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Optimization of Zinc Oxide nanoparticles synthesis to fabricate glucose oxidase sensor

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ABSTRACT

Nanostructured Zinc oxide (ZnO) is inherently electro-catalytic by nature, thus finding application in the field of enzyme immobilization while also keeping its biological activity undamaged and therefore these nanomaterials are utilized for the construction of biosensors. The electrochemical behavior of zinc oxide is the result of the combined characteristics of electrochemistry as they have high speed, sensitivity, simplicity & also have lower limits of detection. Metal oxide nanoparticle modified electrochemical interfaces provides with larger electrochemically active surface areas which improves the performance of the biosensor because these metal oxide nanoparticles demonstrate higher ratios of surface area to volume as compared with their other counterparts in bulk form. In this particular study we prepared the nanoparticles from metal oxide first and then these zinc oxide nanoparticles are combined with glucose oxidase (GOx). After this step the UV- Spectrum helps in the structural confirmation of GOx is preserved after conjugation with ZnO nanoparticles. The main reason behind combining zinc oxide nanoparticles with glucose oxidase (GOx) is to enhance the current sensitivity of the GOx enzyme electrode. In 10 mM solution of β-D-glucose we observe the current response of ZnO nanoparticle containing the enzyme electrode increasing from 0.78 to 22 μA cm^{-2}. Response surface optimization of current response is done using Design expert. The optimum conditions for effective current response were pH, working potential and temperature. The fabricated electrode showed 79.6% stability after 1 month of successive uses.

Key words: Glucose oxidase; Zinc oxide nanoparticles; Biosensor; Scanning electron microscopy

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INTRODUCTION

Glucose concentration acts as an important indicator in diseases like endocrine metabolic disorder and diabetes. Therefore, faster and perfect determination of glucose is required to diagnose the diseases properly at an early stage. Using electrochemical, chemiluminescence and other similar methods many methods have been developed in the recent past to develop enhanced glucose biosensors. Enzyme involved electrochemical glucose biosensor has been the most studied and used because of its simplicity, high selectivity and comparatively lower at cost among all the other available methods. Enzyme immobilization is the keyword behind this particular technique. The performance of a biosensor mostly depends upon the supporting materials. Therefore it is required that the material provides environmental conditions for proper loading of enzyme and also for the maintenance of its bioactivity.

In amperometric glucose biosensor and most other types of glucose biosensor, the glucose oxidase (GOx) is widely used as it has desirable properties like stability and high selectivity towards glucose.

In order to increase the sensitivity of enzyme electrodes, many advanced materials have been used in biosensor fabrication. Zinc oxide (ZnO) nanostructures are nontoxic, biologically compatible [1,2], have faster electron transfer rates [3,4] and the isoelectric point (IEP) of ZnO is around 9.5 which make it suitable for absorption of proteins with low IEPs, by electrostatic interaction and thus find good application in the field of biosensor [5].

Based on ZnO nanoparticles we can describe the fabrication, characterization and analytical performance of a glucose biosensor. Using the UV- spectrum we are able to examine the secondary structure of pure GOx, GOx/ZnO. Experiments demonstrate that zinc oxide nanoparticles are able to markedly improve the current sensitivity of GOx enzyme electrode. The fabrication procedure of GOx enzyme electrode can be used for making a highly sensitive electrode as it is very easy and effective so the conditions for current response must be optimized. Generally hit and trial method is used for optimization of conditions in which different parameters are varied one by one keeping other parameters constant and response is analyzed, but it is a time consuming process and too many experimental errors are generated. With advancement in statistical methods, new methodologies are emerging for optimization of process variables. One of such method is Response Surface Methodology (RSM)[6,7]. Many researchers had used this method for optimization purpose in many researches like bioprocess [8,9,10], enzyme immobilization [11,12] etc. In Response Surface Method, the relation between parameter variables, which varies during a process such as temperature, pH etc., and response variable such as output of a process, is investigated. It calculates level of parameters variables which produces an optimum response.

In present study, first Zinc oxide nanoparticles were made and then glucose oxidase (GOx) was immobilized on Pt. electrode with ZnO nanoparticles. The electrode was further analyzed for pH, working potential and temperature for higher current response [13]. These conditions for current response were optimized by the help of Response Surface Methodology (RSM).

MATERIAL AND METHODS

Chemicals and reagents

All products were purchased from SIGMA-ALDRICH. Glucose oxidase (GOx) from *Aspergillus niger*, b-D (+)-Glucose, Polyvinylbutyral (PVB), gluctaraldehyde and Nafion (5 wt%). 0.1M phosphate buffer (PB) solution was prepared from K_2HPO_4 and KH_2PO_4 (Sigma–Aldrich), the pH was adjusted to 7.0 by H_3PO_4. All reagents were used without further purification. Zinc nitrate were from SISCO Research Lab., Mumbai, India. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used throughout this work.

Preparation of ZnO nanoparticles

A beaker containing 100 ml of 0.9 M sodium hydroxide (NaOH) solution was prepared and heated at 55 ^{o}C. Before preparing the solution of NaOH a solution of 0.45 M zinc nitrate ($Zn(NO_3)_2 \cdot 4H_2O$) was prepared using double distilled water and 0.9 M NaOH was added slowly drop wise to the heated solution, under high speed stirring using stirrer. The beaker was sealed at this condition for 2 h. The dried ZnO NPs were cleaned with deionized water and ethanol and then air dried at 60 ^{o}C [14,15].

Preparation of enzyme electrodes

For this a platinum electrode was taken, which was then first boiled in nitric acid for few minutes and then again washed in double distilled water. After this 12 U GOx was added to different concentration of ZnO suspensions to form a mixture. This mixture was made in a glass beaker. Then 2 ml of PVB 2-propanal solution (w = 2%) was added to the beaker, which was used as an auxiliary membrane matrix. 1µl aqueous solution containing 2.5% glutaraldehyde was also added to the beaker for carrying out the cross-linking procedure. All the contents in beaker were stirred uniformly by the platinum electrode. And then the platinum electrode was dipped into the mixture to a depth of 1.5 cm for 12 min and then taken out for drying. After drying at room temperature, 1.5µl of 0.5% Nafion solution was further dropped onto the enzyme electrode surface to prevent possible enzyme leakage and eliminate foreign interferences. (As a covering membrane, Nafion has been reported to provide biocompatible environment for enzyme and also enhances the anti-interference of the biosensor. Finally, to remove the unimmobilized enzymes the electrode was immersed in deionized water. These electrodes were stored at 4 ^{o}C for overnight before measurement.

Preparation of three electrode cell

Amperometric measurements were carried out using a three electrode cell consisting of an enzyme working electrode, a counter electrode of platinum wire, and a reference electrode of Ag/AgCl. Measurements were conducted in a 5 mL phosphate buffer (Na_2H-PO_4-KH_2PO_4- KCl, pH 6.8) cell at 35 ºC. A fixed potential of 0.4 V was applied to this electronic cell. Firstly, working electrode and reference electrode were put into a phosphate solution at 35 ºC. When background current reached a constant value, different concentrations (from 1.4 to 22 mM) of b-D-glucose solution were added. Then response current was noted down, and background current was deducted, and the correlation between response currents and different concentrations of glucose solution was obtained.

Optimization of Current response Parameters using Response Surface Methodology (RSM)

Current response parameters were again optimized by Response Surface Methodology (RSM). RSM enables us to investigate interaction between different factors at different levels simultaneously. For this type of optimization Design Expert 8.0 was used to generate and analyze the experimental design. The full 2^3- factorial central composite design (CCD) with three variables over four levels: plus and minus alpha (axial points), plus and minus 1 (factorial points) and the center points was used for generation of response surface model (Table 1).

Table 1: Process variables in coded and actual units

Factor	Parameters	Units	Low	0	High
A	pH		4	9	6.5
B	Working potential	V	0.20	0.70	4.5
C	Temperature	ºC	20	75	47.50

Total twenty experiments were carried out in which six replicates of central points had been taken. Value of alpha was set at 1.68179. Initial glucose oxidase (GOx) concentration was kept constant (highest) in all experiments. The value of pH (A), working potential (B), and temperature (C) were taken as variable parameters which affect the response variable, relative current response. For statistical calculations, the variable X_i were coded as given in equation (1),

$$X_i = (x_i - x_0)/\Delta x_i, \ldots\ldots\ldots\ldots (1)$$

where X_i is the coded value of the i^{th} independent variable. X_i (dimensionless) is coded value of the real variable x_i, x_0 is the real value of X_i at the center point (zero) level, and the Δx_i is the step change value. A second degree polynomial equation (2) was used to calculate the predicted response (relative current response)

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_j + \sum_i \beta_{ij} X_i X_j \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where Y represents response variable, β_0 is the interception coefficient, β_i, coefficient of the linear effect, β_{ii}, the coefficient of quadratic effect and β_{ij}, the coefficient of interaction effect. To check the reliability of the response surface model, the predicted values and experimental data were compared. The results were analyzed using the SAS analysis of variance (ANOVA) function.

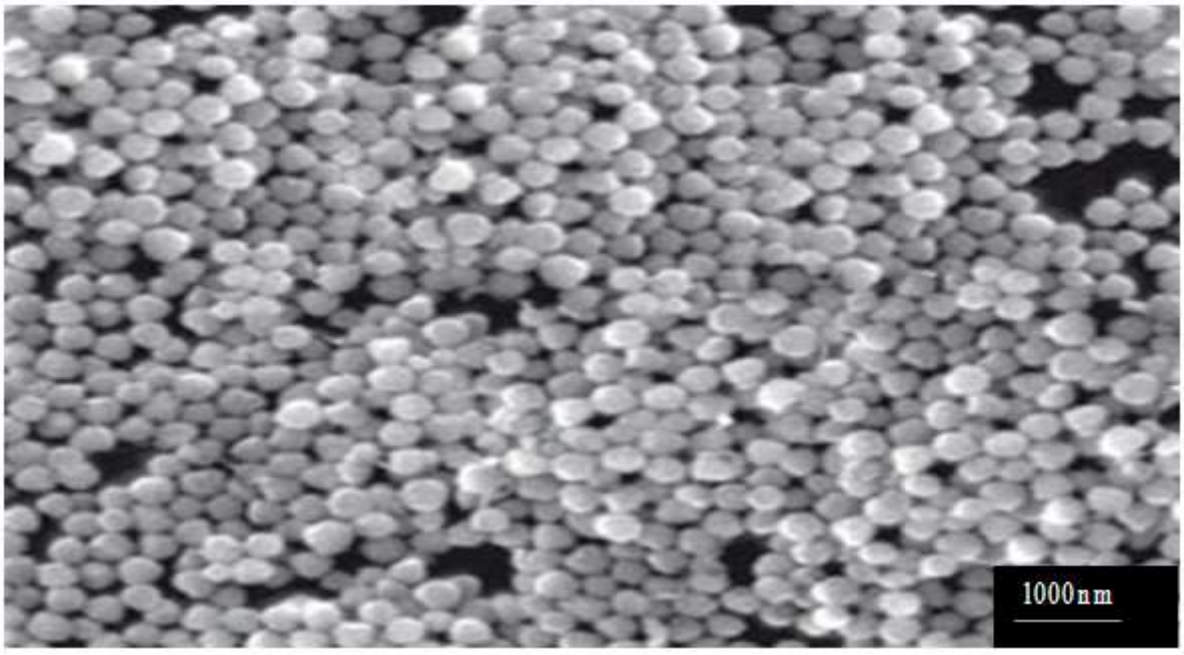

Figure 1: SEM image of the ZnO nanoparticles

RESULTS AND DISCUSSION

Preparation of ZnO nanoparticles

The scanning electron microscope (SEM) of the ZnO nanoparticles is done, which shows that the sample comprises of a large quantity of well-dispersed spherical nanoparticles (Fig.1).

The average size of these nanoparticles estimated from the SEM image is about 120 nm. The surface of every article is rough and with many smaller particles. This shows they are suitable for the immobilization of the biomolecule.

Characterization of GOx/ZnO bioconjugate

Fig. 2 shows the absorption spectrums of GOx, ZnO and bioconjugation of GOx with Zn separately. Pure GOx had absorption in the visible region with maximum values at 380 and 452 nm. ZnO nanoparticles had its own strong absorption at 378 nm. After bioconjugation of GOx with ZnO, the absorption value at 452 nm remains the same, and the absorption value at 380 nm deviates between 380 and 378 nm, which was the due to conjugation between GOx (380 nm) and ZnO nanoparticles (378 nm). The results demonstrated that the enzyme GOx was firmly immobilized on ZnO nanoparticles [9].

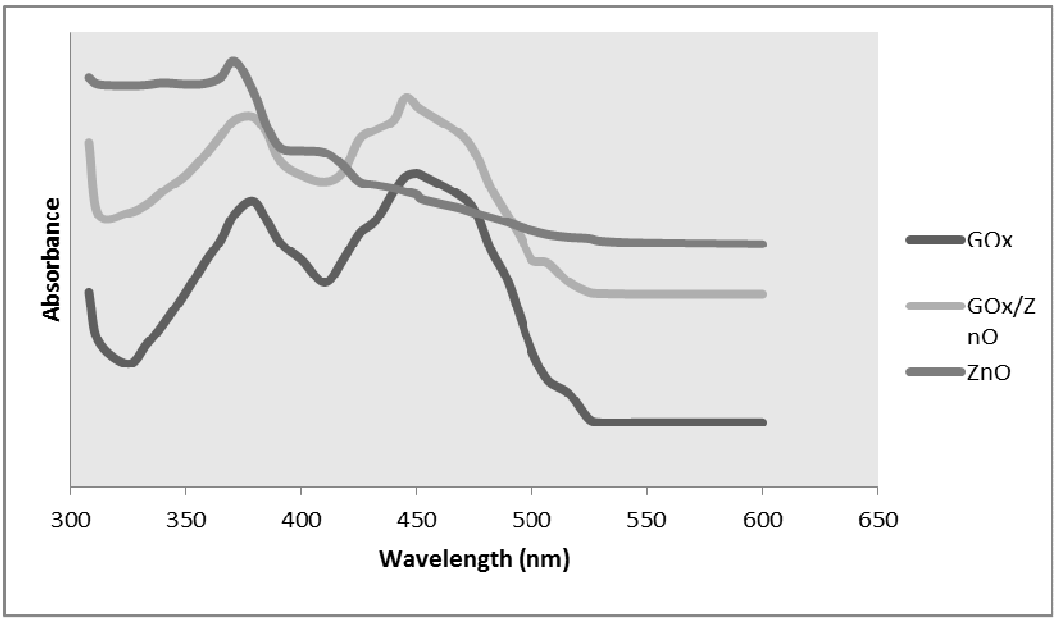

Figure 2:-UV–vis spectra of GOx, ZnO and GOx/ZnO bioconjugates.

The current response curves of the immobilized GOx electrode with ZnO nanoparticles

By using the amperometric measurements the enzyme electrodes containing ZnO nanoparticles were tested to know the effect of the ZnO nanoparticles on the sensitivities of the glucose biosensor and the enzyme electrodes. The current response curves of GOx which GOx gets easily adsorbed on the surface of nanoparticles. This clearly shows that electrodes with and without ZnO nanoparticles were shown in Fig. 3. The current response of the electrode without ZnO nanoparticles was found to be 0.78 µA cm^{-2} when the glucose concentration was 10 mM, while the current response of the electrode with ZnO nanoparticles was 22 µA cm^{-2}. It was observed that the ZnO nanoparticles were able to markedly enhance the current response of the electrodes. ZnO nanoparticles had a large surface area due to surface of nanoparticles leads to the immobilization of the enzyme, and leads to the improvement in the activity and stability of the enzyme.

Optimization of Current Response Parameters using RSM

For model construction, twenty experiments (Table 2) were carried out in random order to minimize errors due to possible systematic trends in the variables.

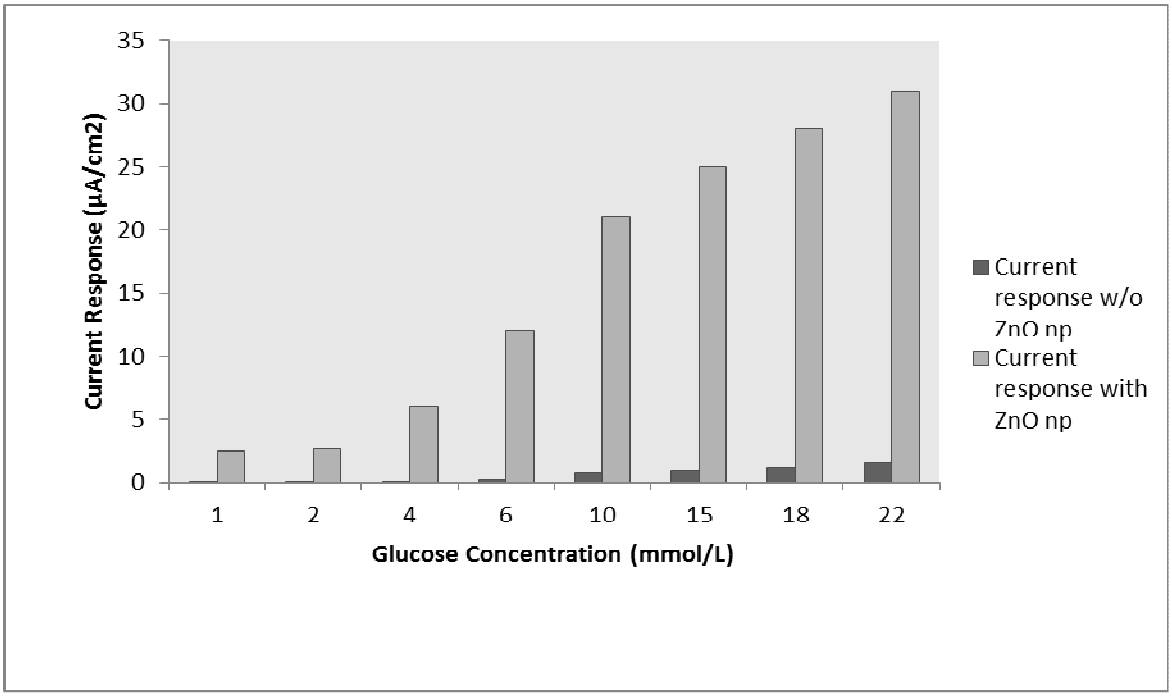

Figure 3:- Effect of ZnO nanoparticles on the GOx enzyme electrode current response.

Table 2: Design matrix for enhancement of current response

Run	A: pH	B:Working Potential (V)	C: Temperature (ºC)	Current response
1	6.5	0.45	47.5	34
2	6.5	0.45	47.5	33.5
3	4	0.2	20	0.9
4	6.5	0.45	47.5	34
5	4	0.7	75	1.4
6	6.5	0.02	47.5	0.7
7	6.5	0.45	47.5	33.2
8	4	0.2	75	3.2
9	4	0.7	20	3.5
10	6.5	0.45	47.5	33.3
11	2.29	0.45	47.5	5
12	9	0.7	20	17
13	9	0.2	20	1
14	10.7	0.45	47.5	13.3
15	6.5	0.87	47.5	11.2
16	6.5	0.45	47.5	32.6
17	9	0.7	75	8.2
18	6.5	0.45	93.7	0.4
19	6.5	0.45	1.25	0.3
20	9	0.2	75	88

The concentration of glucose was 23 mmol/L throughout. At center point, coded as ‘0’, six experiments were carried out to minimize experimental error. For fitting of experimental data linear, two factor interaction (2FI), quadratic and cubic models were tested. Significant “p-value” was found in quadratic model (Table 3) and it was used for further model construction. Also its predicted R-squared value was 0.9943 which is in reasonable agreement with the “adjusted R-squared” value (0.9982).

Table 3: Fit summary of various model generated by Design Expert

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared
Linear	0.7982	< 0.0001	-0.1167	-0.27315
2FI	0.9445	< 0.0001	-0.3363	-1.30058
Quadratic	< 0.0001	0.3227	0.998182	0.959328*
Cubic	0.2260	0.4829	0.99872	

* Suggested

For calculation of relative enzyme activity, the second order polynomial equation used was:

Current Response = 33.42605693 + 2.303523192 * A + 3.101653129 * B + 1.210506378 * C + 2.8875 * A * B - 1.2125 * A * C -1.5625 * B * C - 8.537899635 * A^2 -9.669270484 * B^2 -10.87135201 * C^2

Where, A is pH, B is Working Potential in V, C is pH and C is temperature in °C. Experimental data were then fitted to the model by performing ANOVA. The generated mean square, F-values and p-values for the response surface quadratic models are given in Table 4. p-value (<0.0001) of the model suggests that there is less than 0.01% chance that a "model F- value" (402.04), this large, could occur due to noise, which implies that the suggested model is significant. Also, P-value for lack of fit test was 0.2260 which suggests it as insignificant and the model constructed was quite good. The high F-value of all three parameters suggests that these variables affect the current response. High F-value for AB suggests it as interacting parameters.

Table 4: ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	p-value (Prob > F)
Model	3754.307	9	417.1452	1160.302	< 0.0001*
A-pH	72.46626	1	72.46626	201.567	<0.0001
B-Potential	131.3824	1	131.3824	365.444	< 0.0001
C-Temperature	20.01174	1	20.01174	55.66324	< 0.0001
AB	66.70125	1	66.70125	185.5315	< 0.0001
AC	11.76125	1	11.76125	32.71426	0.0002
BC	19.53125	1	19.53125	54.32675	<0.0001
A^2	1050.522	1	1050.522	2922.057	< 0.0001
B^2	1347.381	1	1347.381	3747.779	< 0.0001
C^2	1703.217	1	1703.217	4737.548	< 0.0001
Residual	3.595144	10	0.359514		
Lack of Fit	2.181811	5	0.436362	1.543734	0.3227**
Pure Error	1.413333	5	0.282667		
Cor Total	3757.902	19			

**Significant **Not-Significant*

The second order polynomial equations were used to generate Surface Response plots and to determine optimum conditions for current response at which maximum current can be retained. Response surface and contour plots were generated for interacting parameters. Figure 4 represents variation in current response due to changes in pH and potential. At low value of working potential and pH, current response was less but with increase in the values of these parameters current response was also increased and reached a maximum. The value of current response depends on both the pH and working potential. Lowering the values of any of these parameters will result in decrease of response. Figure 5 represents other two parameters, pH and temperature. Since current response also depends on the temperature, variation in temperature was result in variation in current response. With the decreases in temperature the current response decreases but after some point with the increase in temperature the current response again decreases. The main parameter which affects the current response more was working potential. Current response was directly proportional to the working potential. The value of current response increases with increase in the value of working potential to some point, after which the value of current response decreases with increase in working potential. Figure represents other two parameters, working potential and temperature. The current response also depends on these two parameters as one of them was working potential which was an interacting parameter which effects the value of current response to a greater extend. Numerical tools present in Design Expert 8.0 were used to determine the optimum conditions. For better current response optimum conditions obtained were: pH 6.5, working potential 0.45 and temperature 47.5°C. An optimum condition as predicted would give maximum current response for making a highly sensitive Glucose biosensor.

Stability of the enzyme electrode

The stability of the biosensor was investigated by amperometric measurements in the presence of 23 mmol/L glucose. Stored at 4ºC, the current response of biosensor was retained about 79.6% of its original response after one month.

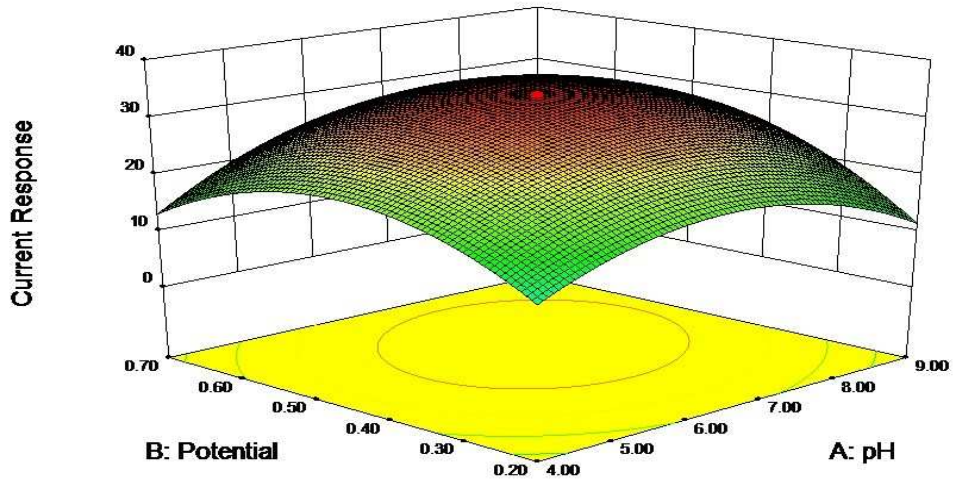

Figure 4: Effect of pH and Working Potential on current response. The other parameter temperature was kept constant at 47.5°C

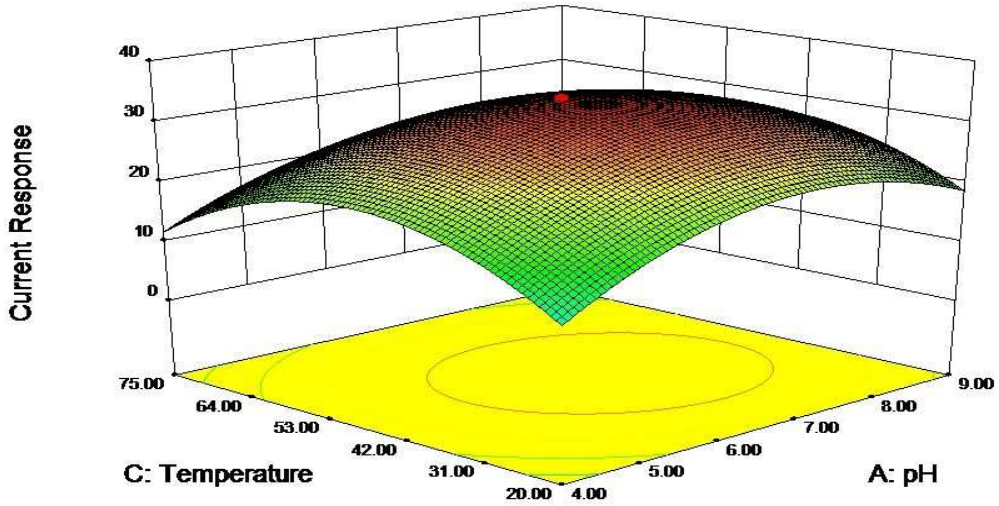

Figure 5: Effect of pH and Temperature on current response. The other parameter working potential was kept constant at 0.45 V.

CONCLUSION

We have developed an effective operational technique for the fabrication of enzyme biosensor developed on ZnO nanoparticles. The structure of GOx can be maintained after bioconjugation with ZnO which can be shown with the help of the UV-spectrum. It was observed that the enzyme electrode containing ZnO nanoparticles improves the current response as compared with the electrodes with no nanoparticles. The optimization of current response was done with the help of RSM.

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