DIRECT CONVERSION OF CELLULOSIC BIOMASS TO ETHANOL BY MIXED CULTURE FERMENTATION OF *CLOSTRIDIUM THERMOCELLUM* AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM*

by

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GEORGE COSTAS AVGERINOS

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ABSTRACT

A process utilizing a mixed culture of thermophilic and anaerobic bacteria, *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*, has been investigated for the single step direct conversion of agricultural residues such as corn stover and wheat straw to ethanol. However, in addition to ethanol, wild type strains also produce a variety of other products including acetic and lactic acids. Deregulated high ethanol yielding strains of both microorganisms have been isolated. These mutants were used in mixed culture for the conversion of refined cellulose such as Solka floc to ethanol attaining 80% of theoretical maximum yield. However, it has been shown that lignin in biomass substrates such as corn stover results in a recalcitrant structure in which a much lower rate and extent of carbohydrate degradation and ethanol yield are obtained. Extracted lignin from corn stover has been shown to be toxic to some strains of *Clostridium thermosaccharolyticum* and precipitates the cellulases of *Clostridium thermocellum*.

In order to overcome this drawback, a selective solvent pretreatment process with alkaline/ethanol/water mixture at low temperature has been examined. The delignification process, performed under appropriate conditions, is accompanied with very little loss of fermentable carbohydrates. Up to 69% delignification of corn stover has been obtained while 95% of the cellulose and pentosan carbohydrates remain insoluble. The kinetics of delignification, substitution of bases and methods for extracted solvent recycle have been examined. The kinetics of subsequent mixed culture fermentation of this

treated corn stover has shown a 40% increase in the rate of degradation with greater than 85% utilization of the carbohydrates. In spite of these increased rates and extents of carbohydrate degradation through pretreatment, reduced ethanol yields to approximately 65% of theoretical maximum were obtained during mixed culture fermentation on this substrate.

A correlation using batch and continuous culture data on the effect of growth rate on the ethanol yield of each organism was obtained. The results demonstrate that these strains, selected for high ethanol yield at high growth rates, have characteristically reduced ethanol yields at decreased specific growth rates. A detailed analysis of the kinetics of insoluble carbohydrate degradation and uptake during growth on corn stover was also performed. These results demonstrate a strong dependence of specific carbohydrate hydrolysis rate on the extent of substrate degradation. Under the carbohydrate degradation rate limited condition of growth during mixed culture fermentation, a kinetic model for this performance has shown that the ethanol yields observed are identical to those predicted at the low specific growth rates.

A final limiting factor to this process was shown to be the production of a growth inhibiting extracellular component by *Clostridium thermocellum*. This factor was shown to be resistant to Pronase, Trypsin, DNAase or RNAase treatment and had a molecular weight between approximately 500 and 10,000 as determined by dialysis and ultrafiltration studies. This factor was produced by *Clostridium thermocellum* at times corresponding to the termination of growth in cellobiose or Solka floc fermentation. This factor was shown to inhibit further cell growth and caused rapid lysis and loss of viability of the cells. A similar rapid loss of cell viability was observed during mixed and monoculture growth of *Clostridium thermocellum* on corn stover.

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1. INTRODUCTION

The fermentation technology for the production of ethanol by yeast was reported by Demain to have been practiced for over 2,000 years[1]. Today, similar to the past, the primary raw material for ethanol production, as well as many other industrially important fermentations, are carbohydrates in the form of soluble monomers or easily hydrolyzed starch polymers. However, for longer range considerations, one cannot neglect the use of alternate raw materials as fermentation feedstocks. A variety of feedstocks, including methanol, synthesis gas, and hydrocarbons, have been examined in many new fermentation processes. In particular, cellulosic biomass, such as agricultural, forestry and municipal residues, represent a large quantity of carbohydrates potentially utilizable as a fermentation feedstock. The production of ethanol, a highly raw material cost intensive fermentation, stands to benefit greatly from the use of these significantly less expensive alternate raw materials.

Agricultural cellulosic residues, such as corn stover, wheat straw and rice straw, are particularly attractive alternate renewable feedstocks due to their concentration and the technology and equipment for their collection. A growing need for alternate sources of transportation fuels and chemicals has

---

[1] Not first-hand knowledge, of course

fostered a renewed interest in processes for the hydrolysis and fermentation of these materials. For example, in the United States, 6.4 x 10^9 bushels of corn were produced in 1979. Corn stover, which consists of the leaves and stalks of the plant after removal of the corn, is produced in approximately equal amounts. If converted to ethanol at theoretical yield and using 100% of the available carbohydrates, this amount of biomass represents 14,000 million gallons of ethanol: roughly 10% of the domestic gasoline consumption in 1979. However, there are numerous potholes on the road to realization of this potential. If a yeast such as Saccharomyces cerevisiae is used for ethanol production, only the hexose portion of the biomass (approximately 55%) can be fermented. In addition, these sugars must be hydrolyzed prior to fermentation. Earlier studies have used high temperature or concentrated acid pretreatment or, more recently, by the addition of cellulases produced by fungi such as Trichoderma reesei. However, recurring drawbacks to these multi-step approaches have been high costs and low yields of fermentable sugars.

As an alternative to these multi-stage methods, we have considered a single step hydrolysis and fermentation process of insoluble cellulosic biomass to ethanol with the thermophilic, cellulolytic, anaerobic Clostridium thermocellum. Both the cellulose and hemicellulose fractions making up 32 and 37%, respectively, of a substrate such as corn stover can be hydrolyzed by this organism.

The hexoses produced are metabolized predominantly to a mixture of ethanol, acetate and lactate by the wild type strain of *Clostridium thermocellum*. However, this organism, like yeast, cannot ferment the pentoses from hemicellulose. In order to increase the economic potential of this feedstock intensive fermentation to achieve higher utilization of all of the available carbohydrates in corn stover, a second thermophilic and anaerobic bacterium, *Clostridium thermosaccharolyticum*, able to metabolize these pentoses, can be included in a mixed culture. In this manner, a single step direct conversion process for the production of ethanol in high yield from insoluble cellulosic biomass can be envisioned.

The ultimate objective of this thesis research has been the investigation and development of this promising concept for the direct production of ethanol from renewable cellulosic biomass. In order to accomplish this objective, a detailed examination of the limitations of real biomass utilization by these organisms has been required. This has entailed an examination of biomass toxicity and an investigation of the physical and chemical properties limiting the potential for enzymatic utilization of this material. A better understanding of these factors has enabled us to logically examine the pretreatment of this biomass substrate in order to promote its utilization. In addition, a detailed understanding of the kinetics of growth and substrate hydrolysis and utilization by these organisms during mixed and

monoculture growth on these "multiple insoluble substrate" carbon sources is an important objective. In this manner, strain improvement programs can be more accurately directed to overcoming the limitations in this system to achieve the desired goal.

2. LITERATURE SURVEY

2.1. Historical Perspective

The first observation of the thermophilic anaerobic fermentation of cellulose is generally attributed to MacFayden and Bloxell in 1899. A number of investigators subsequently isolated organisms with these properties from ruminant fecal samples [1,2]. Vigorous promotion of thermophilic cellulolytic fermentation for the production of useful chemicals has resulted from the work of Langwell and Lynn in the 1920's [3]. These investigators isolated numerous thermophilic mixed cultures from nature capable of degrading waste cellulosics to acetic acid, ethanol, and fuel gas. However, they could not obtain a practical cellulose fermentation with "purified" cultures. Their use of unknown mixed isolates, however, demonstrated ethanol yields of 27% from sulfite pulp, 8% from filter paper, 15% with xylose from rice straw, and 89% from corn cob medium during pilot plant trials. As could be expected, however, reproducible fermentations were difficult to achieve with these ill-defined cultures, and further industrial interest in this approach waned. Meanwhile, attempts to isolate pure cultures were continued by other investigators in the 20's and 30's. In 1926, Viligoen, Fred, and Peterson isolated a thermophilic, cellulolytic, anaerobe and designated it *Clostridium thermocellum* [4]. Subsequent attempts by Tetrault,

Snieszko, and Imsenecki, to verify this isolate's purity or to obtain one themselves were all unsuccessful [5,6]. Improvements in anaerobic techniques finally brought about by Hungate in 1944 [7] subsequently enabled Enebo and McBee to independently isolate and characterize an organism with similar properties identified as Clostridium thermocellulaseum and Clostridium thermocellum, respectively [8,9].

Meanwhile, the fermentative production of ethanol from cellulosics had been commercialized in an alternate manner. Chemical hydrolysis of woodchips with dilute H_2SO_4 followed by yeast fermentation was patented in 1913. A 5,000 gallon ethanol/day plant at 95% concentration was built in the United States. Unfortunately, the availability of sawdust was soon exhausted, and transportation cost proved to be too high thus forcing abandonment of the process by the mid 1920's.

Widespread raw material shortages during World War II revived interest in this technology in both Germany and the United States. Numerous process improvements were added by Schoeller. However, with the war coming to an end, fermentatively derived ethanol rapidly lost ground to ethanol obtained from ethylene hydration. In 1979 98% of industrial alcohol was produced from ethylene. However, in 1981, this figure is expected to drop to 85% due to competition by cheaper ethanol obtained by corn fermentation processes [10]. It is evident that the supply picture for petroleum based feedstocks is again changing, making it increasingly important to examine alternate

technology for ethanol production with the aid of today's better understanding of these biological systems.

2.2. Thermophilic Anaerobic Fermentation

2.2.1. Properties of Clostridium thermocellum

Although first named in 1926, the definitive characterization of this obligately, anaerobic, thermophilic and cellulolytic bacterium was not accomplished until the 1950's by McBee, Enebo, and subsequent workers. C. thermocellum (ATCC 27405) is described as a gram negative rod 0.6 x 4 microns in size forming terminally spherical spores on solid but not in liquid medium. The optimum temperature for growth has been reported between 58 and 64°C and pH between 6.4 and 7.4. The organism has been reported to grow on cellulose, cellobiose, glucose, fructose, and mannitol by Patni and Alexander [11]. Glucokinase activity was shown to be inducible by glucose but absent during growth on cellobiose or cellulose resulting in accumulation of the disaccharide. Fructose and mannitol utilization was shown to occur through phosphoenopyruvate (PEP): phosphofructotransferase and PEP: phosphomannotranserase/dehydrogenase activity also inducible in the presence of these substrates. Ng, Weimer, and Zeikus, however, reported that C. thermocellum was only capable of growth on cellulose and cellobiose [12]. Although these reports may be due to actual strain variations, it should also be pointed out

that they may arise from two unusual features of this organism. These include the relative ease in which single colony isolates can sporadically contain morphologically similar non-cellulolytic contaminants (such as Clostridium thermosaccharolyticum) and the very long lag time in the presence of at least 0.4% yeast extract which was required to demonstrate growth on glucose, mannitol, or fructose. A defined media has been developed for C. thermocellum to include thiamine, riboflavin, pantothenate, pyridoxine, biotin, folic acid, para aminobenzoic acid, and the amino acids phenylalanine, tryosine, tryptophan, cystine, cysteine, and methionine [13]. However, more recent media studies have shown that these requirements can be reduced to only biotin, pyridoxamine, B_{12}, and p-amino benzoic acid in minimal salts media [14].

The major fermentation products of the wild type organism are acetate, ethanol, lactate, CO_2 and H_2, along with smaller amounts of butyric acids [15]. However, through a program of serial transfer in media containing increasing concentrations of ethanol a strain has been developed which produces ethanol as the major product [16]. Dissimilation of fermentable carbohydrate occurs primarily through the Embden-Meyerhoff pathway to pyruvate; however, the enzymes of the hexose monophosphate path have also been demonstrated in lesser amounts [17]. Pyruvate can be further metabolized to lactate with NAD^+ linked lactate dehydrogenase or decarboxylated

to acetyl CoA with ferredoxin oxidoreductase. This enzyme has been shown in C. acidi urici to contain 1 mole of thiamine pyrophosphate, iron, and sulfur and catalyzes the decarboxylation of pyruvate with a concomittant two electron transfer to oxidized ferredoxin [18]. The reduced ferredoxin can be reoxidized with a hydrogenase liberating hydrogen or by reducing NAD^+ or $NADP^+$ [19]. Acetyl CoA can be subsequently converted to acetyl-P and acetic acid with the production of ATP or twice reduced with NADH to form ethanol.

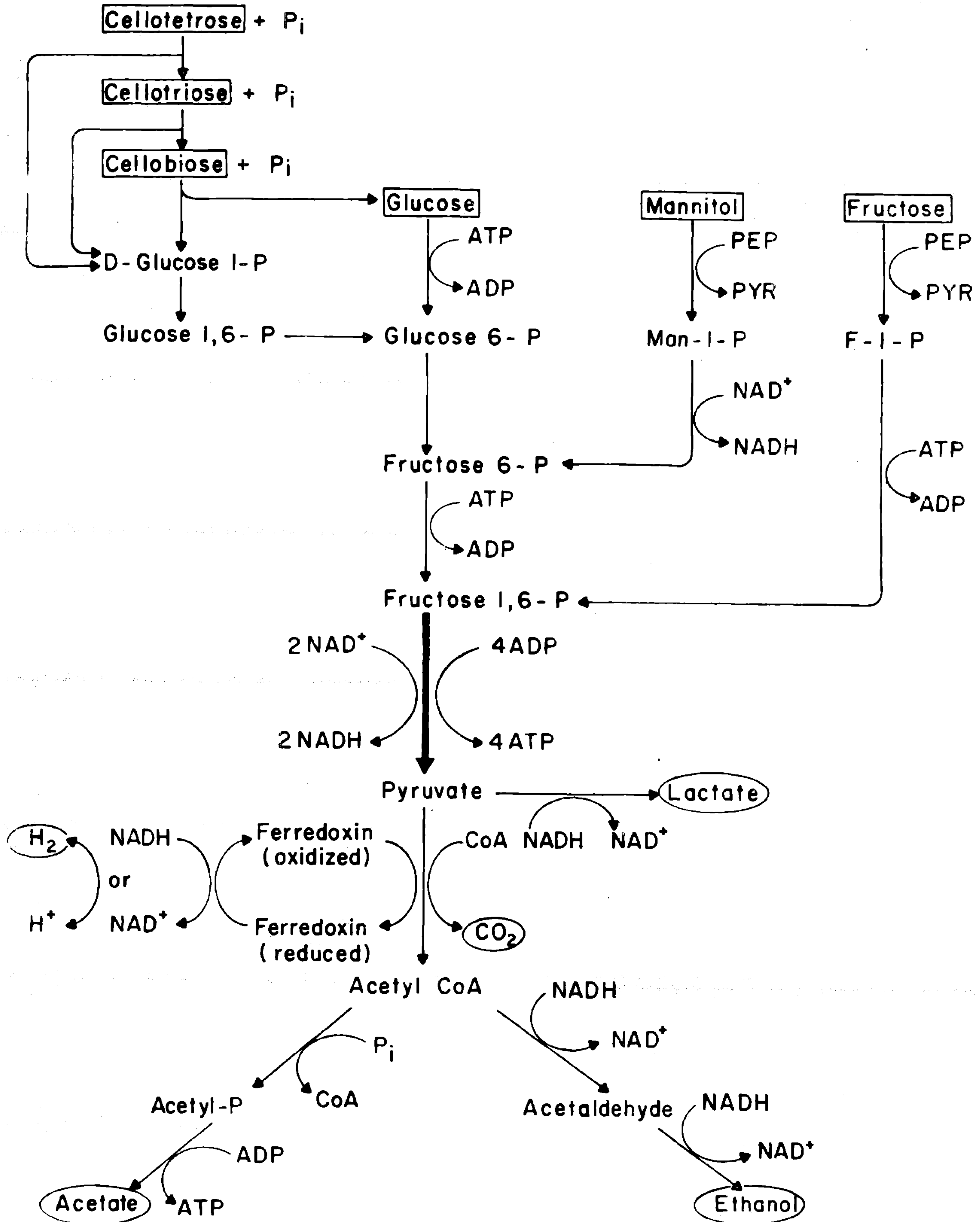
In summary, the overall balanced fermentation from 1/2 mole of glucose can be theoretically made through any of the reactions shown below:

	$\Delta G / \frac{1}{2}$ mole glucose	Net ATP Yield by Substrate Level Phosphorylation
$\frac{1}{2}$ glucose → 1 Lactate	23.7 Kcal	+ 1 ATP
→ 1 Ethanol + $1CO_2$	27.0 Kcal	+ 1 ATP
→ 1 Acetate + $1CO_2$ + $2H_2$	24.7 Kcal	+ 2 ATP

The various pathways are shown in Figure 1.

Figure 1

PROPOSED PATHWAYS OF CARBOHYDRATE METABOLISM IN C. THERMOCELLUM



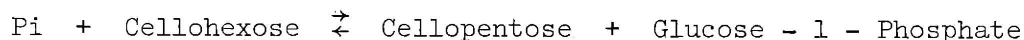
2.2.2. Cellulase

C. thermocellum produces a large quantity of extracellular cellulase recently purified by Ait *et al*. [20]. These investigators demonstrated that after 13 hours of growth on purified cellulose (MN300) only 10% of the total cellulase activity could be detected in the supernatant. After 60 hours, approximately 30% could be freed after centrifugation of the cells from the substrate. Although strong adsorption of cellulase to the substrate was evident, it could not be discerned whether this was the result of saturation of cellulose with a single enzyme or the production of different enzymes during the course of the fermentation.

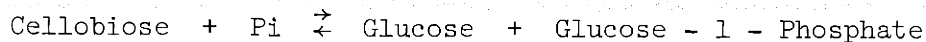
Gel filtration of the protein remaining in solution at the end of the fermentation on Bio Gel P-200 (1 x 80 cm) revealed a single protein peak of 125,000 $\pm$ 10,000 MW. Subsequent sodium dodecyl sulfate gel electrophoresis of this peak yielded at least five discrete bands (some with cellulase activity) forming this unusually large complex. Reducing sugar analysis of the H_2SO_4 hydrolyzed protein revealed a carbohydrate content of the complex to be 9.9% (W/W). Whether this result indicates the presence of glycoproteins stabilizing this thermophilic cellulase, or represents cellodextrins strongly bound in the active sites of a cellulase complex of homogeneous molecular weight was not concluded by these authors. The cellulase from this organism is also unique as compared to fungal cellulase in that it appears to be constituitively produced.

However, recent studies by Johnson et al. with minimal media have shown a significantly lower cellulase activity when cells are grown on sorbitol. The addition of yeast extract or growth using cellobiose or cellulose increases the level of cellulase production by a factor of approximately five [21].

Although the entire mechanism of sugar hydrolysis and transport in C. thermocellum is not completely understood, a number of intermediate enzyme activities have been isolated. Sheth and Alexander partially purified a cellodextrin phosphorylase activity from cell sonicates with the following activity [22]:



This enzyme also displayed analogous activity with cellopentose, cellotetrose, and cellotriose, but was unable to cleave cellobiose. However, a separate enzyme, cellobiose phosphorylase, was also isolated from sonicated cells and catalyzed the phosphorolytic cleavage of cellobiose [23]:



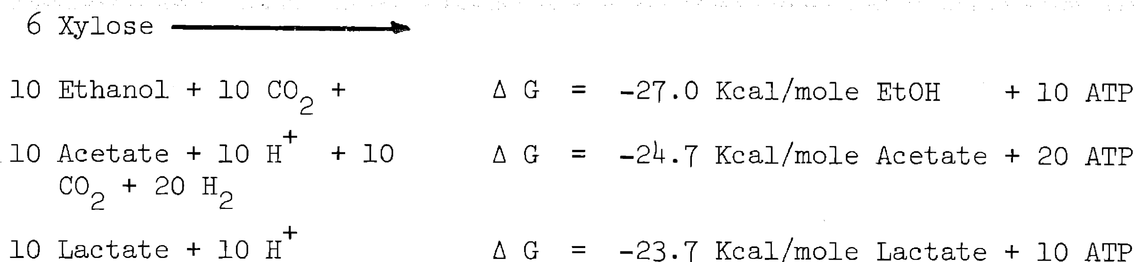
The km for cellobiose during phsophorolysis was 7.3 mM, 29 mM for phosphate, and a K_I of 1.2 mM was reported for glucose. This

enzyme was also capable of using a variety of glucosyl-P acceptors in the reverse reaction including D-xylose. Recently, Ait and co-workers have reported the isolation and purification of a β-glucosidase from the periplasmic space of C. thermocellum [24]. Studies by Gordon at M.I.T. have also indicated the presence of an alternate cellobiose hydrolase activity although the K_M reported for this "β-glucosidase" is very high (28 g/l glucose) [25]. From this evidence, it seems reasonable to speculate that cellodextrins up to 6 glucose units in length in the periplasmic space can be phosphorolytically cleaved conserving the ATP equivalent of each glycosidic bond by being transported (?) into the cell. Studies with Cellovibrio gilvus growing on cellodextrins show an increased cellular yield as the size of the cellodextrin is increased and thus further supporting the above hypothesis [26]. The final cleavage of each cellodextrin by cellobiose phosphorylase results in the formation of one mole of glucose-1-phosphate and a free non-phosphorylated D-glucose molecule. Due to the uninduced state of glucokinase under these conditions, D-glucose produced from this reaction or through the action of B-glucosidase may further inhibit the action of cellobiose phosphorylase allowing cellobiose to accumulate. Thus, D-glucose, and to a lesser extent cellobiose, are free to diffuse from the cell and are found in the medium during growth on cellulose [30]. Since these reactions are reversible in vivo, the accumulation of polysaccharides is also likely and was demonstrated during growth of C. thermocellum on glucose by S. Wang [27].

2.2.3. Clostridium thermosaccharolyticum

C. thermosaccharolyticum was first isolated by McClung in 1935. This obligately and anaerobic thermophile (ATCC 7956, NCIB 9385) displays a temperature optimum similar to C. thermocellum and a pH optimum between 5.8 and 7.0. This gram negative motile rod 0.4 x 7 μ in size forms terminal spores under conditions of restricted growth [28]. C. thermosaccharolyticum has been shown to ferment a variety of carbohydrates including glucose, cellobiose, fructose, xylose, arabinose, maltose, mannose, and lactose, as well as being capable of growing on starch and xylan. The major products of fermentation are acetate, ethanol, and lactate, with smaller amounts of butyrate occasionally detected [29]. Mutation and selection of this organism for increased tolerance to ethanol has resulted in a strain HG-4 in which ethanol is the predominant product of the fermentation [30]. The fermentation of hexoses is believed to occur primarily through the Embden-Meyerhoff pathway in which all of the enzymes have been demonstrated [31]. The metabolism of pentoses by some Clostridia has been thoroughly investigated by Cynkin and Giggs [32]. The fermentation of C^{14} labeled D-xylose and D-ribose by C. perfringens, C. beijerinckii, and C. botulinum was demonstrated to result from the action of transaldolase and transketolase in the pentose phosphate cycle followed by dissimilation of the fructose -6- phosphate through the Embden-Meyerhoff path. In this respect, 6 xylose units are

effectively equivalent to 5 glucose units resulting in the net stoichiometry shown below:



6 Xylose $\longrightarrow$

10 Ethanol + 10 CO_2 +	ΔG = -27.0 Kcal/mole EtOH	+ 10 ATP
10 Acetate + 10 H^+ + 10 CO_2 + 20 H_2	ΔG = -24.7 Kcal/mole Acetate	+ 20 ATP
10 Lactate + 10 H^+	ΔG = -23.7 Kcal/mole Lactate	+ 10 ATP

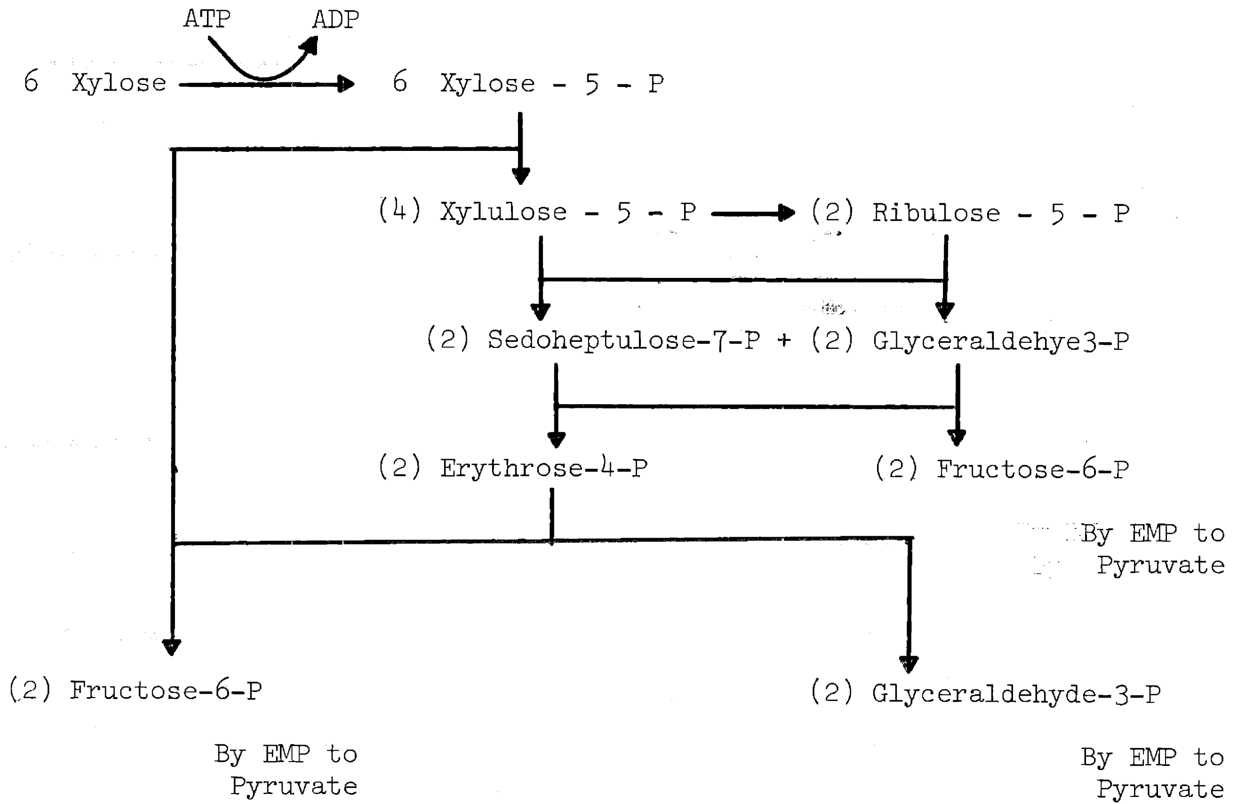
The thermodynamics of this conversion are similar to that seen from glucose as are the yields of ATP per mole of end product produced. The sequence of reactions leading to intermediates in the Embden-Myerhoff pathway are shown in Figure 2.

2.2.4. Growth Inhibitory Factors of Clostridia

Microorganisms produce a vareity of substances which have been shown to limit their subsequent growth. The production of metabolic end products of fermentation, especially by anaerobes often represents such a limiting factor. However, numerous examples of growth inhibition and cell lysis have been demonstrated where inhibition cannot be attributed to the major fermentative end products or nutrient limitations in the media. For Clostridia, in general, a number of lytic enzymes, autolysins or bacteriocins, as well as defective phage particles have been implicated in such autoinhibitory events.

Figure 2

PENTOSE PHOSPHATE PATHWAY



By Pentose Phosphate Path =

$$6 \text{ Xylose} + 10 \text{ NAD}^{+} + 10 \text{ ADP} \longrightarrow 10 \text{ Pyruvate} + 10 \text{ NADH} + 10 \text{ ATP}$$

By Embden Myerhoff Path:

$$5 \text{ Glucose} + 10 \text{ NAD}^{+} + 10 \text{ ADP} \longrightarrow 10 \text{ PYR} + 10 \text{ NADH} + 10 \text{ ATP}$$

In the commercially important acetone/butanol producing organism, C. acetobutylicum, an autolytic enzyme has been isolated [33]. The appearance of this protein in the fermentation broth occurs towards the end of exponential growth phase and is accompanied by cell lysis and cessation of solvent production. The active factor is shown to be a glycoprotein having a molecular weight of 28,000 daltons. It has been purified to homogeneity by acetone precipitation of the broth followed by Sephadex chromatography and polyacrylamide gel electrophoresis (PAGE). The protein has no activity on DNA, RNA, or protein synthesis but was shown to rapidly lyse isolated cell walls of C. acetobutylicum. The protein was partially inactivated by protease addition.

In C. botulinum another autolysin has been shown to be released from the cell walls in late logarithmic growth phase as the cells lyse. At the same time botulinum toxin also appears in the medium [34]. These autolysins have been purified by PAGE gels and shown to contain 2 major and 2 minor bonds [35]. Their enzymatic activity is highly specific for C. botulinum cell walls and corresponds to N-acetyl muramyl-L-alanine amidase and an N-acetyl-glucosaminidase activity. Soluble reducing sugars are produced from the cell wall breakdown products. These enzymes were shown to be insensitive to protease treatment by Nagarase, Trypsin, or Pronase.

In another Clostridial species, C. perfringens, autolytic enzyme activity has also been demonstrated. An endo-

β-N-acetylglucosamidase and an endo-β-N-acetylmuramidase activity have been demonstrated in the type strain NCTC 3237 [36]. However, in *C. perfringens* strain BP6KA the presence of this autolytic activity was not observed. Studies with *C. perfringens* K2 219 have also shown an inducible autolysin activity [37]. In this strain, the addition of Mitomycin C or exposure to U.V. light induces autolysis. The addition of chloramphenicol prevented subsequent autolysis activity. The activity was found in a presumably phage free lysate obtained by centrifugation at 100,000 x G for 90 minutes. The lytic activity was heat labile and partially inactivated by proteases. The autolysin was shown to be highly species specific. Fifty strains of *C. perfringens* type A-E were susceptible while only 5 of 90 strains of 52 other Clostridial species were shown to be affected. Further research with *C. perfringens* has shown that at least 10 different autolysins with various activity spectra are produced [38]. In many cases, stable spheroplasts, practically devoid of cell wall can be formed if autolytic enzyme treatment is accomplished under isotonic conditions [39]. Autolysin activity has also been reported in *C. sporogenes* and *C. butyricum* although less extensive characterization studies are reported [40,41].

Other characterization of autolysins of Clostridia has included a number of "bacteriocin like" activities which have been ascribed to defective phage particles. In *C. botulinum* at least two publications of defective phage have

been reported [42,43]. The autolytic activity of these particles is apparent as well.

The requirement of cell wall cleaving enzymes is obviously a necessity for cell growth and division. Whether or not the autolysin activities described are manifestations of an overabundance of this activity is not known. The mechanism for "induction" of the autolytic function has only been described in a few cases as discussed previously. Nevertheless, the identification of this lysis mechanism is found to be widespread among Clostridia and many other gram (+) organisms as well. This information is well summarized in a review by J. Tagg *et al*. [44].

2.3. Renewable Biomass Substrates

2.3.1. Introduction

The attractive features of agricultural biomass residues as alternate carbohydrates for the production of chemicals lie in their low cost and renewable nature. Estimation of costs for a variety of biomass substrates such as corn stover, wheat straw or wood range from $30 to $60 per ton at the plant site. In general, these residues contain approximately 70% total carbohydrate made up of about one half pentosans and one half cellulose polymers of glucose. These carbohydrates represent a unit cost of from 2¢ to 4¢ per pound of carbohydrate equivalents. When this is contrasted to a typical starch dex-

trose value of from 8 to 15¢ per pound, a significant economic margin for a number of raw material cost intensive fermentations becomes readily apparent.

However the realization of the low cost potential of these feedstocks is hampered by a number of negative factors. In general terms, the diffuse nature and seasonal availability of agricultural residues requires transportation and appropriate feedstock storage expenses leading to detrimental effects on plant scale economy factors. These drawbacks could be potentially mitigated to a large extent through plant siting in climates capable of supporting a year round growing season and/or through the use of a multiple alternate feedstock strategy. However, limiting factors of a more fundamental and specific nature arise from properties of the substrate itself. These include the diversity of monomeric carbohydrate types present in various biomasses which must all be utilized in order to realize the potentially low feedstock cost. Secondly, the difficulty in hydrolyzing these carbohydrate polymers which is required prior to their fermentative utilization has caused a major economic barrier.

In the past, chemical pretreatment has been investigated for the production of readily fermentable glucose and pentose syrups from biomass. However, present advances in our ability to manipulate microorganisms with both hydrolytic and fermentation capacities gives us a new opportunity for direct

utilization of both cellulose and hemicellulose polymers as fermentation feedstocks. One recognizes, however, a general need for biomass pretreatment primarily for the removal of lignin in order to directly use these polymers at high rates and yields.

In this section summaries will be presented on: (1) the pertinent features of general biomass structure and composition which limit their utilization; (2) pretreatment processes described for hydrolysis and the production of soluble carbohydrates; and (3) a review of alternate processes for preparation of biomass residues prior to direct enzymatic hydrolysis by cellulolytic enzymes or microorganisms.

2.3.2. Composition of Natural Ligno-Cellulosic Materials

A large variety of agricultural residues and cellulosic process wastes have been considered as potential sources of fermentable carbohydrates. Phylogenetically, these plant products fall into two major groups based on the morphology of their fruiting structures, the angiosperms and gymnosperms (Table 1). The general composition for both groups is made up in bulk of cellulose, hemicellulose, and lignin. The remainder of the plant (≈ 10-20%) is a diverse mixture of high variability between sources composed of tannins, coloring compounds, oils, sugars, starches, pectins, gums, nitrogen compounds, free organic acids, and minerals. Typical analyses of corn stover (Zea mays) (leaves and stalks) a monocotylodenous angiosperm, hardwoods

Table 1

SPERMATOPHYTO		
ANGIOSPERMAE (Ovules borne in closed cavity, i.e., fruit)		GYMNOSPERMAE (Ovules borne unprotected on surface, i.e., cones)
Monocotyledonea (1 Seed Leaf)	Dicotyledonea (2 Seed Leaves)	
Zea Mays (corn)		
Cereals	Oak	Spruce
Fodder grasses	Elm	Pine
Bananas	Beech	
Palms	Fruit trees	
Lilys	Cabbage	
Tulip		
Orchids		

(dicotylodenous angiosperms), and softwoods (gymnosperms) are shown in Table 2.

The bulk of cellulose, hemicellulose, and lignin is found in the walls of adjacent plant cells referred to as the compound middle lamella. Each cell is surrounded by a thin primary wall of loose fibrillar structure supporting a thicker secondary wall. The secondary wall consists of three layers designated S1, S2, and S3 in which cellulose fibers are wrapped helically, longitudinally, and helically with respect to the cell lumen axis. Hemicellulose and lignin are found tightly associated with cellulose in this wall.

2.3.2.1. Cellulose

The fact that cellulose is a β 1-4 linked polymer of glucose units is well established although the exact crystalline structural arrangement of these chains is still not absolutely certain. Exhaustive methylation of free hydroxyl groups followed by hydrolysis and the detection of the 2,3,4,6 tetramethyl end groups of native cellulose reveals a typical degree of polymerization between 700 and 2,000 units [45,46]. However, this average chain length is highly dependent on the biomass type and pretreatment. The structure of cellulose in plant tissue has been shown to be in two major forms of crystalline and amorphous structure. Cellulose chains aggregate in a parallel fashion to form regions of very high order. Adja-

Table 2

REPRESENTATIVE COMPOSITION OF POTENTIAL
PLANT RAW MATERIALS

	Corn Stover	Hardwoods	Softwoods
Cellulose	32%	40-45%	40-45%
Hemicellulose	38	15-35	20
Lignin	13	17-25	25-35
Ash	9	3	3

cent chains are thought to run in an antiparallel fashion in order to maximize hydrogen bonding between them [47]. Cellulose is defined depending on the exact bond pairing type I (natural) or type II (regenerated). However, these primary chains may be up to 30 times the length of the crystalline region and may emerge from either end or side to form the tangled heterogeneous zones referred to as amorphous cellulose [48]. X-ray data have shown that water is unable to penetrate beyond the surface of the crystalline micelle but can adsorb onto the hydroxyl groups of the cellulose chains in the heterogeneous or amorphous region [49]. The reactivity of cellulose is therefore directly related to the ratio of crystalline to amorphous regions. The degree of methylation of exposed hydroxyl groups of diazomethane verifies this behavior [50]. In dry cotton, diazomethane treatment yields 0.4% methylation while in wet cotton, up to 9.5% of the hydroxyl groups are methylated. The kinetics of this and many other cellulose reactions are typified by a constantly decreasing rate as the amorphous sites are consumed decreasing to a very low basal rate on the residual crystalline material. The treatment of cotton with NaOH has been shown to cause cellulose swelling accompanied by 23% methylation after 15% NaOH treatment [51]. Three forms of cellulose are empirically characterized by their stability in 17.5% NaOH at 25°C. Insoluble cellulose under these conditions is termed α-cellulose. Any cellulose which precipitates upon adjustment to pH 7.0 is termed β-

cellulose, while the remainder is γ-cellulose. The three forms, α, β, and γ, correspond respectively to greater than 90, 90 to 15, and less than 15 units of glucose β 1-4 linkages [52]. The β and γ fractions are alternatively and ambigiously referred to as hemicellulose.

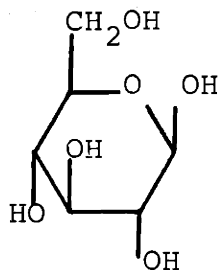
2.3.2.2. Hemicellulose

Hemicellulose is a complex polysaccharide distinguishable from cellulose by a water solubility between that of cellulose and starch. Hemicellulose is readily soluble in dilute alkali and easily hydrolyzed to free sugars by acid. This is in contrast to the much higher energy of activation observed for cellulose hydrolysis by acid. Alkali solubilized hemicellulose polymers will partially precipitate in 50% ethanol or dilute acid and have little or no reducing properties in solution. The polysaccharide composition and molecular weight distribution of hemicellulose shows a large degree of diversity between plant families although at least three basic groups, xylan, mannan, and galactan have been identified.

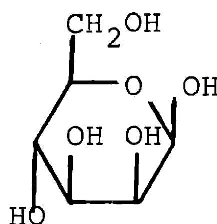
In coniferous woods (gymnosperms) the mannans predominate while in deciduous species and many straws (angiosperms) the xylans are most prevalent. The structures of the sugars found in hemicellulose are shown in Figure 3. Corn stover hemicellulose hydrolyzed with 4 to 6 Kg of 2%

Figure 3

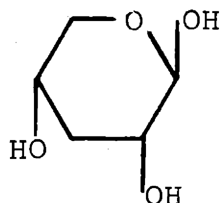
THE SUGARS OF HEMICELLULOSE



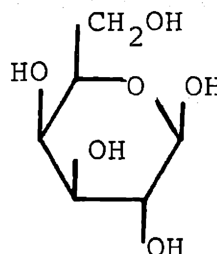
GLUCOSE



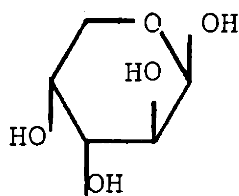
MANNOSE

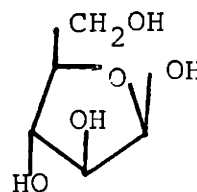


XYLOSE

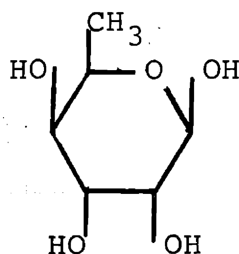


GALACTOSE

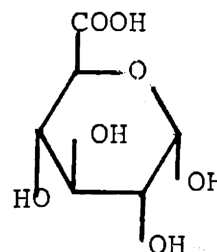




ARABINOSE



RHAMNOSE



GLUCURONIC ACID

H_2SO_4 per Kg of stover has been reported to yield the mixture of sugars shown below [53]:

65%	D-xylose
12%	D-Glucuronic acid (measured as the Δ^{4-5} uronic acid)
13%	L-arabinose
7%	D-Glucose
3%	D-Galactose

2.3.2.3. Xylans

The polymer generally referred to as xylan makes up the bulk of the hemicellulose of many straws and grasses including corn stover. Xylans are polymers of 4-0 methylglucronoxylan and 4-0 methylglucurono-arabinoxylan. They have a basic backbone of β 1-4 linked D-xylose units similar to the glycosidic linkages of cellulose. However, in contrast to cellulose, the hydroxyl groups of xylan are substituted. The substituents include 4-0 methylglucuronic acid at the 2 position, arabinose at the 3 position, and acetyl residues at either position [54]. These bonds are readily cleaved in alkaline solutions above 120°C. Heating 4-o methylglucucronoxylans above 120°C with 5% NaOH results in rapid demethylation of the methylglucuronate residue to a Δ^{4-5} unsaturated uronic acid residue although little reaction occurs in lower temperature alkaline treatment [55]. Alkaline heating also results in rapid hydroly-

sis of the acetyl substituents although these groups are stable towards acid treatment [56]. Acetyl substituted xylans are also found to be the most readily water soluble [57]. Xylans from birch and elmwood show an average of 190 β 1-4 linked D-xylose units with a substituent every 7 to 11 residues [58].

2.3.2.4. Mannans

Less alkali soluble than the xylans are the mannans. Mannans are found predominantly in coniferous woods and are composed of β 1-4 linked glucose, mannose, and galactose units in a ratio of 1:3:1 with an overall degree of polymerization between 50 and 100. This hemicellulose fraction is unusual in that it is believed to contain a number of covalent cross links with lignin, although the evidence for this is disputed [59].

2.3.2.5. Arabinogalactan

Arabinogalactan hemicellulose is a gum of industrial significance purified from larchwood. It consists of β 1-6 and β 1-3 linked arabinose and galactose units in a ratio of 1 to 6. The degree of polymerization of this polymer lies between 44 and 500 residues and a variety of branched forms are believed to exist. Arabinogalactan is also substituted with numerous acetyl groups [60].

2.3.2.6. Lignin

Lignin, the third major component found in the cell wall of most cellulosic materials has been shown to be a heterogeneous, amorphous, three dimensional polymer of phenylpropane units. Coniferyl alcohol units are found predominantly in gymnosperms while both coniferyl and synapyl alcohol units are found in angiosperms. Purified lignin samples have a density of 1.37, a refractive index of 1.61, and in general, a strong adsorption at 280 nm. Various isolation procedures for lignin yields polymers with molecular weight distributions from 900 to over 100,000; however, most soluble lignins range between 1,000 and 4,000 MW. Lignin is isolated in one of two general manners differing by whether it is the lignin or the balance of the substrate, the holocellulose, that is dissolved. The Klason and Wilsatter procedures were developed to hydrolyze all of the carbohydrate with 75% H_2SO_4 or 45% HCl leaving a brown insoluble lignin precipitate [61,62]. Methods of lignin solubilization include treatment with sodium hydroxide with and without sulfite, dioxane and HCl, phenols, organic acids, alcohols, and thioglycolic acids [52]. Methods for lignin extraction, however, also solubilize the bulk of the hemicellulose and thus additional separation is required. The presence of lignin, associated with cellulose and hemicellulose in the plant cell wall is the most important factor responsible for the resistance of these materials to decomposition by many agents. The subject

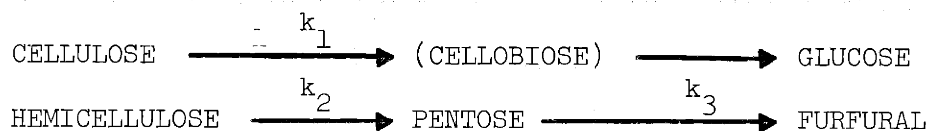
of pretreatment of these cellulosic materials to render them more susceptible to degradation has focused mainly on weakening or removing this supporting lignin matrix.

2.3.2.7. Other Components of the Biomass

The composition for the balance of these plant materials has a high degree of variability between species and seasons. For this reason, only a limited amount of information is available on the specific content of these compounds, in particular, species of interest. However, the significance of these materials in the context of microbial degradation of plants has been reported on numerous occasions to inhibit microbial growth.

2.3.3. Acid Hydrolysis of Biomass

The classical method for hydrolysis of cellulose and hemicellulose polymers to their constituent monomers has been through the use of H_2SO_4 or mineral acids such as HCl. A large number of processes have been proposed and patents granted in order to optimize the yield and concentration of sugar obtained with low levels of acid consumption. However frustrating, these efforts are the intrinsic kinetics of the acid hydrolysis reactions summarized in general as [63]:



The rate constant k_1 for cellulose hydrolysis is typically 100 to 1,000 times slower than the rate of hemicellulose hydrolysis k_2. However, the rate constant k_3 for the degradation of pentose to the toxic product furfural is quite similar to k_2. This non-fermentable end product must be removed, for example, by distillation, prior to fermentation. Due to these reaction kinetics a number of alternate schemes to improve the hydrolysis reaction selectivity have been examined through variations in temperature, time, and acid concentration or by the use of multiple step reaction and separation techniques.

Another intrinsic feature of acid hydrolysis technology addressed by numerous process improvements is the requirement for concentrated sugar syrups for production of concentrated fermentation end products. Although multiple effect evaporation and crystalization have been employed for concentration of sugar solutions, newer high solids density biomass contactors have also been investigated. These general approaches to biomass hydrolysis are illustrated in a number of the major process examples as described below.

One of the earliest acid hydrolysis processes used in the U.S. is the Madison process. In this dilute acid process 0.1% H_2SO_4 is incubated with wood chips at 170°C for up to 1 hour. Under those conditions a maximum glucose yield of 55% from the cellulose can be obtained. The bulk of the pentose polymers are converted to furfural. A recent modification of

this process utilizes SO_2 pretreatment followed by acid hydrolysis to increase the glucose yield [64].

The Hokkaido process developed in Japan in the 40's is a two step acid hydrolysis process. The biomass is first contacted with 1.2 to 1.5% H_2SO_4 at 180°C for short times (0.5 to 5 min.) followed by solid liquid separation of the soluble xylose. The second step uses an 80% H_2SO_4 treatment of the remaining cellulose at 25°C. The concentrated acid hydrolysis step is conducted at very high wood to acid ratios with a mechanical crusher and spray drier. The H_2SO_4 is recovered for re-use by an ion exchange resin process. In Nippon Mokuzai Kogaku Co.'s version of this process calcium oxide (CaO) is used to neutralize the concentrated acid/sugar solution generating $CaSO_4$ (Gypsum).

An improvement of this scheme is represented by the Bergius-Rheinau process which utilizes 40% HCl to generate a 10% sugar solution. The sugar/acid mixture is re-used to contact fresh biomass solids to achieve a 40% sugar solution. Low temperature (36°C) vacuum distillation is employed to recover HCl gas in 80% yield while concentrating the sugar solution (a mixture of 50% glucose and 50% dimers and trimers) up to 60-65% without degradation [65].

Chisso Corp. (Japan) has refined the mineral acid process further by using HCl gas to treat dampened sawdust. A 90 min. high temperature treatment is reported to yield glucose in 95% of theoretical yield [66].

Another low temperature HCl process reported isthe Batelle Geneva process. Here concentrated HCl treatment of biomass at low temperature is reported to give hexose and pentose in high yield, however, the details are proprietary. Low temperature HCl recovery is also employed in this process as well [67].

A new type of high temperature short contact time extrusion reactor is currently under investigation for acid hydrolysis by Grethlein _et al_. at Dartmouth and Rugg at N.Y.U. [68]. A continuous plug flow reactor is operated at 240°C with 1% acid with a residence time of 10 to 20 seconds. Glucose yields of 50 to 60% are currently reported. Of course, significant hemicellulose hydrolysis and decomposition also occurs under these conditions as well.

Perhaps the only large scale acid hydrolysis facility currently operating is in the Soviet Union. There a modified "Madison" process is utilized. Over 90% of the pentosans are hydrolyzed after 1-2 hours treatment with 0.1 - 0.2% H_2SO_4 at 170°C. From 1 ton of wood 0.28 tons of hexose and 0.12 tons of furfural are reportedly obtained [69].

It is clear that a number of innovative improvements have been made in the process technology to achieve concentrated hydrolyzed sugar syrups by acid hydrolysis. Optimized approaches use multistep hydrolysis with pentosan separation to achieve higher total sugar yields. Low temperature

distillation for sugar concentration and acid recovery are also effective. Nevertheless, the costs of acid and its recovery for reuse have remained critical factors to process economics. The technology is also hindered by severe corrosion problems associated with the hydrolysis condition employed which significantly add to capital costs as well.

2.3.4. Biomass Properties Hindering Microbial Degradation

Direct utilization of carbohydrate polymers of cellulose and hemicellulose by microorganisms with these hydrolytic potentials is an extremely attractive option to decrease the costs associated with conventional acid hydrolysis processes. Nevertheless a general need for a different type of pretreatment to make these carbohydrates available as substrates has been found to be necessary []00]. The properties of biomass forming the basis for resistance to microbial attack and a survey of pretreatment methods to overcome these effects are summarized in the following sections.

2.3.4.1. Physical Factors Limiting Enzymatic Degradation

There is an obvious prerequisite for actual physical contact between cellulases and cellulose prior to enzymatic hydrolysis. Therefore, it is not surprising that the basis for resistance of natural cellulosic materials

to enzymatic hydrolysis is derived primarily from the close physical association of lignin with cellulose as well as the crystallinity of insoluble cellulose itself [70]. Multiple anisotropic layers of cellulose give the plant strength and rigidity, while hydrophobic lignin residues offer chemical protection primarily against water hydration. Hemicellulose is believed to further stabilize this structure by hydrogen bonding to both lignin and cellulose. In a number of surveys, the lignin content of the plant cell wall has been shown to correlate closely with the resistance of a variety of agriculture materials to the action of cellulolytic organisms and the yield of free sugar obtained [51].

The influence of cellulose crystallinity has been demonstrated as another limiting factor to cellulase action [71]. X-ray diffraction measurements show that crystallinity increases as the substrate is enzymatically cleaved. The rate of hydrolysis of amorphous regions has been demonstrated to occur much faster than that of crystaline ones [72].

The moisture content of cellulosic materials also effects their susceptability to enzymatic degradation. The presence of lignin effectively prevents moisture swelling of carbohydrate fibers. Hydration of cellulose molecules causes their fine structure to open up making the surface more accessible to cellulases as well as other chemical reagents [73]. The fiber saturation point for hydration is between 24 and 32% of the oven dry weight of many native agricultural sub-

strate materials. This value can be dramatically increased by suitable pretreatment. The total surface area exposed in water saturated cellulosics comprises gross capillaries greater than 200 A in diameter as well as substantially finer cell wall capillaries formed from spaces between microfibrils and cellulose molecules. The surface area of cellulose including the gross capillaries is approximately 2×10^3 cm^2 per gram of wood or cotton while the area including the fine capillaries ranges up to 3×10^6 cm^2 per gram [74]. Determination of the pore size distribution before and after the pulping of sprucewood shows a shift from a narrow distribution centered around 10 Å to a much broader distribution centered from 50 to 100 Å. An estimation of cellulase size compiled from various sources by Whitaker _et al_. reveals an equivalent average size of 59 Å in diameter for many cellulases [75]. Thus, pretreatment procedures which increase water hydration and physical penetration by cellulase proteins are essential for increasing the rates and extents of lignocellulosic carbohydrate uitlization.

In contrast, it should be pointed out that the shorter chain length water soluble hemicellulose oligosaccharides are potentially susceptible for extremely rapid enzymatic hydrolysis in free solution. However the rates of degradation of this primarily pentose fraction are hindered in the same manner as insoluble cellulose carbohydrates due to binding to lignin found in the native state. Analagous physical

hinderances to hydration are also found in the native configuration of hemicellulose in the plant cell wall.

2.3.4.2. Effects of Natural Cellulosics on Microorganisms and Cellulase

It has been repeatedly shown that physical characteristics of natural cellulosics are generally responsible for limiting the rate of cellulolytic degradation and subsequent microbial growth. However, there is also evidence that tannins, coloring compounds, and lower molecular weight phenolics related to lignin may inhibit cellulase action as well. In addition, many of these compounds are believed to be toxic or inhibitory to the growth of many microbial species [101]. While few generalizations of these events as yet appear warranted, as is clearly the case with phsyical limitations to degradation, many specific reports have appeared.

2.3.4.3. Inhibition of Cellulase Activity

Isolation of powerful natural inhibitors of β-amylase and pectinase activity have been reported in wheat, cereals, and grape leaves [76,77,78]. These inhibitory compounds have been shown to be simple phenolics such as trans-cinnamic acid, salicylic acid, leucoanthocyanins, or unidentified complex tannins. The β-amylase inhibitor of grape leaves has also been determined to inhibit cellulase as well [79]. This

non-protein inhibitor was non-dialyzable, resistant to autoclaving at pH 5 or 9 and was strongly adsorbed by activated charcoal. Mandels and Reese examined over 500 species of plants for the presence of cellulase inhibitors and found them in widespread occurrence [80]. Plants were extracted with methanol and water (10 ml/gm) and assayed for inhibition of Trichoderma viride cellulase activity. Of the plant species tested, 17% of the extracts were capable of inhibiting 20 units of cellulase by at least 50% (1 International Unit = 1 μ mole glucose/min). In particular, the inhibition by extracts of bayberry leaves and berries was over 200 times this level and was further characterized. The activity was shown to be non-dialyzable, alkali labile over pH 8, but relatively stable in acid. The inhibitor complexed strongly with gelatin, polyamides, charcoal, sephadex, and various forms of cellulose. From U.V. spectra and chemical tests, it was shown to be polyphenolic in character, probably a leuco-anthocyanin. However, its complete structure was not elucidated. Inhibition kinetics suggest that it acts in a non-competitive manner, reversibly forming a complex with the enzyme.

Inhibition of T. viride cellulase was also examined with two types of polymeric leuco-anthocyanins isolated from pears and persimmon. Both of these inhibitors were as potent as the isolate from bayberry and possessed similar chemical and physical properties. Inhibition studies with these extracts on cellulases from the various other fungal sources

demonstrated that T. viride possessed the most sensitive enzyme studied. In addition, the relative potency of each inhibitor tested remained proportional with respect to each different cellulase tested. Inhibition of cellulases, β-amylases, and pectinases, by leuco-anthocyanins and oxidized catechins, was also reported by Williams [81]. While many reports of cellulase inhibition in vitro have been made, little evidence regarding the importance of these effects on the overall rate of enzymatic decomposition of the substrate has been presented. Inhibition of cellulase by higher molecular weight phenolic polymers may not be due to active site binding or even allosteric binding affecting the activity site. Instead, the binding of the phenolic to the cellulase may simply result in a sterically hindered complex unable to effectively orient on the substrate surface. In terms of the final activity, however, the difference between this inhibition and "physical" inhibition by sterically hindered cellulase may only be one point of view.

2.3.4.4. Microbial Inhibition by Natural Cellulosics

In addition to cellulase inhibition, the inhibition of microbial growth by natural products of real biomass has also been reported for a variety of plant extracts and microbial species [82]. As with cellulase inhibition, each case is usually demonstrated with one microbial

strain and particular plant extract *in vitro*. These results often cannot be justifiably generalized to explain the resistance of plants to pathological infection since an extract toxic to one strain has sometimes proven to be stimulatory to another. In addition, very seldom has a mechanism of action been offered. A well-documented case, however, demonstrated that two simple phenols, protocatechuic acid and catechol at a concentration of 2 mM and found in the outer scales of harvested, pigmented onions inhibited the germination of the fungus *Colletotrichum circinans* [83]. Alternatively, white onions not containing these phenols were susceptible to infection. The same effect was also demonstrated with *Diplodia natalensis* and *Botrytis allii* [84,85]. On the other hand, these compounds or crude extracts had little effect on *Aspergillis niger*, a pathogen to both of these strains of onions.

Extracts of tannins from a variety of plants have been shown *in vitro* to inhibit species of Fusarium, Gloeosporium, Colletotrichum, Cladosporium, and Penicillium and provide some degree of protection from infection *in vitro* [86]. However, many other plant species containing large amounts of tannins are readily attacked by these fungi. Rust fungus resistance of wheat species has also been shown to correlate with the content of phenolic compounds (mainly flavones, protocatechuic acid, and catechols) while in susceptible strains pyrogallol has been shown to predominate [87]. Other phenolics,

chlorogenic, and isochlorogenic acid and their oxidation products, D and L, catechin were isolated from a variety of seed plants and subsequently shown to strongly inhibit nitrogen fixing strains of Azotobacter, Rhizobium, Nitrosomonas, and Nitrobacter [88]. Gallotannin and gallic acid were also shown to inhibit these strains. These compounds were also implicated to the differential resistance of various types of apples to rotting infection by _Sclerotinia fructogena_; however, in this case it was believed that microbial inhibition was due to inactivation of extracellular fungal pectinases rather than any direct toxicity to the organism. In addition, these compounds could not be detected in whole apples but were shown to be produced by oxidation of polyphenols with host enzymes following infection of the apple [89]. Schaal and Johnson correlated inhibition of _Streptomyces scaries_ in potatoe tubers with the intensity of green color produced by reaction of extracts with 5% $FeCl_3$ (indicator of phenols) [90]. However, _in vitro_ tests with chlorogenic acid, caffeic acid, catechol, and p-hydroxybenzoic acid, the predominate phenolics detected all failed to retard growth at concentrations less than 10^{-2}M.

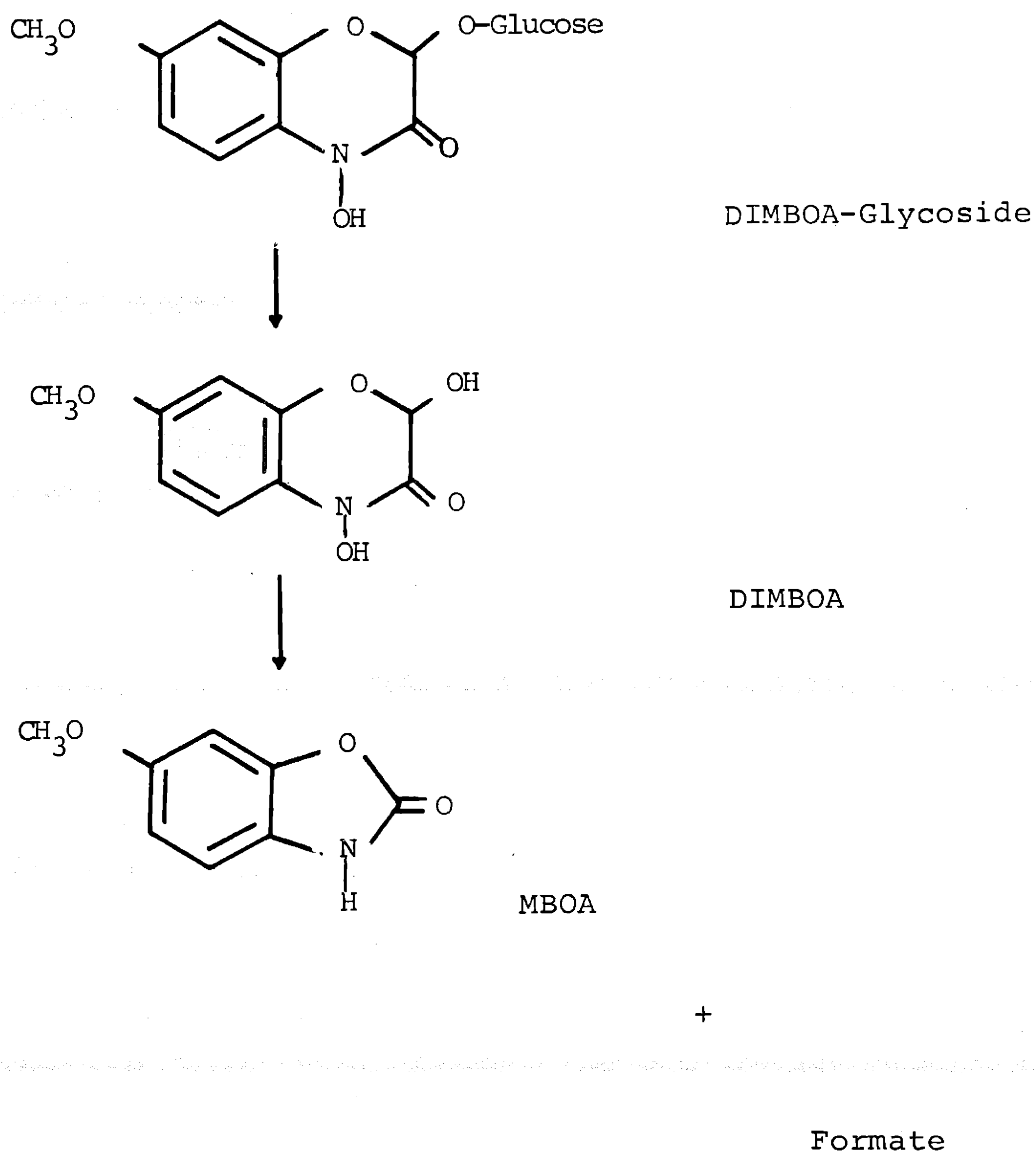
Perhaps the most relevant example of microbial inhibition by plant extracts was recently shown in the case of _Erwinia carotovora_, a causative agent of stalk rot of corn. Aqueous extracts of hydroxamic acids from hardy corn strains were shown to strongly inhibit the growth of two

species of Erwinia in vitro [91]. A number of related hydroxamic acids, principly a glycoside of 2,4-dihydroxy-7-methoxy-2H-1, 4-benzoxazin-3 (4H)-one (DIMBOA Figure 4) can make up over 1% of the dry weight of hardy corn varieties [92]. Extracts of hybrid strains of corn unable to make DIMBOA and susceptible to stalk rot are not able to inhibit Erwinia carotovora. Inhibition of these strains was demonstrated with less than 0.1 gms/liter of DIMBOA extracted from plant material and resulted in an increased lag time of up to 46 hours over that of the control. The final growth rate, however, was unaffected by this compound. Subsequent investigators pointed out that DIMBOA was unstable in solution decomposing to MBOA (Figure 4) with a half life of five hours at 25°C [93]. Growth of Erwinia species in the presence of MBOA showed no lag but showed a strong effect on the growth rate decreasing the maximum specific growth rate by 50% at 0.5 gms/l MBOA. Further examination of the eventual growth of Eprwinia in the presence of DIMBOA could not be mechanistically accounted for by its decomposition, however [94]. The effect of benzoxazolinone and its 6-methoxy derivative (Figure 4) on a number of microbial strains was also tested on glucose agar. Complete inhibition of Fuasrium nivale, Selerotinia trifoliarum, Aspergillus niger, Mucor k., Penicillium roquefortii, and Pesudomonas flourescens, was observed with 0.5 to 1.0 mg/ml [95,96].

From these results it is seen that microbial inhibition by plant polyphenolics is a widespread

Figure 4

STRUCTURE OF BENZOXAZINONES



occurrence [97]. What effect these materials have on a mechanistic level is at best poorly understood in a few instances. Whether or not this is a major mechanism of resistance of plants to pathogenic infection is also unclear. However, any effort to use these materials as substrates for fermentation would be well advised to investigate the possibility of this effect.

In addition to toxic materials found in biomass, it should also be pointed out that many biomass pretreatments can potentially generate toxic components as degradation products of carbohydrates. This is especially problematical in the acid catalyzed production of furfural or the promoted production of saccharinic acids.

2.3.5. Biomass Pretreatment Prior to Enzymatic Hydrolysis

In order to overcome the major obstacles limiting the rapid enzymatic degradation and yield of soluble sugar from natural cellulosics, pretreatment procedures comprising several steps can be employed [102]. Some degree of mechanical size reduction is required in order to ensure shorter diffusion paths for pretreatment chemicals. The surface area for cellulolytic attack can be increased by lignin removal and carbohydrate hydration. Lignin can be decomposed into smaller units better able to elute from this structure. The crystalinity of the cellulose itself can be reduced. Thus, reagents able to cause simultaneous swelling of the carbohydrate struc-

ture and reduction of the crystalinity are particularly effective in creating pores for chemical attack, lignin removal and subsequent cellulose hydrolysis [98,99].

2.3.5.1. Mechanical Methods

Mechanical size reduction by grinding has been examined at Natick Labs as a pretreatment to increase carbohydrate surface area prior to enzymatic cellulose hydrolysis. Although 50% increases in the rates and extents of cellulose utilization are observed, the cost of fine particle ball milling is very expensive making this approach economically untenable.

An alternate form of mechanical combined with chemical treatment is afforded by the Iotech process for explosive decompression. Biomass held at 45 to 52 atmospheres of steam pressure under acidic, basic or neutral conditions is flashed through a small nozzle. The resulting decompression "explodes" the cellular structure. Lignin after treatment can be more readily solvent extracted. Hexose yields of 80% of cellulose are subsequently obtained by enzymatic hydrolysis; however, only 30% pentose yields are observed. This is due to extensive destruction and loss of pentosans at these temperatures. The production of toxic compounds has also been shown to result in inhibited fermentation performance [103].

2.3.5.2. Chemical Pretreatment Methods

Conventional pulping operations such as the Sulfite and Kraft or Soda methods are effective in delignification for the production of pulps in the $300 to $500/ton price range. Experiments with isolated lignin show that aryl-alkyl ether cleavage of the lignin polymer occurs at the para position of the phenyl ring at temperatures between 120 and 160°C under the alkaline conditions employed during Kraft pulping. The addition of sulfite to this reaction allows the cleavage to occur at lower temperatures. Hydrogen sulfide facilitates the lignin disolution by reacting with the cleaved ethers and preventing possible recondensation reactions which could otherwise occur to hinder the diffusion of the lower molecular weight lignin from the structure [104]. This Kraft process has displaced the older sulfite process and is most widely used today. In addition to effectively removing lignin, however, pulping procedures result in rapid hemicellulose solubilization and further degradation occurs to potentially toxic, non-metabolizable saccharinic acids. Although this type of pretreatment is too costly for fermentation substrates, the treatment of straws with dilute alkali or ammonia at low temperature has been employed in the Beckmann process (1919) for upgrading straws as animal feeds [105, 106]. This approach has also been investigated for fermentation substrate pretreatment as well.

Treatment of sugar cane bagasse with 0.1 N NaOH at 120°C for 15 minutes has been shown to result in greatly increased susceptability (10 Fold) of the substrate to the action of cellulase and "hemicellulase" from *Penicillium variable* [107]. The kinetics of enzymatic sugar hydrolysis demonstrated more rapid xylose production than release of glucose or cellobiose residues by this treatment. Increased rates of degradation and yield were also shown by Ghose *et al*. on studies with bagasse pretreated with 1% NaOH (1:7 w/w) at 80°C for 3 hours followed by washing [108]. Under these conditions, the bulk of the hemicellulose was reported to be removed [109].

Pretreatment of wheat straw with a vareity of alkaline delignifying and swelling agents was shown to increase the yield of glucose achieved by saccharification with cellulases [110]. As seen in Table 3, there is a strong correlation between the amount of lignin removed and cellulose hydrolyzed. Under these conditions, however, significant losses of potentially utilizable hemicelluloses are also related with lignin removal. The effect of temperature during treatment on the rate of hydrolysis of this material has also been reported. In Table 4 one observes a positive effect of increasing temperature during NH_3 and NH_4OH treatment, but little effect during ethylene-diamine treatment.

Numerous chemical methods have been reported for biomass pretreatment prior to enzymatic saccharifi-

Table 3

EFFECT OF PRETREATMENT OF WHEAT STRAW ON COMPOSITION AND ENZYMATIC HYDROLYSIS [110]

Treatment at 30°C 4 Hours	% Loss of Cellulose	Hemicellulose	Lignin	% Enzymatic Conversion of Residual Cellulose
None (H_2O)	-	-	-	10
24% NH_3 (w/v)	2	46	20	40
28% ethylene diamine	4	51	60	80
2% NaOH	13	32	7	55
5% H_2SO_4	2	15	6	40
5% HCl	6	15	10	44

Table 4

EFFECT OF TEMPERATURE ON VARIOUS PRE-TREATMENT AGENTS ON WHEAT STRAW [110]

72 Hour Pretreatment of Wheat Straw with:	% Enzymatic Conversion of Cellulose after Treatment at: 25°C	40°C	55°C
2 x H_2O (w/w)	8%	10%	10%
24% NH_3 (w/v)	26	26	40
25% NH_4OH	25	28	41
28% ethylene-diamine	70	75	76

cation. Acid treatment extensively used for hydrolysis of cellulose has been employed at lower concentrations with mechanical disruption of biomass in short contact time high temperature extrusion reactors (10 sec. at 200°C). Subsequent enzymatic hydrolysis demonstrates higher rates and yields as compared to non-treated biomass [111]. Unfortunately, high temperature acid treatment also degrades the rapidly hydrolyzed hemicelluloses as described previously.

A batch pretreatment utilizing 0.2% H_2SO_4 at 180°C for 30 seconds has also been reported. The degree of cellulose polymerization was reportedly reduced by this approach. After removal of the hydrolyzed pentosans, 90% of the theoretical glucose yield was reportedly achieved after enzymatic saccharification [112].

Although many of these procedures have shown some degree of effectiveness for increasing residual cellulose utilization, in general this is usually accomplished with either concomittant loss of hemicellulose carbohydrates or production of inhibitory and toxic materials. The economic utility of these approaches remains to be demonstrated.

2.3.5.3. Cellulose Solubilization

Effective methods have recently been reported by Tsao *et al*. at Purdue for solubilization of cellulose [113]. After a chemical pretreatment with dilute

acid to remove hemicellulose, the residual cellulose can be solubilized using cadoxen. (Tris ethylenediamine cadmium hydroxide) in this solvent up to 10% (w/w) cellulose can be dissolved at room temperature. The cellulose can be reprecipitated with methanol or ethanol in a form extremely susceptible to enzymatic attack. Although highly effective, this approach is not commercially feasible due to cadmium toxicity and recovery problems. Another cellulose solvent, CMCS (aqueous sodium tartrate, ferric chloride, sodium sulfite, sodium hydroxide solution), has also been examined by this group although it is not as effective, dissolving only 4% cellulose at room temperature.

A useful cellulose solvent has been shown to be 60-70% H_2SO_4 [114]. Cellulose highly susceptible to saccharification can be reprecipitated with alcohols, and the acid can be readily recovered. However, ethanol/acid separation and recovery as well as corrosion and hemicellulose recovery problems all limit the commercial feasibility of these approaches.

2.3.5.4. Organic Solvent Pretreatments

Another delignification pretreatment strategy reported has been through organic solvent extraction of lignin. An Organosolv solution containing 50% butanol to extract lignin has been examined at the University

of Pennsylvania and General Electric. At 5 atmosphere steam (150°C) and pH 8.2 with carbonate buffer a 50% aqueous butanol solution can delignify wood to the extent of approximately 50%. After treatment, the cellulose can be enzymatically utilized accompanied by 80-90% yield. Under these conditions, however, the bulk of the hemicellulose carbohydrates are also solubilized and partially degraded.

A simple two step pretreatment procedure of wheat straw by autohydrolysis at 160 to 200°C with water at solid to water ratio of 2:1 (w/w) followed by extraction of lignin with 50% aqueous ethanol at 120 to 160°C has also recently been reported [115]. At these conditions, lignin is first depolymerized accompanied by the solubilization of the hemicellulose. This is then followed by the extraction of the depolymerized lignin. Subsequent analysis of enzymatic hydrolysis kinetics reveals twice the initial yield of glucose after the first treatment and four times the yield of glucose following lignin extraction.

1.3.5.5. Miscellaneous Methods

A novel method of substrate pretreatment has been recently reported which utilizes 2 meV electron irradiation of ligno-cellulosic materials. Rice straw exposed to greater than 5×10^8R shows an increase in reducing sugar yield from 2 to 12% after cellulase digestion [116].

Although novel, this process approach is not believed to be economically attractive at present.

A final method of biomass pretreatment which has been examined uses microbial delignification. The use of Chrysosporium pruinosum has been reported by Bellamy et al. (1977) to achieve partial delignification [117]. A 40 day aerobic fermentation by this organism demonstrated that approximately 50% of the lignin could be solubilized after 30 days of fermentation. However, both cellulose and hemicellulose carbohydrates are more effectively utilized by this organism. Since aerobic metabolism converts these materials to CO_2 and water, their value is rapidly lost. Although an anaerobic microbiological delignification process could be conceivably more attractive, this is not believed to occur in nature. Therefore, the technological basis and prospects for this approach are poor.

2.4. Economics of the Ethanol Fermentation

Traditional yeast fermentations are capable of converting glucose and many other hexoses, but not pentoses, to ethanol at over 95% of the theoretical yield of 0.5 gms of ethanol per gram of glucose. At this yield, approximately 13 lbs. of sugar are required per gallon of ethanol produced. With industrial ethanol currently selling at approximately $1.60 per gallon and sugar priced between 9 and 15 cents per pound (with government price supports), the raw material cost alone repre-

sents at least 70% of the selling price. Due to the substrate intensive nature of this fermentation, molasses and various sources of starch have been under investigation as alternate substrates for the production of ethanol. The starches require hydrolysis, usually with amylases prior to fermentation. A comparison of the manufacturing cost of ethanol obtained from these alternative substrates along with credits obtained from the sale of by-products is presented in Table 5. The relatively small quantities of these substrates, their volatile price behavior (especially if diverted for industrial ethanol production), as well as ethical questions regarding the use of foodstuffs for industrial ethanol production all serve to limit the potential of these carbohydrate sources. It has long been recognized, however, that wood or waste cellulosics form a pentiful, inexpensive, and unutilized source of carbohydrate. A comparison of the availability of these materials is given in Table 6. Utilization of this biomass has fostered research into a variety of chemical and enzymatic means of hydrolysis. Although considerable research into both of these routes of saccharification has been conducted, these processes have not in the long run fulfilled their economic promise. Consistently, one of the major drawbacks has been the low yield of fermentable sugar produced per pound of cellulosic material processed. This has been due in part to the inability of the yeast cells to ferment the pentose fraction of the biomass (up to 35% of the

Table 5

RAW MATERIAL COSTS AND BY-PRODUCT CREDITS FOR VARIOUS FEEDSTOCKS

Raw Material	Salable By-Product	By-Product Credit (per Gal. EtOH)	Net Manufacturing Cost (per Gal. EtOH
Sugar	--	--	$1.16
Molasses	Molasses stillage	$0.12	0.85
Corn	Corn distillers dry solubles	0.36	0.60
Wheat	Wheat distillers dry solubles	0.44	0.85
Potatoes	Potato distillers by-product	0.03	0.66
Sugar Beets	Sugar beet distillers by-product	0.60	0.68

Table 6

U.S. AGRICULTURAL PRODUCTION OF POTENTIAL FEEDSTOCKS[1]

Feedstocks	Annual Production	Equivalent Ethanol[2] Production (million gallons)
Cane Sugar	5.2×10^{9} lbs.	416
Molasses	1.64×10^{8} gallons	65.6
Corn	6.357×10^{9} bushels	24,450
Wheat	2.026×10^{9} bushels	7,790
Potatoe	3.52×10^{10} lbs	507
Sugar Beets	25.1×10^{6} tons	510
Bagasse	2×10^{10} lbs.	800
Corn Stover	7×10^{9} bushels[3]	14,000
Wheat Straw	2.0×10^{9} bushels[3]	4,000
Industrial Ethanol Production (million gallons)		300
Gasoline Consumption (U.S. million gallons)		150,000

[1] Statistical Abstract of the United States (1978) [118] U.S. Department of Commerce

[2] At theoretical yield utilizing all carbohydrate available

[3] Estimates

substrate weight) as well as the low yields of sugar and resultant concentration required during pretreatment and hydrolysis. Thus, the final cost per pound of utilizable glucose has been too high.

Corn stover, for example, contains 32% cellulose and 38% hemicellulose, and may be obtained for 1.5 to 3.0 cents per pound. If only the cellulose portion of this carbohydrate is utilizable, it represents sugar at 4.7 to 9.4 cents per pound plus pretreatment and hydrolysis costs. It is clear, however, that an intrinsic process advantage could be gained if both the cellulose and hemicellulose could be utilized for ethanol production. Raw materials under these conditions represent 2.1 to 4.2¢ per pound of carbohydrate equivalent or as little as 27 cents per gallon of ethanol plus pretreatment, hydrolysis, and fermentation costs. Thus, by combining the cellulolytic and hexose fermenting capability of C. thermocellum with the ability of C. thermosaccharolyticum to ferment pentoses, this process strives to take advantage of two major factors. These are the potential for complete utilization of the substrate, as well as elimination of the need for separate acid or enzymatic hydrolysis prior to the fermentation.

3. MATERIALS AND METHODS

3.1. Fermentation and Culture Methods

3.1.1. Organisms

The wild type parent organism, *Clostridium thermocellum* ATCC 27405, used in this research was obtained from the American Type Culture Collection. Through a program of serial transfer and adaptation to increasing concentrations of ethanol, S. Wang [16] isolated an ethanol tolerant strain S-4. Further strain screening was conducted by I. Biocic for a mutant with decreased production of lactic acid [119]. An enzymatic assay using lactate dehydrogenase coupled with a colorometric tetrahyolium assay for detection of reduced nicotinamide (NADH) cofactor produced was used to detect lactic acid production on petri plates. In this manner S-6 of *C. thermocellum* was isolated. Further ethanol adaptation of this strain resulted in the isolation of strain S-7. This strain was deposited in the American Type Culture Collection and given strain designation 31924. The ability of this strain to reproducibly produce ethanol in high yield, and its ethanol tolerance however were subsequently shown to be unstable during storage in liquid media at $4^{\circ}C$. Unfortunately, this required the isolation of new higher ethanol yielding strains. This was accomplished by detection of low acid producers on plate media supplemented with

pH indicator dyes (see Results and Discussion). The host isolate S-7-19 was used for subsequent fermentation studies described.

The second thermophilic, anaerobic organism used in mixed culture studies was initially isolated by Herrero and Gomez as a xylose fermenting contaminant in a culture of C. thermocellum-H1 obtained from Zeikus at the University of Wisconsin. This strain, designated as HG-2, had Clostridial characteristics and sugar fermentation utilization profile similar to that of Clostridium thermosaccharolyticum ATCC 7956 and was subsequently referred to accordingly. A program of strain selection and adaptation for ethanol tolerance conducted by Fang resulted in isolation of HG-3, HG-4, HG-5 and HG-6. These strains showed increased ethanol tolerance and capability for the production of increasing levels of ethanol at higher yields (see M.S. Thesis, Fang [30]).

A subsequent sequence of strains were obtained by Dalal from HG-6 through the use of penicillin counterselection for the isolation of cells unable to ferment pyruvate. These pyruvate negative clones were shown to produce more ethanol and less acetic acid. A number of markedly superior strains of C. thermosaccharolyticum in this regard including HG-7 and HG-6-62 were used. Further selection of HG-7 with this technique by Kim yielded a new isolate HG-8 [21]. These strains were all employed as they became available in the thesis work described here.

3.1.2. Media and Culture Conditions

Fermentation studies with these thermophilic anaerobic strains in mixed and mono culture were conducted in liquid media at 60°C under N_2 gas in a pH range from pH 6.2 to 7.1. The basic CM-4 medium described in Table 7 was used. To avoid precipitation of Mg and Ca phosphates, the salts were segregated into "A" components and "B" components which were autoclaved separately. For better pH control, 8 g/l of $NaHCO_3$ was incorporated into the media with "A" components and incubation conducted under CO_2 gas.

Soluble carbon sources were autoclaved or filter sterilized with "B" components while insoluble substrates were sterilized with "A" components. Fermentation studies were conducted in Hungate tubes, anaerobic flasks and an 8-liter New Brunswick fermentor. The fermentor was operated at a 6-liter working volume with pH control at a value of 6.5 with automatic NaOH (5N) addition. Agitation speed was maintained at below 100 RPM.

Fermentation and transfers were typically inoculated with 10% (v/v) of exponentially growing cells of *C. thermocellum* or *C. thermosaccharolyticum*. Inoculation of *C. thermocellum* at higher cell density was accomplished by harvesting cultures of exponentially growing cells by centrifugation and transferring the cell pellet with a small quantity of prereduced medium. Typically, through this concentration technique,

Table 7

COMPOSITION OF CM-4 MEDIUM

	Final Concentration (g/l)
A. Salts (10X Stock Solution)	
NaH_2PO_4	1.5
$K_2H\ PO_4$	2.9
$(NH_4)_2\ SO_4$	1.3
B. Salts (10X Stock Solution)	
$MgCl_2$	0.75
$CaCl_2$	0.01
$FeSO_4$	1.25×10^{-6}
Yeast Extract	5.0
Cysteine HCl or Na thioglycholate	0.5
Carbon Source (soluble)	5 - 20
Carbon Source (insoluble)	10 - 80
$NaH\ CO_3$ (optional) (CO_2 ATM required)	8

high density cells in equivalent to a 50% (v/v) inoculum was achieved.

Strain isolation and selection studies were conducted on plate media containing the CM-4 nutrients with the addition of 20 g/l agar or agarose. Surface plating of cultures on soluble carbohydrate medium was conducted in a Coy anaerobic glove box containing an atmosphere of 90% N_2, 5% CO_2 and 5% H_2. Growth of _C. thermocellum_ on insoluble substrates on plate medium was accomplished by inoculation into molten agar containing the insoluble substrate. This mixture was subsequently mixed gently and poured into sterile petri plates, allowed to harden, and incubated at 60 C. Insoluble substrate plate pouring and incubation were accomplished in the anaerobic glove box.

3.1.3. Insoluble Substrates

Corn stover or wheat straw, obtained courtesy of A. E. Staley Co., was dried at 50 C and ground by a Wiley mill equipped with 0.5 mm screen. This particle size material was usually employed for fermentation or pretreatment study. To obtain a different fraction size material, milled and non-milled corn stover was sieved through Tyler standard mesh screens. A model insoluble delignified cellulose substrate Solka Floc BW40 was also used (Brown Co., NH). This material was reported to contain approximately 10% hemicellulose and 90% cellulose [25].

3.1.4. Corn Stover Extract Fermentation

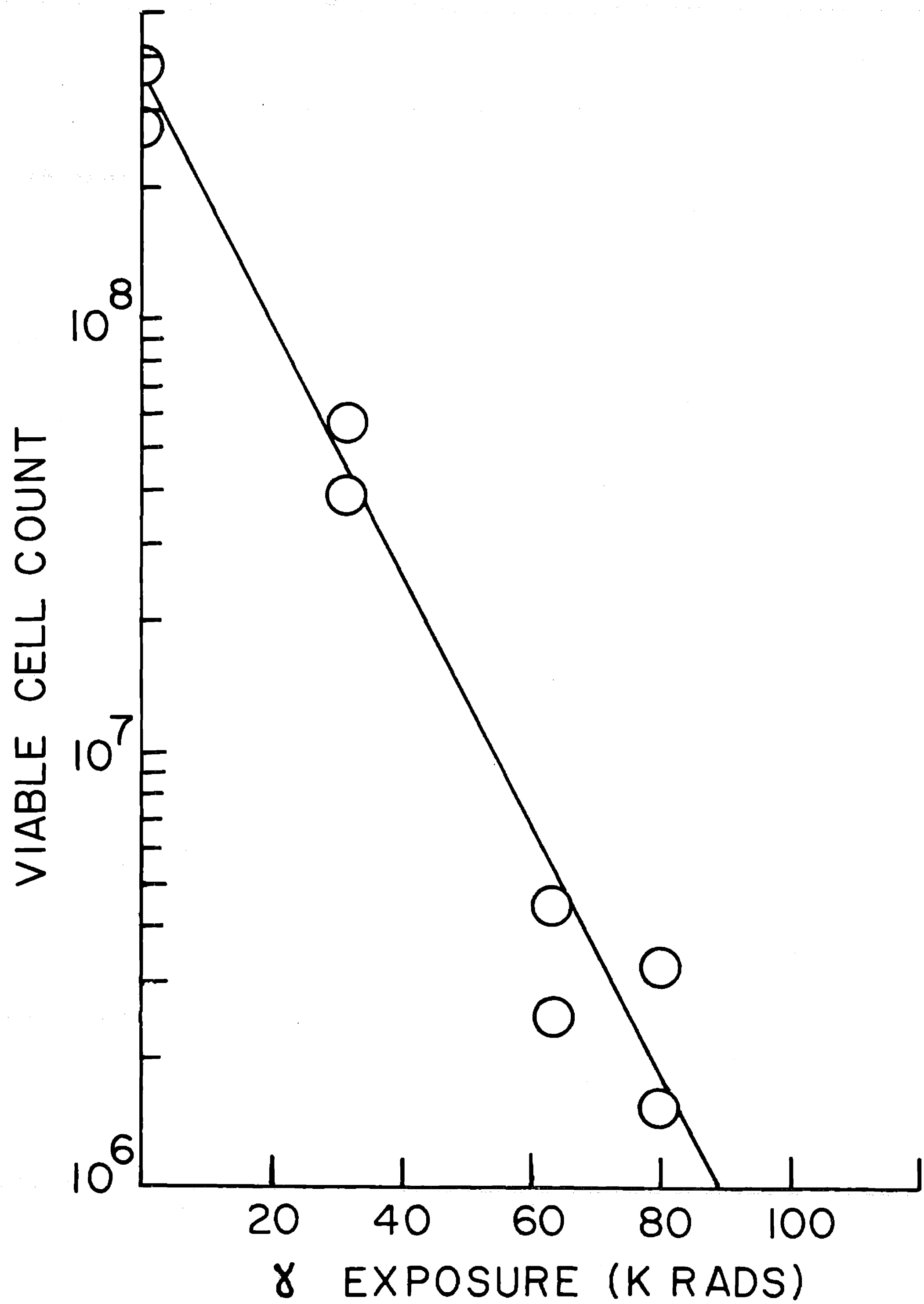
Fermentations were conducted with extracts of corn stover in order to determine the potential toxicity of this material towards the microorganism. Hot water extraction of corn stover was accomplished by autoclaving milled corn stover at 30 to 50 g/l in anaerobic flasks at 121 C for 15 minutes. The hot supernatant was immediately filtered on Whatman #1 filter paper and used in place of water in the preparation of fermentation media. In other experiments as described in the text non pretreated milled corn stover toxicity was examined by the addition of whole corn stover to the media with soluble carbon source added.

3.1.5. Growth Inhibitory Factor

Experimentation to characterize a soluble growth inhibitory factor of *C. thermocellum* was undertaken. Extracellular proteins from *C. thermocellum* fermentations precipitated as described in 3.2.4 were added at various levels to CM-4 medium in Hungate tubes prepared as described previously. Experiments with whole broth were achieved by centrifugation of the broth from Solka Floc or cellobiose fermentations of *C. thermocellum* S7-19 at various times during the fermentation. Sterile capped centrifuge bottles were used. The cell free supernatant was pipetted aseptically into sterile tubes (8 ml/ tube). One ml of filter sterilized cellobiose and 1 ml of

Figure 5

LOSS OF CELL VIABILITY BY EXPOSURE OF CLOSTRIDIUM THERMOSACCHAROLYTICUM TO γ IRRADIATION



yeast extract were added to yield a final concentration of 10 g/l of each respectively. These tubes were inoculated with fresh actively growing cultures of C. thermocellum at various cell densities. Optical density at 660 nm, reducing sugar consumption, product formation and viable count were monitored during subsequent incubations at 60 C. Pretreatment of the purified and whole broth inhibitor were accomplished by 4-6 hr preincubation with Pronase (0.4 μ/ml), Trypsin (0.08 mg/ml), RNAse (0.06 mg/ml), and DNAase (0.06 mg/ml). Control medium was preincubated with identical additions as well.

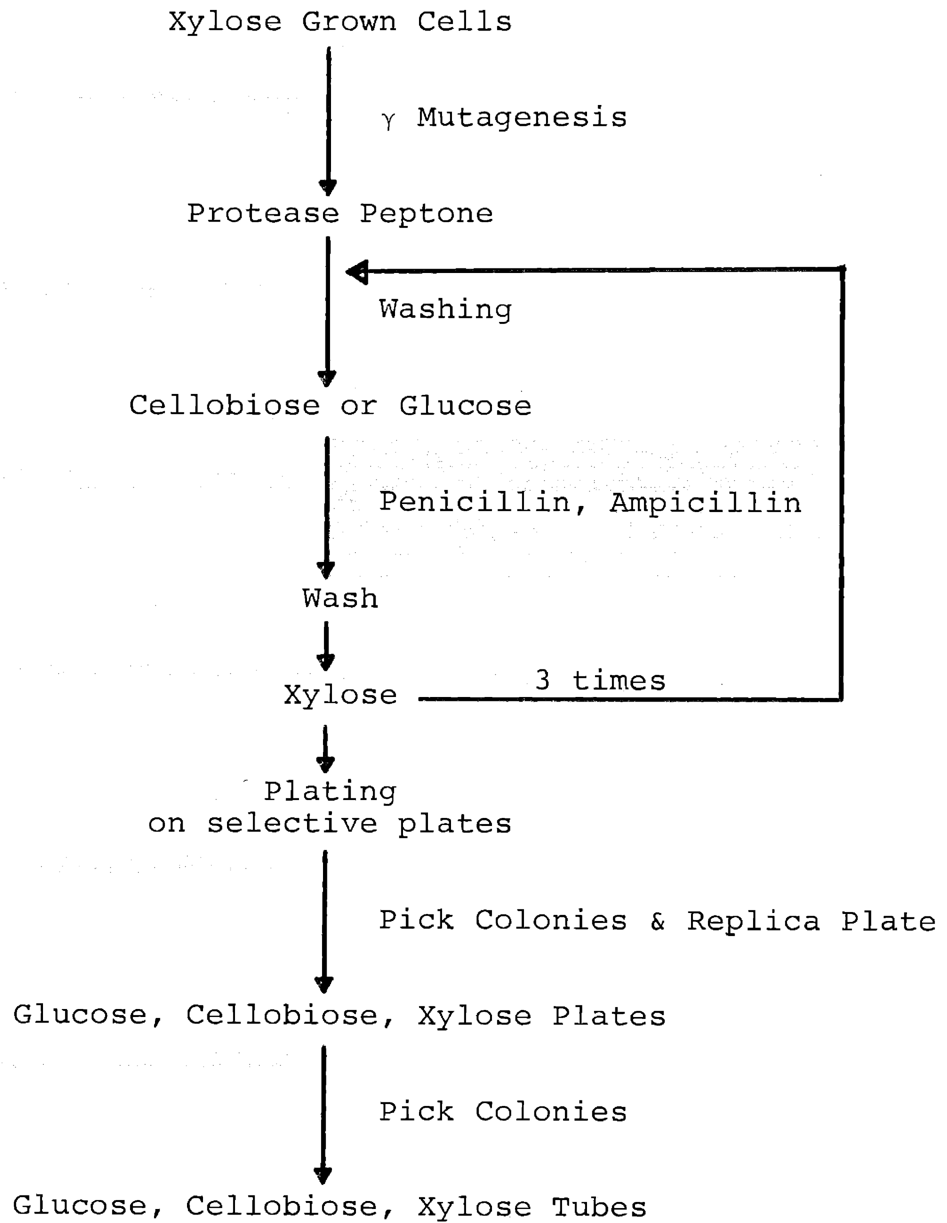
3.1.6. Mutation and Counterselection

Mutation of Clostridium thermocellum and Clostridium thermosaccharolyticum was accomplished routinely by exposure to γ irradiation. A Cobalt 60 source of 4 Kilo Rads/min was used. Exponential growth phase cells in Hungate tubes were exposed for appropriate times to γ irradiation. The cells were then centrifuged and resuspended in rich CM-4 medium with the addition of 5 g/l protease peptone. After one doubling, the cells were centrifuged and resuspended as required. The loss in cell viability of C. thermosaccharolyticum is shown in Figure 5. Exposures equivalent to 90 to 99% high were typically employed to obtain cells for subsequent studies.

A schematic diagram for the selection of hexose strains of C. thermosaccharolyticum is shown in Figure 6.

Figure 6

PROTOCOL FOR ISOLATION OF HEXOSE (-)
C. THERMOSACCHAROLYTICUM



Penicillin counter selection of C. thermosaccharolyticum HG-8 was conducted after γ-mutagenesis through growth in a glucose containing CM-4 medium. After two cell doublings Penicillin G (200 μ/ml) and Ampicillin (200 μg/ml) were added. Following 8 to 15 more hours of incubation the cells were well washed, centrifuged and re-grown in xylose containing CM-4 medium. The cells were centrifuged, washed and transferred to glucose media for a subsequent counter selection. A total of four counter selections on glucose were performed followed by an additional counter selection on cellobiose. These cells were finally grown on xylose and colonies were plated on CM-4 medium with 0.5 g/l yeast extract, 0.5 g/l xylose and 5.0 g/l cellobiose. Small colonies and large colonies were picked for examination in liquid culture.

3.2. Analytical Methods

3.2.1. Cell Mass

Cell mass determinations in soluble carbohydrate fermentations were performed by measuring optical density (O.D.) due to light scattering by the bacteria at 660 nm. One O.D. unit was shown to correspond to approximately 0.67 g/l dry weight of C. thermocellum or C. thermosaccharolyticum cells [16,30]. Unfortunately, this method is only a semiquantitative determination of cell mass during growth on insoluble substrates due to the attachment of C. thermocellum to the insoluble cellulosic substrates.

3.2.2. Protein

Cell and supernatant protein was measured by Bio-Rad protein assay. The protein from centrifuged cell pellets (5,000 x g) was first released by boiling in 0.2 N NaOH for 10 minutes. Soluble protein was diluted with water into the appropriate range from 0.2 to 1.4 mg/ml and 0.1 ml of the solution was assayed with 5.0 ml of the reagent. Optical density was measured at 595 nm using Bovine serum albumin as the standard. The standard curve was found to be linear between 0.2 and 1.4 mg/ml protein and up to 2.0 O.D. A strong interference for this as well as other protein assay methods was shown in any medium containing lignin or related phenolic materials and therefore could not be used.

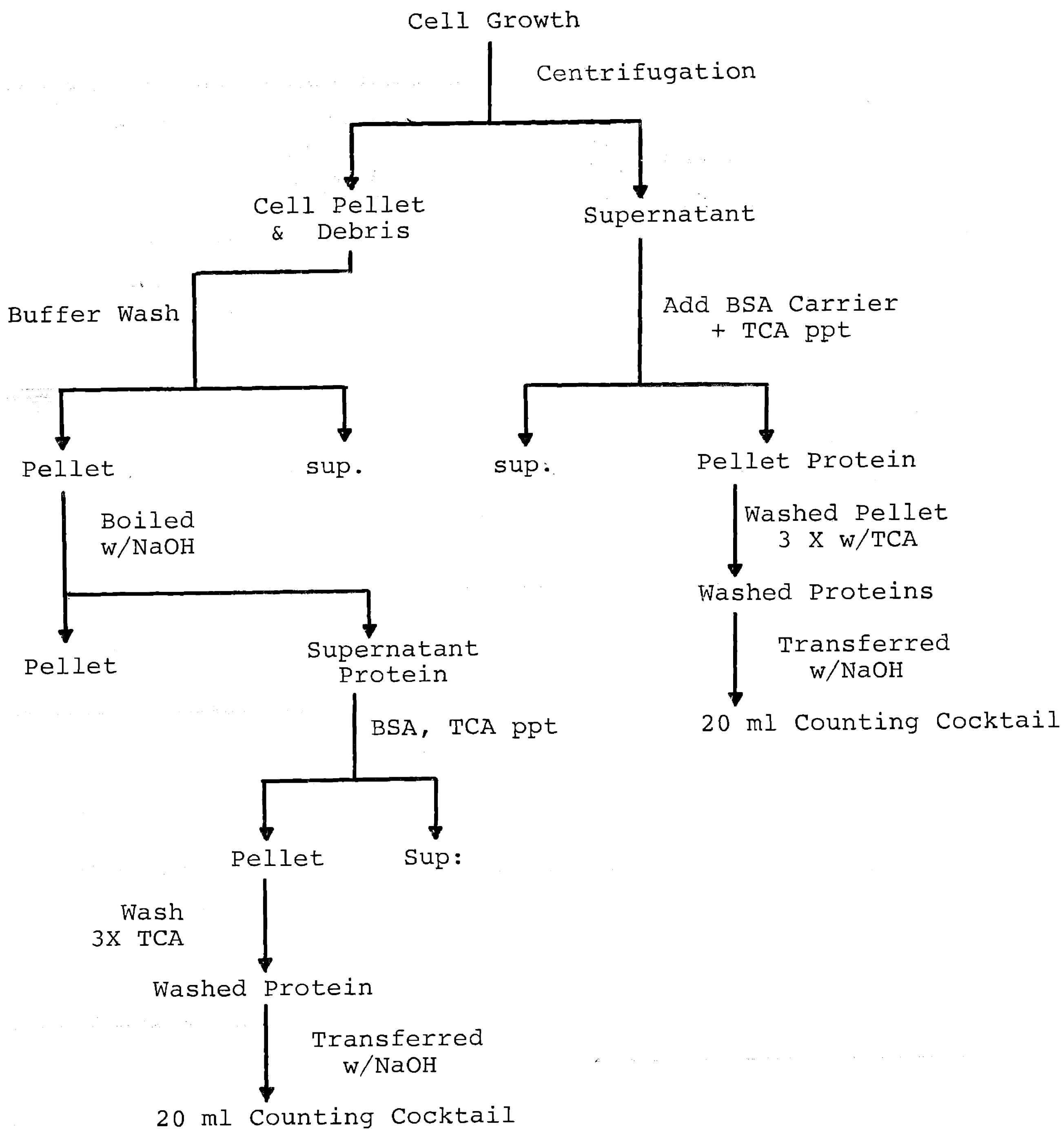
3.2.3. Radioactive Label Incorporation into Protein

In order to determine the relative effect of soluble lignin on protein production, the effect of soluble lignin on incorporation of radioactive amino acid into protein was determined. A schematic diagram of the procedure employed is shown in Figure 7.

Monoculture fermentation of *C. thermocellum* (ATCC 31924) was conducted in Hungate tubes with bicarbonate buffer in CM-4 medium with the addition of extracted corn stover solids and 10 g/l cellobiose. Radioactive C^{14} labeled L-Leucine (10 μC/ml) and cold L-leucine (80 μg/ml) were included.

Figure 7

SCHEMATIC DIAGRAM FOR DETERMINING ^{14}C-LEUCINE CARBON INCORPORATION



After 24 hours of growth 0.5 ml samples were centrifuged for 15 minutes at 4,000 X G to separate cells and insoluble debris. Label incorporation into supernatant protein was measured by the addition of 0.1 ml of centrifuged supernatant to 1.0 ml of 10 mg/ml Bovine serum albumin (BSA) solution to which 1.0 ml of cold 12% trichloroacetic acid (TCA) was added. Precipitated protein was separated by centrifugation at 4,000 X G for 10 minutes. The precipitate was decanted and washed 3 times with TCA. The final pellet was resuspended in 0.5 ml of 0.2 N NaOH and transferred into 20 ml of Liquiflour counting cocktails.

Label incorporation into cell debris was assayed after washing the cell pellet with 1.5 ml of CM-4 salts buffer. The washed pellets were resuspended in 1.5 ml 0.2 N NaOH and boiled 10 minutes. The mixture was pelleted at 4,000 X G for 10 minutes and the supernatant decanted into 1.0 ml of 10 mg/ml BSA. The solution was precipitated with 12% cold TCA, centrifuged and washed 3 times as described previously. The final washed pellet was transferred to 20 ml Liquoflour counting cocktails with 0.5 ml 0.2 N NaOH. Counting was performed with a Packard Tri Carb Scintillation Counter. Net incorporation of label in TCA precipitable protein was corrected for background counts obtained at the time of inoculation (i.e., t = 0). Total label was determined by the addition of 0.5 ml of non centrifuged media to 1.5 ml 0.2 N NaOH containing 10 mg/ml of BSA boiling for 10 minutes. A 0.5 ml sample was taken

for counting. Protein concentration based on label incorporation into TCA precipitable proteins was reported assuming an average protein composition of 5% leucine by the formula:

$$\frac{\mu g\ \text{Protein}}{ml} = \left(\frac{\text{CPM in TCA ppt. Protein/ml}}{\text{Total CPM/ml}}\right)\left(\frac{80\ \mu g\ \text{L-LEU}}{ml}\right)\left(\frac{20\ \mu g\ \text{Protein}}{\mu g\ \text{L-LEU}}\right)$$

3.2.4. Cellulase Concentration and Assay

Cell free supernatant protein for cellulase assays was obtained by centrifugation of fermentation broths at 5,000 X G for 20 minutes. These supernatants were used directly or concentrated by ammonium sulfate or ethanol precipitation. Ammonium sulfate (561 g/l) was added to the supernatant at 4° C with stirring. The precipitated protein is harvested by centrifugation at 10,000 X G for 30 minutes and reconstituted in CM-4 salt solution with 1 mM Dithiothreitol (DTT) and stored under N_2 at 4° C prior to use. An alternate precipitation method by the addition of 95% ethanol (-40 C) to a final concentration of 80% (v/v) was also employed. After storage overnight at -40 C the precipitate is harvested and reconstituted in the same manner.

Carboxymethyl cellulose (CMC) hydrolysis activity (CMCase) was measured by the addition of 1 ml of centrifuged fermentation broth or reconstituted protein to 1 ml of assay solution containing 50 mg/ml CMC, 10 mM DTT, and salts at

2 times the concentration as found in CM-4 media at pH 6.5. Hydrolysis was conducted in Hungate tubes at 60° C under N_2 atmosphere. Samples were taken for analysis of initial rate of reducing sugar release.

Cellulase assays were also performed on insoluble substrates (10-50 g/l) such as pretreated and non-treated corn stover. These assays were performed in the same manner as CMCase with substitution of the desired insoluble substrate which was autoclaved prior to use.

3.2.5. Soluble Carbohydrate Determination

Reducing sugars were assayed by a modification of the method (DNS) of Miller (1959) [120]. To 1 ml of DNS reagent (Table 8) 25 λ of sample containing 1 to 10 mg/ml reducing sugars. The solution was vortexed and boiled for 10 minutes, cooled and the absorbance determined at 550 nm. The reagent was frequently recalibrated (Table 8).

Although this reagent is useful for determination of monomeric reducing carbohydrates, dimers such as cellobiose give only 67% of the expected response. In order to give a more accurate indication of total soluble carbohydrate content including soluble oligomers, a dilute acid pre-hydrolysis was developed. To 0.5 ml of a supernatant sample, 0.1 ml of 35% (conc) HCl was added. This mixture was vortexed and boiled for 10 minutes. The hydrolyzed samples were assayed by DNS as pre-

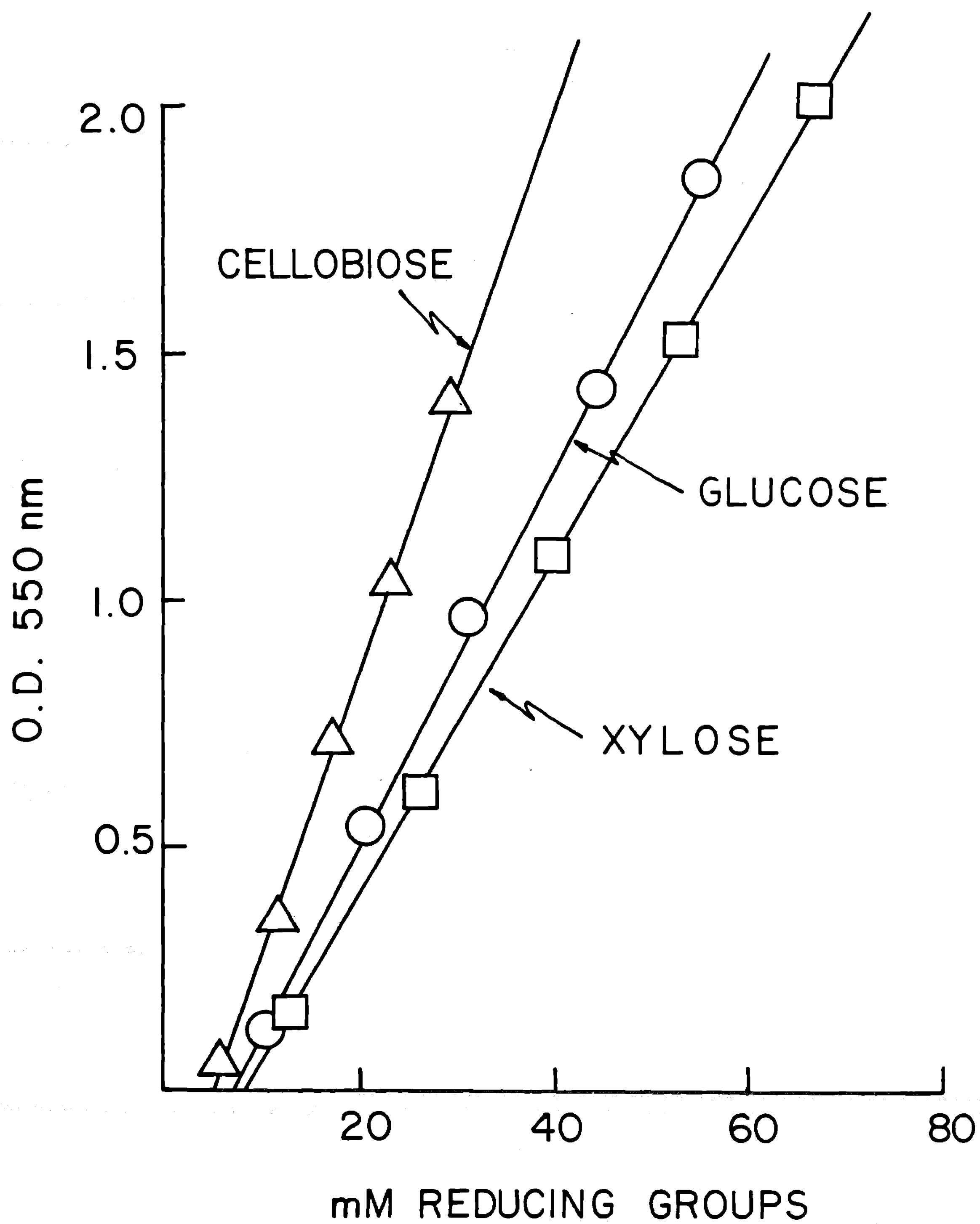
Table 8

COMPOSITION OF DNS REAGENT

Component	Amount
3,5 dinitrosalicylic acid	1 %
Phenol	0.2 %
NaOH	1 %
K Na Tartrate	5 %
Na Sulfite	0.05%

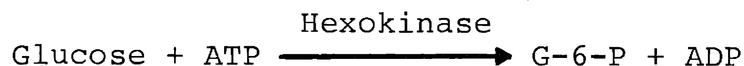
Figure 8

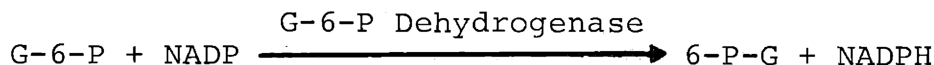
REDUCING SUGAR ASSAY



viously described and acid hydrolyzed reducing sugars were reported with appropriate correction for acid dilution as shown for typical calibration curves in Figure 9.

An alternative but specific enzymatic assay for glucose and cellobiose was also developed. Glucose was determined as described by Sigma method 15-UV. The reactions of this assay are summarized below:



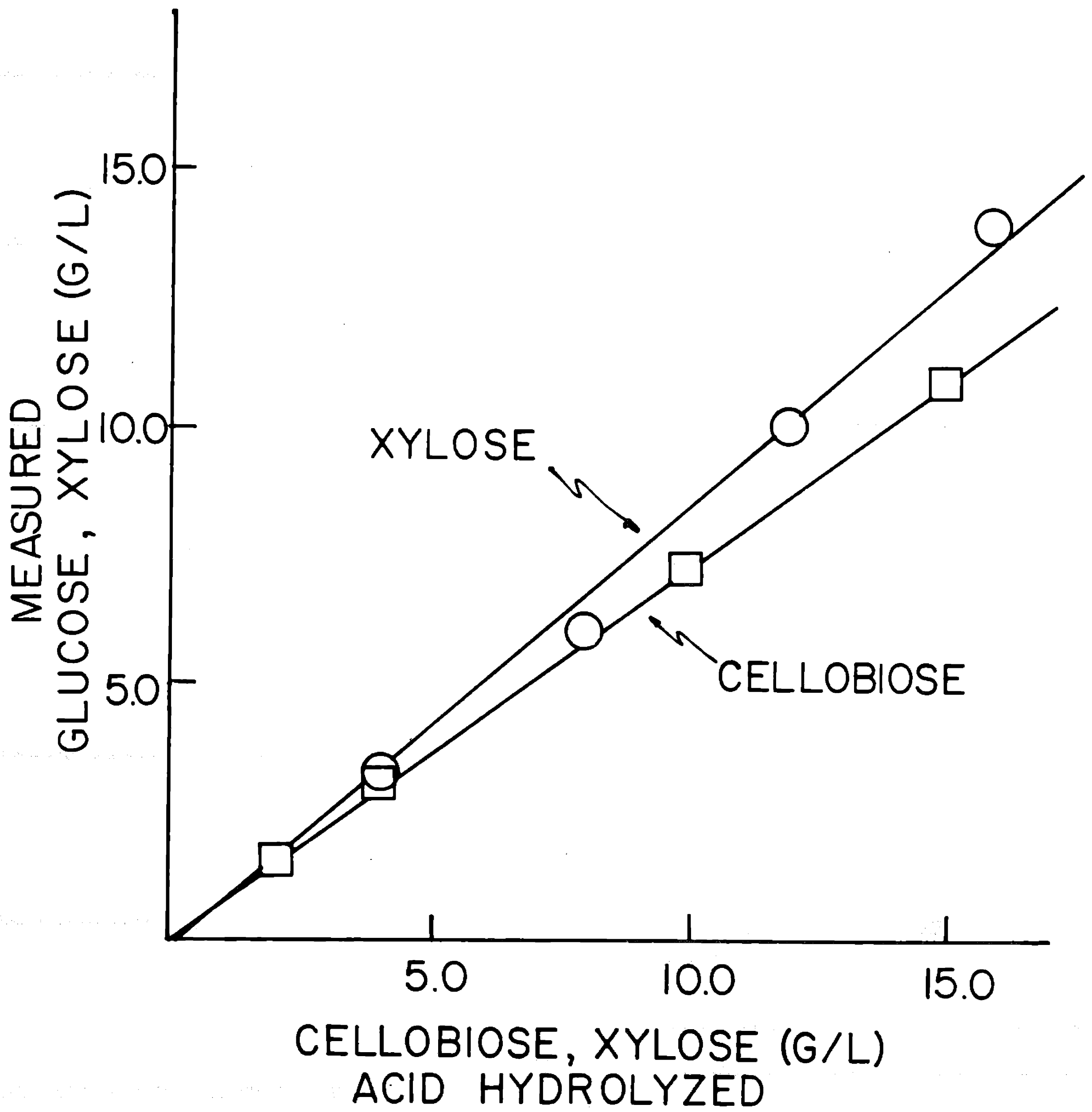


The NADPH produced is measured by optical density at 340 nm. The assay involves reconstituting the Sigma reagent kit with 31 ml of H_2O. To 1 ml of the reconstituted assay solution 10 μλ sample containing 0 to 6 g/l glucose is added. After 5 minutes at room temperature, the O.D. change at 340 nm was determined.

Cellobiose was assayed by the increase in glucose concentration after treatment with β-glucosidase. The β-glucosidase protein is made up with 0.1 M Na acetate buffer (pH 5) at 10 mg/ml. To 500λ of cellobiose containing sample, 100λ of β-glucosidase is added and incubated at 37 C for 2 hrs. The increase in glucose is monitored as described above (Figure 10).

Figure 9

ACID HYDROLYZED DNS SUGAR ASSAY



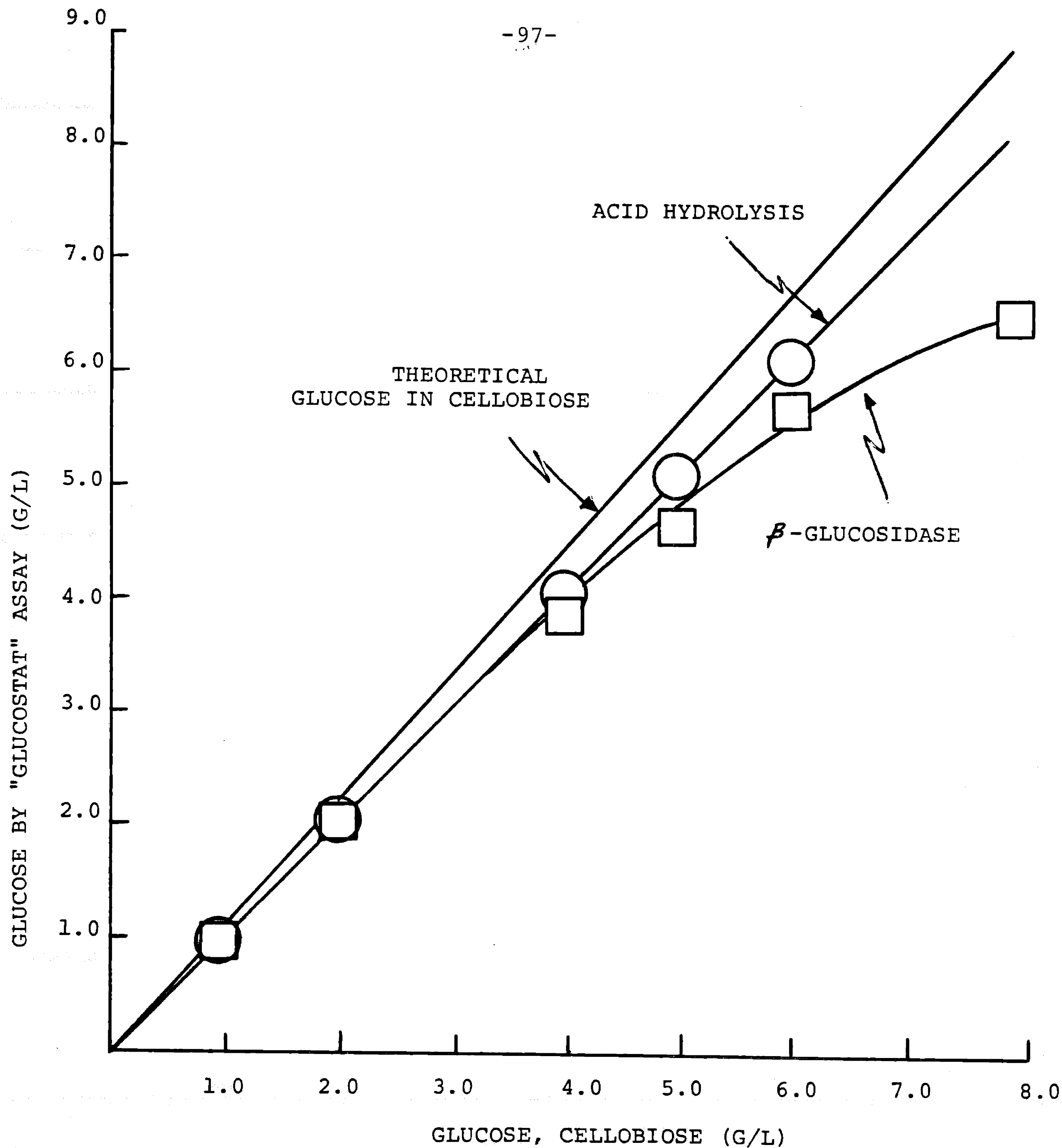


Figure 10

GLUCOSTAT AND β-GLUCOSIDASE ASSAY
FOR GLUCOSE AND CELLOBIOSE

Another method for determination of oligomers by high pressure liquid chromatography (HPLC) was also employed.

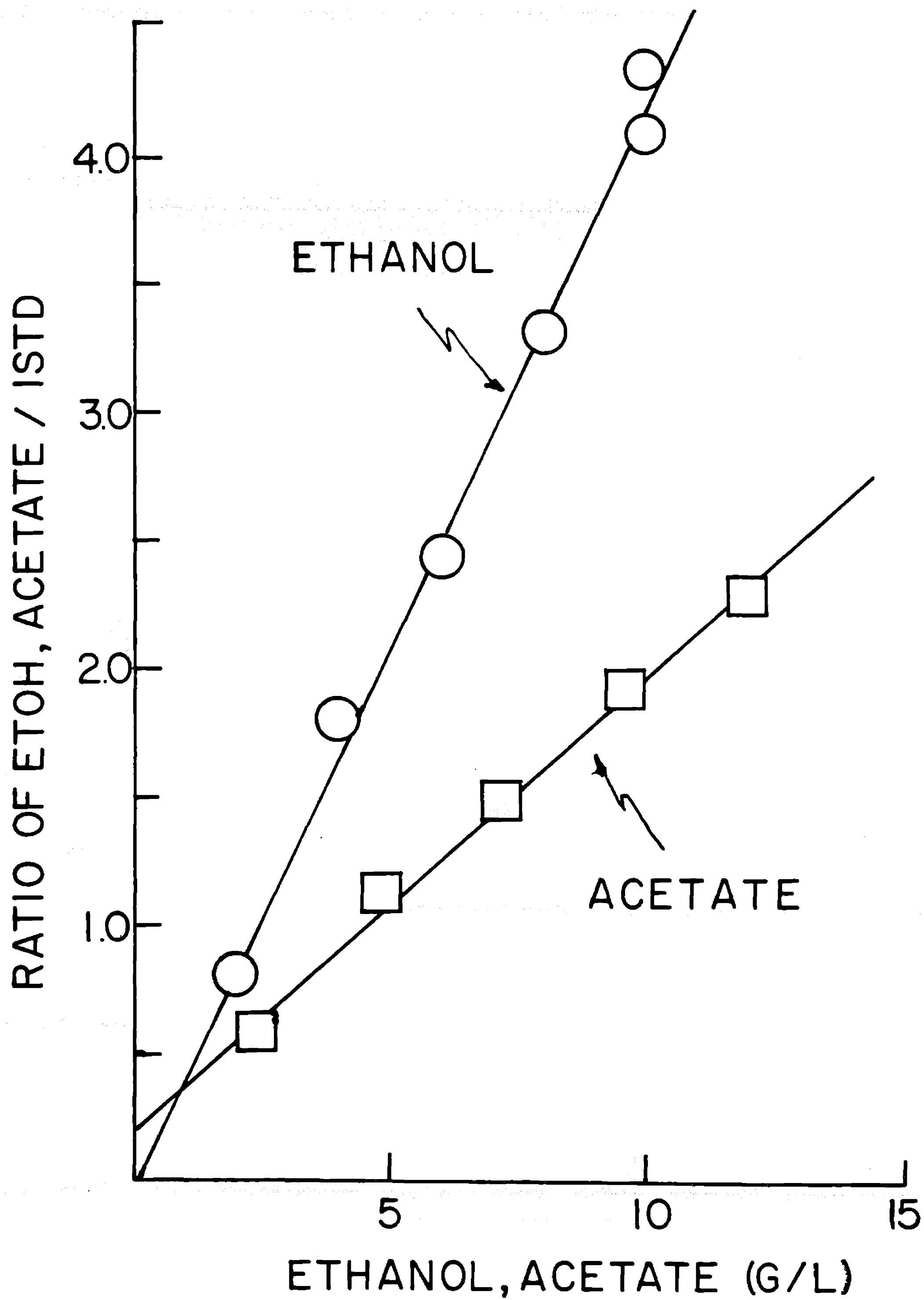
HPLC sugars were assayed after passage of centrifuged fermentation supernatant through Bio Rad SEP-PAK C18 cartridges with AG 11 AB resin (1 ml per 250 mg resin) for 30 minutes at room temperature to remove interferring phenolic substances. The filtered samples were then ultrafiltered through Amicon CF 25 membranes (mol weight cut off 25,000) to partially remove protein (50%) and particulate matter. A sample of 2 to 3 $\mu\lambda$ was injected into a Waters HPLC equipped with HPX-85 heavy metal column from Bio Rad. Column temperature was 85 C and samples were eluted with water at a flow rate of 0.6 ml/min. Detection was accomplished with a differential refractometer and peaks compared with known standards.

3.2.6. Fermentation Products: Ethanol, Acetate and Lactate

Ethanol and acetate in the fermentation broth were assayed by gas-liquid chromatography using samples acidified with 1.5% HCl. Chromosorb 101 columns (6') were used with helium as a carrier gas. Injector temperature of 200 C, column temperature of 150 C and F.I.D. detector temperature at 250 C were used. An internal standard of N-propanol was added for accurate quantitation. Between each sample formic acid was injected to remove residual acetic acid which thus allowed a more accurate determination of samples at low concentrations. Typical calibration curves are shown in Figure 11.

Figure 11

GLC OF ETHANOL AND ACETATE ON CHROMOSORB 101



An alternate assay procedure for more accurate quantitation of acetic acid was performed by GLC on thermon columns. To 1 ml sample, 0.25 ml of 50% H_2SO_4 solution containing 1 g/l Propionic internal standard and 1 g/l distilled formic acid were added. The solution is extracted with 2 mls of diethyl ether and 1 to 2 μl of the ether layer was injected into the GLC column. The chromatograph conditions are similar to those for Chromosorb 101; however, the column temperature is programmed at 80 C for 2 minutes and increased at a rate of 5 C/min up to 150 C. Much better linearity at low acetate levels is observed with this method as shown in the calibration curve in Figure 12.

Determination of lactic acid was performed enzymatically with the use of Sigma lactic acid Kit No. 826-UV. In this assay lactate dehydrogenase (LDH) is used to oxidize lactate to pyruvate while NAD is reduced to NADH. The increased optical density of NADH is determined at 340 nm. To each vial of NAD supplied in the test kit, 6 ml of water, 3 ml of glycine buffer (kit) and 0.2 ml LDH are added. For assay, 100λ of a lactic acid sample in the range of 0 to 0.5 g/l is added to 1,000λ of reconstituted assay mixture. After 30 minutes of incubation at 37 C, the optical density at 340 nm is read, and compared with a standard curve and blank containing 8% perchloric acid (Figure 13).

Figure 12

GAS LIQUID CHROMATOGRAPHY OF
ACETIC ACID ON THERMON

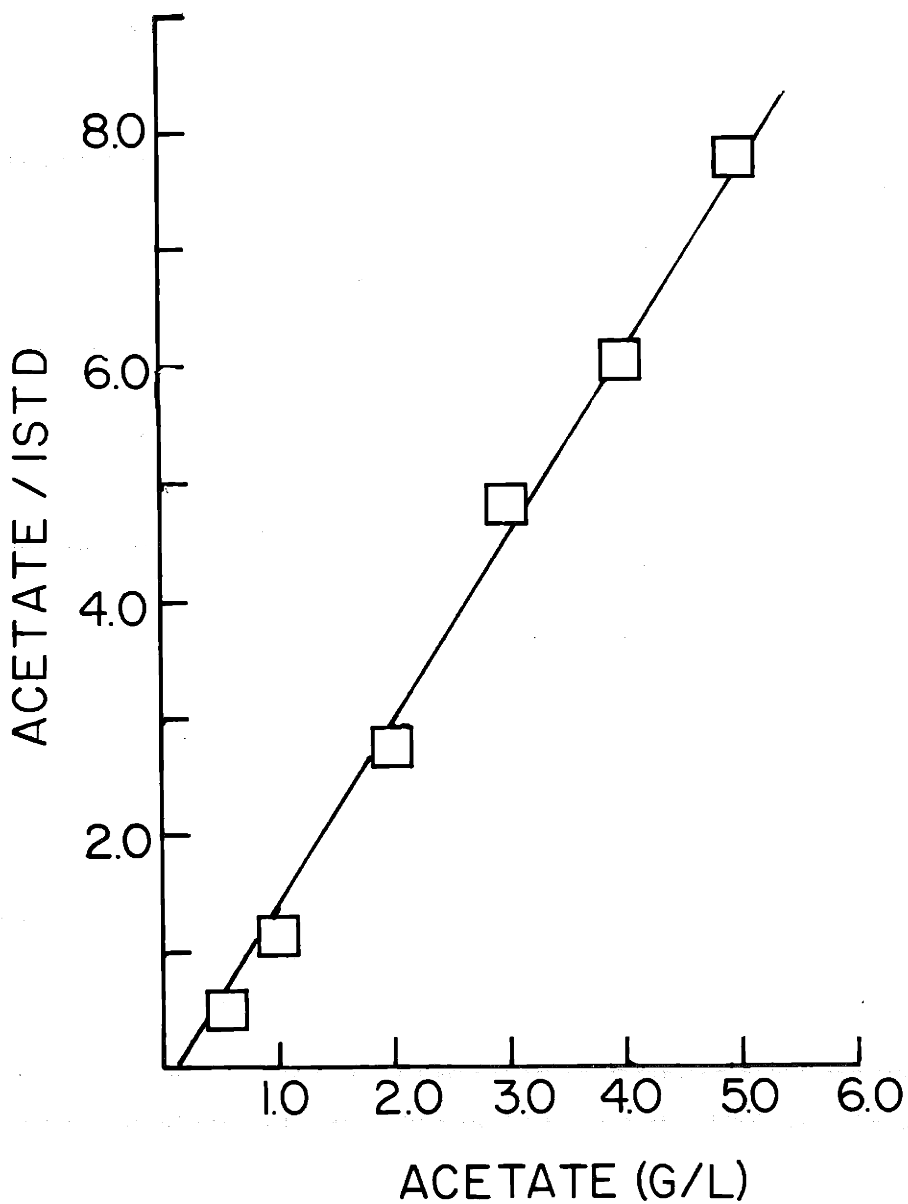
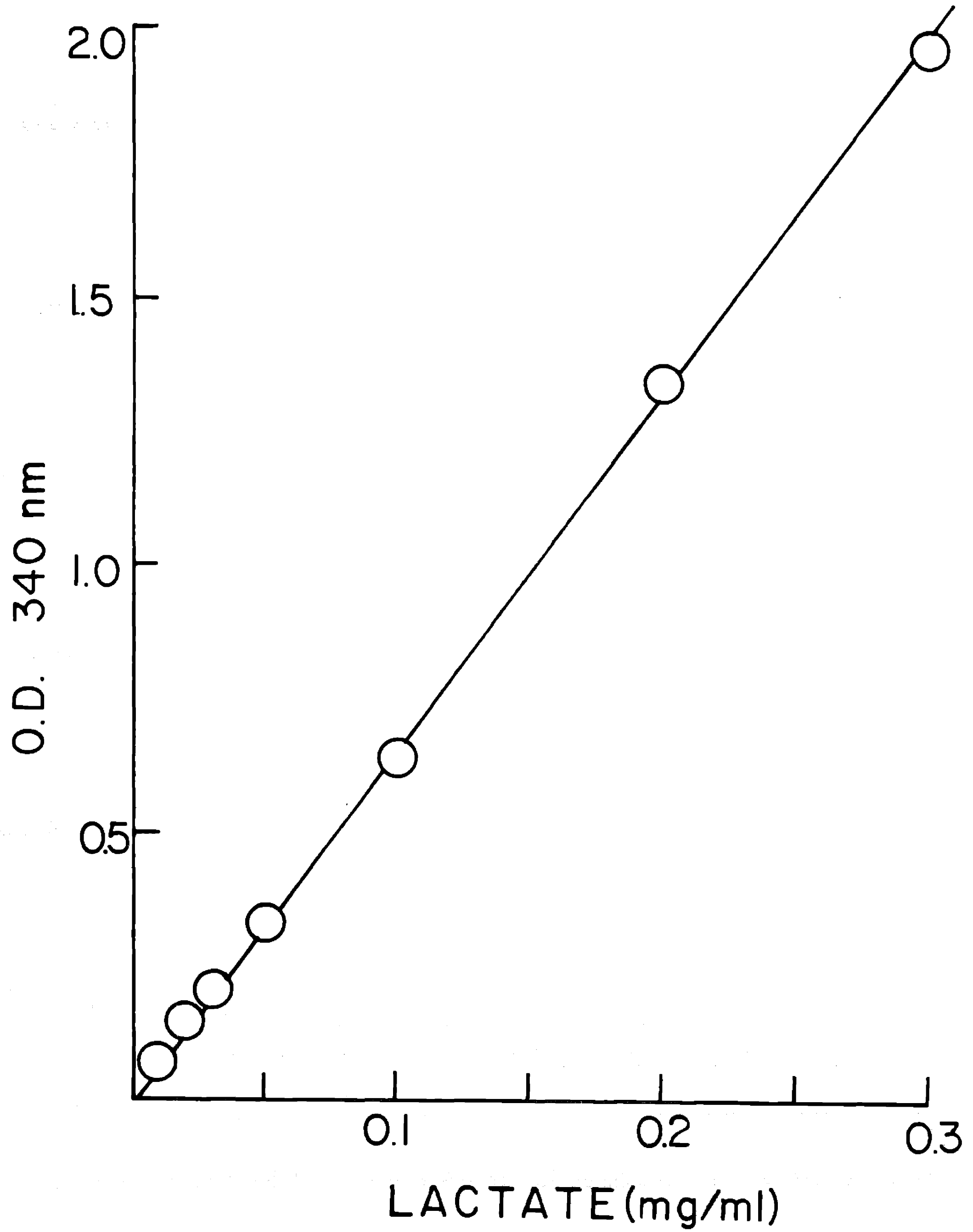


Figure 13

LACTIC ACID ASSAY



3.2.7. Analyses of Biomass Composition

3.2.7.1. Pentosan

Analyses of biomass composition were conducted on oven dried samples at 50 C with a particle size between 0.5 and 1.0 mm. Pentosans in solid or liquid samples were determined by destructive distillation to furfural in 12% HCl according to the A.O.A.C. pentosan method [121]. In this procedure, the furfural produced is determined gravimetrically after precipitation as a phloroglucide derivative. A detailed protocol is summarized below:

DETERMINATION OF PENTOSANS

Two solutions are prepared:

12% w/w HCl.

11 g. phloroglucinol/1500 ml. 12% HCl with the diresorcin impurities crystallized out at 5 C.

- Place in 300 ml distilling flask an amount of sample such that the phloroglucide obtained will not exceed 0.300 g.

- Add 100 ml. 12% HCl, connect condenser and 500 ml. receiving flask.

- Bring to a boil at a rate of about 30 ml/min, and add more HCl through sep. funnel to keep level up.

- Continue until there are 360 ml distillate, then remove receiving flask and add 40 ml phloroglucin solution.

- Stir: the solution will turn yellow, green, then opaque.

- Let stand overnight.

- Filter on tared medium porosity Gooch crucible and wash with 150 ml dist. H_2O, keeping some water on the precipitate until the end.

- Dry at 100 C for 4 hours and weigh (a = furfural phloroglucide).

$$\text{Pentose} = (a + 0.0052) \times 1.0075$$

$$\text{Pentosans} = (a + 0.0052) \times 0.8866$$

3.2.7.2. α-Cellulose Assay

The α-cellulose content of corn stover, defined as the fraction of cellulose remaining insoluble in 24% KOH at 25 C, was determined by the following procedure [122]. The samples are first pre-extracted in 200 ml of 0.5% ammonium

oxalate boiled under reflux for 3 hours. After suspension of the filtered solids in 100 ml of 1% acetic acid, 0.5 g of sodium chlorite is added to solubilize the lignin. After 45 minutes of reaction at 70 C, 0.5 g of ascorbic acid is added to stop the reaction. The filtered solids are washed with water to stop the reaction. The filtered solids are washed with water and extracted with 200 ml of 25% KOH for 2 hours. After refiltration, the residual solids are washed with 25 ml of 24% KOH, water, 10% acetic acid, and finally water again. The weight loss upon washing is assumed to be α-cellulose.

Due to the dependence of this analysis on the exact method, a detailed description is provided below:

DETERMINATION OF ALPHA CELLULOSE

- Accurately weigh corn stover in the vicinity of 0.5 gram and place into a 500 ml ground glass Erlenmeyer flask.

- Add 200 ml of 0.5% ammonium oxalate solution and boil under reflux for two hours.

- Filter on <u>coarse</u> scintered glass crucibles, washing with hot H_2O (200 ml)

- Suck dry, then add to 100 ml 1% (v/v) acetic acid in a 250 ml Erlenmeyer.

- Heat to 70 C on a water bath in a hood and add 0.5 g sodium chlorite.
- Keep at 70 C for 45 min, stirring frequently.
- Add ascrobic acid (up to 0.5 g) to stop the oxidation. The solution will turn from bright yellow to clear.
- Filter on a <u>medium</u> scintered glass crucible and wash 2 times with distilled H_2O.
- Place solids in 250 ml Erlenmeyer and add 200 ml 24% w/v KOH.
- Bubble with N_2 for 10 min. to remove O_2.
- Stir for 110 min.
- Filter on a <u>coarse</u> Gooch crucible.
- Wash with 25 ml 24% KOH, then with water.
- Wash with 25 ml 10% v/v acetic acid.
- Wash with 50 ml dist. H_2O.
- Dry at 50 C, ash, and weigh again. The weight loss is assumed to be alpha cellulose.

3.2.7.3. Lignin Assay

In these studies, lignin was analyzed by a modification of the T13 TAPPI standard method developed at Wisconsin [123]. The water preextraction was omitted and lignin was reported as the weight lost after ashing of the H_2SO_4 treated residual solids. A detailed description of the assay is provided below:

DETERMINATION OF LIGNIN

- 0.5 g of 0.5 mm Wiley screen corn stover dried at 50 C is accurately weighed.
- Sample extracted four hours on Soxhlet apparatus with 95% ethanol (v/v).
- Re-extract with 2:1 benzene-ethanol (95% v/v) for six to eight hours and dried at 50 C.
- The stover is then transferred to a small beaker and 10 ml 72% sulfuric acid (w/w) is added.
- Mixture stirred for one minute and let stand for two hours, stirring frequently.
- Transfer to a 500 ml Erlenmeyer and dilute to 3% with 373 ml distilled water and boil under reflux for four hours.

- Filter on coarse Gooch crucible, washing free of acid with 500 ml hot water (boiling).
- Dry at 50 C, weigh, and ash for 2 hours at 500 C.
- Weigh again. The difference is assumed to be lignin.

3.2.7.4. Analysis of Miscellaneous Biomass Components

The protein, ash, and acetyl content of biomass residues were also assayed by the following methods. Crude protein was determined by the micro-kjeldahl procedure and reported as N x 6.25 [124]. Ash was determined as the residue following 3 hour treatment in a muffle furnace at 550 to 600 C. The content of acetyl ester residues in corn stover was determined by hydrolysis of the solids in 5% NaOH at 121 C for 15 minutes. After acidification to pH 2 with concentrated HCl, the free acetic acid was extracted into ether and quantitated by GLC as described previously.

3.3. Biomass Pretreatment

3.3.1. Selective Solvent Extraction of Biomass

Corn stover and wheat straw were pretreated by a variety of solvent systems prior to fermentation. Residues were dried at 50 C and then ground in a Wiley mill with a 0.5 mm screen. Extraction of these materials was conducted without

mixing in stoppered Erlynmeyer flasks at 40 g/l solids with 0.2 N NaOH and up to 95% (V/V) ethanol. Extraction conditions evaluated at 121 C were performed in screw capped Hungate tubes in an autoclave. Agitated conditions at room temperature were achieved on a rotary shaker with a 4 cm stroke at 200 RPM in 500 ml Erlynmeyer flasks. Residual biomass solids after extraction were separated by centrifugation or filtration using Schliecher and Scheull #595 filter paper. Solids were washed with minimal volumes of extraction solvent at the same composition without alkali and dried at 50 C to constant weight prior to analyses of pentosan, α-cellulose, lignin, ash, and protein as described previously. Consumption of alkali during extraction was determined by titration of the centrifuged extraction supernatants with 0.1 N HCl while monitoring pH changes by combination electrode (Sensorex S900C).

In order to obtain an "equilibrium" curve for characterization of the effect of extracted lignin on subsequent delignification, 2 liters of 50% (V/V) ethanol-water solution with 0.2 N NaOH were used to extract 40 g/l corn stover at 25 C with mixing as described above. After 48 hrs the mixture was centrifuged to recover the liquid. This supernatant was titrated and sufficient NaOH added to make up 0.2 N NaOH. The solution was then used in a subsequent extraction of 40 g/l fresh corn stover under the same conditions. This process was repeated until less than 150 ml of liquid remained after centrifu-

gation due to losses with the wetted solids. Lignin content in solution at each extraction step was calculated by difference based on lignin determinations of the residual washed solids as previously described.

3.3.2. Lignin Recovery

A number of methods were examined for the recovery of lignin from the extraction solvent. Adsorption to charcoal was measured by statically contacting 5.31 grams of activated carbon (DARCO) with 10.0 ml of 50% V/V aqueous/ethanol solvent containing 36.6 g/l lignin. Loss of lignin from solution was measured by change in otpical density at 280 nm. Alternate methods of recovery were evaluated by the addition of $CaCl_2$ to the same lignin containing solvent. Optical density changes of the supernatant were also monitored.

4. RESULTS AND DISCUSSION

4.1. Objectives

The ultimate objective of this thesis research has been the development of a single step mixed culture fermentation process for the direct conversion of cellulosic biomass to ethanol. One of the first steps required to achieve this goal has been the examination of this concept with a refined model cellulosic substrate such as Solka floc. In this manner the performance of the different mixed culture strains can be assessed. Although this investigation of "baseline" performance represents a necessary prerequisite for progress towards the overall goal, it is certain that the subsequent translation of the fermentation to realistic biomass substrates will represent some major obstacles to this process.

Translation studies to realistic biomass residues have focused primarily on corn stover and, to a lesser extent, on wheat straw. The objectives of these studies are to elucidate the biological factors such as biomass toxicity as well as the physical factors that limit the microbial utilization of these substrates. All of these studies were directed towards finding means to overcome these limitations. Some of the approaches which were taken include biomass pretreatment as well as strain improvement to achieve higher cell densities, overcome biomass toxicity, and increase ethanol yields.

4.2. Preliminary Fermentation Studies

4.2.1. Mono Culture Studies

Our knowledge of C. thermocellum's ability to degrade cellulose was well established at the inception of this project. However, this organism's fermentative capacity for the production of ethanol was not as clearly known. Through a program of adaptation and selection of C. thermocellum ATCC 27405 for tolerance to ethanol, S. Wang was able to isolate a strain (S-4) capable of producing reducing sugars and ethanol as its major products when grown on Solka floc [16]. In batch fermentations using S-4 on 15 g/l Solka floc, the average rate of substrate degradation was 0.21 g/hr. In the course of 70 hours, 4.5 g/l of reducing sugars, 3.2 g/l ethanol, 0.5 g/l acetate and 0.5 g/l lactate were produced. The yield of ethanol based on substrate consumed was 0.24 g ethanol/g Solka floc as compared to a theoretical maximum value of 0.55 g/g. This ethanol yield demonstrated a significant improvement over that obtained with the wild type of C. thermocellum ATCC 27405 of 0.13 g ethanol/g Solka floc.

The growth of S-4 on corn stover, however, resulted in a much different fermentation profile. Fermentation of 36 g/l stover resulted in the production of 2 g/l ethanol, 4 g/l acetate, and 8 g/l reducing sugars after 200 hours. Approximately 70% of the dry weight of the substrate

was degraded which corresponds to a low ethanol yield of 0.07 g ethanol/g corn stover consumed and an average degradation rate of 0.13 g/l-hr.

4.2.2. Mixed Culture Studies

The two major drawbacks to the use of real substrates by C. thermocellum as compared to the performance with Solka floc are the low rate of degradation and the low yield of ethanol. The composition of corn stover has been analyzed to contain 32% cellulose and 37% hemicellulose (Table 9). However, the major fraction of the hemicellulose in stover represents polymers of xylose, arabinose, glucuronate, and galactose, which are not metabolized by C. thermocellum. Thus, the maximum ethanol yield theoretically obtainable with C. thermocellum in monoculture is 0.18 g ethanol per g stover. However, during the course of cellulose hydrolysis by C. thermocellum S-4, a considerable quantity of hemicellulose was solubilized since up to 70% of the weight of the corn residue is lost. If the hemicellulose fraction liberated could be simultaneously metabolized by C. thermosaccharolyticum, the maximum theoretical ethanol yield would be increased to 0.38 g ethanol/g stover consumed. To test the ability of this organism to increase the ethanol yield experiments were performed where 25 g/l corn stover was initially inoculated with C. thermocellum, S-4, in duplicate flasks. After 24 hours of growth,

Table 9

COMPOSITION OF CORN STOVER

α Cellulose	32.0%
Hemicellulose	37.7
Pentosan Hemicellulose	(29.4)
Non-Pentosan Hemicellulose*	(8.3)
Lignin	13.3
Ash	9.0
Protein	4.2
Closure	96.2%

* From hydrolyzed hemicellulose composition analysis by Flickinger *et al.* [53]

one flask was then inoculated with C. thermosaccharolyticum (HG-2). As can be seen in Figure 14, the mixed culture is able to consume the reducing sugars accumulated through the hydrolytic action of C. thermocellum on corn stover. Although the mixed culture resulted in an effective doubling of the ethanol yield over monoculture, the product distribution was unfavorable since more acetate than ethanol was produced. One approach to overcome this product distribution was through further mutation, adaptation and selection of each strain for higher ethanol tolerance. In this manner a series of strains of C. thermocellum and C. thermosaccharolyticum with increased ethanol resistance were obtained (I. Biocic, H.Y. Fang, DOE Reports 9 and 10). The effects of ethanol on the maximum cell density of C. thermocellum and C. thermosaccharolyticum cultures grown on cellobiose are shown in Figures 15 and 16. A steady increase in ethanol tolerance was achieved as compared with both parent strains.

The subsequent use of the best isolates of C. thermocellum S-7 and C. thermosaccharolyticum HG-4 in mixed culture has resulted in an improved fermentation profile on Solka floc. Batch fermentations of 30 g/l substrate have yielded 7 g/l ethanol, 1.5 g/l acetate, and 0.6 g/l lactate. In a number of fed batch fermentations ethanol concentrations as high as 29 g/l have been achieved with the concommittant production of 6.8 g/l acetate and 0.6 g/l lactate. The average

Figure 14

MIXED VS MONO CULTURE ON CORN STOVER

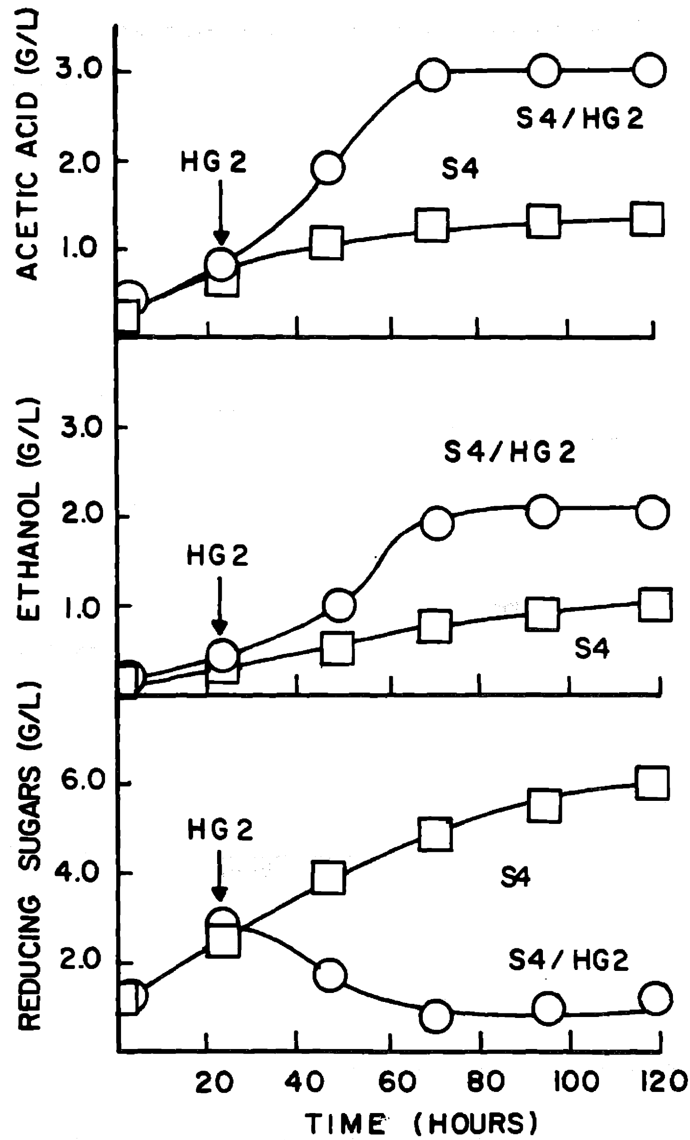


Figure 15

GROWTH OF C. THERMOCELLUM ISOLATES S-4, S-6, S-7, AND WILD TYPE ATCC 27405 IN THE PRESENCE OF ETHANOL

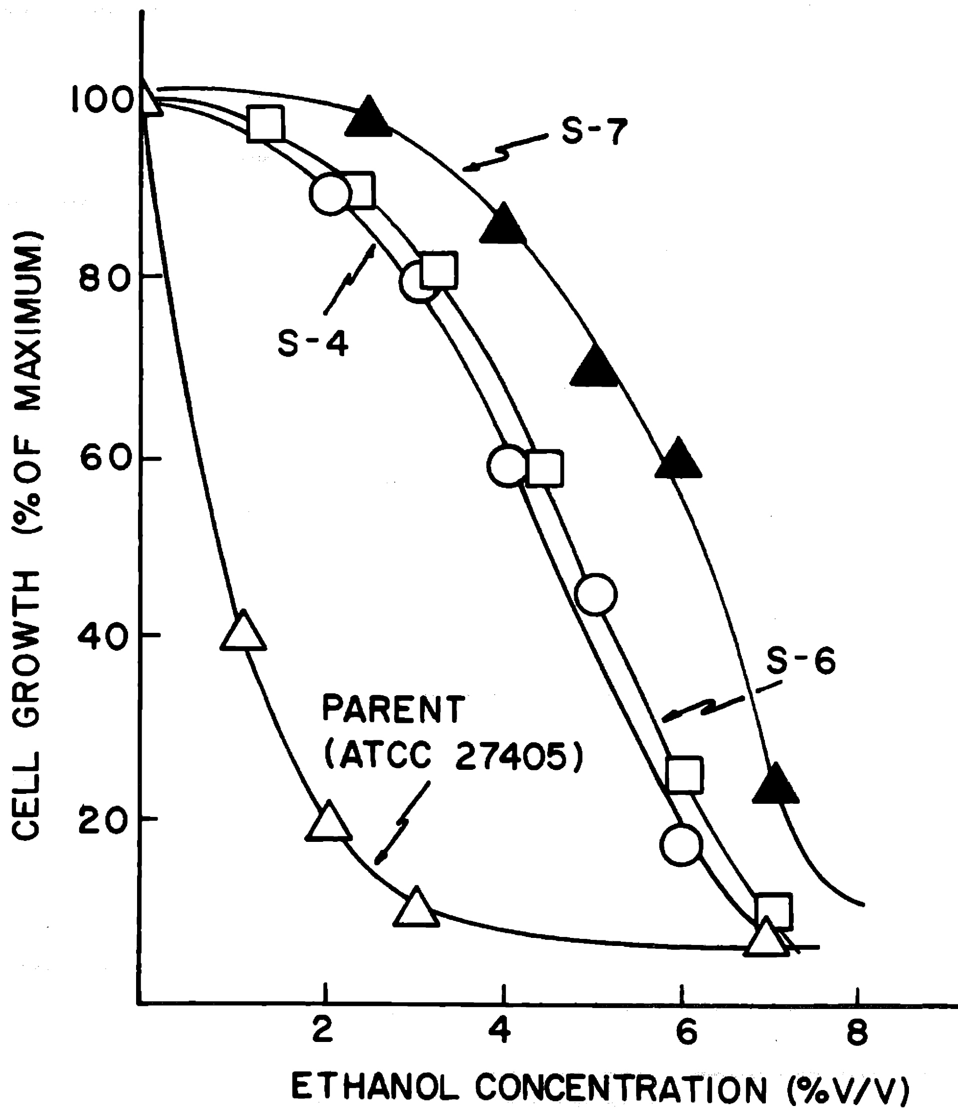


Figure 16

COMPARISON OF ETHANOL TOLERANCE FOR DIFFERENT STRAINS OF *CLOSTRIDIUM THERMOSACCHAROLYTICUM*

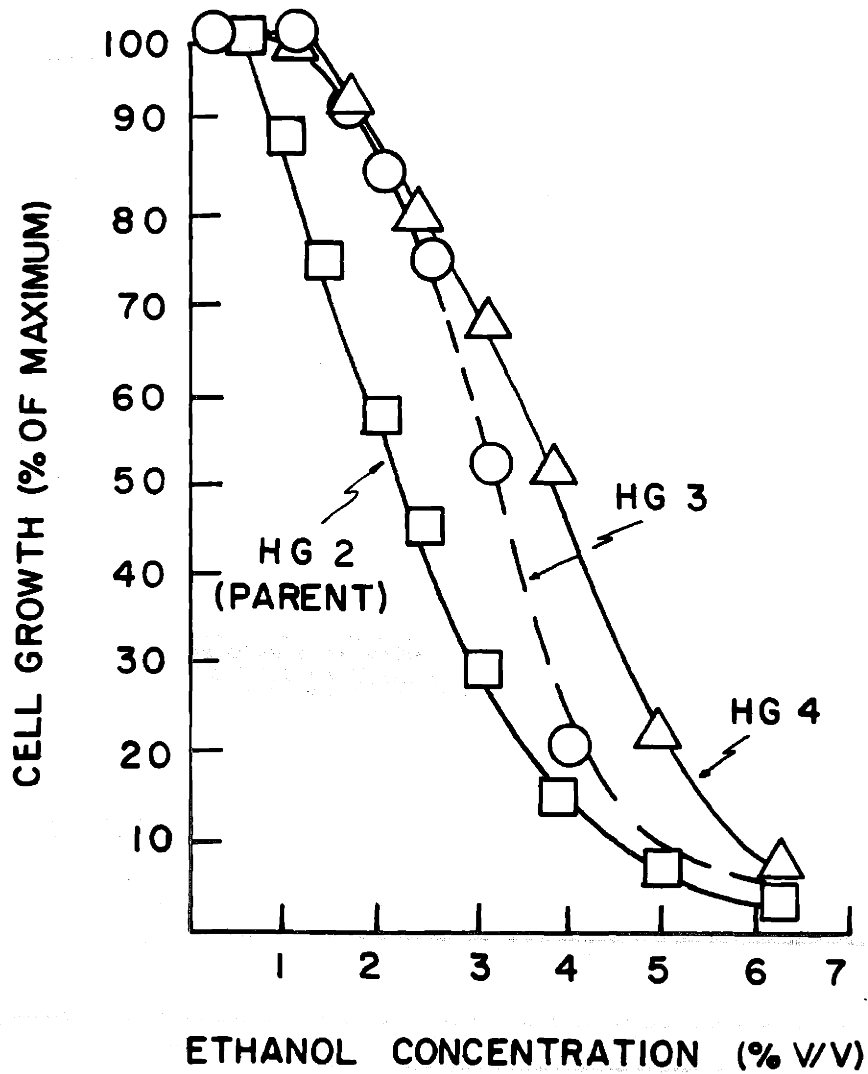


Figure 17

FERMENTATION PROFILE OF MIXED CULTURE OF C. THERMOCELLUM S-7 AND C. THERMOSACCHAROLYTICUM S-7 ON SOLKA FLOC (100 g/l)

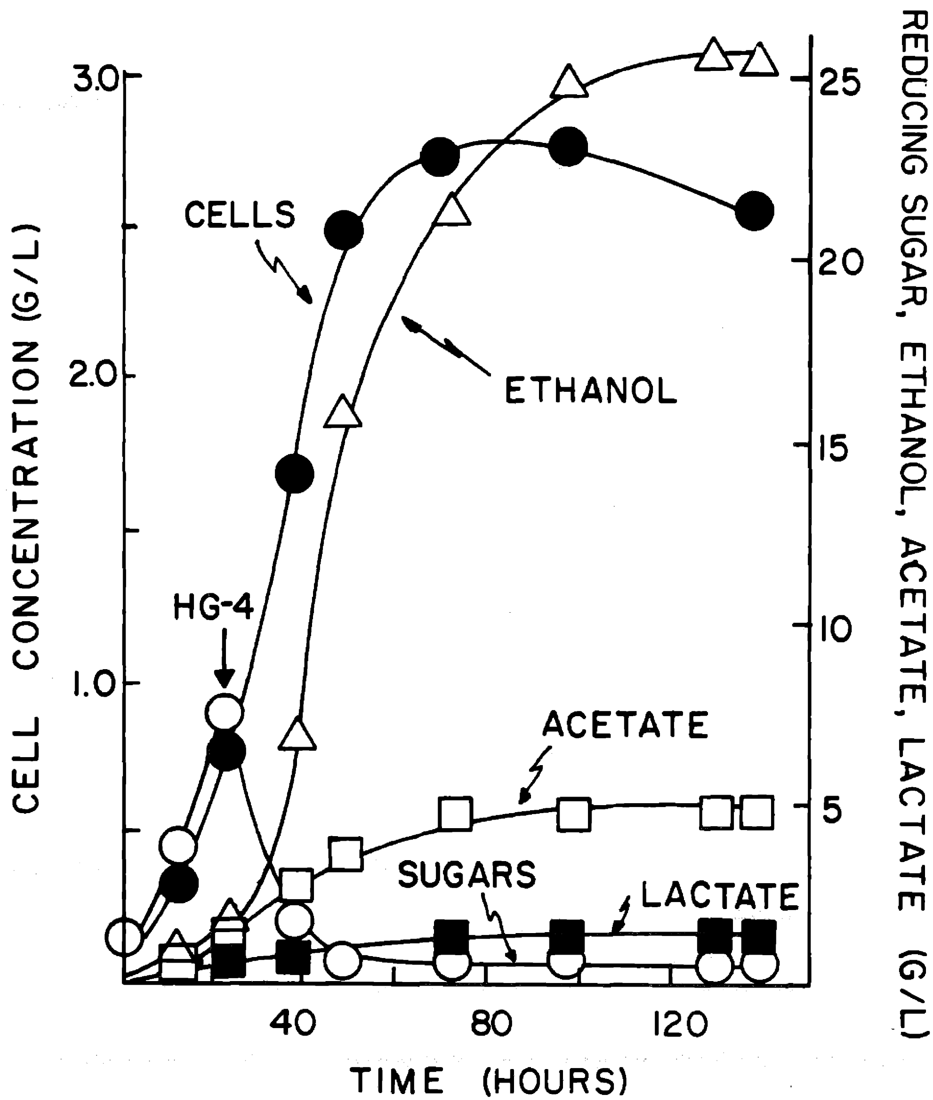
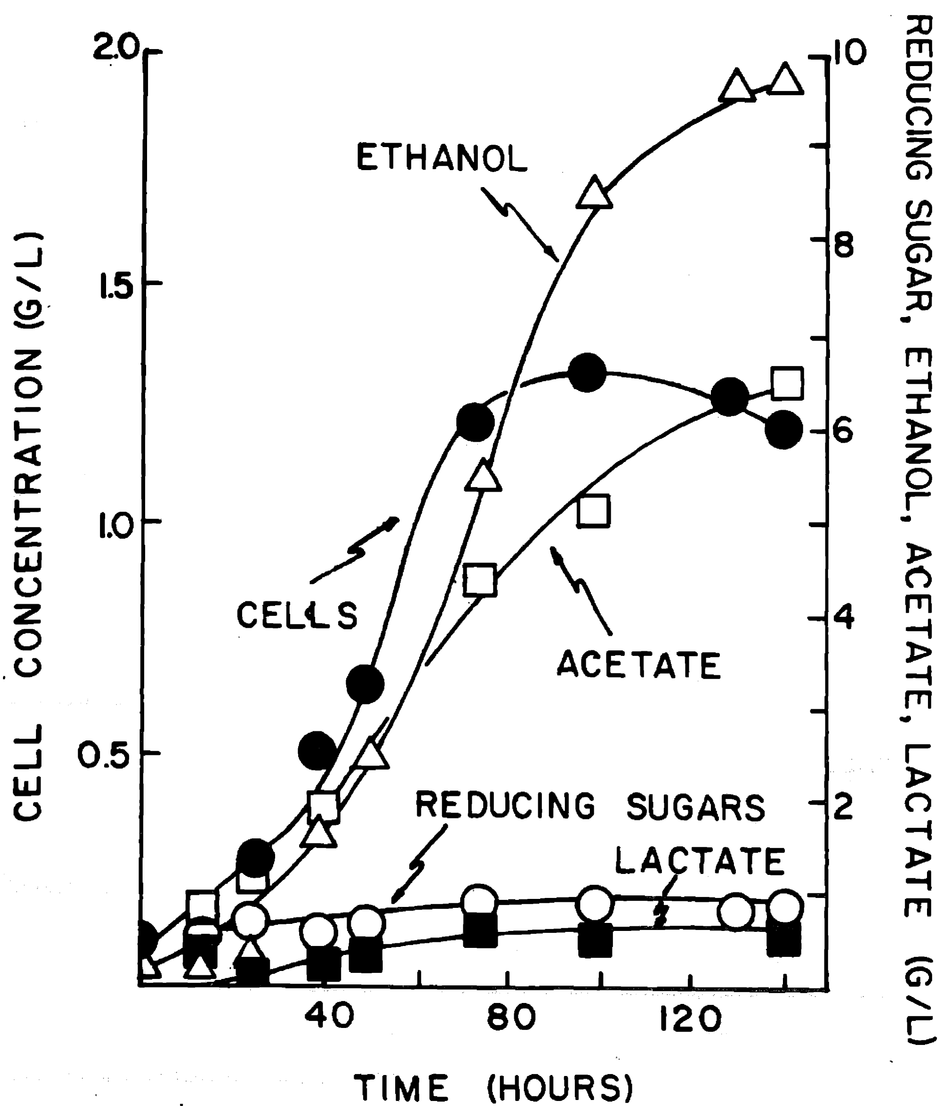


Figure 18

MIXED CULTURE OF C. THERMOCELLUM (S-7) AND C. THERMOSACCHAROLYTICUM (HG-4) ON CORN STOVER (120 g/l)



rate of Solka floc degradation has been as high as 0.8 g/l-hour. The resulting ethanol yield of the mixed culture has been as high as 0.44 g ethanol per gram of Solka floc consumed (e.g. Figure 17).

Unfortunately, however, the use of these new isolates on corn stover have not produced results comparable to those achieved with Solka floc. Results from a number of fed batch fermentations of 30 g/l corn stover has shown an average production of 3 g/l of ethanol, 2 g/l of acetate and 0.6 g/l of lactate. Fed batch fermentations using up to 100 g/l corn stover have shown an average ethanol production of 8 g/l, 6 g/l of acetate and 0.6 g/l of lactate. The average rate of dry matter loss is 0.26 g/l-hr. Although the product distribution and subsequent yield of ethanol has increased over that achieved with the parent strains on corn stover, both the rate of substrate dry weight loss and product distribution remain unfavorable when compared to those obtained on Solka floc (e.g. Figure 18). The presence of lignin, hemicellulose, and other unknown components in an entirely different physical form may all contribute to limit the degradation and subsequent fermentation of this substrate. In the following sections the results of experiments aimed at delineating and overcoming these factors contributing to the altered fermentation profile of mixed culture on corn stover are presented.

4.3. Biological Toxicity of Corn Stover

4.3.1. Effect of Corn Stover Extract on Mixed Culture

The presence of toxic compounds to microbial growth in many extracts of plant biomass is well documented. Thus, one of our first priorities given was to examine this possibility with respect to growth of *C. thermocellum* S-7 and *C. thermosaccharolyticum* (HG-4) when grown on corn stover. Since any potential inhibitor was postulated to be at least partially water soluble, the ability of S-7 and HG-4 to grow in mixed culture was initially examined with the addition of aqueous extracts of corn stover. The performance of mixed cultures on corn stover and Solka floc without extract addition were examined as controls. The aqueous extract of corn stover was prepared as described in Materials and Methods by autoclaving 30 g/l corn stover in distilled water at 120°C for 15 minutes. These conditions are representative of normal sterilization procedures. The resulting hot supernatant was filtered and added to a fresh flask with 30 g/l Solka floc and appropriate CM4 nutrients. As can be seen in Figure 19, the mixed culture fermentation of Solka floc results in the production of 7 g/l ethanol, 1.5 g/l acetate, and 0.6 g/l lactate, as compared to the fermentation of corn stover (Figure 20), which produces only 3 g/l ethanol, 1.6 g/l acetate, and 0.5 g/l lactate. How-

Figure 19

GROWTH OF C. THERMOCELLUM S-7 AND C. THERMOSACCHAROLYTICUM HG-4 ON SOLKA FLOC (30 g/l)

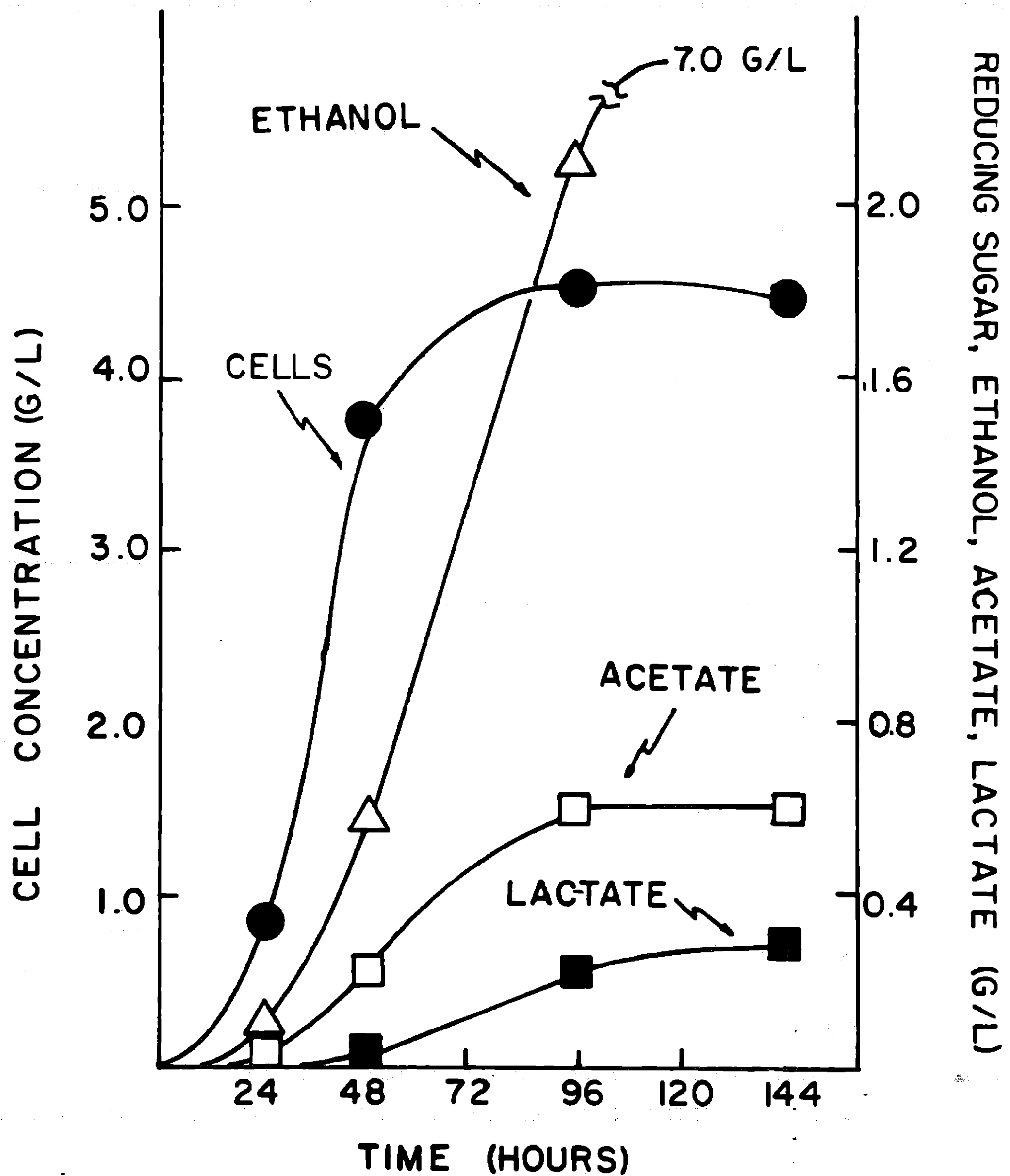


Figure 20

GROWTH OF C. THERMOCELLUM S-7 AND C. THERMOSACCHAROLYTICUM HG-4 ON CORN STOVER (30 g/l)

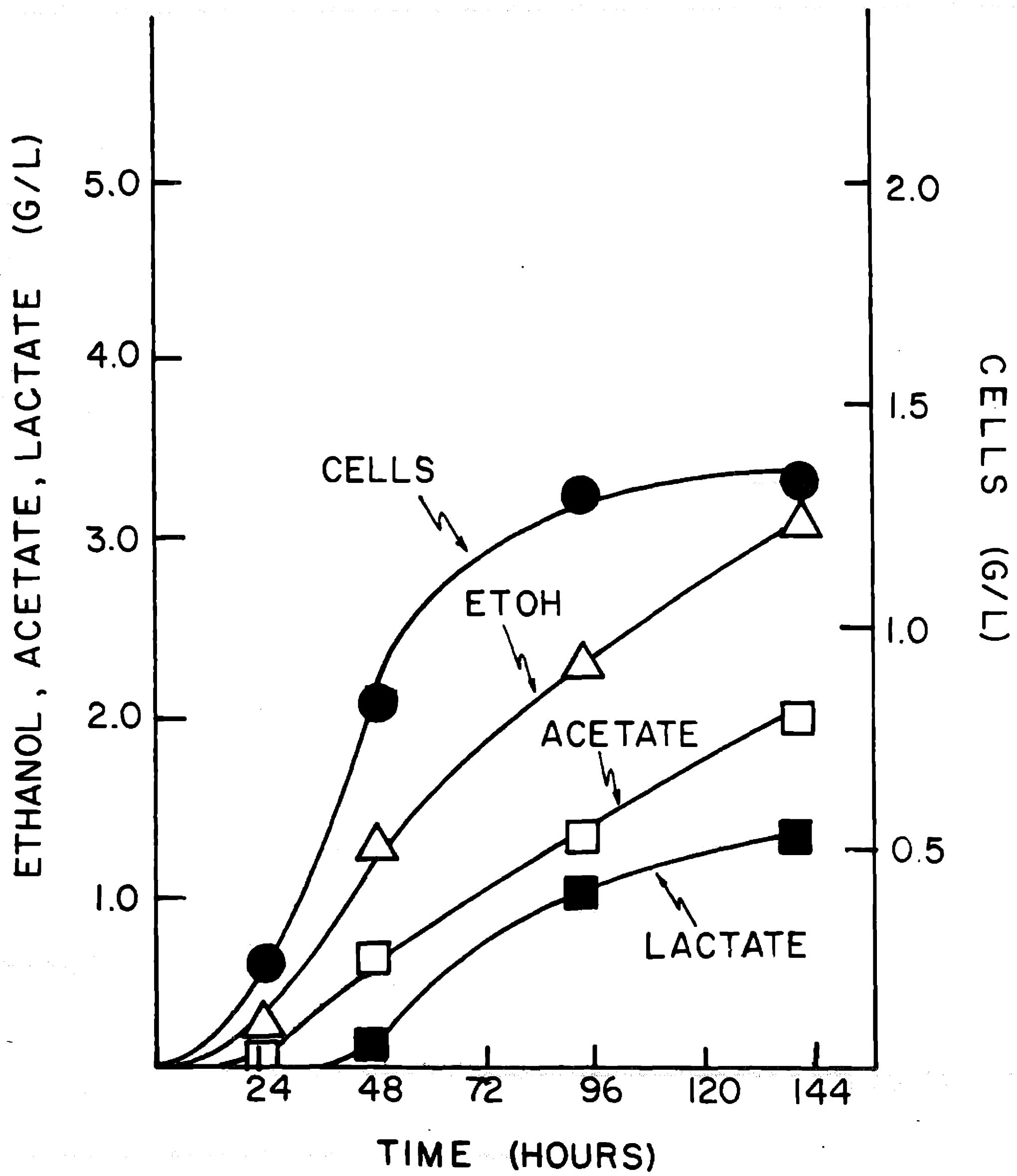
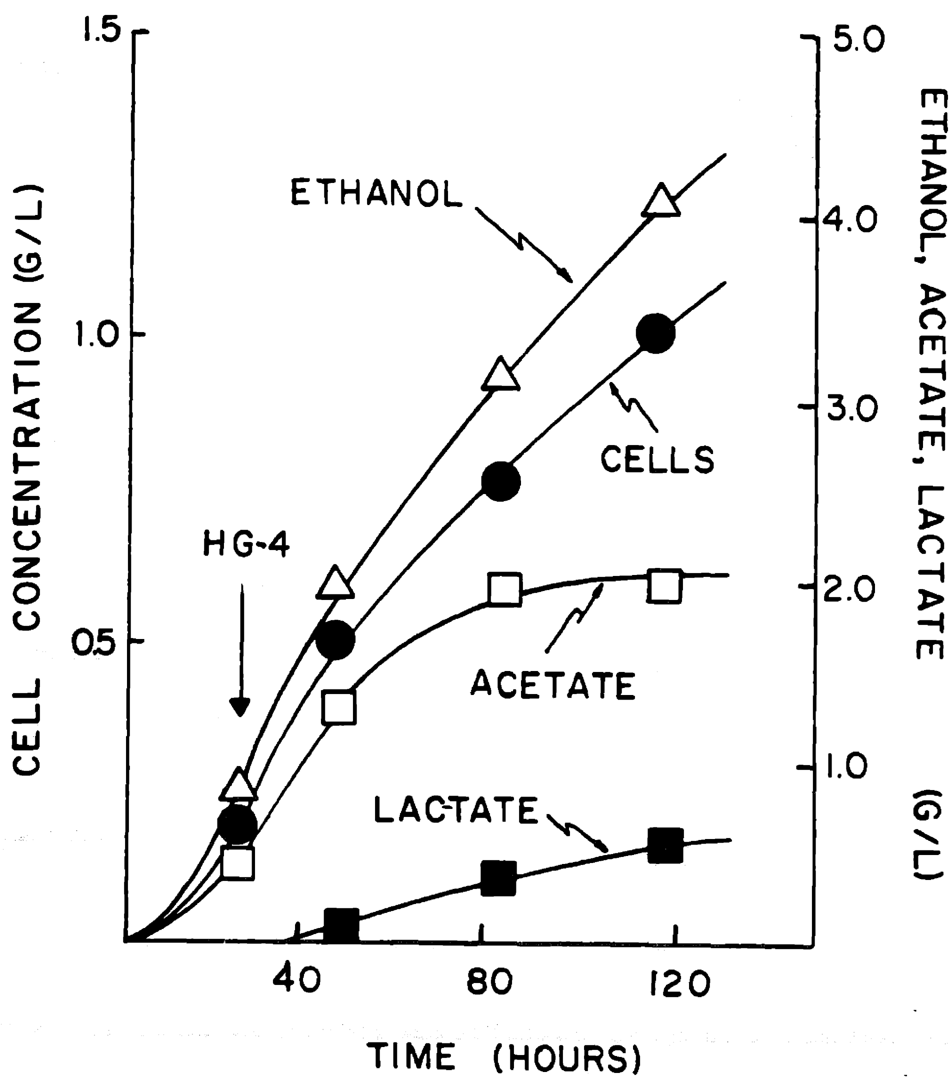


Figure 21

GROWTH OF C. THERMOCELLUM S-7 AND C. THERMOSACCHAROLYTICUM HG-4 ON SOLKA FLOC (30 g/l) WITH AQUEOUS EXTRACT FORM CORN STOVER ADDED



ever, the fermentation of Solka floc in the presence of aqueous extract (Figure 21) results in a strongly inhibited fermentation producing only 3 g/l ethanol, 1.5 g/l acetate, and 1.2 g/l lactate which is similar to that obtained with corn stover. Although the Solka floc in the control is 98% degraded, the fermentation of Solka floc in the presence of aqueous extract shows only 52% degradation after 140 hours. In each case a maximum of 1.5 g/l residual reducing sugars are observed. In conclusion, both the rate of substrate degradation and the ratio of the products produced are apparently affected by the addition of corn stover extract.

4.3.2. Effect of Corn Stover Extract on *Clostridium thermosaccharolyticum* HG-4

In order to further evaluate the effects of this extract on the mixed culture fermentation, the growth of each organism in the presence of extract was examined in monoculture. The results of xylose fermentation by *C. thermosaccharolyticum* HG-4 with the addition of aqueous extract shows an 80% reduction in the maximum specific growth rate (μ_{max}) accompanied by a 40% decrease in ethanol production as compared to the control (Table 10). In spite of this inhibitory effect of corn stover extract on HG-4, no reducing sugars are shown to accumulate during mixed culture fermentations of corn stover. Presumably even at this inhibited growth rate sufficient cell

Table 10

PRODUCT FORMATION BY *C. THERMOSACCHAROLYTICUM* HG-4 GROWN ON 25 G/L XYLOSE WITH ADDED CORN STOVER EXTRACT

	Control	+ Extract
Ethanol	7.7 g/l	4.5 g/l
Acetate	1.4	2.3
Lactate	0.95	1.35
μmax	0.25 hr^{-1}	0.05 hr^{-1}

mass of C. thermosaccharolyticum is present to consume all of the excess reducing sugars produced by S-7. Assuming an average rate of soluble reducing sugar production of S-7 on corn stover of 0.1 g/l-hr [ds/dt], the cell density of HG-4 required to prevent sugar accumulation during mixed culture growth can be calculated from:

$$X = \left(\frac{ds}{dt}\right) \frac{Y_{x/s}}{\mu}$$

Assuming a yield of 0.1 g cells/g substrate and a growth rate of 0.05 hr^{-1}, the cell concentration (X) required to prevent sugar accumulation is calculated to be equal to 0.2 g/l. This amount of cell mass is usually present in the innoculum. Therefore, even though inhibited, the growth of HG-4 does not represent a limiting step to sugar consumption with the mixed culture process on corn stover. On the other hand, the alteration of the product ratios resulting in a decreased ethanol yield by HG-4 in the presence of extract is representative of the major problem seen with real biomass.

The factors in the extract responsible for the inhibition and alteration of product ratios are undoubtedly highly complex. However, progress towards overcoming this problem does not necessarily depend on the identification of the components or mechanisms involved. Strain selection for ethanol

tolerance and subsequent studies in the presence of extracts can be employed without knowledge of "extract" action. This approach is examined in Section 4.5.1. However, one property of natural celluloses not amenable to improvement by strain alteration would be acetate overproduction as a result of hydrolysis of acetylated hemicellulose residues. Morris and Bacon have shown that the acetylated xylose residues are the last to be degraded during rumen fermentation of various grasses [125]. On the other hand, xylans are usually the most water soluble components of natural hemicellulose. These sugars could be inhibitory toward carbohydrate uptake or could yield acetate through hydrolysis prior to fermentation.

Clearly both the acetyl content of xylan in the hemicellulose of corn stover and its extraction into the aqueous extract could play an important role in interpretation of the fermentation results. In order to evaluate this possibility, the acetyl content of corn stover was determined. The results indicate that 30 g of corn stover contains 1.1 g of acetyl residues which are readily released by alkali hydrolysis. This could account for virtually all of the acetate production observed during fermentation of corn stover. However, extraction of these residues into "aqueous solution", shows that only 20% of the total acetyl content is removed from corn stover by this treatment. Therefore, the presence of these acetyl groups could only account for about 25% of the acetate

overproduction observed during growth on xylose in the presence of aqueous extract. It is therefore concluded that the presence of the extract alters the fermentation of C. thermosaccharolyticum with respect to the ethanol to acetic acid ratio. One must therefore devise other means to overcome this problem. This will be addressed in more detail later in Section 4.6.1.

4.3.3. Effect of Extract on S-7

In order to examine the potential biological toxicity of components in corn stover extracts on C. thermocellum, the isolate S-7 was grown in monoculture on Solka floc with and without the addition of aqueous extract. The fermentation results shown in Table 11 indicate that the addition of aqueous extract decreases the final extent of substrate degradation from 66 to 40%. The production of ethanol is similarly decreased by 50% with a slight increase in the production of acetate and soluble reducing sugars.

In order to differentiate between direct inhibition of C. thermocellum (S-7) or cellulose inhibition by corn stover extracts, the growth of S-7 on cellobiose in the presence of up to 50 g/l equivalent aqueous extract was examined in Hungate tubes. No inhibition of growth, as measured by either cellobiose consumption, or ethanol or acetate production, was observed in this case (Table 12). Furthermore,

Table 11

PRODUCT FORMATION BY *C. THERMOCELLUM* S-7 GROWN ON SOLKA FLOC WITH ADDED EXTRACTS FROM CORN STOVER

	Control	Aqueous Extract
μ_{max}	0.08 hr^{-1}	0.05 hr^{-1}
cells	1.3 g/l	1.0 g/l
Ethanol	4.0	1.9
Acetate	1.1	1.3
Lactate	0.6	0.5
Reducing Sugars	5.2	6.0
% Degradation of Solids	66%	40%

Table 12

GROWTH OF <u>CLOSTRIDIUM</u> <u>THERMOCELLUM</u> S-7 ON CELLOBIOSE WITH THE ADDITION OF VARIOUS CONCENTRATION OF AQUEOUS EXTRACT FROM CORN STOVER

Concentration of Aqueous Extract Equivalent Corn Stover (Solids Basis) (g/l)	Cellobiose Consumed (g/l)	Ethanol Produced (g/l)	Acetate Produced (g/l)
0	5.7	2.0	0.8
17	5.3	2.1	0.6
35	5.4	1.8	0.7
52	5.8	2.0	0.8

little effect of this extract on the ratio of ethanol to acetate was noted. These results were also reconfirmed in larger scale anaerobic flask experiments as well. As can be seen in Figure 22, the growth rates of C. thermocellum S-7 as measured by optical density changes are not affected by the addition of up to 50 g/l corn stover extract.

Although no growth inhibition of C. thermocellum was detected on cellobiose in the presence of corn stover extract, a strong effect of this extract on cellulase activity was observed. The experiments were performed in the following manner. C. thermocellum was grown using cellobiose as the carbon source. In addition, corn stover aqueous extracts were added to these cellobiose fermentations at increasing extract concentrations. The supernatants from the end of the fermentations were collected through centrifugation of the broth. The CMCase activity as well as the protein contents in the centrifuged pellet and supernatant were then performed. The results are presented in Figure 23.

As seen in Figure 23, the supernatant CMCase activity was decreased significantly with increased extract addition. The effect of cellulase inhibition by the extracts was also considered. This was achieved by examining the effect of aqueous extract on the initial CMCase activity from the supernatant of the cellobiose control fermentation where no extract was added. No inhibition of this activity was observed.

Figure 22

GROWTH OF CLOSTRIDIUM THERMOCELLUM ON CELLOBIOSE
(25 G/L) WITH EXTRACT FROM 25 and 50 G/L OF CORN STOVER
(333 KLETT UNITS = 1 G/L CELLS)

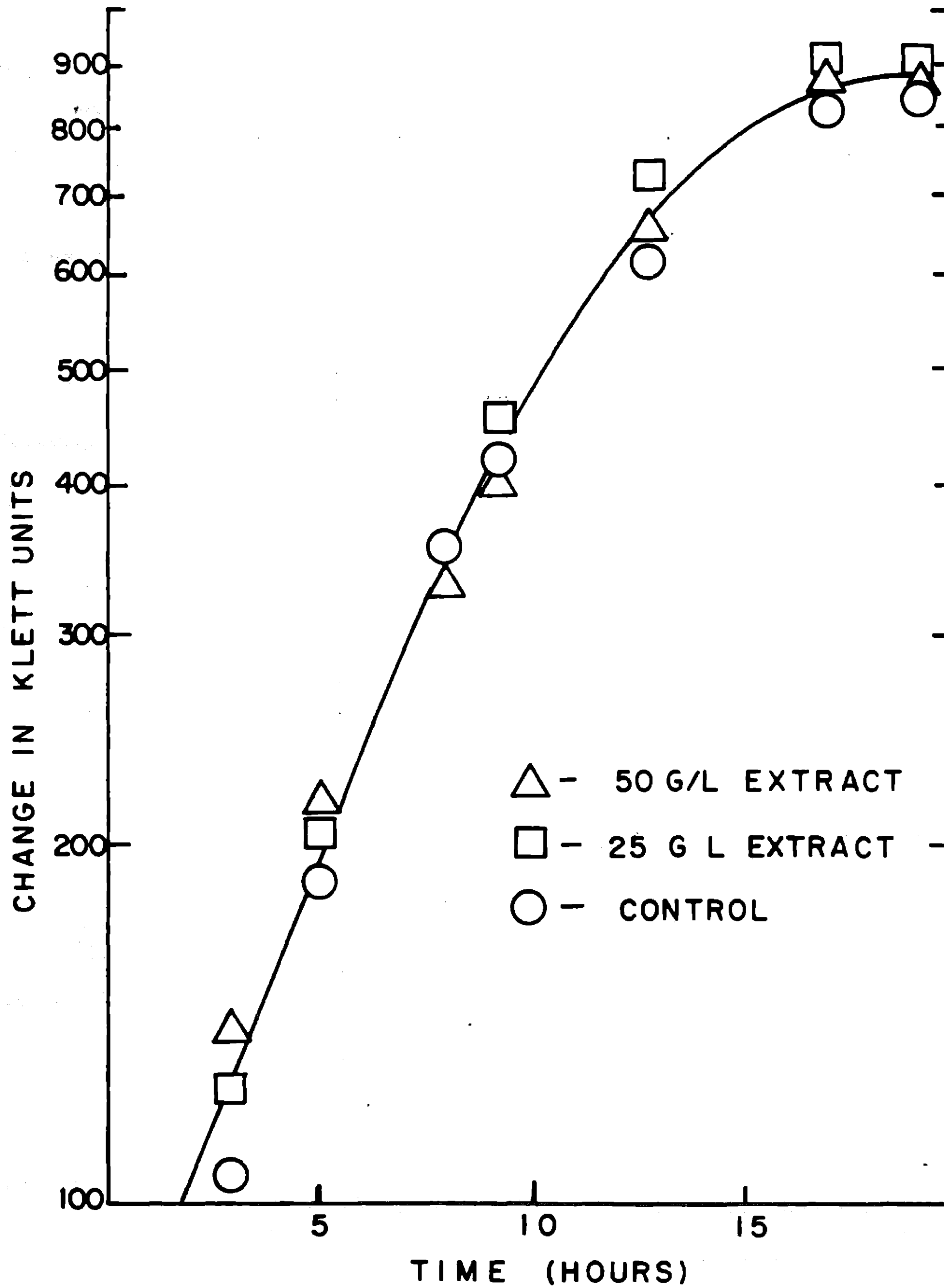
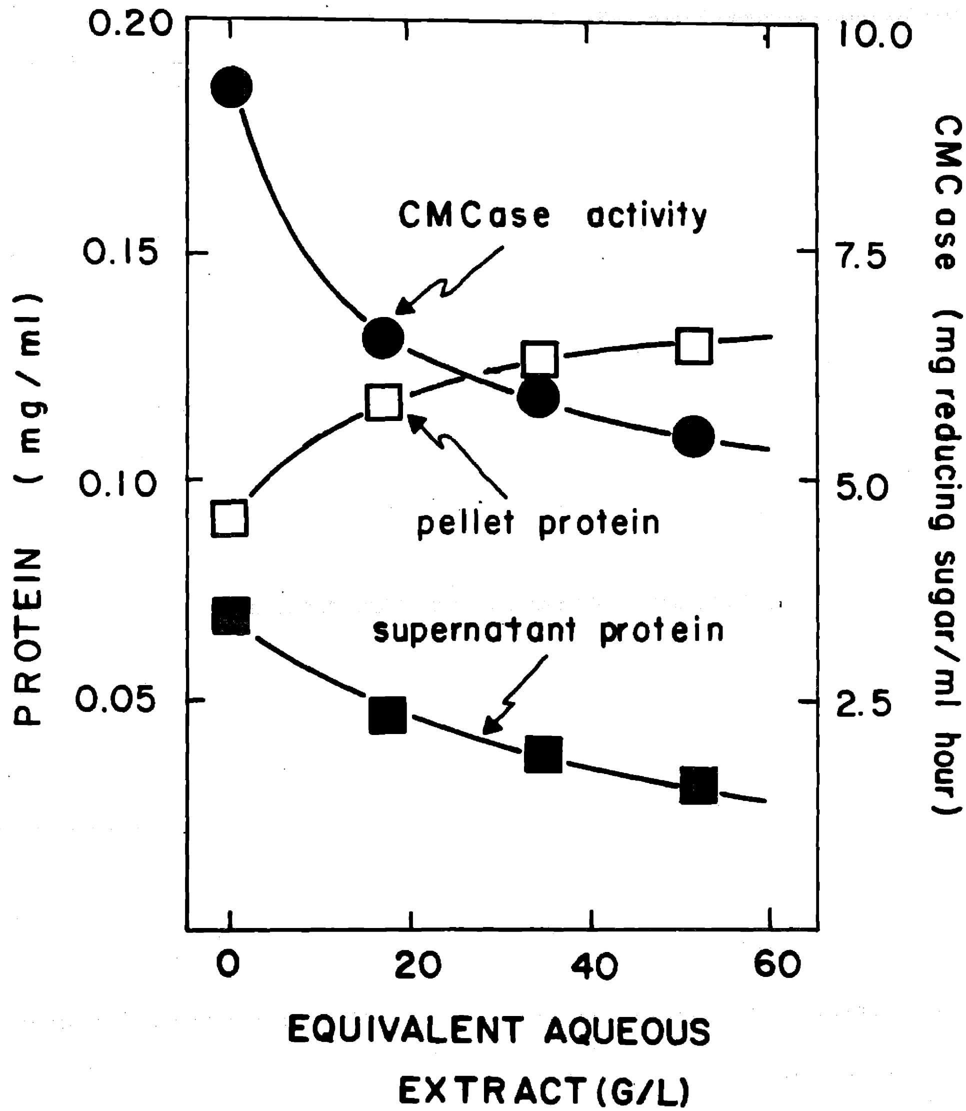


Figure 23

CELLULASE AND PROTEIN PRODUCTION BY CLOSTRIDIUM THERMOCELLUM S-7 GROWN ON CELLOBIOSE WITH AQUEOUS EXTRACT OF CORN STOVER ADDED



The hemicellulose in the extract was also hydrolyzed at an initial rate of 0.3 g/l-hr.

A simple explanation for the decrease in supernatant cellulase activity may arise from the precipitation of material observed during growth in the presence of extract. It is likely that extracellular proteins have a high affinity for lignins in the extract and are thus effectively removed from solution. Due to the difficulty in assaying for protein in the presence of lignin, this hypothesis was indirectly examined by measuring the incorporation of ^{3}H leucine into TCA precipitable protein. C. thermocellum S-4 was grown on CM4 media with cellobiose and with various concentrations of aqueous extract of corn stover added. After 15 hours of growth on cellobiose, samples of whole broth, centrifuged supernatants and pellets were assayed for label incorporation into precipitable protein. At every concentration of aqueous extract tested, the total protein production (supernatant and cells) was approximately constant as seen in Figure 23. However, the percentage of label found in the supernatant versus that found in the pellet dropped rapidly with increasing extract concentration. From these data, it is apparent that the decrease in cellulase activity during growth in the presence of aqueous extract is due to precipitation of cellulase in the presence of the extract. This type of phenomenon is probably the major reason for the decreased rate and extent of Solka floc degrada-

tion by mixed culture in the presence of the corn stover extract. Although cellulase binding to insoluble lignins can occur during growth on corn stover in mixed culture, we cannot conclude that this is the primary rate limiting mechanism from this type of experiment without further information.

4.4. Physical Factors Limiting the Rate of Biomass Degradation

A number of limiting factors have been proposed to explain the resistance of natural lignocellulosic materials to enzymatic degradation (see Literature Survey). By far the most frequently cited, however, is the tight association of lignin with the carbohydrates in lignocellulose. Since we have not observed any direct inhibition of *C. thermocellum* (S-7) or its cellulase by extracts of corn stover, subsequent efforts have been directed at an examination of the physical factors that might influence the degradation of this lignocellulosic substrate. These factors have included the influence of cellulase loading, substrate loading, biomass surface area, and cell loading on the rate of biomass degradation. In order to assess the importance of these factors on the rate of degradation, a number of studies were performed as described below.

4.4.1. *In vitro* Cellulose Hydrolysis of Corn Stover

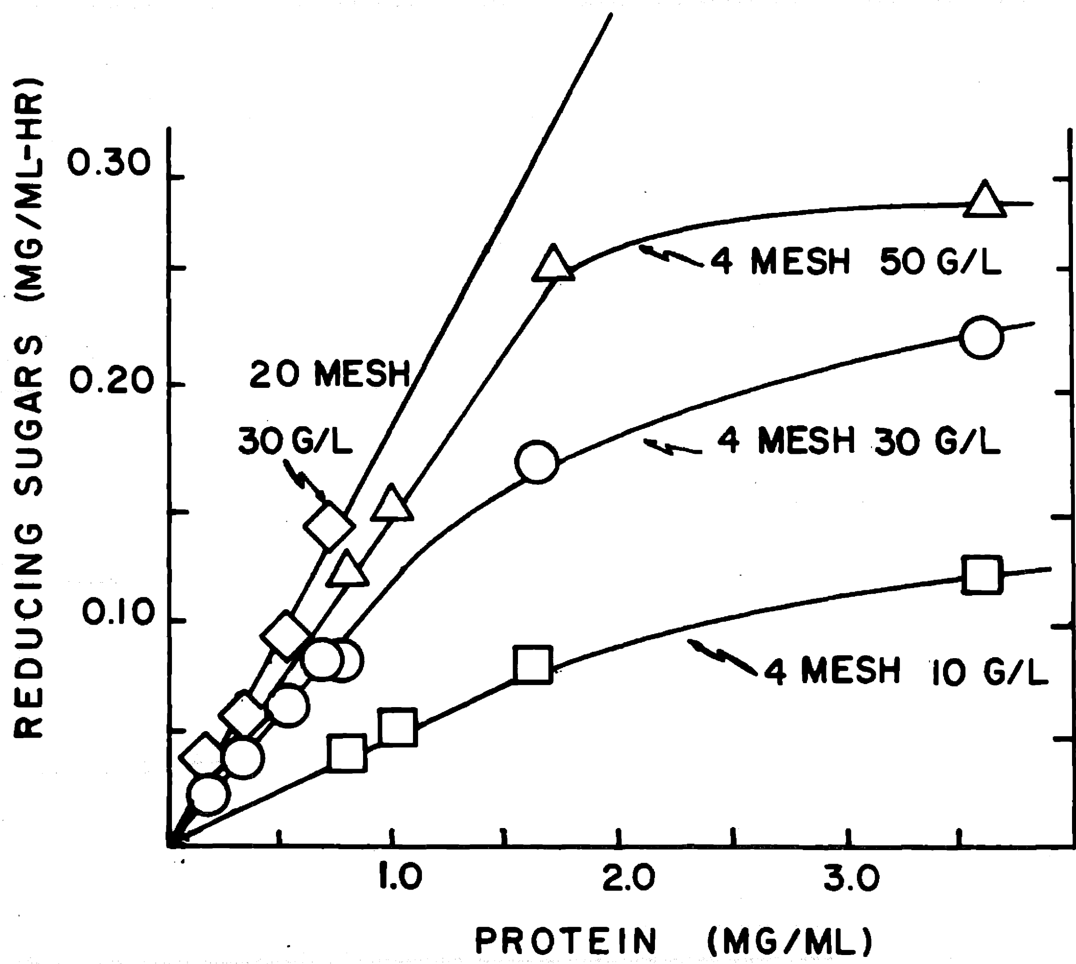
A cell free cellulase preparation was used to examine the effects of protein and substrate loading on the

rate of biomass degradation. The supernatant from C. thermocellum (S-4) grown cells on cellobiose was recovered by centrifugation and the protein precipitated with 40% ethanol at -20°C. Virtually 100% of the initial CMCase activity was recovered using this technique. Corn stover was ground in a Waring blender, sieved, and sterilized in CM4 salt solution with reducing agent prior to addition of the cellulase. Samples were incubated anaerobically at 60°C and periodically assayed for the production of soluble reducing sugars by the DNS method. The initial rates of reducing sugar formation were measured during the first six hours of hydrolysis at various protein concentrations. In addition, different particle sizes and corn stover concentrations were also examined with respect to the initial rate of reducing sugar formation at different protein concentrations. The results from these experiments are shown in Figure 24.

For each substrate condition examined, the specific activity is constant at low protein concentrations. However, a rapid decrease in specific activity is observed at cellulase concentrations greater than 1.5 mg/ml. It is apparent that saturation kinetics of the enzymic activity with respect to the substrate occurs as seen in Figure 24. This is not surprising if we assume that there exists a finite number of readily degradable sites in a substrate such as corn sto-

Figure 24

CELLULASE ACTIVITY ON CORN STOVER



ver. Furthermore, the number of these "sites" decreases as the material is degraded.

From these results, a number of conclusions can be drawn with respect to the degradation of non-treated corn stover by the cellulase enzyme from C. thermocellum. For example, when this organism (S-4) is grown on Solka floc or cellobiose, typically 2 to 3 mg/ml of cell mass is produced. It will be shown later in this thesis that the excretion of extracellular protein by C. thermocellum (parental strain ATCC 27405) is typically 0.26 mg protein/mg of cell mass when grown on cellobiose. Combining the maximum cell mass production and extracellular protein production, one would expect a range of 0.52 to 0.78 mg of protein excreted per ml of fermentation broth. If one further assumes that these excreted proteins have similar enzymic activity on corn stover as shown in Figure 25, the following conclusions are then offered.

If one examines the results in Figure 24 for a given particle size (4 mesh) and different corn stover concentrations (10 to 50 g/l), it can be seen that deviation from the linear relationship between enzyme concentration and rate of degradation occurs at approximately 1.5 mg protein/ml. It is therefore concluded that above this protein concentration and at all particle sizes there is a diminishing return with respect to increasing the rate of corn stover degradation with increases in the enzyme concentration. This arises from the

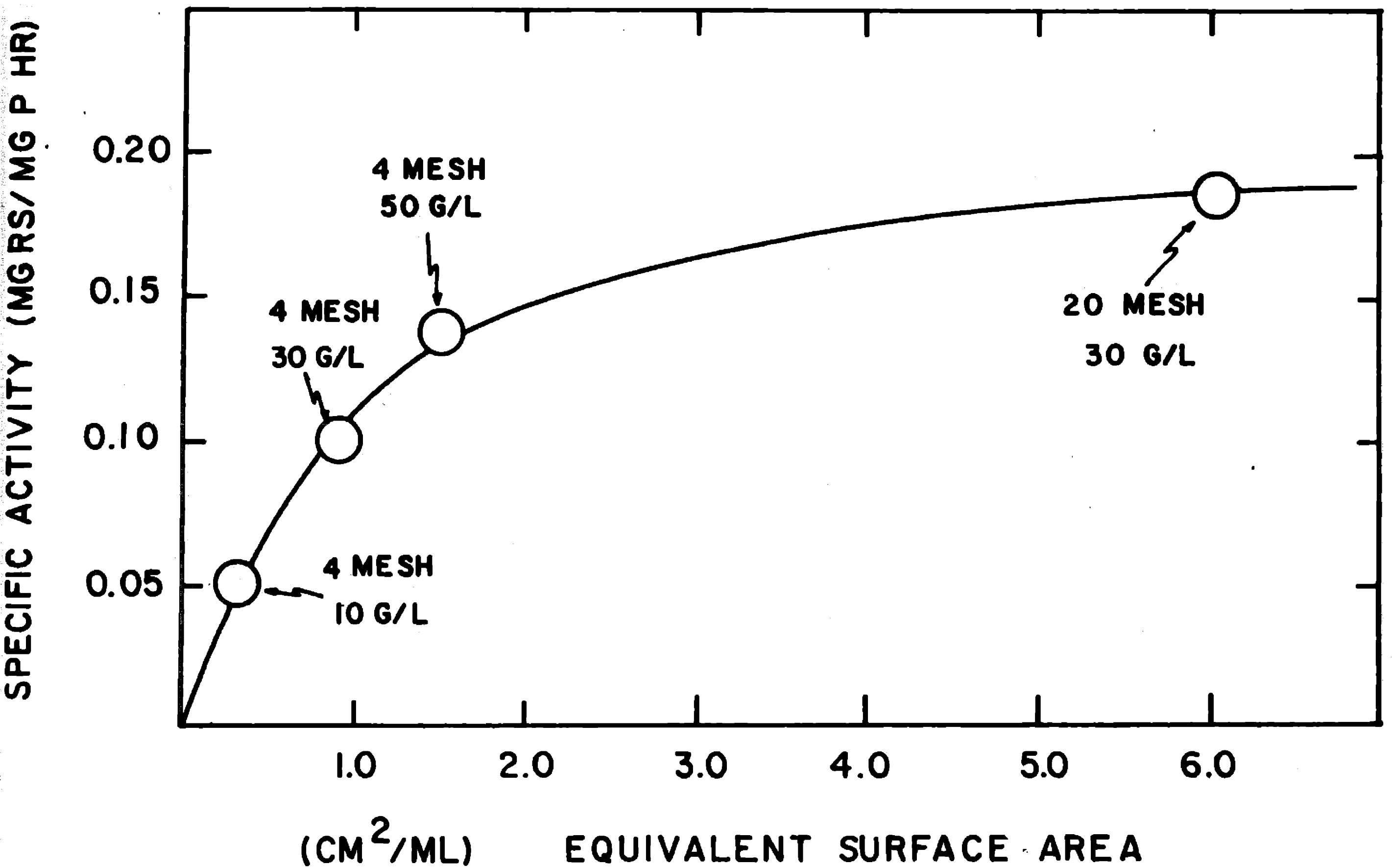
saturation kinetics as stated previously. On the other hand, there is an increased rate of degradation in the region of the linear kinetics as the solids concentration is increased. Therefore, there is a benefit to be derived at a given enzyme (protein) concentration through operating the fermentation at high solids loading (corn stover concentration). Unfortunately, the increase in the rate of degradation is not linearly related to the corn stover concentration.

A quantitative analysis of the effect of increasing substrate concentration or decreasing the particle size on the rate of corn stover degradation can be performed using the data in Figure 24. In this analysis the linear region of the volumetric rate of sugar formation (mg/ml-hr) versus protein concentration (mg/ml) was employed. From the slope of the data in Figure 24, the specific rate of reducing sugar formation (mg RS/mg protein-hour) can be calculated for the different corn stover concentrations and particle sizes. As a first approximation, it was assumed that the corn stover particles are spherical having a diameter equivalent to the particle size. In this manner an equivalent specific surface area (cm^2/ml) of the corn stover particles was then calculated. The specific rate of reducing sugar formation was then plotted versus the equivalent surface area and shown in Figure 25.

It can be seen from Figure 25 that there is an increased rate of reducing sugar production with an in-

Figure 25

EFFECT OF TOTAL SURFACE AREA PER UNIT VOLUME ON THE INITIAL SPECIFIC HYDROLYSIS RATE OF CORN STOVER



creased equivalent surface area. However, this relationship is not linearly related to the increasing surface area. This observation would suggest that there exists a limiting value of available "degradable sites" in a real lignocellulosic biomass such as corn stover. When these degradable sites are completely occupied by the enzyme, further increase in the external surface area will not influence the specific rate of sugar formation.

4.4.2. *In vivo* Hydrolysis of Corn Stover

Electron micrographs of corn stover and Solka floc during mixed culture fermentation by *C. thermocellum* (S-4) and *C. thermosaccharolyticum* (HG-4) have indicated an attachment of cells onto the biomass solids (Figures 26 and 27). The possibility exists that the presence of cells in close proximity to the point of substrate degradation may enhance the rate. In addition, attachment of cells to insoluble substrate could provide a means for achieving "internal" cell recycle and thus enabling the maintenance of a higher cell mass leading to an increased productivity during fermentation.

In view of the observed cell attachment on the solid biomass, it was hypothesized that if one increased the inoculum concentration an increased rate of fermentation might result. The inherent assumption being the high cell inoculum can attach to a greater extent to the corn stover solids

Figure 26

ELECTRON MICROGRAPH OF *CLOSTRIDIUM THERMOCELLUM* S-7 AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM* HG-4 DURING GROWTH ON SOLKA FLOC

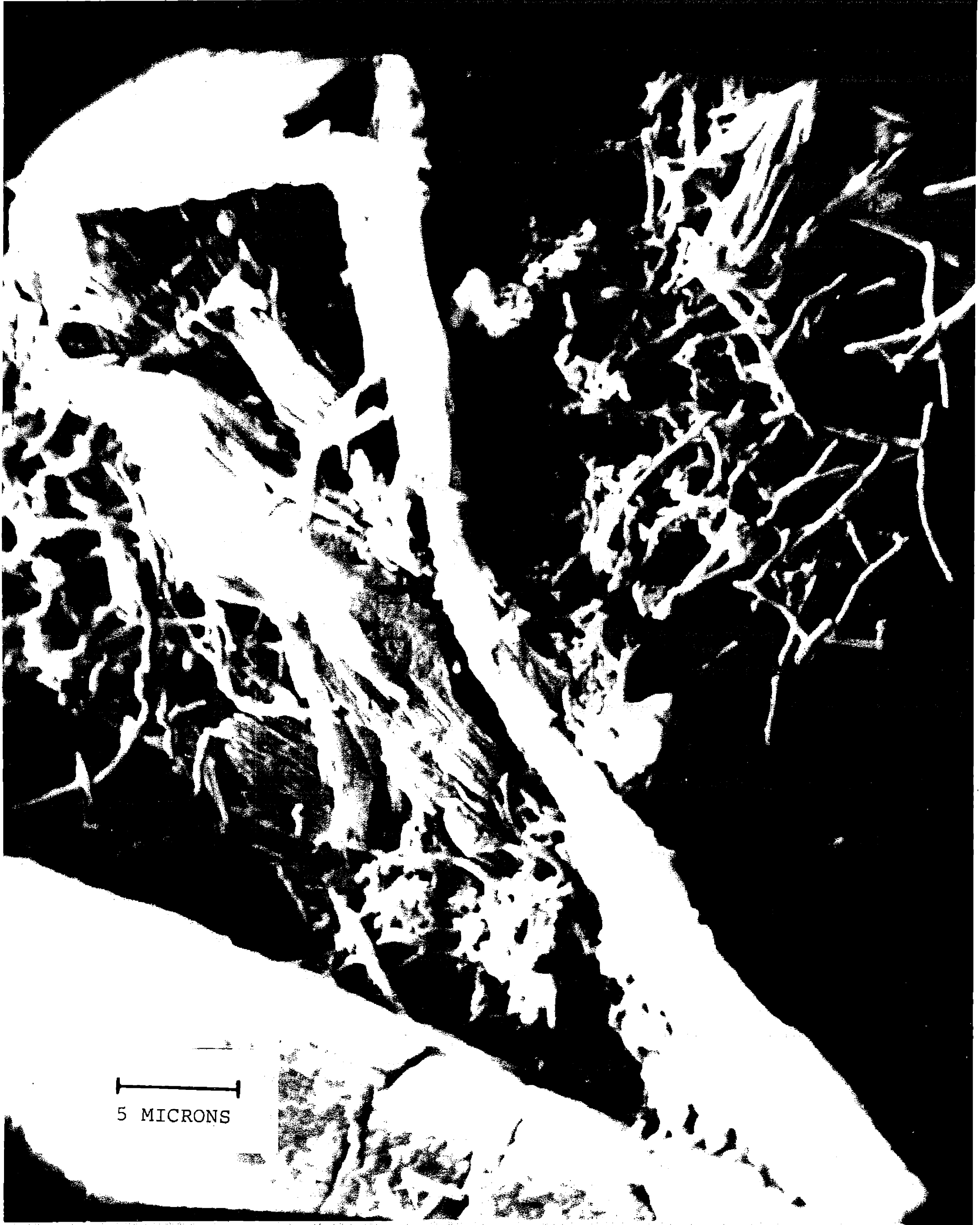
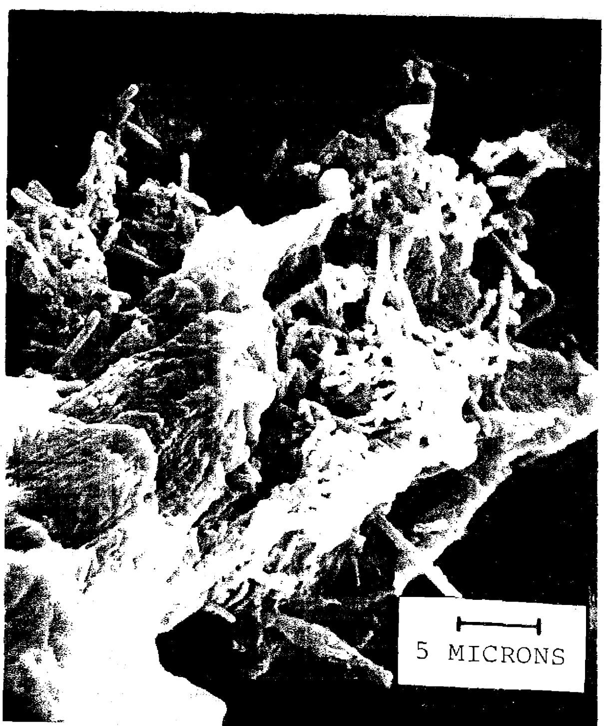

5 MICRONS

Figure 27

ELECTRON MICROGRAPH OF *CLOSTRIDIUM THERMOCELLUM* S-7 AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM* HG-4 DURING GROWTH ON CORN STOVER

25 MICRONS


5 MICRONS

and ultimately lead to a higher rate of degradation. To test this hypothesis, C. thermocellum (S-4) was grown on cellobiose and harvested by centrifugation. The harvested cells were then reconstituted and used as inoculum for untreated corn stover fermentations at 27 g/l and 4 mesh particle size. The increased inoculum concentration was designed to equal 1.22, 2.44 and 3.66 g/l of cells. These values correspond to an increase in the inoculum concentration of 10, 20 and 30 times, respectively, when compared to the conventional inoculum of 0.122 g/l (10%). The results from these studies are shown in Figures 28 and 29.

It can be seen from Figure 28 that the rates or amount of reducing sugar formation do not correspond to the proportional increases in the inoculum concentration. The rates and amount of ethanol production as seen in Figure 29 also do not show significant differences at different inoculum concentrations. At a first glance, one might feel these experiments were not properly conducted. However, a more detailed analysis will show that this behavior is to be expected and is presented below.

In these experiments, the inoculum was concentrated through centrifugation. In this procedure, the extracellular protein (enzymes) was discarded. In order to initiate corn stover degradation, previous results (Figure 24) have shown that the action is through the extracellular enzymes. If one assumes that the cells in these concentrated

Figure 28

PRODUCTION OF REDUCING SUGARS BY CLOSTRIDIUM THERMOCELLUM (S-4) ON CORN STOVER USING CONCENTRATED CELL INOCULUM

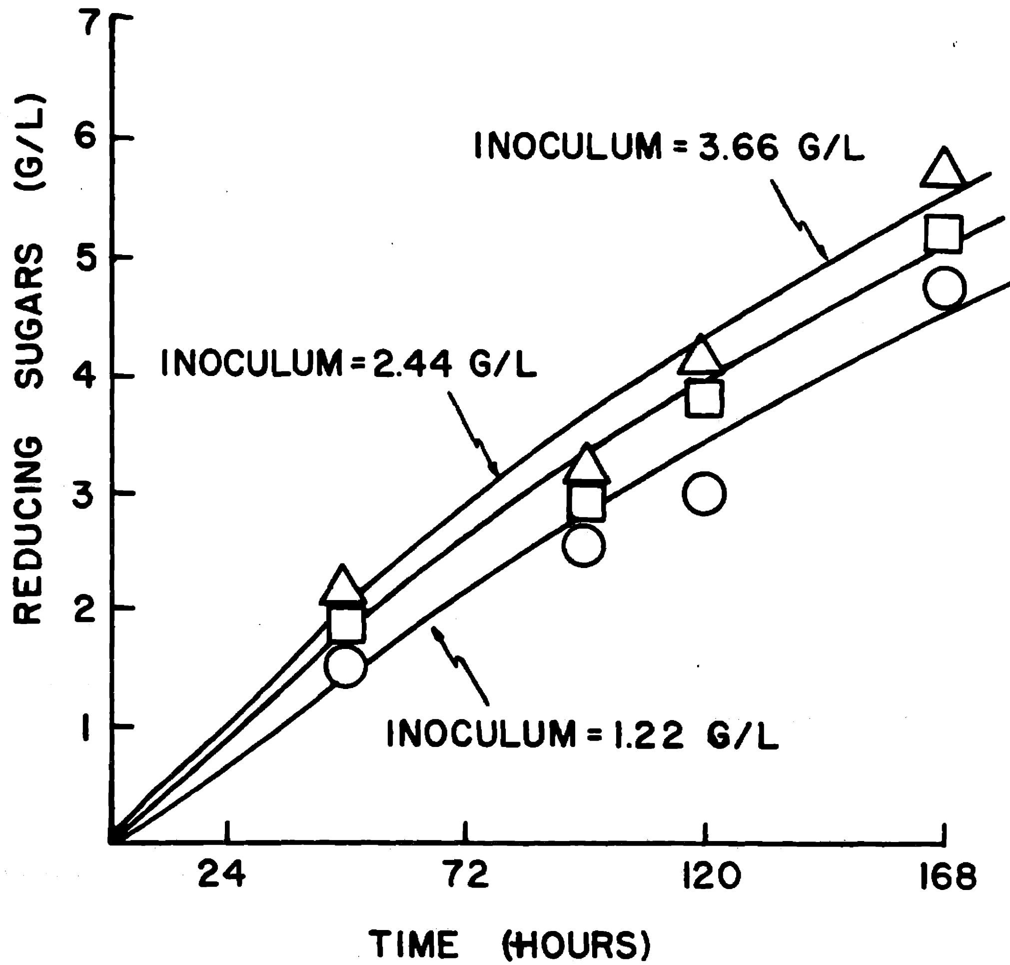
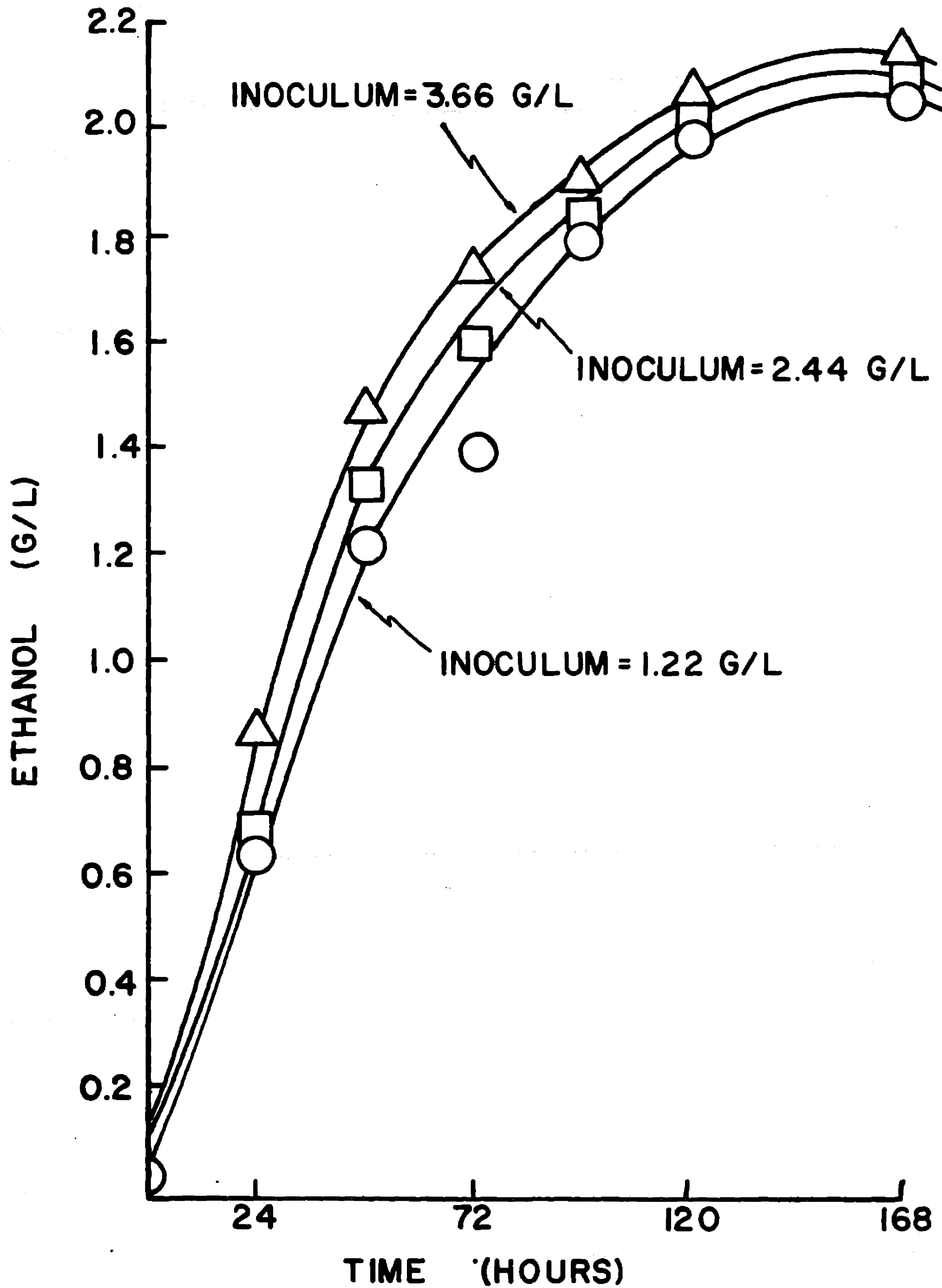


Figure 29

ETHANOL PRODUCTION BY *CLOSTRIDIUM THERMOCELLUM* (S-4) ON CORN STOVER USING CONCENTRATED CELL INOCULUM



inoculum can instantaneously produce extracellular enzymes, from the previous findings one would anticipate the following behavior. As stated before, it has been found that 1 mg/ml (or 1 g/l) of cells will produce 0.26 mg/ml of extracellular enzymes. If the above assumption is correct, at an initial inoculum of 1.22 g/l of cells, in a short time period 0.32 mg/ml of enzyme should be produced. In examining the results in Figure 8 (4 mesh, 30 g/l solids) at this enzyme concentration the rate of reducing sugar formation is still in the linear response region. Furthermore, at the highest inoculum concentration (3.66 g/l) one would have expected a proportional increase in rate of sugar formation since this is still in the linear rate region. However, the results in Figures 28 and 29 do not follow these behaviors. It is therefore concluded that the inoculum cannot instantaneously produce enzymes to effect corn stover degradation. Furthermore, even if cell attachment to solid is increased due to increased inoculum concentration, there is no benefit to be gained since these cells are unable to produce the enzymes which are ultimately responsible for biomass degradation.

4.5. Biomass Pretreatment

The research findings thus far have identified a number of problems in the use of corn stover for the production of ethanol. The results (Figure 24) from increasing the cellulase

enzyme concentrations have shown that a limiting value exists where a saturation of the rate of reducing sugar hydrolysis is to be expected. The reduction of particle size or the increased loading of biomass solids both of which are designed to increase the external surface area for cellulose hydrolysis has also shown saturation kinetics (Figure 25). The use of high inoculum concentration (Figures 28 and 29) also shows that the microorganisms do not adjust instantaneously to an increased enzyme production. Thus far, all of these studies have not shown any monumental increase to enhance the rate of corn stover degradation.

It was therefore concluded that the corn stover lignacious composition must be altered if a significant enhancement in the rate of degradation is to be achieved. It was therefore decided that biomass pretreatment is necessary if this objective is to be attained. However, there are a number of constraints which must be considered in view of other studies that have been performed. For example, it has been shown in Section 4.3.3. that lignin in solution will adversely affect the enzymatic activity of the cellulase through the precipitation of the enzymes. Therefore, the avoidance of soluble lignin materials during pretreatment must be considered. In addition, the major premise in using biomass such as corn stover is its relative cost with respect to other feedstocks. Therefore, the pretreatment process must at all cost

avoid losses of the different carbohydrates such as those derived from cellulose and hemicellulose in biomass. Other constraints during pretreatment include the prevention of the formation of toxic compounds which could have adverse effects on microbiological activities. It is with these constraints that the pretreatment studies described in this section of the thesis were performed.

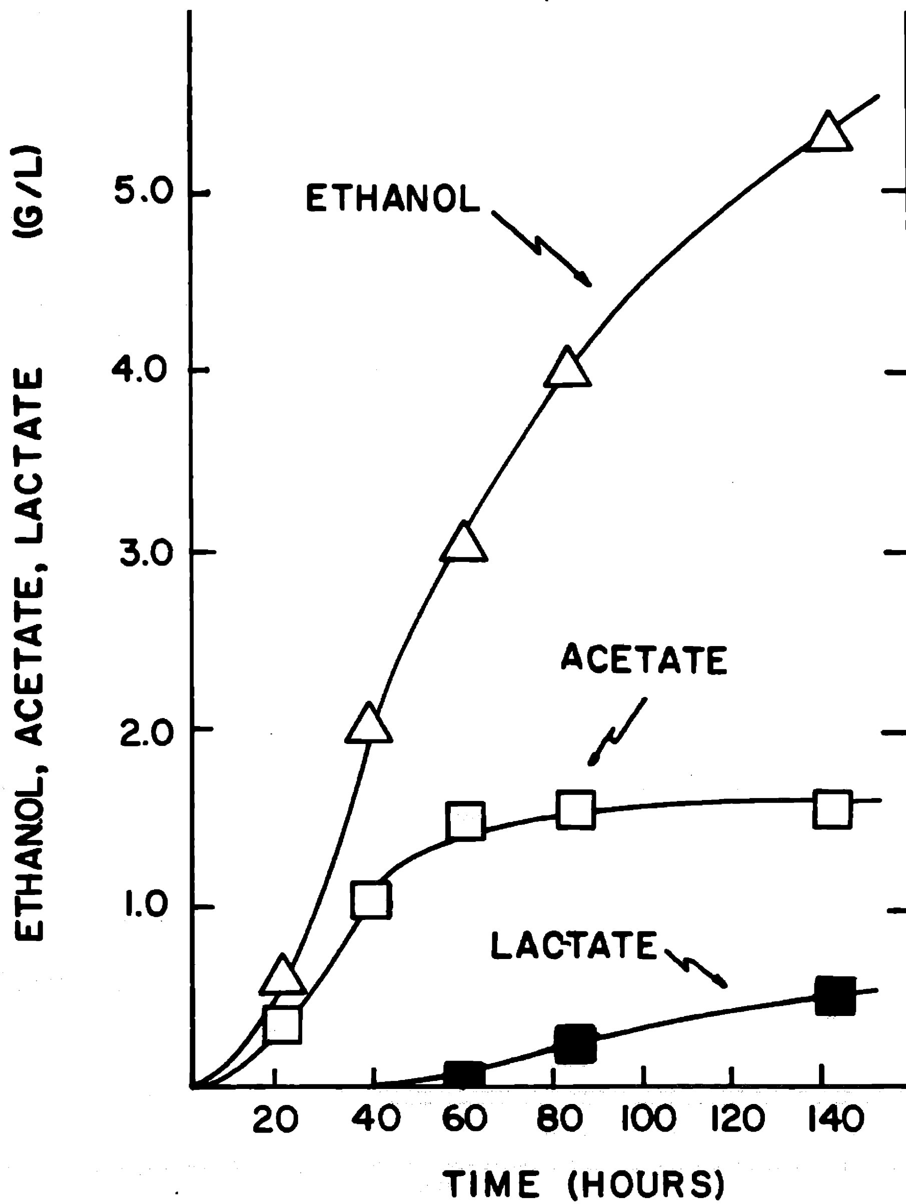
4.5.1. Alkaline Extraction of Corn Stover

Corn stover (40 g/l) was pretreated by contacting with 0.20 N NaOH for 15 minutes at 120°C. The hot alkaline extract was separated by filtration and the residual corn solids were washed three times with 100 ml distilled water. The solids were resuspended in distilled water, supplemented with CM4 nutrients and fermented with a mixed culture of *C. thermocellum* (S-7) and *C. thermosaccharolyticum* (HG-4). The fermentation of this pretreated substrate yielded dramatically improved performance both with respect to the average rate of degradation and ethanol yield as shown in Figure 30. The production of 5.5 g/l ethanol and 1.5 g/l acetate represents a significantly improved product ratio. Furthermore, this pretreated corn stover was able to attain 70% of the degradation rate and ethanol productivity as compared with Solka floc.

The usefulness of alkaline extraction as a pretreatment, however, must be considered within the constraints stated previously. One of the constraints is to retain as much

Figure 30

FERMENTATION OF NaOH PRETREATED CORN STOVER (30 G/L)
WITH *CLOSTRIDIUM THERMOCELLUM* S-7 AND
CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-4



as possible the carbohydrates for subsequent utilization during fermentation. It was found during alkaline extraction, approximately 50% of the corn stover dry weight was lost. On the other hand, the extracted corn stover was found to contain very little lignin. In order to have a better understanding of this extraction process, additional studies were therefore performed on the residual biomass composition.

An examination of the pentosan and lignin losses during alkaline extraction of stover at 121°C, 60°C, and 25°C are shown in Table 13. Delignification in each case is extensive and ranges from 62 to over 90%. However, the pentosan carbohydrate losses were also high, ranging from 38 to 42%. These findings are disappointing since the pentosan losses could represent an increased raw materials cost.

One could, however, consider the utilization of the soluble pentosan in the alkaline extract in order to offset this increased feed stock cost. It should be remembered, however, studies in Section 4.3.2 have shown a toxic effect of aqueous extract on the growth of _C. thermosaccharolyticum_ (HG-4). For the sake of completeness, fermentation studies were performed using _C. thermosaccharolyticum_ (HG-4) in the presence and absence of added alkaline extract from corn stover. The results are shown in Table 14. As to be expected, it can be seen from these results that the presence of the extract indeed inhibited the growth of _C. thermosaccharolyticum_. This

Table 13

COMPOSITION OF LIGNIN AND PENTOSAN RESIDUAL CORN STOVER AFTER PRETREATMENT WITH 0.2 N NaOH

Temp.	Time	% Delignification	% Loss of Pentosan
121°C	15 min	91	42
60°C	24 hrs	81	38
25°C	72 hrs	62	38

Table 14

GROWTH AND PRODUCT FORMATION BY *CLOSTRIDIUM THERMOSACCHAROLYTICUM* HG-4 GROWN ON 25 G/L XYLOSE AND XYLOSE WITH ADDED ALKALINE EXTRACT FROM 30 G/L CORN STOVER PRETREATMENT

	Control	+ Neutralized Alkaline Extract
μ_{max}	0.25 hr^{-1}	0.01 hr^{-1}
Ethanol	7.7 g/l	0.2 g/l
Acetate	1.4	0.4
Lactate	0.9	1.0

result would suggest that the effective utilization, for example, in a second stage following extraction, is not possible. These findings further suggest that other means than alkaline extraction alone must be found.

4.5.2. Selective Solvent Alkaline Extraction of Corn Stover

As reported in the previous section, alkaline delignification was shown to remove a major portion of hemicellulose in addition to lignin. This lack of selectivity represents a major drawback to this approach for increasing the susceptibility of cellulose and hemicellulose to enzymatic attack. In order to overcome this problem, a new method for selectively delignifying corn stover was examined as an alternate pretreatment method. The rationale of this approach is based on the observation that ethanol limits hemicellulose solubility in water.

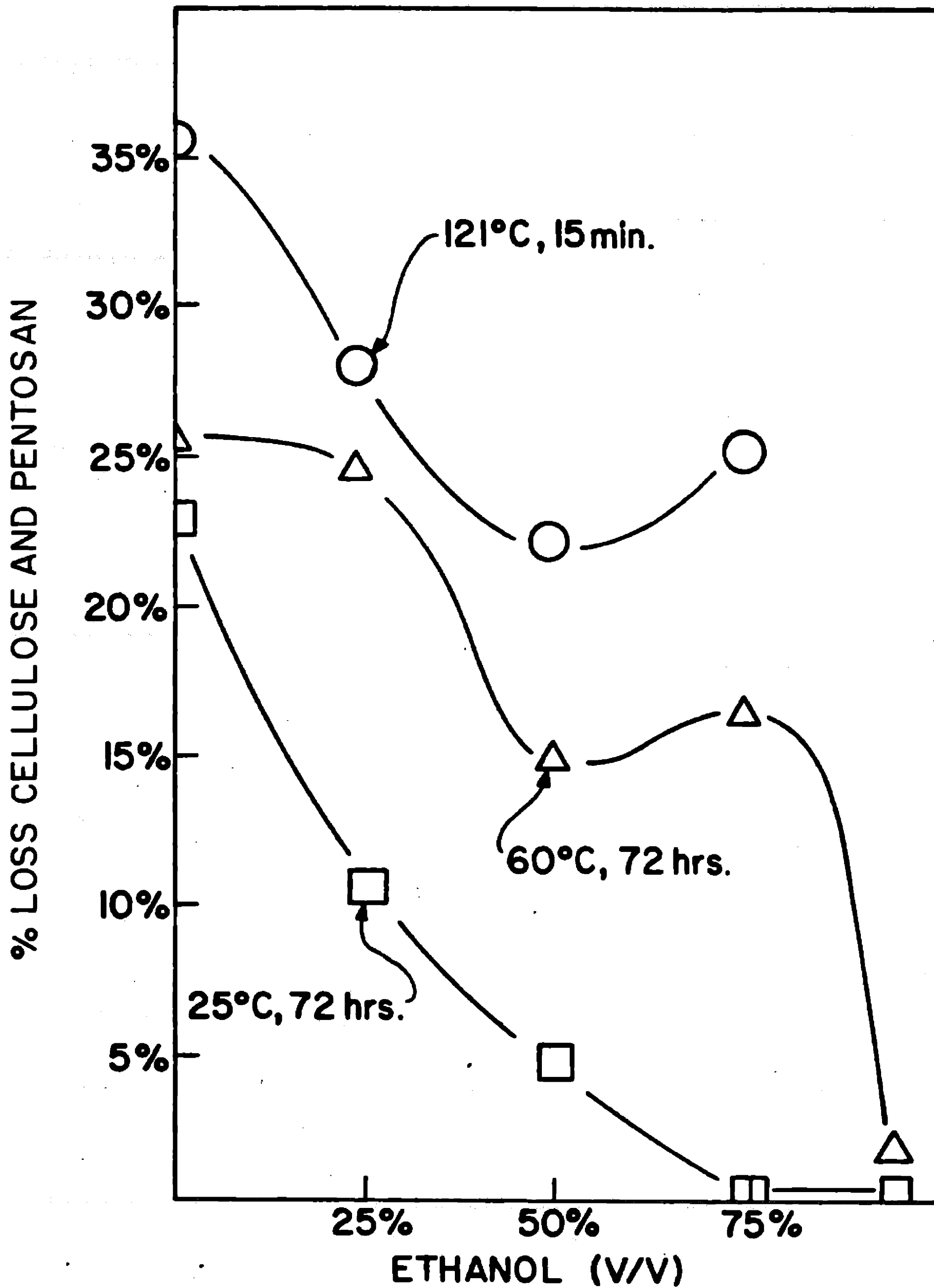
In order to optimize this selective delignification approach, a number of ethanol concentrations and temperatures, at equal alkali and corn stover concentration were experimentally examined. The ethanol concentration was varied from 0 to 95% (V/V) and temperature during delignification varied from 25°C to 121°C. All of the studies were performed using 8 g/l of NaOH and 40 g/l of corn stover solids which had been ground using a Wiley mill and screened through a 0.5 mm sieve.

In view of the constraints previously stated, there were a number of objectives to which this new solvent delignification process were directed. In order to assess the pretreatment effectiveness in these studies, the loss of cellulose and hemicellulose (as pentosan) as well as the degree of delignification during solvent treatment were measured. The effect of these pretreatment conditions studied on holocellulose are shown in Figure 31. At the highest delignification temperature (121°C) and at a treatment time of 15 minutes, it can be seen that there is a decrease in the loss of α-cellulose and pentosans as the concentration of ethanol is increased from 0 to 50% (V/V). For example, at 0% ethanol, there is a 35% loss of the carbohydrates from corn stover. However, at 121°C, as one increases the ethanol to 50% (V/V), the loss of these two carbohydrates is reduced to 22%. Further increase in the ethanol concentration to 75% (V/V) was accompanied by an increased loss of the α-cellulose and pentosan. In view of the minimal carbohydrate loss in excess of 20% at 50% (V/V) ethanol, it was felt this condition would still not fulfill the constraint of retaining maximal carbohydrates in the corn stover for subsequent microbiological utilization. For this reason, lower temperatures for delignification were examined.

Also shown in Figure 31 are the results of delignification at 60°C and 25°C for 72 hours. An increased contact time of 72 hours was used because it was believed at

Figure 31

EFFECT OF ETHANOL ON % LOSS OF α CELLULOSE AND HEMICELLULOSE CARBOHYDRATES AFTER EXTRACTION WITH 8 G/L NaOH AT 121, 60, 25°C



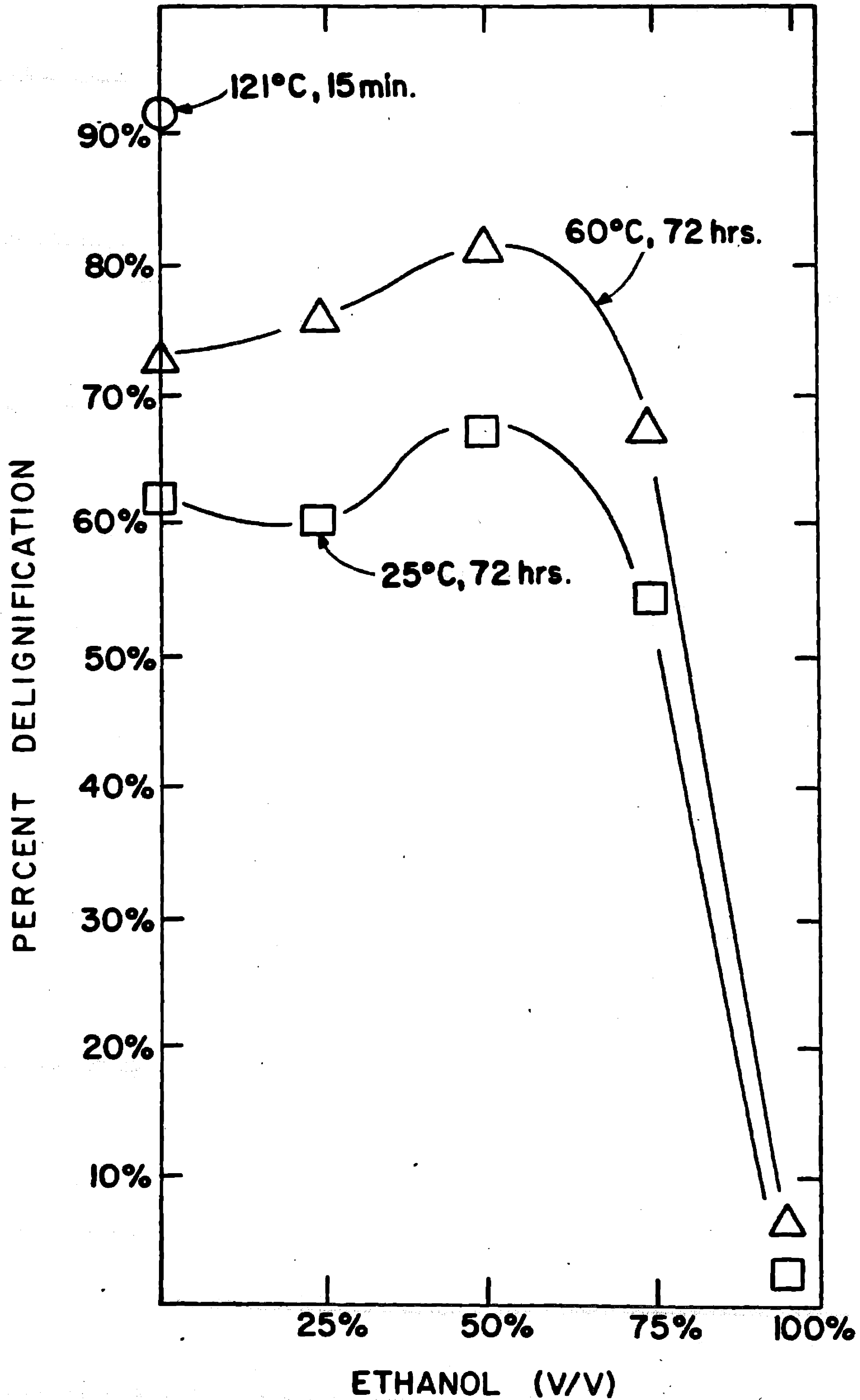
the lower temperatures longer times will be required to achieve equilibrium. (The kinetic properties of this extraction process will be presented later in this Section).

It can be seen from Figure 31 that at the lower temperatures there is a decreased loss of α-cellulose and pentosans as the ethanol concentration is increased. The most encouraging results were those performed at a delignification temperature of 25°C. At zero ethanol concentration, the combined loss of the α-cellulose and pentosans was 23%. However, as one increases the ethanol concentration there is a continual decrease in the loss of the α-cellulose and pentosans from the corn stover solids. For example, at 50% ethanol, the total loss of these carbohydrates is only 5%. At 75% and 100% ethanol there is virtually no loss of either α-cellulose or pentosans. These results are very encouraging since they are within the constraint of retaining to a maximal extent the potentially fermentable carbohydrates in the corn stover solids. However, the minimal loss of α-cellulose and pentosans during solvent treatment is only one part of the ultimate objective. The other important fact is the degree of delignification which can be achieved at the different solvent treatment conditions. The results on the degree of delignification are shown in Figure 32.

At the highest temperature tested (121°C), lignin loss from the corn stover solids was in excess of 90%.

Figure 32

EFFECT OF ETHANOL ON % LOSS OF LIGNIN AFTER EXTRACTION WITH 8 G/L NaOH AT 121, 60, 25°C



This was achieved in the absence of ethanol. However, it has been already stated that the loss of α-cellulose and pentosans at this temperature is prohibitively high and thus offsets the high degree of delignification that can be attained.

On the other hand, as one lowers the temperature to 60°C and 25°C, it can be seen that the removal of lignin remains essentially constant as the ethanol is increased from 0 to 50% (V/V). At both temperatures as the ethanol concentration is increased beyond 50% (V/V), there is actually a decrease in the degree of delignification. Combining the findings of α-cellulose and pentosan losses together with the degree of delignification, it was concluded that 50% (V/V) and 25°C using 8 g/l of NaOH in the presence of 40 g/l of corn stover are the optimal conditions that fall within the initial constraints set forth for solvent delignification of corn stover.

The detailed analysis of corn stover before and after extraction at 25°C using 50% (V/V) ethanol in water are shown in Table 15. Corn stover solids were extracted for 72 hours using 0.2 N NaOH (8 g/l) at a 40 g/l corn stover solids loading. After extraction, 76.8 grams of solids were recovered per 100 g treated. It can be seen from the analyses of the α-cellulose and pentosans that the recovery of these materials was in excess of 95%. Lignin after solvent extraction was reduced from 13.2 grams per 100 grams (13.2%) to 4.4

Table 15

CORN STOVER COMPOSITION BEFORE AND AFTER EXTRACTION WITH 50% ETOH/H_2O (V/V) AND 0.2 N NaOH AT 25°C FOR 72 HRS (BASIS: 100 G)

	Before Extraction	After Extraction
Dry Weight	100 g	76.8 g
α cellulose	32.0	31.6
Pentosan	29.4	26.9
Lignin	13.2	4.4
Ash	9.0	7.7
Crude Protein	4.2	-
Unaccounted	12.2	6.2

grams per 76.8 gram (5.7%). It should be noted that the material balances were not closed since not all components in the solids were assayed. The important facts to be noted are the reduction in the lignin content and the retainment of the α-cellulose and pentosans.

In view of these encouraging results using corn stover, it was decided that wheat straw also be tested using identical procedures. The results from the wheat straw delignification are shown in Table 16. It can be seen that the quantitative behavior of wheat straw delignification is quite similar to that found for corn stover. This is very rewarding since it demonstrates that this selective solvent pretreatment is probably applicable to a number of agricultural residues.

The use of cold alkaline ethanolic solutions for biomass treatment has been examined previously by a number of investigators. Buston (1934) employed such a solvent system for the isolation of polyuronides from cocksfoot grass [126]. Norman (1935) examined the use of alkaline ethanolic solutions for the preparation of pure hemicelluloses from straws [127]. Although similar data for the reduction of hemicellulose solubility in cold alkaline ethanol solutions were obtained, the extents of delignification observed in these early experiments were 2 to 3 times lower than those found here with corn stover.

One difference which may account for the discrepancy in extent of delignification observed may be the time

Table 16

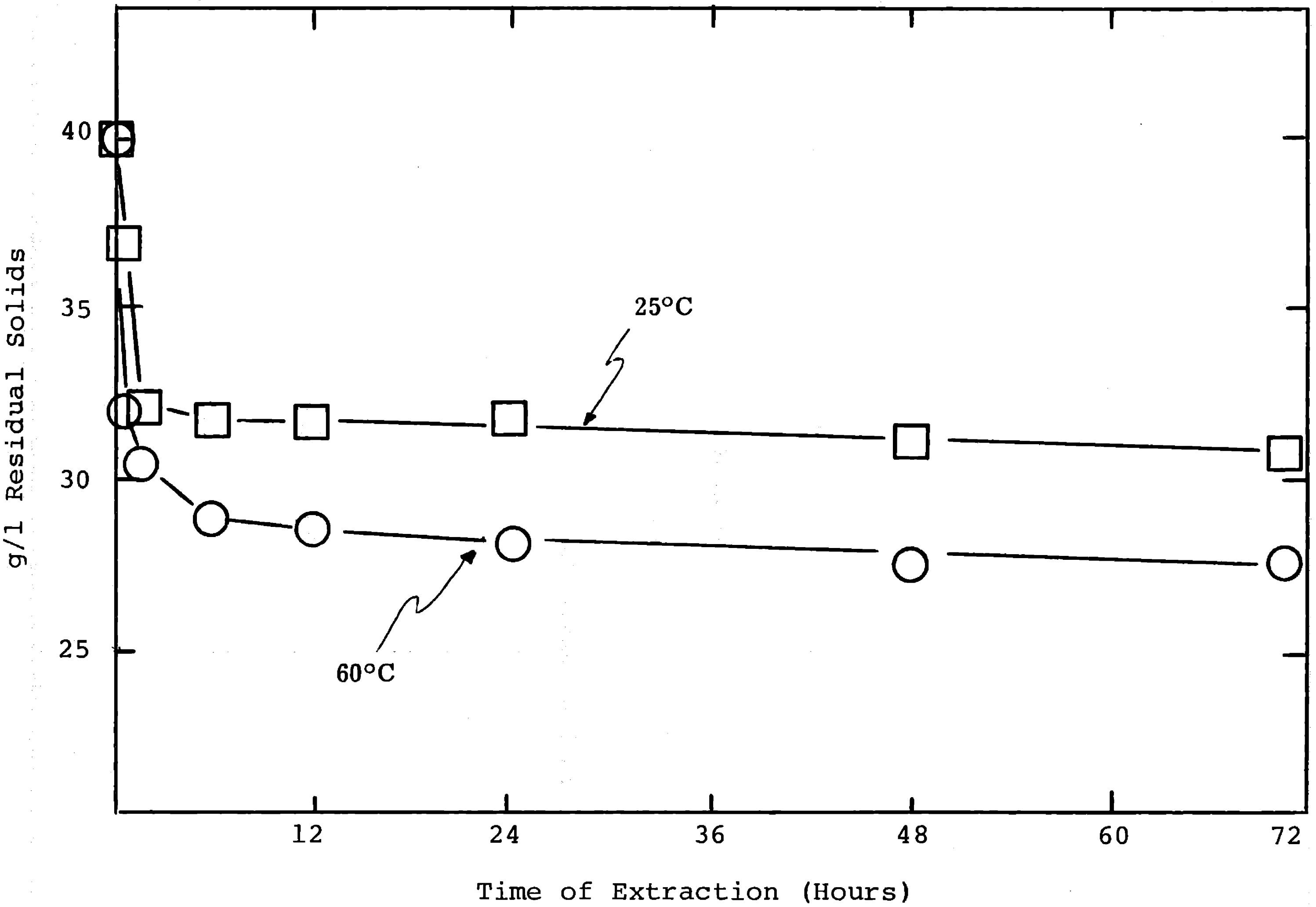
WHEAT STRAW COMPOSITION BEFORE AND AFTER EXTRACTION WITH 50% ETOH/H_2O (V/V) AND 0.2 N NaOH AT 25°C FOR 72 HRS

	Before Extraction	After Extraction
Dry Weight	100 g	83.5 g
α cellulose	33.4	33.0
Pentosan	28.9	27.1
Lignin	15.6	6.1
Ash	7.2	6.9
Unaccounted	14.9	10.4

of treatment. Norman employed 2 hour delignification times, with 50% ethanol and 1% alkali and observed an average of 23% delignification and 28% loss of solids for a number of cereals and grasses. This is in contrast to the 67% delignification of corn stover we have observed after 72 hours of treatment associated with a 23% loss of total solids under similar conditions. The kinetics of the delignification process were examined to see whether this factor can resolve these discrepancies. The first series of experiments were performed at 25°C and 60°C using a mixture of 50% (V/V) ethanol/water containing 8 g/l of NaOH and 40 g/l of corn stover solids. These extractions were performed under non-agitated, stagnant conditions similar to those employed by Norman (1935). The loss in the residual corn stover solids was measured as a function of extraction time. These results are shown in Figure 33. It can be seen that the loss of solid is quite rapid. For example, at 25°C the residual solid reached approximately 90% of the final value in approximately two hours. This represents a 20% loss of solid which is similar to that found by Norman of 28% solids loss. On the other hand, the question arises whether the extraction of lignin from corn stover solids is reflected totally in the solid loss data. To answer this question, the kinetics of lignin extraction were also examined.

Figure 33

KINETICS OF SOLID WEIGHT LOSS DURING EXTRACTION
WITH 50% (V/V) EtOH, 8 G/L NaOH



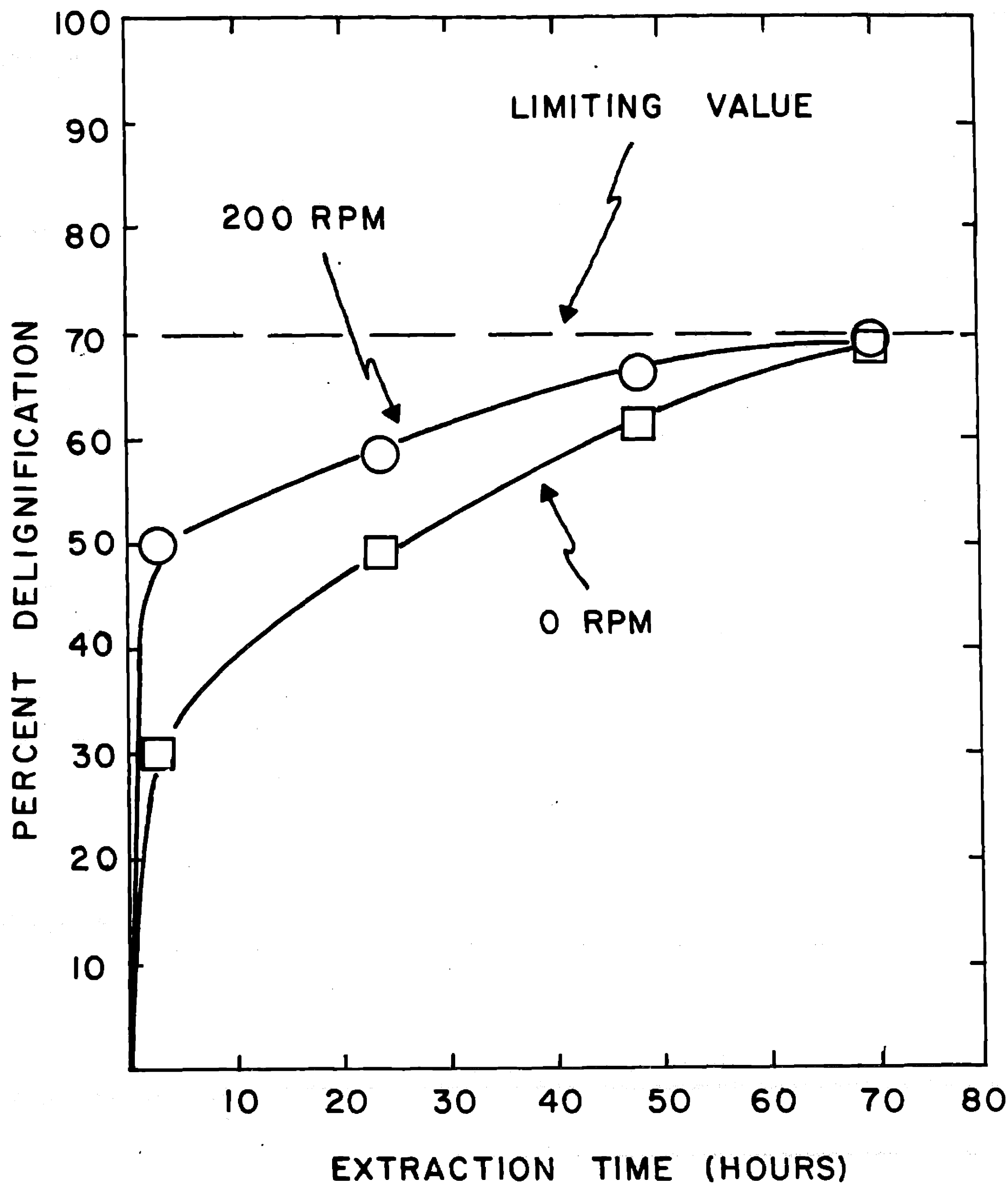
To obtain delignification kinetics, experiments were performed using 250 ml Erlenmeyer shake flasks. Stationary extraction along with extraction under agitated condition were performed. The latter experiment was conducted on a reciprocating shaker at 200 RPM with a 4 cm stroke. The results from these studies are shown in Figure 34.

It can be seen that the initial rate of delignification is extremely rapid. For example, 50% of the lignin of corn stover is removed within 2 hours during shaking at 200 RPM. On the other hand, only 30% delignification is observed in the stationary flask in 2 hours. This decreased extent of delignification under stationary conditions is consistent with the observation of Norman in the earlier work. In spite of the fact that little further loss of total solids are observed after approximately 2 hours, one sees that extraction of the corn residue for longer times under either agitated or stationary conditions results in a slower continued delignification. Eventually a maximum of 69% delignification is achieved after approximately 72 hours in either case. Therefore, the time of treatment especially under non-agitated conditions is a major factor for high delignification extent and hence high selectivity.

It is believed that the increased rate of delignification at 200 RPM is a result of increased mass transfer due to the agitated conditions employed. How-

Figure 34

KINETICS OF LIGNIN EXTRACTION BY ALKALINE/ETHANOL (50% V/V) SOLUTION FROM CORN STOVER UNDER STATIONARY AND MIXED CONDITIONS



ever, at 25 hrs of extraction, the rate of delignification of the non-agitated extraction is higher than that of the agitated condition. In order to interpret this apparently contradictory result we must recognize that lignin is a heterogeneous polymer. After the initial extraction period, the instantaneous rate of delignification should be compared at comparable extent of delignification. As can be seen in Table 17, the instantaneous rates of delignification at 50% lignin extraction are approximately the same under agitated and stationary conditions. From these results we conclude that after 50% extraction, the rate controlling mechanism is no longer due to external mass transfer. However, we cannot distinguish whether the rate controlling step is due to intrapellet diffusion or chemical reaction.

However, it is reasonable to conclude that further increasing external mass transfer will not increase the rate of delignification when the extent of delignification reaches a value of 50%. In order to increase the rate of delignification when external mass transfer is not controlling, smaller particles might be used as a solution.

4.5.3. Alkali Consumption and Alternate Alkalis for Extraction

The use of ethanol/water mixtures with NaOH has been shown to be an effective system for selective pretreatment. However, the quantity of alkali used and choice of

Table 17

COMPARISON OF DELIGNIFICATION RATES WITH AND WITHOUT AGITATION

INSTANTANEOUS RATE OF DELIGNIFICATION AFTER 50% LIGNIN EXTRACTION CALCULATED FROM FIGURE 34	
Stationary Flask	0.042%/Hour
Agitated Flask	0.040%/Hour

NaOH as a base represents only one method for achieving high pH. In order to examine the alkali requirements of this process, the consumption of NaOH was measured by titration of supernatants after pretreatment. In addition to the total quantity of alkali required, it was hypothesized that a number of alternate bases could be used to replace NaOH. Compared to NaOH, less expensive sources of alkali could be $Ca(OH)_2 < Na_2CO_3 < NH_3$. The results of these investigations to quantitate alkali consumption and the potential for base substitution are summarized in the following sections.

Total consumption of alkali was measured by HCl titration of a supernatant after 72 hours treatment of corn stover at 25°C. From the titration behavior of a number of extractions, a total of 100 mg of NaOH was typically shown to be consumed per gram of corn stover extracted to completion (Figure 35). From the inflection points and plateaus of the titration curve, a general idea of the soluble "acidic" groups consumed by the alkali can be determined. As shown in Table 18, 75 mg of the total alkali required is used in the titration of a number of groups with pKa's between values of approximately 6 and 10. Presumably many of these groups are phenolic constituents on the lignin. A smaller portion of NaOH was shown to be required for the titration of lower pKa groups at pH 4-5 and 2-3. These may represent acetic acid and glucuronic acid groups. Recent work by Higuchi _et al_. on the presence of uronic acid esters in straws supports this hypothesis [128].

Figure 35

TITRATION BEHAVIOR OF SUPERNATANTS FROM CORN STOVER PRETREATMENT

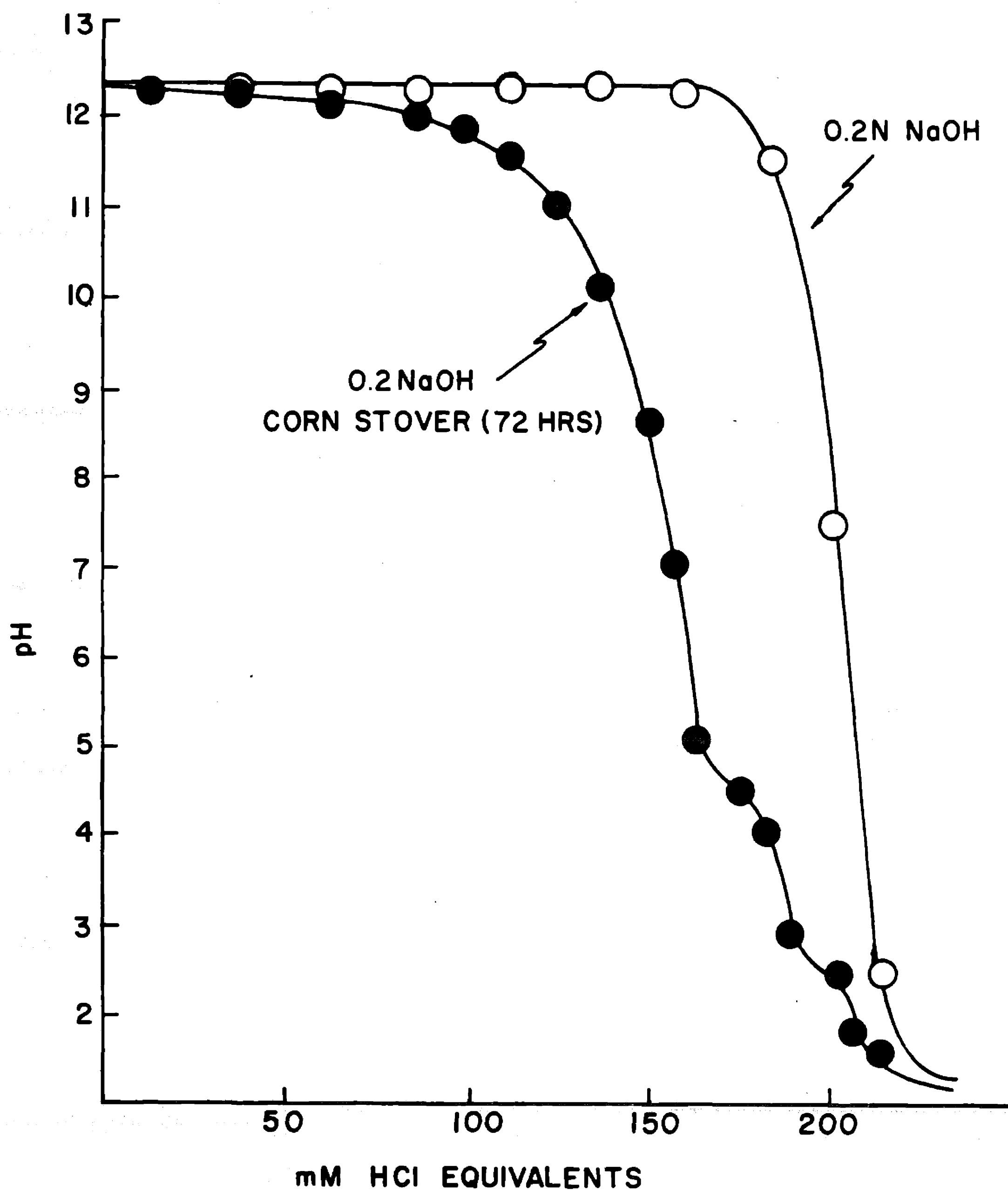


Table 18

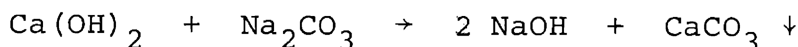
POTENTIAL ACIDIC GROUPS FOR NaOH CONSUMPTION DURING CORN STOVER PRETREATMENT

Titratable Groups	mg NaOH/gm Corn Stover
~6-10 (phenolic groups)	75
~4- 5 (acetic acid)	16
2- 3 (glucuronic acid)	9
Total NaOH Consumed	100

Due to the cost of NaOH, a number of alternate bases were examined for their ability to directly substitute for NaOH in the extraction process. Unfortunately, as shown in Table 19, the use of 0.2 M $Ca(OH)_2$, 0.2 M NH_4OH or even 1.75 M NH_4OH all failed to yield more than 24% delignification.

It is also interesting to note that the substitution of ammonium hydroxide also decreased the selectivity of the solvent system causing almost a 30% pentosan loss.

On the other hand, the substitution of 0.2 M NaOH by a mixture of 0.1 M Na_2CO_3 and 0.1 M $Ca(OH)_2$ was shown to be nearly as effective as 0.2 M NaOH in the extraction process catalyzing 57% delignification. Presumably, this is due to the reaction generating NaOH:



$$Ca(OH)_2 + Na_2CO_3 \rightarrow 2\ NaOH + CaCO_3 \downarrow$$

Unfortunately, a decreased selectivity of the solvent resulting in 17% pentosan loss was also noted.

Previously, it was shown that 0.1 M NaOH (4 g) is consumed per 40 gms corn stover extracted. In all studies conducted so far, a 0.1 M excess of NaOH has been maintained. In order to test whether or not this is actually required, 0.1 M NaOH and 0.1 M Na_2CO_3 were tested for the extraction of 40 g/l corn stover. In this case, only 44% delignification of the corn

Table 19

DELIGNIFICATION AND PENTOSAN LOSS OF CORN STOVER EXTRACTED AT 25°C WITH 50% (V/V) ETHANOL/WATER AND VARIOUS BASES*

	% Delignification	% Pentosan Loss	pH after Extraction
0.2 M NaOH	67	8.5	12.7
0.2 M $Ca(OH)_2$	8	0	-
0.2 M NH_4OH	18	29	-
1.75 M NH_4OH	24	27	11.0
0.1 M $Ca(OH)_2$ 0.1 M Na_2CO_3	57	17	-
0.1 M NaOH 0.1 M Na_2CO_3	44	8	12.0

* 48 Hr extraction at 25°C of 40 g/l corn stover shaken

stover was observed. Thus, it appears from this preliminary work that an excess of a strong base such as NaOH must be maintained during the extraction and that a weaker base such as sodium carbonate or ammonia cannot effectively be substituted.

4.5.4. Effect of Particle Size on Selective Solvent Delignification

In the course of the studies in biomass pretreatment previously described, 0.5 mM Wiley mill ground corn stover was generally used. However, an economic advantage can be gained by using larger particle size material. Data on power consumption for grinding of dried material supplied by A. E. Staley Co. (personal communication) indicates a 0.010 to 0.014 H.P./lb/hr requirement for milling corn stover with a Fitz mill with 1/2 to 1/4" screen size. On the other hand, a significantly higher power requirement of 0.10 H.P./lb/hr was required for grinding to an average size of approximately 0.5 mM (1/50") with an Alpine Mill.

In order to test the delignification efficiency of the selective solvent extractant as a function of particle size, these different samples were sieved yielding the size distribution shown in Figure 36. These samples were subsequently extracted and the resulting delignification performances shown in Table 20. It can be seen that the use of larger particle size material causes a slight reduction in

Figure 36

CORN STOVER PARTICLE SIZE DISTRIBUTION

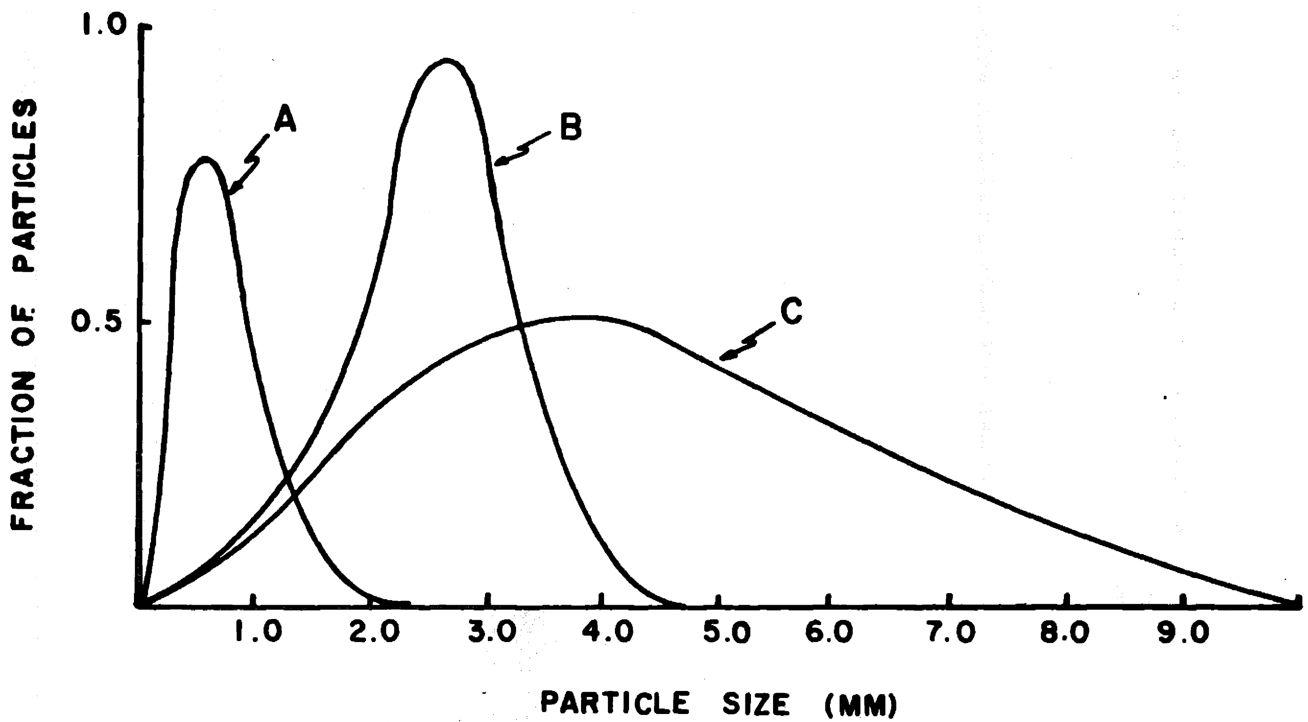


Table 20

EXTRACTION OF VARIOUS SIZE CORN STOVER PARTICLES (40 G/L) IN 50% ETOH/H_2O AND 8 G/L NaOH 50 HRS AT 25°C

	Corn Stover	Predominant Particle Sizes	% Lignin Extracted
A.	0.5 mM Wiley Milled Brand "C"	0.25 - 0.75 mM	69
B.	Brand "C" Corn Stover	2.0 - 3.5 mM	63
C.	Coarse Ground Staley Corn Stover	2.0 - 8.0 mM	62

delignification extent from 69% to 63 and 62% with larger size materials after 50 hrs of agitated extraction. These results show a surprisingly small dependence of delignification on particle size. The subsequent fermentation performance of these treated materials will be addressed in Section 4.7.2.

4.5.5. Effect of Lignin Solubility on Extraction Efficiency

In studies to characterize lignin extraction of corn stover with alkaline ethanol water solutions we routinely employed a solids content of 40 g corn stover per liter of extracting solution. However, more economical attractive operation for such an extraction scheme would result if a higher solid to liquid rate can be used. Although a maximum of approximately 140 g/l of corn stover (depending on particle size) can be completely contacted in any single stage, this limitation can be overcome through operation in a multistage countercurrent fashion. In this manner, as long as sufficient alkali is added the only limitation to extraction will be lignin solubility. In order to quantitatively assess this limitation, the influence of solubilized lignin on extraction efficiency has been examined and is discussed in the following section.

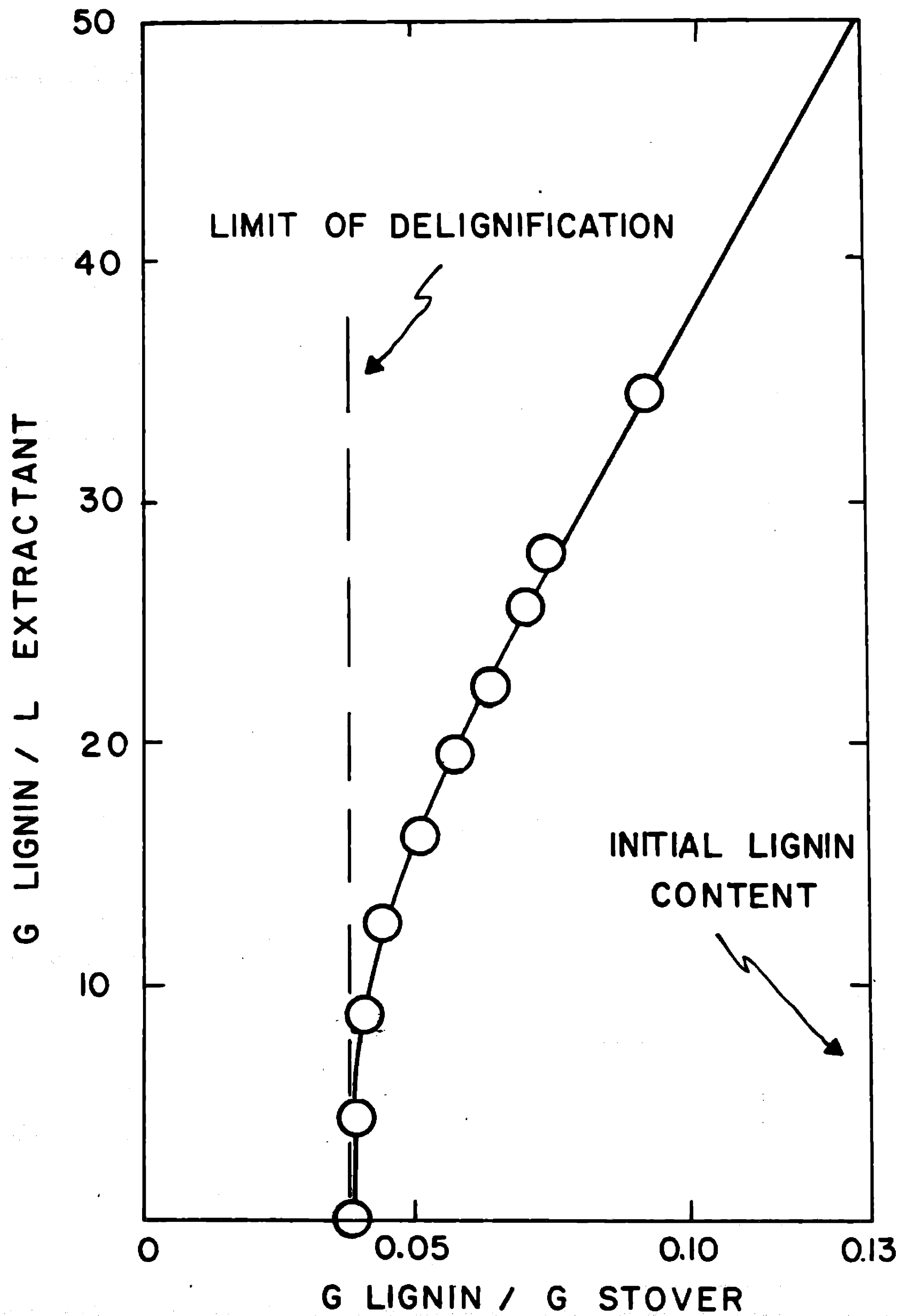
In order to assess the influence of dissolved lignin concentration on extraction efficiency the supernatant from a corn stover extraction was titrated, readjusted to 0.2

M NaOH and re-used for the extraction of fresh corn stover in a subsequent 48 hr extraction step (see Materials and Methods). In this manner, the equilibrium diagram depicted in Figure 37 was constructed. In this diagram one observes the final limit of delignification is 70% or approximately 0.04 g lignin/g corn stover as reported previously. However, it should be noted that the presence of up to 10 g/l lignin in solution has little effect upon reaching this maximum extent of delignification. By the same token, the extraction of 69% delignified material with fresh extraction solvent has little effect on further delignification. Increasing concentrations of lignin greater than 10 g/l in the extracting solvent have an increasing effect on limiting the extent of delignification observed after 48 hr treatment. By suitably extrapolating the equilibrium line in Figure 37, we can calculate the maximum quantity of corn stover that may be contacted per liter of extracting solvent in an optimum countercurrent fashion.

From the slope of this equilibrium line we calculate that a maximum of 430 g of corn stover may be delignified per liter of extracting solution used. This can be accomplished in three 48 hr "equilibrium" contacting stages operated at 143 g/l corn stover each. Fresh extraction solvent containing less than 10 g/l lignin is required and filtrate solvent containing 50 g/l lignin is removed for regeneration and recycle.

Figure 37

EFFECT OF SOLUBLE LIGNIN ON DELIGNIFICATION OF CORN STOVER AFTER 48 HRS MIXING WITH 50% (V/V) ALKALINE/ ETHANOL EXTRACTANT



4.5.6. Solvent Recovery After Extraction

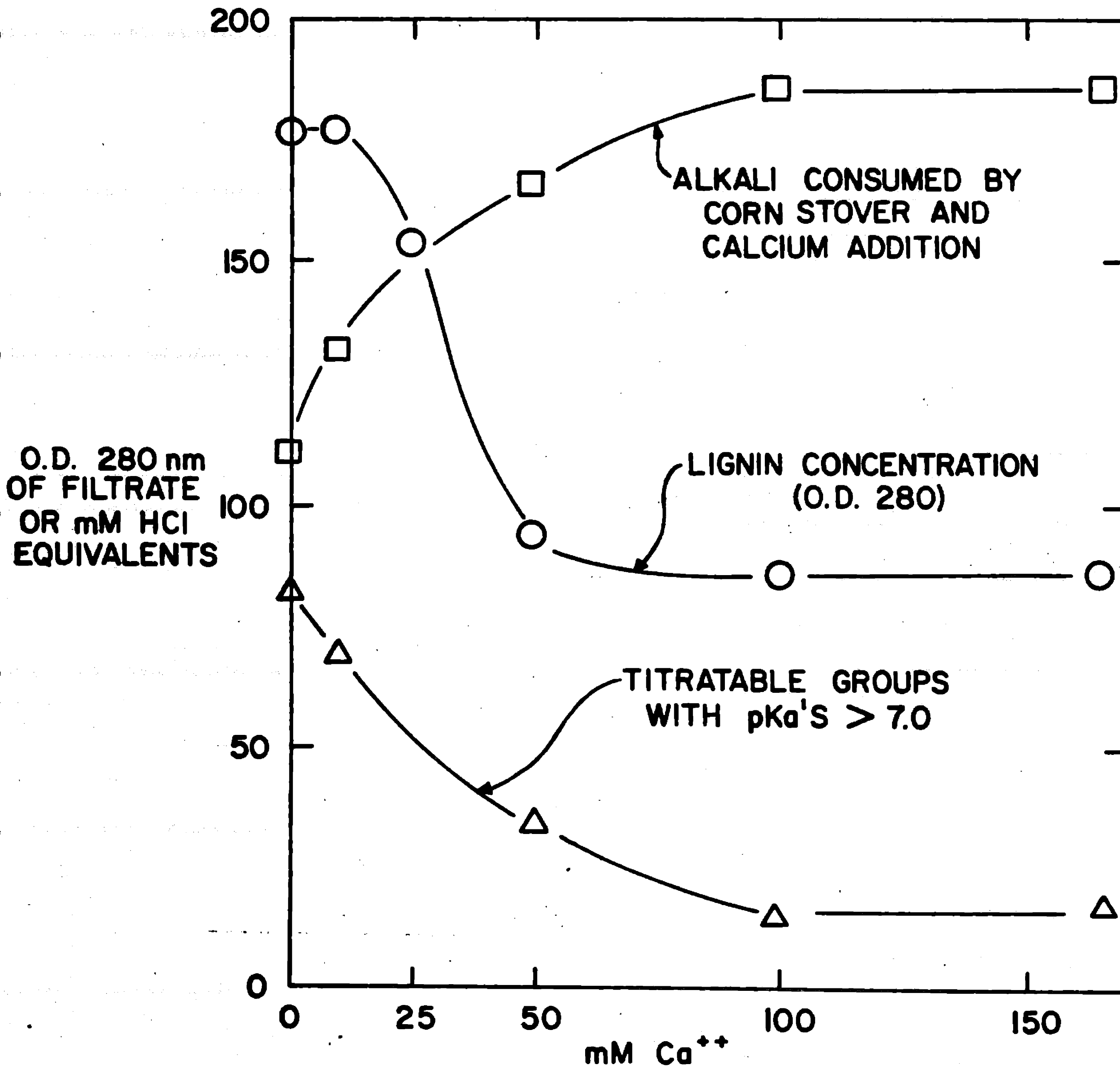
In addition to use of multistage contacting and the choice of an inexpensive alkali, an economical extraction pretreatment for biomass will require an appropriate method for solvent recovery for recycle operations. Although lignin in the solvent could be removed by distillation, a number of alternate methods could conceivably be employed. These include precipitation of lignin at low pH or with calcium. Alternatively, the adsorption of lignin onto activated carbon is also possible. The results of preliminary investigations into these methods are presented below.

The effect of calcium ion addition to alkaline ethanol extraction solvent containing 3.7 g/l of lignin is shown in Figure 38. The addition of 50 mM Ca^{++} results in the precipitation of approximately 50% of the lignin as measured by decrease in optical density (O.D.) at 280 nm. However, in addition to lignin precipitation, colloidal $Ca(OH)_2$ precipitate was also noted due to the presence of NaOH. The addition of excess Ca^{++} ions (beyond 50 mM) does not appear to be able to increase the extent of lignin precipitated above 50% as measured by O.D. 280 nm. The dry weight or characteristics of the lignin species which are not calcium precipitable, however, have not been determined.

An alternate method for lignin removal from the extraction solvent was examined by adsorption with activated

Figure 38

CALCIUM PRECIPITATION OF CORN STOVER EXTRACT
(24 HR EXTRACTION OF 40 G/L CORN STOVER W/
50% V/V EtOH AND 8 G/L NaOH AT 25°C)



carbon. An alkaline/ethanol solution obtained after sequential contacting of 550 g/l corn stover was mixed with activated carbon. After 24 hrs, the lignin content had decreased from 36 g/l to 3 g/l representing an adsorption of 62 mg of lignin per gram activated carbon. Using this procedure, no adsorption of ethanol or free alkali could be detected.

The results of these investigations demonstrate that a number of alternate means are available for removal of lignin from the pretreatment solution. However, in order to achieve a realistic overall process it is felt that further work may be required in this area. Some suggestions of the potential avenues are described in Section 6, Suggestions for Future Research.

4.6 Strain Improvement

During the course of this research, a number of strain improvement programs on *C. thermocellum* and *C. thermosaccharolyticum* for increased ethanol tolerance and yield were conducted by different workers. The new strains isolated provided the "baseline" performances for mixed culture fermentation using Solka floc. However, translation studies to realistic cellulosic biomass such as corn stover demonstrated a number of problems such as "extract toxicity" of corn stover on *C. thermosaccharolyticum* HG-4 (Section 4.3.2). Further improvement of *C. thermocellum* (S-7) was also required in order to attain

higher ethanol yields on corn stover. In addition, as will be shown later, a need was demonstrated for a hexose non-fermenting strain of *C. thermosaccharolyticum*. Therefore, a number of more targeted strain improvement programs were conducted and the results of these investigations are described in this Section.

4.6.1. Selection and Adaptation of *C. thermosaccharolyticum* HG-4 to Overcome "Extract" Toxicity

Selection and adaptation of *C. thermosaccharolyticum* HG-4 to overcome extract toxicity was accomplished by sequential transfer of the culture in xylose tubes containing corn stover aqueous extract and ethanol. The hypothesis for this selection was that during sequential transfer in an inhibitory medium, spontaneous mutations leading to a more resistant strain might arise. By growing at a slightly faster rate, these strains would eventually be selected to dominate the population. In order to accomplish this selection, aqueous extracts from 30 g/l corn stover were prepared as previously described. Xylose (15 g/l), CM-4 nutrients and sodium bicarbonate were filter sterilized and added to extracts in 10 ml Hungate tubes. The growth of *C. thermosaccharolyticum* (HG-4) was monitored by optical density at 660 nm in these tubes and subsequently transferred during the logarithmic growth phase. After two weeks of serial transfers (approximately 20 times),

ethanol at 5 g/l was added to the xylose/corn stover extract containing medium and the transfers were continued. The level of ethanol in the medium was increased an additional 5 g/l when the exponential growth rate of the adapted culture was similar to that without ethanol addition. The program was continued up to an ethanol concentration of 25 g/l. This procedure was achieved in approximately 5 weeks and 30 transfers.

The adapted strain obtained was given the designation as _C. thermosaccharolyticum_ strain HG-4A. The growth of HG-4 and HG-4A were compared during growth on xylose with and without addition of aqueous extract. As can be seen in Table 21, the growth rate of the adapted strain is no longer inhibited by the addition of the extract as seen with the parent. However, when grown on either xylose or xylose plus extract, strain HG-4A produces ethanol and acetate at a lower ratio of approximately 2:1 as compared to the parent having a ratio of approximately 5:1. Nevertheless, this method demonstrated that aqueous extract inhibition can be readily overcome through strain development and encouraged us to believe that further adaption to increased extract resistance could be subsequently accomplished if necessary.

However, efforts in continual strain development with _C. thermosaccharolyticum_ HG-4A were not carried forth for the following reason. A simultaneous mutation and selection program, quite different in principle from those previously

Table 21

GROWTH OF HG-4 AND HG-4A ON XYLOSE WITH AND WITHOUT THE ADDITION OF AQUEOUS EXTRACT OF CORN STOVER*

	HG-4		HG-4A	
	Xylose	Xylose + Aqueous Extract	Xylose	Xylose + Aqueous Extract
μ_{max}	0.25 hr^{-1}	0.05 hr^{-1}	0.25 hr^{-1}	0.31 hr^{-1}
Cells	1.7 g/l	0.9 g/l	1.2 g/l	1.9 g/l
Ethanol	7.7	4.5	7.0	8.9
Acetate	1.4	2.3	3.5	4.5
Lactate	0.9	1.4	1.8	1.8
$\frac{\text{Ethanol}}{\text{Acetate}}$	5.5	2.0	2.0	2.1

* Growth was conducted in anaerobic flasks under pH control with the periodic addition of 10% NaOH.

presented, was able to isolate a high ethanol yielding strain. This new strain appeared to be resistant to corn stover extract as well. The characterization on the properties of this strain will be presented in the following section.

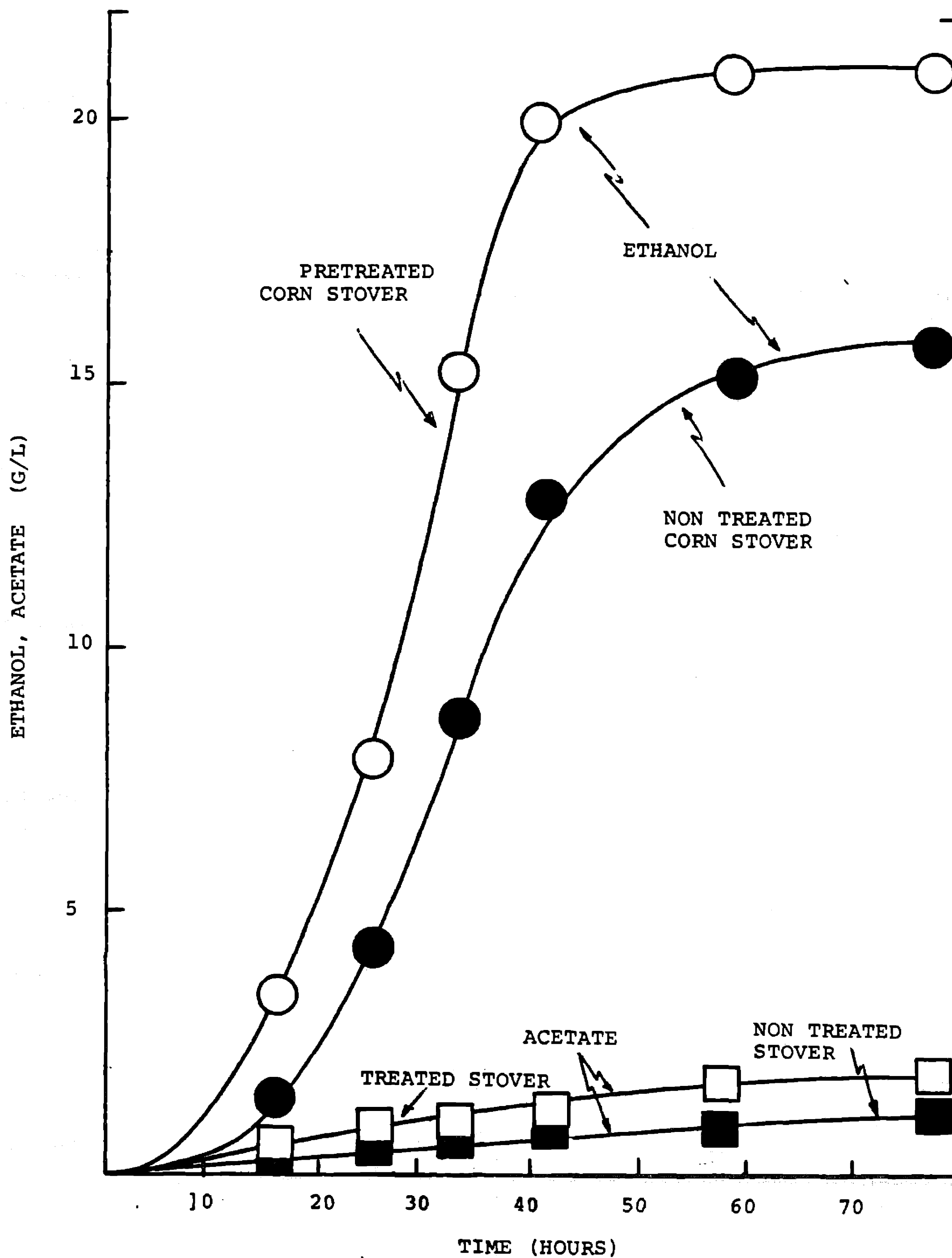
4.6.2. Effect of Residual Lignin on Ethanol Yield by New Strains of *Clostridium thermosaccharolyticum*

In previous sections, it was demonstrated that significant inhibition of *Clostridium thermosaccharolyticum* HG-4 occurred during growth on xylose in the presence of added "lignin" from corn stover. In addition, a significant decrease in the ethanol yield was also observed under these conditions. However, during the course of these studies, new techniques were developed for isolation of acetate negative organisms by counterselection for the inability to grow on pyruvate. This has resulted in the isolation of a new generation of very high ethanol yielding strains of *Clostridium thermosaccharolyticum* [129]. With the development of these new strains and the selective pretreatment of corn stover the effect of residual "lignin" on growth and ethanol yield was retested.

A high ethanol yielding strain of *Clostridium thermosaccharolyticum*, HG-6-62, was tested by fermentation on xylose medium with the addition of 40 g/l pretreated corn stover. As shown in Figure 39, HG-6-62 produced 21 g/l ethanol and less than 2 g/l acetate and lactate in the presence of the

Figure 39

GROWTH OF CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-6-62 ON XYLOSE WITH TREATED AND NON-TREATED CORN STOVER (40 G/L)



pretreated corn stover, while consuming 52 g/l of xylose in 48 hrs. The xylose consumption rate in this fermentation of 1.1 g/l hr shows virtually no inhibitory effect of pretreated corn stover. In addition, the ethanol yield of 80% of the theoretical maximum is similar to that observed with xylose alone. Thus, no significant toxicity of residual "lignin" is observed with this new strain on pretreated corn stover. The growth of HG-6-62 on xylose in the presence of non-treated stover is also shown in Figure 39. In this case, a slight inhibition of growth and decrease in both the rate and extent of ethanol accumulation are observed. However, the performance associated with this strain in the presence of corn stover, is markedly improved over that previously observed with the parent HG-4 in the presence of corn stover extracts or the performance seen by mixed culture on corn stover (Section 4.7). In view of these results, further strain development effort was better spent trying to overcome other problems described as follows.

4.6.3. Isolation of Hexose Negative Strains of *C. thermosaccharolyticum*

From the mixed culture modeling studies which will be described in detail later in Section 4.8, it will be shown that a strain of *C. thermosaccharolyticum* unable to metabolize either glucose or cellobiose would be advantageous when cultivated with *C. thermocellum* on corn stover. In order to isolate this strain, a protocol following γ-mutagenesis and

counterselection with ampicillin and penicillin for enrichment on glucose/cellobiose media was used. Selection of the population enriched with glucose/cellobiose negative strains was accomplished on plates containing high concentrations of cellobiose and glucose and limiting concentrations of xylose (see Section 3.1.6).

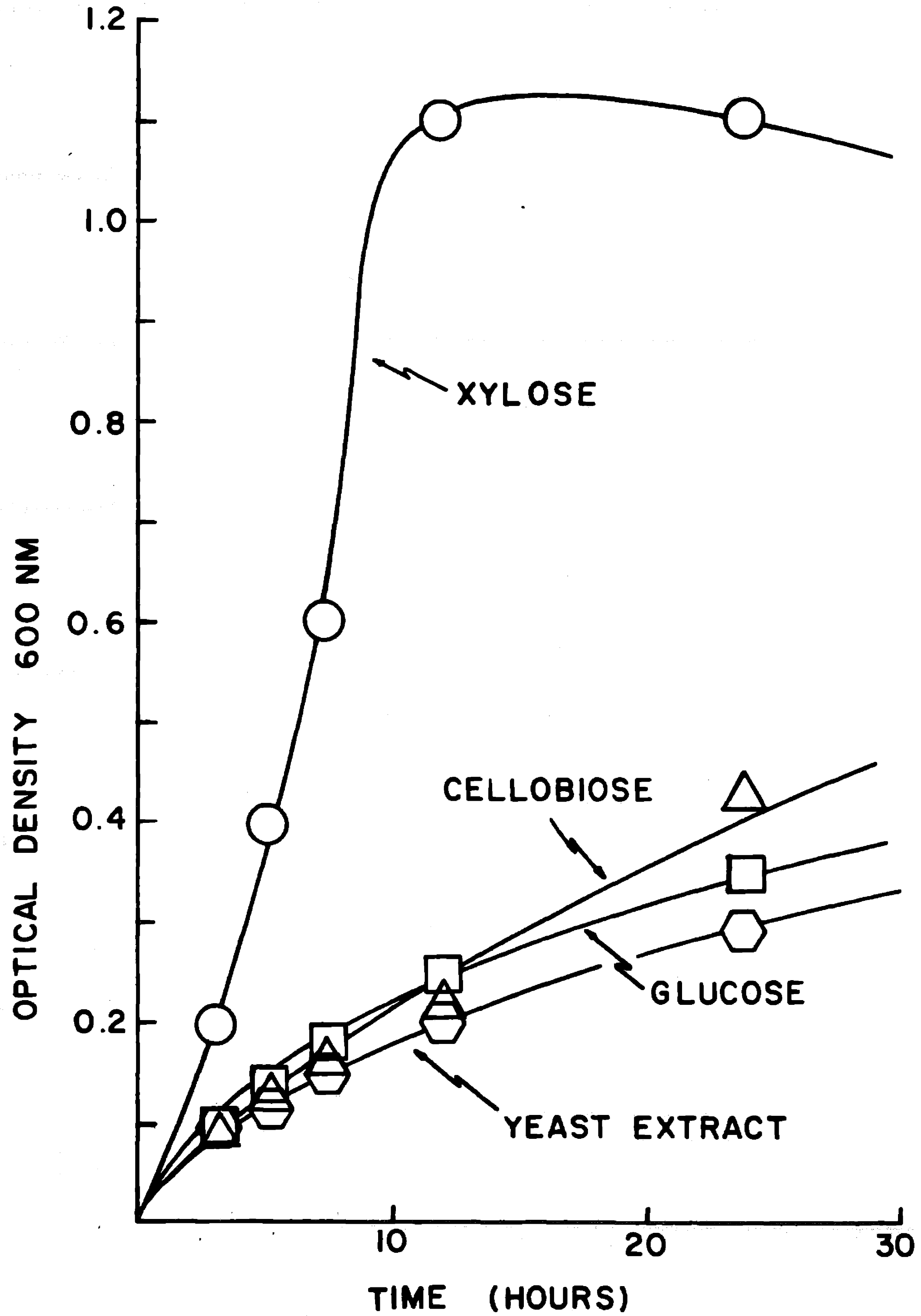
On this medium, hexose negative strains appear as small (< 2 mm) after 2 days incubation. After enrichment, the plated colonies were roughly equally divided into small and large clones. Three large clones and 33 small clones were picked and transferred to plates containing xylose and cellobiose. The three large isolates grew on both xylose and cellobiose. However, only 4 of the 33 small colony isolates grew on cellobiose and all 33 grew on xylose. These cellobiose negative clones were transferred to xylose, glucose, and cellobiose liquid media and tested for growth and production of ethanol. The best isolate, C. thermosaccharolyticum HG-8-19, had as good an ethanol to acetate ratios as its parent (≈ 15:1). Some backround growth on glucose and cellobiose was observed, however, and was primarily due to backround growth on yeast extract (see Figure 40).

4.6.4. Strain Development of C. thermocellum (S-7) for Increased Ethanol Yield

Pretreatment of corn stover to remove preferentially the lignin component results in significant increases

Figure 40

GROWTH OF CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8-19 ON XYLOSE, CELLOBIOSE, GLUCOSE, AND YEAST EXTRACT



in both the rate and extent of substrate utilization. Although Clostridium thermosaccharolyticum isolates such as HG-6-62 can achieve high ethanol yields in the presence of pretreated corn stover, ethanol yields by monocultures of C. thermocellum S-7 on corn stover did not improve. In fact, a degeneration of S-7 performance with regard to ethanol yield even during growth on Solka floc and cellobiose was observed. This was shown to be due to strain instability under storage conditions in liquid culture at 4°C. In order to re-establish good baseline performance it was necessary to conduct a new selection program with C. thermocellum S-7 with the aid of pH sensitive indicator medium for the isolation of new superior ethanol yielding strains.

4.6.4.1. Selection of pH Indicator Dyes

A number of pH sensitive indicator dyes were examined in cellobiose plate media for detection of acid production by C. thermocellum S-7. As shown in Table 22, of the dyes examined growth occurred only with Bromcresol Purple (BCP), Bromothymol Blue and Litmus. Of these dyes, only Bromcresol purple was found to be useful as a pH change indicator. Bromothymol blue and Litmus were shown to be reduced to colorless compounds during growth of C. thermocellum on plates and became unresponsive to changes in the pH of the medium.

Table 22

GROWTH OF *CLOSTRIDIUM THERMOCELLUM* AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM* ON CM-4 pH INDICATOR DYE MEDIUM

Dye Tested	Concentration	Growth	
		Clostridium thermocellum	*Clostridium thermosaccharolyticum*
Bromcresol Purple (purple pH 6.8 - yellow pH 5.)	20 mg/l	+	-
Bromthymol Blue (blue pH 7.6 - yellow pH 6.0)	25 mg/l	+	+
Chlorophenol Red (red pH 6.8 - yellow pH 5.2)	15 mg/l	-	+
Litmus (blue pH 8.3 - red pH 4.5)	100 mg/l	+	+
Methyl Red (yellow pH 6.2 - red pH 4.2)	25 mg/l	-	+
Neutrol Red (yellow pH 8.0 - red pH 6.8)	15 mg/l	-	+
Phenol Red (red pH 8.4 - yellow pH 6.8)	20 mg/l	-	+

4.6.4.2. Isolation of *Clostridium thermocellum* S7-19

A log phase culture of *C. thermocellum* S-7 was mutagenized by exposure to γ-irradiation (50 K Rads), allowed to incubate an additional hour, serially diluted, and plated on BCP-cellobiose CM4 medium. After 40 hours of growth, light halos due to acid production were observed on most of the colonies. Approximately 1,000 colonies were examined and 10 large colonies with small or no halos were picked for further evaluation. Purple colonies without yellow halos yielded average ethanol to acetate ratios of 3.1 ± 0.7 to 1 when grown in cellobiose tubes, while a control group of yellow colonies yielded ethanol to acetate ratio of 1.5 ± 0.4 to 1 (Table 23). Two of the best clones, S7-19 and S7-21, were recovered and examined during fed batch fermentations on cellobiose (Figures 41 and 42). These isolates both produced approximately 8 g/l of ethanol and 1 g/l of acetate and negligible amount of lactate production. When tested on Solka floc, however, S7-21 showed very slow growth characteristics and therefore was not further examined. *C. thermocellum* S7-19, however, proved to be a vigorous grower on Solka floc, as well as corn stover. The results of batch fermentations of pretreated corn stover with this strain are shown in Figure 43. During the course of the fermentation the corn stover was degraded at a maximum rate of 0.6 g/l hr while 8.4 g/l of DNS

Table 23

BEHAVIOR OF MUTAGENIZED *CLOSTRIDIUM THERMOCELLUM* S-7 CLONES ON BROMCRESOL PURPLE-CM4 CELLOBIOSE PLATES*

S-7 Clone	Colony Color	Halo	Ethanol/Acetate Ratio
13	Purple	-	2.9
14	Purple	-	3.5
15	Purple	-	2.5
16	Purple	-	-
17	Purple	-	3.1
19	Purple	-	4.5
21	Purple	-	4.4
22	Purple	-	3.5
23	Purple	Yellow	3.3
25	Purple	-	2.1
		Average	3.3 ± 0.7
18	Yellow	Yellow	2.0
20	Yellow	-	1.3
24	Yellow	Yellow	1.2
		Average	1.5 ± 0.4
S-7	-	-	1.9

*Bromcresol Purple
Purple - pH 6.8
Yellow - pH 5.2

Figure 41

GROWTH OF CLOSTRIDIUM THERMOCELLUM S7-19 ON CELLOBIOSE

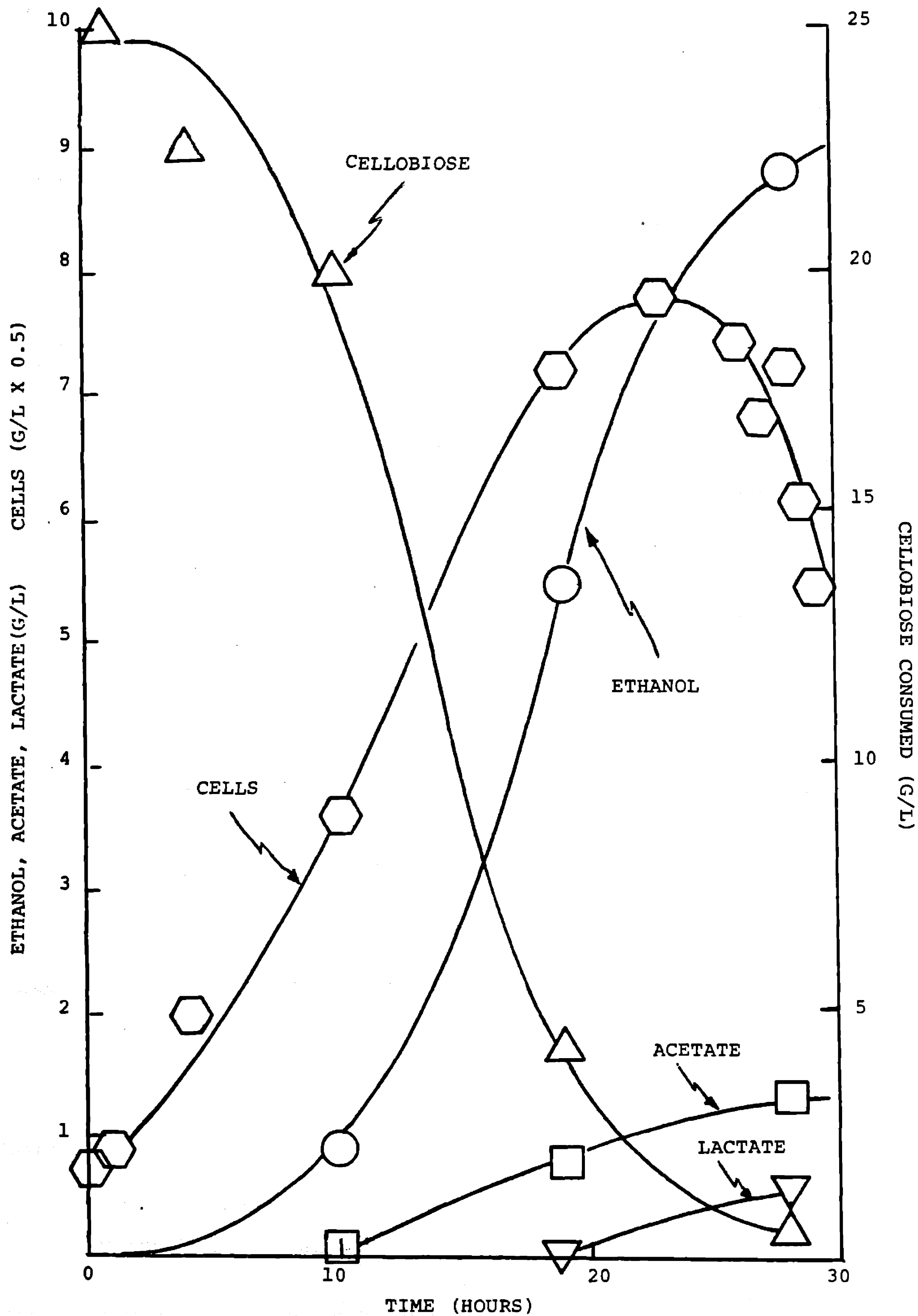


Figure 42

GROWTH OF CLOSTRIDIUM THERMOCELLUM ON S7-21 ON CELLOBIOSE

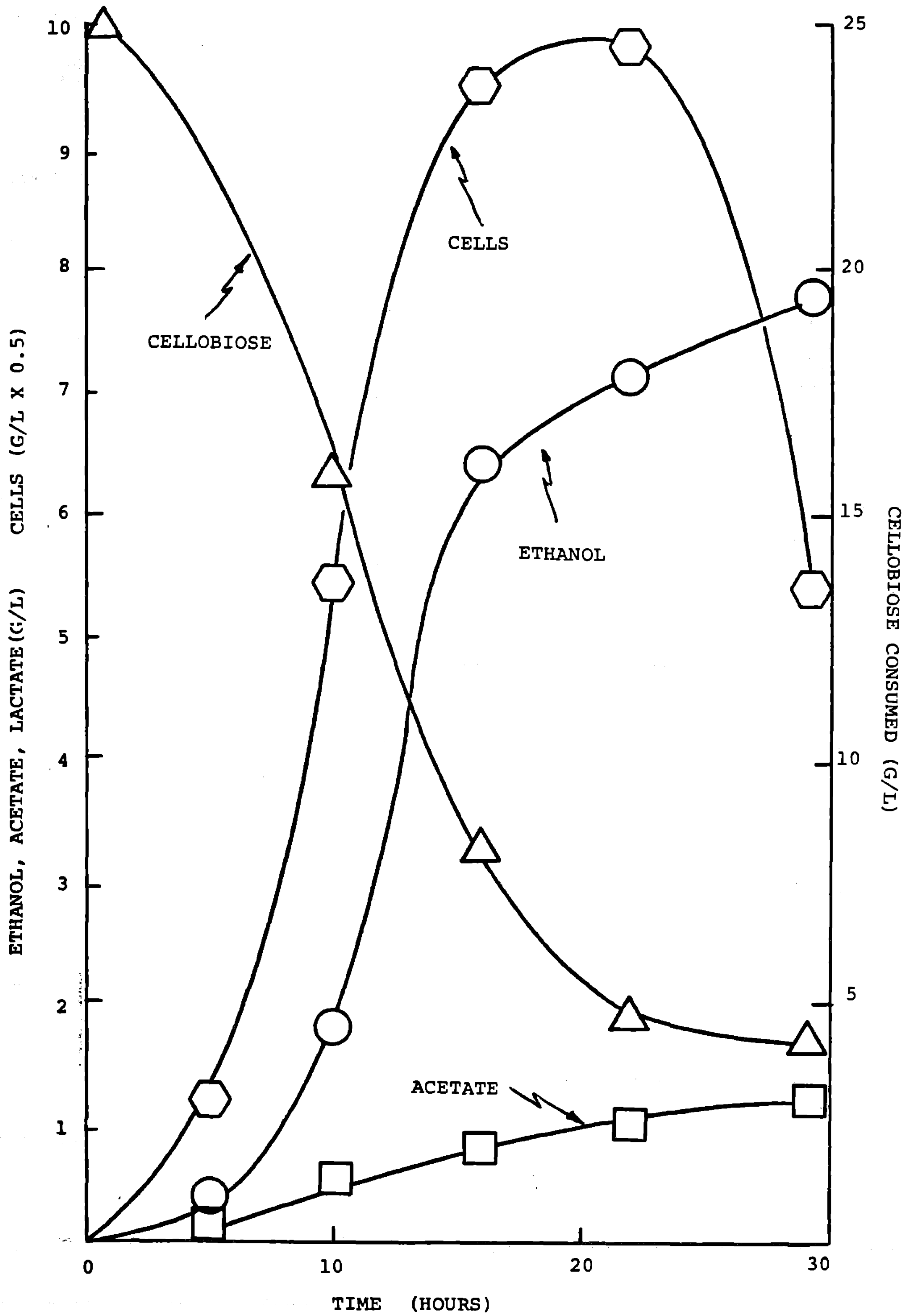
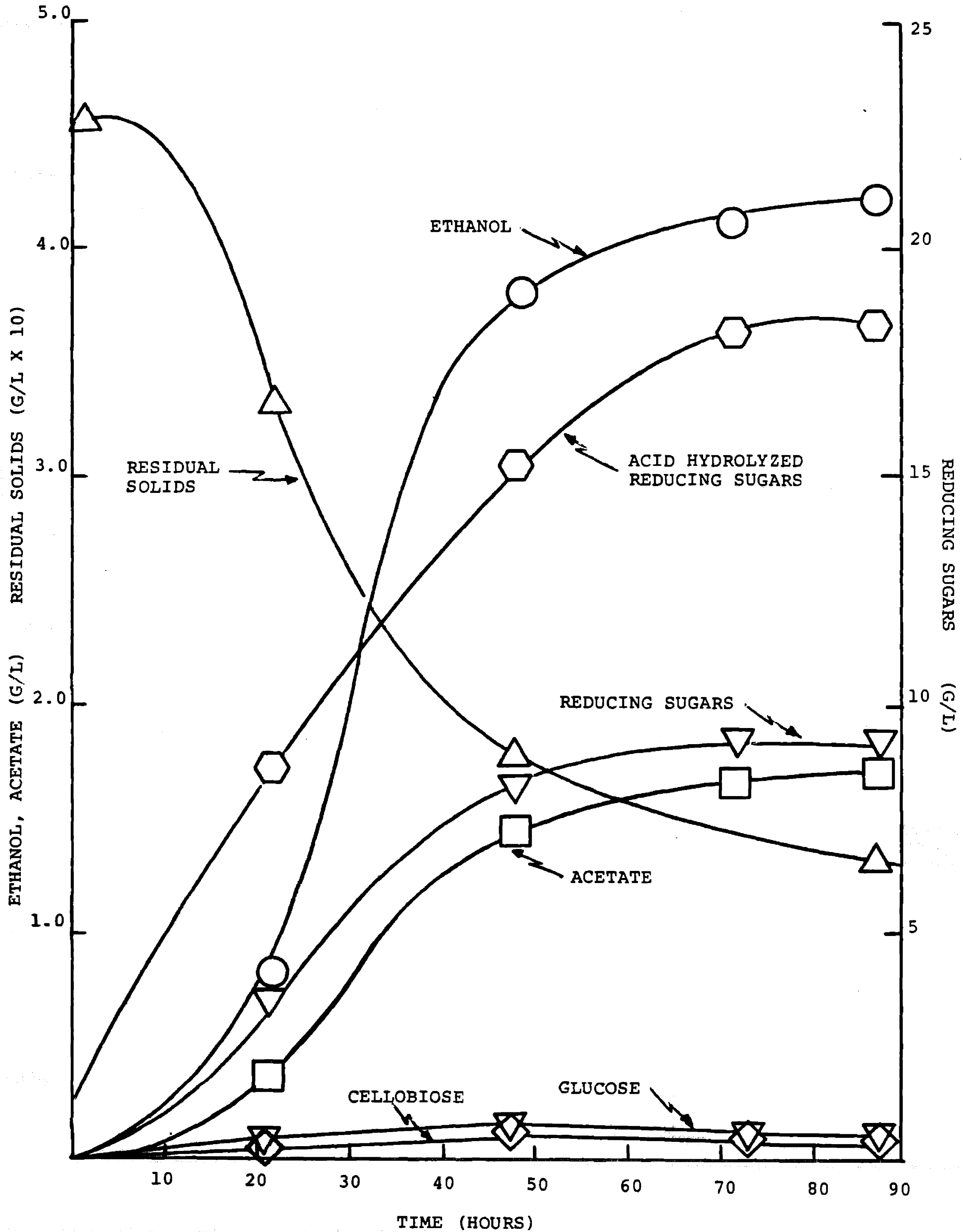


Figure 43

GROWTH OF CLOSTRIDIUM THERMOCELLUM S7-19 ON PRETREATED CORN STOVER (45 G/L)



reducing sugars accumulated (18.2 g/l after acid hydrolysis). Examination of these sugars by glucose specific enzymatic assay before and after β-glucosidase treatment showed virtually no glucose or cellobiose accumulation during monoculture growth on pretreated corn stover. This is in contrast to the performance of this strain's parent on Solka floc where substantial quantities of cellobiose and to a lesser extent glucose are known to accumulate. The growth of this strain on pretreated corn stover is therefore limited by the rate of cellobiose and glucose cleavage from α-cellulose, while pentosans from hemicellulose continue to accumulate. Under these conditions the best ratio of ethanol to acetic acid achieved by this strain on pretreated corn stover is only about 2.3 to 1.

4.6.4.3. Hydrolysate Plate Selection Method

In view of the continued difficulty in observing a high ethanol yield from C. thermocellum when grown on corn stover, a more direct approach to overcome this problem was initiated. One easily testable hypothesis to explain this low E/A ratios observed for S7-19 on corn stover may be the influence of the hydrolyzed pentosan carbohydrates or some other soluble factor of the hydrolyzed corn stover. In order to examine this possibility, synthetic hydrolysates of corn stover were prepared by hydrolyzing 50 g/l of pretreated and non-treated corn stover with cellulose from T. viride.

The hydrolyzed mixtures were shown to contain a total of 21 and 9.5 g/l reducing sugars by DNS assay, respectively.

Growth of S7-19 was examined in Hungate tubes on these hydrolysates as well as on Solka floc and cellobiose to which increasing concentrations of xylose have been added. The results in Table 24 illustrate lower E/A's demonstrated by growth on enzymatically hydrolyzed corn stover and on Solka floc or cellobiose with added pentose.

In view of these findings, a selection scheme was designed to isolate low acid producing colonies of S7-19 on dye indicator plate medium containing corn stover hydrolyzate as carbon source. In this manner, mutagenized clones of S7-19 were screened on Bromcresol purple CM4-hydrolysate plate media. Clones with small halos or preferably purple colonies were picked, grown in corn stover hydrolysate tubes and assayed for E/A ratios. The best isolates were subsequently grown in pretreated corn stover tubes and then anaerobic flasks for final verification. The results in Table 25 show hydrolysate tube performances from 45 of the most promising isolates selected. It should be noted that in this selection step on corn stover hydrolyzate indicator plates over 2,000 colonies were examined, but no clone was found to have as small an acid halo as seen previously on BCP-CM-4-cellobiose media. The final selection from pretreated corn stover tubes yielded 3 promising candidates S7-19-7, 32, and 36 with E/A's of 3.1, 3.8 and 5.0, respectively.

Table 24

RATIO OF ETHANOL TO ACETATE DURING GROWTH OF *CLOSTRIDIUM THERMOCELLUM* S7-19 ON HYDROLYZED CORN STOVER, SOLKA FLOC + XYLOSE, AND CELLOBIOSE + XYLOSE IN HUNGATE TUBES

Hexose (glucose + cellobiose)		Pentose (balance of reducing sugars)	E/A
Hydrolysate	7 g/l	14 g/l	2.2
	3 "	6.5 "	1.6
	13 "	14	2.0
	9 "	6.5 "	2.2
15 g/l Solka floc		0	4.0
"		2.5	4.0
"		5.0	3.8
"		7.5	2.8
"		10.0	3.0
10 g/l Cellobiose		0	4.2
"		0	5.2
"		2.5	4.0
"		5.0	3.6
"		7.5	3.2
"		10.0	3.0
10 g/l Glucose		0	4.0

Table 25

ETHANOL AND ACETATE PRODUCTION BY MUTAGENIZED CLOSTRIDIUM THERMOCELLUM S7-19 CLONES SELECTED ON BCP-CM4-HYDROLYSATE MEDIA

	Hydrolysate Tubes			Pretreated Corn Stover		
S7-19 Clone	Ethanol	Acetate	E/A	EtOH	Acetate	E/A
S7-19- 1	1.8	0.53	3.4	3.4	1.4	2.5
- 2	5.0	1.25	4.0		No Growth	
- 3	0.8	0.56	1.4			
- 4	1.4	0.61	2.3			
- 5	1.0	0.31	3.2			
- 6	1.9	0.71	2.7			
- 7	2.1	0.58	3.6	3.6	1.2	3.1
- 8	0.3	0.56	0.6			
- 9	1.1	0.57	1.9			
-10	0.8	0.24	3.3			
-11	0.8	0.5	1.6			
-12	1.4	0.6	2.5			
-13	3.8	1.4	2.7			
-14	0.7	0.6	1.3			
-15	0.5	0.7	0.7			
-16	1.4	0.9	1.5			
-17	1.2	0.5	2.2			
-18	2.1	0.4	5.1	3.0	1.2	2.5
-19	1.1	0.3	3.4	3.2	1.2	2.6
-20	1.3	0.52	2.5			
-21	1.7	0.7	2.4			
-22	3.6	1.7	2.1			
-23	4.4	1.6	2.8			
-24	2.0	1.65	1.2			
-25	3.2	1.0	3.2	2.8	1.7	1.6
-26	0.9	0.8	1.1			
-27	3.0	1.2	2.5			
-28	1.2	0.9	1.3			
-29	2.7	1.0	2.7			
-30	3.1	1.3	1.2			
-31	3.0	1.4	2.1			
-32	2.3	0.7	3.2	1.9	0.5	3.8
-33	2.8	1.2	2.4			
-34	0.9	0.7	1.2			
-35	3.0	1.2	2.6			
-36	2.5	0.8	3.1	3.5	0.7	5.0
-37	2.5	1.0	2.5			
-38	0.9	0.6	1.4			

continued ...

Table 25

(continued)

S7-19 Clone	Hydrolysate Tubes Ethanol	Acetate	E/A	Pretreated Corn Stover EtOH	Acetate	E/A
S7-19-39	1.9	0.6	1.4			
-40	0.9	0.8	1.1			
-41	1.3	0.8	2.3			
-42	2.5	0.9	2.7			
-43	1.4	1.5	0.9			
-44	1.9	1.2	1.3			
-45	2.3	1.3	1.8			
S7-19				4.0	1.4	2.9

These strains were examined along with S7-19 on pretreated corn stover at 45.5 g/l in anaerobic flask fermentations. The results shown in Figures 43, 44, 45 and 46 show none of the new isolates to have any significant advantage over the parent S7-19 with regard to higher ethanol yield.

At a first glance, one is led to conclude that the strain selection program, although capable of yielding high ethanol to acetate mutants on model substrates, cellobiose and Solka floc, it is not useful for realistic biomass such as corn stover. It will, however, be shown that this conclusion can be improperly interpreted as the result of the detailed analysis of the results to be presented later (Section 4.8). In view of these analyses to be presented, no further discussions will be made at this time.

Figure 44

GROWTH OF CLOSTRIDIUM THERMOCELLUM S7-19-7 ON PRETREATED CORN STOVER (45 G/L)

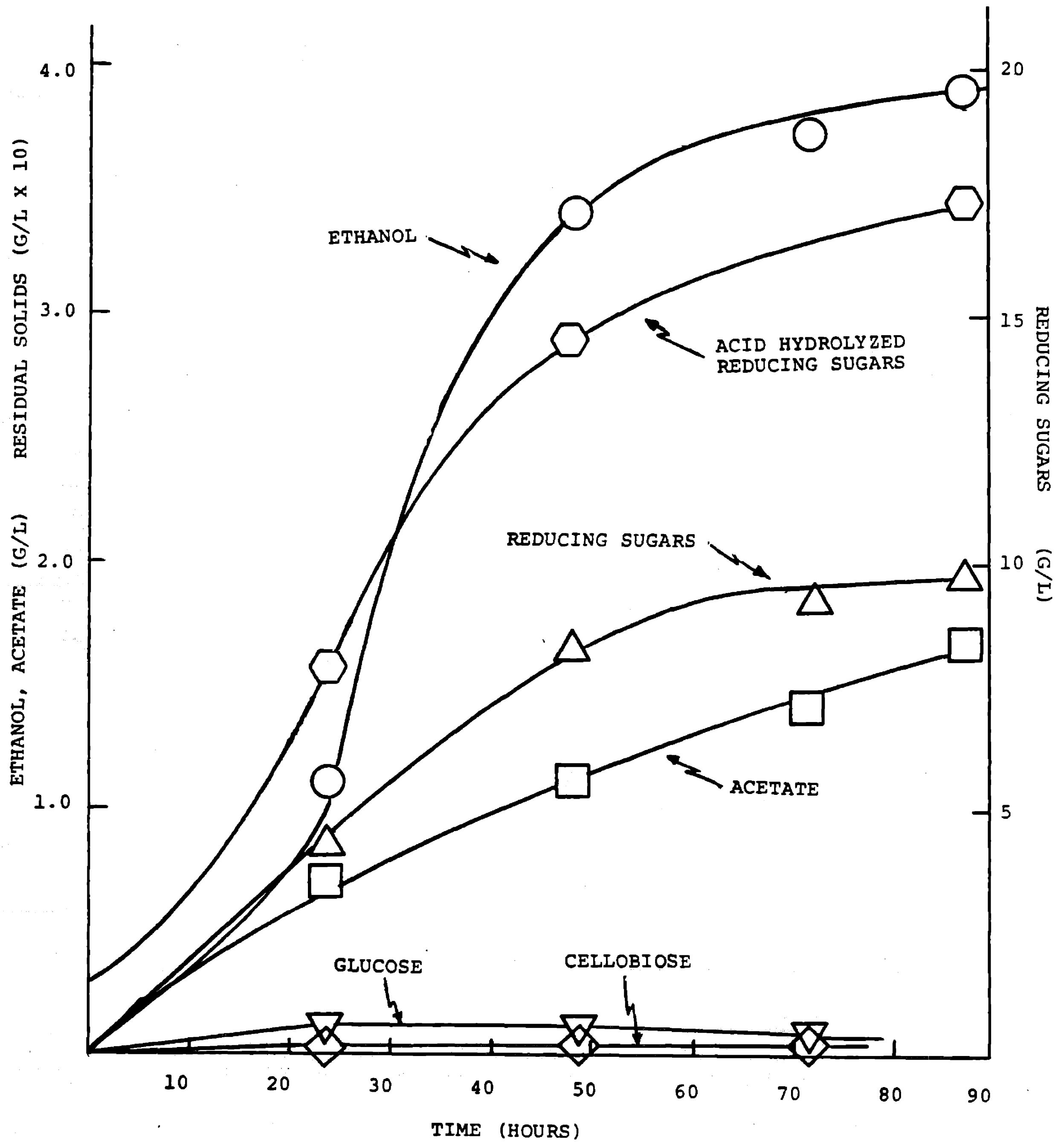


Figure 45

GROWTH OF CLOSTRIDIUM THERMOCELLUM S7-19-32 ON PRETREATED CORN STOVER (45 G/L)

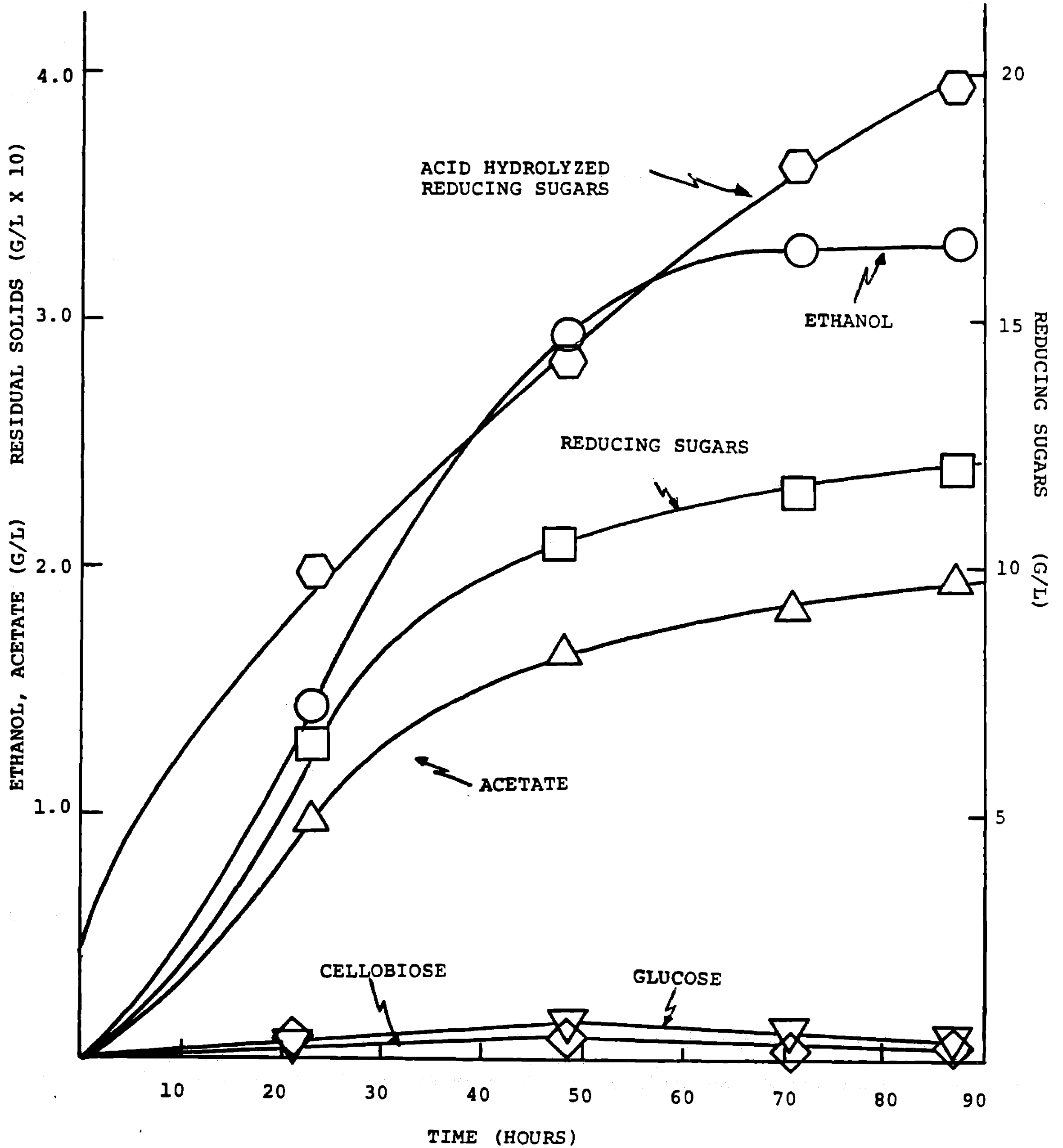
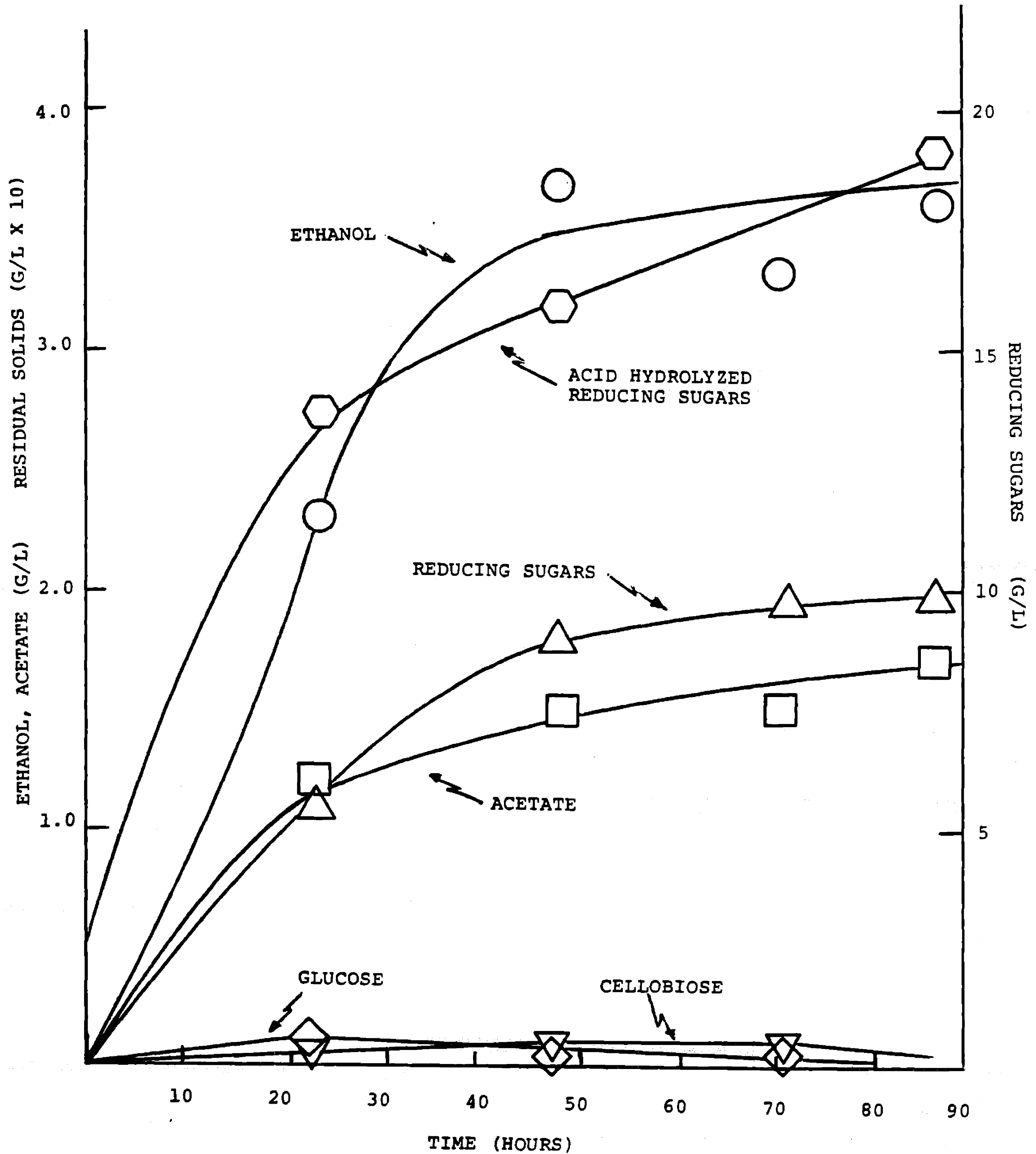


Figure 46

GROWTH OF CLOSTRIDIUM THERMOCELLUM S7-19-36 ON PRETREATED CORN STOVER (45 G/L)



4.7. Fermentation Studies with Pretreated Corn Stover

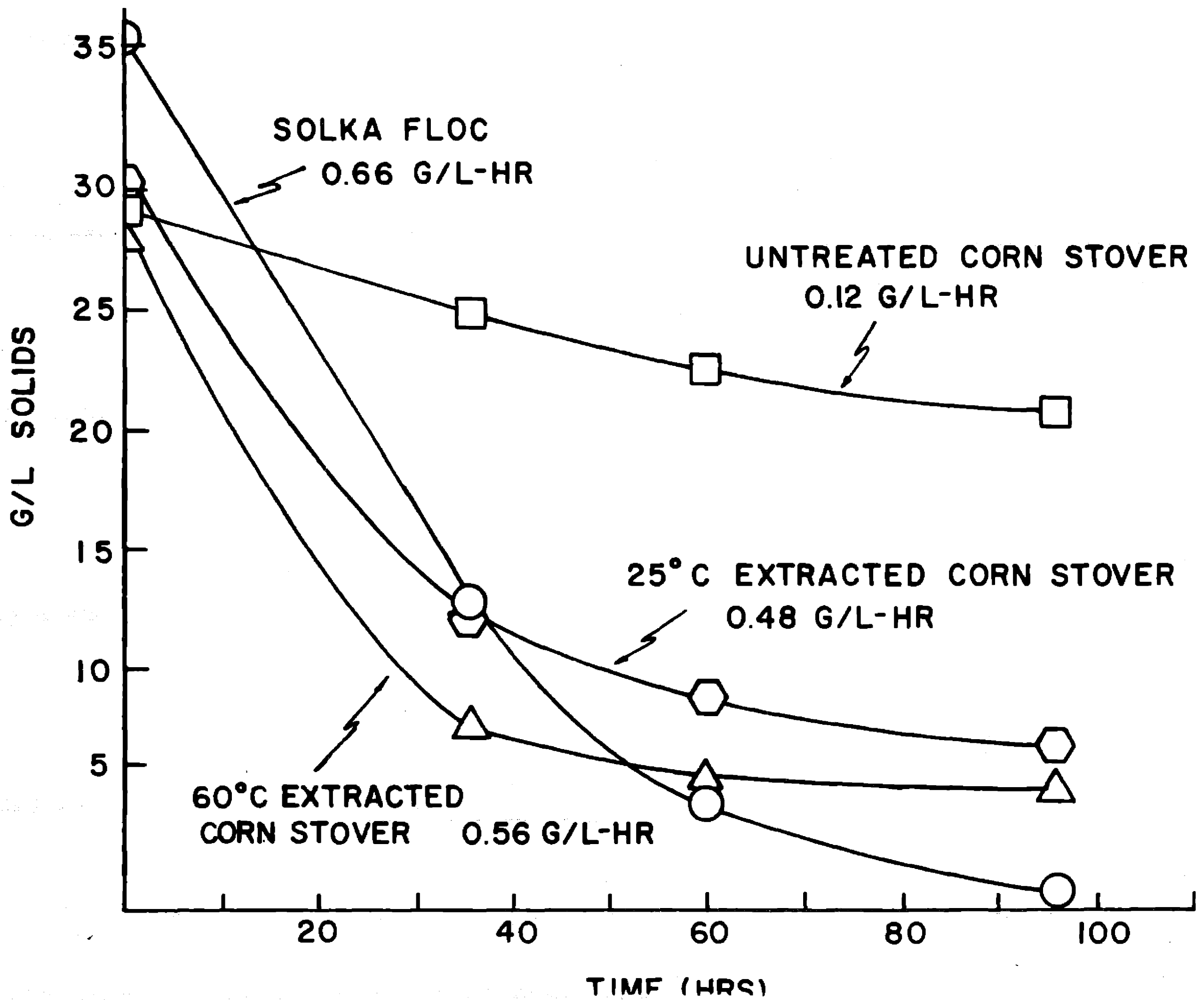
As was described in Section 4.5, biomass pretreatment by selective solvent delignification represents an effective means for biomass delignification without extensive losses of the fermentable carbohydrate fractions. Using different biomass pretreatments, together with high ethanol yielding and lignin tolerant strains, a series of fermentations were conducted to ascertain the optimum pretreatment conditions for high ethanol yield and substrate utilization from corn stover.

4.7.1. Fermentation of Selective Solvent Extracted Corn Stover by Mixed Culture - Kinetics of Substrate Utilization

The fermentation performance of alkaline pretreated corn stover was initially examined with mixed cultures of *Clostridium thermocellum* S-7 and *Clostridium thermosaccharolyticum* HG-4. As shown in Figure 47, untreated corn stover (30 g/l) is slowly degraded at an initial rate of 0.12 g/l hr consuming only 28% of the total solids after 100 hrs. By contrast corn stover pretreated at 25 or 60°C with 50% (V/V) ethanol/water mixture and 8 g/l NaOH is degraded much faster. Initial rates of 0.48 and 0.56 g/l hr substrate hydrolysis at 25 and 60°C pretreatment conditions, respectively, were observed. In addition, the extentsof solids consumption after 96 hours of fermentation are seen to increase.

Figure 47

SUBSTRATE DEGRADATION DURING MIXED CULTURE FERMENTATION OF SOLKA FLOC AND CORN STOVER WITH AND WITHOUT TREATMENT BY _CLOSTRIDIUM THERMOCELLUM_ S-7 AND _CLOSTRIDIUM THERMOSACCHAROLYTICUM_ HG-4



In Figure 48, a relationship on the extent of substrate utilization (at 96 hours) and the initial rate (0 to 36 hours) versus the percent of lignin remaining after pretreatment are presented for corn stover. As a reference, Solka Floc was used for comparison and was assumed to contain no lignin. Linear correlations were observed on the rate, as well as the extent of degradation. These data clearly demonstrate the importance of lignin in limiting the enzymatic utilization of corn stover and show the effectiveness of selective solvent delignification for overcoming this limitation.

A more detailed analysis of α-cellulose and pentosan carbohydrate degradation during fermentation using 25°C treated corn stover was performed. Untreated corn stover following fermentation was also analyzed for direct comparisons. In Table 26 one observes that only 34% of the α-cellulose of non-treated corn stover is consumed after 60 hrs of fermentation. On the other hand, 85% of the cellulose was utilized after pretreatment. A similar increase in pentosan utilization is also observed; from 41 to 90% useage with non-treated and treated materials, respectively. Therefore, by summing the carbohydrate fractions utilized, we see that after 60 hrs of fermentation a total of 87% of the carbohydrate from treated corn stover was consumed whereas less than 37% of the α-cellulose and pentosan from non-treated corn stover could be consumed.

Figure 48

INITIAL RATE (0-36 HR) AND EXTENT (0-96 HR)
OF SUBSTRATE DEGRADATION VS % LIGNIN
REMAINING IN CORN STOVER

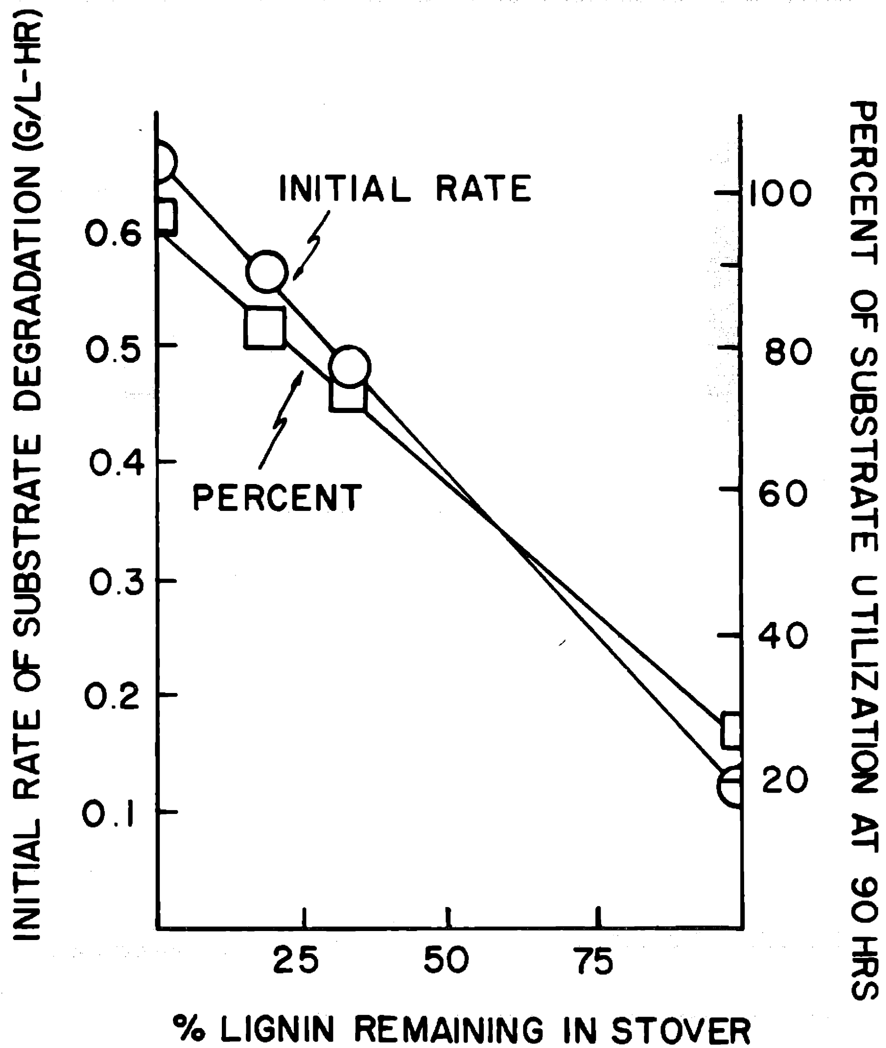


Table 26

ANALYSIS OF SOLIDS AFTER 60 HRS OF FERMENTATION OF TREATED AND NON-TREATED CORN STOVER USING MIXED CULTURE OF CLOSTRIDIUM THERMOCELLUM (S-7) AND CLOSTRIDIUM THERMOSACCHAROLYTICUM (HG-4)

	Non-Treated Corn Stover			Selective Solvent Extracted Corn Stover (25°C, 48 Hrs)		
	t = 0	t = 60 Hrs	% Util.	t = 0	t = 60 Hrs	% Util.
Total Solids	29.1	22.5	-	30.0	11.7	-
α-cellulose	11.3	7.5	34	12.3	1.9	85
Pentosan	9.9	5.8	41	10.5	1.0	90

Effect of Particle Size

Selective pretreatment of different particle size corn stover was shown previously to result in virtually the same extent of delignification regardless of particle size (Section 4.5.4). For example, the largest particles of 2 to 8 mm diameter were shown to attain 62% delignification as compared to 69% delignification observed with the smaller 0.5 mm diameter material. These extracted samples were subsequently fermented by monocultures of Clostridium thermocellum S-7-19. The fermentation profiles in Figures 49, 50 and 51 show the degradation and accumulation of ethanol, acetate and reducing sugars for the three particle size ranges centered at approximately 0.5, 2.5 and 4.0 mm diameter.

A summary of the respective product concentrations at the end of the fermentation is shown in Table 27. One observes very little variation in the total accumulation of acid hydrolyzed reducing sugars or acetic acid with the use of different particle size corn stover. However, the extent of ethanol production appears to be reduced from 4.0 to 3.2 and 2.8 g/l with increasing average particle size from 0.5 to 2.5 and 4.0 mm diameter, respectively. This represents a 20 to 30% decrease in total ethanol production and also reflects a decrease in the ethanol to acetic acid ratio from 2 to 1.4. Also summarized in Table 27 are integral rate data calculated between 40 and 100 hrs for ethanol, acetate and reducing sugars.

Figure 49

FERMENTATION OF EXTRACTED CORN STOVER BY CLOSTRIDIUM THERMOCELLUM S-7-19 (0.5 mm DIAMETER PARTICLE SIZE)

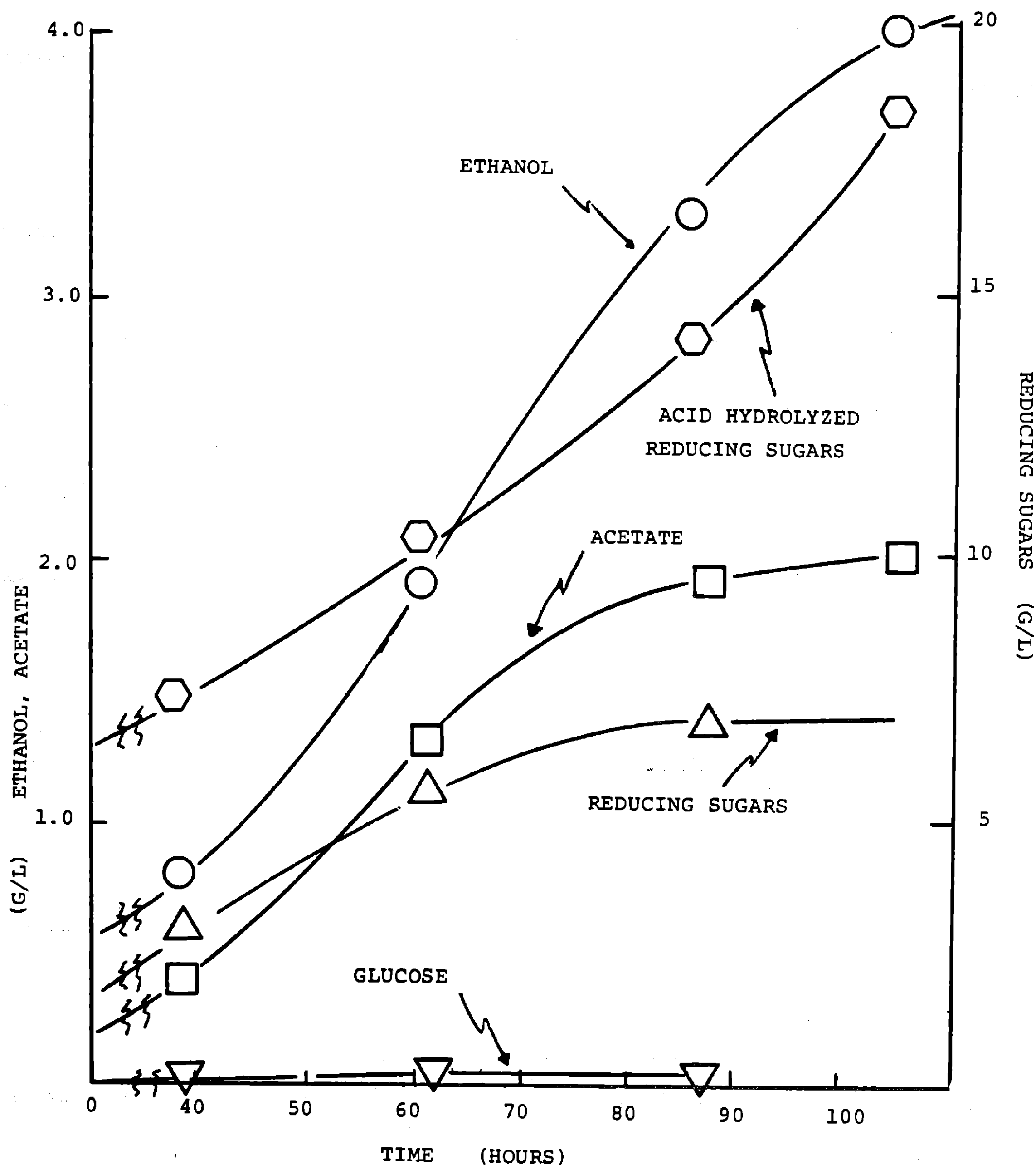


Figure 50

FERMENTATION OF EXTRACTED CORN STOVER BY CLOSTRIDIUM THERMOCELLUM S-7-19 (2.5 mm DIAMETER PARTICLE SIZE)

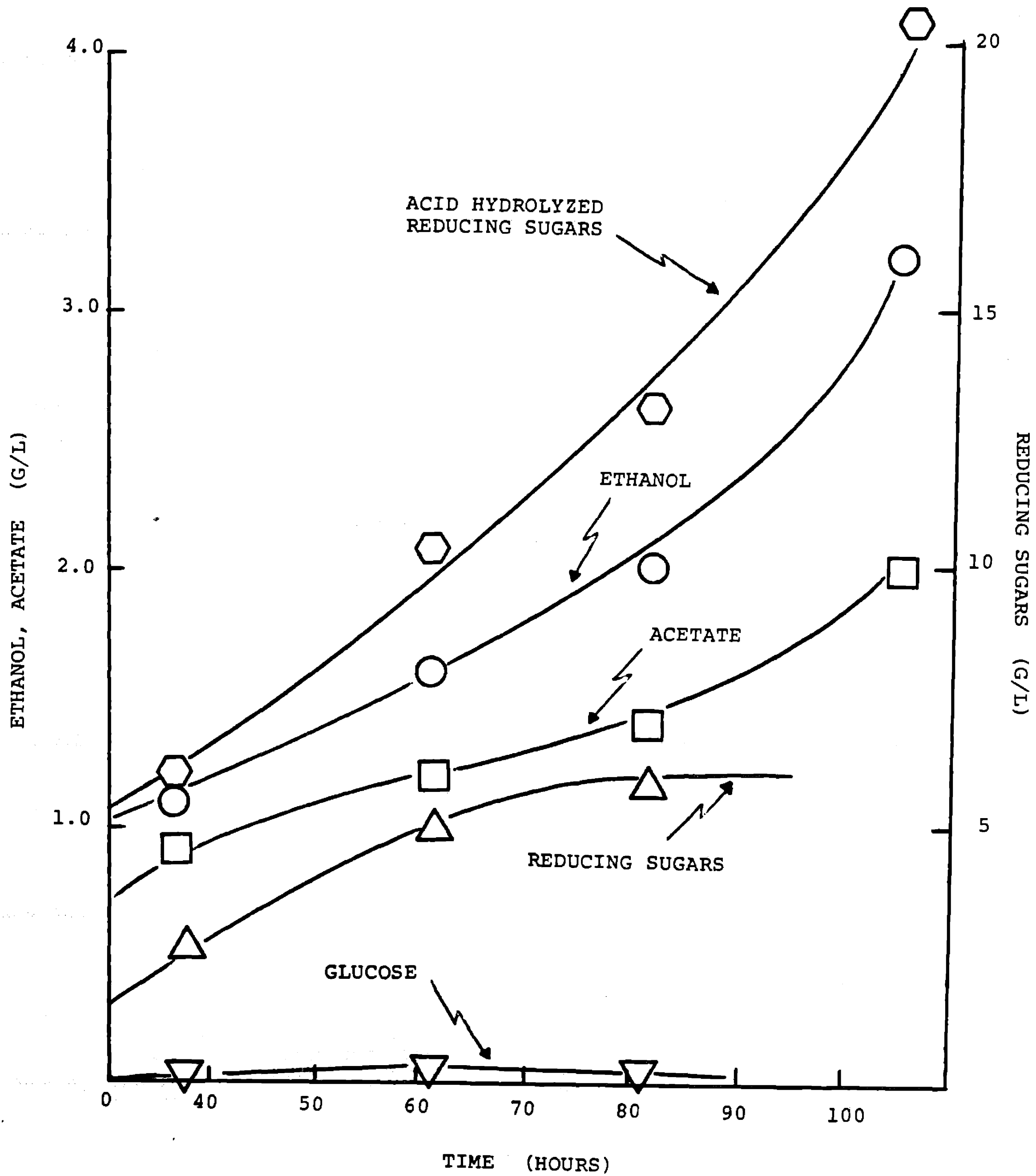


Figure 51

FERMENTATION OF EXTRACTED CORN STOVER BY CLOSTRIDIUM THERMOCELLUM S-7-19 (4.0 mm DIAMETER PARTICLE SIZE)

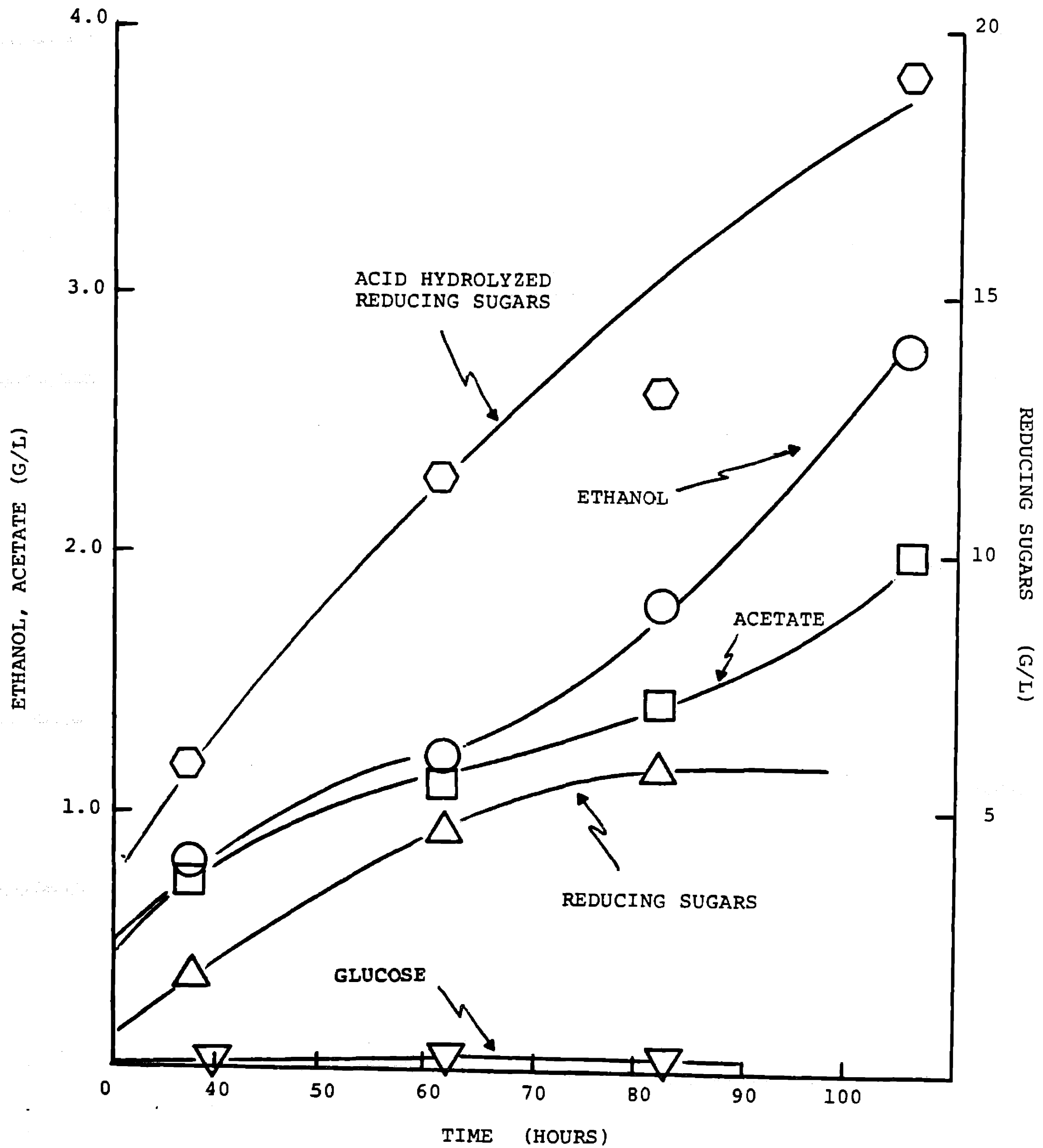


Table 27

COMPARISON OF CORN STOVER OF DIFFERENT PARTICLE SIZES PRETREATED AND FERMENTED BY MONO-CULTURE OF CLOSTRIDIUM THERMOCELLUM (S-7-19) (45 G/L CORN STOVER)

	Average Particle Size of Pretreated Corn Stover		
	0.5 mm	2.5 mm	4.0 mm
Production at 110 hrs:			
Ethanol (g/l)	4.0	3.2	2.8
Acetate (g/l)	2.0	1.8	2.0
Ethanol/Acetate	2.0	1.8	1.4
Acid Hydrolyzed Reducing Sugars (g/l)	18.0	20.0	19.0
Integral rate at 40-100 hrs:			
Ethanol (g/l hr)	0.053	0.035	0.033
Acetate (g/l hr)	0.028	0.018	0.020
Acid Hydrolyzed Reducing Sugars (g/l hr)	0.18	0.23	0.22

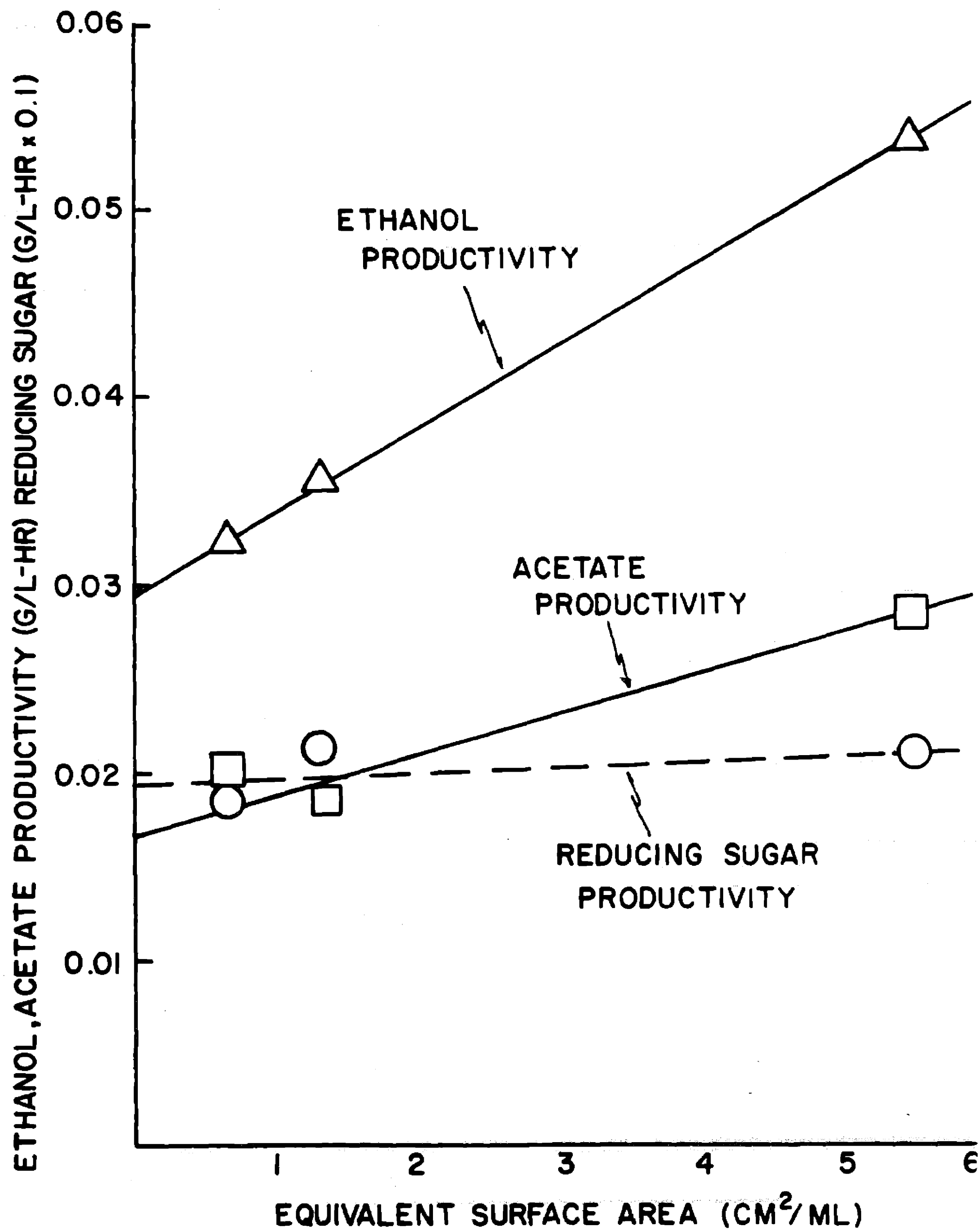
Only a minor difference is observed in the rate of acid hydrolyzed reducing sugar accumulation indicating that the particle size of the treated material has very little effect on either the rate or extent of hemicellulose hydrolysis after treatment.

On the other hand, both the ethanol and acetate production rates are seen to decrease with increasing particle size. The rate of ethanol production with 2.5 and 4.0 mm corn stover is approximately 64% of the smaller 0.5 mm diameter material rate and acetate production is 69% of the small particle size rate.

It is quite reasonable to assume that decreasing the particle size should offer more surface area for the enzymatic attack of the corn stover. If one plots the data of the rates of product formation, as shown in Figure 52, the following graphic picture emerges. It can be seen that there is no effect of particle size on the rate of reducing sugar production. This is interpreted in the following manner. It has been previously shown thatthe extent of delignification for all partizle sizes is identical. Since this is a mono-culture of *Clostridium thermocellum*, all of the reducing sugars are derived from the hemicellulosic fraction of the corn stover. This is further substantiated from the data in Figures 49, 50 and 51 where no six carbon sugars (glucose or cellobiose) were shown to accumulate during fermentation. One is therefore led to conclude that the delignification has now provided accessibility to the hemicellu-

Figure 52

ETHANOL, ACETATE AND REDUCING SUGAR PRODUCTIVITY OF CLOSTRIDIUM THERMOCELLUM S-7-19 AS A FUNCTION OF EQUIVALENT SURFACE AREA OF PRETREATED CORN STOVER PARTICLES



lose to enzymatic hydrolysis which is no longer surface area dependent. One could hypothesize that the removal of the lignin has now rendered the physical state of the hemicellulose to be very "amorphous". In this fashion, there is an increased number of sites for enzymatic hydrolysis. Due to the high availability of hydrolytic sites, enzyme saturation cannot be achieved and therefore one does not observe a difference in rate as the solids specific surface area is increased.

On the other hand, the cellulosic fraction in pretreated corn stover still contains restrictive sites for enzyme hydrolysis. It is only through the solubilization of the cellulose that Clostridium thermocellum can effectively utilize these products for the formation of ethanol and acetic acid. When one examines the rates of these catabolite formation versus the specific surface area (Figure A), the following picture emerges. Although the large particle (4 mm) is nearly twice the size of the medium size particle (2.5 mm), the specific surface area only increases by 60%. However, the smallest particle (0.5 mm) has nearly a ten fold increase in the specific surface area. As seen in Figure 52, there does indeed show an increasing rate with increasing specific surface area. One therefore concludes that the cellulosic fraction through pretreatment still retains a recalcitrant structure where the sites for enzymatic hydrolysis is surface area dependent. Unfortunately, the earlier studies (Section 4.4.1) on the effect of particle

size using untreated corn stover were performed using free enzymes. Therefore, a direct comparison from these fermentation studies cannot be performed with those previously presented.

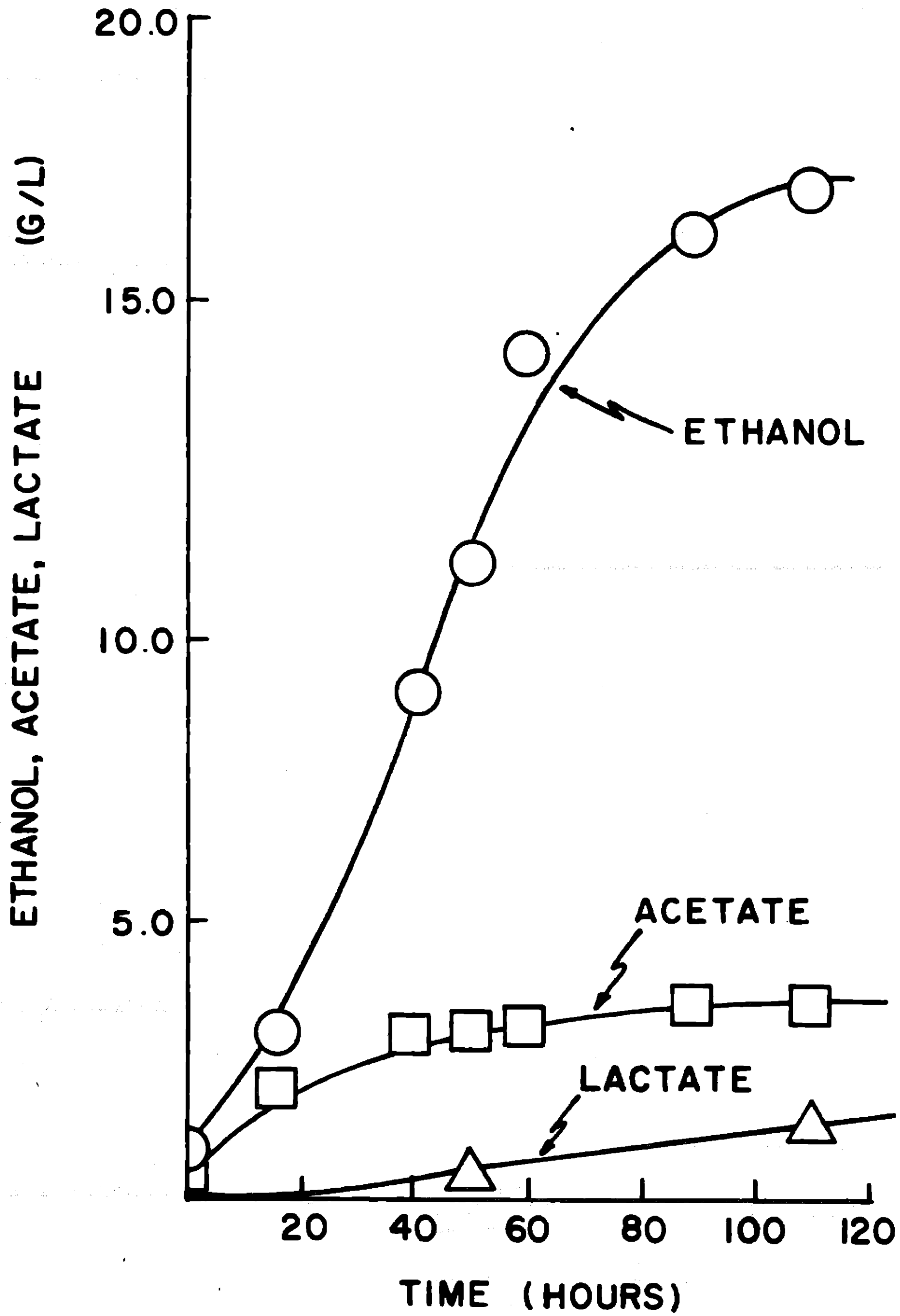
4.7.2. Fermentation of Selective Solvent Extracted Corn Stover by Mixed Cultures - Ethanol Yield

In addition to the rate and extent of corn stover degradation, an equally important factor is the ethanol yield. A mixed culture of fermentation using high ethanol yielding strains of *Clostridium thermocellum* S-7-19 and *Clostridium thermosaccharolyticum* HG-8 was tested on pretreated corn stover. The use of these strains was necessitated by the problems of strain degeneration with respect to maximum ethanol yield observed for *Clostridium thermocellum* S-7, as well as to overcome the problem of lignin toxicity demonstrated by *Clostridium thermosaccharolyticum* HG-4. In addition to the fermentation of pretreated corn stover, these cultures were also grown on Solka floc as a control. The results of these fermentations are compared with the previous results obtained for the high ethanol yielding strains of *Clostridium thermocellum* S-7 and *Clostridium thermosaccharolyticum* HG-4 grown on both Solka floc and non-treated corn stover (Figures 17 and 18). In this manner, the effectiveness of both strain improvements and substrate delignification on the ethanol yield can be examined.

The ethanol and acetate production by the new isolate of *Clostridium thermocellum* S-7-19 and *Clostridium thermosaccharolyticum* HG-8 on 50 g/l Solka floc are shown in Figure 53. On the Solka floc, 17.7 g/l ethanol and 3.5 g/l acetate are produced in approximately 80 hours of fermentation. This is equivalent to an ethanol to acetate ratio of 5.1:1. As

Figure 53

PRODUCTION OF ETHANOL AND ACETATE BY MIXED CULTURE FERMENTATION OF SOLKA FLOC (50 G/L) BY CLOSTRIDIUM THERMOCELLUM S7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8



summarized in Table 28, this performance by these new strains on Solka floc is quite similar to that previously observed for the parent strains, Clostridium thermocellum S-7 and Clostridium thermosaccharolyticum HG-4, on Solka floc. In this case, essentially the same ethanol to acetic acid (E/A) ratio of 5.0 to 1 and ethanol and acetate productivity of 0.25 and 0.05 g/l hr, respectively, were obtained for growth on Solka floc. Therefore, for the fermentation of Solka floc, little difference in performance is observed. However, a significantly different fermentation performance of pretreated corn stover by these new strains is obtained as shown in Figure 54. In this case, mixed culture fermentation of selective solvent treated corn stover results in the production of 9 g/l ethanol and 3.7 g/l acetate in 80 hrs. This corresponds to an E/A ratio of 2.4 to 1. When contrasted with the previous performance obtained for non-treated corn stover (Table 28) one observes that an improvement in the average batch ethanol productivity from 0.08 to 0.11 g/l hr and slight decrease in acetate productivity of from 0.05 to 0.04 g/l hr are obtained for pretreated corn stover with these strains. These factors combine to yield the significant improvement in the E/A ratio from 1.5 to 2.4 to 1 observed.

It was shown previously that the solvent delignification of corn stover increased the rate of degradation to values comparable to those observed during mixed culture

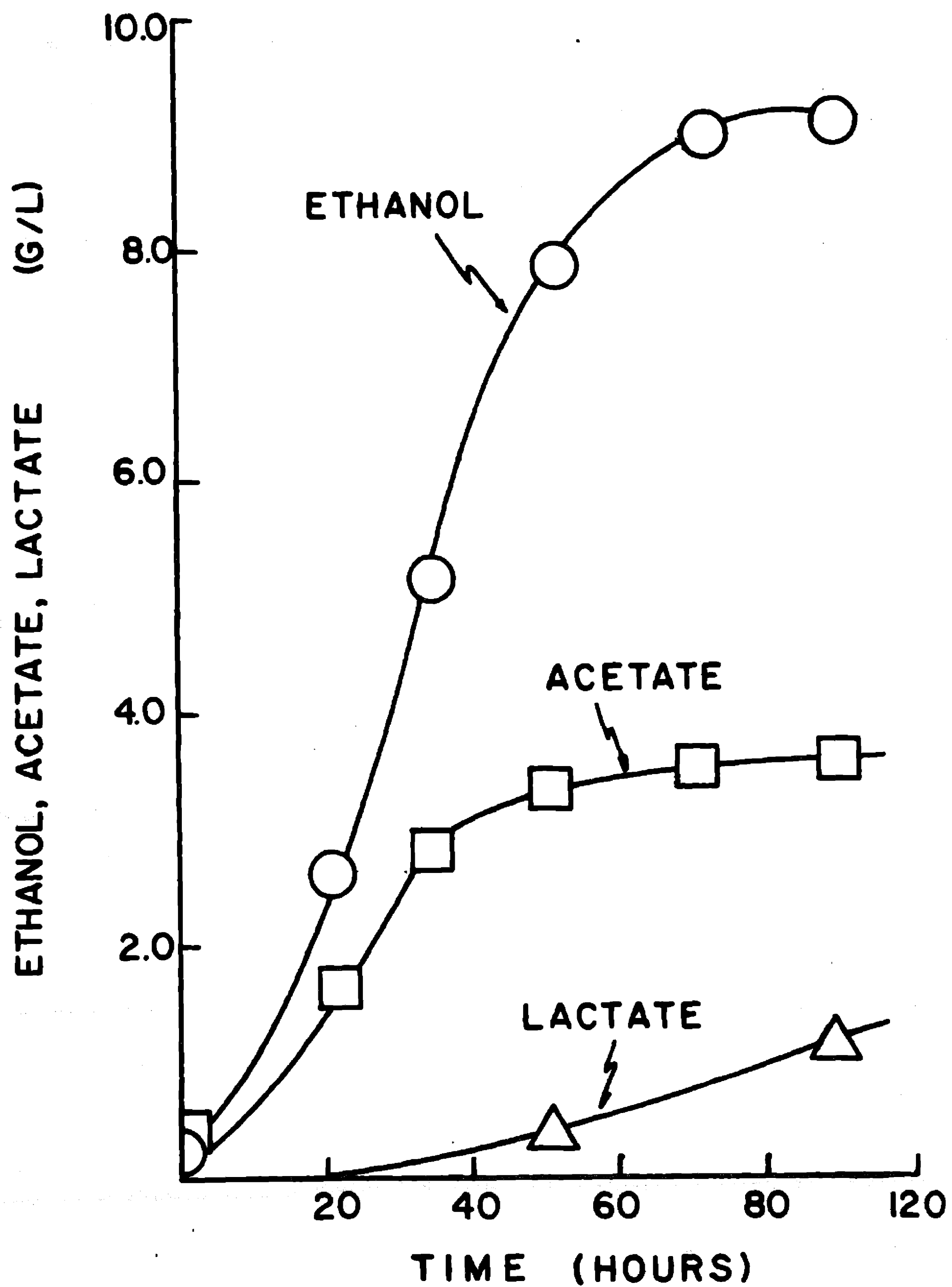
Table 28

COMPARISON OF STRAIN PERFORMANCE IN MIXED CULTURE FERMENTATION OF SOLKA FLOC, SOLVENT EXTRACTED CORN STOVER, AND NON-TREATED CORN STOVER

Strains	Substrate	Average Batch Ethanol Productivity	Average Batch Acetate Productivity	E/A Ratio
<u>C. thermocellum</u> S7-19 and <u>C. thermosaccharolyticum</u> HG-8	Solka floc	0.25 g/l hr	0.05 g/l hr	5.1
<u>C. thermocellum</u> S7-19 and <u>C. thermosaccharolyticum</u> HG-8	Solvent Extracted Corn Stover	0.11	0.04	2.4
<u>C. thermocellum</u> S-7 and <u>C. thermosaccharolyticum</u> HG-4	Solka floc	0.25	0.05	5.0
<u>C. thermocellum</u> S-7 and <u>C. thermosaccharolyticum</u> HG-4	Non-Treated Corn Stover	0.08	0.05	1.5

Figure 54 .

PRODUCTION OF ETHANOL AND ACETATE BY MIXED CULTURE FERMENTATION OF CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 ON PRETREATED CORN STOVER (80 G/L)



growth on Solka floc (Section 4.7.1). Increases in initial rates from approximately 0.1 - 0.2 g/l hr with non-treated corn stover to 0.48 g/l hr with treated stover were observed. This value is comparable with initial rates of approximately 0.66 g/l hr observed for the rate of Solka floc consumption. Nevertheless, this increase in initial rate of degradation of over 4 times by solvent pretreatment only results in a 40% increase in ethanol productivity with the high yielding new strains. In addition, only a 60% increase in E/A rates was observed. As shown in Figure 55, these values are still substantially below the performance observed for the model substrate Solka floc.

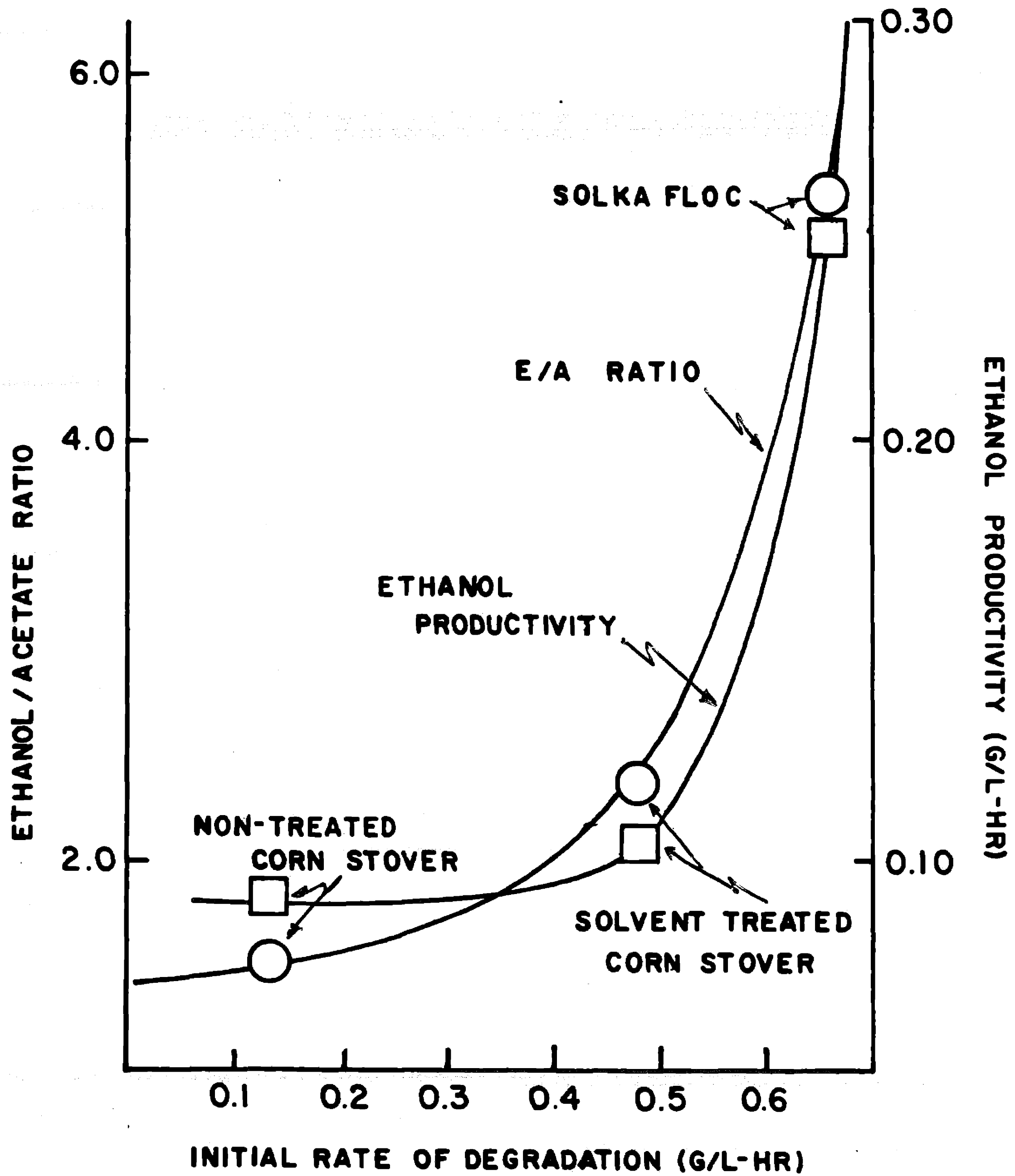
In order to identify the basis for this behavior, an in depth analysis of the kinetics of carbohydrate utilization by the mixed culture was performed. The experimental basis for this analysis will be detailed in the following section. The subsequent kinetic analysis and conclusions are described in detail in Section 4.8.

4.7.3. Analyses of Sequential Monoculture Fermentations of Pretreated Corn Stover by *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*

Unfortunately, in spite of these larger increases in rate and extent of substrate utilization afforded by selective delignification, the yield of ethanol in mixed culture on treated corn stover is still not as good as the baseline case on Solka floc or cellobiose. In order to delineate

Figure 55

EFFECT OF INITIAL RATE OF SUBSTRATE DEGRADATION FOR SOLKA FLOC, TREATED CORN STOVER, AND NON-TREATED CORN STOVER ON E/A RATIO AND ETHANOL PRODUCTIVITY OF MIXED CULTURE



this behavior in greater detail, two sequential monoculture fermentations of *Clostridium thermocellum* S-7-19 and *Clostridium thermosaccharolyticum* HG-8 were conducted on pretreated corn stover.

The typical fermentation of pretreated corn stover by monocultures of S-7-19 was previously shown in Figure 43 and resulted in the production of 4.2 g/l ethanol and 1.7 g/l acetate from 45.5 g/l treated corn stover yielding an ethanol/acetate ratio of 2.5:1. However, in addition to these products from the corn stover, a significant quantity of pentosan sugars are shown to accumulate. In order to characterize the consumption of solids in greater detail, a material balance on carbohydrates of the pretreated corn stover from this monoculture fermentation was made as shown in Table 29. From the pretreated corn stover composition data, it is known that 45.5 g/l corn stover contains 18.7 g/l of cellulose and 15.9 g/l pentosan carbohydrates. If these sugars were enzymatically hydrolyzed to completion, they would represent an equivalent 20.6 g/l and 17.8 g/l glucose and pentose, respectively. The increase is due to the weight gain associated with hydrolysis. From the material balance, we see that 18.3 g/l acid hydrolyzed reducing sugars are formed in the medium at 88 hours of fermentation.

The extent of cellulose utilization during this fermentation can be estimated from the major products, ethanol, acetate, and lactate. If one assumes that the con-

Table 29

ACCOUNTABLE SOLIDS FOR MONOCULTURE FERMENTATION OF CLOSTRIDIUM THERMOCELLUM S7-19 ON PRETREATED CORN STOVER

Fermentation Time	22 Hrs	48 Hrs	88 Hrs
Average Growth Rate (Hr^{-1})	0.094	0.045	0.004
Ethanol	0.8	3.8	4.2
Acetate	0.7	1.4	1.7
Lactate	0.0	0.8	1.6
Acid Hydrolyzed Reducing Sugars	8.6	15.0	18.3
Glucose + Cellobiose	0.4	0.9	0.2
Calculated Glucose in Cell Mass*	0.1	0.6	0.7
Calculated Glucose Consumption	3.2	12.0	13.5
Pentose Accumulation	8.6	15.0	18.3
Calculated Cellulose Hydrolyzed	2.9	10.8	12.1
Calculated Pentosan Hydrolyzed	7.7	13.5	16.5
Calculated Solids Hydrolyzed (Cellulose + Pentosan)	10.6	24.3	28.6
Actual Solids Hydrolyzed	12.5	28.5	32.5
% Closure of Predicted Solids Hydrolysis	85%	85%	88%

* See Text

version of glucose is achieved at the theoretical yield from the known pathways (see Literature Survey), the values for glucose or cellulose consumption shown in table can be calculated. However, it is clear that not all "glucose" metabolized by the cells is represented by soluble products. Data for carbon source dissimilation into cells and into products during anaerobic growth of Streptococcus faecilis and Saccharomyces cerevisiae have been reported by Bauchop and Elsden and Kormancickova et al.[130,131]. These investigators demonstrated a 95 to 98% carbohydrate metabolism into products and a 2 to 5% incorporation of carbohydrate into cell mass. Therefore, in addition to glucose consumed to produce the fermentation products, a minor correction should be made for glucose incorporation into cell mass. For the purposes of this material balance, this was accomplished by assuming a typical anaerobic yield of 0.1 g cells/g substrate consumed. An additional incorporation of glucose into cell mass of 0.5 g glucose/g cells was assumed. The net effect of these assumptions gives a total glucose uptake representing a 96% carbohydrate dissimilation into products and a 4% incorporation into cell mass.

If one compares the expected total hydrolyzed solids calculated by this method with that actually observed at 22, 48, and 88 hours of fermentation, an average of 86% closure of the material balance is obtained. This closure is reasonable if one bears in mind that a fraction of the solids such

as lignins and ash which are not metabolized but may become solubilized as the surrounding carbohydrate material is degraded

4.7.4. HPLC Analysis of Sugars Produced

Since the major product of this monoculture fermentation is pentosan carbohydrate, an analysis of the sugars released from the solids during Clostridium thermocellum S-7-19 fermentation was made by high pressure liquid chromatography (HPLC). As shown in Table 30, the predominant soluble reducing sugar is xylobiose, with lesser amounts of arabinose and xylose released. The total sugars analyzed by HPLC were 7.1 g/l. This number is in reasonable agreement with the value obtained using the DNS reducing sugars assay of 9.0 g/l before acid hydrolysis from this fermentation. Presumably, the balance of the soluble sugars are present as soluble oligimers which are either removed by the HPLC preparation or not detected by HPLC. The ratio of the predominate hemicellulosic carbohydrates reported for corn stover are xylose:arabinose:glucuronic acid at a ratio of 5:1:1. The ratio of xylose to arabinose in the soluble carbohydrates detected is slightly lower at 3.4 to 1. However, glucuronic acid could not be detected with the HPLC column and the procedures used. Thus the presence of glucuronic acid remaining after selective solvent treatment as well as the soluble hemicellulose oligomers which are assumed to represent

Table 30

HPLC OF SUGARS PRODUCED BY *CLOSTRIDIUM THERMOCELLUM* S7-19 MONOCULTURE FERMENTATION OF PRETREATED CORN STOVER

	Supernatant
Xylobiose	3.7 g/l
Xylose	1.0
Arabinose	1.5
Cellobiose	0.8
Glucose	0.1
TOTAL	7.1

the remaining 11 g/l of total soluble sugars (60%) were not detected. The ability of Clostridium thermosaccharolyticum HG-8 to metabolize these carbohydrates was then examined.

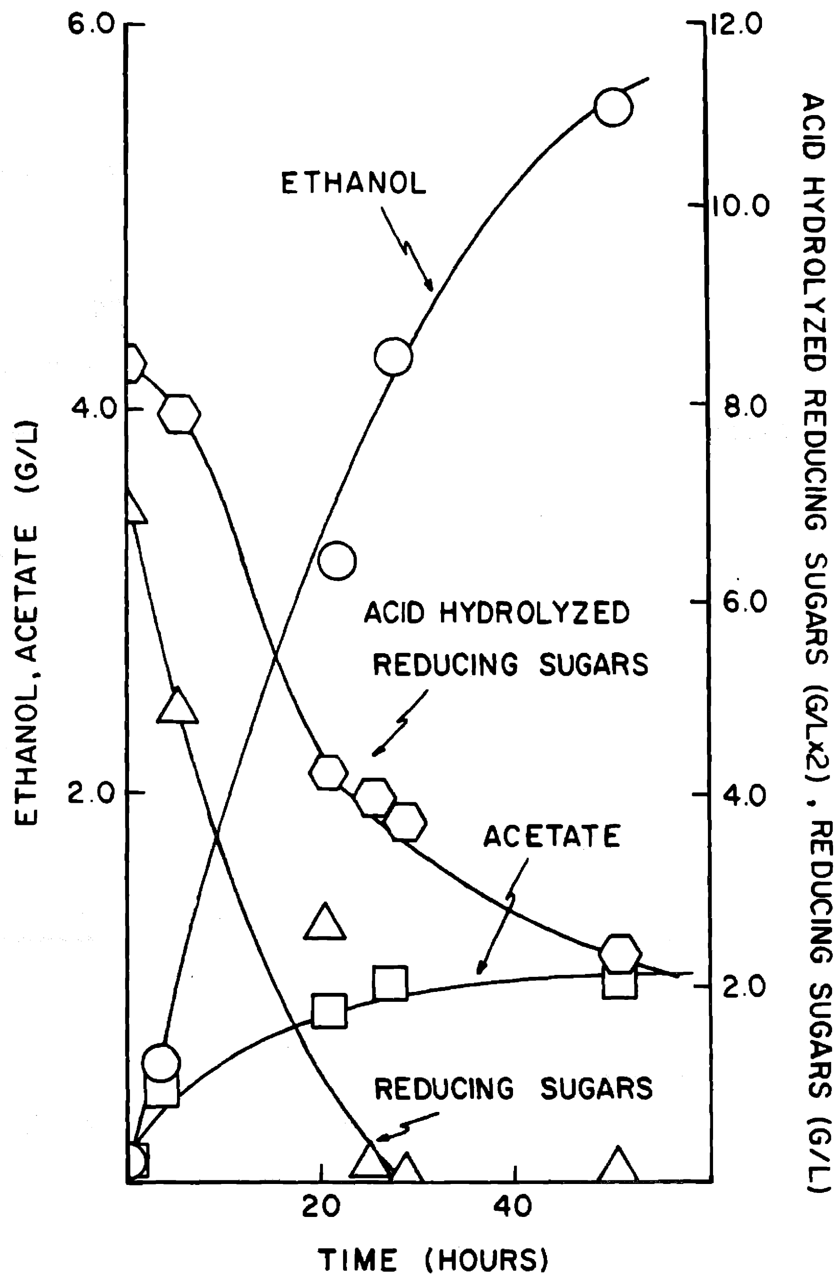
4.7.5. Sequential Fermentation of Hydrolyzed Corn Stover Carbohydrates by Clostridium thermosaccharolyticum

The ability of new strains of Clostridium thermosaccharolyticum, such as HG-8 and HG-6-62, to ferment the solubilized hemicellulosic carbohydrates obtained through the monoculture fermentation of Clostridium thermocellum S-7-19 on pretreated corn stover was performed to determine the maximum yield of ethanol which could be achieved from this carbohydrate fraction. It was previously shown that from a typical monoculture fermentation of Clostridium thermocellum S-7-19 on 45 g/l pretreated corn stover 18 g/l of hemicellulose carbohydrate will accumulate. After 120 hrs of fermentation by Clostridium thermocellum where no further fermentation activity was observed, Clostridium thermosaccharolyticum HG-8 was inoculated into the broth. A continued net fermentation profile as shown in Figure 56 is obtained.

The acid hydrolyzed sugar content of the supernatant is rapidly reduced from 17 g/l to less than 5 g/l in 48 hours of fermentation by Clostridium thermosaccharolyticum HG-8. From these sugars, 5.5 g/l ethanol and 1.0 g/l acetate are produced. The maximum volumetric rate of sugar consumption

Figure 56

FERMENTATION BY CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 INOCULATED AFTER 100 HRS FERMENTATION OF CLOSTRIDIUM THERMOCELLUM S7-19 ON 5 G/L PRE-TREATED CORN STOVER



is reached at approximately 15 hours of fermentation at a value of 0.5 g/l acid hydrolyzed sugars consumed per hour. At 30 hours of fermentation, the free reducing sugar decreases to zero. At this point, the acid hydrolyzed reducing sugar shows a value of 8 g/l but the rate of consumption has decreased to 0.13 g/l hr. From the increased ratio of acid hydrolyzed to non-acid hydrolyzed reducing sugars, these acid hydrolyzed carbohydrates which remain are presumably longer chain polymeric species. It is reasonable that Clostridium thermosaccharolyticum assimilates the shorter chain carbohydrates first and then degrades the longer chains but at a decreased rate. One cannot conclude, however, whether the extracellular enzymes of Clostridium thermocellum which were responsible for the initial solubilization of these polymers are responsible for their subsequent degradation; or whether the extracellular enzymes of Clostridium thermosaccharolyticum are capable of hydrolyzing these carbohydrates. However, growth of Clostridium thermosaccharolyticum HG-4 in monoculture has been shown using hemicellulosic carbohydrates of larchwood xylan [30]. From these results, one might conclude that at least some of the xylose oligomer hydrolysis is achieved by this strain.

It is apparent that Clostridium thermosaccharolyticum HG-8 can grow in monoculture on the previously hydrolyzed carbohydrates of corn stover. It is also capable of quite high yield with an E/A ratio of 5.5 to 1. If one sums

the performances for these strains growing on pretreated corn stover, either sequentially or in mixed culture, a different picture emerges as summarized in Table 31. As shown previously, Clostridium thermocellum S-7-19 typically produces ethanol and acetate on pretreated corn stover in a ratio of 2.5:1. For example, from 45 g/l corn stover, 12 g/l hexoses are consumed resulting in the production of approximately 4.2 g/l ethanol and 1.7 g/l acetate. If Clostridium thermosaccharolyticum HG-8 is used to consume 12 g/l pentoses in a sequential monoculture, then 5.8 g/l ethanol and 1.0 g/l acetate are accumulated. If one sums these two separate fermentations, the net accumulation is 10.0 g/l ethanol and 2.7 g/l acetate. Therefore, one observes in a sequential fermentation the production of ethanol and acetate at a ratio of about 4 to 1. These results demonstrate that no intrinsic limitation exists to the production of ethanol in high yield by Clostridium thermosaccharolyticum HG-8 from the solubilized carbohydrates of corn stover. This behavior is in marked contrast to the fermentation previously shown for simultaneous mixed culture which is also shown in Table 31. The average ratio of ethanol and acetate produced under these conditions is lower and at a value of 2.0:1. This is about one half the value attained by sequential monoculture fermentations. The causes for this reduction in the ethanol to acetic acid ratio are examined in greater detail in the following section.

Table 31

SUMMARY OF ETHANOL AND ACETATE PRODUCTION BY CLOSTRIDIUM THERMOCELLUM S7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 GROWN ON PRETREATED CORN STOVER (45 G/L) IN SEQUENTIAL MONOCULTURE* AND SIMULTANEOUS MIXED CULTURE**

	Ethanol (g/l)	Acetate (g/l)	E/A
Sequential Mixed Culture *			
(1) t=0 hrs C. thermocellum S7-19	4.2	1.5	2.8
(2) t=120 hrs C. thermosaccharolyticum HG-8	5.8	1.0	5.8
Total Production from Sequential Monoculture	10.0	2.5	4.0
Simultaneous Mixed Culture**	9.1	4.7	2.0

* Average of 2 fermentations of 45.5 g/l pretreated corn stover.

** Average of 3 mixed culture fermentations run in fed batch fashion.

4.8. Correlation of Ethanol Production and Substrate Consumption Rate in Mixed and Monoculture

From the previous studies in Section 4.7, it was clearly shown that there was no intrinsic limitation for achieving high ethanol yields from hydrolyzed corn stover carbohydrates by *Clostridium thermosaccharolyticum* HG-8. By the same token, *Clostridium thermocellum* S-7-19 was shown to produce high ethanol yields when grown on cellobiose but exhibited reduced E/A ratios when grown on more slowly metabolized substrates such as treated and non-treated corn stover. In order to quantitate these effects in greater detail, the following analysis was performed.

4.8.1. Effect of Growth Rate on Ethanol Yield

One recurring characteristic of high ethanol yield fermentations with both *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* has been shown to be high specific growth rates. From a large collection of data from batch fermentations for *Clostridium thermocellum* strains S-7-19, S-7 and S-4, it can be shown that all isolates were capable of achieving E/A ratios ranging from 5/1 to 8/1 on cellobiose. A plot of differential ethanol yield versus the instantaneous specific growth rate measured was constructed and shown in Figure 57. These data were collected from a large number of batch growth experiments under conditions where the specific growth

rates of Clostridium thermocellum could be directly calculated through optical density measurement. In the same intervals the changes in ethanol and acetate as measured by gas-liquid chromatography yielded the "instantaneous" E/A ratio shown. In addition to the batch growth data, a number of points from continuous culture experiments conducted by M. Mation were also included [132]. From this data, plotted in Figure 57, we recognize the importance of achieving specific growth rates greater than 0.05 hr^{-1} in order to achieve an E/A ratio above values of 4:1.

A smaller collection of data obtained in a similar fashion from monoculture fermentation of Clostridium thermosaccharolyticum HG-6-62 and HG-8 shows a similar dependence on E/A ratio on specific growth rate as shown in Figure 58. The best Clostridium thermosaccharolyticum strains appear to be able to achieve significantly higher ethanol yields than Clostridium thermocellum S-7-19 (Figure 57).

The alteration of fermentative end products as a function of growth rate is a common phenomenon observed with many microorganisms. A detailed study by DeVries showed a shift between production of lactate, ethanol and acetate as the growth rate of this lactobacillus was shifted in glucose limited continuous culture [133]. In this case, intracellular accumulation of fructose, 1,6 diphosphate at low growth rates was shown to activate the lactate dehydrogenase of this organism.

Figure 57

DIFFERENTIAL ETHANOL/ACETATE RATIO VS. SP. GROWTH RATE OF CLOSTRIDIUM THERMOCELLUM

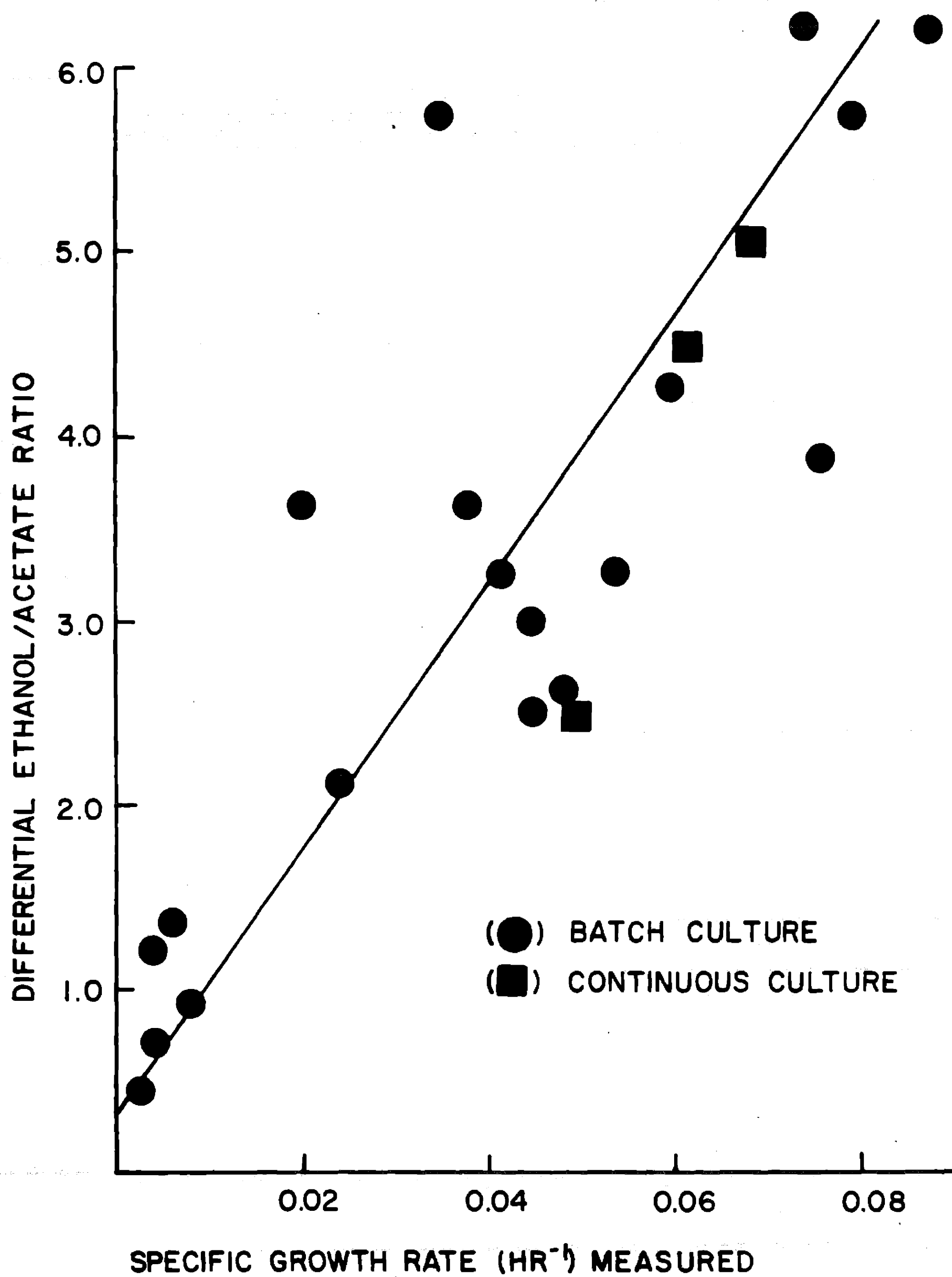
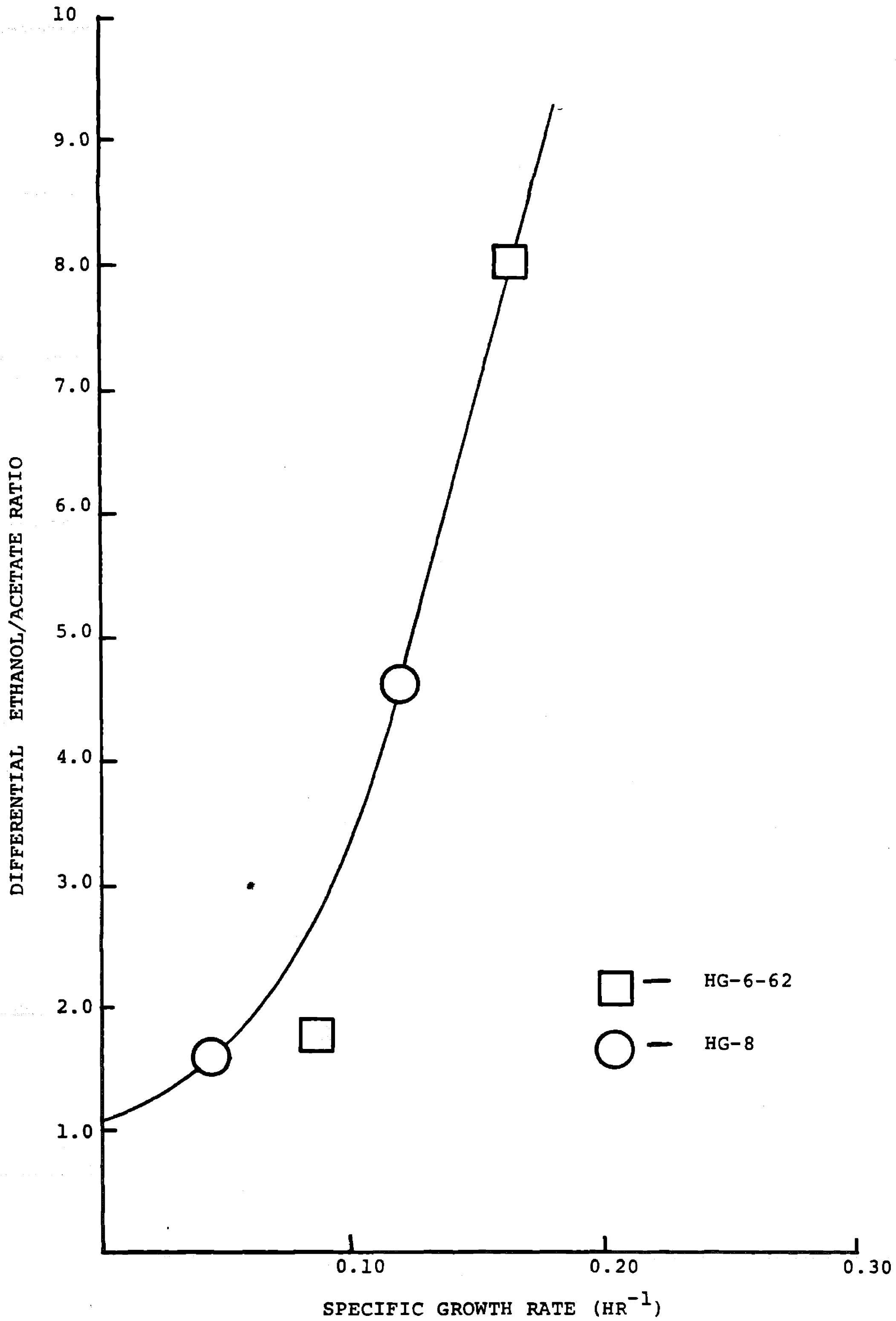


Figure 58

DIFFERENTIAL ETHANOL/ACETATE RATIO VS SP. GROWTH RATE OF CLOSTRIDIUM THERMOSACCHAROLYTICUM



The regulation of ethanol and acetate production in Clostridia is a significantly more complex system than lactate production due to the degree of oxidation/reduction differences in the end products (see Literature Survey). This difference necessitates the evolution of hydrogen as an end product, when acetic acid is produced. The interplay between hydrogenase and reduced and oxidized NAD, NADP, and ferredoxin cofactors has recently been investigated by Zeikus _et al_. [134]. The results of these investigations showed that electron flow and consequently end product production was greatly influenced by the activity and regulation of pyridine nucleotide cofactor oxidoreductases. However, no correlation of any of these activities with growth rate was demonstrated. Experiments at General Electric Labs have also shown a strong influence of H_2 partial pressure on the activity of hydrogenase and consequently acetate production with wild type strain of _Clostridium thermocellum_ [135]. These investigators proposed that a growth rate control of ethanol yield was possible due to the diffusivity of H_2, i.e., at slower growth rates the local H_2 concentration around the cell was lower than at higher growth rates where H_2 mass transfer limitations increased the local H_2 levels and resulted in higher ethanol yield. Unfortunately, preliminary experiments to test this mechanism with high ethanol yielding strains of both _Clostridium thermocellum_ S-7-19 and _Clostridium thermosaccharolyticum_ HG-8 in mixed culture have shown little or

no effect of H_2 up to 1 ATM during growth on corn stover or Solka floc. The results from these latter studies are shown in Table 32.

Alternatively, studies by Thauer *et al*. on energy conservation in chemosynthetic anaerobes provides a different explanation for the effect of growth rate on product ratios. At low growth rates, a greater portion of total ATP production is required to meet the maintenance as opposed to the direct requirements of the cell for net growth.

One way in which the selectively higher maintenance energy requirement at low growth rates can be met is through the production of higher ratios of acetic acid. This is due to the substrate level yield of ATP per mole of acetate which is twice that for ethanol by the pathways postulated for this organism (see Literature Survey).

During strain selection of the organism for higher ethanol yield, a number of mutations may have occurred which could effect any one or more of these parameters. In addition to these possibilities, a phenotypic response of these organisms to adaptation to higher ethanol tolerance has also been required in order to achieve the production of higher ethanol yields than those attainable by the present wild type strains. Nevertheless, in spite of this intrinsic capability for high ethanol yield demonstrated at high growth rates, a decreased ethanol yield appears to be strongly correlated with

Table 32

EFFECT OF FERMENTATION ATMOSPHERE ON PRODUCTION OF ETHANOL AND ACETATE BY MIXED CULTURE OF *CLOSTRIDIUM THERMOCELLUM* S7-19 AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM* HG-6-62 ON *SOLKA FLOC* AND PRETREATED CORN STOVER

Atmosphere	Substrate	Ethanol/Acetate Ratio
N_2	Solka Floc	4.4
H_2	Solka Floc	4.8
N_2	Pretreated Corn Stover	2.3
H_2	Pretreated Corn Stover	2.6

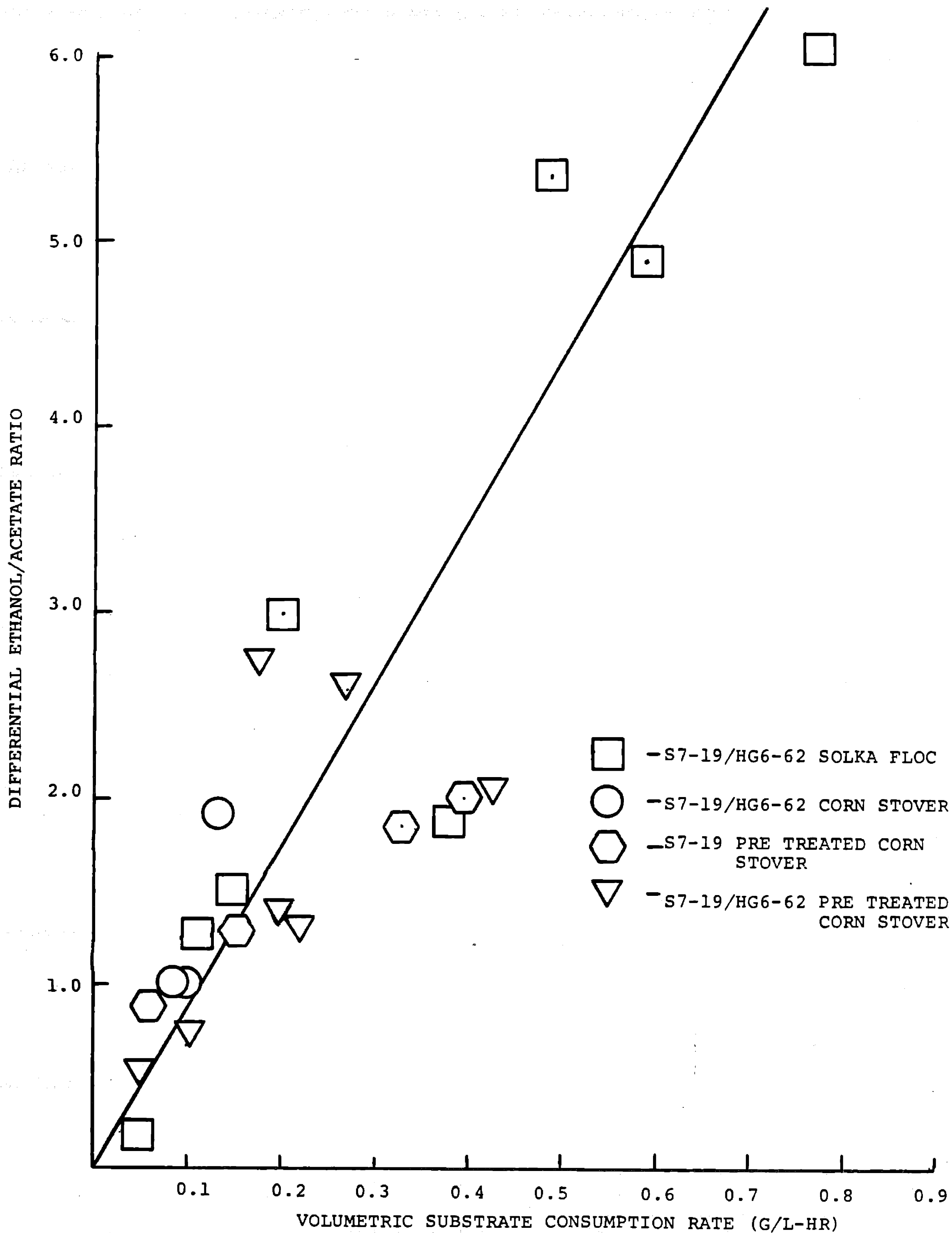
reduced specific growth rate. Whether altered cofactor oxido-reductase regulation, hydrogenase regulation, or ATP maintenance requirements are ultimately responsible for this shift on a mechanistic basis cannot be concluded from these experiments alone.

The relationship between substrate hydrolysis rate, specific growth rate and subsequently the ethanol yield ultimately observed may explain the performance obtained by mixed culture on the insoluble substrates tested. The differential ethanol yield (E/A ratio) observed for mixed and monoculture growth of these intrinsically high ethanol yielding strains is plotted versus the observed volumetric rate of substrate hydrolysis in Figure 59. Although volumetric substrate degradation rate is only an indirect measure of growth rate, a strong positive correlation is observed between the substrate consumption rate and differential E/A ratio which further supports the previously stated hypothesis. However, in order to directly verify this hypothesis, a method for directly evaluating the specific growth rate of each organism growing on insoluble substrate will be required. An indirect approach was taken to assess the specific growth rate on insoluble substrates and is presented in the following paragraphs.

During growth of mixed culture on insoluble biomass such as corn stover, the specific growth rate of each organism is difficult to measure by optical density due to the

Figure 59

DIFFERENTIAL ETHANOL/ACETATE RATIO VS. VOLUMETRIC SUBSTRATE CONSUMPTION RATE



presence of large quantities of solids. Although cell mass cannot be directly measured by optical density, it is possible to indirectly calculate the specific growth rate by an examination of the hydrolysis products of Clostridium thermocellum during growth on insoluble substrates in monoculture. Under these conditions, pentose carbohydrates accumulate but virtually no free residual glucose or cellobiose has been detected (Figure 43). The amount of hexose consumed at any given time in the fermentation could then be calculated from the total soluble products, ethanol, acetate, and lactate produced through the use of the appropriate yield from glucose based on the theoretical pathways as shown previously (Section 4.7).

From a number of batch growth experiments of Clostridium thermocellum S-7-19 and S-4 on cellobiose, data for specific hexose uptake rates and specific growth rates were obtained. From this data, a double reciprocal plot of cell yield on cellobiose versus instantaneous specific growth rate was plotted as shown in Figure 60.

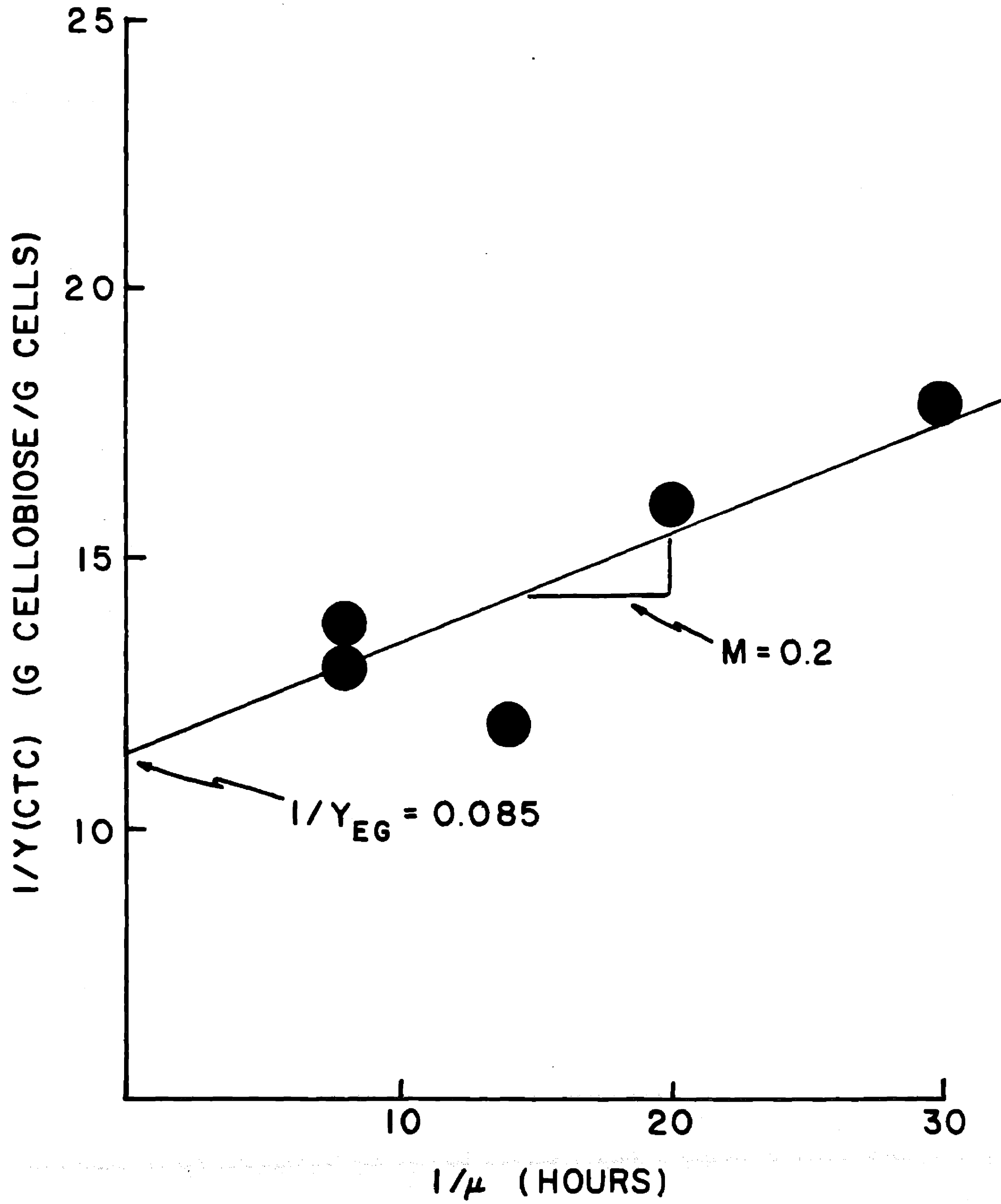
i.e.,

$$\frac{\frac{1}{X}\frac{dH}{dt}}{\frac{1}{X}\frac{dX}{dt}} = \frac{dH}{dX} = m/\mu + \frac{1}{Y_{EG}} \qquad (1)$$

The intercept ($1/Y_{EG}$) represents a maximum cell yield of 0.087 g cells/g substrate consumed and the slope represents a value for the maintenance coefficient (m) of 0.2 g cellobiose/g cell

Figure 60

YIELD OF CLOSTRIDIUM THERMOCELLUM FROM CELLOBIOSE AS A FUNCTION OF SPECIFIC GROWTH RATE



hr. From the rate of hexose consumption predicted by product data, and the cell yield on hexose predicted by the results of Figure 60, an estimate of the cell mass production during fermentation may be calculated.

This is accomplished by the simultaneous solution of the equations for specific growth rate predicted by substrate uptake rate and the equation for growth rate predicted by material balance on cells.

i.e.,
$$\frac{dH}{dt} = [\mu/Y_{EG} + m] \quad X \tag{2}$$

may be written in term of μ:

$$\mu = [\frac{1}{X}\frac{dH}{dt} - m] \quad Y_{EG} \tag{3}$$

which is solved simultaneously with

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{4}$$

and the appropriate initial conditions for X. A computer program was written to speed this data analysis as shown in Appendix A.

Using the calculated specific growth rates and observed differential ethanol yield at each growth rate, a plot relating these parameters was constructed. As we see in

Figure 61, the calculated values correlate well with the observed values previously obtained for *Clostridium thermocellum* growth on soluble sugars (Figure 57). Thus, we conclude that the major factor to attain high ethanol yields with intrinsically high ethanol yielding strains may simply be the growth rate of the organism on insoluble substrate.

4.8.2. Substrate Hydrolysis Kinetics

The extracellular proteins produced by *Clostridium thermocellum* represent the catalyst for the solubilization of the cellulose and hemicellulose polymers. The rate of the enzymatic hydrolysis in turn limits the growth of these organisms. These proteins are believed to be produced in a fully induced manner on CM-4 medium. This assumption is supported by the correlation of extracellular proteins versus dry cell weight obtained from a number of fermentations on soluble carbon sources such as glucose or cellobiose (Figure 62).

From this correlation, a yield of 0.24 mg extracellular protein per mg dry cell weight is obtained. Using this correlation and the calculated cell mass of *Clostridium thermocellum* present, a means to estimate the enzyme content at any time in the fermentation becomes available. From the enzyme production along with the observed volumetric hexose and pentose degradation rate one is able to construct a profile of specific pentose and hexose solubilization through the course

Figure 61

DIFFERENTIAL ETHANOL/ACETATE RATIO VS. SP. GROWTH RATE OF CLOSTRIDIUM THERMOCELLUM CALCULATED

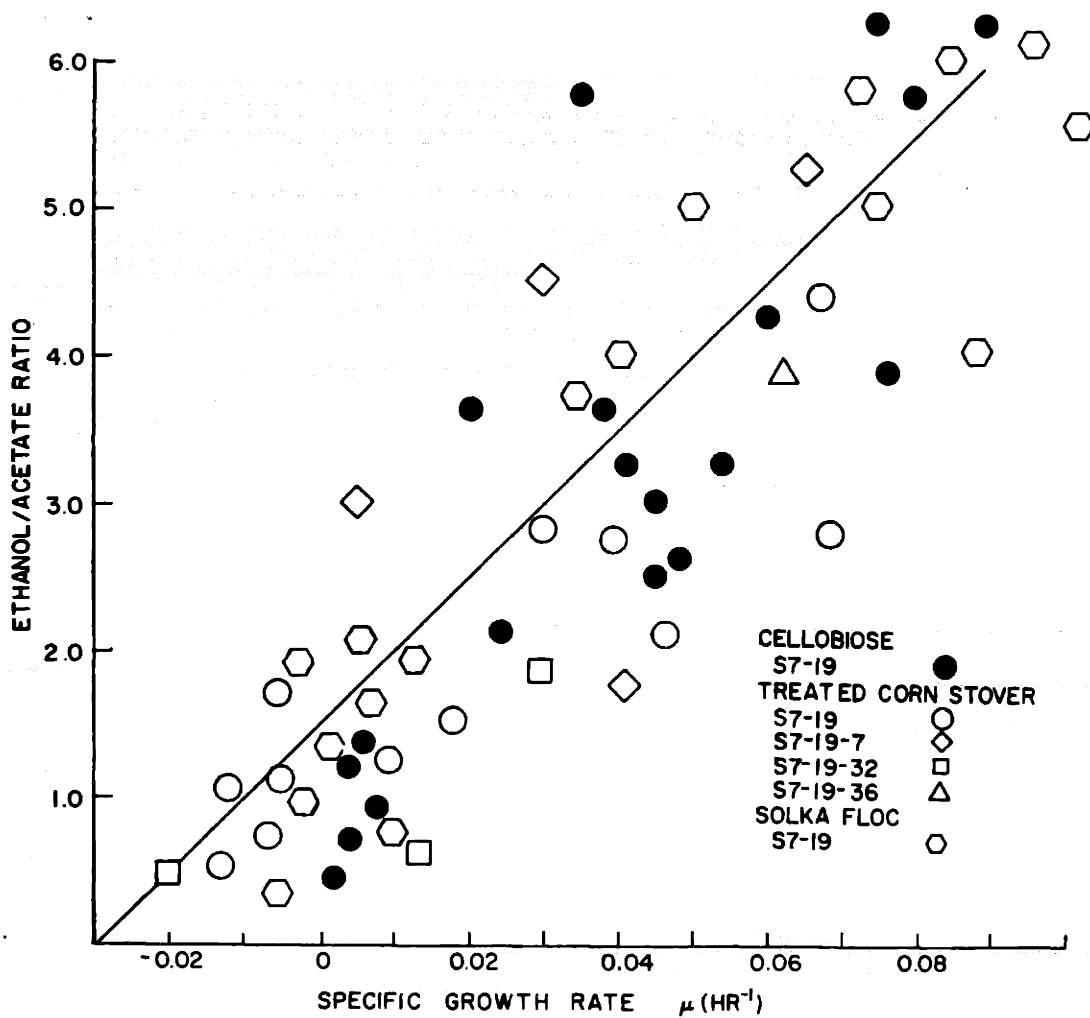
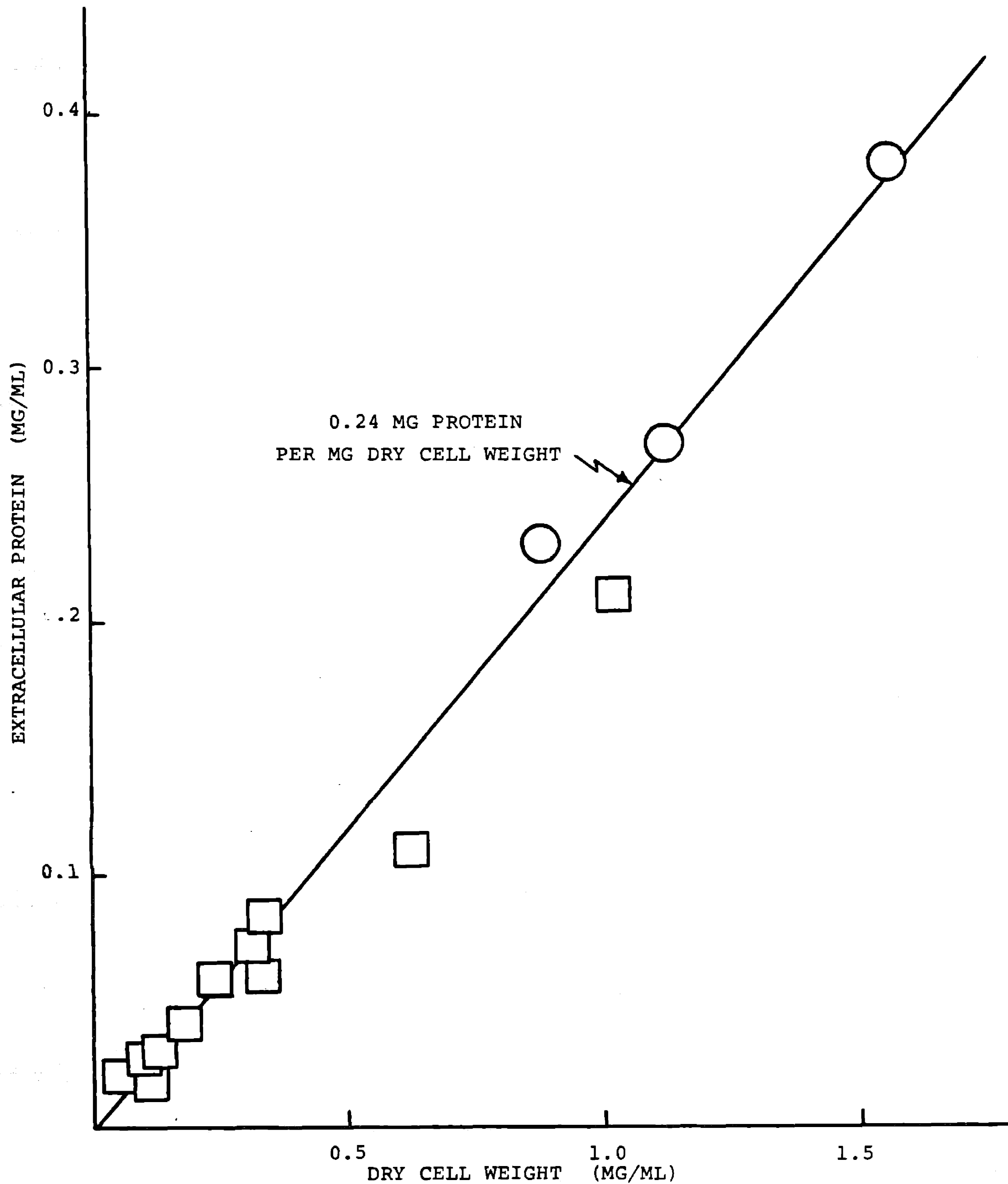


Figure 62

PRODUCTION OF EXTRACELLULAR PROTEIN BY CLOSTRIDIUM THERMOCELLUM ON GLUCOSE AND CELLOBIOSE



of the insoluble substrate fermentation. This has been done for *Clostridium thermocellum* S-7-19 on pretreated corn stover as shown in Figure 63. Both of these rates reach maximal values early in the fermentation corresponding to 4.0 mg hexose/mg protein per hour and 7.5 mg pentose/mg protein per hour when grown on pretreated corn stover. These values then decrease as the fermentation progresses. This behavior with cellulosic biomass has been commonly observed and can be ascribed to a "shrinking site" model previously described. Plots of the specific rate of cellulose degradation as a function of the extent of degradation for both Solka floc and pretreated corn stover have been constructed. The data from these fermentations were taken when no residual cellobiose is present to cause end product inhibition. The results are summarized for a number of fermentations in Figures 64 and 65.

The initial specific rate of Sola floc utilization is shown to be two times greater than that observed for the solvent treated corn stover. The residual lignin, particle size, and the cellulose structure in the corn stover evidentally results in a more recalcitrant material to cellulolytic degradation. In addition to the decreased initial rate of degradation, the utilization of treated corn stover becomes more difficult than Solka floc with increased extent of degradation. For example, at 50% cellulose utilization, Solka floc can be degraded at a specific rate of 2.5 g/mg hr. The degradation rate of

Figure 63

SPECIFIC CELLULOSE AND HEMICELLULOSE CONSUMPTION IN
CLOSTRIDIUM THERMOCELLUM S7-19 ON CORN STOVER

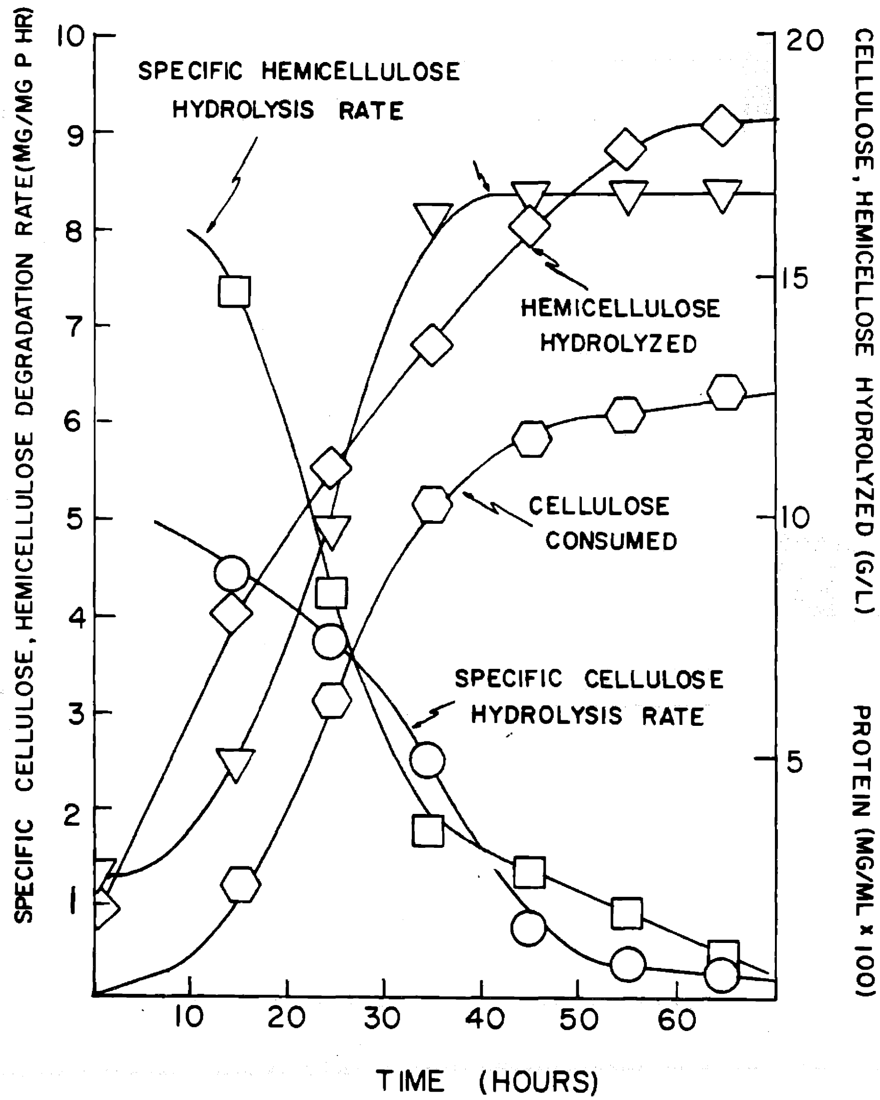


Figure 64

SPECIFIC CELLULOSE HYDROLYSIS OF SOLKA FLOC

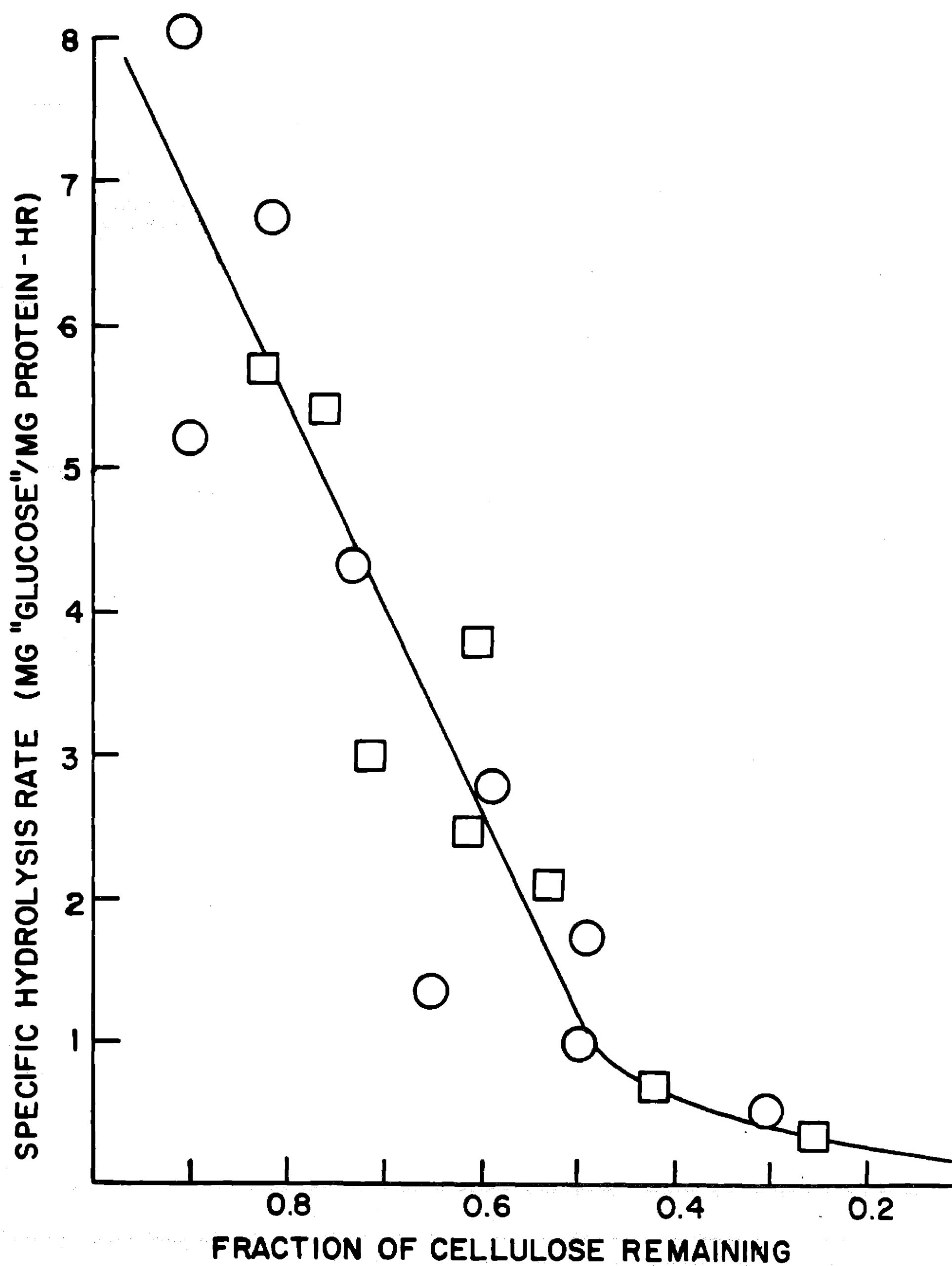
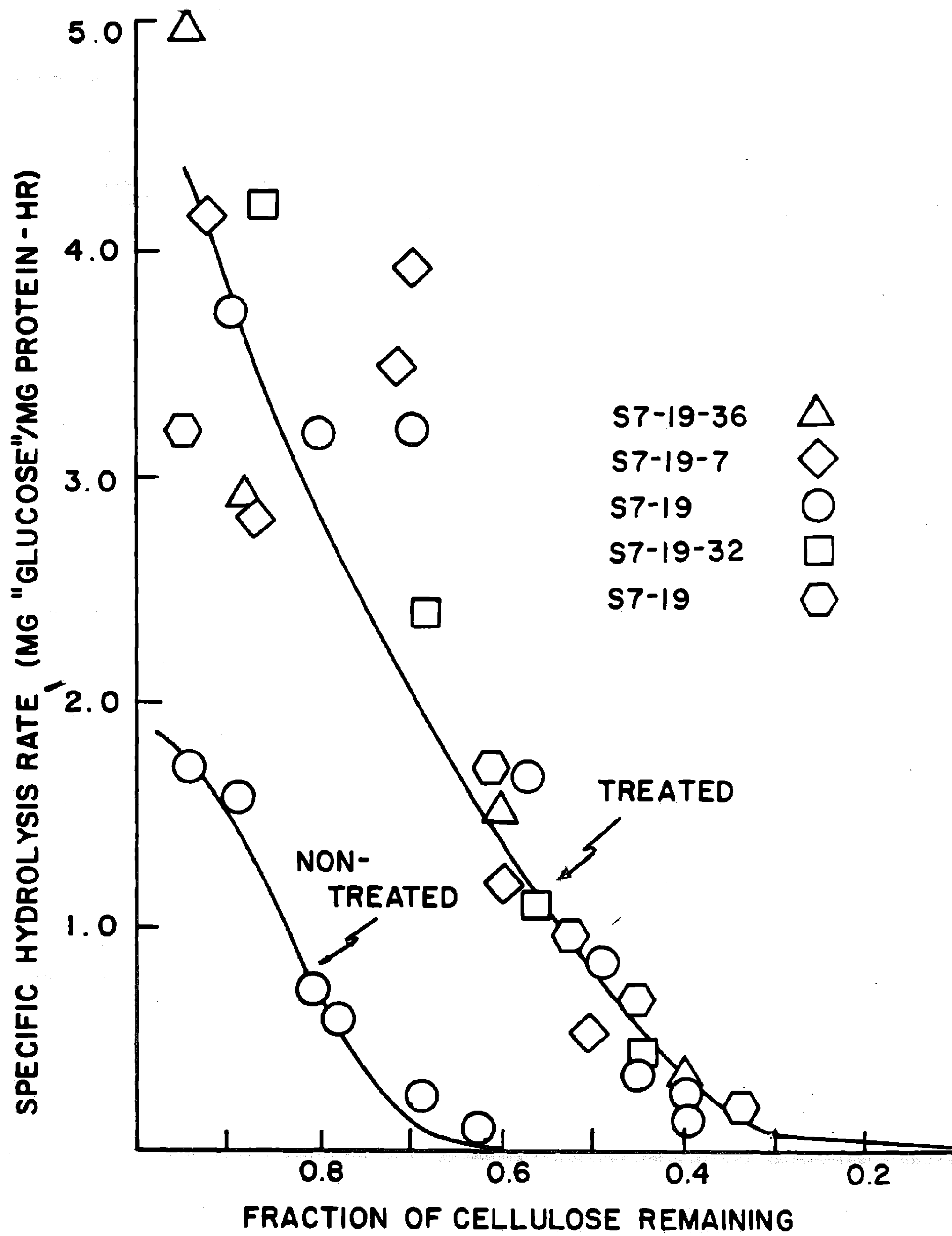


Figure 65

SPECIFIC CELLULOSE HYDROLYSIS OF PRETREATED CORN STOVER



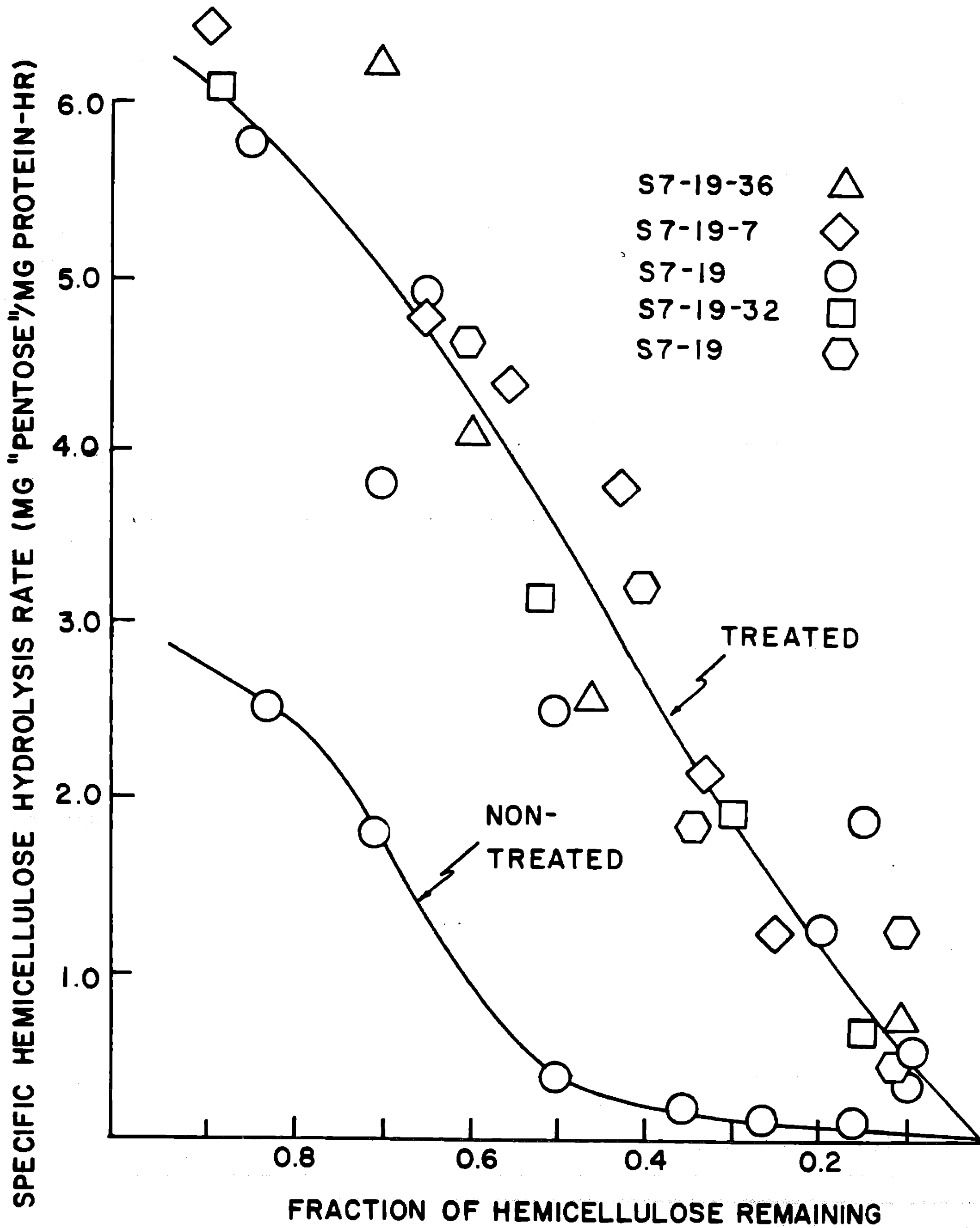
treated corn stover is approximately 5 times lower than Solka floc on a specific basis at a similar extent of utilization.

Evidently, the "core" cellulose material of pretreated corn stover may contain residual lignin which would sterically protect the cellulose and thus render it unaccessible to the cellulase enzymes. On the other hand, the specific rate of hemicellulose hydrolysis shown in Figure 66 is significantly greater than the specific rate of cellulose hydrolysis at all extents of degradation of pretreated corn stover. Whereas the specific cellulose hydrolysis rate drops to less than 90% of its initial value at 60% utilization, the specific rate of hemicellulose hydrolysis decreases in nearly a linear fashion to virtually complete utilization. Although a further detailed analysis of these reaction kinetics is beyond the scope of this discussion, an excellent mechanistic analysis of insoluble enzymatic substrate hydrolysis has been made by Fan *et al*. [136].

A similar analysis of the specific rates of cellulose and hemicellulose utilization are also shown in Figures 65 and 66 for untreated corn stover. In each case, the initial activities are approximately one-half of those compared to treated material. In addition, the rates are seen to decrease more rapidly with increasing extent of conversion. This result is not surprising and can be directly attributed to the increased content of lignin in the untreated material previously demonstrated.

Figure 66

SPECIFIC HEMICELLULOSE HYDROLYSIS RATE
OF PRETREATED CORN STOVER



From the results on the specific rate of degradation versus the extent of degradation, one can present a number of predictions with respect to achieving high ethanol to acetic acid ratio fermentations. Since it has been shown that the E/A ratio is directly related to the specific growth rate, hyper-cellulase mutants must be isolated. These mutants should produce higher levels of cellulase which in turn would reflect in a higher specific growth rate for Clostridium thermocellum. However, if the hyper-cellulase mutants do not exhibit any changes in the make-up of the different cellulases, one can state the following behavior to result. In batch culture, one would anticipate a high E/A ratio when the extent of cellulose degradation is low. As the fraction of cellulose utilized increases, a decrease in the growth rate will result. This will be accompanied by a decreasing E/A ratio due to the lower specific growth rate. In this case, there is a trade-off between the desired E/A and the degree of utilization of the corn stover. Ideally, one would like to isolate mutants which have higher enzymatic activities on the cellulose "core" when significant fraction of the cellulose has already been utilized.

4.8.3. Mechanistic Kinetic Model of Mixed and Mono Culture Growth on Insoluble Biomass

In the previous chapters, correlations were obtained on the effect of growth rate to the yield of ethanol observed in high ethanol yielding strains of Clostridium ther-

mocellum and *Clostridium thermosaccharolyticum*. In addition, calculated data for cellulase enzyme production (total extracellular protein) was correlated with volumetric substrate degradation rates. This provided the necessary information on the intrinsic substrate hydrolysis kinetics. These correlations can be combined into a simple kinetic model for mixed and monoculture growth on insoluble multi-substrate biomass to provide significant fundamental conclusions.

During monoculture growth of *Clostridium thermocellum* (CTC) on soluble carbohydrates, we have shown that extracellular protein production (E) can be expressed as

$$\frac{dE}{dC} = k_E \left(\frac{d\ CTC}{dt}\right) \quad \text{or} \quad E = k_E\ CTC \tag{5}$$

where k_e is 0.24 mg protein/mg dry cell weight. The specific activity of these proteins on Solka floc, and treated or non-treated corn stover was found to be proportional to the extent of degradation of each substrate. The maximum specific activity and the proportionality constant for each substrate has been shown to be characteristically different. Thus, for soluble hexose production, (H) can be expressed as:

$$\frac{dH}{dt} = K_H\ E \tag{6}$$

and for pentose production, (P) can be expressed as:

$$\frac{dP}{dt} = K_P E \qquad (7)$$

(where K_H and K_P are functions of the substrate type and extent of degradation). Typical maximum specific activities for K_H and K_P were 4.0 and 5.0, respectively, for treated corn stover. A maximum specific activity for K_H of 8.0 for Solka floc was found (Figures 64, 65 and 66). In this simplified treatment, no loss of enzyme activity due to instability, or lignin and cellulose binding has been included.

The volumetric activities of these enzymes on the respective substrates (dH/dt or dP/dt) have been expressed as the specific activity (K_H or K_P) times the enzyme concentration (E). While this relationship is expected to apply for low enzyme concentrations (E), it should be remembered that saturation kinetics were observed with increasing enzyme loading even at low extents of substrate degradation. For example, enzyme concentrations between 1.5 to 2.0 mg/ml protein exhibited saturation kinetics with non-treated corn stover (Figure 24). Thus if enzyme loadings below these values are used, a linear relationship between the degradation rate to the protein loading for low extents of degradation can be expected.

The rate of growth of _Clostridium thermocellum_ (d CTC/dt) on the hexose carbohydrate hydrolyzed can be

expressed as:

$$\frac{d\ CTC}{dt} = [\frac{dH}{dt} - m\ (CTC)]\ Y_{CTC/H} \quad (8)$$

This expression is valid when the hydrolysis rate limits the rate of growth on corn stover. In this expression, a value for the maintenance coefficient (m) of approximately 0.2 g glucose/g cell hr has been found from a data analysis of a number of batch fermentations of cellobiose as previously described. The yield coefficient ($Y_{CTC/H}$) of Clostridium thermocellum cell mass (gram of cell) per gram of hexose has also been reported previously and is approximately 0.1 gm cell/gm hexose. Re-writing this expression in terms of specific growth rate (μ_{CTC}) and substituting the expressions for dH/dt and E yields:

$$\mu_{CTC} = [K_H\ K_E - m]\ Y_{CTC/H} \quad (9)$$

Thus we see that the specific growth rate, which correlates with ethanol yield in high ethanol yielding strains is dependent only on the constants K_E and m and the specific "cellulase" activity K_H which has been shown to vary with substrate type and its extent of degradation. Substituting typical values for $Y_{CTC/H}$ and K_E into equation (9) we obtain an expression for the specific

growth rate of Clostridium thermocellum as a function of the intrinsic specific rate of degradation of hexose (K_H):

$$\mu_{CTC} = (0.24\ K_H - 0.2)\ (0.1) \qquad (10)$$

From the initial rate data, the value for K_H was 8.0 as observed in Figure 64 for Solka floc. Substituting this value for K_H in equation (10), a maximum specific growth rate of 0.17 hr^{-1} is calculated. For the initial phase of Solka floc degradation, this value is theoretically independent of the inoculum size as long as the ratio of enzyme to cells remain constant and the substrate is not saturated with respect to enzyme loading. This analysis can be performed for both treated and non-treated corn stover. Using the specific hydrolysis data (K_H) as shown in Figure 65 for the substrates, initial maximum specific growth rates in monoculture for Clostridium thermocellum of 0.07 hr^{-1} and 0.04 hr^{-1}, respectively, for treated and non-treated corn stover can be theoretically attained. However, in each case the specific growth rate attainable will decrease due to the decrease in K_H resulting from the increased extent of substrate conversion. In fact, below a value of 0.83 for K_H, a negative growth rate is obtained. At this point, an insufficient amount of substrate is hydrolyzed to meet the maintenance demands of the cells.

In order to quantitate the effects of different fermentation conditions on ethanol yield, computer simulations can be performed. In this manner, the influence of the substrate degradation rate, which controls the growth rate, can be combined with the correlation relating the growth rate to ethanol to acetate ratio by Clostridium thermocellum S-7-19 (Figure 61).

Numerical integration of these relationships will yield the ethanol and acetate production expected during batch growth of Clostridium thermocellum S-7-19 on pretreated corn stover. This type of simulation can be used to test the various assumptions incorporated and aid in delineating potential improvements.

Shown in Table 33 are the results from the simulation of growth of Clostridium thermocellum S-7-19 on 45 g/l pretreated corn stover. The initial conditions were 0.1 g/l cells as inoculum and proportional enzyme levels associated with the inoculum. The values of MCTC represent the instantaneous specific growth rate achieved during the fermentation. An initial specific growth rate of 0.076 hr^{-1} was calculated and this value decreases according to the model. A maximum production of 0.7 g/l cells of Clostridium thermocellum (CTC) is achieved accompanied by 12.6 g/l of hexose (HEX) from cellulose utilized. In 100 hrs of fermentation, virtually 100% of the pentose is solubilized. The level of extracellular protein (ENZ)

Table 33

SIMULATION OF MONOCULTURE FERMENTATION OF *CLOSTRIDIUM THERMOCELLUM* S-7-19 ON 45 G/L PRETREATED CORN STOVER

MIXED CULTURE SIMULATION STUDY

HEXOSE= 21 PENTOSE= 19 CTC= .1 CTS= 0 KH= 4
KP= 5 KE= .24
ENZYME 0= .024

TIME	CTC	MCTC	CTS	MCTS	ENZ	HEX	PEN	ETOH	ACET
***	0.1	0.076	0.0	0.000	0.02	21.0	19.0	*****	*****
10	0.2	0.066	0.0	0.000	0.05	19.6	17.3	0.5	*****
20	0.4	0.049	0.0	0.000	0.09	17.5	14.4	1.4	0.3
30	0.5	0.029	0.0	0.000	0.13	14.8	10.8	2.4	0.6
40	0.7	0.011	0.0	0.000	0.16	12.4	7.4	3.2	0.9
50	0.7	-.002	0.0	0.000	0.16	10.8	4.8	3.7	1.2
60	0.6	-.009	0.0	0.000	0.16	9.8	3.1	3.9	1.5
70	0.6	-.012	0.0	0.000	0.16	9.2	2.0	4.0	1.7
80	0.5	-.015	0.0	0.000	0.16	8.9	1.3	4.1	1.8
90	0.4	-.016	0.0	0.000	0.16	8.7	0.8	4.1	1.9
100	0.4	-.018	0.0	0.000	0.16	8.6	0.5	4.1	2.0
110	0.3	-.018	0.0	0.000	0.16	8.5	0.4	4.1	2.0
120	0.3	-.019	0.0	0.000	0.16	8.5	0.2	4.1	2.1
130	0.2	-.019	0.0	0.000	0.16	8.4	0.1	4.1	2.1
140	0.2	-.019	0.0	0.000	0.16	8.4	0.0	4.1	2.1
150	0.1	-.020	0.0	0.000	0.16	8.4	0.0	4.1	2.1
160	0.1	-.020	0.0	0.000	0.16	8.4	0.0	4.1	2.1
170	****	-.020	0.0	0.000	0.16	8.4	0.0	4.1	2.1
180	****	-.020	0.0	0.000	0.16	8.4	0.0	4.1	2.1
190	****	-.020	0.0	0.000	0.16	8.4	0.0	4.1	2.1
200	****	-.020	0.0	0.000	0.16	8.4	0.0	4.1	2.1

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increases from 0.024 up to a maximum of 0.16 mg/ml. These values are within the linear specific activity region previously reported. The consumption of hexose by the organism results in the total production of 4.1 g/l ethanol and 1.8 g/l acetate in 80 hours. These products represent an E/A rate of 2.3 to 1 which is in excellent agreement with that previously reported. A graphic representation of this simulation for ethanol, acetate, and reducing sugar production from hemicellulose is shown by the solid lines in Figure 67. The data points shown on this plot were obtained from 2 monoculture fermentations of _Clostridium thermocellum_ S-7-19 on 45 g/l treated corn stover. Good agreement between predicted and observed results are obtained for fermentation times below 80 hrs.

The predicted production of cell mass of _Clostridium thermocellum_ by this model was shown in Table 33 to peak at 0.7 h/l cells at 50 hours of fermentation. At longer fermentation times, a negative growth rate and decrease in cell mass of _Clostridium thermocellum_ is predicted. This is due to the maintenance energy demands of these cells and the decreased specific hydrolysis rate.

Additional studies on the viable counts of _Clostridium thermocellum_ were made during the course of monoculture fermentations of the treated corn stover. These results are presented at this time to see whether the maintenance concept is valid. As shown in Figure 68, an increase in viable

Figure 67

SIMULATED AND OBSERVED DATA FOR ETHANOL, ACETATE AND REDUCING SUGAR PRODUCTION IN MONOCULTURE FERMENTATION OF PRETREATED CORN STOVER (45 G/L) ON *CLOSTRIDIUM THERMOCELLUM* S-7-19

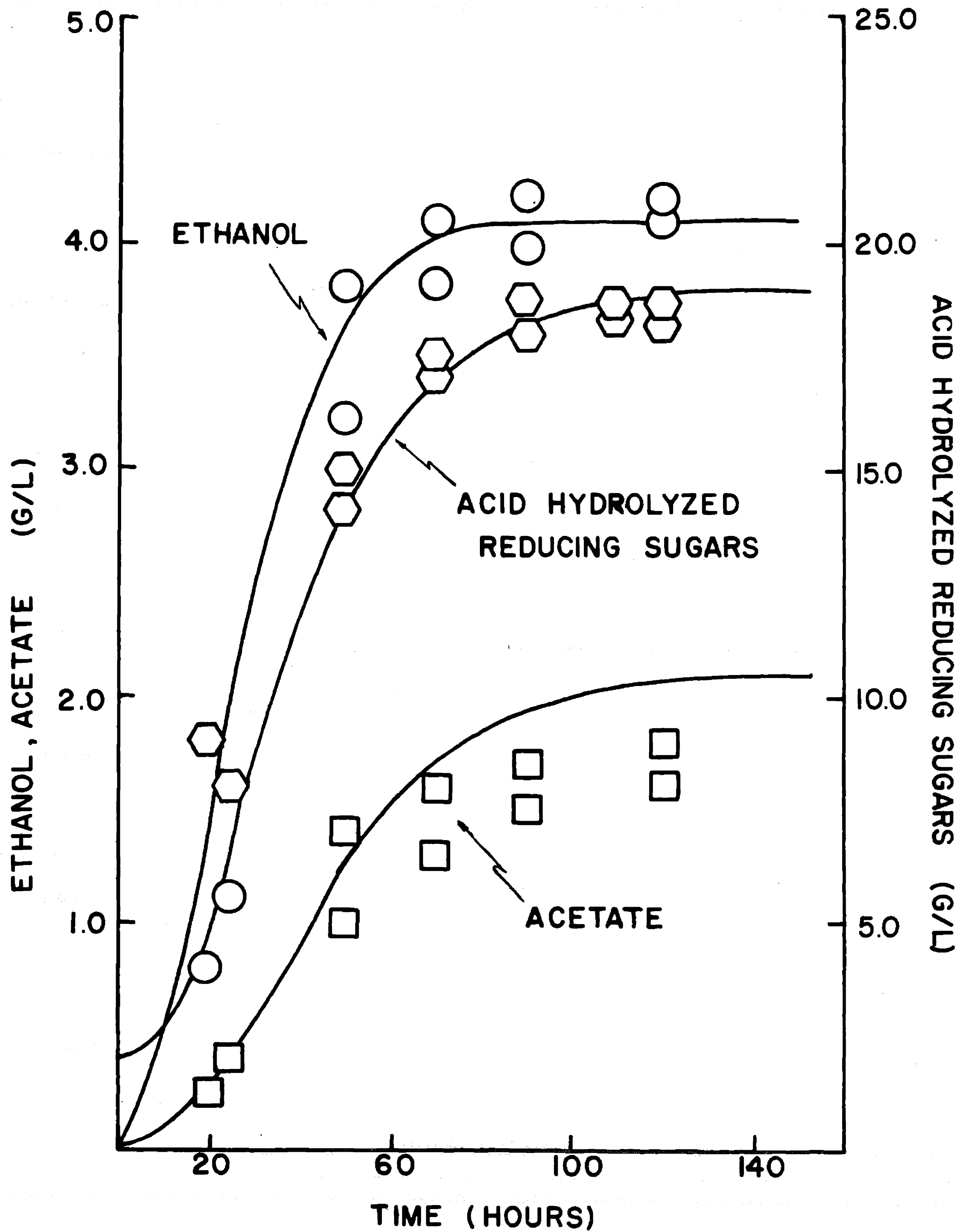
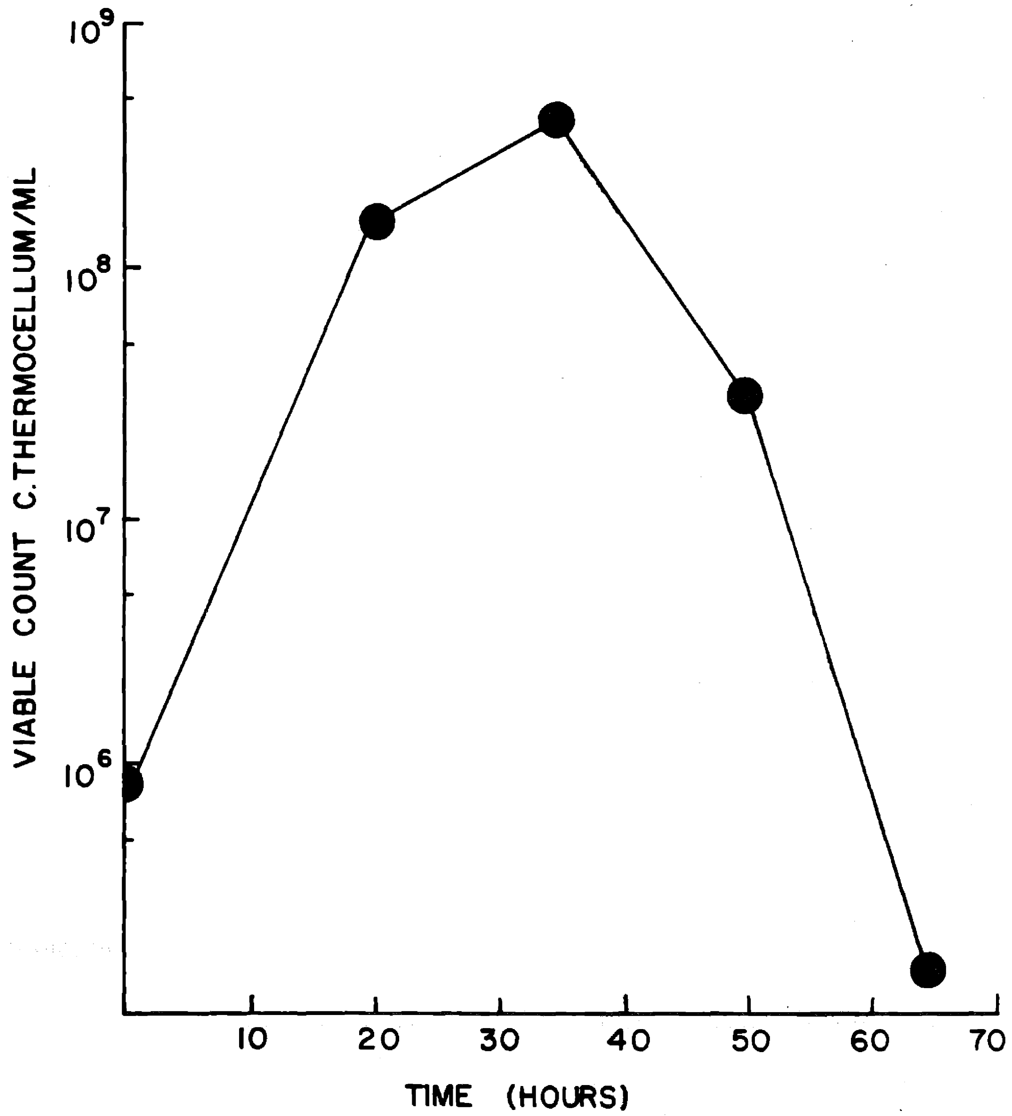


Figure 68

VIABLE COUNT OF CLOSTRIDIUM THERMOCELLUM S-7-19 DURING MONOCULTURE FERMENTATION OF PRETREATED CORN STOVER



count up to a maximum of 4×10^8 cells/ml at 37 hours of fermentation is observed. If we assume cell dimensions of 2×1.5 μ reported for this organism and the cells contain average dry weight of 20%, a maximum of 0.28 g/l cell mass is expected at 40 hours of fermentation.

The calculated cell mass is in reasonably good agreement with that predicted. The viable count in Figure 68 is also shown to decrease rapidly after 40 hours of monoculture fermentation. This behavior of *Clostridium thermocellum* is also predicted by the model which might indicate that the concept of decreased hydrolysis rate insufficient to satisfy the minimum maintenance energy requirement of these cells after approximately 45 hours could very well be correct. On the other hand, from the cell mass data in Table 33, the model predicts tht only a slight decrease of 28% in viable cell mass is required in order to balance maintenance requirements with available substrate between 50 and 80 hours of fermentation. However, from the viable count data in Figure 68, we observe that a greater than 3 order of magnitude decrease of viable cells of *Clostridium thermocellum* occurs. It is possible that the stress imposed on these cells due to energy starvation results in decreased plating efficiency in this period. It should also be pointed out that the recent evidence of an "autolytic" activity produced under late log phase conditions may explain this behavior equally well. This behavior will be described later in greater detail in Section

4.10. Nevertheless, the general agreement of ethanol, acetate, and cell growth kinetics predicted with those observed further supports the assumptions of this model. These facts reinforce the correlation for growth rate control of ethanol yield in intrinsically high ethanol yield strains.

4.8.4. Mechanistic Kinetic Model for Mixed Culture Growth on Insoluble Biomass

Although simulation of substrate hydrolysis and ethanol yield during monoculture fermentations were readily accomplished, mixed culture growth on mixed hexose and pentose sugars from corn stover represents a more complex situation. This is primarily due to competition between _Clostridium thermocellum_ and _Clostridium thermosaccharolyticum_ for the hexose sugars from corn stover. The growth rates of _Clostridium thermocellum_ (CTC) at low concentrations of hexose can be modelled by the classical Monod model:

$$\frac{d\,CTC}{dt} = \mu_{CTC}\,CTC = \frac{H\,\mu_m}{(K_{S_H} + H)}\,(CTC) \qquad (11)$$

where μ_{CTC} = growth rate of _Clostridium thermocellum_

CTC = cell mass of _Clostridium thermocellum_

H = concentration of limiting hexose species, e.g. cellobiose

μ_m = maximum specific growth rate of *Clostridium thermocellum*

K_S = saturation constant for limiting hexose species

In the case of *Clostridium thermosaccharolyticum*, however, both hexose and pentose carbohydrates can be metabolized. However, it is known at low growth rates for many microorganisms corresponding to low concentration of multiple carbohydrates, simultaneous utilization of the substrates can occur. Continuous culture data of Fang with *Clostridium thermosaccharolyticum* HG-4 has shown tht this organism exhibits little preference for glucose or xylose at growth rates below 0.15 hr^{-1}. On the other hand, glucose was shown to repress xylose uptake at higher dilution rates at increased concentrations during continuous culture [30]. In view of the behavior in continuous culture, we can expect this strain to exhibit little preference with regard to glucose vs. xylose catabolism in hydrolysis rate limited mixed culture on insoluble biomass. Incorporation of this assumption into a mixed substrate Monod equation yields an expression for growth rate on hexose and pentose:

$$\frac{d\,CTS}{dt} = \mu_{CTS}\,CTS = \left(\frac{H}{k_{S_H} + H} + \frac{P}{k_{S_P} + P}\right)(\mu_{max})\,(CTC), \qquad (12)$$

subject to the condition that

$$\left(\frac{H}{k_{S_H} + H} + \frac{P}{k_{S_P} + P}\right) \leq 1.0 \qquad (13)$$

where the values for k_{S_H} and k_{S_P} are assumed to be approximately equal.

where

CTS	=	cell mass of <u>Clostridium</u> <u>thermosaccharolyticum</u>
μ_{CTS}	=	specific growth rate of <u>Clostridium</u> <u>thermocellum</u>
P	=	concentration of growth limiting pentose
μ_{max}	=	maximum specific growth rate of <u>Clostridium</u> <u>thermosaccharolyticum</u>
k_{S_H}	=	saturation constant for hexose
k_{S_P}	=	saturation constant for pentose

Unfortunately, however, the specific hexose uptake rate for each strain or, more specifically, values of k_{S_H} for *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*, for glucose or cellobiose are not known with certainty from the previous continuous culture studies. Under growth rates controlled by cellulose hydrolysis, the relative magnitude of these values will dictate the specific uptake rate of hexose by each strain. Therefore, in order to proceed with this analysis a simplifying assumption for the relative magnitude of these constants must be made. Basically, three simple limiting cases for the relative values of these constants can be intuitively examined. If

the value for the ratio of k_{S_H} for Clostridium thermocellum to the k_{S_H} for Clostridium thermosaccharolyticum, (k_{S_H} (CTC)/k_{S_H} (CTS)) is very large (i.e., >> 1) then we would not expect a coculture to be stable. Under these conditions in a well mixed system, Clostridium thermocellum will not be able to grow due to the higher affinity of Clostridium thermosaccharolyticum for virtually all the hexose hydrolyzed. Under these conditions, little additional cellulase would be produced. Mixed culture fermentations of Solka floc would only occur very slowly. Since we have observed many times that the rate of fermentation of mixed culture on both treated and non-treated corn stover, as well as Solka floc increases after an initial lag phase, it is concluded that the ratios of the k_{S_H}'s cannot be extremely large.

A second case of mixed culture behavior assumes the ratio k_{S_H}(CTC)/k_{S_H}(CTS) to be approximately equal to unity. Under these conditions, the growth of Clostridium thermocellum in mixed culture would be expected to be reduced with respect to that observed in monoculture due to the competition by Clostridium thermosaccharolyticum for the hexose sugars hydrolyzed. Under these conditions (i.e., k_{S_H} (CTC) = k_{S_H} (CTS)), the hexose uptake by each organism will be proportional to the amount of that organism present. Thus:

$$\frac{1}{CTC}\frac{d\,CTC}{dt} = \left[\frac{dH}{dt}\left(\frac{CTC}{CTC + CTS}\right) - M_{CTC}\right] Y_{CTC/H} \qquad (14)$$

and

$$\frac{1}{CTS} \frac{d\,CTS}{dt} = [\frac{dH}{dt} (\frac{CTS}{CTS + CTC}) - M_{CTS}]\ Y_{CTS/H} + \frac{1}{CTS} \frac{dP}{dt} (C_{CTS/P}) \quad (15)$$

This expression can be incorporated into a mixed culture model for growth and production of ethanol and acetate on corn stover.

Finally, a third case can be represented by assuming that the ratio of k_{S_H} (CTC)/k_{S_H} (CTS) is very small (<< 1). Under these conditions, _Clostridium thermocellum_ is capable of rapidly metabolizing all of the available hexose and would prevent _Clostridium thermosaccharolyticum_ from competing for significant quantities of hexose. This case also represents a stable mixed culture condition due to the simultaneous hydrolysis of hemicellulose pentose of the corn stover by the extracellular enzymes of _Clostridium thermocellum_. The pentose sugars can only be metabolized by _Clostridium thermosaccharolyticum_. In summary, carbohydrate consumption under this limiting condition is characterized by complete utilization of hydrolyzed hexose (H) by _Clostridium thermocellum_ and pentose (P) by _Clostridium thermosaccharolyticum_. Thus,

$$\frac{1}{CTC} \frac{d\,CTC}{dt} = [\frac{1}{CTC} \frac{dH}{dt} - M_{CTC}]\ Y_{CTC/H} \quad (16)$$

and

$$\frac{1}{CTS} \frac{d\,CTS}{dt} = [\frac{1}{CTS} \frac{dP}{dt} - M_{CTS}]\ Y_{CTS/P} \quad (17)$$

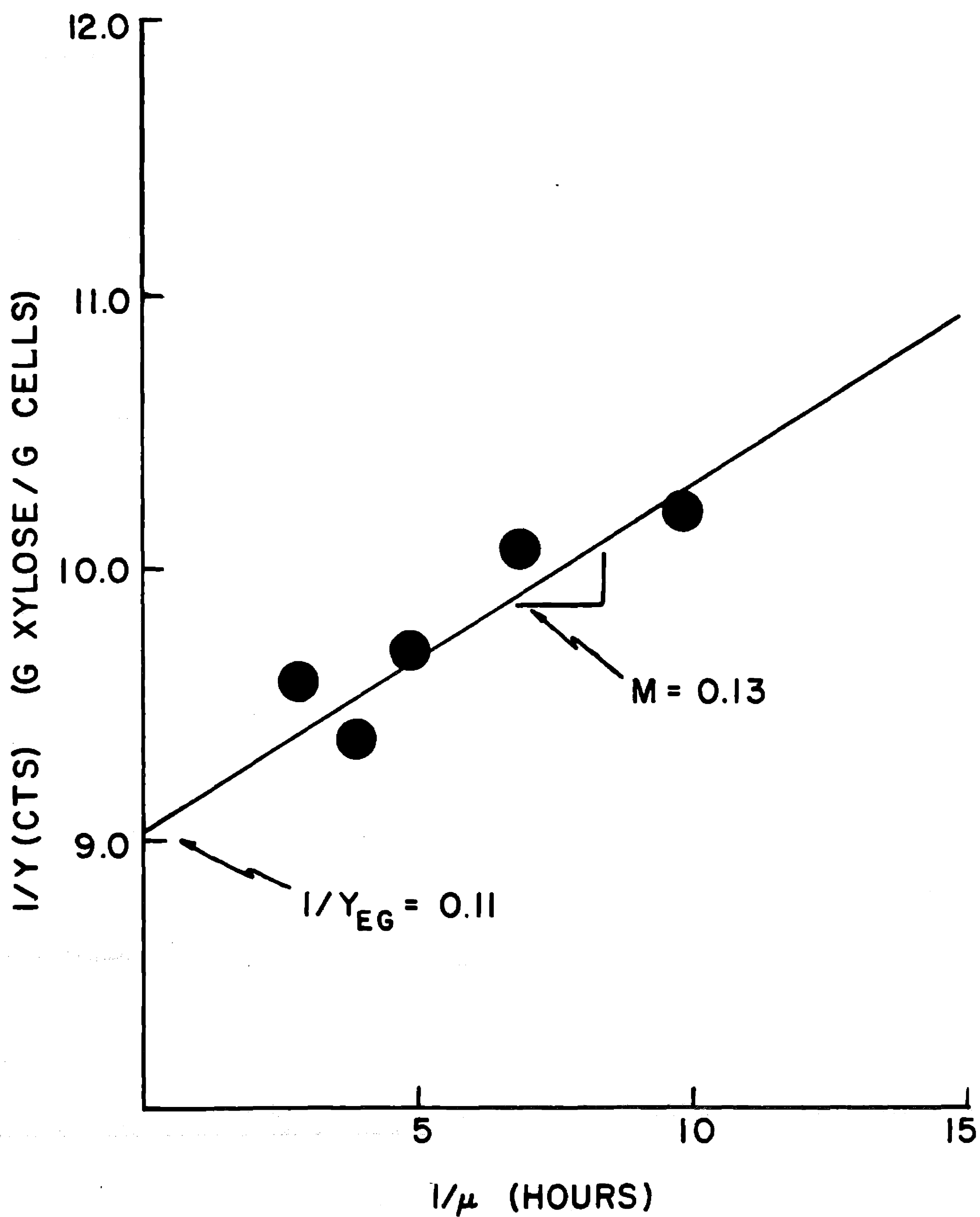
Values for cell yield ($Y_{CTS/P}$) and maintenance coefficient of 0.09 g cells/gm xylose and 0.25 g xylose/gm cell hour, respectively, have been obtained from continuous culture data of <u>Clostridium</u> <u>thermosaccharolyticum</u> HG-4 as replotted in Figure 69 [30].

To simulate mixed culture performance on pretreated corn stover, we can incorporate the carbohydrate uptake assumption discussed into the kinetic model. The ethanol and acetate productivity and yields predicted can be compared with those observed under various experimental conditions in order to verify (1) the assumption for carbohydrate uptake and (2) the applicability of the "growth rate controlling ethanol yield" hypothesis for mixed cultures. Finally, if the model shows a reasonable agreement with the experimental data, sensitivity analysis of the system can be made. This analysis will allow the assessment as to the probable potential for various strain and engineering improvements.

CASE A: Numerical integration of mixed culture performance was initially conducted with the assumption that k_{S_H}'s for <u>Clostridium</u> <u>thermocellum</u> and <u>Clostridium</u> <u>thermosaccharolyticum</u> were equal. With an initial inoculum concentration of 0.1 g/l <u>Clostridium</u> <u>thermocellum</u> with appropriate enzyme and 0.1 g/l <u>Clostridium</u> <u>thermosaccharolyticum</u>, the results shown in Table 34 were obtained. The major feature of this mixed culture performance is the accumulation of only 0.1

Figure 69

CELL YIELD OF CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-4 FROM CONTINUOUS CULTURE DATA



of *Clostridium thermocellum* as opposed to the net growth of 0.7 g/l of *Clostridium thermosaccharolyticum* cells. The model predicts this behavior due to the competition of *Clostridium thermosaccharolyticum* for hexose. Due to the rapid initial hydrolysis of pentose predicted and observed, the cell mass of the pentose utilized increases rapidly, aggravating this competition.

CASE B: On the other hand, incorporation of the second assumption for carbohydrate into the model results in simulation of fermentation performance shown in Table 35. In this case, k_{S_H} for *Clostridium thermocellum* was assumed to be significantly lower than that for *Clostridium thermosaccharolyticum*. This assumption yields an entirely different profile for the growth of *Clostridium thermocellum*. In this case, an increase of 0.7 g/l cells for *Clostridium thermocellum* with a net growth of 0.9 g/l *Clostridium thermosaccharolyticum* were obtained. Under these conditions, a significantly greater production of enzymes to a maximum level of 0.16 mg/ml as opposed to only 0.03 mg/ml obtained in the previous case is observed. This increased level of enzyme allows a significantly greater volumetric rate of total carbohydrate degradation averaging 30.9 g/l in 100 hours (0.31 g/l hr) as opposed to 17.7 g/l in 100 hrs (0.18 g/l hr) in the previous case (A).

Graphical comparisons for the two cases (A & B) and experimental data obtained for a number of mixed culture fermentations of 50 g/l pretreated corn stover are shown

Table 34

MIXED CULTURE SIMULATION OF CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 ON 45 G/L PRETREATED CORN STOVER - CASE A

MIXED CULTURE SIMULATION STUDY

HEXOSE= 21 PENTOSE= 19 CTC= .1 CTS= .1 KH= 4
KP= 5 KE= .24
ENZYME 0= .024

TIME	CTC	MCTC	CTS	MCTS	ENZ	HEX	PEN	ETOH	ACET
***	0.1	0.028	0.1	0.146	0.02	21.0	19.0	*****	*****
10	0.1	0.008	0.3	0.059	0.03	20.0	17.7	0.9	0.2
20	0.1	-.001	0.4	0.031	0.03	18.9	16.4	1.7	0.5
30	0.1	-.006	0.5	0.018	0.03	18.0	15.2	2.4	0.9
40	0.1	-.008	0.6	0.011	0.03	17.2	14.1	3.0	1.2
50	****	-.010	0.6	0.006	0.03	16.4	13.0	3.6	1.6
60	****	-.011	0.7	0.003	0.03	15.7	12.1	4.0	2.0
70	****	-.012	0.7	0.001	0.03	15.0	11.2	4.4	2.4
80	****	-.012	0.7	*****	0.03	14.4	10.3	4.8	2.8
90	****	-.013	0.7	-.002	0.03	13.9	9.6	5.1	3.1
100	****	-.013	0.6	-.003	0.03	13.4	8.9	5.4	3.4
110	****	-.014	0.6	-.004	0.03	13.0	8.2	5.6	3.8
120	****	-.014	0.6	-.004	0.03	12.6	7.6	5.9	4.1
130	****	-.014	0.6	-.005	0.03	12.2	7.0	6.1	4.3
140	****	-.014	0.5	-.005	0.03	11.9	6.5	6.3	4.6
150	****	-.015	0.5	-.006	0.03	11.6	6.0	6.5	4.8
160	****	-.015	0.5	-.006	0.03	11.3	5.6	6.6	5.1
170	****	-.015	0.5	-.006	0.03	11.0	5.2	6.8	5.3
180	****	-.015	0.4	-.006	0.03	10.8	4.8	6.9	5.5
190	****	-.015	0.4	-.007	0.03	10.6	4.4	7.0	5.6
200	****	-.015	0.4	-.007	0.03	10.4	4.1	7.1	5.8

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in Figures 70 and 71. One observes the best agreement for ethanol and acetate production when case B is used.

Although case B fits the overall experimental data more closely than case A, there appears to be a deviation between predicted and observed acetate production at prolonged fermentation times. However, in actuality at these longer times, an increased production of lactic acid was also experimentally observed. The production of lactate can be equated in a similar manner to the production of acetate. For the purposes of this model, however, no provision was made for the production of lactate. If one corrects the observed acetate production by adding the lactate produced (1 g lactate is equivalent to 1.5 g acetate), values of corrected acetate shown in Figure 71 are obtained. In this case, excellent agreement for acetate production in case B is observed at the prolonged fermentation times.

Thus, from this analysis, we can tentatively conclude tht the apparent "Michaelis" constant, k_{S_H}, for hexose uptake in the Monod growth model is probably significantly lower for Clostridium thermocellum than for Clostridium thermosaccharolyticum. In addition, the hypothesis for "growth rate control of ethanol yield" in intrinsically high ethanol yielding strains of both Clostridium thermocellum and Clostridium thermosaccharolyticum also appears to remain essentially applicable for mixed culture.

Table 35

MIXED CULTURE SIMULATION OF CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 ON 45 G/L PRETREATED CORN STOVER - CASE B

MIXED CULTURE SIMULATION STUDY

HEXOSE= 21 PENTOSE= 19 CTC= .1 CTS= .1 KH= 4
KP= 5 KE= .24
ENZYME 0= .024

TIME	CTC	MCTC	CTS	MCTS	ENZ	HEX	PEN	ETOH	ACET
***	0.1	0.076	0.1	0.100	0.02	21.0	19.0	*****	*****
10	0.2	0.066	0.2	0.074	0.05	19.6	17.3	1.2	0.2
20	0.4	0.049	0.5	0.054	0.09	17.5	14.4	3.1	0.7
30	0.5	0.029	0.7	0.033	0.13	14.8	10.8	5.4	1.4
40	0.7	0.011	0.9	0.015	0.16	12.4	7.4	7.4	2.2
50	0.7	-.002	1.0	0.002	0.16	10.8	4.8	8.6	3.1
60	0.6	-.009	0.9	-.006	0.16	9.8	3.1	9.3	3.9
70	0.6	-.012	0.9	-.010	0.16	9.2	2.0	9.6	4.4
80	0.5	-.015	0.8	-.013	0.16	8.9	1.3	9.8	4.8
90	0.4	-.016	0.7	-.015	0.16	8.7	0.8	9.9	5.1
100	0.4	-.018	0.6	-.016	0.16	8.6	0.5	9.9	5.3
110	0.3	-.018	0.5	-.017	0.16	8.5	0.4	9.9	5.4
120	0.3	-.019	0.4	-.018	0.16	8.5	0.2	10.0	5.5
130	0.2	-.019	0.3	-.018	0.16	8.4	0.1	10.0	5.6
140	0.2	-.019	0.3	-.020	0.16	8.4	0.0	10.0	5.6
150	0.1	-.020	0.2	-.020	0.16	8.4	0.0	10.0	5.6
160	0.1	-.020	0.2	-.020	0.16	8.4	0.0	10.0	5.6
170	****	-.020	0.2	-.020	0.16	8.4	0.0	10.0	5.6
180	****	-.020	0.1	-.020	0.16	8.4	0.0	10.0	5.6
190	****	-.020	0.1	-.020	0.16	8.4	0.0	10.0	5.6
200	****	-.020	****	-.020	0.16	8.4	0.0	10.0	5.6

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Figure 70

CASE A: SIMULATED AND OBSERVED MIXED CULTURE GROWTH OF CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 ON 50 G/L PRETREATED CORN STOVER

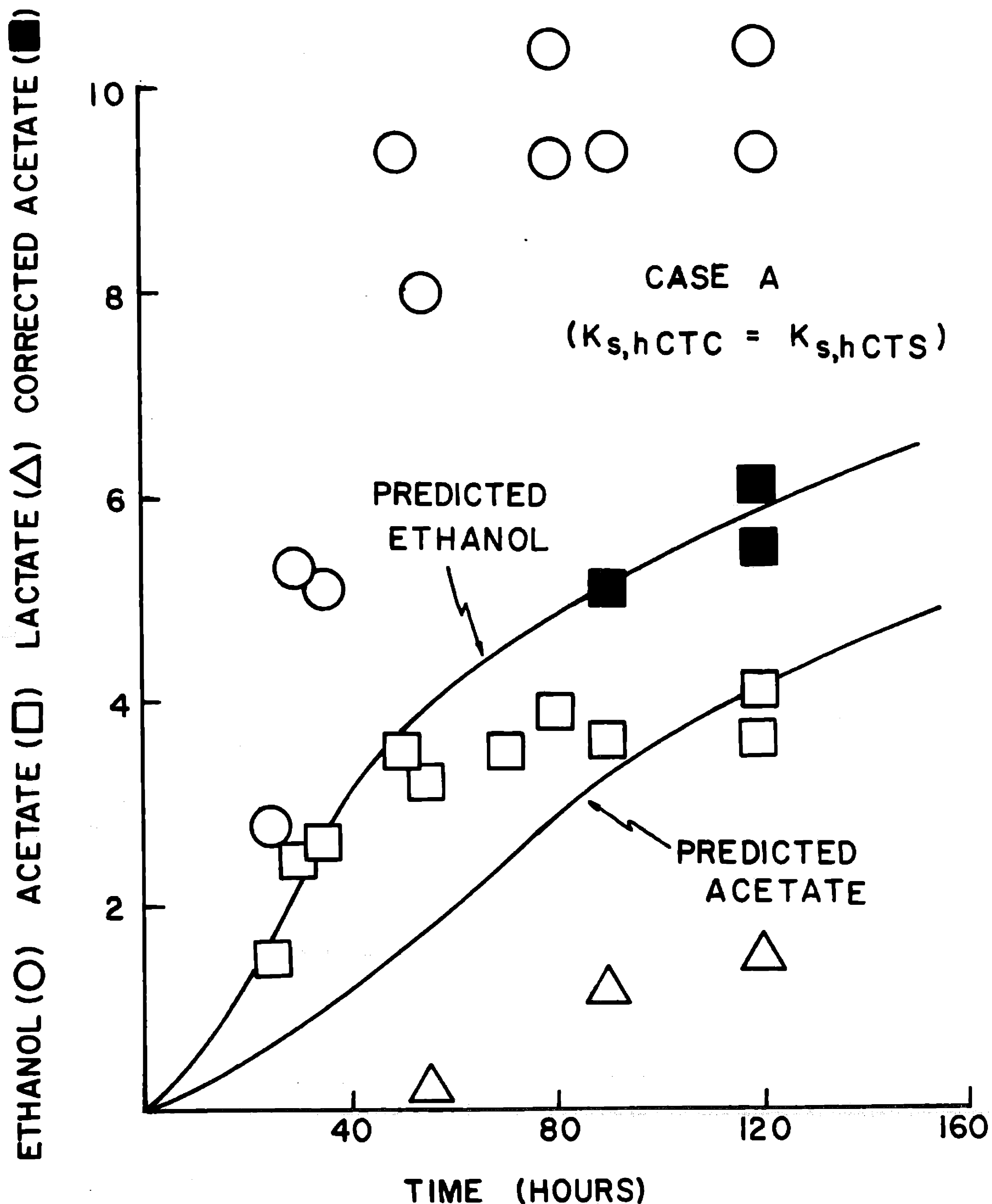
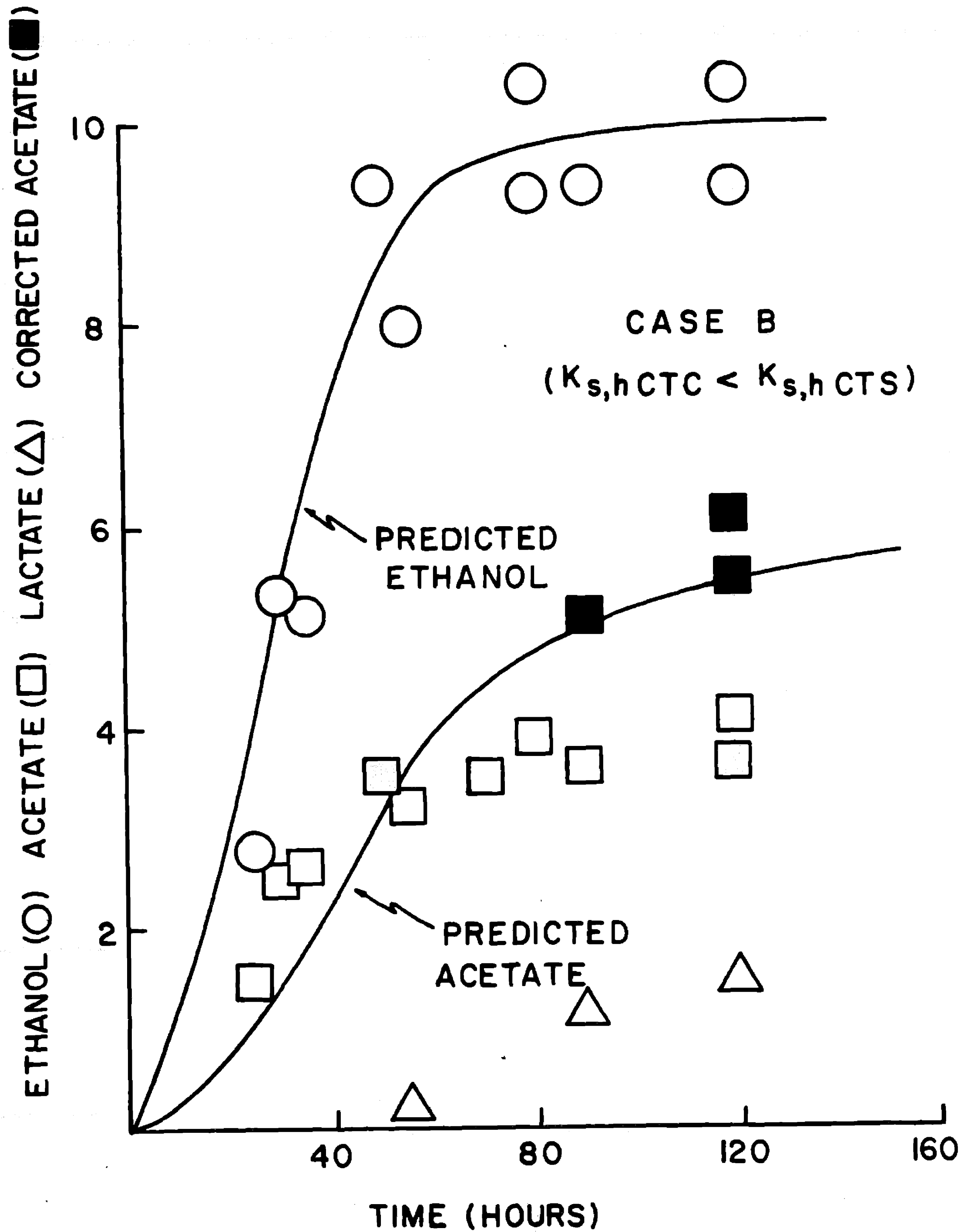


Figure 71

CASE B: SIMULATED AND OBSERVED MIXED CULTURE GROWTH OF CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 ON 50 G/L PRETREATED CORN STOVER



4.8.5. Model Examination of Potential Avenues for Process Improvement

A potential avenue for improvement of this mixed culture process can be hypothesized if one increases the cellulase activity of *Clostridium thermocellum*. If we assume that a new strain capable of producing a 100% increased enzyme activity (i.e., K_E from 0.24 to 0.48 mg protein/mg cell) and this increased protein maintains the same specific activity on the substrate, the behavior of this strain with respect to the production of ethanol and acetate can be simulated.

Shown in Tables 36 and 37 are the results of simulations of mixed culture fermentation incorporating such a hyper cellulase producing *Clostridium thermocellum* strain with the assumptions contained in model A and model B. The data for each case are compared with the normal cellulase strain and summarized after 100 hours of fermentation in Table 38.

One shows that in Case A where little difference between the Michaelis constants for hexose uptake by each strain was assumed, only a slight benefit is noted for increased cellulase activity. Although an increase in ethanol productivity from 0.08 to 0.17 g/l hr resulted, only a slight increase in E/A ratios from 1.6 to 1.7 is observed. From cell growth data, we see the effects of rapid pentose solubilization at both low and high cellulase yield result in a much greater accumulation of cell mass by *Clostridium thermosaccharolyticum*

Table 36

MIXED CULTURE SIMULATION OF 100% INCREASED CELLULASE PRODUCING *CLOSTRIDIUM THERMOCELLUM* S-7-19 AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM* ON 45 G/L PRETREATED CORN STOVER - CASE: A

MIXED CULTURE SIMULATION STUDY

HEXOSE= 21   PENTOSE= 19   CTC= .1   CTS= .1   KH= 4
KP= 5   KE= .48
ENZYME 0= .024

TIME	CTC	MCTC	CTS	MCTS	ENZ	HEX	PEN	ETOH	ACET
***	0.1	0.075	0.1	0.312	0.05	21.0	19.0	*****	*****
10	0.1	0.017	0.5	0.080	0.07	18.7	16.1	2.1	0.3
20	0.2	*****	0.8	0.033	0.08	16.5	13.2	3.9	0.9
30	0.2	-.007	1.0	0.015	0.08	14.8	10.8	5.3	1.6
40	0.1	-.010	1.1	0.006	0.08	13.4	8.8	6.3	2.3
50	0.1	-.013	1.2	*****	0.08	12.3	7.2	7.0	2.9
60	0.1	-.014	1.1	-.003	0.08	11.5	5.9	7.6	3.5
70	****	-.015	1.1	-.006	0.08	10.8	4.8	7.9	4.0
80	****	-.016	1.0	-.008	0.08	10.3	3.9	8.2	4.5
90	****	-.016	0.9	-.009	0.08	9.9	3.2	8.4	4.9
100	****	-.017	0.8	-.011	0.08	9.6	2.6	8.6	5.2
110	****	-.017	0.7	-.011	0.08	9.3	2.1	8.7	5.5
120	****	-.018	0.7	-.012	0.08	9.1	1.7	8.8	5.7
130	****	-.018	0.6	-.013	0.08	9.0	1.4	8.9	5.9
140	****	-.018	0.5	-.013	0.08	8.8	1.2	8.9	6.1
150	****	-.018	0.4	-.014	0.08	8.7	1.0	9.0	6.2
160	****	-.018	0.4	-.014	0.08	8.7	0.8	9.0	6.3
170	****	-.019	0.3	-.015	0.08	8.6	0.6	9.0	6.4
180	****	-.019	0.3	-.015	0.08	8.6	0.5	9.1	6.5
190	****	-.019	0.2	-.015	0.08	8.5	0.4	9.1	6.5

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Table 37

MIXED CULTURE SIMULATION OF 100% INCREASED CELLULASE PRODUCING *CLOSTRIDIUM THERMOCELLUM* S-7-19 AND *CLOSTRIDIUM THERMOSACCHAROLYTICUM* ON 45 G/L PRETREATED CORN STOVER - CASE: B

MIXED CULTURE SIMULATION STUDY

HEXOSE= 21 PENTOSE= 19 CTC= .1 CTS= .1 KH= 4
KP= 5 KE= .48
ENZYME 0= .024

TIME	CTC	MCTC	CTS	MCTS	ENZ	HEX	PEN	ETOH	ACET
***	0.1	0.172	0.1	0.219	0.05	21.0	19.0	*****	*****
10	0.4	0.113	0.5	0.124	0.21	17.1	14.0	3.7	0.4
20	0.9	0.028	1.2	0.036	0.42	11.5	5.9	9.0	1.5
30	0.9	-.008	1.3	-.003	0.45	9.1	1.8	11.0	2.7
40	0.8	-.017	1.2	-.014	0.45	8.6	0.6	11.3	3.4
50	0.7	-.019	1.0	-.018	0.45	8.4	0.2	11.4	3.7
60	0.5	-.020	0.8	-.020	0.45	8.4	0.0	11.4	3.7
70	0.5	-.020	0.7	-.020	0.45	8.4	0.0	11.4	3.7
80	0.4	-.020	0.6	-.020	0.45	8.4	0.0	11.4	3.7
90	0.3	-.020	0.5	-.020	0.45	8.4	0.0	11.4	3.7
100	0.2	-.020	0.4	-.020	0.45	8.4	0.0	11.4	3.7
110	0.2	-.020	0.3	-.020	0.45	8.4	0.0	11.4	3.7
120	0.2	-.020	0.3	-.020	0.45	8.4	0.0	11.4	3.7
130	0.1	-.020	0.2	-.020	0.45	8.4	0.0	11.4	3.7
140	0.1	-.020	0.2	-.020	0.45	8.4	0.0	11.4	3.7
150	****	-.020	0.1	-.020	0.45	8.4	0.0	11.4	3.7
160	****	-.020	0.1	-.020	0.45	8.4	0.0	11.4	3.7
170	****	-.020	****	-.020	0.45	8.4	0.0	11.4	3.7
180	****	-.020	****	-.020	0.45	8.4	0.0	11.4	3.7
190	****	-.020	****	-.020	0.45	8.4	0.0	11.4	3.7
200	****	-.020	****	-.020	0.45	8.4	0.0	11.4	3.7

READY

Table 38

SUMMARY OF MIXED CULTURE SIMULATION OF PRETREATED CORN STOVER FERMENTATION AFTER 100 HRS. WITH CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8

	Case A		Case B	
Enzyme Production	Normal	100% Greater Enzyme	Normal	100% Greater Enzyme
Maximum Cells of *Clostridium thermocellum*	0.1	0.2	0.7	0.9
Clostridium thermosaccharolyticum	0.7	1.2	1.0	1.3
Ethanol (g/l)	5.4	8.6	9.9	11.4
Acetate (g/l)	3.4	5.2	5.3	3.7
Ethanol Productivity (g/l hr)	0.08	0.17	0.15	0.36
E/A ratio	1.6	1.7	1.9	3.1

over *Clostridium thermocellum*. This behavior diminishes the effectiveness of the higher cellulase yielding strain. However, in Case B, the incorporation of an increased cellulase activity by *Clostridium thermocellum* in mixed culture results in an increased ethanol productivity from 0.15 to 0.36 g/l hr. In addition, a 60% increased E/A ratio from approximately 2.0 to 3.6 is also achieved.

Thus, the assumed magnitudes of k_{S_H} for *Clostridium thermocellum* being much lower than that for *Clostridium thermosaccharolyticum* shows a significant improvement in both ethanol yield, as well as productivity from the use of an increased cellulase producing stream. In the next section, both the assumptions of case "B" and the prediction for the use of increased cellulase are experimentally examined.

4.9. Experimental Verification of Mixed Culture Model Assumptions and Predictions

The two assumptions for carbohydrate uptake represented by Case "A" and Case "B" in the kinetic models described in the previous section leads to significantly different predicted results. In case "A", where the Michaelis constants for hexose uptake by each strain were assumed to be approximately equal, little benefit was observed for the mixed culture with a hyper cellulose yielding strain. On the other hand, the predictions for case "B" where the k_{S_H} for hexose uptake by *Clostridium*

thermocellum was assumed to be much lower than that for Clostridium thermosaccharolyticum, a significant advantage would be realized from a higher cellulose production strain. From a comparison of simulated and observed data, it was tentatively concluded tht model "B" was more likely.

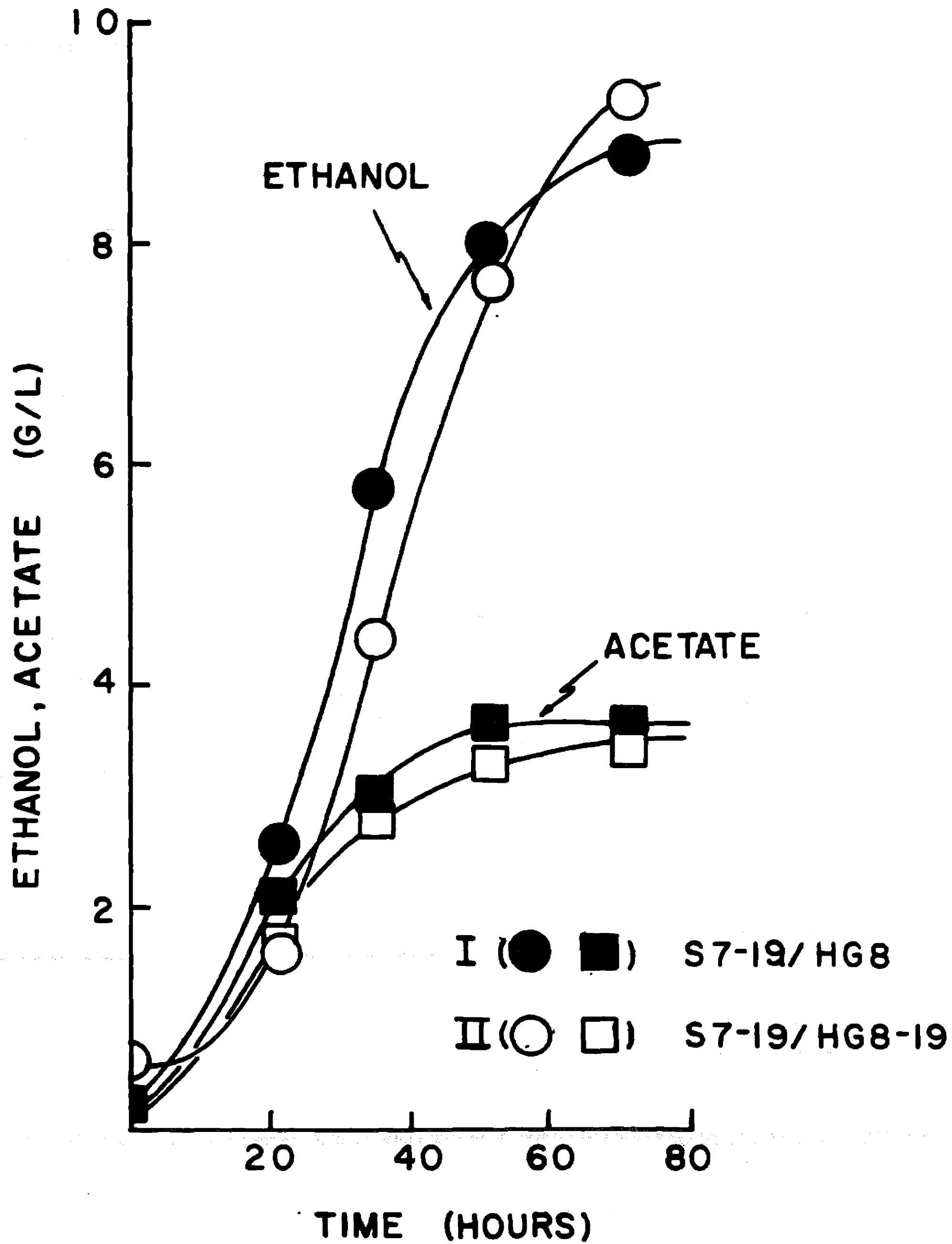
4.9.1. Fermentation of Treated Corn Stover by Mixed Culture of Clostridium thermocellum S-7-19 and Hexose (-) Clostridium thermosaccharolyticum HG-8-19

In order to verify in an alternate manner the accuracy of the modelling conclusions of Case B and to insure an increased ethanol yield and hydrolysis capability for mixed culture, we used a hexose non-fermenting mutant strain of Clostridium thermosaccharolyticum HG-8. In theory, a strain unable to ferment hexoses would have the same properties in hydrolysis rate limited mixed culture with Clostridium thermocellum as a strain with relatively high k_S for hexose as compared to Clostridium thermocellum. The isolation of such a strain has been described previously (Section 4.6). This mutant was designated as strain HG-8-19 and was shown to be unable to ferment either glucose or cellobiose.

The results of two mixed culture fermentations of Clostridium thermocellum S-7-19 with Clostridium thermosaccharolyticum HG-8 and the hexose negative strain HG-8-19 are summarized in Figure 72. From the similar appearance of the ethanol

Figure 72

MIXED CULTURE FERMENTATIONS OF PRETREATED CORN STOVER (80 G/L) BY CLOSTRIDIUM THERMOCELLUM S-7-19/CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 AND CLOSTRIDIUM THERMOCELLUM S-7-19/CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8-19



and acetate profiles for each mixed culture fermentation, we can conclude that the use of a hexose (-) strain offers little advantage for improvement of either the ethanol yield or productivity in mixed batch culture. By the same token, this lack of a significant difference in mixed culture performance with the hexose (-) strain further substantiates our previous conclusion regarding the relative K_S's for hexose uptake for _Clostridium thermocellum_ and _Clostridium thermosaccharolyticum_. These results are all consistent with the assumption of case "B".

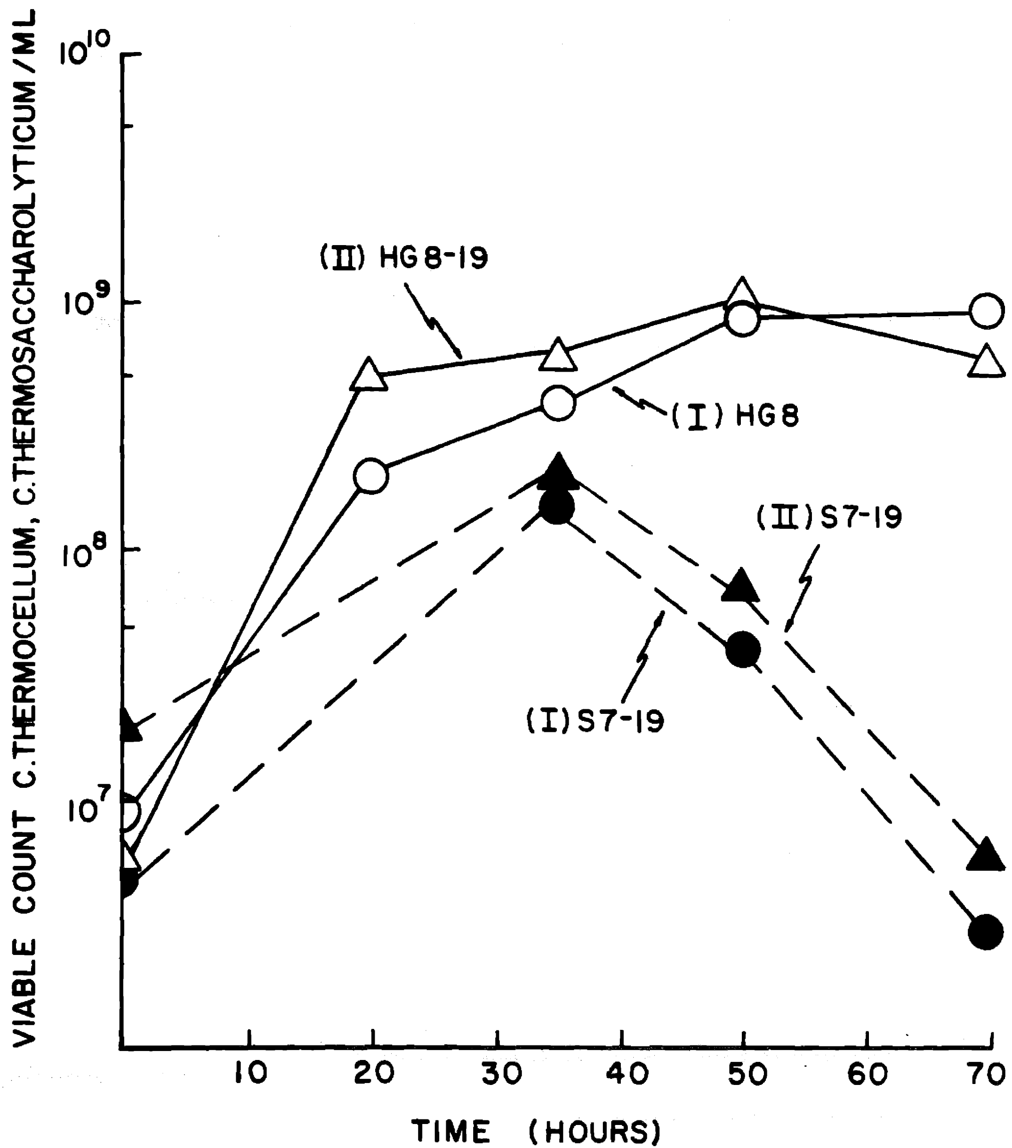
In addition to the production of ethanol and acetate, the viable counts of _Clostridium thermocellum_ and _Clostridium thermosaccharolyticum_ in each mixed culture fermentation were also performed. This was accomplished by selective plating on respective xylose and cellobiose agar as described previously. The growth of both _Clostridium thermosaccharolyticum_ strain HG-8 and HG-8-19 in mixed culture are seen to rapidly increase to maximum values of approximately 9×10^8 cells/ml after 50 hrs of fermentation (Figure 73). By contrast, _Clostridium thermocellum_ only achieves approximately 2×10^8 viable cells/ml and rapidly loses viability at later times. This maximum cell density of 2×10^8 cells/ml and loss of viability of _Clostridium thermocellum_ in mixed culture is very similar to the maximum cell density of 4×10^8 cells/ml and subsequent viability loss observed for monoculture fermentations of treated corn stover

Figure 73

VIABLE COUNT OF CLOSTRIDIUM THERMOCELLUM AND CLOSTRIDIUM THERMOSACCHAROLYTICUM IN MIXED CULTURES OF:

I) CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8, AND

II) CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8-19



by this strain. This result further supports the hypothesis that the presence of Clostridium thermosaccharolyticum has little influence on the growth of Clostridium thermocellum with regard to hexose uptake. The dry cell densities of Clostridium thermosaccharolyticum and Clostridium thermocellum can be estimated from viability data with the assumption of cell volume and dry weight previously reported. Using a cell volume of 3.5 μ^3/cells and dry weight of 20%, a maximum of 0.64 g/l Clostridium thermosaccharolyticum and 0.14 g/l Clostridium thermocellum were calculated. The maximum cell densities of 1.0 and 0.7 predicted by the model of case "B" for Clostridium thermosaccharolyticum and Clostridium thermocellum, respectively, are in moderately good agreement with the observed cell estimates considering that the cells which are bound to solids are not counted and the plating efficiencies are likely to be below 100%.

The model of case B also predicts a slight decrease of viable cell mass of 10-15% for both Clostridia between 50 and 70 hours of fermentation. A much greater loss of viability is expected at a later time, barring the formation of spores which both strains are capable of making. The viable count results demonstrate a much greater than predicted loss of viability for Clostridium thermocellum in this interval. This behavior is similar to that previously observed in monoculture fermentations as well. The behavior of Clostridium thermosac-

charolyticum, on the other hand, shows, as expected, little change in viable count.

4.9.2. Addition of Cellulase to Mixed and Mono-Culture Fermentations of Pretreated Corn Stover

The addition of crude cellulase to cultures of *Clostridium thermocellum* S-7-19 and *Clostridium thermosaccharolyticum* HG-8 during fermentations of pretreated corn stover were performed. This was done to obtain an independent verification of the hypothesis that low specific growth rates limit the maximum ethanol yields in fermentations due to the rate of hydrolysis of corn stover. Using the previous relationships one obtains the respective specific growth rates as:

$$\mu_{CTC} = \left(\frac{1}{CTC}\frac{dH}{dt} - M_{CTC}\right) Y_{CTC/H} \tag{18}$$

and

$$\mu_{CTS} = \left(\frac{1}{CTS}\frac{dP}{dt} - M_{CTS}\right) Y_{CTS/P} \tag{19}$$

where

$$\frac{dH}{dt} = K_H . E \tag{20}$$

and

$$\frac{dP}{dt} = K_P . E \tag{21}$$

Thus, by increasing the initial enzyme concentration (E), an increase in both the volumetric rates of hexose and pentose hydrolysis should result. This in turn should reflect in an increase in the specific growth rate of each organism. Furthermore, if one observes an increased ethanol yield, we will be more confident as to the original hypothesis as well as encouraging the isolation of hyper cellulase producing strains to increase ethanol yield.

The cellulase for this experiment was obtained by ethanol precipitation of the extracellular protein produced in a monoculture fermentation of Clostridium thermocellum S-7-19 grown on cellobiose. A quantity of this concentrated protein at 0.8 mg/ml was added to monoculture fermentations of Clostridium thermocellum S-7-19, Clostridium thermosaccharolyticum HG-8, and a mixed culture of both organisms growing on 50 g/l pretreated corn stover. In addition to these monoculture and mixed fermentations with enzyme addition, control of mixed and monoculture fermentations without enzyme addition were also performed. A summary of the results from this experiment with regard to ethanol, acetate, and reducing sugar production after 80 hrs of fermentation is shown in Table 39.

The mixed culture fermentation without enzyme addition was shown to produce 10.4 g/l ethanol and 3.7 g/l acetate at a ratio of 2.8 to 1. It can be seen that no free re-

Table 39

ETHANOL, ACETATE AND REDUCING SUGAR PRODUCTION OF CLOSTRIDIUM THERMOCELLUM S-7-19 AND CLOSTRIDIUM THERMOSACCHAROLYTICUM HG-8 AFTER 80 HRS MIXED AND MONOCULTURE GROWTH ON PRETREATED CORN STOVER WITH AND WITHOUT CELLULASE ADDITION*

	Ethanol (g/l)	Acetate (g/l)	E/A Ratio	Reducing Sugars (g/l)
Mixed Culture Clostridium thermocellum S-7-19/ Clostridium thermosaccharolyticum HG-8	10.4	3.7	2.8	0
MonoCulture Clostridium thermocellum S-7-19	4.6	1.8	2.6	9.2
MonoCulture Clostridium thermosaccharolyticum HG-8	5.5	2.0	2.8	0
Fermentation with Added Enzyme (0.8 mg/ml)				
Mixed Culture Clostridium thermocellum S-7-19/ Clostridium thermosaccharolyticum HG-8	12.9	2.6	5.0	0
MonoCulture Clostridium thermocellum S-7-19	1.2	0.8	1.5	12.7
MonoCulture Clostridium thermosaccharolyticum HG-8	10.8	2.9	3.7	2.2

* Average of duplicate Hungate tube fermentations of 50 g/l pretreated corn stover.

ducing sugars were found after 80 hours of fermentation. In contrast, the mixed culture conducted with the addition of 0.8 mg/ml enzyme was shown to be capable of producing 12.9 g/l ethanol and 2.6 g/l acetate at a ratio of 5.0 to 1.

This fermentation behavior using treated corn stover and with exogenous enzyme addition is very similar to that previously achieved by mixed cultures on Solka floc. Mixed culture fermentation of this latter model substrate resulted in E/A ratios of approximately 5 to 1 achieved by Clostridium thermosaccharolyticum HG-8 during sequential monoculture fermentation of previously hydrolyzed carbohydrates of treated corn stover. These results support the conclusion that the intrinsic capacities of these strains for achieving high ethanol yield can be readily attained on corn stover carbohydrates provided high specific growth rates can be achieved.

In addition to mixed culture, the performance of each organism was examined with and without enzyme addition in monoculture. As shown in Table 39, Clostridium thermosaccharolyticum HG-8 was shown to readily ferment the hemicellulose carbohydrates of pretreated corn stover when grown in monoculture without benefit of any cellulose addition. In this case, 5.5 g/l ethanol and 2.0 g/l acetate were shown to accumulate after 80 hours of fermentation. However, the low E/A ratio of 2.8 to 1 indicates that the xylanase activity of this strain is insufficient to degrade and to provide sufficient sugars to

maintain the necessary growth rate to achieve high ethanol yield. A calculation of hemicellulose consumed based on product accumulation, however, shows that the enzymes of this organism are capable of degrading 13.4 g/l hemicellulose in corn stover. This represents 63% of the total pentosans (21 g/l) in the pretreated corn stover (50 g/l). It should also be noted that the growth, characteristically exhibited by this organism on pretreated corn stover, is in marked contrast to that observed on non-treated stover. In a number of attempts, no significant growth of *Clostridium thermosaccharolyticum* in monoculture has been observed on the non-treated material. Presumably, only the easily solubilized hemicellulose pentosans of the treated stover are susceptible to degradation by the "xylanase" of this organism.

The growth of *Clostridium thermosaccharolyticum* HG-8 on treated corn stover with the addition of 0.8 mg/ml cellulose demonstrates substantially improved performance as compared to that without enzyme addition. In this case, 10.8 g/l ethanol and 2.9 g/l acetate are produced. The E/A ratio increased from 2.8 without cellulose addition to 3.7 with the enzyme addition. In this monoculture, as well as in the mixed culture, an increased specific growth rate resulted due to the increased specific rate of substrate hydrolysis and ultimately led to an improvement in the ethanol yield.

However, the addition of extracellular protein from Clostridium thermocellum to Clostridium thermocellum monoculture fermentation of pretreated corn stover resulted in an unusual behavior. Without protein addition, the results in Table 39 after 80 hours of fermentation on pretreated corn stover by Clostridium thermocellum showed the accumulation of 4.6 g/l ethanol, 1.8 g/l acetate and 9.2 g/l reducing sugars. These values are quite typical and expected from this strain. However, the addition of 0.8 mg/ml of ethanol concentrated protein from the previous Clostridium thermocellum fermentation on cellobiose results in virtually total inhibition of this subsequent pretreated corn stover fermentation. Although 12.7 g/l reducing sugars are shown to accumulate due to the action of the added enzyme, only 1.2 g/l ethanol and 0.8 g/l acetate are produced.

The effects of the ethanol precipitate from Clostridium thermocellum on the inhibition of growth of this strain could be quite problematic. For example, if the inhibition factor is associated with the cellulase a significant potential drawback to the application of hyper-cellulose strains for improving the fermentation process could result. In order to assess this "inhibition" in more detail, a series of experiments were undertaken and will be described in Section 4.10.

4.9.3. High Cell Density

The use of high cell density inoculum of *Clostridium thermocellum* to improve the rate and extent of biomass utilization was previously examined with non-treated corn stover in Section 4.4.2. However, the results from these experiments demonstrated little difference with respect to the increased cell loading. It was pointed out that the inoculum in that case was obtained by centrifugation of exponentially growing cells. Under these conditions, the bulk of extracellular enzyme was removed from the inoculum. From the modeling studies we now realize the important nature of the rates of volumetric substrate degradation rate with respect to the quantity of cell mass present on the growth rate, i.e.,

$$\left[\frac{E\ K_H}{CTC} - M\right] Y_{CTC/H} = \mu_{CTC} \qquad (22)$$

where the ratio of $E\ K_H/CTC$ in equation (22) in large measure controls the growth rate and consequently ethanol yield obtainable.

In order to further verify this behavior with the more readily degradable solvent treated biomass, a high cell density fermentation was conducted. A five fold concentrated inoculum of *Clostridium thermocellum* S-7 and usual 10% inoculum of *Clostridium thermosaccharolyticum* HG-6-62 were both

added at the start of the fermentation. An anaerobic flask with 120 g/l of pretreated corn stover and bicarbonate buffer were used. As seen in Figure 74, the pretreated corn stover solids were rapidly degraded at a maximum rate of 0.8 g/l hr consuming 71 g/l solids in approximately 80 hours of fermentation. This solids consumption represents 67% of the total carbohydrate calculated to be potentially available in this material. However, in spite of the high volumetric rate of substrate degradation achieved, 22 g/l ethanol and 13 g/l acetate were produced. This amount of products reflects an E/A ratio of 1.7 to 1. On the other hand, the high volumetric rate of substrate degradation and the low E/A ratio are consistent with the modeling analysis previously presented. In Table 40, a numerical integration of this high cell density fermentation as modeled similarly is shown. An assumption is required in this analysis for the initial concentration of enzymes which is carried over with the concentrated cell inoculum pellet at the start of the fermentation. This value was chosen based on the volume of fermentation broth calculated to be present in the inoculum cell pellet volume and the concentration of enzyme (based on cells) calculated to be present in the inoculum. Using this initial enzyme concentration, the numerically integrated performance of the high cell density fermentation is quite similar to the experimental results obtained. In Table 40 , an E/A ratio of 2.0 to 1 and volumetric rate of substrate degradation of 0.9

Figure 74

FED BATCH FERMENTATION OF PRETREATED CORN STOVER BY MIXED CULTURE OF <u>CLOSTRIDIUM THERMOCELLUM</u> S-7 AND <u>CLOSTRIDIUM THERMOSACCHAROLYTICUM</u> HG-6

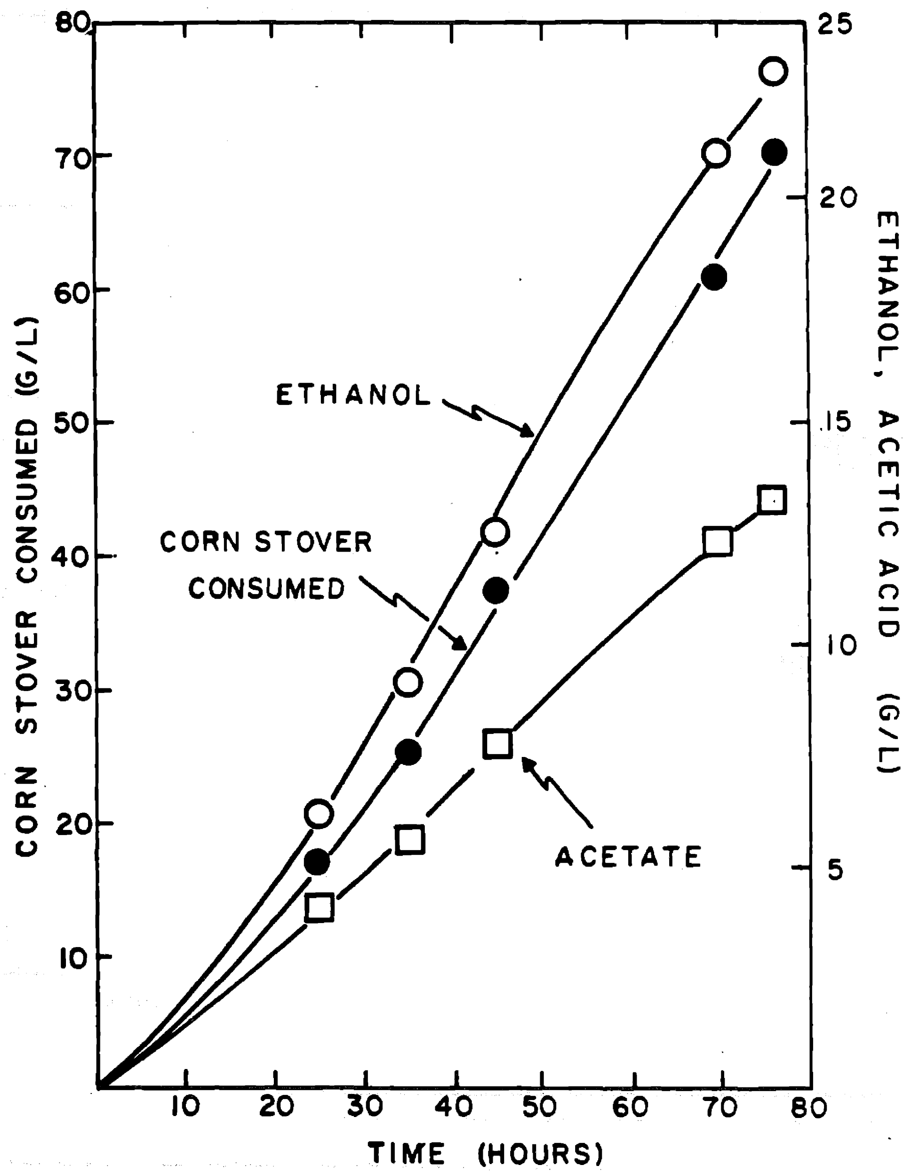


Table 40

SIMULATION OF MIXED CULTURE FERMENTATION OF 120 G/L TREATED CORN STOVER WITH 5X INOCULUM OF *CLOSTRIDIUM THERMOCELLUM* AND 10% *CLOSTRIDIUM THERMOSACCHAROLYTICUM*

MIXED CULTURE SIMULATION STUDY

HEXOSE= 56 PENTOSE= 53 CTC= .5 CTS= .1 KH= 4
KP= 5 KE= .24
ENZYME 0= .05

TIME	CTC	MCTC	CTS	MCTS	ENZ	HEX	PEN	ETOH	ACET
***	0.5	0.020	0.1	0.225	0.05	56.0	52.9	*****	*****
10	0.6	0.028	0.4	0.085	0.08	53.4	49.8	2.2	0.5
20	0.9	0.032	0.8	0.058	0.14	49.7	44.9	5.4	1.4
30	1.2	0.028	1.2	0.042	0.21	44.6	38.1	9.8	2.7
40	1.5	0.018	1.7	0.027	0.29	38.8	30.1	14.6	4.6
50	1.7	0.007	2.1	0.013	0.33	33.7	22.4	18.8	6.7
60	1.7	-.002	2.3	0.003	0.34	30.0	16.3	21.7	8.8
70	1.6	-.007	2.3	-.003	0.34	27.4	11.8	23.4	10.6
80	1.5	-.011	2.2	-.007	0.34	25.8	8.5	24.5	12.2
90	1.3	-.013	2.0	-.010	0.34	24.6	6.2	25.2	13.4
100	1.1	-.015	1.8	-.012	0.34	23.9	4.5	25.6	14.3
110	1.0	-.016	1.6	-.013	0.34	23.4	3.2	25.8	15.0
120	0.8	-.017	1.4	-.014	0.34	23.1	2.3	25.9	15.6
130	0.7	-.017	1.2	-.015	0.34	22.8	1.7	26.0	16.0
140	0.6	-.018	1.0	-.016	0.34	22.7	1.2	26.1	16.3
150	0.5	-.018	0.9	-.017	0.34	22.6	0.9	26.1	16.5
160	0.4	-.019	0.7	-.017	0.34	22.5	0.6	26.2	16.6
170	0.3	-.019	0.6	-.018	0.34	22.5	0.5	26.2	16.7
180	0.3	-.019	0.5	-.018	0.34	22.5	0.3	26.2	16.8
190	0.2	-.019	0.4	-.018	0.34	22.4	0.2	26.2	16.9
200	0.2	-.019	0.4	-.018	0.34	22.4	0.2	26.2	16.9

g/l hr are attained and both values are quite similar to the values observed.

4.10. Inhibition of *Clostridium thermocellum* by an Extracellular Factor

In previous results we observed a rapid loss of viability of *Clostridium thermocellum* S-7-19 after approximately 35 hours of mixed or monoculture growth on pretreated corn stover. At approximately this time into the fermentation a net negative growth of *Clostridium thermocellum* was also predicted by the simulation studies. However, this loss of viability through the model prediction is significantly lower than that observed experimentally. We have also observed that nearly total inhibition of growth of *Clostridium thermocellum* occurs when its extracellular "proteins" are concentrated and added back to corn stover fermentation medium. At the same time, little effect of this inhibitory extracellular product is observed for the growth of *Clostridium thermosaccharolyticum*.

In view of this significant observation for this mixed culture process, the effects of this growth inhibitory factor were examined in greater detail. The results of these investigations are summarized in the following section.

4.10.1. Inhibition of *Clostridium thermocellum* S-7-19 by Concentrated "Protein"

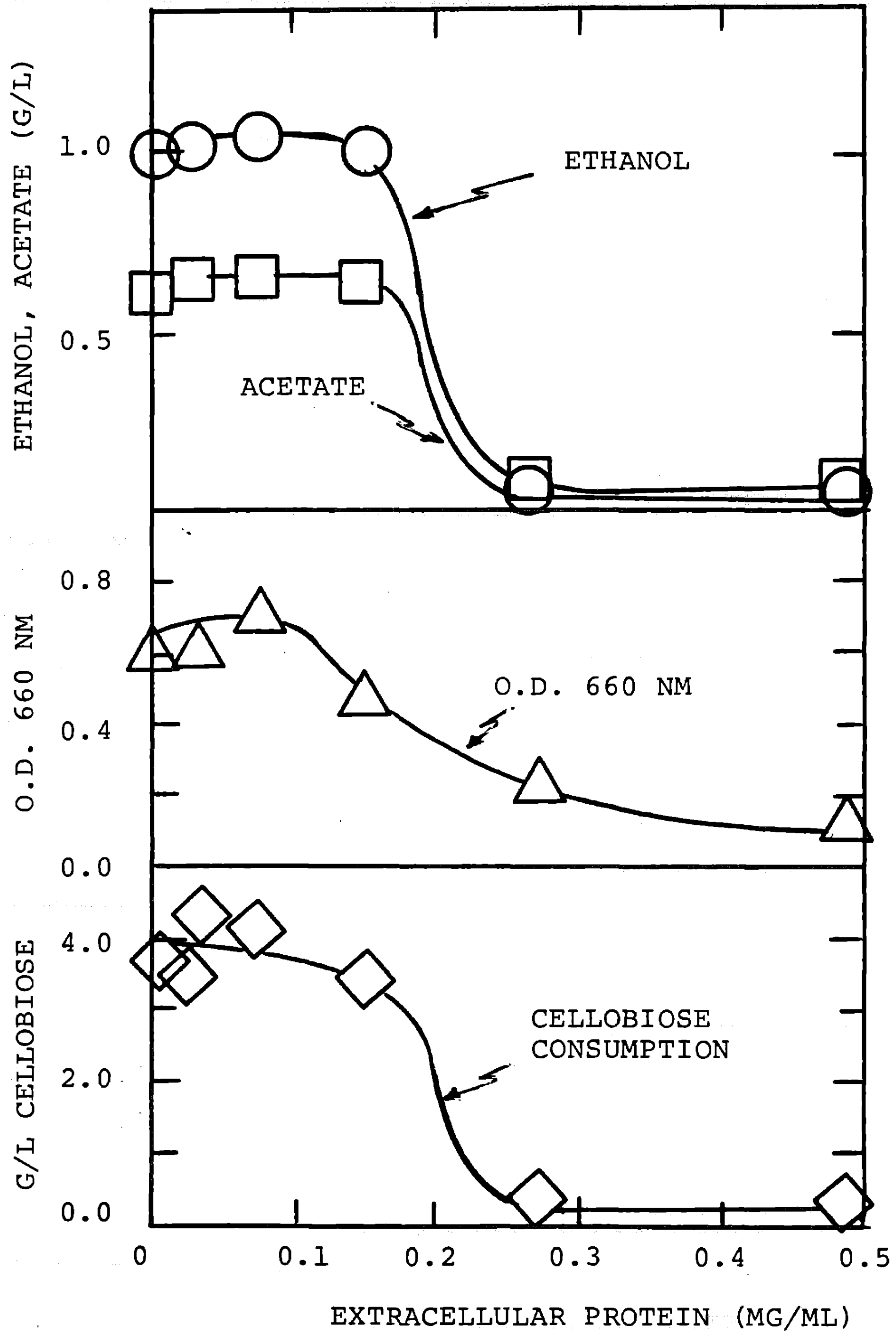
The ability of crude "proteins" from *Clostridium thermocellum* to totally inhibit the growth of this

strain was re-examined on cellobiose medium using either ethanol or ammonium sulfate precipitated material. As shown in Figure 75, the addition of 0.25 mg/ml ethanol precipitated protein or greater prevented further growth of Clostridium thermocellum S-7-19 as measured by changes in optical density, consumption of reducing sugars and appearance of products such as ethanol and acetate. This result confirmed our previous observation of inhibitor by the addition of 0.8 mg/ml extracellular protein. This result was repeated with extracellular proteins from Clostridium thermocellum S-7-19 grown on Solka floc. This protein had been precipitated by ammonium sulfate as described in Materials and Methods. A similar inhibitory result was observed; however, in this case maximum inhibition occurred with the addition of 0.1 mg/ml protein or greater.

Although the extracellular protein clearly prevented the growth of Clostridium thermocellum, it cannot be concluded as to its effect on cell viability. In order to answer this question, cells from an exponentially growing culture of Clostridium thermocellum S-7-19 on cellobiose medium were collected after centrifugation and washed with buffer. The cells were then resuspended in fresh media containing various concentrations of ammonium sulfate precipitated "protein". The changes in optical density and viable count of these cultures after 20 hours incubation at 60°C were then determined.

Figure 75

GROWTH OF CLOSTRIDIUM THERMOCELLUM S-7-19 ON CELLOBIOSE WITH ADDED EXTRACELLULAR PROTEIN FROM CLOSTRIDIUM THERMOCELLUM S-7-19



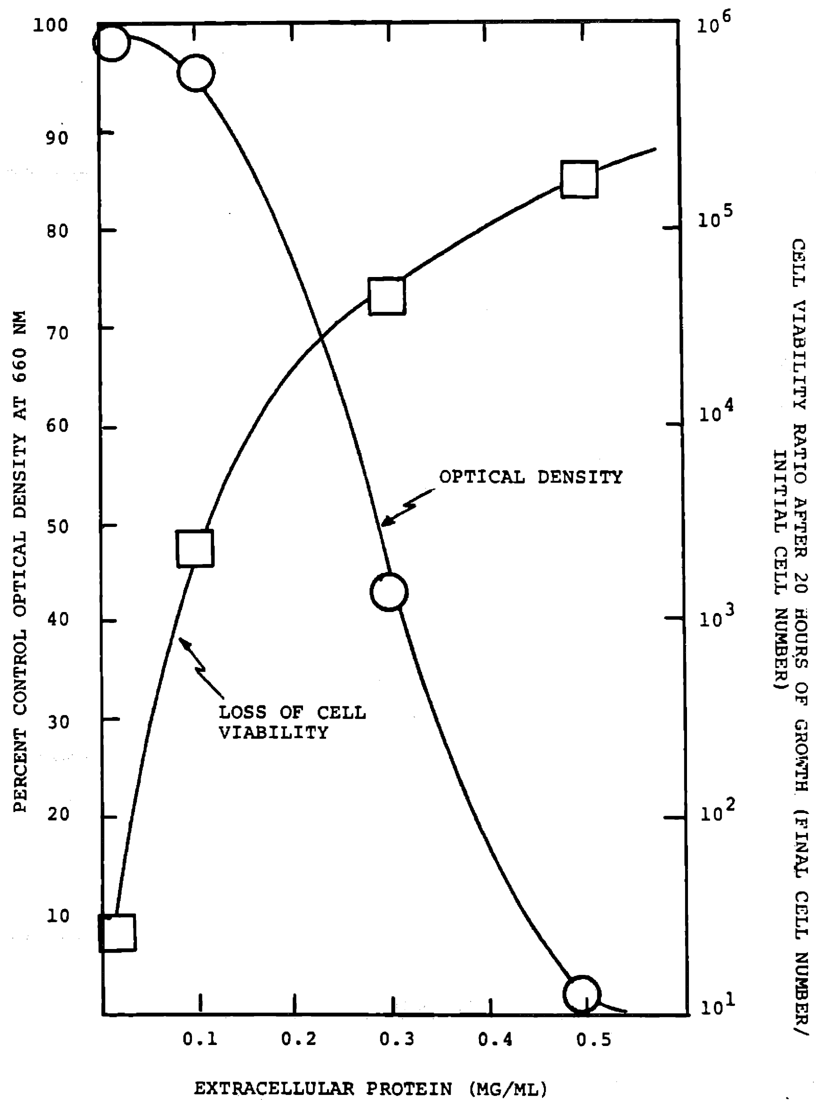
In Figure 76, a decrease in optical density of cells is seen due to the addition of protein. Almost a 100% decrease in cell turbidity is observed after 20 hours of incubation at 60°C with 0.5 mg/ml protein. The viable count of cells after 20 hours of incubation at 60°C at various protein concentrations also correlates with the decreased turbidity. For example, the ratio of final to initial cell viability was 10^{-5} after 20 hrs of incubation in medium containing 0.5 mg/ml protein. These results show that not only is initial cell growth prevented by this extracellular material, but cell lysis appears to occur as well.

The inhibitory protein precipitate obtained for use in these studies just described was harvested from cellobiose or Solka floc fermentation (> 30 g/l) of Clostridium thermocellum S-7-19 after more than 48 hours of fermentation. In order to determine the kinetics of this inhibitory factor with respect to its relationship to cell growth and extracellular protein excretion, the following experiment was conducted.

A batch fermentation of Clostridium thermocellum S-7-19 was run under pH controlled conditions on cellobiose medium in a fermentor. Cellobiose was fed to maintain greater than 5 g/l at all times and a total of 13 g/l yeast extract was periodically fed. At various times during the fermentation, a large volume of broth was harvested and the protein isolated from the supernatant by ammonium sulfate precipitation.

Figure 76

EFFECT OF EXTRACELLULAR PROTEIN FROM *CLOSTRIDIUM THERMOCELLUM* S-7-19 ON *CLOSTRIDIUM THERMOCELLUM* S-7-19 INCUBATED 20 HRS AT 60°C

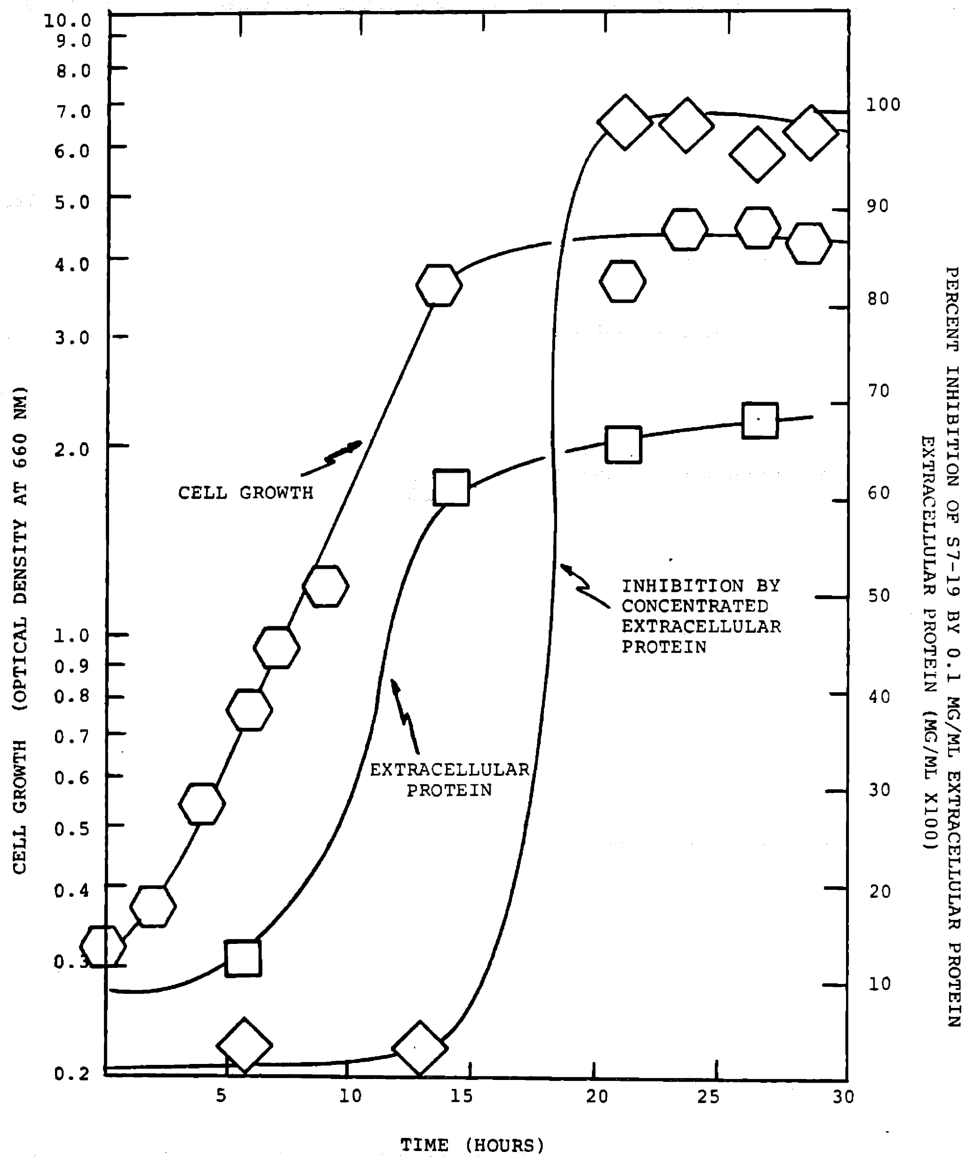


The precipitated material isolated in this manner was then added to fresh cellobiose tube medium adjusted to contain 0.1 mg/ml of the precipitated protein. As shown in Figure 79, exponential growth is observed in the cellobiose fermentation untilapproximately 15 hours of growth where a rapid decreased growth resulted leading to the stationary phase. Precipitated samples of broth proteins reconstituted to equal 0.1 mg/ml from 6 and 13 hours of fermentation appeared to have little inhibitory effect on the subsequent growth of Clostridium thermocellum in fresh media. However, protein precipitates added to the flask cellobiose media at the same total protein concentration from after 22 hours of growth all are seen to cause virtually 100% inhibition of growth.

Also shown in Figure 77 is the extracellular protein produced by Clostridium thermocellum during the fermentation. From 6 to 13 hours of fermentation, where inhibition of growth by the added protein occurs, there was also between 0.1 to 0.6 mg/ml amount of additional extracellular protein produced. From this latter observation where there were high concentrations of excreted proteins in the fermentation, we conclude that the total quantity of extracellular protein is not necessarily the important variable with respect to growth inhibition. However, it is possible that a specific inhibitory protein is produced at the onset of stationary phase which limits further growth. On the other hand, it is also clear that

Figure 77

PRODUCTION OF "AUTOLYSIN" DURING FERMENTATION OF CLOSTRIDIUM THERMOCELLUM S-7-19 ON CELLOBIOSE



one cannot presuppose this inhibitory factor to be a protein at all. This is due to the fact that other materials including oligosaccharides and nucleic acids are all susceptible to precipitation by these methods which were used to isolate the proteins.

Finally, although a growth inhibitory factor can be isolated through precipitation after the onset of stationary phase, one cannot conclude that this factor is necessarily primarily responsible for this cessation of growth or rather is a consequence of it. Nevertheless, it should be pointed out that the identification of the primary causitive factor for the limitation of growth of Clostridium thermocellum at higher cell densities where no substrate or nutrient limitation in the medium is thought to exist has not yet been demonstrated by any other factor.

Initially, the most likely candidates for inhibition of the growth of this strain were thought to be the major end products, ethanol, acetate and lactate. However, the addition of 12 g/l ethanol, 3 g/l acetate and 3 g/l lactate, the levels found at 30 hours of the cellobiose fermentation shown in Figure 77, caused no inhibition of the growth of Clostridium thermocellum S-7-19 as shown in Table 41.

However, as demonstrated previously, these end products usually account for between 80 and 90% of the total carbohydrate fermented. It is possible that the production

Table 41

EFFECT OF THE MAJOR FERMENTATION END PRODUCTS OBTAINED AT 30 HRS OF FERMENTATION* OF CELLOBIOSE (FIGURE 71) ON THE GROWTH OF <u>CLOSTRIDIUM THERMOCELLUM</u> S-7-19 ON CELLOBIOSE

	OD 660 (18 hrs)	Consumption of Cellobiose (18 hrs)
Control	1.3	10 g/l
Solvent Addition	1.2	10 g/l

* Ethanol, 12 g/l; Acetate, 3 g/l; Lactate, 3 g/l

of much smaller quantities of minor end products such as formic acid, butyric acid, or an as yet unidentified product, may be ultimately responsible for the inhibition of further growth of this strain. However, this hypothesis has not as yet been investigated.

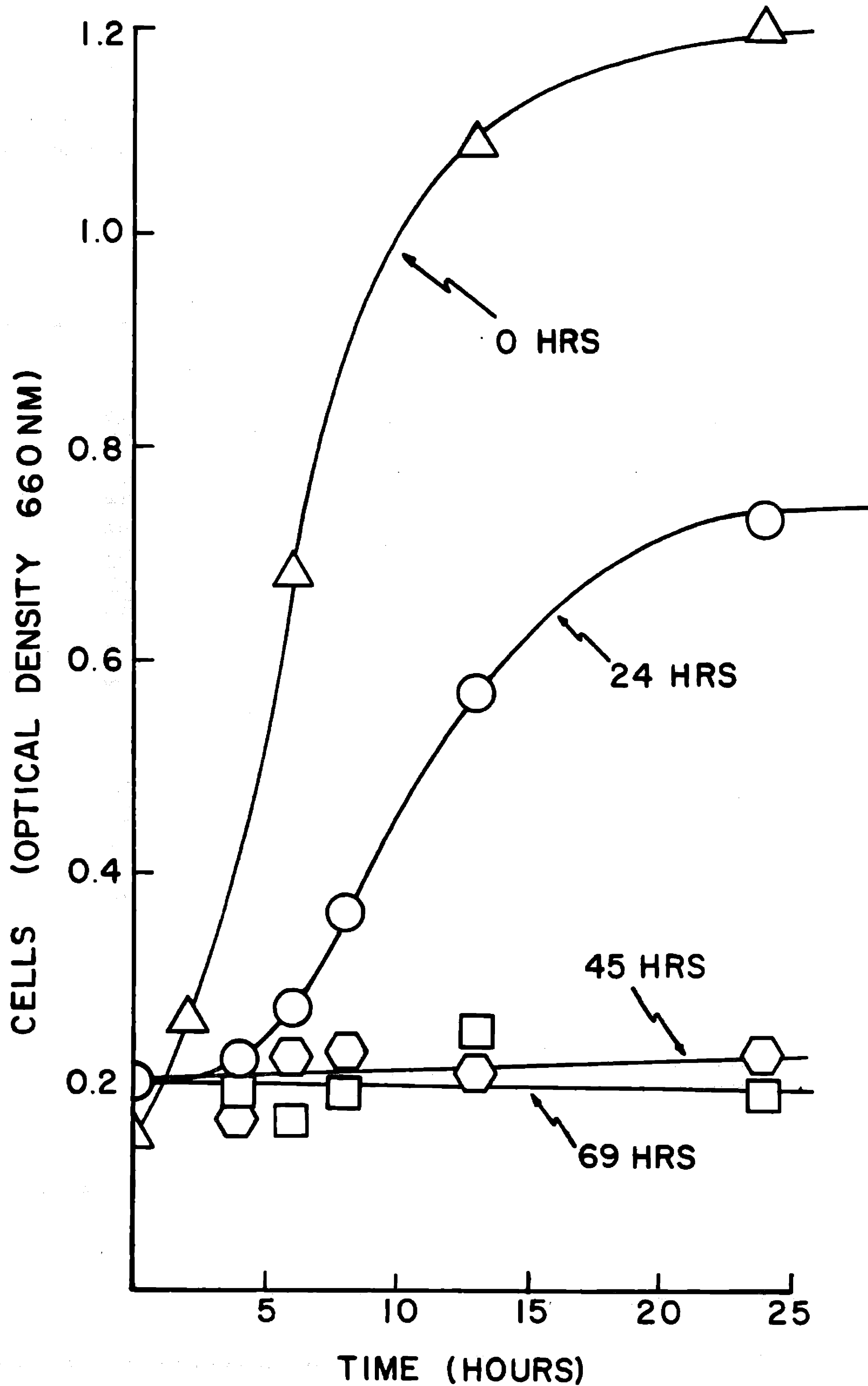
4.10.2. Inhibition by Whole Broth Supernatants

The apparent lack of a relationship between inhibition demonstrated by protein precipitates and the protein produced during growth prompted us to examine growth inhibition by whole broth from *Clostridium thermocellum* fermentations. The purpose of these experiments was to determine whether or not the ethanol or ammonium sulfate precipitated material reflected some component of actual fermentation or an artificially concentrated substance not normally at an inhibitory level in normal fermentation broths.

A monoculture fermentation of *Clostridium thermocellum* S-7-19 on 40 g/l Solka floc was partially harvested at 0, 24, 45 and 69 hours and centrifuged at 10,000 x g for 20 minutes. The supernatants were aseptically transferred to Hungate tubes and supplemented with 10 g/l cellobiose, 2 g/l yeast extract, and 1 mM dithiothreitol (DTT). The pH of the broths were also readjusted to 6.8-7.1. The fermentation profiles of *Clostridium thermocellum* S-7-19 on the supernatant are shown in Figure 78 and exhibits complete inhibition of growth

Figure 78

GROWTH OF S-7-19 IN CENTRIFUGED SUPERNATANT FROM SOLKA FLOC FERMENTATION OF S-7-19 (SUPPLEMENTED WITH 10 G/L CELLOBIOSE + 2 G/L YEAST EXTRACT, pH 7.0)



after 45 hrs of fermentation and partial inhibition at 24 hrs. In order to determine the potency of this broth inhibitor, broths from 69 hrs of fermentation were diluted and prepared with fresh media, supplemented with cellobiose and yeast extract as above, and inoculated with Clostridium thermocellum S-7-19. As seen in Table 42, maximal growth was attained in tubes containing less than 40% of the original broth while total inhibition occurred at 60 and 80% concentrations.

The observed complete inhibition of Clostridium thermocellum after 45 hrs of growth on Solka floc provides an interesting alternate explanation for the kinetic profile of reducing sugar accumulation during monoculture growth of this organism on this substrate. Under pH controlled conditions during monoculture fermentations of Solka floc by Clostridium thermocellum, experiments by Gordon and Wang have shown that a rapid accumulation of cellobiose occurs late in the fermentation which this strain is unable to metabolize [25, 16]. This observation has been puzzling and is inconsistent with the predictions intuitively expected from the model analysis previously shown. However, these results are entirely consistent with a fermentation in which a growth inhibitory substance is produced. Under these conditions, cellobiose metabolism by the cells will rapidly decrease. However, enzymatic degradation of Solka floc is expected to continue at a rate only limited by the intrinsic specific hydrolysis factors and end product inhibition effects.

Table 42

GROWTH OF _CLOSTRIDIUM THERMOCELLUM_ S-7-19 IN CENTRIFUGED BROTH FROM _CLOSTRIDIUM THERMOCELLUM_ S-7-19 FERMENTATION OF SOLKA FLOC (40 G/L) (69 HRS)*

% Broth	ΔOD_{660}
80%	~ 0
60%	~ 0
40%	1.2
20%	1.2
10%	1.2
0%	1.2

* Centrifuged broth supplemented with 2 g/l yeast extract, 10 g/l cellobiose, and pH adjusted to 6.9 - 7.1.

4.10.3. Characterization of Growth Inhibitory Factor

Attempts to characterize the inhibitory component of the broth from *Clostridium thermocellum* fermentations were also performed through various enzymatic treatments of the broth. Samples of centrifuged inhibitory broth supplemented with cellobiose (10 g/l) and yeast extract (2 g/l) were treated with Trypsin, Pronase, RNAase and DNAase. Hungate tubes containing inhibitory broth, as well as controls with fresh media, were incubated with these enzymes at 37°C for 4 hours. The tubes were then heated to 60°C and inoculated with *Clostridium thermocellum* S-7-19. The results in Table 43 show that no reversal of inhibition occurs by this treatment with various Proteases or Nucleases.

In order to completely eliminate the possibility that the active factor in the broth was a low molecular weight compound and in order to ascertain an approximate molecular size for the active principle(s), a number of dialysis experiments were conducted. Both cellulose acetate dialysis bags and ultrafiltration membranes were used. A sample of inhibitory supernatant from a 69 hour Solka floc fermentation of *Clostridium thermocellum* S-7-19 was dialyzed at 4°C overnight against 100x volume of CM4 salt solution at pH 6.7 in a cellulose acetate dialysis bag.

In addition, another sample of this supernatant was ultrafiltered through an Amicon PM-10 ultrafiltration

Table 43

GROWTH OF *CLOSTRIDIUM THERMOCELLUM* S-7-19 ON CENTRIFUGED BROTH FROM 69 HRS *CLOSTRIDIUM THERMOCELLUM* S-7-19 FERMENTATION OF 40 G/L SOLKA FLOC*

Treatment		ΔOD_{660} in.	
		Control Media	69 Hr Broth Media
None	(0.008 mg/ml)	1.20	0.40
Trypsin	(0.4 U /ml)	1.10	0.60
Protease	(0.06 mg/ml)	1.10	0.58
DNAase	(0.06 mg/ml)	1.2	0.48
RNAase	(0.06 mg/ml)	1.2	0.60

* pH of broth was re-adjusted to 7.0 and supplemented with 10 g/l cellobiose and 2 g/l yeast extract.

membrane with a 10,000 molecular weight cut off. Both the dialyzed and the ultrafiltrated broths were collected and supplemented with 10 g/l cellobiose and 2 g/l yeast extract as described before. These tubes and controls, as shown in Table 44, were inoculated with *Clostridium thermocellum* S-7-19. The resulting changes in optical density and reducing sugar consumption due to growth indicates that both dialysis and ultrafiltration treatments slightly improve the extent of growth on the broth. However, in each case, this level of growth is still 40% below that of the control.

These results tend to indicate that the active principle is probably of approximate molecular size below the exclusion limit of the 10,000 mw cut off the Amicon ultrafiltration device but non-dialyzable through cellulose acetate dialysis membranes (i.e., $> \approx 300$-10,000 mw). In any event, the retention of significant inhibition after dialysis appears to rule out the effects of a low molecular weight product of primary metabolism.

It is clear that the elucidation of an "auto" inhibitory component produced by *Clostridium thermocellum* which is not active against *Clostridium thermosaccharolyticum* may play a large role in the rapid loss of viability observed for these cells late in the fermentation of corn stover. The failure of Pronase or Trypsin to eliminate the inhibitory activity of the broth should not be taken as positive evidence

Table 44

GROWTH OF CLOSTRIDIUM THERMOCELLUM S-7-19 ON CENTRIFUGED FERMENTATION BROTH FROM CLOSTRIDIUM THERMOCELLUM S-7-19 (69 HRS SOLKA FLOC) AFTER DIALYSIS AND ULTRAFILTRATION

	ΔOD_{660} (18 hrs)	Consumption of Cellobiose
Control	1.3	10 g/l
Centrifuged Broth	0.32	2.6 g/l
Dialyzed Broth	0.49	4.8 g/l
PM10 Ultrafiltered Broth	0.52	5.8 g/l

* pH of broths were re-adjusted to 7.0 and media supplemented with 10 g/l cellobiose and 2 g/l yeast extract.

that the growth inhibitory factor is not a protein. A number of Clostridial autolysins of approximately 20,000 mw have been isolated of which roughly one-half are not Pronase-Trypsin labile (see Literature Survey). The results of ultrafiltration/dialysis experiments are consistent with a molecular size roughly equivalent to 1,000 to 10,000 mw. However, it is clear that further experimentation will be required in order to completely characterize the composition of the inhibitor.

The lack of a correlation between growth inhibitory activity and protein concentration appears to indicate that the active principle is probably not a component of the cellulase complex. In addition, the molecular size of the inhibitory factor of less than 10,000 molecular weight is significantly smaller than the size of the cellulase aggregate of 125,000 ± 10,000 mw. reported for this organism [20]. Although this complex was reported to consist of five discrete proteins, separation could only be accomplished under sodium dodecyl sulfate denaturing conditions. Thus, the native enzyme complex is believed to be significantly larger than the inhibitor. From these conclusions, there is no reason to believe that attempts to isolate hypercellulase producing strains will in any way be limited by the presence of this growth inhibitory factor.

5. SUMMARY AND CONCLUSIONS

The overall objective of this project has been the investigation of a mixed culture process for directly converting cellulosic biomass such as corn stover to ethanol. Mixed cultures of new strains of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* have been obtained which are capable of directly converting insoluble cellulose carbohydrates of Solka floc (> 85% cellulose) directly to ethanol in high yield and productivity. On Solka floc, 88% of the theoretical maximum ethanol yield based on substrate consumed has been achieved. However, less than half of the theoretical maximum ethanol yield and one-fourth the rate of substrate degradation are typically observed during growth of these cultures on realistic cellulosic feedstocks such as corn stover.

A number of approaches have been examined to increase both the rates of substrate degradation and ethanol yield of this mixed culture during growth on corn stover. The results of these investigations have demonstrated that the "lignin" component of corn stover exerts a toxicity towards *Clostridium thermosaccharolyticum* (HG-4). Lignin also presents a major physical and chemical resistance to substrate degradation by the enzymes of *Clostridium thermocellum*. These factors accounted for the low rates and extents of degradation and ethanol yields attained on this substrate. On the basis of these

findings, we proposed to develop new lignin resistant strains of Clostridium thermosaccharolyticum and examine alternate means for selective removal of lignin from corn stover.

5.1. Process Improvements

5.1.1. Corn Stover Pretreatment

Progress has been made on the development of an alkaline/ethanolic solvent extraction scheme for biomass pretreatment prior to fermentation. The best solvent system performed at 25°C containing 50% (V/V) ethanol in water and 0.2 N NaOH is capable of delignifying corn stover greater than 65% with less than a 5% loss of fermentable cellulose and pentosan carbohydrates. Mixed culture fermentation of stover pretreated in this manner has shown approximately a four-fold increase in rate of substrate degradation with greater than 80% utilization of carbohydrates.

5.1.2. Lignin Toxicity of Clostridium thermosaccharolyticum

A program of strain improvement during the course of the research resulted in a new generation of very high ethanol yielding strains of Clostridium thermosaccharolyticum. An examination of ethanol yields during xylose and corn stover hydrolysate fermentation by these new isolates in monoculture revealed only a slight toxic effect from residual lignin in

solvent extracted corn stover. Due to this finding, further strain selection to overcome residual lignin toxicity was not required.

5.1.3. Clostridium thermocellum Strain Degeneration

On the other hand, the performance of the cellulolytic strains of Clostridium thermocellum, such as S-7, varied erratically with respect to both ethanol yield and tolerance. This behavior was shown to be due to instability of the strains' properties during prolonged storage in liquid culture at 4°C.

In order to re-establish and maintain the desired strain characteristics, new methods of selection for high alcohol yielding isolates were developed. The most successful approach taken uitlized selection of low acid producing clones on solid agar medium with pH sensitive bromocresol purple indicator dye. The best isolate obtained by this method, S-7-19, performed at least as well as its parent, S-7, on cellobiose and in mixed culture on Solka floc with Clostridium thermosaccharolyticum HG-6-62. No variation in strain performance was observed during storage of freeze dried cultures below 4°C.

5.1.4. Strain Performance on Pretreated Corn Stover

The growth of Clostridium thermocellum S-7-19, examined in monoculture on pretreated corn stover, demon-

strated a high overall degradation rate of approximately 0.4 to 0.6 g/l hr and an ethanol to acetate ratio of approximately 2.6 to 1.0. This ratio, although improved considerably, is still below that achieved by S-7-19 on cellobiose of 6.5 to 1 and on Solka floc of 4.5 to 1. The growth of this strain in monoculture on the treated biomass was shown to be limited by the rate of hexose hydrolysis from cellulose.

Fermentation with new strains, *Clostridium thermocellum* S-7-19 and *Clostridium thermosaccharolyticum* HG-8, in mixed culture on non-treated stover, however, also show the typically low rates of substrate degradation of 0.15 g/l hr and ethanol to acetate ratio of approximately 1.5 to 1. The use of pretreated corn stover greatly improves the rate of overall substrate degradation to as high as 0.48 g/l hr, however, the ratio of ethanol to acetate has improved only to 2.5 to 1.

The ability of new strains of *Clostridium thermosaccharolyticum* to produce ethanol in high yield on the soluble pentosan carbohydrates produced by the growth of *Clostridium thermocellum* S-7-19 on extracted corn stover was also examined. These carbohydrates were shown by HPLC to be predominantly xylobiose with smaller amounts of xylose and arabinose. Fermentations of these sugars by *Clostridium thermosaccharolyticum*, HG-8, gave ethanol/acetate ratios of from 5 to as high as 12, showing that no intrinsic limitation exists

for high yields when this organism is rapidly consuming these previously solubilized sugars of corn stover.

5.2. Correlation of Ethanol Yield and Specific Growth Rate

One general feature characteristic of high ethanol yield with both *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* has been shown to be related to high specific growth rate. A large collection of batch data for *Clostridium thermocellum* S-7-19, S-7, and S-4 were examined. These strains were all shown to be capable of achieving to 5/1 to 8/1 E/A ratios when high specific growth rates can be achieved on cellobiose. From this data a plot of differential ethanol yield versus specific growth rate was generated. From the resulting correlation of ethanol yield with sp. growth rate shown by this plot we recognized the importance of operation at greater than approximately 0.05 hr^{-1} sp. growth rate in order to achieve greater than a 4:1 E/A ratio or 80% theoretical maximum ethanol yield with high yielding strains. A collection of similar data for *Clostridium thermosaccharolyticum* HG-6-62 and HG-8 showed a similar dependence of E/A on specific growth rate at approximately the same value. However, at higher growth rates, the new *Clostridium thermosaccharolyticum* strains appear to be able to achieve significantly higher ethanol yields than the *Clostridium thermocellum* S-7-19.

An examination of the specific growth rate achieved by each organism in mixed culture on pretreated corn stover was

made by calculations from a number of substrate hydrolysis experiments. The results indicate that the whole system is limited by the low rates of hexose solubilization on treated corn stover. At constant cellulase yield per cell, the level of "cellulase protein" produced and consequently its activity on the hexose fraction of the biomass became intrinsically limited. Thus, during monoculture growth on treated corn stover, the rate of reducing sugar accumulation is very high; however, no soluble glucose or cellobiose was observed. This behavior may be further aggravated in mixed culture where the higher relative rates of pentose production results in much greater production of *Clostridium thermosaccharolyticum* than *Clostridium thermocellum*. Here competition for the hexoses produced can result in lower cellulase levels and consequently diminished ethanol yields.

The competitive behavior of these two Clostridial strains for the solubilized hexoses produced is expected to be a strong function of the relative affinity for hexoses by each strain during growth under hydrolysis rate limited conditions. The relative magnitude of these saturation constants was examined by the fit to batch growth data obtained for a numerically integrated kinetic model which incorporated various values for the saturation constants. Excellent agreement was obtained with the assumption that the saturation constants for hexose are much lower for *Clostridium thermocellum* than for *Clostridium thermosaccharolyticum*. Subsequently, experimental results were

obtained for mixed culture fermentation of pretreated corn stover using a hexose non-fermenting strain of Clostridium thermosaccharolyticum. The performance demonstrated virtually identical behavior to that observed during mixed culture fermentation of the parent hexose utilizing strain. This result further substantiates our assumption for the relative values of the hexose uptake saturation constants.

The mixed culture kinetic model was further employed to examine the effectiveness for the use of hyper-cellulose producing mutants of Clostridium thermocellum with regard to ethanol yield achieved during mixed culture. In this case, it was shown that significant increases in the E/A ratio up to 4.0 to 1 could be achieved.

In order to confirm these conclusions in an independent fashion, the addition of higher levels of cellulase to mixed and monoculture fermentations of pretreated corn stover was tested. In these cases, high ethanol yields corresponding to E/A ratio of 4.8:1 directly from corn stover were obtained. These results supported our previous conclusions.

One should be cautious not to misinterpret the correlation obtained for ethanol yield with specific growth rate to mean that all strains of Clostridium thermocellum and Clostridium thermosaccharolyticum produce high ethanol yields when growing rapidly, and poor ethanol yields when growing slowly. For example, the parent strainsof Clostridium thermocellum ATCC 27405 and Clostridium thermosaccharolyticum HG-2 grow at quite

high rates (0.2-0.25 hr^{-1}) on soluble sugars, cellobiose and xylose, respectively, but are accompanied by low ethanol yields. The correlation was obtained with strains which all had previously been selected for the ability to achieve high ethanol yields under conditions of high growth rate (i.e., on soluble carbon sources). The decreased ethanol yields observed during slow growth on hydrolysis rate limiting substrates demonstrate that the regulatory alterations selected for are simply insufficient for the lower growth rate conditions presently achieved in mixed culture.

This conclusion, however, suggests a promising avenue for future research through the isolation of high ethanol yielding strains at lower growth rate. A number of methods to achieve this result are described in Section 6.

5.3. Growth Inhibitory Factor of *Clostridium thermocellum*

In the course of experimentation in relating the cellulase activity to growth rate and ethanol production, another potentially significant factor to the growth of *Clostridium thermocellum* was found. It has been previously shown that during *Clostridium thermocellum* fermentation of soluble or insoluble carbohydrates, a maximum upper limit in cell concentration of 2-3 g/l cell mass was routinely encountered. This was shown not to be due to a nutrient limitation or an end product toxicity. Instead, it appears that a soluble extracellular cell

product having an approximate molecular weight between 500 and 10,000 is released by Clostridium thermocellum at higher cell densities.

This ethanol or ammonium sulfate precipitable product inhibits further cell growth and causes a rapid lysis of the cell mass. A similar phenomenon has been observed with a number of different Clostridial species and has been described to the action of a "bacteriocin" or "autolysin". If the observations can be further substantiated and confirmed, they represent the first report of such an activity in Clostridium thermocellum. The presence of such an "autolytic" activity explains the rapid loss of cell viability observed during growth of Clostridium thermocellum S-7-19 on pretreated corn stover in both mixed and monoculture at the point in the fermentation where the diminished hexose hydrolysis rate is calculated to be insufficient to sustain growth.

From these results, it appears that this "autolytic" factor is the causative agent for the rapid viability loss, but it might be produced in response to unfavorable growth conditions (i.e., substrate starvation). On the other hand, this "autolytic" and "growth inhibitory" factor was also shown to be produced in cellobiose medium fermentations where growth is not apparently terminated by any discernable factors such as nutritional limitation or product toxicity. In monoculture fermentations of Solka floc, a strongly inhibitory fermentation broth

component is produced after approximately 45 hours of fermentation which totally prevents further growth of *Clostridium thermocellum*. This behavior may explain the unusually large production of soluble reducing sugars of predominantly cellobiose observed in the broth at later times of monoculture fermentations [16, 25].

6. RECOMMENDATIONS FOR FUTURE RESEARCH

6.1. Pretreatment

It is apparent that some degree of chemical pretreatment of biomass feedstocks will be required in order to achieve the utilization of a significant quantity of the utilizeable carbohydrates. The pretreatment process decided here was developed to optimize the selective delignification of cereal grasses and retain as much of the holocellulose carbohydrate as possible to serve as fermentation substrate. The pretreatment accomplishes these objectives and the subsequent fermentation performance is significantly improved with respect to the non-treated case.

However, one can hypothesize a number of potential avenues for further improvement of this extraction scheme. The successful removal of extracted lignin from the solvent for recycle operation is an important aspect of this process. This removal of lignin can be accomplished by a variety of alternate means such as distillation, calcium ion precipitation, or carbon adsorption. However, another interesting option may be found in the use of flocculation. Preliminary studies with flocculants, such as primafloc C-3, indicates a significant potential for lignin removal leading to solvent regeneration. However, the choice of ionic conditions, flocculant type, and concentration and temperature all remain to be optimized. In addition, one has to examine the effect of non-precipitable

lignin accumulation in the extraction solvent on subsequent extraction and fermentation performance after a number of recycles have been completed.

On the other hand, a different concept for selective pretreatment could be hypothesized. In principle, the use of a strong base in this process is required for two reasons. First, the polyphenolic lignin species are required to be in their deprotonated form to render them water soluble. Secondly, hydroxy anions are believed to be required as a catalyst for the deesterification reactions necessary for lignin removal from the surrounding carbohydrate matrix. At the same time, ethanol is required to prevent the alkali enhanced dissolution of hemicellulose from this structure. In contrast to this approach, a potential alternative selective pretreatment scheme could be envisioned which relies on the hydrophobic character of lignin for solubilization. Under these conditions, an organic solvent would be employed for extraction. In order to keep the phenolic groups protonated, an acid catalyst would be required for deesterification. A small amount of water would also presumably be required to drive this reaction. This approach to delignification is essentially embodied in the acid/dioxane extraction of lignin which has been described previously [137]. However, an economical and more practical choice of solvent system and conditions such as benzene at higher temperature may be shown to replace dioxane for partial selective delignification. The

choice of conditions will, of course, be strongly influenced by the kinetics of acid catalyzed hemicellulose hydrolysis and degradation which must be avoided. On the other hand, a low solubility of carbohydrate in these types of solvents is expected.

6.2. Increased Cellulase Activity/Yield

It has been shown that even after solvent pretreatment, corn stover is not converted into ethanol in as high a yield as the mixed culture organisms are capable of achieving from soluble carbohydrates or sulphite pulped cellulose such as Solka floc. It has been shown that the rate of hydrolysis of the material controls the growth rate attainable and thus the subsequent ethanol yield observed with these strains. From this conclusion and the results of modelling studies and cellulase addition experiments, we feel that the isolation of strains with higher specific cellulase activity will be useful to increase the specific rate of degradation and subsequent ethanol yield.

Presumably this could be accomplished by the isolation of strains with either increased specific cellulase yield (mg protein/g cell) or by the isolation of strains with altered cellulase specific activity (mg hexose/mg protein hour). It is logical to assume that the various enzymatic activity reported for the extracellular proteins of this organism are the result

of the primary activities of different enzymes in a mixture. If this is the case, the isolation of a strain with enhanced production of the limiting cellulolytic activity component for cellulose utilization seems to be a reasonable objective.

A rapid decrease was also observed for the specific rate of degradation of the cellulosic portion of pretreated corn stover with increasing extent of conversion. Due to this behavior, reductions in the specific growth rate occur with increasing fermentation time. Another desirable objective for improving cellulase production lies in increasing specific activity for degradation of this more recalcitrant portion of the substrate. An obvious method for isolation of strains with such improved cellulase properties may be achieved by using the more residual "core" cellulose remaining as substrate for further selection.

6.3. Improvements in Ethanol Yield

A number of programs have been successfully conducted for the improvement of ethanol yields achieved by both *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* when growing at high specific growth rates (Section 4.6) [16,22,129]. However, a clear conclusion from the results of both mixed and monoculture fermentations of corn stover under hydrolysis rate limiting conditions is that much lower rates of growth are ac-

tually achieved. Under these conditions, lower ethanol yields are also observed. If we assume that no intrinsic energetic limitation exists to the production of ethanol at high yield at low growth rates, then the reduction in yields observed might only be the product of regulatory events. If this is indeed the case, then the isolation of extremely useful high ethanol yielding strains of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* should be conducted at *low* growth rates. Of course, the most direct way in which this can be achieved with *Clostridium thermocellum* is on the substrate of interest. A typical protocol might be as follows. First, a large number of pyruvate negative colonies could be selected for (presumptive low acetate producers). These clones could subsequently be screened on acid visualizing BCP-dye media with corn stover or corn stover hydrolysate substrates as described in Section 4.6. In this manner, a high ethanol yielding strain at low growth rates may be selected. Alternative means of achieving reduced growth rates by reduction of the incubation temperature might be employed. In this case, larger numbers of both *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* may be more rapidly screened on cellobiose plate media with acid indicator dyes. A more powerful combination of methods may further be envisioned by the simultaneous use of low growth rate conditions and pyruvate selection. Acid indicator plate media containing high

concentrations (10-15 g/l) of pyruvate and low concentrations of cellobiose (~1 g/l) are used. On this differential media, pyruvate utilizers should be large colonies while clones only able to use cellobiose should be substantially smaller. After a counterselection step for enrichment for non-pyruvate utilizers, the survivors may be plated on this media and incubated at suboptimal temperatures. Under these conditions, small colonies with no acid halos represent ideal candidates for further mixed/ monoculture fermentation studies on corn stover.

6.4. Autolytic Activity

Methods such as those described previously for the isolation of higher ethanol yielding strains at low growth rates may be directly applied to hyper-cellulose producing mutants. However, the demonstration of "autolytic" behavior of *Clostridium thermocellum* through the release of a growth inhibitory factor may represent another limitation to achieving this objective. "Autolysis" may become especially problematical if the activity is produced under conditions of very slow substrate limited growth.

A rapid lysis of *Clostridium thermocellum* was demonstrated under substrate limiting conditions during both mixed and monoculture growth of *Clostridium thermocellum* on corn stover after approximately 50 hours. However, the fermentation broth from corn stover has not been tested further for "auto-

lytic" activity. In addition, a question still remains as to whether the "autolytic" component is the primary cause of rapid viability loss or is triggered by an as yet unknown factor. In any event, it is clear that isolation of strains of *Clostridium thermocellum* which are able to grow under the slow conditions afforded by real biomass substrate hydrolysis without "inducing" the production of this component and rapidly lysing may be useful. The problematic nature of autolysis may become even more apparent during fermentation at increased substrate density. In order to achieve higher ethanol concentrations, *Clostridium thermocellum* must be able to grow to higher cell densities on larger quantities of substrate. A reasonable goal for this fermentation process is the production of ethanol at 50 g/l in the broth. In order to achieve this, approximately 200 g/l pretreated corn stover must be fermented at 80% ethanol yield and with 80% utilization of carbohydrate. If 60% of the cellulose is consumed (48 g/l) over 4 g/l of cell mass of *Clostridium thermocellum* should be produced (assuming a yield of 0.1 g cells/g substrate). To date, the highest cell density achieved by *Clostridium thermocellum* has been approximately 2.5 g/l cell mass during rapid growth on cellobiose.

There are two basic approaches which one could envision to overcome this "autolysis" phenomenon. Through a straightforward method of adaptation and selection of a mutagenized population it may be possible to achieve resistance to

the effects of this growth inhibitory substance. If this factor is indeed an "autolysis" typical of clostridia, mutation of cell wall lipopolysaccharides or the formation of a "slime" capsule may provide the necessary degree of protection from this activity. Alternatively, strains may be isolated which are unable to produce the active component. If this activity is indeed an autolytic cell wall digestion enzyme, a number of screens for non-producers may be envisioned. One such procedure developed by Woods, relies on the synergistic effects of penicillin and autolysin [138]. By using solid medium containing sublethal levels of penicillin it may be possible to isolate strains which do not produce autolysin. Autolysin producers under these conditions would rapidly lyse due to the combined action of penicillin and enzyme. Using this technique, Woods isolated an autolysin deficient strain of Clostridium acetobutylicum. Another method for isolation may be the use of autolysin indicator reagents. Isolated cell walls of Clostridium thermocellum may be easily chemically coupled to Reactive Red or Reactive Blue dyes. These cell wall preparations may then be incorporated into agar media and used as an indicator for autolysis activity. Colonies which do not produce red or blue zones of clearing may be autolysin deficient. Using this approach, Forsberg and Rogers isolated a number of mutants of Bacillus licheniformis which also lacked this autolysis activity [139].

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APPENDIX A

A number of computer programs were written to aid in data analysis and interpretation. A brief explanation of each program is given below. A complete listing for each program may be found in the following pages.

DATAAN

This program was used for the analysis of batch experimental data from monoculture fermentation of *Clostridium thermocellum*. Experimental values of ethanol, acetate, acid hydrolyzed reducing sugars produced during the fermentation are used with the initial cell density to calculate cell mass, enzyme production, and specific cellulose and hemicellulose hydrolysis rates at various points in the fermentation. Assumption for cell yield and maintenance as well as enzyme yield from cells are used as described in Section 4.8.3.

GEOMM2

This program is a batch monoculture fermentation simulation program for *Clostridium thermocellum* growth on pretreated corn stover as described in Section 4.8.3.

GEOMMA

This is a mixed culture batch fermentation simulation program for the fermentation of pretreated corn stover by *Clostridium*

<u>thermocellum</u> and <u>Clostridium</u> <u>thermosaccharolyticum</u>. The model incorporates an assumption for the saturation constants for hexose uptake for <u>Clostridium</u> <u>thermocellum</u> equal to that of <u>Clostridium</u> <u>thermosaccharolyticum</u> as described in Section 4.8.4.

<u>GEOMMB</u>

GEOMMB is identical to GEOMMA with the exception that a saturation constant for hexose uptake by <u>Clostridium</u> <u>thermocellum</u> is assumed to be much lower than that for <u>Clostridium</u> <u>thermosaccharolyticum</u>.

```
LIST

DATAAN  08-JAN-82  BASIC/CAPS  V01-01

10 REMDATA ANALYSIS PROGRAM FOR THE DETERMINATION OF CELL MASS, ENZYME
11 REM PRODUCTION,AND SPECIFIC HEXOSE AND PENTOSE DEGRADATION RATES
13 REMFROM BATCH PRODUCT AND REDUCING SUGAR PRODUCTION ON INSOLUBLE SUBSTRATES
14 REM THIS ROUTINE FOR CELL MASS CALCULATION  ITERITIVELY  FINDS THE
15 REMGROWTH RATE IN EACH INTERVAL FROM MATERIAL BALANCING EQUATIONS WHICH
16 REM INCLUDE A CORRECTION FOR THE MAINTAINANCE ENERGY CONSUMPTION
20 REM ITERATIVE CONVERGENCE ROUTINE
30 T0=0
32 H0=21 \ P0=19 \ X1=.1
35 P1=0 \ H1=0 \ O1=0 \ A1=0
36 E1=.24
39 PRINT
40 PRINT "T2,P2,O2,A2"
41 INPUT T2,P2,O2,A2
42 PRINT "DATA CORRECT? (1/0)" \ INPUT K
43 IF K=1 THEN  GO TO 45
44 GO TO 39
45 O5=O2-O1 \ A5=A2-A1 \ P5=P2-P1 \ Z5=O5/A5
48 H5=((O5*2)+(A5*1.5))/.85
50 T3=T2-T1 \ H2=H1+H5
80 Z=999
84 M=.2
90 Y2=.01
95 I=0
100 REM SECTION FOR CONVERGENCE ROUTINE
105 GO TO 210
110 M3=10*(H5/(T3*X1)-M)
111 IF M3<=0 THEN M3=.05
112 IF M3>.25 THEN M3=.25
120 D2=(X1/2)*(1+EXP(M3*T3))
125 M4=10*(H5/(T3*D2)-M)
126 PRINT M3,M4
128 Y1=ABS((M3-M4)/M4)
129 IF Y1<Y2 GO TO 150
130 X2=X1*EXP(M1)
132 I=I+1
133 IF I>Z GO TO 150
134 Y1=ABS((X3-X4)/X3)
135 IF Y1<Y2 GO TO 15
140 GO TO 210
150 REM FINISHED ITERATION
151 D2=X3
153 IF X2>X1 THEN E1=.24*D2
154 PRINT "ITERATIONS TO CONVERGENCE"I
155 T4=(T1+T2)/2
160 REM SECTION FOR UPDATING VALUES
162 H9=H5/(T3*E1)
163 P9=P5/(T3*E1)
164 H8=1-(H2/H0)
165 P8=1-(P2/P0)
170 REM SECTION FOR UPDATING INITIAL CONDITIONS
172 T1=T2 \ P1=P2 \ H1=H2 \ O1=O2 \ A1=A2
173 X1=X2
200 REM SECTION FOR PRINTING INTERMEDIATE RESULTS
202 PRINT "TIME",T4,"CTC AVG",D2
204 PRINT "ENZ AVG",E1
205 PRINT "MU CTC AVG",M3,"DE/DA",Z5
206 PRINT "1/H DH/DT",H9,"H/H0",H8
208 PRINT "1/P D/DT",P9,"P/P0",P8
209 GO TO 309
210 D1=H5/T3
211 X3=X1
222 M3=(D1-M*X3)/(10*X3)
225 M1=M3*(T2-T1)
230 X2=X1*EXP(M1)
231 I=I+1
232 IF I=Z THEN  GO TO 150
233 X4=(X1+X2)/2
234 Y1=ABS((X3-X4)/X3)
235 IF Y1<Y2 GO TO 150
236 PRINT X1,X2,X3,X4
237 X3=(X1+X2)/2
240 GO TO 222
309 PRINT "*********************************************************"
310 PRINT "CONTINUE? (1/0)" \ INPUT N
311 IF N=1 THEN  GO TO 39
312 PRINT "*********    END OF DATA ANALYSIS FOR THIS FERMENTATION    **********"
313 END
```

```
GEOMM2  08-JAN-82  BASIC/CAPS  V01-01

1 REM THIS IS A BATCH CULTURE SIMULATION PROGRAM FOR THE GROWTH OF
2 REM CTC ON PRETREATED CORN STOVER
3 REM THE PROGRAM IS DIVIDED INTO FIVE SECTIONS
4 REM 1) INITIALIZATION SECTION -10
5 REM2)CALCULATION OF DERIVATIVES -100
6 REM 3)CALCULATION OF INTERMEDIATE VALUES -200
7 REM4)INTEGRATION BY EULERS FMETHOD -300
8 REM5)STEPPING OF PROGRAM -400
9 REM&PRINTING OF INTERMEDIATE RESULTS -500
10 REM SECTION FOR INITIALIZATION OF THE PROGRAM VARIABLES

20 REM FERMENTATION CONDITIONS
21 REM SUBSTRATE
22 H0=21
23 P0=19
25 REM CELL CONCENTRATION ATT TO
26 C1=.1
27 C3=0
28 E1=.024
29 A5=0 \ A4=0 \ A2=0
30 O5=0 \ O4=0 \ O2=0
31 K1=4
32 K2=.24
33 K3=5
40 REM PROGRAM INITIALIZATION MAINTAINANCE
41 T2=0
42 D=0
43 T1=.1
44 REM T1 IS STEP SIZE
81 H1=H0
82 P1=P0
88 J$="MIXED CULTURE SIMULATION STUDY    G. AVGERINOS      RUN #"
90 REM PRINT INITIALIZATION VALUES
91 PRINT J$ \ PRINT  \ PRINT
92 PRINT "HEXOSE="H0,"PENTOSE="P0,"CTC="C1,"CTS="C3,
95 PRINT "KH="K1,"KP="K3,"KE="K2
96 PRINT "ENZYME 0="E1
97 PRINT  \ PRINT  \ PRINT
98 F$="TIME   CTC  MCTC    CTS  MCTS   ENZ    HEX    PEN    ETOH    ACET"
99 PRINT F$ \ PRINT  \ PRINT
100 REM  CALCULATION OF DERIVATIVES
105 S1=K1/.6
106 B1=K1-S1
108 Y1=S1*H1/H0+B1
115 H2=Y1*E1
120 Y2=K3*P1/P0
122 F2=E1*Y2
```

```
125 REM CALCULATION OF CELL MASS OF CTC
127 H5=H2-.2*C1
128 C2=H5*.1
130 C8=C2/C1
150 G2=H2*.9
153 R2=1.2+60*C8
154 IF R2>10 THEN R2=10
155 A2=G2/(1.5+2*R2)
160 O2=A2*R2
165 G4=C4*9.5
167 R4=1+50*C9
168 IF R4>25 THEN R4=25
170 A4=G4/(1.5+2*R4)
175 O4=A4*R4
200 REM SECTION FOR CALCULATION OF INTERMEDIATE VARIABLRS
300 REM SECTION FOR CALCULATION OF SUMS
302 C1=C1+T1*C2
303 C3=C3+T1*C4
305 IF C2>0 THEN E1=K2*C1
311 H1=H1-T1*H2
322 P1=P1-T1*P2
354 IF O2<0 THEN O2=0 \ IF O4<0 THEN O4=0
355 O5=O5+T1*(O2+O4)
365 IF A2<0 THEN A2=0 \ IF A4<0 THEN A4=0
366 A5=A5+T1*(A2+A4)
388 IF H1<.1 THEN LET H1=0
399 IF P1<.1 THEN P1=0
400 REM SECTION FOR STEPPING OF FPROGRAM
411 T2=T2+T1
415 IF T2>210 THEN  GO TO 600
422 D=D+1
433 IF D=100 GO TO 500
440 IF T2=.2 GO TO 500
444 GO TO 100
500 REM SECTION FOR PRINTING OF INTERMEDIATE RESULTS
503 A$="-##   -#.#   -.###   -#.#   -.###   -.##   -##.#   -##.#   -##.#   -##.#"
505 PRINT USING A$,T2,C1,C8,C3,C9,E1,H1,P1,O5,A5
511 D=0
599 GO TO 100
600 END
705 Z=500
```

```
LIST

GEOMMA  08-JAN-82  BASIC/CAPS  V01-01

1 REM BATCH MIXED CULTURE SIMULATION FOR CTC AND CTS ASSUMING KS FOR
2 REM HEXOSE FOR EACH STRAIN IS APPROXIMATELY EQUAL
3 REM THE PROGRAM IS DIVIDED INTO FIVE SECTIONS
4 REM 1) INITIALIZATION SECTION -10
5 REM2)CALCULATION OF DERIVATIVES -100
6 REM 3)CALCULATION OF INTERMEDIATE VALUES -200
7 REM4)INTEGRATION BY EULERS FMETHOD -300
8 REM5)STEPPING OF PROGRAM -400
9 REM&PRINTING OF INTERMEDIATE RESULTS -500
10 REM SECTION FOR INITIALIZATION OF THE PROGRAM VARIABLES
20 REM FERMENTATION CONDITIONS
21 REM SUBSTRATE
22 H0=21
23 P0=19
25 REM CELL CONCENTRATION AT INITIAL CONDITIONS
26 C1=.1
27 C3=.1
28 E1=.024
29 A5=0 \ A4=0 \ A2=0
30 O5=0 \ O4=0 \ O2=0
31 K1=4
32 K2=.24
33 K3=5
40 REM PROGRAM INITIALIZATION MAINTAINANCE
41 T2=0
42 D=0
43 T1=.1
44 REM T1 IS STEP SIZE
81 H1=H0
82 P1=P0
88 J$="MIXED CULTURE SIMULATION STUDY    G. AVGERINOS      RUN #"
90 REM PRINT INITIALIZATION VALUES
91 PRINT J$ \ PRINT  \ PRINT
92 PRINT "HEXOSE="H0,"PENTOSE="P0,"CTC="C1,"CTS="C3,
95 PRINT "KH="K1,"KP="K3,"KE="K2
96 PRINT "ENZYME 0="E1
97 PRINT  \ PRINT  \ PRINT
98 F$="TIME   CTC  MCTC    CTS  MCTS   ENZ   HEX    PEN    ETOH   ACET"
99 PRINT F$ \ PRINT  \ PRINT
100 REM  CALCULATION OF DERIVATIVES
105 S1=K1/.6
106 B1=K1-S1
108 Y1=S1*H1/H0+B1
115 H2=Y1*E1
120 Y2=K3*P1/P0
122 P2=E1*Y2
123 H3=H2*(C1/(C1+C3))
124 P3=H2*(C3/(C1+C3))
```

```
125 REM CALCULATION OF CELL MASS OF CTC
126 P3=P3+P2
127 H5=H3-.2*C1
128 C2=H5*.1
130 C8=C2/C1
132 P5=P3-.2*C3
134 C4=P5*.1
140 C9=C4/C3
142 G4=P2*.9
150 G2=H3*.9
153 R2=1.2+60*C8
154 IF R2>10 THEN R2=10
155 A2=G2/(1.5+2*R2)
160 O2=A2*R2
165 G4=P3*.9
167 R4=1+50*C9
168 IF R4>25 THEN R4=25
170 A4=G4/(1.5+2*R4)
175 O4=A4*R4
200 REM SECTION FOR CALCULATION OF INTERMEDIATE VARIABLRS
300 REM SECTION FOR CALCULATION OF SUMS
302 C1=C1+T1*C2
303 C3=C3+T1*C4
305 IF C2>0 THEN E1=K2*C1
311 H1=H1-T1*H2
322 P1=P1-T1*P2
354 IF O2<0 THEN O2=0 \ IF O4<0 THEN O4=0
355 O5=O5+T1*(O2+O4)
365 IF A2<0 THEN A2=0 \ IF A4<0 THEN A4=0
366 A5=A5+T1*(A2+A4)
388 IF H1<.1 THEN LET H1=0
399 IF P1<.1 THEN P1=0
400 REM SECTION FOR STEPPING OF FPROGRAM
411 T2=T2+T1
415 IF T2>200 THEN  GO TO 600
422 D=D+1
433 IF D=100 GO TO 500
440 IF T2=.2 GO TO 500
444 GO TO 100
500 REM SECTION FOR PRINTING OF INTERMEDIATE RESULTS
503 A$="-##   -#.#  -.###  -#.#  -.###  -.##  -##.#  -##.#  -##.#  -##.#"
505 PRINT USING A$,T2,C1,C8,C3,C9,E1,H1,P1,O5,A5
511 D=0
599 GO TO 100
600 END
```

```
LIST

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1 REM BATCH MIXED CULTURE SIMULATION PROGRAM FOR CTC AND CTS
2 REM ASSUMES KS FOR HEXOSE FOR CTC MUCH LOWER THAN KS FOR HEXOSE FOR CTS
3 REM THE PROGRAM IS DIVIDED INTO FIVE SECTIONS
4 REM 1) INITIALIZATION SECTION -10
5 REM2)CALCULATION OF DERIVATIVES -100
6 REM 3)CALCULATION OF INTERMEDIATE VALUES -200
7 REM4)INTEGRATION BY EULERS FMETHOD -300
8 REM5)STEPPING OF PROGRAM -400
9 REM&PRINTING OF INTERMEDIATE RESULTS -500
10 REM SECTION FOR INITIALIZATION OF THE PROGRAM VARIABLES
20 REM FERMENTATION CONDITIONS
21 REM SUBSTRATE
22 H0=21
23 P0=19
25 REMINITIAL CONDITIONS OF CELL MASS, ENZYME
26 C1=.1
27 C3=.1
28 E1=.024
29 A5=0 \ A4=0 \ A2=0
30 O5=0 \ O4=0 \ O2=0
31 K1=4
32 K2=.24
33 K3=5
40 REM PROGRAM INITIALIZATION MAINTAINANCE
41 T2=0
42 D=0
43 T1=.1
44 REM T1 IS STEP SIZE
81 H1=H0
82 P1=P0
88 J$="MIXED CULTURE SIMULATION STUDY    G. AVGERINOS      RUN #"
90 REM PRINT INITIALIZATION VALUES
91 PRINT J$ \ PRINT  \ PRINT
92 PRINT "HEXOSE="H0,"PENTOSE="P0,"CTC="C1,"CTS="C3,
95 PRINT "KH="K1,"KP="K3,"KE="K2
96 PRINT "ENZYME 0="E1
97 PRINT  \ PRINT  \ PRINT
98 F$="TIME   CTC  MCTC    CTS  MCTS   ENZ   HEX    PEN    ETOH   ACET"
99 PRINT F$ \ PRINT  \ PRINT
100 REM  CALCULATION OF DERIVATIVES
105 S1=K1/.6
106 B1=K1-S1
108 Y1=S1*H1/H0+B1
115 H2=Y1*E1
120 Y2=K3*P1/P0
122 P2=E1*Y2
```

```
125 REM CALCULATION OF CELL MASS OF CTC
127 H5=H2-.2*C1
128 C2=H5*.1
130 C8=C2/C1
132 P5=P2-.2*C3
134 C4=P5*.1
140 C9=C4/C3
142 G4=P2*.9
150 G2=H2*.9
153 R2=1.2+60*C8
154 IF R2>10 THEN R2=10
155 A2=G2/(1.5+2*R2)
160 O2=A2*R2
165 G4=P2*.9
167 R4=1+50*C9

168 IF R4>25 THEN R4=25

170 A4=G4/(1.5+2*R4)
175 O4=A4*R4
200 REM SECTION FOR CALCULATION OF INTERMEDIATE VARIABLES
300 REM SECTION FOR CALCULATION OF SUMS
302 C1=C1+T1*C2
303 C3=C3+T1*C4
305 IF C2>0 THEN E1=K2*C1
311 H1=H1-T1*H2
322 P1=P1-T1*P2
354 IF O2<0 THEN O2=0 \ IF O4<0 THEN O4=0
355 O5=O5+T1*(O2+O4)
365 IF A2<0 THEN A2=0 \ IF A4<0 THEN A4=0
366 A5=A5+T1*(A2+A4)
388 IF H1<.1 THEN LET H1=0
399 IF P1<.1 THEN P1=0
400 REM SECTION FOR STEPPING OF PROGRAM
411 T2=T2+T1
415 IF T2>210 THEN  GO TO 600
422 D=D+1
433 IF D=100 GO TO 500
440 IF T2=.2 GO TO 500
444 GO TO 100
500 REM SECTION FOR PRINTING OF INTERMEDIATE RESULTS
503 A$="-##   -#.#  -.###  -#.#  -.###  -.##  -##.#  -##.#  -##.#  -##.#"
505 PRINT USING A$,T2,C1,C8,C3,C9,E1,H1,P1,O5,A5
511 D=0
599 GO TO 100
600 END
```

BIOGRAPHICAL SKETCH

George Avgerinos was born March 1, 1953, in Wilton, Connecticut. After graduating Wilton public schools, he attended the University of Connecticut. In a chemistry class, he met a coed named Stephanie, who always seemed to have the right homework answers, so he graduated in 1975. At the University of Connecticut, two exciting summers were spent learning chemistry and, in his spare time, working in the lab with Professor Bob Vinopal researching energetics of amino acid uptake by *Salmonella typhimurium* by genetic analysis.

Taking Bob's and his colleague, Antonio Romono's, advice, he attended M.I.T. in 1976. There, working with Professor D. I. C. Wang on hollow fiber reactor technology for enzymatic Gramicidin S synthesis, he received a Master of Science degree in Biochemical Engineering in 1979, taking time out to marry Stephanie in 1977.

Continuing his studies, he received a Master of Science degree in Chemical Engineering in 1979 and Ph.D. in January 1982.