

Establishment of a biosystem engineering process for production of fructose syrup using immobilized glucose isomerase

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Abstract

The current interests of biotechnological ventures are improving enzyme catalytic activity and developing unique techniques to extend their shelf life. These necessities are unavoidable in facilitating an enormous scale and commercial formulation. Immobilization of enzymes provides a phenomenal base for extending the availability of enzymes to the substrate with more noteworthy revenue over a significant time frame. Different ordinary and artificial supports have been estimated as excellent matrices for enzyme immobilization. Currently, immobilized enzymes are favored over their free counterpart because of their extended availability that reduces repetitive downstream and purification processes. Current investigations should attempt to adopt strategic and sensible immobilization techniques along with unique modification of supports to improve the condition of enzyme immobilization. It offers new perspectives to Bangladesh's industrial sectors. The main aim of the current research is to develop a bio-system engineering process based on immobilized glucose isomerase for the industrial production of fructose. In this research, the glucose isomerase enzyme was immobilized on different support matrices, including sodium alginate, native industrial waste cellulosic materials (rice bran, sawdust, sugarcane bagasse, coconut coir), and conjugates of alginate and cellulosic materials. The activity of immobilized enzymes for the production of fructose from glucose was determined. Enzymes immobilized in sodium alginate beads produced 39.01 mg/mL of fructose. In different cellulosic materials, enzymes immobilized in sawdust produced the highest amount (56.80 mg/mL) of fructose. Among all matrices, enzymes immobilized in sawdust-sodium alginate conjugate produced the maximum amount (69.13 mg/mL) of fructose. All the enzyme immobilized matrices were reused up to the seventh or eighth cycles. Free enzymes manufactured 14.44 mg/mL of fructose in a single cycle only. The conversion of fructose from glucose using immobilized glucose isomerase enzymes was much greater than that of free enzymes. A fixed-bed immobilized enzyme bioreactor had been established, which might be used for the manufacture of numerous valuable products on an industrial scale. Therefore, this study can generate a new prospect for the application of immobilized enzymes in many areas of industrial biotechnology.

Keywords: Glucose isomerase, cellulosic materials, enzyme immobilization, fructose, bioreactor.

INTRODUCTION

The enzyme is a protein that functions as a catalyst and speeds up reactions by decreasing activation energy. In the usual chemical process, enzyme catalysts do not alter the reaction's equilibrium. They catalyze the creation and breakage of biochemical building

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blocks and macromolecules, the diffusion of genetic information, the transport of different components across the membranes, and motility in organisms or metamorphosis concerning chemical energy. One characteristic function of enzymes is their specificity. The elements performed upon by enzymes are substrates. Substrate is usually a small molecule or a macromolecule which is like an enzyme itself. The enzyme catalytic activity involves binding its substrates to the active site, the substrate is transformed into the product of the reaction and then released from the enzyme (Schomburg *et al.*, 2012). All the known enzymes are classified into six basic groups on the basis of how the enzyme works on a molecular level. The enzyme categories are as follows: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

Glucose isomerase catalyzes the isomerization of glucose into fructose. This is a thermostable intracellular enzyme, and various microorganisms can produce it to draw up available sugar and transport it to their metabolic channels. Glucose isomerase is one of the most popular enzymes in the market because of its expanding uses in healthcare products. High Fructose Corn Syrup (HFCS) is formed by transforming corn starch into glucose and further isomerizing it to fructose. It is a tremendous sweet and pure syrup, refined by carbon and ion exchange systems to meet the highest food standards in terms of color, transparency, composition, flavor and ash. It is used in therapeutic syrups, beverages, baking, canning and confectionery items as sweetening agents (Liu *et al.*, 2015). The molecular weight of glucose isomerase is around 173 kDa and composed of four indistinguishable polypeptide chains of 43 kDa each. There are two metal binding sites in every monomer for Mg^{2+} , Co^{2+} or Mn^{2+} which are required for catalytic activity and thermostability (Bhosale *et al.*, 1996). Glucose isomerase catalyzes the forward and reverse isomerization reaction: (i) glucose to fructose isomerization occurs between 55 to 65°C, whereas (ii) fructose to glucose isomerization occurs at low temperature at 30 to 59°C (Carrell *et al.*, 1989). Glucose isomerase is more stable between pH 6.0 and 8.0 and crystallizes well. The isoelectric point and a molar extinction coefficient of this enzyme are ~3.0 and $45660 M^{-1} cm^{-1}$ (Kozak, 2005; Fan *et al.*, 2011).

In order to make enzyme employment in biotechnological methods more favorable, various strategies for cost decrease have been tried, and immobilization is one of them. The term 'immobilized enzyme' alludes to enzymes physically localized or confined in a specific, defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously. Immobilization gives an effortless partition of the enzyme from the product, hence protein contamination of the item is minimized or avoided altogether (Asuri *et al.*, 2006; Saifuddin *et al.*, 2013). Immobilization of the enzyme by connection to a network additionally bestows a few included benefits, for example, (1) rapid arrest of the reaction by removal of the enzyme from the reaction solution and (2) enzyme stability improvement against pH, temperature, solvents, contaminants and impurities. It also helps for effective recovery and reuse of expensive enzymes and permits their application in continuous fixed-bed operation. (Xie *et al.*, 2009). The enzyme immobilization process can be affected by the support materials, whereby the properties of both the support material and enzyme will dictate the properties of the supported enzyme preparation. Ideal support properties are described to include hydrophilicity, inertness

towards enzymes, biocompatibility, resistance to microbial attack, resistance to compression and readily accessible at a low cost (Mohamad *et al.*, 2015). Support material selection depends on some properties such as reaction media, type of enzyme, reaction conditions and safety policy in the field of hydrodynamic conditions (Khan & Alzohairy, 2010). The current production of sugar in Bangladesh is only about 5% of total demand, and the rest of the requirement is fulfilled by importation. If fructose syrup can be produced from glucose using a glucose isomerase enzyme immobilized method, the cost will be greatly reduced. Besides, fructose syrup can be a great alternative to sucrose, and it can fulfil the demand of required sugar. It is 1.3 times sweeter than table sugar and 2.3 times sweeter than glucose, and is cheaper than both sugar and glucose as well. It has many advantages compared to sucrose that make it attractive to different industries and these include its sweetness, solubility, acidity, cheap cost and many others (Hanover & White, 1993). Fructose syrup is also safe for diabetes patients because it is completely metabolized in the liver, but it releases energy more slowly than glucose.

In Bangladesh, enzyme immobilization technology is not widely used in both small and industrial scales, and there is no research on the production of high fructose syrup by using the immobilized enzymes. Therefore, it will be very much economical for the production of fructose syrup in our country using immobilized enzymes, and this can be used in various industries of Bangladesh in the near future.

MATERIALS AND METHODS

The whole research was done in the Bioresources Technology and Industrial Biotechnology laboratory of the Department of Biotechnology and Genetic Engineering, Jahangirnagar University and Wazed Miah Science Research Center, Jahangirnagar University.

Preparation of D-fructose solution: D-fructose powder sample (3.5 g) was taken in a 100 mL volumetric flask and distilled water was added up to the mark and dissolved by using a magnetic stirrer. The concentration of the solution was 194.4 mM. Various concentrations of D-fructose solution were prepared by diluting the stock solution (194.4 mM). The molarity of newly prepared solutions were 4.86 mM, 9.72 mM, 14.58 mM, 19.55 mM, 24.3 mM, 29.16 mM, 34.02 mM, 38.88 mM, 43.74 mM, 48.6 mM, 53.46 mM, 58.82 mM, 63.18 mM, 68.04 mM, 72.9 mM, 77.76 mM, 82.62 mM, 87.48 mM, 92.34 mM and 97.2 mM.

Preparation of resorcinol solution: For the preparation of resorcinol solution, 0.1% resorcinol was needed to dissolve in 3 N HCl. We used a stock HCl solution, which had a concentration of 37% and the molarity was 12 M. 100 mL of 3 N HCl was prepared. 0.1 g of resorcinol was dissolved in 100 mL of 3 N HCl.

Addition of resorcinol reagent into D-fructose solution: Various concentrations of fructose solutions (a total of 2 mL) were taken into 20 test tubes. Freshly prepared 4 mL of resorcinol solution was added to each test tube. In a test tube, only 2 mL of distilled water was taken and used as a negative control. All the test tubes were heated in a water bath at a temperature of 80°C for 10 min. Then the test tubes were removed from the water bath

and cooled, and absorbance was measured within 30 min. The absorbance of the solutions of the test tubes were taken using a spectrophotometer at a wavelength of 520 nm (Janee *et al.*, 2024).

The resorcinol reagent consists of resorcinol and concentrated HCl (or H_2SO_4 or CH_3COOH). The acid hydrolysis of polysaccharide and oligosaccharide ketoses yields simpler sugars furfurals or their subsidiaries. The dehydrated ketose (furfural) then reacts with two equivalents of resorcinol in a series of condensation reactions to produce a molecule with a deep cherry red color. Furfural is an organic compound with the formula $\text{C}_4\text{H}_3\text{OCHO}$. It is a colorless or brown liquid. It consists of an aldehyde group attached to the 2-position of furan. Aldoses may react slightly to produce a faint pink color. Fructose and sucrose are two common sugars which give a positive test.

Immobilization of glucose isomerase on sodium alginate and determination of the activity of immobilized enzyme

Preparation of glucose isomerase enzyme solution: The glucose isomerase enzyme was purchased and stored at -20°C . The enzyme was in powder form and white in color. 100 mL of glucose isomerase enzyme solution was prepared by dissolving 7 g of enzyme powder (70 mg/mL) in distilled water and making up the volume to 100 mL with distilled water.

Optimization of sodium alginate concentration: 10 mL of 2%, 3%, 4%, 5%, 10%, 15% and 20% of alginate solutions were prepared, and 0.35 g glucose powder was added to each solution. Glucose solution (3.5%) was used with matrix substances so that the active side of the glucose isomerase enzyme was not blocked with matrix substances. Seven beakers were taken containing a 0.2 M CaCl_2 solution. Then the beads of different concentrations of sodium alginate were prepared by dripping drop by drop into 0.2 M CaCl_2 solution using 10 mL syringes into these different beakers. The beads were kept for 30 min in the CaCl_2 solution without any disturbances. The beads were then separated by using filter paper and washed twice with distilled water. Then, the seven types of enzyme immobilized beads were transferred into seven different falcon tubes containing 10 mL of 3.5% glucose solution. In another falcon tube, 10 mL of 3.5% glucose solution was added to 10 mL of distilled water. It was used as negative control. All the falcon tubes were incubated at 37°C for 20 min. After incubation, from each falcon tube 2 mL of solution was taken into eight separate test tubes. 4 mL of resorcinol solution was added to all test tubes. Then the tubes were heated in a water bath at 80°C for 10 min. The test tubes were cooled to room temperature for up to 20 min, and the absorbance of all the solutions were measured at 520 nm using a UV-Vis spectrophotometer.

Optimization of the concentration of CaCl_2 the solution: To determine the optimized concentration of CaCl_2 , 100 mL of 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M of CaCl_2 solutions were prepared. Then, 50 mL of sodium alginate solution (1.5 g of sodium alginate was dissolved in 50 mL of enzyme solution, and 1.75 g of glucose powder was added to it) was prepared. Each time, 10 mL of alginate solution was taken into a syringe, and beads were prepared by dripping alginate solution into different concentrations of CaCl_2 solution

respectively. After 30 min, the beads were separated and washed. Then, various types of enzyme immobilized beads were transferred to different falcon tubes containing 10 mL of 3.5% glucose solution. In another falcon tube, 10 mL of 3.5% glucose solution was added to 10 mL of distilled water. It was used as a negative control. All the falcon tubes were incubated at 37°C for 20 min. After incubation, from each falcon tube, 2 mL of solution was taken into eight separate test tubes. 4 mL of resorcinol solution was added into all test tubes. Then the tubes were heated in a water bath at 80°C for 10 min. The test tubes were cooled to room temperature for up to 20 min, and the absorbance of all the solutions were measured at 520 nm using a UV-Vis spectrophotometer (Janee *et al.*, 2024).

Optimization of incubation period: To optimize the incubation period, 80 mL of 3% sodium alginate solution was prepared, and 2.8 g of glucose was added. Eight beakers were taken, and each was contained 100 mL of 0.2 M CaCl_2 solution. Then, the beads were prepared into each beaker and the beads were filtered, washed and transferred into eight falcon tubes containing 10 mL of 3.5% glucose solution. To optimize the incubation period, each of the falcon tubes containing glucose isomerase enzyme immobilized beads were incubated for different times, such as 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min and 120 min in an incubator at 37°C. After completion, the specific incubation time from each falcon tube, 2 mL solution was transferred and, 4 mL resorcinol was added. All the test tubes were heated at 80°C for 10 min and cooled to room temperature for 20 min, and the absorbance of all the solutions were measured at 520 nm using a UV-Vis spectrophotometer (Janee *et al.*, 2024).

Preparation of immobilized alginate beads: Sodium alginate (3%) beads were prepared for immobilization of enzymes. 0.3 g of sodium alginate was dissolved in 10 mL of glucose isomerase enzyme solution and stirred for 25 min using a magnetic stirrer. Then 0.35 g of glucose powder was added into the stirring solution, and the stirring continued for another 5 min. Alginate beads were prepared by dripping the alginate solution onto 0.2 M CaCl_2 using a 10 mL syringe. After dripping the alginate solution into CaCl_2 solution drop by drop, spherical beads were formed immediately. The beads were kept in this CaCl_2 solution for 30 min without any disturbances. The beads were then separated by using filter paper and washed properly twice with distilled water. The beads were soaked in distilled water in a falcon tube and stored at 4°C for a long time (Figure 1).

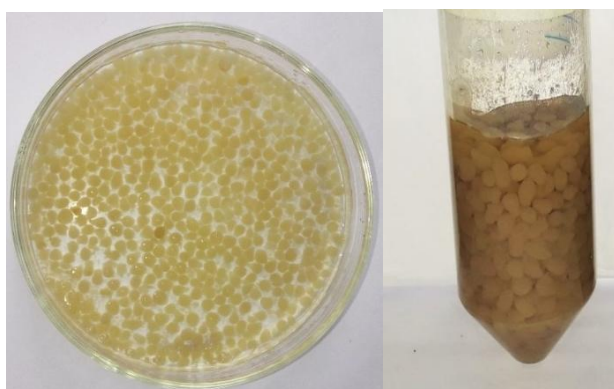


Fig. 1. Enzyme immobilized in sodium alginate beads

Determination of the activity of enzyme immobilized in alginate beads : For the determination of the activity of enzyme-immobilized alginate beads, the prepared beads were taken into a new falcon tube. 10 ml of 3.5% glucose solution was added to the enzyme-immobilized beads as a sample solution. In another falcon tube, 10 mL of 3.5% glucose solution was added to 10 mL of distilled water. It was used as a negative control. For positive control, 10 mL of glucose isomerase solution was added to 10 mL of 3.5% glucose solution. Then three falcon tubes with solutions were incubated for 20 min at 37°C. After incubation, from each falcon tube, 2 mL of solution was taken into three separate test tubes. 4 mL of prepared resorcinol solution was added to all test tubes. Then the tubes were heated in a water bath at 80°C for 10 min. The test tubes were cooled to room temperature for up to 20 min, and the absorbances of all the solutions were measured at 520 nm using a UV-Vis spectrophotometer (Jancee *et al.*, 2024).

Determination of reusability of immobilized alginate beads: The reusability test of immobilized sodium alginate beads was tested for up to several cycles. 0.3 g of sodium alginate was dissolved in 10 mL of enzyme solution, and 0.35 g of glucose powder was added. Alginate beads were prepared, separated and washed. Alginate beads were kept in a falcon tube, and 10 ml of 3.5% glucose solution was added to the enzyme-immobilized beads as a sample solution. In another falcon tubes, negative and positive controls were prepared as per the above method. Then three falcon tubes with solutions were incubated, heated in a water bath, cooled at room temperature, and the absorbance of solutions were measured at 520 nm (Jancee *et al.*, 2024). After finishing the first cycle, the beads were recovered very carefully by using a filter and washed with distilled water twice. The beads were taken into a new falcon tube, and 10 mL of 3.5% glucose solution was added to it and incubated for 20 min. Then 2 mL of the solution was transferred to a new test tube, and an enzymatic activity test was performed as before. When the second cycle was completed, the beads were recovered carefully, and the previous process was repeated. Then, after the completion of the third cycle, the beads were recovered again and the fourth cycle was continued. Thus, several cycles of reusability tests of immobilized beads were performed until the beads were completely broken and did not show any enzymatic activity.

Determination of the stability of enzyme beads after washing the beads: For testing the stability of enzyme immobilized beads due to different washing agents, 40 mL of 3% sodium alginate beads were prepared, and 1.4 g of glucose powder was added to it. 10 mL of alginate solution was dripped into a beaker containing 100 mL CaCl_2 solution. Thus, beads were prepared into four beakers and kept in CaCl_2 solution, and filtered and washed with four different types of washing agents, such as distilled water, PBS buffer, 2 M NaCl and 3 M NaCl, respectively. The beads were transferred into four different falcon tubes, heated, and the absorbance of all the solutions were measured at 520 nm using a UV-Vis spectrophotometer (Jancee *et al.*, 2024).

After the first cycle, the beads were recovered and washed with different washing reagents (distilled water, PBS buffer, 2 M NaCl and 3 M NaCl) and transferred into four different falcon tubes and the previous process was repeated. After the completion of the second cycle, the beads were recovered again, washed with different agents, and the above process

was continued. Thus, several cycles were performed on the beads until the beads were completely broken and did not show any enzymatic activity.

Immobilization of glucose isomerase enzyme in cellulosic materials (rice bran, sawdust, sugarcane bagasse and coconut coir)

Collection and purification of cellulosic materials: Different cellulosic materials such as rice bran, sawdust, sugarcane bagasse and coconut coir were collected from the local market. All the materials were washed properly, cut into very small pieces and dried in a hot air oven until dry. After drying, they were ground finely using a grinder and stored at room temperature.

For further purification of these cellulosic materials, acid treatment and base treatment were needed. For 10 g of each material, 250 mL of 1.25% H_2SO_4 and 250 mL of 1.25% NaOH were needed. In total, for four materials, 1 L of 1.25% H_2SO_4 (12.72 mL of 98% H_2SO_4 was added drop by drop into 987.28 mL distilled water) and 1 L of 1.25% NaOH (12.5 g of NaOH was dissolved into 1 L distilled water) were prepared. 250 mL of 1.25% H_2SO_4 was poured into a beaker and heated. 10 g of cellulosic material was taken into the beaker, mixed properly, and heated until boiling. Then the material is transferred to a new beaker containing 250 mL of 1.25% NaOH. It was heated as before. After heating, it was washed with distilled water several times. Then the treated material was kept on petri plates for oven drying. After drying properly, these were stored at room temperature (Saha *et al.*, 2019).

Oxidation of cellulosic materials: For oxidation, 5 g of each dried purified cellulosic material and 375 mL of 0.03 M periodic acid (sodium metaperiodate and sulphuric acid) were needed. Therefore, four types of samples, 1.5 L of periodic acid, were needed. For the preparation of 1.5 L of periodic acid, 9.90 g of sodium metaperiodate was taken in 1.5 L of distilled water and stirred until dissolved. Then the pH of the solution was adjusted to 3.0 using H_2SO_4 . 5 g of each cellulosic material was taken into 375 mL of periodic acid separately. These solutions were kept in a shaking incubator at 60°C and 150 rpm for 24 hr. After 24 hr, these materials were filtered and washed with distilled water and transferred to separate petri plates for drying in a hot air oven. After drying, they were ready for immobilization. This oxidation with periodic acid helps to insert a number of aldehyde groups into cellulosic materials, and these aldehyde groups covalently bind to the amino group of enzymes (Saha *et al.*, 2019; Janee *et al.*, 2024).

Immobilization of glucose isomerase enzyme in cellulosic materials: 1 g of each oxidized dried sample (rice bran, sawdust, sugarcane bagasse and coconut coir) was immersed in 12.5 mL of glucose isomerase enzyme solution separately and incubated at 37°C in a shaking incubator for 1 hr. The samples were filtered with filter paper to remove excess enzyme, and this was followed by washing five times with distilled water. Then the samples were taken into clean falcon tubes and stored at 4°C for further reactions.

Determination of the activity of the enzyme immobilized in cellulosic materials: 12.5 mL of 3.5% glucose solution was added into each falcon tube containing immobilized enzyme in cellulosic materials such as rice bran, sawdust, sugarcane bagasse and coconut

coir. For positive control, 12.5 mL of glucose isomerase enzyme solution was added into a falcon tube containing 12.5 mL of 3.5% glucose solution. For the negative control, 12.5 mL of distilled water was added to a falcon tube containing 12.5 mL of 3.5% glucose solution. All the tubes were incubated at 37°C for 20 min. Then 1 mL from each tube was taken into new test tubes and 2 mL of resorcinol solution was added to all test tubes and heated in water bath at 80°C for 10 min. After that, the test tubes were cooled at room temperature, and the absorbance of all the solutions were measured at 520 nm.

Determination of the reusability of immobilized enzyme in cellulosic materials: After completing the first cycle of the above section, all immobilized enzymes in cellulosic materials were recovered and were taken into new falcon tubes, and 12.5 mL of 3.5% glucose solution was added to each falcon tube. All the tubes were incubated at 37°C for 20 min. Then 1 mL from each tube was transferred to new test tubes, and 2 mL of resorcinol solution was added to each test tube. All the test tubes were heated at 80°C for 10 min. After heating, all the test tubes were cooled, and the absorbance of all the solutions were measured at 520 nm (Janee *et al.*, 2024). After the completion of the second cycle section, all the cellulosic materials were recovered by filtration and washed properly several times. The previous steps were repeated, and the same process was repeated again up to several cycles.

Establishment of biosystem engineering process for the production of fructose by using immobilized enzyme

Preparation of different types of fixed-bed enzyme reactor systems: Preparation of different types of fixed-bed enzyme reactor systems as per the schematic illustration (Figure 2). To prepare fixed bed enzyme reactor systems, 13 syringes (50 mL) were taken. 13 different types of samples were prepared. First, 1 g of sodium alginate was taken in 12.5 mL of glucose isomerase enzyme solution and dissolved. Then 12.5 mL of 0.2 M CaCl_2 was added to the solution to make a lump. Second, 1 g of purified and oxidized rice bran was taken in 12.5 mL of distilled water. Third, 1 g of purified and oxidized rice bran was taken in 12.5 mL of glucose isomerase enzyme solution. Fourth, 0.5 g of sodium alginate was taken in 12.5 mL of glucose isomerase enzyme solution and dissolved it. 0.5 g of rice bran was also added to it. Then 12.5 mL of 0.2 M CaCl_2 was added to the solution to make a lump. Fifth, 1 g of purified and oxidized sawdust was taken in 12.5 mL of distilled water. Sixth, 1 g of purified and oxidized sawdust was taken in 12.5 mL of glucose isomerase enzyme solution. Seventh, 0.5 g of sodium alginate was taken in 12.5 mL of glucose isomerase enzyme solution and dissolved it. 0.5 g of sawdust was also added to it. Then 12.5 mL of 0.2 M CaCl_2 was added to the solution to make a lump. Eighth, 1 g of purified and oxidized bagasse was taken in 12.5 mL of distilled water. Ninth, 1 g of purified and oxidized bagasse was taken in 12.5 mL of glucose isomerase enzyme solution. Tenth, 0.5 g of sodium alginate was taken in 12.5 mL of glucose isomerase enzyme solution and dissolved it. 0.5 g of bagasse was also added to it. Then 12.5 mL of 0.2 M CaCl_2 was added to the solution to make a lump. Eleventh, 1 g of purified and oxidized coconut coir was taken in 12.5 mL of distilled water. Twelfth, 1 g of purified and oxidized coconut coir was taken in 12.5 mL of glucose isomerase enzyme solution. Thirteenth, 0.5 g of sodium alginate was taken in 12.5 mL of glucose isomerase enzyme solution and dissolved it. 0.5

g of coconut coir was also added to it. Then 12.5 mL of 0.2 M CaCl_2 was added to the solution to make a lump. Thirteen different types of samples were incubated in a shaking incubator at 37°C for 1 hr with constant shaking. After 1 hr, the lump was filtered and washed with distilled water twice and was taken into thirteen different syringes.

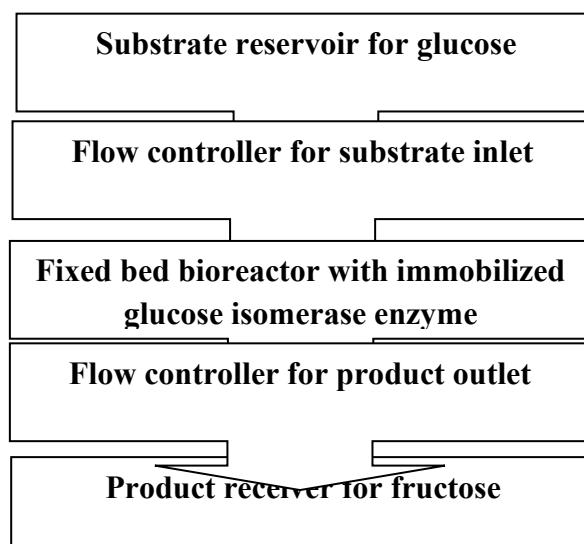


Fig. 2. Schematic illustration of a fixed-bed enzyme reactor

Determination of the activity of immobilized enzyme in different bioreactors

12.5 mL of glucose solution (3.5%) was flowed through thirteen different types of reactors (5 mL/min) and collected in thirteen new falcon tubes. The total necessary time for the solution to pass through each bed was measured. From each bioreactor, 1 mL of product was taken in thirteen test tubes, and then 2 mL of resorcinol was added. These test tubes were heated in a water bath at 80°C for 10 min. After heating, all the test tubes were cooled and the absorbance of all the solutions were measured at 520 nm using a UV-Vis spectrophotometer (Janee *et al.*, 2024).

Determination of the reusability of immobilized enzyme in different bioreactors

After the reaction of the first cycle, some distilled water flowed through different bioreactors for washing. Then 12.5 mL of glucose solution (3.5%) flowed through all the reactors (1 mL/min). The product was collected for all bioreactors in different new falcon tubes. 1 mL of product from all reactors were taken in different test tubes and then 2 mL of resorcinol was added. These test tubes were heated in a water bath at 80°C for 10 min. After heating, all the test tubes were cooled and the absorbance was measured at 520 nm using a UV-Vis spectrophotometer. After the completion of the second cycle, the same process was repeated for several cycles (Janee *et al.*, 2024).

RESULTS AND DISCUSSION

Immobilization of glucose isomerase on sodium alginate and determination of the activity of immobilized enzyme

Optimization of sodium alginate concentration: The concentration of sodium alginate determines the porosity, which greatly affects the immobilization yield. When the alginate concentration was 2%, the beads were not formed in the proper shape and they were also so fragile and difficult to handle. In the case of 3% and 4% alginate concentration, the beads were formed in proper shape and were not so rigid. On the other hand, with more than 4% alginate concentration, the beads were so rigid. The highest immobilization yield was observed in 3% alginate beads. The concentration of alginate was lower and higher than 3% the immobilization yield was decreased (Figure 3). This was probably due to the larger pore size and leakage of enzymes from the bead matrix (Crueger & Crueger, 2000). To optimize the sodium alginate concentration, three independent experiments were carried out for each concentration. The mean values and standard deviations were calculated.

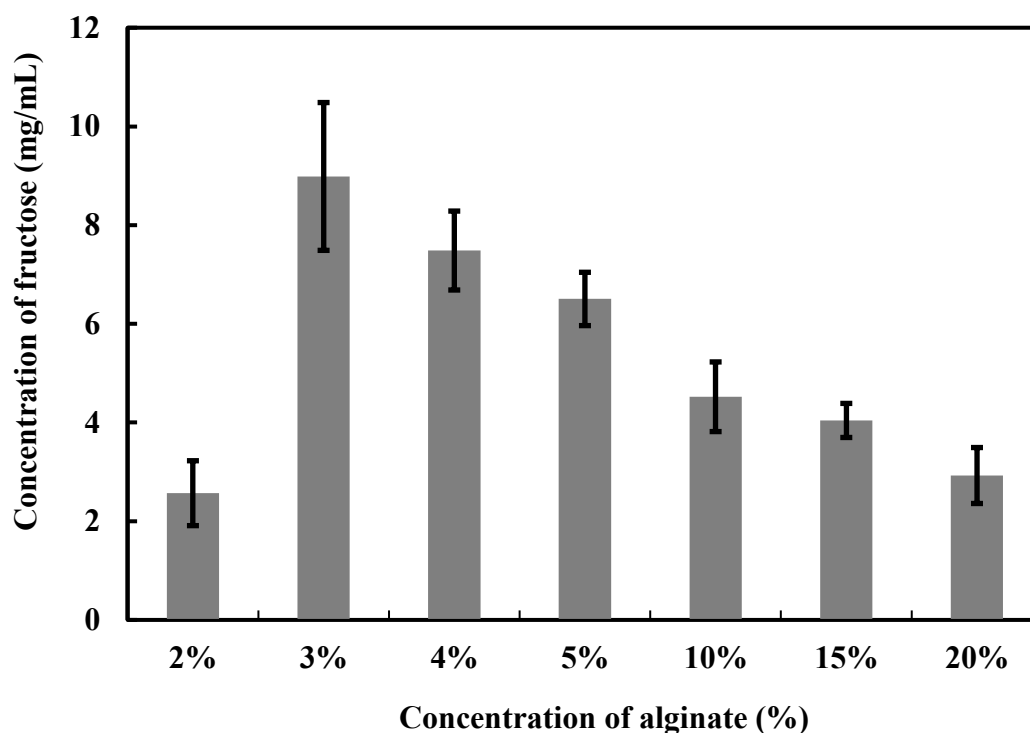


Fig. 3. Activity of immobilized beads prepared from various alginate concentrations. To conduct the experiment, solutions of 2%, 3%, 4%, 5%, 10%, 15% and 20% of alginate concentration with the same unit of enzyme were used. The mean values and standard deviations (N=3) are shown.

Optimization of the concentration of CaCl_2 solution: Concentration of CaCl_2 was prepared from 0.1 M to 0.5 M. Stable beads were formed at 0.2 M CaCl_2 and showed the highest immobilized enzyme activity. Beads were fragile at lower concentrations of

CaCl_2 and while the CaCl_2 concentration was higher than 0.2 M, the immobilization was decreased (Figure 4).

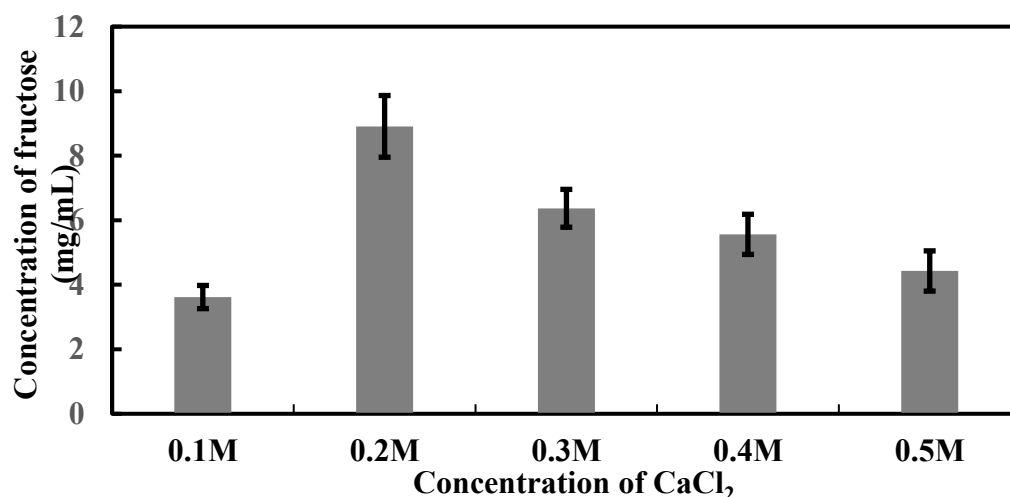


Fig. 4. Activity of immobilized beads formed in various CaCl_2 concentrations. From left to right, the concentrations of CaCl_2 were 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M. The mean values and standard deviations (N=3) are shown.

Optimization of incubation period: The result shows that after 5, 10 and 15 min of incubation, the rate of conversion of glucose to fructose was not sufficient. After 20 min, the conversion rate was the highest, and after 30 min, the conversion rate was also reasonable. In the case of incubation time more than 45 min, the results were not good because after incubation for such a long period, the enzyme activity reversed back, which means fructose may be reversed back to glucose again (Figure 5).

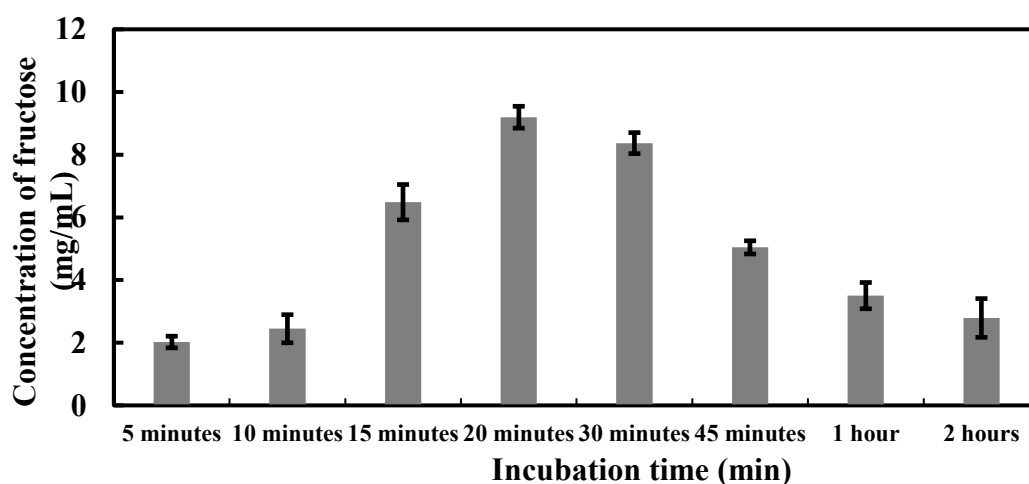


Fig. 5. The effects of incubation time on immobilized enzyme activity. After 20 min incubation, immobilized enzyme beads showed the highest activity. The mean values and standard deviations (N=3) are shown.

Determination of the activity of enzyme immobilized in alginate beads: The results showed that the absorbance of the negative control (without enzyme) is 0.00 while the absorbance of the sample (immobilized enzyme beads) and the positive control (free enzyme) were 0.483 and 0.761. From the result, it was observed that the glucose was converted to fructose due to the enzymatic activity. As a result, the color of the solution was changed. There was no enzyme present in the negative control, and the color did not change, but in the case of the positive control and sample, the color in the solution turned into a cherry red color. The change of color indicated the presence of fructose. The amount of fructose (mg/mL) produced by free enzymes and immobilized enzymes were determined using the standard calibration curve. Free enzymes produced more fructose than immobilized enzymes (Figure 6). However, after one cycle, the reaction of the free enzyme was not possible to use again. However, enzyme immobilized beads were easily recovered after reaction, and they can be used for several reaction cycles, making the process more efficient and decreasing enzyme loss (El-Shora, *et al.*, 2025).

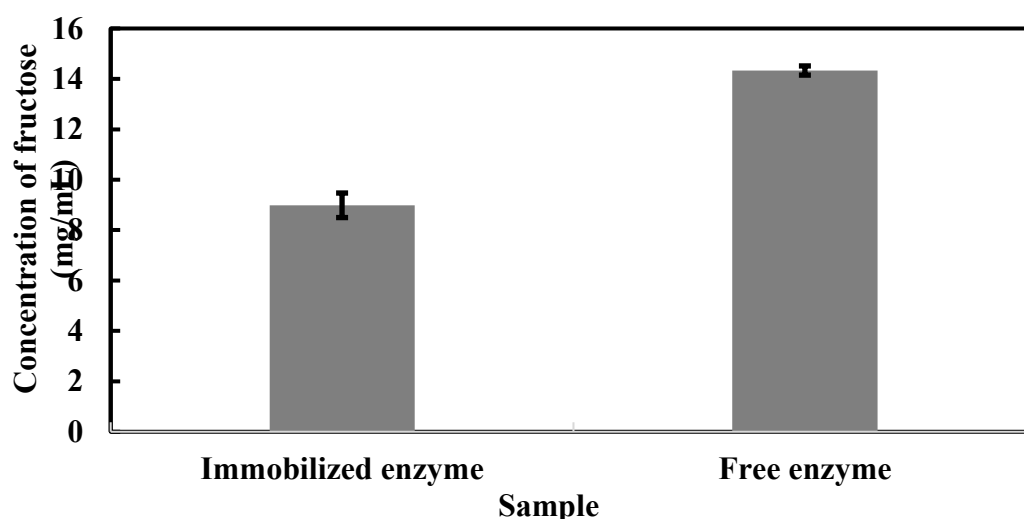


Fig. 6. The amount of fructose (mg/mL) produced in a single cycle by immobilized enzyme and free enzyme. The mean values and standard deviations (N=3) are shown.

Determination of the reusability of enzyme-immobilized alginate beads: As the immobilized enzyme was used for several cycles, the sum of the results was greater than that of the free enzyme. Besides, the product separation from immobilized enzymes was also easier than that of the free enzyme and wastage of the enzyme was greatly reduced (Jancee *et al.*, 2024). It was found that the enzymatic activities of the beads were very good up to the fourth cycles. In total immobilized enzymes produced $(9.235 + 8.196 + 7.218 + 6.08 + 5.003 + 2.445 + 0.829)$ mg/mL = 39.003 mg/mL of fructose. Free enzyme 15.101 mg/mL of fructose was produced once (Figure 7). Therefore, the total products of the immobilized enzyme were better than those of the free enzyme.

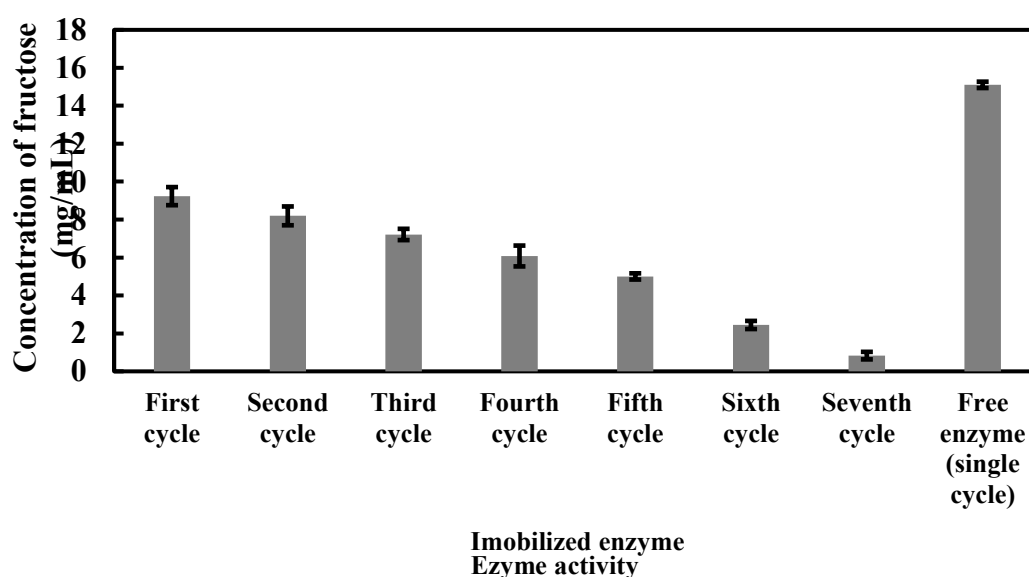


Fig. 7. Reusability of alginate-immobilized enzyme beads in different cycles. The mean values and standard deviations (N=3) are shown.

The enzyme activity was decreased during the reuse because of the leakage of the enzyme from the alginate beads during washing after each cycle (Geethanjali & Subash, 2013). During the reusability test of immobilized enzymes, the activity of the enzymes was decreasing every time. It was probably due to the leakage and wastage of some enzymes in every cycle.

Determination of the activity and reusability of immobilized enzyme in cellulosic materials (rice bran, sawdust, sugarcane bagasse and coconut coir): Free enzymes produced 14.44 mg/mL of fructose and could not be reused. The sum of the products after finishing all cycles, the enzyme immobilized in cellulosic materials showed a better amount of fructose conversion than the free enzyme (Table 1).

Table 1. The amount of fructose (mg/mL) produced from glucose in different cycles using enzyme-immobilized rice bran, sawdust, bagasse and coconut coir.

Cycle	Concentration of fructose (mg/mL)				Free enzyme
	Rice bran	Sawdust	Bagasse	Coconut coir	
First	5.138	9.35	8.677	9.042	14.44
Second	4.811	9.062	8.177	8.369	
Third	4.157	8.581	7.754	7.792	
Fourth	3.503	8.081	7.35	7.523	
Fifth	2.965	7.427	6.927	6.657	
Sixth	2.561	7.177	5.869	5.676	
Seventh	1.772	4.33	4.272	4.33	
Eighth	0.829	2.79	2.618	2.368	
Total	25.736	56.798	51.644	51.757	14.44

In the case of immobilized enzymes in cellulosic materials, the activity of the enzymes gradually decreased in every cycle. This was due to leakage and wastage of enzymes during filtering and washing after every cycle. From different results, it was observed that the enzyme immobilization yield in different cellulosic materials was found to be better than in sodium alginate alone. On the other hand, the cost of alginate is much higher than that of the cellulosic materials (Janee *et al.*, 2024). It would be very economical if waste cellulosic materials could be used instead of sodium alginate for enzyme immobilization.

Determination of the activity of immobilized enzyme in different bioreactors: The fixed-bed enzyme reactor containing the sawdust + alginate (with enzyme) conjugate showed the greatest activity, indicating it converts the highest amount of fructose (11.274 mg/mL) in a single cycle. The reactors with bagasse + alginate (with enzyme) conjugate and coconut coir + alginate (with enzyme) conjugate also showed very good activities. Enzyme reactors with only sawdust (with enzyme), coconut coir (with enzyme), bagasse (with enzyme) and alginate (with enzyme) showed moderate results. The bioreactors with rice bran + alginate (with enzyme) conjugate and only rice bran (with enzyme) showed poor activities. Free enzymes produced 14.44 mg/mL of fructose. The result was shown in figure (Figure 8). The activity of the free enzyme was always better than that of the immobilized enzyme, but the free enzyme cannot be reused, while the immobilized enzyme can be reused up to several cycles (Janee *et al.*, 2024).

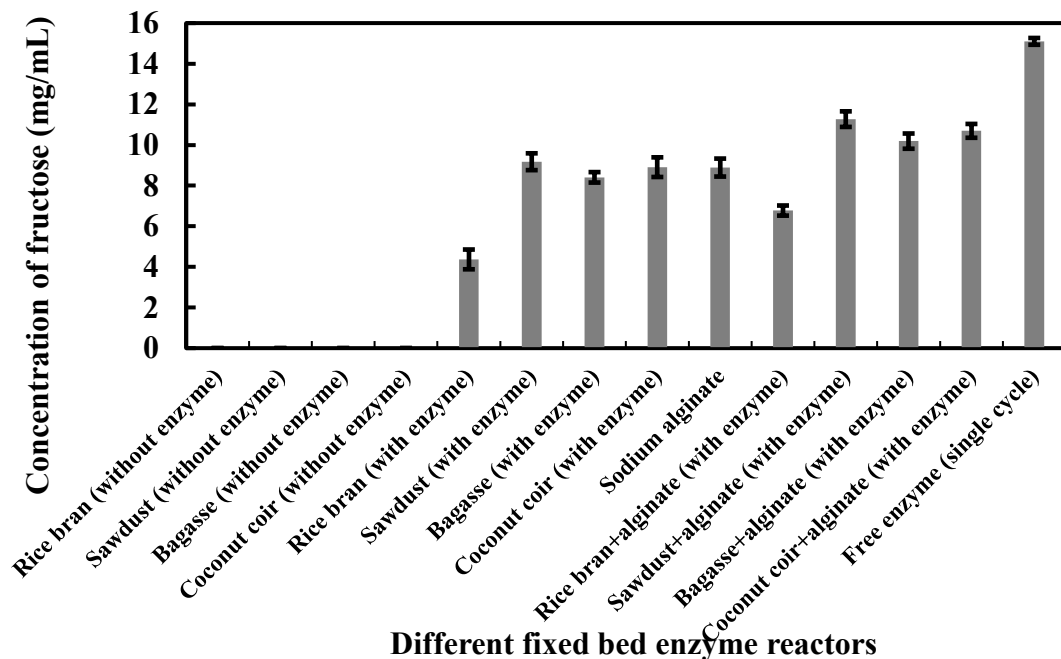


Fig. 8. Production of fructose (mg/mL) from different types of fixed-bed enzyme reactors. The mean values and standard deviations (N=3) are shown.

Determination of the reusability of immobilized enzyme in different bioreactors: All the bioreactors were used till the eighth cycle. The best result was found from the sawdust+alginate (with enzyme) bioreactor. They produced 69.128 mg/mL of fructose in total. All bioreactors produced a good amount of fructose in total. Free enzymes produced 14.44 mg/mL of fructose. The free enzyme was used only once. All the immobilized enzymes in bioreactors were reused up to several cycles and produced more fructose in total than the free enzyme (Table 2).

Table 2. Reusability of immobilized enzyme in different fixed-bed enzyme reactors

Sl	Amount of converted fructose (mg/mL)								Total
	First cycle	Second cycle	Third cycle	Forth cycle	Fifth cycle	Sixth cycle	Seventh cycle	Eighth cycle	
1	8.889	8.196	7.177	6.023	5.215	2.811	1.580	0.502	40.392
2	4.368	4.08	3.58	3.003	2.791	2.310	1.560	0.945	22.638
3	6.773	6.273	5.677	5.042	4.368	3.503	2.214	1.253	35.103
4	9.177	8.639	7.985	7.408	6.927	6.234	5.003	2.022	53.394
5	11.274	10.812	10.254	9.273	8.658	7.792	6.638	4.426	69.128
6	8.408	7.965	7.679	6.927	6.407	5.119	3.503	1.137	47.145
7	10.191	9.235	8.715	7.985	7.427	6.580	4.792	2.176	57.100
8	8.909	8.446	7.946	7.215	6.657	5.676	4.619	2.118	51.587
9	10.697	10.312	9.87	9.139	8.369	7.504	6.253	4.176	66.320
10	14.44								14.44

NB: 1. Sodium alginate, 2. Rice bran (with enzyme), 3. Rice bran + alginate (with enzyme), 4. Sawdust (with enzyme), 5. Sawdust + alginate (with enzyme), 6. Bagasse (with enzyme), 7. Bagasse + alginate (with enzyme), 8. Coconut coir (with enzyme), 9. Coconut coir + alginate (with enzyme), 10. Free enzyme

Fixed-bed bioreactors were used for several cycles for the conversion of fructose from glucose. They showed satisfactory results. However, in every cycle, enzyme activity decreased due to leakage of some enzymes. In all bioreactors, fructose production was very good from the first to the fifth cycle. After the fifth cycle the rate of fructose production started to decrease. In the eighth cycle, the rate decreased drastically. After the eighth cycle, all the bioreactors showed low conversion of fructose from glucose due to leakage of immobilized enzymes.

The enzyme immobilized in the cellulosic materials process was more reliable than other processes (Sheldon & van Pelt, 2013). The reusability test indicates that free enzymes can be used only once, and after one cycle, they cannot be reused. However, enzymes immobilized in cellulosic materials were used in several cycles. The total amount of fructose production (mg/mL) from glucose solution using enzyme-immobilized cellulosic materials was higher than that of free enzymes. Here, some cheap local cellulosic materials were used for the immobilization of glucose isomerase enzymes. The cellulosic materials need to undergo an oxidation process. This oxidation process added an extra aldehyde group to the cellulosic materials that covalently binds with the amino group of enzymes (Varavinit *et al.*, 2001). The periodic acid (0.03 M) was used for oxidizing the cellulosic materials and its use as a decent binder for the enzyme immobilization (Jancee *et*

al., 2024). Immobilized enzymes in cellulosic materials with sodium alginate showed better results than free enzymes and single cellulosic material or sodium alginate, and can be used for several cycles. Immobilization enzymes in different cellulosic materials or combined matrices can open a new field in biotechnology. This enzyme immobilization technique will be helpful for the production of fructose at a lower cost. Therefore, this biosystem engineering process can open a new door in the industrial sector of Bangladesh for the production of fructose in the near future.

Conclusion: Immobilized enzymes have great potential in the field of biotechnology. In spite of lower activity in some cases, the immobilized enzymes have some extraordinary characteristics over free enzymes. Some of them are (a) ease of separation of substrates and products from the reaction medium, (b) ease of separation of enzymes and (c) repeated use of enzymes. Therefore, the immobilized enzymes have great potential at the industrial level. In the present research, glucose isomerase immobilized in sodium alginate showed very good activity. The amount of fructose production (mg/mL) was about the same as that of free enzymes. The most interesting matter is that immobilized enzymes can be used for several cycles, and fructose production was found to be good in all cycles. Therefore, it can be said that the immobilization of glucose isomerase in sodium alginate can create a great opportunity in Bangladesh. One drawback of using sodium alginate was the high cost. Therefore, some cheap local cellulosic materials were used as an immobilization enzyme matrix. Cellulosic materials such as rice bran, sawdust, bagasse and coconut coir were used. Immobilized enzymes in these materials showed very good results up to several cycles. When cellulosic materials were mixed with alginate, the production was enhanced compared to using only alginate. On the other hand, the immobilized enzyme did not mix with the product, and as a result, the separation of the product from the enzyme was easier. In this study, a fixed-bed immobilized enzyme bioreactor was established, which could be used for the production of fructose on an industrial scale. Therefore, the application of immobilized enzymes can create a new opportunity in many sectors of industrial biotechnology.

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