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Use of Different Plant Derived Peroxidases for the Removal of Phenol from Water

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The aim of this study is to investigate alternative vegetal enzymatic phenol removal systems and to compare the strength of the determined enzymes in terms of removal phenol. In addition, the utilization of these enzymes in industry is discussed. For this purpose, some form of vegetal milk that enables the enzymes to be protected in a safe matrix is used. Peroxidase enzymes from *Ficus caricas*, *Ficus elasticas* and maize milk are partially purified 11.33, 12.92 and 8.11 fold, respectively, by the ammonium sulfate precipitation technique. The purified maize milk peroxidase removed all the phenol residues present in the water and *Ficus caricas* and *Ficus elasticas* removed 95.53 and 98.21 % of the phenol, respectively. As a result of this investigation, it is seen that peroxide based enzymes in vegetal milk matrix of *Ficus caricas*, *Ficus elasticas* and maize plants successfully remove phenol from water. The phenol removal effectiveness of maize is higher than that of ficus caricas and ficus elasticas peroxidases. The results of the analyses indicate that the use of peroxidases may find themselves a suitable area of application in the removal of phenol from water.

Key Words: Enzymes, Environmental biotechnology, Plant, Water.

INTRODUCTION

Phenol and phenolic compounds are ubiquitous pollutants which come to the natural water resources from the effluents of a variety of chemical industries such as cool refineries, phenol manufacturing, pharmaceuticals and industries of resin, paint, dying, textile wood, petrochemical, pulp mill as well as iron and steel manufacturing[1-3].

Phenol, present in the water, is a chemical substance that causes serious threat concerning people's health and the environment. Wastewaters of settlement areas that are not refined or are partially refined directly pollute the drinking waters and the environment. Phenolic substances must be removed from accessible potable water in order to preserve public and environmental health[2,3].

Peroxidases (EC 1.11.1.7, *etc*.) belong to a large family of enzymes that are ubiquitous in fungi, plants and vertebrates. These enzymes oxidize several substrates in the presence of hydrogen peroxide[4]. Peroxidase is one of the key enzymes controlling plant differentiation and development. It is known that this enzyme participates in the construction, the rigidification and the eventual lignification of the cell walls, in the biosynthesis of H_2O_2, in the protection of plant tissues from damage and infection by pathogenic microorganisms and in wound healing[5].

So far, peroxidases have been isolated from *Coprinus cinereus*, *Co-prinus macrorhizus*, *Artromyces ramosus* and soybean and they have been used for the removal of phenol from wastewater[6].

Horseradish peroxidase is a common source of the peroxidase enzyme[7]. They are cultivated generally in small quantities, are propagated through root cutting, thus making it difficult to scale up the production. The limited availability of the horseradish root extract coupled with the shortage of the enzyme has created a very expensive market for such enzymes[8].

Plant peroxidases can catalyze the oxidative coupling of phenolic compounds using H_2O_2 as the oxidizing agent. The reaction is a three-step cyclic reaction[9]. AH is phenolic compound[9].

(i) $\underset{\text{Native state}}{HRPC[(Fe(III))Porph^{2-}]^{+}} + H_2O_2 \rightarrow$

$$HRPC[(Fe(IV)=O)Porph^{\bullet -}]^{\bullet +} + H_2O$$

Compound **I**

(ii) $HRPC[(Fe(IV)=O)Porph^{\bullet -}]^{\bullet +} + AH \rightarrow$

$$HRPC[(Fe(IV)=O)Porph^{2-}] - H^{+} + A^{\bullet}$$

Compound **II**

(iii) $HRPC[(Fe(IV)=O)Porph^{2-}] - H^{+} + AH \rightarrow$

$$HRPC[(Fe(III)Porph^{2-}]^{+} + H_2O + A^{\bullet}$$

This work presents the use of various enzymes for phenol removal from water. The peroxidase enzymes partially purified from maize and *Ficus caricas* and *Ficus elasticas* milk were used for this purpose. The sources of peroxidase are available in abundance in Turkey. However, they are seasonal plants, thus the use of their milk is very important to keep their stability during storage. Peroxidase activities of the enzymes were determined by the ABTS chromogenic substrate method. Their activities indicating their phenol removal performance were determined in the presence of hydrogen peroxide and potassium ferricyanide. Following the determination of this activity, the enzymes are used for the removal of phenol from water.

EXPERIMENTAL

All chemicals are commercially available and are of analytical grade. Chemicals and phenol were obtained from Sigma Corp. Peroxidases were prepared and partially purified in our laboratory.

Ficus caricas, *Ficus elasticas* and maize milk were used as the source of the peroxidase enzyme. These plants were damaged with a knife and the oozing milk was collected in a test tube. The milk was stored in the freezer (-20 ºC) until they were used.

Purification steps

Homogenization: Plant milk was used as the raw material for obtaining the peroxidase enzyme. They were collected from the flora of Turkey in spring time. The milk was drawn from cut branches of the plants and was collected in glass containers. Its gummy phase was removed by centrifugation and the supernatant was used.

Ammonium sulfate precipitation: These supernatant samples were brought to 60 % saturation with solid ammonium sulfate under continuous stirring. After 1 h, the solution was centrifuged at 35000 rpm for 0.5 h and the pellet was discarded. Additional ammonium sulfate was added to the clear supernatant to give 75 % saturation and it was stirred for 1 h. After doing the activity assay, the amount of enzyme in the precipitates was determined. Latex homogenization, centrifugation and dialysis were carried out at 4 ºC.

Measurement of peroxidase activity: Peroxidase activity assay was carried out following the procedure of Shindler. This method is based on the oxidation of 2,2'-azino-*bis*(3-ethylbenzthiazoline-sulfonic acid) diammonium salt (ABTS) as a chromogenic substrate, by means of H_2O_2 and the absorbances of the resultant coloured compounds were measured at 412 nm. For this purpose, 2.8 mL of 1 mM ABTS in 0.1M phosphate buffer pH: 6.8 was mixed with 0.1 mL of enzyme in 1 mM phosphate buffer, pH: 6.8 and 0.1 mL of 3.2 mM H_2O_2 solution. The absorbance was measured at 412 nm as a function of time for 1-2 min.

$$H_2O_2 + \underset{\text{Reductive form}}{\text{ABTS}} \xrightarrow[\text{PO}]{} OH_2 + \underset{\text{Oxidative form}}{\text{ABTS}}$$

During the calculation of the purification rate of PO (peroxidase), one unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 mmol of ABTS min^{-1} at 293 K[10].

Determination of the protein content: The protein concentration was determined according to Bradford's method using bovine serum albumin as a standard[11].

Reaction mixture: All of the reactions with peroxidases were carried out at 25 ºC in 100 µL (5 µg/mL) phenol solution containing 50 mL H_2O_2. 100 µL purified peroxidase enzyme solution was placed in the reaction tubes. The reaction tubes were incubated under aerobic conditions. After 5 min, the samples were analyzed for residual phenol.

Estimation of the amount of phenol: The amount of residual phenol was determined after treating the water using different peroxidases. The water containing (5 µg/mL) phenol was treated with different kinds of peroxidases in the presence of hydrogen peroxide. 100 µL of peroxidase treated sample was added to 2.5 mL of 0.5N ammonium hydroxide to adjust the pH to 7.9, 1 mL of potassium ferricyanide was added to this mixture and the absorbance was read at 550 nm. A standard curve was created in a range of 0-500 µg phenol.

RESULTS AND DISCUSSION

Peroxidase enzyme from maize milk was partially purified; approximately 8.11 times by the use of the ammonium sulfate reagent technique and the results are presented in Table-1.

Peroxidase enzyme from *Ficus caricas* milk was partially purified; approximately 11.33 times by the use of the ammonium sulfate precipitation technique and the results are presented in Table-2.

Peroxidase enzyme from ficus elasticas milk was partially purified; approximately 12.92 times by the use of the ammonium sulfate reagent technique and results are presented in Table-3.

TABLE-1
PROPERTIES OF THE PARTIALLY PURIFIED PEROXIDASE ENZYME FROM MAIZE MILK

	Volume (mL)	Activity (EU/mL)	Total activity EU	Total activity %	Protein (mg/mL)	Specific activity (EU/mg)	Purification fold
Homogenate	10	0.900	9.00	100.00	0.595	1.51	–
After purification with 75 % ammonium sulfate reagent	5	0.980	4.90	54.44	0.080	12.25	8.11

TABLE-2
PROPERTIES OF THE PARTIALLY PURIFIED PEROXIDASE ENZYME FROM *ficus caricas* MILK

	Volume (mL)	Activity (EU/mL)	Total activity EU	Total activity %	Protein (mg/mL)	Specific activity (EU/mg)	Purification fold
Homogenate	10	1.52	15.20	100.00	0.593	2.56	–
After purification with 75 % ammonium sulfate reagent	5	1.77	8.85	58.22	0.061	29.01	11.33

TABLE-3
PROPERTIES OF THE PARTIALLY PURIFIED PEROXIDASE ENZYME FROM *Ficus elasticas* MILK

	Volume (mL)	Activity (EU/mL)	Total activity EU	Total activity %	Protein (mg/mL)	Specific activity (EU/mg)	Purification fold
Homogenate	10	0.251	2.51	100.00	0.308	0.814	_
After purification with 75 % ammonium sulfate reagent	5	0.800	4.00	159.36	0.076	10.52	12.92

The graph of mg of phenol response to 550 nm absorbance was displayed (Fig. 1) when using the standard phenol solution. Then the standard calibration chart was used to estimate the quantity of phenol in water.

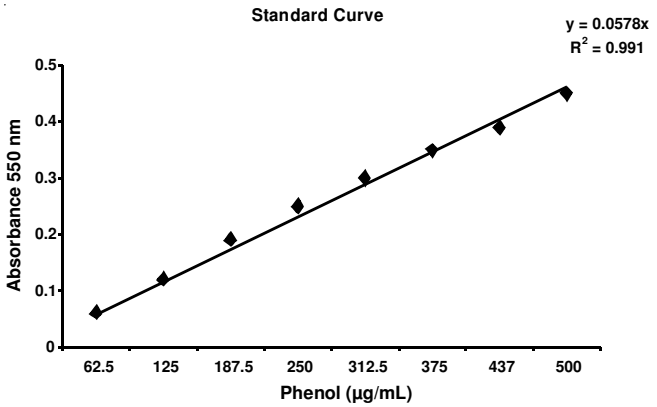

Fig. 1. Standard curve for the determination of the amount of phenol

Table-4 shows the difference between the phenol removal performances using three different plant peroxidases.

TABLE-4
PHENOL REMOVAL PERFORMANCE OF THE LATEX PROTEASES

	Phenol residue (%)	Phenol removal (%)
Maize milk	Not detected	100
Ficus caricas milk	4.46	95.53
Ficus elasticas milk	1.78	98.21

Singh and Singh[2] used an enzymatic method for the removal of phenol from industrial wastewater. Using native and immobilized turnip peroxidase, they were removed with a performance of 81 and 95 % phenol, respectively[2]. Partially purified peroxidase from horseradish root was used for removing the phenol through the precipitation following polymerization and it was determined that this process removed (95-99 %) phenol successfully from water[2,12].

In this study, the peroxidases partially and simply purified with ammonium sulphate (Tables 1-3). The purified maize milk peroxidase removed all the phenol residues in the wastewater; while ficus caricas and *Ficus elasticas* removed 95.53 and 98.21 %, respectively. It is observed that the phenol removal strength of the peroxidase obtained from maize is higher than that of *Ficus caricas* and *Ficus elasticas* peroxidases. Thus, the result clearly shows that the maize peroxidase removed more phenol than the *Ficus caricas* and the *Ficus elasticas* peroxidases (Table-4).

Extraction, adsorption with active charcoal, flowing distillation, oxidation with chemical or bacterial techniques have been used for removing phenols from industrial wastewater. Chemical oxidation with chlorine is not preferred due to the fact that it causes toxic chlorophenol compounds. These techniques have high cost and they require additional high purification. This pioneered the need to seek new alternative methods for the removal of phenol from wastewater[13-16].

Accordingly, there is a necessity for abundant and relatively inexpensive source of peroxidase in the market place[17]. The results show why these plant peroxidases were selected specifically for this purpose. The plant milk has more peroxidase activity among all parts of the plants. In addition, they can easily be cultivated in almost all locations and climates in the world.

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