COO-4198-7
Distribution Category UC-61

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

Progress Report

for Period June 1, 1978 to August 31, 1978

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August 1978

Prepared for

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

DISCLAIMER

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 $(NH_4)_2SO_4$. 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 for 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 acetic acid 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.

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.

Dr. Charles L. Cooney
Associate Professor of Biochemical Engineering
(30% of time academic year and 50% of time in summer)

Dr. Arnold L. Demain
Professor of Industrial Microbiology
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Dr. Reinaldo F. Gomez
Assistant Professor of Food Microbiology
(25% of time academic year and 50% of time in summer)

Dr. Anthony J. Sinskey
Professor of Applied Microbiology
(25% of time academic year and 40% of time in summer)

Dr. Daniel I. C. Wang
Professor of Biochemical Engineering
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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 cellodextrins) 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 CO_2). 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)

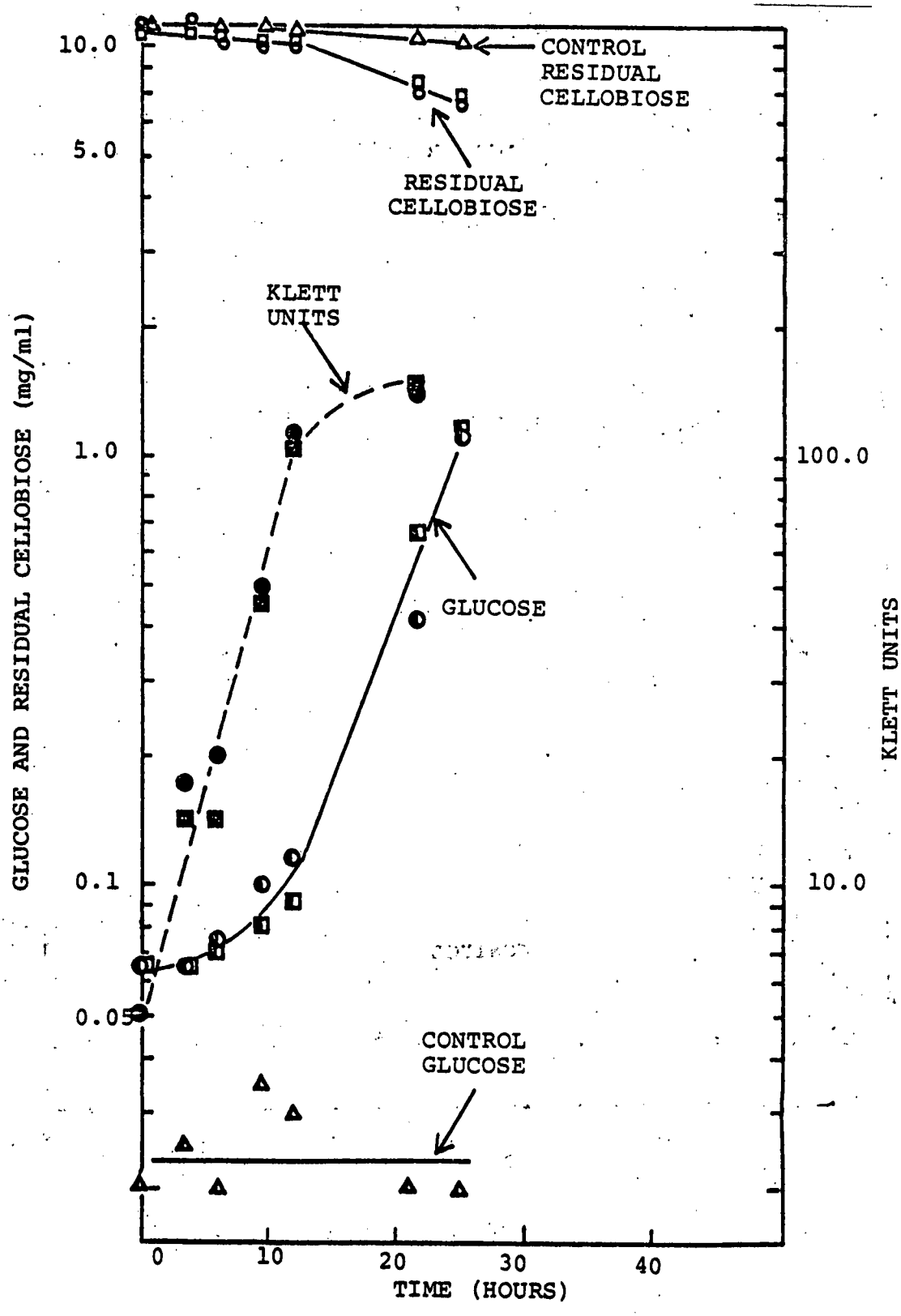
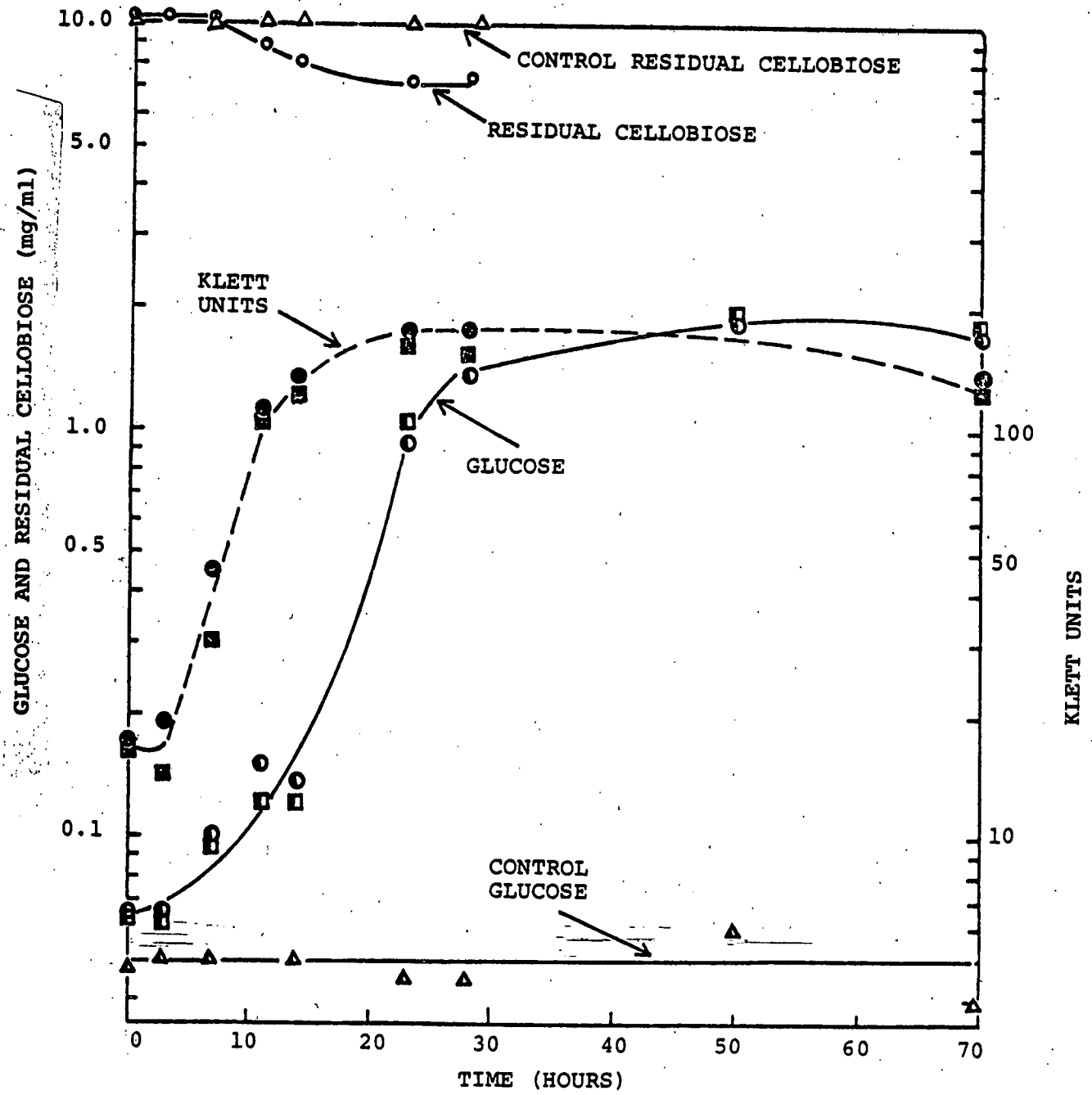


FIGURE I.A.2: KINETICS OF C. THERMOCELLUM GROWN ON CM3/ CELLOBIOSE MEDIUM (FERMENTATIONS D AND E)



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)

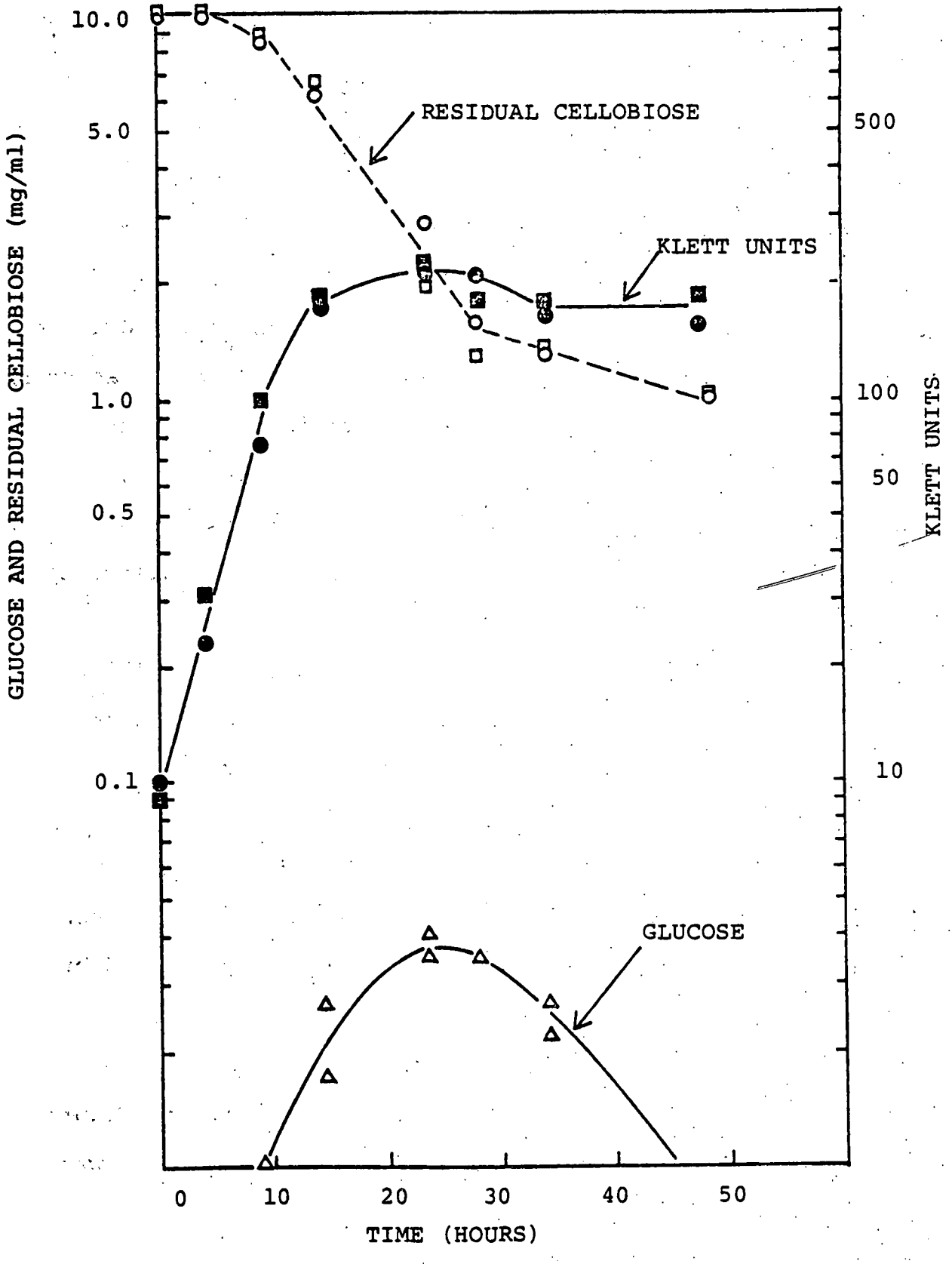


FIGURE I.A.4: KINETICS OF C. THERMOCELLUM GROWN ON CM3/ SOLKA FLOC MEDIUM (CELLULOSE FERMENTATION)

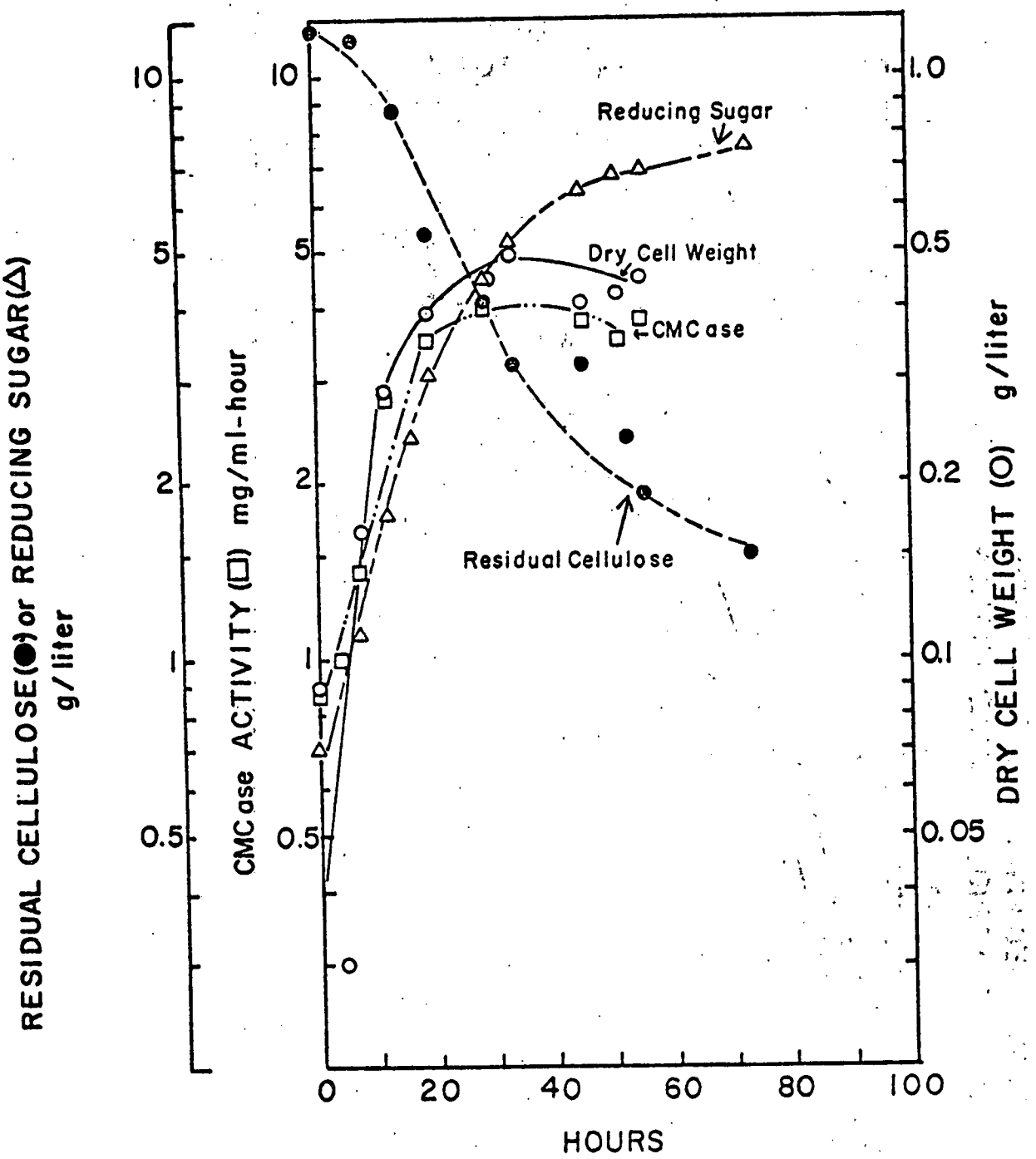
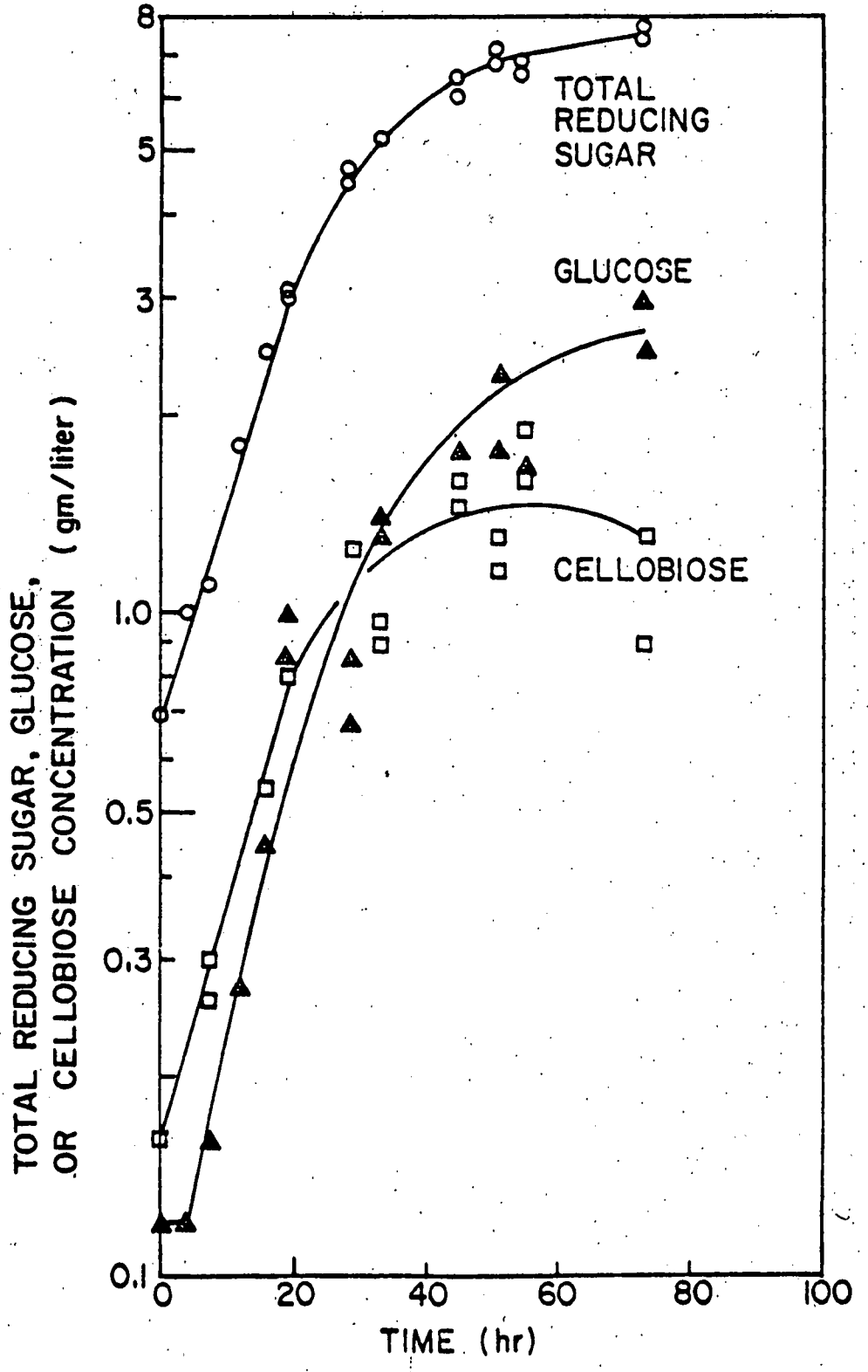


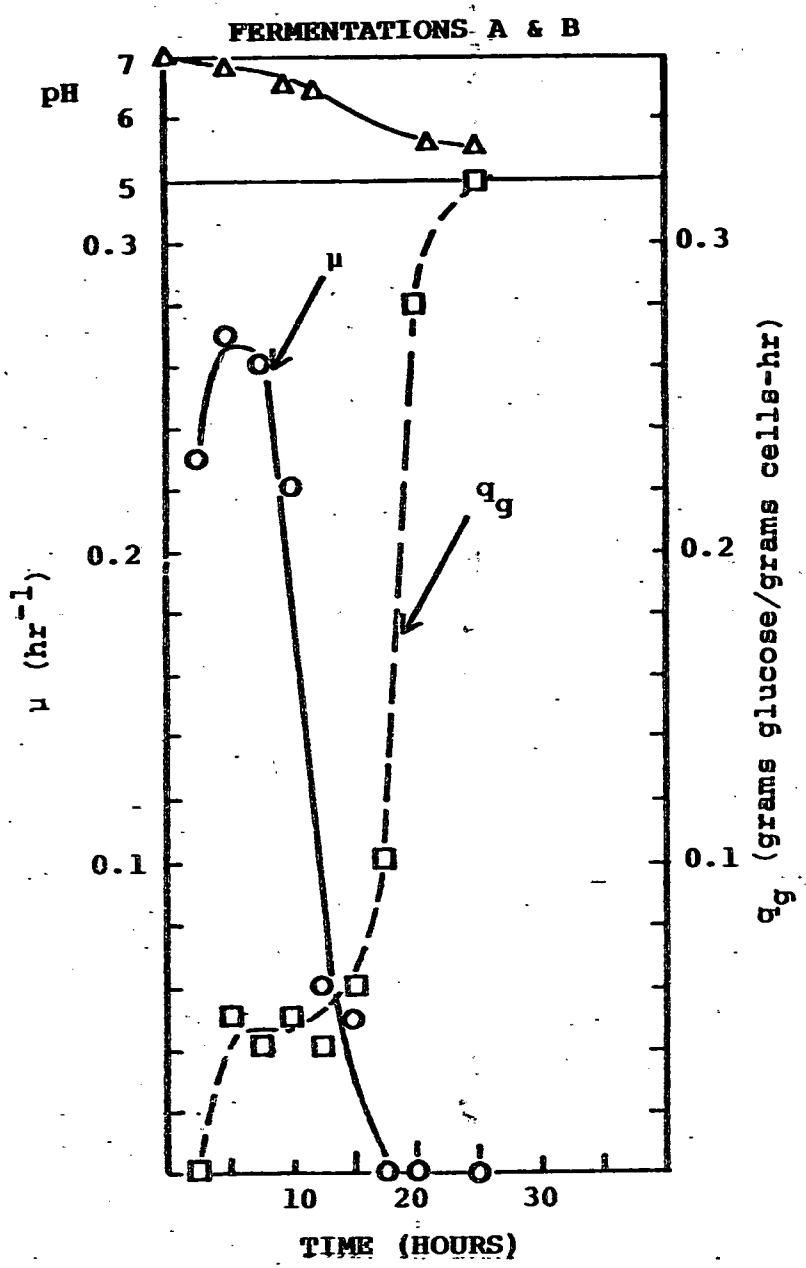
FIGURE I.A.5: SUGAR ACCUMULATION DURING CELLULOSE FERMENTATION BY C. THERMOCELLUM

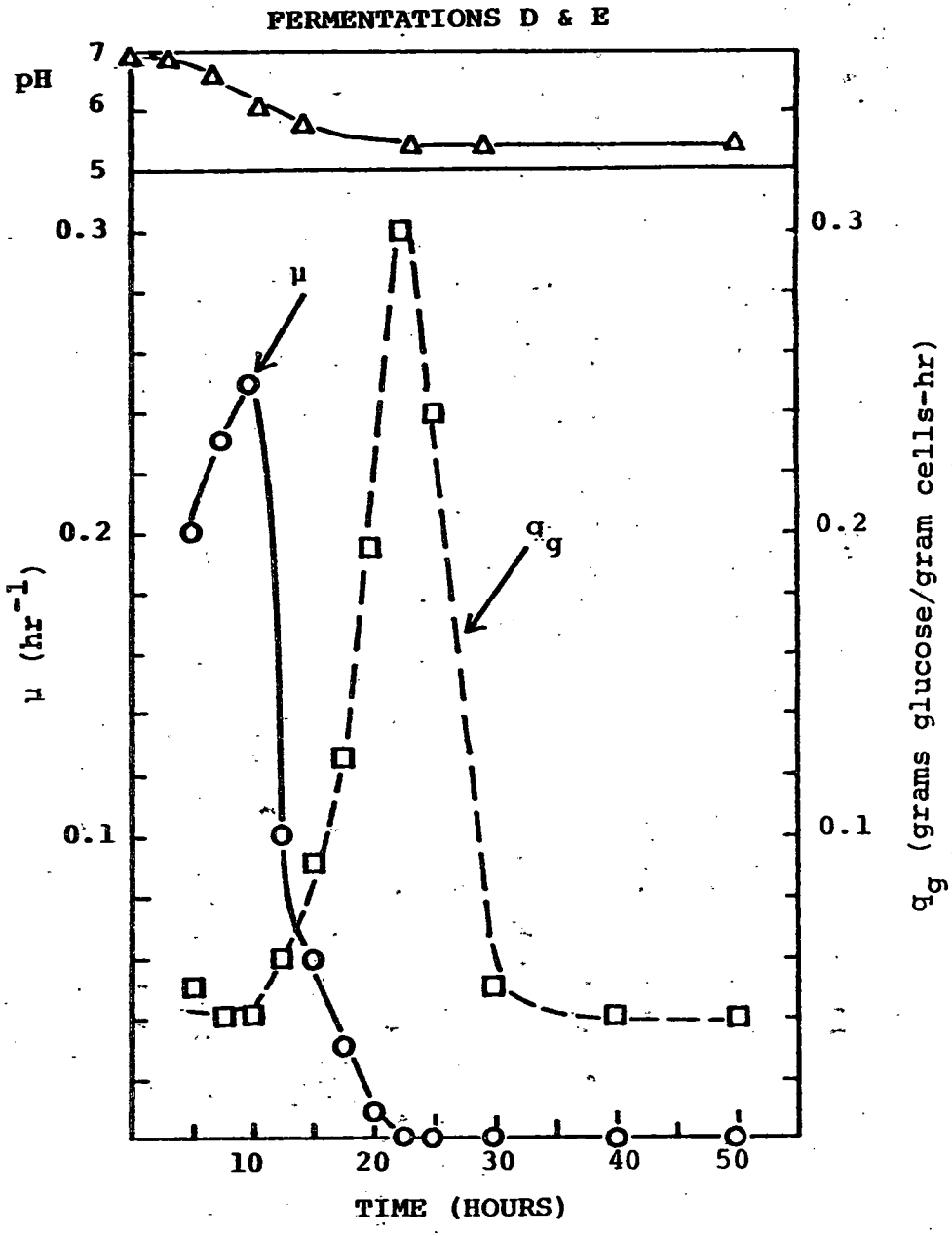


determined by the DNS assay while the curves for glucose and cellobiose accumulation were generated from HPLC data. What is most noteworthy about Figure I.A.4 are the changes that have occurred in the kinetic behavior of C. thermocellum since we began working with it a year ago (see Figure I.A.9 in Progress 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



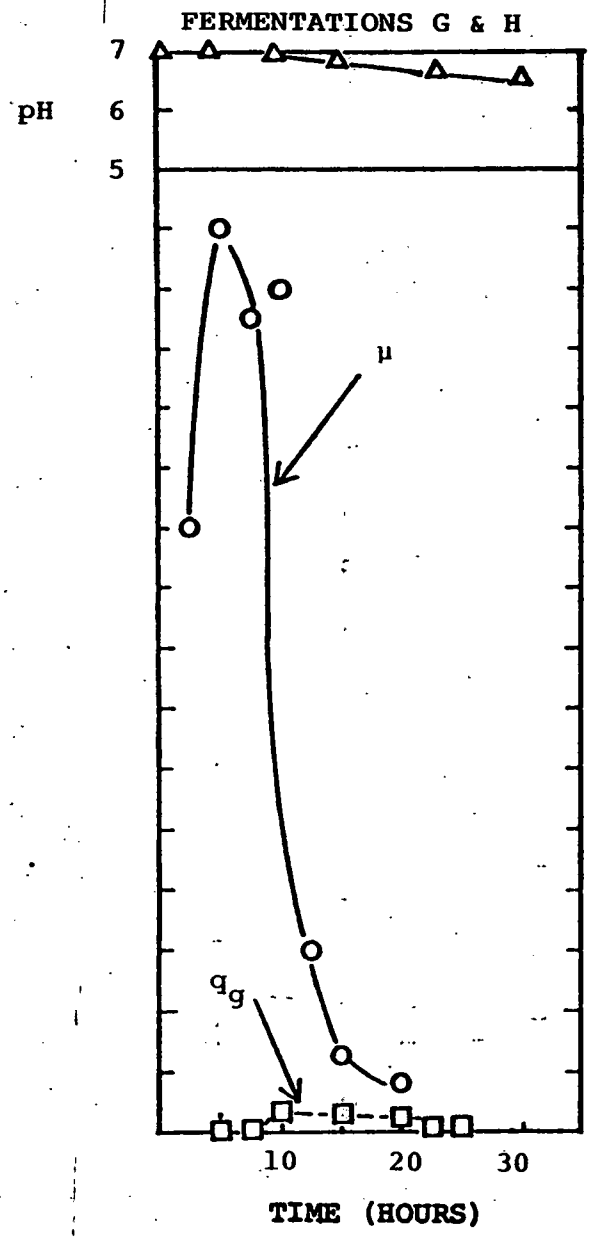


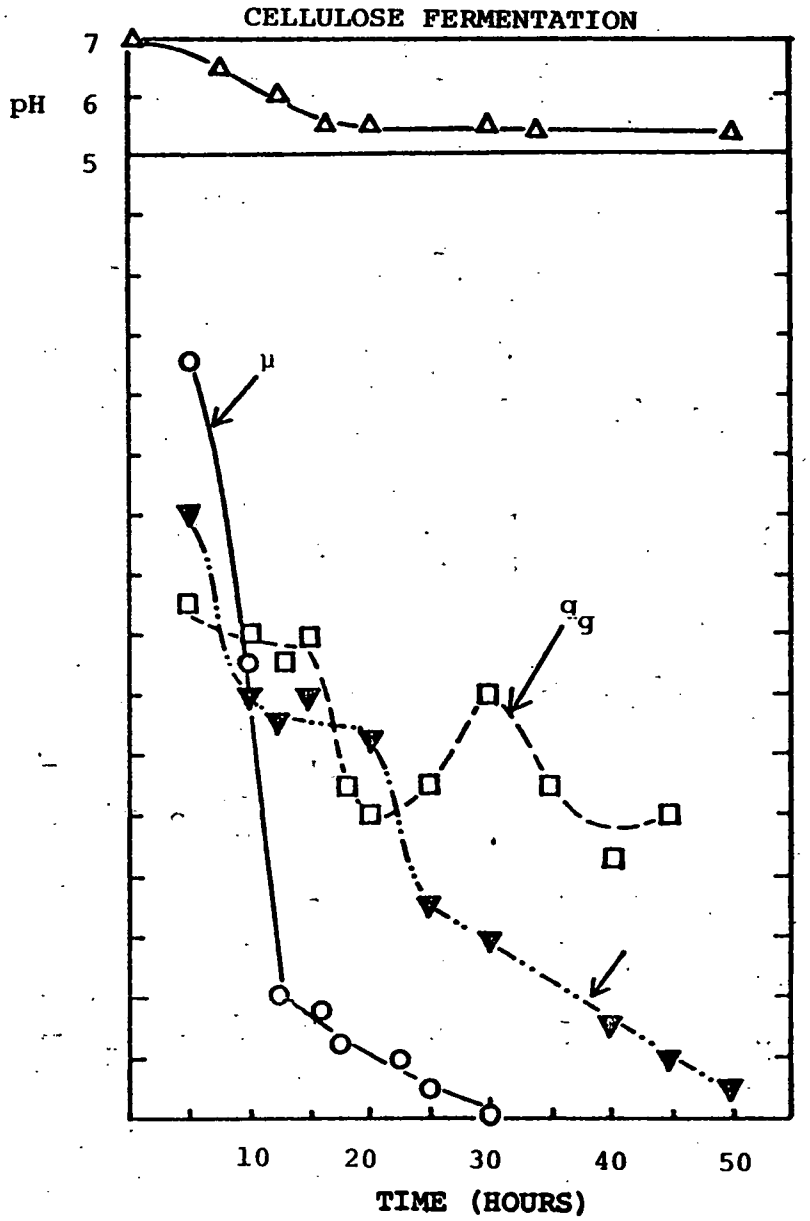
during the fermentation. Whereas the maximum specific growth rate occurs between the 5th and 10th hours, the specific glucose accumulation rate reaches a maximum value between the 20th and 25th hours, a time when the pH has dropped to its minimum value of 5.4. The accumulation of glucose in these cellobiose-grown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

$$\text{cells and proteins (C, H, N, O, S, P) + catalyst} \xrightarrow{\text{Heat}} CO_2 + H_2O + SO_2 + P_2O_7 + CO + NH_4 + HSO_4$$

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.

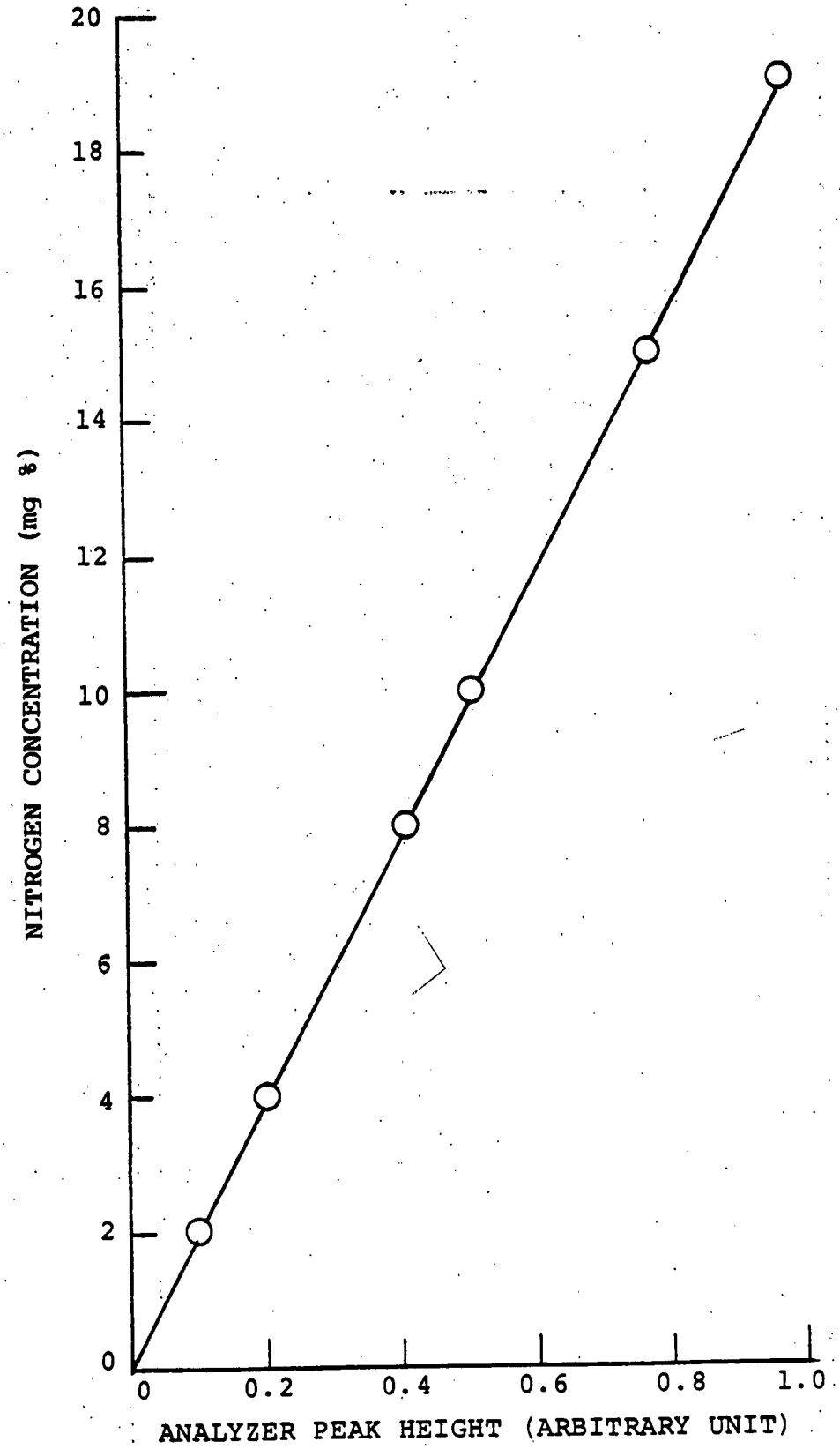
The calibriation curve for the color reaction is presented in Figure I.B.1.1. The reaction is automatically processed on an enzyme analyzer (Technicon). The recorder readout is correlated to nitrogen content in mg per cent. The calibration is linear in the range of 0.5 mg% to 20 mg% nitrogen using $(NH_4)_2SO_4$ 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

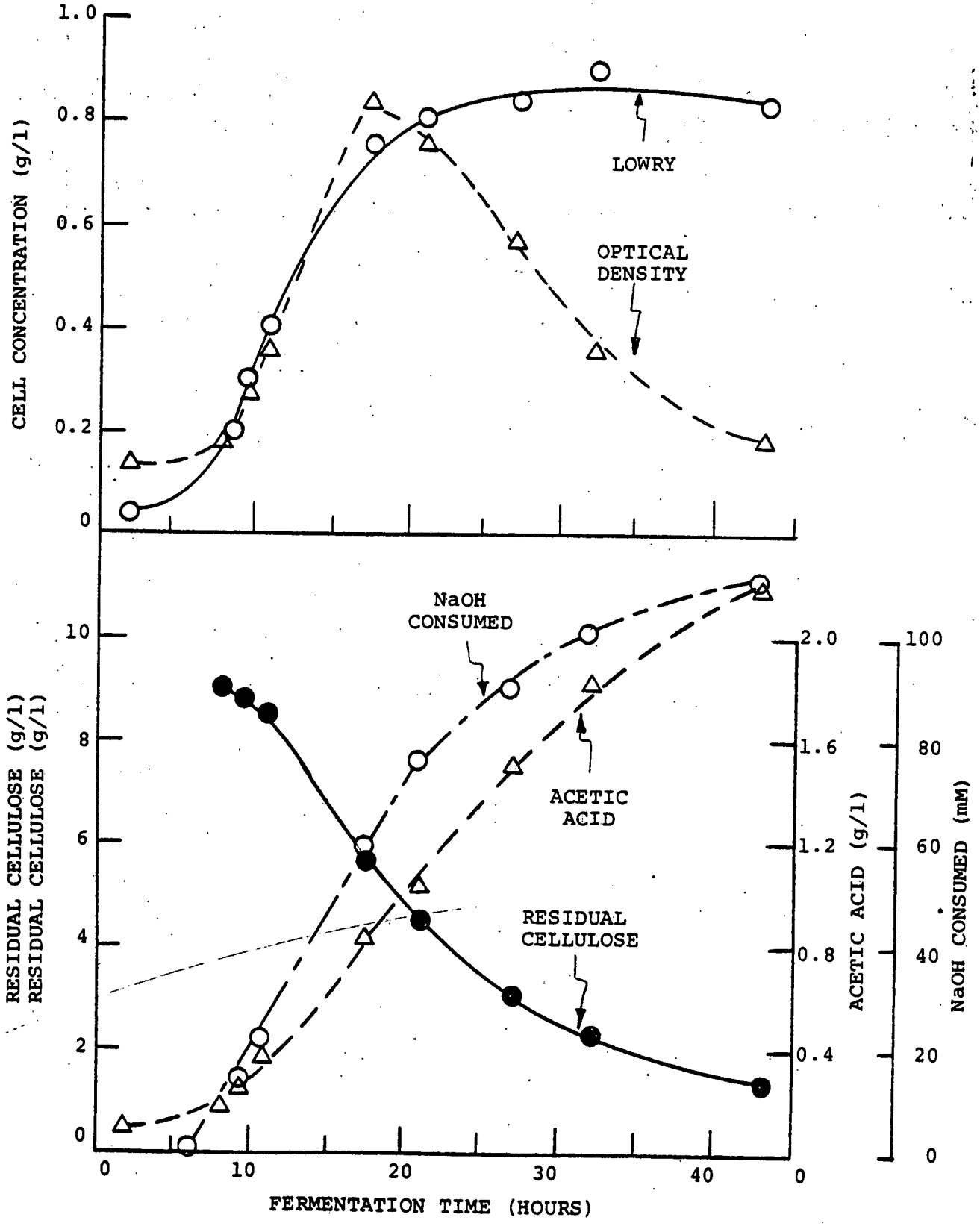
tested in a stirred tank fermentation where removal of homogenous samples is possible.

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 contrifuging 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

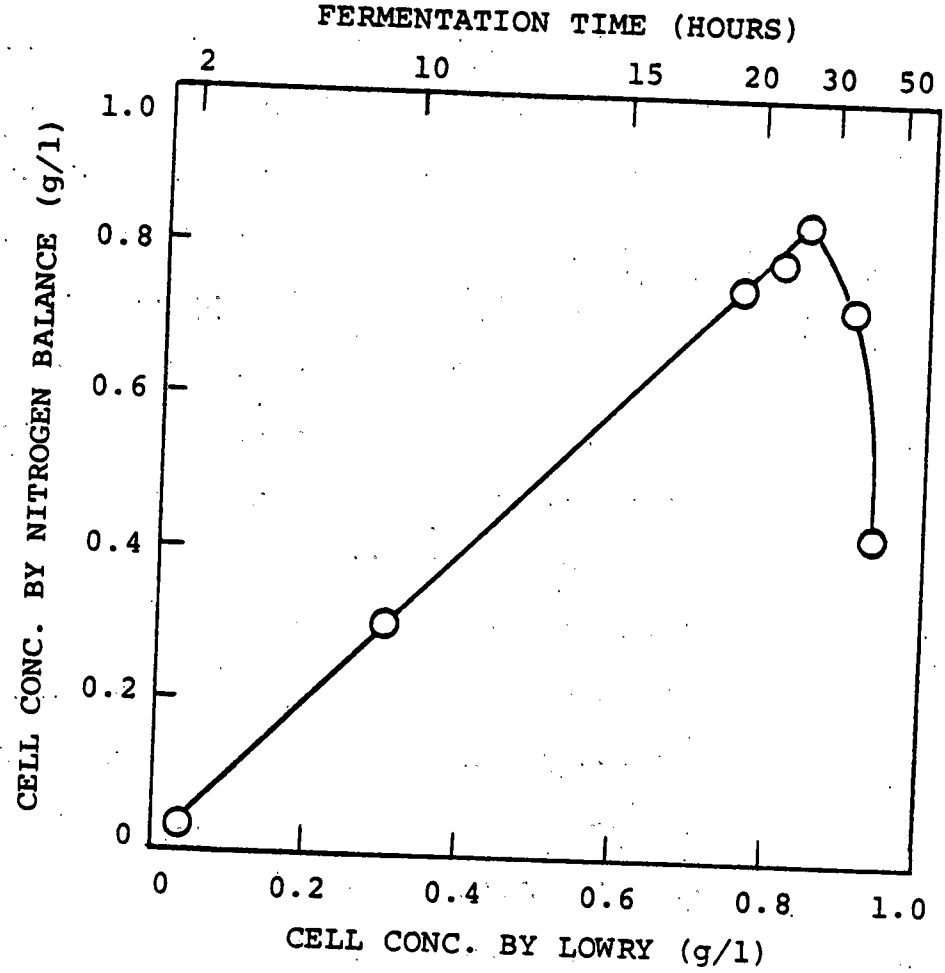
FIGURE I.B.1.2: FERMENTATION PROFILE OF C. THERMOCELLUM GROWN ON SOLKA FLOC IN AGITATED FERMENTOR



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%.

FIBURE 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

Fermentation Time (hours)	Dry Cell Weight (g/l) Calculated from Supern.	Dry Cell Weight (g/l) Measured actually on perc.	% Deviation
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. Thses 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.

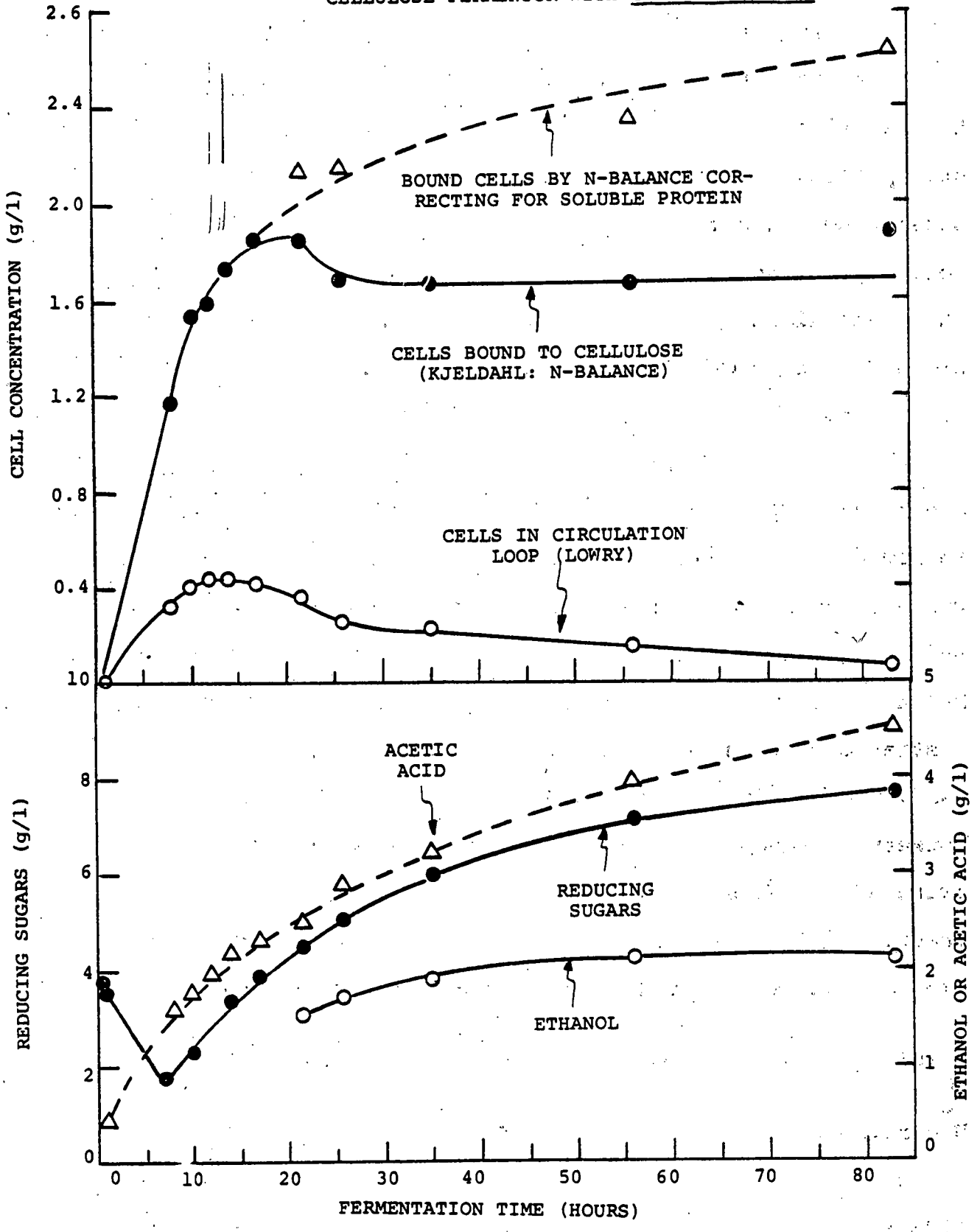
Cell mass accumulation in a packed-bed cellulose fermentor.

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 t_1

[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 s/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 (N_2) 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 cellulose. 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)

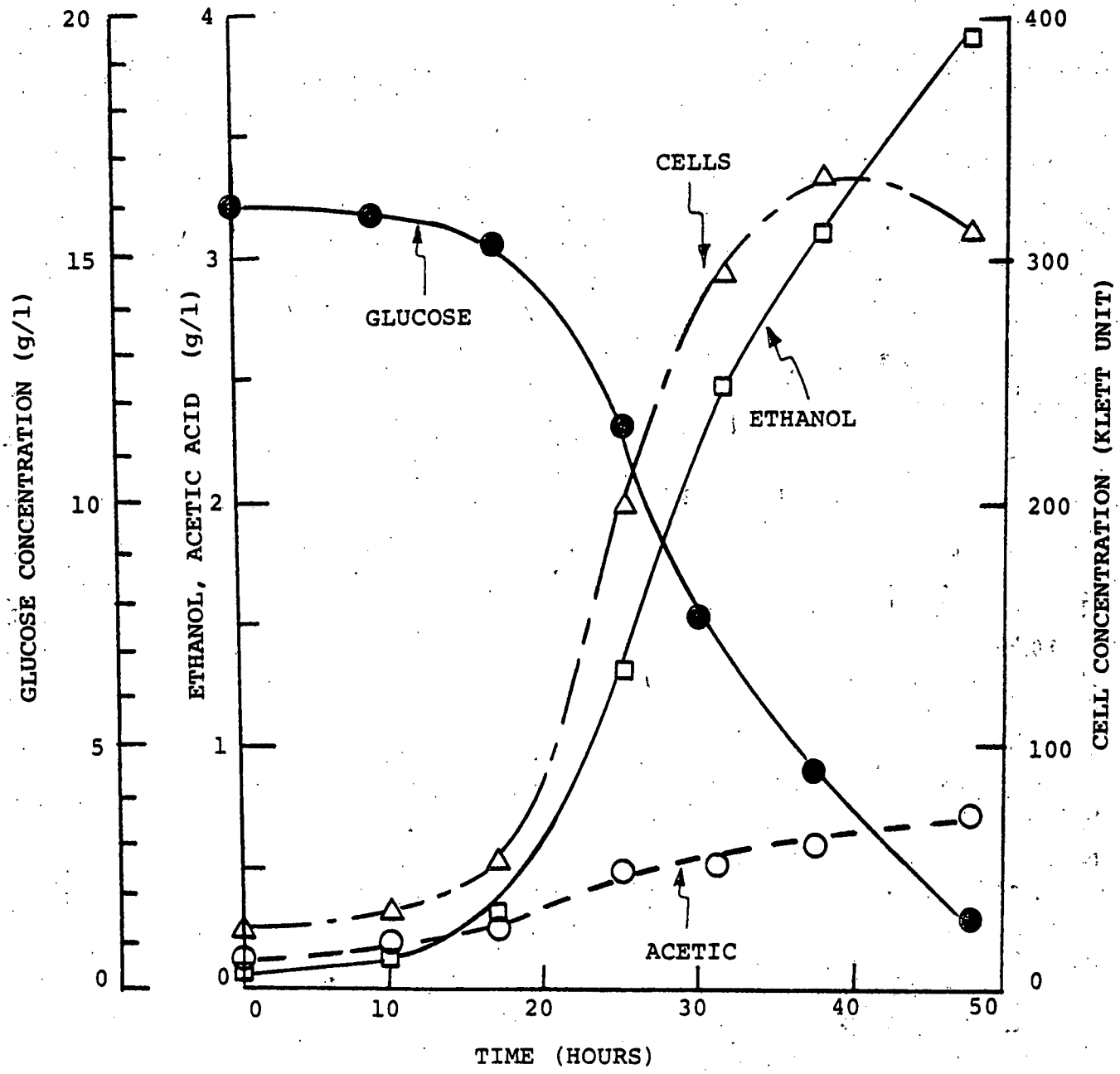


FIGURE I.C.1.2: FERMENTATION PROFILE OF FRUCTOSE BY C. THERMOCELLUM (S-4)

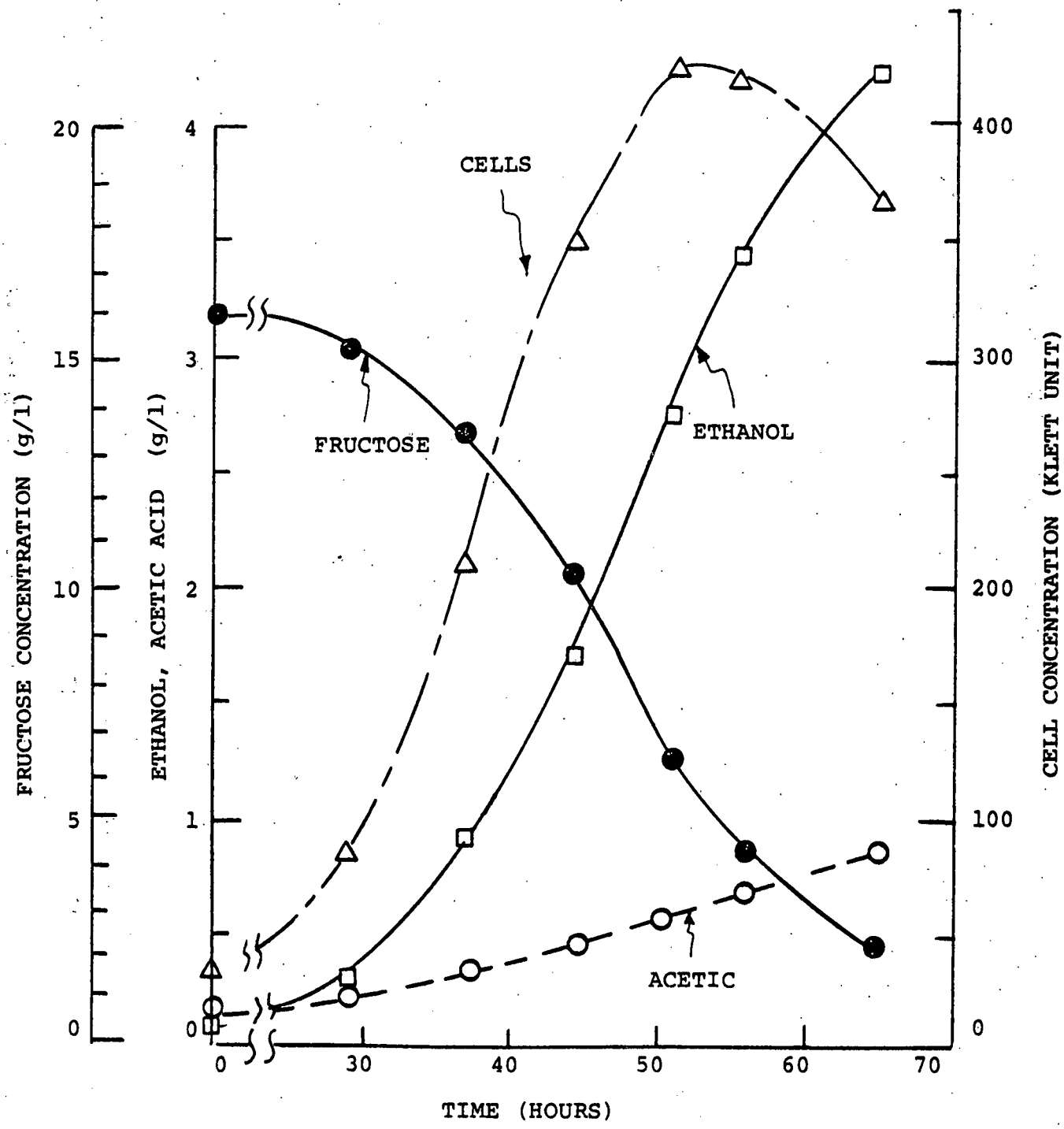


FIGURE I.C.1.3: FERMENTATION PROFILE OF CELLOBIOSE BY C. THERMOCELLUM (S-4)

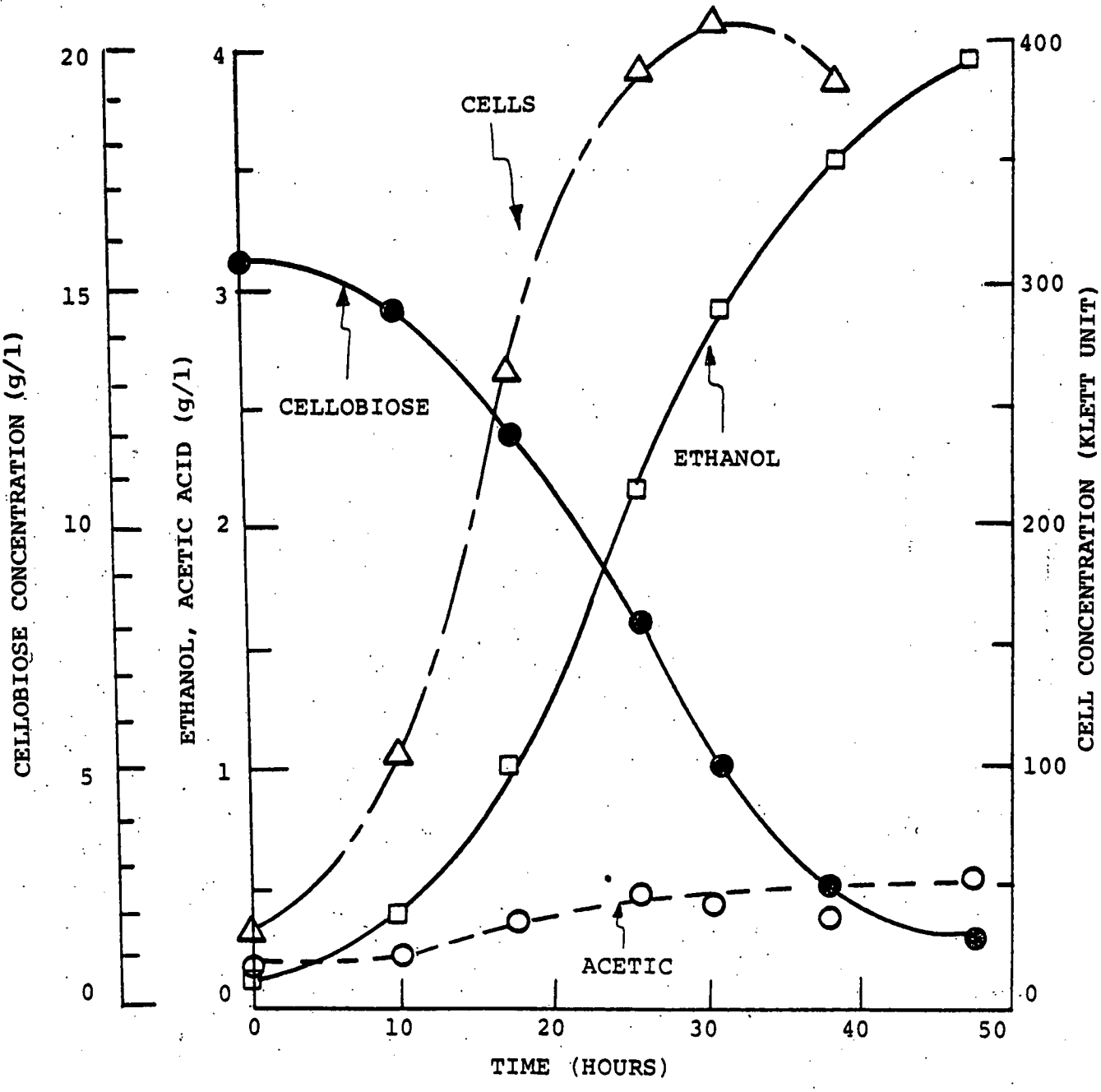
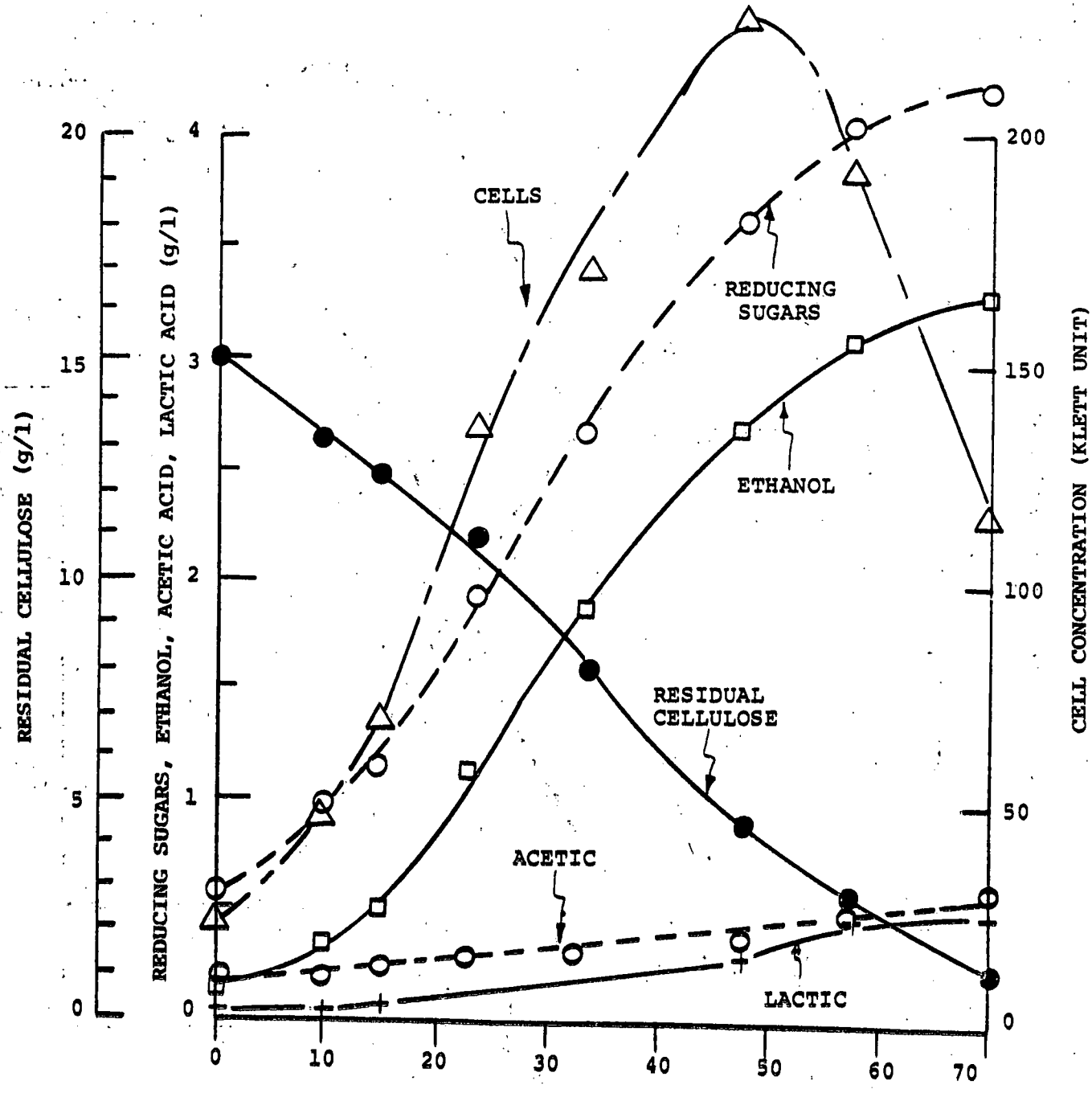


FIGURE I.C.1.4: FERMENTATION PROFILE OF CELLULOSE (SOLKA FLOC) BY C. THERMOCELLUM (S-4)



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 desireable 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 H_2 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 CO_2 and two moles of H_2. The fermentation can be represented by the following equation (1).

$$C_6H_{12}O_6 + H_2O \longrightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 \qquad (1)$$

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

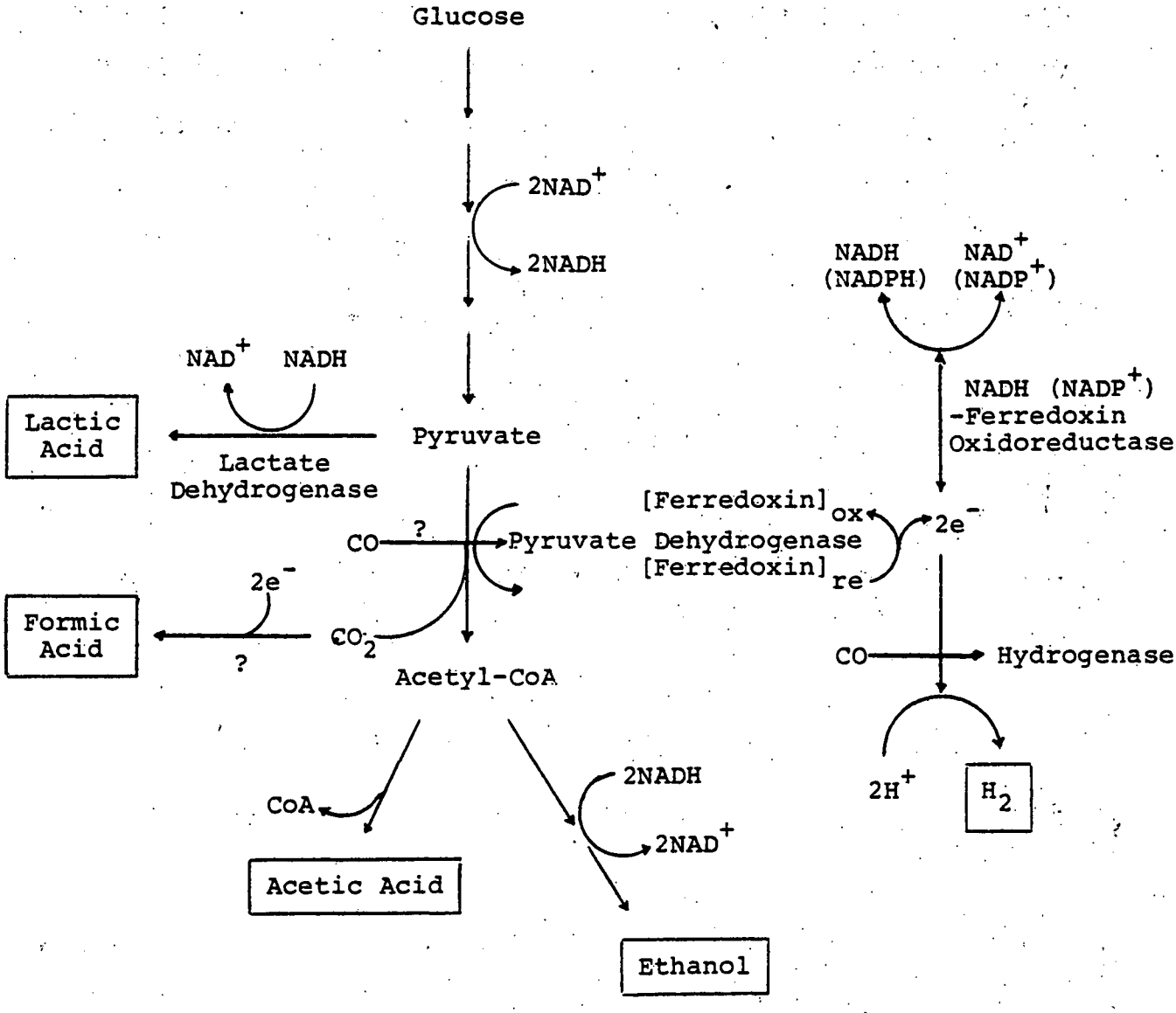


TABLE I.C.1.1: CARBON AND OXIDATION-REDUCTION BALANCES FOR FERMENTATION OF CELLULOSE BY CLOSTRIDIUM THERMOCELLUM S-4

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	2.088	0	0	0
Acetic Acid	0.42	7	0.168	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		+151.6 - 213.4	+151.6 - 137.6
Cellulose Degraded	13.57	83.8	5.971	0		
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 CO_2 evolution: Assume 1 mole of CO_2 produced for each mole of Ethanol or Acetic

3 Acid produded.

Calculated H_2 evolution: Case I : Assume 1 mole of H_2 produced for each mole of CO_2 evolution.

4 Case II: Assume no H_2 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).

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3CH_2OH + 2CO_2 \qquad (2)$$

or

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3CHOH\ COOH \qquad (3)$$

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

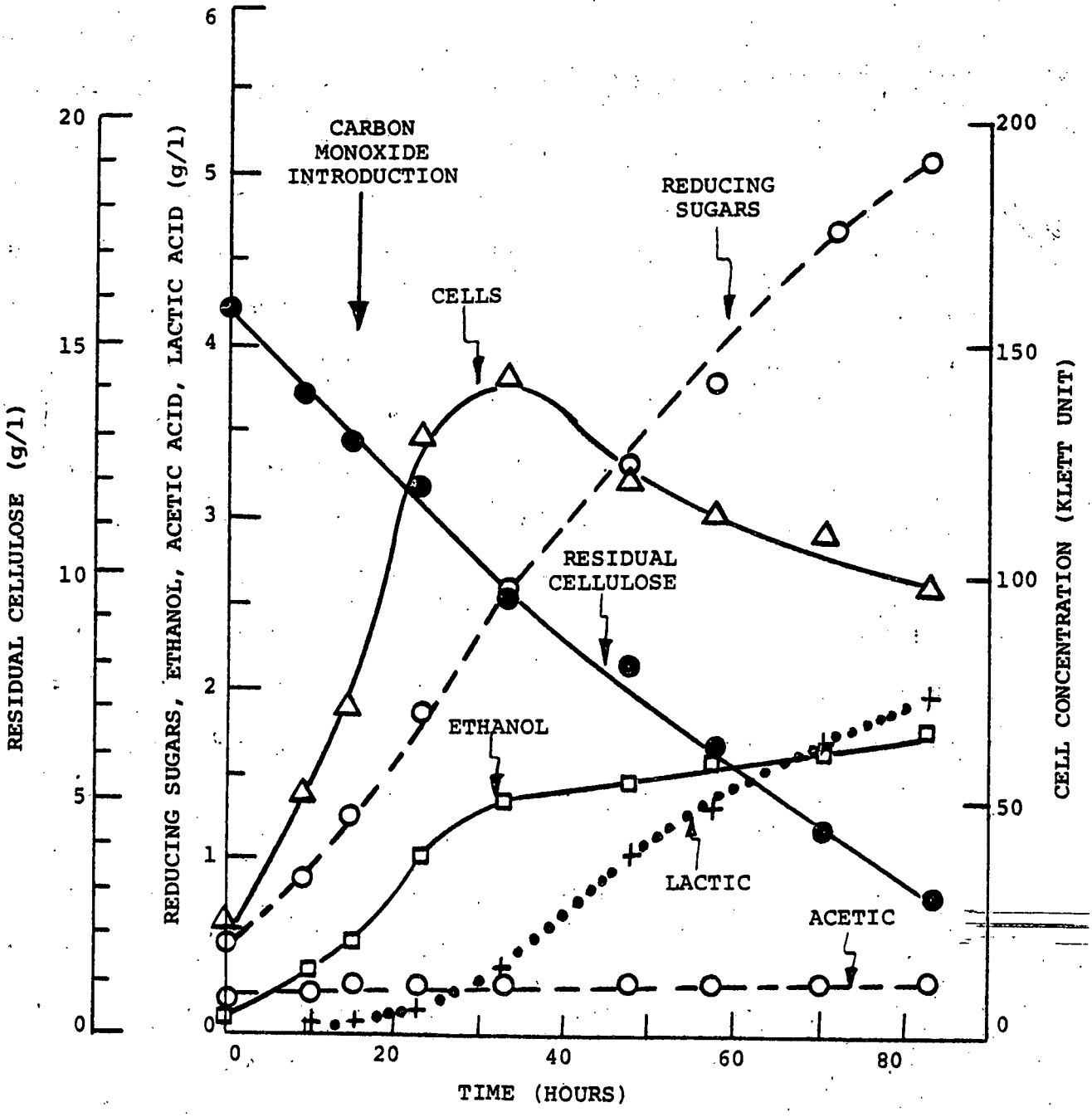


TABLE I.C.1.2: CARBON AND OXIDATION-REDUCTION BALANCES FOR FERMENTATION OF CELLULOSE BY CLOSTRIDIUM THERMOCELLUM S-4 UNDER CO ATMOSPHERE

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

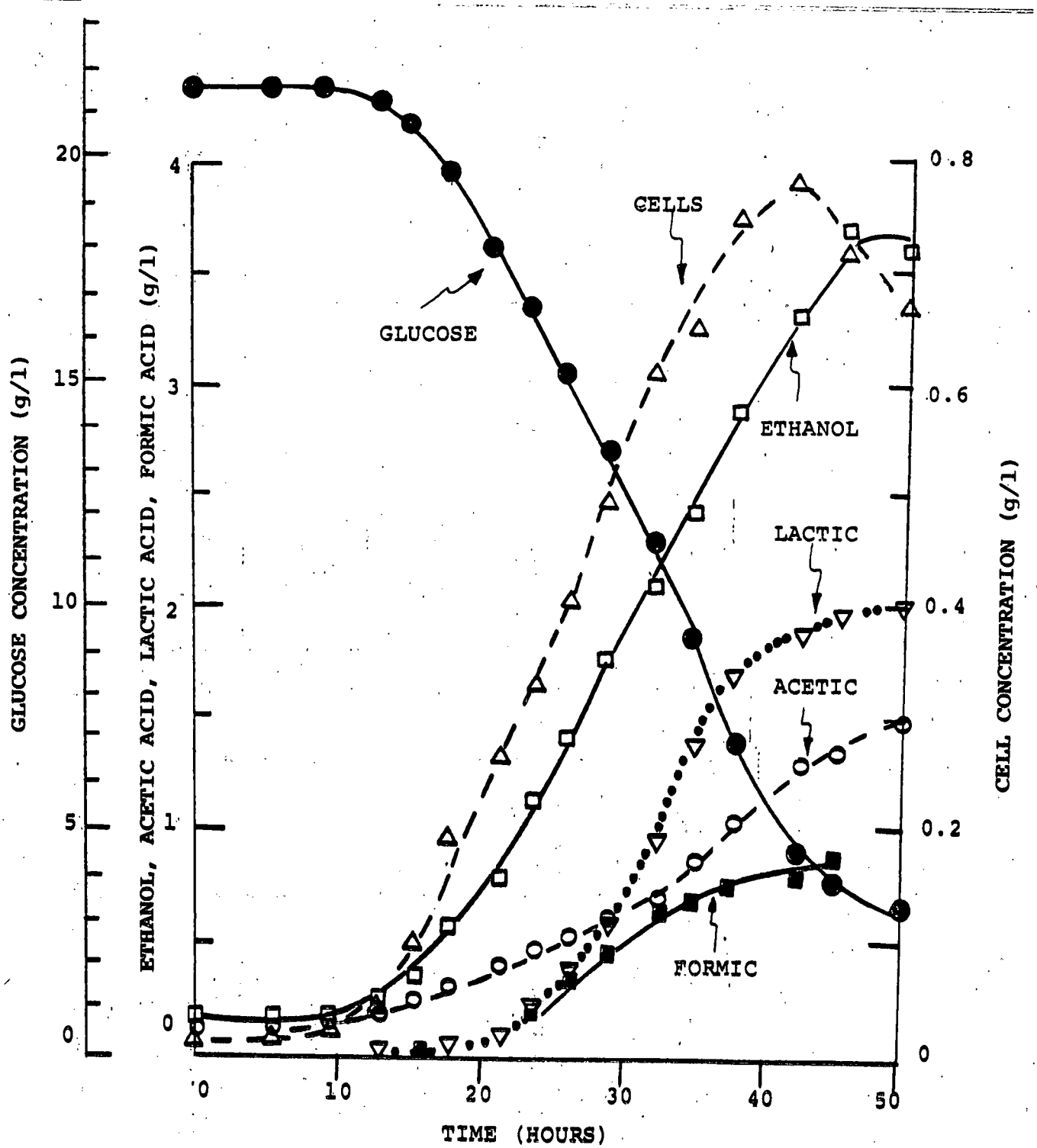


Figure I.C.1.7 showed the product profiles for this fermentation were similar to the previous results. The production of reduced products (i.e. ethanol and lactic acid) is significantly greater than oxidized product (acetic acid). The lactic acid measured by lactate dehydrogenase method and p-hydroxydiphenyl-sulfuric 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 thermocellum will produce cellobiose phosphorylase and cellodextrin phosphorylase which catalyzes the following reactions:

$$\text{Cellodextrin} + \text{Pi} \underset{\text{phosphorylase}}{\overset{\text{cellodextrin}}{\rightleftharpoons}} \text{Glucose-1-P} + \text{Cellobiose} \qquad (4)$$

$$\text{Cellobiose} + \text{Pi} \underset{\text{phosphorylase}}{\overset{\text{cellobiose}}{\rightleftharpoons}} \text{Glucose-1-P} + \text{Glucose} \qquad (5)$$

Both enzymes are reversible, and *in vitro*, cellobiose phosphorylase is favored in the direction of phosphorolysis, but cellodextrin phosphorylase is favored in the direction of synthesis. It is very possible that, under high glucose concentration, cellobiose and cellodextrin 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. N_aOH 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:H_2O
4:2:1

STAINING REAGENT: Aniline Hydrogen Phthalate (10%)

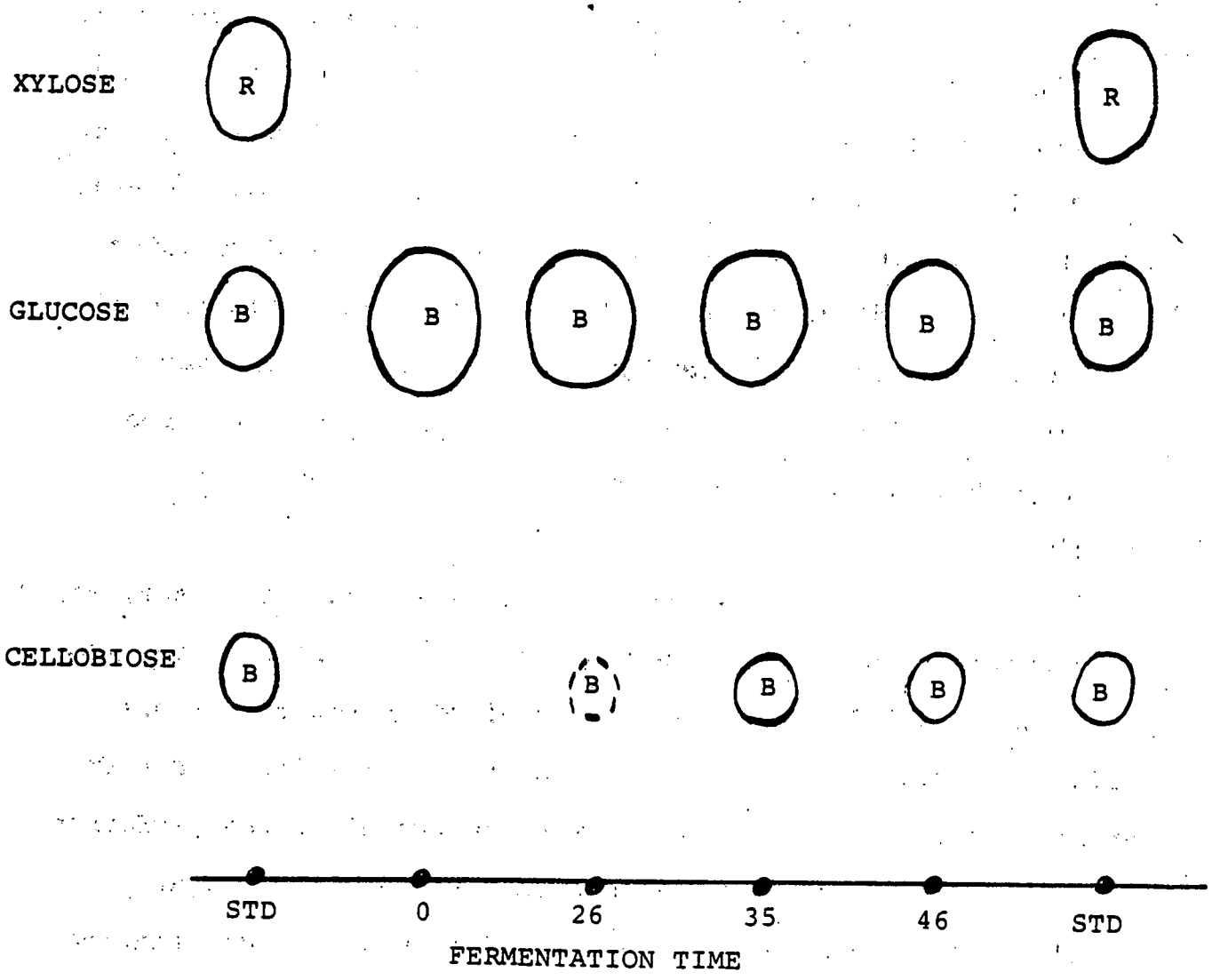
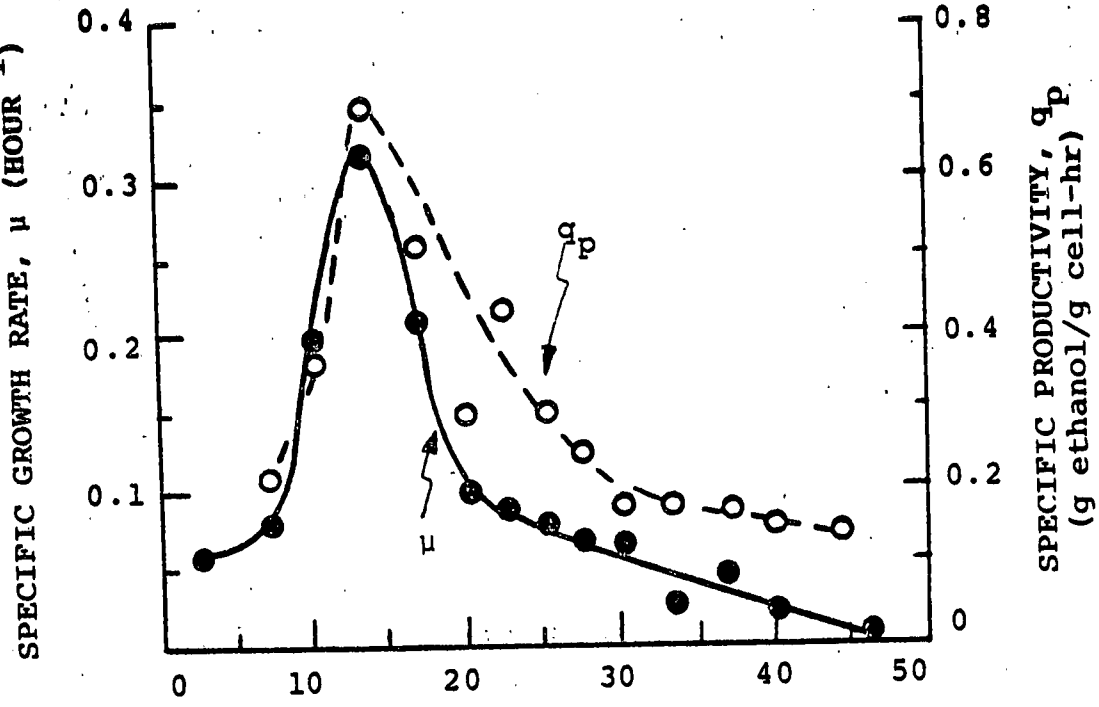


FIGURE I.C.1.9: KINETIC RELATIONSHIP OF GROWTH AND PRODUCT FORMATION BY C. THERMOCELLUM (S-4) GROWN ON GLUCOSE



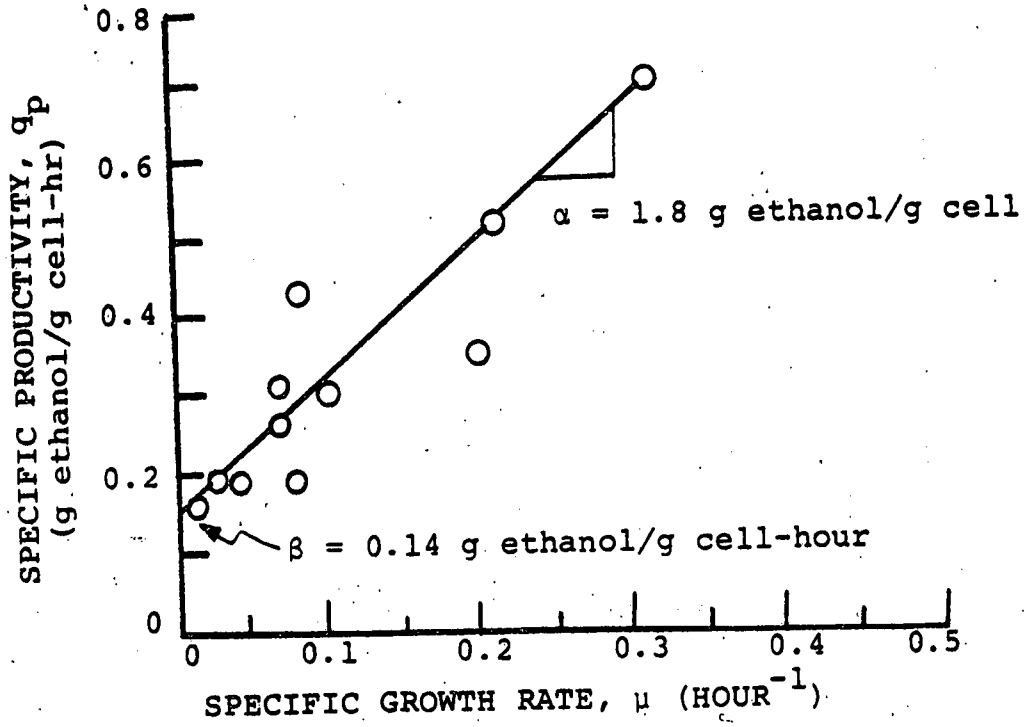


FIGURE I.C.1.10: EFFECT OF INITIAL CORN STOVER CONCENTRATION ON VOLUMETRIC PRODUCTIVITY OF PRODUCTS BY CLOSTRIDIUM THERMOCELLUM (S-4)

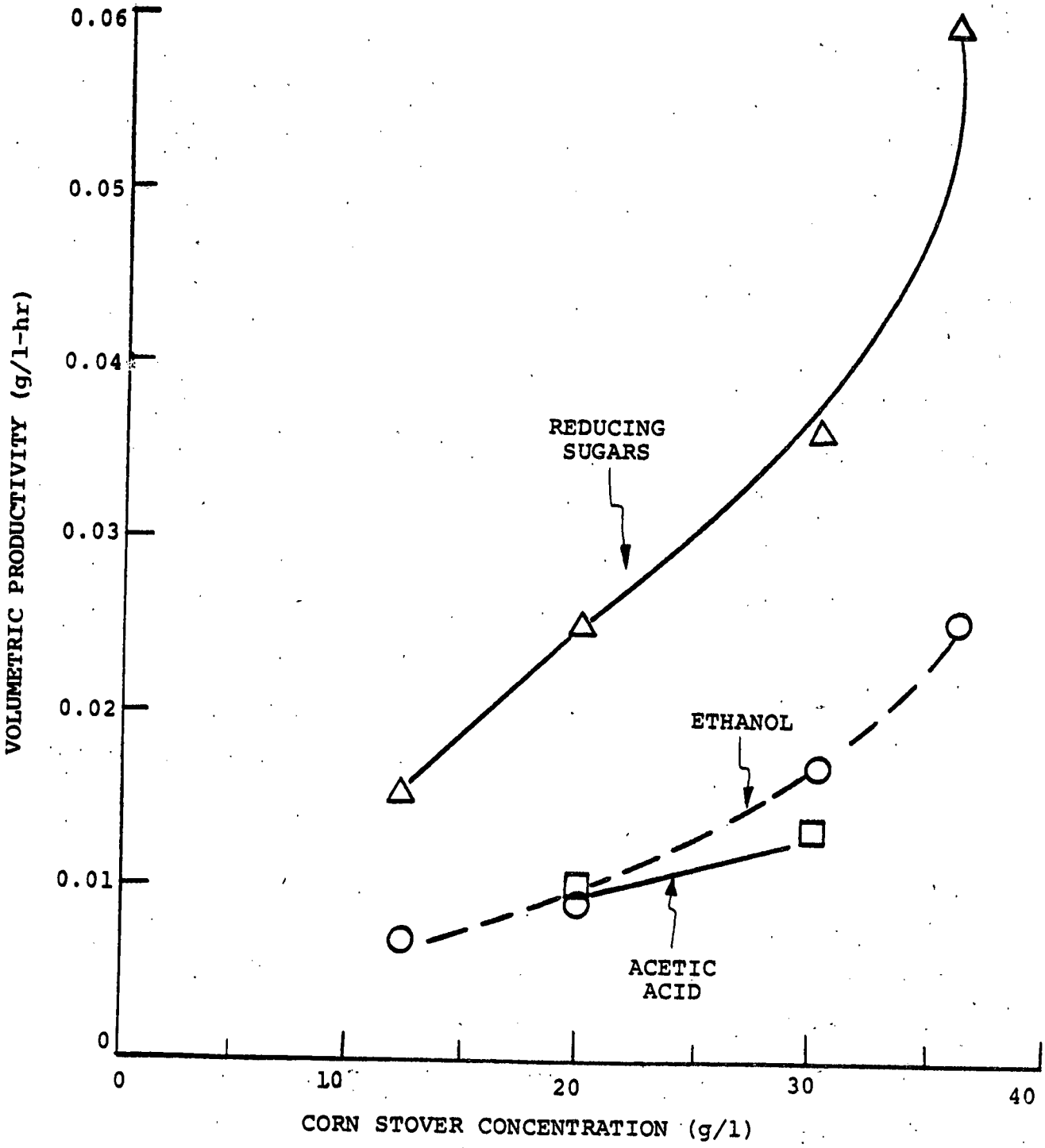
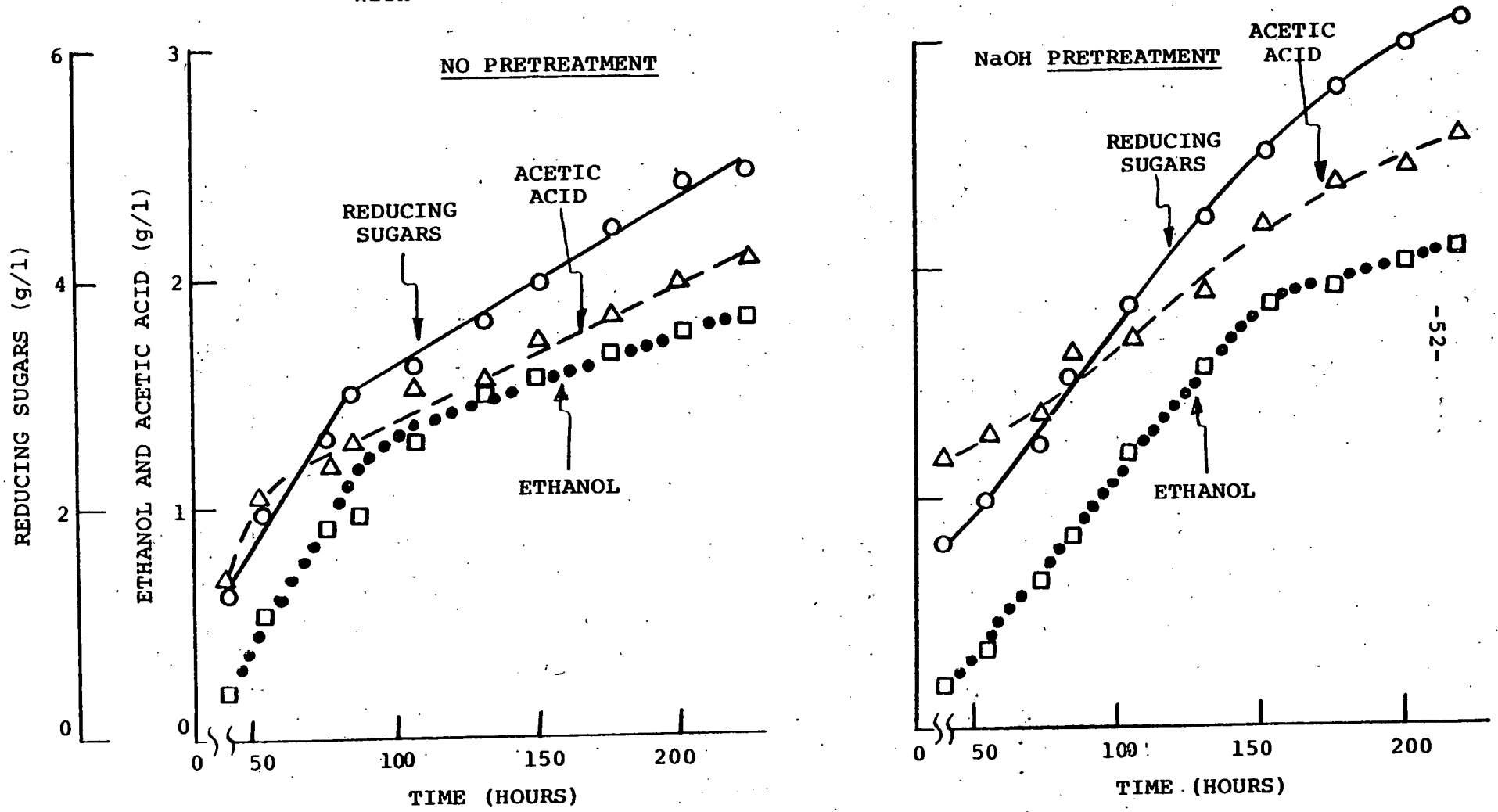


FIGURE I.C.1.11: PRODUCT FORMATION FROM CORN STOVER BY CLOSTRIDIUM THERMOCELLUM (S-4) WITH NO PRETREATMENT AND NaOH PRETREATMENT (CORN STOVER = 30 g/l)



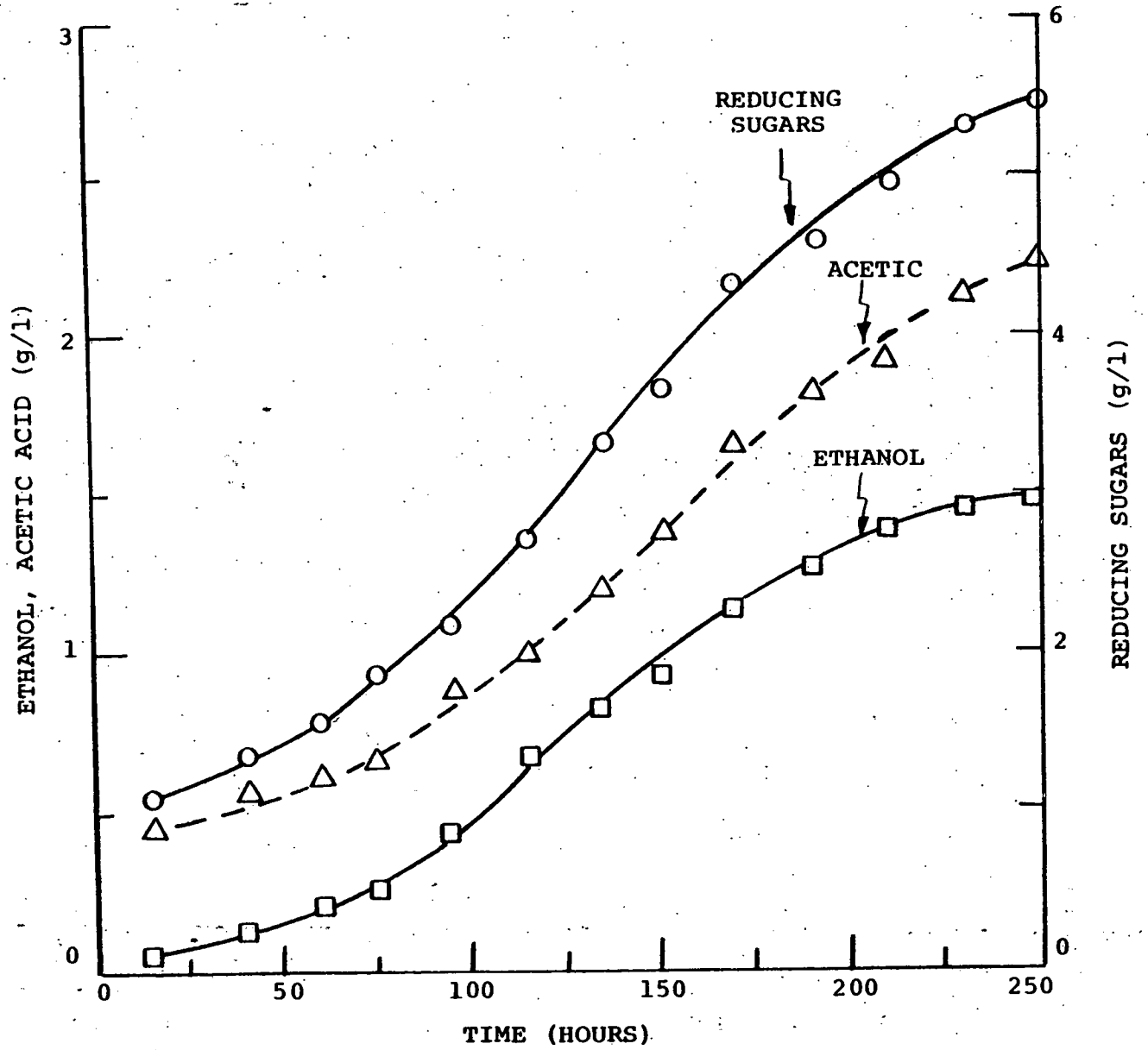
left in the Figure represents no pretreatment and on the right represent NaOH pretreatment. The rates of product formation (ethanol, acetic acid and reducing sugars) are all higher when the stover was pretreated. Further studies with corn stover along with pretreatment will be performed.

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

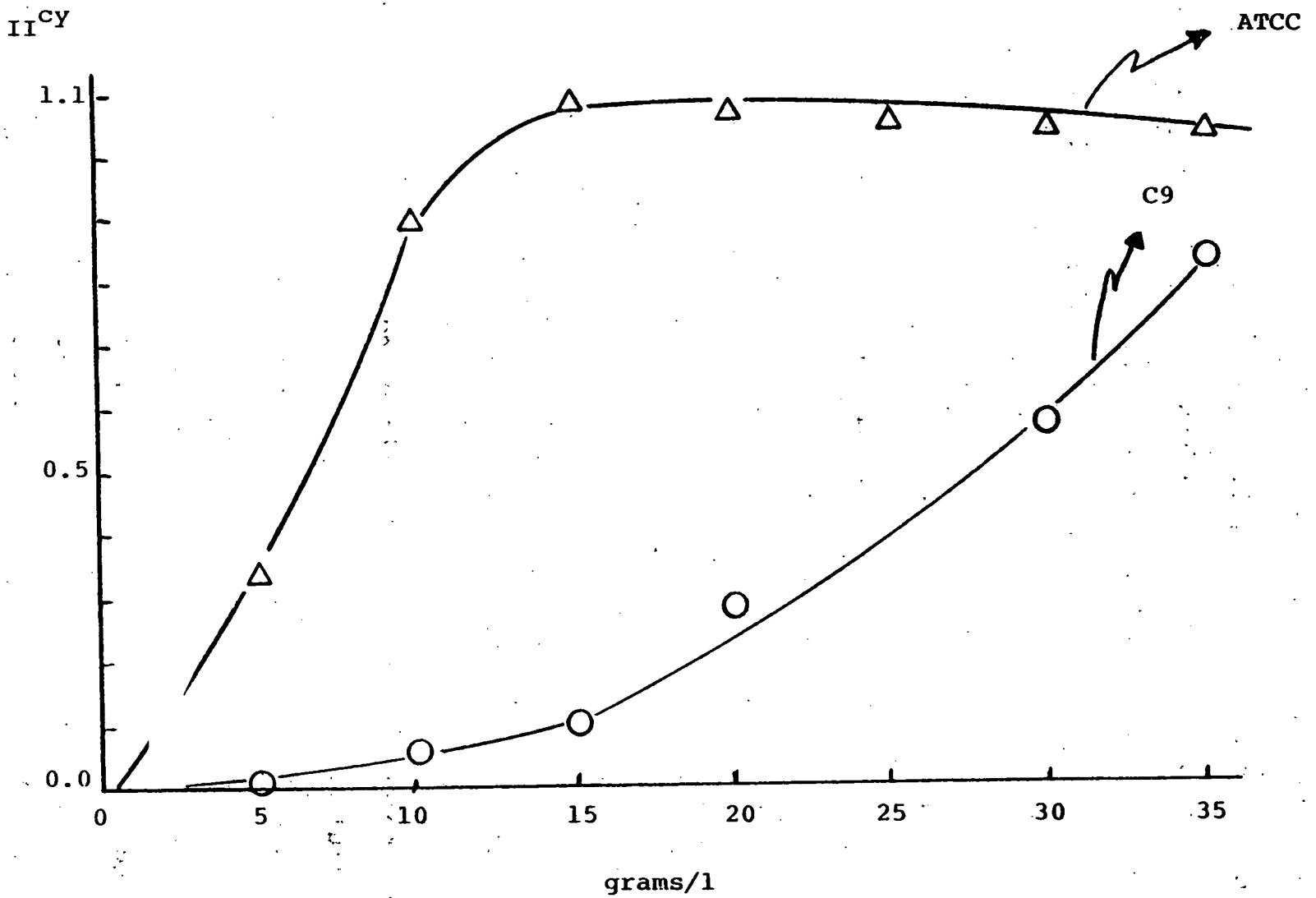
i. enrichment procedure:

An ethanol resitant 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.



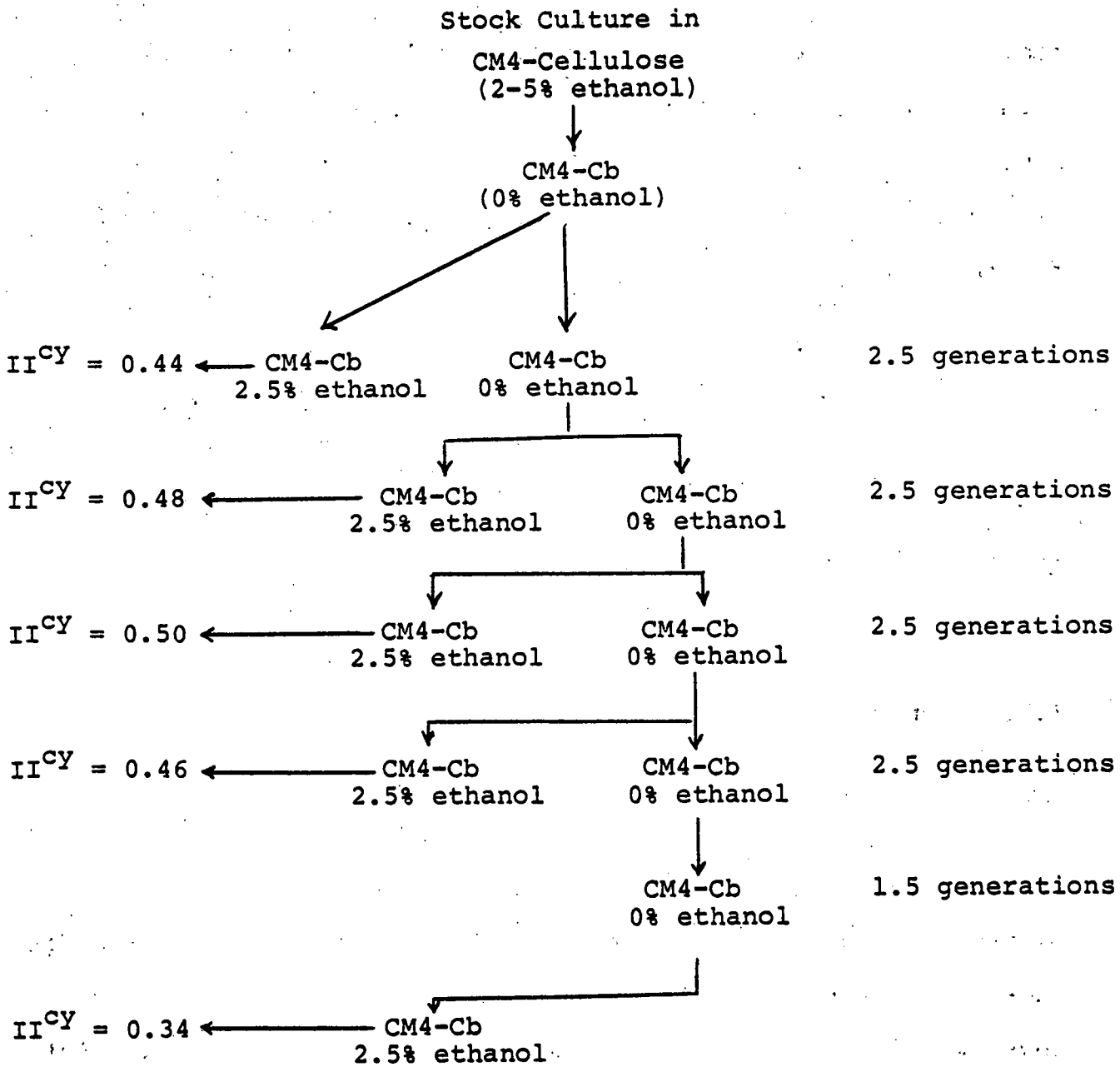
CM4 - cellulose (Solka-floc) broths in the absence of ethanol. After 48 hrs of growth at 60°C in CM4-cellulose, stock tubes were prepared containing 25 grs/lt of ethanol. To ascertain whether the resistance of ethanol of C. thermocellum C9 was due to a mutational change or if it was the phenotypic expression 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.

	$\frac{\text{mg R.S}}{\text{ml.hr. mg.prot.}}$		Ratio
	ATCC	C9	C9/ATCC
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 60°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.

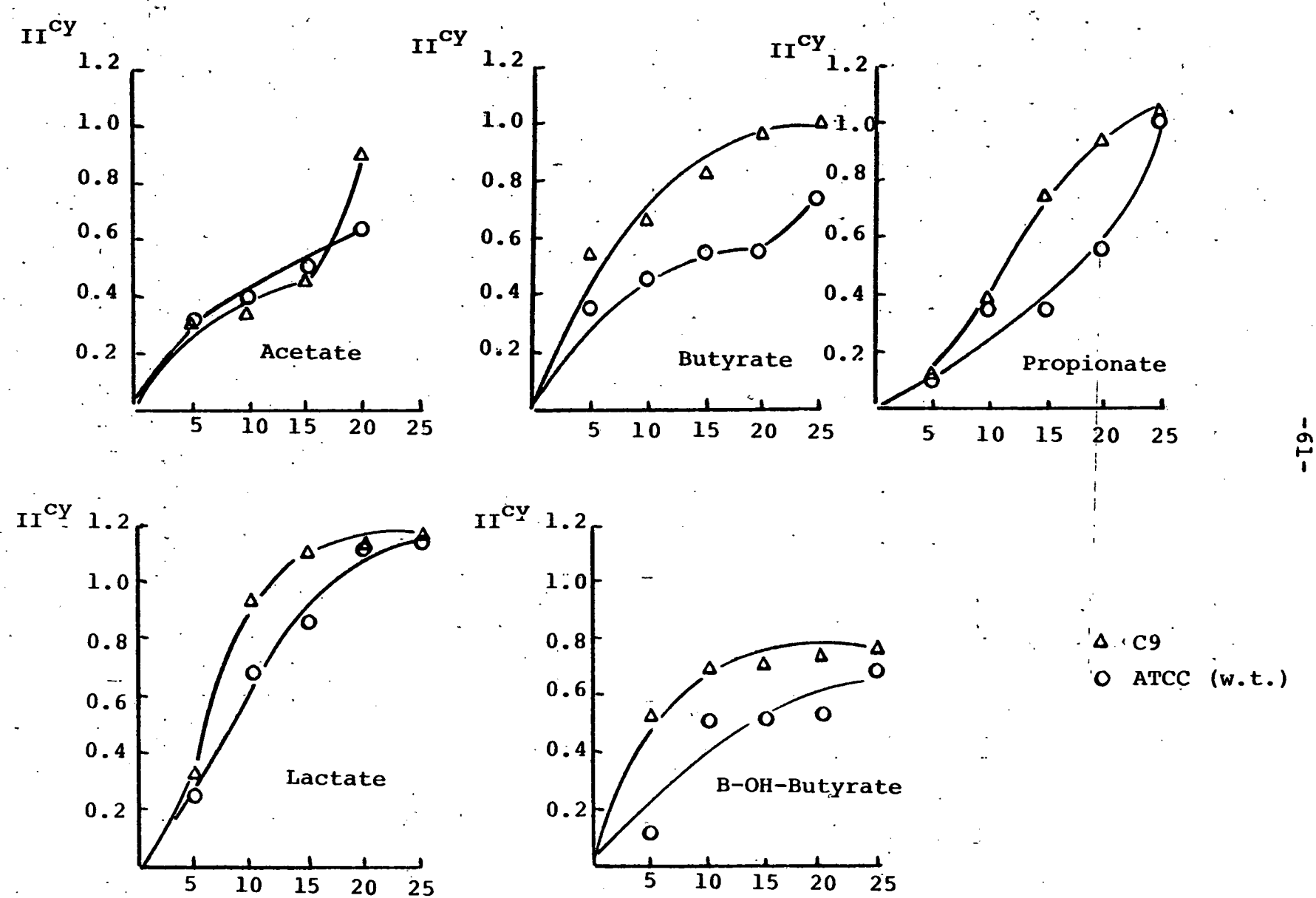
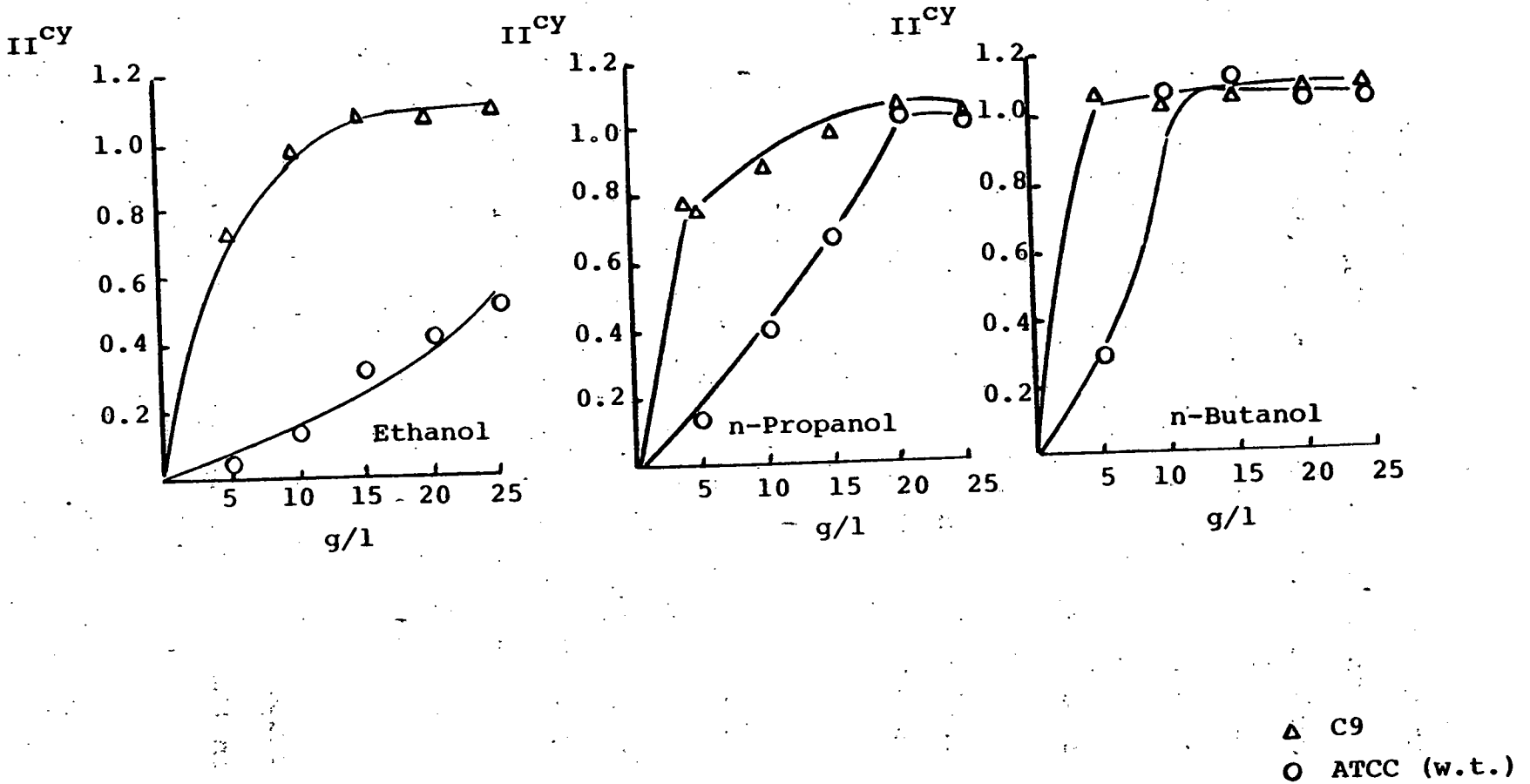


Figure I.C.2.4 Growth Inhibition By Alcohols. Plot of Inhibition Index (Cell Yield) vs. Alcohol Concentration in Grams/L.



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.

Dilution	Cellobiose Growth at 24-48hrs.	Glucose Growth at 80-100hrs.
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 OD_{660} 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

	Lsozyme Treated culture		Control Culture	
	-PEG	+5%PEG	-PEG	+5%PEG
plate count, $\frac{cfu}{mg}$	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 membrance-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

Worked 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 N_2 (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
$(NH_4)_2SO_4$	1.3 g	-
Urea	-	2.14 g
$MgCl_2 \cdot 6H_2O$	1.0 g	1.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g	0.15 g
$FeSO_4 \cdot 6H_2O$	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 substracted from the experimental value. The TNP-CMCase 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 N_2. 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-

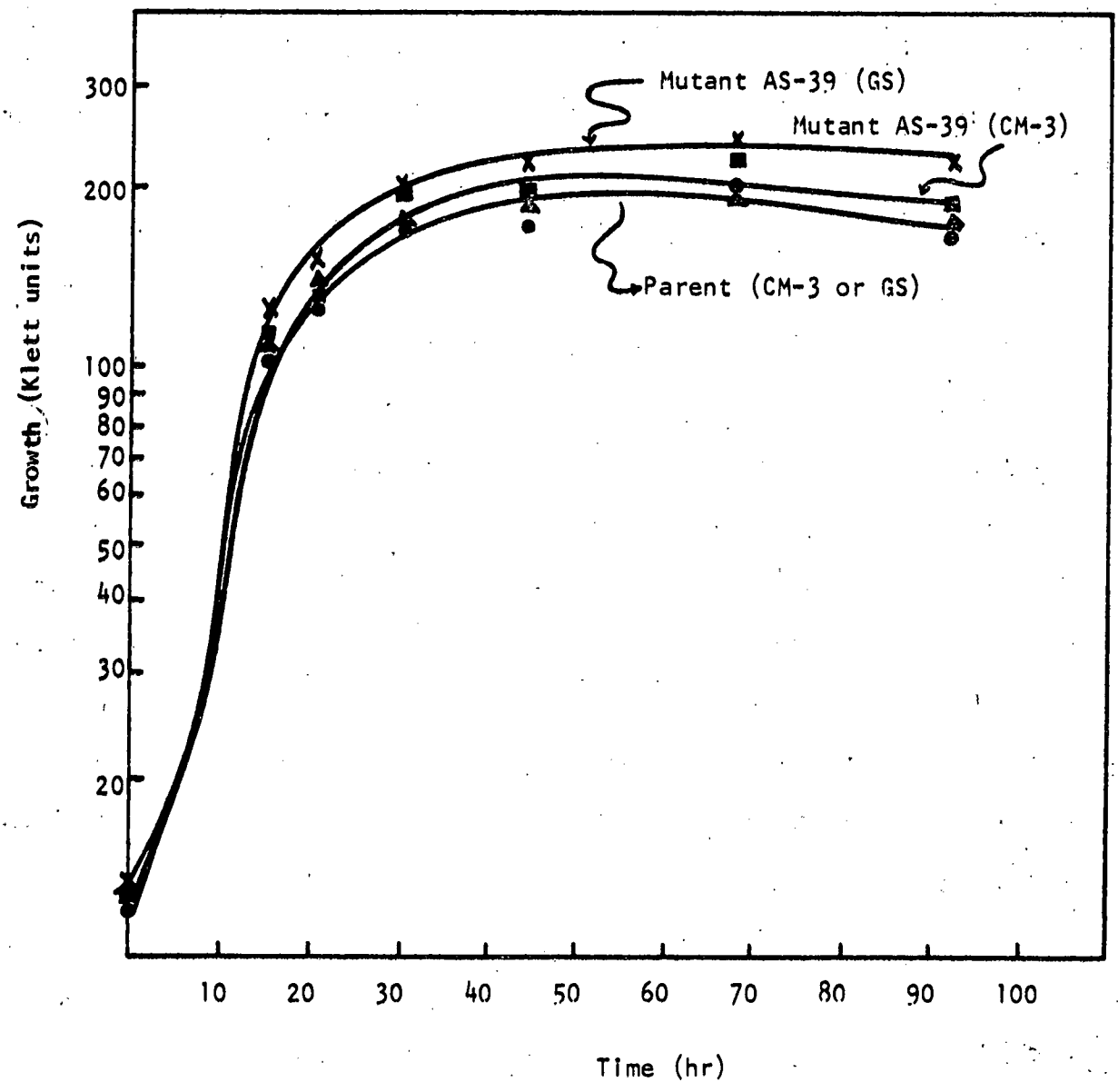


Fig. I.E.1. Growth of C. thermocellum ATCC 27405 (parent) and mutant AS-39 in CM-3 and GS media.

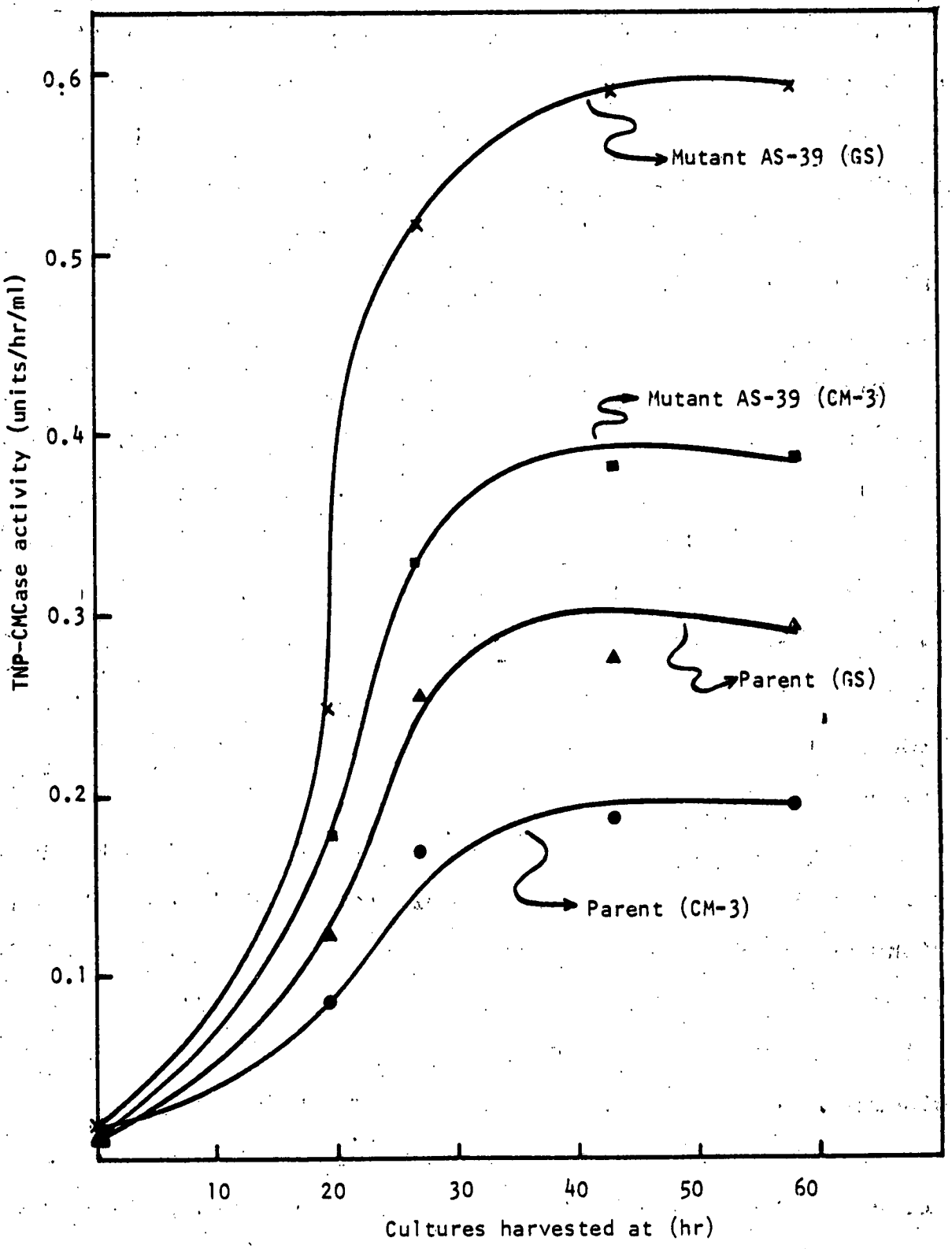


Fig. I.E.2. TNP-CMCase production by strains ATCC 27405 and mutant AS-39 in CM-3 and GS media.

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

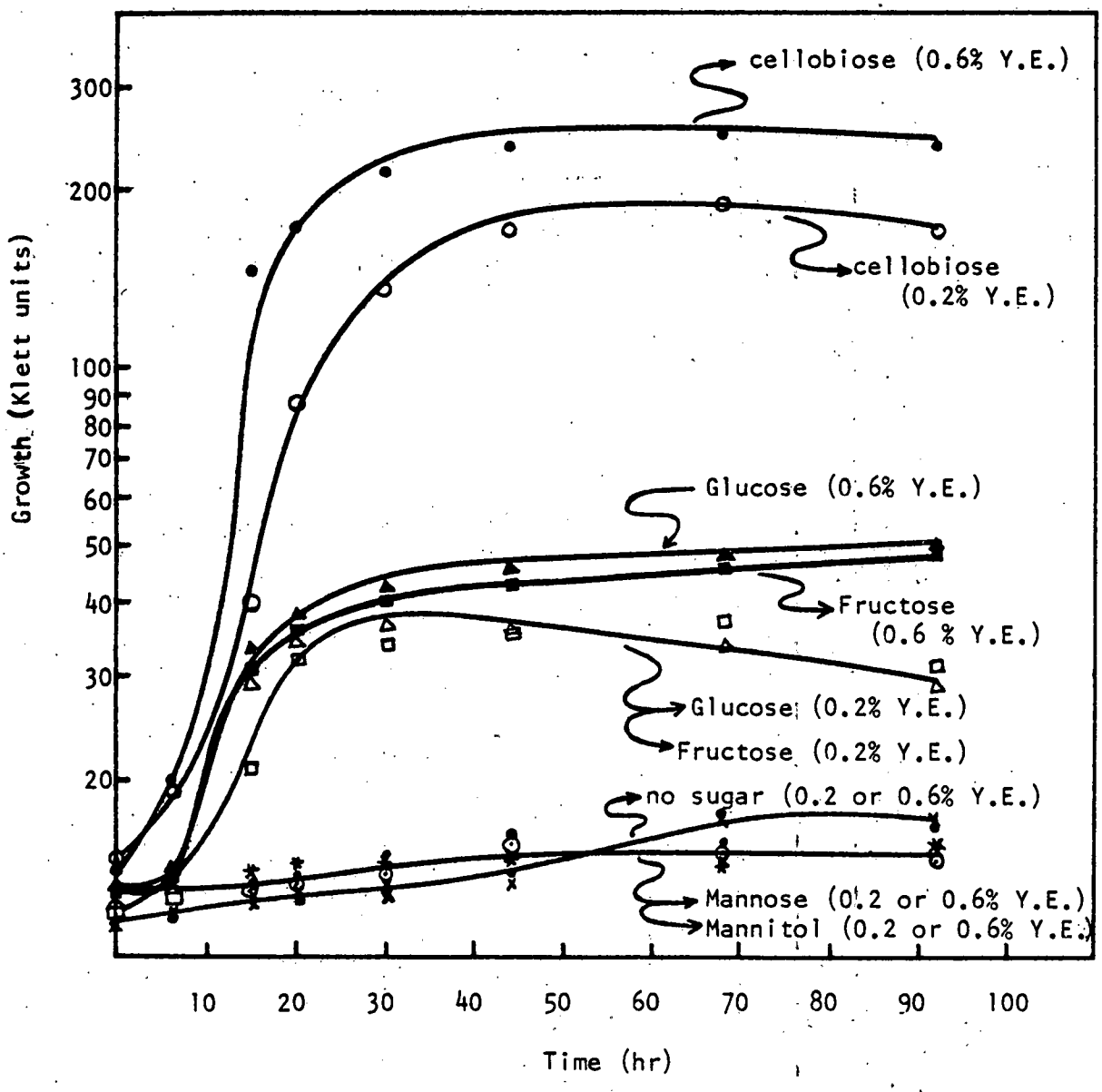


Fig. I.E.3. Growth of C. thermocellum AS-39 on carbon sources at two levels of yeast extract.

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.

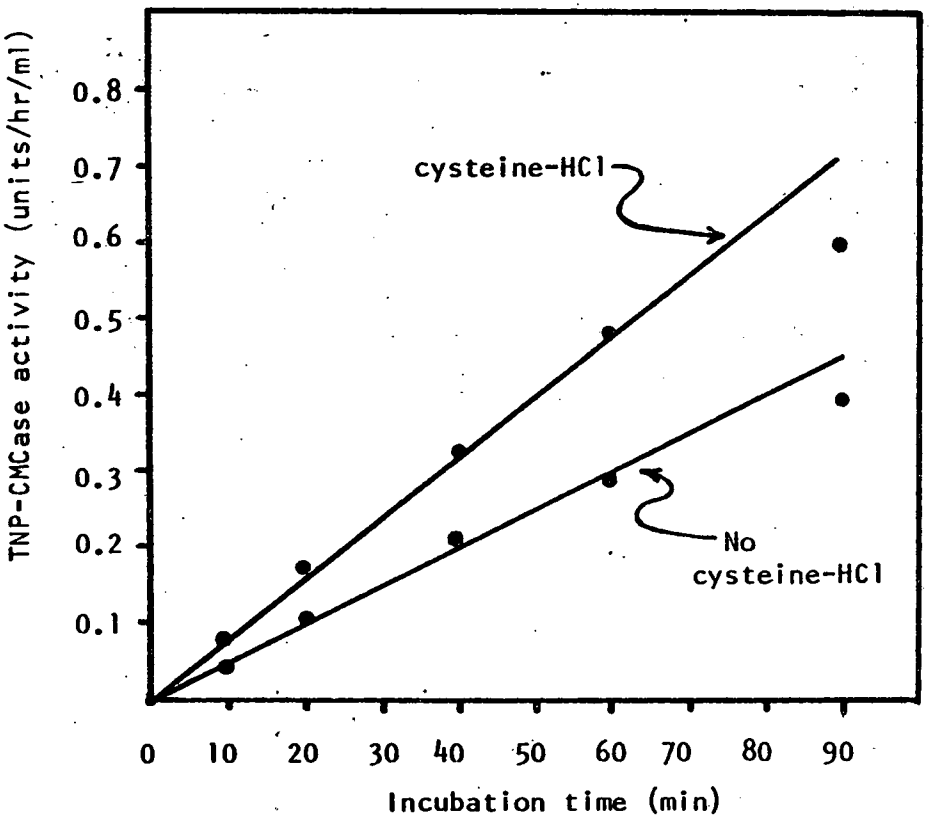


Fig. I.E.4. Effect of 1 mg/ml cysteine-HCl on TNP-CMCase activity

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 2.01 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.1 Standard Filter Paper Assay

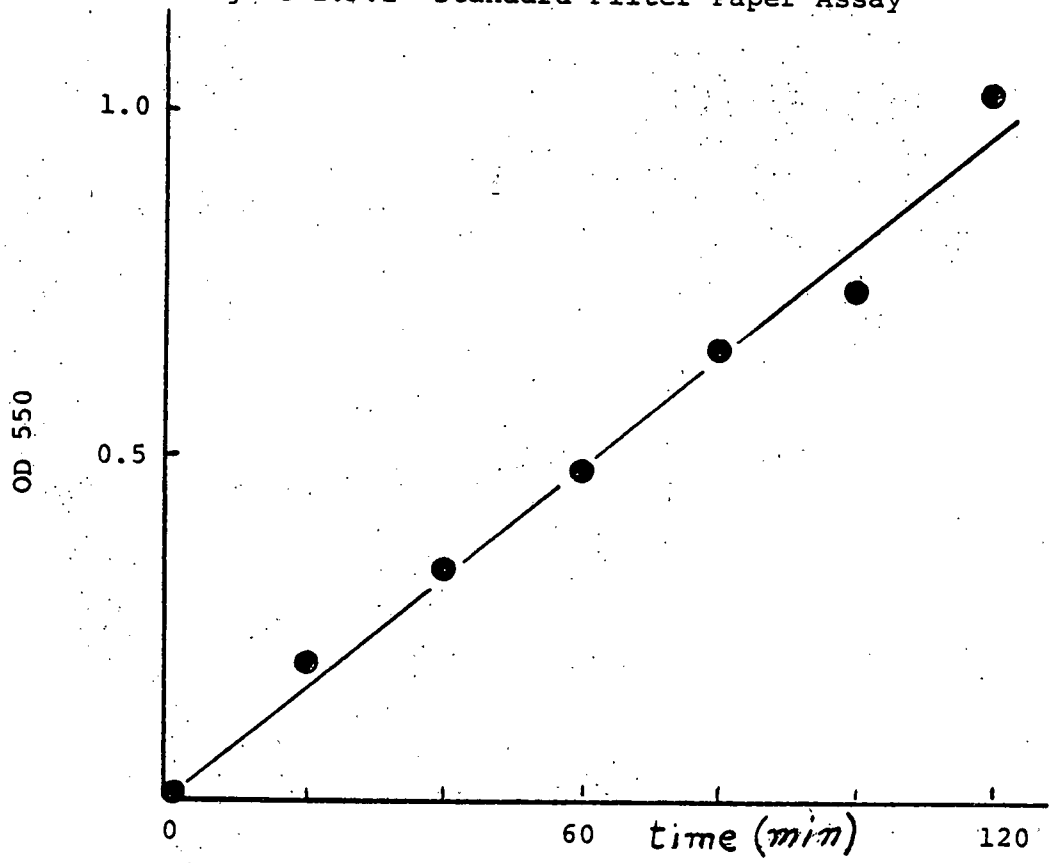


Figure I.F.2 Standard CMC Assay

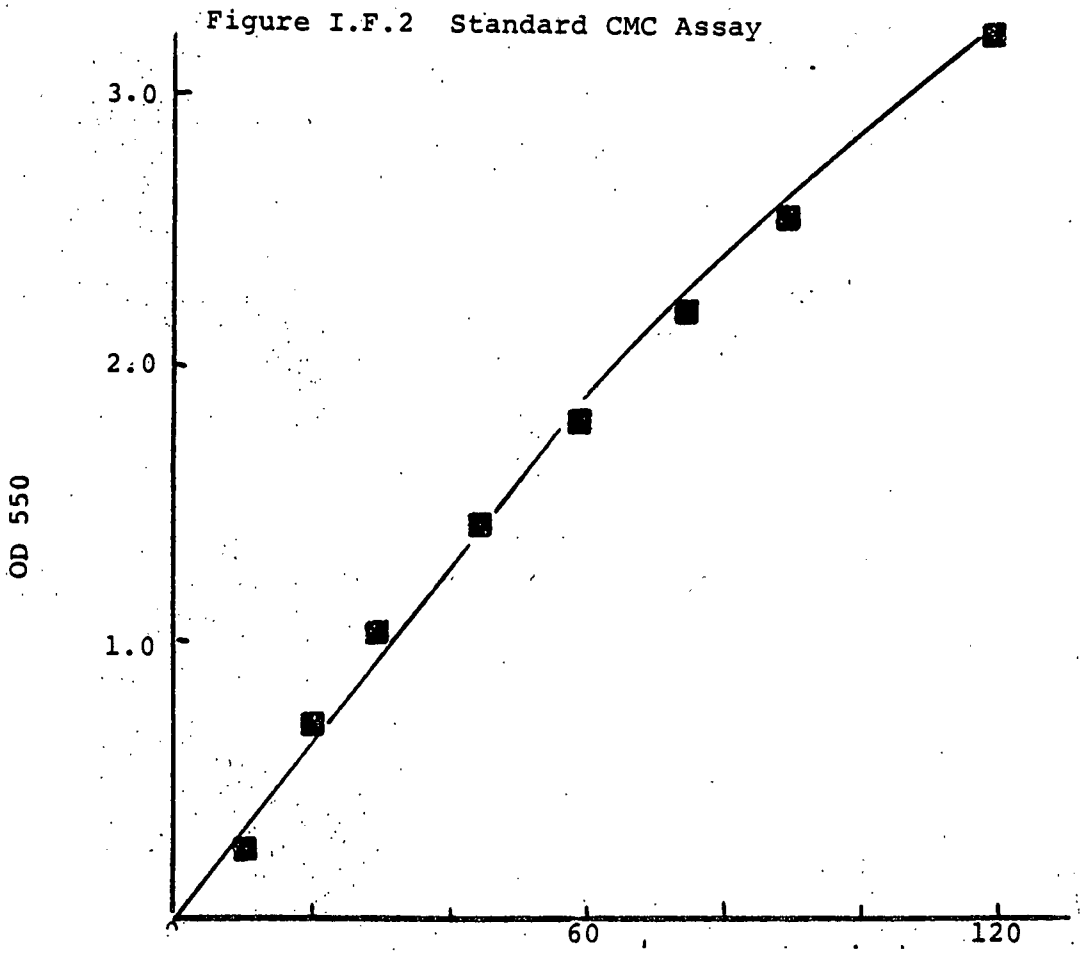


Figure I.F.3 Filter Paper Activity vs. Enzyme Concentration Over Time

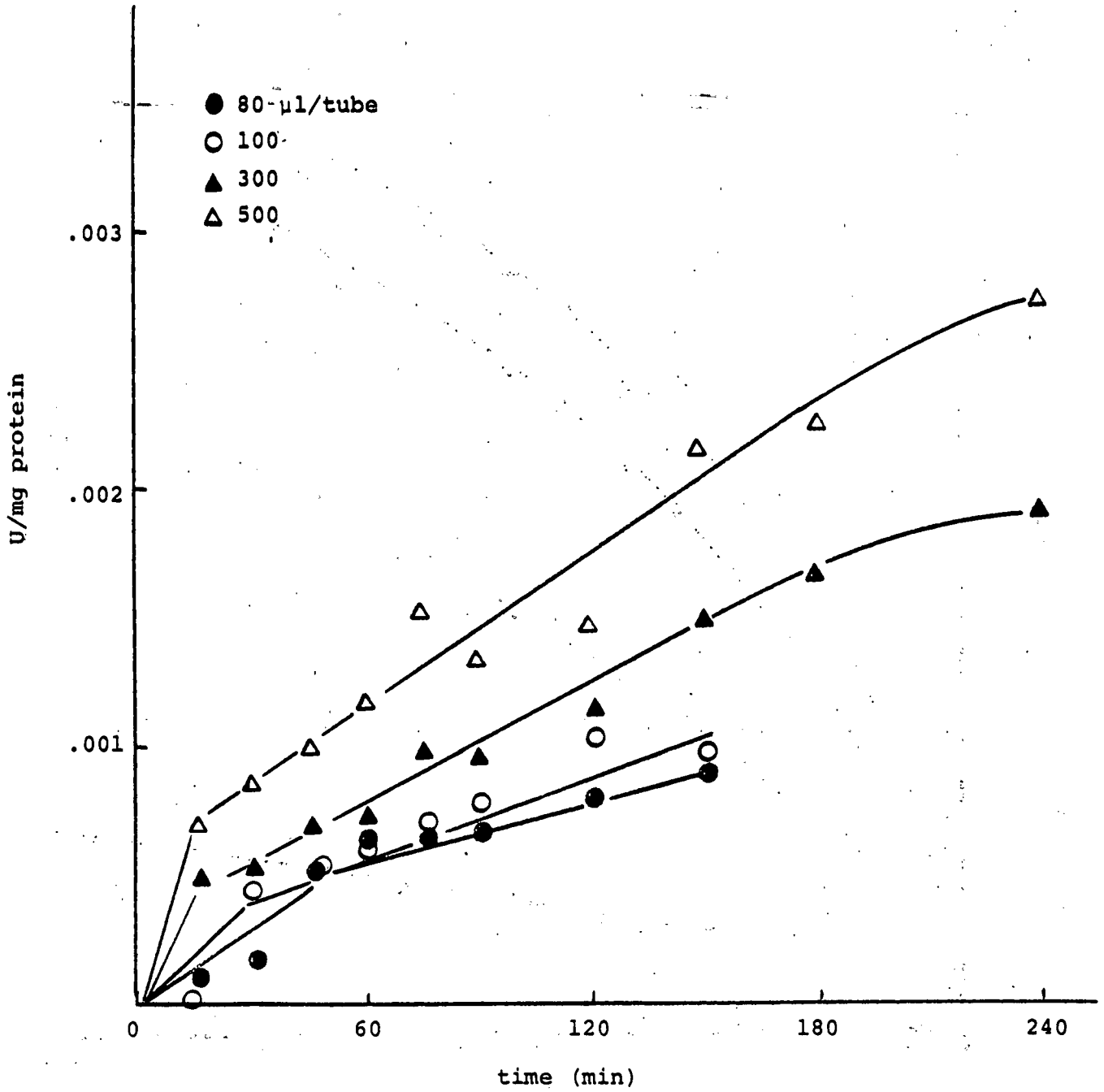


Figure I.F.4 CMCase Activity vs. Enzyme Concentration Over Time

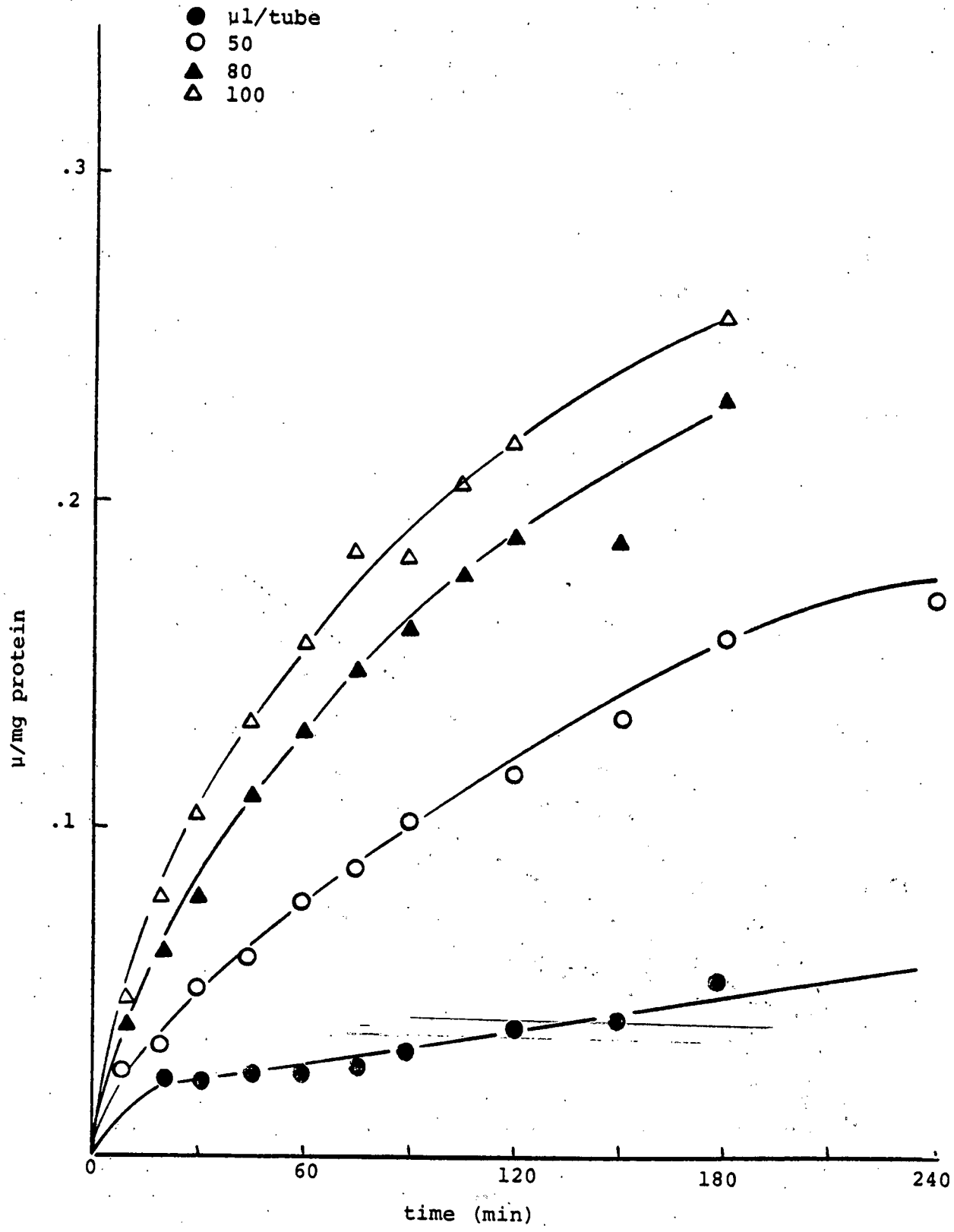


Figure I.F.5 Reaction Rate vs. Enzyme Concentration

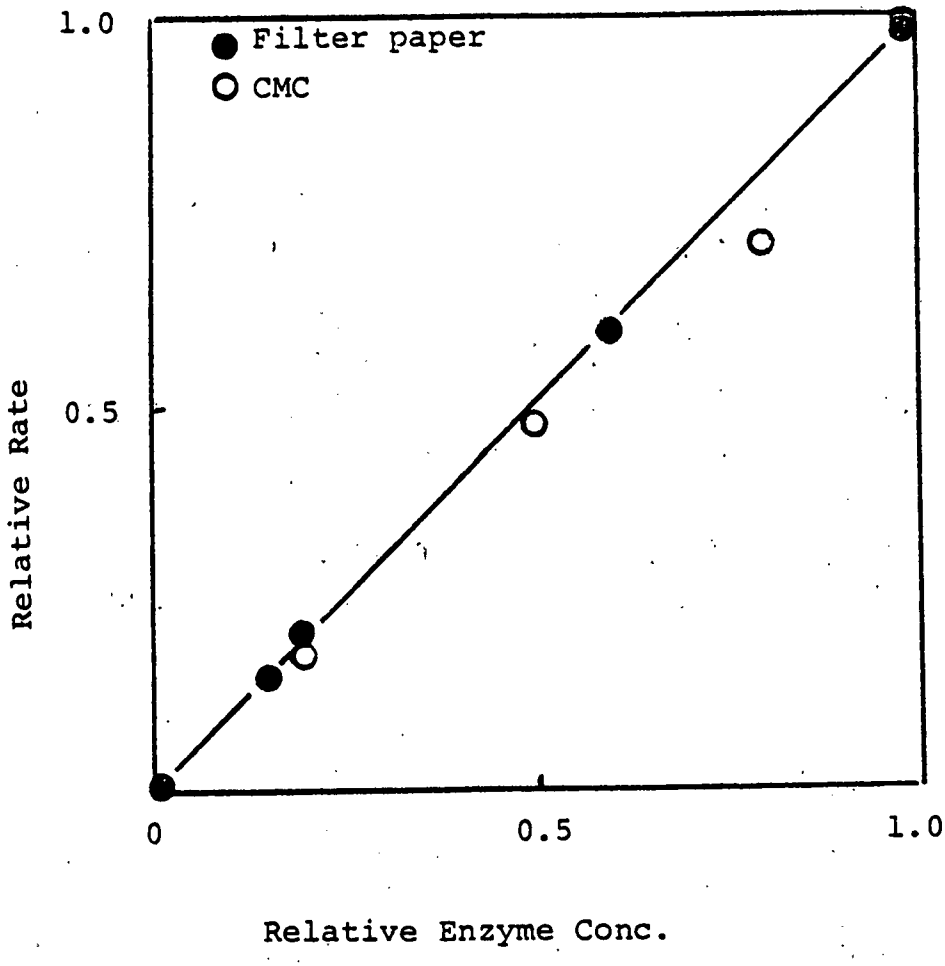


Table I.F.1. Filter Paper Activity vs. Enzyme Concentration

Vol. Enzyme (ml)	Initial Rate (V/ml)	Relative Volume	Relative Activity
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

Vol. Enzyme (ml)	Initial Rate (V/ml)	Relative Volume	Relative Activity
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.

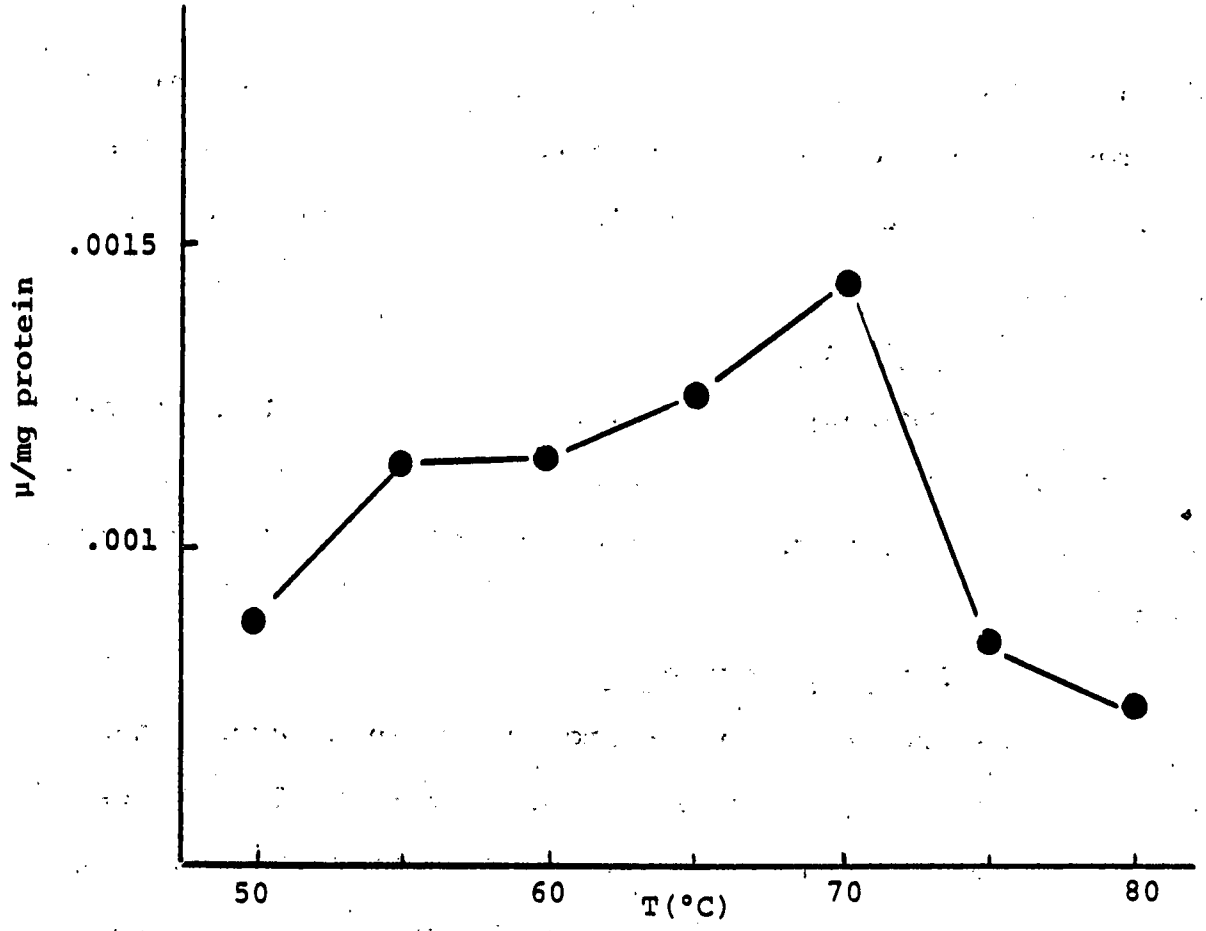
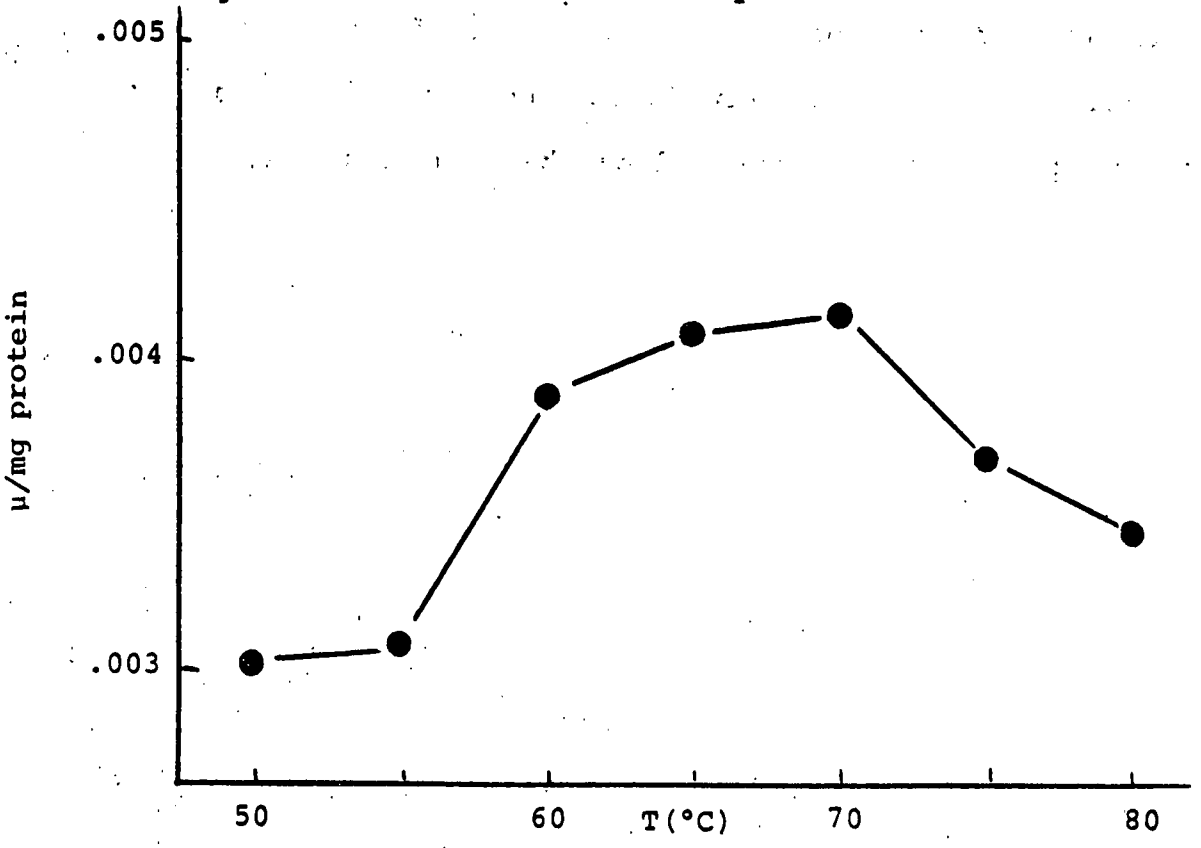


Figure 1.F.1.7 CMCase Activity vs. T.



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 activies 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 centrifugated, 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/in^2. 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 pippetted 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 electrophroesis 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% Coomasie 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 activies 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

Stain	Activity	Solka Floc	MN300	Xylan	Cellobiose	Y.E.	Glucose	Xylose
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 hemicellulose present in these. But because activities increase when the culture is growing on xylan compared with those obtained when the cutlure 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.

Substrate	Inhibition Index
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 : $\frac{\text{mg glucose or xylose}}{\text{ml-HR}}$ / 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, magnefite 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.

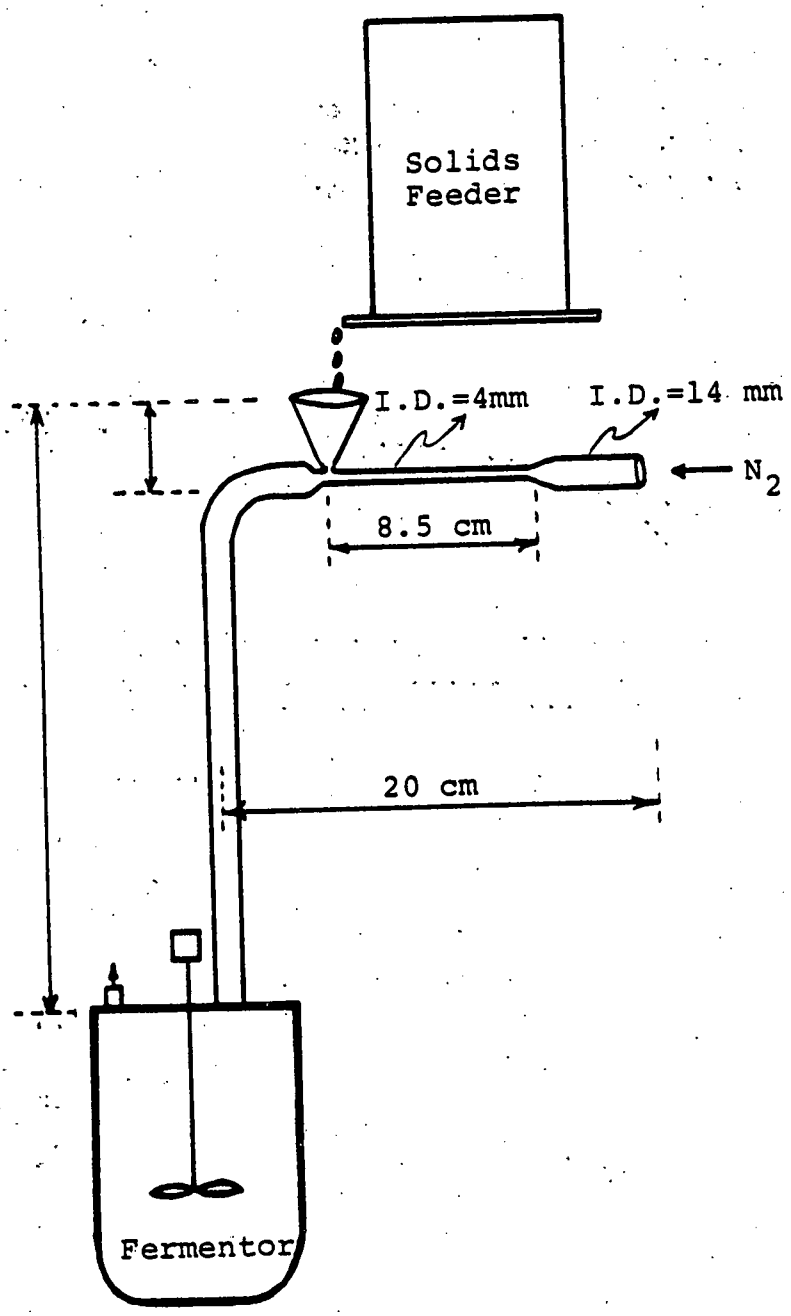


FIGURE I.G.1: DESIGN OF SOLIDS FEEDING APPARATUS FOR CONTINUOUS CULTURE SET-UP

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

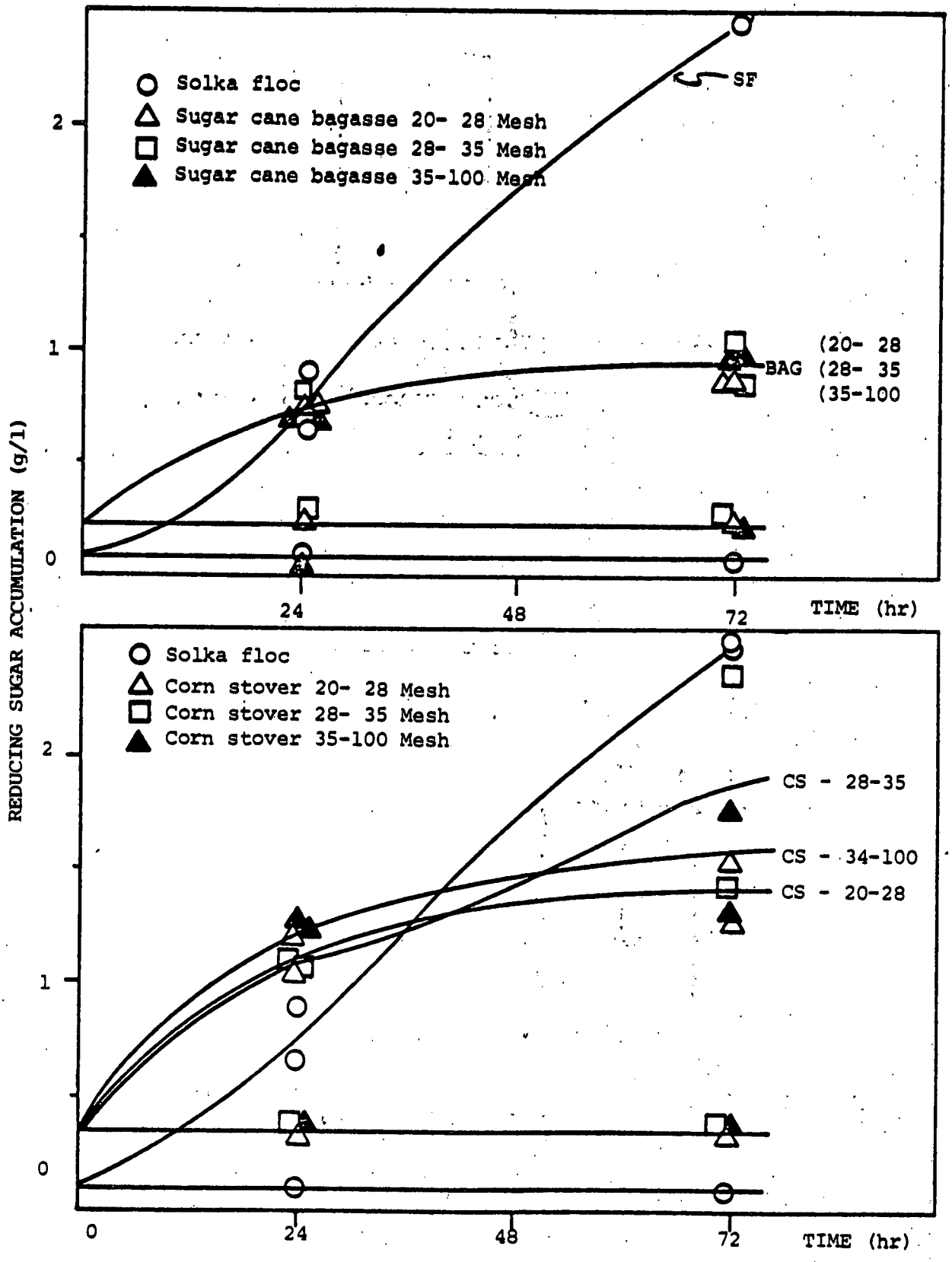
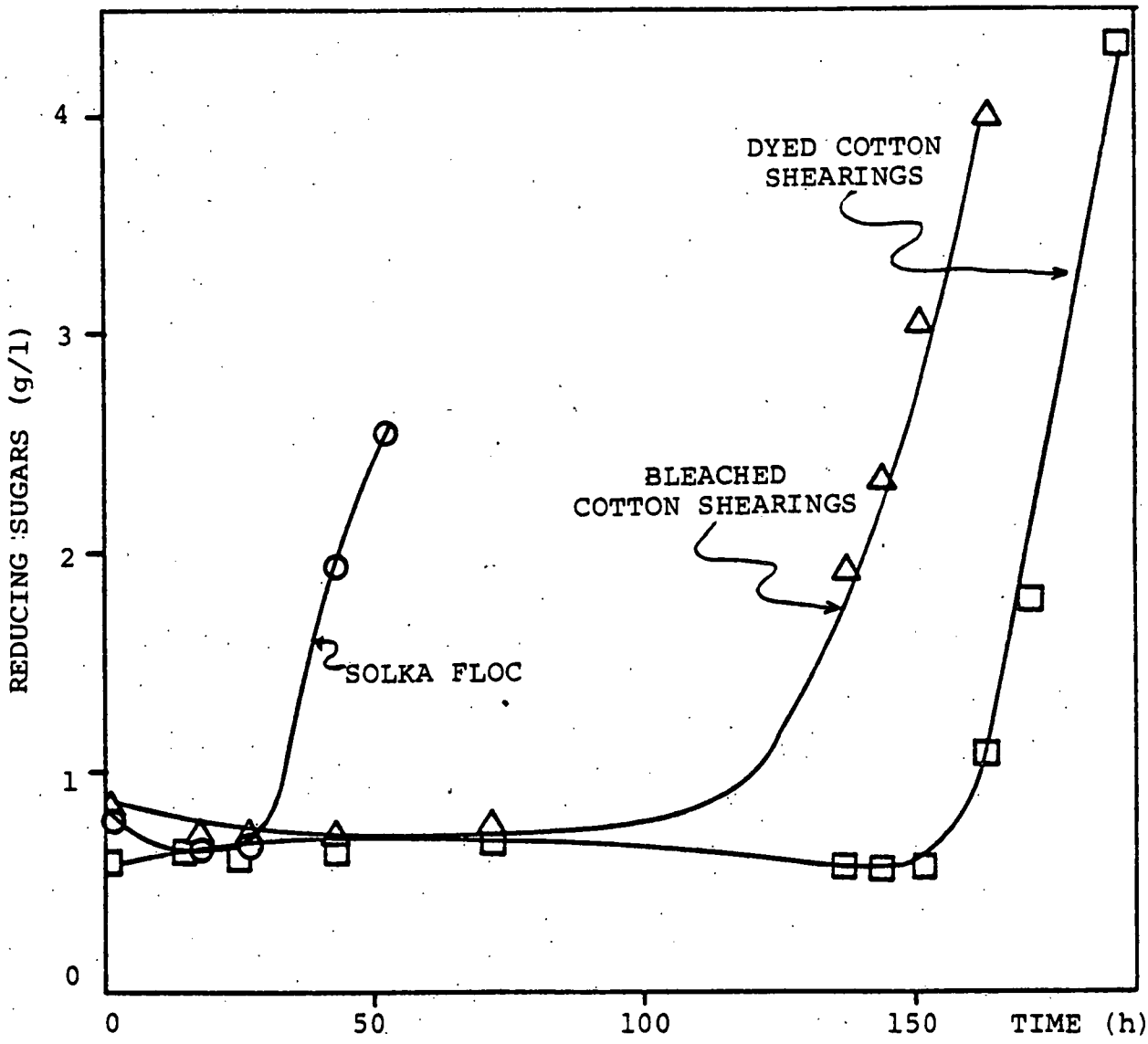


FIGURE I.G.3: REDUCING SUGAR ACCUMULATION FOR CULTURE OF C. THERMOCELLUM GROWN ON BLEACHED AND DYED COTTON SHEARINGS AND ON SOLKA FLOC



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 1 mM $MgSO_4$ (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 4°C. Then 0.25 ml 10% ammonium persulfate solution was added to the mixture followed by 0.5 ml of a 10% tetramethylethylene-

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 µl) were taken at each time interval and transferred to a tube containing 40 µl of 50% H_2SO_4

—o— 0 mM lactate

—●— 10 mM lactate

—Δ— 20 mM lactate

—▲— 50 mM lactate

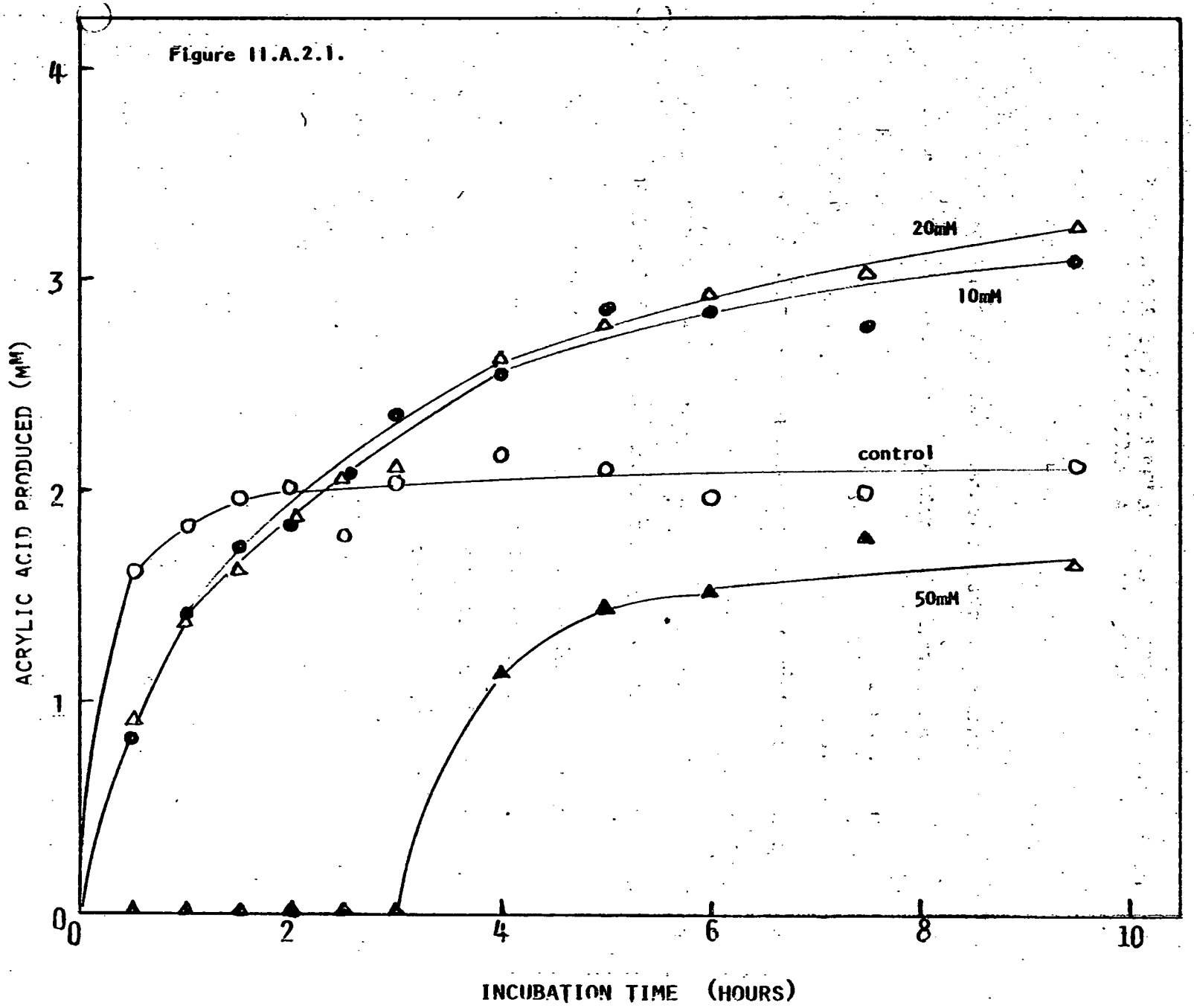


Figure II.A.2.1.
ACRYLIC ACID PRODUCED (MM)
INCUBATION TIME (HOURS)
20mM
10mM
control
50mM
0
1
2
3
4
0
2
4
6
8
10

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 µl) were taken at each time interval and transferred to a tube containing 40 µl of 50% H_2SO_4

—o— 0 mM lactate (control)

—Δ— 10 mM lactate

—●— 20 mM lactate

—□— 30 mM lactate

—▲— 50 mM lactate

—■— 100 mM lactate

Figure II.A.2.2.

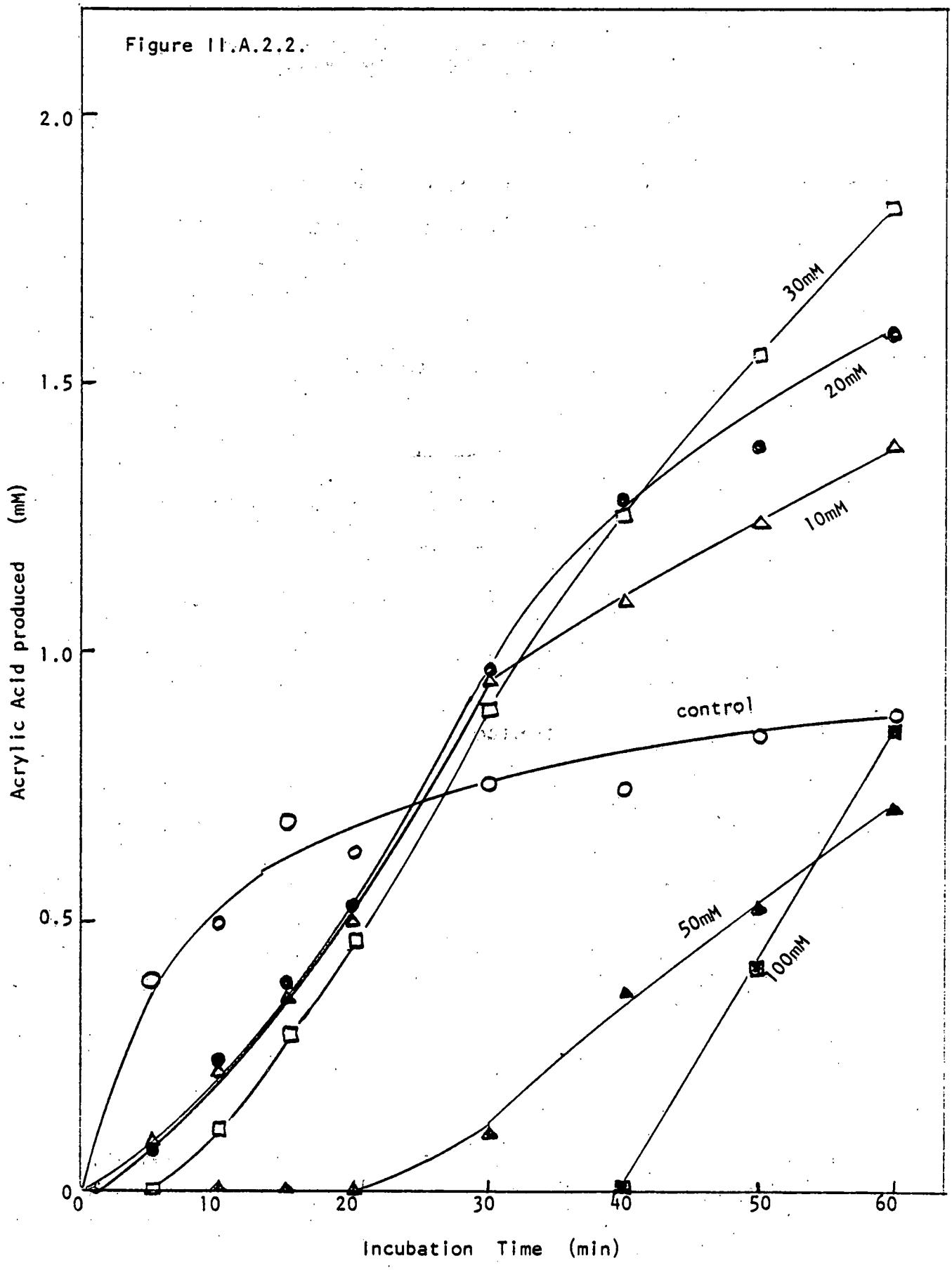


FIGURE II.A.2.3: EFFECT OF LACTATE CONTRATION ON THE PRODUCTION OF ACRYLATE AS A FUNCTION OF PROPIONATE CONCENTRATION

- Reaction mixture (total volume 200 µl) 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 µl H_2SO_4 (50%)

—○— 0 mM propionate
—■— 25 mM "
—□— 50 mM "
—▲— 75 mM "
—△— 100 mM "
—●— 150 mM "
—○— 200 mM "

Figure II.A.2.3

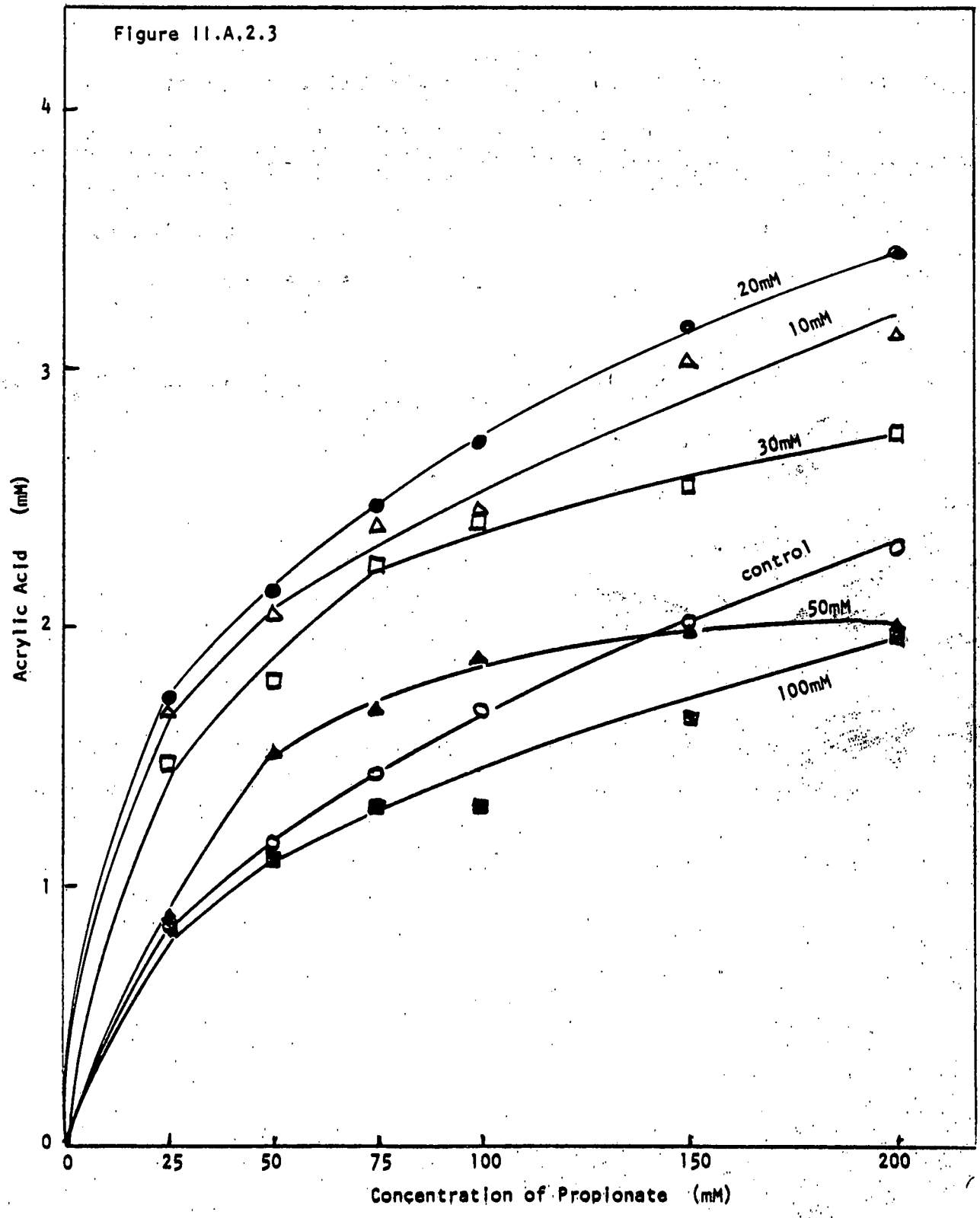


FIGURE II.A.2.4: EFFECT OF PROPIONATE CONCENTRATION ON THE PRODUCTION OF ACRYLATE AS A FUNCTION OF LACTATE CONCENTRATION

- Reaction mixture (total volume 200 µl) 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 µl H_2SO_4 (50%)

—○— 0 mM lactate

—△— 10 mM lactate

—●— 20 mM lactate

—□— 30 mM lactate

—▲— 50 mM lactate

—■— 100 mM lactate

Figure II.A.2.4

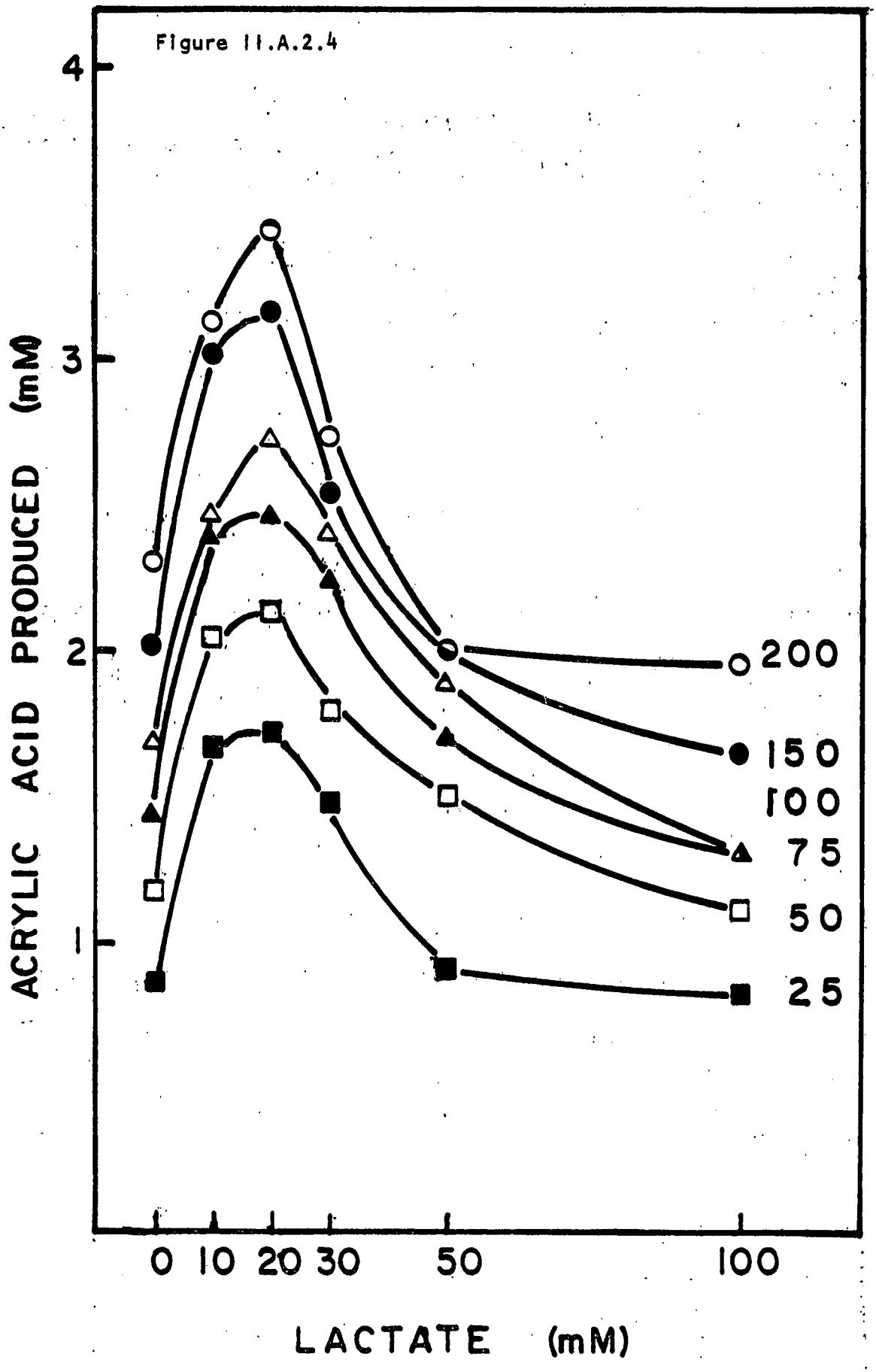


FIGURE II.A.2.5: EFFECT OF LACTATE ON THE PRODUCTION OF ACRYLATE FROM PROPIONATE

- 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 for 8 hours under air atmosphere with mechanical agitation
- Samples (200 µl) were drawn after 8 hour incubation and placed in a tube containing 40 µl of 50% H_2SO_4

Figure II.A.2.5

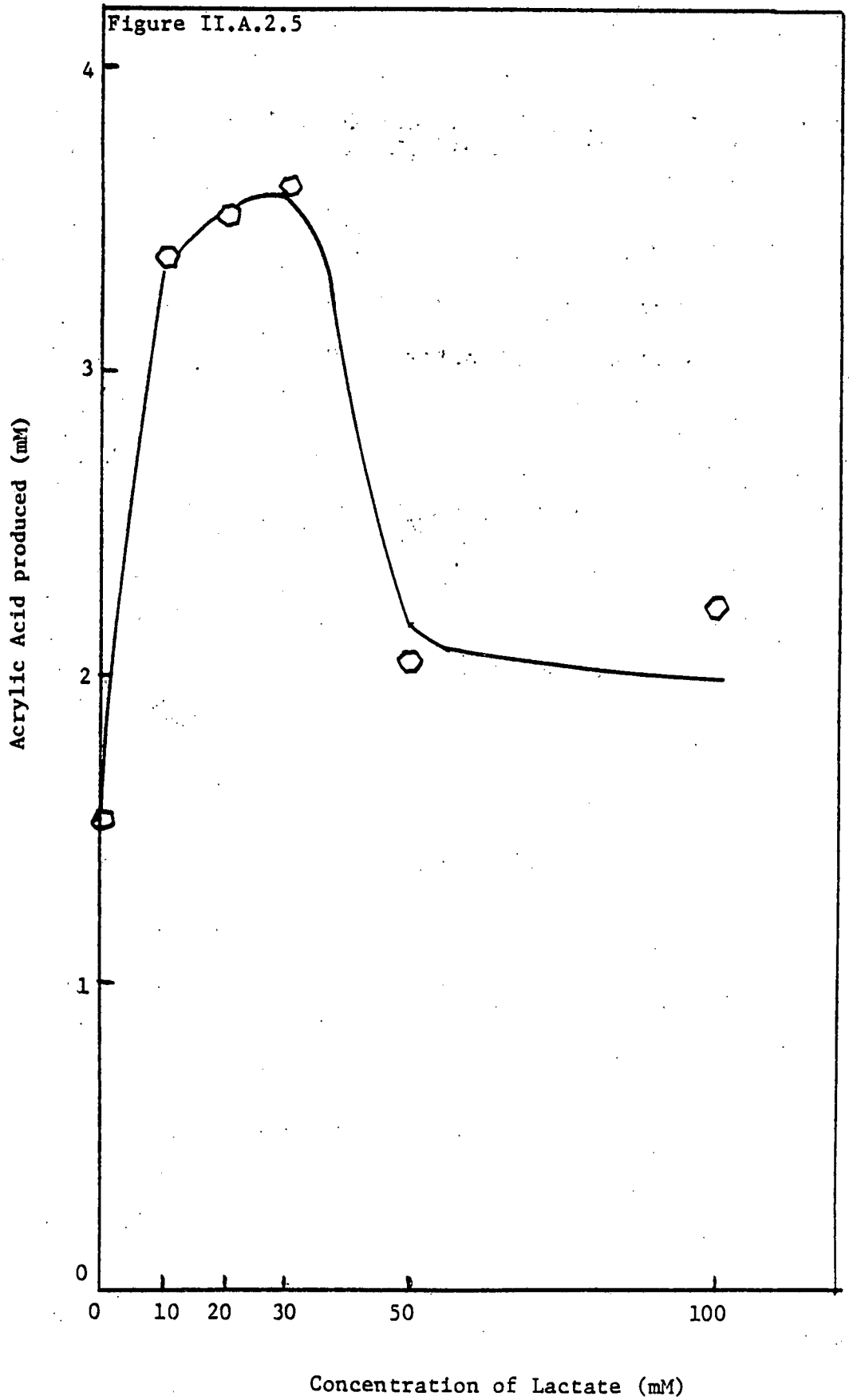


FIGURE II.A.2.6: EFFECT OF pH ON THE OXIDATION OF PROPIONATE TO ACRYLATE

- Reaction mixture (total volume 200 µl) contained:

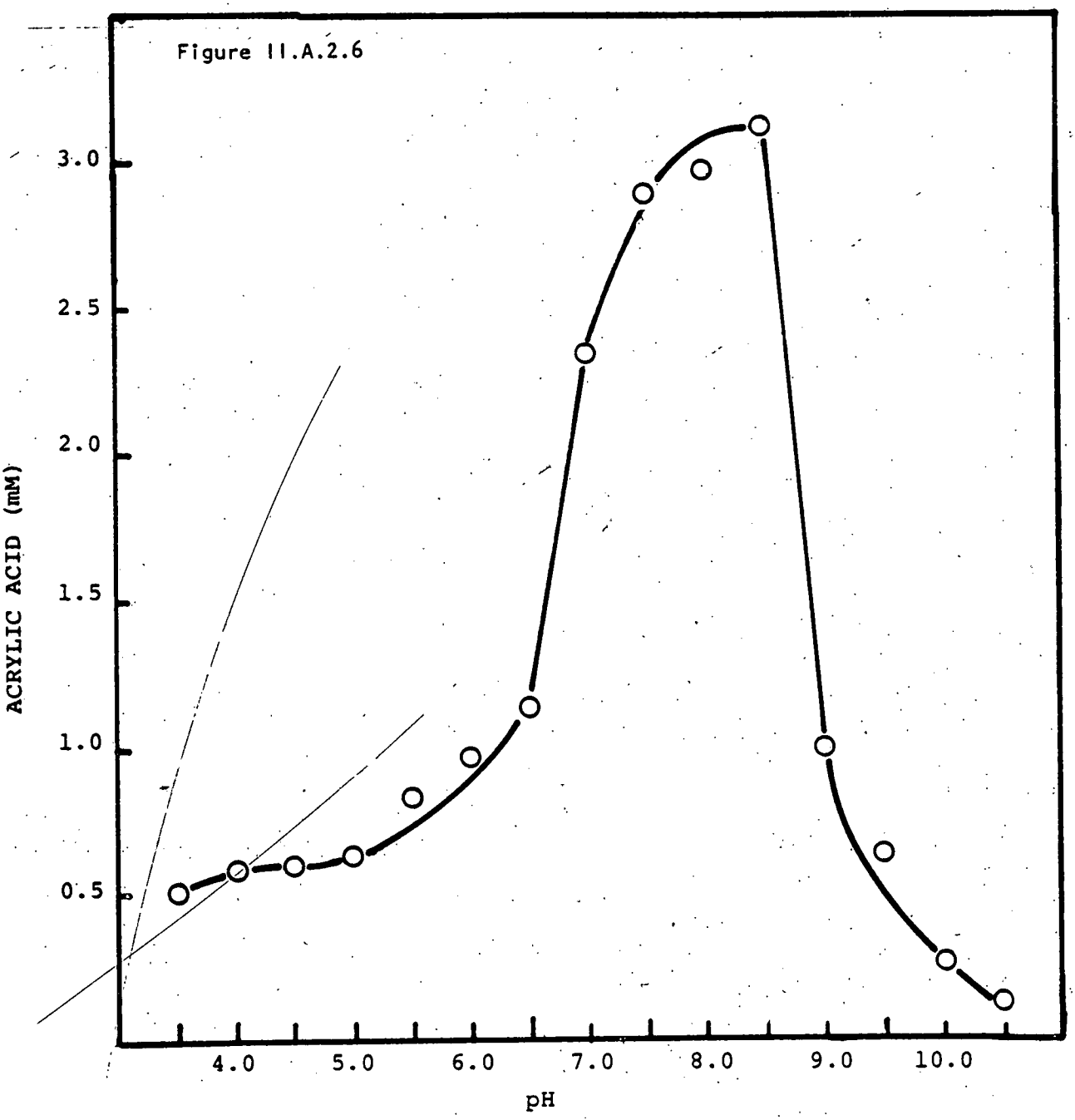
pH buffer*	50 mM
propionate	200 mM
lactate	25 mM

- Incubated at 37°C under air atmosphere with mechanical agitation for 8 hours
- Reactions were stopped with the addition of 40 µl 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



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 N_2 atmosphere with mechanical agitation
- Samples (200 µl) were taken at each time interval and reaction was stopped with 40 µl of 50% H_2SO_4

—○— 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.2.7

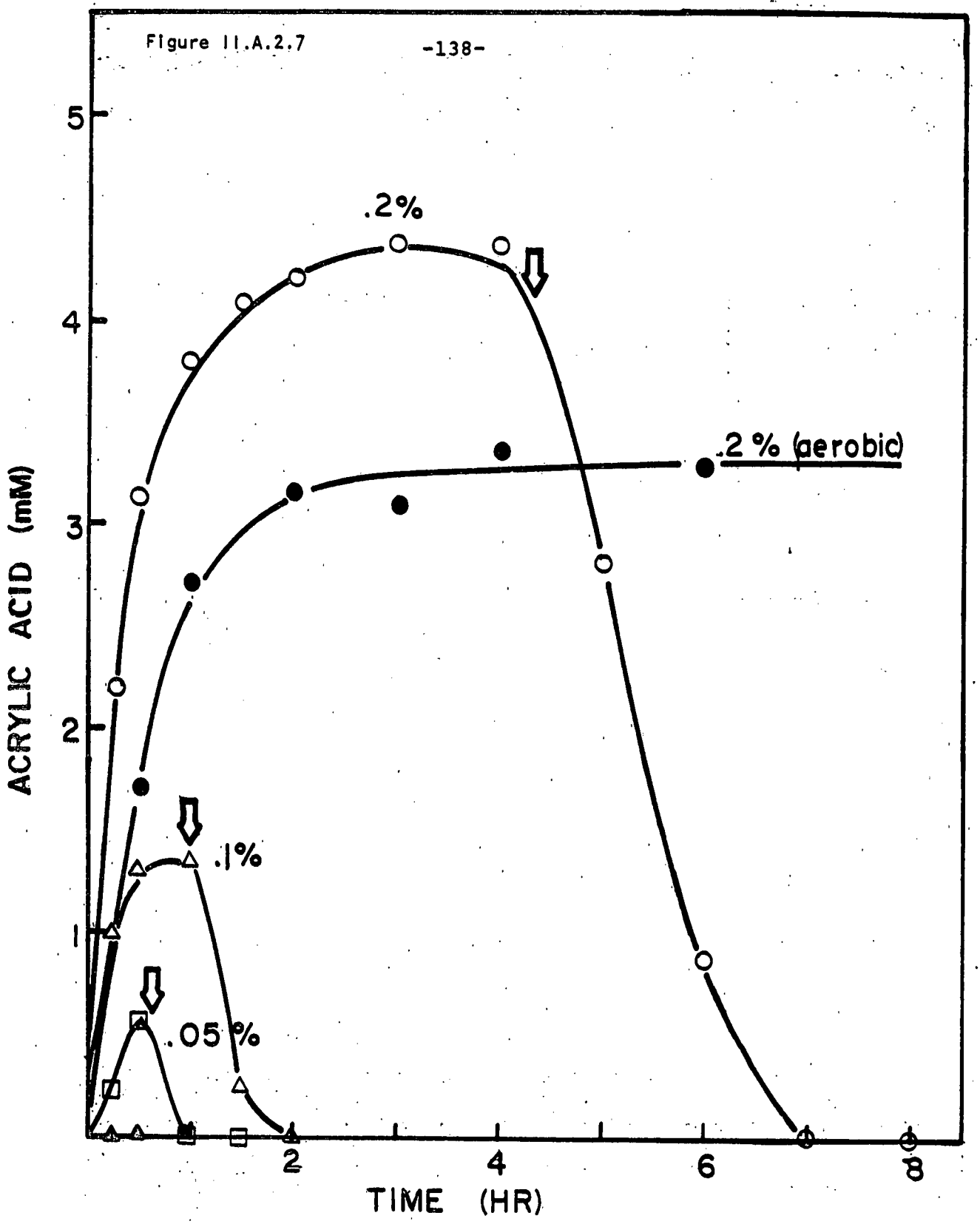


FIGURE II.A.2.8: EFFECT OF METHYLENE BLUE ON THE OXIDATION OF PROPIONATE TO ACRYLATE UNDER <u>AEROBIC</u> CONDITION

- 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% H_2SO_4

—○— 0 % methylene blue (control)

—□— 0.05 % methylene blue

—△— 0.1 % methylene blue

—●— 0.2 % methylene blue

Figure II.A.2.8

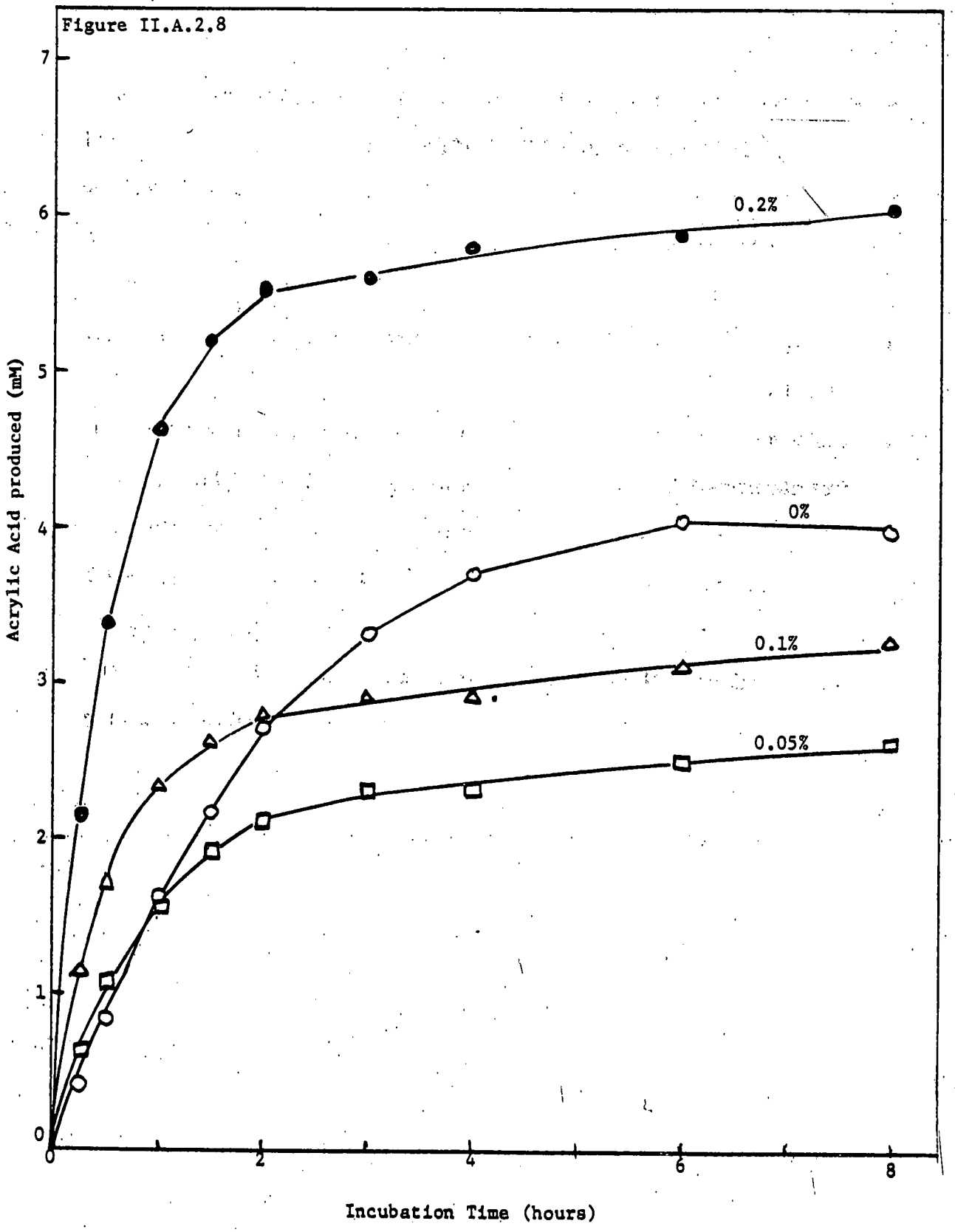


FIGURE II.A.2.9: PRODUCTION OF ACRYLIC ACID FROM PROPIONATE IN THE PRESENCE OF METHYLENE BLUE AND LACTATE

- 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 µl) were taken at each time interval and transferred to a tube containing 40 µl 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.2.9

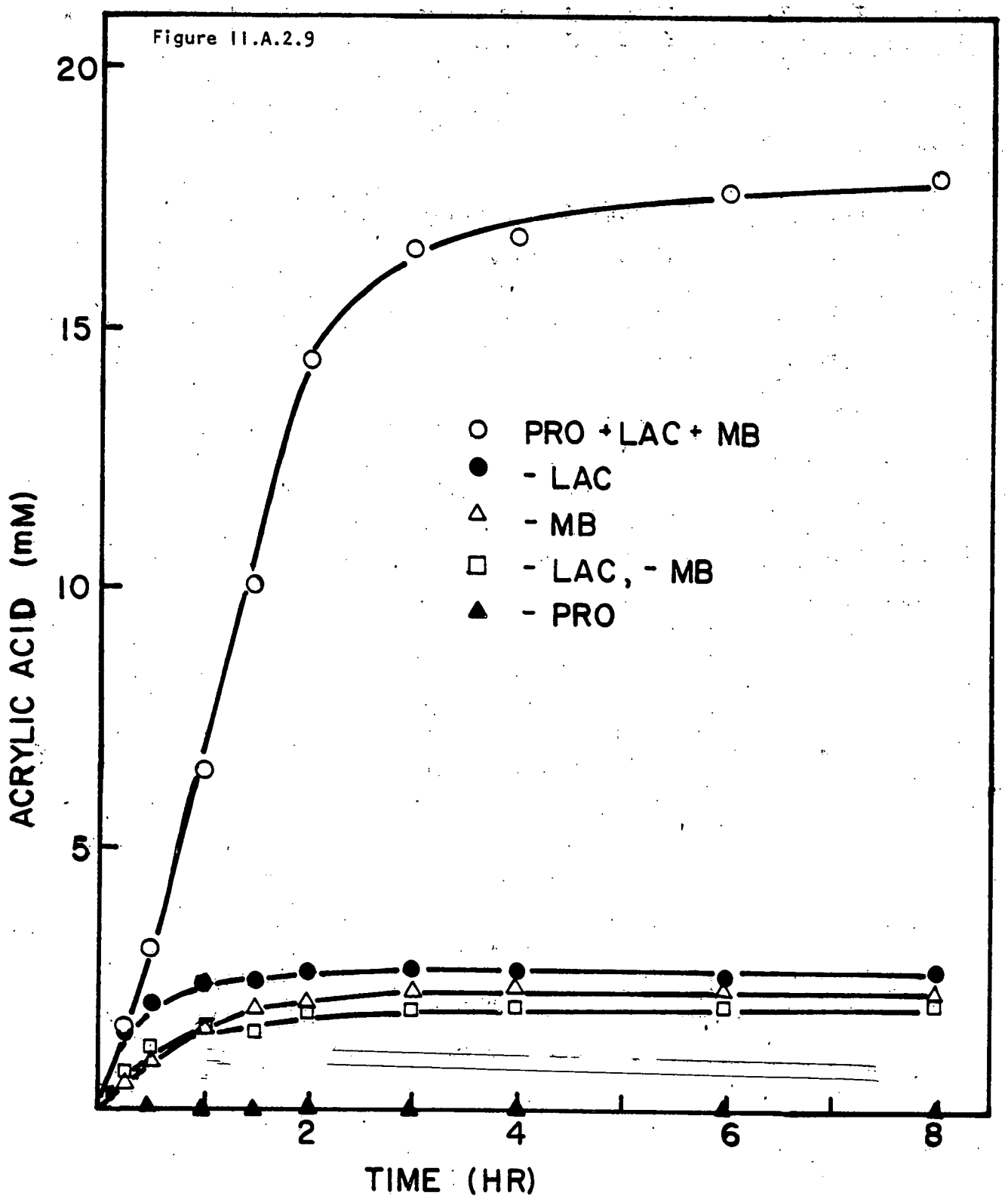


FIGURE II.A.2.10: EFFECT OF AIR (OR OXYGEN) ON THE PRODUCTION OF ACRYLATE FROM PROPIONATE

- 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

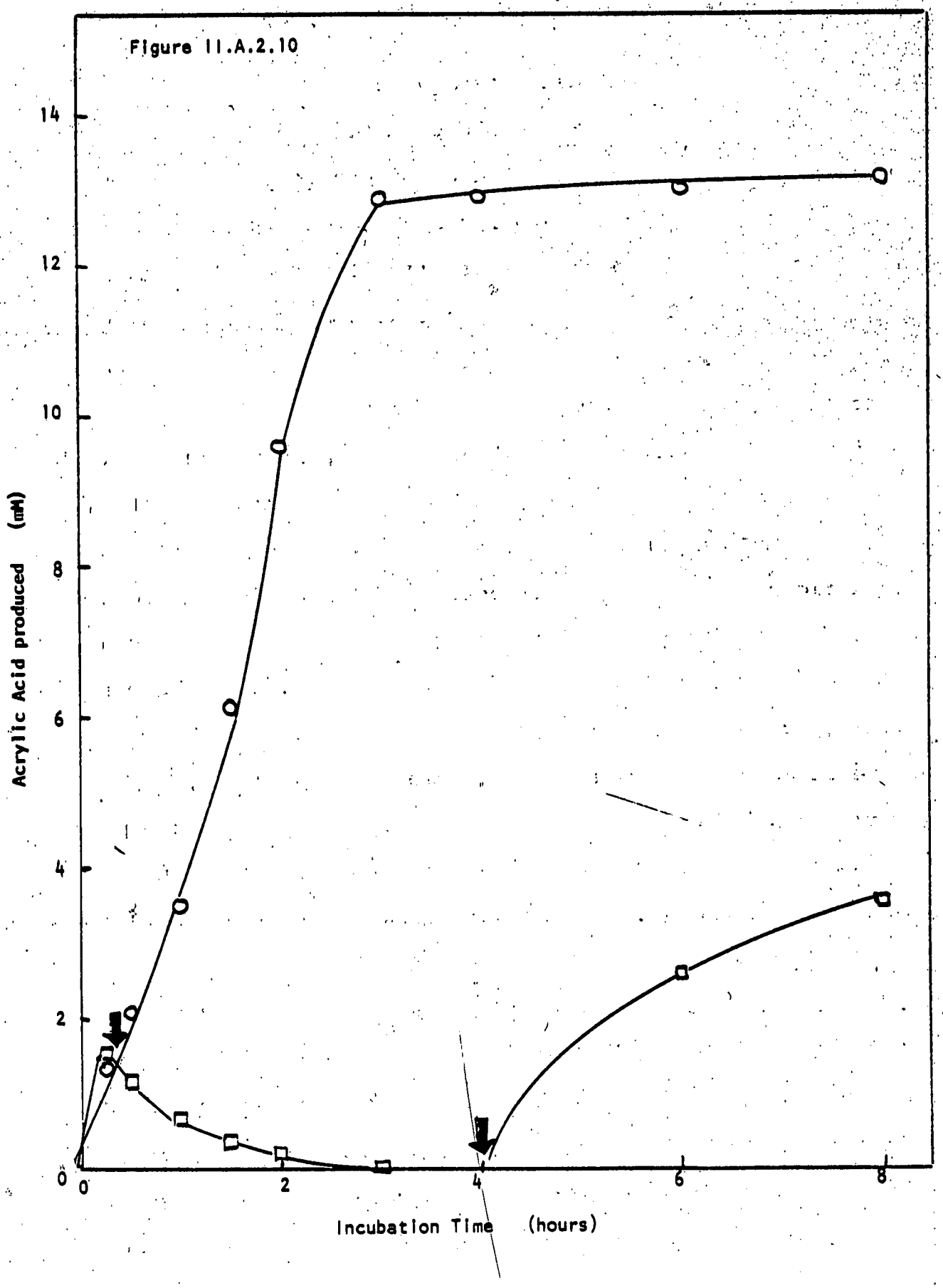
—o— under aerobic condition

—□— 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 II.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 experiement. 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° ofr 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 H_2O 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 KH_2PO_4/K_2HPO_4 buffer was added to the tubes and they were innoculated 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 detremine 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

	Final conc.	Reason
Na_2HPO_4	7 g/l	Buffer
KH_2PO_4	3 g/l	Buffer
NH_4Cl	1.0 g/l	N for amino acid biosynthesis
$MgSO_4 \cdot 7H_2O$	0.25 g/l	Mineral
$FeSO_4 \cdot 7H_2O$	.01 g/l	Mineral
$CaSO_4 \cdot 2H_2O$	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:

	(20 x)
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 ω (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 glyoxylate cycle,

phospho (enol) pyruvate carboxylase or via α-hydrony glutarate. 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 bioconversion.

b. Materials and Methods

The analytical procedures and conditions for growth, mutagenisis, 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 preceeded 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 ω 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 succrinate, 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 matabolism, 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, propiolic 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 mutagenisis 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 mutagenisis.

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, isobutarate, 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
Cystxeine	0.1
Thioglycollate	0.5
KH_2PO_4	3.0
K_2HPO_4	4.4
$(NH_4)_2SO_4$	1.3
$MgCl_2$	1.0
$CaCl_2$	0.15
$FeSO_4 \cdot 7H_2O$	1.25 mg/l
$(NH_4)_3$ 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	
$MgSO_4 \cdot 7H_2O$	11.5
$FeSO_4 \cdot 7H_2O$	0.68
$MnSO_4 \cdot 2H_2O$	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 transfered 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"

Sample	Incubation Conditions	Growth[1] (K.U)	pH	Lactic Acid (gm/l)	Catalase[2] (Units/ml)
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°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

Sample No	Age (Hrs)	Cell Growth (gm/l)	Lactic Acid (gm/l)	Acetic Acid (gm/l)	Ethanol (gm/l)	Res. Xylose (gm/l)
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"

Sample No	Age (Hrs)	Glucose (gm/l)	Red. Sugars (gm/l)	Cellobiose (gm/l)	Lactic Acid (gm/l)
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 cellobioose as carbon sources because it was assumed that C. thermocellum can grow on cellogiose 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 ecperiment 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"

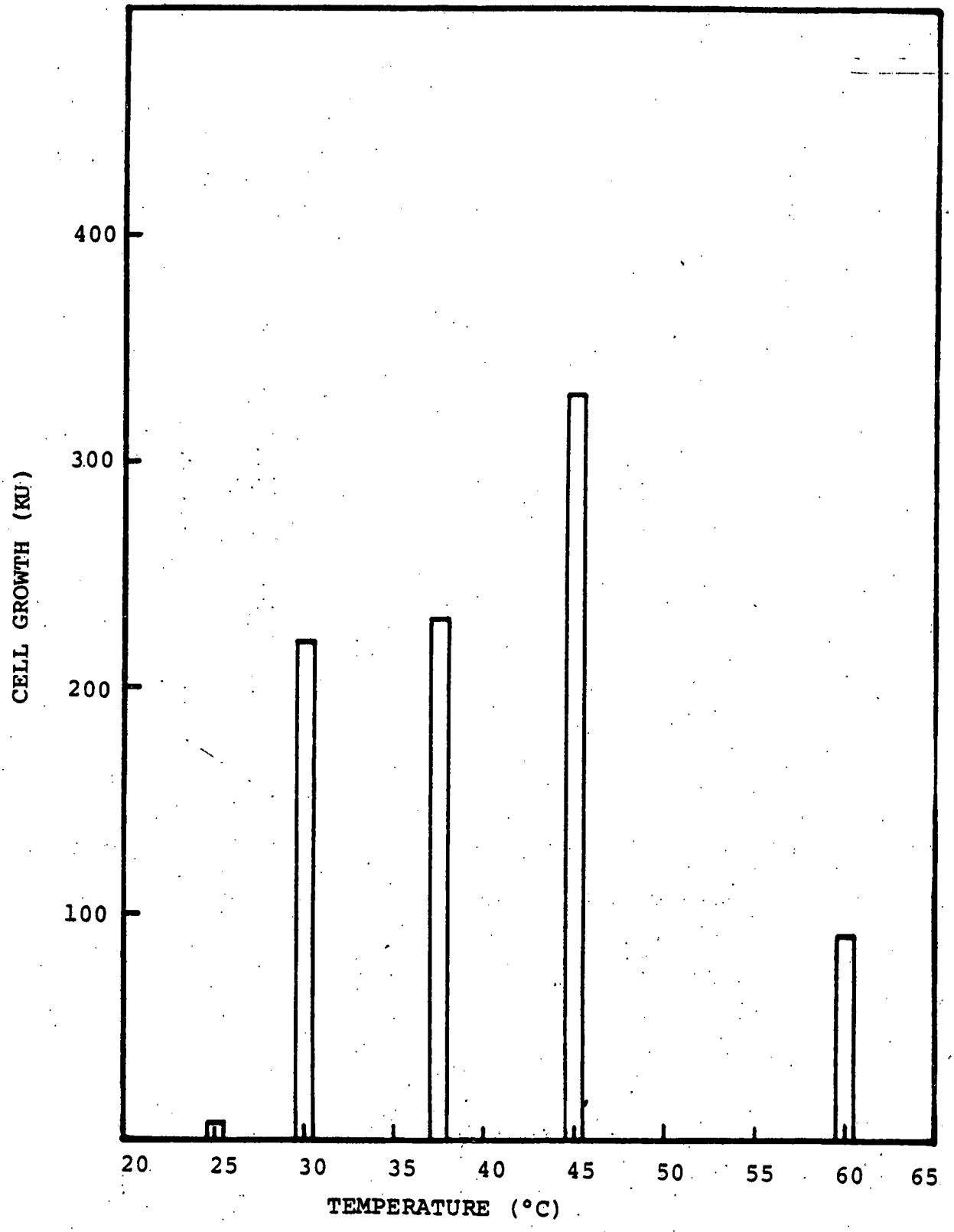


FIGURE II.A.4.2: SCHEMATIC PROCEDURE FOR ADAPTATION OF "T. BACTERIUM" TO GROW ON XYLOSE

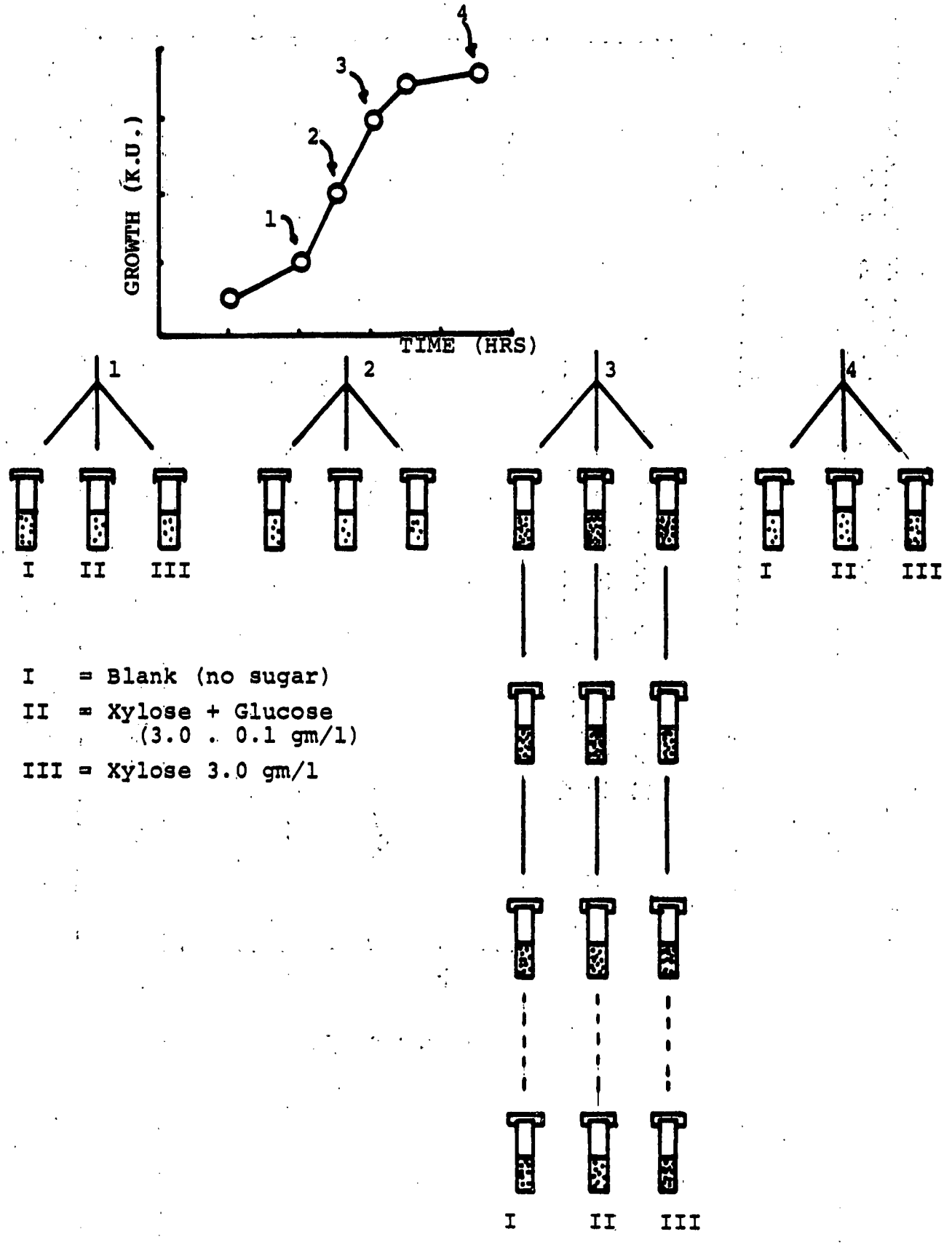


FIGURE II.A.4.3: ADAPTATION OF "T. BACTERIUM" TO GROWTH ON XYLOSE AT 60°C. SERIAL TRANSFERS WERE DONE EVERY 12 HRS.

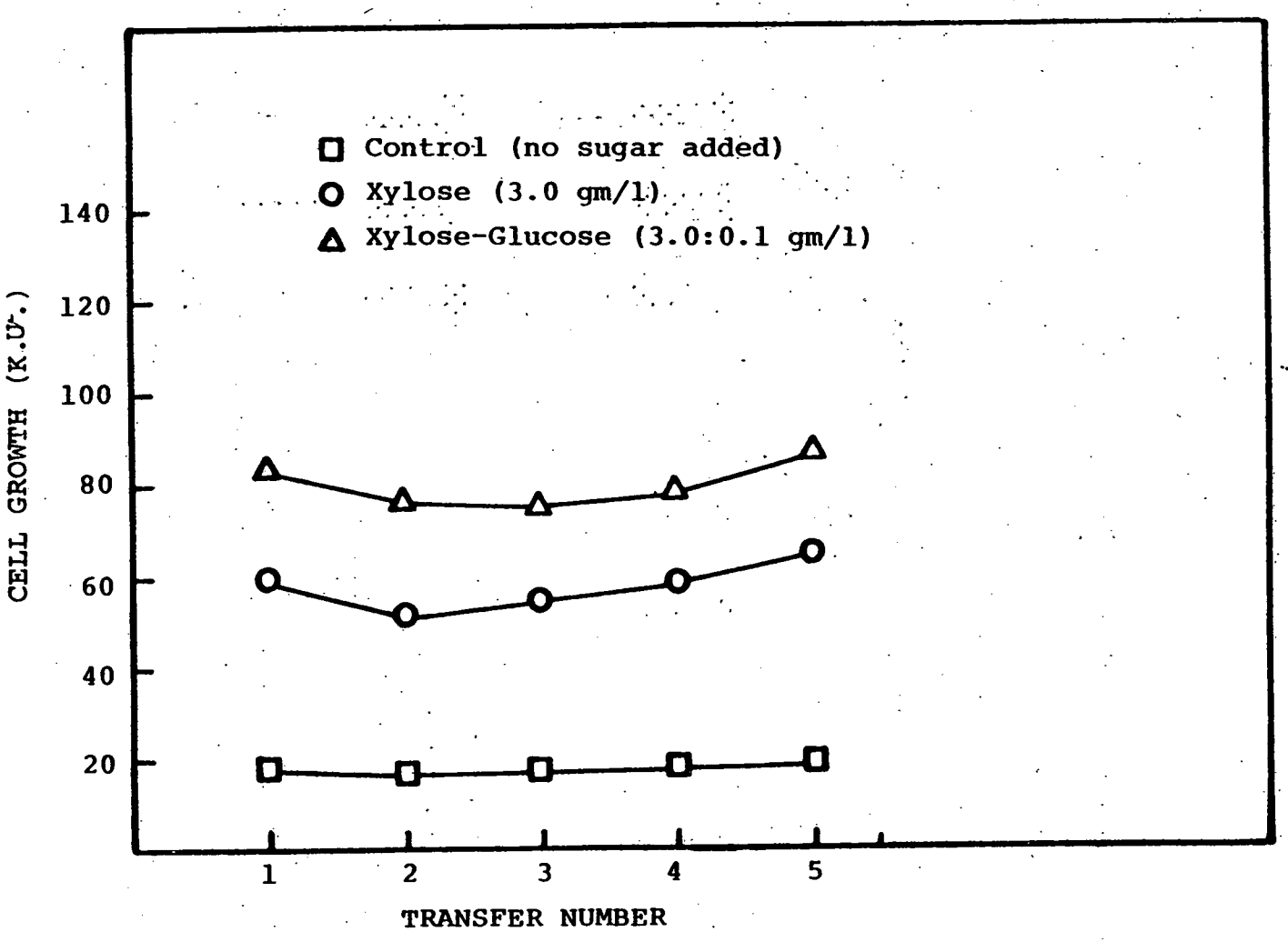


FIGURE II.A.4.4: ADAPTATION OF "T. BACTERIUM" TAKEN FROM LOGARITMIC PHASE OF GROWTH ON CELLOBIOSE

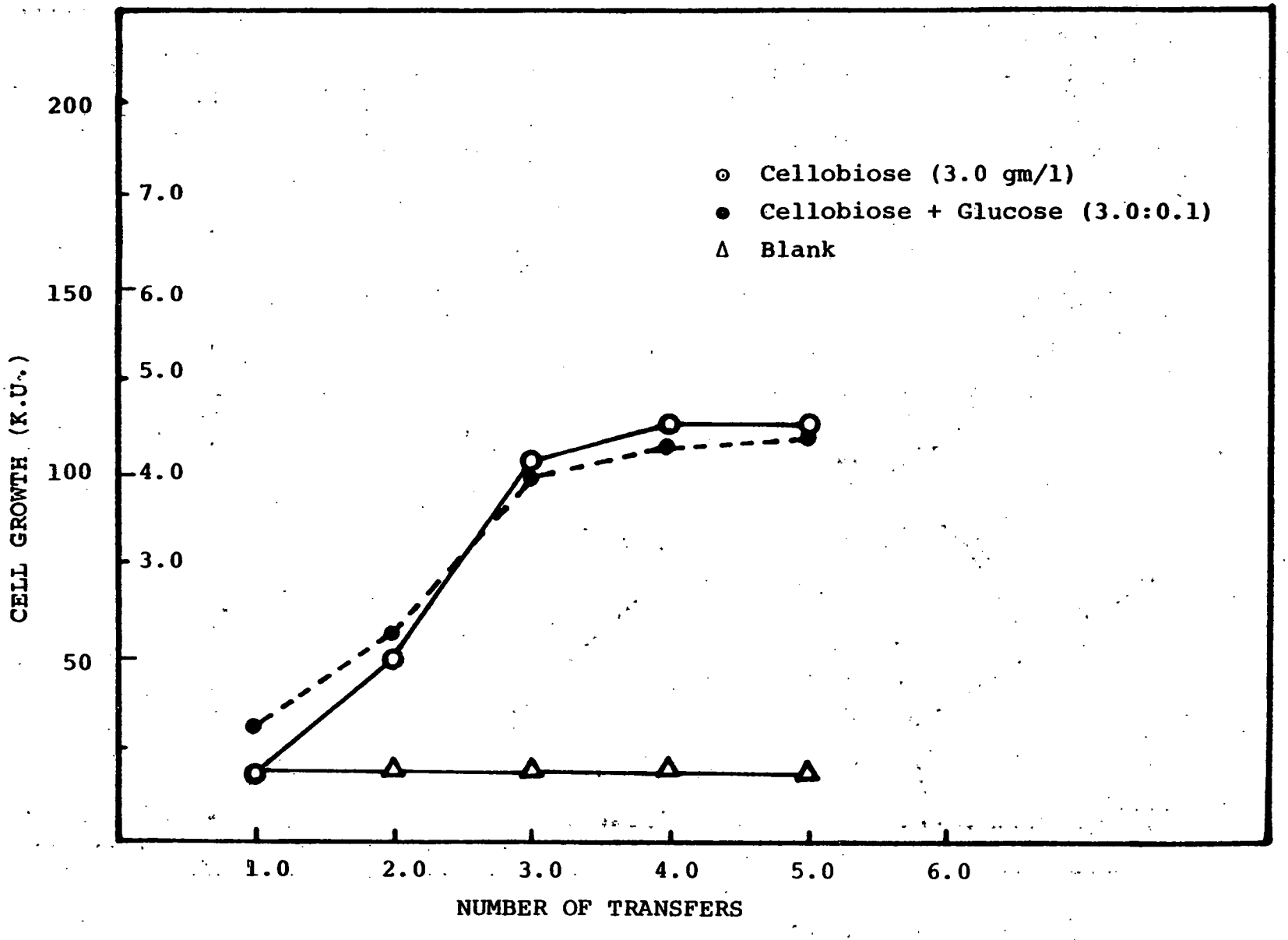


FIGURE II.A.4.5: MIXED SUBSTRATE UTILIZATION BY "T. BACTERIUM" (GLUCOSE-CELLOBIOSE) IN BATCH CULTURE AT 60°C

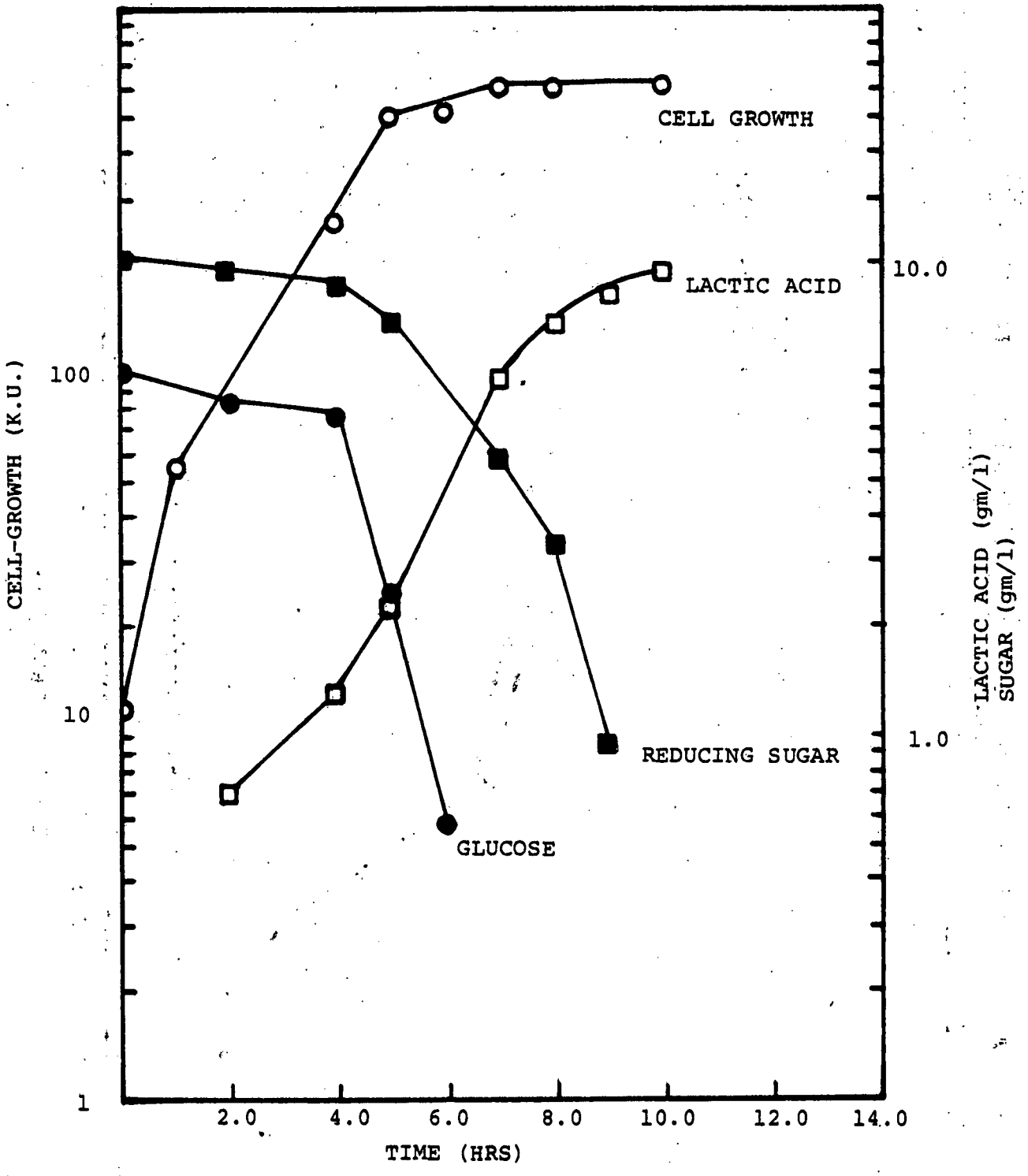
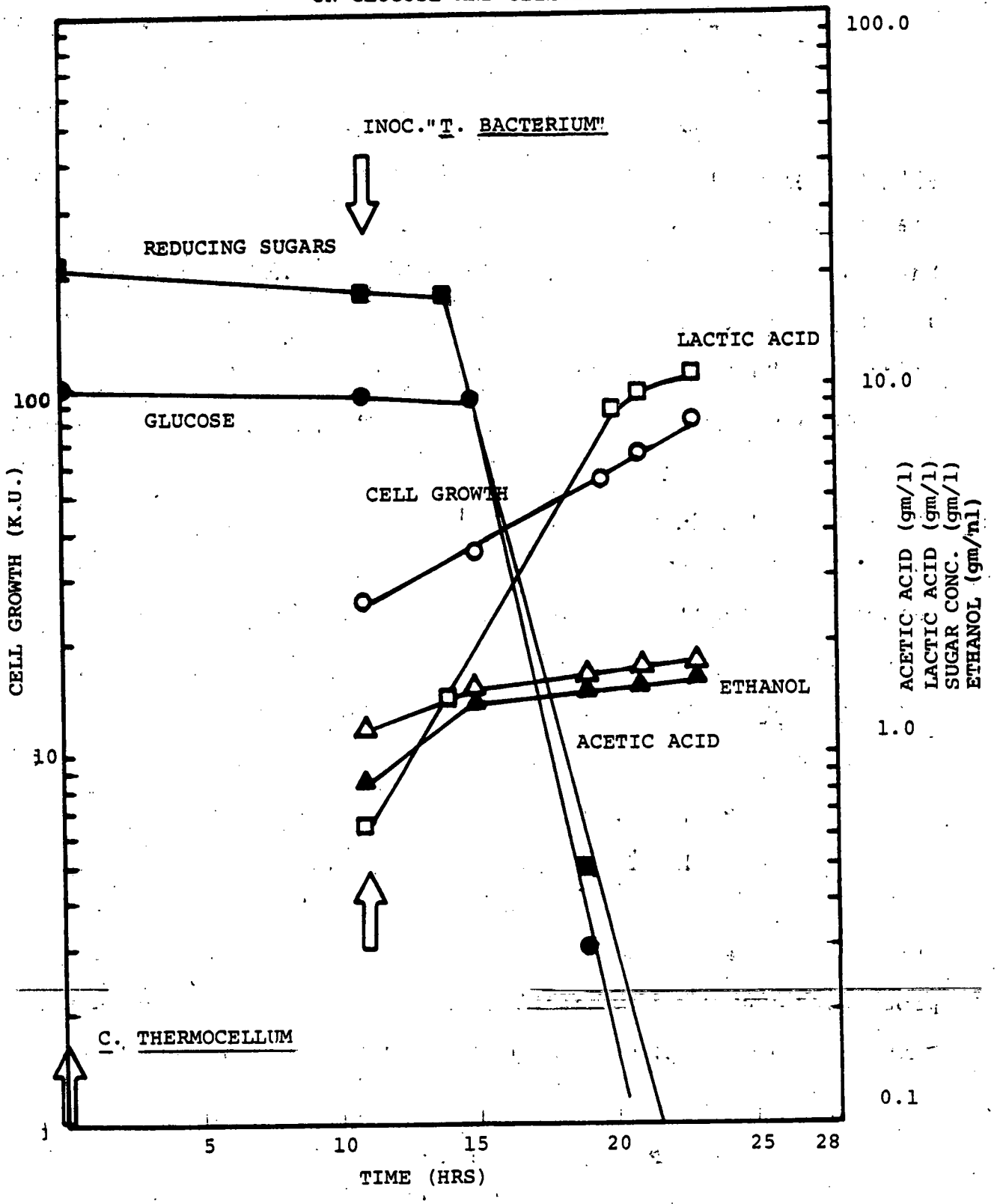


FIGURE II.A.4.6: MIXED CULTURE OF C. THERMOCELLUM AND "T. BACTERIUM" ON GLUCOSE AND CELLOBIOSE IN BATCH CULTURE



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:

	g/l
KH_2PO_4	0.75
K_2HPO_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:

	g/l
KH_2PO_4	0.75
K_2HPO_4	0.75
$MgSO_4$	0.20
$MgSO_4.7H_2O$	0.01
$FeSO_4.7H_2O$	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

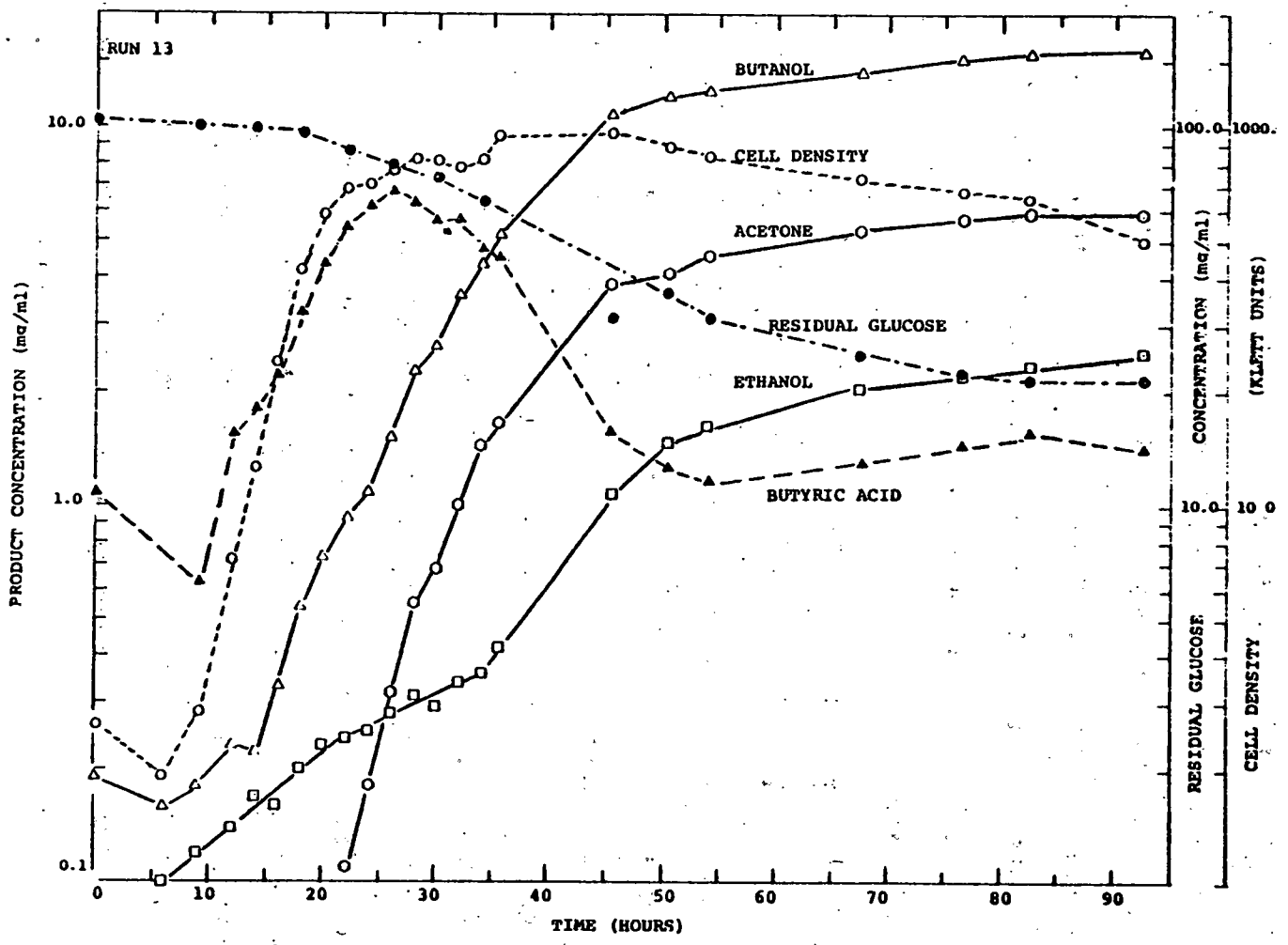


FIGURE II.B.1: pH CONTROLLED FERMENTATION OF C. ACETOBUTYLICUM ATCC 824 GROWING IN SOLUBLE MEDIUM WITH GLUCOSE AT 30°C (RUN NO. 13)

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

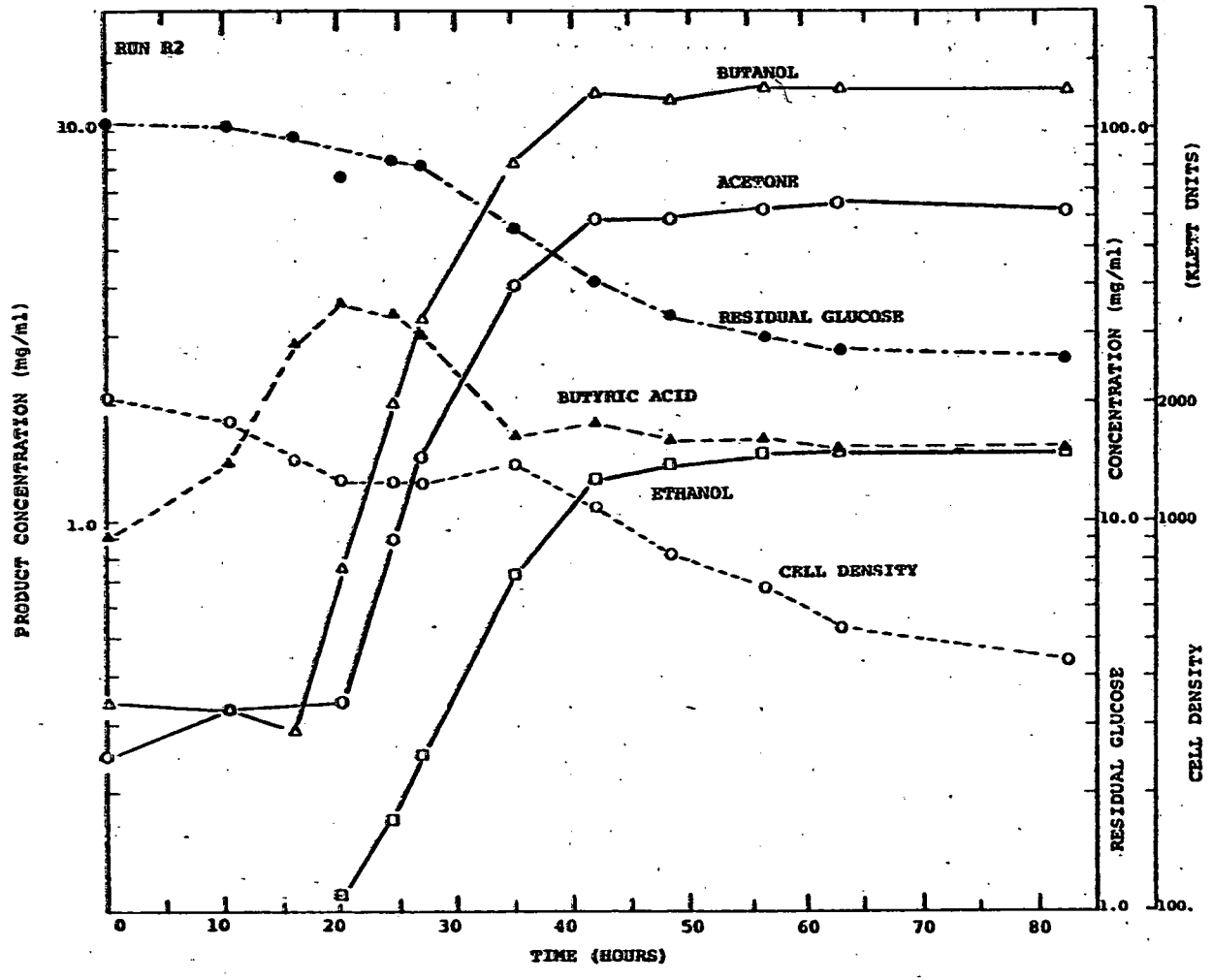


FIGURE II.B.2: RESTING CELL EXPERIMENT OF C. ACETOBUTYLICUM ATCC 824 AT 37°C (CELLS WERE OBTAINED FROM FERMENTATION RUN NO. 12)

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.

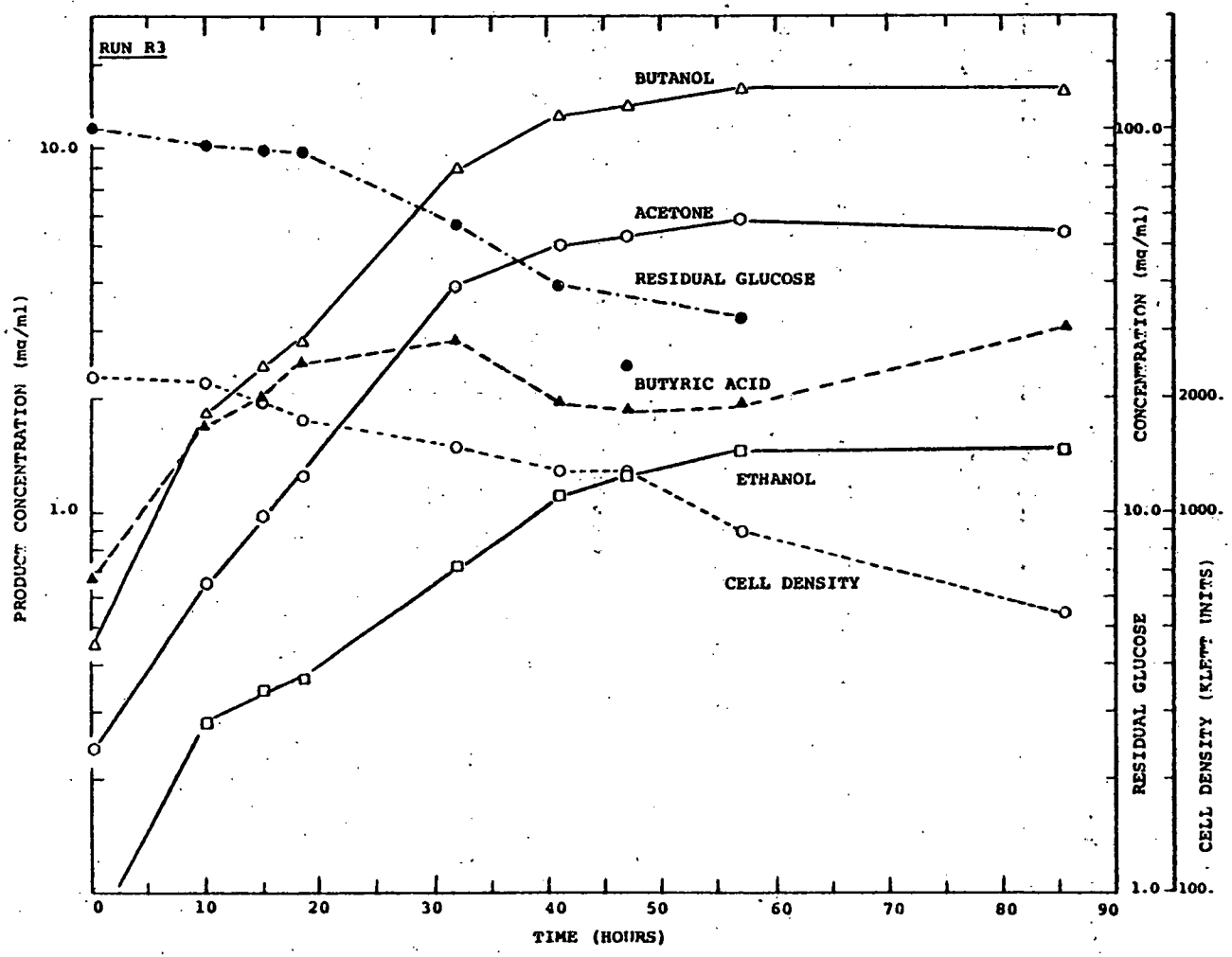


FIGURE II.B.3: RESTING CELL EXPERIMENT OF C. ACETOBUTYLICUM ATCC 824 AT 30°C (CELLS WERE OBTAINED FROM FERMENTATION RUN NO. 13)

FIGURE II.B.4: THE DECAY OF THE CELL DENSITIES IN THE RESTING CELL EXPERIMENTS (RUN NO. R2 AND RUN NO. R3)

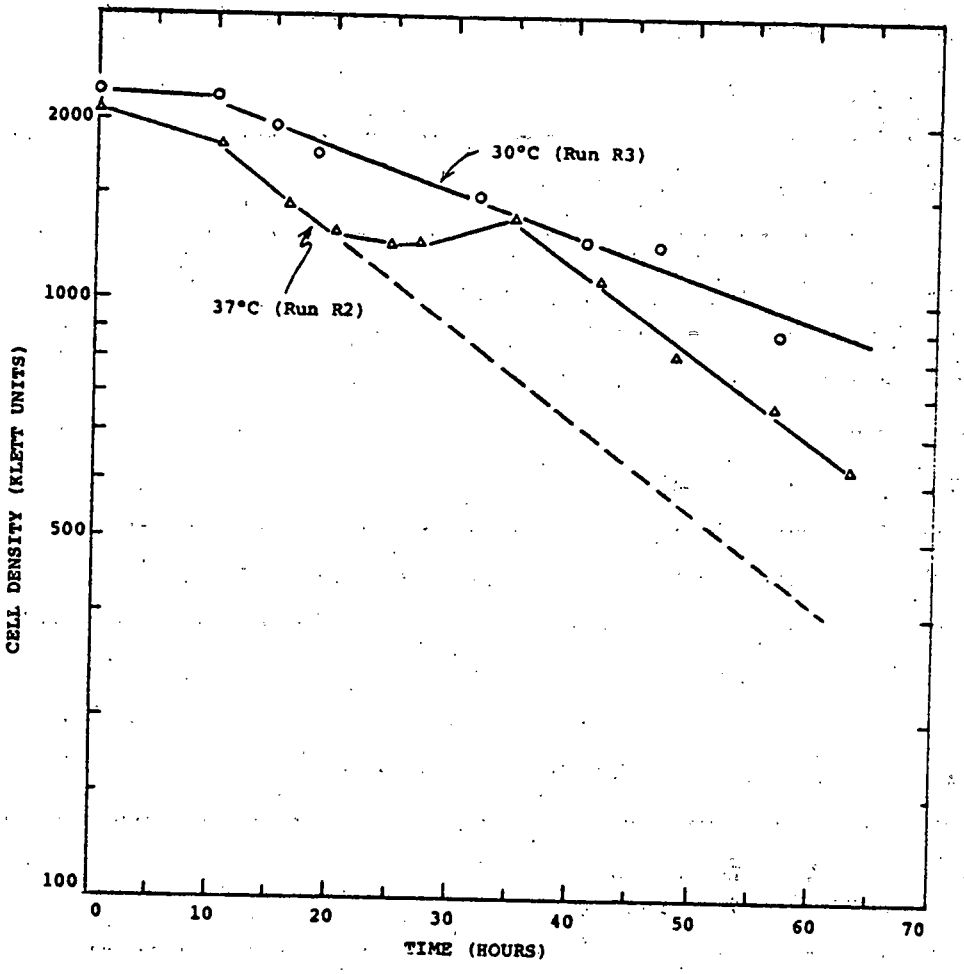


TABLE II.B.2: SUMMARY OF RESULTS USING RESTING CELLS OF C. ACETOBUTYLICUM

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 CO_2 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.

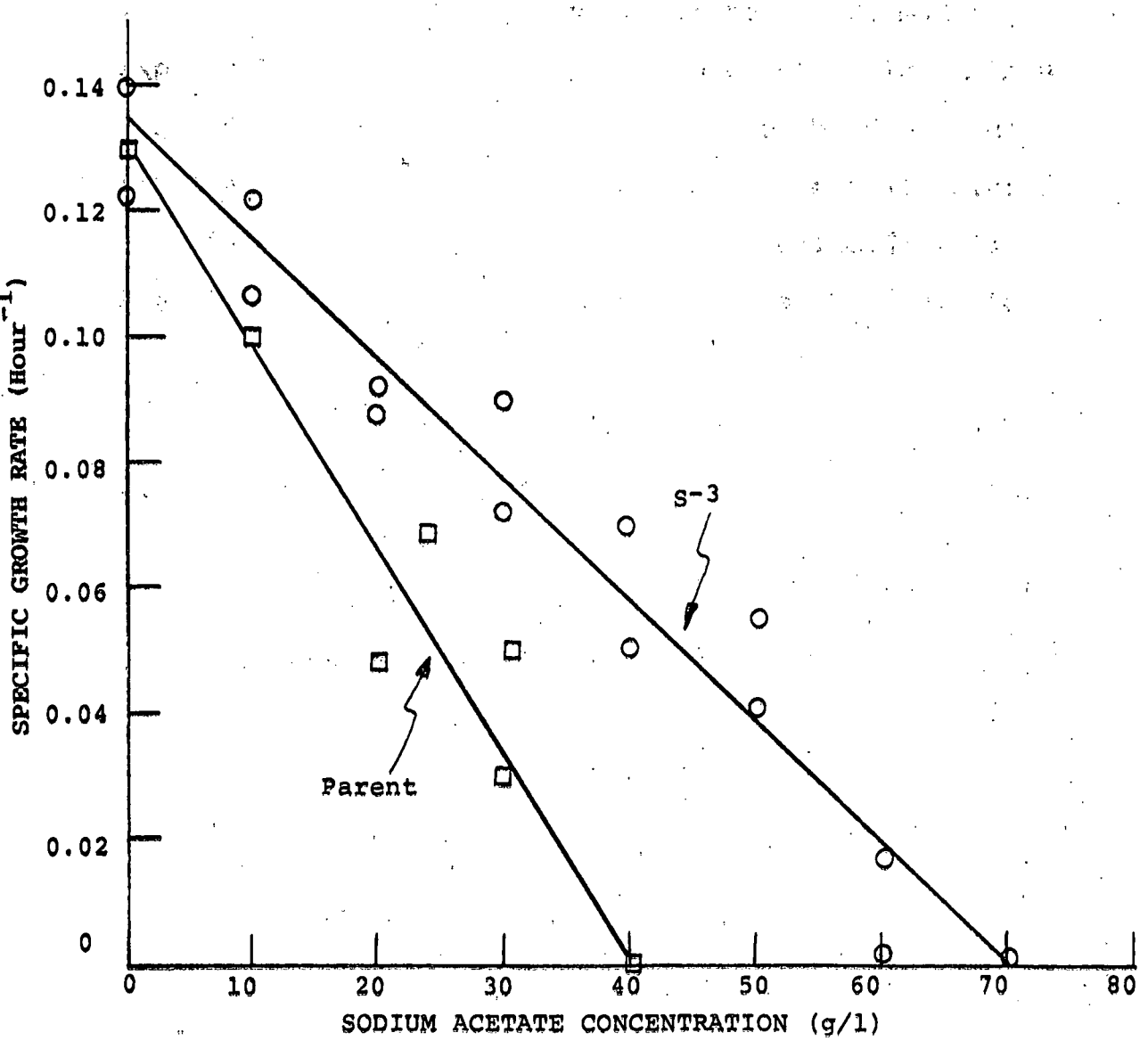
3. Results and Discussions

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)

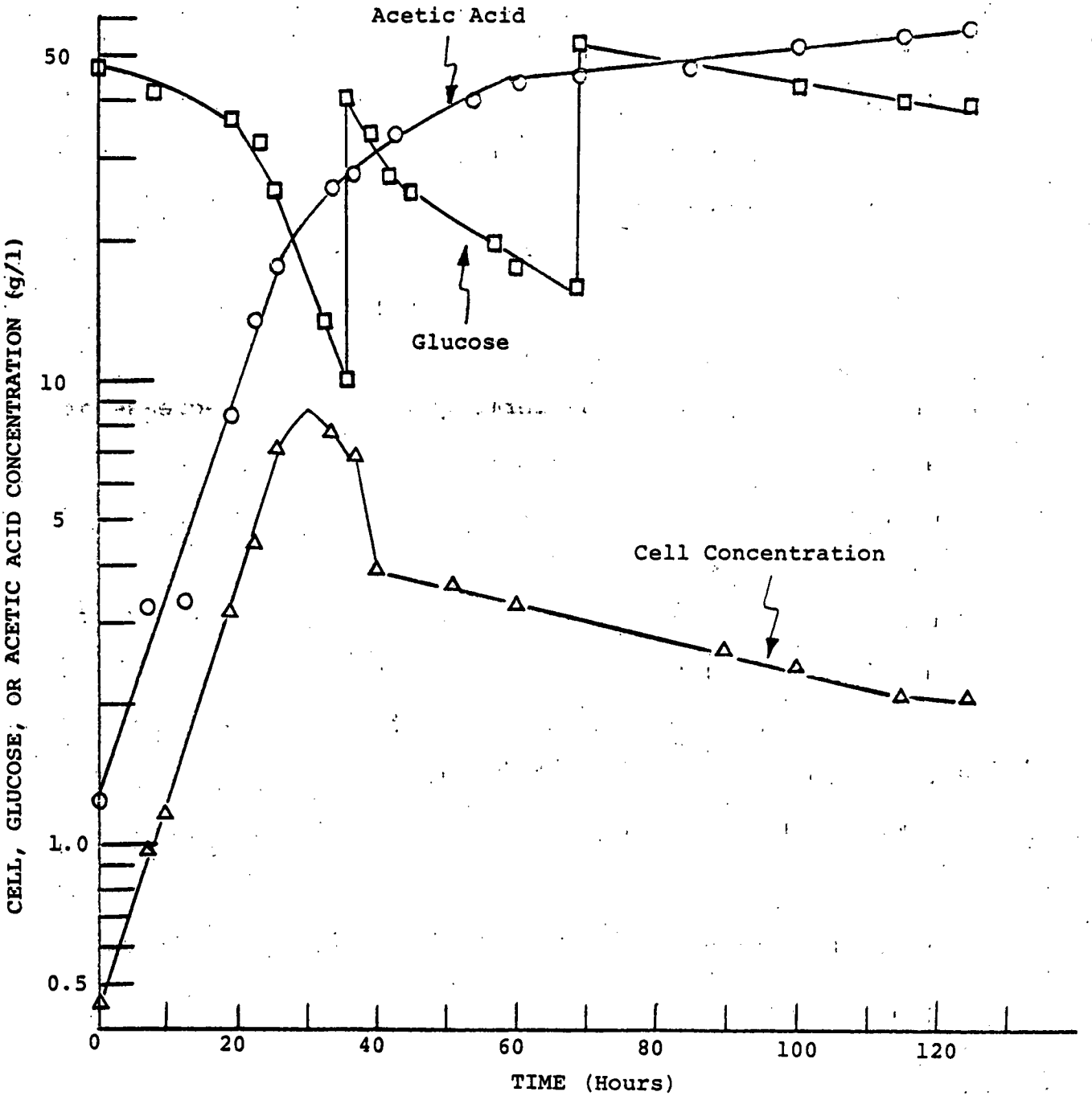


FIGURE II.C.2: FERMENTATION PROFILE OF CLOSTRIDIUM THERMOACETICUM S-3 (pH = 7.0)

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 theobservation 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 (α) 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

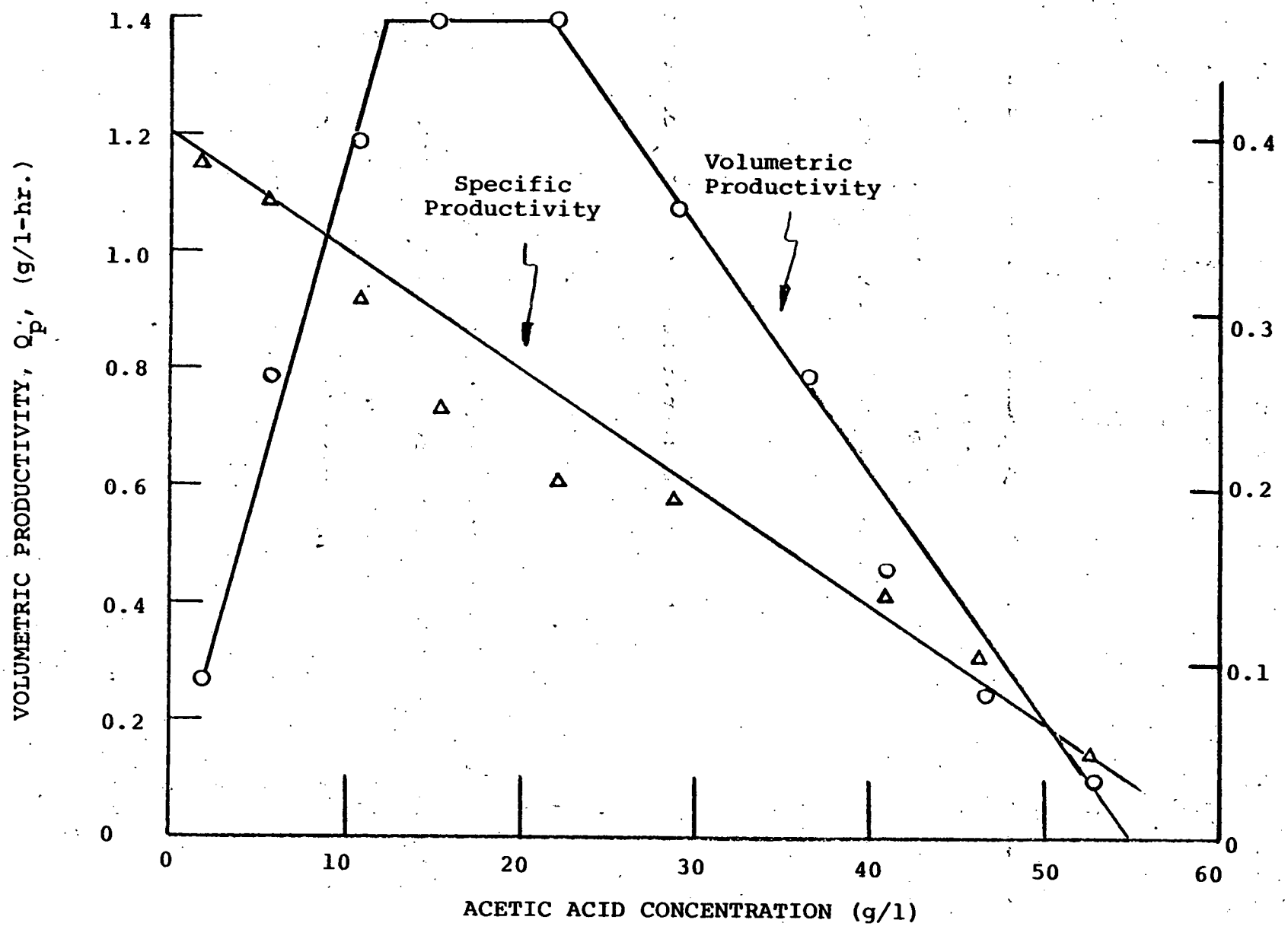
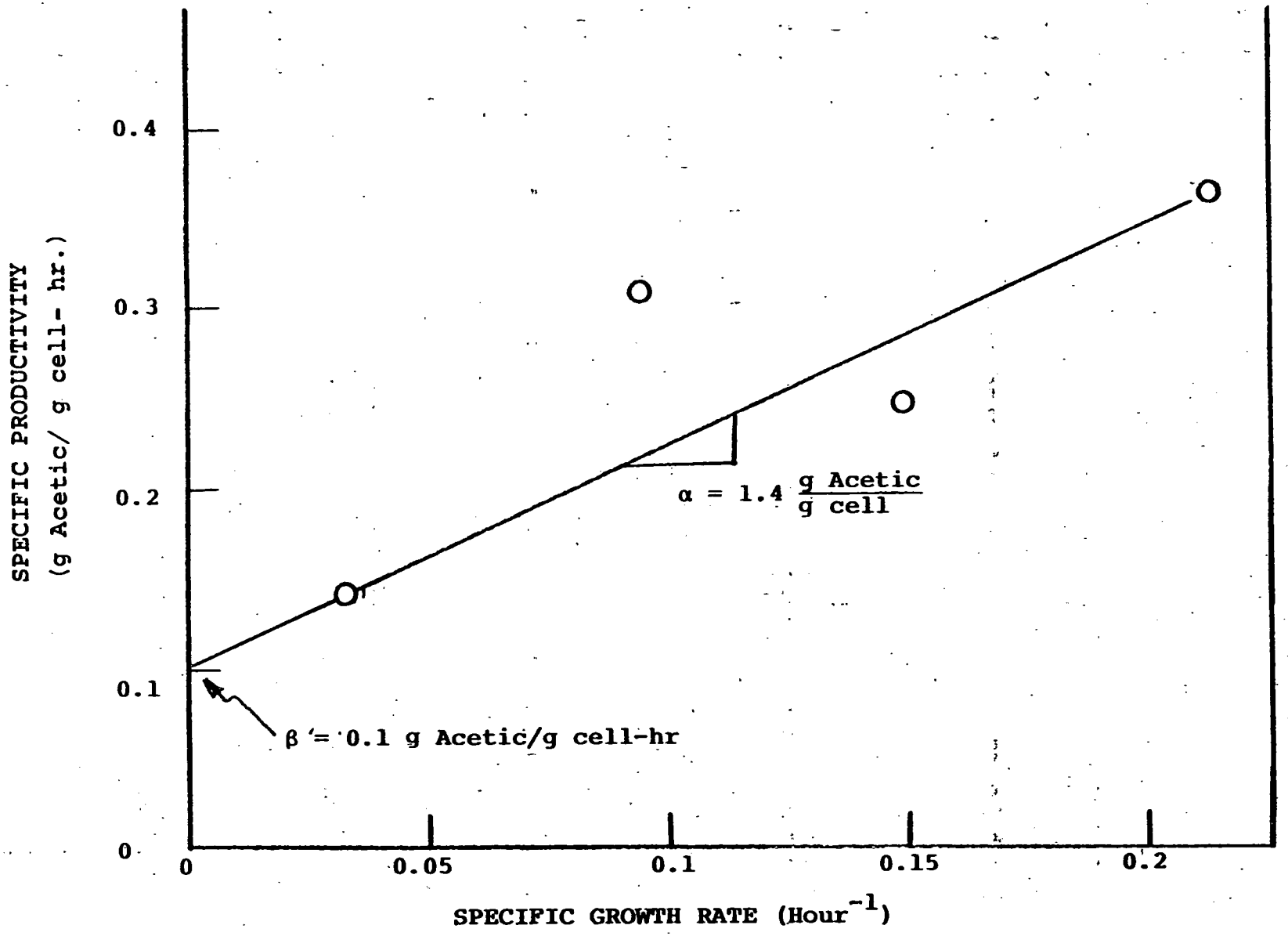


FIGURE II.C.4: RELATIONSHIP BETWEEN SPECIFIC GROWTH RATE TO SPECIFIC PRODUCTIVITY



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

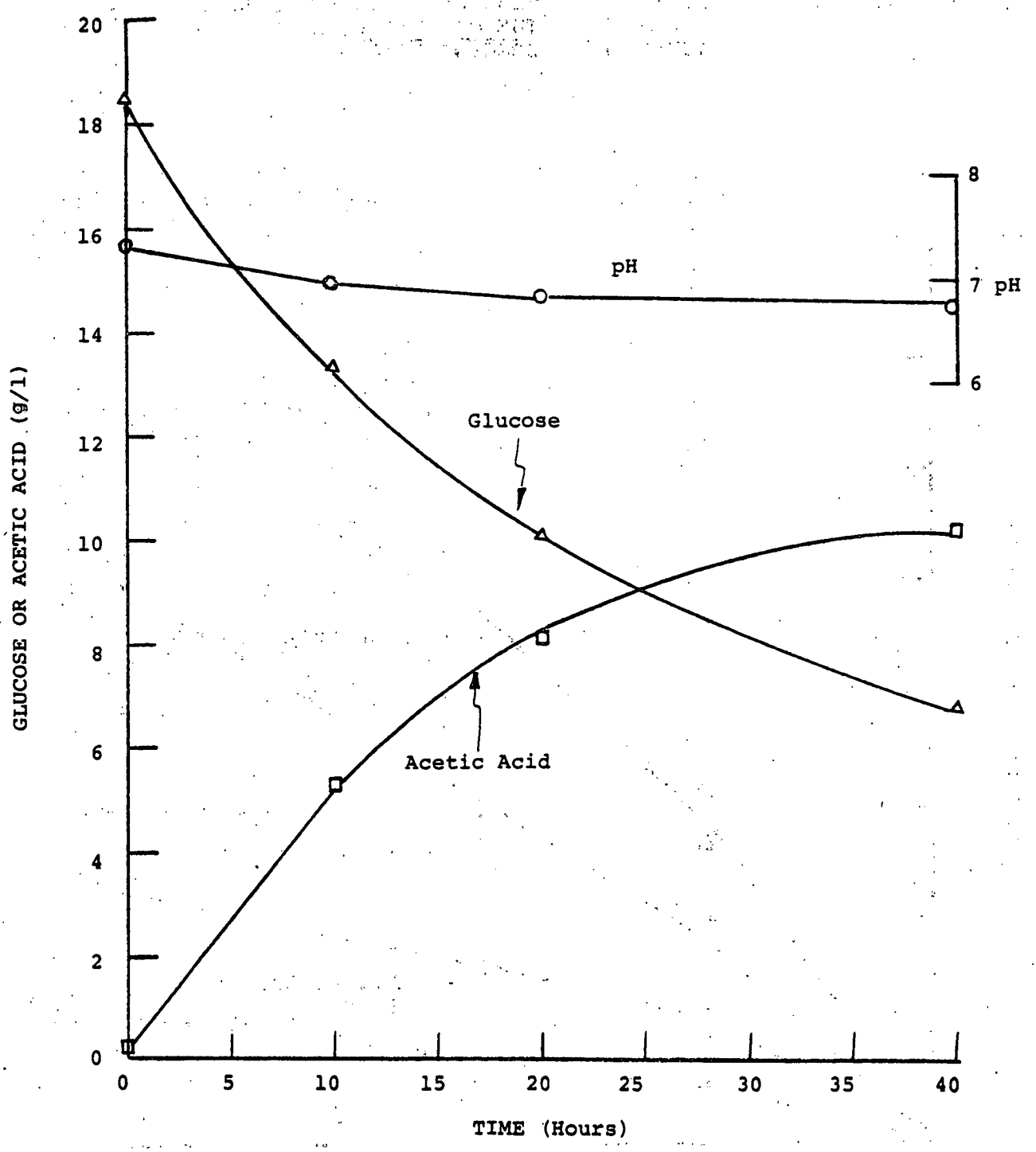


FIGURE II.C.6: KINETICS OF GLUCOSE UTILIZATION BY RESTING CELLS OF C. THERMOACETICUM AT DIFFERENT ACETIC ACID CONCENTRATIONS

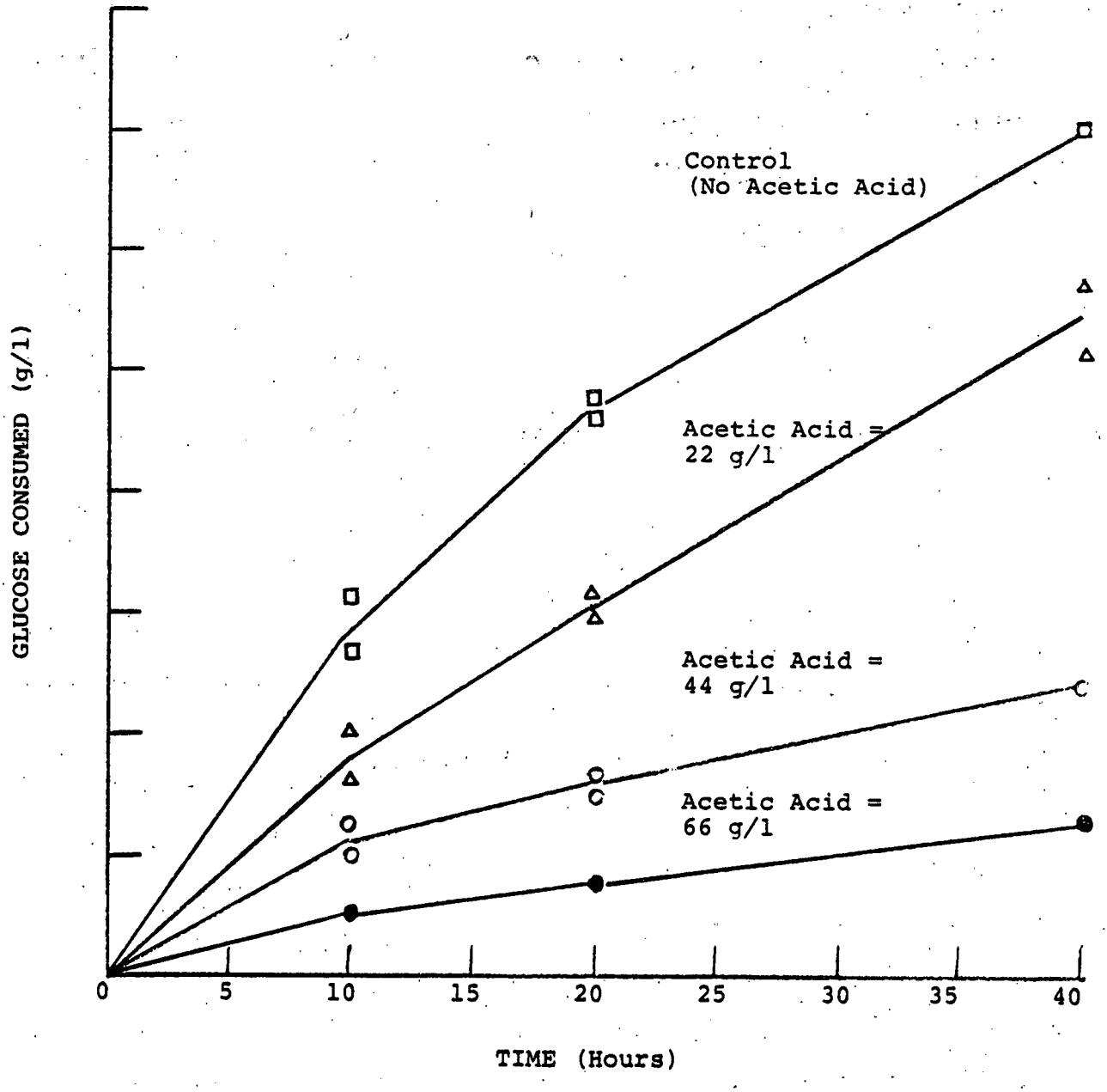
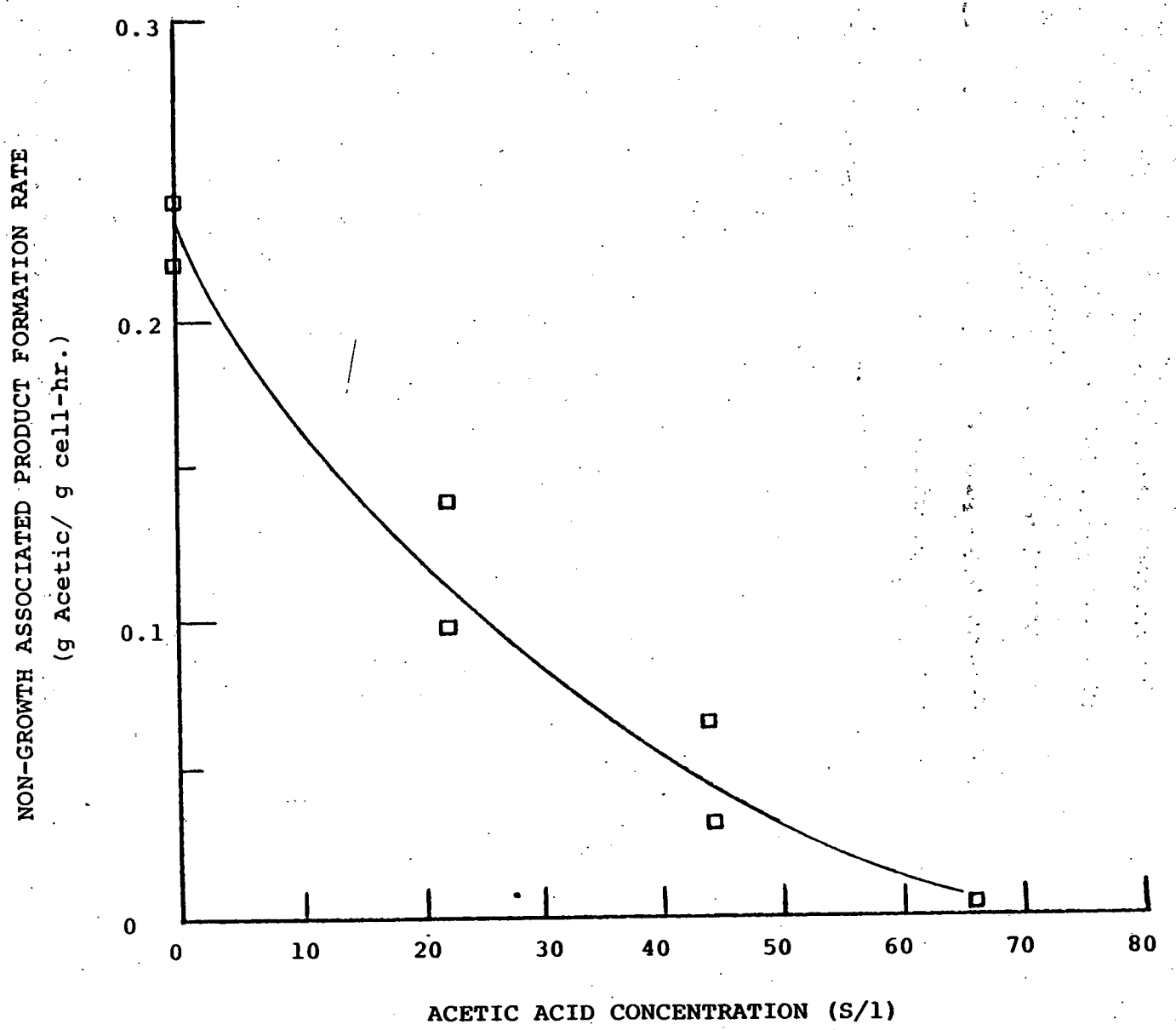


FIGURE II.C.7: RELATIONSHIP BETWEEN ACETIC ACID CONCENTRATION AND NON-GROWTH ASSOCIATED PRODUCT FORMATION RATE USING RESTING CELLS OF C. THERMOACETICUM



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.

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