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Oxidation of Electron-rich Pollutants Using Peroxidase from Fenugreek Waste

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Authors' contributions

This work was carried out in collaboration between all authors. Author MA designed and performed the experiments and wrote the first draft of the manuscript. Author UM conceptualized the study and managed the statistical analyses of the study. Author HD managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To assess the capacity of peroxidase from non-edible fenugreek waste for removal of phenol, a model electron-rich pollutant, from synthetic wastewater.
Study Design: This study involved one-factor-at-a-time analysis. Reaction parameters such as pH of reaction mixture and concentrations of phenol and hydrogen peroxide were optimized one at a time.
Place and Duration of Study: Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Mumbai between 2014 and 2015.
Methodology: The oxidative removal of the model pollutant, phenol, was assessed at ambient temperatures. The pH as well as concentrations of phenol and hydrogen peroxide were varied to optimize each reaction parameter. Each parameter was optimized singly while maintaining the other parameters constant.
Results: The maximum activity (in guaiacol units) of peroxidase was observed at pH 6.0 in the presence of 6 mmol L^{-1} hydrogen peroxide. The removal of phenol achieved was maximum, 64.29% in 24 h, when the initial phenol concentration was 2 mmol L^{-1}. Phenol removal proceeded through

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oxidative polymerization and settling of flocs.
Conclusion: The non-edible waste from fenugreek is a rich source of crude peroxidase from which the enzyme can be easily extracted. The use of vegetable waste as a source of a bioremediative enzyme is the primary focus of this work. This crude enzyme has potential for use in wastewater treatment by the oxidative degradation of electron-rich pollutants, such as phenols, amines, and azo dyes.

Keywords: Fenugreek; peroxidase; enzymatic wastewater treatment; bioremediation; electron-rich pollutants.

1. INTRODUCTION

Peroxidases are enzymes classified as "oxidoreductases" [1]. These enzymes are highly conserved and are widely distributed across kingdoms in the living world. They are found in bacteria, fungi, plants, and animals [2]. These enzymes have been conserved because they protect organisms from the oxidative damage caused by the oxygen-rich atmosphere [3]. In plants, peroxidases have numerous physiological roles, including the lignification of cell walls and metabolism of indole-3-acetic acid [1,4]. Enzymes are being explored for their potential as catalysts in various fields. Enzymatic treatment is considered as an effective alternative to conventional effluent treatment methods. They represent "green chemistry" in effluent treatment [5,6]. Several enzymatic reactions typically occur at ambient temperatures. These reactions often require near neutral pH and low reactant concentrations [7]. Hence, the use of enzymes enables working under mild reaction conditions [8]. Oxidative enzymes like peroxidases and laccases are particularly important in the oxidative degradation of electron-rich pollutants because of their wide range of substrates. Peroxidases, such as horseradish peroxidase (EC 1.11.1.7), require hydrogen peroxide (H_2O_2) as the cosubstrate [9], whereas laccases (EC 1.10.3.2) require molecular oxygen [10]. However, the redox potential of peroxidases in the presence of H_2O_2 (oxidized peroxidase) is sufficiently high to enable it to accept electrons from a variety of electron-rich substrates. Peroxidases such as lignin peroxidase and horseradish peroxidase are stronger oxidizing agents than is laccase [10]. This makes peroxidases suitable for the oxidation of oxidizable, electron-rich pollutants, such as phenols, amines, azo dyes, and triarylmethane dyes, that are commonly present in effluents from various industries.

In this study, we have selected phenol as a representative of electron-rich pollutants. Phenols and their derivatives are present in effluents from industries manufacturing and processing paper, textiles, plastics, and dyes. Phenol is a pollutant associated with anthropological activities including paper manufacture, coal mining, and wood processing. Natural sources of phenol include forest fires and natural decay of lignocellulosic material. Phenols impart an undesirable odor and taste to water, which are perceivable even at low concentrations. Phenols are suspected to show carcinogenic activity in humans and other organisms.

Phenol and its derivatives are considered toxic because they can affect the central nervous system, irritate mucus membranes, and cause bleaching and erosion of skin.

Therefore, the permissible limit of phenols is 1–2 µg L^{-1} for drinking water [11]. Moreover, phenols can adversely affect several organisms that inhabit the water bodies. Hence, the removal of phenols from effluents, before they meet natural water bodies, is necessary. The conventional methods of removal phenols from effluents include advanced oxidation processes, such as electro-oxidation, coagulation, and the Fenton process along with its several variations [12]. Although these methods effectively remove phenols from effluents, some of these methods, such as electro-oxidation require sophisticated, expensive equipment [13]. The Fenton process, often used for its cost-effectiveness and ease of handling also has certain drawbacks such as a low operational pH and the need for replenishing iron salts to sustain the oxidation reaction [13]. In this study, we investigated phenol removal using peroxidase enzyme. Peroxidases can oxidize electron-rich substrates, such as phenols [12,14], and prepare the phenol molecules for polymerization [6]. Consequently, molecules like phenol form a water insoluble, large molecular weight polymer that forms a precipitate and can be separated by filtration [15-17].

Fenugreek seeds and dried leaves have been used for centuries as condiments and spices [18]. The leaves and tender stems are commonly used as a vegetable. The seeds have been reported to contain peroxidase [19]. We found that the non-edible stems and roots (vegetable waste) of fenugreek plants are also rich in peroxidases. The present study investigated the oxidative removal an electron-rich pollutant, such as phenol, using peroxidase extracted from fenugreek vegetable waste. The waste constitutes a large fraction of the fresh weight of fenugreek plants. Using vegetable waste as the source of enzyme adds to the utility of this plant, without adversely affecting the edible yield.

2. MATERIALS AND METHODS

2.1 Chemicals

Phenol was obtained from Merck, India Ltd. (Mumbai) Guaiacol (2- methoxy phenol) was purchased from Sisco Research Laboratories, India. Hydrogen peroxide was purchased from Qualigens Fine Chemicals, Navi Mumbai, India. Chemicals for buffer preparation, such as Potassium dihydrogen phosphate, KOH, Citric acid, and Trisodium citrate, were purchased from S.D. Fine Chemicals (Boisar), India, while Tris–HCl was purchased from Loba Chemie, Mumbai.

2.2 Enzyme Extraction

Vegetable waste of fenugreek plants was collected from the local vegetable market. The roots and stems were washed thoroughly with tap water to remove the adhering soil, laid out on sheets of blotting paper, and air dried at 30℃. The vegetable waste was divided into 5 sets depending on the source (vendor) from which the waste was obtained. The fresh weight of each set was recorded. The plant material was macerated in a pre-chilled mortar and pestle with an appropriate volume of chilled extraction buffer (0.1 mol L^{-1} KH2PO4–KOH, pH 5.8) to obtain a material to liquor ratio of 1:10. The suspension was centrifuged at 2500 rpm (1610 ×g) for 10 min. The supernatant was used as the crude enzyme extract.

2.3 Activity of Peroxidase Extract

The activity of the crude peroxidase extract was estimated using the method of [20] with guaiacol as a substrate. One unit (1 U) was defined as the amount of enzyme that converts 1 µmol L^{-1} of substrate into product in 1 min. The activity (in U mL^{-1}) of the crude peroxidase was calculated using the following formula:

$$\text{Activity (U mL}^{-1}) = \Delta \text{ Absorbance min}^{-1} \times \text{FV.EV}^{-1} \times \text{dilution. } (\varepsilon 470)^{-1}$$

where FV is the final volume of the reaction mixture (mL), EV is the volume of crude enzyme (mL), and ε470 is the molar absorptivity of guaiacol (mL $\mu mol^{-1}.cm^{-1}$).

2.4 Enzymatic Removal of Phenol

The removal of phenol was assessed by measuring the initial and final absorbance of the reaction mixture at 270 nm [21,22], using a JASCO V-530 spectrophotometer, after incubation for 24 h. The absence of interference by nucleic acids was ascertained by measuring the spectra of the enzyme extract in the absence and presence of phenol. All phenol removal experiments were performed at ambient temperatures (30 ± 2℃). A negative control reaction mixture, which did not contain crude enzyme was prepared to verify that phenol removal occurred because of the crude peroxidase. The percentage removal of phenol was calculated using the following formula:

$$\% \text{ Decolorization} = (Ai - Af) \times Af \times 100$$

where Ai and Af represent the initial and final absorbance, at 270 nm, of the reaction mixture.

2.5 Optimization of Reaction Conditions

2.5.1 Effect of pH

The pH of the reaction mixture was varied between 3.0 and 8.0 using a variety of buffers in the reaction mixture. The pH range 3.0–5.0 was obtained using 0.1 mol L^{-1} citrate buffers. Phosphate buffers were used to obtain the pH range 6.0–8.0 (0.1 mol L^{-1}). The reaction mixture contained an initial phenol concentration of 2 mmol L^{-1}, approximately 0.4 ± 0.01 U of enzyme, and 1.0 mmol L^{-1} of H_2O_2 in addition to the buffer for each respective pH.

2.5.2 Phenol concentration

The initial concentration of phenol was varied between 1 and 4 mmol L^{-1}. The other components of the reaction mixture were as previously mentioned. The optimization of initial phenol concentration was performed at pH 6.0

because the enzyme activity was maximum at pH 6.0. A control that did not contain phenol in the reaction mixture was maintained to check for the inadvertent presence of another electron-rich substrate in the reaction mixture.

2.5.3 Hydrogen peroxide concentration

The concentration of H_2O_2 used to initiate the reaction was varied between 1 and 10 mmol L^{-1}. The other components of the reaction mixture are as previously mentioned. The optimization of initial phenol concentration was performed at pH 6.0. A control that did not contain H_2O_2 was maintained to account for spontaneous oxidation of phenol by enzymes other than peroxidases.

2.6 Statistical Analysis

All experiments were performed using triplicates. All data are reported as mean ± standard deviation (SD). All optimizations were performed per the one-factor-at-a-time analysis design. The effects of varying different reaction were compared using ANOVA and Duncan's multiple range test (DMRT). Categories that have been assigned the same alphabet are not significantly different from each other at $P = .05$.

3. RESULTS

3.1 Activity of Enzyme Peroxidase

The activity of the crude peroxidase extract in terms of guaiacol units was 2.01 ± 0.06 U mL^{-1}. Considering that the crude extract was prepared from 1.0 g of fresh fenugreek waste, on average, the peroxidase content of the fenugreek waste is 20.1 U g^{-1}. Various dilutions of the crude extract were used in different experiments.

As illustrated in Fig. 1, the highest activity (2.10 U mL^{-1}) was observed at pH 6.0. The maximum activity of the crude peroxidase is observed between pH 5.0 and pH 7.0. We used pH 6.0 as the optimum pH while optimizing the other reaction parameters.

3.2 Effect of Hydrogen Peroxide on Peroxidase Activity

As depicted in Fig. 2, the activity of crude fenugreek peroxidase at different concentrations of the cosubstrate H_2O_2. The highest activity of the extract is obtained at pH 6.0. An excess of H_2O_2 has been reported to inhibit the activity of plant peroxidases [1]. This effect is observed in Fig. 2.

3.3 Removal of Phenol

As seen in Fig. 3, the highest removal of phenol was observed when the initial concentration was 2 mmol L^{-1}. The removal of phenol decreases progressively with an increase in the initial concentration of phenol. However, the removal of phenol at higher initial concentrations of phenol was approximately 50%. The enzymatic oxidation method has potential application as a pretreatment method for phenol removal because it may effectively reduce the load of phenol (pollutant) before further processing.

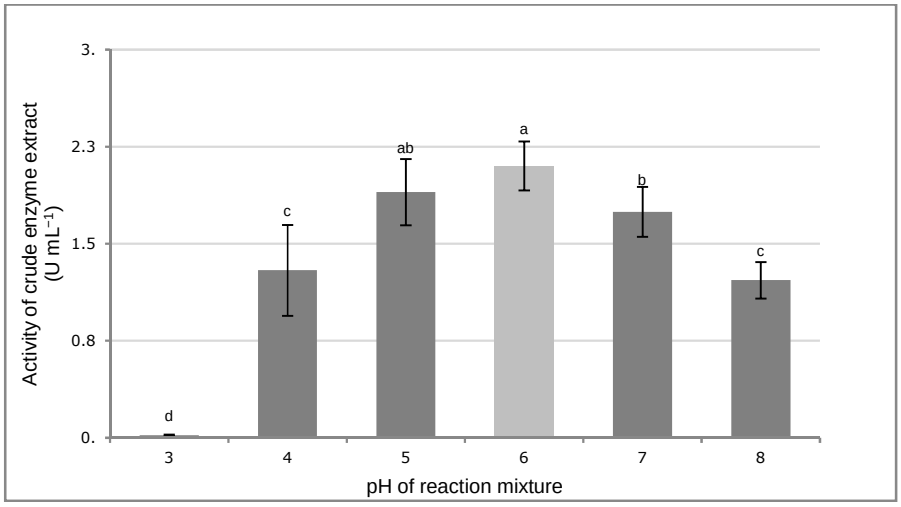

Fig. 1. Guaiacol activity of crude peroxidase extracts of fenugreek at different pH, based on the method of Kim and Yoo (1996)

P = .05; Mean ± SD = Mean values ± Standard deviation (n = 5)

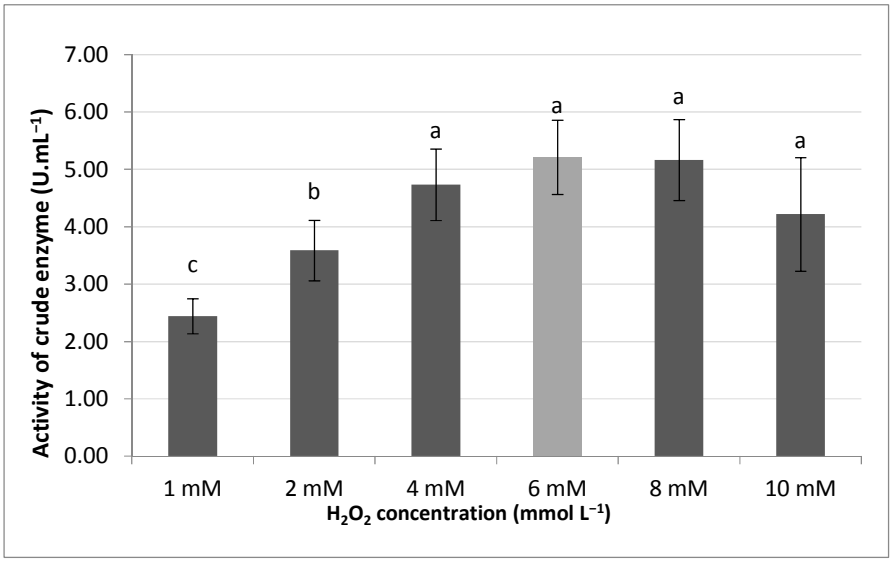

Fig. 2. Activity of crude peroxidase at different concentrations of hydrogen peroxide (pH 6.0)
P = .05; Mean ± SD = Mean values ± Standard deviation (n = 5)

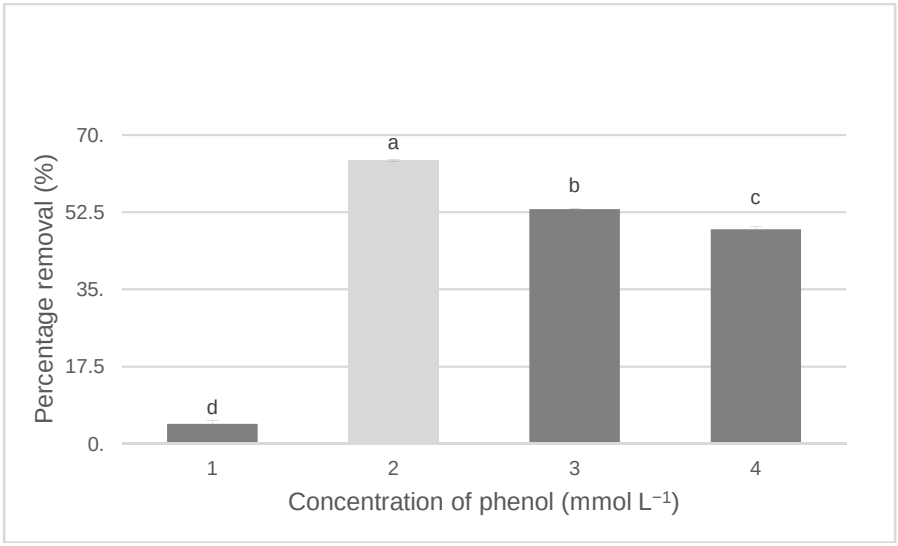

Fig. 3. Phenol removal by crude peroxidase over 24 h (pH 6.0 and 6.0 mmol L^{-1} H_2O_2)

4. DISCUSSION

The activity of peroxidase was highest at pH 6.0 (Fig. 1). The formation of phenolic polymers is not favored at very low pH [23] (Fig. 5). The oxidation of phenols by oxidative enzymes also leads to polymer formation. Therefore, the oxidation of phenols by the enzyme peroxidase is higher at pH 6.0 than at pH 3.0 and 4.0. Although the polymerization of phenols also occurs under alkaline conditions (Fig. 4), the activity of peroxidase is significantly reduced above 6.0. Hence, the pH of the reaction mixture was maintained at pH 6.0 for all further reactions.

Phenols tend to polymerize on oxidation and form flocs. The flocs can be separated from solution by simple settling or filtration [6]. Hence oxidative removal is a method of choice for pollutants such as phenols. The enzymatic method of phenol removal may be an effective pretreatment method to reduce the pollutant load of wastewater.

The oxidation of phenol by peroxidase requires H_2O_2 as a cosubstrate. In fact, increasing the concentration of H_2O_2 does increase the rate of the oxidation reaction (Fig. 2). However, an excess of H_2O_2 has been reported to inhibit the activity of oxidative enzymes, such as peroxidases [1,24]. This effect is depicted in Fig. 2. The activity of the crude enzyme increases steadily until the concentration of H_2O_2 reaches 6 mmol L^{-1}. Thereafter, the activity of crude peroxidase decreases steadily above 6 mmol L^{-1}. Therefore, for the phenol-removal experiment, the concentration of H_2O_2 was maintained at 6 mmol L^{-1}.

One of the factors that required further optimization is the reaction time. The time required for a reaction using a highly purified form of an enzyme like peroxidase would be considerably more rapid. Decolorization reactions using pure enzymes, such as standard horseradish peroxidase, have been reported to reach completion in 5 min [25]. The phenol removal was measured after 24 h. The removal of phenolic pollutants using conventional techniques, such as electro-oxidation and Advanced Oxidation Processes, typically occurs in 0.5 to 2 h. Enzymatic reactions for the removal of pollutants involving pure enzymes reach completion within 5 to 30 min [26]. However, because we have used a crude enzyme extract in the current study, the contact time was significantly longer. Crude extracts often contain a consortium of enzymes. For instance, fenugreek roots contain ascorbate peroxidase [27], which has a considerably higher affinity for H_2O_2 than guaiacol peroxidase (k_m = 3 µM vs. k_m = 50 µM), which is the major enzyme present in the extract [4,28]. Thus, the reaction times with crude extracts are not as short as those with purified enzymes. The use of crude peroxidase achieved 64.9% removal of phenol in 24 h. The proposed method is cost effective primarily because of the use of a crude enzyme extract. The primary expenditure associated with the use of enzymes is that of purification. The use of a crude enzyme, obtained from vegetable waste, considerably reduces the cost of using enzymes.

The novelty of the present study lies in the source of the enzyme peroxidase. The non-edible parts of the fenugreek plant are the source of peroxidase. Considering that these parts are usually disposed as waste, acquiring the source does not involve expenditure. Extraction the crude enzyme is also relatively simple and does not require either specialized equipment, fine chemicals, or skill. The crude enzyme could engender a removal of 64.9% phenol when the initial concentration of phenol was 2 mmol L^{-1}.

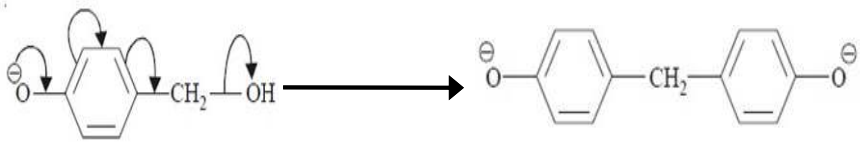

Fig. 4. Oxidation of phenol under alkaline conditions
(Source: Mukherji, 2012)

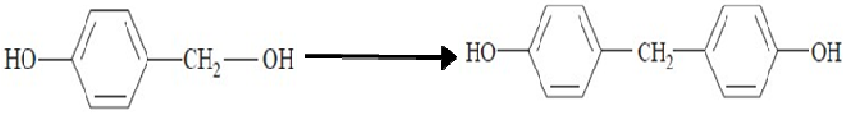

Fig. 5. Oxidation of phenol under acidic conditions
(Source: Mukherji, 2012)

The phenol-removal process can be made more efficient, in terms of enzyme usage, by immobilizing the enzyme in the form of beads or through adsorption on to a support [29,30]. Adsorption of enzymes to the supporting material is subject to reaction conditions. Drastic reaction conditions, such as very high or low pH, high temperature, and high substrate concentrations, can cause the desorption of the enzyme from the support. Bead formation or encapsulation of enzymes is likely to provide greater reusability of the enzymes.

Polymerized phenols produced because of enzyme-mediated oxidation have been reported to have applications such as photolithography (integrated circuits), manufacture of rechargeable battery material, and non-linear optics [31]. Optimizing the enzyme-mediated removal of phenols to obtain the desired degree of

polymerization may enable the use of removed phenol for the aforementioned applications, after appropriate processing.

5. CONCLUSION

The non-edible stems and roots of fenugreek plants are a rich source of enzyme peroxidase. The crude peroxidase enzyme extracted from fenugreek waste can oxidize electron-rich substrates, such as phenols, from synthetic wastewater. In the present study, crude peroxidase could remove between 50%–64% of phenol when the initial concentration was 2–4 mmol L^{-1}. Fenugreek peroxidase may have potential application for the preliminary treatment of wastewater. Additional experiments are required to achieve and optimize reusability of the crude enzyme in treatment of wastewater.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Veitch NC. Horseradish peroxidase: A modern view of a classical enzyme. Phytochemistry. 2004;65:249-59. DOI: 10.1016/j.phytochem.2003.10.022
2. Zámocký M, Hofbauer S, Schaffner I, Gasselhuber B, Nicolussi A, Soudi M, et al. Independent evolution of four heme peroxidase superfamilies. Arch Biochem Biophys. 2015;574:108-19.
3. Zamocky M, Obinger C. Molecular phylogeny of heme peroxidases. In: Torres E, Ayala M editors. Biocatalysis based on heme peroxidases. Heidelberg: Springer; 2010.
4. Penel C, Castillo FJ. Oxidoreduction at the plasma membrane: Relation to growth and transport. Crane FL, Morré DJ, Löw HE, editors. Boca Raton (USA): CRC Press; 1991.
5. Gitsov I, Simonyan A, Tanenbaum S. Hybrid enzymatic catalysts for environmentally benign biotransformations and polymerizations. ACS Polym. 2009;50(2):40.
6. Buchanan ID, Nicell, JA. A simplified model of peroxidase-catalyzed phenol removal from aqueous solution. J. Chem. Technol. Biotechnol. 1999;74:669-74. DOI:10.1002/(SICI)10974660(199907)74:7<669:AID-JCTB100>3.0.CO;2-Z
7. Nelson C, Cox M. Principles of biochemistry. 4th ed. New York: WH. Freeman; 2004.
8. Gioia L, Rodríguez-Couto S, Menéndez MDP, Manta C, Ovsejevi K. Reversible covalent immobilization of Trametes villosa laccase onto thiolsulfinate-agarose: An insoluble biocatalyst with potential for decoloring recalcitrant dyes. Biotechnol. Appl. Biochem. 2015;62(4):502-513.
9. Zille A, Ramalho P, Tzanov T, Millward R, Aires V, Cardoso MH, et al. Predicting dye biodegradation from redox potentials, Biotechnol. Progr. 2004;20:1588-92. DOI: 10.1021/bp049963i
10. Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammar B, Kirk TK. Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem. J. 1990;268 (2):475-80.
11. Bureau of Indian Standards. Indian standards for drinking water; 2009. Available:www.bis.org.in
12. Naghibi F, Pourmorad F, Honary S, Shamsi M. Decontamination of water polluted with phenol using Raphanus sativus root, Iran. J. Pharmaceut. Res. 2003;2(1):29-32.
13. Abo-Farah SA. Comparative study of oxidation of some azo dyes by different Advanced Oxidation Processes, Fenton, Fenton-like, photo-Fenton and photo-Fenton- like. J Am Sci. 2010;6:128-42.
14. Stanisavljević M, Nedić L. Removal of phenol from industrial wastewaters by horseradish (*Cochlearia armoracia* L.), Work. Liv. Env. Prot. 2004;2(4):345-49.
15. Kobayashi S, Higashimura H. Oxidative polymerization of phenols revisited. Prog. Polym. Sci. 2003;28(6):1015-48.
16. Gupta SK, Gupta SK, Hung YT. Treatment of pharmaceutical waste. In: Wang LK, Hung YT, Howard HL, Yapijakis C, editors. Waste treatment in the process Industries. New York: CRC Taylor and Francis; 2006.

17. Mukherji SM. Phenols. In: Organic chemistry, New Delhi: New Age International Publication. 2012;2.
18. Spices Board India. Fenugreek Booklet No. 482; 2012. Available:http://www.indianspices.com/html/spices_spfarm_fenu.html
19. Husain Q, Karim Z, Banday ZZ. Water Air Soil Pollut. 2010;212:319. DOI: 10.1007/s11270-010-0345-9
20. Kim YH, Yoo JY. Peroxidases production from carrot hairy root cell culture. Enzyme Microb Tech. 1996;18:531-53.
21. Zhang Q, Gao L, Guo J, Effects of calcination on the photocatalytic properties of nanosized TiO2 powders prepared by TiCl4 hydrolysis. Appl. Catal. B-Environ. 26:207-215.
22. Trinh DTT, Le STT, Khanitchaidecha W, Nakaruk A. Investigation of photocatalytic degradation of phenol using titanium dioxide particles J. Ind. Pollut. Contr; 2015.
23. Xavier JLN, Ortega E, Feraierra JZ, Bernades AM, Pérez-Herranz V. An electrochemical study of phenol oxidation in acidic medium. Int. J. Electrochem. Sci. 2011;6:622-36.
24. Hollmann F, Arends I. Enzyme initiated radical polymerizations. Polymers. 2012; 4:759-793. DOI: 10.3390/polym4010759
25. Maddhinni VL, Vurimindi HB, Yerramilli A. Degradation of azo dye with horseradish peroxidase (HRP). J Indian Inst Sci. 2006; 86:507-14.
26. Ambatkar M, Mukundan U. Enzymatic decolourisation of Methyl Orange and Bismarck Brown using crude peroxidase from *Armoracia rusticana*. Appl. Water Sci. 2015;5(4):397-406.
27. Elleuch A, Chaâbene Z, Grubb DC, Drira N, Mejdoub H, Khemakhem B. Morphological and biochemical behavior of fenugreek (*Trigonella foenum-graecum*) under copper stress. Ecotoxicol Environ Saf. 2013;98:46-53.
28. Chaurasia PK, Singh SK, Bharati SL. Study of peroxidase obtained from *Daucus carota* (carrot) juice extract J. Applicable Chem. 2013;2:1123-1131.
29. Adam W, Lazarus M, Saha-Moller C, Weichold O, Hoch U, Haring D, et al. Biotransformation with peroxidase. Adv Biochem Eng/Biot. 1999;63:73-107.
30. Kandelbauer A, Maute O, Kessler RW, Erlacher A, Gübitz GM. Study of dye decolorization in an immobilized laccase enzyme-reactor using online spectroscopy. Biotechnol Bioeng. 2004;87(4):552-563. DOI: 10.1002/bit.20162
31. Ayyagari M, Akkara JA, Kaplan DL. Characterization of phenolic polymers synthesized by enzyme-mediated reactions in bulk solvents and at oil-water interfaces. Mater. Sci. Eng. C. 1996;C4:169-73.

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