Tuijin Jishu/Journal of Propulsion Technology
ISSN: 1001-4055
Vol. 44 No. 5 (2023)

Fe_3O_4 Nanoparticles as Nanozyme for the Quantification of Hydrogen Peroxide using 3-Methyl-2-Benzothiazolinonehydrazone Hydrochloride with N-(1-Naphthyl) Ethylenediamine Dihydrochloride as Chromogenic Probe.

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Abstract: A easy and sensitive spectrophotometric method is described for determining the 3-methyl-2-benzothiazolinonehydrazone hydrochloride as the chromogenic co-substrate using a spectrophotometer, the suggested nanozyme approach for detecting hydrogen peroxide in different environment samples, the tolerance limit was determined as the quantity that resulted in a 3% absorbance error in the determination of 2.199 µM of H_2O_2. Less interference was observed for various metal ions. However, it was discovered that several potent oxidizing or reducing species interfered with the suggested technique. the Results revealed that most of the common ions of water constituents have lesser effect on the determination of H2O2 in Water samples, after a day of precision study it is demonstrated that the relative standard deviation values within a day ranged from 2.09 to 7.04%. According to the average of five measurements made over five days, the relative standard deviation of the different H_2O_2 between-day values ranged from 1.56 to 5.9%.

1. Introduction

Nanomaterials comprising magnetic materials, such as magnetite (Fe_3O_4), are especially helpful for imaging and separation techniques [1]. These nanoparticles often have coatings with metal catalysts, antibodies, or enzymes to further enhance their ability to function as isolation agents because they are typically considered biologically and chemically inert [2]. Here, we report that natural peroxidases, frequently used to oxidize substrates made of organic matter in wastewater treatment or as detection tools, have an intrinsic enzyme-stimulating activity comparable to that in magnetite nanoparticles. Based on this finding, we developed a new immunoassay in which magnetite nanoparticles modified with antibodies serve three purposes: capture, separation, and detection. Due to their stability, ease of manufacture, and adaptability, these nanoparticles are effective for various potential uses, including medicine, biotechnology, and environmental chemistry.

Since natural enzymes possess significant complications, such as easily inhibiting catalytic activity and protease digestion [3], so artificial enzyme mimics are currently the focus of the research [4]. Though Magnetic Nanoparticles are typically considered biologically and chemically inert, Yan et al.'s recent study has demonstrated that Fe_3O_4 magnetic nanoparticles (MNPs) have an intrinsic enzyme mimic activity like natural peroxidases enzyme [5]. In the current study, we take advantage of the distinctive H_2O_2 detection capabilities of Fe_3O_4 MNPs as peroxidase mimics, as described by Yan et al. A coprecipitation approach has been employed to form Fe_3O_4 MNPs [5]. The 2,2'-casino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), a peroxidase substrate, had been subsequently utilized to catalyze the oxidation of H_2O_2 to the oxidized colored product by providing a colorimetric detection of H_2O_2. Our team's approach is to allow for the detection of H_2O_2 at concentrations as low as 3 X 10^{-6} mol/L with a linear range of 5 X 10^{-6} to 1 X 10^{-4} mol/L. Furthermore, by employing the enzyme glucose oxidase (GOx) and the as-prepared Fe_3O_4 Magnetic NPs, a sensitive and specific approach to glucose detection was developed. The H_2O_2 and glucose detection platform was developed. The current study offered additional evidence that Fe_3O_4 MNPs have been associated with peroxidase-like activity. Still, they also demonstrated their great potential for use in various future simple, dependable, and easy-to-use analytical methods.

2. Experimental

Instrumentation

All absorbance measurements were carried out using a Systronics UV-Vis spectrophotometer -610 with 1.0 cm paired cells.

Reagents and Solutions

Throughout the entire test, distilled water had been used and all the chemicals were of analytical grade. The solution that had been prepared was wrapped in carbon paper to protect it from the sun to avoid photochemical reactions. 3-Methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) (Sigma Aldrich) (430µM) was prepared by dissolving 10 mg of MBTH with 10 ml of distilled water. N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDA) (S.D. Fine Chem. Ltd, India.) (190 µM) was prepared by dissolving 5 mg of in 10 ml of distilled water. A (30 % v/v) Hydrogen peroxide (H2O2) (E Merck, Mumbai, India) (100 mM) had been prepared, and the solution was standardized using the potassium permanganate titrimetric method [6]. By dilution with distilled water, working standard solutions were created from the stock solution. To prepare the stock solution of Fe-nps, 300 mg of synthesized Fe-nps had to dissolve in 3 ml of glycerol, and the total volume was then increased to 10 ml using distilled water. The solution of a buffer with a pH of 4.0 was prepared by mixing 40 mL of Acetic acid (CH3COOH) (Merck, Mumbai) (0.5 M) with 10 mL Sodium acetate (CH_3COONa) (Merck, Mumbai) (0.5 M). Every day fresh preparations of solutions were made and stored in -4^{0}C till used.

Determination of H_2O_2 in soil sample

2.5g of soil specimens representing the topmost layer were measured, dried, and powdered before mixed well with distilled water. The resulting mixture was permitted to stand for the next day before filtering through the Whatman No. 1 filter paper. With distilled water, the precipitates were washed multiple times. the final volumes of the sample solutions were made to be 25.0 ml. The same solution was analyzed by the suggested method. **Determination of H_2O_2 in plant samples**

The sample plants (Azadirachta indica and Pungemia) were rinsed and washed in distilled water to remove any soil clinging to the plant. It was thoroughly and attentively cleaned using filter paper before weighing. 0.50 g of plant sample was taken and gently crushed in a 50 ml mortar with a pestle containing distilled water. Employing a Whatman no. 1 filter paper, the contents were subsequently filtered. The precipitates were thoroughly cleaned, and the final volumes were adjusted to 50.0 ml using distilled water. The proposed method was then used to analyze the prepared sample.

General procedure for the determination of Hydrogen Peroxide

Lagenaria siceraria leaf extract

The leaves of *Lagenaria siceraria* were taken at Mysuru, Karnataka, India. Botanical Survey of India (BSI/ SRC/5/23/2010-11/Tech-1585) acknowledged the plant's identity. For all the studies, fresh leaves were employed. The LS plant components were cleaned and kept in storage at -4 °C. For the extraction process, around 5 g of ground, air-dried LS samples (seed, leaf, and fruit) were boiled with 100 ml of water that had been double distilled in an Erlenmeyer flask and swirled constantly for 15 minutes. The resulting substance was filtered and then cooled to ambient temperature before being kept at - 4 °C for further use.

Synthesis of Fe Nps

Iron oxide nanoparticles were synthesized through the combination of the LS extract in a 1:1 volume ratio with a 0.01 M FeC_{l3} $6H_2O$ solution. With the reduction method, Fe_3O_4-NPs were produced. To create a suspension of colloidal particles, the liquid was agitated for 60 minutes and then permitted to remain at room temperature for 30 minutes. The mixture was centrifuged, multiple times washed with ethanol, and then dried at 40 °C under vacuum to produce the Fe_3O_4-NPs, LS leaves have the best reduction capacity against ferric chloride, as seen by the exterior color change, compared to other plant components like seeds and fruit. For the following processes, leaves were chosen based on this finding. Following the confirmation test, the Fe_3O_4-NPs were used for further investigation.

Hydrogen peroxide quantification

Into a 3 mL reaction solution comprising 340 µM MBTH, 64 µM NEDA, and 64 µM Fe NPS in a 3 mM acetic acid/sodium acetate buffer at pH 3.8, the linearity of the reaction for the detection of H2O2 by rate method was tested. The range of H2O2 values used was 147 to 2933 µM. At one-minute intervals, a change in absorbance was frequently measured at 580 nm. The initial rate of reaction was established by the slope of the curve, which was then plotted against the H2O2 concentration to produce a calibration graph. According to **Figure 1**, It has been determined that the H2O2 assay's linearity ranges from 10 to 330 µM. **Figure 1** depicts the absorbance-time curves of the catalytic system in the presence of different concentrations of hydrogen peroxide (H_2O_2). To carry out the kinetics method, absorbance was continually measured.

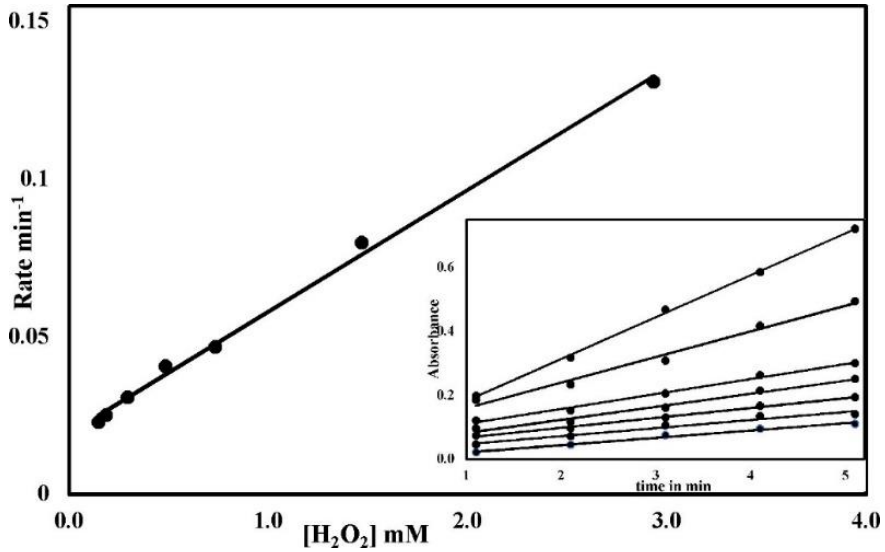


Fig 1: Calibration graph for the quantification of peroxidase by the rate method. The inset shows the absorbance-time curves of the catalytic system in presence of different concentrations of Hydrogen peroxide (H_2O_2)

The calibration curve for the fixed time approach was established by employing a 3 mL reaction solution consisting of 340 µM MBTH, 64 µM NEDA, and 64 µM Fe NPS in a 3 mM acetic acid/sodium acetate buffer with a pH of 3.8. The Concentration range of H2O2 employed was 0.733–4.4 mM. The change in absorbance at 580 nm was recorded while the solution remained at room temperature for 15 minutes. Plotting absorbance versus H2O2 concentration yielded linearity. **Figure 2** illustrates the linear approach for H2O2 concentrations between 0.733 and 4.4 mM. The measurements of 2.199mM H2O2 and apparent molar absorptivity of H2O2 were both 0.32 X 104 L/mol/cm. For H2O2, 1.376 mol/L and 4.128 mol/L were the limits of detection (LOD) and quantitation (LOQ).

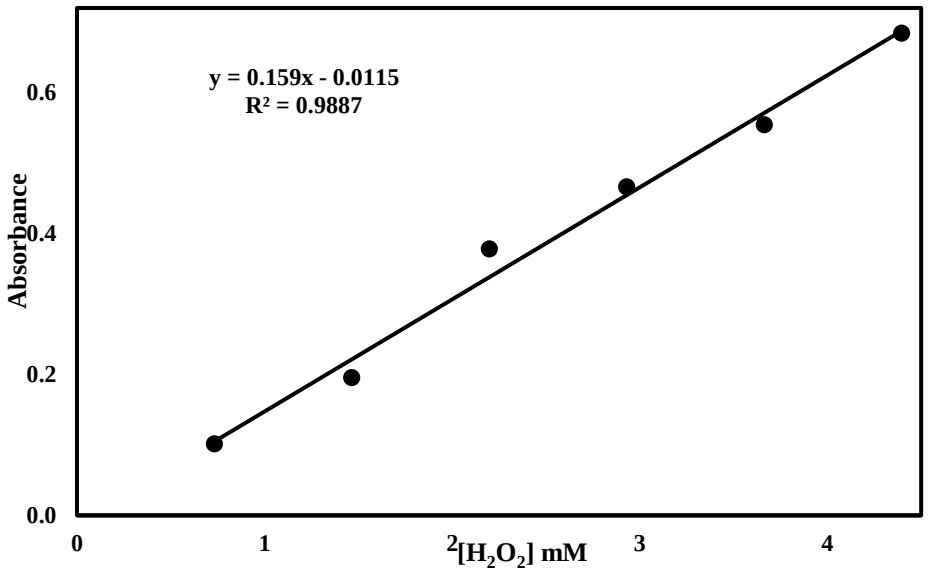


Fig 2: Calibration graph for the quantification of Hydrogen peroxide by the fixed time method after the incubation of the reaction mixture at room temperature for 15 min.

3. Results And Discussion

At a temperature of 25 ^{0}C, MBTH is oxidized by H2O2 to produce diazonium cation, which rapidly couples with NEDA in an acidic environment to produce an intensely blue-colored species.

Absorption spectra

The suggested approach involves the development of species with instance, blue color. By inspecting the sample throughout the visible range of 400 - 750 nm in 3 ml of the solution 340 µM MBTH, 64 µM NEDA, and 64 µM Fe nps in 3 mM, acetic acid/sodium acetate buffer of pH 3.8 at three distinct concentrations of H2O2, the wavelength of the colored product's maximum absorbance was determined. The maximum absorbance was at a wavelength of 580 nm when the reagent blank showed a minor absorption as shown in **Figure 3**.

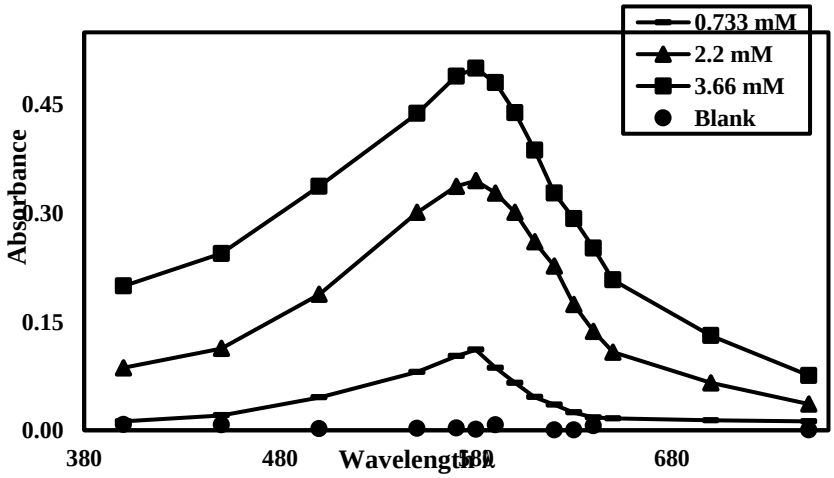


Fig 3: Absorption spectra Blank and three different concentrations of H_2O_2 with 340 µM MBTH, 64 µM NEDA and 64 µM Fe nps in 3 mM acetic acid/sodium acetate buffer of pH 3.8in 3 ml of solution

Optimization of reaction conditions

The effectiveness of the suggested method for determining H2O2 has been improved via the study of the effects of reagent concentrations.

Effect of temperature

The influence of temperature on the reaction was investigated using 3 ml of reaction solution having 340 μM MBTH, 64 μM NEDA, 64 μM Fe NPs over 3 mM acetic acid/sodium acetate buffer of pH 3.8 and 1.4 mM H2O2 in the temperature range of 5 - 60 ^{o}C. The results indicated that the coloured product showed maximum absorbance at 25 ^{o}C thereafter showed a constant or very negligible change in absorbance and the results were reproducible. We observed that as the temperature of the reaction mixture decreases, the rate will also decrease. Hence, all the analyses for the fixed-time approach were done at the optimal temperature of 25 ^{o}C. **Figure 4** illustrates how temperature has an impact on the reaction result.

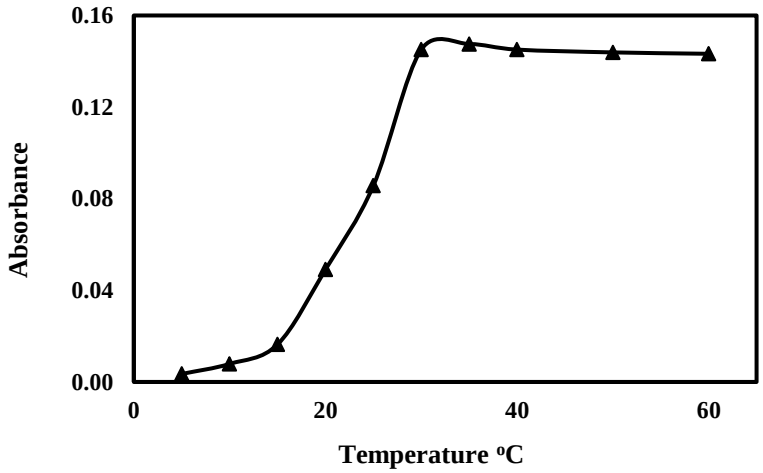


Fig 4: Effect of temperature on reaction

pH for maximum absorbance

Different buffers, ranging in concentration from 0.1 to 100 mM, have been employed to conduct the reaction. Citric acid/potassium citrate at pH 3.6 to 5.6 was employed, acetate/acetic acid at pH 3.5 to 5.5, potassium dihydrogen phosphate/sodium hydroxide at pH 6.0 to 8.0, and potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate at pH 6.0 to 7.8. Acetate/acetic acid at pH 3.8 exhibited the maximum reaction rate. Therefore, further study was conducted using 0.1 ml of 0.1 M Acetate/acetic acid solution with a pH of 3.8 as a buffer.

Effect of order of addition of the reactants

Using different sequences and the optimum concentration of the reactants, the proposed method's influence on reactant addition order was investigated. The experiment results demonstrated that the absorbance values are greatly affected by the order in which the reactants are added, as indicated in **Table 1**., The order of addition depicted in **Table 1** under serial No.1 showed maximum absorbance. All other orders enlisted in **Table 1** under serial No.2, 3, 4 and 5 showed very low colour, blank giving colour and sometimes no colour development. Hence all further experimental methods followed the same order of addition depicted in **Table 1** under serial No.1

Table 1: Effect of order of addition of the reactants

	tion[(a)]	or 2.2 mM
1	D + E	0.375
2	E + D	0.141
3	D + E	0.121
4	A + D	0.019
5	C + D	0.031

A = MBTH, B = NEDA, C = Fe Nps, D = Buffer, E = H_2O_2

Effect of time on the color

The reaction mixture was optimized for maximum and constant absorbance. Initial development of colour was slow but gradually increased with an increase in time up to 15 min and thereafter no significant change was observed over a period. The colored product formed was stable for 6 h. Hence, for routine analysis, it is sufficient to leave the reaction mixture for 10 min at room temperature to attain the required results. The result is as shown in **Figure 5.**

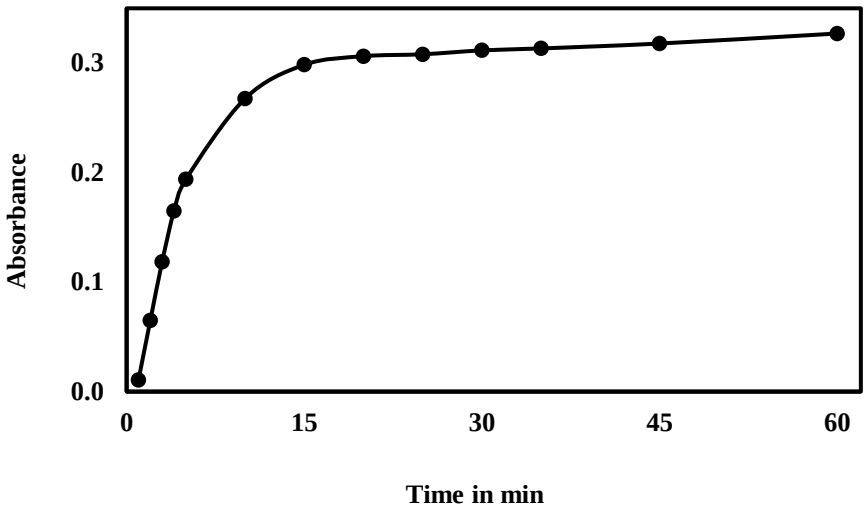


Fig 5: Effect of temperature on reaction.

Evaluation of kinetic constants

All reagents' Michaelis-Menten constant values have been determined by employing Equation 1. As a result of each reagent concentration (M_o = MBTH, N_o = NEDA, F_o = Fe Nps, and H_o = H2O2), the starting velocities (V_o) were calculated. In one experiment, N_o and F_o were kept constant while M_o was altered; in another, Mo and N_o were kept constant while F_o was altered; and in a third, M_o and F_o were kept constant while N_o was altered. Further experiments were repeated for PPDD and BHA at different creatinine concentrations.

Assuming the initial rate as V_o, A general equation for the mechanism in the forward direction is given as a function of all substrate concentrations [7].

$$\frac{1}{V_o} = \frac{1}{V_{\max}} + \frac{K_m^{H_2O_2}}{V_{\max}} \cdot \frac{1}{[H_2O_2]_o} + \frac{K_m^{A}}{V_{\max}} \cdot \frac{1}{[A]_o} \quad \text{----------(1)}$$

Where $V_{\max}$ is the maximum velocity; $[H_2O_2]_o$ is the initial concentration of hydrogen peroxide; $K_m^{H_2O_2}$ and K_m^{A} are the apparent Michaelis-Menten constants for H_2O_2 and MBTH, respectively; and $[A]_o$ is the initial concentration of MBTH.

The double-reciprocal plot of M_o vs N_o and F_o produced a constant slope, as shown in **Figure 6**, **Figure 7 and Figure 8** respectively at different concentrations of creatinine substantiated the catalytic mechanism of the reaction. The $K_{M,}$ K_N and K_F were found to be -0.039 µM, -0.09 and -0.01 µM.

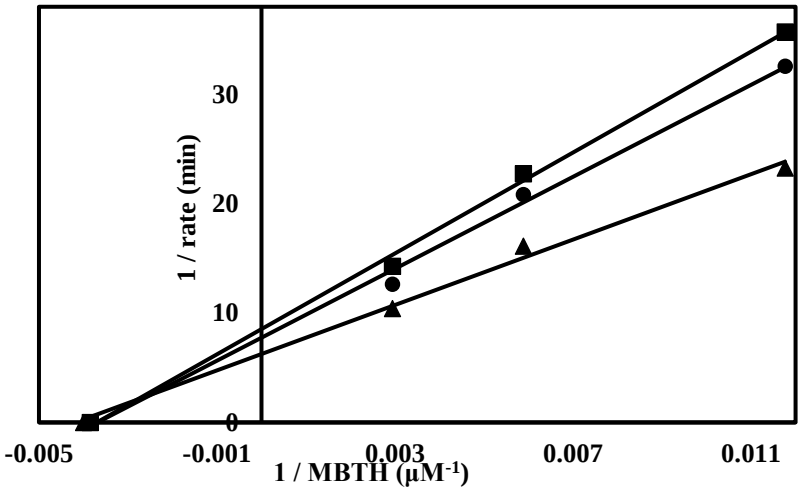


Fig 6: Michaelis-Menten constant for MBTH.

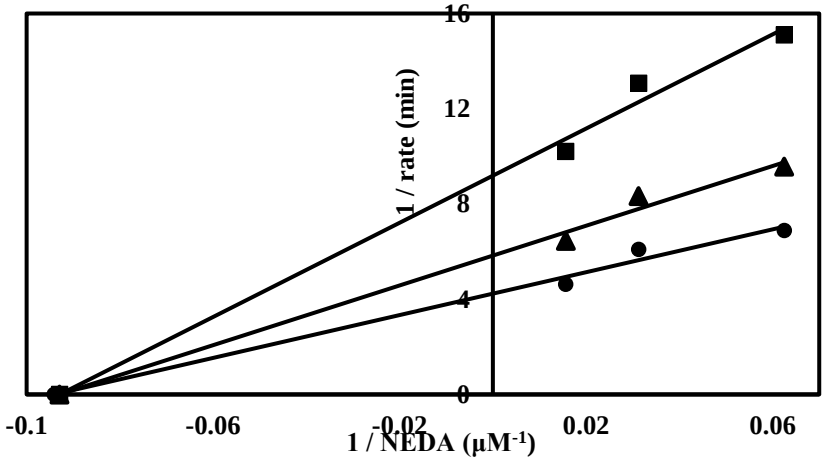


Fig 7: Michaelis-Menten constant for NEDA

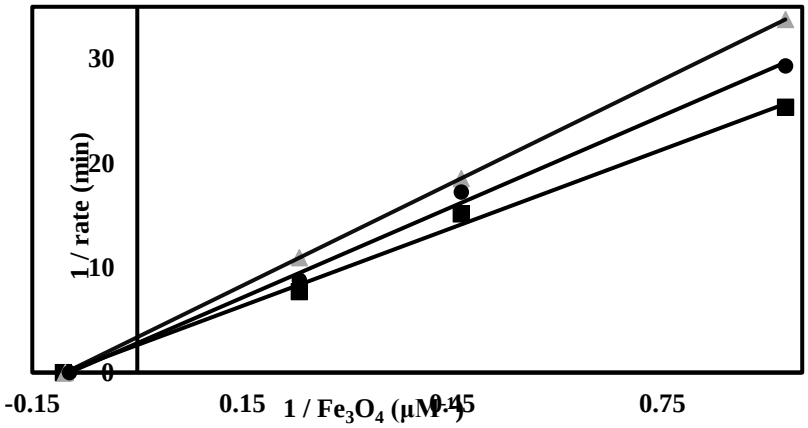


Fig 8: Michaelis-Menten constant for Fe_3O_4

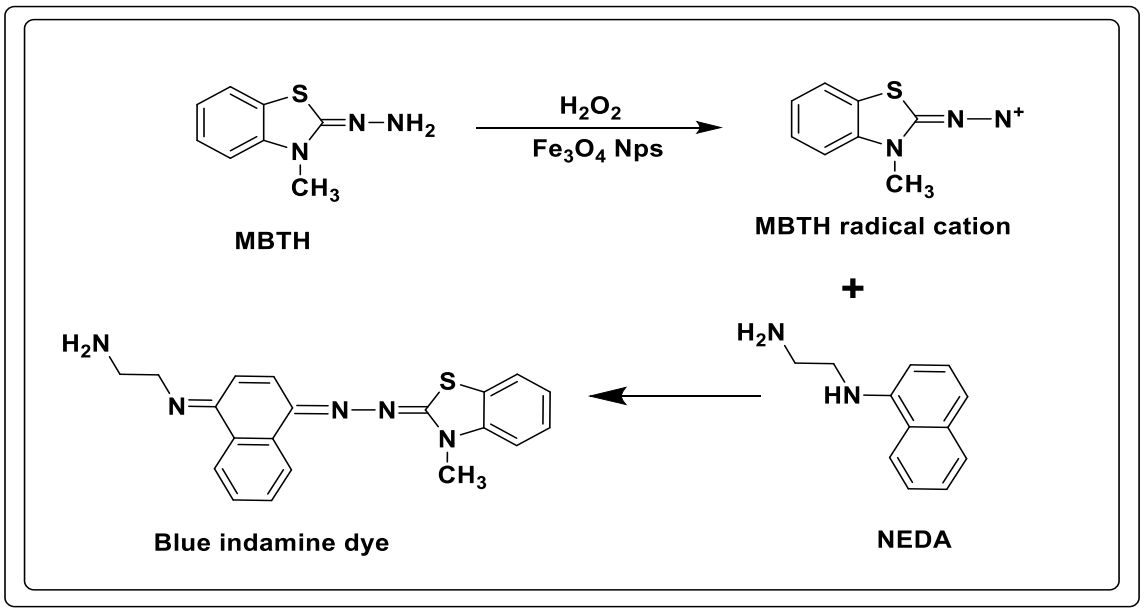
Evaluation of kinetic parameters of the indicator reaction Fe_3O_4 Nps catalyzed hydroxylation of MBTH

Kinetic parameters for the nanozyme assay have been studied under the optimized experimental protocol conditions. The catalytic parameters such as Michaelis-Menten constants for H_2O_2, ($K_m^{H_2O_2}$) and for MBTH, (K_m^A); maximum velocity, (V_{max}); catalytic constant, (K_{cat}) and specificity constant, $\left(\frac{K_{cat}}{K_m^{H_2O_2}}\right)$ for

H_2O_2 and MBTH have been calculated. The catalytic parameters such as $K_m^A, V_{\max}$, K_{cat} and $\frac{K_{cat}}{K_m^{H_2O_2}}$ for Fe_3O_4 assay was found to be 483.8 mM, 0.0921 µM min^{-1}, 0.0213 × 10^3 min^{-1} and 0.0142 $\mu M^{-1}min^{-1}$, respectively. The values of catalytic efficiency and catalytic power of the proposed method by Lineweaver-Burk plot were K_{eff}^A = 0.4103 × 10^5 $M^{-1}min^{-1}$ and K_{pow}^A = 1.90 × 10^{-5} min^{-1}, respectively. It is evident from the data that the catalytic efficiency by the proposed method is significantly higher.

Reaction mechanism

According to Caramyshev et al., the process is like HRP-catalyzed polyaniline synthesis [8]. Under the experimental conditions, the oxidation of H2O2 leads to the release of the free radical through a Ferrell intermediate (FeIV=O-porphyrin -cation radical) of the Fe3O4 Nps. Whenever MBTH is exposed to H2O2, it oxidizes by losing two electrons and two protons during the reaction. This electrophilic intermediate, the oxidative coupling species MBTH radical cation, is likely to develop. Like NEDA, this intermediate interacts with amines by electrophilically targeting the most nucleophilic site on the aromatic ring that contains amines (i.e., ortho or para locations if the amine is substituted in the para positions). The resultant intermediate spontaneously undergoes oxidation in the presence of an oxidant to produce a product with an intense blue color. The MBTH-NEDA oxidation process is catalyzed by the help of Fe3O4 Nps, as indicated in Scheme 1.



Scheme 1: Probable reaction pathway for the formation of colored product by MBTH and NEDA

Interference studies

2.199 µM H2O2 concentrations were used to study the interference caused by any standard components in water, soil, and plant samples. The concentrations of interferants are summarized and presented in **Table 2.** The tolerance limit was determined as the quantity that resulted in a 3% absorbance error in the determination of 2.199 µM of H2O2. Less interference was observed for various metal ions. However, it was discovered that several potent oxidizing or reducing species interfered with the suggested technique. the Results revealed that most of the common ions of water constituents have lesser effect on the determination of H_2O_2 in Water samples. Whereas ions present in plant extract and soil filtrate show notable interferences. Since sulphamic acid and sodium fluoride also showed the interference, the plan of executing the masking was overwhelmed.

Table 2: Effect of foreign ions on the determination of H_2O_2

Foreign ions	**Tolerance limit (µg ml^{-1})**	**Foreign ions**	**Tolerance limit (µg ml^{-1})**
Na^+, K^+	20000	Zr^{4+}, Ba^{2+}	500
Cl^-, Br^-, NO_3^-, Acetate	15000	Leucine, Gluthathione	300
Zn^{2+}, Co^{2+}, Mn^{2+}, Al^{3+}	10000	Tartarate, Oxalate	200
Ca^{2+}, Mg^{2+}	8000	Sulphamic acid	75
HCO_3^-, CO_3^{2-} , SO_4^{2-},	7000	Cr^{3+}, Cu^{2+}	100
EDTA, Citrate,	5000	Ascorbic acid	25
NO^{2-} , NO_3	2000	Ce^{4+}, Fe^{3+} [(a)], Fe^{2+}, F^-	10

Within-day and between-day precision study

Five replicate analyses with different H2O2 concentration levels were carried out to test the robustness of the proposed method. The results demonstrated that the relative standard deviation values within a day ranged from 2.09 to 7.04%. According to the average of five measurements made over five days, the relative standard deviation of the different H_2O_2 between-day values ranged from 1.56 to 5.9%. Based on the previous observation demonstrated that the suggested technique possesses high repeatability inter-day and intraday, as shown in **Table 3.**

Table 3: Within-day and between-day precision study on the determination of H_2O_2

H_2O_2 added (µM)	Inter day			Intra day		
	Found (µM)	% RSD	CV	Found (µM)	% RSD	CV
1.466	1.35	1.56	0.015	1.36	2.09	0.021
2.199	2.28	5.90	0.059	2.24	7.04	0.07
3.665	3.64	3.67	0.036	3.68	4.97	0.049

[(b)] Mean value of five determinations carried out over five days.

[(a)] Mean value of ten determinations carried out in one day within a 1 hr time interval.

Application and Recovery studies

Recovery tests were conducted by adding a known amount of standard H2O2 to the water, soil filtrate, and leaf extract.

[(final concentration - beginning concentration)/added concentration] was used to compute the recovery percent. The results are depicted in **Table 4**.

Table 4: Application of the proposed method for determination of H_2O_2 in various samples.

Sample number	**Sample**	**Added µM**	**Found µM**	**Recovered µM**	***Recovery %**
1	Tap Water	1.466	1.47	1.46	99.74
2		2.199	2.21	2.20	100.06
3		3.665	3.66	3.65	99.46
4	Soil	1.466	1.38	1.27	86.59
5		2.199	2.62	2.51	113.94
6		3.665	3.45	3.34	91.14
7	Azadirachta indica	1.466	1.35	1.32	89.99
8		2.199	2.27	2.27	103.04
9		3.665	3.65	3.62	98.85
10	Pungemia	1.466	1.33	1.27	86.40
11		2.199	2.26	2.20	99.85
12		3.665	3.69	3.63	98.99

*= (Recovered H_2O_2 / Added H_2O_2) X 100

4. Conclusions

Using 3-methyl-2-benzothiazolinonehydrazone hydrochloride as the chromogenic co-substrate using a spectrophotometer, the suggested nanozyme approach for detecting hydrogen peroxide in different environment samples has been described for the first time. This method allows measuring hydrogen peroxide at micromolar levels in various environmental samples. The method is cost-effective since just a small number of reagents is required; it is straightforward, requires little assay run-time, and provides high analytical throughput. One of the unique advantages of the established approach is the strongly blue-colored chromogenic product produced by combining MBTH with NEDA, which is stable, requires less time, and delivers accurate and repeatable results. Furthermore, absorption at longer wavelengths improves how to eliminate background interference from environmental components [6].

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