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Immobilization of Glucose Oxidase on Iron Oxide Nanoparticles to Enhance Its Stability for Industrial Applications

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Abstract
Glucose oxidase is an oxidoreductase flavoprotein which has high range applications in food, pharmaceutical and in clinical chemistry. However, enzyme applications are prohibited due to some limitations of enzymes. Hence, there is a need to overcome these limitations by immobilization techniques. This study mainly focuses on magnetic nanoparticles-based immobilization of an enzyme that is successfully used because this method provides the greater stability and support to enzyme due to the high surface area and small size of nanoparticles. Different immobilization conditions were also optimized. Co-precipitation method was followed to synthesize the iron oxide nanoparticles and iron oxide nanoparticles was characterized using various spectrophotometric and analytical techniques like UV-Vis, SEM, FTIR, Zeta Sizer and XRD. Moreover, the role of magnetic and immobilized iron oxide nanoparticles as decolorizing agent was examined. Present results showed the successful synthesis of magnetic nanoparticles and confirmed by their characterization. Best immobilization conditions were achieved at temperature of 10°C, pH 6 and immobilization time of 2.5 hours while GOx/nanoparticles ratio of 3000U/g. From the present findings it is concluded that immobilization of glucose oxidase is an effective way for creating bioconjugates to develop economically feasible and high-performance catalysts with improved stability and reusability for a variety of applications in medical and food industries.

Keyword: Glucose oxidase, Enzyme immobilization, IONPs/Magnetite

1. Introduction

Glucose Oxidase is a glycoprotein that catalysis the oxidation of β-D-glucose using O_2 molecule that act as an electron acceptor for D-glucono-1, 5-lactone which later on, hydrolyzed to make D-gluconic acid and H_2O_2. In previous reaction, later one is catalytic product that act as an anti-fungal and anti-bacterial agent [1-3]. Glucose oxidase enzyme was first introduced by Muller in 1925 [4]. Glucose oxidase enzyme is currently getting much consideration because of diverse commercial applications in different areas such as in chemical, pharmaceutical, synthetic, food, beverages, biotechnology, life sciences and in clinical chemistry. In current years, novel use of glucose oxidase is immobilization that expanding its demand [5, 6]. At present time, nanomaterials are used as a support for immobilization of biocatalysts because this method provides stability and greatest support to enzyme [7, 8].

Among various nanomaterials, magnetic nanoparticles are very popular that use for enzyme immobilization. These magnetic materials are attracted because of their non-toxicity, super paramagnetic property, high capacity for enzyme loading, highest compatibility with other systems and oxidative stability [9-11]. IONPs causes to reduce the economic burden due to reusability of iron oxide based nano-material [12-14]. The chemical co-precipitation method to synthesize the IONPs is an extra facile and cost-effective method which can control the size of NPs through varying the reaction conditions and surface modification [15, 16].

For manipulation of different kinds of MNPs significant attention has developed as they are widely studied for their applications in the field of biotechnology, biomedical, material science, environmental and in engineering [17, 18]. Decolorization ability of iron oxide nanoparticles can also be studied using reactive textile dye (λmax: 475nm). Adsorption is the most efficient and promising way for the treatment of dye effluents as adsorbents used are cost effective and easily available such as IONPs [19, 20]. Enzyme immobilization is a powerful tool that is intensively used to prepare various superior and economically feasible biocatalysts with improved activity, stability and reusability for the recovery of enzyme limitations [21-23]. Additionally, immobilization technique reduces the operation cost and produce contamination free product that are useful for industrial applications. In the past few years, adsorption method was used to immobilize the glucose oxidase using the magnetic support [24, 25].

The objectives of the current research work were the successful synthesis of iron oxide nanoparticles as well as their characterization by SEM, UV-Vis spectroscopy, Zeta sizer, XRD and FTIR techniques. The aim of the present study was the successful immobilization of glucose oxidase using the magnetic support of IONPs and the treatment of dye effluents to assess the decolorization ability of chemically synthesized iron oxide nanoparticles.

2. EXPERIMENTAL METHOD

2.1. Synthesis of Iron Oxide Nanoparticles

Iron oxide nanoparticles were chemically synthesized using well known co-precipitation method following procedure [19] with slight modifications. For this purpose, solutions of 0.1M $FeCl_2.4H_2O$ and 0.2 M $FeCl_3.6H_2O$ were prepared using 1:2 molar ratios respectively. $FeCl_2.4H_2O$ was added dropwise to ferric chloride solution under continuous magnetic stirring. Then completely covered solution was subjected for heating in water bath for 15-20 minutes. After that, pH of the reaction solution was measured using pH meter which was 4.3. 1N NaOH was prepared and added dropwise to master solution up to 14 ml, then instant black precipitates of magnetic nanoparticles were formed. Significant change was noticed in pH (9.8) after the addition of alkaline solution.

$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$……. (i)

The solution was centrifuged at 7000 rpm for 15-20 minutes and the pallet was formed that was collected through washing with 70% ethanol. After centrifugation, supernatant liquid was discarded while precipitates were dried in oven at 50°C [26-28] and isolated with the help of magnet. Hence, the magnetic nanoparticles were synthesized. Magnetic power of chemically synthesized iron oxide nanoparticles was checked by putting a magnet near black nanoparticles that showed the strong magnetic behavior of newly synthesized iron oxide nanoparticles.

2.2. Immobilization of glucose Oxidase

The adsorption method was used to immobilize glucose oxidase on chemically synthesized magnetic nanoparticles following procedure [14]. For this purpose, IONPs-I (1g) were dissolved in 10ml of phosphate buffer solution (0.1M, pH 6) referred as NPs solution that was used as a control and the same amount of these IONPs-I were dispersed in 10ml phosphate buffer solution containing glucose oxidase enzyme that was named as enzyme solution.

Both solutions were put for about 2.5 hours in shaker-incubator. During immobilization process, pH of both solutions was also monitored. The precipitates containing magnetic nanoparticles (Enzyme nanoparticles conjugates) were washed by phosphate buffer solution to remove excess GOx and then used for further analytical experiments.

2.3. Optimization of GOx immobilization conditions

During immobilization of enzyme, different immobilization conditions were optimized. To measure optimum conditions of immobilized glucose oxidase, influence of different kinetic parameters was measured such as pH, temperature, enzyme/nanoparticles ratio and immobilization time. The activity of immobilized enzyme was used as the criterion for successfully immobilized enzyme amount [26, 29].

2.4. Assay of enzyme activity

Enzyme activity of glucose oxidase was measured quantitatively by spectrophotometric analysis. After setting the blank rate for enzyme assay, 100 µ litter/ 0.1 ml glucose oxidase enzyme was added in buffered-substrate solution in cuvette and incubated in spectrophotometer for 3-5 minutes to get temperature equilibration then optical density was measured on spectrophotometer at 460 nm [30, 31].

2.5. Dye Decolorization

Adsorption is the most efficient and promising way for the treatment of dye effluents as adsorbents used are cost effective and easily available such as IONPs-I [32] . Decolorization ability of iron oxide nanoparticles was studied using reactive textile dye (λmax: 475nm). The experimental flasks 1 contain IONPs (0.1g), 50ml water and 50µL dye while the experimental flasks 2 contain E-IONPs-I (0.1g) under the same conditions. In addition, parallel blank experiment was performed with deionized water and dye without IONPs [31]. The experimental and control flasks were incubated at 35°C temperature on rotary shaker (150 rpm) for 24 hours. Samples were taken after every 2h and centrifuged (3,000×g, 10 min) to eradicate the suspended particles. Residual dye concentrations in the supernatants were determined by measuring the absorbance at corresponding wavelength λ_{max}= 475 nm of original dye solution. The following equation was used to determine the dye decolorization in percentage.

$$Decolorization\ (\%) = \frac{(Ai) - (At)}{Ai} \times 100$$

2.6. Reusability of Immobilized Enzyme

Freshly prepared GOx/IONPs-I sample (0.1g) was washed 20 times with 100 ml distilled water and the remaining activity was performed after each run as a measure of enzyme adherence to magnetite [33].

3. Results and discussion

3.1. Synthesis of Iron Oxide Nanoparticles

Ferrous chloride tetrahydrate and ferric chloride hexahydrate were used as a source of iron for the chemical synthesis of IONPs-I. Both the salts were mixed by means of magnetic stirring. Deprotonation of the two salts occurred as ferric and ferrous chloride solutions were prepared in deionized water and finally the hydroxides of these salts were formed. Sodium

hydroxide (NaOH) was added as a precipitating agent and the precipitates were synthesized and confirmed by the color change from brownish to black that was further investigated through spectrophotometric analysis and pH changes. It was observed that, the sodium hydroxide causes the reduction of iron and started to form the black precipitates of ferric oxide.

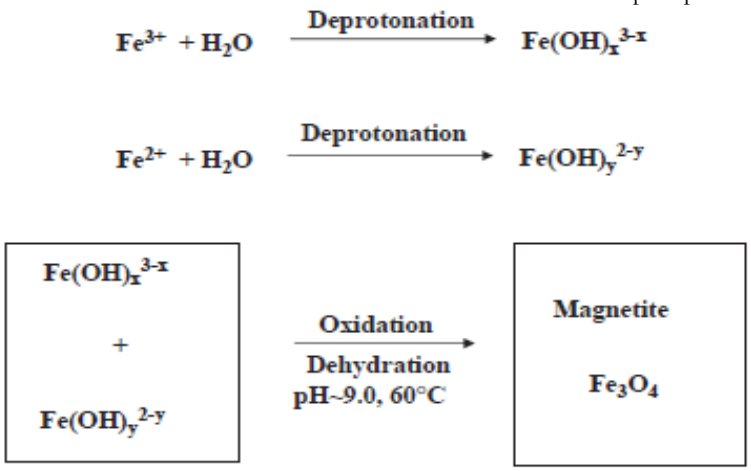

Figure 1. Display of reaction mechanism for IONPs formation

Hariani *et al.* [19] also found the similar visual and spectrophotometric effects by noticing the color changes from brownish to black which showed the formation of iron oxide nanoparticles. The complete equation is:

$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$

Magnetic power of chemically produced iron oxide nanoparticles was checked using magnet. The magnetic nanoparticles were gained a particular shape as magnet was brought near to it. Furthermore, magnetic nature of iron oxide nanoparticles was also confirmed by some other studies [20, 34, 35].

(a) Black nanoparticles of iron oxide **(b) Magnetism checked with the help of magnet**

Figure 2. Displaying (a) Black nanoparticles of iron oxide (b) Magnetism checked with the help of magnet

3.2. Characterization of iron oxide nanoparticles

Chemical synthesis of IONPs was confirmed using spectrophotometric and analytical techniques such as UV-visible spectroscopic analysis, Fourier transform infra-red spectroscopy, Scanning electron microscope, Zeta sizer and X-ray diffraction.

3.2.1. UV-Vis Spectroscopy

UV-Visible double beam spectrophotometer was utilized (200nm-800nm) to monitor chemically produced IONPs. Powdered type of iron oxide nanoparticles was mixed in deionized water and then the sample was investigated for optical characteristics of IONPs. By applying the Lambert-Beer law, the concentration of analyte can be measured at specific wavelength. UV-Visible spectrum of IONPs-I showed the characteristic peak at 382nm (fig. 3) which is in accordance with the previously reported results in which IONP shows characteristic absorption peak at 359nm, 367nm [36-38].

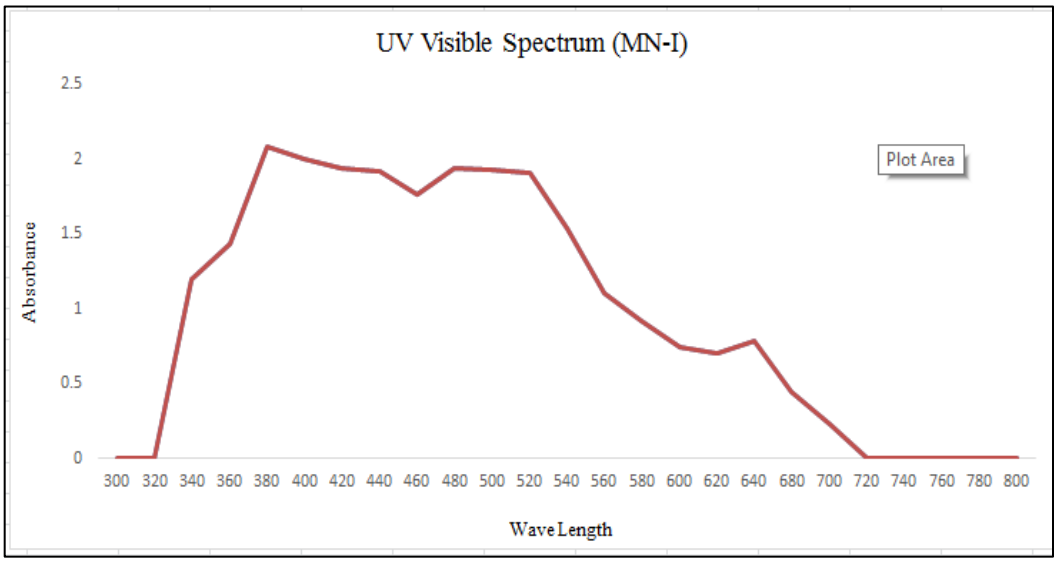

Figure.3. UV-Visible spectrum of nanoparticles of iron oxide- I (IONPs–I)

3.2.2. Scanning Electron Microscopy of IONPs–I (SEM)

Scanning electron microscope (SEM) FEI-Quanta 250 was used to determine the particle size and external morphology of the Fe_3O_4 nanoparticles synthesized from ferric chloride and ferrous chloride which is magnified at 1000X, 2000X and 4000X exhibiting the size of particles in the range of 10µm-40µm due to different magnification power as shown in fig.4. The current results are in accordance with previous studies [39, 40] who displayed the micrograph of SEM prepared through carbo-thermal reduction which showed spherical shape and agglomerates up to some extent.

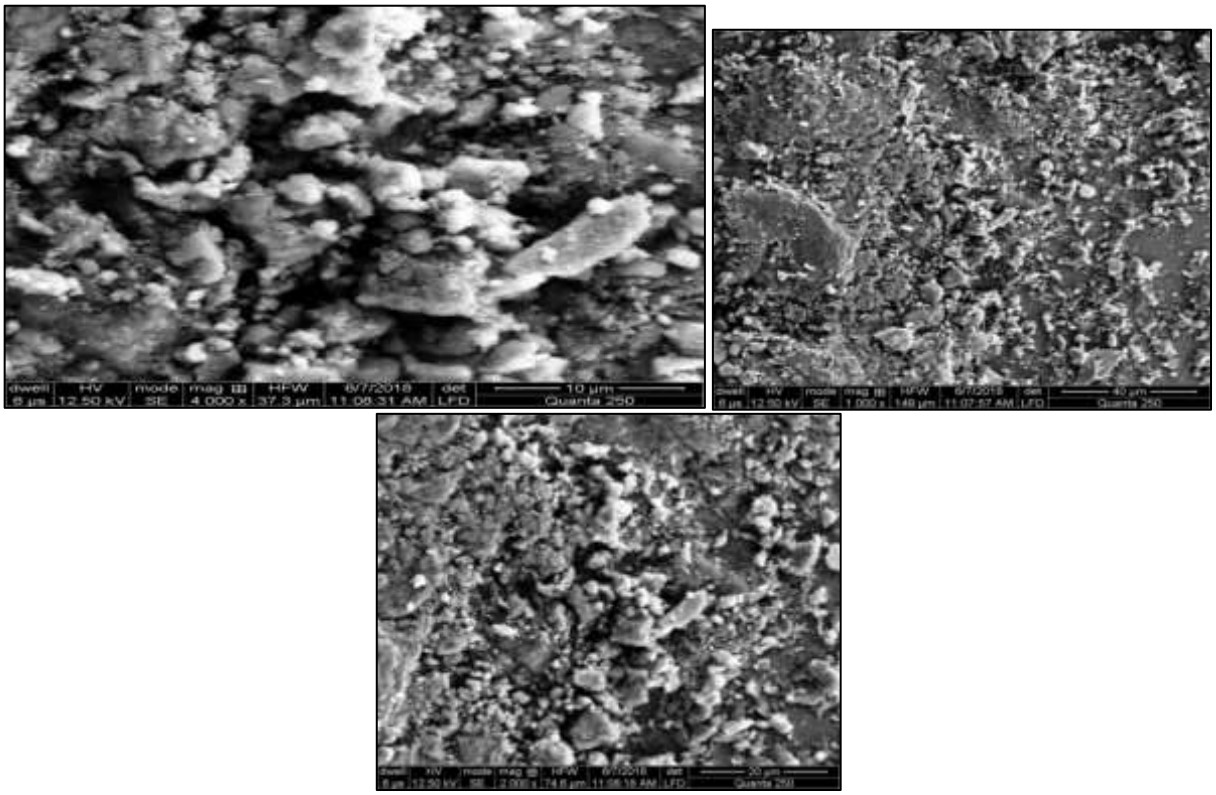

Figure.4. SEM Image of nanoparticles of iron oxide- I (IONPs-I)

3.2.3. Zeta Sizer of nanoparticles of iron oxide (IONPs–I)

The size of IONPs-I was measured using Malven zeta sizer analysis. Zeta analysis is shown in fig. 5 (A) that displayed the particle size distribution and poly dispersity index (PdI) of IONPs-I as 362.5 (d.nm) and 0.557 respectively. Three peaks were identified of different sizes 1163, 207.8, 5082 with percentage intensities 60.7, 37.8, and 0.9 respectively. Similar findings were also determined by [41, 42], their results showed that the particles average size was 2.6 µm and polydispersity index was 0.156 of ZnO micro-particles. Zeta sizer analysis for E-IONPs-I is shown in Fig. 5 (B), exhibited the good quality of average zeta size and polydispersity index which were 247.2 (d.nm) and 0.491 respectively. The sizes of other two peaks were 299.1 and 4823 and their intensities were 96.7 and 3.3 respectively. The current result is in accordance with results reported by [43, 44].

Their results represented the zeta average size of 38.4 units/mg nanoparticles for immobilized glucose oxidase on magnetic nanoparticles.

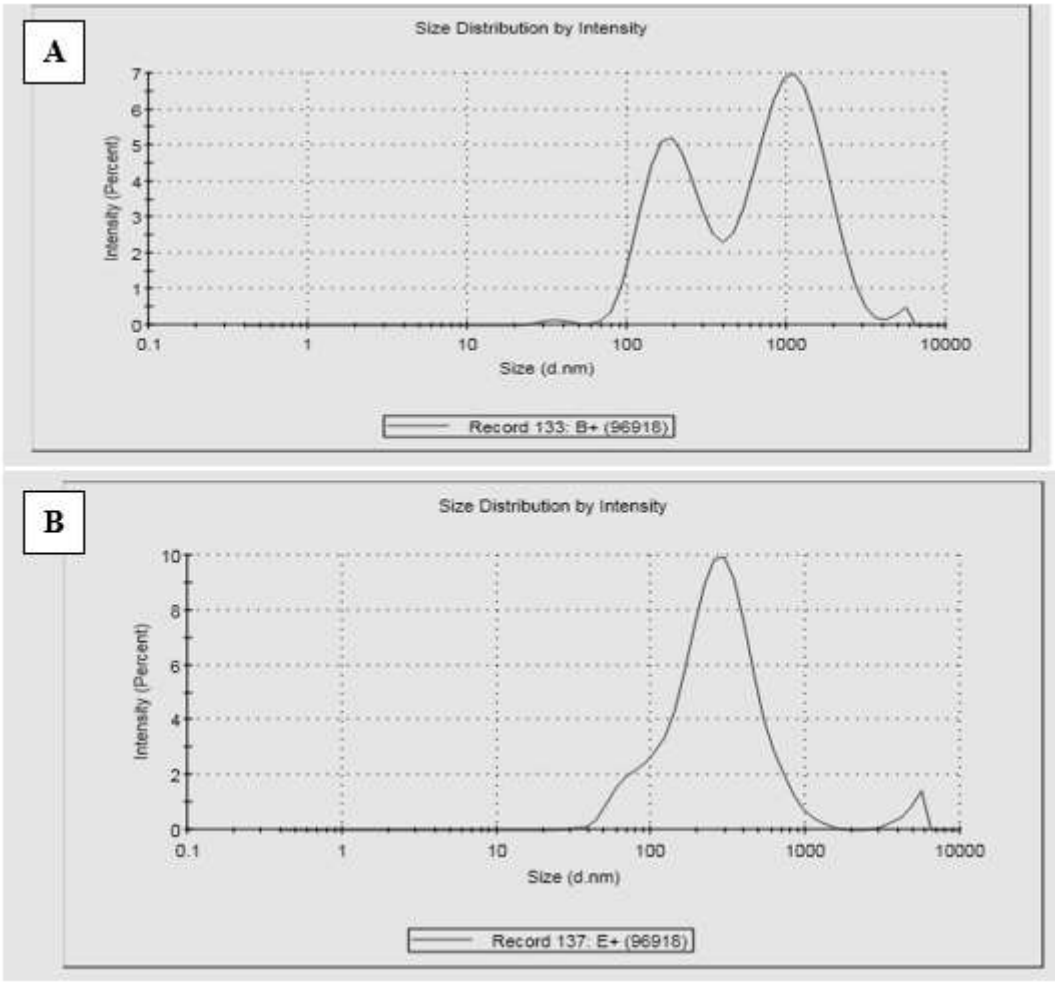

Figure.5. Zeta Sizer of (A) IONPs-I and (B) E-IONPs-I

3.2.4. X-ray Diffraction analysis of IONPs-I

The XRD pattern of chemically synthesized IONPs-I was observed at 2θ=10–90° (fig.6). Three different peaks appeared at 2θ = 32.245°, 34.135° and 36.985° that was also reported by [12-14, 32]. Crystal size of chemically synthesized IONPs-I was calculated (23.74) by Scherer equation.

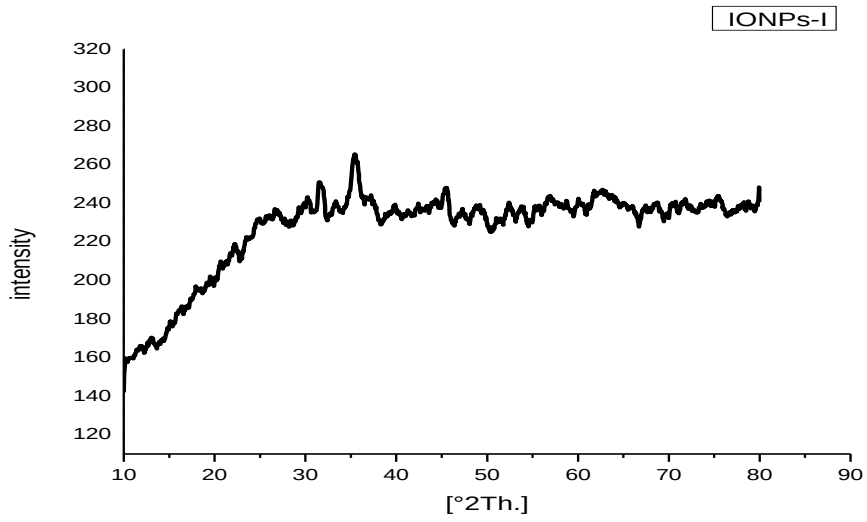

Figure.6. XRD graph of iron oxide nanoparticles

3.2.5. FTIR analysis of chemically synthesized IONPs-I

FTIR analysis of IONPs was recorded using the IR spectrophotometer to identify the functional groups available on the surface of IONPs. The FTIR analysis of IONPs-I was performed using (Bruker, Germany) in the wave number range from 4000 to 650cm^{-1}. FTIR measurement was performed for one batch of samples on KBr matrix and is shown in Fig. 7. Number of peaks such as 3445.60cm^{-1}, 546.42 cm^{-1}, 1456.63 cm^{-1} and 848.34 cm^{-1} were observed. A broaden peak at about 546.42 cm^{-1}observed is attributed to Fe-O-Fe bending vibration of magnetite due to reaction of iron salts with base which is the confirmation of formation of magnetite particles. Similar results are found by Puvvada *et al.* [45]. Current peaks are also related to Hua and Zeng [46] who showed a peak at 580cm^{-1} attributed to Fe-O stretching vibration of magnetite. Moreover, such results are also related with peaks 465-686cm^{-1} which were shown by Goswami [47]. The peak at 1456.63 cm^{-1} is attributed to the stretching vibration of H-O-H which was also observed by Kandpal *et al.* [26] around 1429.6cm^{-1}. While the peak at 3445.60cm^{-1} corresponds to hydroxyl group (OH) which is present due to existence of water molecules [28, 29].

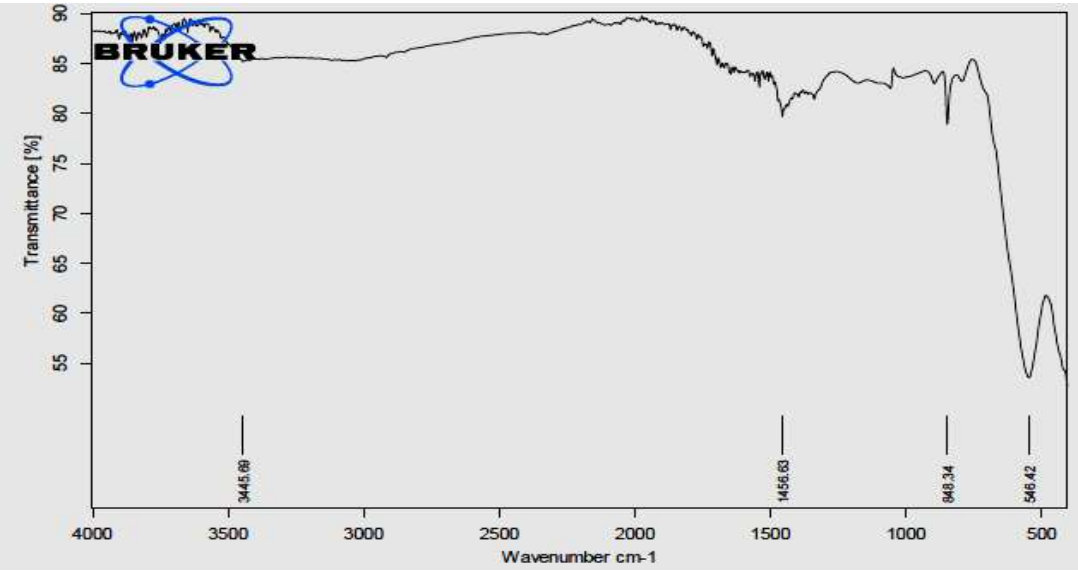

Figure 7. FTIR of iron oxide nanoparticles

3.3 Immobilization of glucose oxidase

Glucose oxidase enzyme was successfully immobilized on magnetic support of chemically synthesized IONPs. Effects of various parameters were determined to find the optimum conditions of the immobilization of GOx. The activity of the immobilized enzyme was used as the criterion for the successfully immobilized enzyme amount.

3.3.1 Effect of pH

Immobilized enzyme efficiency was checked at pH ranges vary from 4, 5, 6, 7 and 8. Glucose oxidase was stable at pH 6 as shown in fig. 8 (A). Similar results were reported in the works of [48, 49]. They found the pH 6.15 as the best pH value for GOx immobilization on TiO_2/polyurethane.

3.3.2. Effect of Temperature

The immobilization process was carried out at different temperature values ranging from 10°C to 50°C with each 10^{o}C interval to find the optimum temperature. The highest immobilization efficiency was obtained at 10°C (fig.8.B). This similar findings were reported by previous studies [9, 31] that GOx became inactive at temperature values higher than 20.

3.3.3. Effect of Enzyme/nanoparticles Ratio

IONPs were dissolved in enzyme solution to measure the effect of enzyme/nanoparticles ratio. According to the present result, best enzyme/support ratios were achieved at 3000u/g as presented in fig.8 (C). current result is related to work reported by previous researchers [31, 50] they noticed the maximum enzyme/support ratios at 1800u/g.

3.3.4. Effect of Time

Immobilized enzyme effectiveness was measured at different time values vary from 2, 2.5, 3, 3.5 and 4 hours. The highest immobilization efficiency was obtained at 2.5 hours (Fig.8.D). Similar finding was also stated by [14, 31].

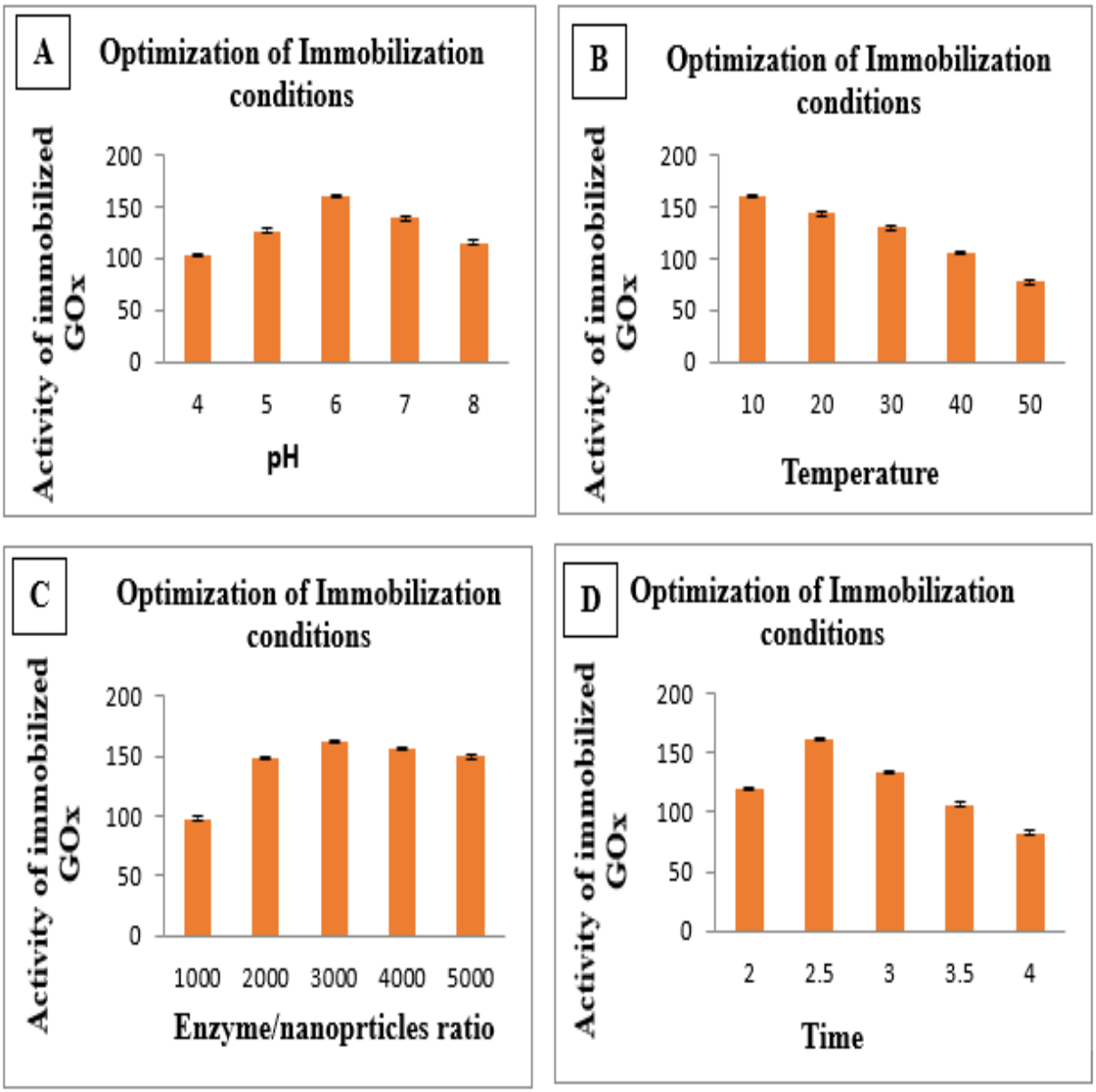

Figure.8. Effect of (A) pH (B) Temperature (C) Enzyme/nanoparticles Ratio (D) Time on Immobilized glucose oxidase

3.4. FTIR analysis of Free glucose oxidase enzyme

FTIR analysis of free glucose oxidase was analyzed. This spectrum displayed the various absorption peaks of GOx. Fig. 9 showed the closely related two peaks at 1074.94cm^{-1} and at 1094.57cm^{-1} that represented the C-O-C group stretching. Similar outcomes were also explained by [51]. The peak near to 1120.05cm^{-1} was signaled for C-N stretching vibration. Same peak was also observed by [52, 53]. Some absorbance bands were formed at 1306.50cm^{-1}, 1340.42cm^{-1}, 1353.06cm^{-1}, 1395.85cm^{-1}. These peaks were related to the carboxylate group of enzyme. Similar results were also noticed by [43, 44].

The peak at 1513.2 cm^{-1} revealed the NH_2 bending vibration. The two peaks at 1604.71cm^{-1} and at 1625.93cm^{-1} corresponds to the presence of Amide-I stretching and C=C group vibration respectively. Identical findings were too mentioned by [48, 49]. The peak near to 2954.68cm^{-1} indicated the C-H stretching vibration. Two peaks that were appeared in the spectrum at 3255.44cm^{-1} and 3404.5cm^{-1} exhibited the N-H band and O-H group stretching respectively. Such peaks are related to the results reported by [14] respectively.

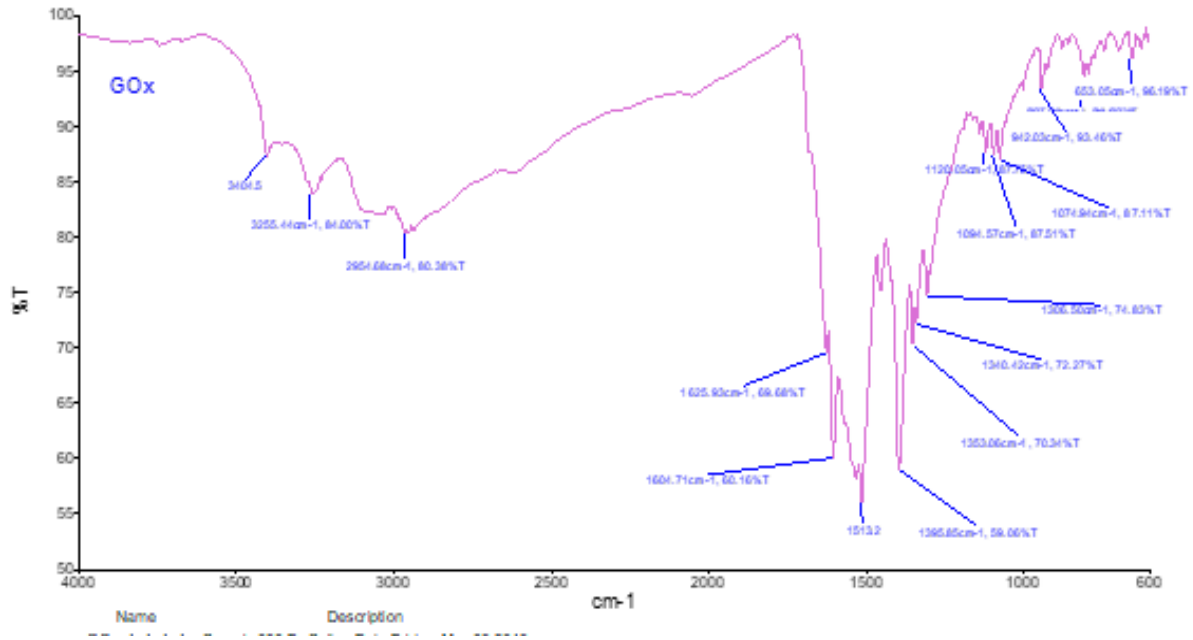

Figure.9. FTIR analysis of Free glucose oxidase

3.5. FTIR analysis of E-IONPs-I

The FTIR analysis was also observed for E-IONPs-I. The FTIR spectrum is shown in figure 10. The absorption spectra of E-IONPs-I revealed several peaks at 1405cm^{-1}, 1040.92cm^{-}1, 1629.6cm^{-1}, 2856cm^{-1} 2923.9cm^{-1} and at 3218.17cm^{-1}. A sharp peak at 1405cm^{-1} indicated the carboxylate group of enzyme. Same result was also noticed by [43, 44]. The peak near to 1040.92cm^{-1} displayed the C-O-C group stretching which was analogous to the results of [51]. Absorbance band at 1629.6 cm^{-1} of E-IONPs-I was appeared which is related to the NH^{4+} bending vibration. Actually, ammonium group is the part of enzyme structure. Thus, FTIR spectrum proves the immobilization of glucose oxidase enzyme on magnetic support. While the two peaks at 2856cm^{-1}, 2923.9cm^{-1} exhibited the C-H stretching vibration. Present result is in accordance with the peaks observed by [54-56]. The peak at 3218.17cm^{-1} demonstrated the N-H stretching vibration. Moreover, N-H group is the main part of enzyme structure. So, it confirms the enzyme immobilization. Similar findings were also stated by [1, 57].

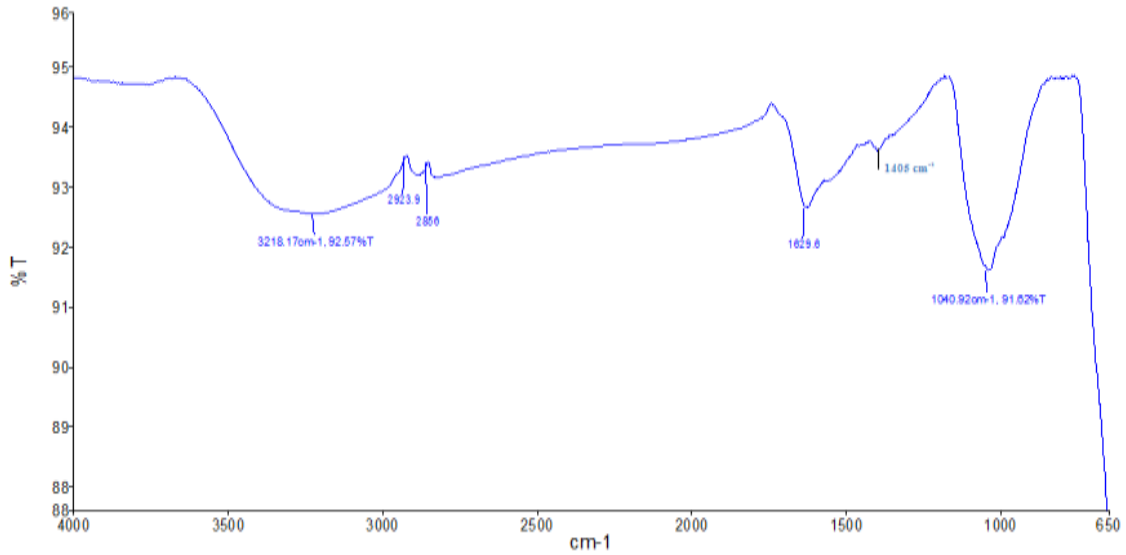

Figure.10. FTIR analysis of E-IONPs-I

3.6. IONPs as dye decolorizing agent

In order to examine the dye adsorption ability of IONPs, E-IONPs and free enzyme, reactive textile dye was used. The whole experiment was performed in triplicate 250 ml Erlenmeyer flask and their decolorization percentages were calculated. The dye decolorization progress was monitored with the passage of time through measuring the dye absorbance on (UV-Visible) spectrophotometer. E-IONPs showed significant high percentage efficiency of dye decolorization (84%) compare to IONPs (68%) and free enzyme (57.78%) as shown in fig 11. Current findings are related to the results observed by [58, 59]. They showed percentage efficiency more than 90% of immobilized chloroperoxidase (I-CPO) and immobilized Gox 62.7% respectively [9, 31].

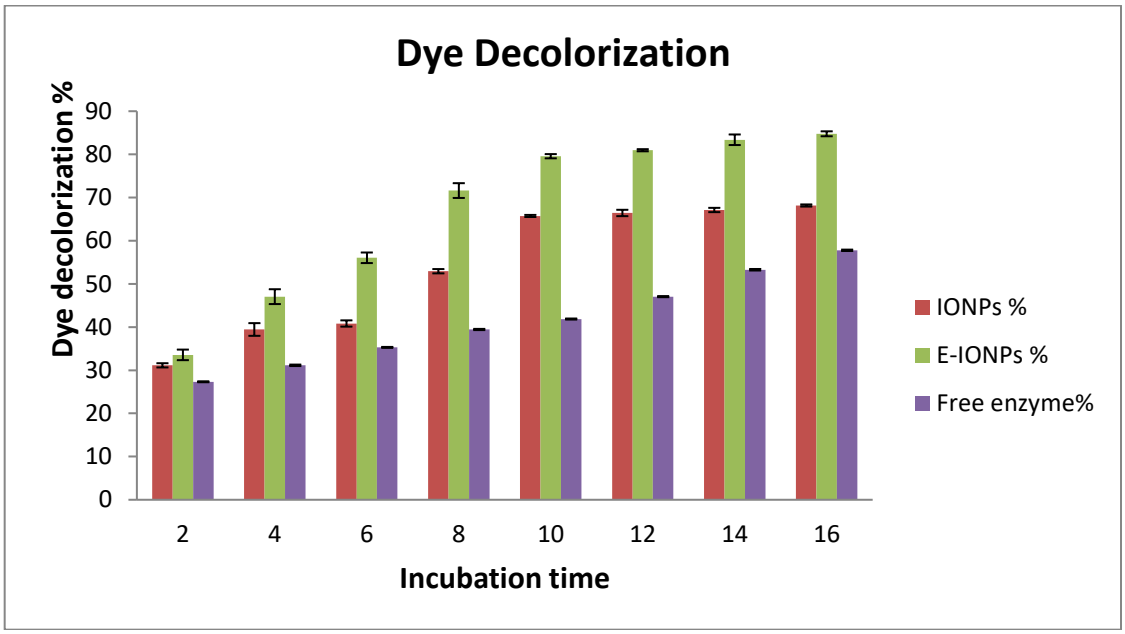

Figure.11. Results of IONPs, Free Enzyme and E-IONPs for dye degradation

3.7. Reusability of Immobilized Enzyme

Immobilized glucose oxidase was washed 17 times with deionized water and their remaining efficiency was observed after each run to measure the enzyme adherence with IONPs. Initial activity of enzyme was reduced just 6% after the 17 washing cycles as presented by figure 12. The present results show the strong attachment of free GOx to IONPs surface. The immobilized glucose oxidase on (MnO_2) retained 92 % of its activity after 14 washing runs related to the results observed by previous research [33, 60].

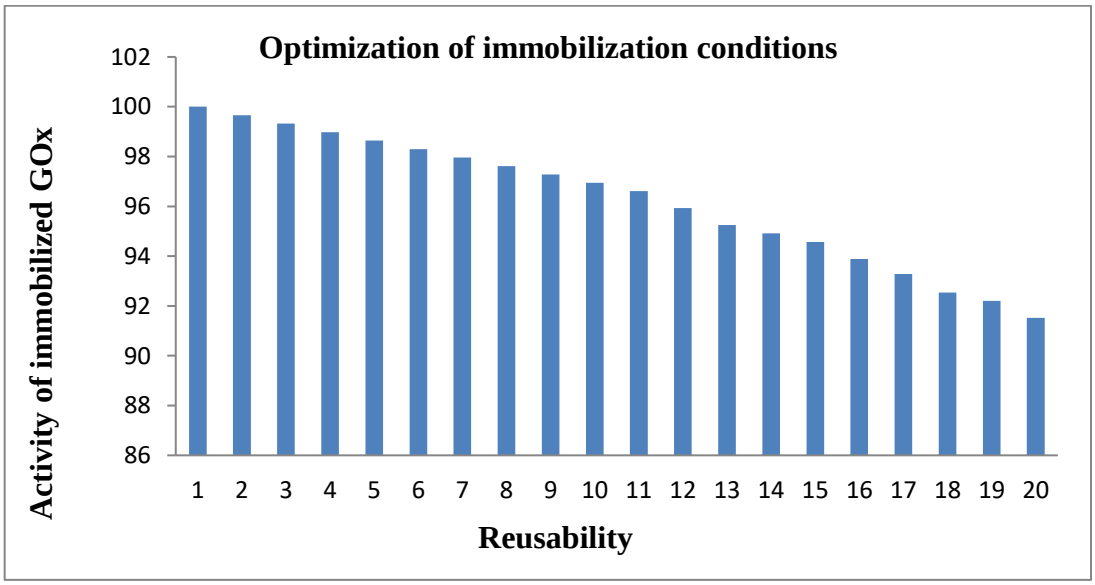

Figure.12. Reusability of Immobilized enzyme

4. Conclusion

Chemically synthesized IONPs were used as a support for immobilization of glucose oxidase which was further confirmed by optimizing the immobilization conditions. Present results showed the highly efficient immobilized enzyme efficiency with increased stability and reusability. Moreover, E-IONPs can adsorb the dye from effluents more effectively compare to that of IONPs only. From the current findings, it is concluded that immobilized glucose oxidase has higher stability and can effectively be used for various industrial applications.

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Conflict of Interest

Authors declares no conflict of interest

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