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Removal of Phenols from Synthetic Wastewater by Horseradish Peroxidase (HRP)

Nurul Zakiah Md Salleh[1], Siti Amira Othman[1]*

[1] *Faculty of Applied Sciences and Technology*
Universiti Tun Hussein Onn Malaysia, Pagoh, 84600, JOHOR

*Corresponding Author: sitiamira@uthm.edu.my
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Abstract

Plant peroxidase has a solid potential to remove phenol from wastewater. However, large-scale use of these enzymes for phenol removal requires a source of cheap, abundant, and easily accessible peroxidase-containing material. In this study, the horseradish peroxidase (HRP) removes the phenolic compound from the phenolic environment. The enzymatic method was used to show a reduction of toxicity between the treated and untreated phenolic solution. The HRP, with the addition of hydrogen peroxide (H_2O_2), reacted with the phenolic compound and produced a treated solution containing about 20% of phenol compared to the initial acidic solution. The concentration of the treated solution was then determined using UV-Vis and FTIR spectroscopy. The effects of treating the acidic solution with HRP enzyme and H_2O_2 were studied. The HRP enzyme decreases the amount of phenol present in an acidic solution by 80 %. The most effective concentrations for treating the acidic solution were HRP at 1.0 M and 1.0 M of H_2O_2 at neutral pH.

1. Introduction

Phenol is one of the aromatic compounds that are toxic organic pollutants even at low concentrations. They are widely used in industrial manufacturing and present in industrial wastewater from petroleum refining, plastics, textiles, drugs, pesticides, papers, iron, and steel [1]. The wastewater is very difficult to treat. It contains phenolic compounds that are toxic and some even are carcinogens [2]. These compounds are difficult to separate from wastewater. This could cause severe problems if the wastewater containing the phenolic compound is discharged untreated or partially treated into water bodies or land sites. The disposal of wastewater into the water bodies without any treatment causes undesirable effects on the water environment and also harms aquatic life. At levels of 0.0045-0.05 mg/L, the phenol is already toxic to the fish. Therefore, the decontamination of phenols from wastewater is essential [3]. The aromatic and phenol elements are the leading cause of the critical toxicity of wastewater.

Most methods used for treating wastewater are physical, chemical and biological. Chemical transformations involve the applications of reagents and reaction conditions to transform and treat target species. Conventional processes have proven to be efficient in detoxification of phenolic compounds. However, these processes have certain disadvantages of solvent extraction, precipitation, chemical degradation, and activated carbon adsorption. It requires a lot of cost to handle the experiments. Horseradish peroxidase (HRP) is a promising candidate as a bioremediation catalyst. It was reported that horseradish peroxidase was able to be used for removing phenolic compounds and aromatic amines and decolorizing textile effluents from aqueous solutions. Horseradish peroxidase is one of the most essential enzymes obtained from a plant source. It continues to attract the attention of researchers from various disciplines because of its practical and commercial applications. Advances in understanding the structure and catalytic mechanism of horseradish peroxidase have been made using protein

engineering and other techniques. The physiological role of the enzyme is now being investigated in the context of new information on the plant peroxidase gene family of Arabidopsis thaliana [4].

Another way to treat wastewater containing phenolic compounds is by using horseradish peroxidase in enzymatic treatment. Phenols are oxidized to catalyst in the presence of the peroxidase, producing the corresponding radicals where the radicals spontaneously react rapidly to form insoluble polymeric phenolic aggregates [5]. Several researchers have reported enzymatic removal of phenols. It has been shown that aqueous phenolic compounds can react with peroxidase and non-soluble materials will be formed and could be easily removed from aqueous solution [6]. Today, different methods are used to remove phenolic compounds from wastewater, such as chemical, physical, or biological. However, chemicals may worsen the situation and cause more contamination, and hence, biologicals are preferred in achieving this purpose. Using the pure horseradish peroxidase enzyme can provide safe ways of producing treated wastewater and cutting the high-cost method used by most industries. Biological processes use the natural metabolism of cells to break down and remove oil and aromatic from the wastewater. The metabolic processes occur because of reactions conducted inside the cell that are catalyzed by proteins called enzymes [5].

The significance of this study is to study the importance of enzyme horseradish peroxidase in removing the phenolic compound from contaminated water bodies. Disposing wastewater into water sources containing phenol has become a serious matter since it already influences humans, even aquatic life, from water bodies [7]. This problem could not be solved individually nevertheless, it required cooperation from the industry itself. In this research, recommendations on improving the performance of horseradish peroxidase in removing the phenol from the water waste were studied. In addition, this study also provides ways for industrial manufacturers to dispose of their waste in the water source in the right way. And importantly, this research will also educate the industry in realizing the effect of disposing of untreated wastewater into the water resources of the aquatic life, society, and the country itself.

2. Methodology

HRP and H_2O_2 with a concentration of 30 wt. % in H_2O and phenols pellets are used for analysis of the toxicity of phenol and the activity of HRP. At the same time, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were used as soluble solutions for pH test. All the chemicals were purchased from Sigma Aldrich (United States). The resultant samples then undergo characterization with an Ultraviolet-visible (UV-Vis) spectrophotometer to determine the enzyme activity, Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FIR) to determine the functional group exist in the sample of horseradish peroxidase and lastly Scanning Electron Microscope (SEM) for studying the morphology of horseradish peroxidase enzyme after treatment.

The test solution was prepared by diluting stock phenol to the desired concentration. About 250 ml volumetric flasks are labelled from 0.2 M to 1.0 M according to the phenol concentration needed. Phenol pellets are filled into the 250 ml beaker. Next, distilled water is added into the beaker until it reaches 100 ml level. The pellet of phenol and distilled water are mixed using the magnetic stirrer for 10 minutes at 300 rpm. After the phenol pellets are wholly dissolved, carefully fill the flask to the 250 ml mark. Then, distilled water was added a drop at a time until the bottom until the solution reached the single mark accurately using a dropper. Dilution was repeated for other concentrations of phenol, which are 0.4 M, 0.6 M, 0.8 M and 1.0 M. Chemicals with high acid concentration or corrosive elements, such as phenol and HCl, were handled in the fume chamber.

The removal of phenols is carried out in polypropylene (PP) tubes containing the reaction mixture composed of 10 ml of diluted phenol of specified concentration, 10 ml of HRP enzyme and 10 ml of H_2O_2 solution in a total of 30 ml. The solution was then transferred into the 50 ml beaker. The reactions are stirred using the magnetic stirrer for 30 minutes. After stirring, the residual phenol will be measured spectrophotometric using a UV-Vis spectrophotometer. In obtaining the optimum conditions of phenol can be removed, the effect of several variables, such as the phenol, H_2O_2, HRP enzyme concentration and pH, are investigated. To optimize the percentage of phenol removal, we determined the efficiency of phenol removal in reactions by preparing 0.6 M of horseradish peroxidase enzyme solution. The HRP solution was then added to 10 ml of phenol solution. To optimize the phenol concentrations, phenol solutions will be mixed with 0.6 M of HRP and then the reaction was initiated by adding 10 ml of diluted H_2O_2. To assess the effect of pH on the efficiency of phenol removal, the reactions are performed using different pH values of phenol from pH 1 to 7 as the reaction medium. The pH was adjusted using concentrated NaOH and HCl. The pH is adjusted before and after adding phenol into the solution.

The phenol solution pH varies using the hydrochloric acid. The electrode from the pH probe was removed from the bottle of storage solution and rinsed with distilled water. The electrode was immersed in phenol solution, and the swirled solution was used to saturate the electrode with the solution thoroughly. After the reading was stable, the pH reading was taken. Removed the phenol solution and rinsed the electrode using distilled water. Repeat the experiment for another pH.

The pH value of the optimum phenol concentration from the previous experiment is taken before being varied into the other pH. 10 ml of 0.6 M of phenol solution from the previous experiment is filled into 7 PP tubes. The PP

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tubes are labelled pH 1 to 7. Then, each PP tube varied according to the pH value stated. Next, 10 ml of H_2O_2 are poured into the PP tubes and the pH values are retaken. Finally, 10 ml of HRP enzyme is poured into the PP tubes. The pH values are retaken for the last time. The mixture was then poured into the 100 ml beaker and stirred for 30 minutes using a magnetic stirrer at 300 rpm. This allows the phenol to be oxidized by H_2O_2 in the presence of the HRP enzyme. The steps are repeated for each pH value from pH 1 to pH 7. All these steps are carried out in the fume chamber because high-acidity solutions are used. Removal of phenolic compounds is dependent on the amount of catalyst added. To study the effect of enzyme concentration on phenol removal, the optimum phenol concentrations from the previous experiments are used to compare the efficiency of HRP enzymes at different concentrations. The optimal dose of enzyme concentration for the experimental condition was figured out.

The optimum phenol concentration and pH from the previous experiments are poured into the 5 PP tubes with a volume of 10 ml. The enzyme concentration is varied and the diluted HRP enzyme is obtained from 0.2 M to 1.0 M. Each of the PP tubes is labelled according to their concentration. Then, 10 ml of H_2O_2 with a concentration of 0.6 M are poured into the PP tubes. The mixture was then poured into the 100 ml beaker and stirred for 30 minutes using a magnetic stirrer at 300 rpm. This allows the phenol to be oxidized by H_2O_2 in the presence of the HRP enzyme. The steps are repeated for each peroxidase concentration from 0.2 M to 1.0 M. The enzyme activity is observed and compared for each concentration of HRP reacted with phenol and H_2O_2.

To determine an optimum H_2O_2 concentration on phenol removal in an acidic solution, a diluted H_2O_2 solution of 0.2 M to 1.0 M is added to optimal phenol concentration and the phenol content is observed. To determine the optimal H_2O_2 concentrations, the mixture of phenol, horseradish peroxidase and acidic solution is supplemented with different concentrations of H_2O_2 from 0.2 M to 1.0 M. The mixture was then poured into the 100 ml beaker and stirred for 30 minutes using a magnetic stirrer at 300 rpm. This allows the phenol to be oxidized by H_2O_2 in the presence of the HRP enzyme. The steps are repeated for each HRP concentration from 0.2 M to 1.0 M.

3. Results and Discussion

These experiments were conducted to study the effect of phenol concentration in removing phenol from the wastewater using HRP enzyme and H_20_2. The phenol was varied into different concentrations from 0.2 M to 1.0 M at normal pH and at room temperature, 37 °C.

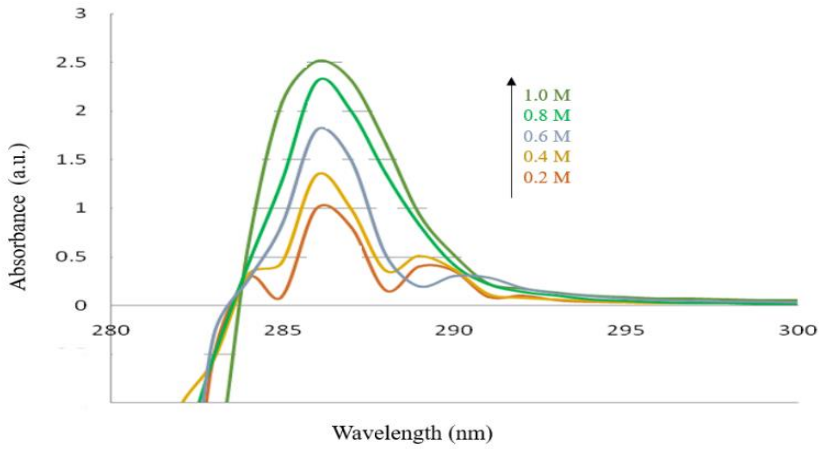

Fig. 1 *The graph of the effect of phenol concentration with HRP enzyme*

From Figure 1, it shows the absorbance of light increases as the concentration of phenol increases. The absorbance of light at 0.2 M has the lowest light absorbance at wavelength 286 nm, which is 1. The next phenol concentration at 0.4 M at wavelength 286 nm absorbs light at 1.343. The 1.0 M phenol concentration has a light absorbance of 1.805 at 286 nm. Next, the phenol concentration at 0.8 M has a light absorbance of 2.3 at the same wavelength of 286 nm. The highest peak of light absorbance is at 2.512 at wavelength 286 nm. The graph shows that the phenol concentration at 0.6 M is the optimum concentration for the condition where the enzyme can react with the phenol.

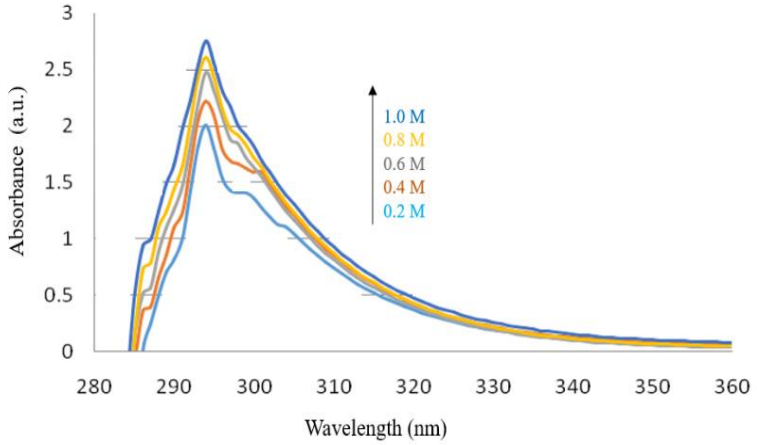

Fig. 2 *The graph of the effect of phenol concentration with HRP enzyme and hydrogen peroxide (H_2O_2)*

From Figure 2, the graph shows as the concentration of phenol with HRP and H_2O_2 increases, the absorbance of light also increases. The lowest concentration at 0.2 M of phenol has the lowest light absorbance at 2.006 at a wavelength of 294 nm. The phenol concentration at 0.4 M has a light absorbance 2.218 at wavelength 294 nm. Next, the phenol concentration at 0.6 M at light absorbance 2.48 at wavelength 294 nm. The light absorbance of 0.8 M phenol concentration is 2.543 at a wavelength of 294 nm. The highest peak of light absorbance is at 2.756 at wavelength 294 nm. The optimum concentration to remove phenol using HRP and H_2O_2 at 1.0 M phenol concentration because the phenol is removed the highest at 1.0 M.

High concentrations of phenol cause the wastewater to become acidic [8]. The acidic environment killed the enzyme, for instance, as the enzyme can only exist at specific conditions at the normal condition [9]. As the concentration increased from 0.2 M to 1.0 M, the activity of the HRP enzyme decreased. It is shown by the graph plotted between the absorbance versus the wavelength. As the phenol concentration increases, the absorbance of light also increases. HRP combines with H_2O_2 and produces the resultant HRP-H_2O_2 complex. This resultant complex oxidize a variety of hydrogen atoms, including phenol. The horseradish peroxidase undergoes a cyclic reaction when reacting with the phenolic substrates. The conclusion is as the phenol concentration increases, the higher the removal of phenol from the wastewater, but 0.6 M phenol is the optimal condition as it has the highest peak in phenol content that has been removed [5].

The experiment was conducted to study the effect of pH in removing phenol from the wastewater using HRP enzyme and H_2O_2. The pH of the phenol solution was varied from pH 1 to pH 7 and treated with the HRP with the addition of H_2O_2. The horseradish peroxidase enzyme decreases the amount of phenol in an acidic solution by 50% at a molarity of 0.6 M and pH 7.

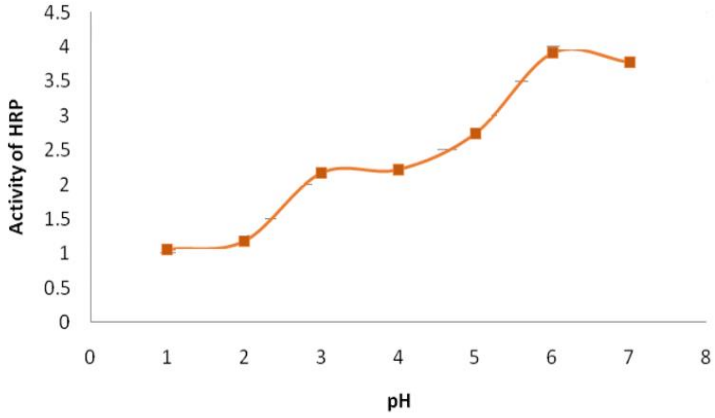

Fig. 3 *The graph of the effect of pH on phenol removal*

Figure 3 shows the effect of pH on removing phenol using the HRP with the addition of H_2O_2. The graph showed the optimum pH for the HRP to remove phenol is between pH 6 and 7. The activity of HRP at the removal of phenol decreases as the acidity increases from pH 7 to pH 1. HRPs are primarily active in neutral conditions

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[10]. Aside from temperature changes, an alteration in the acidity, or pH, of the enzyme's environment inhibits enzyme activity. One of the types of interactions that hold an enzyme's tertiary structure together is ionic interactions between amino acid chains. A positively charged amine group is neutralized when interacting with a negatively charged acid group. A change in pH, which is a change in the number of protons, can change the charges of these two groups, making them unattracted to each other. It should be noted that each enzyme functions within a specific pH range, some liking very acidic environments, others very alkaline, or basic, environments. The conclusion is as the pH of the phenol increases, the content of the phenol removed also increases as pH 7 showed the highest peak in the graph [8].

The test was conducted to determine the optimum HRP concentration. It was determined using the optimum phenol concentration and pH from the previous set of experiments. The horseradish peroxidase was added at 20 ml per test tube to determine the effect of horseradish peroxidase concentration on the phenol content in the phenol-polluted wastewater sample after enzymatic treatment. These solutions are mixed for 1 hour. Scanning Electron Microscope (SEM) can be used to study the morphology of the horseradish peroxidase membrane surface. SEM images show the surface morphology of lyophilized horseradish peroxidase and peroxidase with phenol. SEM images of horseradish peroxidase with phenol show that the entire membrane surface appeared to be enclosed by phenol molecules [11].

Figure 4 shows the morphology of the immobilized horseradish peroxidase, which has a porous surface. The porous structure of the horseradish peroxidase surface causes the phenol to react with the enzyme vigorously [12]. The hydrogen peroxide and the peroxidase enzyme oxidize the enzyme to an intermediate enzymatic form. The enzyme then accepts the phenol compound into its active site and oxidizes. A free radical is released into the solution, leaving the enzyme in the compound state [13,16]. This compound oxidizes a second aromatic molecule, releasing another free radical product and returning the enzyme to its native state, thereby completing the cycle. The free radicals formed during the cycle diffuse from the enzyme into the bulk solution, where they react to form polycyclic aromatic hydrocarbon products.

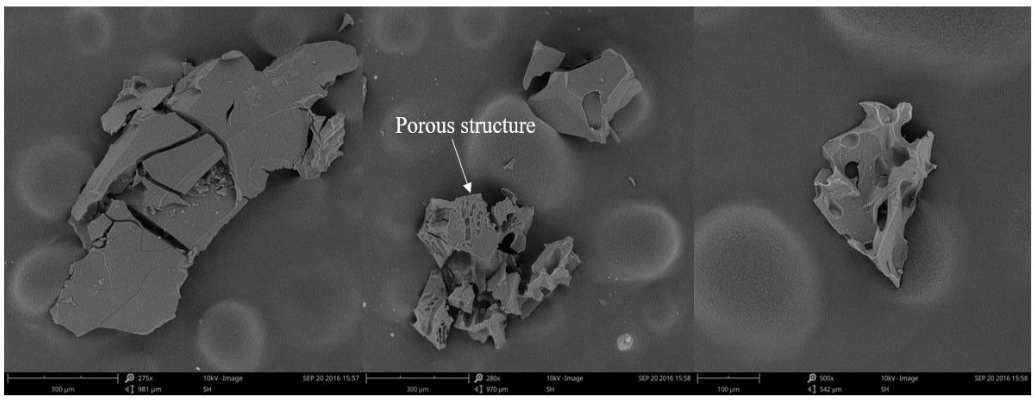

Fig. 4 *Morphology of HRP*

The enzymatic reactions can still occur too slowly if the concentration of enzymes in a reactive system is low [14,17]. In most situations, decreasing enzyme concentration has a direct influence on enzyme activity because enzyme molecules can catalyse only one reaction at a time. The molecule to which an enzyme binds is called a substrate. Generally, one enzyme binds to one substrate to lower the activation energy for one chemical reaction. If all the enzymes in a system are bound to substrates, additional substrate molecules must wait for an enzyme to become available following the completion of a reaction. The conclusion is the rate of reactions decreases as enzyme concentration decreases.

The experiment was conducted to determine an optimum hydrogen peroxide concentration at the previously determined optimum conditions phenols at pH 6 and concentration of 0.6 M with HRP enzyme at 0.6 M). Figure 5 shows that as the H_2O_2 concentration was increased, the absorbance increased at a constant wavelength of 288 nm. The lowest peak showed 0.2 M has the lowest light absorbance, 1.227. Then, at 0.4 M of H_2O_2, the light absorbance is at 1.429. The absorbance at 0.6 M of H_2O_2 concentration, the light absorbance increases at 1.651. At 0.8 M of H_2O_2 concentration, the absorbance is at 1.896. Finally, the highest peak in the graph is shown by the 1.0 M of H_2O_2 concentration, as it has a value of 2.214 of absorbance. The optimum concentration for H_2O_2 concentration in phenol removal is 1.0 M.

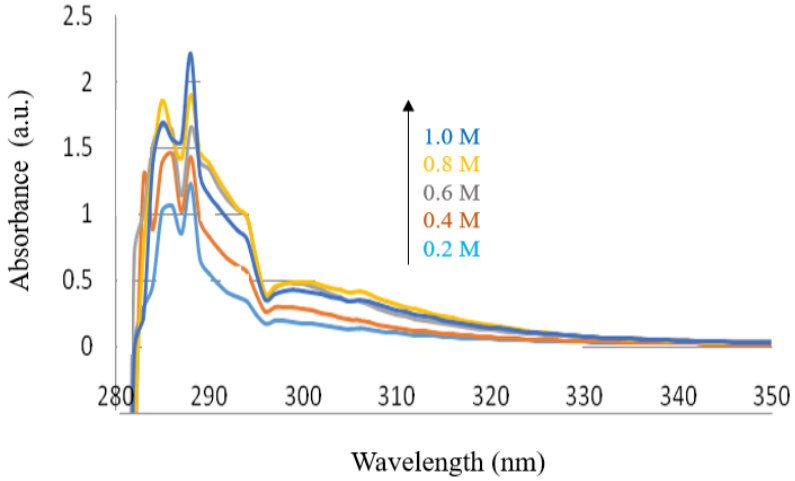

Fig. 5 *The graph of absorbance versus wavelength for the effect of H_2O_2 concentration*

The increased the concentration of H_2O_2, the decreased the phenol content. The high dose of H_2O_2 was due to the enzymatic oxidation of phenol. Hydrogen peroxide makes the HRP catalyze hydroxyl group of phenolic structure and become unstable and generate phenoxyl radicals. These radicals then, by oxidative self-coupling, stimulated the polymerization of phenolic compounds [15,18].

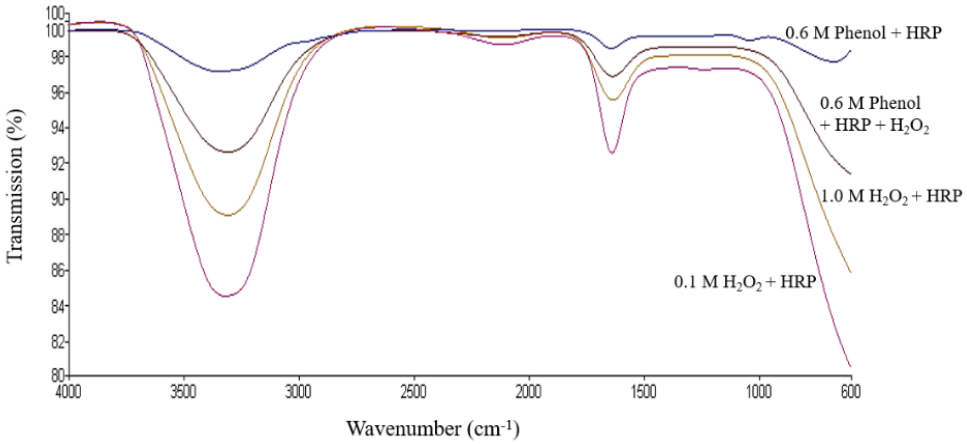

Fig. 6 *ATR-FTIR spectra of treated and untreated phenol solution*

Figure 6 shows that phenol concentration decreases when compared between the treated phenol wastewater and the untreated phenol wastewater. The graph depicted in Figure 6 shows the vibration that occurs in the ATR-FTIR. The bond remains in the solution after undergoing several experiments where the phenol bond consists of 2 double bonds and 1 hydrogen bond. The bond decreases, as stated in the graph between the pink line, yellow line, brown line and blue line. The blue line represents the phenol at 0.6 M, which oxidizes with only HRP. The brown line represents the phenol at 0.6 M, which oxidizes by HRP and H_2O_2. The yellow line represents the phenol after oxidizing with 1.0 M of H_2O_2 and 1.0M HRP. The pink line represents the phenol after oxidizing with 0.1 M of H_2O_2 and 1.0 M HRP. From these results, it may be stated that the optimum concentration and conditions for enzymatic treatment of wastewater were at pH 7.0, an HRP concentration of 1.0 M, a H_2O_2 concentration of 1.0 M, which collectively achieved a final phenol concentration of 0.6 M corresponding to a phenol removal of phenol 80%.

4. Conclusion

The phenol concentration at 0.6 M (pH 6) showed the highest phenol removed after being treated with horseradish peroxidase and hydrogen peroxide. Next, the effects of increasing the horseradish peroxidase and hydrogen peroxide have been studied. The optimum concentration in removing the phenol is obtained at the end of the study. The phenol compound decreases as the concentration of horseradish peroxidase and hydrogen

peroxide increases from 0.2 M to 1.0 M. At pH 6, with 1.0 M of horseradish peroxidase and 1.0 M hydrogen peroxide, the phenol removed has the highest peak in ATR-FTIR spectra. Finally, the morphology of the horseradish peroxidase surface was observed using Scanning Electron Magnification (SEM) at a magnification of 970 mm. The horseradish peroxidase combines with hydrogen peroxide and produces the resultant complex. This resultant complex oxidize phenol.

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Conflict of Interest

Authors declare that there is no conflict of interests regarding the publication of the paper.

Author Contribution

The authors confirm contribution to the paper as follows: ***study conception and design:*** *Nurul Zakiah Md Salleh, Siti Amira Othman;* ***data collection:*** *Nurul Zakiah Md Salleh;* ***analysis and interpretation of results:*** *Nurul Zakiah Md Salleh, Siti Amira Othman;* ***draft manuscript preparation:*** *Nurul Zakiah Md Salleh, Siti Amira Othman. All authors reviewed the results and approved the final version of the manuscript.*

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