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Stabilization of Glucose Oxidase Enzyme in Liquid Reagent

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ABSTRACT

Attempts were made to improve the stability of glucose oxidase (GOD) enzyme to expand its feasibility of application using sodium gluconate (1%, 2%, 3% and so on), disaccharide trehalose (1%, 2%, 3% etc.), glycerol (1%, 2%, 3% etc.) and sorbitol (1%, 2%, 3% and so on) in liquid form, having a pH of 7.4 and containing phosphate buffer saline at a concentration of 50 mM. Different combinations of these stabilizers were applied to the glucose oxidase preparation and the stabilizing effects of these combinations were verified and discussed. The stability of the enzyme preparation were checked with respect to concentration of the stabilizing agent for 7 days at 37°C. For all stabilizers, an increasing activity of the enzyme was found with increasing percentage of stabilizer in the stabilizing solution up to a certain limit. Among the formulations top three were selected for the 'degradation of enzyme activity with respect to time' tests to find the most effective formulation. Results showed that, formulation containing 5% sodium gluconate with 5% trehalose could retain at least 99% initial activity of glucose oxidase enzyme during 28 days of assay. So, it was concluded that, sodium gluconate together with trehalose is the most appropriate for stabilization of glucose oxidase in liquid reagent at a certain concentration.

1. Introduction

Glucose oxidase (GOD) is an enzyme which catalyzes the following reaction:

$$\text{Glucose} + O_2 \rightarrow \text{Gluconic acid} + H_2O_2$$

Glucose oxidase is a dimer, every monomer of it contains FAD molecule which is strongly tied, but not in covalent bond. The protein is glycosylated and comprises 11%-30% carbohydrates most of which are amino-sugars and neutral-sugars. Molecular weight of glucose oxidase is 160 kDa [1, 2].

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Glucose oxidase is a naturally produced enzyme by a range of different fungi mainly from the genus Aspergillus [3, 4] and Penicillium [5, 6], and insects such as honey bee (*Apis mellifera* L.)—salivary secretion from hypopharyngeal gland [7], Larva (*Spodoptera exigua*, *Mamestra configurata*)—salivary secretion from labial glands [8]. Currently this enzyme is obtained mainly from *Aspergillus Niger* and is used in various applications such as the removal of oxygen (for example from foods), in determination of glucose (in biological liquids, foods), as a natural preservative and probiotic support, as a resistant to bacteria, as natural immune booster and so on [9].

As in the case of all enzymes, GOD in liquid form is poorly stable as they are subjected to a number of denaturation reactions during production, storage and use [10]. The instability of glucose oxidase enzyme necessitates the use of refrigeration during storage and application. This makes glucose oxidase expensive and the field of its application becomes unfeasible.

Therefore, for commercial purposes various efforts were taken in the past to overcome this drawback. For example, thermostabilization of GOD by thermal inactivation mechanism of the enzyme using appropriate additives [11], enzyme immobilization by various techniques such as immobilization on porous silicon for biosensing applications [12], using magnetic nanoparticles to immobilize enzymes in order to enhance stability [13] etc. Nevertheless, an appropriate balance between structural stability and enzyme activity was not obtained by all those attempts. Different immobilization techniques had different drawbacks along with many advantages. For instances, during the immobilization process, inactivation of functional groups present on the enzyme surface may occur due to bonding with the supporting material. Configurational changes may cause kinetic variations during enzyme inactivation which is unfavorable. Moreover, additional procedures like- recovery of enzymes and contamination removal may be required if enzyme molecules leach out from the surface [14].

However, evolutionary changes were brought out by some compatible solutes for their efficiency to stabilize cellular structures without destroying enzyme activity [15, 16]. Addition of polyols and sugars to aqueous solutions of proteins could enhance thermostability [17]. Resistance against the denaturating-working conditions could be increased and long shelf-life could be induced to enzyme molecules by applying stabilizing additives [18]. Favorable result was found in the presence of physiological concentrations of trehalose to stabilize glucose oxidase [19].

This study aimed at providing a method for stabilizing a glucose oxidase preparation in liquid form such that it is economical to produce, stable over time and simple to use compared with previously proposed preparations.

In this work, sodium gluconate, trehalose, glycerol and sorbitol were applied separately as stabilizing agents for glucose oxidase which were found to be effective. Finally, we obtained a dramatic improvement in stabilization when sodium gluconate was used together with trehalose.

2. Materials and Methods

2.1 Reagents

Glucose oxidase (GOD) and peroxidase (POD) enzymes were the products of BBI enzyme, USA. D (+) glucose was the laboratory grade chemical and obtained from Fluka (USA). Sodium gluconate and trehalose were obtained from LOBA CHEMIE, India. Glycerol and sorbitol were from Unique Pharmaceuticals LTD. Dhaka, Bangladesh and MC&B, Norwood, Ohio, US respectfully. ProClin 300 and 4-amino antipyrine (4-AA) were purchased from Sigma, USA and Merck, India respectively. Phenol and sodium hydroxide were obtained from BDH chemicals LTD., England and Merck, India respectively. All other chemicals were of analytical grade and used without further purification.

2.2 Analytical

The concentration or absorbance was taken on T60 UV-Vis spectrophotometer (UK).

2.3 Preparation of stabilizer

Five different stabilizers were prepared and applied for stability verification of GOD. Ingredients of those stabilizers and their sub-divisions were as follows:

Stabilizer-1: Different percentages of sodium gluconate were prepared

Ingredients		Amount
Sodium gluconate		1%, 2%, 3%, 4% and so on.
Phosphate buffer saline, pH 7.4	:	50 mM
ProClin 300		0.01%

Stabilizer-2: Different percentages of trehalose were prepared

Ingredients		Amount
Trehalose		1%, 2%, 3%, 4%, 5%, 6%, 7%, 8% etc.
Phosphate buffer saline, pH 7.4		50 mM
ProClin 300	:	0.01%

Stabilizer-3: Different percentages of glycerol were prepared

Ingredients		Amount
Glycerol	:	1%, 2%, 3%, 4%, 5% and so on.
Phosphate buffer saline, pH 7.4		50 mM
ProClin 300		0.01%

Stabilizer-4: Different percentages of sorbitol were prepared

Ingredients		Amount
Sorbitol		1%, 2%, 3% etc.
Phosphate buffer saline, pH 7.4	:	50 mM
ProClin 300		0.01%

Stabilizer-5: Percentage of trehalose and sodium gluconate combination was varied

% of sodium gluconate	% of trehalose	
1	1	In 50 mM Phosphate buffer saline, pH 7.4 containing 0.01% ProClin 300
2	2	
3	3	
4	4	
5	5	
6	6	

2.4 Determination of the effect of stabilizer on the activity of glucose oxidase enzyme

Glucose oxidase (GOD) enzyme (100 micro gram) was suspended in 10 mL of various phosphate buffer saline solutions each containing different types of stabilizer. The final preparation was incubated at 37°C. At specific intervals of time, discrete formulations were tested for determining enzyme activity [20].

2.5 Glucose oxidase assay

After incubation, GOD samples were removed from incubator and diluted with phosphate buffer saline. 100 micro liter of the diluted GOD suspension was mixed with 10 mL of glucose oxidase assay solution and with standard glucose solution in a test tube and shaken for 30 min at room temperature. The resultant color was immediately measured at 546 nm. The GOD assay solution was prepared using the ingredients as mentioned in the Table 1 [20].

Table 1: Ingredients of glucose oxidase assay solution

Ingredients	Amount
Phosphate buffer saline	100 mmol/L
Peroxidase (POD) enzyme	>2 KU/L
4-aminoantipyrine (4-AA)	1 mmol/L
Phenol	4 mmol/L

2.6 Construction of calibration curve for glucose by uv-visible spectrophotometric method

To construct calibration curve several solutions of standard glucose solutions of different concentrations (25-200 mg/dL) were prepared by dilution technique from the stock solution.

1 mL of glucose kit containing mixture of glucose oxidase, peroxidase, 4-aminoantipyrine, phenol in phosphate buffer saline was taken in each of the test tubes containing standard glucose solutions of different concentrations. After incubation period for 30 min at 25°C the resultant color was immediately measured for absorbance readings at 546 nm by UV-visible spectrophotometer. A calibration curve was prepared by plotting absorbance readings against their respective concentrations of the solutions on a graph paper which is shown in figure 1.

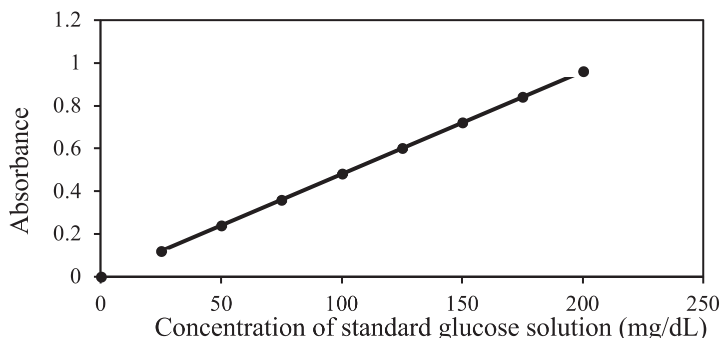

Figure 1: Calibration curve for glucose solution.

2.7 Measurement of percentage activity of glucose oxidase enzyme

The following steps were followed:

Step-1: Glucose concentration was determined for different types of stabilizers using calibration curve.

Step-2: Percentage (%) error was determined from the difference between actual concentration of glucose and that of the experimental value using the following formula:

$$\text{Percentage of error (\%)} = ([\text{glucose}]_{actual} - [\text{glucose}]_{experimental}) \times 100 / [\text{glucose}]_{actual} \quad (1)$$

Here,

[glucose] = concentration of glucose

Step-3: Percentage (%) activity of glucose oxidase enzyme was determined as follows:

$$\text{Percentage of activity (\%)} = (100 - \%\text{Error})\ \% \quad (2)$$

2.8 Determination of degradation of activity of glucose oxidase enzyme with respect to time

For each of the stabilizers, one sub-divided stabilizer was found to be most effective. These were then ranked according to their effectivity. From the rank top three effective sub-divided stabilizers were selected for degradation of activity with respect to time test.

Percentage activity of GOD enzyme was checked during 28 days of GOD assay period with 7 days of intervals. The degradation of activity of the enzyme obtained for each stabilizer was then noted.

3. Results and discussion

The instability of glucose oxidase enzyme necessitates the use of refrigeration during storage and use, making glucose oxidase expensive and field of application unfeasible. The purpose of this work was to improve the stability of the enzyme at higher temperature using stabilizing agents such as sodium gluconate, trehalose, glycerol and sorbitol in liquid form.

The stability was measured in terms of percentage activity of the enzyme. The relation between activity of glucose oxidase and concentration of the stabilizing agents was examined.

Then the degradation of the enzyme activity with respect to time was also analyzed. The results were then reported.

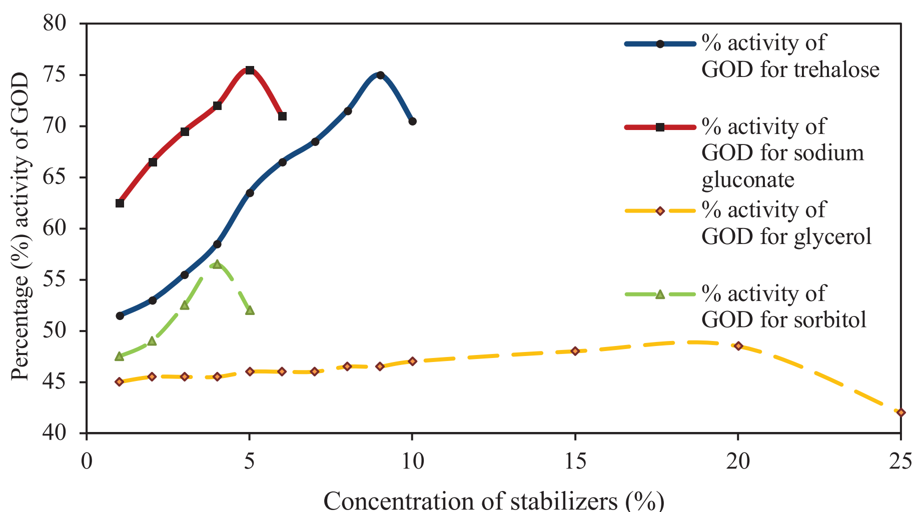

Figure 2.a: Activity of glucose oxidase enzyme with respect to percentage of sodium gluconate, trehalose, glycerol and sorbitol separately.

The graph of figure 2.a shows the activity of GOD at 37°C for the concentrations of stabilizer-1, stabilizer-2, stabilizer-3, and stabilizer-4. The horizontal axis of the curves represents the concentrations of stabilizers and the vertical axis the remaining activity of the enzyme calculated as a percentage of its initial activity at the moment it was placed in solution, following the measurement being stated in section 2.7.

This graph shows that the enzyme stabilization increases with increase in stabilizers concentrations (sodium gluconate, trehalose, glycerol and sorbitol) in the stabilizing solution, the maximum effectiveness being 75.5% for 5% sodium gluconate, 75% for 9% trehalose, 56.5% for 4% sorbitol and 48.5% for 20% glycerol. Then, for each of the stabilizers, the effectiveness decreases as the concentration increases beyond the maximum concentration, so demonstrating the actual stabilization of the enzyme with the compositions mentioned. It is noticeable that, not much significant results were being found by increasing the concentration of glycerol by 1% (from 1% to 10% glycerol); so, to obtain more significant difference in activities, the concentrations of glycerol were increased by 5% (from 10% to 25% glycerol).

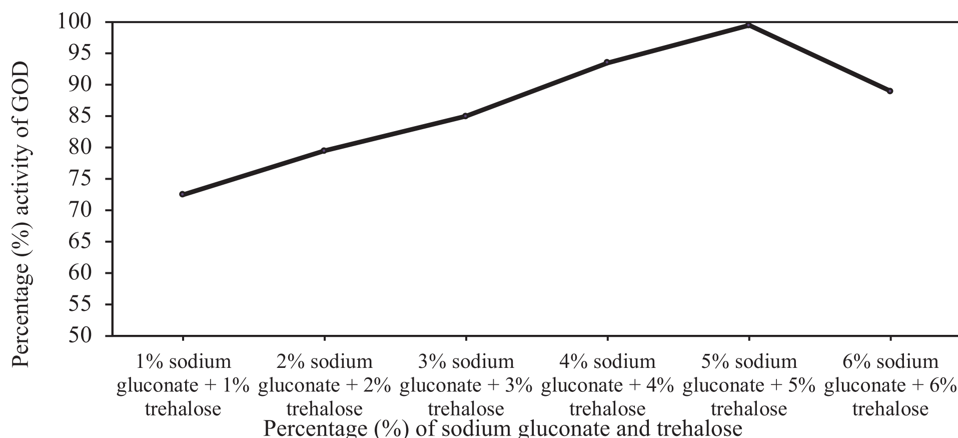

Figure 2.b: Activity of glucose oxidase enzyme with respect to percentage of sodium gluconate and trehalose in combination.

The graph of figure 2.b, in which the vertical axis represents the same units as in figure 2.a, shows the combined effect of sodium gluconate and trehalose. From figure 2.b it is clear that, stabilizing effect was greater when sodium gluconate and trehalose were used in combination than in separately. The maximum effectivity was found to be 99.5% for a stabilizing media containing 5% sodium gluconate with 5% trehalose. Further increase in stabilizer concentration made the formulation dense which in turn caused loss of workability.

While comparing the effects of different stabilizers as cryoprotectors for GOD enzyme it was obtained that, stabilizer-5 had a cryoprotectant effect 1.3 times higher than that of the stabilizer- 1 and stabilizer- 2 and around 2 times and 1.8 times than that of the stabilizer- 3 and 4 respectively.

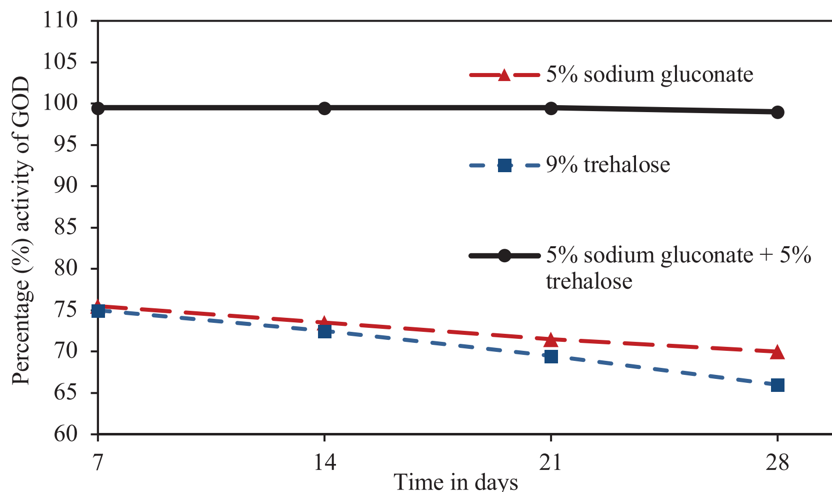

Figure 3: Degradation of activity of glucose oxidase with respect to time.

For stabilizer- 1, 2 and 5, 5% sodium gluconate, 9% trehalose and combination of both at 5% were found to be most effective sub-divided stabilizers respectively. Their 'degradation of activity with respect to time' test results are shown in the figure 3.

The graph of figure 3 is of particular importance in demonstrating the characteristics of the present study. It shows the GOD residual activity during 28 days of assay period at 37°C with 7 days of intervals. It is observed from figure 3 that, all the selected stabilizers except that containing 5% sodium gluconate together with 5% trehalose were unable

to retain the activity of glucose oxidase enzyme with the passage of time while that containing 5% sodium gluconate with 5% trehalose could retain minimum 99% initial activity of glucose oxidase enzyme and did not let the enzyme to degrade with time significantly.

The stabilization of glucose oxidase by the described method is particularly useful in preparing liquid preparations ready for use in determining glucose in biological liquids.

By grafting to polysaccharides or polysaccharide attachment method, the protease enzyme could retain 70% of the original activity at 50°C when pre-incubated at pH 9.0 for 180 min [10], partially similar to the case of this study where, 75.5% effectiveness was found for 5% sodium gluconate and 75% for 9% trehalose at 37°C, incubation period of 7 days at pH 7.4 (figure 2.a).

Addition of polyols and sugars such as 50-70% glycerol made possible for protease enzyme to be stored for 23 days at 33°C [10]; 100% storage stability of GOD at 4°C remained for first 20 days by adding 30% concentrated dextran [18]; whereas this work showed that, for GOD enzyme, stabilization was possible for 28 days at 37°C on addition of 5% sodium gluconate with 5% trehalose (figure 3). However, 48.5% effectiveness was found with 20% glycerol for 7 days at 37°C for GOD enzyme.

Thermal stabilization of GOD enzyme (incubated in 20 mM phosphate buffer, pH 6.0) by trehalose was examined at different temperatures ranging from 50 to 70°C by calculating the inactivation rate constant of the enzyme and it was observed that, inactivation rate constant was largely decreased to half the values observed in the absence of trehalose; thermal stability of GOD was increased by the presence of 0.6 M concentration of trehalose in the incubation media [19]. In this respect, it can be compared with the results found from this study as about 1.5 times increase in the stability of GOD was obtained by increasing the concentration of trehalose in the stabilizing media from 1% to 9% (figure 2.a).

After observing some recent works in this regard it is obvious that, the stabilizing effects of the reagents depend on the enzyme selected, concentrations of the stabilizing agents, pH, temperature and time applied.

The stabilization of glucose oxidase enzyme by the described method is particularly useful in preparing liquid preparations ready for use in determining glucose in biological liquids primarily to the laboratory handling and/or purification of labile enzymes.

4. Conclusion

The enzyme undergoes different kinds of denaturation processes during production, storage and application. It can be reversed if the denaturing influence is removed. This study evaluates the stabilization efficiency of stabilizing agents at various concentrations on the biocatalyst activity of glucose oxidase. Sodium gluconate with trehalose appeared to be appropriate for stabilization of glucose oxidase subjected to extreme environmental condition. The experiments revealed that the stabilizing agents provided much better long term storage stability at pH 7.4, by stabilizing enzyme activity. The application of stabilizing agents improved the temperature profile of enzyme activity, and shifted the activity optimum toward the region of higher temperature. The noticeable increase in enzyme activity in the presence of sodium gluconate with trehalose could be of great biotechnological importance while performing enzymatic conversions at low concentrations.

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