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RECOVERY OF CELLULASE ENZYMES
BY COUNTERCURRENT ADSORPTION

Stephan T. Orichowskyj, Charles R. Wilke,
and Harvey W. Blanch
(M.S. thesis)

October 1982

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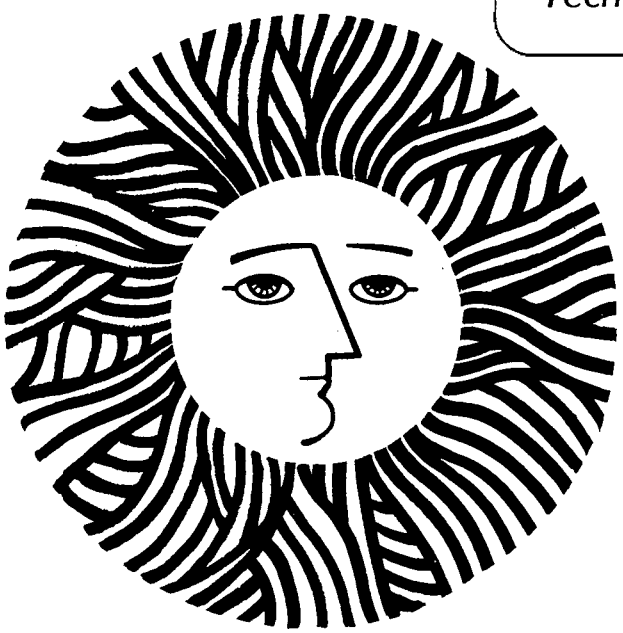

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RECOVERY OF CELLULASE ENZYMES BY COUNTERCURRENT ADSORPTION

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*M.S. Thesis

October 1982

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ABSTRACT

The cost of enzyme production accounts for as much as 60 % of the total cost for enzymatic hydrolysis of glucose. The purpose of this study was to evaluate countercurrent adsorption as a means of recovering enzyme from hydrolyzate. Results from adsorption studies and hydrolysis experiments were used to design a system for enzymatic hydrolysis of steam exploded corn stover operated with two stages of countercurrent adsorption.

The effects of temperature, enzyme loading, and pretreatment on the adsorption of cellulase enzymes were studied. Adsorption is greatest at low temperatures and low enzyme loadings. Enzyme is adsorbed quickly; most of the adsorption is complete within five minutes. Steam explosion is superior to acid treatment and Wiley milling in increasing the susceptibility of the substrate to enzymatic attack.

Adsorption of filter paper activity, activity towards cotton, activity towards carboxymethylcellulose, and cellobiase activity onto steam exploded corn stover were measured and fitted to a Langmuir isotherm. The enzyme responsible for activity toward cotton is adsorbed to the greatest extent. Adsorption of cellobiase is weak.

Glucose yields as high as 94 % are achieved by enzymatic hydrolysis of steam exploded corn stover. High conversions permit release of enzyme so that it may be

recovered from the liquid. The glucose yield decreases with increasing solids concentration, this is due to product inhibition by glucose and cellobiose.

A 10 wt% suspension of steam exploded corn stover was hydrolyzed with an enzyme loading of 25 FPU/gm at 45 ^{o}C for 24 hours. Enzyme in the hydrolyzate was recovered by two stages of countercurrent adsorption, each stage operated at 25 ^{o}C for 45 minutes. The make up enzyme requirement, measured as filter paper activity, was 35 % of that required for the control experiment (batch hydrolysis without enzyme recovery). Compared to the control experiment, the release of filter paper activity and the rate of sugar production were considerably slower. This is attributed to poor recovery of cellobiase.

A preliminary economic evaluation showed that the enzyme production cost can be reduced by 61 % if enzyme is recovered by countercurrent adsorption. This includes the cost of operating the countercurrent adsorption system.

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I am most thankful for the reassurance provided by my family.

TABLE OF CONTENTS

I. INTRODUCTION AND OBJECTIVES..... 1

II. BACKGROUND..... 5

2.1 Elements of Enzymatic Hydrolysis Processes..... 5

2.1.1 Cellulose..... 5
2.1.2 Pretreatment..... 6
2.1.3 Cellulase Enzymes..... 12
2.1.4 Hydrolysis..... 23
2.1.5 Enzyme Recovery..... 28

2.2 Characteristics of Cellulase Adsorption..... 33

2.2.1 Factors Affecting Cellulase Adsorption.. 33
2.2.2 Adsorption of Individual Cellulase Activities..... 33
2.2.3 Rate of Adsorption..... 35
2.2.4 Desorption of Enzyme..... 36
2.2.5 Models of Adsorption..... 37

III. MATERIALS AND ANALYTICAL METHODS..... 40

3.1 Materials..... 40

3.1.1 Cellulase Enzyme..... 40
3.1.2 Substrate..... 42

3.2 Analytical Methods..... 44

3.2.1 Sugar Assays..... 44
3.2.2 Enzyme Assays..... 49

IV. EXPERIMENTAL PROCEDURES..... 55

4.1 Adsorption..... 55

4.1.1 Rate of Adsorption..... 55
4.1.2 Adsorption Isotherms..... 56
4.1.3 Reversibility of Adsorption..... 57

4.2 Hydrolysis..... 58

4.3 Hydrolysis with Countercurrent Adsorption..... 59

V. RESULTS AND DISCUSSION..... 62

5.1 Adsorption..... 62

5.1.1 Rate of Adsorption..... 62
5.1.2 Adsorption Isotherms..... 69
5.1.3 Reversibility of Adsorption..... 81

5.2 Hydrolysis..... 86

5.3 Hydrolysis with Countercurrent Adsorption..... 94

VI. PROCESS DESIGN AND ECONOMIC EVALUATION..... 104

6.1 Process Design..... 104
6.1.1 Enzyme Recovery..... 104
6.1.2 Enzyme Production..... 107

6.2 Economic Evaluation..... 111

VII. CONCLUSIONS..... 115

VIII. RECOMMENDATIONS FOR FUTURE WORK..... 117

BIBLIOGRAPHY..... 119

APPENDIX

Equipment Design and Utilities..... 128

I. INTRODUCTION AND OBJECTIVES

The realization that our supply of fossil fuels is limited has inspired a search for energy resources that are easily replenished. Cellulose, the main constituent of plants, is the most abundant renewable resource, and is available in various forms; agricultural residues, by-products from the production of other materials, and wastes generated by industrial and community activity (29). It can be hydrolyzed to sugars which can then be fermented to ethanol. Commercial scale processes for hydrolysis of cellulosic materials, with subsequent fermentation to ethanol, could help solve waste disposal problems, diminish pollution of the environment, and reduce our dependence on fossil fuels (22).

Cellulose can be hydrolyzed to sugars by using either acids or enzymes. Acid hydrolysis processes are less practical than enzymatic methods because of additional heat requirements, formation of by-products, reversion of compounds, and need for corrosion proof equipment (2,29,30,39).

Several enzymatic hydrolysis processes have been developed (106). A process developed at the University of California at Berkeley consists of several stages; cellulose pretreatment, enzyme recovery, hydrolysis, and enzyme production (Figure 1-1). The sugar product is concentrated, fermented, and distilled to give 95 wt% ethanol. In the pretreatment stage the cellulose is made more susceptible to

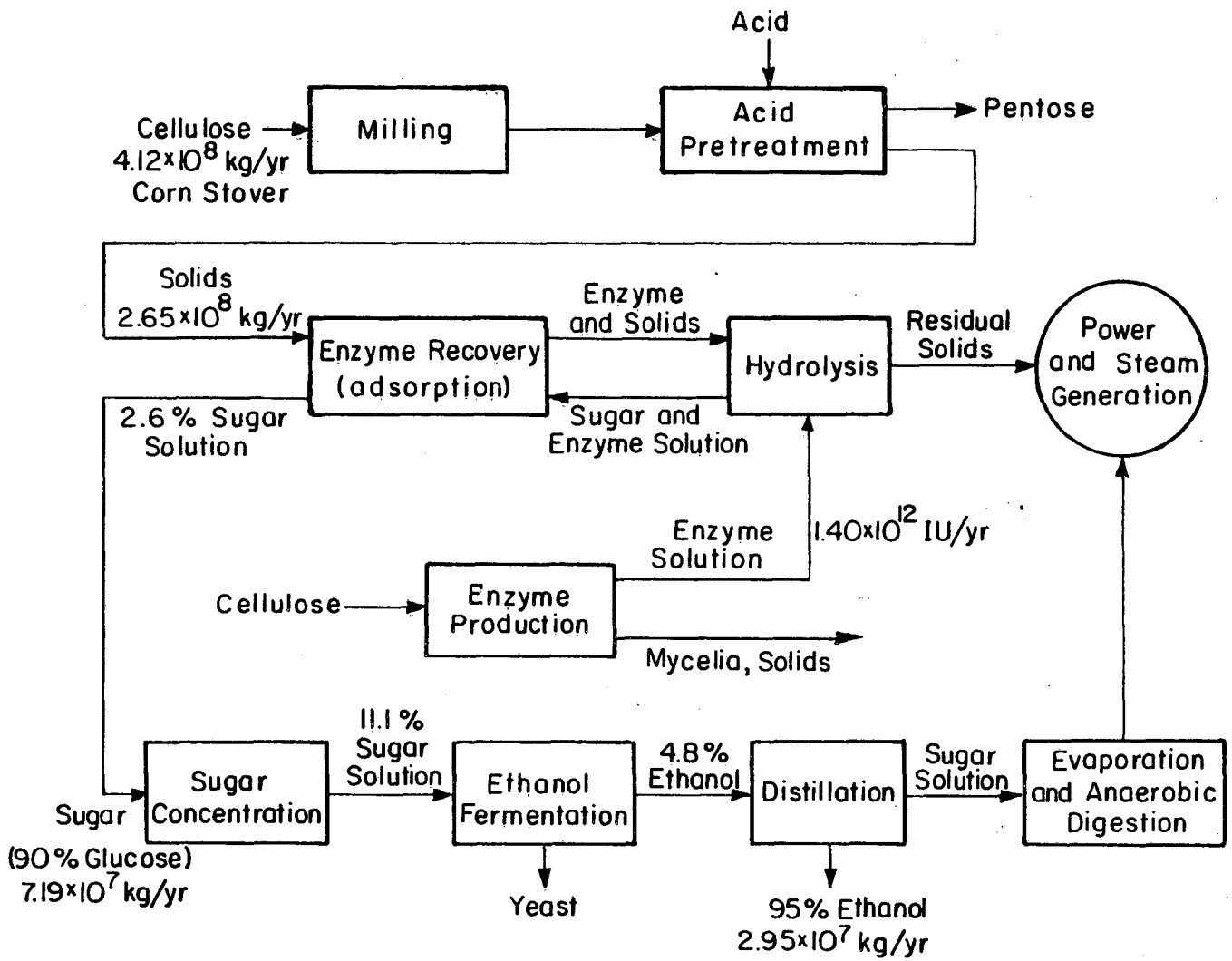

Figure 1-1. Berkeley process for enzymatic hydrolysis of corn stover.

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enzymatic attack so that the rate of hydrolysis and the extent of conversion is increased. The enzyme recovery stage helps to decrease the cost of enzyme production by separating enzyme from the sugar solution, then recycling the enzyme to the hydrolysis stage. The effectiveness of the hydrolysis stage is dependent on the type of substrate and its pretreatment, and on the strength and balance of the cellulase enzyme system. In the enzyme production stage a selected mutant strain of the fungus Trichoderma reesei is grown on cellulose to produce the extracellular cellulase enzyme.

The cost of enzyme production accounts for as much as 60 percent of the total processing cost for enzymatic hydrolysis of cellulose to sugar (109). In an attempt to decrease this cost, various enzyme recovery methods have been investigated. These include acetone precipitation (43) or ultrafiltration (33) of enzyme from the hydrolysis product, addition of urea to the hydrolysis stage (17), and countercurrent adsorption of enzyme onto fresh substrate (66). Studies to date indicate that countercurrent adsorption is the most economical method of enzyme recovery (17,43,72).

The purpose of this study was to more thoroughly evaluate countercurrent adsorption as a means of enzyme recovery. In addition, explosive steam decompression was investigated as a possible pretreatment method. The adsorption of

cellulase enzyme onto steam exploded corn stover was studied, and the results used to predict the make-up enzyme requirement for batch hydrolysis operated in parallel with two stages of countercurrent adsorption. The effects of enzyme loading and solids concentration on batch hydrolysis of steam exploded corn stover were also studied.

II. BACKGROUND

2.1 Elements of Enzymatic Hydrolysis Processes

2.1.1 Cellulose

All cellulosic materials found in nature are comprised of carbohydrates, lignin, and ash. The carbohydrates consist of cellulose and hemicellulose. Cellulose, the main constituent of plants, is a linear homopolymer of anhydroglucose units linked by β-1,4-glucosidic bonds. Individual cellulose molecules may contain from 1000 to 10000 anhydroglucose units. Hemicellulose is a branched heteropolymer of galactose, mannose, xylose, arabinose, other sugars and their uronic acids. Lignin is formed from the enzyme-initiated polymerization of three primary precursors: trans-p-coumaryl alcohol, trans-coniferyl alcohol, and trans-sinapyl alcohol (80).

Cellulose molecules are associated in fibrils, long threadlike bundles, which are stabilized laterally by hydrogen bonds between the hydroxyl groups of adjacent molecules (19). These fibrils are arranged into crystalline regions, where the high degree of order makes it difficult for water or enzyme to enter the structure (102), and less ordered amorphous regions which are easily hydrolyzed. Lignin binds adjacent fibrils and acts with hemicellulose as an additional barrier against enzymatic attack.

2.1.2 Pretreatment

The crystalline structure of cellulose fibrils and the lignin barrier can be disrupted to make the cellulose more vulnerable to the hydrolytic action of cellulase enzymes. Various methods to pretreat cellulose have been developed to increase the effectiveness of subsequent hydrolysis.

All pretreatment methods attempt to increase the susceptibility of cellulosic material to enzymatic attack by altering it in one or more of the following ways: increasing the surface area, decreasing the degree of crystallinity, removing the hemicellulose, and removing the lignin. A pretreatment method should maximize the yield of cellulose, hemicellulose, and lignin (69), and avoid significant conversion of cellulose to degradation products.

Physical Pretreatments:

Physical preatments are effective in increasing the available surface area of cellulose but do not necessarily increase its susceptibility to enzymatic attack. Conventional physical pretreatments use size reduction equipment, such as grinders and mills, to increase surface area. Mandels, Hontz and Nystrom (54) compared the effectiveness of mulchers, shredders and various types of mills. They found that hammer milling gives good size reduction and increases bulk density, but is ineffective in increasing

susceptibility. Fluid energy milling reduces particle size and increases susceptibility, but only after extensive treatment at high cost (29). Ball milling increases susceptibility more efficiently; it reduces crystallinity and increases both contact surface area and bulk density (26). Two roll compression milling is effective in increasing both susceptibility and bulk density (97) and apparently consumes less energy than ball milling (60). Simultaneous wet milling and enzymatic hydrolysis has also been demonstrated as a practical method of pretreatment (45); crystalline material is continuously disrupted as it becomes exposed by enzymatic action.

The most promising physical pretreatment of cellulosic material appears to be explosive steam decompression. Biomass is subjected to saturated steam at 500-1000 psi for 5-300 seconds, then explosively released to atmospheric pressure. The cellulosic material is thus fractionated into three main components: highly accessible cellulose which retains the basic crystalline structure, hemicellulose rendered hot water soluble, and thermoplastic lignin soluble in aqueous ethanol and dilute sodium hydroxide solution (69). The method was developed by the Iotech Corporation (69) as a pretreatment of cellulosic material. The same concept had been used previously, on a commercial scale, by the Masonite Company (24) for production of an animal feed supplement. As a pretreatment method, explosive steam decompression is highly effective. Enzymatic hydrolysis of steam exploded

aspen, done at Iotech, showed glucose and xylose yields of 90 and 75 percent of theoretical, respectively. Perez (72), at Berkeley, observed glucose yields of 80 percent from hydrolysis of steam exploded corn stover, compared to a maximum conversion of 55 percent from the hydrolysis of acid treated corn stover.

Chemical Pretreatments:

Chemical pretreatments are, in general, more effective than conventional physical methods in increasing the susceptibility of cellulosic material to enzymatic hydrolysis. Many of the methods that have been developed cannot be easily classified by their direct effect on biomass because it is not always clear what the primary effect is. Delignification treatments can cause definite alterations in cellulose structure. Intracrystalline swelling methods which disorder the crystalline structure can promote hydrolysis of the ether cross-links of lignin. Methods to increase accessibility of cellulose by removal of hemicellulose often cause partial delignification. The multiplicity of effects observed with most pretreatments reflects the close association of cellulose with hemicellulose and lignin.

Treatments to delignify cellulose often decrease its degree of crystallinity or its degree of polymerization, or both. Kleinert (48) significantly decreased the lignin content of wood by treating it with 50 v% alcohol at 180 ^{o}C for

one hour. When a similar method was applied to samples of wheat straw (104), it was found that the decrease in lignin content did not fully account for the significant increase in hydrolysis yield. This suggests that the cellulose structure had been modified.

Cellulosic material can also be delignified by selective solvent delignification with aqueous ammonium hydroxide/organic solvent solution followed by solids swelling with sodium hydroxide/solvent solution (74). Microbial delignification has also been investigated (47,78).

Intracrystalline swelling methods cause disorder of the crystalline structure of cellulose by penetrating swelling agents into crystalline regions. Toyama and Ogawa (98) used sodium hydroxide as an alkaline swelling agent to increase the susceptibility of rice straw and bagasse. Researchers at Berkeley (84) concluded independently that this method was uneconomical because of excessive consumption of alkali, noting that alkali would also be consumed in depolymerization to solubilize lignin. Other intracrystalline swelling methods include use of ammonia as a swelling agent (103) and use of an oxygen atmosphere over alkaline solutions.

Degradation and removal of hemicellulose has been performed by various methods. Miron and Ben-Ghedalia (63) treated cotton straw with ozone and found decreases in lignin and hemicellulose content of 40 and 54 percent, respectively. They also used sulphur dioxide at 70 ^{o}C for 72

hours and observed a decrease in hemicellulose content of 48 percent; no decrease of lignin was observed. Treating an aqueous slurry of biomass with air or oxygen at elevated temperatures and pressures, known as wet oxidation (5,81), causes partial hydrolysis and solubilization of hemicellulose, and may also degrade and solubilize lignin (62). Borrevick, Wilke and Brink (10) reacted wheat straw with nitric oxide and air at atmospheric pressure, then extracted the material with water. The extraction liquor was rich in xylose and also contained a sizable fraction of the lignin. Further extraction with a dilute alkali solution resulted in additional delignification.

Dilute acid treatment of biomass will hydrolyze the hemicellulose. This process has been used in the pulping industry for many years. Researchers at Berkeley (84), using the work of Dunning and Lathrop (18), developed a dilute acid pentosan extraction pretreatment to increase the susceptibility of cellulose to enzymatic hydrolysis. Milled agricultural residues were boiled at 6-8 wt% suspensions in 0.09 molar sulfuric acid for 5.5 hours. Treatment of corn stover and rice straw by this method gave pentose conversions of 83 and 70 percent, respectively. Lignin was also solubilized. Enzymatic hydrolysis of the treated material resulted in overall combined carbohydrate conversions of 61 percent for corn stover and 64 percent for rice straw. A short time, high temperature dilute acid pretreatment developed at Dartmouth College (49) is also effective.

Enzymatic hydrolysis of corn stover pretreated by this method gave nearly quantitative conversion of glucose.

Another method of increasing the susceptibility of cellulose to enzymatic hydrolysis is to isolate the cellulose by selective solvent extraction and then to precipitate the cellulose (100). Warm alkaline solution or dilute acid solution is used to remove hemicellulose and other extractable materials and to reduce the degree of polymerization of cellulose. The wet residue is then extracted with Cadoxen (a solution of cadmium oxide in aqueous ethylene diamine) or CMCS (an aqueous solution of sodium tartrate, ferric chloride, caustic, and sodium sulphite) to dissolve cellulose. Lignin remains as a solid residue. The cellulose solution is mixed with water to precipitate cellulose in an amorphous form. Enzymatic hydrolysis of Avicel that had been treated by this method gave a 90 percent glucose yield, compared to a 30 percent yield for untreated Avicel. The economics of this treatment, however, are not attractive because of solvent costs, low bulk density of product, necessity of keeping the substrate wet, and generation of chemical waste streams (54).

2.1.3 Cellulase Enzymes

Many microorganisms are able to synthesize an enzyme that will hyrolyze the β-1,4-glucosidic linkage found in cellulose. Those that are truly cellulolytic can utilize native cellulose, not just its soluble derivatives.

The Components of Cellulase and Their Mode of Action:

Realizing the distinction of truly cellulolytic organisms from those that can utilize only soluble derivatives of cellulose, Reese and his colleagues (76) postulated that the classical enzyme "cellulase" (75) - which presumably converts native cellulose to sugars - consists of at least two systems (Fig 2.1.3-1). It was hypothesized that native cellulose is degraded to shorter linear polyanhydroglucose chains by a system designated as C_1; the precise action of the C_1 system was not known. These chains are hydrolyzed into soluble sugars by a second component, termed C_x. The assumption that C_x hydrolyzes the β-1,4-glucosidic linkage of cellulose was based on results that showed that C_x attacks this linkage in carboxymethylcellulose (CMC). Thus, organisms that could grow on only soluble derivatives of cellulose lacked the ability to synthesize C_1 enzymes.

The existence of a C_1 component in the enzyme systems of truly cellulolytic organisms was confirmed many years after the original C_1-C_x concept had been proposed. The

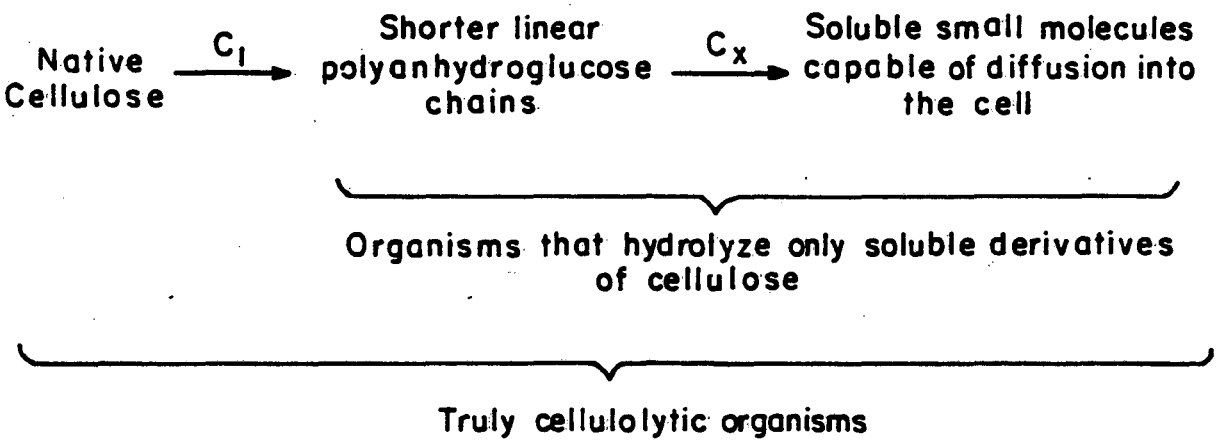

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Figure 2.1.3-1 The original C_1-C_x concept of enzymatic hydrolysis of cellulose, as proposed by Reese et al. (76).

precise action of the C_1 component, however, is still a subject of great controversy; some of the more important observations are discussed in a paper by Bisaria and Ghose (8).

Attempts to verify the C_1-C_x concept have led to a clearer, but not complete, understanding of the cellulase enzyme system. It is agreed that the system is based on the synergistic action of three distinct types of cellulases; endo-β-glucanases, exo-β-glucanases, and cellobiases (Figure 2.1.3-2). Endo-β-glucanases hydrolyze the glucose polymer in a random fashion to expose new chain ends and to produce both glucose and cellobiose. Exo-β-glucanases remove cellobiose units from the nonreducing ends of chains. Cellobiases split cellobiose into glucose molecules. The endoglucanases and exoglucanases are both inhibited by cellobiose and glucose. Cellobiases are inhibited by glucose (9,34). Product inhibition by glucose is mild, inhibition by cellobiose is much stronger (53). An extensive review of accumulated papers on the mode of action of cellulase is presented in Lee and Fan (51)

There have been many efforts to separate, purify, and characterize the cellulase components (3,4,34,35,86,87, 110,111). Methods used to fractionate cellulases into their constituent components include ultrafiltration, gel filtration, ion-exchange chromatography, adsorption chromatography, and iso-electric focusing (106). Twelve cellulase components have been identified, their synergistic effects are

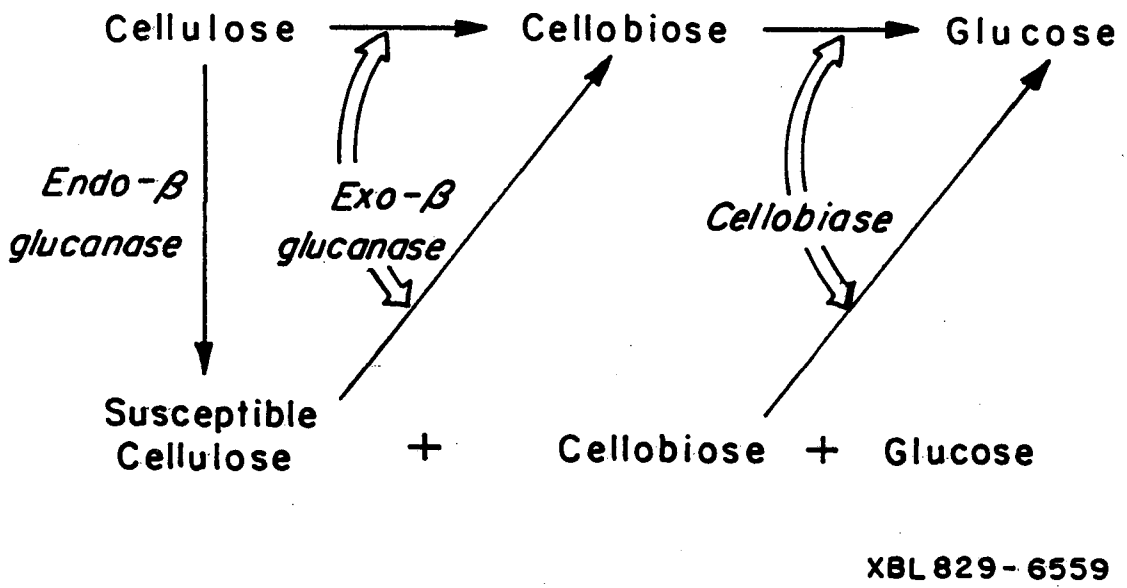

Figure 2.1.3-2 Present understanding of the cellulase enzyme system. Redrawn from Ryu and Mandels (79).

reported by Wood and McCrae (112).

Measurement of Cellulase Activities:

Several methods have been developed to estimate the ability of cellulase enzymes to hydrolyze various forms of cellulose. The methods presented herein are acceptable for general comparison of enzyme preparations; they do not measure activities of individual cellulase components. In all of these assays the enzyme is reacted with the substrate for a specific amount of time so that the rate of sugar production can be measured.

The two most informative assays are activity towards Whatman No. 1 filter paper and activity towards cellobiose. The filter paper activity (FPA) assay measures the ability of the enzyme complex to hydrolyze moderately crystalline cellulose into reducing sugars. Filter paper is used for this assay because it is the most convenient approximation of real substrates (61). The test for cellobiase activity measures production of glucose from cellobiose.

Two additional assays are commonly used to characterize the cellulase system. These have evolved as a result of attempts to verify the C_1-C_x concept. Activity towards highly crystalline material, termed C_1 activity, is measured by production of reducing sugars from cotton. C_x activity is determined by action on carboxymethylcellulose.

It is sometimes useful to determine the amount of soluble protein in an enzyme preparation. Dividing the filter paper activity by the amount of soluble protein will give the specific activity of the enzyme. This information is especially useful in studies to produce a more efficient enzyme complex.

Sources of Cellulase Enzymes:

Many studies of the enzymatic hydrolysis of cellulose have used the extracellular cellulase produced by the fungus Trichoderma reesei, also known as T. viride. This cellulase complex contains all the enzymes required to hydrolyze native cellulose, is resistant to chemical inhibitors (59), and is stable in stirred tank reactors at pH 4.8, 50 ^{o}C for 48 hours or longer. The disadvantages of using Trichoderma are that it will not digest lignin and that its cellulase has a low specific activity, is product inhibited, contains low levels of cellobiose, and is rapidly inactivated at temperatures higher than 50 ^{o}C (79).

Two mutant strains of Trichoderma, namely, MC-G77 and RUT-C30, produce a superior cellulase enzyme complex. Both strains were developed by successive mutation of wild-type QM6a (Figure 2.1.3-3), which is the parent Trichoderma strain isolated at U.S. Army Natick Research and Development Command. Montenecourt and Eveleigh (67) developed the RUT-C30 strain by using a rapid semiquantitative plate screening

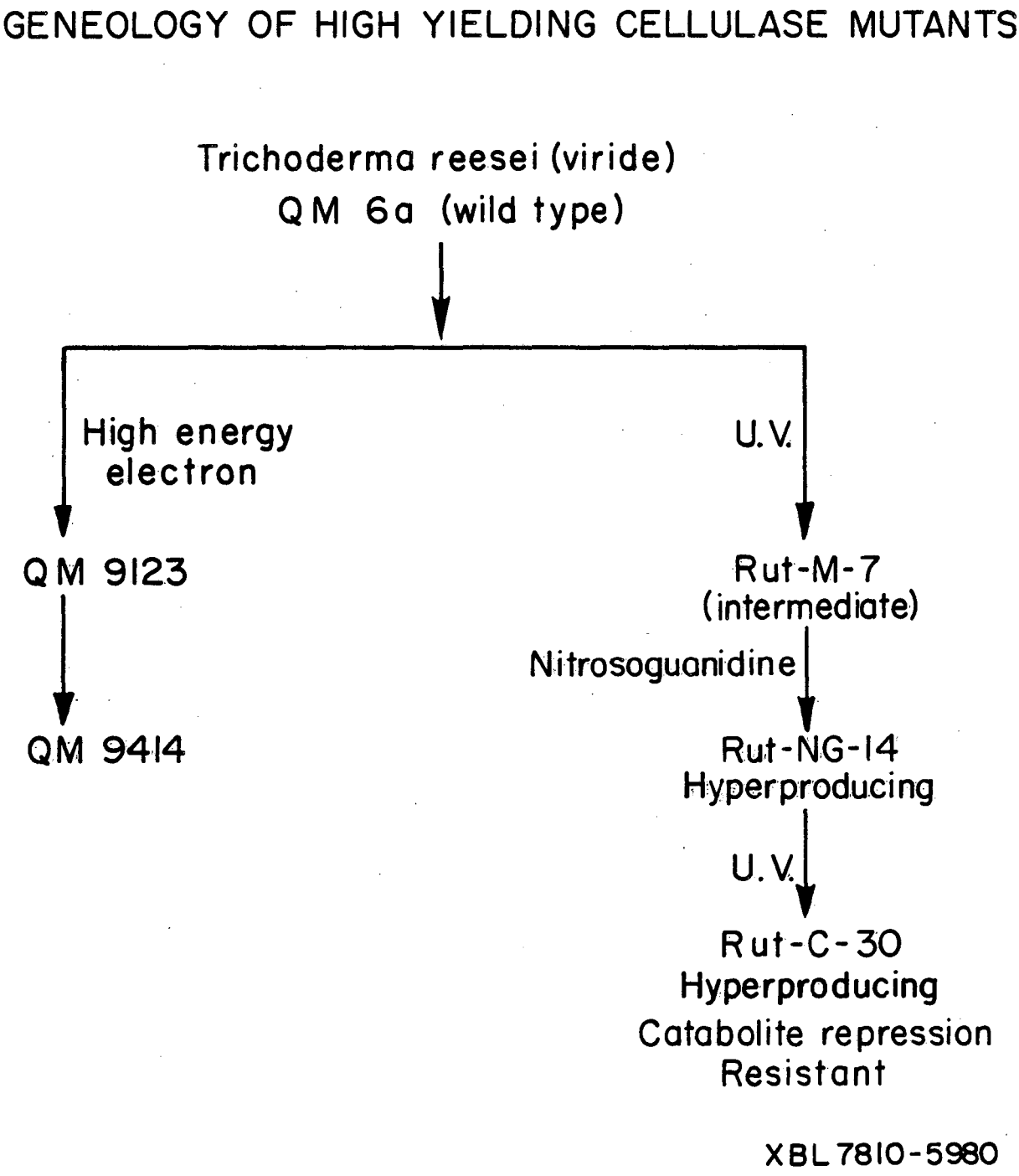

Figure 2.1.3-3 Genealogy of the RUT-C30 mutant strain of *Trichoderma reesei*.

technique which allowed easy isolation of high yielding mutants. In addition to being both hyper-cellulase producing and catabolite repression resistant, this strain produces high xylanase and cellobiase activities (68).

Trichoderma reesei MCG80, a mutant of the RUT-C30 strain (23), produces cellulase at an earlier fermentation time and at a higher cellulase titer than the MCG77 and RUT-C30 strains. Other mutants, of the QM9414 strain, have also been developed recently. The D1-6 strain (31) has a higher filter paper productivity than the RUT-C30 and MCG-77 strains. Mutant strain L27 (88) produces a higher proportion of the most active endoglucanase components than do other strains. The C-5 mutant (64) is a constitutively cellulase producing strain -it produces enzyme in the absence of an inducing substrate.

Thermophilic organisms are also of interest as sources of cellulase enzymes. They produce cellulases that are relatively stable at temperatures above 50 ^{o}C and therefore can be used in a mixed culture with other thermophilic organisms that will ferment sugars to ethanol. The advantages of high temperature mixed culture systems are that product inhibition is reduced and that the rate of hydrolysis is increased.

A process of simultaneous cellulose hydrolysis and alcohol production being developed at MIT (16) uses a mixed culture of Clostridium thermocellum, a thermophilic

cellulolytic anaerobe, and Clostridium thermosaccharolyticum, a thermophilic anaerobe that ferments both hexoses and pentoses. The extracellular enzymes produced by C. thermocellum will degrade both cellulose and hemicellulose, and are resistant to end product inhibition and repression. Researchers at SRI (6) are hoping to introduce the cellulase producing genes of Thielavia terrestris, a thermophile, into a thermophilic bacterium or yeast. T. terrestris produces a cellulase complex that has a peak activity at 65 ^{o}C and a half life of about one day at temperatures between 70 and 80 ^{o}C.

Production of Cellulase Enzymes:

The cost of sugars obtained by enzymatic hydrolysis of cellulose can be reduced by minimizing the cost of enzyme production, which accounts for as much as 60 percent of the total processing cost (109). Improvements in enzyme production can be made by selecting superior producers of cellulase and by optimizing conditions for cellulase production.

Control mechanisms within microbial cells prevent production of enzymes and other metabolites beyond levels necessary for growth and survival. Yields of cellulase enzymes from wild-type cellulolytic organisms in their native state are relatively low because of such biochemical control mechanisms.

Three mechanisms are involved in the production of cellulase enzymes; induction, catabolite repression, and end product inhibition. Several carbon sources will induce production of cellulase enzymes, cellulose gives the best balance of enzymes (79). The synthesis of cellulase enzymes will be repressed by excessive levels of glucose and metabolites of glucose. Production of endoglucanases and exoglucanases is subject to end product inhibition by cellobiose; cellobiase production is inhibited by glucose. Efforts to isolate mutant strains with superior cellulase systems have considered resistance to catabolite repression and end production inhibition. Most of the strain selection work and studies of conditions affecting cellulase production have been with *Trichoderma reesei*.

The conditions that affect cellulase production include medium composition, pH, temperature, and aeration capacity. Optimum conditions for cell growth are not the same as those for enzyme production.

The composition of the medium required for maximal cellulase production in a batch culture of *T. reesei* has been determined by Mandels and Weber (61). The ratio of carbon source to nitrogen source is important. Native cellulose is the most economical carbon source. If the cellulose is delignified, then the activity of cellulase grown on it may be increased (38,96). Addition of 0.01-0.02 percent Tween 80 increases cellulase production, higher concentrations

(greater than 0.1 percent) decrease cellulase production by 40 percent (106). The mechanism of enhancement by Tween 80 is not understood, but it is hypothesized that it may be related to the increased permeability of the cell membrane, allowing for more rapid secretion of enzyme, which leads to greater enzyme synthesis (96).

Cellulose concentrations greater than 0.75 percent result in decreased enzyme yields when pH is not controlled (91). This is probably because of increased acid levels, which cause slow growth (12) and inactivation of enzymes (89). Increases in enzyme yields at higher cellulose concentrations can be achieved if the pH is controlled so that it does not fall below 3.0-4.0. (90)

A combination of temperature and pH profiling may improve yield of enzyme in a batch system. The conditions depend on the strain used. For T. reesei RUT-C30 the optimum conditions are to maintain temperature at 25 °C and pH above 5.0 for the entire fermentation (106). The optimum conditions for other T. reesei strains are a temperature of 31 °C and pH of 4.5 for the first 48 hours, then a temperature of 28 °C and pH greater than 3.3 for the remaining fermentation time (96,105).

Continuous production of cellulase enzymes is best done with a two stage system. The first stage is operated at conditions optimum for cell growth, the second stage is optimized for enzyme production. Typical conditions for the

growth stage are a temperature of 32-35 ^{o}C and a pH of about 4.0. Conditions for the enzyme production stage are 25-28 ^{o}C and a pH of about 3.0 (79).

Adequate oxygen transfer is important during cell growth and enzyme production. An insufficient rate of oxygen transfer will cause cellulase biosynthesis to cease (79). The dissolved oxygen should be maintained at a level greater than 20 percent of saturation (96).

2.1.4 Hydrolysis

Kinetics:

When describing the kinetics of enzyme-substrate interaction one must consider two types of systems; homogeneous systems in which both enzyme and substrate are soluble, and heterogeneous systems in which either the enzyme or the substrate is insoluble. Physical contact of the enzyme with the substrate precedes formation of the intermediate enzyme-substrate complex. Thus, the kinetics of enzymatic hydrolysis of cellulose are complicated by the ability of the enzyme to physically contact the substrate. In both homogeneous and heterogeneous systems the kinetics may be affected by product inhibition.

The kinetics of enzymatic hydrolysis of cellulose are further complicated by the structural features of cellulose (a heterogeneous substrate) and the synergistic action of

the cellulase complex. Inhibition by substrate and noncellulosic material, as well as product inhibition, may also affect the kinetics (106).

The structural features of cellulose that affect its digestibility include the degree of crystallinity, particle size, available surface area, and association with hemicellulose and lignin. The relative importance of these features has not been established.

The synergistic action of cellulase enzymes on cellulose is the basis of a kinetic model proposed by Okazaki and Moo-Young (70). They concluded that the factors that affect the synergistic action of the cellulases include the degree of polymerization of cellulose, the ratio of endoglucanase to exoglucanase, the concentrations of endoglucanase and exoglucanase, end product inhibition, and substrate concentration.

Interaction of the enzyme system with cellulose involves the mechanisms of diffusion, adsorption, desorption, surface reaction, and product inhibition (19).

During hydrolysis the diffusion of cellulase enzymes becomes increasingly difficult because the degree of crystallinity of the unhydrolyzed material increases as amorphous material is degraded. The increased mass transfer resistance can decrease the rate of formation of an enzyme-substrate complex, but this resistance can be reduced

substantially by mixing (19).

The accessibility of the cellulose also affects adsorption of cellulase enzymes. Adsorption of cellulase enzymes onto various substrates has been modelled as a Langmuir isotherm (71). Linear models (46,65,108,113) have also been used to relate the adsorbed enzyme concentration to the concentration of enzyme in solution. Desorption of enzyme occurs as product is formed, but enzyme can be readsorbed onto freshly exposed material.

The rate of enzymatic hydrolysis may be proportional to the available surface area of cellulose, although it has been shown that the action of the cellulase is not exclusively a surface phenomenon (19).

The mechanism of product inhibition is controversial. Both competitive (28,42,58) and non-competitive (19,70) inhibitions have been observed. It is agreed, however, that inhibition by cellobiose is much stronger than inhibition by glucose.

Effectiveness:

Improvements in the effectiveness of enzymatic hydrolysis of cellulose have been made by realizing the significance of the structural features of cellulose and by understanding the action of the cellulase complex and the mechanism of enzymatic hydrolysis.

The structural features of the cellulose substrate are determined by the original source of the cellulose and the pretreatment used. The most effective pretreatment will maximize the digestibility of cellulose, increasing the rate and extent of hydrolysis, at minimal cost.

The efficiency of the cellulase system is greatly affected by the relative amount of cellobiase. It is necessary to have a sufficient amount of cellobiase to prevent accumulation of cellobiose, which strongly inhibits endoglucanases and exoglucanases. Howell and Mangat (40) observed that inhibition by cellobiose controls the initial fast stage of hydrolysis. Enzyme systems deficient in cellobiase activity can be supplemented with cellobiase from *Aspergillus phoenicis*, *Botriodiplodia theobromae* or other sources (41). Yamanaka (113) found that the effect of supplemental cellobiase is much more significant when the accessibility of the substrate is high and when the overall cellulase activity of the original enzyme system is low. Systems with immobilized cellobiase have been used with *Trichoderma reesei* cellulase to avoid cellobiose inhibition (44,92,94).

The ratio of cellulase activity (measured as filter paper activity) to solids concentration also affects the initial rate of hydrolysis. The optimum ratio of enzyme to solids for hydrolysis is about 25 filter paper activity units per gram of solid (72,101). This is based on economic evaluations and the consideration that fast initial rates of

hydrolysis will decrease residence time in reactors, minimizing deactivation of enzyme. Greater amounts of enzyme will not significantly increase the yield.

The concentration of substrate to be used also needs to be considered. Because of viscosity limitations continuous systems cannot exceed solids concentrations of about 10-15 percent, the limit depending on the bulk density of the material. Effective substrate concentrations as high as 25 percent can be achieved in batch systems by stepwise addition of substrate (72,101). The hydrolysis is initiated at a solids concentration of 10 wt%; as cellulose is degraded the viscosity decreases and additional substrate can be added. Use of high substrate concentrations, however, may result in decreased sugar yields. At high sugar concentrations the effects of product inhibition by glucose may become significant.

Glucose inhibition can be avoided in the hydrolysis stage by continuous removal of glucose. Membrane filtration allows selective removal of glucose. Mixed culture processes combine cellulolytic organisms with organisms that ferment glucose to ethanol, thus avoiding inhibition of both cellulase production and enzymatic hydrolysis.

2.1.5 Enzyme Recovery

In the enzymatic hydrolysis of cellulose to sugars the significant cost of enzyme production can be decreased by recycling enzyme. The success of an enzyme recovery scheme depends on the stability of the enzyme under process conditions, the availability of enzyme for recovery after hydrolysis, and the effect of the recovery method on process operations downstream of it. Because the cellulase enzyme system is a balanced complex of enzymes the recovery of each enzyme component should be the same.

If the extent of conversion in the hydrolysis stage is low then a significant amount of enzyme remains adsorbed onto cellulose. It is also possible that enzyme is adsorbed, perhaps by a different mechanism, onto noncellulosic material.

Mitra and Wilke (66) developed an enzymatic hydrolysis process in which adsorbed enzyme is recovered from hydrolyzed solids by countercurrent wash with process water fed to the hydrolysis stage. They observed that most of the C_1 activity remains adsorbed while all of the C_x activity is desorbed. Yamanaka (113) found that the recovery of enzyme activity is enhanced by increasing the wash temperature from 45 ^{o}C to 50 ^{o}C, probably because of weaker adsorption at higher temperatures.

Desorption of cellulase enzymes from solids is also enhanced by addition of urea or other reagents (17,77). Dove (17) evaluated a process design in which hydrolyzed solids are washed with a concentrated urea solution to remove adsorbed enzymes. The washed solids are filtered, then burned for power generation. Enzyme in the filtrate is concentrated by ultrafiltration and is returned to the hydrolysis stage. Urea is also recycled. An alternate method to concentrate enzyme in the filtrate is to precipitate the enzyme with acetone, but the economics of this method are less attractive.

Dove also evaluated a modification of the Berkeley process in which urea is added directly to the hydrolysis stage. Urea is permitted to continue through the major unit operations downstream of hydrolysis, and is recovered as part of the bottoms leaving the ethanol distillation column. The optimum concentration of urea in the hydrolysis stage is 1.0 molar; 65 percent of the enzyme is retained in the hydrolyzate after 40 hours of hydrolysis. To yield a concentrated ethanol product from the fermentation stage the sugar/urea solution from hydrolysis has to be concentrated by a factor of 5-7 before it is fermented. This results in a urea concentration well above the 1.0 molar limit where *Saccharomyces cerevisiae*, the fermenting yeast, is no longer able to function in a stable manner. No economical method to remove urea before the fermentation stage has been found.

Enhanced enzyme recovery with urea is uneconomical mostly because of the high cost of urea. A similar reagent must be found that is less expensive and is effective at low concentrations (17).

A high conversion of cellulose in the hydrolysis stage permits release of adsorbed enzyme into solution; most of the enzyme available for recovery is in the liquid phase, not on unhydrolyzed solids. Several schemes to recover cellulase enzymes from the hydrolysis product stream have been developed from established laboratory techniques of protein concentration and purification.

Ghose and Kostick (33) developed a model system in which rapid hydrolysis of a high cellulose suspension in concentrated enzyme is combined with quick removal of products soon after they are formed. Polymeric membranes of suitable molecular cutoff values are used to separate products from the reacting system and to concentrate enzyme for reuse in hydrolysis. They hydrolyzed a 23 wt% suspension of Solka Floc in their semicontinuous system for ten days, and achieved a substrate concentration of 71 percent. Actual recovery of enzyme in the system was not reported, but in a preliminary ultrafiltration experiment with membranes of molecular weight cutoffs at 10,000, 20,000, and 30,000 they observed that a five-fold concentration of enzyme gave maximum recoveries of 46.7 and 56.7 percent for filter paper and C_x activities, respectively. Total protein recovery

from ultrafiltration of T. reesei cellulase was correlated with individual enzyme activities by Mitra and Wilke (65).

Another technique to concentrate and purify proteins that has been developed as a method of cellulase enzyme recovery is that of enzyme precipitation. Inorganic salts such as ammonium sulphate are used to purify proteins on a large scale. A disadvantage of continuous salt precipitation processes is that they require long contact times for complete salt precipitation. A more extensive review of salting out methods is presented in Ige (43).

Organic solvents will also precipitate proteins. Askonas (1) separated enzymes from aqueous rabbit muscle extracts by using ethanol, methanol, n-propanol, and acetone as precipitating agents. Acetone appeared to be the superior solvent for enzyme separation, giving enzyme recoveries near 100 percent.

Acetone precipitation of cellulase enzymes was investigated by Ige (43) as a possible method of enzyme recovery. The effects of acetone concentration, temperature, and total soluble protein on the recovery of cellulase were examined. More that 85 percent of the original enzyme activity can be recovered with an acetone concentration of 2.0 v/v or greater. Using acetone precipitation as an enzyme recovery process is not economical, primarily because of its large energy consumption for the solvent recovery step (113).

The most promising method of enzyme recovery is adsorption of enzyme from hydrolyzate by countercurrent contacting with fresh cellulosic solids. Halliwell (36) was one of the first to observe that mixing cellulase and its substrate resulted in almost immediate adsorption of enzyme. Enzyme is slowly released only as cellulose is solubilized. Mandels et al. (55) also observed the strong adsorption of cellulase at 25-50 ^{o}C, pH 4.0-5.0. They found that adsorbed enzyme was sufficient for digestion of cellulose without replenishment of enzyme, even though the liquid phase containing the sugar was continuously removed. They proposed that hydrolysis with adsorbed cellulase offered a promising approach to a practical continuous system.

The newsprint hydrolysis process developed by Mitra and Wilke (66) includes recovery of enzyme from hydrolyzate by countercurrent contacting with fresh newsprint at 50 ^{o}C. When combined with recovery of adsorbed enzymes from hydrolyzed solids by countercurrent wash the total predicted recovery is 85 percent. Others have also predicted the effect of enzyme recovery by countercurrent adsorption on the enzyme requirements and process economics (14,15,107).

2.2 Characteristics of Cellulase Adsorption

2.2.1 Factors Affecting Cellulase Adsorption

The adsorption of cellulase onto cellulose is affected by the temperature of the system, the substrate, the concentration of the substrate, and the enzyme loading. Adsorption of enzyme is decreased as temperature is increased from 5 to 50 ^{o}C (7,71). The effect of accessibility of cellulose on adsorption was studied by Yamanaka (113) and Peitersen et al. (71). The effective concentration of cellulose for adsorption is a function of available surface, which means that removal of lignin and particle size reduction can increase adsorption and digestibility (55). Pretreatment can prevent adsorption of enzyme onto undigestible materials that may cause chemical inactivation of the enzyme (11). The quantity of enzyme adsorbed is increased if the substrate concentration is increased (36,55). At higher enzyme loadings a smaller percentage of the original enzyme is adsorbed (71), probably because of a limited number of adsorption sites.

2.2.2 Adsorption of Individual Cellulase Activities

The relative adsorption of the individual cellulase enzymes is determined by their affinity for the substrate. Enzymes with a high affinity for the substrate will adsorb the fastest and to the greatest extent (15). It has also

been observed that active cellulase enzymes are held more strongly than nonactive ones (7). The pattern of adsorption is affected by the condition of the substrate and thus may vary during hydrolysis as the substrate is modified.

In one study (108) it was observed that the initial adsorption of C_1 activity onto newsprint was the same as adsorption of C_x activity. Other studies with newsprint (14,15,65) showed a more selective pattern, with preferential adsorption of C_1 activity. The differences in these observations may be due to variations in the treatment of the newsprint or differences in the enzyme stocks.

Ghose and Bisaria (27) studied the significance of synergism on the adsorption of endoglucanases, exoglucanases, and xylanases onto bagasse. They found that each component adsorbed to a greater extent if it was isolated from the enzyme mixture, and suggested that cellulose contains common sites at which all cellulase components can attack.

Ghose and Bisaria also observed that the adsorption of cellobiase activity onto bagasse was very weak. Mandels et al. (56) adsorbed cellulase onto Solka Floc and Avicel and evaluated the effectiveness of the adsorbed enzyme and the free enzyme by comparing sugar production with that of original enzyme solution. They found that free enzyme was at least as effective as predicted, but adsorbed enzyme was less effective than was expected. They suggested that the poor performance of adsorbed enzyme and the superior

performance of free enzyme was due to lack of cellobiase adsorption.

2.2.3 Rate of Adsorption

Most workers have observed that the adsorption of cellulase onto cellulose at 40-50 °C is characterized by an initial rapid adsorption followed by a slower uptake of enzyme. Halliwell (36) found that when enzyme was mixed with swollen cellulose powder the adsorption was immediate. Similar observations were made by other workers using milled Solka Floc (55), Wiley milled newspaper (14), and ball milled newspaper (11). Ghose and Bisaria (27), using delignified bagasse as a substrate, determined the rate of adsorption of purified endoglucanase, exoglucanase, and xylanase at temperatures as low as 5 °C. At 5 °C the initial adsorption of each enzyme took about five minutes. They were able to compute the activation energy of each component by measuring the rate of adsorption at various low temperatures. Mitra (65) observed a slow adsorption of filter paper activity onto a 0.1 wt% suspension of ball milled newspaper at 50 °C ; the enzyme had an activity of 2.85 filter paper units.

2.2.4 Desorption of Enzyme

Once adsorbed, the enzyme may or may not desorb. Desorption of enzyme from cellulose can occur only as the cellulose is solubilized. Many workers using various substrates have observed that as cellulose is hydrolyzed the adsorbed enzyme is released back into solution (11,36,55,108). Wald (101) observed no release of enzyme activity after hydrolyzing acid treated rice straw for 50 hours. It is possible that desorbed enzyme had been inactivated by thermal or physical effects, but this could not be confirmed because the observations were based on activity measurements alone. Castanon and Wilke (14,15), using Wiley milled newsprint, concluded that adsorbed enzymes remain immobilized on the substrate, even after extensive digestion. This conclusion was drawn from measurements of enzyme activities and total protein, and from evidence obtained by SDS polyacrylamide gel electrophoresis that established that the initial adsorption was followed by a slow continuous uptake of enzyme.

Yamanaka (113) evaluated the effect of washing out adsorbed enzyme from Wiley milled newspaper and observed that enzyme did desorb, but concluded that the adsorption was not reversible. Wald (101) also observed desorption of enzyme in a similar experiment with acid treated rice straw.

2.2.5 Models of Adsorption

Designs of countercurrent adsorption systems for enzyme recovery have been based on linear models of adsorption (65,108,113); the distribution of enzyme between the two phases was described by a constant partition coefficient.

Peitersen et al. (71), in studying the effects of pH, temperature, and type of substrate on the adsorption of cellulase, assumed that the adsorption of enzyme can be described by a Langmuir Isotherm (50):

$$\frac{E_{ads}}{E_{ads,max}} = \frac{K \cdot E}{1 + K \cdot E}$$

where:

E = enzyme activity in solution at equilibrium, units/ml

E_{ads} = enzyme adsorbed onto solids at equilibrium, units/gm

$E_{ads,max}$ = maximum level of enzyme adsorption, units/gm

K = constant, ml/units.

Because the adsorption of enzyme is usually characterized by a fast initial adsorption followed by a much slower uptake of enzyme, it was assumed that equilibrium was

achieved one hour after adding enzyme to the substrate.

The Langmuir isotherm is usually used to model chemical adsorption (chemisorption) of gases, which differs from physical adsorption in that electrons are transferred between adsorbent and adsorbate. Chemisorption, being a chemical reaction, implies formation of a single monolayer. Because chemisorption is exothermic it decreases continuously with increasing temperature.

The conditions implied in kinetic and statistical derivations of the Langmuir isotherm are that (20,37):

(a) Adsorption is localized and takes place only through collision of gas molecules with vacant sites.

(b) Each site can accommodate only one adsorbed molecule.

(c) The energy of an adsorbed molecule is the same at any site on the surface, and is independent of the presence or absence of nearby adsorbed molecules.

These conditions do not always apply. The second part of condition (a) applies to slower chemisorptions where a small activation energy is involved. Condition (b) is obeyed for many chemisorptions. Condition (c) is rarely obeyed; it requires that forces of attraction and repulsion

between adsorbed molecules are negligible.

Adsorption of enzyme can be tested for fit to the Langmuir isotherm by plotting $1/E_{ads}$ against $1/E$. The result should be a straight line, with the slope equal to $1/(E_{ads,max} \cdot K)$ and the intercept equal to $1/E_{ads,max}$.

III. MATERIALS AND ANALYTICAL METHODS

3.1 Materials

3.1.1 Cellulase Enzyme

The cellulase enzyme used in all experiments was produced by the RUT-C30 mutant strain of *T. reesei*. Viable cultures were maintained on potato dextrose agar (PDA) slants for short term storage at 4°C. The slants were used to inoculate 200 ml of mineral salts medium containing 1 % glucose, 0.01 % Tween 80, and 0.1 % antifoam. Lactose (1 %) was added as an inducer to yield a more active culture. The mixture was incubated at 28 °C for 5-6 days, at which time it was used as the inoculum for a 10 liter batch fermentation (97) in a 14 liter fermentor (New Brunswick Scientific Company, Magnaferm Model MA114, or Chemapec Inc., Chemap Type LF). The medium composition (Table 3.1.1-1), developed by Mandels and Reese (57), was modified for the RUT-C30 strain by Tangnu et al. (97). Temperature was controlled at 28 °C, agitation was kept at at approximately 400 rpm, and the aeration rate was 2.0 vvm. The pH was maintained above 5.0 by controlled addition of 2 N sodium hydroxide. After 7 days the enzyme solution was harvested and filtered through glass wool to remove mycelia. The solution was buffered to pH 5 with 0.05 M sodium acetate and was stored at 4 °C to minimize inactivation of the enzyme. Sodium azide (0.02 %) was added to prevent contamination. The original activities

Table 3.1.1-1

Cellulase Production Media (61,101)

Component (gm/L)	Cellulose Concentration (gm/L) 10	50
$(NH_4)_2SO_4$	1.4	11.6
KH_2PO_4	2.0	38
$MgSO_4$	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

of the enzyme are listed in Table 3.1.1-2.

Some experiments required a stronger enzyme solution than was obtained from fermentation. Enzyme solution was freeze dried in a Labconco Freeze Dry-5 freeze dryer. The freeze-dried enzyme was added to the stock enzyme solution to increase the activity of the solution. The relative activity of each component was approximately the same after addition of freeze-dried enzyme.

Although the enzyme complex produced by the RUT-C30 strain is high in cellobiase activity, the cellobiase component is relatively unstable, having a half life of about two weeks at 4 ^{o}C (83). In a commercial integrated hydrolysis process enzyme would be continuously produced and would be used almost immediately. It was impractical to produce fresh enzyme for each experiment, but the relative amount of cellobiase activity was not important for adsorption studies. In hydrolysis experiments the cellobiase activity of the enzyme solution was supplemented with freeze-dried β-glucosidase obtained either from almonds or from *Aspergillus phoenicis*.

3.1.2 Substrate

Corn stover was obtained from the University of California at Davis fall 1980 crop. A portion of this material was steam exploded by the Iotech Corporation of Ottawa, Ontario, Canada. Steam exploded corn stover was used in all

Table 3.1.1-2 (101)

Original Acitivities of Cellulase Enzyme

Activity	Units/ml
Filter paper	4.1
C1 (Cotton)	0.39
Cx (CMC)	140
Cellobiase	8.6

experiments. In addition, Wiley milled corn stover and acid treated corn stover were used in experiments to determine the effect of pretreatment on enzyme adsorption. The Wiley mill pretreatment consisted of hammermilling through a 6 mm screen, followed by Wiley milling through a 2 mm screen. A portion of this Wiley milled material was acid treated with 0.09 M sulphuric acid, according to the method developed by Sciamanna et al. (84).

Samples of each substrate were analyzed for carbohydrate, lignin, and extractives by the method of Freitas et al. (21) (Tables 3.1.2-1,2,3). The percentage of solids totalled to less than 100 percent because some of the components, such as sugar derivatives and protein, were not assayed. Also, the ash content measurement is is inaccurate because of oxidation and loss of volatile inorganics at 600 °C.

3.2 Analytical Methods

3.2.1 Sugar Assays

Glucose:

The concentration of glucose was measured with an Instrumentation Laboratory Glucose/BUN/Creatinine analyzer model 919, which analyzes glucose automatically according to the procedure developed by Trinder (99). The rate of glucose oxidation is measured over an interval of a few seconds, allowing quick determination of glucose

Table 3.1.2-1

Composition of Wiley Milled Corn Stover
University of California, Davis - Fall 1980 Crop

Carbohydrate		Sugar Equivalent	
36.7 %	Glucan	40.7 %	Glucose
17.0	Xylan	19.3	Xylose
2.2	Arabinan	2.5	Arabinose
55.9 %	Carbohydrate	62.5 %	Sugar Equivalent

11.4 %	Lignin
18.5	Ash (21.5 as actual silicates)
6.5	Azeotropic benzene/alcohol extractives
0.4	Acid insolubles
---	Protein (not determined)
95.7 %	

Table 3.1.2-2

Composition of Acid Treated Corn Stover (72)
University of California, Davis - Fall 1980 Crop

Carbohydrate		Sugar Equivalent	
55.8 %	Glucan	62.0 %	Glucose
11.0	Xylan	12.5	Xylose
66.8 %	Carbohydrate	74.5 %	Sugar Equivalent

15.1 %	Lignin
5.4	Ash (7.3 as actual silicates)
6.0	Azeotropic benzene/alcohol extractives
---	Protein (not determined)
95.2 %	

Table 3.1.2-3

Composition of Steam Exploded Corn Stover (85)
University of California, Davis - Fall 1980 Crop

Carbohydrate & Sugar (a)		Sugar Equivalent	
32.8 %	Glucan	43.4 %	Glucose
7.0	Polyglucose (b)		
5.6	Xylan	15.3	Xylose
8.9	Polyxylose (b)		
0.40	Polyarabinose (b)	0.40	Arabinose
0.14	Polyribose (b)	0.14	Ribose
0.60	Sucrose	0.60	Sucrose
55.4 %	Carbohydrate & Sugar	59.8 %	Sugar Equivalent

11.4 %	Lignin with distribution:	0.242 hot water soluble 0.758 water insoluble
18.2	Ash (21.5 as actual silicates)	
6.9	Azeotropic benzene/alcohol extractives (c)	
---	Protein (not determined)	
95.3 %		

(a) 37.0 % of dry corn stover is hot water soluble with distribution after secondary acid hydrolysis:

0.189 glucose
0.240 xylose
0.011 arabinose
0.004 ribose
0.015 sucrose
0.075 lignin
0.467 acids and resins.

(b) Poly-sugar is the monomer and oligomers including hot water-soluble pentosans and hexosans and substituted pentosans/hexosans.

(c) Total azeotropic benzene/alcohol extractives was 33.05 %, with a distribution: 0.791 hot water soluble
0.209 water insoluble.

concentration.

Reducing Sugars:

Two modifications of the dinitrosalicylic acid (DNS) method (85) were used to measure reducing sugars.

For enzyme activity measurements the reducing sugars concentration should not exceed 1 gm/L. To accurately determine reducing sugars in this range 5 ml of DNS reagent are added to 2 ml of sample in a test tube. The mixture is vortexed, covered with a steel cap, and boiled for ten minutes. At that time it is cooled in an ambient water bath and vortexed again. If necessary, the solution is cleared of cellulose debris by pushing a Kim-Wipe plug to the bottom of the tube with a glass rod. The cleared solution is transferred to a new tube.

Absorbance is measured at 600 nm with a Bausch and Lomb Spectronic System 400 spectrophotometer, using distilled water as a blank. The absorbance is compared with a glucose standard curve to determine reducing sugars concentration.

Samples from hydrolysis usually contain much higher concentrations of reducing sugars; diluting them to 1 gm/L would be both time consuming and inaccurate. A modified procedure allowed accurate determination of reducing sugars as high as 15 gm/L. In this method 0.1 ml of sample is added to 1.5 ml of DNS reagent in a test tube. As in the

other method, the mixture is vortexed, capped, and boiled. The boiled mixture is diluted with 9 ml of distilled water and mixed by inversion. Absorbance at 600 nm is compared with an appropriate glucose standard curve to determine reducing sugars concentration.

Other Sugars:

The concentration of cellobiose and other sugars was determined by liquid chromatography. Carbohydrates were separated with a Partisil PAC column (Whatman Inc.) and analyzed with a Waters Associates Carbohydrate analyzer. Azeotropic acetonitrile-water (83.7 % acetonitrile) was the carrier at a flow rate of 1 ml/min.

3.2.2 Enzyme Assays

Because cellulase enzyme is actually a complex of enzymes its activity cannot be determined by one assay. In this laboratory the activity of the cellulase complex is determined by measuring its activity towards four different substrates; Whatman No. 1 filter paper, Red Cross cotton, carboxymethylcellulose, and cellobiose.

Filter Paper Activity:

In the filter paper assay 1 ml of diluted enzyme is added to 1 ml of 0.05 M acetate buffer in a test tube. A 1 x 6 cm strip (50 mg) of Whatman No. 1 filter paper is coiled

and added to the solution. The tube is capped and incubated at 50 °C for one hour. The amount of reducing sugars produced is determined by the DNS method, using glucose as a standard. A background tube, containing enzyme/buffer solution but no filter paper, is analyzed for reducing sugars that are present in the enzyme sample before enzymatic hydrolysis of the filter paper. The filter paper background is also determined; a coil of filter paper is added to 2.0 ml of buffer and the reducing sugars are measured.

The net production of reducing sugars is converted into international units per milliliter (IU/ml) by calculating the micromoles of glucose produced per minute, taking into account prior dilution of the enzyme:

$$\frac{IU}{ml} = \frac{c \cdot d}{t} \cdot \frac{1000 \text{ micromol/mmol}}{180 \text{ mg/mmol}}$$

where:

c = net production of sugars, mg/ml

d = total enzyme dilution

t = hydrolysis time, minutes.

One should realize that the DNS method measures all reducing sugars, not just glucose, so the accuracy of this method is limited.

C_1 Activity:

The C_1 assay measures activity towards Red Cross cotton. The procedure is similar to the filter paper assay; 50 mg of cotton is used in place of filter paper, and the incubation time is 24 hours. The long incubation period is necessary because this assay measures degradation of highly crystalline material, which is hydrolyzed much more slowly than filter paper. To prevent excessive evaporation, rubber stoppers or serum caps should be used in place of steel caps.

C_x Activity:

The C_x assay measures activity towards carboxymethylcellulose (CMC), a soluble derivative of cellulose. The activity tubes for this assay contain 1.0 ml of 2.25 % CMC (CMC 7L, Hercules, DP 400, DS = 0.65-0.85) in 0.025 M acetate buffer. Capped tubes are placed in a 50 ^{o}C water bath and, at timed intervals, 0.1 ml of diluted enzyme solution is quickly added and the solution swirled. After 30 minutes DNS is quickly added to determine reducing sugars. Enzyme background is determined by adding 0.1 ml enzyme to 1.0 ml 0.025 M acetate buffer. CMC background is determined by adding 0.1 ml water to 1.0 ml CMC.

Cellobiase Activity:

The cellobiase assay differs from the other three enzyme assays in two ways; the activity of a specific enzyme component is measured, and the production of glucose, not reducing sugars, is measured. In this assay the activity tubes contain 1.0 ml of 2.0 % cellobiose ("Baker" grade, JT Baker Chem. Co.). Capped tubes are placed in a 50 ^{o}C bath. At timed intervals 1.0 ml of diluted enzyme is added, and the solution swirled. After 30 minutes 50 microliters of concentrated sulphuric acid is added to inactivate the enzyme. Enzyme background is determined by adding 1.0 ml enzyme to 1.0 ml acetate buffer. Cellobiose background is determined by adding 1.0 ml cellobiose to 1.0 ml acetate buffer. Glucose concentrations are measured with the glucose analyzer.

Sample Preparation:

In all of these assays the enzyme must be diluted to produce a net sugar concentration of about 0.5 gm/L or less. Above 0.5 gm/L the assays are not linear (Fig 3.2.2-1). At concentrations much less than 0.5 gm/L the assays are less accurate because errors in activity and background measurements are much more significant. The most accurate way to determine enzyme activity is to assay the sample at several dilutions within the linear limit. The linear portion should extrapolate through the origin, and the slope will

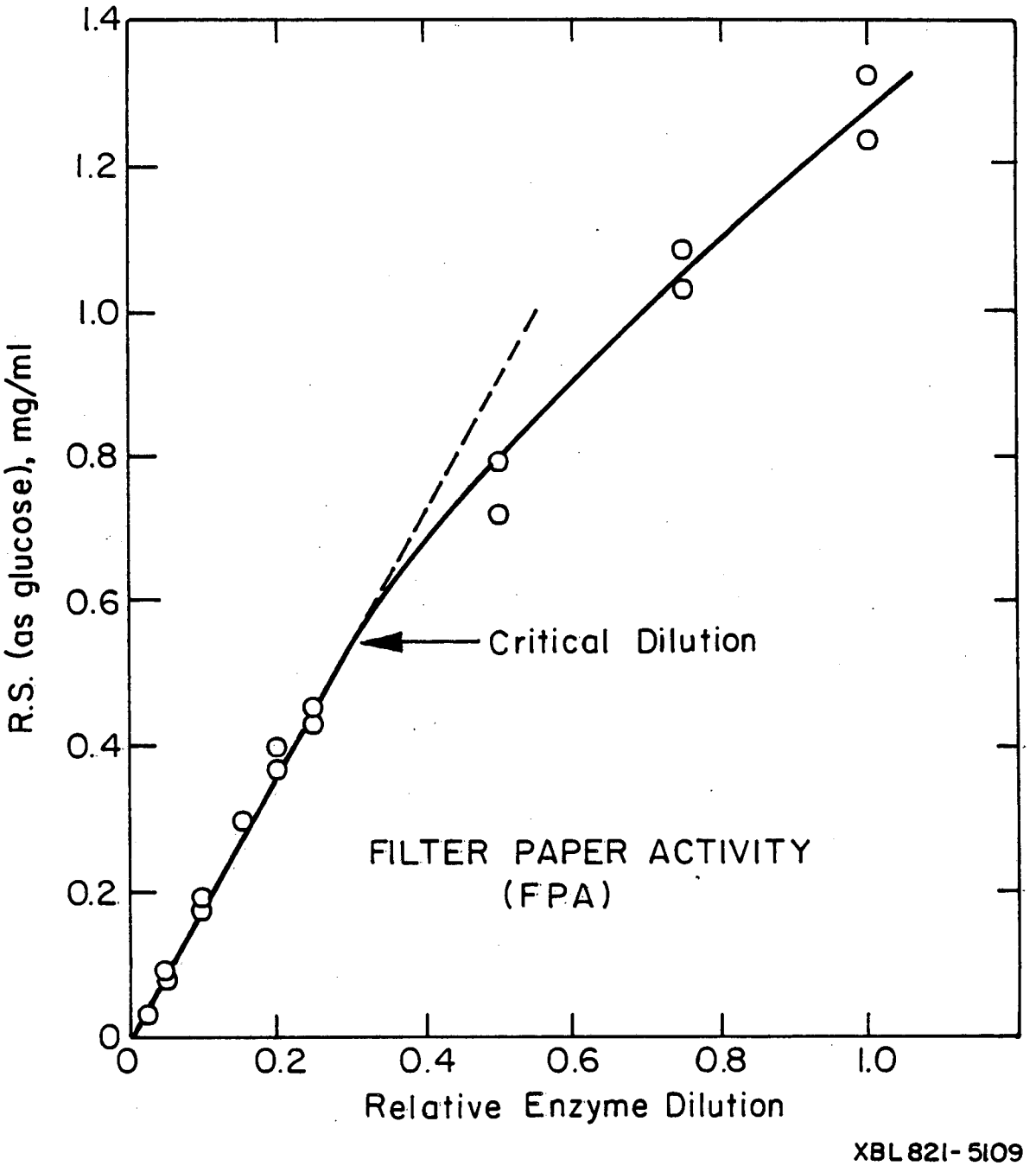

Figure 3.2.2-1 Dilution curve for filter paper activity assay. Reducing sugars as a function of relative dilution.

give an accurate determination of enzyme activity. This method is impractical, however, if a large number of samples must be assayed.

In some cases the amount of background sugars present in the enzyme sample is so large that the additional sugars produced by the enzyme assays will be insignificant. This makes it impossible to accurately determine the net activity. This is especially true with samples from an enzymatic hydrolysis. High background sugars can be separated from the enzyme by mixing one part enzyme with three parts acetone. Most of the enzyme will precipitate and most of the sugars will remain in solution. The mixture is then centrifuged at 10,000 rpm for ten minutes and the supernatant carefully decanted. Any remaining acetone is removed by applying vacuum to the tubes for 2-3 minutes. Acetone must be removed as completely as possible because it interferes with the enzyme assays. The precipitated enzyme is resuspended in acetate buffer. Recovery of enzyme is not complete, and varies with concentration. Standard curves relating activity precipitated to activity recovered must be constructed for each enzyme assay used. The standard curves should not be considered universal, they apply only to the enzyme stock with which they were prepared. It may be necessary to dilute the sample prior to acetone precipitation to improve separation of enzyme from background sugars, but the accuracy of the standard curve for acetone precipitation at low enzyme activities must be considered.

IV. EXPERIMENTAL PROCEDURES

4.1 Adsorption

4.1.1 Rate of Adsorption

The effects of temperature and enzyme loading on the rate of cellulose adsorption were studied. Adsorption onto a 5 wt% (dry basis) suspension of steam exploded corn stover with 25 filter paper units per gram of solid (FPU/gm) was measured at 15, 25, 35, and 45 ^{o}C. Adsorption with enzyme loadings of 5, 15, 25, 50, and 150 FPU/gm was studied at 15 ^{o}C. Adsorption of enzyme was monitored by intermittent sampling during the first 45 minutes of adsorption.

Steam exploded corn stover was added to a 600 ml Berzelius beaker which was equipped with a marine impeller and tight fitting rubber stopper. If needed, 0.025 M acetate buffer was also added. The beaker was immersed in a water bath of the desired temperature. The solids were stirred at 70 rpm and allowed to reach bath temperature. Enzyme, equilibrated to bath temperature in a separate vessel, was added to the solids. The total mass was 150 gm. Representative samples, containing both solid and liquid, were withdrawn with an inverted pipette. Samples were transferred to a centrifuge tube immersed in ice and were centrifuged as soon as possible in a refrigerated centrifuge. Enzyme in the supernate was precipitated with acetone (refer to

section 3.2.2) to separate it from high background sugars that are released by steam exploded corn stover. Precipitated enzyme was resuspended in acetate buffer and analyzed for activity.

Adsorption over shorter time intervals (less than 5 minutes) was studied by an alternate method. Enzyme/substrate solution (50 gm total) was stirred in a 150 ml fritted glass funnel (medium frit) immersed in a water bath. A vacuum tube was tightly fitted to the bottom of the funnel. At the desired time vacuum was applied to the system, allowing quick separation of enzyme from substrate. This method, however, altered reaction conditions with respect to solids concentration; only one representative sample could be obtained for each experiment.

4.1.2 Adsorption Isotherms

Enzyme activity remaining in solution after 45 minutes of contacting with 5 and 10 wt% suspensions of steam exploded corn stover at 25, 35 and 45 ^{o}C was measured. Initial enzyme loadings ranged from 5 to 150 FPU/gm solid. Samples were assayed for filter paper, C_1, C_x, and cellobiase activity. Enzyme activity adsorbed was calculated by subtracting activity in solution from activity in control samples at similar conditions, but without solids. Similar experiments were done with Wiley milled corn stover and acid treated corn stover; samples were analyzed for filter paper

activity only. All results were fitted to a Langmuir adsorption isotherm.

For these adsorption studies, the solids were placed in 250 ml shake flasks fitted with steel caps. Acetate buffer was added if necessary. The flasks were placed in a rotary shake bath of the proper temperature and swirled at 200 rpm. Enzyme, at the same temperature, was added to the solids. Total mass was 100 gm. Samples were withdrawn after 45 minutes and analyzed for enzyme activity (refer to section 4.1.1).

4.1.3 Reversibility of Adsorption

The reversibility of enzyme adsorption onto steam exploded corn stover at 25 ^{o}C was investigated with respect to filter paper activity and cellobiase activity. After 45 minutes of contacting, the enzyme solution was removed and the solids resuspended in buffer. Activity in solution, due to desorption of enzyme, was monitored for 24 hours. Reducing sugars and glucose were also measured.

A 5 wt% suspension of steam exploded corn stover was mixed with 25 FPU/gm of enzyme (both equilibrated to 25 ^{o}C) in a 600 ml fritted glass funnel (medium frit). Total mass was 200 gm. A 6-blade turbine impeller stirring at 40 rpm provided good mixing with minimal splashing. A tight fitting rubber stopper was fitted to the top of the funnel, a plug at the bottom prevented leakage through the glass frit.

After 45 minutes the vessel was removed from the water bath and the solids filtered to approximately 27% moisture content. Filtrate was analyzed for enzyme activity, reducing sugars, and glucose. Acetate buffer, equal to the volume of the filtrate, was added to the solids. The contents of the vessel were stirred at 25 ^{o}C for the next 24 hours. Duplicate samples, one for enzyme activity measurements and one for sugar analysis, were withdrawn intermittently. Samples for enzyme analysis were prepared and analyzed as described in section 4.1.1. Samples for sugar analysis were boiled for 5 minutes, then centrifuged. The supernatant was analyzed for reducing sugars and glucose.

4.2 Hydrolysis

Steam exploded corn stover was hydrolyzed for 48 hours at 45 ^{o}C by cellulase enzyme. Solids concentrations of 5 to 25 wt% and enzyme loadings of 5 to 50 FPU/gm were used. Production of reducing sugars and glucose were measured. Cellobiose concentration at 24 hours was also measured.

Solids were placed in a 600 ml Berzelius beaker which was equipped with a marine impeller and a tight fitting rubber stopper. Acetate buffer (0.025 M) was added if needed. Solids and enzyme were equilibrated separately to 45 ^{o}C in a water bath. The cellulase enzyme, after several months storage at 4 ^{o}C, was deficient in cellobiase activity; it was supplemented with freeze-dried β -

glucosidase to give a ratio of 1.1 cellobiase units per filter paper unit. Enzyme was added to the cellulose solids and the mixture stirred at 70 rpm. Total mass was 150 gm. For solids concentrations greater than 10 wt% the hydrolysis was started with a 10 wt% suspension. The additional solids required were added during the next 2-3 hours as the viscosity decreased.

Representative samples were withdrawn with an inverted pipette to avoid altering the reaction conditions. Samples were boiled for 5 minutes to stop hydrolysis and to denature the enzyme, then centrifuged. The supernatant was analyzed for sugars.

In addition, the enzyme activity in solution was monitored during hydrolysis of 10 wt% steam exploded corn stover with an enzyme loading of 25 FPU/gm. Samples for enzyme analysis were prepared as described in section 4.1.1 and were analyzed for filter paper, C_1, C_x, and cellobiase activities.

4.3 Hydrolysis with Countercurrent Adsorption

Batch hydrolysis of steam exploded corn stover at 45 ^{o}C with an enzyme loading of 25 FPU/gm was operated in parallel with two stages of countercurrent adsorption. The residence time of the hydrolysis stage was 24 hours. Adsorption stages were operated at 25 ^{o}C, each with a residence time of 45 minutes. The amount of fresh solids added to the final

adsorption stage corresponded to the amount needed for a 10 wt% suspension in the hydrolysis stage. Make-up enzyme requirements were based on adsorption of filter paper activity, predicted by the Langmuir isotherm. Samples from the final adsorption stage were analyzed for reducing sugars, glucose, filter paper activity, and cellobiase activity.

The effect of enzyme recovery on the hydrolysis profile was observed by taking representative samples for sugar and enzyme analysis from the hydrolysis stage during the eighth cycle of operation. The effectiveness of each adsorption stage was observed after the eighth hydrolysis was completed.

Steam exploded corn stover was placed in a 600 ml fritted glass funnel (medium frit) which was equipped with a 6-blade turbine impeller, a tight fitting rubber stopper, and a plug at the bottom which prevented leakage. Acetate buffer was added and the mixture stirred at 30 rpm in a 45 ^{o}C water bath. Enzyme was added to give a total mass of 300 gm with a 10 wt% suspension of steam exploded corn stover and an enzyme loading of 25 FPU/gm. The solids were hydrolyzed for 24 hours, at which time the contents were filtered to approximately 35 % moisture content. Solids were discarded. The filtrate was mixed with fresh steam exploded corn stover for 45 minutes at 25 ^{o}C (first adsorption stage), this mixture was filtered to approximately 25 %

moisture content. The solid cake was supplemented with the predicted requirement of make-up enzyme, brought up to a 10 wt% suspension with acetate buffer, and hydrolyzed at 45 ^{o}C for 24 hours. The filtrate was stored overnight at 4C. The next day, before completing the second hydrolysis, the filtrate was contacted with fresh steam exploded corn stover for 45 minutes at 25 ^{o}C (final adsorption stage), this mixture was filtered to approximately 25 % moisture content. The solids were contacted with filtrate from the second hydrolysis. Filtrate from the final adsorption stage was analyzed for sugars and enzyme activity as described in section 4.2. This operation was repeated for eight cycles. After the first cycle the solids in the first adsorption stage were solids which had been contacted with enzyme/sugar solution in the final adsorption stage. In the eighth cycle the adsorption stages were scaled down to reflect losses due to sampling in previous stages.

V. RESULTS AND DISCUSSION

5.1 Adsorption

In developing a scheme for cellulase enzyme recovery by countercurrent adsorption the factors that affect the rate and extent of adsorption need to be understood. The relative adsorption of the different cellulase enzymes must also be known. The effects of temperature, enzyme loading, and pretreatment on the adsorption of cellulase enzymes were studied. The reversibility of enzyme adsorption was also investigated.

5.1.1 Rate of Adsorption

The relative adsorption of filter paper, C_1, C_x, and cellobiase activity onto steam exploded corn stover at 15 and 45 ^{o}C is shown in figures 5.1.1-1 and 5.1.1-2. An initial enzyme loading of 25 filter paper units per gram of solid (FPU/gm) was used with a 5 wt% (dry basis) suspension of solids.

The initial adsorption of enzyme is rapid at either temperature; much of the adsorption occurs within two minutes. The trend in the extent of adsorption reflects the solubility of the substrate with which activity is measured. Activity towards crystalline material, measured by the C_1 assay, is adsorbed to the greatest extent, leaving 13-17 % of the original activity in solution after 45 minutes.

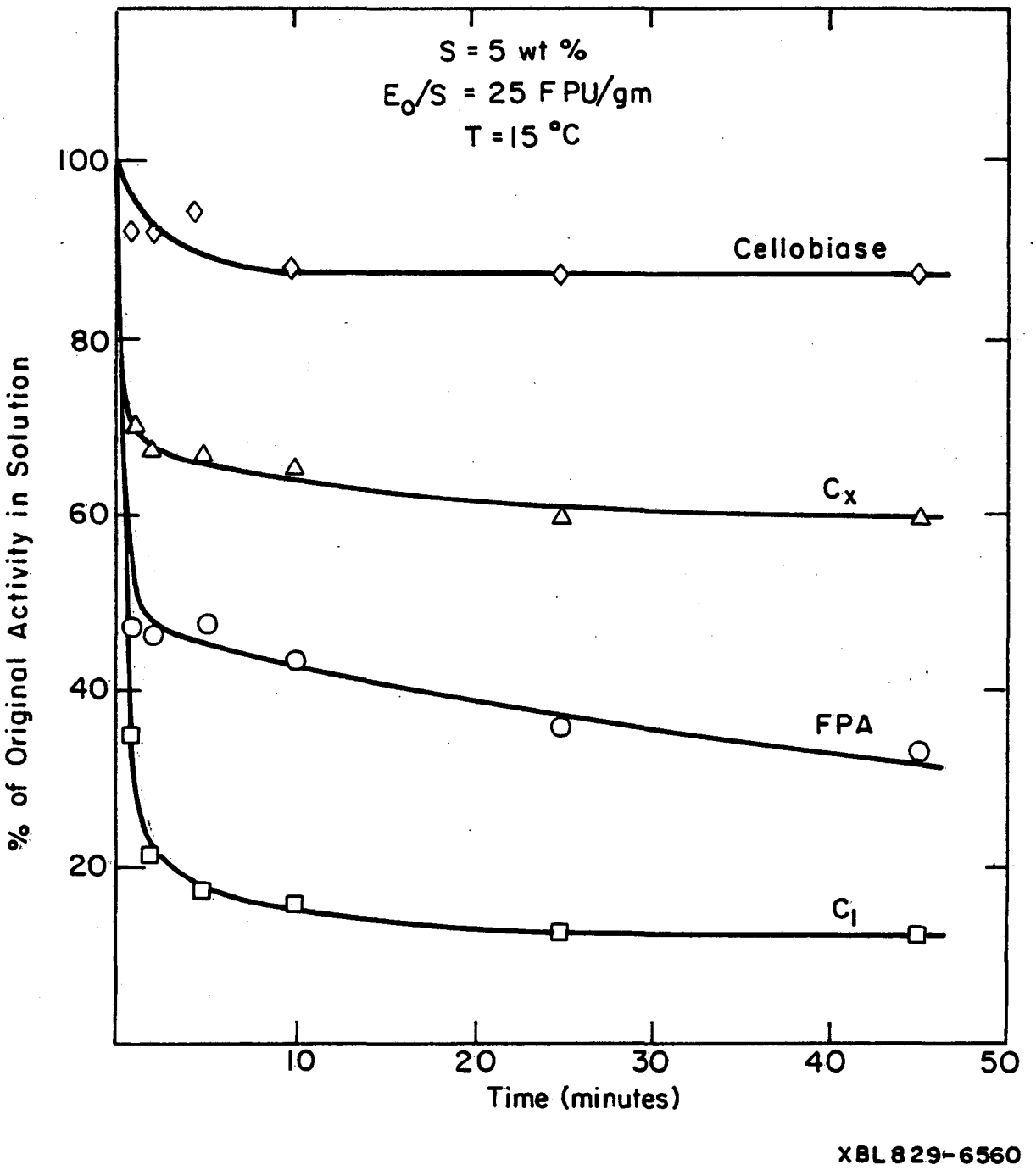

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Figure 5.1.1-1 Adsorption of cellulase enzyme activities onto 5 wt% steam exploded corn stover at 15 °C and E_o/S = 25 FPU/gm.

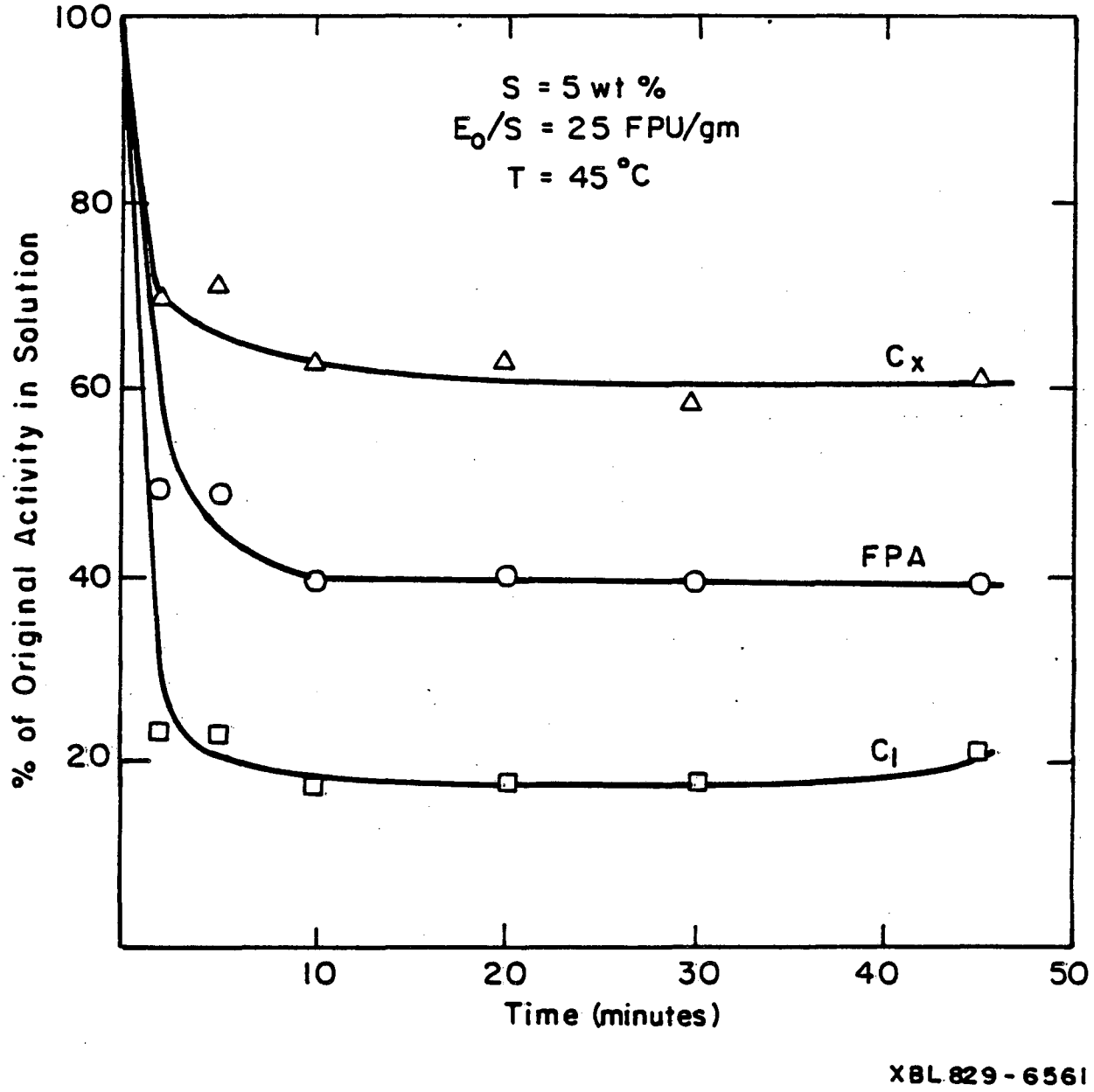

Figure 5.1.1-2 Adsorption of cellulase enzyme activities onto 5 wt% steam exploded corn stover at 45 °C and E_o/S = 25 FPU/gm.

Activity towards cellobiose, the most soluble substrate, is adsorbed the least; at 15 ^{o}C, 87 % of the original activity remains in solution after 45 minutes, no adsorption was observed at 45 ^{o}C. Adsorption of filter paper activity leaves 33-40 % of the original activity in solution. Adsorption of C_x (amorphous) activity leaves 60 % of the original activity in solution.

Preferential adsorption of C_1 activity, which was also observed in studies with newspaper (14,15,65), can be explained by considering a simplified mechanism of enzymatic hydrolysis; crystalline material is first broken down into less ordered amorphous material which is hydrolyzed to produce glucose.

The initial fast rate of adsorption at 15 and 45 ^{o}C could not be accurately measured. The experimental procedures used did not separate the liquid and solid phases quickly enough to measure the activity in solution at times less than 2 minutes. Ghose and Bisaria (27) were able to determine the initial rate of adsorption at 5 ^{o}C, using delignified bagasse as a substrate. For the purposes of enzyme recovery, however, quantitative measurement of the initial rate of adsorption is not important.

The effect of temperature on adsorption of enzyme after the fast initial adsorption could be determined. At lower temperatures further uptake of enzyme is slower, but the extent of adsorption at 45 minutes is greater. This effect

is most significant for adsorption of filter paper activity.

The initial adsorption of enzyme is faster and possibly greater at higher temperatures because adsorption of enzyme onto cellulose is a chemical reaction which requires a minimum activation energy. Further uptake of enzyme is greater at lower temperatures because binding of enzyme is an exothermic reaction.

The effect of enzyme loading on adsorption of filter paper activity at 15 ^{o}C is shown in figure 5.1.1-3. At an enzyme loading of 5 FPU/gm no additional adsorption of enzyme is observed between 5 and 45 minutes. At higher enzyme loadings adsorption of enzyme between 5 and 45 minutes is more significant, but the extent of adsorption is lower. Although a smaller percentage of the original enzyme is adsorbed at higher enzyme loadings, the amount of activity adsorbed is greater. The results suggest that contacting times greater than 10 minutes would significantly increase recovery of enzyme at loadings greater than 15 FPU/gm.

The combined effects of enzyme loading and temperature on the adsorption of filter paper activity are shown in figure 5.1.1-4. The rate of adsorption within the first 5 minutes could not be accurately determined. Regardless of enzyme loading, adsorption of enzyme after 5 minutes is slower at the lower temperature. The extent of adsorption at 45 minutes, however, is greater at lower temperatures.

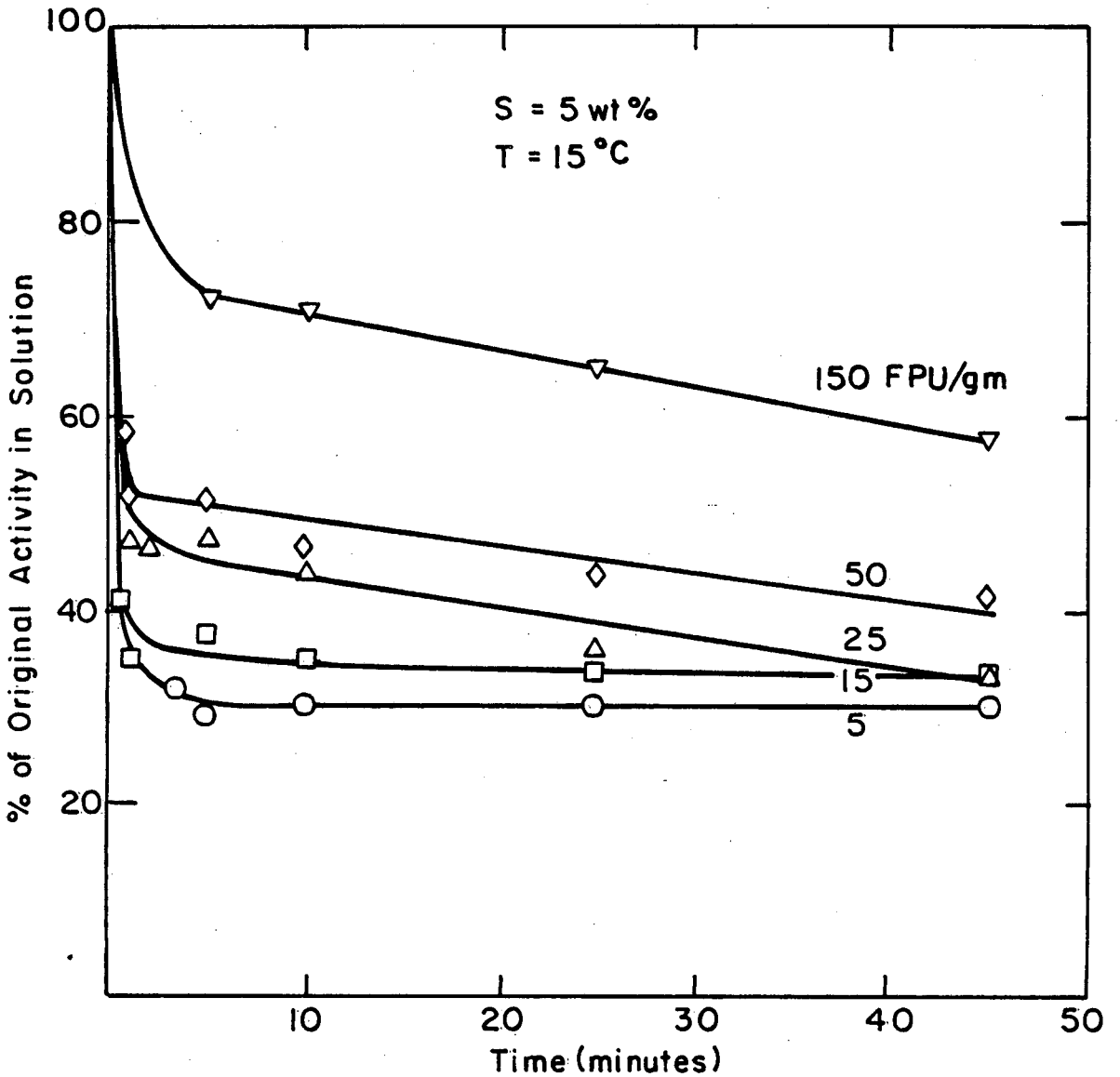

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Figure 5.1.1-3 Adsorption of filter paper activity onto 5 wt% steam exploded corn stover at 15°C and various enzyme loadings.

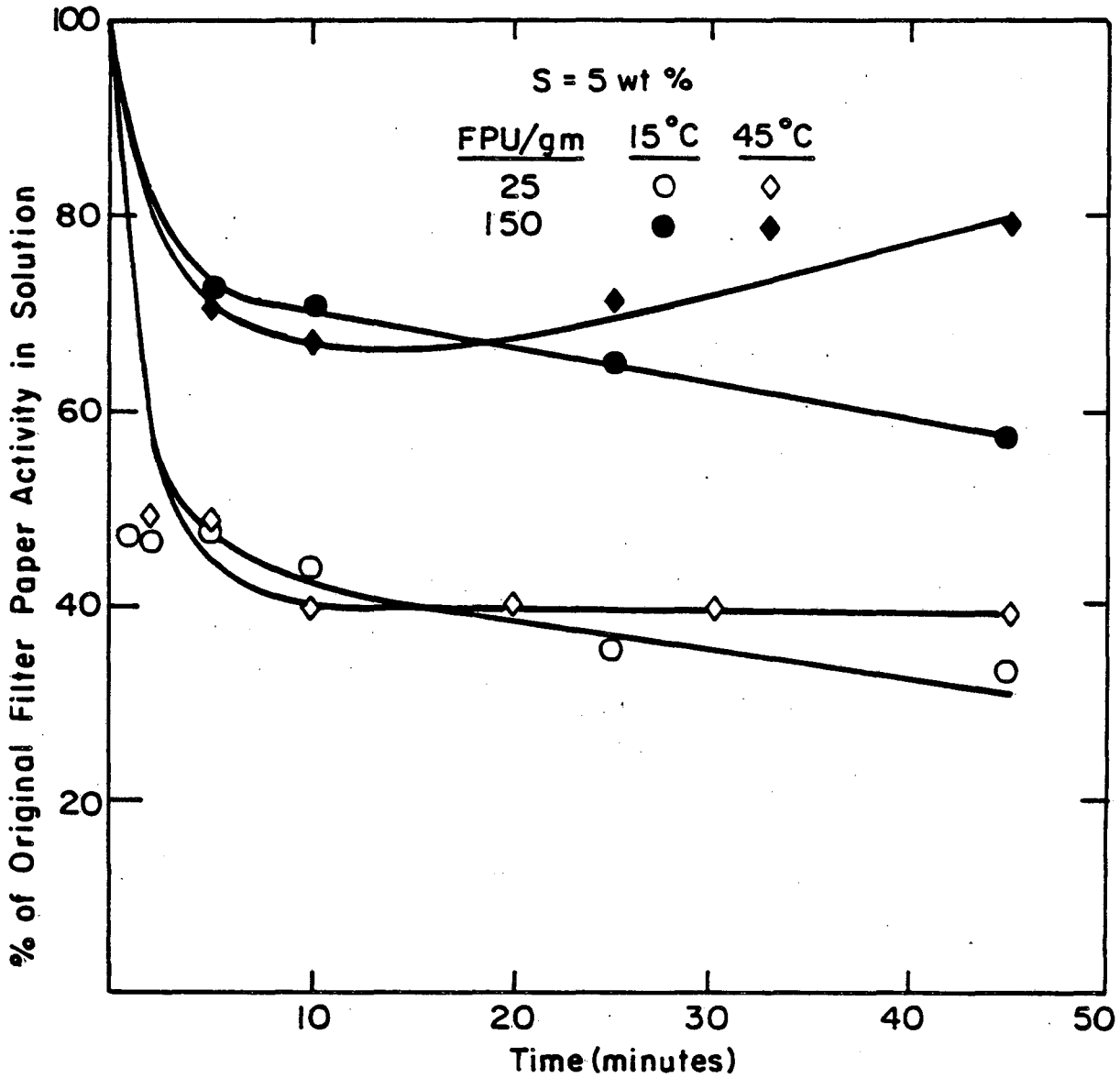

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Figure 5.1.1-4 Combined effects of temperature and enzyme loading on the adsorption of filter paper activity.

The combination of high enzyme loading (150 FPU/gm) and optimum hydrolysis temperature (45 ^{o}C) has an interesting effect on the adsorption behavior. After 10 minutes enzyme is slowly released back into solution. This is probably because these conditions promote rapid hydrolysis of cellulose. Similar results would be observed at lower enzyme loadings and temperatures if the experiments were conducted for a longer time.

5.1.2 Adsorption Isotherms

Design of a countercurrent adsorption scheme for enzyme recovery requires that the distribution of enzyme between the liquid phase and solid phase at various conditions be known. If the distribution is not known then the make-up enzyme requirement cannot be predicted.

The adsorption of cellulase enzyme onto steam exploded corn stover was modelled with a Langmuir isotherm (50). Based on observations made on the rate of adsorption, it was assumed that equilibrium was achieved after 45 minutes of contacting. Further adsorption of enzyme is significant only when low temperatures and high enzyme loadings are used. Peitersen (71) had also modelled cellulase adsorption with a Langmuir isotherm, using contacting times of one hour. The assumptions of the Langmuir isotherm are discussed in section 2.2.5.

The Langmuir isotherm can be rearranged to the form:

$$\frac{1}{E_{ads}} = \frac{1}{K \cdot E_{ads,max}} \frac{1}{E} + \frac{1}{E_{ads,max}}$$

A plot of $1/E_{ads}$ against $1/E$ should give a straight line with slope $1/(K \cdot E_{ads,max})$ and intercept $1/(E_{ads,max})$. The value of E_{ads} cannot be directly measured, but can be calculated if the original amount of enzyme added and the solids concentration are known:

$$E_{ads} = (E_o - E) \cdot (1-w)/w$$

where:

E_o = enzyme activity in solution before adsorption, IU/ml

w = weight fraction of solids.

Thus, when $1/E_{ads}$ is plotted against $1/E$ the measured variable E appears in both terms. It is best to isolate the measured variable to one of the two terms, but this could not be done. The accuracy of E_{ads} is affected not only by the accuracy of E but also by the extent of adsorption. The calculated value of E_{ads} is most accurate when the extent of adsorption is great. This is because errors in E are less significant when the difference between E and E_o is large.

Adsorption of filter paper, C_1, C_x, and cellobiase activity onto steam exploded corn stover after 45 minutes of contacting was fitted to the Langmuir isotherm by the method described. Figure 5.1.2-1 shows the double reciprocal plot used to determine the constants for adsorption of filter paper activity at 25 ^{o}C. Points farther from the origin were not considered because the accuracy of the standard curve for acetone precipitation is poor at low enzyme activity. Points very close to the origin are not very accurate for two reasons; they represent a small extent of adsorption, and they might not represent equilibrium. A similar plot for adsorption of filter paper activity at 45 ^{o}C has much more scatter (Figure 5.1.2-2). This is because the extent of adsorption is lower at higher temperatures. All plots for adsorption of cellobiase activity had a great amount of scatter. The cellobiase component does not adsorb to a great extent, if at all.

The values of K and $E_{ads,max}$ were determined for adsorption of the four activities at 25, 35, and 45 ^{o}C (Table 5.1.2-1) and were used to construct the adsorption isotherms shown in figures 5.1.2-3,4,5,6. The isotherms for adsorption of filter paper activity show a consistent increase throughout the curves; as temperature increases $E_{ads,max}$ decreases and K increases. The trends shown by the isotherms for C_1 activity suggest that these isotherms might not be as accurate. One would expect that these should be

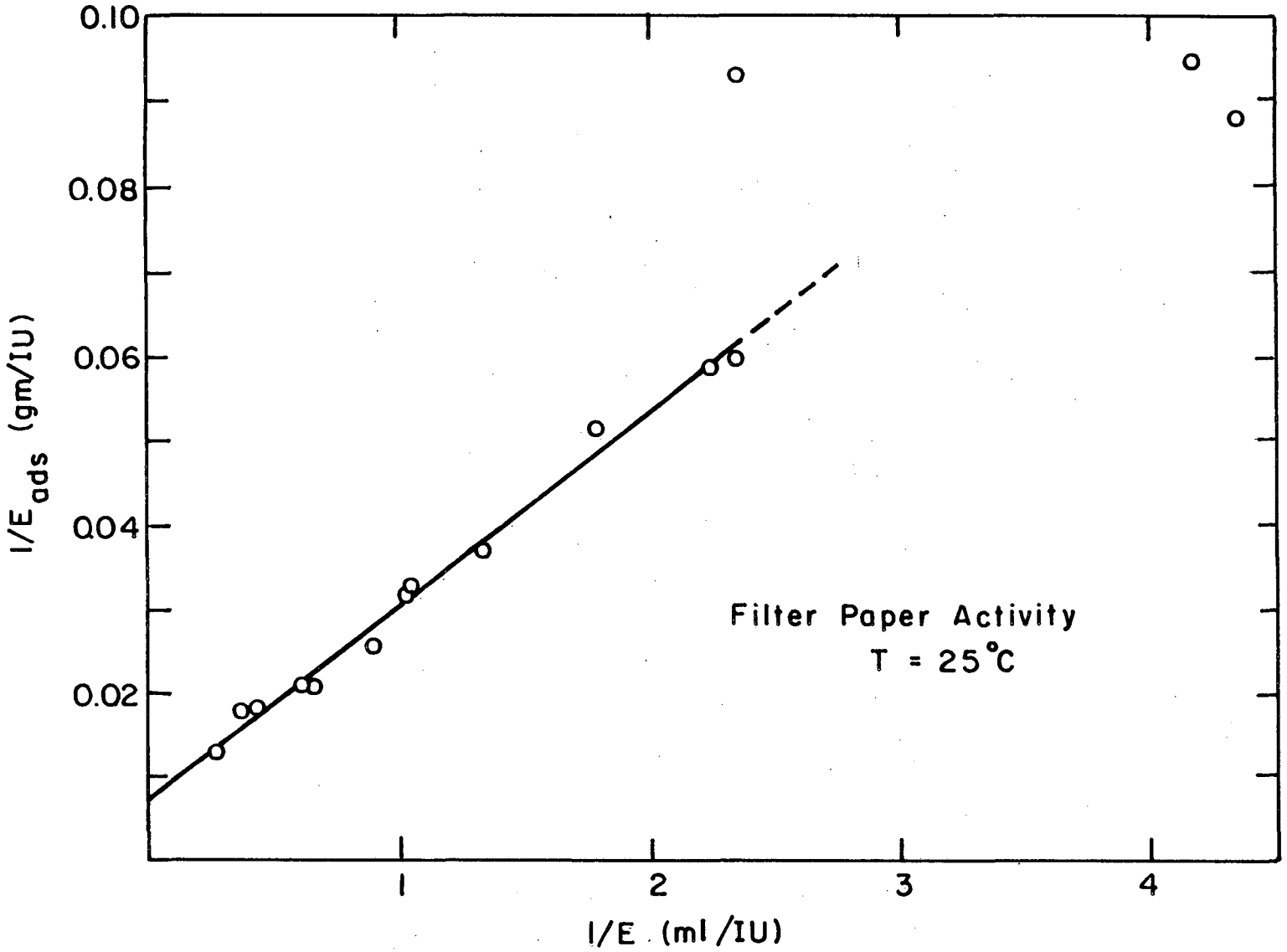

Figure 5.1.2-1 Double reciprocal plot used to evaluate Langmuir constants.

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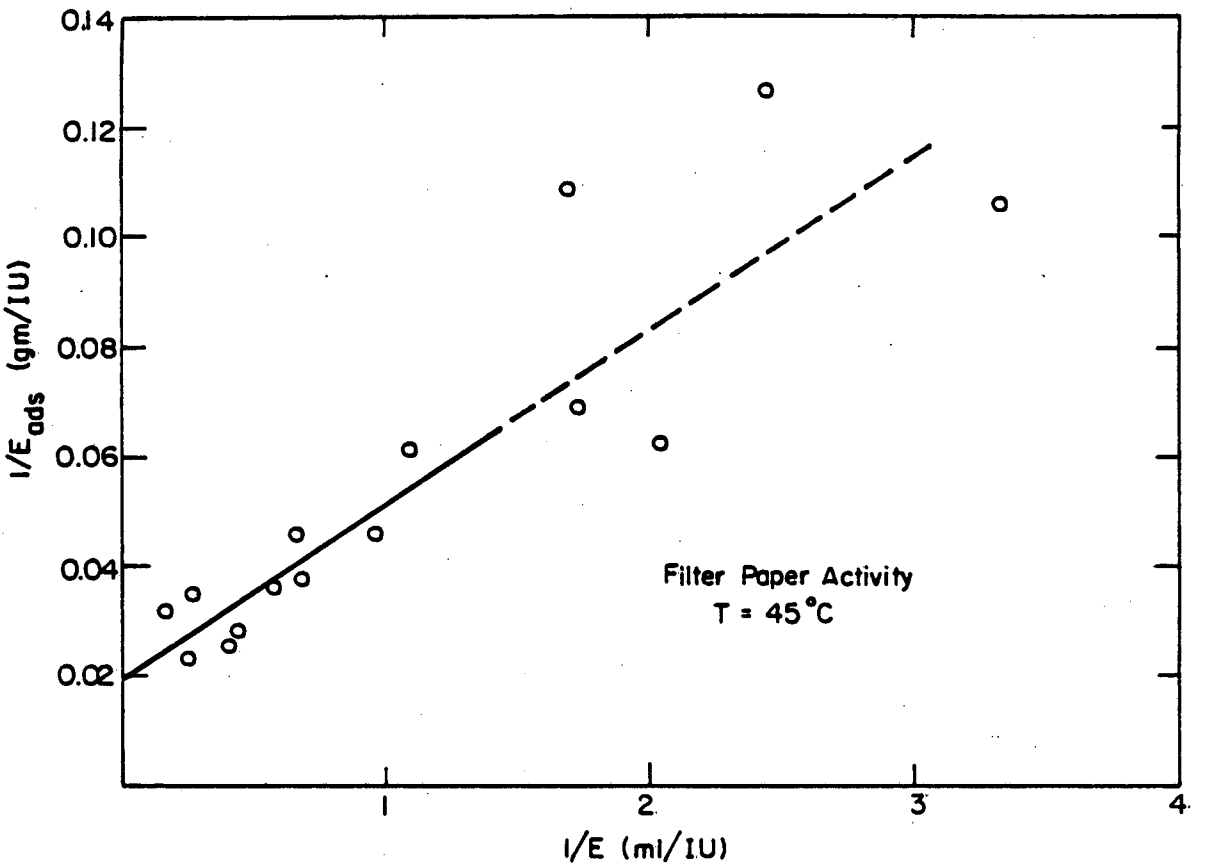

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Figure 5.1.2-2 Double reciprocal plot used to evaluate Langmuir constants.

Table 5.1.2-1

Langmuir Constants for Adsorption of Cellulase Enzymes onto Steam Exploded Corn Stover

Filter Paper Activity

T, °C	$E_{ads,max}$, IU/gm	K, ml/IU
25	133	0.329
35	89.8	0.406
45	50.5	0.615

Activity Towards Cotton

T, °C	$E_{ads,max}$, IU/gm	K, ml/IU
25	3.97	32.4
35	3.33	42.0
45	3.10	23.5

Activity Towards Carboxymethylcellulose

T, °C	$E_{ads,max}$ IU/gm	K, ml/IU
25	1388	0.0148
35	1170	0.0182
45	480	0.0470

Cellobiase Activity

T, °C	$E_{ads,max}$ IU/gm	K, ml/IU
25	84.3	0.102
35	66.2	0.806
45	5.43	0.962

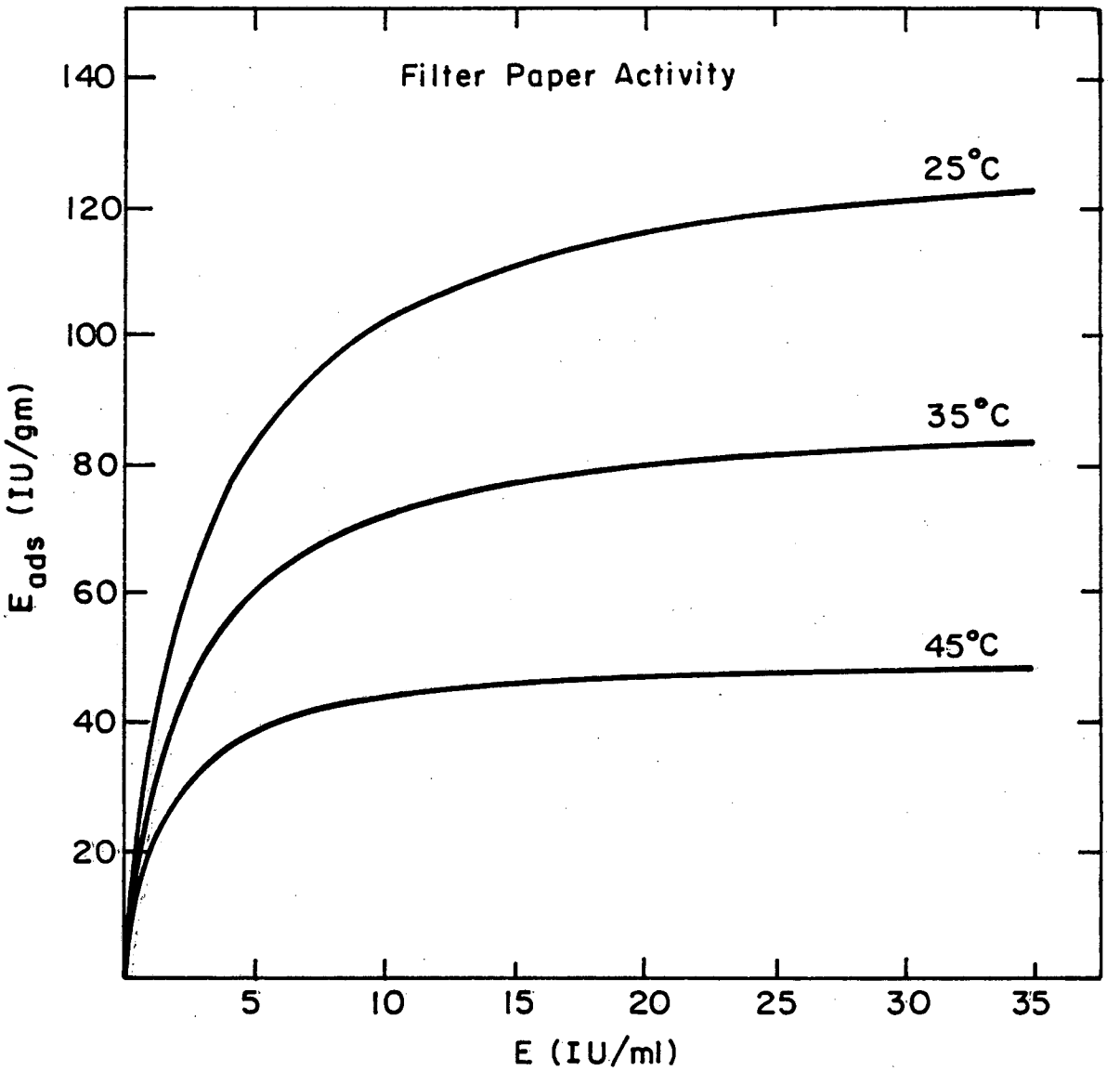

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Figure 5.1.2-3 Langmuir isotherms for adsorption of filter paper activity onto steam exploded corn stover.

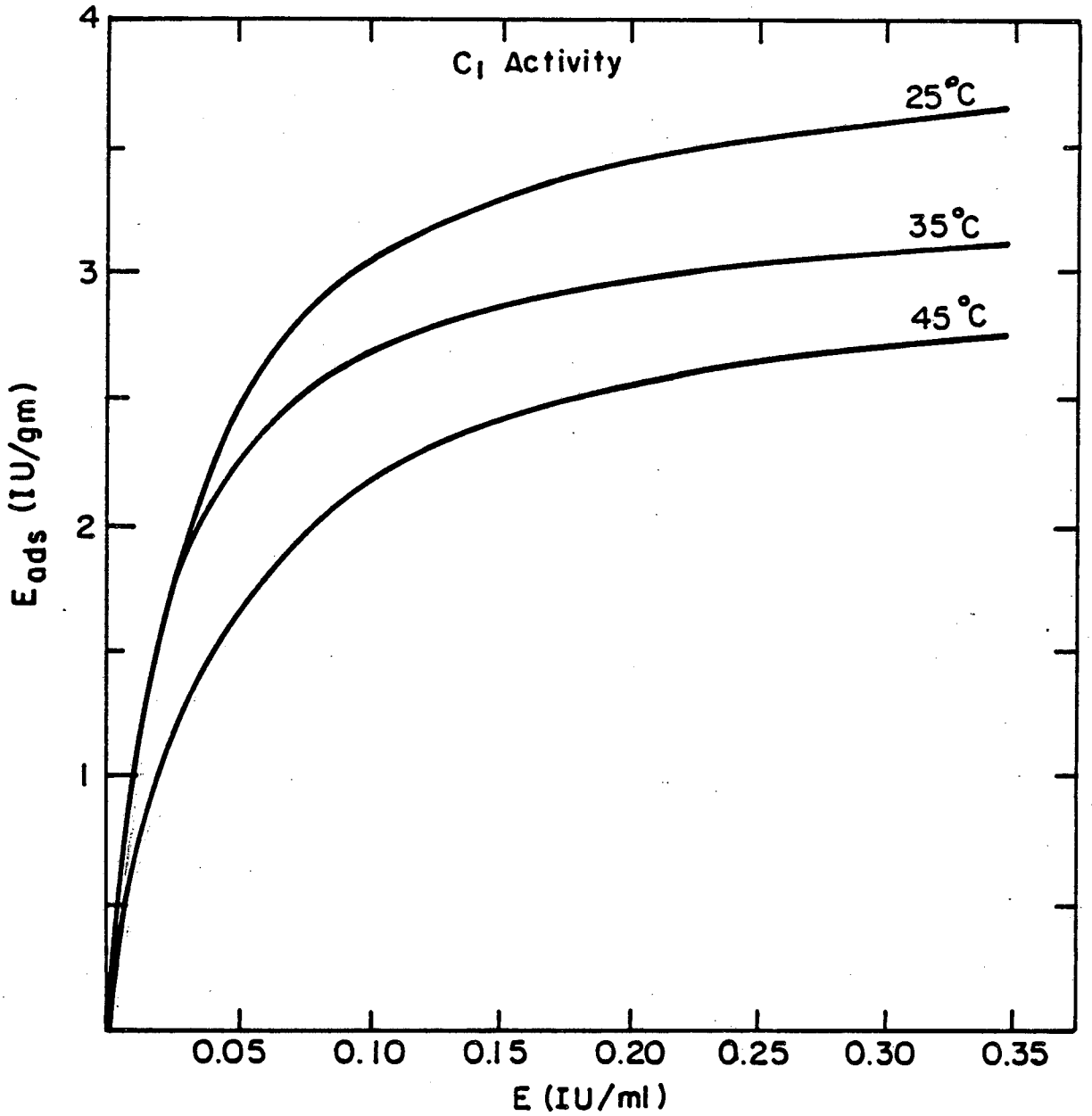

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Figure 5.1.2-4 Langmuir isotherms for adsorption of C_1 activity onto steam exploded corn stover.

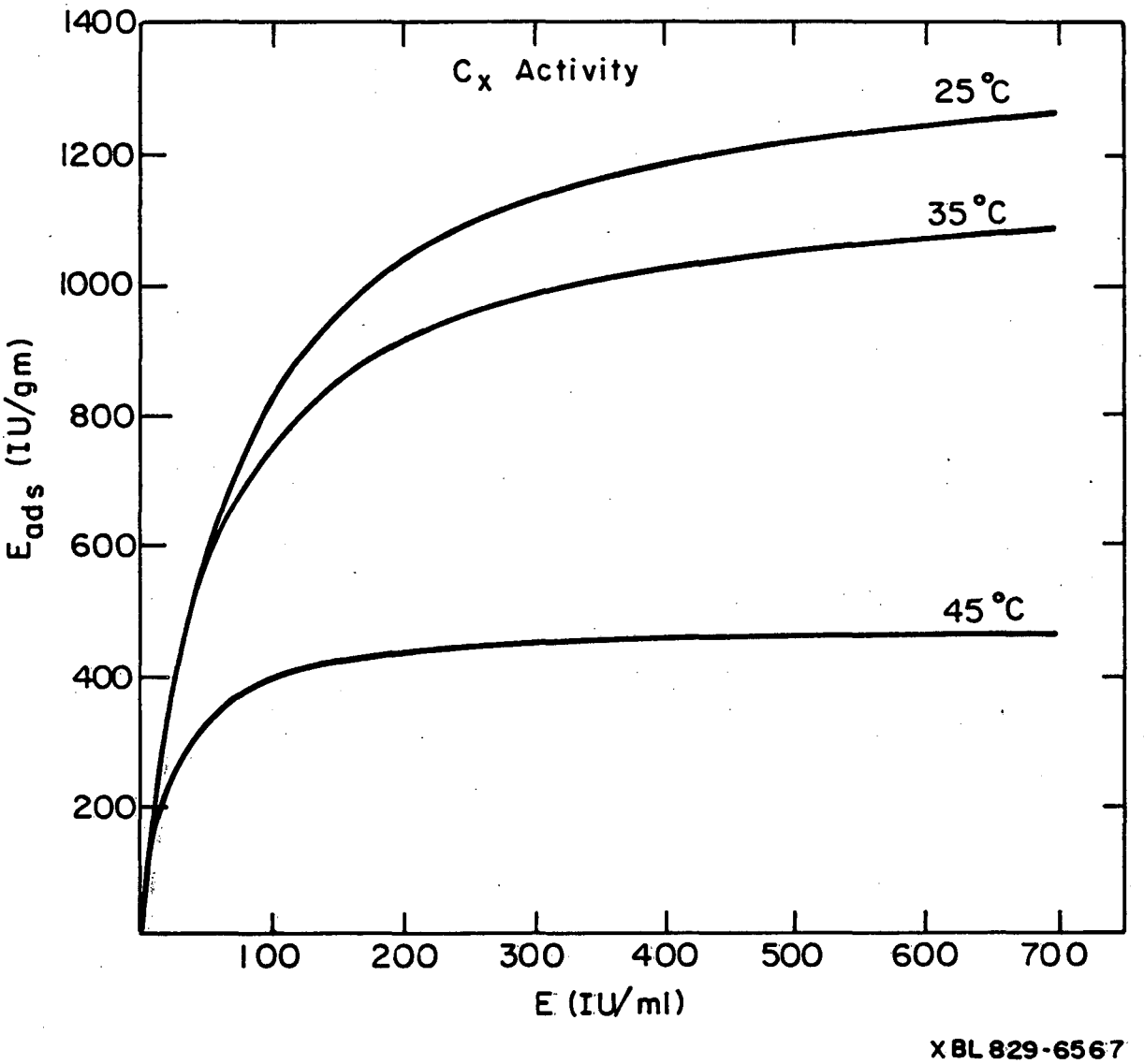

Figure 5.1.2-5 Langmuir isotherms for adsorption of C_x activity onto steam exploded corn stover.

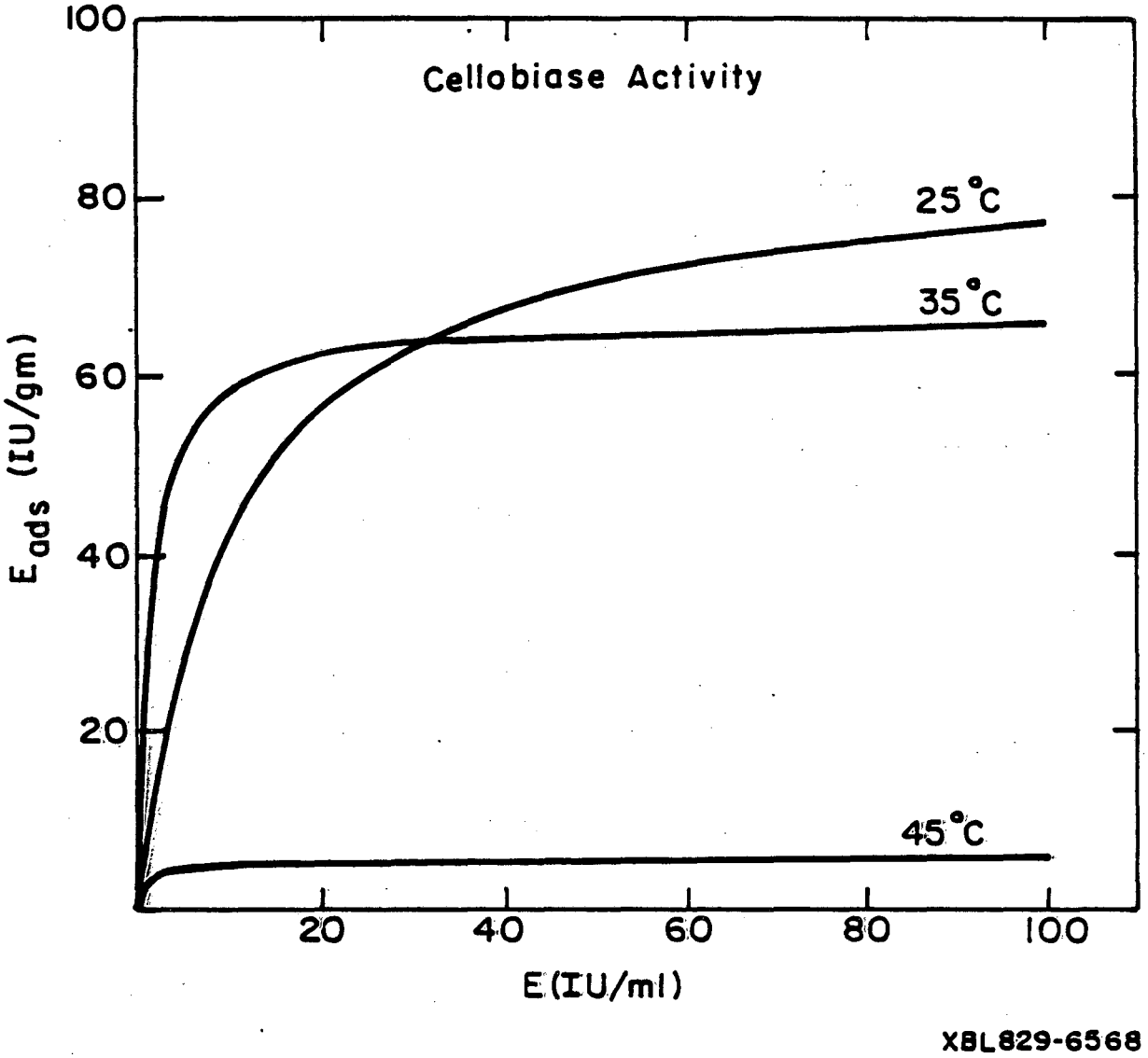

Figure 5.1.2-6 Langmuir isotherms for adsorption of cellobiase activity onto steam exploded corn stover.

more accurate because adsorption of C_1 activity is greater than that of filter paper activity. Measurement of C_1 activity, however, was less accurate.

The isotherms for adsorption of C_x activity show an even less consistent trend. This cannot be explained by inaccuracies in measuring C_x activity, the C_x assay is much more more accurate than the filter paper and C_1 assays. The extent of adsorption of C_x activity is less, which would account for greater inaccuracies in evaluating K and $E_{ads,max}$. The primary effect is probably denaturation. No significant denaturation of filter paper and C_1 activities was observed at 45 ^{o}C. Denaturation of C_x activity at 45 ^{o}C was significant. The value of E_o was corrected for denaturation, thus the calculated value of E_{ads} was decreased, resulting in a lower value for $E_{ads,max}$.

The isotherms for adsorption of cellobiase activity are not consistent, perhaps because adsorption of cellobiase activity is weak, making it difficult to determine E_{ads}. This enzyme is also significantly denatured at 45 ^{o}C. The plots used to determine K and $E_{ads,max}$ for cellobiase adsorption show a great amount of scatter, the poor trend of the isotherms is not unexpected.

The temperature dependence of K and $E_{ads,max}$ for adsorption of filter paper activity was fitted to an Arrhenius type plot (Figure 5.1.2-7). If the product of K and $E_{ads,max}$ is fitted to such a plot then a better fit is

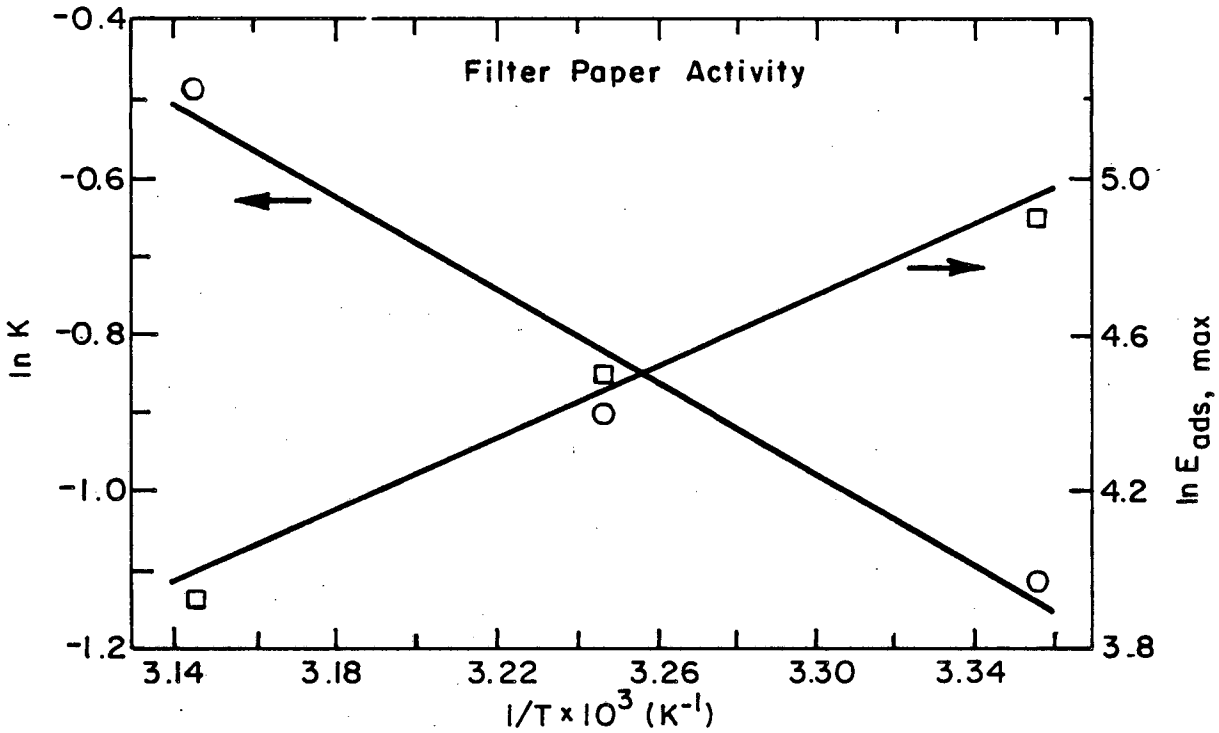

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Figure 5.1.2-7 Arrhenius type plot of Langmuir constants for adsorption of filter paper activity onto steam exploded corn stover.

obtained. This is because the method used to determine K and $E_{ads,max}$ gives an accurate slope and a less accurate intercept. The product of K and $E_{ads,max}$ is determined more accurately than the separate terms.

The effect of pretreatment on the adsorption of filter paper activity onto corn stover at 25 ^{o}C was observed by comparing the adsorption isotherms for steam exploded, acid treated, and Wiley milled corn stover. K and $E_{ads,max}$ were determined and used to plot the Langmuir isotherms. The results (Table 5.1.2-2 and Figure 5.1.2-8) show that steam explosion is the best pretreatment for increasing adsorption of filter paper activity. This is probably because steam explosion makes the cellulose highly accessible by increasing the surface area and rendering the hemicellulose hot water soluble. No significant adsorption onto Wiley milled corn stover was observed in the range tested.

5.1.3 Reversibility of Adsorption

The reversibility of cellulase adsorption is of interest for design of enzyme recovery schemes. Desorption of enzyme during hydrolysis will dictate the amount of enzyme available for recovery from solution. There have been conflicting reports on the release of enzyme back into solution during hydrolysis, these observations are discussed in section 2.2.4.

Table 5.1.2-2

Langmuir Constants for Adsorption
of Filter Paper Activity onto Corn Stover
($T = 25\ ^{o}C$)

Pretreatment	$E_{ads,max}$ IU/gm	K, ml/IU
Steam Exploded	133	0.329
Acid Treated	16.9	2.69

Note: No significant adsorption of filter paper activity onto Wiley milled corn stover was observed.

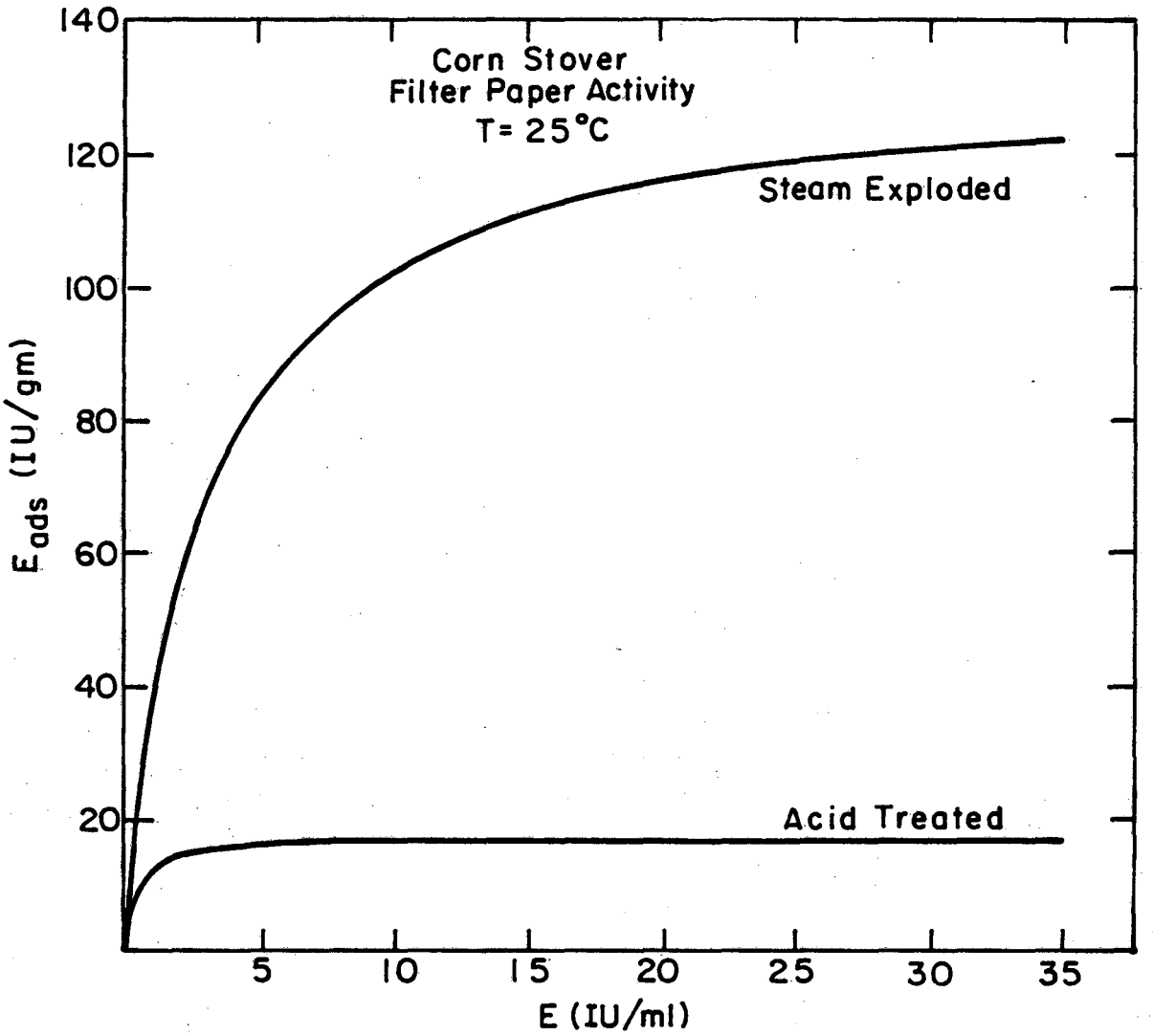

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Figure 5.1.2-8 Effect of pretreatment on the Langmuir isotherms for adsorption of filter paper activity onto corn stover at 25°C.

Cellulase enzyme was adsorbed onto steam exploded corn stover at 25 ^{o}C for 45 minutes. The mixture was filtered to 27 % moisture content and the liquid replaced with an equal amount of acetate buffer. The filter paper activity of the filtrate was within 2.7 % of that predicted by the Langmuir isotherm. Desorption of filter paper activity and cellobiase activity at 25 ^{o}C was monitored for the next 24 hours. Production of glucose and reducing sugars was also measured.

Initial desorption of filter paper activity was rapid within the first hour and was followed by a much slower, gradual release of enzyme (Figure 5.1.3-1). Desorption of cellobiase activity was also rapid in the first hour. No additional desorption of cellobiase activity was observed after 3 hours. After 16 hours there apparently had been some denaturation of cellobiase activity.

The Langmuir isotherm predicts that 28 % of the adsorbed enzyme would desorb. After 24 hours of desorption only 16 % of the adsorbed enzyme had desorbed.

The adsorbed enzyme was sufficient to digest the substrate, as shown by the continual production of glucose and reducing sugars. Similar observations were made by Mandels (55) and Wald (101).

It is concluded that the adsorption of cellulase enzyme onto steam exploded corn stover is not reversible. Yamanaka (113), using Wiley milled newspaper as the substrate, made

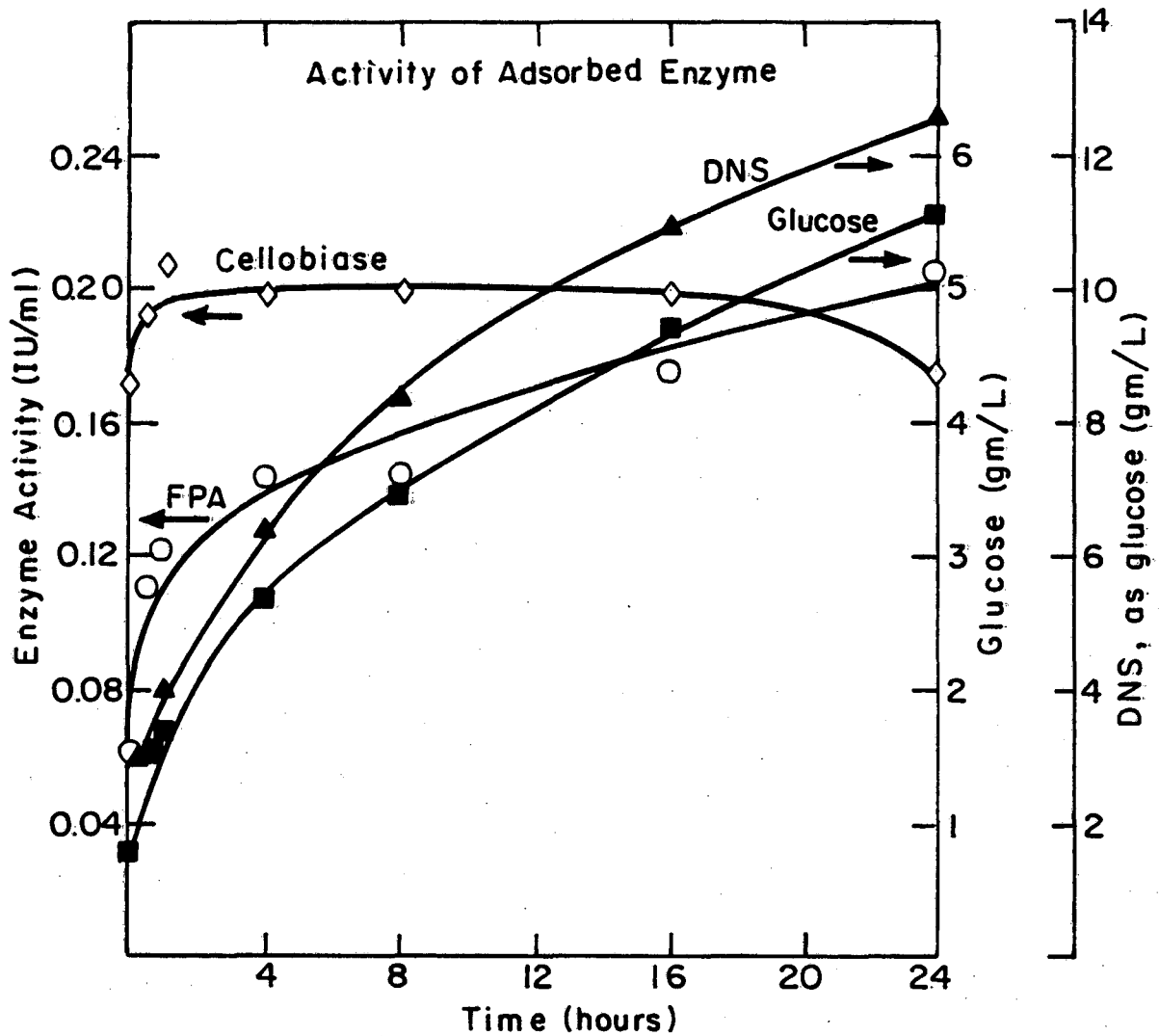

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Figure 5.1.3-1 Desorption of adsorbed cellulase enzymes and production of sugars at 25°C.

the same conclusion.

5.2 Hydrolysis

The effect of pretreatment on the adsorption of cellulase enzyme was shown by comparing the adsorption isotherms with steam exploded corn stover, acid treated corn stover, and Wiley milled corn stover as substrates. It is evident that steam explosion is the most effective in enhancing adsorption. If adsorption is enhanced then it is likely that hydrolysis will also be enhanced.

Steam exploded corn stover was hydrolyzed at various conditions to observe the effectiveness of steam explosion as a pretreatment and to study the effects of enzyme loading and solids concentration on the extent of conversion. The uptake and release of cellulase enzyme during hydrolysis was monitored to determine the amount of enzyme available for recovery after hydrolysis is completed.

Steam exploded corn stover was hydrolyzed at 5 to 25 wt% suspensions with enzyme loadings of 5 to 50 filter paper units per gram of solid (FPU/gm) for 48 hours at 45 ^{o}C. The glucose concentrations obtained after 24 hours are shown in figure 5.2-1. For solids concentrations of up to 15 wt% the optimum enzyme loading is 25-30 FPU/gm. At higher solids concentrations the production of glucose within 24 hours would benefit from even higher enzyme loadings. The strength of enzyme required for enzyme loadings greater than

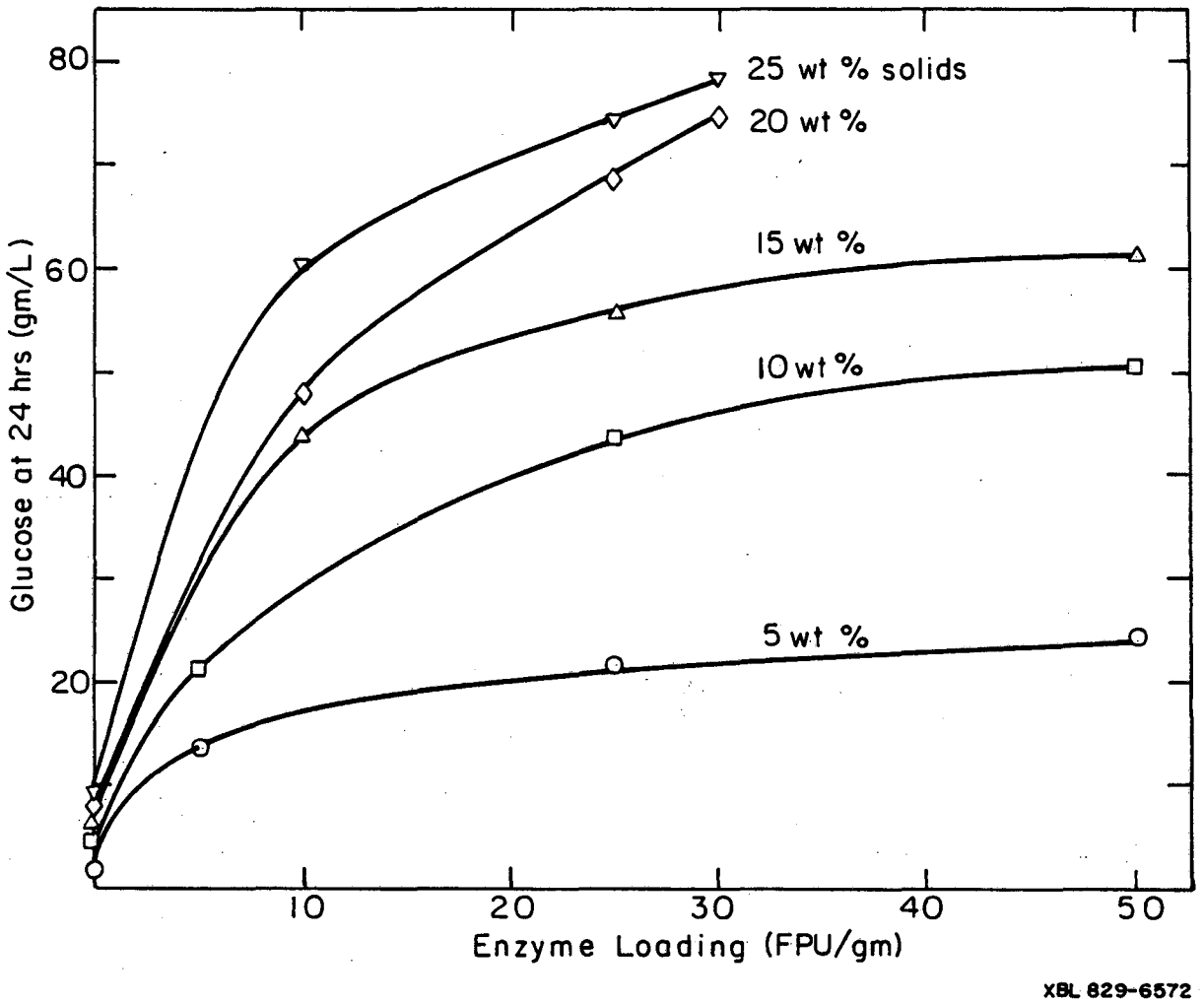

Figure 5.2-1 Effect of enzyme loading on glucose produced from hydrolysis of steam exploded corn stover.

30 FPU/gm, however, is unrealistically high if solids concentrations greater than 15 wt% are used. The higher enzyme loading requirement at high substrate concentrations is probably due to significant product inhibition at glucose concentrations greater than 50 gm/L.

The effect of solids concentration on the yield of reducing sugars and glucose at 24 and 48 hours was observed at an enzyme loading of 25 FPU/gm (Figure 5.2-2). For solids concentrations of 10 wt% or less there would be no benefit in hydrolyzing the material for more than 24 hours. Yield of glucose at 48 hours is linear with solids concentration up to 10 wt% solids. Beyond that point the yield decreases, probably because of product inhibition. The yield of reducing sugars at 48 hours is linear up to 25 wt% solids. This suggests that the decrease in glucose yield is due to glucose inhibition. If the yield of reducing sugars had dropped with glucose yield then it would have indicated that inhibition by cellobiose was significant. It can be concluded that the relative amount of cellobiase activity, which was 1.1 times the filter paper activity, was sufficient to prevent cellobiose inhibition. The glucose yields and cellobiose concentrations at 24 hours are shown in figure 5.2-3. Interference by other sugars made it difficult to accurately measure the cellobiose concentration.

Hydrolysis of a 5 wt% suspension of steam exploded corn stover with an enzyme loading of 50 FPU/gm is compared in

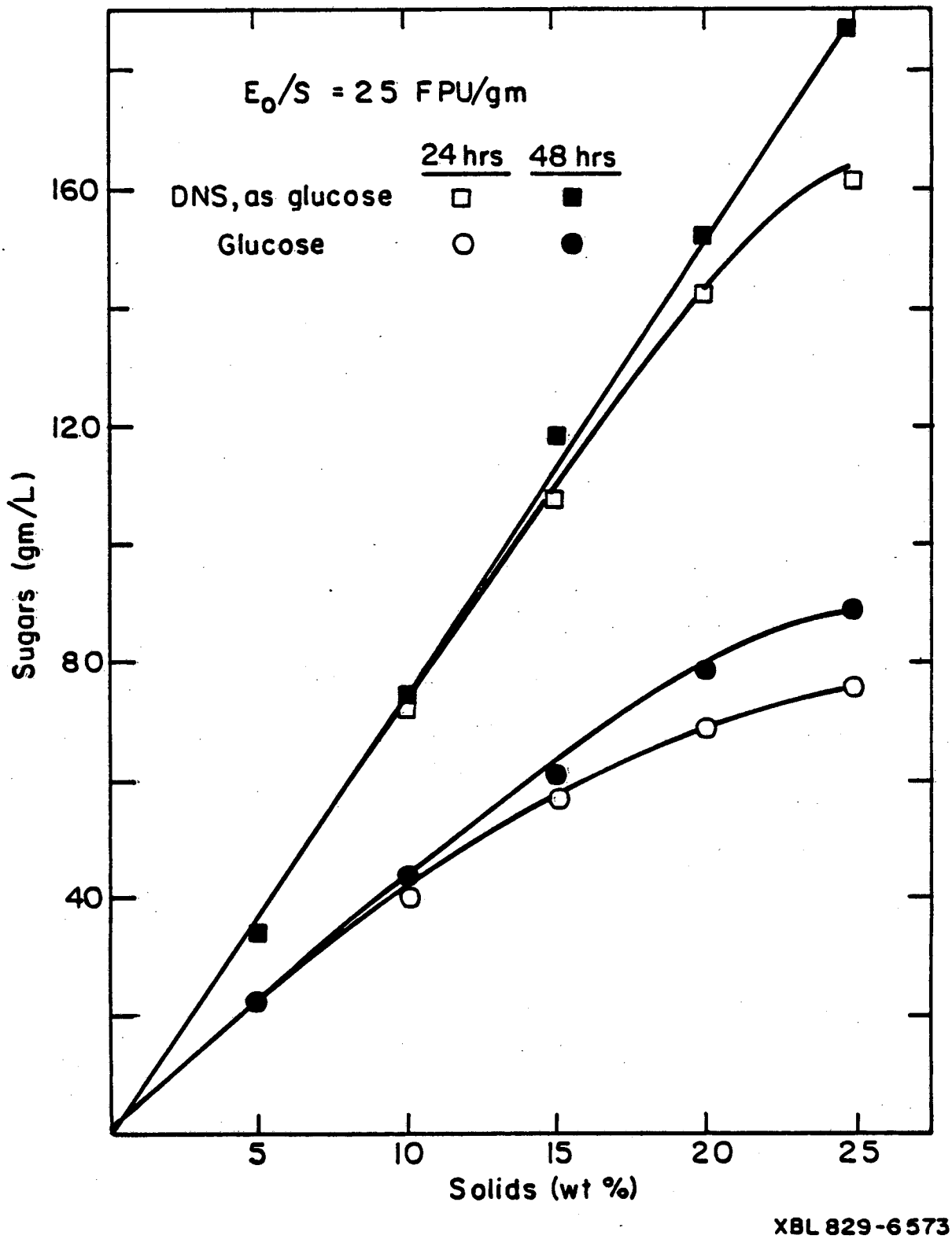

Figure 5.2-2 Effect of solids concentration (steam exploded corn stover) on sugar production.

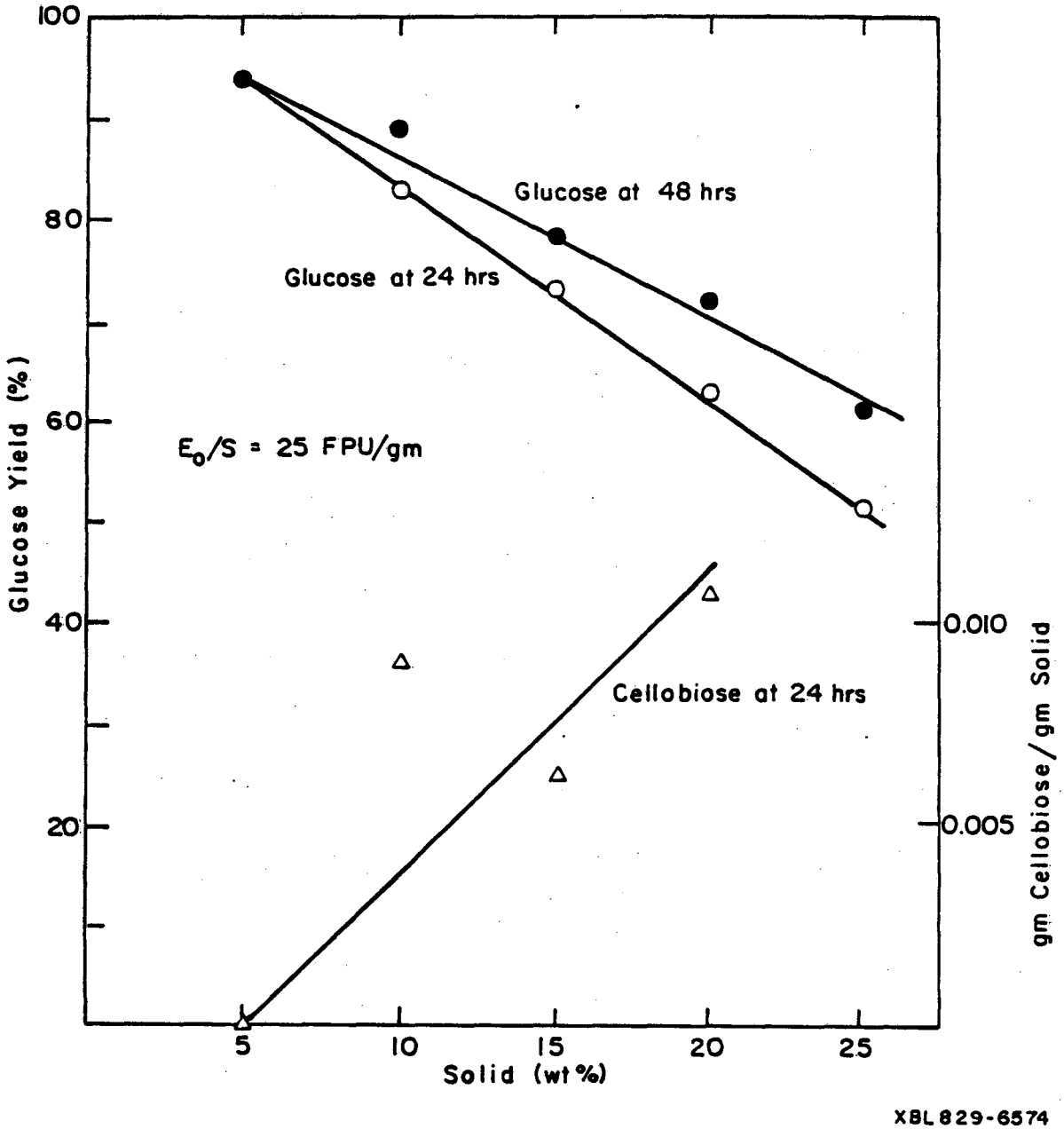

Figure 5.2-3 Glucose yields and cellobiose concentrations from hydrolysis of steam exploded corn stover -constant enzyme loading.

figure 5.2-4 to hydrolysis of acid treated corn stover at similar conditions. Data for acid treated corn stover hydrolysis are from Perez (72). It is clear that steam explosion increases the initial hydrolysis rate and the extent of conversion.

The uptake and release of enzyme in solution was monitored during hydrolysis of 10 wt% steam exploded corn stover with 25 FPU/gm at 45 ^{o}C (Figure 5.2-5). As shown in the adsorption experiments, uptake of enzyme was rapid, with preferential adsorption of C_1 activity. Desorption of the four activities follows the trend exhibited during adsorption; C_1 activity is released at the fastest rate, desorption of the other activities is much slower. This is probably because once the crystalline material is broken down into amorphous material enzyme components exhibiting C_1 activity are no longer required for hydrolysis. Other components are released at a much slower rate as amorphous material is hydrolyzed to glucose.

With respect to enzyme recovery, the optimum hydrolysis time for the conditions used is 24 hours. At that time the amount of enzyme activity in solution is maximized. A longer residence time will result in significant denaturation of C_1, C_x, and cellobiase activities.

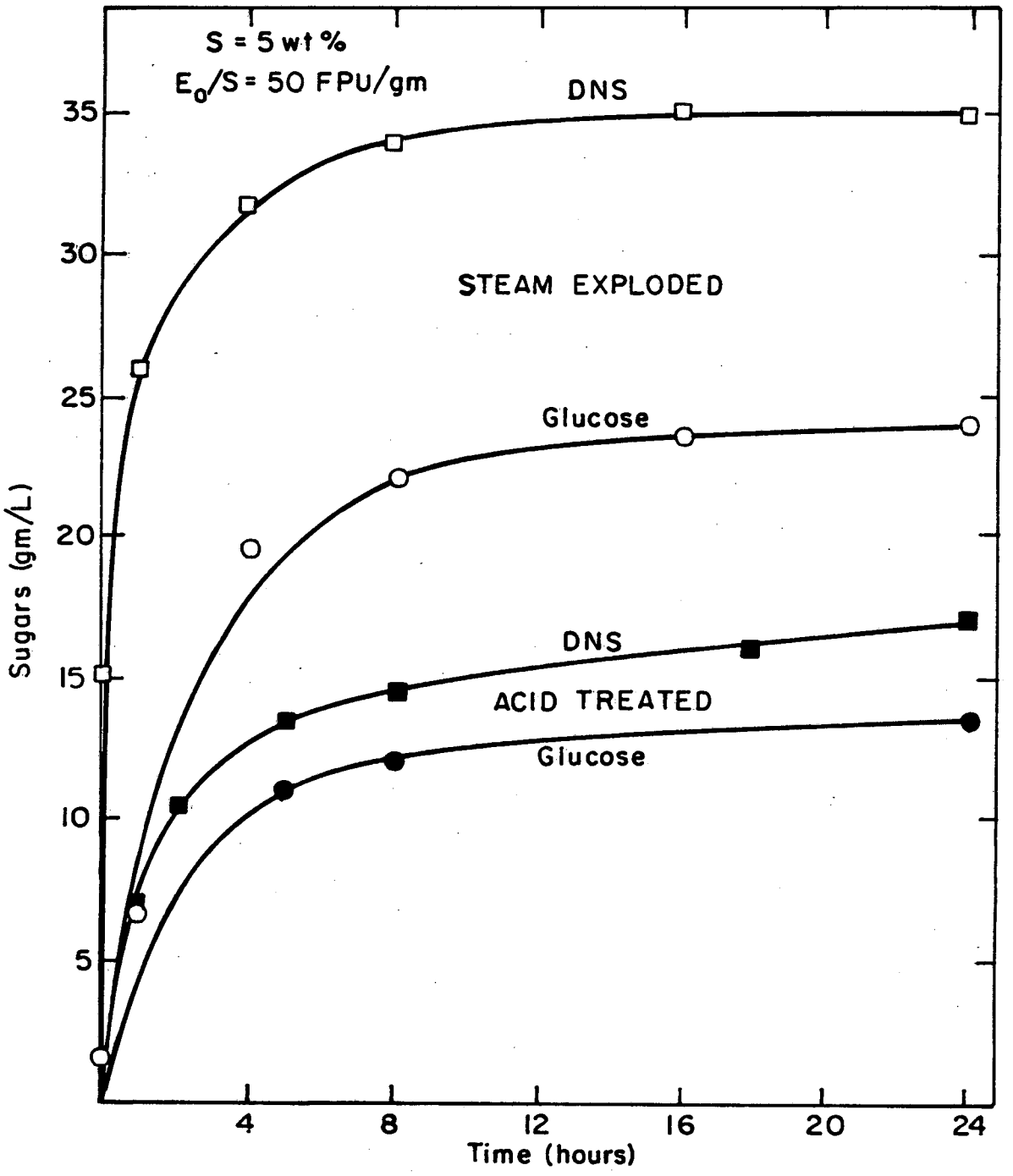

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Figure 5.2-4 Effect of pretreatment on hydrolysis of corn stover. Data for acid treated corn stover from Perez (72).

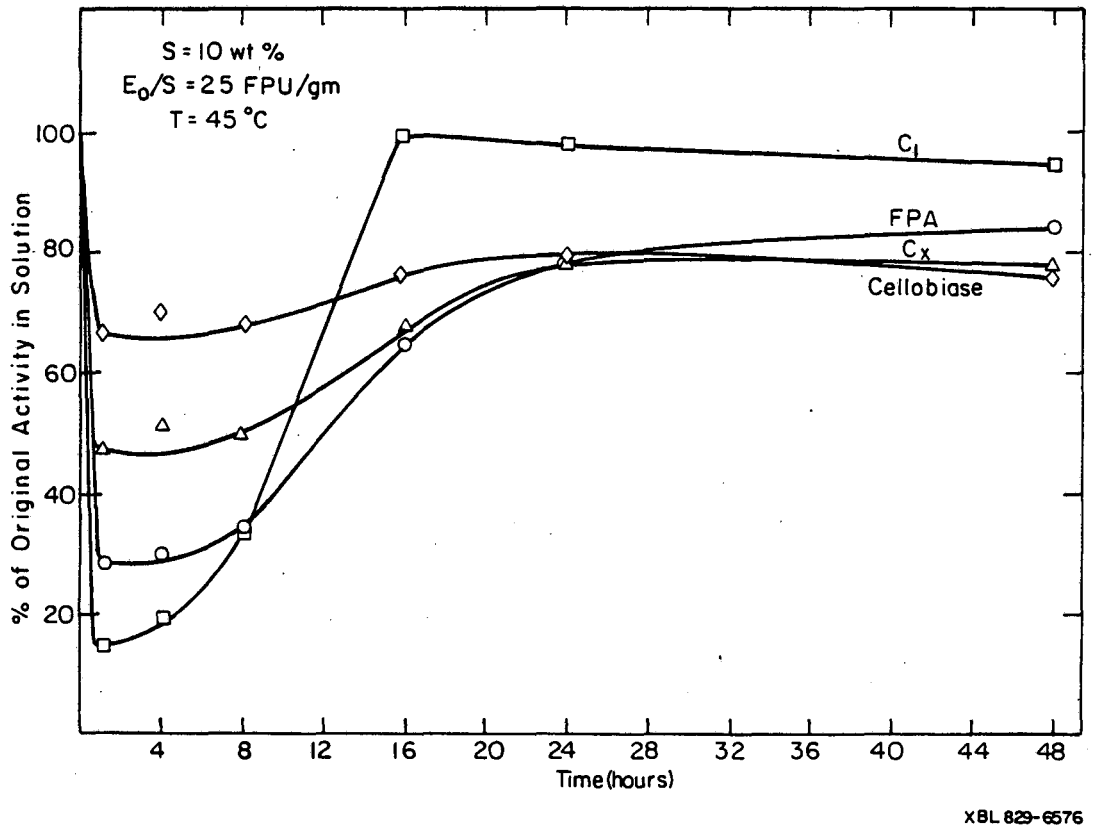

Figure 5.2-5 Adsorption and desorption of cellulase enzymes during hydrolysis of steam exploded corn stover.

5.3 Hydrolysis with Countercurrent Adsorption

Based on the experiments discussed previously, several predictions can be made on the performance of a system for enzymatic hydrolysis of cellulose in which enzyme is recovered by countercurrent contacting of hydrolyzate with fresh substrate.

The optimum conditions for adsorption of enzyme onto cellulose are low temperature, low enzyme loading, and a residence time of 10 to 45 minutes. The adsorption of enzyme onto the substrate can be predicted by the Langmuir isotherm. Recovery of cellobiase will be poor. Adsorbed enzyme will digest cellulose; the effectiveness of adsorbed enzyme is not known.

Optimum conditions for hydrolysis of 10 wt% steam exploded corn stover at 45 ^{o}C are an enzyme loading of 25 FPU/gm and a residence time of 24 hours. These conditions optimize sugar production and availability of enzyme for recovery.

Enzymatic hydrolysis of steam exploded corn stover was operated in parallel with two stages of countercurrent adsorption. A 10 wt% suspension of steam exploded corn stover was hydrolyzed with an enzyme loading of 25 FPU/gm solids at 45 ^{o}C for 24 hours. Adsorption stages were operated at 25 ^{o}C and a residence time of 45 minutes. The

make-up enzyme requirement was based on adsorption of filter paper activity, predicted by the Langmuir isotherm. It was assumed that 75 % of the original filter paper activity was available for recovery after hydrolysis; this was based on previous hydrolysis experiments.

The system was operated for eight complete cycles (Figure 5.3-1). During the second and third cycles the predicted enzyme loading in the hydrolysis stage was less than 25 FPU/gm because of errors in estimating the enzyme requirement. This is the reason for the unusually high make-up enzyme requirement at the beginning of the fourth and fifth hydrolyses. The enzyme requirement for the next three hydrolyses was relatively constant at 35 % of that required for batch hydrolysis without enzyme recovery.

The filter paper activity of the solution leaving the final recovery stage was measured and compared to that predicted by the Langmuir isotherm (Figure 5.3-2). During the last four cycles of operation the actual filter paper activity averaged 0.19 FPU/ml; the predicted activity averaged 0.054 FPU/ml. If it is assumed that the enzyme loading in the hydrolysis stage was maintained at 25 FPU/gm and that 75 % of this activity was available for recovery, then the actual recovery was 91 % and the predicted recovery was 97 %.

The cellobiase activity leaving the final adsorption stage was not predicted because the accuracy of the Langmuir

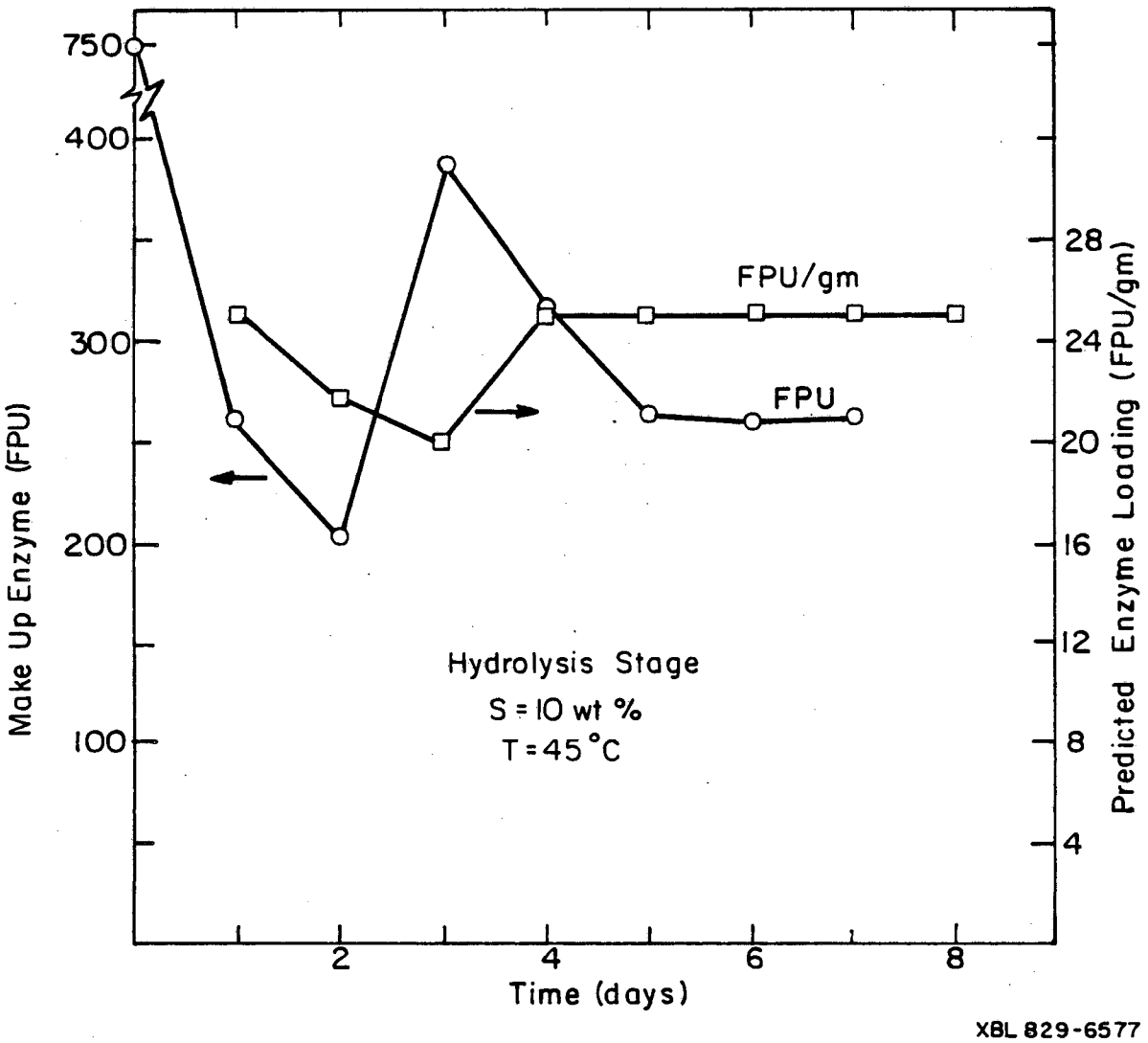

Figure 5.3-1 Batch hydrolysis of steam exploded corn stover with enzyme recovery by two stage batch countercurrent adsorption.

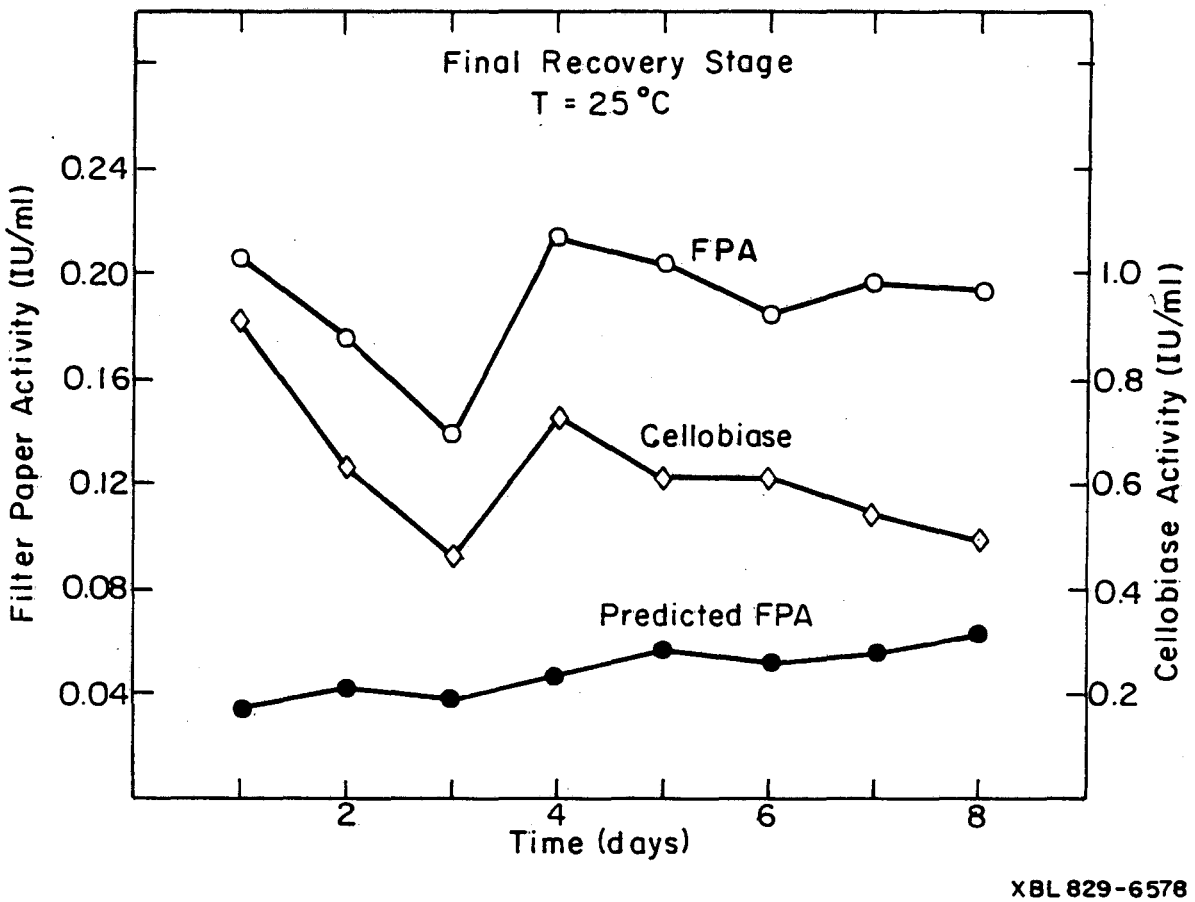

Figure 5.3-2 Enzyme activity of solution leaving final recovery stage.

model for cellobiase adsorption is poor. Actual activity leaving the final recovery stage averaged 0.11 IU/ml during the last four cycles of operation. There was a decrease in cellobiase activity after the seventh and eighth cycles, at this point it was not known if this decrease would continue in subsequent cycles.

The concentration of glucose and reducing sugars (measured as glucose) in the liquid leaving the final recovery stage were measured (Figure 5.3-3). The significant drop in reducing sugars after the first cycle was expected; in the countercurrent adsorption scheme the soluble sugars are washed off of steam exploded corn stover, solids for the first hydrolysis cycle had not been contacted countercurrently with enzyme/sugar solution. The reducing sugars concentration averaged 76 gm/L during the last four cycles of operation. Glucose concentration averaged 38 gm/L during the last four cycles, and was relatively constant during the entire experiment.

The hydrolysis stage of the eighth cycle of operation was monitored for filter paper activity, cellobiase activity, reducing sugars, and glucose, and was compared to a control experiment (batch hydrolysis of washed solids at similar conditions, but without enzyme recovery) (Figure 5.3-4).

The net glucose production at 24 hours was 30 gm/L, compared to 33 gm/L for the control. The concentration of

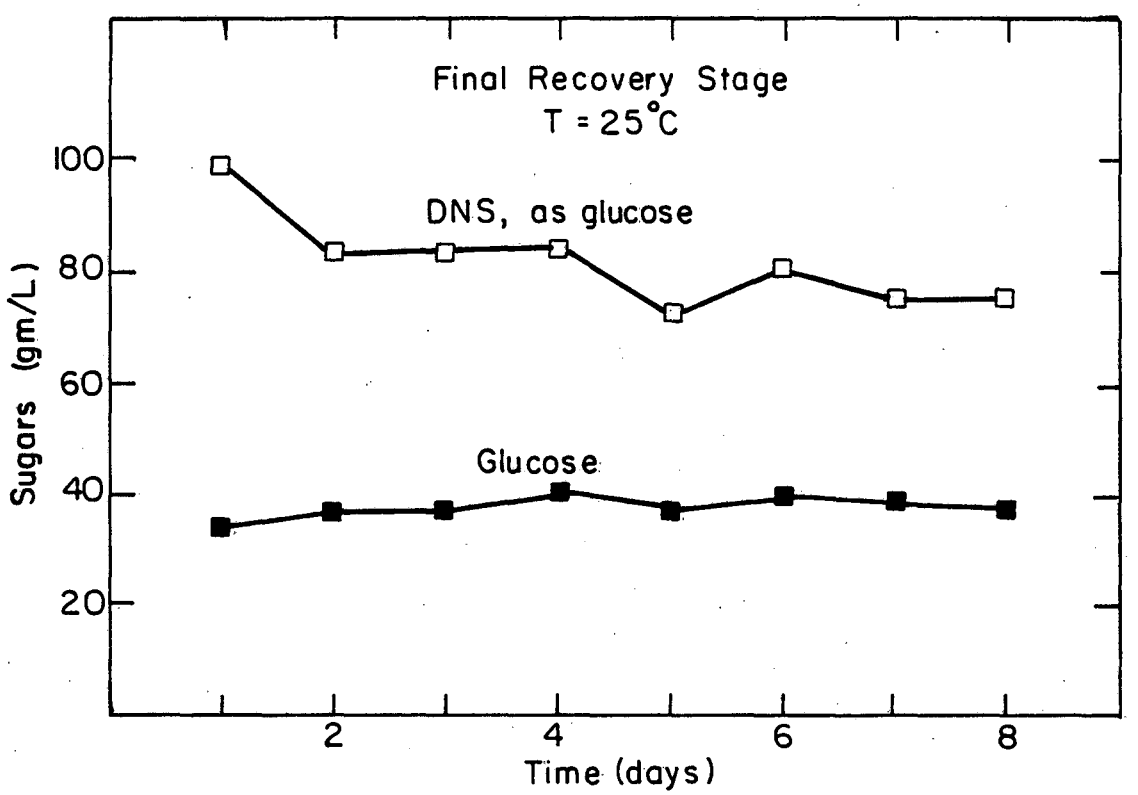

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Figure 5.3-3 Sugars leaving final recovery stage.

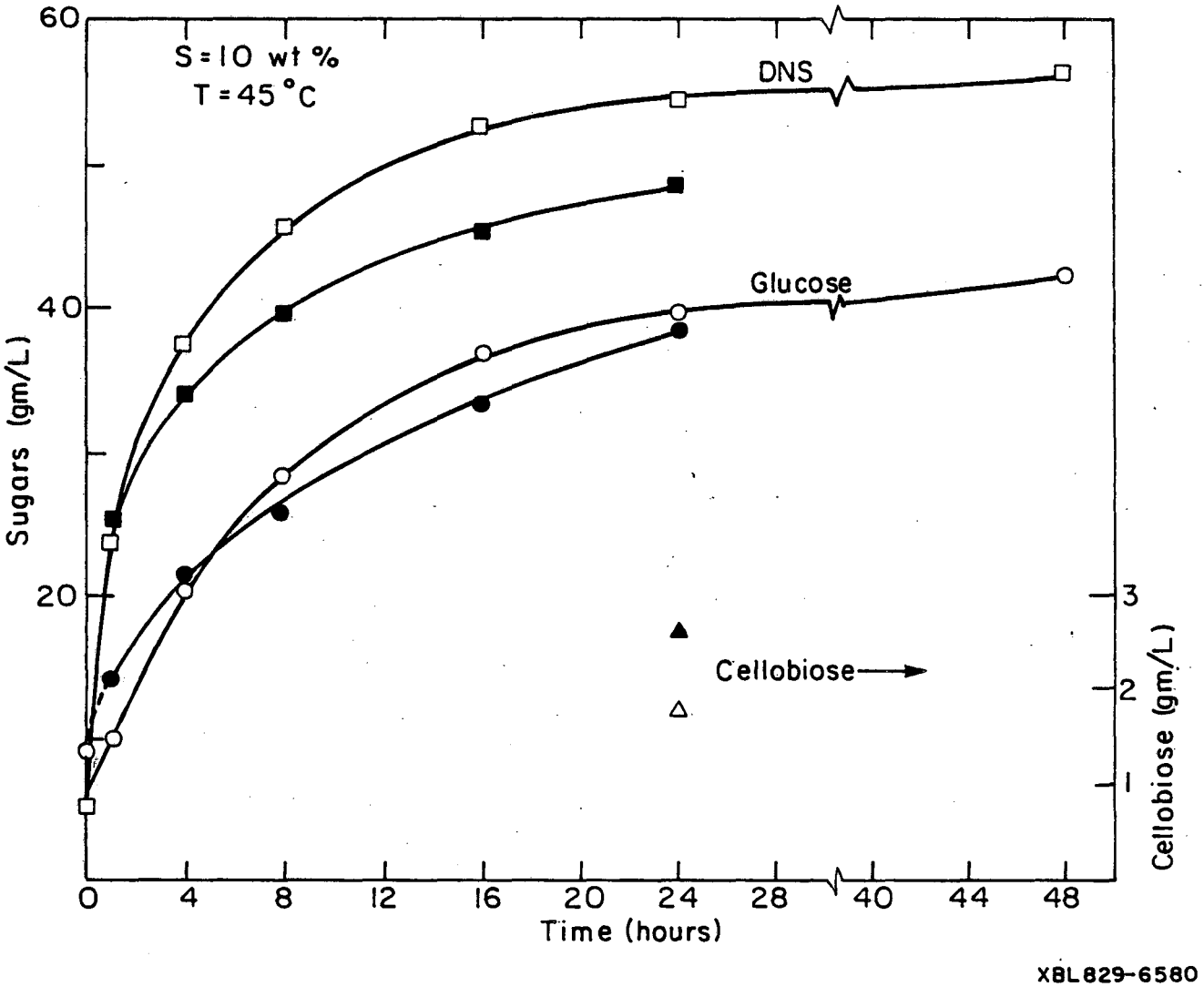

Figure 5.3-4 Effect of enzyme recovery on sugar production.

(○) glucose, control
(●) glucose, enzyme recovery
(□) reducing sugars, control
(■) reducing sugars, enzyme recovery
(△) cellobiose, control
(▲) cellobiose, enzyme recovery

reducing sugars is significantly lower with enzyme recovery. At 24 hours the cellobiose concentration was 30 % greater than in the control experiment. The increase in cellobiose concentration and the decrease in net production of glucose and reducing sugars are probably due to insufficient cellobiase activity.

At 24 hours the cellobiase activity in the eighth hydrolysis stage was 56 % lower than in the control experiment (Figure 5.3-5). Filter paper activity at 24 hours was 29 % lower than in the control experiment, and was released much more slowly. This is probably due to insufficient cellobiase activity, which caused an increase in cellobiose concentration. A high cellobiose concentration would inhibit exoglucanases and endoglucanases. The result would be a slower hydrolysis rate, as shown in figure 5.3-4, and a slower release of filter paper activity. Unless additional cellobiase activity is added to the system, hydrolysis with enzyme recovery would probably benefit from a longer residence time in the hydrolysis stage; net production of glucose would be increased and more enzyme would be released into solution.

The filter paper activity of the solution leaving the first enzyme recovery stage of the eighth cycle was 0.45 FPU/ml, the solution leaving the final stage had 0.19 FPU/ml. Based on 1.54 FPU/ml actually leaving the hydrolysis stage, this corresponds to 71 % recovery in the first

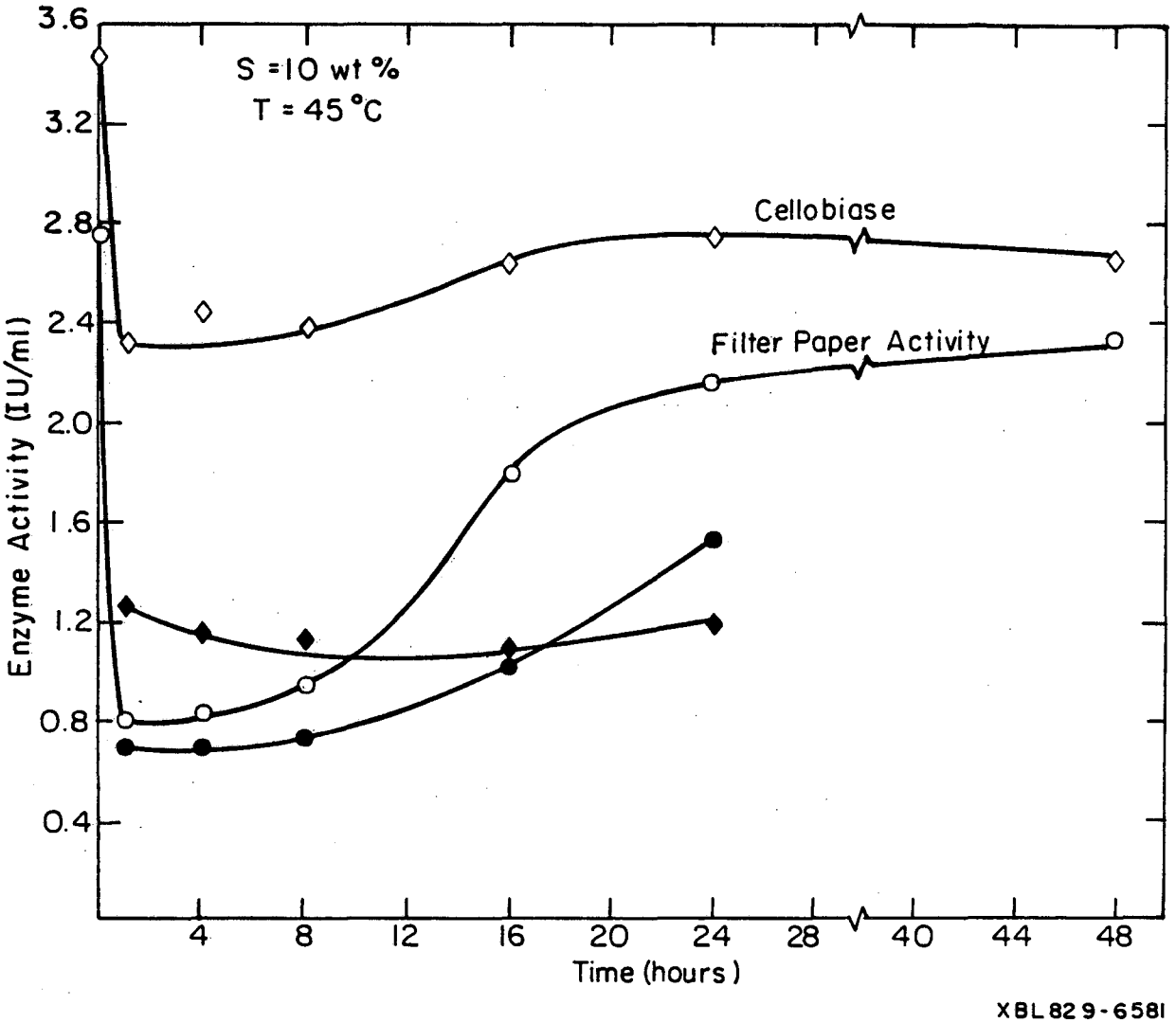

Figure 5.3-5 Effect of enzyme recovery on release of enzyme activity in the hydrolysis stage.

(●) filter paper activity, enzyme recovery
(○) filter paper activity, control
(◆) cellobiase activity, enzyme recovery
(◇) cellobiase activity, control

stage, and a total recovery of 87 %. The predicted total recovery was 98 %. The cellobiase activity was 0.77 IU/ml leaving the first stage, and 0.50 IU/ml leaving the final stage.

Results from the eighth cycle hydrolysis and adsorption stages can be used to predict the cellobiase activity in subsequent hydrolyses. The average cellobiase activity during the eighth hydrolysis was approximately 1.2 IU/ml. The next hydrolysis would be supplemented with make-up enzyme equal to 35 % of that required for batch hydrolysis without enzyme recovery. This corresponds to a cellobiase activity of 1.1 IU/ml. Solids from the first enzyme recovery stage were filtered to 36 % moisture content and would be used for the next (ninth) hydrolysis. The cellobiase activity of the liquid carried over with solids is 0.77 IU/ml. When this liquid is added to the make-up enzyme in the ninth hydrolysis stage the total cellobiase activity would be 1.2 IU/ml, the same as in the eighth hydrolysis. Based on this calculation it can be concluded that cellobiase activity would not decrease in subsequent hydrolyses.

VI. PROCESS DESIGN AND ECONOMIC EVALUATION

The cost of enzyme recovery by countercurrent adsorption was estimated and compared to the savings in enzyme production costs. Design of the enzyme recovery system was based on two stages of countercurrent adsorption operated in parallel with hydrolysis of 10 wt% steam exploded corn stover at an enzyme loading of 25 FPU/gm. Results from the enzyme recovery experiments were used to predict the stream compositions. The cost of enzyme production was based on the economic evaluation done by Perez (72), who found that batch production was the most economical method.

6.1 Process Design

6.1.1 Enzyme Recovery

The size of the countercurrent adsorption system is based on a glucose production rate of 214 tons/day. Figure 6.1.1-1 shows the mass balance used to design each adsorption stage. Steam exploded corn stover (35.5 % moisture content) is fed to Adsorption Stage 1, where it is contacted with the liquid from Adsorption Stage 2. The additional liquid from the steam exploded solids dilutes the glucose solution, decreasing its concentration by 24 %. Previous hydrolysis experiments showed that a 10 wt% suspension of steam exploded corn stover has a glucose concentration of

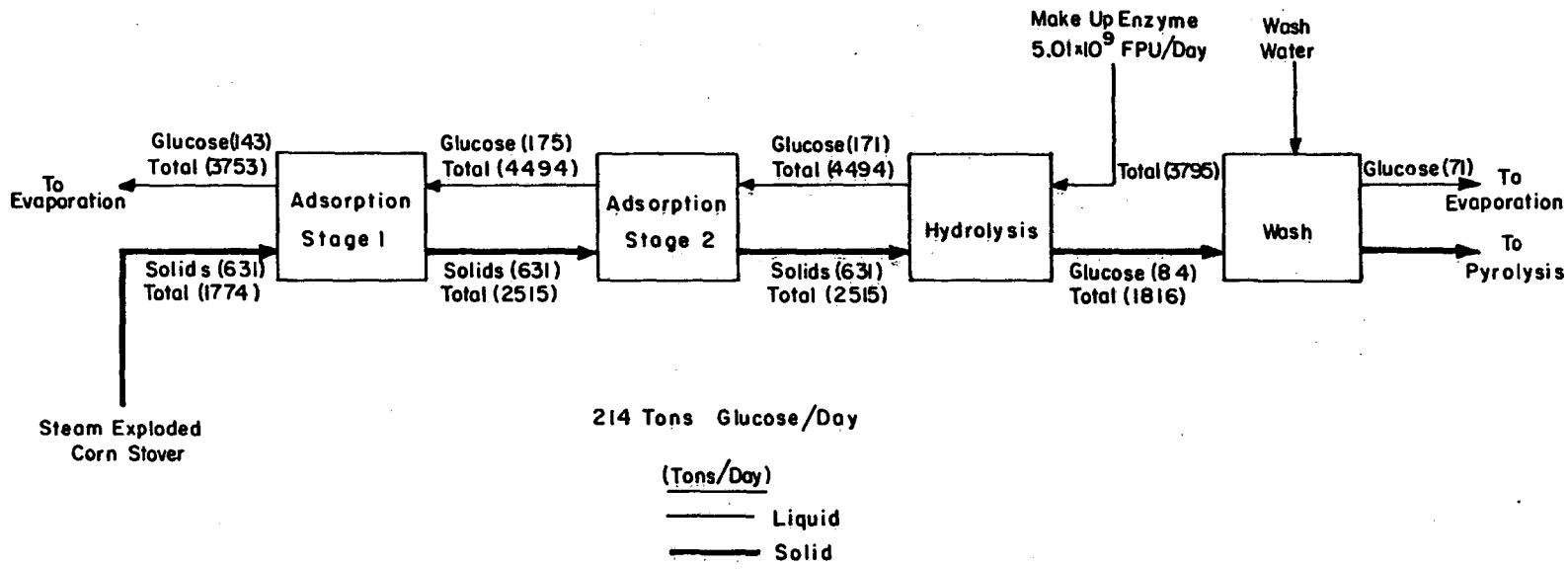

Figure 6.1.1-1 Process flow diagram for enzymatic hydrolysis with enzyme recovery.

5-8 gm/L. This is the reason why the glucose stream is actually diluted by only 6 %. The mixture is stirred at 25 ^{o}C and a residence time of 45 minutes. The residence time could be decreased to 30 minutes or less, but the purpose of this evaluation was to simulate the conditions that were observed experimentally. The mixture is filtered to 25 % moisture content and the liquid sent to the evaporation stage. The solids are fed to Adsorption Stage 2. The high moisture content of the solids is advantageous in that it allows soluble cellobiase activity to be recycled. In Adsorption Stage 2 the solids are contacted with the hydrolyzate at 25 ^{o}C and a residence time of 45 minutes. The mixture is filtered to 25 % moisture content and the liquid sent to Adsorption Stage 1. The solids are fed to the hydrolysis stage. Make-up enzyme, equal to 35 % of that required for an enzyme loading of 25 filter paper units per gram of solid (FPU/gm), is added to the hydrolysis stage. The ratio of cellobiase activity to filter paper activity in the make-up enzyme is 1.1.

The hydrolysis stage is operated at 45 ^{o}C and a residence time of 24 hours. The solids suspension is 10 wt% (based on dry weight of solids entering Adsorption Stage 1, actually lower because some soluble solids are washed off in the adsorption stages). For a predicted enzyme loading of 25 FPU/gm the cellobiase activity to filter paper activity ratio is approximately 0.43. The mixture is filtered and the liquid sent to Adsorption Stage 2. The solids are

washed with water to recover sugars. It is assumed that 85 % of the sugars are recovered from the solids in the wash (72). The liquid from the wash is sent to the evaporation stage. Spent solids are pyrolyzed to generate power and steam.

The equipment required for the enzyme recovery system is shown in figure 6.1.1-2. Steam exploded corn stover is continuously fed to the mixing tank by a screw conveyor. A continuous belt rotary vacuum filter separates solids from liquid. The vacuum system consists of a vacuum pump, filtrate receiver, and filtrate pump. Reciprocating piston-type vacuum pumps achieve very high vacuums and maintain good volumetric efficiencies at these levels (82).

6.1.2 Enzyme Production

Perez (72) evaluated three processes for enzyme production; batch, continuous, and continuous with cell recycle. It was concluded that under current processing conditions batch production is the most economical method, with cellulase cost as low as $4 per million filter paper units (Figure 6.1.2-1). The system for batch enzyme production is shown in figure 6.1.2-2. The economic evaluation was based on the following processing assumptions:

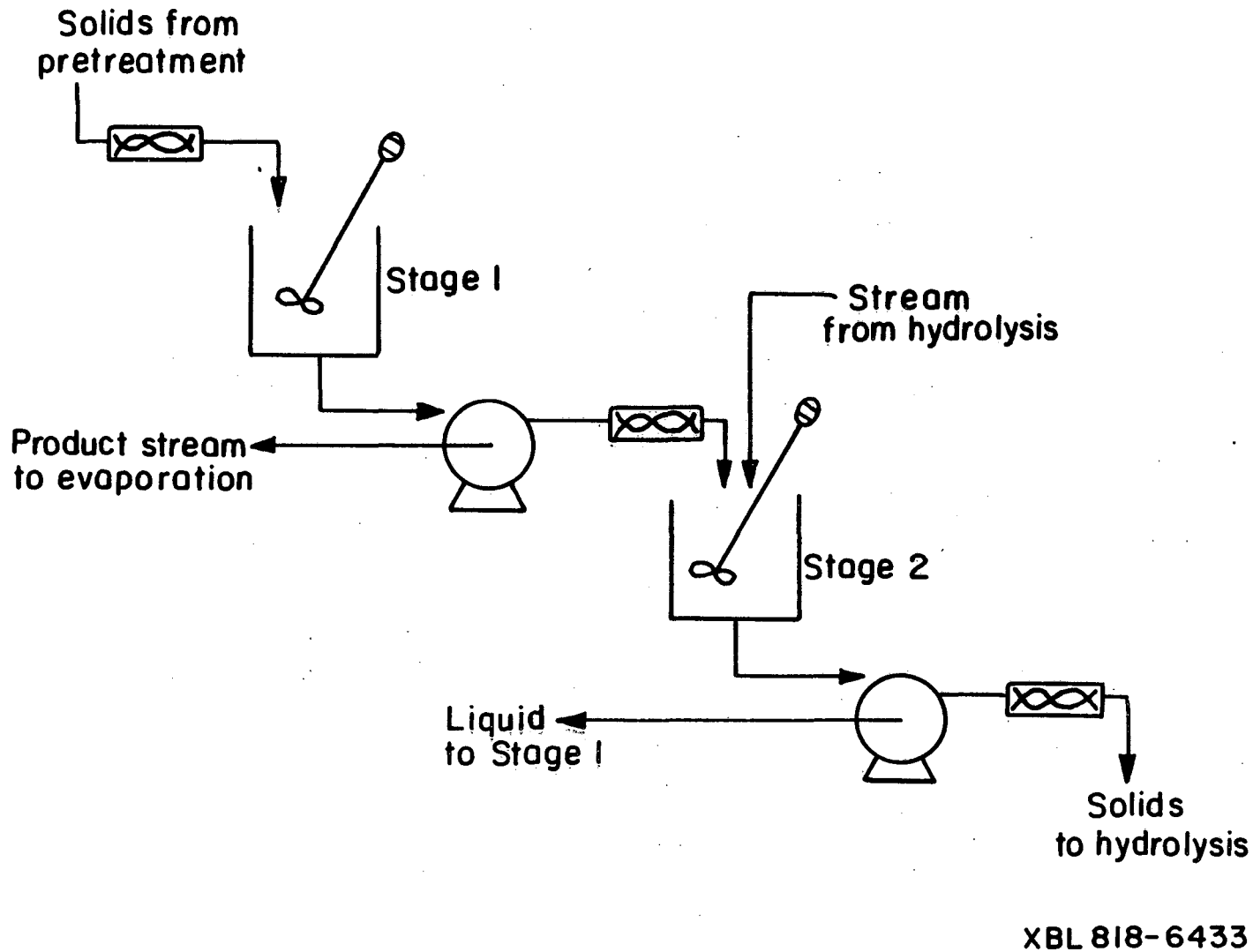

Figure 6.1.1-2 Flow diagram for enzyme recovery by countercurrent adsorption.

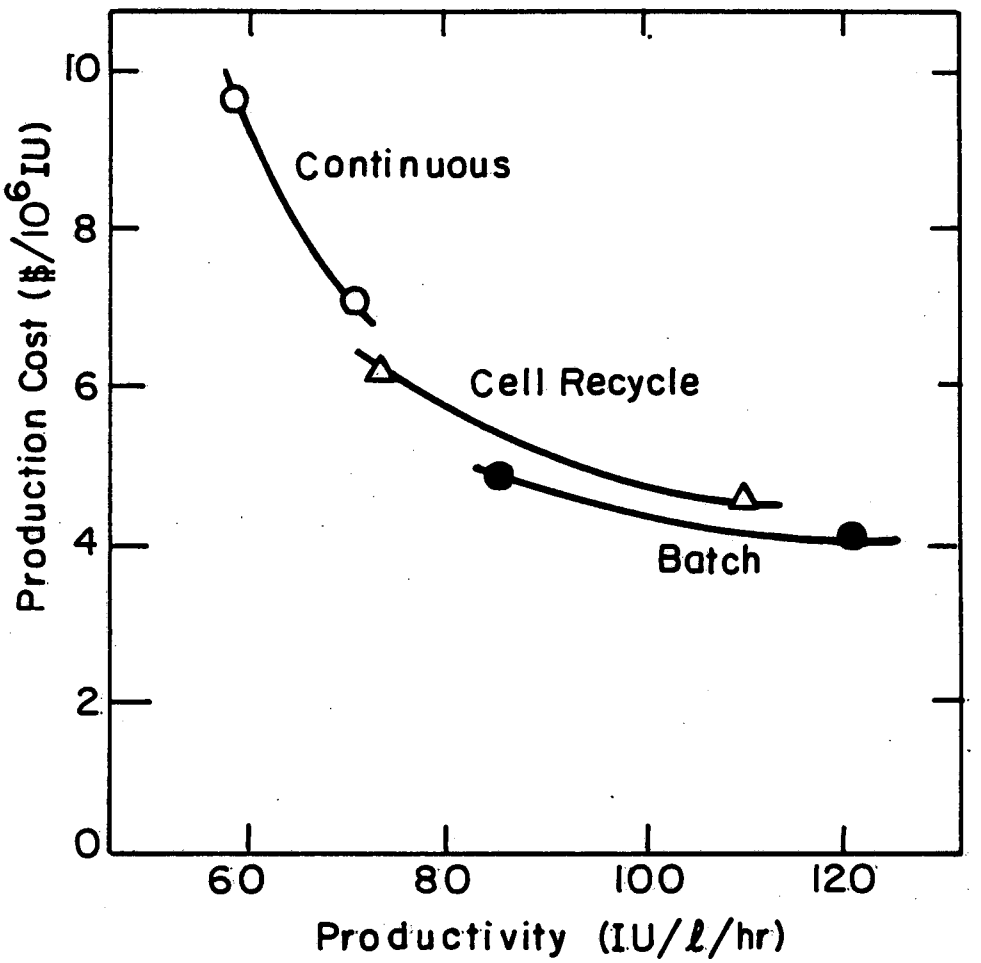

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Figure 6.1.2-1 Cellulase production cost as a function of productivity. From Perez (72).

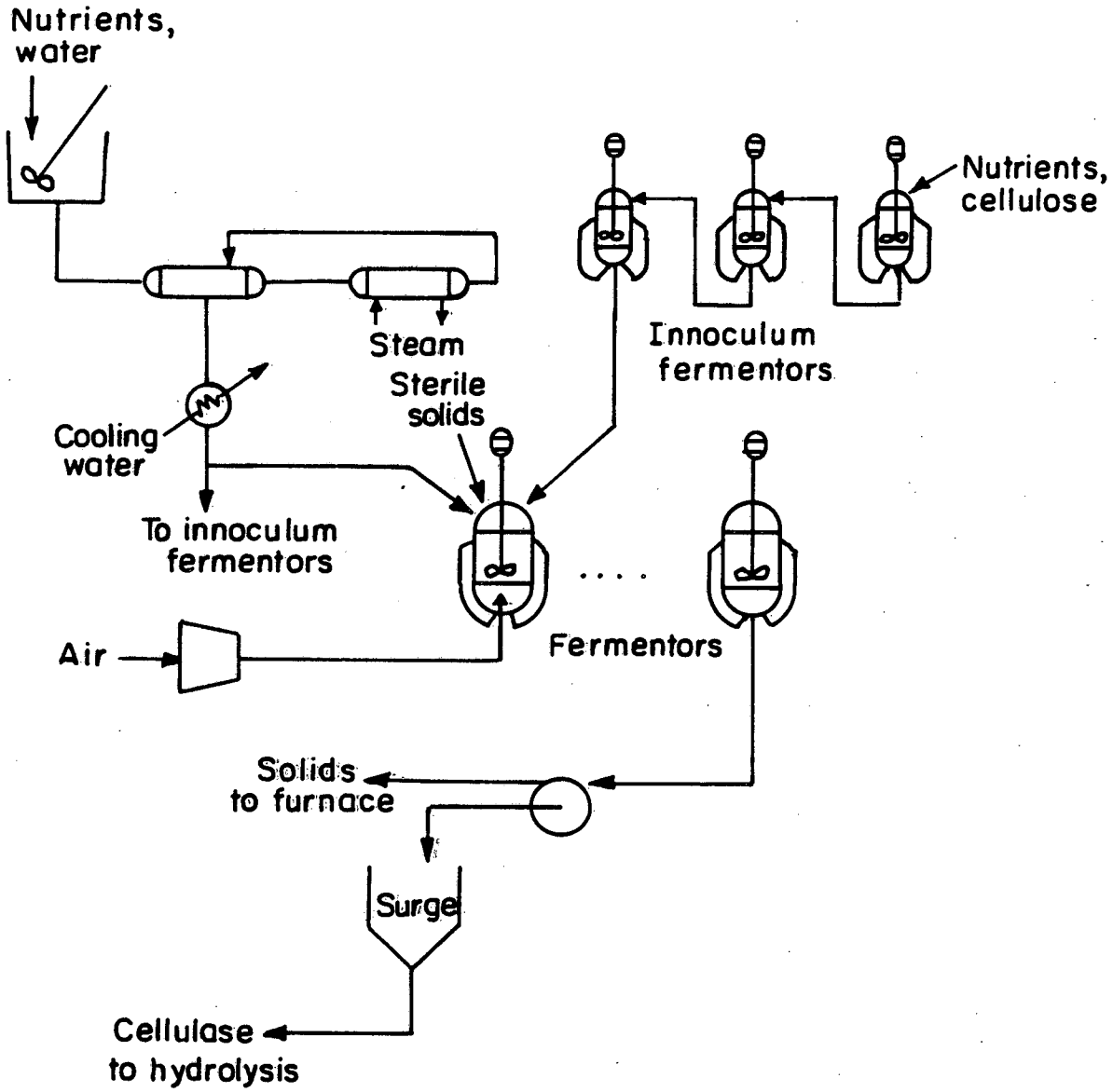

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Figure 6.1.2-2 Flow diagram for batch production of enzyme.

1. Cellulase is consistently produced at an activity of 12 FPU/ml.

2. Unspecified inexpensive delignified cellulose is substituted for solka floc.

3. An unspecified inexpensive nitrogen source is substituted for proteose peptone.

4. There are no oxygen transport limitations in the fermentor.

6.2 Economic Evaluation

A preliminary cost estimate for enzyme recovery by countercurrent adsorption was made and compared to the savings in enzyme production costs. Enzyme production costs were assumed at $4/million FPU -the minimum cost for batch enzyme production predicted by Perez (72).

The total cost for enzyme recovery consists of capital related, labor related, and utilities costs. Table 6.2-1 outlines the basis for cost analysis. The fixed capital cost is estimated at 3.9 times the purchased cost of principal items of equipment (72). The cost basis was the same as that of Perez (72) so that the enzyme recovery cost could be compared directly with the enzyme production cost. Details for equipment design and utilities costs are in Appendix C.

The annual manufacturing cost for enzyme recovery by countercurrent adsorption is $796,950 (Table 6.2-2). The annual savings in enzyme production costs, based on a 65 % decrease in enzyme requirement, is $12,289,700. The

Table 6.2-1

Basis for Cost Analysis (72)

Capital Related Costs:

Item	Factor
Depreciation	0.1
Interest	0.06
Maintenance	0.06
Insurance	0.01
Plant Supplies	0.01
Taxes	0.00
Total	0.24

Annual Capital Related Cost = 0.24 x Fixed Capital

Labor Related Costs:

Labor Rate	$ 22,000 / Operator
Operators / Shift	2
Shifts / Day	4
Labor Factor	1.65

Utilities:

Electricity	$ 0.044 / kWh

Marshall and Stevens Index	707 (4th Quarter 1980)
Stream Factor	0.9 (330 Days / Year)

Table 6.2-2

Cost Summary for Enzyme Recovery

Fixed Capital Costs	$1,954,800
Annual Capital Related Costs	$ 469,150
Annual Labor Related Costs	290,400
Annual Utilities Cost	37,400
Total Annual Cost	$ 796,950

corresponding unit cost for enzyme recovery is $0.48/million FPU. The net annual savings is $11,492,800, which corresponds to a 61 % net reduction in enzyme production costs.

VII. CONCLUSIONS

Studies on the adsorption of cellulase enzymes onto steam exploded corn stover indicate that enzyme recovery by countercurrent adsorption is best at low temperatures and low enzyme loadings (high solids concentrations). The optimum contacting time is 5 to 45 minutes. Recovery of cellobiase activity will be much poorer than recovery of other cellulase activities.

Steam explosion is superior to acid treatment and Wiley milling in increasing the susceptibility of the substrate to enzymatic attack. This was concluded from studies on the adsorption behavior and from hydrolysis experiments.

Hydrolysis experiments with steam exploded corn stover also showed that, for a 10 wt% suspension of solids at 45 ^{o}C, the optimum conditions are an enzyme loading of 25 filter paper activity units per gram solid (FPU/gm) and a residence time of 24 hours. These conditions permit high conversion of substrate and maximize release of enzyme so that it may be recovered from solution. If higher solids concentrations are used, then glucose yields can be improved by increasing the residence time, increasing the enzyme loading, or both. The decrease in glucose yield at higher solids concentrations is attributed to significant product inhibition at glucose concentrations greater than 50 gm/L.

Two stages of batch countercurrent adsorption were operated in parallel with hydrolysis of steam exploded corn stover. The hydrolysis was operated at a 10 wt% solids suspension, an enzyme loading of 25 FPU/gm, and a residence time of 24 hours. Each adsorption was operated for 45 minutes at 25 ^{o}C. In this experiment it was demonstrated that the enzyme requirement, measured as filter paper activity, could be reduced to 35 % of that required for hydrolysis without enzyme recovery. Compared to the control experiment the release of filter paper activity and the rate of sugar production in the hydrolysis stage is considerably slower, probably because of poor cellobiase recovery. This suggests that the hydrolysis stage may benefit from a longer residence time or from supplemental cellobiase enzyme.

Results of the countercurrent adsorption experiment were used to make a preliminary economic evaluation. The enzyme production cost, assumed at $4/million FPU (72), can be reduced by 61 %. This includes the cost of operating two countercurrent adsorption stages and all associated equipment.

VIII. RECOMMENDATIONS FOR FUTURE WORK

This study showed that steam exploded corn stover is an ideal substrate for enzyme recovery by countercurrent adsorption; high conversions in the hydrolysis stage permit release of enzyme back into solution, enzyme in the hydrolyzate is readily adsorbed onto fresh substrate. Several problems and process alternatives should be considered in future studies.

Steam explosion solubilizes hemicellulose and lignin. Pentose sugars and lignin are thus found, along with glucose, in the product stream from the adsorption stages. *Clostridium thermohydrosulphuricum* (a thermophilic, ~~facultative~~ anaerobe) converts glucose, xylose, and other pentose sugars to ethanol (52). This is a promising alternative to *Saccharomyces cerevisiae*, which ferments glucose only. The effect of soluble lignin on fermentation with *Clostridia* or *Saccharomyces* is not known.

In the Berkeley process spent solids from the hydrolysis stage are pyrolyzed to generate power and steam. If higher conversions are achieved in the hydrolysis stage then less material is available for subsequent power generation.

The conditions for enzymatic hydrolysis with enzyme recovery by countercurrent adsorption should be optimized. Higher solids concentrations would yield a more concentrated

glucose solution, thus reducing the cost of subsequent evaporation. Yields at higher solids concentrations, however, are lower. The percentage of enzyme available for recovery would also be lower. Because cellobiase recovery is poor, it would be advantageous to increase the residence time of the hydrolysis stage or to supplement the hydrolysis with additional cellobiase enzyme.

The economic evaluation of enzyme recovery by countercurrent adsorption did not consider the cost of the substrate, this cost is associated with the hydrolysis stage. Perez (72) showed that the economics of enzymatic hydrolysis of acid treated corn stover are dominated by the corn stover cost. Enzymatic hydrolysis of a less expensive substrate that can be pretreated by steam explosion should be investigated.

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APPENDIX

A.1 Equipment Design

Unless noted, individual equipment items were designed and priced (Table A.1-1) according to information in Peters and Timmerhaus (73).

A.1.1 Mixing Tanks

It is assumed that the specific gravity of the liquid is 1.0. Tanks are sized for a residence time of 45 minutes. Working volume is 80 % of nameplate capacity. Tanks are constructed of carbon steel.

A.1.2 Vacuum Filters and Associated Pumps

Filters are sized with respect to filtration area, which is calculated as:

$$\text{AREA} = \text{FLOWRATE} / \text{FILTRATE} .$$

The filtration rate for 5 wt% corn stover is estimated at 166 gal/ft^{2}hr (72). The relative filtration rate for 10 wt% corn stover is estimated at 60 % of that for 5 wt% corn stover (Figure A.1.2-1). Continuous belt rotary vacuum filters are used because they are ideal for a slurry in which the liquid phase is saturated with a slightly soluble substance and which filters relatively slowly (82). Costs for filtration units are estimated according to Perez (72).

Table A.1-1

Equipment List

Item	Quantity	Capacity	Cost
Mixing Tanks	1	59,000 gal.	$ 49,150
	1	66,000 gal.	50,410
Agitators	1	5 hp	5,040
	1	6 hp	5,290
Vacuum Filters	1	378 ft^2	132,330
	1	453 ft^2	146,190
Vacuum Pumps	1	13 hp	39,070
	1	15 hp	47,890
Screw Conveyors	2	16 in x 20 ft	16,130
Pumps	4	22 hp	7,460
Motors	4	31 hp	2,270
Total Purchased Cost			$ 501,230

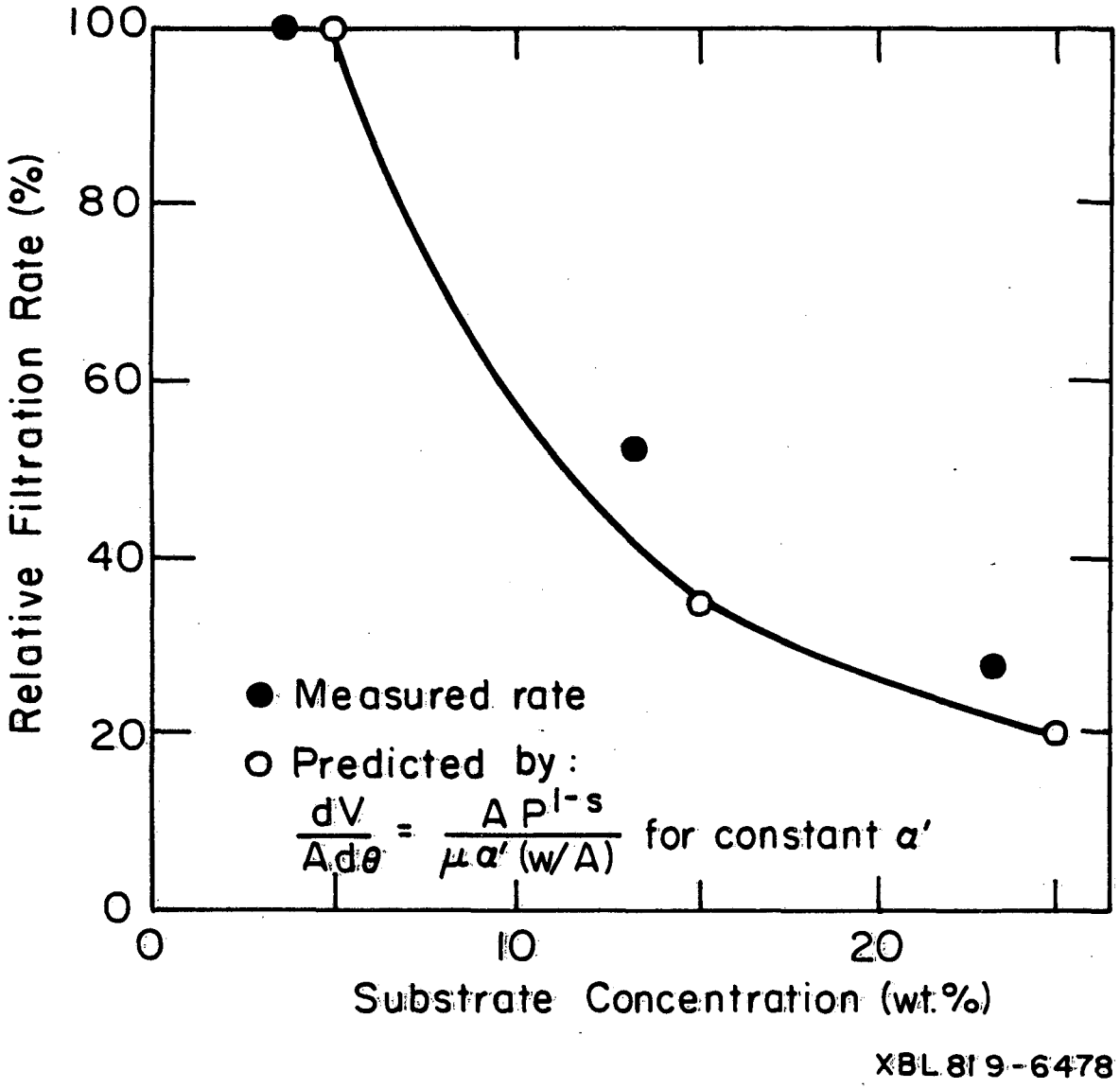

Figure A.1.2-1 Relative filtration rate for corn stover. From Perez (72).

Vacuum pumps are sized for a head of 50 ft and an efficiency of 70 %. Reciprocating piston-type pumps are used to achieve high vacuums and maintain good volumetric efficiencies at these levels (82).

A.1.3 Screw Conveyors

Screw conveyors are sized at a screw diameter of 16 inches and a length of 20 feet. This corresponds to a capacity of 110 tons of grain/hour and a power of 3 hp.

A.1.4 Pumps and Motors

General purpose centrifugal pumps are sized for a head of 50 feet. They are cast iron, bronze filled. Motors are squirrel cage, induction type, with an efficiency of 70 %. Pumps and motors are specified in duplicate.

A.1.5 Agitators

Agitators were sized according to the method of Gates et al. (25). The equivalent volume of the tank (V_{eq}) is equal to the specific gravity of the slurry ($[S_g]_{SL}$) times the working volume of the tank. A 10 wt% suspension of steam exploded corn stover in water has a $[S_g]_{SL}$ of 1.04 at 25 ^{o}C. The terminal settling velocity (u_t) of the solids is determined from the specific gravity of the solids ($[S_g]_S$), the specific gravity of the solids-free liquid ($[S_g]_L$), and the particle size (Figure A.1.5-1). $[S_g]_S$ for steam

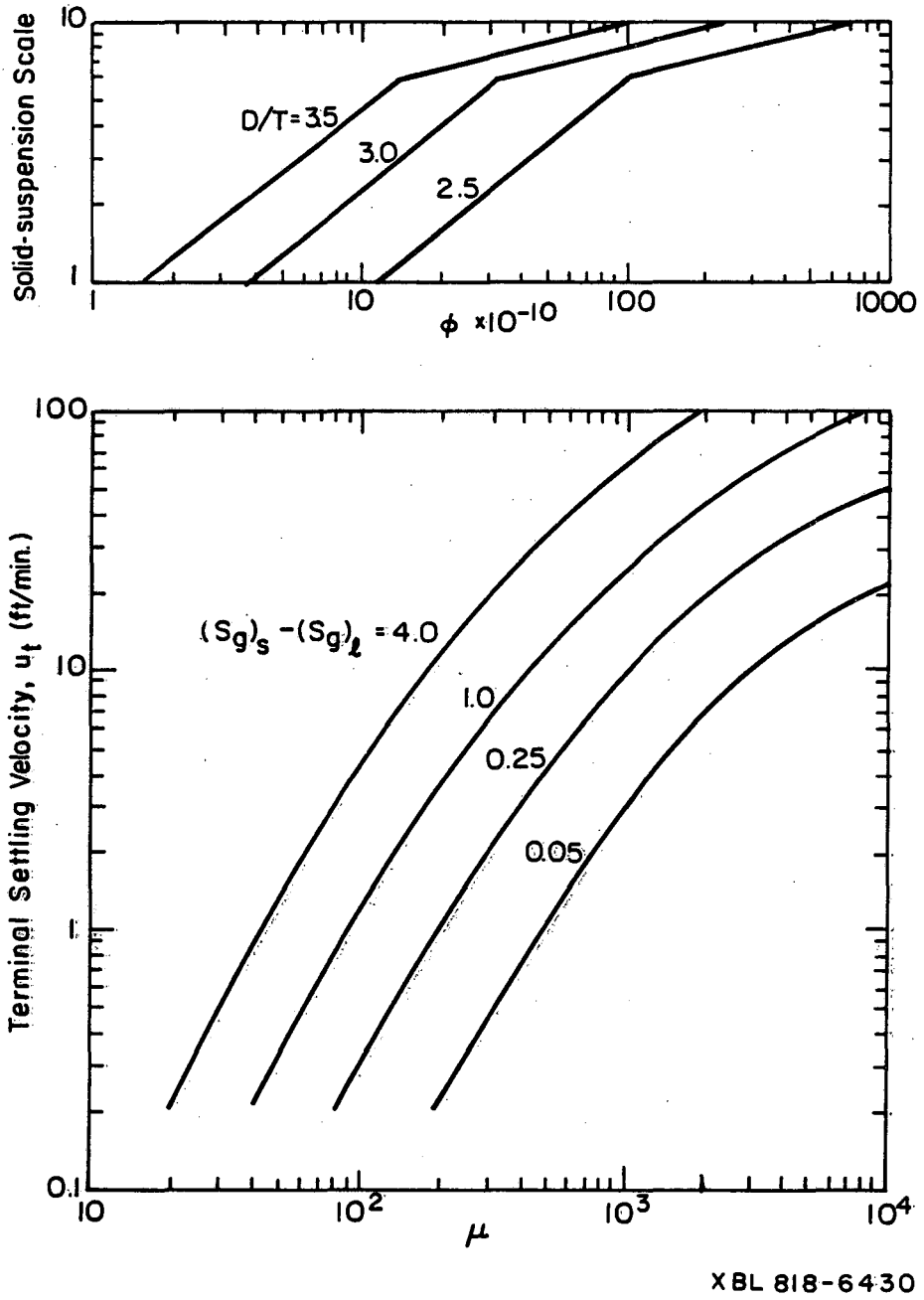

Figure A.1.5-1 Specifications for sizing agitators. From Gates et al. (25).

exploded corn stover is 1.29. The particle size of steam exploded corn stover could not be determined, but is estimated at 37 microns. This is based on the observation that the hydrolysis rate of steam exploded corn stover is comparable to that of -400 mesh ball milled corn stover (83). The terminal settling velocity is multiplied by a correction factor (f_w) to obtain the design settling velocity (Table A.1.5-1).

Scale of agitation (ranked from 1 to 10) is assumed at 7. This provides concentration uniformity of solids to 95 % of the fluid-batch height (25). The slurry depth in the vessel (Z) is assumed equal to the vessel diameter (T). Impeller diameter (D) is 0.3 times vessel diameter. Based on these constraints and the design settling velocity the required shaft speed (N) is determined:

$$\phi = N^{3.75} D^{2.81} / u_d$$

where:

ϕ = determined from figure A.1.5-1

N = required shaft speed, rpm

D = impeller diameter, inches

u_d = design settling velocity, ft/min.

Table A.1.5-1 (25)

Correction Factor for Settling Velocity of Solids in Slurries

Solids, %	Factor, f_w
2	0.8
5	0.84
10	0.91
15	1.0
20	1.1
25	1.2

The power associated with this speed is determined as:

$$H_p = \left(\frac{D}{394}\right)^5 n N^3 [S_g]_{SL}$$

where:

H_p = prime mover power, (hp).

n = number of impellers.

Single turbine impellers constructed of carbon steel are used to agitate the mixture.

A.2 Utilities

Utility requirements for enzyme recovery consist solely of electric power. Power requirements were estimated for individual equipment items (Table A.2-1). It is assumed that electricity is bought from a utility company at 4.4 cents per kWh (73). Power requirements for vacuum pumps, screw conveyors, and agitators were corrected by an efficiency factor of 85 %. The total power requirement was multiplied by a factor of 1.25 to account for line losses and contingencies (73). Total annual utilities cost is estimated at $37,420.

Table A.2-1

Electric Power Requirements

Item	Quantity	Rated Horsepower	Efficiency
Agitators	1	5	0.85
	1	6	0.85
Vacuum Pumps	1	13	0.85
	1	15	0.85
Screw Conveyors	2	3	0.85
Centrifugal Pump Motors	2	31	1.0

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