

Original Research Article

Oxidation of Electron-Rich Pollutants Using Peroxidase from Fenugreek Waste

ABSTRACT

Aims: To assess the removal of phenol from synthetic wastewater using peroxidase from non-edible fenugreek waste.

Study design: One-factor-at-a-time analysis of reaction parameters.

Place and Duration of Study: Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Mumbai between 2014 and 2015.

Methodology: The 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.

Results: The maximum activity (in guaiacol units) was observed at pH 6.0 in the presence of 6 mmol L⁻¹ hydrogen peroxide. The maximum removal of phenol (initial concentration 2 mmol L⁻¹) achieved was 64.29% in 24 h.

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.” (Veitch, 2004) These enzymes are highly conserved and are widely distributed across kingdoms in the living world. They are found in bacteria, fungi, plants, and animals (Zamocky and Obinger, 2010). These enzymes have been selected for by nature because they protected organisms from the oxidative damage caused by an oxygen-rich atmosphere (Zamocky and Obinger, 2010). In plants, peroxidases have numerous physiological roles, including the lignification of cell walls and metabolism of indole-3-acetic acid (Veitch, 2004, Penel and Castillo, 1991). Peroxidases can oxidize a variety of electron-rich substrates in the presence of hydrogen peroxide (Zille et al, 2004). 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. Phenols impart an undesirable odor and taste to water, which are perceivable even at low concentrations. Therefore, the permissible limit of phenols is 1–2 µg L⁻¹ for drinking water (Bureau of Indian Standards, 2009). Moreover, phenols are suspected carcinogens and can adversely affect humans as well as 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 (Naghibi et al, 2003). Although these methods effectively remove phenols from effluents, some of these methods, such as electro-oxidation require sophisticated, expensive equipment (Abo-Farah, 2010). The Fenton process, often used for its cost-effectiveness and ease of handling also has certain drawbacks such as a low optimum pH and the need for replenishing iron salts during the oxidation reaction (Abo-Farah, 2010). Enzymes are being explored as potential alternative means for oxidizing pollutants in effluents. They represent “green

strategies” of effluent treatment (Buchanan and Nicell, 1999; Gitsov et al, 2009). The use of enzymes permits working under mild conditions. For instance, several enzymatic reactions typically occur at ambient temperatures (Nelson and Cox, 2004). These reactions often require near neutral pH and low reactant concentrations. Oxidative enzymes like peroxidases and laccases are of particular interest for the oxidative degradation of electron-rich pollutants because of the wide range of substrates that these enzymes can oxidize. Peroxidases, such as horseradish peroxidase (EC 1.11.1.7), require hydrogen peroxide (H_2O_2) as the co-substrate, whereas laccases (EC 1.10.3.2) require molecular oxygen. However, the redox potential of peroxidases in the presence of H_2O_2 (oxidized peroxidase) is sufficiently 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 (Kersten et al, 1990) In our study, we extracted peroxidase from the non-edible stems and roots (vegetable waste) of fenugreek plants, which constitute a large fraction of their fresh weight. Fenugreek seeds and dried leaves have been used for centuries as condiments and spices (Spices Board of India, 2012). The leaves and tender stems are commonly used as a vegetable. By contrast, the vegetable waste is rich in peroxidases. Using vegetable waste as the source of enzyme adds to the utility of this plant without adversely affecting the edible yield.

2. MATERIAL AND METHODS

2.1 Chemicals used

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 KH_2PO_4 , 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 Crude 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 °C. The vegetable waste was divided into 5 sets. 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 ($0.1 \text{ mol L}^{-1} KH_2PO_4$ -KOH, pH 5.8) to obtain a material to liquor ratio of 1:10. The suspension was centrifuged at 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 Kim and Yoo (1996) with guaiacol as a substrate. One unit (1 U) was defined as the amount of enzyme that converts $1 \mu\text{mol L}^{-1}$ of substrate into product in 1 min. The activity (in U mL^{-1}) of the crude peroxidase was calculated using the following formula:

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

where FV is the final volume of the reaction mixture (mL), EV is the volume of crude enzyme (mL), and ϵ_{470} is the molar absorptivity of guaiacol ($\text{mL } \mu\text{mol}^{-1}.\text{cm}^{-1}$).

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, using a JASCO V-530 spectrophotometer, after incubation for 24 h. All phenol removal experiments were performed at ambient temperatures ($30 \pm 2 \text{ }^\circ\text{C}$). 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:

$$\% \text{ Decolorization} = (A_i - A_f) \times A_f \times 100$$

where A_i and A_f represent the initial and final absorbance, at 270 nm, of the reaction mixture.

88 2.5 Optimization of reaction conditions

89 2.5.1 Reaction mixture pH

90 The pH of the reaction mixture was varied between 3.0 and 8.0 using a variety of buffers in the reaction
91 mixture. The pH range 3.0–5.0 was obtained using 0.1 mol L⁻¹ citrate buffers. Phosphate buffers were
92 used to obtain the pH range 6.0–8.0 (0.1 mol L⁻¹). The reaction mixture contained an initial phenol
93 concentration of 2 mmol L⁻¹, approximately 0.4 ± 0.01 U of enzyme, and 1.0 mmol L⁻¹ of H₂O₂ in
94 addition to the buffer for each respective pH.

95 2.5.2 Phenol concentration

96 The initial concentration of phenol was varied between 1 and 4 mmol L⁻¹. The other components of the
97 reaction mixture were as previously mentioned. The optimization of initial phenol concentration was
98 performed at pH 6.0. A control that did not contain phenol in the reaction mixture was maintained to
99 check for the inadvertent presence of another electron-rich substrate in the reaction mixture.

100 2.5.3 Hydrogen peroxide concentration

101 The concentration of H₂O₂ used to initiate the reaction was varied between 1 and 10 mmol L⁻¹. The
102 other components of the reaction mixture are as previously mentioned. The optimization of initial phenol
103 concentration was performed at pH 6.0. A control that did not contain H₂O₂ was maintained to account
104 for spontaneous oxidation of phenol by enzymes other than peroxidases.

105

106 2.6 Statistical analysis

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

112

113

114 3. RESULTS AND DISCUSSION

115

116 The activity of the crude peroxidase extract in terms of guaiacol units was 2.01 ± 0.06 U mL⁻¹.
117 Considering that the crude extract was prepared from 1.0 g of fresh fenugreek waste, on average, the
118 peroxidase content of the fenugreek waste is 2.01 U g⁻¹. Various dilutions of the crude extract were
119 used in different experiments.

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

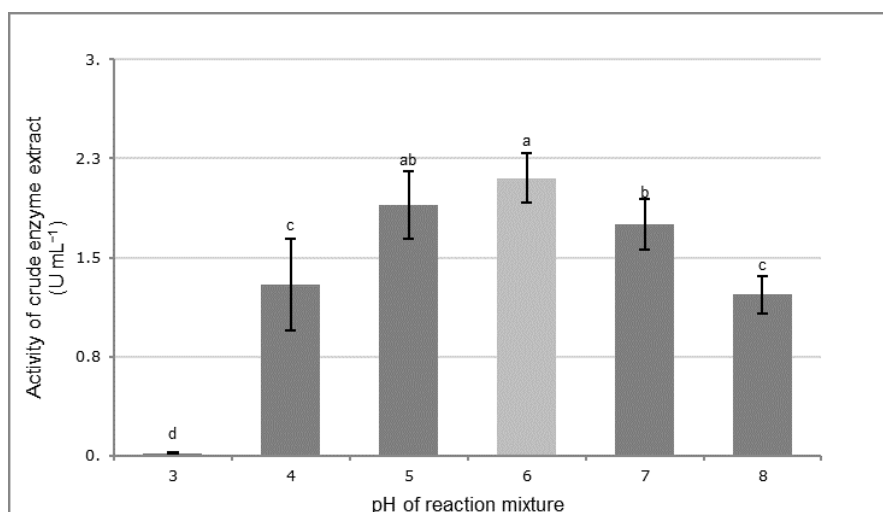


Fig. 1. Guaiacol activity of crude peroxidase extracts of *Trigonella foenum-graecum* at different pH

$P = .05$; Mean $\pm$ SD = Mean values $\pm$ Standard deviation ($n = 5$)

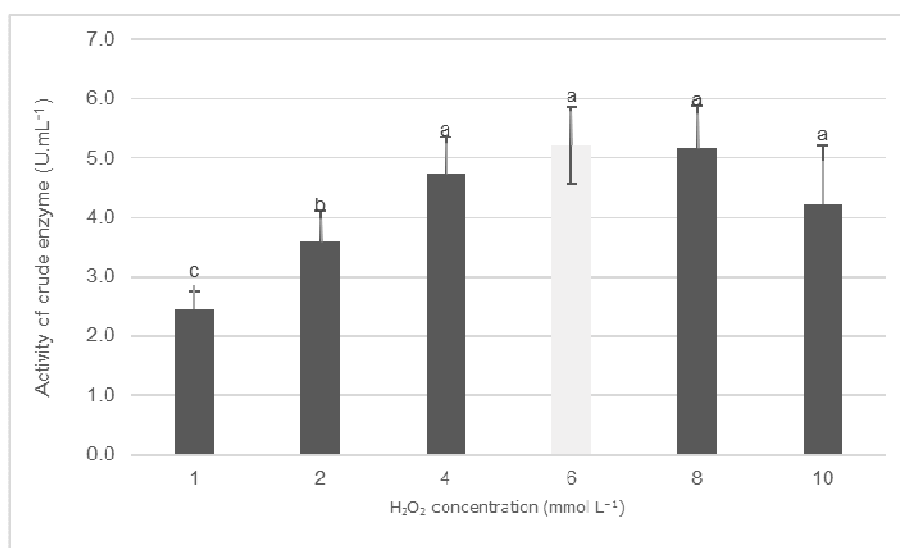


Fig. 2. Activity of crude peroxidase at different concentrations of hydrogen peroxide

$P = .05$; Mean $\pm$ SD = Mean values $\pm$ Standard deviation ($n = 5$)

As depicted in Fig. 2, the activity of crude fenugreek peroxidase at different concentrations of the cosubstrate H₂O₂. The highest activity of the extract is obtained at pH 6.0. An excess of H₂O₂ has been reported to inhibit the activity of plant peroxidases (Veitch, 2004). This effect is observed in Fig. 2.

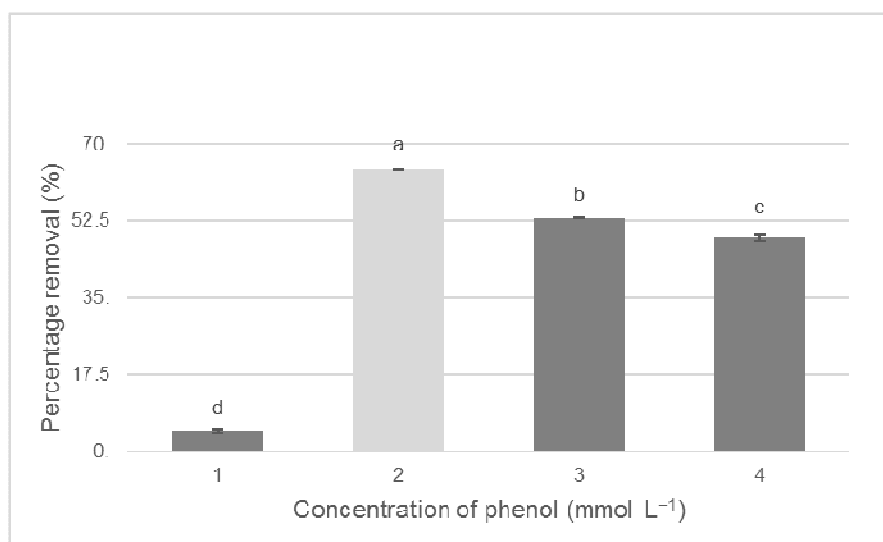


Fig. 3. Phenol removal by crude peroxidase over 24 h

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. These assays were performed using the optimum values of pH and concentration of H₂O₂.

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. 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.

Oxidative enzymes predominate bioremediation techniques. These enzymes can oxidize electron-rich substrates, such as phenols (Naghibi et al, 2003; Stanisavljević and Nedić, 2004), and prepare these molecules for polymerization (Buchanan and Nicell, 1999). Consequently, molecules like phenol form a water insoluble, large molecular weight polymer that forms a precipitate and can be separated by filtration (Kobayashi and Higashimura, 2003; Gupta and Gupta, 2006; Mukherji, 2012).

The formation of phenolic polymers is not favored at very low pH (Xavier et al, 2011). The oxidation of phenols by oxidative enzymes occurs by the same mechanism. Therefore, the oxidation of phenols by the enzyme peroxidase is higher at pH 6.0 than at pH 3.0 and 4.0. Furthermore, the activity of the crude peroxidase extract was highest at pH 6.0. Hence, the pH of the reaction mixture was maintained at pH 6.0 for all further reactions. The polymerization of phenols under alkaline and acidic conditions is depicted in Fig. 4 and Fig. 5, respectively.

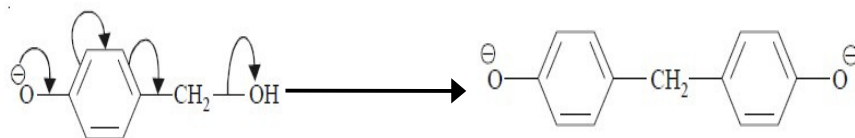


Fig. 4. Oxidation of phenol under alkaline conditions [Source: Mukherji S.M. (2012) *Organic Chemistry, Volume 2*]

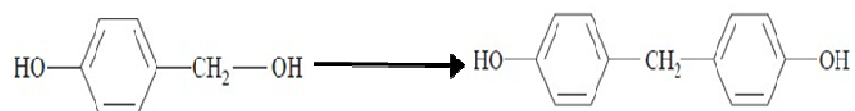


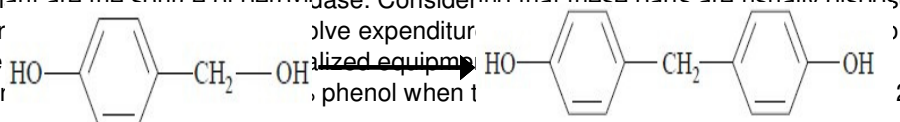
Fig. 5. Oxidation of phenol under acidic conditions [Source: Mukherji S.M. (2012) *Organic Chemistry, Volume 2*]

Phenols tend to polymerize on oxidation and form flocs. The flocs can be separated from solution by simple settling or filtration (Buchanan and Nicell, 1999).

Excess H_2O_2 has been reported to inhibit the activity of oxidative enzymes, such as peroxidases (Veitch, 2004; Hollman and Arends, 2012). 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} . For the phenol-removal experiment, the concentration of H_2O_2 was maintained at 6 mmol L^{-1} .

For the phenol-removal experiment, the pH and concentration of H_2O_2 were fixed at 6.0 and 6 mmol L^{-1} , respectively. The concentration of phenol was varied from 1–4 mmol L^{-1} . The reaction time was maintained at 24 h. The removal of phenolic pollutants using conventional techniques, such as electrooxidation and Advanced oxidation processes, are typically 0.5 to 2 h. However, because in the current study, we have used a crude enzyme extract, the contact time significantly longer. The use of crude peroxidase achieved 64.9% removal of phenol in 24 h. The present 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, acquire relatively simple enzyme could be a cost-effective method for phenol removal. The chemical structure of 4,4'-methylenebis(phenol) is shown below.



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 (Adam et al, 1999; Kandelbauer, 2004). 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.

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 (Maddhinni et al, 2006). However, a crude extract contains a consortium of enzymes, in addition to peroxidase, which may interfere with the interaction of the substrate and enzyme. Hence, reactions using crude peroxidase require longer durations of contact than those involving pure peroxidase.

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 (Ayyagari et al, 1996). 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.

4. 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. Additional experiments are required to achieve and optimize reusability of the crude enzyme in treatment of wastewater.

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