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

Progress Report

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Daniel I.C. Wang, Principal Investigator
Charles L. Cooney, Co-Principal Investigator
Arnold L. Demain, Co-Principal Investigator
Reinaldo F. Gomez, Co-Principal Investigator
Anthony J. Sinskey, Co-Principal Investigator

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Department of Nutrition and Food Science
M.I.T.
Cambridge, MA 02139

DISCLAIMER

Scope of Program

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

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

ABSTRACT

The microbial degradation of cellulosic biomass has focused on the use of a thermophilic (55-60°C), anaerobic microorganism, Clostridium thermocellum. When this organism is grown with a crystalline cellulose, the cellulases produced are mainly extracellular. This same organism when grown on solka floc, high specific growth rates are exhibited as well as the ability to produce high concentrations of soluble reducing sugars. The rate of soluble sugar production appears to be growth associated. Studies on acrylic acid production are focused on two organisms: Peptostreptococcus elsdenii and Clostridium propionicum. An economic analysis on the acetone/butanol fermentation has been completed. The results show that continuous operation can reduce significantly the production cost compared to batch operation with the cost of raw material being major fractions for both processes. An increase in solvent concentration will effect substantial cost reduction. The production of acetic acid by Clostridium thermoaceticum has been shown to occur rapidly by this organism. Acetic acid concentration between 15 to 20 gm/liter have been achieved, corresponding to 86% of the theoretical maximum yield.

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.

Dr. Charles L. Cooney
Associate Professor of Biochemical Engineering

Dr. Arnold L. Demain
Professor of Industrial Microbiology

Dr. Reinaldo F. Gomez
Assistant Professor of Food Microbiology

Dr. Anthony J. Sinskey
Professor of Applied Microbiology

Dr. Daniel I.C. Wang
Professor of Biochemical Engineering

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

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A.) Enzyme Production by Clostridium thermocellum

1.) Introduction

An anaerobic, thermophilic bacterium, Clostridium thermocellum, has been chosen for the production of the cellulohydrolytic enzymes necessary to convert cellulosic biomass to fermentable sugars. In the initial phase of our program, we have sought to evaluate the fermentation kinetics of C. thermocellum grown on insoluble cellulosic substrates. Results from batch fermentations of the organism on a commercially available cellulosic powder are reported. Acquisition of batch data has been an important first step toward: 1) determining the cellulolytic potential of this organism, 2) elucidating the bottlenecks in enzyme production to be overcome by environmental or genetic means, and 3) providing us with an initial source of enzymes to be used in assay development and hydrolysis studies.

Thus far, enzyme activities found in broth supernatants are not comparable to those reported for fungal systems. Nevertheless, the short fermentation times and the rapid in vivo rate of cellulose degradation by C. thermocellum suggest the presence of "tappable" cellulolytic capabilities that will compare well with more fully developed systems. Consequently, some preliminary experiments aimed at localization of the enzyme(s) in the presence of insoluble cells and residual cellulose have been performed. Other investigators have proposed that cellulase systems particularly bacterial ones are cell-bound and

that the presence of cellulase activity in solution is the result of autolysis (Hofsten, 1975). The fact that cellulase enzymes bind tightly to their substrates is also widely reported. Thus, in order to assess the true "extracellularity" of the cellulases of C. thermocellum, several experiments have been conducted involving extraction of additional enzyme activity from broth pellet fractions. Some preliminary adsorption experiments with cellulase have been performed as well. The results are presented herein.

2. Materials and Methods

Organism: Clostridium thermocellum obtained from the American Type Culture Collection

Growth of the Organism: C. thermocellum was grown in CM 3 medium. Its composition is as follows: KH_2PO_4, 0.15%; K_2HPO_4, 0.29%; $(NH_4)_2SO_4$, 0.13%; $MgCl_2$, 0.10%; $CaCl_2$, 0.15%; $FeSO_4$ (5% solution), 25 µl/l; Resazurin (0.2% solution), 1 ml/l; Yeast Extract, 0.2%; MN 300 cellulose (TLC grade, obtained from Brinkmann Instruments), 0.9%; cysteine hydrochloride, 0.1%. Medium pH was adjusted to 7.6 before autoclaving with NaOH, 4N. Medium was reduced by gassing with prepurified N_2 or CO_2.

Tube Culture: Stock cultures are grown in Hungate anaerobic tubes containing 10 ml of CM 3 medium containing 1 ml inoculum at 60°C until cellulose is visibly degraded and turbidity appears. They are then refrigerated at 4°C and can be stored for up to 5 weeks. Transfers are made every three weeks.

Flask Culture: 500 ml Erlenmeyer flasks converted for anaerobic use are used to grow up inoculum cultures (from the stock tubes) for the fermentor (250 ml - 300 ml cultures grown for 72 hr. on CM 3 medium are employed). Shaking is not necessary.

Fermentor cultures: A 2 liter fermentor with a working volume of 1.5 liters is employed. Agitation is set at 100 rpm. Anaerobiosis is maintained by sparging N_2 or CO_2 during the fermentation. The CM 3 medium is autoclaved for 35 minutes in the fermentor.

Growth Measurement: A procedure for the determination of pellet protein has been developed and is used to follow growth of the organism: 2 ml of whole broth sample is centrifuged at 20,000 xg for 15 minutes. The supernatant is removed. The pellet is resuspended in 2 ml or distilled water and is again centrifuged and supernatant is removed. This distilled water wash is performed once more followed by centrifugation and supernatant removal. The pellet is then resuspended in 2 ml of 0.2 N NaOH and boiled for 15 minutes. The suspension is centrifuged for a final time after which the Lowry assay for protein is performed on the supernatant to determine solubilized protein.

Protein Determination: All protein measurements were performed according to the Lowry method using bovine serum albumin as a standard.

Determination of Residual Cellulose: 5 ml of whole broth samples were centrifuged at 20,000 xg for 15 minutes. Supernatants were

withdrawn with a Pasteur pipette. The pellet was resuspended in 5 ml of 8% formic acid to effect cell lysis. Then the residual solids were filtered through preweighed metrical GA-6 Nucleopore filters (diameter = 25 mm, pore size = 0.45 µm) and were allowed to dry overnight at 75°C. The dried filter plus cellulose was weighed and the residual cellulose was determined as the difference.

Reducing Sugars Assay: Broth reducing sugars were determined on supernatant fractions using a colorometric dinitrosalysilic acid reagent as described by Miller (1959). 0.2 ml of supernatant was added to 1.0 ml of sodium citrate buffer (0.05 M, pH 4.8) followed by addition of DNS reagent. Glucose solutions served as standards.

Measurement of Cellulolytic Activity: CMC assay: Whole broth is centrifuged as described above. 0.2 ml of supernatant is added to duplicate tubes containing 1 ml of a 2% carboxymethyl-cellulose (sigma) solution dissolved in 0.05 M sodium citrate buffer pH 4.8. 0.2 ml of supernatant is added to a third tube containing 1 ml of the citrate buffer as a background control. Incubation is allowed to take place for 1 hour at 60°C after which time the DNS reagent is added as mentioned before and reducing sugars are measured. Controls containing just the CMC solutions are used as standards.

Filter Paper Assay: This assay has been described in detail by Mandelss et al (1975).

Treatments to Liberate Additional "Cellulase" from Pellet Fractions of Fermentation Broths into Free Solution: The following procedures were performed on broths from the same fermentation. Sonication: A measured volume of whole broth was centrifuged at 20,000 xg for 15 minutes. The supernatant was removed and saved for the CMC and filter paper assays. The pellet was resuspended in 2.0 ml of distilled water before sonication in a Branson sonifier. Sonication times were one minute and five minutes, followed by microscopic examination. The sonified suspension was centrifuged and the supernatant was measured for CMC and FP activities.

Detergent Treatments: Measured volumes of whole broth were centrifuged as before. The pellets were resuspended in Triton X-100 solutions of varying concentrations. These suspensions were swirled for 10 minutes at 60°C. They were then centrifuged and the supernatants were assayed for enzyme activity as before.

Lysozyme Treatment: A measured volume of whole broth was centrifuged as before. The pellet was resuspended in 0.03M tris buffer pH 8 containing a 20% sucrose solution. EDTA 0.2M, pH 7 was added to a concentration of 10 mM. 13 mg of lysozyme (Sigma Co.) was added as well. The total volume was 20 ml. The mixture was incubated for 20 min. at 37°C, spun down and supernatant was assayed for enzyme activity. The pellet from this mixture was again suspended in the tris-sucrose solution (to a volume of 4 ml.) and dialyzed against 0.05 M citrate buffer for 30 minutes with vigorous mixing. After dialysis, citrate

buffer was added to make a total volume of 20 ml. Again the suspension was spun down and the supernatant was analyzed for enzyme activity.

Triton Extraction Performed on C. thermocellum Cells (unlike the above experiments in which whole pellets had been used):

C. thermocellum was grown in flasks with CM 3 medium in which MN 300 was replaced by Solka floc. This was used since Solka floc is a coarser substrate and cells can be separated from cellulose by simple filtration. The cell filtrates were centrifuged (20,000 xg, 30 minutes) and the cell pellets were resuspended in a 2% Triton X 100 solution (in sodium citrate buffer 0.05 M, pH 4.8). These suspensions were incubated at 60°C with swirling for 10 minutes. They were then centrifuged and supernatants were assayed for CMC ase activity. Part of the original filtrate had been saved for dry cell weight determination. Also the extraction w/Triton was performed on the entire pellet fraction.

Adsorption Experiments (Adsorption of enzyme in solution onto added Cellulose): All adsorption experiments reported were performed with the supernatant fraction of the same fermentation broth (C. thermocellum grown on MN 300). Adsorption onto MN 300 cellulase: MN 300 was added to 5 ml of supernatant from a batch fermentor culture) in centrifuge tubes to make final concentrations of 0.2%, 0.5%, 1.0% and 1.5%. Tubes were allowed to incubate at 60°C for 10, 20, 30, 40 and 50 minutes with 3 second vortexing every five minutes. The tubes were centrifuged (20,000 xg, 15') and supernatants were assayed for CMC-ase activity. Tubes of supernatant containing no added MN 300 were also incubated for the times stated and serve as references.

Adsorption onto Solka Floc Cellulose: This experiment was performed in a similar manner to that just described for MN 300.

Extraction of Adsorbed Enzyme: In the MN 300 adsorption experiment, a fermentation supernatant had been mixed and incubated with added cellulose. These mixtures were centrifuged and the supernatants removed for the CMC ase assay. The pellets were also saved. To these pellets, (cellulose and adsorbed enzyme) 5 ml of a 2% Triton x 100 solution was added. The suspensions were incubated at 60°C for 10 minutes with swirling. They were then centrifuged and the CMC assay was performed on the supernatants.

3. Results and Discussion

Visual characteristics of the cultures of C. thermocellum are similar to those reported in the literature for other strains of the organism. As the cellulose first becomes utilized it takes on a more "fluffy" appearance. As the fermentation proceeds, a yellow pigment bound tightly to undegraded cellulose appears. The broth eventually becomes turbid from cell growth.

It will be noted that shaking does not seem to be necessary for the flask cultures. In fact,high agitation may actually be detrimental to the culture. Such an adverse effect was observed with fermentations run with an agitation speed of 200 rpm. In a 2 liter fermentor cells grow poorly (pellet protein values were low compared to cultures agitated at 100 rpm). Microscopic examination of cells and cellulose from cultures

agitated at different speeds revealed a marked difference. More cells appeared to be situated on or in the cellulose fibers from cultures grown at lower agitation speeds. For this reason, 100 rpm is routinely used as the agitation speed.

The Figures 1 and 2 from Run 8 depict the behavior of a typical fermentations of C. thermocellum on MN 300 cellulose. Pellet protein (µg/l), residual cellulose (mg/ml), pH, broth reducing sugars (mg/ml) and CMC ase activity (mg of reducing sugar equivalents released per milliliter of added broth supernatant per hour) are plotted as a function of time.

Slightly higher final CMC ase concentrations are found in Run 8 then is some other experiments. This fermentation has lower broth reducing sugars at the end of the fermentation [Run 8 final CMC ase: @ 4 mg/ml-hr; RS: < 0.1 mg/ml whereas Run 9 final CMC ase: @ 3 mg/ml-hr; RS: @ 0.3 mg/ml]. It is tempting to speculate that the higher broth reducing sugars found in Run 9 may be inhibiting the enzyme and thus less activity is measured.

It should be mentioned at this point that the profile of reducing sugars vs. time appears to be quite different when the organism has been cultured on Solka floc. In these flask fermentations (described elsewhere) reducing sugars accumulate throughout the course of the fermentation to significantly higher values than the maximum reached in Run 8. The presence of broth reducing sugars (in either low or high concentrations) in our fermentations conflicts with findings of investigators at the U. of Wisconsin working with C. thermocellum LQ 8. They report that no cellodextrins or glucose are detected during

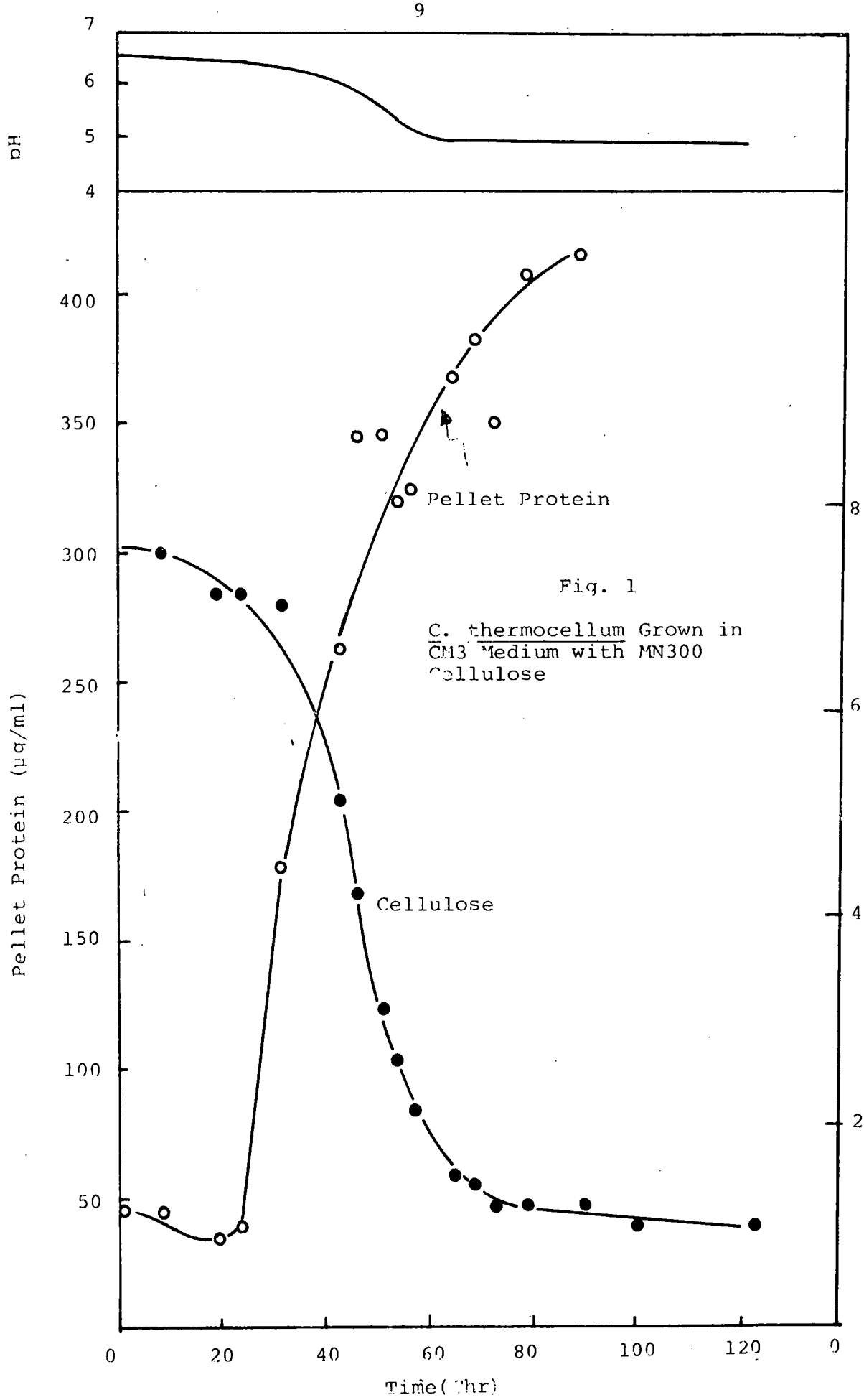
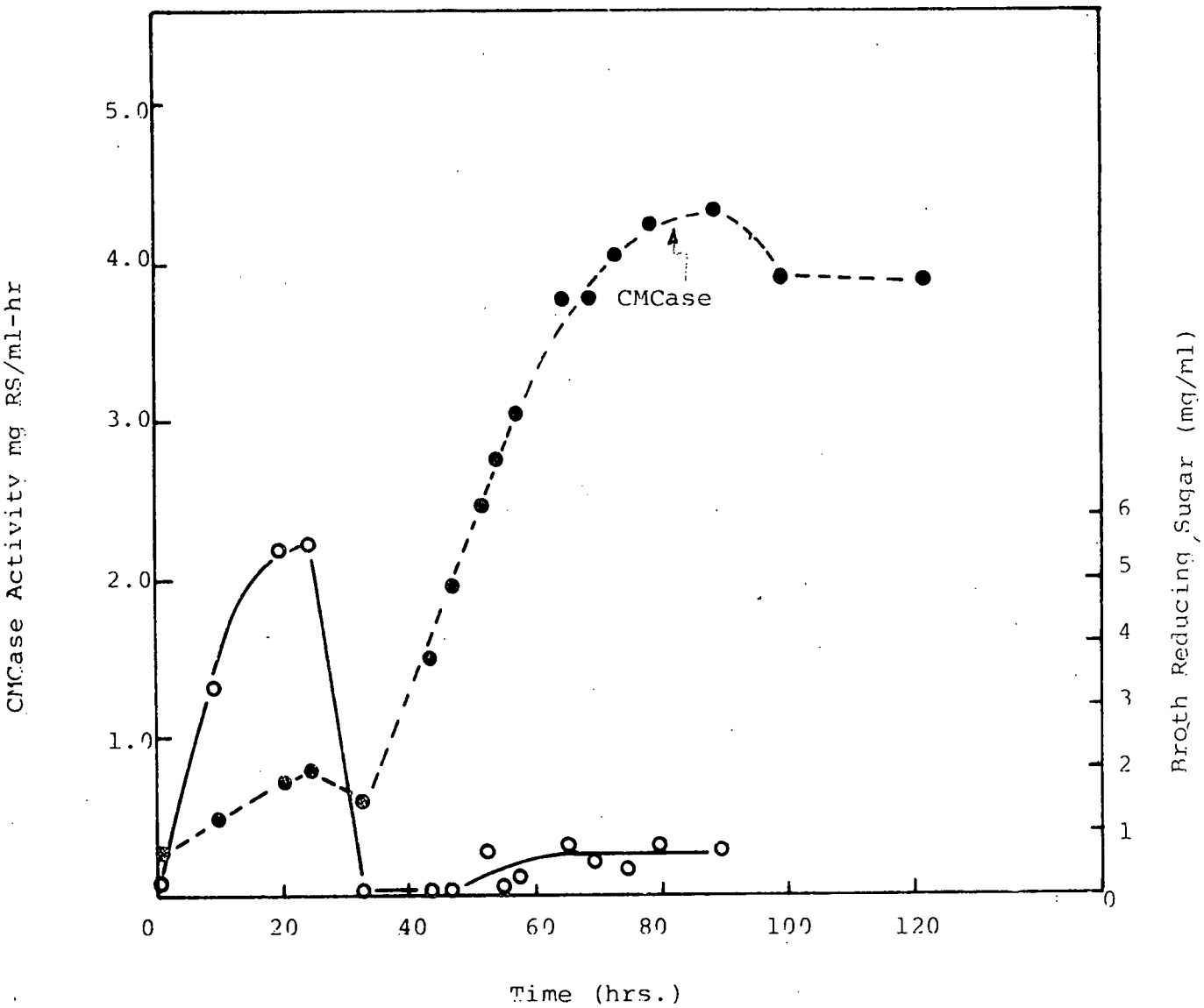


Fig. 1

C. thermocellum Grown in CM3 Medium with MN300 Cellulose

Figure 2: CMCase Activity and Broth Reducing Sugar During Growth of C. thermocellum on Cellulose



growth of their organism (unpublished data) on the same CM 3 medium.

In both runs the following behavioral characteristics are observed. Increase in pellet protein follows a drop in broth reducing sugars. The drastic drop in residual cellulose of Run 8 seems to occur slightly after the initiation of the increase in pellet protein. This may be due to growth of C. thermocellum on yeast extract and/or the initial reducing sugars formed. In Run 8, the amount of broth reducing sugars at 25 hrs. corresponds well with the amount of cellulose degraded. In bour runs, a drop in pH accompanies cellulose degradation and pellet protein formation.

When pellet protein is converted into dry cell weight assuming a cellular protein concentration of 40%, the maximum cell densities obtained are around 1 g/l and the estimated cell yield (g DCW/g cellulose) is around 0.15. This value is reasonable for anaerobes but it is subject to error due to the presence of yeast extract in the medium and also because the "pellet protein" will measure any solids-bound protein.

Figures 3 and 4 depicting the rate of cellulose degradation versus time for Runs 8 and 9. Though the time frame is slightly shifted the behavior is similar. Figures 3 and 4 indicate nearly identical maximal rates of cellulose disappearance (dc/dt). It is interesting to note that the maximal rates (0.2-0.25 g/l. hr) of C. thermocellum grown on the MN 300 are quite comparable with those obtained when the organism is cultured on Solka floc.

Filter paper activity tests have been performed on supernatant of centrifuged broth samples. Very little filter paper

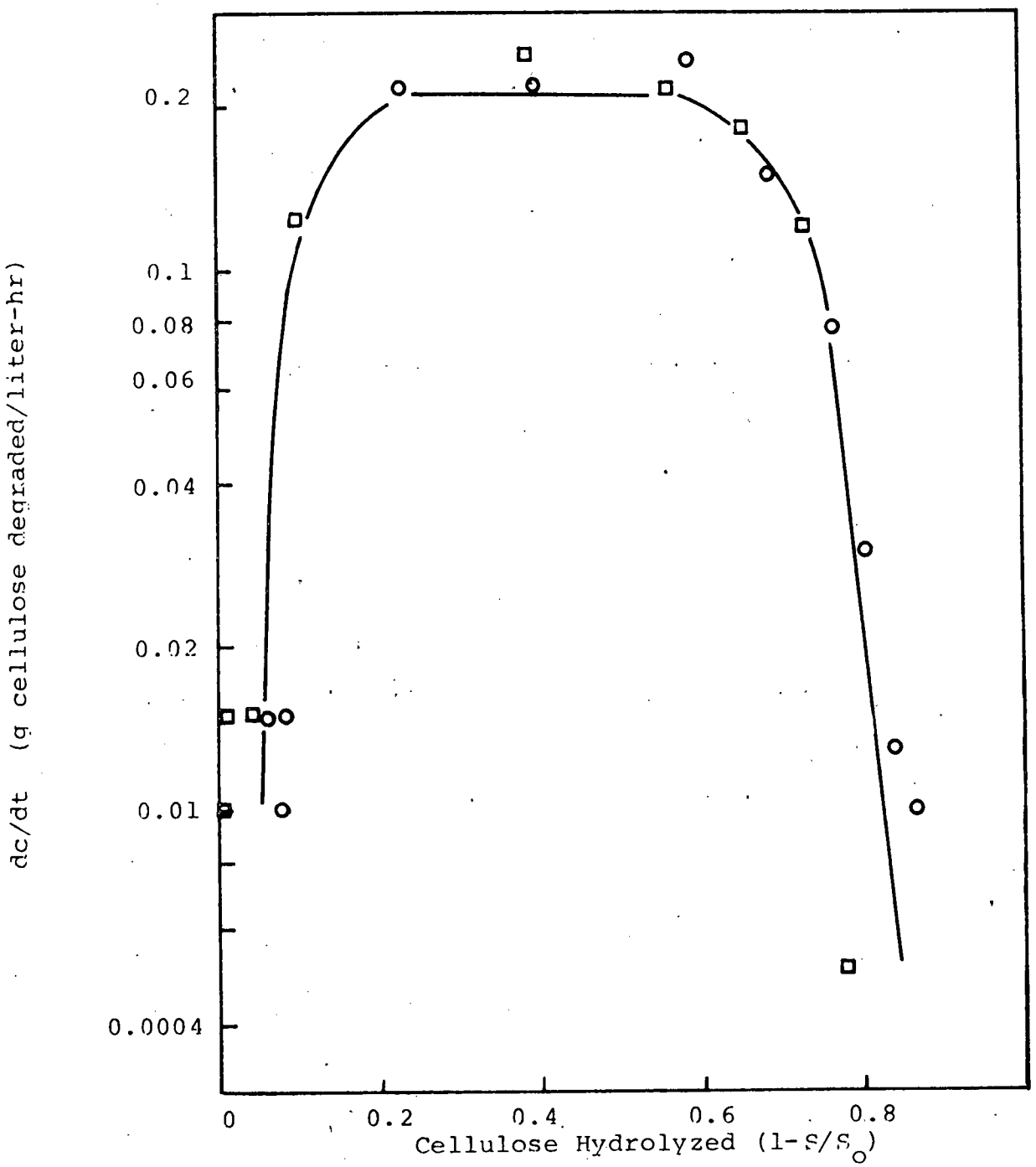


Figure 3: Rate of Cellulose Hydrolysis Versus Fraction of Cellulose Hydrolyzed for C. thermocellum on MN300

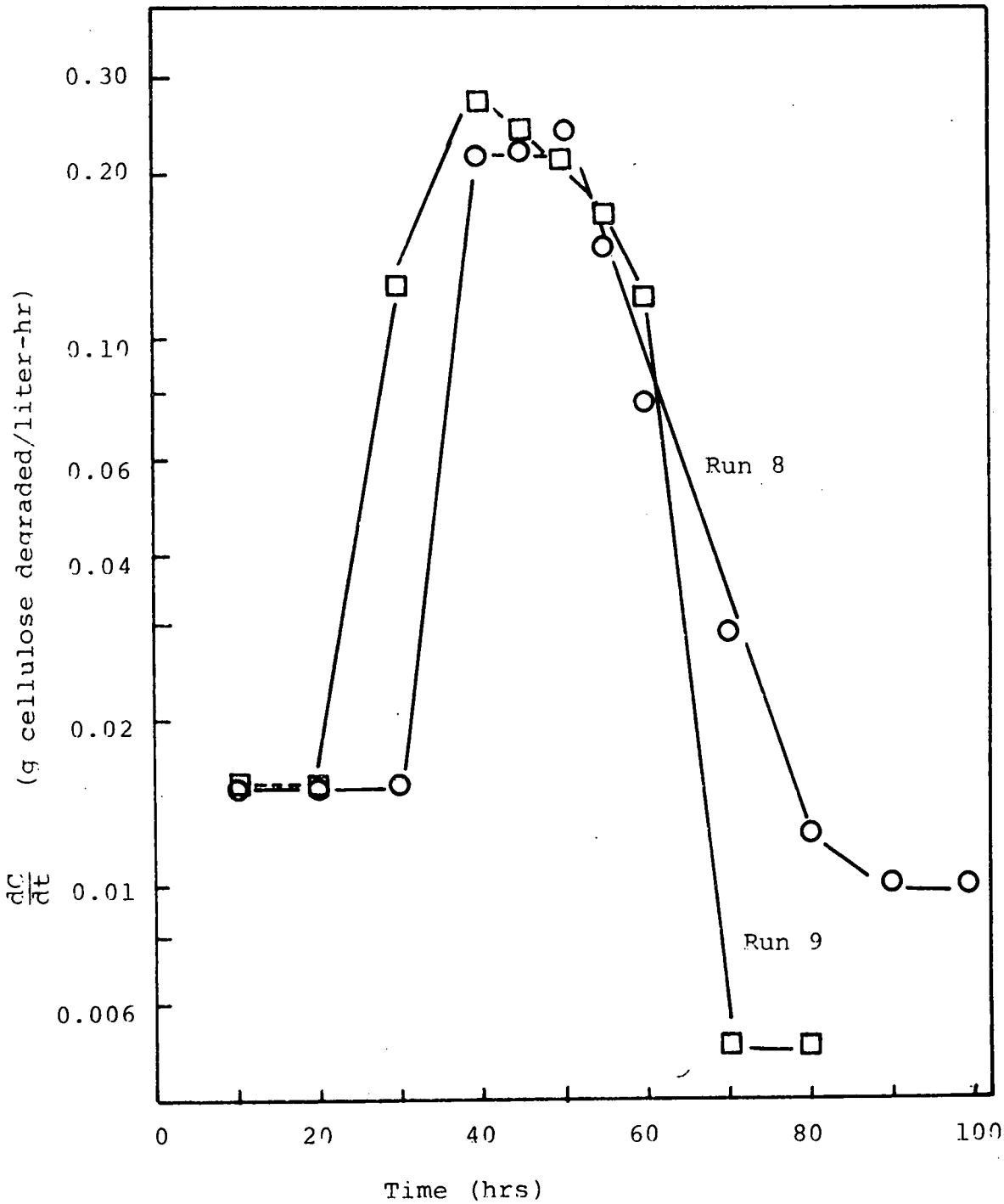


Figure 4: Cellulose Degradation by C. thermocellum as a Function of Fermentation Time

hydrolysis activity is observer (< 0.1 mg RS/ml-hr) indicating that C. thermocellum broths have very low exoglucanase activity.

The next question addressed was whether or not there is any additional enzyme activity associated with the residual broth solids, that can be solubilized.

Results of various pellet treatments are summarized in Table 1. Sonication (for 1 & 5 minutes) is marginally effective. Lysozyme is more effective but the Triton X 100 treatments appear to extract the greatest amount of pellet-bound enzyme. Increasing triton concentrations resulted in increased solubilized CMC ase activity. Filter paper activity was measured for the 68 hour sample but again these values were negligible.

Results of the initial adsorption experiment and subsequent desorption are presented in Figures 5 and 6. As suggested by the drop in measureable activity against CMC, soluble enzyme adsorbed to the freshly added cellulose. This lost enzyme activity could be retrieved with 2% Triton treatment. It appears that the longer the originally soluble enzyme is allowed to incubate with cellulose MN 300, the more difficult it is to completely re-extract it.

Adsorption studies for time periods longer that 10 and 20 minutes were carried out with soluble enzyme (from an MN 300 grown culture) on added MN 300 or Solka floc. The final set of figures (7 and 8) show : 1. units bound (calculated as the difference between the measureable enzyme activity before and after adsorption) per gram of cellulose (either MN 300 or solka floc) vs. time for adsorption to occur and 2. isotherms: units bound/concentration of MN 300 versus concentration.

Table 1

CMC ase Activity Solubilizable from Pellet Fraction of Fermentation Broth from RUN 9 (C. thermocellum Grown on CM 3 Medium with 0.9% MN 300 Cellulose). Pellets were Subjected to Sonication, Resuspension in Triton X100 and Resuspension and Incubation with Lysozyme. Filter paper activities were also assayed for 68 hr. sample but these were negligible.

Age of fermentation broth used (hrs)	Treatment	CMC ase Activity Solubolizable from Pellet RS mg/ml-hr=units	units pellet activity / units supernatant activity
68	Sonication 1 min.	0.16	0.05
	5 min.	0.21	0.07
	Triton X100		
	0.5%	0.77	0.24
	2.0%	1.13	0.35
92	Citrate Buffer Wash	0.53	0.18
	Triton X 100		
	0.083%	0.70	0.23
	0.42%	0.90	0.30
	0.83%	0.94	0.31
	1.6%	1.0	0.33
	Lysozyme	0.62	0.21
	Lysozyme, dialyzed	0.14	0.04

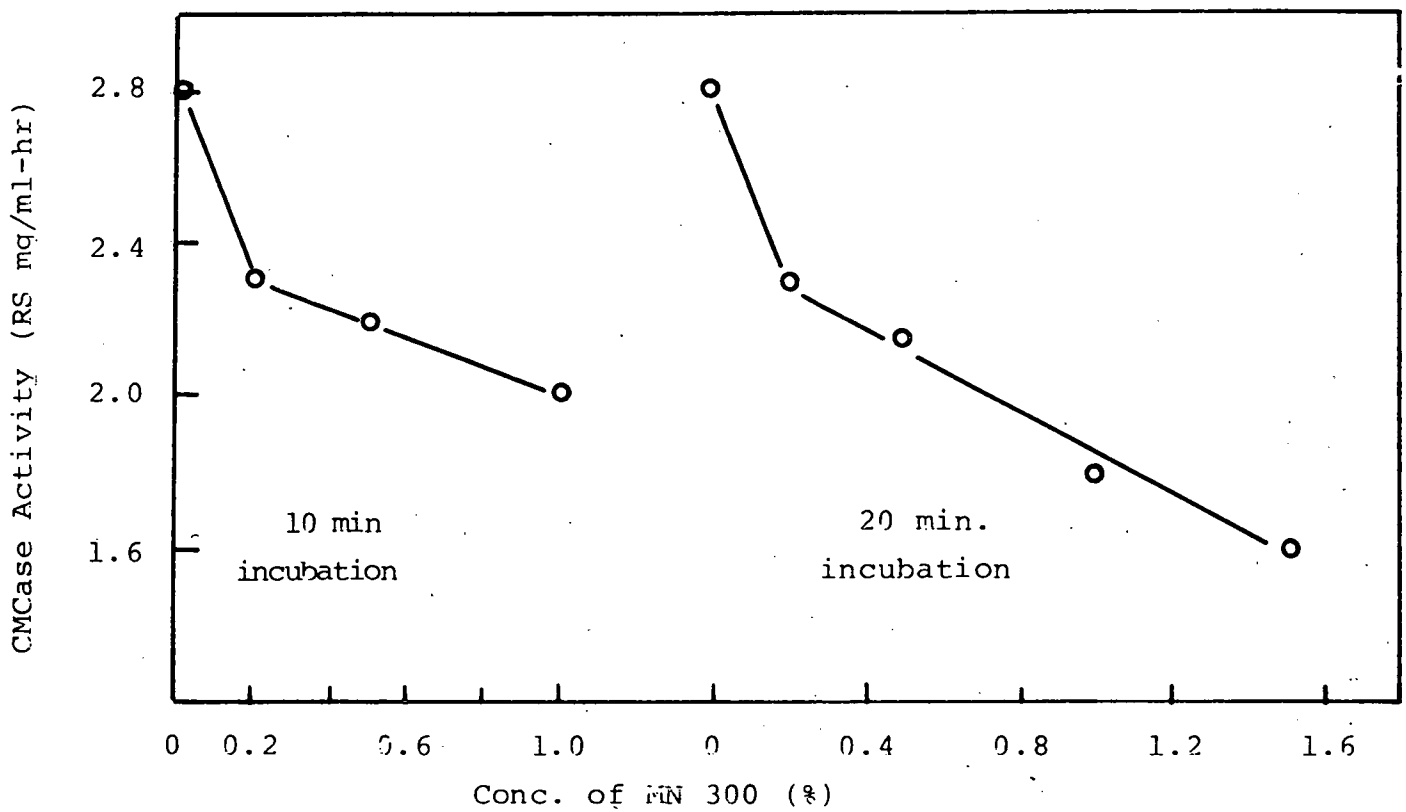


Figure 5: Adsorption of Cellulase onto MN300

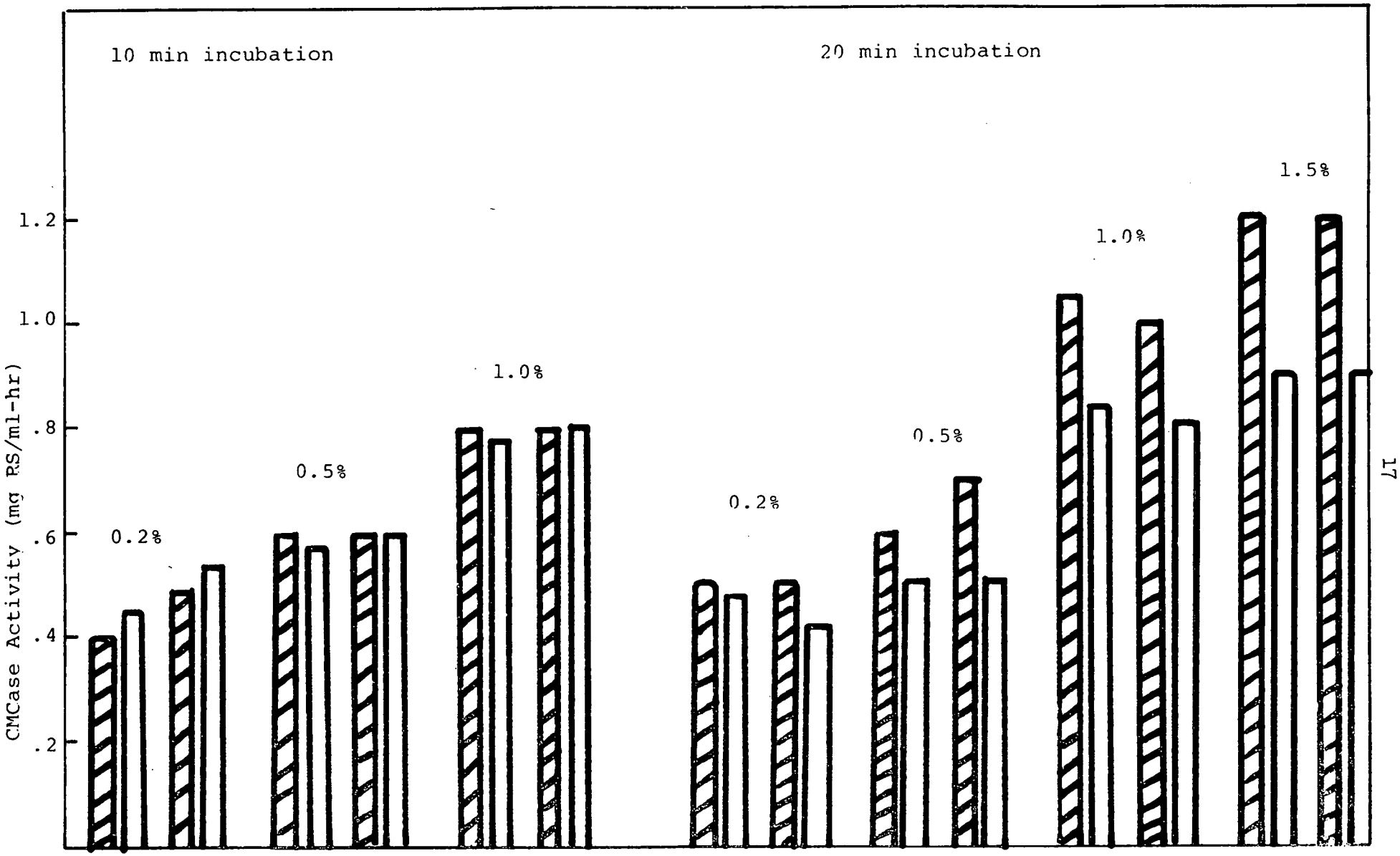


Figure 6: Adsorption and Recovery of Cellulase from MN300 Crosshatch is Activity Added and Clear Bar is Activity Recovered. Concentration of MN300 is Given top of Bar. Each Concentration was Run in Duplicate

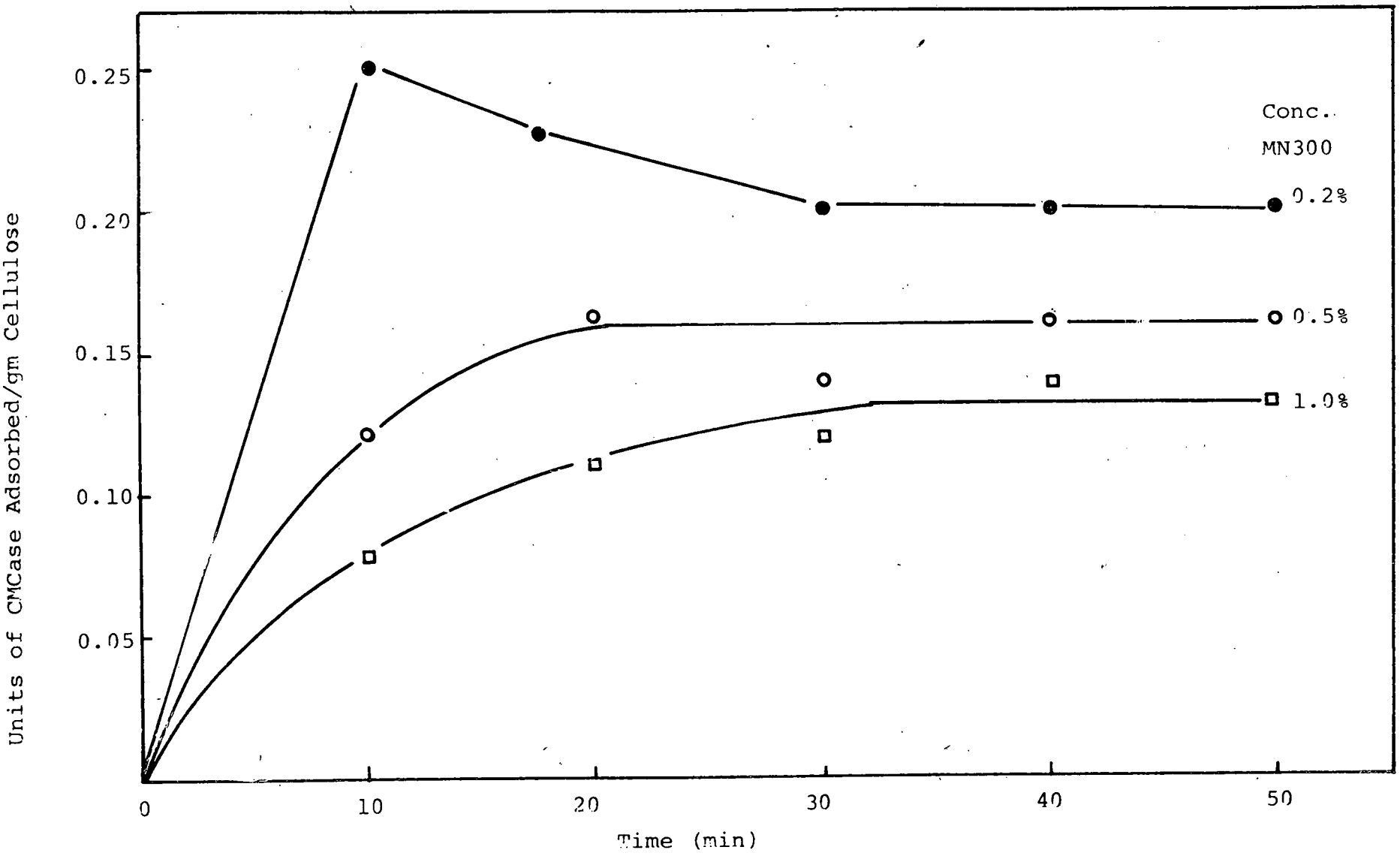
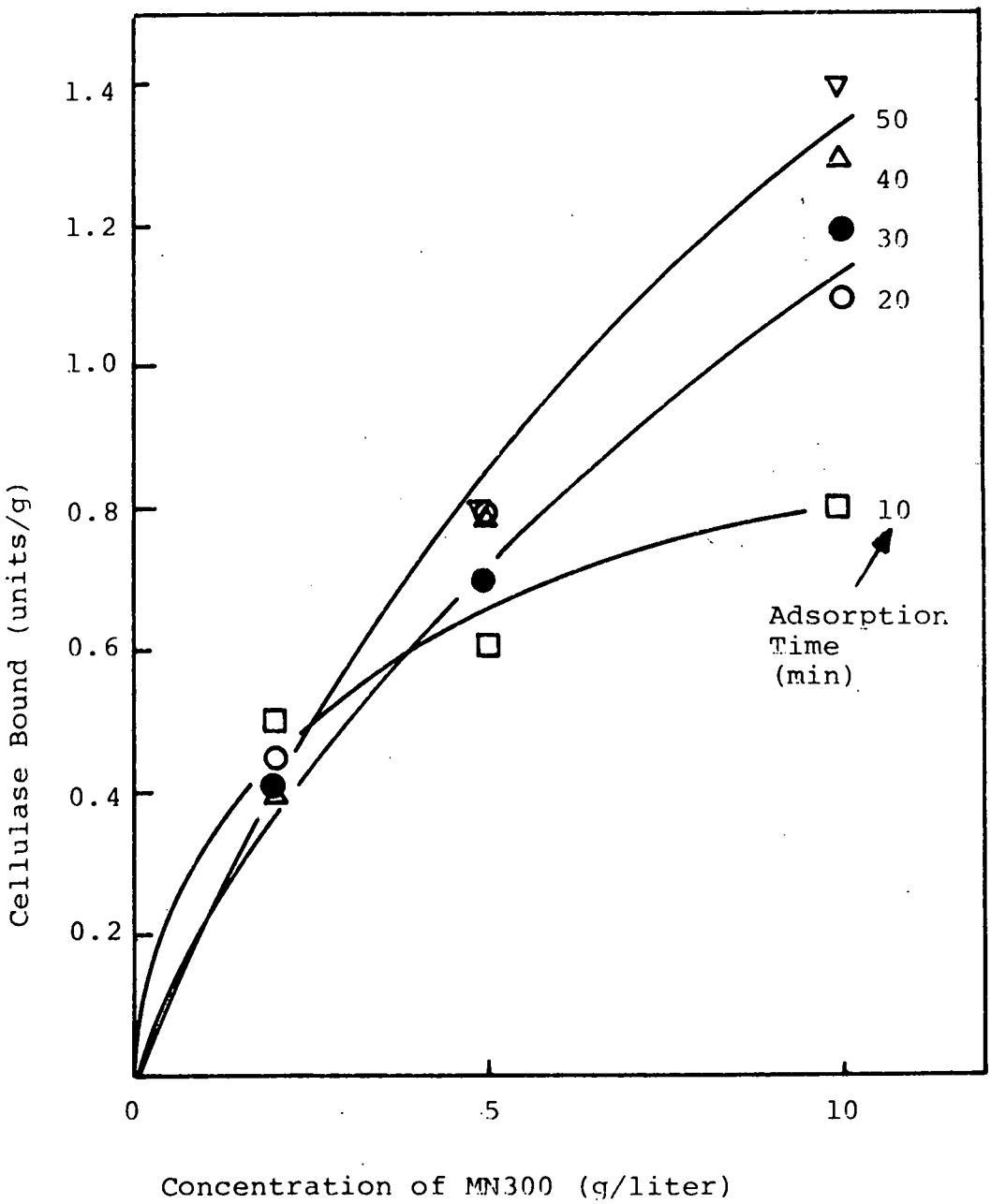


Figure 7a: Kinetics of Cellulase Adsorption onto MN300

Figure 7b: Adsorption Isotherm for Cellulase onto MN300 Cellulose



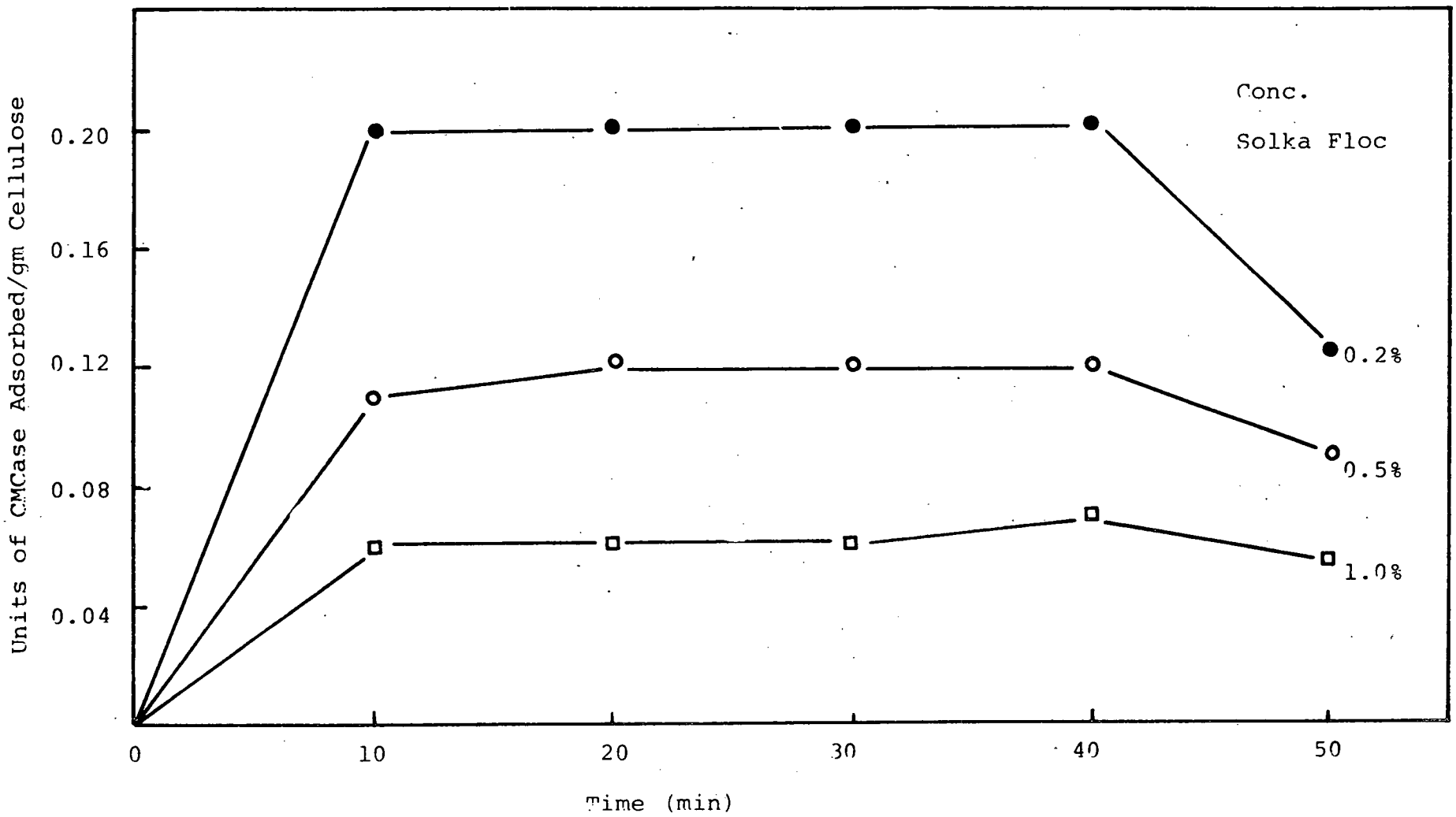
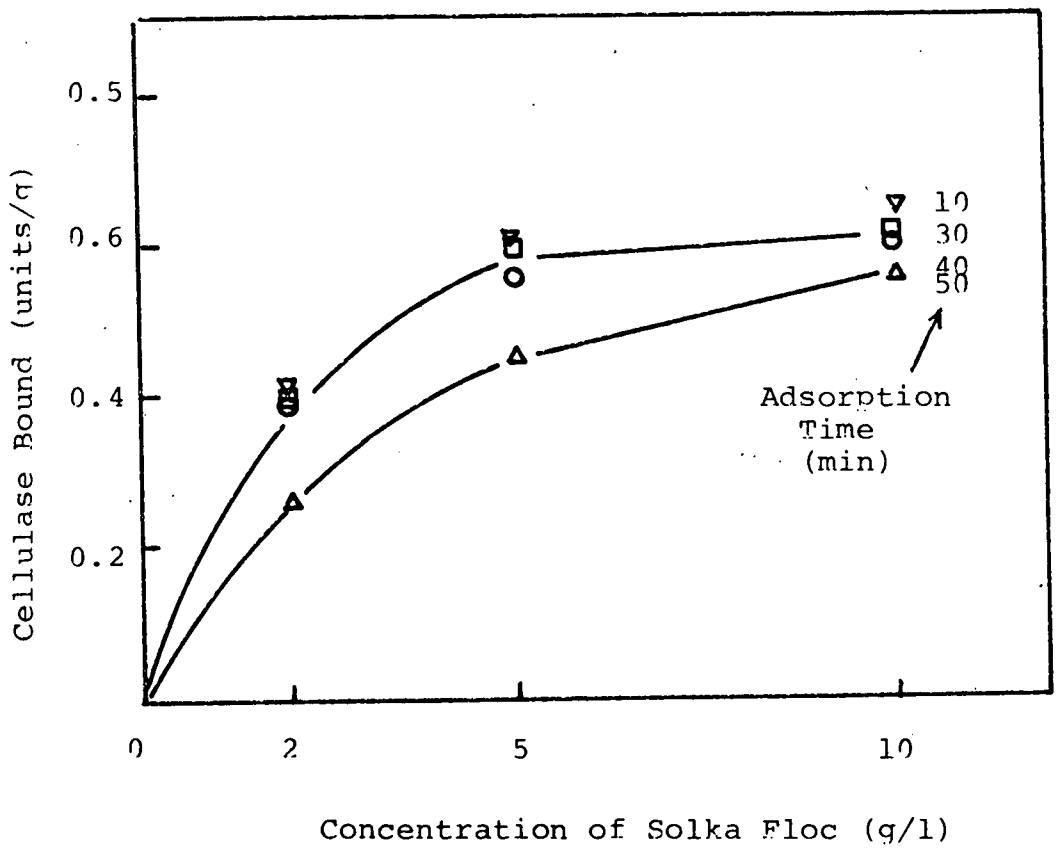


Figure 8a: Kinetics of Cellulase Adsorption onto Solka Floc

Figure 8b: Adsorption Isotherm for Cellulase on Solka Floc



There are obvious differences between the adsorption behavior of the soluble enzyme on the 2 substrates. The solka floc isotherm has approached equilibrium at 1% added cellulose whereas the MN 300 isotherm still suggests a non-equilibrium state at the same concentration.

The plot of units bound (mg RS/ml-hr CMC ase activity lost) per gram cellulose vs. time suggest two things: 1. that after 40 minutes of contact time between soluble enzyme and solka floc, the substrate is changed such that enzyme becomes desorbed, 2. that increasing concentrations of cellulose fail to adsorb a proportional increase in enzyme even though measureable enzyme activity remains in solution. Thus a certain fraction of the soluble enzymes with CMC ase activity in the fermentation broths can adsorb to solid substrates. However, the rest of the enzyme is unable to adsorb and remains in solution. This may indicate two separate components in the C. thermocellum cellulase system.

The most immediate problem brought to attention by the batch fermentation data is the lack of measureable soluble exoglucanase activity in vitro active in the hydrolsis of filter paper, a widely used insoluble substrate in cellulase analysis. Nevertheless, we observe rapid utilization of crystaline cellulose in vivo suggesting that an enzyme active against insoluble (as well as crystalline) cellulose does indeed exist. Adsorption studies indicate that there is some soluble enzyme fraction that can at least bind to a solid cellulosic substrate, a likely first step in hydrolysis, yet it does not effect any measureable hydrolysis. In this light, we are

pursuing the following questions:

1. Is there cell bound (surface or intrawall) exoglucanase?

2. If so, how much?
3. Must exoglucanase be cell bound or associated in order to function most effectively?
4. Can environmental or genetic manipulations make this component truly extracellular and functional?

In the immediate future, we plan to set up an HPLC based assay system for analysis of residual sugar from the fermentation as well as determine the type of cellulase activity that becomes bound to cellulose and that which remains free in solution. It is important to be able to distinguish between the various types of cellulase activities produced and the localization during enzyme production as well as cellulose hydrolysis.

B.) Degradation of Cellulosic Biomass to Produce Soluble Sugars

1.) Introduction

Since one of the primary objectives of this overall research project is the utilization of degraded cellulosic biomass to produce useful substances, it was rationalized that a method to achieve the microbiological solubilization of cellulose would be highly desireable. It is our belief that if a microorganism can be found which will solubilize cellulosic biomass in substantial quantities, one can then use this soluble substrate by a second microorganism directly from this fermentation broth without subsequent processing. For example, the second microorganism can be used either in mixed-culture with the cellulose degrading organism or separately in a staged-wise fashion. In view of the fact that the organisms which we have selected to produce useful chemicals (acrylic acid, acetone/butanol, acetic acid) are all anaerobic, one of the compatibility constraints would be an anaerobe which will produce soluble sugars from cellulose.

To assess the potential of this approach, we have been studying the degradation of cellulose (Solka floc) by an anaerobic and thermophilic microorganism Clostridium thermocellum (ATCC 27405) in shake flasks and laboratory agitated fermentors.

2.) Materials and Methods

The growth of C. thermocellum was conducted in tube cultures, shake flasks and agitated fermentor already described

in the previous section. The medium composition used in these studies is similar to that already reported with the exception of the carbon source. In these studies the cellulose tested was Solka floc and its initial concentration was varied from 2.5 to 10 gm/liters.

Analytical procedures employed in these studies were again similar to those already presented. The main difference in the analytical procedure was in the method used for determining cell growth. In these studies it was possible to employ a spectrophotometric method for the measurement of cell density. This was achieved by first growing Clostridium thermocellum on a soluble carbon source, cellobiose. A calibration curve using gravimetric analysis of cell dry weight versus optical density as measured by a Klett-Summerson spectrophotometer was first constructed. It was found that below 0.3 grams of cells per liter (100 Klett units) linearity between cell concentration and optical density resulted. When the organism was grown on the insoluble substrate (Solka floc), a modification of the optical method had to be devised in order to determine cell growth. This was accomplished by first filtering the fermentation broth through a Whatman No. 1 filter paper. Clostridium thermocellum is able to pass through the filter paper but the unde graded cellulose is retained. The filtrate is then measured spectrophotometrically to determine the cell concentration. However, it is known that portion of the microorganism in solution will be adsorbed onto the undegraded cellulose. To correct for this adsorption experiments were also performed to determine the adsorption of the organism onto cellulose as

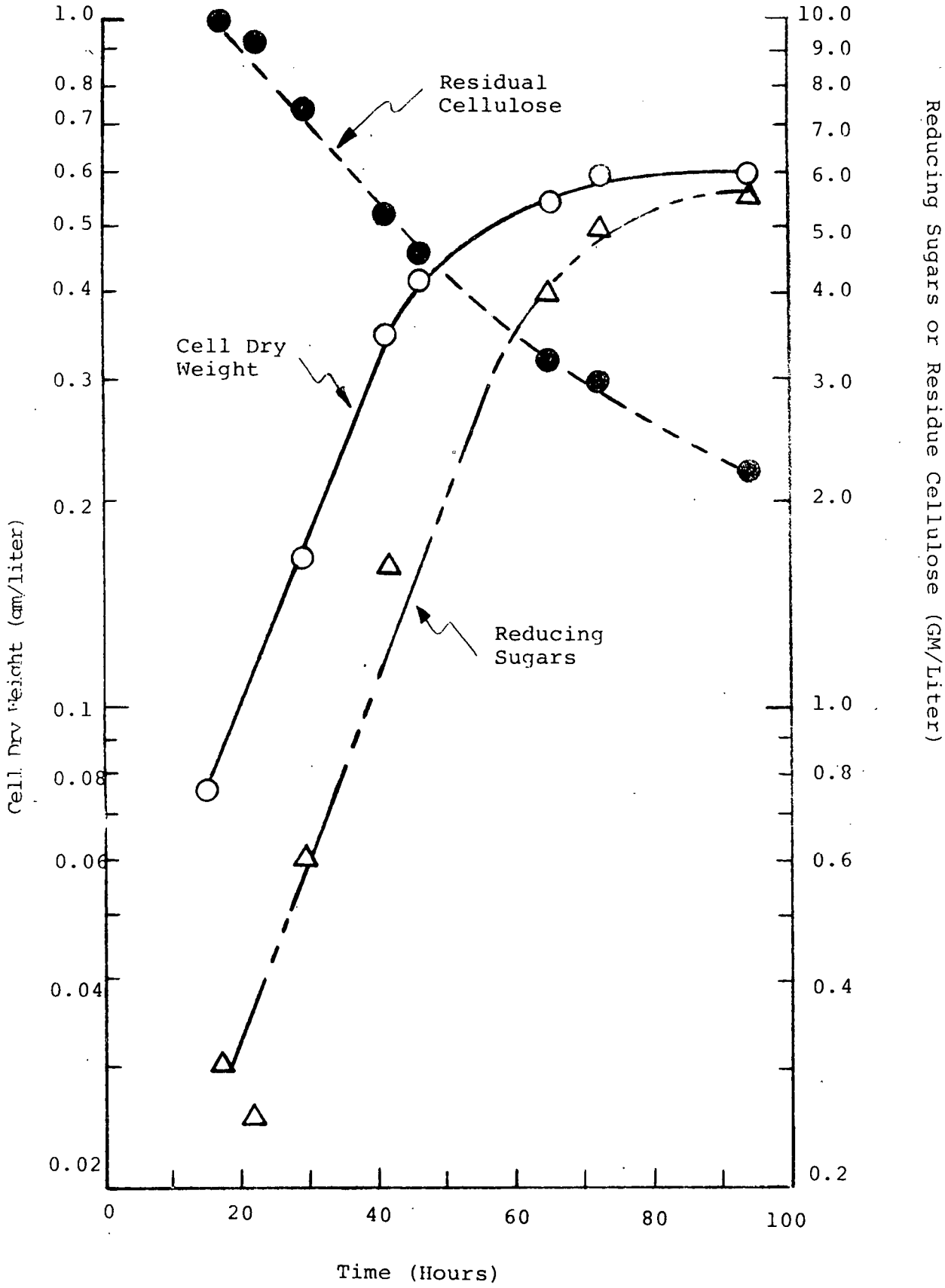
functions of cell concentration and cellulose concentration. These latter relationships allowed a correction of actual cell concentration to accommodate cell-cellulose adsorption.

3.) Results and Discussion

When Clostridium thermocellum was grown at 60°C using Solka floc as the carbon source, typical growth kinetics, reducing sugar production and cellulose degradation are shown in Figure 9. The results in Figure 9 were obtained using an initial cellulose concentration of 10 grams per liter. The organism exhibits an initial exponential rate of growth having a mass doubling time of about 10 hours (specific growth rate $\sim$0.07 hour^{-1}). This high specific growth rate is extremely encouraging since it is significantly greater than aerobic fungi grown on similar substrates. The rate of growth decreases as the concentration of cellulose is depleted from the medium. Further discussions on this behavior will be presented shortly when the detailed kinetic analyses are performed. A final cell concentration of 0.6 grams per liter was obtained.

The production of reducing sugar follows a similar exponential rate as that found for growth. It can be seen from the slopes of the cell growth and reducing sugar production in Figure 9 that they are nearly identical. Here again the rate of sugar production decreases as the substrate becomes exhausted. It should be noted, however, from 10 grams per liter of cellulose approximately 5.5 grams per liter of reducing sugar is accumulated in the broth at the end of the fermentations. The residual cellulose during fermentation is also shown in this figure. The striking feature of these results

Figure 9: Growth, Reducing Sugar and Residual Cellulose during Fermentation by Clostridium thermocellum at 60°C



being that nearly 80% of the initial cellulose is degraded leading to the production primarily of soluble reducing sugars. If one calculates the specific capability of Clostridium thermocellum to degrade cellulose to produce reducing sugars, very impressive values can be obtained. For example using the results in Figure 9, it can be seen that 0.6 grams per liter of dry cell was capable of producing 5.5 grams per liter of reducing sugars. This calculation shows that 9.2 grams of sugar can be produced per gram of cellular biomass. It should be realized that this is the inherent capability of the wild strain of Clostridium thermocellum where no mutation, selection or environmental manipulations has been performed. One could reasonably conclude that efforts in these directions could improve substantially the biosynthetic capabilities of this organism to increase the specific reducing sugar formation.

Identification of the reducing sugars produced by this organism was also performed using silica gel plates and various solvents. Two solvent systems which separated the sugar solutions were:

1.) N-propanol (50%) - ethylacetate (10%) - 25% aqueous ammonia (40%)

2.) N-butanol (60%) - glacial acetic acid (30%) - water (10%)

In both solvents, separation of the sugars produced was achieved. Qualitatively the results showed that the reducing sugars produced were mainly glucose, xylose and cellobiose.

More detailed kinetic analyses were then performed in order to answer the following questions: 1.) Is the production of

reducing sugars growth associated or non-growth associated? 2.) Due to the formation of the reducing sugars in the broth, do they exert a repressive or inhibitory effect on rate of sugar production? 3.) Lastly, what might be the bottleneck which controls the rate of growth and rate of sugar production?

To answer question number 1 and 2, the kinetics of growth and reducing sugar production from two batch cultures were carefully examined. These two experiments were performed at initial cellulose concentrations of 8 and 10 grams per liter. Shown in Figures 10 and 11 are the relationships between specific growth rate ($hour^{-1}$) and specific reducing sugar production rate (gm sugar/gm cell-hour) to the concentration of reducing sugar in the broth. From Figure 9, it can be seen that the specific sugar production is essentially constant when the specific growth rate is constant up to 2 gm/L in broth reducing sugar as sugars accumulate in the broth, both the specific growth rate and specific reducing sugar production rate decreases. At a first glance, one might be tempted to conclude that the accumulation of reducing sugars in the broth exerts a repression or inhibitory effect on growth and product formation. However, one can not conclude with absolute assurity whether these phenomena are truely expressed.

The same kinetic analyses were also performed when the initial cellulose concentration was increased to 10 gm/liter. These results are shown in Figure 11. Here again the qualitative behaviors are similar to those previously presented in

Figure 10: Relationship Between Specific Growth and Sugar Production Rates to Reducing Sugar Concentration for Clostridium thermocellum (T = 60°C; initial cellulose = 8 gm/l)

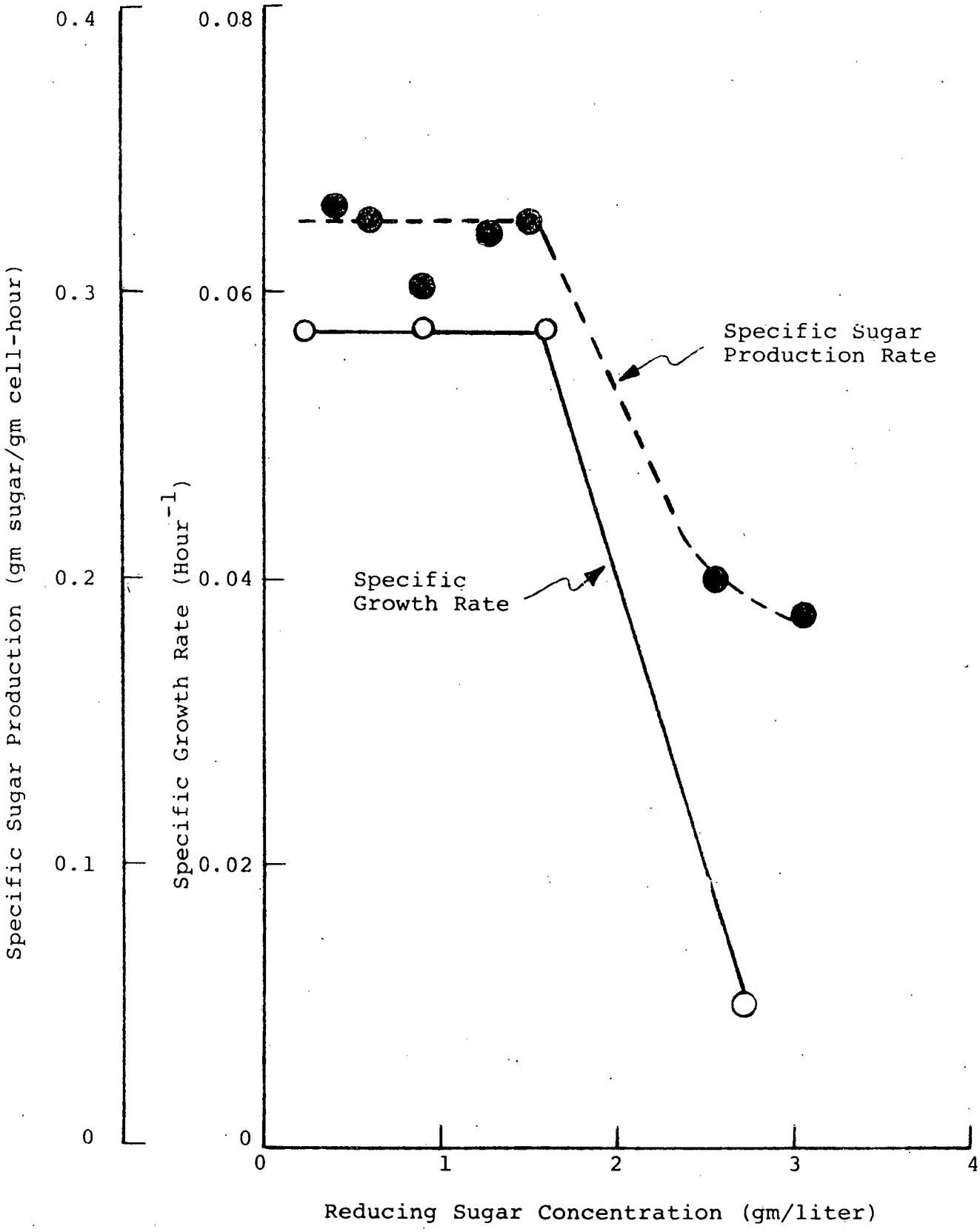


Figure 11: Relationships Between Specific Growth and Specific Sugar Production Rates to Reducing Sugar Concentration for Clostridium thermocellum
(T = 60°C; Initial Cellulose = 10 gm/L.)

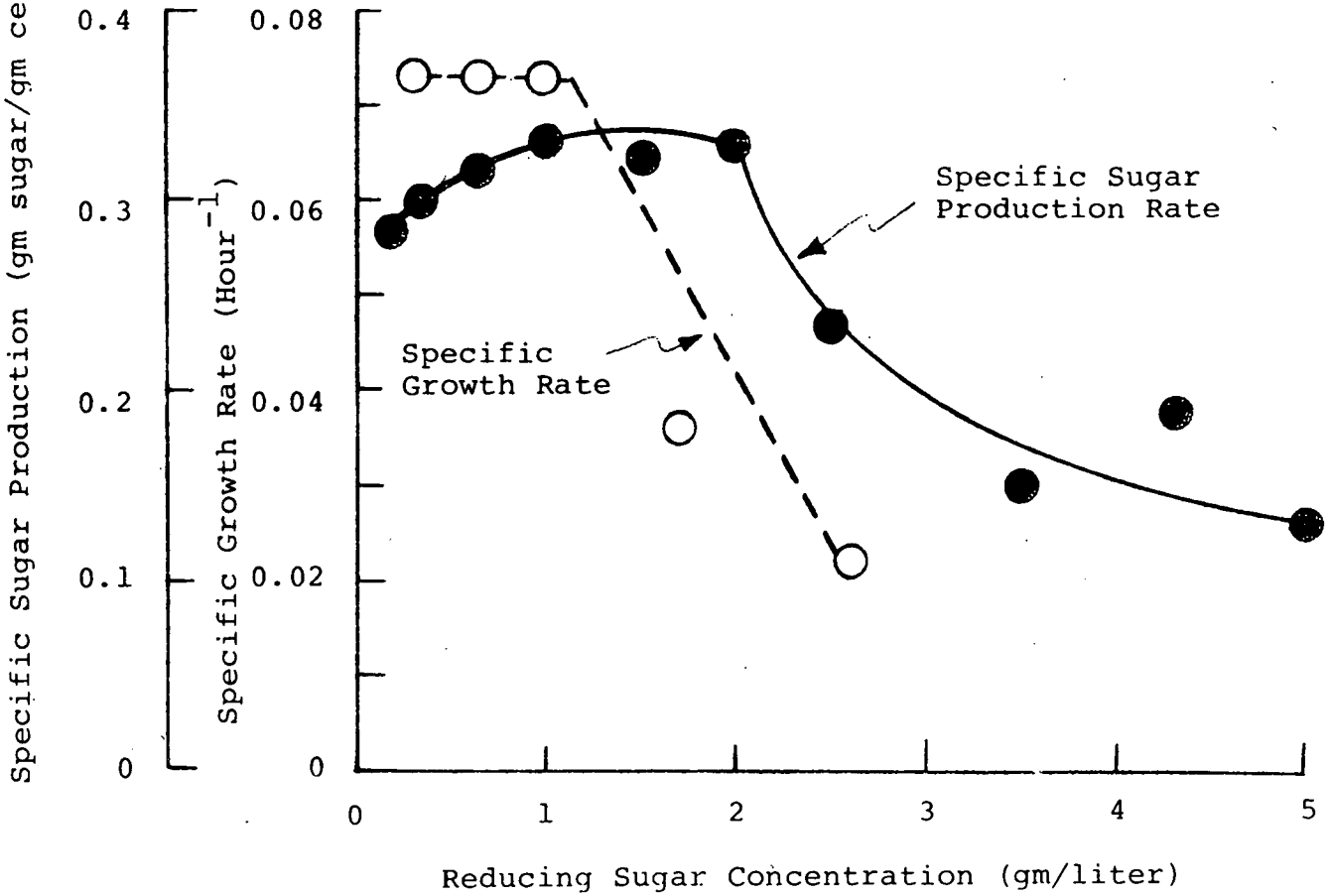
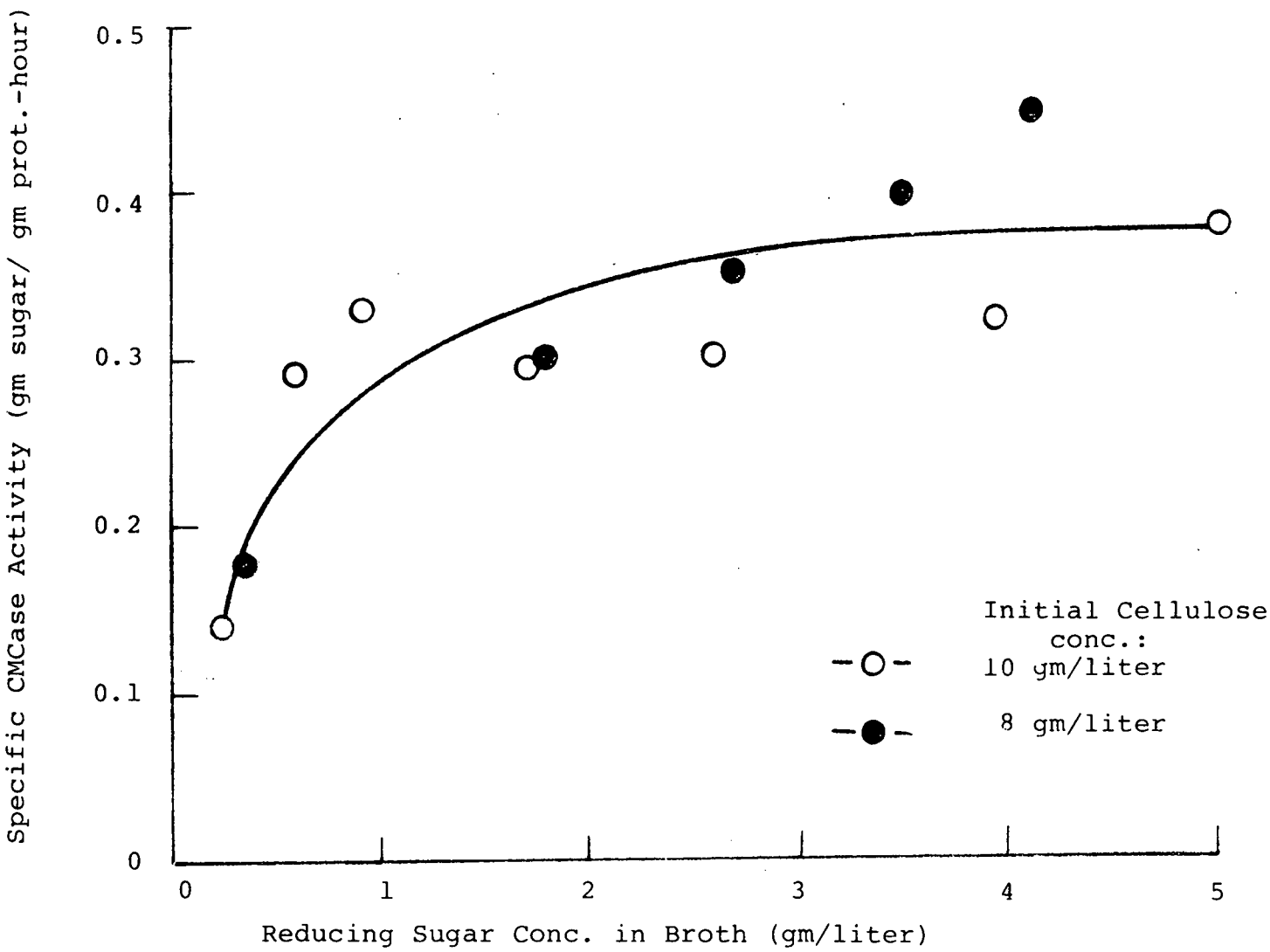


Figure 10. The specific formation rate of reducing sugars appears to be associated with growth; as the specific growth rate decrease, the specific product rate also decreased. It is still unclear from these results whether the decrease in the rate of specific product formation is a result of accumulation of reducing sugar in the broth.

In order to conclude more definitively the influence of accumulated reducing sugars during fermentation on the different rate parameters, we resorted to examine the extracellular cellulase activities as measured by the CMC assays. These results are shown in Figure 12. In this figure the reducing sugar concentration (gm/l) in the fermentation broth was plotted against the specific CMCase activity (gm sugar/gm protein-Hr.) at the two different initial Solka floc concentrations. It can be seen that the specific enzyme activity actually increased as the reducing sugars accumulated in the broth. Furthermore, above a conventration of 1 gm/liter of reducing sugar, it does not appear to effect the extracellular CMCase activity. We therefore conclude that the presence of reducing sugars (at least up to 5 grams/liter) does not exert a repressive or inhibitory effect on enzyme formation. From these results along with those from Figures 10 and 11 we further conclude that the reduction in the specific rate of reducing sugar formation is a direct result of the decrease in specific growth rate.

In an attempt to answer the question as to the reason(s) why the specific growth and product formation rates decreased

Figure 12: Effect of Reducing Sugar on Extracellular CMCase Activity of Clostridium thermocellum



during batch fermentations, the results from these experiments were analyzed in another manner. Shown in Figure 12A are the calculated volumetric rates of cellulose degradation (gm cellulose/liter-hour) plotted against the fraction of cellulose degraded at three different initial cellulose concentrations. One observed a slow decrease in the volumetric rate as the fraction of cellulose degraded is increased. However, for the different initial cellulose concentrations, one observes an abrupt decrease in the volumetric rate at an identical fraction of the initial cellulose degraded. These results are believed to reflect the different types of cellulose which are present in Solka floc. Furthermore, it is reasonable to conclude that the decreases in specific growth and product formation rates are direct consequences of this phenomenon.

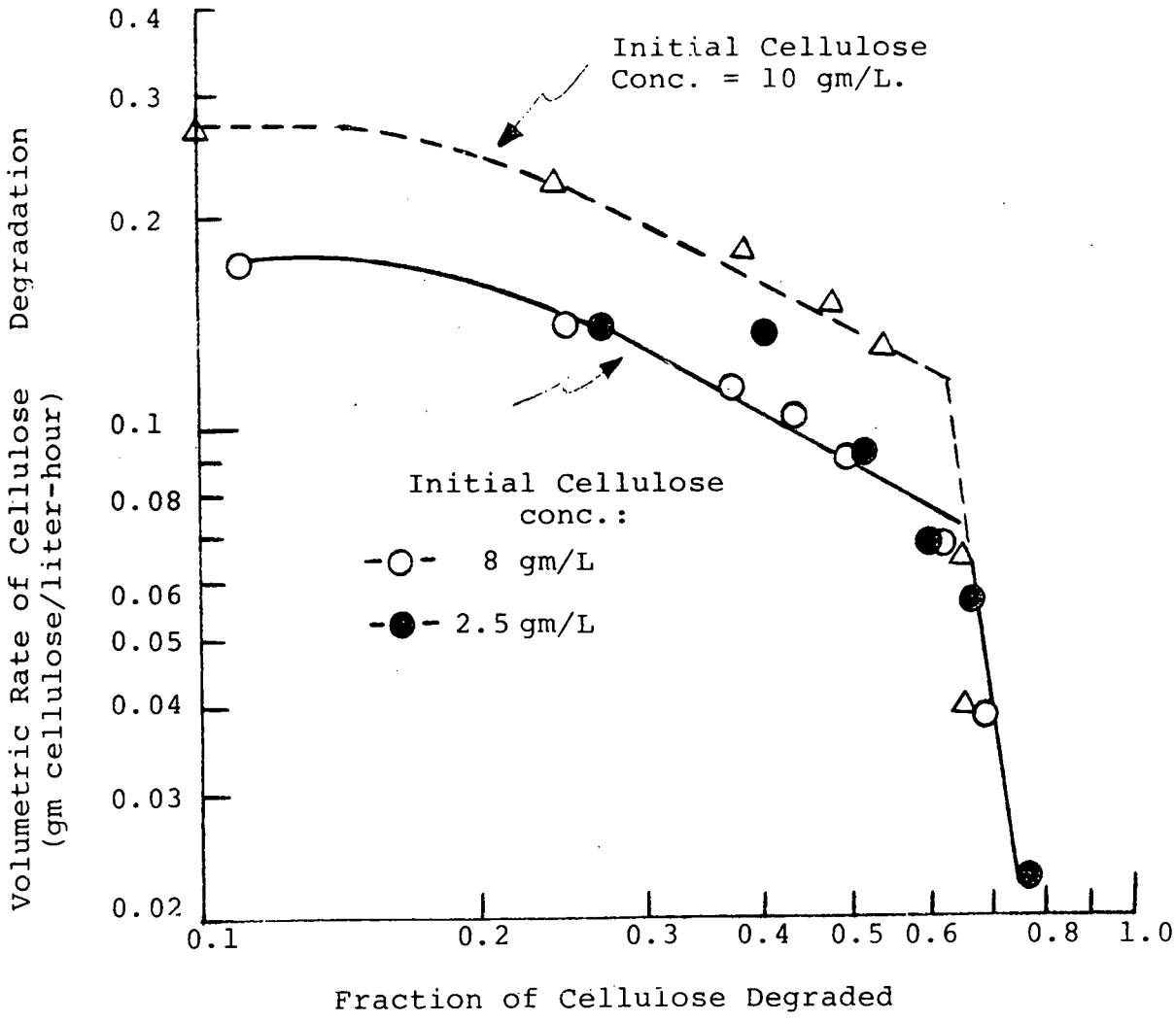
4.) Conclusions and Future Research

From the results presented in this section, the following conclusions can be drawn:

- The specific growth rate of Clostridium thermocellum is sufficiently high to merit it to be used for furthur process considerations.
- The possibility of producing soluble reducing sugars using Clostridium thermocellum exists.
- The formation of soluble reducing sugars, mainly glucose, xylose and cellobiose, appears to be associated with growth.
- The accumulation of reducing sugars does not exhibit repressive or inhibitory effect on enzyme formation.
- Decreases in the different rates are attributed to the different types of cellulose in Solka floc.

Having completed these initial studies, we are now in the

Figure 12A: Volumetric Rate of Cellulose Degradation by Clostridium Thermocellum as Related to Fraction of Cellulose (Solka Floc) Degraded



position to explore the following avenues for the future:

- Mutation and selection programs to increase the cellulytic enzyme levels of C. thermocellum which will increase the rate of soluble reducing sugar formation.
- The examination of this general approach with cellulosic biomass of importance: e.g. primary waste from paper pulp industry, straws, bagasse, and agricultural waste.
- Exploration of continuous culture with cell recycle to increase overall productivity.
- Examination of the soluble reducing sugars as substrate for chemical production.

C.) Deregulation of Clostridial Cellulase

1.) Nutritional Characteristics

The main goal of this part of the program is to deregulate cellulase production in thermophilic, anaerobic cellulase producers. Before attempting to understand regulation, one must properly understand the basic nutrition of the culture. Unfortunately, the nutrition of the culture we have received as Clostridium thermocellum, is poorly understood. The main problem revolves around the use of glucose and fructose by our culture, yet C. thermocellum is supposed to be unable to use either.

According to McBee (Bact. Rev. 14, 51, 1950), C. thermocellum was first isolated by Viljoen, Fred and Peterson. As they transferred the culture on glucose-containing media, it lost the ability to ferment cellulose. McBee later isolated a thermophilic, obligately anaerobic, cellulose digester which he called C. thermocellum. The organism grew on cellulose, cellobiose, D-xylose but not on D-glucose, D-gructose or L-arabinose. Enebo (Royal Inst. Technol. Stockholm, 1, 1954) later isolated a different type of anaerobic, thermophilic cellulose digester which he called C. thermocellaseum which grown on cellulose, cellobiose, L-arabinose, D-xylose, D-glucose and D-gructose. (Cellulose and cellobiose are best for fermentation rate and cellulase production, then in above order for decreasing ability). The enzyme is inhibited by sugars and the fermentation stops due to this inhibition. Some other reported differences between the two cultures:

C. thermocellum	C. thermocellaseum
McBee (1950) reported that the yeast extract could be replaced by thiamine, riboflavin, pantothenate, pyridoxine and biotin, all of which were required	Enebo (1954) reported that only biotin is needed for growth.
McBee (1950) reported that complex nitrogen sources do not stimulate cellulase production	Enebo (1954) reported that casein hydrolysate enhances cellulase production

According to the work of Herrero and Gomez here at M.I.T., the culture we are using ("C. thermocellum ATCC 27405") grows on cellulose, cellobiose, D-glucose, and D-fructose thus behaving in a manner intermediate between C. thermocellum and C. thermocellaseum. Proposed studies on the effect of vitamins and casein hydrolysate should help us decide which culture we are really working with.

2.) Growth Studies

Our initial studies were short time (24 hr.) growth studies in complex medium 1 (see Appendix) less carbohydrate and with yeast extract increased to 1%. In the basal medium, yeast extract served as carbon source to a limited extent. [Growth was determined by Klett value (red filter) and converted to dry cell weight (DCW) by the relationship 100 Klett units = 0.3 mg/ml DCW.] DCW reached only 0.3mg/ml and the pH drop was insignificant. Substrates were used at 10g/liter. Both glucose and cellobiose supported rapid growth. In glucose, DCW reaced 0.7 g/l and the pH dropped to 4.6. In cellobiose, DCW reached 0.8g/l and pH reached 4.7. With carboxymethylcellulose (MCB Na salt - CMC), Solka floc (Brown Co.) and Avicel (FMC RC-591)

no growth over and above that produced without carbohydrate was evident in these short time studies. [With insoluble substrates, Klett determinations were made after 20 min. static incubation of the whole broth.] Longer experiments (6 days) were next done in medium 1 and in a variation in which Solka floc replaced the MN-300 (Macherly, Nagel & Co., No. 36) cellulose. In both media, cellulose supported growth and cellulase activity on CMC and trinitrophenyl cellulose (TNP-C). These assays are described in the appendix. CMCase activity was 0.14-0.17 units/ml and TNP-Case activity was 0.086 OD units/ml.

We also have conducted 7 day experiments in the defined medium of Fleming and Quinn (Appl. Microbiol. 21, 967, 1971; FQD medium, see Appendix) which employs Avicel as carbohydrate. In this medium, growth occurred and cellulase was produced. The values were 0.11 units/ml CMCase and 0.11 OD units/ml TNP-Case. In complex medium D58 (see Appendix) in whicn yeast extract replaces the vitamins and amino acids of FQD medium, the values obtained were 0.17 units/ml CMCase and 0.13 OD units/ml TNP-Case.

D.) Genetic Manipulation

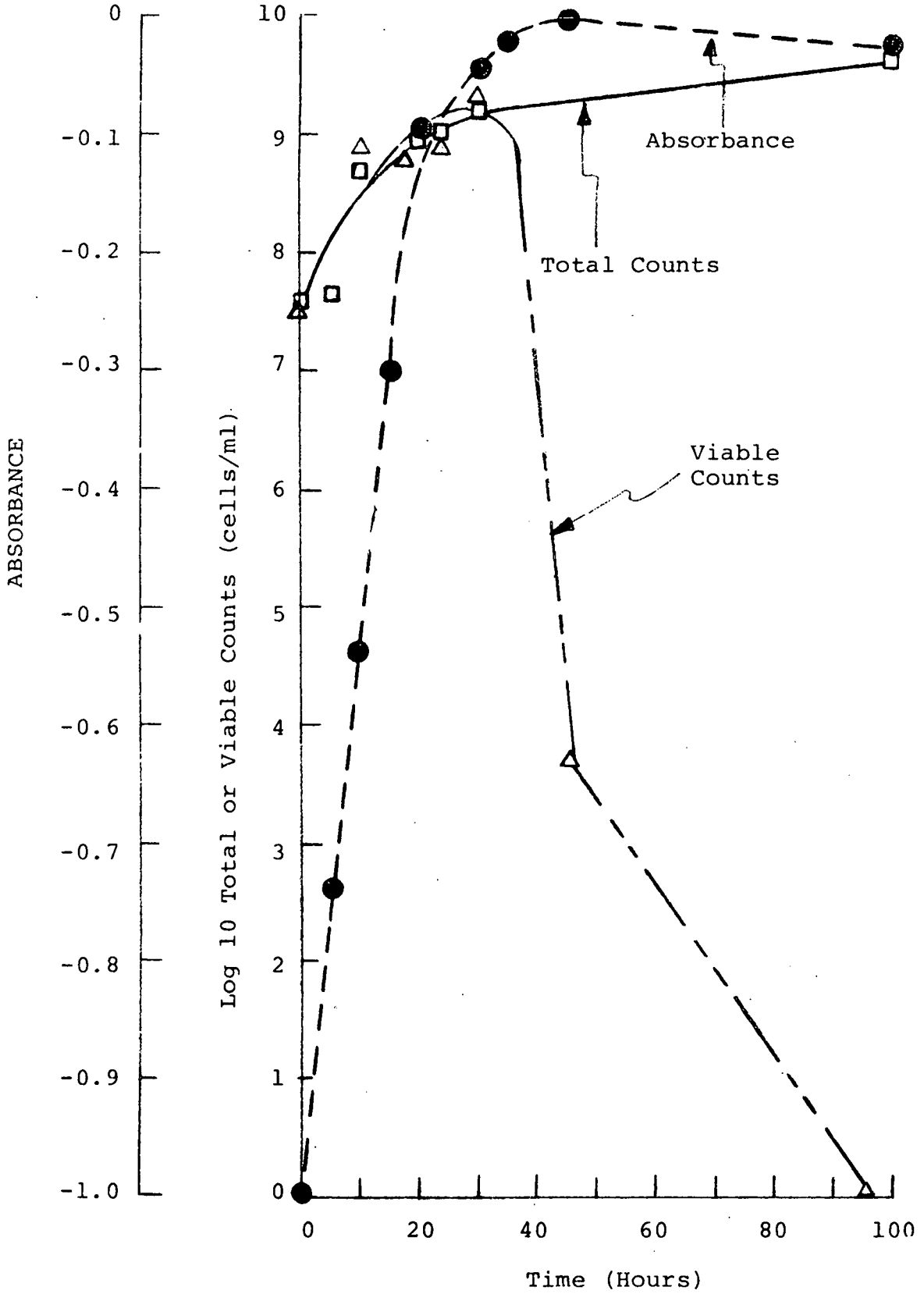
1. Growth and Enumeration

In order to familiarize ourselves with Clostridium thermocellum a growth curve was constructed. The bacteria were grown in CM4 medium with 1 % (w/v) α-cellulose (Sigma) as the carbon source at 60°C. CM4 is composed of:

KH_2PO_4	1.5g/l
K_2HPO_4	2.9g/l
$(NH_4)_2SO_4$	1.3g/l
$MgCl_2$	0.75g/l
$CaCl_2$	0.1g/l
NaCl	1.0g/l
Na-thioglycollate	0.5/gl
Yeast extract	10.0g/l
1.25% $FeSO_4$	0.1ml/l
0.2% Resazurin	1.0ml/l

At various intervals, aliquots were removed and assayed for viability, total counts and optical density. Viability was determined by spreading aliquots of serial dilutions on the surface of prepoured plates containing CM-4 agar medium with 1% cellobiose; plates were incubated anerobically at 60°C for 48 to 72 hours. Total counts (direct microscopic counts) were determined with a Petroff-Hausser counting chamber (± 10% error, 95% confidence). Absorbance was measured at 660 nm. The results are shown in Figure 13. From this figure two important points became clear. First, that we could adequately enumerate C. thermocellum on the surface of agar plates. This technique is required to facilitate mutant

Figure 13. Growth of Clostridium Thermocellum as Determined by Total and Viable Counts and Optical Density



screening and other types of genetic manipulations. Second, it is important to work with exponentially growing cultures since stationary phase cultures could not be recovered on the surface of agar plates under the experimental conditions described.

2.) Substrate Utilization by C. thermocellum

In the past, other investigators have noted that C. thermocellum will loose its ability to utilize cellulose. We have observed this phenomenon also. In addition, the "new variant" acquired the ability to utilize sugars which C. thermocellum is not able to utilize. The pattern of metabolizable sugars is shown in Table 2. The medium used was CM-4, each sugar was added at a concentration of 1%.

This troublesome development has, in the past, been attributed to contamination problems. However, there are various aspects of this phenomenon that makes us suspicious of this simple explanation. For example, its frequency and widespread occurrence. We believe this problem is extremely important if C. thermocellum is to be considreed for large scale cellulose degradation or enzyme production. For these reasons, we have initiated experiments to establish the nature of this observation.

3.) Cellulase Assay

Since one of our goals is to increase cellulose degradation by C. thermocellum, it has become important to choose a convenient and sensitive indicator of cellulase activity. The procedure of Huang and Tang [Analytical Biochemistry 73, 369-377 (1976)] has been evaluated for this purpose. The procedure

TABLE 2

Utilization of Different Carbon Sources
by C. thermocellum at 60°C

Carbon Source	ATCC27405	Variant
L-Arabinose	-	+
D-Arabinose	-	+
Cellobiose	+	+
Cellulose	+	-
L-Fucose	-	+
D-Galactose	-	+
Lactose	-	+
Maltose	-	+
D-Mannose	-	+
Sucrose	-	+
Trehalose	-	+
Xylose	-	+
None	-	-

involves measuring the release of yellow chromophore groups from chemically modified cellulose substrates (TNP cellulose). The substrate is carboxymethyl cellulose coupled through an amide bond to a trinitrophenyl group. Enzymatic hydrolysis of cellulose releases water soluble yellow oligosaccharides that can be monitored spectrophotometrically.

We have measured the kinetics of dye release from TNP-cellulose by cell-free supernatants of C. thermocellum cultures. Figure 14 shows a linear relationship between reaction time and dye released up to 200 min. A Lineweaver-Burke plot indicates that the reaction proceeds with Michalis-Menten Kinetics (Fig. 15). However, there appears to be a non-linear relationship between cellulase concentration and dye released from TNP-cellulose (Fig. 16). Although this non-linearlity would complicate quantitative analyses of cellulase activity, it is felt that this procedure may serve a useful purpose for the rapid screening of new strains and mutants.

4.) Isotope Labeling of DNA

One of our goals is to screen for plasmids in C. thermocellum. For this reason it became necessary to develop the procedures for radioactively labeling DNA. The ability of C. thermocellum to incorporate various precursors of DNA was determined. As shown in Table 3 uracil and adenine are incorporated into polymeric material in C. thermocellum. However, thymidine and thymine are taken up very poorly. In addition, 2'-deoxyadenosine did not stimulate the uptake of thymidine or thymine. Uridine was also incorporated but not as well as uracil or adenine.

Figure 14: Kinetics of Dye Release by C. thermocellum Cellulases.

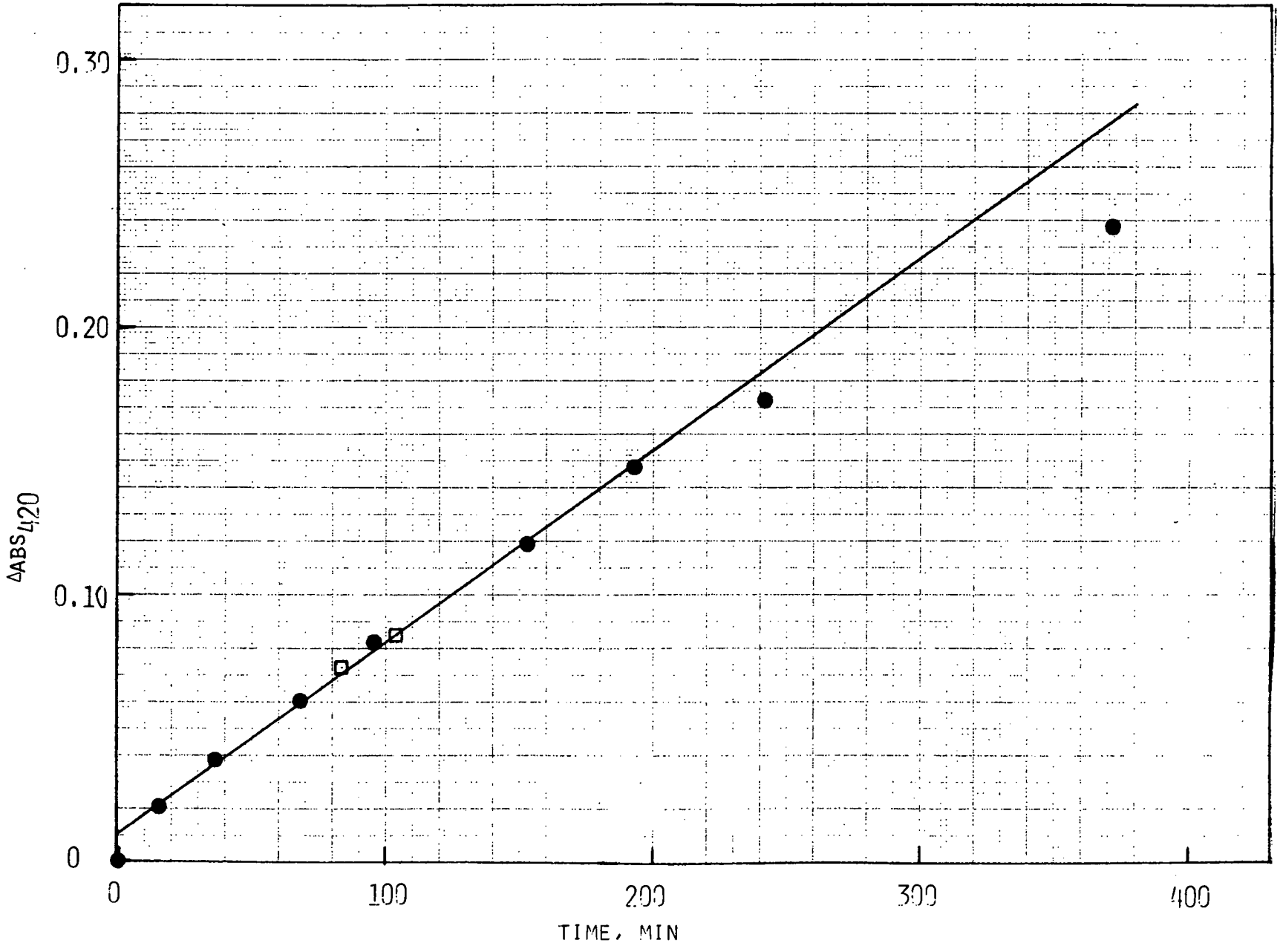


Figure 15: Lineweaver-Burke Plot

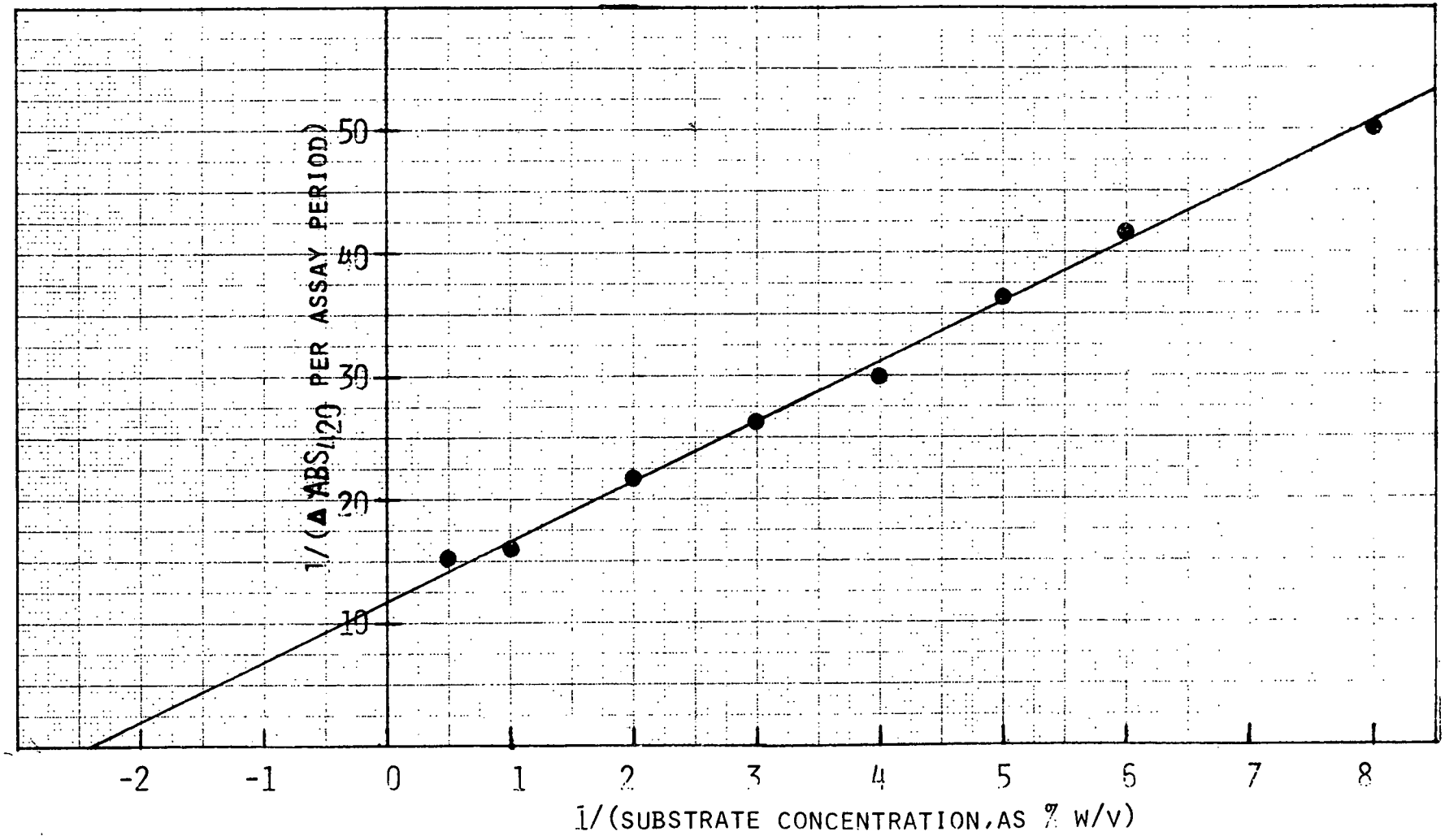


Figure 16: Effect of Cellulase Concentration on Dye Release

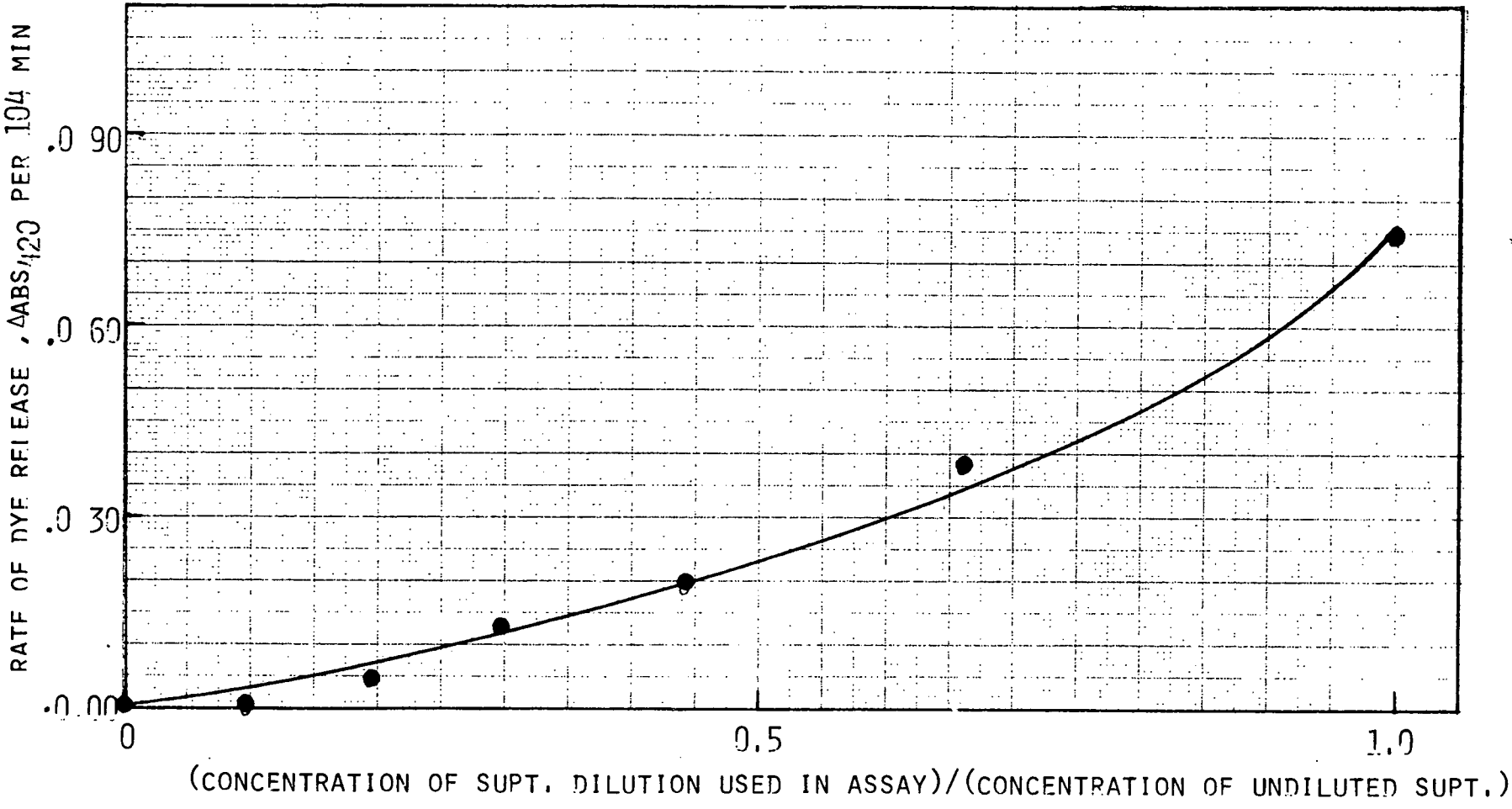


TABLE 3

Incorporation of nucleotides or nucleosides into acid insoluble material of C. thermocellum

Precursor	% CPM of Total Available
^{3}H-thymidine, 20 mg/ml 5 MCi/ml	1.2
^{3}H-thymidine, 20 mg/ml 5 MCi/ml plus 250 mg/ml deoxyadenosine	1.2
^{3}H-thymine, 20gm/ml 10MCi/ml	1.1
^{3}H-thymine, 20 mg/ml 10 MCi/ml plus 250 mg/ml deoxyadenosine	1.4
^{3}H-uridine 20 mg/ml 5 MCi/ml	5.4
^{3}4-uracil, 20 mg/ml 5 MCi/ml	13
^{3}H-adenine10 mg/ml 5 MCi/ml	39.5

Growth was for 48 hours at 60°C in CM 4 medium containing 1% cellobiose as carbon source.

Based on these results we have chosen adenine for future experimentation. The presence of radioactive DNA in C. thermocellum labeled with ^{3}H-adenine was confirmed by CsCl equilibrium density analyses and neutral sucrose sedimentation velocity analyses.

It should also be pointed out that a gentle lysis procedure has been developed. The procedure involves incubation of C. thermocellum in lysozyme followed by membrane solubilization with cationic detergents.

5.) Defined Medium

A medium of the following composition has been utilized.

Difco Yeast Nitrogen Base without amino acids	67g/l
L-phenylalanine	40mg/l
L-tyrosine	40mg/l
L-tryptophan	40mg/l
L-methionine	70mg/l
L-cysteine	500mg/l
Rezasurin	10mg/l
Carbon source	5g/l

In this medium C. thermocellum is able to grow and utilize cellulose as the carbon source. This medium has been used successfully in both liquid and solid form. On the latter C. thermocellum forms visible colonies within 72 hours. This medium will be utilized for isolating auxotrophic mutants.

II.) Production of Chemical Feedstocks

A.) Production of Acrylic Acid by Fermentation

1.) Introduction

Acrylic acid and various esters, which by 1980 will be a billion lb/yr chemical commodity, are made soley by chemical synthesis from aliphatic petrochemicals. A summary of acrylic acid synthesis routes is provided inFigure 17. Our objective is to develop a fermentation process for the production of acrylic acid from glucose and other carbohydrates which can be derived from the renewable resources in the form of cellulose or starch. Such a fermentation process would provide an alternative route for acrylic acid synthesis and permit a reduction in the use of petrochemicals.

From the stoichiometry for acrylic acid production from glucose it may be seen that the theoretical yield on material

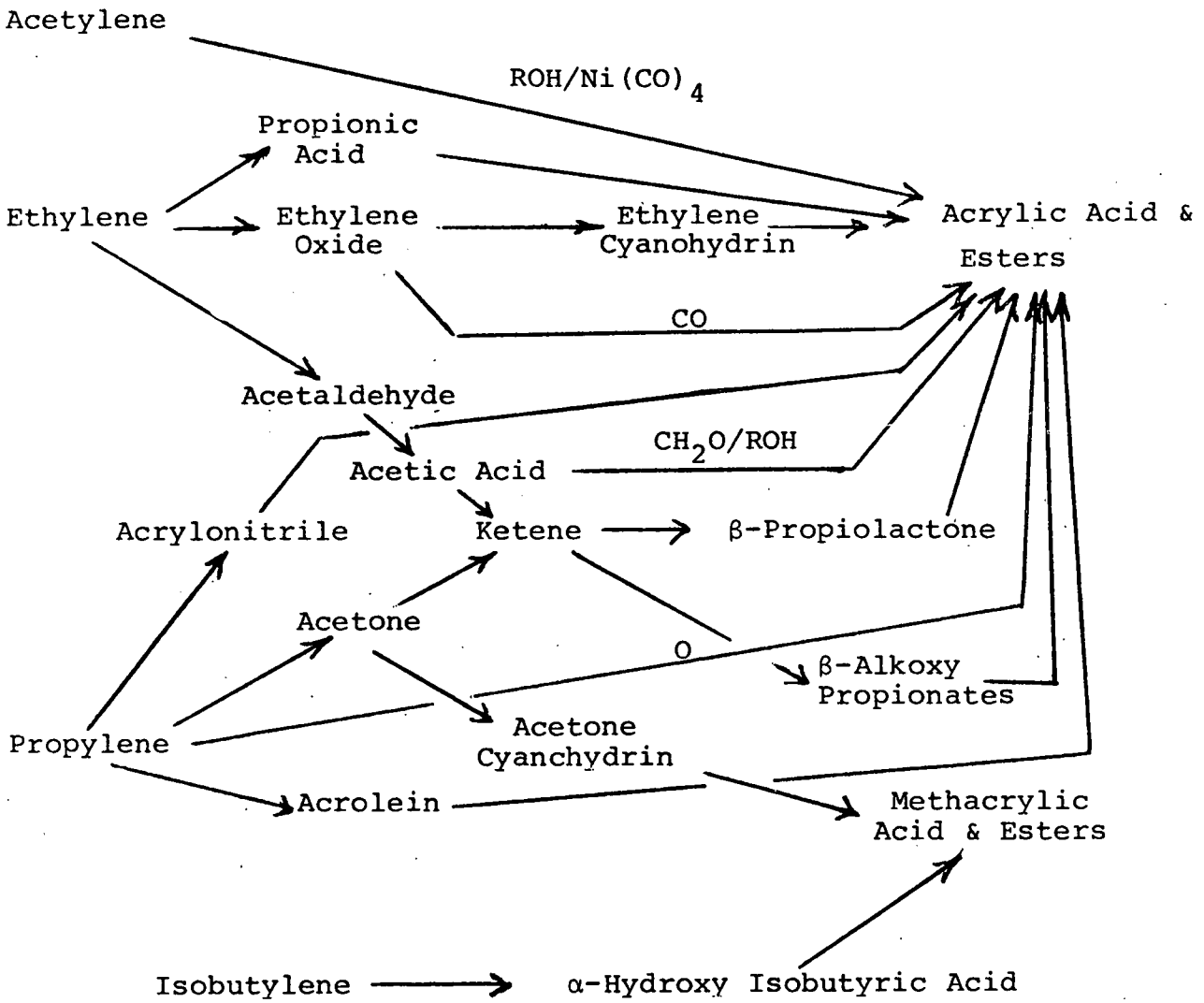
$$C_6H_{12}O_6 \longrightarrow 2CH_2 = CHCOOH + 2H_2O$$

and energy are 0.8 gm acrylic/gm glucose and 0.96 Kcal recovered in acrylic/Kcal in glucose respectively. As a consequence, it is possible to achieve a high degree of conservation of mass and energy. This is a major attribute of anaerobic fermentations.

2.) Biochemistry of Acrylic Acid Synthesis

In biological systems, acrylic acid is found as an intermediate in several pathways. Recently Cerniglia, et.al. (1976) described a strain of Mycobacterium convolutum that accumulate acrylic when growing in the presence of propylene. Some micro-

Figure 17: Inter-related Routes to Acrylic Acids and its Esters



organisms also accumulate trans-3-thioacrylic acid.

As an intermediate, acrylate also occurs in the formation of ethylene by Penicillium digitatum and in the production of propionic acid by several anaerobes. It is the pathway for the formation of propionic acid that is of particular interest here and two mechanisms have been proposed.

a.) The formation of succinic acid and its subsequent decarboxylation, i.e. lactate $\rightarrow$ pyruvate $\rightleftarrows$ oxaloacetate $\rightleftarrows$ malate $\rightleftarrows$ fumarate $\rightarrow$ succinate $\rightarrow$ propionic + H_2O.

b.) The dehydration of lactic acid to acrylic acid which is then reduced to propionate utilizing electrons generated by the oxidation of lactate to acetate via pyruvate. At least three microorganisms, Clostridium propionicum (Cardon and Barker, 1947), Peptostreptococcus (Megasphaera) elsdinii (Lewis and Elsden, 1955; Ladd, 1959) and Bacteroides ruminicola (Wallnofer and Baldwin, 1967) utilize this route.

Formation of propionate by these three microorganisms has been postulated to occur via lactate, acrylate and propionate, or their activated derivatives on the basis of the fact that:

a.) Neither organisms ferment malate, fumarate, or succinate.

b.) They ferment lactate, pyruvate, acrylate, and several amino acids.

c.) They convert position labeled lactate to propionate without randomizing the label.

$$2 \text{ lactate} \longrightarrow 2 \text{ acrylate} + 2 H_2O$$

$$2 \text{ acrylate} + 4H \longrightarrow 2 \text{ propionate}$$

$$\text{lactate} \longrightarrow \text{pyruvate} + 2 H$$

$$\text{pyruvate} + H_2O \longrightarrow \text{acetate} + 2H + CO_2$$

$$3 \text{ lactate} \longrightarrow \text{acetate} + 2 \text{ propionate} + CO_2$$

In Peptostreptococcus (Megasphaera) elsdinii, some of the reducing power is disposed of as molecular hydrogen and the formation of other reduced products such as butyrate and valerate. In Clostridium propionicum, however, there is no hydrogenase, and lactate is reduced primarily to propionate in disposing of the excess reducing power. P. elsdenii is the organism most intensively studied, and it is known that the various transformations involve the co-enzyme A derivatives of lactate, acrylate, and the volatile fatty acids (Ladd and Walker, 1965).

We have choosen to work with both P. elsdenii and C. propionicum in our initial studies to effect acrylic acid production. For this reason, we have reviewed in detail, the literature on lactate reduction to propionate. Most of this work has been done with P. elsdenii and is summarized here.

P. elsdenii is an obligately anaerobic Gram negative bacterium with complex nutritional requirements. Both lactate and glucose are fermented with the production of short chain fatty acids, CO_2, and some H_2 gas. Products from lactate fermentations are acetate, propionate, both straight and branched C_4 acids, valerate, little or no caproate, a large quantity of

CO_2 and a small amount of H_2. Products from glucose fermentation are noteably defferent in that some formate is produced, less acetate, propionate, butyrate, and valerate are formed and caproate is the most copious product.

The enzymatic system involved in the fermentation of lactate by P. elsdenii has been thoroughly investigated. Figure 17a is a summary of the metabolic pathways.

Evidence suggests that the pathway in C. propionicum is similar except that this organism will not ferment glucose and does not produce butyrate, valerate or caproate.

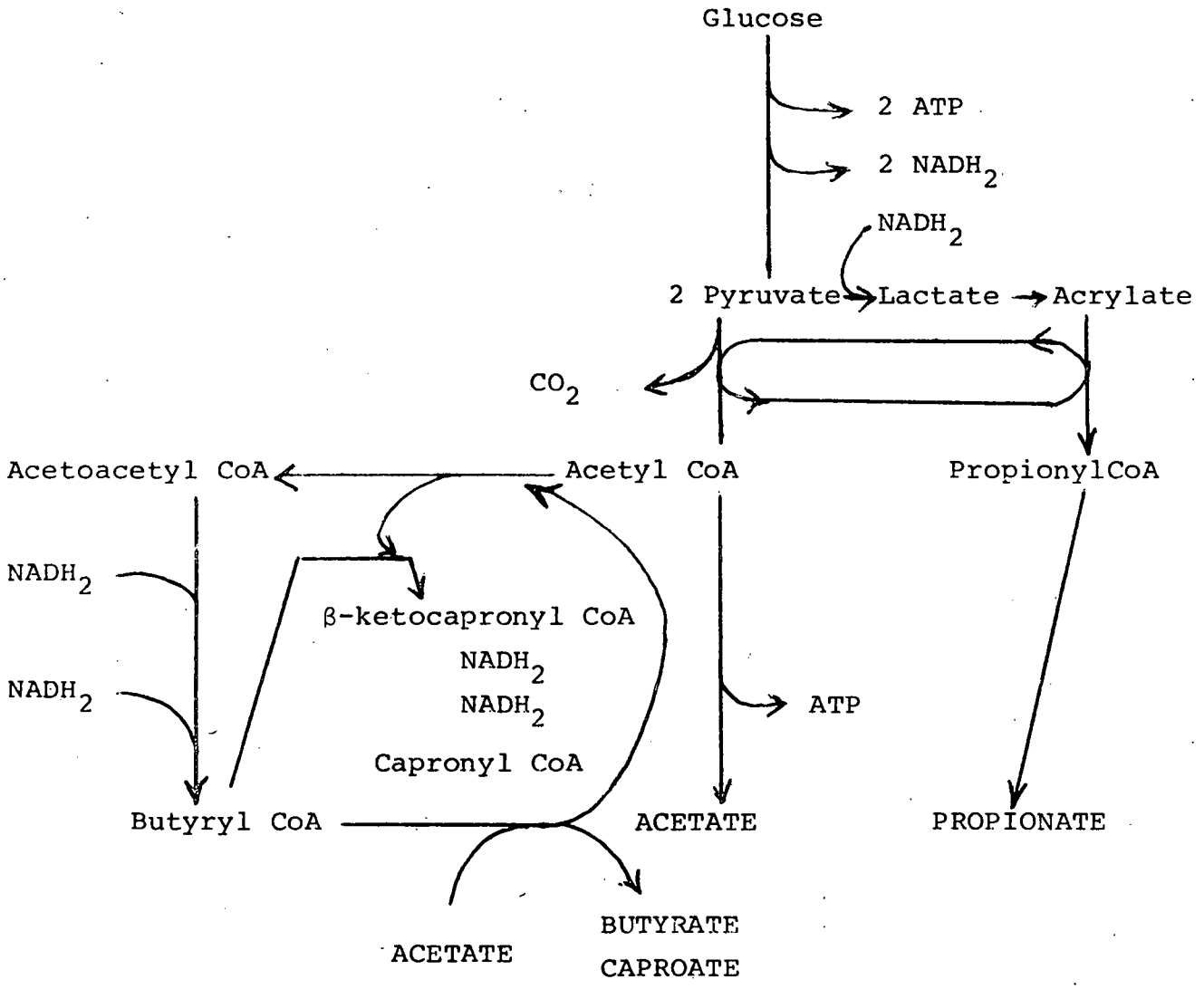
From the biochemical pathway described above, it is possible to see the rational for our approach to this problem. In the fermentations from both lactate and glucose, the conversion to acrylic acid is balanced without the need to dispose of excess reducing equivalents:

$$CH_3CHOHCOOH \longrightarrow CH_2 = CHCOOH + H_2O$$

$$C_6H_{12}O_6 \longrightarrow 2CH_2 = CHCOOH + 2H_2O$$

This is an important point because growing or resting cells of P. elsdenii should be able to produce acrylate from glucose, and resting cells of P. elsdenii and C. propionicum should be able to dehydrate lactate to acrylate. Incidently, the free energy change is in favor of acrylate formation from lactate by -1.4 Kcal/mole. Our approach, therefore, is to block the oxidation of lactate to pyruvate and acetate while at the same

Figure 17a: Possible Route for Glucose Fermentation by P. elsdenii



time opening the pathway to acrylate. We are then left with two possible processes. In one, growing or resting cells of P. elsdenii would be used to convert glucose to acrylate, and in the second, a homolactate fermentation (which gives almost quantitative conversion of glucose to lactate) would be used to convert glucose to lactate and then resting cells of P. elsdenii or C. propionicum would be used for dehydration to acrylate.

3.) Materials and Methods

Microorganisms: Peptococcus elsdenii and Clostridium propionicium were obtained from the American Type Culture Collection. P. elsdenii has complex nutritional requirements and is grown in a medium containing:

KH_2PO_4	1.60 gm
K_2HPO_4	3.20 gm
NH_4Cl	0.50 gm
$CaCl_2$	0.20 gm
$MgCl_2$	0.20 gm
Thioclycollate	0.45 gm
Yeast Extract	4.00 gm
Na-Lactate (60% syrup)	16 ml
Distilled water to	1000 ml

The medium used for C. propionicum contains:

Alanine*	3.0 g
Peptone	3.0 g
Yeast Extract	4.0 g
Cysteine**	0.2 g
$MgSO_4 \cdot 7H_2O$	0.05g
$FeSO_4 \cdot 7H_2O$	0.01g

1 M potassium phosphate buffer, pH 7.1	5.0 ml
Saturated Ca SO_4 solution	2.5 ml
0.2% resuzurin	1 ml
distilled water	1000 ml

* Other substrates when studied or substituted for alanine.

** If cysteine hydrochloride (neutralized) is used, add 0.5 g to the medium instead of 0.2 g cysteine. Cysteine may be replaced as a reducing agent by sodium sulfite (Na_2S) at a final concentration of 0.03%.

The medium is prereduced in Huntgate roll rubes with butyl rubber caps or in anaerobic flasks before sterilization at 15 psi for 15 minutes. Solid medium is prepared by adding agar at a final concentration of 2%.

Phosphate buffer (0.1 M, pH 7.1) with 0.03% cysteine as a reducing agent is used for washing resuspending and diluting cell suspensions.

Growth Kinetics: Growth is monitored by measuring changes in absorbance at 660 nm or in a Klett-Summerson colorimeter with a red filter.

Fermentation Product Analysis: Volatile acids and non-volatile acids are analyzed for in fermentation broths by gas chromatography. Volatile acids routinely examined are:

acetic acid	(bp 118)
propionic acid	(bp 141)
butyric acid	(bp 163)
isobutyric acid	(bp 154)
valeric acid	(bp 186)
acrylic acid	(bp 141)

The acids are extracted from the broth by extraction according to the procedures recommended in the "Anaerobic Laboratory Manual". The culture broth is acidified with 50%

aqueous H_2SO_4 to give a final pH below 2 then extracted with ethyl ether. Non-volatile acids are methylated. The details are presented below:

The following procedure is used for determination of volatile acids.

a.) One milliliter of the culture is pipetted into a centrifuge tube.

b.) 0.2 ml of 50% H_2SO_4 and 0.4 g NaCl are added in order to increase strength.

c.) One milliliter of ethyl ether is then added and the tube is stoppered.

d.) The tube is then mixed gently about 20 times and then centrifuged to break the emulsion.

e.) The sample is then frozen at -70°C and the clear phase is removed into a test tube (12 X 75 mm).

f.) Added to the ether extract is anhydrous $MgSO_4$ equal to one-half the volume of ether and the tube is stoppered.

g.) After 10 minutes approximately 0.5 µ l of the ether extract is injected into the gas chromatograph.

For analysis of the non-volatile acids the following methylation procedure is employed.

a.) One milliliter of the original culture is pipetted into a test tube (12 X 75 mm).

b.) Two milliliters of methanol and 0.4 ml of 50% H_2SO_4 is then added.

c.) The tube is stoppered and incubated in a water bath at 55°C for 30 min. (or held overnight at room temperature).

d.) 1 ml of distilled water and 0.5 ml of chloroform is then added.

e.) The stopper is replaced and the sample mixed gently.

f.) If a emulsion occurs the sample is centrifuged.

g.) For analysis a 5 µl of sample from the chloroform phase is then injected into the gas chromatograph.

For both volatile and non-volatile determinations a column packed with 10% DEGS (Diethylene glycolsuccinate) on Chromosorb W 100-120 mesh in a 1/8 inch Teflon column. Conditions for volatile acid determinations are; injection temperature, 225°C, Detector temperature 300°C and column temperature 95-155°C at 10°C/min. Flow rate of carrier gas is 30 ml/min. Valeric and isocaproic acids are used as internal standards for broths from C. propionicum and P. elsdenii respectively. The same column is employed for non-volatile acid determinations. All operating conditions are the same except that the column is operated isothermally at 125°C amd oxalic acid is used as the internal standard.

The gas chromatograph now employed is Hewlett-Packard 5830A with a flame ionization detector.

A typical chromatogram is presented in Figure 18A showing standards and Figure 18B showing a typical fermention broth from P. elsdenii grown on lactate.

Carbon dioxide production is measured by passing the exit gas through 20% W/V KOH to give K_2CO_3 which can then be precipitated as the Barium salt and measured gravitimetrically. H_2 gas was measured by water dissplacement after removal of CO_2.

4.) Fermentation by Resting Cells

In some experiments resting cells were employed. These were obtained by first growing the cells with alanine at 0.8% for 48 hours at 37°C. Cells were harvested by centrifugation

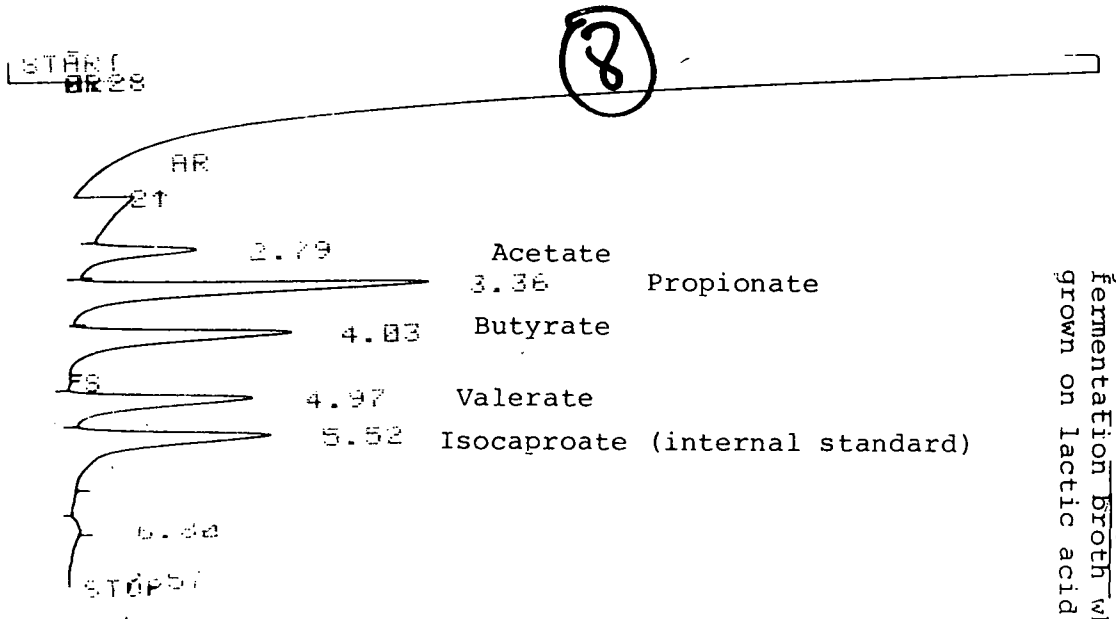


Fig. 18b: Gas chromatogram of P. elsdenii fermentation broth when grown on lactic acid

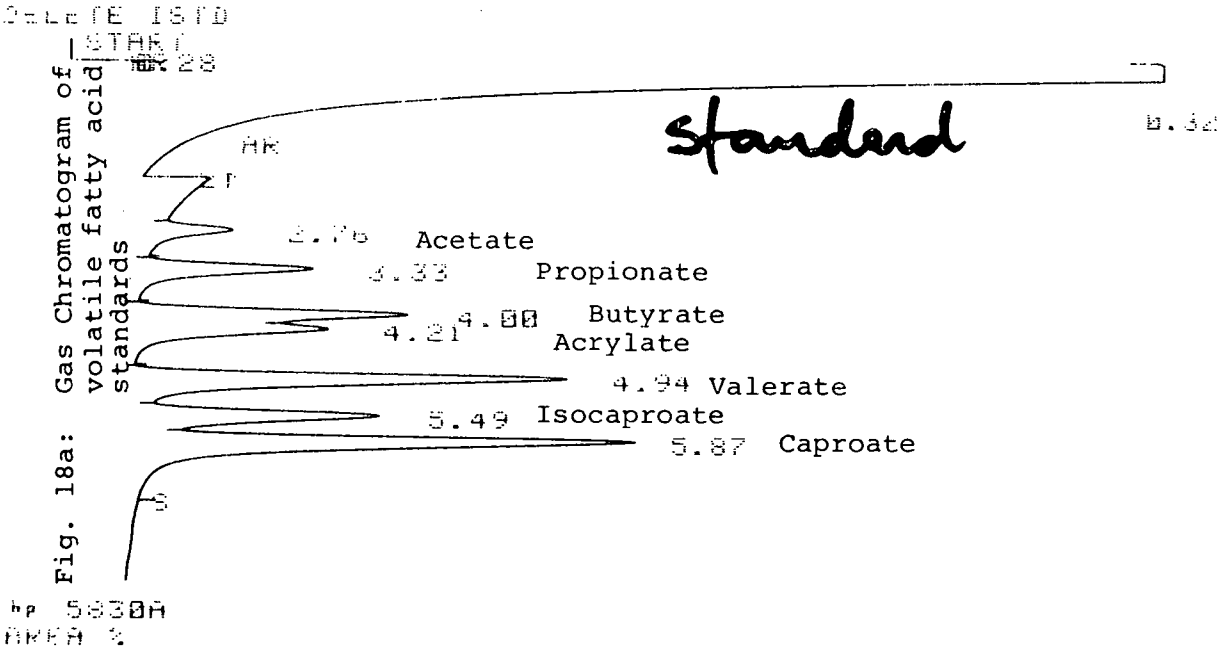


Fig. 18a: Gas Chromatogram of volatile fatty acid standards

and then washed twice with reduced phosphate buffer (0.1M, pH 7.1) and then resuspended in test tubes. Various substrates are then added to the tubes. Usually the substrate concentration is 1% in phosphate buffer (0.1M, pH 7.1). The tubes are incubated at 37°C in a water bath and samples for volatile and non-volatile acid determination is conducted at various time intervals (0.3, 12, 24 and 48 hours). Substrates investigated with C. propionicum include α-alanine (112 mM , β-alanine (112 mM), lactate (114 mM) and pyruvate (132 mM). Acrylate, lactate, and pyruvate are used as the sodium salt (pH 7.1).

Control tubes containing either cells only on substrates were also analyzed. No products are produced without addition of a fermentable substrate and all substrates were found to be pure.

5. Results

a.) Peptostreptococcus elsdenii

P. elsdenii, obtained from ATCC, was grown in the complex medium described earlier. This organism is able to utilize either lactate or glucose as its primary carbon-energy source. The kinetics of growth on glucose (5 g/l) are shown in Figure 19. The specific growth rate is 0.17 hr^{-1}. At the end of the fermentation, the products were butyrate (0.83 g/l), valerate (0.32 g/l), caproate (1.4 g/l), cell mass (1.66 g/l) and little or no acetate or propionate. Because we are particularly interested in the reaction pathway in which acrylate is an intermediate leading from lactate to propionate we have choosen to use lactate as the carbon source in the initial studies. Results from a typical fermentation on lactate as the carbon source are shown in Figure 20. The specific growth rate is 0.32 hr^{-1} at 37°C with the initial pH set at 7.0. Under these conditions, the cell uses the production of propinate as a major sink for disposing of reducing equivalents obtained in the oxidation of lactate. Valerate and butyrate occur late in the fermentation, possibly in response to the decreased pH resulting from acetate and propionate production.

The results from Figure 20 support the hypothesis that during growth on lactate, acrylate is the major mechanism for removal of reducing power. To further test this hypothesis we added acrylate to a fermentation on lactate at a point in late exponential growth and followed the kinetics of its disappearance. Results from the experiment are presented in Figure 21. Lactate consumption and growth are unaffected by the acrylate

Figure 19: Growth and H_2 Production by Peptostreptococcus elsdenii on Glucose

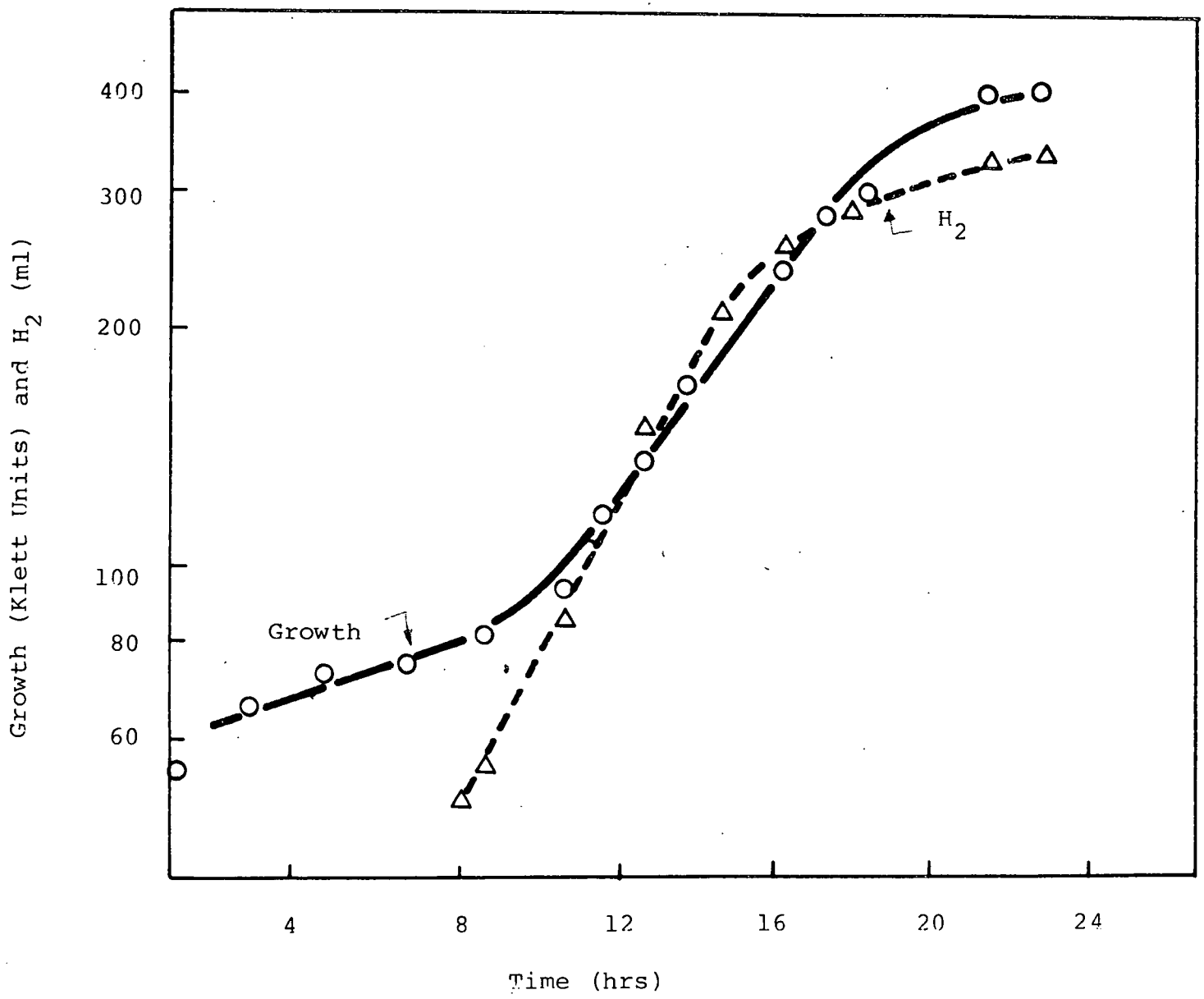
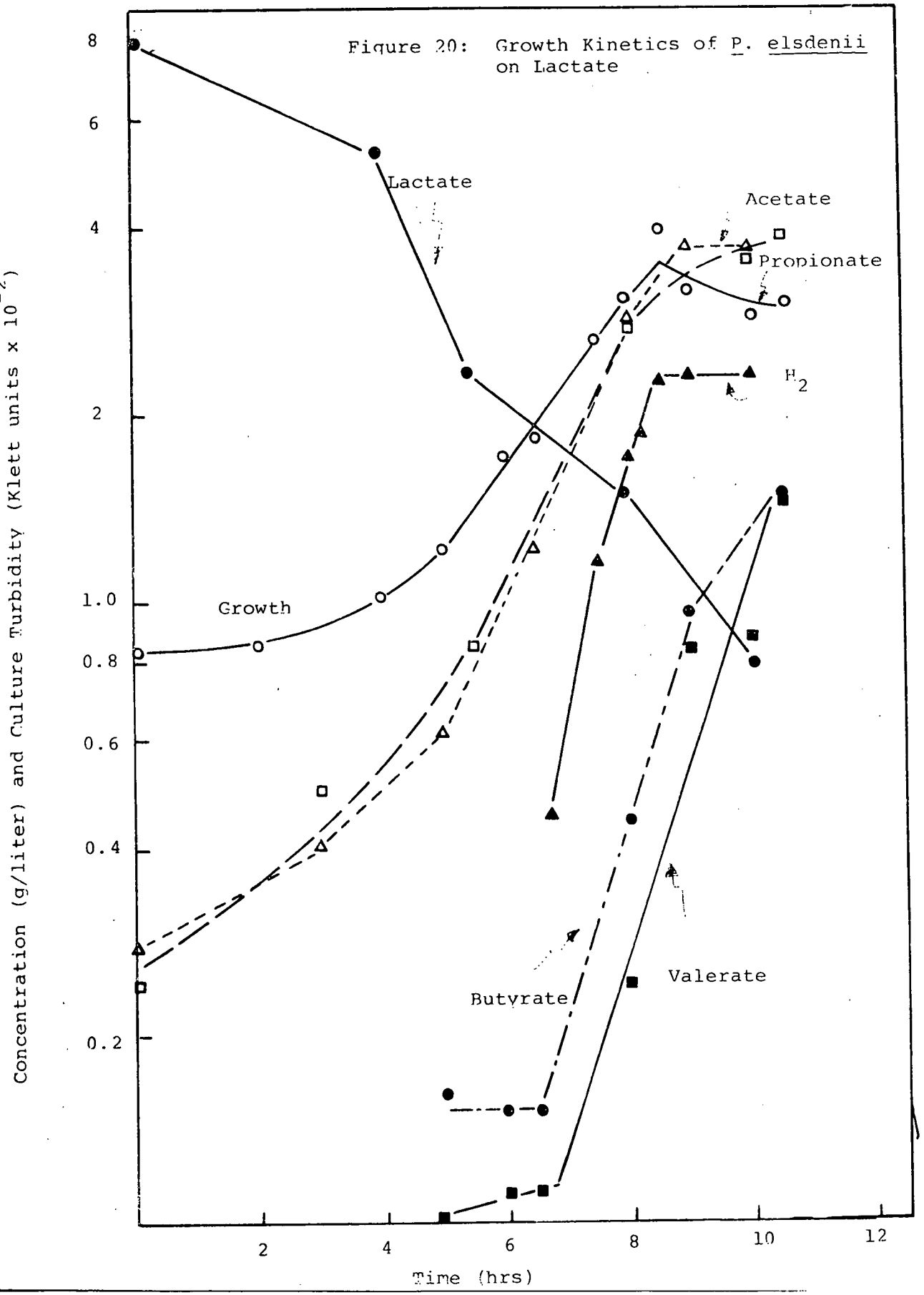


Figure 20: Growth Kinetics of P. elsdenii on Lactate



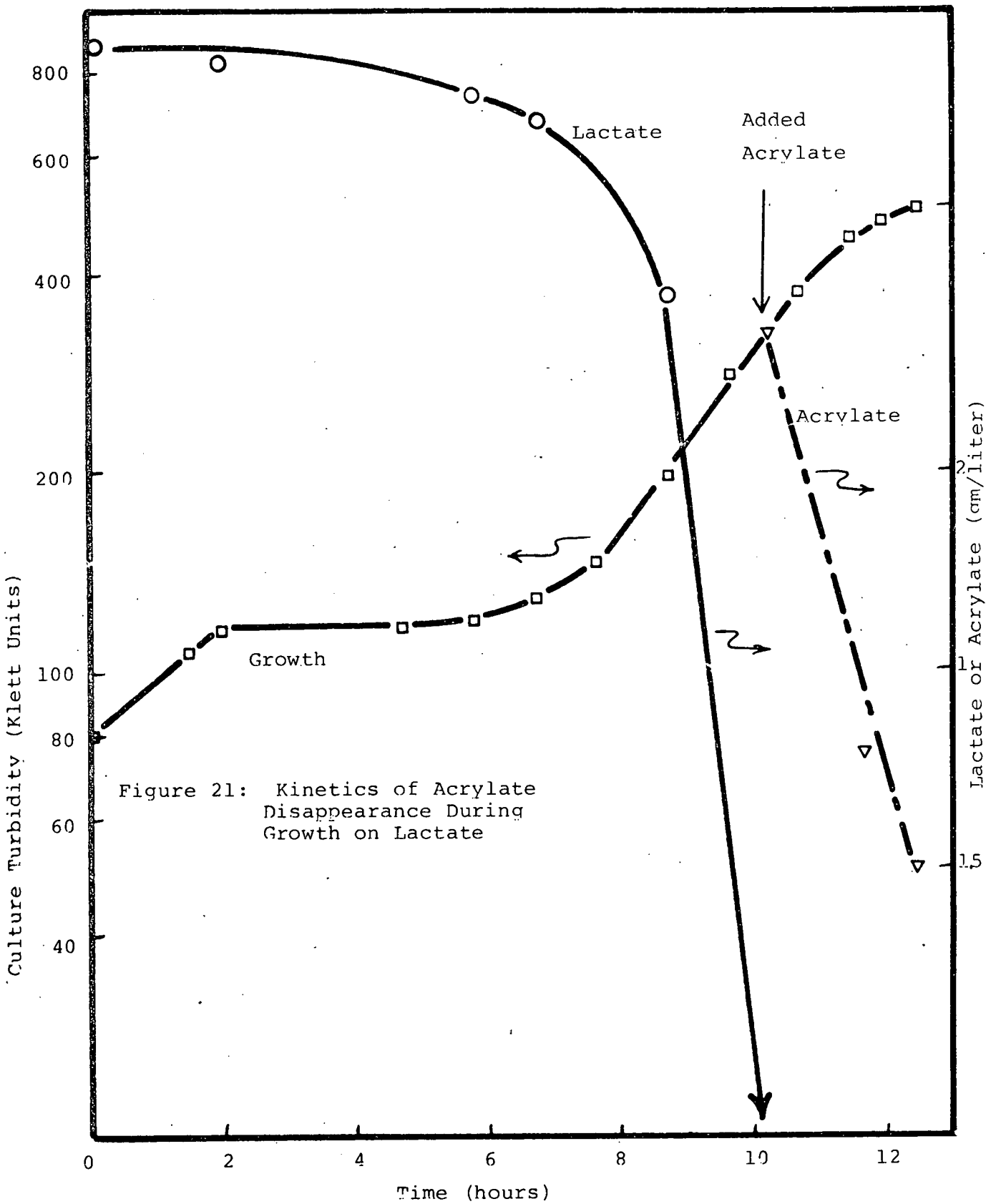


Figure 21: Kinetics of Acrylate Disappearance During Growth on Lactate

addition, and the acrylate disappears rapidly presumably serving as an electron acceptor.

At this point in our studies, we gathered evidence that acrylate is a primary intermediate in the reduction of lactate to propionate. Although we have looked for excretion of acrylic acid during normal fermentation on glucose and lactate, not surprisingly, none has been found. One would expect that under normal conditions, all acrylate, especially as a CoA derivative would readily be reduced by available electrons.

These initial efforts have been done to provide a base line for our future work and we are now proceeding to interrupt the fermentation by the use of selected metabolic inhibition and electron acceptors by blocking the conversion of lactate to pyruvate or acrylate to propionate, we hope to see an accumulation of acrylate. This also needs to be done in the presence of alternative electron acceptors.

Conclusions:

1. Rapid growth and fermentation on glucose and lactate has been demonstrated for P. elsdenii.
2. Evidence supports the major role of acrylate as an intermediate electron acceptor during fermentation on lactate.
3. A good quantitative assay for acrylic acid in the presence of other short chain volatile fatty acids has been worked out.

b.) Clostridium propionicum

Clostridium propionicum ATCC 25522 upon arrival from the ATCC was transfered to the basal medium.

Morphological examination revealed short thick rods occuring singly or more commonly in pairs. The organism is motile and forms subterminal spores upon prolonged incubation. Simple physiological experiments determined that the optimal growth temperature is 37°C and the pH range for growth is between 5.8 to 8.6. The optimal pH is approximately 7.0.

Although the organism is an obligate anaerobe, several experiments were conducted to determine if extreme precautions are required to avoid exposure of the organism to oxygen. For example C. propionicum was streaked on the basal agar medium and incubated at 30°C under N_2 in an anaerobic jar. After four days small, white, translucent, round, flat and smooth colonies appeared. Plates were replicated onto the basal agar in air and then incubated at 30°C under N_2. After 1 day, colonies were visible.

A comparison of viable cell counts was made on agar plates and Hungate tubes. A 4-day culture in basal broth was serielly diluted with reduced phosphate buffer and plated on basal agar plates in air and rolled Hungate tubes render N_2. Plates under N_2 and Hungate tubes were incubated at 37°C. Colonies appeared after 2 days. In Hungate tubes colonies were 2 mm as compared to 1 mm colonies on spread plates. 1.3×10^6 cells/ml were recovered on the spread plate, while 8.5×10^5 cells/ml were recovered by the Hungate method. A microscopic examination indicated a total count of 2.4×10^8 cells/ml. indicating that a viable cell junction of about 0.5%. This is probably due to the fact that the culture was already in the stationary phase. The significant conclusion is however, that the organism can be diluted and plated in air without significant losses in cell

viability.

This organism will not ferment glucose but will ferment lactate, acrylate, and alanine. Because of its sensitivity to lactate, we have used alanine as the carbon source for initial studies. The growth kinetics of C. Propionicum at 37°C with varying amounts of alanine are summarized in Figure 21A Final absorbance values are proportional to the alanine concentration. To date the final absorbance values are low indicating that further nutritional studies will be required to increase yields of C . propionicum cells. Other substrates, that could be described as being more readily available than alanine, were then examined. Although the literature indicates that DL serine, D-L -threonine, pyruvate, acrylate and lactate are fermented by C. propionicum it was determined that the organism cannot grow and utilize pyruvate acrylate and lactate as sole carbon sources for growth.

Next series of experiments were done to determine the degree by which the following acids would inhibit the growth of C. propionicum. Employing the basal medium the growth of the organism was found to be extremely sensitive to the presence of acrylic and lactic acid (Fig 22). Growth was completely inhibited by 0.015 moles/liter acrylic acid and 0.07 moles/liter lactic acid. The growth of the organism is more tolerant to propionic and acetic acids. 0.07 moles/l propionic acid and 0.2 moles/l acetic acid inhibit the growth by 50%. Fermentation experiments were then conducted with resting cells. Substrates examined to date are α-alanine, β-alanine serine, threonine, acrylate, lactate and pyruvate. The

Figure 21A: Growth of Clostridium propionicum on Alanine

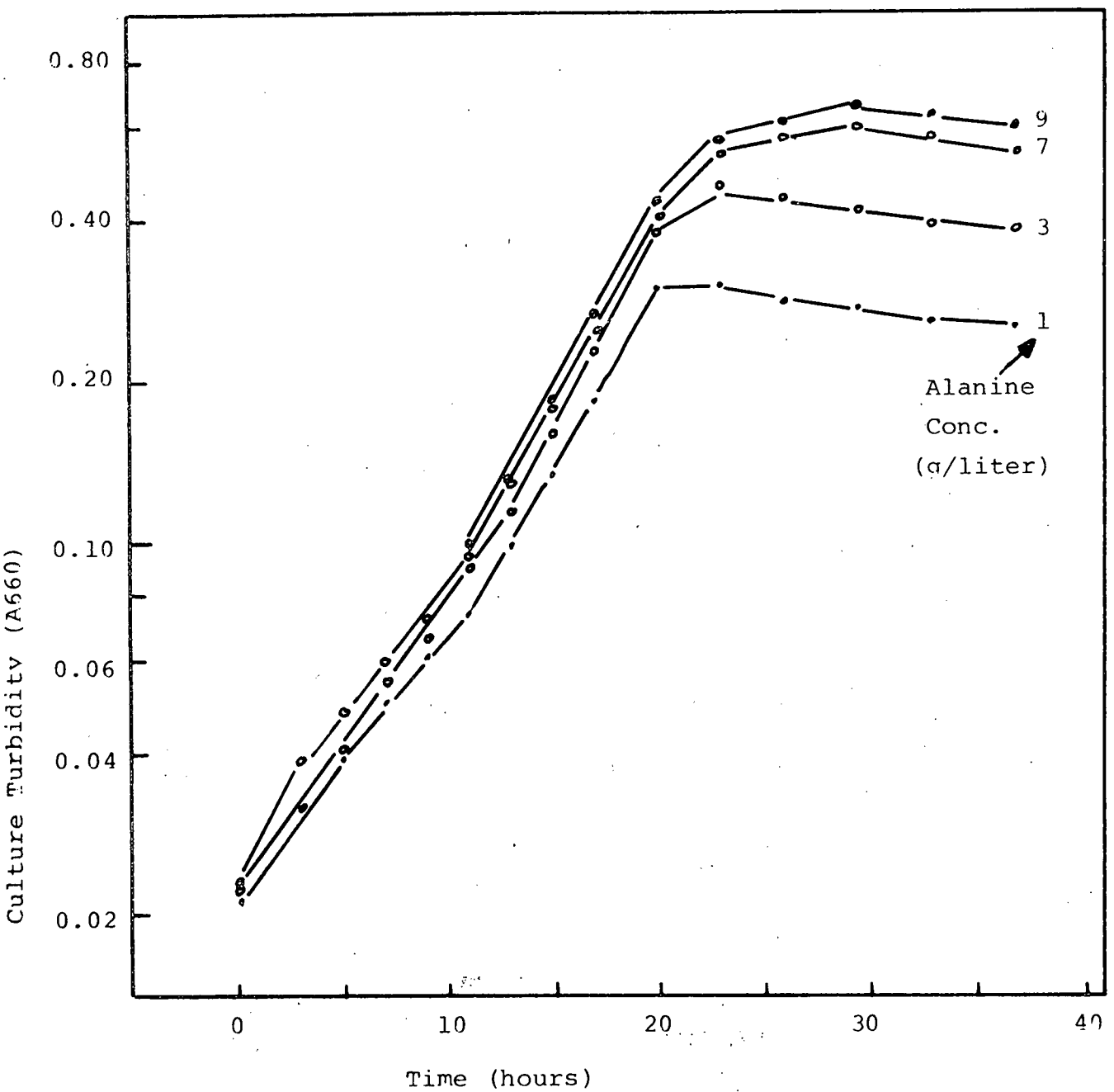
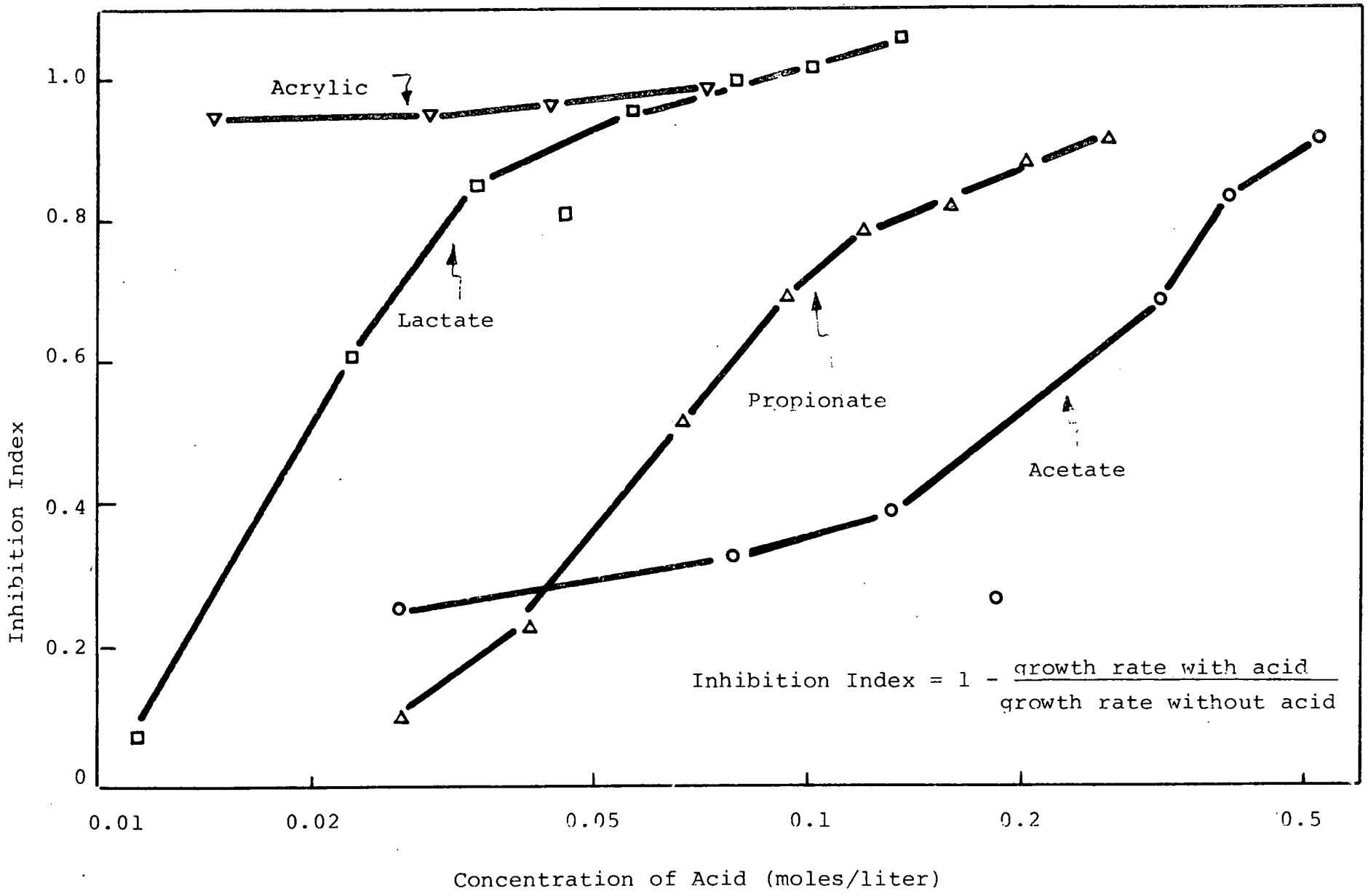


Figure 22: Inhibition of C. propionicum growth by Organic Acids



results of and products produced during a 48 fermentation by resting cells are summarized in Table 4.

At this time some general conclusions can be made. For example even though the organisms cannot grow on several of the substrates it is capable of fermenting them. For example acrylate, acetate and pyruvate may be fermented to acetate and propionate. Note that the results with the non-volatile acids are not satisfactory. No acrylic acid has been detected as a intermediate in the fermentation broths. This is expected under normal fermentation conditions.

Summarized in Table 5 are the molar ratios of acetate propionate after 48 hours of fermentation. Published values are also compared. The agreement is good for lactate, acrylate α-alanine and threonine. With serine and pyruvate more acetate is produced than reported. Such results will be confirmed. It is interesting to point our, however, that in the fermentation of both serine and pyruvate an unknown volatile acid having a retention time of 6.10 minutes is produced. Also during the fermentation of pyruvate and acrylate to unknown non-volatile acid is produced. The unknown fermentation products will be identified in future studies.

Conclusions:

1. Clostridium propionicum can be grown, plated transfered with minimal precautions regarding oxygen toxicity.
2. The organism is limited as to substrates available for growth.
3. Fermentation of a variety of substrates has results in expected fermentation and products. For several

Table 4

Fermentation by Resting Cells

Substrate	Products	3 hr	12 hr	24 hr	48 hr
		Concentration (mM)			
α-alonine	acetate	-	-	-	1.4
	propionate	1.2	2.5	3.1	3.5
β-alanine	acetate	-	-	-	-
	propionate	-	-	-	0.20
Serine	acetate	-	3.1	6.0	9.9
	propionate	.43	1.4	2.2	3.1
	unknown (6.1 min retention)	1.05%[X]		14.2%	25.2%
	pyruvate	-	15.6	77.7	132.
Threonine	propionate	0.12	0.96	2.0	2.9
	butyric	0.06	0.32	0.72	1.2
	unknown (6.8 min. retention)	-		14.7%	58.6%
	unknown non-volatile (1.2 min)	-		16.5%	43.0%
Acrylate		119	116	114	113
	acetate	-	1.7	2.7	3.1
	propionate	1.7	4.4	6.6	7.7
	unknown non-volatiles				
	2.85 min.	7.6%	8.3%	8.0%	8.0%
	3.3 min	3.1%	3.1%	3.4%	3.2%

X = unknown compound, concentration given as % total area on gas chromatogram.

Table 4 (cont.)

Substrate	Products	3 hr	12 hr	24 hr	48 hr
Lactate	-	157	142	134	124
	acetate	-	3.3	5.1	7
	propionate	2.7	7.4	11.0	15
Pyruvate	acetate	146	133	129	119
	propionate	6.4	133	13	15
	unknown (1.6 min. retention)	1.3	2.7	3.3	4.1
	volatile	33.1%	32%	29%	26%
	unknown-non-volatile (3.23 min)	7.1%	7.53%	7.1%	7.0%

Table 5

Production of Acetate and Propionate After 48 hrs. Incubation at 37 C by Resting Cells

Substrate	Acetate:Propionate	Published Values
α-alanine	1.0:2.5	1:2
serine	3.2:1	2:1
threonine	2.4:1	1:2
acrylate	1.0:5	1:2
lactate	1.0:2	2:1
pyruvate	3.8:1	

substrates, pyruvate, and acrylate and serine unknown fermentation products are produced.

Future Studies:

Future studies will concentrate on producing acrylic acid during fermentation. Initial studies will employ various biochemical inhibitors and electron acceptors that may result in acrylic acid formation.

Also additional studies will be conducted on acrylate toxicity and isolation of acrylate resistant strains will be initiated.

B.) Production of Acetone/Butanol By Fermentation

1. Introduction

As mentioned in the original proposal, the acetone/butanol fermentation was practiced industrially in large scale until the 1950's. It was therefore rationalized that research and development on this segment of the overall program should be performed on a sequential fashion. More specifically, since process data on this fermentation are available in the literature, it was felt that preliminary economic analyses can be performed using this information to assess the different cost sensitivities. These analyses can then be used to address the specific areas for research and development priorities which ultimately will lead to cost reduction. In this progress report we will present this economic assessment which we will then use to generate our reserach and development priorities.

2. Economic Analysis of the Acetone/Butanol Fermentation

a. Economic Assessment of Direct Manufacturing Cost for Acetone/Butanol Fermentation (Base Case Considerations)

The published process data from Davis and Stephenson (1941) and Beesch (1952) were used to analyse the direct manufacturing cost for acetone/butanol today if a batch process was to be employed. In addition, Dyr et al (1958) had published process information for this fermentation based on continuous operation. From these publications the following pertinent information shown in Table 6 were extracted for our analysis. It can be seen from Table 6 that the data are quite

Table 6

Process Data Employed for Economic Analysis of Acetone/ Butanol Fermentation (Clostridium acltobutylicum)

Type of System	Solvent Concentration	Solvent Yield gm solvent / gm sugar	Fermentation Cycle (hours)	Turn-Around Time	Reference
Batch	Butanol = 1.4% Acetone = 0.4% Ethanol = 0.08%	0.35	50	20	Davis & Stephenson (1941) Beesch (1952)
Continuous	Butanol = 1.5% Acetone = 0.8% Ethanol = 0.6%	0.35	480	80	Dyr et. al. (1958)

similar and therefore render these two analyses to be compared on a reasonable basis.

The economic analyses for the direct manufacturing cost for these two types of systems are shown in Table 7. It should be mentioned that for convenience the carbon source for acetone/butanol production was assumed to be blackstrap molasses which presently sells for 6 cents per pound of fermentable sugars. The batch fermentation was calculated using one 200,000 gallon fermentor similar to that reported by Beesch (1952). This system is able to produce a mixed solvent at a rate of 3.5×10^6 pounds per year. The total capital investment was estimated to be $\$2.66 \times 10^6$. The yearly operating cost was calculated to be $\$1.64 \times 10^6$. Of this total the major items were raw material, labor and fixed charges. Lastly, the direct manufacturing cost was estimated to be $0.47 per pound of the mixed solvent.

The continuous system employed one 60,000 gallon fermentor operated at a dilution rate of 0.2 $hour^{-1}$, similar to that reported by Dyr et al (1958). However, the annual production rate for the mixed solvent was nearly 4 times greater than the batch system (12.6×10^6 lbs/year.) On the other hand the total capital investment was slightly doubled that of the batch system ($\$5.65 \times 10^6$). The yearly operating cost breakdown showed that a much larger fraction of the total is now associated with the raw materials. Lastly, the direct manufacturing cost can be reduced significantly to be about $0.33 per pound of the mixed solvent.

Table 7

Estimated Direct Manufacturing Cost for Acetone/Butanol (Based on Assumptions in Table 6: Carbon Source - Blackstrap Molasses at 6¢/lb. Sugar)

Type of System	Annual Production (lbs.)	Total Capital Investment (TCI)	Yearly Operating Cost (YOC)	Direct Manufacturing Cost ($/lb)
Batch 200,000 gal.	3.5×10^6 lbs.	TCI = $\$2.66 \times 10^6$	YOC = $\$1.64 \times 10^0$	$0.47/lb
		% of total	% of total	
		Sterilizer & storage = 18.8%	Raw Mat'l = 39.7%	
		Fermentation = 7.9%	Utilities = 0.2	
		Separation = 13.3%	Labor = 26.0	
		Plant, Land, etc. = 42.2%	Fixed charges = 15.7	
		Engr. Constr. = 18.0%	Overhead = 5.7	
			Gen. Exp. = 1.2	
			Interest = 11.3	
Continuous 60,000 gal. Dilution Rate = 0.2 hr^{-1}	12.6×10^6 lbs.	TCI = $\$5.65 \times 10^6$	TYC = $\$4.14 \times 10^6$	$0.33/lb.
		% of total	% of total	
		Sterilizer & storage = 8.9%	Raw Mat'l = 56.7%	
		Fermentation = 2.3	Utilities = 0.2	
		Separation = 28.8	Labor = 17.7	
		Plant, Land, etc. = 42.0	Fixed Chrs. = 13.2	
		Engr. Constru. = 18.0	Overhead = 2.3	
			Gen. Exp. = 0.5	
			Interest = 9.5	

From these analyses one can conclude that significant cost reduction can result if the acetone/butanol fermentation was conducted on a continuous basis. In addition, significant fraction of the direct manufacturing cost is associated with the raw materials (primarily the carbon source). These conclusions offer us guides to potential avenues of research and development that will ultimately lead to cost reduction.

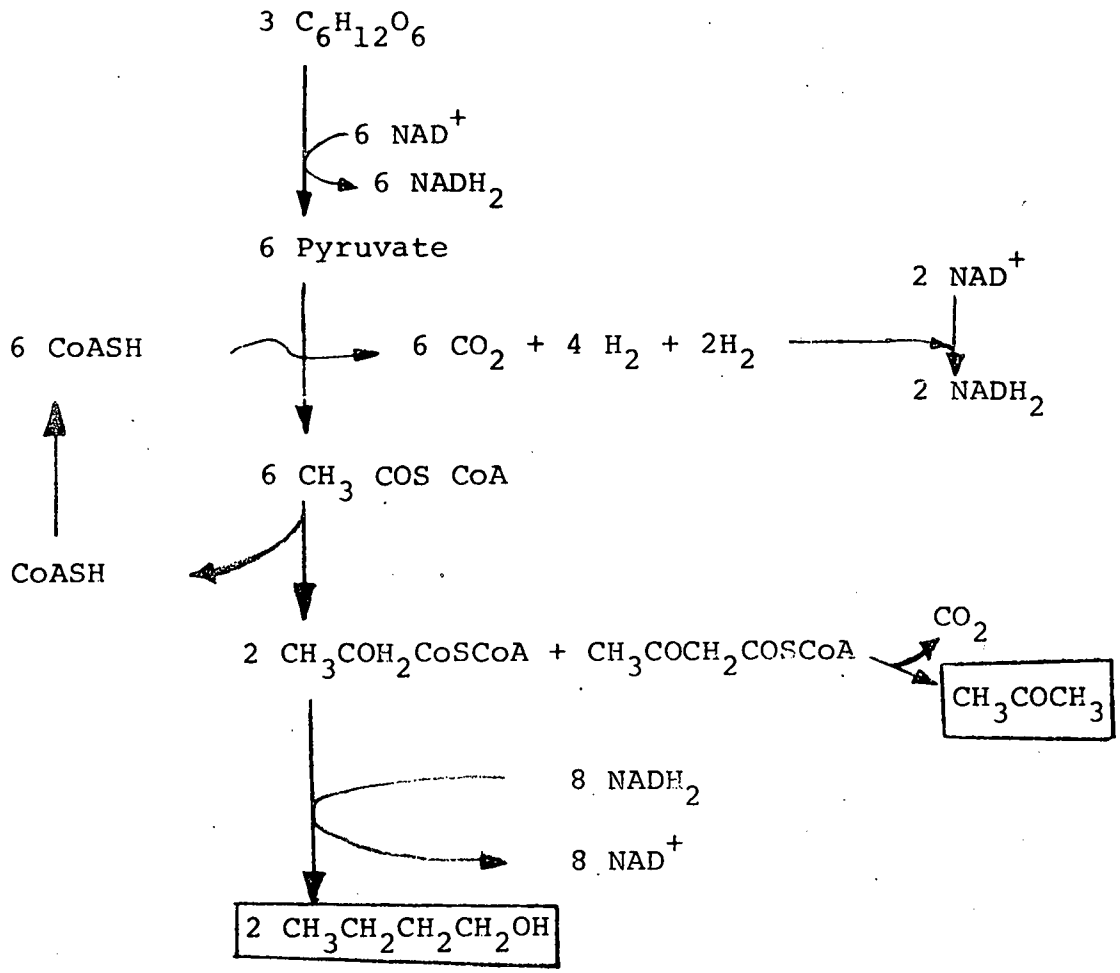
b. Analysis of Solvent Yield

The cost analyses shown in Table 7 indicated that the raw material cost constituted a substantial fraction of the total. It was therefore felt that an analysis on the theoretical yield of the mixed solvent should be made to see whether the assumed yield (0.35 lb. solvent/lb. sugar) can be increased significantly. Based on the fact that the ratio of butanol to acetone has usually been found to be 2:1, the biochemical pathway and stoichiometry were reasoned to be that shown in Figure 22. The pathway appears to be theoretically valid since the oxidations and reductions can be balanced as:

Reactions	$NADH_2$	NAD^+
EMP pathway	6	0
2 Mole But.	0	8
Ferredoxin (red. 2 H_2)	2	0
	8	8

It should be noted that hypothetically it is also possible to suppress the formation of acetone so that only butanol is formed. This type of a pathway was also considered as a alternate scheme

Figure 22: Hypothetical Biochemical Pathway for Acetone/ Butanol Fermentation



OVERALL:

$$3\ C_6H_{12}O_6 \longrightarrow 3\ CH_3(CH_2)_3OH + CH_3COCH_3 + 7CO_2 + 4H_2 + H_2O$$

to examine its effect on solvent yield. A summary of our assessment on the theoretical yields for this solvent fermentation is shown in Table 8.

From these results, it is predicted that for a mixed solvent fermentation containing only acetone and butanol, the theoretical maximum yield is only about 0.38 pounds of mixed solvent per pound ofglucose consumed. It should be noted that the energy recovered in the mixed solvent is about 84%. If one assumes that the formation of acetone can be suppressed, it is possible to increase the yield to 0.41 lb. of butanol per pound of sugar consumed. Here again the energy recovery is excellent accounting for nearly 95% of the energy input. In examining the calculated theoretical maximum yield (0.38 lb. solvent/lb. glucose) for the mixed solvent fermentation and comparing with those experimental values reported in the literature (0.30 to 0.35 lb. solv./lb. glucose), one must conclude that there is not much room for improvement. This is unfortunate since our economic analysis had shown the extreme economic sensitivity of this area if improvements can be achieved. In view of this conclusion one must search for other cost sensitive area(s) for process improvement.

C. Effect of Solvent Concentration on Cost

It was reasoned that an alternate approach to reduce overall production cost for acetone/butanol is by increasing the mixed solvent concentration. Using the batch and continuous processes shown in Tables 6 and 7 as base line

Table 8

Analysis on the Theoretical Yield of the Acetone/ Butanol Fermentation

REACTANTS	PRODUCTS	THEORETICAL YIELD $\frac{\text{lb solvent}}{\text{lb glucose}}$	% ENERGY RECOVERED
3 Glucose	2 Butanol 1 Acetone 7 CO_2 4 H_2 1 H_2	Butanol = 0.274 Acetone = 0.107 Y_{max} = 0.381	84.4%
1 Glucose	1 Butanol 2 CO_2 H_2O	Butanol = 0.411	94.9%

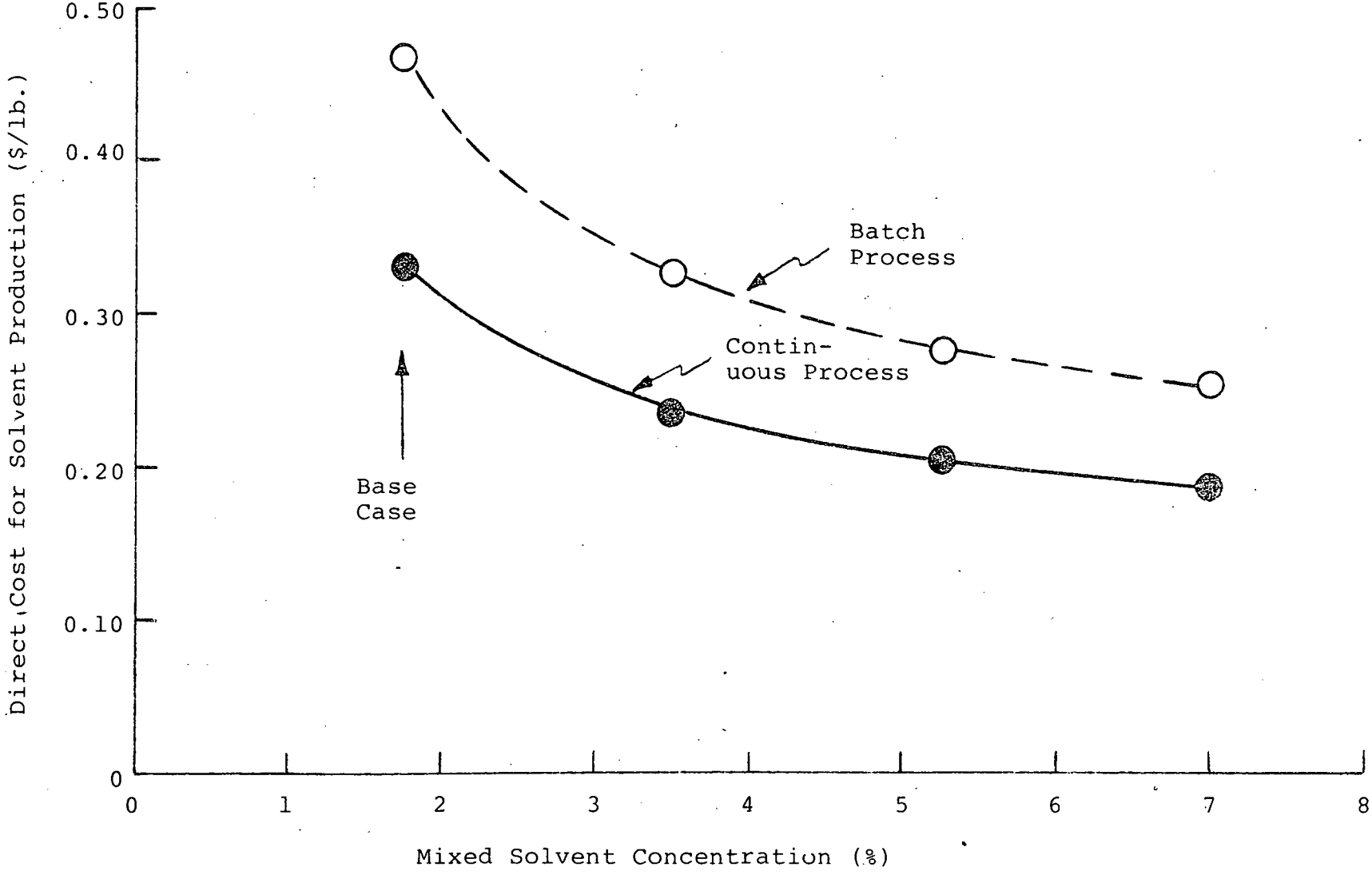
cases for comparison, the effect on increasing the solvent concentration on cost is shown in Figure 23. It can be seen that the reductions in cost are quite dramatic for both the batch and continuous processes. For example, for the batch process if the solvent concentration can be increased from the base case (1.75% solvent) to four times this value (7% solvent), the cost of production is reduced from $0.47/lb to $0.25/lb. The same situation when analysed for the continuous operation shows a reduction in cost of solvent production from $0.33/lb. to $0.19/lb. In view of these calculations it appears that increase in the solvent concentration is definitely a worthy goal to pursue for cost reduction.

d. Research and Development Priorities

As a result of the preliminary economic analysis, there are a number of research and development opportunities which can now be identified. At the present time we have begun to explore some of these avenues. Specifically our immediate future goals include:

- Examine different species of Clostridium which have been reported to produce acetone/butanol with respect to their nutritional requirements.
- Determine the experimental yields of solvent which these different species are capable of achieving.
- Determine the kinetic behavior of growth and solvent production.
- Exploration and determination on the process parameters needed for continuous operation.

Figure 23: Effect of Solvent Concentration (Acetone/Butanol) on the Cost of Production for Batch and Continuous Processes



- Determine the maximum solvent concentration which the best strain is able to produce.
- Mutation and strain selection to obtain culture with high solvent production capability and tolerance.

C.) Production of Acetic Acid By Fermentation

1. Introduction

It is well recognized that the cost of production for any bulk chemical will probably be heavily influenced by the cost of the raw material. In the previous section we had shown this to be true for the production of acetone/butanol. In order to find solutions to overcome this major and general problem, one should find microorganisms which can convert substrates such as glucose to products without the production of carbon dioxide. In this segment of the program we have focused on the unique capability of a thermophilic and anaerobic organism, Clostridium thermoaceticum, which can perform the following biosynthesis:

$$C_6H_{12}O_6 \longrightarrow 3\ CH_3\ COOH$$

Glucose ⟶ 3 Acetic Acid

This organism is unique since it is able theoretically to achieve quantitative conversion of one mole of glucose to three moles of acetic acid. Furthermore this is a homofermentative organism where no other product than acetic acid is produced. Lastly, this organism is capable of utilizing a number of carbon sources including glucose, fructose, xylose and pyruvate.

2. Materials and Method

Microorganism: Two cultures of Clostridium thermoaceticum have been kindly furnished by Dr. L. Ljungjahl

(U. of Georgia) and Dr. H.G. Wood (Case Western Research).

Experimental Equipment: The tube cultures, shake flask and agitated fermentor used in these studies have been reported in section I.A.2. The temperature of fermentation is 60°C. The cell growth can be followed optically using the Klett-Summerson or Bausch and Lomb spectrophotometer and calibrated for dry cell weights through gravimetric analysis. The calibrations show that:

260 Klett units (Red filter) = 1 gm (dry)/liter

Absorbance at 660 nm: 1.54 O.D. unit = 1 gm (dry)/liter

Medium Analytical Procedures: The medium composition used in our studies is shown in Table 9. The glucose concentration is determined using the Glucostat reagent kit (Worthington Biochemicals) or the continuous glucose analyser (Enzymax, Leeds and Northurp Co.)

We are presently completing our experimental protocals for determining the acetic acid concentration by gas chromatography.

3. Result and Discussion

Our preliminary studies using Clostridium thermoaceticum have cautioned us to the following. The organism is very sensitive to oxygen and strict anaerobic procedures must be employed during transfer, inoculation and sample removal. After the organism reaches the stationary phase of growth at

Table 9

Medium Composition for Growth of Clostridium thermoaceticum and Production of Acetic Acid

Ingredient	Concentration (gm/liter)
Glucose	18
$NaHCO_3$	16.8
K_2HPO_4	7
KH_2PO_4	5.5
Yeast Extract	5
Tryptone	5
$(NH_4)_2\ SO_4$	1
$MgSO_4 \cdot 7H_2O$	0.25
$Co(NO_3) \cdot 6H_2O$	0.029
Sodium thioglycolate	0.5
$Fe\ (NH_4)_2\ (SO_4)_2 \cdot 6H_2O$	0.20
$Na\ M_0\ O_4 \cdot 2\ H_2O$	0.12
$CaCl_2$	0.016
Trace Salts	1 ml

Trace Salts in gm/l: $MnCl_2 \cdot 4H_2O = 5$; $NaSe\ O_3 = 0.172$; $H_2BO_3 = 0.1$; $ZuCl_2 = 0.05$; $AlK\ (SO_4)_2 \cdot 12\ H_2O = 0.1$; $NiCl_2 \cdot 6\ H_2O = 0.02$; $CuCl_2 \cdot 2\ H_2O = 0.01$; EDTA = 5

60°C, loss of viability can be expected within 3 to 4 days. Cells from stationary phase of growth cannot be stored at low temperatures (4°C) for extended periods of time (2 - 3 weeks). During storage, anaerobiosis must be maintained. However, cells can be maintained on agar slants at refregerated temperatures for extended periods (> 3 weeks).

A number of tube culture, shake flask and agitated fermentor studies have been conducted in our laboratory. Shown in Figures 24 and 25 are two typical experiments performed in a 3 - liter liquid volume agitated fermentor at 60°C. The organism is able to grow rapidly with reproducible specific growth rate of 0.11 to 0.12 $hour^{-1}$ (mass doubling time of 6 to 6.4 hours). Since our gas chromatography assay for acetic acid is yet to be perfected, these experiments followed the glucose consumption during fermentation. The results in Figure 24 show that the glucose consumption rate is slightly greater than the cell growth rate. This is reasonable since some fraction of the glucose must be used for cell biosynthesis and remainder going to acetic acid production. The final acetic acid concentration was estimated since its value was sufficiently high and the use of GC is reasonably accurate. The results showed that 15.5 gm/liter of acetic acid had accumulated which corresponds to a conversion effeciency of 86% of the theoretical maximum.

Figure 24: Growth and Glucose Consumption (Acetic Acid Production) by Clostridium thermoaceticum (T = 60°C)

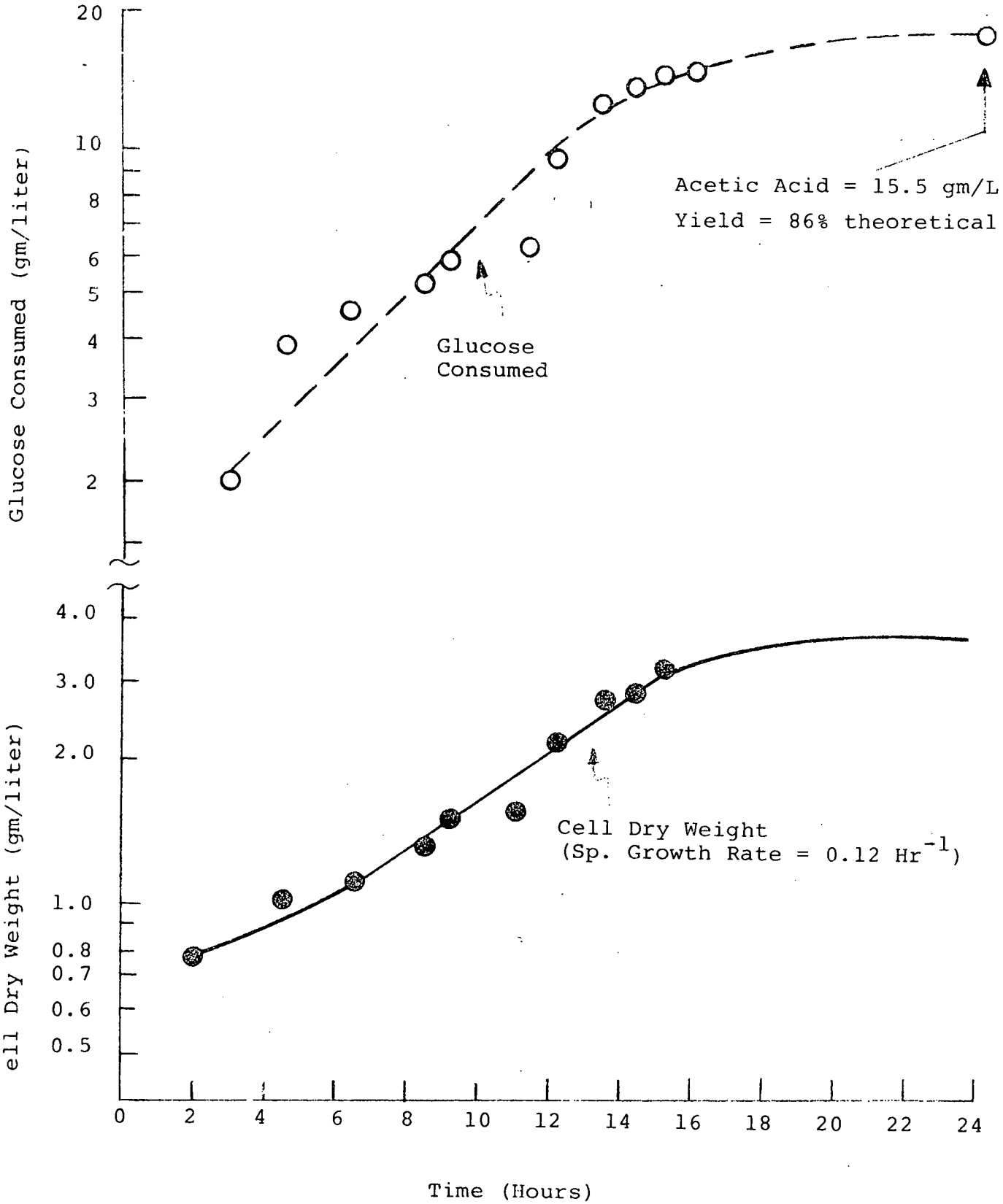


Figure 25: Growth and Glucose Consumption (Acetic Acid Production) By Clostridium thermoaceticum with Glucose Pulse Addition (T = 60°C)

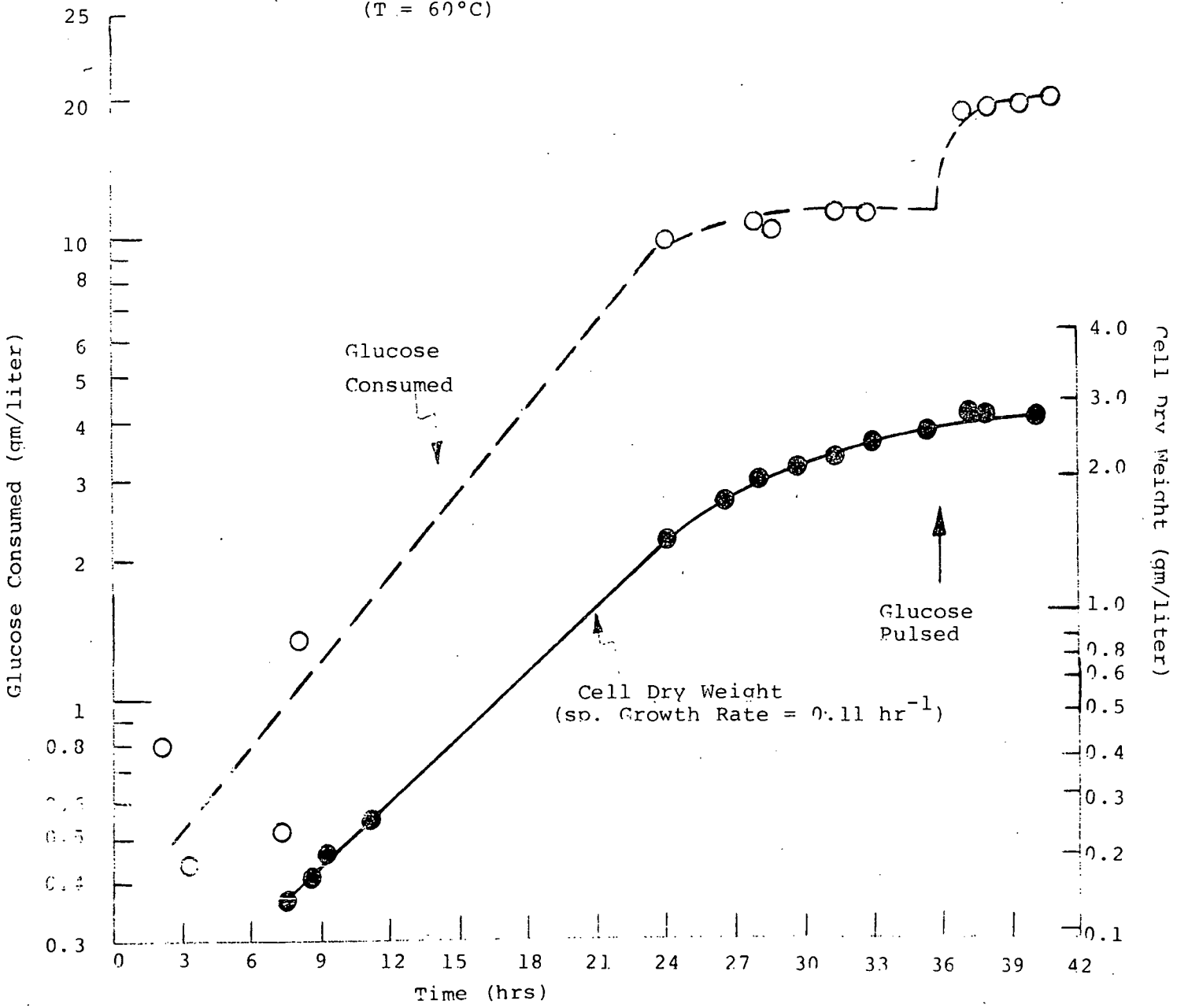


Figure 25 shows a second agitated fermentor study similar to that reported in Figure 24. In this experiment, however, a pulse of glucose was added after the organism had entered the stationary phase of growth (at 36 hours). A nearly instantaneous response in glucose consumption was noted after this addition. On the other hand we did not detect any further increase of cell growth following this glucose addition. These results are encouraging since they indicate that "resting cells" might be able to convert glucose to acetic acid. We are very encouraged with these preliminary results since the wild strain of this organism has already demonstrated its rapid growth characteristics accompanied by moderate acetic acid accumulation.

4. Future Research and Development

The future studies in the production of acetic acid will focus on:

- Quantifying the stoichiometry of the fermentation.
- Kinetic behavior of growth and acetic acid production in batch and continuous culture.
- Nutritional requirement of Clostridium thermoaceticum to obtain a defined medium without organic nitrogen sources.
- Study the potential of cell recycle along with "resting cells" for acetic acid production.
- Examine the use of soluble reducing sugars from cellulosic biomass produced by other organisms as carbon source for acetic acid production: Exploration of mixed-culture studies.

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APPENDIX

1. CMCase assay

To 1ml of 2% Sigma CMC suspended in 0.2M citrate buffer (pH4.5) is added up to 0.2ml broth supernate. Two types of controls are run: A, omitting cellulose and B, substituting buffer for broth supernate. Tubes are incubated at 60 C for 1 h and cooled in ice. Three ml dinitrosalicylic acid reagent (DNS) are added. The tubes are boiled for 5 min, cooled in ice and the absorbance determined at 550nm. Standard curves are also run containing 0, 0.2, 0.4, 0.6 and 0.8 mg/ml glucose in 1.2ml water. The absorbances of the two controls are subtracted from the sample absorbance and the resulting value converted to mg reducing sugar by use of the standard curve. The following calculation is used to obtain units/ml.

$$\text{mg reducing sugar/hr/0.2ml enzyme} \times \frac{5}{60} \times \frac{1000}{180} = \mu \text{ moles reducing sugar per min per ml enzyme}$$

$$= \text{units/ml}$$

2. TNP-Case

To 2ml of a 1% suspension of trinitrophenyl-cellulose in 0.05M citrate buffer (pH 4.5) containing 0.001M ditheothreitol is added up to 1ml of broth supernate. A zero time sample is millipore-filtered immediately. A control is run substituting buffer for broth supernate. The tubes are incubated 1 h at 60 C and millipore filtered. Absorbance is read at 344 nm, the increase in absorbance between 0 and 60 min is calculated, and the control value is subtracted from the sample value. The assay is reported in OD units/hr/ml of enzyme.

3. Media

	1*	D58**	FQD**
Cellulose MN300	9.7g	-	-
Cellulose Avicel	-	13g	13g
KH_2PO_4	1.5g	1.4g	1.4g
$K_2HPO_4 \cdot 3H_2O$	2.9g	7.2g	7.2g
$(NH_4)_2SO_4$	1.3g	1.3g	1.3g
$MgCl_2 \cdot 6H_2O$	1g	2.6g	2.6g
$CaCl_2$	150mg	130mg	130mg
$FeSO_4 \cdot 7H_2O$	1.25mg	1.1mg	1.1mg
Resazurin	20mg	-	-
Yeast extract	2g	4.5g	-
L-cysteine	1g	-	70mg
Na β-glycerophosphate	-	6g	6g
Glutathione	-	250mg	250mg
Thiamine · HCl	-	-	200µg
Riboflavin	-	-	200µg
Ca panththenate	-	-	200µg
Pyridoxine	-	-	200µg
Biotin	-	-	2µg
Folic acid	-	-	8µg
pABA	-	-	4µg
DL-β-Phenylalanine	-	-	80mg
L-Tyrosine	-	-	50mg
DL-Tryptophan	-	-	70mg
L-cystine	-	-	60mg
L-methionine	-	-	70mg
pH adjusted to	7.8	-	-

*Medium 1 is similar to that of Weimer and Zeikus (Appl. Env. Microbiol. 33, 289, 1977) except that it contains 1g/l L-cysteine instead of 500mg/l L-cysteine plus 500mg/l $Na_2S \cdot 9H_2O$.

**Media described by Fleming & Quinn, Appl. Microbiol. 21 967 (1971).

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