Optimization of Differential pH Sensors Device Operation Conditions to Be Used in Quantification of Low Glucose Concentration

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Abstract

The aim of this study is to find optima of operating conditions of phosphorylation enzymatic reaction of glucose within differential pH sensors device. Five variables were studied, i.e., amount of enzyme (7.45-44.7 µl), buffer concentration (20-50mM), pH of buffer (6.8 to 8.1), ATP concentration (0.2-2mM) and Mg^{+2} concentration (1.2 to 6mM).

The kinetic study indicated optima of amount of enzyme of (30µl), buffer concentration of (40mM), pH value of buffer of (7.6), ATP concentration of (1.2mM) and Mg^{2+} concentration of (2.2mM) for phosphorylation of 1g/l glucose concentration sample. A calibration curve of glucose quantification was done for low glucose concentration range i.e. from 0 to 1 g/l. This low range makes the assay of this study efficient to be used in many applications. For instance, the assay can be used in glucose quantification during cultivation of variety kinds of cells at low glucose concentration. The assay was developed by using HEPES buffer as new carrying buffer system.

Keywords: Optimization, Differential pH Sensors Device, Quantification, Low Glucose Concentration

1. Introduction

Determining glucose concentration for many applications was done by using different methods such as gas chromatography-mass spectrometry, HPLC, electrochemical and enzymatic assay (Mosca et al, 1981; Xie et al, 2009; Diessel et al, 2004; Ciantar et al, 2002; Boduroglu et al, 2005; Barrado et al, 1999; Mori et al, 1999; Wahjudi et al, 2010).

Since enzymatic assay needs non expensive apparatus, it is widely applied. Differential pH device of two pH sensors was developed to meet the need of simple and cheap glucose assay. The use of this device depends on measuring the change in pH of solution which is caused by phosphorylation of glucose. Hexokinase enzyme is used to achieve this reaction in the presence of ATP and Mg^{2+} at optimum alkali pH. It is similar to glucose phosphorylation reaction that takes place in virtually all organisms. Also phosphorylation of glucose is the first step of glycolysis process which leads to phosphorylate glucose at 6 position to yield glucose 6-phosphate at the expense of ATP. This reaction is found in most animal, plant, microbial cells (Lehninger, 1987). The mechanism of phosphorylation reaction of glucose can be seen in the following reaction:

$$\text{D-Glucose} + (\text{ATP-Mg}^{+2}) \xrightarrow{\text{HK}} \text{Glucose-6-Phosphate} + \text{ADP} + \text{H}^+ \tag{1}$$

Therefore, librated H^+ will cause change in pH of solution leading to get less pH. As the device includes two pH sensors, one is used to measure the pH of solution before adding enzyme while the second sensor will measure the pH after adding the enzyme. The difference between values of pH, before and after glucose phosphorylation enzymatic reaction, will be monitored by computer Manger program. Figure (1) represents the schematic diagram of differential pH device. Also details on the device work, set up, its applications can be found in different publications (Luzzana and Dossi, 1983; Rovida et al 1984; Luzzana and Agnellini, 2001).

Since many earlier researches of glucose determination by using differential pH device applied published optima of glucose phosphorylation, the aim of this study is to find the optimum operating conditions of glucose quantification within differential pH device. Also the use of this device was limited for high glucose

concentration i.e. above 1g/l. This limitation in concentration makes this device not widely used. Many of kinetics studies of cells cultivation needs to observe glucose consumption below 1g/l. Therefore, it was intended to develop an assay for low glucose concentration samples i.e. below 1g/l. Such low concentration range can be used for assaying high glucose concentration samples too. However, high glucose content samples have to be diluted. Finally, developing the measurement by using new buffering system, HEPES buffer was determined.

2. Experimental Procedure

2.1 Materials

Glucose powder of molecular weight (198.18) was used in preparing standard glucose samples. These samples of different concentrations $(0-1$ g/l) were obtained by dissolving glucose powder in bidest water of 0.005 microsiemens (μS). Adenosine triphosphates (ATP) and hexokinase enzyme (HK) were purchased from Sigma-Aldrich Company. HEPES buffer at different pH was used as carrying reaction solution. Solution of hexokinase enzyme of final activity of 564 U/ml was prepared by dissolving enzyme in a mixture of 25 % of glycerol/water.

2.2 Instruments

2.2.1 Differential pH analyzer system CL10 (Eurochem, Italy).

The main parts of this apparatus are two capillary glass electrodes of pH sensors for detecting pH value of solution. Mixing chamber of 1090μl maximum volume is occurred as small reactor to achieve glucose phosphorylation. Five peristaltic pumps are available for pumping desired solutions e.g. buffer, enzyme and to execute washing step. This device is controlled by using the CL10 Manger program

2.3 Procedure

During this study the temperature of differential pH device was held constant at 30° C. A waiting time of 4 seconds before each measurement was applied. Maximum reaction time of 300 seconds (cycle time) of differential pH device was used. In order to check the stability of device, first runs were done with buffer only. A same procedure was repeated for all variables. This procedure includes injecting of 120μl of glucose sample of 1g/l concentration into 780μl buffer within the mixing chamber of differential pH device. The measurement cycle was started and about 435 μl of this solution were then automatically distributed into electrodes 1 and 2. Defined amount of hexokinase enzyme was added to the remaining solution in mixing chamber according to each run set up. The difference in pH of electrode 1 and electrode 2 was observed. The pH deviation is a result of phosphorylation reaction of glucose which will induce H^+ into solution.

3. Results and discussion

In this optimization study, standard glucose samples of 1 g/l concentration were used. It was intend to make a calibration curve of glucose concentration up to 1g/l in order to be used in assaying glucose samples of low concentration and high concentration as well.

Total enzyme activity is an estimate of the amount of enzyme present if assay conditions, including concentrations of substrates, are kept nearly constant. This is due to the establishment of pseudo-zero-order reactions which means that rates of reactions are dependent solely on the enzyme concentration (Rogers and Gibon, 2009).

Kinetics study was started by varying the amount of used hexokinase enzyme of final activity of 564 U/ml. The range of enzyme amounts was (7.45-44.7 μl) at constant other parameters such as ATP concentration of 1.2mM, 20mM HEPES buffer at pH 7.6 and 2.2mM of Mg^{2+}. Enzyme activity is related to enzyme concentration and it was observed by measuring the obtained change in pH. Enzyme activity was proportional to the amount of enzyme present. The results showed that the required time of reaching end point of glucose phosphorylation was decreased from 240sec to 32sec when enzyme volume was increased from 7.45μ to 44.7μl respectively. This reduction in time was on expense of maximum obtained change in pH as it was decreased also from (-55.2mpH to 17.8mpH) as shown in figure (2). Increasing enzyme volume acted as a diluting solution for samples and caused lowering of obtained change in pH. In order to define the required amount of enzyme (volume), rates of reaction against enzyme amount were studied. Rate of reaction, enzyme activity, was calculated from obtained change in pH with time. In figure (3), rate of reaction, activity, was directly proportional to enzyme amount in the area (7.45-22.35μl). Then the rate of reaction started being nearly constant when the enzyme amount was increased above 22.35μ. In order to provide sufficient enzyme amount for the next variables study, 30μl of enzyme volume was indicated as an optimum value for phosphoration of 1g/l glucose within reaction time of 60 seconds or more.

pH value affects enzyme activity and optimum pH is the point where the enzyme is most active. In order to optimize pH of carrying buffer, enzyme activity was assayed with different pH values from 6.8 to 8.1 at constant at ATP concentration of 1.2mM, 20mM HEPES buffer, 2.2mM of Mg^{2+} and enzyme volume of 30μl. Enzyme activity was indicated by observing rate of reaction which was calculated from obtained change in pH within 60 seconds. The results indicated that the enzyme activity was not efficient to reach the end point of glucose phosphorylation when the pH increased from 6.8 to 7.4. Further increase in pH indicated an end point with constant obtained change in pH for each buffer pH values between 7.6 and 8.1. These results can be shown in figure (4). Buffer capacity of different pH values were plotted in order to see its effect simultaneously. Figure (5) shows that buffer capacity increased with pH till pH value of 7.4. Then buffer capacity remained constant till pH value of 7.6. Buffer capacity started decreasing after pH 7.6. Since this study depend on measuring the change in pH, optimum pH of 7.6 was indicated as the increase in obtained change in pH for pH value above 7.6 could be happened because of lowering in buffer capacity which can make the measurement unstable. This result is agreed with published optimum pH value 7.5 in range (7.5-9.0) (Sols et al. 1958).

Effect of buffer concentration on the obtained change in pH was studied. The higher buffer concentration is the lower obtained change in pH. Variables such enzyme volume of 30μl, pH 7.6, ATP concentration of 1.2mM and 2.2mM of Mg^{2+} were held constant. In figure (6), increase buffer concentration from 30mM to 50mM caused a decrease in obtained change in pH from (-23.74mpH to -12.8mpH) as the buffer capacity proportional with the buffer concentration. Since obtained changes in pH were sufficient for the mentioned above buffer concentrations, 40mM was chosen for the next experiments. This choice was to avoid any effect of varying ATP and Mg^{2+} concentrations on buffer stability during ATP- Mg^{2+} optimizing.

Enzyme volumes of 30μl, 40mM buffer concentration at pH 7.6 were applied to find optimum concentration of ATP and Mg^{2+}. ATP and Mg^{2+} effects have to be studied simultaneously as they form a complex compound ATP-Mg^{2+}. First runs showed that increase the concentration of Mg^{2+} from 1.2 to 6mM had no big effect on the obtained change in pH as shown in figures (7 and 8). The obtained changes in pH were in range between (-15.5 to -14.5mpH). On the other hand increase ATP concentration from 0.2mM to 1mM caused increase in obtained change in pH from (-3.4 to -15mpH). Then the obtained change in pH remained constant till ATP concentration of 1.4mM where the change in pH started to decrease noticeably as shown in figure(9). Therefore, 1mM ATP concentration was determined as optimum value.

Increase Mg^{2+} concentration till 2mM, when the concentration of ATP is 1mM, will increase the probability of ATP- Mg^{2+} compound formation to 89.2% of present ATP concentration. Any further increase in Mg^{2+} concentration didn't increase this probability (Storer and Cornish-Bowden, 1976). 1g/l, 5.5mM, of glucose sample was used in this study. This glucose concentration was diluted to 0.67mM by mixing chamber content i.e. buffer and enzyme. Therefore, ATP- Mg^{2+} compound concentration has to be equal or above 0.67. Thus, 1.2mM ATP and 2.2mM Mg^{2+} were indicated as optima for phosphorylation 1g/l glucose sample.

The mentioned obtained optimum operating conditions were used in calibration curve preparation for glucose concentrations range (0- 1g/l). The results indicated a linear relationship with (-15mpH) maximum obtained change in pH as shown in figure (10).

4. Conclusion

This study develops using differential pH sensors device for low glucose concentration determination. The obtained calibration curve can be used in assaying low glucose concentration samples. Such kinds of samples are occurred often in cell culture experiments such as mammalian cells cultivation. Other advantage was obtained with developing measurement system by using HEPES buffer, which is widely used in cell culture i.e. buffer system of cultivation medium of mammalian cells. The results also showed that there is a need to optimize the required concentration of ATP- Mg^{2+} instead of optimizing ATP and Mg^{2+} separately.

Acknowledgment

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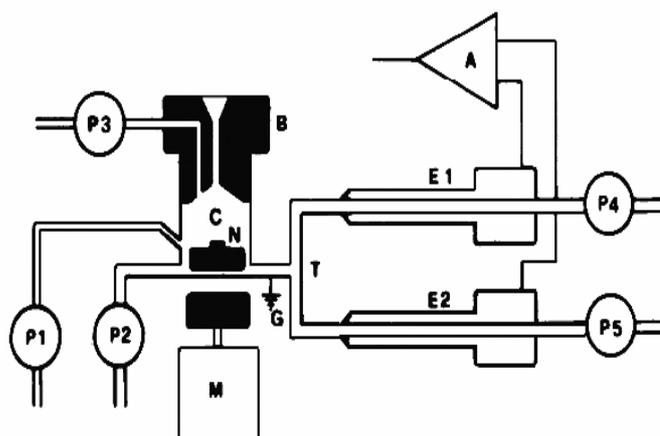
Figure 1. Schematic diagram of the differential pH analyzer system CL10.P1 to P5,peristaltic pumps; C, mixing chamber ;M, stirring motor ; N, magnetic stirrer ; E1 and E2, class capillary electrodes pH sensors ; A, differential amplifier.
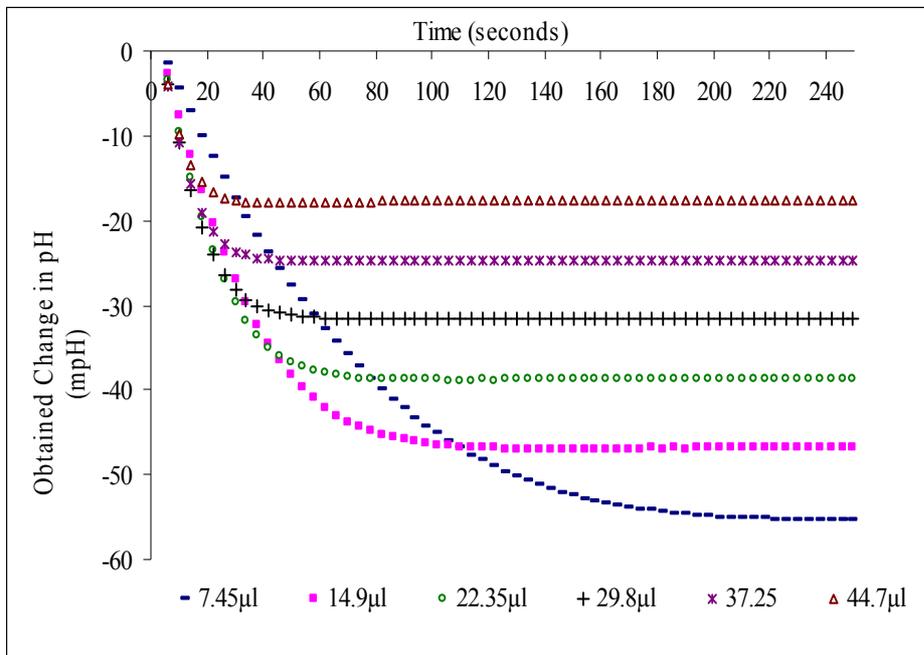
Figure 2. Enzyme volume effect on enzyme activity, obtained change in pH, at constant other operating conditions such as ATP concentration of 1.2mM, 20mM HEPES buffer at pH 7.6 and 2.2mM of Mg^{2+}
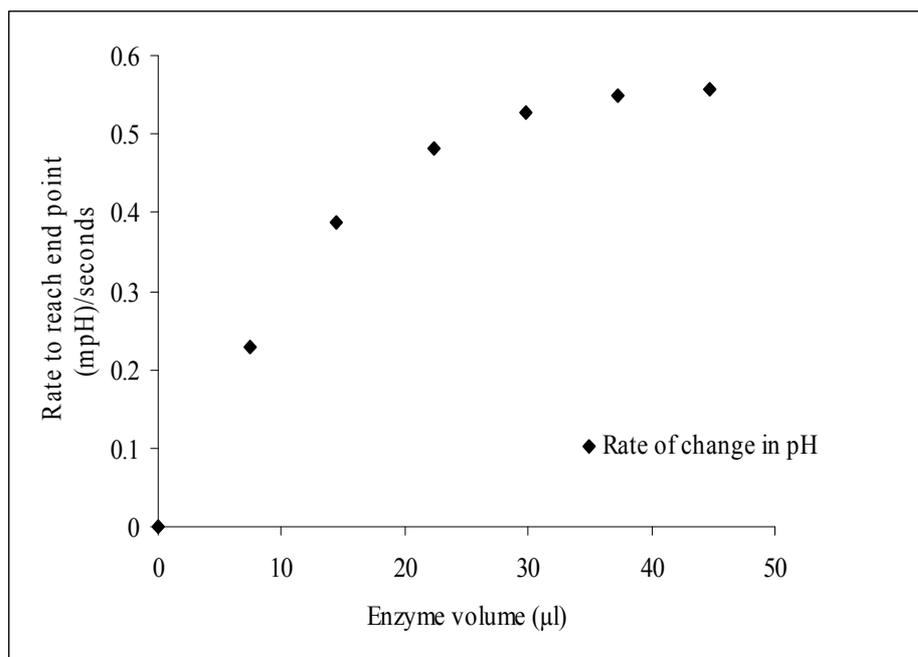
Figure 3. Enzyme volume effect on enzyme activity by measuring rate of obtained change in pH to reach end point of 1g/l glucose phosphorylation, at constant other operating conditions such as ATP concentration of 1.2mM, 20mM HEPES buffer at pH 7.6 and 2.2mM of Mg^{2+}
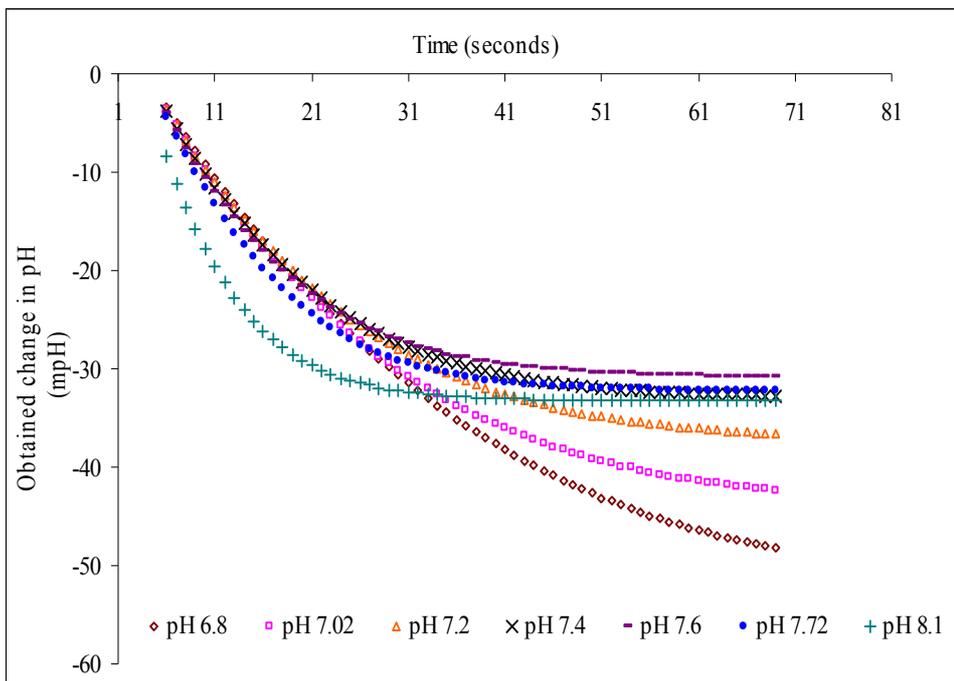
Figure 4. Effect of pH value on enzyme activity, obtained change in pH, at constant other parameters such as ATP concentration of 1.2mM, 20mM HEPES buffer, 30µl enzyme volume and 2.2mM of Mg^{2+}
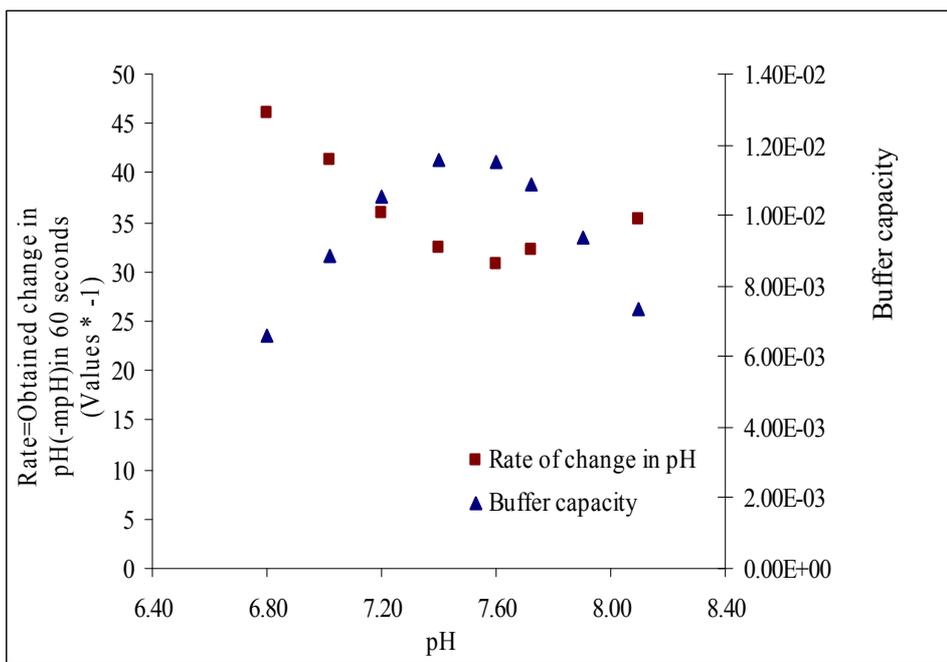
Figure 5. Effect of pH value and buffer capacity on obtained change in pH within 60 seconds reaction time at constant ATP concentration of 1.2mM, 20mM HEPES buffer, 30µl enzyme volume and 2.2mM of Mg^{2+}
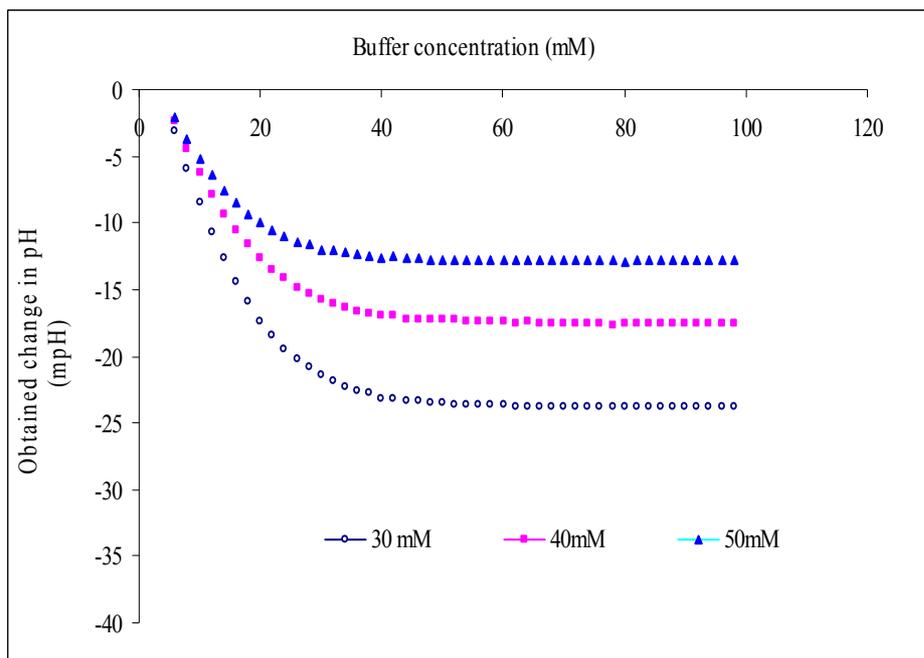
Figure 6. Buffer concentration effect on obtained change in pH at constant ATP concentration of 1.2mM, HEPES buffer of pH 7.6, 30µl enzyme volume and 2.2mM of Mg^{2+}
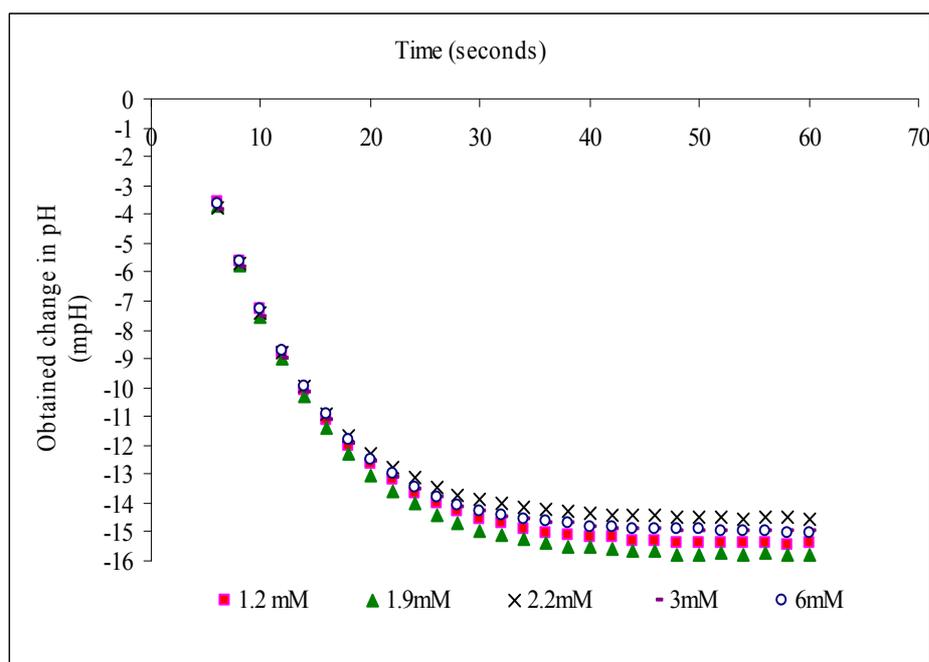
Figure 7. Mg^{2+} concentration effect on obtained changes in pH at constant ATP concentration of 1.2mM, 40mM HEPES buffer, pH 7.6 and 30µl enzyme volume
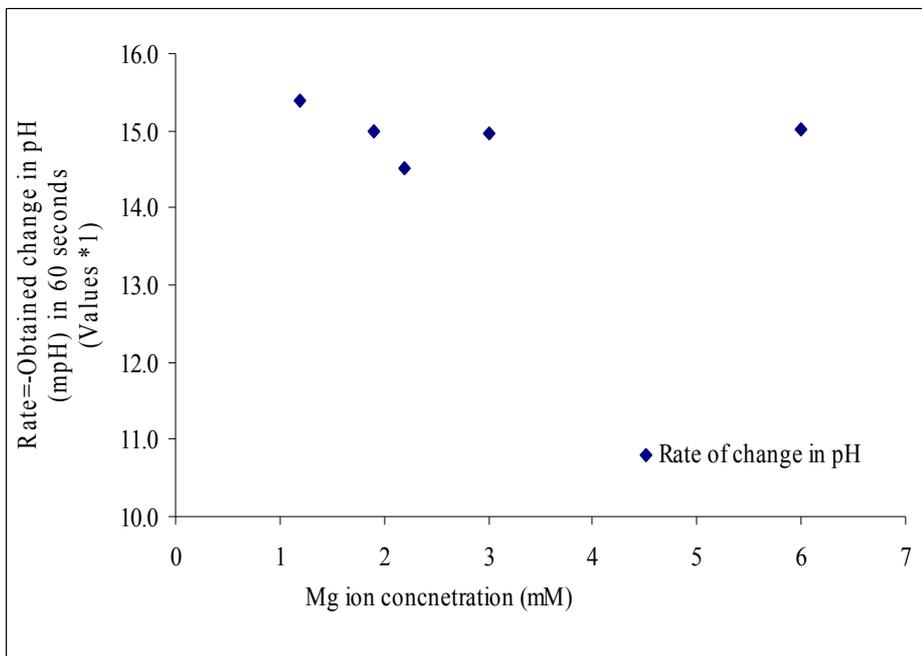
Figure 8. Mg^{2+} concentration effect on obtained change in pH at constant ATP concentration of 1.2mM, 40mM HEPES buffer, pH 7.6 and 30µl enzyme volume
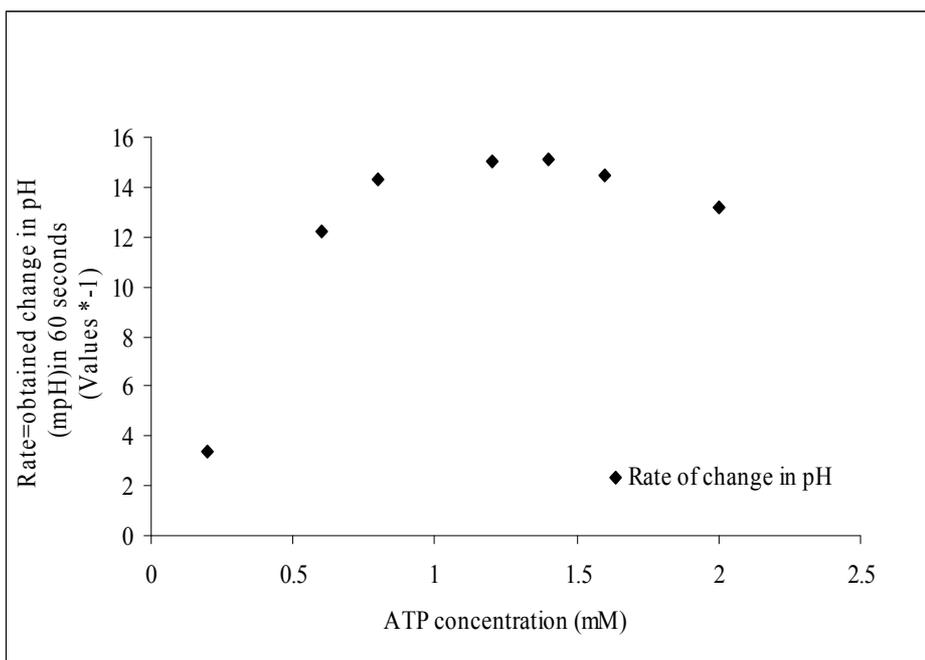
Figure 9. ATP concentration effect on enzyme activity, obtained change in pH, at constant operating conditions of 40mM HEPES buffer concentration, pH 7.6, 30µl enzyme volume
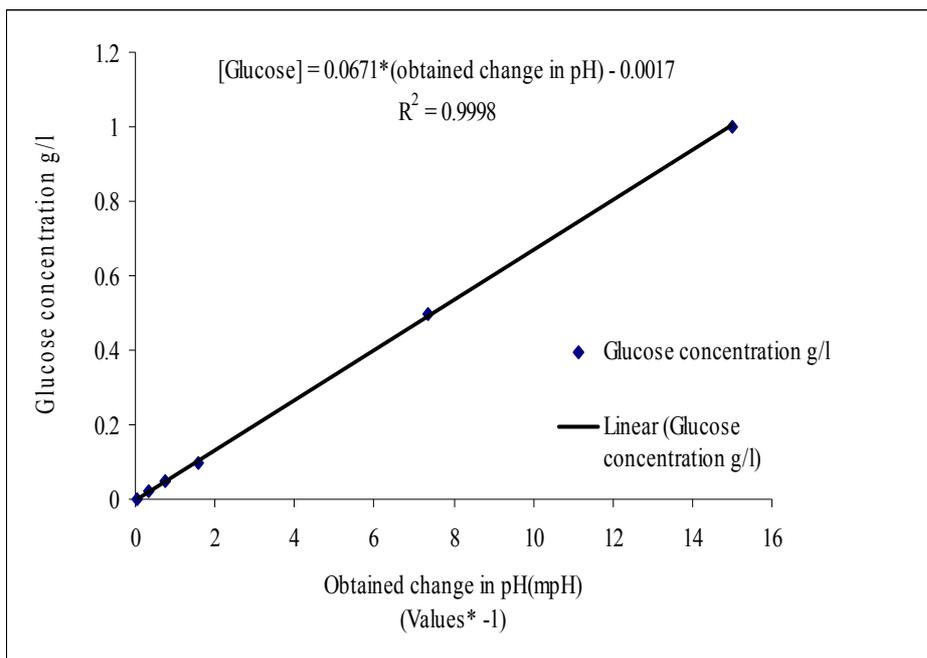
Figure 10. Calibration curve of glucose concentrations (0-1 g/l) against obtained change in pH (mpH) within Differential pH Sensors Device. Optimum operating conditions of ATP concentration of 1.2mM, 40mM HEPES buffer concentration, pH 7.6, 30µl enzyme volume and 2.2mM of Mg^{2+} were applied