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The Use of Horse Radish Peroxidase, an Eco-Friendly Method for Removal of Phenol from Industrial Effluent

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Abstract: *The rising population, increasing urbanization, rapid industrialization and spread of more water intensive life styles are making water resources scarce and polluted. waste waters of various industries such as textile, petroleum, refining, plastics etc. contain phenolic compounds. (1) Due to toxicity of these compounds to aquatic organisms and humans, it is necessary that phenolic compounds are removed from contaminated water before they are discharged into any natural stream of water. In this study, the removal of phenolic contaminants was undertaken biologically by using peroxidase enzyme. This enzyme was extracted from various natural sources like carrot, radish, broccoli, beetroot, soybean as well as some microorganisms like Pseudomonas aeruginosa NCIM 2200, Bacillus subtilis and Staphylococcus aureus NCIM 2079. Peroxidases catalyse the oxidation of phenols by hydrogen peroxide which results in the formation of water insoluble polymers which can be separated by coagulation, sedimentation etc. The prime objective of this work was to develop an eco-friendly, economical and effective biological method of removing phenolic pollutants from the waste water. The most effective peroxidase enzyme was white radish peroxidase which showed maximum enzyme activity. It exhibited above 90% phenol removal potential. This compound could be tested further by making certain formulations.*
Key words: *Hydrogen peroxide, peroxidase, Phenol, polymers*

I. Introduction

Water pollution is a major global problem**.** It is the leading worldwide cause of deaths and diseases and it accounts for the deaths of more than 3 million people annually (WHO world water day report). Water is typically referred to as polluted when it is impaired by anthropogenic contaminants and either does not support a human use, such as drinking water, and/or undergoes a marked shift in its ability to support its constituent biotic communities, such as fish. Waste water from manufacturing or chemical processes in industries contributes to water pollution. Industrial waste water usually contains specific and readily identifiable chemical compounds. Industrial activities should comply with the regulatory norms for prevention and control of water pollution. Rapid industrialisation is having an adverse effect on our environment resulting in the generation of industrial effluents. Due to high organic load and toxic materials, industrial effluents form a major source of water pollution. High concentration of metallic effluents such as, Cadmium, Zinc, Mercury, Manganese, Iron and others generating from the untreated effluents get accumulated in the food chain and thereby affect the human health when the food is consumed. These contaminants can interfere with the formation of red blood cells that may show harmful effects in humans. Presence of phenolic compounds even at low concentrations in the industrial waste water adversely affects aquatic as well as human life directly or indirectly when disposed off to public sewage, river or surface water. Phenolic contaminants are observed in wastewaters of various industries like photo developing chemicals, petroleum refining, coal conversion, textiles, iron and steel manufacturing, plastics, as well as pulp and paper manufacturing. Various phenolic derivatives like 2,4-dichlorophenol, xylenol, cresol, bisphenol form a major part of the phenolic contaminants in industrial effluents (2). The phenol concentrations in these wastewaters range from 1.0 to 10.0 mM (100 to 1000 mg/L). Phenols of anthropogenic origin may enter the environment because of uncontrolled discharges or accidental spills. It may also accumulate as an intermediate during incomplete biodegradation of aromatic compounds and pesticide mixtures. **Peroxidases** (EC number 1.11.1.x) are a large family of enzymes that typically catalyse a reaction of the form (3)

$$\mathbf{ROOR' + electron\ donor\ (2\ e^-) + 2H^+ \square\ ROH + R'OH}$$

Peroxidase also catalyse the phenol removal. (4)

$$\mathbf{E + H_2O_2 \longrightarrow Ei + H_2O}$$
$$\mathbf{Ei + AH_2 \longrightarrow Eii + AH.}$$
$$\mathbf{Eii + AH_2 \longrightarrow E + AH. + H_2O}$$

The native enzyme (E) gets oxidized by hydrogen peroxide (H_2O_2) to an active intermediate enzymatic form called compound I (Ei). Compound I then accept an aromatic compound (AH_2) into its active site and carries out its oxidation. A free radical ($AH^{\cdot}$) is then produced and it is released into solution leaving the enzyme in the compound II (Eii) state.

Compound II further oxidizes a second aromatic molecule, releasing another free radical product and returning the enzyme to its native state, thereby completing the cycle. The overall peroxidase reaction consists of the reactions described by the above Equations. Free radicals formed during the cycle diffuse from the enzyme into the bulk solution where they react to form poly aromatic products. The water-insoluble polymers may be removed by solid liquid operations. Phenol is the most potent industrial pollutant being carcinogenic and genotoxic. MWPCB [Maharashtra Water Pollution Control Board] has norms for discharge of phenol in natural water bodies [1 ppm] Phenol is toxic to fish at a level of 0.05 mg/lit. WHO has set a limit of 0.1mg/ml to regulate the level of phenol in drinking water. The standard processes for phenol removal from industrial wastewaters include extraction, bacterial and chemical oxidation, adsorption on activated carbon, irradiation, electrochemical techniques, etc. These methods have several shortcomings such as high costs, incompleteness of purification, hazardous by-products formation, low efficiency and could be applied to a limited range of concentration. There are certain advantages of using enzyme technique. Over conventional phenol removal system. They are Cost effective. Broad substrate specificity. High reaction rates, Milder reaction condition, No hazardous end products. And it shows activity over a wide range of pH, temperature end salinity ranges.

II. Materials And Methods

1.Extraction of enzyme peroxidase from natural sources and microorganisms

Th**e** natural sources like radish, broccoli, carrot, soybean and beetroot were used for the extraction of peroxidase. These vegetables were washed, cleaned and crushed. The juice was filtered and 60% ammonium sulphate was added in the juice. The centrifugation was carried out at 4000 rpm for 20 min at 4^{0} C. The supernatant was taken and the enzyme peroxidase was extracted using the 90% Ammonium sulphate precipitation. (5)

2.Biodegradation of phenol (6)

The micro-organisms used *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* were assessed to study their potential of biodegradation of phenol. In this assay the ability of the organisms to utilise phenol as a sole carbon source was evaluated. The phenol degrading activity of all the bacterial strains was evaluated. The micro-organisms were grown on a mineral salt agar medium which was supplemented with phenol concentration of 200mg/l at 25ºC for 72 hrs.

The composition of mineral salt agar medium in g/l was: KH_2PO_4 -0.42, K_2HPO_4 – 0.375, $(NH_4)_2SO_4$ – 0.244, NaCl – 0.015, $CaCl_2.2H_2O$- 0.015, $MgSO_4.7H_2O$- 0.05, $FeCl_3.6H_2O$ – 0.054. The significance of the study was the assessment of the above bacteria for their potential of bioremediation.

3.Measurement of peroxidase activity (7)

The principle of the test is: In the peroxidase assay Guaiacol is used as the substrate.

$$\textbf{Guaiacol} + H_2O_2 \longrightarrow \textbf{Oxidized Guaiacol} + 2H_2O$$

The oxidized (dehydrogenated) Guaiacol that is formed is probably more than one compound and its formation depends on the reaction conditions. The rate of formation of Guaiacol dehydrogenation product is a measure of the peroxidase activity and can be assayed spectrophotometrically at 436nm.The chemicals and reagents used in this test were, Phosphate buffer 0.1M (pH 7), Guaiacol solution 20 Mm, Hydrogen peroxide solution (0.042%=12.3 Mm), Enzyme extract.3 ml buffer solution, 0.05 ml Guaiacol solution, 0.1 ml enzyme extract and 0.03ml hydrogen peroxide solution was pipetted out in a cuvette. The resulting mixture of the reactants was assayed spectrophotometrically. The absorbance could increase by 0.05. A stop watch was used to note the time required in minutes (Δt) to increase the absorbance by 0.1. The reaction rate is determined by measuring an increase in absorbance resulting from the decomposition of hydrogen peroxide. One unit results when the decomposition of one micromole of hydrogen peroxide takes place per minute at 25^{0} C and pH 7.0 under specified condition.

As the extinction coefficient of Guaiacol dehydrogenation product at 436nm under the conditions specified is 6.39 per micromole, the enzyme activity per litre of extract could be calculated as follows:

$$\textbf{Enzyme activity units/litre} = 3.18 \times 0.1 \times 1000 / 6.39 \times \Delta t \times 0.1$$
$$= 500 / \Delta t$$

4.Phenol removal assay (8)

The phenol removal assay is a phenol concentration measurement assay. From this assay the ability of each of the extracted peroxidase enzymes to remove phenol is evaluated. A reduction in the concentration of phenol is directly correlated to the efficiency of the extracted peroxidase enzymes from the natural sources and microorganisms. Their efficiency to polymerise and thereby to remove phenol can be assessed from this assay. Phenol concentration is measured by a colour reaction based on the condensation of 4-AAP [4-AminoAntiPyrine] with phenol in the presence of alkaline oxidising reagents like potassium ferrocyanide [K_3FeCn_6]. This results in the formation of antipyrine dyes. The formation of antipyrine dyes is a measure of phenol concentration which is assayed colorimetrically at 510 nm. The chemicals and reagents used in this test were 20.8 mM 4-Aminoantipyrine, 2. 0.25 M sodium bicarbonate, 83.4 mM potassium ferrocyanide, Phenol solution. The assay mixture contains 2ml of phenol, 2ml enzyme solution ,4ml of 0.25M sodium bicarbonate and 0.9ml 20.8 mM 4-AAP. After proper mixing 0.9ml of 83.4 mM potassium ferrocyanide is added and vigorously mixed. The absorbance of the samples was measured at 510 nm, 9 min after the Ferro cyanide addition and converted to concentration using calibration curve. The above phenol removal assay was performed using:1] Synthetic phenol.2] Industrial effluent. The initial concentration of phenol in industrial effluent was measured using the above assay extracted peroxidase enzyme. Synthetic phenol and industrial effluent were reacted with the extracted peroxidase enzyme at different time intervals. A reduction in the phenol concentration was correlated to phenol removal potential of the enzyme.

5.Optimization of pH and temperature

The term "immobilization" refers to the trapping of biomolecules or whole living cells. Immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously." Every enzyme exhibits maximum activity at a specific pH and temperature value. This value is known as the optimum pH and temperature of the enzyme of the most effective peroxidase enzyme was optimised by measuring the activity of enzyme at pH 3, 5, 7 and 9. The temperature of the most effective peroxidase enzyme was optimised by measuring the activity of enzyme at 25 ºC ,37 ºC and at 55 ºC. The enzyme activity of the enzyme was evaluated using the following assay: The chemicals and reagents used were Phosphate buffer 0.1M (pH 3, 5 ,7, 9) Guaiacol solution 20 Mm Hydrogen peroxide solution (0.042%=12.3 Mm) Enzyme extract.3 ml buffer solution, 0.05 ml Guaiacol solution, 0.1 ml enzyme extract and 0.03ml hydrogen peroxide solution was pipetted out in a cuvette. The resulting mixture was then placed in the spectrophotometer. The absorbance could increase by 0.05. A stop watch was used to note the time required in minutes (Δt) to increase the absorbance by 0.1. The pH of the phosphate buffer used was varied in the assay (pH 3, 5 ,7 ,9) The assay mixture was exposed to temperatures of 25 ºC, 37 ºC and at 55 ºC for temperature optimisation.

6.Immobilization of the enzyme peroxidase

The term "immobilization" refers to the trapping of biomolecules or whole living cells. Immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously." For the immobilization of the extracted peroxidase enzyme the Entrapment method was used. The chemicals required were 5%Sodium alginate solution, Enzyme solution, 5ml Syringe, 3% calcium chloride. The Enzyme solution was mixed with 5% aqueous Sodium alginate solution. The resulting mixture was slowly extruded as droplets through a 5ml syringe into chilled Calcium chloride solution. Calcium alginate Beads were stored at 4^0 C in buffer for further use.

7.Lyophilisation of the enzyme peroxidase extracted from radish

Freeze-drying also known as lyophillization or cryodesiccation is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. The enzyme peroxidase extracted from radish showed the maximum activity for phenol removal from industrial effluent. Hence the extracted peroxidase enzyme from radish was subjected to lyophilisation.

III. Results

1.Ammonium sulphate fractionation

The ammonium sulphate fractionation method was used for the extraction of enzyme peroxidase from various natural sources and microorganisms. The extracted enzyme mixture was stored at 4ºC for further use.

2. Biodegradation of phenol

There was no growth seen on the mineral salt agar medium. The organisms could not use phenol as a sole carbon source. Therefore, the isolates used did not exhibit any biodegradation potential.

2.1 Reduction of synthetic phenol

Reduction of synthetic phenol over a time range of 1,2,24 and 48 hours was studied. The O.D. reading of 0.3M phenol solution at 510nm was found to be 0.03 using UV-VIS spectrophotometer. Due to activity of various peroxidase containing enzyme extracts from natural sources and micro-organisms, there was removal of phenol which was correlated by reduction in absorbance.

Table 1: Reduction of synthetic phenol

Sr.no	Natural sources	O.D at 510 nm			
		1 Hour	**2 Hrs**	**24 Hrs**	**48 Hrs**
1.	Radish	0.022	0.017	0.007	0.001
2.	Broccoli	0.026	0.020	0.010	0.003
3.	Soybean	0.025	0.018	0.008	0.004
4.	Carrot	0.024	0.019	0.009	0.006
5.	Beetroot	0.023	0.018	0.006	0.005
	Micro-organisms				
1.	*P. aeruginosa*	0.029	0.027	0.024	0.021
2.	*Staphylococcus aureus*	0.028	0.025	0.023	0.022
3.	*Bacillus subtilis*	0.026	0.024	0.023	0.023

3. Measurement of peroxidase activity

The Guaiacol assay was used for measurement of the activity of the extracted peroxidase enzyme. The rate of formation of Guaiacol dehydrogenation product was a measure of the peroxidase activity and was assayed spectrophotometrically at 436nm.Since the extinction coefficient of Guaiacol dehydrogenation product at 436nm under the conditions specified is 6.39 per micromole, the enzyme activity per litre of extract was calculated as below:

Enzyme activity units/litre = 3.18 x 0.1 x 1000 / 6.39 x Δt x 0.1

= 500/ Δt

Table 2: Measurement of peroxidase activity

Sr. No.	Natural sources	□ t (mins)	Enzyme activity in units/lit
1.	White Radish	2	250
2.	Red radish	2.1	240
3.	Broccoli	2.5	200
4.	Soybean	2.7	185
5.	Carrot	3	166
6.	Beetroot	3.3	150
	Micro-organisms		
1.	*Pseudomonas aeruginosa*	5	100
2.	*Bacillus subtilis*	6.2	80
3.	*Staphylococcus aureus*	6.8	73.5

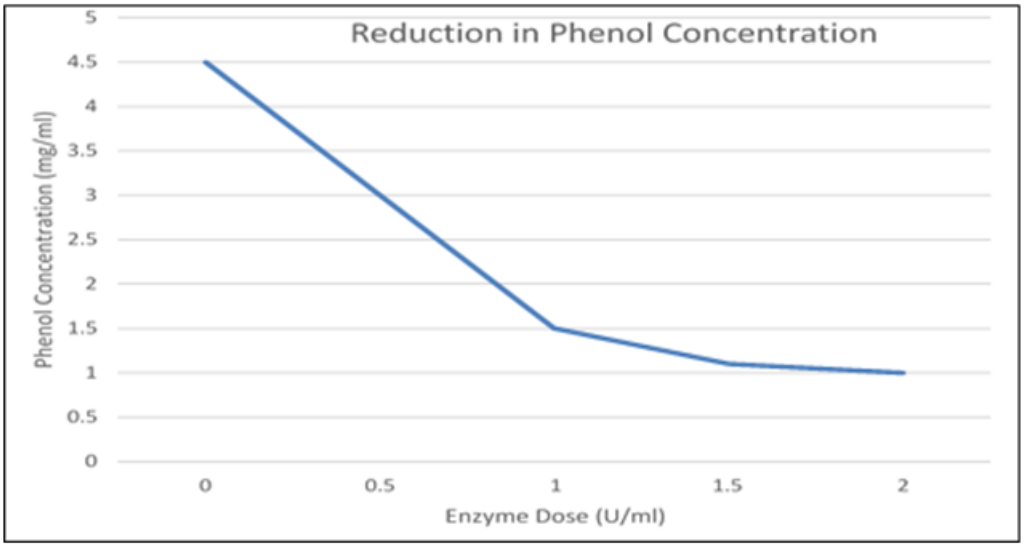
4.Removal of phenol (9)

A phenol concentration measurement is done by the phenol removal assay. From this assay the ability of each of the extracted peroxidase enzymes to remove phenol was evaluated. A reduction in the concentration of phenol was directly correlated to the efficiency of the extracted peroxidase enzymes from the natural sources and microorganisms. Their efficiency to polymerise and thereby to remove phenol was assessed from this assay. The phenol concentration is measured by a colour reaction based on the condensation of 4-aap [4-aminoantipyrine] with phenol in the presence of alkaline oxidising reagents like potassium ferrocyanide [k_3fecn_6]. this resulted in the formation of antipyrine dyes. The formation of antipyrine dyes was a measure of phenol concentration which was assayed colorimetrically at 510 nm.

The samples used in the phenol removal assay were: 1] synthetic phenol 2] industrial effluent. Since the highest peroxidase activity was observed in the enzyme extract from white radish, peroxidase enzyme from white radish was incorporated in the phenol removal assay.

4.1 Synthetic phenol

1] A graph representing reduction in the concentration of synthetic phenol by enzyme peroxidase from white radish was drawn. The graph shows that with an increase in the enzyme dose, the removal of phenol increases. The initial phenol concentration was 4.5mg/l and the final concentration reduced to 1.1mg/l. This shows that there was more than 90% removal of phenol.



2] Synthetic phenol concentration ranging from 1mM to 10 mM were prepared and tested for the reduction in the concentration of phenol after addition of white radish peroxidase. This synthetically prepared phenol was subjected to the phenol concentration measurement assay to measure the reduction in the phenol concentration.

Table 3: Removal of phenol

Test	Phenol Concentration				Phenol Conversion (%)
	Initial		Final		
	mM	mg/l	Mm	mg/l	
1	1	94	0.011	1.0	90
2	2	188	0.003	0.3	99
3	4	376	0.005	0.5	99
4	6	564	0.002	0.2	99
5	8	752	0.012	1.1	99
6	10	940	0.009	0.9	99

From the above result table, it was observed that in all experimental conditions phenol conversion was greater than 90%. The high efficiency of phenol removal observed was in accordance with conditions optimized to guarantee 90% polymerisation of phenol using purified white radish peroxidase.

4.2 Industrial effluent

To evaluate the decrease in concentration of phenol, the initial concentration of phenol in the industrial effluent was determined using the phenol removal assay. The concentration of phenol in the industrial effluent was determined by measuring the absorbance at 510 nm. This was performed to study the efficiency of the enzyme peroxidase from white radish to remove phenol from industrial effluent. The decrease in the phenol concentration at different time intervals was assessed.

Table 4: Concentration of phenol in industrial effluent.

Industrial effluent	O.D. at 510 nm
Phenol	4.000

Table 5: Decrease in the absorbance of phenol from industrial effluent

Time(mins)	O.D at 510 nm
10	3.129
20	0.230
40	0.220
60	0.137

In about 20 mins, phenol conversion higher than 90% was observed by the polymerisation reaction.

5.Optimization of pH and temperature

Since maximum peroxidase activity and maximum phenol removal was shown by white radish enzyme extract optimisation of the pH and temperature of peroxidase from white radish extract was performed.

A] Optimisation of pH

pH of the white radish peroxidase was optimised by measuring the activity of enzyme at pH 3, 5, 7 and 9.

Table 6: Enzyme activity at different pH values

pH	Enzyme Activity (units/l)
3	185
5	200
7	247
9	225

Thus, it was observed that the optimum pH of white radish peroxidase was 7.

B] Optimization of temperature

The temperature of the white radish peroxidase was optimised by measuring the activity of enzyme at 25 ºC ,37 ºC and at 55 ºC.

Table 7: Enzyme activity at different temperatures

Temperature	Enzyme Activity(Units/L)
25 ºC	245
37 ºC	200
55 ºC	175

Thus, it was observed that the optimum temperature of white radish peroxidase was 25 ºC.

6. Immobilization of peroxidase enzyme extracted from white radish

The white radish extract containing peroxidase showed the highest enzyme activity for efficient removal of phenol so it was immobilized by the entrapment method for reusability.

The enzyme was immobilized in the form of calcium alginate beads for phenol removal.

A] Enzyme activity of the immobilized white radish peroxidase

The enzyme activity of the immobilized enzyme was checked by using the Guaiacol assay in a similar way as it was performed for the free enzyme extract and the results obtained were as follows:

Table 8: Enzyme activity of immobilized peroxidase

Natural Source	Enzyme Activity (Units/l)
Radish	245

From the above result, it was observed that the immobilised enzyme had similar enzyme activity as compared to the free enzyme. The immobilised enzyme could be used repeatedly for the removal of phenol from industrial effluent.

B] Phenol removal from industrial effluent by immobilized white radish peroxidase

Table 9: Phenol removal by immobilized peroxidase.

Phenol concentration in industrial effluent	O.D. at 510 nm
Initial	4.000
Final [After Immobilized Enzyme Addition]	0.147

From the above result, it can be concluded that there was above 90% removal of phenol from industrial effluent was observed by using the immobilized white radish peroxidase. Thus, the activity of the immobilised peroxidase enzyme was found to be promising for effective phenol removal.

IV. Discussion

Dec and J.M. Bollag (10) have demonstrated that plant materials like potato tubers, raw soybean hulls, turnip roots, onions and parts of fifty other plant species are effective carriers of peroxidase activity, which is instrumental in removing anilines and other aromatic compounds from water solutions. Bollag and co-workers studied the effectiveness of different enzymes like laccases, tyrosinases, peroxidases for waste water treatment. In this project the removal of phenol from industrial effluent was undertaken using enzyme peroxidase isolated from various natural sources and micro-organisms. Peroxidases catalyse the oxidation of phenols by hydrogen peroxide resulting in the formation of water insoluble polymers which can be separated by coagulation, sedimentation etc. The natural sources used were radish, broccoli, carrot, soybean and beetroot. The micro-organisms used were *Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis*. Firstly, the

enzyme peroxidase was extracted from both the sources using ammonium sulphate fractionation technique. The measurement of peroxidase activity was undertaken by using the Guaiacol assay. The maximum peroxidase activity was exhibited by white radish enzyme extract.S. Chakraborty et .al (11) assessed the biodegradation of phenol by native organisms isolated from coke processing wastewater. In our study the micro-organisms used *Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis* were assessed to study their potential of biodegradation of phenol. In this assay the ability of the organisms to utilise phenol as a sole carbon source was evaluated. The micro-organisms were grown on a mineral salt agar medium supplemented with phenol concentration of 200mg/l at 25ºC for 72 hrs. There was no growth seen on the mineral salt agar medium. The organisms could not use phenol as a sole carbon source. Wu. et al (1993) studied the effects of additives on aromatic compound removing capacity of enzymes. Phenol removal (phenol concentration measurement) assay was performed using white radish POD. The samples used in the phenol removal assay were:1] Industrial effluent 2] Synthetic phenol. For synthetic phenol, it was observed that with an increase in the enzyme dose, the removal of phenol increases. The initial phenol concentration was 4.5mg/l and the final concentration reduced to 1.1mg/l. This shows that there was more than 90% removal of phenol. Decrease in the absorbance of phenol from industrial effluent over a period of 10, 20, 40 ,60 minutes was studied. In about 20 mins, phenol conversion higher than 90% was observed by the polymerisation reaction. Therefore, it was concluded that about 99% phenol from industrial effluent was removed by using the peroxidase enzyme obtained from white radish. When white radish peroxidase was added to synthetic phenol, formation of water insoluble phenolic polymers was seen. Cooper and Nicell (12) and Li, Tonegawa et al, (13) Stanisavljevic (2004) studied the effects of different parameters like enzyme concentration on the efficiency of phenol removal from solutions. Since maximum peroxidase activity and maximum phenol removal was shown by white radish enzyme extract optimisation of the pH and temperature of peroxidase from white radish extract was performed. pH of the white radish peroxidase was optimised by measuring the activity of enzyme at pH 3, 5, 7 and 9 and at pH 7 maximum enzyme activity was seen. Therefore, the optimum pH of white radish peroxidase was found to be 7**.** The temperature of the white radish peroxidase was optimised by measuring the activity of enzyme at 25 ºC ,37 ºC and at 55 ºC. Since highest enzyme activity was observed at 25 ºC**,** the optimum temperature of white radish peroxidase was 25 ºC. Syed Mustapha et al (2004) proposed an economical and high yield procedure for immobilization of peroxidases from turnip roots. The enzyme was immobilized in the form of calcium alginate beads for phenol removal. The enzyme activity of the immobilized enzyme was checked by using the Guaiacol assay in a similar way as it was performed for the free enzyme extract. It was observed that the immobilised enzyme had similar enzyme activity as compared to the free enzyme. The immobilised enzyme could be used repeatedly for the removal of phenol from industrial effluent. There was above 90% removal of phenol from industrial effluent using the immobilized white radish peroxidase. Thus, the activity of the immobilised peroxidase enzyme was found to be promising for effective phenol removal**.**

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