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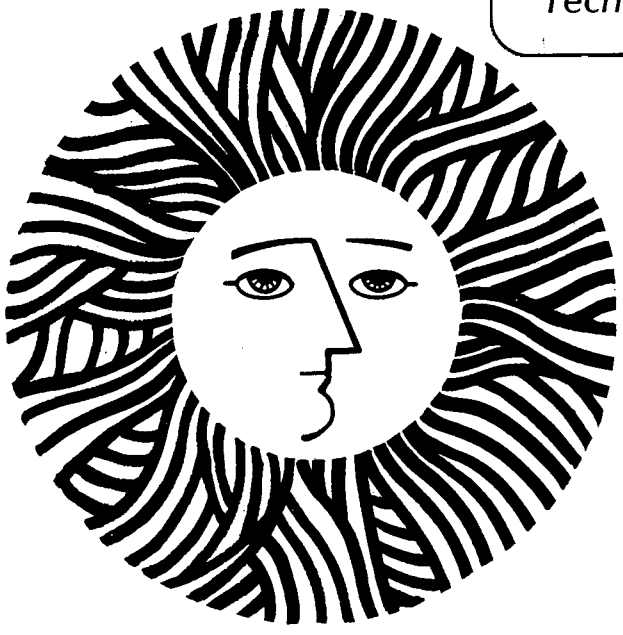
PROCESS DEVELOPMENT STUDIES FOR THE ENHANCED RECOVERY OF CELLULASE IN CELLULOSE HYDROLYSIS

George Brown Dove, III*, Charles R. Wilke, and Harvey W. Blanch
(*M.S. thesis)

August 1981

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PROCESS DEVELOPMENT STUDIES FOR THE ENHANCED RECOVERY OF CELLULASE IN CELLULOSE HYDROLYSIS

George Brown Dove, III*,
Charles R. Wilke and Harvey W. Blanch

*M.S. Thesis

August 1981

Energy and Environment Division
Lawrence Berkeley Laboratory
University of California
Berkeley, CA 94720

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ABSTRACT

The enzymatic hydrolysis of cellulose to a sugar solution (and subsequent fermentation to ethanol) results in a large loss of enzyme due to adsorption on solids. Alternatives were investigated to enhance the recovery of Rut-C-30 cellulase enzyme from a corn stover hydrolysis. Several nitrogenous salts were introduced to the hydrolysis to maximize the fraction of enzyme remaining in solution.

Addition of urea at a concentration of 1 M in the enzyme solution resulted in 65% retention of enzyme in the hydrolyzate. For subsequent fermentation of the hydrolyzate, it is necessary to concentrate the sugar solution to 5-7 M urea. However, at urea concentrations greater than 0.75 M, the yeast Sacchromyces cerevisiae employed in the fermentation exhibited greatly inhibited metabolic activities. Therefore, addition of urea to the hydrolysis is not feasible. No economical means of removing the urea prior to fermentation was found.

Several other alternatives of recovering enzyme were investigated and compared with the present process of countercurrent contacting of the solids. This method results in an estimated 27% recovery of the enzyme entering the hydrolysis(99% of the fraction of enzyme in the

hydrolyzate). A solids wash with concentrated urea solution recovers an estimated 35% of the enzyme entering the hydrolysis from the solids alone. A total recovery of 56% is estimated because losses are attributed to ultrafiltration units employed to remove enzyme from solution. This alternative is marginally uneconomical due to excessive consumption of urea. Replacing the ultrafiltration units with precipitation of enzyme by acetone results in greater costs.

It is concluded that the most economical alternative is countercurrent contacting of the hydrolyzate with fresh solids.

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DEDICATION

To my family, whose encouragement was and is essential to my progress and achievements. Their constant support continues to amaze me daily.

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I. INTRODUCTION.

As our society enters the 1980's, demands for liquid fuels are continually increasing, spurred by the need for mobility. This demand has created the largest industry in the world, the petrochemical industry. Unfortunately, all fossil resources are limited and the raw materials necessary for the production of gasoline are becoming more inaccessible. Critical damage to the natural environment will be required to obtain further resources.

The work described in this thesis is part of a long term project to develop an alternative liquid fuel from a renewable natural resource currently considered a waste product, cellulosic materials. Cellulose may be hydrolyzed to sugars, which then may be fermented to ethanol.

Ethanol (and methanol) has proven its applicability in reducing the load that is now placed upon petroleum in several uses. Current field test studies in Brazil and many regions of the United States(1,2) demonstrate that ethanol in a 10% blend with gasoline, a mixture popularly known as gasohol, may be used in automobiles without major modifications. With engine modifications, ethanol may be used as the sole fuel(3). Deregulation of petroleum prices in the U.S. will allow the price of gasoline to rise to open market levels, perhaps reaching the prices of Europe.

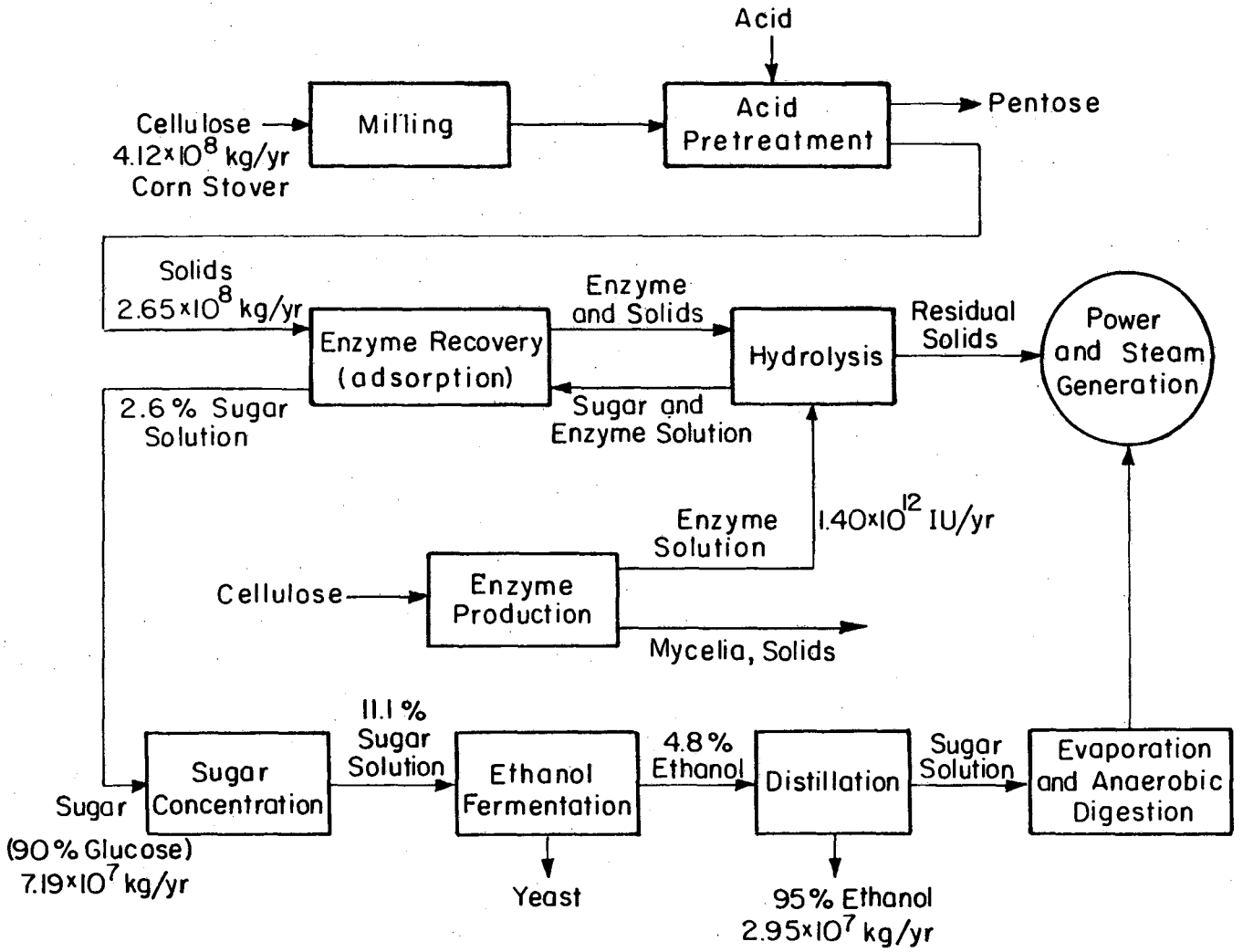
Then, many alternatives will be more than competitive.

In other uses, ethanol may act as a chemical feedstock, reducing the demand for its precursor, ethylene(4).

The diversity of raw materials and processing schemes in the utilization of cellulosic materials dictates that this research project be confined within certain limits. Current work at the University of California focuses on the enzymatic hydrolysis of cellulose, in addition to chemical hydrolysis, to produce a sugar solution. Included amongst the cellulosic substrate studied is corn stover grown in Indiana. The sugar solution resulting from cellulose hydrolysis is fermented to ethanol by the yeast, Sacchromyces cerevisiae. The product beer is distilled to 95% ethanol. A block diagram of the process is illustrated in Figure 1(5), followed by a detailed outline of the current process in Figure 2(6). A detailed description of this scheme may be found in the appendix, Process Design Equations, where the process is subjected to modifications.

Cellulase enzyme is produced by fermentation of the fungus Trichoderma reesei. Two strains are discussed in this paper. Cellulase of strain QM9414 was often utilized in the previous work referenced in this paper. Present work utilizes cellulase of strain Rut-C-30.

Cellulase enzyme is adsorbed onto the substrate during hydrolysis. Since the substrate is subsequently burned, a



Berkeley Process for Corn Stover

Figure 1 Block Diagram for Current Process.

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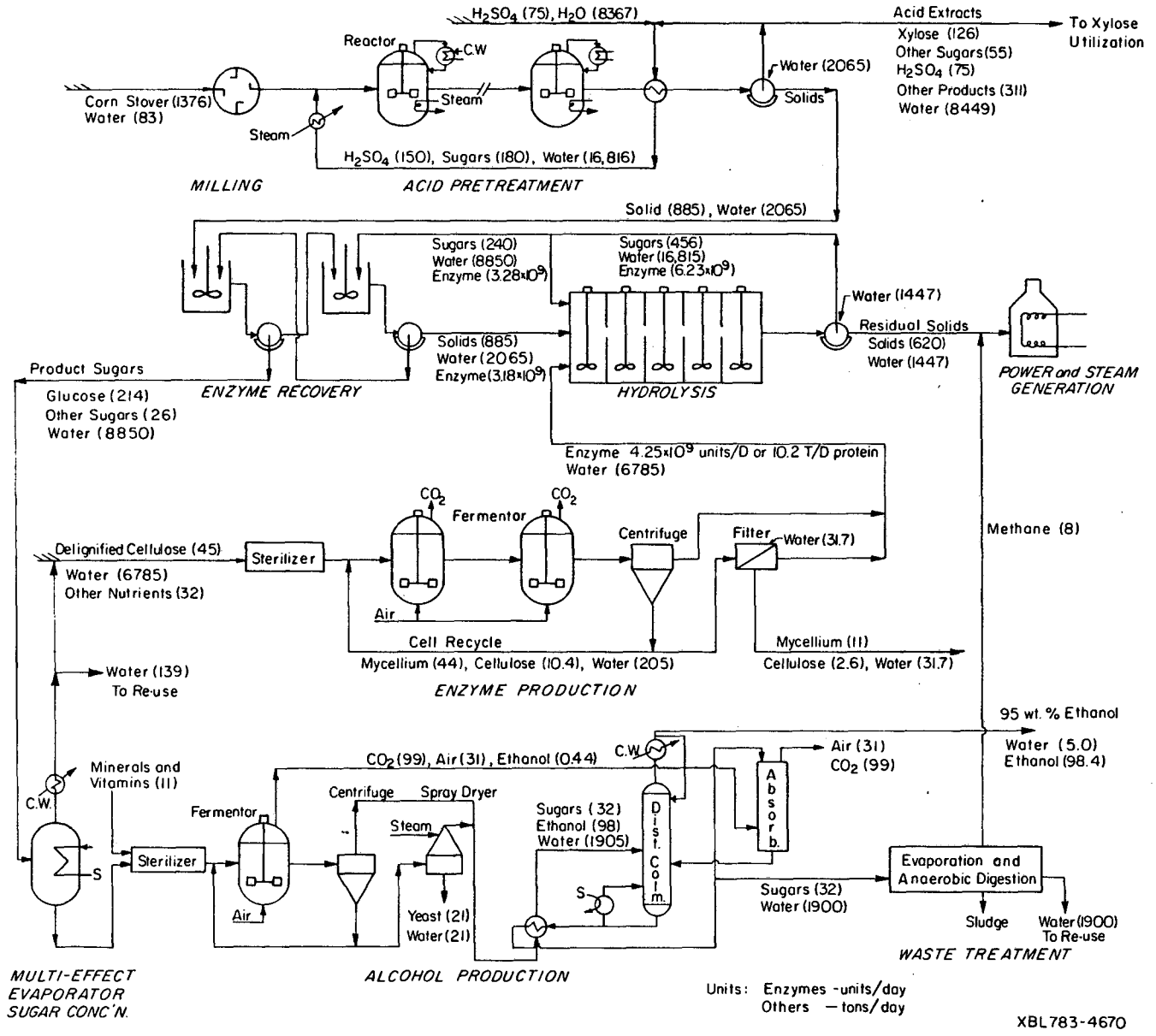


Figure 2 Detailed Flow Scheme of Current Process.

large fraction of the enzyme is lost. Recovery of the expensive enzyme would enhance the economics of the process significantly, considering that roughly 40% of the production cost of ethanol is attributed to enzyme production.

The primary consideration of this thesis is the reduction of the amount of enzyme lost in the hydrolysis. A direct approach to this goal is to maximize the concentration of enzyme remaining in solution during hydrolysis.

Reagents may be introduced to the hydrolysis to disrupt the adsorption process. In this work, the effects of urea, several ammonium salts, and glycine are observed. The highest level of enzyme remaining in solution is achieved with 1 M urea in the hydrolysis, in which 65% of the available enzyme is retained in solution.

The separation of urea from the aqueous sugar solution resulting from the hydrolysis is difficult. It was thought that a closed loop scheme could be employed whereby urea would remain in the aqueous stream through the distillation and be recycled back to the hydrolysis.

The unit operation most sensitive to this proposal is the glucose fermentation, in which the yeast are subjected to high concentrations of urea. Studies conducted in this work establish that the presence of urea is deleterious to normal yeast growth. The most pronounced effect is a

severe decline in the maximum specific growth rate. At concentrations greater than 0.5 M urea, severe metabolic changes occur. Data indicate increased specific ethanol productivities, yields(ethanol/glucose consumed), and maintenance requirements with increasing urea concentrations. The yeast cannot tolerate a concentration of urea greater than 1 M. Therefore, a complete recycle scheme is not feasible.

It is concluded that the most effective recovery alternative consists of washing the cellulosic residue with a concentrated urea solution following hyrolysis. The enzyme is recovered by ultrafiltration and recycled back to the hydrolysis. The urea solution is recycled within the wash operation. However, the economics of this alternative are dominated by the tonnage and cost of urea and it is concluded that the alternative is not economically feasible.

II. CELLULOSE HYDROLYSIS.

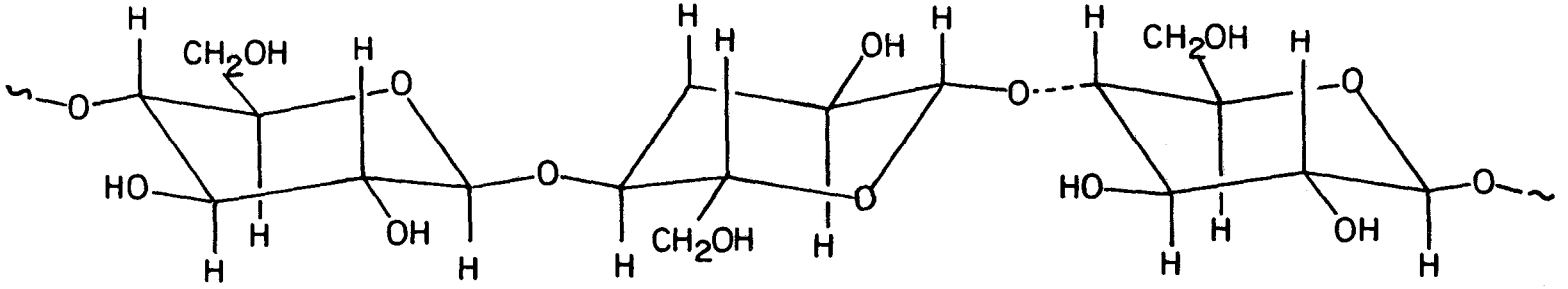
1. Background Information.

1.1. Cellulose.

Plant biomass, such as corn stover, consists of carbohydrate polymers, lignin, and small amounts of ash(7). Carbohydrates dominate the composition of the cell wall in native plant structures whereas lignin is concentrated in the middle lamella between cells(8). The carbohydrates are made up of a-cellulose (primarily hexosans) and hemicellulose (primarily pentosans). In corn stover, hexosans account for 35% of the total dry weight, pentosans 15%, lignin 15%(9).

Cellulose is a hydrophilic linear homopolymer of D-glucose (a hexosan), consisting of several thousand units linked by β,1-4 glucosidic bonds(10). The conformational formula of cellulose is shown in Figure 3. Hydroxyl groups occupy equatorial positions and hydrogen atoms are positioned axially. The glycosidic linkages and hydroxyl appendages act as the primary functional groups for all significant chemical reactions.

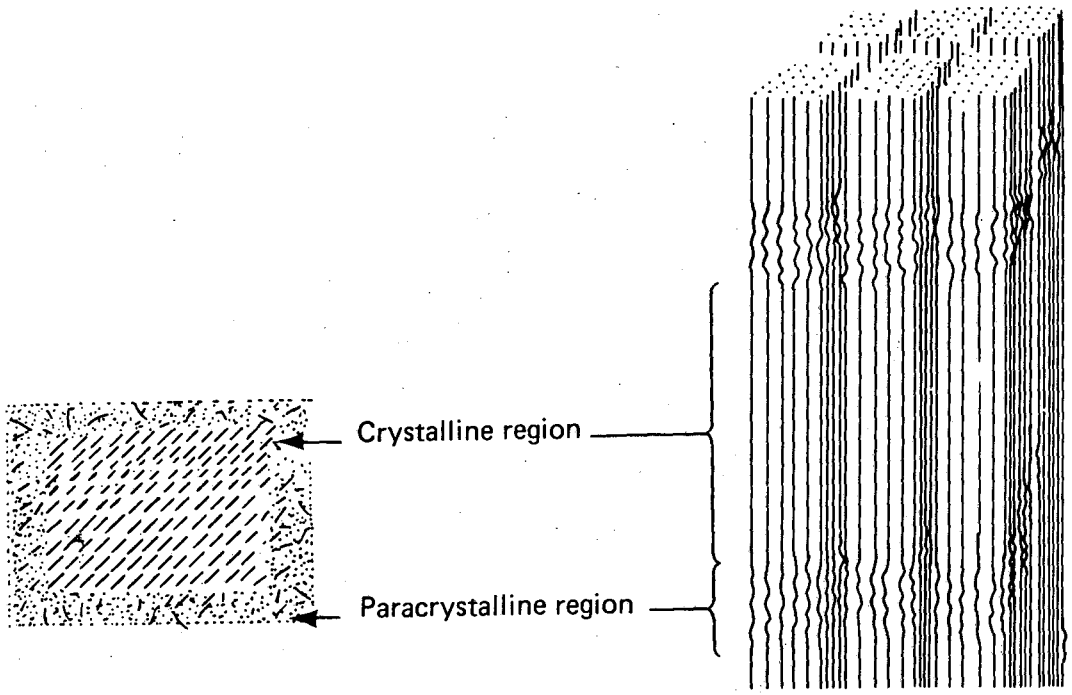
Cellulose molecules are linked together to form elementary fibrils(10). Models involving folded fibrils have been frequently proposed based on observations of chain folding in linear synthetic polymers (Figure 4,

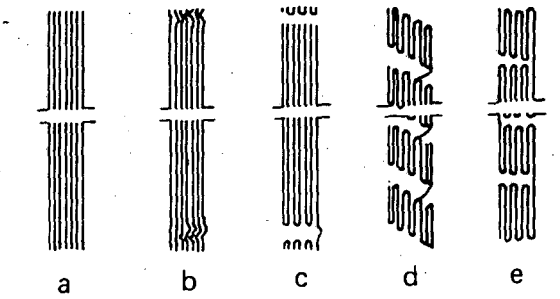


CONFORMATIONAL STRUCTURE OF CELLULOSE POLYMER

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Figure 3 Conformational Structure of Cellulose Polymer.





Models of Polymeric Nature of Cellulose

XBL 818-1087

Figure 4 Models of Polymeric Nature of Cellulose.

(11)). Chang(11) has concluded that model C is supported by most of the facts known about cellulose. Models without folding have been suggested as well.

Within the aggregates of elementary fibrils, referred to as crystalline cellulose because of the highly ordered structure, are regions of less ordered structure (amorphous or paracrystalline cellulose)(10). Surrounding all of these structures and acting like mortar are layers of lignin, which protect the cellulose from enzymatic action.

The amorphous structure is most amenable to enzymatic hydrolysis, provided the enzyme can physically approach the region, Figure 5(12, 13). Nisizawa(14) depicts a degradation sequence, illustrated in Figure 5. In a fibril composed of both crystalline and amorphous regions, the amorphous material in the peripheral structures is attacked quickly, leaving a greater fraction of crystalline material, only a fraction of which is easily accessible. The less accessible regions are degraded systematically: the less ordered structures (amorphous) are weakened, releasing crystalline segments to be dispersed and solubilized. As these segments are broken off, new sites for attack open up. Repetition of this sequence leads to a greater fraction of lignin and other indigestible material. The scenario is highly simplified, considering the structural diversity of natural cellulose.

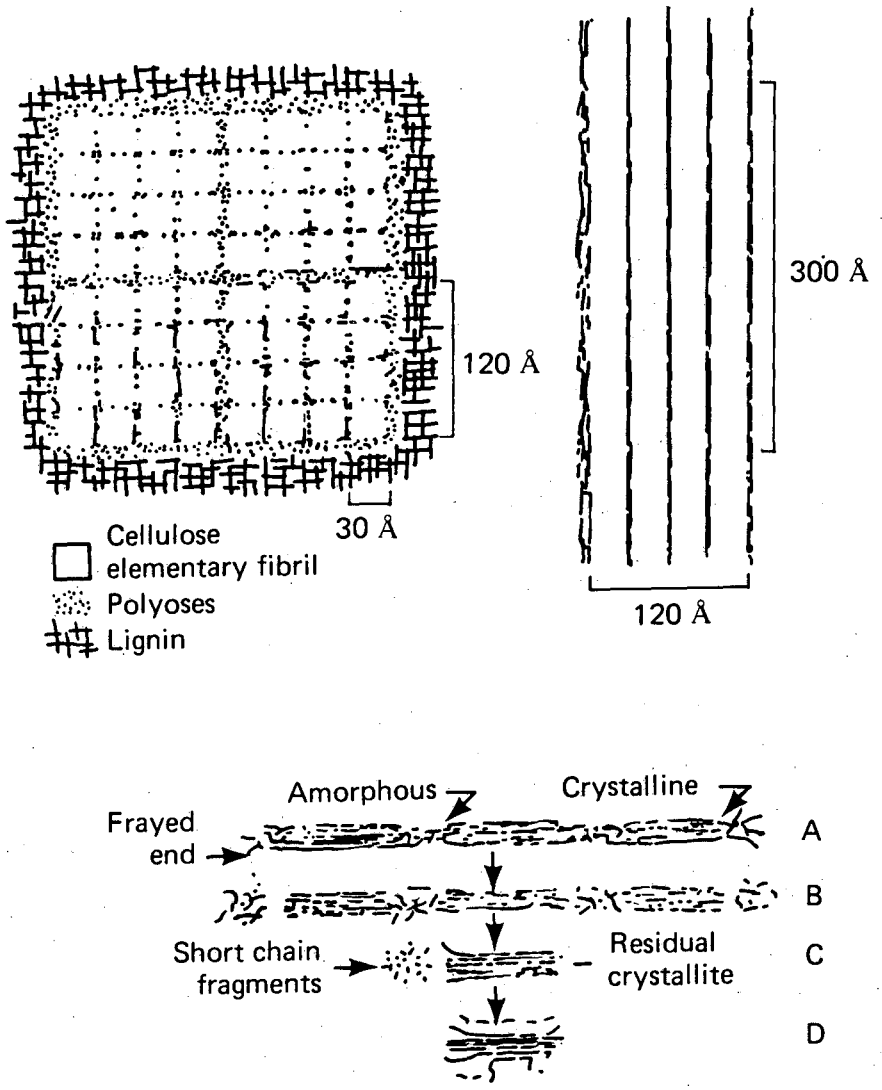


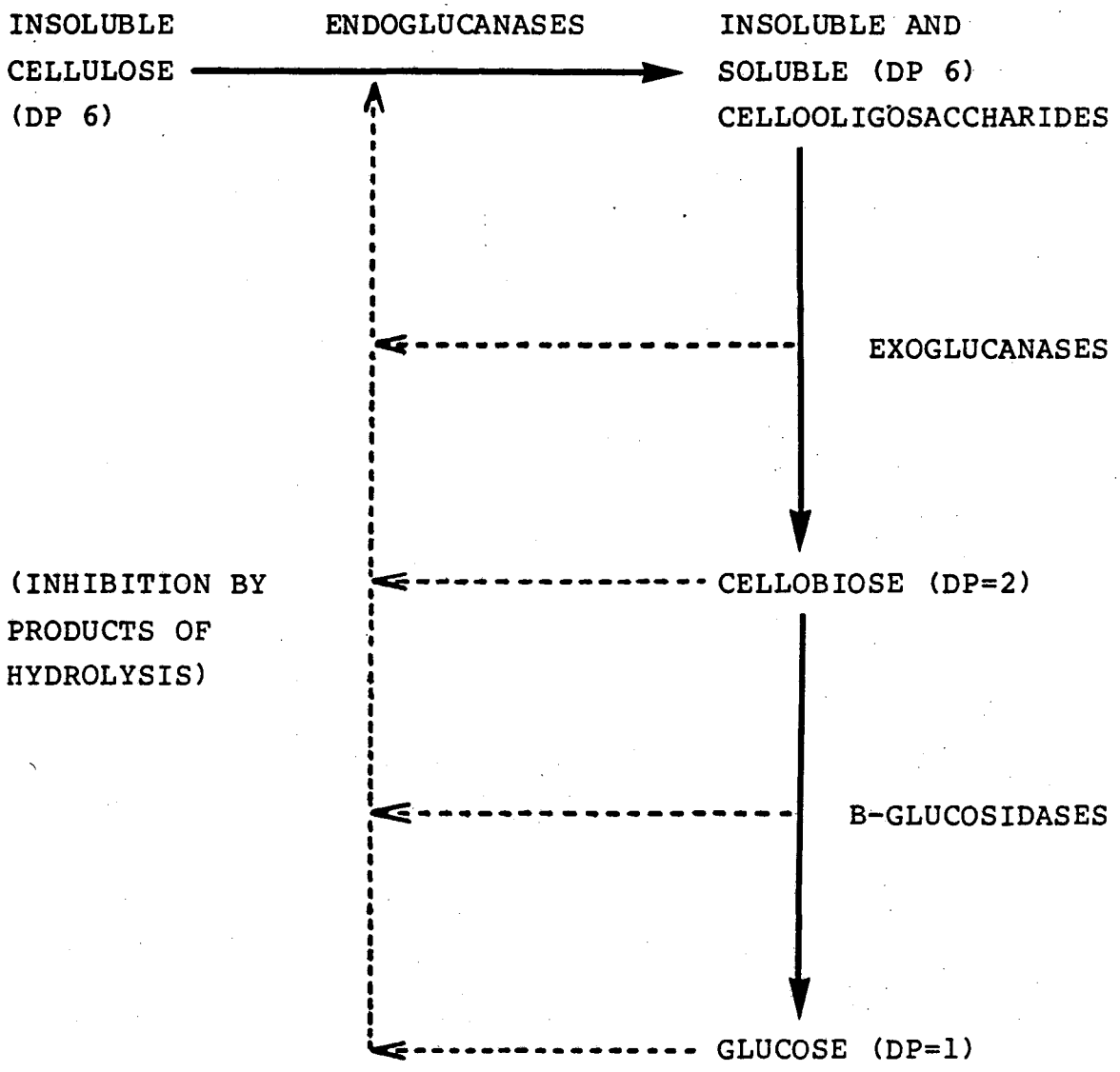
Figure 5 Physical Structure of Cellulose. Degradation Sequence.

1.2. Cellulase.

In recent years, fractionation experiments have permitted the isolation of three major types of cellulolytic activity in the cellulase enzyme system: β-1,4 glucan glucanohydrolase, an endo-enzyme known as C_x; β-1,4 glucan cellobiohydrolase, an exo-enzyme known as C_1; and β-glucosidase, an enzyme acting on cellobiose (dimer of glucose) known as β-G. Within these groups, minor components may be distinguished(15).

Incorporating the specific types of cellulose with experiments on these component enzymes, various enzyme/substrate preferences have been established. A current theory, supported by work in this laboratory and elsewhere(16, 17, 18), postulates that C_x acts randomly on the interior of the polymer to generate new chain ends. Its action is directed primarily toward the amorphous regions which are most easily accessible. C_1 acts on the non-reducing ends of the polymer chain, cleaving off cellobiose units. These soluble dimers and small polymers are hydrolyzed to glucose monomers by β-G.

Synergistic relationships between the various components complicate the separation of the enzymes. Negative feedback responses (e.g. inhibition) are illustrated in Figure 6, indicating that substrates and products, and the presence or absence of enzymes involving these compounds, can influence the entire sequence(15).



DP = degree of polymerization.

Figure 6. Mode of Action of Cellulase(15).

Isolated components often have little hydrolytic effect. C_x alone is not effective in solubilizing cellulose(19). In general, C_1 alone cannot degrade cellulose, especially amorphous cellulose(20). However, several reports indicate that C_1 acting alone can break down crystalline cellulose(20,21). Together, C_x and C_1 components reduce cellulose to cellobiose and minor amounts of larger oligomers and glucose. Cellobiose is a competitive inhibitor of C_1(22).

1.3. Hydrolysis Models.

Brandt, et. al.(23) reported on the hydrolysis of newsprint and proposed a feasible model which was further developed by Castanon and Wilke 24). Mandels, et. al.(25) discusses models of this type. Kim(26) has developed a quantitative model for the hydrolysis of pure cellulose incorporating most of the factors listed below.

Castanon and Wilke's studies(24) were conducted using newsprint as the substrate, degraded by QM9414 cellulase. Some of the factors included were: changing substrate reactivity, accessibility(23, 27), competitive(26, 28, 29, 30) and noncompetitive(30) inhibition, enzyme immobilization/deactivation (Howell and Mangat, 31), and the presence of indigestible materials.

The hydrolysis of cellulose is a heterogeneous reaction, taking place at a solid-liquid interface. Reactions occur at varying rates due to the multiplicity of

substrates and enzymes. There are three distinct rate regimes defined by Brandt, et. al(23).

An initial stage occurs in the first four hours of contact. Fast initial uptake of enzyme corresponds to the adsorption of enzyme on a fraction of newspaper which is highly reactive. This fraction is of low porosity with high surface area and contains loose ends. Adsorption is believed to occur in the first hour of contact. The enzyme proteins with high affinities for their specific substrates are adsorbed most quickly (C_x), while components with lower affinities for the substrate are adsorbed later and more slowly(C_1). Non-cellulase proteins are adsorbed even more slowly. The enzymes are being immobilized within the substrate, but retain their hydrolytic activities. They are able to act only on neighboring sites which, in the initial stage, are still plentiful(23).

A transitional stage (6 hrs.) of slightly less vigorous hydrolysis is observed primarily because of the action of C_x on amorphous cellulose. In the earlier stage of this period, there is more amorphous cellulose than crystalline cellulose. Adsorption of C_x occurs more rapidly than C_1. Also, C_x appears to possess a higher specific reactivity(24). This reactivity may be due to the higher mobility of C_x and lack of strong binding tendencies. Also, several investigators have observed that C_1 is inhibited by cellobiose, present in high

concentrations at this stage (see earlier discussion).

New types of substrates and reactive sites are being created during the hydrolysis(see Figure 5). These sites will be occupied only if they are accessible and there is sufficient free enzyme in solution. The cellulose in the vicinity of the immobilized enzyme is exhausted. Specific adsorption of enzymes, both C_x and C_1, decreases as the easily accessible sites are depleted and access by the enzyme is hindered by the accumulation (enrichment) of crystalline cellulose and indigestible materials, particularly lignin. In short, substrate accessibility becomes an important limiting factor. Concurrently, the enzyme concentration in solution is decreasing(23).

The final stage occurs after the first 10 hrs. Hydrolysis proceeds at a rate that is limited only by the amount of free enzyme in solution and by the rate of enzyme transfer to specific binding sites which depends on substrate accessibility. The available amorphous cellulose has been consumed so that protein uptake is limited almost entirely to C_1. Adsorption of C_1 proceeds slowly due to the structural complexity of crystalline cellulose, which makes susceptible binding sites difficult to be reached. However, this action does provide some new binding sites for other enzymes, accounting for minimal uptake of C_x(23).

No significant amount of enzyme is released into solution. However, it is not possible to distinguish

whether the enzyme is desorbed and immediately binds to an adjacent reactive site or remains adsorbed and moves along the cellulose fibrils. It is also possible that some cellulase is unspecifically adsorbed, especially since this property of cellulose and its derivatives is applied in ion exchange columns and other devices.

Other studies have yielded discrepant results. Brandt,et. al.(23) found that protein concentration increased without displaying enzyme activity. Enzyme deactivation was postulated. However, it may be that non-specific proteins were being released. Wilke and Yang(32, 33) observed a gradual increase in enzyme after its initial strong adsorption.

Studies with purified cellulose have yielded enlightening results. Mandels, et. al.(34) reported that the enzyme desorbed and returned to solution within 24 hrs., because 50% of the cellulose had been digested. Howell and Mangat(31) have observed with solka floc that protein, CMC activity and to a lesser extent, FPA, increase in concentration in solution during the course of a hydrolysis. This suggests that perhaps indigestible materials not found in purified cellulose, such as lignin, may be binding the enzyme during the later stages of digestion.

Product inhibition models based on studies of solka floc are proposed by Howell and Stuck(30) and Mangat and

Howell(35) to explain changes in reaction rates. Castanon and Wilke(24) conclude that competitive inhibition is exhibited only under initial rate conditions. However, inhibition by products such as cellobiose may immobilize the enzyme within the structural matrix of cellulose. This effect may be due to conformational or activity changes (loss of ability to "eat its way out"). Access of new enzyme molecules would be prevented from degrading the substrate, causing loss of enzyme. Sugar production would suffer because of loss of both substrate accessibility and enzyme.

1.4. Immobilization/Adsorption Phenomena: Theory.

Working models of the physical and chemical features of cellulose and the cellulase system have been established. Components of cellulase are bound preferentially in some manner to the heterogeneous substrate, perhaps including regions of indigestible material. This action results in loss of enzyme, an expensive commodity. Thus,it would be economically beneficial to recover the enzyme, thereby reducing the amount of enzyme required per unit of product ethanol.

The recovery of cellulases would be enhanced greatly if the immobilization/adsorption phenomena could be disrupted without interfering with the hydrolytic functions of the enzymes.

Globular protein molecules often exhibit complete unfolding of the tertiary structure to give films, at a phase interface having the thickness of a single polypeptide chain. This action is often referred to as "surface denaturation". Further, desorption is a process with a very high activation energy since all parts of the molecule must leave the interface together. These and other observations have led to a widespread belief in the irreversibility of protein adsorption(MaCritchie, (36)).

Based on work by Dillman and Miller(37), adsorption of proteins on a variety of polymer membranes may be classified by two isotherms. One system is defined by being easily reversible, hydrophilic, and exothermic with ΔH_{ads} values of about -40 kJ mol^{-1}, expected for a condensation change. The other isotherm is defined as being tightly bound, of a hydrophobic nature, and endothermic. ΔH_{ads} ranged from 20-80 kJ mol^{-1}, on the order of chemical bond formation.

Adsorption of cellulase protein has been found to be temperature dependent(38, 39, 40). Maximum specific adsorption of components is reported to occur at 50°C. A decline at temperatures greater than 50°C may be attributed to heat instability of the enzyme. Results in this laboratory and others(41, 42) have shown that cellulase protein adsorbed at lower temperature can be recovered simply by washing the residues.

It may be concluded that perhaps the components are being tightly bound to a hydrophobic substrate, leading to an endothermic reaction. Since cellulose is hydrophilic by nature, the enzyme molecules may be binding to either hydrophobic regions of the polymer or, more likely, to indigestible materials (e.g. lignin) of a hydrophobic character.

1.5. Immobilization/Adsorption:

Techniques For Disruption.

Means to prevent the loss of cellulase during a hydrolysis conducted at 45°C are being explored. Several methods of approaching the problem from a process viewpoint are available.

Factors which can be altered include:

a) the substrate.

b) the enzyme.

c) the environment (e.g. the bulk solution).

Conditions may be altered prior to, during, or following hydrolysis.

1.5.1. Alteration of Substrate. The substrate may be either pretreated or further digested at the end of hydrolysis by means other than cellulase. Studies of pretreatment are being conducted currently(43). Digestion following hydrolysis would remove the material binding the enzyme, releasing it by simple physical means. Mandels, et. al(34) reports that further digestion is successful with purified cellulose, but does not appear to work on substrates containing indigestible compounds. Current methods of degrading lignin and other residues, several of which are applied in the process for pretreatment, are much too harsh for cellulase, leading to complete loss of enzyme.

The cellulosic surface may become coated with the

reagent. The enzyme is prevented from binding permanently because the functional groups on the cellulose structure are bound. Kinetic models such as those developed for solid support structures (e.g. ion exchange resins, enzyme immobilization techniques) may lend insight to the action of the reagent and/or enzyme on the cellulose, since cellulose and its derivatives are often utilized as resins.

The reagent molecules may also block accessible pores of the cellulose matrix, preventing the entrapment of enzyme molecules. This layer action would probably lead to decreased hydrolysis rates since the reagent would act as a physical barrier.

1.5.2. Alteration of Enzyme. Immobilization of B-G component on solid supports has been investigated in detail and has proven effective(45). The application of this technique to C_x and C_1 components has not proven as fruitful. The intermolecular forces and reduction in surface potential due to contact between enzyme and substrate are not strong enough to overcome the shear forces exerted on the bulk cellulosic particles in agitated vessels. Also, immobilization on a support reduces the mobility of the enzyme, isolating it from more intimate contact with the substrate and inhibiting it from entering the pores of substrate particles. However, studies are being conducted to overcome these barriers (e.g. reduction of support size by utilizing magnetic materials.

Techniques for the concentration and purification of enzyme may be applied. Precipitative methods include salt and/or solvent concentration, isoelectric precipitation and concentration by ultrafiltration(45).

Cohn(46) suggested that the solubility of protein could be represented by the linear relationship

$$\log S = B - (K_s \times C_s) \qquad (1)$$

where S is the protein solubility, C_s is the concentration of the salt, B and K_s are constants for the particular system. Further refinement substitutes the salt activity for C_s(47). It should be noted that precipitation methods yield best results when a high concentration of enzyme is present in solution(48).

Dixon and Webb(48) have investigated several theoretical and applied aspects of precipitation of proteins in concentrated salt solutions. The main force governing protein-protein interaction is the surface potential, which is reduced to very low values at high salt concentrations. As an example, "ammonium sulfate solutions are very effective in eluting proteins from even powerful adsorbents"(48).

Ammonium sulfate is commonly used as a precipitating agent in enzyme fractionations. Gray, Dunnill, and Lilly(49) isolated B-G using ammonium sulfate in a

continuous process precipitation. In general, sodium sulfate is somewhat more efficient, but much less soluble and magnesium sulfate is less effective, giving low values of K_s. Phosphates work very well, yielding high values of K_s. Chlorides are totally ineffective(48).

Deformation caused by excessively harsh conditions may lead to irreversible denaturation of the enzyme components. Perhaps partial denaturation would alter the binding ability of the enzyme without eliminating its hydrolytic ability. Controlled restructuring is not likely until more is known about the components, when each can be dealt with individually.

Note that denaturation often occurs with the precipitation of an enzyme. In this paper, denaturation usually refers to irreversible changes leading to impaired hydrolytic ability.

1.6. Studies on Cellulase Recovery.

Interactions in cellulose-cellulase systems may involve one or more of these mechanisms. Observations reported in the production of cellulase may lead to insights in the hydrolysis. An effect of surfactants has been observed in the production of cellulase. Addition of Tween 80 to fungal cultures of QM9414 resulted in a 13-fold increase in cellulase production(24). Observations with Rut-C-30 indicate that a Tween 80 concentration of 0.02% has no effect, but 0.1% reduces extracellular enzyme

activity by 40%(15). Addition of Tween 80 to newsprint hydrolyses resulted in greater sugar production(24). Moreover, the percentage increase in sugar concentration increased over time: at 10hrs., 5.81 % higher; at 20 hrs., 8.11 %; at 48 hrs., 12.0%. The effects are most noticiable as the crystalline cellulose concentration is enriched(24).

Riaz and Wilke(38) introduced several reagents into the hydrolysis of newsprint and solka floc with cellulase of QM9414. Results are listed in Table 1. Riaz and Wilke optimized the concentration of urea to keep a high concentration of enzyme in solution. The most encouraging result was 67% of the original enzyme activity remained in solution using 0.9 M urea in the hydrolysis. Further details are reviewed in later discussions.

The primary consideration of this thesis is the reduction of the amount of Rut-C-30 enzyme lost in the hydrolysis of corn stover. To this end, the amount of enzyme remaining in solution may be maximized. The effects of urea may be extrapolated to this system. Further, other reagents may be applied to seek an alternative to urea that is: 1) more effective, 2) more economical, or 3) more easily recovered.

Material	Recovery from Solids (%) 40 hr. hydrolysis FPA	C_x
a) Triton X-100, octyl-phenoxy-polyethoxy ethanol. 5% solution. Surfactant.	9	16
b) Borate-glycerol buffer. 0.4 M solution. Breaks bonds between proteins and carbohydrates.	7	15
c) pH shift. Optimum for FPA: pH 10. Optimum for C_x: pH 7, 9. Exploits amphoteric nature. Usually, large shift required.	10	18
d) Guanidine-HCl. 0.1 M solution. (With only enzyme in solution, 10% remaining after 24 hrs.)	No protein.	

Table 1. Summary of Results of Riaz and Wilke(38).

2. EXPERIMENTAL PROCEDURE.

2.1. Analytical Procedures.

2.1.1. Reducing Sugar Assay.

Reducing sugar was measured by Long's modifications(50) of the dinitrosalicylic acid(DNS) method of Sumner and Somers(51). See the appendix, Enzyme Assay.

2.1.2. Soluble Protein Assay.

Protein was measured by the method of Lowry, et. al.(52) without precipitation as modified in the Biochemistry Dept., University of California, Berkeley(53) and in this laboratory with crystalline bovine serum albumin as standard. See appendix, Soluble Protein Assay.

2.1.3. Enzyme Assay.

The hydrolytic activity shown by the crude enzyme solution acting on filter paper (Filter Paper Activity, FPA) gives a general estimation of its catalytic strength. Cellulase activities were followed by production of reducing sugars measured as glucose. Assay procedures suggested by Mandels and Weber(54) were followed with minor modifications. See appendix, Enzyme Assay.

2.2. Experimental Apparatus and Methods.

2.2.1. Enzyme Preparation.

The cellulase enzyme system was obtained from culture filtrates of the fungus, *Trichoderma reesei*, strain

Rut-C-30. The Rut-C-30 strain, developed by Montanencourt and Evenleigh(55), is a hyperproducing extracellular cellulase strain resistant to catabolite repression.

Viable cultures of Rut-C-30 were maintained on PDA slants. A modified fermentation media was employed(56), based on that reported by Mandels and Weber(54). Solka floc (cellulose) was the carbon source. Tween 80, a surfactant, was added to increase productivity.

The pH and temperature were controlled at or near optimum levels for the production of cellulase. Optimum values were pH 5, 25°C, and 5% iniitial carbon source, as determined by Tangnu, et.al.(56).

The raw cell culture was filtered through glass wool and refiltered through the cake of resulting mycelium. This solution was then filtered through a Millipore 0.4μM Prefilter. The enzyme solution was preserved with 0.02% sodium azide and stored at 4°C.

2.2.2. Materials.

Urea, ammonium sulfate, and ammonium chloride were of analytial reagent grade, obtained from Mallinckrodt Chemicals. Ammonium oxalate and glycine were of reagent grade, obtained from Matheson, Coleman, and Bell(MCB). Dialysis tubing consisted of regenerated cellulose acetate, with a molecular weight cut-off of 10,000 and average pore radius permeability of 24 Angstroms. The tubing is manufactured by Van Waters and Rogers in a viscose process.

2.2.3. Hydrolysis Method.

The hydrolysis substrate was Indiana corn stover, ball-milled to 2 mm particle size, and pretreated with sulfuric acid. The initial contact between enzyme and substrate was carefully controlled. To equal volumes of a separate substrate solution (10% solids by weight) and enzyme solution (7.0 FPA) are added a specified reagent (e.g. urea at 1 M). The reagent is slowly dissolved and the solutions are allowed to remain for 1 hr.. All solutions are made up with 0.05 M sodium acetate buffer at room temperature.

The solutions were mixed and immediate agitation was imposed. Hydrolyses were conducted in a series of 600 ml. Pyrex beakers sealed with black rubber stoppers with sample ports. Teflon propeller stirrers were inserted, operating continuously at 300 RPM. The vessels were immersed in a 45^{o}C water bath, under non-aseptic conditions.

Samples of 25 ml. were taken at 20, 40, and 80 hrs. during the hydrolysis. Agitation was not stopped during sampling. Samples were centrifuged at 20xG to separate supernatants and solids. Supernatants were subjected to dialysis in cellulose acetate dialysing tubing against distilled water at 4^{o}C for 20 hrs. Dialysis water at a dilution of one volume of sample per 100 volumes of distilled water was agitated in polyethylene tanks by a teflon stirrer which simultaneously agitated the dialysis

tubing segments. Dialyzed samples were diluted to a total volume of 30 ml. and subjected to analysis.

The solids in the samples were crushed with mortar and pestle and washed with a concentrated reagent solution. The solution was centrifuged and the supernatant was treated with the preceeding procedures. At times, the reagent solution was different from the hydrolysis reagent to observe multiple effects of combinations (see Results and Discussion).

Control samples of enzyme (without substrate) with and without reagent solution run in identical hydrolysis conditions and were found to be very reproducible. These samples were not centrifuged.

3. RESULTS AND DISCUSSION.

3.1. Time and Heat Stablity.

Degradation of the cellulose system at temperatures near 4°C is slight. Enzyme can be stored for several weeks with little decrease in activity, although a period of a month or longer results in a slow decline, with specific components changing at different rates(57).

Loss of activity at 45°C, the hydrolysis temperature, is slow, but significant. Over a 40 hr. period, degradation attributed to time and/or temperature is roughly 5-10% and occurs approximately by first-order kinetics. When the enzyme is subjected to dialysis (see Hydrolysis Method), a loss of 5% is observed. Therefore, when a pure enzyme sample is run as a control, losses account for a decrease of about 10-15%. These control samples were produced for every series to account for variations in crude enzyme activity (specific components), dialysis degradation, and enzyme assays.

3.2. Interpretaton of Recovery Data.

Physical properties of the reagents under study are listed in Table 2.

The following tables and graphs present the recovery data. If not noted otherwise, comparisons are made between samples taken at 40 hrs. The percentage recovery is

MATERIAL	MOL. WT.	D.P. (°C)	G N/G SALT	SOLUBILITY (G/L) (0°C)	--(°C)	PRICE ($/TON)
UREA	60.06	D.	0.47	600		160-175
AMM. SULFATE	132.14	D.235	0.21	706	1038^{100}	65-89
AMM. CHLORIDE	53.49	S.340	0.26	297	758^{100}	231
AMM. OXALATE	142.11	D.	0.20	25.4	118^{50}	2300
GLYCINE	75.07	D.262	0.37			3540

KEY.
D. = TEMPERATURE OF DECOMPOSITION.
S. = TEMPERATURE OF SUBLIMATION
SOLUBILITY = SOLUBILITY IN WATER,
PER LITER OF SOLUTION.
PRICE = LOWEST PRICE QUOTED FOR TECHNICAL GRADE,
50 TON CAR LOTS, DELIVERED.
QUALITY MAY BE HIGHER THAN REQUIRED.

TABLE 2. PROPERTIES OF REAGENTS(86).

UREA.

MOLARITY (G-MOL/L)	SPECIFIC GRAVITY 20/20	OSMOLALITY (OS/KG)	OSMOSITY (G-MOL/L)
1.531	1.565	Ø.846	1.Ø238
2.Ø59	2.125	1.138	1.Ø322

KEY:
OSMOSITY = MOLAR CONCENTRATION OF NACL SOLUTION HAVING THE SAME FREEZING POINT OR OSMOTIC PRESSURE AS THE GIVEN SOLUTION, IN G-MOL/L.

TABLE 2, CONTINUED. PROPERTIES OF REAGENTS(86).

defined as the fraction of original enzyme activity at initialization of hydrolysis(time zero), in liquid (hydrolysis supernatant) or in solid (wash solution). The total recovery is calculated by adding the fraction of enzyme retained in the liquid (supernatant) (Tables 3, 4, 5) to the fraction of enzyme washed from the solid residue (Table 6). The data are plotted in Figures 7, 8, 9, 10, 11. In this discussion, the "maximum recovery" refers to the highest recovery observed for a particular salt over the range of salt concentrations explored.

Runs of enzyme without substrate or salt acted as controls. Runs of enzyme with salt alone were made to observe the effects on the enzyme. Occasionally, data indicate that more enzyme is recovered at 40 hrs. than at 20 hrs. This effect may be real, but its actual impact is lessened because the control enzyme runs show a decline in activity, as noted earlier.

3.3. Urea.

Urea is commonly used in the paper pulping industry. Pretreatment with the reagent causes the cellusoic structure to soften(58), permitting the production of a finer, more homogenous product.

Urea, and other amide derivatives such as guanidine, denature proteins. The hydrophobic nature and bi-functional hydrogen bonding activities of urea may be factors in its behavior towards proteins. This type of

Table 3

Recovery of Enzyme with Salts
(fractional recovery)

Run #	Sample Time (hr.)	Compound in Hydrolysis Liquid	Molarity	Recovery in Liquid	Total Recovery Liquid + Solid	Sugar Production (g/l)
1.2	20	none	--	0.174	~~0~~	~~0~~
	40	none	--	0.143	0.207	16.2
1.3	20	urea	0.5	0.294	~~0~~	~~0~~
	40	urea	0.5	0.328	0.405	11.0
1.4	20	urea	1.0	0.515	~~0~~	~~0~~
	40	urea	1.0	0.573	0.648	15.0
1.5	20	urea	2.0	0.776	~~0~~	~~0~~
	40	urea	2.0	0.515	0.570	13.4
1.6	20	urea (no substrate)	2.0	0.111	--	--
	40	urea (no substrate)	2.0	0.020	--	--
1.7	20	ammonium sulfate	0.20	0.152	~~0~~	~~0~~
		ammonium sulfate	0.20	0.134	0.137	11.4
1.8	20	ammonium sulfate	0.38	0.080	~~0~~	~~0~~
	40	ammonium sulfate	0.38	0.079	0.083	1.6
1.9	20	ammonium sulfate	0.76	0.029	~~0~~	~~0~~
	40	ammonium sulfate	0.76	0.033	0.038	0.1
1.10	20	ammonium sulfate (no substrate)	0.76	0.717	--	--
	40	ammonium sulfate (no substrate)	0.76	0.867	--	--

See Table 6 for symbol key.

Table 4

Recovery of Enzyme with Salts
(fractional recovery)

Run #	Sample Time (hr.)	Compound in Hydrolysis Liquid	Molarity	Recovery in Liquid	Total Recovery Liquid + Solid	Sugar Production (g/l)
2.2	20	none	--	0.419	0.471	~~0~~
	40	none	--	0.373	0.396	17.5
2.3	20	glycine	0.2	0.364	0.421	~~0~~
	40	glycine	0.2	0.282	0.323	15.5
2.4	20	glycine	1.0	0.299	0.341	~~0~~
	40	glycine	1.0	0.235	0.268	1.1
2.5	20	glycine	3.0	0.104	0.121	~~0~~
	40	glycine	3.0	0.083	0.089	0.3
2.6	20	glycine (no substrate)	3.0	0.632	--	--
	40	glycine (no substrate)	3.0	~~00~~	--	--
2.7	20	ammonium oxalate	0.1	0.440	0.482	~~0~~
	40	ammonium oxalate	0.1	0.381	0.404	13.7
2.8	20	ammonium oxalate	0.5	0.168	0.203	~~0~~
	40	ammonium oxalate	0.5	0.104	0.112	0.6
2.9	20	ammonium oxalate	0.83	0.095	0.147	~~0~~
	40	ammonium oxalate	0.83	0.073	0.082	0.5
2.10	20	ammonium oxalate (no substrate)	0.83	0.394	--	--
	40	ammonium oxalate (no substrate)	0.83	0.424	--	--

See Table 6 for Symbol Key.

Table 5

Recovery of Enzyme with Salts
(fractional recovery)

Run #	Sample Time (hr.)	Compound in Hydrolysis Liquid	Molarity	Recovery in Liquid	Total Recovery Liquid + Solid	Sugar Production (g/l)
3.2	20	none	--	0.396	0.443	14.6
	40	none	--	0.291	0.323	17.0
3.3	20	ammonium chloride	0.08	0.294	0.327	14.2
	40	ammonium chloride	0.08	0.255	0.282	15.9
3.4	20	ammonium chloride	0.3	0.404	0.440	11.6
	40	ammonium chloride	0.3	0.206	0.235	12.5
3.5	20	ammonium chloride	0.94*	0.310	0.350	0.1
	40	ammonium chloride	0.94	0.172	0.202	0.1
3.6	20	" (no substrate)	0.94	0.787	--	--
	40	" (no substrate)	0.94	0.945	--	--
3.7	20	ammonium oxalate	0.01	0.366	0.406	14.3
	40	ammonium oxalate	0.01	0.310	0.357	16.0
3.8	20	ammonium oxalate	0.04	0.291	0.334	14.0
	40	ammonium oxalate	0.04	0.328	0.357	15.5
3.9	20	ammonium oxalate	0.10	0.215	0.253	12.3
	40	ammonium oxalate	0.10	0.239	0.274	13.7
3.10	20	" (no substrate)	0.10	0.915	--	--
	40	" (no substrate)	0.10	0.988	--	--

*satuarated (salt crystals present).
See Table 6 for Symbols Key.

Table 6

Recovery of Enzyme in Solid
(fractional recovery)

Run #	Sample Time (hr.)	Compound in Wash Solution	Molarity	Recovery in Solid
1.2	20	urea	3.33	-0-
	40	urea	3.33	0.064
1.3	20	urea	3.33	-0-
	40	urea	3.33	0.077
1.4	20	urea	3.33	-0-
	40	urea	3.33	0.075
1.5	20	urea	3.33	-0-
	40	urea	3.33	0.055
1.6	20			--
	40			--
1.7	20	ammonium sulfate	2.02	-0-
	40	ammonium sulfate	2.02	0.003
1.8	20	ammonium sulfate	2.02	-0-
	40	ammonium sulfate	2.02	0.003
1.9	20	ammonium sulfate	2.02	-0-
	40	ammonium sulfate	2.02	0.005
1.10	20			--
	40			--
2.2	20	glycine	2.6	0.052
	40	ammonium oxalate	0.5	0.023
2.3	20	glycine	2.6	0.057
	40	glycine	2.6	0.041
2.4	20	glycine	2.6	0.042
	40	glycine	2.6	0.033
2.5	20	ammonium oxalate	0.5	0.017
	40	glycine	2.6	0.006
2.6	20			--
	40			--
2.7	20	ammonium oxalate	0.5	0.042
	40	ammonium oxalate	0.5	0.023

continued...

Table 6 Continued

Recovery of Enzyme in Solid
(fractional recovery)

Run #	Sample Time (hr.)	Compound in Wash Solution	Molarity	Recovery in Solid
2.8	20	ammonium oxalate	0.5	0.035
	40	ammonium oxalate	0.5	0.008
2.9	20	acetate buffer (pH 5)	0.05	0.052
	40	ammonium oxalate	0.5	0.009
2.10	20			--
	40			--
3.2	20	ammonium chloride	0.94	0.047
	40	ammonium chloride	0.94	0.032
3.3	20	ammonium chloride	1.25	0.033
	40	ammonium	5.00	0.027
3.4	20	ammonium chloride	1.25	0.036
	40	ammonium chloride	5.00	0.029
3.5	20	ammonium chloride	1.25	0.040
	40	ammonium chloride	5.00	0.030
3.6	20			--
	40			--
3.7	20	ammonium oxalate	0.12	0.040
	40	ammonium oxalate	0.24	0.037
3.8	20	ammonium oxalate	0.30	0.043
	40	ammonium oxalate	0.30	0.029
3.9	20	ammonium oxalate	0.30	0.038
	40	ammonium oxalate	0.30	0.035
3.10	20			--
	40			--

Symbols
-- no data possible--refer to previous tables
-0- no data taken
-00- data obvious in error

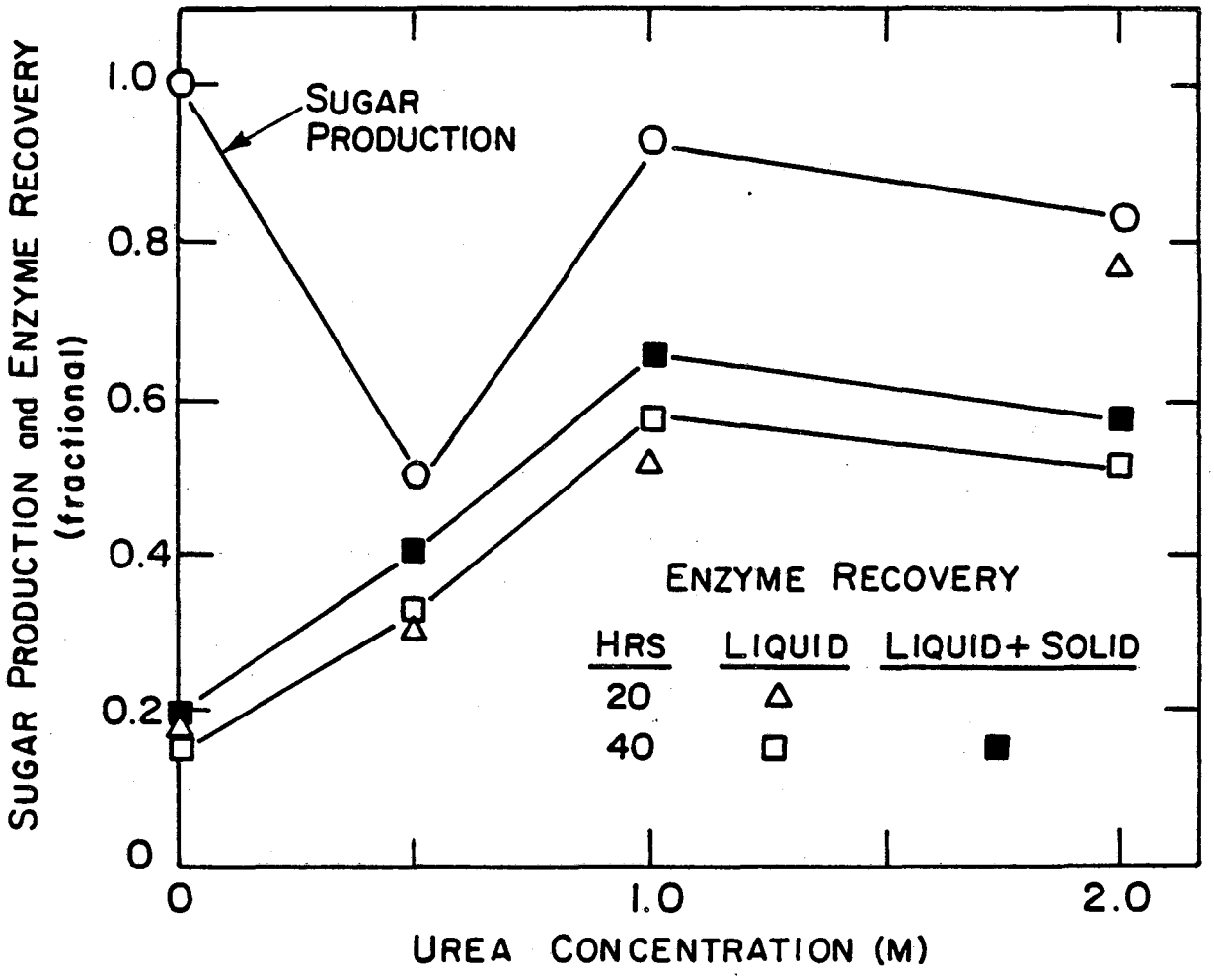


Figure 7 Effect of Urea on Sugar Production and Enzyme Recovery.

XBL 815-5700

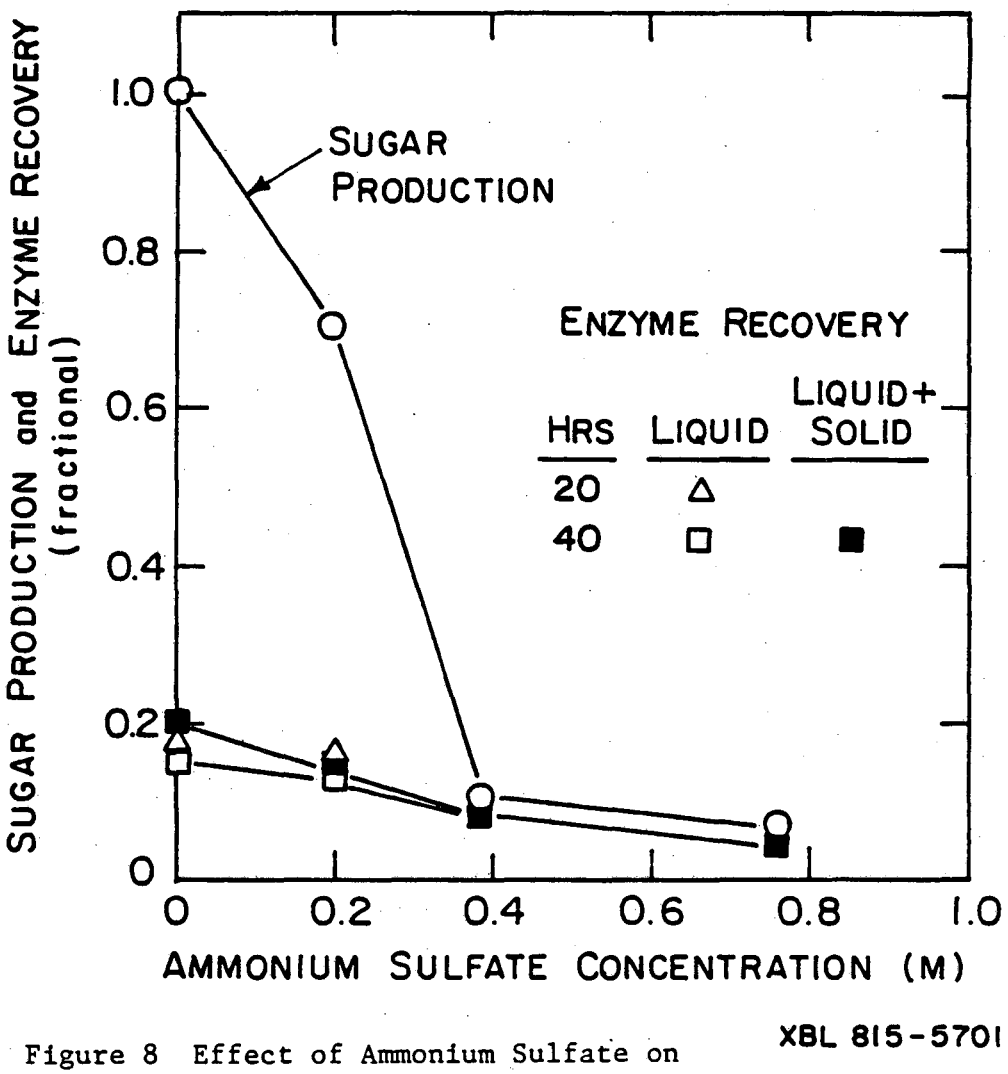


Figure 8 Effect of Ammonium Sulfate on Sugar Production and Enzyme Recovery.

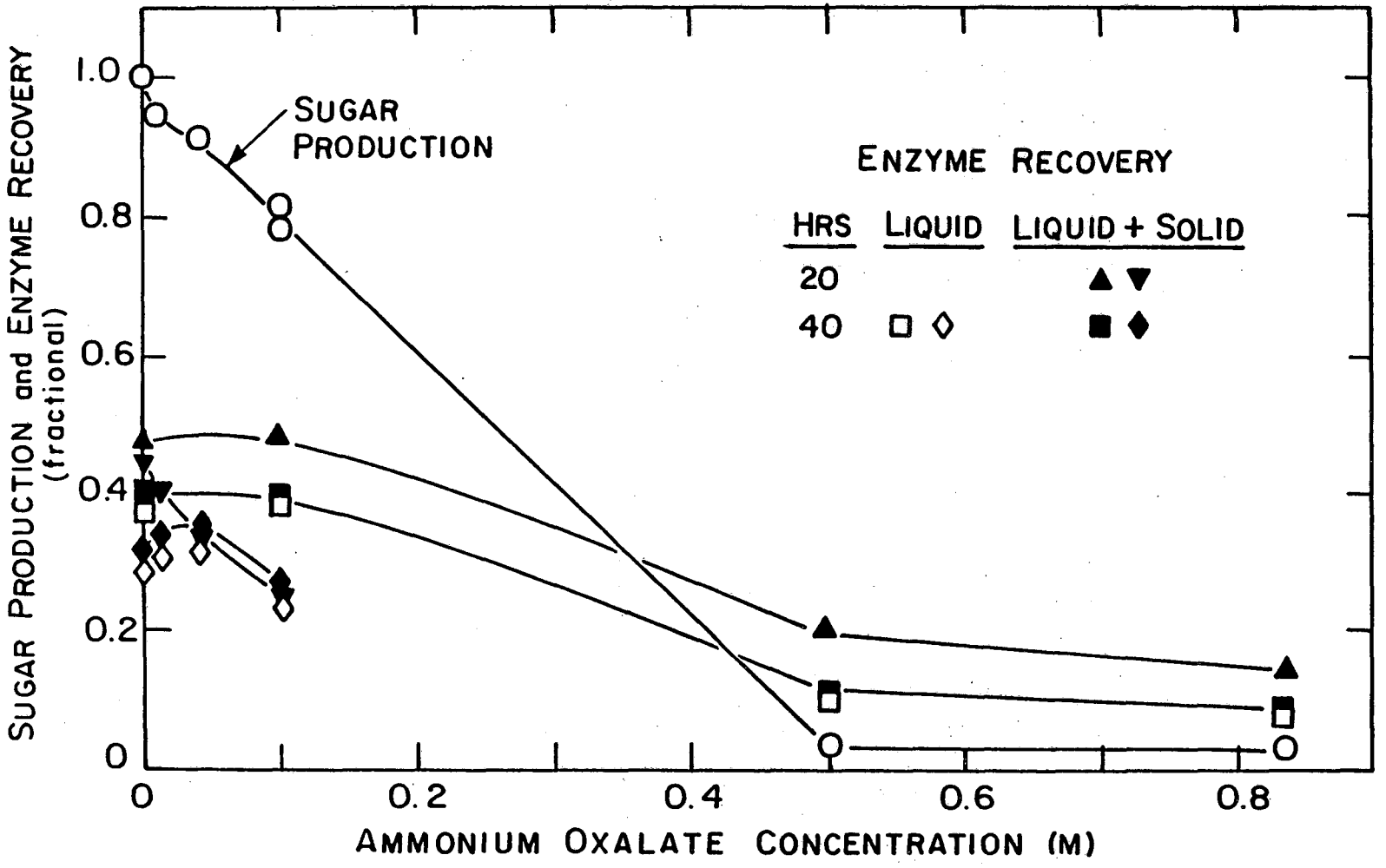


Figure 9 Effect of Ammonium Oxalate on Sugar Production and Enzyme Recovery.

XBL 815-5702

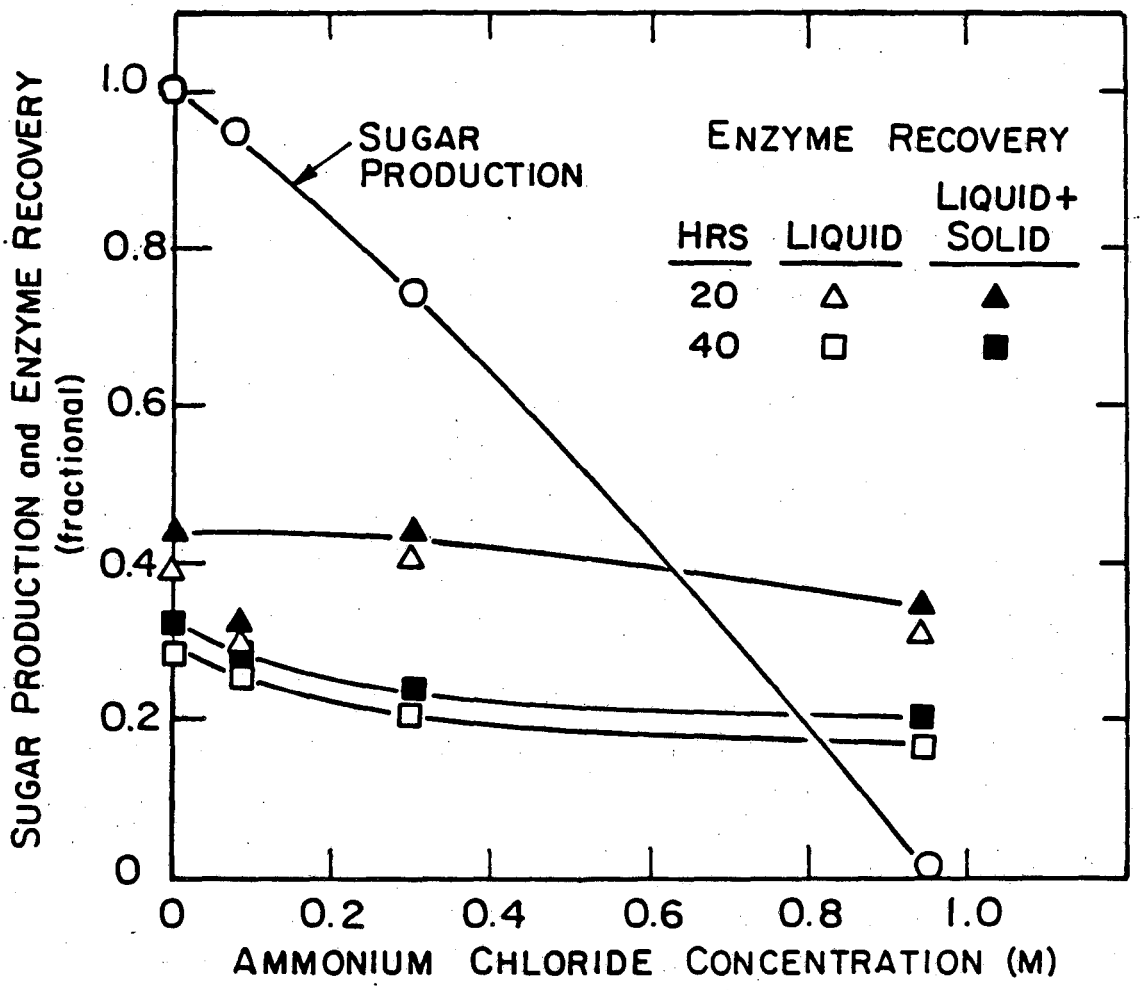


Figure 10 Effect of Ammonium Chloride on Sugar Production and Enzyme Recovery.

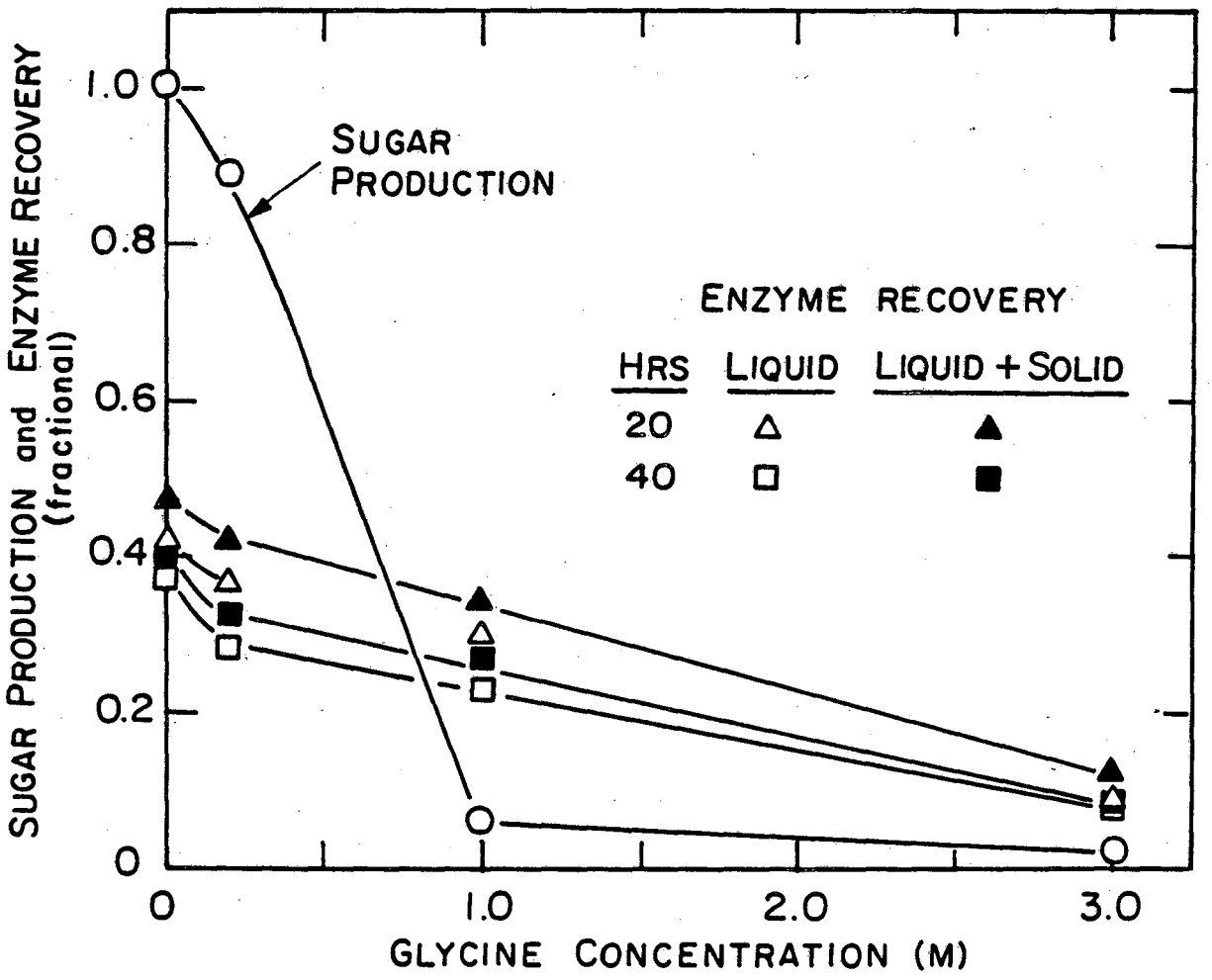


Figure 11 Effect of Glycine on Sugar Production and Enzyme Recovery.

denaturation is characteristic of changes in the tertiary structure, defined primarily by intramolecular hydrogen bonding(38).

Riaz and Wilke(38) conducted experiments using newsprint hydrolyzed by QM9414 cellulase. Urea was found to exhibit exceptional qualities as a recovery agent.

In hydrolyses without urea, it was observed that 25% FPA remained in solution after a 42 hr. hydrolysis. 32% of the original enzyme concentration could be recovered by washing the solids with 6 M urea, yielding 57% total recovery. Assayed activity of the combined fractions yielded 68% recovery, indicating the separate fractions contained varying proportions of a single component (C_1, C_x, etc.). C_x assays showed 40% in the hydrolyzate, 39% in the wash and a combined assay of 77%. Therefore, it appears reasonable to conclude that there was adequate C_1 component in the wash, but insufficient amount in the hydrolyzate, giving low FPA results.

Urea appears to affect control enzyme solutions (without substrate) slightly at 25°C. A linear decrease from 2.7 to 2.4 FPA was observed over the range of 0.0-6.0 M urea where the exposure time was 24 hrs. The presence of 1 M urea in the FPA assay causes no apparent loss in activity. However, 2 M results in a 30% loss and higher concentrations result in little activity.

Two variables were altered by Riaz and Wilke(38) in a

series of hydrolyses: concentration of urea, duration of the hydrolysis. Urea was introduced to the solka floc prior to hydrolysis.

The concentration of urea varied from 0.0-2.2 M. An optimum recovery of 67% in the liquid phase was reported for 0.9 M urea at 48 hrs. At lower concentrations of urea, a linear decline to 17% FPA remaining in solution with no urea was observed. Higher concentrations of urea reduced enzyme activity to 55-60% at 2 M with severe losses occurring beyond this point.

Hydrolyses either with no urea or 0.9 M were observed over periods up to 90 hrs. Enzyme controls (without substrate) without urea lose approximately 10% FPA over the first 20 hrs., with little further change up to 70 hrs. During hydrolysis without urea, FPA drops to 30% in the initial 20 hrs. A slow decline to 25% occurs during the following 30 hrs. A further decline to 10% occurs from 40-90 hrs. With 0.9 M urea in the hydrolysis, a decline to 67% occurs by 20 hrs. with no significant decline during the remainder of the hydrolysis.

Sugar production is indicated in Figure 12. The increase for both hydrolysis is linear from 30-90 hrs., establishing a constant rate of production. Therefore, it may be concluded that the reaction is not substrate-dependent, since substrate consumption is reducing the concentration. If there is a kinetic

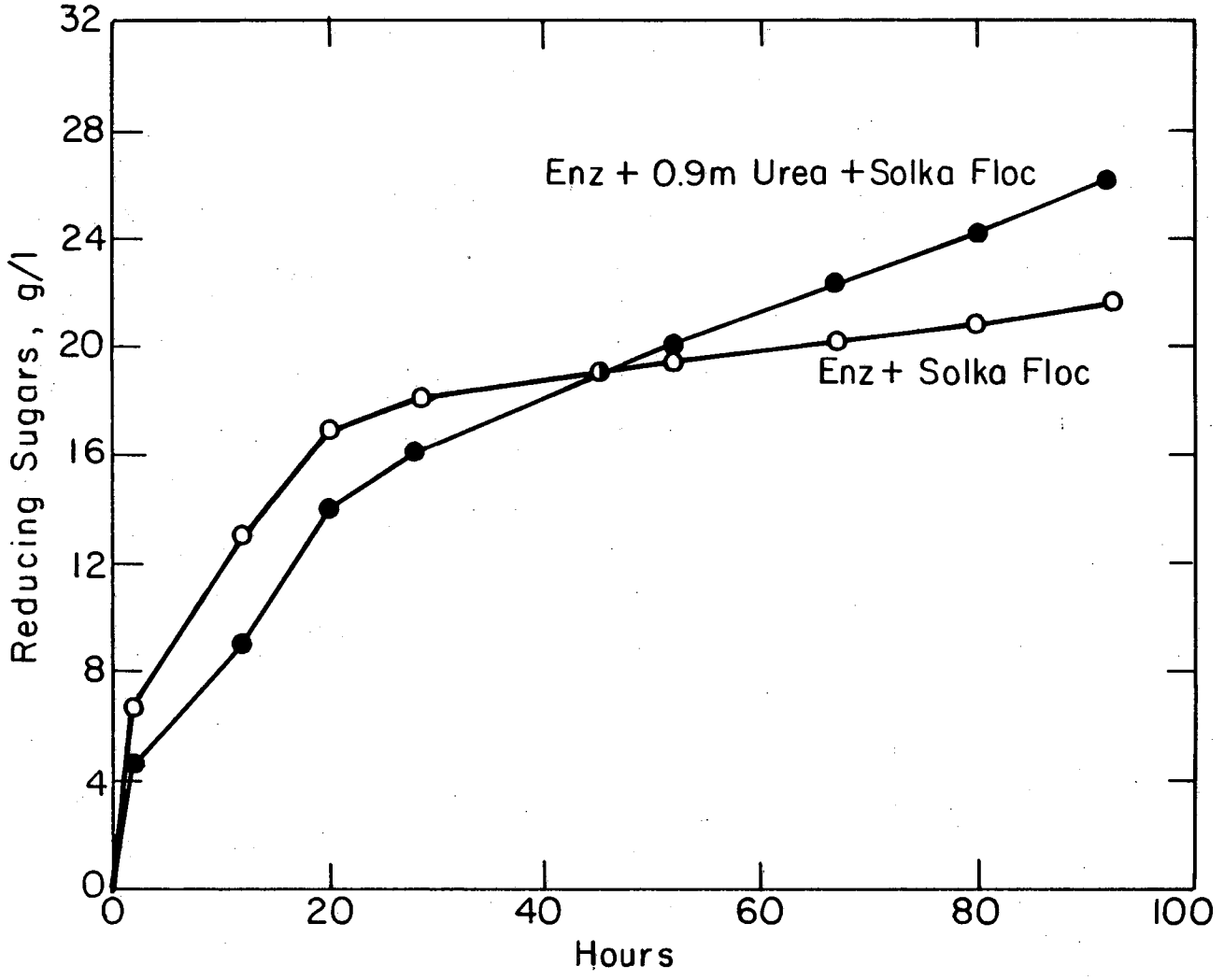


Figure 12 Effect of Urea on Rate of Sugar Production. XBL 789-5738

dependence on substrate concentration, its effect is small due to the system being saturated with substrate (no appreciable change). However, the rate is proportional to the concentration of enzyme remaining in solution, indicating first-order dependence with respect to the free enzyme.

According to the model presented by Brandt, et. al.(23) and Castanon and Wilke(24), sugar production should be limited to the action by C_1 in the later stages of digestion. If the enzyme is totally immobile, the concentration of substrate in its reactive vicinity must either be declining or depleted. If the enzyme is able to move along the fibril, it is not so severely limited. With a linear rate (therefore, substrate cannot be diminishing) apparently proportional to enzyme in solution, it may be concluded that the bound enzyme has little effect on sugar production at this point.

In another experiment, urea was added to the enzyme solution rather than the substrate solution prior to hydrolysis. Results are limited, but it appears that the same effects are observed.

Results with urea in this study of corn stover hydrolyzed by Rut-C-30 cellulase are similiar to those of Riaz and Wilke(38). A maximum concentration of enzyme in solution is achieved at 1.0 M urea. At 40 hrs., 65% of the original enzyme concentration remains in the liquid phase.

A linear decrease to 20% with no urea occurs with lower concentrations. Increasing the reagent concentration to 2.0 M yields a recovery of 57% FPA. It is possible that a concentration of urea slightly greater than 1.0 M may retain an even higher fraction of enzyme in solution.

Sugar production attains a relative maximum of 93% at 1.0 M urea, decreasing to 50% at 0.5 M and 83% by 2.0 M. However, production rises at low concentrations of urea, reaching 16.2 g/l sugar (100%) with no urea. In contrast, Riaz reports an absolute maximum in sugar production at 0.9 M urea.

The effects of urea on the two cases are remarkably similar. It may be concluded that the mode of action imposed by urea is the same. Comparisons of the enzyme QM9414 and Rut-C-30 show few differences in enzyme behavior other than the relative amounts of specific components(59). However, the substrates are quite different, particularly since it has been observed that merely varying the substrate in the reaction can greatly alter the amount of enzyme in solution. It is reasonable to conclude that urea is acting only on the enzyme rather than the substrate. This conclusion will be substantiated as it becomes obvious that urea is indeed unusual.

3.4. Ammonium Salts.

It was thought that the unique nature of urea might be due to its amide groups, particularly the nitrogen bonding.

Therefore, several ammonium salts were tested to observe both the effect of the ammonium cation and the varying anion. It is known that anions often have the stronger effect.

The Hofmeister series of ions(60) suggests a trend that predicts the effectiveness of a salt in determining the solubility of a protein.

Positive ions: magnesium>calcium>lithium
sodium>potassium

Negative ions: sulfate>acetate>chloride>nitrate

Stronger ions reduce the solubility of proteins more efficiently. Dixon and Webb(48) suggest that this effect may be caused by hydration of the ions, removing solvent molecules.

3.4.1 Ammonium Sulfate.

Results in Run 1.10 indicate that enzyme in 0.76 M salt solution is not denatured irreversibly. No visible precipitation or coagulation was observed. This is not surprising since the level of enzyme is rather low. At low levels of salt and/or enzyme, the linear relationship does not predict consequences accurately(48). Note that the enzyme concentration is lower in Run 1.10 (20 hrs.) than 1.10 (40 hrs.). The presence of ammonium sulfate may stabilize cellulase such that control samples (without

substrate or reagents) degrade relatively more quickly.

Results of hydrolyses illustrate the ineffectiveness of ammonium sulfate in preventing the loss of enzyme from solution, presumably due to adsorption or precipitation since the salt does not impair the enzyme irreversibly. The solids do not yield significant enzyme upon elution, confirming either that immobilizaton has not been affected in a positive manner or the enzyme has been precipitated.

Sugar production drops quickly with increasing salt concentration, indicating loss of hydrolytic ability due to binding or precipitation.

3.4.2. Ammonium Chloride.

The effect of ammonium chloride on enzyme in solution without substrate (Run 3.6) is similar to that of ammonium sulfate in terms of stability. An apparent increase in enzyme concentration occurs over time, indicating stabilization of the enzyme. Therefore, irreversible denaturation caused by the salt is very slight.

There is no large drop in enzyme concentration with substrate present as with ammonium sulfate. The loss of enzyme is minor over the entire range of ammonium chloride solubility. Although enzyme concentration in solution is relatively high, sugar production declines and essentially stops by 0.94 M. This would indicate that ammonium chloride:

1) deactivates the binding and hydrolytic site

structures of the enzyme molecule, or

2) coats the substrate with a physical layer.

Details are examined in the Discussion.

3.4.3 Ammonium Oxalate.

By comparison of the effects of the sulfate and chloride salts, it is apparent that the anion does indeed have a profound effect. It was thought that an organic salt might yield results more like those of urea. Ammonium oxalate was chosen for its high solubility. Its atomic configuration is of roughly the same size as urea. Also, the carboxyl group functionality was of interest.

The enzyme controls (Runs 2.10, 3.10) show that the enzyme is stabilized in 0.10 M solution, but is degraded when subjected to 0.83 M.

Data are contradictory at 0.1 M. Run 2.7 indicates recovery of 0.404 at 40 hrs. and Run 3.9 indicates 0.274. Neither is distinctly inconsistent with other data. Sugar production at 40 hrs. for both runs is 13.7 g/l, consistent with sugar production values in other runs. Note that the enzyme concentrations in Runs 3.7 and 3.8 are lower than in Run 2.7, but sugar production is greater in Runs 3.7 and 3.8. This pattern is not followed in any other runs. Therefore, it may be concluded that the values of enzyme in solution reported in Run 2.7 are erroneously high.

3.5. Glycine.

Urea contains two amide functional groups which may

contribute to its behavior. It was thought that amino groups may have a similiar effect. Glycine is the simplest and smallest amino acid. Of the amino acids, it most closely resembles the configuration of urea. However, it possesses only one amino group unlike the dual nature of the two amide groups of urea.

The trend exhibited by glycine is similiar to that of ammonium sulfate. Run 2.6 indicates moderate denaturation in a control solution. In hydrolyses, higher concentrations are required (compared to ammonium sulfate) to effect the same loss of enzyme in solution, but the tendency is the same. Low salt concentrations produce the most severe changes.

3.6. Errors in Data.

Consistency within a particular series of runs appears satisfactory. That is, the data are reasonably precise such that the error is approximately 5%. However, comparisons between runs indicates that reproducibility is rather poor. The variance may be attributed to differences between runs: substrate, enzyme and operating conditions. Of these factors, the lack of homogeneity in both the substrate and the enzyme solution have caused problems in the past.

3.7. Discussion.

The graphs of enzyme recovery and sugar production do offer several comparisons. Extending the results of Riaz and Wilke(38) to a different system confirms that urea is by far the superior agent in retaining enzyme in solution. Over the range of urea concentrations explored, sugar production attains a relative(intermediate) maximum(15.0 g/l) at the same urea concentration(1.0 M) at which the enzyme concentration in solution is highest(57%). This pattern substantiates the hypothesis that mobility of the enzyme has a direct effect on the efficiency of the enzyme. The drastic denaturation power of urea on enzyme alone (without substrate) is still unclear. It is well established that substrates often protect enzymes against denaturation caused by temperature or chemicals. In this case, perhaps degradation proceeds when the enzyme is not able to bind to a solid support which can lend rigidity to the enzyme molecule. The process of enzyme action on substrate may have a renaturing effect. It may be that the presence of substrate and/or product induces configurational changes that are more resistant to denaturation by urea.

The deleterious action of ammonium sulfate, ammonium oxalate, and glycine are most likely caused by simple

precipitation. With the ammonium salts, this effect would be expected, particularly with ammonium sulfate. However, the similiarity in glycine is unclear. Proteins can precipitate proteins, but the effect usually is not as severe as with salts.

The action of ammonium chloride is an anomaly. It was known that it was a poor precipitation agent, which is borne out in the data. However, the high enzyme concentration and lack of sugar production raises several possibilities. It is reasonable to conclude that enzyme and substrate were never able to contact each other, leading to little activity and consequent small loss of enzyme. It may be that ammonium chloride reversibly denatured the enzyme in solution. The effect would not be as severe as precipitation, but would change the configuration enough to hinder its hydrolytic and binding capabilities. In other words, the enzyme is ineffective, but still suspended in solution. One would expect that higher concentration of salt would lead to further inactivation and more permanent denaturation. This is not observed.

Alternatively, the substrate may be coated with a physical layer of the salt. Analogous observations have been made by Antonoplis and others(61, 62) studying wood particles. Exposing wood to gaseous HCl results in adsorption of HCl onto or within the cellulosic matrix, forming an adduct. It is possible that lignin is the

primary binding region for chloride(63). The chloride can be removed by simple elution. In the case of cellulase, particularly at low salt concentrations, the enzyme would eventually bind to open sites, albeit at a lower rate than if no salt was present. This pattern may be indicated in the graph of ammonium chloride(Figure 10). Note that the difference between 20 and 40 hrs. is greater for this salt than other compounds.

The trend exhibited by the ammonium salts does agree well with previous work(48, 60), substantiating the predictions set forth based on the Hofmeister series. In this regard, cellulase and the activity of all of its components appear to respond in the same general trend of most proteins.

It is interesting to note that sugar production often declines before enzyme activity as a function of the salt concentration. In the case of precipitation agents, hydrolytic activity appears to be much more sensitive to agents than precipitation since activity appears to be affected at lower concentrations of salt than precipitation. It may be that reversible conformational changes are occurring in the enzyme prior to precipitation. On the other hand, the agents in general may be coating the substrate, as suggested for ammonium chloride. This action would produce a physical barrier against enzymatic action, leading to lower sugar production rates. Addition of salts

always appears to inhibit sugar production.

5. PROCESS CONCLUSIONS.

This study was conducted to extend previous work. Whereas Riaz and Wilke and others(38, 24) utilized newsprint hydrolyzed by QM9414 cellulase, it was necessary to determine if the results obtained with urea could be extended to corn stover hydrolyzed by Rut-C-30 cellulase. Urea appears to have strikingly similiar effects on this hydrolysis and it is concluded that degradation occurs by the same mechanism. Results with other salts have yielded no applicable results. Urea retains its position as the only exceptional recovery agent in cellulose-cellulase systems.

Recovering urea from aqueous solutions to recycle it within the hydrolysis operation is difficult. Thus, it was envisioned that the entire process might benefit from the presence of urea in the major process streams. This allowance would eliminate the need to remove the reagent. Various unit operations are conducted downstream, most of which could be adapted relatively easily to high salt concentrations in aqueous streams. However, the most sensitive of these involves the yeast in the sugar fermentation.

As shown in the process diagram (Figure 2), the sugar solution is sterilized and concentrated following the hydrolysis step. Then, the sugar enters the fermentation

to provide a carbon and energy source for the production of ethanol. The yeast employed is relatively osmophilic and, therefore, can withstand, and perhaps benefit from, a high salt concentration present in the sugar solution.

Investigations were conducted to determine the effects of urea on the metabolic activities of the yeast. Studies are described in the next section(III. Glucose Fermentation).

III. GLUCOSE FERMENTATION.

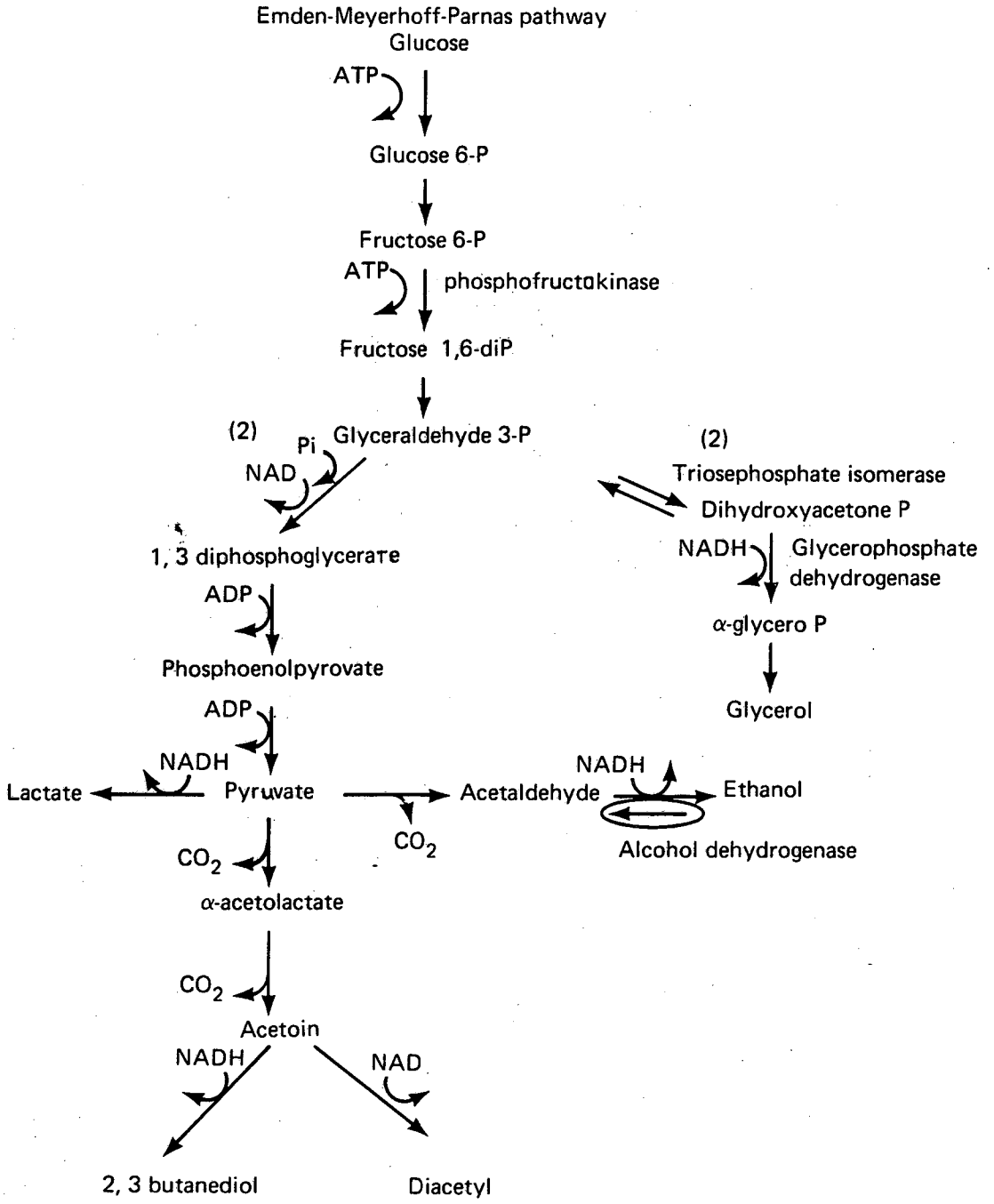
1. BACKGROUND INFORMATION.

1.1. Yeast Metabolism.

Sacchromyces cerevisiae is a facultative anaerobe, grown under essentially anaerobic conditions in these experiments. Glucose is fermented to ethanol and carbon dioxide by the glycolytic (Embden-Meyerhoff-Parnas) pathway and alcoholic fermentation. The important steps are outlined in Figure 13(64). The overall reaction yields two moles of ethanol, carbon dioxide, water and ATP for each mole of glucose.

Under physiological conditions, a theoretical yield of ethanol may not be achieved because the glycolytic pathway is not balanced in terms of the ATP cycle. The formation of ATP serves as a teporary storage of energy, in the phosphate bond of ADP and inorganic phosphate(P_i). The produced ATP must be converted back to ADP and P_i via anabolic pathways.

On a stoichiometric basis, one gram of glucose yields 0.511 grams of ethanol. In practice, the ethanol yield is 90-95% of the theoretical yield, since a fraction of the substrate is utilized in anabolic pathways. In general, about 0.05-0.12 grams of cells are produced per gram of glucose. One of the links between catabolic reactions of



$$\text{Glucose} + 2\,\text{Pi} + 2\text{ADP} \rightarrow 2\,\text{ethanol} + 2\,CO_2 + 2\text{ATP} + 2\text{H2O}$$

Figure 13 Emden-Meyerhoff-Parnas Pathway.

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glucose and anabolic pathways yielding cell mass is provided by the hexose monophosphate pathway(HMP). Carbon units are removed from the cycle and channelled into various biosynthetic schemes. Other pathways yielding co-products different from ethanol will be discussed when applicable.

Early work in microbial studies established that the yield of cells from cultures was integrally related to the way in which the energy substrate is metabolized. Pathways giving high yields of ATP also yielded large amounts of cells(65). Bauchop and Elsden(66) examined the yields of cultures grown anaerobically. They found that the yield $Y_{x/sm}$(g dry weight of cells/mole energy substrate used) varied with different substrates and organisms. However, the yield $Y_{x/ATP}$(g cells/mole ATP synthesized) was nearly constant at 10+2. For S. cerevisiae grown on glucose, $Y_{x/sm}$ was 21 and $Y_{x/ATP}$ was 10.5. Hernandez and Johnson(67) noted that the value of $Y_{x/ATP}$ is constant only if the energy substrate is the one factor limiting growth. In the presence of inhibition (e.g. by other materials), $Y_{x/ATP}$ values are likely to be lower.

Consider the converse of the statement concerning high yields of ATP leading to large amounts of cells. It is reasonable to expect that, if the cell changes its catabolic pathways to less efficient ones, smaller amounts of cells will be produced per unit of glucose, giving a

lower yield ($Y_{x/sm}$ and $Y_{x/ATP}$).

1.2. Yield Coefficients.

To gain an understanding of alterations in metabolism caused by changes in the yeast environment, Pirt(68) examined the fate of glucose. There are three uses to which a substrate such as glucose may be put: a) an energy source for growth-related activities(S_g), b) an energy source for maintenance requirements of the cell(S_m), c) a carbon source for synthesis of the mmetabolic products. It is possible that ATP may be lost in functions not related to growth or maintenance. This discrepancy would usually be included in maintenance energy. The cell yields may be defined as

$$Y_{x/a} = X / (S_g + S_m) = X / S_a \quad (1)$$

$$Y_{x/g} = X / S_g \quad (2)$$

where X is the change in biomass and S_a is the total consumption of glucose for energy purposes only. The maximum specific growth rate (exponential phase under the given conditions) is designated by μ. The maintenance requirement coefficient(m) may be defined as [g glucose consumed/(g cell mass x hr)], indicating the amount of glucose required merely to maintain a viable state. The specific substrate consumption coefficient(q_s) may be defined as ($\mu/Y_{x/a}$).

A mass balance on the energy source gives:

consumption rate, total = consumption rate for growth + consumption rate for maintenance

$$\mu X / Y_{x/a} = \mu X / Y_{x/g} + mX \quad (3)$$

Dividing by X and substituting in q_s,

$$q_s = \mu / Y_{x/g} + m \quad (4)$$

or

$$1 / Y_{x/a} = 1 / Y_{x/g} + m/\mu \quad (5)$$

Equations 4 and 5 will be utilized to estimate the values of m and $Y_{x/g}$.

1.3. Effects of Salts.

Many studies have been conducted on the effects of high concentrations of salts in yeast fermentations. The tolerance of yeast strains varies greatly. Two metabolic factors tending to dominate the tolerance are a mechanism to maintain a low level, or less deleterious species, of salt within the cell and the ability of enzymes to function in the presence of high salt concentrations. The effects of sodium chloride on yeast have been particularly well-documented.

Norkrans and Kylin(69) compared the halotolerance capabilities of S. cerevisiae and Debaryomyces hansenii, a marine yeast, in salts of sodium, potassium, and rubidium. In the presence of high Na^+ concentrations, D. hansenii maintained a higher ratio of K^+ to Na^+, while S. cerevisiae tended to excrete more H^+. It is known that the

uptake of K^+ in yeasts can occur either as an exchange of K^+-Na^+ or as an exchange of K^+-H^+(70). D. hansenii possesses a stronger extrusion mechanism for Na^+.

Norkrans and Kylin conclude that their data and the superior tolerance of D. hansenii support a stronger dominance of K^+-Na^+. However, the total salt level of the cells was not sufficient to overcome the osmotic potential of the environment, so that additional osmoregulatory mechanisms must be involved. It is suggested that compartmentalized concentration gradients within the cell would be maintained by internal osmotic agents.

Studying S. cerevisiae, Watson(71) observed that the maximum specific growth rate and the cell yield with respect to glucose decreased in the presence of 0.25-1.5 M NaCl. The duration of the lag phase in growth increased. Utilizing Eqs. 4 and 5, maintenance requirements of 0.036 [g glucose/(g cell mass x hr)] in the absence of NaCl and 0.36 in 1.0 M NaCl, respectively were calculated.

Cultures grown anaerobically without NaCl produce glycerol(Nordstrom, 72), probably due to the generation of excess $NADH_2$, which is removed through the conversion of dihydroxy-acetone phosphate to glycerolphosphate. Nordstrom showed that the yield of cell mass with respect to glycerol production($Y_{x/glycerol}$) did not vary with dilution rate, giving a constant value of $1/Y_{x/glycerol}$ over a range of 1/D. This trend suggests a surplus of

$NADH_2$ directly proportional to biomass production. The presence of NaCl increased production of glycerol per unit of biomass formed as the dilution rate was decreased, giving a plot of $1/Y_{x/glycerol}$ versus 1/D an approximately linear slope of 0.092 g/(g hr). This value represents the base (maintenance) production level of glycerol. However, the data were somewhat scattered, indicating that glycerol production perhaps is not a simple function of energy metabolism.

The conversion of substrate to glycerol reduces the yield of ATP, by consuming 2 moles of ATP/mole glucose. Nordstrom found a maintenance requirement of 0.52 mmol ATP/(g cell mass hr) (no NaCl) and 2.2 (1.0 M NaCl). In both cases, the value of $Y_{x/ATP}$ was found to be 11 g cell mass/mole ATP, in accord with the range determined by Bauchop and Elsden. Watson(71) concluded that the effect of NaCl on the ATP yield is solely due to an increased maintenance expenditure required for maintaining an electrochemical gradient of sodium and chloride ions across the cell membrane. Conclusions concerning the manifestation of glycerol production primarily as a maintenance effect are tentative. Enzymes of the pathway leading to glycerol may be activated by NaCl(Shoeffenials, 73). Alternatively, $NAD/NADH_2$ regulation may require increased organic acid production resulting in a by-product of glycerol(Armstrong and Rothstein, 74).

Tajima and Yoshizumi(75, 76) observed the effects of 1 M NaCl on S. formosensis in batch growth. Concentrations of intermediate metabolites were monitored. During the first 10 hrs. of fermentation, the concentrations of glucose 6-phosphate(G6P), fructose 6-phosphate(F6P), and fructose 1,6-diphosphate(FDP) were substantially higher in cells in 1 M NaCl than the control without NaCl. Tajima and Yoshizumi concluded that general inhibition of ethanol fermentation was caused by a decline in hexokinase, aldolase and triosephosphate isomerase activities. However, at 10-20 hrs., the concentrations of the intermediates decreased to levels lower than those of the control(no salt). If simple inhibition is occurring, the concentration of these intermediates would be expected to remain at or above the levels of the control since higher concentrations are required to attain the same rate.

Mass balances measured by Tajima and Yoshizumi(75, 76) showed that high salt concentrations primarily increased yields of glycerol and 2,3 butanediol and decreased ethanol production. The control produced 2.0 g/l of glycerol and 0.23 g/l of 2,3 butanediol. In 1 M NaCl, the respective concentrations of glycerol and 2,3 butanediol were 5.4 and 1.7 g/l. Concentrations of other organic acids rose as well. The yield loss of ethanol in terms of glucose was 8.9 g/l.

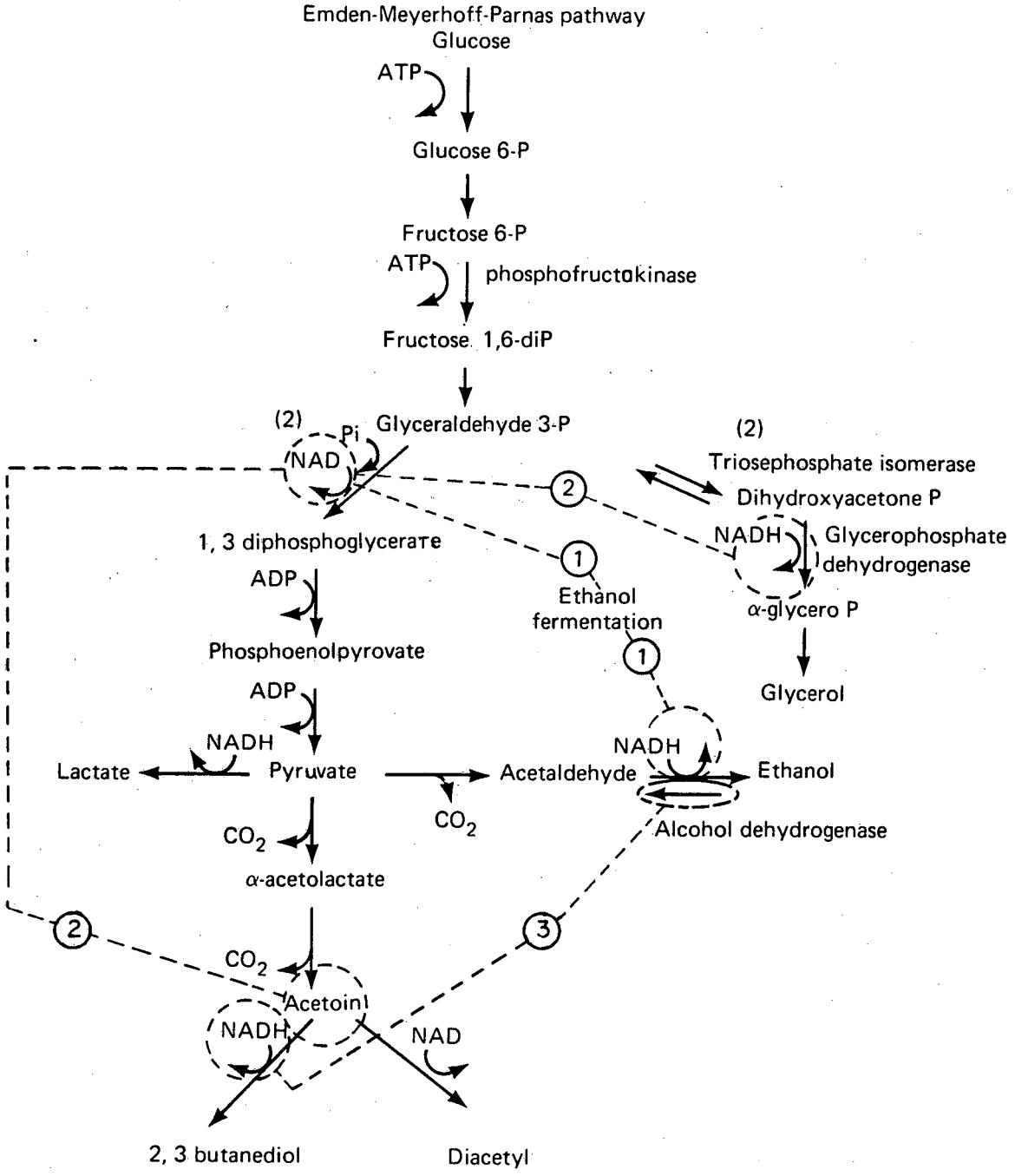
It was observed that the ATP concentration in 0.5 M

NaCl was approximmately 67% of that of the control throughout the entire fermentation. The level of glycerolphosphate was about 10 times higher in 0.5 M NaCl.

Further studies of intermediates(75,76) led to the following conclusions concerning high salt concentrations (see Figure 14). The salt stimulates production of glycerolphosphate dehydrogenase. The observed decline in the level of ATP increases activity of the enzyme. Further, triosephosphate isomerase activity is enhanced, leading to an accumulation of glycerol.

The pool of $NADH_2$ depleted by action of glycerolphosphate dehydrogenase must be replenished by conversion of glyceraldehyde 3-phosphate to 1,3-diphosphate, leading to formation of acetoin. The low level of $NADH_2$ hinders the conversion of acetaldehyde to ethanol by alcohol dehydrogenase such that this enzyme and glycerolphosphate dehydrogenase are competing for $NADH_2$. In the later stage of fermentation, the high concentration of acetoin enhances its conversion to 2,3 butanediol, utilizing $NADH_2$. A redox balance is maintained by coupling to the reactions of ethanol to acetaldehyde and acetoin to diacetyl.

The effects of urea have been observed primarily at low levels. At less than 0.1 M, urea provides a source of nitrogen which is comparable to ammonium sulfate for Sacchromyces strains. However, a high concentration of



$$\text{Glucose} + 2\,\text{Pi} + 2\text{ADP} \rightarrow 2\,\text{ethanol} + 2\,CO_2 + 2\text{ATP} + 2\text{H2O}$$

Figure 14 Feedback Responses of the Catabolic Pathway. XBL 818-1086A

biotin is necessary(77, 78). This is likely to be uneconomical considering that biotin is a high-value nutrient.

The concentration of urea in the fermentation is likely to be very high in the industrial process fermentation, exerting an osmotic effect on the yeast, perhaps similiar to that of simple salts. The industrial process is dependent on the rate of production and yield of ethanol. An increase in the yield of ethanol may be accomplished by either lowering the efficiency of ATP production through glycolysis, or by creating conditions in which the ATP present is "wasted" in uncoupled hydrolytic reactions. Postulates incorporating both pathways have been applied to previous observations involving simple salts.

The diffusion/transport of alkyl-substituted ureas and aliphatic amides across biological membranes, specifically liposomes, has been explored by Cohen and Bergham(79). In a plot of permeability coefficients having a positive slope, the amides are more permeable than the corresponding ureas at similiar partition coefficients. In both series, additional methyl groups lead to progressively smaller increases in permeability. As the molecular weight increases, the log of the value(permeability/partition) decreases linearly. The barriers opposing the transfer of non-electrolytes appear to have the same fundamental nature

and structure in biological membranes and hydrophobic polymer networks.

2. EXPERIMENTAL PROCEDURE.

2.1. Analytical Procedures.

2.1.1. Reducing Sugar Assay.

Reducing sugar was measured by Long's modifications(50) of the dinitrosalicylic acid (DNS) method of Sumner and Somers(51). See the appendix, Enzyme Assay.

2.1.2. Ethanol Determination.

Ethanol samples were injected into an Aerograph 1520 Gas Chromatograph and data were integrated digitally and recorded by a CDS 111 integrator, both manufactured by Varian Associates. An internal standard of 2-butanol was used.

2.1.3. Cell Density.

Cell density was measured optically in a Bausch and Lomb Spectronic System 400 spectrophotometer. This method was used only while following the course of a fermentation. Data reporting cell density were made directly. 50 ml. culture samples were filtered through 0.4 μm Nuclepore polycarbonate membranes under vacuum. The cell filtrates were dried at 70°C for 2 days, followed by 105°C for 4-6 days. Changes in weight upon further drying were negligible.

2.2. Experimental Apparatus and Methods.

2.2.1. Yeast Strain.

The yeast species in the fermentaion was Sacchromyces cerevisiae, ATCC #4126. The yeast was stored at 4°C on agar slants.

Inocula for the fermentations were grown up by transferring a small number of cells with an inoculating loop to 250 ml. shake flasks containing 100 ml. of sterile liquid media. The flasks were incubated at 35°C on a reciprocating shaker.

2.2.2. Media, Materials.

The media composition used in all experiments and inocula, unless otherwise noted, is given in Table 7(80). Glucose and all salts were reagent grade. Glucose was obtained from J.T. Baker, Baker-analyzed. Yeast extract was obtained from Difco Laboratories. Salts were obtained from Mallinckrodt Chemicals.

2.2.3. Batch Cultures.

Shake flasks of 250 ml. volume were filled with 50 ml. of media (at 2x concentration) and sterilized at 124°C for 30 min. An aqueous solution of 2 M urea was filter-sterilized through a 0.2 um Nuclepore polycarbonate membrane. Higher concentrations of urea led to a slow filtration rate, perhaps due to swelling of the membrane. Urea solution was added to the shake flasks and the mixtures were diluted with water. 3% inocula were

MATERIAL	CONCENTRATION (G/L)
GLUCOSE, ANHYDROUS	100
YEAST EXTRACT	8.5
NH_4CL	1.32
$MgSO_4 \cdot 7H_2O$	0.11
$CaCL_2 \cdot 2H_2O$	0.08
TAP WATER	MAKE UP TO 1 LITER

TABLE 7. FERMENTATION MEDIA(80).

introduced. The flasks were sealed with chromium-plated shaker caps and were immersed in a reciprocating shaker water bath at 35°C. Samples were taken by pipet.

2.2.4. Continuous Cultures.

An illustration of the fermentor system is illustated in Figure 15. Continuous fermentation was carried out in a 14 l Chemap-Fermenter, model LF-14, made by Chemap, Inc. The working volume was set by inserting a tube through the top flange of the fermentor to the level of 5 l. Broth was pumped out continuously, creating a sterile outlet port.

Media was fed to the fermenter by a Masterflex (Cole-Palmer, model 7013) peristaltic pump from a mixing bottle. Connected to the mixing bottle were reservoirs of concentrated solutions of glucose, yeast extract, urea, and water. Appropriate amounts of these solutions were mixed in batches by a magnetic stirrer such that the concentrations of glucose and urea could be varied. Glucose solution(200 g/l.), urea solution(180 g/l., 3 M), and water were filter-sterilized through a Pall Prefilter and Final Filter (0.2 μm absolute rating) at room temperature. Yeast extract(42.5 g/l.) was autoclaved at 124°C for 2.5 hrs. The long period was neccesitated by the heat transfer limitations in 30 l. of liquid.

The fermentation tends to produce acids, lowering the pH. One-sided pH control in the range of 4.0-4.4 was maintained by addition of 4 M NaOH. pH was monitored by an

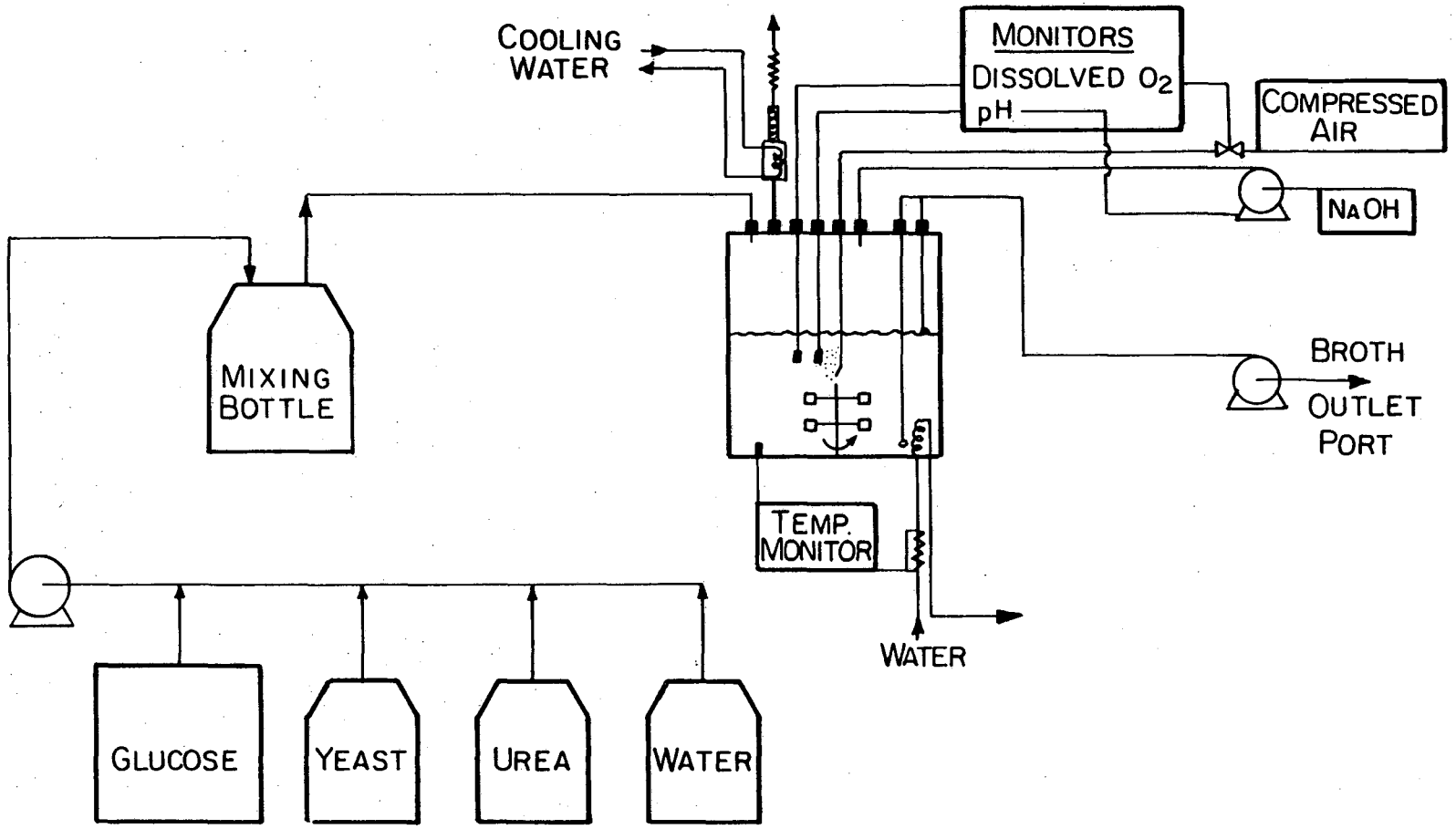


Figure 15 Fermenter System for Continuous Culture.

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Ingold pH probe and controlled by a PEC Fermenter System.

Dissolved oxygen was maintained at 10+2 mm partial pressure of oxygen by sparging in compressed air. Oxygen tension was measured by a 530 System O_2 probe with amplifier 531) of Instrumentation Laboratory and controlled by a Leeds and Northrup Electromax III O_2 controller. Air was filtered through a glass prefilter and final sintered glass filter. The gas leaving the fermentor passed through either a condenser and a sintered glass filter or the outlet port with the broth. A minimum stirring speed of 300 RPM was required in the fermentor to achieve tight dissolved oxygen control, and a speed of 350 RPM was chosen.

Tubing material consisted of silicone, except for black surgical tubing used in the NaOH addition. The sugar reservoir was a 50 gal. polypropylene barrel; all other reservoirs were 40 l Pyrex bottles.

3. RESULTS AND DISCUSSION.

3.1. Batch Cultures.

A preliminary series of runs showed that yeast growth was adversely affected at all concentrations of urea. Cells were harvested following a fermentation period of 15 hrs. Cell yields(dry weight) are plotted as a function of urea concentration in Figure 16. A severe lag phase is induced at concentrations greater than 0.5 M.

A second series produced more detailed results(Table 8). The maximum specific growth rate(Figure 17) declines in an approximately linear fashion. The utilization of glucose and production of cell mass and ethanol decline sharply with increased urea concentration. It appears that eventually glucose is metabolized to ethanol, giving a final broth containing 46-47 g/l ethanol in 0.25 M urea.

Other calculations produce scattered results, owing to errors and small differences in large numbers (e. g. glucose concentration change). Within these errors, yields remain high over the range of urea concentrations. Note that the largest change occurs in cell growth rate and yield.

3.2. Continuous Cultures.

The urea concentration was varied in a step-wise fashion. 1) The fermenter was drained to 0.05 l. 2) A new concentration of urea was introduced to this inoculum. 3) Growth in batch culture was allowed to obtain a value of

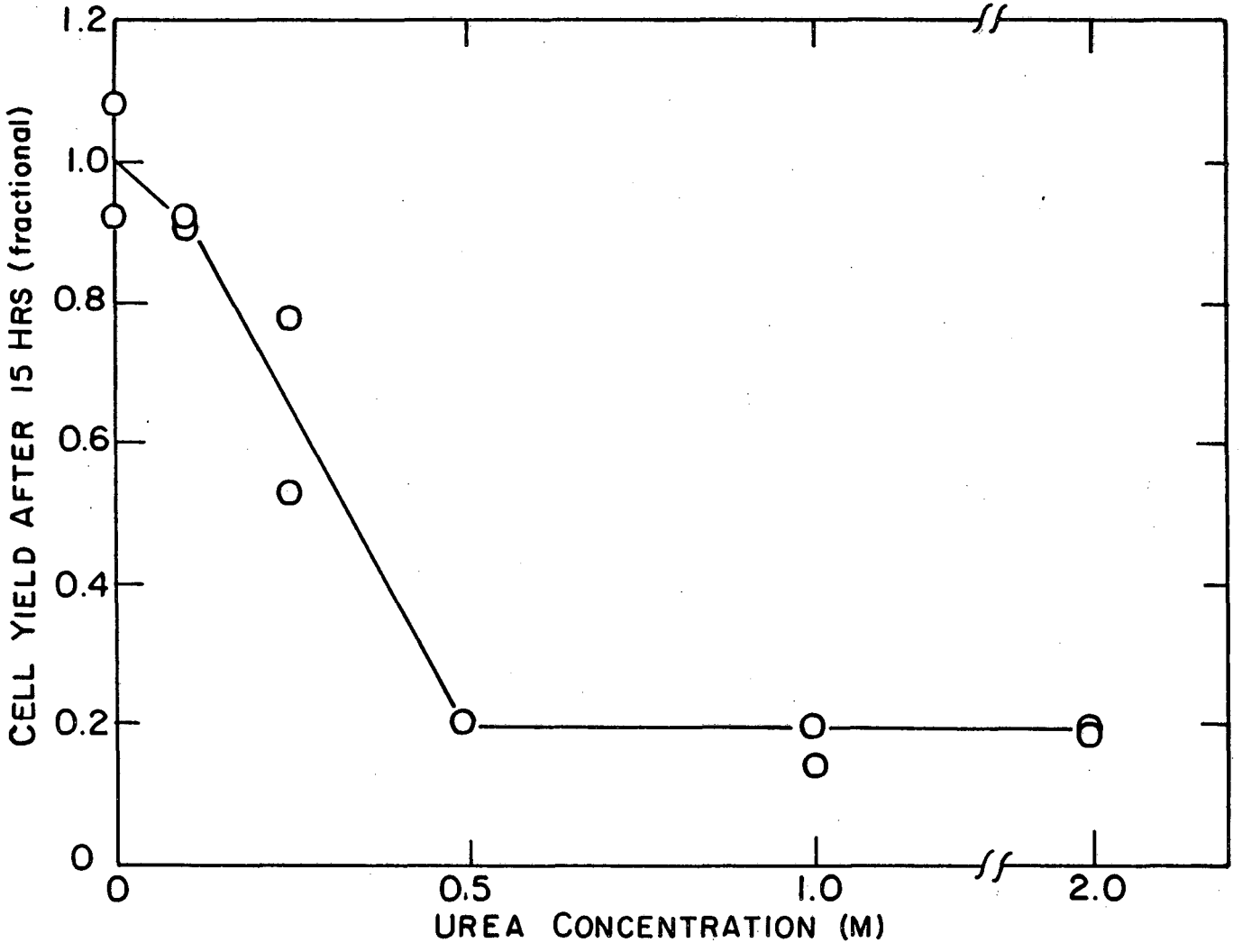


Figure 16 Effect of Urea on Cell Yield.

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Samples taken
At 6 hrs. and
At 11 hrs. after innoculation.

Urea (M)	u_{MAX} (hr^{-1})	Cell Mass (g/l)	Glucose (g/l)	Ethanol (g/l)	$Y_{X/S}$	$Y_{E/X}$	$Y_{E/S}$	Specific Ethanol Product. (g/g/l)	At 26 hrs. Ethanol (g/l)
0.0	0.31	0.53	95.8	4.0	0.059	6.51	0.39	2.29	
		2.50	62.5	16.9					46.
0.25	0.23	0.47	92.5	2.9	0.050	8.79	0.44	2.03	
		1.46	73.0	11.5					47.
1.0	0.11	0.18	96.0	1.3	0.051	5.48	0.28	0.70	
		0.31	93.5	2.0					6.

Key:
X = change in cell mass concentration.
S = change in glucose concentration.
E = change in ethanol concentration.

Table 8. Effect of Urea on
Batch Culture of S. cerevisiae.

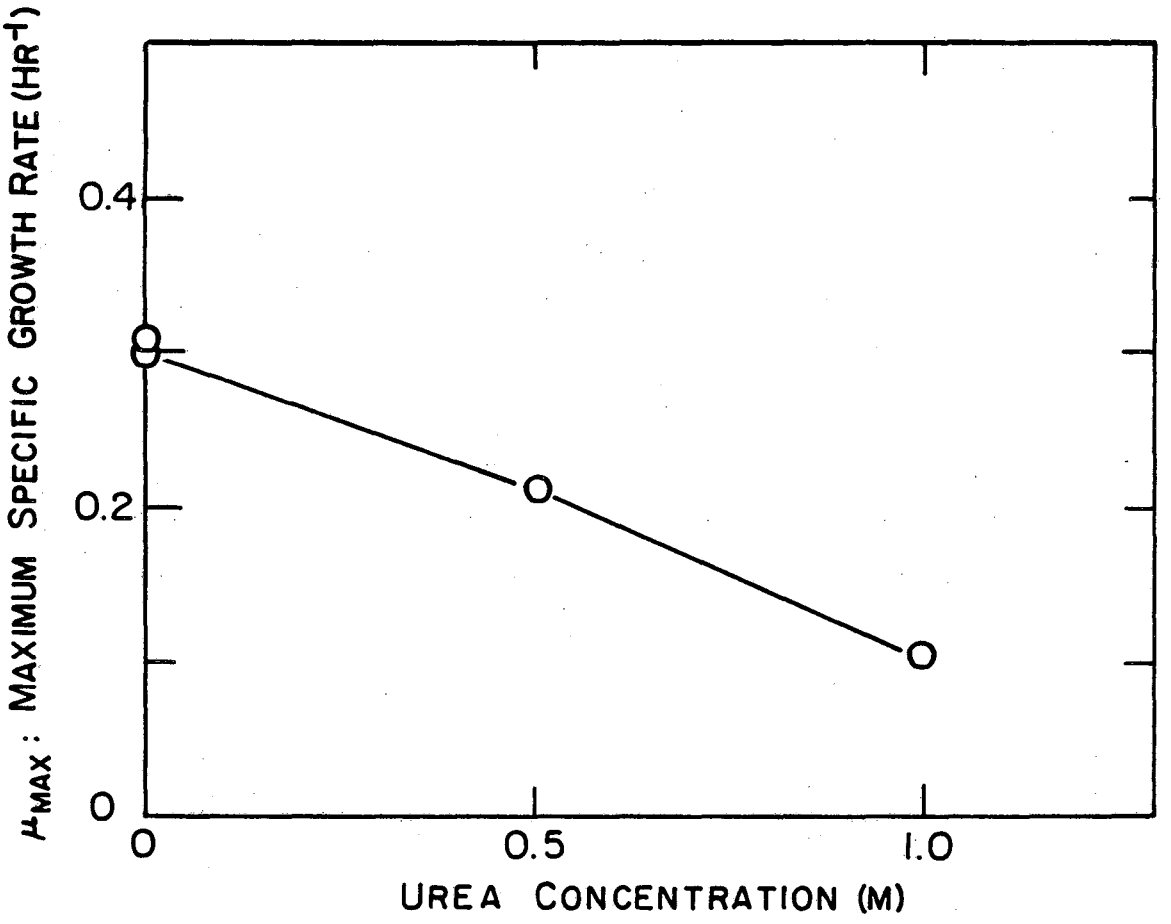


Figure 17 Effect of Urea on Maximum Specific Growth Rate.

the maximum specific growth rate(μ_{MAX}). pH and dissolved oxygen were controlled during this stage. 4) The culture was then subjected to sequentially smaller dilution rates, the maximum being set by μ_{MAX}. Wash-out techniques to determine μ_{MAX} were not successful with this strain because a long lag period resulted in loss of the culture regardless of the wash-out dilution rate. These steps minimized oscillations in cultures reaching steady-state conditions. Steady-state conditions within 95% of true value were achieved in 6-10 fermenter volumes.

The fermenter was operated continuously for approximately three months. A concentration of 0.5 M urea was approached gradually. Contamination required the fermenter to be reinoculated with a new culture. The urea concentration was increased over a period of one month to 0.75 M. Further data were taken up to 1 M.

The urea concentration was more easily controlled than the dilution rate. It was difficult at times to attain a set feed rate to allow a chosen dilution rate. Therefore, the urea concentration was set at a speciific value and the dilution rate was varied over the allowable range. In sequence, the urea concentration was altered to a new value and the dilution rate was varied.

The following plots(Figures 18-29) illustrate several trends observed in the growth of the cultures. In comparing the various graphs, patterns will be highlighted.

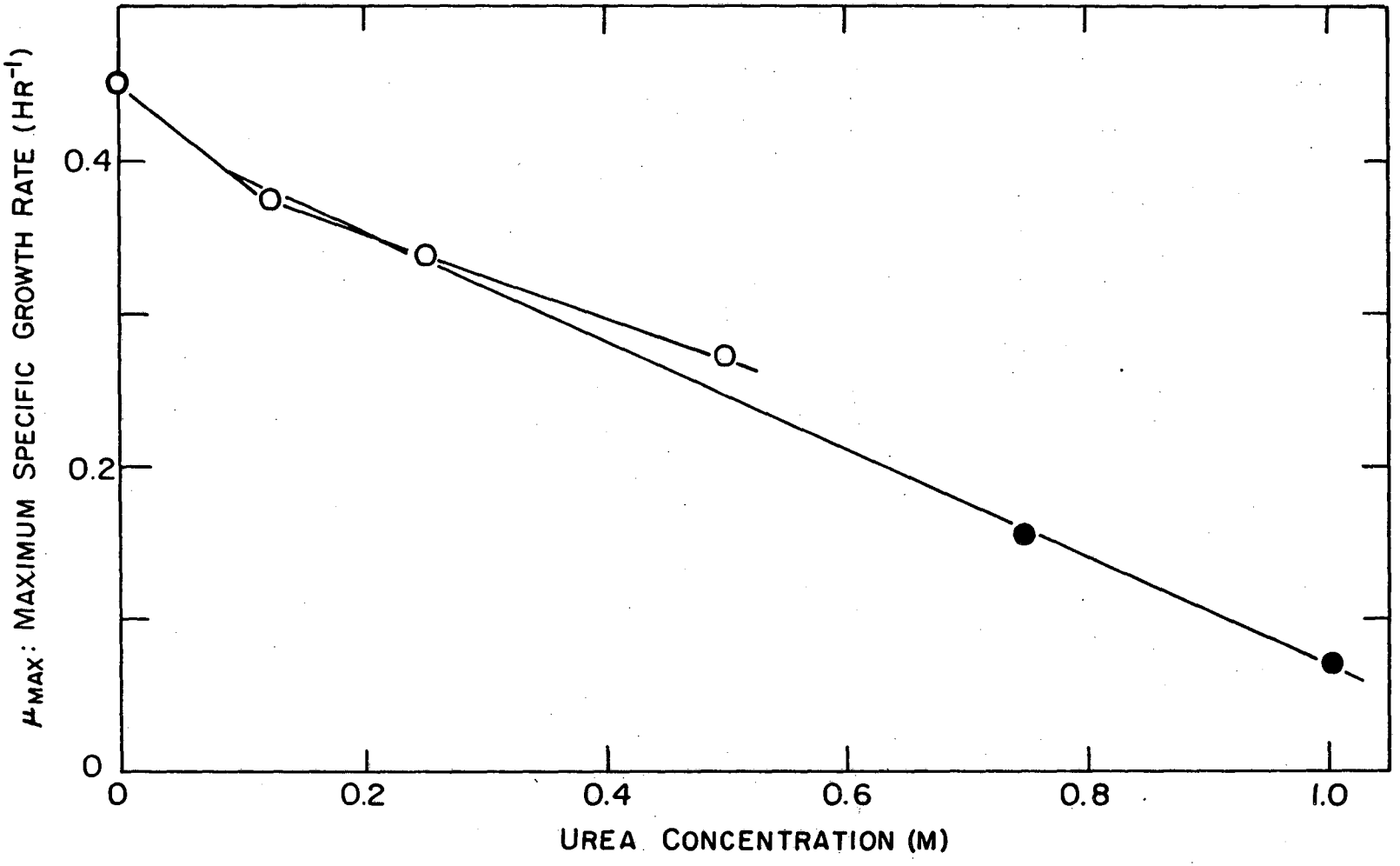


Figure 18 Effect of Urea on Maximum Specific Growth Rate.

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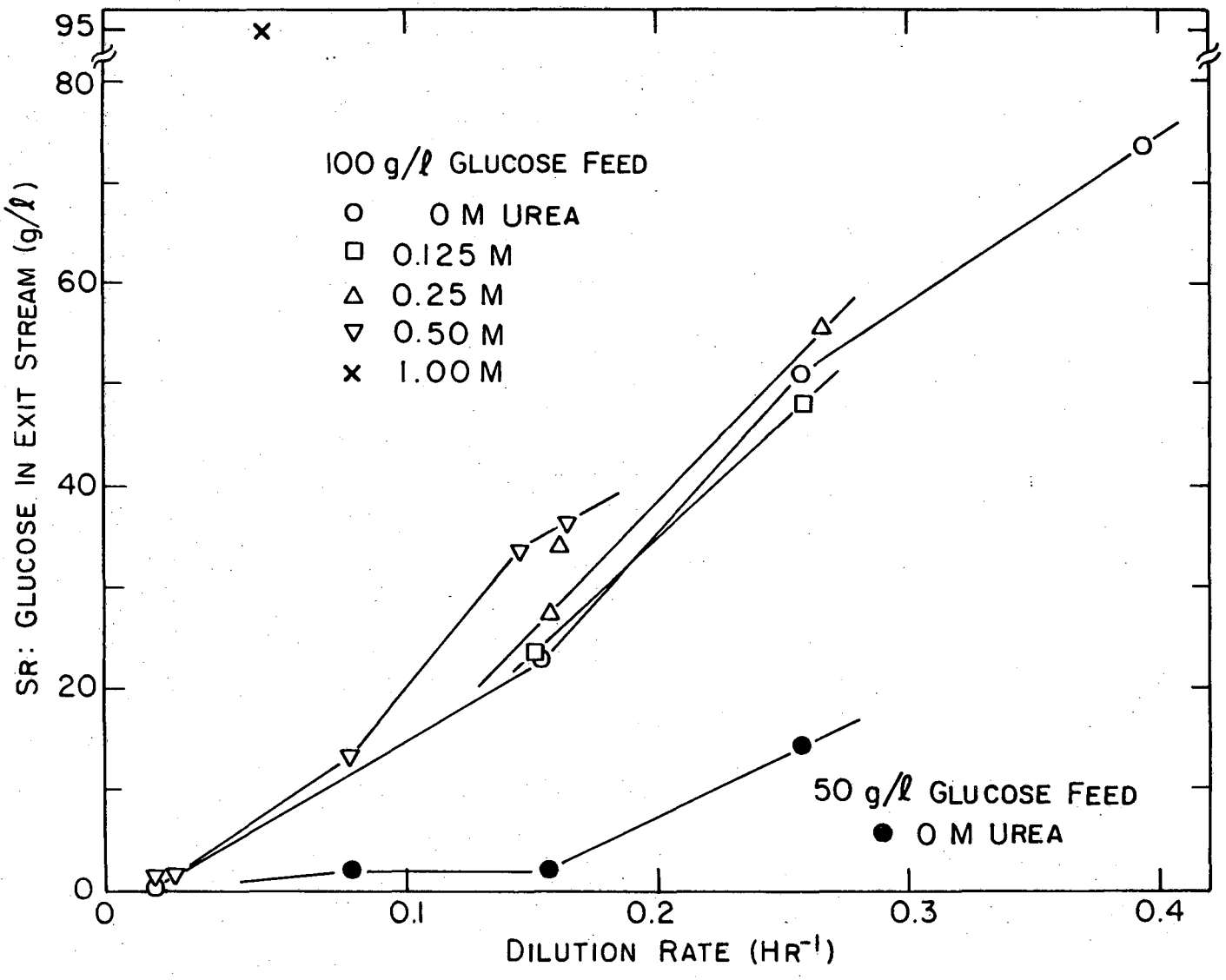


Figure 19 Effect of Urea on Glucose Concentration in Broth as a Function of Dilution Rate.

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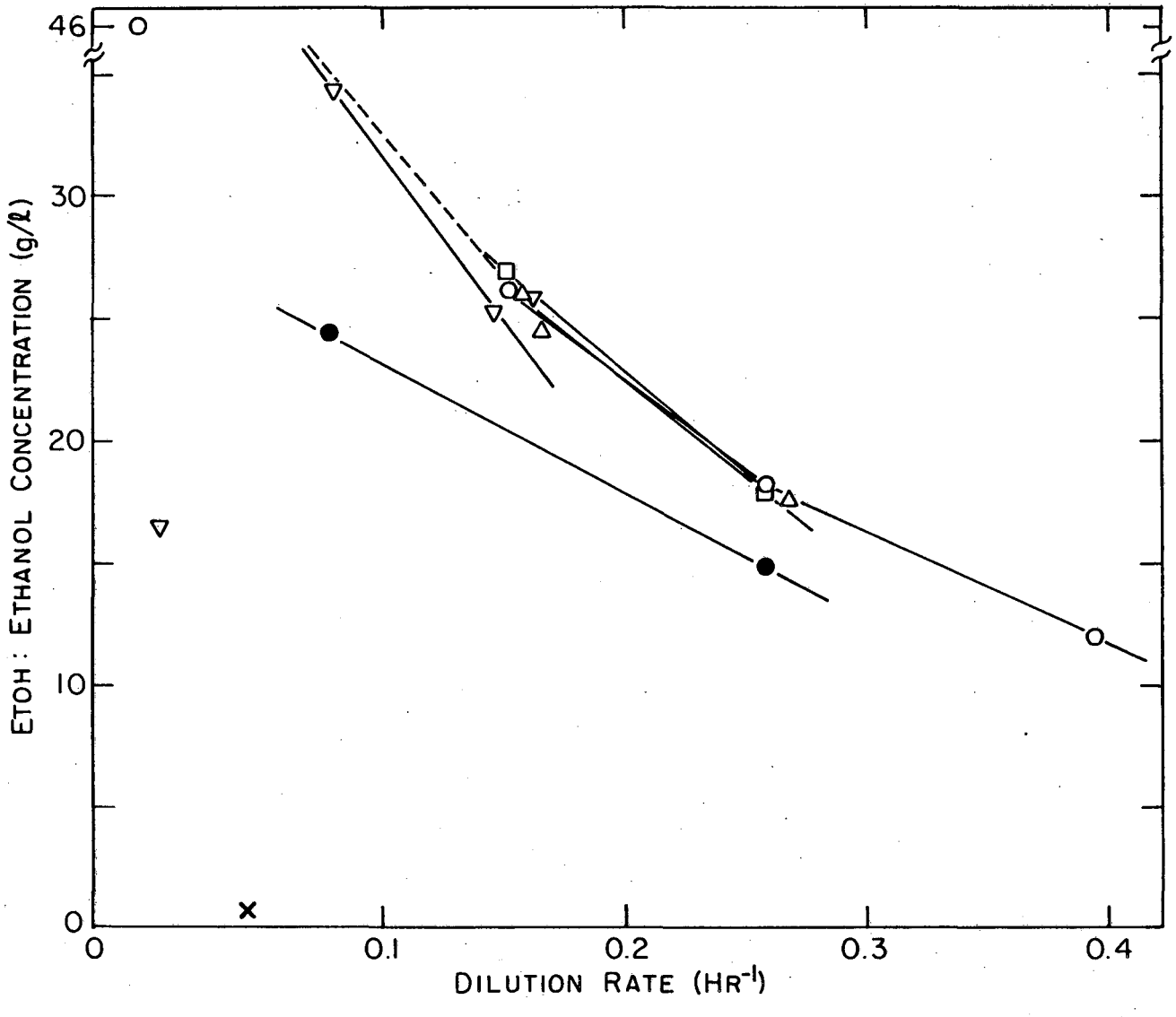


Figure 20 Effect of Urea on Ethanol Concentration in Broth. XBL 815-5709

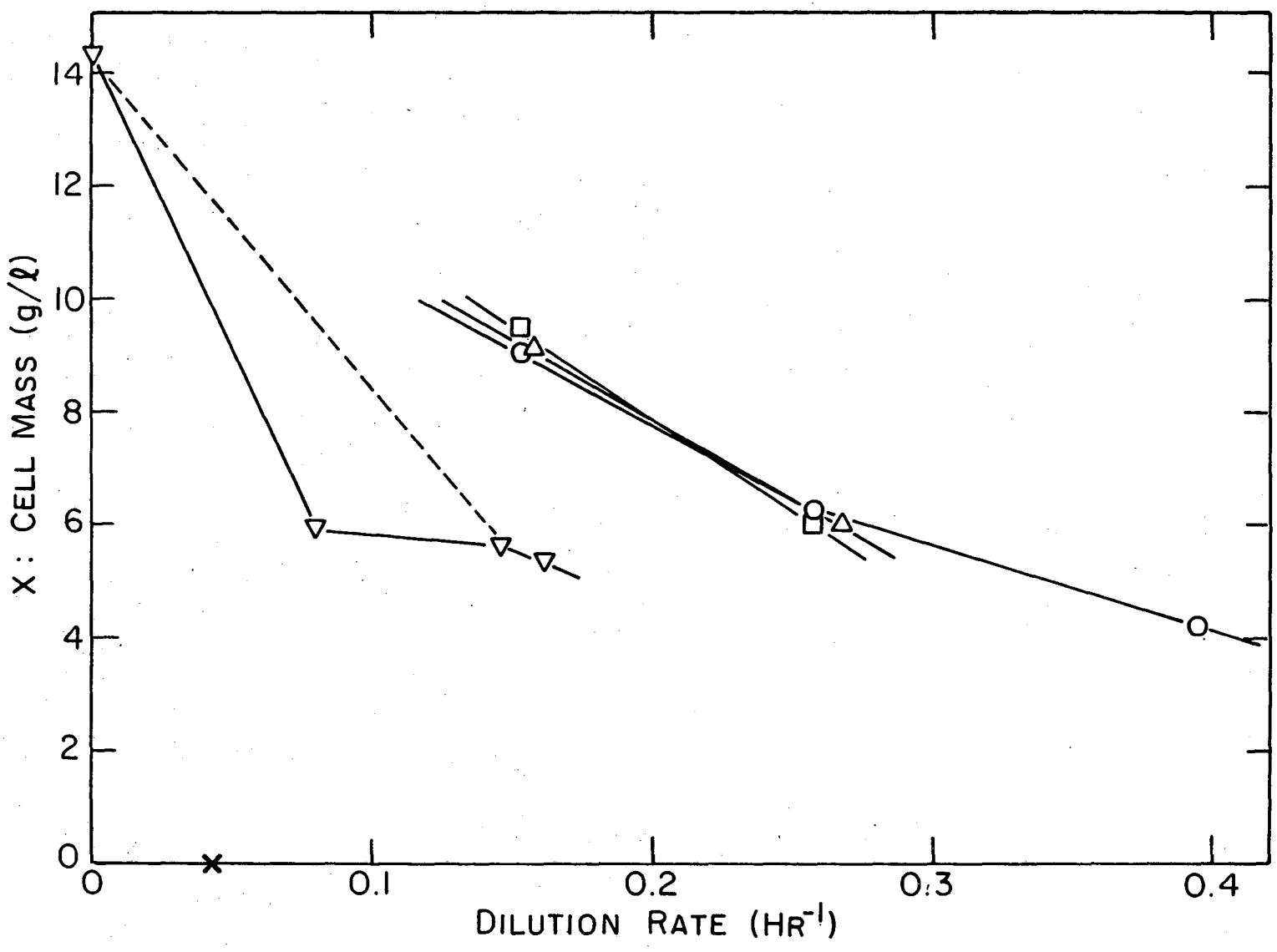


Figure 21 Effect of Urea on Cell Mass in Broth.

XBL 815-5710

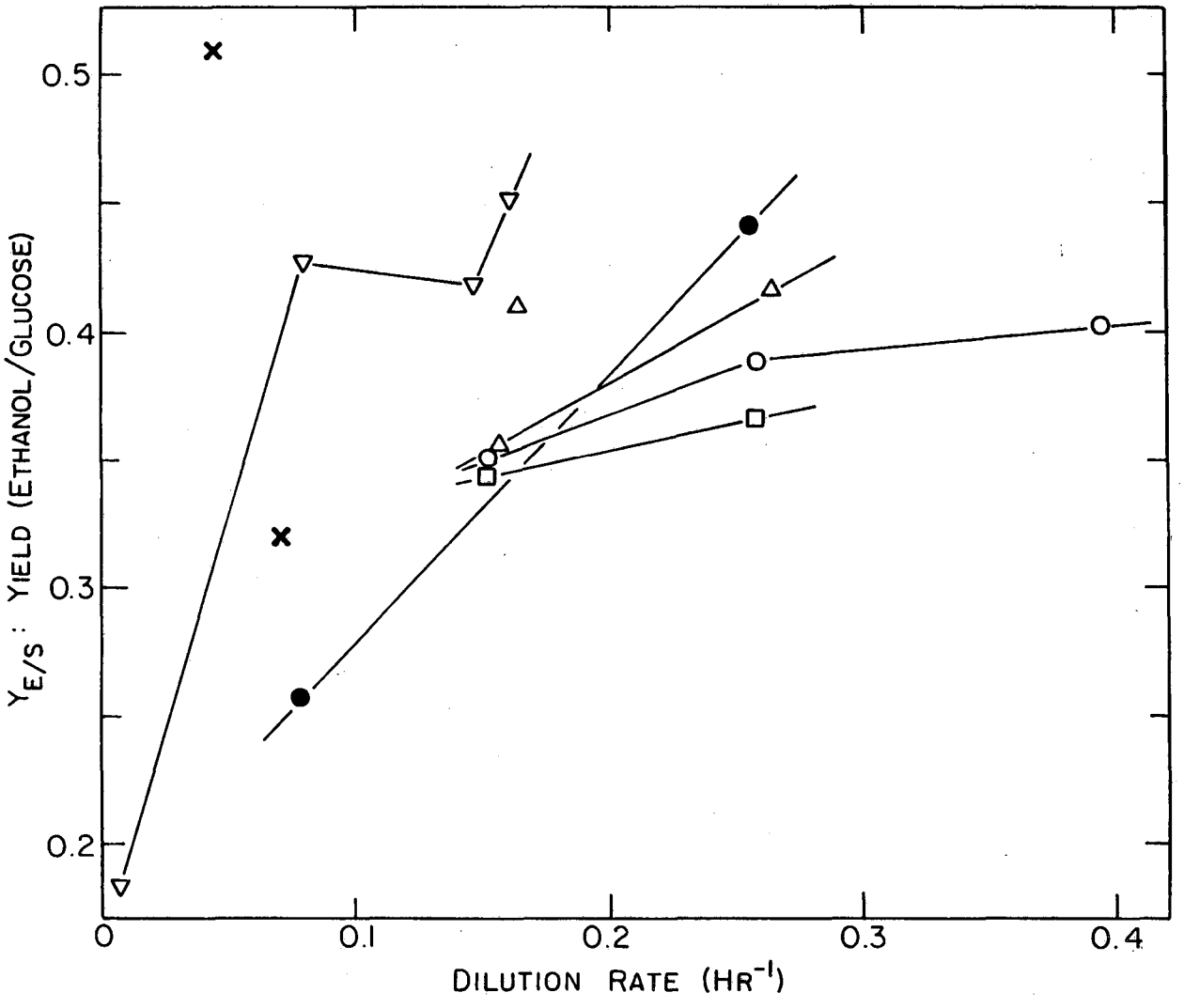


Figure 22 Effect of Urea on Yield(ethanol/glucose). XBL 815-5711

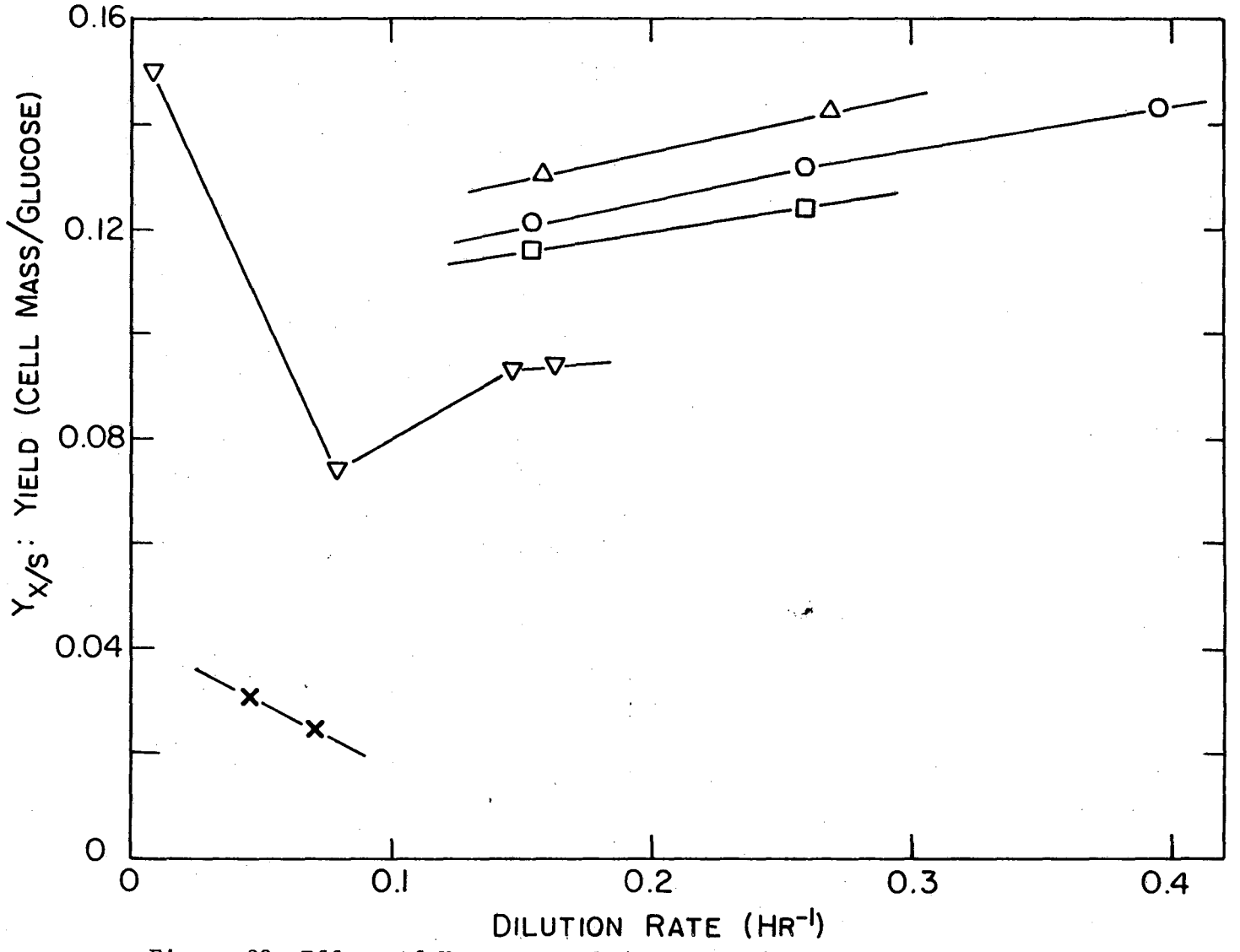


Figure 23 Effect of Urea on Yield(cell mass/glucose).

XBL 815-5712

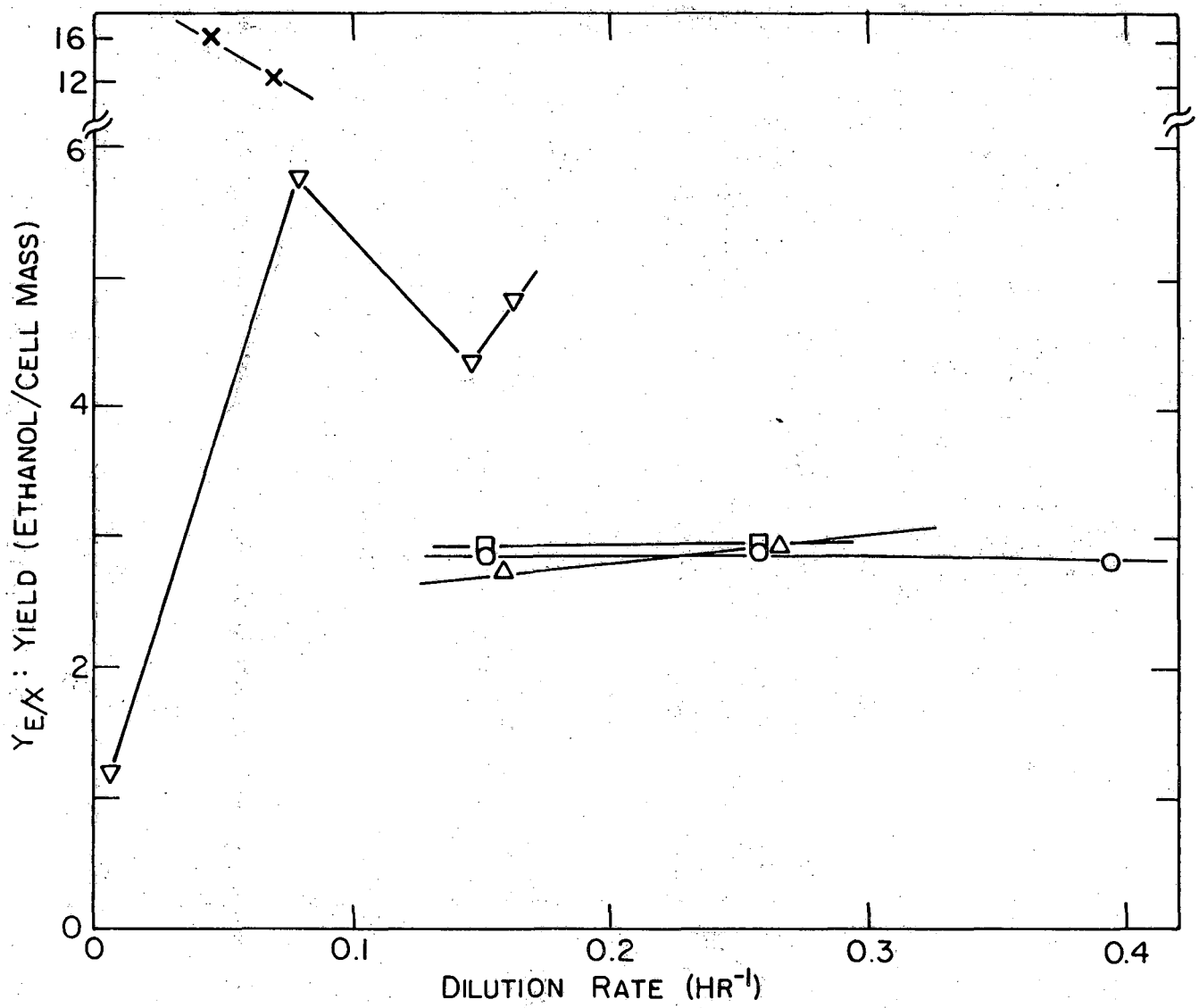


Figure 24 Effect of Urea on Yield(ethanol/cell mass). XBL 815-5713

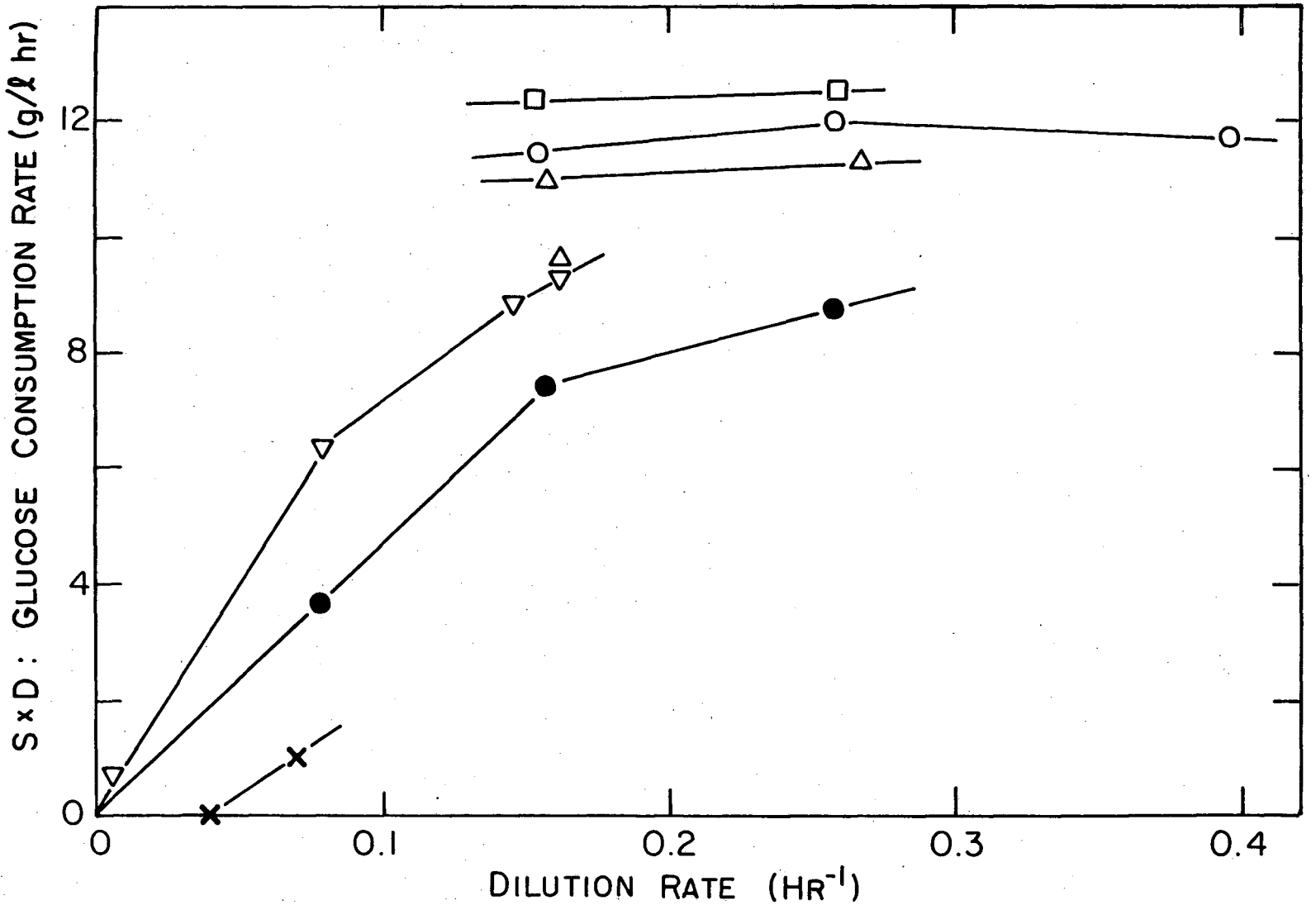


Figure 25 Effect of Urea on Glucose Consumption Rate as a Function of Dilution Rate.

XBL 815-5714

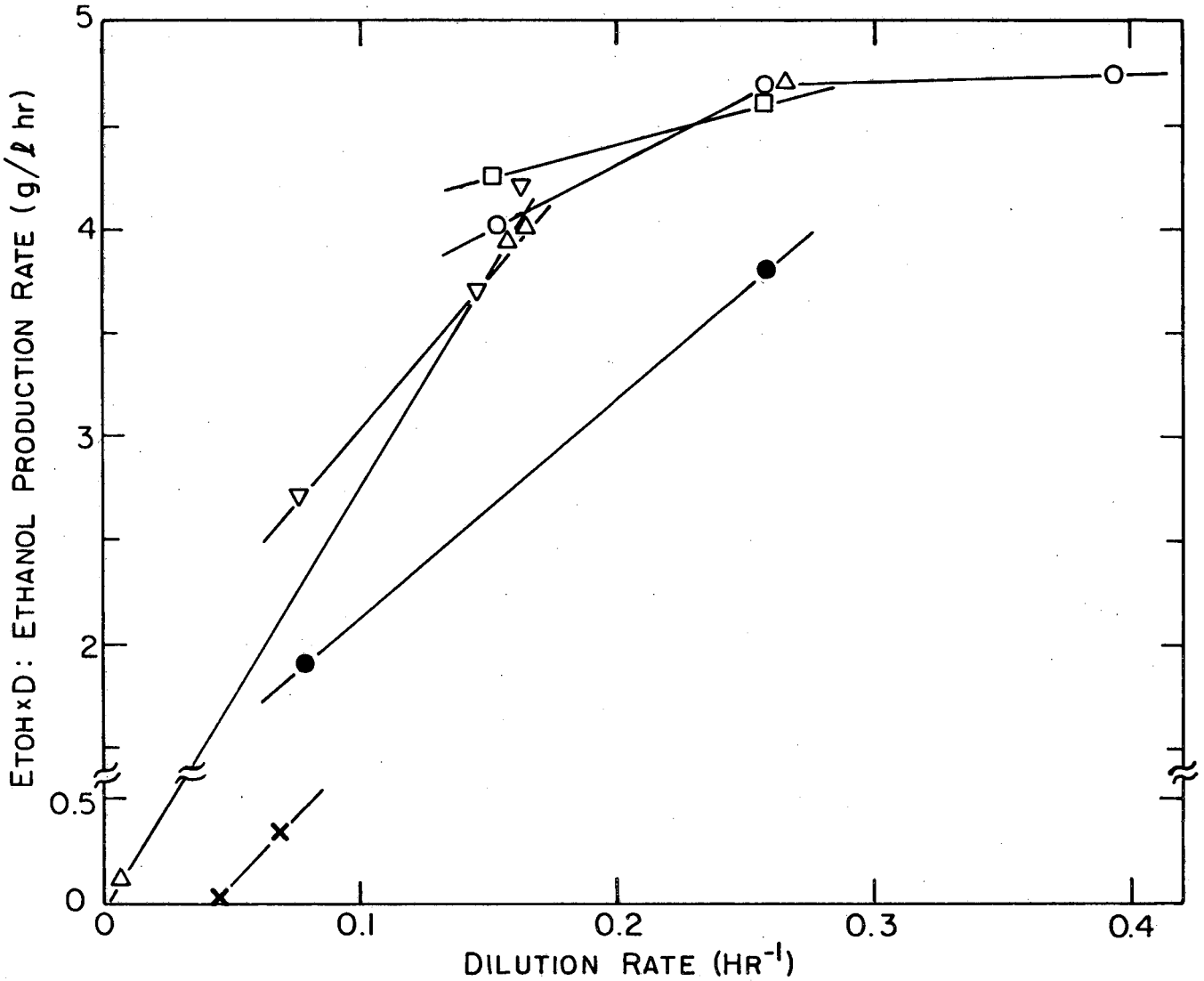


Figure 26 Effect of Urea on Ethanol Production Rate.

XBL 815-5715

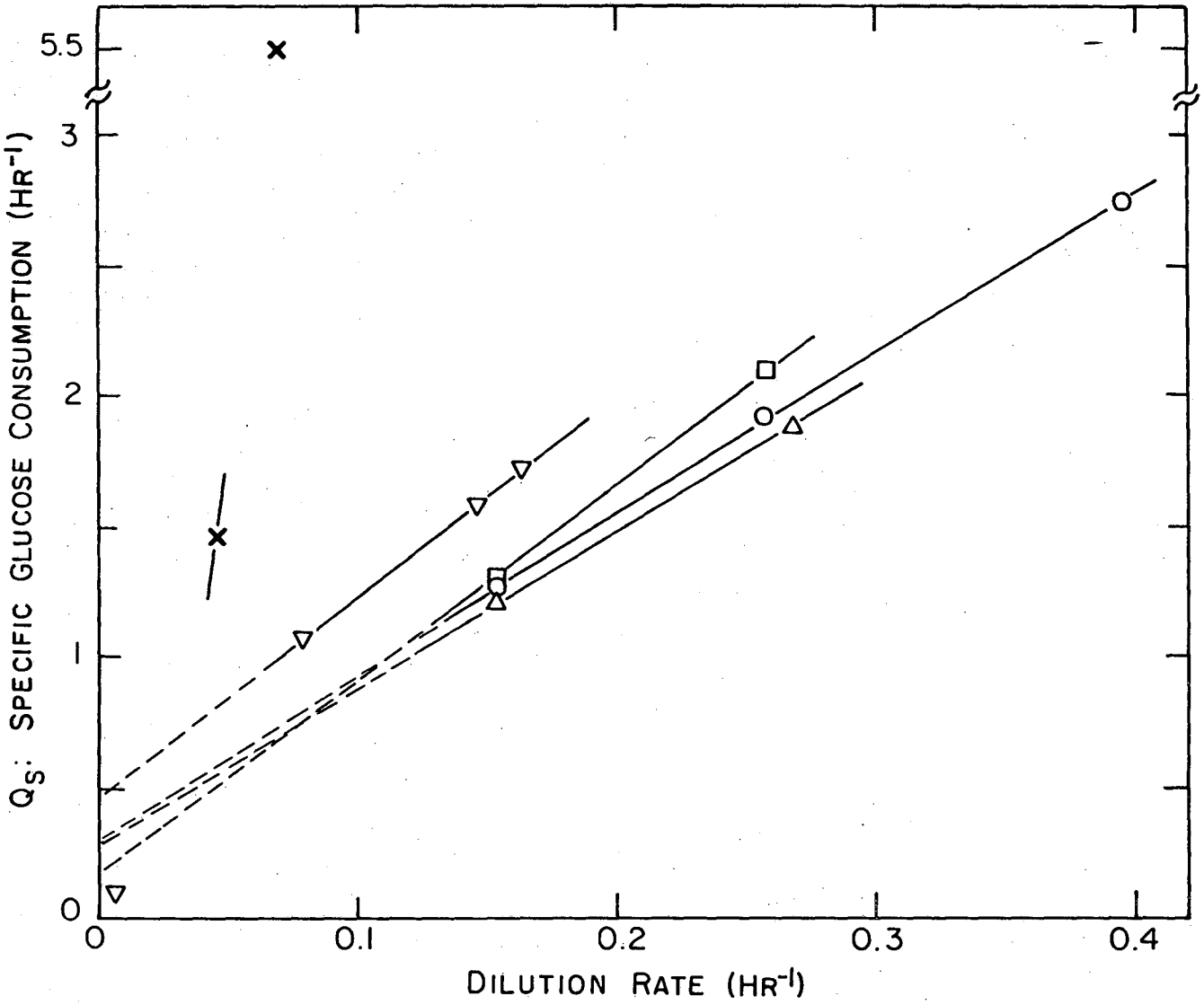


Figure 27 Effect of Urea on Specific Glucose Consumption Rate.

XBL815-5716

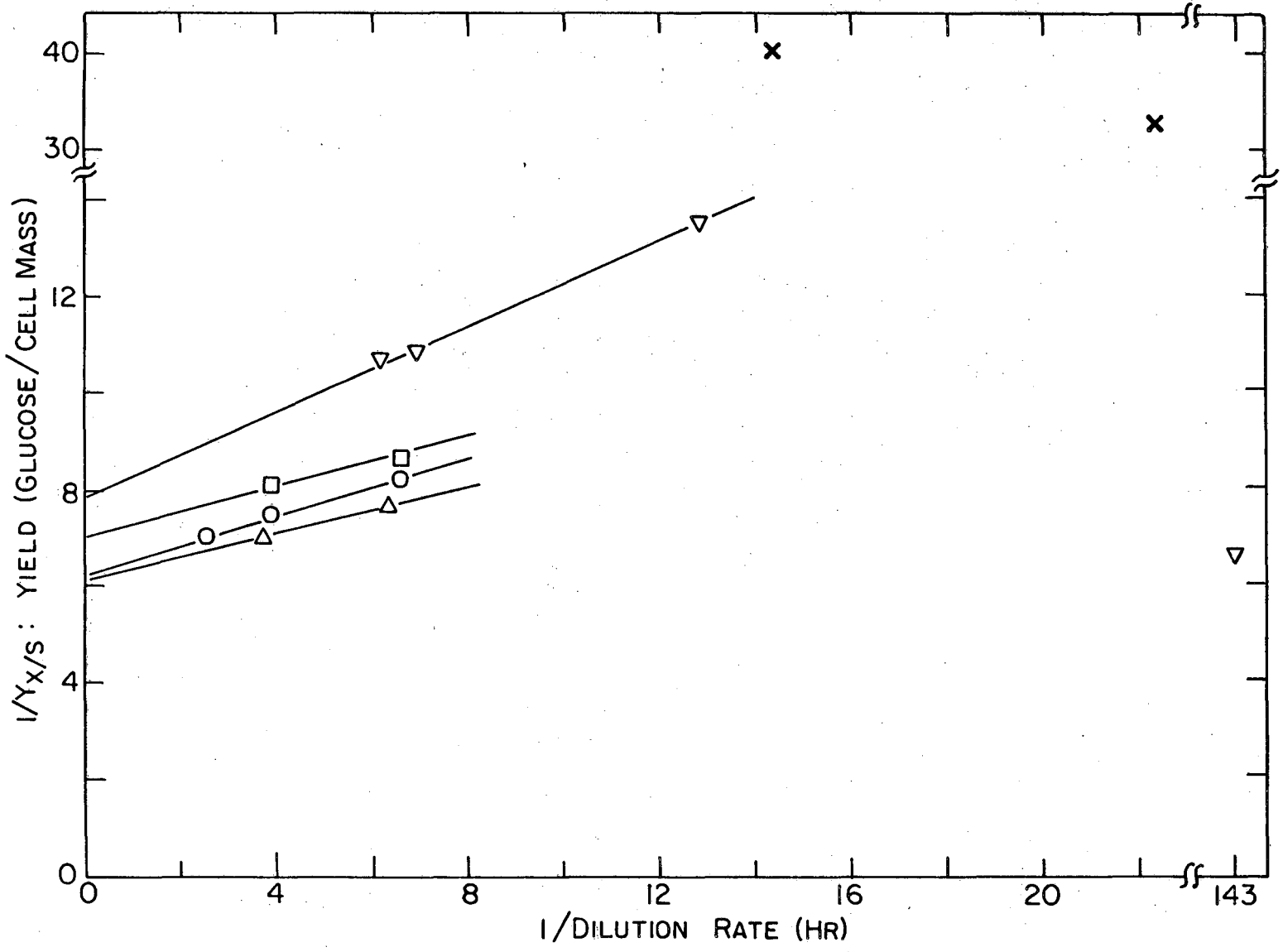


Figure 28 Reciprocal plot: Yield(glucose/cell mass) vs. Dilution Rate. XBL 815-5718

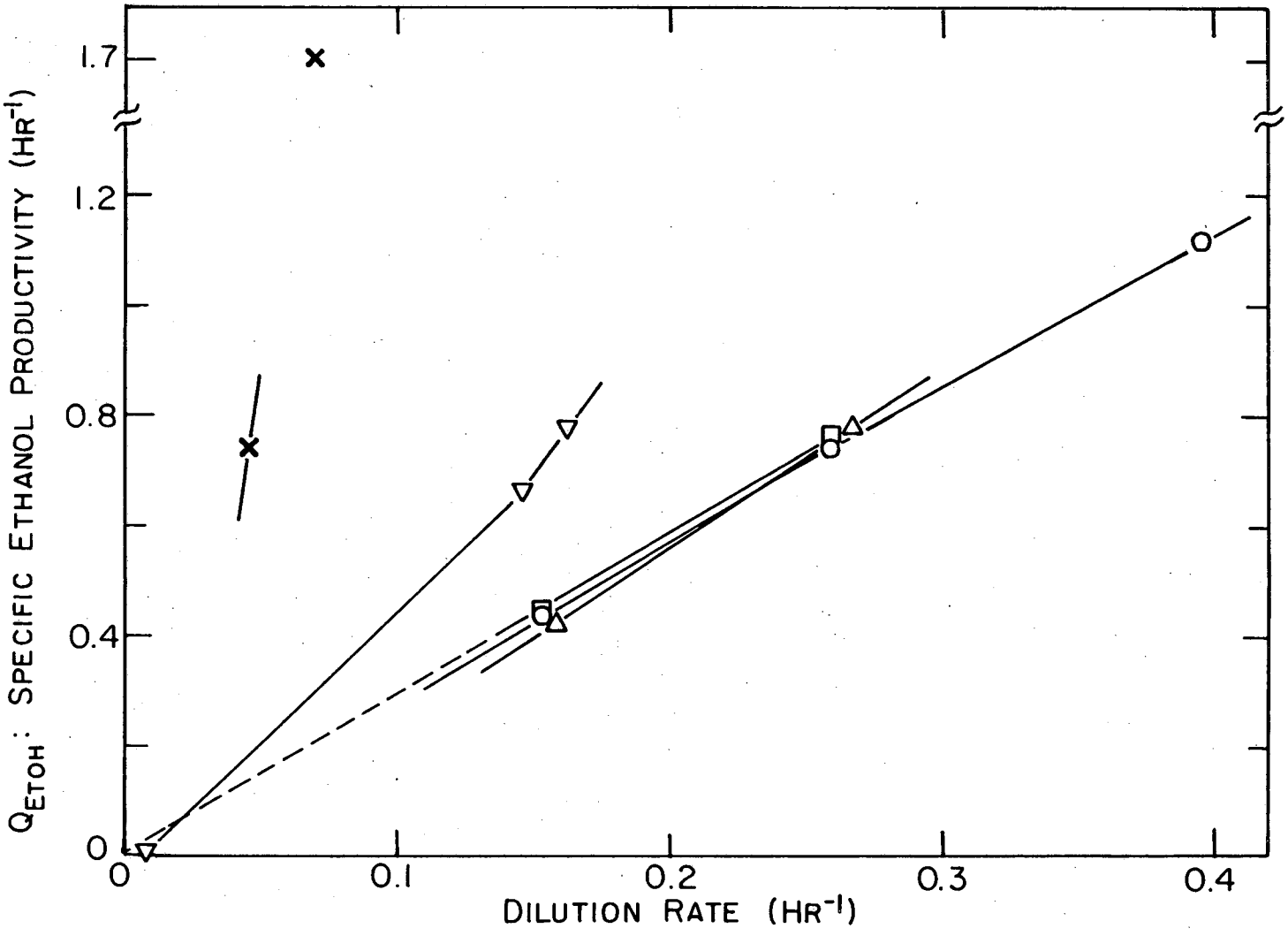


Figure 29 Effect of Urea on Specific Ethanol Productivity. XBL 815-5717

Further, comparisons can indicate whether random scatter or an actual pattern is being observed. In the majority of the plots, data are plotted as a function of the dilution rate(D) for reasons cited above.

Values for 1 M urea are rough approximations because steady-state conditions were difficult to achieve. As observed with batch cultures, low values of cell density and ethanol concentration and small differences in glucose concentrations led to large errors in yields and other calculated values. The data are included to lend further insights, but must be treated with caution. Graph symbols are given in Figure 19.

The maximum specific growth rate, which limits the maxium dilution rate, as a function of urea concentration, is plotted in Figure 18. A severe decline in growth rate is observed. The relatively high value at 0.5 M suggests that adaptation may have been occurring, providing a higher growth rate with the culture exposed to a gradual change in urea concentration.

The three graphs(Figures 19, 20, 21) provide an over-all mass balance of the fermenter by the three major components: glucose, ethanol, and cell mass in the broth leaving the fermenter.

The concentration of glucose in the exit stream as a function of dilution rate, is plotted in Figure 19. A similiar trend is observed at all concentrations except 1

M. At 0.5 M, the sugar concentration rises more quickly as the dilution rate nears μ_{MAX}. Given suffficiently low dilution rates, little glucose remains in the broth, providing for complete consumption of substrate. An inlet substrate concentration of 50 g/l leads to complete utilization at higher dilution rates, as expected.

Trends of the ethanol concentration in the broth essentially support those of the glucose concentration, as illustrated in Figure 20. However, at very low dilution rates, the ethanol concentration declines due to evaporation. This loss occurs primarily through the broth outlet port where no condenser is present. It is negligible except at low dilution rates where the concentration should be highest and production should be lowest. The value of 46 g/l at a D=0.0 indicates the maximum value of ethanol in batch culture, which declines to essentially zero in about 5 days after inoculation.

Uniform results are obtained for cell mass in the broth in the range of 0.0-0.25 M urea, as shown in Figure 21. At 0.5 M, a dilution rate of 0.10 hr^{-1} leads to a decline of 50%. A sharp incline occurs as D approaches zero, giving a cell density comparable to lower levels of urea. Cell density at 1 M indicates virtually no cells.

Conversion efficiencies of glucose indicate whether established pathways of ethanol and cell mass production are being given disproportionate emphasis or whether

alternate paths are being used which are wasteful of stored energy or create new, undetected products.

The yield of most interest from an economic viewpoint is the yield(ethanol/glucose), illustated in Figure 22. Yields increase in general with D. At lower D, ethanol concentrations are higher and production is slower, increasing the effect of evaporation. Alternatively, high ethanol levels may lead to a conversion of ethanol to acetaldehyde to provide $NADH_2$ for the reaction of acetoin to 2,3 butanediol.

Yields(cell mass/glucose) decline with increasing urea concentration, as shown in Figure 23. However, plots indicate a minimum yield occurring at a dilution rate at about 0.8 hr^{-1}.

The yield(ethanol/cell mass) as a function of dilution rate, is shown in Figure 24. The yields remain constant at 2.8 for 0.0-0.25 M over a wide range of D. However, 0.5 M leads to increased yields at D greater than 0.08 hr^{-1} with data slightly scattered. The decline in ethanol at very low D has been discussed. Note that yields for 1 M are consistently high, substantiating the trend of increased yield with increasing urea concentrations.

The effectiveness and efficiency of the paths being used are monitored by the rates of glucose consumption(Figure 25) and ethanol production(Figure26). Inhibition and stimulation of the enzymes of the major

pathways can be observed by the rates of conversion.

At lower D, the concentration of glucose declines, leading to substrate starvation. Effects of starvation are suggested by the data of 50 g/l(glucose entering fermenter). Note from Figure 19 that the glucose level is still at 15 g/l, yet the consumption rate of glucose is lower than that at 100 g/l (glucose entering) with 51 g/l leaving the fermenter, indicating lack of substrate at a level of 15 g/l.

The glucose level (50 g/l entering) drops to 2 g/l by D=0.15. In 0.5 M urea with an exiting glucose concentration of 12 g/l, the sugar consumption rate is lower. In other words, a steady-state culture is established with a higher glucose concentration with urea than without, leading to effects similiar to glucose starvation. This condition may be caused by a decline in ability in the cells to obtain glucose.

The data of ethanol production(Figure 26) follow approximately the same trends over a range of urea concentrations as in Figure 25.

Analytical tools developed by Pirt may be applied to establish maintenance requirements and true yields(see Eqs. 4 and 5). Application of Eq. 4 gives a plot of the specific glucose consuption coefficient as a function of the dilution rate(Figure 27). The slope equals $1/Y_{x/g}$ and the ordinate intercept gives the maintenance

requirement(m). Figure 28 is a reciprocal plot of the relationship between the cell mass/glucose yield)($Y_{x/a}$) and the dilution rate, application of Eq. 5. The slope equals m and the ordinate intercept gives the yield($1/Y_{x/g}$). In the range of 0.0-0.25 M urea, $Y_{x/g}$ varies from 0.14-0.16 and m varies from 0.21-0.32. At 0.5 M, $Y_{x/g}$ equals 0.13 and m equals 0.44.

In Figures 27 and 28, there is a low or negative maintenance energy in 0.5 M, D=0.007 and for both values in 1 M. Although there is substantial experimental error involved, it may be hypothesized that utilization of urea is taking place: a) when glucose starvation is imposed, or b) when the urea concentration is high. However, urea is utilized as a very poor secondary substrate, leading to severely limited growth. Alternatively, urea is being consumed as a nitrogen source rather than as an energy or carbon source.

The specific ethanol productivity, in combination with the yield(ethanol/glucose), dtermine directly the basis for economic evaluation. This is particularly true in the case of cell recycle, where the cell concentration may be enhanced greatly. The specific ethanol production as a function of the dilution rate is illustrated in Figure 29. This plot is analogous to the plot for Eq. 4 in Figure 27 and is similiar to the discussion of Nordstrom's work(Section 1.3)(72). A base (maintenance) rate of

ethanol production and a yield(ethanol/cell mass) may be obtained. The yield is based on the ethanol produced only as a result of growth-related metabolic activities.

In the range of 0.0-0.25 M urea, a maintenance requirement of zero and $1/Y_{x/EtOH}=2.7$ are indicated. In 0.5 M, a maintenance requirement of slightly less than zero (experimental error) and $1/Y_{x/EtOH}=4.8$ are obtained. As illustrated by previous graphs, specific ethanol productivity and specific ethanol yield are similiar for the range of 0.0-0.25 M, but considerably greater for 0.5 M. Values for 1.0 M suggest higher values for these coefficients.

3.3. Conclusions.

General inhibition of growth is the only major change that occurs in this yeast strain at concentrations of urea up to 0.25 M. Other changes appear to occur within the limits of experimental error. At a concentration of 0.5 M, several marked effects may be observed.

At 0.5 M urea, the following system properties decrease substantially compared to those of the control(no urea): 1) maximum specific growth rate, 2) yield(cell mass/glucose), 3) rate of glucose consumption.

The rates of ethanol production(per volume of fermenter) are similiar.

The following values increase at 0.5 M urea:

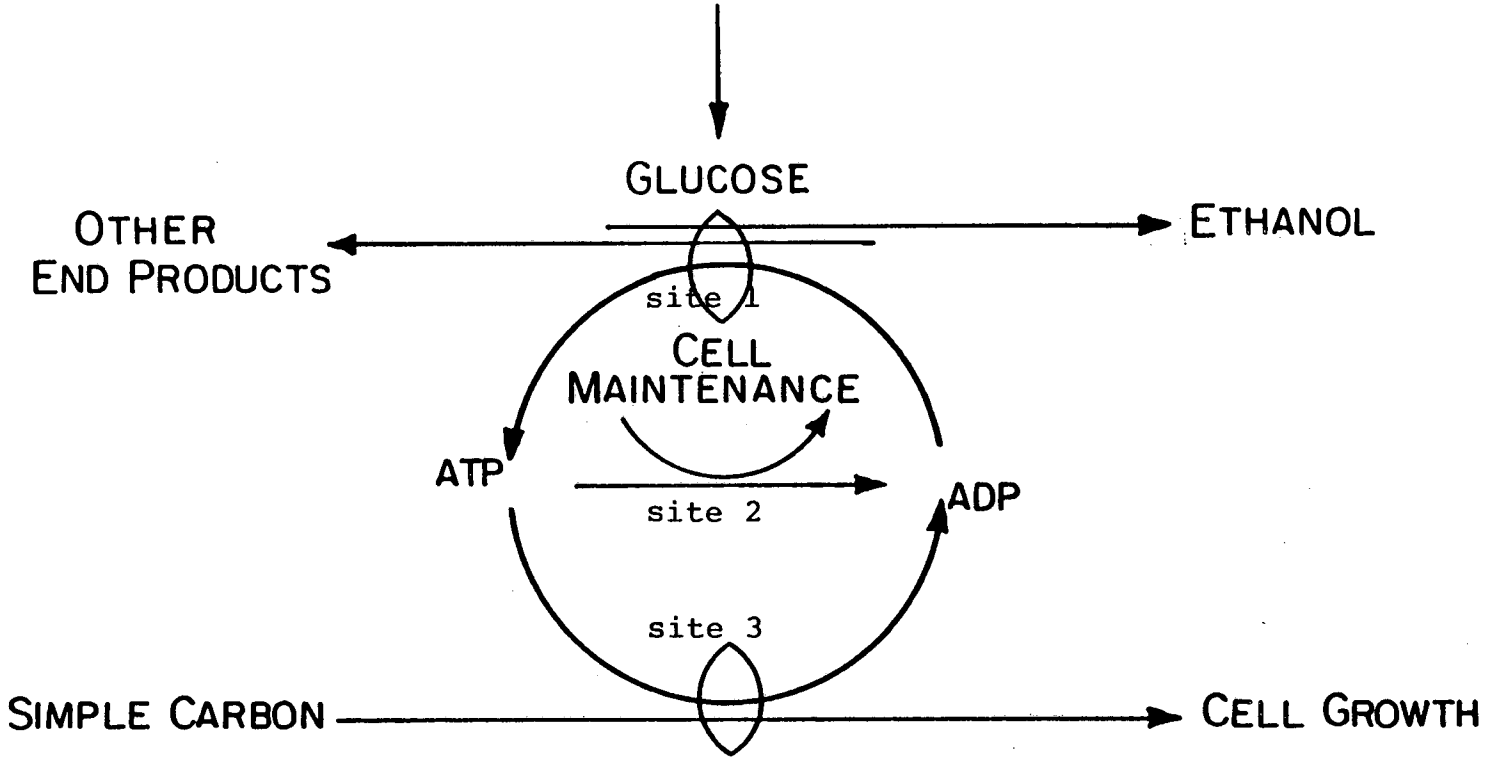
1) yield(ethanol/glucose), 2) yield(ethanol/cell mass), 3) specific glucose consumption rate, 4) specific ethanol productivity. The values at 1.0 M urea tend to substantiate these trends.

It is concluded that the presence of urea exerts a detrimental effect on the metabolic pathways of yeast, particularly at concentrations of 0.5 M or greater. The substrate appears to catabolized less efficiently, producing less ATP per unit of glucose consumed, or the ATP produced is being dissipated. This condition manifests itself as a decrease in $Y_{x/g}$, the yield based on substrate

utilized for growth only. Production of by-products yielding less ATP may be enhanced (Figure 30, reaction site #1).

The decrease in $Y_{x/g}$ is not the primary effect. The maintenance requirement increases substantially (reaction site #2). An increased maintenance expenditure is required by the concentration gradient of urea. The simple osmotic potential is most likely maintained by an increase in the internal concentration of potassium. Perhaps increasing the potassium level(or other osmotic agent) of the media would allow the cell to accumulate potassium more readily. Although urea is somewhat hydrophylic, its size allows penetration of the cell wall. Thus, some energy must be expended to expel urea. Further, the presence of urea may lead to degradation of the cell membrane, requiring constant repair to maintain its integrity. This degradation would impair the cell's ability and reduce its efficiency in maintaining any concentration gradient.

The maintenance requirement acts as a shunt("short-circuit") in decreasing the ATP pool necessary for cellular growth(site #3). Thus, a lower yield(cell mass/glucose) is produced. At the same time, the yield(ethanol/cell mass), specific glucose consumption rate, and specific ethanol productivity increase because the catabolic system is working more rapidly to replenish the ATP pool depleted by the shunt.



XBL815-5719

Figure 30. Schematic Diagram of Anabolic and Catabolic Pathways.

The yield(ethanol/glucose) also increases, suggesting that the maintenance requirement is the primary effect. If the yield had declined, it would be reasonable to conclude that by-product formation is the primary contributor to the decline in efficiency.

IV. PROCESS ALTERNATIVES AND ECONOMICS.

1. CURRENT PROCESS.

The current process is illustrated in Figures 1 and 2. Corn stover is milled and pretreated with sulfuric acid to break down the physical structure of the lignocellulosic material(MILLING, ACID PRETREATMENT). The solids are washed and separated in a rotary filter. The stover is passed through a series of mixing tanks(ENZYME RECOVERY) to contact it with solution containing enzyme and sugar, leaving the hydrolysis. The solids adsorb enzyme from this solution, are removed by rotary filters, and enter another series of mixing tanks(HYDROLYSIS) with additional enzyme and water. Enzymatic action degrades the cellulose to a sugar solution. The solids are burned to generate power. The sugar/enzyme solution passes through the countercurrent mixers(ENZYME RECOVERY) and, then, is concentrated (MULTIEFFECT EVAPORATOR) by a factor of 5-7. Minerals and vitamins are added and the mixture is sterilized and sent to the sugar fermenter(ALCOHOL PRODUCTION). Broth leaving the fermenter is centrifuged to remove the yeast, for partial recycle. The remainder is washed and dried for commercial feed purposes. The liquid leaving the centrifuge is heated by an exchanger before entering the

distillation column. The overhead product is 95 wt.% ethanol. The bottoms product stream, consisting of water and sugar, is split. One stream passes through an absorber to remove ethanol from the gas leaving the fermenter. The stream is returned to the distillation column. The other bottoms stream is sent to an anaerobic digestor(WASTE TREATMENT), utilizing the small amount of sugar for the production of methane.

Enzyme is produced by a fungal fermentation(ENZYME PRODUCTION). Delignified cellulose and nutrients are sterilized and sent to the fermenter. The broth is centrifuged. The enzyme solution is sent to the hydrolysis. Part of the solids are recycled to the fermenter. The remainder is washed and filtered to remove enzyme. The solution is sent to the hydrolysis; the solids to the furnace.

2. ENZYME RECOVERY BY ADDITION OF UREA TO HYDROLYSIS.

The current process may be modified to allow the introduction of urea to the hydrolysis. It is envisioned that urea is added in solid form to the hydrolysis tanks and is recovered as part of the bottoms stream leaving the ethanol distillation column. No major changes in the equipment or flow scheme are required.

The optimum concentration of urea in the hydrolysis is approximately 1.0 M. The effective recovery of enzyme is sensitive to lower concentrations of urea, dropping to 40%

at 0.5 M urea. Thus, a decrease in urea concentration is highly unattractive.

The sugar solution leaving the hydrolysis is concentrated by a factor of 5-7 to allow fermentation to a more concentrated ethanol broth and consequent reduction in distillation costs. This concentration step is particularly critical in modifications of the process currently under development(e.g. vacuum fermentation, flash-fermentation).

Concentration of the sugar/urea solution following hydrolysis would raise the urea concentration in the fermentation to 5-7 M. The maximum concentration of urea in which S. cerevisiae is able to function in a stable manner is approximately 0.75-1.0 M urea. The strain grows reasonably well, if slowly, at 0.75 M, but a dense culture declines at 1.0 M. Therefore, simple addition of urea into the current process is not feasible.

Concentration of the sugar solution would require removal of large amounts of urea. Several methods of removal were investigated (e.g. exchange resins, crystallization, molecular filtration), but all proved uneconomical or infeasible. Removal of urea by degrading it to ammonia and carbon dioxide is fairly easy, but the large loss of material is uneconomical, even though urea is sold as a bulk chemical.

By deleting the concentration step, a scheme allowing

urea to flow through the fermentation may be marginally possible with a dilute sugar stream and resultant ethanol stream. In conclusion, a flow-through scheme does not appear to be attractive from an economic standpoint and may be only marginally feasible.

3. ENZYME RECOVERY BY CONCENTRATED UREA WASH.

An alternative modification of the current process employing a wash of the hydrolysis solids to remove enzyme may be considered. A block diagram of the alternative is illustrated in Figure 31, indicating only those operations which will be affected by modifications of the current process. Quantities listed in parentheses are in units of tons per day, except enzyme given in I.U. per day.

Acid-treated corn stover and make-up enzyme enter the hydrolysis(streams 1 and 2). A rotary filter(RF-1) removes the hydrolysis solids with adsorbed enzyme. Sugar/enzyme solution enters an ultrafiltration unit(UF-1). Enzyme is concentrated by a factor of 10 and returns to the hydrolysis. The sugar solution is sent to the multi-effect evaporator. The solids leaving RF-1 enter a mixing tank where they are combined with 6 M urea. Solids are removed from the solution leaving the mixing tanks by a rotary filter(RF-2) and burned for power generation. The filtrate from RF-2(urea/enzyme solution) enters an ultrafiltration unit(UF-2). Enzyme is concentrated and returned to the hydrolysis. Urea solution is recycled to the mixing tanks.

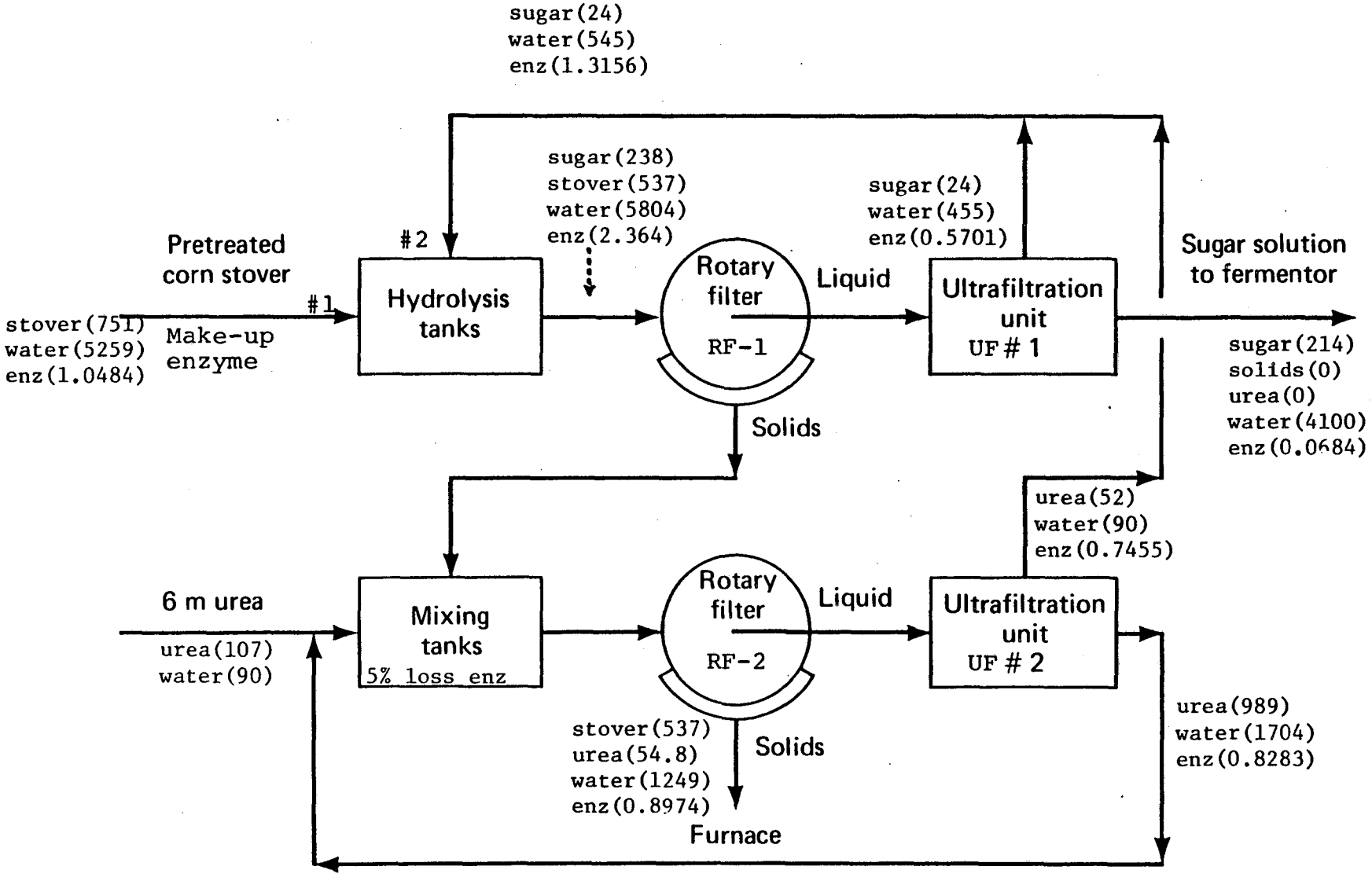


Figure 31. Partial Block Diagram of Process:
Enzyme Recovery By Concentrated Urea Wash.

Enz.: I.U./day
Other quantities: tons/day

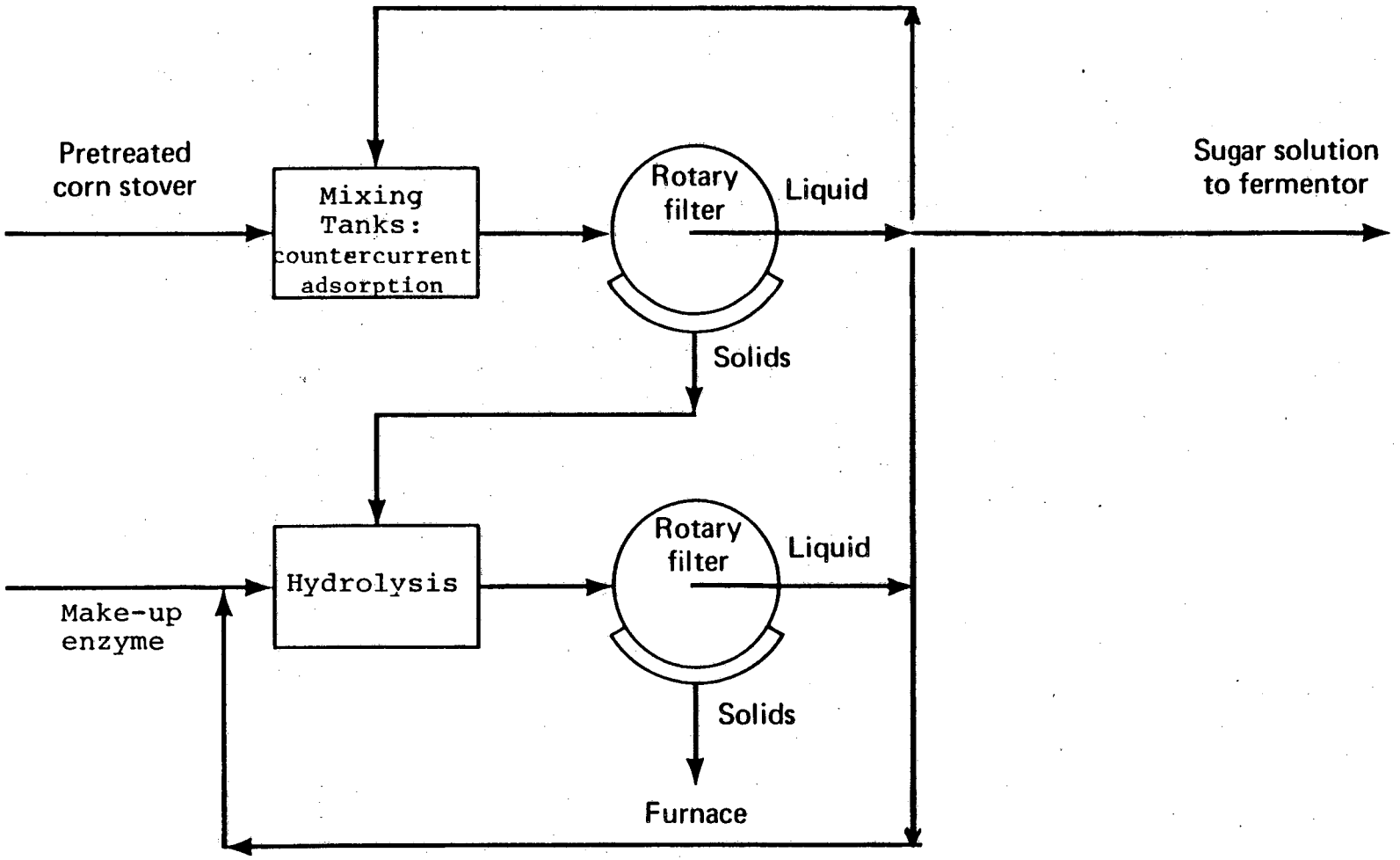
XBL 818-1084

The above alternative may be compared with the process as it presently exists with countercurrent streams of stover providing for recovery. The current process has been redrawn in Figure 32 to facilitate a comparison of the two schemes. Although specific pieces of equipment may be used for different purposes, general use requirements are similiar for all operations except the ultrafiltration units and pumps prior to these units.

The conditions of a urea wash and efficiency of enzyme recovery were investigated by Riaz(38), utilizing QM9414 cellulase in a newsprint hydrolysis. Riaz(38) observed that 25-30% FPA of the enzyme remained in solution following newsprint hydrolysis. The residue solids were washed with varying concentrations of urea. A maximum recovery of 32-39% FPA of the original enzyme concentration in the hydrolysis was reported with a wash solution of 6 M urea. C_x activity approached a broad maximum of 44% over a range of 3-7 M urea. C_1 activity attained a sharp maximum of 34% at 8 M, declining quickly at other concentrations.

Results with this system, involving QM9414 enzyme, may be reasonably extrapolated to a system of Rut-C-30 enzyme since other comparisons of these two investigations have been found to be similiar.

Calculations for the necessary modifications to the current process are given in the appendix, Process Design Equations. Parameters were taken from the most current



XBL 824-9349

Figure 32 Partial Block Diagram of Current Process.

calculations of this general process(6, 43). Equations were taken from a computer program written by Perez(81), the correlations of which were derived from graphs by Peters and Timmerhaus(82) and others(83).

A design basis of 214 tons glucose/day was chosen. Enzyme loading was set at 5.2 I.U./ml. (1 I.U./ml. = 3.5 FPA approximately). Cost of manufactured enzyme was taken at $4.0/million I.U.(Jan. 1981)(81). A recovery gain of 29% between countercurrent and washing schemes was taken, leading to an incremental annual savings of $8.45 million. Annual fixed capital costs of the ultrafiltration units and pumps total $1.47 million and ultrafiltration membranes are $0.76 million. Utilities costs are $0.23 million. A net gain of $5.98 million, exclusive of the cost of urea, is achieved.

The bulk cost of urea is estimated at $165/ton(Jan. 1981)(84). A once-through process for urea (no recycle) consumes 1096 tons/yr. or $59.7 million/yr. Therefore, the break-even point necessitates a recycle fraction of 90.0%. In the rotary filter(RF-2), urea will be lost by surface adsorption to the solid and by being dissolved in the water wetting the surface. Also, urea will be dissolved in the enzyme solution leaving UF-2. Both losses are estimated at 5% of the entering streams. Further, urea will degrade to some extent, particularly if high temperatures(greater than 40^{o}C) are achieved locally("hot spots").

Denaturation of enzyme in concentrated urea solutions is temperature-dependent, with severe degradation above 30°C. However, removal of enzyme from solid residues is most effective at approximately 40°C. The most economic compromise is likely to be in the range at which cooling water is available(15-20°C).

If the processing streams containing urea are maintained below 20°C, a urea loss of less than 5% due to degradation may be feasible. If a reasonably optimistic estimate of 5% is taken, the break-even point is exceeded. The incremental savings over a counter-current process are exceeded by the incremental expense of the additional equipment and, primarily, urea. Therefore, this alternative does not appear to be economically feasible.

4. ENZYME REMOVAL BY ACETONE PRECIPITATION.

The alternative of a concentrated urea wash may be modified to recover enzyme by precipitation with acetone rather than by ultrafiltration. Mitra(85) designed a process converting newsprint to ethanol that is similiar to the current process for stover. However, in the enzyme production stage, enzyme is produced in a dilute aqueous solution and precipitated by acetone("acetone precipitation"). This precipitated enzyme provides the make-up stock for the hydrolysis.

The unit operation of acetone precipitation may be applied in the concentrated urea wash alternative to

replace the ultrafiltration operations(UF-1, UF-2). Calculations are given in the appendix, Process Design Equations. Equipment costs for the "acetone precipitation" are dominated by the reboiler furnace for the acetone distillation column. The newsprint solids are burned in this furnace, eliminating any heating costs. However, this energy can also be used in the distillation of ethanol. If a reasonable cost of this energy is assumed, the cost of the process increases by approximately 100%. The cost of make-up acetone is small, because recovery is estimated at 99.9%.

Scale-up of equipment and other costs allows for a rough comparison. The scaled costs, based on processing 6349 tons water/day are:

"Ultrafiltration":	$2.45 million/yr
"Acetone precipitation":	$5.92 million/yr

Based solely on economics, ultrafiltration is superior to acetone precipitation for removal of enzyme from solution.

5. FINAL CONCLUSIONS.

The most economical alternative of removing enzyme from solution appears to be counter-current contacting of the solution with fresh solids. Ultrafiltration is approximately 3-4 times more expensive. Precipitation by acetone is more expensive by a factor of 6-8.

Enzyme recovery from the solids is marginally feasible

by using urea in the hydrolysis itself. Recovery by a concentrated urea wash of the solids is effective, but marginally uneconomical. It is the tonnage and cost of urea which totally dominates the economics of the alternative.

The inherent uncertainties in critical assumptions, particularly in regard to urea, and the increased complexity of the process make this scheme unattractive. However, the economics of either enzyme production or urea wash may change. The cost of producing enzyme may drop dramatically as continuous fungal fermentations are improved. Further, if the cost of urea changes, it will have a profound effect on the economics.

The equipment costs are a relatively minor fraction of the cost. Urea is sold as a bulk chemical so its price is not extraordinarily high. Losses of both urea and enzyme are not excessive, and are not likely to be reduced significantly, particularly for a reagent that has a high solubility coefficient in water. A reagent acting in a manner similiar to urea must be found that: 1)may be utilized at lower concentrations, 2) is less expensive per unit, or 3) allows for a design with smaller losses. The most promising avenue to explore is a reagent that simply is more effective per ton(or mole). Possibilities may arise as further understanding is achieved of the adsorption phenomena occuring with the cellulase complex.

V . APPENDIX: ENZYME ASSAYS.

BUFFERS, SUBSTRATES AND REAGENTS.

A. Acetate buffer, 0.05M, pH 5 (+ 0.10, check with meter).
   1. 4.35 gm/L(NaAc + $3H_2O$) or 2.62 gm/L(NaAc) and,
   2. 1 ml glacial acetic acid per liter and,
   3. 0.02% NaN_3(0.2 g/L)

B. Filter Paper (for FP Activity)
   1. 1 x 6 cm strips cut from Whatman □1 filter paper

C. Glucose Standard
   1. 4.0 mg/ml glucose ('Baker analyzed' dextrose) in d.$H._2O$ and 0.02% NaN_3

D. DNS (3,5-dinitro salicylic acid)
   1. Dissolve 24 gm NaOH in approximately 200 ml d.H_2O and cool.
   2. Dissolve 8.0 gm DNS (acid, monohydrate or monosodium salt) in approximately 500 ml d.H_2O, as completely as possible.
   3. Dissolve 5.0 gm phenol in approximately 80 ml H_2O.
   4. Add approximately 15 ml (1) to (3) and mix.
   5. Slowly add remainder of (1) to (2) and mix to dissolve.
   6. Add 200 gm NaK tartrate to (5) and mix until dissolved and no further gas bubbles escape.

7. Add 5 gm $NaHSO_3$ (6.1 gm Na_2SO_3)to (4) phenol solution and mix to dissolve.
8. Add (7) to (6) and mix. Bring volume to 1 liter and mix.
9. Filter through glass wool, add 5 additional gms $NaHSO_3$ (6.1 gms Na_2SO_3) and mix well to dissolve.
10. Store in inert container with taped cap and in minimum light until ready to use.

FPA (Filter Paper Activity) ANALYTICAL PROCEDURE.

I. Materials, Reagents, etc.:

1. 50°C bath.
2. 16 x 100 mm test tubes.
3. Filter paper strips, 1x6 cm (50 mg) cut from Whatman □1.
4. 0.05 M acetate buffer, pH 5.0.
5. Glucose standard solution, 2 mg/ml in d.H_2O.
6. DNS

II. Preliminary Preparation:

1. Roll filter paper strips into tight coils.
2. Dilute enzyme sample to give a final FPA of 0.3-0.5 mg/ml.
3. Dilute standard glucose solution to 2.0, 1.0 and 0.5 mg/ml concentrations.

III. Preparation of Test Tubes:

1. Activity Tubes.
   a) Add 1.0 ml of diluted enzyme to each of two test containing 1.0 ml acetate buffer (This is 1:1 dilution

= x 2.

2. Background Tubes (BKGD)

   a) Add 1.0 ml of diluted enzyme to a test tube containing 1.0 ml acetate buffer; cap tubes (enzyme BKGD).

   b) Add filter paper coil to tube containing 1 ml H_2O + 1 ml buffer (filter paper BKGD).

3. Standard Tube

   Add 1.0 ml of standard to each of two test tubes containing 1.0 ml acetate buffer and cap tubes.

IV. Incubation of Test Tubes:

1. Add a filter paper coil to each activity tube, so that the coil lies on its side <u>completely submerged</u> in the enzyme solution. Cap tubes.
2. Place all activity, background and standard tubes in a 50°C bath for 60 minutes
3. Add 5 ml DNS to each tube.
4. Vortex all tubes taking care to disintegrate filter paper in activity tubes.
5. Place all tubes in 100°C bath for 10 min., followed by cooling in ambient bath 3-4 min. Vortex.
6. Clear filter paper debris in activity tubes by pushing Kim-Wipe plug to bottoms of tubes with a glass rod and transfer cleared solutions to new tubes.
7. Read absorbance at 600 nm, using d.H_2O as a blank.

V. Activity Calculation:

1. Plot absorbance vs. mg glucose in standard tubes to obtain standard curve.
2. Determine mg glucose in activity and BKGD tubes by use of standard curve.
3. Subtract mg of enzyme and filter paper BKGD from mg in activity tube to obtain net enzyme activity. Record as:

FPA (x 1/dil).

e.g. 0.50 (x5 x2) if enzyme was initially diluted 1:4 (= x 5) and net activity is 0.5 mg/ml.

NOTE: Dilution of the enzyme includes the dilution by addition of acetate buffer. If enzyme was originally diluted 1:9, the dil used is 1/20, since addition of buffer further dilutes the enzyme by 1/2.

V. APPENDIX: PROCESS DESIGN EQUATIONS.

1. ENZYME RECOVERY BY CONCENTRATED UREA WASH.

A modification of the current process employing a 6 M urea wash of the hydrolysis solids to remove enzyme may be considered. Parameters were taken from the most current calculations of this general process. Many equations were from a computer program written by Perez(81), the correlations of which were derived from graphs by Peters and Timmerhaus(82) and others(83).

A design basis of 214 tons glucose/day was chosen. The product stream consists of 6.1% reducing sugars, of which 80% is glucose, giving a glucose concentration of 4.9%. The water flow is 4100 tons/day.

Stover/Solids Mass Balance.

Raw corn stover is treated with acid, reducing its dry weight to 62% of its original weight. Acid-treated stover contains 57 wt.% glucose. A cellulose-glucose conversion of 50% is estimated. Therefore, the acid-treated stover requirement is 751 tons treated stover/day or 1211 tons raw stover/day.

Enzyme Mass Balance.

A solids suspension of 15 wt.% and enzyme

concentration of 5.2 I.U./ml.(1 I.U./ml. equals 3.5 FPA approximately) are set, based on economic optimization studies(81). These values are significantly higher than concentrations investigated in this paper. Enzyme loading is 3.15×10^7 I.U./ton treated stover and 2.36×10^{10} I.U./day.

Enzyme Mass Balance.

The fraction of enzyme remaining in solution following the hydrolysis is estimated at 27% of the original enzyme concentration. Of the remaining 73%, 48% may be recovered in a concentrated urea wash(35% of original). However, a loss of 5% is set for the mixing tanks.

The enzyme recovery efficiency of the ultrafiltration units is set at 90%. This efficiency is set at a higher value than has been assumed in other works(Mitra(85)) because a low molecular weight cut-off(10,000) and low operating pressures(20 psi) have been set in the design. This allowance decreases the flux rate and, therefore, increases the required surface area of the ultrafiltration membranes.

Water Mass Balance.

A solids suspension of 15wt.% is set in the mixing tanks(wash). The stover/water ratio of the solids stream leaving the rotary filters is set at 0.43.

Urea Mass Balance.

Ultrafiltration unit UF-1 filters and passes 90% of the inlet water stream, leaving 10% in the solids stream.

UF-2 passes 95% of the incoming water. This specification results in a 5% loss of urea. An additional 5% loss is lost in the solids stream of RF-2.

Additional criteria pertinent to specific pieces of equipment will be stated as they are applied.

SAVINGS IN ENZYME COST.

The cost of manufacturing enzyme is estimated at $4.0/million I.U.(Jan. 1981)(81). An enzyme demand of 7.802×10^{12} I.U./yr. is required in the hydrolysis. A recovery of 27% gives an annual savings of $8.43 million. A recovery of 55.8% saves $17.41 million, yielding a net gain of $8.99 million.

ULTRAFILTRATION UNITS: UF-1, UF-2.

Definitions and Equations,

derived from program of Perez(81).

a) 1 I.U./ml. = 1.3 g. protein/l. approximately.

b) PROT1[wt.%] = E2[I.U./day]/W2[tons water/day] x 1.3 [(g. prot./l)/(1 I.U./ml.)] x 1/2000[ton/lbs] x 1/8.35[lbs/gal] x 1/1000[l/ml] x 1/1000[l/g] x 100%.

The protein concentration of inlet stream to UF unit equals the enzyme flow rate/ water flow rate.

c) PROT2[wt.%] = PROT1/0.1.

The inlet stream is concentrated by a factor of 10 with regard to solids.

d) FLUX1[gal/sq.ft. day] = -4.489 x ln(PROT1) + 29.74

FLUX2 = -4.489 x ln(PROT2) + 29.74

FMAX = FLUX2 + (0.667 x (FLUX1 - FLUX2))

FAVE = 0.87 x FMAX x 0.90

Calculations of the fluxes through the membranes are made, based on performance correlations. FAVE is the average flux.

e) WATUF[gal water/day] = WPROD[tons water/day] x 239.6[gal/tons]

The flow rate of water passing through the membrane is calculated by the flow rate in the product stream.

f) UFAREA1[sq.ft./unit] = WATUF/(FAVE x 3)

The ultrafiltration surface area is calculated by dividing

the duty between three individual units.

g) UFNO = 3 + 1

A spare unit is provided.

h) UFCOS[$] = UFAREA x UFNO x 80.[$/sq.ft.] x MSI/707

The cost of the hardware(exclusive of membranes) is calculated, based on an installed cost of $80. per sq. ft.(Jan. 1981). Increases in cost are considered by including the Marshall-Stevens Index(Jan. 1981 = 707).

i) CAPCOS[$/yr] = UFCOS x 1.0 x 0.24

The capital cost of the hardware is calculated . An in-plant cost factor of 1.0 is applied because the unit cost includes installation. A capital cost factor of 0.24 is applied to depreciate the equipment over 10 years by straight line method.

j) RAW[$/yr] = UFAREA1 x UFNO x 10.[$/sq.ft. yr] x MSI/707

The annual replacement cost of membranes is estimated at $10. per sq. ft. per year.

k) UEL[$/yr] = UFAREA1 x 3 x 0.02[kw/sq.ft.] x 0.025[$/kw hr] x MSI/707 x 24[hr/day] x 330[day/yr]

The power requirement is estimated at 0.02 kw/sq.ft. Electrical utilities are estimated to be $0.025/kw hr. and annual consumption is calculated.

Calculations.

Parameter	UF-1	UF-2
E2(I.U./day) x 10^{10}	0.6384	0.8283
W2(tons water/day)	4555	1794
PROT1(wt.%)	0.206	0.680
PROT2(wt.%)	2.06	13.60
FLUX1(gal/sq.ft. day)	36.823	31.47
FLUX2(")	26.50	18.02
FMAX(")	33.38	26.99
FAVE(")	26.14	21.13
WPROD(tons water/day)	4100	1704
WATUF(gal/day)	9823604	408278
UFAREA1(sq.ft./unit)	12527	6440
UFNO	4	4
UFCOS($)	4008610	2060839
CAPCOS($/yr)	962066	494601
RAW($/yr)	501080	257600
UEL($/yr	148821	76507

PUMPS: P-1, P-2.

Calculations are based on correlations by Peters and Timmerhaus(82). The stream entering UF-1 and P-1 is 1.091 x 10^6 gal/day or 758 gal/min. A pressure head of 100 ft. of liquid(43 psi) is estimated. The pump, exclusive of a 40 H.P. motor, costs $1770(carbon steel) x 1.5(steel, stainless steel fittings) gives a cost of $2655(Jan. 1979). An efficiency of 75% is indicated. A motor delivering 40 H.P.(90% efficiency), enclosed, costs $2000(Jan. 1979). The MSI ratio(Jan. 1981)/(Jan. 1979) is 707/569. An in-plant cost factor of 4.1 and a depreciation factor (straight-line, 10 yrs.) of 0.24 bring the total capitalized cost to $5691(Jan. 1981).

The stream entering UF-2 is 4.3 x 10^5 gal/day or 299 gal/min. A pressure head of 100 ft. is estimated. The pump costs $1360 x 1.5 = $2040. An efficiency of 65% is indicated. A motor delivering 15 H.P. is indicated, operating at 87% efficiency. The cost is $600. The total capitalized cost is $3228(Jan. 1981).

COST OF UREA.

A urea solution of 6 M contains 0.36 lb. urea/lb. water. The inlet stream to the washing tank is 3043 tons water/day, giving 1096 tons urea/day x 330 days/yr = 361624 tons/yr. The current price of urea is $160-175/ton(Jan. 1981)(84) for industrial grade, delivered. Agricultural and other grades are more expensive. A value of $165/ton yields an annual cost of $59.7 million with no recycle.

The current mass balance of urea, exclusive of degradation, indicates a consumption of 107 tons/day or $5.8 million/yr.

SAVINGS

Total(55.8% recovery)	$17,413,000
Incremental(28.8% recovery)	$8,426,000

EQUIPMENT/UTILITIES

UF-1	$962,066
Membrane	$501,080
UF-2	$494,601
Membrane	$257,600
P-1	$5,691
P-2	$3,228
Utilities	$225,328
	$2,449,594

Net Savings(exclusive of urea cost)= $5,976,406

The break-even point set by this value on the recycle fraction of urea is

$$\frac{\$5,976,406}{\$59,668,007} = 10.0\%$$

2. ENZYME REMOVAL BY ACETONE PRECIPITATION.

The alternative of a concentrated urea wash may be modified to remove enzyme from aqueous solutions by acetone precipitation rather than ultrafiltration. Economic data may be taken from a design by Mitra(85), similiar to the stover process. However, in the enzyme production operation, enzyme is produced in a dilute aqueous solution and precipitated by acetone("acetone precipitation").

The unit operation of acetone precipitation may be applied to the concentrated urea wash alternative to replace the ultrafiltration operations(UF-1, UF-2). The costs of major pieces of equipment are listed on the following pages. The costs are updated and capitalized. The MSI ratio(Jan. 1981)/(Jan. 1974) is 707/371. An in-plant factor of 4.1 and capitalization factor of 0.24 are used. To allow comparisons between systems, annual costs are normalized by the process water flow rates of each system.

The heating value of the solids is taken as a cost because the credit could be applied to the distillation of ethanol. The heat load is 157 x 10^6 BTU/hr. The heating value and efficiency is set at 50% of the cost of process steam(120 psi: \$3.0/10^6 BTU), resulting in a cost of \$1.865

million/yr.(Jan. 1981) or $504.9/yr/(ton water/day).

The total cost of "acetone precipitation" is $932/yr/(T/D). The concentrated urea wash system designed with ultrafiltration units processes 5804 tons water/day through the hydrolysis and a total of 6349 tons of water/day through both ultrafiltration units. No exponential factor is used in scale-up("0.6 rule") because the large magnitude of the process dictates the use of multiple units. The scaled costs, based on 6349 tons water/day, are:

"Ultrafiltration",	
Equipment/Utilities	$2.45 million/yr
"Acetone precipitation",	
Equipment/Acetone	$2.71 million/yr
Heating Value	$3.21 million/yr
Total	$5.92 million/yr

		Purchased cost, [$] [Jan. 1974]	Capit. cost [$/yr/(T/D] [Jan. 1981]
	"ACETONE PRECIPITATION".		
	Capacity: 3694 tons water/day.		
a)	Distillation column.	43,000	
b)	Heat exchanger.	57,000	
c)	Reboiler furnace.	620,416	
d)	Exchanger.	11,466	
		731,882	371.6
e)	Acetone.	108,385	55.9
f)	Solids Heat Value.		504.9
	157 million BTU/hr.		
			932.4

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