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Distribution Category UC-61

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

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Progress Report

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SCOPE OF PROGRAM

This is a coordinated program to effect the microbiological degradation of cellulosic biomasses and will focus on the use of anaerobic microorganisms which possess cellulolytic enzymes. These studies will attempt to increase the enzyme levels through genetics, mutation and strain selection. In addition, the direct conversion from cellulosic biomasses to liquid fuel (ethanol) and/or soluble sugars by the cellulolytic, anaerobic organism is also within the scope of this program. Process and engineering scale-up, along with economic analyses, will be performed throughout the course of the program.

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

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ABSTRACT

The accumulation of fermentable sugars during the fermentation of Clostridium thermocellum on cellulose is an important and unique ability of our anaerobic process for biomass utilization. We have continued to study and to elucidate the factors responsible for this accumulation of reducing sugars. Previous studies have shown glucose to be a major product accumulated during fermentation on cellulose by C. thermocellum. We have examined the glucose accumulation more closely using an enzymatic assay to allow us to differentiate between glucose and xylobiose accumulation; HPLC analysis will not separate these two sugars. The results have shown that glucose accumulation is entirely non-growth associated, suggesting that xylobiose may accumulate during growth. To elucidate the reason for glucose accumulation, cellobio-hydrolase activity has been measured during growth and stationary phases of C. thermocellum, with negative results showing an absence of such enzyme activity. At this point, the reason for glucose accumulation remains to be identified.

Characterizations on the growth and production of ethanol and other products by Clostridium thermocellum (S-4) have continued. It was found that for C. thermocellum the specific productivity (g ethanol/g cell-hr.) for ethanol is both growth and non-growth associated. The specific ethanol productivity is directly related and increases linearly with the specific growth rate. However, the specific productivity of lactic acid formation is inversely related to increasing specific growth rate. Carbon and oxidation-reduction balances again indicate that the new isolate S-4 is deficient in the hydrogenase enzyme. This behavior again shows that a high ratio of ethanol to acetic acid (14:1) production can be achieved. Lastly, nutritional studies have shown that iron is a critical component leading to increased rates of ethanol production by C. thermocellum.

Controlled fed-batch fermentations have been performed with C. thermocellum using glucose as the carbon source. These experiments were performed to determine the highest concentration of ethanol attainable. Unfortunately, the maximum ethanol accumulated was only 8.5 g/l. Analysis of the residual glucose shows the formation of cellobiose. Detailed studies will be performed to seek answers to this problem.

A number of different biomasses has been tested with strain S-4 of C. thermocellum. These include exploded poplar, sugar cane bagasse, corn cobs, sweet gum, rice straw and wheat straw. Rice and wheat straws were shown to be most readily degraded by C. thermocellum. Calculations with rice straws showed a degradation efficiency of 0.84 gm product/gm wheat straw degraded.

To effect a more complete utilization of biomass, the use of HG-2, an anaerobic thermophile, to produce ethanol from xylose has been studied. HG-2 was found to grow rapidly (doubling time = 2 hours) on xylose and can produce ethanol and acetic acid as catabolic by-products. Adaptation and selection studies on HG-2 have been initiated. An isolate designated as HG-3 has been obtained which has a higher tolerance for ethanol.

Mixed culture studies have been initiated using corn stover as the carbon source. Clostridium thermocellum and HG-2 have been cultivated as a mixed microbial system. Results indicate that the reducing sugars produced through the cellulolytic action of C. thermocellum on corn stover can be effectively metabolized by HG-2 to produce ethanol and acetic acid.

Our attempts to isolate plaque forming units derived from C. thermocellum have continued. None of the strains examined so far contained mitomycin or U.V. inducible prophages. A qualitative examination of the sensitivity of each of the C. thermocellum strains on hand, to a variety of metal salts and antibiotics, revealed differences in sensitivity which could be used as genetic markers. Attempts to achieve inter-species transfer of antibiotic resistance from C. perfringens to C. thermocellum have not been successful.

Efforts in the nutritional and environmental manipulations, as well as deregulation studies on Clostridium thermocellum, have continued. Clostridium thermocellum grew and produced cellulase on cellobiose, on glucose and on a glucose-cellobiose mixture, indicating that cellulase production in this organism is not subject to glucose repression. Glucose utilization was extensive but growth on this hexose was characterized by a long lag phase. For growth on glucose, a higher concentration of yeast extract was required than for growth on cellobiose. The utilization of carbohydrates by C. thermocellum ATCC 27405 was further examined in detail. The maximum cell concentration was obtained on fructose (.45 g/l) followed by cellobiose (.44 g/l) and glucose (0.18 g/l). However, maximum cell yield (g cells/g initial sugar) was obtained in cellobiose (.21) followed by

glucose (.19) and fructose (.07). When the ATCC 27405 was conditioned for growth in glucose and then transferred to cellobiose, glucose and fructose, the same trends as with cells conditioned for growth in cellobiose were obtained. However, maximum cell concentrations and cell yields were highest in all conditions when the cells were conditioned for growth in glucose. The differences in maximum attainable cell concentrations and cell yields are believed to be due to differences in the way these sugars are transported. Yeast extract could be replaced by eight vitamins plus casein hydrolysate, but not by the vitamins alone. By mutagenesis of C. thermocellum AS-39, we have isolated two mutants which produce 3 times as much cellulase as AS-39.

Purification of the cellulase enzyme from C. thermocellum has been initiated. Ammonium sulfate precipitation followed by gel filtration of C. thermocellum extracellular protein has yielded purification factors of 3.7-fold for filter paper activity and 2.0-fold for CMCase activity. Elution profiles from Biogel A-5m indicate a population of heterogenous molecular weights (10,000 to 5,000,000 daltons) for both filter paper and CMCase activities.

Considerable progress has been made in the optimization of conditions for conversion of propionic acid to acrylic acid by free and immobilized cells of Clostridium propionicum. The optimal conditions for propionate dehydrogenation are: initial propionate concentration 200 mM, methylene blue concentration 0.2%, lactate or α-alanine concentration 20-25 mM, pH 8.5, temperature 32°C with cells harvested from the late exponential growth phase. Using these conditions with free cells of C. propionicum, we have achieved concentrations of acrylic acid in excess of 40 mM. Studying the immobilization of whole cells, we have shown that it is possible to prepare polyacrylamide gels containing C. propionicum that retain 70-80% of their biological activity. The use of immobilized cells is of particular importance for the development of reactor systems for production of acrylic acid. Our ultimate goal is to derive acrylic acid from cellulosic biomass. A first step in this conversion is the utilization of a mixed culture of C. thermocellum to produce sugar from cellulose and a thermophilic, homolactate organism converting the sugars to lactic acid. This second organism has been identified as Bacillus coagulans and work is currently in progress to define its behavior in mixed cultures with C. thermocellum.

Detailed studies using resting cells of Clostridium acetobutylicum in the presence and absence of penicillin G have been performed. It has been shown that in the presence of

penicillin G, C. acetobutylicum is unable to metabolize glucose to produce mixed solvents (acetone or butanol). Therefore, redirections will be needed to achieve high productivity solvent fermentations for this system. In situ extractive fermentation appears to be one such solution. Using corn oil as the extractant, total n-butanol in excess of 15 g/l has been achieved. This concentration of butanol has never been achieved in conventional fermentations. Search for additional extractants is now in progress.

The production of acetic acid by resting cells of Clostridium thermoaceticum has focused on the reason leading to cell instability. "Cell lysis" was shown to occur in the presence, as well as the absence, of chloramphenicol. These results indicate lytic phenomenon alone does not explain the loss of the organism's ability to produce acetic acid. Studies will continue to determine the reason and seek answers to increase cell stability. Xylose has been examined as the carbon source for growth and acetic acid formation by C. thermoaceticum. Product yield of about 0.8 gm acetic/gm xylose was obtained.

CONTRIBUTORS OF PROGRAM

This program is under the coordination of Daniel I.C. Wang, Principal Investigator. The research and development within this program are directed by the following individuals. The percentage efforts of the Principal and Co-Principal Investigators on this project are shown along with the names of the different participants.

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

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I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. ENZYME PRODUCTION BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

As a continuation of the work described in the last progress report (COO-4198-7) concerning the conversion of cellulose to glucose by *Clostridium thermocellum* ATCC 27405, studies have been extended to further assess the β-glucosidase, or cellobiase, activity produced by *C. thermocellum* and to determine the kinetics of glucose accumulation during fermentations on cellulose. Since glucose is an important intermediate in the overall scheme of cellulose degradation and subsequent fermentative production of chemical feedstocks, information about the mechanism or enzymes involved in its production must be established so that we may find ways to enhance the final proportion of glucose among the other soluble cellulose hydrolysis end products and so that we may more rationally design mixed-culture fermentations with *C. thermocellum* and other anaerobic thermophiles (e.g., *C. thermoaceticum* or *Bacillus coagulans*) which utilize the glucose (released from cellulose by *C. thermocellum*) as a growth substrate.

Experiments were performed to measure whether extracellular 'cellobiase' is produced by *C. thermocellum*,

during fermentation on cellobiose, and to determine whether an intracellular 'cellobiase' may be released at the end of cellobiose fermentations as a result of cell lysis. In addition, the kinetics of glucose accumulation during cellulose fermentations was followed using the enzymatic Glucostat assay to compare the results with those previously obtained by HPLC analysis.

2. Materials and Methods

a. Growth of Organism

C. thermocellum was cultured in 500 ml flasks containing 300 ml of CM3 medium with 0.5% yeast extract. The carbon sources were either solka floc cellulose (SW40) or cellobiose at a concentration of 1.0%. No pH control was used.

b. Glucose Measurement

For both the cellobiose-grown and solka floc-grown cultures, glucose was measured enzymatically by the Glucostat assay (Worthington Biochemicals).

c. Protein Measurement

Extracellular protein production by *C. thermocellum* was measured with the Bio-Rad Protein Assay, a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. Bovine serum albumin (Sigma) was used to prepare standards.

d. Cellobiase Assay

The extracellular protein in the supernatant of the broth from a cellobiose fermentation was precipitated at 4°C in 40% ethanol overnight. The precipitate was resuspended in 0.05 M sodium citrate buffer, pH 5.4, at one half the original volume of the broth to effect a two fold concentration. A substrate solution containing cellobiose was prepared in the following way. In citrate-phosphate buffer, pH 5.4, (0.1 M citric acid: 0.2 M $Na_2HPO_4.7H_2O$: H_2O, 1:1:2) cellobiose was dissolved to a final concentration of 10 mg/ml (1.0%) and cysteine was added as reducing agent to a final concentration of 2 mg/ml (0.2%). (The pH was chosen as pH 5.4 to match the pH of the medium during fermentation when the specific rate of glucose accumulation is maximal. A citrate-phosphate buffer was selected to allow for a possible phosphorolytic cleavage of cellobiose.) One milliliter of the enzyme solution was added to one milliliter of the substrate (cellobiose) solution and the mixture was incubated, in the presence of air, at 60°C. After one hour, the DNS assay for reducing sugars was performed on the enzyme-substrate mixtures. The absorbance at 550 nm was compared to that of a zero time unincubated control and also to a control containing the substrate solution mixed only with buffer. Any increase in absorbance of the test samples over the controls would indicate a liberation of glucose from cellobiose.

3. Results

Because Clostridium thermocellum is known to possess an intracellular cellobiose phosphorylase and since glucose accumulation has been shown to be primarily a non-growth associated event during cellobiose fermentations, one plausible explanation for glucose accumulation was that autolysis occurred during the organism's stationary phase, releasing an intracellular enzyme that would catalyze the hydrolysis of cellobiose to glucose extracellularly. However, turbidity measurements of cell density did not indicate any significant cell lysis, nor did measurements of extracellular protein accumulation during the fermentation of C. thermocellum on cellobiose suggest that cell lysis had taken place. The results from two fermentations on CM3/cellobiose are shown in Figure I.A.1., where extracellular protein production, culture turbidity (Klett units) and glucose accumulation are plotted against time. For both fermentations it is seen that when culture turbidity reaches a plateau so does the extracellular protein level. There appears to be no stationary phase release of protein to correspond with the accumulating glucose. Assuming that no intracellular cellobiose phosphorylase was escaping, another plausible explanation for the glucose accumulation was that the organism did produce an extracellular cellobiase which functioned when the medium pH dropped from

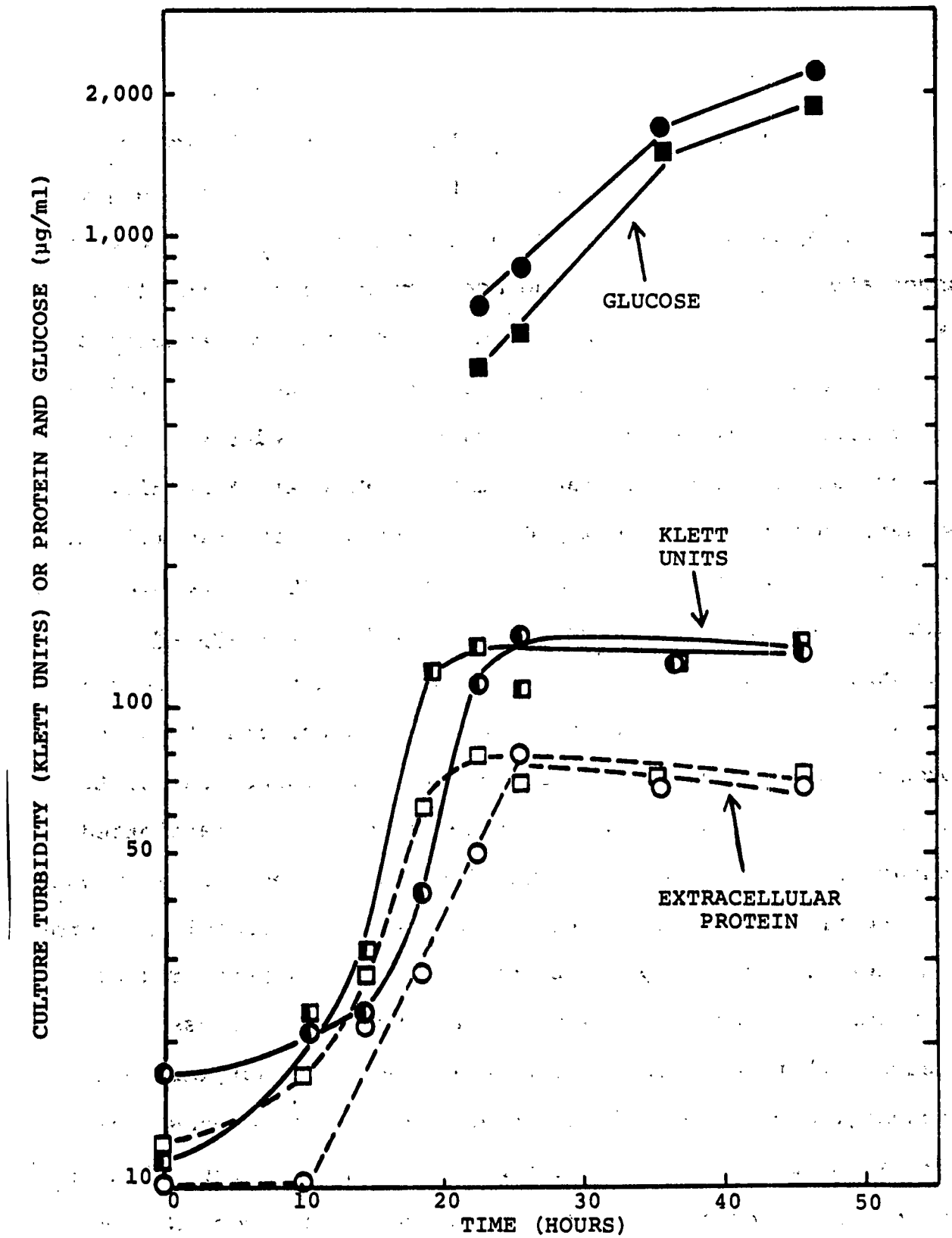


FIGURE I.A.1: KINETICS OF GROWTH, GLUCOSE ACCUMULATION AND EXTRACELLULAR PROTEIN PRODUCTION DURING FERMENTATION OF C. THERMOCELLUM ATCC 27405 ON CM3/CELLOBIOSE

pH 7.0 to pH 5.4, the final pH of the broth. However, when extracellular cellobiase was assayed according to the procedure described above, no activity could be measured as shown in Table I.A.1. In this table, the absorbances at 550 nm, after the DNS assay for reducing sugars, are presented for broth samples taken from a cellobiose fermentation at 20 and 40 hours (times when the pH of the medium was within the range of 5.4-5.6), as well as the zero time point control and the substrate (no enzyme added) control. The absorbance values correspond to the standard cellobiose concentration of 5 mg/ml which is the concentration of substrate used in this assay.

TABLE I.A.1.

Sample Time (Hrs.)	pH	Abs. 550 nm: Substrate Solution Cellobiose	Abs. 550 nm: Zero Time Control	Abs. 550 nm: Incubated at 60°C for 1 hr.
20 a	5.6	1.29	1.28	1.27
b		1.31	1.26	1.27
40 a	5.4	1.29	1.29	1.31
b		1.31	1.33	1.20

In the previous progress report (COO-4198-7), a kinetic analysis of the specific growth rate and specific glucose and cellobiose accumulation rates (determined by HPLC analysis) during a fermentation of C. thermocellum on Solka floc

was presented as shown in Figure I.A.2. It was hypothesized that the growth-associated glucose accumulation, which had not been observed during cellobiose fermentations, may have been the result of an exoglucohydrolytic activity which cleaved glucose units from the ends of larger cellodextrin chains, substrates that would not be present in a cellobiose medium. However, since solka floc contains up to 10% hemicellulose or xylan, and since we have encountered difficulties with the separation of xylobiose from glucose on our HPLC analytical column, it may have been that the 'glucose' which we observed to accumulate in a growth-associated manner during solka floc fermentations was really xylobiose, and that actual glucose accumulation occurred as a non-growth associated event, similar to that of cellobiose fermentations. As a first experiment to check the HPLC measured glucose, C. thermocellum was again grown on CM3/solka floc medium, but glucose accumulation was measured with the enzymatic glucostat assay. Figure I.A.3 depicts the kinetics of cell mass, reducing sugar and glucose accumulation during a fermentation without pH-control. When the enzyme assay is used, it can be seen that glucose does not accumulate until after dry cell weight measurements indicate that growth has leveled off. This separation of glucose accumulation from growth is more clearly shown in Figure I.A.4 where the specific growth rate, μ, and the specific glucose accumulation rate, q_g, (data from Figure

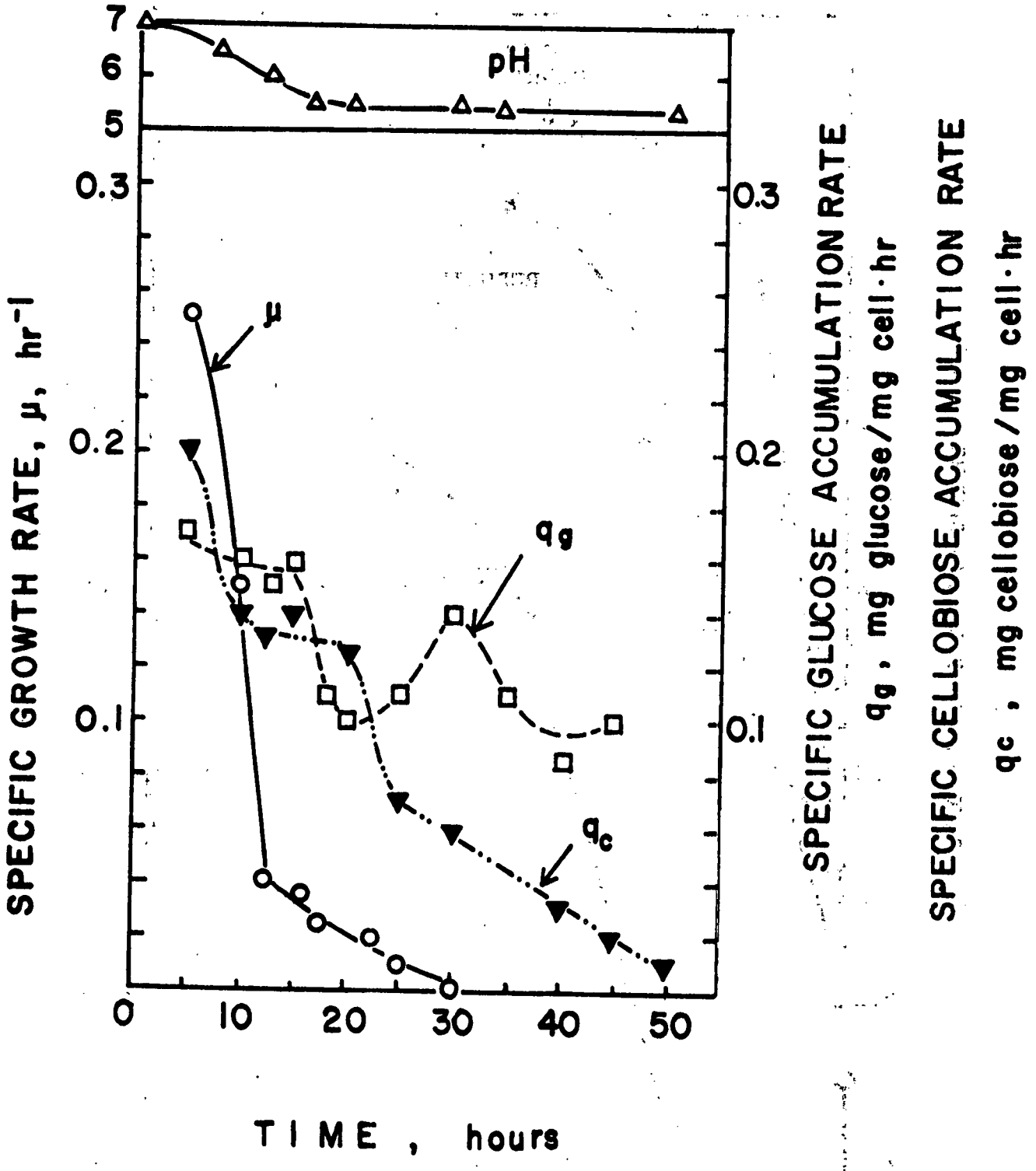


FIGURE I.A.2: SPECIFIC GROWTH RATE, SPECIFIC GLUCOSE AND CELLOBIOSE ACCUMULATION RATES, AND pH PROFILE VS TIME FOR FERMENTATION OF C. THERMOCELLUM ATCC 27405 ON CM3/SOLKA FLOC (HPLC DATA ANALYZED TO OBTAIN q_g, q_c)

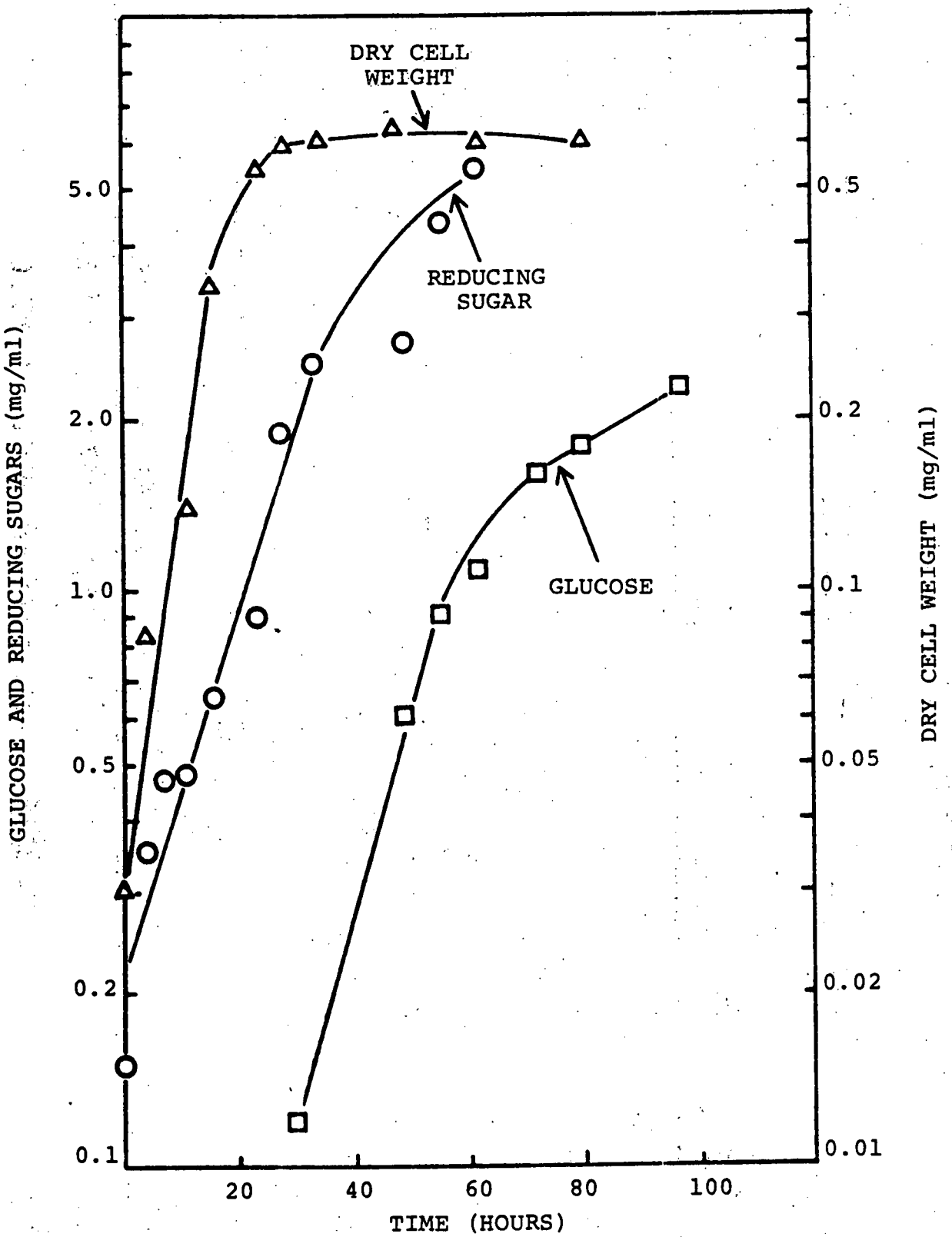


FIGURE I.A.3: KINETICS OF GROWTH, REDUCING SUGAR ACCUMULATION AND GLUCOSE ACCUMULATION (BY GLUCOSTAT METHOD) DURING FERMENTATION OF C. THERMOCELLUM ATCC 27405 ON CM3/SOLKA FLOC

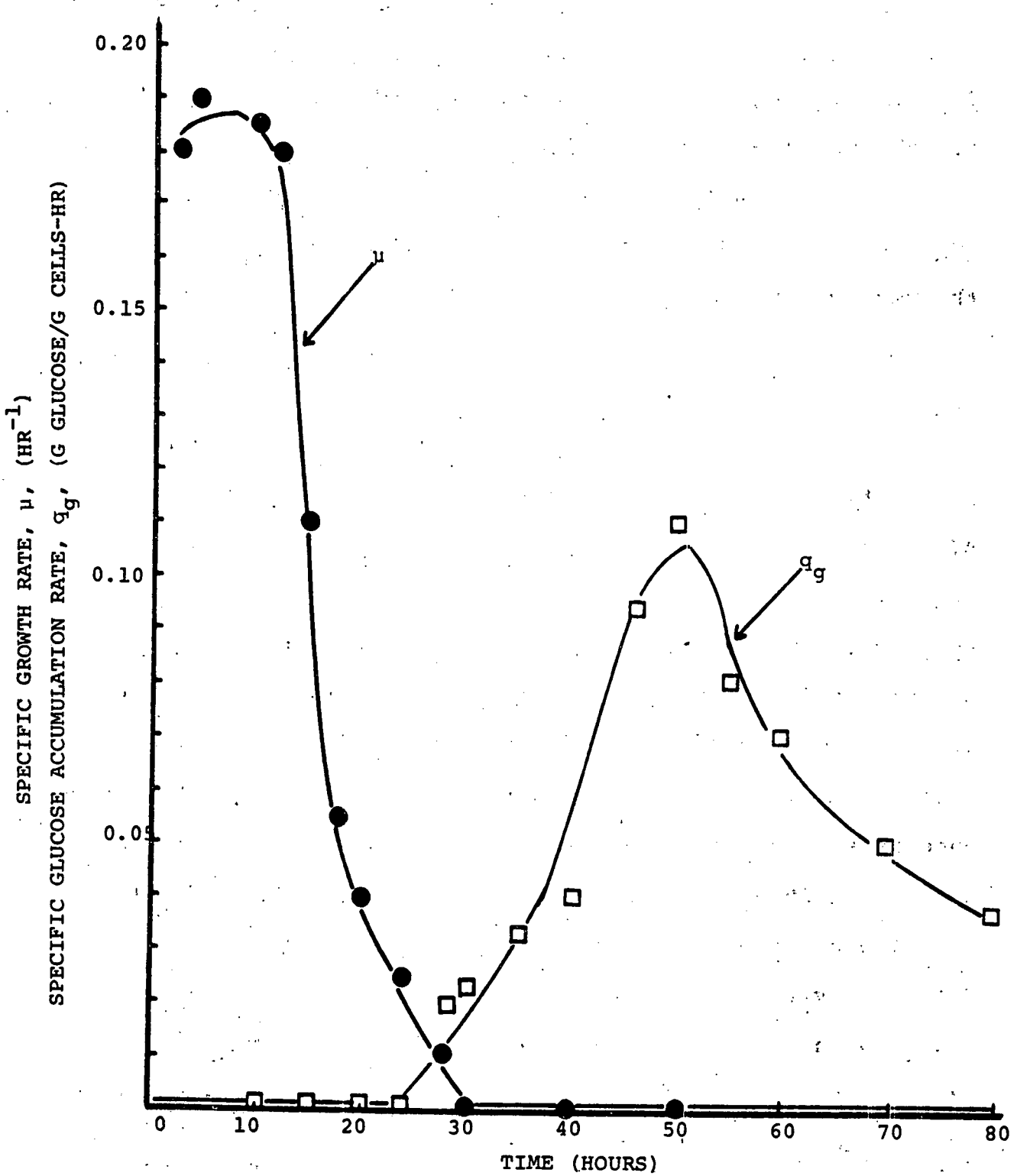


FIGURE I.A.4: SPECIFIC GROWTH RATE AND SPECIFIC GLUCOSE ACCUMULATION RATE VS. TIME FOR FERMENTATION OF C. THERMOCELLUM ATCC 27405 ON CM3/SOLKA FLOC (GLUCOSTAT DATA USED TO OBTAIN q_g)

I.A.3) have been plotted as a function of time. As in the cellobiose fermentations, glucose accumulation during a solka floc fermentation, measured by the glucostat assay, appears to be a non-growth associated event, occurring when the pH of the medium has dropped to 5.4.

4. Discussion

Measurements of culture turbidity and extracellular protein production during fermentations of C. thermocellum ATCC 27405 on cellobiose failed to positively show the occurrence of autolysis which might account for the non-growth associated glucose accumulation via the release of the intracellular cellobiose phosphorylase into the medium where it could act extracellularly. Similarly, an assay to measure extracellular cellobiase production did not indicate the presence of such an enzyme in the fermentation broth. There are several explanations why glucose accumulation occurs during cellobiose fermentations even though an extracellular cellobiase activity has not been measured, be it an extracellularly produced enzyme or a released intracellular enzyme. An obvious explanation is that the conditions of the cellobiase assay were unsuitable for measuring the appropriate enzyme activity. For instance, anaerobiosis may have been necessary. Two alternative possibilities are that a cell-surface-bound enzyme is responsible for the degradation of cellobiose to glucose, or that

the intracellular cellobiose phosphorylase is responsible for the cleavage and that the resulting glucose-1-phosphate is retained within the cell for further catabolism, while the other glucose unit is excreted into the medium. These possibilities will be looked into for future work.

The discrepancy between HPLC and glucostat data for glucose accumulation during solka floc fermentations may be explained, as mentioned in the Results section, if accumulating xylobiose is being artifactually detected as glucose during the active growth phase of the organism. This hypothesis will be tested by collecting the 'xylobiose-glucose' fractions (from samples taken during the active growth phase) eluting from the HPLC column and subjecting them to paper chromatography to determine whether both sugars are present. HPLC will be further used to study the kinetics of cellulose degradation, as a means of measuring whether there is a random or specific time sequence in the breakdown of cellulosic materials to cellodextrins and then to disaccharides and finally to the monosaccharides, glucose and xylose, which are useful substrates for subsequent fermentations.

B. PRODUCTION OF ETHANOL FROM CELLULOSIC BIOMASS BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

In our last progress report (COO-4198-7), we reported that the new strain of Clostridium thermocellum, S-4, produces a significantly higher ratio of ethanol to acetic acid. Carbon and oxidation-reduction balances have led us to conclude that this strain is deficient in the hydrogenase and thus can produce the high ratio of ethanol to acetic acid. The kinetics of ethanol production were examined and found that it is produced through both growth and non-growth associated modes. In this quarter, we have continued to examine the kinetics of production of other products. In addition, the degradation capabilities of C. thermocellum, strain S-4, on various cellulosic biomasses were also examined. The results from these experiments will be presented.

2. Materials and Methods

The microorganism used through this study was C. thermocellum, strain S-4. The basic medium used for growth was CM4. Modifications were made in some cases and these changes will be specified along with the presentations of the results.

The rice straw and wheat straw used were obtained from Professor Charles R. Wilke, Lawrence Berkeley Laboratory, University of California, Berkeley, California. Rice straw is

the crop of Fall, 1975, in Butte county; wheat straw is the crop of Fall, 1975, in Yolo county. Both are 0.25 - 0.35 mm fraction of 2 mm Wiley milled. The sweet gum tree sawdust was obtained from the University of Georgia. The sources of other biomasses have already been reported.

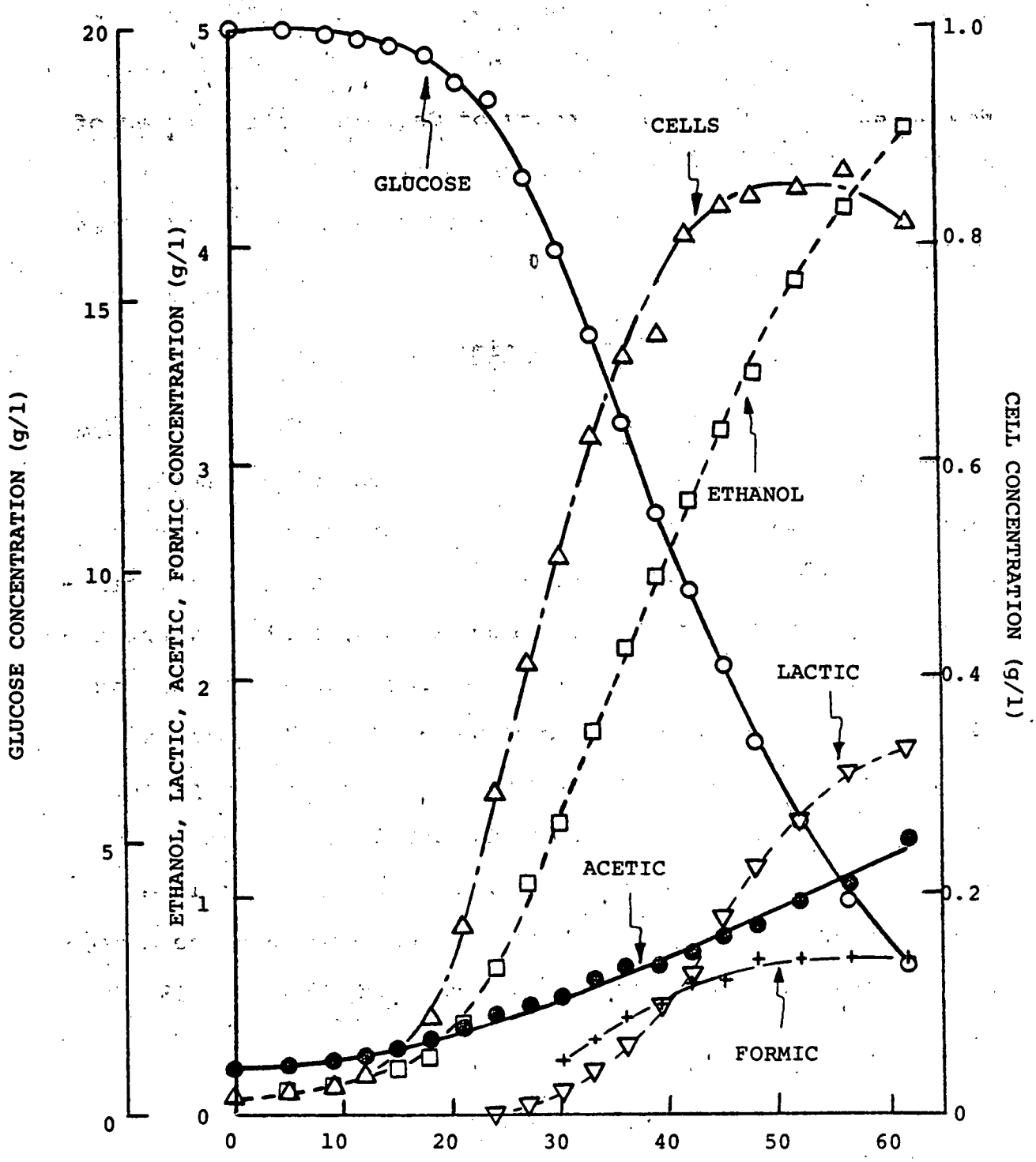
All the analytical procedures have already been reported and will not be reported.

3. Results and Discussion

In order to study the kinetics of products formation and the effects of environmental conditions, several fermentations were performed in a 7-liter fermentor with a 5-liter working capacity. Figure I.B.1 shows a typical fermentation profile with pH controlled at 6.5; initial yeast extract concentration was 3 g/l and glucose concentration was 2%. The production of reduced products (i.e., ethanol and lactic acid) is significantly greater than oxidized product (acetic acid) and is similar to the previous results. The kinetics of growth and ethanol production can be achieved through growth and non-growth associated modes and the mathematical proposed in an earlier progress report (COO-4198-7) was as followed:

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

FIGURE I.B.1: FERMENTATION PROFILE OF *Clostridium thermocellum* (S-4) WITH pH CONTROL (6.5) IN LOW IRON CONCENTRATION MEDIUM



where q_p = specific productivity, gm product/gm cell-Hr.

μ = specific growth rate, Hr^{-1}

α = growth associated product formation constant, gm product/gm cell

β = non-growth associated product formation constant, gm product/gm cell-Hr

A plot of specific growth rate (μ) versus specific productivity (q_p) is shown in Figure I.B.2. The growth associated ethanol formation constant is 1.5 (g ethanol/g cell).

The production of lactic acid was analyzed in the similar manner and the results also shown in Figure I.B.2. In contrast to the positively growth associated mode of ethanol formation, lactic acid production shows a negatively growth associated mode. The specific productivity of lactic acid has an inverse relationship with the specific growth rate. These results indicated that higher growth rate will increase the productivity of ethanol and decrease the productivity of lactic acid, thus increasing the yield of ethanol production.

Because both pyruvate-ferredoxin oxidoreductase and ferredoxin are non-heme iron protein, it was expected that iron might play an important role in the production of ethanol. Therefore, the effect of iron concentration on the kinetic parameters was examined.

Figure I.B.3 shows the fermentation profile with pH controlled at 6.5, $FeSO_4.7H_2O$ concentration was 0.01 g/l:

FIGURE I.B.2: KINETIC RELATIONSHIPS OF GROWTH AND PRODUCT FORMATION BY C. thermocellum (S-4) AT LOW IRON CONCENTRATION

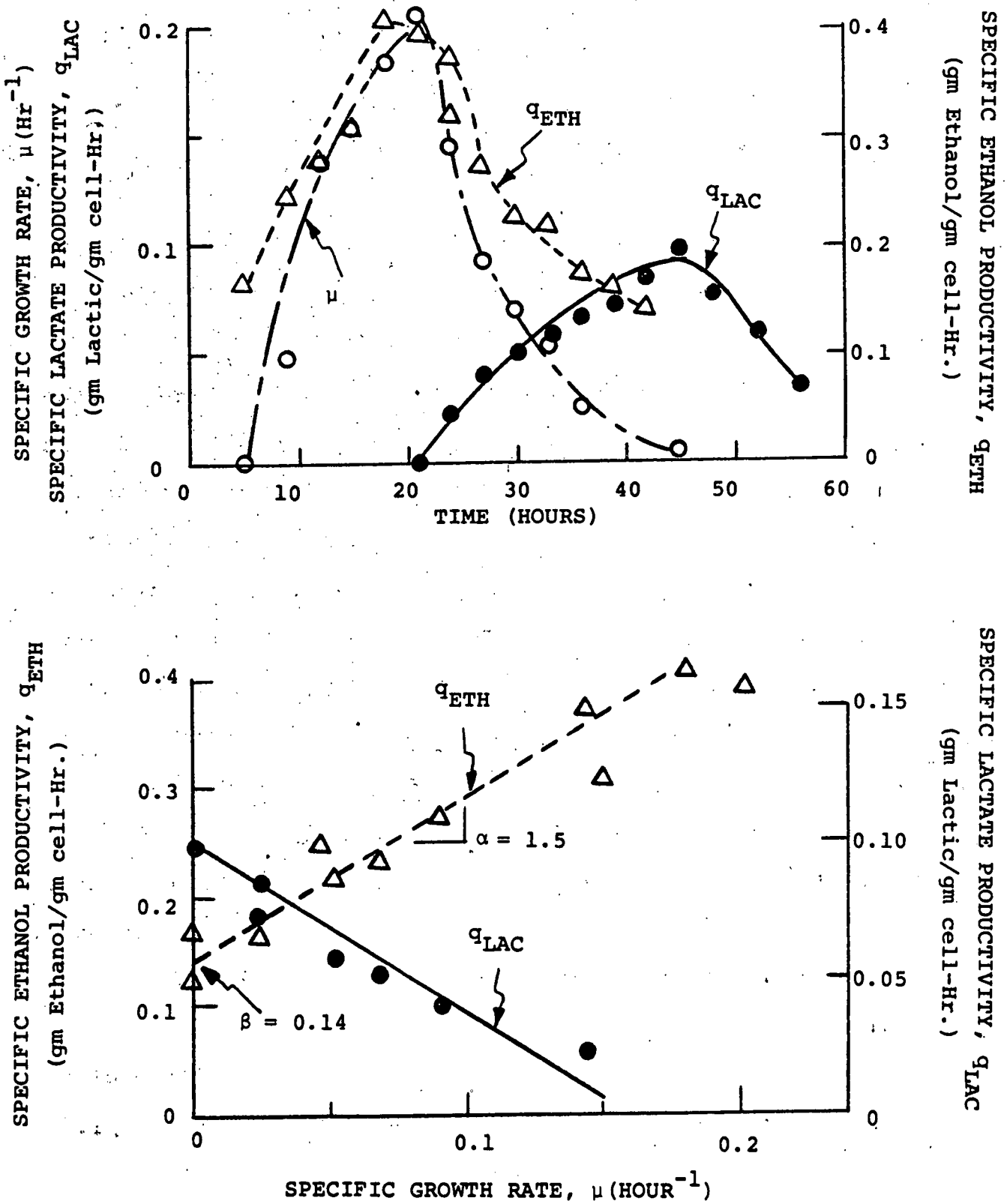
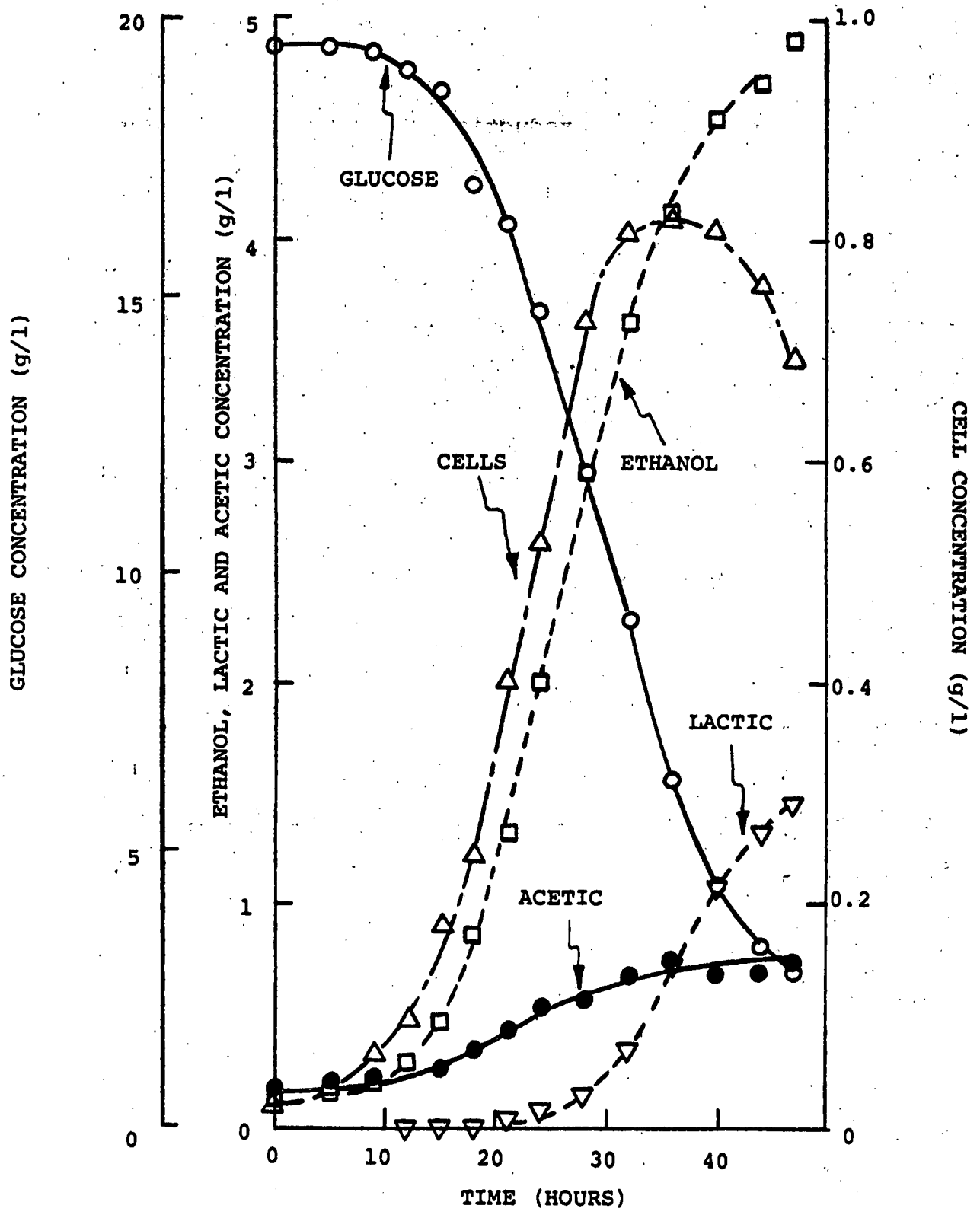


FIGURE I.B.3: FERMENTATION PROFILE OF *Clostridium thermocellum* (S-4) WITH pH CONTROL (6.5) IN HIGH IRON CONCENTRATION MEDIUM



eight times higher than that of CM4 medium. Other conditions are the same as the other experiments. The data from Figure I.B.3 were analyzed in the same way as described previously, and shown graphically in Figure I.B.4.

For ethanol production, the calculated value of α and β from Figure I.B.4 are 2.6 (g ethanol/g cell) and 0.14 (g ethanol/g cell-hr) respectively. It can be seen that growth associated constant α increased significantly as iron concentration increased while non-growth associated constant β remained the same. Again, lactic acid production shows a negatively growth associated mode.

Another important effect of iron concentration was the formation of formic acid. Formic acid, as shown in Figure I.B.5, is the key intermediate of one-carbon unit synthesis in clostridia. Formate accumulated when low iron concentration (1.25 mg $FeSO_4.7H_2O/l$) was used and disappeared when iron concentration was increased to 10 mg $FeSO_4.7H_2O/l$. There are at least 4 different mechanisms for formate production. The mechanism in <u>Clostridium butyricum</u> and <u>Clostridium butylicum</u> which formate is generated from pyruvate by pyruvate formate lyase according to the following reaction:

$$CH_3COCOOH + CoASH \longrightarrow CH_3CO\text{-}SCoA + HCOOH$$

This is the most reasonable one to explain the observed phenomenon.

FIGURE I.B.4: KINETIC RELATIONSHIPS OF GROWTH AND PRODUCT FORMATION BY C. thermocellum (S-4) AT HIGH IRON CONCENTRATION

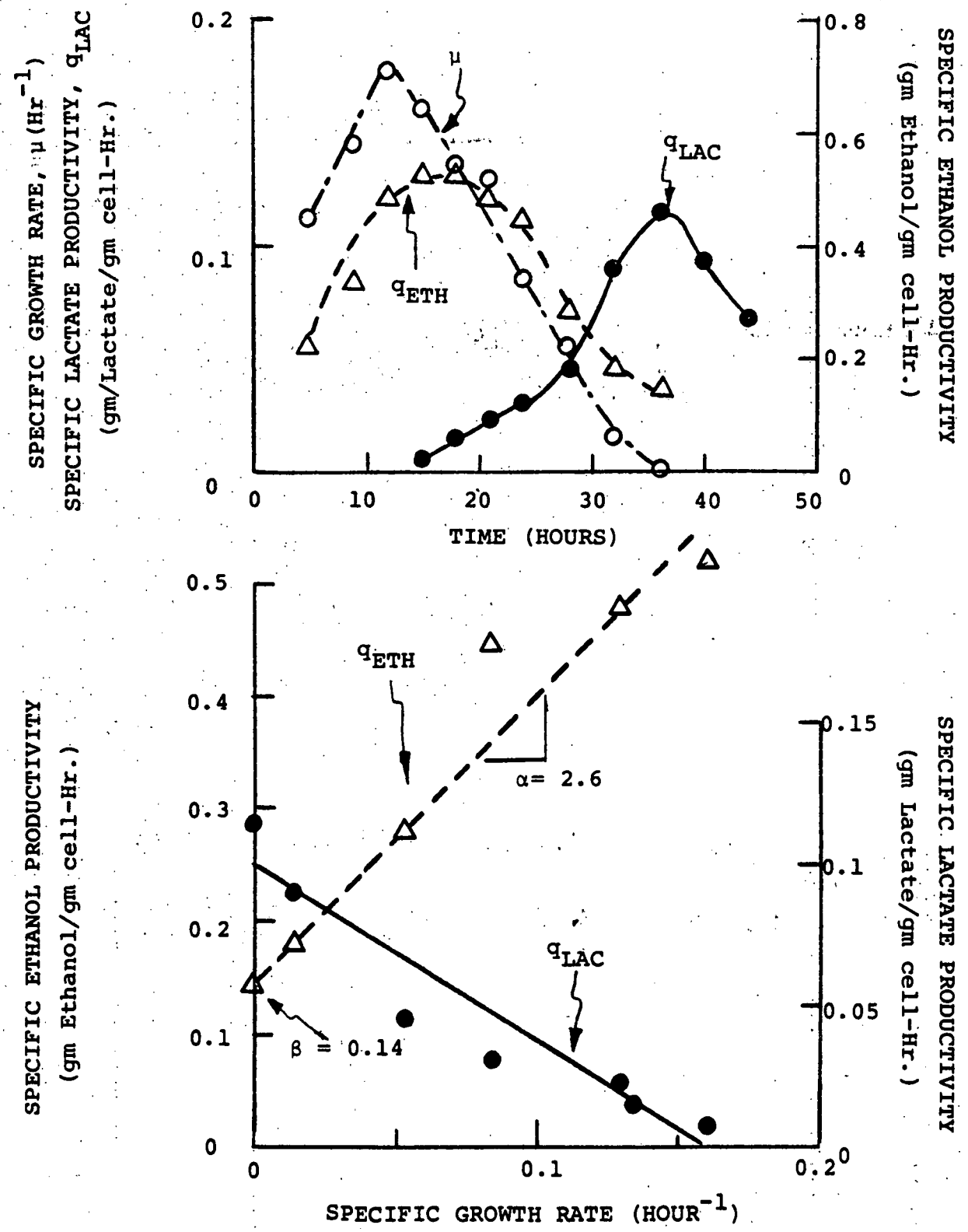
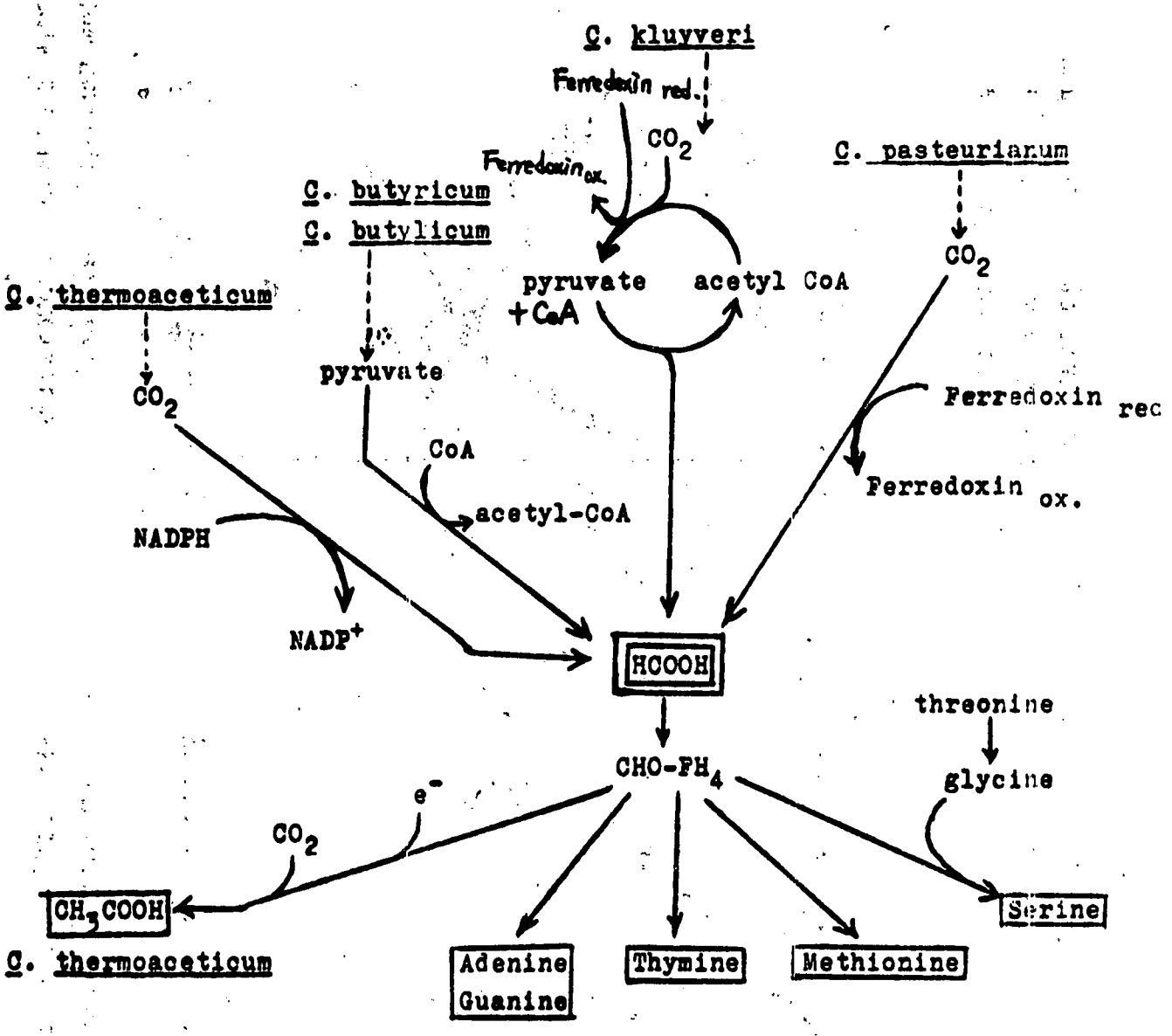


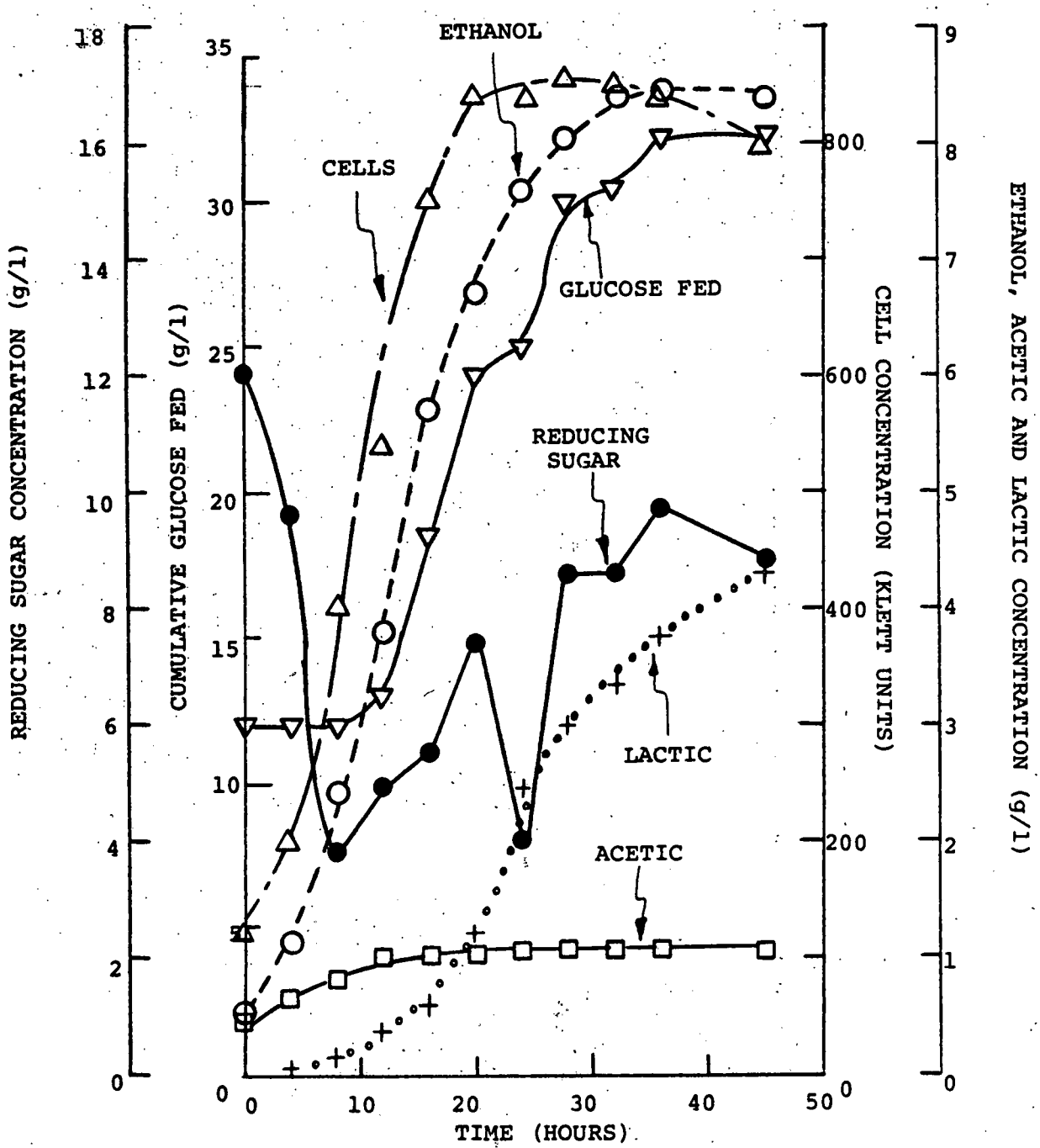
FIGURE I.B.5: PATHWAY OF ONE-CARBON UNIT SYNTHESIS IN CLOSTRIDIA



It is well known that flavodoxin, which has a similar low potential, can replace ferredoxin in certain reactions and are synthesized by many bacteria, especially in media low in iron concentration. It is reasonable to explain the phenomenon of formic acid accumulation in the same way that C. thermocellum can synthesize pyruvate formate lyase replacing pyruvate ferredoxin oxidoreductase to produce acetyl CoA in medium low in iron concentration. In this fashion, C. thermocellum is able to maintain the production of acetyl CoA from pyruvate at a certain level and leading to the accumulation of formic acid. If this is true, then it can be concluded that CM3 and CM4 medium are deficient in iron.

At this point, it still remained to be answered what is the highest ethanol concentration the strain S-4 can produce. A fed batch fermentation was used in seeking this answer. The concentration of glucose was maintained between 5-10 g/l and pH was controlled at 6.5. The results from this experiment are shown in Figure I.B.6. As seen from this figure, the initial rate of cell growth and ethanol production was quite rapid up to 20 hours. The acetic acid production occurred only in the very beginning then stopped while the lactic acid production started only when the growth rate decreased. A final concentration of 8.4 g/l of ethanol, 4.3 g/l of lactic acid and 1.0 g/l of acetic acid were obtained. A total of 32.3 g/l of glucose and 8.4 g/l of yeast extract was added and 8.8

FIGURE I.B.6: FERMENTATION PROFILE OF FED BATCH SYSTEM WITH C. thermocellum (S-4) AT pH 6.5



g/l of reducing sugar remained after 45 hours fermentation. However, from the qualitative analysis of the reducing sugar by using paper chromatography, it was found that a large portion of the reducing sugar was cellobiose (the concentration was about one third of that of glucose). Moreover, from the HPLC analysis, it was revealed that about 2 g/l of cellobiose was accumulated. Although higher oligosaccharides could not be demonstrated due to the sensitivity of these methods, it was believed that some oligosaccharides also accumulated during the fermentation by the enzymatic reaction of cellobiose phosphorylase and cellodextrin phosphorylase.

Due to the presence of oligosaccharides, the reducing sugar concentration was underestimated. Therefore, acid hydrolysis (5% HCl, boiled for one and half hours) of the fermentation broth was performed in order to know the actual sugar concentration. It was found that the final sugar concentration increased from 8.8 g/l to 9.8 g/l. The actual reducing sugar concentration was then used to calculate the carbon and oxidation reduction balance and ethanol yield of this fermentation.

Table I.B.1 shows the carbon and oxidation-reduction (O/R) balance from this experiment. Here again, the analysis of the O/R balance showed that there must be very little or no hydrogen formation during the fermentation.

TABLE I.B.1: CARBON AND OXIDATION-REDUCTION BALANCE FOR FERMENTATION OF GLUCOSE BY STRAIN S-4 (C. THERMOCELLUM)

PRODUCTS	AMOUNT		g carbon/l	UNIT O/R VALUE per MOLE	OVERALL O/R VALUE	
	g/l	mM			CASE I*	CASE II*
Ethanol	8.4	182.6	4.38	-2	-365.2	-365.2
Lactic acid	4.3	47.8	1.72	0	0	0
Acetic acid	0.6	10	0.24	0	0	0
CO_2*	8.47	192.6	2.31	+2	+385.2	+385.2
H_2*	0.385	(192.6)	-	-1	-192.6	0
Cell mass*	2.5	-	1.25	-	-	-
Total carbon recovery			9.90		+385.2 -557.8	+385.2 -365.2
Glucose fermented	22.5	125	9.0	0		
Yeast extract consumed (30%)**	2.52	-	1.08	0		
Total carbon recovery in percent of theoretical value	$\frac{9.9}{9.0 + 1.08} = 98.2\%$					
Oxidation-Reduction Index					$\frac{385.2}{557.8} = 0.69$	$\frac{385.2}{365.2} = 1.05$

* See previous progress report.

** Assume 30% of yeast extract was consumed. 40% of consumed yeast extract was carbon.

The yield of ethanol was calculated to be 37% of fermented sugar. Compared to 45% in yeast alcohol fermentation, those results are very encouraging since it is now almost possible to compete with yeast alcohol fermentation as far as ethanol yield is concerned.

In this fed batch experiment, the fermentation stopped when a total of about 14 g/l of products were accumulated. It is believed that other nutrients were in excess at the end of the fermentation and the concentration of products is not high enough to inhibit the fermentation. Therefore, there are some unknown reasons contributing to the cessation of the fermentation. This might be due to sporulation or it may also be due to capsule formation (i.e. further polymerization of cellodextrin). Lastly, clostridia has been reported to be able to produce bacteriocin and this should be investigated in order to seek answers to this problem.

In the overall process of cellulose degradation, cheap and abundantly available biomasses are required. Therefore, other potential biomasses including rice straw, wheat straw, corn cob, sugar cane bagasse, sweet gum tree sawdust and exploded poplar were examined. These experiments were performed using 25 g/l of biomass, 3 g/l of NaOH. After sterilization, CO_2 was sparged until pH is 7.0, 10 g/l of soybean meal was then added and 10% of seed culture was then inoculated. The results are summarized in Figure I.B.7. It can be seen from

FIGURE I.B.7: FERMENTATION PROFILE OF VARIOUS CELLULOSIC BIOMASS BY C. THERMOCELLUM (S-4)

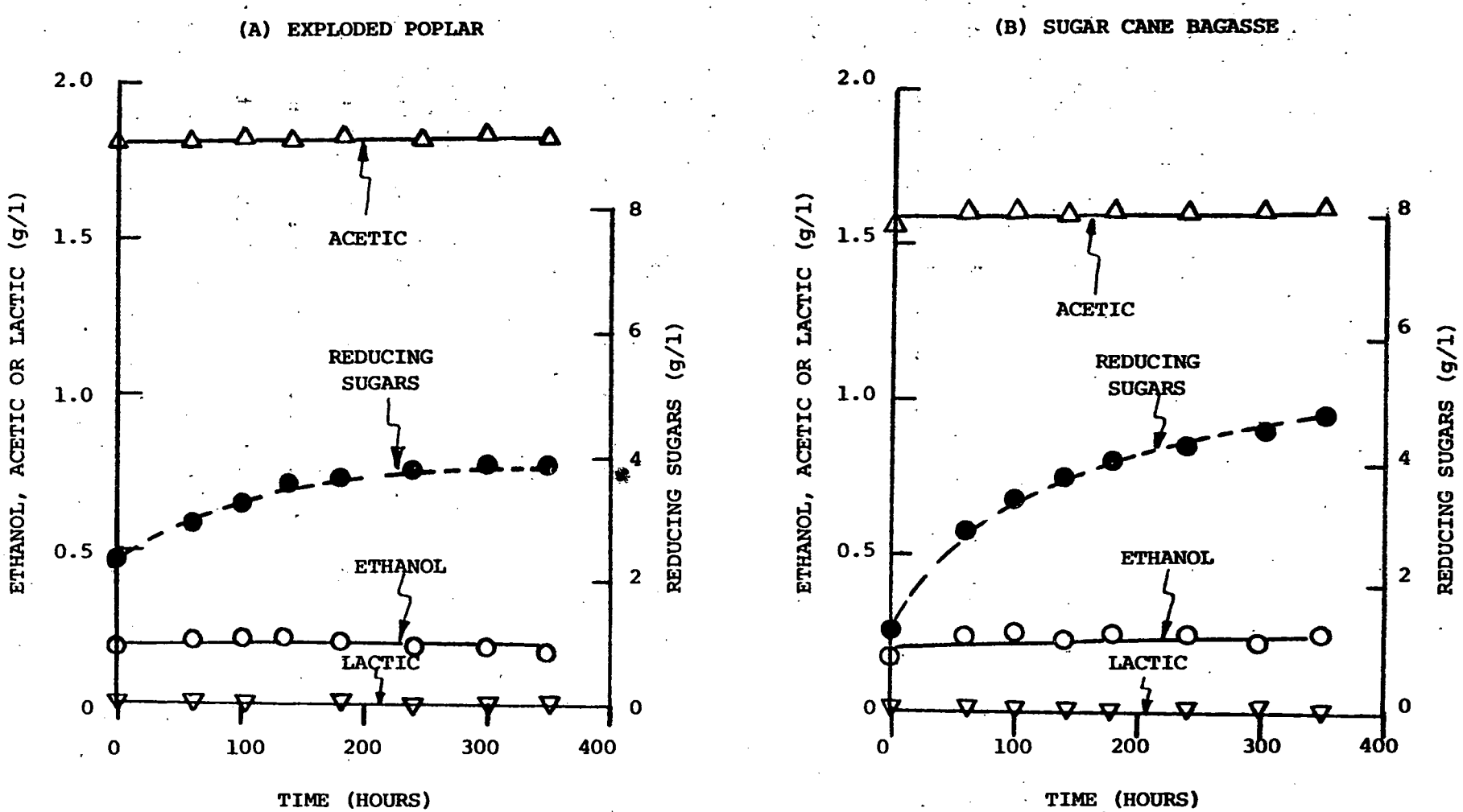
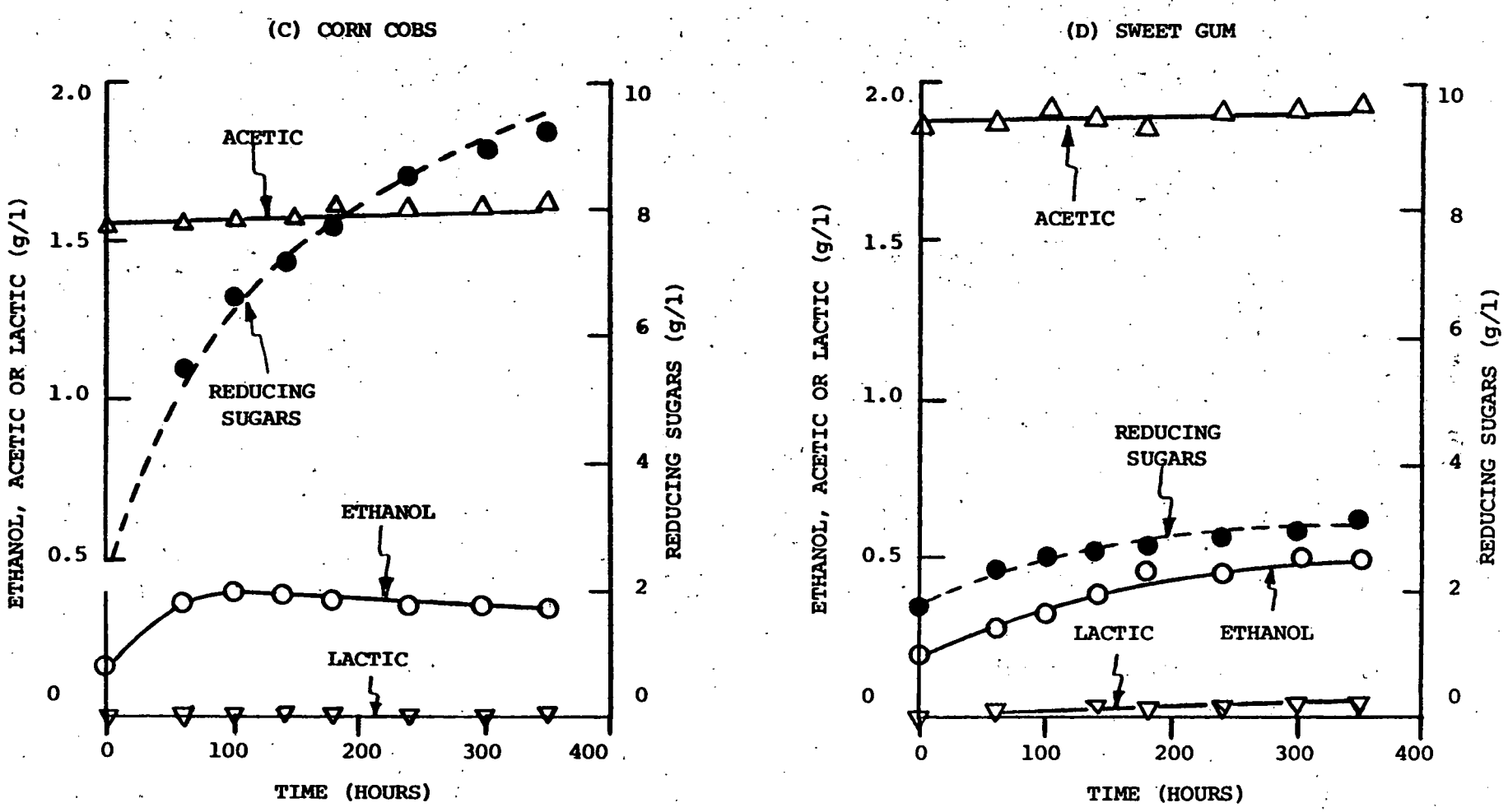
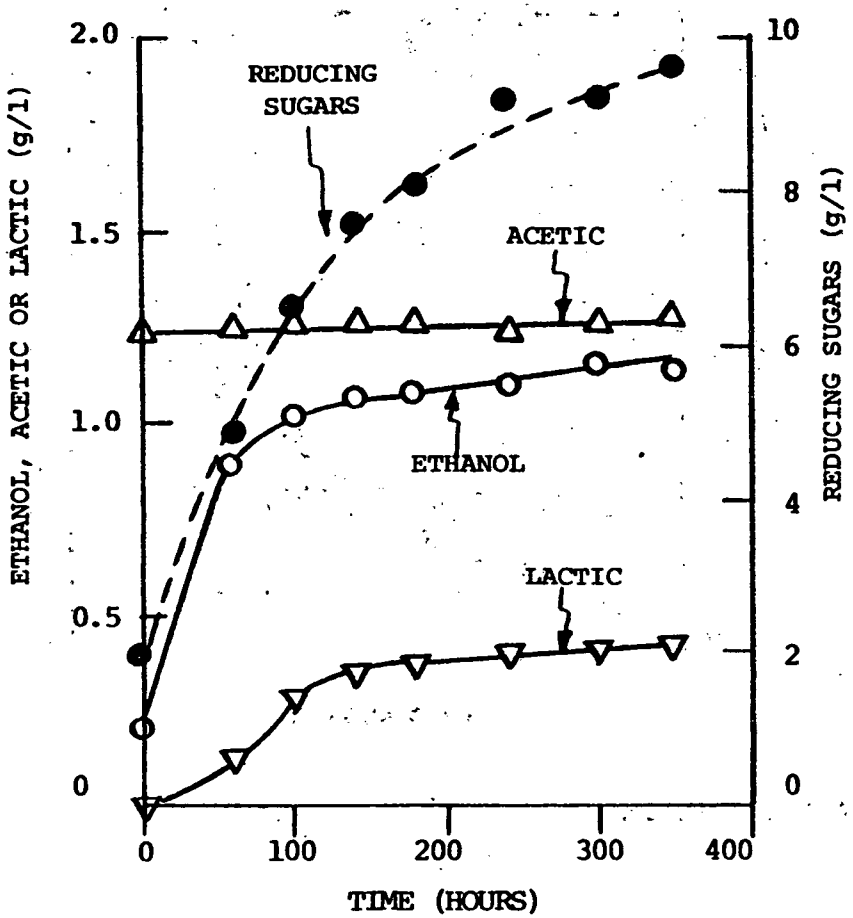


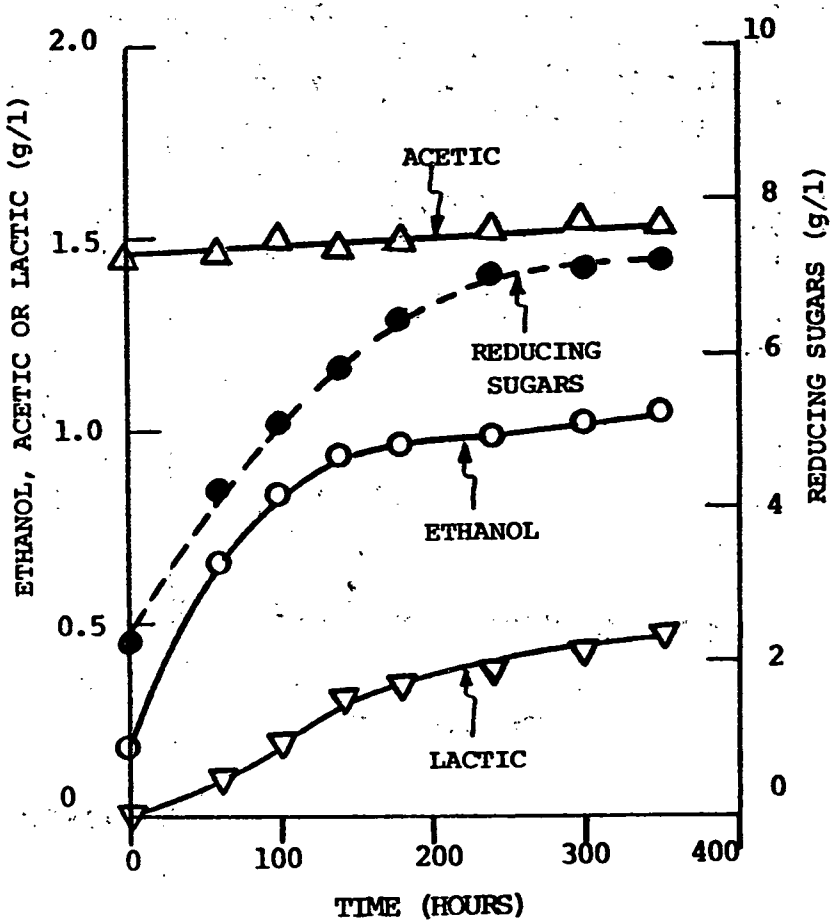
FIGURE I.B.7: CONTINUED



(E) RICE STRAW



(F) WHEAT STRAW



these results that straws are the best substrates among these biomasses tested. Since the production of ethanol is growth associated, one might use this index for growth of the organism. If one follows this rationale, we conclude that strain S-4 cannot metabolize sugar cane bagasse and exploded poplar. This may be due to the high lignin content or other toxic substance in these materials. Similarly, sweet gum tree sawdust and corn cob support only poor growth of strain S-4. The particle size of sweet gum tree sawdust is very large, up to 5 mm or more, and thus it can be explained by the limiting of surface area. The corn cob used in this experiment contains very fine particles and was not limited by surface area but by inhibitory effect of toxic substance, maybe lignin. The rapid accumulation of reducing sugar may be due to the extracellular cellulase reaction.

The best biomass, rice straw, produced 1.17 g/l of ethanol, 0.42 g/l of lactic acid and 9.6 g/l of reducing sugars. After acid hydrolysis, the actual concentration of reducing sugars was found to be 16.5 g/l. This represents a total of 18.1 g/l of products were accumulated. If one assumes that the carbohydrate contents of 70% and 40% for rice straw and soybean meal, respectively, this is equivalent to a total carbohydrate concentration of 21.5 g/l in the medium. From this total, strain S-4 is able to produce 18.1 g/l of products. This is equivalent to a conversion yield of 0.84 gram

of products per gram of carbohydrate. This yield is quite excellent and shows further encouragement for the project objectives.

4. Future Work

- Continue the packed-bed fermentor to achieve high production rate and high product concentration.
- Investigate the reason why fermentation stopped when only 14 g/l of products accumulated. Examine whether sporulation or polymerization of sugars have anything to do with the inhibition, also the possibility of bacteriocin production.
- Further efforts to increase ethanol tolerance.
- Mutation to direct catabolism only to ethanol.

C. ETHANOL PRODUCTION USING XYLOSE BY HG-2 AND CORN STOVER DEGRADATION BY MIXED CULTURE OF C. THERMOCELLUM AND HG-2

1. Introduction

One of the problems associated with the use of *Clostridium thermocellum* to produce ethanol from biomass is its inability to metabolize the degradation products arising from hemicellulose. In order to effect a more complete utilization of biomass, we have initiated studies on the metabolism of xylose using an anaerobic and thermophilic organism designated as "HG-2." This microorganism was isolated by personnel in our laboratory, but has not yet been identified. Some of the interesting properties of isolate HG-2 include its ability to metabolize pentose and hexoses at anaerobic and thermophilic conditions. These latter traits are particularly significant with respect to our overall objective of effective biomass utilization. Specifically, it offers us a potentially compatible mixed microbial system where the cellulolytic activities of *Clostridium thermocellum* are employed to produce ethanol and reducing sugars (xylose) from the cellulosic biomass and the metabolic activities of HG-2 to produce ethanol from hemicellulose degradation products. Therefore, during the past contract period, we have also initiated mixed culture studies using these two organisms to examine the feasibility for such a biomass utilization scheme.

2. Materials and Methods

Pure culture studies using HG-2 were performed in anaerobic shake flasks at 60°C. In these studies, either xylose or glucose was used as the carbon source. Substrates utilization were measured using the DNS method for reducing sugars which has been reported in previous progress reports. Adaptation studies to select higher ethanol tolerant strains of HG-2 were also performed. The protocol for adaptation and selection was identical to that reported in progress report COO-4198-6. Analytical methods such as ethanol and acetic acid concentration determinations were performed through gas chromatography as reported in other progress reports.

Mixed culture studies were performed using the procedure outlined below.

A 10% inoculum of C. thermocellum (S-4) was used in each of two anaerobic flasks containing 300 ml of medium. The medium contained corn stover (25 g/l), soy flour (10 g/l) and salts as in the CM-3 medium. Sodium bicarbonate buffer (12 g/l) was used to maintain the pH at 7.0 and sodium thioglycolate was used to maintain anaerobiosis. After 24 hrs. of growth at 60°C, one flask was re-inoculated with an additional 10% inoculum of HG-2. The second flask served as a control for C. thermocellum alone. Samples were periodically removed and assayed for ethanol, acetic acid, and reducing sugars as described in previous progress reports.

3. Results and Discussion

a. Studies on HG-2 in Pure Culture

Growth and products formation by HG-2 using glucose as the carbon source are shown in Figure I.C.1. Mass doubling time of this organism on glucose was calculated to be about 2 hours (specific growth rate = 0.35 hr^{-1}). As can be seen from Figure I.C.1 that both acetic acid and ethanol are produced and appear to be associated with growth. Final acetic acid and ethanol are produced and appear to be associated with growth. Final acetic acid and ethanol concentrations were respectively 2.8 and 1.5 g/l. In view of this ratio of acetic acid to ethanol, we conclude that other catabolic product(s) must also be produced. Unfortunately, we did not perform analysis on these other products. One could postulate, for example, lactic acid which is at the same oxidation-reduction state as ethanol being one of the other major products of catabolism.

When xylose was used as the carbon source for HG-2, results of growth and products formation obtained are shown in Figure I.C.2. Here again, the mass doubling time was calculated to be about 2 hours (specific growth rate = 0.35 $hour^{-1}$). It is interesting to note that the results in this figure indicate that the final ethanol and acetic acid concentrations were nearly the same. Specifically, a final ethanol

FIGURE I.C.1: GROWTH AND PRODUCT FORMATION BY HG-2 ON GLUCOSE

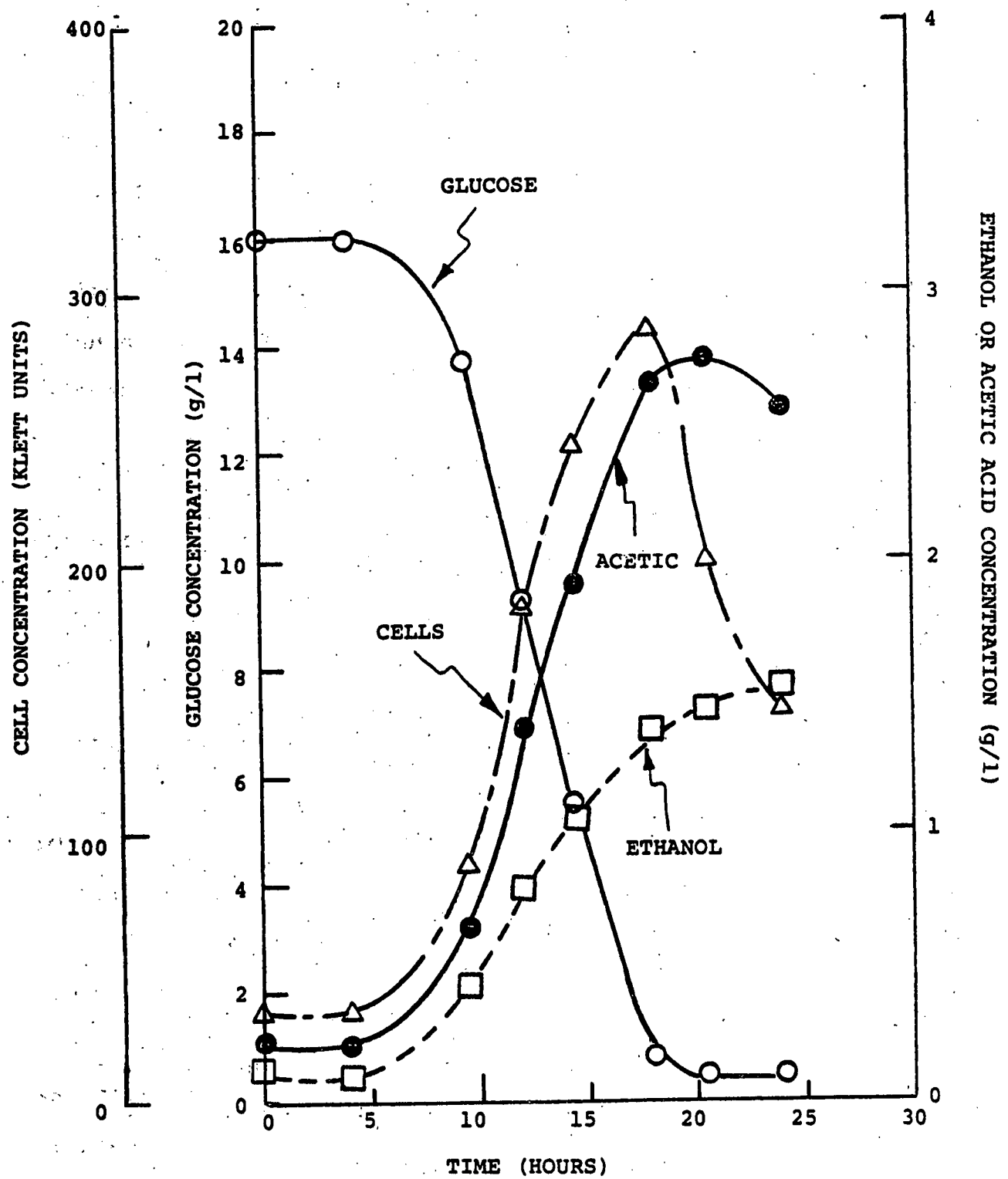
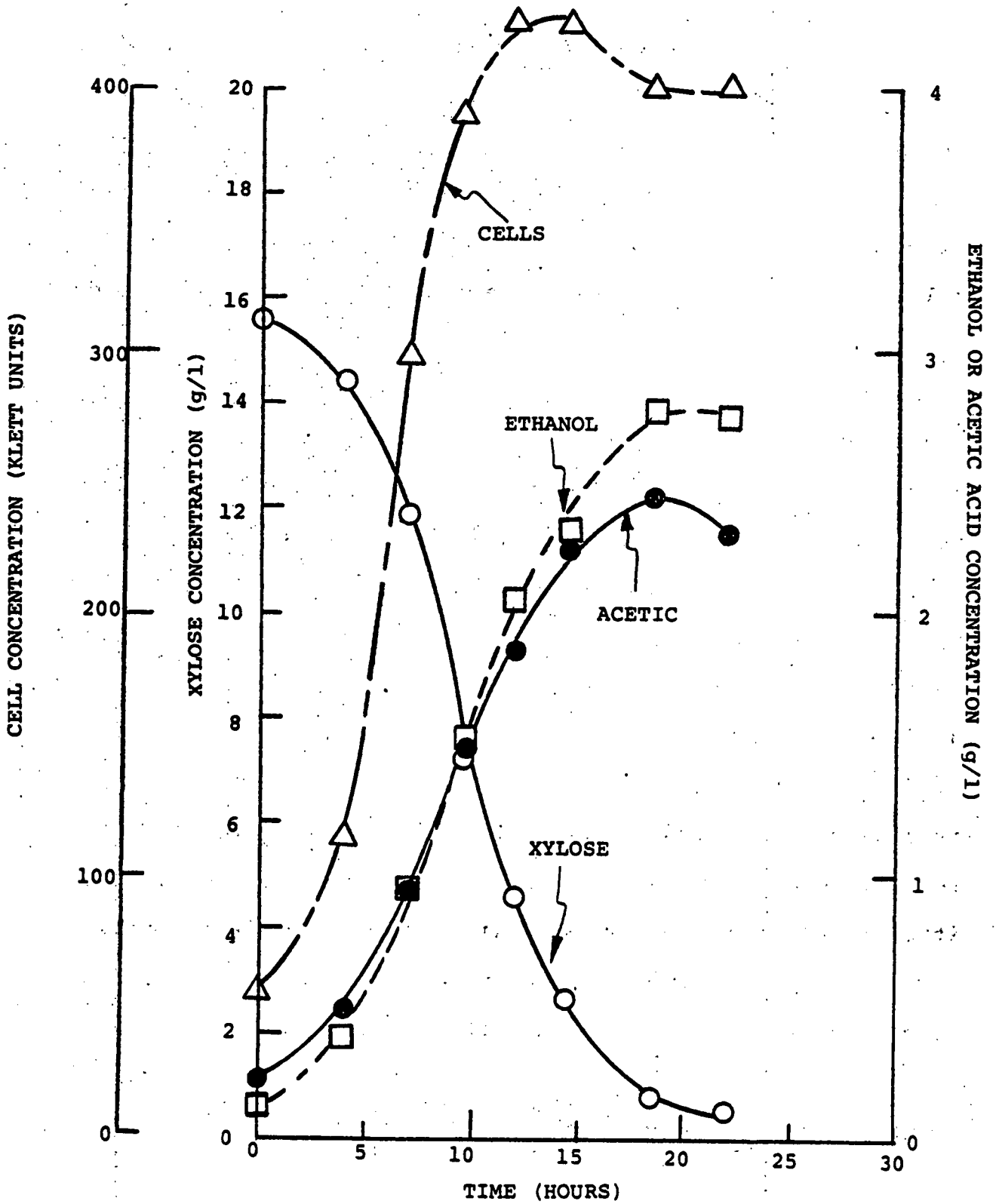


FIGURE I.C.2: GROWTH AND PRODUCT FORMATION BY HG-2 ON XYLOSE



concentration of 2.8 g/l and acetic acid concentration of 2.5 g/l were attained.

In view of our previous experiences with Clostridium thermocellum with respect to its tolerance to ethanol, we decided to embark immediately to a selection program with HG-2 to increase its tolerance to end products. It was rationalized that we should attempt to isolate higher product tolerant strains of HG-2 before attempting to perform more detailed characterizations on this organism. Therefore, an adaptation and selection program was initiated to isolate higher ethanol tolerant strains of HG-2. The results from these studies are shown in Figure I.C.3. It can be seen that the parental strain of HG-2 exhibits a 50% growth inhibition at an ethanol concentration of 1.8% (V/V). After adaptation and six transfers in higher alcohol concentrations, an isolate designated as HG-3 was obtained. This new strain now exhibits a 50% growth inhibition at an ethanol concentration of 2.6% (V/V). We might add that this selection procedure was achieved over a period of three weeks. These results reinforce us as well as being encouraging in demonstrations on rapid capabilities in the isolating mutants with the desireable properties.

During the selection studies with HG-2, the tolerance of this organism to acetate was also examined. These results are shown in Figure I.C.4. It is interesting to note that the parental strain of HG-2 exhibited a higher

FIGURE I.C.3: ETHANOL TOLERANCE OF PARENT (HG-2) AND ISOLATE HG-3

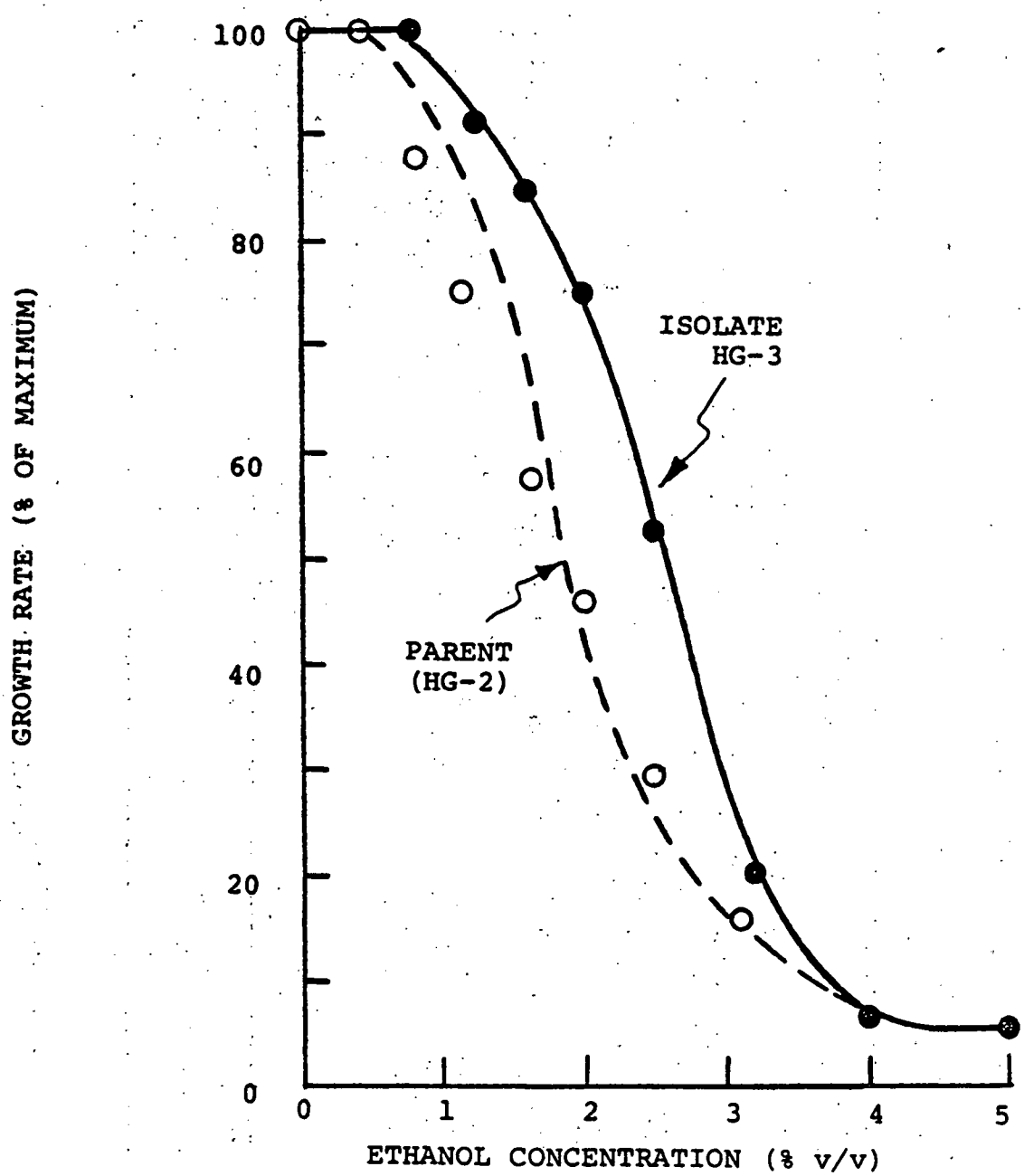
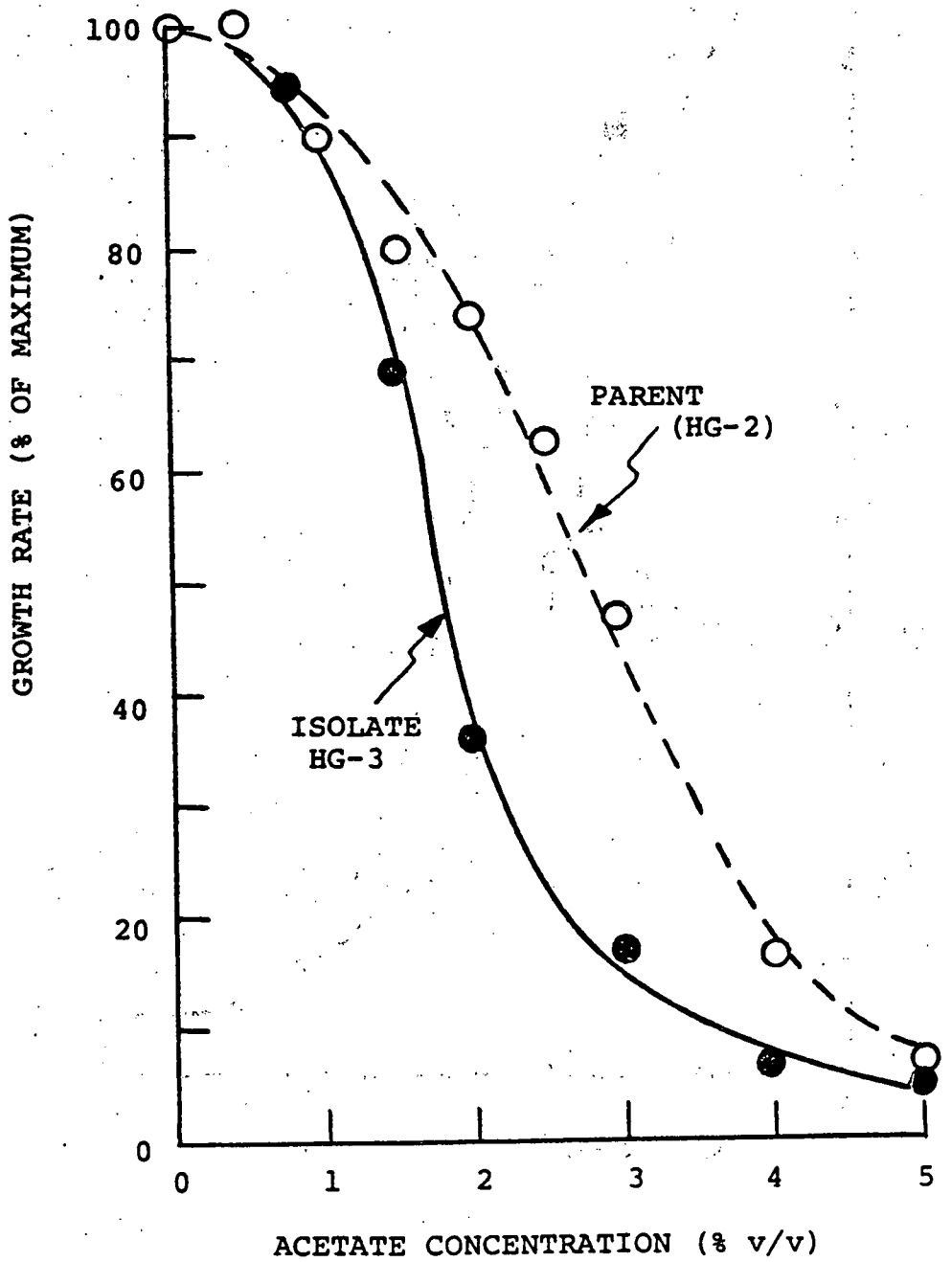


FIGURE I.C.4: ACETATE TOLERANCE OF PARENT (HG-2) AND ISOLATE HG-3



tolerance to acetate than the new isolate HG-3. For example, 50% growth inhibition of the parental strain occurred at an acetate concentration of 2.9% (V/V), whereas the new isolate HG-3 exhibited the same growth inhibition at 1.8% (V/V) of acetate. At this moment, we do not have a logical explanation as to this observation. Further studies will be required to delineate the implication of such a behavior.

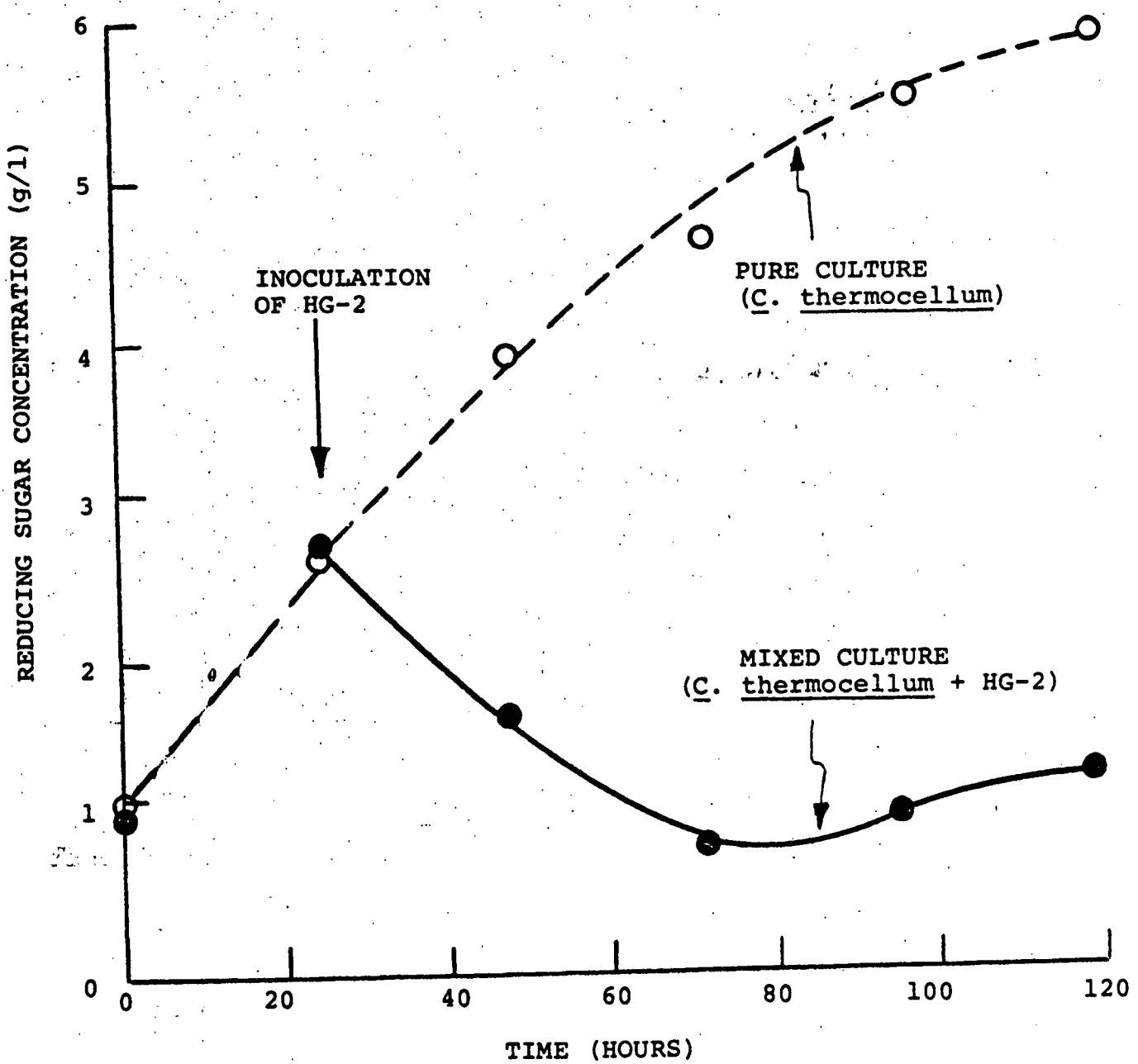
b. Studies on Corn Stover Degradation by Mixed Culture of Clostridium thermocellum and HG-2

Studies using mixed culture of C. thermocellum and HG-2 were performed to obtain preliminary indications as to their environmental compatibilities. It is our objective to pursue this approach in more detail should positive results be found. These preliminary findings will now be presented.

As mentioned in the Materials and Methods section in this report, Clostridium thermocellum (S-4) was allowed to grow in anaerobic shake flasks using corn stover as the carbon source. After 24 hours of growth, HG-2 was then inoculated. The behaviors of the pure culture (C. thermocellum) and mixed culture are shown in Figure I.C.5, I.C.6 and I.C.7.

In Figure I.C.5, the fermentation time profiles with respect to the formation of reducing sugars in pure and mixed culture are shown. It can be seen that the production of soluble reducing sugars by pure culture of C.

FIGURE I.C.5: REDUCING SUGAR ACCUMULATION FROM CORN STOVER BY PURE CULTURE OF C. thermocellum AND MIXED CULTURE OF C. thermocellum and HG-2



thermocellum continues to increase throughout the course of the fermentation. On the other hand, when HG-2 was inoculated, one observes a decrease in the reducing sugars as fermentation progresses. At the termination of the study, the residual reducing sugar in the mixed culture system was less than 1 g/l. These results indicate that the two organisms can maintain their biological activities in a compatible fashion.

During the course of these fermentations, concentrations of acetic acid and ethanol were also determined. Shown in Figure I.C.6 is the time profile of acetic acid formation for the pure and mixed culture systems. It can be seen that for C. thermocellum in pure culture, acetic acid formation reached a maximum value of 1 g/l at about 50 hours. However, for the mixed culture, the rate of acetic acid formation was significantly greater than that found for the pure culture. Furthermore, production of acetic acid continued up to 70 hours of fermentation attaining a final concentration of 3 g/l.

The behaviors on the production of ethanol by the pure and mixed culture are similar to that found for acetic acid. When C. thermocellum was grown as a pure culture, ethanol formation continues up to 100 hours of fermentation. When a mixed culture was used, the rate of ethanol formation as well as the final concentration attained were

FIGURE I.C.6: ACETIC ACID PRODUCTION FROM CORN STOVER BY PURE CULTURE OF C. thermocellum AND MIXED CULTURE OF C. thermocellum AND HG-2

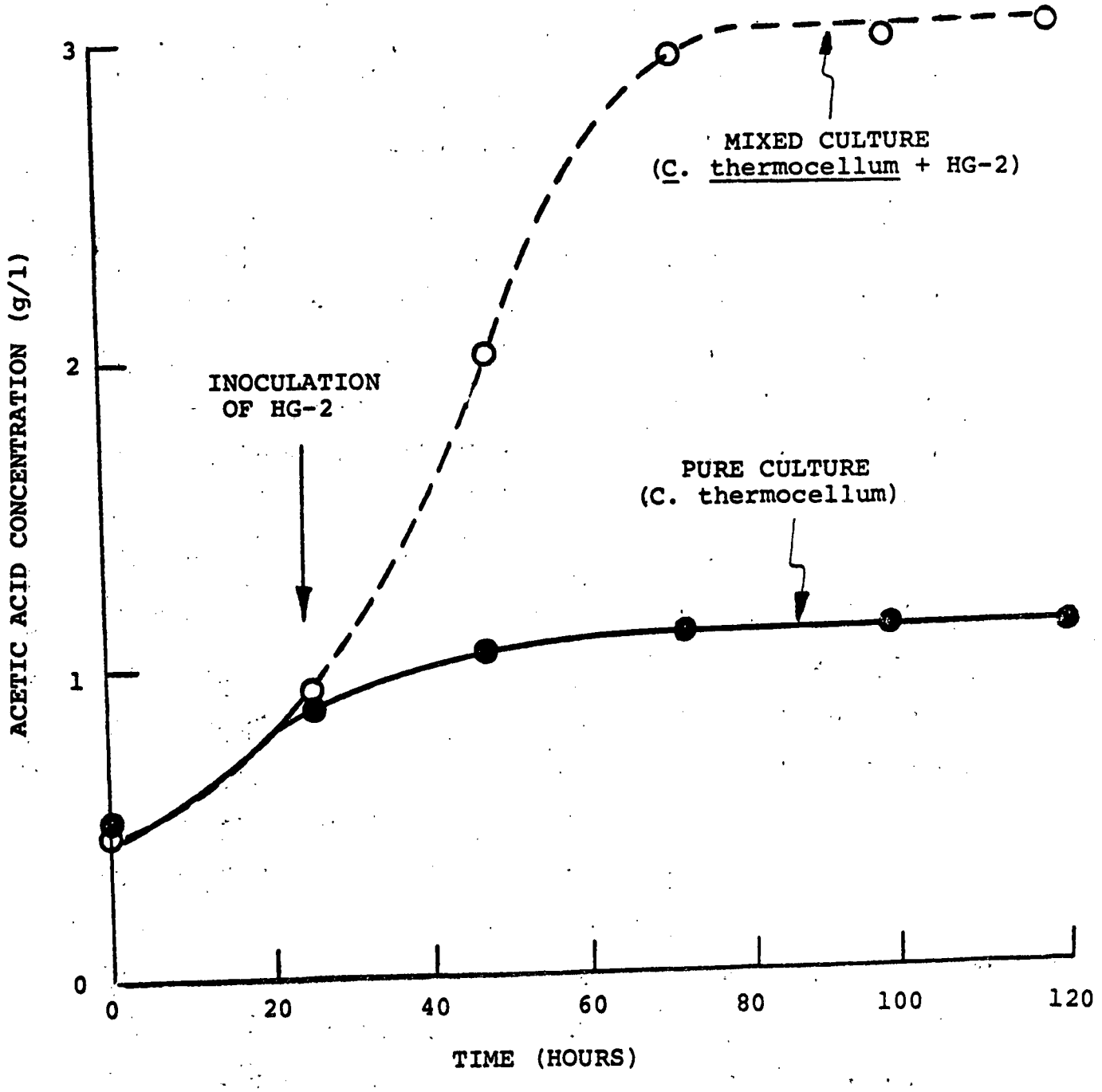
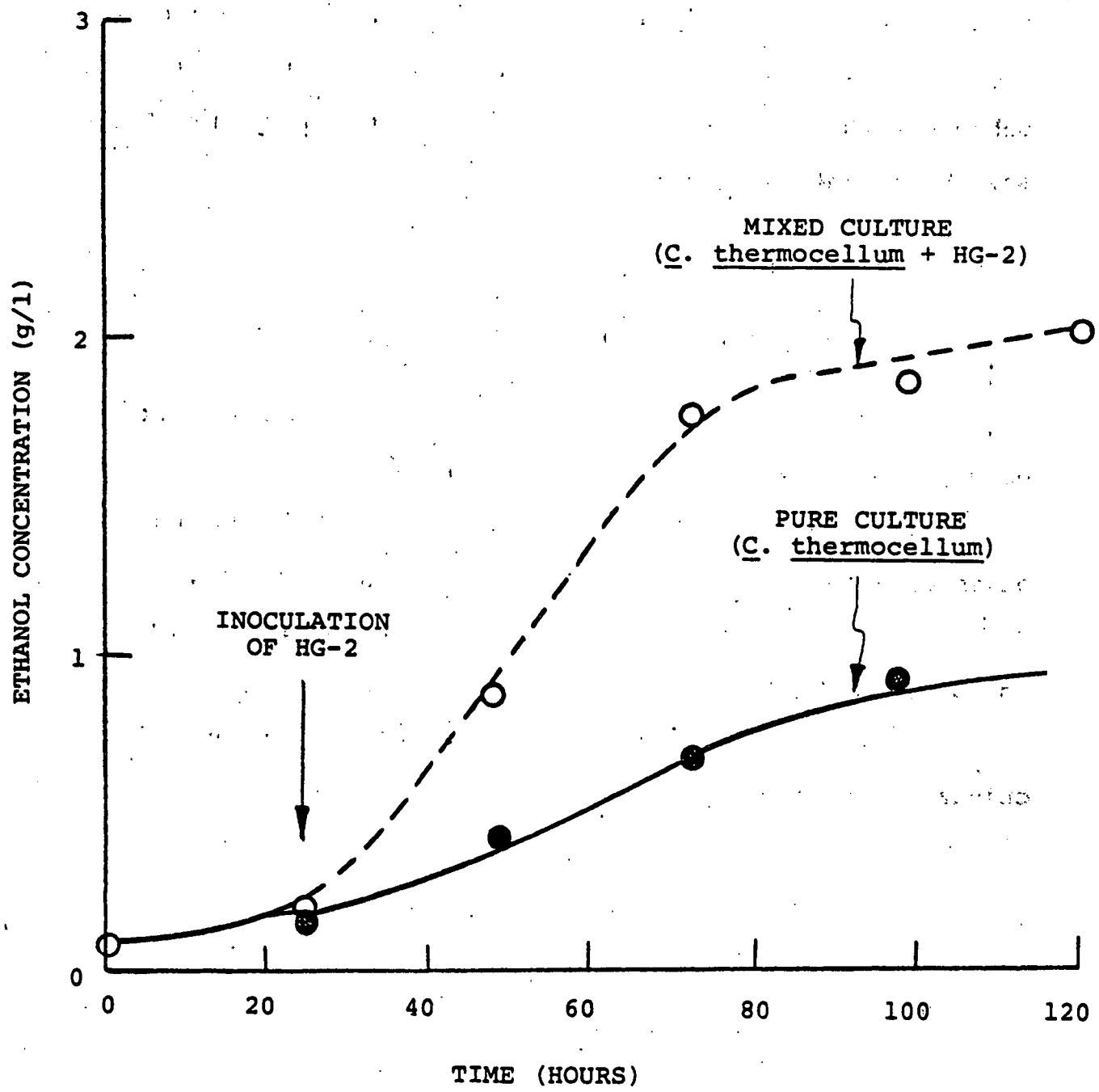


FIGURE I.C.7: ETHANOL PRODUCTION FROM CORN STOVER BY PURE CULTURE OF C. thermocellum AND MIXED CULTURE OF C. thermocellum AND HG-2



both significantly greater than that found in the pure culture system. In view of the results from the reducing sugar time profiles (Figure I.C.5), we conclude that HG-2 can actively metabolize the degradation products from corn stover as a result of the cellulolytic activities of C. thermocellum. These results are encouraging since they indicate no abnormal behaviors when a mixed culture of C. thermocellum and HG-2 are cultivated using a cellulosic biomass.

4. Future Work

Our future studies in this area consist of:

- Further adaptation, mutation and selection of HG-3 to increase its ethanol tolerance.
- A mutation program to effect direct catabolism of HG-3 to produce ethanol as the only product.
- Further exploration using mixed cultures to increase rate and product concentration.
- Determine product yields of HG-2 and mixed culture of C. thermocellum and HG-2.

D. GENETIC MANIPULATIONS

1. Search for Phages

More attempts were made to isolage plaque-forming units to test the transfectability of C. thermocellum, as described in the last progress report (No. 7). The filtrates used as inocula were obtained from two sources. One came from a digestor operated by the Meat Animal Research Center, Nebraska. This digestor is fed with cattle manure and operates at 55°C. The other sample came from a thermophilic digestor in Illinois which utilizes cellulosic agricultural waste. Neither of these yielded plaque formers.

The possibility that a plaque former could be obtained from a lysogenic strain of C. thermocellum was examined also. Filtered supernatants from cultures of C. thermocellum obtained from different sources were cross-checked for plaque-forming ability against each of the strains by an agar spot test. Filtrates from cultures which had been disrupted with chloroform, as well as from cultures which had not, were used. No plaques were observed.

A test was made to determine if any of the strains could be induced to lyse by agents commonly known to induce lysogens in other bacteria. In one experiment a range of mitomycin C concentrations increasing by two-fold steps, between 1/16 to 16 μg/ml was added to growing cultures of each of the C. thermocellum strains. The optical density of these was followed until growth stopped (Figure I.D.1.1).

Figure I.D.1.1. Treatment of growing cultures of C. thermocellum strains with mitomycin C.

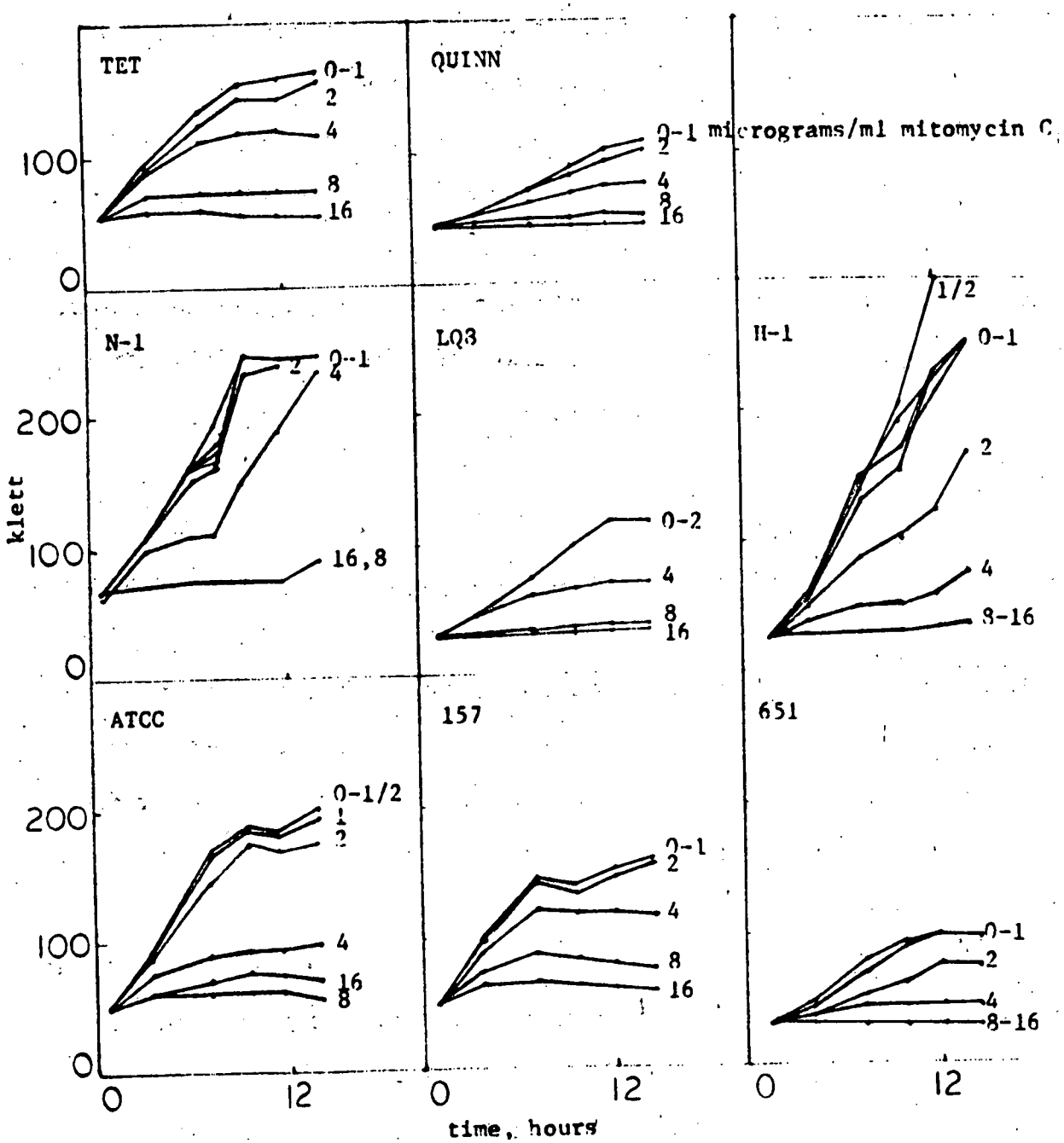
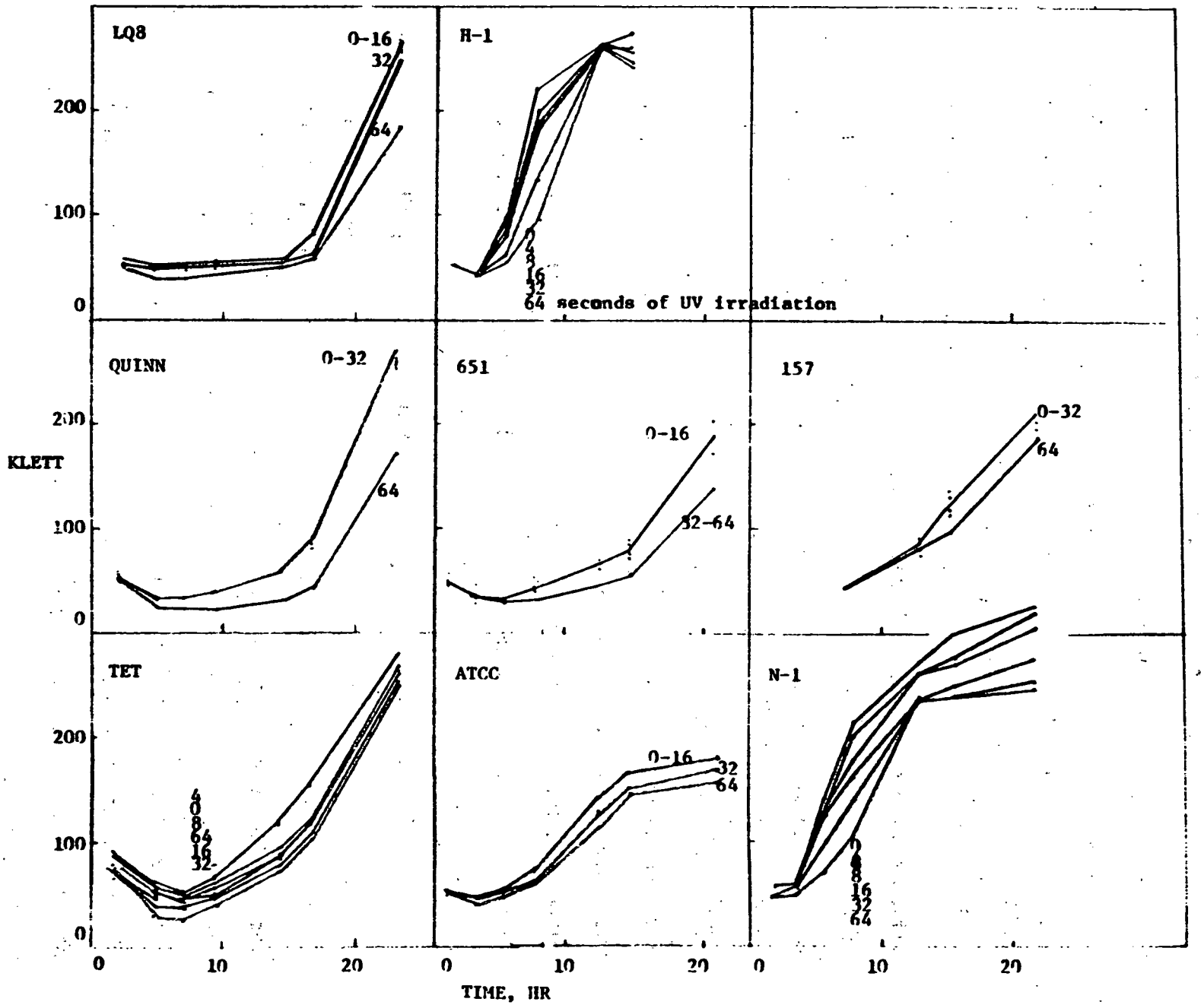


Figure I.D.1.2. Effect of U.V. radiation on the subsequent growth of C. thermocellum strains



In no case was lysis of the culture induced.

The same experiment was carried out using ultraviolet light to irradiate washed cells. The cells were kept in the dark after irradiation and used to inoculate tubes of fresh mediums. No dose given to any of the strains induced lysis (Figure I.D.1.2).

To determine if phage-induced lysis was occurring without gross cultural lysis, the supernatants of mitomycin-treated (4 μg/ml) and untreated C. thermocellum cultures were examined by electron microscopy. The cultures were cleared by low-speed centrifugation and then the supernatants were pelleted and washed by high-speed centrifugation. The pellets were suspended in a small volume of water and negatively stained for examination. While phage-like particles (70 nm icosohedrons) were observed, as has been observed in other occassions, no increase in their numbers resulted from mitomycin C treatment of the cultures.

2. Sensitivity to Antibiotics and Metal Salts

A qualitative examination of the sensitivity of each of the C. thermocellum strains on hand, to 65 metal salts and antibiotics, was made by placing impregnated discs on overlays of seeded agar. After incubation, the presence or absence of an inhibitory zone was noted. Qualitative differences in sensitivity, which could be used as markers or could be extra chromosomally determined are shown in Table I.D.2.1.

Table I.D.2.1

NATURAL RESISTANCE OF C. THERMOCELLUM

COMPOUND	ZONES OF INHIBITION (MM) 651	QUINN	TET	157	ATCC	
SODIUM SILICATE	0	0	0	0	0	
VANADYL SULFATE	9.9	9.6	8.3	10.8	8.5	
ZINC CHLORIDE	8.1	7.4	8.2	0	0	X
SODIUM ARSENATE	N.D.	0	17	0	16.5	X
ANTIMONY POTASSIUM TARTRATE	44	48	38.2	46.6	43	
LEAD NITRATE	0	0	0	0	0	
BERYLLIUM NITRATE	0	0	0	0	0	
FERROUS CHLORIDE	10.3	8	11.2	8.7	10	
FERRIC CHLORIDE	10.2	10.6	10.1	10.3	10.3	
COBALT CHLORIDE	11.2	0	0	0	0	X
NICKEL CHLORIDE	7.6	0	0	0	0	X
CUPROUS CHLORIDE	9.6	9	9.2	9.2	14.4	
CUPRIC CHLORIDE	13.7	14.2	13.6	14.1	8.6	
THALLIUM ACETATE	15.2	14.2	18	13.4	0	X
SODIUM TUNGSTATE	7.2	N.D.	8.4	N.D.	0	X
SULFANILAMIDE	0	0	0	8.4	0	X
KANAMYCIN	0	0	0	25.2	0	X
TRIMETHOPRIM	0	0	0	0	14	X
CERULENIN	0	0	0	0	38	X

ANOTHER 30 CPDS TESTED WITHOUT SIGNIFICANT DIFFERENCES.

3. Mating

Resistance factor transfer by mating on plates has been reported to occur between strains of C. perfringens (Brefort et al., Plasmid 1:52-66, 1977). The possibility that such a resistance could be transferred from a C. perfringens strain (CP600) used by Brefort and co-workers to any of our strains of C. thermocellum by inter-species mating was examined. Mating was carried out on plates at 52°C. Selection for antibiotic resistance in C. thermocellum was done at 60°C. No evidence of resistance factor transfer to any C. thermocellum strain was obtained.

4. Future Work

- Effect of temperature on phage recovery from C. thermocellum.
- Initiation of construction of cloning vectors.

E. DEREGULATION OF CLOSTRIDIAL CELLULASE

1. Introduction

In our previous progress report (COO-4198-7), we presented several interesting results that required further investigation. One was that *Clostridium thermocellum* can grow and produce cellulase on glucose if elevated levels of yeast extract are used in the medium. In this report, we shall present the results of experiments in which a range of yeast extract concentrations were tested in both cellobiose and glucose containing media and growth and cellulase production were measured.

We have further studied growth and enzyme production on glucose. Glucose consumption, growth and cellulase production have been measured in media containing glucose, cellobiose, and mixtures of the two. Our objective is to determine if glucose plays a regulatory role in cellulase production.

Our attempts to devise a chemically defined medium will be described. Various substitutions for yeast extract were tested in cellobiose medium. These include mixtures of vitamins and amino acids and a protein hydrolysate.

Our cellulase-overproducing mutant, AS-39, described in earlier reports, was subjected to further mutation by physical and chemical agents. Two promising second generation mutants have been isolated and characterized.

2. Materials and Methods

a. Organism

C. thermocellum ATCC 27405 and its mutant AS-39 were used in this study.

b. Anaerobic Methods

See progress report COO-4198-7, page 71.

c. Cultural Conditions

The organisms were grown and maintained on GS medium which is described in progress report COO-4198-7, page 71. In the nutritional studies, yeast extract in the GS medium was replaced by various mixtures of vitamins, amino acids, and a protein hydrolysate. Growth was measured as absorbance using a red filter in a Klett instrument.

d. Analysis of Cellulolytic Activity

Cultures were centrifuged at 7850 x g for 10 minutes; cellulolytic activities of the supernatants were determined as follows:

1. TNP-CMCase Activity

One ml of supernatant is added to 2 ml of 1% TNP-CMC suspension in 0.1 M citrate buffer (pH 4.5). Zero-

time activity is determined by Millipore-filtering immediately after the addition of the supernatant. A control is also run in which the supernatant is replaced by 1 ml of the citrate buffer. After thorough mixing, the experimental tubes are incubated at 60°C for 1 hr. The contents are then Millipore filtered; absorbance of the filtrate is measured at 344 nm. The value at zero-time is subtracted from that at 60 minutes. Activity is expressed in units which are defined as follows:

$$1 \text{ unit} = 0.01 \text{ O.D.}_{344} \text{ increase/min.}$$

2. Avicel Hydrolyzing Activity

One ml of supernatant is added to 2 ml of 1% Avicel (Type RC-591, FMC Corp.) suspension in 0.1 M Na-citrate buffer (pH 4.5). Zero-time activity is determined by Millipore-filtering immediately after the addition of the supernatant. A control with 1 ml of buffer instead of supernatant is also run. After thorough mixing, the tubes are incubated in a boiling-water bath for 5 minutes, then cooled immediately; absorbance at 550 nm is read. The increase in absorbance from 0 to 2 hours is measured. The amount of reducing sugar liberated as glucose is read off a glucose standard curve. A unit of Avicel hydrolyzing activity is defined as follows:

$$1 \text{ unit} = 1 \ \mu\text{mole glucose/min.}$$

3. Filter Paper Activity

Filter paper activity was determined using a modified method (Montenecourt *et al.*, *Biotechnol. Bioeng.*, *XX*, 297, 1978) which employs filter paper discs as the substrate. One ml of supernatant is added to an 18 mm test tube containing 0.5 ml of 0.05 M Na-citrate buffer (pH 4.8). The disc, which has a 1/2 inch diameter, weight of 46-48 mg and is 95% α-cellulose, is placed at the bottom of the tube; after vortexing, the tubes are incubated at 50°C for 1 hour. After incubation, 3 ml of DNS-reagent is added; the tubes are then incubated in boiling water for 5 minutes. At that point, 16 ml of distilled water is added to the tubes and absorbance at 550 nm is read. A zero-time sample is prepared in which the DNS-reagent is added immediately after the filter paper disc and boiled. A control tube without a filter paper disc is also run to correct for any reducing sugars present in the supernatant. The increase in absorbance between zero-time and 1 hour is determined. The amount of reducing sugar liberated as glucose is read off a glucose-standard curve prepared by the DNS-reagent method. A unit of filter paper hydrolyzing activity is described as follows:

$$1 \text{ unit} = 1 \ \mu\text{mole glucose/min.}$$

DNS-Reagent Method

To prepare the glucose standard curve, three ml of DNS-reagent is added to 1 ml of glucose solutions whose concentrations range from 0.1 - 1.0 mg/ml. The tubes are incubated in boiling water for 5 minutes. After cooling, absorbance at 550 nm is read.

3. Results and Discussion

A number of experiments were done to study the possibility of glucose repression of cellulase production in C. thermocellum AS-39. We used GS medium described in our previous progress report, replacing or varying the cellobiose content where indicated. The organism grew and produced cellulase in medium containing 1% glucose after a lag phase of 50 h (Figure I.E.1). Increasing the glucose or cellobiose concentration to 2% did not stimulate either growth or cellulase production. There was no inhibition of growth or cellulase production when 1% glucose was added to medium containing 1% cellobiose (Table I.E.1). When the specific production of cellulase was calculated, there was no significant difference between that produced on cellobiose, glucose or cellobiose plus glucose. It thus appears that glucose repression of cellulase production does not exist in this organism. These findings are important because the production of most cellulases is repressed

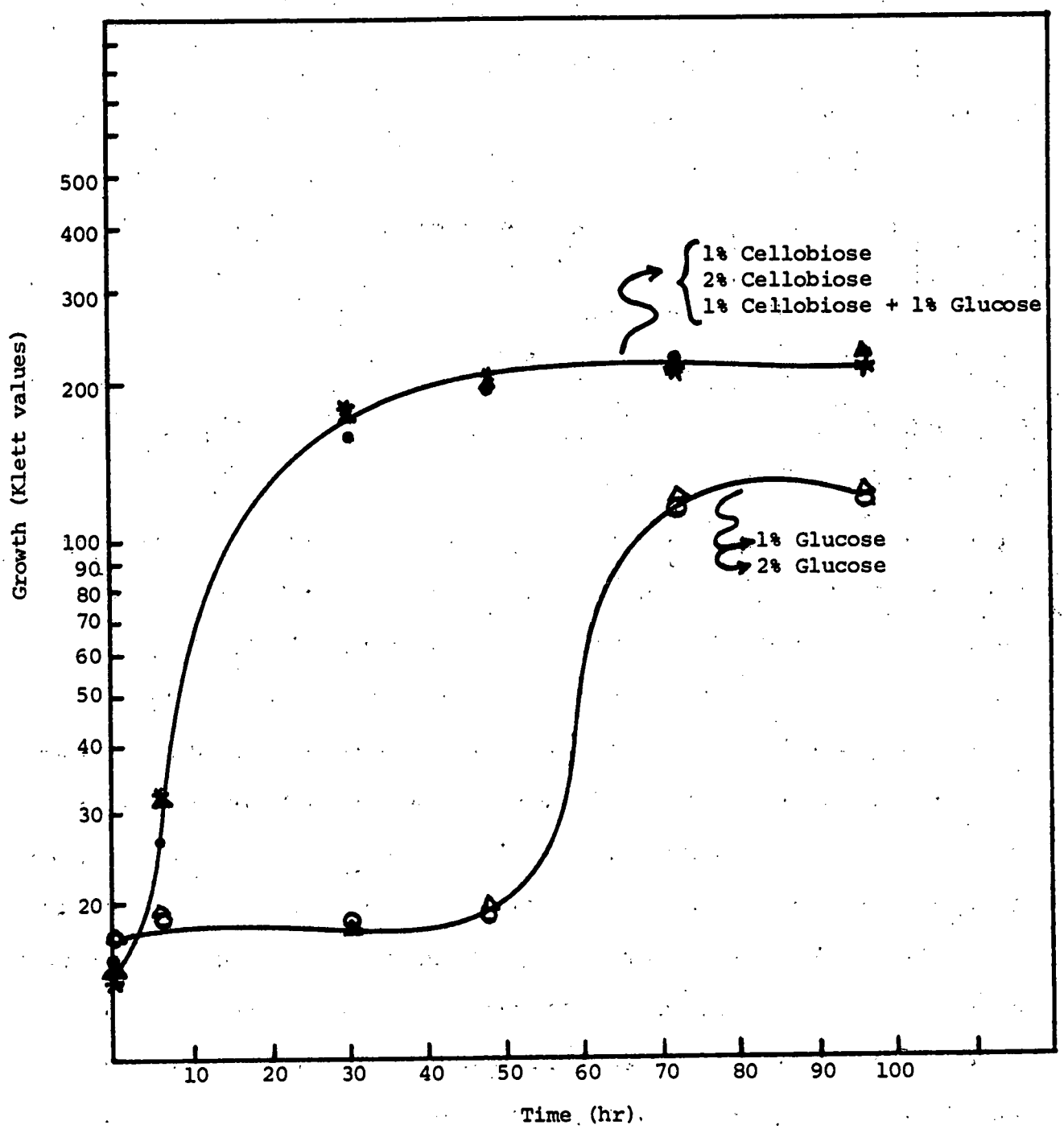


Fig. I.E.1. Growth of Clostridium thermocellum mutant AS-39 on cellobiose, glucose and mixtures of the two.

Table I.E.1. LACK OF GLUCOSE REPRESSION OF CELLULASE[1] IN AS-39

CELLOBIOSE (g/l)	GLUCOSE (g/l)	RESIDUAL SUGAR[2] (g/l)	ENZYME PRODUCTION (units/ml sup.)	GROWTH (Klett units)	SPECIFIC ENZYME PRODUCTION [(u/ml·Ku) x 10^3]
10	0	3.8	1.12	221	5.1
0	10	0.8	0.61	139	4.4
10	10	3.7	0.98	217	4.5
20	0	5.0	1.11	217	5.1
0	20	5.2	0.53	128	4.1

1) TNP-CMCase assay at 168 hr; 1 unit = increase in OD_{344} of 0.01/min.

2) Reducing sugar as glucose at 200 hrs.

3) Maximum Klett values up to 168 hrs.

by the products of the enzymatic reaction. Such repression by glucose, for example, limits cellulase production in Trichoderma reesei.

Carbon source utilization was also studied in this experiment; as can be seen in Table I.E.1, 92% and 75% of the glucose was utilized in the 1% and 2% glucose media respectively. In the case of cellobiose media, reducing sugar utilization was 62% at 1% cellobiose and 75% at 2% cellobiose. In medium containing both glucose and cellobiose, each at 1%, there were no signs of inhibition of sugar utilization; approximately 80% utilization was observed.

We next examined the effect of various concentrations of yeast extract on glucose and cellobiose fermentations. Figure I.E.2 shows that C. thermocellum AS-39 failed to grow on cellobiose when the yeast extract concentration was 0.05%. Twenty and 40% maximum growth was observed when 0.1% and 0.2% yeast extract, respectively, were used. In the case of glucose, the organism failed to grow when 0.05% and 0.1% yeast extract were used. About 70% of maximum growth was observed when 0.2% yeast extract was used. Thus, our results show that the organism requires more yeast extract to grow on glucose than on cellobiose.

The effect of yeast extract concentration on cellulase production is shown in Table I.E.2. The highest volumetric titers were observed at the highest (0.6%) yeast extract

Fig. I.E.2. Growth of Clostridium thermocellum mutant AS-39 on cellobiose and glucose using different concentrations of yeast extract.

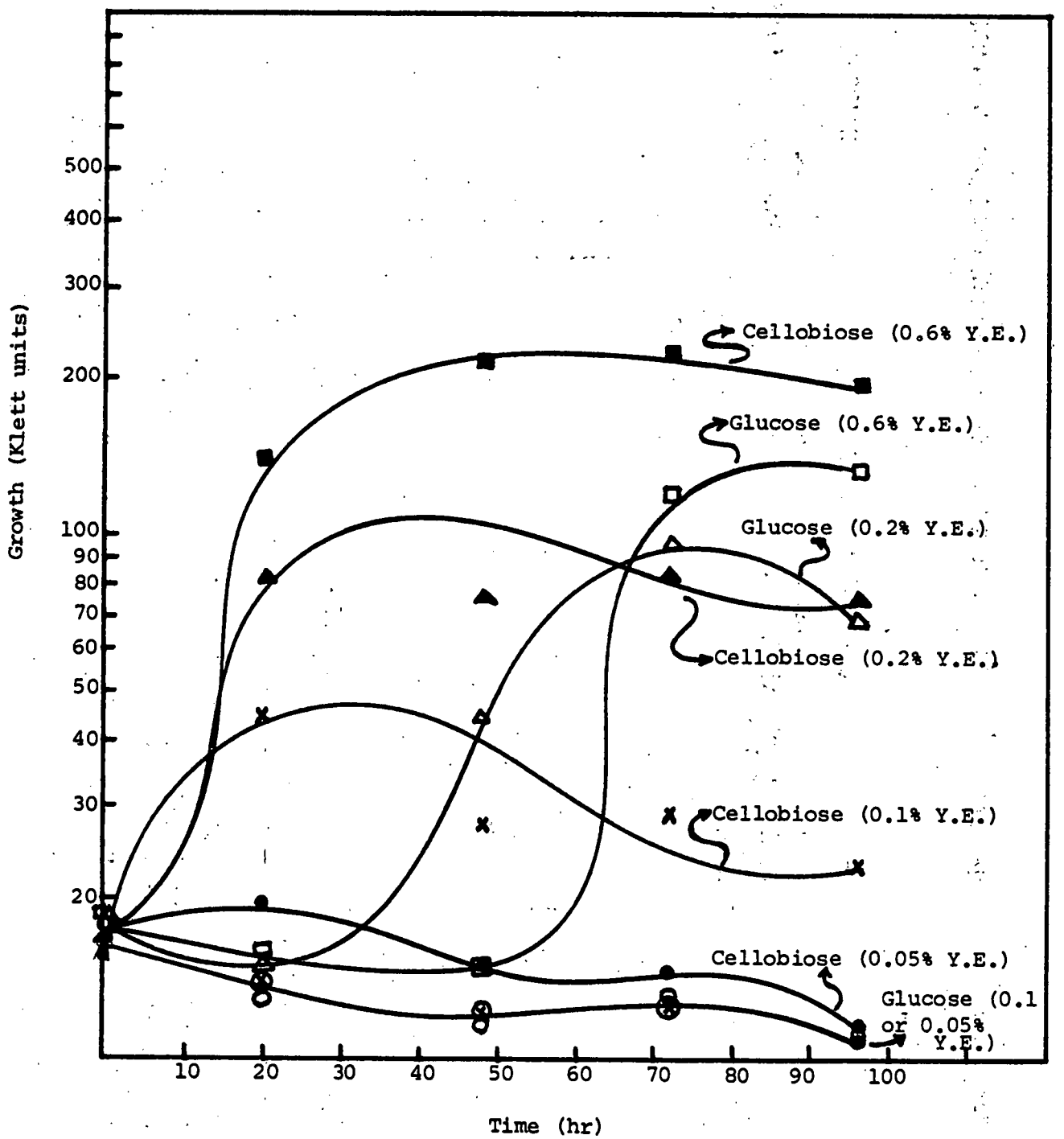


Table I.E.2. TNP-CMCase[1] PRODUCTION BY AS-39 IN CELLOBIOSE AND GLUCOSE

YEAST EXTRACT %	CELLOBIOSE (1%)			GLUCOSE (1%)		
	ENZYME[2] PRODUCTION (units/ml sup.)	GROWTH[3] (KLETT UNITS)	SPECIFIC ENZYME PROD. (units/ml·Ku) x 10^3	ENZYME[2] PRODUCTION (units/ml sup.)	GROWTH[3] (KLETT UNITS)	SPECIFIC ENZYME PROD. [(u/ml·ku) x 10^3
0	0	12	-	0	13	-
0.05	0	20	-	0	13	-
0.1	0.48	45	10.7	0	18	-
0.2	0.87	85	10.3	0.55	98	5.6
0.6	1.74	224	7.8	1.21	120	10.0

1) 1 unit = increase in OD_{344} of 0.01/min.
2) Assays done after 72 hrs of growth.
3) Maximum Klett value up to 72 hrs.

concentrations in the cases of both glucose and cellobiose. Although volumetric titers were higher on cellobiose than on glucose, specific cellulase titers were similar.

To develop a chemically-defined medium, we have continued our nutritional studies testing various defined mixtures as yeast extract replacements. Other authors such as McBee, Quinn, and Alexander have individually reported that C. thermocellum can be grown successfully on yeast extract-free medium supplemented with vitamins alone or together with amino acids. We have tested these yeast extract replacements but have failed to obtain growth after successive transfers of the culture.

The results presented in Table I.E.3 show that, in our hands, McBee's mixture of 5 vitamins failed to support growth in the absence of yeast extract. Furthermore, neither Difco Vitamin-free Yeast Base (glucose NH_4, 3 amino acids, no vitamins) nor Difco Yeast Nitrogen Base without Amino Acids (NH_4, 9 vitamins), no amino acids) replaced yeast extract (Table I.E.4). Poor growth was observed with Quinn's mixture of amino acids and vitamins. The only supplement which gave fair growth was Alexander's mixture No. 2 which contains eight vitamins and no amino acids. Since Alexander has found a simpler mixture of 5 vitamins (Alexander's mixture No. 1) to support growth (personal communication), we used the 120 hr. growth in Alexander's mixture No. 2 to inoculate the experiment described in Table I.E.5.

TABLE I.E.3. Effect of replacement of yeast extract by McBee's vitamin mixture on growth of C. thermocellum AS-39

Supplement	Growth (Klett values)* at hour				
	24	48	72	96	120
None	0	0	0	1	0
Yeast extract	174	218	238	242	228
McBee Vitamin-mixture**	8	8	8	6	4

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

** Thiamine-HCl, 200; riboflavin, 200; calcium pantothenate, 200; pyridoxine, 200; biotin, 2μg/liter.

TABLE I.E.4. Effect of yeast extract replacements on the growth of C. thermocellum AS-39

Supplement***	Growth (Klett values)* at hour				
	24	48	72	96	120
None	11	11	12	7	7
Yeast extract	132	218	218	211	206
Difco Vitamin-free yeast-base	0	0	1	0	0
Difco Yeast-nitrogen base w/o amino acids	6	5	5	3	0
Quinn's mixture****	5	30	39	38	37
Alexander's mixture, No. 2*****	3	48	58	57	71**

* Initial absorbance of the culture medium was subtraced from the Klett values obtained at different times.

** Used as an inoculum for the next experiment (Table I.E.5).

*** All concentrations of vitamins are expressed as µg/liter and the concentrations of amino acids are expressed as mg/liter.

**** Quinn's mixture: Thiamine-HCl (200); riboflavin (200); calcium pantothenate (200); pyridoxine (200); biotin (2); folic acid (8); PABA (4); DL-phenylalanine (80); L-tyrosine (50); L-tryptophan (70); L-cystine (60) L-cysteine (70); and L-methionine (70).

***** Alexander's mixture No. 2: Pyridoxine (1000); niacin (1000); riboflavin (1000); biotin (10); folate (10); thiamine (1000); pantothenate (1000); and B_{12} (10).

TABLE I.E.5. Effect of replacement of yeast extract by Alexander's vitamin mixture on the growth of C. thermocellum.

Supplement	Growth (Klett values)* at hour:				
	24	48	72	96	120
Yeast extract	214	245	235	220	218
Alexander's mixture No. 1***	26	37	40	59	67**

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

** Used as an inoculum for the next two experiments (Tables I.E.6 and I.E.7).

*** Pyridoxine, niacin and riboflavin (1 mg/liter) and biotin and folate at 10 µg/liter.

In Alexander's simple vitamin mixture No. 1, we obtained a low degree of growth (Table I.E.5). Again, the 120 hour growth was used as inoculum for the next experiment. Here we attempted to obtain growth again in Alexander's mixture No. 1, but in addition, we eliminated one vitamin at a time. Table I.E.6 shows that Alexander's mixture No. 1 failed to support growth as did all mixtures containing only 4 vitamins. Thus, sustained growth through multiple passages is not supported by Alexander's mixture of 5 vitamins.

We next returned to Alexander's mixture (No. 2) of eight vitamins which had given a small amount of growth in the experiment of Table I.E.4. As seen in Table I.E.7, we failed to obtain growth in this mixture. On the other hand, when 0.5% casein hydrolysate was added to the vitamins, considerable growth was observed.

In summary, the simplest medium which has supported growth of strain AS-39 is Alexander's mixture No. 1 of eight vitamins plus casein hydrolysate. Further work is underway to confirm this finding and simplify this mixture.

The cellulase-overproducing mutant, AS-39, was subjected to further mutation by ethylmethane sulfonate, nitrosoguanidine, and UV-radiation. Two promising candidates for increased cellulase production were isolated and characterized; they were designated AM-5 and CM-17-3.

TABLE I.E.6: Effects of replacement of yeast extract by Alexander's vitamin mixture on the growth of *C. thermocellum*.

Supplement						Growth (Klett values)* at hour				
Yeast Extract	Pyridoxine	Niacin	Riboflavin	Biotin	Folate	24	48	72	96	120
-	-	-	-	-	-	0	0	0	0	0
+	-	-	-	-	-	162	171	247	241	235
-	-	+	+	+	+	0	0	0	0	0
-	+	-	+	+	+	0	0	0	0	1
-	+	+	-	+	+	1	0	1	0	2
-	+	+	+	-	+	2	0	0	0	1
-	+	+	+	+	+	3	2	0	1	3

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

TABLE I.E.7: Effect of replacement of yeast extract by Alexander's vitamin mixture and casein hydrolysate on the growth of C. thermocellum.

Supplement			Growth (Klett values*) at hour:			
Yeast extract	Alexander's mixture No. 2	Vitamin-free acid hydrolysate of casein	24	48	72	96
+	-	-	238	238	230	196
-	+	-	4	5	2	3
-	+	5 g/liter	9	68	115	137
-	+	10 g/liter	30	69	119	135
-	+	17.5 g/liter	9	65	116	137

* Initial absorbance of the culture medium was subtracted from the Klett values obtained at different times.

** All vitamins were used at the concentrations mentioned in Table I.E.4.

AM-5 was obtained in the following manner. C. thermocellum AS-39 cells grown on CM-3 medium were UV-irradiated for 60 seconds (99% kill) and plated on CM-3 agar containing 2% Avicel and 0.5% cellobiose. After incubation for 8-12 days, 17 colonies showing large clear zones were transferred to Hungate tubes containing 10 ml of CM-3 medium with 0.5% cellobiose. After incubation for 48 hours, growth and TNP-CMCase activity were measured.

CM-17-3 was obtained by EMS mutagenesis followed by the screening of 65,000 colonies on agar plates containing 2% Avicel and 0.05% cellobiose. Thirty colonies were selected for tests in tubes on the basis of clear zone diameters. Of these, CM-17-3 proved to be the best cellulase producer.

Table I.E.8 shows that both CM-17-3 and AM-5 exhibit about 3 times as much TNP-CMCase activity as their parent, AS-39. The table also shows an increase in Avicel and filter paper hydrolyzing activity over AS-39.

4. Future Work

Future studies on this segment of research will include:

- Studying the ability of glucose to support cellulase production after repeated transfers in glucose medium in the absence of cellulose and cellobiose.

Table I.E.8. CELLULASE PRODUCTION BY MUTANT CULTURES

CULTURE	GENERATION	TNP-CMC u/ml. sup.[1]	TNP-CMC specific activity[2]	AVICEL u/ml. sup.[3]	AVICEL specific activity[2]	FILTER PAPER u/ml. sup.[3]	FILTER PAPER specific activity[2]
ATCC 27405	1	0.22	1.1	0.011	0.055	0.012	0.060
AS-39	2	0.61	2.2	0.022	0.080	0.018	0.066
CM-17-3	3	1.6	6.1	0.035	0.14	0.031	0.12
AM-5	3	1.6	5.9	0.041	0.15	0.035	0.13

1) 1 unit = ΔOD of 0.01 per minute

2) [units/ml·Ku] x 10^3

3) 1 unit = 1 μ-mole reducing sugars as glucose per minute

- Continued testing of various vitamins and amino acids to replace the yeast extract requirement.
- Continued mutagenesis and selection for cellulase-hyperproducing mutants.

F. PURIFICATION OF CELLULASE

1. Introduction

As a preface to genetic manipulations and reactor design, efforts are underway to isolate and purify the enzyme(s) responsible for celluloytic activity in *C. thermocellum*.

2. Materials and Methods

a. Preparation of Crude Enzyme

A crude extract of clostridial cellulase was prepared as follows:

Cultures of *C. thermocellum* ATCC 27405 were grown in CM-4 containing 5% cellobiose. Incubation was at 60°C without shaking. After 48 hr , cells were chilled for 15 min and harvested by centrifugation at 18,000 xg for 15 min. The supernatant was decanted and centrifuged again. This clarified broth was filtered through an Amicon Pm-10 filter at ambient temperature. The filter cake was resuspended in 1/10 the original broth volume in 0.05 M sodium citrate pH 5.7, and stored frozen at -16°C. An enzyme so prepared is stable for at least two months.

b. Ammonium Sulfate Precipitation

The enzyme preparation was further concentrated by ammonium sulfate precipitation. The extract was made 80% saturated by addition of a cold, saturated solution, and incubated at 0°C for 2 hr. It was then spun at 18,000 xg for 15 min. The supernatant was discarded. The pellet was resuspended in 1.5 ml of citrate buffer.

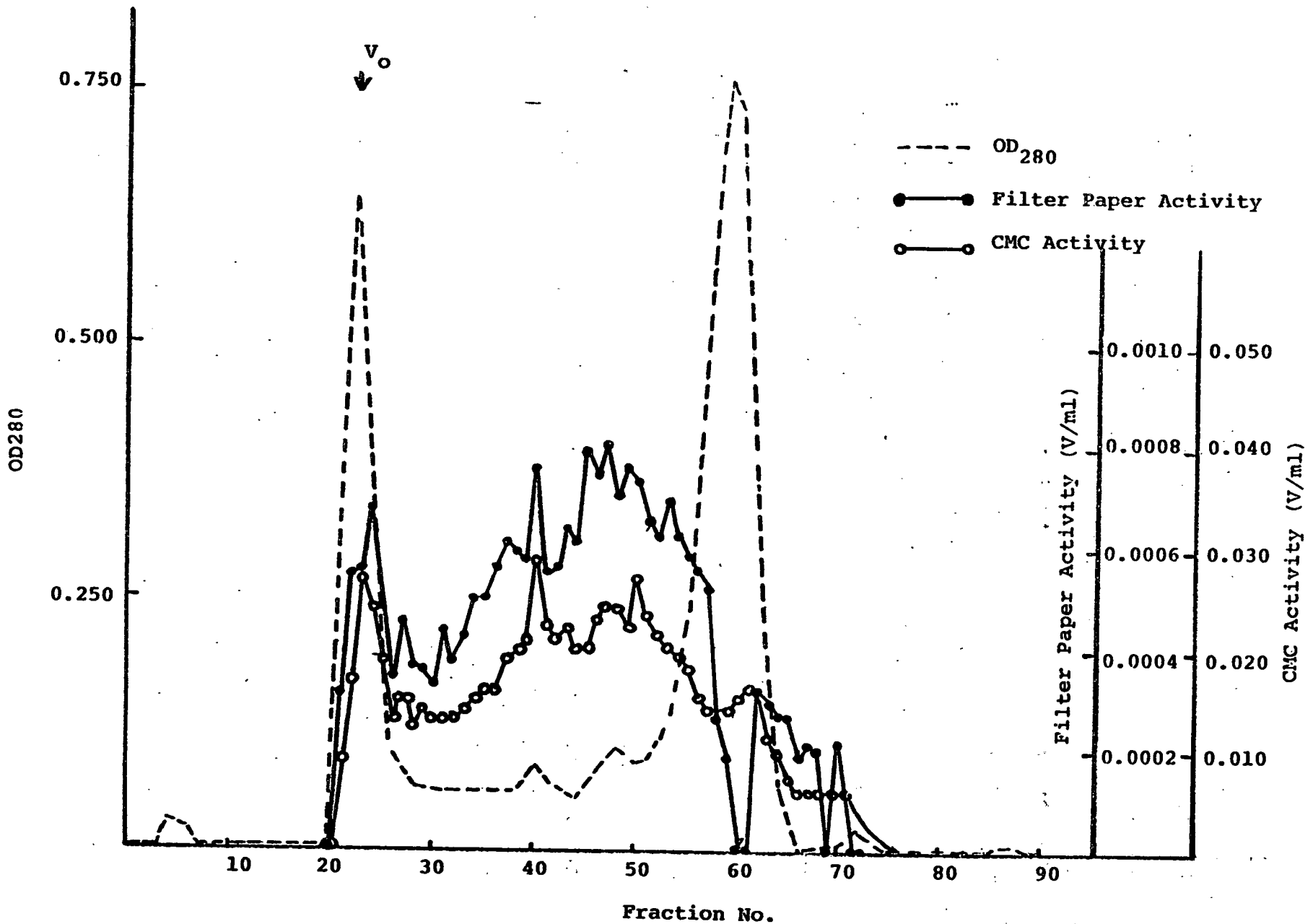
c. Gel Filtration

The ammonium sulfate precipitate was applied to a 2.5 x 35 cm column packed with Biogel A-5m, 100-200 mesh, at ambient temperature. This agarose gel is a molecular sieve of 5×10^6 exclusion limit; its effective operating range is $1 \times 10^4 - 5 \times 10^6$ daltons. The column has been previously equilibrated with 0.05 M Na citrate pH 5.7. The flow rate was maintained at 25 ml/hr. Fractions of 2.3 ml were collected. Absorbance at 280 nm, as well as filter paper and CMC activity in the column fractions, were monitored. Cellulase activity was measured as described previously (Progress Report 6/1 - 8/31/78, p. 85-86). Results are presented in Figure I.F.1.

3. Results and Discussion

No single discrete peak of either enzyme protein or enzyme activity was observed. Instead, there exists a large, heterogeneous population of enzyme complexes which ranges in size from approximately 10,000 to 5 million, or more, molecular weight. Activity profiles for CMC and filter paper are approximately identical, with some variation in the lower molecular weight region (higher fraction numbers). Activities overlap, but do not coincide with the large peak of OD280. These profiles indicate the presence of enzyme aggregates of a monomer of approximately 10,000 daltons. This preparation is substantially contaminated with nucleic acids, which comprise a large proportion of the major protein peak.

Figure I.F.1. Elution profiles of protein and cellulase activity through Biogel A-5m



Three separate enzyme pools, including fractions 20-34, 35-59, and 60-70, were pooled and reanalyzed for protein and nucleic acid content by the Warburg-Christian method, and for filter paper and CMC activity. Results are presented in the form of purification tables (Tables I.F.1 - I.F.3). It is evident that ammonium sulfate precipitation, followed by this chromatographic step provided some purification of the enzyme(s). It is however, of note that endo-and exo-glucanase activities, as represented by CMC and filter paper activity, respectively, are not concentrated to the same degree.

4. Future Work

Pooled fractions from this column will next be subjected to affinity chromatography on a CMC-Agarose column. Ideally, purification will proceed to near homogeneity, permitting further characterization studies. In the event of incomplete purification, other traditional enzymological approaches will be considered.

Table I.F.1 Protein and Nucleic Acid Analysis of Fractions

Fraction	Procedure	Volume (mg)	Protein (mg/ml)	Total Protein (mg)	Protein Recovery (%)	Nucleic Acid Content (%)
I	Crude Broth	344	NA*			NA*
II	Ultra-Filtration	34.4	0.54	19.	100	NA*
IV	Gel Filtration					
20-34		34.4	0.110	3.8	20.	5.5
35-59		57.5	0.077	4.4	23.	15.
60-70		25.3	0.038	0.96	5.0	37.

Protein and nucleic acid content as estimated by the Warburg and Christian Method.

*NA = Not assayed.

Table I.F.2 Purification of Filter Paper Activity

Fraction	Procedure	Volume (ml)	Protein (mg/ml)	Activity (U/ml)*	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification (X)
I	Crude Broth	344	NA**	≤ 0.0005	-	-	-	1
II	Ultra-Filtration	34.4	0.54	0.00118	0.041	0.0022	100	1
IV	Gel Filtration							
20-34		34.5	0.110	0.00038	0.013	0.0035	32.	1.6
35-59		57.5	0.077	0.00056	0.032	0.0073	78.	3.3
60-70		25.3	0.038	0.00031	0.0078	0.0082	19.	3.7

* 1 Unit = Amount of enzyme producing 1 mMole glucose/hr at 60°C.

** NA = Not assayed.

Table I.F.3. Purification of CMC Activity

Fraction	Procedure	Volume (ml)	Protein (mg/ml)	Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification (X)
I	Crude Broth	344	NA*	≤0.005				1
II	Ultra-Filtration	34.4	0.54	0.0836	2.9	0.15		1
IV	Gel Filtration							
20-34		34.5	0.110	0.0120	0.41	0.11	14.	.73
35-59		57.5	0.077	0.0228	1.3	0.30	45.	2.0
60-70		25.3	0.038	0.0098	0.25	0.26	8.6	1.7

G. UTILIZATION OF CARBOHYDRATES BY C. THERMOCELLUM

1. Ability to Utilize Glucose

In order to establish if growth in glucose of *C. thermocellum* is due to the selection of pre-existing mutants having this ability or due to the whole population being able to do so, the following experiment was carried out. *C. thermocellum* grown to mid-exponential phase in CM-4 medium with cellobiose (6g/l) as the carbon source was submitted to a three-tube Most Probable Number (MPN) analysis. The MPN count was obtained in CM-4 with cellobiose (6g/l) as the carbon source and in CM-4 with glucose (6g/l) as the carbon source. The results are shown in Table I.G.1.1. The counts obtained in glucose were slightly lower than those obtained in cellobiose. These results demonstrated that a large proportion (>10%) of the cellobiose-grown culture is able to grow in glucose. This frequency is much too large for it to be due to spontaneous mutation. Thus, we conclude that wild type *C. thermocellum* ATCC 27405 is able to grow on glucose as the sole carbon source. This conclusion has to be viewed in light of the fact that our current strain is a single colony isolate from cellulose plates.

2. Effect of Carbohydrate Concentration on Cell Concentration And Yields

The effect of initial carbohydrate concentration on maximum cell concentration and apparent cell yields was determined. Apparent cell yield is defined as grams of cells per grams of initial carbohydrate concentration.

Table I.G.1.1 Three-tube MPN counts of *C. thermocellum* ATCC 27405 obtained in glucose and cellobiose. Inoculum was grown in cellobiose.

	MPN Count (cells/ml)		
Sugar	Minimum[1]	Mean	Maximum[1]
Cellobiose	7.0×10^8	4.3×10^9	2.1×10^{10}
Glucose	1.5×10^8	9.3×10^8	3.8×10^9

[1]Minima and maxima are the extremes of the 95% condifence interval.

Stationary cultures grown in cellobiose (6g/l) for approximately 10 generations were transfered to CM-4 medium containing cellobiose, glucose or fructose at various concentrations. Optical density at 660 nm was followed until the maximum O.D was reached. This O.D value was converted to g cells dry weight/l from previous constructed standard curves, and plotted against the initial carbohydrate concentration (Fig. I.G.2.1). The results indicate that C. thermocellum under conditions where carbon is not limiting, is able to reach a higher cell concentration when grown in cellobiose than when grown in glucose at equivalent sugar concentrations. In addition, growth in fructose is not observed until the initial carbohydrate concentration is 6 g/l. At higher concentrations of fructose the final cell concentration continues to increase until it reaches a level equivalent to that obtained in cellobiose.

The same experiment was repeated with cultures which were grown on glucose prior to the experiment for at least 10 generations. The results are shown in Figure I.G.2.2. This time, although the maximum cell concentration in glucose was still lower than in cellobiose under conditions where carbon is not limiting, the maximum cell concentration in glucose was higher than that obtained with cellobiose-grown cells (Fig. I.G.2.1). Growth in fructose was similar for both cellobiose and glucose grown cells. However, in the latter, growth was observed at a lower fructose concentration. Table I.G.2.1 summarizes the results obtained in Figures I.G.2.1 and

Figure I.G.2.1 Effect of carbohydrate concentration on maximum cell concentrations of cellobiose-grown cells of ATCC 27405.

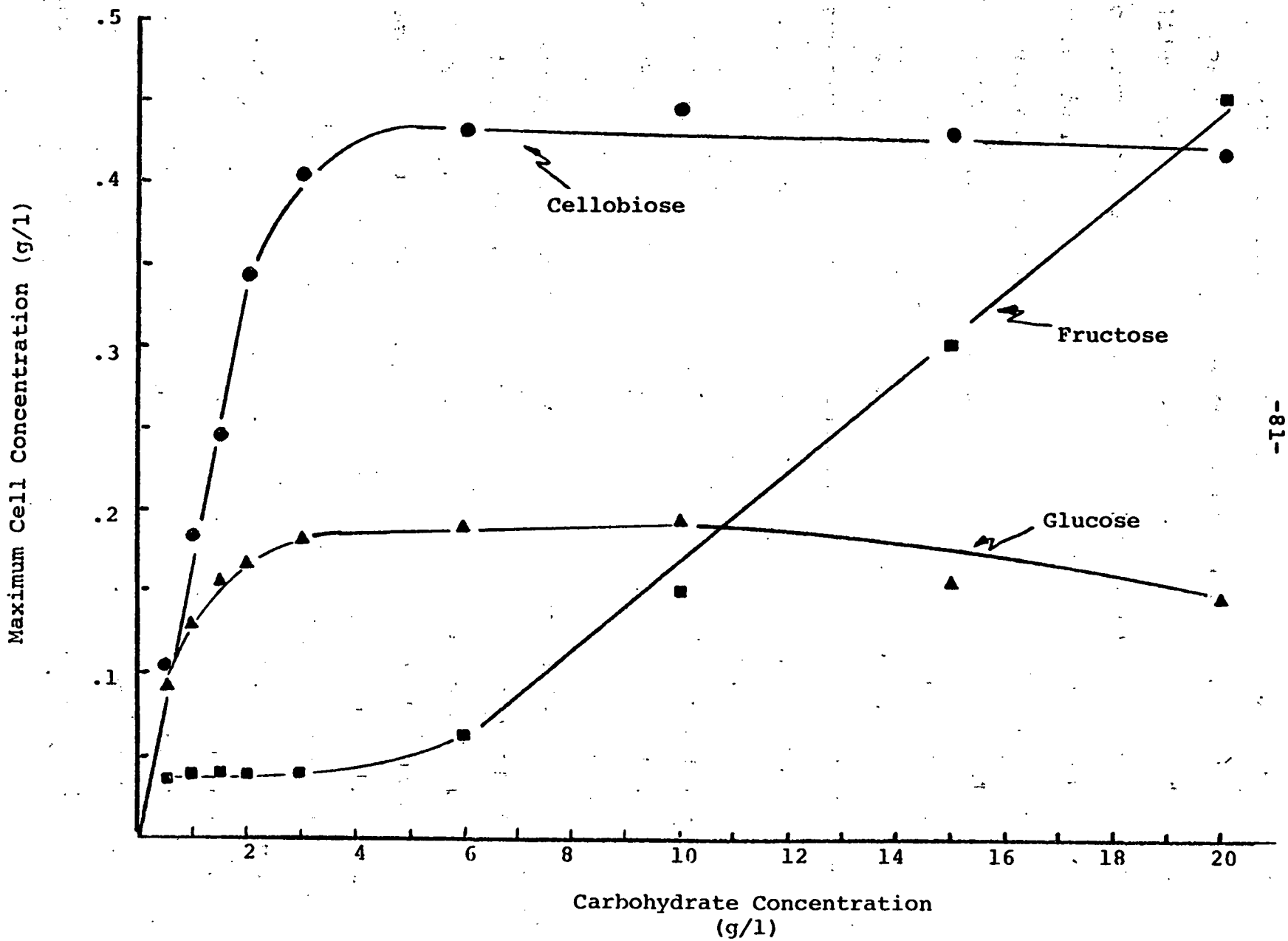


Figure I.G.2.2. Effect of carbohydrate concentration on maximum cell concentrations of glucose-grown cells of ATCC 27405.

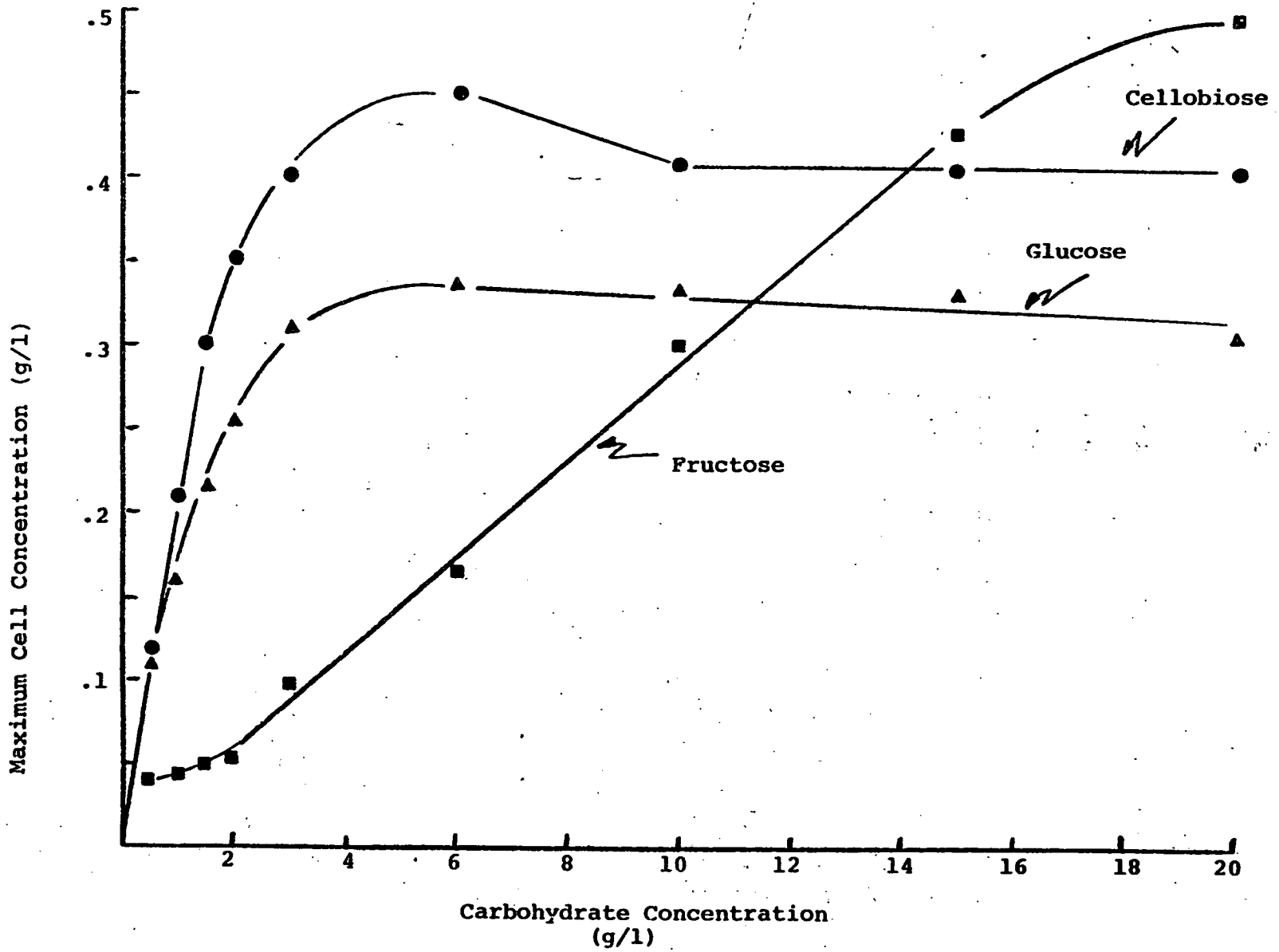


Table I.G.2.1. Apparent cell yields[1] obtained for *C. thermocellum* ATCC 27405 grown in various carbon sources. Inocula were grown either in celloboise or glucose at 6 g/l and then transferred to glucose, cellobiose or fructose at various concentrations.

Inoculum	g Sugar / l	Cell Yields[1] Cb	Glu	Fru
Cellobiose Grown	0.5	0.210	0.190	0.070
	1.0	0.185	0.130	0.040
	1.5	0.160	0.103	0.027
	2.0	0.170	0.080	0.018
	6.0	0.072	0.031	0.011
	20.0	0.021	0.008	0.023
Glucose Grown	0.5	.240	.230	.080
	1.0	.210	.160	.045
	1.5	.200	.143	.033
	2.0	.175	.128	.028
	6.0	.075	.056	.028
	20.0	.021	.015	.025

[1] Cell yields are expressed as g of cells (dry weight) per g of initial sugar.

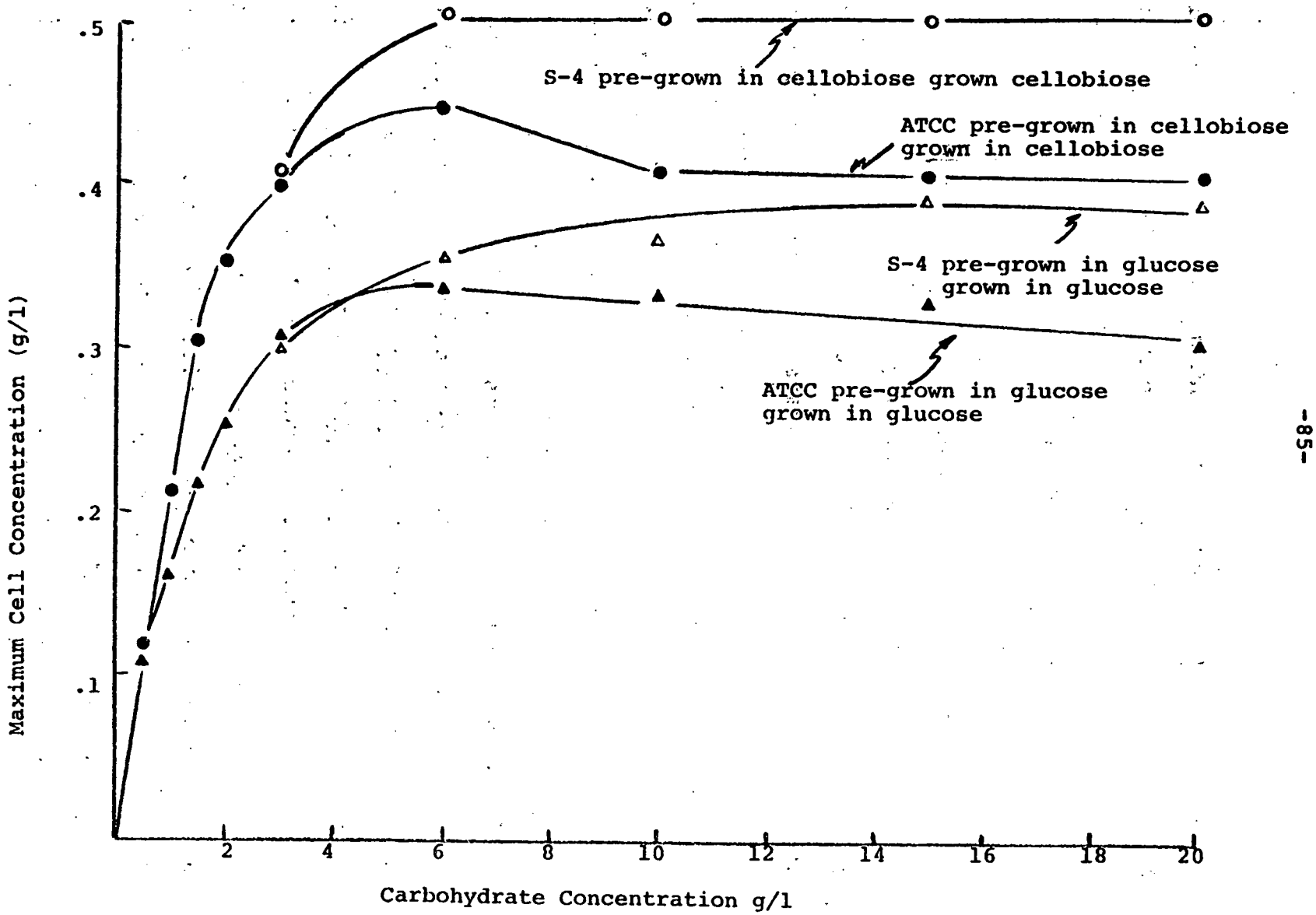
The ethanol resistant strain (S-4) was compared to ATCC 27405 (Figure I.G.2.3). The results demonstrated that S-4 can reach higher cell concentrations in both glucose and cellobiose. However, as with ATCC 27405, with strain S-4 the maximum attainable cell concentration in cellobiose is greater than that obtained in glucose.

There are essentially two reasons why apparent cell yields may change depending on carbon source, one is dependent on the manner in which the sugars are transported into the cell and the other is dependent on final catabolic products obtained. The data obtained so far does not permit us to differentiate between these two alternatives. However, in view of the large differences obtained and previously conducted end product analyses it is difficult to build a hypothesis based on differences in catabolic products. At this time we would like to propose that the differences observed are due to differences in the way in which C. thermocellum transports its sugars.

3. Effect of Yeast Extract on Carbohydrate Utilization

The current literature dealing with the utilization of glucose by C. thermocellum alludes to the fact that this microorganism will only grow in glucose at high yeast extract concentrations. We have tested this claim with our strain. C. thermocellum ATCC 27405 was grown in CM-4 medium containing cellobiose or glucose at 6 g/l and varying concentrations of yeast extract. Optical density was followed until a maximum

Figure I.G.2.3 Comparison of ATCC 27405 and S-4 according to their growth on cellobiose and glucose



was obtained. The results obtained are shown in Figure I.G.3.1. From this graph ratios of maximum cell concentration in cellobiose to glucose, at various yeast extract concentrations were calculated and are shown in Table I.G.3.1. The results indicate that whenever growth in cellobiose occurred it also was seen in glucose. However, it appears that yeast extract does have a stimulatory effect on the growth of C. thermocellum on glucose compared to cellobiose.

4. Future Work

- To determine true cell yields (g cells/g of carbohydrate utilized) of C. thermocellum grown in glucose, cellobiose and fructose.
- To determine the dependence of the distribution of fermentation end products on type of substrate.
- To initiate studies to investigate the mode of transport of glucose, fructose and cellobiose by C. thermocellum.

Figure I.G.3.1 Effect of yeast extract concentration on the growth of C. thermocellum ATCC 27405.

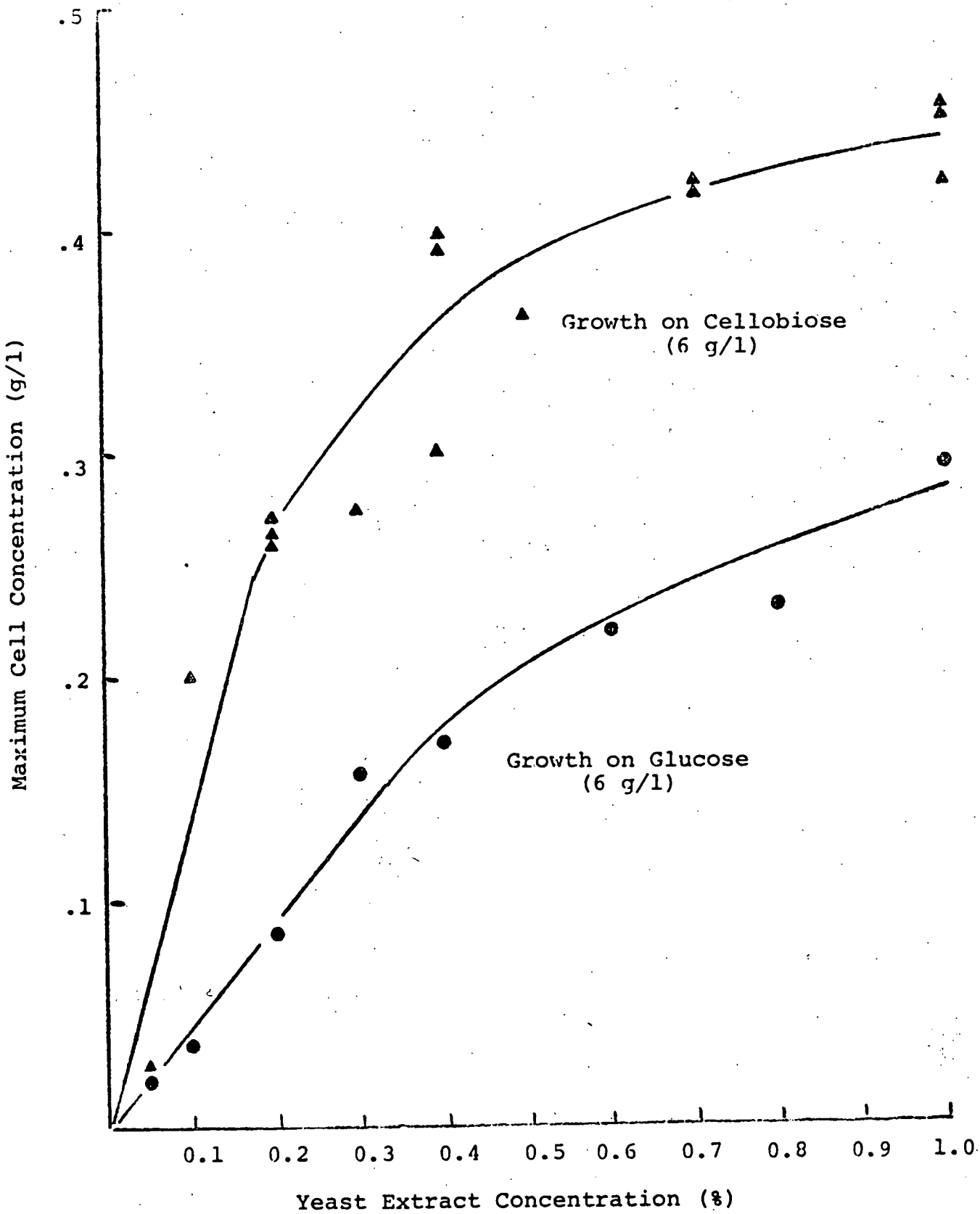


Table I.G.3.1. Ratios of maximum cell concentrations obtained in cellobiose (6 g/l) and glucose (6 g/l) at various yeast extract concentrations.

[YE]%	Cb/Glu
0.05	3.25
0.1	3.00
0.2	3.18
0.3	2.40
0.4	2.03
0.5	1.88
0.6	1.79
0.7	1.71
0.8	1.63
0.9	1.59
1.0	1.53

II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. PRODUCTION OF ACRYLIC ACID BY FERMENTATION

1. Introduction

Four aspects of acrylic acid production were investigated during this recent period of research. They were: (1) determination of the optimum conditions for conversion of propionic acid to acrylic acid with cell suspensions of *Clostridium propionicum*; (2) examination of the role of α-alanine and lactate on acrylic acid accumulation from propionic acid; (3) examination of cell immobilization procedures for production of acrylic acid; and (4) conversion of cellulose to lactic acid as an intermediate in acrylate production.

2. Materials and Methods

Experimental procedures for work with *Clostridium propionicum* were the same as previously reported. Modifications in procedures are described in figure legends or tables. The following is an update of the methods used in pure and mixed culture studies devoted to lactate production from cellulose:

a. Microorganisms

1. *Clostridium thermocellum* ATCC 27405 and *Bacillus coagulans*, a thermophilic, facultative homolactic bacterium.

b. Culture Media

The basic medium used for growth and inoculum preparation of B. coagulans was the MIXED MEDIUM described previously (COO-4198-6).

For sporulation experiments, done for identification purposes, the media used were:

1. Duncan and Strong modified medium.

Ingredient	Concentration (gm/l)
Yeast Extract	4.0
Trypticase	15.0
Soluble Starch	10.0
Sodium thioglycollate	1.0
$Na_2HPO_4 \cdot 7H_2O$	10.0
Agar	20.0

2. Bacto-Tam agar (modified)

Ingredient	Concentration (gm/l)
Yeast Extract	5.0
Trypticase	5.0
Dextrose	2.0
K_2HPO_4	0.5
$MnSO_4$	0.2
Agar	20.0

3. Mixed-Medium agar

The composition of this medium has been previously described (see page 166, report COO-4198-7).

c. Biochemical Tests

For identification purposes, we used commercial kits with micro tests for metabolic products. These kits are manufactured by BBL Products under the trademarks of BBL MINITEK:

1. Procedure for differentiation of Enterobacteria
2. Procedure for differentiation of Anaerobes

3. Results

a. Determination of the Optimum Conditions for the Conversion of Propionic Acid as Acrylic Acid with Cell Suspensions of Clostridium propionicum

Parameters examined for their influence on the oxidation or dehydrogenation of propionic acid to acrylic acid included: temperature, pH, methylene blue concentration, and growth phase of cells used in the experimental system.

1. Effect of Methylene Blue

Determination of the role of methylene blue represents a continuation of experiments reported on in the last progress report. The reaction mixture was contained in a total volume of 2 ml, 50 mM triethanol-amine HCl buffer (pH 8.5), 200 mM propionate, 20 mM lactate and concentrations of methylene

blue that varied from 0 to 0.5%. Samples were incubated at 37°C in air with mechanical agitation. 200 µl samples were collected at the times indicated in Figure II.A.1 and analyzed for acrylic acid as described in previous progress reports. Highest concentrations of acrylic acid were detected in the samples containing 0.2% methylene blue. However, initial rate of production was maximal at 0.4% methylene blue (Figure II.A.2).

2. Effect of Temperature

Samples containing standardized reaction mixture, 0.2% methylene blue and 20 mM α-alanine (experiments to be described later show that 20 mM α-alanine is optimal) were incubated at different temperatures. The cell concentration corresponded to 2.8 mg protein/ml of reaction volume.

The samples were incubated at temperatures between 10° and 60°C and assayed for acrylic acid after 4 hr of incubation. As seen in Figure II.A.3, the optimal temperature for dehydrogenation of propionate to acrylate is between 30 to 35°C. The experiment was repeated with a higher cell concentration (5.19 mg protein/ml), and the results indicated that 32°C is the optimal temperature (Figure II.A.4). Note that, under these conditions, the final concentration of acrylic acid was 41 mM. The resulting specific productivity is 2.0 mM acrylic acid/mg protein/ml per hr.

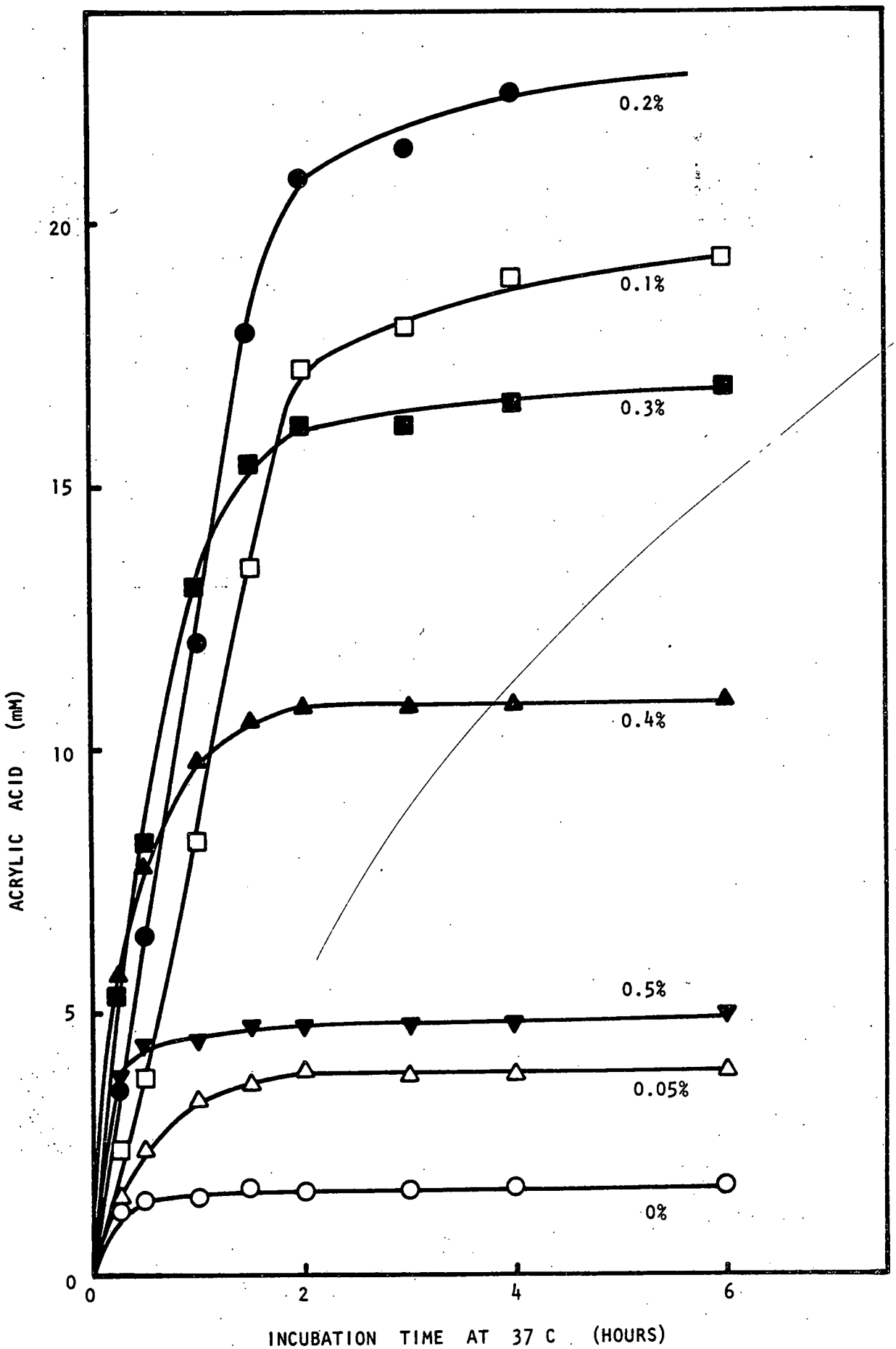


FIGURE II.A.1: EFFECT OF METHYLENE BLUE CONCENTRATION ON ACRYLIC ACID PRODUCTION

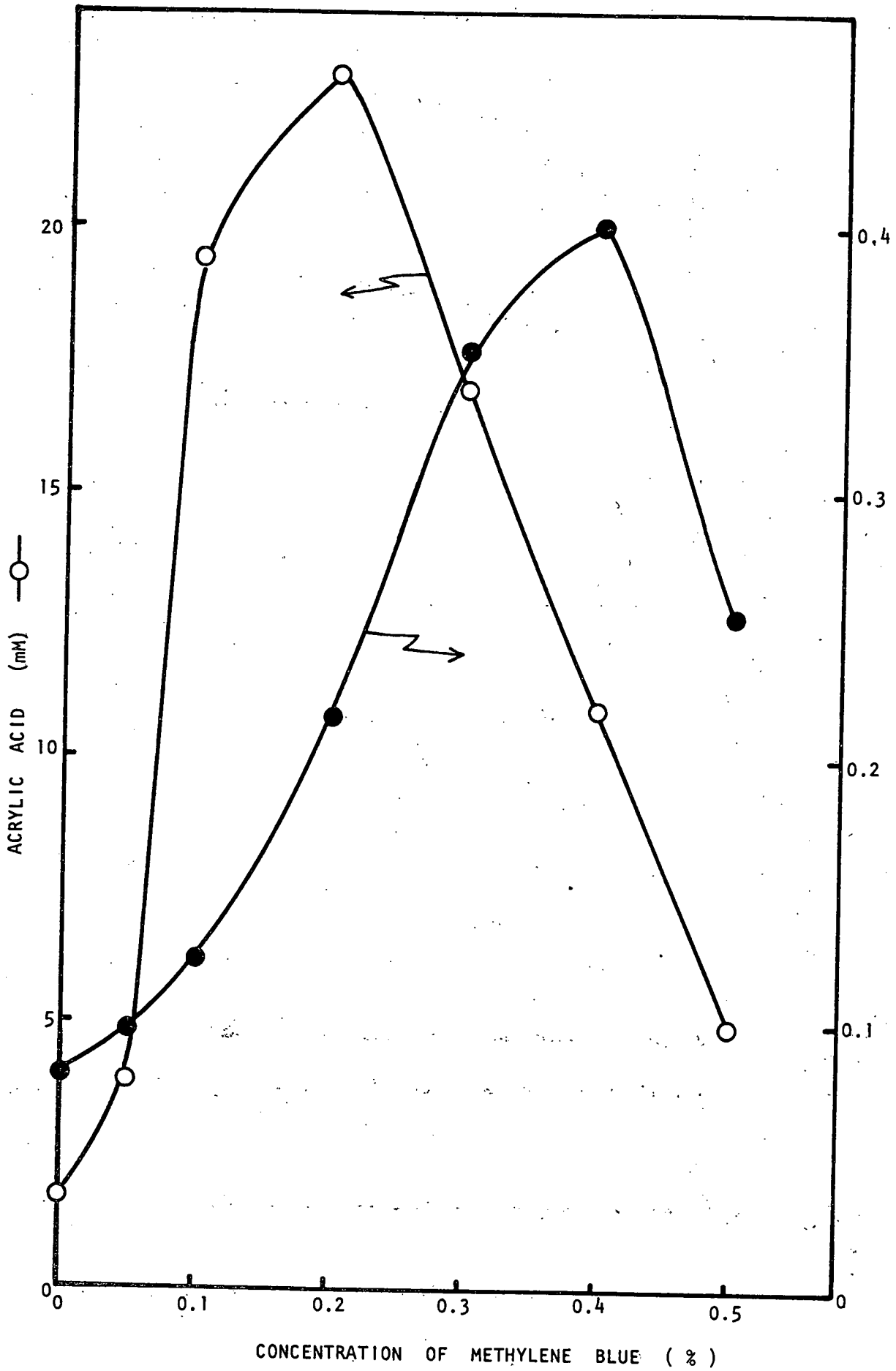


FIGURE II.A.2: EFFECT OF METHYLENE BLUE ON ACRYLIC ACID PRODUCTION

FIGURE II.A.3: EFFECT OF TEMPERATURE ON THE CONVERSION OF PROPIONIC ACID TO ACRYLIC ACID

- Reaction Mixture (total volume 500 µl) contained:

triethanolamine-Hcl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
α-alanine	20 nM
cellular protein	2.87 mg/ml

- Incubated under ait atmosphere with mechanical agitation for 4 hours in either water bath or constant temperature room.

FIGURE II.A.3

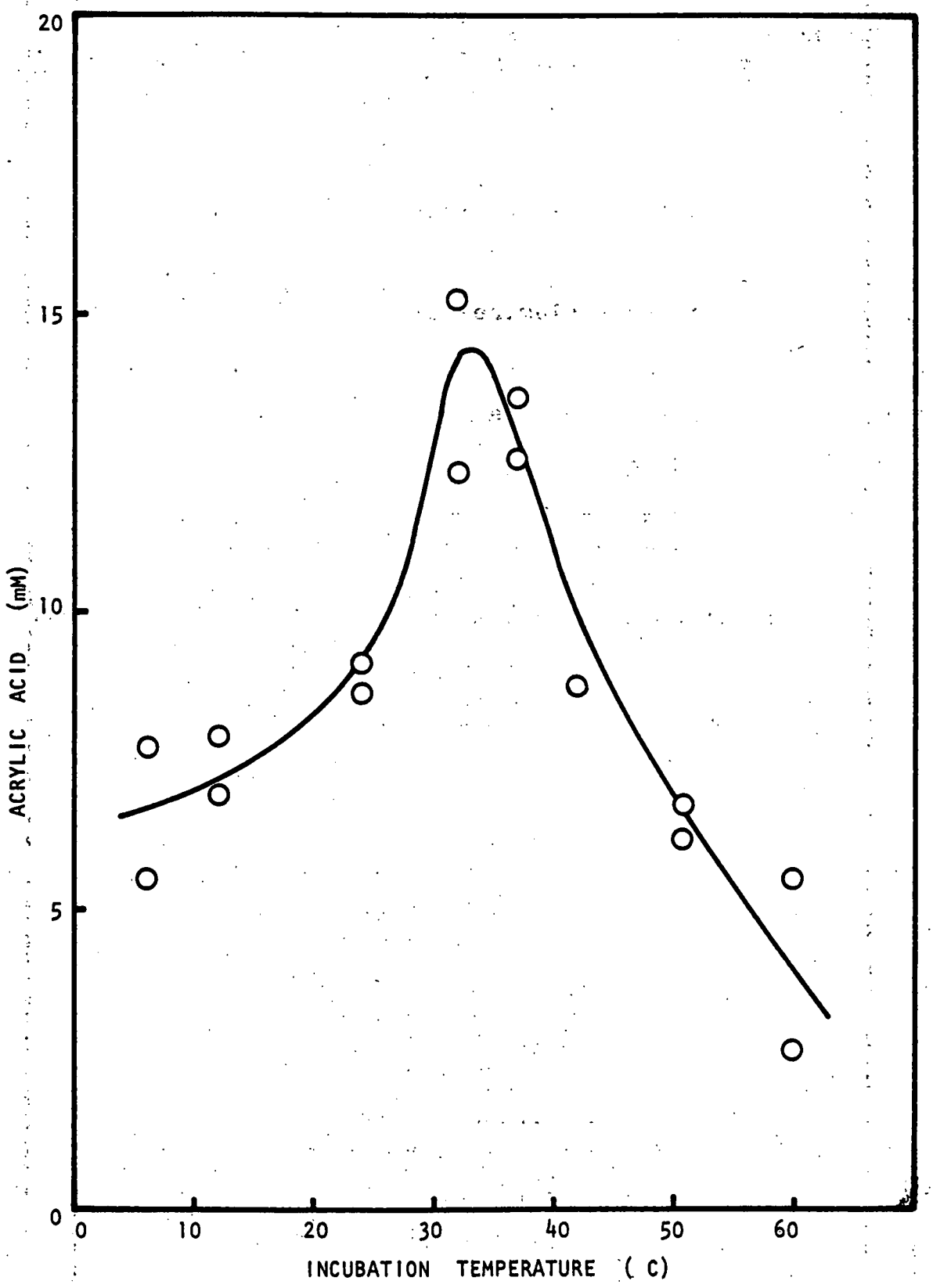

20
15
10
5
0
ACRYLIC ACID (mM)
0
10
20
30
40
50
60
INCUBATION TEMPERATURE (C)

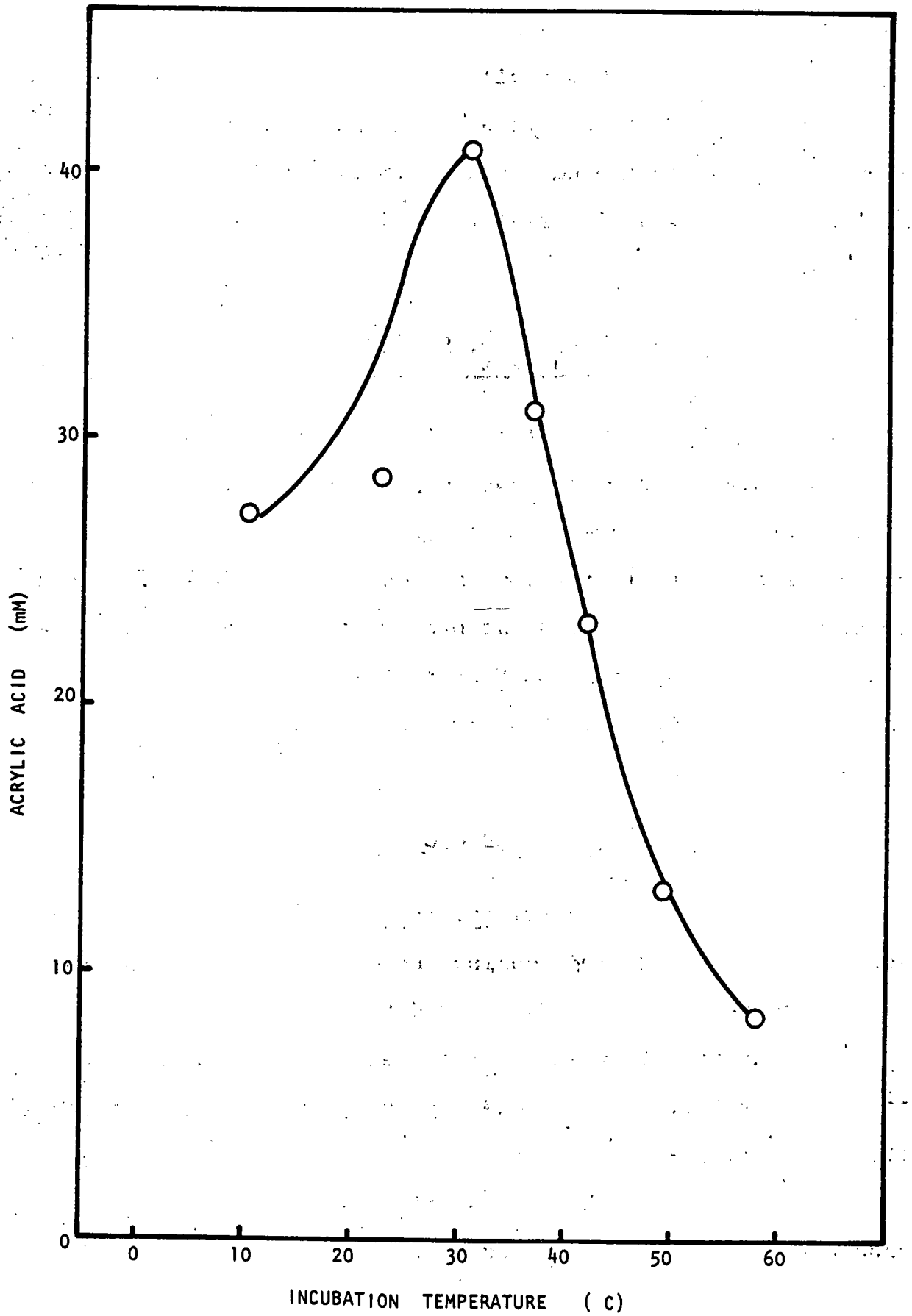
FIGURE II.A.4: EFFECT OF INCUBATION TEMPERATURE ON THE CONVERSION OF PROPIONIC ACID TO ACRYLIC ACID

- Reaction mixture (total 500 µl) contained:

triethanolamine-Hcl buffer	50 mM
propionate	200 mM
methylene blue	0.2 %
α-alanine	20 mM
cellular proteins	5.19 mg/ml

- Incubated under air atmosphere with mechanical agitation for 4 hours in either water bath or constant temperature room.

FIGURE II.A.4


40
30
20
10
0
ACRYLIC ACID (mM)
0
10
20
30
40
50
60
INCUBATION TEMPERATURE (C)

Since all other previous experiments were conducted at 37°C, it can be assumed that the efficacy of the system was underestimated. For example, the final concentration of acrylic acid was 30% greater at 32°C (41 mM) than at 37°C (31 mM).

3. Conversion of Propionic Acid to Acrylic Acid as A Function of Cell Concentration

The effect of cell concentration on final level of acrylic acid from 200 mM propionate was determined at 37°C; protein concentrations were used as a measure of cell concentration. As summarized in Figure II.A.5, the final concentration of acrylic acid was proportional to cell concentration. It also appears from Figure II.A.5 that cell concentration is the limiting reactant in the system presently being characterized.

4. Effect of Growth Phase

Clostridium propionicum cells were harvested at different times during the growth phase. Results are summarized in Figure II.A.6 and Figure II.A.7. This data confirms earlier observations that cells harvested shortly before the onset of the stationary phase produced the most acrylate from propionate.

The optimal conditions for propionate dehydrogenation to acrylate appear to be as follows:

FIGURE II.A.5: CONVERSION OF PROPIONIC ACID TO ACRYLIC ACID AS A FUNCTION OF CELL CONCENTRATION

- Reaction mixture (total 500 µl) contained:

triethanolamine-Hcl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
α-alanine	20 mM

- Incubated at 37°C under air atmosphere with mechanical agitation for 4 hours.

- Cell concentrations were expressed as total cellular proteins per ml. Proteins were assayed by modified Lowry method with Bovine Serum Albumin (BSA) as standard.

FIGURE II.A.5

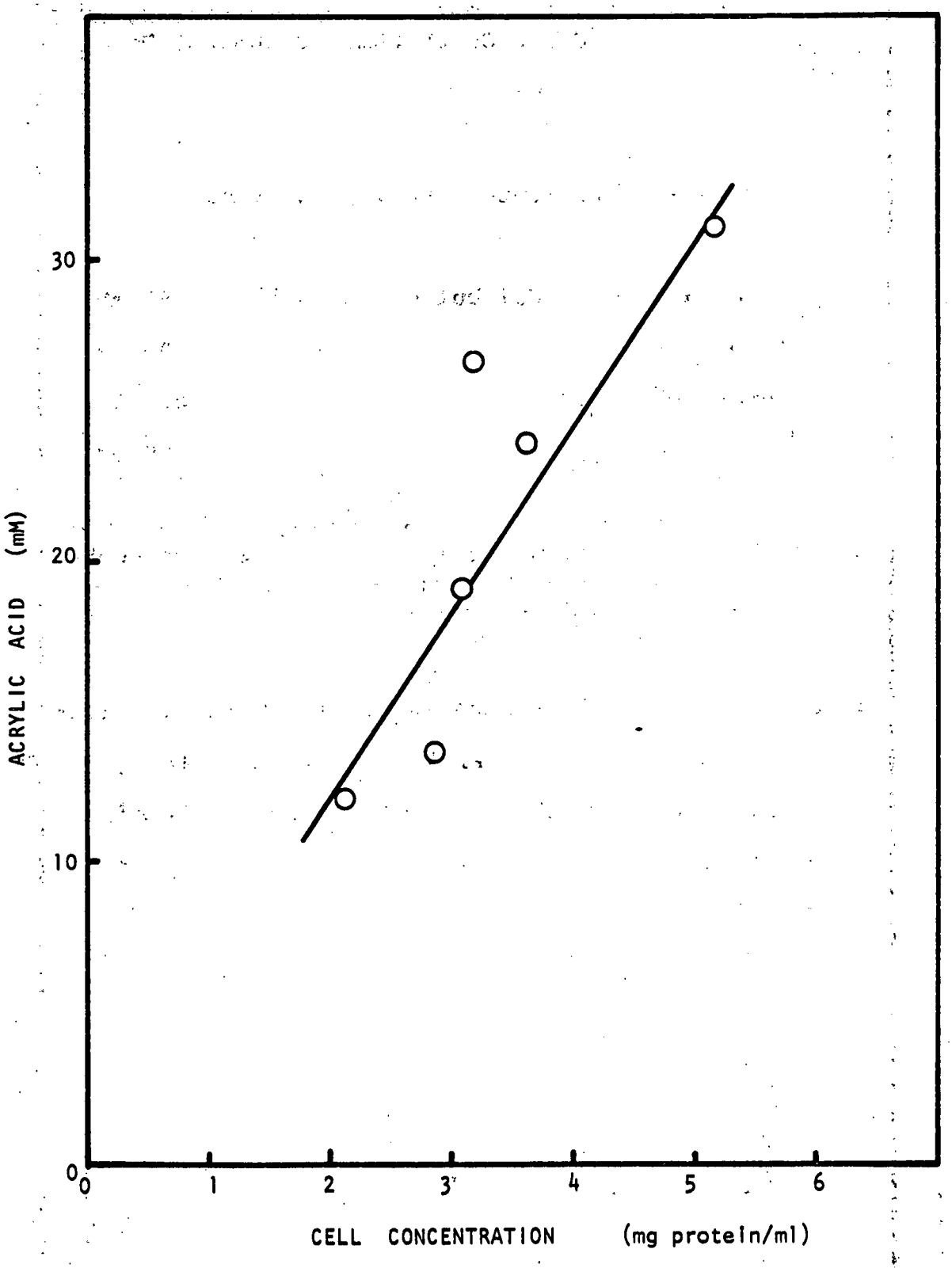

ACRYLIC ACID (mM)
30
20
10
0
0
1
2
3
4
5
6
CELL CONCENTRATION (mg protein/ml)

FIGURE II.A.6: ACRYLIC ACID PRODUCTION AS A FUNCTION OF GROWTH PHASE OF CELLS

●——● Optical density of growing cells measured in Klett units.

▲——▲ Wet weight per liter of cells.

o——o Acrylic acid produced at 4 hours after initiation of bioconversion using cells harvested at various growth phases.

The medium was inoculated with a 5% (V/V) inoculum of an actively growing pure culture.

FIGURE II.A.6

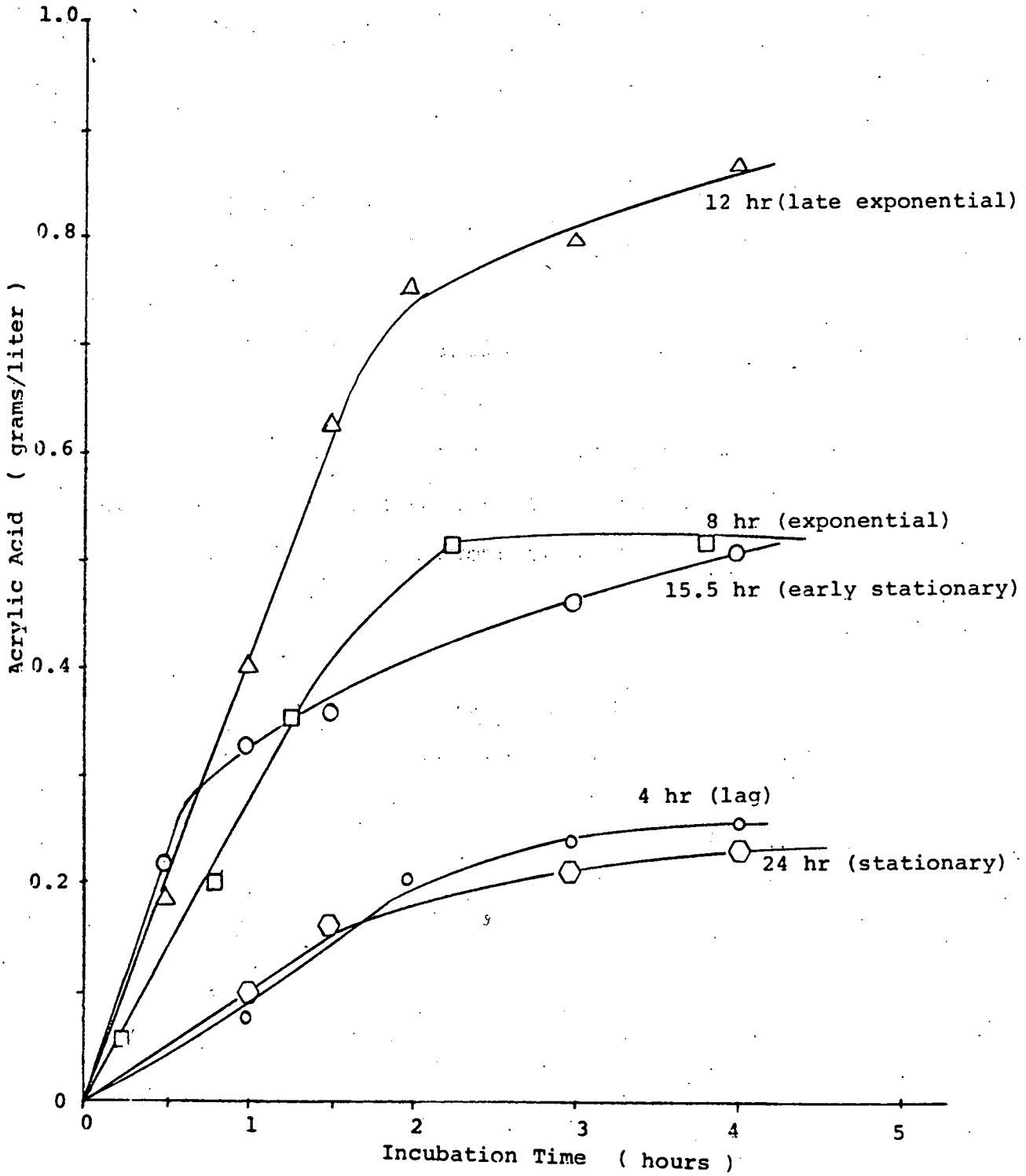
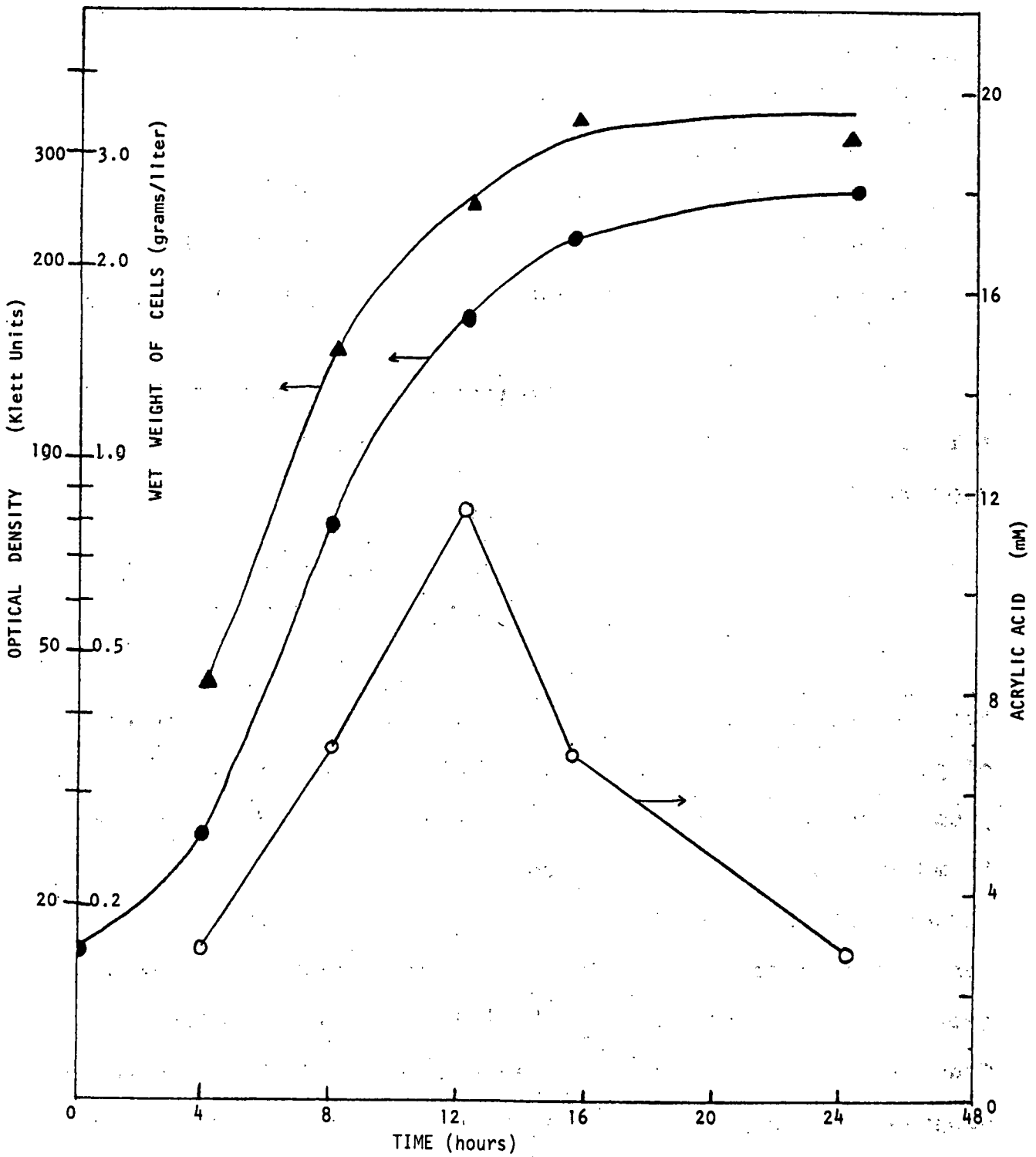

1.0
0.8
0.6
0.4
0.2
0
Acrylic Acid (grams/liter)
12 hr (late exponential)
8 hr (exponential)
15.5 hr (early stationary)
4 hr (lag)
24 hr (stationary)
0
1
2
3
4
5
Incubation Time (hours)

FIGURE II.A.7: EFFECT OF THE HARVEST TIME ON ACRYLATE PRODUCTION. RESULTS ARE TAKEN FROM FIGURE II.A.6.



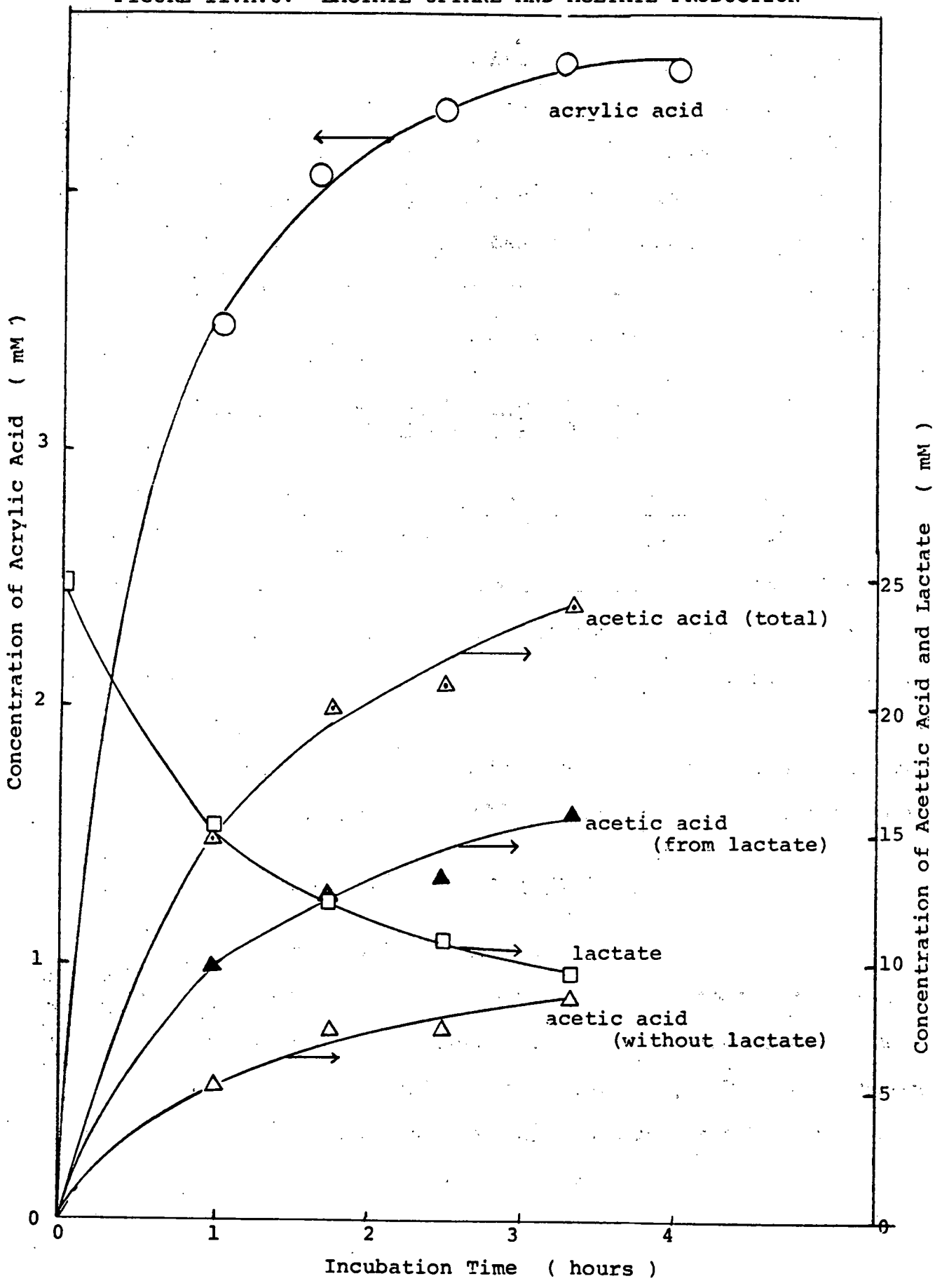
propionate concentration	200 mM
Methylene blue	0.2 %
lactate or α-alanine	20-25 mM
pH	8.5
temperature	32°C
growth phase	late exponential
cell concentration (measured by protein)	>5 mg/ml cellular protein

These optimization experiments have resulted in the use of standardized conditions which lead to reproducible results.

b. Effect of Biochemical Intermediates on Acrylic Acid Accumulation from Propionic Acid

As previously described, the addition of lactate to the reaction mixture greatly stimulated the conversion of propionate to acrylate. A standard reaction mixture (200 mM propionate, 0.2% methylene blue with or without 25 mM lactate in 50 mM Tris buffer, pH 8, and containing 20% net weight of cell suspension) was used at 37°C. The reaction was followed as a function of time. As summarized in Figure II.A.8, it was observed that the lactate is converted entirely to acetic acid. Thus, it is hypothesized that lactate is stimulating the dehydrogenation reaction by generating acetyl CoA. In view of this possibility, intermediates other than lactate were evaluated for

FIGURE II.A.8: LACTATE UPTAKE AND ACETATE PRODUCTION



their ability to stimulate dehydrogenation. As summarized in Table II.A.1, α-alanine and pyruvate have a stimulating effect identical to lactate, as expected. β-alanine and β-hydroxy propionate were ineffective as stimulants. Additional experiments (data not shown) indicated that β-alanine and β-hydroxy propionate were taken up by the cells and metabolized. However, this did not affect the amount of acrylic acid produced.

c. Immobilization of Clostridium propionicum for Production of Acrylic Acid

Immobilized whole cells may have advantages over free resting cell suspensions of C. propionicum, including facilitation of separation of the "biocatalysts" in stirred tank reactors, greater mechanical support and packaging properties necessary for a tubular flow reactor and increased stabilization of catalytic activity. During the course of this progress report period, two different organic immobilizing systems were studied and some preliminary results were obtained.

1. Polyacrylamide Gels (PAG)

Encouraging preliminary results with this system were reported in the previous progress report. Since then, crosslinking of the gel, loading of cells on the polymer, rates of acrylate production in presence of any of the components of the reaction mixture have been investigated.

TABLE II.A.1: EFFECT OF INTERMEDIATES ON THE CONVERSION OF PROPIONIC ACID TO ACRYLIC ACID

Intermediates	m moles acrylic acid/g cellular protein	
	α-alanine grown	β-alanine grown
none	0.91	0.15
α-alanine	4.34	3.25
lactate	2.24	0.49
pyruvate	2.47	0.65
β-alanine	0.84	0.23
β-OH-propionate	0.90	0.17

- Reaction mixture (total volume 500 µl) contained:

triethanolamine-HCl buffer (pH 8.5)	50 mM
propionate	200 mM
methylene blue	0.2 %
intermediate compound	20 mM

- Incubated at 37°C under air atmosphere with mechanical agitation for 4 hours.
- Amount of cellular proteins were measured by modified Lowry method.

i. Toxicity of Polymerization Reactants

With PAG gels system, TEA-Mg buffer was replaced by Tris pH 8.0 and initial experiments evaluated the toxicity of the various components of the reaction (Figure II.A.9). N, N, N', N'-tetramethylethylendiamine (TEMED) and N,N'-methylene bis (acrylamidie) (Bis), had essentially no effect on the cell system. On the other hand, ammonium persulfate caused a slight reduction in rate and reduced the final amount of acrylate produced by 30%. Acrylamide was the most toxic decreasing the rate of production. Final level of acrylate was the same as that observed for persulfate added to the system. The important finding, however, is that a significant fraction of cellular activity remains after treatment with any of the reagents and the chemicals required for immobilizing cells will not inactivate cellular activity.

ii. Effect of Degree of Cross-linking on Cellular Activity

Percent cross-linking is defined as the percent of cross-linking agent added with the monomer. A 1% cross-linked gel was rubbery, elastic and difficult to homogenize and retained only 50% catalytic activity (Figure II.A.10). 15% cross-linked gels were brittle and easy to homogenize. Though Figure II.A.10 shows a maximum activity at 2.5% cross-linking, repeated experiments indicate that except for 1% cross-

FIGURE II.A.9: TOXICITY OF POLYACRYLAMIDE GEL COMPONENTS ON ACRYLIC ACID PRODUCTION

●——● Control bioconversion, without gel components. Contained 0.2 gm Na-propionate, 50 mM tris buffer pH 8.0, 25 mM Na-lactate, 0.2% methylene blue. Agitated under air atmosphere.

All other runs contained above components in the reaction mixture, plus below mentioned gel component.

Δ——Δ Plus potassium persulfate, 2.5 mg/ml (100 µl of 2.5% fresh solution).

∇——∇ Plus "BIS" (N, N'-methylene-bis acrylamide) 8 mg/ml

□——□ Plus 5 µl TEMED (N, N, N', N'-Tetramethyl-ethylenediamine).

○——○ Plus acrylamide monomer 150 mg/ml.

FIGURE II.A.9

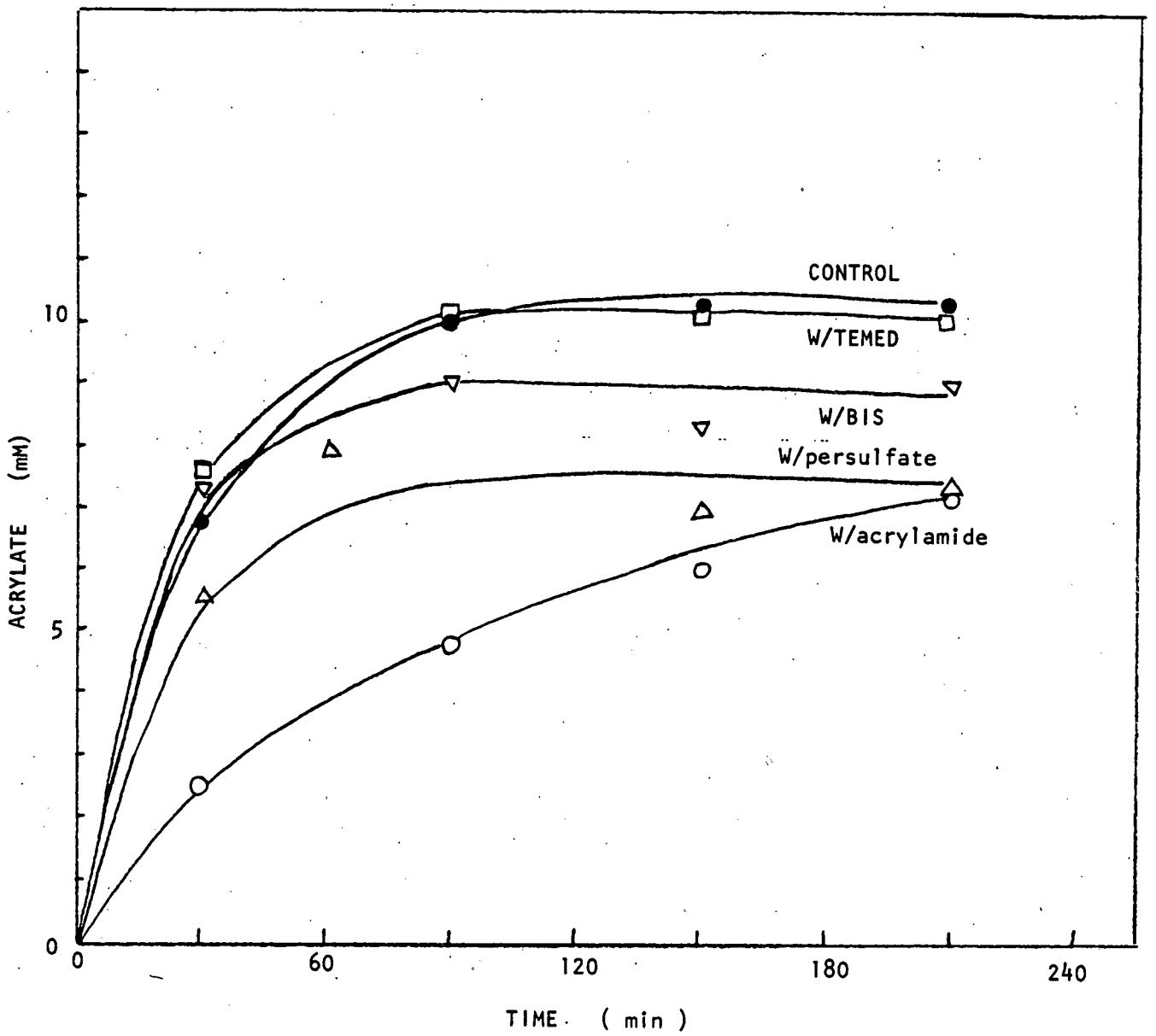
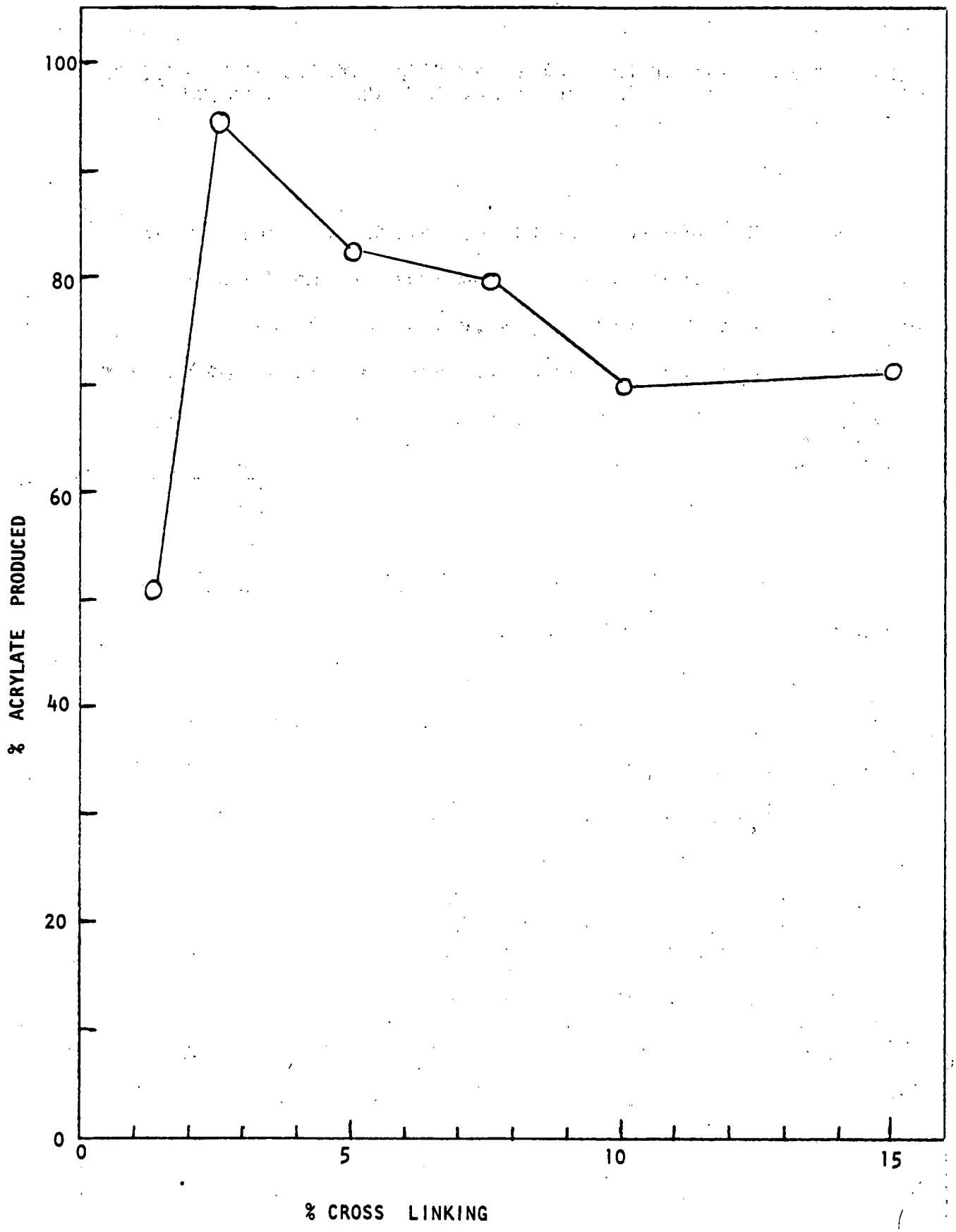

CONTROL
W/TEMED
W/BIS
W/persulfate
W/acrylamide
ACRYLATE (mM)
10
5
0
0
60
120
180
240
TIME (min)

FIGURE II.A.10: EFFECT OF PERCENTAGE CROSSLINKING OF THE GEL ON ACRYLIC ACID PRODUCTION

o——o Represents acrylic acid produced after 3.5 hrs. of bioconversion using standard conditions for bioconversion, as a percentage of acrylic acid produced by a control reaction using immobilized resting cells.

FIGURE II.A.10



linking which is consistently less active, gels between 2.5% and 15% cross-linking typically retain 70-80% biological activity.

iii. Cell Loading of the Gels

Cell concentrations ranging from 500 mg/ml net wt to 50 mg net wt/ml resulted in a wide range of gel activities. With 50 mg net wt/ml, no activity was detected. With higher cell concentrations, acrylic acid could be detected. It was found that a cell concentration of 400 mg net wt/ml had 13% of activity of 200 mg net wt/ml of control cells.

2. Polyacrylamide-CO-N-acrylory Succinimide Gels (PAN)

PAN gels were prepared according to the method of Whitesides et al. (J. Am. Chem. Soc. 100, 302, 1978).

PAN, a water soluble polymer was dissolved in 4 ml (4-(2-hydroxyethyl-)-1-piperazeneethanesulfonic acid) (HEPES) buffer 300 mM, pH 7.5 succinimide buffer. The solution is stirred and allowed to polymerize after the addition of 50 µl 500 mM dithiothreitol, 400 µl triethyltetramine, (TET), and 0.5 ml cell suspension containing variable amounts of cells (typically 300 gms wet wt/liter) with continuous stirring at 25°C. The resulting gel is crushed in a mortar and

worked five times with a total of 100 ml TRIS buffer, 10 mM, pH 8.0. The washings are normally analyzed for protein. Results obtained with this type of gel will be presented in the next progress report.

d. Microbial Degradation of Cellulose for The Production of Lactic Acid

Continuing with efforts to convert cellulosic materials into lactic acid by means of mixed cultures, we conclude with the final characterization and identification of the second microorganism of the system. This lactic acid producer is a thermophilic, homolactic facultative bacterium. The physical and biochemical characteristics show that it belongs to the genera of *Bacilli* and its metabolic properties are very close to the species *coagulans*. Therefore, we believe it to be a strain of *Bacillus coagulans*.

1. Growth in Defined Medium

It is desireable to grow *B. coagulans* on a chemically defined medium, to study its metabolic performance and its behavior in a mixed culture. The results obtained on such a medium are shown in Table II.A.2 compared to growth on the normal, complex medium. The growth was poor and future work will focus on the need for vitamins and amino acids.

TABLE II.A.2: GROWTH OF B. COAGULANS ON A CHEMICALLY DEFINED MEDIUM

	AGE (HRS)	pH	GROWTH (K.U.)	LACTIC ACID (gm/l)
Control	24	4.58	225	2.3
Defined Medium	24	6.1	45	0.6

2. Classification

Table II.A.3 presents a summary of the physical and biochemical characteristics of B. coagulans. By comparing these properties to those reported for gram positive bacilli in Bergey's manual, we found that our microorganism fits very close the description of Bacillus coagulans. The key characteristics considered for its classification were: It is a rod, gram(+), spore forming catalase positive, homolactic, and facultative with no gas production.

Sporulation of B. coagulans is low in frequency and difficult to observe. Three different agra media were tested before spores were detected. The results are shown in Table II.A.4. Mix-media agar plates under aerobic conditions gave the positive results with respect to sporulation. Poor growth and no sporulation were found when cells were incubated under anaerobic conditions. Sporulation was difficult to see under the microscope, but was easily detected by the heat

TABLE II.A.3: SUMMARY OF THE CHARACTERISTICS OF B. COAGULANS

MORPHOLOGY

Form	Rods, slightly curved
Size	0.5 - 0.7 μm width and 3 - 5 μm length
Arrangement	Single, mostly pairs and short chains
Motility	Non-motile when grown on liquid or agar medium
Staining	Young cells are gram(+), gradually gram(-) with age
Spore Formation	Hard to sporulate. No sporulation in liquid medium. Spores observed in Agar plates low frequency. Hard to notice on the microscope. Detected only by heat test (80°C/10 min).

CULTURAL CHARACTERISTICS

Liquid Agar	Fast growth in 15 hrs in microaerophilic or anaerobic conditions.
Liquid Medium	Long lag (12 hrs) when inoculum entered stationary phase. Log phase inoculum grow without a lag with specific rates up to 0.7 1/hr.
Agar Plates	No growth under anaerobic conditions by streaking technique. Excellent growth under aerobic conditions, colonies were round, white, opaque, 2-3 mm.

BIOCHEMICAL CHARACTERISTICS

Gas Formation	No gas is produced
Oxygen Relation	Anaerobic facultative
Temperature Relationship	Maximum growth between 45-55°C. Grows between 30-63°C at slower rates on the extremes.
Fermentation Products	Homolactic fermentation when growing on hexoses. Acetic acid and ethanol are also produced when growing on pentoses.
Catalase	Positive
Indol	Negative

TABLE II.A.3 (Contd)

BIOCHEMICAL CHARACTERISTICS (Contd)

Nitrate Reductase	Negative
H_2S	Negative
Esculine	Positive
Grows on	Glucose, Fructose, Arabinose, Xylose, Salicin, Rhamose
Low Growth	Lactose, maltose, mannitol
No Growth	Glycerol, Inositol, starch, sucrose, Citrate malonate, Citrate
Lysine, Phenyl-alamine, or-nithine	Not decomposed
ONPG	No β-galactosidase produced
Urea	Urease negative
Azide	No growth at 0.2 gm/l

BIOCHEMICAL TESTS

These tests were done by using the MINITEK kits from BBL Products. These are microtests on filter paper circles, which detect the appearance of a metabolic product, upon growth of the microorganism. Inoculum was prepared by suspending cells obtained from colonies on agar plates. The results were analyzed after 24 hrs at 60°C.

TABLE II.A.4: SPORULATION OF BACILLUS COAGULANS

MEDIUM	CONDITION	AGE (HRS)	GROWTH	SPORES
Duncán	Aerobic	72	(-)	-
	Anaerobic	72	(-)	-
Bacto-Tam	Aerobic	72	(+)	(-)
	Anaerobic	72	(-)	-
Mix-Medium	Aerobic	72	(++)	(+)
	Anaerobic	72	(±)	(-)

test which consisted of heating a cell suspension from agar plates at different temperatures for 10 minutes and then incubating for 24 hrs at 60°C. The results obtained are shown in Figure II.A.11. Cells were able to grow as well as a non-heated control when heated at 80°C which is conclusive of the presence of spores. Growth in the presence of sodium azide, in a test designed to detect the presence of cytochromes or hemo-proteins involved in the energy metabolism of the microorganism. Some growth (Figure II.A.12) was observed in mix-medium after 48 hrs when 100 ppm of sodium azide were present. No growth was observed at 200 ppm, which is conclusive of the sensitivity of the microorganism to sodium azide, and the involvement of hemo-proteins in its metabolism, probably in the mechanism of energy generation.

4. Future Work

- The immobilized cell preparations of C. propionicum will be investigated further to seek optimum reactor concentration and improvement in stability.

- Biochemical experiments to be conducted include:

  i. further refinement of C. propionicum nutrient requirements

  ii. development of mutagenising conditions for preparation of strain improvement program

  iii. initiate cell-free experiments to further elucidate role of lactate or α-alanine in stimulating the conversion of propionate to acrylate

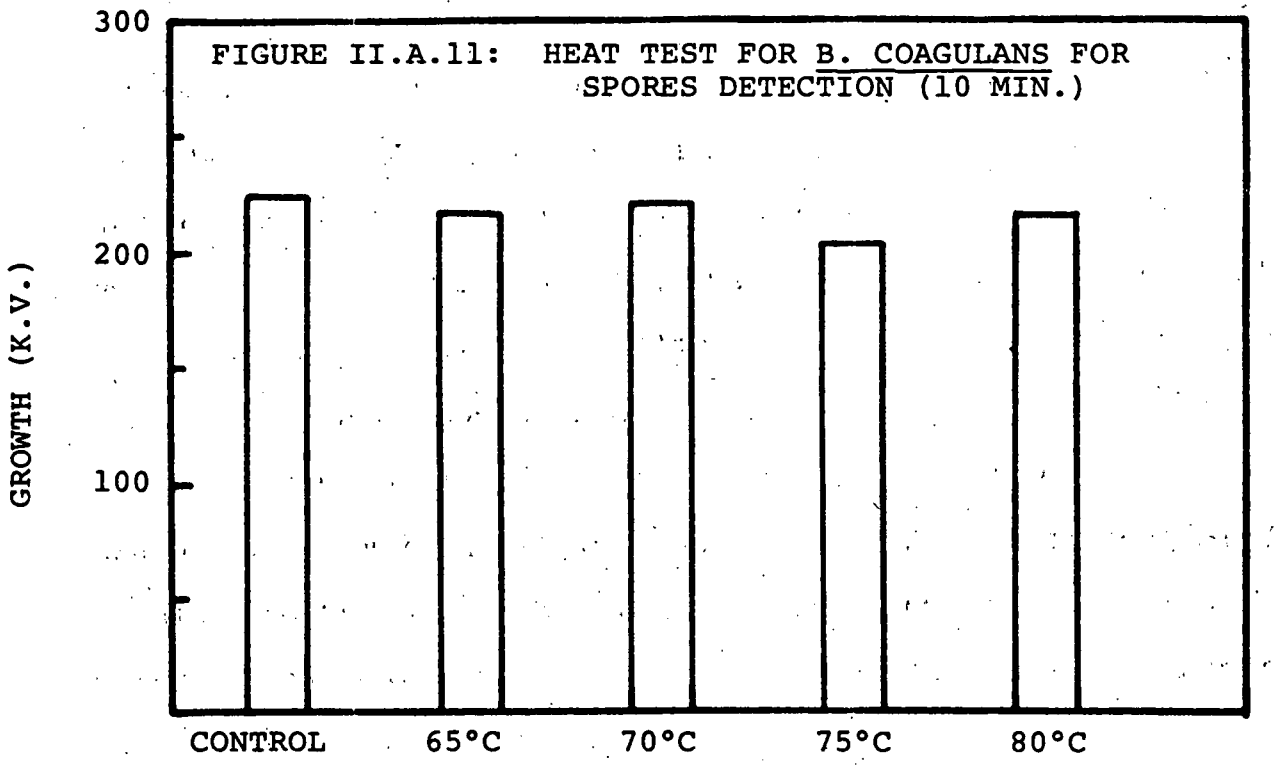


FIGURE II.A.11: HEAT TEST FOR B. COAGULANS FOR SPORES DETECTION (10 MIN.)

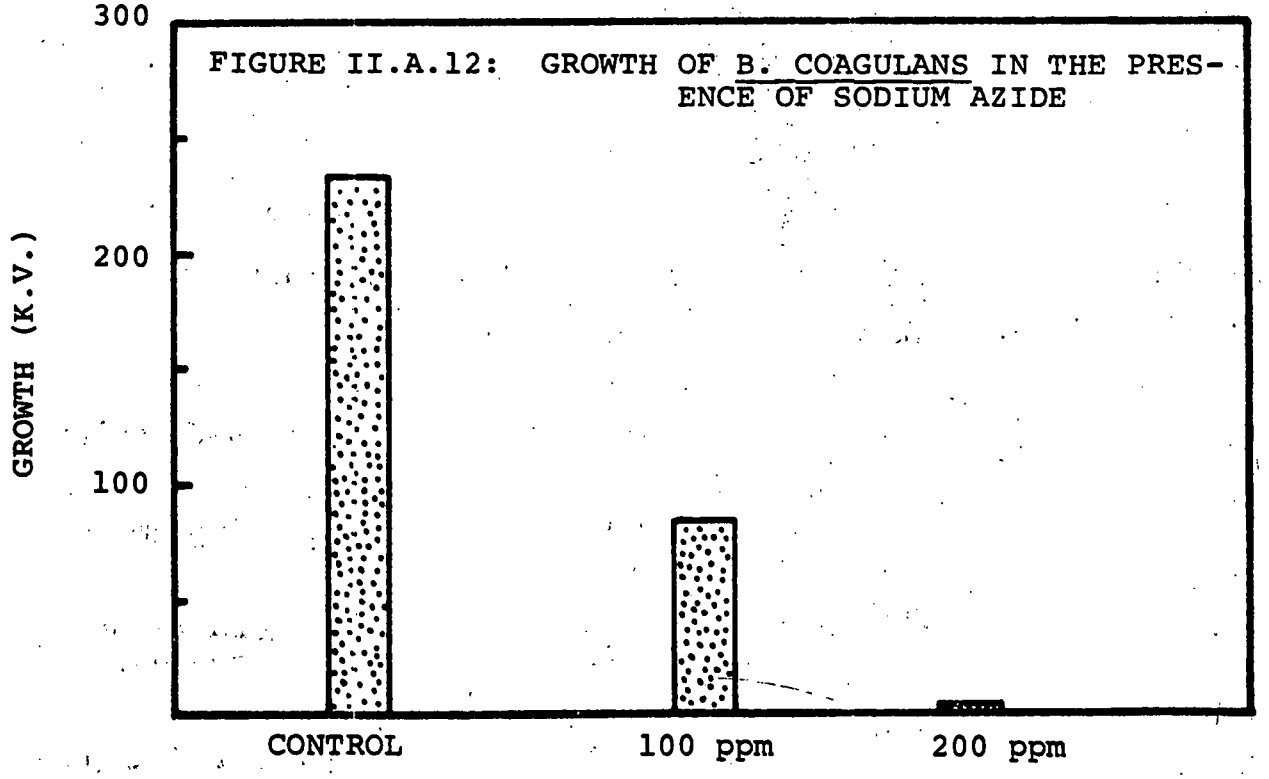


FIGURE II.A.12: GROWTH OF B. COAGULANS IN THE PRESENCE OF SODIUM AZIDE

- We will study the nutritional requirements of C. thermocellum and B. coagulans in an attempt to obtain a chemically defined medium which support rough growth of both microorganisms in mixed cultures.

- Experimental mixed population will be studied on solid substrate looking at the rates of cellulose degradation and the accumulation of lactic acid.

B. PRODUCTION OF ACETONE AND N-BUTANOL BY FERMENTATION

1. Introduction

In the two earlier progress reports (COO-4198-5 and COO-4198-6), we have reported that washed cells of *Clostridium acetobutylicum* were able to utilize glucose for the production of acetone and n-butanol. It was concluded then that such activities were results of the "resting cells." In this report, we performed experimental studies using "resting cells" in the presence of penicillin G to determine whether this type of cell preparation is truly able to produce solvents in the absence of growth. In addition, the study of using corn oil as an extracting agent for n-butanol in a batch fermentation was also performed.

2. Materials and Methods

a. Sensitivity of C. acetobutylicum towards Penicillin G

C. acetobutylicum was grown at various concentrations of penicillin G ranging from 0.1 to 10 units/ml. A soluble medium containing 20 g/l glucose was used. The final growths of the cultures were determined through optical density measurements after 48 hours of incubation at 37°C. The results were compared to a control where no penicillin G was added.

b. Resting Cell Experiment of C. acetobutylicum in Presence of 100 units/ml Penicillin G

The procedures and apparatus used were described in the progress report designated as COO-4198-6. The only difference in this experiment was that penicillin G was added to the suspending medium. The final concentration of penicillin G was 100 units/ml.

c. Resting Cell Experiment using Cells Prepared under Strict Anaerobic Conditions

The cells harvested from a fermentation were subjected to similar treatment as in the above experiments. However, these experiments were performed to ensure that the cells were not exposed to oxygen. The washing solution and suspending medium in these experiments were degassed before use. All the washing and suspensions were prepared inside an anaerobic hood containing N_2:CO_2 (95:5). The centrifugations were performed using air-tight bottles.

The cell pellets after washing were resuspended in medium and distributed to the following tubes. Five milliliters of the cell suspension were added to 5 ml medium. The final cell concentration was about 12 g/l. Types of incubation medium are shown below.

Tube	Medium
I	Phosphate, 10mM; cysteine, 0.5 g/l; pH, 6.7
II	Phosphate, 10mM; cysteine, 0.5 g/l; glucose, 20 g/l; pH, 6.7
III	Phosphate, 10mM; cysteine, 0.5 g/l; glucose, 20 g/l; yeast extract, 1 g/l; pH, 6.7
IA	Same as I, and penicillin G, 100 units/ml
IIA	Same as II, and penicillin G, 100 units/ml
IIIA	Same as III, and penicillin G, 100 units/ml

d. Extractive Fermentation with Corn Oil

A 5-liter fermentor was used in this experiment. The description of the fermentation system was reported earlier (COO-4198-4). The total working volume of the fermentor was 3 liters. Equal volume of a solubles medium containing 50 g/l of glucose was added to corn oil. The soluble medium and corn oil were sterilized separately. Temperature of the fermentation was controlled at 37°C, and the pH was controlled at above 5.0 by addition of 2N NaOH. Agitation of the fermentor was maintained at about 500 rpm. The high agitation rate was used to ensure an efficient contact between the two phases as well as providing means to withdraw equal volumes of the two phases during fermentation.

The samples taken at different time intervals were allowed to settle into two separate phases on ice for

about 10 minutes. From the aqueous phase, the cell density was determined by optical density measurement. The supernatants of the aqueous samples after centrifugation were frozen for subsequent substrate and product analyses. The oil phase was also extracted with equal volume of distilled water for n-butanol concentration determination. This serves as a double check on the measurement of the concentration of n-butanol in the oil phase. Cell dried weight of the samples could not be obtained due to the interferrence caused by the corn oil.

3. Results and Discussion

a. Sensitivity of C. acetobutylicum towards Penicillin G

The inhibition of growth by penicillin on C. acetobutylicum is shown in Figure II.B.1. The growth of C. acetobutylicum was inhibited by penicillin G at all concentrations above 1 unit/ml.

b. Resting Cell Experiment in Presence of 100 Units/ml of Penicillin G

The resting cell suspension in the presence of 100 units/ml penicillin G had no activity. Glucose was not metabolized during the 50 hours period and no product formation was observed.

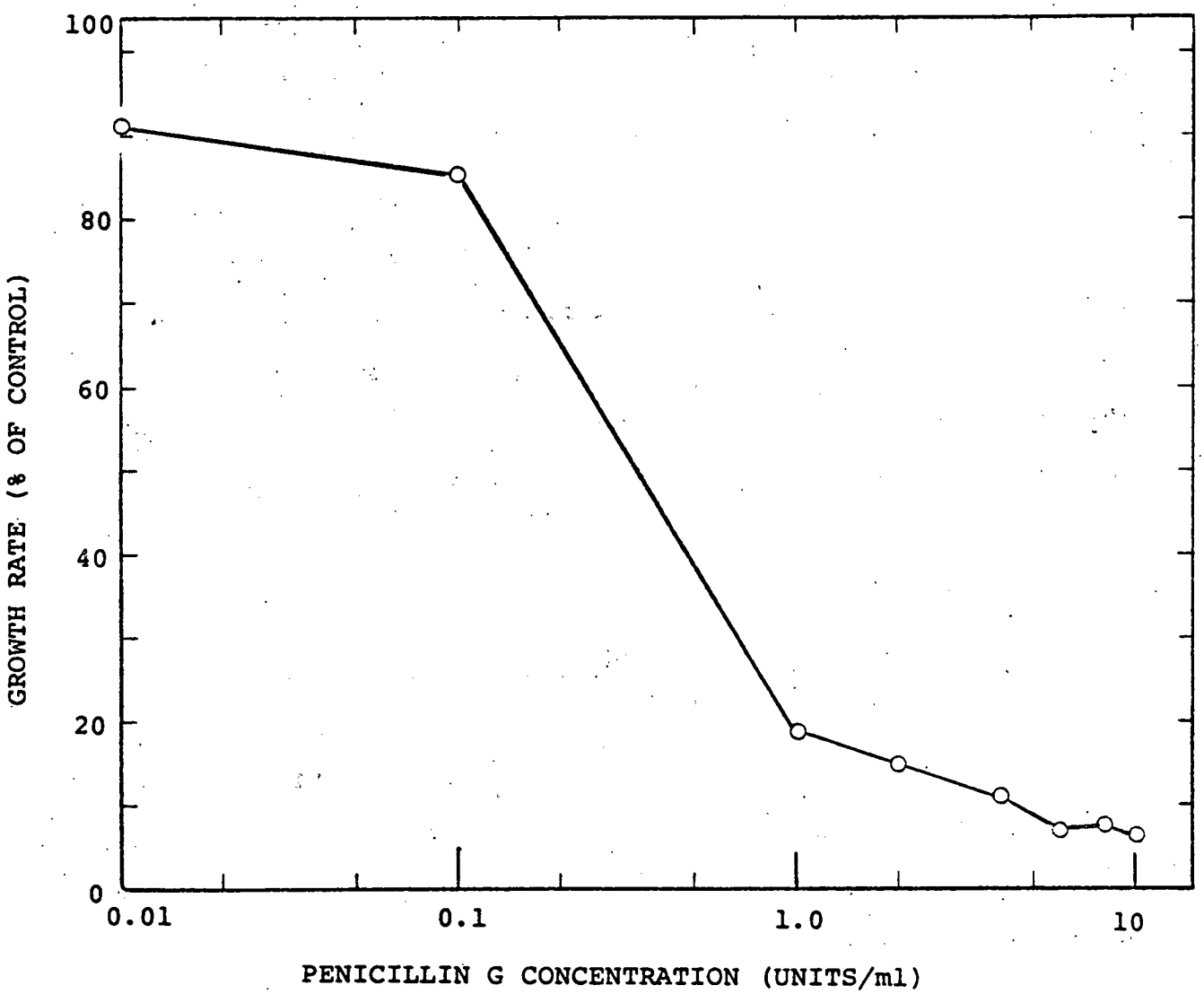


FIGURE II.B.1: EFFECT OF PENICILLIN G ON THE GROWTH OF *Clostridium acetobutylicum* (ATCC 824)

The mode of action of penicillin is the inhibition of enzymes relating to cell wall synthesis in gram positive microorganisms. In the presence of penicillin G, there is no new cell synthesized. Assuming that penicillin has no effect on cells that were intact, these results suggested that "resting cells" are not able to produce solvents.

c. Resting Cell Experiment using Cells Prepared under Strict Anaerobic Conditions

The results from the previous experiment suggests that either the resting cells do not have the capabilities to produce the solvents, or the resting cells ere damaged during the preparation stage. One of the most possible antagonizing factors is oxygen which could inhibit the growth of this organism. However, the results of the resting cell experiment performed using the greatest care to exclude oxygen exposure to the cells suggested that oxygen is not the inactivation factor. These results are shown in Table II.B.1. Again, in the absence of penicillin G, significant formation of products were observed, and in the presence of penicillin G no product accumulation was noted.

From these results, we conclude that the microorganisms that are not growing are incapable of producing acetone and n-butanol. The reported activities in earlier experiments are probably due to the regrowth of the cells due to

TABLE II.B.1: RESTING CELL EXPERIMENT. CELLS PREPARED IN ABSENCE OF OXYGEN. EXPERIMENT CARRIED OUT IN HUNGATE TUBES. CELL CONCENTRATION OF EACH TUBE WAS ABOUT 12 g/l d.c.w.

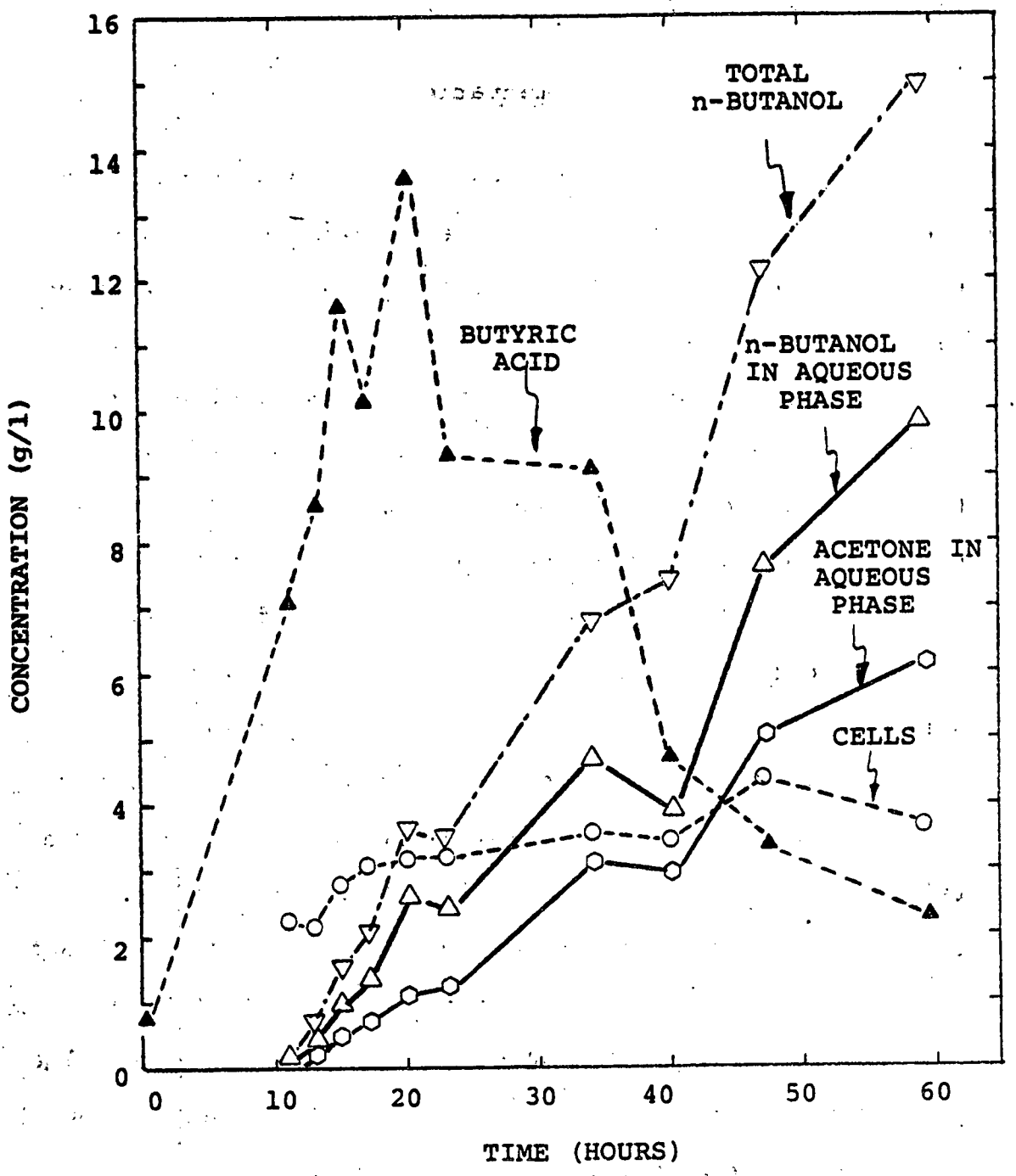
Medium	Penicillin Conc. (unit/ml)	Product Conc. (g/l) after 48 Hrs.			
		acetone	ethanol	n-butanol	butyric acid
I	0	0.03	0.05	0.22	0.32
II	0	0.08	0.07	0.34	1.00
III	0	0.26	0.12	0.98	1.01
IA	100	0.03	0.05	0.06	0.32
IIA	100	0.03	0.04	0.07	0.43
IIIA	100	0.04	0.07	0.05	0.26

endogenous metabolism. This is also supported by the observation of a slight increase of cell density in one of the resting cell experiments in report COO-4198-6.

d. Extractive Fermentation with Corn Oil

The results of this batch fermentation using *in situ* extraction are shown in Figure II.B.2. The general pattern of the fermentation are similar to the batch fermentations reported in the earlier reports. The butyric acid in the aqueous phase increased up to a concentration of 8 g/l before acetone and n-butanol began to appear. The final concentration of n-butanol in the aqueous phase was 10 g/l, and the acetone concentration was about 6 g/l. The n-butanol in the oil phase was about 5 g/l at the end of the fermentation. Thus, a total concentration of 15 g/l n-butanol was produced. The concentration of acetone in the oil phase was not determined. However, using a distribution coefficient of 0.3, the total acetone concentration was claculated to be 7.8 g/l. The fermentation ended due to the depletion of glucose which was about 2 g/l. The total glucose consumption was 93 g/l. (About 50 g/l of glucose was added to the fermentor at the 15th hour.) Therefore, the solvent yield from glucose was 0.24. This value is comparable to values obtained in other fermentations. However, compared to a conventional batch fermentation, the extractive fermentation takes about 2.5 times as much time to

FIGURE II.B.2: TIME PROFILE OF *in situ* EXTRACTIVE FERMENTATION (CORN OIL) USING *Clostridium acetobutylicum*



consume 100 g of glucose (60 hours vs 25 hours). Nonetheless, these results demonstrate the feasibility of increasing the final product concentration by extraction of n-butanol into an inert phase. Thus, if an extracting agent with a higher distribution coefficient for n-butanol is used, the final concentration can be increased even further. These results are quite encouraging in the use of in situ extraction to overcome product toxicity.

4. Future Work

- Extend study of extractive fermentation by selection of better extracting agent.
- Initiate study of high-cell-density packed column reactor for production of solvent.

C. PRODUCTION OF ACETIC ACID BY FERMENTATION

1. Introduction

In the previous progress reports, it has been shown that the production of acetic acid by *Clostridium thermoaceticum* is through both growth associated and non-growth associated modes. The production of acetic acid using resting cells was reported in progress report COO-4198-7. It was observed in growing and in resting cell experiments, cell lysis occurred and could be one of the factors causing the decrease of acetic acid production. Further studies were carried out to determine the cause of cell lysis, and also its effect on the production of acetic acid by resting cells.

Since xylose is one of the major reducing sugars produced by degradation of cellulosic biomass, preliminary studies were performed to determine the ability of *C. thermoaceticum* to use xylose as carbon source for acetic acid production.

2. Materials and Methods

The resting cell experiment was carried out in a 1-liter fermentor with a working volume of 750 ml. Anaerobic environment was maintained by passing gaseous CO_2 through the broth. The temperature was controlled at 60°C and pH was controlled at 7.0 by using 5N NaOH.

The experiments for xylose utilization were carried out in hungate tubes, using the complex medium as reported earlier, except that glucose was replaced by xylose.

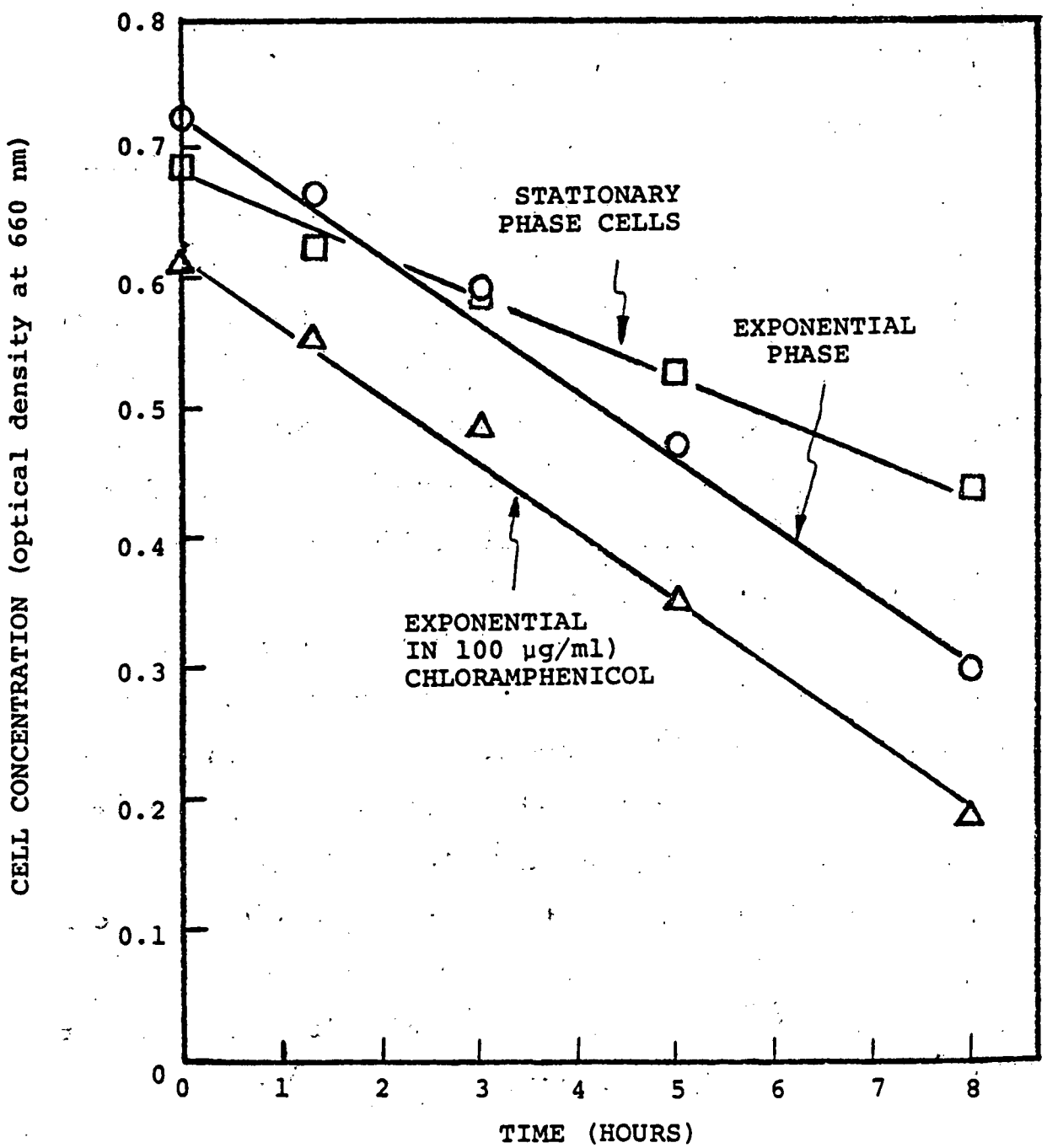
The other analytical methods have already been presented in the previous progress reports.

3. Results and Discussion

In the fermentation of Clostridium thermoaceticum under pH control, cell lysis was observed at high acetate concentration. Similarly, cell lysis was observed in the resting cell experiments. One possible explanation is the involvement of autolytic enzymes, similar to systems found widely in various gram-positive and gram-negative bacteria, such as in Bacillus licheniformic (Forsberg & Rogers, 1974, J. Bacteriol. 118, 358), or in Streptococcus faecalis (Cornett, Redman & Shockman, 1977, J. Bacteriol. 133, 631).

When cells are harvested, washed and resuspended into buffer of relatively high ionic strength (e.g. 0.3 M sodium phosphate, pH 7.1), rapid decrease of optical density was observed. This decrease of optical density is interpreted as cell lysis. Exponentially grown cells have a significantly higher rate of cell lysis than stationary grown cells, as shown in Figure II.C.1. If an autolytic system is causing the cell lysis, one method to verify such a phenomenon is to see whether this decrease of OD would be stopped with the addition of

FIGURE II.C.1: DECREASE OF OPTICAL DENSITY OF *Clostridium thermoaceticum* IN PHOSPHATE BUFFER



chloramphenicol. Chloramphenicol at concentrations between 50-200 μg/ml was shown to inhibit cell growth, and was used for this experiment. However, no significant effect on the lysis rate was observed as shown in Figure II.C.1. More efforts will be spent to study this "apparent" cell lysis behavior.

The production of acetic acid by resting cells under pH control is shown in Figure II.C.2. The kinetic profile is similar to the results presented in a previous report. The production rate is highest at the beginning and decreases with time. The cell mass, measured optically, also decreases steadily during the experiment from an initial value of 1.8 g/l to the final value of 0.7 g/l. The rate of decrease of acetic acid fermentation, however, is faster than the rate of decrease of cell mass. These results that the decrease of acetic acid production by resting cells is probably due to a combination of cell lysis and cell instability.

The production of acetic acid and cell mass at different concentrations of xylose is shown in Figure II.C.3. The conversion yield was found to be 0.76 gm acetate/gm xylose consumed, and the cell yield is about 0.21 gm cell/gm xylose consumed. The specific growth rate is .14 hr^{-1}. These results indicate that C. thermoaceticum can use xylose efficiently for acetic acid production with high conversion yield.

FIGURE II.C.2: PRODUCTION OF ACETIC ACID BY RESTING CELLS OF C. thermoaceticum AT pH 7.0

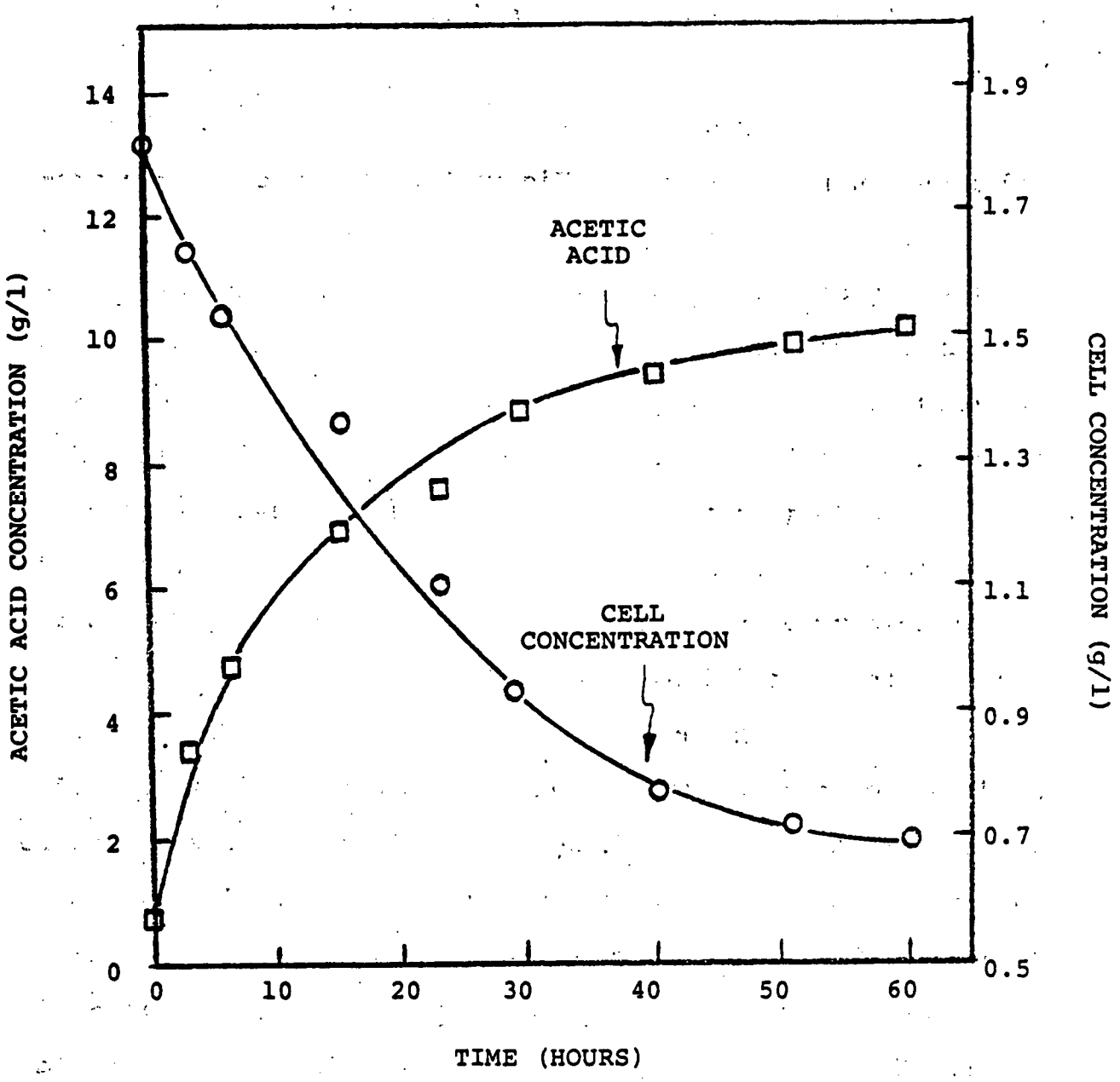
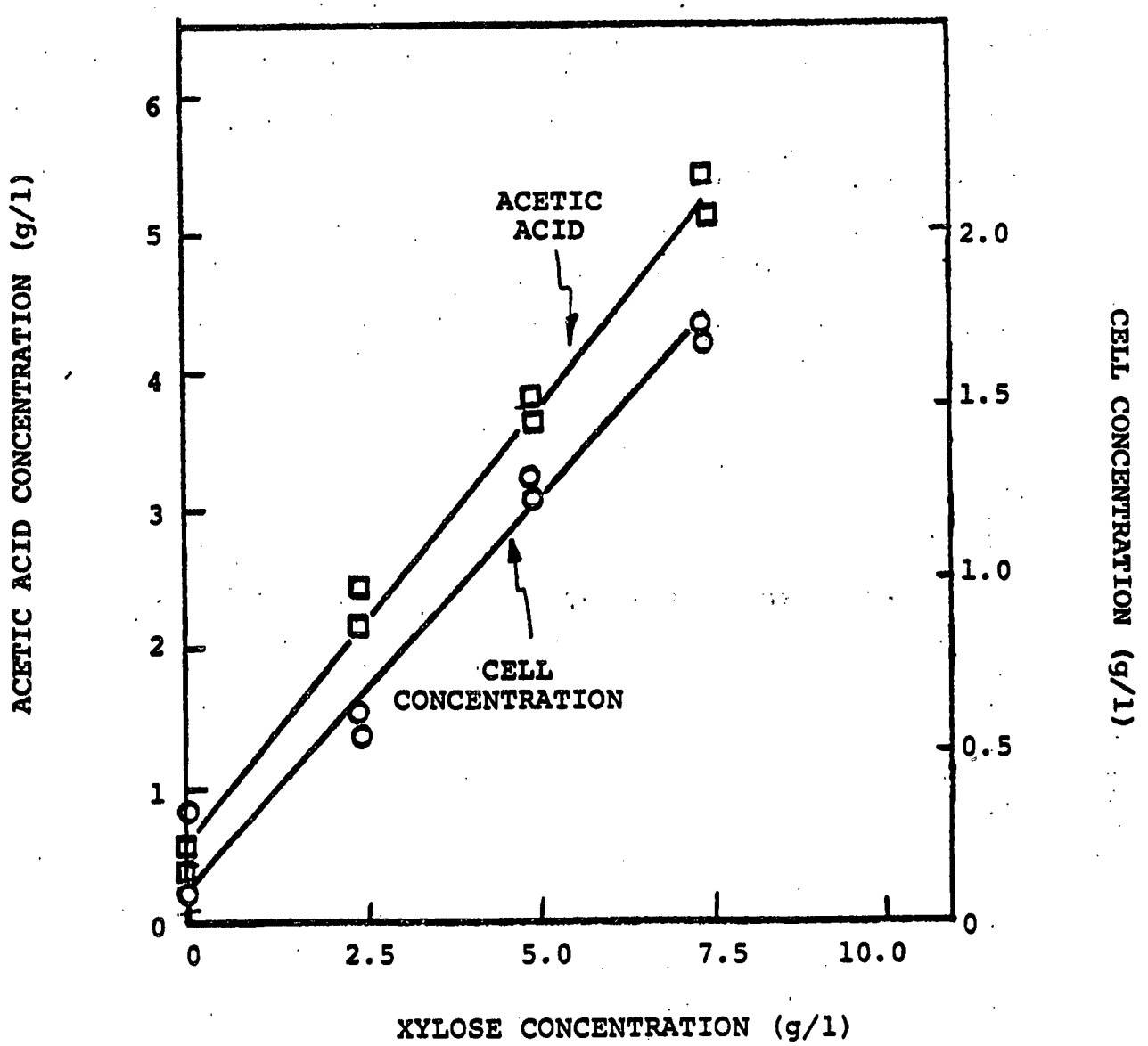


FIGURE II.C.3: PRODUCTION OF ACETIC ACID BY *Clostridium thermoaceticum* ON XYLOSE



4. Future Work

- Continue to study the causes of cell lysis, and find the appropriate means to eliminate it.
- Cell immobilization to increase stability and productivity of resting cells.
- Design of high productivity bioreactor.

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