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Enhanced Glucose Production from Cellulose Using Coimmobilized Cellulase and β-Glucosidase

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ABSTRACT

β-Glucosidase was covalently immobilized alone and coimmobilized with cellulase using a hydrophilic polyurethane foam (Hypol® FHP 2002). Immobilization improved the functional properties of the enzymes. When immobilized alone, the K_m for cellobiose of β-glucosidase was decreased by 33% and the pH optimum shifted to a slightly more basic value, compared to the free enzyme. Immobilized β-glucosidase was extremely stable (95% of activity remained after 1000 h of continuous use). Coimmobilization of cellulase and β-glucosidase produced a cellulose-hydrolyzing complex with a 2.5-fold greater rate of glucose production for soluble cellulose and a four-fold greater increase for insoluble cellulose, compared to immobilized cellulase alone. The immobilized enzymes showed a broader acceptance of various types of insoluble cellulose substrates than did the free enzymes and showed a long-term (at least 24 h) linear rate of glucose production from microcrystalline cellulose. The pH optimum for the coimmobilized enzymes was 6.0. This method for enzyme immobilization is fast, irreversible, and does not require harsh conditions. The enhanced glucose yields obtained indicate that this method may prove useful for commercial cellulose hydrolysis.

Index Entries: β-Glucosidase; cellulase; polyurethane foam; enzyme immobilization.

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INTRODUCTION

Cellulose is the storage form of about half of the photosynthetic biomass produced annually, and represents, therefore, a vast renewable energy source that has yet to be fully used (*1*). The key to exploitation of this resource is the development of technology for breaking down cellulose into its component sugars, sugars that can be used in a variety of food industry and biotechnological processes (*2*). Extensive current research is focused on the enzymatic, rather than chemical, degradation of cellulose as the most promising method of cellulose processing (*2*).

Cellulases are multienzyme systems that perform the net conversion of cellulose to glucose. The system is composed of three enzyme types: endoglucanases, cellobiohydrolases, and β-glucosidases (*3*). Endoglucanase randomly hydrolyzes the β-1,4 links of the cellulose chains. Cellobiohydrolase sequentially cleaves cellobiose, a disaccharide, from the nonreducing end of the cellulose chain. β-Glucosidase (or cellobiase) then breaks the β-1,4 linkage of cellobiose to yield two glucose molecules (*1*).

Natural cellulases from most fungal sources are limited in their overall activity by low amounts of β-glucosidase (*4*) and by cellobiose inhibition of cellobiohydrolase (*1*). Although advantageous to the growth of the fungus, these characteristics are inconvenient for the industrial use of cellulase in cellulose processing. Improved cellulose hydrolysis and increased glucose yield can be achieved under laboratory or industrial conditions by combining additional β-glucosidase with fungal cellulases (*1*). However, β-glucosidase is an expensive enzyme to produce and, if used in a soluble form that cannot be recycled, is not cost effective for industrial use. Techniques for the immobilization of β-glucosidase appear, therefore, to be critical to the development of a practical system for cellulose hydrolysis.

Previous studies have presented techniques for the immobilization of β-glucosidase, but these had major limitations owing to the use of support media and enzyme sources that were impractical for cellulose hydrolysis (*1,5,6*). This work presents a method for the immobilization of β-glucosidase in a polyurethane foam (Hypol® FHP 2002; W. R. Grace Co.), a support that is not only resistant to enzymatic and bacterial degradation, but has a variety of advantageous mechanical properties (flexibility, high additive loading capacity, high water absorbance, wide porosity range, adaptability of size and shape) (*7,8*; W. R. Grace Co. product information). We have recently used this foam for the immobilization of cellulase with results that show improved kinetic properties, increased physical stability, and relatively increased activities with insoluble cellulose substrates by the immobilized enzyme, compared to free cellulase (*9*). The present study also reports the coimmobilization of cellulase and β-glucosidase and demonstrates the improved kinetic properties of the enzyme system and the improved glucose yield possible over cellulase function alone.

MATERIALS AND METHODS

Chemicals

Biochemicals and coupling enzymes were purchased from Sigma Chemical Co., J. T. Baker Chemical Co. or Boehringer Mannheim, Corp.

Polymer

The foamable hydrophilic prepolymer (Hypol® FHP 2002) was graciously supplied by W. R. Grace and Co., Lexington, MA. This polymer, a derivative of toluene diisocyanate, is easy to use because polymerization is water activated (W. R. Grace Co., product information). The isocyanate group of the polymer reacts with water to produce an unstable carbamic acid. The carbamic acid spontaneously breaks down to form an amine and simultaneously generate carbon dioxide, which serves as the inflating agent for the foam. The amine group reacts with other prepolymer isocyanate groups to produce urea linkages, with the process repeating itself to produce extensive crosslinking (W. R. Grace Co., product information).

Immobilization of β-Glucosidase and Cellulase

β-Glucosidase (Novozym™ 188) from *Aspergillus niger* and cellulase (Celluclast®) from *Trichoderma reesei* were kindly provided by Novo Industries, Bagsvaerd, Denmark. The typical amounts of enzyme immobilized were: 2 U for β-glucosidase and 30 U for cellulase (where 1 U=1 μmole glucose produced/min under standard conditions defined by Novo Industries).

The standard buffer used throughout was 20 m*M* sodium acetate at pH 4.5. Enzyme(s) was dissolved in 2 mL of buffer in a petri dish. A 1.0 g aliquot of prepolymer was then added and mixed by vigorous agitation until extensive polymerization was detected visually and by increased viscosity. The resulting foams were allowed to cure at room temperature for at least 20 min.

After polymerization was completed, the foams were washed with 6 mL of the acetate buffer. The foams were then squeezed repeatedly to ensure complete absorption of the wash into the foam.

Cellulase and β-Glucosidase Activity

Cellulase and β-glucosidase activity were measured as glucose liberated from various forms of cellulose and cellobiose, respectively. Standard reactions were carried out at 23°C. Substrates were added to 20 m*M* sodium acetate buffer, pH 4.5 at concentrations of 3.0 m*M* for cellobiose and 1% (w/v) for carboxymethylcellulose (CMC), unless otherwise indicated. The CMC (CMC(7LF)), a soluble cellulose derivative (mw 70–110 000), was kindly provided by Hercules Inc., Wilmington, DE.

To carry out a reaction, the foams were first squeezed to remove the wash buffer, then 4 mL of the appropriate substrate was added and foams were again squeezed to aid absorption of the substrate. At timed intervals, foams were again squeezed and an aliquot of reaction mixture (0.1 mL) was removed for the determination of glucose. Samples were mixed with Tris buffer (pH 9.5) in a 1:1 ratio. The resulting pH change stopped any β-glucosidase and cellulase activity. If necessary, excess Tris buffer was added to dilute samples with high glucose concentrations before assay of glucose content. Glucose was determined enzymatically by measuring the reduction of NAD^+ at 340 nm using the hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme assay of Lowry and Passonneau (*10*). Hexokinase (0.28 U) was added to a 1-mL solution of buffer (100 m*M* Tris, pH 8.0), NAD^+ (0.50 m*M*), $MgSO_4$ (5.0 m*M*), ATP (0.50 m*M*), glucose-6-phosphate dehydrogenase (0.10 U) and the test sample (0.100 mL).

Reaction conditions for the free enzymes used the equivalent amount of enzyme to that added to foams (2 U β-glucosidase, 30 U cellulase) added to 4 mL acetate buffer containing the appropriate substrate.

Protein Determination

Protein was measured by the Coomassie Blue dye binding method using the BioRad Laboratories prepared reagent. Total protein (before addition to the foam) and protein recovered in the wash buffer were measured; protein bound to the foam was determined by subtraction. Direct measurements of foam-bound protein were not possible since the foam reacted with the dye. Foams polymerized without added protein provided controls to show that dye-reacting material from the foam was not released into the wash buffer.

Kinetic Parameters

The kinetic parameters for β-glucosidase were determined using cellobiose as the substrate. Glucose production after 30 min at room temperature was determined for reactions using 11.7, 5.8, 2.9, 2.0, 1.5, 1.0, and 0.7 m*M* cellobiose for both the free and immobilized enzymes. K_m and V_{max} values were calculated from Lineweaver-Burke (double reciprocal) plots.

Polyethylene Glycol (PEG) Effects

Kinetic parameters of free β-glucosidase and cellulase were determined in the presence or absence of 10% (w/v) PEG 8000 (Sigma). The PEG was mixed with the substrate prior to addition to the enzyme.

Salt Effects

Free and immobilized β-glucosidase were tested for the ability to breakdown cellobiose in the presence and absence of 2 *M* KC1. The salt solution was mixed with the substrate prior to addition to the enzyme.

Insoluble Substrates

The ability of coimmobilized β-glucosidase and cellulase to break down insoluble celluloses was tested using four substrates: cellulose acetate, microcrystalline cellulose, cellulose (boiled in acid) (all obtained from Sigma Chemical Co.), and Solka Floc (BW 40) (obtained from Iogen, Ottawa). All were added as 4 mL of 1% (w/v) suspensions.

Optimum Temperature

Glucose production after 1 h was measured at a variety of temperatures in order to establish the optimum temperature for β-glucosidase function.

Operational Half-Life

Foams containing immobilized β-glucosidase or coimmobilized cellulase and β-glucosidase were tested for their ability to hydrolyze cellobiose and CMC, respectively, over a 6-wk period. Glucose production after 1 h at 23°C was measured. Foams were thoroughly washed before each use and then fresh substrate was added. Between uses foams were stored at 4°C in buffer containing 0.04% sodium azide.

Optimum pH

Foams were washed with 6 mL of 20 m*M* acetate buffer of the appropriate pH. Free enzyme samples and CMC or cellobiose substrates were also prepared in buffer of the desired pH. Glucose release was measured after 30 min (free) or 1 h (immobilized) at 23°C.

Data Calculations

Data are reported as means ± SEM, $n = 3$. Where SEM bars are not shown in figures, these values were within the dimensions of the symbol used.

RESULTS

Enzyme Properties of Immobilized and Free β-Glucosidase

Relative to an equal amount (2 U) of β-glucosidase free in solution, 74 ± 0.6% of the total β-glucosidase activity was immobilized into the foam. Remaining activity was recovered in the first 6 mL wash of the foam with no activity found in subsequent washes. Measurements of protein agreed closely with 73% of total protein bound, 27% in the wash. The recovery of 167% of the added activity suggests that the apparent activity of the enzyme rises when it is immobilized.

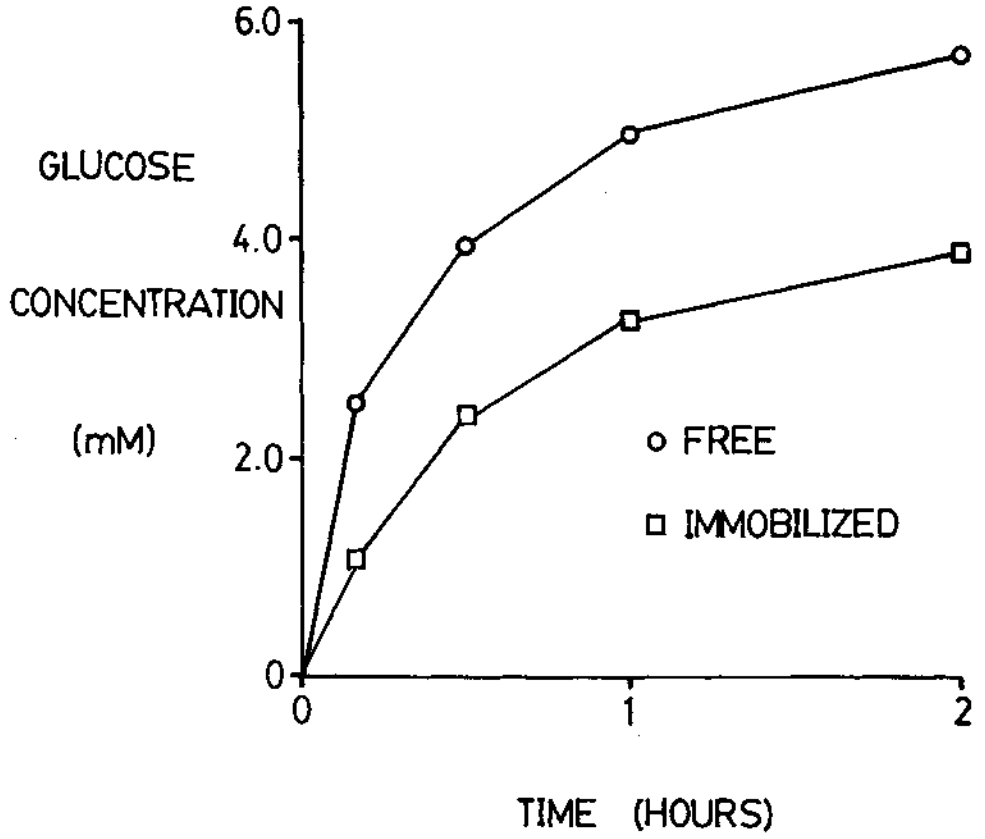

Fig. 1. Time course of glucose production by free vs immobilized β-glucosidase. Reaction conditions were 4 mL of 3.0 m*M* cellobiose as substrate in 20 m*M* acetate buffer, pH 4.5 and incubation at 23°C. Data are means ± SEM, $n = 3$. Where error bars are not shown, SEM values were within the dimensions of the symbols used.

The kinetic parameters of β-glucosidase were significantly altered by immobilization. The K_m for cellobiose dropped by approximately 35% upon immobilization (from 7.11 m*M* ± 0.82 to 4.59 m*M* ± 0.18). The calculated V_{max} also decreased by about one-half (from 410 ± 40 to 190 ± 4 nmoles glucose min^{-1} mL^{-1}) when immobilized, which is partially accounted for by the percentage activity bound (74%). The K_{cat} ($= V_{max}/K_m$) value was unchanged for the free vs the immobilized enzymes.

The time course of cellobiose hydrolysis using free vs immobilized β-glucosidase is shown in Fig. 1. Half-times for maximal glucose production were about 15 min for the free enzyme and 30 min for the immobilized enzyme, consistent with V_{max} values for the enzymes noted above.

Table 1 shows that the activity of both free and immobilized β-glucosidase increased with increasing temperature up to 50°C; i.e., no thermal inactivation was observed.

Table 1
Effect of Temperature on β-Glucosidase Activity

Temperature	Free enzyme	Immobilized enzyme
	nmol glucose min^{-1} mL^{-1}	
1°C	52± 1.0	32±1.3
20°C	108± 0.3	55±2.0
40°C	139± 7.3	86±1.3
50°C	202±11.0	120±2.7

The effect of pH on β-glucosidase activity is shown in Fig. 2. Immobilization resulted in a small shift in optimum pH from 4.25 to 4.50. More importantly, the immobilized enzyme exhibited increased activity at more basic pH values. Free β-glucosidase showed no activity at pH 7.0 or above, whereas the immobilized enzyme showed activity even at pH 9.0.

High salt concentrations are often present in industrial cellulose products such that enzymes of a cellulose hydrolyzing complex must be capable of functioning in the presence of high salt. Free and immobilized β-glucosidases were tested to see how their activity would be affected by 2 *M* KCl. Immobilization offered no protection against the inhibitory effects of salt on β-glucosidase; the same result was found for immobilized cellulase (*9*). Indeed, the immobilized enzyme was inhibited by 2 *M* KCl to a slightly greater extent than the free enzyme (60% activity loss vs 45%). This may have been owing to physical shrinking of the foams in the presence of KCl, causing increased diffusional barriers to substrate permeation of the foam.

Figure 3 shows the changes in activity noted for immobilized β-glucosidase over a 6-wk period. There was almost no decrease in β-glucosidase activity observed (95% activity remained after 6 wk). β-Glucosidase, therefore, appears to be quite stable once immobilized.

Coimmobilization of Cellulase and β-Glucosidase

Cellulase from *T. reesei* was supplemented with *A. niger* β-glucosidase and the two enzymes were coimmobilized in the same foam. Compared to the CMC-hydrolyzing activity of a mixture of the two enzymes (30 U cellulase, 2 U β-glucosidase) in solution, coimmobilization of cellulase and β-glucosidase resulted in 65.8±2.7% of the enzymatic activity bound to the foam. Protein measurements indicated that 74.5% of total protein was immobilized.

Table 2 shows the effect of coimmobilization on the hydrolysis of soluble (CMC) and insoluble (microcrystalline cellulose) cellulose. Supplementation of cellulase with β-glucosidase greatly improved cellulose breakdown, particularly the hydrolysis of the insoluble cellulose substrate.

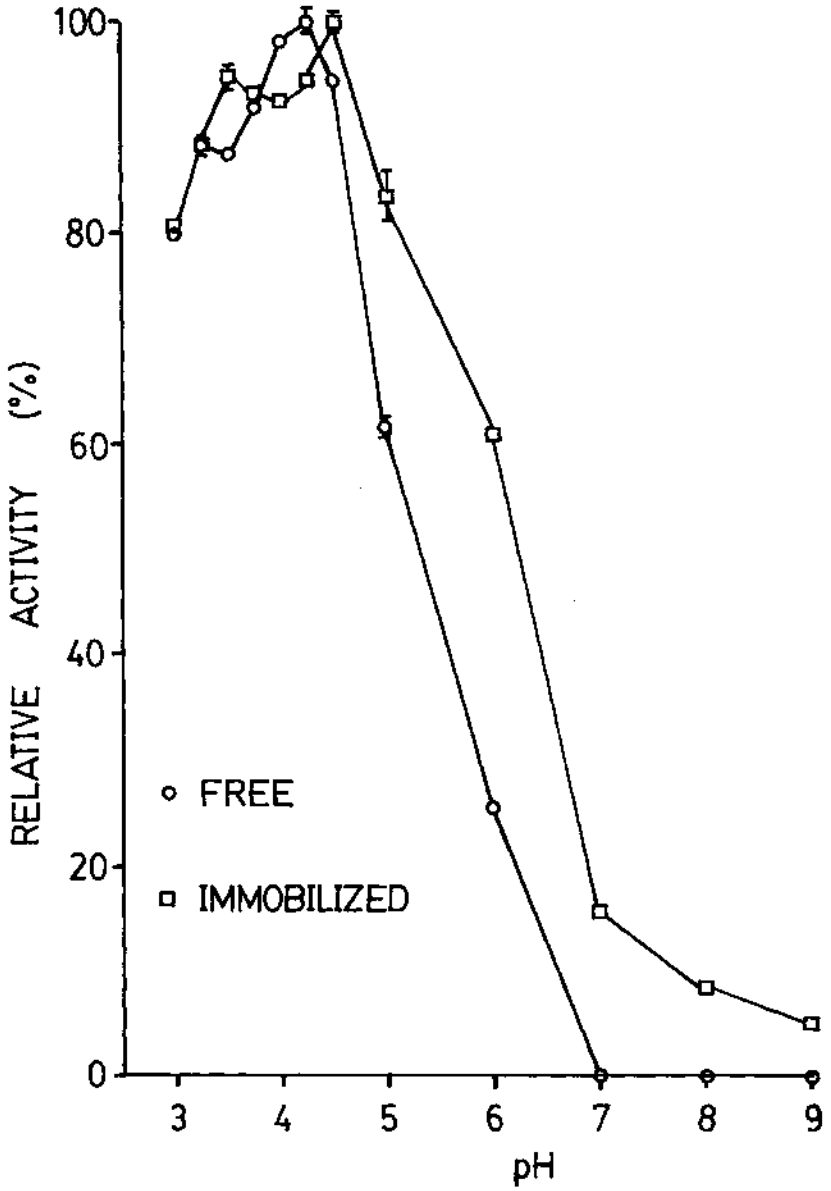

Fig. 2. Effect of pH on the activity of free versus immobilized β-glucosidase. Reaction conditions were 4 mL of 3.0 m*M* cellobiose as substrate in 20 m*M* acetate buffer and incubation at 23°C for 30 min (free) or 60 min (immobilized).

Addition of β-glucosidase increased the glucose yield by approximately three-fold for the free and immobilized enzymes using the soluble cellulose substrate (CMC). A four-fold increase was noted for the immobilized enzyme using the insoluble substrate (microcrystalline cellulose), whereas a 70-fold increase in the glucose yield was observed for the corresponding free enzymes. The soluble cellulose could apparently be utilized by cellulase with or without additional β-glucosidase. However, the insoluble

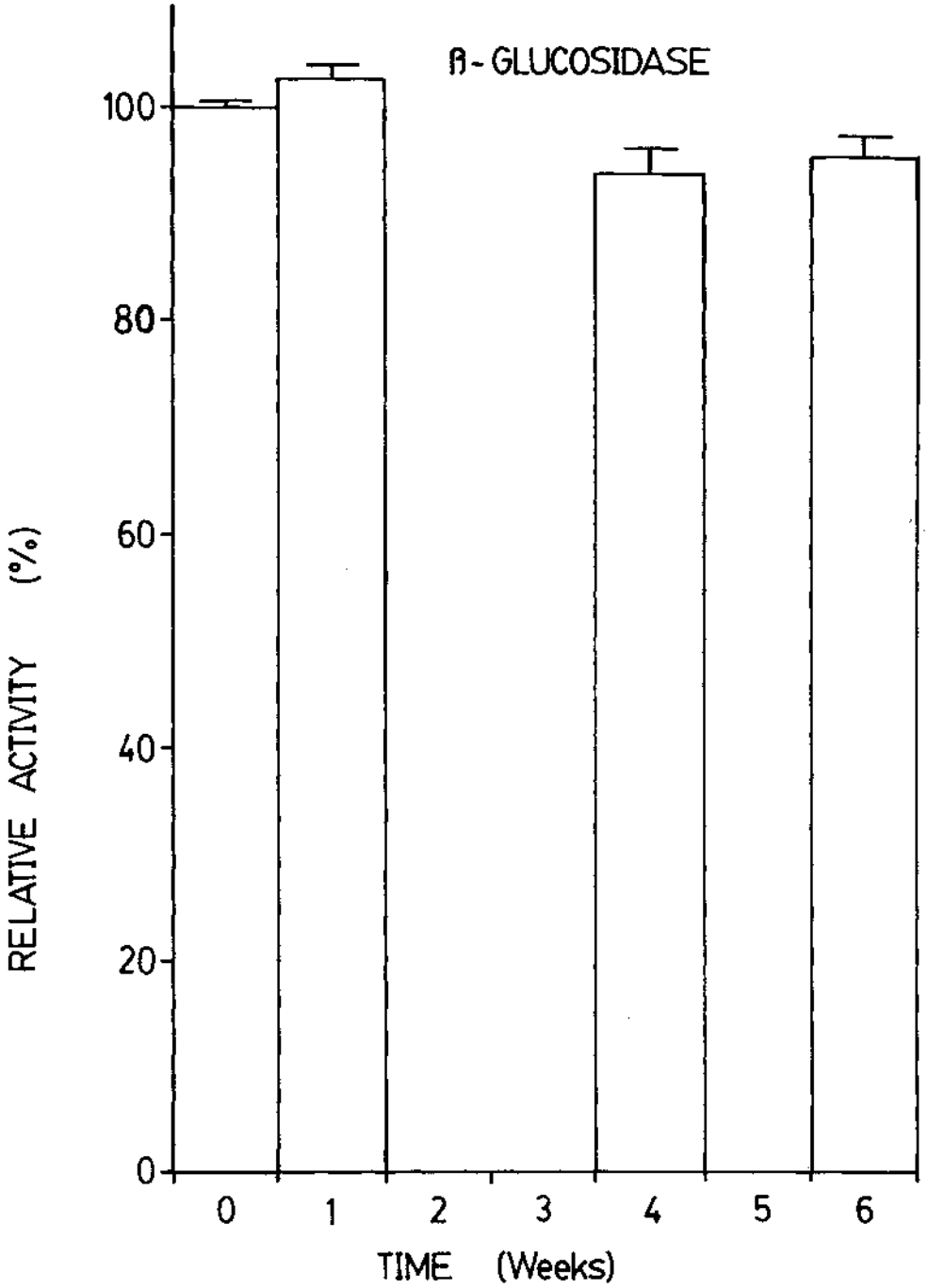

Fig. 3. β-Glucosidase activity over a 6-wk period. Activity was determined at 23°C using 3.0 m*M* cellobiose as substrate. Foams were stored at 4°C in substrate containing 0.04% (w/v) sodium azide between activity measurements.

Table 2
Supplementation of Cellulase with β-Glucosidase:
Cellulose Breakdown by Both Free and Immobilized Enzymes[a]

	Carboxymethyl cellulose	Microcrystalline cellulose
	μmol glucose released per mL per 2 h	
Cellulase		
Free	1.14±0.03 (20.2%)	0.08±0.003 (1.4%)
Immobilized	0.92±0.01 (16.3%)	0.25±0.01 (4.4%)
Cellulase + β-glucosidase		
Free	3.48±0.08 (61.6%)	5.65±0.07 (100%)
Immobilized	2.31±0.06 (40.9%)	0.97±0.03 (17.2%)

[a]Data are means±SEM, $n=3$, assay temperature 23°C. 1% (w/v) solutions of cellulose substrates were used. Numbers in brackets are percentages relative to maximum activity observed.

cellulose required the presence of both cellulase and β-glucosidase for significant hydrolysis to occur.

Figure 4 shows the time courses of degradation for CMC (soluble) and microcrystalline cellulose (insoluble) by mixtures of cellulase and β-glucosidase in free vs coimmobilized states. Neither the free nor the immobilized enzymes made effective use of CMC as a substrate; although initial rates of hydrolysis were similar to those with microcrystalline cellulose as the substrate, total accumulation of glucose represented a degradation of only about 6% of the total cellulose substrate (1% w/v cellulose = 56 m*M* as glucose). However, hydrolysis of microcrystalline cellulose was linear over the 24 h for both the free and immobilized enzymes. The rate of glucose production was substantially higher for the free enzymes, but this reflects the percentage of added cellulase and β-glucosidase that are immobilized in the foam (*see above and* (*9*)).

Polyethylene glycol (PEG) is a large inert polymer that crowds proteins by restricting water availability (*11*). The kinetic parameters of a free mixture of cellulase plus β-glucosidase were determined in the presence and absence of PEG. In the presence of PEG, both parameters decreased by about 20–25% (K_m decreased from 24.4 to 19.2 mg/ml and V_{max} decreased from 120 to 90 nmoles glucose min^{-1} mL^{-1}). The effect on K_m values indicates that enzyme crowding can result in more efficient catalysis.

The pH profiles for the mixture of cellulase and β-glucosidase are given in Fig. 5. The immobilized enzymes, at both 60 and 20°C, and the free enzymes at 60°C all showed maximum activity at pH 6.0. The immobilized enzymes were again more active at neutral and basic pH values than the free enzymes.

Preliminary experiments were conducted to assess the relative effectiveness of mixtures of cellulase and β-glucosidase in hydrolyzing various insoluble forms of cellulose. Of the insoluble celluloses, enzyme activity

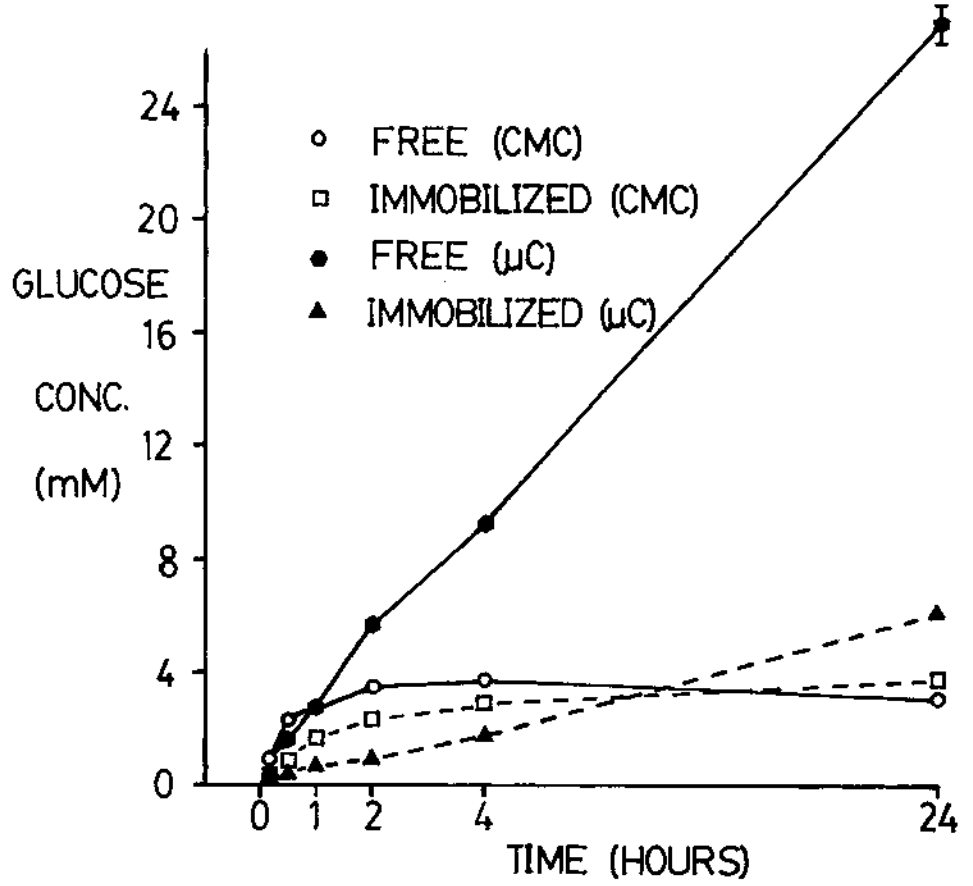

Fig. 4. Time course of glucose production by cellulase plus β-glucosidase, free vs immobilized, with either carboxymethylcellulose (CMC) or microcrystalline cellulose (μC) as the substrate. Reaction conditions were 4 mL 1% cellulose, 20 m*M* acetate buffer, pH 4.5 and 23°C. Data are means ± SEM, $n = 3$. Where error bars are not shown, SEM values were within the dimensions of the symbols used.

was highest with microcrystalline cellulose for both the free and immobilized enzymes. Activity with this substrate for the free enzymes was about threefold higher than that with Solka Floc or cellulose boiled in acid, whereas cellulose acetate was not hydrolyzed by the free enzymes. The immobilized enzymes, however, hydrolyzed all four substrates tested at relatively high rates, indicating broader substrate acceptance.

Figure 6 shows the declines in activity noted for coimmobilized cellulase and β-glucosidase over a 6-wk period. The half-life for the coimmobilized enzymes was approximately 3 wk (500 h). A sharp decline in activity was noted after 1 wk (only 69% if the initial activity remained). However, after 2 wk the activity remained quite stable at about 50% of the initial value.

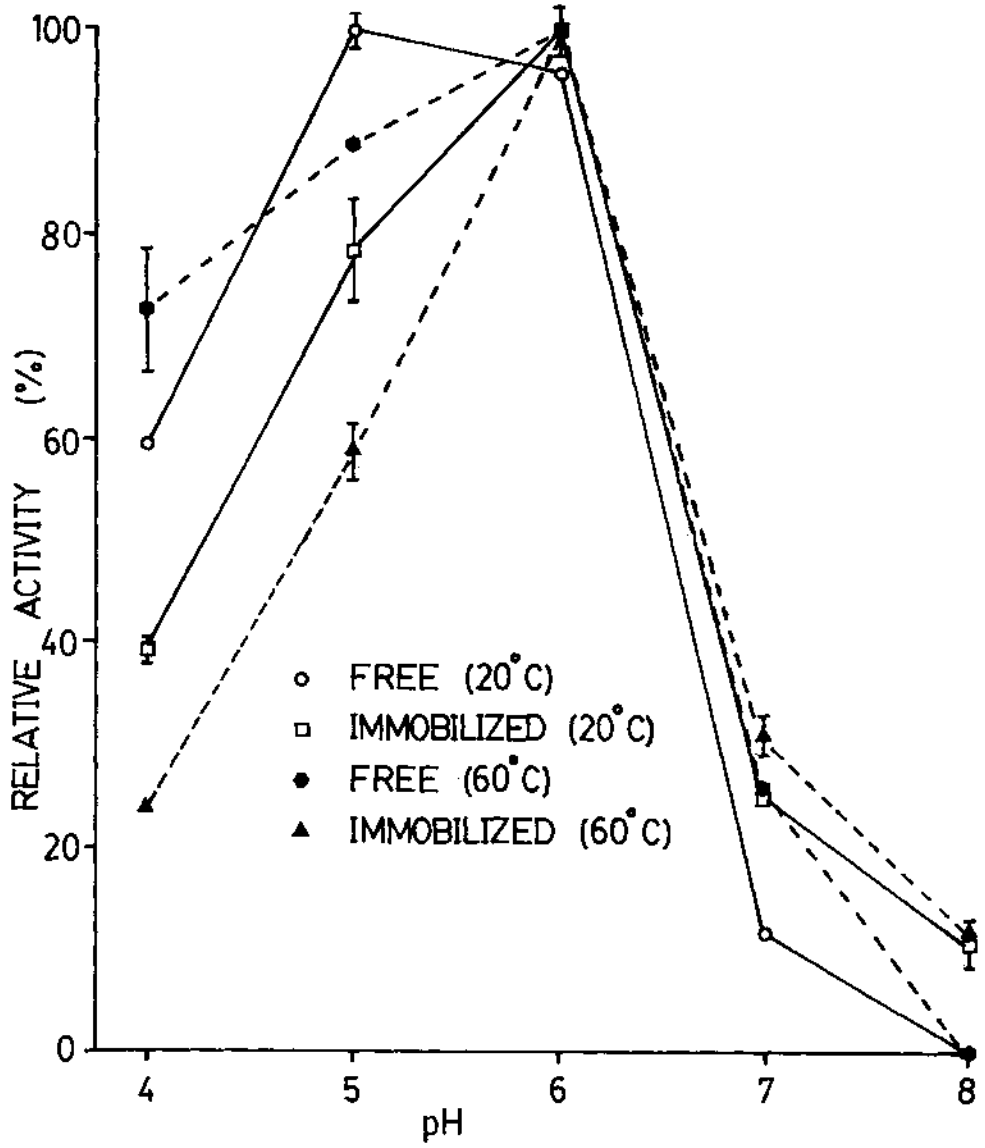

Fig. 5. Effect of pH on the activity of cellulase plus β-glucosidase, free vs immobilized, at two temperatures 20 and 60°C. Reaction conditions were 4 mL of 1% carboxymethylcellulose in 20 m*M* acetate buffer.

Loss of cellobiohydrolase (the enzyme in the cellulase complex responsible for cleaving cellobiose from cellulose) activity could account for the shortened half-life of the coimmobilized enzymes (*9*). β-Glucosidase would become substrate limited as a result, and, therefore, the combined overall activity of the two enzymes would decline.

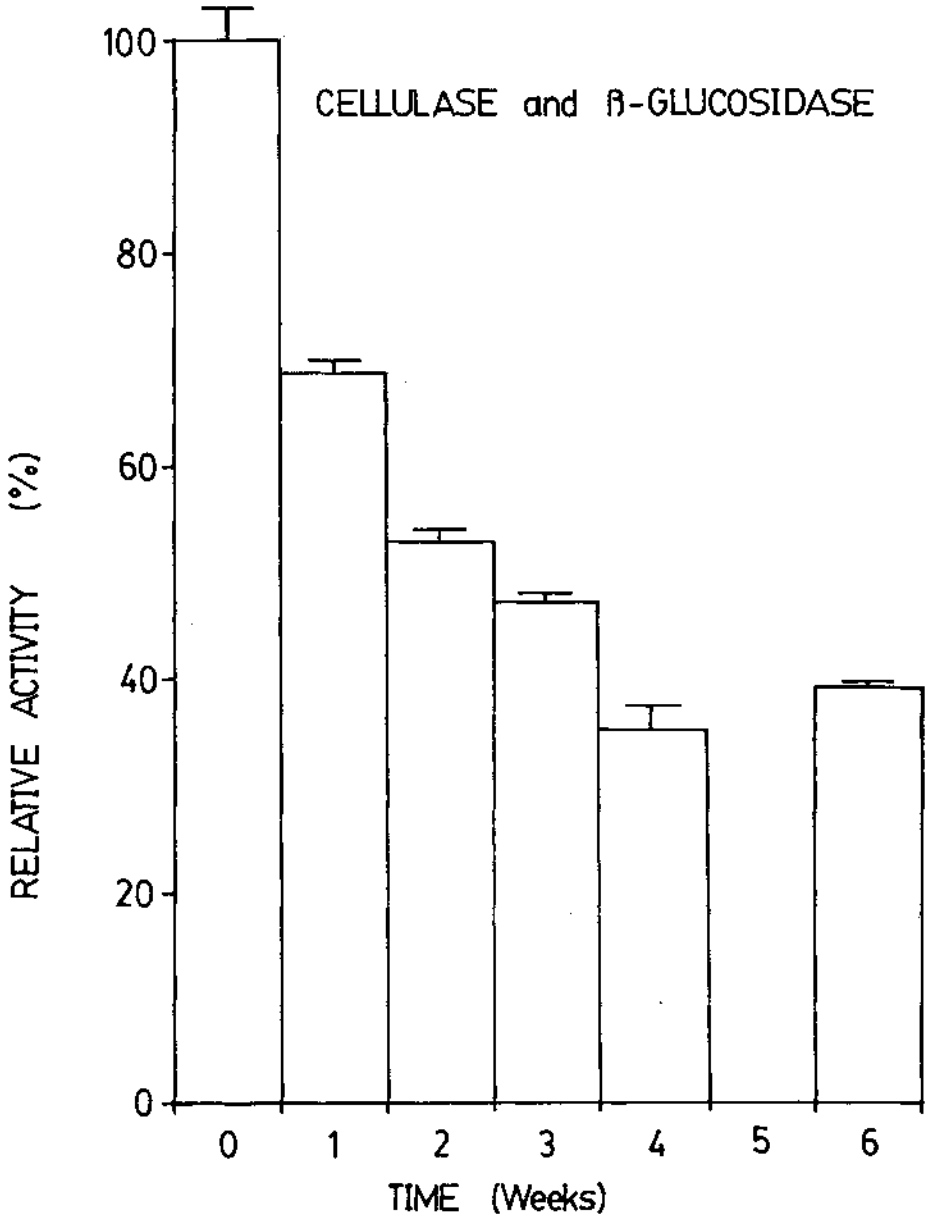

Fig. 6. Changes in the combined activities of cellulase and β-glucosidase over a 6-wk period. Activity was determined at 23°C using 1.0% carboxymethylcellulose as substrate. Foams were stored at 4°C in substrate containing 0.04% (w/v) sodium azide between activity measurements. Data are means ± SEM, $n = 3$.

DISCUSSION

Covalent attachment of enzymes to the foam prepolymer is thought to take place via a primary amino group on the enzyme reacting with a foam isocyanate group (*9;* W. R. Grace, product information). This new method for immobilizing β-glucosidase presents several advantages over methods previously used (*1,5*).Immobilization can be achieved quickly (within several minutes) and without the use of harsh conditions of temperature or pH. This method is also better than or equal to other techniques in terms of the amount of enzyme immobilized and effects of immobilization on enzyme kinetic parameters and pH optima. The polyurethane foam is also resistant to enzyme or microbial attack, unlike previously used supports. The retention of added β-glucosidase activity within the foam (74% of total activity, 73% of total protein) was substantial and similar to that found in previous studies. Sundstrom et. al. (*1*) reported about 60% retention of β-glucosidase activity using alumina adsorption, whereas Matteau and Saddler (*5*) reported 67% retention using encapsulation of mycelial-associated β-glucosidase in calcium alginate beads.

Immobilization improved the kinetic parameters of β-glucosidase, decreasing the K_m for cellobiose by 35%, the K_m value of 4.6 m*M* being similar to that reported for β-glucosidase immobilization onto alumina (K_m=2.7 m*M*) (*1*). Hence, the polyurethane foam-immobilized enzyme appears to possess similar substrate affinity to that of other free and immobilized β-glucosidases (*12*).

Other advantageous properties of the foam-immobilized β-glucosidase included the long term stability of enzyme activity during storage (approx 95% activity remaining after 1000 h) and the improved enzyme functioning at higher pH values (both a shift in pH optima and a retention of activity above pH 7). Long term stability has also been reported for other forms of β-glucosidase immobilization, extending up to 500 h with less than 10% loss of activity (*1,5*). Other mechanisms of immobilization, however, did not alter the pH optimum of β-glucosidase; an optimum of pH 3.5 was reported for the enzyme immobilized on alumina (*1*). Foam immobilization may be particularly advantageous, therefore, when it is necessary to carry out substrate hydrolysis at higher pH values, as when coimmobilization of enzymes is used (*see below*).

All these observations make our method for β-glucosidase immobilization appear to be equal or superior to most other techniques used. However, the primary goal of our research was to improve the efficiency of cellulose hydrolysis by coimmobilizing cellulase and β-glucosidase into the same foam. To date, such coimmobilization has not been reported. Other studies focused on the immobilization of β-glucosidase alone or the use of β-glucosidase to supplement cellulase function in a free (soluble) enzyme system (*1,5,13*). We feel, therefore, that we are not only presenting a new method for immobilizing β-glucosidase, but are actually developing a new strategy for approaching cellulose hydrolysis.

Previous work with cellulase alone (*9*) had established that immobilization in the polyurethane foam system used created no significant limitations to the diffusion of substrate (cellulose). Both Arrhenius and Lineweaver-Burke plots supported this conclusion (*9*). An Arrhenius plot for β-glucosidase alone showed a linear relationship over a broad range of temperatures (1–50°C), indicating an absence of diffusional limitations. Hence, it was concluded that diffusional limitations imposed by the foam played an insignificant role on cellulose hydrolysis by coimmobilized cellulase and β-glucosidase.

Preliminary experiments established that 2 U of β-glucosidase was optimal for enhancing the glucose yield from cellulase (30 U) function in a free mixture of the enzymes. This represents a fivefold increase in the total β-glucosidase content of the system; Klyosov (*4*) reported that the β-glucosidase content of *T. reesei* cellulase was about 0.4 U. Addition of supplemental β-glucosidase, therefore, not only speeds cellobiose hydrolysis but relieves the cellobiose inhibition of cellobiohydrolase (*13*), thereby improving the activity of that enzyme and of the entire enzyme complex. Coimmobilization of the two enzymes in the polyurethane foam greatly enhanced the rate of cellulose hydrolysis and the net yield of glucose, compared with the results when cellulase was immobilized alone (*9*). Rates of glucose production using CMC or microcrystalline cellulose as substrates were 2.5- or 4-fold greater, respectively, for the coimmobilized enzymes compared to immobilized cellulase alone (*9*; Table 2). Part of the advantage of coimmobilization may be simply owing to the closer packing of the enzymes within the foam. Thus, crowding the enzymes by limiting water availability with the addition of polyethylene glycol slightly improved the kinetic properties for cellulose hydrolysis by the free enzymes, K_m for CMC decreasing by 20%.

The coimmobilization of β-glucosidase and cellulase had several other advantages. Firstly, all four types of insoluble celluloses were hydrolyzed by the coimmobilized enzymes, indicating the use of insoluble substrates by the enzymes was possible. In addition, only the immobilized enzymes could hydrolyze cellulose acetate. Thus, the immobilized enzymes have broader substrate acceptances, perhaps indicating that immobilization positions the enzymes (individually or in a complex) such that they can better bind to all types of insoluble cellulose substrates. The coimmobilized enzymes were also able to maintain a long term linear rate of hydrolysis of microcrystalline cellulose (as were the free enzymes), indicating that coimmobilization overcomes the problems of cellobiose feedback inhibition of the cellulase complex and permits a long term, high level accumulation of glucose endproduct. Indeed, Ward (*14*) has shown that the activity of cellobiohydrolase with insoluble substrates is greater than that with soluble cellulose when cellobiose inhibition has been removed owing to β-glucosidase addition. Increasing the rate of cellulose hydrolysis by removal of cellobiose is not the only important function served by the addition of β-glucosidase. Cellobiose removal is also essential if fermenta-

tion of the glucose product is anticipated; Tan et al. (*15*) have shown that the presence of cellobiose makes fermentation difficult.

The data also show that coimmobilization has an important effect on the pH optima for cellulose hydrolysis. Immobilization shifts the pH optimum to a more basic value for both individual components. The pH optimum of cellulase shifts from a rather sharp optimum between pH 5.0 and 5.5 in the free state to a broad, flat optimal range between 5.0 and 6.0 in the immobilized state with continuing high activity above pH 6 (*9*). β-Glucosidase has an optimum of 4.25 when free and 4.5 when immobilized (Fig. 2). Together, the two enzymes have a clear pH optimum of 6.0 (Fig. 4) as well as significant activity at pH values of 7 and 8. Such a basic shift in the optima of the coimmobilized enzymes, compared to the free enzymes or the individually-immobilized enzymes, is potentially key to a further step in the processing of cellulose: addition of glucose isomerase to the immobilized enzyme complex to allow complete conversion of cellulose to fructose. The resulting foam reactor could convert cellulose into fructose (a valuable sweetening agent) using moderate conditions and with the potential for complete enzyme recovery and reuse. Development of just such a "one-step" reactor is now underway.

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