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RESEARCH ARTICLE OPEN ACCESS

Phenol Removal from Aqueous solutions by Crude peroxidase extract from Indigenous Bean varieties

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ABSTRACT
Phenolic compounds can be removed from industrial wastewater systems by the action of peroxidase enzymes from different sources. Additional and readily available sources of this enzyme continue to be explored. The ability of crude enzyme extracts from two local bean varieties, Black eyed bean and Bambara bean to remove phenol from aqueous solutions was demonstrated in this study. The optimum pH for highest activity for these two extract was found to be 6 and the optimum temperature was 40°C, similar to other enzyme sources. It was also shown that the Bambara bean extract retained its activity over a wider range of pH. Phenol removal efficiencies of more than 90% were achieved in the presence of PEG additive.
Keywords- Bambara, Black-eyed, peroxidase, Phenol, PEG

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I. INTRODUCTION

Phenolic compounds and their derivatives are common recalcitrant pollutants found in wastewaters from several industries [1]. These compounds may present detrimental impacts on human health and the ecosystem in general though they also perform various beneficial roles in plants and contribute to particular properties in some foods [2]. They are generated and found in found in wastewaters from such industries as petroleum refineries, coal processing, plastics, textiles, iron and steel manufacturing and also in the pulp and paper industries. They may also be produced naturally in the environment and also find their way into the soil through application of chemicals in agricultural practices [3]. Phenols are considered very toxic, some have even been classified as hazardous waste and others have been suspected of containing carcinogens or enhancing the carcinogenic ability of other precursors [4]. They are also reported to have long-term effects on ecosystems related to the release of toxic components over a prolonged period [5] including shortened lifespan, reproductive problems, lower fertility and changes in behavior [6].

The increased exploration and production activities in the oil and gas industry in Ghana has the potential for the discharge of large quantities of phenolic compounds as waste. There is the need therefore to develop innovative and efficient methods of removal of these pollutants especially based on local and readily available materials. Several methods are available for phenol removal from waste waters such as direct extraction, bacterial and chemical oxidation, adsorption on activated carbon, electrochemical techniques, irradiation and a host of others [7]. Chemical oxidation is on the expensive side for high strength waste treatment due to the high cost of chemicals. Sometimes, hazardous by-products are formed during the processes, the purification is incomplete and even low efficiencies have been observed. Enzyme-based treatment has emerged as the highly desirable method and has been reported in several studies for many years [8,9,10,11,12].

Enzymes tend to be more convenient to handle and store than micro-organisms used in biological oxidations and are greatly selective when they act, making it easy to know the particular group of contaminants which is being removed. Peroxidases (EC.1.11.1.7.) are a large group of heat stable enzymes found mostly in plants which catalyse redox reactions between hydrogen peroxide (H_2O_2) as an electron acceptor and a variety of substrates in which oxygen, water or both are liberated (depending on the substrate). The proposed mechanism is well studied and documented [8]. When phenols are present, they act as the electron acceptor and are eventually polymerised into insoluble polymers with high molecular weight. This makes it possible to remove them from the water through sedimentation and filtration.

High levels of this enzyme was initially found in horseradish but it is also present in other plants such as tomato, banana, dates, strawberry, wheat, and soybeans. In our earlier study [13], we demonstrated the presence of peroxidase in four varieties of locally grown beans and the current study seeks to apply the crude enzyme from two of these sources in the removal of phenols from aqueous solutions. The activity of the enzyme from these sources was optimized at temperatures close to 50°C suggesting significant thermal stability.

II. MATERIALS AND METHODS

2.1 Materials and Chemicals

Two (2) locally grown bean varieties, namely Black-eyed beans (*Vigna unguiculata*), and Bambara beans (*Vigna subterranean*) were obtained from the local market (Kumasi, Ghana).

Polyethylene glycol (PEG 3,500 and PEG 10,000), hydrogen peroxide (30% v/v) solution and phenol were obtained from Sigma-Aldrich, USA. 4-aminoantipyrine (4-AAP), potassium ferricyanide, sodium phosphate dibasic, sodium phosphate monobasic, sodium citrate, o-dianisidine dye solution and Bradford protein assay kit were obtained from Thermo-Fisher Scientific, UK.

2.2 General Statistical Analysis

All experiments were conducted in at least triplicates and the reported data points are the means of these measurements.

2.3 Crude Enzyme Extraction

All procedures were carried out on the selected bean varieties at room temperature. 50 g of each bean seed variety were soaked for 12 hours in 200 mL of distilled water. The mixture was blended to homogenise it and the solution was centrifuged at 10,000 rpm for 15 minutes. The clear supernatant, containing the peroxidase enzyme was placed in a water bath at 65°C for 3 minutes to inactivate any catalase enzyme present [14]. The extract was finally stored at 4^oC. The total protein in the extract was determined by the Bradford method [15], using bovine serum albumin (BSA) as the standard.

2.4 Measurement of Enzyme Activity

The assay on the peroxidase activity was carried out by the method of Malik and Singh [16] with slight modifications. 4.0 mL of 0.05M phosphate buffer (pH 7) and, 0.1 mL of o-dianisidine dye solution were mixed and 1.0 mL of 0.2 mM H_2O_2 solution and 0.1 mL of the enzyme extract were added. A control was set up with 0.1 mL distilled water in place of the hydrogen peroxide solution. The absorbance at 460 nm of the mixture was immediately read and also at 30 second intervals for 5 minutes. The enzyme activity is a reflection of the rate of the reaction and is measured by the change in the concentration of the H_2O_2 substrate per unit time and was calculated using the extinction coefficient of o-dianisidinedye
($11.3\ mM^{-1}cm^{-1}$) at 460 nm. The unit of activity is defined as the amount of enzyme extract that decomposes 1 μmole of H_2O_2 per minute.

2.5 Effect of pH on activity

A set of reaction mixtures as used in the determination of activity in 2.4 were prepared but the buffer solution was substituted with buffer of varying pH from 3 to 9. 0.1 mL of enzyme extract was added to the reaction mixture and the absorbance at 460 nm was measured at 30 second intervals for 5 minutes. The procedure was repeated for all the reaction mixtures.

2.6 Effect of Temperature on activity

5 mL of the enzyme solution in a test tube with a known activity (measured previously) was placed in different test tubes and dipped in water bath maintained at temperatures ranging from 30^oC to 80^oC at the optimum pH for each extract sample. The enzyme solutions were removed after 30 minutes and the activity test conducted on the samples as described earlier in 2.4.

2.7 Phenol Concentration Assay

The concentration of the phenol was determined by a colorimetric method which uses 4- aminoantipyrine (AAP) and ferricyanide as colour generating substances when combined with phenolic compounds. The method of Stanisavljević and Nedić [17] was followed with slight modifications. One mL of the assay mixture consisted of 100 μL of 20.8 mM 4-AAP, 100 μL of 83.4 mM potassium ferricyanide, 750 μL of 0.25 M sodium bicarbonate and 50 μL of the phenolic solution (post enzymatic reaction). After 10 min, the absorbance was measured at 510 nm. The generated color was directly proportional to the concentration of the phenolic compound.

2.8 Effect of Enzyme, H_2O_2, PEG doses and pH on Phenol removal

Varying concentrations of the enzyme, H_2O_2 and polyethylene glycol (average molecular weights of

3,500 and 10,000) were evaluated for their effect on the phenol removal efficiencies. For these set of experiments, the tested concentration of phenol was 0.2 mM and the optimal temperature of 40°C was used. The rate of change of activity (absorbance) at varying H_2O_2 concentrations is a reflection of reaction velocity and was used to determine the kinetic parameters of the enzyme extracts.

III. RESULTS AND DISCUSSION

3.1 Ideal Enzyme Concentration

A series of dilutions of the original enzyme extracts were prepared and the initial reaction rates were determined by measuring the absorbance at 510 nm. These are shown in Fig. 1. For the Black-eyed beans, dilutions up to 0.4 are appropriate for the subsequent activity and phenol removal experiments. The change in absorbance for the Bambara beans remained linear up to dilutions of 0.2 which defined the appropriate concentration ranges in the subsequent experiments. A dilution of 0.125 was chosen corresponding to total protein concentrations of 6.56 mg/mL and 8.13 mg/mL for the black eyed and Bambara beans respectively.

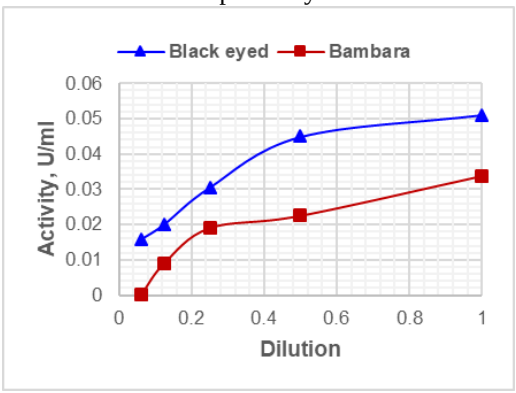


Figure 1: Effect of dilution on enzyme activity

3.2 Effect of Temperature and pH on Activity

The relative thermal stability of the peroxidase from the two bean varieties studied is shown in Fig. 2.

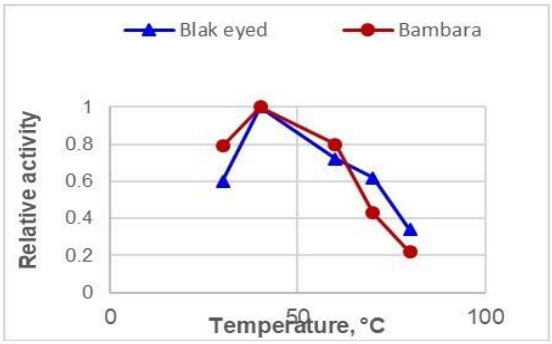


Figure 2: Effect of temperature on enzyme activity

The enzymes from the two bean varieties show higher activities at elevated temperatures than at room temperature. The optimum activities for the two extracts were recorded between 40 and 50°C. It is also observed that all samples show retention of more than 60% of the maximum activity at 60°C. In applications, this allows for the higher operating temperatures at which most substrates are more soluble in the aqueous solution. Oztekin and SeymaTasbasi [18] have recently shown similar activity dependence on temperature for Runner beans. At 80°C, the enzyme from the two varieties of beans were virtually inactivated completely. Similar results were obtained for peroxidase from Long beans where it was shown that the enzyme lost all activity at a temperature of 95°C [19]. All phenol removal experiments were subsequently performed at 40°C in this study.

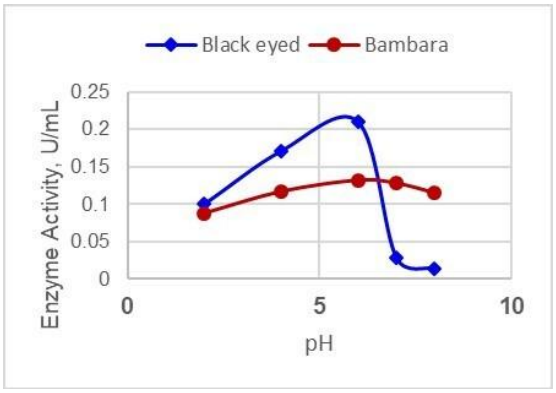


Figure 3: Effect of pH on enzyme activity

The effect of pH on the activities of the enzyme from the two bean varieties is shown in Fig. 3. The activity of the Bambara bean extract is seen as significantly lower than that of the black-eyed bean but its activity is maintained over a wider range of pH values. In each case, the optimum pH for the highest activity is about 6. Several studies have reported pH optima of between 5.5 and 7.5 for peroxidases from different plant sources [20,21,22,23,24]. In general, the system pH dictates the extent of ionization of residues at the active site of the enzyme. At very low and very high pH values, the weakening of the attachment of the heme group responsible for interactions at the active site of the enzyme presumably leads to lower activities [25]. A pH of 6 was therefore chosen for all subsequent studies unless otherwise specified for both enzyme extracts.

3.3 Kinetic Parameters

The Michaelis-Menten model was assumed for the action of the peroxidase enzyme and used to determine the maximum velocity (V_{max}) and the

Michaelis constant, K_m. A linearized form of the model equation is presented in equation 1as:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}} \quad (1)$$

where V is the initial reaction rate, S, the substrate concentration, K_m, the Michaelis constant and V_{max} is the maximum reaction rate. The plots are shown in Fig. 4. And the results summarized in Table1.

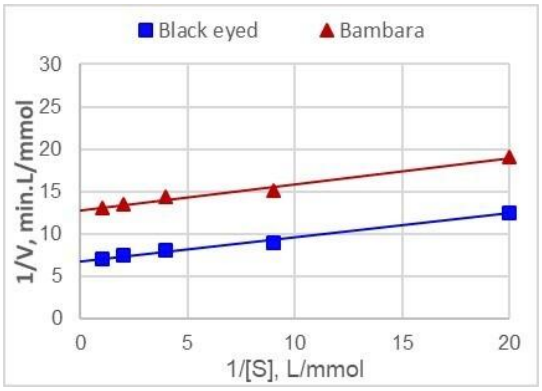


Figure 4: Lineweaver-Burk Plot

The lower value of K_m for Bambara beans shows the enzyme from this source has a higher affinity for phenol within the concentration range under consideration though the maximum reaction rate observed is about half that of the black eyed bean extract. In the crude state, the presence of residual bean tissue may offer different environments to either enhance or diminish the activity of the enzyme. Km is a dissociation constant and lower values imply a high affinity for the substrate (phenol).

A value of 0.765 mM was reported by Ghaemmaghami *et al* [26] for soybean peroxidase. Using H_2O_2 as substrate, Bania and Mahanta reported K_m values of 0.25, 0.37 and 0.277 mM respectively for peroxidases from tobacco, cabbage and radish [22]. The high affinity for the phenol substrate by the bambara bean extract compared to the black-eyed extract could be advantageous in applications where selective removal of phenol will be required.

Table 1: Kinetic Parameters

Bean variety	**Kinetic Parameters**	
	V_{max} (mM/min)	**K_m (mM)**
Black Eyed	0.1475	0.414
Bambara	0.0778	0.241

3.4 Phenol Removal

3.4.1 Effect of H_2O_2 Concentration

Hydrogen peroxide is known to inhibit a number of enzymes and the effect of its concentration on phenol removal rates has been reported. A 2:1 ratio was reported by Kingsley and Nicell, [27] and Deva et al [28] as optimum for the maximum removal of phenols. In the current study, the enzyme from the two bean varieties seem to be affected differently. Bambara bean extract retained a higher activity than the extract from black eyed bean as the concentration of H_2O_2 was increased as shown in Fig. 5. The optimal concentration of H_2O_2 for the phenol removal recorded in this study was 0.5 mM for the black-eyed bean extract and about 1.0 mM for Bambara bean extract. Peroxidases are thought to play the critical role of removing lethal levels of reactive oxygen species (ROS) such as H_2O_2 during regular metabolism of the plant and the enzyme might have evolved differently in these cellular systems to scavenge the high levels of ROS [29,30]. These activities are based on the crude extract and it is possible that the enzyme is more shielded in the Bambara bean extract just by the nature of the different cellular tissues.

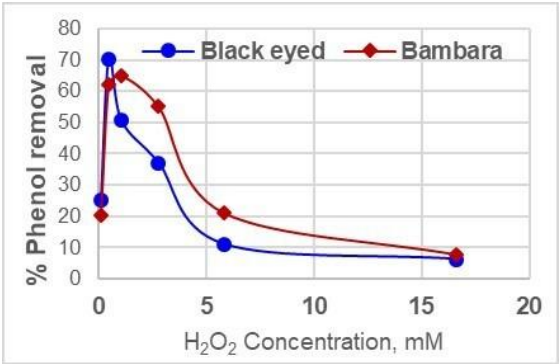


Figure 5: Effect of H_2O_2 concentration on phenol removal

3.4.2 Effect of Enzyme Concentration

The effect of enzyme concentration on phenol removal is shown in Fig. 6. For a 10 mM phenol solution, the percentage removed increased with enzyme concentration up to about 3 U/mL for both extracts though the black eyed sample achieved higher removal efficiency than the Bambara extract. Higher enzyme concentrations seem to have no significant effect on the phenol removal rate. Similar trends are also reported by other studies in the past [31, 32]. It has been suggested that the enzyme could be inactivated due to stabilization as the enzyme molecules at higher concentrations tend to adsorb onto the polymeric end product of the overall

reaction creating a diffusion limitation for the substrate to the active site [33].

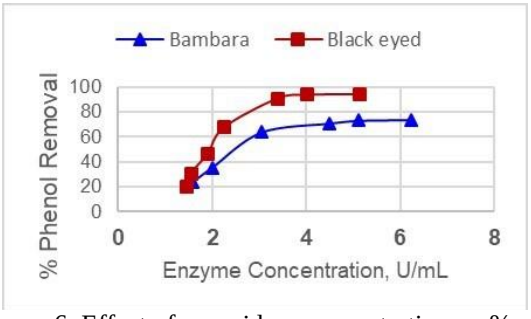


Figure 6: Effect of peroxidase concentration on % phenol removal

3.4.3 Effect of pH

The effect of pH on phenol removal was studied at the optimal temperature and H_2O_2 concentrations and the results shown in Fig. 7.

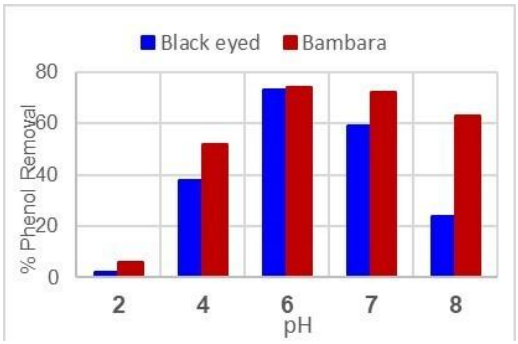


Figure 7: Effect of pH on phenol removal

The highest removal efficiencies of more than 70% were observed at a pH of 6 for both enzyme extracts as expected. Almost no removal was observed at the low pH of 2. At pH 8, phenol removal efficiency for the blackeyed extract dropped significantly but the Bambara bean extract maintained a value of over 60%. The source of the enzyme determines activity and each source tends to have different pH optima.

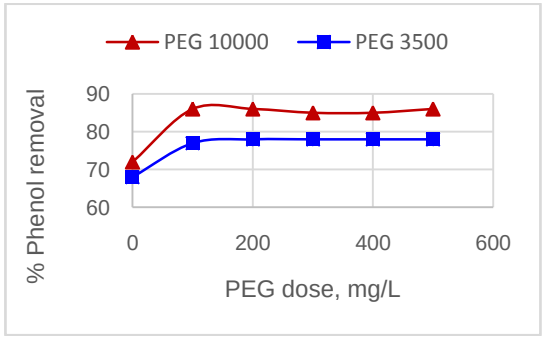
Crude enzyme extracts may still be protected differently by different cellular tissue types found in the source [34]. Several different pH optima have been reported for different peroxidase systems. Mizobutsi*et al*. [33] reported phenol removal efficiencies of >80%, 99% and >90% at pH of 4, 6, and 8 respectively using partially purified peroxidase from litchi pericarp and an initial phenol concentration of 2 mM. Using soybean peroxidase, removal efficiencies of between 75 and 80% have been reported at pH values between 6 and 8 [36, 37]. The extended pH range of high activity of the extract from bambara bean suggests its applications in systems where large fluctuations in pH are expected.

3.4.4 Effect of PEG Dose

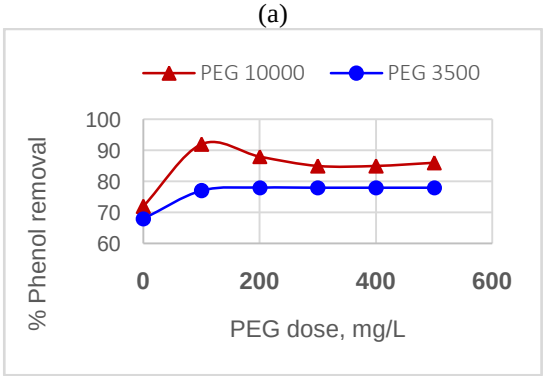
In phenol removal applications, it is known that the enzyme becomes quickly inactivated and to protect the enzyme from inactivation, a number of additives have been applied to extend the catalytic life of the enzyme [38,39]. The most common and one of the most studied of these additives is polyethylene glycol (PEG) which is relatively cheap and considered non-toxic. One of the proposed mechanisms of inactivation of the enzyme involves the adsorption of the enzyme onthe precipitated phenolic polymers which may shield the active site of the enzyme. [40].

PEG has the ability to bind to several water molecules and significantly swells to create a large hydrated volume [41]. The competitive adsorption of the polymers onto this swollen hydrated molecules will decrease the rate of adsorption onto the enzyme surface. The uptake of several water molecules also implies the enzyme is partially shielded from water molecules. The swelling ability of PEG is dependent on its molecular weight and it has been shown that high molecular weight PEG is more effective in stabilizing different peroxidase enzymes [27,32,42]. In this study the effect of molecular weight and dose of PEG was studied using PEG 3,500 and PEG 10,000.

Fig. 8 shows the extent of phenol removal by the two enzyme extracts in the presence of PEG. In line with other findings, the dose of PEG enhances the phenol removal rate at an optimum dosage of 100mg/L. It appears the effect is more pronounced in the bambara bean extract than the black eyed bean extract. At high concentrations, the attachment of PEG to the surface of the insoluble polymeric product is also believed to repel specific proteins leading to inactivation [8,21,43]. In addition, excessive swelling may also result in enzyme entrapment reducing their availability.



(a)



(b)

Figure 8: Effect of PEG dose on phenol removal (a) Black eyed beans (b) Bambara beans

IV. CONCLUSION

The applicability peroxidase enzyme found in two indigenous varieties of beans (Black eyed and Bambara) for removal of phenol from aqueous solutions was demonstrated. The enzyme from both sources showed maximum activity at a pH of 6 and a temperature of 40°C. In the presence of PEG, the phenol removal capability of the Bambara bean extract was very pronounced though its absolute activity was lower than the extract from the black-eyed bean.

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