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# SPECTROPHOTOMETRIC ANALYSIS OF HYDROGEN PEROXIDE WITH ITS APPLICATIONS ILLUSTRATED BY PEROXIDASE ASSAY

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**ABSTRACT**

Hydrogen peroxide ($H_2O_2$) is an essential compound in food, pharmaceutical, clinical, industrial and environmental analysis. $H_2O_2$ has been widely used for preservation of food products due to its bactericidal properties. However, excess of $H_2O_2$ can be deleterious on the nutritional value of food. In the present research work a simple and sensitive spectrophotometric assay was developed for the quantification of $H_2O_2$ in different samples. This method involves the reaction of Iso-nicotinic acid hydrazide (INH) and pyrocatechol (PC) as co-substrates in presence peroxidase enzyme and $H_2O_2$ to form a colored product, which has strong absorbance at $\lambda_{max}$ = 520 nm. The effects of different parameters on the sensitivity of the proposed assay were studied. The Michaelis-Menten constant and catalytic efficiency were also calculated. Some of the important advantages of the proposed method are, use of inexpensive reagents, simple operation conditions, rapid analysis, wide linear range, high sensitivity, free from interfering substances and precision comparable to the reference Gilliland method. The proposed method was used for analysis of different samples.

**KEYWORDS**

Peroxidase, Hydrogen peroxide, Iso-nicotinicacidhydrazide and Pyrocatechol.

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## INTRODUCTON

Hydrogen peroxide is the two-electron reduction product of $O_2$. It is not a free radical, but potentially reactive oxygen[1]. Hydrogen peroxide produced in plants chloroplast and mitochondria via electron transport, where oxygen is reduced to superoxide, which is further dismuted into $H_2O_2$ spontaneously or catalyzed by dismutase[2].

There is increasing interest for rapid and accurate detection of hydrogen peroxide because it exists as the product of reactions catalyzed by many selective

oxidases, besides it is also an essential compound in food, pharmaceutical, clinical, industrial and environmental analysis[3]. $H_2O_2$ has been widely used for preservation of raw foods due to its bactericidal properties[4]. However, excess of $H_2O_2$ can be deleterious on the nutritional value of food due to degradation of essential vitamines[5].

A variety of analytical methods have been developed for the assay of $H_2O_2$ including titrimetry[6], spectrofluorometry[7-9], chemiluminescence[10,11], high performance liquid chromatography[12] and electrochemistry[13-18]. All these methods have achieved great success as well as some shortcomings like lack of sensitivity, susceptibility to interference by foreign substances in samples, need for waste expensive biocatalyst and long time analysis. The spectrophotometer is of great use to overcome these drawbacks because of its inherent simplicity, inexpensiveness, facile nature and wide availability.

Some of the common chromogenic co-substrates are used for determination of hydrogen peroxide includes guaiacol[19], 2, 2-azinobis(3-ethylbenzothiazoline-6-sulphonicacid)[20], benzidine, p-phenylenediamine[21], pyrogallol[22], 3, 3, 5, 5-tetramethylbenzidine (TMB)[23], o-phenylenediamine, catechol, o-dianisidine[24], tetramethyl-p-phenylenediamine (TMPD)[25], p-phenylenediamine-3-dimethylaminobenzoic acid[26] and 3-methyl-2-benzothiazolinonehydrazone hydrochloride with 10, 11- dihydro-5H-benz (b, f) azepine[27]. But use of these reagents have some limitations such as carcinogenicity, mutagenicity, poor solubility, poor sensitivity. In order to overcome the some of the limitations of the above co-substrates INH and PC are used as chromogenic co-substrates, which are easily soluble in water.

In this paper, we have developed a simple colorimetric method to quantify $H_2O_2$ based on biocatalysis of POD. The technique is based on the reaction of $H_2O_2$ with Iso-nicotinic acid hydrazide (INH) and pyrocatechol (PC) to form a colored compound, which is monitored at 520 nm. This analytical method can be employed to determine $H_2O_2$ in different samples.

## MATERIAL AND METHODS

### Reagents and apparatus

Peroxidase (EC.1.11.1.7, 100units/mg) was purchased from Himedia Laboratories (Mumbai, India). The stock solution of the enzyme was prepared by dissolving 2mg in 10ml of 100mM $KH_2PO_4$/ NaOH buffer of pH 6.0. Working solutions were prepared from the stock solution by appropriate dilution with water. $H_2O_2$ stock solution was prepared by diluting the commercial reagent (30%, v/v E. Merck, Mumbai, India). Pyrocatechol solution was prepared by dissolving 30 mg sample in 10 ml of distilled water. Iso-nicotinic acid hydrazide solution was prepared by dissolving 30 mg sample in 10 ml distilled water. All other reagents used were of analytical grade.

A Jasco model UVIDEC-610 ultraviolet-visible (UV-Vis) spectrophotometer with 1.0 cm matched cells was used for all absorption measurements. Temperature controlled thermostat (model 206-88950-93, Shimadzu, Japan) was used for maintaining the reaction temperature. All pH measurements and adjustments of pH were done by a digital pH meter (model EQ-614, Equip-tronics, Mumbai, India).

### Hydrogen peroxide assay protocol

The concentration of $H_2O_2$ was measured in 3 ml solution containing 650µM INH, 845µM PC and 9.5nM horseradish peroxidase with 100mM $KH_2PO_4$/NaOH buffer at pH 7. The reaction was initiated at 30°C by adding 100µL of different concentrations of $H_2O_2$. Data of progress curve was resolved in 5 min against the corresponding control containing all the reagents except $H_2O_2$. The initial rate of reaction was calculated from the time domain of 1 min at 520 nm. The calibration graph was obtained by plotting rate against concentration of $H_2O_2$. In fixed time method, the linearity of $H_2O_2$ was quantified by incubating the reaction mixture for 10 min at 30°C, and absorbance was measured for the colored solution.

## RESULTS AND DISCUSSION

### Optimization of experimental variables

Various factors influencing the activity of $H_2O_2$ and horseradish peroxidase were investigated, which

includes pH of the solution, temperature of the reaction mixture, concentration of PC and INH solution. Optimization of pH was done by using different buffer solutions such as citric acid/potassium citrate buffer (pH 3.6-5.6), potassium dihydrogen orthophosphate/sodium hydroxide buffer (pH 6.0-8.0), acetate/acetic acid buffer (pH 3.6-5.6), and potassium dihydrogen orthophosphate/ dipotassium hydrogen phosphate buffer (pH 6.0-7.5). The highest activity was observed in 100mM $KH_2PO_4$/NaOH buffer of pH 7.0 (Figure No.1).

The concentration of PC on the reaction rate was studied in the range of 113 - 3632µM. The result showed that the rate of reaction increased with increase in the concentration of the PC up to 845µM, beyond which the rate was independent of the concentration of PC. Hence, for further assays PC concentration of 845µM was selected. Similarly, the effect of concentration of INH on the reaction rate showed that the rate of reaction increased with increasing concentration of INH up to 650 µM, beyond which the rate was inhibited. Hence, 650µM INH was chosen as the optimum concentration for all further assays. (Data were not shown)

Figure No.2 shows the temperature curve for reaction. As the temperature was raised above 30°C the colored product intensity decreased; this can be attributed to a gradual decline in HRP activity due to heat inactivation. Based on this result, temperature of 30°C was chosen for further assays. At this temperature enzymatic assay reached maximum activity.

**Horseradish Peroxidase assay**

The concentration of POD was quantified in a 3 ml solution containing 650µM INH, 845µM PC, 128µM $H_2O_2$ in 100mM $KH_2PO_4$/NaOH buffer at pH 7. The reaction was initiated at 30°C by adding 100µL of different concentrations of HRP. The POD concentrations, studied for the assay were in the range of 0.039 to 151nM. The progress curve was measured in a period of 5 min against the corresponding control containing all the reagents except POD. The initial rate of reaction was calculated from the time domain of 1 min at 520 nm. The calibration graph was obtained by plotting rate against concentration of HRP. In fixed time method, the linearity of HRP was quantified by incubating the reaction mixture for 10 min at 30°C, and absorbance was measured for the colored solution. The results showed that the rate of the reaction increased with increase in the concentration of HRP up to 19nM. The calibration graph for HRP assay shows linearity in the range of 0.59 - 18.92nM and 0.59 - 9.5nM (Figure No.3) by kinetic and fixed time methods respectively.

**Hydrogen peroxide assay**

$H_2O_2$ assay was carried out in a series of $H_2O_2$ concentrations ranging from 0.5 to 200µM. It is found that the rate of the reaction increased with increase in the concentration of $H_2O_2$ up to 110 µM, beyond which the rate of the reaction decreased. The calibration graph for $H_2O_2$ exhibited linearity in the range of 0.5-65 µM (Figure No.4). The linearity of $H_2O_2$ assay in the proposed method was observed in a comparatively wider range. Moreover, the initial concentration of $H_2O_2$ is as low as 0.5µM, the detection limit, quantification limit and molar extension co-efficient determined by fixed time assay were 0.62 µM, 2.13 µM and $0.381\times10^5$ L/mol/cm, respectively. Low detection limit and wide linearity range demonstrate that the established method may be an excellent method for the determination of $H_2O_2$ in different samples. Moreover, the reagents used are simple and the operation procedures are very simple.

**Interference Study**

The optimal reaction conditions and 30 µM hydrogen peroxide was used for the interference study, the feasibility of the method for application in samples was studied by taking various proteins, ions, and other commonly accompanying amino acids in sample. Except ascorbic acid none of the other foreign species studied were not interfered in the quantification. The tolerance ratios are tabulated in Table No.1, which correspond to the concentration of interfering species that cause an interference of ±3% in its quantification. The result showed that large number of ions, amino acids, and carbohydrates examined did not interfere in the assay.

**Samples Analysis**

**Milk sample analysis**

The feasibility of the proposed colorimetric method was tested for measuring $H_2O_2$ in different animal milk samples. The analyses of $H_2O_2$ in these samples were carried out by standard addition method (Table No.2) shows the typical spectrophotometric determination of $H_2O_2$ in 5 different milk samples using the proposed method. The results show that Goat milk samples contained minimum amount of $H_2O_2$, whereas commercial (market) milk sample has maximum $H_2O_2$ concentration. Because $H_2O_2$ has been widely used for preservation of raw milk due to its bactericidal properties[4]. However, excess of $H_2O_2$ can bring deleterious effects on the nutritional value of milk such as the degradation of folic acid, which is an essential vitamin to human body. Hence goat and sheep milks may be good for drinking purpose than commercial milk. The feasibility of the developed method was further examined by recovery tests. The results (Table No.2) indicated that the proposed method possesses reasonable selectivity and produces satisfactory recovery of 98.5- 101.0 % as compared to 98.5 - 102.0 % by reference ABTS method.

**Rain water sample analysis**

The proposed method was applied for the analysis of $H_2O_2$ concentration in different rain water samples. The results obtained by the proposed method are comparable favorably with those of a reference method. From the data it is observed that the industrial region rain water contained high concentration of $H_2O_2$ as compared to urban and rural region rain water samples. The areas with higher concentrations of $H_2O_2$ are regions with a high photochemical activity due to intense levels of solar radiation and with a large scale transport of polluted air[28]. The feasibility of the developed method for the determination of $H_2O_2$ in rain water samples was further confirmed by conducting recovery tests. The results indicated that the proposed method possesses reasonable selectivity and produces satisfactory recovery results with recovery range of 98.32 to 101.9%.

**Mineral waters analysis**

In the 1950's, hydrogen peroxide was first used for drinking water disinfection in Eastern Europe. It is known for its high oxidative and biocidal efficiency. Hydrogen peroxide has not been used often for drinking water disinfection. But it's popularity seems to increase. It is often used combined with ozone or silver. Eight different mineral waters were analyzed by using standard addition method. The relative standard deviations (RSD) were all below 3.0% for ten successive measurements, which indicated that the proposed assay possesses a good reproducibility (Table No.4). The recovery was preferable, indicating that the proposed method could be used for the determination of $H_2O_2$ in real samples.

**Packed fruit juice sample analysis**

Ascorbic acid and its derivatives are used in many foods for various purposes. They are added to foods, including fruit juices, to improve the nutritional quality and to prevent enzymatic browning reactions. Ascorbic acid is also used as an index of the nutrient quality of fruit and vegetable products. This is because, as compared to other nutrients, it is much more sensitive to various modes of degradation in food processing and subsequent storage. Therefore, it is assumed that, if the ascorbic acid is well retained during processing and storage, other nutrients would be as well.

Ascorbic acid degradation in packaged fruit juices depends mainly on storage temperature, dissolved oxygen level, residual $H_2O_2$ left after the sterilization of packaging material and trace metal ions. $H_2O_2$ is the primary chemical for the sterilization of plastic packaging material used in aseptic systems. FDA (Food and Drug Administration) regulation currently limits residual $H_2O_2$ to 9.7 micromolar, leached into distilled water, in finished food packages. However, during the sterilization of packaging materials with $H_2O_2$, residues left on the packaging material or vapours generated during drying may get trapped inside the package upon sealing. Therefore, residues left inside packages may occasionally be over the legal limit and particularly cause the degradation of ascorbic acid[29].

In this study, orange, sour cherry, grape and pomegranate juices were selected. Orange juice is an important source of ascorbic acid in the human diet (Table No.5). The result shows that one of the orange juice sample contains more than the limiting value of hydrogen peroxides.

**Table No.1: Influence of interfering species for the quantification of hydrogen peroxide**

| S.No | **Interference species** | **Tolerance Ratio*** |
|---|---|---|
| 1 | Ascorbic acid | 1.38 |
| 2 | L-Cystein | 5.75 |
| 3 | Folic acid, Ferric Oxide | 8.91 |
| 4 | Urea, L-glutamic acid, D-Fructose | 23.92 |
| 5 | Lacto globulin, Lacto albumin | 33.79 |
| 6 | Lactose | 47.85 |
| 7 | Cholesterol | 63.79 |
| 8 | Calcium, Potassium | 71.11 |
| 9 | Glucose | 95.70 |
| 10 | Sodium, Magnesium(II), Zinc(II) | 101.21 |
| 11 | Sucrose | 114.84 |
| 12 | Uric acid | 133.98 |
| 13 | Citric acid | 574.20 |

**Table No.2: Analytical recovery of Hydrogen peroxide in different animal milk samples**

| **S.No** | **Milk Samples** | **Proposed method** | | | | **Standard method** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **$H_2O_2$ (µM)** | **Added (µM)** | **Found (µM)** | **Recovery* (%)** | **$H_2O_2$ (µM)** | **Added (µM)** | **Found (µM)** | **Recovery* (%)** |
| 1 | Market available | 8.3 | 20<br>100 | 28.1<br>108.5 | 99.0<br>100.2 | 8.7 | 20<br>100 | 28.6<br>109.3 | 99.5<br>100.6 |
| 2 | Buffalo | 7.3 | 20<br>120 | 27.2<br>128.5 | 99.5<br>101.0 | 7.7 | 20<br>120 | 28.1<br>127.9 | 102.0<br>100.1 |
| 3 | Cow | 7.1 | 32<br>128 | 38.7<br>134.8 | 98.7<br>99.7 | 6.9 | 32<br>128 | 50<br>148.4 | 101.5<br>102.2 |
| 4 | Sheep | 5.6 | 82<br>32 | 88.1<br>37.9 | 100.6<br>100.9 | 6.1 | 82<br>32 | 88.5<br>38.6 | 100.4<br>101.5 |
| 5 | Goat | 3.3 | 20<br>100 | 23<br>103.8 | 98.5<br>100.5 | 3.9 | 20<br>100 | 23.6<br>104.9 | 98.5<br>101 |

*[(Found $H_2O_2$ concentration − initial $H_2O_2$ concentration)/added $H_2O_2$ concentration] ×100

**Table No.3: Analytical recovery of Hydrogen peroxide in rain water samples**

| S.No | Rain water Samples | Proposed method | | | | Reference method | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $H_2O_2$[a] (µM) | Added (µM) | Found (µM) | Recovery* (%) | $H_2O_2$[a] (µM) | Added (µM) | Found (µM) | Recovery* (%) |
| 1 | Industrial area | 13.1 | 25 | 38.5 | 101.6 | 13.24 | 25 | 37.82 | 98.32 |
| 2 | Urban area | 8.65 | 50 | 58.1 | 98.9 | 8.15 | 50 | 59.13 | 101.9 |
| 3 | Rural area | 4.88 | 75 | 78.94 | 98.7 | 5.01 | 75 | 81.2 | 101.5 |

[a] Mean of triplicate measurement.

*[(Found $H_2O_2$ concentration − initial $H_2O_2$ concentration)/added $H_2O_2$ concentration] ×100

**Table No.4: Analytical table for Hydrogen peroxide in mineral water samples**

| S.No | Samples | Add $H_2O_2$ (µM) | Found $H_2O_2$ (µM) | RSD | Recovery |
|---|---|---|---|---|---|
| 1 | Bisleri | 25 | 25.09 | 1.2 | 100.1 |
| 2 | Kinley | 25 | 25.06 | 1.8 | 102.2 |
| 3 | Aquafina | 25 | 26.1 | 2.2 | 101.5 |
| 4 | Bailey | 25 | 25.08 | 1.6 | 102.8 |
| 5 | Himalayan Mineral Water | 50 | 51.3 | 2.1 | 100.7 |
| 6 | Kingfisher Mineral Water | 50 | 51.1 | 1.5 | 101.2 |
| 7 | Qua Mineral Water | 50 | 51.7 | 1.9 | 103.1 |
| 8 | Manikchand Oxyrich Mineral Water | 50 | 52.0 | 2.5 | 102.9 |

**Table No.5: Analytical table for Hydrogen peroxide in packed fruit juice samples**

| S.No | Samples | | Concentration of $H_2O_2$ (µM) |
|---|---|---|---|
| 1 | Orange Juice | Sample 1 | 10.0 |
| | | Sample 2 | 9.3 |
| 2 | Sour cherry Juice | Sample 1 | 9.7 |
| | | Sample 2 | 7.1 |
| 3 | Grape Juice | Sample 1 | 6.8 |
| | | Sample 2 | 6.3 |
| 4 | Pomegranate juice | Sample 1 | 9.5 |
| | | Sample 2 | 8.9 |

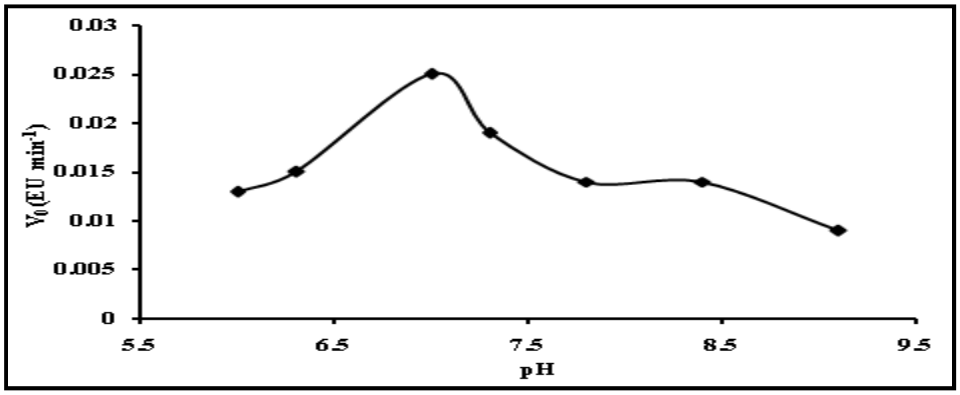


**Figure No.1: Effect of pH on the activity of enzyme**

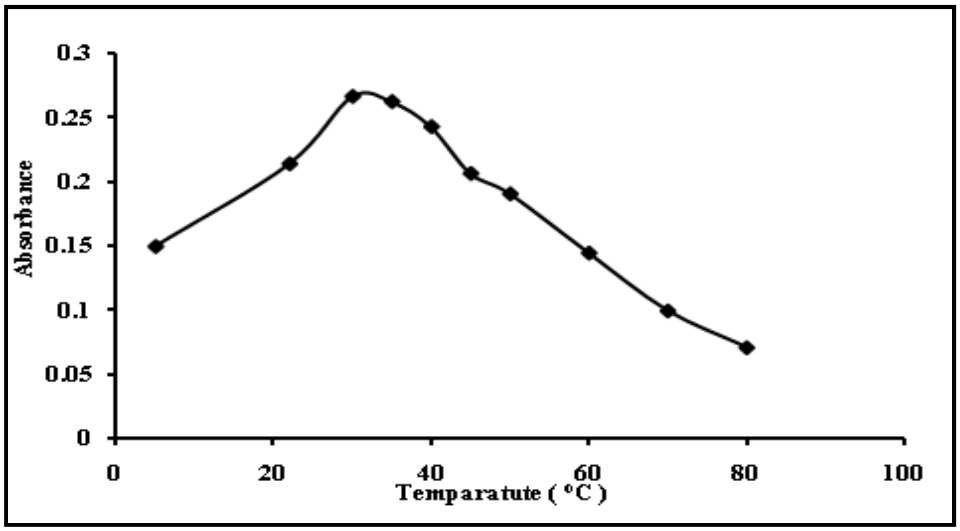


**Figure No.2: Effect of temperature on the enzyme activity**

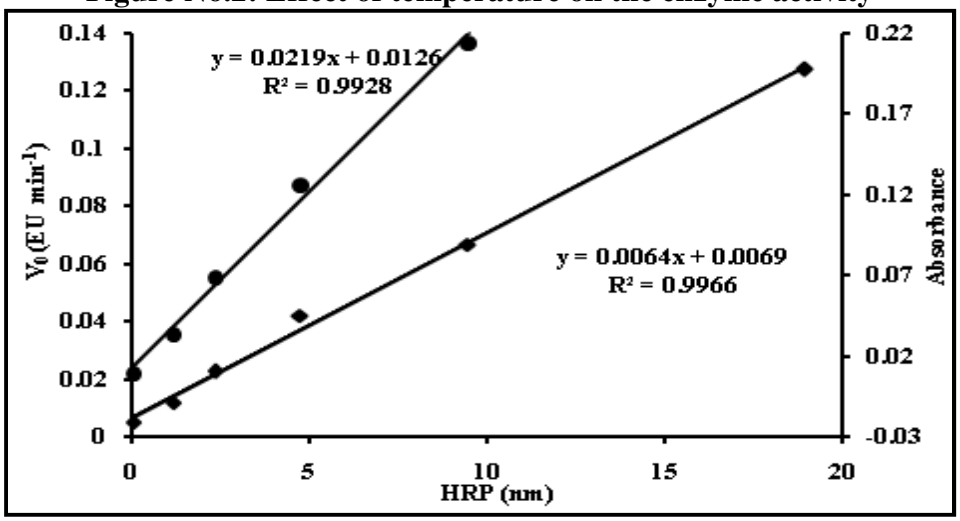


**Figure No.3: Analytical curve for the quantification of POD via (♦) kinetic assay and (●) fixed time assay**

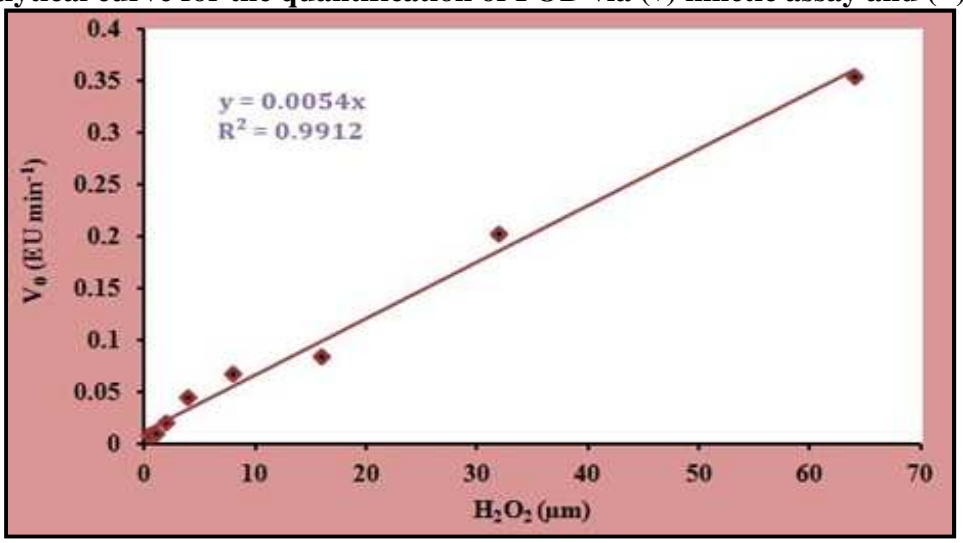


**Figure No.4: Analytical curve for the quantification of Hydrogen peroxide**

## CONCLUSION

We have successfully developed a new spectrophotometric method, which afforded a new route for the determination of ultra-trace amounts of $H_2O_2$ in different samples. The method is based on the HRP - catalyzed reaction of INH and PC with the biocatalytic self-coupling of the reactants in the presence of $H_2O_2$. The proposed method has the almost widest linear range and lowest detection limit. Such a wide linear range and a low detection limit demonstrate that the established method may be an excellent method for the determination of $H_2O_2$ in different samples. This method has merits of use of cheap reagents, simple operation conditions, rapid analysis, wide linear range, high sensitivity, free from interfering substances and precision comparable to the reference method. Hydrogen peroxide is not mentioned in the European Drinking Water Standard 98/83/EC. In the USA, hydrogen peroxide is registered as a pesticide by the EPA in 1977. But still many companies using the Hydrogen peroxide for sterilization purpose in packing material.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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