Peroxidase extraction from jicama skin peels for phenol removal

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Abstract. Phenol and its derivatives exist in various types of industrial effluents, and are known to be harmful to aquatic lives even at low concentrations. Conventional treatment technologies for phenol removal are challenged with long retention time, high energy consumption and process cost. Enzymatic treatment has emerged as an alternative technology for phenol removal from wastewater. These enzymes interact with aromatic compounds including phenols in the presence of hydrogen peroxide, forming free radicals which polymerize spontaneously to produce insoluble phenolic polymers. This work aims to extract peroxidase from agricultural wastes materials and establish its application for phenol removal. Peroxidase was extracted from jicama skin peels under varying extraction conditions of pH, sample-to-buffer ratio (w/v %) and temperature. Experimental results showed that extraction process conducted at pH 10, 40% w/v and 25^{o}C demonstrated a peroxidase activity of 0.79 U/mL. Elevated temperatures slightly enhanced the peroxidase activities. Jicama peroxidase extracted at optimum extraction conditions demonstrated a phenol removal efficiency of 87.5% at pH 7. Phenol removal efficiency was ~ 97% in the range of 30 – 40^{o}C, and H_2O_2 dosage has to be kept below 100 mM for maximum removal under phenol concentration tested.

1. Introduction

Rapid development and blooming of various industries such as petroleum refineries, wood preservation, resins and plastics, pulp and paper, dyes and other chemicals contribute significantly to the economics of many countries. However, these industries also generate huge amount of industrial effluents, of which if discharged untreated or partially treated, could cause serious ecological problems to aquatic lives and human health. This is due to the presence of xenobiotic and recalcitrant organic pollutants in the wastewaters, which include phenols and its derivatives. Phenolic compounds are mostly toxic and have been classified as hazardous pollutants [1]. Therefore, developing effective and sustainable treatment technologies for industrial wastewaters is of vital importance and continues to be one of the greatest challenges to the environmental engineering and science.

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Conventional treatment methods for phenol removal from wastewater include microbial degradation [2, 3], adsorption on activated carbon [4, 5], and chemical oxidation such as ozonation [6, 7] and Fenton reaction [8, 9]. The aforementioned methods, though showing high removal efficiencies for phenol, suffer certain disadvantages such as lengthy start-up for microbial acclimatization [10], microbial growth inhibition due to high concentrations of phenolic compounds [11], intensive cost and energy for carbon regeneration [12], and disposal concern of reagents used during reaction process [12]. Due to the challenges associated with conventional phenol removal methods, research focuses have been geared towards finding an alternative method that is versatile for a wide range of reaction conditions with little or no environmental impacts for phenol removal. Enzymatic approach has been proposed by researchers for phenol bioremediation owing to its potential advantages over conventional treatment methods. These include application to bio-refractory compounds, operation at high and low contaminant concentrations, operation over a wide range of pH, temperature and salinity, absence of delays associated with the acclimatization of biomass, reduction in sludge volume (no biomass generated) and ease of controlling the process [13].

Among the various types of enzymes, peroxidases have been identified as a suitable candidate for the treatment of phenolic contaminants and related compounds. Activated by hydrogen peroxide, peroxidases catalyze the oxidation of aromatic compounds, forming free radicals which undergo spontaneous polymerization. The polymerized product precipitates out from the solution and can be readily separated by sedimentation and filtration techniques [14]. Peroxidases are ubiquitous in nature and can be found in plants, microorganisms and animals. Horseradish peroxidase (HRP) has been explored extensively for its potential in decontamination of phenolic compounds from wastewater [15-20]. However, most of the studies conducted were using purified HRP which imposed high cost to the removal process. This has triggered the search for peroxidases from other less expensive sources with comparable or better stability and efficiency in phenol removal. Peroxidases from soybean seed hulls [1,14,21], turnip [22], bitter gourd [23], radish [24], cauliflower [25] and potato [24] have been evaluated for their performances in phenol removal, and the results obtained were encouraging and promising. Other peroxidases that have been identified, extracted and characterized include oil palm leaves [26], rice leaves [27], sweet potato [28] and tomato [29].

The aim of this study is to explore the possibility of using jicama skin peels as the source of peroxidase for phenol removal. The skin of jicama is not edible and therefore is discarded as waste. Our preliminary study has shown that peroxidase exists in the skin peels of jicama. The use of this agricultural waste for peroxidase extraction is not in competition with human food consumption. This approach offers a chain of green technology as waste from one source is transformed into a useful product to treat waste from a different source. The extraction of peroxidase from jicama skin peels will be studied from the aspect of pH, sample-to-buffer ratio and temperature. Jicama peroxidase extracted at optimum conditions will then be evaluated for its performance efficiency in phenol removal under varying reaction conditions which include pH, temperature and hydrogen peroxide (H_2O_2) concentration.

2. Materials and methods

2.1. Preparation of crude extract

Jicama purchased from local market was washed thoroughly with distilled water, and then the skin peeled off and chopped into small pieces. The chopped jicama was weighed and mixed with buffer solutions based on weight (g)/volume (mL) percentage (w/v %). Homogenization of enzyme mixtures was carried out at 400rpm for 1 h at room temperature (unless stated otherwise). The enzyme extract was then filtered through four layers of cheesecloth before being subjected to centrifugation and sonication. The enzyme collected was considered as crude extract and stored at 4^0C until further use.

2.2. Analytical procedures

Enzyme activity of jicama peroxidases was measured using a colorimetric assay containing phenol, 4-aminoantipyrene (4-AAP) and hydrogen peroxide. This assay is a modification of that developed by Wu *et al.* [20] in which the assay mixture consists of 250µl of 9.6mM 4-AAP, 100µl of 100mM phenol, 100µl of 2mM hydrogen peroxide, 450-500µl of 100mM phosphate buffer (pH 6.0) and 50-100µl of enzyme solution. Prior to significant substrate depletion, activity was proportional to the rate of formation of a coloured product which absorbs light at a peak wavelength of 510nm with an extinction coefficient of 7100 L/mol.cm based on the conversion of H_2O_2.One unit of activity is defined as the number of micromoles of H_2O_2 consumed per minute at pH 6.0 and 25^{o}C.

Phenol concentrations were determined colorimetrically using 4-AAP and potassium ferricyanide in an alkaline buffer medium. Phenolic compounds react with 4-AAP under alkaline conditions to yield an intermediate species which is oxidized in the presence of the potassium ferricyanide reagent. The resulting compound is a quinone-type dye which absorbs light at 510nm. The colour intensity is linear with respect to phenol concentration, provided that this concentration does not exceed 0.1mM in the cuvette [30]. The absorbance was measured at 510nm after 5 minutes.

2.3. Extraction of jicama peroxidase

2.3.1. Effect of pH on peroxidase extraction. The effect of pH on peroxidase extraction from jicama peels was evaluated by mixing buffer solutions (0.1M) of different pH values to the weighed amount of jicama. The range of pH being studied was from pH 3 to 10. One gram of jicama peels was added to 10ml of buffer solution at certain pH, and homogenization was carried out as previously described. Enzyme sample-to-buffer ratio (w/v %) in this aspect was 10%.

2.3.2. Effect of sample-to-buffer ratio on peroxidase extraction. Sample-to-buffer ratio (w/v %) for jicama peroxidase extraction was investigated at 10%, 20% and 40%. The maximum w/v % that can be achieved was based on the minimum amount of buffer solution needed to cover all the pre-weighed quantity jicama peels for proper homogenization.

2.3.3. Effect of temperature on peroxidase extraction. Jicama peroxidase extraction was carried out at 25^{o}C, 35^{o}C and 45^{o}C. For 35^{o}C and 45^{o}C, the extraction process was carried out in water bath with constant stirring. The temperatures of the water bath were monitored to ensure constant temperature throughout the extraction process.

2.4. Batch treatment of aqueous phenol by using jicama peroxidase

The efficiency of jicama peroxidase in removing phenol from aqueous solution was evaluated under varying reaction conditions such as pH, temperature and H_2O_2 concentration. Jicama peroxidase was prepared based on the optimum conditions in extraction process. The reaction mixture consisted of buffer solution, phenol solution and enzyme. The enzymatic reaction was initiated through the addition of H_2O_2 into the mixture, and the process was carried out in incubator with constant shaking for 24 h to ensure maximum phenol removal. Phenol concentration in the reaction mixture before and after reaction process was assayed according to section 2.2, and percentage of phenol removal calculated.

2.4.1. Effect of pH on phenol removal.

Phenol removal processes were carried out in buffer solutions of various pH ranging from pH 4 to 9. The molarity of the buffer solutions was kept constant at 0.1M. The reaction mixtures were incubated at 30^{o}C with constant shaking for 24 h.

2.4.2. Effect of temperature on phenol removal.
Reaction mixtures containing 1 mL of 10mM phenol were treated with 1.5 mL jicama peroxidase in the presence of 0.2 mL of 10mM H_2O_2 at the optimum pH. Reaction mixtures were incubated at temperatures 30, 40, 50 and 60^{o}C respectively for 24 h with constant shaking.

2.4.3. Effect of H_2O_2 concentration on phenol removal.
The effect of H_2O_2 concentration on phenol removal was performed by varying the concentrations of H_2O_2 added into the reaction mixtures while keeping other reaction conditions (pH and temperature) optimum. The range of concentration of H_2O_2 being evaluated was from 0.2mM to 4mM.

3. Results and discussion

3.1. Effect of pH on peroxidase extraction

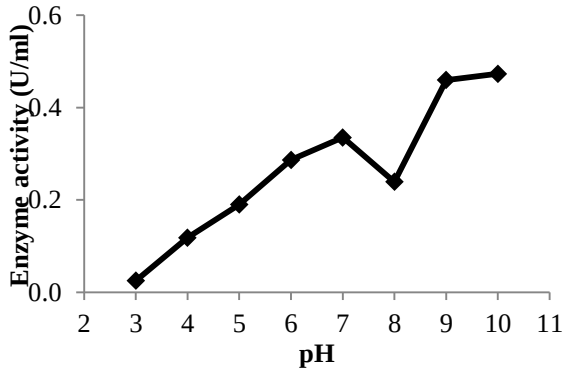

Figure 1. Peroxidase extraction as a function of pH. Extraction conditions: 1g of jicama peels was mixed with 10 mL of buffer solutions of various pH (10% w/v) and homogenized at 400 rpm and 25^{o}C for 1 h.

The pH condition of the medium plays an important role in enzyme extraction process as it affects the ionization state of enzyme amino acids side chain. As shown in figure 1, low pH did not favour the extraction process for jicama peroxidase. This could be due to the detachment of haem prosthetic group from the poly-peptide chain [31]. Crude enzyme of jicama extracted at high acidic regions showed low enzyme activities. Peroxidase activity increased as the pH value approached neutral. However, there was a dip in peroxidase activity at pH 8, which is slightly basic. Peroxidase activity increased again at higher alkalinity regions. The maximum peroxidase activity for jicama was observed at pH 10, with an activity of 0.473 U/mL. This could be due to most of the amino acids side chain in jicama peroxidase molecules are positively charged. In a highly basic medium, the positively charged amino acids are attracted to hydroxide ions, hence releasing the peroxidase molecules into the medium.

3.2. Effect of sample-to-buffer ratio on peroxidase extraction
As can be seen from Figure 2, enzyme activity obtained increased when the amount of jicama used in the homogenization medium was increased. This is because the extracts are more concentrated with peroxidase molecules. The highest sample-to-buffer ratio that can be achieved with jicama was 40%. Beyond this ratio, the volume of buffer solution was not sufficient for proper homogenization. Sample-to-buffer ratio of 40% (w/v) will thus be used for subsequent parameter studies.

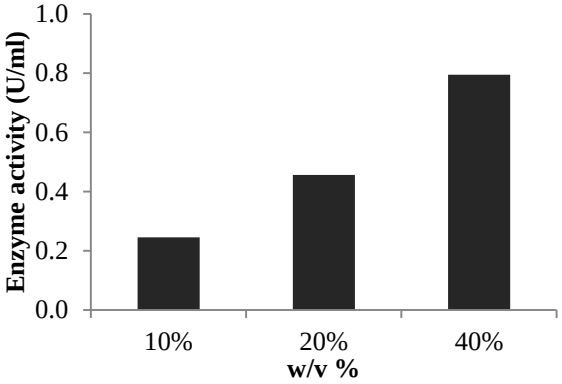

Figure 2. Peroxidase extraction as a function of sample-to-buffer ratio (w/v %). Extraction conditions: jicama peels was mixed with 0.1M buffer pH 10 at various w/v % and homogenized at 400 rpm and 25°C for 1 h.

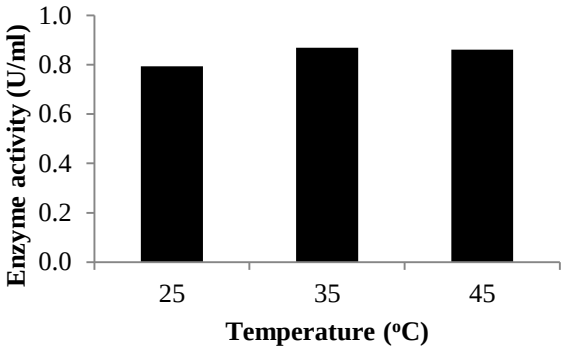

Figure 3. Peroxidase extraction as a function of temperature. Extraction conditions: jicama peels was mixed with 0.1M buffer pH 10 at 40% w/v and homogenized at 400 rpm at various temperatures for 1 h.

3.3. Effect of temperature on peroxidase extraction

From figure 3, it can be seen that extraction of jicama peroxidase at temperatures of 35°C and 45°C enhanced enzyme activity for ~ 9.6 %. However, in view of the extra cost incurred for extraction process at higher temperatures, it is therefore more economical to extract jicama peroxidase at room temperature (~25°C).

3.4. Effect of pH on phenol removal

Figure 4 shows the dependence of phenol removal efficiency on pH for jicama peroxidase. When 1 mM stock phenol solution was treated with jicama peroxidase, phenol removal efficiency of more than 80% was observed at pH values 6 to 7, with the optimum occurring at pH 7. Removal efficiency decreased to less than 40% below pH 5 and above pH 8. This is a result of variations in enzyme protein structure in response to varying pH. The enzyme molecules undergo structural modifications as a result of protonation and hydroxylation effects which could potentially obscure the enzyme active sites before causing denaturation and permanent loss of functionality. Moreover, the decrease in removal efficiency at pH above 8 could be attributed to the formation of phenol conjugated base since the pK_a of phenol at 25°C is 10. This conjugated basic form does not permit the phenolic compounds to act as hydrogen donors, thus hindering binding onto the surface of the enzyme active sites.

Previous studies on phenol removal catalyzed by horseradish peroxidase demonstrated optimal operating pH at pH 8 [17, 18], which was slightly basic. Another work by Wright and Nicell [21] showed that nearly complete removal of phenol was observed over a pH range of 5 to 9, with the maximum removal at pH 6 by using high dose of soybean peroxidase. Other studies using soybean peroxidase exhibited optimum pH for phenol removal at pH 6 [1] and pH 7 [32]. For jicama peroxidase, subsequent enzymatic reactions will be conducted using buffer pH 7.

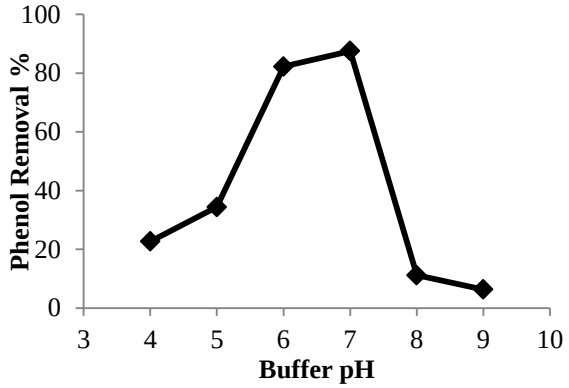

Figure 4. Phenol removal using jicama peroxidase at different pH conditions. Experiments were performed using 1.5 mL jicama peroxidase and 1 mL of 1mM phenol in buffer solutions of various pH at 30°C in the presence of 0.2mM H_2O_2.

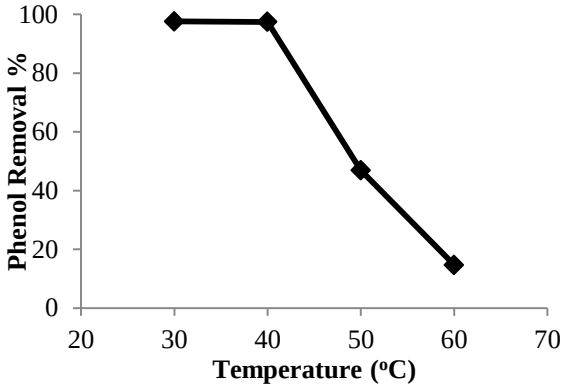

Figure 5. Phenol removal using jicama peroxidase at different operating temperatures. Experiments were performed using 1.5 mL jicama peroxidase and 1 mL of 10mM phenol in buffer pH 7 in the presence of 0.2mM H_2O_2.

3.5. Effect of temperature on phenol removal

The effect of temperature on phenol removal was examined by incubating the reaction mixtures containing 1 mL of 10 mM phenol stock solution at various temperatures, ranging from 30°C to 60°C. As depicted in figure 5, phenol removal efficiency of greater than 90% was observed over the temperature range of 30 – 40°C. Moderate increases in temperature from ambient conditions results in enhanced collision of the enzyme molecules with substrate molecules to form more products. However, further increase in temperature to above 50°C caused a decrease in removal efficiency to less than 50%. This is attributed to thermal denaturation of enzyme molecules, causing loss of its active sites to catalyze substrate molecules. The thermal stability of peroxidases is governed by the haem prosthetic group, which under elevated temperatures is released to form apoenzyme. The transient enzyme formed is less stable and more susceptible to thermal inactivation as compared to the native enzyme [33]. Higher temperature conditions distort the structure of the enzyme, causing a limited binding capacity of its active sites onto substrate molecules.

3.6. Effect of H_2O_2 concentration on phenol removal

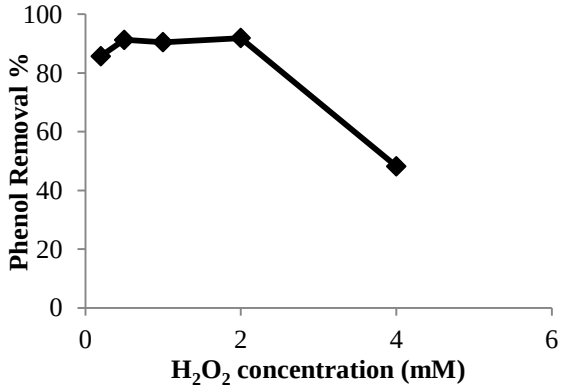

Figure 6. Phenol removal using jicama peroxidase at different H_2O_2 concentrations. Experiments were performed using 1.5 mL jicama peroxidase and 1 mL of 10mM phenol in buffer pH 7 at 30°C.

Hydrogen peroxide (H_2O_2) is required in peroxidase-catalyzed reactions to oxidize the native enzyme molecules into Compound I which then accepts an aromatic compound into its active site and carries out its oxidation [18]. Low concentrations of H_2O_2 limit the enzymatic reaction rate while excessive amounts could result in inhibitory effect on the enzyme activity [34]. According to figure 6, phenol removal efficiencies of more than 85% were recorded over a wide range of H_2O_2 concentrations, from 0.2 mM to 2 mM. However, enzymatic inhibition effect was observed at H_2O_2 concentration beyond 2 mM where the efficiency dropped to less than 50%. High concentrations of H_2O_2 inhibit peroxidase catalytic activity by irreversibly oxidizing the enzyme ferriheme group which is vital for peroxidase catalysis [22]. Therefore, phenol removal process using jicama peroxidase has to be carried out at H_2O_2 concentration less than 2 mM to ensure maximal removal.

4. Conclusions

Present study demonstrated that plant peroxidase can be extracted from agricultural and bio-wastes for phenol removal application. The peroxidase extraction process established in this work is simple and does not require the usage of many chemicals. The crude enzyme from jicama peels was subjected to phenol removal reaction and the results showed that the enzymatic process was dependent on pH, temperature and concentration H_2O_2. The extracted jicama peroxidase was not subjected to any further purification, and its activity in the homogenous crude enzyme solution was uniform. Relatively, enzyme volume was used in place of enzyme concentration as one of variable parameters in phenol removal reaction. However, its effect on phenol removal efficiency was not significant for the range being studied (data not shown).

The phenol removal efficiencies exhibited by jicama peroxidase was encouraging and this revealed the potential of jicama peroxidase in enzymatic wastewater treatment. The use of low cost materials and processes for peroxidase extraction coupled with moderate enzymatic reaction conditions and limited use of harsh chemicals creates a sustainable and environmentally friendly operation with low process economics. Nevertheless, further studies into optimization of reaction conditions such as reaction time and substrate dosages are essential to better understand the factors affecting the performance of this enzyme. The effects and interactions between various extraction conditions such as pH, sample-to-buffer ratio and temperature as well as the effect of H_2O_2 on enzymatic activities should also be addressed.

5. References

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