Energy and Environment Division

Utilization Of Immobilized B-Glucosidase In The Enzymatic Hydrolysis Of Cellulose

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M.S. thesis

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Prepared for the U.S. Department of Energy under Contract W-7405-ENG-48 and for the National Science Foundation (RANN) under Grant No. AER75-2386.

LBL-7857

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This work was done with the support of the U. S. Department of Energy

Table of Contents

Acknowledgments

Many people have been of aid to me in my endeavors to produce this work. Dr. C. R. Wilke has been helpful both in his direction of the research and for providing the opportunity for this learning experience.

I am grateful to the members of Dr. Wilke's laboratory for their constant help and guidance. Mr. Aldo Sciamanna has been an invaluable aid for suggestions on experimental procedures and for getting through red tape. Special thanks should go to Dr. Mohammed Riaz who also provided much direction and suggestions.

I thank Dr. A. C. Olson at Western Regional Laboratories for his guidance on methods of enzyme immobilization.

The financial support of this research by the National Science Foundation is gratefully acknowledged.

Finally, I would like to thank Liz, Terry, Tree and Richelle, who provided the will to continue.

UTILIZATION OF IMMOBILIZED β-GLUCOSIDASE IN THE ENZYMATIC HYDROLYSIS OF CELLULOSE

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ABSTRACT

Several process schemes which enzymatically convert cellulose to glucose in order to ferment the glucose to ethanol for use as a fuel have been proposed. Work performed by various researchers have indicated the possibility of inhibition of the cellulase hydrolysis process by cellobiose. The addition of an immobilized β-glucosidase reactor, to continuously convert cellobiose to glucose, may reduce the processing cost of ethanol by increasing the hydrolysis yields.

Purified, concentrated β-glucosidase obtained from Trichoderma viride was successfully immobilized via a crosslinking technique using tannic acid and glutaraldehyde, and the kinetic characteristics of the immobilized enzyme were studied. However, this immobilization method was abandoned due to the difficulty in producing a suitable

enzyme reactor due to the amorphous nature of the immobilized enzyme.

β-glucosidase obtained from Aspergillus phoenicis was immobilized onto phenol formaldehyde resin using glutaraldehyde as a fixing agent, and kinetic characteristics such as pH optimum, temperature stability and Michaelis-Menton constants were determined. The crystalline nature of the support allowed ready use in a fixed-bed reactor. Experimental and theoretical studies predicted the influence of film diffusion of substrate to resin surface on the rate of reaction at various operating conditions.

Three experiments were performed where a batch hydrolysis of a cellulosic source was carried out with a recycle stream through an immobilized β-glucosidase column in order to continuously remove cellobiose. The first two experiments using pretreated corn stover as the substrate showed no increase in hydrolysis over that of a control system, presumably because the cellobiose production was too low for cellobiose inhibition to occur. The third experiment, using Solka Floc as the substrate, which produced as high as 8.8 grams per liter of cellobiose,

showed only a slight increase in soluble sugar production over that of the control system. Since the current process indicates the use of corn stover or a similiar substrate, it does not appear useful to include an immobilized enzyme reactor in this manner.

Since the fermentation part of the process cannot use cellobiose to preduce ethanol, the use of the immobilized β-glucosidase reactor to convert the cellobiose to glucose may have economic significance by increasing the ethanol yield in this fashion. A computer program was produced in order to simulate a fixed-bed reactor with diffusion limitations and to determine the cost per pound of glucose for a given reactor design. After many trials covering various parameters the optimal design was found to be a 2 x 3 column arrangement (2 columns in parallel, each bank having three columns in series), each column having a diameter of 1.2 meters and a length of 3.5 meters. The column would be regenerated with fresh enzyme every 65 days. A cost analysis of the current hydrolysis process with the addition of the immobilized β-glucosidase reactors indicated that glucose could be produced for 9.45 cents per

pound. Therefore use of the immobilized enzyme system results in a savings of 0.53 cents per pound of glucose, which results in a corresponding savings of 7.2 cents per gallon of ethanol upon subsequent fermentation of the hydrolyzate.

1. Introduction

The use of cellulose as a fuel source is an aid to the ever increasing demand for energy and the ever decreasing supply of natural resources. Several attempts to utilize cellulose directly as supplies of food, chemicals and energy have been reported[1] however, the utility of the cellulose is enhanced if first hydrolyzed to soluble sugars.

Several process schemes which convert various cellulosic substrates first to glucose and then to ethanol for use as a fuel have been proposed. The latest process design proposed by Wilke et. al. is shown in Figure 1.1[2]. Corn stover, the cellulosic substrate (derived from corn husks and stalks) is milled, pretreated with dilute acid, enzymatically hydrolyzed to glucose and other sugars and the glucose is fermented to ethanol. A cost analysis of this process indicates a price of 179.4 cents per gallon of 95% ethanol which is not competitive with the current production price of 84.3 cents per gallon (produced from ethylene, January 1978 price). 75.7 % of the ethanol cost is attributed to the production of glucose (10 cents

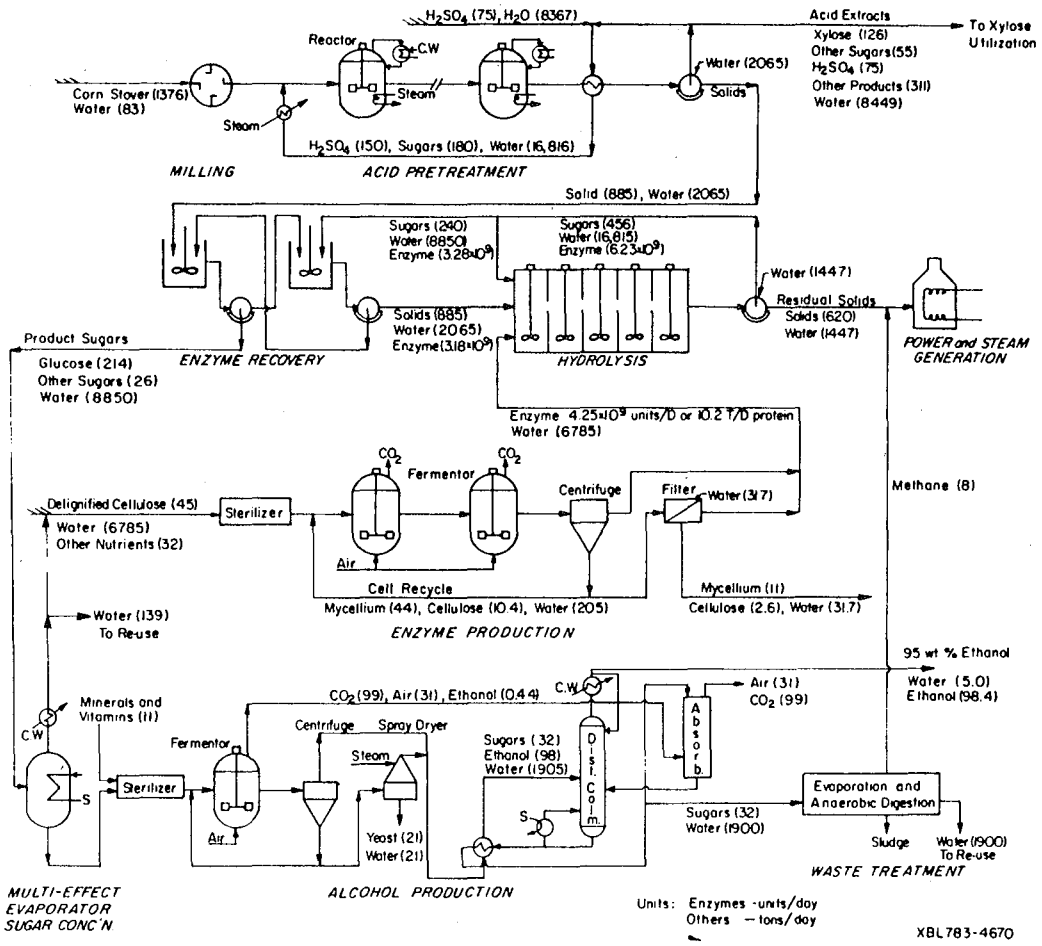


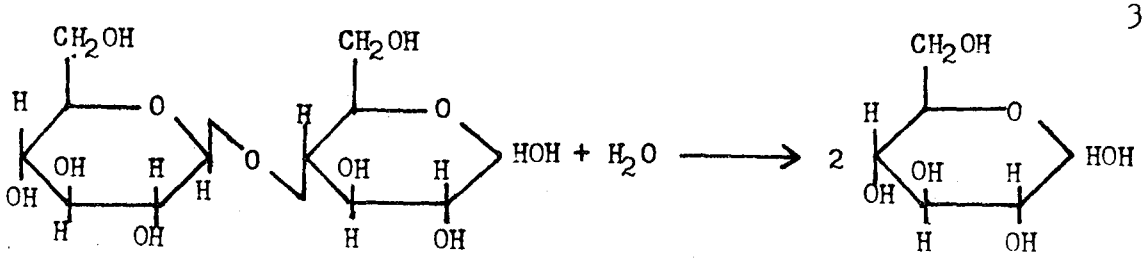
Fig. 1.1 Flowsheet of latest process design for enzymatic conversion of cellulose to ethanol. Reprinted from Wilke et. al.[2]

per pound). Decreasing the cost of glucose by increasing the glucose yield per pound of substrate or by decreasing the processing cost (such as the cost of the cellulase enzyme) will significantly decrease the cost of ethanol.

The kinetics of the hydrolysis of cellulose with the cellulase enzyme system is complicated and as yet not completely understood. Cellulase derived from soil and rumen microorganisms such as Trichoderma viride, appears to be composed of various enzymes with three basic types of activities:

endo-β-1,4 glucanases
exo-β-1,4 glucanases
β-glucosidase

The sequential and synergistic action of these enzymes convert the cellulose into soluble sugars[3], mainly glucose and cellobiose, a disaccharide of glucose. The endo-β-1,4 glucanases open up chain ends randomly over the polymer structure. The exo-β-1,4 glucanases are composed of two types which remove glucose or cellobiose from the nonreducing end of the chains. β-glucosidase converts cellobiose to glucose as shown below:



Work performed by various researchers have indicated the possibility of inhibition of the cellulase hydrolysis process by cellobiose.[4,5,6,7] Wilke and Yamanaka have indicated that the addition of soluble β-glucosidase from various sources may enhance the breakdown of cellulose to soluble sugars, presumably by removing cellobiose and its inhibitory effect. The addition of β-glucosidase from fungal sources such as Aspergillus phoenicis which are known to produce a stronger strain of the enzyme than that found as a component of the cellulase system to a process such as shown in Figure 1.1 may have the effect of increasing sugar yields and decreasing the cost of glucose.

Unlike theinsoluble cellulosic substrate, cellobiose is soluble and can therefore be catalytically hydrolyzed to glucose by an immobilized form of the β-glucosidase. There are many reasons for the immobilization of an enzyme for industrial use, some of which are summarized below:

1) On a per milligram protein basis, enzymes may be

the most expensive and difficult material to obtain in reasonable quantities. Unlike inorganic catalysts, most enzymes are soluble and, unless immobilized, reuse of the enzyme is impractical due to the difficulty of recovery from the product.

2) When the enzyme is immobilized, a clean separation from product can occur and the product will not be contaminated with the enzyme, which may be a factor in food and pharmaceutical processes.

3) By immobilizing the enzyme, processes that normally would need to be performed in a batch mode can be converted readilly to a continuous process.

4) In some applications the enzymic reaction is not desired to continue to completion. With an insolublized enzyme, the reaction may be terminated by the separation of the phases and not by denaturation of the enzyme.

5) Enzymes are generally unstable and the immobilization of the enzyme may enhance stability due to maintenance of the tertiary structure, antiturbulence, etc.

Numerous articles and reviews on immobilization techniques and immobilized enzyme kinetics have been published.[8,9]

There are three major methods of enzyme immobilization: crosslinking, physical techniques, and covalent attachment. Crosslinking involves covalently coupling enzyme molecules to each other with an agent such as glutaraldehyde. The enzyme particles become macroscopic and insoluble. Physical techniques include adsorption onto ion exchange resins and use of the large size of the enzyme molecule as a means of containment. The enzyme molecule is generally much larger than the substrate or product and can be kept contained by a substance such as fibrous tubing or polymeric shells which will allow product and substrate transport, but not enzyme transport. Covalent attachment includes a wide variety of supports and techniques where the enzyme molecule becomes covalently bound to the solid support. Porous glass, aluminum oxide, synthetic polymers and even sand have been used as media for covalent attachment. The immobilization method will depend generally on the nature of the enzyme, the product and substrate, and the process.

The first portion of this study was to find a method of immobilization of a strong strain of the β-glucosidase enzyme suitable for industrial applications and to charac-

terize the kinetics of the enzyme on this support for scale up to an industrial-size reactor. Once this had been done, the effect of the addition of an immobilized β-glucosidase reactor on the hydrolysis process was studied by performing batch hydrolyses of cellulosic substrates with recycle through the immobilized enzyme column. Finally, in the last section of this work, a computer aided design and cost estimation was performed in order to show the feasibility and relative cost savings per gallon of ethanol by the addition of the immobilized enzyme reactor to the current process.

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2. Analytical Procedures

This section summarizes the analytical procedures that are common in this study. Variations to the procedures as well as the procedures for individual experiments will be described in later sections.

2.1 Reducing Sugar (DNS) Assay

Sugar concentrations for the filter paper assay were measured as reducing sugar equivalent to glucose,[1] as the reagent reduced to a 3-amino-5-nitrosalicylic acid.[2] The assay procedure was as follows:

1) 3.0 milliliters of DNS reagent was added to 1.0 milliliter of sugar sample containing 0.35 to 3.5 milligram per milliliter of reducing sugar. Necessary dilutions were performed with 0.05 M pH 5.0 acetate buffer.

2) The sample was mixed with a vortex mixer and placed in a boiling water bath for exactly five minutes.

3) The sample was cooled in an ice bath for at least two minutes with occasional motion on the sample tube to effect rapid cooling.

4) 20. milliliters of distilled water was added to the sample and the sample was mixed by inverting end over end.

5) The absorbance of the sample was measured against a distilled water blank at a wavelength of 600 nanometers.

6) Milligrams of reducing sugar as glucose produced was determined against a standard curve which was produced with the above procedure using known quantities of glucose.

The dinitrosalicylic acid (DNS) reagent was prepared as follows:

1) 13.5 grams of NaOH was dissolved in 300 milliliters of distilled water and allowed to cool.

2) 8.8 grams of 3,5-dinitrosalicylic acid was dissolved in 880 milliliters of distilled water.

3) The above two solutions were mixed together and 255 grams of Rochelle Salt (Na-K-tartrate·$4H_2O$) was added.

4) In a 100 milliliter graduated cylinder 10 grams of phenol and 65 milliliters of distilled water were added and shaken to dissolve. 22 milliliters of 10% NaOH was

then added, the mixture was dilluted to 100 milliliters and shaken again to mix.

5) 6.9 grams of sodium bisulfite were dissolved in 69 milliliters of the above phenol solution and added to the dinitrosalicylic acid solution, and mixed well.

Rochelle Salt was added to prevent the reagent from dissolving oxygen and losing reducing capacity.[3] Phenol was added to increase color produced and bisulfite was added to stabilize the color. Alkali was required for the reducing action of sugars on dinitrosalicylic acid.[3]

2.2 Filter Paper Activity (FPA) Assay

The activity of the cellulase enzyme system was determined by the filter paper activity (FPA) assay. The assay measured the amount of reducing sugar produced by an enzyme solution in contact with a 50. milligram sample of Whatman #1 filter paper. The procedure was as follows:

1) 50. milligrams of Whatman #1 filter paper (1.0 x 6.0 centimeters strip) was added to 1.0 milliliter of enzyme solution plus 1.0 milliliter of 0.05 M acetate buffer at pH 5.0.

2) The tube was vortexed in order to cause the filter paper strip to curl and form a ring completely immersed in the enzyme solution. A 1.0 milliliter pipet was used as an aid in positioning the filter paper.

3) After incubation at 50°C for one hour 1.0 milliliter of clear liquid was extracted from the tube by pipet and the reducing sugar was measured with the DNS assay procedure.

4) Enzyme activity was recorded as units of activity equivalent to milligrams of reducing sugar produced by 1.0 milliliter of the undiluted enzyme.

5) An enzyme blank (a sample containing enzyme but no filter paper) was run with each sample in order to determine the amount of background reducing sugar already present in the enzyme solution. This background sugar was subtracted from the sample sugar in order to determine the filter paper activity.

2.3 Protein Assay

The protein assay used was a modified version of the protein method described by Lowry.[4] The procedure is as

follows:

1) To 0.5 milliliter of protein sample containing up to 150 micrograms of protein was added 5.0 milliliters of protein assay reagent, followed by thorough mixing with a vortex mixer.

2) After exactly 10.0 minutes incubation at room temperature, 0.5 milliliter of 1 N phenol solution was added followed by thorough mixing with the Vortex mixer.

3) The absorbance was determined after an additional ten minutes at a wavelength of 660 nanometers against a distilled water blank.

4) The protein content in micrograms per milliliter of the sample was determined by comparison to a standard curve prepared from dilutions of a stock solution of 1.0 milligram per milliliter bovine albumin in distilled water. This standard curve was prepared for each assay since the reaction was carried out at room temperature.

The protein assay reagent was prepared from the following three stock reagents:

Reagent A: 60 grams Na_2CO_3 + 8 grams of NaOH diluted to 2 liters with distilled water
Reagent B: 2% $CuSO_4 \cdot 5H_2O$ in distilled water
Reagent C: 4% sodium tartrate in distilled water

To prepare the protein assay reagent 1.0 milliliter of each of reagents B and C were added to 98.0 milliliters of reagent A. The combined reagent was stable for a period of four days. 1 N phenol solution was prepared fresh for each protein determination by diluting 1:1 with distilled water a stock solution of 2 N phenol solution. The diluted phenol solution was stable for only a period of up to six hours.

2.4 Glucose-Oxidase Peroxidase (G.O.P.) Procedure:

Glucose concentration was determined using the glucose-oxidase peroxidase (G.O.P.) procedure. Preliminary work has shown the G.O.P. to be specific for glucose in solutions of cellobiose and glucose. Merthiolate, an inhibitor of bacterial growth, has been found to inhibit the G.O.P. reactions and therefore can not be present in the solutions being analyzed. The G.O.P. procedure was as follows:

1) 8.0 milliliters of G.O.P. assay solution was added to 2.0 milliliters of sample containing up to 150 micrograms per milliliter glucose.

2) After incubation at 40°C for one hour the reaction was terminated by the addition of 2 drops of concentrated HCl followed by vigorous mixing with the Vortex mixer.

3) The absorbance was determined against a reagent blank at a wavelength of 420 nanometers.

4) Glucose content was determined by comparison with a standard curve prepared with each set of samples. This standard curve consisted of samples containing 0, 75, 100, and 150 micrograms per milliliter of glucose with the 0 microgram per milliliter sample being used as the reagent blank.

5) A cellobiose blank was included in order to subtract any contribution to absorbance due to glucose impurities in the stock cellobiose solution.

The G.O.P. assay reagent was prepared as follows:

1) A stock solution was prepared consisting of 4% w/v O-dianicidine-dihydrochloride in distilled water. This

was stable for at least a month when refrigerated.

2) Tris buffer was prepared by dissolving 10.8 grams of tris(hydroxymethyl)aminomethane in 85 milliliters of distilled water, bringing to a pH of 7.0 with concentrated HCl and then bringing the total volume to 100 milliliters with distilled water.

3) G.O.P. reagent was prepared by adding to 100 milliliters of the Tris buffer: 600 microliters of the 4% O-dianicidine-dihydrochloride solution; 1051 units of peroxidase (5 milligrams of peroxidase obtained from horseradish supplied by Sigma); and 120,000 units of glucose oxidase (200 microliters of stock glucose oxidase obtained from Aspergillus niger supplied by Sigma). The combined reagent was stable for a few hours only and was prepared immediately prior to use.

2.5 β-glucosidase Activity Assay:

The general scheme for the assay of β-glucosidase activity consisted of contacting the enzyme with a buffered cellobiose solution for a set time at a certain temperature and pH. The enzymic reaction was then halted either by

inactivation with Tris buffer in the case of the soluble enzyme or by removal of the aqueous sugar solution from the insoluble immobilized enzyme. The glucose produced was then determined using the G.O.P. assay procedure described in section 2.4.

Standard conditions of temperature, buffer and pH were established as a basis for β-glucosidase activity although these were varied for some experiments in order to determine the kinetic dependancies. Standard conditions for β-glucosidase obtained from Trichoderma viride were 0.015 M cellobiose solution in pH 5.0 0.05 M acetate buffer at 40°C. Standard conditions for β-glucosidase obtained from Aspergillus phoenicis were 0.01 M cellobiose solution in pH 4.0 0.05 M acetate buffer at 40°C. The standard conditions were chosen as such because the β-glucosidase activities were approximately at a maximum at these conditions, as will be shown in later sections.

In order to determine the activity of the soluble β-glucosidase 1.5 milliliters of the appropriate buffered cellobiose solution was placed in a test tube which was placed in a water bath. After at least 5 minutes 0.5 milliliter

of the enzyme solution was added with thorough mixing. After 15 minutes a portion or all of the 2.0 milliliters (depending on the anticipated strength of the enzyme) was added to 8 milliliters of the G.O.P. assay solution. The volume was brought to 10.0 milliliters with the buffer which was used for the cellobiose solution and the G.O.P. assay procedure was performed. An alternate method was to add the portion or all of the 2.0 milliliters to 4.0 milliliters of Tris buffer in order to halt the β-glucosidase reaction. This solution was then frozen for storage. The glucose content was determined at a later time by thawing the solution, adding 4.0 milliliters of double strength G.O.P. assay solution (produced by adding twice the normal amount of peroxidase, glucose oxidase, and O-dianicidine-HCl solution to the Tris buffer), bringing to a volume of 10.0 milliliters with buffer and then performing the G.O.P. assay procedure. An enzyme blank was performed with each sample in order to subtract the sugars already present in the enzyme solution. Soluble β-glucosidase activity was reported as units of activity equivalent to micrograms of glucose produced per minute per milliliter of ori-

ginal enzyme solution.

In order to determine the β-glucosidase activity of the β-glucosidase-TAG (β-glucosidase immobilized with tannic acid and glutaraldehyde), a volume (3 to 8 milliliters) of β-glucosidase-TAG was transferred by pipet from a well-stirred suspension to a 15 milliliter centrifuge tube. The sample was washed several times with pH 5.0 0.05 M acetate buffer by centrifugation and resuspension. After the final wash the liquid was drawn off and 2.0 milliliters of the cellobiose solution was added. The tube was tightly sealed with a rubber cap and agitated by rotating end over end in a 40°C water bath. The enzymic reaction was terminated after 15 minutes by rapid cooling in an ice bath followed by centrifugation and the glucose content of the supernatant liquor was determined by the G.O.P. assay method. β-glucosidase activity was reported as TAG units of activity equivalent to micrograms of glucose produced per minute per sample. In some experiments in which several samples were assayed at several temperatures, the enzyme-substrate contacting was performed by magnetic stirrer and stir-bar agitation in 50 milliliter glass centrifuge tubes

which were immersed in the appropriate temperature bath.

The β-glucosidase activity of the enzyme immobilized onto the phenol formaldehyde resin was determined by transferring a known amount of the resin (0.5 to 1.0 gram on a dry basis) to a 250 milliliter flask. At time zero 50.0 milliliters of buffered cellobiose solution at 40°C was added and the flask was placed in a water bath at 40°C equipped with a reciprocal shaker. A rotation rate of 110 rpm was found to be adequate such that the kinetics of the enzymic reaction rather than diffusion controlled the production of product. At certain time intervals (every 1 to 2 minutes) a 1.0 milliliter sample was removed from the flask by syringe and the glucose content was determined by the G.O.P. method. The β-glucosidase activity was reported as Resin units of activity equivalent to the initial rate of glucose production (micrograms glucose produced per minute per gram of dry resin) by plotting the glucose produced at at least four different times.

2.6 G.L.C. Sugar Determination

In order to quantify the amount of cellobiose as well

as the glucose present in a sugar solution or when merthiolate, which was found to interfere with the G.O.P. procedure for measuring glucose, was present in the solution to be tested, a gas-liquid chromatograph (G.L.C.) was utilized.

The sugar solution was prepared for G.L.C. analysis as follows:

1) 1.0 milliliter of the solution to be tested, which was previously boiled and centrifuged to remove denatured protein, was quantitatively transferred to a 1 dram vial.

2) The vial and contents were frozen with liquid nitrogen with the vial slanted such that the liquid-air interface touched the vial bottom. This orientation allowed splattering due to escaping gases during the freezing process to be avoided.

3) The sample was then freeze-dried overnight.

4) 1.0 milliliter of dimethyl sulfoxide solution (containing 5 grams per liter of 2-hydrolypyridine and 2 grams per liter of myo-inositol) was added to the freeze-dried residue. The myo-inositol served as an internal

standard.

5) The vial was capped with a teflon-lined screw top and placed in a 40°C oven for at least 4 hours. This step allowed the isomers of the sugars to equilibrate.

6) After equilibration, 0.5 milliliter of silylating agent (2 parts by volume hexamethyl disilazane and 1 part by volume of chlorotrimethyl silane) was added, the vial capped tightly, and mixed well on a Vortex mixer every 20 minutes for 1 hour.

7) The lower phase in the vial was discarded by pipet and the upper phase was washed once with 1 milliliter of distilled water. The bottom phase was again discarded.

8) Several small portions of anhydrous sodium sulfate was added to the vial in order to absorb the remaining water.

9) The sample was then injected into the G.L.C. and the glucose, inositol and cellobiose peakes were recorded on paper. The glucose and cellobiose content was determined by comparison of the peak areas to that of the inositol standard. The calculations for glucose and

cellobiose are illustrated below:

$$\text{grams per liter of glucose} = 1.286 \times \frac{\text{area of second glucose peak}}{\text{area of inositol peak}}$$

$$\text{grams per liter of cellobiose} = 1.812 \times \frac{\text{second cellobiose peak area}}{\text{area of inositol peak}}$$

The constants 1.812 and 1.286 were determined from standard solutions of cellobiose and glucose, respectively.

The reason for the equilibration step was due to the overlapping of peaks of various sugars. Equilibration of the sample allowed an accurate determination to be made from the area of only one peak per sugar.

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3. Tannic Acid-Glutaraldehyde (TAG) Method of Immobilization

Crosslinking of β-glucosidase with tannic acid and glutaraldehyde (to be referred to as the TAG method of' immobilization) was chosen as the first immobilization method for investigation due to its relative ease and inexpense. The immobilization reaction required only a contacting of the enzyme with glutaraldehyde and tannic acid and did not require exotic conditions and chemicals or specially prepared support media such as glass beads. The following sections describe results of experiments designed to determine the kinetic characteristics of the tannic-acid glutaraldehyde immobilized β-glucosidase (β-glucosidase-TAG) in order to determine suitable operating conditions and scale-up parameters as well as attempts to produce a continuous immobilized enzyme reactor.

3.1 Previous Work

Much work has been performed on the reaction between glutaraldehyde and proteins, although due to the reaction complexity, the reaction is far from being completely un-

derstood. When glutaraldehyde and a soluble protein are combined crosslinking of the protein occurs forming an insoluble product. An amino acid analysis of the product after the reaction shows only a change in the lysine values, with a loss of up to about 50 to 60% of the original lysine level.[1] Although there were exceptions, the most rapid insolubilization of proteins occurred near their isoelectric point[2] which indicates that intermolecular crosslinking is dependant on the protein charge; the optimum is at the isoelectric point where repulsive charges between protein molecules is at a minimum.

Light scattering methods were used in the study of the formation of crosslinks between α-chymotrypsin and glutaraldehyde.[3] Scattering increased rapidly with time initially, slowed, and increased again in a linear fashion at longer times. The first increase is attributed to intermolecular crosslinking with the reactive ε-amino groups of lysine residues on or near the molecule surface. The second increase is due to a crosslinking of these initially formed polymers to form larger polymers, whose shapes appear to be flexible, branched coils.

Treatment of crosslinked α-chymotrypsin with 6 M urea showed no solubilization, indicating that the insolubilization is due to chemical crosslinking rather than aggregates of smaller, soluble polymers formed by crosslinking.[2]

Work has been performed on the insolubilization of proteins with tannic acid and glutaraldehyde. It has been reported that invertase could be precipitated with tannic acid and used in a packed column when mixed with a filter aid[4] but other work has shown that use with tannic acid alone caused a resolubilization of the complex when subjected to succesive washings.[5] However, difficulty was had forming an insolubilized product with glutaraldehyde alone.[6]

Olson and Stanley have described a procedure for the insolubilization of enzymes with glutaraldehyde and tannic acid.[6] The reaction is carried out at a pH at which the enzyme is stable and soluble using tannic acid and glutaraldehyde in excess, 10 to 50 parts per part of enzyme. The reaction can be carried out in water at a temperature of 1°C to 40°C. Lactase was insolubilized and packed into a

column after mixing with a filter aid, "Celite", at a ratio of 1 part insolubilized enzyme to 2 parts of Celite. The column was jacketed with dimensions of 8 millimeters in diameter and 2 centimeters long. When operated at 45°C, pH 4.5 and 0.6 milliliters per minute flow rate, 90% hydrolysis of a 3% lactose solution was attained. This column was run continuously for 72 hours with no detectable loss in ability to hydrolyze the lactose. Olson and Stanley also report succesfully immobilizing invertase and glucoamylase with the tannic acid-glutaraldehyde method.[5]

3.2 Immobilization Procedure

Two batches of β-glucosidase-TAG were produced for use in this work. β-glucosidase from Trichoderma viride was obtained in a purified, concentrated form from this laboratory. The β-glucosidase was separated from the cellulase complex by fractionation on a column packed with the microcrystalline cellulose preparation Whatman CC31.[7] β-glucosidase was not absorbed on Whatman CC31 and came off with theinitial eluting buffer (0.02 M phosphate) whereas the cellulase complex was retained. The α-gluco-

sidase was washed in order to remove phosphate and concentrated on an Amicon UM-2 ultrafilter membrane.

The immobilization reaction for both batches consisted of contacting a small portion (8.0 milliliters) of soluble enzyme with 20.0 milliliters of 10% w/v tannic acid solution buffered to pH 5.0. The activities of the soluble enzyme used in the first and second batches were 93.9 and 195.2 units per milliliter of enzyme at standard conditions. The contacting was performed witha magnetic stirrer and stir-bar in a 125 milliliter flask at 4°C. After five minutes 2.4 milliliters of glutaraldehyde (25% stock solution) was added. After approximately 24 hours the complex was washed four times with pH 5.0 0.05 M buffer by centrifugation and resuspension. 500 milligrams of Celite (used as a dispersing agent) was mixed with the complex and the β-glucosidase-TAG was packed into an 8 millimeter in diameter column. Glass wool was used as plugs to position the complex in the column. The β-glucosidase-TAG was washed with 1 to 2 liters of acetate buffer at 40°C by passing the buffer through the column. This washing step was necessary in order to remove excess glu-

taraldehyde which was not eluted from the column at room temperature during the initial washing procedure. The β-glucosidase-TAG was then ready for use either in the column or in a stirred batch reactor.

The amount of enzyme activity that was immobilized in the second batch was quantified and was found to be 569 units. This corresponded to a 36.5% yield of activity taken from the soluble state.

3.3 Kinetic Characteristics

3.3.1 Assay Linearity and Reproducibility

Two sets of experiments were performed in order to ascertain that the enzyme-substrate contacting methods utilized for the determination of β-glucosidase-TAG activity was reproducible and linear in amount of enzyme.

In order to test for assay reproducibility, four 15 milliliter centrifuge tubes each received 5 milliliters of the well-stirred β-glucosidase-TAG suspension and the activity was determined according to the procedure described in section 2.5. The results shown in Table 3.1 indicate that the assay procedure was reproducible. The dif-

Table 3.1 Results of assay reproducibility experiment

Sample	Activity units/TAG sample
1	28.53
2	28.53
3	27.47
4	28.40

ference between the glucose produced in sample 3 and the other samples was 3.7%.

Figure 3.1 shows the results of the tests for assay linearity. The three points which comprize the upper line in the figure represent 2.5, 5.0 and 7.5 milliliters of β-glucosidase-TAG suspension assayed in 15 milliliter centrifuge tubes using end-over-end agitation. The three points comprising the bottom line represent 3.5, 5.0 and 7.5 milliliters of β-glucosidase-TAG suspension assayed in 50 milliliter centrifuge tubes using stir-bar agitation. Duplicate samples were run for both cases. The results in Figure 3.1 indicate that over this range the difference in β-glucosidase-TAG mass prsent did not seem to effect the kinetics of the reaction as an incremental increase in the enzyme amount gave rise to the same increase in the glucose produced. The activity levels for both agitation methods were not expected to coincide as a different batch of enzyme-suspension was used for each.

3.3.2 pH Optimum

The pH optimum of an enzyme depends on many factors

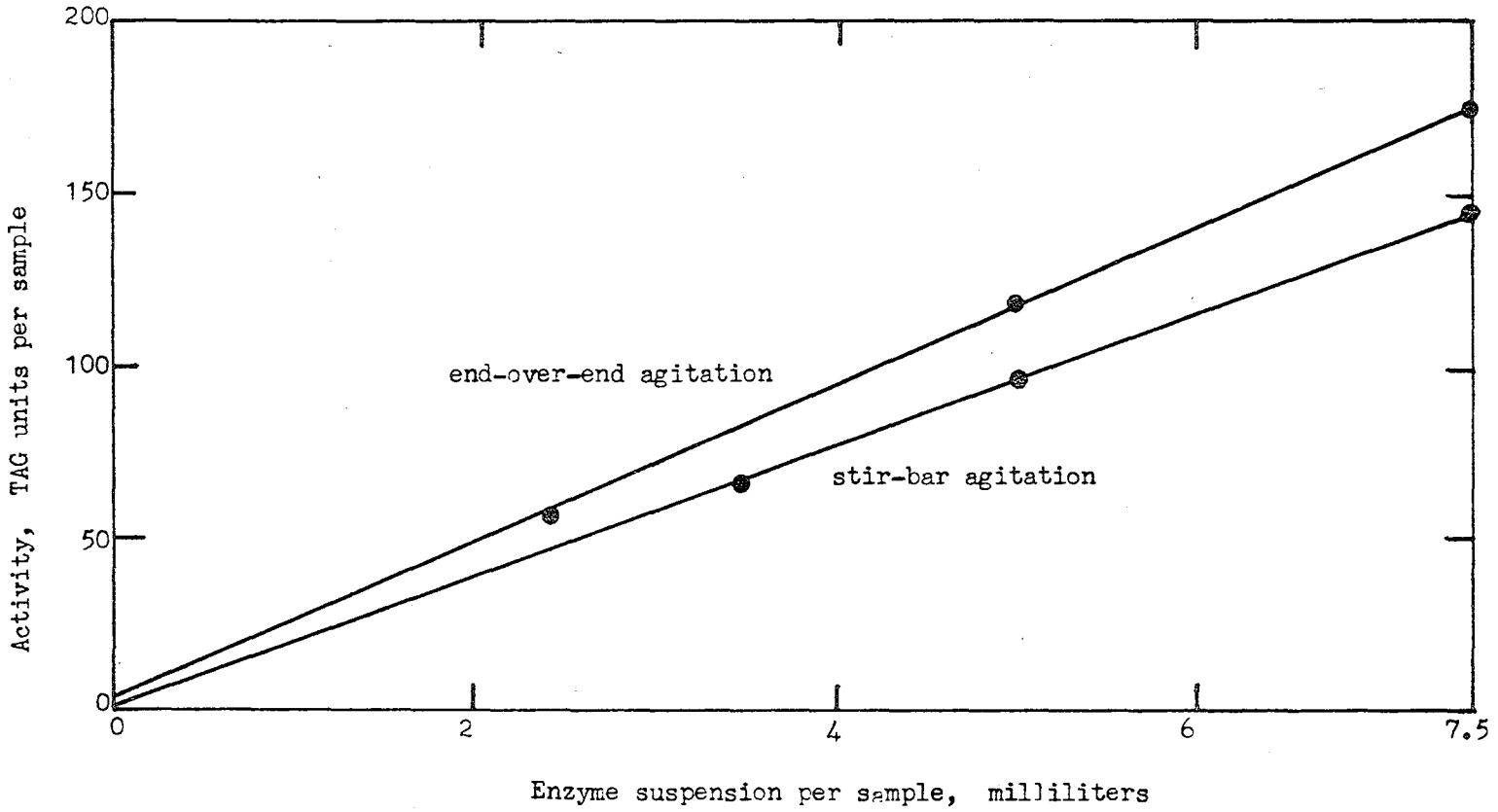

Fig. 3.1 Test for assay linearity for the tannic acid and glutaraldehyde immobilized β-glucosidase.

XBL 788-9889

including temperature, nature and concentration of substrate, nature and concentration of buffer and ionic strength.[8] When these conditions are held constant the pH optimum at those conditions can be determined. Michaelis-Menton constants for β-glucosidase obtained from various organisms have been determined in citrate-phosphate buffer[9] and since citrate-phosphate buffer has a broad buffering range (2.6 to 7.0)[10] it was chosen at a strength of 0.05 M for the pH optimum determination. However, it was not until long after the use of Trichoderma viride β-glucosidase and the TAG method of immobilization had been abandoned in this work was it discovered that the citrate ion has an inhibitory effect on the β-glucosidase obtained from Aspergillus phoenicis (see section 4.3.1). Therefore the pH optimum reported in this section is that optimum for citrate-phosphate buffer and the optimum may differ in acetate buffer.

When an enzyme is immobilized the microenvironment surrounding the enzyme is changed from that of the homogeneous solution to that of the support. The pH optimum of the immobilized enzyme may vary from that of the soluble

enzyme if the support is charged. Also in the case of the product and substrate of the enzymic reaction having different charges, diffusion influences will change the microenvironment from that of the bulk solution, causing an apparent change in the pH optimum from that of the soluble enzyme at the macroscopic level.[11]

0.015 M cellobiose solution was prepared in citrate-phosphate buffer at various pH ranging from 3.0 to 7.0 and the activity of both the soluble and immobilized enzyme was determined using end-over-end agitation at 40°C. The results are plotted in Figure 3.2 and indicate a maximum activity at pH 4.8 for both the soluble and immobilized enzyme. Apparently the pH optimum is not changed by the immobilization of the β-glucosidase with tannic acid and glutaraldehyde. Diffusional influences were expected to be negligible with the end-over-end method of agitation. The microenvironment of the enzyme is apparently not greatly changed by the crosslinking with the glutaraldehyde and tannic acid.

Mandels and Weber reported a pH optimum for production of glucose by Trichoderma viride cellulase as pH 4.8.[12]

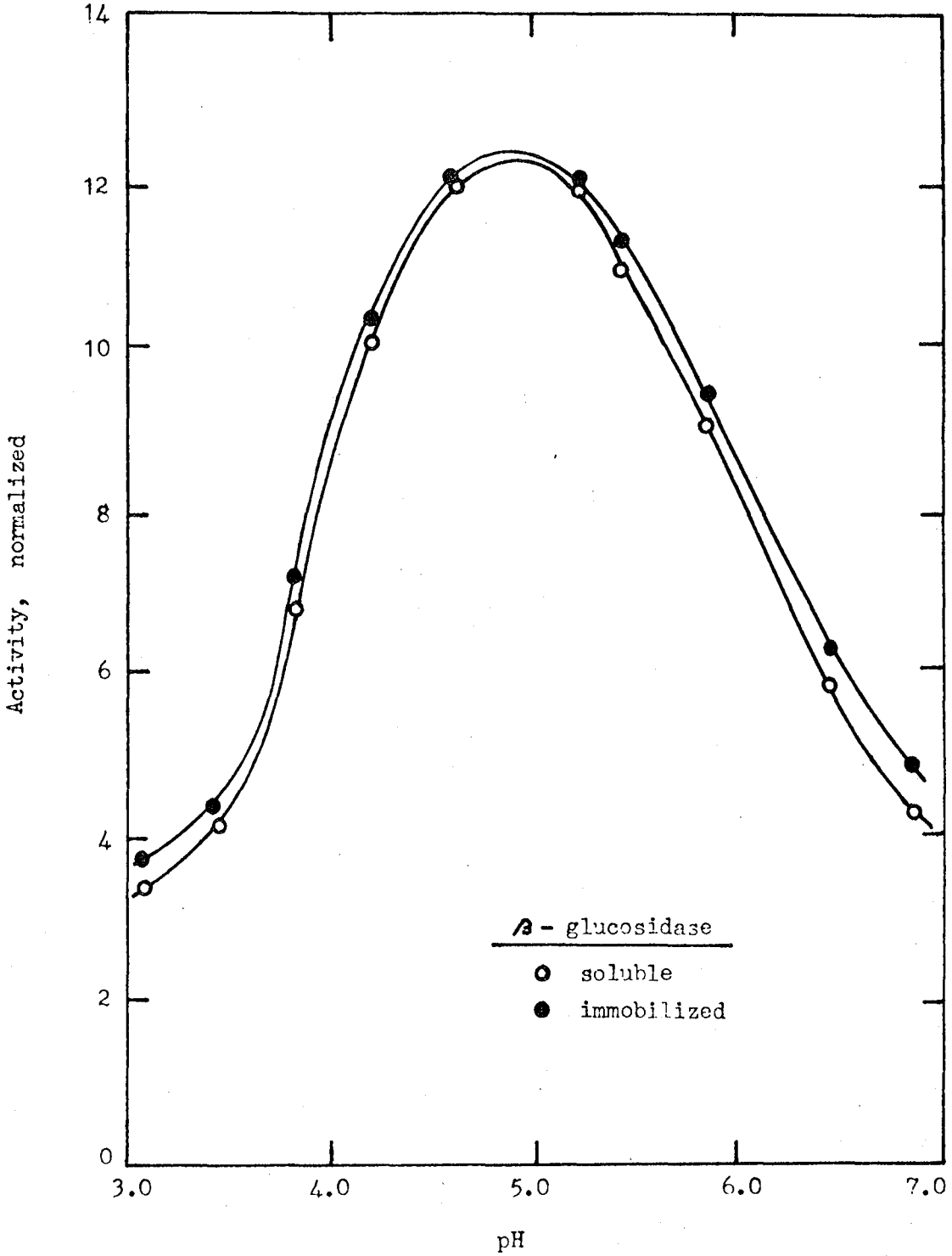

Fig. 3.2 Activity versus pH profile for soluble and immobilized enzyme obtained from Trichoderma viride. XBL 788-9890

Therefore the β-glucosidase-TAG produced in this work would be compatible with the Trichoderma viride cellulase for use in the hydrolysis of cellulose.

3.3.3 Temperature Stability

The stability of an enzyme is a function of many factors including pH, ionic strength and nature of the buffer, presence or absence of substrate and enzyme concentration and purity.[8] Therefore in determining the temperature stability it is necessary to keep these conditions constant and well defined. The rate of heat denaturation will normally follow a first order process:

$$R_d = k_d(E), \qquad k_d = Ae^{(-E_d/RT)} \qquad 3.1$$

where (E) is the active enzyme concentration, R_d is the rate of deactivation, k_d is the rate constant, A and R are constants and E_d is the activation energy for the denaturation process. E_d normally ranges from 50 to 150 kilocalories per mole whereas the activation energy of the enzyme reaction normally ranges from 6 to 15 killocalor-

ies per mole. Once the half-life, or the time it takes to lose one half of the original enzyme activity, is determined, the amount of remaining activity of an enzyme preparation at any time can be determined:

$$\% \text{ activity remaining} = 100/2^n \qquad 3.2$$

where n is the number of half lives that has past.

The temperature stability of the β-glucosidase-TAG was determined at 40°C, 45°C and 50°C in order to determine a suitable operation temperature for an immobilized enzyme reactor. 40 milliliters of the enzyme suspension in 0.05 M acetate buffer was transferred to each of three 125 milliliter flasks. The flasks were placed in separate water baths at the appropriate temperature. Agitation was provided by magnetic stirrers. At various times 5.0 milliliter samples were transferred by pipet to 15 milliliter centrifuge tubes and the sample activities were determined using end-over-end agitation. The results are shown in Figure 3.3. It is difficult to determine over the eighty hour period whether the 40°C and 45°C samples followed a zero or first order denaturation

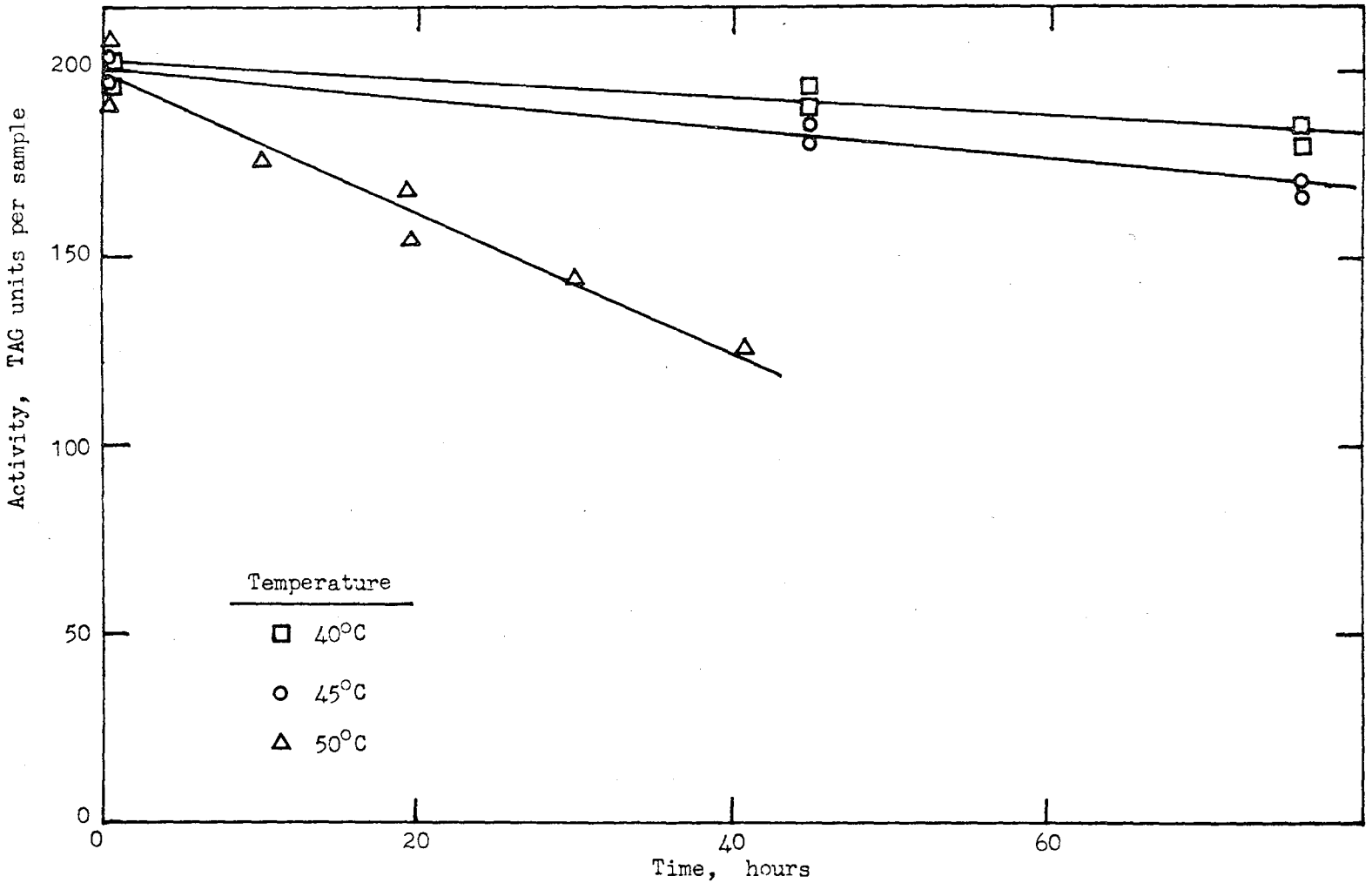

Fig. 3.3 Results of temperature stability experiment for β-glucosidase-TAG.

XBL 788-9891

rate. Assuming a first order rate the half lives were calculated to be 536 and 307 hours for 40°C and 45°C respectively. The 50°C sample appears to follow a zero order denaturation rate but this may be due to scatter and lack of long-term data points (due to equipment failure after 40 hours). Assuming a zero and first order denaturation rate the half life was calculated to be 53 and 59 hours respectively. Regardless of the order of denaturation, it is readilly apparent that operation of the enzyme reactor at 50°C would necessitate recharging of the enzyme very often and that operation at temperatures of 45°C or lower would be more desireable from an economic viewpoint.

3.3.4 Temperature Effect on Activity Determination

An experiment was performed in order to determine the effect of temperature on the glucose produced by a sample of β-glucosidase-TAG over a 15 minute time interval. As the reaction temperature is increased there will be an increase in the rate of denaturation of the enzyme as discussed in the previous section. Also, the rate con-

stant, which has a temperature dependancy given by

$$k = Ae^{(-E_a/RT)} \qquad 3.3$$

where A and R are constants, k is the rate constant, T is the temperature in degrees Kelvin and E_a is the energy of activation, will increase with an increase in temperature. Since the rate of reaction is proportional to the rate constant, the glucose production will increase with an increase in temperature.

5 milliliters of β-glucosidase-TAG suspension were transferred to a 50 milliliter centrifuge tube and washed three times. After the last wash the tube was centrifuged and the liquid was removed. 4.0 milliliters of 0.015 M cellobiose solution in 0.05 M pH 5.0 acetate buffer, which was preheated to the water bath temperature, was added to the tube and the tube was placed in the water bath at the desired temperature. Agitation was provided by magnetic stirrer and stir-bar. Duplicate samples were performed in water baths set to various temperatures ranging from 40°C to 80°C. After 15 minutes the reaction was stopped

by rapid cooling in an ice bath followed by centrifugation. The glucose in the supernatant liquor was determined using the G.O.P. method.

The results in Figure 3.4 show an increase in the glucose production during the 15 minute time interval from 40°C to 65°C after which the glucose production dropped greatly. Between 40°C and 65°C the increase due to increased enzyme activity was greater than the decrease due to enzyme inactivation. However, at temperatures greater than 65°C, the effect of enzyme denaturation was so great that there was a decrease in glucose production as temperatures increased. No attempt was made to determine the initial rates of reaction at the various temperatures since the available methods and equipment made sampling at times less than 15 minutes difficult and undependable. Therefore from these results and from the results of section 3.3.3, 40°C was chosen as the standard temperature for the β-glucosidase assay method.

3.3.5 pH Stability

Many enzymes denature readily upon exposure to very

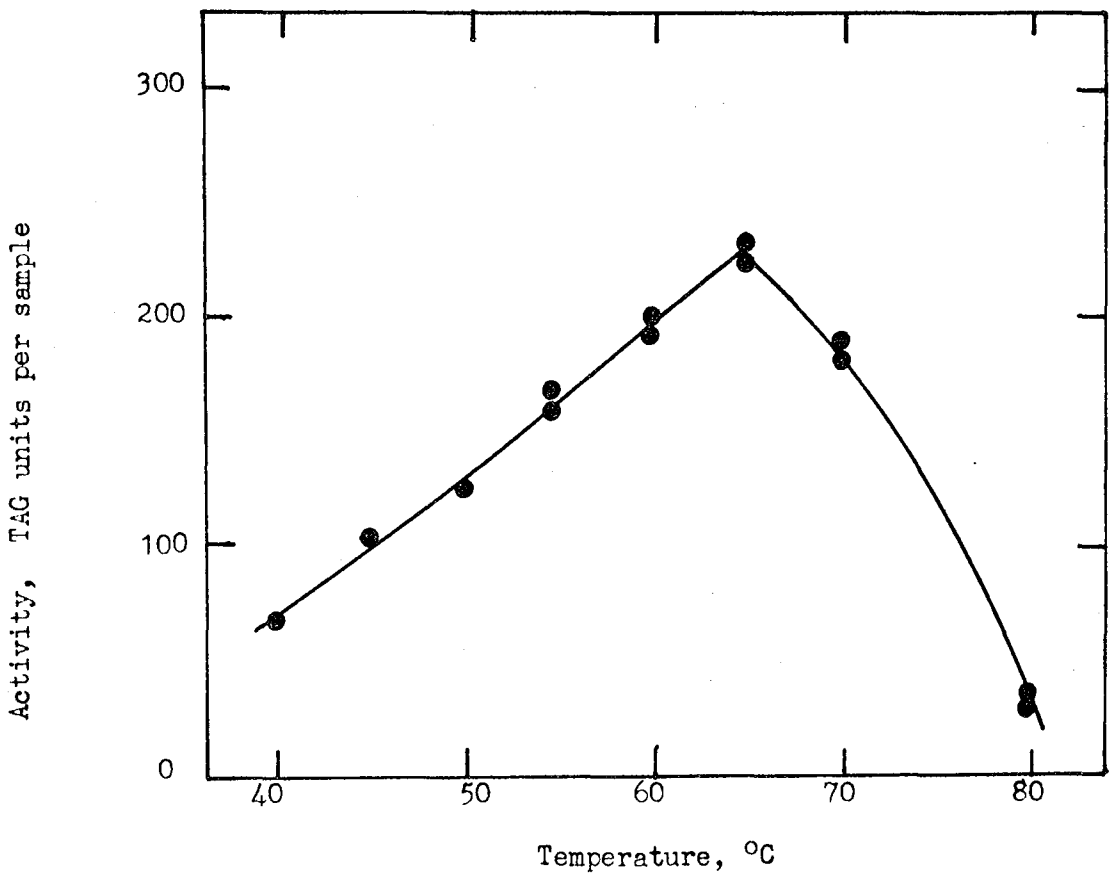

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Fig. 3.4 Effect of temperature on the assay procedure for β-glucosidase-TAG.

alkaline or very acidic conditions. The pH at which denaturation occurs varies not only with the nature of the enzyme but with other conditions such as temperature, presence or absence of substrate, etc.[8] Immobilization may enhance the stability of an enzyme by stabilizing the enzyme's secondary, tertiary, or quaternary structures.[11] The approximate effect of the pH on the stability of the β-glucosidase-TAG was determined at room temperature in order to determine a suitable pH range for operation of a reactor. Room temperature was chosen to minimize temperature denaturation effects.

40 milliliters of β-glucosidase-TAG was transferred to each of four 125 milliliter flasks. Citrate-phosphate buffer was added to each flask at various pH ranging from 3.0 to 5.0. The final buffer concentration was 0.05 M. Agitation was provided by magnetic stir-bars and 5 milliliter samples were removed by pipet at times for activity determination. The activity was determined 15 milliliter centrifuge tubes by first washing several times to remove the citrate-phosphate buffer. The activity assay was performed with acetate buffer as described in

section 2.5 using end-over-end agitation. Results are shown in Figure 3.5.

Over the 45 hour contacting time no significant difference in denaturation rate was observed over this range of pH. The rate of denaturation for the four samples was approximately constant and therefore attributable to factors other than pH. Although these results are not indicative of stability at other conditions such as at a higher temperature, qualitatively they indicate that there are no drastic instability effects associated with pH over this range.

3.3.6 Enzyme-Substrate Interaction

The enzyme-substrate interaction for both the soluble and immobilized β-glucosidase was investigated using the simple kinetic model proposed by Michaelis and Menton,[8] who suggested the following sequence for the reaction between an enzyme and substrate molecule:

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} P + E \qquad 3.4$$

In their treatment, Michaelis and Menton proposed that

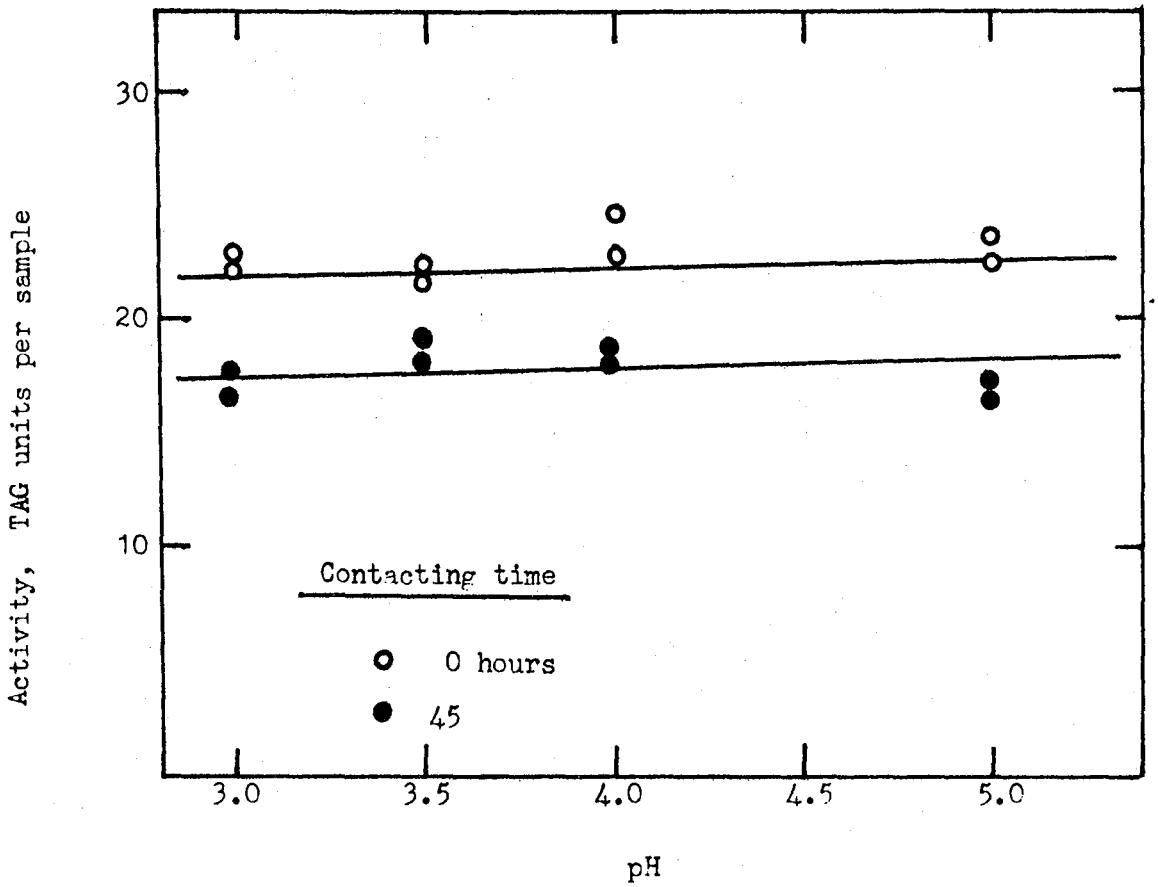

Fig. 3.5 Results of pH stability experiment for β-glucosidase-TAG.

the equilibrium was reached very rapidly among the enzyme, E, substrate, A, and enzyme-substrate complex, EA, and that k_2 was small compared to k_{-1} and k_1. P is the product of the reaction.

If the substrate concentration is much greater than the total enzyme concentration and assuming steady state of the enzyme-substrate complex, i.e. the rate of formation of EA is equal to the rate of dissappearance, then the Michaelis-Menton Equation is observed

$$r = \frac{Vm \cdot A}{Km + A} \qquad 3.5$$

where r is the reaction rate, Vm is the maximum reaction rate, A is the substrate concentration and Km is the Michaelis-Menton constant.

From this kinetic model it is apparent that at low substrate concentrations the reaction is first order in the substrate concentration:

$$r = \frac{Vm}{Km} \cdot A, \qquad Km \gg A \qquad 3.6$$

and at high substrate concentrations the reaction is zero order

$$r = Vm, \qquad A \gg Km \qquad 3.7$$

This follows from the standpoint of the reacion model; as substrate concentration is increased from low values the amount of substrate combined with enzyme will increase. However, once the enzyme is saturated with substrate, further increases in substrate concentration will not increase the reaction rate; the rate is at the maximum, Vm.

Taking the reciprical of the Michaelis-Menton equation,

$$1/r = Km/Vm(A_o) + 1/Vm \qquad 3.8$$

the Lineweaver-Burke equation is obtained. (A_o) is the initial substrate concentration. A plot of 1/r versus $1/(A_o)$ would yield a line with an x intercept equal to -1/Km and a slope equal to Km/Vm. In this manner a value can be determined for Km for the particular system. The Km value is a useful parameter in observing effects of enzyme immobilization on the enzyme kinetics as it is

inversely proportional to the chemical affinity of the enzyme for the substrate.

In order to determine the Km value for both the soluble and immobilized β-glucosidase, substrate solutions were prepared in 0.05 M pH 5.0 acetate buffer ranging in cellobiose concentration from 0.0 to 0.02 M. The activity of the soluble and immobilized enzyme was determined in these solutions according to the methods described in section 2.5. The assay for β-glucosidase-TAG was performed with 5 milliliters of enzyme suspension in 15 milliliter centrifuge tubes with end-over-end agitation. The results are shown in Figure 3.6. For both enzyme systems the maximum activity was found to occur at about 0.0125 M cellobiose. 0.015 M cellobiose was chosen as the standard cellobiose concentration for the Trichoderma viride β-glucosidase for this reason, as at this point the enzyme is saturated with substrate and the activity is at the maximum.

Two factors may cause a change in the Km value of an enzyme upon immobilization. In an assay system where diffusion of substrate to the immobilized enzyme site may

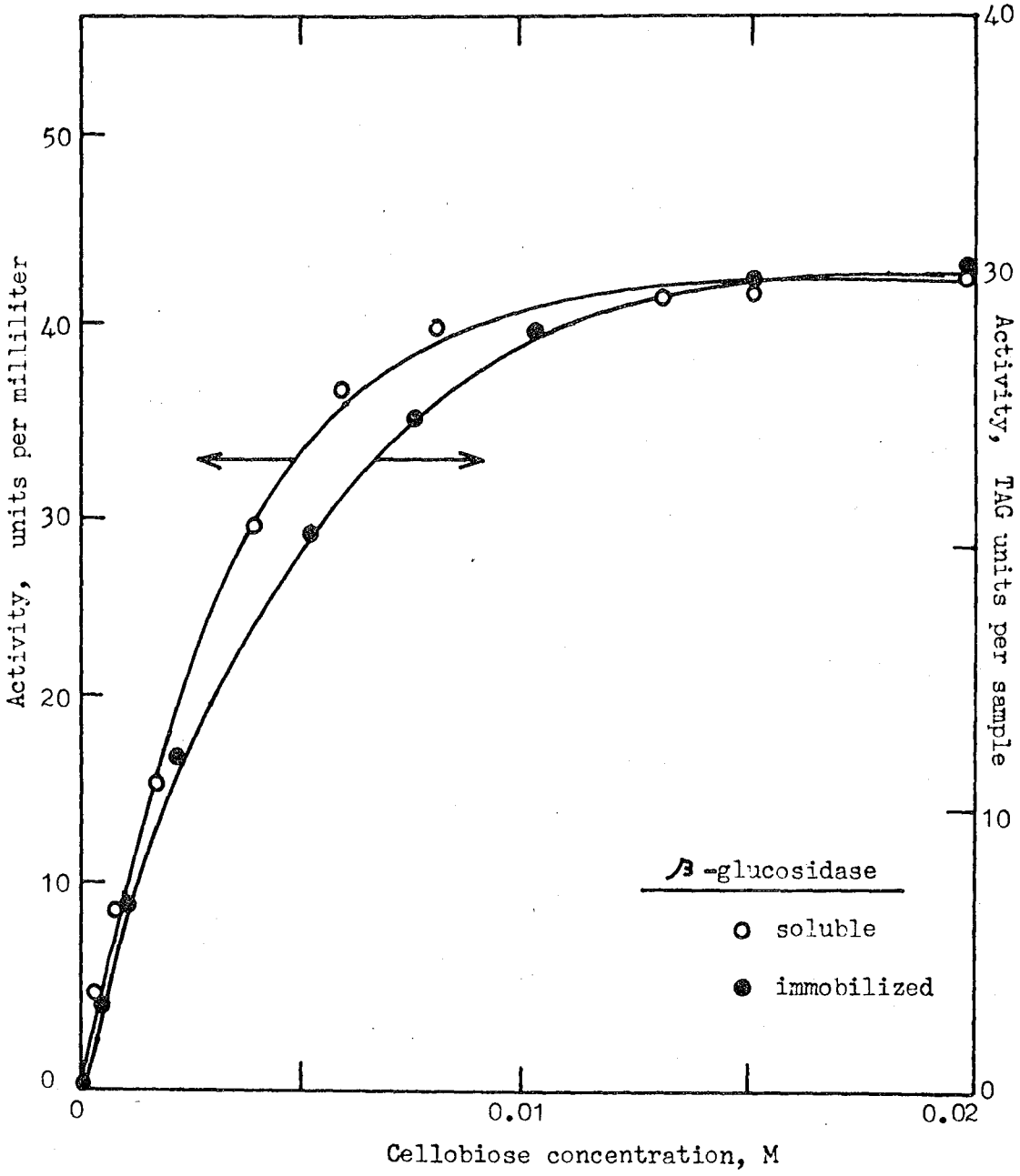

Fig. 3.6 Activity versus substrate concentration for both soluble and tannic acid-gluteraldehyde immobilized enzyme obtained from Trichoderma viride.

limit the reaction, the Km value may appear to be different due to the substrate concentration in the enzyme micro-environment being different from that of the bulk phase.[13] Figures 3.7 and 3.8 show the Lineweaver Burke plots for both the soluble and immobilized enzymes, respectively. From the x intercepts the Km value for the soluble enzyme was found to be 4.76×10^{-3} M and for the β-glucosidase-TAG, 4.22×10^{-3} M. These two values are very close within experimental error and indicate that the immobilization has not greately effected the chemical affinity of the enzyme for the substrate.

3.4 Use of β-glucosidase-TAG in an Immobilized Reactor

Several attempts were performed in order to determine a suitable method for the utilization of the β-glucosidase-TAG in a reactor system. The β-glucosidase-TAG was composed of amorphous particles with a diameter on the order of 0.045 millimeter. The density of the particles was not high enough for use in a fluidized bed reactor. When the enzyme was used alone in a fixed bed reactor or when held in place by a filter in a continuous stirred

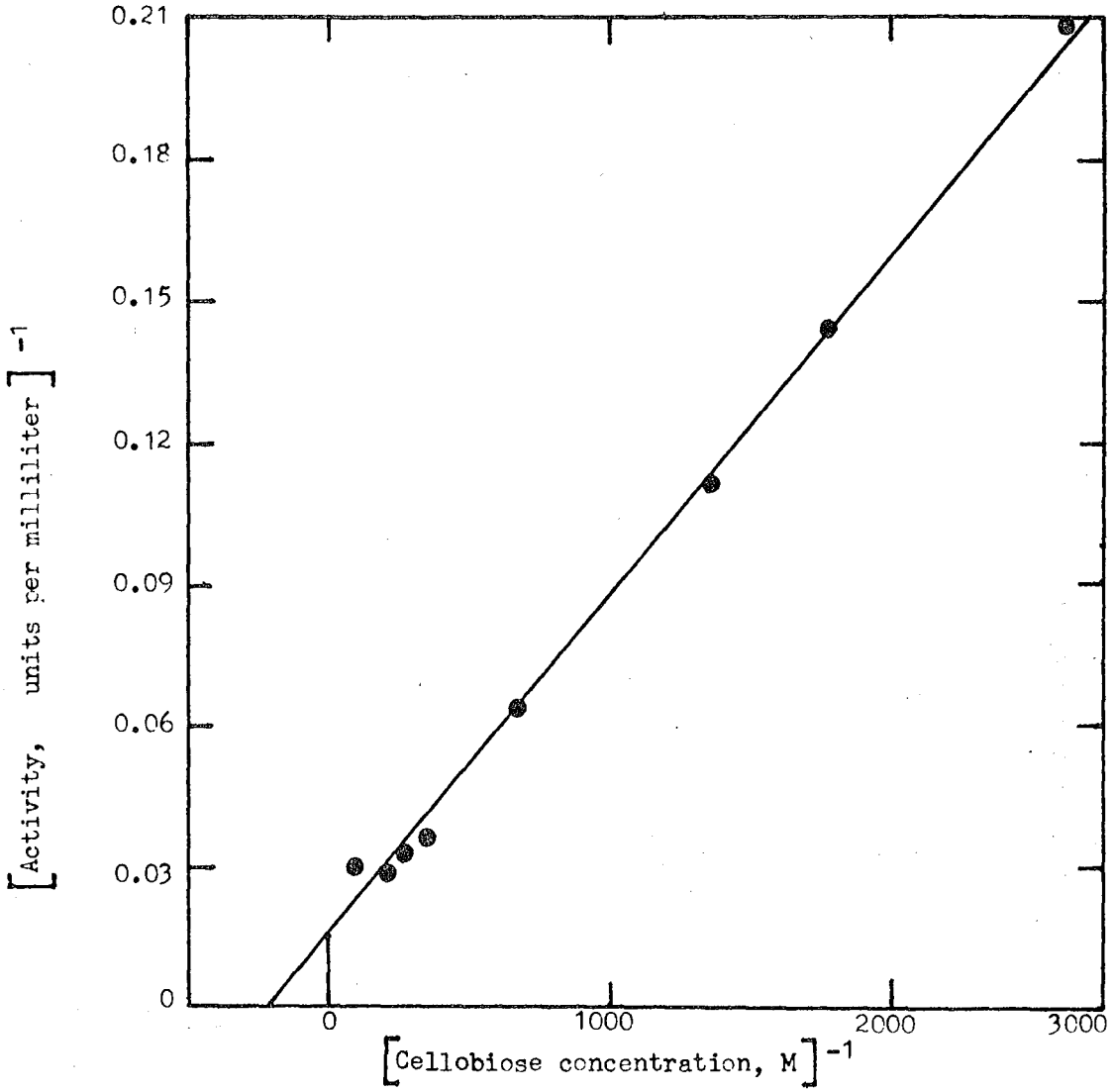

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Fig. 3.7 Lineweaver-Burke plot for soluble β-glucosidase obtained from Trichoderma viride.

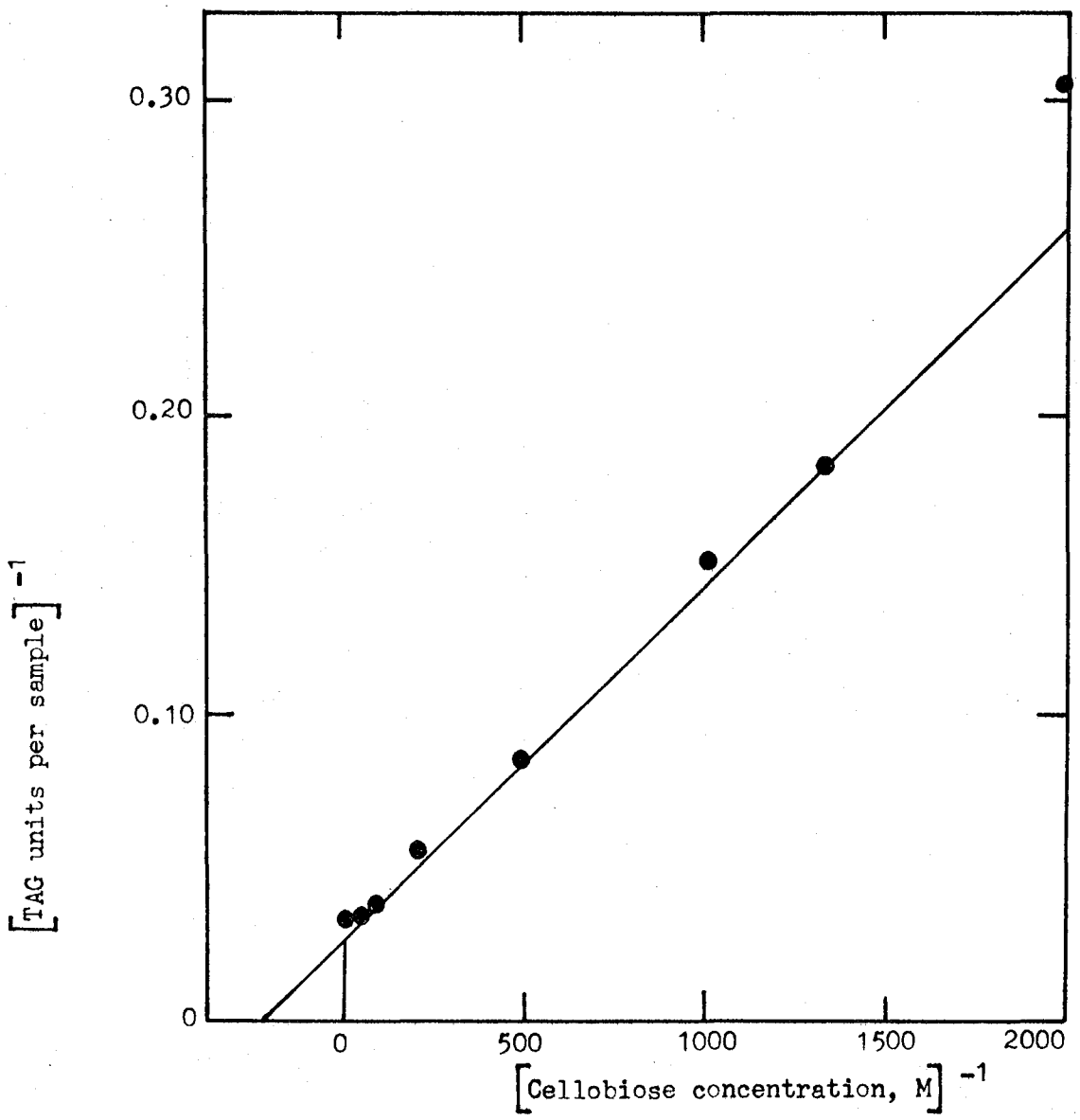

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Fig. 3.8 Lineweaver-Burke plot for β-glucosidase-TAG obtained from Trichoderma viride.

tank reactor, plugging occurred within a short time and caused the passage of liquid to be slow and difficult.

Several dispersing agents with rigid shapes were used with the β-glucosidase-TAG in order to produce a complex to be used in a fixed bed reactor which would allow the easy passage of liquid. "Celite" (crystalline particles with a diameter of about 0.045 millimeter) was used during the washing stage described in section 3.2 but pressure drops of up to 10 psi were necessary to pass 0.5 to 1.0 liter of liquid per day. When silica gel powder (diameter of 0.08 to 0.25 millimeter) was mixed with the β-glucosidase-TAG, liquid passed easilly through a column initially, but at long operation times the β-glucosidase-TAG particles travelled through the silica gel medium and packed at the column bottom, causing plugging of the column. This phenomena was also found to occur when using sand (diameter of 0.5 to 0.9 millimeter).

Because of information from Western Regional Laboratories[14] it was decided to seek a new method of immobilization that would be readilly used in a fixed bed reactor rather than perform further experimentation on reactor de-

sign with the β-glucosidase-TAG. The binding support chosen was phenol formaldehyde resin and the results will be discussed in the remainder of this work.

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4 Phenol Formaldehyde Resin Method of Immobilization

Due to the success of other workers and to the relative ease of the immobilization procedure, phenol formaldehyde resin was used as a support for the β-glucosidase. A stronger activity of β-glucosidase than that produced by Trichoderma viride was found to be produced by Aspergillus phoenicis and was utilized in the remainder of this work. The following sections describe results of experiments designed to determine the kinetic characteristics of the soluble and immobilized enzyme which will be needed to determine suitable operating conditions and scale-up parameters for an immobilized enzyme reactor. The influence of diffusion on conversion in a packed bed reactor was also studied and is included in the following sections.

4.1 Previous Work

Phenol formaldehyde resins have been available commercially for a number of years as absorbants for removal of proteins and colored materials from solutions.[1] Olson and Stanley have reported successfully immobilizing lac-

tase and other enzymes onto two grades of phenol formaldehyde resin, Duolite S-30 and a sized grade called Duolite Enzyme Support, both obtained from Diamond Shamrock Chemical Company.[1,2,3] They have been able to adsorb 2 to 3 milligrams of protein per gram of drained resin (drained resin contained about 60% water) onto the Duolite S-30. Adsorption was 90 to 95% complete within 1 to 2 hours. The enzyme was found to be bound firmly to the resin when treated with 1 to 3% glutaraldehyde. Lactase was adsorbed onto the resin at a specific activity of 200 micromoles of lactose hydrolysed per minute per gram of drained resin at conditions of 45°C in 0.4 M lactose solution at pH 4.0 in 0.10 M sodium acetate. A specific activity of 500 micromoles of lactose hydrolysed per minute per gram of drained resin was adsorbed onto the Duolite Enzyme Support resin. This corresponded to 10 to 11 milligrams of protein per gram of drained resin, a considerably higher figure than that for the Duolite S-30.

The stability of the glutaraldehyde treated immobilized lactase was determined in column operation. Two

columns were operated with a feed of 4% lactose solution for 7 days. There was a 10% loss of activity in one of the columns, which did not receive glutaraldehyde treatment. The other column, which did receive glutaraldehyde treatment, showed no change in activity.

The immobilization procedure reported by Stanley and Olson typically involved storing the resin overnight in 0.1 N NaCl followed by thorough washing with water. An aqeous solution of the enzyme was added followed by addition of enough 25% glutaraldehyde such that the final concentration was 1 to 3% in glutaraldehyde. Contacting time was overnight and contacting temperature was reported to be able to vary between 1°C and room temperature. No difference in the resultant enzyme-resin activity was found whether the glutaraldehyde was added before, during or after the application of the soluble enzyme.

Olson and Stanley have also reported various resin activities with the enzymes invertase, pronase, α-chymotrypsin and glucoamylase, as well as the results of several column studies with the immobilized lactase.[3]

4.2 Immobilization Procedure and Optimization Results

There is a maximum amount of activity that can be immobilized onto the phenol formaldehyde resin as there are only a finite amount of adsorption sites. In an actual immobilization procedure this maximum may bot be attained either due to enzyme inactivation upon adsorption or due to incomplete utilization of the adsorption sites. This incomplete utilization may be due to factors such as inadequate enzyme to resin ratio, inadequate enzyme concentration or inadequate contacting time. The five experiments described in this section were designed to qualitatively study some of the parameters that may influence the activity retained on the resin.

A general immobilization procedure was followed for all of the experiments. 0.5 gram of dried resin was transferred to a flask and immersed overnight in 0.1 N NaCl. The resin was quantitatively transferred to a sinterred glass filter, washed and the water was removed with a syringe. A quantity of enzyme (β-glucosidase from Aspergillus phoenicis) was added and contacting was performed at 4°C on a rotational shaker. After one half hour glutaraldehyde (if any)

was added. After a contacting time of 20 to 95 hours the resin was transferred to a sinterred glass filter where it was washed free of glutaraldehyde and unadsorbed enzyme with distilled water. The resin was then ready for transfer to a 250 milliliter flask for assay using the methods described in section 2.5.

The data and results for Experiment 1 are shown in Table 4.1. The soluble enzyme used in this experiment had an activity of 667 units per milliliter of enzyme and a protein content of 310 micrograms per milliliter. Contacting time was 20 hours. In addition to determining the activity of the resin after contacting, the washings of samples 4, 5 and 6 were assayed for β-glucosidase activity since no glutaraldehyde was present in these washings to interfere with the assay. From column 6, the activity on the resin samples which received glutaraldehyde was found to be consistantly higher than that of the samples that did not receive glutaraldehyde. This may be due to an enhancement of the adsorption process by glutaraldehyde (crosslinking to adsorbed molecules may have the effect of increasing the number of adsorption sites) but most

Table 4.1 Results of optimization experiment #1

	1	2	3	4	5	6	7	8	9	10
Sample number	Glutaraldehyde volume, ml	Enzyme volume, ml	Total volume, ml	Original activity, units/ml x 10^{-3}	Original protein, mg/ml	Resin activity, units x 10^{-3}	Theoretical resin activity, units x 10^{-3}	Theoretical resin protein, mg	Δ activity, units x 10^{-3}	% retained
1	1.0	20.0	21.0	0.667	0.310	4.88	-	-	-	36.6
2	2.0	40.0	42.0	0.667	0.310	4.36	-	-	-	16.3
3	3.0	60.0	63.0	0.667	0.310	3.87	-	-	-	9.7
4	0	20.0	20.0	0.667	0.310	3.29	4.87	6.84	1.58	24.7
5	0	40.0	40.0	0.667	0.310	3.09	3.92	5.32	0.83	11.6
6	0	60.0	60.0	0.667	0.310	2.80	3.42	3.18	0.62	7.0

likely is due to the retainment of the adsorbed molecules during the washing stages. Column 7 lists the theoretical activity immobilized onto the resin samples 4, 5 and 6 as determined by subtracting the wash activity from the total initial activity. The difference between this theoretical activity and the actual (column 9) may be due either to inactivation during immobilization or loss during subsequent washing steps. As the volume of enzyme (and thus the total liquid volume) per sample increased, the amount of resin activity decreased. This phenomena can be explained by the difference in contacting efficiency in the samples. All samples were contacted in a rotational shaker at the same rotation rate in 125 milliliter flasks. At the volume range and shaker speed utilized in Experiment 1, the mixing, and therefore the contacting of resin, with enzyme, was more efficient in the flasks with the smaller liquid volumes.

In Experiment 2 (Table 4.2) various flask sizes were used to help eliminate the contacting efficiency problem encountered in Experiment 1. The contacting time was 24 hours. In order to determine the effect of enzyme concen-

Table 4.2 Results of optimization experiment #2

	1	2	3	4	5	6	7
Sample number	Glutaraldehyde volume, ml	Enzyme volume, ml	Total volume, ml	Original activity, units/ml x 10^{-3}	Total system activity, units x 10^{-3}	Resin activity, units x 10^{-3}	% retained
1	0.5	5.0	5.5	0.581	2.91	2.48	85.4
2	0.8	8.0	8.8	0.581	4.65	3.43	73.8
3	1.6	16.0	17.6	0.581	9.30	3.49	37.5
4	2.4	24.0	26.4	0.581	13.94	3.30	23.7
5	1.6	5.0	17.6	0.581	2.91	2.58	88.7
6	1.6	8.0	17.6	0.581	4.65	3.42	73.5
7	0	5.0	5.0	0.581	2.91	2.06	70.8
8	0	8.0	8.0	0.581	4.65	2.79	60.0
9	0	16.0	16.0	0.581	9.30	2.40	25.8
10	0	24.0	24.0	0.581	13.94	2.39	17.1

tration on the immobilization, samples 5 and 6 received 16.0 milliliters total liquid volume composed of enzyme diluted with distilled water (columns 2 and 3). As in Experiment 1, the activity (column 6) and the percentage activity immobilized (column 7) was greater for the samples which received glutaraldehyde. Studying the results of samples 1 through 4, the maximum activity of approximately 3400 units was obtained using 9.0 milliliters of enzyme. An increase in the volume of enzyme beyond this did not increase the resin activity. The highest percentage of activity retained was from sample 1 (85.4%) but this did not yield the maximum activity on the resin. A comparison of the resin activity of the sample pairs 1 and 5, and 2 and 6 indicates that the enzyme concentration does not seem to influence the immobilization as the activities of these pairs of samples are similiar.

From Experiment 2, 6 to 8 milliliters of soluble enzyme seem to be sufficient in order to saturate the resin with activity. Experiment 3 was designed to study further the effects of enzyme concentration. In order for the contacting efficiency to be equal for all samples, each was

contacted in a 125 milliliter flask with a total liquid volume of 20 milliliters. Contacting time was 24 hours. Two trends are apparent from the results in columns 6 and 7 of Table 4.3. As the enzyme concentration was increased, the percentage retained of the enzyme decreased and the amount of resin activity increased. A maximum of retained activity of approximately 3200 units occurred at sample 5, a one to four dilution.

To study the possibility of a tradeoff between enzyme concentration and contacting time in order to achieve a certain level of activity on the resin, Experiment 4 was performed. This experiment was designed to determine if an increase in time of contacting would allow the maximum loading of the resin to be attained at low enzyme concentrations. The data and results are shown in Table 4.4. From samples 1 through 3, using 20.0 milliliters of full strength enzyme, a maximum loading of 3300 units was obtained sometime between a contacting time of 18 and 42 hours. This maximum was not reached even after a contacting time of 52 hours for the dilute enzyme samples, 4, 5 and 6.

Table 4.3 Results of optimization experiment #3

	1	2	3	4	5	6	7
Sample number	Glutaraldehyde volume, ml	Enzyme volume, ml	Total volume, ml	Original activity, units/ml x 10^{-3}	Total system activity, units x 10^{-3}	Resin activity, units x 10^{-3}	% retained
1	3.0	4.0	23.0	0.552	2.21	1.70	77.0
2	3.0	6.0	23.0	0.552	3.31	1.90	57.3
3	3.0	8.0	23.0	0.552	4.42	2.38	53.9
4	3.0	10.0	23.0	0.552	5.52	2.53	45.8
5	3.0	15.0	23.0	0.552	8.28	3.27	39.5
6	3.0	20.0	23.0	0.552	11.04	3.12	28.3

Table 4.4 Results of optimization experiment #4

	1	2	3	4	5	6	7	8
Sample number	Glutaraldehyde volume, ml	Enzyme volume, ml	Total volume, ml	Contacting time, hours	Original activity, units/ml x 10^{-3}	Total system activity, units x 10^{-3}	Resin activity, units x 10^{-3}	% retained
1	3.0	20.0	23.0	18	0.552	11.04	2.57	23.2
2	3.0	20.0	23.0	42	0.552	11.04	3.33	30.2
3	3.0	20.0	23.0	52	0.552	11.04	3.23	29.3
4	3.0	8.0	23.0	18	0.552	4.42	1.89	42.8
5	3.0	8.0	23.0	42	0.552	4.42	2.37	53.6
6	3.0	8.0	23.0	52	0.552	4.42	2.78	62.9

Experiment 5 (Figure 4.5) was performed as an expansion of Experiment 4. Three enzyme concentration levels were utilized over a contacting time range of 22 to 95 hours. The enzyme used for this experiment was from a new batch of Aspergillus phoenicis fungi and had a much lower soluble enzyme concentration (196 units per milliliter of enzyme). A maximum loading of approximately 1800 units of activity was reached for the samples which utilized full strength enzyme after contacting for 67 hours. The other two dilutions did not reach this maximum even after 95 hours of contacing, however the resin activity steadilly increased in both cases with increasing contacting time.

Experiments 1 and 2 and the work of Stanley and Olson indicate that the use of glutaraldehyde increases the amount of activity retained by the resin due to the fixation of the enzyme to stop desorption. From Experiment 2 it is seen that there is an enzyme to resin ratio beyond which an increase in soluble enzyme will not increase the adsorbed activity. For an initial soluble enzyme strength of 581 units per milliliter of enzyme the maximum resin activity was found to be approximately 3500 units per 0.5

Table 4.5 Results of optimization experiment #5

Sample number	1 Glutaraldehyde volume, ml	2 Enzyme volume, ml	3 Total volume, ml	4 Contacting time, hours	5 Original activity, units/ml x 10^{-3}	6 Total system activity, units x 10^{-3}	7 Resin activity, units x 10^{-3}	8 % retained
1	3.0	5.0	23.0	22	0.196	0.98	0.58	59.2
2	3.0	10.0	23.0	22	0.196	1.96	0.89	45.6
3	3.0	20.0	23.0	22	0.196	3.91	1.18	30.2
4	3.0	5.0	23.0	46	0.196	0.98	0.69	68.4
5	3.0	10.0	23.0	46	0.196	1.96	1.20	61.5
6	3.0	20.0	23.0	46	0.196	3.91	1.43	36.6
7	3.0	5.0	23.0	67	0.196	0.98	0.63	64.3
8	3.0	10.0	23.0	67	0.196	1.96	1.25	64.1
9	3.0	20.0	23.0	67	0.196	3.91	1.84	47.1
10	3.0	5.0	23.0	95	0.196	0.98	0.83	84.7
11	3.0	10.0	23.0	95	0.196	1.96	1.55	79.5
12	3.0	20.0	23.0	95	0.196	3.91	1.87	47.9

gram of dry resin. This enzyme preparation was not purified and the maximum activity is expected to increase when a purified soluble enzyme preparation is used. However, due to the higher costs involved in producing purified enzyme preparations the level of activity produced in these experiments may be suitable for industrial use. From Experiments 3,4 and 5, a tradeoff is apparent between enzyme concentration and contacting time. In the determination of the optimal conditions for the immobilization procedure on an industrial scale, the high cost of enzyme concentration may be in part removed by an increase in reaction time, or the immobilized enzyme may be produced at less contacting time by an increase in the level of enzyme concentration.

4.3 Resin-immobilized β-glucosidase Kinetic Characteristics.

The following sections describe the results of experiments designed to determine the kinetic characteristics of both the soluble and immobilized β-glucosidase from Aspergillus phoenicis. Due to the inaccuracy inherent in the

portioning by weight of a moist sample of the resin-bound enzyme, one sample of the resin-bound enzyme was used to produce the data points of each experiment. The resin-bound enzyme samples were obtained from various mixtures of the samples produced in the experiments of the previous section.

4.3.1 pH Optimum

The effect of pH on the activity of the soluble and resin-bound β-glucosidase was determined at 40°C in 0.01 M cellobiose. Figure 4.1 shows that the pH profile of the soluble and resin-bound enzyme in 0.10 M citrate-phosphate buffer both exhibit optima at pH 4.3. Figure 4.2 shows the results of an experiment designed to determine the reproducibility of the pH optima experiments. Since the same sample of resin-bound enzyme was used to determine all the data points of each experiment, it was possible that the shape of the resultant curve may be influenced by enzyme denaturation. Figure 4.2 shows that the pH curve has the same shape regardless of whether the data points were determined from low to high or high to low pH, indicating

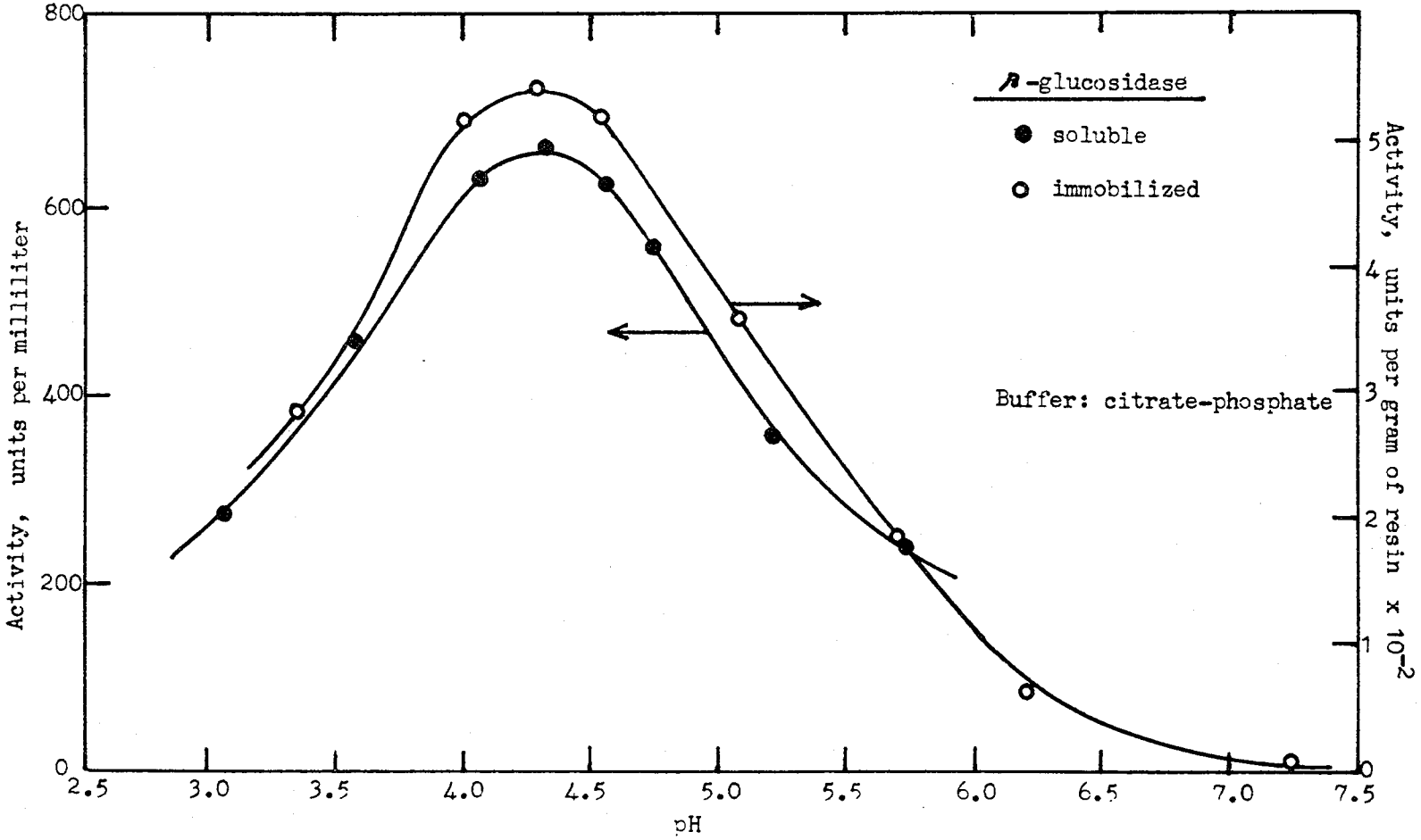

Fig. 4.1 pH profile for soluble and resin-bound enzyme in citrate-phosphate buffer. Enzyme obtained from Aspergillus phoenicis.

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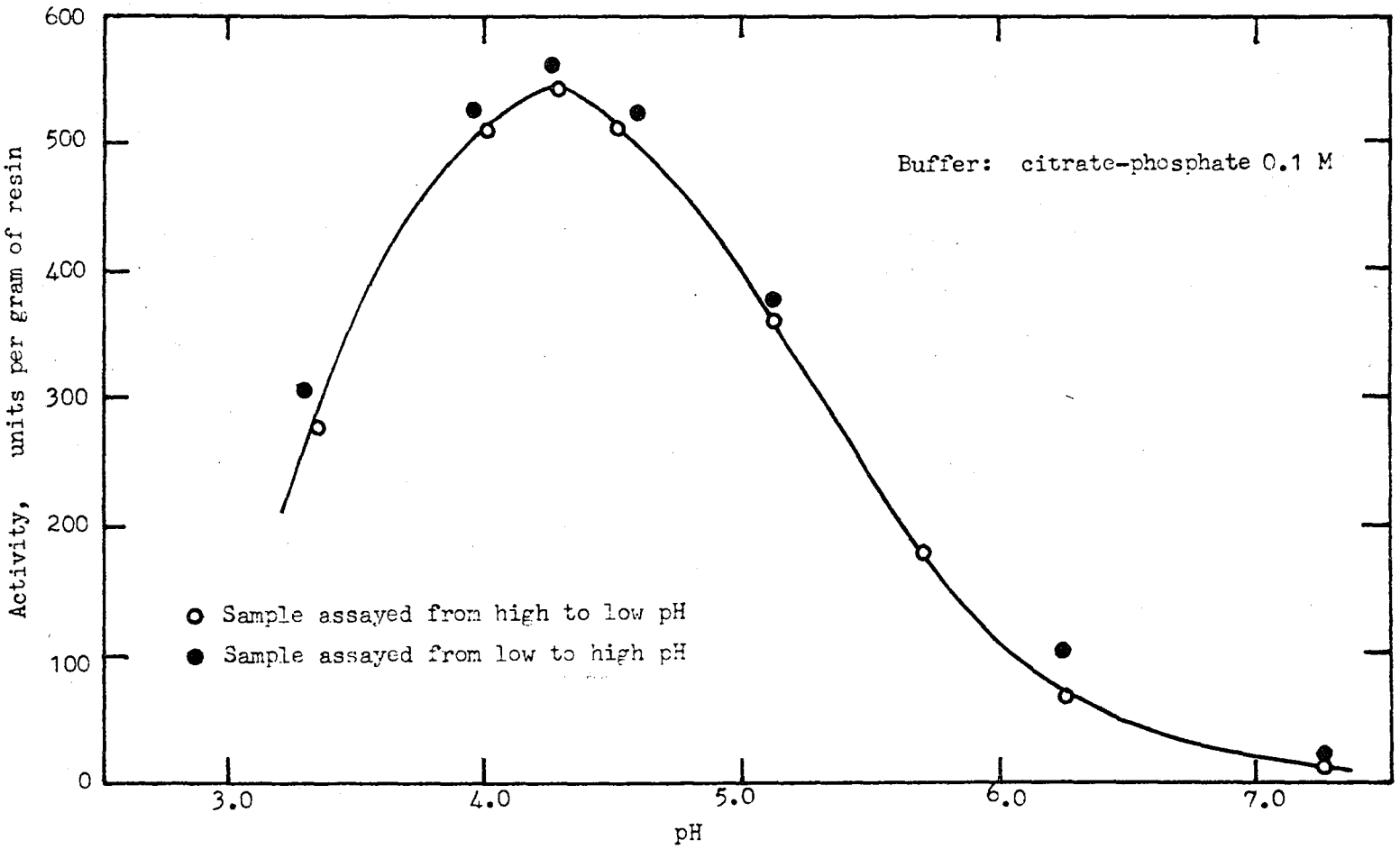

Fig. 4.2 pH profile of resin-bound enzyme in citrate-phosphate buffer. Results of experiment designed to show independance of profile on the sample order.

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there was little if any denaturation during the course of the experiment.

Figure 4.3 shows a pH optimum of 4.7 for the soluble enzyme in citrate buffer. Since it was felt at this point that citrate may be having an inhibitory effect on the β-glucosidase activity, an experiment was designed to indicate this and the results are shown in Figure 4.4. Identical samples of the soluble enzyme were assayed in cellobiose solutions prepared at four differnt pH in citrate buffer concentrations of 0.10 and 0.05 M. There was a greater glucose production from the 0.05 M citrate buffer samples and the difference in the two concentrations increased as pH decreased, indicating that the level of citrate ion influenced enzyme activity.

The pH optima was determined in acetate buffer due to the apparent inhibitory effect of citrate and due to the widespread use of acetate buffer in the hydrolysis process. Figure 4.5 shows the effect of acetate buffer strength on the enzyme activity. More scatter was observed in the pH profile of the soluble β-glucosidase in 0.1 M acetate buffer and since 0.05 M acetate buffer pro-

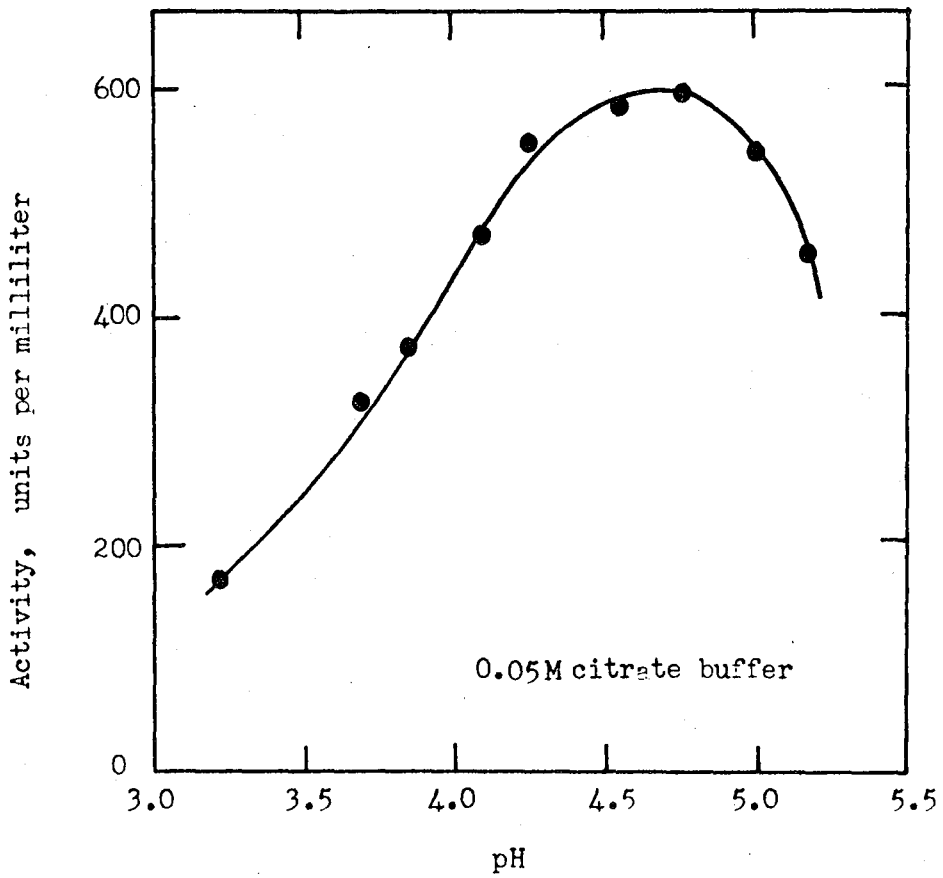

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Fig. 4.3 pH profile of soluble Aspergillus phoenicis β-glucosidase in citrate buffer.

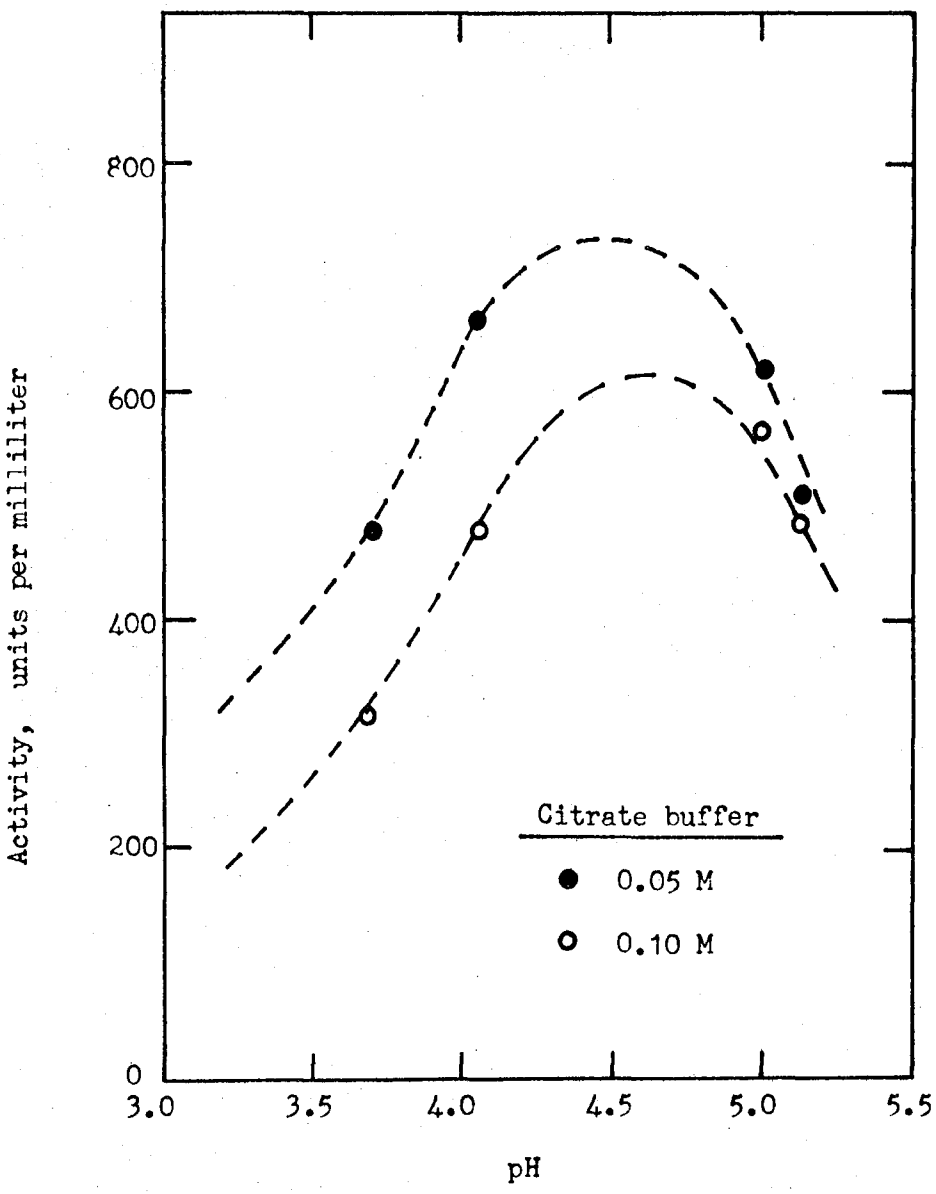

Fig. 4.4 Results of experiment to study the inhibitory effect of citrate ion on enzyme activity.

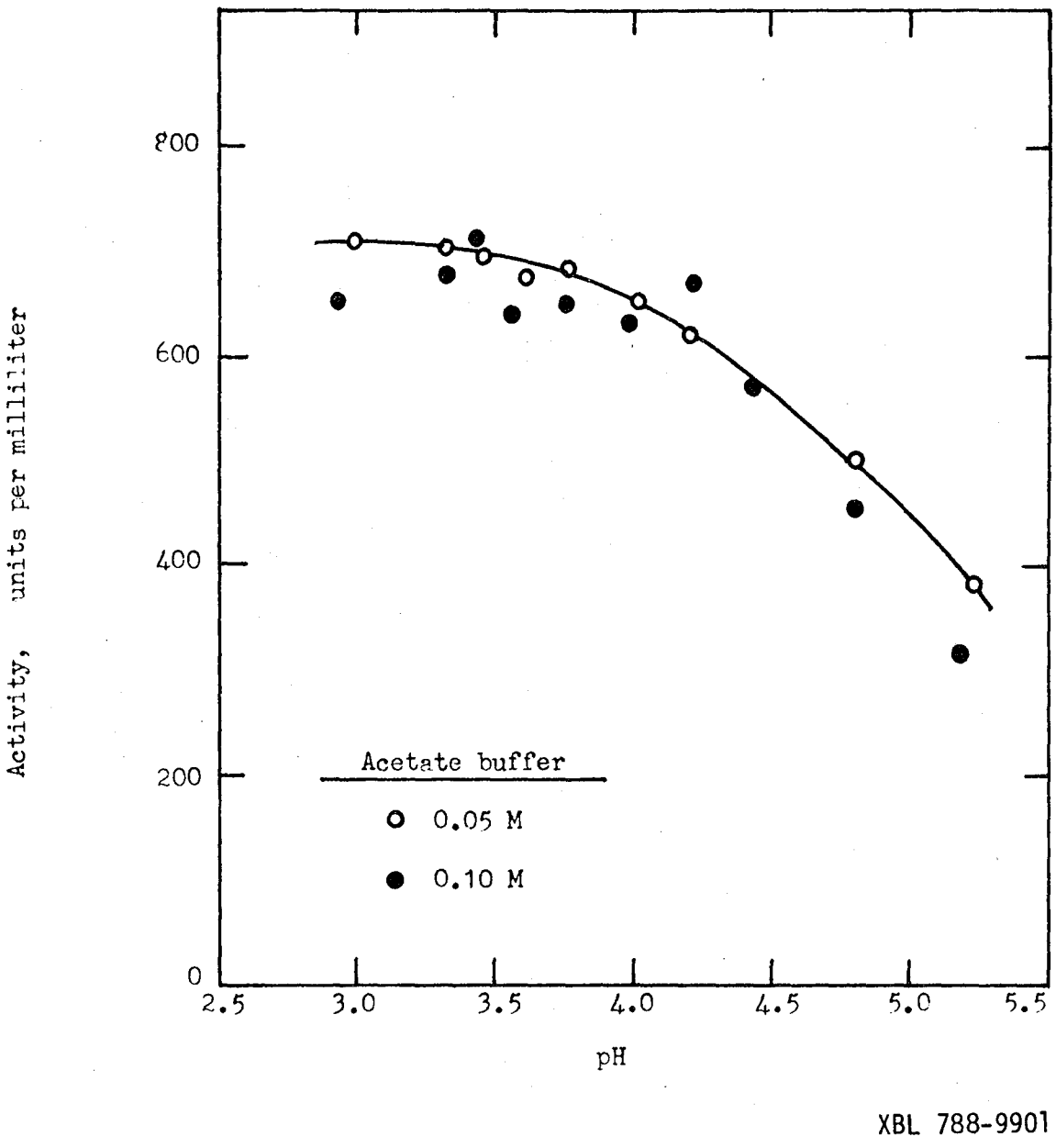

Fig. 4.6 Results of experiment designed to study the effect of buffer strength of acetate buffer on enzyme activity and pH profile.

vided a smoother curve, this strength was chosen for future experiments. Figure 4.6 shows that the pH profiles for both the soluble and immobilized enzyme are identical and exhibit maximum activity at a pH below the buffering range of the acetate buffer (pH 3.5 to 5.5). pH 4.0 was chosen as the standard pH for the assay of β-glucosidase obtained from Aspergillus phoenicis as at this pH the activity is almost at the maximum and the pH is well within the buffering range of the acetate buffer.

The pH profiles for both the soluble and immobilized enzyme in acetate and citrate-phosphate buffers (Figures 4.1 and 4.6) exhibit the same pH optima, indicating that microenvironmental effects from the adsorption of the enzyme onto the resin has no apparent effect on the enzyme kinetics.

4.3.2 Enzyme-Substrate Interaction

Section 3.3.6 discussed the substrate-enzyme interaction from the standpoint of the classical Michaelis-Menton model

$$r = \frac{VmA}{Km + A} \qquad 4.1$$

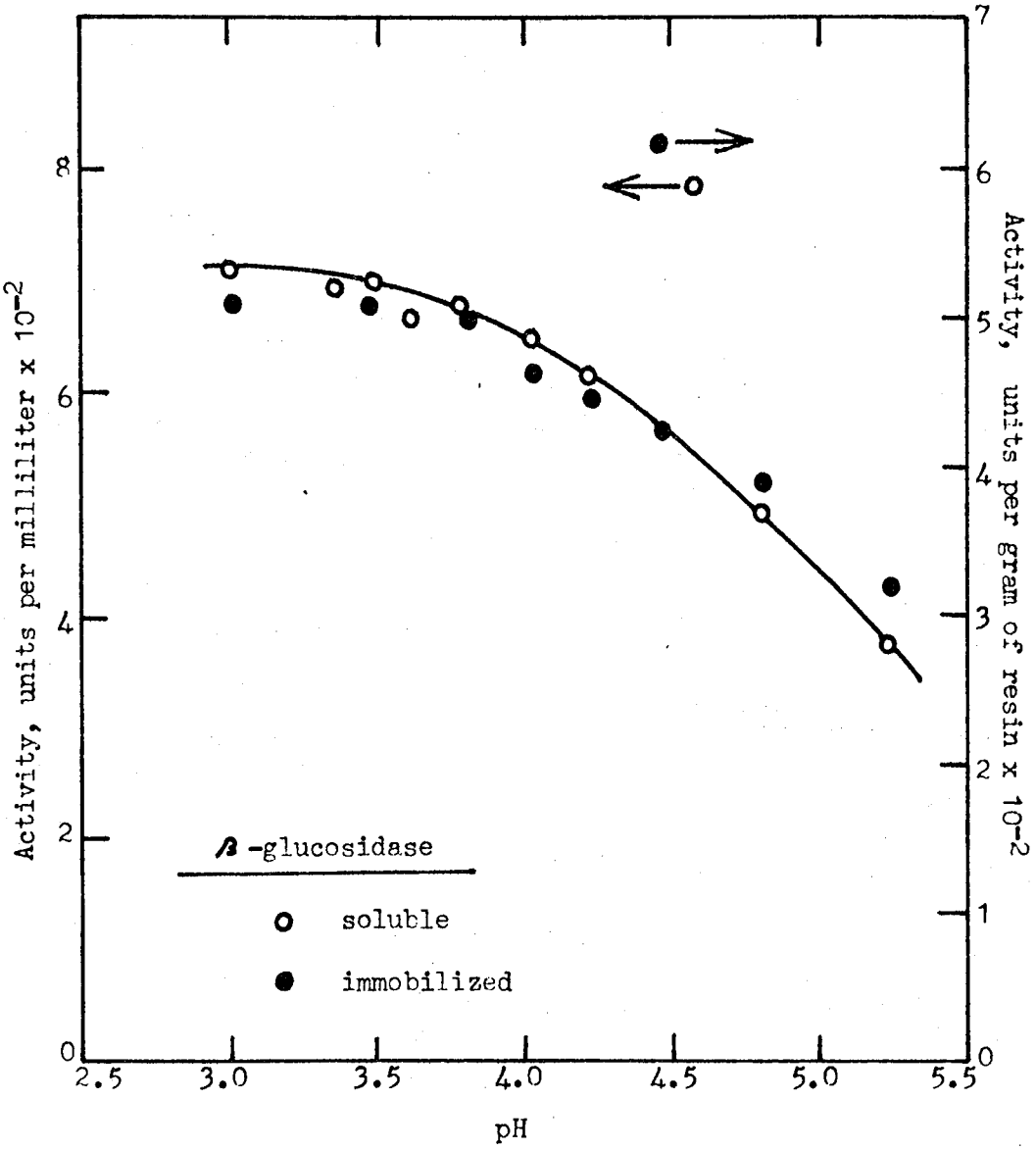

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Fig. 4.6 pH profile of soluble and resin-bound β-glucosidase in 0.05 M acetate buffer.

which does not take into account substrate or product inhibition. In this work on kinetic characteristics, product inhibition can be assumed negligible as the initial reaction rates are being determined, when product formation is small. Substrate inhibition was not observed in the work on β-glucosidase obtained from Trichoderma viride as the highest substrate concentration studied was 0.02 M (Figures 3.7 and 3.8 actually do show the beginnings of substrate inhibition by the points corresponding to the highest substrate concentrations). Substrate inhibition was observed in the work with β-glucosidase from Aspergillus phoenicis as the substrate concentration range was expanded to 0.4 M.

In a number of single site enzymes there is evidence for the binding of a second molecule of substrate at a location near the active site of the enzyme.[4] This second substrate molecule does not react with the enzyme to form product, but may influence the ability of the enzyme to bind with the first substrate molecule or influence the formation of product, or both. The substrate is considered an activator if its presence leads to an enhanc-

ment of product production and is considered an inhibitor if production is decreased. The Michaelis-Menton model can be modified to account for substrate inhibition by the addition of a term

$$r = \frac{Vm}{1 + Km/A + A/Ks}$$

where Ks is the substrate inhibition constant.

The activity versus substrate concentration profiles for both the soluble and immobilized enzyme are shown in Figures 4.7 and 4.8. The resin bound enzyme data points were all determined from the same sample of resin at 40°C in pH 4.0 0.05 M acetate buffer according to the procedure described in section 2.5. The glucose production for each sample was determined at four different times and the initial rate of reaction was determined from the slope of the resultant curve at time zero for both the soluble and immobilized enzyme samples in order to account for substrate depletion at lower concentrations. Both enzyme systems follow classical Michaelis-Menton kinetics at substrate concentrations below 0.005 M and exhibit substrate inhibition at greater concentrations as

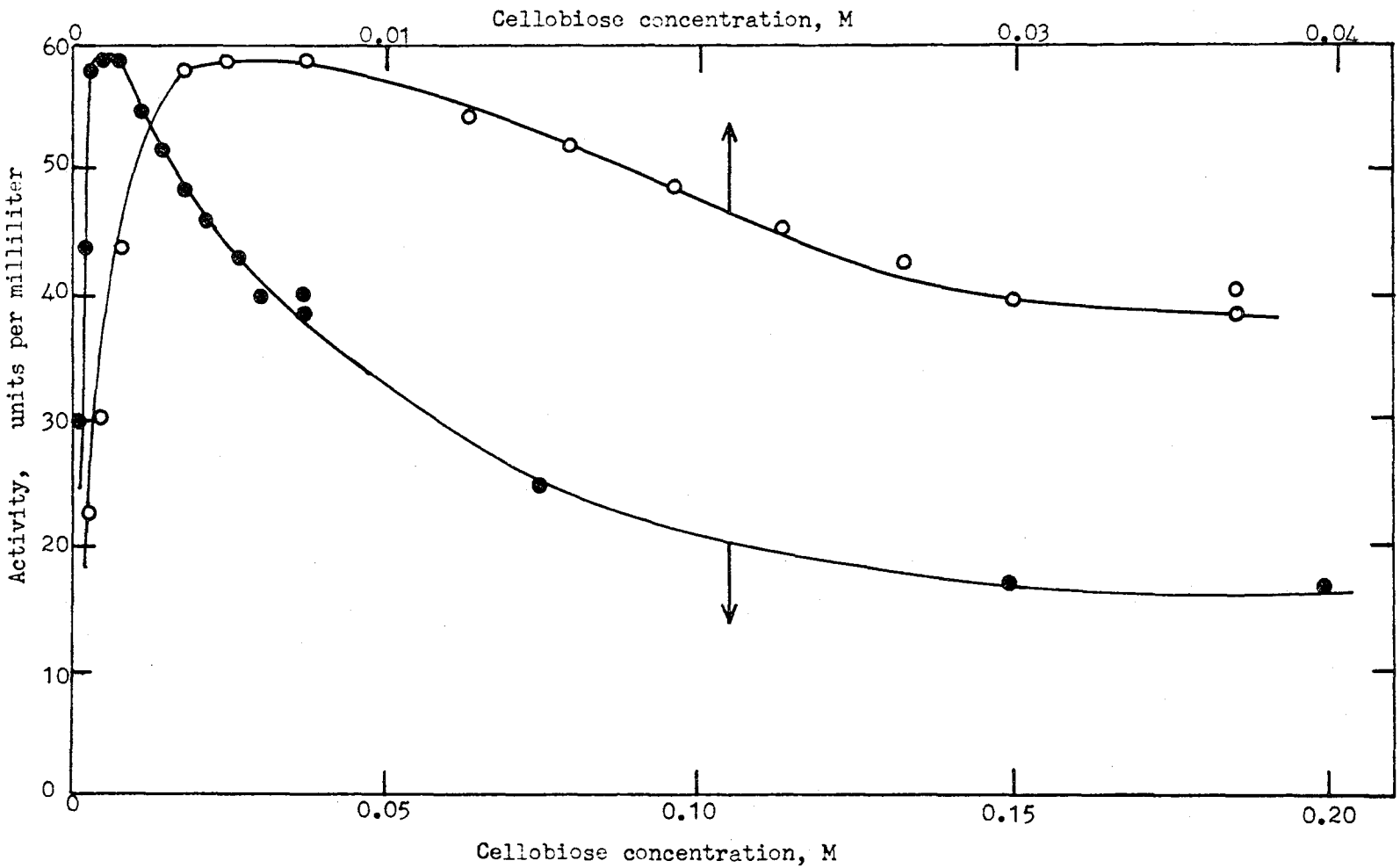

Fig. 4.7 Activity versus substrate concentration for soluble β-glucosidase. Top scale is the expanded profile of the bottom scale.

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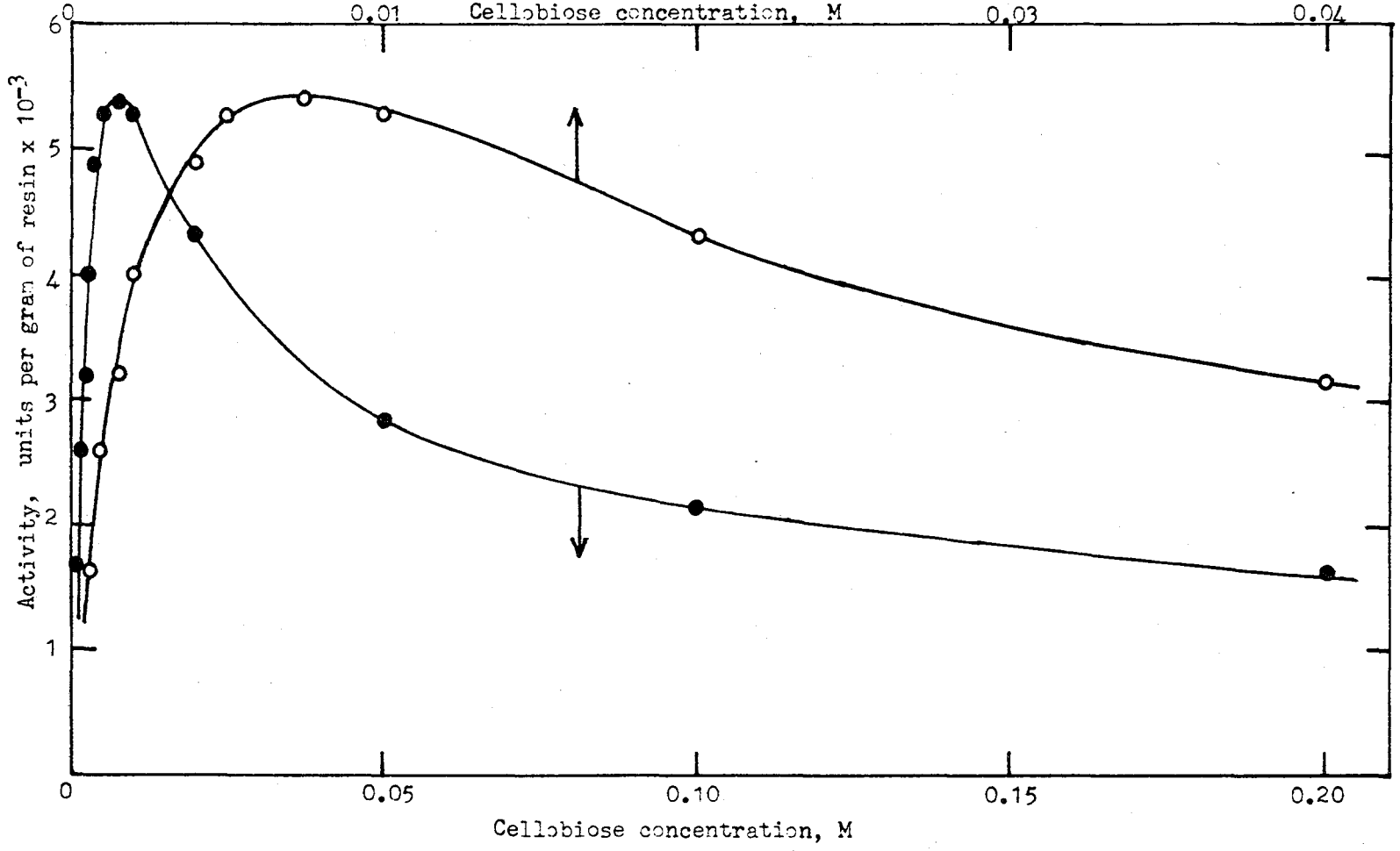

Fig. 4.8 Activity versus substrate concentration for resin-bound β-glucosidase. Top scale is the expanded profile of the bottom scale.

XBL 788-9904

seen by the decrease in activity. A value for Ks of 0.35 to 0.04 moles per liter causes an approximate fit of the rate equation to the profiles in Figures 4.7 and 4.8. An accurate value of Ks could not be determined as the profiles do not exactly follow the above reaction model. The Km constants were determined to be 1.39×10^{-3}M and 1.56×10^{-3} M for the soluble and resin-bound enzyme, respectively, from the x-intercepts of the Lineweaver-Burke plots shown in Figures 4.9 and 4.10. The trailing upwards of the data points on these plots at high substrate concentrations is a characteristic of the substrate inhibition.

4.3.3 Temperature Dependance

The effect of temperature on the rate determining step in conversion of enzyme-substrate complex to products can be determined in the presence of saturating amounts of substrate and when the effect of temperature on the enzyme stability is accounted for.[4] The dependance of the rate constant, k, on temperature is given by the Arrhenius equation

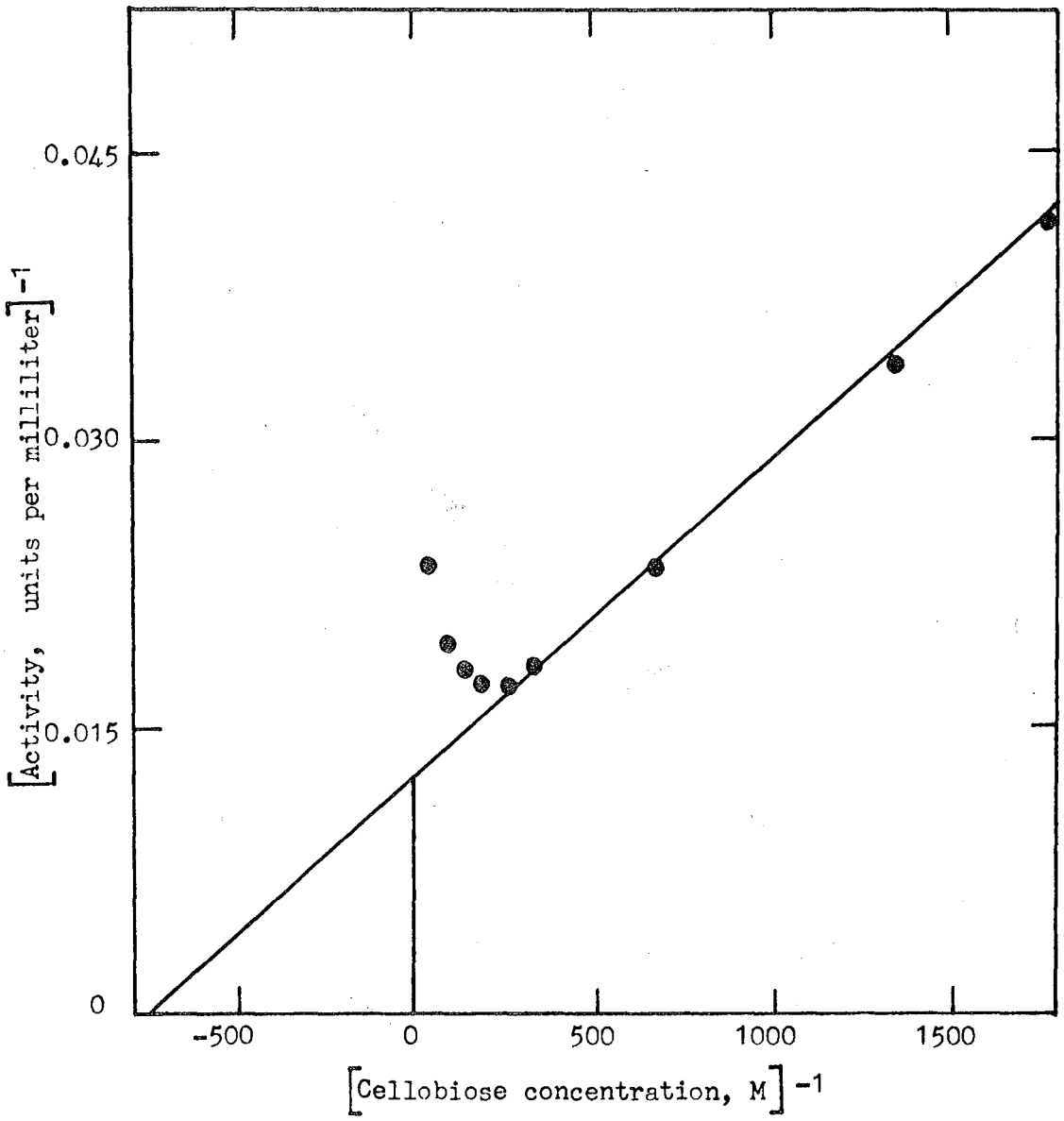

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Fig. 4.9 Lineweaver-Burke plot for soluble enzyme obtained from Aspergillus phoenicis.

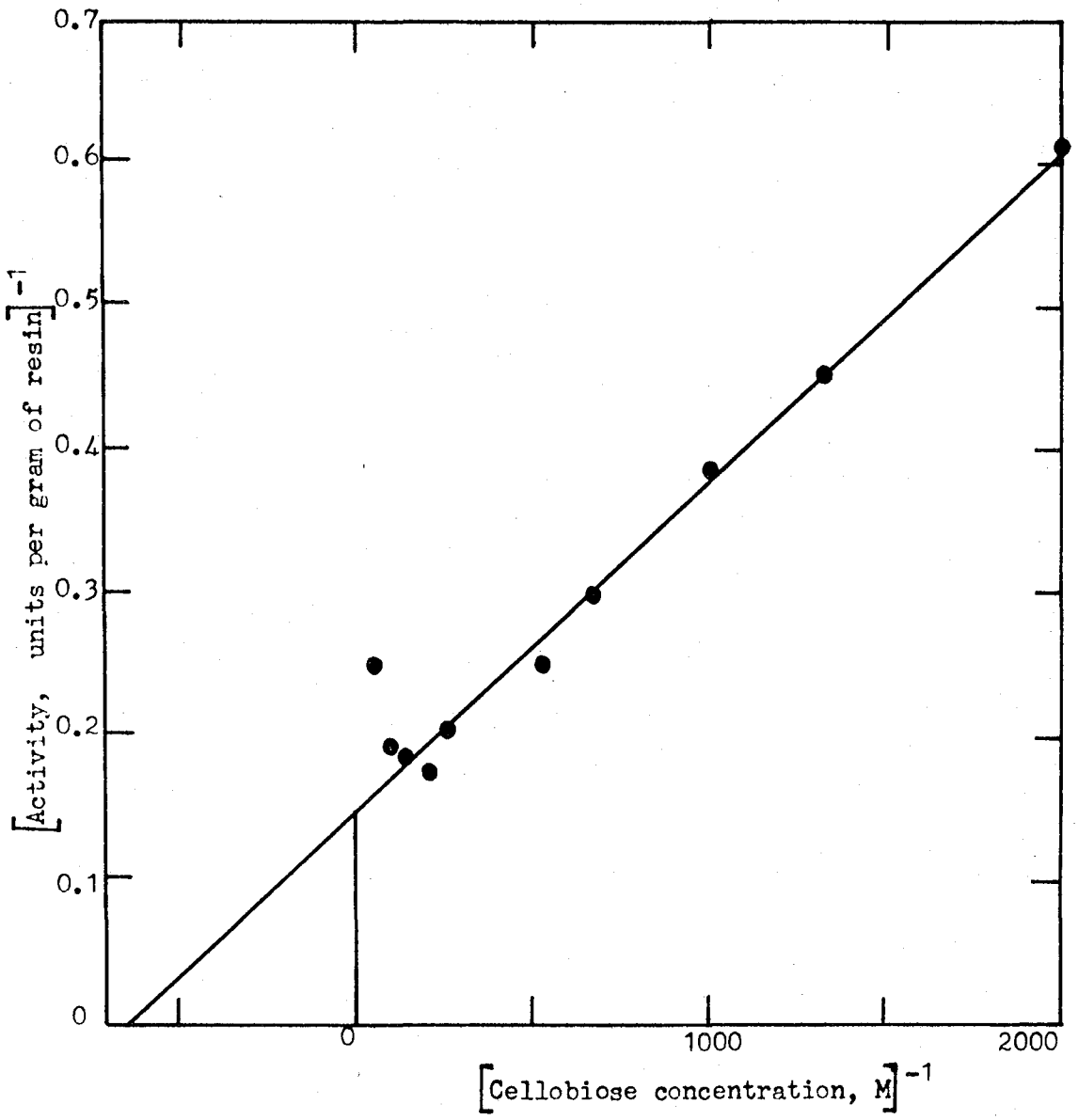

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Fig. 4.10 Lineweaver-Burke plot for resin-bound enzyme obtained from Aspergillus phoenicis.

$$k = Ae^{-Ea/RT} \qquad 4.2$$

as discussed in section 3.3.4. Once Ea is known for an enzymic reaction, the reaction rate at any temperature can be predicted from the reaction rate at another temperature. Taking the logarithm of the Arrhenius equation

$$\ln k = \ln A - Ea/RT \qquad 4.3$$

the activation energy, Ea, can be determined from the slope of a plot of the logarithm of reaction rate versus inverse temperature, since the reaction rate is directly proportional to the rate constant, k. Figure 4.11 shows this plot for both the soluble and resin-bound β-glucosidase prepared by performeing the standard enzyme assays in 0.01 M cellobiose solution in pH 4.0 0.05 M acetate buffer at various temperatures. In Figure 4.11 the activities have been normalized such that the soluble and immobilized samples assayed at 40°C coincide. From the slope of the plot the activation energy for the soluble and immobilized enzyme is the same, 11.63 killocallories per mole, another indication that the enzyme kinetics are

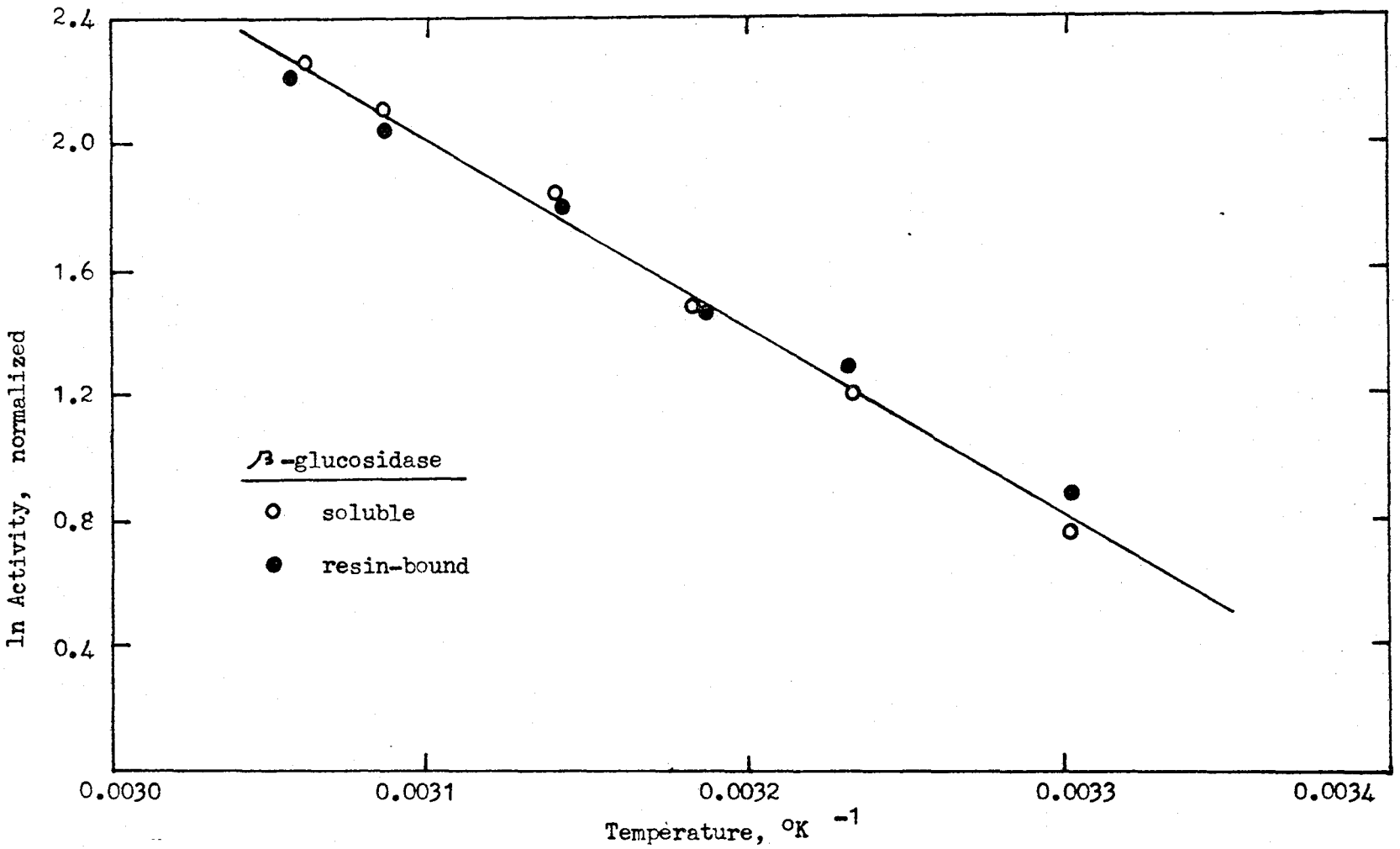

Fig. 4.11 Arrhenius plot for the soluble and resin-bound β-glucosidase. Ordinate has been normalized to allow the 40°K data points to coincide.

not noticeably effected by the immobilization.

4.3.4 Temperature Stability

The temperature stability of the resin-bound β-glucosidase was determined at both 40°C and 45°C in order to determine a suitable temperature for reactor operation. 0.7 gram of the moist resin was placed in each of two 250 milliliter flasks. The resin in each flask was immersed in 50 milliliters of 0.05 M pH 5.0 acetate buffer and one flask each was placed in a 40°C and 45°C water bath. At various times the resin was assayed according to the procedure described in section 2.5 with 0.01 M cellobiose solution in pH 4.0 0.05 M acetate buffer. As a comparison, a well-capped vial of the soluble enzyme was also stored in each of the water baths and samples of the enzyme were assayed at various times.

Figure 4.12 shows the activity versus time profiles for the soluble and immobilized enzyme at each temperature. The soluble enzyme was found to be much less stable when stored at 45°C rather than at 40°C. Of the samples stored at 45°C, greater stability is seen in the immobilized enzyme, and this stabilization may be due to the immobili-

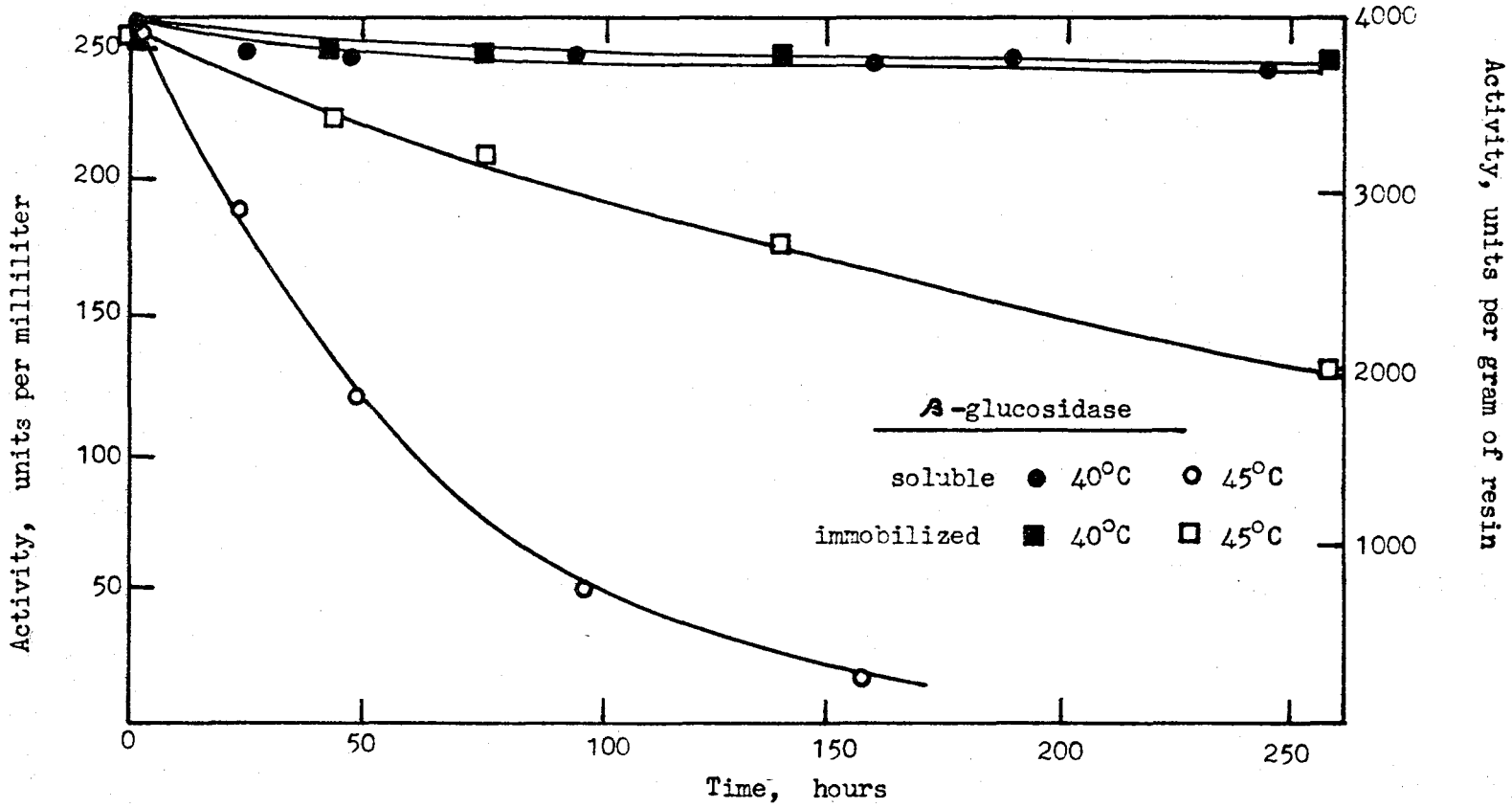

Fig. 4.12 Results of temperature stability experiments for both soluble and resin-bound β-glucosidase.

XBL 788-9908

zation. As discussed in section 3.3.3, the rate of heat denaturation will normally follow a first order process:

$$R_d = k_d(E) \ , \qquad k_d = Ae^{-E_d/RT} \quad 4.4$$

Assuming this form for the denaturation kinetics, from the data in Figure 4.12 approximate values for k_d for the immobilized enzyme at 40°C and 45°C were found to be 0.0002 $hour^{-1}$ and 0.0026 $hour^{-1}$, respectively. These values were determined from the average of values of k_d determined for several points as the curves do not exactly follow a first order rate of denaturation.

4.4 Packed Bed Immobilized Enzyme Reactor Studies

4.4.1 Phenol Formaldehyde Resin Characteristics

The phenol formaldehyde resin was obtained from Diamond Shamrock Chemical Company and was a specially sized resin, called Duolite ES-762 Enzyme Support. The void fraction, ε, was determined to be 0.36. This value was determined by packing the resin in a column filled with distilled water. The water was forced through the

column with compressed air and collected in a vessel. The void fraction was calculated by dividing the volume of eluted water by the total column volume.

A sample of the resin was fractionated by size on a set of screens and the size fractions are listed in Table 4.6. An average particle diameter of 0.0261 centimeter was determined by the summation of the products of the weight fractions (column 3) and the median diameters (column 2) of each size range.

Once enzyme had been immobilized onto the resin, the resin had to remain moist or denaturation would occur. Accurate weight determinations of resin samples therefore were difficult to obtain. 1.0 gram of resin was found to weigh 2.7 to 3.0 grams when wet (slight water removal with vacuum), 1.8 grams when drained (most water removed by strong vacuum) and had a total volume (voids plus resin) of 2.28 milliliters.

From the void fraction, the total volume of voids and resin and the amount of retained water when drained, the density of the resin was estimated at 1.415 grams per cubic centimeter. Assuming the resin particles to be

Table 4.6 Weight fraction of ES-762 Enzyme Support phenol formaldehyde resin

Diameter range, microns	Median diameter, microns	Weight fraction
351/300	326	0.153
300/246	273	0.575
246/210	228	0.177
210/175	193	0.069
175/105	140	0.006

spherical, the average surface area and volume per particle are estimated to be 2.1×10^{-3} square centimeters and 9.3×10^{-6} cubic centimeters, respectively.

4.4.2 Diffusion Studies in a Packed Bed Reactor

Five steps are involved in the conversion of a substrate molecule to product catalyzed by an enzyme immobilized onto a solid surface: (1) diffusion of the substrate to the solid surface, (2) diffusion to the domain of the enzyme, (3) enzyme catalyzed reaction to product, (4) and (5) reversal of steps 2 and 1 for the product.[5] Steps 1 and 5 are external or film diffusional effects, steps 2 and 4 are internal or pore diffusional effects, and step 3 is the kinetic reaction step. Any one or more may be the limiting factor in the overall rate of conversion to product.

In the assay procedure described in section 2.5 the rate limiting step was determined to be the enzyme reaction as a change in the agitation rate (which would cause a change in the mass transfer rate) did not cause a change in the rate of glucose formation. The studies

of this section were performed in order to determine the influence of diffusion for the immobilized enzyme when used in a packed bed reactor. For the phenol formaldehyde resin, diffusional influences were expected to be in the form of film diffusion of the substrate to the solid surface (step 1 above) from information received from Western Regional Laboratories.[6] The transport of substrate to a solid surface through a liquid media is commonly expressed in terms of the mass transport coefficient,

$$N = k_l(C_b - C_s) \qquad 4.5$$

where N is the flux of substrate, C_b and C_s are the bulk and surface substrate concentrations, and k_l is the mass transport coefficient.[7]

Chilton and Colburn suggested a correlation of the mass transport coefficient with the reactor variables of flow rate, reactor diameter, etc. may be obtained by the definition of a dimensionless group

$$j_d = \frac{k_l \rho}{G} \left(\frac{\mu}{\rho D_f}\right)^{2/3} \qquad 4.6$$

where ρ and μ are the density and viscosity of the liquid, D_f is the molecular diffusivity of the diffusing substrate and G is the superficial velocity (given by reactor corss-sectional area divided by the flowrate).[8] j_d is a function of the Reynolds number, Re, which is defined as

$$Re = d_p G/\mu \qquad 4.7$$

where d_p is the diameter of the solid particles. Many methods have been reported for correlating the mass transport coefficients around submerged solid objects and most have used the above formulation.[9] Rovito and Kittrel have shown the correlation proposed by McCune and Wilhelm[10] to be effective in predicting diffusion in immobilized enzyme packed bed reactors.[11]

The mass transport coefficient, k_l, was determined from three correlations for a variety of flow rates. These three correlations are listed in Table 4.7 (the McCune

Table 4.7 Three examples of correlations used to determine the mass transport coefficient, k_l, of cellobiose.

Correlation number	Packing type	Correlation	Effective range of Reynolds number
1	spherical particles	$\varepsilon j_d = 1.09 \cdot Re^{-2/3}$	0.0016 - 55
2	granular solid	$St \cdot Sc^{0.6} = 0.45 \cdot Re^{-0.5}$ $Sc = \mu/D_f$, $St = k_l/G$	<10
3	spherical and flaked shaped particles	$j_d = 1.625 \cdot Re^{-0.507}$	<120

and Wilhelm model is correlation 3). D_f for cellobiose in water at 40°C was determined to be 7.94×10^{-6} square centimeters per second from the Wilke-Chang correlation[12] for a dilute solute at low concentration

$$D_f = 7.4 \times 10^{-8} \left[(XM)^{0.5} T/\mu V_b^{0.6}\right] \qquad 4.8$$

where T = temperature = 313°K

X = association parameter of solvent = 2.6

M = molecular weight of solvent = 18

μ = viscosity of solvent = 0.006529 grams/cm·sec

V_b = molal volume of solute (from charts[12])
= 299 cm^3/gram-mole

The calculated values for the mass transport coefficient are listed in Table 4.8 and differ within an order of magnitude for the various correlations. The minimum value for k_l, that for a stagnant fluid, given by

$$k_l = \frac{2D_f}{d_p} \qquad 4.9$$

is 6.1×10^{-4} centimeter per second. The McCune and Wilhelm model, correlation 3, approaches this value at

Table 4.8 Calculated values for the mass transport coefficient, k_1, from three mass transport correlations

Flow rate, ml/min.	Superficial velocity, cm/min.	Reynolds number	Correlation # 1 $(sec.)^{-1}$	Correlation # 2 $(sec.)^{-1}$	Correlation # 3 $(sec.)^{-1}$
0.2	0.255	0.0170	0.00200	0.00026	0.000621
0.5	0.637	0.0425	0.00271	0.000413	0.000976
1.0	1.273	0.0849	0.00342	0.000583	0.00137
2.0	2.546	0.1697	0.00430	0.000825	0.00193
5.0	6.366	0.4244	0.00548	0.00130	0.00305
10.0	12.732	0.8488	0.00736	0.00185	0.00427

low flow rates, whereas correlations 1 and 2 yield values that are too high and too low, respectively.

If steady state is assumed between the transport of substrate to the resin surface (eq. 4.5) and the rate of reaction of substrate to product at that surface (eq. 3.5), and assuming the Michaelis-Menton form for the reaction kinetics, the following equation is obtained:

$$k_1(C_b - C_s) = \frac{Vm \cdot Cs}{Km + C_s} \qquad 4.10$$

where C_b and C_s are the bulk phase and surface concentrations of substrate, Vm is the saturation (maximum) rate of reaction in units of Moles per surface area per second, and Km is the Michaelis-Menton constnat.[13] An estimate of the relative speeds of the diffusion and reaction steps can be obtained by calculating both sides of the above equation independantly, assuming both sides to be at the maximum rate. The maximum rate of diffusion is determined by assuming C_s is equal to zero and the maximum rate of reaction is determined by assuming C_s is equal to C_b. Table 4.9 lists values for the maximum diffusion and

Table 4.9 Relative values of the maximum rate of mass transport to the resin surface and maximum rate of reaction at the resin surface under various conditions

Bulk phase concentration, C_b, Moles/liter	Mass transport coefficient, k_l, $(seconds)^{-1}$	Maximum mass transport rate, $k_l(C_b-0)$, $Moles/cm^2 \cdot sec$	Maximum reaction rate, $VmC_b/(Km+C_b)$, $Moles/cm^2 \cdot sec$	Surface concentration, C_s, Moles/liter	% Efficiency
0.001	2.6×10^{-4}	2.6×10^{-10}	3.23×10^{-10}	3.8×10^{-4}	50.0
0.001	5.0×10^{-4}	5.0×10^{-10}	3.23×10^{-10}	5.6×10^{-4}	67.6
0.001	7.4×10^{-3}	7.4×10^{-9}	3.23×10^{-10}	9.6×10^{-4}	97.4
0.01	2.6×10^{-4}	2.6×10^{-9}	7.15×10^{-10}	7.4×10^{-3}	95.4
0.01	5.0×10^{-4}	5.0×10^{-9}	7.15×10^{-10}	8.6×10^{-3}	97.8
0.01	7.4×10^{-3}	7.4×10^{-8}	7.15×10^{-10}	9.9×10^{-3}	99.8

reaction rates (columns 3 and 4) for a resin activity of 3000 units per 0.5 gram dry resin, which translates to an activity of $8{,}26 \times 10^{-10}$ moles of cellobiose hydrolyzed per second per square centimeter of resin surface. These values are within an order of magnitude of each other, indicating that both film diffusion and enzyme kinetics may limit the overall reaction rate.

When the steady-state equation (eq. 4.10) is solved for the surface concentration, equation 4.11 is obtained:

$$C_s = \left[-\left(\frac{Vm}{k_1} + Km - C_b\right) + \sqrt{\left(\frac{Vm}{k_1} + Km - C_b\right)^2 + 4 \cdot C_b \cdot Km} \right] / 2 \qquad 4.11$$

In Table 4.9, the values for C_s calculated in this manner are tabulated in column 5, and in column 6 are listed the percentages of the maximum activity (found in column 4) that are achieved when the cellobiose concentration is that of the calculated surface concentration. From column 6 diffusion through the external film will limit the rate at conditions of low bulk phase substrate concentration and low mass transport coefficient (low flow rates).

Two experiments were performed in order to verify this influence of substrate diffusion on the reaction

rate in a packed bed reactor. The reactor in both experiments consisted of a jacketed glass column. The resin was held in place in the column with glass wool plugs and water was circulated around the columns to keep temperature constant at 40°C. Substrate solution was pumped through the column with a variable-speed peristaltic pump. The column lengths for Experiments 1 and 2 were 7.2 and 3.85 centimeters, respectively, and both had an inside diameter of 1.0 centimeter.

The results of both experiments are shown in Tables 4.10 and 4.11. The substrate concentration and flow rate, listed in columns 1 and 3, were the independant variables and the exit cellobiose concentration (column 7) was found by determination of the glucose content using the G.O.P. assay procedure described in section 2.4. The residence time, τ, superficial velocity, G, and Reynolds number, Re, were determined from the following equations:

$$\tau = \frac{\pi R^2 L}{Q} \quad , \qquad G = \frac{Q}{\pi R^2} \quad , \qquad Re = \frac{d_p G}{\mu} \qquad 4.12$$

where Q is the volumetric flow rate, ε is the void fraction,

Table 4.10 Results of diffusion experiment #1; column length = 7.2 centimeters

1 Cellobiose inlet concentration, moles/liter	2 Flow rate, ml/min.	3 τ, sec.	4 G, cm/sec.	5 Re	6 Conversion, %	7 Cellobiose outlet concentration, moles/liter	8 Vm, moles/liter-second
0.001	12.6	9.7	0.267	1.07	65.0	0.00035	2.36×10^{-4}
0.0025	12.6	9.7	0.267	1.07	52.8	0.00118	2.57×10^{-4}
0.005	12.6	9.7	0.267	1.07	42.2	0.00289	3.06×10^{-4}
0.01	12.6	9.7	0.267	1.07	26.8	0.00732	3.27×10^{-4}
0.001	9.0	13.6	0.191	0.76	72.0	0.00028	1.99×10^{-4}
0.0025	9.0	13.6	0.191	0.76	60.0	0.00100	2.16×10^{-4}
0.005	9.0	13.6	0.191	0.76	53.6	0.00232	2.86×10^{-4}
0.01	9.0	13.6	0.191	0.76	36.5	0.00635	3.21×10^{-4}
0.001	4.3	28.4	0.090	0.37	73.0	0.00027	0.98×10^{-4}
0.0025	4.3	28.4	0.090	0.37	72.0	0.00070	1.33×10^{-4}
0.005	4.3	28.4	0.090	0.37	65.0	0.00175	1.72×10^{-4}
0.01	4.3	28.4	0.090	0.37	45.1	0.00544	1.94×10^{-4}

Table 4.11 Results of diffusion experiment #2; column length = 3.85 centimeters

1 Cellobiose inlet concentration, moles/liter	2 Flow rate, ml/min.	3 τ, sec.	4 G, cm/sec.	5 Re	6 Conversion, %	7 Cellobiose outlet concentration, moles/liter	8 Vm, moles/liter-second
0.001	4.88	13.3	0.103	0.414	69.9	0.000301	1.94×10^{-4}
0.0025	4.88	13.3	0.103	0.414	63.6	0.000911	2.38×10^{-4}
0.005	4.88	13.3	0.103	0.414	50.9	0.00246	2.75×10^{-4}
0.01	4.88	13.3	0.103	0.414	36.7	0.00633	3.31×10^{-4}
0.001	11.1	5.9	0.236	0.942	45.0	0.00055	2.35×10^{-4}
0.0025	11.1	5.9	0.236	0.942	42.7	0.00143	3.32×10^{-4}
0.005	11.1	5.9	0.236	0.942	31.2	0.00344	3.65×10^{-4}
0.01	11.1	5.9	0.236	0.942	21.6	0.00789	4.21×10^{-4}
0.001	17.6	3.7	0.374	1.494	47.0	0.00063	2.94×10^{-4}
0.0025	17.6	3.7	0.374	1.494	32.8	0.00168	3.88×10^{-4}
0.005	17.6	3.7	0.374	1.494	24.0	0.00383	4.38×10^{-4}
0.01	17.6	3.7	0.374	1.494	13.9	0.00861	4.37×10^{-4}

R is the colum radius, d_p is the resin particle diameter, and μ is the viscosity of water at 40°C.

The design equation for a plug-flow reactor with a reaction rate in the form of Michaelis-Menton kinetics is

$$\tau = -\int_{C_i}^{C_f} \frac{dC}{r} \qquad 4.13$$

where C, C_i, and C_f are the substrate, inlet substrate, and outlet substrate concentrations, r is the reaction rate, Vm is the maximum reaction rate and Km is the Michaelis-Menton constant. When this equation is integrated the following equation is obtained upon re-arrangement:

$$Vm = (Km \cdot \ln(C_f/C_i) + C_f - C_i)/\tau \qquad 4.14$$

For a plug flow reactor in which the reaction rate is not influenced by diffusion the above equation will determine Vm for the resin when the parameters, C_f, C_i, Km and are experimentally determined. When diffusion does limit the reaction rate the column efficiency decreases and the calculated Vm' will be an apparent value of the maximum reaction rate, somewhat lower than the actual Vm.

The Vm' values are tabulated in column 8 in units of moles of cellobiose hydrolysed per second per liter of liquid volume and are plotted against flowrate in Figure 4.13. Two trends are apparent that are explained from a diffusion limitation model: (1) Vm' increased as flowrate increased since an increase in the superficial velocity caused an increase in the mass transport coefficient (see correlations in Table 4.7) and (2) Vm' increased as the inlet substrate concentration increased since this caused an increase in rate of diffusion to the resin surface created by a greater driving force, (C_b-C_s). The dotted lines in Figure 4.13 are the values for the actual Vm for each experiment, determined from a stirred-reactor experiment and translate to 6200 and 7960 units of activity per gram dry resin. As seen in the figure these two lines are approached asymptotically as flowrate and substrate concentration increase.

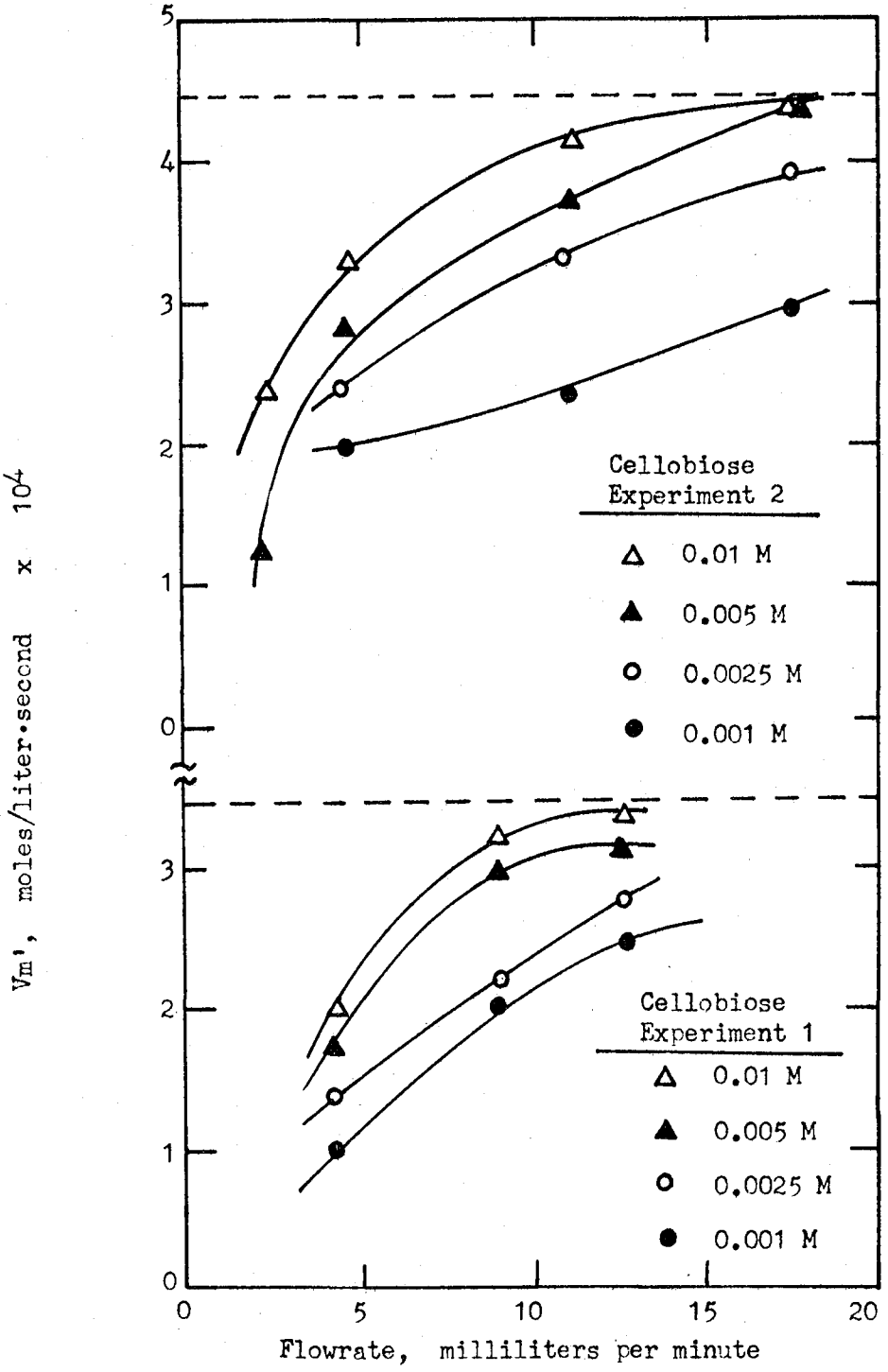

Fig. 4.13 Results of plug-flow reactor studies with the resin-bound enzyme. Data points obtained from Tables 4.10 and 4.11.

XBL 788-9909

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5 Hydrolysis Experiments

5.1 Previous Work

Quite often the rate of reaction catalyzed by an enzyme is inhibited by the accumulation of the products of that reaction. Cellobiose has been found to inhibit the cellulase of many organisms.[1,2,3,4] Castanon and Wilke performed a set of experiments where various amounts of cellobiose were added to batch hydrolyses using newsprint as the substrate and the sugar production was determined at various times.[5] The addition of 3.42 grams per liter cellobiose showed little inhibitory effect but 10 grams per liter addition showed a significant decrease in total sugar production as seen in Figure 5.1.

The inhibition of cellobiose on cellulase activity not only varies with the organism from which the cellulase was obtained, but also varies from batch to batch from the same organism as indicated by work performed by Yamanaka and Wilke.[6] They added glucose and cellobiose to the cellulase-cellulose system in order to determine the inhibitory effect on initial reaction rates. A Lineweaver-

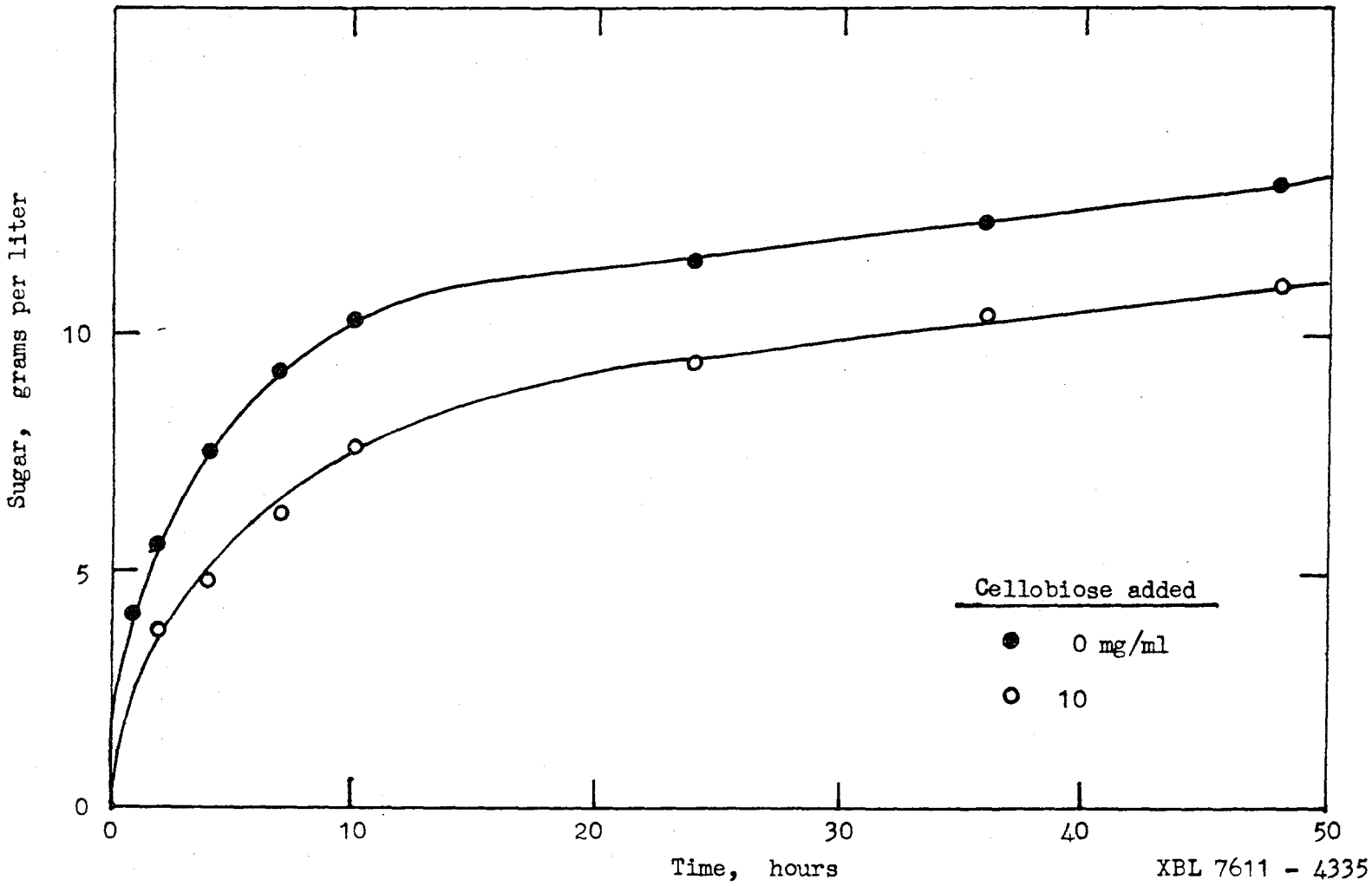

Fig. 5.1 Effect of the addition of cellobiose on the hydrolysis of cellulose. Figure reprinted from Castanon and Wilke.[5]

Burke plot of the results using one batch of cellulase obtained from Trichoderma viride yielded a cellobiose inhibition constant of 4.63 grams per liter. Another batch also obtained from Trichoderma viride showed less cellobiose inhibition and the data suggested an inhibition constant of 10.3 grams per liter. The batch with the lesser cellobiose inhibition had a slightly greater β-glucosidase activity (227 units per milliliter versus 187 units per milliliter) which appears to cause a considerable decrease in the inhibitory effect of cellobiose.

The results of several experiments performed by Yamanaka and Wilke indicate that the conversion of cellobiose to glucose during the course of a batch hydrolysis will enhance the hydrolysis.[6] In one experiment β-glucosidase obtained from almond emulsin was added to the cellulase enzyme obtained from Trichoderma viride used in the hydrolysis of ball-milled Solka Floc. The concentration of both glucose and total sugar was found to increase as the amount of β-glucosidase added was increased. Figure 5.2 shows the effect of the added β-glucosidase on the percent of ball-milled Solka Floc hydrolyzed for

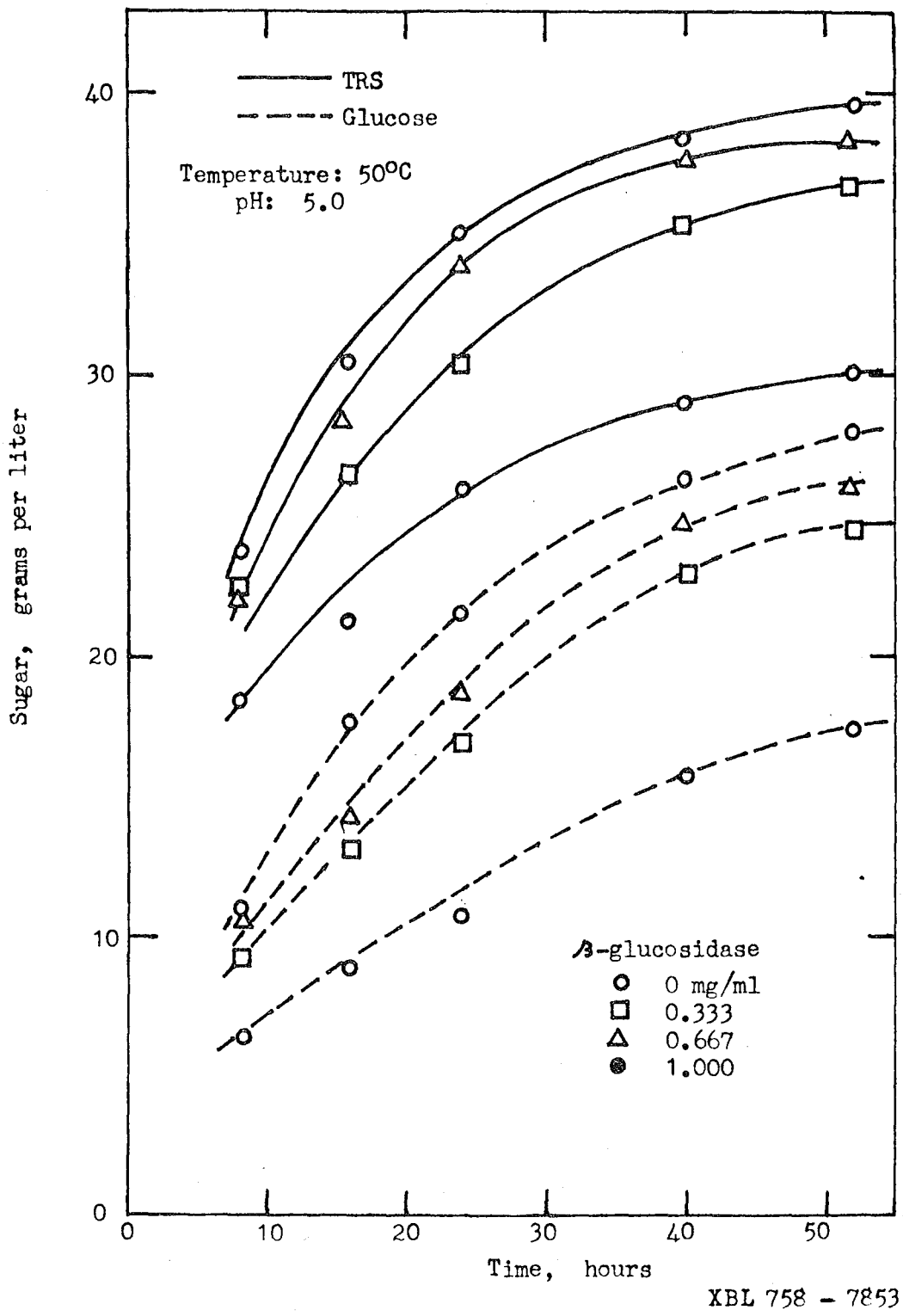

Fig. 5.2 Effect of the addition of almond emulsin β-glucosidase on the hydrolysis of cellulose. Figure reprinted from Yamanaka and Wilke.[6]

this experiment. TRS refers to the amount of total reducing sugar as measured by the DNS reagent (a discussion of TRS and the assay methods are found in the report by Yamanaka and Wilke[6]). An increase in hydrolysis was also achieved with the addition of β-glucosidase obtained from the fungal source, Botryodiplodia theobromae. An increase of total sugar production (measured as glucose) from 30 to 34 grams per liter after 40 hours was obtained with an enzyme mixture of 15% β-glucosidase and 85% cellulase (from Trichoderma viride). However, when this β-glucosidase was used in a batch hydrolysis along with cellulase from Trichoderma viride which contained a higher β-glucosidase component, there was no significant increase in the total sugar production. Similiar results were obtained using newsprint as the cellulosic substrate.

5.2 Batch Hydrolysis Experiments: Apparatus and Procedure

In order to determine the effect of continuously converting cellobiose to glucose during a batch enzymatic hydrolysis using an immobilized β-glucosidase reactor, three experiments were performed. The cellulosic substrate for two of the experiments was Wiley milled and then acid pretreated corn stover and the substrate for the third experiment was Wiley milled Solka Floc. Cellulase enzyme was obtained from Trichoderma viride and β-glucosidase was obtained from Aspergillus phoenicis and was immobilized onto the phenol formaldehyde resin according to procedures described in section 4.2.

The apparatus for each experiment consisted of two of the systems depicted in Figure 5.3 operated in tandem. Each system was operated under identical conditions except that glass beads were used in place of the resin-bound β-glucosidase in one of the columns. In this manner any effect of the immobilized enzyme reactor on the batch hydrolysis of the substrate can be determined by direct comparison to the control system. The enzymatic hydrolysis of the substrate took place in a 350 milliliter fine-pore

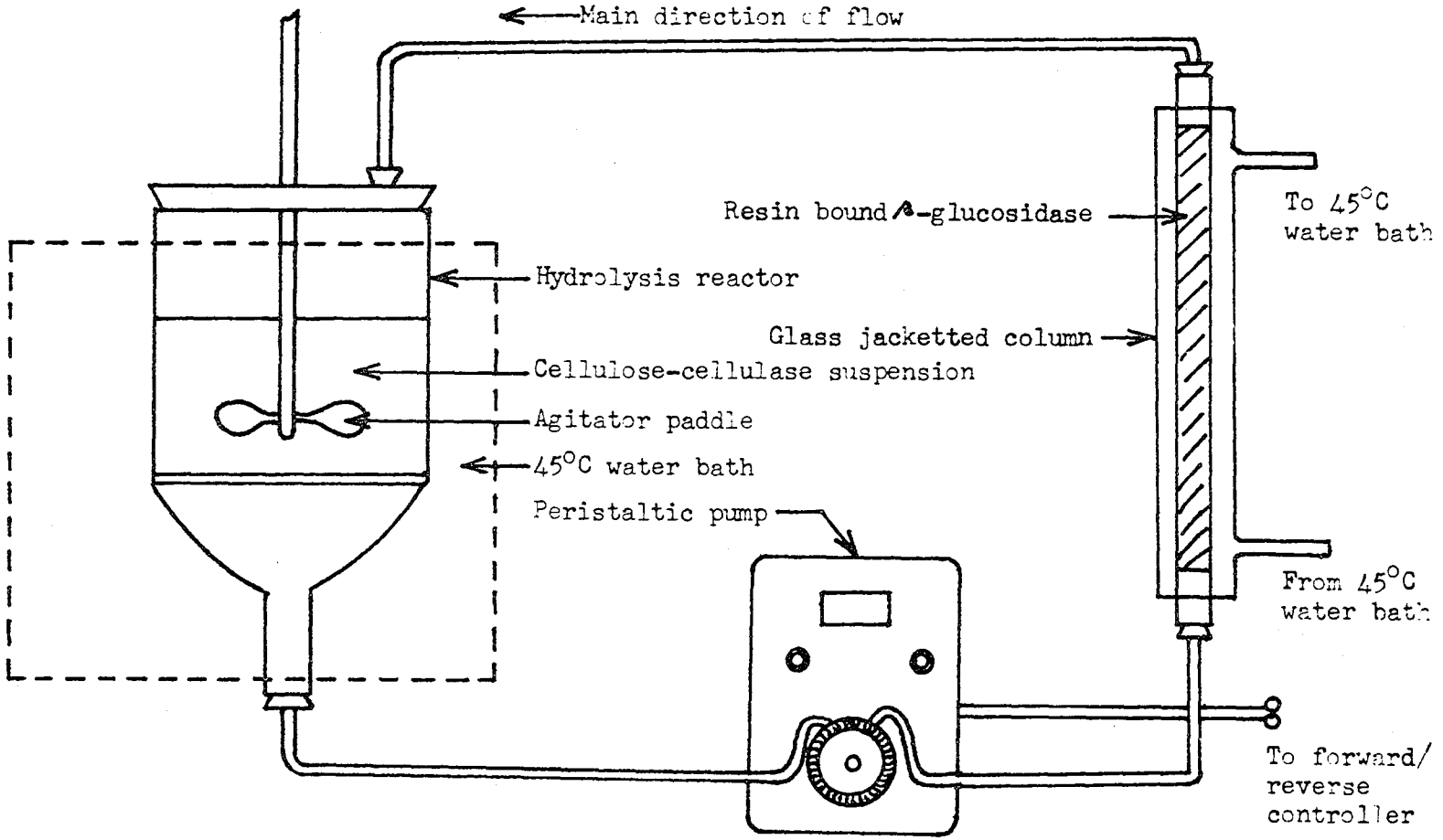

Fig. 5.3 Experimental apparatus for the batch hydrolysis of cellulose using a recycle through an immobilized enzyme plug-flow reactor.

XBL 788-9910

sinterred glass filter which was immersed in a water bath held at 45°C. The immobilized enzyme and inert glass reactors consisted of 1.0 centimeter in diameter jacketed glass columns. The water bath water was circulated around the columns to maintain a 45°C temperature. The resin and glass beads were held in place with glass wool plugs. The cellulase enzyme-sugar solution from the hydrolysis reactor (sinterred glass filter) was circulated through the recycle system by means of a peristaltic pump, with the direction of flow being down through the filter and up through the column. The peristaltic pumps were modified to flow in reverse direction at full speed intermittantly to back flush the sinterred glass filters free of trapped cellulosic particles. In this manner a continuous separation and cycling of the hydrolysis liquor was achieved for periods of 20 to 30 hours. When complete plugging of the filters occurred the system was temporarily stopped and the filters were changed.

Recent studies have shown that acid pretreatment of corn stover increases the enzymatic hydrolysis yield,[7] so this pretreatment was performed on the corn stover uti-

lized in this study. 87 grams of corn stover (containing 6.5% by weight water) was combined with 1 liter of 0.09 M H_2SO_4. After boiling with stirring for 5.5 hours this mixture was cooled and washed on a double layer of Whatman #42 filter paper to remove acid and solubilized sugars. The corn stover was then air dried and a portion was oven dried for determination of percent water content.

At various times during each experiment samples were taken mainly from the hydrolysis section with an occasional sample taken before and after the β-glucosidase column. Approximately 5 milliliters of sample were collected, boiled for five minutes to denature cellulase enzyme, cooled, and centrifuged. The sugar content of the supernatant liquor was determined using the G.L.C. procedure described in section 2.6. The solids removed with the samples taken from the hydrolysis section were returned after the centrifugation step.

All three experiments were performed at pH 5.0 by the addition of acetic acid and sodium acetate such that the final acetate buffer concentration was 0.05 M. The

recycle flowrate for the three experiments was 4 to 7 milliliters per minute in the forward direction (the flowrate decreased as the filters plugged) and with the intermittant backflush, the average forward flowrate was 2 to 5 milliliters per minute.

5.3 Hydrolysis Experiment Results

The cellulase enzyme used for Experiment 1 had an FPA of 2.5 and the resin-bound β-glucosidase had an activity of 5800 units per gram of dry resin. The column length of the β-glucosidase reactor was 26.3 centimeters. The total initial liquid volume in the system was 270 milliliters of cellulase, of which 150 milliliters was in the hydrolysis section. The remainder of the cellulase resided in the column, tubings, and void space under the sinterred glass filter. The hydrolysis section was initially charged with 19.4 grams of the 73.2% dry pretreated corn stover in order to achieve an overall substrate suspension of 5%. This gave an 8.7% suspension of the solids in the hydrolysis section.

Figure 5.4 shows the production of glucose and cello-

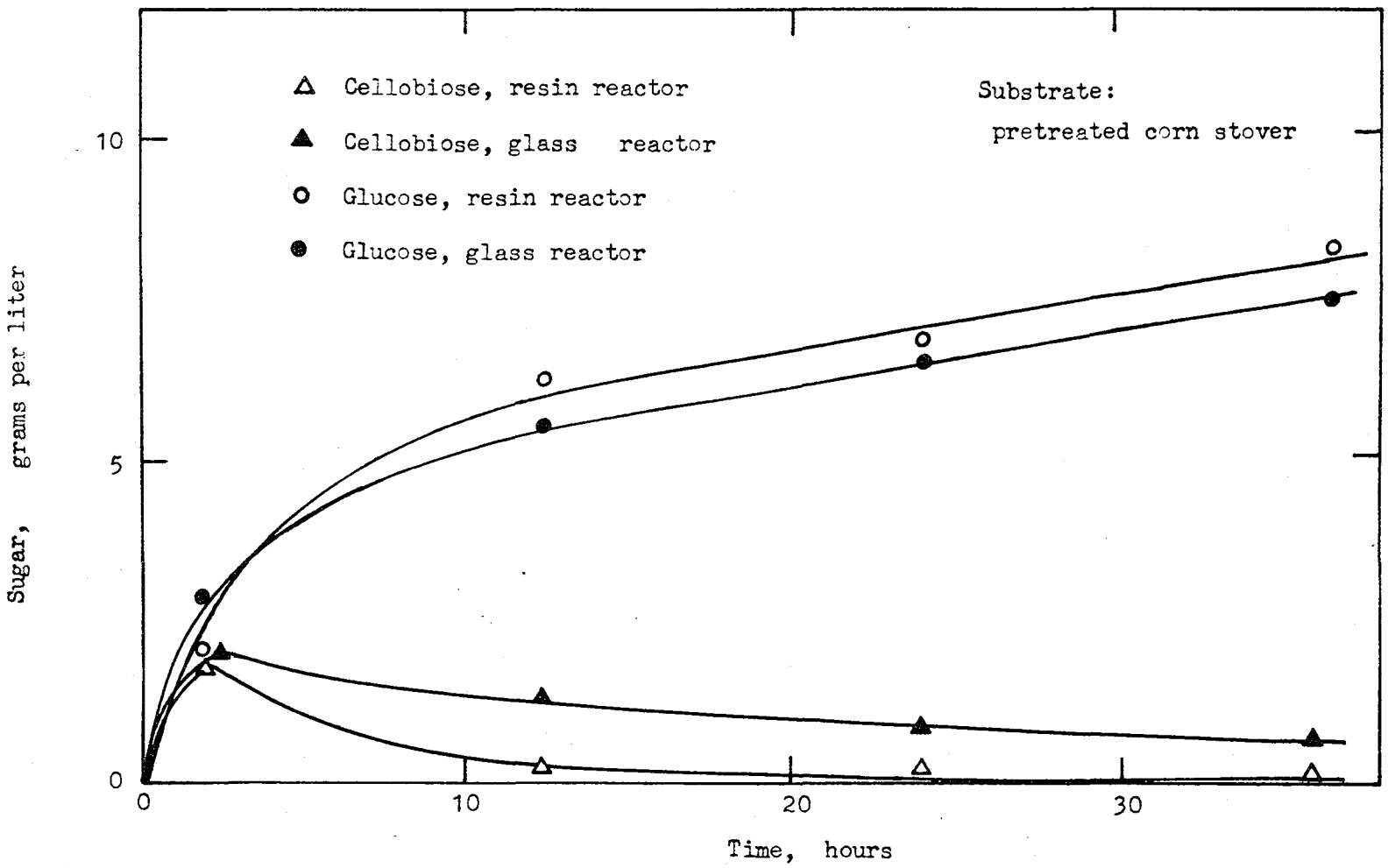

Fig. 5.4 Results of Hydrolysis Experiment 1, using acid pretreated corn stover as the cellulosic substrate.

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biose versus time for Experiment 1. Initially, the rate of production of glucose in both systems is large, and this rate tapers off to almost a constant level at long times. After 36 hours the glucose level in the immobilized enzyme system was 8.3 grams per liter while the level in the control system was 7.5 grams per liter. The level of cellobiose in both systems exhibit an initial rapid increase followed by a steady decrease after two hours of reaction time. This behavior is explained by the fact that the circulation rate of the hydrolysis liquor is slow compared to the initial rate of formation of cellobiose and the cellobiose concentration becomes more equally distributed throughout the entire system after two or three hours of operation. As expected, the cellobiose level in the immobilized enzyme system dropped to a level of less than 0.1 gram per liter while the level in the control system was 0.7 gram per liter after 36 hours. A constant conversion of cellobiose to glucose was expected in both systems due to a small β-glucosidase component in the cellulase enzyme, but it is apparent that the presence of the recycle through the β-glucosidase reactor caused almost

a complete conversion of the cellobiose after ten to twelve hours. Figure 5.5 shows the total sugar production during the batch hydrolysis measured as grams per liter of glucose. These two curves are within experimental error of each other and indicate no increase in the overall hydrolysis of the corn stover due to the constant conversion of cellobiose to glucose.

The cellulase enzyme used for Experiment 2 was stronger, with an FPA of 3.5, such that greater effects due to the removal of cellobiose may be seen over a 40 hour reaction time. The column length for this experiment was 28.0 centimeters and the resin-bound β-glucosidase had an activity of 4800 units per gram of dry resin. Some of the void volume in the lower portion of the sinterred glass filter was removed by filling the space with glass beads. The overall volume was 235 milliliters of cellulase, of which 180 milliliters was in the hydrolysis reactor. 16.9 grams of corn stover was added to the reactor to obtain an overall suspension of 5% (6.4% suspension in the hydrolysis reactor). The decrease in void volume by the addition of the glass beads served to de-

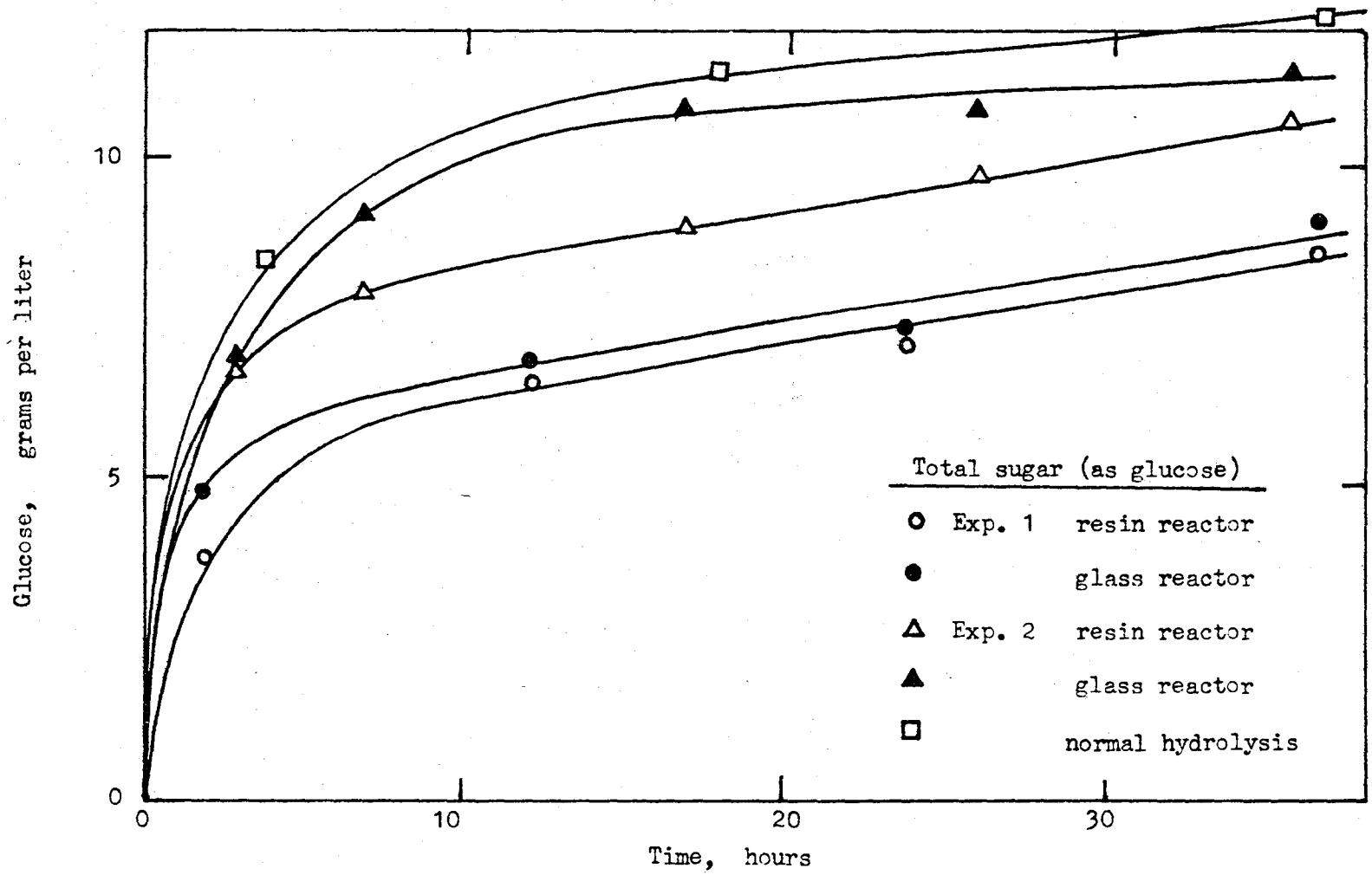

Fig. 5.5 Total sugar production measured as glucose for Hydrolysis Experiments 1 and 2.

XBL 788-9912

crease the time needed to circulate a reactor volume through the immobilized enzyme column and also to bring the substrate concentration in the hydrolysis reactor closer to the overall concentration. In addition to the immobilized enzyme and control systems, 60 milliliters of cellulase and 4.3 grams of corn stover was placed in a 250 milliliter hydrolysis vessel which was placed in a 45°C water bath. In this manner a normal batch hydrolysis (no recycle) was performed with a 5% suspension of cellulosic solids as a means of comparison of the recycle system to normal methods of carrying out a batch hydrolysis.

The results of Experiment 2 are shown in Figure 5.6. Once again the initial rate of glucose formation is rapid followed by a tapering off to about a constant production level at long times. After 36 hours of operation the glucose levels for the immobilized-enzyme, inert glass, and normal systems were 10.1, 10.5, and 11.2 grams per liter, respectively. The cellobiose levels of the glass and normal systems rapidly increased to 3.1 and 3.5 grams per liter, respectively, followed by a slow decrease to

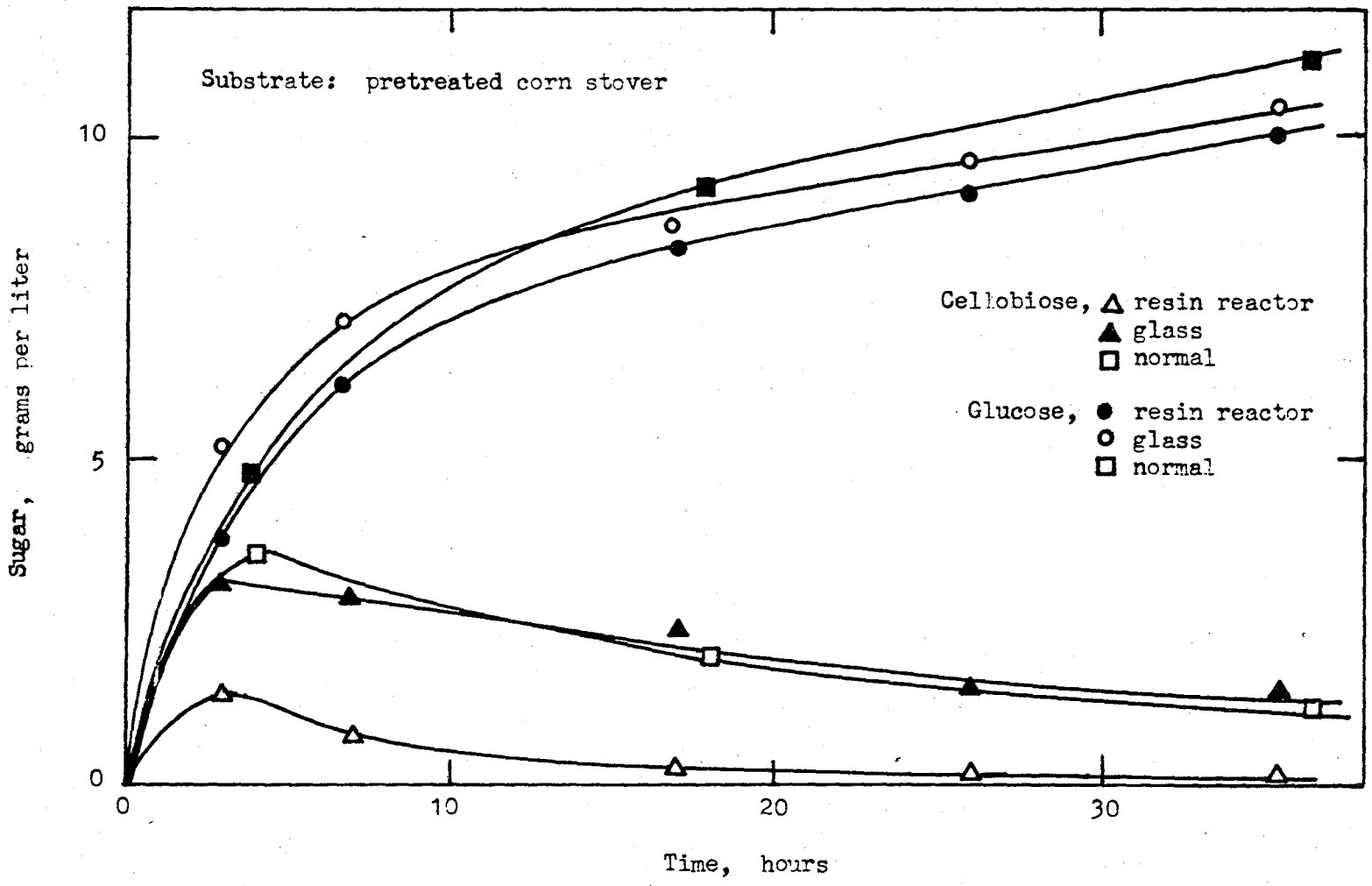

Fig. 5.6 Results of Hydrolysis Experiment 2.

XBL 788-9913

1.5 and 1.3 grams per liter after 36 hours. The cellobiose concentration in the immobilized enzyme system reached a maximum of 1.3 grams per liter and decreased to less than 0.1 gram per liter after 36 hours. The total sugar production as glucose versus time was also plotted in Figure 5.5. The total glucose equivalent produced with the normal hydrolysis after 36 hours was 12.3 grams per liter, while the total glucose production in the inert glass control system was not far behind at 11.4 grams per liter. The total glucose equivalent produced with the β-glucosidase system was only 10.6 grams per liter after 36 hours, and was significantly lower than the control at earlier times.

Experiment 3 was performed using Wiley milled Solka Floc as substrate rather than the pretreated corn stover. Solka Floc was known to produce higher levels of cellobiose during enzymatic hydrolysis than corn stover and therefore any effect of cellobiose removal would be enhanced. The cellulase enzyme used for this experiment had an FPA of 3.5. Resin-bound β-glucosidase was packed into a column with a length of 27.5 centimeters and had

an activity of 4100 units per gram of dry resin. 14.4 grams of 94.8% dry Solka Floc was charged to the hydrolysis section which contained 180 milliliters of the cellulase enzyme. Total liquid volume for the system was 260 milliliters, and the Solka Floc suspension in the hydrolysis section and in the overall system was 7% and 5%, respectively. As in Experiment 2, a normal hydrolysis (no recycle) was performed as a comparison, using 5.6 grams of Solka Floc and 100 milliliters of the cellulase enzyme in a 250 milliliter hydrolysis vessel placed in a 45°C water bath.

The concentrations of cellobiose produced in the normal hydrolysis and inert glass control system after 36 hours were much higher than that produced with corn stover and were about twice the concentrations of glucose produced after 36 hours (Figure 5.7). The concentration of cellobiose in the immobilized enzyme system showed the same trend as in the previous experiments; an initial increase in the first few hours (to a maximum level of 1.9 grams per liter) followed by a decrease to a very small level (0.3 gram per liter after 36 hours). There was significantly

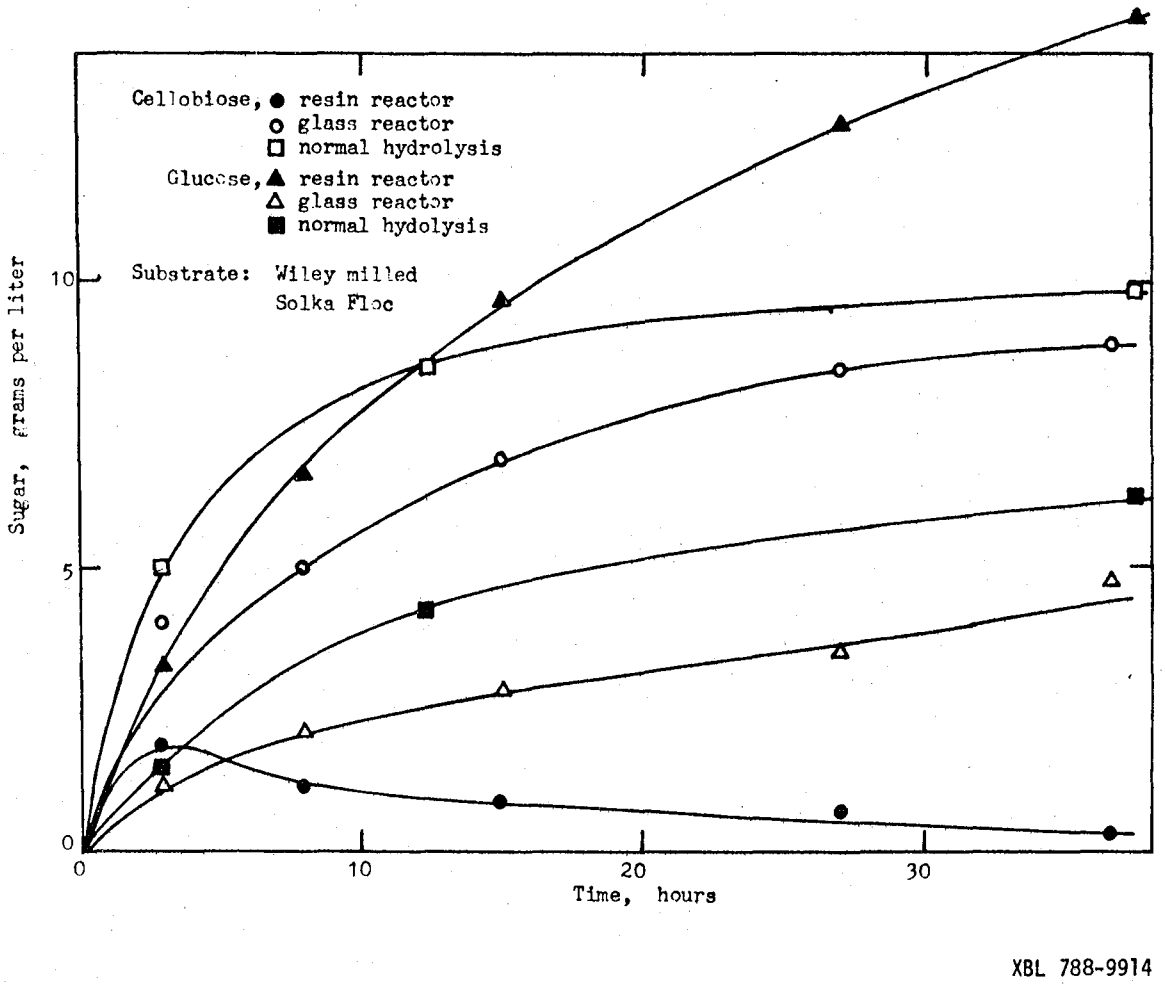

Fig. 5.7 Results of Hydrolysis Experiment 3. Wiley milled Solka Floc was the cellulosic substrate.

more glucose present in the immobilized enzyme system, which was as high as 14.5 grams per liter after 37 hours. The total glucose equivalent for all three systems is plotted versus time in Figure 5.8. The sugar production in the normal hydrolysis is again slightly greater than either the recycle systems. The interesting observation is that although there was a great difference in the concentration of cellobiose in both recycle systems, there was only about a 1 gram per liter increase in the total glucose equivalent in the immobilized enzyme system.

No noticeable increase in the total production of sugars was observed using the β-glucosidase recycle reactor during the batch hydrolysis of pretreated corn stover in Experiments 1 and 2. However, the maximum concentration of cellobiose produced in the first two experiments at the described conditions was only about 3.6 grams per liter (normal hydrolysis, Experiment 2, after 4 hours). Since Castanon and Wilke reported no significant inhibitory effects on cellulose hydrolysis with a cellobiose background of 3.42 grams per liter, it is conceivable that the corn stover hydrolysis does not produce a high enough

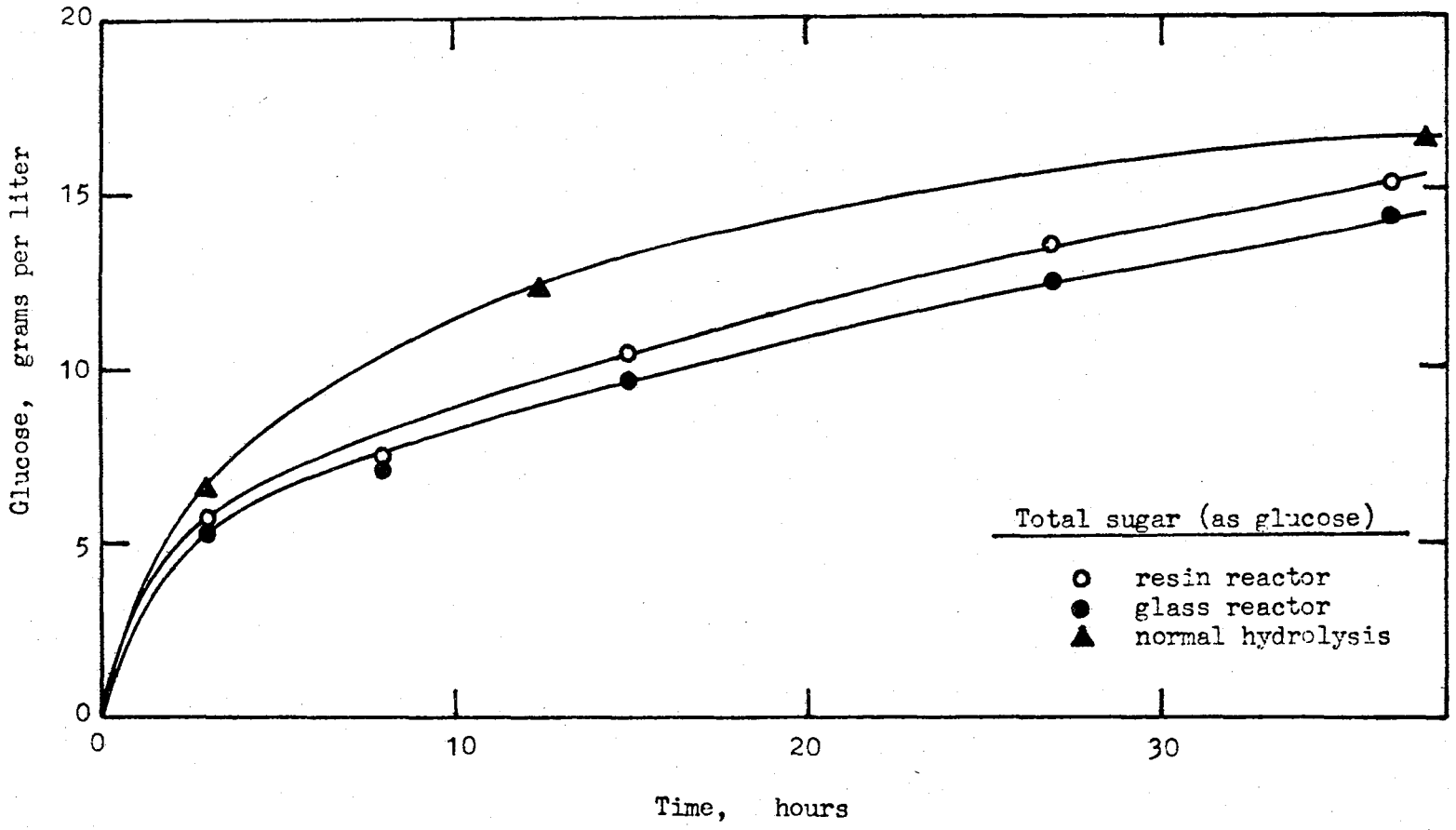

Fig. 5.8 Total sugar production measured as glucose for Hydrolysis Experiment 3.

XBL 788-9915

level of cellobiose for the inhibition of cellobiose to become a limiting factor. When the cellobiose background was as high as 8.8 grams per liter as with the Solka Floc hydrolysis in Experiment 3, only a slight increase in the total sugar production was observed with use of the β-glucosidase reactor recycle. This may be due to the particular cellulase used for this experiment having little inhibitory effect by cellobiose (high cellobiose inhibition constant). The latest design on the overall hydrolysis process indicates a cellobiose concentration in the hydrolysis reactors of 1.58 grams per liter using pretreated corn stover as a substrate. Therefore, from the results of the three experiments described in this section and from previous studies, it appears not to be feasible to incorporate an immobilized enzyme recycle system to enhance the hydrolysis.

In all of the three experiments, however, the use of the β-glucosidase reactor enabled the sugars in the hydrolyzate to be mainly glucose, with very little sugar present in the form of cellobiose. Since the glucose to ethanol fermentation cannot utilize the cellobiose, it

may be worthwhile to position an immobilized β-glucosidase reactor between the cellulose hydrolysis and ethanol fermentation sections of the overall process in order to "purify" the hydrolyzate stream and increase the amount of glucose being fed into the fermentation system. The economics of this approach will be examined in the next section of this study.

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6 Design Cost and Analysis

From the conclusions of the previous section, the most feasible use of an immobilized β-glucosidase reactor in the cellulose hydrolysis process appears to be as a "sugar purification" unit (conversion of cellobiose to glucose) and not as a means of enhancing the cellulose degradation. The next sections will present an estimate of the decrease in the cost of ethanol per gallon (by causing a decrease in the cost of glucose per pound) by addition of such a unit to the process proposed by Wilke et. al.[1] At the same time the design of the immobilized reactor will be determined that produces the glucose at the lowest cost.

6.1 General Design Basis

Referring to Figure 1.1, the diagram of the current process design, the optimal location of the immobilized β-glucosidase reactor would be after the multieffect evaporator sugar concentration step and before the sterilizer for the ethanol fermentation section. The flowrate to the immobilized enzyme reactor based on a stream 10% in glucose

assuming 100% conversion of the cellobiose to glucose, would be 21.78 liters per second or 2052 tons per day. 14 tons per day of cellobiose would be fed to the reactor with a concentration of 0.019 M.

The current annual manufacturing cost of the glucose in the process is 14.1×10^6 dollars to produce 1.412×10^8 pounds per year of glucose, which gives a cost of glucose of 9.98 cents per pound. Letting R equal the additional annual manufacturing cost of glucose associated with the addition of the immobilized enzyme column, the cost per pound of glucose is given by the following equation:

$$\text{¢/lb.} = \frac{100 \times (14.1 \times 10^6 + R)}{\left[214 + \left(\frac{\%\ \text{conversion}}{100}\right)(14)(1.053)\right](330)(2000)} \qquad 6.1$$

The numerator is equal to the total annual manufacturing cost and the denominator is equal to the total yearly production of glucose in pounds. % conversion refers to the percentage of the 0.019 M inlet cellobiose concentration converted to glucose and the factor 1.053 is a weight conversion factor to convert cellobiose to glucose. The minimum cost that can be attained for glucose, assuming

zero cost due to the addition of the enzyme reactor (R = 0) and 100 % conversion, is 9.33 cents per pound, for a savings of 0.65 cent per pound. Since 13.59 pounds of glucose is required for the production of each gallon of ethanol, this yields a savings of 8.83 cents per gallon of ethanol. The added cost to the process due to the addition of the immobilized enzyme reactor is due to the manufacturing and installation cost of the reactor, cost of the resin, enzyme and glutaraldehyde, and cost due to labor for the regeneration of the reactor.

From Cysewski and Wilke[2] the cost of a column shell is given by

$$\text{Cost} = \text{MSI} \times 0.000953 \times L^{.812} \times D^{.879} \qquad 6.2$$

where L and D are the column length and diameter measured in centimeters. MSI is the Marshal and Steven Index used to adjust a base price to the current year inflation level. From the same source the cost of a pump is given by

$$\text{Cost} = \text{MSI} \times (2.64 + .0068 \times \text{Size}^{.718}) \qquad 6.3$$

where Size is the flow rate in gallons per minute multiplied by the pressure drop in pounds per square inch (psi). An MSI of 518 corresponding to the fourth quarter of 1977 was chosen to remain consistant with the cost estimation presented by Wilke et. al.[1] in the current process. A Lange multiplier of 3.1 was used to obtain the total manufacture and installation cost of the pump and column shell. Annual cost due to the pump and column shell was determined by multiplication by 0.24. The Lange multiplier and cost factor was estimated as described in Wilke et. al.[1] to remain consistant with the economics of the current process, and a breakdown of these factors is shown in Tables 6.1 and 6.2.

The cost of the resin has been estimated to be 2.83 dollars per liter of column volume from an estimate obtained from the Diamond Shamrock Chemical Company. On the assumption that 20 man hours of labor at 5.60 dollars per hour is required to regenerate a column with a diameter of 1 meter and a length of 2 meters (which includes immobilization of the enzyme, quality control and column repacking) the added cost due to the labor was estimated

Table 6.1 Lange multiplier for estimation of fixed capital investment from equipment cost

Direct costs (D)	Factor
Equipment cost	1.0
Equipment installation	0.5
Piping	0.4
Instrumentation	0.3
Insulation	0.05
Electrical	0.2
Building/facilities	0.4
Land/yard improvement	0.1
Total	2.95
Indirect cost (I)	
Engineering and Construction	0.25D
Contractor's fee and contingency	0.15(D + 0.25D)
Lange multiplier	(1.15)(1.25)(2.15) = 3.09

Table 6.2 Capital related cost factors
(Annual cost = factor x fixed capital)

Item	Cost factor
Depreciation	0.10
Interest	0.06
Maintenance	0.06
Insurance	0.01
Plant supplies	0.01
Taxes	0
Total	0.24

at 0.07 dollars per liter of column volume. In this cost estimation it has been assumed that fresh resin is utilized each time the column is regenerated. Additional studies may indicate the ability to clean and reuse the resin which would decrease regeneration costs considerably.

A cost of 3.28 cents per gallon of β-glucosidase from Aspergillus phoenicis has been estimated.[3] From the sample #2 of optimization experiment #2 described in section 4.2, 16 milliliters of enzyme would be required to saturate 1 gram of resin, which would add a cost of 0.00866 dollars per liter of column volume. From the same experiment, 1.6 milliliters of 25% glutaraldehyde was used per gram of resin. At a cost of 515 dollars per 55 gallon drum (cost estimate obtained from Eastman Kodak) this would add a cost of 2.70 dollars per liter of column volume. The glutaraldehyde is used in the immobilization procedure in excess and this cost my decrease with studies to optimize the use of the glutaraldehyde in an industrial scale immobilization process.

From kinetic and diffusional data, for a given column diameter, length, inlet cellobiose concentration and flow

rate, temperature, and resin activity, the percent conversion in the column can be calculated, and with the reactor cost estimations the price per pound of glucose can be determined. 40°C has been chosen as the operation temperature due to the greater stability of the enzyme as described in section 4.3.4.

In the optimization experiments of section 4.2, several of the samples have exceeded a resin activity loading of 7000 units per gram of dry resin and this level approximately corresponds to the level bound to sample #2 of optimization experiment #2 which was used for the estimate on enzyme requirement. Therefore this level of activity loading has been used in the following design and costing program. From Figure 4.6, the activity versus pH profile for this enzyme, this activity translates to 4880 units per gram of dry resin at pH 5.0, the operational pH of the process.

The effect of one additional parameter has been studied in this cost estimation, that of the arrangement of multiple immobilized enzyme columns. Due to the constant rate of denaturation of the resin activity, and

the necessity to recharge the resin during the course of operation, it may be desirable to arrange multiple columns either in series, parallel, or a combination of series and parallel. Additional columns would add a larger capital cost due to the cost of the pumps and the column shells, but by recharging only one column at a time a lesser volume of resin would be required for each recharge and there would be a more uniform output of glucose concentration over the course of time.

In the next section will be described the computer program used to determine the cost per pound of glucose for a given design.

6.2 Costing Program Description

A listing of the program utilized to cost the price per pound of glucose is shown in Figure 6.1. The computer system used was the EDIT subsystem of the interactive SESAME system of the Lawrence Berkeley Laboratory computer system. A mild form of Fortran programming language is recognized by this system, hence the absence of any formatting and the presence of statement numbers.

```
1.  DIMENSION C(10),VR(15)
2.  3 READ,D,L,VV0,COLN,SCC,DAYS
3.  VR(1)=21777/(3.1416*COLN*((D/2)**2))
4.  VR(2)=2.0*.36/VR(1)
5.  VR(4)=0.02612*VR(1)/0.006529
6.  VR(3)=VR(1)+1.625*SQRT(1/VR(4))+0.0113299
7.  VR(5)=(SCC*COLN)+1
8.  VR(14)=0.0
9.  DO 2 I = 1,COLN
10.  CS=.00001975
11.  DO 4 J=1,SCC
12.  VV=VV0*EXP(-.0042*DAYS*(COLN*(J-1)+I))
13.  LL=0.0
14.  VR(6)=VV/VR(3)+.00000156
15.  30 IF(LL.GT.1.) GO TO 40
16.  CS=(SQRT(((VR(6)-CS)**2)+(4*CR*.00000156))-(VR(6)-CS))/2.
17.  CR=(1.-VR(2)*VV*405/(1+1.56E-6/CS+CS/3.7E-5)
18.  LL=LL+2.0
19.  GO TO 30
20.  40 VR(2)=100*(1.-CR/.00001975)
21.  2 CONTINUE
22.  4 VR(14)=VR(14)+CR
23.  CR=VR(14)/COLN
24.  VR(11)=.0112*L*(VR(1)**2)*EXP(2.3)3*(2-ALOG10(VR(4)))
25.  VR(8)=100*(1.-CR/.00001975)
26.  VR(7)=((L/2)**2)*3.1416*L/1000.
27.  C(1)=VR(5)*365*.000253*EXP(.812*ALOG(L))*EXP(.979*ALOG(D))
28.  C(3)=(330/DAYS)*VR(7)*5.60866
29.  C(6)=365*VR(5)*(2.64+6.8E-3*EXP(.718*ALOG((544/COLN)*VR(11))))
30.  C(7)=C(6)+C(1)+C(3)
31.  C(9)=(14000000+C(7))*.05/(330*(214+((VR(6)/100)*14.736)))
32.  PRINT,VR(4),VR(9),C(1),C(3),C(6),C(7)
33.  PRINT,C(9),VR(11)
34.  READ,VR(10)
35.  IF(VR(10).GT.0) GO TO 3
36.  STOP
```

XBL 788-9888

Fig. 6.1 Listing of program used to cost the optimal immobilized-enzyme reactor design.

Due to limitations of the system, the number of program lines must be kept to below 40 to 50 and the variable names must be kept low in number. Therefore the program is not easilly understandable and many of the variables have been put into array form.

The program is divided into two parts. The first part inputs the parameters of diameter, length, days between column regenerations, resin activity and column arrangement and determines the overall conversion. Because of the possibility of film diffusional limitations this conversion could not be calculated analytically and the program simulates the plug flow reactor as a series of small, well mixed reactors. The second portion of the program calculates the costs due to the introduction of the immobilized enzyme reactor and calculates the cost per pound of glucose.

A list of the program variables and what they stand for is given in Table 6.3. The trial parameters are inputted in line 2 and lines 3 through 8 set other operational parameters. In line 3 the superficial velocity, G, is set equal to the inlet flowrate divided by

Table 6.3 Definitions of costing program variables

Symbol	Parameter	units
D	column diameter	centimeters
L	column length	centimeters
VMO	maximum initial resin activity	moles/cm^2·sec
COLN	# of columns operated in parallel	columns
SCC	# of columns operated in series	columns
DAYS	# days between column regeneration	days
VR(1)	superficial velocity, G	cm/second
VR(2)	step volume residence time	seconds
VR(3)	mass transport coefficient, k_1	cm/second
VR(4)	Reynolds number, Re	dimensionless
VR(5)	total number of columns	columns
VR(14)	calculation parameter	moles/cm^3
CB	bulk phase cellobiose concentration	moles/cm^3
CS	surface cellobiose concentration	moles/cm^3
VM	maximum resin activity	moles/cm^2·sec
LL	calculation parameter	centimeters
VR(8)	% conversion of cellobiose	percent
VR(11)	pressure drop across each column	psi
VR(7)	volume of each column	liters
C(1)	column shell cost	dollars/year
C(3)	resin, enzyme, gluteraldehyde and labor cost	dollars/year
C(6)	pump cost	dollars/year
C(7)	total cost due to enzyme reactor	dollars/year
C(8)	cost per pound of glucose	cents/pound

the cross sectional area per column and divided by the number of columns operated in parallel. Line 4 sets the step size for the simulation of the plug flow reactor by a series of well mixed reactors. In line 5 the Reynolds number, Re, is determined using a particle diameter of 0.02612 centimeter and the viscosity of water at 40°C. The mass transport coefficient, k_l, is determined in line 6 using the McCune-Wilhelm model, again using liquid parameters at 40°C. Line 7 sets the total number of columns and pumps that must be costed later in the program.

Lines 9 through 22 perform the column simulation. In line 10, the inlet concentration of cellobiose is reset to 0.00019 moles per milliliter for each parallel bank of columns. Line 12 determines the maximum resin activity, Vm, for each column using the equation

$$Vm = Vm_o \times e^{-k_d t} \qquad 6.4$$

where the denaturation rate constant, k_d, is taken at 40°C to be 0.0048 $days^{-1}$ (from section 4.3.4). The time, t, is set such that the activity of a column is equal to

that activity that would be remaining at the end of a regeneration interval. For example, if a column is to be regenerated after DAYS days, the first column would have an activity equal to that remaining after (1 x DAYS) days, the second an activity equal to that remaining after (2 x DAYS) days, etc. The columns with the higher activities are always positioned first in each parallel bank. At high inlet concentrations of 0.019 M where some substrate inhibition does occur, it would be desirable to to have the greatest amount of film diffusional limitations and the diffusional limitations will be greater in those columns with the greater activity. Line 17 determines the decrease in the cellobiose concentration due to reaction in a small well mixed reactor. The equation for this line is

$$C_b = C_b - VR(2) \times \frac{Vm'}{(1 + Km/C_s + C_s/Ks)} \quad 6.5$$

where C_b and C_s are the bulk and surface cellobiose concentrations. Km is the Michaelis Menton constant equal to 1.56×10^{-6} moles per milliliter, Ks is the substrate

inhibition constant, approximated in section 4.3.2 as 3.7×10^{-6} moles per milliliter and Vm' is the maximum reaction rate in units of moles per cubic centimeter per second. The factor 408 is used to convert the input value of Vm in units of moles per square centimeter of surface area per second to units of moles per cubic centimeter of void volumer per second, estimating the resin surface area as that of a sphere. VR(2), set in line 4, is the amount of time spent in the reactor volume. C_s is the actual concentration at the reacting resin surface and is calculated from the equation

$$C_s = \left[\left(\frac{Vm}{k_1} + Km - C_b\right) + \left(\left(\frac{Vm}{k_1} + Km - Cb\right)^2 + 4Km.C_b\right)^{0.5}\right]/2 \qquad 6.6$$

as described in section 4.4.2. VR(6), set in line 14, is used to simplify the calculations.

Line 24 calculates the pressure drop across a column from an equation derived from Perry's Handbook.[4] Using a shape factor of 0.9 since the resin particles are not completely spherical and using the physical constants for a temperature of 40°C, this equation reduces to

$$\Delta P = 0.0119 \times L \times G^2 \times 10^{(2-\log Re)} \qquad 6.7$$

where the pressure drop is in units of psi. This equation is accurate for Reynolds numbers less than 10. The length, L, and superficial velocity, G, are in units of centimeters and centimeters per second, respectively. The volume for each column in liters is determined in line 26. The overall conversion of cellobiose is calculated in line 25.

The remainder of the program determines the various costs as described in section 6.1. Line 27 determines the annual manufacture and installation cost of the column shells. Line 29 does the same for the pumps. The cost of the resin, glut raldehyde and enzyme is calculated in line 28. The factor (330/DAYS) is equal to the number of times a column is regenerated per year and the factor 5.60866 is the cost per liter of column volume of the resin, glutaraldehyde, enzyme and labor. The cost per pound of glucose is calculated in line 31.

6.3 Design Results

The inlet flowrate and cellobiose concentration was fixed by the current process and the operation temperature and resin activity was set to 40°C and 4880 units per gram of dry resin at pH 5.0 as discussed earlier. Design parameters to be optimized were the column diameter and length, number of columns in series and in parallel and the number of days between column regenerations. Preliminary computer trials indicated that regardless of column configuration, the glucose cost was lowered as the column pressure drop was increased, in optimizing the various parameters. Therefore, the length was chosen for each diameter such that the pressure drop was 100 psi across each column, which would be a reasonable value for industrial operation since many column shells are rated to 120 psi. Figure 6.2 shows the plot of length versus diameter for 2 and 3 columns in parallel that was used to determine the length for the computer trials. The lines are approximately linear, as expected from the equation for the pressure drop discussed in section 6.2.

The following column arrangements were optimized for

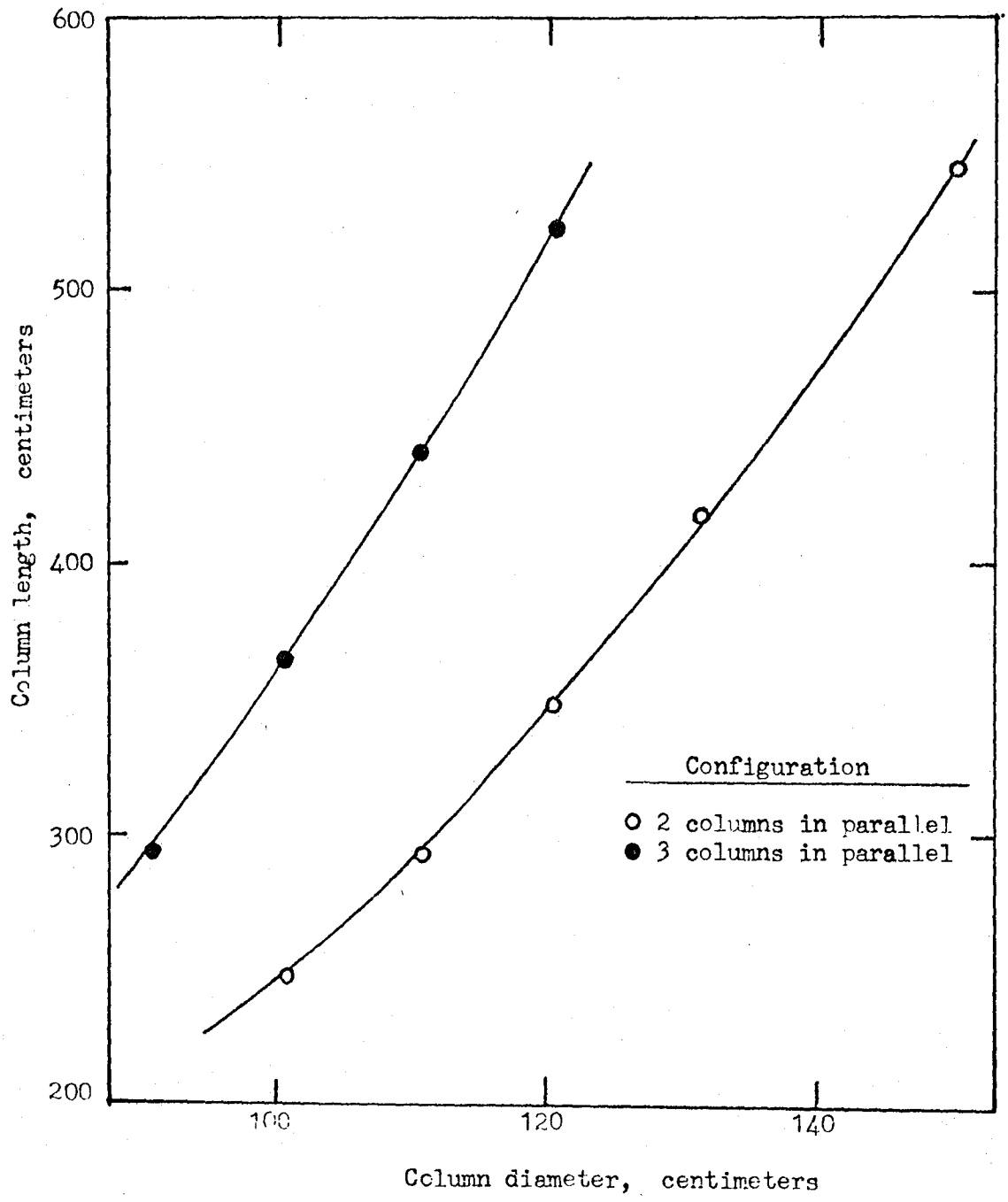

Fig. 6.2 Plot of the length required for each diameter to achieve a pressure drop of 100 psi across a fixed-bed reactor produced with the phenol-formaldehyde resin.

XBL 788-9916

the parameters of diameter and regeneration interval: 2 x 2, 2 x 3 and 3 x 2 (in this report, the first number always refers to the number of columns in parallel). Preliminary studies have shown that arrangements with greater than six columns did not yield a lower glucose price than those with six or less columns. At least 2 columns were required in parallel to maintain a realistic length to diameter ratio with a reasonable pressure drop.

For each column arrangement the regeneration time was varied for various diameters (and respective lengths). The results are plotted in Figures 6.3 through 6.5 as price of glucose versus regeneration time. The minimum prices of glucose for each diameter from these plots are plotted versus diameter in Figure 6.6. From this figure the lowest cost of glucose of 9.453 cents per pound is obtained with a 2 x 3 column arrangement each having a diameter of 1.2 meters and length of 3.5 meters. From Figure 6.4, the regeneration interval for these conditions is 65 days. This design would produce a savings of 0.53 cents per pound of glucose or 7.2 cents per gallon of ethanol over that of the current process. In Figure 6.7,

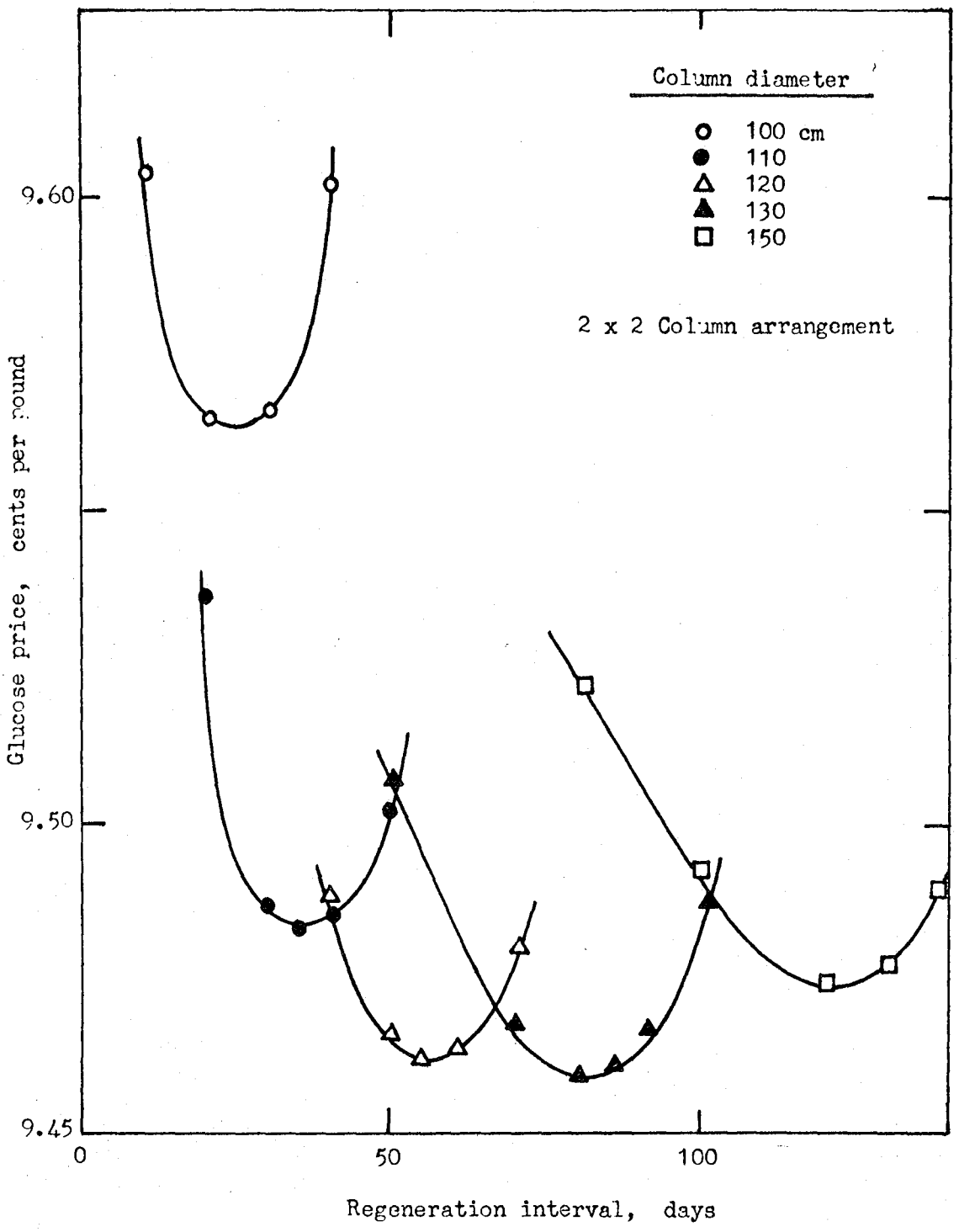

Fig. 6.3 Costing program results for a 2 x 2 column arrangement.

XBL 788-9917

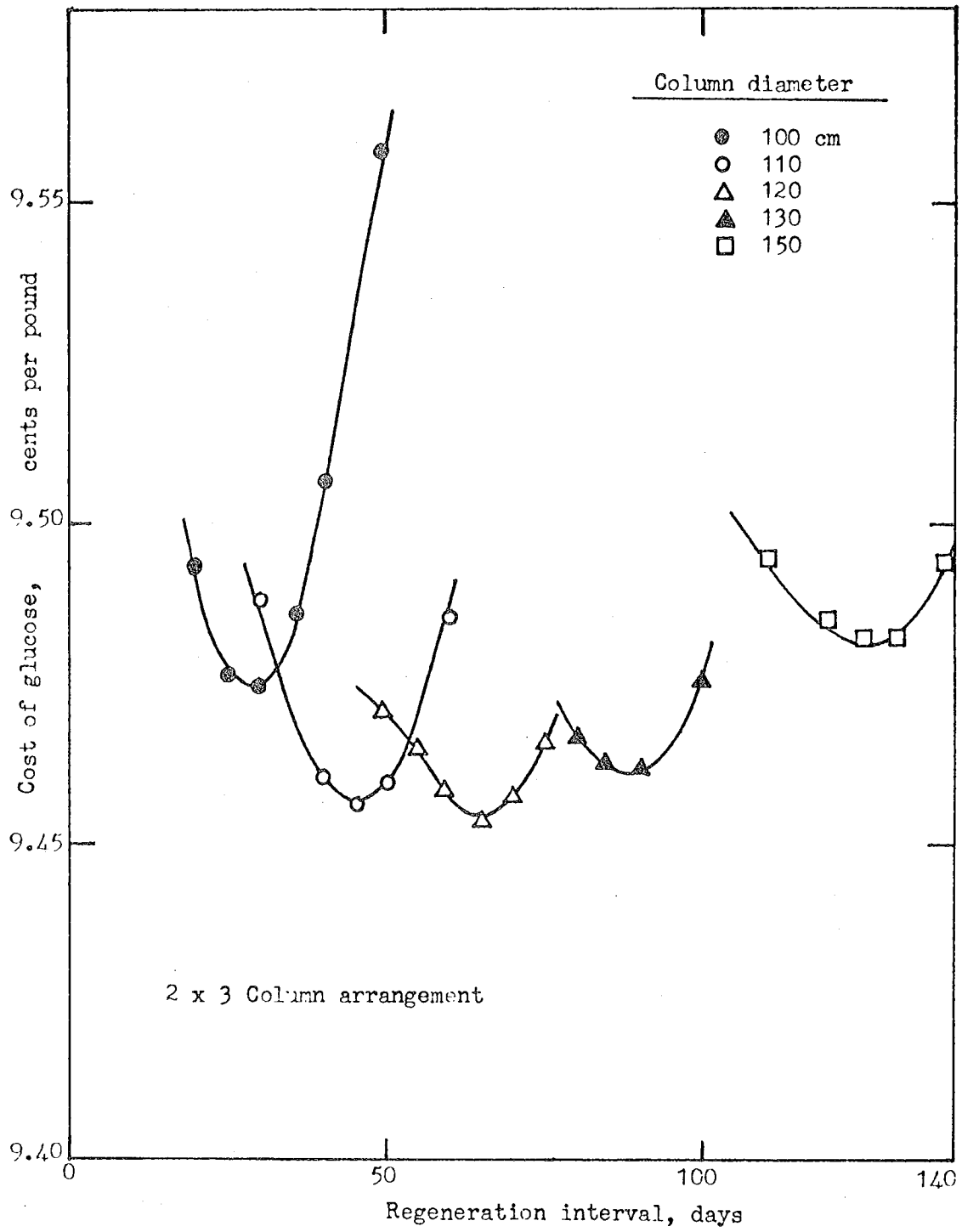

Fig. 6.4 Costing program results for a 2 x 3 column arrangement.

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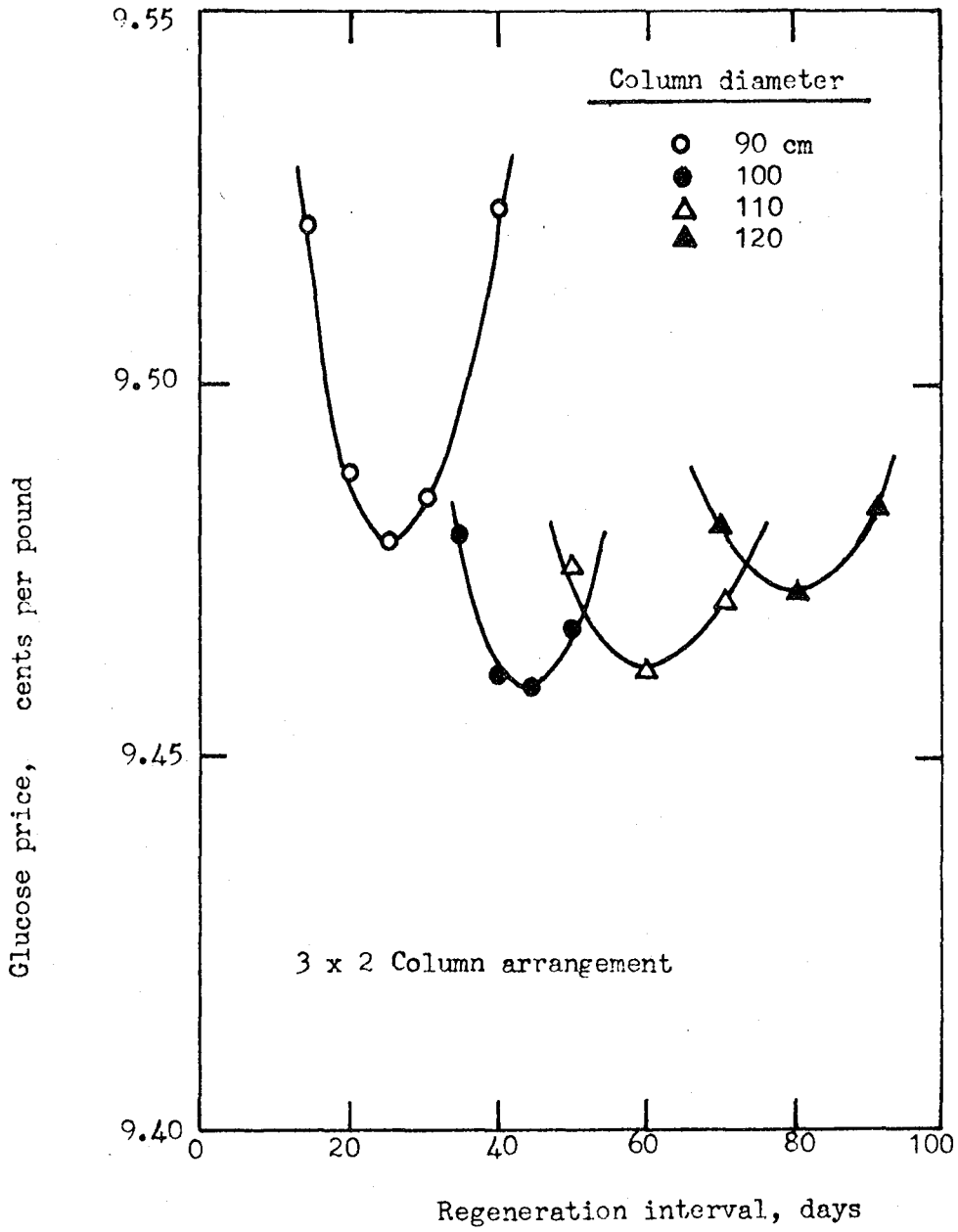

Fig. 6.5 Costing program results for a 3 x 2 column arrangement. XBL 788-9919

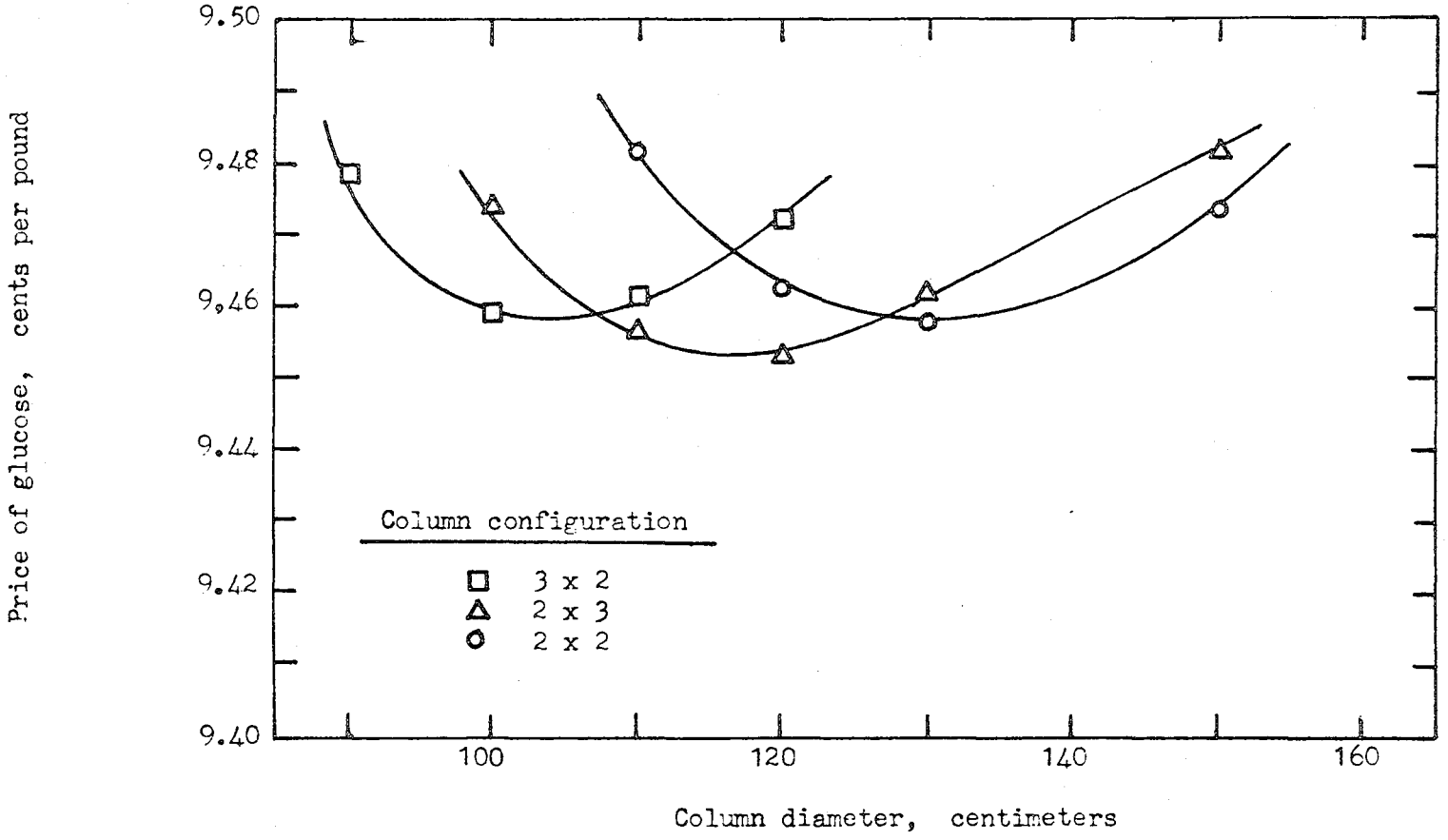

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Fig. 6.6 Plot of the minimum costs of glucose from Figs. 6.3 through 6.5 versus column diameter.

the shematic of this optimal design is presented along with the material balance around each column.

Table 6.4 shows the cost distribution of the optimal design of the immobilized β-glucosidase reactor. The total cost of this design was estimated as 1.64×10^5 dollars per year. The annual cost due to glucose production in the current process is 1.41×10^7 dollars per year. Therefore the added cost due to the addition of the immobilized enzyme reactor is 1.14% of the new total glucose manufacturing cost.

6.4 Conclusions

The introduction of an immobilized β-glucosidase reactor to the current process for the production of ethanol from corn stover would, from the design estimates presented in this work, decrease the price of ethanol from 179.4 cents per gallon to 172.2 cents per gallon for a savings of 7.2 cents per gallon. This is due to a 5.3% decrease in the cost of glucose which yields a 4.0 % decrease in the price of ethanol. Figure 6.8 shows that the savings in the cost of ethanol per gallon increases linearly with

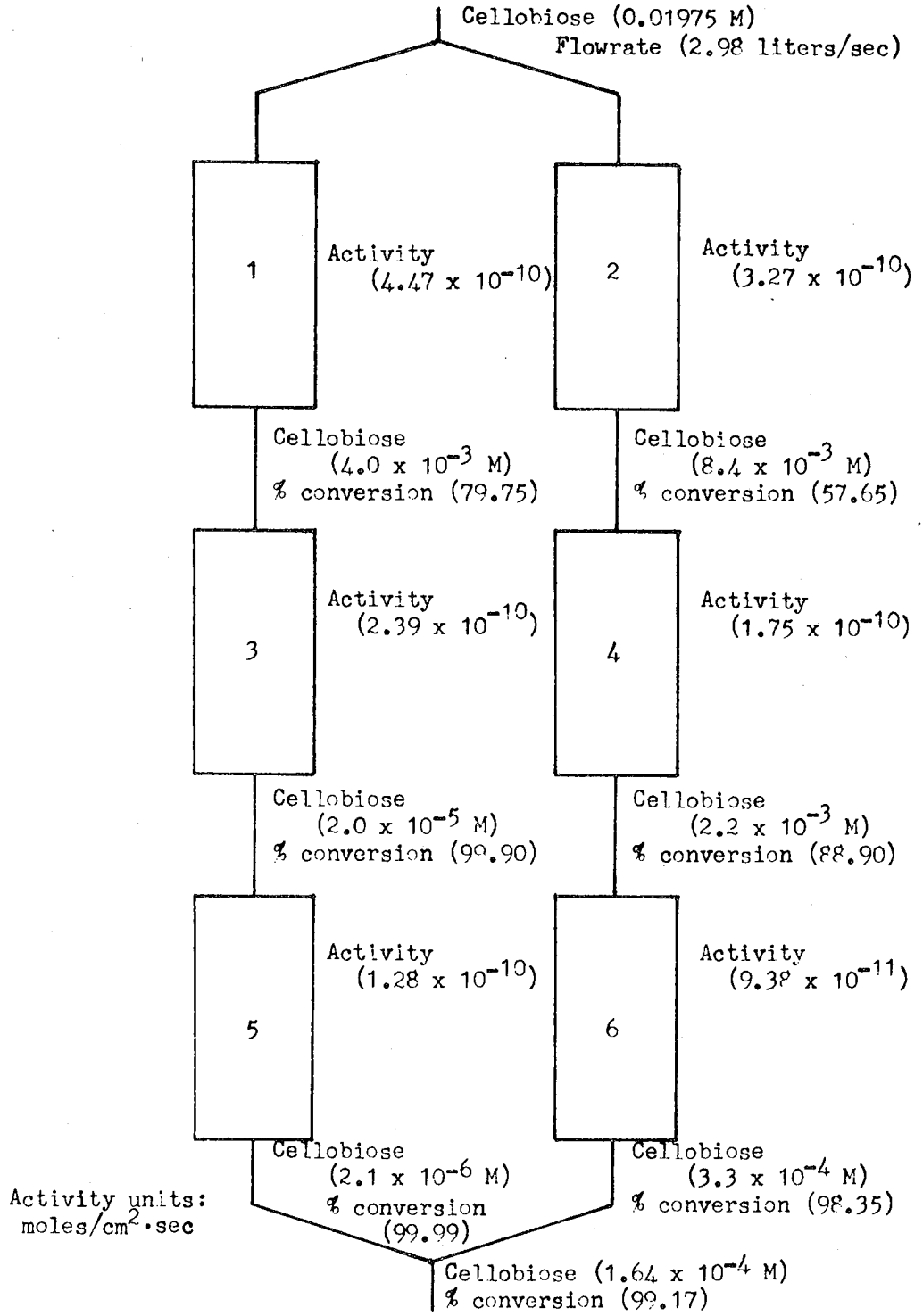

Fig. 6.7 Schematic of optimal design of immobilized β-glucosidase reactor. XBL 788-9921

Table 6.4 Processing cost breakdown due to the immobilized β-glucosidase reactor

Item	Annual cost $/year	% of total
Column shells	1.963×10^4	12.0
Pumps	3.459×10^4	21.1
Enzyme	1.691×10^2	0.1
Resin	5.525×10^4	33.7
Gluteraldehyde	5.271×10^4	32.2
Labor	1.366×10^3	0.8
Total	1.637×10^5	100

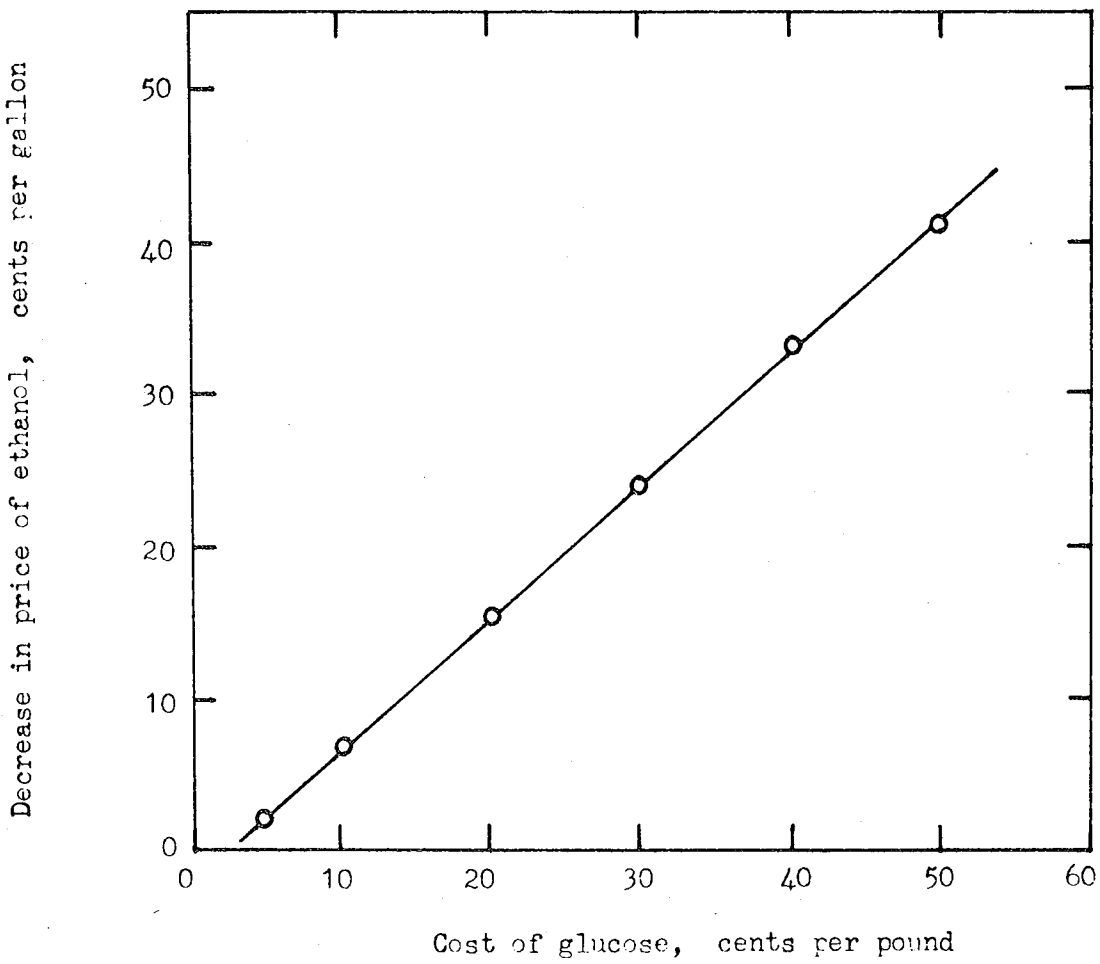

Fig. 6.8 Savings in the cost of ethanol versus the production price of glucose in cents per pound.

XBL 788-9922

what glucose would cost per pound if no immobilized enzyme reactor was used in the process. This shows that even if the current process estimates are inaccurate, a savings in the price of ethanol would still be realized.

The results of this work should be treated as an initial estimate of the feasibility of the use of such a reactor with the current process. Further work may indicate a greater reduction in the price of glucose by 1) finding a method to reuse the phenol formaldehyde resin, 2) optimizing the use of the glutaraldehyde in the immobilization process, or 3) increasing the activity per gram of resin either by immobilization optimization, enzyme purification prior to immobilization, or introduction of buffers to decrease the pH of the stream to the enzyme reactors. Table 6.3 indicated that over 65% of the annual cost of the immobilized enzyme reactor was attributed to the cost of the resin and glutaraldehyde. For this case the price per liter of column volume of the resin and glutaraldehyde were 2.83 and 2.70 dollars, respectively. Figure 6.9 shows the effect of decreasing the cost of the resin and/or glutaraldehyde via a plot of the price per

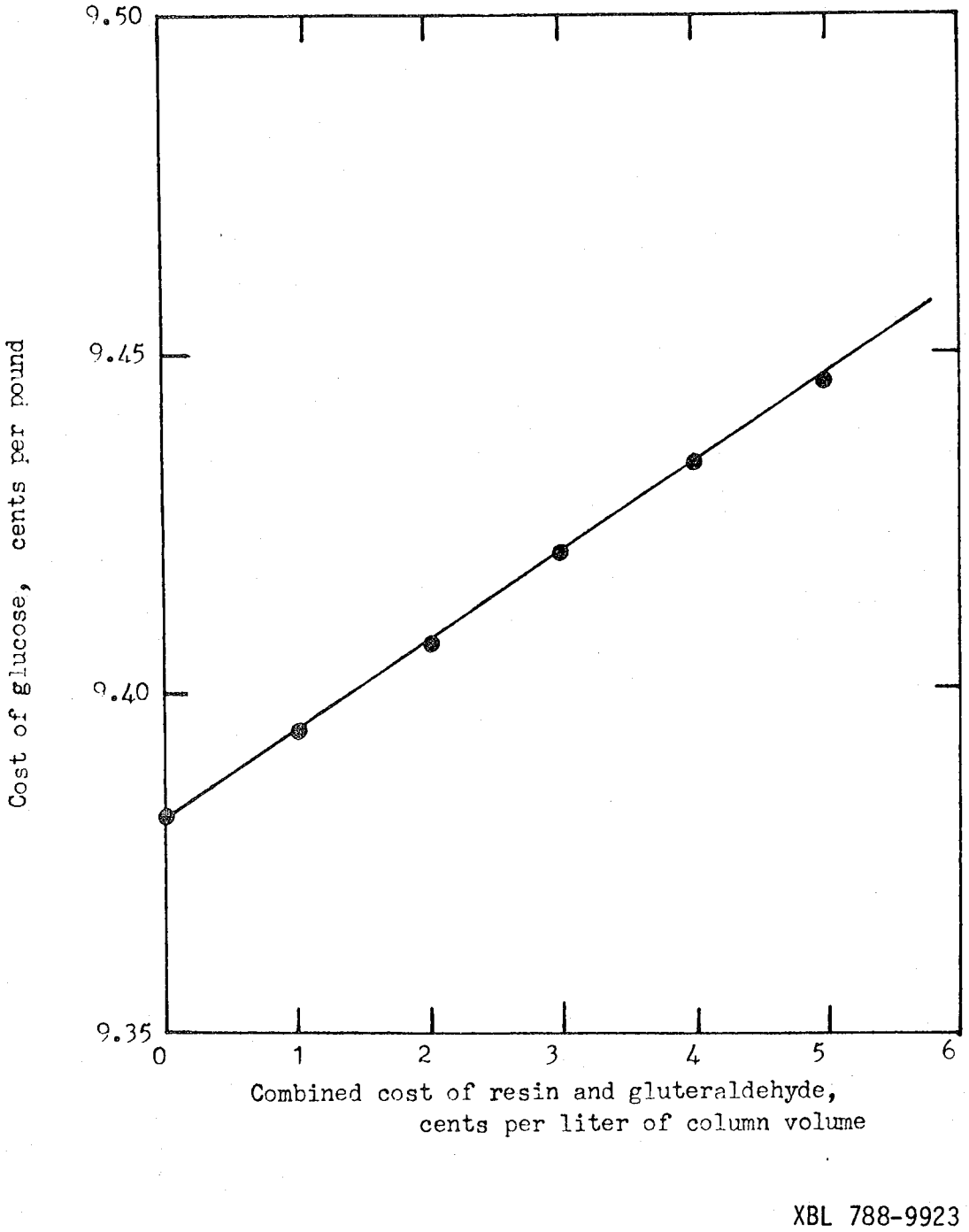

Fig. 6.9 Production price of glucose for the optimal design of the β-glucosidase reactor versus the cost per column volume of glutaraldehyde and phenol formaldehyde resin.

pound of glucose for the reactor design of Figure 6.7 versus the combined price (cents per liter of column volume) of the resin and glutaraldehyde. A decrease from the current combined price of 5.53 cents per liter to 2 cents per liter, for example, either by reuse of the resin or optimizing the use of glutaraldehyde, or both, would drop the price of glucose to 9.407 cents per pound. This would result in a further decrease in the price of ethanol by 0.6 cents per gallon.

The presence of glucose inhibition on the activity of the immobilized β-glucosidase will reduce the effective activity of the resin and should therefore be expected to decrease the savings in the price of ethanol. Studies have not as yet been performed along this line due to the complexity of the experimental proceedures due to the complex nature of the enzyme kinetics.

Finally, as more research is performed on pretreatment and delignification of cellulosic substrates, allowing greater hydrolysis of the cellulose, more cellobiose would be produced, which may result in greater cost decreases with the addition of an immobilized β-glucosidase reactor.

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This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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