COO/4198-3
Distribution Category UC-61

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

Progress Report
for Period June 1, 1977 - August 31, 1977

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SEPTEMBER, 1977

UNDER CONTRACT NO. EG-77-S-02-4198.AOOO

Prepared for
ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

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DISCLAIMER

Scope of Program

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

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

Abstract

Studies on the microbial degradation of cellulose biomass continues to be centered around Clostridium thermocellum. The effect of surfactants on growth and cellulase production by C. thermocellum was investigated. Triton X100, shown to be the most effective surfactant for releasing cellulase from residual cells and cellulose, was found to release additional endoglucanase (CMC'ase) activity but not activity against solid cellulose. Incubation of the cellulases from C. thermocellum under anaerobic conditions yields about a 15% increase in activity on CMC over aerobic incubation.

The effect of pH on growth and reducing sugar accumulation rate of Clostridium thermocellum on solka floc was evaluated. With a pH = 6.8-controlled system a two-fold increase in dry cell weight was obtained when C. thermocellum was cultured on cellobiose (1%). When it was cultured on solka floc (1%) and cellobiose (0.16%) the dry cell weight increased 1.5 times. Consequently the cellulase degradation and sugar accumulation rate increased 1.5 - 1.7 times. Extracellular cellulase on CMC values increased to 22 $\frac{mgRS}{ml\ hr}$. A mutagenic procedure has been established to attempt to isolate strains with an enhanced ability to accumulate reducing sugars from cellulose.

Activity of extracellular cellulase of Clostridium thermocellum ATCC 27405 was examined using TNP-CMC and Avicel as substrates. The pH optima are 5 and 4.5 respectively. Hydrolysis of either substrate is not inhibited by cellobiose, xylose, or glucose. The enzyme appears to be quite stable under reaction conditions at 60°C. Thus far, regulation studies indicate that CMCase formation is not repressed by cellobiose.

The search for plasmids in C. thermocellum was continued. The presence of plasmids was confirmed by cesiumchloride ethidium bromide gradient centrifugation and electron microscopy. Two plasmids were detected, one with an approximate molecular weight of 1×10^6 daltons and the other 3.5×10^6 daltons. At least one of the plasmids appears to be present in multiple copies per cell. This characteristic, if indeed true, would make this plasmid ideal for gene amplification. Our pursuit of a system of gene transfer continues. The feasibility of transformation in this organism is being evaluated and cell fusion techniques are being explored.

Studies on the fermentation of lactic acid to propionic acid have shown the pathway in C. propionicum to be simpler than in M. elsdenii and hence more amenable to manipulation for acrylate production. Using Lactobacillius delbrueckii, we are able to convert glucose, cellobiose, and cellulose hydrolysates to lactic acid rapidly and quantitavitely. This fermentation, coupled to C. propionicum should be quite suitable as an approach to acrylate production. Studies to evaluate the aerobic oxidation of propionate to acrylate as an alternative approach have been initiated.

Fermentations of C. acetobutylicum growing in soluble media were performed. The formation of cell mass and end products especially n-butanol and acetone seem to be limited by the supply of glucose and inhibited by the accumulation of acids. Batch cultures of C. acetobutylicum have shown no significant butanol production at 2.0 - 3.4 g/l glucose. Production is significant at 10g/l glucose. Initial pH in weakly buffered media has no significant effect on the fermentation. Tolerance of butanol lies in the range of 1.6 - 2.4%. Acetopyruvate inhibits growth completely at 5mM. At lower levels butanol production is stimulated. Initial studies have been done on immobilization of cells.

Detailed studies of Clostridium thermoaceticum have shown that pH is the primary limiting factor in the production of acetic acid. pH-controlled fermentations have indicated accumulations of over 30 gm/l of acetic acid.

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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 each name.

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Professor of Biochemical Engineering
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This progress report acknowledges the contribution by these individuals listed above.

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PROGRESS REPORT

I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. Effect of Surfactants on Growth and Cellulase Production by C. thermocellum

1. Introduction

In our last progress report (No. 2, March 1 - March 31, 1977) we presented the results of an experiment aimed at determining the effect of surfactants on growth and cellulase production by C. thermocellum. At the concentrations used, all the surfactants (Triton X100, Tween 20, and Tween 80) were inhibitory to growth and thus enzyme levels were lower than controls. Nevertheless, specific total activities, i.e. total enzyme units per gram of dry cell weight were higher. Since Tween 80 was least inhibitory to growth, the organism was again cultured in the presence of lower concentrations of this surfactant to determine whether a level could be achieved which allows growth and, simultaneously, an increased release of cellulase enzymes. Even at the lowest concentration used, cell growth was still inhibited.

2. Materials and Methods

C. thermocellum was cultured in Hungate anaerobic tubes (10 ml volume) on CM3 medium containing Tween 80 (polyoxyethylene monooleate) at concentrations of 0.01, 0.025 and 0.05 per cent. Control tubes containing no surfactant were run as well. Cultures were harvested at 48 hrs at which time pellet protein was determined (as described in earlier reports) as measurement of cell growth. The CMC assay was performed on supernatant fractions. The pellet was not assayed for CMCase activity.

3. Results and Discussion

The data from this experiment are presented in Table I.A.1. The primary purpose of this follow-up experiment was to see whether or not growth would occur at lower Tween 80 concentrations, comparable to control values. As can be seen in the table, even at 0.01%, Tween 80 inhibits growth of the organism. Soluble CMCase activity is not significantly enhanced by the surfactant though specific activities remain higher for these cultures. The inhibitory effect on growth precludes the use of surfactants as an environmental means of increasing soluble enzyme yields during growth. Nevertheless, surfactants may be used post-fermentation to release enzyme adsorbed onto residual fermentation solids.

Table I.A.1. Effect of Tween 80 on growth and soluble enzyme levels of C. thermocellum

%Tween 80 (v/v)	Tube #	Pellet Protein ug/ml	Soluble CMCase RS mg/ml · hr	Sp. activity units/ug pro
0.01	1	75	1.70	0.023
	2	95	2.10	0.022
0.025	1	85	2.70	0.032
	2	82	2.25	0.027
0.05	1	80	2.40	0.030
	2	73	2.40	0.033
0.00	1	127	2.35	0.019
	2	120	2.35	0.019

B. Degradation of Cellulosic Biomass to Produce Soluble Products

1. Introduction

Clostridium thermocellum when grown in batch culture at 60°C on solka floc as a carbon source has been shown to have greater hydrolytic activity than is required for growth. As a consequence it is able to accumulate reducing sugars in the broth. In light of this (see earlier progress reports for more details) one of our objectives is to define the conditions required to obtain high rates of sugar production.

From the previous kinetic analysis, it has been shown that sugar production is growth related; therefore, batch fermentations have been run in which an attempt has been made to generate a dense growing cell mass to increase the rate of sugar production. This has been achieved through the use of pH control, the addition of initial low concentrations of cellobiose, and increased yeast extract (10g/l).

2. Materials and Methods

Organism - Clostridium thermocellum ATCC 27405

Growth of Clostridium thermocellum is on the basic CM3 medium containing solka floc as a carbon source with or without cellobiose. Yeast extract was increased to 1%. The organism was cultured in anaerobic flasks and in a 7 liter fermentor with the agitation at 100 rpm and the temperature at 60°C. pH was continuously recorded and controlled at 6.8 with a radiometer pH controller and when needed NH_4 OH (10% solution) was added automatically. Anaerobiosis was maintained by passing a N_2-gas stream through the head space of the fermentor during the fermentation.

Broth samples were analyzed for its content in reducing sugars by

the DNS Method. Growth was determined by the filtration-optical method described in the first progress report (Dec. 1, 1976 - Feb. 28, 1977).

Cellulase activity was measured with 1 ml of 2% CMC (Sigma) in citrate buffer 0.05 M pH 4.5 as a substrate and 0.2 ml of centrifuged broth. After incubation for 30 min at 60°C the reducing sugars liberated were measured by the DNS photocolorimetric method. Glucose solutions were used as a standard.

3. Results and Discussion

From our previous results with batch fermentation we observed that the pH dropped from 6.8 to 5.5. To determine if this change in pH had an effect on the growth rate and product accumulation, we designed an experiment with pH control using cellobiose (1%) as the carbon source to quantify the effect of pH on cell accumulation.

Our results showed us that we could indeed increase the cell density by at least twice the amount over that obtained with no pH control. These results are in Table I.B.1.

Table I.B.1. Growth of Clostridium thermocellum in Batch Fermentation with 1% cellobiose.

	Dry Cell Weight	Sugar Consumption	Yield Coefficient
	gr/lt	gr/lt	$\frac{\text{gr Cells}}{\text{gr Substrate}}$
with pH = 6.8 control	1.22	8.4	0.14
without pH (5.5 final) control	0.67	4.2	0.16

With these results in hand, we proceeded to run the cellulose fermentation with pH control. In Fig. I.B.1, the kinetic profile of residual cellulose and dry cell weight are shown. 1.6 gr/lt of cellobiose at the beginning of the fermentation to initiate rapid growth. biose was added at the beginning of the fermentation to initiate rapid growth. In this manner the amount of dry cell weight was increased to endoglucanase activity (CMCase) are in this case 3 times higher, however it is important to point out that in a different run, values as high as $\frac{40 \text{ Mg RS}}{\text{ml hr}}$ of endglucanase activity have been obtained.

The rate of cellulase degradation also increased, as shown in Fig. I.B.2, when the different values were obtained with and without pH control. The same figure depicts the reducing sugar concentrations for comparison. It can be seen that C. thermocellum uses the soluble sugar first, and when it starts to use cellulose, reducing sugars begin to accumulate at a rate slightly higher than without pH control.

4. Future Work

   a. Kinetics of growth and cellulose degradation in continuous culture.

   b. Examination of real cellulosic wastes or residue, e.g. corn residue.

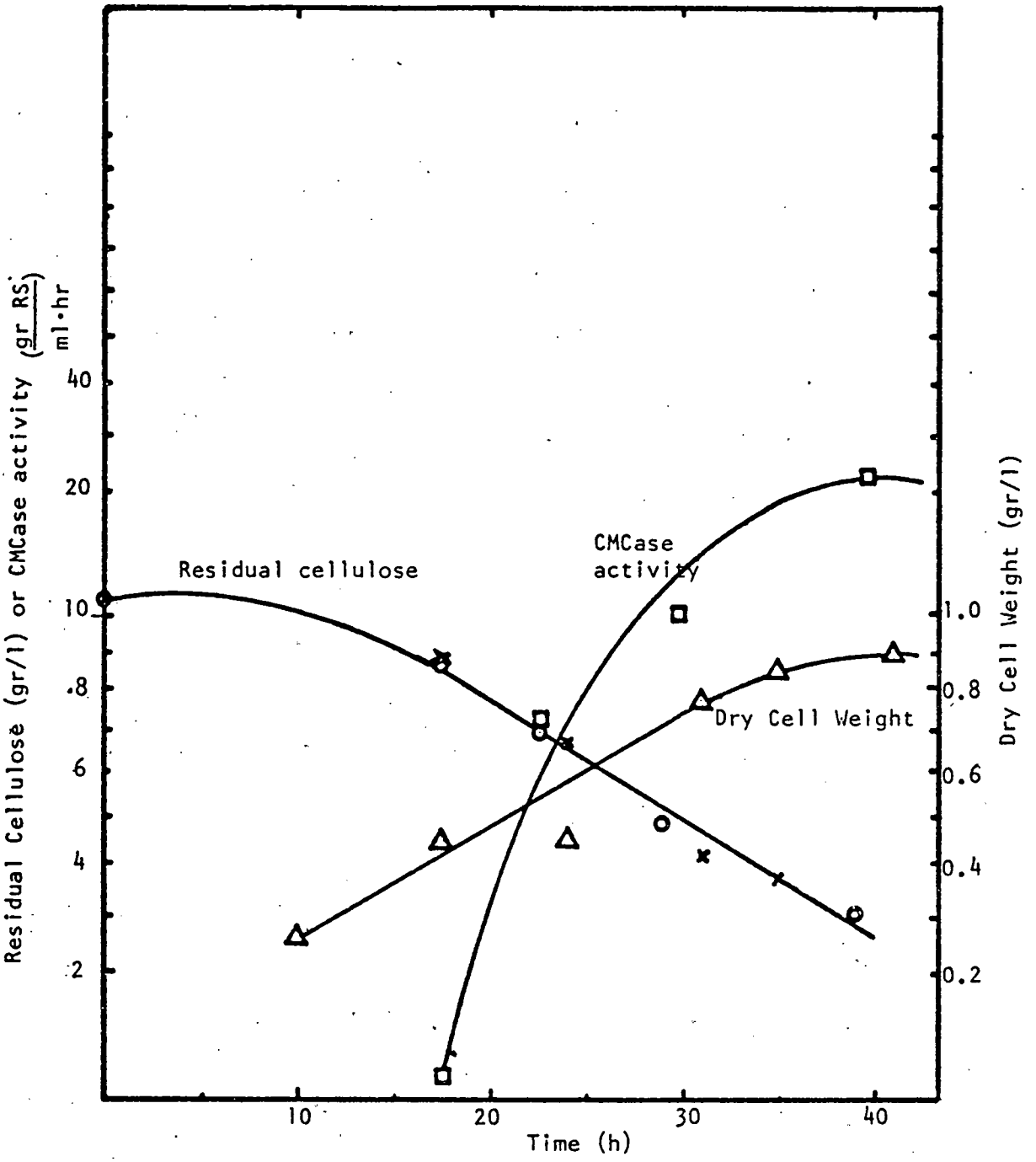


Fig. I.B.1: Growth, Reducing Sugar and Residual Cellulose During Fermentation by Clostridium Thermocellum With pH Control at 6.8.

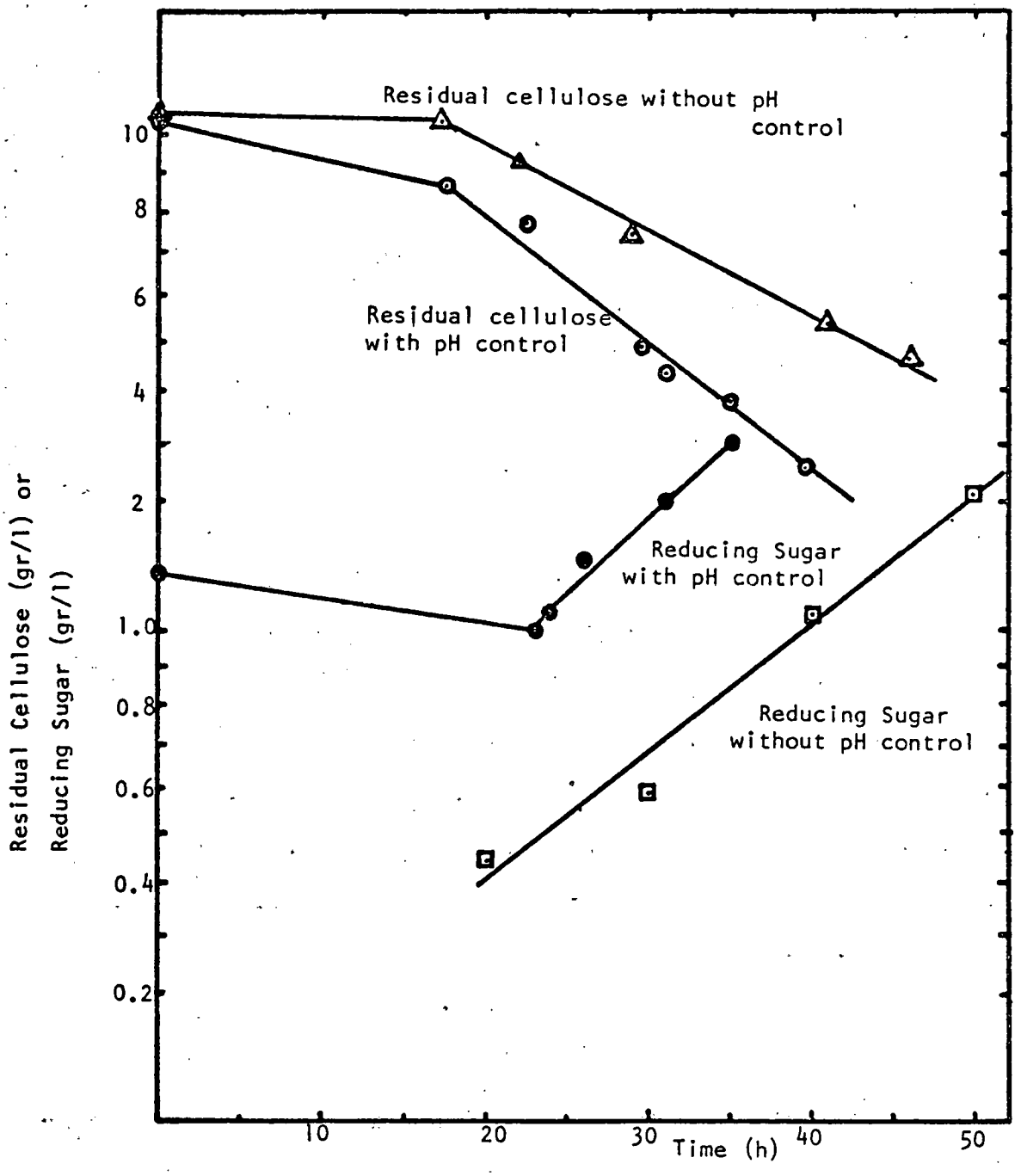


Fig. I.B.2: Cellulose Degradation and Sugar Accumulation Rates With and Without pH Control.

C. Strain Improvement by Mutation to Improve Reducing Sugar Accumulation

1. Introduction

Another approach to increase rate of sugar accumulation is by genetic manipulation of strain characteristics. To initiate a mutation program we have choosen nitrosoguanidine as a first mutagenic agent. Our criteria for mutant selection will be halo formation on agar plates containing cellulose as the carbon source. This will permit us to observe mutants with high hydrolytic activity.

2. Materials and Methods

A culture of Clostridium thermocellum, growing on cellobiose was harvested by centrifugation and then washed with citrate buffer (0.05 M with 0.5 gr/lt of thioglycollate). It was centrifuged again and resuspended in citrate buffer. Klett reading was adjusted to 65 units and nitrosoguanidine at 25, 50 and 100 ug/ml was added in different tubes. A control with no nitrosoguanidine was prepared at the same time. After 30 min at room temperature, the culture was diluted to stop the mutagenic action and then plated to determine viability. Different dilutions were plated using 1 ml each and pouring 20 ml of agar--cellulose medium with Avicel (1%) as a carbon source. The plates were incubated in an anaerobic jar at 60°C. Dilutions were made with a solution of thioglycollate (0.5gr/lt) and peptone (0.1%).

3. Results and Discussion

Fig. I.C.1 shows the killing curve obtained with nitrosoguanidine. From these results it is concluded that 100 ug/ml is

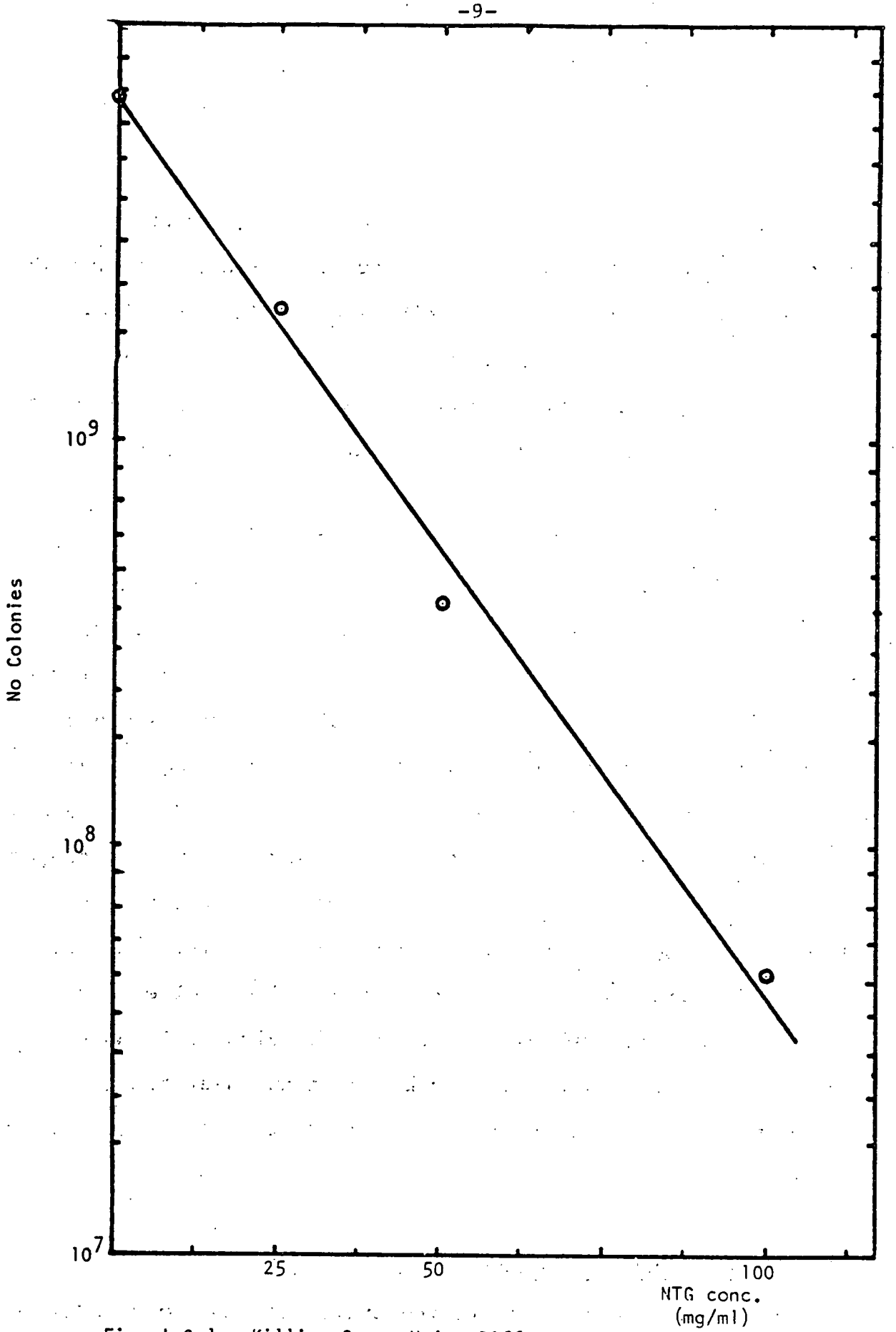


Fig. I.C.1: Killing Curve Using Different Nitrosoguanidine Concentrations.

the dose to obtain 99 % kill. This dose will be used to continue the mutant selection.

Fig. I.C.2 shows the plates obtained with C. thermocellum using Avicel and solka floc as a carbon source.

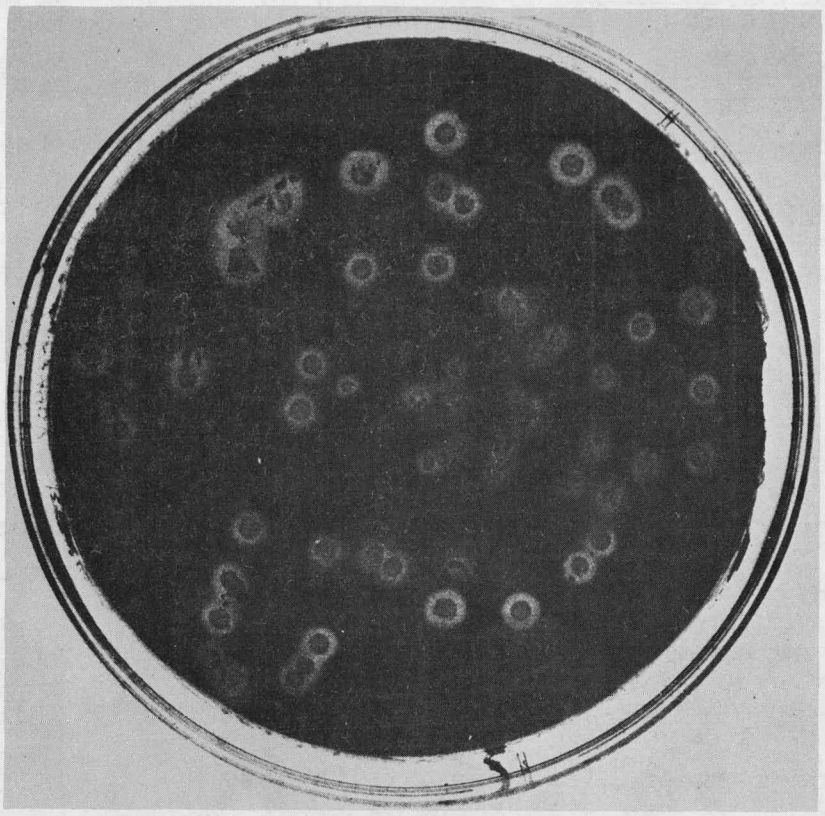
4. Future Work

a. Selection of mutants

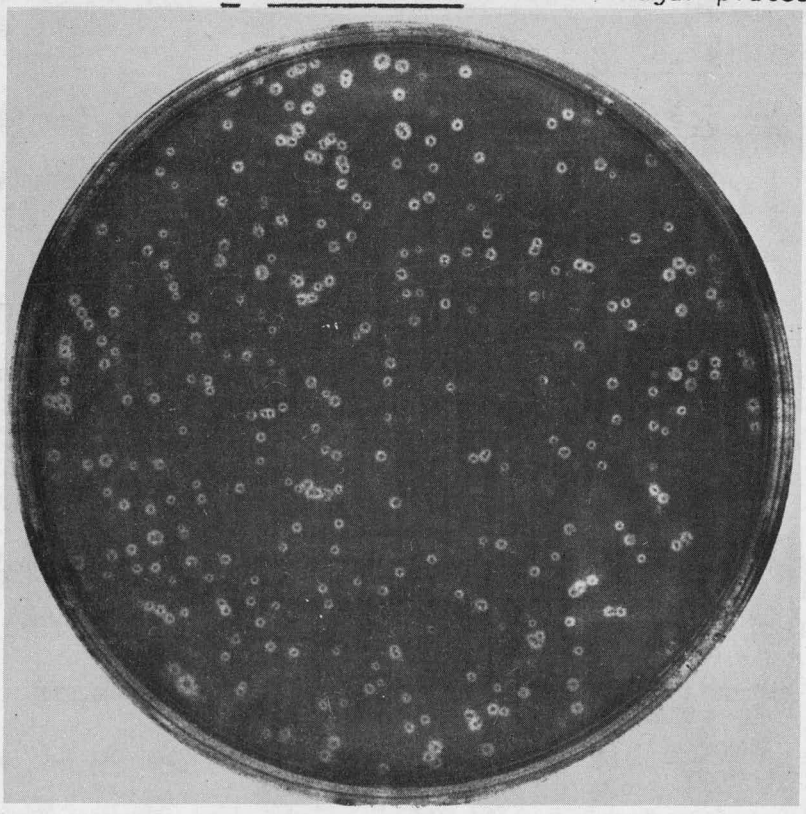
b. Use of other mutagens

Fig. I.C.2

a. Colonies of C. thermocellum on solka floc agar plates



b. Colonies of C. thermocellum on Avicel agar plates



D. Clostridium thermocellum cellulase: Effect of anaerobic assay conditions on enzyme activity.

I. Introduction

Because Clostridium thermocellum is an obligately anaerobic bacterium, the possibility that it produces oxygen-sensitive cellulases had to be considered. To determine whether or not additional enzymatic activity, be it exoglucanase, endoglucanase or β-glucosidase, could be detected in the absence of oxygen, the filter paper and carboxymethyl-cellulose assays were performed under anaerobic conditions. Since the filter paper activity present in fermentation broths has in the past been low, we were particularly interested in the effect of anaerobiosis on exoglucanase activity. The results presented here indicate that it is endoglucanase (CMCase) activity which is slightly stimulated under anaerobic assay conditions.

2. Materials and Methods

C. thermocellum was grown in a two-liter fermentor on CM3 medium as previously described (Progress Report, December 1, 1976 - February 28, 1977). The filter paper assay was performed on a sample taken from the fermentor at 92 hours in the following manner. In addition to the usual assay tubes (described elsewhere), quadruplicate tubes containing enzyme + buffer + + filter paper that were sparged with nitrogen for approximately 10 minutes prior to the 60 minute incubation were included. After reaction with the DNA colorimetric reagent for measuring reducing sugars, the Absorbance 550_{nm} values for these N_2-sparged assay mixtures were noticeably higher than those exposed to air during incubation. This was a first indication that enzyme activity might

be enhanced under anaerobiosis; however, since N_2-sparged controls had not been included, the experiment was repeated.

At 114 hours a sample from the same fermentation was withdrawn from the fermentor into a small flask into which N_2 was bubbled. While continuing to bubble N_2, the broth was pipetted into centrifuge tubes which were then capped. Centrifuged for 15 minutes at 20,000 x g. Unfortunately, the capping of the centrifuge tubes failed to maintain anaerobiosis as was apparent from the color change of the resazurin used as O_2 indicator. Nevertheless, the supernatants were removed and bubbled with nitrogen until anaerobiosis was once more achieved. The supernatant was then ready to be used in both the filter paper and CMC assays. All assay reagents, that is, the sodium citrate buffer, the 2% CMC solution and the standards were prepared containing resazurin so that anaerobiosis or lack thereof could be observed during the assay. Assay mixtures were run in quadruplicate in the Hungate anaerobic tubes used to culture the organism. Aerobic assay mxitures were also run. As is normally done, controls to detect background activity, i.e. tubes containing enzyme + buffer but no substrate were included and incubated. These controls pick up the hydrolysis of oligomers already in solution in the fermentation broth. Also, reducing sugars present in this 14hr-old sample, prior to incubation at 60°C for 60 minutes, were measured.

3. Results and Discussion

The results from this experiment are summarized in Table I.D.1.

Table I.D.1. Activities of extracellular C. thermocellum cellulases assayed aerobically and anaerobically

Quantity measured	Aerobic conditions	Anaerobic conditions
1.*Reducing sugars (mg/ml) in background controls	0.45 ± 0.02	0.66 ± 0.0
2. Filter paper activity (mg reducing sugar/ml·hr)	0.24 ± 0.0	0.21 ± 0.04
3. CMCase activity (mg reducing sugar/ml · hr)	3.0 ± 0.0	3.43 ± 0.02

*The concentration of reducing sugars originally present in the sample, i.e. in an aliquot that was not incubated at 60°C for 60 minutes was 0.43 mg/ml.

As indicated in Table I.D.1, anaerobiosis had no effect on improving filter paper activity. It should be noted that these filter paper activities are comparable to the maximal in vivo rates of cellulose degradation reported for C. thermocellum in our first progress report. However, the anaerobic assay conditions did seem to enhance CMCase activity slightly, an increase of roughly 15%. Whether the oxygen removal was beneficial for a single enzyme with endoglucanase activity or several enzymes cannot be determined until we have better characterized the components of the C. thermocellum cellulase system.

The most significant change under anaerobic conditions is in the concentration of background reducing sugars. Whereas under

aerobic conditions the amount of reducing sugars after incubation of the sample in the absence of substrate (filter paper or CMC) is essentially equal to the amount originally present, the concentration of reducing sugars has increased by roughly 50% after incubating anaerobically. This suggests at least two possibilities. It may be reflective of enhanced endoglucanase activity (as observed with CMCase activity) which is hydrolyzing soluble oligomers present in the sample. It may possibly be reflective of "cellobiase" activity which is otherwise oxygen sensitive. Since filter paper and CMC were the only substrates tested here nothing conclusive can be said in this vein. The experiment can be repeated with cellobiose as assay substrate to try to assess the existence of an enzyme with 0_2-sensitive β-glucosidase activity. Since we have shown that this strain of C. thermocellum utilizes glucose, such an enzyme with β-glucosidase activity may indeed exist.

If the increased background sugars are due to endoglucanase activity, one can total this value with the CMCase activity. That is, if the original sugar concentration was 0.43 mg/ml an extra 0.23 mg/ml of sugars were released in an hour of anaerobic incubation at 60°C. Added to the CMCase activity of 3.43 mg/ml · hr, this gives $\sim$ 3.7 mg sugars/ml·hr compared to 3.0 under aerobic conditions, or an effective increase of > 20%.

As mentioned in the materials and methods section, anaerobiosis was not maintained during centrifugation of the broth. Whether this exposure to air caused any significant damage that was irreversible with the post-centrifugation N_2 gassing cannot be said. The addition of a reducing agent to the broth or to the assay buffers can be investigated in a similar experiment. Bubbling of pure 0_2

into the assay mixtures can also be tried to determine whether this effects decreased enzyme activity.

E. Deregulation of clostridial cellulase

1. Optimum pH of C. thermocellum cellulase

The optimum pH of extracellular cellulase from C. thermocellum was determined using both TNP-CMC and Avicel as substrates. As shown in Fig. I.E.1, TNP-CMCase was most active at pH 5 whereas the activity against Avicel was optimal at 4.5. A temperature of 60°C was used for both determinations.

2. Resistance of C. thermocellum cellulase to sugar inhibition

In our last report, we presented data from our initial experiments indicating that TNP-CMCase was not inhibited by sugars such as cellobiose, glucose or xylose. This finding is extremely important since most cellulases are inhibited by these breakdown products of waste cellulosic substrates; in fact, such inhibition is what limits the usefulness of Trichoderma viride cellulase. For this reason, further experiments were done using other cellulosic substrates to insure that the effect was not specific for TNP-CMC. Increasing amounts of glucose were added to CMC; then, C. thermocellum extracellular cellulase was added. As shown in Fig.I.E.2, glucose up to 1g/l failed to inhibit CMCase activity. Further evidence is shown in Fig. I.E.3 where CMC was prepared with a final concentration of 0.2mg/ml of cellobiose, xylose or glucose and extracellular cellulase was added. Again, none of the sugars inhibited CMCase activity. Fig. I.E.4 shows that Avicel hydrolysis is likewise uninhibited by the three sugars at concentrations of 0.05mg/ml.

3. Effect of substrate concentration on hydrolyses of CMC and Avicel

We previously reported that TNP-CMCase was not saturated with substrate until 14mg/ml substrate was used. In view of the fact that Fig. I.E.3 indicated a continual decrease in hydrolysis with time when 6.7mg/ml CMC was used as substrate, we attempted to determine whether substrate limitation was the problem.

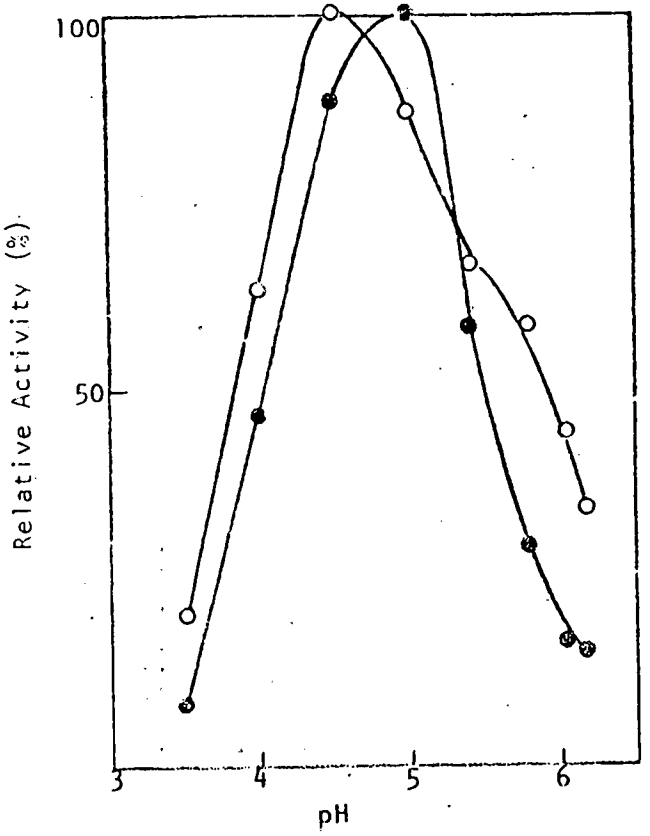


Fig. I-E-1. pH vs. activity curves of cellulase.

o: Avicel hydrolyzing activity (60°C, 2hr)

●: TNP-CMCase (60°C, 1 hr)

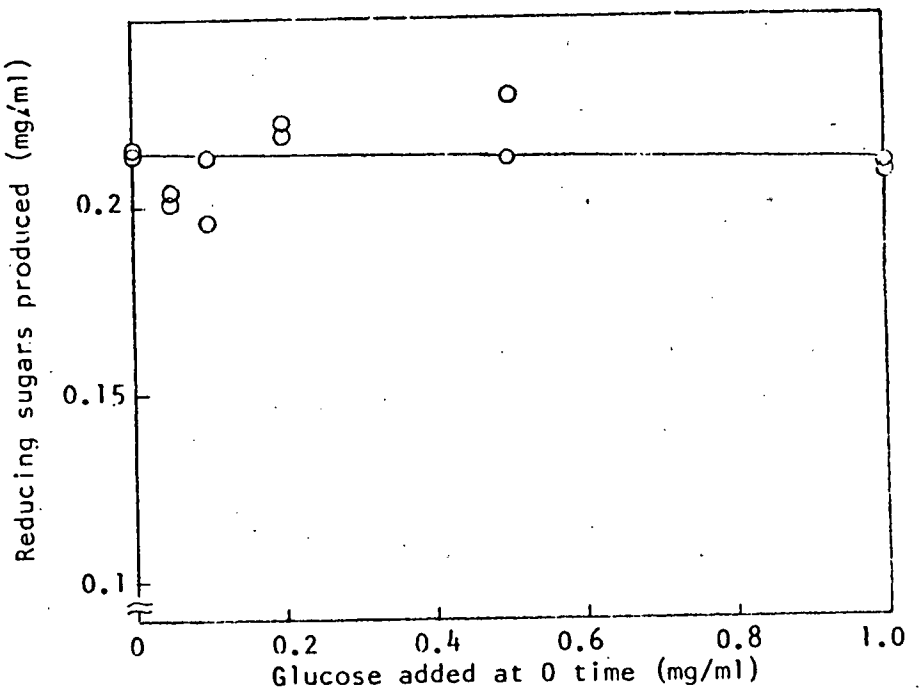


Fig. I-E-2. Effect of glucose on CMCase activity. (1 hr reaction time)

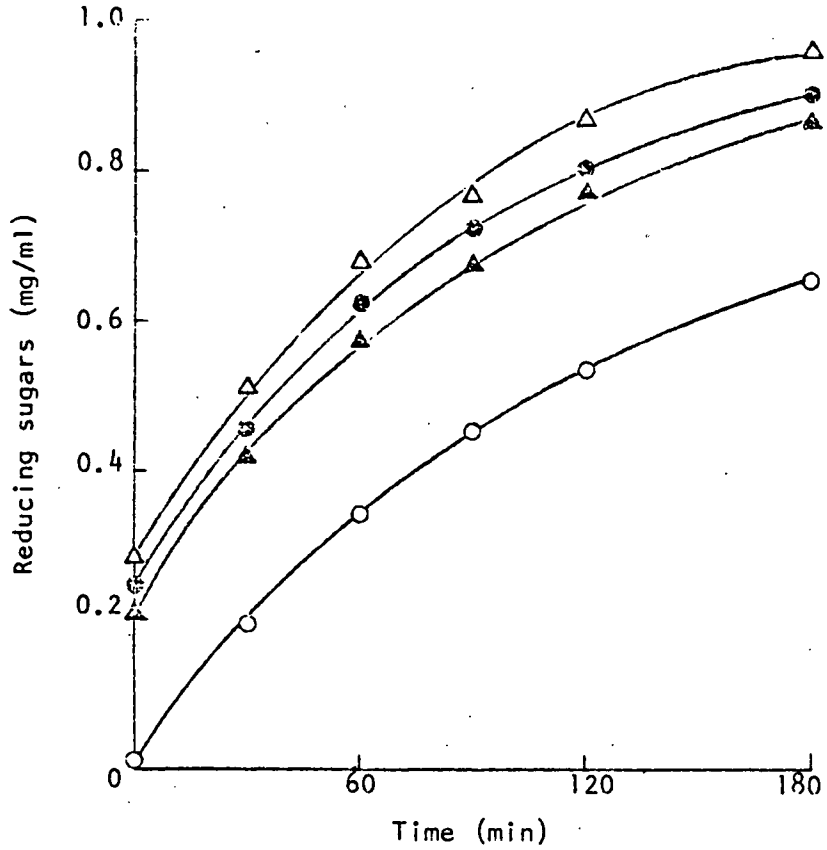


Fig. I-E-3. Effect of sugars on CMCase activity. At 0 time, glucose (⊖), xylose (Δ), or cellobiose (▲) was added to a final concentration of 0.2mg/ml. Open circles show the control run without addition.

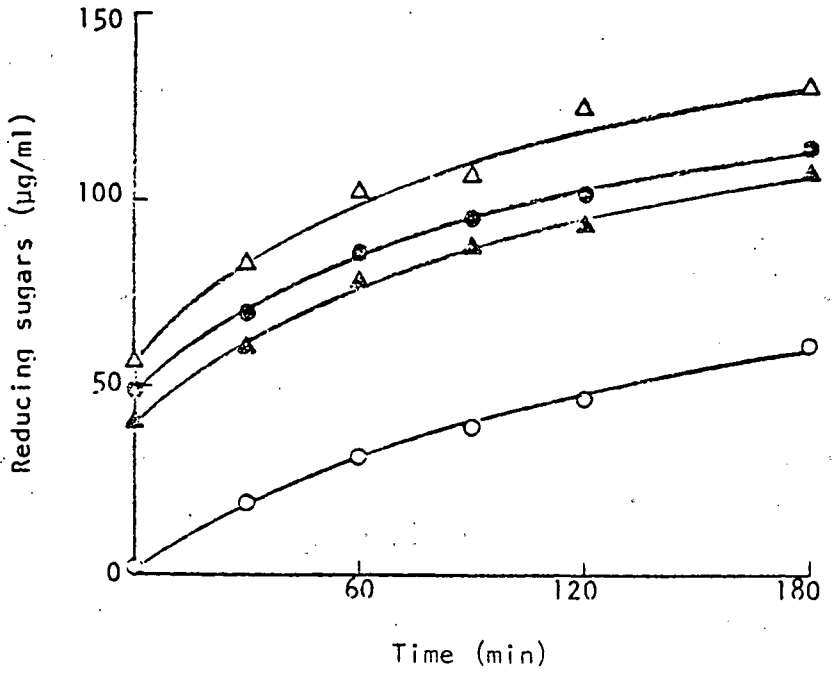


Fig. I-E-4. Effect of sugars on Avicel hydrolysis. Experiment was the same as Fig. I-E-3.except sugars were added to a final concentration of 50µg/ml.

Fig. I.E.5 shows that this is the case; up to 20mg/ml CMC, the rate and extent of the CMCase reaction increased. A Lineweaver-Burk plot (Fig. I.E.6) showed a Km of 3.6mg/ml, very similar to that of TNP-CMCase reported earlier. A drop off in rate of hydrolysis is shown in Fig. I.E.7. Here additional substrate (10mg/ml) was added at 180 min to a reaction mixture which initially contained 10 mg CMC/ml. This addition resulted in a renewed rate of hydrolysis equal to the initial rate. This experiment also points out the stability of the enzyme since after 3 hours, the initial hydrolytic rate could be achieved by adding more substrate.

4. Preparation of a chromogenic reagent from Avicel

In order to devise a chromogenic assay for Avicel hydrolysis, Remazol Brilliant Blue (RBB) was complexed with Avicel according to the procedure of Finderknecht et al. (Experimentia 23, 805, 1967). This substrate was exposed to C. thermocellum extracellular cellulase but unfortunately the solubilization of blue color was too low to be useful. Our results indicate that only 1 mole of RBB was liberated per 31 moles of glucose released. This agrees fairly well with the data of Leisola and Linko (Analyt. Biochem. 70, 592, 1976) who found one dye group released per 40 glucose groups released. Furthermore, we find that the rate of sugar release from RBB-Avicel is only 28% of that from Avicel. We thus conclude that RBB-Avicel is not a useful chromogenic substrate at this point in our studies.

5. Induction of cellulase

A refrigerated cellulose grown (1% MN 300) culture was transferred (10% v/v inoculum) into MN 300 and grown for 48 hours. This was inoculated (3% inoculum) into 1% cellobiose and grown for 24 hours as seed. The seed was inoculated (3% inoculum) into CM 3 medium containing different carbon sources (Fig. I.E.8).

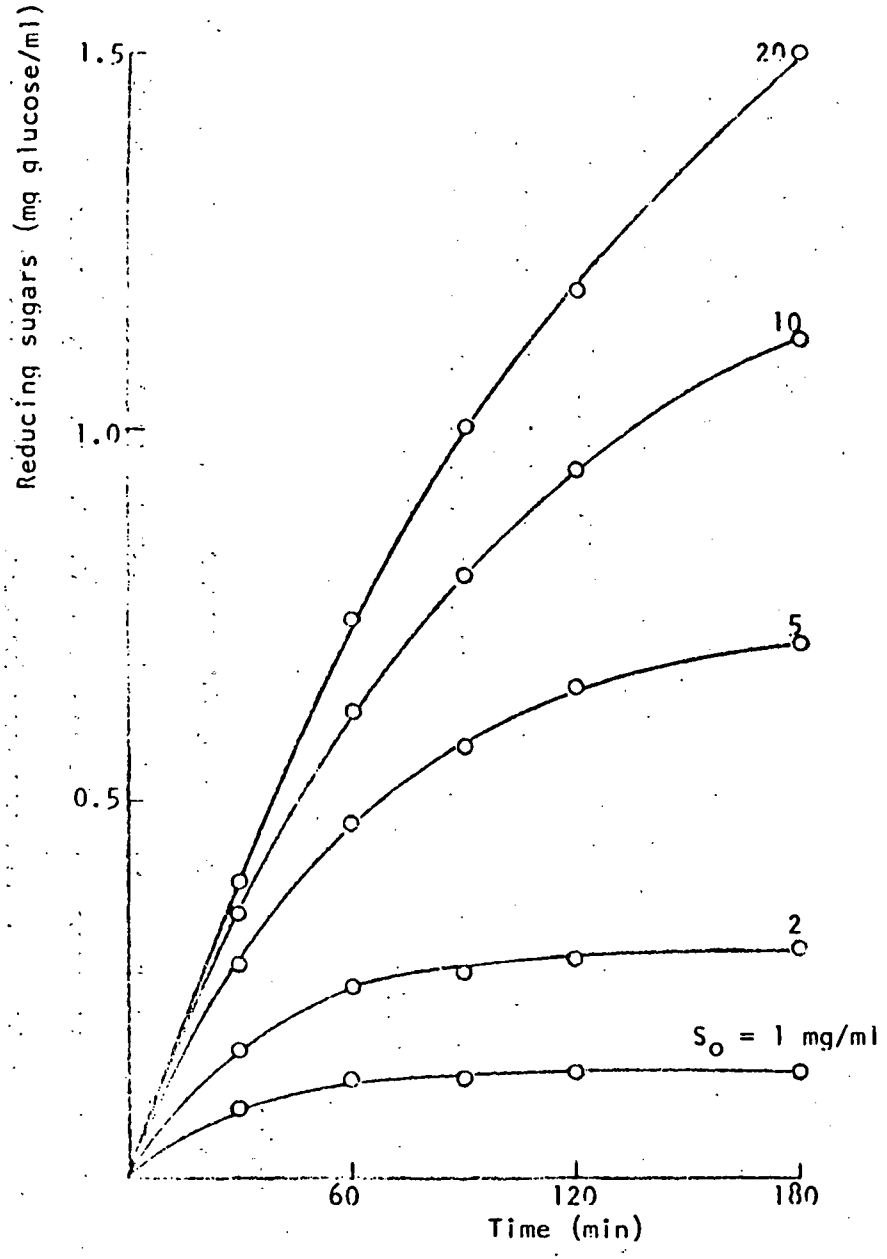


Fig. 1-E-5. Time course of CMCase reaction varying CMC concentration.

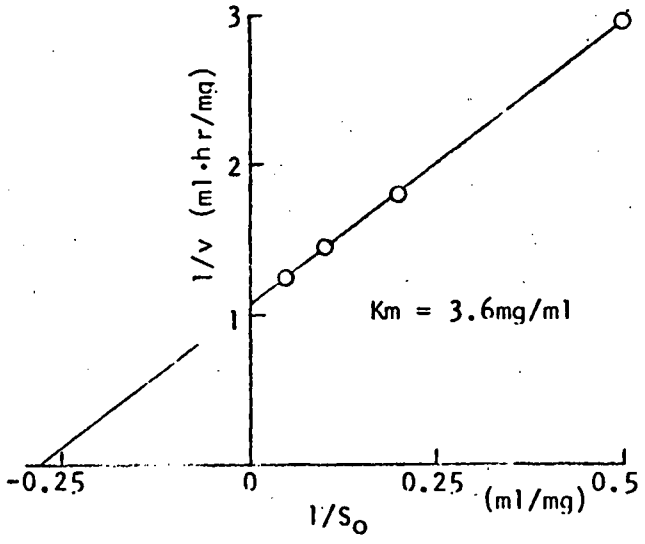


Fig. 1-E-6. Lineweaver-Burk plot of CMCase reaction.

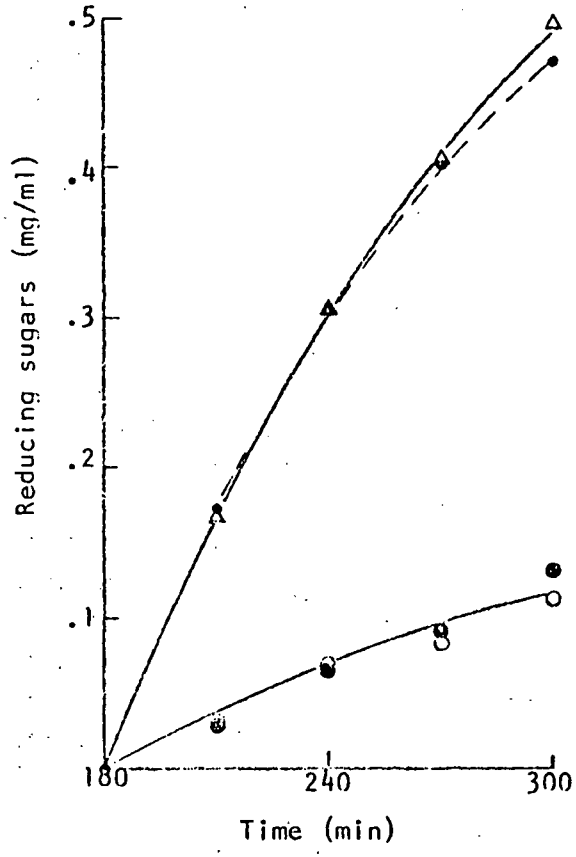


Fig. I-E-7. Effect of substrate addition at 3 hrs on CMC hydrolysis.

o: none, ●: buffer
Δ: CMC (10mg/ml)

Broken line shows the time course of original reaction mixture from 0 to 2 hr.

Since the reaction mixture was diluted by the addition of buffer or CMC solution, observed values of reducing sugars were corrected to those in original mixture.

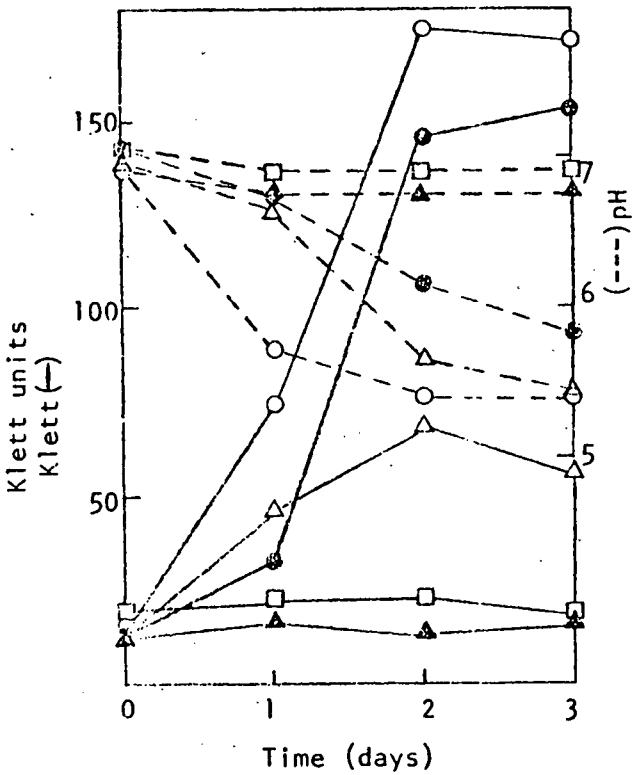


Fig. I-E-8. Growth of C. thermocellum on various carbon sources.

□: None, Δ: cellobiose, ▲: glucose
●: MN300, o: MN300 + cellobiose

1% carbon source was added to CM3 medium.

No growth took place in the CM3 basal medium (containing yeast extract) in the presence or absence of glucose, as would be expected from literature reports of C. thermocellum. Growth did occur in 1% cellobiose (70 Klett units) and in 1% MN 300 (145 Klett units) and TNP-CMCase activity was produced in both. Growth in MN 300 plus cellobiose reached 175 Klett units suggesting that cellobiose does not repress cellulase formation. In fact, in this experiment, TNP-CMCase activity was higher on cellobiose than on cellulose. Since this result does not agree with literature reports on another strain of C. thermocellum (which indicates no enzyme production during growth on cellobiose), we are repeating the experiment.

F. Genetic Manipulations

During this research period we continued our search for plasmids in C. thermocellum and initiated studies to attempt to establish a transformation system.

1. Plasmids from C. thermocellum

In the last progress report (March 1 - May 31, 1977) we indicated that plasmids may be present in C. thermocellum and that these plasmids were revealed by CsCl-ethidium bromide gradients when the deoxyribonucleases are inactivated by heat treatments. However, we expressed some reservations as to the true identity of the observed plasmid peaks. The following experiments were designed to reproduce these early observations and confirm the presence of plasmids.

A streptomycin resistant mutant of C. thermocellum was grown as previously described (Progress Report March 1 - May 31, 1977). The cell pellet was essentially lysed as described in the March 1 - May 31, 1977 Progress Report, except that the lysozyme treatment was omitted since it has been found unnecessary. One portion of the cell pellet was lysed in hot (80°C) TES buffer (see Progress Report March 1 - May 31, 1977) and cooled rapidly with the addition of cold 0.67M KCl, final KCl concentration was 0.45M. The second portion was lysed in hot TES buffer containing 0.45M KCl and cooled slowly. The third portion was lysed in hot TES buffer, heated, cooled rapidly, 0.67M KCl added and filtered through a Millipore membrane (HAWP 25mm; 0.45u). Again the final KCl concentration was 0.45M KCl. The purpose of the KCl is to prevent denaturation of

the DNA at 80°C. Hypothetically, heating at 80°C in low ionic strength buffer, such as TES, followed by filtration through a Millipore membrane should selectively remove chromosomal DNA. This is due to the reported tendency of DNA with single-stranded portions to stick to Millipore filters. This procedure can be used to enrich lysates for covalently-closed-circular (CCC) DNA.

All lysates were then subjected to CsCl-ethidium bromide density gradient analysis (Progress Report March 1 - May 31, 1977).

Figure I.F.1 shows the results obtained for lysates which were heated and cooled rapidly, no attempt was made to selectively remove chromosomal DNA since the lysates were not filtered. The chromosomal DNA peak is observed from fractions 33 to 40, CCC CNA (plasmids) from fractions 40 to 60 and the dense RNA peak from fraction 60 onwards. This figure clearly indicates the presence of plasmids and confirms our previous results. The ratio as percent of radioactivity in the satellite peak to that in the chromosomal peak is approximately 40 to 50%. This is an uncommonly high ratio but not unheard of. Clowes (Bacteriol. Rev. 36:361, 1972) reported on ratios as high as 40% for plasmid R6K in E. coli K-12. This is potentially a significant finding since it indicates a relaxed mode of replication for these plasmids. Obviously, these plasmids are ideal for gene amplification and consequently for enhanced activities of enzymes coded by them.

Figure I.F.2 shows that when the lysate is cooled slowly the satellite peaks disappear. This is indicative that heat treatment does not irreversibly inactivate deoxyribonuclease function but rather inhibits its action. If the lysate is allowed to cool slowly the nucleases are exposed to a range of temperatures for extended

Fig. I.F.1: Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. Cells were lysed, the lysate was heated and cooled rapidly.

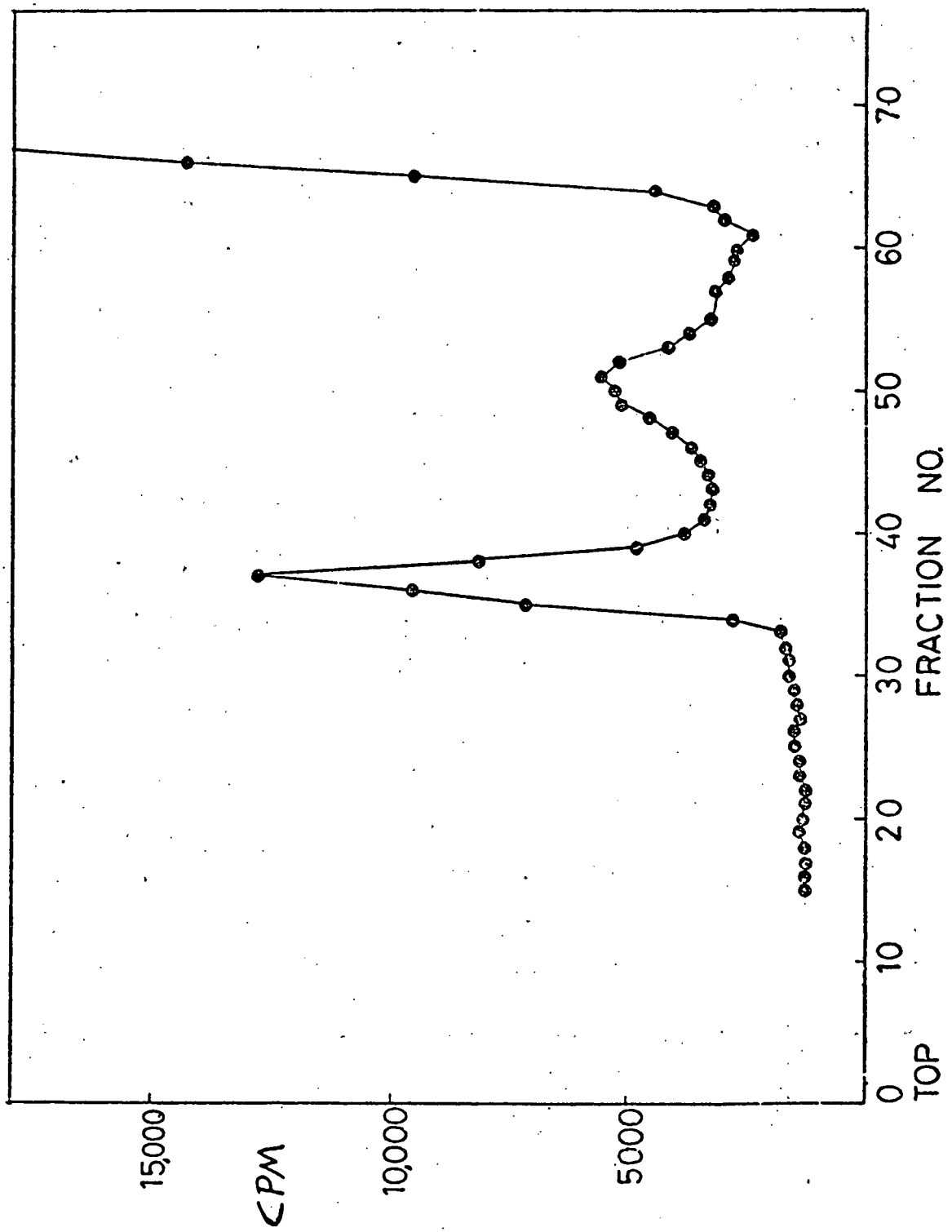
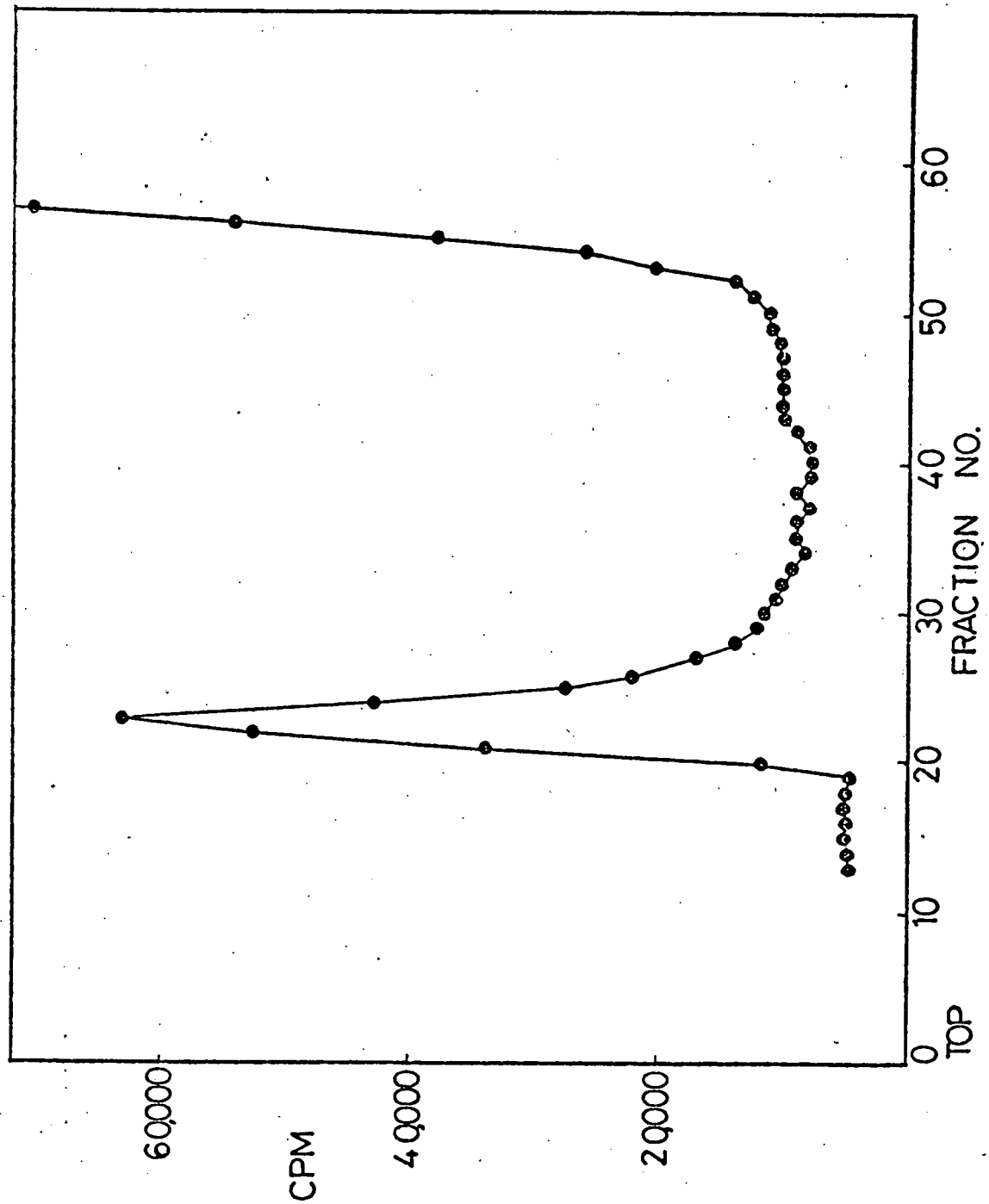


Fig. I.F.2: Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. Cells were lysed, and the lysate was heated and cooled slowly.



periods of time at which they are active and consequently introduce nicks into the CCC DNA. Under these conditions the plasmid DNA will behave exactly as chromosomal DNA.

Figure I.F.3 again demonstrates the presence of satellite DNA. However, it also indicates that the procedure for selective enrichment of plasmid DNA does not apply in our case. This conclusion stems from the fact that the ratio of CCC DNA to chromosomal DNA is approximately the same as seen in Figure I.F.1. In the latter no attempt was made to select for plasmid DNA.

In order to confirm the presence of plasmids a preparation run was made by collecting every other fraction and the CsCl-ethidium bromide gradient profile is shown in Figure I.F.4. The fractions indicated by the arrows in Figure I.F.4 were pooled and dialyzed against TES buffer overnight. Recovery after dialysis, based on radioactivity recovered, was 42%. The pooled and dialyzed fractions were examined by electron microscopy according to the procedure of Kleinschmidt (Methods in Enzymology 12B, pp. 361 - 377, Norris and Ribbons, eds., Academic Press, New York, 1968). The specimens were viewed with an RCA electron microscope at 50Kv. Some of the resulting preparations are shown in Figure I.F.5. On the upper left hand corner, a plasmid from a preparation which had been exposed to 12 Krads of Co^{60} irradiation is seen. This procedure is used to create a small number of single strand breaks in order to relax the plasmids and extend them to a circular conformation. The magnification of this photograph is 40,000X. It appears that this plasmid has a molecular weight of approximately 3.5×10^6 dal s. On the upper right hand and lower left hand corners photographs of a smaller plasmid are shown. The magnification of these pictures

Fig. I.F.3: Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. Cells were lysed, and the lysate was heated, cooled rapidly and chromosomal DNA selectively removed.

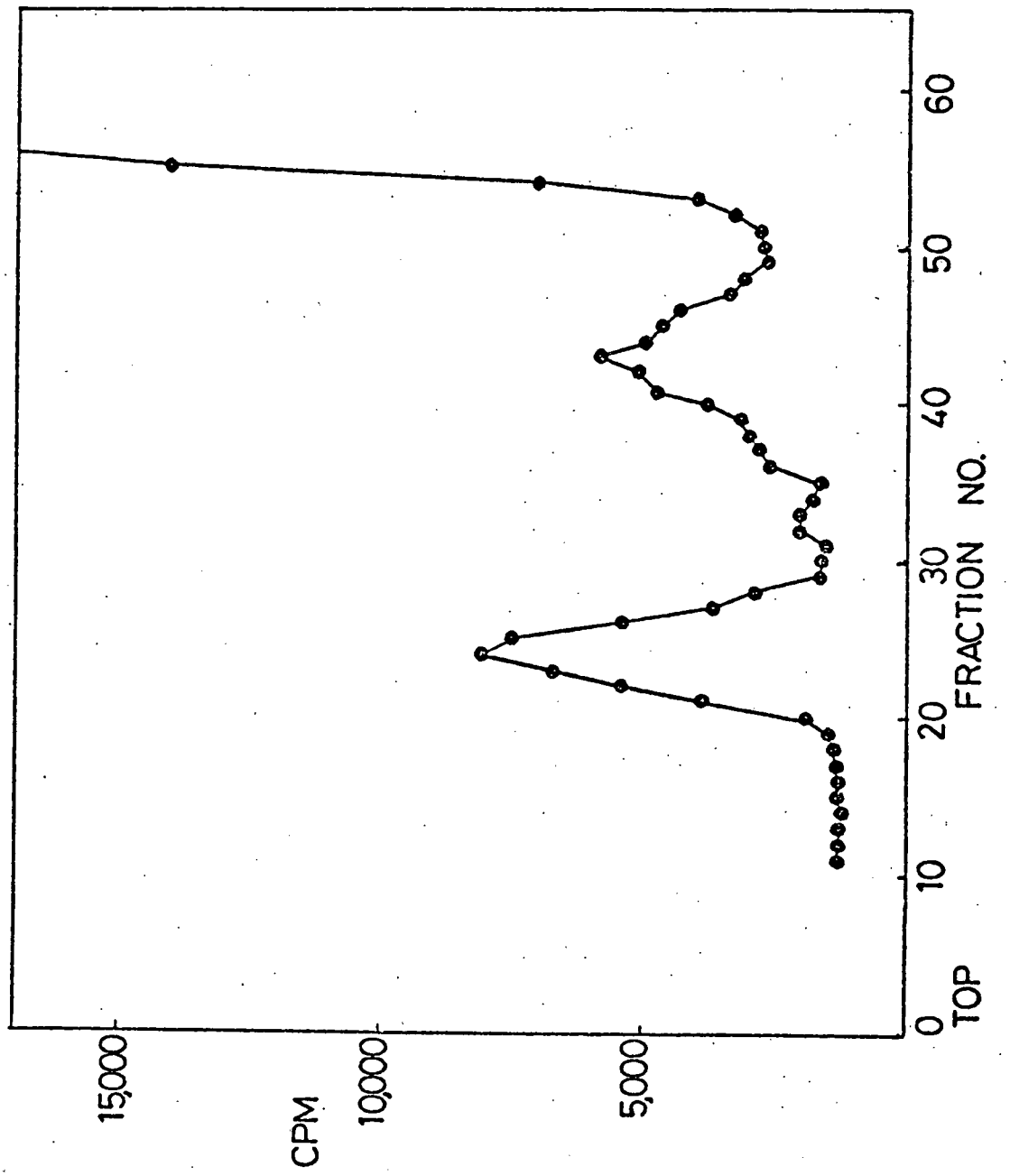


Fig. I.F.4: Cesium chloride-ethidium bromide gradient of ^{3}H-adenine-labelled lysate of C. thermocellum. The lysate was treated as described in Figure I.F.3.

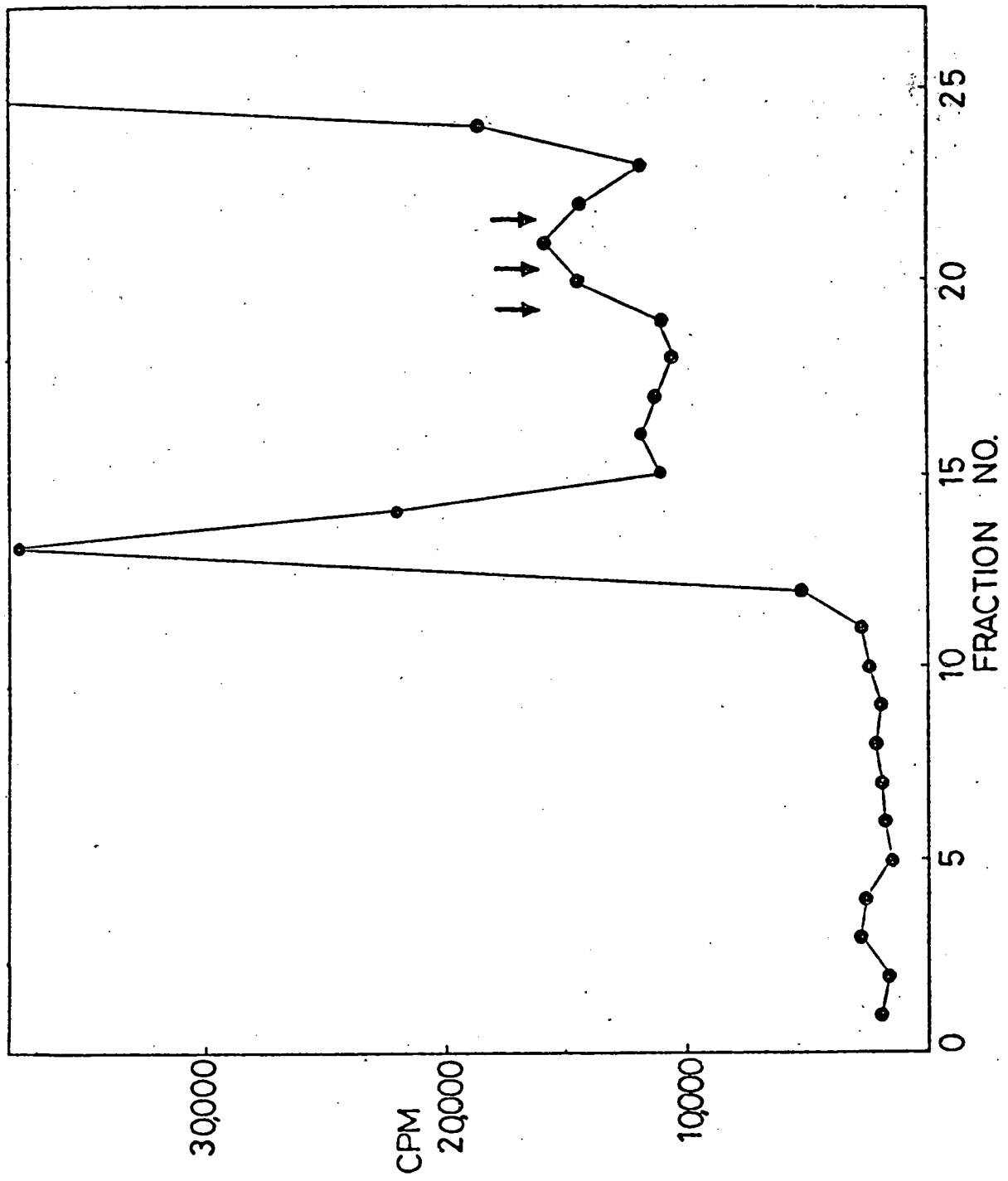
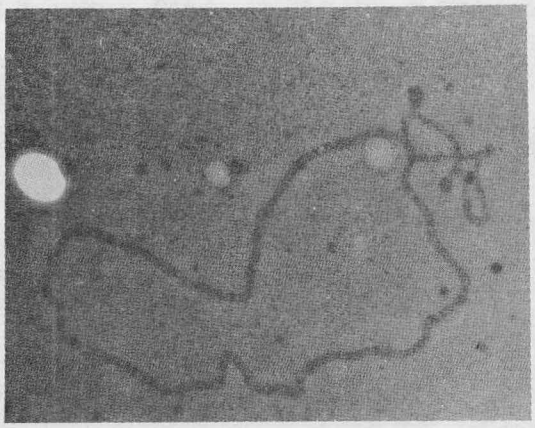
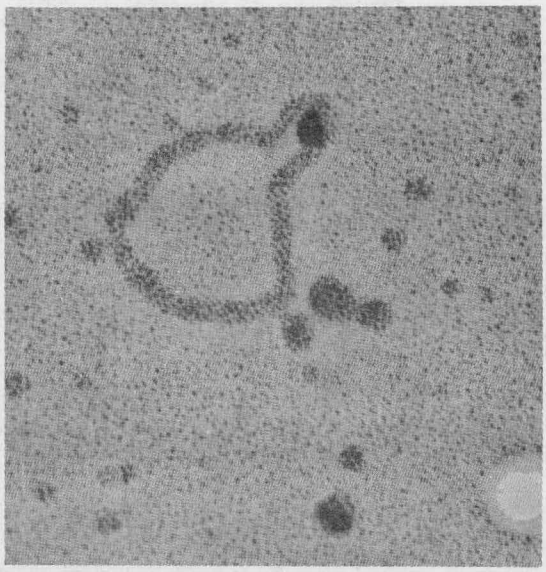
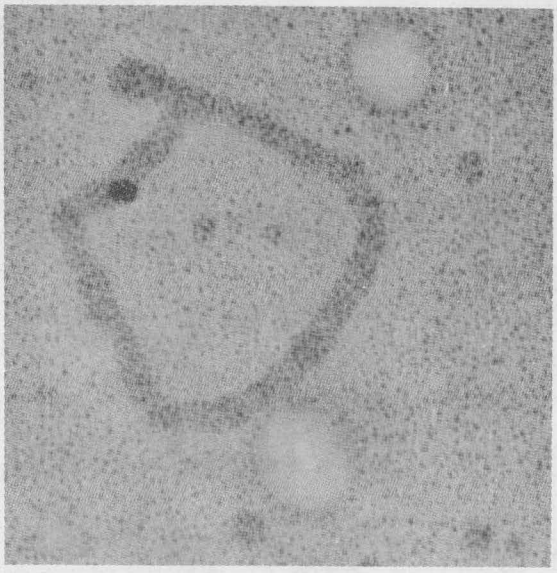
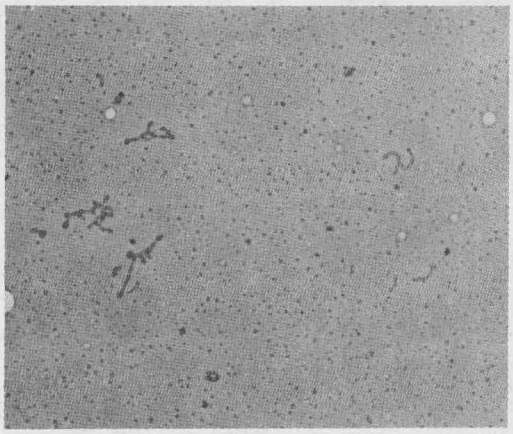


Figure I.F.5.









are 96,000X and 120,000X, respectively. This plasmid has a molecular weight of approximately 1 X 10^6 daltons. Finally on the lower right hand corner a picture of unrelaxed plasmids is seen. This photograph has a magnification of 17,000X.

Thus, we have confirmed the presence of plasmids in C. thermocellum. It appears that there are at least two distinct plasmids and that these plasmids are probably under relaxed replication control and consequently are found in multiple copies per cell. These characteristics make them ideal for genetic manipulations.

2. Transformation experiments

In order to start our attempts to establish a transformation system in C. thermocellum DNA from this microorganism was extracted and purified. Calbiochem "free of nuclease" Pronase was treated at 80°C for 10 min and then added to a cell lysate at a concentration of 1mg/ml and incubated at 37°C for 1 hr. The lysate is gently extracted in a rotary evaporator (60 rpm) with 1/2 volume of 3X TES-saturated phenol at atmospheric pressure and room temperature for 30 min. After extraction, the aqueous phase is centrifuged and the supernatant collected. Any gel remaining at the interface is extracted with a small amount of TES buffer and recentrifuged. The combined supernatants are twice extracted with 1 volume of ether in the same manner as above, but for only 5 min. The aqueous phase is then layered with 2 volumes of 95% ethanol and the precipitate is spooled onto a glass rod with gentle mixing of the two phases. The precipitate is resuspended in 0.3X TES buffer and RNAase (heat treated at 80°C for 10 min) is added to a concentration of 100ug/ml. After incubation at 37°C for 75 min, the solution is made 0.3M

sodium acetate. The DNA is selectively precipitated using 0.54 volumes of isopropanol. The precipitate is spooled and soaked overnight in 70% ethanol and suspended in sterile 0.3X TES buffer after removing the ethanol.

An exponential culture growing on CM4-cellobiose (CM4-Cb) obtained from a 10% inoculum of the regular stock in CM4-cellulose, is inoculated into CM4-Cb (also 10% inoculum) in the presence of:

a. 100 ug/ml of wild type DNA from C. thermocellum.

b. 100 ug/ml of DNA from a streptomycin resistant mutant of the same origin.

c. 100 ug/ml of w.t. DNA plus spermine (2×10^{-4} M).

d. 100 ug/ml of Sm^R DNA plus spermine (2×10^{-4} M).

The cultures are allowed to go into the early stationary phase (about 18-20 hrs) at 60°C and then subcultured separately (10% inoculum) in CM4-Cb broth. When the late exponential phase is reached, (about 12-14 hrs) the cultures are plated onto:

i) CM4-Cb (10^{-2} to 10^{-7} dilutions)

ii) CM6-Cb + Streptomycin, 0.5 grs/lt, (10°, 10^{-1}, 10^{-2} dilutions).

The results are shown in Table I.F.1. The recovery (numbers in parentheses) is expressed as % of viable counts on CM4-Cb w/out Sm over direct Petroff Hauser direct microscopic counts (± 10%, 95% confidence).

Table I.F.1: Recovery of C. thermocellum after attempt at transformat

Incubation with wild type DNA		Incubation with SM^R DNA	
w/out Sm	w/Sm	w/out Sm	w/Sm
7.5×10^7	<1	2.5×10^8	<1
(20)		(100%)	

Incubation with wild type DNA and spermine		Incubation with Sm^R DNA and spermine	
w/out Sm	w/Sm	w/out Sm	w/Sm
9.7×10^7	<1	8.1×10^7	<1
(16)		(9)	

These results clearly indicate that these experimental conditions are not adequate to obtain functional transformation.

It was decided to determine if under similar conditions C. thermocellum is able to physically take up DNA.

Cells were grown in CM4-Cb until early exponential phase in 5 ml of broth and in the presence of spermine (Petroff-Hauser counts = 2×10^8). At this time 1 ug/ml of ^{14}C DNA (NEN; 2.015 uCi/ml, 0.062 uCi/µg) was added and the temperature is maintained at 60°C during all the manipulations of the experiment.

At 6, 15, 30, 60 and 112 min., 0.5 ml aliquots (duplicates) are taken out. To these samples 0.5 ml of DNAase solution (200 ug/ml in 0.1 M Tris + 0.015 M $MgCl_2$, pH 8.5) are added and the final mixture allowed to incubate for 10 min at 37°C. Procedure was followed according to Bodmer, W. F. and A. T. Ganesan (1964), Genetics, 50, 717-738.

After DNAase digestion, 1 ml of cold 10% TCA is added; and the tubes placed in an ice bath for 30 min. The mixture is then filtered

through Millipore filters HAWP 02500 (0.45u). The tubes were then washed with 1 ml of cold 10% TCA and this volume is also passed through the filter. The incorporated DNA, as a percent of the total radioactivity per aliquot is given in Table I.F.2.

Table I.F.2.

Time (min)	% Incorporated
6	16.2
10	10.8
30	20.9
60	16.1
112	13.0

These percentages are not considered significantly different compared to controls in which the cells were excluded from the reactions. Therefore it is indicative that, under these experimental conditions, C. thermocellum is not able to significantly take up exogenous DNA.

Since it is important that exogenous DNA is not attacked by extracellular nucleases, we performed an experiment to determine the extracellular nuclease activity of C. thermocellum.

A culture grown in CM4-Cb was inoculated (10%) into After 3 hrs at 60°C growth had occurred and 0.1ml of ^{14}C DNA per 5 ml of broth was added.

At times 0, 1, 2, 4, 5, 7, 10 and 19 hr, aliquots were removed and put in cold 10% TCA (equal volume). After 30 min at 0°C, the mixtures are filtered through glass fiber filters, the tube washed with cold 10% TCA and the radioactivity on the filters and in the filtrates measured.

Figure I.F.6 gives the results. The calculated percentages are given as % of the total radioactivity present in each aliquot.

The results indicate that in the midexponential phase of growth there is nuclease activity. However, DNA degradation ceases with the onset of stationary phase. Thus, it would appear that during exponential phase the observed nuclease activity may be a source of difficulties in establishing a transformation system. It is not clear to us why nuclease activity ceases at the stationary phase. There is little to be gained by speculating regarding this observation.

Another attempt at finding functional transformation has been made. E. coli genetic transformation has been found with the highest frequency after a treatment with Ca Cl_2 described by Cosloy and Oishi (PNAS 70 (1), 84-97, 1973). We have investigated the effect of such a procedure with some minor modifications. Our protocol can be described as follows:

a. grow 10ml cells in CM4-cellobiose until $A6s^{660}$ is about 0.6 - 0.7. Centrifuge and resuspend the pellet in 13 ml of fresh broth; allow 60 minutes for enough gas production to reduce indicator and maintain anaerobic conditions.

b. Centrifuge and resuspend the cells in 10 ml of cold 0.1mM $CaCl_2$ in 0.1% cysteins. Allow 20 minutes at 0°C with strong N_2 flushing.

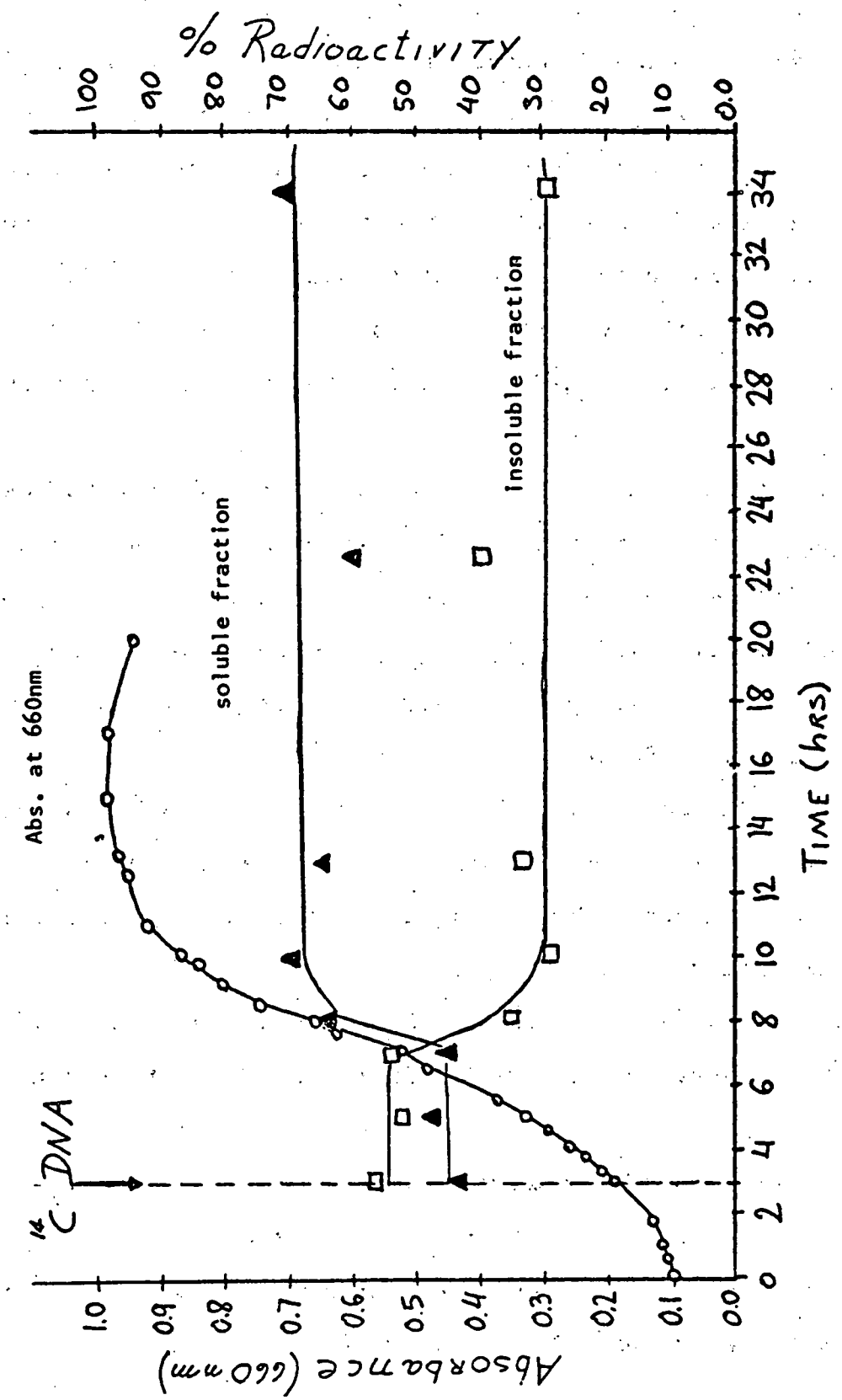
c. Centrifuge; add sequentially:

i) 1 ml of 10^{-4} M spermine in 0.1% cysteine.

ii) 2 ml of a solution containing 50mM $CaCl_2$ in 0.1% cysteine.

iii) 1 ml of Sm^R DNA solution (0.6mg/ml).

Fig. I.F.6: Extracellular nuclease activity of C. thermocellum



d. Incubate 45 minutes at 0°C under strong N_2 flushing, followed by 15 minutes at 60°C. After this time, the viability of the cells was 3.5 to 7.0% compared to direct microscopic counts. This indicates that the above procedure does not inactivate enough cells to make it unworkable. Unpon subculturing the solutions for 2 generations to allow for expression of potential transformants the cultures were enumerated on CM 4-Cb plates with and without streptomycin. No transformants were found. However, the expression time may not have been long enough to significantly dilute the intracellular wild type ribosomal population.

3. Future Studies

During the next research period we will further characterize the plasmids isolated from C. thermocellum. We would like to know more accurately what their molecular weights and the plasmid copy numbers are. In addition, we will attempt to "cure" C. thermocellum of these plasmids and determine whether there is any correlation with the microorganism's ability to grow on cellulose as the sole carbon source.

Furthermore, we will try to increase our stock of mutants of C. thermocellum (auxotrophs and cellulase deficient). These we feel will be necessary to more adequately attack the problem of gene transfer. With regards to the latter we will continue to investigate the possibility of establishing a functional transformation system in C. thermocellum. However, it is felt that it is time that we started to consider alternate routes. To this end we will initiate studies dealing with cell fusion. Recently,

there have appeared in the literature reports of protoplast fusion in bacteria. This procedure would very adequately, with some modifications, provide us with an alternative to transformation.

If time permits, we will resume our search for phage particles in C. thermocellum. We have preliminary, but unconfirmed, evidence by electron microscopy that "phage-like" particles are present in supernatants of C. thermocellum cultures.

II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. Production of acrylic cid by Fermentation

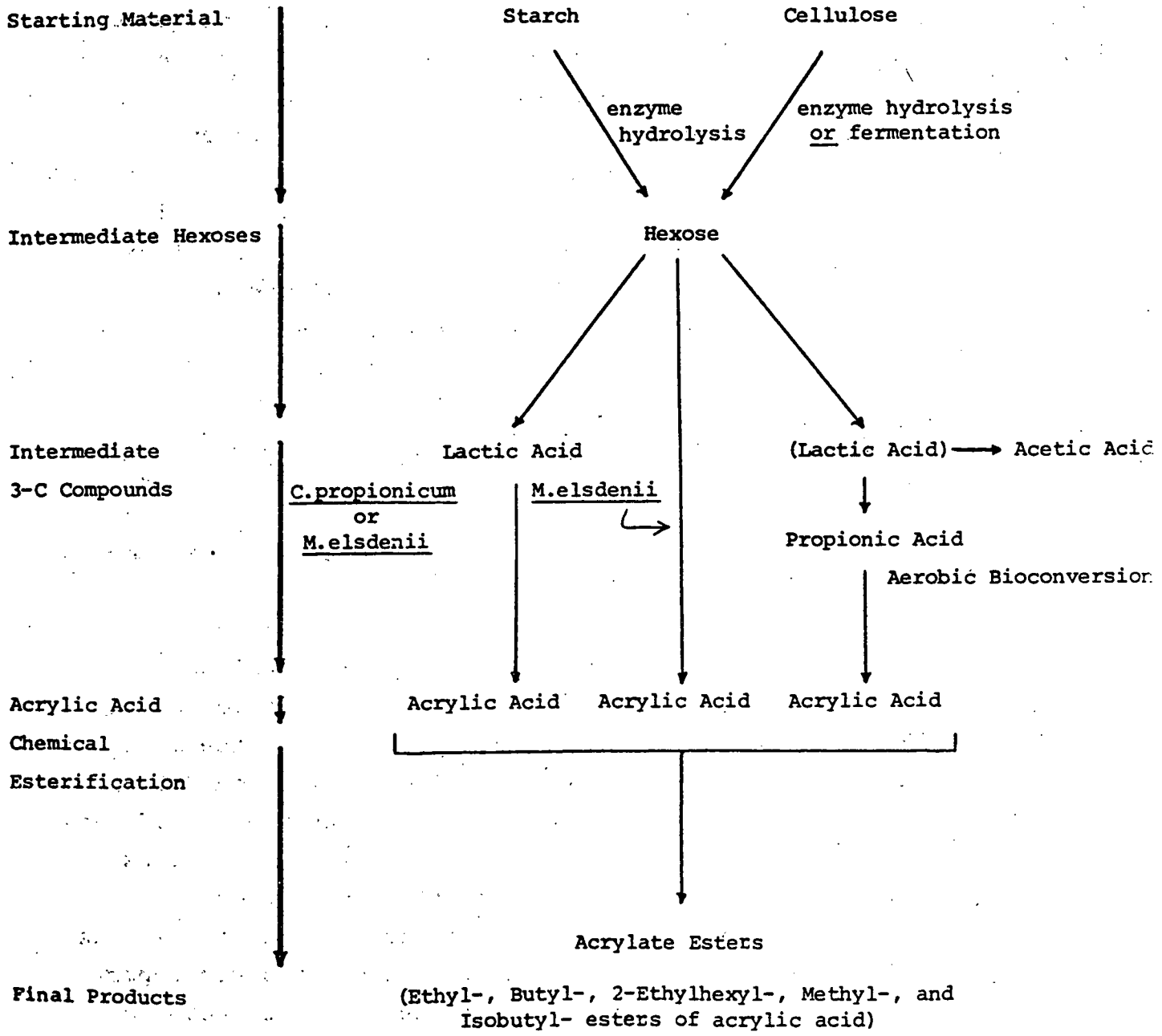
1. Introduction

Acrylic acid and its various esters (methyl, ethyl, isopropyl, butyl, etc.) are major chemical commodities with an annual production of 800 million pounds and a projected 1980 production of 1 billion pounds per year; as a consequence, its production requires a substantial amount of energy and material. In this phase of our program, we are working on the development of a fermentation process for the production of acrylic acid from renewable resources. The alternative approaches which have been identified are summarized in Figure II.A.1. Acrylic acid exists as a coenzyme A thioester in both the pathway for anaerobic reduction of lactic acid and the pathway for aerobic oxidation of propionic acid. As a consequence, we can approcach the problem by anaerobically fermenting hexoses to lactic acid and then carrying out an anaerobic dehydration to acrylic acid. An alternative is fermenting lactic acid to propionic and acetic acids (mole ratio of 2:1) followed by the aerobic oxidation of propionate to acrylate. In this latter approach, acetic acid is a desirable by-product since its demand is approximately 2 billion pounds per year.

A major advantage of anaerobic fermentation processes is the high conservation of mass and energy in the final products. The theoretical yield of glucose to 2 moles of acrylic acid is 80% on a mass basis and 96% on an energy basis. If the anaerobic route to propionate and acetate followed by aerobic oxidation of propionate to acrylate is used, then the conservation of mass in one mole each of acrylate and acetate falls to 73%. Thus, while the anerobic route is most favorable, the use of an aerobic step at the end is still very reasonable and results is only a slight decrease in mass. The following progress report outlines our results in pursuing the paths outlined above to the production of acrylic acid by fermentation.

Fig. II.A.1

FERMENTATION ROUTES TO ACRYLIC ACID FROM BIOMASS



2. Fermentation by Megasphaera elsdenii

a. Introduction

Megasphaera (Peptostreptococcus) elsdenii was choosen for investigation because it will ferment glucose and and lactic acid through acrylic acid to give propionic acid. As a consequence it offers the possibility of producing acrylic acid in a one step fermentation. However, its disadvantage is that under normal fermentation conditions it not only produces propionate and acetate but also condenses 2- and 3-carbon units to yield butyric, valeric, and caproic acids. As will be shown, the mix of products is observed in both batch and continuous culture. Studies in continuous culture were initiated to try to minimize the production of C_4-C_6 acids. while maximizing propionic acid production.

b. Materials and Methods

The conditions for growth and the growth medium used for M. elsdenii are given in the progress report from December 1 through February 28. Continuous culture studies were done in a one-liter Bioflow fermentor (New Brunswich Scientific Co., New Brunswick, NJ) with 375 ml of liquid. The production of fatty acids were monitored by gas chromatography as described in the previous report. Other methods for measuring cell growth and lactic acid concentration are described in the previous reports.

c. Results and Discussion

M. elsdenni was grown in continuous culture to examine its patterns of product formation. Because acrylic acid lies in the reductive pathway of lactic acid to propionic acid and acrylate is the election acceptor coupled with acetate fermation from lactate, we sought to maximize the production of propinate and acetate. Results from two dilution rates are presented in Table II.A.1. The feed concentration of lactic acid was 10.3 g/liter. While it was intended that the chemostat

TABLE II.A.1

Results from continuous culture experiments with *M. elsdenii*

Dilution Rate (hr^{-1})	Product Concentration (g/l)				
	Acetate	Propionate	Butyrate	Valerate	Caproate
0.05	1.6	0.5	2	0.7	0
0.10	0.82	0.4	1.4	0.8	0.15

be carbon-limited, it is not clear that it was since residual lactate was about 3 g/l at a dilution rate of 0.1-hr^{-1}. Because of the use of yeast extract in the medium, definition of the limiting nutrient is difficult.

A time plot of the results in continuous culture over a 36 hr interval, representing almost 4 turn over volumes, is presented in Figure II.A.2. As seen in this plot, it is difficult to obtain a truely stable steady state in terms of constant production formation. This, we believe, is a result of their being a branched catabolic pathway such as shown schematically in Figure II.A.3. In such a pathway, there are multiple points for control of metabolic flow. Each control could be dependent on not only environmental factors such as pH and temperature, but also the concentrations of the various products present in the broth. In such a case, one is not surprised by the appearance of oscillations and instabilities in the pattern of products.

It is interesting to note that the C_3-C_6 acids are the major products compared to acetate and propionate at both dilution rates, 0.05 and 0.1-h^{-1}. This is in opposition to the results typically observed in batch culture where propionate is the major product (see Figure II.A.1 in progress report for March 1 - May 31, 1977). Even if valerate (a condensation product of a C_2 and a C_3 acid) is added to the propionate, the proportion of C_3 acid formation is low. This suggests that lactate is preferentially going to acetate and the available elections are going to the formation of reduced aliphatic acids. e.g. butyrate, valerate, caproate. Possible explanations could be that the cell is trying to make less acid in response to decreasing pH or it may be able to obtain more ATP via this route. There is the possibility of obtaining one ATP per acetate formed and if the cell can use the electrons for lactate oxidation to form aliphatic acids via acetate and or propionate condensations rather than using two moles of lactate to propionate per mole of acetate, then it would probably try to take advantage of the improved energetics. This is most likely to occur when the cells are limited by the carbon source and have become energy limited.

Fig. II.A.2

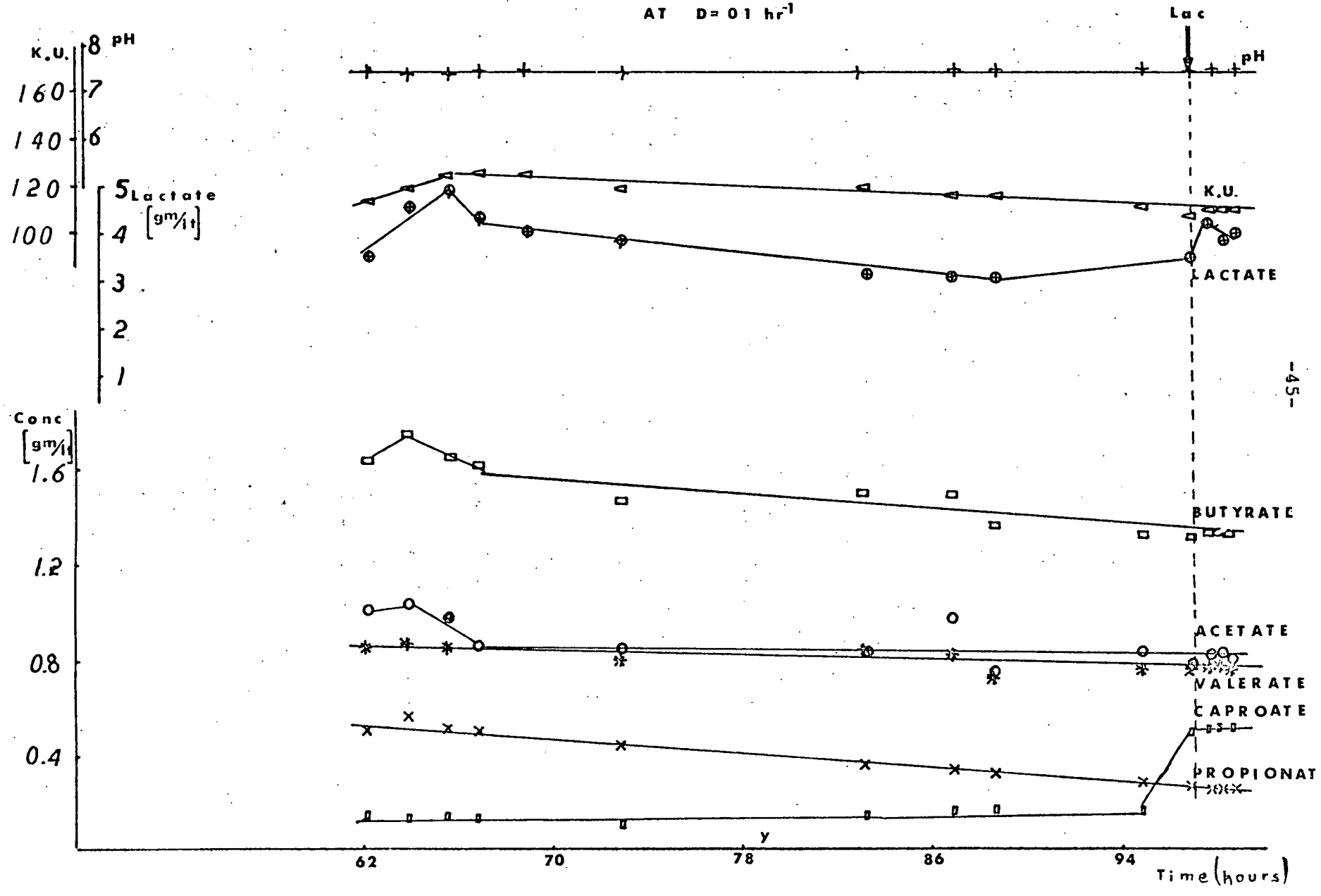
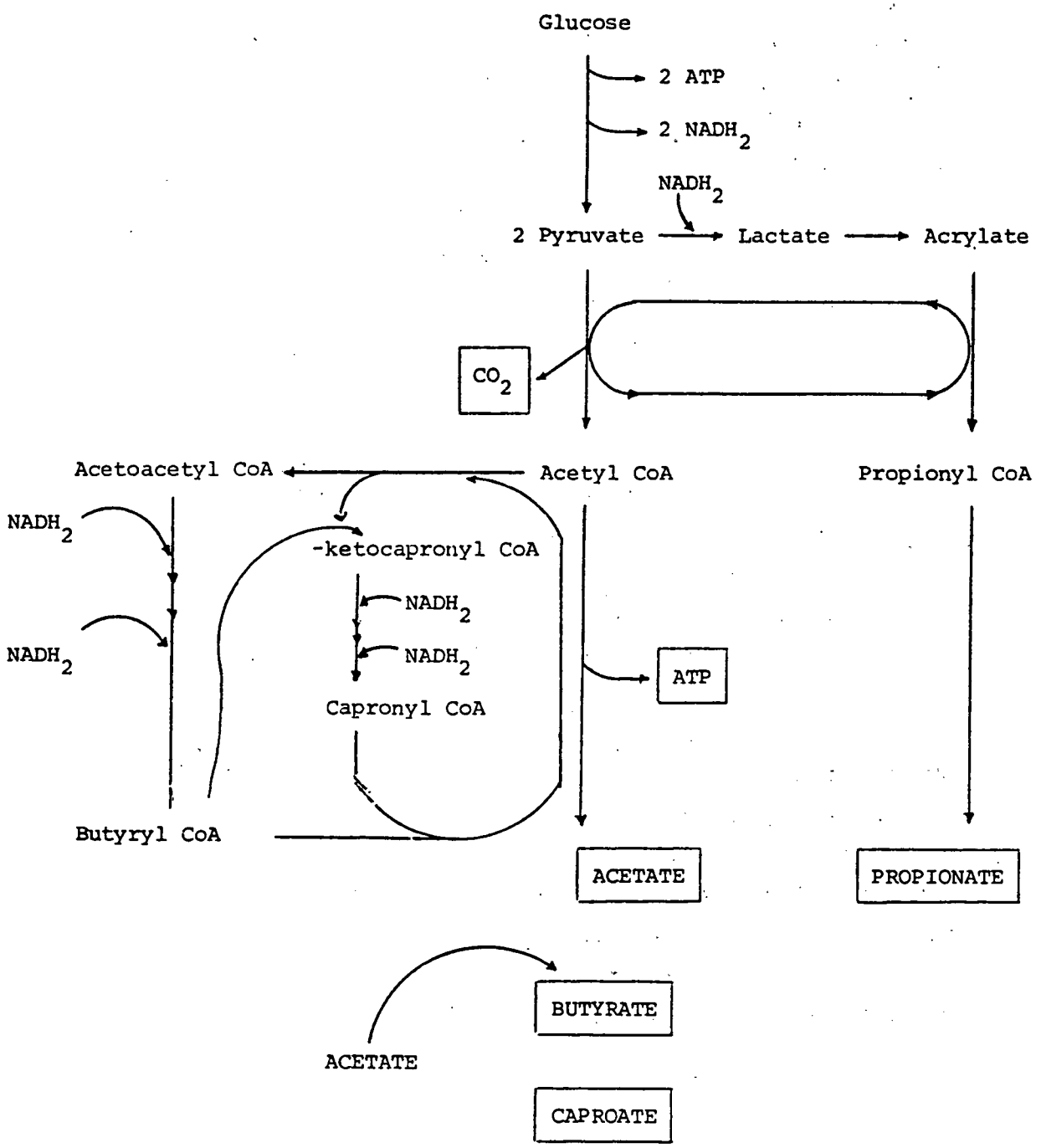

CONTINUOS CULTURE OF MEGASPHAERA ELSDENII
AT D= 0 1 hr-1
Lac
pH
K.U.
LACTATE
BUTYRATE
ACETATE
VALERATE
CAPROATE
PROPIONAT
K.U.
160
140
120
100
8
7
6
5
4
3
2
1
Lactate [gm/lt]
Conc [gm/lt]
1.6
1.2
0.8
0.4
62
70
78
86
94
Time (hours)

FIGURE II.A.3

BRANCHED CATABOLIC PATHWAY FOR GLUCOSE AND LACTATE FERMENTATION BY M.elsdenii



These results suggest that with M. elsdenii, it may be necessary to use non-carbon limiting conditions (e.g. N-or PO_4^- orS-limited growth), and high pH (around 7-7.5) to maximize the flow of carbon through the lactic-acrylate-propionate pathway.

One problem that has occurred is repeated contamination by a motil, rod shaped organism. Since very few anaerobes will ferment lactate, it is possible that the contaminate is growing on the amino acids in the medium. For this reason, as well as the need to reduce the cost of the medium, we need to develop a simpler medium with few if any amino acids.

d. Future Work

- Grow M. elsdenii under P-, N-, and S-limited conditions to enhance acid production at controlled pH values of 7.0 or above.
- Develop a simpler medium to reduce the fermentation cost and minimize the possibilities of contamination.
- Examine the use of resting cells to effect the dehydration of lactic acid to acrylic acid.

3. Fermentation by Clostridium propionicum

a. Introduction

C. propionicum will ferment a restriced group of compounds to propionic acid and acetate as discussed in the progress report for Dec. 1, 1976 to Feb. 28, 1977. These include alanine, pyruvate and lactate. Interestingly, C. propionicum will not grow on lactate but will ferment it to propionate and acetate as the sole products in the ratio 2:1 respectively. These fermentations occur through acrylate as an intermediate. Because it is a "cleaner" system, not giving C_4-C_6 acids, we have choosen to look at this organism in more detail. During

the past period, we have sought to develop cell-free extract systems that will convert lactate to propionate and acetate; these systems can then be used to screen for chemical blocking agents that will prevent acrylate reduction to propionate. Furthermore, an understanding of the kinetics from these cell-free systems can then be used to better direct the fermentation through enviromental control.

b. Materials and Methods

Cell-free extracts prepared by sonic oscillation did not show any fermentation activity for acid production. For this reason other methods for cell lysis were employed. The combination of lysozyme and EDTA, and glass beads showed good activity for acid production. These techniques are relatively mild and one can maintain anaerobiosis during cell-free extract preparation. Cells were grown in alanine (0.8%) medium (see previous program report for details) for 2 days at 37°C in a water bath (125 rpm) and then harvested by centrifugation. They were washed twice with a 0.03% Na_2S and then frozen as a pellet under N_2 at -20°C. Frozen cells are resuspended in buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 75 mM EDTA) to give 1 g wet cells/5 ml buffer. Lysozyme (2 mg/ml) is added to the cell suspension and stirred at 0°C for 30 min and the temperature is then raised to 30°C for 35 min. Glass beads (~100 μ) are added to the mixture and vortexed at moderate speed. We checked cell breakage under the phase contract microscope and almost 100% of the cells, but not the spores are broken with the first vortex. These suspensions are centrifuged at 18,000 g for 15 min and the supernatant fluid (greenish yellow) is transferred to a tube under N_2. Extracts are stored at -20°C.

Neither method, lysozyme plus EDTA or glass beads plus vortex give quick cell lysis but the combination of both methods gave us good results.

c. Results and Discussion

To test the retention of acid forming ability of cell extracts, we compared crude and cell-free extracts for their ability to convert lactate to acetate and propionate. The reaction mixture (1 ml) contained:

potassium phosphate buffer	0.1 M, pH 7.5
lactate	20 mM
acetyl-coA	0.2mM
acetyl-P	10 mM
methylene blue (except control)	0.02%
MgCL2	5.5mN
DTT (dithiothreitol)	10 mM
extracts	9.9 mg protein in crude/ml 5.3 mg protein in cell-free/ml

The extracts were incubated at 37°C with stiring and 100 samples were drawn by syringe at 5, 10, 15, 20, 25, 30 and 40 min into a tube containing 20 uL of 50% H_2SO_4 in order to stop the reaction. Samples were kept frozen until extracted for fatty acids

Both crude broken cells preparation and cell-free extracts showed good activities on acetate and proprionate production. These extracts could be frozen and stored quite well with a retention of 95% of the initial activity.

Using this system, we proceeded to examine a variety of electron acceptors as substitutes for acrylate. In these experiments DTT was not added since it acts as a reducing agent. The following were added to the cell-free extracts.

potassium ferricyanide
2,6 - dichloro-indo-phenol (Na salt)
2,3,5 - triphenyl-tetrazolium chloride (TTC)
phenazine methosulphate
methylene blue
NAD

Acrylate was not detected in any of reaction mixtures. The Electron acceptors seem to stimulate the initial rate of acetate production compared with controls which did not contain any electron acceptors. This is seen in a typical kinetic profile shown in Figure II.A.4 in which 2, 3, 5 -triphenyl-tetrazolium chloride was added to the cell-free extract. Propionic acid production was relatively unaffected. The electron acceptors which are dyes did show a color change as the reaction proceeded indicating that they were active. The problem may lie with the need to uncouple acrylate from its coenzyme A thioester. This will be tried in subsequent work. In summary, cell-free extracts did show propionic acid production, which is derived from lactate via acrylyl-CoA. Electron acceptors, however, did not shut off the reaction for acrylyl-Coa ⟶ propionyl-CoA.

d. Future Work

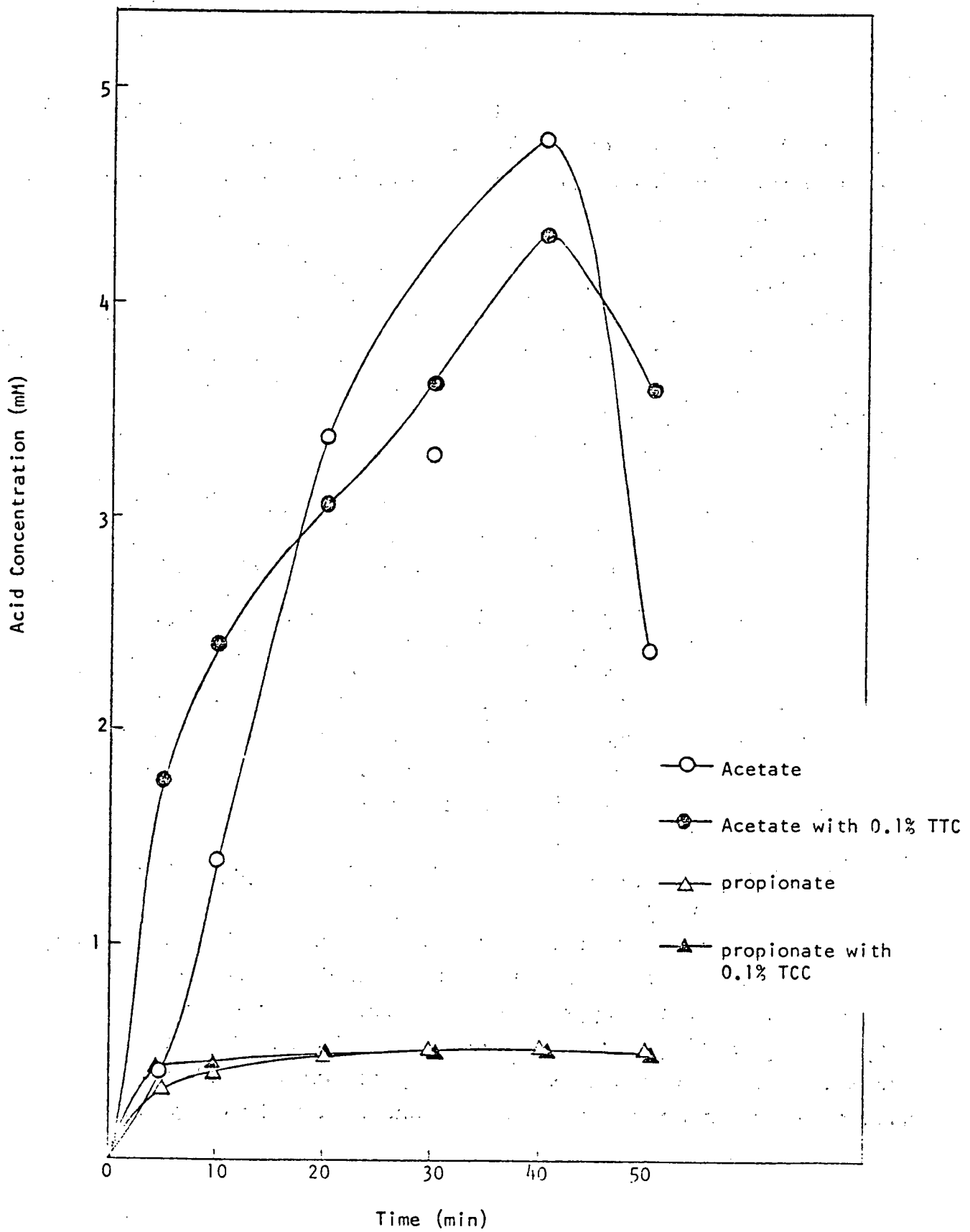
- Inhibitors of acetate production will be added in addition to electron acceptors
- Labeled (C^{14}) lactate and cold acrylate will be added to the extract and permeabilized cells to detect the production and release of labeled acrylate. This system will provide a basis for initial optimization of acrylate production.
- Immobilized cells will be tried to effect the dehydration of lactate.

4. The Production of Lactic Acid From Soluble Sugars

a. Introduction

As discussed above, there are a number of alternative approaches to the production of acrylic acid by fermentation. One of these approaches utilizes the organism Clostridium propionicum, which will anaerobically ferment lactate to propionic acid via the acrylic pathway. This organism has a number of advantages; one major advantage is

Fig. II.A.4: Production of Acetate and Propionate by cell-free extracts of C. propionicum



that its pathway of fermentation is much simpler than other organisms examined. However, in order to effectively use this organism, one needs to convert glucose or other soluble sugars to lactic acid. This can be done in essentially quantitative conversion yields to obtain 1 gram of lactic acid per gram of starting glucose. The homo-fermentative lactobacilli will generate two moles of lactic acid per mole of glucose and the achievement of 95% of theoretical conversion yield is common.

With this rationale in mind, we proceeded to explore the production of lactic acid from glucose. Since our biological pretreatment of cellulose to give us soluble sugars occurs at the thermophilic temperatures, we are particularly interested in the Lactobacillus species which grow at elevated temperatures, e.g. 45°C or above.

The objectives of this phase of our program are: (1) to study important variables in the lactic acid fermentation in order to achieve maximal conversion yields to lactic acid and a rapid rate; (2) evaluation of resting cells of Lactobacillus species for conversion of reducing sugars to lactic acid; (3) ultimately couple the cellulase hydrolysis process with lactic acid production for eventual conversion to acrylic acid; and (4) evaluate cell recycle in order to obtain high rate of lactic acid production as well as high concentrations of lactic acid.

b. Materials and Methods

(1) Microorganism: Several strains of lactobacilli were examined for their ability to rapidly produce lactic acid. These are Lactobacillus delbrueckii ATTC 9649, ATCC 1744, NRRL-B-445, and L. bulgaricus ATCC-11834. Particular emphasis is given to Lactobacillus delbrueckii NRRL-B-445.

(2) Growth Medium: The medium used for the growth of all of the lactobacilli is shown in Table II.A.2.

TABLE II.A.2

INGREDIENT	CONCENTRATION (GM/ℓ)
Trypticase	10.1
Yeast Extract	5.0
Tryptose	3.0
Glucose	60.0
Cysteine	0.2
K_2HPO_4	3.0
KH_2PO_4	3.0
$(NH4)_3$ Citrate	2.0
Tween 80	1.0 ml
Sodium Acetate	1.0 gm/ℓ
Salt Solution	5.0 ml
Water	1.0 ℓ
$MgSO_4$ $7H_2O$	11.5 gm
$FeSO_4$ $7H_2O$	0.68 gm
$MnSO_4$ $2H_2O$	2.4 gm
H_2O	100.0 ml

(3) Inoculum Preparation: The cultures were stored on agar slants. Inoculum was prepared by the addition of 0.1 ml of cell suspension into a Hungate tube under anaerobic conditions. These tubes were incubated at 45°C for 15 hours. This provided an inoculum for flasks or fermentors.

(4) Fermentation: A bench scale fermentor (5 liters) was used in this work. pH and temperature were controlled.

(5) Resting Cell Experiments: For study on resting cells, the cells were separated from the fermentation broth by centrifugation and suspended in glucose-buffer solutions.

(6) Assays: Glucose was measured by the Glucostat procedure (Worthington Biochemicals Corporation). Lactic acid was determined by either lactate dehydrogenase or gas chromatography. Cell density was measured by turbidity or dry cell weight.

c. Results and Discussion

Evaluation of Lactobacillus Species

Shown in Table II.A. 3 is a summary of avialable kinetic data for several Lactobacilli.

TABLE II.A.3

Microorganism	Optimum Temperature (°C)	Carbon Source	Yield gm Lactic (gm subst.)	Sp. Growth Rate (1/hr)	Maximum Productivity (log Phas (gm lactic/ℓ-hr)
L. delbrueckii ATCC-9648	48	Glucose	0.95	0.4	6.5
L. delbrueckii ATCC-1744	48	Glucose or Cellobiose	-	-	-
L. delbrueckii NRRL-B-445	48	Glucose	0.95	0.48	7.5
L. bulgaricus ATCC-11834	45	Glucose	0.90	0.30	6.25

It is interesting to note the relatively high optimum temperatures for growth, as well as high volumetric productivities for lactic acid that are observed for these organisms. These strains were initially evaluated for growth and carbon conversion using medium described above at 45°C for 28 hours. The results are shown in Fig. II.A.5. From these results, we obtained the best carbon utilization from L. delbrueckii NRRL-B445. As a consequence, subsequent work is done primarily with this organism.

The kinetic profile for this organism, showing growth, pH changes, glucose consumption and lactic acid production, are shown in Fig. II.A.6 and II.A.7. It is apparent that, when the pH falls to a value near 4, growth and lactic acid production cease. In this particular experiment, roughly 30 grams per liter of lactic acid were produced. As shown by the data in Table II.A.4, the conversion yield to lactic acid was approximately 93%. Also in

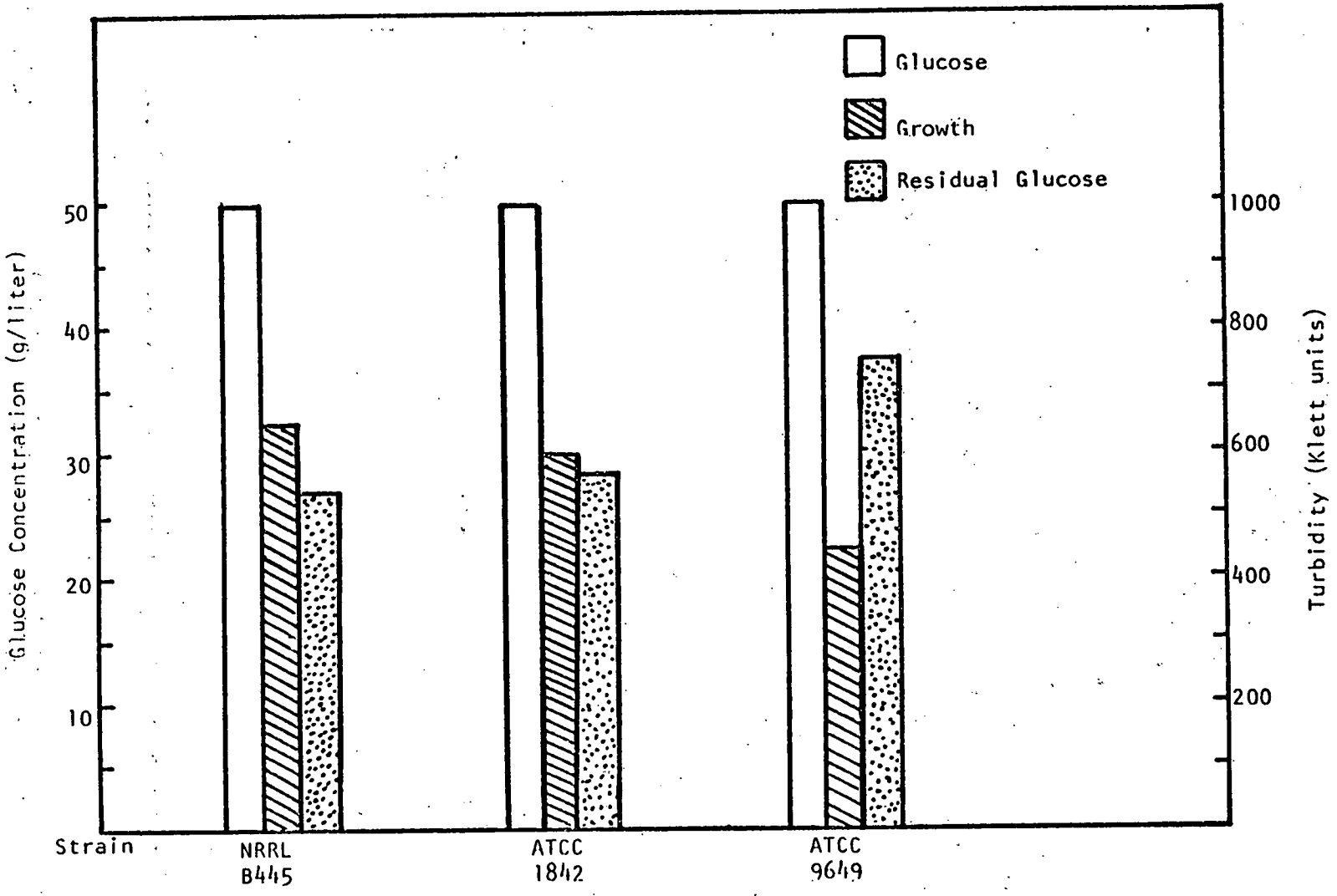


Fig. II.A.5: Evaluation of growth of lactobacilli on glucose

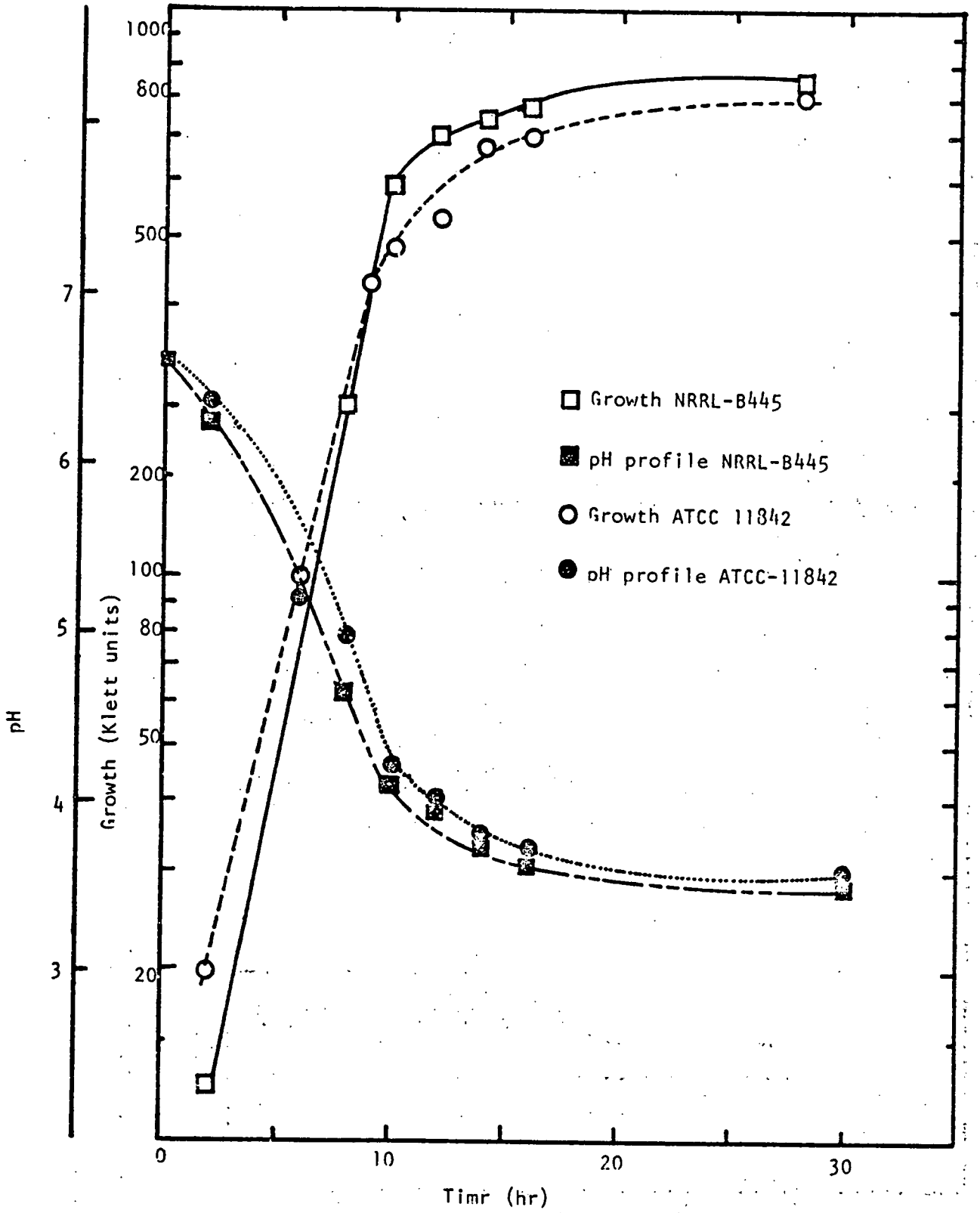


Fig. II.A.6: Growth of Lactobacillus delbrueckii NRRL-B445 and L. bulgaricus ATCC-11842 on glucose.

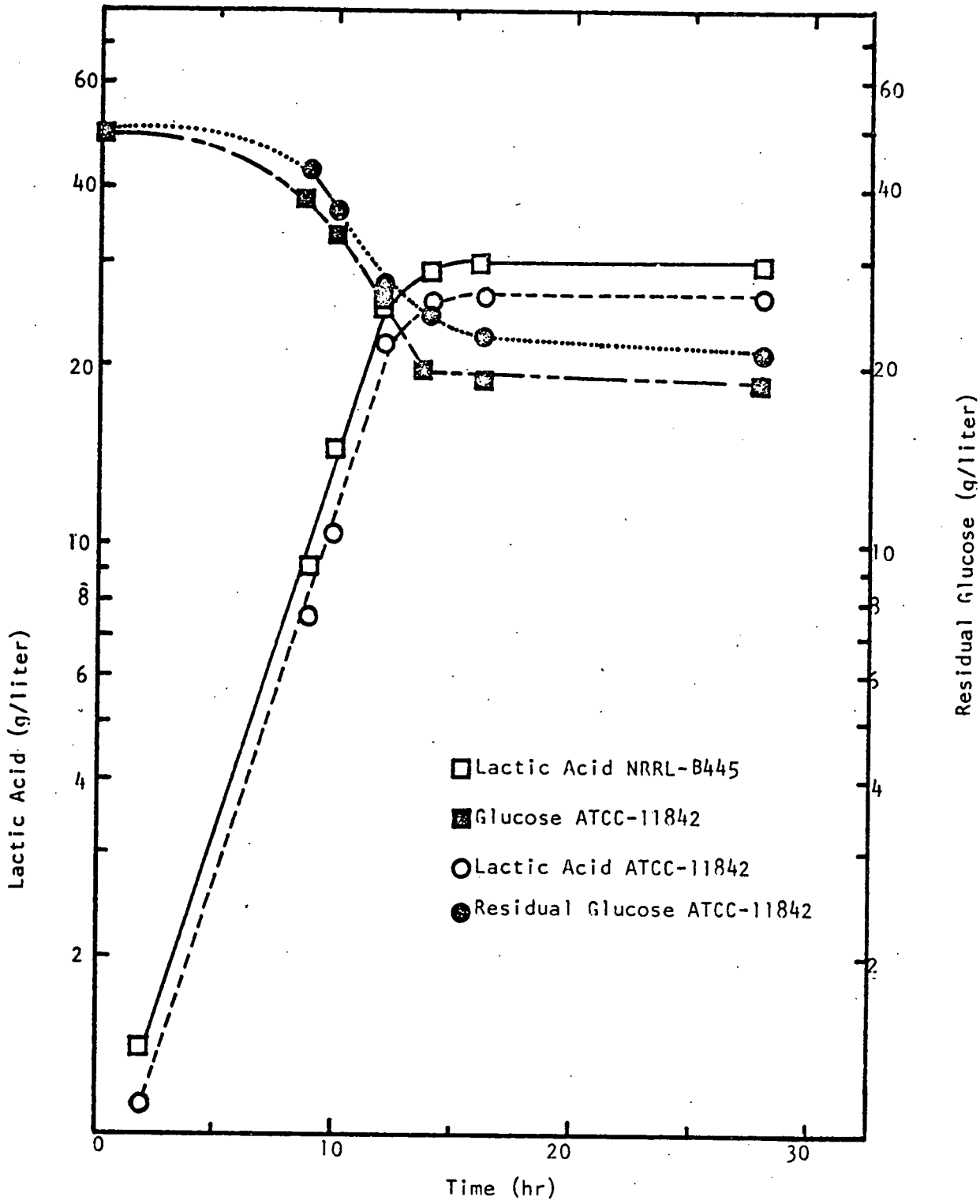


Fig. II.A.7: Glucose Consumption and Lactate Production by L. delbrueckii NRRL-B445 and L. bulgaricus ATCC-11842.

TABLE II.A.4

Strain	Initial Glucose	Age	Final Klett	Specific Growth Rate	Volumetric Productivity	Conversion
				Mmax	9 max	gm lactic
	(gm/l)	(hrs)	(K.U)	(1/hr)	(gm/l-hr)	gm glucose consum.
NRRL-B-445	50.0	3.0	6.50	0.35	4.65	0.925
ATCC-11842	50.0	3.0	6.30	0.34	4.88	0.91

Strain	T_D (hrs)	Final Cell Conc (gm/l)	Sp. prod. formation gm lactic / gm cell-hr
NRRL-B445	1.98	1.74	4.35
ATCC-11842	2.01	1.68	4.54

Table II.A.4, this organism is compared with L. bulgaricus ATCC-11842.

We are also interested in the ability of this organism to use carbon sources other than glucose. In particular, cellobiose is particularly important since C. thermocellum produces a significant amount of cellobiose as an end product of hydrolysis. A comparison of growth of several organisms on cellobiose is shown in Table II.A.5. This experiment was run at 45°C for 24 hours. All strains were able to use cellobiose; however, L. delbrueckii NRRL-B445 had the highest growth. Lactic acid production from glucose and cellobiose is very close. This is shown in Table II.A.6, where conversion yields on glucose are shown to be 92% and on cellobiose 89%.

Having demonstrated the ability of L. delbrueckii to utilize both glucose and cellobiose, the next step is to evaluate its performance on a mixture of reducing sugars resulting from cellobiose hydrolysis via cellulases from C. thermocellum. A hydrolysis mixture was prepared from sulka floc as described in an earlier section on the production of soluble sugars during fermentation of this organism. The resulting broth contained 4.9 grams per liter of reducing sugars expressed as units of glucose. This was concentrated four times to give a value of 19.8 grams per liter of reducing sugars. This concentrate was then added to fermentation media to give a variety of concentrations of sugars from 0.5 to 8 grams per liter. The broth was inoculated with L. delbrueckii NRRL-B445 and growth and lactic acid production were monitored. Shown in Fig. II.A.8 are the results of this experiment. We can see that there was

TABLE II.A.5

Strain	Initial Cellobiose or Initial Glucose (gm/l)	Growth Glucose K.U	Cellobiose K.U
L. delbrueckii NRRL-B445	30.0	425	410
L. bulgaricus ATCC-11842	30.00	440	325
L. delbrueckii ATCC-94649	30.0	380	175

TABLE II.A.6

Results from L. delbrueckii at 45°C for 24 hr.

Carbon Source	Final Cell Conc. gm/l	pH	Lactic Acid (gm/l)	Residual Glucose (gm/l)	Conversion gm Lactic Acid / gm carbohydrate	Sp. Growth Rate (1/hr)
Glucose	1.92	3.65	29.60	10.00	0.915	0.40
Cellobiose	1.84	3.83	28.10	19.5	0.890	0.30

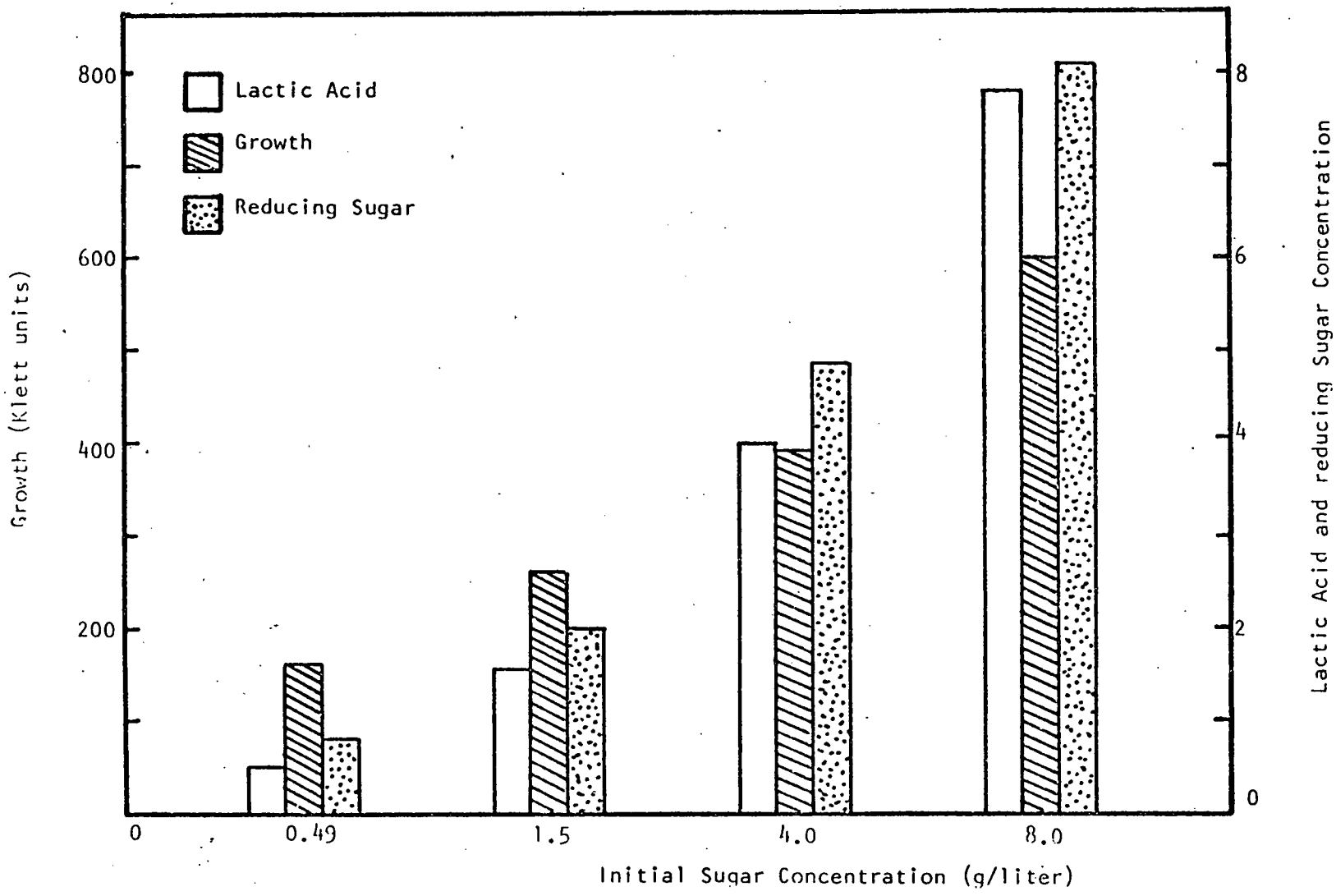


Fig. II.A.8: Growth of L. delbrueckii on Soluble Sugars from Cellulase Hydrolysis.

essentially quantitative conversion of the reducing sugars to lactic acid. The calculated conversion yields range from 95% to 107%. From these results, we can see that it is possible to rapidly produce lactic acid from not only sugar solutions of glucose and cellobiose, but also quantitatively from reducing sugars produced by the hydrolysis of cellulose with C. thermocellum. The kinetics of lactic acid production in this experiment, alone with a control having no added carbon source, are shown in Fig. II.A.9.

As noted earlier, the production of lactic acid in a fermentation with uncontrolled pH ceased when the pH fell to a value of 4. To examine the benefits of controlling pH, a fermentation was run and controlled at pH 5.5. The results of this experiment are shown in Fig. II.A.10. One can see that the residual glucose concentration was reduced much further and lactic acid accumulation reached 55 grams per liter in 20 hours. The overall conversion yield in this experiment was 92%, and the maximum productivity that occurred during the fermentation reached 4.5 grams of lactic acid per liter per hour. The average productivity during exponential growth was approximately 3.6 grams of lactic acid per liter per hour, with a specific growth rate of 0.4 gm acid/gm cell-hr.

It is well known that lactic acid production has both a growth associated and non-growth associated component. Using the model shown in Equation 1, we can plot the specific productivity versus the specific growth rate as shown in Fig. II.A.11. In this equation d is lactic acid acid concentration, t is time, μ is the specific growth rate, x is the cell concentration and α and B are growth and

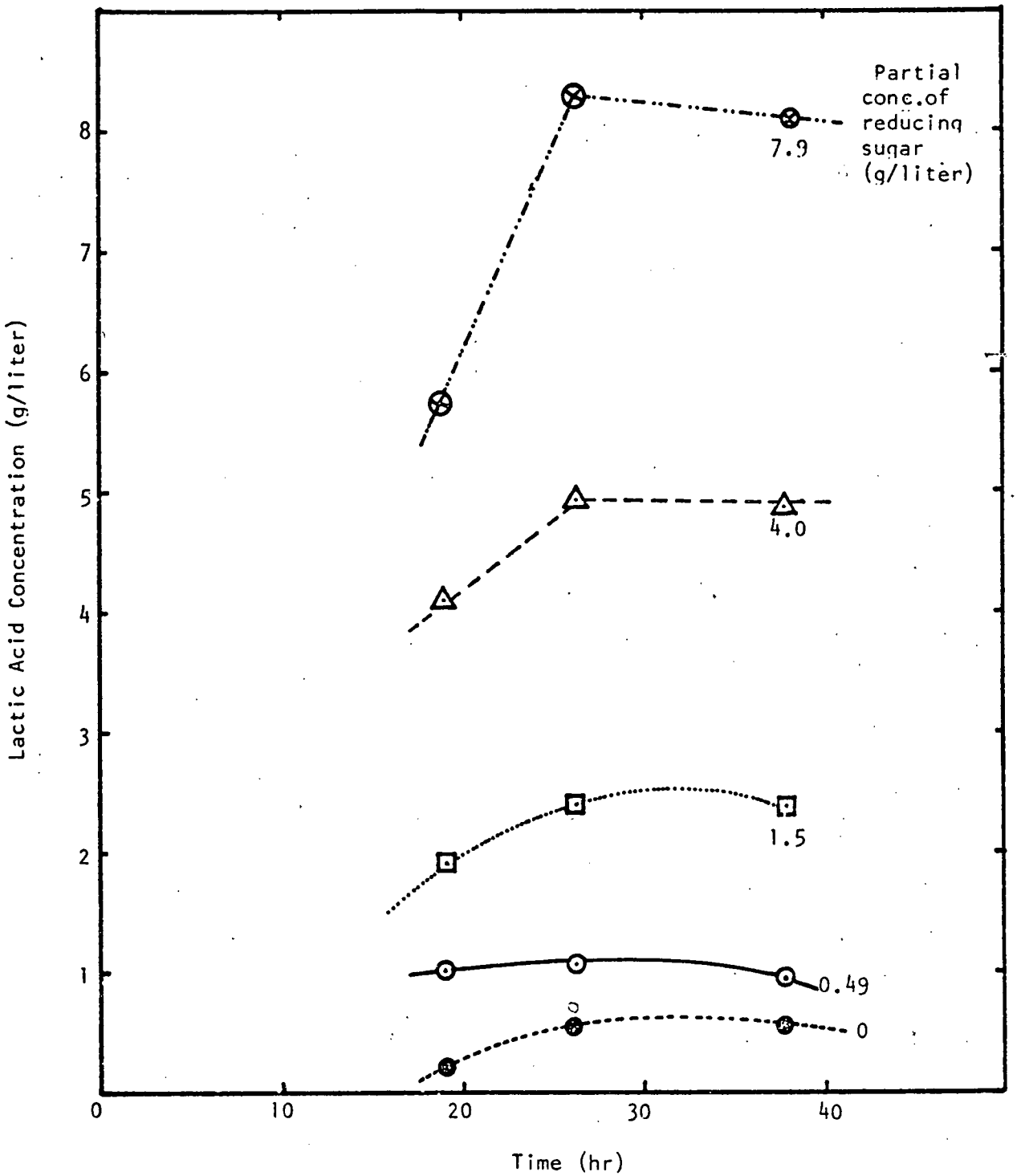


Fig. II.A.9: Lactic Acid Production by L. delbrueckii NRRL-B445 from soluble sugar produced by cellulose hydrolysis.

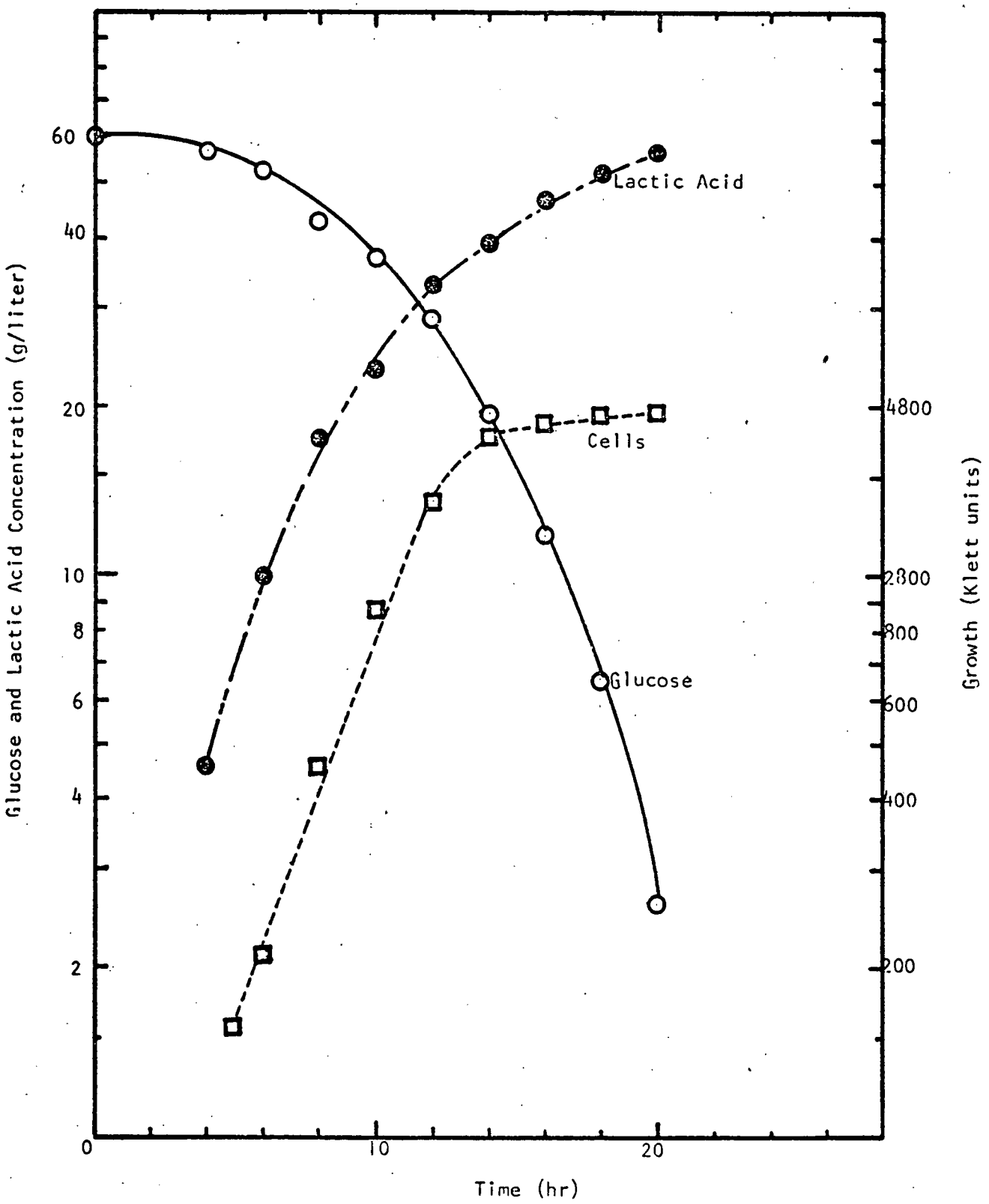
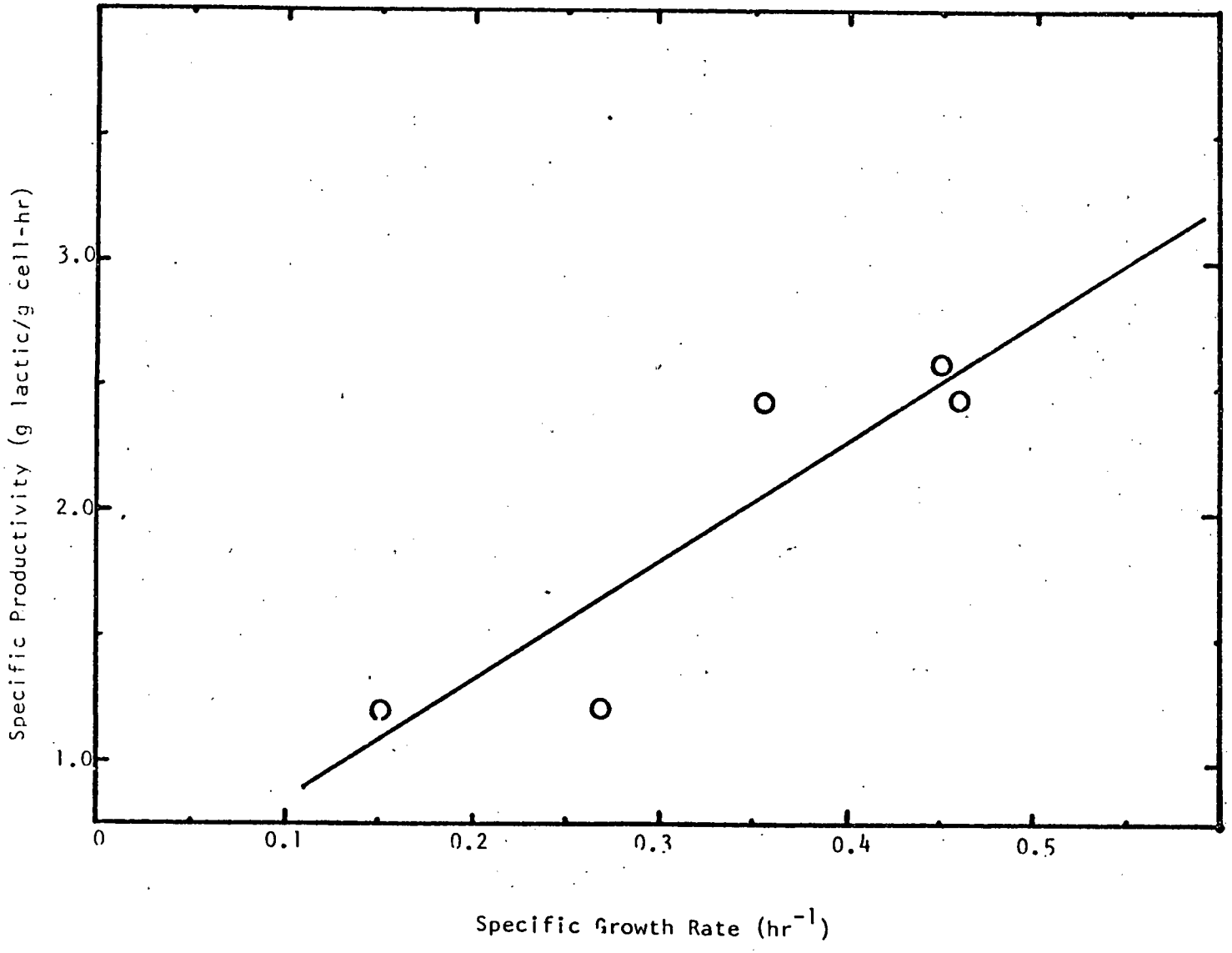


Fig. II.A.10: Kinectis of L. delbrueckii grown on glucose at controlled pH of 5.5.

Fig. II.A.11: Mixed Kinetic Model for Lactic Acid Production



non-growth associated terms, respectively:

$$\frac{dP}{dt} = \alpha uX + BX \qquad (1)$$

When this is done for the data shown in Fig. I.A.10, we can see that the growth associated term with a slope α is 4.91. The value of the intercept is equal to 0.751. Thus, while most of the lactic acid production is growth associated, there is significant non-growth associated component. This suggests that one could use resting cells of lactobacilli in order to effect lactic acid production. Such an experiment was run, cells were grown in their normal medium, after growth the cells were harvested by centrifugation and resuspended in buffer with an initial glucose concentration of 37 grams per liter. This system was evaluated under both batch and continuous flow conditions. The results are shown in Fig. II.A.12. Under batch conditions, one can see the accumulation of lactic acid at the expense of glucose. In the continuous flow mode, a dilution rate of 0.08 h^{-1} was maintained.

A summary of the kinetic data for both batch and continuous operation is given in Table II.A.7. During batch operation, a conversion yield of 98% was achieved with a productivity of 1.48 grams of lactic acid per liter per hour. During continuous operation, the conversion yield was between 96 and 99%. As can be seen we evaluated both the use of sodium hydroxide and calcium hydroxide as a neutralizing agent in this bioconversion. The volumetric productivity ranged from 0.6 to 1.1 grams of lactic acid per liter per hour. However, it is more significant to compare the specific productivity in both the batch and continuous system. From the

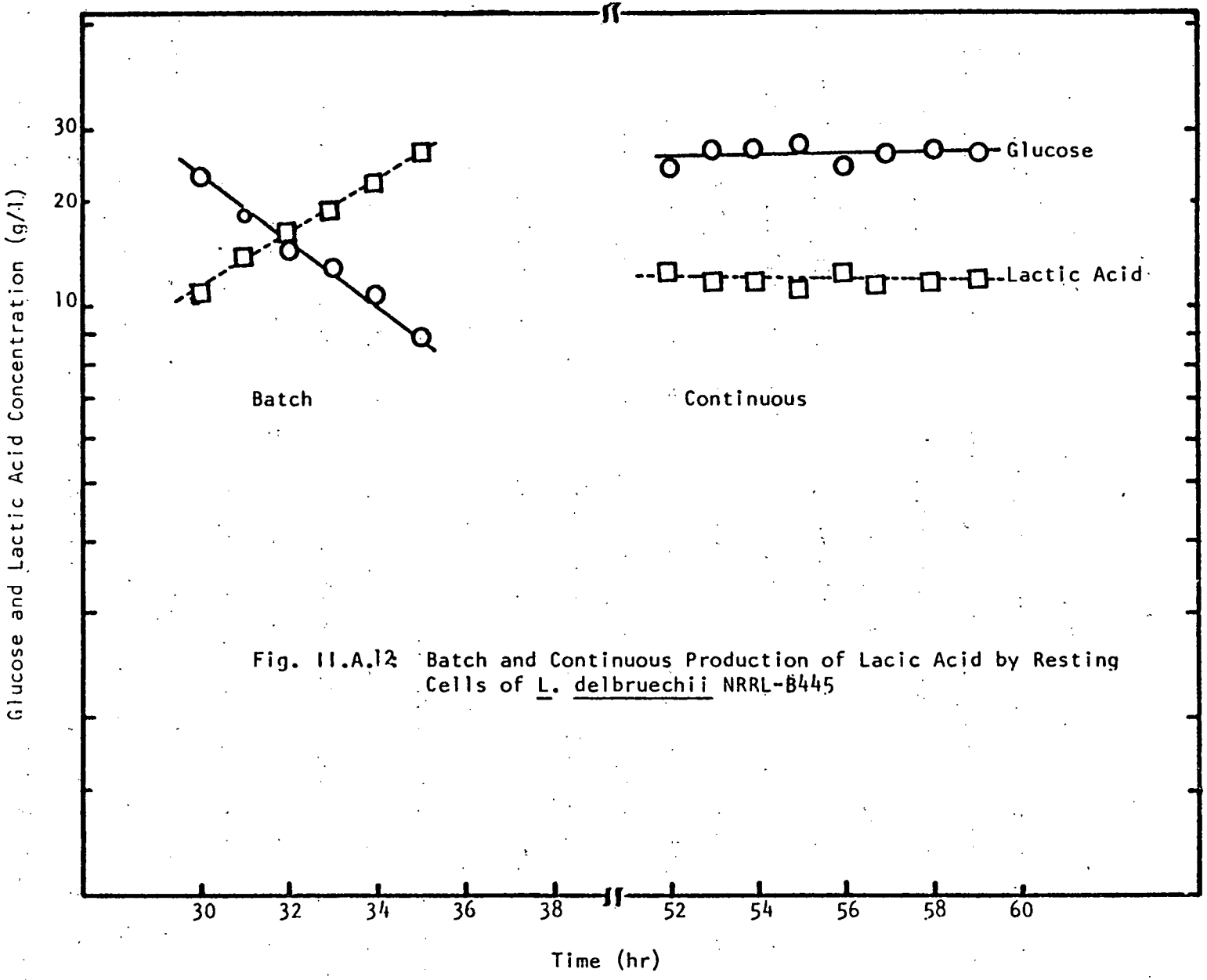


Fig. II.A.12 Batch and Continuous Production of Lacic Acid by Resting Cells of L. delbruechii NRRL-B445

TABLE II.A.7

LACTIC ACID PRODUCTION BY RESTING CELLS IN BATCH MODE

	Run N	Time (HRS)	Cells (gm/l)	Lactic Ac. (gm/l)	Glucose Used (gm/l)	Conversion (gm Lactic) / gm Glu	Productivity (gm Lactic) / L-hr	Sp. Productivity (gm Lactic) / gm cell
*	1	5	10.3	7.4	7.5	0.98	1.48	0.141

LACTIC ACID PRODUCTION BY RESTING CELLS IN CONTINUOUS MODE

	Run N	Time (HRS)	Cells (gm/l)	Lactic Acid (gm/l)	Initial Glucose (gm/l)	Dil. Rate (1/HR)	Conversion gm Lactic / gm glucose	Productivity gm Lactic / l-hr	Sp. Product. gm Lactic / gm-cell
**	1	30	3.0	7.5	37	0.08	0.99	0.60	0.20
*	2	8	10.3	10.3	30	0.10	0.96	1.10	0.11

* Same batch of cells using NaOH, 20% as pH control agent. NaOH showed a negative effect on cell capacity for Lactic acid accumulation. Shown in batch fermentation and resting cells.

** Used $Ca(OH)_2$ as neutralizing agent. Specific productivity is better in cells that used NaOH.

results, we see that values of 0.11 to 0.2 grams of lactic acid per gram of cell per hour were obtained under both batch and continuous operation. It is important to note that operation of the resting cell system was maintained for approximately 60 hours with no significant loss in lactic acid production ability.

In conclusion, we have shown that it is possible to obtain quantitative conversion of carbohydrates to lactic acid at 45°C using L. delbrueckii. Experiments using defined carbohydrates, as well as reducing sugars from cellulose hydrolysis were equally effective. The production of lactic acid is described by a growth model using a growth associated and non-growth associated component. Using resting cell experiments, we have been able to achieve results with volumetric rate of 1.5 grams of lactic acid per liter per hour. This value and the conditions are certainly not optimal and will require further work. However, results on the stability of the system are quite encouraging and one might expect long-term performance to permit the continued achievement of conversion yields approaching 1.0. In batch fermentation, concentrations of lactic acid approaching 60 grams per liter were obtained.

d. Future Work

The effect of important fermentation parameters in particular, temperature, pH, ionic strength will be examined on lactic acid production from cellulose hydrolysates.

The resting cell system for lactic acid production will be examined in more detail with efforts to optimize operating conditions, i.e. with respect to temperature, pH, etc. and

maximize the specific productivity of lactic acid production.

The resting cell system will be evaluated with both cellulose hydrolysis as well as combined with the fermentation of cellulose by C. thermocellum to attempt to get rapid conversion of soluble reducing sugars to lactic acid during the fermentation of cellulose.

The longer term objective is to combine the production of lactic acid with its fermentation using Clostridium propionicum for acrylic acid production.

5. Aerobic Oxidation of Propionic Acid to Acrylic Acid

a. Introduction

It has been reported in the literature that a number of organisms have the ability to aerobically oxidize propionic acid via the propionyl-CoA derivative to acrylyl-CoA. Organisms utilizing this pathway include Escherichia coli and Pseudomonas aeruginosa. Acrylic acid in these organisms exist, like in the obligate anaerobes, in the form of the CoA-thioester and it is a normal route for the degradation of propionate to acetate. In this pathway, it is important to convert propionate to propionyl CoA; this requires one mole of ATP. However, the oxidation of priopionyl-CoA to acrylyl-CoA results in the formation of a reduced nucleotide which, through the electron transport chain, will give more than sufficient ATP for the activation of propionate.

Our objective is to utilize aerobic organisms having this pathway to effect the oxidation of propionate to acrylate followed by the subsequent release of acrylic acid. Since this is a one-step bioconversion, there is the possibility of using not only growing cells, but also resting cell suspensions. For this purpose, it would be desirable to isolate organisms able to oxidize propionate and then

select the mutants unable to further consume acrylate. Such organisms are likely to be blocked between propionate and acrylate. In this phase of our efforts, we have begun to isolate aerobic organisms able to oxidize propionate and acrylate.

(b) Materials and Methods

Microorganisms were isolated from soil by incubation in growth medium containing propionate or acrylate as the sole carbon source. The medium was a mineral salts synthetic medium with 0.15 gram of yeast extract/liter. When growth was observed, the cultures were plated on agar plates containing propionate or acrylate. Those organisms with an ability to use both of these acids were selected for further study.

(c) Results

There was only one organism capable of utilizing both propionate and acrylate isolated (530-40, a yeast). Organisms capable of growing on propionate only are:

530-10 - yellow colony, gram neg. cocci

530-11 - gray-green colonies, gram neg. rod

530-50 - gray colonies, gram positive, in between cocci and bacilli

530-30 - gray colonies, gram neg. rods

530-601- small gray colonies, cocci

526-4S1- red colonies, cocci

In addition, various mycelial organisms capable of growing on both propionate and acrylate were isolated. Initial studies, however, have not used these organisms.

Microorganisms isolated for their ability to use acrylate are likely to have acrylate as an intermediate in a metabolic pathway. However, organisms that will grow only on propionate may or may not involve acrylate as an intermediate. The fact that these organisms do not use acrylate could result from acrylate toxicity or the lack of a transport system to take it into the cell. We examined the ability of the seven isolates noted above to grow in media containing propionate alone or propionate plus acrylate with the intent of seeking cooxidation. Propionate alone was added in a concentration of 4 grams per liter and mixtures of propionate and acrylate contained 2 grams per liter of each. Results shown in Fig. II.A.13 for two of these organisms are typical of the entire group. The organism 530-10 is a gram negative cocci. One can see that, in the presence of propionate alone, it grows quite rapidly reaching a final cell density at approximately 12 hours.

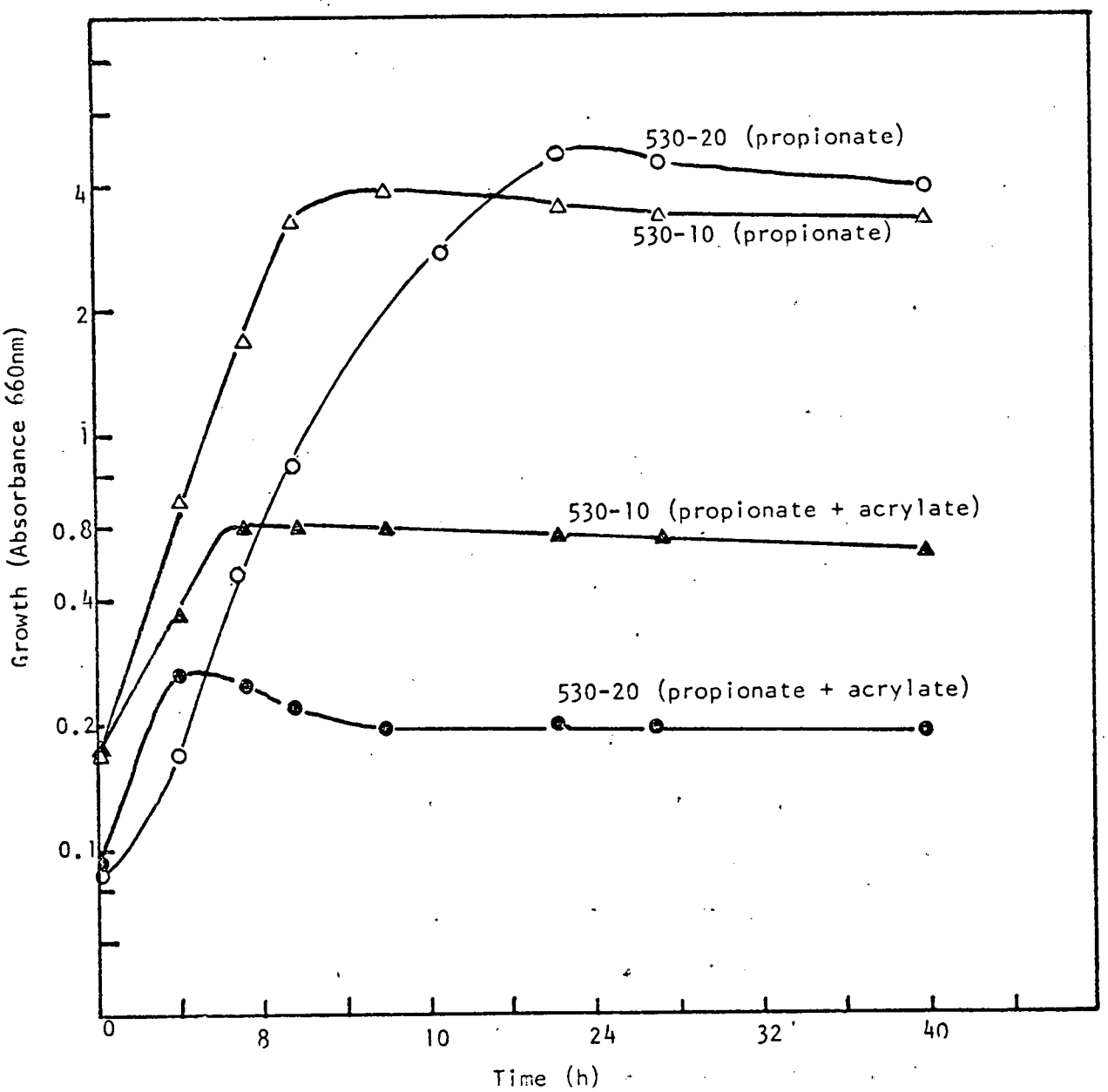


Fig. II.A.13: Growth of soil isolates on propionate and propionate plus acrylate as the sole carbon sources.

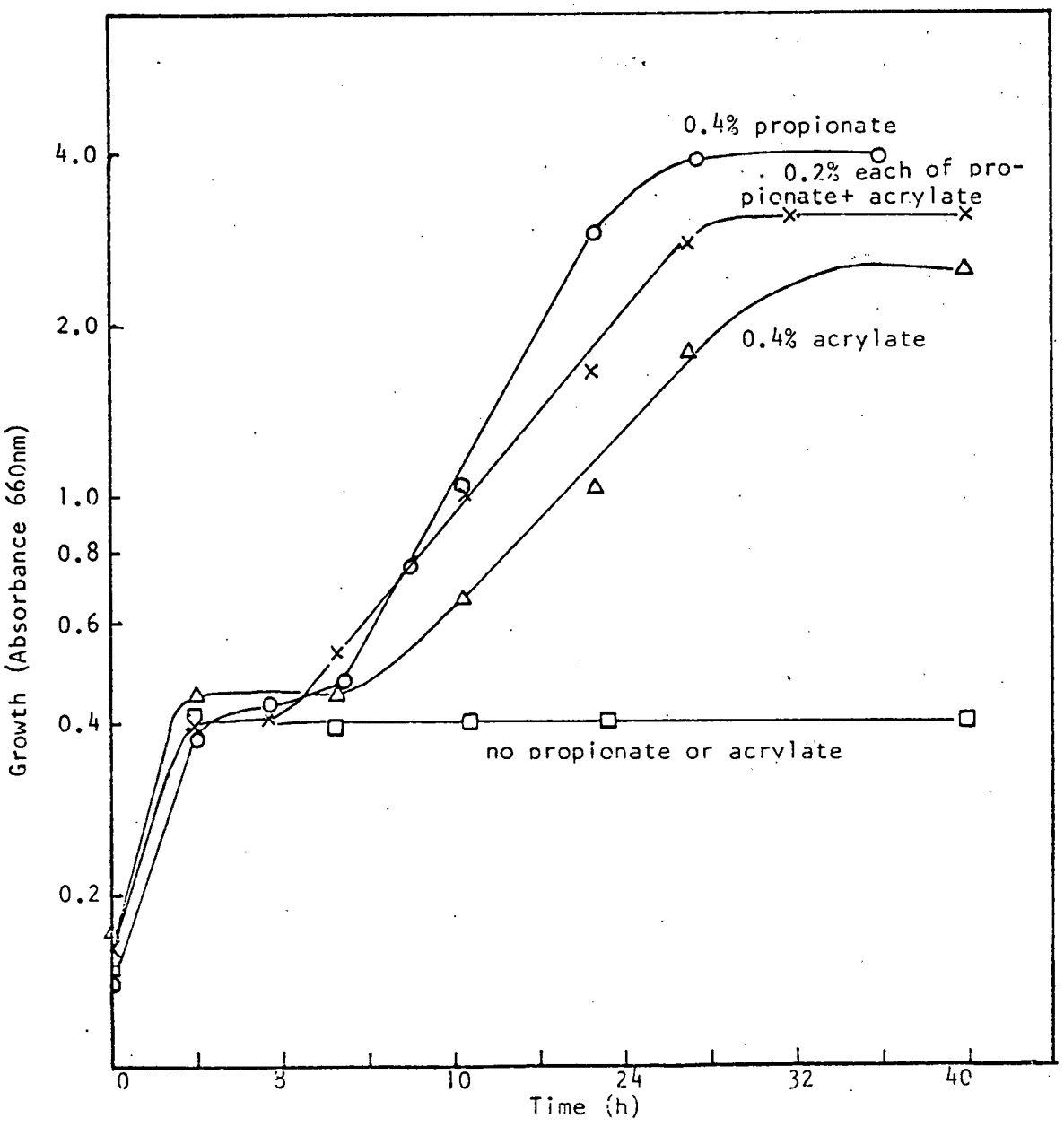
When propionate and acrylate are both present, the cell density is less than half of the previous growth curve, indicating that acrylate is probably not metabolized and it may express some toxicity. A similar phenomena is noted for organism 530-20 but, in this case, one can see that the toxicity of acrylate appeared to be even more severe. In some cases, the addition of acrylate is completely inhibitory to growth.

The most promising isolate and one which will be examined in detail is a yeast, isolate 530-40. A growth curve for this organism is shown in Fig. II.A.14. These results were obtained at 30° using 100 ml of medium in a 500 ml shaken flask. When the organism is grown in the presence of both acrylate and propionate, its growth rate and final cell density is intermediate between growth on propionate or acrylate alone. In all cases, there appears to be diauxic growth. The two phases of growth appear to coincide with the same point at which the cells run out of some nutrient in yeast extract. This organism appears to use acrylate almost as well as propionate and, as a consequence, we feel is an attractive organism with which to explore the aerobic oxidation of propionate to acrylate. In addition, there have been discussed in the literature a number of organisms which have acrylyl-CoA as an intermediate in the aerobic oxidation of propionate. We will proceed to examine some of these systems as well.

d. Future Work

i. An examination of growing and resting cells of yeast culture 530-40 on propionate to achieve the bioconversion of propionate to acrylate.

Fig. II.A.14: Growth of a yeast isolate 530-40 on propionate and acrylate.



ii. The examination of cell-free extracts of the yeast isolate 530-40 for its ability to oxidize propionate.

iii. The examination of appropriate strains of E. coli and Pseudomonas aeruginosa known to aerobically degrade propionate for their ability to produce acrylate both as resting cells and in cell-free extracts.

iv. The optimization of conditions by these cultures for the aerobic oxidation of propionate.

v. The isolation of mutants which are able to oxidize but not grow on propionate or acrylate, but are able to grow on lactate which is the step immediately beyond acrylate in the propionate degradative pathway.

B. ACETONE-n-BUTANOL FERMENTATION

1. Introduction

Continuing efforts are being made to study the batch culture growth kinetics of Clostridium acetobutylicum in soluble medium. The soluble medium used has been reported in the previous progress report (3/1/77 - 5/31/77). It contains glucose, yeast extract, asparagine and salts. The purpose of this study is to clarify the relationship between the growth of the culture and product formation. Fermentation runs were made with minimal environmental manipulation. The results of these runs will serve as a base for further studies and as a guide for further experiments.

Initial studies in batch culture have also been started to determine effects of initial pH, butanol, butyric acid and acetopyruvate (an acetoacetate analog) on the fermentation.

At the same time, a different aspect of research was initiated. Attempts to immobilize whole cells of Cl. acetobutylicum were made. A polymer, poly (acrylamide-co-N-acryloxysuccinimide) or PAN, with free ester groups were used for the immobilization. Gel formation was accomplished by using the cells with a α, ω-diamine as a cross-linking agent (C.L.A.). The cells will be covalently bonded to the polymer by the C.L.A. This immobilization technique was developed by a group in the Chemistry Department at M.I.T. This technique has been demonstrated to be successful in immobilizing a wide variety of enzymes (1). However, immobilization of whole cells with this technique have not been reported. Our attempt was an explorational trial. The advantages of this technique are that the reaction is

not exothermic and no free radicals are involved. By immobilizing the whole cells, the productivity of solvents per unit cell mass may be increased; and the stability of the whole cell may also be increased.

2. Materials and Methods

a. Microorganisms: As reported in the previous report, Cl. acetobutylicum strain ATCC 824 was determined to be the high-producer of solvent when growing in corn mash. Therefore strain ATCC 824 was chosen for further studies.

b. Media: See previous quarterly reports.

c: Inoculation procedure: See previous quarterly reports.

d: Methods of analysis: See previous quarterly reports.

e: Fermentation of soluble medium: Three batches of soluble medium were prepared, each containing approximately 2.0, 3.0 and 10.0 g/l glucose.

In the experiments involving additions such as butanol, butyric acid and acetopyruvate, sterilization was carried out separately and the component was added prior to inoculation. Ph was adjusted using HCl prior to sterilization. These media were dispensed into three series of Huntgate tubes. These tubes were then inoculated with 1% 24 hours old culture growing in soluble medium. Duplicate tubes from each series were harvested at intervals of time. After the turbidity and pH measurements, the cells were spun down by centrifugation. The supernatant was frozen. At the end of the run, the samples were thawed and analyzed at the same time for residual glucose and accumulated products.

f. Immobilization of whole cells of Clostridium acetobutylicum: Strain ATCC 824 was grown in soluble medium with 20 g/l glucose for 24 hours. The cell mass was about 1 g/l dry cell weight. The cells were harvested by centrifugation. They were washed once with 0.01 M phosphate buffer at pH 6.0. The buffer also contained 0.1 M glucose as a stabilizing agent, and 1mM dithiothreitol, DTT, to maintain an oxygen free environment. The pellet was then resuspended in the same buffer to a concentration of about 500 mg dry cell weight/ml.

The immobilization was performed in a 3-dram (about 12 ml in volume) vial, which can be capped with a Huntgate tube serum stopper and screw cap. The reaction was carried out under nitrogen. To immobilize the whole cells, 220 mg of polymer PAN was weighed out in the vial. Seven-tenths of a millimeter of 0.3M phosphate buffer containing 0.1 M glucose and 0.1 mM DTT at pH 7.5 was added. Mixing was achieved by using a magnetic stirrer. After two minutes of mixing, 80 microliters of the cross linking agent, 0.5 M triethylinetetramine was added. One-tenth of a millimeter of cell suspension was then added at about 20 seconds after the cross-linking agent was added. The gel point was noted when the magentic stirrer slowed down suddenly and all the liquid in the vial disappeared. Three minutes after the gel point, the gel was suspended in 5 ml of suspending solution. The vial was sparged with nitrogen before it was capped. Incubation was at 37°C. Samples were taken from the vial using a 1 ml syringe through the serum stopper.

The suspending solutions used for the gel are listed in Table II.B.1.

TABLE II.B.1: Suspending Solutions For The Immobilization Of *Clostridium Acetobutylicum*.

	Concentration Of Components (Molar)				
Solution	Phosphate	Glucose	Acetate	Cysteine	PH.
1	0.1	0.10	-	0.008	6.0
2	0.1	0.25	-	0.008	6.0.
3	0.1	-	0.1	0.008	6.0
4	-	-	0.1	0.008	5.0
5	-	0.25	0.1	0.008	4.0
6	-	0.25	0.1	0.008	5.0
7	Soluble medium with 5000 units of penicillin /6.2 ml.				

3. Results and Discussion

a. Fermentation: The fermentations with 2.0 g/l and 3.4 g/l initial glucose concentration are very similar (Fig. II.B.1, II.B.2). Cell growth occurs in the first twenty hours. All the glucose is depleted in that period. The butyric acid production follows closely with the cell growth. This is reflected by the rapid drop of pH in the first 20 hours. The amounts of n-butanol, acetone and ethanol accumulated are small compared to the accumulation of butyric acid. The concentration of n-butanol in both cases is about 0.2 g/l. Acetone and ethanol concentrations are below 0.1 g/l. In the fermentation with initial glucose concentration equal to 10 g/l, a marked difference is noted after the first twenty hours (Fig. II.B.3). The cell growth continues until the 40th hour. The pH value drops rapidly in the first twenty hours and remains as such for the remainder of the run. However, the amount of butyric acid keeps on increasing until the 70th hour when the glucose is near complete depletion. The n-butanol concentration reaches 0.2 g/l at the 20th hour. This is the same as the other two fermentations. Then the concentration starts going up to about 1.2 g/l 80 hours later. Acetone accumulation remains low at about 0.1 g/l throughout the run. The differences between this run and the other two runs are the accumulation of n-butanol and the concentration of the residual glucose along the course of the fermentation. In the fermentation when the initial glucose concentrations are low, the culture consumes the glucose for growth. Butyric acid is formed as a result of the growth. Glucose is consumed before the mechanism for converting butyric acid to n-butanol is being triggered. However, in the fermentation which has high initial glucose concentration, there is excess glu-

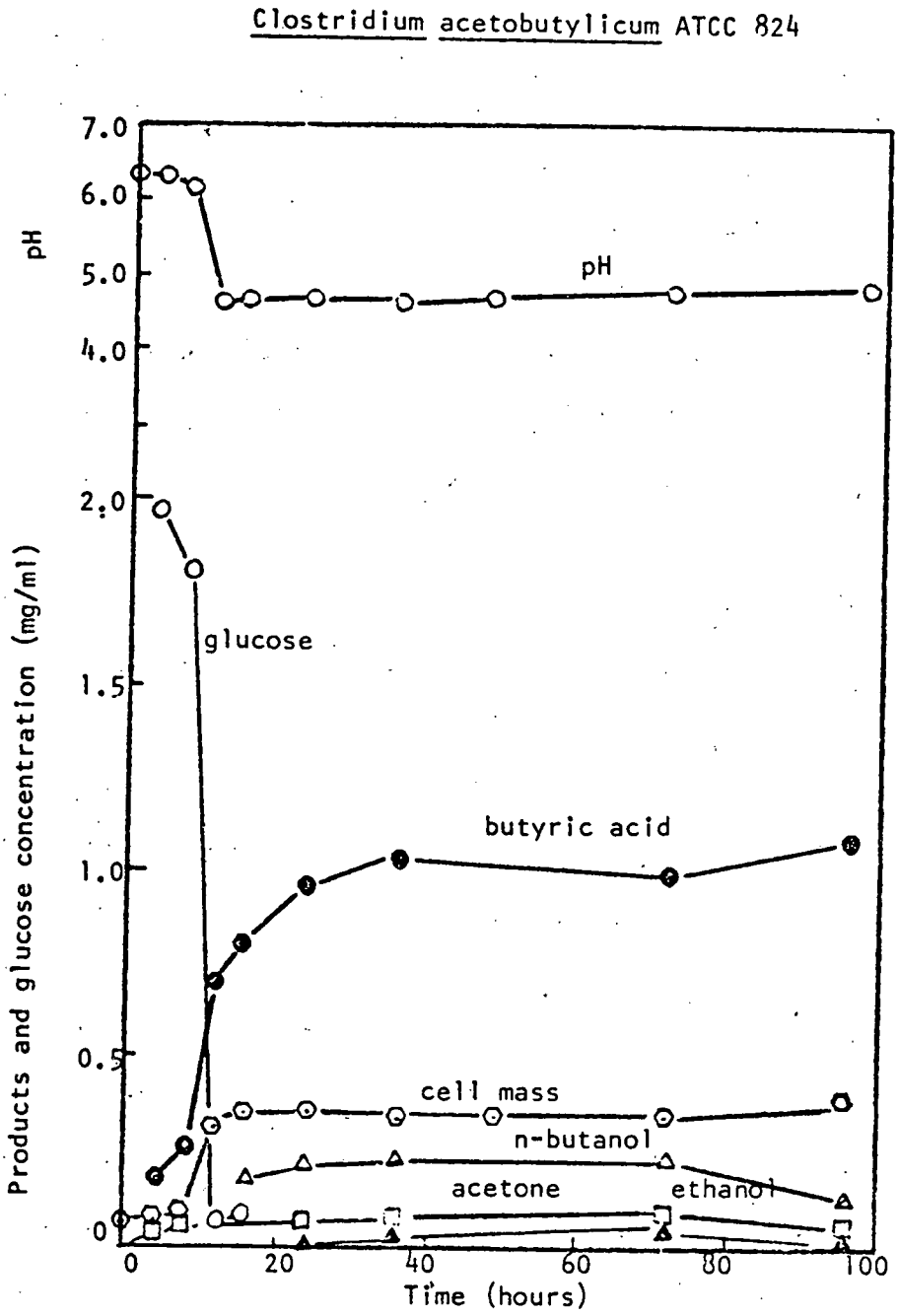


Fig. II.B.1: Time course of fermentation of ATCC 824 growing in soluble medium with 2.0 g/l initial glucose concentration.

Clostridium acetobutylicum ATCC 824

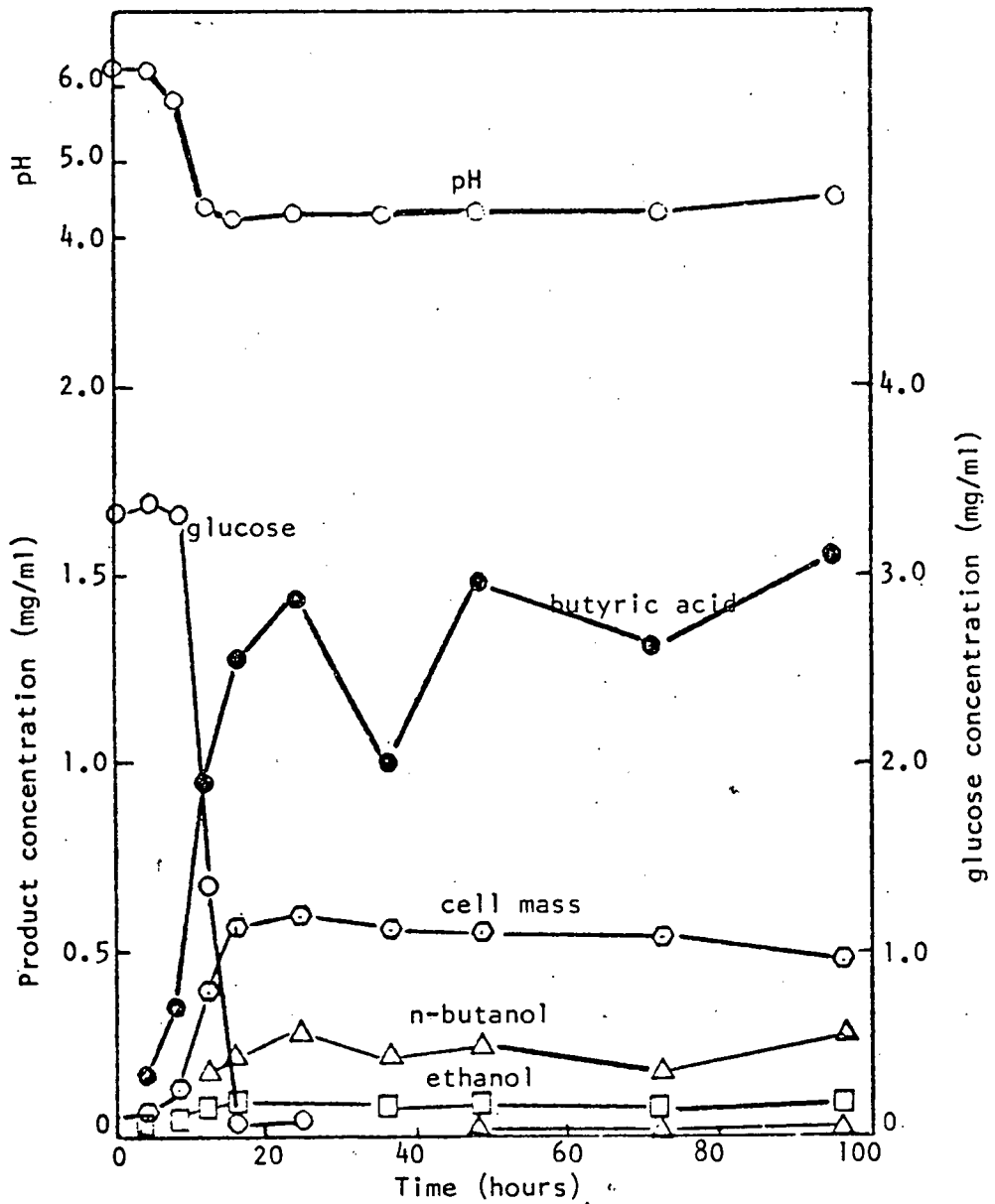


Fig. II.B.2: Time course of fermentation of ATCC 824 growing in soluble medium with 3.4 g/l initial glucose concentration.

Clostridium acetobutylicum

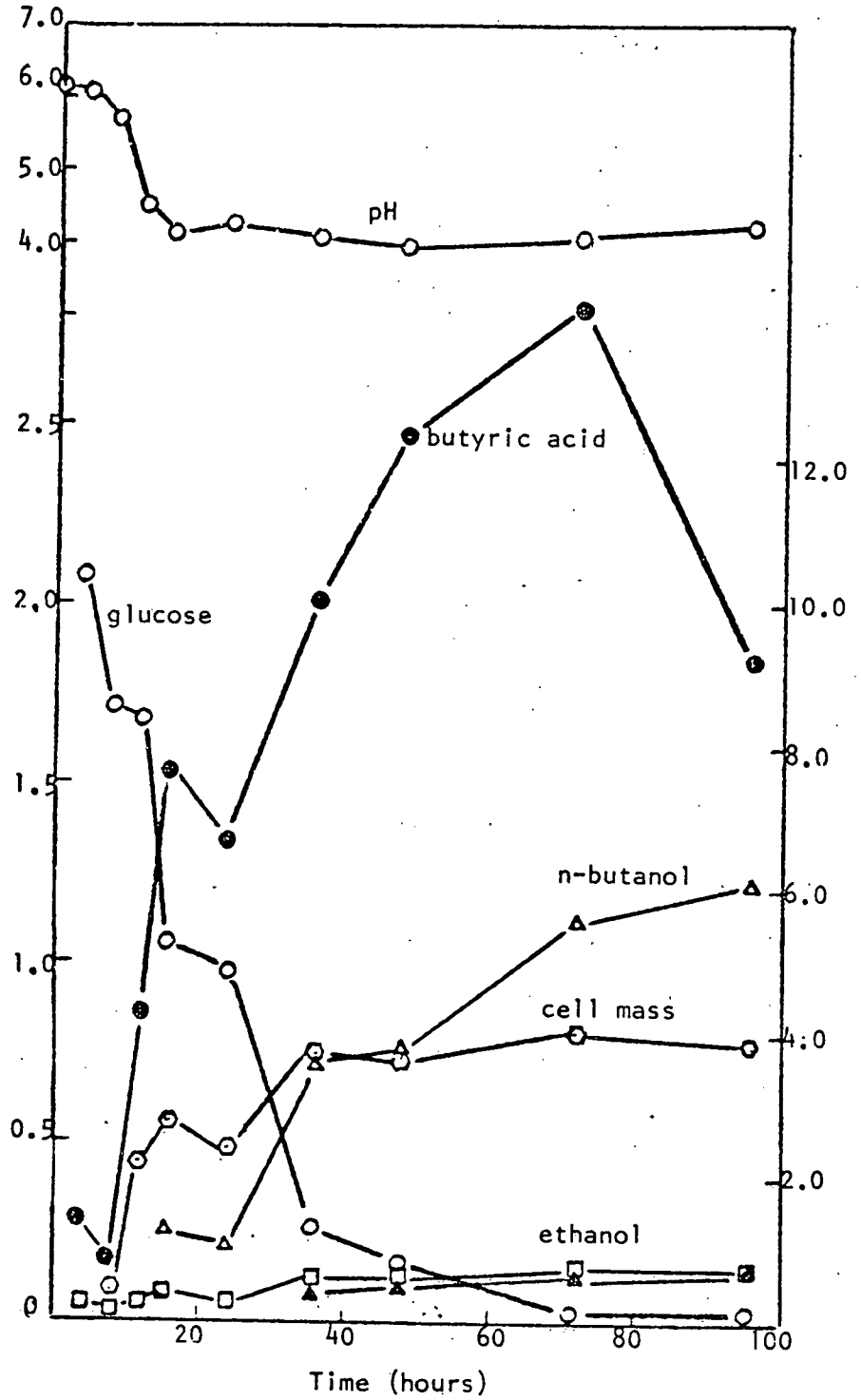


Fig. II.B.3: Time course of fermentation of ATCC 824 growing in soluble medium with 10.0 g/l initial glucose concentration.

cose when such mechanism is switched on. The excess glucose supplies reducing power for the conversion of butyric acid to n-butanol. The control of such mechanism is not known. There could be several possibilities. The enzyme system for such conversion may be induced by the presence of butyrate. It is also possible that such enzyme systems may be repressed or inhibited by metabolites from glucose along the pathway. Moreover, the reaction may be set off by an equilibrium relationship between the butyrate and n-butanol, thus a presence of excess butyrate will drive the reaction to form n-butanol. These postulates could explain the fermentation patterns, but further research have to be done for confirmation as to which one is correct. However, the conclusions that can be drawn from these studies are that the fermentation is slowed down by the accumulation of acids or by the low pH environment. Also the results confirmed what was reported in the last progress report that the bottle neck in the fermentation is the conversion of acids to solvents.

b. Effect of Initial pH: Initial pH has no effect on final solvent yields even though final pH varies slightly (Table II.B.2). The variation in pH may not be significant since they all lie below the pK of butyric acid.

c. Butanol Toxicity: Growth inhibition by butanol falls in the range of 1.6 - 2.4% (w/v) (Table II.B.3). The maximum tolerance will have to be determined more accuately as the basis for further work on development of high tolerance strains. These strains are necessary to increase productivity and ease of butanol recovery.

GC data which will be obtained will show if there is any significant effect on butanol production and may be able to test the hypothesis that butanol inhibits its own synthesis.

TABLE II. B. 2. Effect of Initial pH on Final Solvent Yields

pH (Initial)	OD_{660}	pH (Final)
6.6	.34,.37	4.20,4.21
6.0	.53,.48	4.11,4.0
5.5	.61,.62	3.94,3.9
5.2	.67,.68	3.81,3.81
5.0	.68,.69	3.71,3.71
4.8	.68,.70	3.71,3.71
4.5	.70,.70	3.61,3.61
4.3	.68,.67	3.61,3.61

(g/l)

pH (Initial)	Acetone	EtOH	Acetate	Butanol	Butyrate	Butanol/Butyrate	Butanol & Butyrate
6.6	.2	.11	1.23	1.4	1.3	1.11	2.7
6.0	.4	.15	2.28	1.4	1.3	1.01	2.7
5.5	.35	.14	1.22	1.7	1.2	1.41	2.8
5.2	.161	.09	1.0	1.2	1.4	.881	2.6
5.0	.251	.091	.85	1.2	.9	1.41	2.7
4.8	.251	.12	1.1	1.4	1.3	1.11	2.8
4.5	.191	.12	1.1	1.4	1.3	1.01	2.6
4.3	.091	.11	1.2	1.2	1.2	1.00	2.5

CAS Medium is Titrated with HCL. 1% Inoculum is added.
Incubation is at 37 C for 2 days

TABLE II.B.3 Butanol Toxicity

%Butanol (w/v)	OD_{660}	pH
0	1.1;0.73	3.7,3.81
1.6	0.95;0.64	4.7,4.2
2.4	-	
3.2	-	
4.0	-	
4.9	-	

To 10 ml of CAS soluble medium is added 1% inoculum and butanol. Incubation is at 37 C.

Broth was clarified by centrifugation at 10K for15'. Supernatant was frozen at -20 C until GC performed.

d. Growth Inhibition by Acetopyruvate (AP): Growth inhibition is complete at 5mM acetopyruvate. Since at lower concentrations of AP acetone is still produced, it appears that the primary block by AP is not acetone production.

At lower concentrations of AP the amount of butanol produced is significantly increased (Table II.B.4). AP must have some effect on the regulation of butanol dehydrogenase probably by changing the level of its regulators. AP may inhibit reduction of acetoacetate to butyrate or acetoacetate formation from acetate. An increase in NADH, acetyl CoA or other metabolites may induce or derepress the dehyrogenase system. Currently, experiments have been performed to see if the effect is a kinetic one which increases butanol productivity (i.e. butanol is made early in fermentation) or one of duration (i.e. butanol continues to be made for longer periods of time).

e. Immobilization of Whole Cells: After 50 hours of incubation, samples were taken and analyzed. The results of the immobilization experiment are summarized in Table II.B.5.

TABLE II. B. 4: Effect Of Acetopyruvate On Cl. Acetobutylicum Fermentation

g/l

Acetopyruvate (mM)	OD_{660}	Final pH	Acetone	EtOH	Acetate	Butanol	Butyrate	Butanol Butyrate	Butanol + Butyrate
0	.97	3.9	.19	.07	1.2	1.1	1.3	.8 >.6	2.6
	.95	3.9	.11	.06	.88	.7	1.6	.40	2.3
.1	1.0	4.3	.28	.11	1.1	1.1	1.7	1.0 >1.7	3.4
	1.2	4.16	.58	.19	1.5	3.0	1.2	2.5	4.3
.2	1.1	4.20	.35	.16	1.3	2.6	1.3	2.0 >2.1	4.0
	1.1	4.2	.24	.09	.76	2.0	.9	2.2	2.9
.3	1.1	4.3	.44	.27	1.7	3.7	1.6	2.3 >2.6	5.3
	1.05	4.4	.45	.19	1.6	3.5	1.2	3.0	4.7
.4	1.2	4.4	.40	.2	1.8	3.6	1.5	2.4 >2.2	5.1
	1.1	4.4	.39	.17	1.5	3.1	1.5	2.1	4.6
.5	1.1	4.3	.28	.14	1.5	2.6	1.8	1.4 >1.7	4.4
	1.1	4.4	.23	.12	1.3	2.4	1.2	1.9	3.6
.6	1.2	4.4	.43	.18	1.5	3.0	.96	3.2 >3.0	4.0
	1.1	4.3	.21	.13	1.0	2.2	.76	2.9	

Acetopyruvate is added in various amounts to CAS medium. Medium is inoculated w/1% fresh culture of ATCC 824 and inculated at 37 C for 2 days.

Table II.B.5. Results of Immobilization of C. acetobutylicum with PAN.

Reactor	t_c(sec)[a]	t_g(sec)[b]	suspending solu.[c]	gas formation	pH Int.	pH Fin
0	1% cell suspension in soluble medium			+	6.2	4.2
1	20	65	7	+	6.2	5.8
2	20	15	1	+	6.0	5.6
3	10	70	2	+	6.0	5.5
4	15	120	3	+	6.0	5.9
5	20	105	7	+	6.2	5.8
6	20	55	4	-	5.0	5.0
7	20	57	5	-	4.0	4.0
8	20	57	6	-	5.0	5.0

a. t_c: time between addition of cross linking and cells.
b. t_g: time between addition of cells and gel formation.
c. refer to Table II.D.1.

Gas-chromatograph assays show that except for reactor 0 and 1, the other reactors do not contain any products. Reactor 0 is the control showing that the cells are capable of growing and producing solvents in the scluble medium. In reactor 1, there was trace amount of acetone, 0.58 g/l of acetic acid and 0.42 g/l of butyric acid. There is no n-butanol detected. The gas formed in the vial was not determined, since technique for analyzing gas products has not been fully developed to date.

The results, though mostly negative, do show that the immobilization technique is a worthwhile avenue to be pursued. The formation of gas in these vials shows that there is reaction going on in the gel. The failure to detect products of interest may be due to mass transfer limitation which was not dealt with in this experiment. Future experiments will be designed to look at the immobilization with crushed gel. Cells harvested at different times during the fermentation will be used. Also, different immobilization techniques will be explored.

4. Future Research

Fermentation of ATCC 824 on soluble medium will be studied further. Control of environment, such as pH control will be used.

Immobilization of whole cells will be further investigated as mentioned in the discussion section. A more detailed analysis of the effects of acetopyruvate on final yields of butanol will also be explored.

C. PRODUCTION OF ACETIC ACID BY FERMENTATION

1. INTRODUCTION

Acetic acid production by Clostridium thermoaceticum has been studied during the past quarter in order to delineate the pertinent parameters which will allow us to establish the proper process conditions. The primary objective of these studies was to determine those conditions which will allow us to maximize the rate of acetic acid production as well as to attain high concentration of this product. From our preliminary studies, the growth and acid production by this organism appears to be affected by the pH. This results from the decrease in pH during acid production. Detailed studies on the effect of pH as well as other parameters on the growth and acid production were performed.

2. MATERIALS & METHODS:

Growth Medium:

The medium is prepared in 3 parts

A	glucose	18 grams
B	$NaHCO_3$	16.8 gms
	K_2HPO_4	7 "
	KH_2PO_4	5.5 "
C	Yeast Extract	5 gms

Tryptone	5 gms
$(NH_4)_2SO_4$	1 "
$MgSO_4 \cdot 7H_2O$	0.25
$Co(NO_3) \cdot 6H_2O$	.029
Nathioglycolate	.5
$Fe(NH_4)_2(SO_4)2 \cdot 6H_2O$	.20
$NaMoO_4 \cdot 2H_2O$	.12
$CaCl_2$	.016
Trace Salts	1 ml
Resazurin (.2%)	1 ml

Trace Salts in gm/l

$MnCl_2 \cdot 4H_2O$,5; $NaSeO_3$, ·172; $H_2BO_3 \cdot 0.1$; $ZnCl_2$, .05; $AlK(SO_4)_2 \cdot 12H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$ 0.02; $CuCl_2 \cdot 2H_2O$, 0.01; EDTA, 5.

The media is prepared using freshly boiled distilled water. A, B, and C are prepared separately. A is dissolved in 150 ml H_2O, B in 250 ml and C in 600 ml. The three parts are autoclaved separated and CO_2 is bubbled through them as they are allowed to cool. After cooling the three parts are combined.

Agar Medium

To one liter of freshly boiled distilled water the following are added:

Agar	20 grams
Yeast Extract	5 "
Tryptone	5 "
$(NH_4)_2SO_4$	1 "
$MgSO_4 \cdot 7H_2O$	0.5 "
Na-thioglycolate	0.5 "

One ml of 0.2% Resazurin is added as an indicator. The agar medium is boiled until the red color of the dye has disappeared, CO_2 is bubbled through the medium during this process. The agar is then transfered under CO_2 to culture tubes and allowed to solidify. It is stored at 4°C and autoclaved just before it is to be used.

AGITATED FERMENTOR STUDIES:

Controlled pH studies can be readily performed in laboratory scale agitated fermentors. In our studies a 5-liter total volume New Brunswick fermentor was used containing 3.5 liters of medium. pH was controlled with 10N NaOH.

3. RESULTS AND DISCUSSION:

a. Effect of glucose concentration

The effect of initial glucose concentration on the growth and acetic acid production by Clostridium thermoaceticum was studied in shake flasks. The initial glucose concentrations examined were 20, 40 and 100 gm/liter. The results of these studies are shown in Figures II.C.1, II.C.2 and II.C.3. It can be seen that the final cell concentration and final acetic acid concentration are essentially

FIGURE II.C.1 - Effect of glucose concentration (20 gm/L) on growth, acid and pH

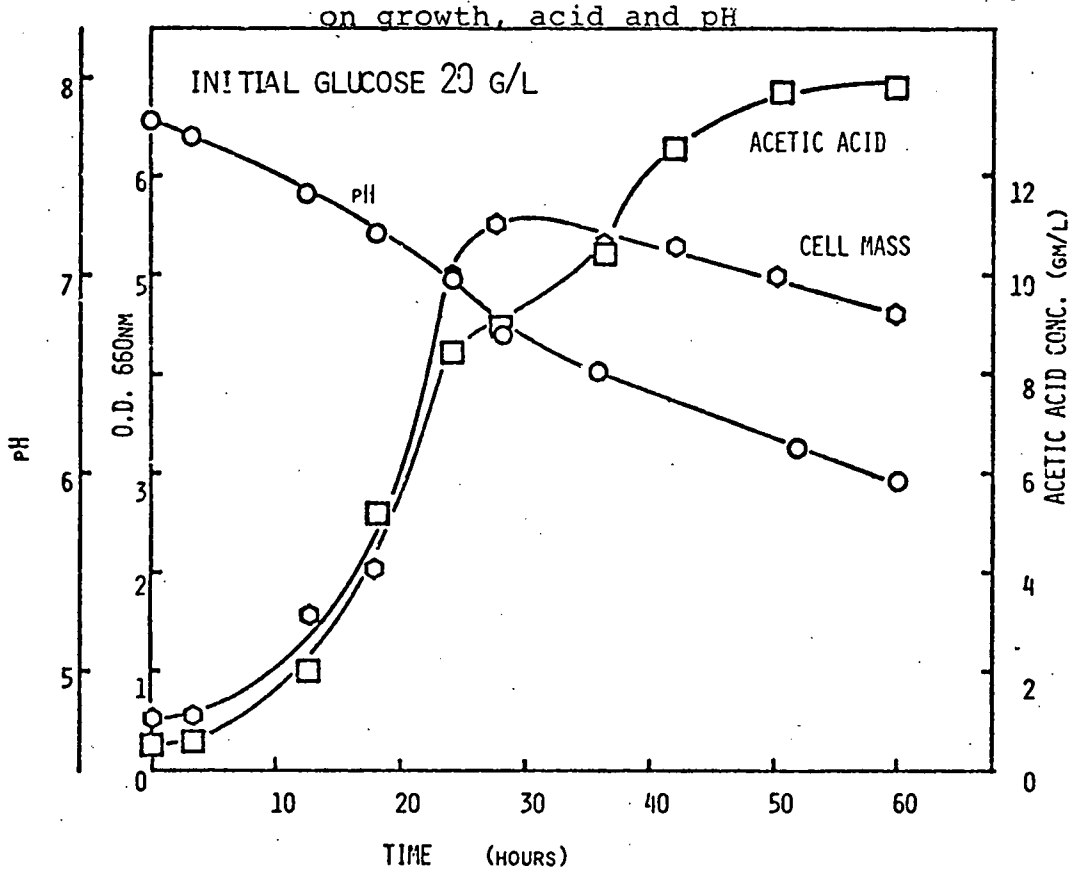


FIGURE II.C.2 - Effect of glucose concentration (40 gm/L) on growth, acid and pH

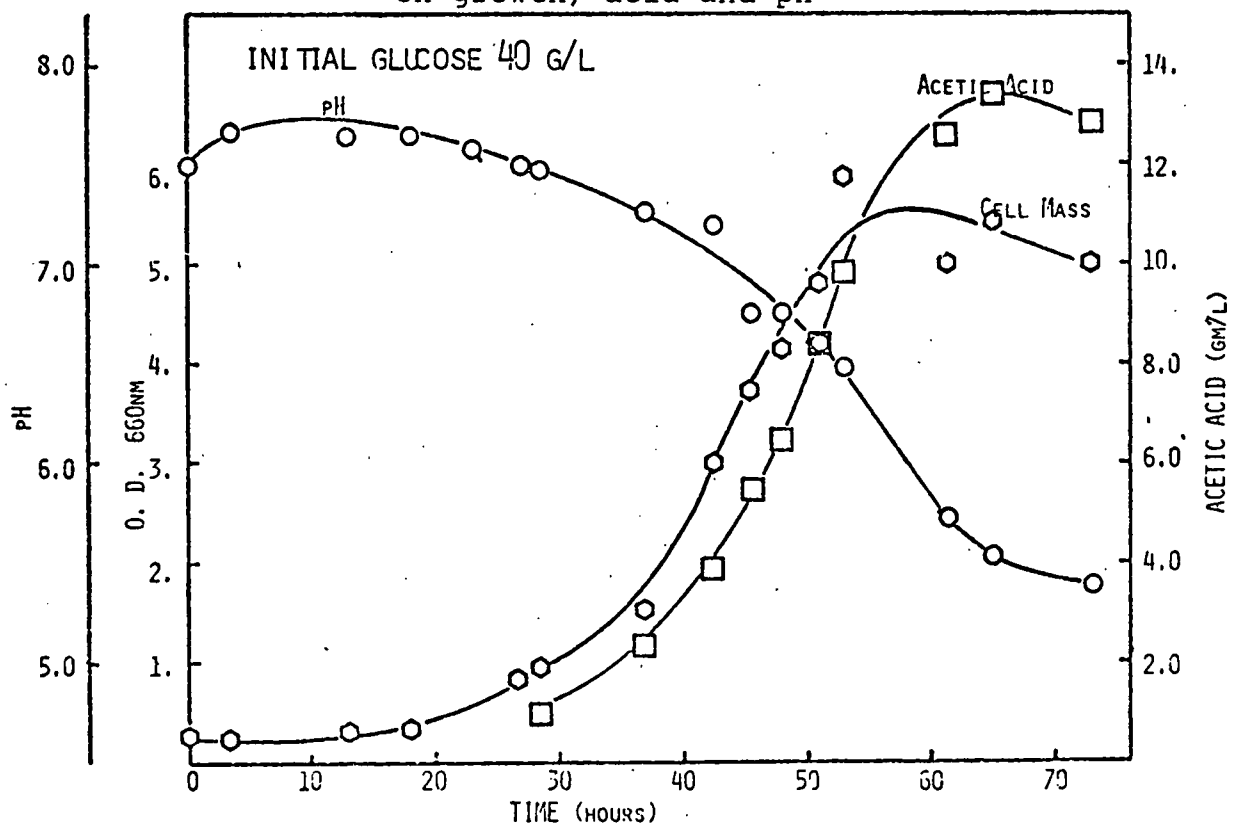
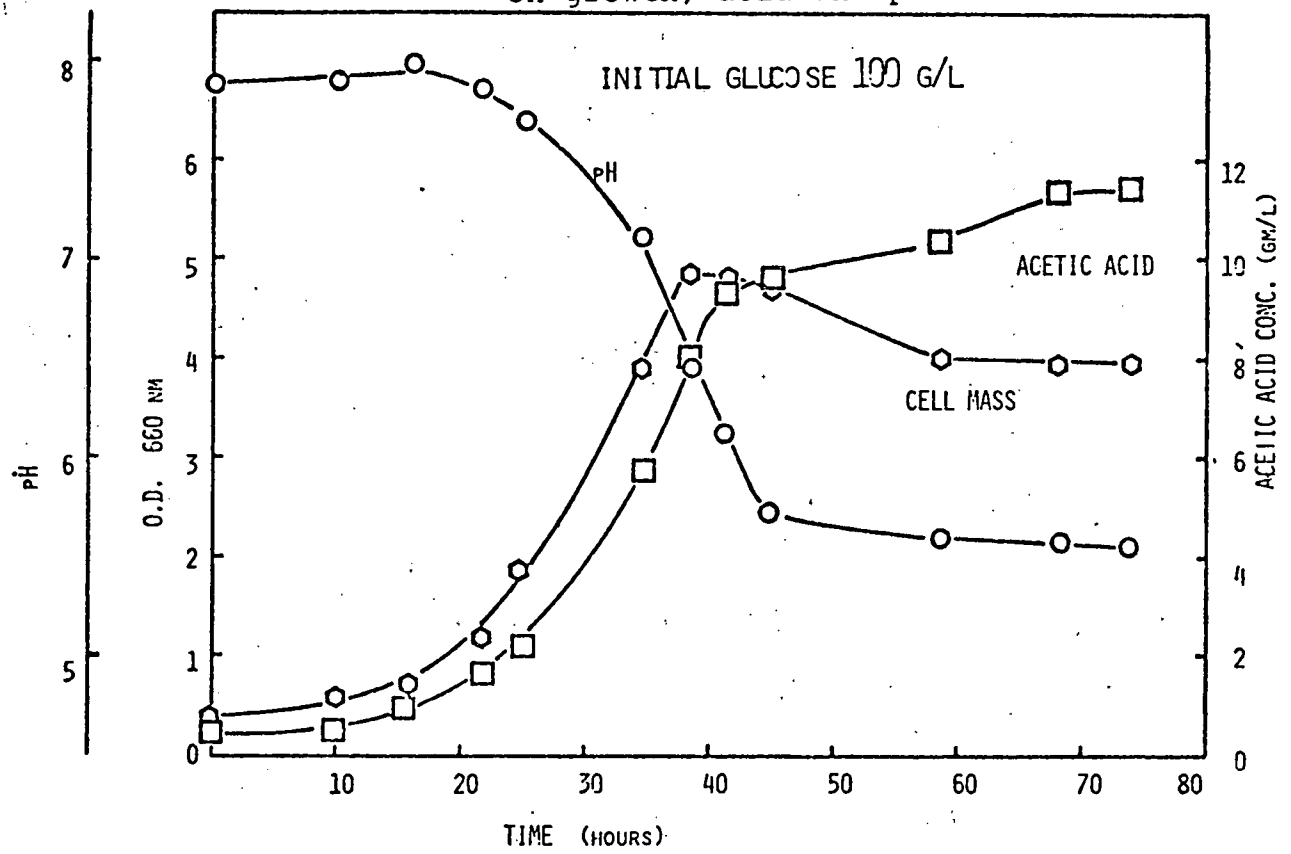


FIGURE II.C.3 - Effect of glucose concentration (100 gm/L) on growth, acid and pH



unaffected by the initial glucose concentration. However, the cessation of growth and acid production appears to be influenced by the pH. One observes, for example, at the three different initial glucose concentrations the organisms' growth rate essentially stops when the pH reached 6.4 to 6.8. It is interesting to note that even though the growth stops, acetic acid continues to be produced. However, when the pH decreased further to about 5.6 to 5.8, the organism is unable to continue producing acetic acid. These results strongly hint that the pH (H^+ concentration) might be the primary parameter in controlling growth and acid production. However, two other explanations must also be considered; one, is that acetate not pH is inhibiting the production of cell mass and acetic acid by C. thermoaceticum. The second, is that some nutrient other than glucose has been depleted and is limiting cell growth and acid production.

b. Effect of sodium acetate on the growth of Clostridium thermoaceticum

One possible method to overcome the effect of pH on the growth and product formation is through controlled pH fermentation. In order to neutralize the acid form, an alkali such as NaOH might be employed. However, the effect of the salt, sodium acetate, on growth must be known. Therefore a series of experiments were performed in tube cultures where the concentration of sodium acetate was varied. Shown

in Figure II.C.4 are the growth kinetics of Clostridium thermoaceticum where the sodium acetate concentration was varied between 0 to 50 gm/liter. It can be seen that as the sodium acetate concentration is increased, there was a noticeable lag before cell growth starts. This is reasonable since the organism must adjust to a new environment when faced with increasing sodium acetate concentration. Using the kinetic data from Figure II.C.4, the specific growth rate of this organism can be calculated. These results are shown in Figure II.C.5. It can be seen that sodium acetate has an inhibitory effect on the growth of the organism. At 100 gm/liter of sodium acetate, no growth was observed. This inhibitory effect on growth appears to be linearly related to sodium acetate concentration.

In order to delineate which ion (sodium or acetate) exists, the inhibitory effect on growth, a series of experiments were conducted where sodium chloride was added to the medium. The growth kinetics when NaCl was varied between 0 to 16 gm/liter are shown in Figure II.C.6. The effect of NaCl on the specific growth rate is shown in Figure II.C.7. It should be mentioned that 25 gm/liter of NaCl was also tested and total inhibition of growth was encountered. At a first glance one might conclude from Figures II.C.5 and II.C.7 that the sodium ion might be the inhibitory substance. However, a more detailed examination of these results does not support this conclusion. For

FIGURE II.C.4 - Kinetics of growth of Clostridium thermoaceticum at different sodium acetate concentrations

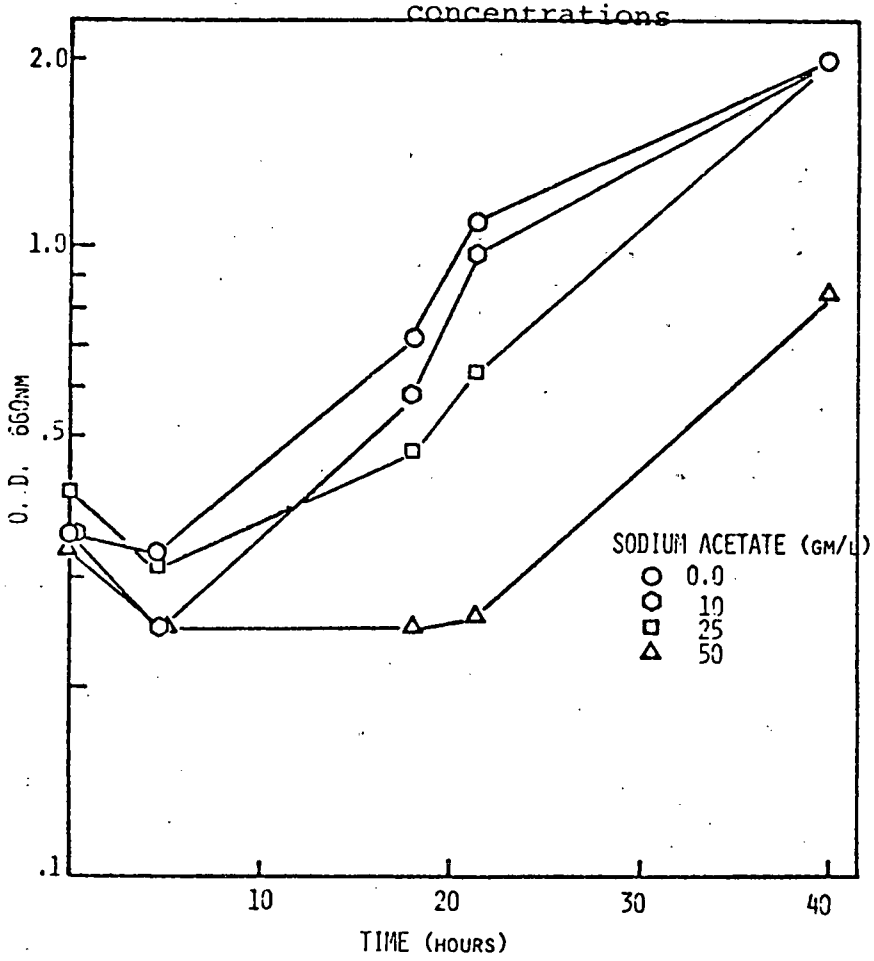
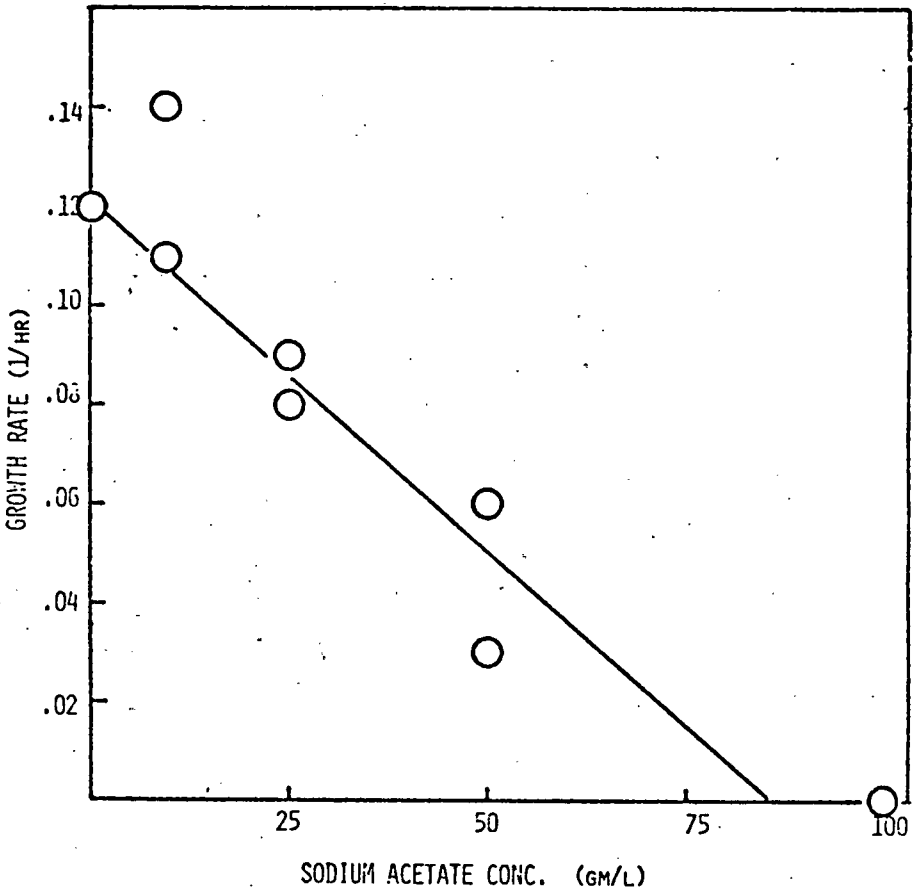
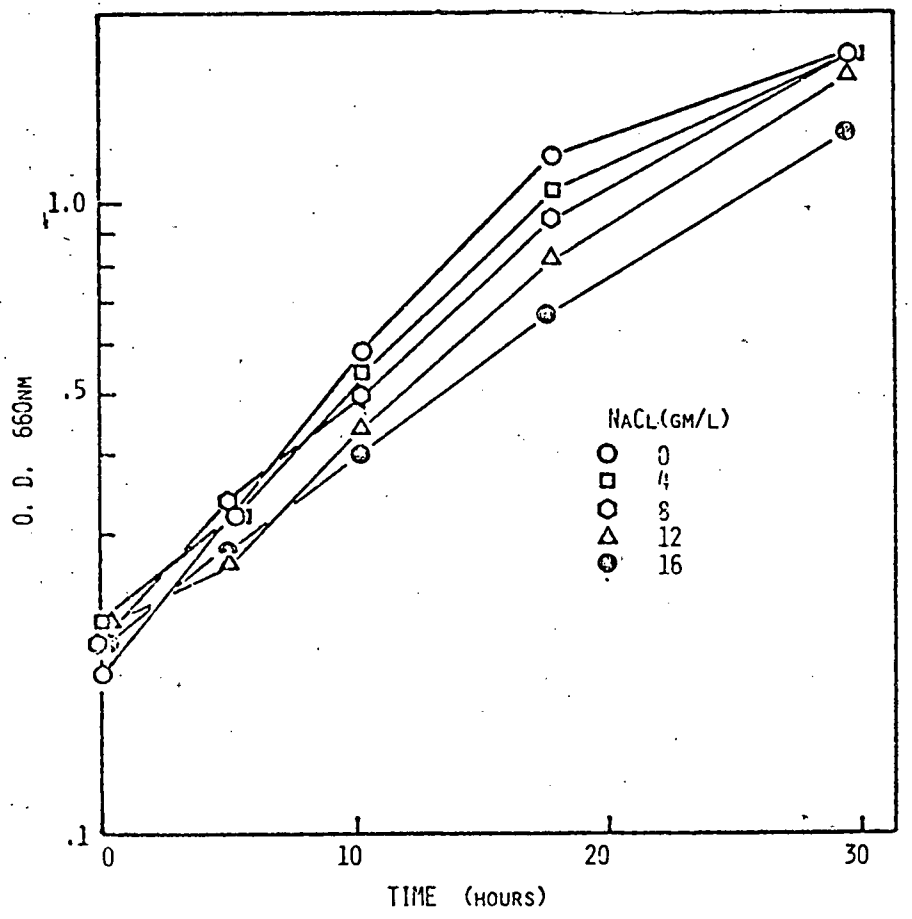


FIGURE II.C.5 - Relationship between sodium acetate concentration on specific growth rate



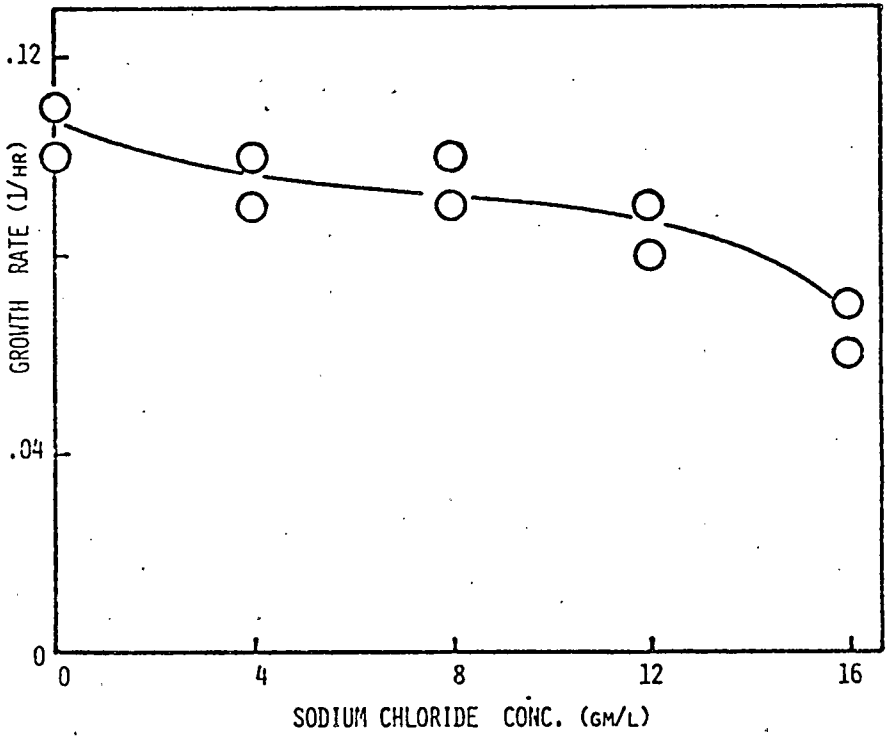
EFFECT OF SODIUM CHLORIDE ON CELL GROWTH

FIGURE II.C.6 -



Kinetics of growth of Clostridium thermoaceticum at different sodium chloride concentration

FIGURE II.C.7 - Relationship between sodium chloride concentration on specific growth rate



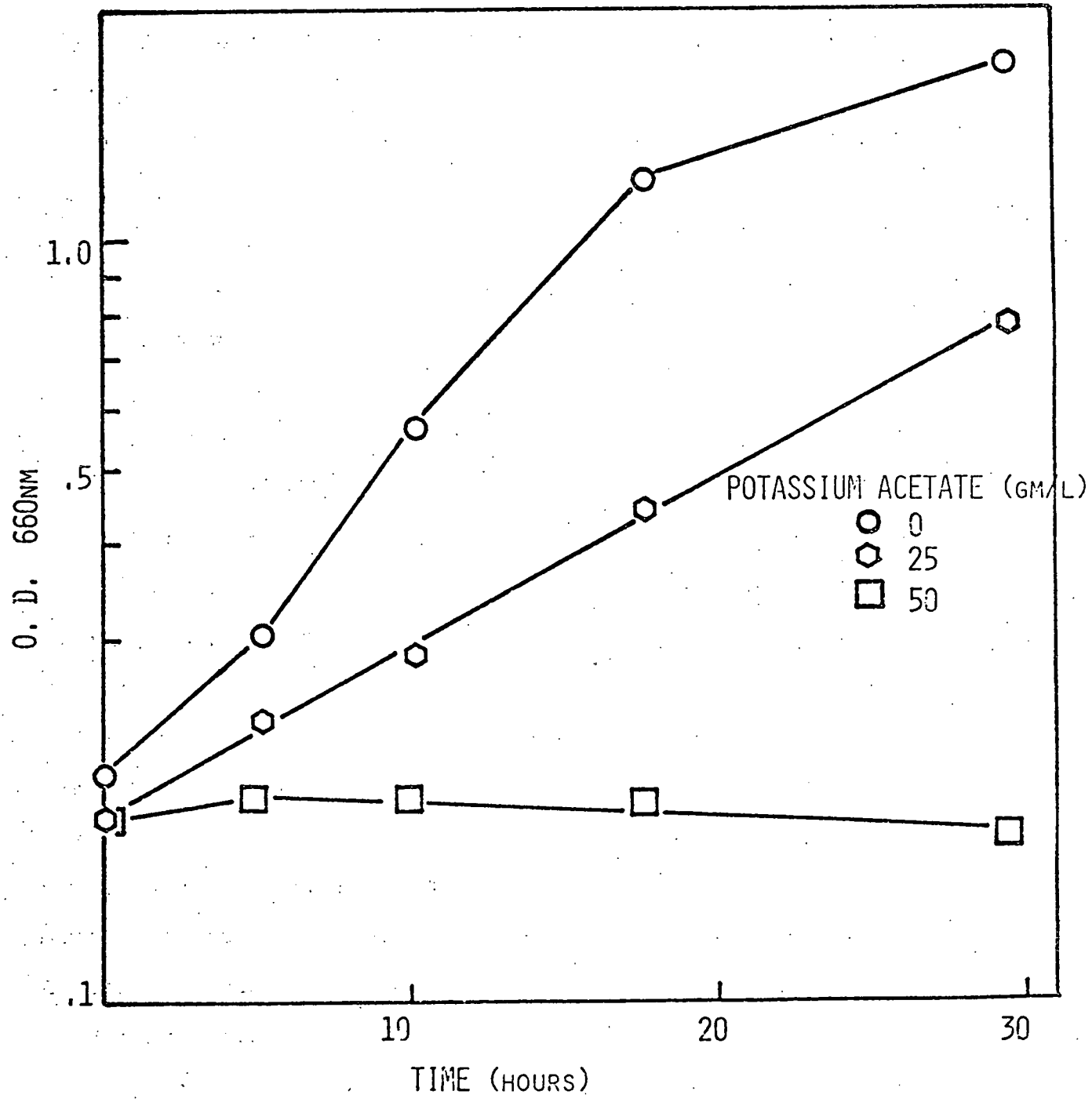
example, at a sodium acetate concentration of 50 gm/liter and sodium chloride at 16 gm/liter, the specific growth rate was reduced to about 0.05 hour^{-1} for both cases. However, at this reduced growth rate the sodium ion concentrations are respectively 0.54 and 0.27 molar. It therefore appears that the drastic reduction in the specific growth rate as shown in Figure II.C.7 is a result of the chloride ion. These results unfortunately still do not allow us to state whether the inhibition of growth (as shown in Figure II.C.5) is due to sodium or acetate.

To further elucidate the role of sodium and acetate, experiments were performed where potassium acetate was added from 0 to 50 gm/liter. These results are shown in Figure II.C.8. It can be seen that at 50 gm/liter of potassium acetate total growth inhibition was noted. Furthermore, a drastic reduction in the growth rate from 0.12 hour^{-1} at 0 gm/liter potassium acetate to 0.04 hour^{-1} at 25 gm/liter of potassium acetate was noted. These results show that potassium is more toxic than sodium in the growth of this organism. Again these results can not be used to state the exact role of sodium or acetate on growth inhibition. Studies are in progress where this question can be clearly answered.

Using this data to interpret the results obtained from the study of the effect of glucose concentration (Section A), we can observe from Figures II.C.1 - 3 that production of cell mass and acid stopped at acetic acid concentrations of about 10 and 12 g/l respectively. These values represent approximately 14 and 16.5 g/l sodium acetate and it is

EFFECT OF POTASSIUM ACETATE ON GROWTH

FIGURE II.C.8 - Kinetics of growth of Clostridium thermoaceticum at different potassium acetate concentration

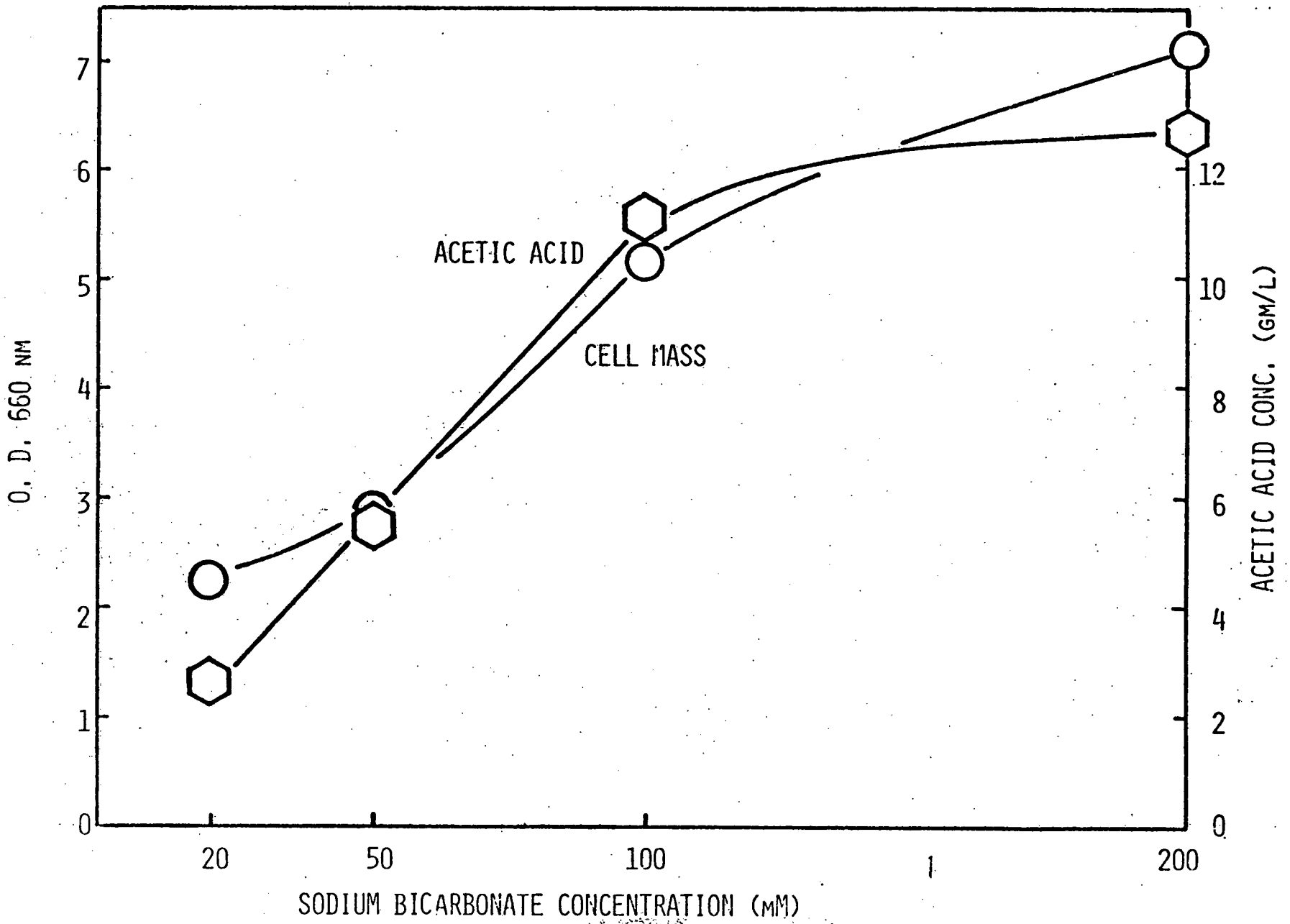


evident from Figure II.C.5 that this level of acetate would not account for the cessation of growth or production of acid (inhibition of growth at 25 g/l sodium acetate was only 33%).

c. Effect of buffer capacity on growth and acid production

It is conceivable that the results observed from the study on the effect of glucose concentration (Section 4) are the result of a nutrient limitation. In order to differentiate between a nutritional effect and a pH effect a series of culture tubes were prepared such that only the concentration sodium bicarbonate was varied. The effect of increasing the buffer strength is to maintain the pH within a range acceptable to the organism for a longer period of time. The results from this study are shown in Figure II.C.9. It should also be noted that the final pH in all of the culture tubes varied between 5.2 and 5.7. It can be shown in Figure II.C.9 that there is a direct relationship between the final cell mass and acetic acid concentration to the strength of the buffer. These results give very firm evidence that the hydrogen ion concentration plays a major role in regulating the growth and production of acetic acid by C. thermoaceticum.

FIGURE II.C.9 - Relationship of sodium bicarbonate buffer on growth and acetic acid production



d. Controlled pH Fermentation

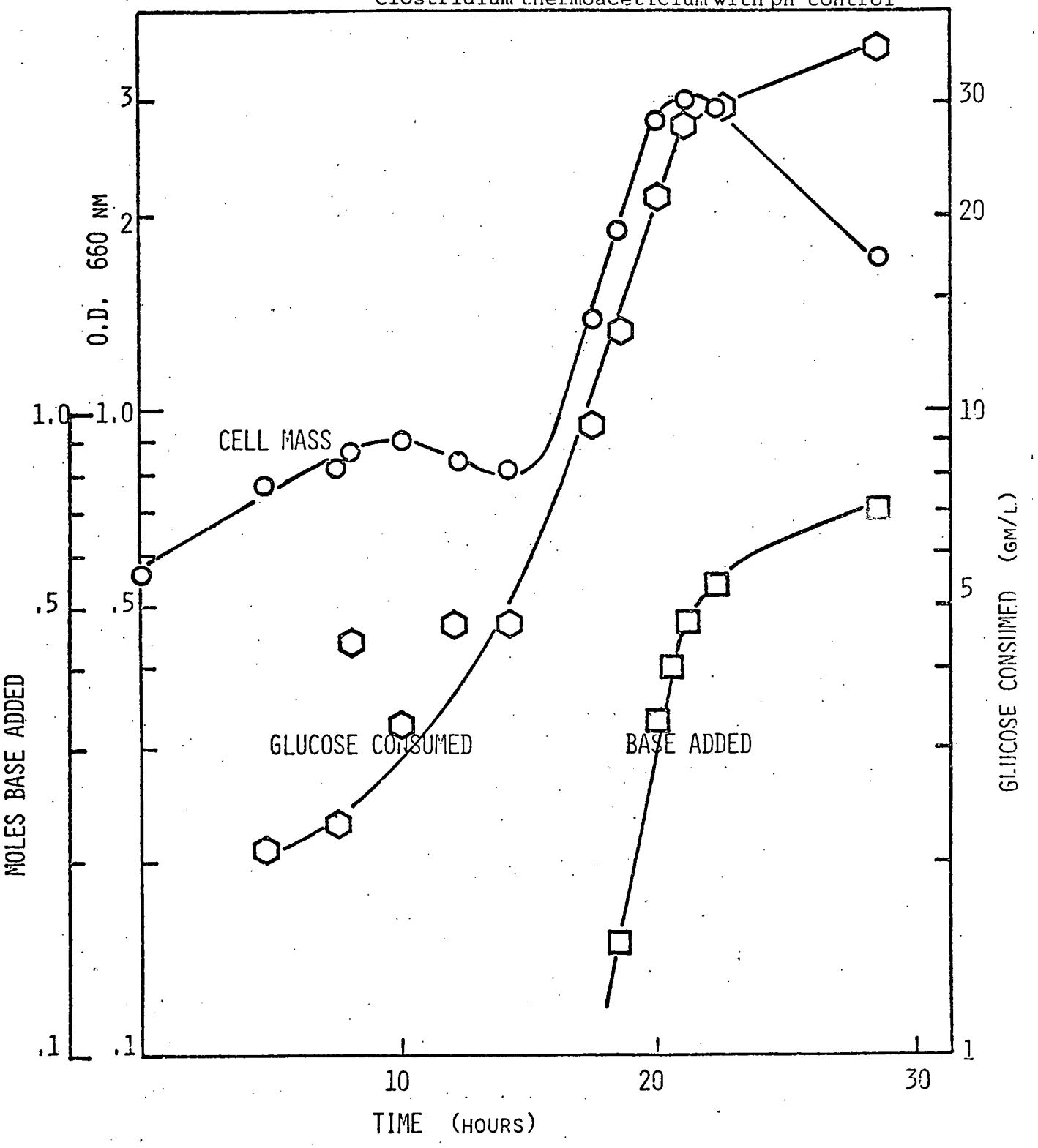
A number of fermentations using pH control in 5-liter agitated fermentors have been performed. These fermentations are generally performed using 3.5 liter working volume. Due to the increase in volume over tube cultures and shake flasks as well as continuous pH control, one is able to obtain in more quantitative fashion the kinetics of this fermentation using such a system. A typical time cause of this fermentation is shown in Figure II.C.10. The pH was controlled at a value of 7.0 using 10 N NaOH. The cell growth was shown at the start of the fermentation presumably because of the initial high glucose concentration (55 gm/liter). Following an adjustment period of about 10 hours, the organisms achieved its maximum specific growth rate of about 0.3 $hour^{-1}$. The glucose consumption appears to increase steadily even though the cells were not growing substantially in the first 10 hours of fermentation. However, as the maximum growth rate was attained, the glucose consumption paralleled that of growth.

Since the NaOH addition to maintain constant pH can be quantitatively measured, it offers an indirect method of estimating the acid production. Shown in Figure II.C.11 is a relationship between amount of NaOH added to the glucose consumed. From this plot one is able to calculate the yield of acetic acid from glucose. A value of 0.83 gm acetic acid per gm of glucose consumed was found.

From the kinetic data in Figure II.C.10, it is also pos-

FERMENTATION AT CONSTANT pH, 7.0

FIGURE II.C.10 - Fermentation profile in 5-liter fermentor of Clostridium thermoaceticium with pH control



sible to calculate the specific rate of glucose consumption. One can assume that this specific consumption rate is directly proportional to the specific acetic acid production rate. When the specific rates are compared as shown in Figure II.C. 12, a very interesting and encouraging phenomenon is observed. The results in this figure show that the production of acetic acid occurs through both growth association and non-growth association. This observation had been stated in our first quarterly report (12/1/76-2/28/77). However, the results in Figure II.C.12 are more quantitative. For example, the results show that at zero growth rate, the specific rate of product formation is quite substantial (about 1/3 of maximum). These results are encouraging since the possibility now exist where non-growing cells might be useable for the production of this chemical.

4. FUTURE STUDIES

The future studies in the production of acetic acid include:

- Increase the tolerance of the organism to H^+ ion through mutation and selection
- Further assessment on the inhibitory effect of acetate salt on growth
- Continuous culture studies using cell recycle to increase productivity
- Examination of immobilized whole cells or resting cells for acetic acid production

FIGURE II.C.11 - Relationship between amount of base added to glucose consumed

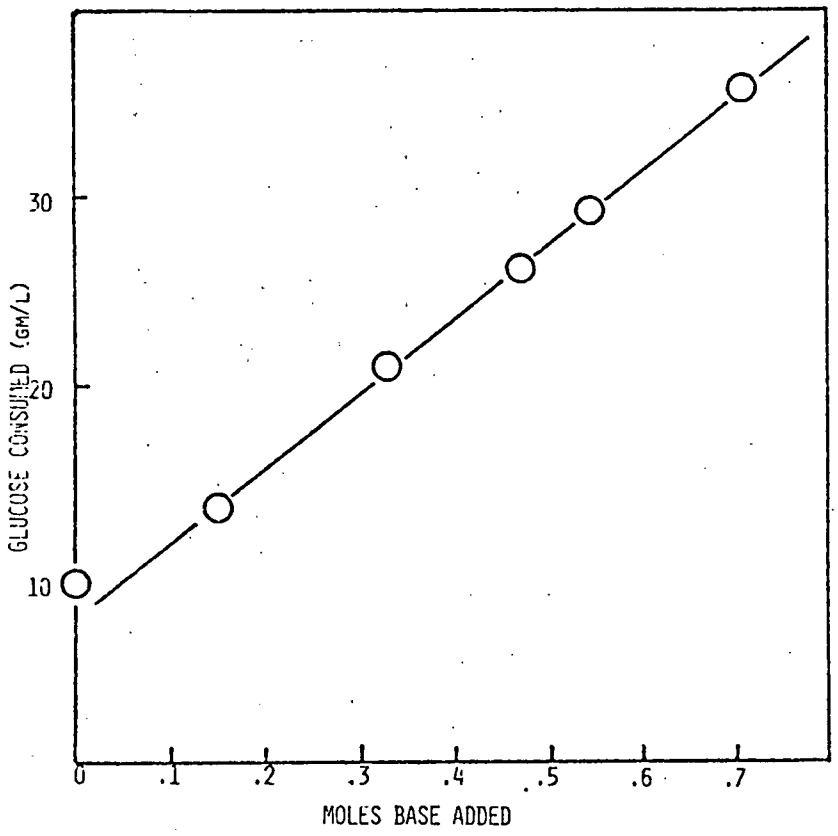
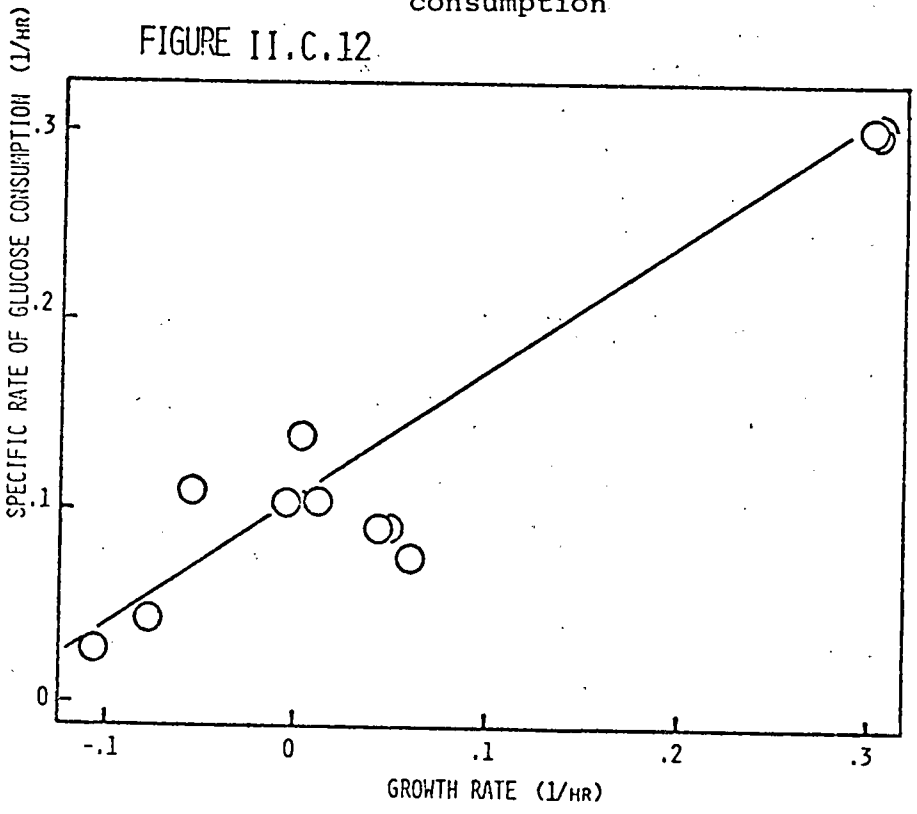


FIGURE II.C.12 Relationship between specific growth rate to specific sugar consumption



- Examination of the use of soluble sugars from cellulose degradation for acetic acid production
- Complete the economic analysis for acetic acid production by Clostridium thermoaceticum.

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