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A Novel Method to Increase the Stability and Activity of Enzyme through Immobilization on Green Synthesized ZnO Nanoparticles

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Abstract:
It is essential to increase the stability of the enzyme.The activity of the enzyme is also required to be maintained. In this paper, these were achievedthrough immobilization techniques. Adsorption on nanoparticleswas used for this purpose. Glucose oxidase (GOx) and horseradish peroxidase (HRP) were studied for this purpose. ZnO nanoparticles were synthesized via a green method for the immobilization process. The research focuses on standardization techniques to optimize various parameters, including temperature, pH, concentration, and incubation time, in enzyme immobilization. The study concludes with a comparative analysis between green synthesized ZnO nanoparticles and commercially available native ZnOnanoparticles.

Keywords: Enzyme, Green Synthesization, Stability, Activity, ZnO Nanoparticles

1. INTRODUCTION

In our day-to-day lives, enzymes play an important role. It is basically a biological catalysts. It is used in pharmaceuticals (Sun et al., 2018; Panesar et al., 2012), food processing (Fernandes, 2010), research, and the chemical industry. As enzymes are used in many places, if we can control its reaction rates (Choi et al., 2015), it will be very effective. Glucose oxidase (GOx) is a good example, commonly used in diagnostic tests to measure glucose levels influids of human body, like blood and urine. GOx is an oxidoreductase,whichis used to do oxidation of glucose into hydrogen peroxide and gluconate (Wong et al., 2008), plays an important role in diabetes mellitus management.

Peroxidase enzymes are also used to detect glucose. Hydrogen peroxideis produced by the glucose-GOx reaction. Peroxidase changes the Hydrogen peroxide into reactive hydroxyl radicals. Hydroxyl radicals react with secondary metabolites like potassium iodide (Song et al., 2010) and generate colorimetric signals. So, both GOx and peroxidase are important to detect glucose.

In spite of its usefulness, there are some disadvantages of soluble enzymes in the sectors of industrial and medical applications. Some of the disadvantages are instability, non-reusability, low recovery purity, and high costs associated with single-use (Basso et al., 2019). Enzyme immobilization can be one possible solution. This process offers a few benefits like reusability, easy separation from reaction mixtures, and enhanced stability (Homaei et al., 2013; Mohamad et al., 2015). Immobilized enzymes have not only the advantage of stability, but there are other advantages like the nature of interactions, binding positions and conformational flexibility within the matrix. There are several immobilization techniques, like adsorption, crosslinking, entrapment, covalent binding, and microencapsulation (Datta et al., 2013).

One very simple and ancient method for immobilization is Adsorption. It is attaching enzymes to a carrier matrix, for example, nanoparticles, Sepharose, starch, resins, or aluminum oxide. The attaching is done by using weak bonds, for example, hydrogen bonds, ionic bonds, and van der Waals forces (Hernandez et al., 2011; Jesionowski et al., 2014). The main advantage of this method is the lack of pore diffusion limitations, as enzymes are bound to the external surface of the support material. There are some other advantages, too, for example, execution, no need for reagents, minimal activation steps, cost-effectiveness, and reduced enzyme disruption compared to chemical methods. The immobilization method of enzymes onto nanomaterials has various applications, like, offering a large surface area, high catalytic efficiency, and strong adsorption capability (Ahmad et al., 2015). Due to these characteristics, we can have more enzyme loading per unit mass of particles and more enzyme stability as there is a multipoint attachment to the nanomaterial surface.

In this paper, mainly immobilizing ofGOx and HRPare done. These are two crucial enzymes in blood glucose diagnostics, onto ZnO nanoparticles. Moreover, if the synthesis of ZnO nanoparticles is donevia biological methods, we can have cost-cuttingand more efficiency. The synthesized ZnO nanoparticles were observed and tested by UV-visible spectrophotometry. Characteristics of haltedGOx and HRP were observed and optimized under various conditions.

2. METHODOLOGY

2.1 Enzymes and Reagents

All of the chemicals present in the tests were of analytical rating. GOx and HRP enzymes were procured from TCI, Japan, and Zinc Nitrate Hexahydrate (Zn(NO3)2·6H2O) was sourced from Merck, USA. The glucose oxidase assay kit was also obtained from Sigma Merck, USA. Native ZnOnanopowderwas purchased from TCI, Japan.

2.2 Synthesis of ZnO Nanoparticles from Leaves

ZnO nanoparticles were synthesized using an aqueous extract from the leaves of C. roseus. Briefly, the extract was prepared by heating the leaves for 30 minutes at 65°C with continuous stirring. After filtration, the extract was centrifuged, and Zn(NO3)2·6H2O was added to the supernatant. This mixture was stirred for 40 minutes to form ZnO nanoparticles, indicated by a color change from pale brown to greenish-yellow. The synthesized nanoparticles were then used for immobilization studies.

2.3 Immobilization of Horseradish Peroxidase (HRP) on ZnO Nanoparticles

The enzymes were standardized under different pH levels, concentrations, temperatures, and incubation times as summarized in Table 1. To immobilize HRP, 1 mg of the enzyme was adsorbed onto 0.5 g of synthesized ZnO nanoparticles. HRP was melted in a 0.1 M of 10 ml buffer having a pH of 7 of potassium phosphate, followed by the addition of ZnO nanoparticles. The combination was rearedfor 2 hours at 4°C, then with at 10,000 rpmcentrifugation for the duration of 5 min at room temperature. The supernatant was collected, and the sediments were eroded a total of 3times with the same buffer to eliminate non-specifically gathered enzymes. Supernatants remained used to determine enzyme loading, and solid was kept at -20°C for auxiliary use.

Table 1: Standardization Parameters for Enzyme Immobilization

Sl. No.	Parameters	Standardization Values/Compounds
1	pH	3, 5, 7, 9
2	Concentration	Enzyme, nanoparticle
3	Temperature	4°C, 28°C, 37°C, 60°C
4	Incubation Period	1, 2, 3, 4 hr

2.4 Determination of HRP Activity (ZnO Nanoparticles-HRP)

HRP activity was measured through a phenol oxidation reaction. The halted HRP was mixedto an assay solvant containing phenol 60 mM of 1 ml, 4-AAP of 14.38 mM, 1.21 mM hydrogen peroxide, and pH 7.0 of phosphate buffer 0.1 M. Reaction remained conducted at 30°C through 5 min, the subsequent red color product remained monitored by measuring absorbance at 510 nm.

2.5 Immobilization of Glucose Oxidase (GOx) on ZnO Nanoparticles

GOxwas immobilized onto ZnO nanoparticles via adsorption. For this, 1 mg of GOxwas dissolved in pH 5 sodium acetate buffer of 10 ml of 0.1 M, next by the adding of 0.5 g of ZnO nanoparticles. The mixture was incubated overnight at 30°C with constant stirring. The immobilized enzyme was then collected by centrifugation at 10,000 rpm for 5 minutes at ambient temp. The solid pellets were eroded a total of 3 times with 0.1 M sodium acetate buffer (pH 5), supernatants from each wash were used to estimate enzyme loading on nanoparticles. The immobilized GOx was stored at -20°C.

2.6 Determination of GOx Activity (ZnO Nanoparticles-GOx)

The activity of immobilized GOx was determined using a glucose oxidase assay kit. This assay measured the hydrogen peroxide produced by the enzymatic reaction of GOx on glucose, with the reaction product being quantifiedcolorimetrically at 570 nm using a fluorescent peroxidase substrate.

2.7 Optimization of Immobilization Conditions

The adsorption method for enzyme immobilization was optimized by varying several parameters: pH, temperature, incubation time, and concentrations of ZnO nanoparticles and enzymes (HRP/GOx). Initial conditions were based on previous literature. The optimum activity was set as 100% under the selected conditions. The activity of immobilized enzymes was then compared relative to this optimum activity. The pH effect was tested using buffers with a 3.0–9.0 pH variety, including sodium acetate of 5.0 pH, 3.0 pH glycine-HCl, 7.0 pH sodium phosphate, 9.0 pH Tris-HCl, all at 0.1 M. Temperature effects were studied at 4°C, 28°C, 37°C, and 60°C. Incubation times were varied from 1 to 4 hours. Aliquots remainedkept at -20°C and were taken to ambient temp for activity testing. Enzyme activity was also assessed at different concentrations of ZnO nanoparticles (0.3, 0.5, and 0.7 g) and enzymes (0.5, 1, and 1.5 mg).

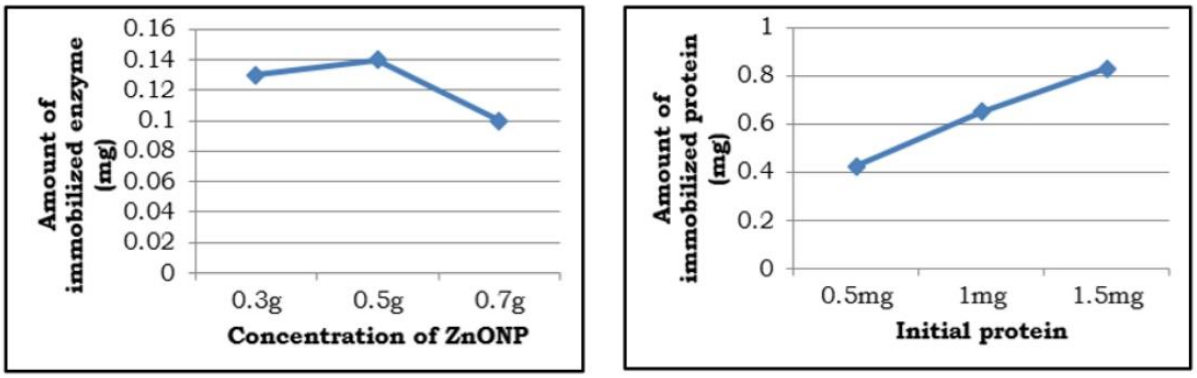
3. RESULTS AND DISCUSSION

3.1 UV-Visible Spectrophotometry

The initial confirmation of ZnO nanoparticle synthesis was indicated by a visual color change from pale brown to greenish-yellow. This color change suggests the successful formation of ZnO nanoparticles. Comprehensive characterization of the synthesized ZnO nanoparticles, as discussed in the previous chapter, affirmed their successful production. The powdered ZnO nanoparticles, along with the purchased ZnOnanopowder, were utilized for enzyme immobilization.

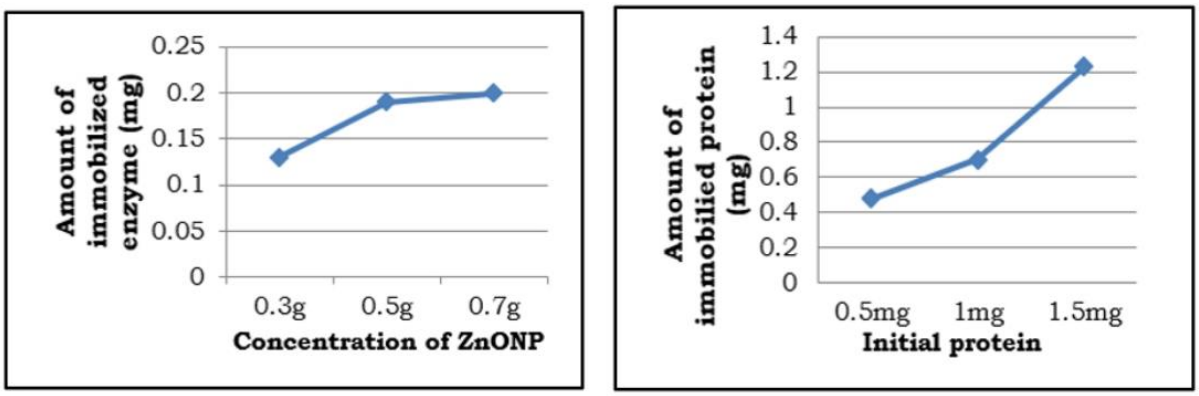
3.2 Determination of Enzyme Loading

Enzyme loading on ZnO nanoparticles was analyzed by varying the concentrations of both enzymes and ZnO particles. Enzyme loading refers to the amount of enzyme bound to the immobilized scaffold post-immobilization.



(a) (b)

Fig. 1: Impact of HRP loading on ZnO nanoparticles with varying concentrations of (a) ZnO nanoparticles and (b) initial protein.



(a) (b)

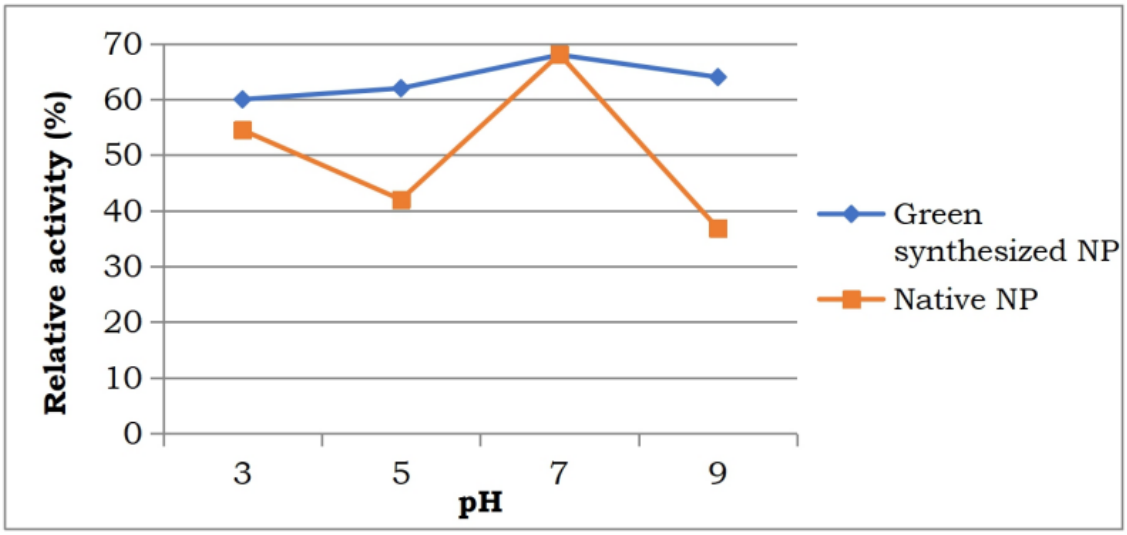
Fig. 2: Impact of GOx loading on ZnO nanoparticles with varying concentrations of (a) ZnO nanoparticles and (b) initial protein.

The effect of varying ZnO nanoparticle concentrations on HRP loading is illustrated in Fig. 1(a), and the effect of varying HRP concentrations is revealed in Fig. number 1(b). Similarly, Fig. number 2(a) and Fig. number 2(b) demonstrate the amount of GOx loaded on nanoparticles at different nanoparticle and enzyme concentrations, respectively. Observations indicate that increasing the ZnO nanoparticle concentration from 0.3g to 0.5g results in a steady increase in GOx loading, with minimal changes beyond 0.5g. This suggests that 0.5g is the optimal concentration of ZnO nanoparticles for GOx adsorption. For HRP, enzyme loading increased exponentially with higher concentrations of HRP, reaching a peak at 0.5g of ZnO nanoparticles.

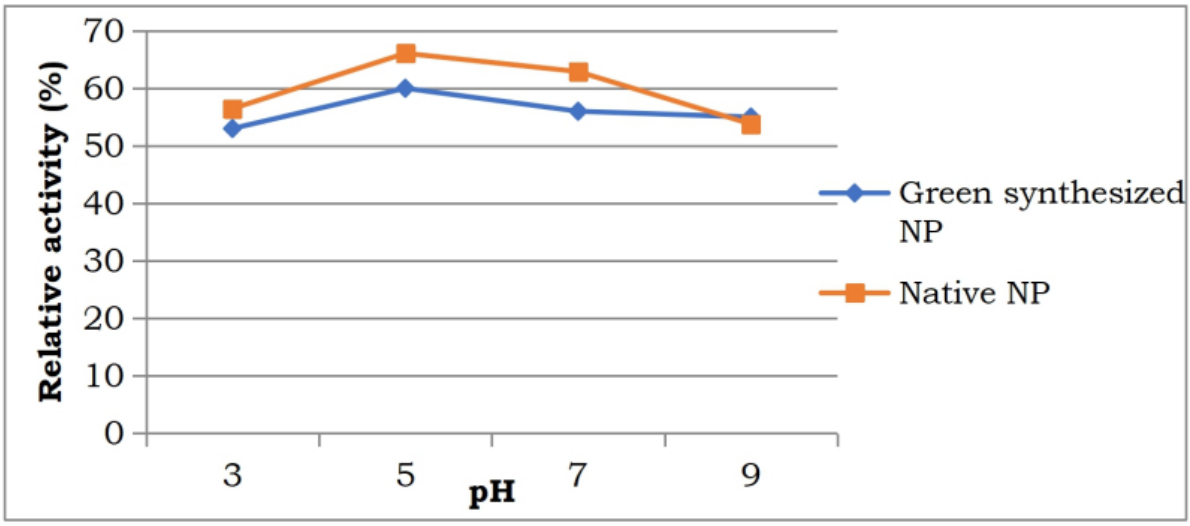
3.3 Optimization of Immobilization of GOx and HRP

Effect of pH

The optimization of HRP and GOx adsorption on ZnO nanoparticles was conducted. The optimal pH for immobilized HRP and GOx was found to be 7 and 5, respectively. At pH 7, immobilized HRP exhibited about 68% activity, as depicted in Fig. 3(a). There was a noted decrease in catalytic activity at both higher and lower pH values. A comparative study with native ZnOnanopowder (purchased) revealed a similar activity profile for HRP, with native nanoparticles showing 67% activity. For GOx, all tested pH levels resulted in over 50% activity, with the highest activity of about 60% at pH 5, as shown in Fig. 3(b). Synthesized nanoparticles demonstrated higher activity for HRP, whereas native nanoparticles were more effective for GOx.



(a)

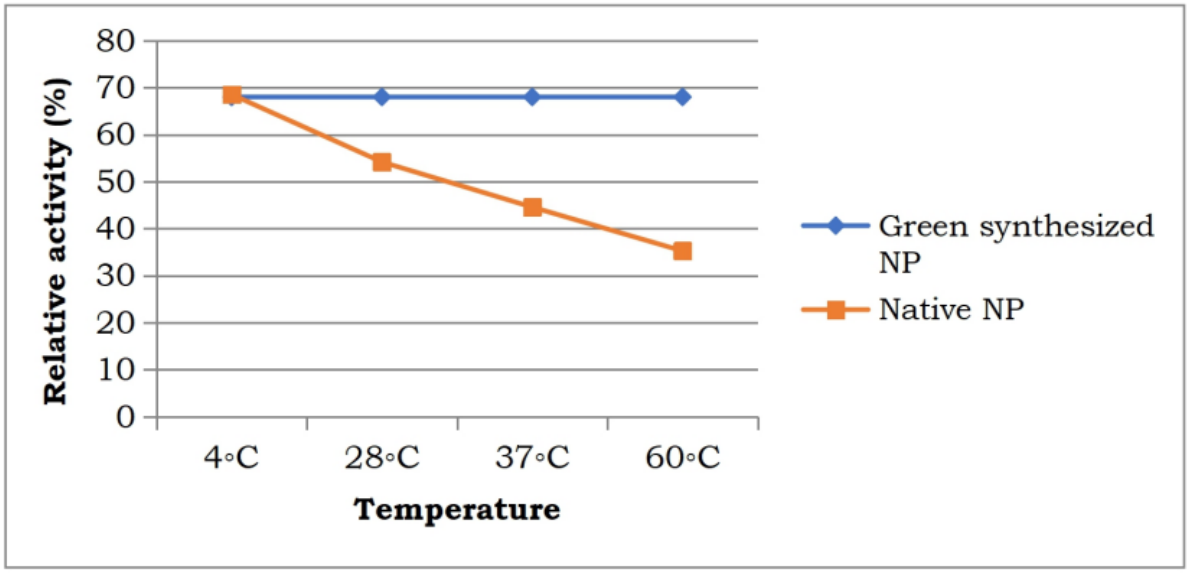


(b)

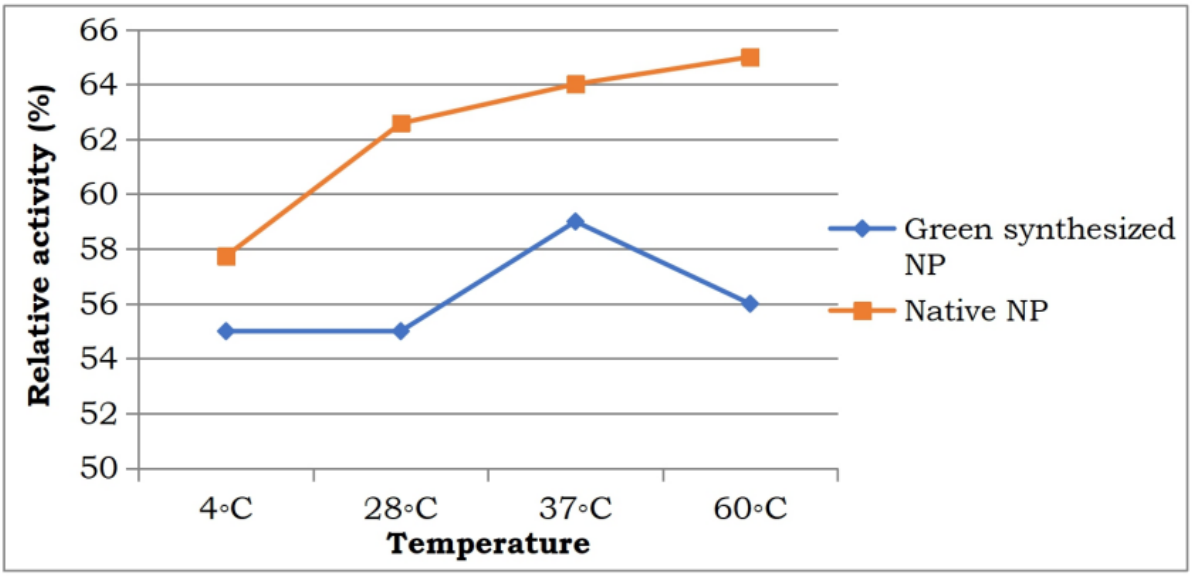
Fig. 3: Enzyme activity profile at different pH levels using green synthesized and native nanoparticles for (a) HRP and (b) GOx.

Influence of Heat

Heat activity summary of immobilized GOx and HRP on green synthesized ZnO nanoparticles was compared with that on purchased ZnOnanopowder. Immobilized HRP on green synthesized nanoparticles maintained consistent catalytic activity across all temperatures, showing nearly 70% activity, indicating thermal stability.



(a)



(b)

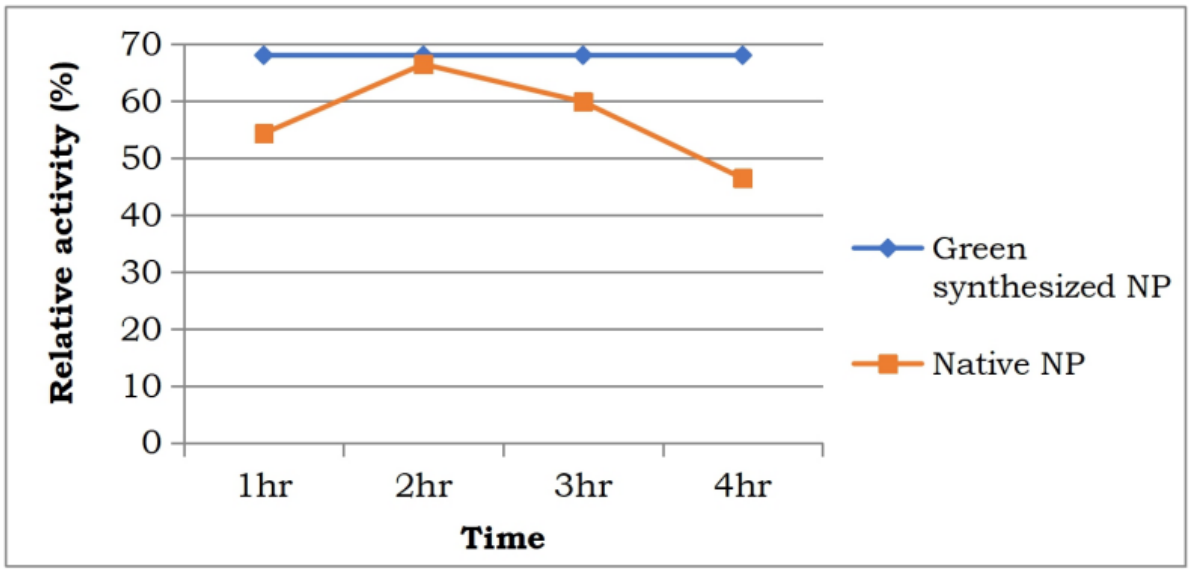
Fig. 4: Enzyme activity profile at different temperatures using green synthesized and native nanoparticles for (a) HRP and (b) GOx.

The temperature activity analysis for HRP is shown in Fig. 4(a). When compared to native ZnO nanoparticles, the green synthesized nanoparticles showed more stable activity, whereas the activity of HRP on native nanoparticles decreased with increasing temperature. For GOx, the optimal temperature was determined to be 37°C, with 59% activity for green synthesized ZnO nanoparticles. Below 37°C, the immobilized enzyme exhibited a constant 55% activity, while slightly higher activity (56%) was observed above 37°C. Therefore, 37°C was established as the optimal temperature for GOx immobilization on ZnO nanoparticles, as illustrated in Fig. 4(b). Conversely, GOx immobilized on native nanopowder displayed higher activity with increasing temperature.

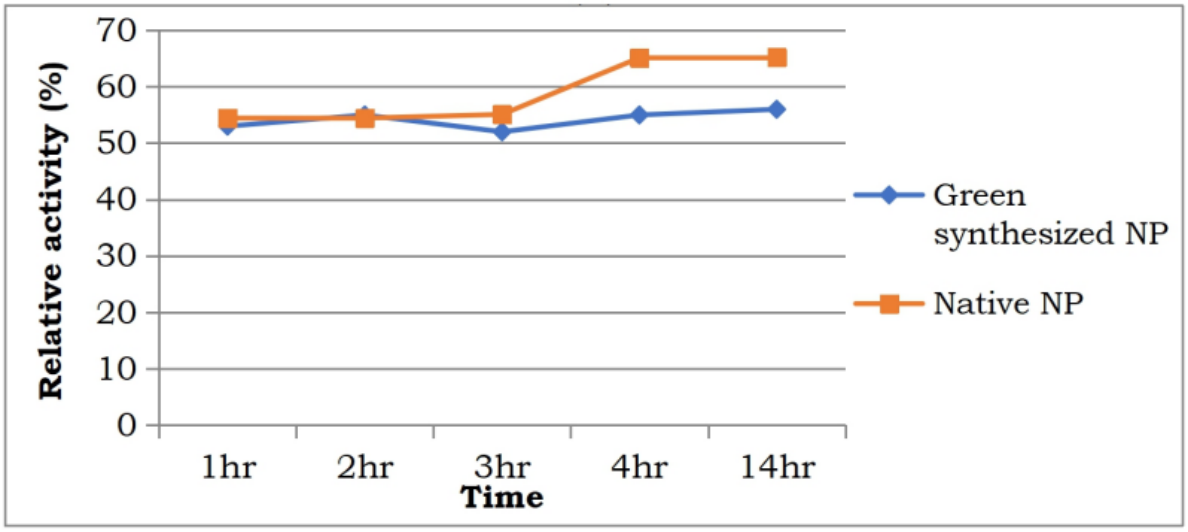
Influence of Duration

Varying incubation times on enzyme adsorption to ZnO nanoparticles was investigated. For synthesized nanoparticles using plant extracts, enzyme activity remained constant at 70%

across all tested time intervals. Figure 5(a) shows that the incubation period did not significantly impact the activity of immobilized HRP when using green synthesized nanoparticles.



(a)



(b)

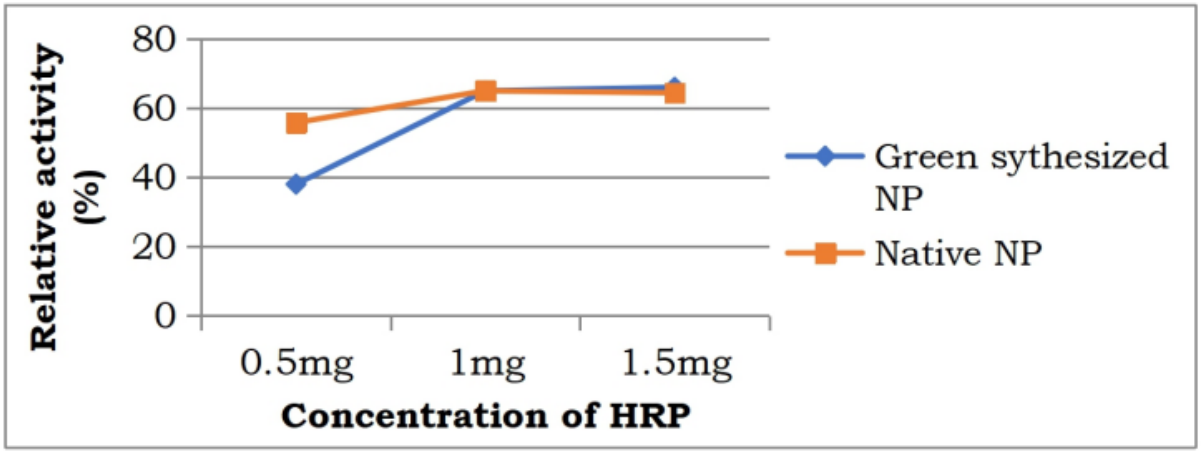
Fig. 5: Enzyme activity profile at different incubation times using green synthesized and native nanoparticles for (a) HRP and (b) GOx.

When HRP was immobilized on native ZnOnanopowder, a 2-hour incubation yielded the highest activity at 66%. In contrast, GOx exhibited varying enzyme activity with different incubation times, as depicted in Fig. 5(b). The highest activity (56%) was recorded after overnight incubation, with 55% activity observed at both 2 and 4 hours for green synthesized nanoparticles. The lowest activity (52%) was seen at a 3-hour incubation. GOx immobilized on native ZnOnanopowder achieved 65% activity after overnight incubation.

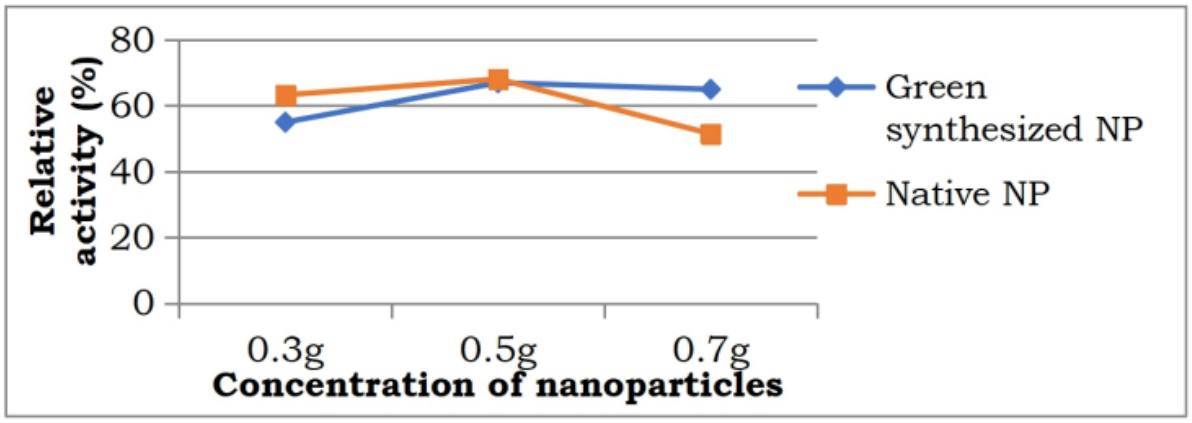
Effect of Concentration

The activity of immobilized enzymes varied with changes in enzyme and nanoparticle concentrations. This trend was observed for both green synthesized and chemically synthesized nanoparticles. Figure 6(a) illustrates that HRP activity increased exponentially

with higher initial HRP concentrations, reaching a plateau beyond 1 mg for both green synthesized and native ZnO nanoparticles. Figure 6(b) shows that HRP activity peaked at 68% with 0.5g of nanoparticles, slightly decreasing with further concentration increases.



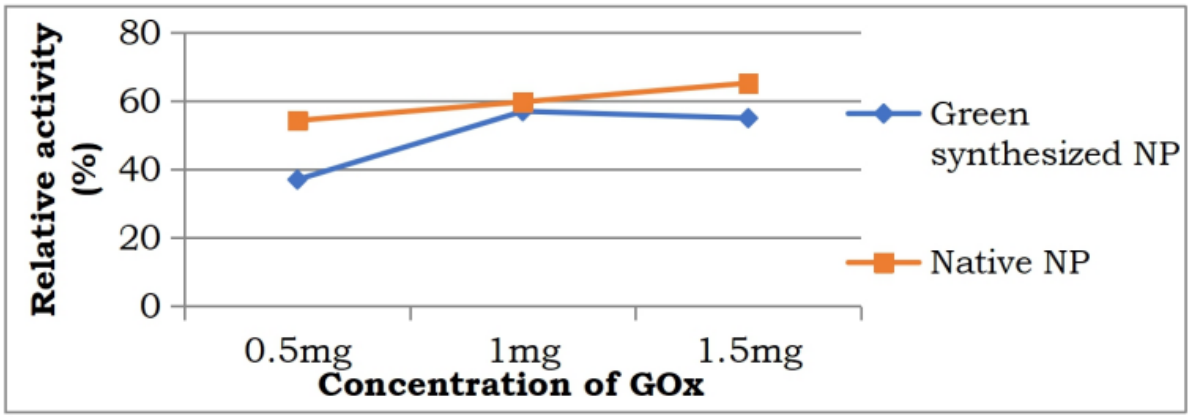
(a)



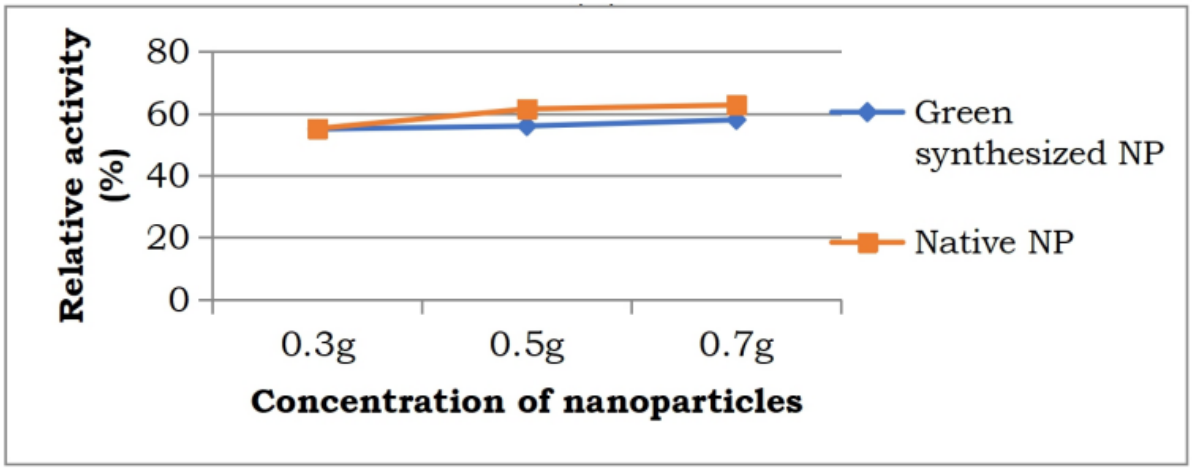
(b)

Fig. 6: Enzyme activity profile of immobilized HRP using green synthesized and native nanoparticles with varying concentrations of (a) HRP and (b) nanoparticles.

For GOx, Fig. 7(a) indicates the highest activity at 1 mg, followed by 1.5 mg and 0.5 mg. Figure 7(b) reveals that GOx activity increased with higher concentrations of green synthesized ZnO nanoparticles. However, GOx immobilized on native ZnOnanopowder exhibited the highest activity (62%) across different nanoparticle concentrations.



(a)



(b)

Fig. 7: Enzyme activity profile of immobilized GOx using green synthesized and native nanoparticles with varying concentrations of (a) GOx and (b) nanoparticles.

Conclusion

Optimal conditions for enzyme immobilization on ZnO nanoparticles were determined by evaluating various parameters, including concentration, pH, temperature, and incubation time for both HRP and GOx. The optimized conditions for the highest activity of immobilized HRP on green synthesized and native ZnO nanoparticles are summarized in Table 2, while those for immobilized GOx are detailed in Table 3.

Table 2: Optimal parameters for highest activity of immobilized HRP

Sl. No.	Parameters	Optimum value of ZnO nanoparticles	
		Green synthesized	Native
1	pH	7	7
2	Temperature	4°C	4°C
3	Incubation time	1 hour	2 hours
4	Concentration of enzyme	1.5 mg	1 mg
5	Concentration of nanoparticles	0.5 g	0.5 g

Table 3: Optimal parameters for highest activity of immobilized GOx

Sl. No.	Parameters	Optimum value of ZnO nanoparticles	
		Green synthesized	Native
1	pH	5	5
2	Temperature	37°C	60°C
3	Incubation time	4 hours	14 hours
4	Concentration of enzyme	1 mg	1.5 mg
5	Concentration of nanoparticles	0.7 g	0.7 g

4. SUMMARY

This study demonstrated that ZnO nanoparticles can be effectively synthesized using plant extracts, presenting a sustainable alternative to chemical synthesis. The biosynthesized nanoparticles were successfully utilized for enzyme immobilization, showing promising results. Horseradish peroxidase (HRP) immobilized on the green synthesized ZnO nanoparticles exhibited remarkable thermal stability across various time intervals. In contrast, glucose oxidase (GOx) showed variability in its activity profile under different incubation times and temperatures. The optimal pH for immobilizing HRP and GOx was determined to be 7 and 5, respectively. These findings offer valuable insights for developing enzyme-nanoparticle immobilization techniques, which could pave the way for creating more stable and durable enzyme test strips for extended applications.

REFERENCES:

Ahmad, R., &Sardar, M. (2015). Enzyme immobilization: an overview on nanoparticles as immobilization matrix. Biochemistry and Analytical Biochemistry, 4(2), 1.

Basso, A., &Serban, S. (2019). Industrial applications of immobilized enzymes - A review. Molecular Catalysis, 479, 110607.

Choi, J. M., Han, S. S., & Kim, H. S. (2015). Industrial applications of enzyme biocatalysis: Current status and future aspects. Biotechnology advances, 33(7), 1443-1454.

Datta, S., Christena, L. R., &Rajaram, Y. R. (2013). S. Enzyme immobilization: an overview on techniques and support materials, 3 Biotech, 3(1), 1–9.

Fernandes, P. (2010). Enzymes in food processing: a condensed overview on strategies for better biocatalysts. Enzyme research, 2010(1), 862537.

Hernandez, K., & Fernandez-Lafuente, R. (2011). Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. Enzyme and microbial technology, 48(2), 107- 122.

Homaei, A. A., Sariri, R., Vianello, F., &Stevanato, R. (2013). Enzyme immobilization: an update. Journal of chemical biology, 6(4), 185-205.

Jesionowski, T., Zdarta, J., &Krajewska, B. (2014). Enzyme immobilization by adsorption: A review. Adsorption, 20(5-6), 801-821.

Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., &Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnology & Biotechnological Equipment, 29(2), 205-220.

Panesar, P. S., & Kennedy, J. F. (2012). Biotechnological approaches for the value addition of whey. Critical reviews in biotechnology, 32(4), 327-348.

Song, Y., Qu, K., Zhao, C., Ren, J., & Qu, X. (2010). Graphene oxide: intrinsic peroxidase catalytic activity and its application to glucose detection. Advanced Materials, 22(19), 2206-2210.

Sun, H., Zhang, H., Ang, E. L., & Zhao, H. (2018). Biocatalysis for the synthesis of pharmaceuticals and pharmaceutical intermediates. Bioorganic & medicinal chemistry, 26(7), 1275-1284.

Wong, C. M., Wong, K. H., & Chen, X. D. (2008). Glucose oxidase: natural occurrence, function, properties and industrial applications. Applied microbiology and biotechnology, 78(6), 927-938.