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Preparation and Characterization of β-D-Glucosidase Immobilized in Calcium Alginate

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CHEMICAL TECHNOLOGY DIVISION

PREPARATION AND CHARACTERIZATION OF β-D-GLUCOSIDASE IMMOBILIZED IN CALCIUM ALGINATE

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ABSTRACT

Enzymatic hydrolysis of biomass to produce glucose may become feasible if an inexpensive method to reuse the enzyme can be found. This study investigated one such method whereby β-D-glucosidase (E.C. 3.2.1.21) was immobilized in calcium alginate gel spheres, which were shown to catalyze the hydrolysis of cellobiose to glucose. There was a loss of 49% of the enzyme from the alginate slurry during gelation. After gelation, in the stable gel spheres, there was a 37% retention of the enzyme activity that was actually immobilized. The reason for the loss in activity was investigated and may be caused by inhibition of the enzyme within the sphere by the calcium cations and the alginate anions also present. Mass transfer effects were minimal in this sytem and were not responsible for the activity loss.

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Contents

1. SUMMARY

Cellulose is being considered as a possible renewable source of raw material for the production of fuel and chemicals. In the envisioned scheme, cellulose is separated from biomass and hydrolyzed to glucose, which can be subsequently converted to fuel and chemicals using appropriate fermentations. Hydrolysis of cellulose can be achieved enzymatically using cellulase. However, the cost of enzyme production necessitates its recovery and reuse. Cellulase, in actuality, is a complex of at least 3 enzymes: endoβ-glucanase, exoβ-glucanase, and β-D-glucosidase. This study examines immobilization of β-D-glucosidase produced by the fungus Aspergillus niger in calcium alginate gel spheres.

We have shown that β-D-glucosidase immobilized in calcium alginate gel spheres can be used to hydrolyze cellobiose, a glucose dimer, to glucose. The maximum amount of activity entrapped in 25 gel spheres (≡ 0.5 ml of soluble enzyme) was 108 m units. A standard set of conditions was used for comparison throughout the study. The actual activity of the spheres under the conditions was 39 m units, which represents 37% of the amount of the activity actually entrapped, compared with free enzyme in solution. The reason for this loss of activity was investigated.

The extent of any external mass transfer effects was determined by varying the rate of stirring during the assay. The results, however, showed that the observed activity was largely independent of the stirring rate, indicating that external mass transfer was unimportant.

The extent of internal mass transfer effects was determined by assaying the gel-entrapped enzyme in two different sizes of spheres, whose surface area-to-volume ratio differed. Increasing the surface area-to-volume ratio of the spheres did not result in an increase in activity. It was concluded, therefore, that mass transfer effects had little influence on the activity of β-D-glucosidase entrapped within calcium alginate gel spheres.

The inhibitory effect of the calcium cations and alginate anions (the components of the gel matrix) was also investigated. It was found that calcium is, in fact, an inhibitor of the soluble β-D-glucosidase enzyme. However, the inhibition by calcium alone was not sufficient to account for the total loss in activity found on immobilization. We also found that sodium alginate inhibited β-D-glucosidase and suggest that the combined inhibitory effect of the calcium cations and anions may be responsible for the loss in activity. Preliminary investigation showed that strontium may be a suitable noninhibitory replacement anion for calcium.

The effect of temperature and pH on the activity of the immobilized enzyme was investigated. The immobilized enzyme apparently possessed a broad temperature optimum between 30 and 60°C. Initial results on the effect of pH on activity showed no increase in activity above pH 5.0.

2. INTRODUCTION

2.1 Background

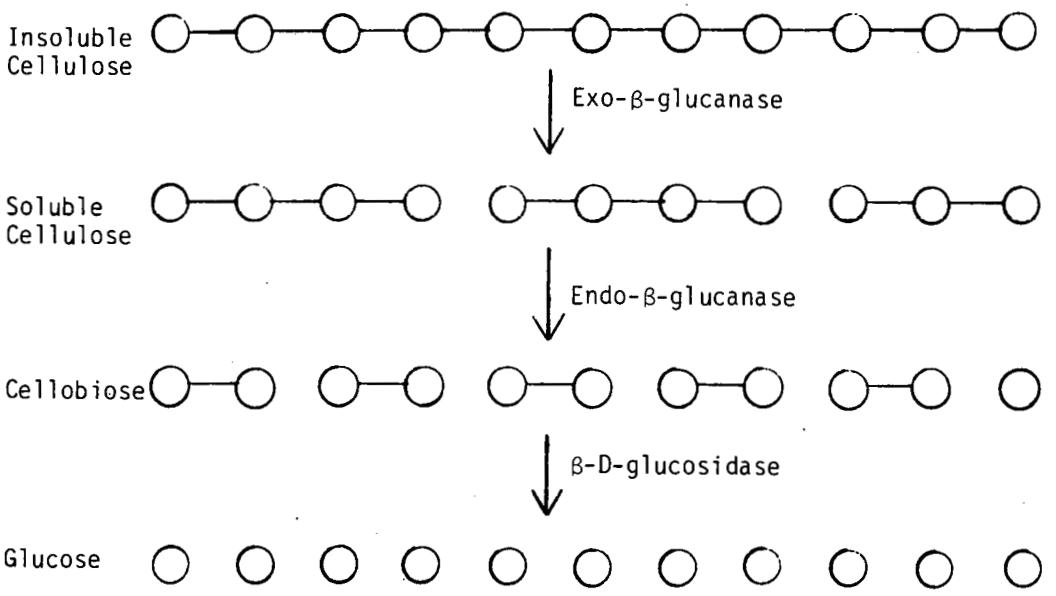
Cellulose, abundantly available as a major component of municipal, agricultural, and forest wastes, has enormous potential utility as a renewable carbon source for large-scale fermentation to produce ethanol. Ethanol can be used as a fuel or chemical feedstock to supplement the nonrenewable reserves of petroleum (1). Prior to fermentation, the nonfermentable polymer cellulose must be hydrolyzed to its fermentable monomer glucose as shown in Fig. 1. This hydrolysis, or cleavage of carbon-oxygen-ether linkages, with the addition of water, can be catalyzed either by inorganic acids or by a group of enzymes known as cellulases.

Enzymes are particularly well-suited to cellulose hydrolysis. The high degree of regularity in celluloses, where the carbon-oxygen bonds between successive glucose moieties are virtually all in the β-1,4-stereochemical configuration (as shown in Fig. 1), make this possible. The cellulase known as β-1,4-glucosidase, for example, will catalyze the hydrolysis of this type of bond almost exclusively, so that very high conversions of cellulose to glucose may be possible, without the simultaneous but undesirable hydrolysis of glucose to nonfermentable by-products. Mandels gives a more detailed explanation of cellulose hydrolysis using cellulase (6).

Economical evaluation of the enzyme-catalyzed hydrolysis of cellulose reveals that the largest single obstacle to an economically viable process is the cost of enzyme manufacture (6). Thus, enzymes must be produced inexpensively or reused.

Cellulases are produced by many microorganisms. One prolific source is the fungus Trichoderma reesei, which was first isolated during World War II as a major deteriorating agent of cellulosic components in military clothing and equipment used in tropical environments. Since T. reesei secretes these enzymes extra-cellularly, cell lysis is not necessary during enzyme production, resulting in low cost separation of enzymes from intercellular material. However, other difficulties are encountered when this cellulase complex is used.

The cellulase, secreted by T. reesei, contains enzymes known as exo-glucanases and endo-glucanases, which synergistically break down insoluble cellulose chains into the glucose dimer cellobiose. The cellulase complex has little of the enzyme β-D-glucosidase, which catalyzes the further hydrolysis of cellobiose to glucose. As a result, in vitro procsses using only cellulase from T. reesei experience a rapid buildup in the concentration of cellobiose. This feedback in turn inhibits endo- and exogluconase, greatly reducing the rate of cellulose hydrolysis to cellobiose. To alleviate this problem, this cellulase can be enriched with β-D-glucosidase, which allows greater conversion of cellobiose to



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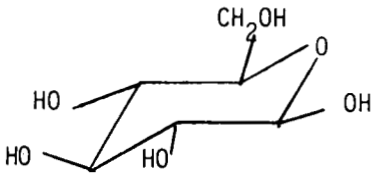

β-D-glucose

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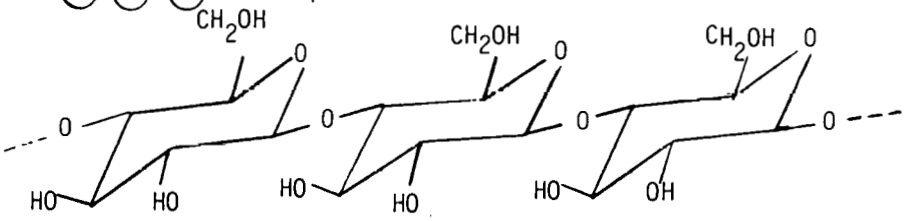

β-D-glucose in β-1-4 bondage

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CELLULOSE DEGRADATION BY CELLULASE ENZYMES (10)			
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glucose, eliminating inhibition by cellobiose and improving overall productivity (12).

Endo-β- and exo-β-glucanases strongly adsorb directly onto cellulosic substrates and may be recovered efficiently using continuous chromatographic processes (2). β-D-glucosidase does not adsorb as strongly and must be immobilized within an insoluble support to be efficiently recoverable.

There are three principal methods of enzyme immobilization: covalent bonding of the enzyme onto a support, adsorption onto a support, or physical entrapment within a support. Each method has advantages, and all have successfully immobilized β-D-glucosidase (12). Large-scale inexpensive immobilized enzyme processes require retention of enzyme stability and activity, and an inexpensive support, which is available in large quantities and is compatible with fermentation bacteria. One immobilization process potentially capable of meeting these criteria is physical entrapment within calcium alginate gel spheres. This has been used successfully to immobilize other enzyme systems (4).

Calcium alginate is a naturally occurring polysaccharide polymer, composed primarily of the calcium salts of D-mannuronic acid and L-guluronic acid. It is a major structural component of the cell walls of brown seaweed (class Phaeophyceae) and is used industrially for thickening, stabilizing, emulsifying, film-formation, and gelation. Alginic acid is produced commercially in the United States by extraction from the giant kelp, Macrocystis pyrifera, which contains roughly 15% alginic acid on a dry weight basis. The toxicological properties of alginates have been extensively investigated, and both sodium and calcium alginate are included in the list of food stabilizers, which are generally recognized as safe (GRAS). The estimated average annual world production of alginic acid salts is 10,000 metric tons (5). In this study, water soluble sodium alginate is converted into the insoluble calcium alginate gel to entrap the enzyme. The following section explains some of the pertinent theory necessary for analysis of the experimental data.

The focus of this study is measurement of the activity of β-D-glucosidase converting cellobiose to glucose, both when free and immobilized within calcium alginate gel spheres, and quantification of any losses in activity which may result from inhibition, physical loss, or mass transfer limitations.

2.2 Theory

One simple yet general method to characterize the kinetics of an enzyme-catalyzed reaction is the Briggs-Haldane steady-state modification of the Michaelis-Menton equation (8). This approach has found widespread use in the reduction of biochemical data. Although this simple treatment gives information only about initial rates of reaction, it has the same general form as more rigorous derivations, allowing modification to account for higher conversion of substrate, inhibition, and multiple reactions.

The simple model assumes that the enzyme and substrate join reversibly to form an intermediate complex, which breaks down to form product and regenerates the enzyme:

$$[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_2} [E] + [P] \qquad (1)$$

where [E] is the enzyme concentration, [S] is the substrate (cellobiose) concentration, [ES] is the intermediate complex concentration, [P] is the product (glucose) concentration, and k_1, k_{-1}, and k_2 are kinetic rate constants.

Several simplifying assumptions were used. Enzyme and substrate were assumed to be in equilibrium with the complex, and the change in substrate concentration and the rate of the reverse reaction from product to complex were assumed to be zero.

The concentration of the enzyme-substrate complex was assumed to rapidly attain a constant steady-state value:

$$0 = \frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_{-1})[ES] \qquad (2)$$

where all the enzyme is assumed to be either free or complexed with substrate, or:

$$[E] = [E]_{total} - [ES] \qquad (3)$$

Combining Equations (2) and (3) gives:

$$0 = k_1([E]_{total} - [ES])[S] - (k_2 + k_{-1})[ES] \qquad (4)$$

which can be rearranged to give the substrate concentration:

$$[ES] = \frac{k_1[E]_{total}[S]}{k_1[S] + (k_2 + k_{-1})} \qquad (5)$$

The reaction velocity or rate of product formation is then given by:

$$v = \frac{d[P]}{dt} = k_2[ES] \tag{6}$$

Substituting Eq. (5) into Eq. (6) and dividing by k_1 give the result:

$$v = \frac{k_2[E]_{total}[S]}{[S] + (\frac{k_2 + k_{-1}}{k_1})} \tag{7}$$

This is commonly abbreviated as:

$$v = \frac{v_{max}[S]}{[S] + k_m} \tag{8}$$

where

$$v_{max} = k_2[E]_{total}$$

and

$$k_m = (\frac{k_2 + k_{-1}}{k_1})$$

It is important to note that when $[S] = k_m$, $v = v_{max}/2$.

Experimental determination of reaction velocity at several substrate concentrations should therefore give two empirical parameters; v_{max}, the maximum possible rate of reaction when all of the enzyme is complexed with substrate; and k_m, the substrate concentration necessary for half maximum velocity.

These parameters can be estimated from a plot of reaction velocity versus substrate concentration (Fig. 2a), but the hyperbolic nature of this relationship makes accurate determination difficult. A more accurate method for determining v_{max} and k_m uses the Lineweaver-Burk plot as shown in Fig. 2b. If the velocity equation is recast in reciprocal form, the intercepts at $[S]^{-1} = 0$ and $v^{-1} = 0$ respectively yield v_{max} and k_m directly:

$$\frac{1}{v} = \frac{1}{v_{max}} + (\frac{k_m}{v_{max}}) \frac{1}{[S]} \tag{9}$$

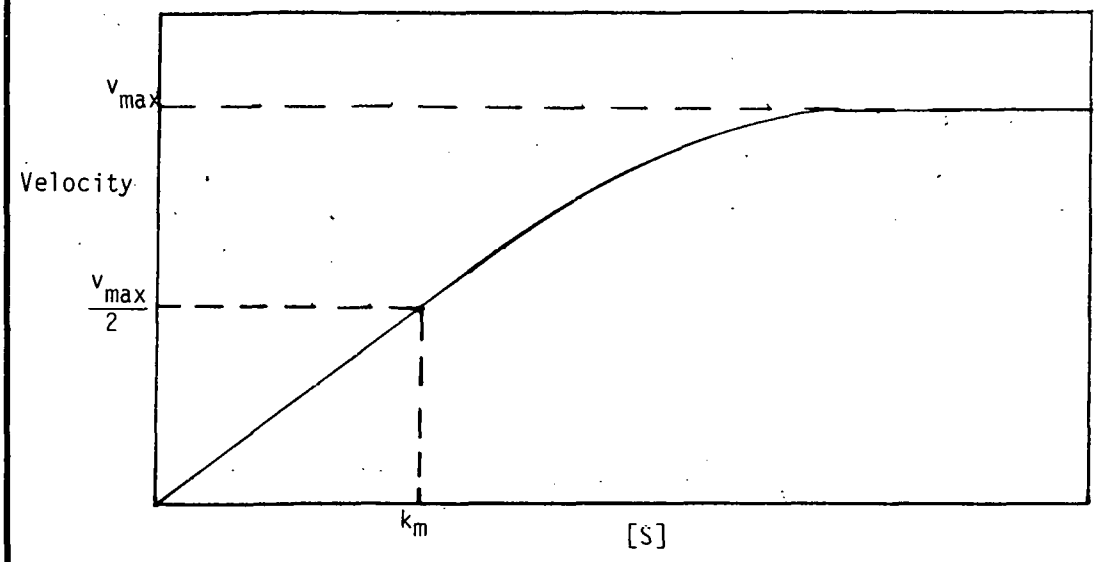

Fig. 2a. Velocity vs Substrate for Enzymatic Reaction

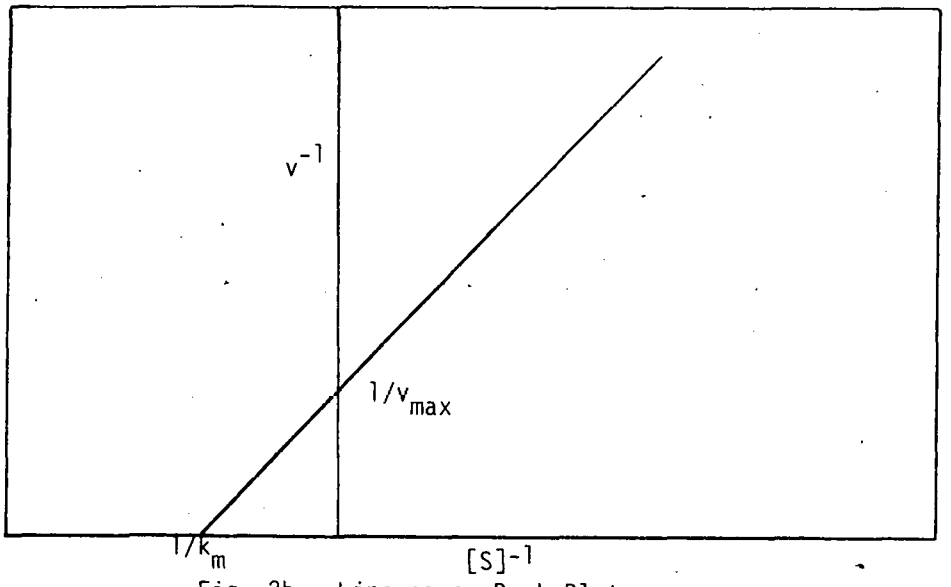

Fig. 2b. Lineweaver-Burk Plot

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CHARACTERISTIC KINETIC CURVES
FOR ENZYMATIC REACTIONS

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Inhibition of enzyme reactions takes a number of forms (8). The Lineweaver-Burk plot can give an indication of what phenomena are at work. The influence of the presence of a particular species on v_{max} and k_m can also indicate its inhibitory influence on the enzyme and can give an indication of the mechanism of inhibition.

Figure 3a illustrates simple noncompetitive inhibition where the enzyme is irreversibly deactivated by the inhibitor. The inhibitor decreases v_{max} but has no effect on the k_m value. The degree of inhibition depends only on the inhibitor concentration [I], and the enzyme complex formation rate constant k_i, where the following reaction is assumed at equilibrium:

$$[E] + [I] \underset{\leftarrow}{\overset{k_i}{\rightleftharpoons}} [EI] \tag{10}$$

When this relationship is substituted into Eq. (2) and simplified into reciprocal form, the following equation results:

$$\frac{1}{v} = \frac{k_m}{v_{max}} \left(1 + \frac{[I]}{k_i}\right) \frac{1}{[S]} + \frac{1}{v_{max}} \left(1 + \frac{[I]}{k_i}\right) \tag{11}$$

At zero inhibitor concentration, this reduces to Eq. (9).

Figure 3b illustrates simple competitive inhibition where the inhibitor and the substrate compete for active sites on the enzyme. This acts to increase the apparent k_m requiring higher substrate concentrations to achieve the same v_{max}. The reciprocal form exhibiting this type of inhibition is:

$$\frac{1}{v} = \frac{k_m}{v_{max}} \left(1 + \frac{[I]}{k_i}\right) \frac{1}{[S]} + \frac{1}{v_{max}} \tag{12}$$

In this study the enzyme is β-D-glucosidase, the substrate is cellobiose, and the product is glucose.

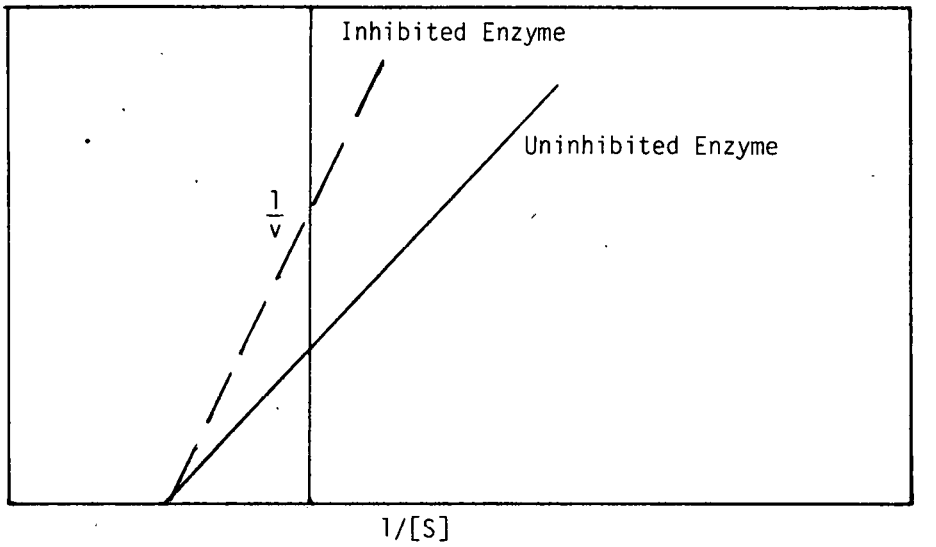

Fig. 3a. Simple Noncompetitive Inibition

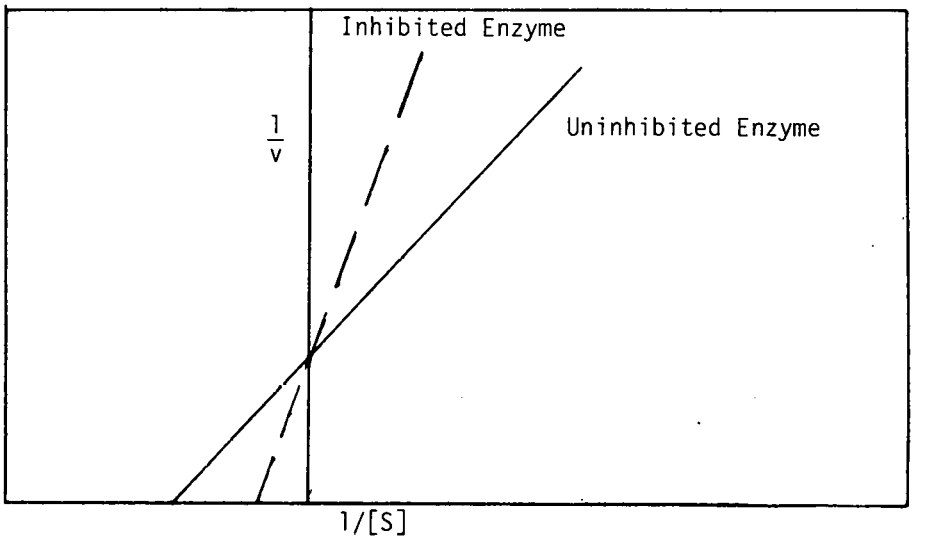

Fig. 3b. Simple Competitive Inhibition

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LINEWEAVER-BURK PLOT OF
TWO SIMPLE INHIBITION MODELS

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2.3 Objectives

As part of a larger program to evaluate and scale up the alginate encapsulation process, the following objectives were pursued:

1. The enzymatic activity of β-D-glucosidase from Aspergillus niger entrapped in calcium alginate gel spheres was measured to establish the causes of any loss of activity relative to the free enzyme.

2. New immobilization or reaction procedures were developed to circumvent the causes of lost activity of the immobilized enzyme.

3. Optimal reaction conditions and immobilized enzyme lifetimes were investigated as a preliminary step to pilot-plant scaleup.

Completion of the objectives required manufacturing calcium alginate gel spheres and quantifying their stability, followed by examining inhibition caused by physical loss of the enzyme during gelation, by calcium, by alginate, and by internal or external mass transfer (varying sphere surface area or agitation, respectively). Finally, a preliminary investigation of temperature and pH was begun.

3. EXPERIMENTAL APPARATUS AND PROCEDURES

3.1 Chemicals and Assays

A commercial preparation of β-D-glucosidase from Aspergillus niger (NOVO Laboratories Inc., Wilton, Conn.) in a 200:1 aqueous dilution, with 10 mM sodium acetate added as a buffer (pH 5.0), was used as the enzyme stock solution. A 100 mM aqueous solution of β-D-(+) cellobiose (Sigma Chemicals) with 10 mM sodium acetate buffer (pH 5.0) was used as the substrate stock solution. These standard solutions were used throughout the experimental series.

Glucose concentrations, resulting from conversion of cellobiose, were measured using a glucose 10 assay vial (Sigma) that contained hexokinase, glucose-6-phosphate dehydrogenase, ATP, Mg^{2+}, and NADP. An aliquot (0.1 ml) was added to 1.0 ml of the glucose assay reagent. Ultraviolet absorbance at 340 nm was monitored to determine the concentration of NADPH, which is formed quantitatively in a one-to-one stoichiometric ratio with glucose. The reaction was stopped by virtue of the dilution of the enzyme in the glucose assay reagent, which also effected a pH change from 4.8 to 7.7. where the β-D-glucosidase from A.niger is inactive.

The standard unit for reporting enzyme activity is the International Unit (U), which is defined as that amount of enzyme which catalyzes the

fermentation of one micromole of product per minute under defined reaction conditions. The defined reaction conditions usually are a fixed temperature, optimal assay conditions, and saturating concentrations of the substrates. Thus, the specific activity represents v_{max}/mg enzyme.

3.2 Reaction Procedures and Apparatus

The reactions for each experiment were carried out in three bench-top, constant-stirred batch reactors at a constant temperature of 30°C. The reactors were 30-ml water-jacketed flasks with magnetic stirrers, which ran at a minimum agitation (120 rpm) to ensure mixing without damaging the gel spheres. Glass covers were placed on the reactors between sampling to protect against contamination.

Three reactors were required to account for U.V. absorbance contributions by the enzyme and substrate present. The first reactors, the cellobiose-to-glucose conversion reactor, contained aliquots of enzyme and substrate solutions, with 10 mM sodium acetate buffer used to bring the total volume to 5 ml. The second and third reactors contained aliquots of the stock enzyme solution and the stock substrate solution, respectively, with 10 mM sodium acetate buffer added to give a volume of 5 ml.

The absorbance values of the enzyme and substrate solutions from the second and third reactors were subtracted from the absorbance value of the cellobiose conversion reactor to give the true optical density of the glucose in the cellobiose conversion reactor solution. The concentration of glucose was then read from a calibrated linear plot of glucose concentration versus adsorbance.

In assays with immobilized enzymes, the number of spheres added to the cellobiose conversion and enzyme reactors was such that the volume of the spheres equaled the volume of the enzyme stock solution added to the reactors during free enzyme experiments. This may have led to some error, because the concentration of enzyme in the gel sphere is not known. Further, the enzyme is not a pure chemical, and therefore the molar concentration of enzyme in any solution is unknown. These uncertainties are discussed in Sect. 4.

3.3 Immobilization of β-D-Glucosidase in Calcium Alginate Spheres

Sodium alginate (Type IV, Sigma Chemicals, 2% by weight in 10 mM sodium acetate buffer aqueous solution, pH 5.0) was stirred at room temperature with a 200:1 dilution of β-D-glucosidase. The β-D-glucosidase-sodium alginate slurry had a honey-like consistency (∿14,000 cp) (5). This slurry was added dropwise into a flask containing 0.5 M calcium chloride aqueous solution (20-mesh anhydrous, Allied Chemicals, in 10 mM

sodium acetate buffer, pH 5.0). A 1-ml Gilson pipetman with a 1-ml pipet tip was used to drop the alginate into calcium chloride solution at a rate of 30 drops/min from a height of 15 cm. The average drop size was 0.03 ml. A 5-ml Gilson pipetman with a 5-ml pipet tip cut at the taper of the tip was used to make 0.21-ml spheres at a rate of 10 drops/min. The spheres formed on contact with the calcium chloride solution, floated on the solution surface for 15 min, then settled to the bottom of the flask. During this time, the spheres turned from clear, while floating, to translucent as the precipitation reaction proceeded to completion.

The gelation occurs, as shown in Eq. (13), where R is an alginate anion:

$$CaCl_2 + NaR \text{ (soluble)} \longrightarrow NaCl + CaR_2 \text{ (insoluble)} \qquad (13)$$

when sodium in the water soluble sodium alginate is replaced by calcium cations to form the water insoluble calcium alginate gel.

The spheres were stored in the calcium chloride supernatant at 4°C. The time between production and use varied between 1 hr and 10 days. No variation in the activity of spheres stored for different periods was observed. Prior to use, the spheres were removed from the calcium chloride, sorted to obtain approximately the same size spheres and washed with distilled water. The volume of the spheres were calculated by difference as shown in Appendix 9.2.

Four types of experiments were performed to characterize the immobilized enzymes. The experimental procedures are explained in the next four sections.

3.4 Physical Loss of Enzyme

Physical loss of enzyme from the gel spheres was determined by measuring the catalytic activity of the $CaCl_2$ supernatant solution after the spheres were removed from the supernatant. One-half milliliter of sodium alginate/enzyme slurry was dropped into 4.0 ml of 0.5 M $CaCl_2$ and left in a reactor for 1 hr to complete gelation. The calcium alginate gel spheres were then removed, and the reactor volume was made up to 5.0 ml by addition of 0.5-ml cellobiose solution and 0.5-ml sodium acetate buffer. The production of glucose was monitored, using the techniques described in Sects. 3.1 and 3.2 to determine the enzymatic activity of the $CaCl_2$ supernatant.

Additional experiments were performed to determine the maximum amount of enzymatic activity that may be retained in the gel spheres. Enzyme concentrations of 200:1 and 100:1 in the sodium alginate slurry were added to $CaCl_2$ supernatant solutions containing 200:1 and 20:1 (v/v) concentrations of enzyme. Enzyme was added to the $CaCl_2$ supernatant to prevent diffusion

of enzyme from the spheres into the supernatant. All other reaction conditions as described in Sects. 3.1 and 3.2 remained the same.

3.5 Enzyme Inhibition

The effect of calcium inhibition on free enzyme activity was determined by measuring the activity of the free enzyme in 0.05, 0.1, and 0.5 M calcium chloride aqueous solution using the techniques explained in Sects. 3.1 and 3.2. The glucose concentration was corrected for the U.V. absorbance of 0.5 M $CaCl_2$.

The inhibition of enzymatic activity from alternate divalent alkaline metal earth cations was studied. Enzyme in 0.5 M aqueous $MgCl_2$ or $SrCl_2$ was stored at 4°C for 12 hr, then diluted to a 200:1 enzyme concentration (10 mM salt concentration) and examined using the standard assay technique.

Magnesium alginate is water-soluble, but strontium alginate balls were successfully made by adding 2% sodium alginate to 0.5 M $SrCl_2$. Strontium alginate gelled more slowly than calcium alginate. The enzymatic activity of the $SrCl_2$ supernatant and strontium alginate spheres were then examined using the standard techniques of Sects. 3.1 and 3.2.

The effect of sodium alginate inhibition on free enzyme activity was determined by reacting 0.5 ml of the sodium alginate/enzyme slurry with 4.0 ml of sodium acetate buffer and 0.5 ml substrate solution. A standard assay, as described in Sects. 3.1 and 3.2,was then performed to determine enzymatic activity.

3.6 Mass Transfer Characterization

Two experiments were performed to quantify the effect of mass transfer on immobilized enzymatic activity. Internal mass transfer was examined by measuring the activity of 0.5 ml of calcium alginate gel spheres with radii 0.176 cm and 0.331 cm at the standard reaction conditions.

External mass transfer was quantified by varying the amount of agitation in the reactor vessels for identical spheres, while monitorng the conversion rate of cellobiose to glucose. The approximate rotation speeds of the magnetic stirrers in the reactors were 120 and 500 rpm for low and high agitation, respectively.

3.7 Temperature and pH Optimization

Assays using the standard methods described in Sects. 3.1 and 3.2 were performed to determine what effect temperature and pH had on enzyme

activity. Experiments were performed at 50, 60, 65, and 70°C compared with 30°C for the other experiments. Ten millimolar sodium hydroxide was used to raise the pH of sodium acetate from the standard of 5.0 to 5.5 and 6.0. These preliminary experiments should be followed by future detailed study to optimize the process reaction conditions.

4. RESULTS

4.1 Immobilized and Free Enzyme Activity

Calcium alginate gel spheres can effectively immobilize β-D-glucosidase from A. niger with retention of enzyme activity. Our standard method of immobilization, described in Sect. 3.3, results in retention in the spheres of 37% of the enzyme activity compared with the same amount of free enzyme. This is shown in Fig. 4. The free enzyme line is the average of four separate experiments, taken on separate days, totaling 20 data points. The average and standard deviation of the slope of the four lines are 211 and 15 m units, respectively. Similarly, for the immobilized enzyme, the line on Fig. 4 is the average of three separate experiments. The average and standard deviation of the slope are 39 and 8 m units, respectively. This corresponds to an immobilized activity of 39 m units in a total volume of gel spheres of 0.5 ml, compared with 106 m units available in an equivalent amount of the free enzyme.

In addition to the enzyme activity lost within the spheres, activity is also lost during the sphere manufacture process. Table 1 shows the distribution of enzyme activity between the gel spheres and $CaCl_2$ supernatant. This results in a retention of enzyme activity within the spheres calculated on the basis of total enzyme used to be 18% of the free enzyme activity. Forty-nine percent of the total possible activity is lost into the calcium chloride salt solution prior to gelation of the spheres. A total of 211 m units of enzyme activity is present in the free enzyme used. An activity of 105 m units was measured in the salt solution after the spheres were made and removed, leaving a maximum expected activity within the spheres of only 106 m units (assuming no enzyme inactivation in the supernatant).

Loss of enzymatic activity, which occurs with the immobilized enzyme, is logically divided into three groups: (1) the physical loss of enzymatic activity into the $CaCl_2$ supernatant solution, (2) the loss of activity resulting from inhibition or inactivation of the enzyme which remains entrapped within the spheres, and (3) the activity loss due to limitations of the rate of reaction by mass transfer of products or reactants to the enzyme. These groups are discussed in the following sections.

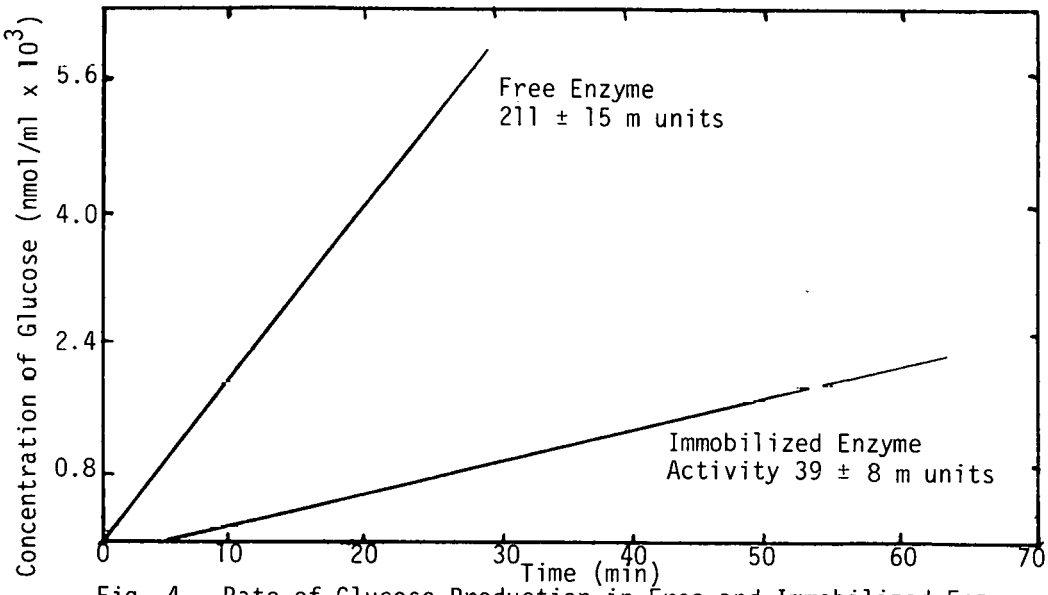

Fig. 4. Rate of Glucose Production in Free and Immobilized Enzyme

Table 1. Distribution of Enzyme Activity

	Immobilized in Spheres		Lost in $CaCl_2$ Supernatant	Total
	Active	Inactive		
Activity (units)	39	67	105	211
% of Total Activity	19	32	49	100
% of Immobilized Activity	37	63	-	100

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ACCOUNT OF ENZYMATIC ACTIVITY			
DATE 9-18-81	DRAWN BY SRK	FILE NO. CEPS X 336	FIG. 4

4.2 Physical Loss of Enzyme

The physical loss of activity reported in Table 1 occurred within the first hour after the sodium alginate slurry containing the enzyme was dropped into the $CaCl_2$ supernatant. Of the 211 m units in 0.5 ml of sodium alginate/enzyme slurry, 105 m units were lost into the $CaCl_2$ supernatant from 0.5 ml of calcium alginate gels. This is 49% of the activity in 0.5 ml of sodium alginate/enzyme slurry. After storing the gel spheres for 12 hr in the $CaCl_2$ supernatant at 4°C, there was no further loss of activity into an agitated buffer solution at 30°C for 3 hr. Thus, even with substantial initial loss of activity, reproducible kinetics studies can be conducted at a later time.

Increasing the enzyme concentration in both the sodium alginate slurry and in the aqueous $CaCl_2$ solution resulted in an increase in total enzyme activity remaining within the gel spheres. The resulting activity in the gel spheres due to increased enzyme concentrations are summarized in Table 2. The corresponding activity of enzyme dilutions (in buffer) are shown on the right.

Table 2. Immobilized Enzyme Activity

Enzyme Dilution in Sodium Alginate \ Enzyme Dilution in $CaCl_2$ Supernatant	0	200:1	20:1	Free Enzyme Activity
200:1	42.2[a]	44	97	211
100:1	-	73	108	422[b]

[a]Standard conditions.

[b]This entry is assumed twice the activity of the 200:1 dilution activity.

An addition of 200:1 dilution of enzyme to the $CaCl_2$ supernatant increased the immobilized activity by 1.8 m units. A tenfold dilution increase of enzyme in calcium chloride increased the amount of immobilized activity to 97 m units. The maximum activity obtained in 0.5 ml of calcium alginate gel spheres was 108 units at enzyme dilutions of 100:1 and 20:1 in the sodium alginate slurry and calcium chloride supernatant, respectively. This corresponds to an increase in activity per unit volume of a factor of 2.5 over the standard immobilization technique.

4.3 Enzyme Inhibition

The second major cause of activity loss in the calcium alginate gels appears to be due to inhibition of the amount of measured activity in the calcium alginate gels. This study focused on calcium and alginate inhibition of the free enzyme.

4.3.1 Calcium Inhibition

Calcium chloride was shown to act as a competitive inhibitor of the free β-D-glucosidase. Calcium chloride concentrations as low as 0.01 M inhibit the enzyme under the conditions of our standard kinetic assay using 10 mM cellobiose. The activity of 0.5 ml of free enzyme in calcium chloride is shown on Fig. 5a. A Lineweaver-Burk plot made at several substrate concentrations in the presence of 0.5 M calcium chloride, compared with a similar plot for the free enzyme alone, shows that v_{max} is unchanged, but that $K_{m_{app}}$ increases, indicating simple competitive inhibition (see Fig. 5b). Since 0.5 M calcium chloride does not increasingly inhibit the enzyme at concentrations greater than 0.1 M, this indicates that calcium chloride is a partially competitive inhibitor.

4.3.2 Other Alkaline Earth Cations

Preliminary experiments of the effect of other alkaline earth cations on the activity of free enzyme show no inhibition. The activity of 0.1 ml of free enzyme with 10 mM cellobiose in the presence of 0.01 M magnesium chloride and 0.01 M strontium chloride was measured under our standard assay conditions. Magnesium and strontium, unlike calcium, did not inhibit the activity of free enzyme, as shown in Table 3.

Table 3. Activity (m units) of 0.1 ml Free Enzyme in the Presence of Alkaline Earth Cations

Cation	$CaCl_2$ (0.01 M)	$MgCl_2$ (0.01 M)	$SrCl_2$ (0.01 M)	No Cation Present
Enzyme Activity	33.6	44.1	46.0	42.2

Our preliminary analysis indicates that there is little difference in the performance of strontium gels, as compared with calcium gels (see Table 4). Roughly the same amount of enzyme is measured in the strontium chloride supernatant as is lost into the calcium chloride supernatant. Virtually the same amount of activity is retained in the gel spheres in either case. Further study is required to determine which cation is superior.

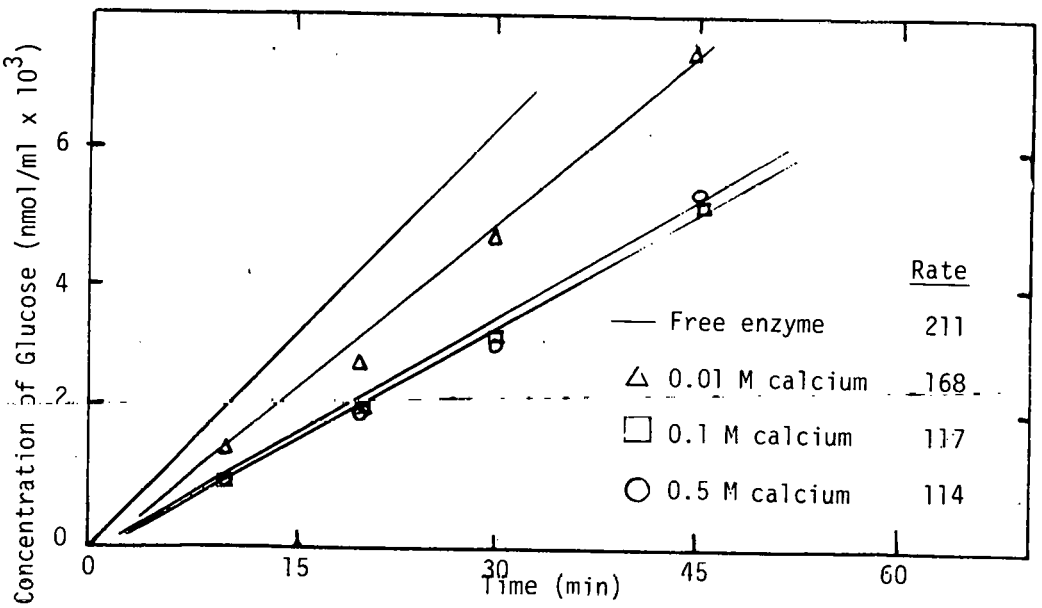

Fig. 5a. Effect of Calcium on Activity

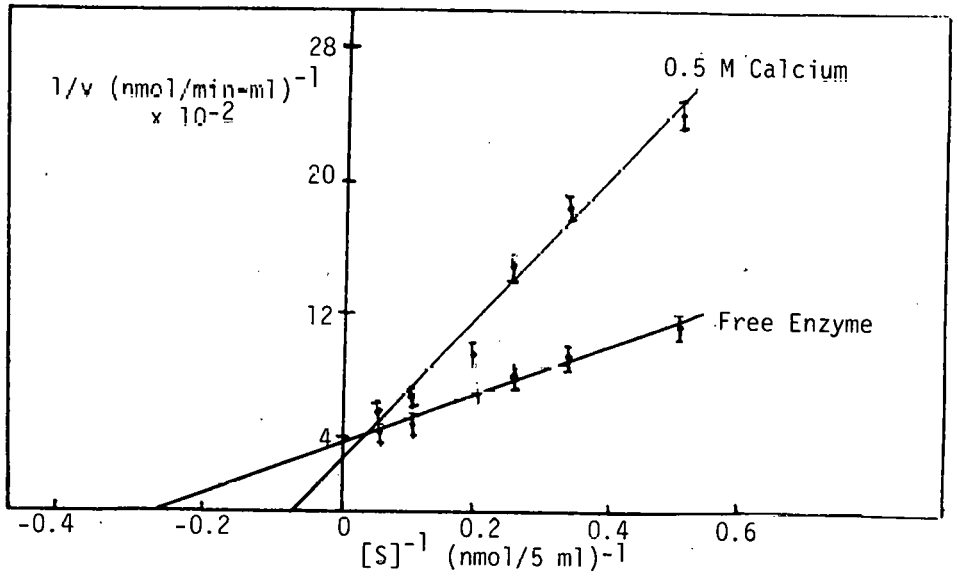

Fig. 5b. Lineweaver-Burk Plot for Calcium Inhibition

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INHIBITION OF β-D-GLUCOSIDASE BY CALCIUM

DATE	DRAWN BY	FILE NO.	FIG.
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Table 4. Comparison of Activity Distribution (m units) for Sphere Manufacture Using $CaCl_2$ and $SrCl_2$

	Activity in Supernatant	Activity in Spheres
0.5 M $CaCl_2$	105	39
0.5 M $SrCl_2$	100	40

4.3.3 Alginate Inhibition

The activity of 0.5 ml of free enzyme in the presence of 2.0×10^{-3} g/ml of sodium alginate was 124 m units, as compared with 211 m units for free enzyme. This concentration corresponds to the concentration of sodium alginate that would be in the reactor if 0.5 ml of gel spheres made of 2% sodium alginate dissolved completely into the 5-ml reactor volume.

This inhibition appears to be due to alginate rather than sodium. The activity of 0.1 ml of free enzyme in 10 mM sodium acetate buffer was 42.2 m units, while the activity in 50 mM sodium acetate buffer was 40.9 m units. This suggests that increased concentrations of sodium does not inhibit the free enzyme.

The alginate may be sterically hindering the active site of the enzyme, or the alginate may be ionically bonding to the enzyme. Further study is required to elucidate the inhibitory mechanism.

4.4 Mass Transfer

The activity measured in a standard volume of spheres (0.5 ml) agitated at the minimum practical agitation rate of approximately 120 rpm (magnetic stir bars barely turning) was 39 m units. The activity measured in identical spheres agitated at the maximum practical agitation rate of approximately 500 rpm (fully-developed vortex extending to the bottom of the reaction vessel) was 45.5 m units (see Fig. 6a). This is a good indication that, although external mass transfer may not be negligible, it is not a major cause of the loss in enzyme activity in the gel spheres. The effect of internal mass transfer on activity was small.

The activity measured in 25 small spheres (radius 0.17 cm, total volume 0.5 ml, surface area 8.9 cm^2) was 39 m units, while the activity measured in 3 large spheres (radius 0.33 cm, total volume 0.46 ml, surface area 4.1 cm^2) was 45.7 m units (see Fig. 6b). This indicates that there is no mass transfer limitations within the spheres for the standard immobilization conditions. As techniques are discovered to improve the activity retained in the spheres, further study will be required to determine if mass transfer becomes important.

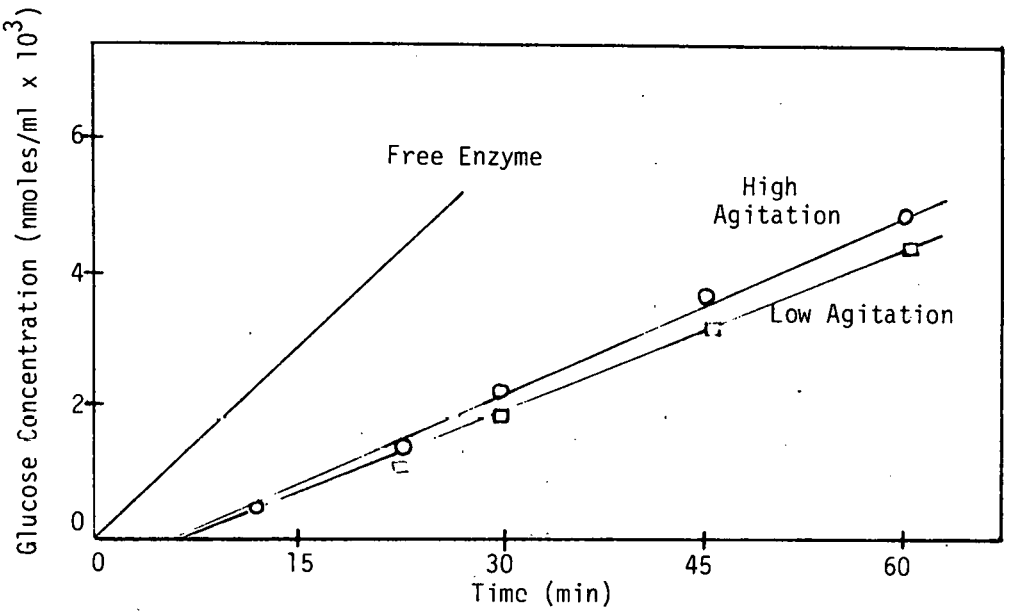

Fig. 6a. Effect of Agitation on Activity

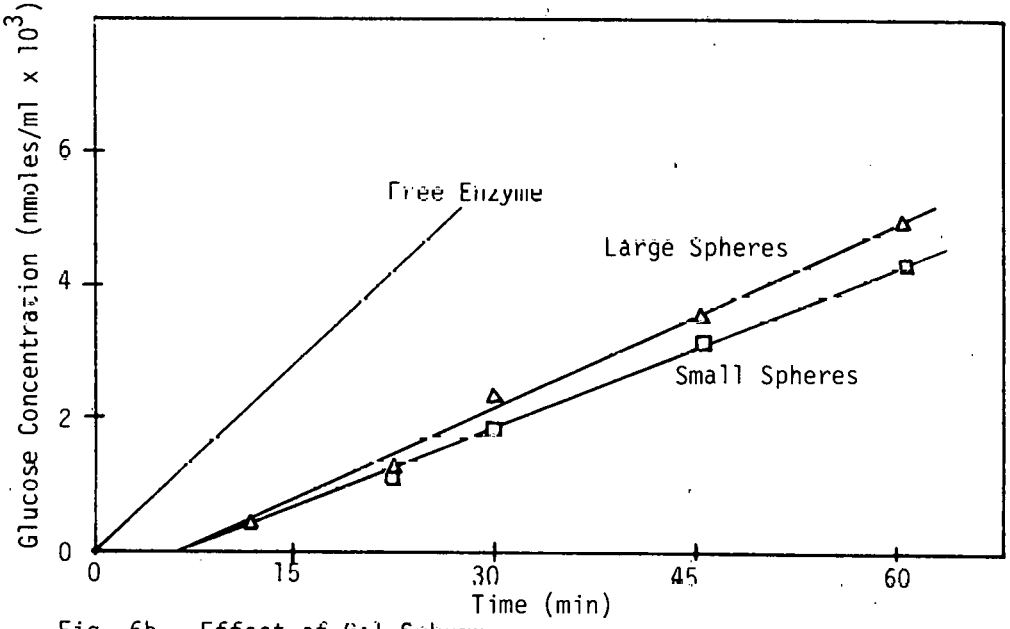

Fig. 6b. Effect of Gel Sphere Size on Activity

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EFFECT OF MASS TRANSFER ON ACTIVITY			
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4.5 Temperature and pH Optimization

The relative activity as a function of temperature of free and immobilized enzyme is shown in Fig. 7a. Initial results show that there is a broad optimal temperature range around 45°C. This is unlike the free enzyme which has a narrow optimal temperature range around 65°C.

The effect of pH on activity of free enzyme is plotted in Fig. 7b. These data show that the optimal pH for maximum activity occurs below 4.5. Initial data on activity as a function of pH for immobilized enzyme is inconclusive. There appears to be no increase in activity at pH greater than 5.0. It is anticipated that the activity of the immobilized enzyme will increase at pHs lower than 5.0. Additional studies are required to determine the pH for maximum activity.

5. DISCUSSION OF RESULTS

5.1 Improvements in Gel-Sphere Production

5.1.1 Physical Loss

At present, physical loss of enzyme activity into the $CaCl_2$ supernatant during sphere manufacture is the greatest single cause of diminished enzyme activity, calculated on the basis of total enzyme added to the sodium alginate slurry prior to gelation. However, if the spheres are manufactured in a continuous process, enzyme lost into the salt supernatant could be purified and recycled, or it could be allowed to build up in the supernatant until a limiting concentration is achieved. In either case, the fraction of enzyme lost into the salt solution could be unimportant if the amount of enzyme activity retained per volume of gel sphere is maximized.

The total amount of enzyme physically remaining in the spheres should be related to the amount of enzyme present initially in the slurry, the rate of enzyme diffusion out of the spheres, and the rate of gelation of the alginate in the spheres by the calcium ions. The initial enzyme concentration is related to concentrations of enzyme in the slurry and salt solution. Diffusion is related to the concentration of sodium alginate in the slurry and the slurry temperature (which determines the slurry viscosity) and enzyme concentration, while the rate of gelation is related to the concentration of calcium chloride in the salt solution and the solution temperature, the size of the slurry droplets, and the rate at which they are added to the salt solution. It is probable, however, that manufacturing conditions which maximize the amount of enzyme retained in the spheres will also maximize the diffusional resistances to cellobiose and glucose movement through the spheres, so that a balance between these two effects may be necessary to optimize overall productivity. Further investigation of all the above variables is required before scaleup can proceed.

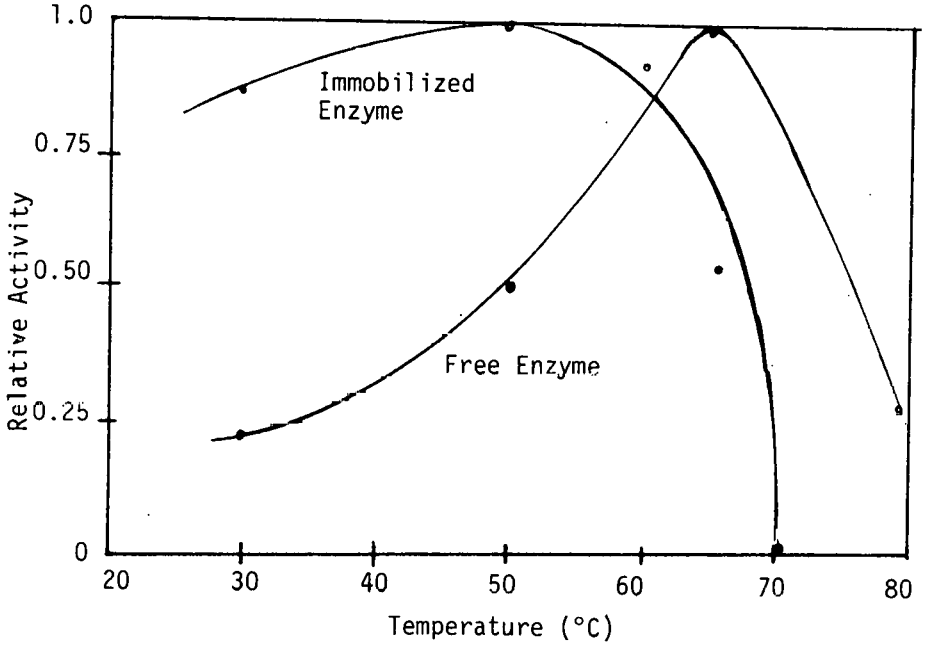

Fig. 7a. Relative Activity as a Function of Temperature

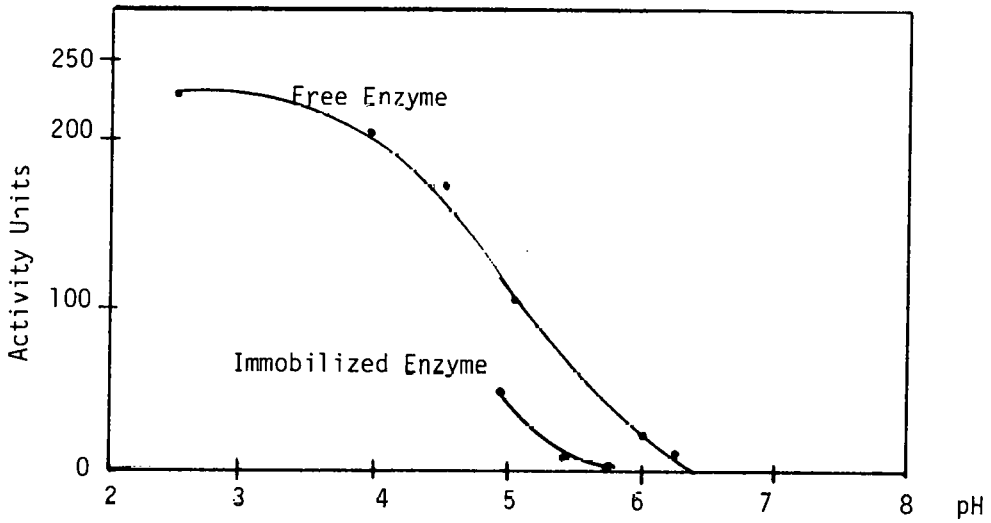

Fig. 7b. Activity as a Function of pH

Free enzyme data from Woodward (12)

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EFFECT OF pH AND TEMPERATURE ON ACTIVITY			
DATE 9-22-81	DRAWN BY SRK	FILE NO. CEPS-X-336	FIG. 7

Kiersten and Bucke (4), using calcium alginate gels to immobilize the enzyme inulase (m.w. > 100,000) for the hydrolysis of inulin (m.w. = 3000 - 5000),showed that although substantial amounts of inulase were lost when a 1 % (w/w) aqueous sodium alginate slurry was added to a 0.1 M calcium chloride solution, virtually no enzyme activity was lost when a 2% sodium alginate slurry was added to a 0.5 M calcium chloride solution. In addition, the substrate inulin was able to diffuse through the gel spheres made under either conditions. For this reason, we chose the higher concentrations of sodium alginate and calcium chloride used in this study for the manufacture of our gel spheres.

Further increases in sodium alginate concentration are possible but are limited by processing conditions. Although the slurries are said to be pseudoplastic for shear rates between 10 - 10,000 s^{-1} (5), the low shear viscosity of a 2% sodium alginate slurry in water at 25°C is 14,000 cP (5), and a 5% solution is reported to be poured only with difficulty out of a wide-mouthed bottle (10).

Droplet processing could be done at higher temperatures or pressures to reduce the slurry viscosity; however, this is somewhat limited by inactivation of the enzyme above 80°C.

Calcium chloride concentration can also be increased, since the maximum solubility of calcium chloride in water is approximately 5 M (59 g/100 ml) at 0°C and 9 M (102 g/100 ml) at 20°C (7); however, only 0.15 g/100 ml of solution of calcium is required on a stoichiometric basis for complete substitution of all the sodium in 100 ml of sodium alginate (5) compared with 5.9 g/100 ml (0.5 M) used in the standard method. Methods for removal of any excess calcium cations over stoichiometric from the gel spheres should be investigated.

5.1.2 Maximum Activity Possible

The preliminary experiments into the maximum possible enzyme loading for our system were complicated by the enzyme and calcium chloride solution forming a precipitate. Possibly the precipitate was the result of impurities in the crude enzyme preparation, and the enzyme activity in the supernatant was unchanged. Detailed studies are needed to determine if this is so and why inhibition of the enzyme by calcium chloride occurs.

It appears that there is a maximum amount of enzyme that can be put into 0.5 ml of calcium alginate gel. The existence of a limiting concentration for activity in 0.5 ml of calcium alginate gel can be understood on theoretical grounds, since it is well-known that the increased chemical potential within a swollen elastic-charged network can maintain a substantial concentration gradient between the inside of the gel and the bulk solution (10). The limiting enzyme concentration in the gel should be determined in future studies.

5.1.3 Alternate Cations

The experiments listed in Sect. 4.3.2 indicated that magnesium and strontium divalent alkaline earth cations, which are above and below calcium on the periodic table, do not inhibit the free enzyme as strongly as calcium. Inhibition of the free enzyme by calcium chloride is probably due to the calcium cation rather than to the chloride anion.

Magnesium alginate is reported to be water-soluble (5), but strontium alginate is not. This noninhibiting salt should allow manufacture of gel spheres with improved retention of enzyme activity if calcium is indeed inhibiting the immobilized enzymes. We have successfully made gel spheres immobilizing β-D-glucosidase in strontium alginate but have not conducted extensive testing to determine if strontium gels are superior to calcium gels. Further study is required to determine to what degree the enzyme is inactivated by either cation, the rate of alginate gelation with the two cations, and the diffusivity of cellobiose and glucose through the different gels.

5.1.4 Calcium and Alginate Inhibition

Sections 4.3.1 and 4.3.3 describe the experiments with the inhibition of calcium and alginate with free enzyme. Use of these results to explain the loss of enzyme activity within the gel spheres is therefore qualitative at best, since the local environment of the enzyme within the spheres is as yet unknown. It is also entirely possible that the enzyme within the spheres is somehow physically inactivated, or prevented sterically from achieving the precise conformation necessary for catalysis. This result would not readily be apparent from free enzyme studies.

The results of the study of other alkaline earth cations indicate that the same amount of activity is in the strontium chloride supernatant as in the calcium chloride supernatant. Nearly the same amount of activity is retained in the gel spheres in either case. Although the calcium may inhibit the free enzyme more than strontium, it is somehow less inhibiting in the immobilized enzyme compared with strontium. This may be due to the calcium cations within the spheres being associated primarily with alginate anions, therefore being unavailable for inhibition.

Inhibition studies of the free enzyme by soluble sodium alginate may give an indication as to whether the alginate may be inhibiting the enzyme; however, crosslinking during gelation changes the chemical species present. This may greatly change the mechanism of inhibition. Study of other gel supports may elucidate the mechanism of inhibition.

5.2 Mass Transfer

Internal or external mass transfer was unimportant for the reaction conditions studied here. However, this conclusion is not generally

extendable to the reaction conditions that may occur in a larger-scale process. Higher concentrations of substrate and product, changes in gelation procedures, or larger spheres may make internal mass transfer important. External mass transfer may become important if the gel spheres are used in a fixed-bed reactor. Thus, during each step of future process studies, mass transfer limitations should be characterized to allow accurate kinetic data interpretation.

5.3 Process Scaleup

An industrial process will require a procedure for mass production of the gel spheres. One possible technique to investigate could incorporate a pressurized vibrating nozzle capable producing large numbers of spheres of uniform diameter (3). A study of the variables affecting gelation should accompany the previous study to determine the manufacturing conditions required to produce tough, durable spheres with good enzyme retention. Once spheres can be produced, a process reactor will need to be designed.

Internal and external mass transfer characterization of the spheres can be used to determine what type reactor can be used. If mass transfer proves unimportant, relatively large spheres can be used in a packed-bed plug-flow reactor to achieve high product conversion. A possible hindrance to use of this type reactor is end-product inhibition of the enzyme by the glucose produced. Two possible methods of eliminating end-product inhibition, by removal of the glucose, are co-isomerization of glucose to fructose and co-fermentation of glucose to ethanol.

Isomerization of glucose to fructose by glucose isomerase could occur with the gel sphere if isomerase were immobilized along with β-D-glucosidase. Fructose does not inhibit β-D-glucosidase (10). If this were successful, and mass transfer remained unimportant, a packed-bed reactor could still be used. Alternately, fermentation of the glucose to ethanol could proceed using Zymomonas mobilis, a bacteria growing on the gel support. Growth of Z. mobilis could interfere with mass transfer in a packed-bed reactor. This process could be successful if a tapered fluid-bed bioreactor were substituted for the packed-bed. Z. mobilis has been grown successfully in this type of reactor using anion exchange resin as a support (9), This unified approach could effectively convert cellobiose to ethanol in one piece of process equipment.

6. CONCLUSIONS

1. β-D-glucosidase was immobilized in calcium alginate gel spheres to obtain a maximum activity of 108 m units/0.5 ml of gel when a 20:1 enzyme dilution was added to the $CaCl_2$ supernatant and a 100:1 enzyme dilution was added to a 2% sodium alginate slurry.

2. Forty-nine percent of the enzyme activity was measured in the initially enzyme-free $CaCl_2$ supernatant after sphere gelation (less than 10 min). This may mean that well over 50% of the enzyme leaches from the gel during gelation. The exact amount is unknown since some of the enzyme in the supernatant is inactivated by calcium.

3. Calcium and alginate inhibit the hydrolysis of cellobiose by β-D-glucosidase in free solution. It is not known if these substances also inhibit the enzyme immobilized within the gel.

4. For the conditions of this study, mass transfer did not inhibit immobilized enzyme activity.

7. RECOMMENDATIONS

1. Determine the immobilized enzyme loading that gives the maximum amount of activity per volume of calcium alginate gel.

2. Completely characterize the kinetic behavior of β-D-glucosidase immobilized in calcium alginate gels, including inhibition at high product conversions.

3. Compare strontium with calcium for alginate gelation.

4. Investigate the kinetic behavior of β-D-glucosidase when glucose inhibition is removed by glucose fermentation or isomerization to fructose.

5. Determine the reactor design and reactor conditions necessary to maximize the hydrolysis of cellobiose by immobilized β-D-glucosidase The conditions include temperature, pH, feed concentration, and flow rate.

8. ACKNOWLEDGMENTS

The authors thank T.L. Donaldson and S.E. Shumate for their helpful discussions and constructive suggestions, and F. Spielberg for her assistance in the laboratory. Special thanks go to J. Woodward for his guidance and good humor during the project.

9. APPENDIX

9.1 Location of Original Data

The original data are located in ORNL Databooks A-9182-G, pp. 68-100, and A-9909-G, pp. 1-27, on file at the MIT School of Chemical Engineering Practice, Bldg. 1505, ORNL.

9.2 Sample Calculations

9.2.1 Spectrophotometry

Beckman DU and Varian Cary 219 spectrophotometers were used to determine the absorbance of the reactor sample. The optical density (OD) was obtained by correcting the test sample absorbance for the absorbance due to the enzyme, substrate, and buffer. From the optical density, the concentration of glucose was calculated from a best fit line of known concentrations of glucose vs absorbance. The velocity of the reaction (nmole glucose/ml-min) was then the best-fit line slope of the curve of glucose concentration as a function of time. Table 5 shows a sample calculation of glucose concentration from absorbance.

9.2.2 Gel Volume

The volume of the immobilized sphere gels were calculated by difference. A known number of spheres were placed in a graduated cylinder with a known volume of water, and the volume difference was measured. This volume was divided by the number of spheres to give the volume of one sphere. For example, if 133 spheres was 2.7 ml or 0.0203 ml/gel sphere, then for a 0.5-ml total volume of spheres,

$$0.5 \text{ ml}/0.0203 \text{ ml/gel sphere} = 24.6 \text{ gel spheres}/0.5 \text{ ml}$$

Thus, 25 gel spheres need to be added to the reactor.

9.3 Nomenclature

$[E]$ concentration of uncomplexed enzyme, mol/l

$[ES]$ concentration of enzyme-substrate complex, mol/l

$[E]_{total}$ total concentration of enzyme, mol/l

k_1, k_2, k_{-1} kinetic rate constants, s^{-1} or l/(mol/l)-s

Table 5. Determination of Glucose Concentration

Absorbance

Time (min)	Test	Enzyme (0.5 M)	Substrate (10 mM)	Blank
5	0.740	0.209	0.048	0.045
10	1.310	0.208	0.050	
15	1.731	0.212	0.050	

optical density = absorbance of test - average enzyme absorbance - average substrate absorbance + blank absorbance

= test - 0.210 - 0.049 + 0.045

= test - 0.214

Optical densities

5	0.526
10	1.096
15	1.517

From glucose standard, nmole/ml = OD(1901.2) - 26.2

Glucose Concentration (nmol/ml)

5	973.8
10	2057.5
15	2857.9

With a zero intercept, the slope of this line is 193.1 nmol/min-ml.

k_m substrate concentration which gives half of maximum velocity, mol/l

m units 10^{-3} International Standard unit of enzyme activity, defined in Sect. 3.1.

[P] concentration of product, mol/l

[S] concentration of substrate, mol/l

t time, s

v d[P]/dt = rate of product formation velocity, mol/l-s

v_{max} maximum velocity, mole/l-s

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