A

Dissertation report

On

“Development of Enzymatic paper strip for detection of heavy metal Mercury in water”

submitted by

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CANDIDATE'S DECLARATION

I hereby, declare that the work, which is being presented in this evaluation report, entitled "**DEVLOPMENT OF ENZYMATIC PAPER STRIP FOR DETECTION OF HEAVY METAL MERCURY IN WATER"** was carried out by me as a part of my M. Tech dissertation, under the supervision of **Dr. S. P. SINGH, Professor, Department of Paper Technology, Indian Institute of Technology Roorkee.** The matter embodied in this dissertation report has not been submitted by me for the award of any other degree in any other institute.

Date :

Place : Saharanpur BHANU PRATAP

This is to certify that the above statement made by candidate is correct to the best of my knowledge

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Finally, I am highly obliged to all authors whose papers, reports have helped me for completion of report and also Google and its random contents which help me to collect data on various subjects.

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ABSTRACT

This thesis report is based on the research work in the field of application of enzymatic reaction for the detection of mercury in water. My research is mainly focused on the detection of mercury in water as mercury ion is among the common pollutant with ability to affect the environment. For environmental analysis determination of heavy metals gaining an increasing importance. Heavy metals determination by instrumental methods is highly selective and sensitive, but many of them require expensive and specialized equipment and are fairly complicated. Enzymatic methods are characterized by simpler methods, very cheap, simple device and give results very quickly.

Metal ions may be act as inhibitor, cofactor or activator of bio-catalysis. Mercury is an inhibitor of horseradish peroxidase and influence the catalytic activity of enzyme. This property of enzyme can be used for determination of mercury concentration in water. The O- dianisidine oxidation catalyzed by horseradish peroxidase is used as an indicator reaction to develop a procedure as a visual test for the determination of mercury concentration in waters sample.

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NOMENCLATURE

HRP - Horseradish peroxidase

UV – Ultra Violet

Hg – Mercury

Conc – Concentration

H_2O_2 - Hydrogen Peroxide

WHO – World health organization

MCLG – Maximum contamination level goal

CHAPTER 1: INTRODUCTION

Background

Mercury is one of the hazardous pollutants that render water unusable. Bioaccumulation1 of mercury has toxic effects on the both human health and environment. Because of high degree of toxicity many heavy metals like Mercury, Arsenic, Chromium, Cadmium, and Lead rank among the priority metals that are of public health significance. Metal detection required in various industries like, pharmaceutical, beverage, mining, plastics, textile, chemicals, beverage, packaging and food industries. To reduce damage to human health and also to control mercury pollution, regular detection of mercury is important.

Mercury detection through instrumental method is highly sensitive and selective, but also requires expensive and specialized equipment. Simple device and experimental techniques are the characteristics of enzymatic methods. Metal ions may be act as inhibitor, cofactor or activator of bio-catalysis. So catalytic activity of enzyme is influence by metal ions. This property of enzyme can be used in determination of metal ion in water.

1. Bioaccumulation means increase in chemical concentration overa time in a biological organism as compared to concentration of chemical in the environment.

Objective

This chapter will give a brief description about motivation, objectives and importance of this master thesis. A supposition as an initial starting point is also given in this chapter to further investigate the problem

Motivation and objective

This master thesis entitled as “Devlopment of Enzymatic paper strip for detection of heavy metal Hg in water” aims to determine the concentration of mercury in water through immobilized paper strip.

The instruments to detect the heavy metal in water are far more costly like inductively coupled plasma mass spectrometry (*ICP-MS*), Atomic absorption spectroscopy (AAS) etc. Further they have some disadvantaged like they requires ultra pure reagents and the maintenance and operation are far more complicated. So enzymatic methods extremely proposing for monitoring environment which characterized as simple cheap experimental techniques for detection of metal in water on a regular basis.

The objective is to synthesize paper strip just like a pH paper which will give color on dissolving in sample water. The time taken by the strip to change the color gives the idea of amount of metal present in the water. The detection method was developed using inhibitive enzymatic reaction. Study of previous work showed was limited to detection with the help of solution of enzyme prepared regularly and the lab is in need to test the mercury contamination in water should pose the necessary chemicals and enzyme or need to carry the solutions.

For this enzyme, (Dolmanova et al. 1979)most efficient inhibitor was Mercury (II) .In a reaction presence of thiourea enhanced the HRP inhibition that had been developed as highly selective and sensitive mercury(II) determination method.

Set Goal by WHO for MCLG

For mercury, the MCLG (Maximum Contamination Level Goal) is 0.002 mg/l or 2ppb. Contaminants may be radiological substances, biological, chemical or physical matter in water

The Major Flows of Mercury within the Biosphere

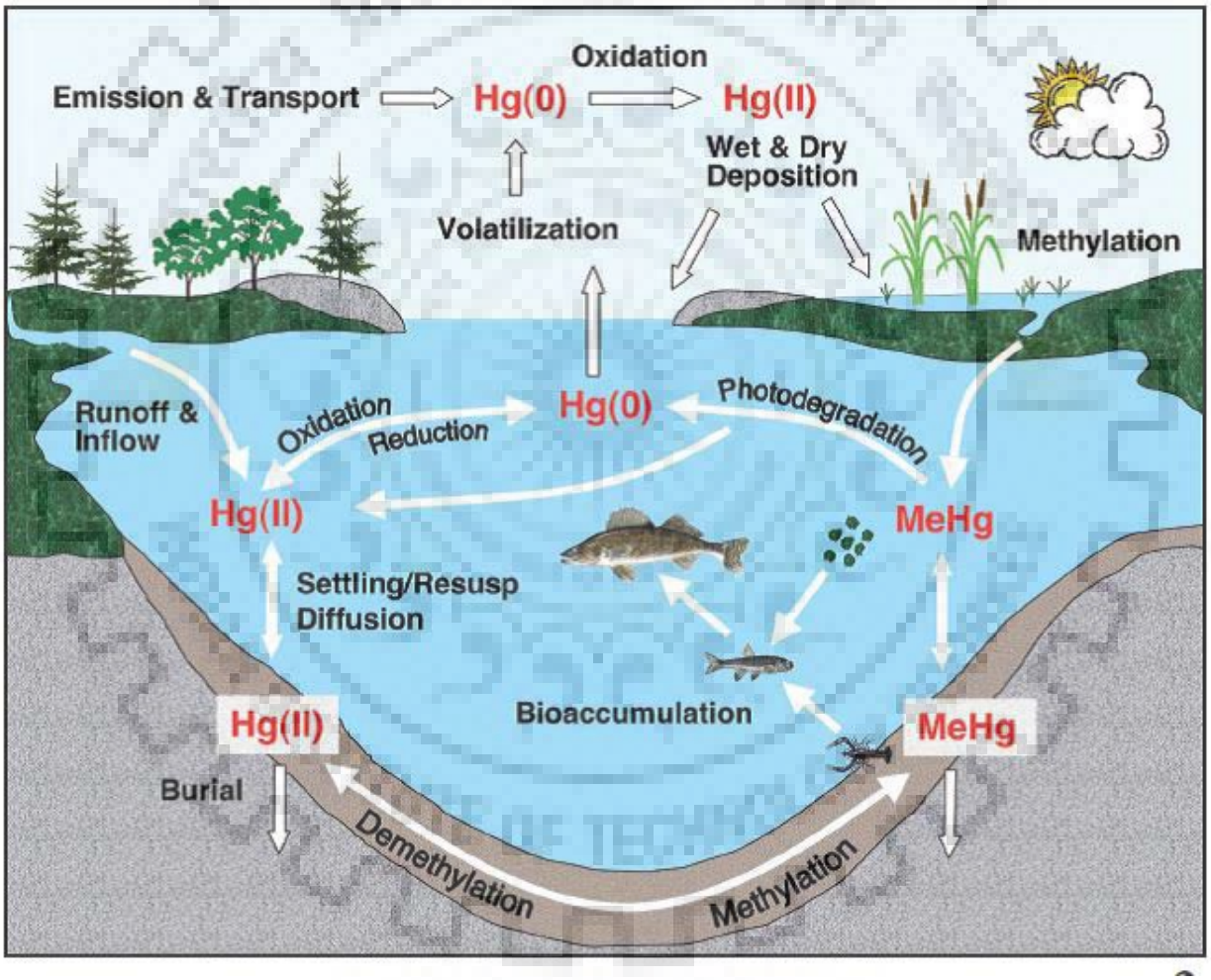
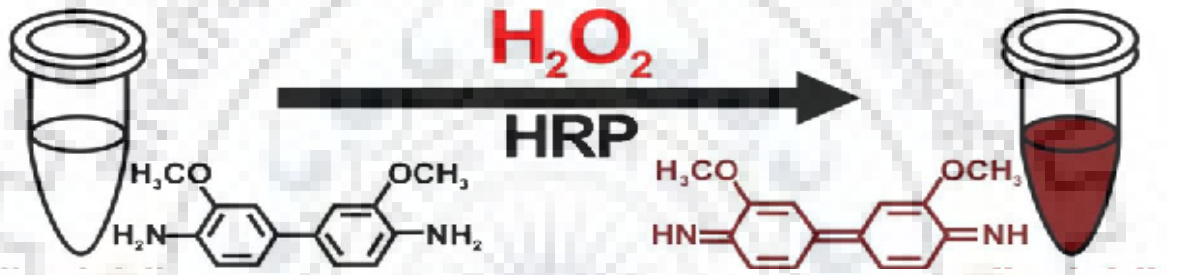


Figure 1: The major flows of mercury within the biosphere.

CHAPTER 2 : LITERATURE REVIEW

A general overview about literature background relevant to this master thesis which is useful to interpret results of various experiment trials

Oxidation of O-dianidisine (3, 5, 3', 5'- Tetramethylbenzidine) catalyzed the Horseradish Peroxidase (Enzyme)



O-Dianisidine **Oxidised O-Dianisidine**

(Red colour)

Figure 2: O-Dianisidine Oxidation in presence of peroxidase.

O-Dianisidine is a colorimetric substrate for peroxidase. For the detection of various metals, nitrites and thiocyanates, it is used as a reagent. This aromatic amine initially pale red but turns to red upon oxidation

Inhibition of Horseradish Peroxidase activity by Mercury

For this enzyme, (Dolmanova et al. 1979) most efficient inhibitorwasMercury (II) .In a reaction presence of thiourea enhanced the HRP inhibition that had been developed as highly selective and sensitive mercury (II) determination method. Peroxidases are hemoproteins which contain calcium ions and glycan chains and oxidation of inorganic and organic compounds catalyzed by it in presence of hydrogen peroxide. However, for a particular enzyme, the accessibility to enzyme active site is specific. Further surrounding peptide chain conformation, and alsoglycannumbers are specific. This results in different sensitivity, different stability and different profiles of substrate peroxidase specificity towards action of effector compounds.

Factors influencing the activity of peroxidise.

In the presence or contamination of Sulfur leads to deactivation process of horseradish peroxidase (HRP). Sulfur destabilizes the tertiary structure of enzyme to linear structure.

Effect of Immobilisation of enzyme.

The observations suggested that for stabilization of HRP, immobilization are useful because HRP contain 18-20% carbohydrate (w/w) in form of oligosaccharide chains on surface of protein carbohydrate moieties and this helps in stabilizing the HRP's conformational structure which is more resistant to denaturation which induced by extremes of pH and heat.

1). From the literature review, the application of paper for immobilization of HRP allows retaining the catalytic activity for 1 year and most stable enzyme preparation is obtain.

2).The intermediate and final product shows the maximum contrast (Pale red-green-red).

3).The inhibition of horseradish peroxidase by mercury immobilized on paper gives most Effective and reproducibility for determination of Mercury.

Enzyme Immobilization Methods

Immobilization means restricting the enzyme mobility in a fixed space

Covalent Bonds Formation

One of the most widely used method for immobilization of enzymes based on covalent bonds formation.

This method having the main advantage that when immobilized enzyme is used, it is not released into solution because bonds between matrix and enzyme formed are stable.

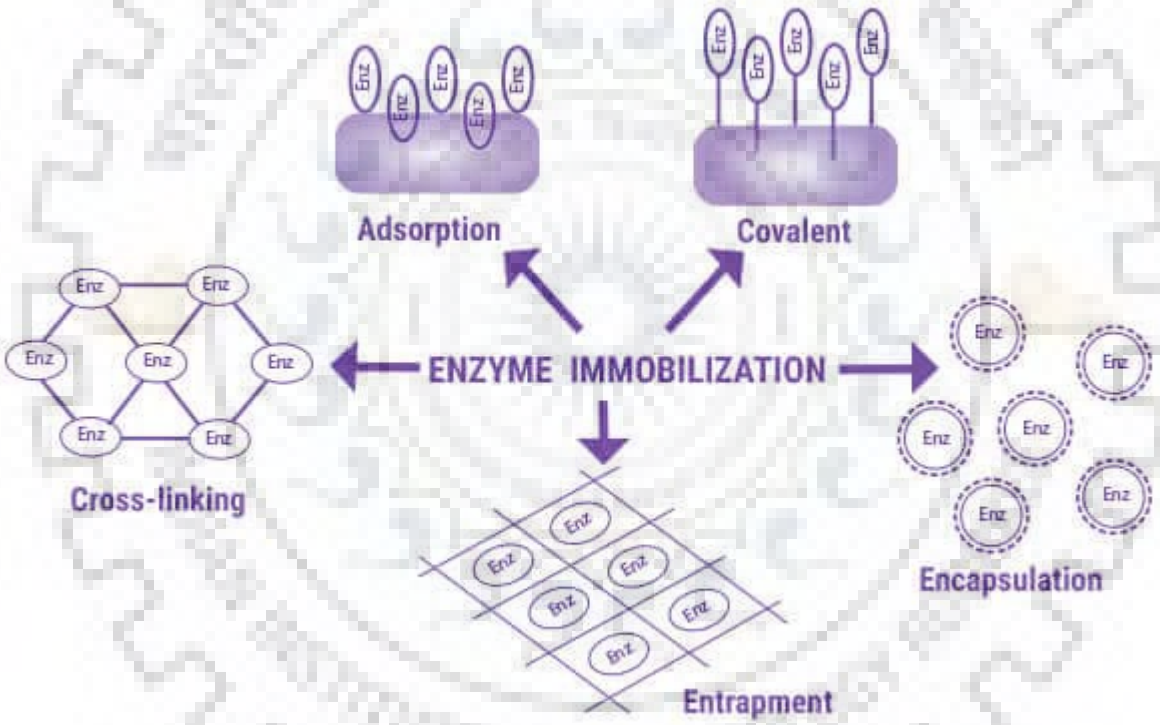


Fig 3 : Approaches to Enzyme immobilization

Advantage

1. Enzymes increase rates of chemical reactions without being themselves permanently altered Consumed by the reactions.
2. Time efficient process.
3. Enzymes can be reuse for many reaction cycles.
4. Easy separation of enzyme from the product.
5. Requires mild reaction condition.
6. Cost effective process

2.5 Influence of chitosan and Starch as the binding agent on HRP to paper

1. From the literature review, Chitosan is necessary for holding the enzyme on the paper. Else it will lead to dissolution of HRP in the water sample from paper. And the presence of chitosan gives better result for measuring time of red color appearance as compared in absence of chitosan. Also chitosan have the antimicrobial activity which prevents the contamination of HRP.
2. It provides stability to HRP.
3. In absence and presence of chitosan, the degree of peroxidase sorption on paper was 28.1% and 86.3 % respectively.
4. Binding agent starch giving the best results as compared to chitosan because starch is more hydrophilic then chitosan which increases the rate of reaction time.

CHAPTER 3: EXPERIMENT AND METHODOLOGY

Overview

Solution o-dianisidine prepared by the dissolving weighed amount of it in water daily. Small amount of concentrated HNO (3–4 drops) used for dissolving weighed amounts of Mercury (II) nitrate (1g/100ml) and then serial diluted with distilled water. The diluted solutions of metal salts had pH 3.0.

Chemicals and material required

1. **Polyurethane Foam –** It will act as a solid support.

2. **Chitosan** - It will entrap the enzyme onto a solid support. For this, using open cell polyurethane foam.0.1% Chitosan is dissolved in 1% acetic acid for 1 hour at 60oC in magnetic stirrer for completely dissolves the chitosan and enzyme solution is added.

3. **Starch**- Also used as binding agent for enzyme.

4. **O-Dianisidine** - is a colorimetric peroxidase substrate. Initially it is pale red but turns to red upon oxidization and is spectrophotometrically

5. **Thiourea** - It enhance the inhibitory effect of metal ions.

6. **Enzyme** – **Horseradish peroxidase –** catalyzes the oxidation of O-Dianisidine.

7. **Borate Buffer (pH 7)** –used for dissolving enzyme.

8. **Hydrogen peroxide –** required forthe oxidation of O-Dianisidine

9. **Potassium Hydrogenpthalate buffer (pH 5.0) :** maintaining the pH of the reaction

10. Instruments used:

- UV Spectrophotometer: For measuring absorbance at 420nm wavelength
- Potentiometer: For measuring pH of buffer solution
- Weighing machine
- stopwatch

Experimental procedure (a) (Using Enzyme Solution)

The Enzyme solution was prepared mixing Borate buffer at pH (7.0)

following chemicals added sequentially in test tube

Buffer Potassium Hydrogenpthalate (pH 5.0)

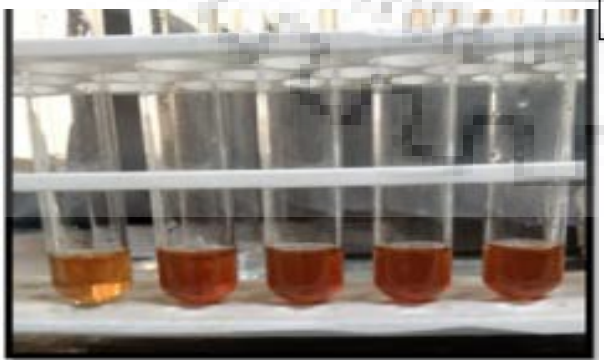
Thiourea

Mercury solution of known concentration

O-Dinaisidine

Hydrogen Peroxide

At the moment when H_2O_2 was added, the time for the **red color** appearance was measured by stopwatch



A calibration graph was plotted for the time of red colour appearance vs Mercury concentration

Experimental Procedure (b) (Using paper strip with immobilised enzyme)

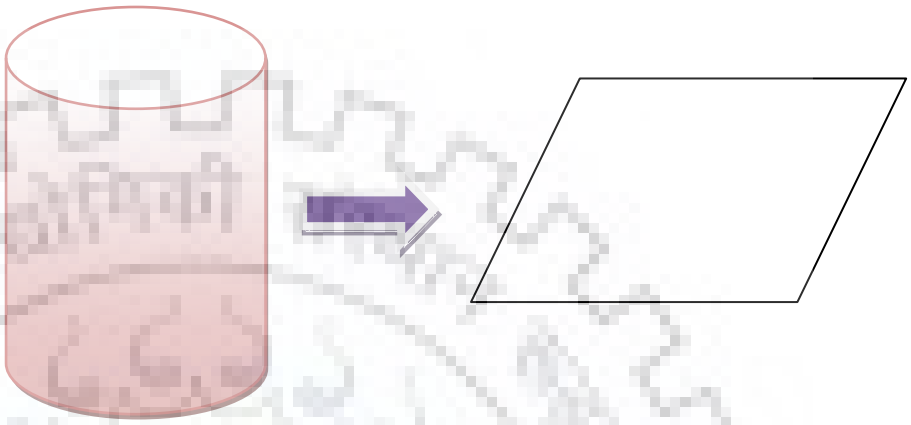
Enzyme solution+

Phthalate buffer+

Thiourea+

O-Dinisidine+

Starch (binding



Paper strip dipped in the solution so that

Enzyme immobilized on the paper

Air dried fo 2 -4 hours

Dip in sample water containing H_2O_2

The time for red colour appearance was measured

Effect of Temperature

All these experiments were done at room temperature and the rate of O-Dianisidine oxidation would change up to 2% on the change of surrounding temperature by 1 degree C.

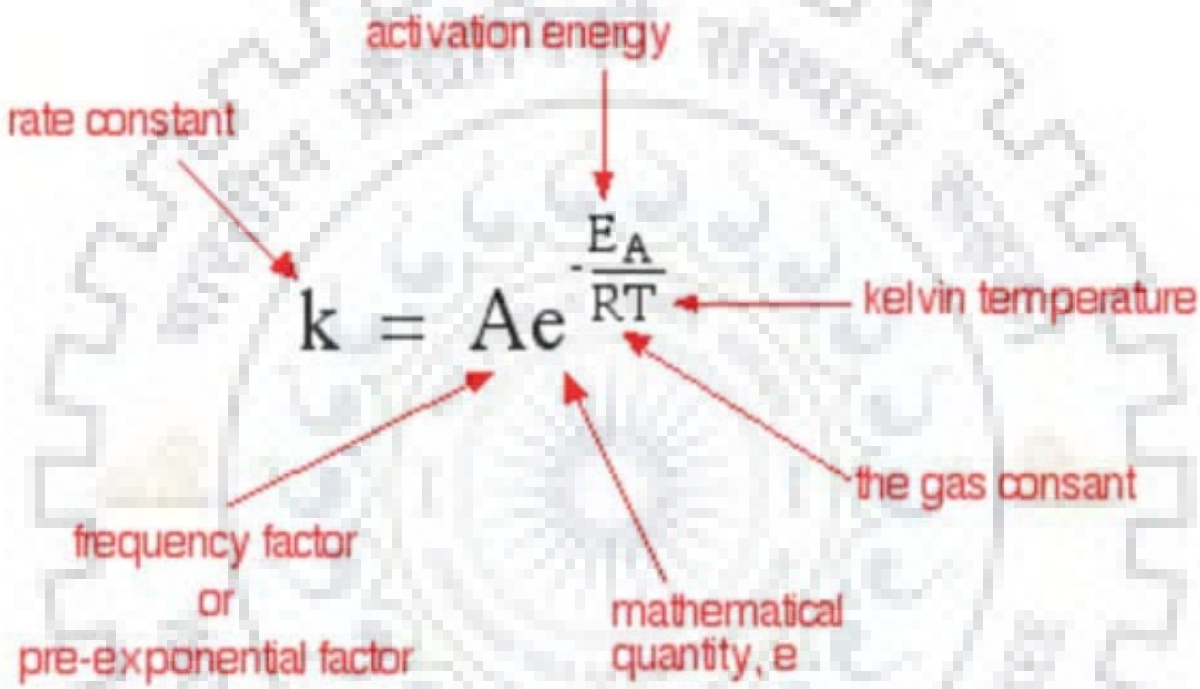


Figure 4: Effect of Temperature.

According to Arrhenius theory of dependency of rate contant on temperature the value of constant K increases on increasing value of Temperature T.

UV spectroscopy of samples

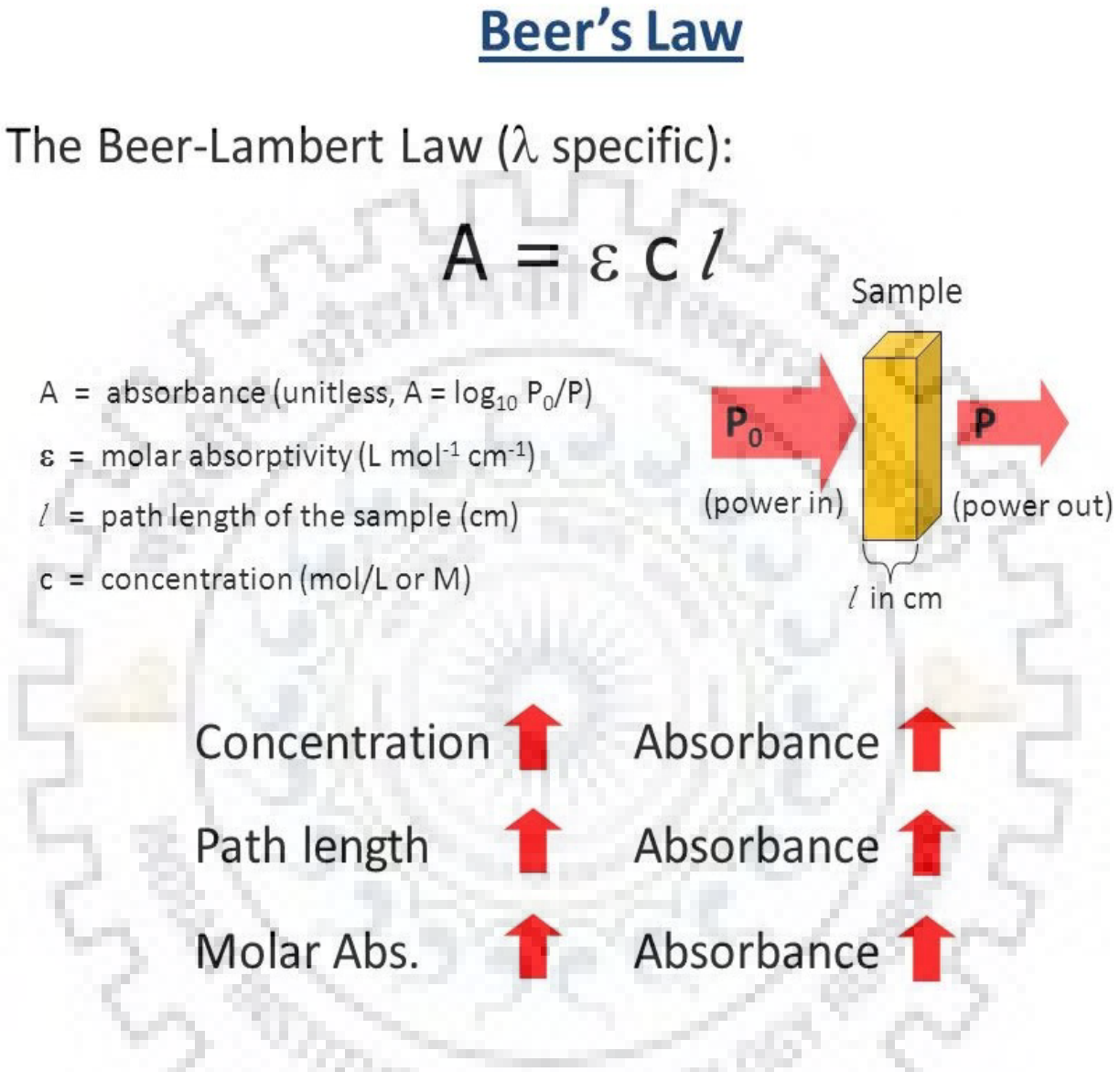


Fig. 5 : UV spectroscopy working principle

Spectrophotometer used for quantitative determination of concentration of solution containing absorbing species which creates the difference in initial intensity of light enters into the cuvette and final intensity of light coming out. From this, absorbance of the solution is calculated. Therefore with increase in concentration, absorbance increases.

CHAPTER 4 : RESULTS & DISCUSSIONS

Mercury determination by using horseradish peroxidase (HRP)

Based on inhibitory effect of mercury on HRP's catalytic activity, different test procedures and test devices developed. Here mercury is the most effective inhibitor of HRP catalyzing the o-dianisidine oxidation in presence of oxidizing agent H_2O_2. In case of horseradish, presence of thiourea - another enzyme inhibitor, the inhibitory action of mercury significantly increases. This procedure having detection limits 1 and 0.0001 mg/l, respectively. The procedures developed for determining mercury were applied to samples prepared. It was observed that dependence of velocity of indicator reaction on mercury concentration has a linear dependence. As the mercury concentration decreases, inhibitory effect from mercury would also decrease.

Choice of the type of paper

Bloating paper is used for the purpose because of its high porosity and proper wet strength. Following are the characteristics of different types of blotting paper.

Paper 1 ; IPT Blotter paper

Paper 2 : CPPRI Blotter paper

Paper 3 : Whatman filter apper

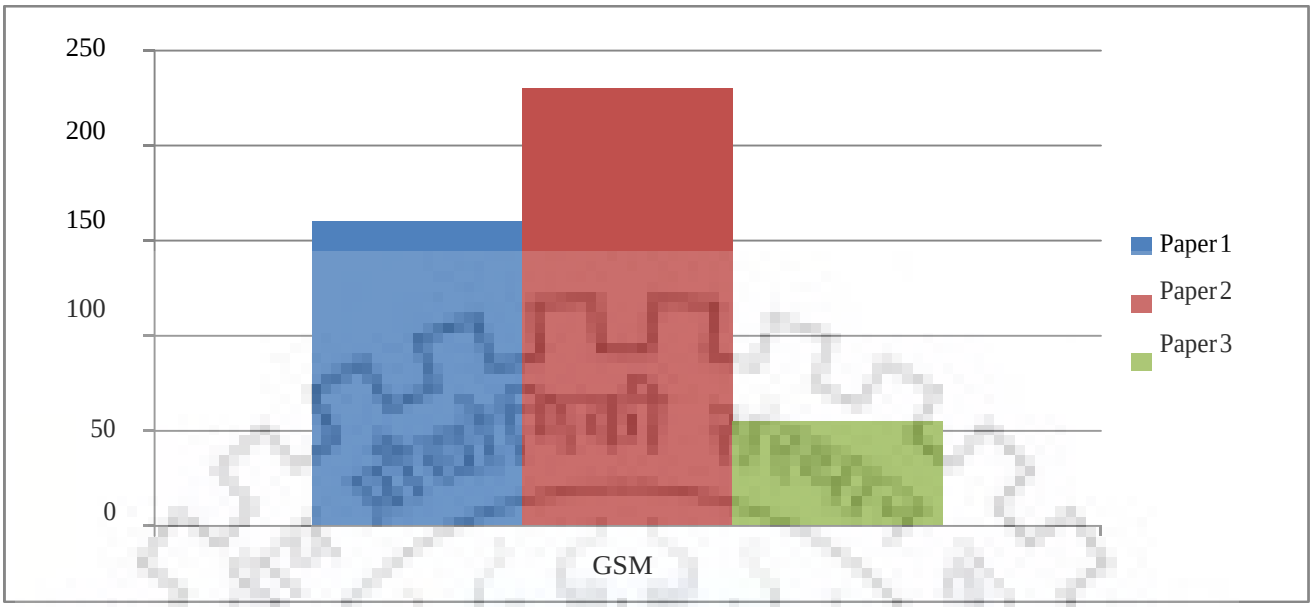


Figure 6: Graph showing the grammage of three different papers.

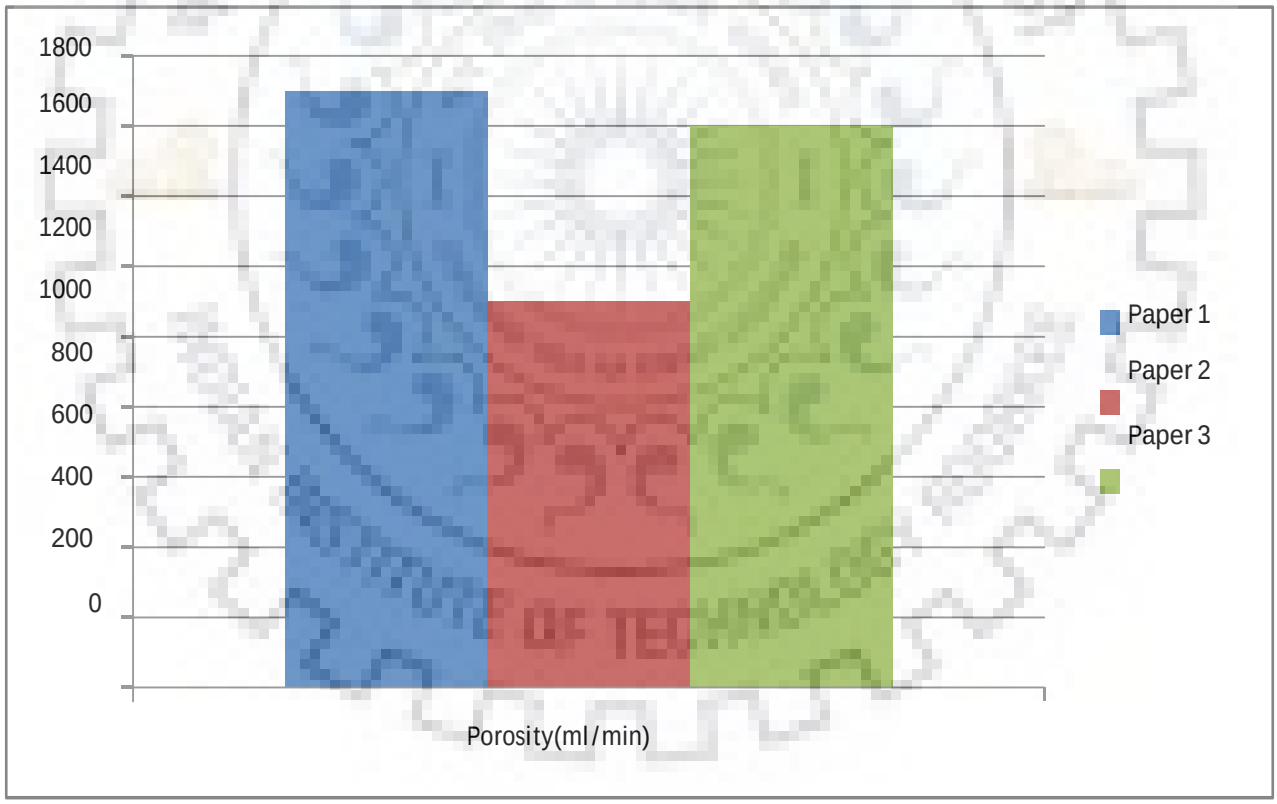


Figure 7: Graph showing the porosity of three different papers.

Paper 1 was preferred as compared to paper 2 & 3 as paper 1 shows highest porosity which help the enzyme to be entrap in the pores of paper and high grammage which holds the enzyme on paper when dip in water and giving uniform color to the paper strip.

While paper 2 because of its low porosity and paper 3 of its low grammage were not giving proper result

Kinetics of O-dianisidine Oxidation

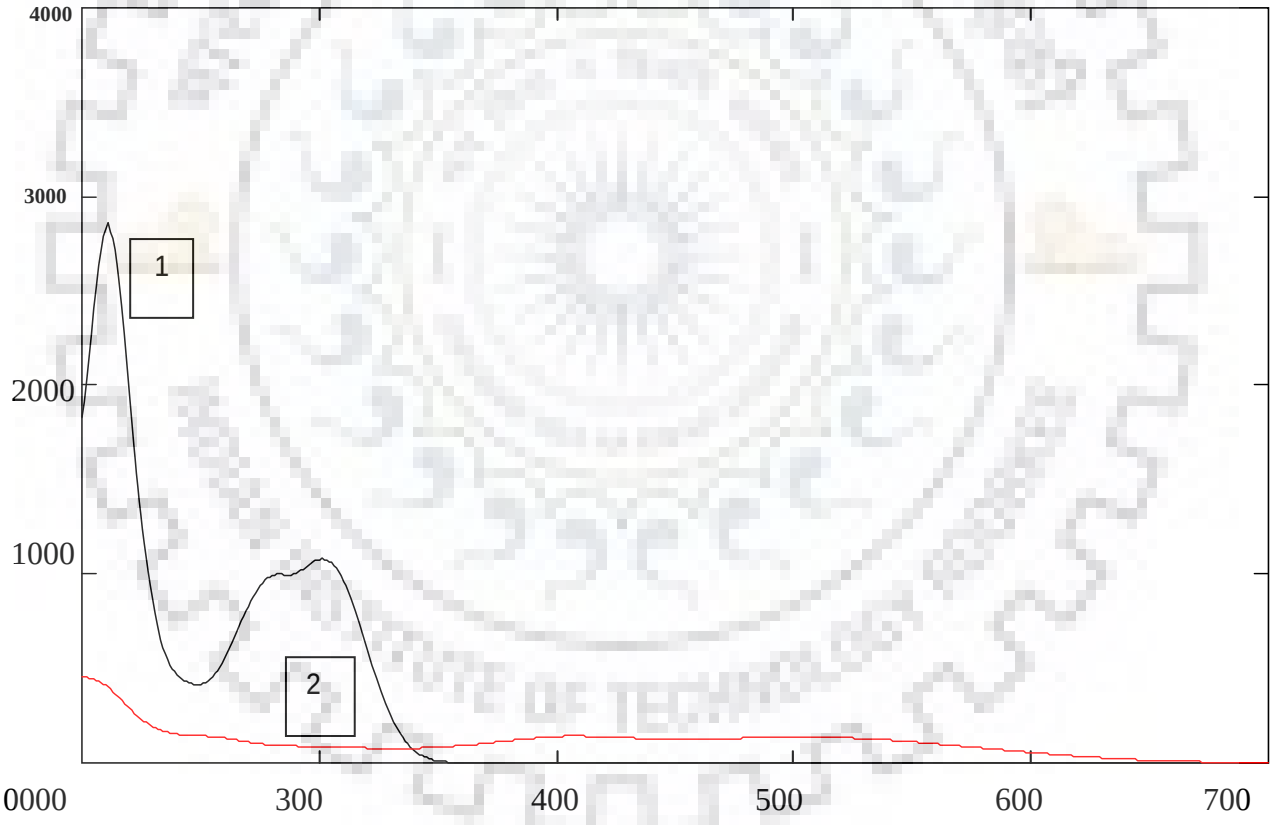


Figure 8: Dependence of rate of O-Dianisidine oxidation in presence (1) and in absence (2) of Horseradish Peroxidase

The reaction of O-dianisidine with hydrogen peroxide takes 2-3 days to complete the reaction while in presence of horseradish peroxidase enzyme, the reaction completed within fractions of seconds. Since enzyme increases the rate of reaction

Determination of mercury using UV spectrophotometer

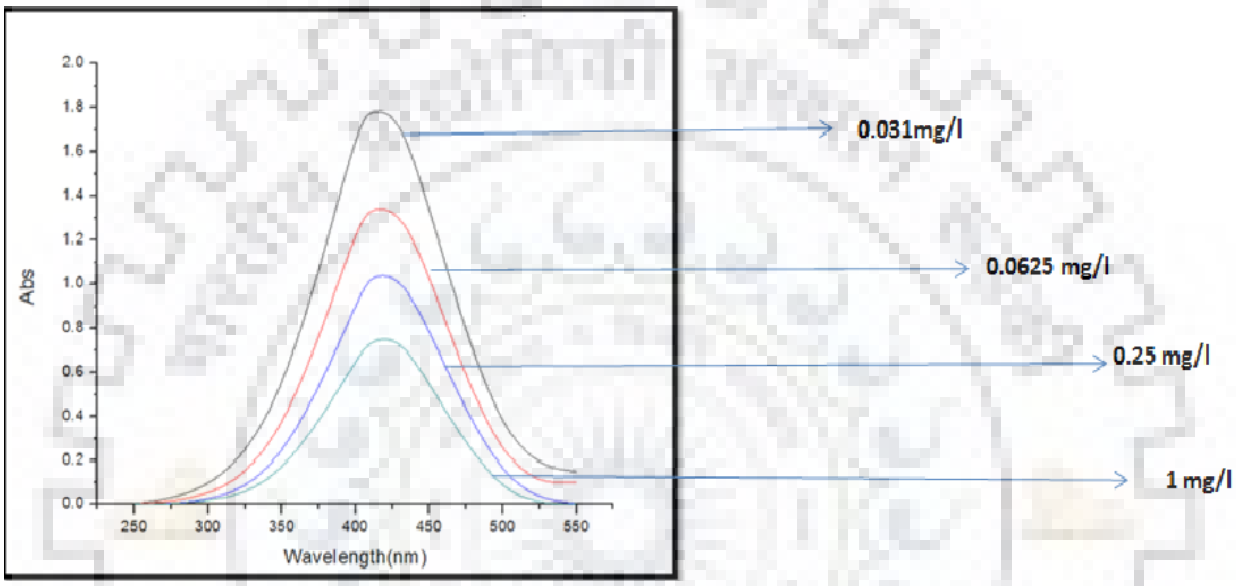


Figure 9: Dependence of absorbance at 420nm wavelength of O-Dianisidine oxidation at different concentration of mercury using UV spectrophotometer.

When the rate of O-Dianisidine oxidation catalyzed by HRP is measured by UV spectrophotometer, the peak of absorbance is found at a wavelength of 420nm.

The absorbance was 0.76, 1.04, 1.34 and 1.77 at concentration 1, 0.1, 0.01 and 0.001 (mg/l) of mercury respectively

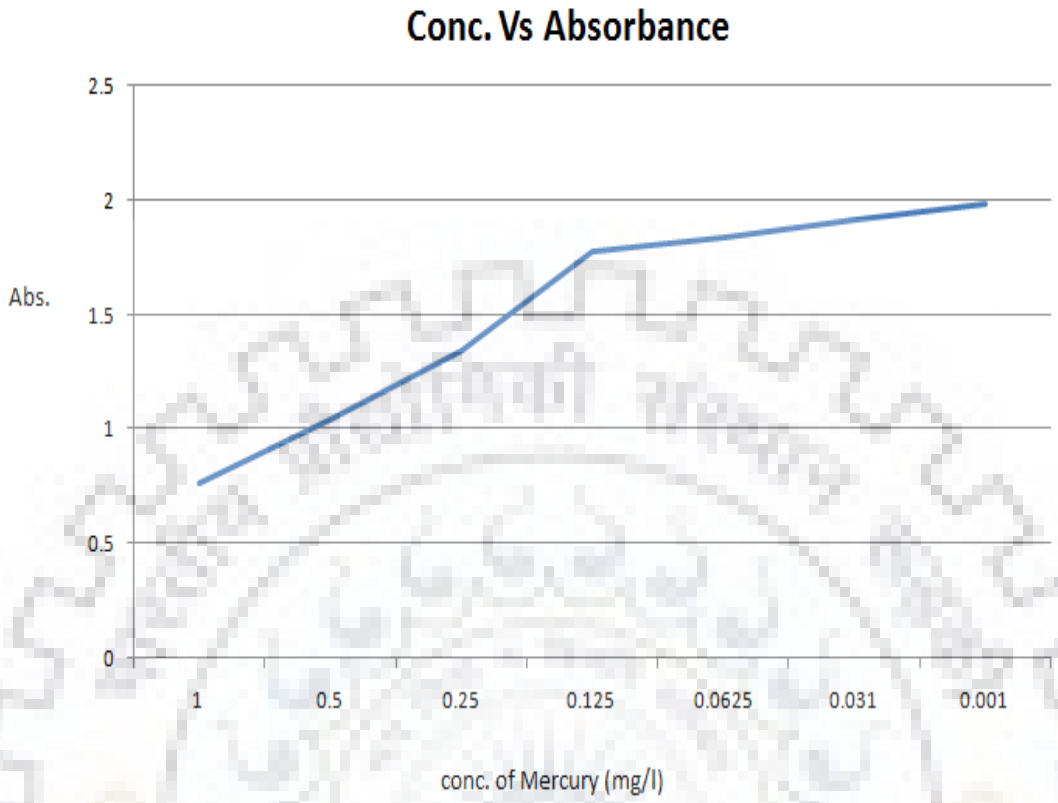


Figure 10: Plot of maximum absorbance vs. concentration of mercury used at wavelength 420 nm.

Here, we see a straight line relationship between absorbance of o-dianisidine oxidation and the concentration of Mercury used.

The higher the concentration of mercury present in water lower will be the absorbance of the reaction product because of the inhibitory effect of Hg.

Determination of Mercury using Enzyme solution

Hg Conc. (mg/l)	Time (sec) for red colour appearance
1	122
0.5	108
0.25	92
0.125	82
0.0625	70
0.03125	35
0.015625	30
0.0078125	20

Table 1 : Determination of mercury using enzyme solution

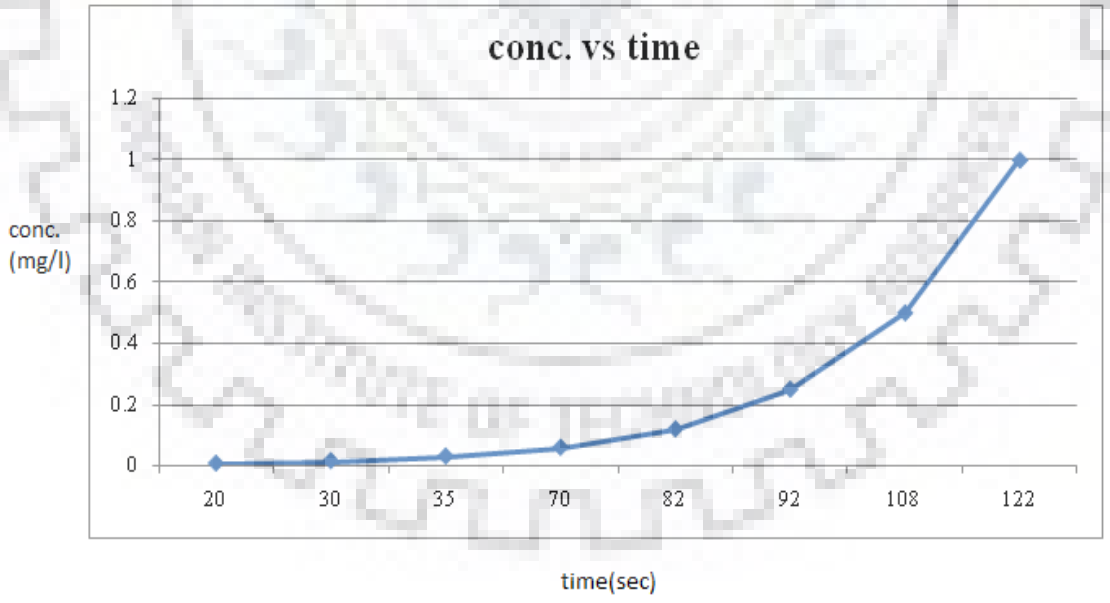


Figure 11 : Plot of time versus mercury concentration using enzyme solution.

CHAPTER 5: CONCLUSION

The procedures developed on enzymatic determination are based on the inhibiting effect of mercury ions on catalytic activity of horseradish peroxidase in oxidation of o-dianisidine with hydrogen peroxide. This procedure having detection limits 1 and 0.00001 mg/l, respectively. The Paper 1 having high grammage and very high porosity is good for holding the enzyme into the pores of the matrix. Binding agent starch giving the best results as compared to chitosan because starch is more hydrophilic then chitosan which increases the rate of reaction time. Based on the three methods for mercury determination i.e. using spectrophotometric method, enzyme solution method and paper strip method draws the same conclusion that with increase of mercury concentration in water the oxidation rate of O-dianisidine decreases by enzyme. Besides the proposed procedures are less expensive and significantly simpler as compared to instrumental methods for determination of mercury and this procedure can be used for field analysis of samples collected from different sources successfully.

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