Bioremediation of Wastewater using Plant-based Peroxidase

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Abstract

This project aims to investigate the methods of treating wastewater produced by textile dyeing industries through the use of plant-based peroxidases, as well as create a viable treatment water treatment filter using easily accessible recyclable materials. In this study, chitosan beads were crosslinked in pH 7.0 phosphate buffer containing glutaraldehyde solution and used to immobilise horseradish peroxidase(HRP). This HRP was used to degrade phenolic dye (phenol red) commonly found in wastewater into harmless substances, reducing the overall toxicity of the wastewater and alleviating its harmful effects on the environment. To conduct the experiment, phenol red, a common indicator dye used in laboratories and a textile dye, was added to water to create simulated wastewater. Chitosan beads immobilised with HRP were used to treat the wastewater for a period of time. To analyse the concentration of dye remaining in the wastewater, a UV-Vis spectrophotometer was used to read the absorbance of light of the wastewater and the decolourisation efficiency of the immobilised HRP was determined. To determine the toxicity of treated wastewater, the wastewater was used to germinate mung bean seeds and the toxicity was determined by calculating the LC50 value of the results. Our findings showed that the chitosan-HRP beads made using HRP at concentration of 5µg/ml had the highest decolourisation efficiency.

1. Introduction

Dyes from the fashion industries are responsible for 17-20% of industrial water pollution (Kant, 2012). Factories get rid of wastewater through dumping it in nearby water bodies without processing it as this does not incur much cost. The World Bank identified 72 toxic chemicals originated from textile dyeing that may contribute to water pollution problems (Kant, 2012). Phenolic dyes are one of the toxic substances commonly used in the dye industry. Phenol red, also known as phenolsulfonphthalein, is a pH indicator dye, and is a commonly used acid-base

indicator (Held, 2018). It has been used as a pH indicator dye in tissue culture media for decades (Held, 2018). However, it is also a finishing textile dye, which enters water bodies through industrial effluents. Due to its toxic nature, phenol red present in drinking water can be harmful to human health (Alebachew, Yadav & Lokesh, 2017), and is able to inhibit seed germination in plants (Muscolo, Panuccio & Sidari, 2001). Hence, there is an imperative need for accessible methods to effectively treat wastewater, to reduce its harmful effects to the environment and to humans.

Enzymes are used in some industries to control and catalyse reactions in order to quickly and accurately obtain a final product (Martinez, 2018). According to Pomar et al.(2003), peroxidases are heme-containing enzymes that catalyse the one-electron oxidation of several substrates at the expense of hydrogen peroxide. They are a diverse group of oxidoreductases, which use hydrogen peroxide as an electron acceptor (Buchert & Autio, 2007) for oxidation of a wide variety of substrates.

To carry out our study on bioremediation of dye-contaminated water, we will use horseradish peroxidase (HRP) as it is able to catalyse the breakdown of phenolic dyes to produce harmless substances (Regalado et al., 2004). Peroxidases have been widely praised for its availability, affordability and efficiency in pollutant removal especially in dye removal (Mousa et al. 2011). Peroxidases from various plant sources like horseradish, turnip, tomato and soybean have been used in the removal of commercial dyes (Osuji et al., 2014). HRP specifically is well known for its effectiveness in degradation of industrial dyes, being widely used in textile industries in countries like Brazil where there is a growing market for this enzyme (Silva et al., 2011). Hence, it has the potential to be used in wastewater treatment. Building on previous research dedicated to the treatment of other industrial dyes in wastewater using HRP, we aim to determine the enzyme's effectiveness in treating phenolic dyes when it is immobilised onto chitosan beads, and hence study its potential in managing environmental pollution.

Something unique about our project is that we will build a simple column made out of common recyclable materials, packed with chitosan beads with immobilised HRP, to treat simulated wastewater containing phenol red as the contaminant. This will enable us to see whether an

effective water treatment system can be made from easily obtainable materials. The treated water will be used to germinate mung bean seeds to determine its toxicity.

2. Objectives and Hypothesis

Hypothesis

The horseradish peroxidase and peroxidases extracted from watermelon and lemon peels are effective in removing phenol red from simulated wastewater.

Objectives

1. Immobilise horseradish peroxidase onto chitosan beads.
2. Study the effectiveness of immobilised horseradish peroxidase and peroxidases extracted from watermelon and lemon peels in the removal of phenol red from simulated wastewater.
3. Determine the toxicity of the treated simulated wastewater by studying its effect on the germination rate of mung bean seeds.

3. Materials and Methods

Apparatus

Vortex mixer, pH indicator paper, 250ml/500ml/1L Beakers , Pipette, Spectrophotometer, 50ml Centrifuge Tube, Retort stand, 15ml and 50ml centrifuge tubes

Materials

Chitosan (Sigma-Aldrich 448869), Glutaraldehyde (Sigma-Aldrich G6257), Phenol red (Sigma-Aldrich P0290), Horseradish peroxidase (Thermo Scientific 31490), pH 7.0 phosphate buffer, 1M NaOH solution, Acetic acid solution , Ethanol, Deionised water, Mung bean seeds

Methods

(A) Preparation of Chitosan beads (Chagas, Torres, Silva & Correa, 2015)

5g of chitosan was dissolved in 250 ml of 3.0% (v/v) aqueous acetic acid solution in a 500ml beaker. Using a dropper, the chitosan solution was dropped into a 350ml aqueous 1 M NaOH solution and 125ml ethanol in a 1L beaker for the chitosan beads to precipitate immediately to form gelatinous beads. After hardening for 3h, the resulting beads were thoroughly washed with

deionised water until neutrality was reached. 20g of beads was then cross-linked by immersing and mixing the chitosan beads in a 500ml beaker with 50mM, pH 7.0 phosphate buffer containing 1% (v/v) glutaraldehyde solution for 30 minutes (or at 25ºC for 24h). The resultant beads were washed with deionised water to remove excess glutaraldehyde solution before use.

(B) Immobilisation of peroxidase onto crosslinked chitosan beads (Aybastier, Sxahin, Isxix Demir, 2011)

10ml of a peroxidase solution containing 100µg/ml of a specific activity 307U mg^{-1} of horseradish peroxidase (HRP) in pH 7.0 50mM phosphate buffer was added to 3g of chitosan beads in a 50ml plastic centrifuge tube. To make up the final volume to 25ml, phosphate buffer was added. This step was repeated but with HRP concentrations 75µg/ml, 50µg/ml, 10µg/ml and 0µg/ml. The mixtures were mixed on a mixer for 24h at 25ºC.

The resulting chitosan-HRP beads were filtered out for further use. To create the preserving solution for the beads, 0.8775g of sodium chloride and 1.2114g of trihydroxymethylaminomethane were mixed together in 100ml of water. The chitosan-HRP beads were stored in a refrigerator at 4ºC in the preserving solution when the beads were not used immediately.

(C) Treatment of simulated wastewater

600ml of phenol red solution with concentration of 2g/L was prepared using deionised water. This solution served as the simulated wastewater. 15ml plastic centrifuge tubes were filled with 1g of the chitosan-HRP beads, prepared with varying concentrations of HRP in (B), and 10 ml of the simulated wastewater. The centrifuge tubes were placed on a mixer for 1h at room temperature. Chitosan-HRP beads were filtered off from the treated wastewater using a filter funnel and filter paper. The absorbance readings at 480 nm of the filtered simulated wastewater samples were determined using a spectrophotometer as detailed in (D) to compute the decolourisation efficiency of the chitosan-HRP beads.

(D) Determination of decolourisation efficiency of chitosan-HRP beads

The absorbance at 480 nm of the treated wastewater samples was determined using a

spectrophotometer. To calculate the decolourisation efficiency, the absorbance readings of treated and untreated wastewater was inserted in the formula:

Decolourisation efficiency = $[(A_u-A_t)/A_u] \times 100\%$,

where A_u is the absorbance reading of the untreated solution and A_t is the absorbance reading of the treated solution.

A graph of decolourisation efficiency against HRP concentration was plotted to determine the optimal concentration of HRP for the treatment of wastewater.

(E) Toxicity Study of Treated Simulated Wastewater

To determine the toxicity of the treated simulated wastewater, 15 mung bean seeds were soaked in 5ml of each treated wastewater sample in a petri dish and allowed to germinate for 2 days. In 2 control setups, 15 mung bean seeds were soaked in 5ml of tap water and untreated simulated wastewater, and allowed to germinate. The germination rate in the control and test setups were compared to determine the toxicity of the treated wastewater samples whereby the germination rate in the test setups should have a higher germination rate. Afterwards, experimental procedures (B) to (F) were repeated 2 more times to check for consistency of the results.

(F) Preparation of Watermelon and Lemon Peroxidase (Mathkor, Hasan, & Daham, 2019)

If given additional time, we would also like to extract watermelon and lemon peroxidase by soaking lemon peels and watermelon rinds in 100ml of distilled water for 5 min, then ground the peels in water at a ratio of 1:3 (phosphate buffer pH7.0). Next, the peels and rinds would be blended separately in a blender for 5 min and the chunks of peels or rinds would be filtered away using a filter paper and filter funnel. The resulting filtrate was centrifuged at 3000g at 4℃ for 5 minutes and the resulting supernatant would be used as the source of peroxidase. Unfortunately, due to restrictions imposed by COVID-19, limited access to the laboratories prevented us from carrying out this part of our project.

4. Results and Discussion

A) Decolourisation Efficiency of Chitosan-HRP beads

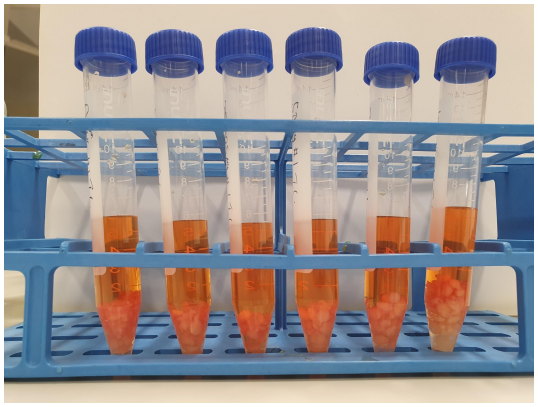


Fig. 1. Appearance of simulated wastewater before the treatment with chitosan-HRP beads made using HRP solutions with concentrations from left to right as follows: 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 5 µg/ml, 0µg/ml

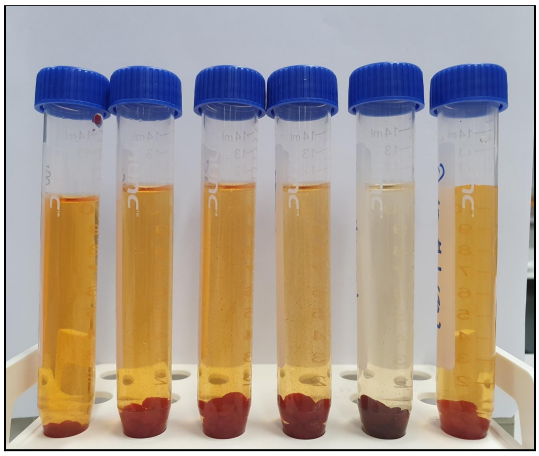
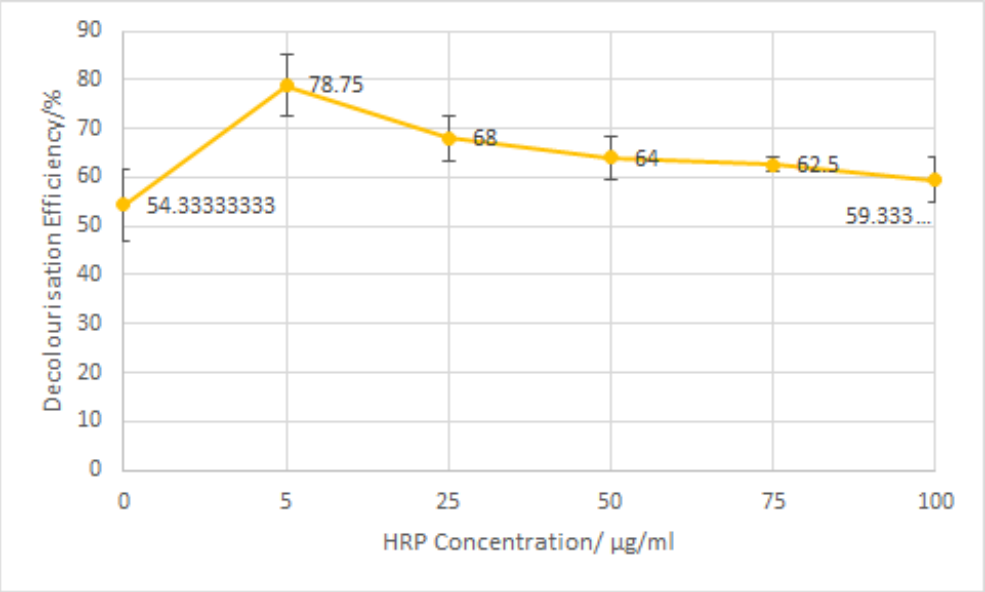


Fig. 2. Appearance of simulated wastewater after treatment with chitosan-HRP beads made using HRP solutions with concentrations from left to right as follows: 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 5 µg/ml, 0µg/ml

Fig. 3. Decolourisation efficiency of chitosan-HRP beads made with varying concentrations of HRP



p = 0.00001

n = 9

From Fig. 3, it can be seen that chitosan-HRP beads, made using HRP solution of 5ug/ml, has the highest decolourisation efficiency. Kruskal-Wallis test showed that using varying concentrations of HRP solutions to make chitosan-HRP beads, which were then used to treat the simulated wastewater affected the decolourising efficiency of the beads. The decolourisation efficiency of the beads is as follows: 5µg/ml, followed by 100µg/ml, 50µg/ml, 25µg/ml, and 75µg/ml.

B) Toxicity study of treated simulated wastewater

At the start of germination

Trial A

Trial B

Tap water control

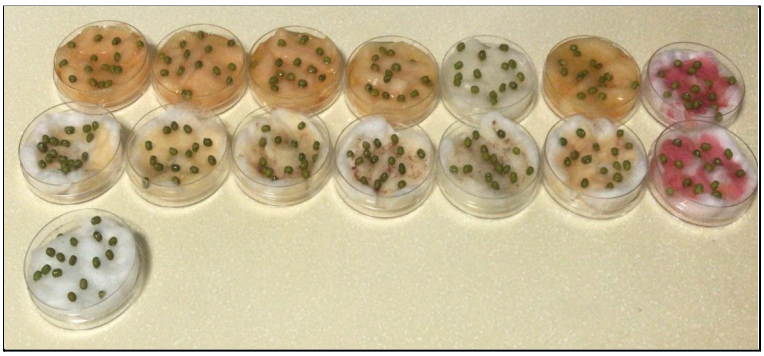


Fig. 4. Picture of mung bean seeds from Trials A and B before germination. Concentrations of HRP solutions used to make chitosan-HRP beads to treat wastewater used for germination are, from left to right: 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 5 µg/ml, 0µg/ml, untreated sample.

After 2 days of germination

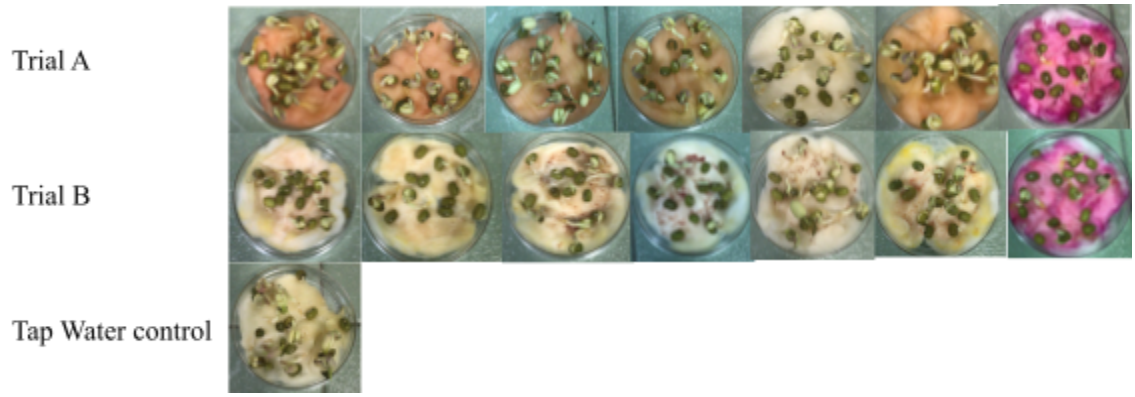
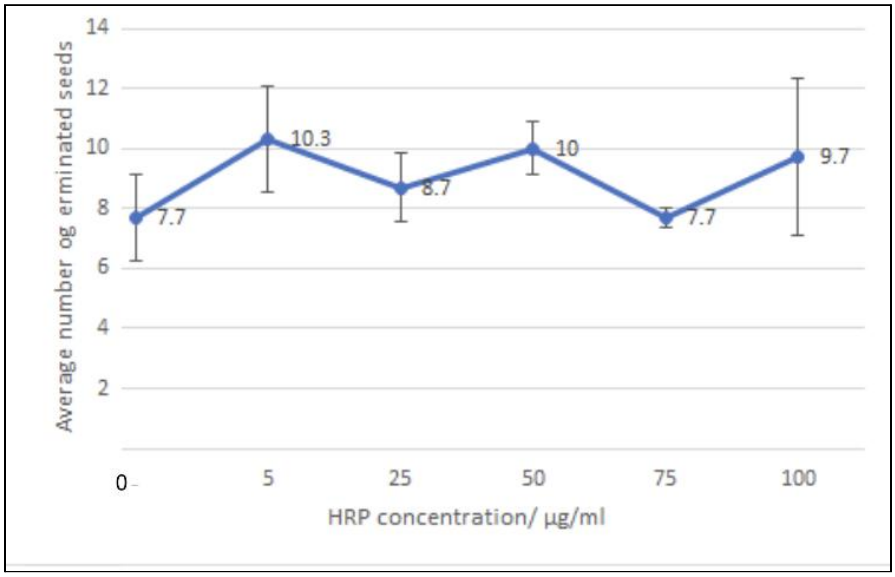


Fig. 5. Picture of germinated seeds from Trials A and B. Concentrations of HRP solutions used to make chitosan-HRP beads to treat wastewater used for germination are: 100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 5 µg/ml, 0µg/ml, untreated sample.

Fig. 6 . Number of germinated seeds in each treated simulated wastewater sample



p = 1.88

n = 3

Germination rates showed an overall increase as HRP concentration of chitosan-HRP beads increased (Fig. 6), but this observation is only preliminary and is not statistically significant based on the Kruskal Wallis test due to the small sample space (n=6). More replicates need to be done to verify the trend observed. Our preliminary findings showed that over 50% of the green bean seeds germinated for every concentration, and HRP concentration which caused the most germination was 100µg/ml, followed by 5µg/ml, 50µg/ml, 0µg/ml, 25µg/ml, and 75µg/ml. We observed that HRP solution of concentration 5µg/ml is the preliminary optimal concentration of HRP for making chitosan-HRP beads to remove phenol dye treatment in wastewater, while 100µg/ml HRP solution follows closely behind. Similarly, past research reported that increasing concentration of enzyme caused a gradual increase in dye removal and after that, the amount of phenol dye remained fairly constant (Gholami-Borujeni et al., 2011).

5. Conclusions and Recommendations for Future Work

It can be concluded that with the optimal concentration of peroxidase in the chitosan-HRP beads, phenolic substances in wastewater can be removed effectively. This method may be suitable to treat wastewater that may end up in rivers and streams, preventing phenolic substances from

harming animals and affecting plant growth. Our findings showed that chitosan-HRP beads made using 5ug/ml HRP solution had the highest decolourisation efficiency and consequently simulated wastewater treated with these chitosan-HRP beads had the highest germination rate (needs to be verified with more experiments).

This study can be extended by making chitosan-HRP beads using HRP extracted from watermelon and lemon. This may allow unconsumed fruits to be recycled to treat wastewater.

We have attempted to construct a wastewater treatment filter with a recycled mineral water bottle as shown below:

Fig 7. Wastewater treatment filter

The filter can be filled with chitosan-HRP beads and experiments can be carried out to determine the efficiency of the wastewater filter in removing phenolic dyes from wastewater.

Annexes

Annex A: Table of values for Fig. 3.

	Decolourisation Efficiency/ %			
HRP Concentration of beads (µg/ml)	**Trial A**	**Trial B**	**Trial C**	**Average**
100	67	60	51	59.3
75	61	64	67 (5 µg/ml due to insufficient HRP)	62.5
50	65	71	56	64
25	71	74	59	68
5	94	84	70	78.8
0	65	58	40	54.3

Annex B: Table of values for Fig. 6.

	Number of germinated seeds			
HRP Concentration of beads (µg/ml)	**Trial A**	**Trial B**	**Trial C**	**Average**
100	12	10	7	9.7
75	11	5	7 (5 µg/ml due to insufficient HRP)	7.7
50	12	8	10	10
25	10	7	9	8.7
5	11	10	10	10.3
0	12	8	3	7.7
Untreated wastewater	3	5	3	3.7
Tap Water	13			

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