0273-2289/91/3601—0063$02.40

# Fructose Production

## Coimmobilized Amyloglucosidase, Pullulanase, and Glucose Isomerase on BIOBONE™

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Received February 19, 1992; Accepted April 7, 1992

## ABSTRACT

Amyloglucosidase, pullulanase, and glucose isomerase were co-immobilized onto granular chicken bone (BIOBONE™). Enzyme ratios showing optimum glucose and fructose production (0.7:10:22.3 U amyloglucosidase: pullulanase: glucose isomerase) resulted in 14.4±1.9% of activity bound relative to an equal amount of free enzyme. The estimated specific activity for these enzymes decreased 4.6-fold with immobilization. Reaction pH strongly influenced the yield and ratio of glucose and fructose produced. Net hexose production from the immobilized system was optimal at pH 6.5 and 55°C with a fructose yield of about 20%.

**Index Entries:** Amyloglucosidase; chicken bone; pullulanase; enzyme coimmobilization; glucose isomerase; high fructose syrup.

## INTRODUCTION

High fructose syrups (HFS), derived from the enzymatic breakdown of various inexpensive and abundant starchy crops, have rapidly developed as alternatives to sucrose for use in sweetening food products (*1*). Presently, industrial starch hydrolysis and the isomerization of the resulting glucose syrup is conducted in two separate stages using very different

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reaction conditions. The preliminary step, which involves the use of amylases for the saccharification of starch to glucose, is conducted at pH 4–5, followed by the isomerization of glucose to fructose in a second step performed at pH 7–9 (*2*). Both of these enzyme reactions have been developed as immobilized enzyme systems bound to various solid supports. In industrial applications these immobilized enzymes offer the benefit of recovery and reuse of expensive enzyme catalysts (*3*). Further improvements to the process of HFS production could be gained by developing a single step conversion process that carries out the reactions of both saccharification and isomerization at a single pH. An immobilized enzyme system combining both of these reactions and utilizing a food-compatible solid support would be particulary advantageous.

Previous work in our lab has successfully developed immobilized enzyme systems bound to granular chicken bone (BIOBONE™), a by-product of the poultry industry. Systems of BIOBONE™-immobilized glucose isomerase, amyloglucosidase, and amyloglucosidase plus pullulanase have been developed (*4–6*). The mechanism of enzyme binding to BIOBONE™ is via adsorption, apparently largely as a result of ionic interactions with both the hydroxyapatite and the amino acid side chains of collagen; hydrophobic interactions with other side groups on collagen may also occur. Binding is strong with no enzyme desorption at salt concentrations of up to 1*M* and only pullulanase showing some loss of activity of the immobilized enzyme in the presence of high salt (*4–6*).

Immobilized glucose isomerase could function at very broad pH values (pH 6–9), and maintained high operating temperatures (> 55°C) (*8–11*). Coimmobilized amyloglucosidase and pullulanase were also functional over a broad pH range (pH 3–6.5) and showed enhanced rates of glycogen and starch hydrolysis over amyloglucosidase alone (*4,6*). These immobilized enzyme systems, therefore, seemed to have good potential for developing a coimmobilized system involving all three enzymes bound to BIOBONE™. Similar trials were involved in the development of a system of coimmobilized cellulase, $\beta$-glucosidase, and glucose isomerase bound to a polyurethane foam support for cellulose hydrolysis. We found that immobilized cellulase and $\beta$-glucosidase retained activity at higher pH values than did the free enzymes and this allowed them to be combined with glucose isomerase to create an effective one-step conversion process (*11*).

Presented here are studies of a coimmobilization system involving amyloglucosidase, pullulanase, and glucose isomerase bound to BIOBONE™ and an analysis of the system for fructose output from polysaccharide substrates. Development emphasized the determination of specific temperature and pH values for obtaining optimal glucose and fructose production.

## MATERIALS AND METHODS

### Materials

Amyloglucosidase from *Aspergillus niger* was obtained from Sigma Chemical Co.; 1 unit is defined as the amount of enzyme that liberates 1.0 mg of glucose from starch in 3 minutes at pH 4.5 and 55°C. Pullulanase from *Bacillus sp.* was donated by Novo Industries, Bagsvaerd, Denmark; 1 U is defined as the amount of enzyme that hydrolyses pullulan under standard conditions, releasing reduced carbohydrates equivalent to 1 μmol glucose per minute. Glucose isomerase from *Streptomyces rubiginosus* was donated by Finnsugar Chemical Co.; 1 U is defined as the amount of enzyme that converts glucose to fructose at an initial rate of 1 μmol/min under specified conditions, which include 2.0 mol/L glucose substrate and pH 7.0 at 60°C. Other biochemicals were purchased from Sigma Chemical Co.,J. T. Baker Chemical Co., or Boehringer Mannheim Corp.

Clean granular chicken bone, which had been previously stripped using hot aqueous NaOH followed by washing and sizing to between 10 and 20 mesh (BIOBONE™), was donated by Protein Foods Research, Guelph, ON.

### Enzyme Coimmobilization

Before use, pullulanase and glucose isomerase were passed through columns of Sephadex G-25 equilibrated with acetate (100 m*M*, pH 4.5) or phosphate (50 m*M*, pH 8.0) buffer, respectively, to remove low mol wt contaminants. The standard amounts of amyloglucosidase, pullulanase, and glucose isomerase used in all experiments was 0.7, 10, or 22.3 U, respectively. For enzyme immobilization onto bone, standard amounts of amyloglucosidase and pullulanase in acetate buffer (pH 4.5, 100 m*M*) were added to 100±1 mg bone, and pre-equilibrated in this buffer, in a capped tube. Following mixing for 30 min, the buffer was poured off and replaced with phosphate buffer (pH 8, 50 m*M* containing 2 m*M* bisulfite and 1.5 m*M* $Mg^{2+}$) containing the standard amount of glucose isomerase. Following a second 30-min mixing, excess buffer was poured off and the bone was washed four times in succession with 1 mL phosphate buffer (pH 6.5, 50 m*M*).

#### *Amyloglucosidase, Pullulanase, and Glucose Isomerase Assay*

The standard experimental conditions, unless stated otherwise, consisted of a 60-min reaction time at high temperature (55°C) with the enzyme (either free or bone-bound) added to 1 mL phosphate buffer (pH 6.5, 50 m*M*) containing 2 m*M* bisulfite, 1.5 m*M* $Mg^{2+}$, and glycogen (12%

w/v) as the substrate. Reactions were carried out in capped tubes that were gently shaken throughout. At the end of the reaction period, a 0.1 mL aliquot of the reaction mixture was removed and mixed with 0.1 mL of 1*N* NaOH to stop enzyme activity.

The hexose concentrations of samples were determined by monitoring the reduction of $NAD^+$ at 340 nm using a coupled enzyme assay (*7*). For glucose assay,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. After the glucose reaction had run to completion, fructose concentrations in the same samples were determined by the subsequent addition of phosphoglucose isomerase (0.07 U) to the reaction cuvet.

### *Experimental Protocol*

Details of the methodology for studying optimal enzyme amount, and the temperature and pH optima of the immobilized enzymes can be found in Schafhauser and Storey (*4*).

## RESULTS

Using conditions previously determined for the optimal immobilization to BIOBONE™ of amyloglucosidase + pullulanase (*6*) or glucose isomerase (*5*), the two-step binding procedure outlined in the Materials and Methods was developed to first immobilize amyloglucosidase + pullulanase during incubaton at pH 4.5 followed by the addition of glucose isomerase to the system during a second incubation at pH 8. The result of this procedure, following 10 washes of the bone with 50 m*M* phosphate buffer pH 6.5 (4 washes was sufficient for subsequent practical use), was a net coimmobilized activity of amyloglucosidase, pullulanase, and glucose isomerase activity (measured as total glucose + fructose produced) of $14.4 \pm 1.9\%$, compared with equal amounts of the enzymes combined and assayed free at the same pH. Activity eluted into the washes amounted to $44.2 \pm 5.1\%$ of the total, leaving a net activity loss of 41.4%. Amounts of the individual enzymes immobilized were impossible to determine in the coimmobilized system owing to the interconversion of the glucose and fructose products but are probably similar to the amounts immobilized when the enzymes were assessed individually (*4–6*). The specific activity of the bone-immobilized activities was $2.9 \pm 0.6$ nmol hexose produced/min/mg protein, a decrease compared with $13.4 \pm 1.8$ nmol hexose/min/mg for the free enzymes.

Bisulfite and magnesium are necessary cofactors for glucose isomerase activity. To determine if they had any adverse effects on the amyloglucosidase or pullulanase reactions, the activities of these two enzymes were

Table 1
Effect of Bisulfite and Magnesium on Free and Immobilized Amyloglucosidase and Pullulanase Activity

| | Activity (nmoles glucose produced/mL/min) | |
|---|---|---|
| | Free | Immobilized |
| Control (no addition) | 355±11 (100%) | 76.7±9.8 (100%) |
| 2 m*M* Bisulfite | 368±6 (103%) | 84.1±4.0 (110%) |
| 1.5 m*M* $Mg^{2+}$ | 367±23 (103%) | 65.1±5.8 (85%) |

Data are means±SEM, $n=3$. Biobone™ containing immobilized amyloglucosidase+ pullulanase was prepared as described in the Materials and Methods. Reactions were run at 55°C for 30 min in phosphate buffer, pH 6.5 with glycogen as the substrate. Relative activities are given in parentheses.

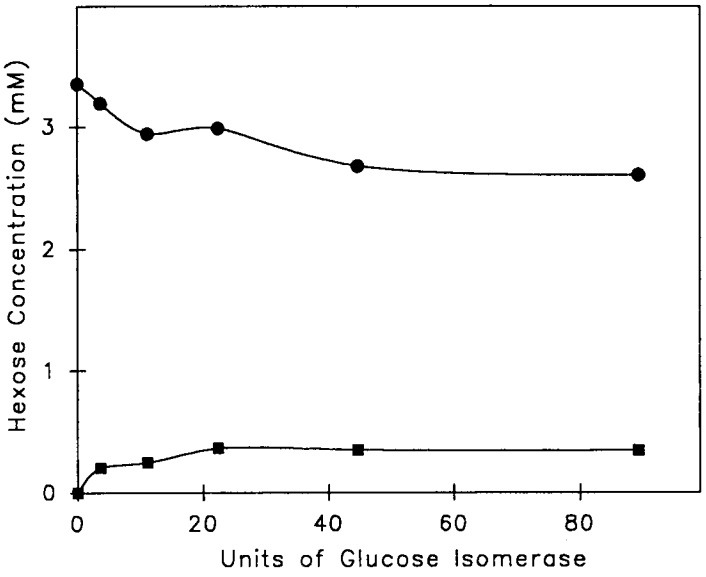


Fig. 1. Effect of the addition of increasing amounts of glucose isomerase to set amounts of amyloglucosidase (0.7 U) and pullulanase (10 U), on the final output of glucose and fructose from the system. Glycogen was used as the substrate with phosphate buffer, pH 6.5 and 55°C. Data are means±SEM, $n=3$. Where error bars are not shown, SEM values are within the dimensions of the symbols used. ●, glucose; ■, fructose.

tested in the presence or absence of these ions. As Table 1 shows, however, neither ion had a significant effect on the free or immobilized amyloglucosidase and pullulanase activities.

Figure 1 shows the effect on glucose and fructose production of increasing the amount of glucose isomerase coimmobilized with the set amounts of immobilized amyloglucosidase (0.7 U) and pullulanase (10 U). Fructose output increased with increasing glucose isomerase activity

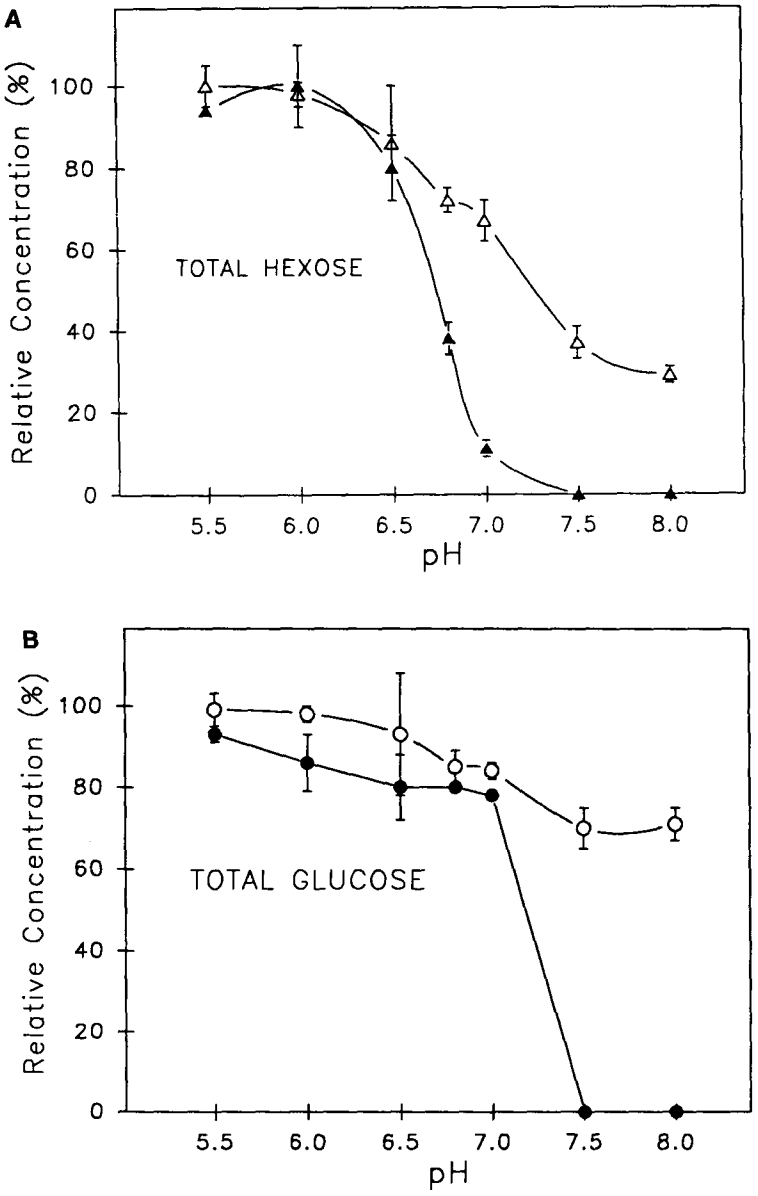


Fig. 2. Effect of pH on the total hexose (A) glucose; (B) fructose; (C) output by free and coimmobilized amyloglucosidase, pullulanase and glucose isomerase. Open symbols represent the free enzymes; filled symbols represent the immobilized enzymes. Total hexose production from glycogen is shown relative to the maximal output obtained at pH 5.5 whereas glucose and fructose levels show the percentages of each sugar produced at the different pH values. Data are means ± SEM, $n = 3$. Where error bars are not shown, SEM values are within the dimensions of the symbols used.

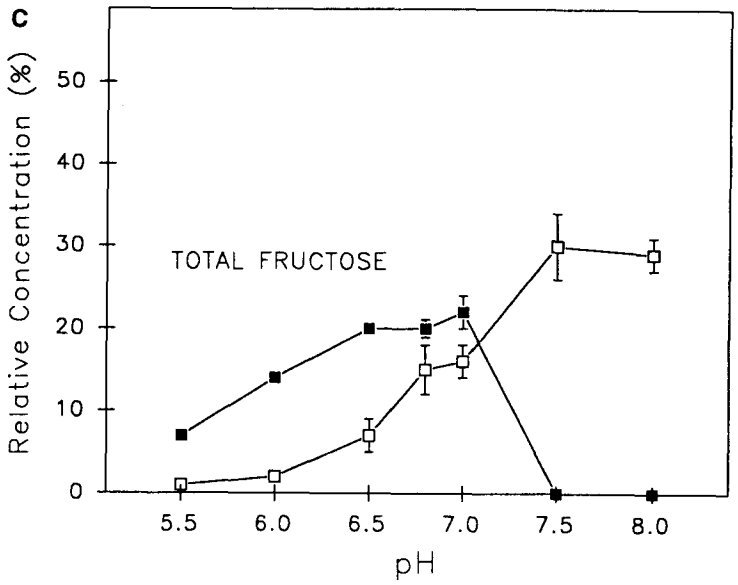


Fig. 2. (*cont'd*)

to an optimum in the presence of 22 units. This was the standard amount used for all other experiments.

Figure 2(a,b,c) shows the effect of varying pH on the total hexose output of the coimmobilized three-enzyme system and the relative amounts of glucose vs fructose produced. The total hexose sugar yield for both the immobilized and free enzymes decreased with increasing pH, dropping rapidly at pH values greater than 6.5. Indeed, the immobilized enzyme system showed no activity at pH 7.5 or higher. However, higher pH values favored fructose production by the glucose isomerase reaction. Fructose levels increased steadily from pH 5.5 to 7.0 for both the free and immobilized enzymes. The greatest percentage of fructose product for the immobilized system was 20% of the total hexose over the pH range of 6.5–7.0. Over the same pH range, however, the total hexose sugar yield of the immobilized system dropped from 80 to 12%, relative to the output at pH 5.5. Therefore, because of the high total hexose output, pH 6.5 was considered to be optimal for the immobilized system. For the free enzyme system, a maximum fructose yield of 30% occurred over the pH range 7.5–8.0. However, the total hexose output for this pH range was only 29–37% of the maximum at pH 5.5. When both the absolute amounts of hexose sugars produced and product ratios were considered, pH 6.5–6.8 was considered to be optimum for the free enzyme system, for although fructose made up only 8–15% of the product, the total hexose yield was 72–86% of the maximal. All subsequent experiments were done in pH 6.5 buffer, favoring the optimum for the immobilized enzyme system.

Table 2
Effect of Temperature on the Activity of Free and Immobilized Amyloglucosidase, Pullulanase, and Glucose Isomerase

| Temperature, °C | Activity (nmoles glucose produced/mL/min) | | | |
|---|---|---|---|---|
| | Free | | Immobilized | |
| | Glucose | Fructose | Glucose | Fructose |
| 45 | 416±20 | 42.4±5.5 | 57.8±6.9 | 5.9±0.1 |
| 55 | 488±25 | 40.9±1.6 | 66.3±9.7 | 7.1±0.4 |
| 60 | 317±42 | 46.4±6.0 | 11.0±1.6 | 3.1±0.1 |
| 67 | 214±9 | 51.9±4.7 | 11.0±1.2 | 4.7±0.5 |
| 75 | 124±15 | 19.6±2.0 | 7.1±0.7 | 1.4±0.2 |
| 82 | 134±6 | 15.7±1.2 | 7.1±0.9 | 1.3±0.3 |

Data are means ± SEM, $n = 3$. Reactions were run for 1 h using glycogen as substrate in phosphate buffer (50 m*M*, pH 6.5).

The effect of temperature on glucose and fructose production is shown in Table 2. The relative fructose output was highest at 67°C for both free and immobilized enzyme systems. However, both systems showed maximal hexose production at 55°C with a particularly sharp decrease in overall hexose production for the immobilized enzymes at temperatures higher than 55°C. Thus, subsequent experiments were performed at 55°C.

The time courses of glucose and fructose production by the free and immobilized enzyme systems at 55°C and pH 6.5 are shown in Fig. 3. Good rates of glucose production were maintained for both immobilized and free enzymes over 5 h. Long term fructose production was slow for both immobilized and free systems, representing a fraction of the total hexose output.

In an attempt to improve the output of fructose from the immobilized enzyme system, a dual pH system was tested. After an initial 1-h incubation at pH 6.0 to facilitate glycogen breakdown, the pH of the reaction mixture was raised to pH 7.2 to promote the glucose to fructose conversion. Figure 4 shows the results of this procedure for both the free and immobilized enzyme systems. Adjusting the pH to the higher value improved the fructose output in both systems. For the immobilized system, the product ratio shifted from 89:11 glucose:fructose at pH 6 to 75:25 at pH 7.2. For the free enzymes, the ratio changed from 98:2 at pH 6.0 to 88:12 glucose:fructose at pH 7.2. Therefore, reactor pH could be favorably manipulated to increase fructose output.

## DISCUSSION

Previous experiments establishing the pH and temperature optima for coimmobilized amyloglucosidase and pullulanase, and for glucose

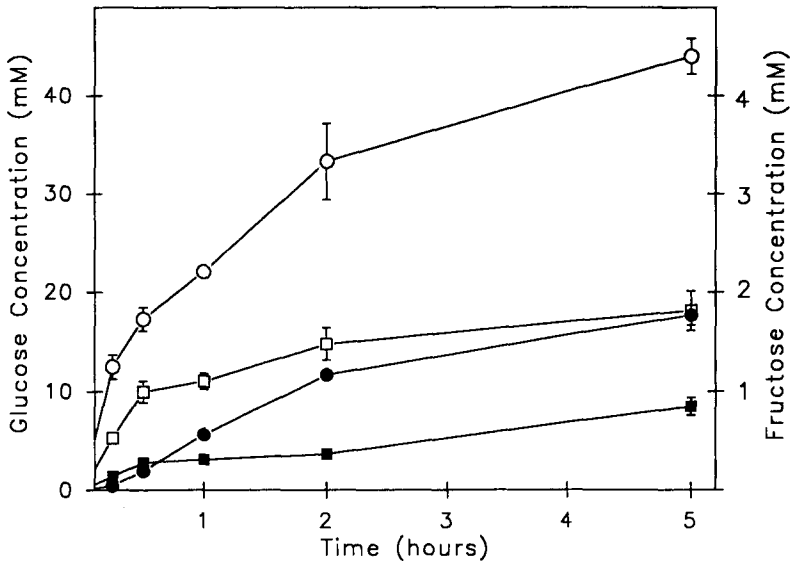


Fig. 3. Time course of glucose and fructose production from glycogen at 55°C and pH 6.5 by free and coimmobilized amyloglucosidase, pullulanase, and glucose isomerase. Data are means ± SEM, $n = 3$. Where error bars are not shown, SEM values are within the dimensions of the symbols used. ○, glucose, free; ●, glucose, adsorbed; □, fructose, free, ■, fructose, adsorbed.

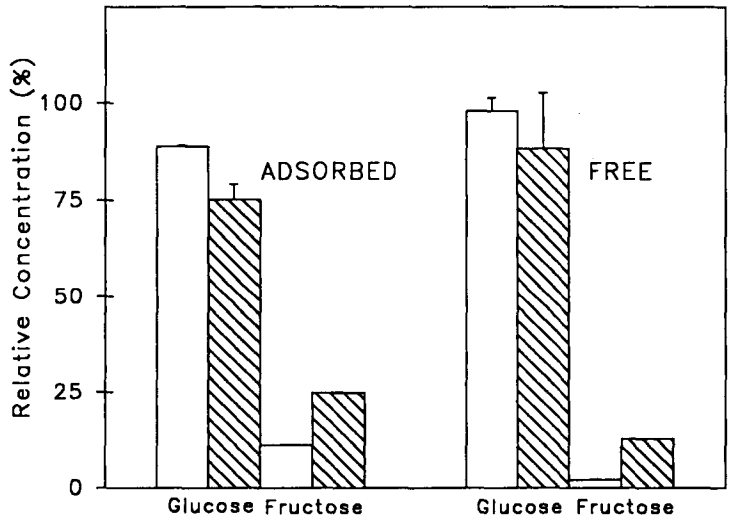


Fig. 4. Effect of pH increase on fructose output during saccharification by free and coimmobilized amyloglucosidase, pullulanase, and glucose isomerase. Following glycogen hydrolysis for 1 h at pH 6.0, the reaction pH was increased to 7.2 for another 1 h period of hydrolysis. Data are means ± SEM, $n = 3$. □, pH 6.0; ▨, pH 7.2.

isomerase bound alone onto BIOBONE™ (*4–6*), suggested that the three enzymes could be successfully coimmobilized on bone to create a functional system for a one-step conversion of polysaccharide to fructose. The potential for creating a three-enzyme immobilized system was further enhanced by the demonstration that neither amyloglucosidase nor pullulanase activities were adversely affected by the presence of bisulfite or magnesium, additives needed to ensure maximum glucose isomerase stability and activity (Table 1) (*8*). However, amyloglucosidase/pullulanase and glucose isomerase showed optimum binding to bone at different pH values (pH 4.5 and 8.0) (*5,6*). Thus, the use of a two-step procedure was necessary to ensure maximum enzyme binding by first immobilizing amyloglucosidase and pullulanase at pH 4.5 and then binding glucose isomerase during incubations at pH 8. Under these conditions, the net enzyme activity bound to bone was 14.4± 1.9%, compared with equivalent amounts of the free enzymes mixed together. Unbound enzyme eluted from the bone amounted to 44% of the total activity, indicating that substantial amounts of enzyme were bound to bone in conformations unfavorable to catalysis. This could account for the 4.6-fold drop in specific activity of the immobilized compared with the free enzymes.

The specific pH used when all 3 enzymes were coimmobilized strongly influenced the yield and ratio of glucose:fructose in the sugar product. Lower pH values increased the total hexose yield by favoring glycogen breakdown by amyloglucosidase and pullulanase but reduced the rate of conversion to fructose because of low pH inhibition of glucose isomerase. Raising pH increased the percentage of fructose product but at pH 7.5 and above the amyloglucosidase and pullulanase reactions were halted. The best compromise was pH 6.5 where total hexose prodcution was high and the percentage of fructose was about 20%. Use of this pH could also be advantageous in an industrial application because color and byproduct formation, which can occur at the high isomerization pH values of 8–9 that are typically used (*9*), would be reduced at pH 6.5.

It would have been ideal to run the reactions at temperatures higher than 55°C to enhance fructose output, particularly since glucose isomerase functions well at high temperatures and optimal glucose:fructose output occurred at 67°C (Table 2) (*5*). However, reactions carried out at high temperatures appeared to be limited by the hydrolyzing enzymes, which rapidly lost activity at higher temperatures, particularly with the bound system (Table 2). Thermoinactivation of enzymes typically results from a combination of covalent and conformational processes (*10*). At present, industrial HFS production optimizes production capacity, economy, and syrup quality by maintaining low temperature reactions (55–57°C), despite the fact that the equilibrium shifts in favor of fructose production at higher temperatures (*9*).

One of the key goals of the companies involved in the production of HFS is to increase the degree of conversion of glucose to fructose without

resorting to complicated separation procedures (*9*). Producers of HFS concentrate fructose by chromatographically separating it from glucose (*9*). In this study, efforts to increase fructose yield were done by first adjusting the pH to 6 to favor glucose output, followed by pH adjustment to 7.2 to favor fructose output (Fig. 4). Such pH manipulation resulted in a 5.8- and 2.2-fold increase in fructose levels for free and immobilized enzymes, compared with reactions run at a constant pH 6.5. Iso-syrups consisting of 42% fructose are more easily achieved by starting with as high a glucose level as economy allows (*8*).

The present study is not the first to outline a "one-step" reactor for use in biomass conversion. Chakrabarti and Storey (*11*), coimmobilized cellulase, β-glucosidase, and glucose isomerase for converting cellulase into fructose. However, the work presented here is more industrially relevant, because of the characteristics of BIOBONE™. Manufacturers of HFS require simple, cheap, efficient and durable immobilization systems. BIOBONE™ is certainly an inexpensive alternative to other common support materials; its current cost is US$3.40 per pound compared with US$86 per pound for DEAE-cellulose from Sigma Chemical Co. The natural source of BIOBONE™ is also important, for the exhausted enzyme plus support material may be included in animal feed fractions (*12*). BIOBONE™ is, therefore, an ideal support matrix because of its food grade quality and low cost.

## ACKNOWLEDGMENTS

We are very grateful to C. J. Findlay, Protein Foods Research Corp., Guelph, Ont. for donating BIOBONE™. Thanks also to J. M. Storey for critical reading of the manuscript. Supported by an operating grant from the N.S.E.R.C. Canada to K.B.S.

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