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ENZYMATIC HYDROLYSIS OF RICE STRAW FOR ETHANOL PRODUCTION

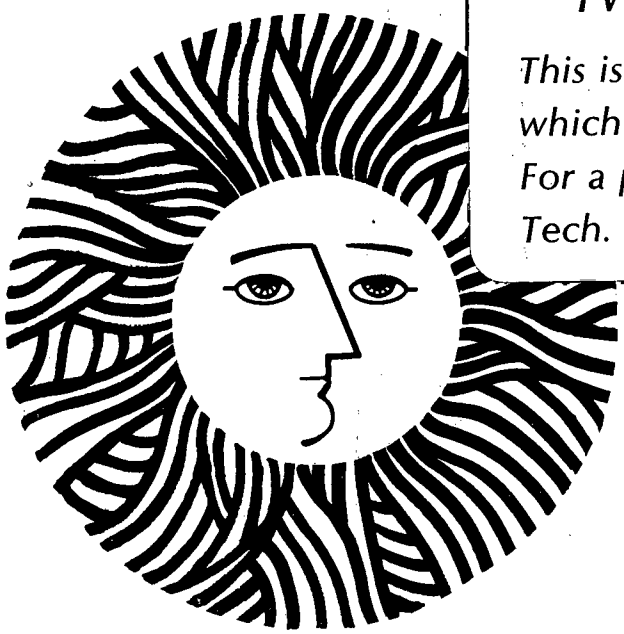
Stephen Alan Wald*, Charles R. Wilke,
and Harvey W. Blanch
(*M.S. thesis)

July 1981

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ENZYMATIC HYDROLYSIS OF RICE STRAW FOR ETHANOL PRODUCTION

Stephen Alan Wald*

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*M.S. Thesis

July 1981

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ABSTRACT

This study is concerned with an agricultural residue, rice straw, and its potential as a feedstock for ethanol production. Disposal of the rice straw is required to control the fungal infection called stem rot. The objective of this research was aimed at demonstrating the technical feasibility of ethanol production from rice straw and providing an economic evaluation of a proposed processing scheme.

Enzymatic hydrolysis experiments indicated that up to 60% of the available cellulose can be converted to a fermentable sugar solution. A kinetic model was developed to aid in understanding the hydrolysis process and for use in process optimization studies. The model incorporates an enzyme adsorption mechanism, product inhibition, and considers a multiple enzyme and substrate system.

Economic evaluation of the proposed processing scheme shows that ethanol can be produced for $2.56 per gallon with an additional raw material cost of $1.43 per gallon. This was based on nominal capacity of 10 million gallons of 95% ethanol annually and a rice straw cost of $30 per dry ton.

It is recommended that future research should focus on improving pretreatment and enzyme production techniques. In addition, more emphasis should be placed on effective utilization of all the components of the lignocellulosic material.

Enzymatic Hydrolysis of Rice Straw for Ethanol Production

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I. INTRODUCTION

It has been estimated that the annual worldwide production of cellulose through photosynthesis may approach 10^{11} metric tons, with up to 25% of this available for processing (35). The potential of this renewable resource has been recognized and has spurred a large amount of research activity in the field. The hydrolysis of the cellulose for sugar production can be accomplished by acid, enzymes, or direct microbial attack. The main product of microbial attack is single-cell protein. Acid hydrolysis yields a sugar solution but is subject to decomposition products because of the harsh reaction conditions. Enzymatic hydrolysis is preferable because of its ability to reuse the enzyme, the mild operating conditions, and the specificity of the enzymes.

A significant fraction of the available cellulose occurs as agricultural and municipal wastes. This study is concerned with an agricultural waste material, rice straw, and its potential as a feedstock for the production of ethanol. About 1.5 million tons of rice straw are produced each year in the Sacramento Valley. Disposal of the rice straw is crucial in the control of stem rot disease, a fungal infection caused by Schlerotium oryzae. Rice losses of 10 to 12 percent are common and have been as high as 50 percent as a result of this disease. The current disposal method is field burning of the straw. Environmental considerations are making it necessary to find an

alternative to open field burning. The production of ethanol from rice straw has been proposed.

The objective of this study was aimed at demonstrating the technical feasibility of ethanol production from rice straw and providing an economic evaluation of a proposed processing scheme. Hydrolysis experiments were conducted to evaluate the effect of several design parameters on the system. Results of these experiments, in conjunction with fermentation studies, were the basis for a preliminary process design. An economic evaluation of this design was used to evaluate the feasibility of this disposal option and identify key areas for further investigation.

II. BACKGROUND

2.1. Chemical Constituents of Rice Straw

Rice straw is composed of four main classes of components: 1)carbohydrates, 2)lignins, 3)extractives, and 4)inorganics. Table 2.1 shows the complete composition of native and acid treated rice straw (99). Cellulose, the main constituent, is an unbranched polymer of glucose units linked by β(1-4) glucosidic bonds. The degree of polymerization varies widely depending on the origin of the cellulose. The average degree of polymerization has been reported as 1000-2000 for wood pulp, 3000-5000 for cotton linters, 3000 for wheat straw (108), and 10000-14000 for α-cellulose (83).

The other main carbohydrate is hemicellulose, a branched heteropolymer of glucose, xylose, galactose, mannose, and arabinose, of relatively short chain length. Lignin is an aliphatic-aromatic polymer of oxygen substituted phenylpropane units. It acts as binding agent between adjacent fibers and sheaths the carbohydrates. Extractives can be divided into three groups: terpenes, resins, and phenols. Inorganic compounds, in the case of rice straw, comprise up to 12% of the dry weight. They are largely alkali carbonates and silicates (97).

2.1.1. The Structure of Cellulose:

The structure of cellulose fibers has been studied extensively for wood products. Wood fibers have a primary

Table 2.1. Composition of rice straw (99).

	Rice Straw	Acid Treated* Rice Straw
Glucan	0.37 (0.41)+	0.51 (0.57)
Xylan	0.13 (0.15)	0.05 (0.06)
Arabinan	0.04 (0.05)	0.02 (0.02)
Lignin	0.10	0.14
Extractives	0.04	0.06
Ash	0.12	0.17

+ Sugar equivalents in parentheses
* See Section 3.4.1

wall that surrounds a relatively thick secondary wall. The secondary wall consists of three layers, where the cellulose and other constituents are aggregated into long bundles called microfibrils. There are ordered (crystalline) and less ordered (amorphous) regions in these bundles. Highly ordered regions are possible because the β-configuration makes the hydroxyl bonds align in the same plane. This allows hydrogen bonding to occur.

There are several theories relating to the structure of the microfibrils. Preston and Cronshaw proposed that the microfibrils are about 50 x 100A in cross section and consist of a "crystalline core" surrounded by a "paracrystalline sheath", which contains hemicellulose and lignin (88). Hess et al. suggested that the cellulose molecules are segmented into crystalline and paracrystalline regions that alternate along the length of the microfibril (41). Manley proposed that the cellulose molecule is first folded into a flat ribbon which in turn is wound into a helix (72). According to Chang, single cellulose molecules are folded back and forth in the (101) lattice plane to form "platellites". The glucosidic bonds at the folds are the weak links due to the loop configuration of the chain (17). There is some controversy about the real struture because the microfibrils are not visible in wood fibers.

Regardless of the model chosen, cellulose is made up of crystalline or accessible regions and less ordered,

amorphous regions. Measurement of the ordered fraction of cellulose has been attemped, however, different methods yield different values. Table 2.2 shows this variablility. The ordered fraction ranged from 40 to 90% depending upon the method. All methods, however, rank cotton, wood pulps, mercerized cotton and regenerated cellulose in decreasing levels of order. X-ray diffraction will delineate well ordered regions above a minimum size, while density measurements allow the detection of regions of intermediate order. Chemical and sorption methods, which depend on hydrogen breaking ability, yield values dependent on the reagent used. Water vapor will be able to penetrate farther into ordered regions than liquid systems (111). Nevertheless, these independent methods may be combined together to yield a relative degree of crystallinity.

The characterization of cellulose into regions of order and disorder is thus a function of the method used. In addition, the terminology is not clearly defined. For example, crystallographic studies refer to Cellulose I and II as the crystalline regions with respect to their lattice structure. For the purposes of this study of enzymatic degradation of cellulose, cellulose can be divided in two regions, differing in their resistivity to enzymatic degradation. This may result from differences in crystal structure or accessibility to the enzyme.

2.1.2. The Effect of Pretreatment:

Many lignocellulosic wastes in their natural state

Table 2.2. Measurement of the degree of crystallinity by various techniques (114).

Technique	Cotton	Mercerized cotton	Wood Pulp	Regenerated celluloses
X-ray diffraction	0.73	0.51	0.60	0.35
Density	0.64	0.36	0.50	0.35
Moisture regain	0.58	0.41	0.45	0.25
Acid hydrolysis	0.90	0.80	0.85	0.70
Periodate oxidation	0.92	0.90	0.92	0.80
Iodine sorption	0.87	0.68	0.85	0.60
Formylation	0.79	0.65	0.75	0.35

are quite resistant to enzymatic hydrolysis. Studies of the digestibility of cellulosic materials by ruminants reflect this problem. This resulted in a search for an effective and inexpensive pretreatment to increase digestibility. Studies showed that fine grinding or ball milling (74) and alkali treatment (7) were effective. This early work demonstrated two different approaches to pretreatment--physical and chemical.

Physical Treatments-

The two major techniques used in this area are roll and ball milling. Ball milling increases surface area and destroys a portion of the ordered structure of the cellulose. Ball milled newspaper yielded about 84% carbohydrate conversion compared to 25% conversion for untreated material (100). The major reason for the increased susceptibility, according to Caulfield and Moore, is decreased particle size and increased surface area (16), while Walseth argued that crystallinity is the most important factor (113).

Two roll compression milling was investigated by Tassinari and Macy (107). This treatment has demonstrated increases in susceptibility over untreated controls in the range from eleven fold for cotton to 1.25 fold for newsprint. The wet density of the product is also increased to permit higher slurry concentrations during hydrolysis.

Another technique studied is simultaneous wet milling and enzymatic hydrolysis (53). This process is an

improvement over ball milling. As new surface area is exposed during saccharification, the crystalline structure is continuously disrupted. These physical treatments are successful in increasing the hydrolysis rate and conversion; however, their economic and industrial feasibility requires further investigation.

Chemical Treatments-

Chemical methods are aimed at disrupting the crystalline structure by intracrystalline swelling or partial delignification. It has been established that the association of lignin with cellulose decreases its accessibility to enzymatic attack (14). Swelling by sodium hydroxide and ammonia are two methods under investigation. Toyama and Ogawa delignified rice straw by using 1% sodium hydroxide at 80^{o}C for three hours (109,110). They reported complete delignification and the susceptibility of the holocellulose to hydrolysis was increased markedly. Work by Sciamanna et al. on rice straw showed that base treatment of acid treated material removed only 60% of the lignin and about 40% of the sodium hydroxide was consumed by the depolymerization reaction (100). The carbohydrate yield of the base treated material increased to 60.8% from 52.0% for the control.

Chlorite-acetic acid (14), ethanol (117), ethylene glycol (117), and nitric oxide with alkali (10) have been studied as delignifiers. In addition, cadoxen, a solution of 5-7% cadium oxide in 38% aqueous ethylene diamine, has

been studied to dissolve the cellulose (58). The reprecipitated cellulose has been enzymatically hydrolyzed with glucose yields up to 90%. However, all of these delignification procedures exhibit dubious economic feasibilty due to high reagent costs.

Acid Pretreatment-

The use of dilute acid to hydrolyze the hemicellulose fraction has been common in the pulping industry. The accessibilty of the cellulose can be increased by the removal of the hemicellulose fraction (30). The work of Dunning and Lathrop (23) was the basis for the pretreatment method employed in the Berkeley process (100). Milled rice straw was treated with 0.09M sulfuric acid at 100°C for 5.5 hours. Over 70% of the pentosans are hydolyzed to yield mainly xylose. This process increases the reaction rate and the wet density of the material. In addition, the cellulose concentration is higher (57%) in the solids resulting in reduced material handling requirements.

Another dilute acid treatment is a high temperature, short contact time process developed at Dartmouth College. The short contact time minimizes degradation and subsequent hydrolysis results in almost complete cellulose conversion (57).

Steam Explosion-

A new approach to pretreatment that exhibits great promise is explosive steam decompression. The Masonite Company developed a process that heated wood chips with 600

psi steam for 10 minutes and quickly released the pressure to atmospheric (28). This resulted in a material suitable as an animal feed supplement. The Iotech Corporation has developed a similar process with 500-1000 psi steam and contact times of 5-300 seconds that has resulted in saccharication yields of over 60% from populus wood (49). Preliminary work at Berkeley with Iotech exploded corn stover has demonstrated glucose yields of 70-85% compared to 50-60% yields for acid treated material (86). Control of decomposition products (furfural and hydroxymethylfurfural) and the effect of the solubilized lignin on fermentation are problems that must be addressed.

2.2 The Cellulase Enzyme System

2.2.1. Strain Selecton and History:

About 1950, Reese and his coworkers at the Natick Laboratories isolated a *Trichoderma* strain which had an active and well-balanced extracellular cellulase complex. Cellulolytic activity was also found in cultures of *Pencillium funiculosum* (5), *Irpex lacteus* (52), *Sporotrichum pulveruleatum* (25), *Trichoderma koningii* (120), *Thermomonospora* (27), and many others (64). Most studies, however, have utilized *Trichoderma reesei* (*T. viride*). The *Trichoderma* strain has the advantage of a complete enzyme system required for the degradation of crystalline cellulose and is stable at 45^{o}C and pH 5 for 48 hours or longer. The disadvantages are that the strain does not digest lignin,

the enzyme has a low specific activity and it is product inhibited.

The wild type of T. reesei was isolated at Natick (QM6a) and mutant selection was conducted by Montanencourt and Everleigh (78) at Rutgers University. Figure 2.1 shows the geneology of the strains examined. These strains were compared when grown on 6% rolled milled cotton and controlled at above pH 3.0. Table 2.3 shows the results. The productivity of the Rutger's strains are three times greater than those of the parent. This improvement has been due to the development of a rapid, semiquantitative plate screening technique that allows easy isolation of high yielding mutants (78). The Rutgers C-30 strain is resistant to carbon catabolite repression in addition to being hyper-cellulase producing (79).

2.2.2. The Mode of Action of Cellulase:

The mechanism of cellulose degradation has been the subject of extensive investigation. A theory must explain the physical changes in the cellulose, in addition to the production of soluble sugars. These changes include fragmentation, loss in tensile strength, transverse cracking and lowering of the degree of polymerization (59).

In an effort to explain this process, Reese and his coworkers proposed the C_1-C_X concept (92). The conversion of native cellulose to soluble sugars was envisioned as a two-step process. They proposed the cellulose is converted to reactive cellulose by the C_1 component that activates or

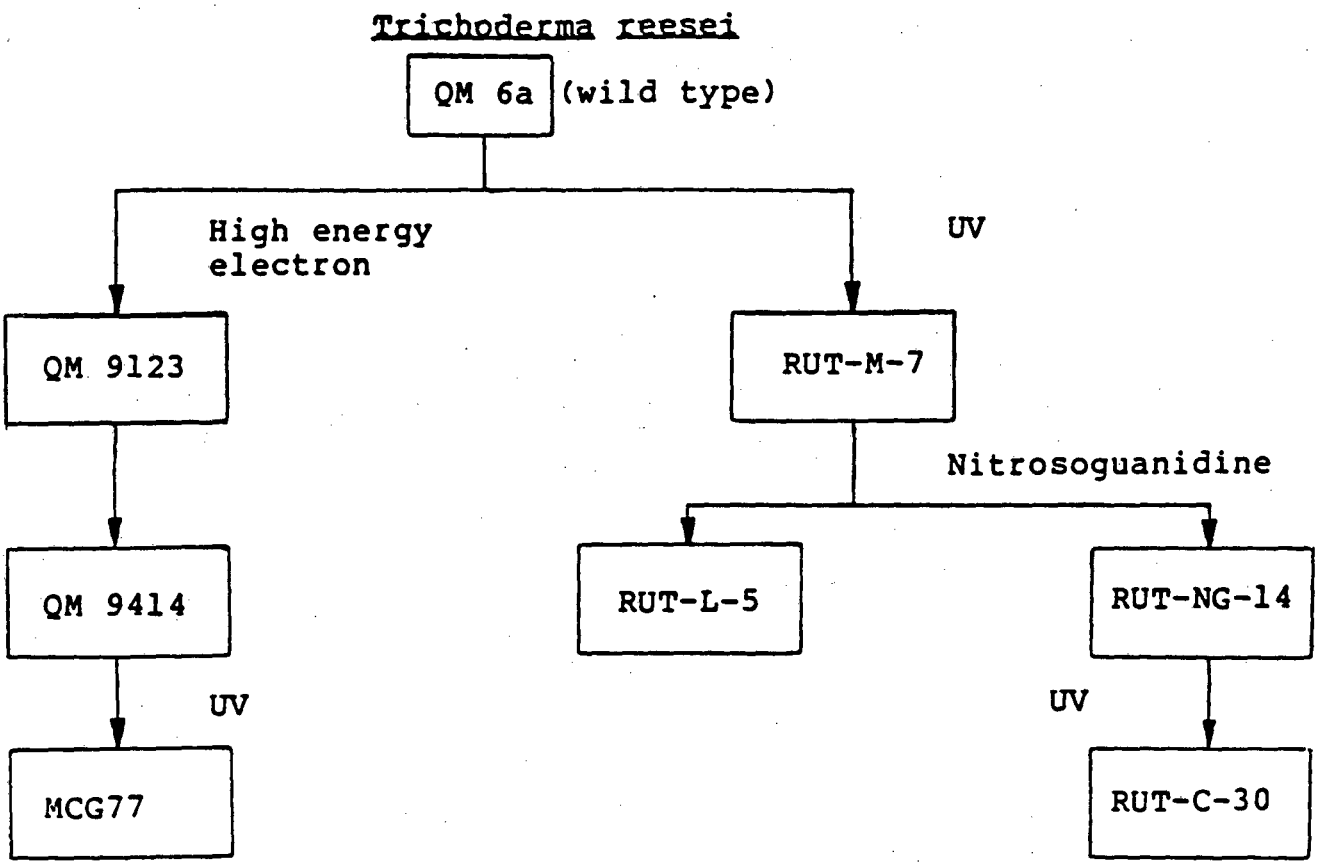


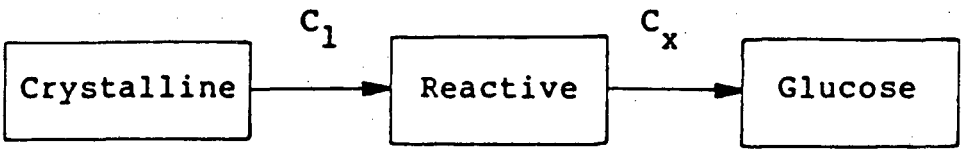
Figure 2.1. Strain improvement by mutation (118).

Table 2.3. Cellulase production by mutant strains of T. reesei. Adapted from (98).

Strain	Filter Paper (units/ml)	β-Glucosidase (units/ml)	Productivity (FPU/l/hr)	Soluble Protein (mg/ml)
QM6a	5	0.3	15	7
QM9414	10	0.6	30	14
MCG77	11	0.9	33	16
C30	14	0.3	42	19
NG14	15	0.6	45	21

Grown on 6% roll-milled cotton, pH greater than 3.0, 14 days
Assay procedures outlined in Section 3.2.

de-aggregates the cellulose chain. The C_X enzymes are responsible for the depolymerization.



This is based on the fact that some organisms possess incomplete cellulase systems that could not utilize highly ordered forms of cellulose. They were assumed to possess only the C_X components.

This concept stimulated a great deal of fractionation work to separate and isolate the different enzyme components. There are several review papers available that summarize this research (59,98). The idea of the C_1 component having a non-hydrolytic function was supported by several investigators (55,60,65,101).

Recent enzyme fractionation studies suggest that this concept should be abandoned and a new mechanism be developed. The cellulase system has been shown to possess three main enzyme groups: 1) β-1,4-glucan glucanohydrolase (EC 3.2.1.4), an endoglucanase, 2) β-1,4-glucan cellobiohydrolase (EC 3.2.1.91), an exoglucanase, and 3) β-glucosidase (EC 3.2.1.21).

A cellobiohydrolase, the primary exoglucanase, has been isolated by several workers (8,40,121). The enzyme catalyzes the cleavage of a cellobiose unit from the non-reducing end of the molecule. It is end product

inhibited (8) and is required for hydrolysis of highly ordered substrates (121). In addition, exo-β-1,4-glucanases have been shown to exist; however, they have not been so thoroughly studied (55,60).

Endoglucanases hydrolyze cellulose in a random fashion, resulting in a rapid reduction of the degree of polymerization together with a slow increase in reducing sugars. Several different endoglucanases have been isolated (62,39); however, this multiplicity could be due to proteolysis (36). β-Glucosidases hydrolyze cellobiose and other short β-1,4-oligoglucosides to glucose. They act by a noncompetitive mechanism with end product inhibition (37).

The different enzyme components have been isolated and synergism among the components has been demonstrated on cotton and Avicel (36,121). Figure 2.2 shows a current model of cellulose degradation. The endo- and exo-glucanases act synergistically to degrade crystalline cellulose to primarily cellobiose. The amorphous region of the cellulose is attacked by the exoglucanases. The resultant soluble cellobiose is hydrolyzed by the β-glucosidase to glucose.

2.2.3. Activity Measurement:

The task of designing an assay procedure to measure the cellulase activity of a culture filtrate is a difficult one. Since the substrate may be insoluble and the enzyme solution is a system of enzymes, the selection of a representative substrate and reaction conditions is not

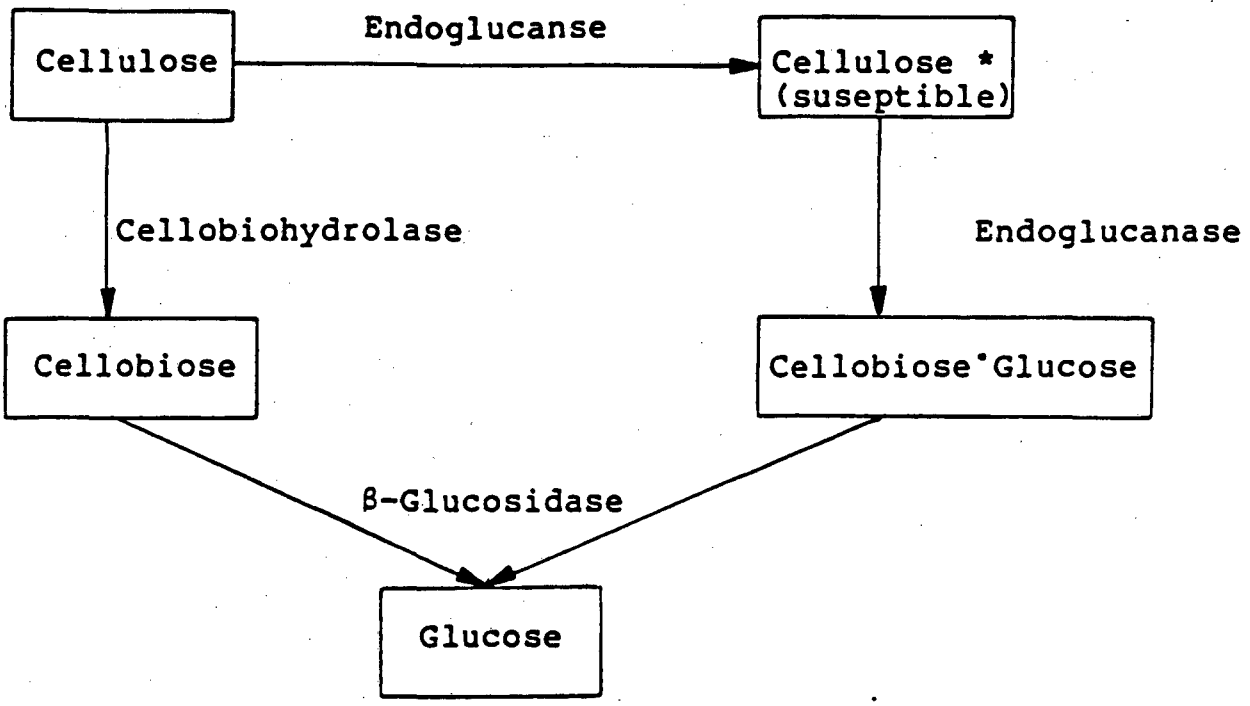


Figure 2.2. Mode of action of cellulase enzyme system on cellulose. Adapted from (98).

straightforward. As a result, the established assay conditions were designed to approximate closely the actual uasge conditions for the enzyme.

The most widely used assay is the filter paper test (66). The substrate is universal and easily available. It is neither too susceptible nor too resistant to hydrolysis and can be measured by area thus avoiding tedious weighing. Other substrate include resistant celluloses, like cotton and Avicel, and susceptible substrates, like carboxymethylcellulose. These assay procedures are described in detail in the Section 3.2. It should be emphasized that these assays were not designed to represent the activity of specific components but a lumped measurement of the activity of an enzyme mixture.

2.3. Kinetic Modeling of Cellulose Hydrolysis

The kinetics of cellulose degradation are affected by: 1) the nature of the enzyme, 2) the physical stucture of the substrate, and 3) the interactions between the two. Table 2.4 summarizes the previous work in modelling saccharification based on the above criteria. The first important factor to consider is the nature of the enzyme. The composition of cellulase has been outlined in the previous section. Many of the early models have been simplified because the enzyme was from the QM9123 strain of *T. reesei*. This enzyme system is deficient in β-glucosidase activity, hence, the predominant hydrolysis product, for short to moderate reaction times, was cellobiose.

Table 2.4. Existing models of cellulose hydrolysis.

Structure of the Substrate	Nature of the Enzyme	Enzyme-Substrate Complex Formation
Single:	Single Component:	Michaelis-Menten:
Howell and Stuck (43) Mangat and Howell (44,71)	Mangat and Howell (44) also (2,43,44,46,47)	Amemura and Terui (2 Ghose and Das (31)
Multiple:	Multiple Components:	Adsorption:
Amemura and Terui (2) Van Dyke (112) Brandt (11) Huang (47) Peiterson and Ross (85)	Okazaki and Moo-Young (83) Kim (54) Humphrey (48) also (85)	Huang (46,47) Kim (54) Humphrey (48)

The use of a strain possessing a more balanced enzyme mixture requires recognition of the different enzyme components and their synergistic action. Several workers have recognized this complexity and have accounted for it by using lumped activities on the crystalline and amorphous fractions (48,54,85). The most ambitious approach to model this situation was undertaken by Okazaki and Moo-Young based on three types of enzymes: endoglucanse, cellobiohydrolase and β-glucosidase (83). The model was used to study the synergistic effect among these components. They also accounted for the dependency of the reaction rate on the degree of polymerization of the cellulose. There have been some recent efforts to use a more fundamental approach by studying the kinetics of purified enzyme fractions on model substrates, like soluble cellodextrins (45,58).

The enzyme activity is also affected by product inhibition and enzyme deactivation. Ghose and Das were the first to study the effect of inhibition for the T. reesei cellulase (31). They observed severe inhibition by cellobiose and mild inhibition by glucose, both acting by a competitive mechanism. Howell and Stuck assumed a non-competitive mechanism dominates the kinetics (43). They predicted the kinetics of Solka Floc degradation up to 65% conversion, for low substrate concentrations (less than 15 grams/liter), by using Michaelis-Menten kinetics.

Mangat and Howell observed that product inhibition kinetics overpredicted their data after the first 12 hours

(44). The model was modified to include first order deactivation of the enzyme-substrate complex that blocks further hydrolysis. While this may be an important factor for long reaction times and enzyme recycle situations, one must be careful not to exaggerate this effect to fit the data without including the other factors, such as substrate multiplicity.

The second major factor that affects saccharification is the physical structure of the substrate. The structural features of cellulose and lignocellulose have been outlined in Section 2.1. Native cellulose is not homogeneous; it is composed of highly ordered, crystalline regions and easily accessible, amorphous regions. This multiplicity of the substrate was first incorporated into a model by Amemura and Terui in their work with the cellulolytic enzyme from P. variable (2). They introduced an effective substrate concentration into the Michaelis-Menten equation to account for the accessibility of the cellulose.

Much of the subsequent work either assumed this multiplicity was not important or used a model substrate. For instance, Huang used Walseth (phosphoric acid swollen) cellulose which is highly accessible to hydrolysis (46). Kim (54) and Peitersen (85) accounted for the dual nature of the substrate. Perhaps the most important improvement to modeling was the recognition that the bulk concentration of cellulose does not represent the effective concentration

(48,85). Since the cellulose-cellulase system is heterogeneous, the bulk concentration must be raised to a power to simulate a surface area effect.

This leads into the third major factor in cellulose kinetics, the interactions between the enzyme and the substrate, that is, the enzyme-substrate complex formation. The formation of the enzyme-substrate complex is dependent on an adsorption process. The adsorption of cellulase was studied by Mandels (66) and Peitersen (84). Peitersen observed that the absorption was largely independent of pH but strongly dependent on temperature and the type of cellulose. They used a Langmuir isotherm type equation to fit the data for contact times of 60 minutes.

$$E_{ads} = \frac{E_{ads,m}\, E}{K_E + E}$$

where E_{abs} = absorbed enzyme (FPU/gram)

E = free enzyme (FPU/ml)

$E_{abs,m}$ = maximum enzyme absorbed (FPU/gram)

K_E = constant (FPU/ml)

The differences in absorbability of the enzyme components was studied by Mandels (66), Castanon and Wilke (15) and Ghose and Bisaria (30). They observed that simultaneous absorption of the components occurs very rapidly and this absorption is the causative factor of hydrolysis. It was also noted that the different enzyme components had different patterns of adsorption.

The incorporation of adsorption into modelling was largley ignored by most workers; a Michaelis-Menten model was generally used. This model may be applicable at low substrate concentrations when the substrate is not close to maximum loading. As higher substrate concentrations are attained, due to a better balanced enzyme solution, an absorption process must be included. McLaren and Packer commented on this situation in an early review paper of enzyme kinetics in heterogeneous systems (73). Huang was the first to model cellulose degradation by taking into account adsorption (46). He first assumed fast adsorption followed by slow reaction and susequent product inhibition. This predicted the rate of hydrolysis of Walseth cellulose up to 70% conversion. Humphrey also utilized a Langmuir isotherm in his model for SCP production from cellulose (48).

2.4. Ethanol Fermentation

2.4.1. Yeast Metabolism:

The enzymatic hydrolysate contains mostly glucose with low levels of cellobiose and xylose. The yeast, Saccharomyces cerevisiae is used to ferment this glucose to ethanol. This organism is a facultative anaerobe. Under aerobic conditions, cell mass and carbon dioxide are produced through a respiratory mechanism. Under anaerobic conditions ethanol and carbon dioxide are produced by glycolysis. The overall reaction produces 2 moles of

ethanol and carbon dioxide per mole of glucose consumed. In practice, a portion of the glucose is used for the synthesis of new cell mass so the actual yield is about 90% of the theoretical yield.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 + \text{Cell Mass}$$

Glucose	Ethanol	Carbon Dioxide	
(1.0gm)	(0.46gm)	(0.46gm)	(0.054gm)

The rate of ethanol production is strongly effected by the ethanol concentration in the fermenting beer (1,6). The tolerance of various yeast to ethanol depends on the strain but ethanol production and cell growth generally cease at ethanol concentrations of 9 to 12%. Secondary metabolites, such as fusel oils, acetaldehyde and organic acids, can also inhibit the metabolism of the yeasts (62).

The fermentation of enzymatic hydrolysates from bagasse has been studied by Ghose and Tyagi (32,33). It was observed that high levels of sugars inhibited cell growth and ethanol production. This result was attributed to the other sugars in the hydrolysate (mainly xylose and cellobiose) that are not fermentable by the yeast (33). However, studies at the University of California at Berkeley with the cellulase from T. reesei, the QM9414 strain, indicated that a metabolic by-product in the hydrolysate may inhibit the fermentation (119).

2.4.2. Fermentation Processes:

The United States has about 100 million gallons per year of fermentative alcohol capacity (94). Almost all of

this production is by batch fermentation. This is a slow process. Figure 2.3 shows ethanol production by a highly productive yeast strain that fermented a 10% sugar solution. About 94% of the sugar has been utilized and 46 grams of ethanol per liter have been produced in 14 hours. The fermentor must be emptied, cleaned and refilled for another cycle. This results in an overall productivity of about 1.8 to 2.5 grams per liter of fermentor volume per hour (96).

In order to improve the productivity of the alcoholic fermentation, high rate fermentations have been studied extensively. Few processes, however, have been advanced to the point of pilot plant testing (118). The most basic process to increase productivity is a simple continuous stirred tank fermentor (CSTR). Continuous culture has the advantages of operating at a single optimum condition, reducing labor costs as a result of the elimination of downtime for cleaning, and the potential for a consistent product with more control over the processing conditions. However, strict aseptic conditions are necessary and the potential for adaptation or mutation of the organism is greater. A simple C.S.T.R. will have an ethanol productivity of about 6 grams per liter-hour (20). This is limited by cell densities of only about 10-12 grams per liter. Improvement can be attained by using a series of stirred tanks. The first stage will have a high productivity due to a low ethanol concentration with the second fermentor finishing the conversion. Overall, a

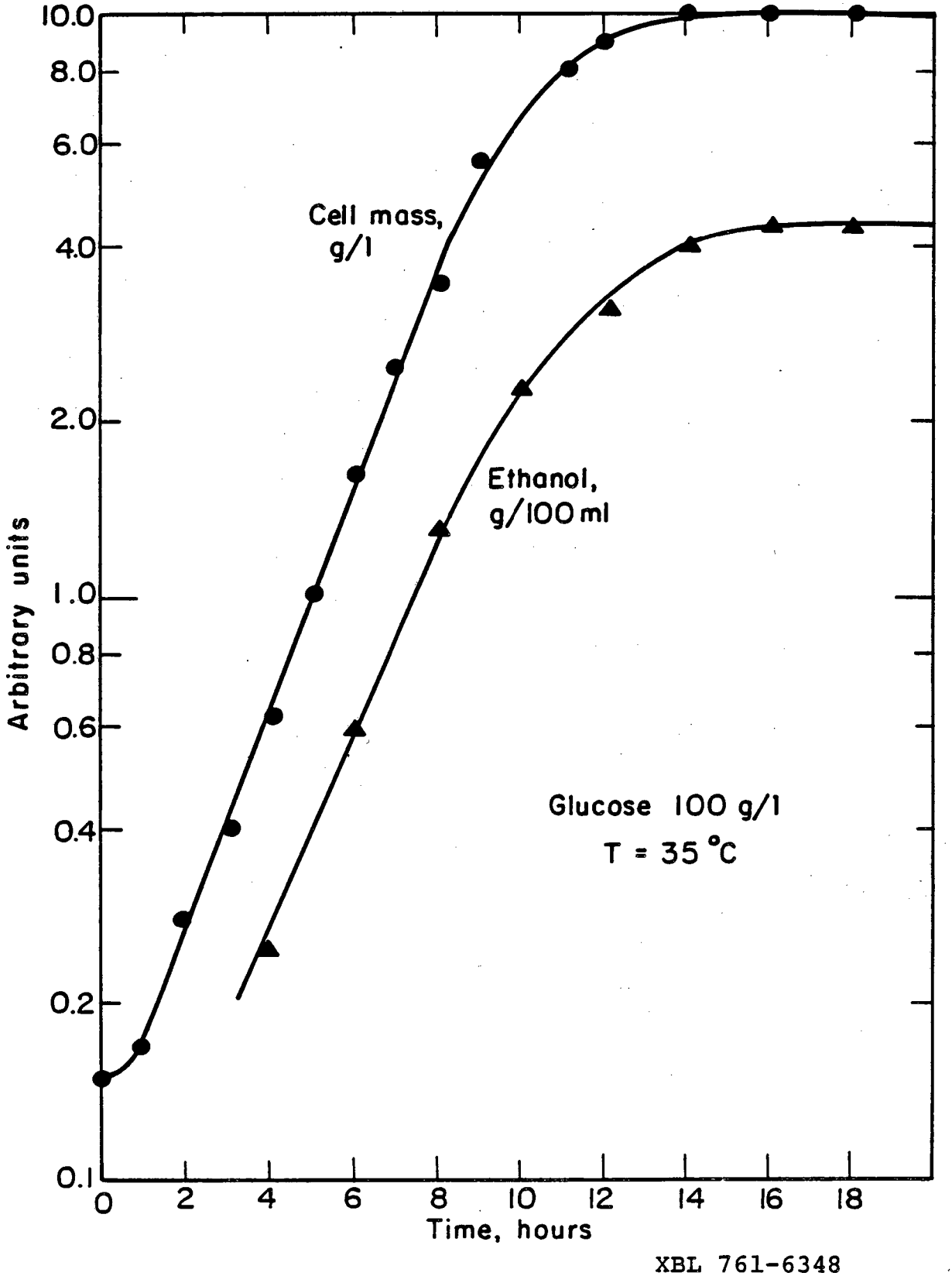


Figure 2.3. Batch fermentation with Saccharomyces cerevisiae (21).

two-stage system can increase the productivity by 2.3 times over a single C.S.T.R. (33).

The addition of a cell recycle capability to a simple C.S.T.R. allows very high cell concentrations to be attained. Cell densities as high as 83 grams per liter have been maintained (21). This type of system can achieve productivities of 30-40 grams per liter-hour (33). The use of a mechanical centrifuge adds complexity as well as additional capital and utility costs. There has been research to develop systems for recycle that do not require mechanical centrifuges (115). Tower fermentors take advantage of high cell densities by letting the cells settle against the flow. However, long startup times and difficulties in oxygen supply are major problems (118).

Another approach to high rate fermentation processes is the continuous removal of ethanol from the fermenting beer to minimize inhibition. Removal by vacuum (21,62,91), solvent extraction (15) and membrane extraction (38) has been proposed. The flash-ferm process is a modification of the original vacuum fermentation process (62). Flash-ferm carries out the fermentation at atmospheric pressure and cycles the broth to a flash chamber to remove the ethanol. The carbon dioxide is removed at atmospheric pressure and does not need to be compressed. Figure 2.4 shows a process design for this type of system.

In conclusion, simple continuous, series continuous, cell recycle, and tower fermentors have been

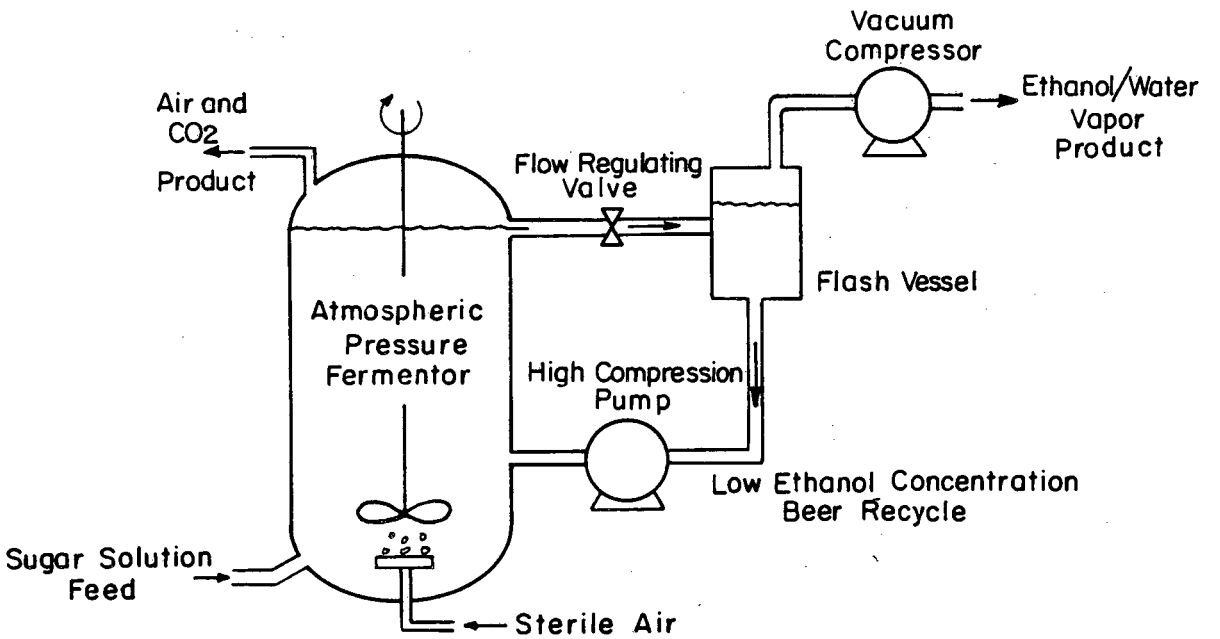


Figure 2.4. Continuous flash fermentation (118).

operated in large scale and exhibit considerable savings over a batch process. The processes utilizing continuous removal of ethanol have great potential but pilot plant testing is required.

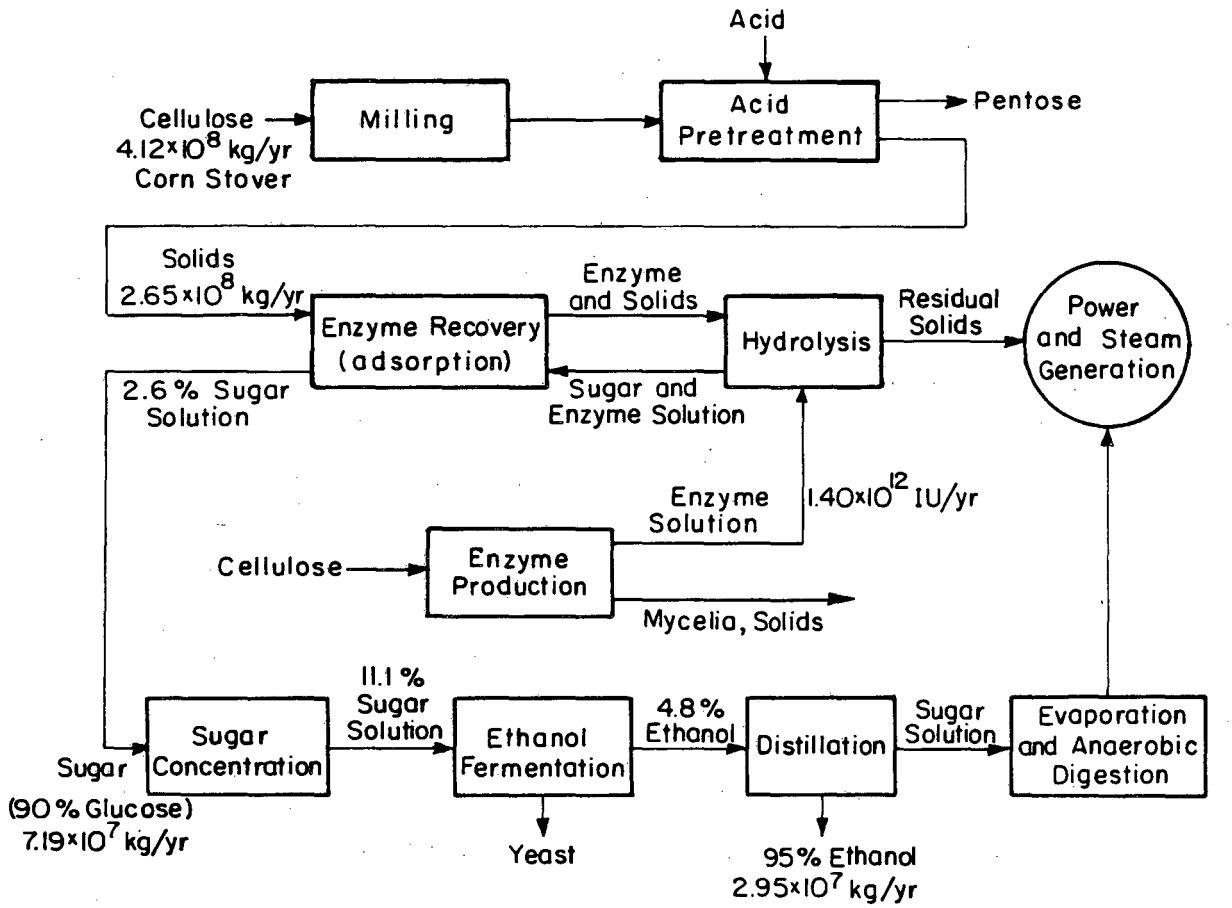
2.5. Process Design for the Bioconversion of Cellulosic Materials to Ethanol

The different processing schemes proposed for the bioconversion of cellulosic wastes all have several aspects in common. These processes all contain enzyme production, hydrolysis, and ethanol fermentation steps. The major differences are how these steps are integrated together.

2.5.1. Separate Enzyme Production, Hydrolysis and Ethanol Fermentation:

The two major processes in this category have been developed by the University of California at Berkeley and the U.S. Army Natick Research and Development Command. The Berkeley process has been developed for corn stover (119) and newsprint (76), while the Natick process uses urban waste (102). The basic design for these processes is illustrated in Figures 2.5 and 2.6

The Natick process has the advantage of high enzyme productivity and concentrated sugar solution from the hydrolysis sector. This approach simplifies the process by eliminating an evaporation step. Nevertheless, the high solid suspension in the hydrolysis vessel that is required causes problems in recovering the sugars from the solid



Berkeley Process for Corn Stover

XBL 803-4876

Figure 2.5 Berkeley process for bioconversion of corn stover (118).

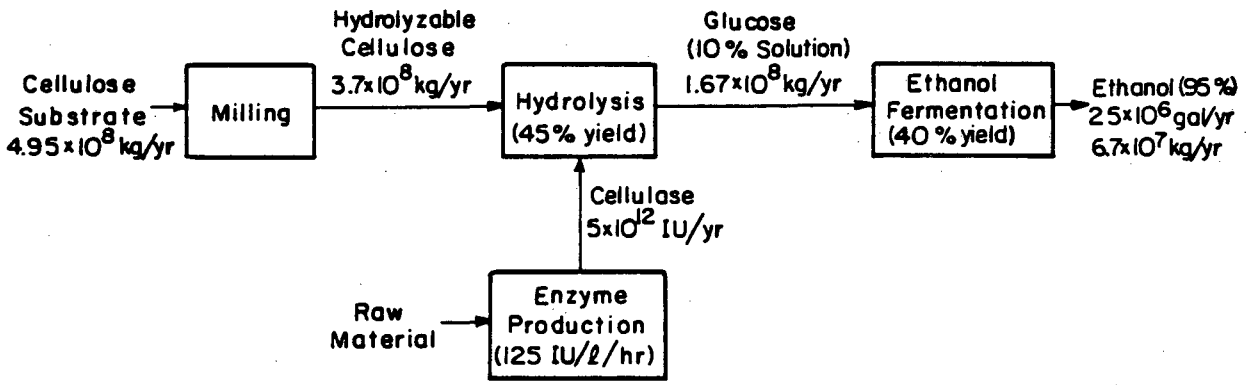


Figure 2.6. Natick process for ethanol production from cellulosic materials. Adapted from (102).

residue (without diluting the product by washing).

The Berkeley process offers the advantage of low enzyme usage by utilizing an adsorption train to recover the enzyme. The corn stover, which has a sizable amount of hemicellulose, requires an acid pretreatment step to hydrolyze the pentosans. This process will be covered in more detail in Section 6.1.

2.5.2. Simultaneous Saccharification and Fermentation:

A process for the simultaneous hydrolysis of cellulose and fermentation to ethanol has been developed by Gulf Oil Chemical Company and later transferred to the University of Arkansas (24). This process was designed to use about two-thirds municipal solid waste (MSW) and one-third pulp mill waste. About 15% of the MSW is proposed as the substrate for the continuous enzyme production unit that uses a mutant strain of T. reesei. The 8% cellulose feed is continuously hydrolyzed to glucose and fermented to a 3.6% ethanol beer by a yeast.

The advantages of this process are twofold. One, the glucose level is kept low, thereby minimizing the inhibition of cellulose hydrolysis by glucose. Two, this design reduces the equipment size and requirements for strict asepsis by intergrating the hydrolysis and fermentation in one operation. The primary disadvantage is that the optimum temperature for yeast fermentation is 30 to 35^{o}C while 45 to 50^{o}C is the optimum for the cellulase system.

2.5.3. Mixed Culture for Saccharification and Fermentation:

This approach expands on the Gulf process by utilizing organisms that are capable of hydrolyzing the cellulose to sugars, and fermenting the sugars to ethanol and side products. This eliminates the need for a separate enzyme production unit. This type of process was developed by Massachusetts Institute of Technology (18) and General Electric Corporate Research and Development (GE/CRD, 12). Figure 2.7 shows the Battelle design of the M.I.T. process (51) and Figure 2.8 shows the GE/CRD process design.

Both processes use a mixed culture of thermophilic, anaerobic bacteria. Clostridium thermocellum can hydrolyze cellulose and hemicellulose to glucose, cellobiose, and xylose. It will also ferment the glucose and cellobiose to ethanol, acetic acid, and lactic acid. Clostridium thermosaccharolyticum can ferment all the sugars to ethanol, acetic acid, and lactic acid.

These processes differ in the feedstock used, pretreatment, and ethanol recovery. The M.I.T. process uses corn stover while the GE/CRD process is based on populus wood, pretreated with sulphur dioxide under a steam pressure of about 300 psi for 10 to 15 minutes. The GE/CRD process also proposes the use of a flash-ferm process to recover the ethanol.

The advantages of these processes are that further equipment savings are realized and smaller probability of contamination occurs because of the high temperature and

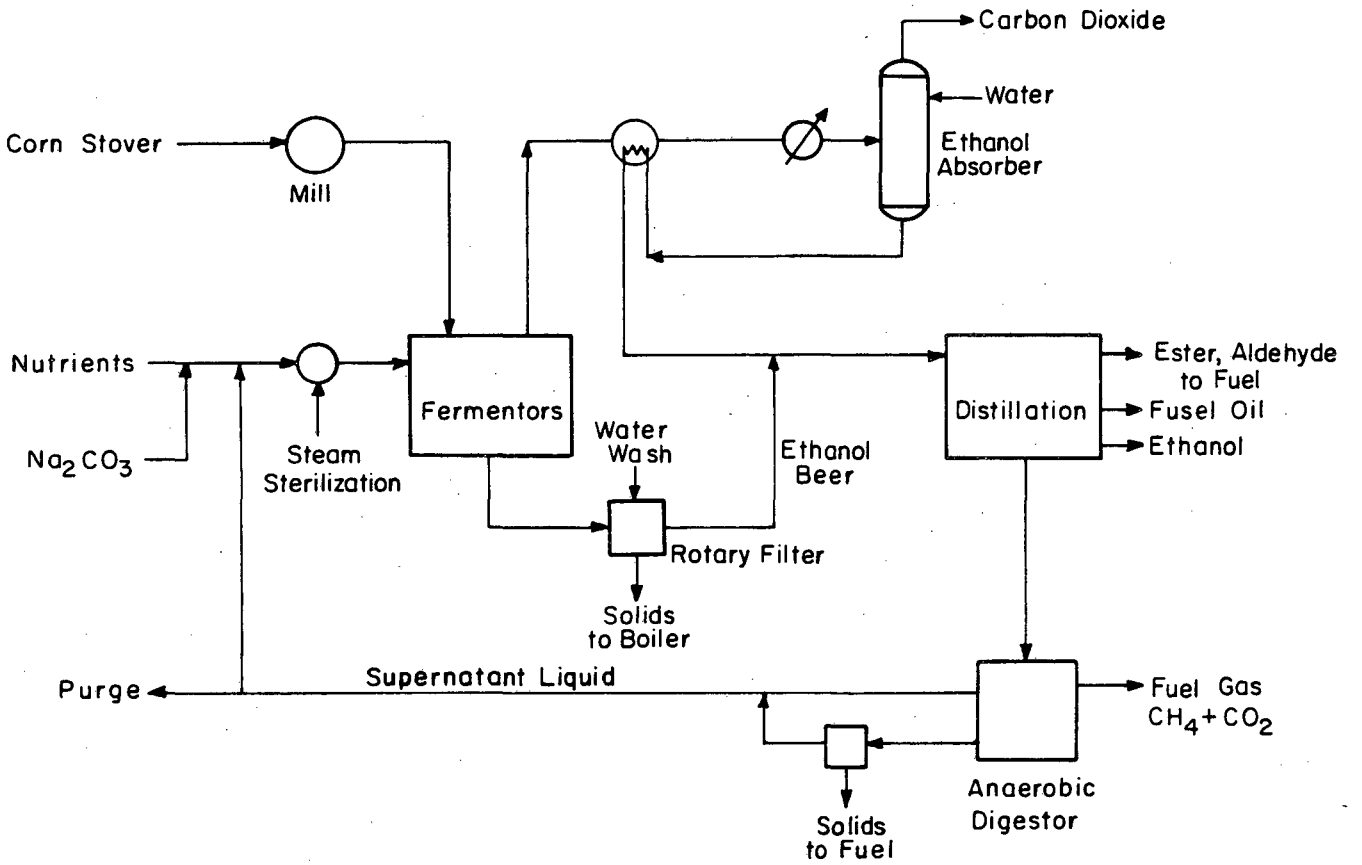


Figure 2.7. Battelle design for simultaneous hydrolysis and fermentation of corn stover to ethanol. Adapted from (51).

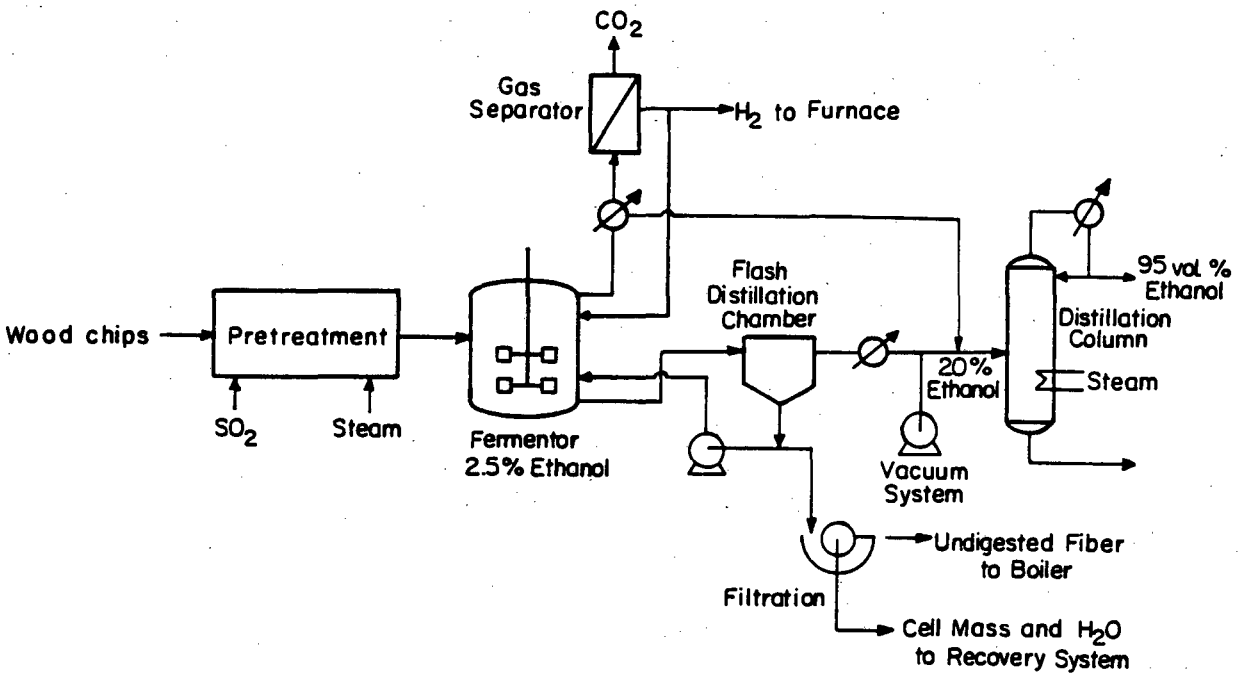
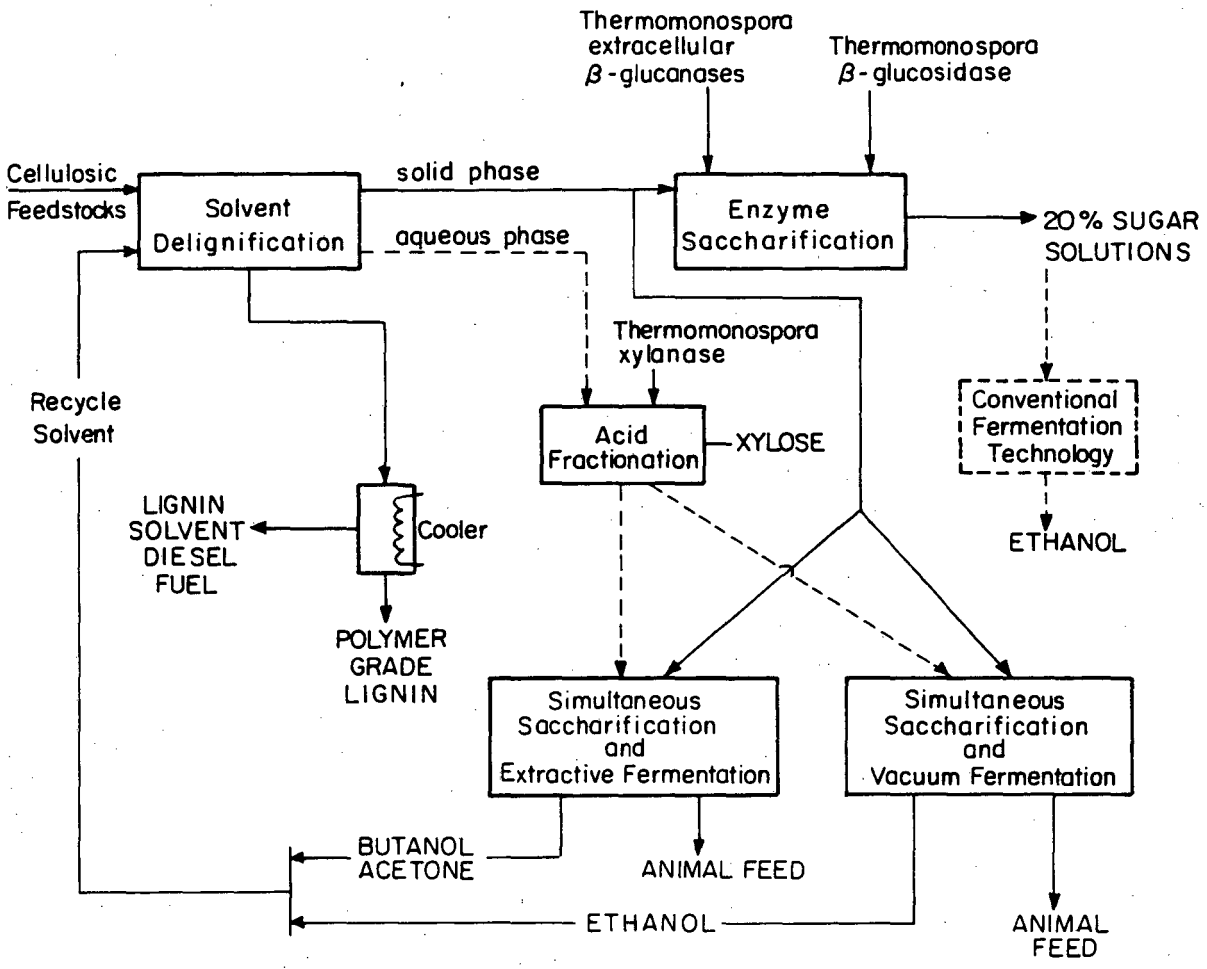


Figure 2.8. General Electric Research and Development process for ethanol production. Adapted from (12).

XBL-803-4885

anaerobic conditions. Maintainance of low oxygen tension and minimization organic acid formation are the major problems facing these processes.

The University of Pennsylvania and General Electric are developing an integrated process that converts all the biomass to liquid fuels and valuable side-products (90). The process utilizes the cellulase from Thermomonospora to produce high sugar solutions. Figure 2.9 shows the processing options considered. The process has the flexibility to produce ethanol, butanol, acetone, lignin-butanol fuel, and animal feed. This process is still essentially conceptual; however, the flexibility and total biomass utilization are attractive features of this process.



University of Pennsylvania / General Electric (PENN/GE)

XBL 803-4886

Figure 2.9. University of Pennsylvania/General Electric process for total utilization of biomass. Adapted from (89).

III. EXPERIMENTAL

3.1. Analytical Procedures

3.1.1. DNS Sugar Assay:

The standard method for the determination of reducing sugars is the dinitrosalicyclic acid (DNS) method (104). This assay measures all reducing sugars so it was used as a relative measure of the rate of sugar production. The samples are clarified by centrifugation at 10,000 RPM for 5 minutes. 1.5 ml of the DNS reagent are added to 0.5 ml of diluted sample, containing 0.5 to 3.0 grams/liter of sugar. This mixture is heated in a boiling water bath for 5 minutes and cooled in an ambient water bath for 3-4 minutes. The mixture is diluted with 10.0 ml of distilled water and mixed well by inverting the tube several times. The absorbance is measured with a Bausch and Lomb Spectronic System 400 spectrophotometer at 600 nm using a distilled water blank. The sugar concentration is determined with glucose as the standard.

3.1.2. Glucose Oxidase-Peroxidase Glucose Assay (GOP):

The GOP method is highly specific for the glucose monomer since it is based on an enzymatic reaction. The reagent is made by adding 0.5 ml of glucose oxidase (Sigma Chemical Company, Type V, 1200 units/ml) to 100 ml of Tris(hydromethyl)aminomethane (Sigma Chemical Co.), pH 7.0. 5 mg of peroxidase (Sigma Chemical Co., Type II, 125-200 purpurogallin units/mg) and 0.5 ml of 4% o-Dianisidine

dihydrochloride in water are added to the solution. 4.0 ml of this GOP reagent are added to 1.0 ml of sample, containing 20 to 100 µg of glucose. The tubes are incubated at 35-40°C for 1 hour. Two drops of concentrated HCl are added to stop the reaction and clear the solution. The solution is left to stand for 15 minutes and the absorbance is read at 400.0 nm.

3.1.3. Sugar Determination by High Pressure Liquid Chromatography:

The concentration of xylose and cellobiose in the enzyme hydrolysates was determined by liquid chromatography. Carbohydrates were separated using a Partsil Pac column (Whatman Inc.) with a refractive index detector. Azeotropic acetonitrile-water was the carrier at a flow rate of 2 ml/min.

3.1.4. Ethanol Concentration:

Ethanol was measured by gas chromotography using an Aerograph 1520 G-L Chromatograph. A column packed with Chromosorb 101 was used with a flame ionization detector. The injector and detector temperatures were 250°C and the column was maintained at 170°C. The output was analyzed using a digital intergrator (Varian CDS 111) and compared to standard ethanol samples.

3.1.5. Cell Mass Concentration:

The cell mass concentrations were estimated by measuring the optical density using a Beckman DU-2

Spectrophotometer at 650 nm and a slit width of 0.5mm. The samples were diluted to give absorbance readings in the range 0.1 to 0.4. Optical density was used as a relative measure of cell mass concentration. Cell mass was determined accurately by measuring the dry weight of the cells. The samples were filtered through a tared 0.4 micron Nucleopore filter, washed, and dried overnight at 70°C.

3.2 Enzyme Activity Determination

As described previouly, cellulase is a system of enzymes that act in a synergistic manner to degrade insoluble cellulose to produce glucose as the final product. In an effort to lump the individual activities together, an overall activity of the culture filtrate is measured with respect to different substrates (67).

3.2.1. Filter Paper Activity:

This is a common assay to measure the overall activity of an enzyme with respect to filter paper. The assay was originally conceived to use full strength enzyme with a measured amount of filter paper. Strain improvement has necessitated dilution of the enzyme prior to reaction to yield a meaningful initial reaction rate. Since the system is heterogeneous, the reaction rate is not first order with respect to enzyme concentration. The assay is simplified, though, by operating with diluted enzyme so that the reaction is linear in enzyme strength. The procedure is as follows (34): 25 mg of Whatman #1 Filter Paper (1 x 3 cm)

is coiled and added to 1.0 ml of diluted enzyme. The capped tubes are placed in a 50°C water bath for 60 minutes. After incubation, 5 ml of DNS reagent are added to each tube and mixed well. The tubes are placed in a boiling water bath for 10 minutes and cooled in an ambient temperature water bath for 3-4 minutes. The filter paper debris is cleared by pushing a Kim-Wipe plug to the bottom of the tubes with a glass rod. The cleared solutions are transferred to a new tube and the absorbance is measured at 600 nm using a distilled water blank. The sugar produced is determined from a standard glucose curve, accounting for background sugar in the samples and in the filter paper itself.

The enzyme is diluted to yield a final sugar concentration of 0.2 to 0.6 grams/liter. Figure 3.1 shows a typical dilution curve for an enzyme. The activity must be calculated from the linear region and extrapolated back to full strength. The activity is measured in Filter Paper Units, that is, 1.0 FPU is the amount of enzyme required to produce 1.0 micromole of reducing sugar per minute. For example, if 0.4 mg/ml of sugar is produced in the assay by diluting the enzyme 50 times, then the activity is calculated as follows:

$$\frac{0.4\text{mg}}{\text{ml}} \quad \frac{\text{mmol}}{180\text{mg}} \quad \frac{1000\,\mu\text{mol}}{\text{mmol}} \quad \frac{1}{60\text{min}} \quad 50 = 1.9 \text{ FPU/ml}$$

It should be emphasized that an enzyme of 8 FPU/ml is <u>not</u> twice as "strong" as an enzyme of 4 FPU/ml. Only at high dilutions will these different enzymes show this relationship.

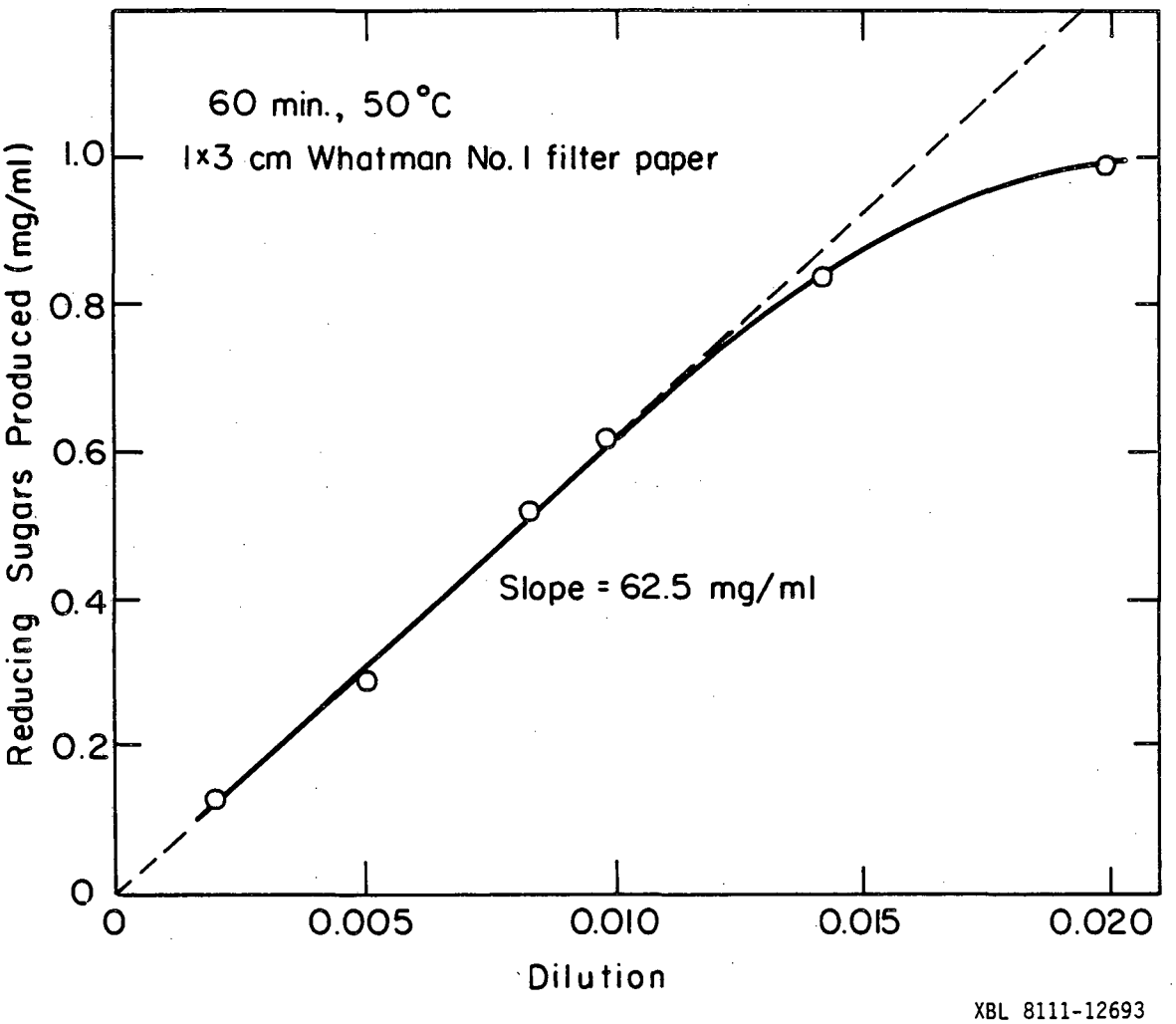


Figure 3.1. Dilution curve of the cellulase enzyme used for determination of the filter paper activity.

3.2.2. C_1 (Crystalline) and C_x (CMCase) Activity:

The C_1 assay measures the ability of the enzyme to degrade a crystalline cellulose, cotton. This procedure is the same as the filter paper assay except 25 mg of Red Cross cotton is used as the substrate and the incubation time is 24 hours (34).

The C_x assay measures the rate of degradation of a soluble cellulose derivative, carboxymethyl cellulose (34).

3.2.3. β-Glucosidase Activity:

This assay measures the cellobiase activity of the enzyme solution (34). 0.1 ml of diluted enzyme is added to 1 ml of 1.25% cellobiose ("Baker" grade, J.T. Baker Chem. Co.) in 0.025M acetate buffer. The tubes are placed in a 50°C bath for 15.0 minutes. After incubation 4.0 ml of GOP reagent are added and the procedure then follows the GOP glucose assay.

3.2.4. Soluble Protein Assay:

This assay does not measure the activity of the enzyme but provides a technique to measure the amount of soluble protein in the culture filtrate. The test was proposed by Lowry et al. (61) and modified for this system (34). The amount of protein is measured with bovine serum albumin (Pentex) as the standard.

3.3 Cellulase Production

3.3.1. Inoculum:

Viable cultures of T. reesei, mutant strain Rutgers C-30, were maintained on potato dextrose agar (PDA) slants for short term storage. A more stable medium for long term storage that ensures retention of the hydrolytic activity is with 1% Avicel PH-105, modified Vogel salts , and 2% agar (78). All slants were stored at 4°C until use.

These slants were used to inoculate 200 ml of mineral salts medium containing 1% glucose, Tween-80 (0.01%), and antifoam (0.1%). This was incubated for 96 hours at 28°C. This solution acted as the inoculum (10%) for 200 ml mineral salts medium containing 1% Solka Floc, Tween-80 (0.01%), and antifoam (0.1%). This was incubated for 5-6 days at 28°C. This served as the inoculum for the fermentor (106). There were several variations on the scheme used to improve the induction process to yield a more active inoculum. The addition of 1% lactose as an inducer appeared to be effective.

3.3.2. Fermentation:

The enzyme was produced by batch growth in a 14-liter fermentor (New Brunswick Scientific Co., Magnaferm Model MA 114 or Chemapec Inc., Chemap Type LF) with an operating volume of 10 liters. The medium composition was based on Mandels and Reese (68) and modified for this strain by Tangnu et al. (106). The medium is shown in Table 3.1.

Table 3.1. Cellulase production media. Adapted from (106).

Components (mg/ml)	Cellulose Concentration (g/l) 10	50
$(NH_4)_2SO_4$	1.4	11.6
KH_2PO_4	2.0	3.8
$MgSO_4$	0.15	0.3
$CaCl_2 \cdot 2H_2O$	0.4	0.8
Peptone	1.0	2.9
Tween-80 (ml)	0.2	0.2
Trace Elements (mg/l):		
$FeSO_4 \cdot 7H_2O$	5.0	5.0
$MnSO_4 \cdot H_2O$	1.6	1.6
$ZnSO_4 \cdot 7H_2O$	1.4	1.4
$CoCl_2$	2.0	2.0

The temperature was controlled at 28°C, the agitation at about 400 RPM, and the aeration at 0.2 vvm. The pH was controlled with 2N NaOH to prevent it from falling below 5.0 units.

The enzyme solution was harvested at the end of 7 days. Mycelia were removed by filtration through glass wool and the enzyme was stored at 4°C with 0.02% sodium azide added to prevent contamination. The solution was buffered with 0.05M sodium acetate to a pH of 5.0.

3.4. Hydrolysis Experiments

3.4.1. Milling and Pretreatment:

About 5 pounds of rice straw were received from John Dobie (University of California, Davis) from the fall harvest of 1979. A portion was hammermilled, first through a 5mm screen and then through a 2mm screen. The remainder was Wiley milled to the same size. The milled rice straw was acid treated in batches of 300 grams in 3.5 liters of 0.09M sulfuric acid. The procedure was based on work by Dunning and Lathrop (23) and modified by Sciamanna et al. (100).

3.4.2. Enzymatic Hydrolysis:

The hydrolyses were conducted in 600 ml Berzelius beakers with 3-bladed stirring shafts. The beakers were immersed in a 45°C water bath and sealed with a rubber lid. The amount of enzyme was usually 150 ml with the amount of pretreated rice straw varied from 7.9 to 50 grams. For

example, a 10% (by weight) solid suspension was 16.7 grams of straw in 150 ml of enzyme. A stepwise addition of the substrate is required to exceed a 10% level because of viscosity limitations. As the reaction proceeds rapidly at first, there is a significant decrease in viscosity that allows more solids to be added. A solid suspension of 25% can be acheived in 2.5 hours. Figure 3.2 shows a typical addition scheme. Samples of 3-5 ml were withdrawn with an inverted pipette to obtain a representative sample. The slurries were centrifuged at 10,000 RPM for 5 minutes and the supernatant was analysed for sugar and enzyme activity. The glucose yield was based on a cellulose content of acid treated rice straw of 57% (99) and adjusted for volume changes of the supernatant due to the solubilization of the rice straw.

3.4.3. Enzyme Precipitation for Analysis:

High sugar concentrations of the enzymatic hydrolysates interfere with the enzyme assays. As a result, the protein was precipitated prior to analysis. One part enzyme was mixed with three parts acetone to precipitate the enzyme (99). The solutions were centrifuged at 10,000 RPM for 10 minutes and the acetone was decanted. The tubes were subjected to a vacuum for several minutes to evaporate the remaining acetone. Care was taken to remove all the acetone because it interferes with the DNS reagent. The protein was resuspended in 0.05M acetate buffer.

There are two problems that must be recognized.

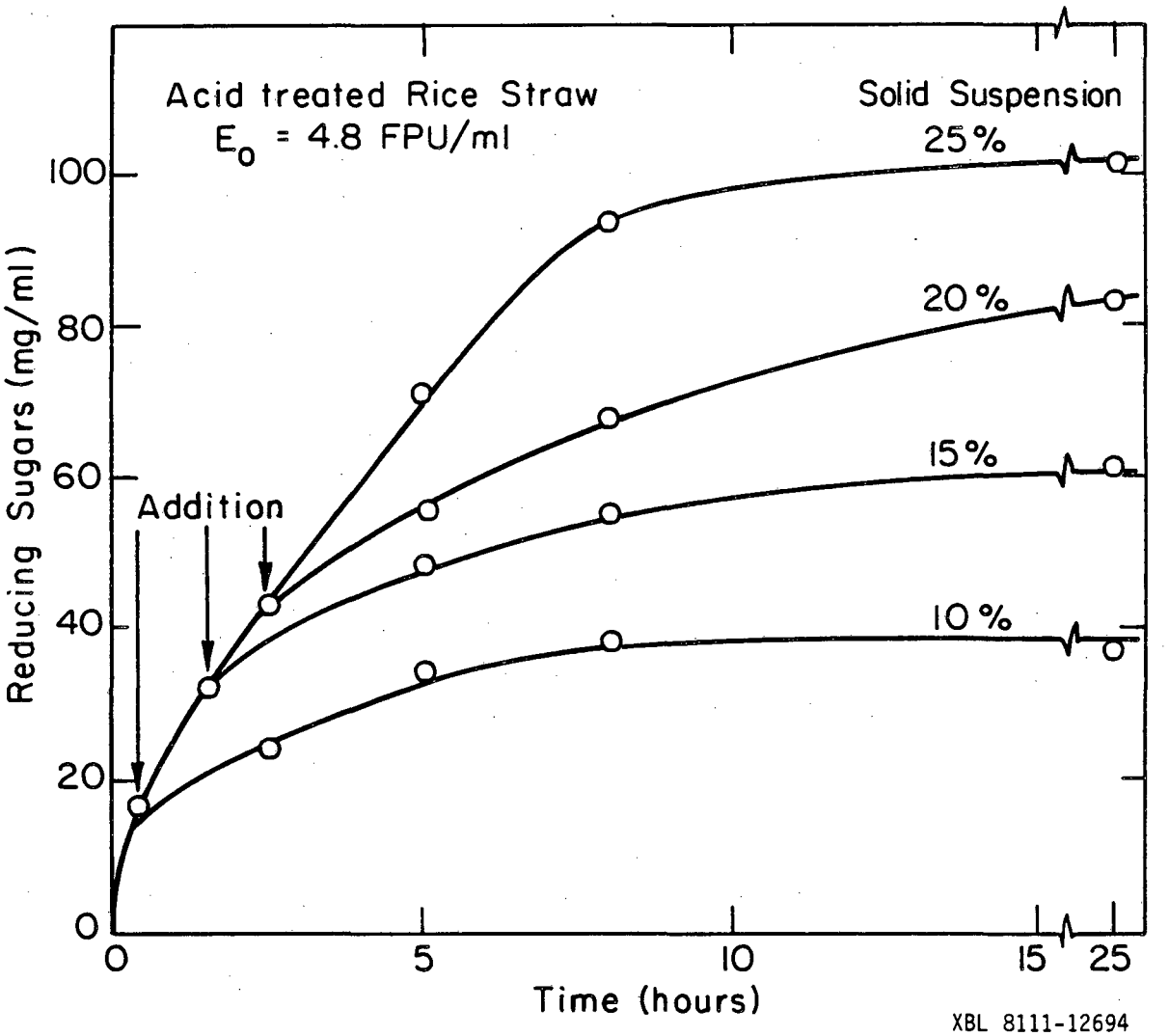


Figure 3.2. Addition scheme for a high substrate concentration in the hydrolysis vessel.

One, there are enzyme losses due to a finite solubility of the protein in the acetone-water mixture and, two, the sugar has a finite solubility in this solution and may come out of solution at high concentrations. This requires careful dilution of the enzyme prior to precipitation to balance these factors. Standard curves were prepared to account for these losses. Figure 3.3 shows that a linear relationship exists between the amount of enzyme precipitated and the amount recovered. Equations were fitted for each activity assay. These relaionships are summarized in Table 3.2.

3.4.4. Kinetic Studies:

The initial rate of hydrolysis of rice straw was measured as follows: 50 to 557 mg of rice straw were added to 5 ml of enzyme solution (preheated to 45^{o}C). The test tubes were incubated in a water bath shaker (Magni Whirl Constant Temperature Bath, Blue M Electric Co.) at 45^{o}C. The reaction was stopped by immersion in boiling water for two minutes to denature the protein. The solids were centrifuged out and the sugars were analyzed by DNS and corrected against a reagent blank. Figure 3.4 shows a typical progress curve.

The initial reaction rate for cellobiose hydrolysis required a more precise technique. The conditions were determined to give glucose formation that was linear with respect to both time and enzyme concentration. The enzyme solution was diluted by a factor of 220 (about 25 β-glucosidase units/liter) and the time interval was between

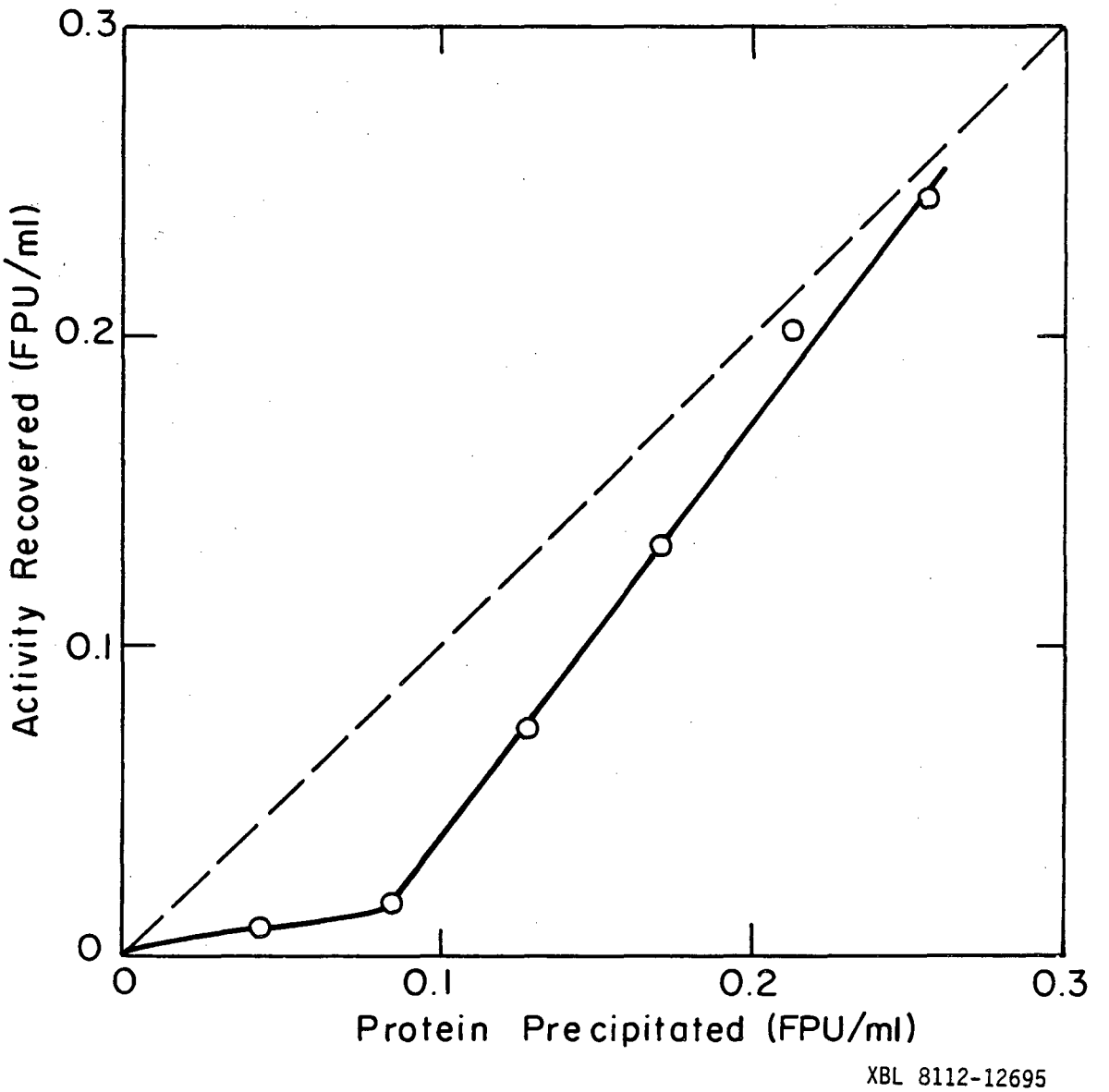


Figure 3.3. Protein losses from the acetone precipitation technique.

Table 3.2. Enzyme losses by acetone precipitation.

$$(\text{Activity Measurement})_{ppt} = m(\text{Activity Measurement})_{recovered} + b$$

Activity Measurement	Range (units/ml)	m	b
FPA	FPA less than 0.255	0.722	0.074
C_1	C_1 less than 0.025	0.768	0.00773
CMCase	CMCase less than 7.9	0.757	2.37
Cellobiase	CB less than 0.41	0.717	0.132

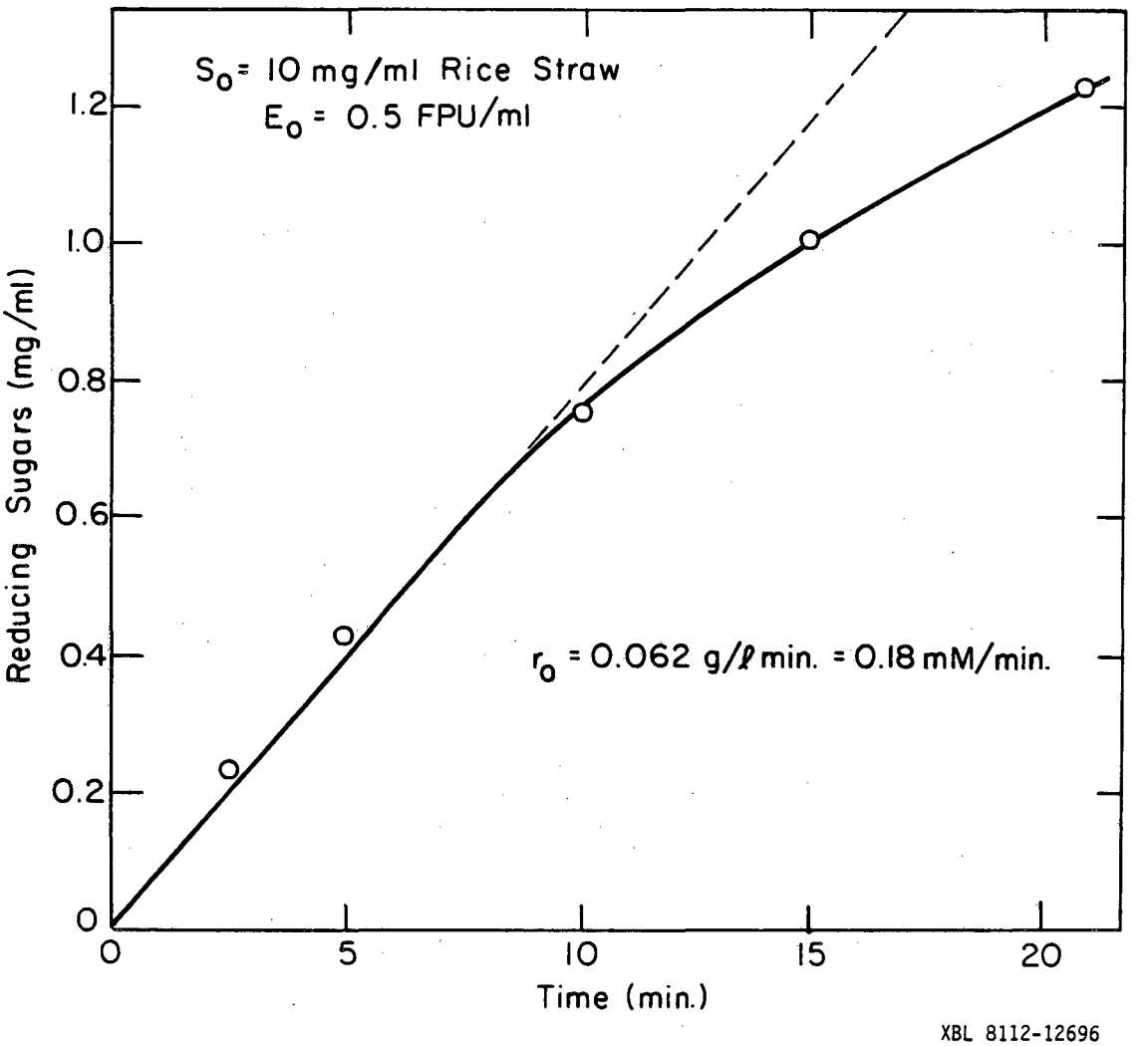


Figure 3.4. Initial reaction rate determination for rice straw hydrolysis.

5 and 20 minutes. The procedure was the same as for rice straw except the reaction was stopped by adding TRIS buffer (95) and the amount of glucose produced was measured by the GOP assay procedure.

3.5. Ethanol Fermentation

3.5.1. Yeast Strain and Culture Medium:

The organism used was Saccharomyces cerevisiae (ATCC #4126). The yeast was stored at 4°C on nutrient agar slants. Inocula were prepared by transferring aseptically a few loops of cells to a 250 ml shake flask containing 150 ml of sterile medium shown in Table 3.3. The flasks were incubated at 35°C for about 15 hours to ensure exponential growth.

3.5.2. Batch Fermentation:

An one liter fermentor (MiniFerm, New Brunswick Scientific Co.) was used for these studies. The jar was immersed in a 35°C bath and agitation was supplied by a magnetic stirring bar. The pH was controlled with 2N NaOH to prevent it from falling below 4.0 units.

The fermentor was sterilized and charged with 600 ml of filter sterilized medium. The medium was sterilized by vacuum filtration through a 0.2 micron Nucleopore filter. Table 3.3 shows the media used. The analytical grade glucose was substituted by glucose produced in enzymatic hydrolysis. The hydrolysates were concentrated from 4% glucose to 10% by freeze drying. The remaining nutrients

Table 3.3 Media composition for S. cerevisiae. Adapted from (21).

Component (g/l)	Agar Support	Fermentation
Glucose (anhydrous)	20	100
Yeast Extract (Difco)	2.2	8.5
NH_4Cl	0.33	1.32
$MgSO_4 \cdot 7H_2O$	0.03	0.11
$CaCl_2 \cdot 2H_2O$	0.02	0.08
Antifoam (General Electric AF20)	-	0.2 ml
Agar (Difco)	15	-
Tap Water	Make up to 1 liter	

were added to the concentrated hydrolysate.

The medium was sterilized by filtration through a glass wool filter, saturated with air by sparging for several hours. The fermentor was inoculated with 15 ml of active culture through a silicon septum with a syringe. Samples of 15 ml were withdrawn with the syringe which was kept in methanol and flamed before sampling. Strict asepsis was not necessary for the active growth of the yeast.

IV. RESULTS AND DISCUSSION

4.1. Enyzme Production

The enzyme requirements for this study were supplied by the batch cultivation of T. reesei. Figure 4.1 shows a typical fermentation profile of this organism. The cellulase production was monitored by the filter paper assay, and cellular activity was followed by the ammonia consumption or base uptake to maintain a constant pH (82). Table 4.1 shows a more detailed characterization of the cellulase activity of the culture filtrate (enzyme A). This enzyme was used for the hydrolysis studies. High strength enzyme solutions were obtained by concentration. Freeze drying was used to minimize denaturation of the protein.

Table 4.1 also compares this enzyme with the results of previous work conducted at the University of California, Berkeley. The original fermentation studies with the Rutgers C-30 strain by Tangnu et al. (106) were reproduced by Wiley (116) and yielded enzyme solutions in excess of 9 FPU/ml. Early efforts to duplicate this work resulted in an enzyme (enzyme B) deficient in C_1 activity, as evidenced by a C_1 to soluble protein ratio of 1.9. This ratio is low compared to an average ratio of 5.6 reported for earlier work.

A new slant produced an enzyme solution of 4.1 FPU/ml. The specific activities for this enzyme were not significantly different from previous work. This indicates that the protein yield was diminished. This may be a result

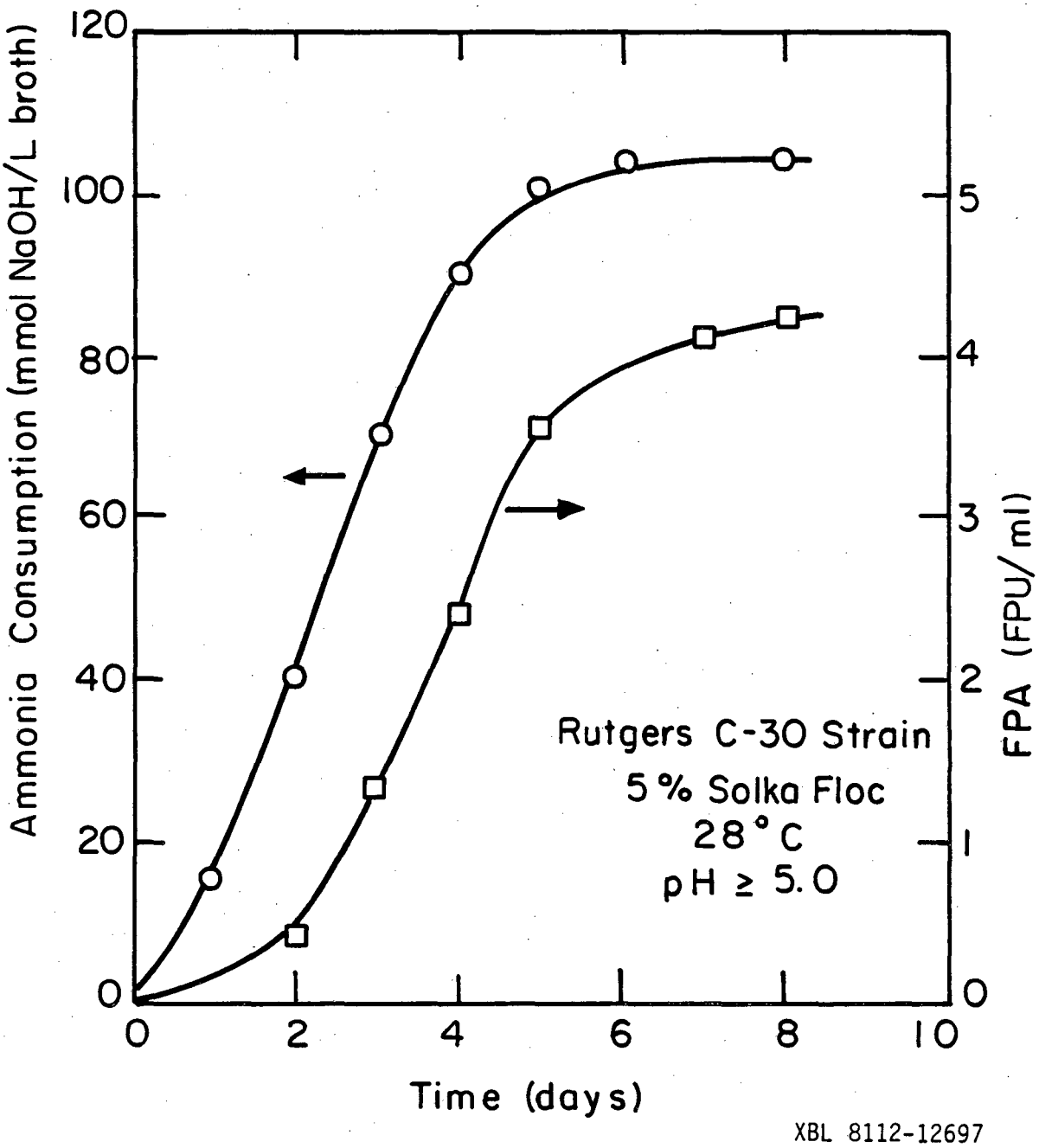


Figure 4.1. Fermentation profile of T. reesei.

Table 4.1 Comparison of enzyme activities of different *T. reesei* culture filtrates.

Enzyme	A	B	C	D	E
Source	-	-	116	106	106
Date	9/80	4/80	12/79	8/79	7/79
Substrate (%)	5	5	5	2.5	5
FPA (units/ml)	4.1	5.6	9.4	4.4	14.1
C_1	0.39	0.18	0.67	0.47	1.19
CMCase	140	103	150	137	313
Cellobiase	8.6	10.5	13.7	9.2	22.8
Specific Activities:					
FPA/SP	0.48	0.67	0.79	0.55	0.64
C_1/SP	4.5	1.9	5.6	5.8	5.4
CMCase/SP	13	17	13	17	14
Cellobiase/SP	0.9	1.0	1.2	1.1	1.0

Growth conditions: Section 3.3

of instability in the strain or mass transfer limitations of the fermentation equipment. It was noted that a yellow pigment was produced after 4 days. This metabolite could have been responsible for the yield loss. It was beyond the scope of this work to investigate this phenomenon further.

It is recommended that future investigation should focus on increasing the activity of the inoculum to minimize the lag phase, developing a continuous process using cell recyle to increase productivity, and studying alternate, inexpensive carbon and nitrogen sources to reduce raw material costs.

4.2. Hydrolysis Experiments

Enzyme catalysis is characterized by turnover numbers of 10^{-3} to 10^{3} molecules substrate/sec.-molecule enzyme, at mild operating conditions. This is due to the high specificity of the protein structure for the substrate. The best operating conditions for an enzyme usually fall within a relatively narrow range of temperature and pH that result in the optimal configuration and charge on the functional groups at the active site. It has been established that the cellulase system, as a whole, operates most efficiently in the temperature range of 45 to 50^{o}C (3) and a pH of 4.5 to 5.0 (67).

Figure 4.2a shows a typical hydrolysis progress curve. The distinctive features of cellulose hydrolysis are evident. The first region is characterized by the rapid production of sugars, in the range of 10 to 40 grams per

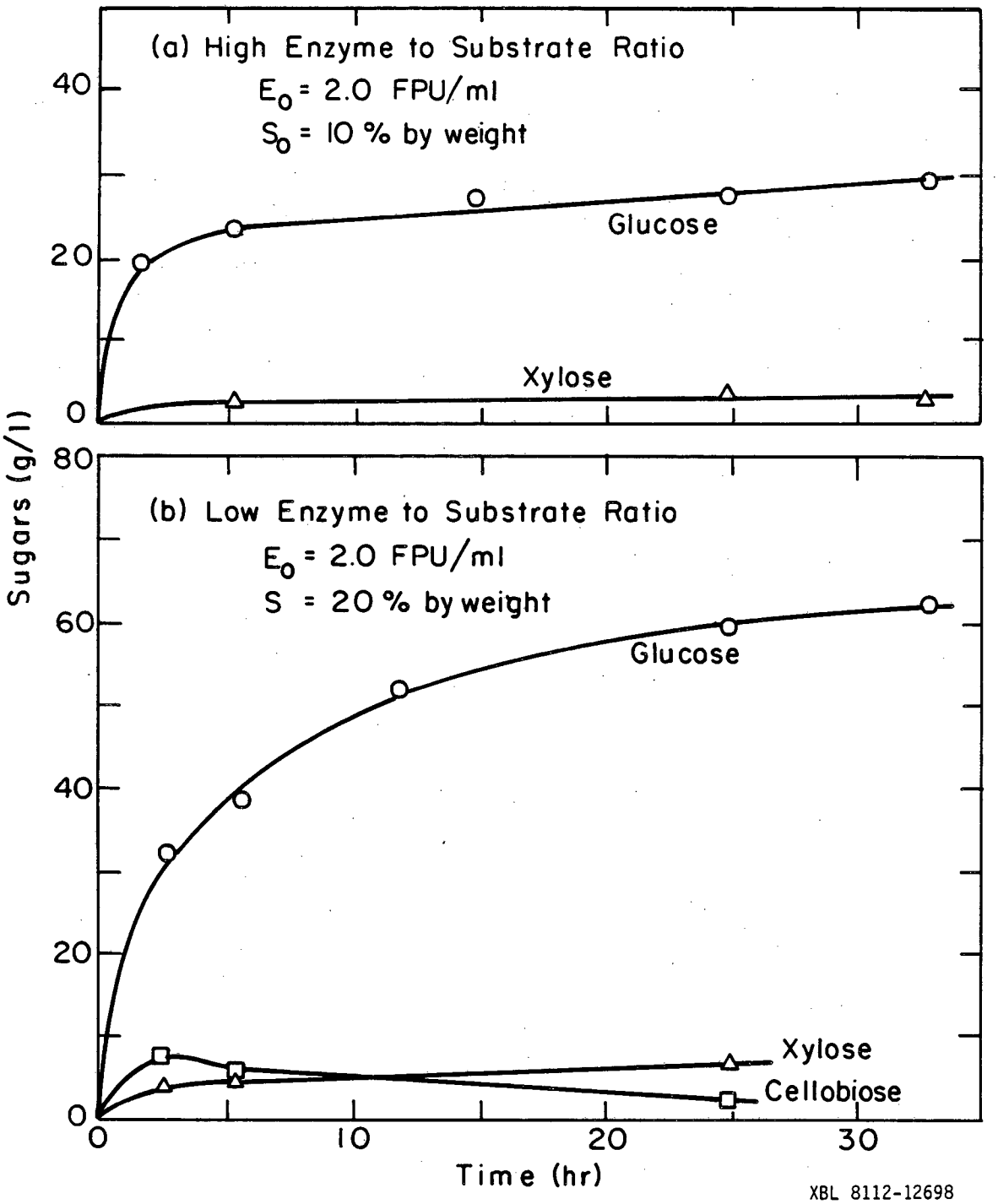


Figure 4.2. The influence of the enzyme to substrate ratio on the sugar production from rice straw.

liter-hour. This occurs typically in the first 2 to 3 hours of hydrolysis. A transition occurs which is followed by a region of slow reaction (0.1-0.5 grams per liter-hour). This behavior results from variations in the accessibility of the substrate to the enzyme or crystallinity of the substrate.

Figure 4.2a also shows the production of the major sugars, glucose and xylose, during hydrolysis. Since the cellulose-cellulase system is heterogeneous, the enzyme:substrate ratio will provide information concerning the adsorption properties of the system. This run corresponds to an initial loading of 18 FPU/gram of rice straw. This behavoir can be contrasted to a low enzyme loading shown in Figure 4.2b. An enzyme:substrate ratio of 8 FPU/gram shows a similar initial rise in glucose, however, cellobiose accumulates. The latter results when the cellobiase activity of the enzyme becomes rate limiting. Figure 4.3 shows that cellobiose accumulation exceeds 1 gram/liter in 5.5 hours when the initial enzyme:substrate ratio (in this case, β-glucosidase units per grams of rice straw) decreases below about 30 units/gram. This is an important design constraint because the presence of cellobiose will inhibit the enzymes that degrade the insoluble cellulose. In addition, cellobiose can not be fermented to ethanol by S. cerevisiae.

4.2.1. Effect of Acid Treatment:

All of the hydrolysis experiments were conducted

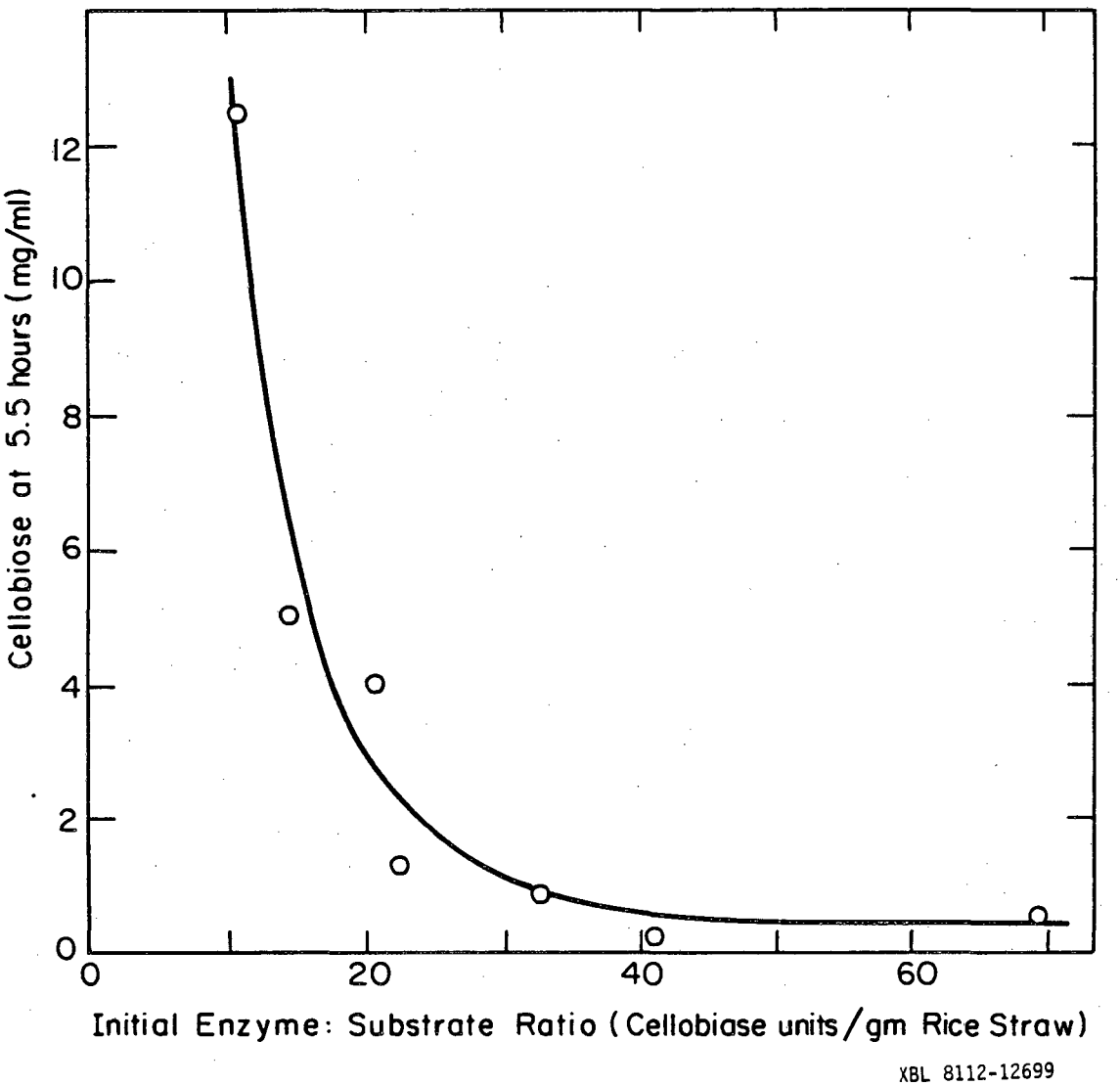


Figure 4.3. Effect of cellobiase activity on cellobiose accumulation.

with acid treated material. The purpose of acid treatment is to extract the pentosans and make the substrate more susceptible to hydrolysis. Figure 4.4 shows the enhancement of hydrolysis due to pretreatment. The wet density is substantially increased to allow higher solid suspensions. In addition, the cellulose content is increased from 40 to 57% of the dry material, thus reducing the material handling requirements.

4.2.2. Effect of Enzyme and Substrate Concentration:

For a given enzyme concentration, an increase in solid suspension will increase the sugar concentration. Figure 4.5 shows this trend and the inverse relationship between substrate concentration and glucose yield. The yield loss with increasing solid suspension is explained by examining the enzyme:substrate ratio. Figure 4.6 shows the glucose yield as a function of the initial enzyme loading at 8 and 47 hours. Both curves exhibit a similar pattern. The yield increases with enzyme loading until about 10 to 20 FPU/gram. Beyond this point the yield remains relatively constant. The region where the glucose yield is directly proportional to the enzyme:substrate ratio is a result of hydrolysis being limited by either low cellulase concentrations or cellobiose inhibition due to low β-glucosidase concentration. The glucose yield will reach a maximum because the substrate becomes saturated with enzyme. Since the rate of hydrolysis is proportional to the amount of enzyme adsorbed, the rate also reaches a maximum.

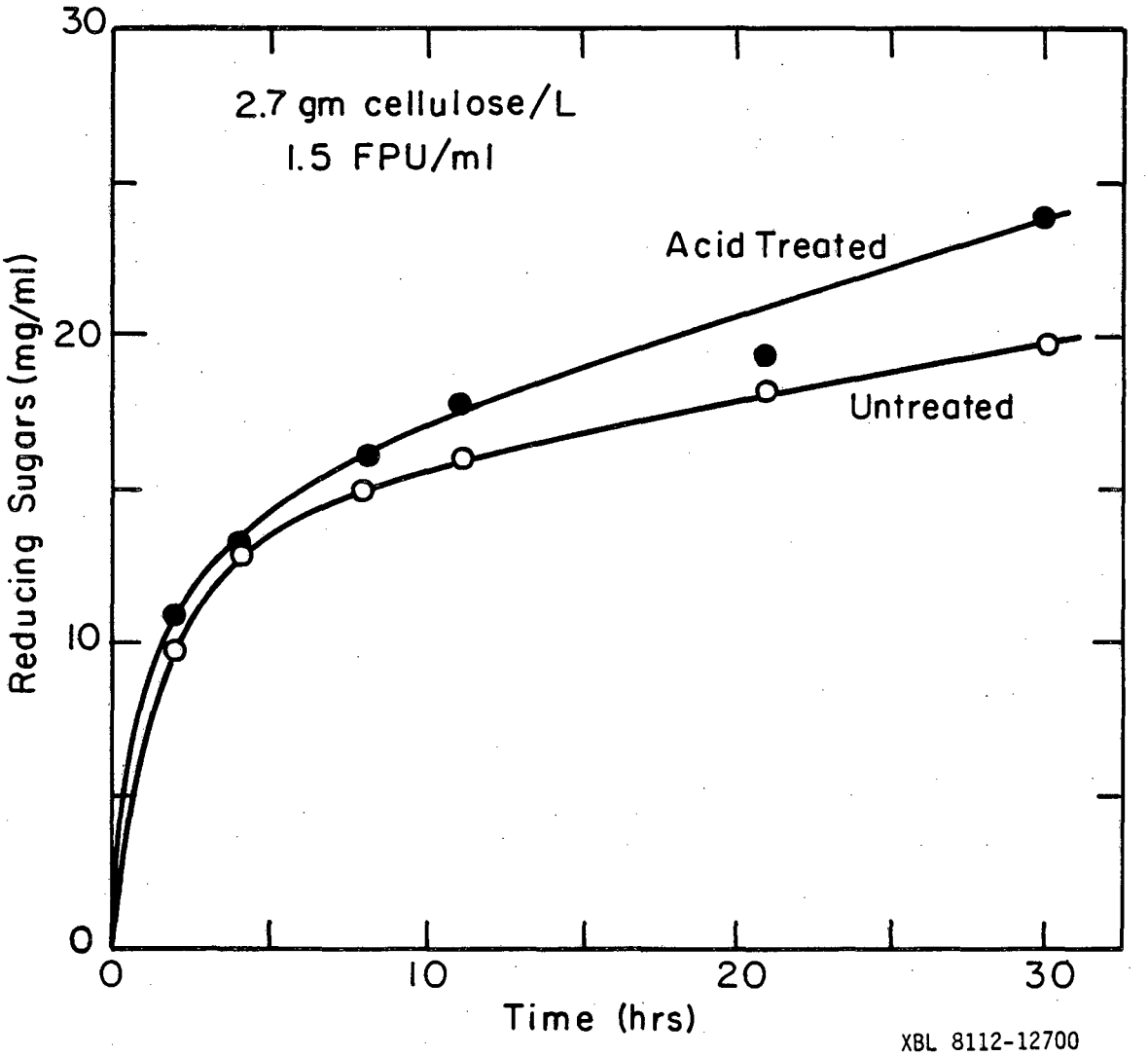


Figure 4.4. Effect of acid treatment on hydrolysis behavior.

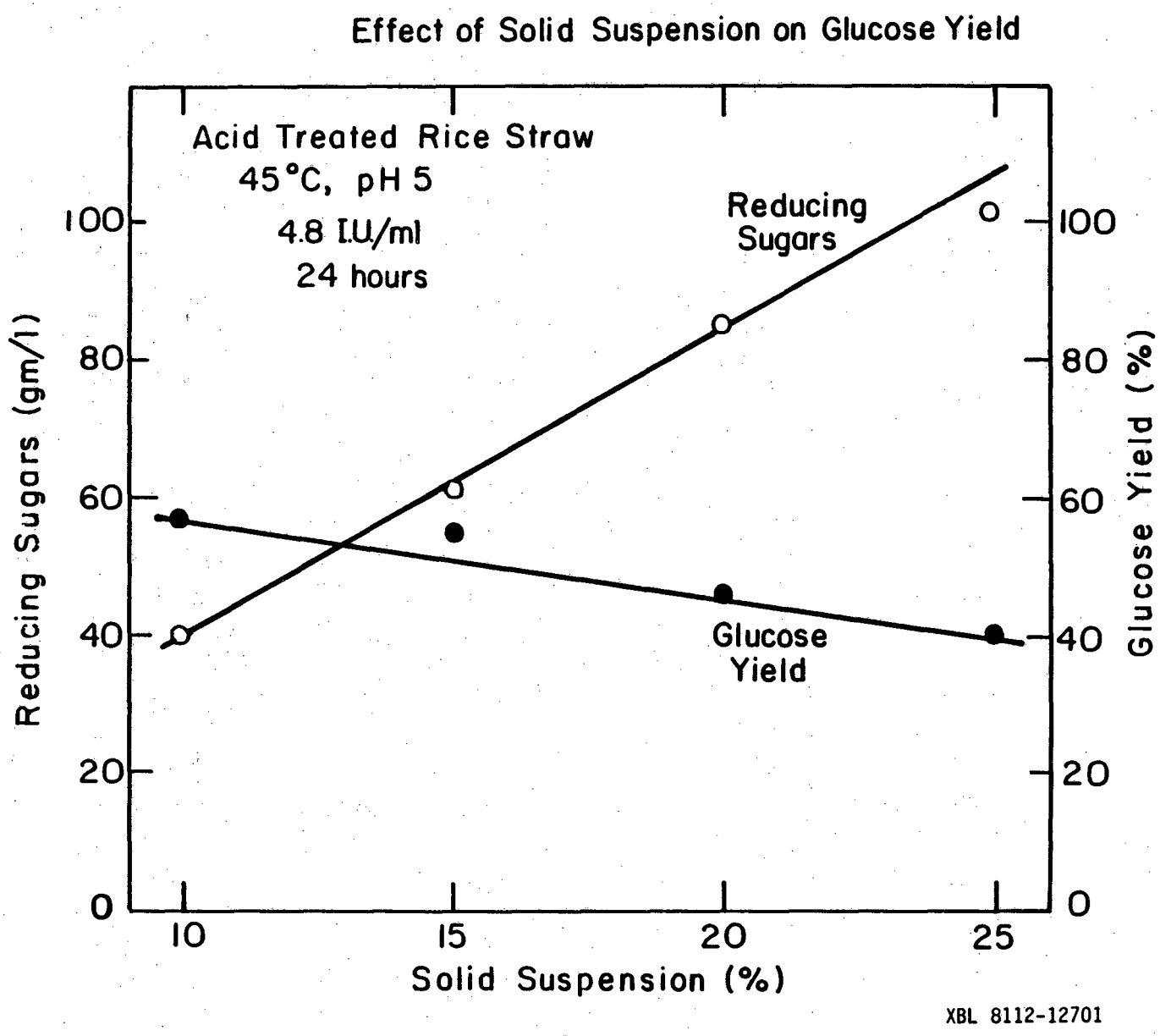


Figure 4.5. Effect of solid suspension on glucose yield.

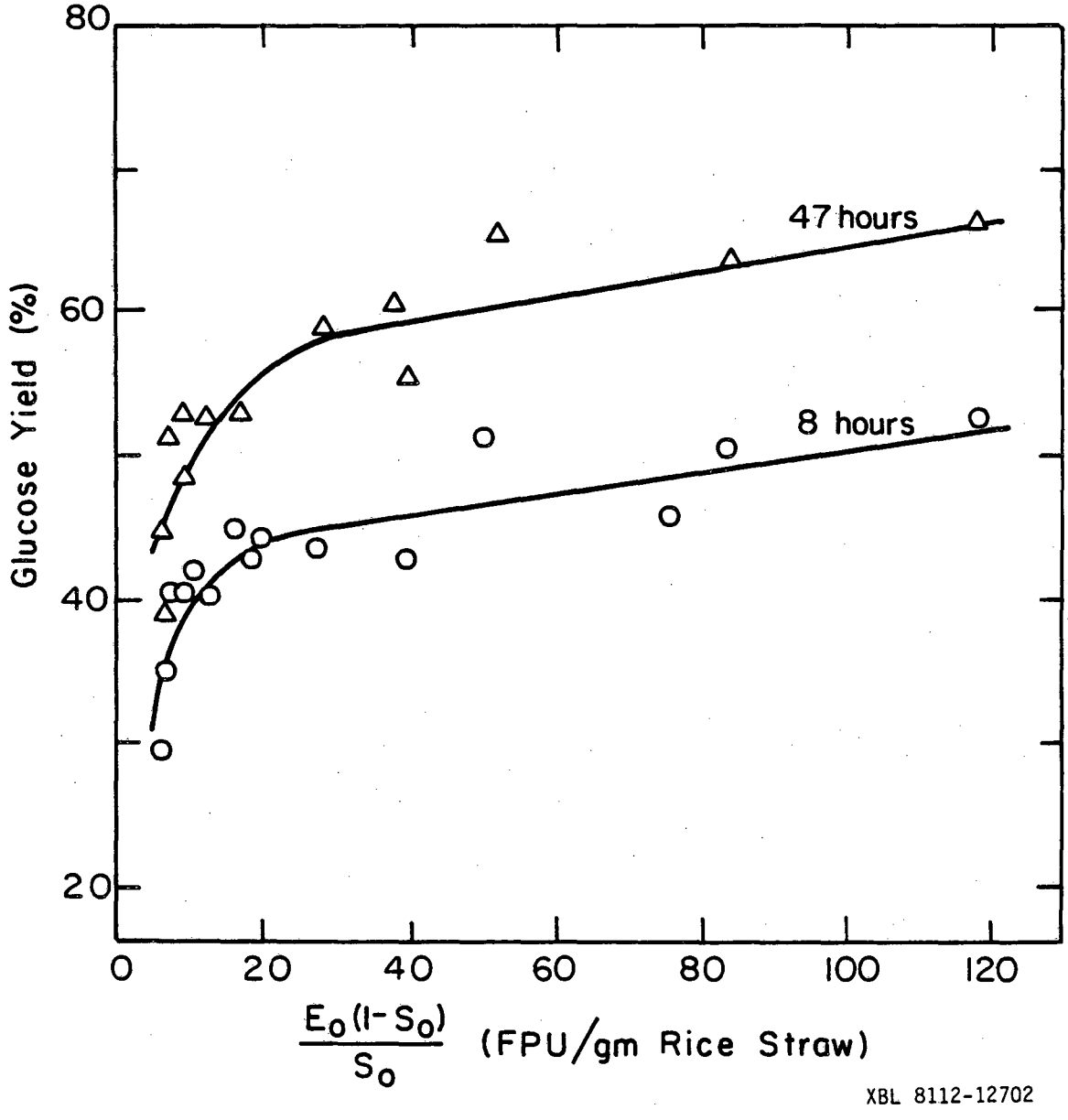


Figure 4.6. Non-linear dependence of glucose yield on enzyme concentration. Solid concentration in weight fraction, S_o.

Additional enzyme does not result in a significant increase in yield. This is unlike a homogeneous system where the reaction rate is first order in enzyme concentration. Hence, in the case of a heterogeneous system of this kind, an adsorption process is controlling.

It should be noted that this manner of correlating the data obscures the effect of concentration of the products. For example, a 5% solid suspension with 0.5 FPU/ml has about the same enzyme:substrate ratio as a 20% suspension with 2.5 FPU/ml. While both cases may have about the same yield at 24 hours, the concentration of inhibitors, glucose and cellobiose, will differ because of the dilution.

4.2.3. Enzyme Adsorption:

The adsorption of cellulase on a cellulosic material was observed first by Halliwell (40). He reported that the aqueous phase becomes relatively free of enzyme immediately after mixing cellulase and substrate. This phenomenon is the basis for the enzyme recovery operation in the Berkeley process (119). As a result, the amount of enzyme adsorbed is a primary consideration in the process economics. Figure 4.7 shows the fraction of filter paper activity retained in solution after 33 hours as a function of solid suspension and enzyme strength. The percentage of enzyme adsorbed increases with increasing rice straw concentration and decreasing enzyme strength.

Figure 4.8 shows the various activity profiles during hydrolysis. Variations in the adsorption of the

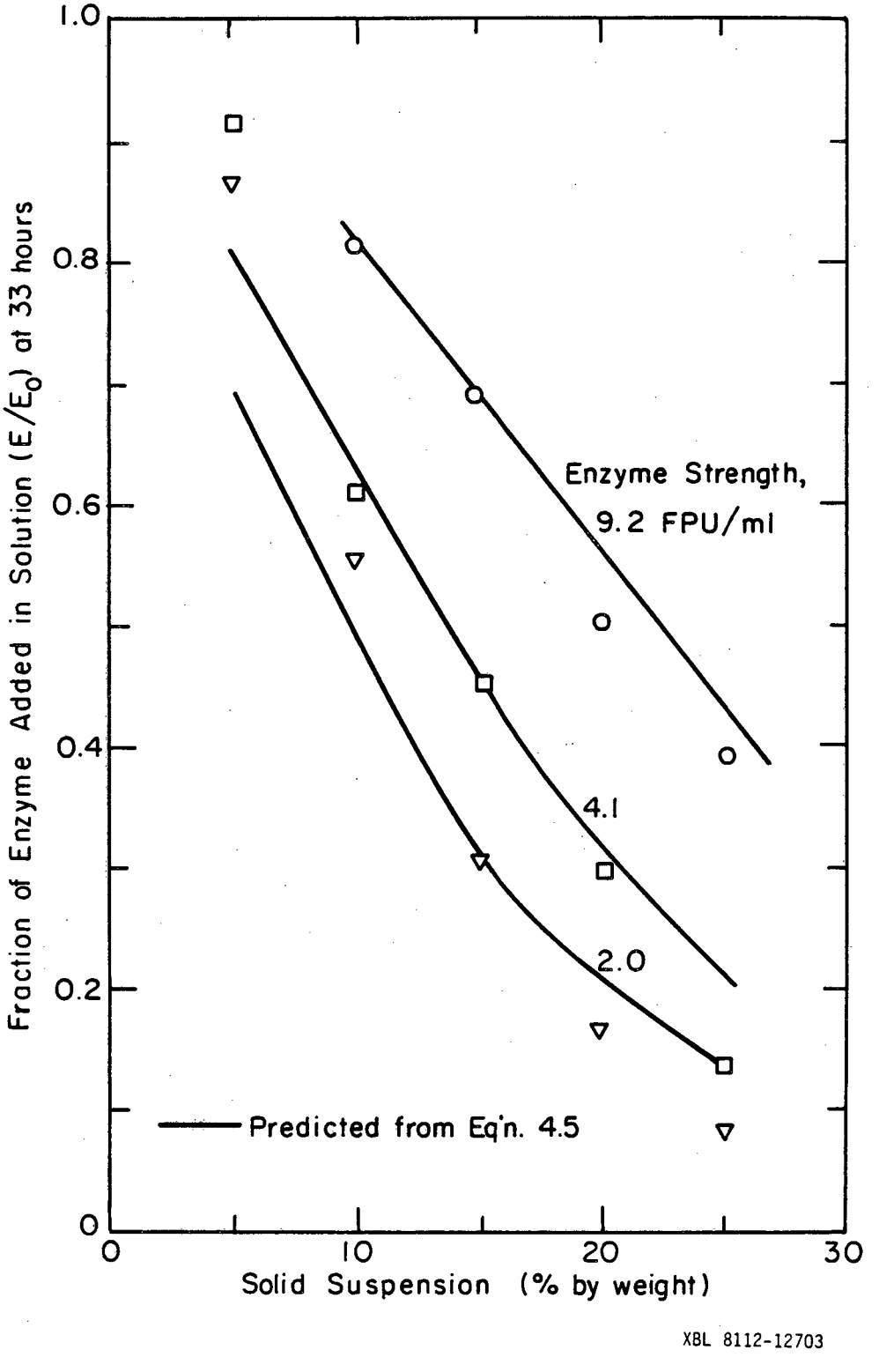


Figure 4.7. Effect of solid suspension and enzyme concentration on the amount of free enzyme during hydrolysis.

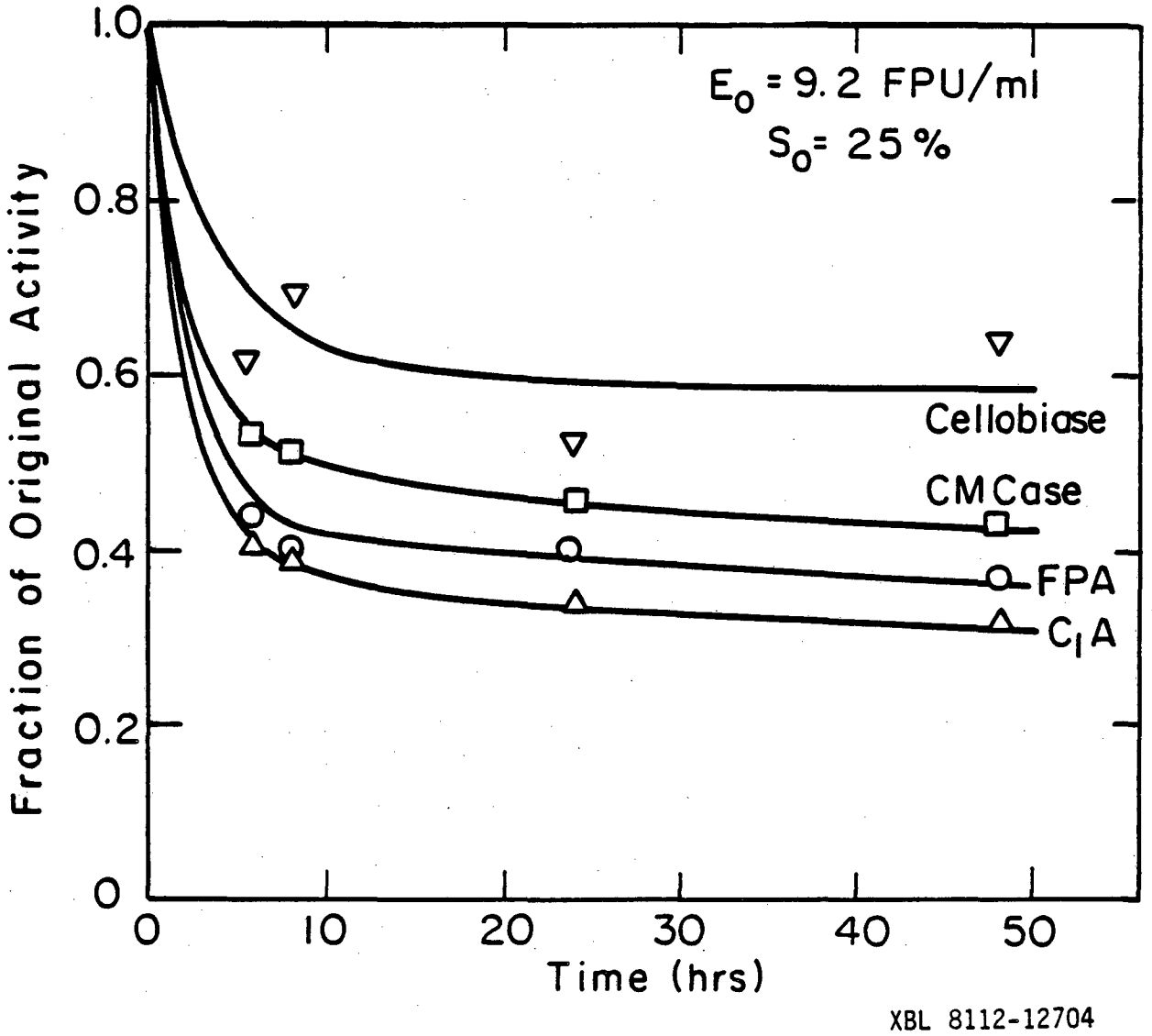


Figure 4.8. Absorption patterns of cellulase during hydrolysis.

enzyme components are expected because they possess different affinities for different regions of the cellulosic material. Each activity measurement has a common trend of rapid initial adsorption followed by a gradual decrease in the activity remaining in solution. Mandels (69) observed that after 24 hours of hydrolysis some of the enzyme had gone back into solution because more than 50% of the cellulose had been digested. No conclusion concerning release of enzyme back into solution can be drawn from these data since there may be two opposing forces responsible for the continual decrease in activity of the solution--release back in solution and inactivation as a result of thermal or physical effects.

Table 4.2 shows the difference in activity retained in solution at 33 hours for several different hydrolysis conditions. C_1 and FPA activites follows roughly the same pattern while CMCase demonstrates less of a change in activity. The cellobiase components show significantly less ability to adsorb on cellulose. While these differences in adsorptive capacity have important implications in a continous enzyme recovery operation, this study will be based on retention of filter paper activity. This may well be an oversimplification but it is beyond the scope of this work to consider the dynamics of the adsorption-hydrolysis system. For instance, if β-glucosidase does not significantly adsorb on cellulose (this is reasonable since it is specific for soluble oligoglucosides) then an

Table 4.2 Adsorption patterns for various hydrolysis conditions.

	A	B	C	D
Enzyme Concentration (FPU/ml)	4.1	2.0	2.0	1.0
Substrate Concentration (%)	10	5	15	5
Percent Activity Remaining in solution (E/E_o)				
FPA	65	69	29	78
CMCase	88	83	35	60
C_1	62	67	41	76
Cellobiase	82	81	63	91

Reaction time: 25 hours

ultrafiltration system or immobilized β-glucosidase reactor (50) may be neccessary to maintain a stable system.

The adsorption of cellulase can be better understood by fitting the data to an equilibrium adsorption model, similar to a Langmuir isotherm (84). This is based on a reversible, adsorption-desorption of the cellulase. Castanon and Wilke proposed that the enzyme, once adsorbed will remain immobilized within the molecular and supramolecular organization of the substrate (15). This finding was based on two observations: 1) The enzymatic activities in solution decreased during the course of hydrolysis, and 2) SDS polyacrylamide gel electrophoresis studies established that a slow but continuous uptake of enzyme occurred after the rapid initial adsorption.

Nevertheless, desorption was evident from the following experiment. An enzyme hydrolysis was conducted for 2 hours and the slurry was filtered by vacuum through glass wool filter paper. The cake was washed with two volumes of 45^{o}C, 0.05M acetate buffer. The volume of wash was equivalent to the water in the cake. The wash solution was the same temperature and pH as the cake in order to maintain an equilibrium situation. The solids were resuspended in buffer and the hydrolysis was continued. Table 4.3 shows the FPA after resuspension. The FPA showed significant change in 22.8 hours, indicating the enzyme was released back into solution.

This experiment also measured the sugar production

Table 4.3 Reversibility of adsorption.

	Low Enzyme Loading	High Enzyme Loading
E_o	1.0 FPU/ml	4.0
S_o	10%	10
$E_o(1-S_o)/S_o$	9 FPU/gm rice straw	36
Free Enzyme: (FPU/ml)		
Time: 2.0 hrs	0	0.26
22.8	0.23 (0.17)	0.50 (0.61)

The numbers in parenthesis are estimated from Equation 4.5

by the adsorbed enzyme. Figure 4.9 shows the adsorbed enzyme still retains its hydrolytic activity. The differences are due to the shift in equilibrium, thus reducing the amount of adsorbed enzyme, and a reduction in cellobiase activity.

This experiment supports an equilibrium adsorption model of the form:

$$E_{ads} = \frac{E_{sat} E}{E + K_E} \qquad (4.1)$$

where E_{ads} = adsorbed enzyme (FPU/mmol cellulose)

E = free enzyme (FPU/l)

E_{sat} = maximum adsorptive capacity (FPU/mmol cellulose)

K_E = equilibrium constant (FPU/l)

This equation will be derived in more detail in Section 5.2. The collection of the data at 33 hours resulted in two simplifications: 1) the reaction rate is very slow at this time so the flux at the surface is negligible, and 2) the surface area is about the same for all points because the data correspond to a narrow range of conversions (50-55%). This allows the simplification that the adsorbed enzyme can be expressed in activity per millimole of cellulose rather than activity per unit surface area. It should be noted that the amount adsorbed could be based on either rice straw or the cellulose content of the straw. This study, however, does not attempt to investigate the microscopic qualities of adsorption.

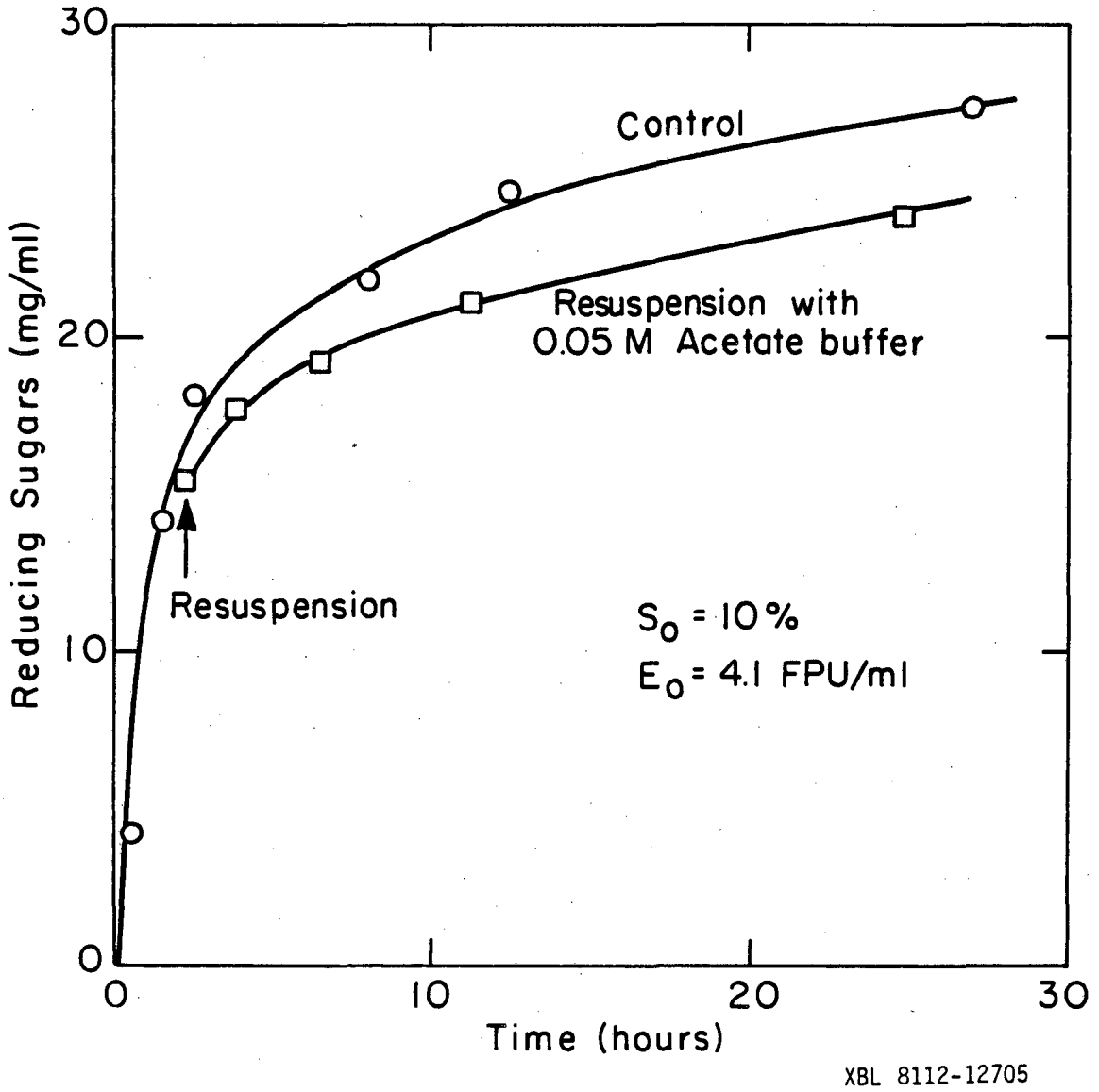


Figure 4.9. Hydrolytic activity of adsorbed enzyme.

Figure 4.10 shows the data from Figure 4.8 replotted to fit the form of equation 4.1. The constants can be found from a linear form of the equation:

$$\frac{E}{E_{ads}} = \frac{1}{E_{sat}} E + \frac{K_E}{E_{sat}} \qquad (4.2)$$

This rearrangement was chosen to minimize the error inherent in reciprocal plots. The constants could be found with a double reciprocal plot but this magnifies the errors on both axes. Figure 4.11 shows the determination of the maximum adsorptive capacity, E_{sat}, and the equilibrium constant, K_E.

$$E_{ads} = \frac{26.8E}{E + 1.050} \qquad (4.3)$$

This equation can be combined with an enzyme balance to predict the fraction of the activity remaining in solution.

$$E_{ads}S + EV = E_oV \qquad (4.4)$$

where S = rice straw at 33 hours (mmol of cellulose/l)

V = volume of solution (l)

This yields an equation of the form:

$$e = E/E_o = \frac{26.8S+1.05V-E_oV+[(26.8S+1.05V-E_oV)^2-3.32E_oV^2]^{1/2}}{2VE_o} \qquad (4.5)$$

The above relationship is shown in Figure 4.10. The largest deviations occur for the low substrate concentrations since the activity measurements are subject to the most uncertainity in this region. The model was also used to estimate the new equilibrium conditions for the resuspension experiments. The predicted values agree quite closely with

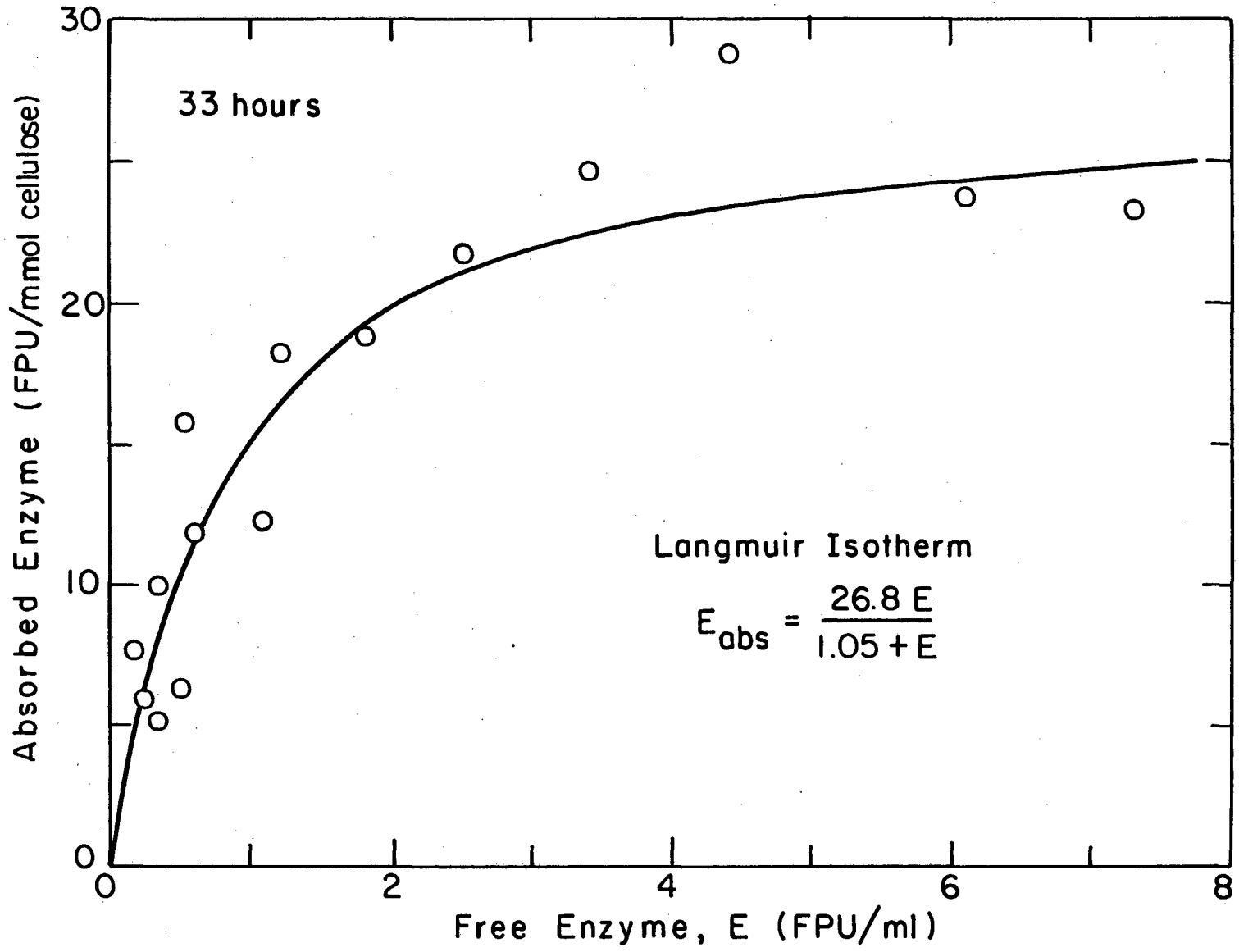


Figure 4.10. Adsorption isotherm for cellulase on cellulose.

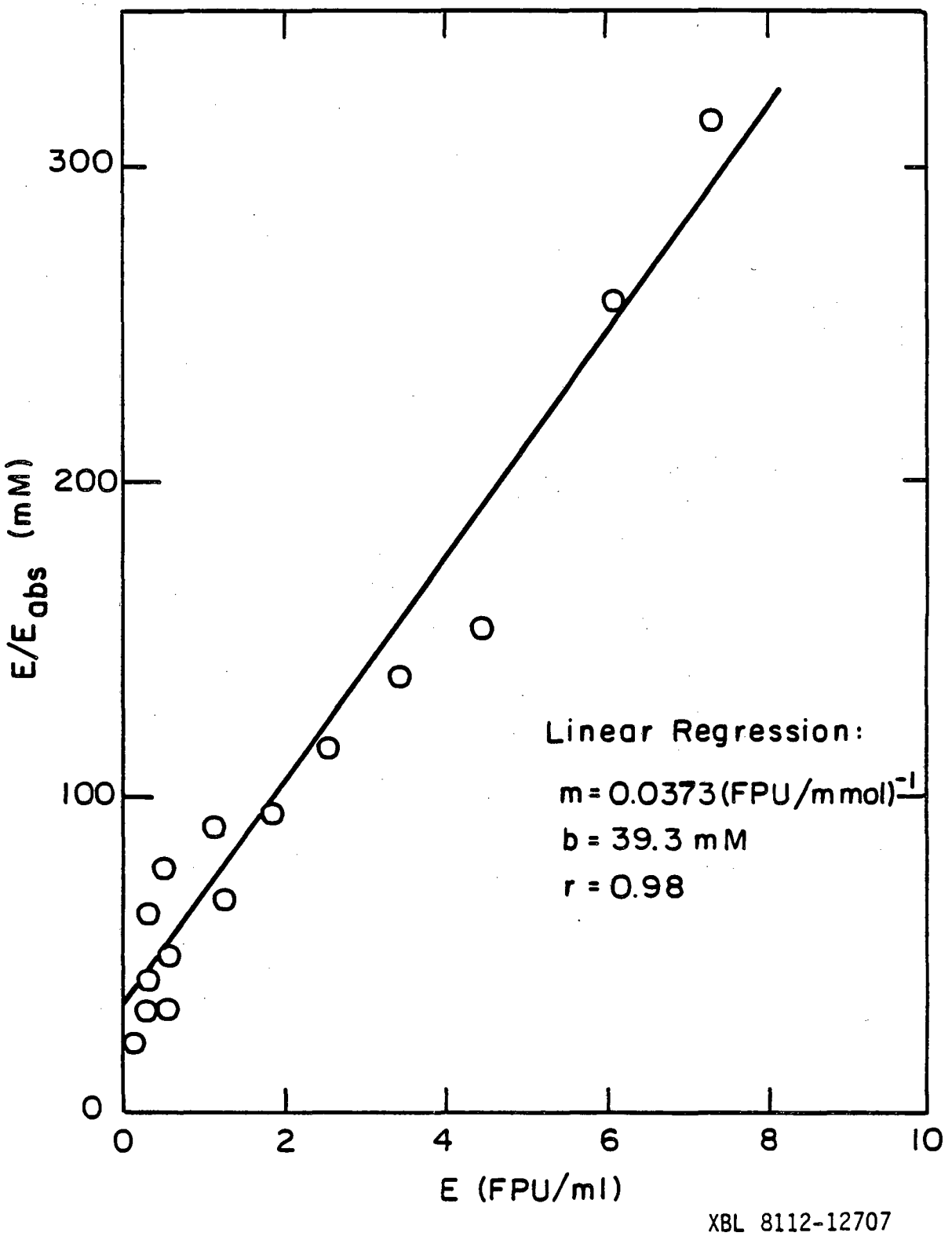


Figure 4.11 Determination of the parameters in the adsorption model.

the experimental results.

The parameters in equation 4.3 were derived from data at 33 hours of hydrolysis time. Extrapolation should be done with care for two reasons. One, adsorption is a surface phenomenon and the parameters hold for a narrow range of conversions. Two, the equilibrium constant is a function of the enzyme-substrate interactions, so regions where the surface properties differ significantly (e.g. the accessible cellulose) will have different binding constants.

In summation, cellulose hydrolysis is controlled by an adsorption process. A Langmuir isotherm models the data well at long reaction times. The maximum adsorptive capacity of the rice straw, 27.7 FPU/gram, coincides closely to the point where the glucose yield levels out with respect to initial enzyme:substrate ratio (see Figure 4.7). This supports the conclusion that a saturation of the substrate with enzyme limits the reaction rate.

4.3. Ethanol Fermentation of Enzymatic Hydrolysates

The sugar solution resulting from rice straw hydrolysis will be concentrated to a level that yields the optimum overall productivity and cost effectiveness for the plant. A 10% glucose solution was chosen as the first approximation (21). A more complete study is required to optimize the ethanol inhibition effects, distillation requirements, and evaporation costs. The hydrolysate will contain xylose (about 1.4%), cellobiose, higher oligomers, and other solubilized compounds from the rice straw. There

was some concern that toxic substances from the rice straw (pesticides or herbicides) may be concentrated in this solution and drastically affect the yeast metabolism.

Figure 4.12 shows a typical batch fermentation profile for rice straw hydrolysate. No major inhibition was exhibited and the maximum specific growth rate was 0.36 hr^{-1}. A control with laboratory glucose and nutrients demonstrated a specific growth rate of 0.45 hr^{-1}. The product yields were the same. Upon completion of the fermentation, 5.3 grams of cells and 46 grams of ethanol were produced from 100 grams of glucose. These results indicate that the overall ethanol productivity will be slightly lower for hydrolysate sugars.

Future investigation is required to determine whether the inhibition is a result of a metabolite in the enzyme solution, a compound associated with the rice straw from agricultural practices, or the non-fermentable sugars in the solution. In addition, the nutritional requirements for organisms fermenting rice straw hydrolysate should be studied. Certain trace growth factors may be present in the straw or could be supplemented inexpensively from other rice by-products.

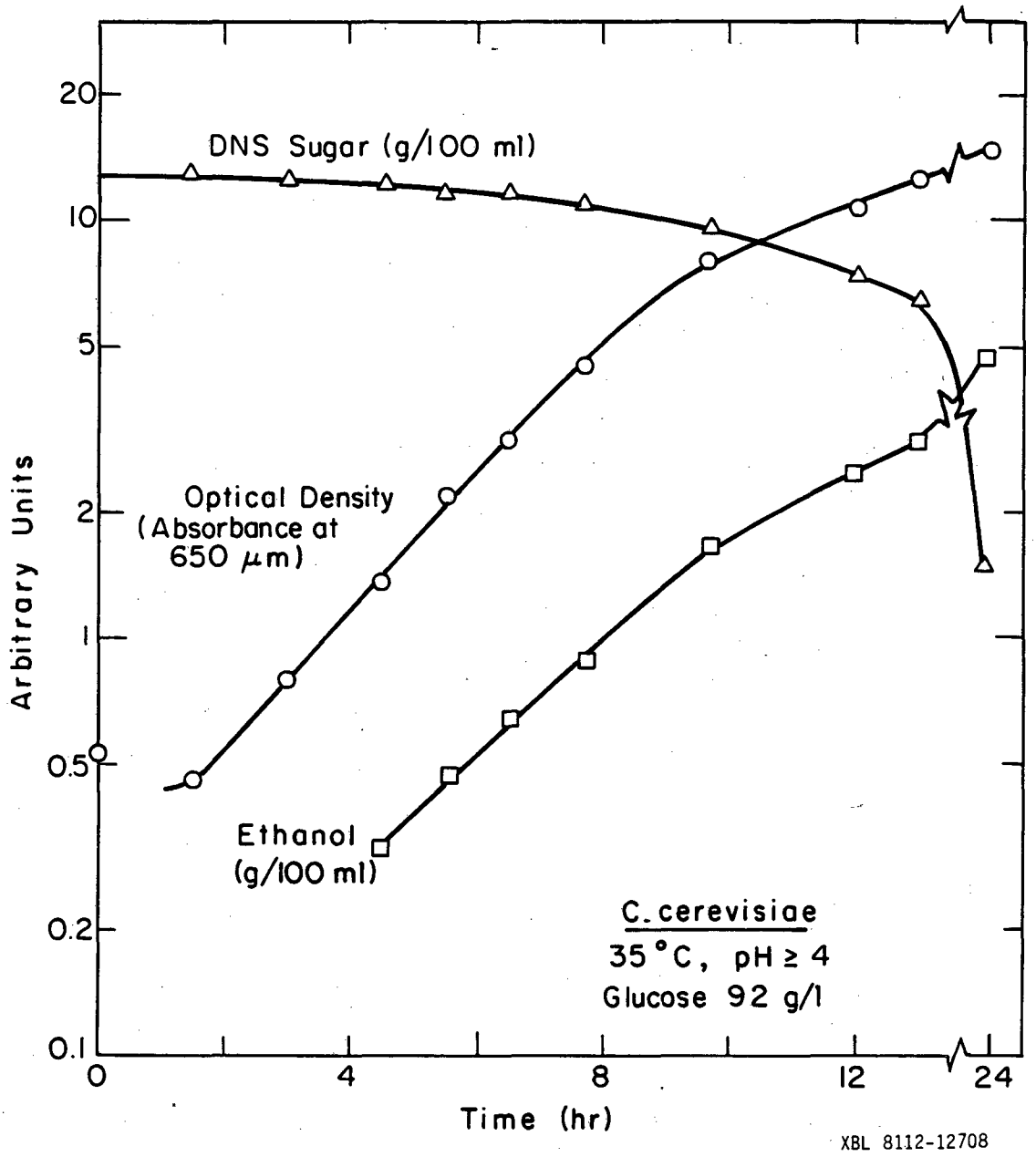


Figure 4.12. Batch fermentation of enzymatic hydrolysate.

V. KINETIC MODELLING

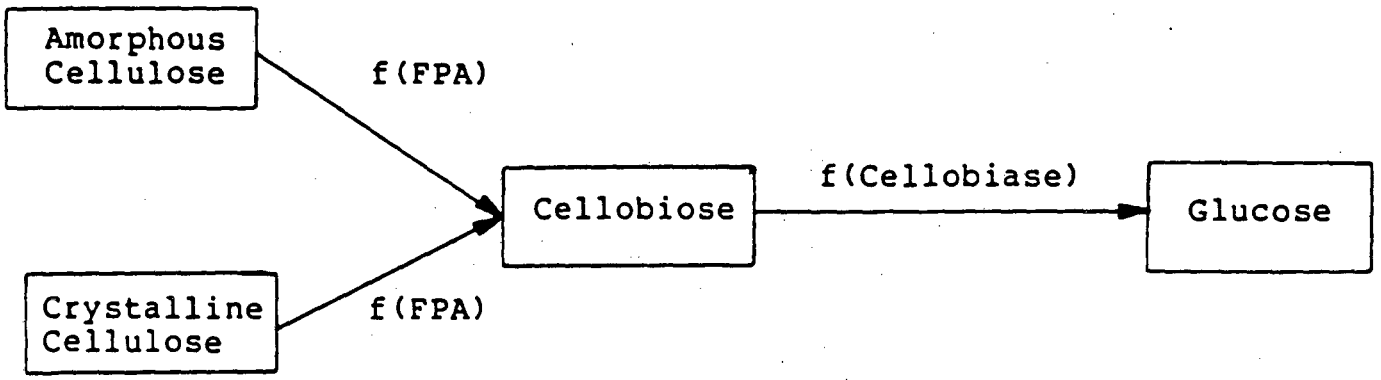
5.1. Principle Assumptions

The major objective in developing a kinetic model of cellulose hydrolysis was to incorporate the concepts of heterogeneous catalysis into the cellulose-cellulase system. The model will aid in understanding the mechanism of hydrolysis and be used to simulate saccharification of rice straw at high concentrations of substrate and enzyme, for process design and optimization purposes. Previous work in modelling cellulolytic systems was summarized in Section 2.3. It should be noted that earlier studies were generally restricted to low substrate concentrations (less than 50 grams per liter), delignified cellulosic substrates (Solka Floc), and low strength enzyme solutions (less than 2 FPU/ml with the QM9414 strain). This study will consider substrate concentrations up to 333 grams of rice straw per liter and enzyme solutions with activities up to 9.2 FPU/ml.

Enzymatic hydrolysis depends on three major factors: 1) the structure of the substrate, 2) the nature of the enzyme system, and 3) the interactions between the enzyme and substrate. This model will make the following simplifying assumptions with regard to these considerations:

1) The cellulose consists of two regions--crystalline and amorphous--that exhibit different properties with respect to enzyme adsorption and hydrolysis.

Table 5.1 Proposed reaction network for cellulose hydrolysis.



2) The multiple enzyme system can be represented quantitatively by two overall activity measurements--filter paper and cellobiase.

3) Adsorption of cellulase on the cellulose surface is rapid and equilibrium is established at all times.

4) Both the crystalline and amorphous fractions of the cellulose are degraded by a heterogeneous enzymatic reaction to cellobiose. The rate determining step in each of these pathways is a function of the filter paper activity of the enzyme. The resultant cellobiose is hydrolyzed to glucose by a homogeneous reaction and is a function of the β-glucosidase activity of the enzyme solution. Table 5.1 shows the proposed reaction network.

5.1.1. Assumptions Concerning the Structure of Rice Straw:

The complex nature of lignocellulosic materials was outlined in Section 2.1. Experimental results confirm the existence of two regions of reactivity of the cellulose (Figure 4.2). These regimes correspond to differences in the structure of the cellulose. They will be called amorphous and crystalline for simplicity. It should be emphasized that the observed differences in reactivity and adsorption (15) may result from variations in crystal structure (26,113), in accessibility to the enzyme

(16,19,104), or in the degree of polymerization (83). This study will not attempt to distinguish the predominant effect that causes differences in reactivity. It will differentiate on the basis of hydrolysis behavior with the cellulase enzyme.

The degrading rice straw particles will be modelled as shrinking spheres with an amorphous shell and a crystalline core. This is a first approximation for the intricate structure of the lignocellulosic material based on the assumption that the majority of amorphous material is formed as a result of the attrition process. The disorientation of the exterior of the particle from mechanical forces and chemical treatment is responsible for the amorphous nature of the substrate.

An alternative model of interspersed amorphous and crystalline material throughout the material could be proposed, but this would necessitate significant pore diffusion to degrade a majority of the amorphous cellulose. This seems improbable because of the macromolecular nature of the enzyme molecules (54).

5.1.2. Assumptions Concerning the Nature of the Enzyme System:

The cellobiohydrolase and endoglucanases present in cellulase have different affinities (15,30) for the different regions of the substrate. This leads to a complex adsorption process between the components. The established activity measurements do not distinguish between the

components but reflect rather their combined activity toward a particular substrate. As a result, one activity measurement--filter paper activity--is used to simplify the adsorption process on the amorphous and crystalline fractions. This will also account for the synergism among the enzyme components. A more detailed model that accounts for these complex interactions would have to be based on fractionation studies that yield the kinetic and adsorptive properites of the individual enzymes, a procedure which is beyond the scope of this present study.

The protein structure of the enzymes is sensitive to temperature and shear and is subject to denaturation. For the time frame of this model, less than 48 hours, this effect is assumed to be negligible. It should be noted that this factor could become significant when reuse of the enzyme is considered for a continuous system.

5.1.3. Assumptions Concerning the Enzyme-Substrate Interactions:

The amount of enzyme adsorbed is a function of the number of available sorption sites and, in turn, the amount of accessible surface area. A distinction must be made between accessible surface area and total surface area. Total surface area has been determined for water swollen Solka Floc by applying the BET equation to nitrogen adsorption data (26).

The surface area available for a nitrogen molecule will be different than for a cellulase molecule. The size

of the adsorbing molecule will influence the amount of surface area accessible. Steric constraints will result from the orientation of the cellulose and lignin within the molecular organization of the substrate. As a result, direct measurement of the accessible surface area is difficult. Nevertheless, the relationship between surface area and adsorption was supported by Mandels et al. (66). They observed that adsorption increased as particle size decreased from 50 to 6.7 micron average diameter.

5.1.4. Other Assumptions:

The depolymerization reaction to cellobiose involves a detailed reaction pathway consisting of several intermediates and complex interactions among the enzyme components. This model assumes a parallel mechanism for the conversion of crystalline and amorphous cellulose to cellobiose where the global rates are proportional to the filter paper activity adsorbed on each surface. This reaction scheme, however, neglects glucose formation directly from cellulose by exo-β-1,4-glucanase glucohydrolase or by endo-β-1,4-glucanase.

The cellulase enzymes are subject to inhibition by cellobiose (31,70). It has been proposed that cellobiase acts by a competitive mechanism (competition between the substrate and the inhibitor for the active sites on the enzyme). This observation was based on a Michaelis-Menten analysis (31). For an adsorption process, a non-competitive mechanism appears more applicable. In other words, a

reversible reaction occurs between the enzyme-substrate complex and cellobiose to yield an inactive complex. The basis for this assumption is the fact that the enzyme is adsorbed rapidly at the start of hydrolysis when the cellobiose concentration is at a low level. The subsequent inhibition occurs at the surface with the enzyme-substrate complex.

5.2. Derivation of the Rate Equations

Table 5.2 shows the proposed reaction scheme, which is based on the modelling assumptions described above. The equations are similar in form to the Michaelis-Menten model; however, the substrate is not in excess. The rate of enzyme-substrate complex formation is not a direct function of the total substrate concentration but related to the amount of sorption sites available.

The amount of enzyme-substrate complex or adsorbed enzyme will be derived in a general form below since it is applicable to both the amorphous and crystalline regions.

5.2.1. Heterogeneous Reactions:

The amount of enzyme adsorbed in determined by an equilibrium between the adsorption and desorption reactions.

$$r_a = k_1 \left(\frac{E}{\beta M_E}\right)(1-\theta) a n_s$$

$$r_d = k_{-1} \theta a n_s$$

The application of the quasi-steady state assumption, that is, the rate of adsorption equals the rate of desorption,

Table 5.2 Reaction Scheme for Cellulose Hydrolysis.

Heterogeneous Reactions:

$$E_C + S_A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \xi_A \xrightarrow{k_2} E_C + G_2$$

$$E_C + S_C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \xi_C \xrightarrow{k_4} E_C + G_2$$

$$\xi_A + G_2 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \xi_A G_2$$

Homogeneous Reactions:

$$E_B + G_2 \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E_B G_2 \xrightarrow{k_7} E_B + 2G$$

$$E_B + G \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} E_B G$$

$$E_B G_2 + G \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} E_B G_2 G$$

results in the following expression:

$$\xi = \theta n_s a = \frac{n_s a \Sigma}{E + K} \qquad \text{where } K = \frac{k_{-1}}{k_1} \beta M_E \tag{5.1}$$

The amount of enzyme adsorbed is based on reactor volume, rather than the weight of adsorbent, since it is constant with time.

The interfacial area, a, is proportional to the number of particles. While fragmentation has been observed during hydrolysis, it is predominately free fiber formation (59). It is assumed that the area of the particles, rather than that of the fibers, controls the reaction rate. As a result, the number of particles per unit volume, N_o, is constant with time. The interfacial area is equal the the product of the surface area per particle and the number of particles per unit volume.

$$a = \pi d_p^2 N_o$$

The total number of particles is related to the cellulose concentration as follows:

$$C = \frac{\pi}{6} d_p^3 \rho_c N_o$$

These relationships yield an expression for the interfacial area that is a function of the initial particle size, the particle density, and the bulk concentration of cellulose. It should be noted that this is an equivalent surface area for a shrinking sphere.

$$a = \frac{6}{\rho_c d_{p_o}} C_o^{1/3} C^{2/3} \tag{5.2}$$

The surface area will change with the cellulose concentration to the two-thirds power. This relationship combined with Equation 5.1 will yield an expression relating the amount of enzyme adsorbed at any time to the substrate concentration and the amount of free enzyme.

$$\xi = \frac{6n_s}{\rho_c d_{p_o}} C_o^{1/3} C^{2/3} \frac{E}{E + K} \qquad (5.3)$$

This equation can be related to the initial enzyme concentration by an enzyme balance, $E_o = E + bM_E\xi$. The simplification common to gas-solid heterogeneous catalysis, that the amount adsorbed is negligible with respect to the initial concentration, is not applicable for this system. Figure 4.7 shows that 10 to 90% of the enzyme can be adsorbed on the solid. A quadratic equation in ξ results that can be solved as shown:

$$\xi' = \frac{1}{2}[bC_o^{1/3}C^{2/3} + K + E_o - [(bC_o^{1/3}C^{2/3} + K + E_o)^2 - 4bC_o^{1/3}C^{2/3}E_o]^{1/2}]$$

$$\text{where } b = {}^{6E_{sat}}/_{\rho_c d_{p_o}} \qquad (5.4)$$

The amount of enzyme adsorbed is expressed in filter paper units.

These expressions apply for adsorption on one surface. However, as hydrolysis proceeds, crystalline and amorphous portions of the material will be exposed at the same time. This results in a modified form of Equation 5.3.

$$\xi_T' = \xi_A' + \xi_C' = \frac{b_A C_{Ao} C_A^{1/3} C_A^{2/3} E}{E + K_A} + \frac{b_C C_{Co} C_C^{1/3} C_C^{2/3} E}{E + K_c} \qquad (5.5)$$

A cubic equation in ξ results when combined with an enzyme balance.

The existence of amorphous and crystalline regions of cellulose simultaneously can be explained by the fact that the substrate consists of a distribution of particle sizes. Assuming that each particle contains the same percentage amorphous material, the amorphous content of the smaller particles will be deplete more rapidly than the larger ones. Hence, amorphous and crystalline substrate will be present at the same time. The particle distribution can be simulated by subdividng the population in subsets that correspond to the different screen fractions (Tyler Series). Each subdivision can be regarded as a separate substrate. The total surface area for the amorphous and crystalline regions can be estimated with Equation 5.2 and the total amount of enzyme adsorbed can be computed from Equation 5.5.

The reaction rate is proportional to the amount of enzyme adsorbed. This results in a rate expression of the form:

$$r_i = k_i \xi_i' \left(\frac{1}{1 + K_I G_2}\right) \quad \text{where } K_I = k_5/k_{-5} \qquad (5.6)$$

The rate of hydrolyis is a non-linear function of enzyme strength and substrate concentration. It should be noted that the proportionality factor, k_i, is not a rate constant

in the strict sense. Since the activity values are actually initial rate measurements, expressed in micromoles of substrate per minute, k_i is a conversion factor to account for differences in the hydrolysis conditions. This form of the rate equation is applicable to both the amorphous and crystalline reactions, while the crystalline reaction is assumed to exhibit negligible cellobiose inhibition.

5.2.2. Homogeneous Reactions:

The hydrolysis of cellobiose occurs in the aqueous phase and the classical Michaelis-Menten equation for mixed-type inhibition is applicable. This type of inhibition has been observed by Gong et al. with purified β-glucosidase components from the QM9414 strain of T. reesei (37). This proposed reaction sequence is shown in Table 5.1. The application of the quasi-steady state assumption results in a rate expression of the form:

$$\frac{dG}{dt} = \frac{2k_7 E_o G_2}{(1+G/K_1)G_2+(1+G/K_2)K_m} \qquad (5.7)$$

where $K_1 = k_{-9}/k_9$

$K_2 = k_{-7}/k_7$

$K_m = (k_6+k_{-7})/k_6$

This equation will be used to model the cellobiose hydrolysis in the overall scheme of cellulose saccharification.

5.3. Parameter Estimation

The development of a kinetic model specifies several

parameters that are characteristic of the entire system. These parameters can be estimated from the data set by using a non-linear optimization routine. The use of specific, independent experiments to estimate individual or groups of related parameters will produce estimates with more certainity, since the number of degrees of freedom are reduced.

5.3.1. Amorphous to Cellobiose Reaction:

The parameters for the hydrolysis of amorphous cellulose to cellobiose can be estimated from initial rate measurements of the hydrolysis of rice straw. Intial rate studies allow the following assumptions:

* The rate of sugar production represents amorphous cellulose hydrolysis since $\frac{dA}{dt} >> \frac{dC}{dt}$ at small time.

* Cellobiose inhibition is negligible since $K_I G_2 >> 1$.

This reduces equations 5.4 and 5.6 to the form:

$$r_{AG_2} = -\frac{dA}{dt} = \frac{k_2}{2\beta M_E} \left[b_A C_o + K_A + E_{Co} - \left[(b_A C_o + K_A + E_{Co})^2 - 4 b C_o E_{Co} \right]^{1/2} \right] \quad (5.8)$$

The values of the parameters were optimized in a non-linear least squares routine by minimizing the error function of the form:

$$L = \sum_{i=1}^{n} (r_{ip} - r_i)^2$$

The number of parameters to be estimated can be reduced by recognizing that the initial reaction rate will be a maximum

when the cellulose surface is saturated with enzyme. Equations 5.3 and 5.6 will reduce to the form:

$$(r_o)_{max} = \frac{k_2}{\beta M_E} bC_o = k^* C_o$$

Figure 5.1 shows the initial rate as a function of enzyme and substrate concentration. This behavior is in sharp contrast to a homogeneous system where the Michaelis-Menten model would predict that the initial rate is first order in enzyme concentration and zeroth order in substrate concentration. This difference arises from the assumption that in a homogeneous system the substrate is in excess while, in this heterogeous system, the enzyme is in excess (73).

Figure 5.2 shows that k^* is about 0.019 min^{-1} for this system. The values of the parameters in equation 5.8 were estimated. Figure 5.1 also shows the predicted curve in comparision to the experimental data.

The value for b is a function of the surface mean particle diameter for the particle distribution. Figure 5.3 shows the cumulative size distribution, from screen analysis, for this set of experiments. The surface mean particle diameter is calculated by integrating between $\phi = 0$ to $\phi = 1$ and yields a value of 0.16 mm. The particle density is assumed uniform for all particles and estimated from a bulk density of 0.19 grams of rice straw per milliliter. Assuming a porosity of 0.5 the cellulose concentration is:

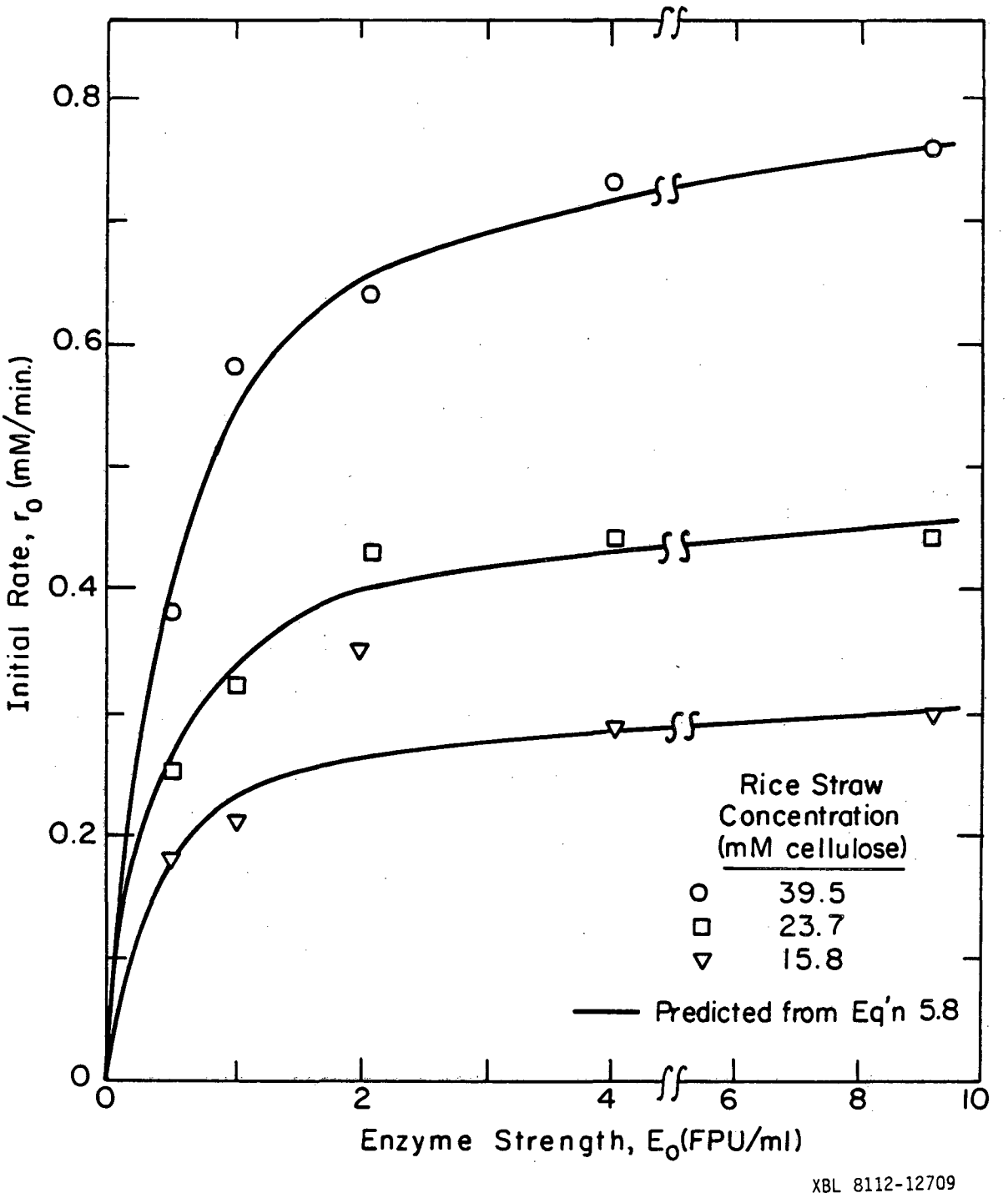


Figure 5.1. Non-linear relationship between initial reaction rate and enzyme concentration.

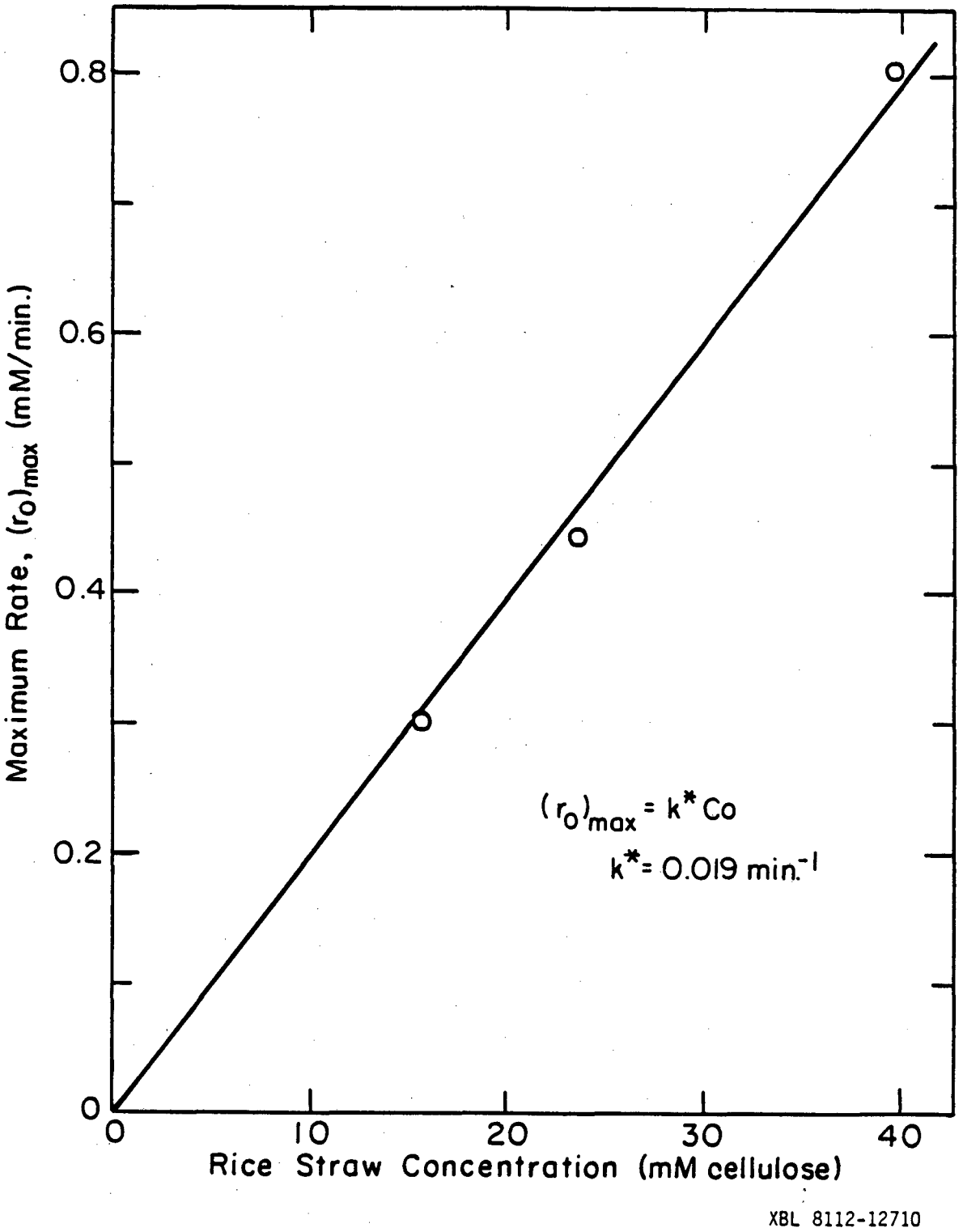


Figure 5.2. Determination of the rate constant for rice straw hydrolysis.

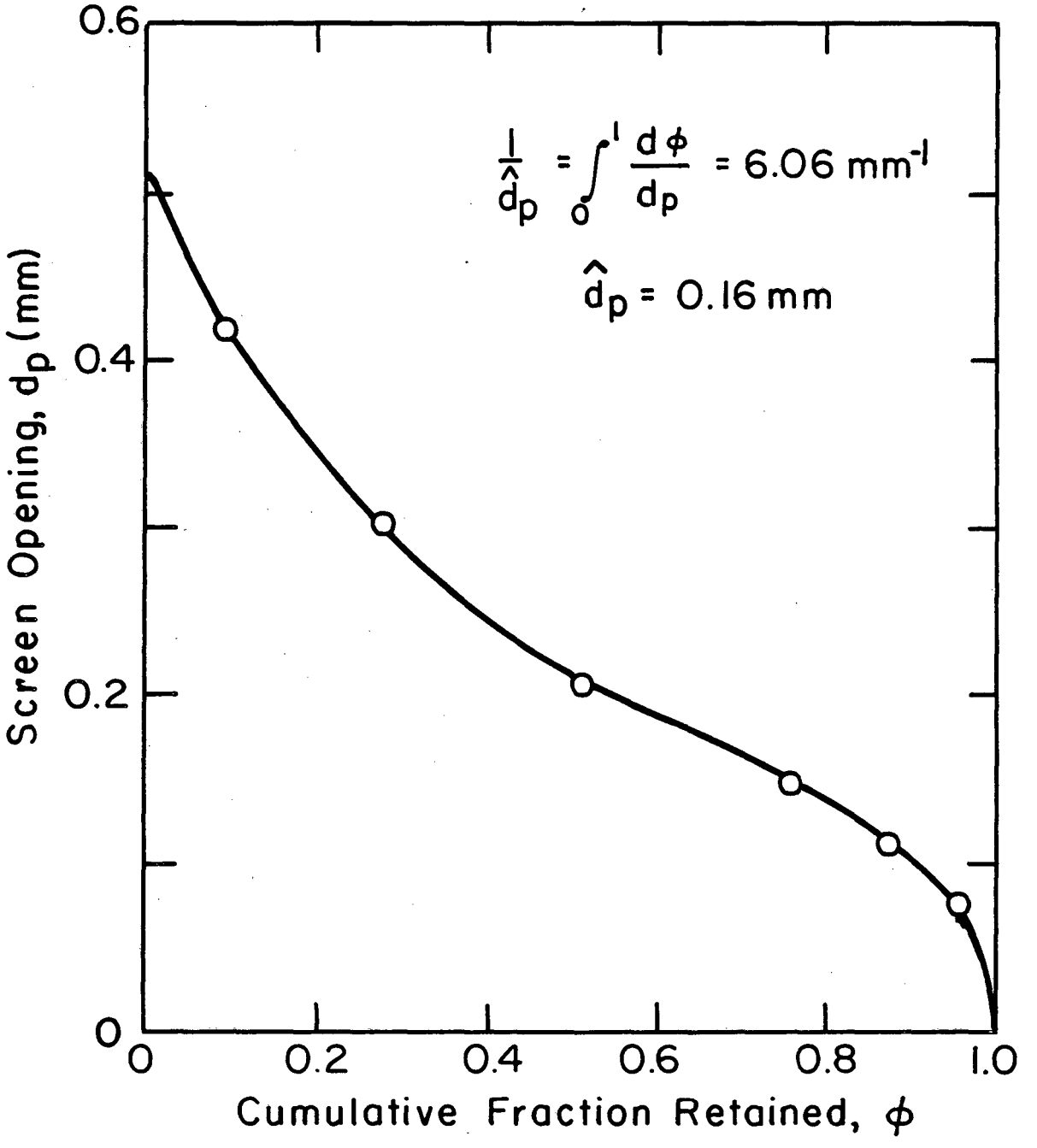


Figure 5.3. Cummulative particle size distribution for rice straw.

$$\rho_c = \left(\frac{0.19\text{gm rice straw}}{\text{ml}}\right)\left(\frac{0.57\text{gm cellulose}}{\text{gm rice straw}}\right)\frac{\text{mol}}{342\text{gm}} = 158\ \frac{\text{mol}}{(\text{m}^3)\text{P}}$$

since $b=\rho_c\frac{E_s}{d_p}$ = 9.29 FPU/mmol, the adsorptive capacity of the amorphous fraction, E_s, is 41.4 FPU/m^2. This yields a rate expression of the form:

$$r_{AG_2}=2.11\times10^{-3}\left(\frac{b-(b^2-4c)}{2}\right)(1/1+K_I G_2) \qquad (5.9)$$

$$\text{where}\quad b=41.4a_A+314+E_o \qquad a_A=\frac{6}{160}\sum_{k=1}^{n} C_{ok}^{1/3}C^{2/3}/d_{pk} \qquad (5.9a)$$

$$c=41.4a_A E_{Ao}$$

This equation will be used to model the cellobiose production from amorphous cellulose. The inhibition coefficient can not be found directly from experiment. The estimation of this quantity will be covered in Section 5.4.

The degree of crystallinity of rice straw was estimated from hydrolysis behavior. The transition between amorphous and crystalline is characterized by a bend in the hydrolysis curve. A procedure for determining the location of this transition is extrapolation of the linear region of the hydrolysis curve back to zero time (81). Figure 5.4 illustrates this technique. The mean fraction of amorphous for acid treated rice straw was estimated to be 0.38. This method is specific for this cellulase enzyme system, the molecular organization of this substrate, and its pretreatment.

5.3.2. Crystalline to Cellobiose Reaction:

Intial rate studies for crystalline hydrolysis are

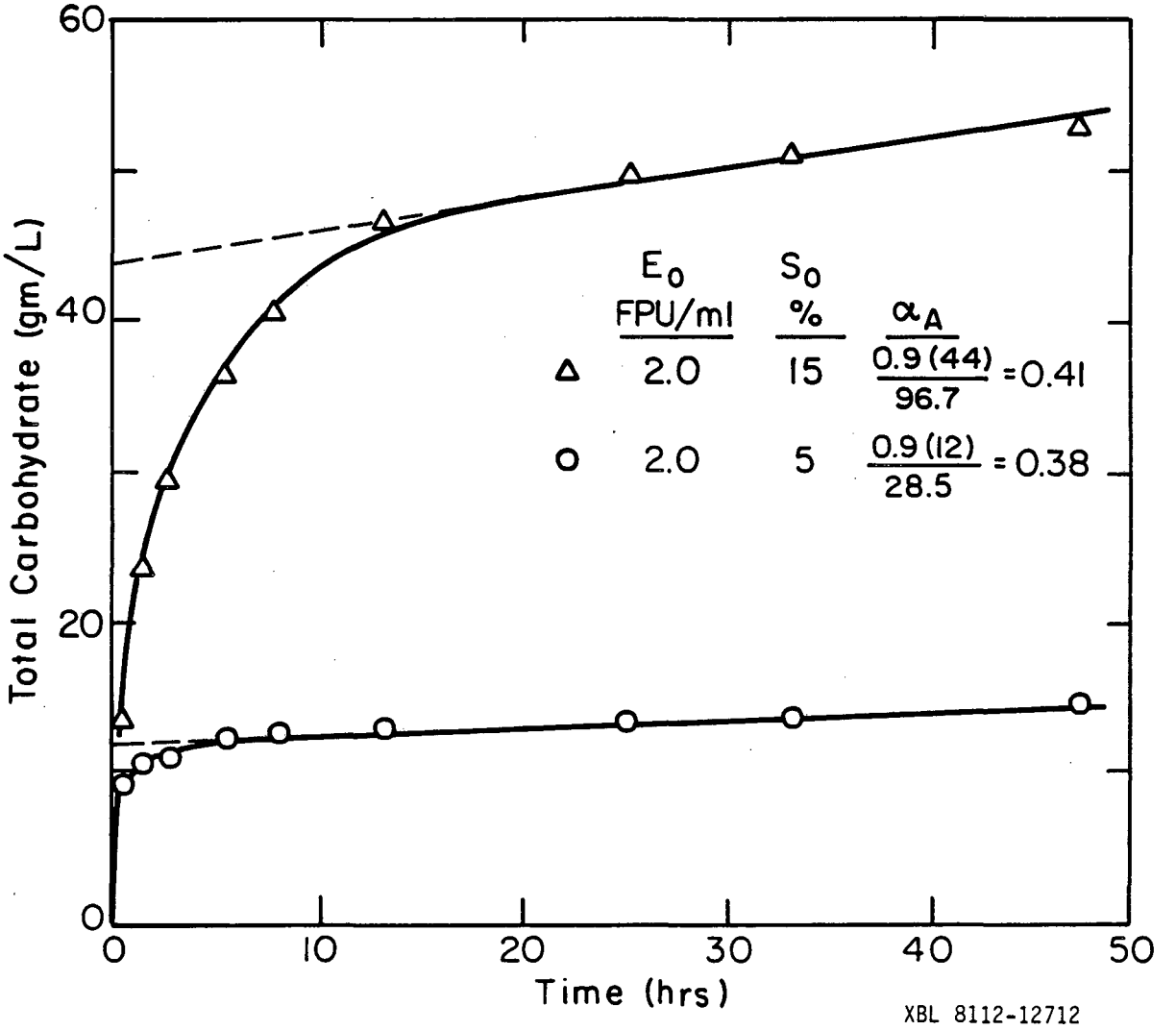


Figure 5.4. Determination of the degree of crystallinity from hydrolysis behavior.

difficult because of problems in obtaining a representative substrate and measuring the initial rate (an order of magnitude slower than the amorphous rate). As a result, the parameters were estimated from the crystalline regions (hydrolysis time greater than 10 hours) of the hydrolysis data presented in Section 4.2.

The adsorptive properties of cellulase on crystalline cellulose were analyzed in Section 4.2.4. Equation 4.1 is of the form:

$$\xi' = \frac{26.8CE}{E + 1.050}$$

The adsorptive capacity, E_s, is 26.8 FPU/mmole of cellulose. The capacity on a surface area basis can be estimated by calculating the surface area from the shrinking sphere model. The material used for the hydrolysis experiments had a surface mean diamter of 0.34mm (See Appendix I). The diameter that would correspond to the crystalline core is found as follows:

$$f_c = 1 - f_A = \left(\frac{d_C}{d_C + d_A}\right)^3$$

$$0.62 = 1 - 0.38 = \left(\frac{d_C}{0.34}\right)^3 \qquad d_C = 0.29\text{mm}$$

Equation 5.4 is used to calculate the adsorptive capacity of 203.4 FPU/m^2 for crystalline cellulose.

The rate of hydrolysis for different amounts of adsorbed enzyme were found from the hydrolysis data in Section 4.2.4. Cellobiose inhibition was assumed negligible. Figure 5.5 shows the relationship between

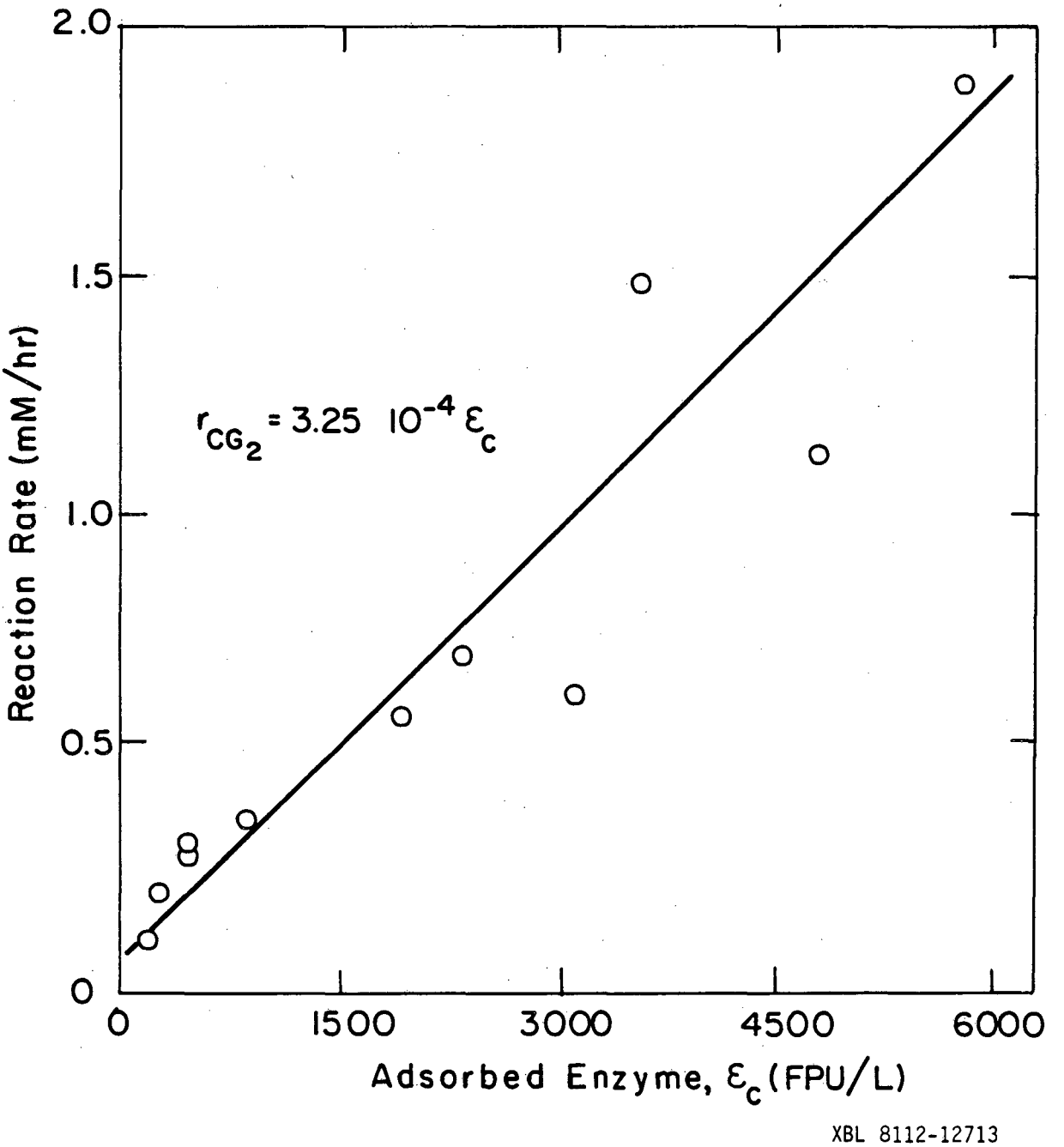


Figure 5.5. Relationship between the rate of crystalline hydrolysis and the amount of adsorbed enzyme.

hydrolysis rate and the amount of enzyme adsorbed. This yields of rate constant of 3.25×10^{-4} FPU/mmol-min. Hence, the equation for the rate of hydrolysis is of the form:

$$r_{CG_2} = 3.24 \times 10^{-4} \left(\frac{b-(b^2-4c)}{2}\right) \qquad (5.10)$$

where $b = 203.4a_C + 1050 + E_o$

$c = 203.4a_C E_o$

The amount of surface area is calculated from a similar expression to Equation 5.9a.

5.3.3. Cellobiose to Glucose Reaction:

The kinetic constants for cellobiose hydrolysis can be evaluated by initial rate studies. Figure 5.6 shows a Lineweaver-Burk plot for various levels of inhibitor.

$$\frac{1}{v} = \frac{K_m(1 + G/K_2)}{V_{max}} \frac{1}{G_2} + \frac{1 + G/K_2}{V_{max}} \qquad (5.11)$$

Caution in using a double reciprocal plot must be taken because measurement errors are magnified and yield larger uncertainity in the value of the slope. The approach to ensure the greatest degree of certainity is to find V_{max} from the intercept. The points with the least uncertainity cluster around the origin, giving a reliable value for V_{max}. Since the Michaelis constant is equal to the substrate concentration when the velocity is one-half of V_{max}, K_m can be found by interpolation (4). Figure 5.7 shows the determination of K_m.

The intersecting patterns obtained from the initial rate data in Figure 5.6 indicate that a mixed type of

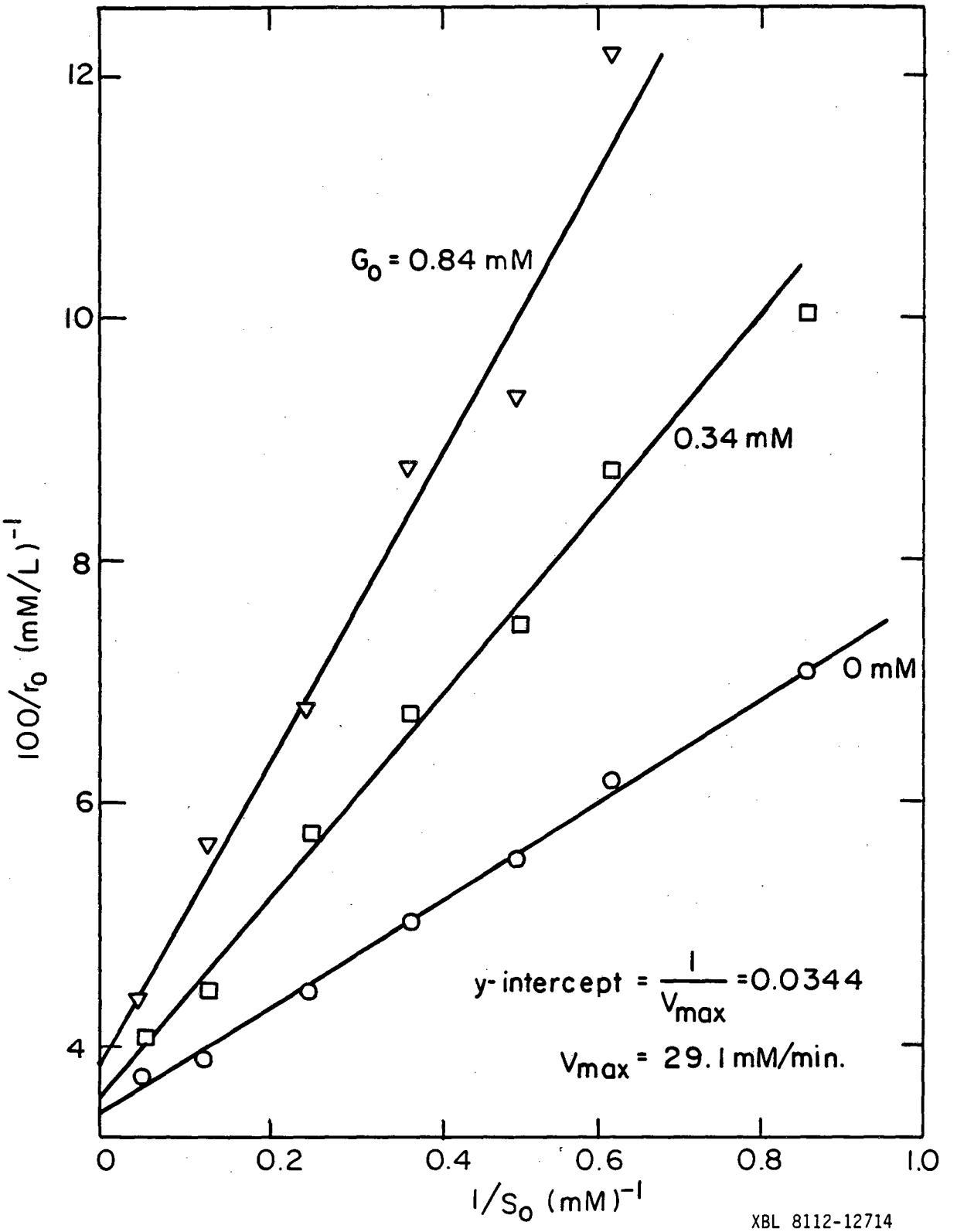


Figure 5.6 Lineweaver-Burk plot for cellobiose hydrolysis.

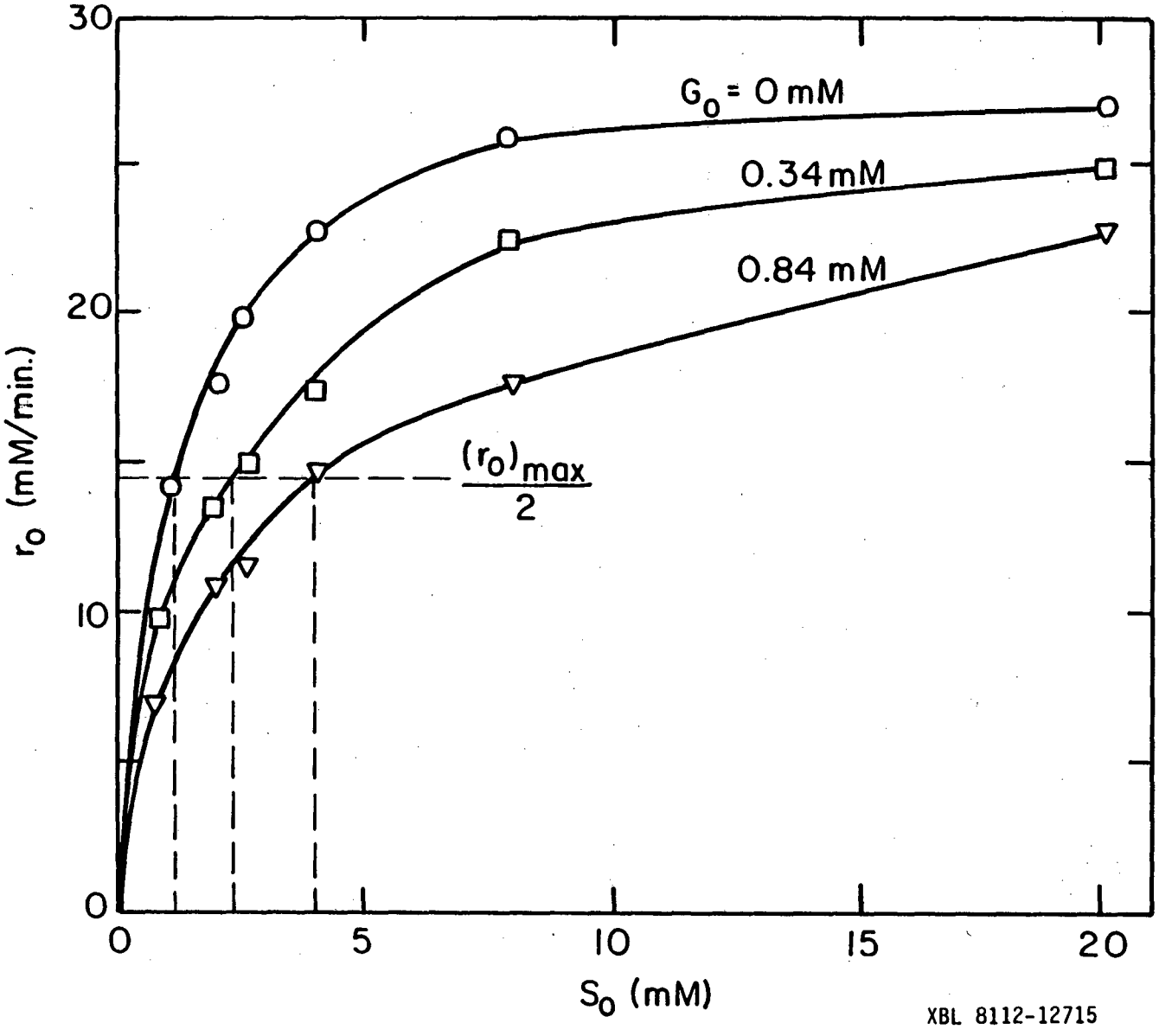


Figure 5.7. Initial rate of cellobiose hydrolysis as a function of substrate concentration.

inhibition by the product glucose is present (37). Since the slope exhibits a greater degree of sensitivity to the inhibitor concentration than the intercept, a competitive mechanism dominates in this range of glucose concentrations. As a result, the inhibition constant, K_2, can be estimated from the apparent Michaelis constant for various levels of glucose. The apparent K_m is equal to:

$$(K_m)_{app} = K_m(1 + G/K_2)$$

The values for $(K_m)_{app}$ were determined (Figure 5.7) and K_2 was calculated to be 0.38mM for this enzyme.

The non-competitive term can be determined from the intercept of the Lineweaver-Burk plot. A plot of the intercept term in Equation 5.11 as a function of glucose concentration should be linear with the x-intercept corresponding to K_1. Figure 5.8 shows this type of plot and K_1 is computed to be 7.0mM.

These initial rate studies were conducted at a low enzyme concentration to ensure that the reaction rate was linear in time. Since V_{max} is proportional to the cellobiase activity, the rate constant was obtained at high enzyme strength to minimize the errors in measurement. Glucose production was non-linear in time and the integrated rate expression was used.

$$V_{max}t = k_b E_{Bo} t = \frac{G^2}{2K_1} + (1 - \frac{2K_m}{K_2})G - K_m(2 + \frac{4G_{2o}}{K_m}) \ln(1 - \frac{G}{2G_{2o}})$$

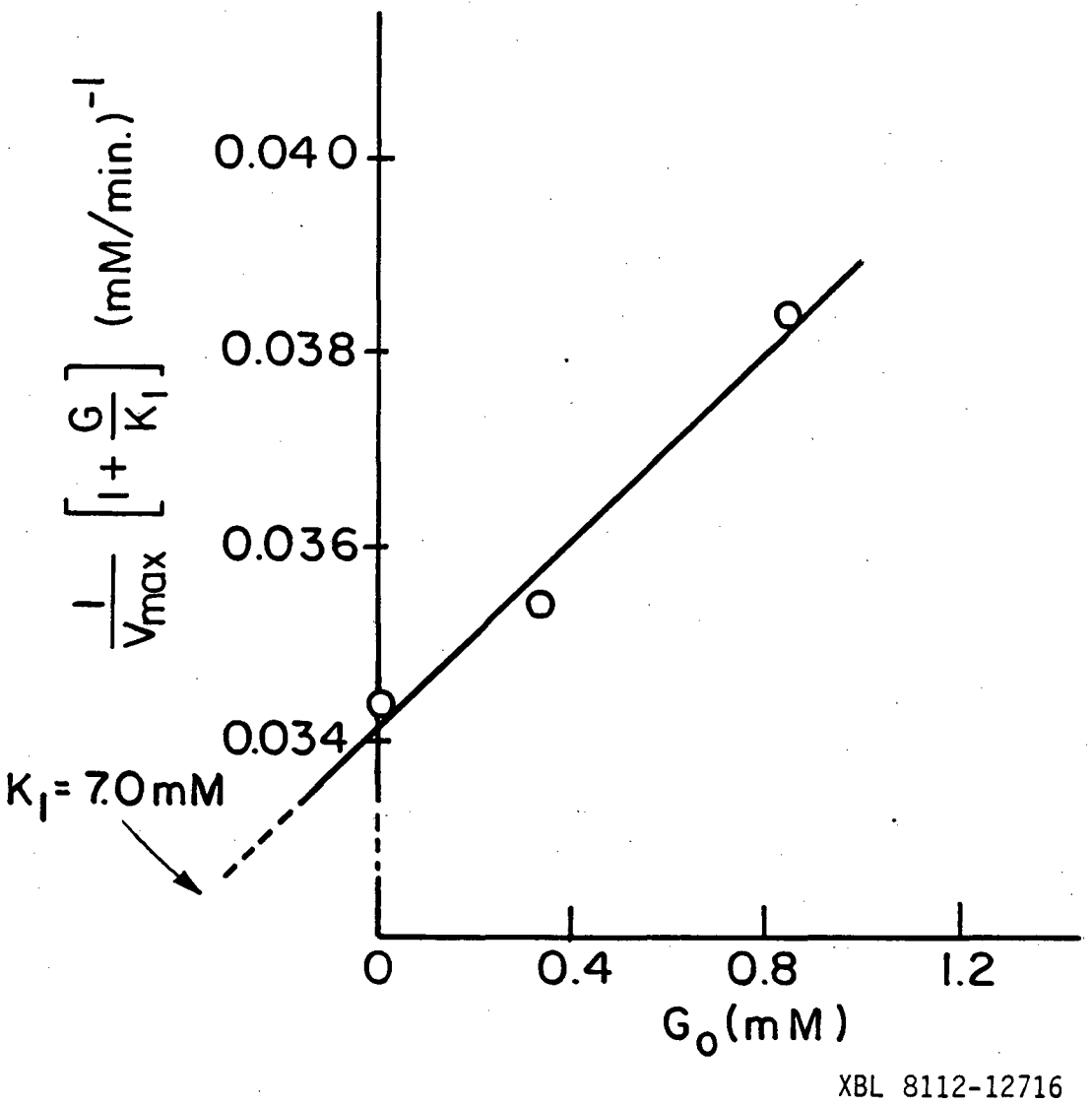


Figure 5.8. Determination of the non-competitive inhibition constant.

Since all the constants on the right hand side are known, it reduces to: $k_b t = f(G, E_{Bo})$. A linear plot of time versus the function of glucose concentration and cellobiase activity will have a slope equal to k_b. Figure 5.9 shows this type of plot. This analysis estimated the parameters in Equation 5.7 and results in the following rate expression for glucose production.

$$\frac{dG}{dt} = \frac{1.21 \times 10^{-3} E_{Co} G_2}{(1 + G/7)G_2 + (1 + G/0.38)1.3} \qquad (5.12)$$

5.4. Mathematical Aspects

Equations 5.9, 5.10 and 5.12 are rate expressions for the hydrolysis of amorphous cellulose, crystalline cellulose, and cellobiose, respectively. This set of differential equations are coupled and highly non-linear, resulting in the necessity for a numerical method of integration. Gears' method was adopted because of its ability to handle stiff equations and the variable step size allowed rapid computation (29,42). This technique was coded in FORTRAN and available as an University of California Math Library program (IMSL Routine DGEAR).

The unspecified parameters, P_k, of the model can now be estimated from experimental data for rice straw saccharification. The best values for the parameters were found by minimizing the sum of the squares of the weighted deviation between the model predictions and experimental

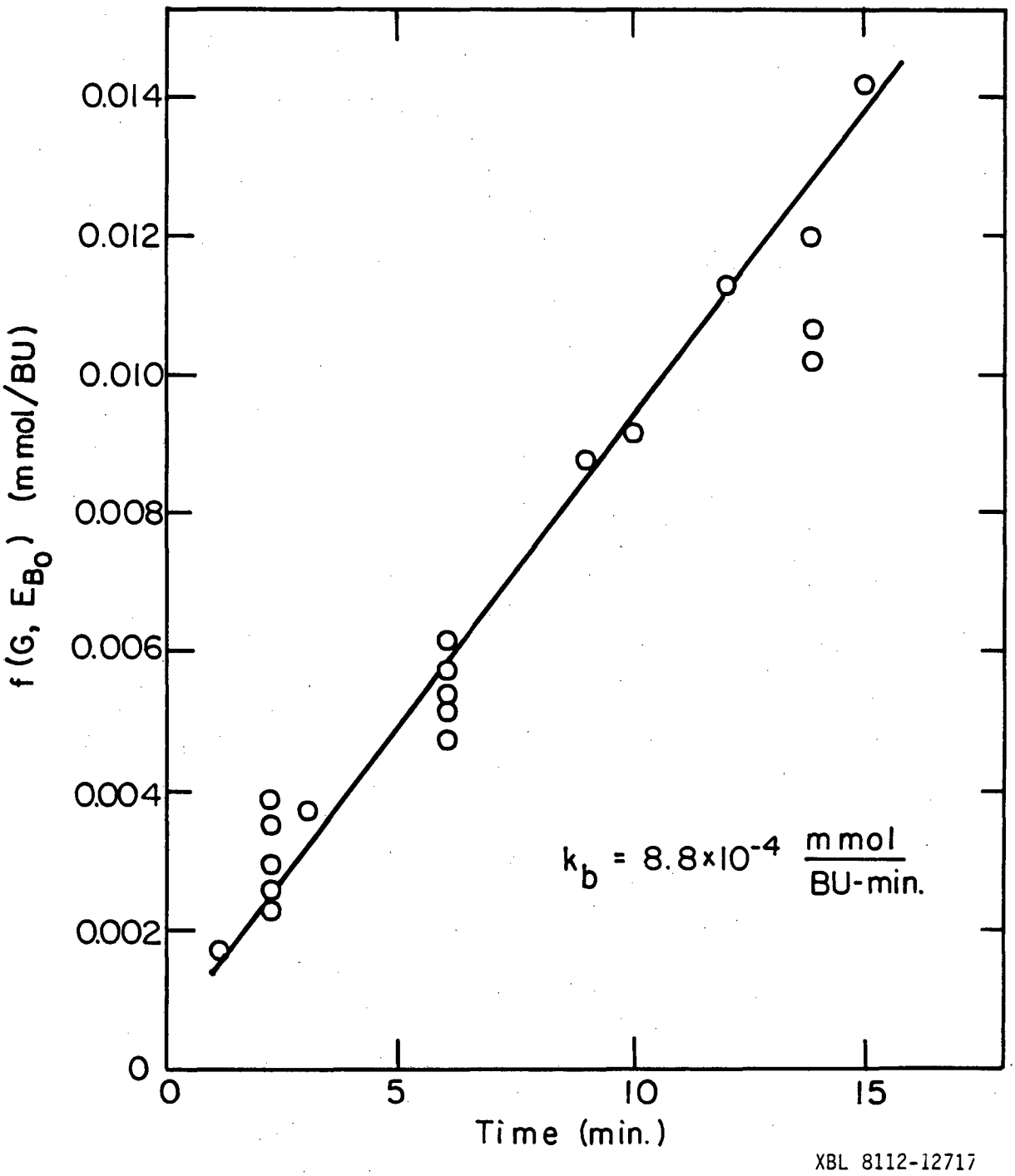


Figure 5.9. Determination of the rate constant for cellobiose hydrolysis.

measurements.

$$L = L(P_k) = \sum_i \sum_j W_i (y_{ij} - y^p_{ij})^2$$

The weighting factors most frequently used are: (1) $W_i = 1$, equal weighting for each deviation, (2) $W_i = 1/y_{ij}$, relative deviation, or (3) weighting factors related to the variance of y_{ij}. The resultant estimates will be a function of the weighting convention chosen. After some investigation, equal weighting was adopted to avoid placing too much importance on the initial points (the relative deviation approach, $W_i = \frac{y_{ij}-y^p_{ij}}{y_{ij}}$, will tend to have large W_i, for small y_{ij}).

The minimization technique used was the Simplex method of Nelder and Mead (80). The parameters estimated were K_I, the inhibition constant for amorphous cellulose hydrolysis, and k_b, the rate constant for cellobiose hydrolysis. While k_b was estimated in Section 5.3.2, preliminary investigation indicated that the model was overpredicting the accumulation of cellobiose. The simplification of no direct glucose formation from cellulose causes a greater flux through the cellobiose reaction. As a result, the previous value for k_b was relaxed and re-estimated by the above procedure.

A FORTRAN computer program was developed to rapidly carry out the integration and parameter estimation procedures. This basic program does the numerical integration of the state equations, given a set of initial

conditions. The output consists of tables depicting the time history of glucose and cellobiose production. Appendix II contains the code with comments on the logic adopted.

The program is easily modified for parameter estimation by the addition of the non-linear optimization program (103) and initial values for the search variables. The initial guesses for each parameter were based on literature values. Before the optimization was started, various combinations of the parameters were evaluated (find the sum of the squares) to narrow the region for search. Figure 5.10 shows the results for various combinations of the parameters.

Because of the model structure, only certain subsets of the data can affect the estimation of the desired parameters. More specifically, the inhibition constant and cellobiose rate constant will strongly influence the size and shape of the cellobiose curve. As a result, runs with large amounts of cellobiose accumulation (less than 30 β-glucosidase units per grams of rice straw) were used for the parameter estimation procedure.

5.5 Model Evaluation

5.5.1. Comparson with Experimental Data:

The paramters estimation procedure resulted in values of K_I = 0.14 $(mM)^{-1}$ and k_b = 0.54 mmol/BU-hr. Testing the model for validity required experiments that varied the initial conditions with respect to the main

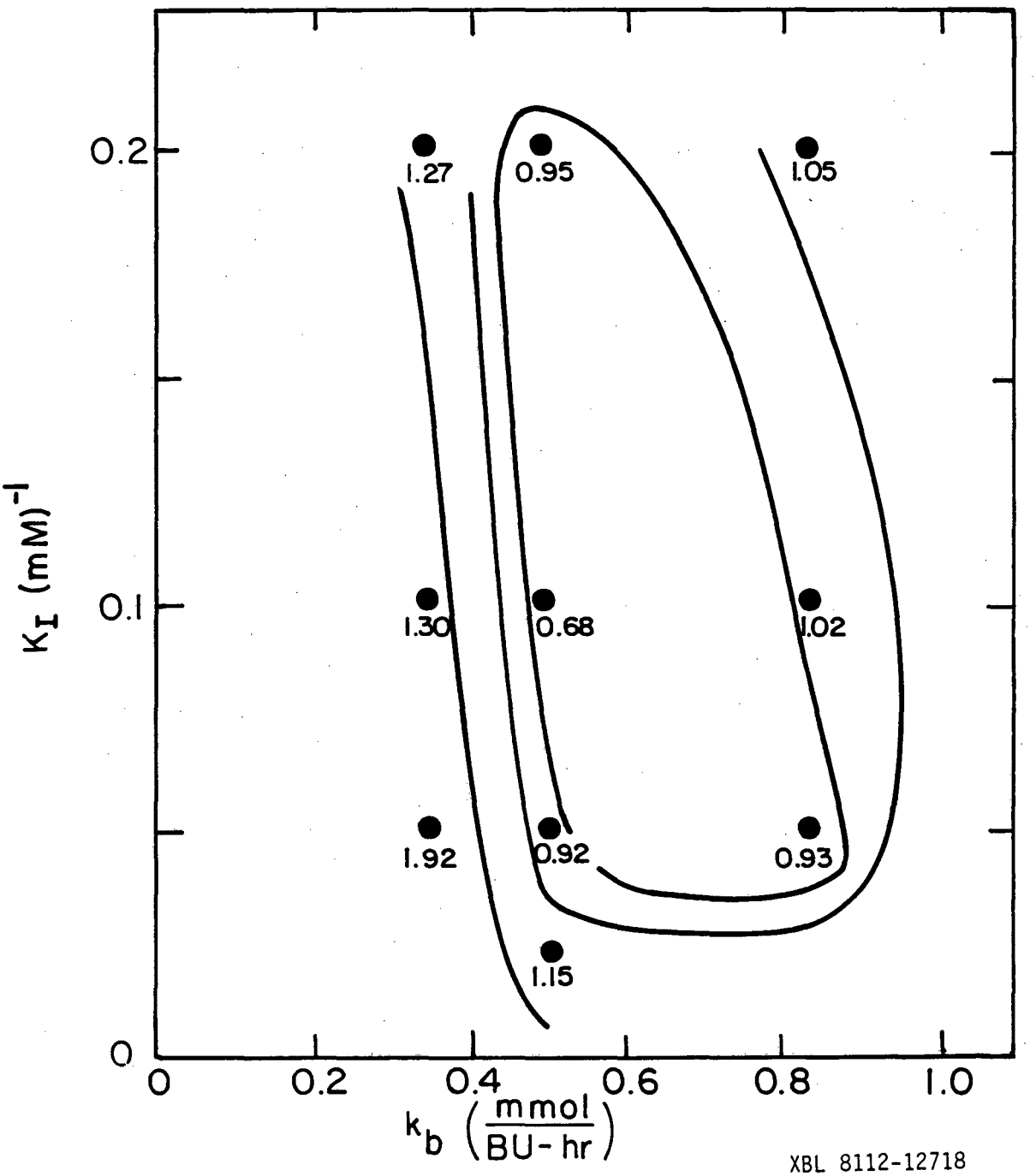


Figure 5.10. Contour map of the model performance, the sum of the squares, for different parameters.

dependent variables. Figure 5.11 compares the predictions of the proposed model and experimental data for low substrate concentrations and two different levels of enyzme strength. The model is in good agreement with the data for glucose production. The cellobiose concentration was not recorded for these runs because of measurement limitations.

High substrate concentrations, in excess of 10%, requires semi-batch operation. Figures 5.12 and 5.13 show the results for this manner of operation. Figure 5.12 represents a situation of low β-glucosidase activity. The accumulation of cellobiose is predicted well in terms of magnitude and the shape of the curve. However, at long reaction times, the model predicts lower levels of cellobiose than experiment. This could result from neglecting enzyme deactivation, especially since β-glucosidase components are the most sensitive to temperature and pH.

The effect of adding the major inhibitors, cellobiose or glucose, at the start of hydrolysis is demonstrated in Figure 5.14. In both cases, the final glucose yield (at 47 hours) is not significantly effected. While the initial rate of hydrolysis is reduced, the cellobiase activity is high enough to overcome the inhibition in several hours. The model is consistent with this behavior.

5.5.2. Conclusions:

The proposed kinetic model incorporates the enzyme

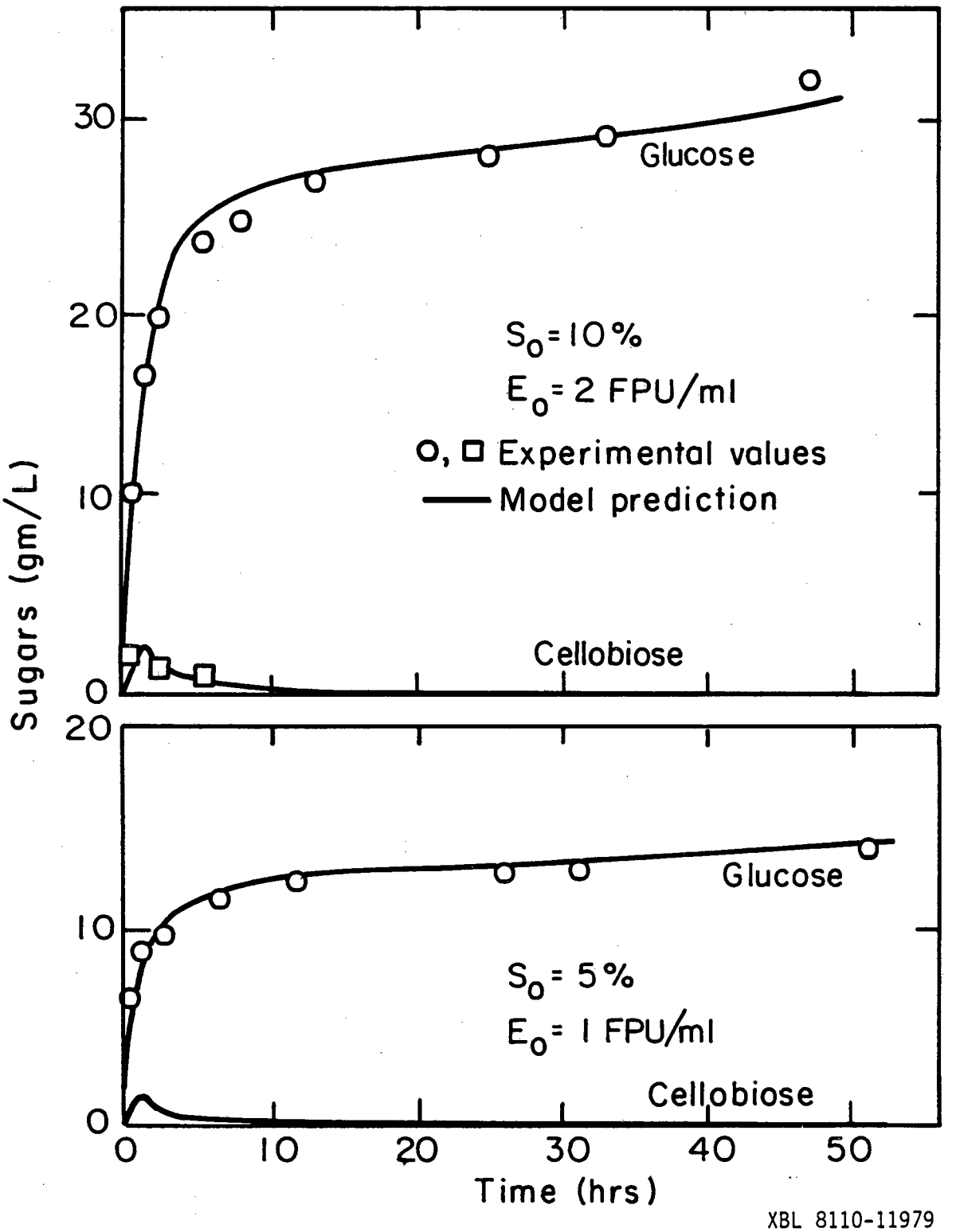


Figure 5.11. Model performance for low substrate concentrations.

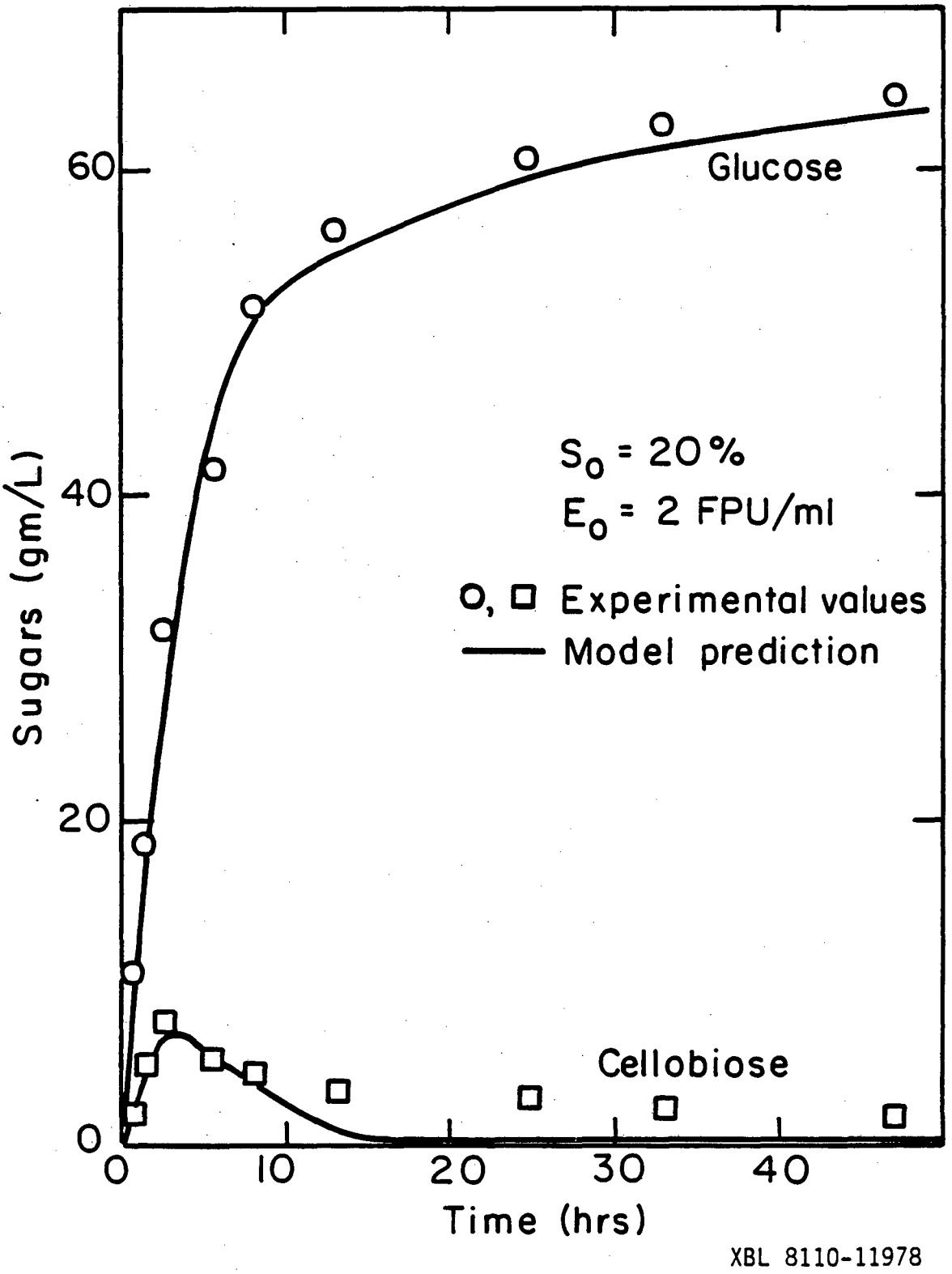


Figure 5.12. Model prediction of cellobiose accumulation.

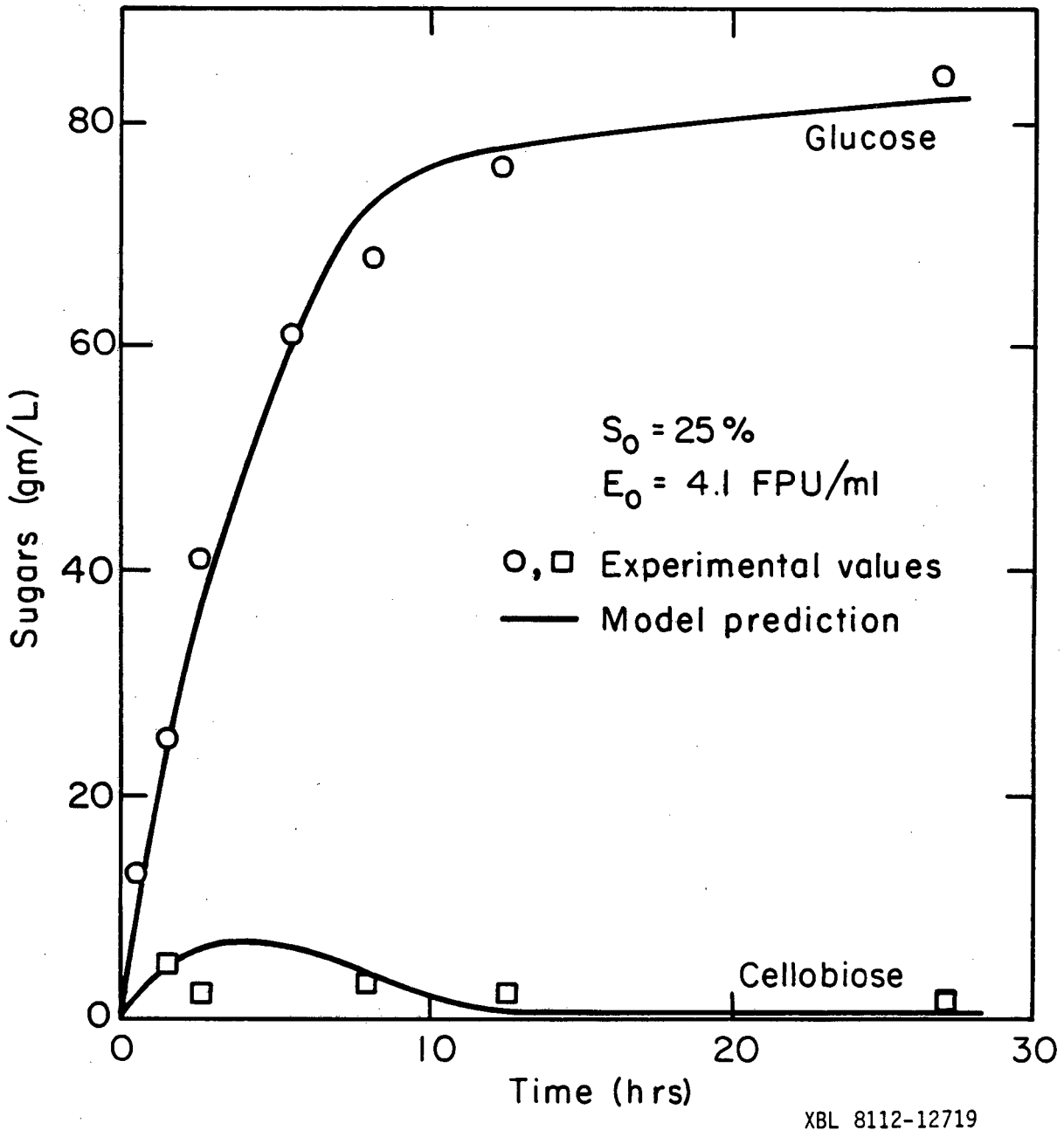


Figure 5.13. Model performance for high substrate concentration.

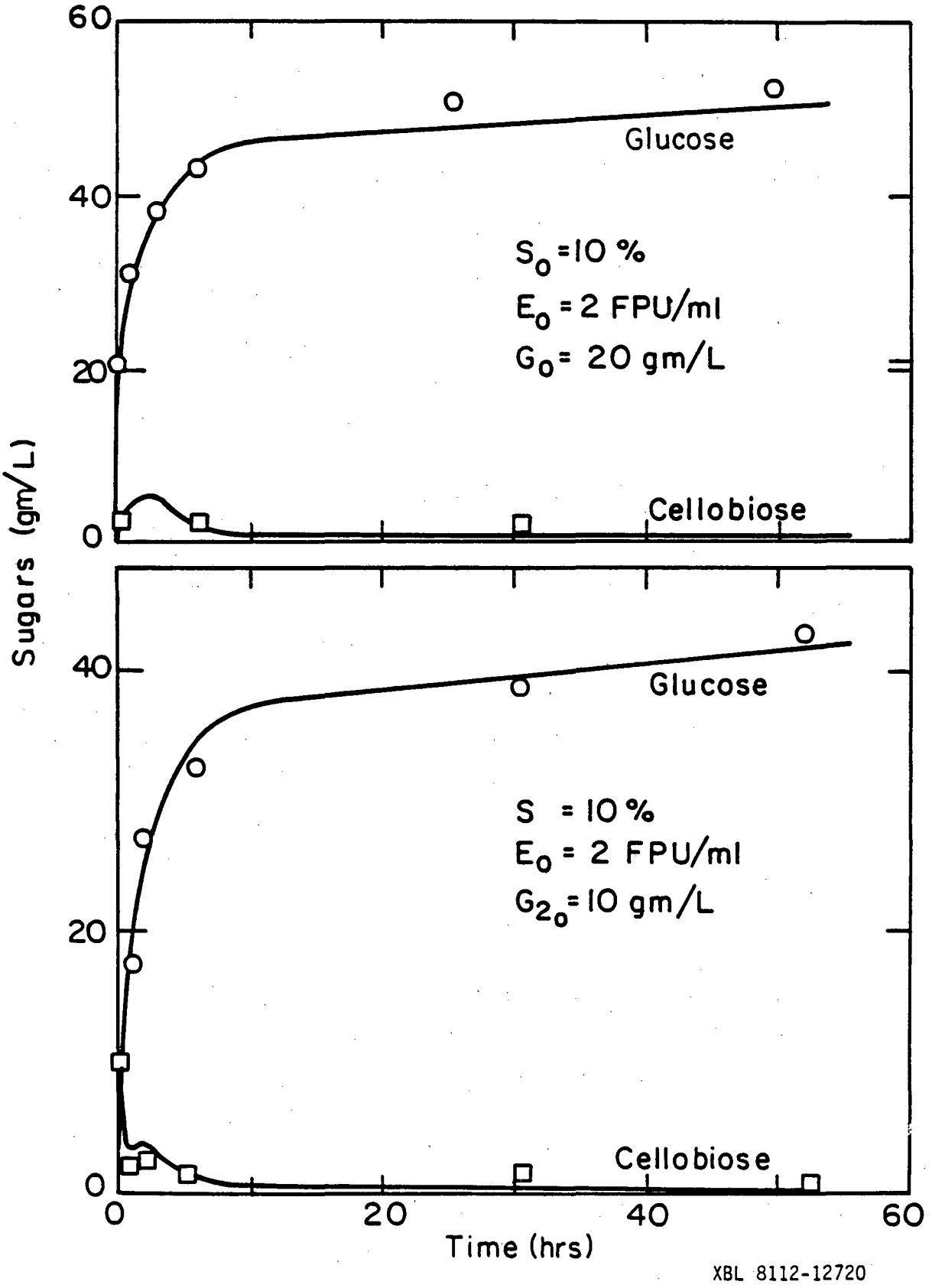


Figure 5.14. Model performance for initial addition of the primary inhibitors, glucose and cellobiose.

adsorption process, product inhibition, and considers a multiple enzyme and substrate system. Preliminary assessment indicates good agreement with experimental data. An especially important feature of the model is its ability to predict semi-batch operation, since high substrate concentrations have important economic advantages. On the other hand, the assumption of negligible enzyme inactivation may have resulted in the overprediction of cellobiose hydrolysis.

This model was used for economic optimization in Section 6.2. As with any model, care was taken in extrapolation of the model to different systems or conditions. For instance, only one stock enzyme solution was used. This fixed the ratio of the enzyme components for each dilution. Hence, the use of filter paper activity as a measure of cellulase activity has limitations since it is not a sensitive indicator for various combinations of the exo- and endo-glucanases. Development of more specific assay procedures and refinement of fractionation techniques will allow improvement in this area.

Nevertheless, conclusions concerning improvements in enzymatic hydrolysis can be made based on the results of this model and associated experiments. The general trend in cellulose hydrolysis is as follows: The majority of the amorphous content of the cellulosic material is hydrolyzed in the first 2-5 hours of hydrolysis. Because of rapid cellobiose production, low levels of β-glucosidase activity

(less than 30 units per grams of rice straw) cause the inhibitor to accumulate and slow the preceding reactions. The hydrolysis of the remaining crystalline material is slow, even at high concentrations of enzyme. This indicates that the best approach to increase the efficiency of rice straw usage is to reduce the degree of crystallinity or increase the amount of accessible surface area. In other words, the focus of research should be in the pretreatment area.

Preliminary work with corn stover steam exploded by the Iotech process, shows glucose yields of 70-85% in comparison to yields for acid treated material of 50-60%. This improvement will also result in high rates of cellobiose production. Enzyme solutions with high levels of β-glucosidase or an immobilized enzyme system may be necessary.

In summation, cellulolytic enzymes posses the ability to rapidily hydrolyze the amorphous or easily accessible portions of a substrate. Therefore, pretreatments to make the lignocellulosic materials more accessible, at a low cost and minimal energy requirement, is the best avenue for improvement.

5.6. Nomenclature

a	Equivalent spherical interfacial area, m^2/L
C	Cellulose concentration, mM
d_p	Surface mean particle diameter, m
E	Free enzyme concentration, FPU/ml
E_{sat}, E_s	Absorptive capacity, FPU/ml, FPU/mmol
f_A	Fraction of amorphous cellulose in total cellulose
G	Glucose concentration, mM
G_2	Cellobiose concentration, mM
k, k'	Rate constant, min^{-1}, FPU/mmol-min
K	Equilibrium constant for enzyme and substrate, FPU/L
K_1, K_2 K_m	Inhibition constant for cellobiose hydrolysis, mM
M_E	Molecular weight for the enzyme, mg/mmol
n_s	Site density, $mmol/m^2$
N	Number of particles, L^{-1}
r_i	Reaction rate, mM/min
t	Time, min
V_{max}	Maximum rate of cellobiose hydrolysis, mM/min
W_i	Weighting factor
y_{ij}	Sugar concentration, mM
β	Specific Activity, FPU/mg enzyme
$\varepsilon, \varepsilon'$	Adsorbed enzyme, mM, FPU/L
θ	Fraction of absorption sites occupied
ρ_c	Particle density, mol cellulose/m^3
p	Particle porosity

Subscripts:

A	Referring to amorphous cellulose
C	Referring to crystalline cellulose
o	Intial value at time zero

VI. PROCESS DESIGN AND ECONOMIC EVALUATION

6.1. Process Description

6.1.1. Requirements for Rice Straw Utilization:

The various processing options for bioconversion of cellulosic materials into ethanol were outlined in Section 2.5. The Berkeley process will be adapted to the requirements for rice straw utilization (119). The use of any agricultural residue results in two major problems; one, availability of substrate, and, two, storage considerations. Since rice is a seasonal crop, the availabilty of rice straw will coincide with the fall harvest. The design of a processing facility requires year round operation to utilize efficiently the capital equipment. This presents a problem for a plant that strictly utilizes rice straw. Several alternatives are possible. One, the rice straw can be left on the field and continually harvested throughout the winter. The soft ground in a rice field requires the development of high floatation equipment. In addition, the moisture content of the rice straw will be higher. Another alternative is large storage facilities. Since the bulk density of rice straw is about 10 lbs/ft^3, the sheer storage volume needed makes this alternative unrealistic. Possibily the best approach is to design a flexible facility that can utilize other cellulosic materials; for example, wheat straw in the summer or orchard prunings in the spring.

The supply of rice straw in the Sacramento Valley is

about 1.5 million tons per year (13). This is based on a yield of 3.0 tons per acre (75). About three-quarters of the rice produced in California is concentrated in four counties in the northern part of the valley. Table 6.1 shows the geographic distribution of the straw. A processing facility in the area between the four counties would have access to about 1.2 million tons of rice straw annually within a 40 mile radius. On the average only about 40% of the straw can be harvested because of weather conditions (75). As a result, this plant could be designed to handle a maximum of 1500 tons per day of straw or produce about 10 million gallons of ethanol annually.

6.1.2. Enzymatic Hydrolysis:

Figures 6.1 to 6.4 show the proposed flow sheet for the process. The design basis for the material balances in Figure 6.1 will be discussed at a later point in this chapter. The feed preparation consists of two major steps, milling and acid treatment. The rice straw is fed by belt conveyors to a series of hammermills. Milling allows convenient handling in an aqueous solution and increases the surface area for reaction. It is possible, though, that the rice straw could be densified into cubes after harvesting to reduce transportation and handling costs. This improvement would eliminate the need for milling onsite but more research is required to make the densification process economical.

The milled straw is fed by screw conveyor to the

Table 6.1 Rice straw production in northern California (13).

County	Area (sq. miles)	Annual production (tons/year)
Colusa	78	378,000
Butte	104	309,000
Sutter	36	252,000
Glenn	79	225,000
Yuba	52	87,000
Yolo	65	69,000

Yield: 3 tons rice straw/acre (75).

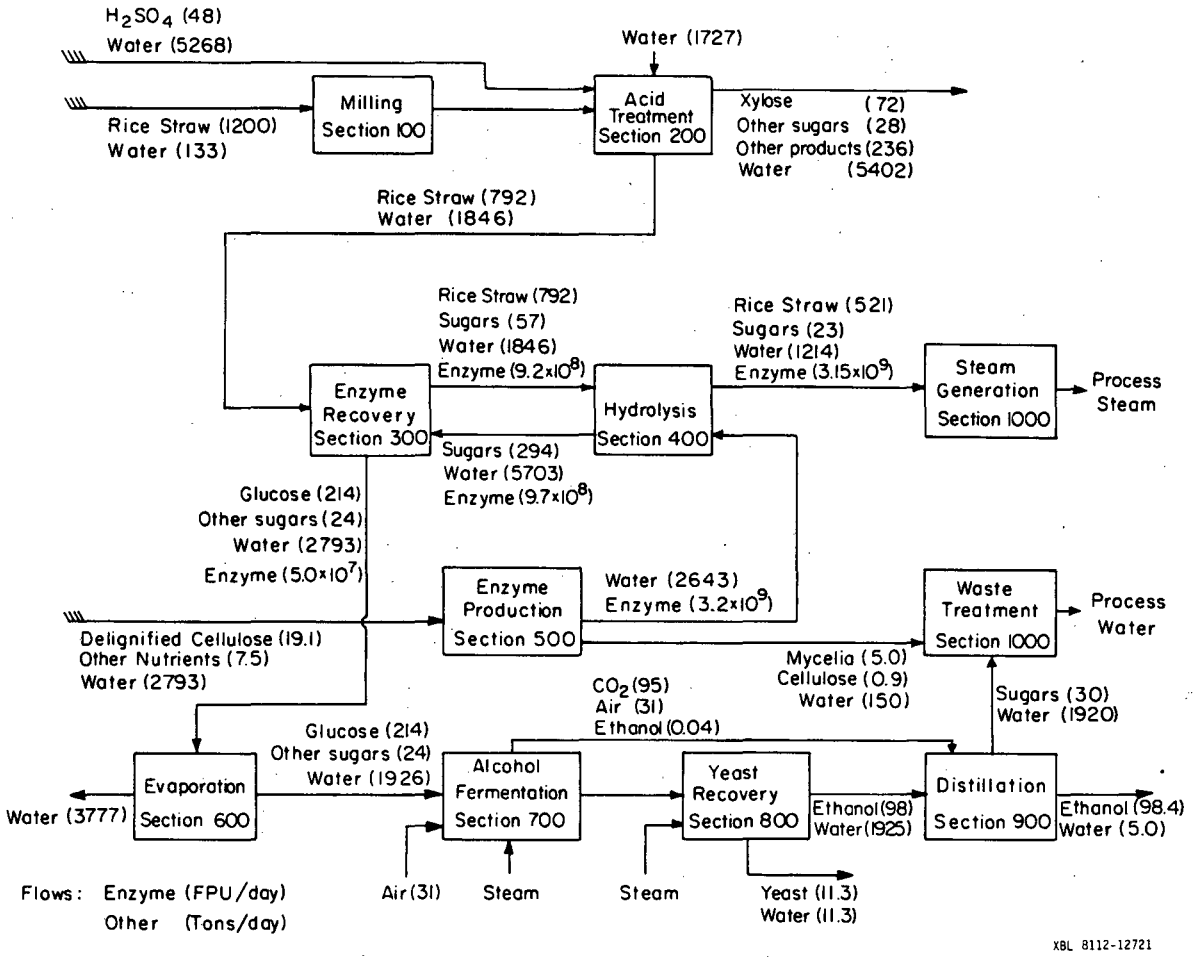


Figure 6.1 Block flow diagram and material balances for ethanol production from rice straw.

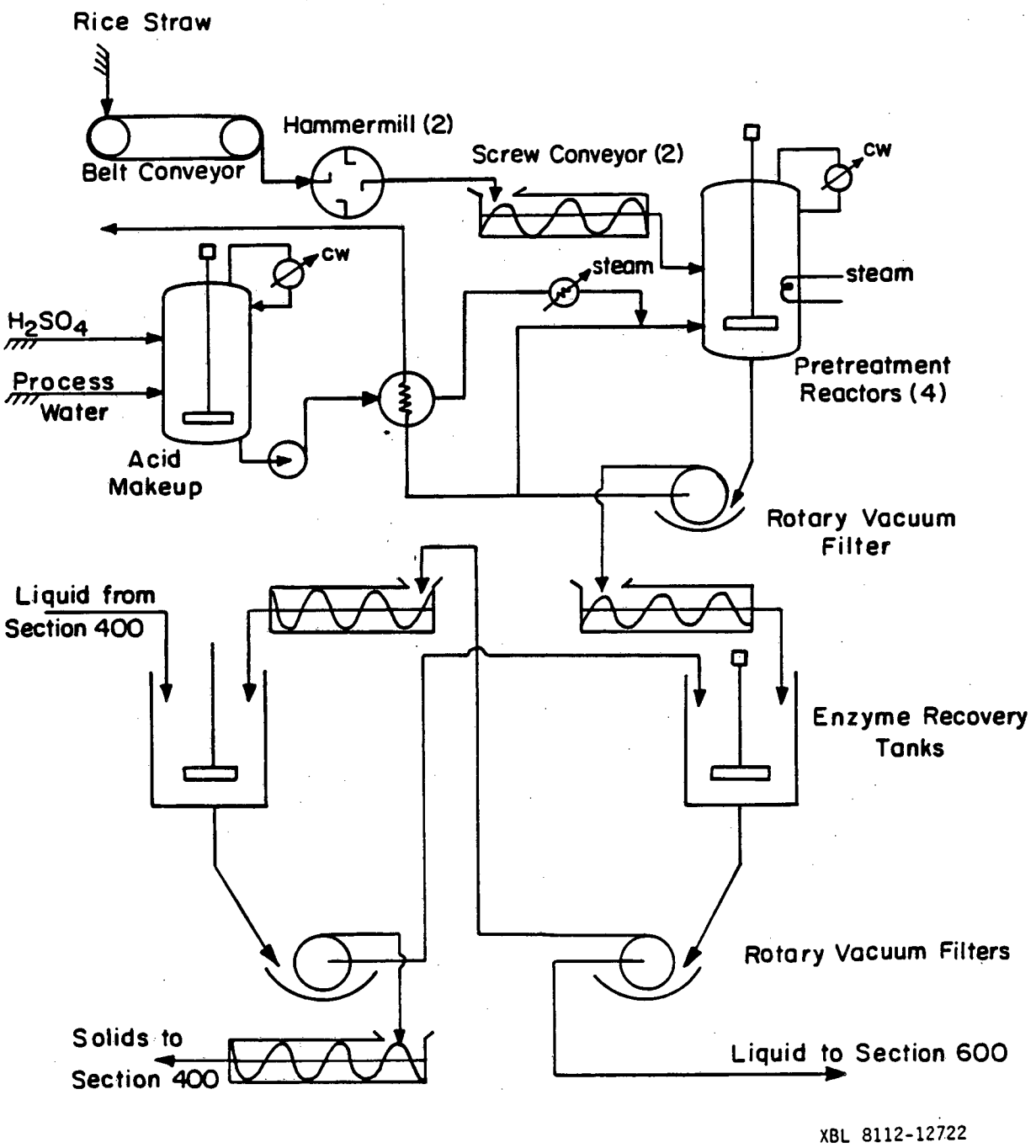


Figure 6.2. Process design for Sections 100 to 300.

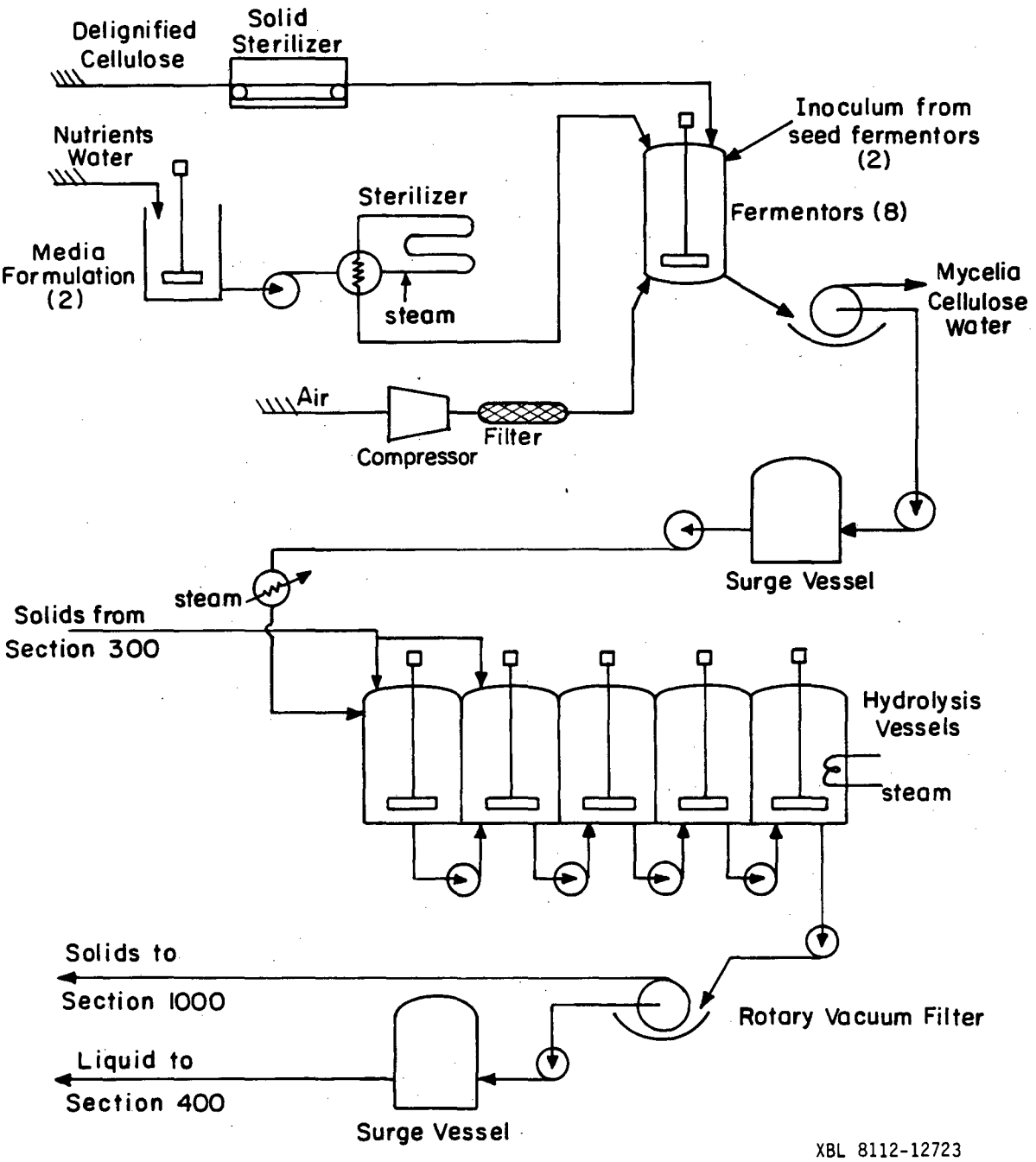


Figure 6.3. Process design for Sections 400-500.

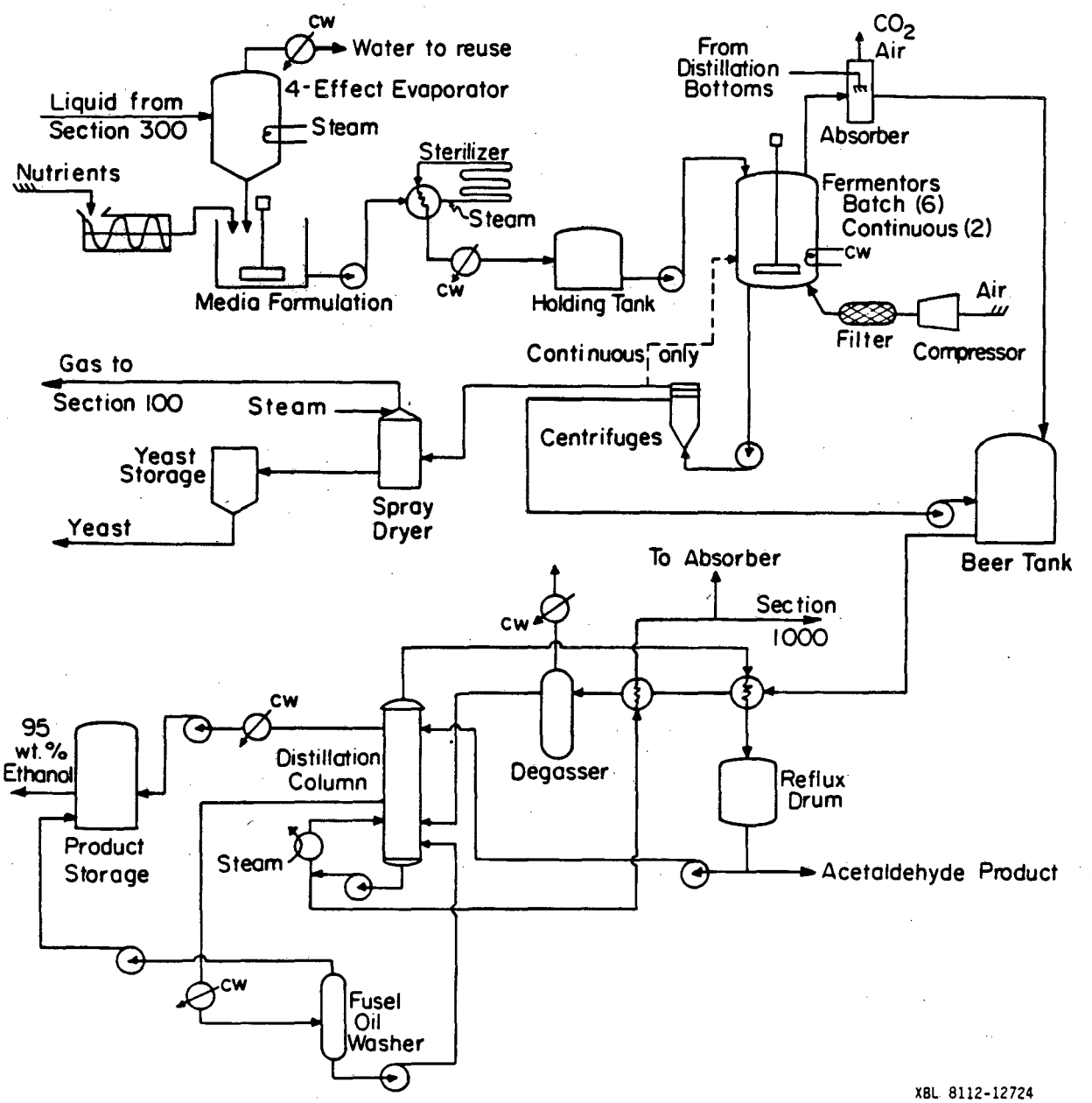


Figure 6.4. Process design for Sections 600 to 900.

acid treatment reactors. The treatment is conducted for 5.5 hours at 100°C. A solid suspension of 7.5% by weight is employed in the first stage. Acid strength is maintained at 0.09M H_2SO_4 with makeup acid and a recycle ratio of 0.5. The product stream contains 1.5% xylose. The xylose is a potential carbon source for ethanol production; however, early work at the University of California with Bacillus macerans shows low ethanol tolerance and high by-product formation (22). Two types of obligate anaerobes, C. thermohydrosulfuricum (63) and C. thermosaccholyticum (18), appear to be promising. Further work is underway to increase the ethanol tolerance and the ethanol yield of these organisms.

The pretreated solids are separated by a rotary vacuum filter and washed. This wash must remove the acid from the solids to ensure a pH of 5.0 in the hydrolysis vessel. A slightly basic wash solution may be necessary. The rice straw is transferred to the enzyme recovery section. Here, the adsorption properties of the cellulose are used to recover the free enzyme in the hydrolysate. The solids are contacted countercurrently in two mixer-filter stages with the sugar solution from the hydrolysis reactor. The process is based on retention of filter paper activity, while the β-glucosidase activity will not be considered. Section 4.2.3 discusses this problem.

The rice straw, with adsorbed enzyme, is added to the hydrolysis reactor with makeup enyzme. Hydrolysis will

take place in a series of cylindrical carbon steel tanks that are agitated and controlled at 45°C. Other reactor conditions will be determined, based on economic considerations, in Section 6.3. The reaction will be conducted as a continuous operation with staging and multiple feeds to minimize inhibition effects. The addition of a biocide may be required to minimize sugar losses by growth of contaminants (93).

The makeup enzyme is produced by batch fermentation of T. reesei, Rutgers C-30 strain, following the procedure outlined in Section 2.4. The fungus is grown aerobically at 28°C with the pH controlled above 5.0. The media containing delignified cellulose and other nutrients are sterilized in a continuous manner. Batch growth is assumed to yield enzyme solutions of 10 FPU/ml based on recent pilot plant work (123).

The slurry from the hydrolysis vessel is filtered and washed. The wash is critical, here, to recover the maximum amount of sugars without dilution of the hydrolysate. High substrate concentrations in the hydrolysis reactor will leave a large percentage of the sugar solution wetting the filter cake. Large amounts of wash water are required to recover the sugar but will cause some dilution of the product.

The unconverted solids are sent to a furnance for steam generation. The hydrolysate will be transferred to the enzyme recovery area and, then, concentrated in a

4-effect evaporator to give a 10% solution of fermentable sugars.

6.1.3. Ethanol Production:

Two processing schemes have been considered for fermentation--conventional batch and a more conceptual continuous process with cell recycle. The overall ethanol productivity is much higher in the cell recycle process allowing smaller fermentor volumes. This is traded off against the less expensive materials of construction of the batch fermentors, which require no sterilization and are mechanically more simple.

The sugar solution from the evaporators is mixed with nutrient supplements and sterilized in a continuous manner. Batch fermentation utilizes carbon steel fermentors that operate for 16 hours and are down for five hours for cleaning and refilling. Seed fermentors are required to provide an active inoculum. The continuous process uses stainless steel vessels operating at a dilution rate of $0.65hr^{-1}$ with cell recycle maintaining a cell concentration of 50 grams of dry weight per liter.

The fermented beer, about 5% ethanol, is sent to the yeast recovery section. Continuous centrifuges are utilized to remove the yeast. The cell recycle process has an increased load on the centrifuges due to the higher cell concentration. The yeast cells are dried and stored for sale as a protein supplement.

The distillation section is based on a design by

Raphael Katzen Associates (77). The energy efficient design uses about 20 pounds of steam per gallon of ethanol. It consists of a stripper/rectifier, fusel oil washer, and several heat exchangers.

6.1.4. Waste Treatment:

While a detailed analysis of waste treatment is beyond the scope of this study, a general scheme will be proposed. The unconverted solids will be used as a fuel for the steam plant. This facility will use boilers similar to the bagasse boilers used in the sugar cane industry to provide high pressure steam. The solids have a relatively high heating value because of the lignin content and can supply the process steam requirements for about 52 cents per 1000 pounds.

The liquid stream from the distillation bottoms will be concentrated by evaporation, allowing a majority of the process water to be recycled. The nutritional value of the concentrate should be evaluated for sale as a feed supplement. Other options are anaerobic digestion to produce methane or incineration by spraying the concentrate into a furnace, as is the case with black liquor in a sulfite mill.

6.2. Economic Evaluation

A preliminary economic evaluation of the proposed processing scheme was made to estimate the direct fixed capital and the manufacturing costs of ethanol production.

This preliminary cost estimate was used as the basis for evaluating the different processing options available and for identifying the key areas for future investigation. The economics are based on an annual production of 10 million gallons of 95% ethanol. The output must be fixed to provide a common basis for comparison of the processing alternatives. This requires, by material balance, production of 214 tons of glucose per day.

The cost estimation procedure recommended by Peters and Timmerhaus (87) was used. The manufacturing cost is composed of capital related, labor related, utilities, and raw material costs. The assumptions used in estimating these costs are summarized in Table 6.2. Taxes have been omitted on the assumption that the plant will be tax exempt. The allocated capital charges for the offsites, steam generation and waste treatment, are not included because of uncertainity in the processing options to be employed.

6.2.1. Cost of Rice Straw:

The manufacturing cost is highly dependent on the raw material costs, especially the rice straw cost. While the demand for rice straw is negligible, except for its potential value as a fuel, there are significant capital and transportation costs associated with delivery of rice straw to a central processing facility. Work has been performed at the University of California, Davis to evaluate these costs. The minimum cost required to deliver the rice straw would be about $25 per ton; this includes collection,

Table 6.2 Basis for cost analysis.

Capital Related Costs:	
Multipliers (DFC = Multiplier x Purchased Equipment Cost	
Milling	3.4
Pretreatment	4.0
Enzyme Recovery	3.9
Hydrolysis	3.6
Enzyme Production	4.0
Evaporation	3.3
Storage	3.3
Process Equipment Ethanol Prod.	3.1
Fermentors	4.2
Factor (Annual Cost = Factor x DFC)	0.24
Labor Related Costs:	
Labor Rate	$22,000/operator
Factor	1.65
Utility Rates:	
Electricity	$0.044/kW-hr
Process Water	$0.14/1000 gal
Steam	$0.55/1000 lbs. (residual solids only)
Raw Material Costs:	See Appendix III
Marshall and Stevens Index	707 (4^{th} quarter 1980)
Stream Factor	0.9 (330 days/yr)

Source: J. Perez (86)
G. Cysewski (21)

hauling to the roadside and transportation to a plant (75). Densification may increase this cost by 20%. In addition, modifications are necessary in the harvesting techniques to cut the rice straw at ground level rather than leaving the straw for field burning. Currently, custom operators harvest wheat straw for horse bedding or the mushroom industry at $40 to 50 per ton (75). Legislation to prohibit agricultural burning practices in California will increase the supply and reduce the price. This study will assume a price of $30 per dry ton and, because of the uncertainity in the price, analyze the sensitivity of the manufacturing cost of ethanol to the rice straw cost.

6.2.2. Economic Model:

The ethanol manufacturing cost, based on the above assumptions, is a function of the three main design variables--enzyme concentration, solid suspension, and the residence time in the hydrolysis reactor. An economic model was developed to determine the optimum economic design. It was based on a more detailed program developed at the University of California, Berkeley that provides material and energy balances, equipment lists, and manufacturing costs for a variety of processing alternatives (86).

This model calculates the capital related, labor related, utility, and raw material costs for different conditions in the hydrolysis section. Since there is a logarithmic relationship between purchased equipment costs and capacity, capital related costs can also be related to a

characteristic flow rate in a similar manner, while the other costs are directly proportional to the flow rate. Table 6.3 summarizes the relationships used in the model. Each flow rate is related to the design variables as follows:

(1) Solids input: $W_o = 637/Y_G$

where $Y_G = f(E_o, S_o, t_r)$ from kinetic model developed in Chapter V.

(2) Liquid flow rate: $W_w = 0.64W_o(1-S_o)/S_o$

(3) Enzyme production rate:

$$E_p = 8.42 \times 10^5 W_w E_o (1-0.94e)$$

where $e = E/E_o$ from Equation 4.5

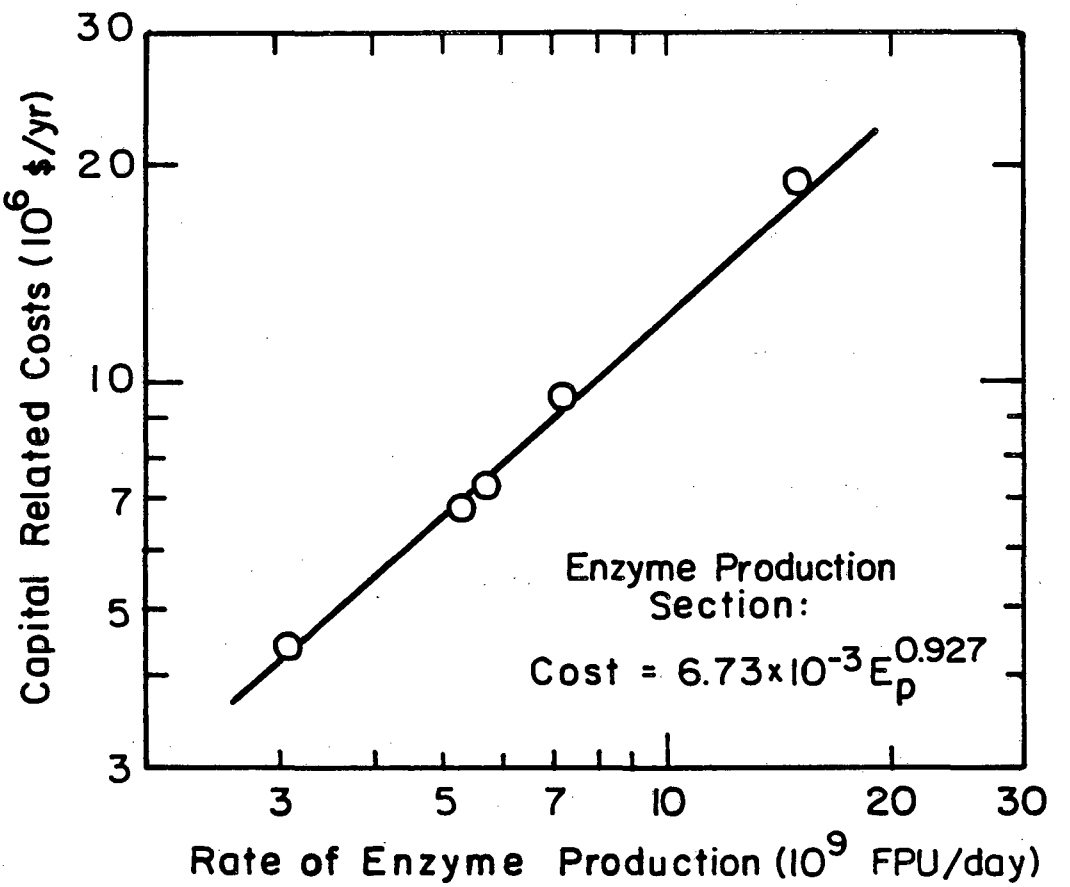
Appendix III includes the derivation of these expressions.

The detailed economic program, developed by Perez (86), was adapted to the properties of rice straw. Several runs were conducted to obtain cost data as a function of flow rate. Figure 6.5 shows a typical logarithmic relationship between the capital related costs and the characteristic flow quantity for the section. The data were fitted to an equation of the form: Cost = a(flow rate)^n. This analysis resulted in a simple model for estimating the ethanol manufacturing cost. The sugar transfer cost and the ethanol cost can be computed based on the initial enzyme strength and solid suspension, and the glucose concentration for a particular residence time. A program was written for operation on a microcomputer, allowing rapid analysis of experimental results. It was also used for process

Table 6.3 Basis for economic model.

Design Variables:	E_o, S_o, t_r	
Capacity Measures:	W_o	Solids Input
	W_w	Liquid Flow
	E_p	Enzyme Production
	S_c	Steam Cost

Operation	Capital Related	Labor Related	Utility	Raw Material
Milling	$f(W_o)^n$	constant	$f(W_o)$	-
Pretreatment	$f(W_o)^n$	"	$f(W_o)$	$f(W_o)$
Enzyme Recovery	constant	"	constant	-
Hydrolysis	$f(W_w,t_r)^n$	"	$f(W_w,t_r)$	-
Enzyme Production	$f(E_p)^n$	"	$f(E_p)$	$f(E_p)$
Evaporation	$f(W_w)^n$	"	$f(W_w,S_c)$	-
Fermentation	constant	"	constant	constant
Yeast Recovery	constant	"	$f(S_c)$	-
Distillation	constant	"	$f(S_c)$	-
Storage	$f(E_p)^n$	-	-	-



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Figure 6.5. Logarithmic relationship between capital related costs and characteristic flow rates. Data from (86).

optimization purposes in conjunction with the kinetic model developed in Chapter V and the enzyme adsorption properties (Equation 4.5). Appendix III outlines the development of the program and contains a listing in BASIC (for use on a Commodore PET 2001 microcomputer)

6.3. Optimum Process Design

The optimum process design will be based on the minimum unit cost for this particular plant size and substrate. This could be found, mathematically, by a non-linear optimization technique; however, a certain degree of subjectivity must be included to account for practical operating considerations and flexibility requirements.

Figures 6.6 and 6.7 show the effect of substrate concentration and enzyme strength on the major components of the ethanol cost. For a given enzyme strength, as the solid suspension increases, the hydrolysis vessel size decreases and enzyme usage is improved. The enzyme production costs decrease until the enzyme:substrate ratio drops below about 25 FPU/gram and the yield begins to fall. In addition, high substrate concentrations leave less enzyme in solution, thus reducing the amount that can be recycled. A similar explanation accounts for the trend in Figure 6.7.

Figure 6.8 shows the effect of residence time on the ethanol cost. The incremental increase in yield with longer reaction time is balanced by the increase in vessel size and utility costs for the hydrolysis section. A comparison between two levels of enzyme strength demonstrates that the

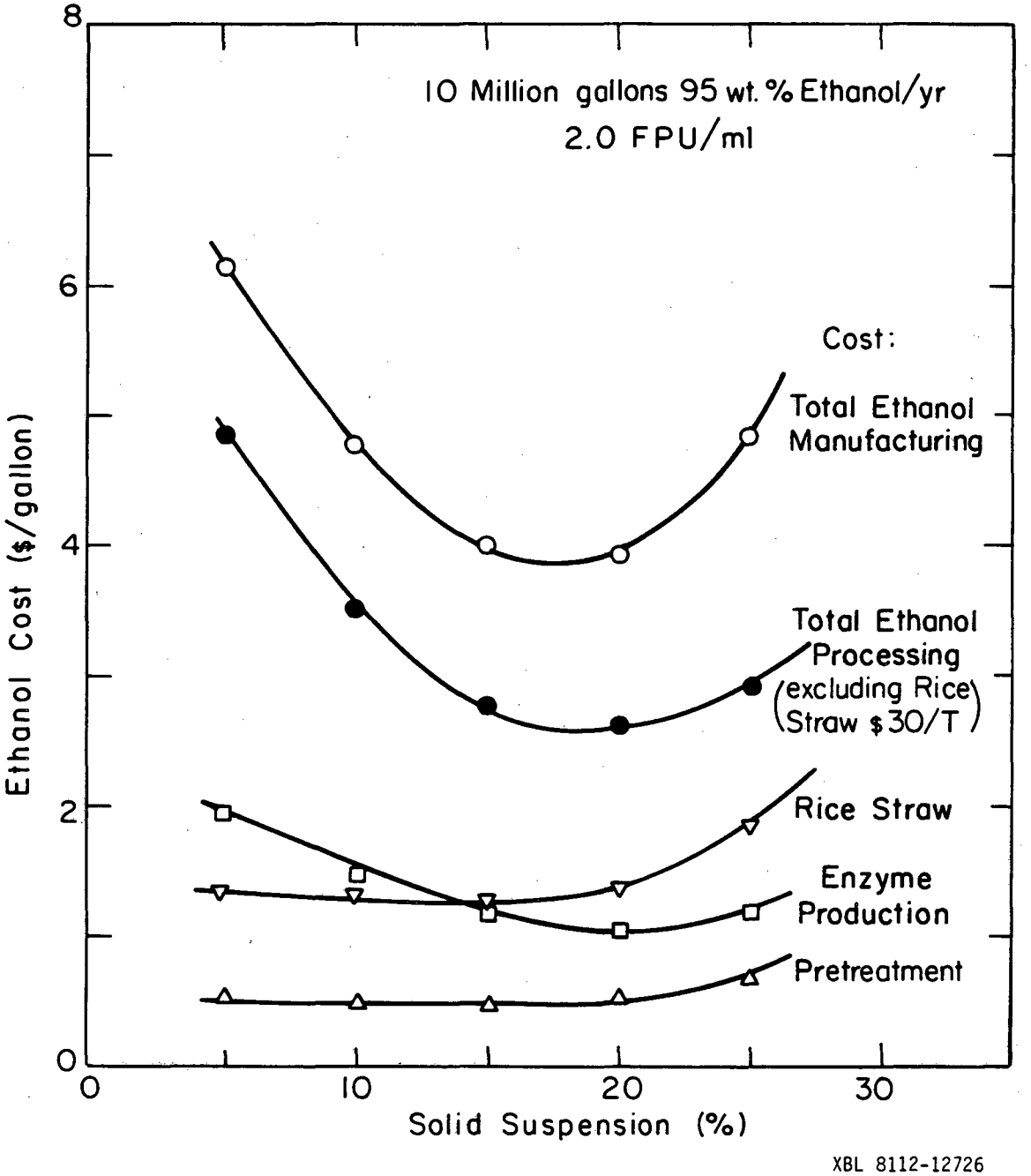


Figure 6.6. Effect of solid suspension on ethanol cost.

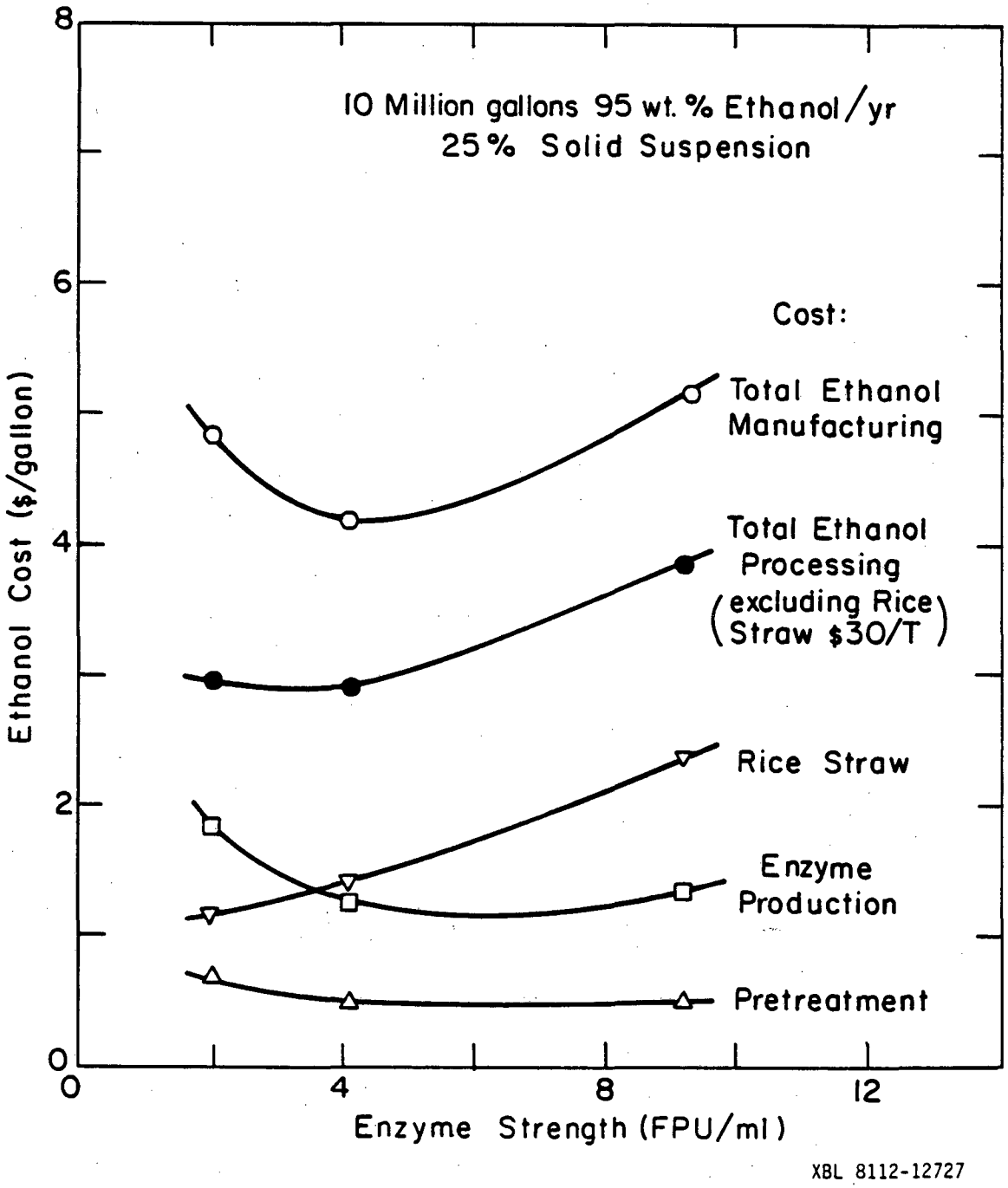


Figure 6.7. Effect of enzyme strength on ethanol cost.

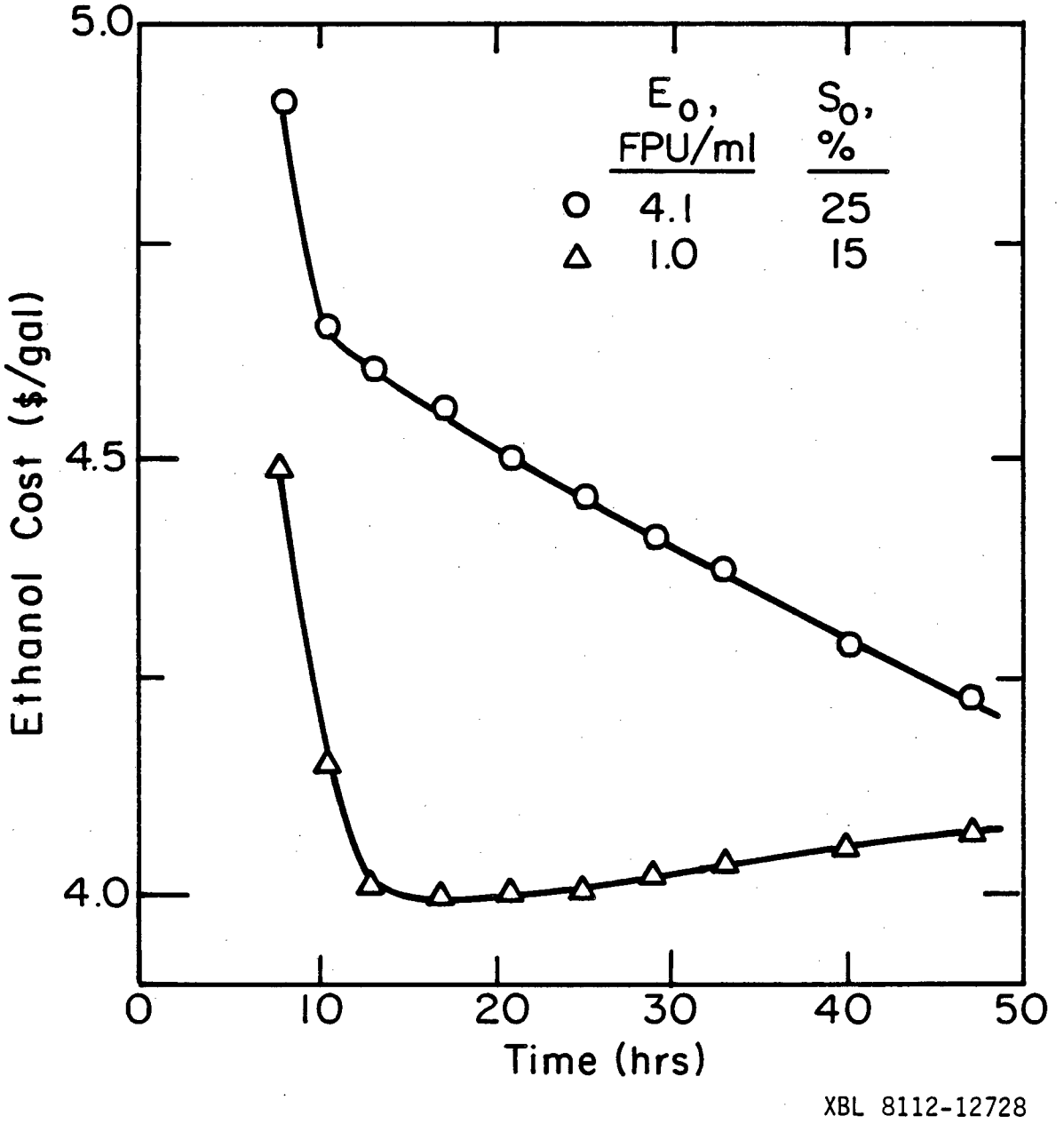


Figure 6.8. Effect of enzyme strength on optimum residence time.

higher enzyme concentrations result in a more rapid rate of hydrolysis; causing the optimum residence time to occur at longer reaction times.

Long residence times may also have the disadvantage of increasing the retention time of the enzyme in a continuous system with enzyme recycle. Enzymes are sensitive to high temperatures for prolonged times. The cellulase enzyme at 50°C will lose 14% of its activity in 48 hours (93). While the optimum temperature for reaction is 50°C, hydrolysis is conducted at 45°C in this process to reduce the amount of denaturation. As a result, the residence time was limited to 25 hours to minimize this effect.

Figure 6.9 is used to determine the optimum economic design based on the above considerations. The minimum ethanol cost occurs at about a 15% solid suspension, an enzyme strength of 1.0 FPU/ml, and a 25 hour residence time in the hydrolysis vessel. Table 6.4 outlines the conditions for optimum design in more detail. These conditions have the primary advantage of low enzyme usage. This offsets the sacrifice in glucose yield (45% versus 51% for the highest enzyme:substrate ratio). The equipment size is also reduced compared to a 5 to 10% solid suspension.

Table 6.5 shows a complete cost analysis of this case, delineating the major cost areas in ethanol production. The ethanol manufacturing cost is $3.88 per gallon including a 12 cent per pound yeast credit for sale

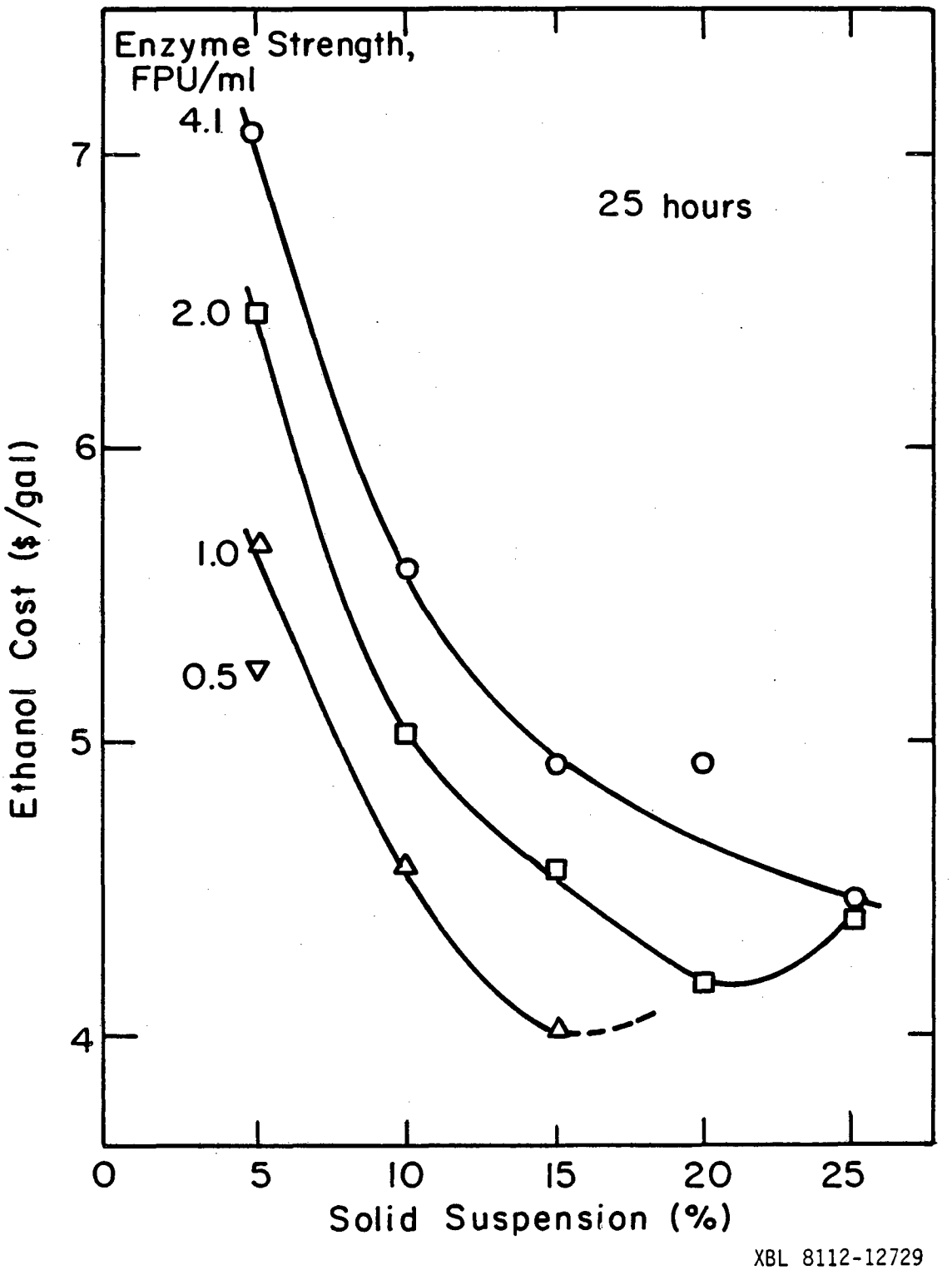


Figure 6.9. Determination of optimum processing conditions on the basis of minimum ethanol cost.

Table 6.4 Design basis.

Glucose Production	214 tons/day
Ethanol Production	10×10^6 gal/yr
Substrate	Rice Straw
Cellulose Content	40% 57% (acid treated)
Hydrolysis Conditions:	
Substrate Concentration	15% (170 gm/l)
Enzyme Strength	1.0 FPU/ml (1.8 β-g/ml)
Residence Time	25 hours
Glucose Yield	45%
Enzyme Recovery	24%
Cellulase Production:	
Operation	Batch
Conditions	28°C, pH greater than 5.0
Enzyme Concentration	10 FPU/ml
Productivity	55.5 FPU/l/hr
Ethanol Production:	
Operation	Continuous with Cell Recycle
Conditions	35°C, pH greater than 4.0
Ethanol Concentration	4.6%
Productivity	30 grams ethanol/l/hr

Table 6.5 Cost analysis for ethanol production.

	Capital Related	Labor Related	Utilities	Raw Materials	Total Processing
Milling	4.5	1.5	9.7	-	15.7
Pretreatment	29.2	3.0	10.5	9.9	52.6
Enzyme Recovery	4.9	1.5	1.1	-	7.5
Hydrolysis	18.3	3.0	14.5	-	35.8
Enzyme Production	46.2	8.8	12.2	10.7	77.9
Evaporation	19.2	1.5	2.9	-	23.5
Alcohol Fermentation	5.2	4.4	4.3	15.0	28.9
Yeast Recovery	3.5	4.3	0.6	-	5.6
Distillation	3.7	1.1	1.1	-	7.8
Chemical Storage	1.1	-	-	-	1.1
Total	135.8	28.2	56.9	35.6	256.5
				Rice Straw ($30/ton)	142.8
				Manufacturing Cost	399.2
				Yeast Credit ($0.12/lb)	(11.2)
MSI = 707 (4^{th}Q.1980)					388.0

All numbers in cents/gallon of ethanol

as a feed supplement. This facility requires a direct fixed capital investment of $55 million. The major component of the ethanol cost is the rice straw cost, amounting to $1.43 per gallon. This is a function of the glucose yield. Without this raw material charge, the processing accounts for $2.56 per gallon. For this level of production, the unit cost for enzyme is about $2.38/10^3 FPU, which amounts to $0.78 per gallon of ethanol. The other major cost is acid pretreatment, at about $12 per ton of rice straw, accounting for $0.53 per gallon.

Table 6.6 compares batch and continuous fermentation for ethanol production. Continuous culture with cell recycle is more economical due to higher ethanol productivities (about 15 fold improvement) that more than offsets the higher material, labor, and centrifugation costs. Nevertheless, batch is the proven process with continuous processing just beginning to emerge for industrial applications.

6.3.1. Sensitivity Analysis:

Since all economic evaluations contain various assumptions and estimations that possess varying degrees of uncertainity, an analysis of the sensitivity of the ethanol cost to the major assumptions is necessary. Because of the significance of the rice straw cost, Figure 6.10 shows the effect the rice straw cost has on the ethanol cost. As the price changes, the optimum operating conditions for the hydrolysis section may change. In the range of rice straw

Table 6.6 Comparison of batch and continuous ethanol production.

	Batch	Continuous with Cell Recycle
Ethanol Productivity (gm/l/hr)	2.2	30.0
Manufacturing Costs (cents/gal)		
Alcohol Fermentation	46.3	28.9
Yeast Recovery	5.4	5.6
Distillation	7.8	7.8
Total	59.5	42.3

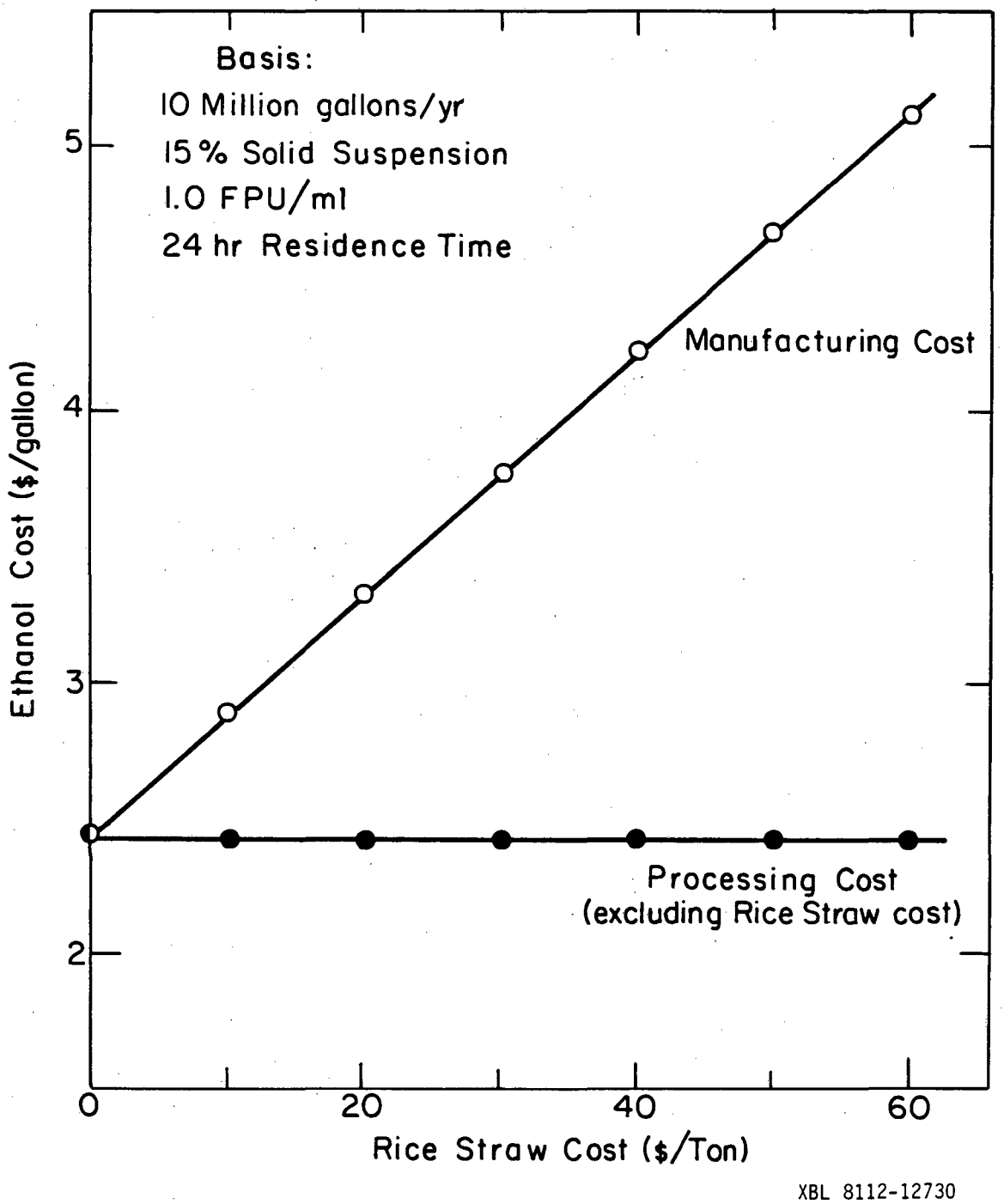


Figure 6.10. Effect of rice straw cost on ethanol cost.

costs investigated, a 15% suspension and 1.0 FPU/ml remains the best conditions; however, the optimum residence time will increase with the rice straw cost. The selection of a 25 hour residence time includes some flexibility for variations in this cost (the optimum residence time, for $30/ton would be 17 hours).

Another process variable that may be subject to change is the enzyme productivity. This design was based on producing 55.5 FPU/liter/hour. Process improvements in this area will have a significant effect on the process economics. Figure 6.11 shows the effect of increasing enzyme productivity for batch operation. Improvement by continuous operation with cell recycle will further decrease costs; however, changes in equipment, labor requirements, and raw materials must be considered.

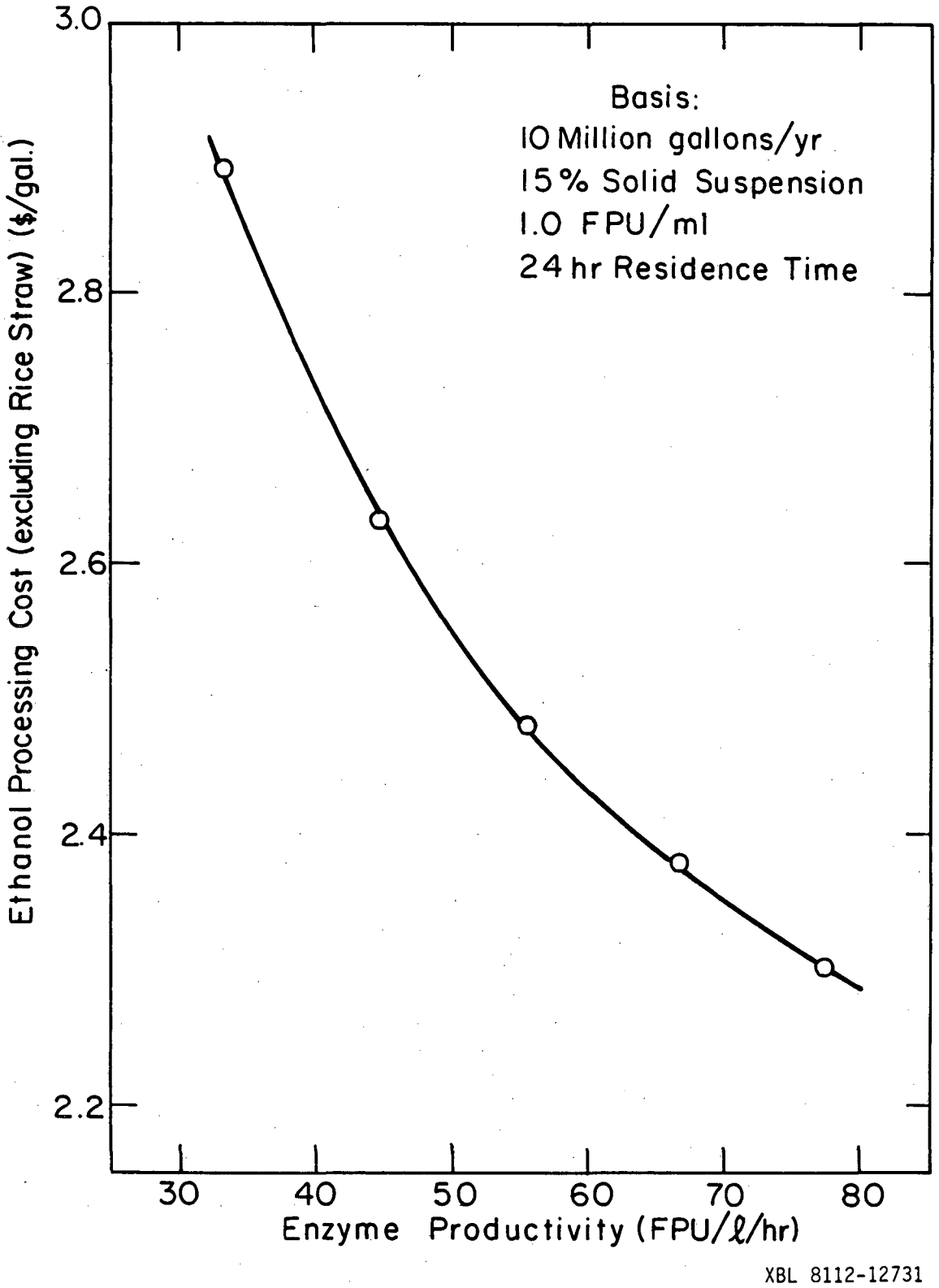


Figure 6.11. Effect of enzyme productivity on ethanol cost.

VII. CONCLUSIONS

This study has demonstrated the technical feasibility of producing ethanol from rice straw. Enzymatic hydrolysis converts up to 60% of the available cellulose to a fermentable sugar solution. A kinetic model was developed to aid in understanding the hydrolysis process and for use in process optimization studies. The model incorporated an enzyme adsorption mechanism, product inhibition, and considers a multiple enzyme and substrate system.

Economic evaluation indicated that the minimum ethanol processing cost is $2.56 per gallon with an associated raw material cost for rice straw of $1.43 per gallon. This was based on a conservative raw material cost of $30 per dry ton. This facility will produce 10 million gallons of 95% ethanol annually and requires a $55 million investment for direct fixed capital.

It is recommended that future investigation should focus on increasing conversion by reducing the degree of crystallinity via pretreatment, improving enzyme productivity by continuous culture with cell recycle, and utilizing all of the components of the lignocellulosic material. Preliminary work in the pretreatment area has indicated significant process improvement is possible. The current glucose yield is limited to about 55% in 24 hours because of the properties of rice straw and its pretreatment. Current research at Berkeley on the enzymatic hydrolysis of a similar substrate, corn stover, that was

pretreated using a steam explosion process rather than acid treatment, has resulted in glucose yields of 70 to 85%. This improvement could reduce the ethanol cost by 30% (86).

In summation, the production of ethanol from cellulosic materials, based on the present technology, does not appear to be competitive with petrochemicals. Yet, genetic improvements in cellulolytic and fermentative organisms, new pretreatment methods, and advances in bioreactor design are making the future prospects more promising.

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APPENDIX I. Particle Size Distribution for Rice Straw.

Tyler Mesh	Diameter	Weight Fraction	
Material 1: Hydrolysis experiments			
+20	1.079 mm	0.12	
20-28	0.711	0.22	
28-35	0.503	0.20	
35-48	0.356	0.23	$\frac{d\phi}{dp} = 2.95\ mm^{-1}$
48-65	0.252	0.12	$d_p = 0.34$ mm
65-100	0.178	0.06	
100-150	0.125	0.02	
150-200	0.089	0.02	
-200	0.037	0.01	
Material 2: Initial rate experiments			
+35	0.589	0.09	
35-48	0.356	0.18	
48-65	0.252	0.23	$\frac{d\phi}{dp} = 6.06\ mm^{-1}$
65-100	0.178	0.25	$d_p = 0.16$ mm
100-150	0.125	0.12	(See Table 5.3)
150-200	0.089	0.09	
-200	0.037	0.04	

APPENDIX II. Computer Program for Kinetic Modelling

```
      program waldmd (input,output,tape5=input,tape6=output)
c
c  this program executes a numerical integration routine, in conjunction
c  with a non-linear optimization package, to estimate the unknown
c  parameters in a model of cellulose hydrolysis.  the model is based
c  on 2 substrates, 2 enzymes and an equilibrium adsorption process.
c  a shrinking sphere is used to simulate the cellulose particles and
c  the particle size distribution is included
      dimension alo(15),ahi(15),so(20)
      dimension conib(10),vmax(10),rad(10)
      common x(18,15),para(10),kount
      common/b1/sa(20),sc(20),pki,vm,g1o(10),g2o(10)
      common/b3/ea(20),eb(20)
      common/b4/g1p(20,20),g2p(20,20),t(20,20),np(20),nds,adt(20),
     1del(10,10)
      common/b5/g1(20,20),cb(20,20),f(20),wf(10),spar(10)
c
c  set intial values for search variables.
c  alo and ahi are not bounds on variable but set intial size of simplex
      alo(1)=0.14
      ahi(1)=0.16
      alo(2)=0.5
      ahi(2)=0.60
      read(5,101)nds
c
c  read in particle size distribution
      do 150 i=1,7
      read(5,103)wf(i),rad(i)
      spar(i)=0.036/rad(i)
  150 continue
c
c  read in intial conditions for hydrolysis and data
      do 250 i=1,nds
      read(5,102)so(i),ea(i),bdp,adt(i)
      read(5,103)g1o(i),g2o(i)
      f(i)=so(i)/(1-so(i))
      if(so(i).eq.0.10) so(i)=so(i)+((adt(i)-1)*.05)
      so(i)=100*so(i)
c
c  cellobiase activity is fixed ratio of fpa based on stock enzyme
      eb(i)=ea(i)*1.83
      n=bdp
      np(i)=n
      do 200 k=1,n
      read(5,104)g1(i,k),cb(i,k),t(i,k),del(i,k)
  200 continue
  250 continue
c
c  begin optimization
      call simpx(2,alo,ahi)
```

```
c
c  output results
      write(6,113)pki,vm
      do 350 i=1,nds
      write(6,110)so(i),ea(i)
      n=np(i)
      write(6,111)
      do 300 k=1,n
      g1p(i,k)=g1p(i,k)*.18
      g2p(i,k)=g2p(i,k)*.342
      write(6,112)t(i,k),g1(i,k),g1p(i,k),cb(i,k),g2p(i,k)
  300 continue
  350 continue
  101 format(i4)
  102 format(4f10.3)
  103 format(2f10.3)
  104 format(5f10.3)
  105 format(3f10.3)
  110 format(//5x,*solid suspension     *,f10.2,* percent*,/5x,
     1*enzyme concentration *,f10.2,* fpu/l*)
  111 format(//10x,*time (hrs)*,6x,*glu expt*,8x,*glu prd*,8x,
     1*cb expt*,9x,*cb prd*)
  112 format(/10x,f10.2,4x,3(f10.2,5x),f10.2)
  113 format(//5x,*inhibition constant*,f7.3,/5x,*vmax glucose prod*,
     1f9.2)
      stop
      end


      subroutine objtv(irow,area4,kpnt)
c
c  this subroutine is called from the optimization program.  input
c  includes guesses for search variables.  output includes performance
c  measure, sum of the squares, based on given parameters
      dimension iwk(3),wk(42) ,yo(3),ada(4)
      common x(18,15),para(10),kount
      common/b1/sa(20),sc(20),pki,vm,g1o(10),g2o(10)
      common/b2/rate(3),g2,i,jx,finb
      common/b3/ea(20),eb(20)
      common/b4/g1p(20,20),g2p(20,20),t(20,20),np(20),nds,adt(20),
     1del(10,10)
      common/b5/g1(20,20),cb(20,20),f(20),wf(10),spar(10)
      common/b6/amold,amnew,crold,crnew,jadd,rso(4,10),rst(4,10),
     1coeff(4,10)
      external eqn,dumi
      data fam/ 0.38/
c
c  check if parameters are within constraints
c  pki = inhibition constant for amorphous cellulose hydrolysis
c  vm = rate constant for cellobiose hydrolysis
      pki=x(irow,1)
      vm=x(irow,2)
      if(pki.lt.0.001)pki=0.001
```

```
      if(vm.lt.0.0007)vm=.0007
      ada(2)=106.5
      ada(3)=112.9
      ada(4)=131.8
      sum=0.
      do 150 i=1,nds
      sumc=0.
      sumg=0.
      nadd=adt(i)
      n=nadd
      ada(1)=1583.3*f(i)
      sa(i)=fam*ada(1)
      sc(i)=(1-fam)*ada(1)
c
c  calculate amount of total cellulose and crystalline cellulose
c  in each subdivision
      do 60 k=1,n
      do 50 l=1,7
      rso(k,l)=wf(l)*ada(k)
      coeff(k,l)=spar(l)*rso(k,l)**0.333
      rst(k,l)=rso(k,l)
      rso(k,l)=rso(k,l)*(1-fam)
   50 continue
   60 continue
      jadd=1
      satcr=0.
c
c  set intial conditions for integration
      tin=0.
      tout=del(i,1)
      yo(1)=sa(i)
      yo(2)=sc(i)
      yo(3)=glo(i)
      jx=0
c
c  integrate state equations by gears method using variable step size.
c  do integration for region of additions.
c  subscripts- (1) amorphous (2) crystalline (3) glucose
      do 210 j=1,n
      jx=jx+1
      ho=.0001
      index=1
      call absorb
   70 amold=yo(1)
      crold=yo(2)
      call dgear(3,eqn,dumi,tin,ho,yo,tout,.001,2,2,index,iwk,wk,ier)
      jadd=0
      amnew=yo(1)
      crnew=yo(2)
      call absorb
c
c  must call dgear several times between data points to include
c  recalculated enzyme equilibrium
```

```
      tout=tin+del(i,j)
      if(tout.gt.t(i,j))go to 75
      go to 70
   75 g1p(i,j)=yo(3)
      g2p(i,j)=g2
      tout=t(i,j)+del(i,j+1)
      if(j.eq.n) go to 210
      sa(i)=sa(i)+ada(j+1)*fam
      sc(i)=sc(i)+ada(j+1)*(1-fam)
      yo(1)=yo(1)+ada(j+1)*fam
      yo(2)=yo(2)+ada(j+1)*(1-fam)
      jadd=1
  210 continue
      n=np(i)
      nadd=nadd+1
c
c  continue integration for  remainder of hydrolysis
      do 220 k=nadd,n
   80 amold=yo(1)
      crold=yo(2)
      call dgear(3,eqn,dumi,tin,ho,yo,tout,.001,2,2,index,iwk,wk,ier)
      if(yo(1).lt.0.)yo(1)=0.
      amnew=yo(1)
      crnew=yo(2)
      call absorb
      tout=tin+del(i,k)
      if(tout.gt.t(i,k))go to 85
      go to 80
   85 g1p(i,k)=yo(3)
      g2p(i,k)=g2
      kend=k+1
      if(kend.le.n)tout=t(i,k)+del(i,k+1)
  220 continue
c
c  calculate the sum of squares
      do 140 m=1,n
      d1=((g1(i,m)*5.55)-g1p(i,m))
      d2=((cb(i,m)*2.92)-g2p(i,m))
      sumg=sumg+d1**2
      sumc=sumc+d2**2
  140 continue
      sum=sum+sumc+sumg
      write(6,250)sumg,sumc
  150 continue
      area4=-sum
c
c  print each guess and the sum of the squares
      write(6,300)pki,vm,sum
  250 format(35x,2(1pe15.3))
  300 format(5x,3(1pe15.3),/)
      return
      end
```

```
      subroutine absorb
c
c  calculate amount of enzyme adsorbed on crystalline and amorphous area
      dimension sacr(4,10),saam(4,10)
      common/b2/rate(3),g2,i,jx,finb
      common/b3/ea(20),eb(20)
      common/b6/amold,amnew,crold,crnew,jadd,rso(4,10),rst(4,10),
     1coeff(4,10)
      common/b7/eaam,eacr
      data as,ak,cs,ck/41.4,314.0,216.3,1050.0/
c
c  calculate amount of cellulose remaining for each subdivision
      delcr=0.
      delam=0.
      if(jadd.eq.1)go to 38
      if(amnew.le.0.)satam=0.
      if(satam.eq.0.)go to 30
      delam=(amold-amnew)/satam
      if(satcr.eq.0.)go to 32
   30 delcr=(crold-crnew)/satcr
   32 continue
      do 36 m=1,jx
      do 34 n=1,7
      rst(m,n)=rst(m,n)-saam(m,n)*delam-sacr(m,n)*delcr
      if(rst(m,n).lt.0.)rst(m,n)=0.
   34 continue
   36 continue
   38 satcr=0.
      satam=0.
c
c  calculate surface area for each region
      do 60 m=1,jx
      do 50 n=1,7
      sfar=coeff(m,n)*rst(m,n)**0.667
      if(rst(m,n).gt.rso(m,n))go to 40
      sacr(m,n)=sfar
      saam(m,n)=0.
      satcr=satcr+sfar
      go to 50
   40 continue
      saam(m,n)=sfar
      sacr(m,n)=0.
      satam=satam+sfar
   50 continue
   60 continue
      if(amnew.le.0.and.jadd.ne.1)satam=0.
   72 if(satcr.eq.0.)go to 80
      if(satam.eq.0.)go to 85
c
c  calculate the amount of enzyme adsorbed on both  regions
c  use newton-rhapson to solve cubic equation
      b=as*satam+cs*satcr+ak+ck-ea(i)
```

```
      c=as*ck*satam+cs*ak*satcr+ak*ck-(ak+ck)*ea(i)
      d=-1*ak*ck*ea(i)
      kntr=1
      e1=ea(i)-eaam
   75 fe=e1**3+b*e1**2+c*e1+d
      fedot=3*e1**2+2*b*e1+c
      e2=e1-(fe/fedot)
      err=(e2-e1)/e1
      e1=e2
      if(abs(err).lt.0.02.or.kntr.gt.20)go to 77
      kntr=kntr+1
      go to 75
   77 eaam=as*satam*e1/(e1+ak)
      eacr=cs*satcr*e1/(e1+ck)
      go to 90
c
c  calculate enzyme adsorbed on amorphous cellulose only
   80 b1=(as*satam+ak+ea(i))/2.
      c1=as*satam*ea(i)
      d=b1**2.-c1
      s=b1+d**0.5
      eaam=c1/s
      eacr=0.
      go to 90
c
c  calculate enzyme adsorbed on crystalline cellulose only
   85 b2=(cs*satcr+ck+ea(i))/2.
      c2=cs*satcr*ea(i)
      d=b2**2-c2
      s=b2+d**0.5
      eacr=c2/s
      eaam=0.
   90 continue
      return
      end


      subroutine eqn(neqn,t,y,ydot)
c
c  subroutine called from dgear for evaluating functions
      dimension y(3),ydot(3)
      common/b1/sa(20),sc(20),pki,vm,g1o(10),g2o(10)
      common/b2/rate(3),g2,i,jx,finb
      common/b3/ea(20),eb(20)
      common/b7/eaam,eacr
      data ak,ck,pkm,pk1,pk2/0.1266,3.25e-04,1.3,7.0,0.38/
c
c  calculate amount of cellobiose by mass balance
      g2=g2o(i)+sa(i)+sc(i)-y(1)-y(2)-0.5*(y(3)-g1o(i))
      ydot(1)=0.
      finb=1./(1+pki*g2)
      ydot(1)=-1*ak*eaam*finb
      ydot(2)=-1*ck*eacr
```

```
      b3=pkm*(1+y(3)/pk2)
      c3=g2*(1+y(3)/pk1)
      d3=vm*eb(i)*g2
      ydot(3)=d3/(b3+c3)
      return
      end


      subroutine dumi(n,t,y,pd)
c
c  dummy subroutine necessary for dgear
      dimension y(3),pd(3,3)
      return
      end
Glossary of variable names-


WALDMD
   ADT        number of additions including intial charge
   AHI,ALO    intial values for search variables
   CB         experimental cellobiose concentration, mg/ml
   DEL        time increment for integration
   EA         filter paper activity
   EB         cellobiase activity
   NDS        number of data sets
   NP         number of data points
   RAD        average radius for a screen fraction
   SA         amorphous cellulose concentration
   SC         crystalline cellulose concentration
   SO         solid suspension, weight fraction
   SPAR       quantity = 6/
   T          time, hr
   WF         weight fraction of a screen fraction

OBJTV
   ADA        amount of cellulose added in each addition
   AMNEW,     cellulose concentration after integration
   CRNEW
   AMOLD,     cellulose concentration before integration
   CRNEW
   COEFF      quantity = SPAR x C
   F          conversion factor from weight fraction
   FAM        fraction amorphous
   G1         experimental glucose concentration, mg/ml
   G2,G2P     predicted cellobiose concentration
   HO,INDEX,  see IMSL literature on DGEAR
   IER
   JADD       counter, if JADD=1 addition has just occurred
   NADD       number of additions
   RSO        intial concentration of crystalline cellulose
              for a screen fraction
   RST        concentration of cellulose for a screen fraction
   SUM        sum of the squares
   SUMC,SUMG  sum of the squares for glucose, cellobiose
```

TIN	starting point for integration
TOUT	finishing point for integration
YO	sugar concentration

ABSORB

DELAM,	incremental change in amount of cellulose per unit
DELCR	surface area of amorphous, crystalline
EAAM,EACR	amount of enzyme adsorbed on amorphous, crystalline
SAAM,SACR	equivalent spherical surface area for each fraction
SATCR,	total equivalent spherical surface area
SATAM	

EQN

FINB	cellobiose inhibition term

All concentrations in mM unless otherwise noted

APPENDIX III. Development of economic model for calculation of ethanol cost.

Basis: Economic analysis modified for rice straw requirements from Perez (86) and Cysewski (21).

1. Derivation of characteristic flow rates from Table 6.3.

a) Solids input:

$$w_o = \frac{1.19(214\ T/day)}{0.40Y_G} = \frac{637}{Y_G}$$

b) Liquid flow rate:

$$\text{Since } S_o = \frac{w'_o}{w'_o + w_w} \quad \text{where } w'_o = \text{acid treated rice straw} = 0.64w_o$$

$$w_w = 0.64w_o \frac{1-S_o}{S_o}$$

c) Enzyme production rate:

$$E_p = 0.93(2000lb/T)(454gm/lb)(1gm/ml)$$
$$w_w E_o(1-0.94e)$$
$$= 8.42x10^5 w_w E_o(1-0.94e)$$

All derivations include factors to account for losses in processing and handling.

2. Economic Model for Ethanol Production from Rice Straw.

Operation	Capital Related	Labor Related	Utility	Raw Material	Comments
Milling	$3475w_o^{.67}$	146,000	$670w_o$	-	Hammermills- max. cap. 5T/mill (86)
Pretreatment	$3375w_o^{.93}$	292,000	$730w_o$	$690w_o$	t_R=5.5 hrs; Mat. SS304 (86)
Hydrolysis	$84.6(w_w t_R)^{.85}$	292,000	$32.1w_w t_R^{.67}$	-	Tanks- max. cap. 250,000 gal.; Mat. CS (86)
Enzyme Production	$6.73x10^{-3}E_p^{.927}$	876,000	$3.58x10^{-4}E_p$	$3.13x10^{-4}E_p$	Fermentors- max. cap. 100,000 gal; Mat. SS304; t_d= 24 hrs. (86)
Enzyme Recovery	494,000	146,000	110,000	-	t_R= 0.5 hrs/stage; Mat. CS 90% efficiency (86)
Evaporation	$1.22x10^{-5}(w_w-1926)^{.34}$	146,000	$160C_s(w_w-1926)$	-	4-effect evaporator (86)
Fermentation	518,000	438,000	430,000	1,500,000	Fermentor- max. cap. 100,000 gal.; Mat. Batch-CS; Cont. SS304; t_d = 5 hrs.; Medium (121)
Distillation	367,000	292,000	$2.0x10^{-5}C_s$	-	20 lb steam/gal EtOH (76)

2. Continued

Yeast Recovery	345,000	146,000	$5.0 \times 10^{-4} C_s$	-	(21)
Storage	$31.4 E_p^{.37}$	-	2500	-	for media chemicals and acid (21)

where C_s = 0.55/1000 lbs. if steam produced from unconverted solids

$C_s = 0.55f + 2.5f \quad f = \frac{\text{steam produced from solids}}{\text{steam required}}$

based on coal at $34/ton (86).

3. Medium chemicals for cellulase production (86).

H_2SO_4	$58/ton
$(NH_4)_2SO_4$	65
K_2HPO_4	180
$MgSO_4$	230
$CaCl_2$	90
Corn steep liquor	400
Delignified cellulose	100

4. Program for Economic Evaluation:

```
100 rem economic model
102 open1,8,15
105 open130,4
110 dim c(10)
165 open2,8,2,"1:swdata,s,r"
170 input#2,ns
180 for j=1 to ns
200 input#2,eo,so,np
217 print#130,""
240 for k=1 to np
250 input#2,tr,gl
252 rem
255 rem calculate glucose yield from glucose conc.
260 yg=gl*(1-so)/(541.5*so)
270 cr=1.583*so*(1-yg)/(1-so)
280 b=26.8*cr+1.05-eo
290 c=4.2*eo
300 fe=(-b+(b^2+c)^.5)/(2*eo)
302 rem
305 rem solid input in tons\day
310 wo=637/yg
312 rem
315 rem liquid flow rate in tons\day
320 ww=.64*wo*(1-so)/so
322 rem
325 rem enzyme production in fpu\day
330 ep=8.42e+05*ww*eo*(1-.94*fe)
332 rem
335 rem calculate steam requirement and cost
340 sp=1.82e+05*wo*(1-.4*yg-.03)
350 sr=1.98e+05*ww+1.77e+05*wo+.099*ep+4e+07
360 xs=sr-sp
370 cs=.55
380 if xs<o then 410
390 f=sp/(sp+xs)
400 cs=.55*f+2.5*(1-f)
402 rem
405 rem labor rate-4 operators per shiftat 22,000/year
410 l=146000
412 rem
415 rem cost of rice straw
420 p=30
430 c(1)=31.4*ep^.37+2500
440 c(2)=3475*wo^.67+1+670*wo+330*p*wo
450 c(3)=3375*wo^.93+2*1+1420*wo
460 c(4)=84.6*(ww*tr)^.85+2*1+32.1*ww*(tr^0.67)
470 c(5)=6.73e-03*ep^.927+6*1+6.71e-04*ep
480 c(6)=604000+1
490 c(7)=1.22e+05*(ww-1926)^.34+1+cs*(ww-1926)*160
500 c(8)=3.054e+06
510 c(9)=6.59e+05+2e+05*cs
520 c(10)=5.19e+05+5e+04*cs
```

```
522 rem
525 rem calculate sugar transfer cost in cents/pound
530 mc=0
540 for i=1 to 7
550 mc=mc+c(i)
560 next i
570 sc=mc/1.4124e+06
580 mk=mc+c(8)+c(9)+c(10)
582 rem
585 rem calculate ethanol maunfacturing cost in $/gal
590 ec=mk/1e+07
595 print#130,eo,so,tr,yg,fe,sc,ec
642 print#130,""
650 next k:next j
660 print#130:close130
670 close2:close1
```

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