2

COMPARISON OF ACETATE PRODUCTION BY FREE AND
POLYACRYLAMIDE-GEL-ENTRAPPED CELLS

	Glucose Buffer	Yeast Extract Medium
Free Cell	0.20 gm/gm cell-hr	-
Immobilized Cell	0	0.03 gm/gm cell-hr

No activity was found for immobilized cells using glucose buffer. Using yeast extract medium, only 15% of original activity was found for the first 15 hrs. After 24 hrs, cell growth started in the original clear medium, probably as a result of leakage of cells from the gel. The possible reasons for the low activity of immobilized cell are (1) mass transfer limitation into the gel or (2) toxicity of reagents used for polymerization. Further experiments using crushed gel and different concentration of reagents have to be performed.

The adsorption of cells to the ammonium anion exchange resin, Ambergard XE-352, is dependent on the pH, the ionic strength and the cell concentration of the cell suspension. Under the present conditions, using glucose-bicarbonate buffer at neutral pH, the cell loading was estimated to be 0.004 - 0.01 gm of cells per gram of resin (Table II.C.3).

Using the assumptions that the average cell size is 0.2 micron by 4 microns and that the average cell weight is 10^{-2} gm, the theoretical maximum cell number that can be adsorbed per gram of resin is about $2-5 \times 10^{11}$ cells (or equivalent to 0.2 - 0.5 gm). It is therefore conceivable that by manipulating the environmental conditions, the cell loading per unit of resin can be increased.

Because of the low concentration of cells adsorbed onto the resin, the acetate production in these tube experiments by resting cells alone could not be determined

TABLE II.C.3

CELL LOADING TO AMBERGARD XE-352

Test Number	Initial Cell Concentration (Klett Units)	Final Cell Concentration (Klett Units)	Cell Loaded gm cell/gm resin
1	325	204	0.01
2	250	180	0.006
3	230	178	0.004

accurately. In these experiments, yeast extract was in the medium. The adsorbed cells can desorb and be released into the medium. In this case, both the adsorbed cells and the free cells can produce acetic acid. The only evidence showing that adsorbed cells can produce acetic acid is by comparing the amount of products of cells adsorbed to the resin and that of the control (shown in Table II.C.4). The acetic acid produced was only 30% lower for cells adsorbed to resin, but the cell concentration in the medium was less than four times. We conclude that some of the acetic acid must have been produced by the adsorbed cells. Further experiments will be performed in column operated continuous to study the kinetics of the adsorbed cells with respect to acetic acid production.

4. Future Work

- Continuous to study the optimal conditions for cell immobilization.
- Design for high productivity reactor.

TABLE II.C.4

COMPARISON OF ACETIC ACID PRODUCTION BY FREE CELLS
AND CELLS-ADSORBED ONTO AMBERGARD XE-352

	Free Cells	Cells-Adsorbed to Ambergard XE-352
Acetic Acid (g/l)	12.9 g/l	10.4
Cell (K.U.)	870	226
pH (Final)	5.8	6.0

D. Microbial Degradation of Cellulose for The Production of Lactic Acid

1. Introduction

The objective of this section of the project is to develop the technical methodology to grow a mixed culture of C. thermocellum and B. coagulans to achieve the bioconversion of cellulose into lactic acid. The lactic acid could then provide the ultimate substrate for acrylic acid production. This report describes the experimental results of a mixed culture fermentation on Solka floc and analyzes the conversion efficiency into lactic acid. The results obtained probe the feasibility of the process and provide a baseline for the system. Having demonstrated the feasibility, this line of work will be suspended in the future and a greater emphasis will be placed on the other aspects of acrylate production.

2. Materials and Methods

a. Microorganisms

- (1) Clostridium thermocellum ATCC-27405.
- (2) Bacillus coagulans (refer to Report No. COO-4198-8 for further information).

b. Culture Media

The medium used in this experiment was the "mixed medium". Composition described in Progress Report No. COO-4198-7.

c. Inoculum Preparation

The standard procedure for inoculum preparation was followed (see Report No. COO-4198-4, pages 108-109). Handling and growth of C. thermocellum was done following the methodology and growth conditions described elsewhere (see Report No. COO-4198-1, pages 2-3).

d. Fermentor

A 5.0 liter Magnaferm bench scale (New Brunswick Scientific) fermentor was used with 2.0 liter working volume. pH and temperature were controlled at 6.8 and 60°C respectively unless otherwise indicated.

e. Assays

During the fermentation, the following kinetic parameters were followed. Cell concentration by Klett measurement on supernatant. Lactic acid by lactic dehydrogenase method. Ethanol and acetic acid by gas chromatography. Reducing sugars by the DNS method. The standard methodology has been described in previous reports.

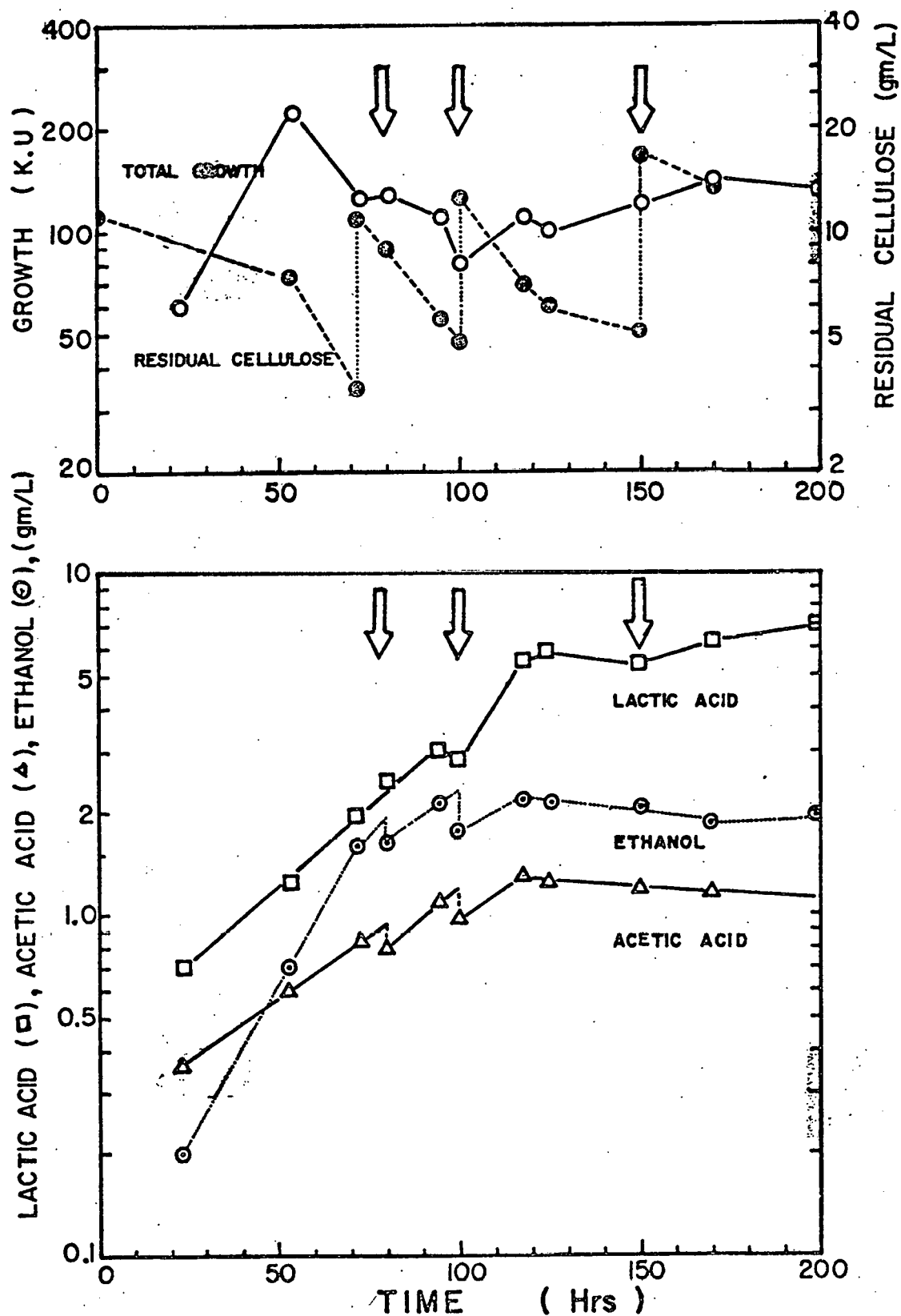
3. Results and Discussion

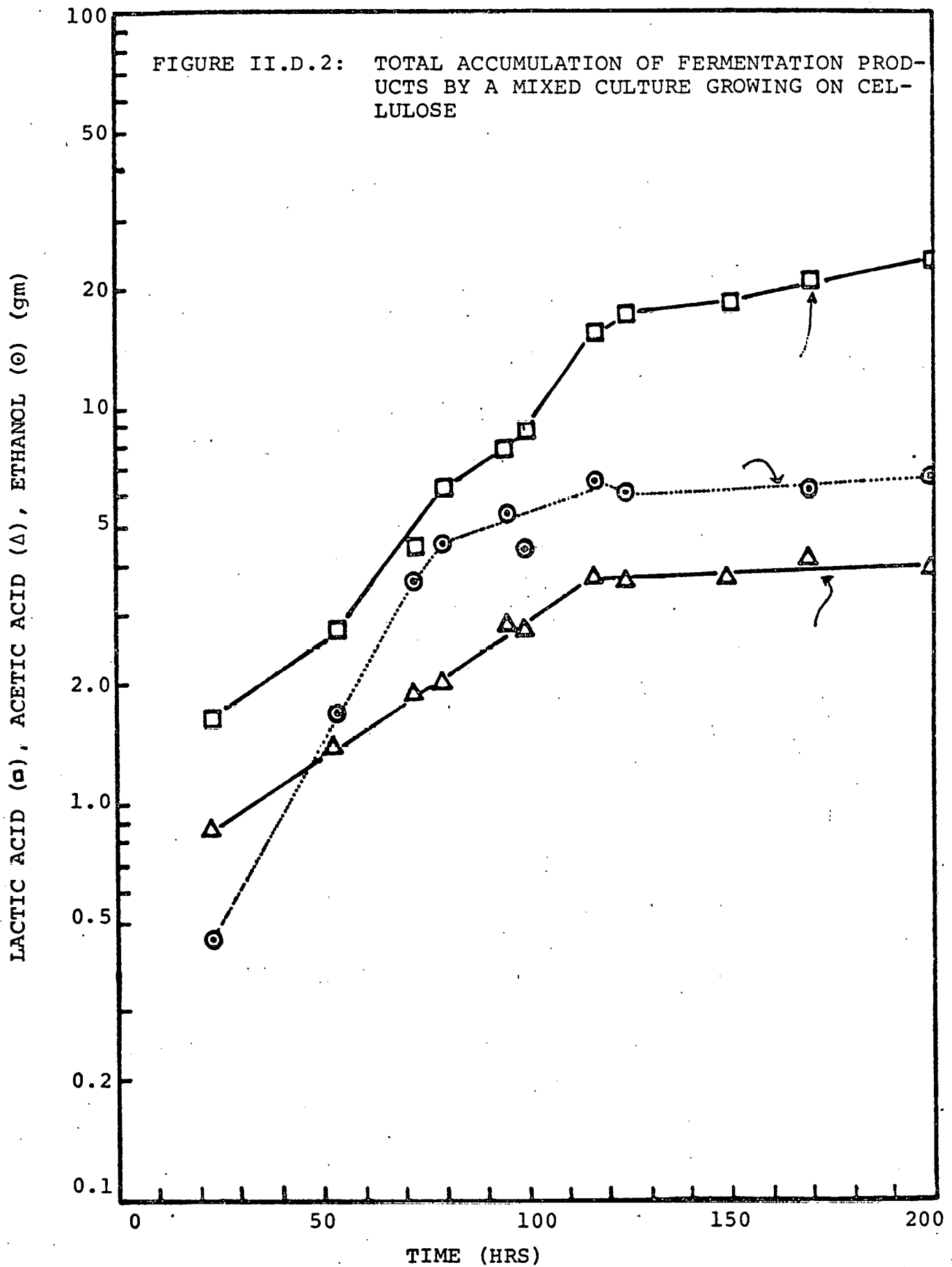
a. Mixed Culture Fermentation of Cellulose

This experiment was designed to evaluate the performance of a mixed culture population of C. thermocellum

and B. coagulans using Solka floc as the carbon source. Lactic acid is expected to accumulate as final product. The results of such experiment are shown in Figure II.D.1. The fermentor was inoculated with C. thermocellum initially and, after 12 hrs, B. coagulans was added to the system. Initial solka floc concentration was 10 gm/l and subsequent additions (10 gm/l each time) were made as indicated by the arrows. Total cell growth was followed by measuring the optical density of sample supernatant. The time profile of lactic acid, ethanol and acetic acid were followed along the fermentation. The results show the degradation of cellulose to fermentation products. Lactic acid is associated with the growth of B. coagulans while acetic acid and ethanol are indicators of C. thermocellum growth. After 200 hrs of fermentation, 69% of the total cellulose added was degraded. The lactic acid concentration was 7.0 gm/l ethanol and acetic acid were 2.0 and 1.0 gm/l respectively. These numbers account for 58% of total cellulose added (111 gm) were degraded and 35% of this fraction was converted into lactic acid. Figure II.D.2 shows the total product accumulation after 200 hrs. It shows 23 gm of lactic acid were produced when 111 gm cellulose were fed to the fermentor. 46 gm cellulose remained after 200 hrs. The total amount of ethanol and acetic acid were 3.5 and 6 respectively. These results demonstrate the feasibility of the bio-conversion of cellulose into lactic acid with at least a yield of 0.35 gm lactic 0.35 gm of lactic acid per gm cellulose consumed.

FIGURE II.D.1: FERMENTATION KINETICS OF A MIXED CULTURE OF C. THERMOCELLUM AND B. COAGULANS ON SOLKA FLOC





b. Fermentation Carbon Balances

The fermentation data were further analyzed by doing the carbon balance of the fermentation. The objective is to obtain more information on the extent of the fermentation and detect other fermentation products not measured. These results are shown in Table II.D.1. The balance was done at 100 hrs of fermentation; 31 gm cellulose were consumed and the products measured are given the table. The carbon content in products was calculated and accounts only for 69% of carbon consumed. A carbon composition for cells was assumed to be 47%. The discrepancy of 31% in the carbon balance can be due to (a) inaccuracy of carbon content in cells, (b) presence of oligosaccharides measured as reducing sugars, (c) presence of other fermentation products not being measured such as formic acid, other fatty acids (short chain).

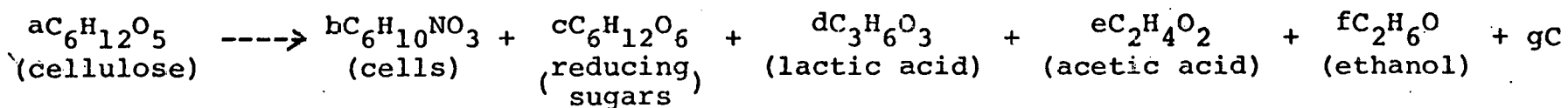
4. Future Work

- Future experiments will be directed to improve the rate of cellulose degradation which is a major limiting step. One alternative is the utilization of a packed bed fermentor.

- Develop the analytical procedures to measure individual cell growth in mixed cultures. Differential plating, or specific enzyme content (cellulose) are initially suggested.

TABLE II.D.1: FERMENTATION BALANCE (CARBON)

General stoichiometry:



			Carbon			Carbon
	(gm)	mMoles	(gm)	(gm)	mMoles	(gm)
Cellulose (consumed)	31.72	195.8	14.04	-	-	-
Cells				1.56	10.8	0.78
Acetic Acid				2.82	47.0	1.13
Lactic Acid				8.12	90.2	3.25
Ethanol				5.10	110.8	2.66
Reducing Sugars				2.07	11.5	0.825
CO ₂				3.88	88.2	1.05

TOTAL CARBON

14.04

9.7

Time Elapsed: 97 hrs.

$$\% \text{ Cellulose Consumed} = 69\%; \quad \% \text{ Recovery} = \frac{9.7}{14.04} \times 100 = 69.0\%$$