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Effect of pH on Simultaneous Saccharification and Isomerization by Glucoamylase and Glucose Isomerase

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Abstract

pH and temperature play critical roles in multistep enzymatic conversions. In such conversions, the optimal pH for individual steps differs greatly. In this article, we describe the production of glucoamylase (from *Aspergillus oryzae* MTCC152 in solid-state fermentation) and glucose isomerase (from *Streptomyces griseus* NCIM2020 in submerged fermentation), used in industries for producing high-fructose syrup. Optimum pH for glucoamylase was found to be 5.0. For glucose isomerase, the optimum pH ranged between 7.0 and 8.5, depending on the type of buffer used. Optimum temperature for glucoamylase and glucose isomerase was 50 and 60°C, respectively. When both the enzymatic conversions were performed simultaneously at a compromised pH of 6.5, both the enzymes showed lowered activity. We also studied the kinetics at different pHs, which allows the two-step reaction to take place simultaneously. This was done by separating two steps by a thin layer of urease. Ammonia generated by the hydrolysis of urea consumed the hydrogen ions, thereby allowing optimal activity of glucose isomerase at an acidic pH of 5.0.

Index Entries: Glucoamylase; glucose isomerase; *Aspergillus oryzae*; *Streptomyces griseus*.

Introduction

In multienzyme systems, the product of one enzymatic reaction becomes the substrate for the other. One such example is the production of high-fructose syrup from low-cost starchy materials. High-fructose syrup, an equilibrium mixture of glucose and fructose, is approximately one to three times sweeter than sucrose. It is preferred in the food industry because it does not pose a problem of crystallization, as happens with sucrose.

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Glucoamylase hydrolyzes starch into glucose, which is converted into fructose by glucose isomerase. Both the enzymes are required for producing high-fructose syrup; however, their optimal pH differs. A simultaneous reaction catalyzed by an immobilized multienzyme system has advantages over that catalyzed by several isolated soluble enzymes in series reactors. Hui et al. *(1)* used simultaneous saccharification and isomerization to convert starch to fructose by attaching different charged groups on polystyrene beads.

In the present study, an attempt was made to conduct such reactions simultaneously, in close proximity at their optimal pH. The technique used to separate an acidic pH environment from that of a basic one was the hydrolysis of urea in a thin layer of immobilized urease. Chen et al. *(2)* have used this pH control technique for conversion of salicin to saligenin and fructose with β-glucosidase and glucose isomerase requiring different pH optima. In the present work, the pH control technique is demonstrated for the following two-step sequential reaction:

$$\text{Starch} \xrightarrow{\text{Glucoamylase}} \text{Glucose}$$

$$\text{Glucose} \xrightarrow{\text{Glucose isomerase}} \text{Fructose}$$

Materials and Methods

Organisms and Enzyme

The source material for glucoamylase, *Aspergillus oryzae* MTCC152, was obtained from Imtech, Chandigarh, India. *Streptomyces griseus* NCIM2020, the source material for glucose isomerase, was obtained from NCL, Pune, India.

Glucoamylase enzyme was produced by *A. oryzae* grown on wheat bran in solid-state fermentation. Wheat bran was supplemented with 3.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 8.7 mg of $FeSO_4 \cdot 7H_2O$, 2.5 mg of $ZnSO_4 \cdot 7H_2O$, and 3.5 mg of $CaCl_2$ for 100 g of dry substrate. Initial pH was 4.8 and moisture content of the substrate was maintained at 60% *(3)*.

Extraction of Enzyme

A weighed quantity of fermented substrate was crushed in a mortar and pestle using distilled water (1:5 ratio). The content was filtered and centrifuged. The clear supernatant containing enzyme was used to assay glucoamylase activity. To study the effect of pH on glucoamylase activity, the assay mixture consisted of culture filtrate with 1% starch in 0.1 *M* buffer at different pHs (acetate buffer, 3.0–6.0; phosphate buffer, 6.5–8.0) at 50°C for 1 h. The reducing sugar produced was determined by the dinitrosalicylic acid method *(4)*. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 μmol of reducing sugar/min under standard conditions.

Production of Glucose Isomerase

Glucose isomerase was produced by *S. griseus* in submerged fermentation. The microorganism was grown in an Erlenmeyer flask containing xylose (0.5%), glucose (0.5%), peptone (1%), meat extract (0.5%), yeast extract (0.25%), NaCl (0.5%), and $MgSO_4 \cdot 7H_2O$ (0.05%) at pH 7.5 and 30°C. To a 500-mL flask containing 100 mL of the same medium 2 mL of broth was added as a seed. After 3 d of incubation, the material was centrifuged and cell pellets were dissolved in a minimum amount of buffer (pH 7.0) and sonicated for 5 min. The material was centrifuged and the supernatant was taken as crude enzyme extract. The enzyme was partially purified by ammonium sulfate precipitation *(5)*.

Immobilization

Glucose isomerase was mixed with aqueous gelatin-cellulose powder. The amount of gelatin added was determined by the desired rigidity of immobilized enzyme product. The mixed paste was then cast and treated with 5% aqueous glutaraldehyde at room temperature for 1 min to form a crosslinked gelatin matrix. The gel was washed thoroughly with distilled water and dried overnight at room temperature. The dried immobilized glucose isomerase was ground in a mortar and sieved to obtain the desired size *(6)*.

Assay of Soluble and Immobilized Glucose Isomerase

The reaction mixture contained 0.2 mL of 1 *M* D-glucose, 0.1 mL of 0.1 *M* $MgSO_4 \cdot 7H_2O$, 0.1 mL of 0.01 *M* $CoCl_2 \cdot 6H_2O$, 0.2 mL of enzyme extract, or 40 mg of glucose isomerase immobilized on gelatin-cellulose matrix. The final volume was made up to 2 mL with 0.1 *M* buffer at different pHs (phosphate buffer, 6.0–7.5; Tris-HCl buffer, 7.5–8.0; glycine NaOH buffer, 8.0–9.0) and incubated at 60°C for 1 h. The cysteine carbazole method was used to estimate fructose *(7)*.

Extraction, Assay, and Coimmobilization of Urease

Fifty grams of *Cajanus indicus* seeds was soaked with 100 mL of buffer (pH 6.5) for 2 h. Seeds were crushed using a mortar and pestle, suspended in phosphate buffer (pH 6.5), and kept overnight under refrigerated conditions. The suspension was centrifuged at 15,000*g* for 20 min at 4°C. The supernatant thus obtained was crude cell-free extract. The enzyme was partially purified by acetone precipitation.

For assay of urease, to 0.1 mL of enzyme 1 mL of 0.2 *M* urea was added and the volume made to 2 mL by adding buffer (pH 7.0) and incubated for 10 min followed by colorimetric analysis using Nesseler's reagent.

For coimmobilizing urease onto the immobilized glucose isomerase, immobilized glucose isomerase was impregnated with urease extract at room temperature for 24 h. Coimmobilized enzymes were removed from the solution and left at room temperature until dry *(2)*.

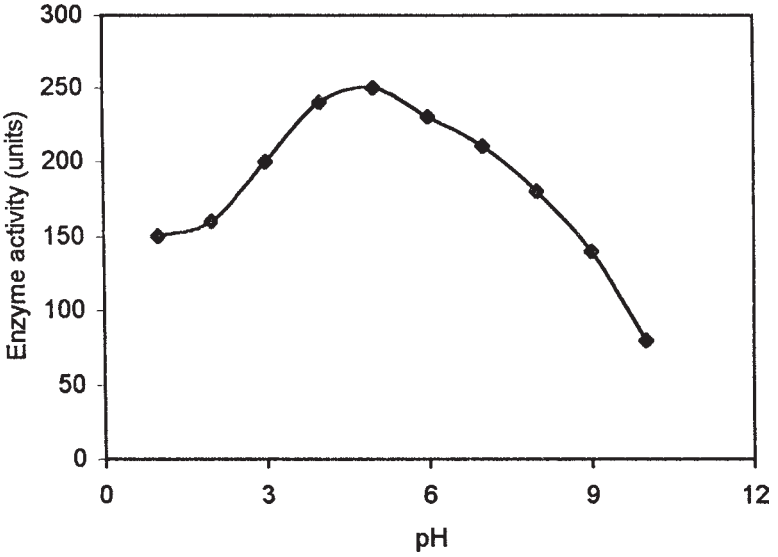

Fig. 1. Glucoamylase activity on starch at different pHs.

Simultaneous Saccharification and Isomerization

Coimmobilized enzyme was placed in a column (23-mm length and 10-mm diameter). This was connected to a flask containing glucoamylase, 1% starch, 0.1 *M* $MgSO_4$, and 20 m*M* urea in 0.1 *M* acetate buffer at pH 5.0. The flask was kept in a water bath at 50°C. The reaction mixture was recycled at 5 mL/min through a column containing coimmobilized glucose isomerase. Twenty-microliter samples taken at different time intervals were analyzed for the concentration of glucose and fructose *(2)*.

Analytical Methods

Fructose (3.25 μ*M*) formed in the presence of urea in 4 h was taken as 100%, and the percentage of relative activity in the absence and presence of urea was thereby determined.

Results and Discussion

Glucoamylase is an important industrial enzyme used in the production of glucose syrups. The material used in solid-state fermentation was wheat bran (60% moisture content). Sufficient nutrient level and the ability to remain loose even under high moisture and to maintain large surface area and aerobic condition make wheat bran a suitable substrate for this purpose. The optimum pH for starch hydrolysis was found to be 5.0 at 50°C (Fig. 1).

Glucose isomerase from *S. griseus* immobilized onto gelatin-cellulose matrix and coimmobilized with a thin layer of urease isomerized glucose to fructose optimally in an alkaline range depending on the type of buffer used. Figure 2 indicates that the enzyme is optimally active at pH 7.5 in phosphate buffer at 60°C.

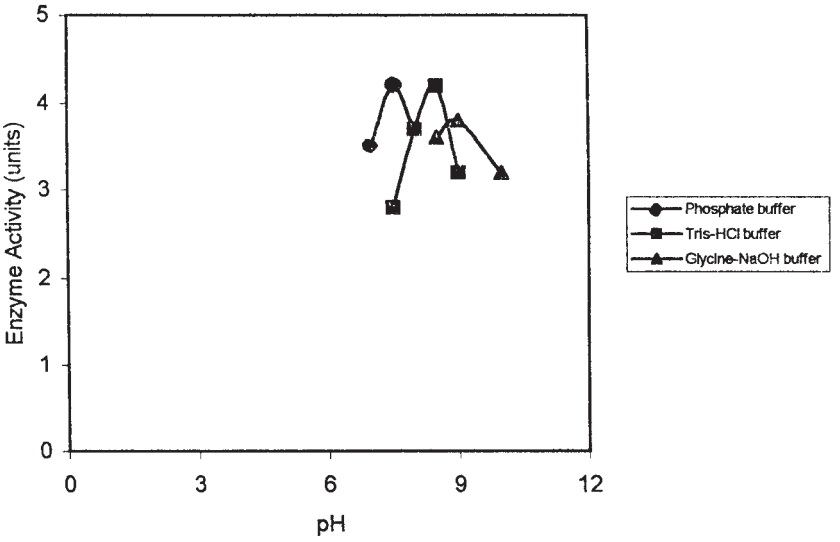

Fig. 2. Immobilized glucose isomerase activity on glucose at different pHs.

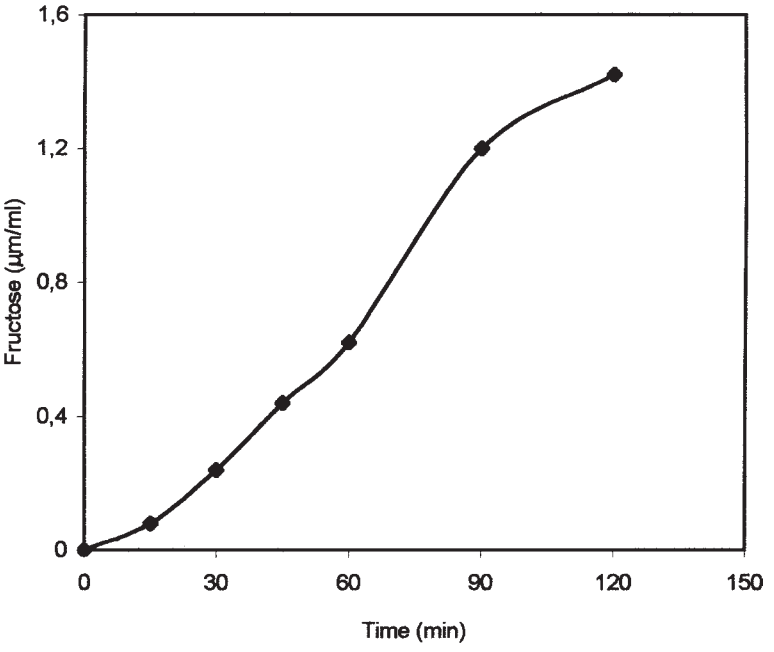

Fig. 3. Simultaneous saccharification and isomerization at pH 6.5 and 50°C.

At 50°C, no appreciable change in the activity and stability of the enzyme was observed (data not shown). Therefore, for sequential reaction, 50°C was considered.

Figure 3 shows simultaneous saccharification and isomerization at a compromised pH of 6.5 and 50°C in the absence of urea. It is evident that

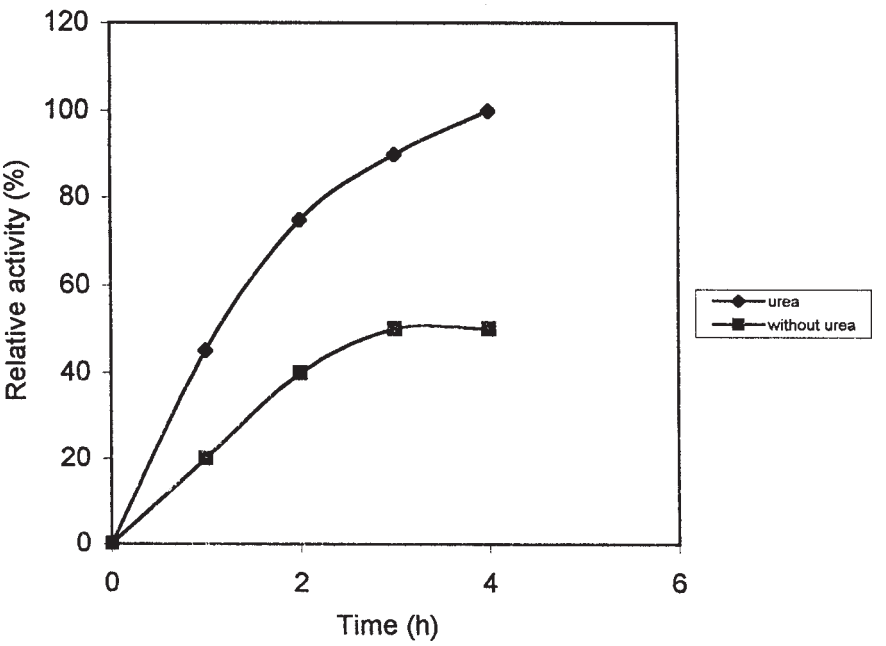

Fig. 4. Simultaneous saccharification and isomerization of starch to fructose at bulk pH 5.0 and 50°C.

at pH 6.5, although glucose conversion increases with increasing incubation period, both the enzymes had lowered activity.

When the sequential reaction took place in the presence of 20 m*M* urea at pH 5.0 and 50°C, high fructose production was recorded (Fig. 4). The concentration of urea (20 m*M*) was found too low to produce any adverse effect on enzyme activity. The generation of NH_3 by the action of urease on urea increases the pH within the coimmobilized enzyme as NH_3 traps hydrogen ions. A pH gradient thus developed across the thin layer of urease on the external surface of coimmobilized enzyme increases enzyme activity. A comparative study of changes in the relative activity (%) with time in the presence and absence of 20 m*M* urea is shown in Fig. 4. It is evident that when no urea was present in the bulk solution, there was very little fructose formation. It would be expected that the pH throughout the coimmobilized enzyme would also be about 5.0, since no ammonia was produced by the thin layer of urease coimmobilized on the glucose isomerase without urea. Chen et al. *(2)* made similar observations for conversion of salicin to saligenin.

Figure 5 shows the relationship between starch (substrate) degradation and product (glucose and fructose) formation with respect to time in the presence of 20 m*M* urea. When column operation was carried out at 50°C and pH 5.0, the required mean residence time (the value of an enzyme bed volume per substrate flow rate) was 4.0 h in order to attain 40% conversion of starch to fructose. A similar observation was made by Hui et al. *(1)*

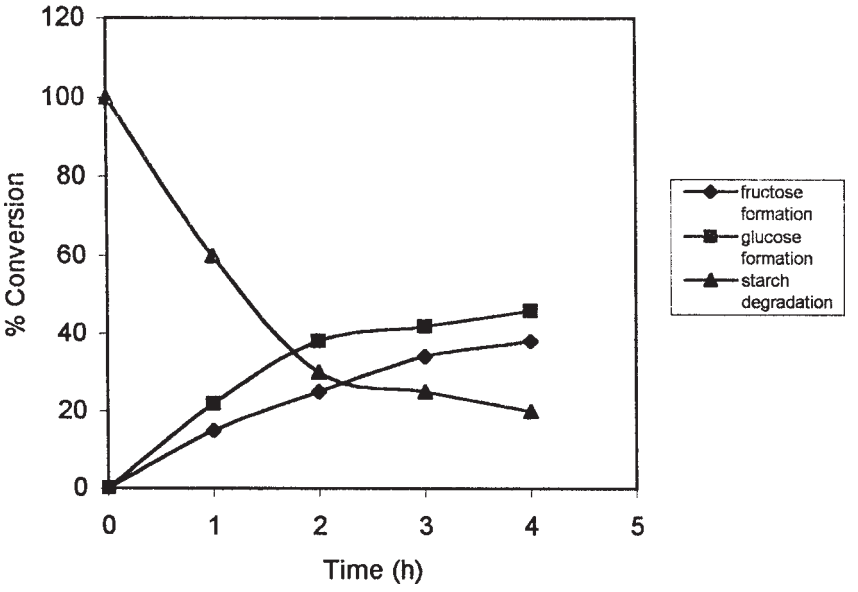

Fig. 5. Simultaneous saccharification and isomerization of starch to fructose.

with glucoamylase and glucose isomerase immobilized on beads of porous anilinosulfonic polystyrene and porous triethanolamine methyl polystyrene, respectively.

Our results indicate that the optimal pH control technique involving urease coimmobilized on gelatin-cellulose immobilized glucose isomerase works well for a two-step reaction system. The immobilized glucose isomerase works optimally in a basic pH range, while urease coating on it hydrolyzes a small amount of urea present in the acidic bulk solution (pH 5.0) and generates ammonia that neutralizes the hydrogen ions diffusing into the immobilized enzyme pellets, thus generating the desired pH gradient. Therefore, this system simultaneously allows starch hydrolysis and isomerization of glucose (hydrolyzed product of starch) into fructose.

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