Assessment and Characterization of Peroxidase Activity in Locally Grown Bean varieties from Ghana

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Abstract- The presence of peroxidase enzyme in four locally grown bean varieties, red beans, Bambara beans, black-eyed beans and soybeans was demonstrated. The enzyme showed a much higher activity at elevated temperatures than at room temperature with peak activity at about 50°C. The activity of the enzyme appears to be highly dependent on the pH of the medium. The optimum pH for the enzyme from all bean extracts is about 6 but the extract from bambara beans was shown to maintain its activity over a wider range of pHs than the rest. Such stability will make peroxidase from Bambara beans a useful enzyme for applications at higher pH as may be found in certain wastewaters.

Index Terms- Peroxidase activity, bean varieties, phenol removal, temperature, pH.

I. INTRODUCTION

Peroxidases (EC.1.11.1.7.) are enzymes which catalyze 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. They are heat stable enzymes and are found in plants and animals. (In: Brill, 1996). Their potential application in the removal of phenols from waste water is well documented in the literature (Aitken, 1993; Nair *et al*, 2007; Hamid and Khalil-ur-Rehman, 2009). Phenol contaminants, alongside other contaminants, are found in various industrial wastewaters such as petroleum refineries, coal processing factories, plastics, textiles, iron and steel manufacturing and also in the pulp and paper manufacturing industries. (Dyer and Mignone, 1983; Michalowicz and Duda, 2007).

The recent discovery of petroleum deposits in commercial quantities in Ghana has generated tremendous activity in the oil sector ranging from intensified exploration to establishment of new refineries. The expected expanded activity in the petrochemical industry will result in the discharge of large quantities of phenolic compounds as waste. Majority of phenols are very toxic, some have even been classified as hazardous waste and others have been suspected of containing carcinogens. About 0.005mg/L of phenol will cause odors and objectionable taste when it combines with chlorine to form chlorophenols (Lanouette, 1977). There are also long-term effects on ecosystems related to the release of toxic components over a prolonged period. (Samanta *et al*., 2002).

Several conventional methods are available for the removal of phenols from wastewaters including extraction, bacterial and chemical oxidation (Mark, 2003), adsorption on activated carbon (Grutsch and Mallat, 1981; Riaz and Abdul, 2002), electrochemical techniques and irradiation. These methods have their individual disadvantages that make them economically unfeasible. Chemical oxidation may be expensive for high strength waste treatment due to the high cost of chemicals. Also, hazardous by-products may be formed during the processes, the purification is incomplete and even low efficiencies have been observed (Stanisavljevic and Nedic, 2004). Enzyme- based treatment has been suggested as alternative method for phenol removal. Peroxidase enzyme extracted from soybeans has been shown to be highly thermo-stable and able to catalyze the removal of phenolic compounds from waste water (McEldoon and Dordick, 1996; Wright and Nicell, 1999). In this report, the presence and characteristics of the peroxidase enzyme in locally grown and readily available bean varieties is assessed as an alternative source of the enzyme for potential phenol removal applications.

II. MATERIALS AND METHODS

Four (4) locally grown bean varieties, namely Black-eyed bean (*Vigna unguiculata unguiculata*), Bambara beans (*Vigna subterranean*), Red bean (*Phaseolus vulgaris)* and soybean (*Glycine max*) were obtained from the local market (Kumasi, Ghana). All chemicals used in this study were of analytical grade and obtained from commercial sources.

Crude Enzyme Extraction

All procedures were carried out on the selected bean varieties at room temperature. 50 g of each bean variety were soaked for 12 hours in 200 mL of distilled water. The mixture was blended to homogenize 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 5 minutes to inactivate any catalase enzyme present. The extract was finally stored at 4 ^{o}C.

Test for the presence of Peroxidase

Two methods were used to demonstrate the presence of peroxidase in the extract, the iodometric titration method and the spectrophotometric method using o-dianisidine dye.

a. Iodometric method

This method is a modification of the Kingzett method for the determination of peroxide (Zabicky, 2006; Kingzett, 1881). The basis of the method is the oxidation of iodide to iodine in the presence of a catalyst, in this case peroxidase enzyme. 0.05 M of sodium thiosulphate was prepared prior to the experiment and kept aside. 0.2 mL of 30% hydrogen peroxide solution was added

to a solution of 5 mL enzyme extract in 45 mL distilled water in each of 4 Erlenmeyer flasks labeled 60 seconds, 120 seconds, 180 seconds and 240 seconds respectively. 10 mL of 1% solution of freshly prepared potassium iodide (KI) was added. At the appropriate time, a drop of concentrated HCl (5M) was then added to each of the flasks and the mixture was titrated with 0.05 M sodium thiosulphate solution using starch indicator. A drop in the concentration of the H_2O_2 is an indication of the peroxidase activity.

b. Spectrophotometric method

The test for the presence of peroxidase was carried out according to the method of Malik and Singh (1980) with modifications. 2mL each of the enzyme extract was added to a mixture of 0.1mL of o-dianisidine dye (extinction coefficient 11.3) and 1mL of 30% H_2O_2 in a test tube. The development of an orange color indicates the presence of the peroxidase enzyme in the sample.

Effect of Enzyme Concentration on Activity

A stock dilution of each enzyme concentration was prepared as follows:

i. 5 test tubes were thoroughly washed with distilled water and labeled 1-5.
ii. 2mL of the enzyme extract was added to test tube 1.
iii. To test tube 2 was added 2mL of the enzyme extract and diluted with 2mL of distilled water.
iv. 2mL of the diluted sample in test tube 2 was taken into test tube 3 and diluted with 2mL of distilled water.
v. 2mL of the diluted sample in test tube 3 was taken into test tube 4 and diluted with 2mL of distilled water.
vi. Finally, 2mL of the diluted enzyme sample in test tube 4 was taken into test tube 5 and diluted with 2mL of distilled water

The reaction mixture for the effect of enzyme concentration on the consumption of H_2O_2 was prepared by mixing 4.0 mL of 0.05M phosphate buffer (pH 7), 0.1 mL of o-dianisidine dye solution, 1.0 mL H_2O_2 solution and 0.1 mL of enzyme extract from each of the test tubes above. A control was set up with 0.1 mL distilled water in place of the hydrogen peroxide solution and enzyme extract from test tube 1.

For each mixture, the addition of the enzyme extract marks the start of the reaction. The absorbance at 460 nm of the mixture was immediately read and also at 30 second intervals for 3 minutes. The enzyme activity is a reflection of the rate of the reaction and is measured by the change in the concentration of the substrate per unit time. The unit of activity is defined as the amount of enzyme extract that decomposes 1 µmole of H_2O_2 per minute.

Effect of Substrate Concentration on activity

The reaction mixtures and reagent blank were prepared as indicated in table 3. 0.1 mL of the undiluted enzyme extract was added to the mixture in tube 5 and the absorbance at 460 nm of the mixture was measured at 30 second intervals for 3 minutes. The procedure was repeated for all the mixtures. The rate of change of absorbance is a reflection of the reaction velocity.

Table 1: Reaction Mixtures for effect of substrate concentration

Tube No.	mL, pH 7 phosphate Buffer	mL of Dye	mL of dH_2O	mL of H_2O_2 solution	mL of Enzyme mixture
1	4.0	0.10	0.90	0.10	0.10
2	4.0	0.10	0.80	0.20	0.10
3	4.0	0.10	0.60	0.40	0.10
4	4.0	0.10	0.40	0.60	0.10
5	4.0	0.10	0.20	0.80	0.10
6	4.0	0.10	----	1.0	0.10
7	4.0	0.10	1.0	----	0.10

Effect of pH on activity

A set of reaction mixtures as used in the effect of substrate concentration were prepared but the buffer solution was substituted with buffer of varying pH from 3 to 9. 0.1 mL of enzyme extract with dilution factor 1 was added to the reaction mixture in test tube 1 and the absorbance at 460 nm was measured at 30 second intervals for 3 minutes. For pH range 3-5, acetate buffer was used and phosphate buffer was used for pH range 6-9. The procedure was repeated for all the reaction mixtures.

Effect of Temperature on activity

5 mL of the enzyme solution with a known activity (measured previously) were placed in different test tubes and dipped in water bath maintained at temperatures ranging from 30ºC to 80ºC at the optimum pH of 6. The enzyme solutions were removed after 30 minutes and the activity test conducted on the samples as described earlier.

III. RESULTS AND DISCUSSION

Presence of Peroxidase

Spectrophotometric Method

O-dianisidine is oxidized by hydrogen peroxide in the presence of peroxidase to an orange colored product according to the following equation:

$$H_2O_2 + \text{Colorless dye (reduced)} \xrightarrow{\text{peroxidase}} H_2O + \text{Colored dye (oxidized)}$$

An orange color was observed for all samples when the extract was added to a mixture of H_2O_2 solution and o-dianisidine dye, indicating the presence of the enzyme in all bean varieties under consideration.

Iodometric Method

H_2O_2 oxidizes iodide to iodine in the presence of peroxidase enzyme. The iodine formed is titrated with thiosulfate solution, using starch indicator. The reaction in the iodometric titration method is as follows:

$$H_2O_2 + 2KI + 2H^+ \rightarrow I_2 + 2H_2O$$
$$I_2 + 2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2I^-$$

The drop in the concentration of the hydrogen peroxide showed the presence of the peroxidase in the extract as compared

with the control where no appreciable change in concentration of H_2O_2 was observed. The change in concentration of H_2O_2 versus time is shown in figure 1 using the extracts of the four bean varieties indicating the presence of peroxidase in all cases. The enzyme from red beans appears to be most active in catalyzing this particular reaction.

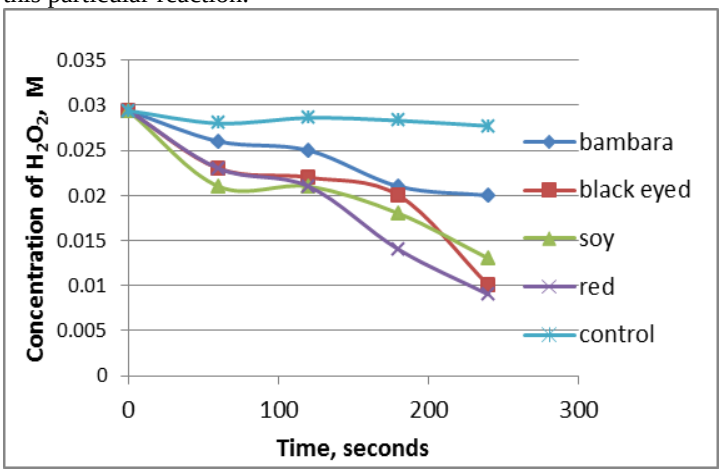

Figure 1: Effect of the various bean extracts on concentration of hydrogen peroxide

Effect of Enzyme Concentration

The enzyme activity is a reflection of the rate of the reaction and is measured by the change in the concentration of the substrate per unit time. At different concentrations of enzyme, the reaction velocities, reflecting the activities of the enzyme from the different bean varieties are shown in figure 2.

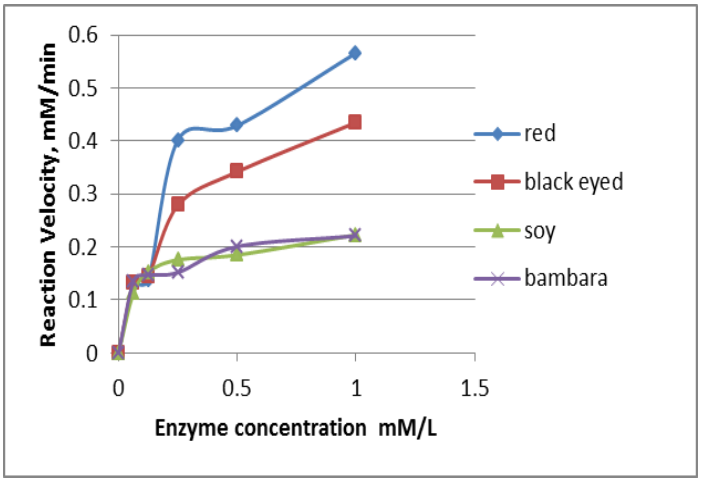

Figure 2: Effect of enzyme concentration on reaction velocity

In general an increase in enzyme concentration results in higher rates of reaction provided the substrate is not depleted in the reaction mixture. In the limit, the active site of the enzyme will be saturated and the reaction rate becomes constant. The enzyme concentration corresponding to this substrate limitation could be a measure of the total concentration of enzyme active sites in the extract. The lower saturation velocities recorded for the extracts from soybeans and bambara beans indicate that they contain a higher concentration of active sites such that the substrate is depleted more rapidly by these enzyme species. The ratio of substrate to the total enzyme concentration is also reported by several workers to affect the activity of the peroxidase enzyme. (Klibanov *et al*, 1980; Nicell *et al*, 1992; Bassi *et al*, 2004; Miland *et al*, 1996).

Effect of Substrate Concentration and kinetic parameters

Enzymes generally follow saturation kinetics with respect to the substrate. It is believed that the substrate occupies specific active sites on the enzyme to be properly oriented for a round of reaction (Rodgers and Gibson, 2009). As the substrate concentration increases, all the active sites might be occupied and any further increase in substrate concentration does not affect the rate of reaction. Figure 3 shows the effect of substrate concentration on the activity of the enzyme in the four bean extracts. Peroxidases from all the bean varieties exhibit saturation kinetics with hydrogen peroxide as the substrate.

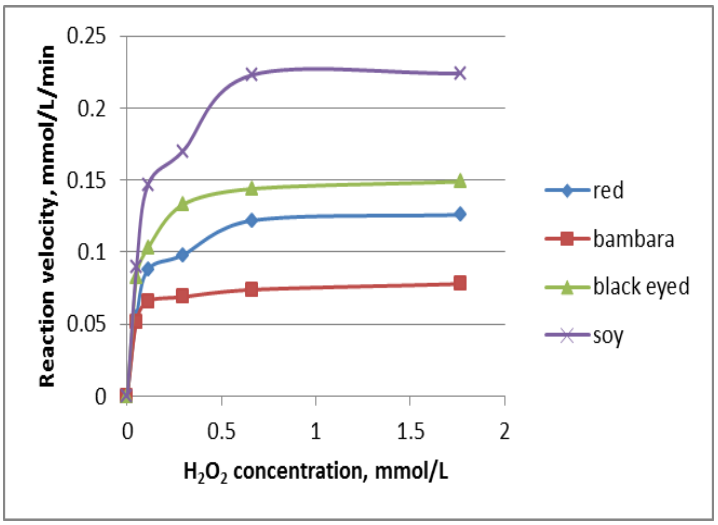

Figure 3: Effect of substrate concentration on reaction velocity

An important deduction about the mechanism of enzyme catalysis is that the substrates bind to specific regions of the enzyme, the active site, forming enzyme-substrate complexes that may eventually decompose to form products (Copeland, 1996). The implication is that, there must be a finite number of enzyme molecules, and consequently active sites, available at any given time. Substrate molecules, if present in enough concentration, should be able to saturate these binding sites.

Figure 4 shows the Lineweaver-Burk plot of the effect of H_2O_2 concentration on the rate of reaction to determine the maximum velocity (V_{max}) and K_m, the equilibrium constant in the Michaelis-Menten model of enzyme kinetics. The results are summarized in table 2. K_m is dissociation constant and indicates the level of affinity of the enzyme towards a particular substrate, in this case H_2O_2. The low value of K_m for bambara beans shows the enzyme from this source has the highest affinity for H_2O_2 within the concentration range under consideration. This may be important in selecting the source of the enzyme for particular applications.

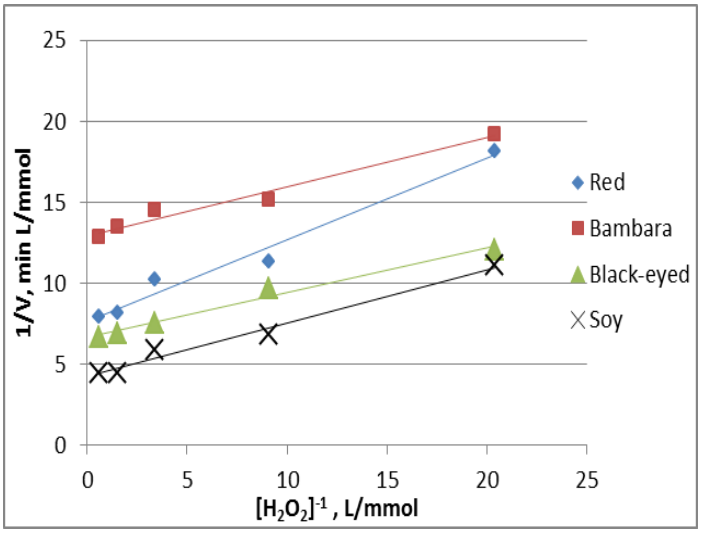

Figure 4: Lineweaver-Burk plot of effect of substrate concentration on velocity

Table 2: Kinetic parameters

Bean variety	Kinetic parameters	
	V_{max} (mmol/L/min)	K_m (mmol/L)
Red	0.131	0.0656
Bambara	0.077	0.0232
Black-eyed	0.151	0.0411
Soybean	0.236	0.0780

Effect of Temperature on relative activity

The thermal stability of soybean peroxidase is reported to be extremely high such that about 80% of its activity is maintained at 60°C (McEldoon and Dordick, 1996). The relative stability of the peroxidase from the four bean varieties studied is shown in figure 5.

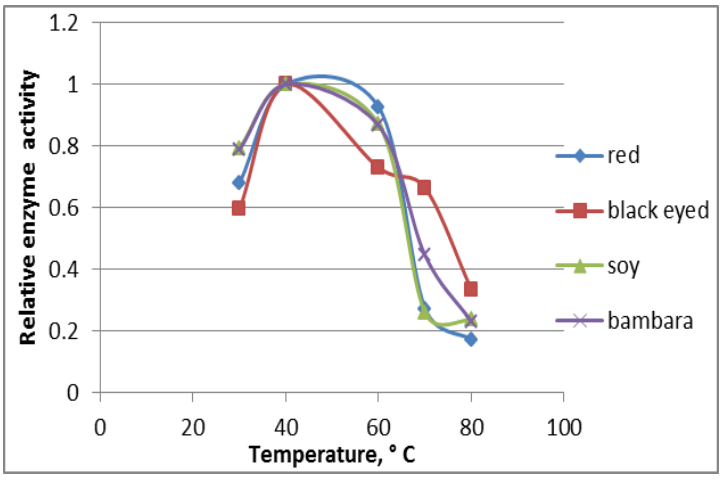

Figure 5: Effect of temperature on enzyme activity

The enzymes from all bean varieties show higher activities at elevated temperatures than at room temperature. The optimum activities for all 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.

Effect of pH

All enzymes display a characteristic range of pH at which they are most active. This pH optimum may be due to several factors involving the structure and ionic state of the enzyme, substrate, or cofactors. The pH dictates the ionization of residues at the active site of the enzyme. Figure 6 shows the effect of pH on the relative activities of the enzymes from the four bean varieties. The activity is equivalent to the change in absorbance with time at the different pHs under consideration.

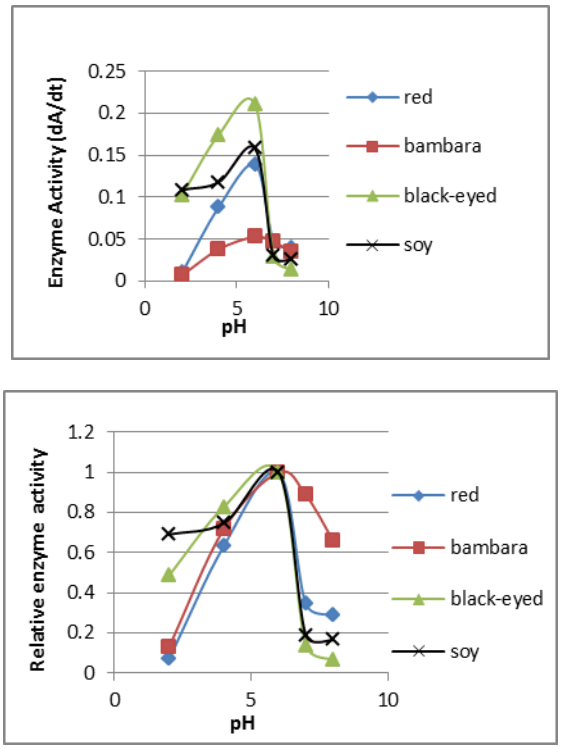

Figure 6: Effect of pH on Activity

Generally, all the enzyme extracts had their optimum pH around 6. Wright and Nicell, (1999) report a pH optimum of 6.4 for soybean peroxidase. The figure also shows that the enzyme from bambara beans maintains its activity over a wider range of pHs than those from other varieties. Though the absolute activity from this extract is low, it is significant that it maintains more than 60% of its activity at a pH of 8 which should make it more versatile over wider applications.

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